

ABSTRACT

KIM, MOO JUNG. Organically Grown Blackberries: Storage Life, Phenolic Composition, and Anti-inflammatory Activity. (Under the direction of Dr. Penelope Perkins-Veazie and Dr. Gina Fernandez).

Organically grown 'Natchez', 'Ouachita', and 'Navaho' blackberries at shiny black (SB) and dull black (DB) ripeness stages were stored at 1°C for 15 days or at 1°C for 13 days and at 20°C for the next two days to analyze storage life and phenolic composition. Anti-inflammatory activity of 'Natchez' and 'Ouachita' blackberries were analyzed in berries frozen immediately after harvest and in berries stored constantly at 1°C for 15 days prior to freezing. Leakage and decay incidence were higher and overall rating was lower in DB berries or in berries stored at 20°C for two days. Weight loss significantly increased during last two days of storage when fruit were exposed to 20°C. After storage, soluble solids content and titratable acidity were generally higher in 'Navaho' and decreased in 'Natchez' and 'Ouachita'. Total anthocyanin and phenolic contents analyzed using spectrophotometer were generally higher in 'Natchez' and 'Navaho' than in 'Ouachita' in berries that were not stored. Total anthocyanin content decreased after storage in 'Natchez' blackberries at both ripeness stages while decreased only in DB berries for 'Ouachita' and 'Navaho' fruit. Total phenolic content decreased after storage in all treatments with the exception of SB 'Ouachita' berries. Four anthocyanins, cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-xyloside, and pelargonidin 3-glucoside, and four non-anthocyanin phenolic compounds, gallic acid, vanillic acid, quercetin 3-galactoside, and quercetin 3-glucoside, were identified using HPLC. Cyanidin 3-glucoside was the predominant anthocyanin in the investigated blackberry

cultivars at both ripeness stages and in all storage conditions, and hydroxybenzoic acids were the major group of non-anthocyanin phenolic compounds. Phenolic profile differed slightly among cultivars, especially in cyanidin 3-xyloside and vanillic acid. The content and proportion of these two compounds were significantly lower in 'Natchez' than in the other cultivars. Anthocyanin and phenolic contents generally increased after storage with a greater increase in berries stored at 20°C for the last two days of storage. Extracts of 'Natchez' and 'Ouachita' blackberries used to determine anti-inflammatory activity significantly inhibited nitric oxide and prostaglandin E₂ (PGE₂) production and COX-2 expression in RAW 264.7 macrophage cells stimulated with lipopolysaccharide. Cyanidin 3-glucoside only inhibited nitric oxide production. The results of these studies indicate that postharvest quality of organically grown 'Natchez', 'Ouachita', and 'Navaho' blackberries were affected by ripeness stages and storage condition, and berries harvested at SB ripeness stage and stored constantly at low temperature had better postharvest quality. Composition slightly differed among cultivars and was affected by storage regime. Total anthocyanin and phenolic contents analyzed using HPLC did not decrease with storage. Organically grown blackberries showed anti-inflammatory activity by reducing nitric oxide and PGE₂ production and suppressing COX-2 expression but the effects decreased in extracts from stored berries.

© Copyright 2014 by Moo Jung Kim

All Rights Reserved

Organically Grown Blackberries: Storage Life, Phenolic Composition, and Anti-inflammatory Activity

by
Moo Jung Kim

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Horticultural Science

Raleigh, North Carolina

2014

APPROVED BY:

Dr. Penelope Perkins-Veazie
Co-chair of Advisory Committee

Dr. Gina Fernandez
Co-chair of Advisory Committee

Dr. Jeanine Davis

Dr. Gabriel Harris

DEDICATION

I dedicate this work to my parents. Thank you for your love, encouragement, and endless support.

BIOGRAPHY

Moo Jung Kim was born in Busan, Korea and grew up in a small town near Busan. Growing up in the suburbs, she found lots of different crops and wild plants everywhere and watched how they grew and changed over time. Her mom used to have a small garden growing vegetables and she loved to spend time with her mom gardening and observing those vegetables. This was where her interest for horticulture started.

Moo Jung Kim earned her BS degree in Plant Science from Seoul National University, Seoul, Korea. The Postharvest Physiology class that she took in her junior year stimulated interest in postharvest physiology of fruits and vegetables and she decided to continue her education. During her MS program at Seoul National University, she got a chance to participate in a research project on physicochemical characteristics of strawberries. For this project, she assisted measuring size and color of strawberries and analyzing secondary metabolites such as anthocyanins and ascorbic acid. For her MS degree, she worked on glucoraphanin and sulforaphane in broccoli as affected by fertilization and postharvest heat treatment. She wanted to get more experience in secondary metabolites that can be health-beneficial, and she was accepted into a graduate program in Horticultural Science at North Carolina State University. For her PhD research, she worked on postharvest quality and phenolic composition of organically grown blackberries at Plants for Human Health Institute at North Carolina State University. To her knowledge, this is the first study on refrigerated storage effects on postharvest quality and phenolic content in organically grown blackberries with the fresh market focus, and she is very proud of this. She received second and first place

awards for the PhD student paper competition at the Southern Region American Society for Horticultural Science in 2012 and 2013, respectively. In addition to her research on postharvest quality and phenolic composition of blackberries, she studied the anti-inflammatory activity of blackberries at cell level. Working and communicating with food scientists and seeing how horticultural crops are used in health researches and can be processed for nutritious products at food science department were exciting experience. Through her research at North Carolina State University, she found her passion for the postharvest and nutritional quality of fruits and vegetables and development of nutritious food products. She believes the power of plants for human health.

ACKNOWLEDGMENTS

I would like to thank my committee. I cannot express my gratitude enough in words. They have encouraged me to learn, challenge, travel, and experience for the last four years. Dr. Penelope Perkins-Veazie brought me to the field of postharvest physiology. She has taught me so many things that she learned from her experience and supported expanding my research spectrum into health research at food science department. I am truly grateful for everything she has done for me. Dr. Gina Fernandez has exposed me to the breeding process and it has been very interesting experience. When going out to field, she always explained me cultivation systems and showed physiological disorders or diseases, and it was great to see how blackberries are grown at commercial farms. I am also very grateful for her interest and advice for my graduate life and study. Dr. Gabriel Harris generously accepted me to work in his lab. When I started cell culture, I had no idea what cell culture is and how to design experiment and analyze data for this kind of work. He has taught all of those and encouraged to finish all steps. I sincerely thank him for his instruction and patience. Dr. Jeanine Davis encouraged me to attend a meeting and learn what is encompassed by organic productions. I appreciate her recommendation to attend the meeting and fieldtrip. It was really great time and I learned a lot.

I would also like to thank Dr. Guoying Ma and Joyce O'Neal at Plants for Human Health Institute for providing lab protocols and technical help. Guoying was a good friend during working at the research campus as well. In addition, I appreciate Ruth Watkins at Harris lab. She provided lab protocols and helped with procedures. She has always been there for me

whenever I needed her. I would also acknowledge Dr. Debra Clare and Evelyn Durmaz for their technical help and interest for my research. Additionally, Mr. John Vollmer and his staff produced wonderful blackberries and I enjoyed visiting his farm to get the samples and watching his beautiful plants.

I would like to acknowledge all people in horticulture department and Harris lab members, and friends at North Carolina State University for their support and friendship. Finally, I sincerely thank my parents and brother. Their support, love, and sacrifice made it possible to finish this challenge.

TABLE OF CONTENTS

LIST OF TABLES	ix
LIST OF FIGURES	xi
CHAPTER 1. Literature Review	1
Blackberries	1
Phenolic compounds in blackberries	6
Health benefits and bioavailability of berry phenolics	12
Anti-inflammatory activities.....	18
Factors affecting phenolic compounds content in berries.....	23
Literature cited	31
CHAPTER 2. Storage life and composition of organically grown blackberries	53
Abstract	53
Introduction.....	55
Materials and methods	57
Results and discussion	61
Conclusion	68
Literature cited	69
CHAPTER 3. Phenolic profile of organically grown blackberry cultivars at varying ripeness stages and storage conditions	80
Abstract	80
Introduction.....	81

Materials and methods	84
Results and discussion	87
Conclusion	97
Literature cited	99
CHAPTER 4. Anti-inflammatory activities of organically grown blackberries as	
affected by storage	123
Abstract	123
Introduction.....	124
Materials and methods	126
Results and discussion	133
Conclusion	142
Literature cited	144
APPENDICES	163
A. Antioxidant capacity of organically grown blackberries	164
B. Phenolic profile of organically grown blackberries	166
C. Phenolic composition in blackberry seeds	168
D. Cell culture	170
E. Western blot.....	173
Literature cited	178

LIST OF TABLES

Table 1-1. Common anthocyanins in plants	47
Table 2-1. Subjective ratings of organically grown shiny black and dull black ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries after storage at two temperature regimes, CL and RT, for 15 days.....	73
Table 2-2. Soluble solids content and titratable acidity of organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries after storage for 0 and 15 days at constant low (CL) and retail temperature (RT)	75
Table 3-1. Anthocyanin contents (mg g ⁻¹ DW) in ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries 0 and 15 days after storage	107
Table 3-2. Anthocyanin composition in organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries	109
Table 3-3. Anthocyanin contents (mg g ⁻¹ DW) in ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries at SB and DB ripeness stages	110
Table 3-4. Phenolic contents in (mg 100 g ⁻¹ DW) ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries 0 and 15 days after storage	111
Table 3-5. Composition of phenolic compounds in ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries	113
Table 3-6. Phenolic content (mg 100 g ⁻¹ DW) in ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries at SB and DB ripeness stages	115

Table 3-7. Pearson’s correlation coefficients for anthocyanins and non-anthocyanin phenolic compounds in ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries	116
Table 4-1. Anthocyanin and non-anthocyanin phenolic compounds in organically grown ‘Natchez’ and ‘Ouachita’ blackberries at 0 and 15 days after storage	150
Table A-1. Antioxidant capacity (mmol Fe ²⁺ equiv. kg ⁻¹) of organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries	179
Table A-2. Anthocyanin content (mg g ⁻¹ DW) in organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries at 0 and 15 days after storage	180
Table A-3. Anthocyanin composition of organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries at SB and DB ripeness stages	181
Table A-4. Phenolic content (mg 100 g ⁻¹ DW) in organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries at 0 and 15 days after storage	182
Table A-5. Main effects and interaction on non-anthocyanin phenolic content in organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries	183
Table A-6. Non-anthocyanin phenolic composition of organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries at SB and DB ripeness stage	184
Table A-7. Phenolic content (mg 100g ⁻¹ DW) in the seeds of organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries	185

LIST OF FIGURES

Figure 1-1. Blackberry flowers	48
Figure 1-2. Blackberry fruit	48
Figure 1-3. Types of phenolic compounds in berries	49
Figure 1-4. Structure of anthocyanins.....	50
Figure 1-5. Possible metabolism of phenolic compounds	51
Figure 1-6. Proposed flavonoid effects on inflammatory responses.....	52
Figure 2-1. Weight loss during storage	77
Figure 2-2. Total anthocyanin content in organically grown blackberries	78
Figure 2-3. Total phenolic content in organically grown blackberries	79
Figure 3-1. HPLC chromatogram of SB ‘Navaho’ blackberries (day 0)	118
Figure 3-2. Scheme of the flavonoid biosynthetic pathway.....	119
Figure 3-3. Schematic pathway of anthocyanins biosynthesis in grapes.....	120
Figure 4-1. Total phenolic content in organically grown ‘Natchez’ and ‘Ouachita’ blackberries at 0 and 15 days after storage	151
Figure 4-2. Viability of RAW 264.7 cells treated with blackberry extracts or cyanidin 3- glucoside followed by LPS treatment.....	152
Figure 4-3. Inhibition of nitrite production by blackberry extracts or cyanidin 3-glucoside treatment	153
Figure 4-4. Effects of blackberry extracts and cyanidin 3-glucoside in COX-2 expression in LPS-stimulated RAW 264.7 cells	156

Figure 4-5. PGE₂ production in LPS-stimulated RAW 264.7 cells.....160

CHAPTER 1

Literature review

Blackberries

Blackberries (*Rubus* spp.) belong to rose family (*Rosaceae*) and genus *Rubus*, and are classified as caneberries, as are raspberries. Blackberries have been collected as wild fruits for about 2,000 years (Bushway et al., 2008). Worldwide, blackberries have historically been used as hedges or for medicinal uses (Jennings, 1988). Cultivation of blackberries in America began in 1850's, with many commercial cultivars developed in 19th and 20th century (Bushway et al., 2008). Blackberries are harvested and consumed for fresh or processed markets and the majority of blackberries grown in the US are for processing markets. Agricultural Marketing Resource Center (AgMRC) (2012) has reported that about 90% of blackberries are consumed in frozen form. Worldwide blackberry production in 2005 was estimated at 20,035 ha and 140,292 ton with concentrated production in Europe and North America (Strik et al., 2008). In the US, Oregon leads blackberry production (Strik et al., 2007), and the United States Department of Agriculture (USDA) (2013b) reported that total 53,520,000 lbs (24,276 ton) of blackberries was produced from 6,800 acres (2,752 ha) in Oregon in 2012. It was also reported that US blackberry production in 2009 was valued at \$30.8 million and increased to \$43.2 million in 2011 (AgMRC, 2012).

The organic food sales in the US reached \$28 billion, and fruits and vegetables accounted for 43% of the total organic sales in 2012 (USDA, 2013a). The Organic Trade

Association (OTA) reported that the organic industry reached \$28.6 billion in 2010 (OTA, 2011), and consumer-driven organic market in 2011 was \$31.5 billion in sales (OTA, 2012). Certified organic production of berries in the US increased from 8,138 acres (3,293 ha) in 2008 to 12,796 acres (5,332 ha) in 2011 (USDA, 2013c).

Crop characteristics

Blackberries are a woody shrub having a perennial root system and biennial canes (Bushway et al., 2008). Depending on growth habit, blackberries can be divided into erect, semi-erect, and trailing types. Trailing type requires trellis system to support canes while trellis is not necessary for erect and semi-erect types. Canes emerge every year from the crown and usually fruit at the second year (floricane-fruiting). First year fruiting type (primocane-fruiting) varieties have recently been developed and utilized for late season or off-season production (Clark et al., 2005; Clark and Perkins-Veazie, 2011).

Blackberry flowers typically consist of five sepals, five petals, and stamens surrounding pistils (Bushway et al., 2008) (Fig. 1-1). Caneberries including blackberries are self-fruiting, and each flower contains 100-125 pistils and two ovules. After pollination, one ovule develops to seed and the other develops to drupelet. Blackberry is classified as aggregate fruit composed of individual drupelets (Fig. 1-2). Blackberry fruits undergo color change during ripening from green to red and then to black (Burdon and Sexton, 1993; Perkins-Veazie et al., 1996). Fully black fruits can be divided into shiny black (fruit sheen, fresh

market ripeness stage) and dull black (no fruit sheen, processing ripeness stage) stages, and fruits at both stages are harvested for commercial uses (Perkins-Veazie et al., 1996). When harvested, the torus (receptacle) remains inside the blackberry fruit, in contrast to raspberries where the torus remains on the stem and separates from the fruit (Bushway et al., 2008).

Blackberry production

Blackberries are grown worldwide, mainly in Europe and North America (Strik et al., 2008). The US is one of the largest blackberry producers in the world, and North Carolina was the 9th largest producer in the US based on area (ha) and 5th based on total production (ton) (Strik et al., 2007). Worldwide commercial blackberry production in 2005 was estimated at 20,035 ha and is expected to increase over 27,000 ha in 2015 (Strik et al., 2007; Strik et al., 2008). The US blackberry production in 2011 was valued at \$43.2 million (AgMRC, 2012). Oregon produces about 65% of total blackberry production in the US, and over 95% of blackberries produced in Oregon is processed (Strik et al., 2007). In Oregon, most cultivated plants are trailing type, particularly cultivars ‘Marion’, ‘Boysen’, and ‘Thornless Evergreen’. In contrast, many cultivars grown in North Carolina are erect and semi-erect, and suitable for fresh market, with the predominant cultivars being ‘Ouachita’ and ‘Navaho’ (G. Fernandez, personal communication).

Organic production of blackberries

Organic agriculture is defined as “an ecological production management system that promotes and enhances biodiversity, biological cycles, and soil biological activity” by the USDA National Organic Standards Board (NOSB) (NOSB, 1995). In addition, minimal use of off-farm inputs and management practice that can restore, conserve, or improve ecological harmony are important in the organic agriculture. The National Agricultural Statistics Service (NASS) (2010) says that unlike conventional agriculture, the use of conventional pesticides, petroleum-based fertilizers, sewage sludge-based fertilizers, herbicides, pesticides, genetic engineering (biotechnology), antibiotics, growth hormones, or irradiation is prohibited in the organic agriculture.

In 2011, the acreage of certified organic farms for blackberries and dewberries production was 308 acres (125 ha) in the US (USDA, 2012). Total production was 2,015,513 lbs (1,008 ton) with the main producers of California and Oregon. Offerings of a wide range of organically produced fruit are now common in most large market chains (USDA, 2014). Organic fruit is both produced in the US and imported from other countries and offerings are very diverse and include many small fruit. Although small in size compared to strawberries, the production of organic blackberries is strong. During the period from June 2010 through June 2012, the retail price for a 5.6-6.0 ounce package of fresh conventional and organic blackberries increased from \$2.56 to \$3.25 per pound (AgMRC, 2012). Other sources indicated that the average retail price of fresh blackberries was reported as \$5.18/lb, higher

than that of blueberries (\$3.91/lb) and strawberries (\$2.28/lb) (Stewart et al., 2011). That of raspberries was \$7.29/lb and the highest among investigated fruits.

Nutritional composition of blackberries

Blackberry fruit are composed of about 88% of water, 1.4% of protein, and 5% of sugars (USDA, 2013d). Glucose and fructose are the major sugars in blackberries, and sucrose content in blackberries are usually very low (Fan-Chiang and Wrolstad, 2010; Kafkas et al., 2006; Perkins-Veazie et al., 1999). Malic acid, citric acid, or isocitric acid were found to be the major organic acids in blackberries, depending on cultivar. Fan-Chiang and Wrolstad (2010) reported that °Brix value and titratable acidity ranged from 6.88 to 16.83 and from 0.52 to 2.24 g citric acid equivalent 100 mL⁻¹, respectively, in 18 cultivars and 21 selections of blackberries. Blackberries are good source of vitamins A, C, and E, folic acid, iron, potassium, and soluble fiber (Bushway et al., 2008). Fresh blackberries (100 g) can provide about 214 IU of vitamin A, 21 mg of ascorbic acid, 25 µg of folate, 162 mg of potassium, and 5.3 g of dietary fiber (USDA, 2013d).

Blackberries are a good source of phytochemicals, especially phenolic compounds, and contain small amount of carotenoids. Phenolic compounds in blackberries include anthocyanins, phenolic acids, flavonoids, and ellagic acids (Cho et al., 2004; Siriwoharn and Wrolstad, 2004; Zadernowski et al., 2005). Marinova and Ribarova (2007) reported carotenoids such as lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and β-carotene in

blackberries. They also found that total carotenoid content was higher in blackberries than other berries investigated including strawberries, red- and black currants, blueberries, and raspberries.

Phenolic compounds in blackberries

Phenolic compounds are a group of plant secondary metabolites possessing at least one aromatic ring and hydroxyl group (Dai and Mumper, 2010; Karakaya, 2004). Phenolic compounds in plants are primarily involved in defense system against pathogens, parasites, insects, or ultraviolet radiation, but they also have an important role in color of leaves, flowers, and fruits, and affect sensory and nutritional quality of foods derived from plants (Bravo, 1998; Dai and Mumper, 2010; Karakaya, 2004). Phenolic compounds are the most abundant plant secondary metabolites with more than 8,000 compounds found. Depending on their structure, this large group can be divided into several sub-groups including phenolic acids, flavonoids, tannins, stilbenes, and lignans (Fig. 1-3.) (Karakaya, 2004; Manach et al., 2004). Among these, phenolic acids and flavonoids are more common, and flavonoids are the most abundant in our diet (Scalbert and Williamson, 2000). Blackberries are rich in phenolic compounds, in particular anthocyanins, which are widespread plant pigments belonging to flavonoids.

Phenolic acids

Phenolic acids are a subgroup of phenolic compounds possessing carboxylic acid group, and include hydroxybenzoic acids, hydroxycinnamic acids, and their derivatives (Robbins, 2003). Depending on the number and position of the reactive groups, types and properties of phenolic acids are determined. However, there are limited literatures on phenolic acid composition of blackberries. Zadernowski et al. (2005) analyzed phenolic acids in six types of berries and reported six hydroxybenzoic acids, six hydroxycinnamic acids, and other phenolic acids in blackberries. Among identified phenolic acids, *m*- and *p*-coumaric acids, 3,4-dimethoxycinnamic acid, and hydroxycaffeic acid were the major phenolic acids in blackberries representing 18.3, 12.9, 15.4, and 16.1% of the total phenolic acids content, respectively. Other phenolic acids including gentisic, gallic, *o*-pyrocatechuic, protocatechuic, salicylic, vanillic acids, caffeic, *p*-hydroxyphenyl-acetic acid, and *p*-hydroxyphenyl-lactic acid were also found in blackberries. Hydroxycinnamic acids were found to be more abundant than hydroxybenzoic acids. These authors also found out that phenolic acids liberated from esters were major form compared to those liberated from glycosides or free forms. Phenolic acid content in blackberries ranges from 7 to 64 mg 100 g⁻¹ FW (Kaume et al., 2012).

Flavonoids

Flavonoids are another subgroup of phenolic compounds. The common structure of flavonoids is diphenylpropanes, consisting of two aromatic rings linked through three carbons (Bravo, 1998). Flavonoids are commonly found as glycosides or their derivatives. Flavonoids can further be divided into several types including anthocyanins, flavonols, flavanols, flavones, and isoflavones. Among these, anthocyanins and flavonols are important flavonoids in blackberries.

Anthocyanins

Anthocyanins are the most important and widely distributed water-soluble pigments responsible for various colors from orange and red to blue and purple (Castañeda-Ovando et al., 2009; Clifford, 2000; Kong et al., 2003). Anthocyanins in sugar-free form are called anthocyanidins, and anthocyanidins consist of two aromatic rings and heterocyclic ring containing oxygen (Fig. 1-4) (Castañeda-Ovando et al., 2009). More than 20 anthocyanidins have been identified to date, varying in number and position of hydroxyl and methoxyl groups attached, but only six anthocyanidins are commonly found in higher plants (Table 1-1) (Castañeda-Ovando et al., 2009; Kong et al., 2003). Among these six, cyanidin, delphinidin, and pelargonidin are the most widespread anthocyanidins in edible plant parts, commonly present in a form of glycoside (Kong et al., 2003). Anthocyanins are usually present in glycosylated forms *in vivo*, and the glycosylation occurs mostly at 3-*O*-position

and often at 5-*O*-position (Springob et al., 2003). The 3-*O*-glycosylation is necessary for further glycosylation, acylation, methylation, and/or prenylation. The glycosylation can stabilize anthocyanins and is occurred by an enzyme UDP-glucose:anthocyanin glycosyltransferase. The common sugar moieties for anthocyanin glycosylation include glucose, galactose, rhamnose, arabinose, xylose, and glucuronic acid (Andersen and Markham, 2005; Shahidi and Naczki, 2004). However, anthocyanins glycosylated with rutinose, sambubiose, or sophorose are also found in blackberries and raspberries (Bradish et al., 2012; Fan-Chiang and Wrolstad, 2005).

Cyanidin is the predominant anthocyanidin in blackberries (Fan-Chiang and Wrolstad, 2005; Kaume et al., 2012). Fan-Chiang and Wrolstad (2005) reported that cyanidin 3-glucoside represented 43.6-96.2% of the total anthocyanin content in blackberries depending on genetic background. Other anthocyanins including cyanidin 3-rutinoside, cyanidin 3-xyloside, cyanidin 3-malonyl-glucoside, and cyanidin 3-dioxalyl-glucoside were also detected in blackberries. It was reported that about 94% of anthocyanins in blackberries is present in non-acylated form, and 90% of anthocyanins is monoglycosides while 10% is found to be diglycosides (Wu et al., 2006). Total anthocyanin content in blackberries ranges from 70 to 240 mg 100 g⁻¹ FW varying with genotype (Cho et al., 2004; Fan-Chiang and Wrolstad, 2005).

Flavonols

Flavonols are another commonly found flavonoid. The chemical structure of flavonols is similar to that of anthocyanins but differs by the presence of a ketone group, the number and position of double bond of C ring, and hydroxyl group replacing R2 of B ring (Kaume et al., 2012). In blackberries, quercetin 3-galactoside was reported as the major flavonol, and quercetin 3-glucose, quercetin 3-rutinoside, quercetin 3-xylosylglucuronide, and quercetin 3-[6''-(3-hydroxy-3-methylglutaroyl)]- β -galactoside flavonols were also found (Cho et al., 2004; Cho et al., 2005). Total flavonol content in blackberries ranges from 99 to 160 mg kg⁻¹ FW depending on genotype. 'Evergreen' and 'Marion', important cultivars in Oregon, were reported to contain 11-20 mg 100 g⁻¹ DW of flavonol (quantified as rutin equivalent) (Siriwoharn and Wrolstad, 2004). Mertz et al. (2007) reported that quercetin glucuronide and quercetin glucoside were main flavonol in *R. adenotricus* and *R. glaucus* blackberries grown in Costa Rica and Ecuador, ranging from 51-58 mg 100 g⁻¹ DW.

Tannins

Tannins are polymerized phenolic compounds with high molecular weight (Bravo, 1998) that can cause tart taste/astringency and color change of foods (Bravo, 1998; Szajdek and Borowska, 2008). Tannins are divided into two groups depending on their structure. As the name indicates, hydrolysable tannins can be easily hydrolyzed by acid, alkali, hot water, or enzymes (Bravo, 1998). Hydrolysable tannins include gallotannins and ellagitannins which

are polymers of gallic acid and its dimeric condensation product hexahydroxydiphenic acid, respectively. Condensed tannins are polymers of flavan 3-ols such as catechin and epicatechin. Condensed tannins are often called proanthocyanidins because they can form red pigments in concentrated acids upon oxidative heating (Herderich and Smith, 2005). Procyanidins and propelargonidins are major proanthocyanidins in blackberries (Kaume et al., 2012). Procyanidin and propelargonidin are polymer of catechin and afzelechin, respectively (Andersen and Markham, 2005). The condensed tannins usually refer oligomeric proanthocyanidins (dimers, trimmers, and tetramers), but the degree of polymerization of proanthocyanidins can be over 50 (Bravo, 1998). Procyanidin content in blackberries was reported as 3.7, 6.7, and 3.6 mg 100 g⁻¹ FW for monomers (catechin glycoside), dimers, and trimmers, respectively, and the total procyanidin content was 27.0 mg 100 g⁻¹ FW (Gu et al., 2004).

Ellagic acid

When ellagitannins are exposed to acids or bases, they can be hydrolyzed and form ellagic acid, a dimeric derivative of gallic acid (Clifford and Scalbert, 2000; Hager et al., 2008). In blackberries, most ellagic acid is present as ellagitannins (Kaume et al., 2012). Ellagitannins in blackberries are found in seeds, torus, and flesh, with seeds as the primary source, and some ellagitannins are only found in seeds (Hager et al., 2008). Ellagitannins and ellagic acid contents decrease as the fruit ripen (Acosta-Montoya et al., 2010). Total

ellagitannin content in fresh 'Apache' blackberries was 23.3 mg 100 g⁻¹ FW (Hager et al., 2010).

Health benefits and bioavailability of berry phenolics

Oxidation

A reduction-oxidation system in living organisms is necessary to maintain healthy balance (Zhao, 2007), and antioxidant is important for preventing and inhibiting reactive oxygen species that can cause damage to DNA, lipids, proteins, and other biomolecules (Halliwell, 1996). Phenolic compounds in a number of berries have shown *in vitro* and *in vivo* inhibitory effects against oxidation. Slatnar et al. (2012) investigated antioxidant activity of berry juices in a yeast cell model. Juices of black currants (*Ribes nigrum*), blueberries (*Vaccinium corymbosum*), chokeberries (*Aronia melanocarpa*), and bilberries (*Vaccinium myrtillus*) significantly inhibited intracellular oxidation in yeast cells, with bilberry having the greatest inhibitory activity. In another study, a bilberry extract applied to human endothelial EA.hy926 cells inhibited intracellular oxidation in a concentration-dependent manner (Bornsek et al., 2012). Juices of blackberries, strawberries, and *Aristotelia chilensis* (maqui berry) delayed oxidation of human low-density lipoproteins and inhibited H₂O₂-induced oxidation in human umbilical vein cells (Miranda-Rottmann et al., 2002). These studies showed that berry extracts that contain phenolic compounds can reduce oxidative stress at cell level.

Cancer

Cancer is an important cause of death in the US and many industrialized countries (Zhao, 2007). Berry extracts containing phenolic compounds can have anticarcinogenic activities by inhibiting cell proliferation, modulating cell cycle arrest, or inducing apoptosis of cancer cells (Seeram, 2008). Raspberry extracts (cvs. 'Anne', 'Goldie', 'Heritage', and 'Kiwigold') inhibited proliferation of liver cancer cells HepG2 (Liu et al., 2002). Extracts of blackberries, black raspberries, blueberries, cranberries, red raspberries, and strawberries showed anti-proliferative activity against human oral (KB, CAL-27), breast (MCF-7), colon (HT-29, HCT116), and prostate (LNCaP) tumor cells (Seeram et al., 2006). These authors also reported that black raspberry and strawberry extracts significantly enhanced apoptosis of HT-29 cells. Sun and Liu (2006) reported induced apoptosis and a concentration-dependent anti-proliferative activity of cranberry extracts in MCF-7 cells. Katsube et al. (2003) also reported that bilberry extract induced apoptosis of human leukemia HL60 cells. Cyanidin 3-glucoside, a major anthocyanin in blackberries, extracted from blackberries showed anticarcinogenic activities by suppressing proliferation of human lung cancer cells A549 or tumor growth in mice implanted with A549 cells (Ding et al., 2006). As shown in these studies, phenolic compounds in berries can suppress cancers by participating in various anti-carcinogenic mechanisms.

Inflammation

It is widely accepted that chronic inflammation can result in various diseases such as cancers, Alzheimer's disease, and atherosclerosis (Wang et al., 2008). Inflammation is a complicated mechanism in which various proteins, cytokines, and other metabolites are involved (Kim et al., 2004). Of those, nitric oxide (NO) is an important signaling molecule in inflammation and participates in regulation of physiological and cellular responses (Conforti and Menichini, 2011; Vuong et al., 2006). NO produced by inducible nitric oxide synthase (iNOS), an enzyme that is rapidly induced when inflammatory process begins, is an important inflammatory mediator. Cyclooxygenase-2 (COX-2) is another enzyme that plays an important role in inflammation. COX-2 is significantly induced by inflammatory stimulus (Seibert et al., 1994) and produces other inflammatory metabolites such as prostaglandins (PGs) and thromboxane A₂ (Yoon and Baek, 2005). Several studies have shown anti-inflammatory activities of phenolic compounds contained in berries. Wang et al. (2008) reported reduced gene expression of iNOS and COX-2 in macrophage THP-1 cells by cyanidin 3-glucoside treatment. Wang and Mazza (2002) reported inhibition of NO production by various phenolic compounds in macrophage RAW 264.7 cells. It was also reported that black currant extract inhibited synthesis of prostaglandin E₂ (PGE₂), a metabolite produced during inflammation by COX-2 (Khoo et al., 2012). These results indicate that berry and berry phenolics can be beneficial for inhibiting inflammation and inflammation-related diseases.

Others

Diabetes is an important metabolic disease with 151 million people reported as diabetic in 2000 worldwide. The number of diabetic patients is increasing and thought to reach 334 million in 2025 (Wild et al., 2004). The World Health Organization (WHO) (1999) defines diabetes as a metabolic disorder characterized by chronic hyperglycaemia resulting from defects in secretion and/or action of insulin. There are two major types of diabetes. Type I diabetes results from destruction of pancreatic β cells, which produce insulin. In contrast, type II diabetes is related to insulin resistance. Berry extracts have shown anti-diabetic activities by inhibiting enzymes α -glucosidase and pancreatic lipase. Inhibiting α -glucosidase can retard carbohydrate digestion and thus important for people with type II diabetes (You et al., 2012). In addition, inhibiting pancreatic lipase, which can result in protecting insulin-producing β cells of pancreas by reducing lipid accumulation in the pancreas, can help type I diabetic patients. Muscadine (cv. 'Carlos') and its seed extracts showed inhibitory activities against α -glucosidase and pancreatic lipase too. Extracts of black- and red- currants, and red- and green- gooseberries also inhibited α -glucosidase in a dose-dependent manner, and red currant showed the highest inhibition (Pinto et al., 2010). Red currants greatly inhibited α -amylase too. Inhibiting α -amylase can retard glucose absorption due to reduced starch hydrolysis and therefore, it can be helpful for type II diabetic patients.

As a group, berries have beneficial effects against age-related neurodegeneration. Shukitt-Hale et al. (2009) reported that 19-month old rats fed with 2% blackberry-

supplemented diet had improved motor and cognitive performance compared to control group. Aged rats (15-month old) fed with blueberry-supplemented diet showed better object recognition memory and had lower expression of nuclear factor kappa B (NF- κ B), an indicator of oxidative stress, in the brain (Goyarzu et al., 2004). In addition, it was also reported that rabbiteye blueberry extracts enhanced memory and reduced DNA damage in mice brain (Barros et al., 2006). As shown above, berry phenolics have been studied for health benefits and shown beneficial effects on various symptoms and diseases including oxidation, cancers, inflammation, diabetes, and age-related neurodegeneration by affecting proteins and metabolites that are involved in those physiological process.

Bioavailability and metabolism of phenolic compounds in berries

Bioavailability is commonly defined as the proportion of the nutrients that are digested, absorbed, and metabolized (McGhie and Walton, 2007). Therefore, the bioavailability can be measured as the level of compounds and/or their metabolites in the plasma or urinary excretion. Absorption and metabolism of phenolic compounds are affected by their chemical structure (Scalbert and Williamson, 2000). In the human diet, phenolic compounds are the most common plant secondary metabolites, but are not the most bioactive *in vivo*, because they are poorly absorbed or quickly eliminated (Manach et al., 2004). Fig. 1-5 is a simplified scheme of polyphenol metabolism.

It was believed for a long time that anthocyanins are hardly absorbed in intact glycoside forms because of hydrophilic sugar moiety (Galvano et al., 2004; Hollman, 2001; Wu et al., 2002). However, several studies have shown that anthocyanin glycosides can be absorbed in intact forms (Felgines et al., 2002; McGhie et al., 2003; Wu et al., 2002). It was reported that cyanidin glycosides can be absorbed by the digestive tract and enter the blood stream in their original forms (Tsuda et al., 1999). Cyanidin glycosides were also detected in methylated forms in urine (Felgines et al., 2002). It was suggested that cyanidin is unstable in plasma and degraded to protocatechuic acid (Tsuda et al., 2000). Cyanidin is either absorbed intact in the gut or conjugated with glucuronide or methylated in the liver or gut (Kay et al., 2004; Wu et al., 2002). Glucuronidation of cyanidin seems mostly to occur at the 3-position (Wu et al., 2002).

In general, the bioavailability of anthocyanins are low. Several studies have shown that less than 3% of total anthocyanins administered was found in human urine when blackberries, strawberries, blueberries, and elderberries were fed as fresh fruit or juice (Felgines et al., 2005; Hollands et al., 2008; Wu et al., 2002). All these authors detected anthocyanin metabolites in the form of methylated or glucuronidated conjugates, or sulfoconjugates. Seeram et al. (2004) detected ellagic acid in human plasma within 4 h after consuming pomegranate juice containing 25 mg of ellagic acid and 318 mg of ellagitannins. Maximum level (31.9 ng mL^{-1} of ellagic acid) was detected after 1 h of consumption.

Espín et al. (2007) found that ellagitannins were hydrolyzed to ellagic acid by the intestinal flora, and then metabolized into urolithin A, B, C, and D by the intestinal flora in

Iberian pig model. Although some studies have shown how anthocyanins and ellagic acid are absorbed and metabolized and how much of them is bioavailable *in vivo*, there is still need of further studies for better understanding of absorption, metabolism, and bioavailability of phenolic compounds in berries.

Anti-inflammatory activities

Inflammation

Inflammation is a body's response to cell damage and vascularized tissues (Kim et al., 2012) with symptoms of redness, edema, fever, pain, and loss of function (Kim et al., 2004). Inflammation is a complex process that involves numbers of proteins, cytokines, and other metabolites (Fig. 1-6). When the inflammatory process is triggered by stimuli such as cytokines, thrombin, or reactive oxygen species (Bogatcheva et al., 2005), serum proteins and leukocytes (neutrophils, eosinophils, and macrophages) move from the blood to the extra-vascular tissues (Santangelo et al., 2007). The inflammatory process is complex but self-limiting so it can prevent severe damage to the host itself. However, when the self-limiting nature is not properly working, the inflammatory response becomes chronic, which can be a cause of many diseases including rheumatoid arthritis, atopic dermatitis, cancers, or Alzheimer's disease (Kim et al., 2004; Wang et al., 2008).

Arachidonic acid pathway

Arachidonic acid (AA) is a polyunsaturated fatty acid derived from linoleic acid by the enzyme phospholipase A₂ (Calder, 2011). AA is released from phospholipids in cell membrane and can be metabolized to prostaglandins (PGs), which are important mediators of inflammatory response, via cyclooxygenase (COX) pathway (Calder, 2011; Yoon and Baek, 2005). COX is present in two major forms (COX-1 and COX-2) and one variant (COX-3). COX-1 is constitutively expressed in various tissues while COX-2 is considered an inducible enzyme significantly induced by inflammatory stimuli such as carrageenan (Seibert et al., 1994). In the presence of inflammatory stimuli, COX-2 is induced and converts AA into PGs and thromboxane A₂ (Yoon and Baek, 2005). COX-2 is closely associated with inflammatory responses (Kim et al., 2004) such as inducing synthesis of interleukin-6, a pro-inflammatory cytokine (St-Jacques and Ma, 2011). Prostaglandin E₂ (PGE₂) produced by COX-2 is involved in inflammation. Zhang et al. (1997) treated carrageenan to induce rat paw inflammation and reported that PGE₂ was rapidly produced in induced tissue and PGE₂ played an important role in hyperalgesia. PGs can cause symptoms such as vasodilation, enhanced blood flow, erythema, fluid exudation from the vasculature, and pain during inflammation (Simon, 1999; Weinberg, 2000). In addition, it was shown that COX-2 increased in the site of inflammation and temporarily correlated with inflammation. Therefore, suppressing COX-2 and PGE₂ can be very important to prevent and/or suppress inflammatory process.

Nitric oxide and inducible nitric oxide synthase

Nitric oxide (NO) is an important signaling molecule synthesized from L-arginine by nitric oxide synthase (NOS) (Vuong et al., 2006) and involved in various physiological and cellular responses (Conforti and Menichini, 2011). NO can easily diffuse in and out of cells, or between cellular compartments because of its small size (30 Da) and absence of charge, and is considered radical due to an unpaired electron carried (Coleman, 2002).

NOS is present in three isoforms, endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). iNOS is rapidly expressed by inflammatory stimuli such as lipopolysaccharide (LPS) and interferon- γ in some type of cells including macrophages while the other two are constitutively expressed (Conforti and Menichini, 2011; Kim et al., 2004; Vuong et al., 2006). NO can be beneficial for homeostasis (Nathan, 1992), but when it is overproduced by iNOS or inappropriately regulated, it can contribute to various diseases including cancer, diabetes, obesity, and hypertension (Conforti and Menichini, 2011; Gallo et al., 1998).

Tazawa et al. (2013) reported that NO converted human colonic adenoma cells into adenocarcinoma cells. However, when the normal adenoma cells were treated with the iNOS-inhibiting agent aminoguanidine, tumor formation was significantly inhibited. Gallo et al. (1998) found that higher levels of total NOS and iNOS occurred in tumor specimens than in normal mucosa specimens from patients with head and neck cancer. If lymph nodes metastasis was involved, total NOS activity was higher and more vascularization was found. As seen above, regulating iNOS-induced NO and agents capable of reducing iNOS activity

can play an important role in anti-inflammatory mechanism and inflammation-related diseases.

Anti-inflammatory activity of phenolic compounds

There are over 8,000 structural variants of phenolic compounds from simple molecules such as phenolic acids to highly polymerized forms such as condensed tannins (Conforti and Menichini, 2011). However, the bioactivity of phenolic compounds like free radical scavenging or metal chelating capacity is attributed to *para*-hydroxyl groups in their structure. Phenolic compounds are thought to interact with a variety of molecular targets and be involved in cell signaling process (Santangelo et al., 2007). Among phenolic compounds, the most widely distributed group is flavonoids (Conforti and Menichini, 2011), and various phenolic compounds have been studied for their anti-inflammatory activities. It is generally thought that phenolic compounds such as flavonoids possess antioxidant activity by scavenging radicals, and they can regulate cellular responses of the inflammation-related cells such as mast cells and macrophages (Kim et al., 2004). Moreover, some phenolic compounds have shown inhibitory capacity of enzymes involved in inflammation such as phospholipase A₂, COX, or NOS.

Kim et al. (2012) have shown that corn bran extract inhibited NO production and iNOS expression in LPS-induced RAW 264.7 macrophages. The authors indicated that the phenolic compounds contained in corn bran extract such as *p*-coumaric acid and ferulic acid

contributed to the inhibition of NO production and iNOS expression. Schreckinger et al. (2010) analyzed anti-inflammatory activity of *Vaccinium floribundum* (commonly known as mortiño) and *Aristotelia chilensis* (maqui berries). They reported that an phenolic-rich extract, which was fractionated using Amberlite XAD-7 column to remove free sugars, pectins, and phenolic acids, of *V. floribundum*, anthocyanin-enriched fraction of *A. chilensis*, and proanthocyanidin-enriched fraction of both berries significantly reduced NO production in LPS-treated RAW 264.7 macrophage cells compared to LPS-treated control. These two berry extracts also inhibited PGE₂ production. Extracts of six blackberry genotypes, including four wild berries, one commercial cultivar, and one breeding line grown and collected from Mexico, showed anti-inflammatory activities by reducing NO and PGE₂ production and inhibiting expression of iNOS and COX-2 (Cuevas-Rodríguez et al., 2010). These effects varied with genotype and concentration of extracts.

Wang et al. (2008) analyzed anti-inflammatory activity of cyanidin 3-glucoside in THP-1 macrophages and found that iNOS and COX-2 were suppressed at both mRNA and protein levels by cyanidin 3-glucoside treatment. In another study, various phenolic standards including phenolic acids, catechin, flavonols, isoflavones, and anthocyanins were tested for their anti-inflammatory activity in RAW 264.7 macrophages (Wang and Mazza, 2002). Anti-inflammatory activity varied with type of phenolic compounds. Some phenolic compounds such as gallic acid, kaempferol, quercetin, daidzein, and genistein inhibited NO production in a dose-dependent manner while chlorogenic acid did not show an inhibitory effect. Glycosylation of flavonols and isoflavones decreased anti-inflammatory activity. Anthocyanins also inhibited NO production in a dose-dependent manner but glycosylation

decreased the inhibitory effect. Concentrated extracts of Saskatoon berries, blueberries, blackberries, and black currants also inhibited NO production.

Factors affecting phenolic compounds content in berries

Genetics

Phenolic composition and content of fruits can be influenced by genetic differences. Cho et al. (2004) found that anthocyanins in red grapes, blueberries, and blackberries differed in profiles and relative amounts. Blackberries had a very simple profile, consisting primarily of cyanidin 3-glucoside, while anthocyanin profile of blueberries and red grapes was more complex. Petunidin 3-glucoside, malvidin 3-glucoside, and malvidin 3-(*p*-coumaroyl)glucoside were found in red grapes, while delphinidin 3-galactoside, petunidin 3-galactoside, and malvidin 3-galactoside were found in blueberries. Borges et al. (2010) reported delphinidin 3-rutinoside and cyanidin 3-rutinoside in black currants, malvidin 3-galactoside and malvidin 3-arabinoside in blueberries, cyanidin 3-sophoroside in raspberries, cyanidin 3-rutinoside and cyanidin 3-(2''-xylosyl) rutinoside in red currants, and peonidin 3-galactoside in cranberries as the major anthocyanins.

Flavonol composition also differs among fruits, particularly in sugar moiety. Quercetin 3-galactoside was found in all three fruits with myricetin 3-galactoside and myricetin 3-glucoside in red grapes, and quercetin 3-glucoside and quercetin 3-rutinoside in blueberries and blackberries (Cho et al., 2004). Total anthocyanin and flavonol contents in red grapes,

blueberries, and blackberries were 380.9-7904.7 and 21.0-322.2, 1435.2-8227.3 and 172.5-327.5, and 1143.9-2415.4 and 102.0-160.2 mg kg⁻¹ FW, respectively.

Within one type crop, phenolic composition can vary greatly among varieties. Although cyanidin 3-glucoside is the predominant anthocyanin in blackberries, cyanidin 3-glucoside content varies with cultivars. Cyanidin 3-glucoside and cyanidin 3-xyloside contents in ‘Apache’, ‘Kiowa’, and ‘Navaho’ blackberries were higher than in ‘Chickasaw’ blackberries (Cho et al., 2004). In contrast, cyanidin 3-rutinoside content in ‘Chickasaw’ was higher than in the other cultivars. Fan-Chiang and Wrolstad (2005) reported that cyanidin 3-glucoside composition calculated as percentage of total peak area in blackberries ranged from 43.6 to 95.2% depending on varieties.

Environmental conditions

Phenolic content can also be affected by environmental conditions during cultivation. Temperature can have a significant impact on phenolic content. ‘Earliglow’ and ‘Kent’ strawberries grown under 30/22°C (day/night) in growth chambers contained higher levels of *p*-coumaroyl glucose, kaempferol 3-glucoside, kaempferol 3-glucuronide, cyanidin 3-glucoside, pelargonidin 3-glucoside, cyanidin 3-glucoside-succinate, and pelargonidin 3-glucoside-succinate compared to other temperature regimes (18/22, 25/12, and 25/22°C) (Wang and Zheng, 2001). Quercetin 3-glucoside, quercetin 3-glucuronide, and pelargonidin 3-rutinoside contents in ‘Kent’ strawberries and dihydroflavonol content in ‘Earliglow’

strawberries increased as the day and night temperature increased. Bilberries grown at 12 or 18°C also differed in levels of phenolic compounds (Uleberg et al., 2012). Epicatechin and catechin contents were higher in the berries grown at 12°C but total anthocyanin content was higher when the plants were grown at 18°C. Total amounts of ten anthocyanins out of the 13 analyzed were also higher in the berries grown at 18°C. However, chlorogenic acid and hydroxycinnamic acid derivatives were lower at 18°C.

Water stress can also affect phenolic content. Strawberries (cvs ‘Maehyang’ and ‘Seolhyang’) grown under water stress (from 94 days after transplant) showed increased anthocyanin and ellagic acid contents in an early stage of being stressed compared to the control (Kim et al., 2009).

Cultural practices

Cultural practices, such as fertilization or mulch application, can affect phenolic content. Quercetin, kaempferol, and ellagic acid contents in ‘Bounty’ strawberries were highest at the fertilization level of 0.6 mS cm⁻¹ (electrical conductivity of the fertilization solution) and slightly decreased as the strength of the solution increased (1.2 or 2.4 mS cm⁻¹) (Anttonen et al., 2006). They also investigated the effects of mulch color and shading. Total phenolic and anthocyanin, and ellagic acid contents in ‘Korona’ strawberries grown with brown mulch were higher than those grown with white mulch. Shading affected the total anthocyanin

content but not total phenolic and ellagic acid contents. Total anthocyanin content was higher in unshaded berries than in 32% shading treatment.

There are studies on phenolic contents in organically grown berries compared to conventionally grown ones. However, whether organically grown produces contain higher levels of phenolic compounds compared to conventionally grown produces is inconclusive. You et al. (2011) reported that total phenolic content in organically grown 'Powder blue' and 'Climax' blueberries was higher than in conventionally grown berries. Total anthocyanin content in 'Powder blue' was higher when grown organically but that in 'Climax' was not different between cultural practices. However, cyanidin 3-galactoside and quercetin 3-glucose contents analyzed by HPLC-MS were higher in organically grown berries of both cultivars. Jin et al. (2011) analyzed total anthocyanin and phenolic contents in strawberries after storage at 0, 5, and 10°C. Total anthocyanin and phenolic contents in both 'Earliglow' and 'Allstar' strawberries were higher in organically grown berries at all of storage conditions, and those in organically grown unstored berries were also higher with the exception of total phenolic content in 'Allstar' strawberries. 'Bluecrop' blueberries grown in five organic and five conventional farms were analyzed for phenolic content, and phenolic compounds including chlorogenic acid, myricetin 3-arabinoside, and quercetin 3-glucoside, and anthocyanins of delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, and delphinidin 3-arabinoside were higher in organically grown berries. Total phenolic content in frozen and air-dried marionberries and strawberries was higher when grown organically, but that in freeze-dried strawberries was not different between organic and conventional production (Asami et al., 2003). Häkkinen and Törrönen (2000) analyzed

phenolic compounds in organically and conventionally grown 'Polka', 'Jonsok', and 'Honeoye' strawberries. Kaempferol and ellagic acid contents in organically grown 'Jonsok' strawberries were higher compared to conventionally grown strawberries, but not in the other cultivars. Quercetin and *p*-coumaric acid contents were not significantly different between two cultural practices in all three cultivars.

Developmental stages

Fruits undergo physicochemical changes during ripening. Wang and Lin (2000) reported that total anthocyanin content in blackberries, black- and red raspberries, and strawberries increased as the fruit matured. In contrast, total phenolic content in blackberries and strawberries decreased with fruit ripening. However, that in raspberries decreased from green to pink stage and then increased in ripe fruit. It was also reported that total anthocyanin content increased as fruit matured from semi-ripe to ripe, and then to slightly over-ripe in 'Tulameen' raspberries (Krüger et al., 2011). But total phenolic content did not significantly change.

Individual compounds change differently with fruit maturation. In strawberries, *p*-coumaric acid content increased as fruit matured from green to pink, and then to red (Kosar et al., 2004). However, *p*-hydroxybenzoic acid content changed differently among genotypes, and ellagic acid content generally decreased at red stage except two genotypes out of total 19 investigated. Cyanidin 3-glucoside and pelargonidin 3-glucoside contents increased at the red

stage in all genotypes. Ellagitannins (lambertianin C and sanguin H-6), flavonols (quercetin), and ellagic acid derivatives contents decreased with fruit maturation, in contrast to increased cyanidin 3-glucoside and cyanidin 3-(6'-malonyl)glucoside contents in tropical highland blackberries (Acosta-Montoya et al., 2010).

Differences among fruit tissues

Kim et al. (2010) reported that the total anthocyanin content in 'Maehyang', 'Seolhyang', 'Keumhyang', and 'Akihime' strawberries was the highest in the epidermis (29-74 mg 100 g⁻¹ FW) followed by achenes (15-40 mg 100 g⁻¹ FW) and flesh (below 30 mg 100 g⁻¹ FW). Ellagic acid content was the highest in achenes. Aaby et al. (2005) also reported higher level of total phenolic, ellagic acid and derivative, ellagitannins, catechin, and flavonol contents in achenes. Anthocyanin profiles and quantity varied within fruit tissues. Cyanidin 3-glucoside content was higher and pelargonidin 3-glucoside content was lower in achenes than in flesh, but pelargonidin 3-rutinoside and cyanidin 3-glucoside-malonate were not detected in the achenes and flesh, respectively.

In blackberries, ellagitannins are present in seeds (pyrenes), torus, and flesh, with the highest content in the seeds (Hager et al., 2008). Some ellagitannins such as castalagin/vescalagin isomers, lambertianin C isomer, and ellagic acid were found only in the seeds. In comparison, gallic acid, catechin, and epicatechin contents in 'Chardonnay' and 'Merlot' grapes were 15 and 10, 358 and 127, and 421 and 115 mg 100 g⁻¹ DW in seeds

while those in skin were 5 and 3, 60 and 16, and 44 and 13 mg 100 g⁻¹ DW, respectively (Yilmaz and Toledo, 2004).

Postharvest handling

Storage

Fruit continue to respire after harvest, consequently metabolic changes occur during storage. Total anthocyanin content in strawberries and raspberries increased during storage at 0, 10, 20, and 30°C, with greater increase at 20 or 30°C than lower temperatures (Kalt et al., 1999). Krüger et al. (2011) reported that total anthocyanin and phenolic contents in red raspberries increased after storage at either 20°C for one day or at 2-4°C for three days and at 20°C for the next day. Total phenolic, anthocyanin, and flavonol contents in 17 Saskatoon berry genotypes changed depending on cultivar during storage at -20°C for 9 months (Bakowska-Barczak and Kolodziejczyk, 2008). However, total hydroxycinnamic acid and procyanidin contents generally decreased after storage. Total anthocyanin and phenolic contents in three grapes also changed during frozen storage at -18°C depending on cultivar (García et al., 2011).

Processing

Berry fruit are used as ingredients of various processed products, and processing can change the resulting phenolic content. Ellagic acid content in strawberry jam was lower than in unprocessed strawberries (Häkkinen et al., 2000), and grapes used in jelly or raisins were reduced in total phenolic and anthocyanin contents (Rababah et al., 2012). After the drying process, using either whole or ground raisins did not change these levels in resulting jams. Total phenolic content in 'Marion' and 'Evergreen' blackberries increased in freeze-dried berries but significantly decreased in canned-in sucrose syrup or jammed blackberries compared to frozen control (-23°C) (Wu et al., 2010). However, total anthocyanin content decreased in all processing treatments except freeze-dried 'Evergreen' blackberries. Ellagitannin content in 'Apache' blackberries increased in canned-in-syrup or water treatment, maintained when individually quick frozen or pureed, and decreased when made as nonclarified or clarified juice (Hager et al., 2010).

In summary, research on the bioactive compounds in blackberries continues to grow. In general, levels of phenolic compounds such as anthocyanins and flavonols can vary as a result of location, climate, cultivar, ripeness stage, and production practices including organic production. Savvy consumers are keen to gather as much information on the health benefits of all that they eat, including blackberries. This study is part of a larger project that is trying to elucidate the health benefits of organically produced blackberries.

Literature cited

- Aaby, K., G. Skrede, and R.E. Wrolstad. 2005. Phenolic composition and antioxidant activities in flesh and achenes of strawberries (*Fragaria ananassa*). J. Agric. Food Chem. 53:4032-4040.
- Acosta-Montoya, O., F. Vaillant, S. Cozzano, C. Mertz, A.M. Pérez, and M.V. Castro. 2010. Phenolic content and antioxidant capacity of tropical highland blackberry (*Rubus adenotrichus* Schltdl.) during three edible maturity stages. Food Chem. 119:1497-1501.
- AgMRC. 2012. Blackberries.
http://www.agmrc.org/commodities__products/fruits/blackberries/
- Andersen, Ø .M. and K.R. Markham, 2005. Flavonoids: Chemistry, biochemistry and applications. CRC Press, Boca Raton, FL.
- Anttonen, M.J., K.I. Hoppula, R. Nestby, M.J. Verheul, and R.O. Karjalainen. 2006. Influence of fertilization, mulch color, early forcing, fruit order, planting date, shading, growing environment, and genotype on the contents of selected phenolics in strawberry (*Fragaria x ananassa* Duch.) fruits. J. Agric. Food Chem. 54:2614-2620.
- Asami, D.K., Y.J. Hong, D.M. Barrett, and A.E. Mitchell. 2003. Comparison of the total phenolic and ascorbic acid content of freeze-dried and air-dried marionberry, strawberry, and corn grown using conventional, organic, and sustainable agricultural practices. J. Agric. Food Chem. 51:1237-1241.
- Bakowska-Barczak, A.M. and P. Kolodziejczyk. 2008. Evaluation of Saskatoon berry (*Amelanchier alnifolia* Nutt.) cultivars for their polyphenol content, antioxidant properties, and storage stability. J. Agric. Food Chem. 56:9933-9940.

- Barros, D., O.B. Amaral, I. Izquierdo, L. Geracitano, M. Raseira, A.T. Henriques, and M.R. Ramirez. 2006. Behavioral and genoprotective effects of *Vaccinium* berries intake in mice. *Pharmacol. Biochem. Behav.* 84:229-234.
- Bogatcheva, N.V., M.G. Sergeeva, S.M. Dudek, and A.D. Verin. 2005. Arachidonic acid cascade in endothelial pathobiology. *Microvasc. Res.* 69:107-127.
- Borges, G., A. Degenève, W. Mullen, and A. Crozier. 2010. Identification of flavonoid and phenolic antioxidants in black currants, blueberries, raspberries, red currants, and cranberries. *J. Agric. Food Chem.* 58:3901-3909.
- Bornsek, S.M., L. Ziberna, T. Polak, A. Vanzo, N.P. Ulrih, V. Abram, F. Tramer, and S. Passamonti. 2012. Bilberry and blueberry anthocyanins act as powerful intracellular antioxidants in mammalian cells. *Food Chem.* 134:1878-1884.
- Bradish, C.M., P. Perkins-Veazie, G.E. Fernandez, G.X. Xie, and W. Jia. 2012. Comparison of flavonoid composition of red raspberries (*Rubus idaeus* L.) grown in the Southern United States. *J. Agric. Food Chem.* 60:5779-5786.
- Bravo, L. 1998. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* 56:317-333.
- Burdon, J.N. and R. Sexton. 1993. Fruit abscission and ethylene production of four blackberry cultivars (*Rubus* spp.). *Ann. Appl. Biol.* 123:121-132.
- Bushway, L., M. Pritts, and D. Handley, 2008. Raspberry and blackberry production guide for the Northeast, Midwest, and Eastern Canada. Natural Resource, Agriculture, and Engineering Service, Ithaca.

- Calder, P.C. 2011. Fatty acids and inflammation: The cutting edge between food and pharma. *Eur. J. Pharmacol.* 668:S50-S58.
- Castañeda-Ovando, A., M.D. Pacheco-Hernández, M.E. Páez-Hernández, J.A. Rodríguez, and C.A. Galán-Vidal. 2009. Chemical studies of anthocyanins: A review. *Food Chem.* 113:859-871.
- Cho, M.J., L.R. Howard, R.L. Prior, and J.R. Clark. 2004. Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* 84:1771-1782.
- Cho, M.J., L.R. Howard, R.L. Prior, and J.R. Clark. 2005. Flavonol glycosides and antioxidant capacity of various blackberry and blueberry genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* 85:2149-2158.
- Clark, J.R., J.N. Moore, J. Lopez-Medina, C. Finn, and P. Perkins-Veazie. 2005. 'Prime-Jan' ('APF-8') and Prime-Jim' ('APF-12') primocane-fruited blackberries. *HortScience* 40:852-855.
- Clark, J.R. and P. Perkins-Veazie. 2011. 'APF-45' Primocane-fruited blackberry. *HortScience* 46:670-673.
- Clifford, M.N. 2000. Anthocyanins - Nature, occurrence and dietary burden. *J. Sci. Food Agric.* 80:1063-1072.
- Clifford, M.N. and A. Scalbert. 2000. Ellagitannins - Nature, occurrence and dietary burden. *J. Sci. Food Agric.* 80:1118-1125.

- Coleman, J.W. 2002. Nitric oxide: A regulator of mast cell activation and mast cell-mediated inflammation. *Clin. Exp. Immunol.* 129:4-10.
- Conforti, F. and F. Menichini. 2011. Phenolic compounds from plants as nitric oxide production inhibitors. *Curr. Med. Chem.* 18:1137-1145.
- Cuevas-Rodríguez, E.O., V.P. Dia, G.G. Yousef, P.A. García-Saucedo, J. López-Medina, O. Paredes-López, E.G. De Mejia, and M.A. Lila. 2010. Inhibition of pro-inflammatory responses and antioxidant capacity of Mexican blackberry (*Rubus* spp.) extracts. *J. Agric. Food Chem.* 58:9542-9548.
- Dai, J. and R.J. Mumper. 2010. Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules* 15:7313-7352.
- Ding, M., R.T. Feng, S.Y. Wang, L. Bowman, Y.J. Lu, Y. Qian, V. Castranova, B.H. Jiang, and X.L. Shi. 2006. Cyanidin-3-glucoside, a natural product derived from blackberry, exhibits chemopreventive and chemotherapeutic activity. *J. Biol. Chem.* 281:17359-17368.
- Espín, J.C., R. Gonzalez-Barrio, B. Cerdá, C. López-Bote, A.I. Rey, and F.A. Tomás-Barberán. 2007. Iberian pig as a model to clarify obscure points in the bioavailability and metabolism of ellagitannins in humans. *J. Agric. Food Chem.* 55:10476-10485.
- Fan-Chiang, H.J. and R.E. Wrolstad. 2005. Anthocyanin pigment composition of blackberries. *J. Food Sci.* 70:C198-C202.
- Fan-Chiang, H.J. and R.E. Wrolstad. 2010. Sugar and nonvolatile acid composition of blackberries. *J. AOAC Int.* 93:956-965.

Felgines, C., S. Talavera, O. Texier, A. Gil-Izquierdo, J.L. Lamaison, and C. Remesy. 2005.

Blackberry anthocyanins are mainly recovered from urine as methylated and glucuronidated conjugates in humans. *J. Agric. Food Chem.* 53:7721-7727.

Felgines, C., O. Texier, C. Besson, D. Fraisse, J.L. Lamaison, and C. Rémésy. 2002.

Blackberry anthocyanins are slightly bioavailable in rats. *J. Nutr.* 132:1249-1253.

Gallo, O., E. Masini, L. Morbidelli, A. Franchi, I. Fini-Storchi, W.A. Vergari, and M. Ziche.

1998. Role of nitric oxide in angiogenesis and tumor progression in head and neck cancer. *J. Natl. Cancer Inst.* 90:587-596.

Galvano, F., L. La Fauci, G. Lazzarino, V. Fogliano, A. Ritieni, S. Ciappellano, N.C.

Battistini, B. Tavazzi, and G. Galvano. 2004. Cyanidins: metabolism and biological properties. *J. Nutr. Biochem.* 15:2-11.

García, S., L.G. Santesteban, C. Miranda, and J.B. Royo. 2011. Variety and storage time

affect the compositional changes that occur in grape samples after frozen storage. *Aust. J. Grape Wine Res.* 17:162-168.

Goyarzu, F., D.H. Malin, F.C. Lau, G. Taglialatela, W.D. Moon, R. Jennings, E. Moy, D.

Moy, S. Lippold, B. Shukitt-Hale, and J.A. Joseph. 2004. Blueberry supplemented diet: Effects on object recognition memory and nuclear factor-kappa B levels in aged rats.

Nutr. Neurosci. 7:75-83.

Gu, L.W., M.A. Kelm, J.F. Hammerstone, G. Beecher, J. Holden, D. Haytowitz, S. Gebhardt,

and R.L. Prior. 2004. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *J. Nutr.* 134:613-617.

- Häkkinen, S.H., S.O. Kärenlampi, H.M. Mykkänen, I.M. Heinonen, and A.R. Törrönen. 2000. Ellagic acid content in berries: Influence of domestic processing and storage. *Eur. Food Res. Technol.* 212:75-80.
- Häkkinen, S.H. and A.R. Törrönen. 2000. Content of flavonols and selected phenolic acids in strawberries and *Vaccinium* species: Influence of cultivar, cultivation site and technique. *Food Res. Int.* 33:517-524.
- Hager, T.J., L.R. Howard, R. Liyanage, J.O. Lay, and R.L. Prior. 2008. Ellagitannin composition of blackberry as determined by HPLC-ESI-MS and MALDI-TOF-MS. *J. Agric. Food Chem.* 56:661-669.
- Hager, T.J., L.R. Howard, and R.L. Prior. 2010. Processing and storage effects on the ellagitannin composition of processed blackberry products. *J. Agric. Food Chem.* 58:11749-11754.
- Halliwell, B. 1996. Antioxidants in human health and disease. *Annu. Rev. Nutr.* 16:33-50.
- Herderich, M.J. and P.A. Smith. 2005. Analysis of grape and wine tannins: Methods, applications and challenges. *Aust. J. Grape Wine Res.* 11:205-214.
- Hollands, W., G.M. Brett, J.R. Dainty, B. Teucher, and P.A. Kroon. 2008. Urinary excretion of strawberry anthocyanins is dose dependent for physiological oral doses of fresh fruit. *Mol. Nutr. Food Res.* 52:1097-1105.
- Hollman, P.C.H. 2001. Evidence for health benefits of plant phenols: Local or systemic effects? *J. Sci. Food Agric.* 81:842-852.
- Jennings, D.L., 1988. Raspberries and blackberries: Their breeding, diseases and growth. Academic Press, San Diego.

- Jin, P., S.Y. Wang, C.Y. Wang, and Y.H. Zheng. 2011. Effect of cultural system and storage temperature on antioxidant capacity and phenolic compounds in strawberries. *Food Chem.* 124:262-270.
- Kafkas, E., M. Koşar, N. Türemiş, and K.H.C. Başer. 2006. Analysis of sugars, organic acids and vitamin C contents of blackberry genotypes from Turkey. *Food Chem.* 97:732-736.
- Kalt, W., C.F. Forney, A. Martin, and R.L. Prior. 1999. Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. *J. Agric. Food Chem.* 47:4638-4644.
- Karakaya, S. 2004. Bioavailability of phenolic compounds. *Crit. Rev. Food Sci. Nutr.* 44:453-464.
- Katsube, N., K. Iwashita, T. Tsushida, K. Yamaki, and M. Kobori. 2003. Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. *J. Agric. Food Chem.* 51:68-75.
- Kaume, L., L.R. Howard, and L. Devareddy. 2012. The blackberry fruit: A review on its composition and chemistry, metabolism and bioavailability, and health benefits. *J. Agric. Food Chem.* 60:5716-5727.
- Kay, C.D., G. Mazza, B.J. Holub, and J. Wang. 2004. Anthocyanin metabolites in human urine and serum. *Br. J. Nutr.* 91:933-942.
- Khoo, G.M., M.R. Clausen, H.L. Pedersen, and E. Larsen. 2012. Bioactivity and chemical composition of blackcurrant (*Ribes nigrum*) cultivars with and without pesticide treatment. *Food Chem.* 132:1214-1220.

- Kim, E.O., K.J. Min, T.K. Kwon, B.H. Um, R.A. Moreau, and S.W. Choi. 2012. Anti-inflammatory activity of hydroxycinnamic acid derivatives isolated from corn bran in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Food Chem. Toxicol.* 50:1309-1316.
- Kim, H.P., K.H. Son, H.W. Chang, and S.S. Kang. 2004. Anti-inflammatory plant flavonoids and cellular action mechanisms. *J. Pharmacol. Sci.* 96:229-245.
- Kim, S.K., R.N. Bae, H. Hwang, M.J. Kim, H.R. Sung, and C. Chun. 2010. Comparison of bioactive compounds contents in different fruit tissues of June-bearing strawberry cultivars. *Korean J. Hortic. Sci. Technol.* 28:948-953.
- Kim, S.K., H.Y. Na, J.H. Song, M.J. Kim, J.E. Son, R.N. Bae, C. Chun, and H.J. Kang, 2009. Influence of water stress on fruit quality and yield of strawberry cvs. 'Maehyang' and 'Seolhyang', p. 177-180. In: LopezMedina, J. (ed.), *Vi International Strawberry Symposium*. Int Soc Horticultural Science, Leuven 1.
- Kong, J.M., L.S. Chia, N.K. Goh, T.F. Chia, and R. Brouillard. 2003. Analysis and biological activities of anthocyanins. *Phytochemistry* 64:923-933.
- Kosar, M., E. Kafkas, S. Paydas, and K.H.C. Baser. 2004. Phenolic composition of strawberry genotypes at different maturation stages. *J. Agric. Food Chem.* 52:1586-1589.
- Krüger, E., H. Dietrich, E. Schöpplein, S. Rasim, and P. Kürbel. 2011. Cultivar, storage conditions and ripening effects on physical and chemical qualities of red raspberry fruit. *Postharvest Biol. Technol.* 60:31-37.

- Liu, M., X.Q. Li, C. Weber, C.Y. Lee, J. Brown, and R.H. Liu. 2002. Antioxidant and antiproliferative activities of raspberries. *J. Agric. Food Chem.* 50:2926-2930.
- Manach, C., A. Scalbert, C. Morand, C. Rémésy, and L. Jiménez. 2004. Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr.* 79:727-747.
- Marinova, D. and F. Ribarova. 2007. HPLC determination of carotenoids in Bulgarian berries. *J. Food Compos. Anal.* 20:370-374.
- McGhie, T.K., G.D. Ainge, L.E. Barnett, J.M. Cooney, and D.J. Jensen. 2003. Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolized by both humans and rats. *J. Agric. Food Chem.* 51:4539-4548.
- McGhie, T.K. and M.C. Walton. 2007. The bioavailability and absorption of anthocyanins: Towards a better understanding. *Mol. Nutr. Food Res.* 51:702-713.
- Mertz, C., V. Cheynier, Z. Günata, and P. Brat. 2007. Analysis of phenolic compounds in two blackberry species (*Rubus glaucus* and *Rubus adenotrichus*) by high-performance liquid chromatography with diode array detection and electrospray ion trap mass spectrometry. *J. Agric. Food Chem.* 55:8616-8624.
- Miranda-Rottmann, S., A.A. Aspillaga, D.D. Pérez, L. Vasquez, A.L.F. Martinez, and F. Leighton. 2002. Juice and phenolic fractions of the berry *Aristotelia chilensis* inhibit LDL oxidation *in vitro* and protect human endothelial cells against oxidative stress. *J. Agric. Food Chem.* 50:7542-7547.
- NASS. 2010. 2007 Census of agriculture - Organic production survey (2008). USDA, Washington D.C.

- Nathan, C. 1992. Nitric oxide as a secretory product of mammalian cells. *FASEB J.* 6:3051-3064.
- NOSB, U. 1995. Oct. 30. 2013. <http://www.nal.usda.gov/afsic/pubs/ofp/ofp.shtml>
- OTA. 2011. U.S. organic industry valued at nearly \$29 billion in 2010. Mar. 30. 2013. http://www.organicnewsroom.com/2011/04/us_organic_industry_valued_at.html
- OTA. 2012. Consumer-driven U.S. organic market surpasses \$31 billion in 2011. Nov.5. 2013. http://www.organicnewsroom.com/2012/04/us_consumerdriven_organic_mark.html
- Paredes-López, O., M.L. Cervantes-Ceja, M. Vigna-Pérez, and T. Hernández-Pérez. 2010. Berries: Improving human health and healthy aging, and promoting quality life - A review. *Plant Food Hum. Nutr.* 65:299-308.
- Perkins-Veazie, P., J.K. Collins, and J.R. Clark. 1996. Cultivar and maturity affect postharvest quality of fruit from erect blackberries. *HortScience* 31:258-261.
- Perkins-Veazie, P., J.K. Collins, and J.R. Clark. 1999. Shelf-life and quality of 'Navaho' and 'Shawnee' blackberry fruit stored under retail storage conditions. *J. Food Qual.* 22:535-544.
- Pinto, M.D., Y.I. Kwon, E. Apostolidis, F.M. Lajolo, M.I. Genovese, and K. Shetty. 2010. Evaluation of red currants (*Ribes rubrum* L.), black currants (*Ribes nigrum* L.), red and green gooseberries (*Ribes uva-crispa*) for potential management of type 2 diabetes and hypertension using *in vitro* models. *J. Food Biochem.* 34:639-660.

- Rababah, T.M., M. Al-u'datt, A. Almajwal, S. Brewer, H. Feng, M. Al-Mahasneh, K. Ereifej, and W.D. Yang. 2012. Evaluation of the nutraceutical, physiochemical and sensory properties of raisin jam. *J. Food Sci.* 77:C609-C613.
- Robbins, R.J. 2003. Phenolic acids in foods: An overview of analytical methodology. *J. Agric. Food Chem.* 51:2866-2887.
- Santangelo, C., R. Varì, B. Scazzocchio, R.D. Benedetto, C. Filesi, and R. Masella. 2007. Polyphenols, intracellular signalling and inflammation. *Ann. Ist. Super. Sanità* 4:394-405.
- Scalbert, A. and G. Williamson. 2000. Dietary intake and bioavailability of polyphenols. *J. Nutr.* 130:2073S-2085S.
- Schreckinger, M.E., J.Z. Wang, G. Yousef, M.A. Lila, and E.G. de Mejia. 2010. Antioxidant capacity and in vitro Inhibition of adipogenesis and inflammation by phenolic extracts of *Vaccinium floribundum* and *Aristotelia chilensis*. *J. Agric. Food Chem.* 58:8966-8976.
- Seeram, N.P. 2008. Berry fruits for cancer prevention: Current status and future prospects. *J. Agric. Food Chem.* 56:630-635.
- Seeram, N.P., L.S. Adams, Y.J. Zhang, R. Lee, D. Sand, H.S. Scheuller, and D. Heber. 2006. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* 54:9329-9339.
- Seeram, N.P., R. Lee, and D. Heber. 2004. Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice. *Clin. Chim. Acta* 348:63-68.

- Seibert, K., Y. Zhang, K. Leahy, S. Hauser, J. Masferrer, W. Perkins, L. Lee, and P. Isakson. 1994. Pharmacological and biochemical demonstration of the role of cyclooxygenase-2 in inflammation and pain. *Proc. Natl. Acad. Sci. U.S.A.* 91:12013-12017.
- Shahidi, F. and M. Naczk, 2004. *Phenolics in food and nutraceuticals*. CRC Press, Boca Raton, Florida.
- Shukitt-Hale, B., V. Cheng, and J.A. Joseph. 2009. Effects of blackberries on motor and cognitive function in aged rats. *Nutr. Neurosci.* 12:135-140.
- Simon, L.S. 1999. Role and regulation of cyclooxygenase-2 during inflammation. *Amer. J. Med.* 106:37S-42S.
- Siriwoharn, T. and R.E. Wrolstad. 2004. Polyphenolic composition of Marion and Evergreen blackberries. *J. Food Sci.* 69:C233-C240.
- Slatnar, A., J. Jakopic, F. Stampar, R. Veberic, and P. Jamnik. 2012. The effect of bioactive compounds on *in vitro* and *in vivo* antioxidant activity of different berry juices. *PLoS One* 7:8.
- Springob, K., J. Nakajima, M. Yamazaki, and K. Saito. 2003. Recent advances in the biosynthesis and accumulation of anthocyanins. *Nat. Prod. Rep.* 20:288-303.
- St-Jacques, B. and W.Y. Ma. 2011. Role of prostaglandin E2 in the synthesis of the pro-inflammatory cytokine interleukin-6 in primary sensory neurons: An *in vivo* and *in vitro* study. *J. Neurochem.* 118:841-854.
- Stewart, H., J. Hyman, J.C. Buzby, E. Frazão, and A. Carlson. 2011. *How Much Do Fruits and Vegetables Cost?* . USDA.

- Strik, B.C., J.R. Clark, C.E. Finn, and M.P. Bañados. 2007. Worldwide blackberry production. *HortTechnology* 17:205-213.
- Strik, B.C., J.R. Clark, C.E. Finn, and M.P. Bañados. 2008. Worldwide production of blackberries. *Acta Hort.*:209-217.
- Sun, J. and R.H. Liu. 2006. Cranberry phytochemical extracts induce cell cycle arrest and apoptosis in human MCF-7 breast cancer cells. *Cancer Lett.* 241:124-134.
- Szajdek, A. and E.J. Borowska. 2008. Bioactive compounds and health-promoting properties of berry fruits: A review. *Plant Food Hum. Nutr.* 63:147-156.
- Tazawa, H., T. Kawaguchi, T. Kobayashi, Y. Kuramitsu, S. Wada, Y. Satomi, H. Nishino, M. Kobayashi, Y. Kanda, M. Osaki, T. Kitagawa, M. Hosokawa, and F. Okada. 2013. Chronic inflammation-derived nitric oxide causes conversion of human colonic adenoma cells into adenocarcinoma cells. *Exp. Cell Res.* 319:2835-2844.
- Tsuda, T., F. Horio, and T. Osawa. 1999. Absorption and metabolism of cyanidin 3-*O*- β -D-glucoside in rats. *FEBS Lett.* 449:179-182.
- Tsuda, T., F. Horio, and T. Osawa. 2000. The role of anthocyanins as an antioxidant under oxidative stress in rats. *Biofactors* 13:133-139.
- Uleberg, E., J. Rohloff, L. Jaakola, K. Trøst, O. Junttila, H. Häggman, and I. Martinussen. 2012. Effects of temperature and photoperiod on yield and chemical composition of Northern and Southern clones of bilberry (*Vaccinium myrtillus* L.). *J. Agric. Food Chem.* 60:10406-10414.
- USDA. 2012. 2011 Certified organic production survey. USDA.

- USDA. 2013a. Growth patterns in the U.S. organic industry. Jan.30. 2014.
<http://www.ers.usda.gov/amber-waves/2013-october/growth-patterns-in-the-us-organic-industry.aspx#.Uusjv5uPLIU>
- USDA. 2013b. Noncitrus fruits and nuts 2012 preliminary summary USDA, Washington D.C.
- USDA. 2013c. Organic production. Nov. 5 2013. 2013. <http://www.ers.usda.gov/data-products/organic-production.aspx#.UnkIP7D3RMs>
- USDA. 2013d. USDA National nutrient database for standard reference, release 26. Nov. 5. 2013. <http://www.ars.usda.gov/nutrientdata>
- USDA. 2014. National fruit and vegetable organic summary. USDA.
- Vuong, T., L. Martin, and C. Matar. 2006. Antioxidant activity of fermented berry juices and their effects on nitric oxide and tumor necrosis factor-alpha production in macrophages 264.7 gamma NO(-) cell line. *J. Food Biochem.* 30:249-268.
- Wang, J. and G. Mazza. 2002. Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN- γ -activated RAW 264.7 macrophages. *J. Agric. Food Chem.* 50:850-857.
- Wang, Q., M. Xia, C. Liu, H.H. Guo, Q.Y. Ye, Y. Hu, Y.H. Zhang, M.J. Hou, H.L. Zhu, J. Ma, and W.H. Ling. 2008. Cyanidin-3-O- β -glucoside inhibits NOS and COX-2 expression by inducing liver X receptor alpha activation in THP-1 macrophages. *Life Sci.* 83:176-184.

- Wang, S.Y. and H.S. Lin. 2000. Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *J. Agric. Food Chem.* 48:140-146.
- Wang, S.Y. and W. Zheng. 2001. Effect of plant growth temperature on antioxidant capacity in strawberry. *J. Agric. Food Chem.* 49:4977-4982.
- Weinberg, J.B. 2000. Nitric oxide synthase 2 and cyclooxygenase 2 interactions in inflammation. *Immunol. Res.* 22:319-341.
- WHO. 1999. Definition, diagnosis and classification of diabetes mellitus and its complications. WHO, Geneva, Switzerland.
- Wild, S., G. Roglic, A. Green, R. Sicree, and H. King. 2004. Global prevalence of diabetes - Estimates for the year 2000 and projections for 2030. *Diabetes Care* 27:1047-1053.
- Wu, R.Y., B. Frei, J.A. Kennedy, and Y.Y. Zhao. 2010. Effects of refrigerated storage and processing technologies on the bioactive compounds and antioxidant capacities of 'Marion' and 'Evergreen' blackberries. *LWT-Food Sci. Technol.* 43:1253-1264.
- Wu, X.L., G.R. Beecher, J.M. Holden, D.B. Haytowitz, S.E. Gebhardt, and R.L. Prior. 2006. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *J. Agric. Food Chem.* 54:4069-4075.
- Wu, X.L., G.H. Cao, and R.L. Prior. 2002. Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. *J. Nutr.* 132:1865-1871.
- Yilmaz, Y. and R.T. Toledo. 2004. Major flavonoids in grape seeds and skins: Antioxidant capacity of catechin, epicatechin, and gallic acid. *J. Agric. Food Chem.* 52:255-260.

- Yoon, J.H. and S.J. Baek. 2005. Molecular targets of dietary polyphenols with anti-inflammatory properties. *Yonsei Med. J.* 46:585-596.
- You, Q., F. Chen, X. Wang, Y.M. Jiang, and S.Y. Lin. 2012. Anti-diabetic activities of phenolic compounds in muscadine against alpha-glucosidase and pancreatic lipase. *LWT-Food Sci. Technol.* 46:164-168.
- You, Q., B.W. Wang, F. Chen, Z.L. Huang, X. Wang, and P.G. Luo. 2011. Comparison of anthocyanins and phenolics in organically and conventionally grown blueberries in selected cultivars. *Food Chem.* 125:201-208.
- Zadernowski, R., M. Naczek, and J. Nesterowicz. 2005. Phenolic acid profiles in some small berries. *J. Agric. Food Chem.* 53:2118-2124.
- Zhang, Y., A. Shaffer, J. Portanova, K. Seibert, and P.C. Isakson. 1997. Inhibition of cyclooxygenase-2 rapidly reverses inflammatory hyperalgesia and prostaglandin E₂ production. *J. Pharmacol. Expt. Ther.* 283:1069-1075.
- Zhao, Y., 2007. *Berry fruit: Value-added products for health promotion.* CRC Press, Boca Raton, FL.

Table 1-1. Common anthocyanins in plants (modified from Kong et al., 2003).

Name	Substitution pattern							Color
	3	5	6	7	3'	4'	5'	
Cyanidin	OH	OH	H	OH	OH	OH	H	Orange-red
Delphinidin	OH	OH	H	OH	OH	OH	OH	Bluish-red
Malvidin	OH	OH	H	OH	OMe	OH	OMe	Bluish-red
Pelargonidin	OH	OH	H	OH	H	OH	H	Orange
Peonidin	OH	OH	H	OH	OMe	OH	H	Orange-red
Petunidin	OH	OH	H	OH	OMe	OH	OH	Bluish-red



Fig. 1-1. Blackberry flowers.



Fig. 1-2. Blackberry fruit.

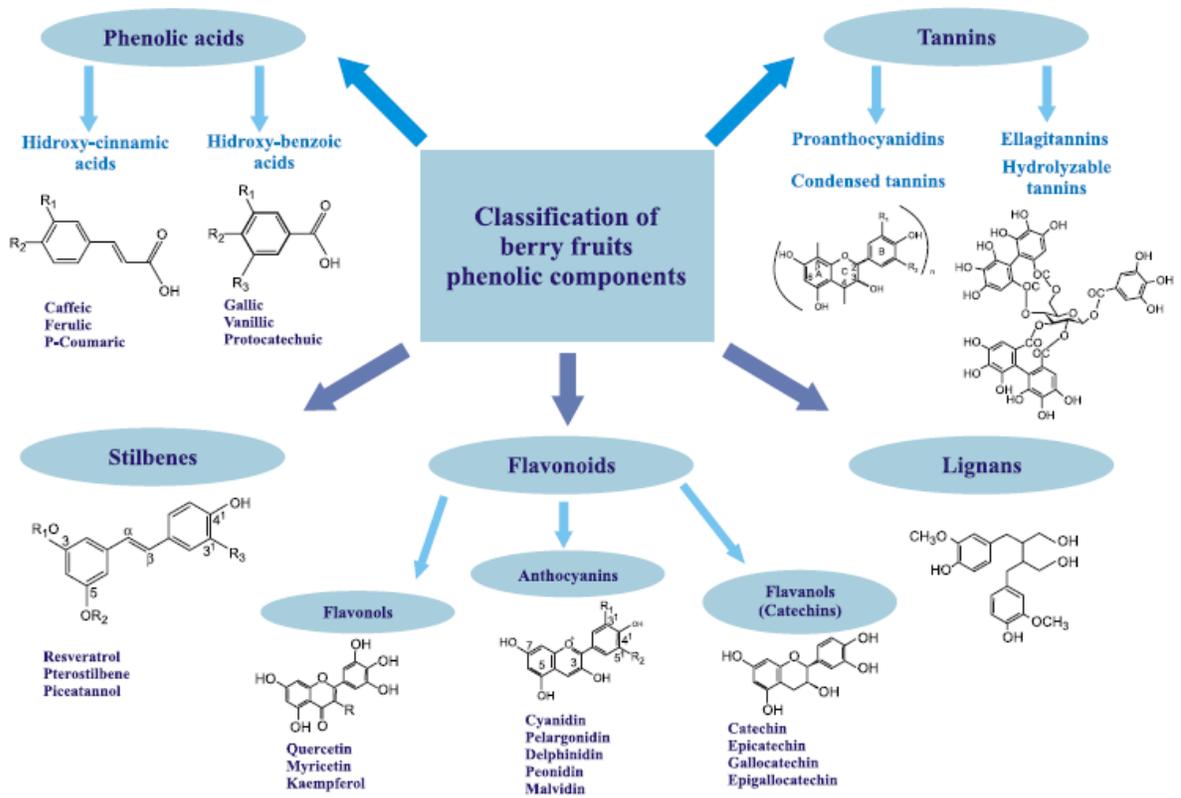


Fig. 1-3. Types of phenolic compounds in berries (From Paredes-López et al., 2010).

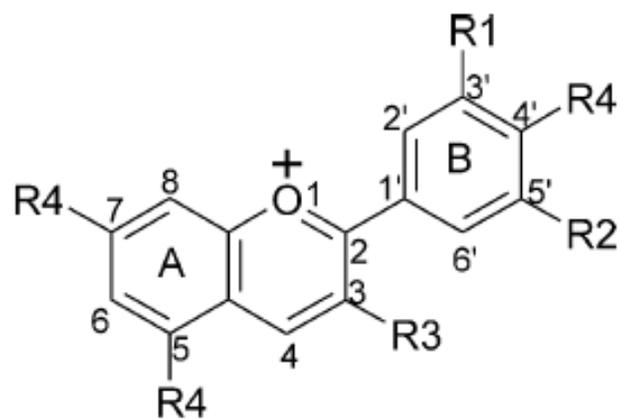


Fig. 1-4. Structure of anthocyanins (From Kong et al., 2003).

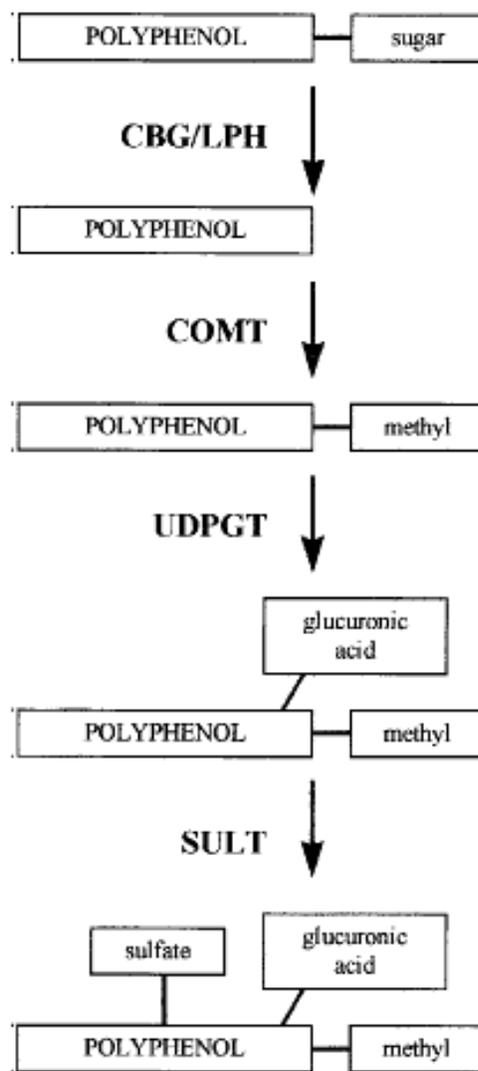


Fig. 1-5. Possible metabolism of phenolic compounds. CBG, cytosolic β -glucosidase; LPH, lactase phlorizin hydrolase; COMT, catechol-O-methyltransferase; UDPGT, glucuronosyl transferase; SULT, phenol sulfotransferases (From Scalbert and Williamson, 2000).

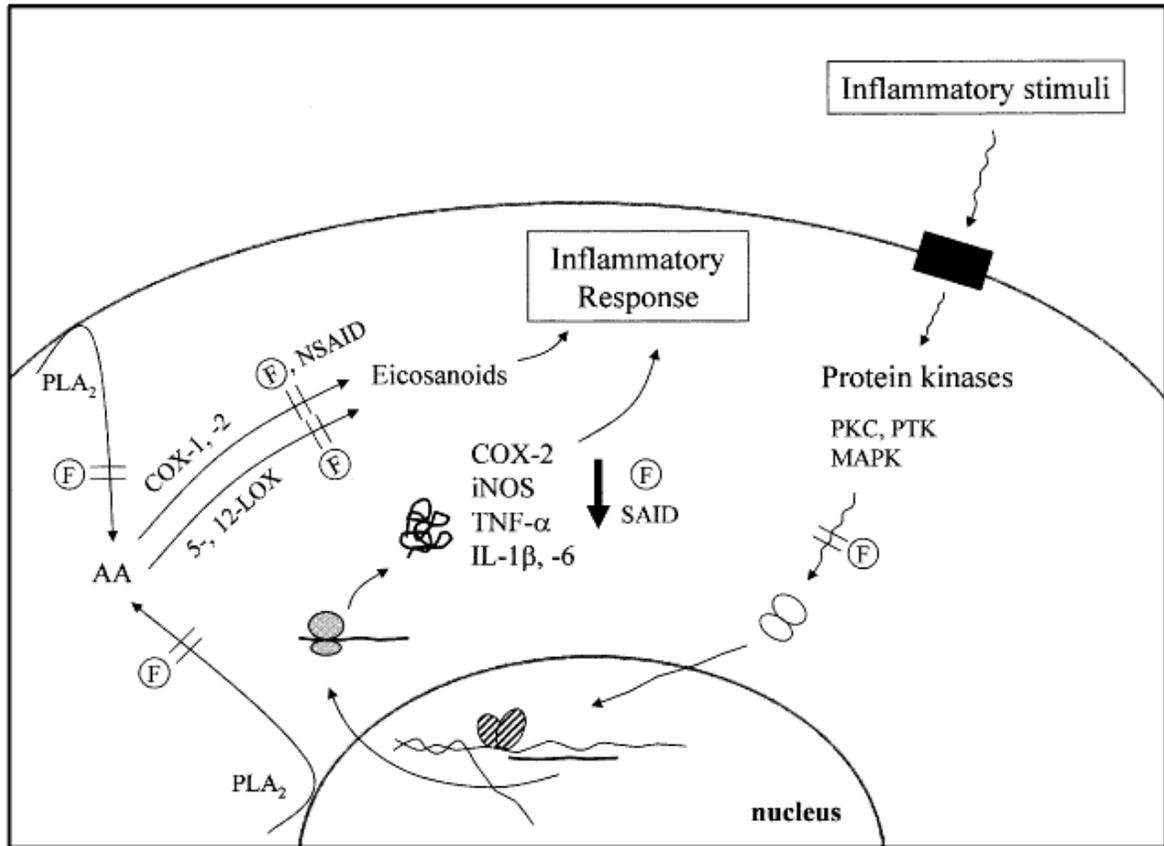


Fig 1-6. Proposed flavonoid effects on inflammatory responses. Flavonoid (F), nonsteroidal anti-inflammatory drug (NSAID), steroidal anti-inflammatory drug (SAID), “=” and “↓” denote enzyme inhibition and down-regulation of the expression, respectively (From Kim et al., 2004).

CHAPTER 2

Storage life and composition of organically grown blackberries

Abstract

Organically grown 'Natchez', 'Ouachita', and 'Navaho' blackberries at shiny black (SB) and dull black (DB) ripeness stages were evaluated for their storage life and composition after storage at 1°C for 15 days or at 1°C for 13 days followed by two days at 20°C. Weight loss was 2-4% in all treatments and significantly higher during the last two days of storage when fruit were exposed to 20°C. Leakage and decay incidence were higher and overall rating was lower in DB berries or when stored at 20°C for two days compared to SB berries or fruit stored constantly at 1°C. Soluble solids content and titratable acidity decreased after storage while pH increased after storage especially when fruit were stored at 20°C for two days with the exception of titratable acidity and pH of DB 'Navaho' berries. Total anthocyanin and phenolic contents of unstored berries were generally higher in 'Natchez' and 'Navaho' than in 'Ouachita'. Total anthocyanin content decreased after storage in 'Natchez' at both ripeness stages while that in 'Ouachita' and 'Navaho' only decreased in DB berries. Total phenolic content decreased after storage in all treatments except SB 'Ouachita' berries. This study established a baseline of postharvest attributes of organically grown 'Natchez', 'Ouachita', and 'Navaho' blackberries. We found that the postharvest attributes were affected by cultivar, ripeness stage, and storage condition. Fruit harvested at SB ripeness stage and stored constantly at 1°C showed better postharvest quality compared to DB fruit or fruit held

at 20°C for two days. Fruit composition was affected by cultivar and storage condition and soluble solids content, titratable acidity, and total anthocyanin and phenolic contents generally decreased after storage especially in berries exposed to 20°C for two days.

Introduction

Blackberries (*Rubus* sp.) are caneberries known for their healthful benefits. One hundred grams of blackberries provide 20 and 5% of recommended daily fiber and potassium, respectively (USDA, 2013b). Additionally, blackberry fruit contain phenolic compounds that have demonstrated activity against oxidation, cancers, inflammation, and diabetes (Bornsek et al., 2012; Cuevas-Rodríguez et al., 2010; Seeram et al., 2006; You et al., 2012).

Blackberries are grown worldwide with concentrated production in Europe and North America, and overall production has increased over decades (Strik et al., 2007; Strik et al., 2008). The US blackberry industry is dominated by Oregon, where the largest proportion of blackberries are used for processing (Strik et al., 2007). In other major blackberry producing states such as California, Texas, and Arkansas, semi-erect or erect cultivars are mainly cultivated with fresh market focus. In 2005, North Carolina was ranked as the 9th largest blackberry producer in the US (Strik et al., 2007), however, recent expansion of the industry has put North Carolina in the top 5 states in terms of acreage for fresh market blackberry production (G. Fernandez, personal communication). Organic berry production has also increased (USDA, 2013a) as the demand for organic foods increases (Zhao et al., 2006). A few studies on yield of organic blackberries have been done (Harkins et al., 2013; Lowe et al., 2012). Floricane-fruiting blackberry cultivar ‘Chickasaw’ and APF-77, primocane-fruiting selection, produced 8,826 and 6,665 kg ha⁻¹, respectively, in 2008 (Lowe et al., 2012).

Blackberries are highly perishable with at most three weeks of shelf life when held at

constant 2°C (Perkins-Veazie et al., 1999). The recommended storage conditions for blackberries is at 0-0.5°C and 90-95% relative humidity (Kader, 2002) and storage life is shortened by leakage, decay incidence, and fruit softening. Storage life of blackberry fruit depends on both storage temperature and cultivar. Perkins-Veazie et al. (1999) reported that ‘Shawnee’ blackberries had higher rate of decay incidence than ‘Navaho’ blackberries when stored at 2°C for 21 days or at 5°C for 14 days and decay incidence increased when fruit was exposed to 20°C for two days in both cultivars. Controlled atmosphere storage at 15 kPa CO₂ and 10 kPa O₂ for 7 days was helpful to reduce decay without off-flavor of ‘Arapaho’ and ‘Navaho’ blackberries (Perkins-Veazie and Collins, 2002).

Fruit composition are commonly taken at harvest for quality control purposes (pH, titratable acidity, and soluble solids content) and can change during storage. Perkins-Veazie et al. (1996) reported increased soluble solids content in ‘Choctaw’, ‘Cheyenne’, ‘Navaho’, and ‘Shawnee’ blackberries and higher titratable acidity in ‘Navaho’ blackberries after storage for 7 days at 2°C. Titratable acidity decreased in ‘Navaho’ and ‘Shawnee’ blackberries during storage at 2 or 5°C for 7 or 14 days and the effect was greater when the berries were exposed to 20°C for two days following low temperature storage (Perkins-Veazie et al., 1999). ‘Arapaho’ blackberries held under controlled atmosphere storage at 2°C had higher titratable acidity compared to those held at ambient atmosphere at 2°C for 3, 7, and 14 days (Perkins-Veazie and Collins, 2002).

Blackberries have been found to have high levels of phenolic compounds (Cho et al., 2004; Hager et al., 2008). Total anthocyanin and phenolic contents have been used as a

general baseline of the phenolic composition of fruit. Phenolic composition can vary with cultivars and storage conditions. Fan-Chiang and Wrolstad (2005) reported that total anthocyanin content ranged from 70.3 to 201 mg 100 g⁻¹ FW in 18 blackberry cultivars and 20 selections. Total anthocyanin and phenolic contents in ‘Marion’ blackberries were higher than in ‘Evergreen’ blackberries and decreased at the end of storage at 2°C for 7 or 9 days compared to those in unstored fruit.

Although a number of studies have been done on shelf life of berry fruits and berry phenolics, studies on storability and phenolic content of organically grown blackberries for fresh market uses are lacking. Therefore the objective of this study was to assess postharvest attributes of organically grown fresh market blackberries. Specifically we measured pH, titratable acidity, soluble solids content, and total anthocyanin and phenolic contents of three blackberry cultivars at harvest and after 15 days of storage. The storage conditions of berries were either held at a constant low temperature or mimicked retail conditions.

Materials and Methods

Plant materials

Freshly harvested fruit of ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries were purchased from a commercial, certified organic farm in Bunn, NC in 2011 and 2012. Blackberries were planted in 2008 and organically grown following the US organic standards. Fruit were harvested at the commercial ripe stage, where berries were firm and

either black with some reflection (shiny black, SB) or slightly riper and dull black (DB). Berries were transported on ice (5°C) to Kannapolis, NC (about 3 h), and sorted into SB and DB. Sorted berries were stored either at constant 1°C for 15 days (CL; constant low) or at 1°C for 13 days and at 20°C for the next two days (RT; retail) in 250 g vented clamshells (No. 1560, Southern Containers, Wilson, NC, USA). A minimum of three containers (replications) of each cultivar, ripeness stage, and storage condition were evaluated in this study.

Subjective ratings

Weight of each clamshell was measured at 0, 7, 13, and 15 days after storage to calculate weight loss. Leakage, decay, and red drupelets were measured as relative ratio of fruit with each characteristic to the total number of fruit. Fruit softness was evaluated by a 5 point scale where 1 was very firm and 5 was disintegrating. Berries in group 1-3 were considered marketable, and the percentage of those in group 4 and 5 was calculated for softness. An overall rating was obtained by subtracting the average of leakage, decay, and softness from 100 (Perkins-Veazie et al., 1999). Berries representing unstored (day 0) fruit were immediately frozen at -20°C after sorting and kept at -80°C until analysis. After subjective ratings, fruit without leakage and decay were kept at -80°C for further analysis. A minimum of three clamshells were used for this part of the study.

Soluble solids content and titratable acidity

For composition analysis, fruits were thawed at 4°C overnight and pureed using a

homogenizer (Polytron PT 10/35 GT, Kinematica, Inc., Bohemia, NY). Soluble solids content was measured as °Brix by placing 0.5 mL of puree onto a digital refractometer (P100, Atago USA Inc., Bellevue, WA). Titratable acidity was analyzed by mixing 2 g of puree with 60 mL water and titrated to a pH endpoint of 8.2 using an automated titrometer (862 Compact Titrosampler, Metrohm AG, Herisau, Switzerland) and 0.1 N NaOH, and calculated as %citric acid equivalent.

Total anthocyanin and phenolic content

For total anthocyanin and phenolic content analysis, 0.3 g of puree was extracted with 10 mL of cold extraction solvent (methanol:DH₂O:formic acid, 60:37:3, v/v/v). Samples were vortexed for 1 min and filtered through VWR qualitative 415 filter paper (VWR International, Randor, PA). Extracts were stored at -80°C until used for total anthocyanin and phenolic contents analysis. For the analysis, microplate spectrophotometer (PowerWave XS-BioTek Instruments, Inc., Winooski, VT) was used.

To determine total anthocyanin content, 30 µL of samples were added into a 96-well microtiter plate (PS-microplate, Greiner Bio-One North America Inc., Monroe, NC) in triplicate, and 120 µL of 0.25 M potassium chloride buffer (pH 1.0) was added to each well. Plates were covered with aluminum foil and gently shaken for 1 min and sample mixtures were incubated for 1 h at room temperature in the dark. Anthocyanins were detected at 510 and 700 nm and quantified as cyanidin 3-glucoside equivalent following the equation:

Total monomeric anthocyanin content (mg L^{-1}) = $((A \times \text{MW} \times \text{DF} \times 100))/((\epsilon \times 1))$

$A = A_{510 \text{ nm}} - A_{700 \text{ nm}}$, MW = 449.2, DF = dilution factor, and $\epsilon = 26,900$

For total phenolic content analysis, 20 μL of samples and 20 μL of 1.25 N Folin-Ciocalteu reagent were added into a 96-well microtiter plate in triplicate, and the plates were covered with aluminum foil and gently shaken for 1 min. After 3 min incubation at room temperature, 20 μL of 1N sodium carbonate was added into each well followed by 1 min of shaking and 6 min of incubation at room temperature. Finally, 120 μL of water was added, and plates were covered with aluminum foil and gently shaken for 1 min followed by 1 h of incubation at room temperature in the dark. Phenolic compounds were detected at 726 nm and standard curve of gallic acid was used for quantitation. Total phenolic content was calculated following the equation:

Total phenolic content (mg L^{-1}) = $A \times \text{slope of standard curve} \times \text{DF}$

$A = A_{726\text{nm}} - A_{\text{blank}}$ and DF = dilution factor

Statistical analysis

Data were subjected to univariate analysis of variance (ANOVA) (SAS 9.2, SAS institute, Cary, NC, USA), and means were separated using Tukey's honestly significant difference test (HSD) or Fisher's least significant difference (LSD) at 95% significance level.

Results and Discussion

Storability

Weight loss was 2-4% in all treatments (Fig. 2-1). In general, berries harvested at DB ripeness stage or stored at 20°C for the last two days of storage had higher weight loss than SB berries or those stored constantly at 1°C. Significantly higher weight loss occurred during the two days of storage at 20°C in all cultivars and both ripeness stages than those held constantly at 1°C. These results are in agreement with those of Perkins-Veazie et al. (1999) who reported higher weight loss in ‘Navaho’ and ‘Shawnee’ blackberries when exposed to 20°C for two days after low temperature storage at 2 or 5°C for 7, 14, or 21 days. Among cultivars, ‘Ouachita’ had lower weight loss than ‘Navaho’ in all treatments.

In general, leakage, decay, and red drupelet were lower and overall rating was higher in ‘Navaho’ except that of DB berries stored at 20°C for the last two days of storage (Table 2-1). There was no significant difference in softness among cultivars within ripeness stages and storage conditions. In all cultivars, SB berries had lower leakage and decay incidence and higher overall ratings in both storage conditions, with the exception of decay incidence of ‘Ouachita’ stored at 20°C for two days. Softness was higher in DB berries of all cultivars at both storage conditions except ‘Natchez’ berries stored constantly at 1°C. Results were similar to those of Perkins-Veazie et al. (1996), who stored conventionally grown ‘Choctaw’, ‘Cheynne’, ‘Navaho’, and ‘Shawnee’ blackberries at mottled, shiny black, and dull black ripeness stages at 2°C for 7 days. They reported that SB berries had significantly lower leakage and decay incidence than DB berries after 7 days of storage at 2°C. In contrast to

Perkins-Veazie et al. (1996), less red drupelet discoloration was found in this study. The cause of red discoloration of fresh blackberry drupelets is not fully understood although it is related to lower pH in frozen fruit (Jennings, 1988). Our samples did not show relationship between red drupelet incidence and titratable acidity or pH, but as discoloration usually affects less than 50% of the berry, and purees were made from affected and unaffected berries, possible acidity and pH effect may have been diluted.

When berries were exposed to room temperature for two days, more leakage and decay were observed and overall rating was lower than those held constantly at 1°C in all cultivars and both ripeness stages (Table 2-1). Red drupelet incidence was not affected by storage condition in ‘Natchez’ and ‘Navaho’ blackberries at either ripeness stages, but SB ‘Ouachita’ berries had more red drupelets when stored at 20°C for two days. These results were similar to those of Perkins-Veazie et al. (1999), who stored ‘Navaho’ and ‘Shawnee’ blackberries at 2 or 5°C for 7 or 14 days and at 20°C for the next two days or at 2°C for 21 days and at 20°C for the following two days. Berries from all storage temperature treatments had increased leakage, decay, and softness and decreased marketable fruits when exposed to 20°C, and the effects were greater for ‘Shawnee’, a cultivar with softer berries than ‘Navaho’. Our results also showed storage effects on postharvest quality and quality deterioration when the fruit were exposed to room temperature. Cultivar, ripeness stage, and storage treatment affected leakage, decay incidence, and overall rating, but only cultivar x year was significant for red drupelets, indicating that cultivar, ripeness stage, and storage treatment were not dependent on each other.

Soluble solids content, titratable acidity, and pH

There was no difference in soluble solids content, titratable acidity, and pH among cultivars in unstored SB berries while stored 'Navaho' was slightly higher in soluble solids content and titratable acidity and lower in pH than the other cultivars (Table 2-2). In unstored fruit, titratable acidity was higher and pH was lower in SB than in DB berries of all three cultivars while there was no difference in soluble solids content between ripeness stages in 'Ouachita' and 'Navaho' blackberries. However, soluble solids content was higher in DB 'Natchez' berries in both storage treatments and 'Navaho' DB berries stored at 1°C for 15 days. In contrast, higher titratable acidity and lower pH was observed in SB 'Ouachita' berries that were exposed to 20°C for two days. In 'Navaho', titratable acidity was higher in SB berries when stored constantly at 1°C. Perkins-Veazie et al. (1996) reported higher soluble solids content and lower titratable acidity in DB 'Navaho' berries than in SB berries. In 'Tulameen' raspberries, titratable acidity decreased as fruit ripened from semi-ripe to ripe and to slightly over-ripe while soluble solids content did not change during ripening (Krüger et al., 2011). Our samples also showed ripeness stage effects on soluble solids content, titratable acidity, and pH.

Soluble solids content decreased after storage in 'Natchez' and 'Ouachita' in both storage conditions while that of 'Navaho' decreased only in fruit held for two days at 20°C (Table 2-2). Titratable acidity decreased when fruit were exposed to 20°C for two days in all three cultivars except the highest level in DB 'Navaho' berries that were stored constantly at 1°C. As titratable acidity decreased, pH increased after storage in all cultivars with greater

increase in the berries exposed to 20°C for two days with the exception of DB ‘Navaho’ berries. Soluble solids content and titratable acidity in blackberries during storage have been reported in several studies and showed differences among cultivars and storage conditions. Perkins-Veazie and Collins (2002) reported decline of soluble solids content and titratable acidity in ‘Arapaho’ and ‘Navaho’ blackberries during 14 days of storage at 2°C. However, controlled atmosphere storage with the same storage temperature increased soluble solids content and titratable acidity. Decreased titratable acidity in ‘Navaho’ blackberries during 7 or 14 days of storage at 2 or 5°C was also reported and greater decrease was observed when the berries were exposed to 20°C for two days following low temperature storage (Perkins-Veazie et al., 1999). These authors also reported reduced sucrose and slightly increased glucose and fructose content after 7 or 14 days of storage at 2°C. Wu et al. (2010) reported that soluble solids content did not change but titratable acidity decreased during 7 days of storage at 2°C in ‘Evergreen’ blackberries and theorized that organic acids were used for respiration during storage, thus titratable acidity decreased. Our results showed reduction of soluble solids content and titratable acidity after storage and the effect was greater when berries were exposed to room temperature and could be explained by respiration during storage and higher respiration rate at warmer temperature. Soluble solids content, titratable acidity, and pH were affected by cultivar, ripeness stage, and storage treatment (Table 2-2). Interactions between cultivar and ripeness stage and cultivar and storage treatment were significant, indicating these three factors were dependent on each other. Unlike subjective rating, fruit composition was affected by year, possibly due to different environmental conditions during fruit production.

Total anthocyanin content

Although total anthocyanin content was generally higher in 'Natchez' than in 'Ouachita', differences between SB and DB fruit were not clear (Fig. 2-2). Total anthocyanin content in 'Natchez' stored at 20°C for the last two days of storage, unstored 'Ouachita', and 'Navaho' stored constantly at 1°C was higher in DB berries and there was no difference between ripeness stages in the other storage treatments. Although it is generally known that berries significantly accumulate anthocyanins during ripening from white and green to red and black (Acosta-Montoya et al., 2010; Pineli et al., 2011; Wang and Lin, 2000) and there is one day interval between SB and DB ripeness stages (Perkins-Veazie et al., 1996), blackberry fruit at both stages are commercial ripe and black in color and there was no significant anthocyanin accumulation. It seems that genetic factors may have greater influence on anthocyanin level than ripeness stage once the berries reach certain level of ripeness stage.

Total anthocyanin content in SB berries decreased in 'Natchez' but did not change in 'Ouachita' and 'Navaho' after storage (Fig. 2-2). However, DB berries of all three cultivars were reduced in total anthocyanin content after storage, with the exception of 'Navaho' stored constantly at 1°C. Perkins-Veazie and Collins (2002) reported increased total monomeric anthocyanin content in 'Navaho' blackberries but not changed in 'Arapaho' blackberries during storage at 2°C. However, both cultivars had decreased total anthocyanin content during controlled atmosphere storage with 15 kPa CO₂ and 10 kPa O₂ at 2°C. 'Marion' and 'Evergreen' blackberries stored at 2°C for 9 and 7 days, respectively, showed decreased total anthocyanin content at the end of storage compared to initial levels (Wu et al.,

2010). This reduction was thought to be from oxidative degradation of anthocyanins accelerated by increased pH. Kalt et al. (1999) reported anthocyanin increase in ‘Kent’ strawberries and ‘Nova’ raspberries during storage at 0, 10, 20, and 30°C for 8 days and theorized that the increase in anthocyanin content of stored raspberries and strawberries could be from use of organic acids for carbon skeletons for anthocyanin biosynthesis.

Total phenolic content

Total phenolic content was generally higher in ‘Natchez’ than in ‘Ouachita’ but was not different among cultivars in SB berries after storage (Fig. 2-3). SB berries of unstored ‘Natchez’ and ‘Navaho’, and ‘Ouachita’ stored at both conditions had higher levels of the total phenolic content than DB berries. Change in total phenolic content during maturation of berry fruit varies in the literature. Wang and Lin (2000) reported that the total phenolic content in ‘Chester Thornless’, ‘Hull Thornless’, and ‘Triple Crown’ blackberries decreased as fruit matured from green to pink and to ripe stage while in the black raspberry ‘Jewel’ and the red raspberries ‘Autumn Bliss’, ‘Canby’, ‘Sentry’, and ‘Summit’, total phenolic content decreased from green to pink and then increased with ripeness. There was no difference in the total phenolic content of ‘Tulameen’ raspberries among semi-ripe, ripe, and slightly over-ripe stages (Krüger et al., 2011). The difference might be due to genetic difference, extraction methods, and fruit composition. The Folin-Ciocalteu reagent, widely used to estimate total phenolics, is not specific to phenolic compounds (Huang et al., 2005). This reagent actually measures the reducing capacity of samples, so the results can reflect non-phenolic

compounds that can reduce the Folin-Ciocalteu reagent such as vitamin C or sugars (Huang et al., 2005; Prior et al., 2005).

Total phenolic content decreased after storage in all cultivars at both ripeness stages except for 'Ouachita' SB berries (Fig. 2-3). Wu et al. (2010) also reported decreased total phenolic content in 'Marion' and 'Evergreen' blackberries after storage at 2°C for 7 or 9 days. Total phenolic content decreased in 'Oso Grande' strawberries during storage at 1°C for 8 days while polyphenol oxidase activity increased (Nunes et al., 2005). In contrast, Kalt et al. (1999) observed that total phenolic content increased in raspberries during storage at 0, 10, 20, and 30°C for 8 days while that in strawberries and blueberries did not change. Krüger et al. (2011) reported increased total phenolic contents in 'Tulameen' raspberries after storage at 20°C for one day or at 2-4°C for three days followed by one day storage at 20°C. Kalt et al. (1999) discussed that the increase in the total phenolic content could be from loss of organic acids during storage as discussed above. Krüger et al. (2011) suggested that increased phenolic content might be related with the concentration effect due to weight loss while Nunes et al. (2005) discussed that water loss could have affected as stress so it might increase polyphenol oxidase activity and resulted in a decreased in total phenolic content. A more precise evaluation of phenolic profile is needed to better estimate storage and ripeness effects among cultivars.

Conclusion

In the present study, we investigated storage life and composition of organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries after storage at 1°C for 15 days or at 1°C for 13 days followed by two days at 20°C. Weight loss during last two days of storage was significantly higher in the fruit exposed to 20°C compared to the fruit stayed constantly at 1°C. Leakage and decay incidence were also higher and overall rating was lower when fruit were stored at 20°C for two days. Storability was generally better in ‘Navaho’ than in the other cultivars. Soluble solids content, titratable acidity, pH, and total anthocyanin and phenolic contents were influenced by genetic factors and storage. Soluble solids content and titratable acidity generally decreased during storage while pH increased especially in the fruit exposed to 20°C for two days. Total anthocyanin content was higher in ‘Natchez’ and ‘Navaho’ than in ‘Ouachita’ and decreased after storage with the exception of SB ‘Ouachita’ and ‘Navaho’ berries. Total phenolic content was also higher in ‘Natchez’ and ‘Navaho’ than in ‘Ouachita’ except stored SB berries. Stored berries were lower in the total phenolic content in both ripeness stages with the exception of SB ‘Ouachita’ berries. This research has established a baseline of postharvest attributes of organically grown blackberries. We have determined that storability and composition of organically grown blackberries were affected by cultivar and storage condition. Further work on storage conditions that can maintain postharvest quality and phenolic content is needed. Consumers should be advised that numerous changes occur in postharvest attributes of fruit, especially shelf life decrease once fruit is brought up to room temperature.

Literature Cited

- Acosta-Montoya, O., F. Vaillant, S. Cozzano, C. Mertz, A.M. Pérez, and M.V. Castro. 2010. Phenolic content and antioxidant capacity of tropical highland blackberry (*Rubus adenotrichus* Schlttdl.) during three edible maturity stages. *Food Chem.* 119:1497-1501.
- Bornsek, S.M., L. Ziberna, T. Polak, A. Vanzo, N.P. Ulrih, V. Abram, F. Tramer, and S. Passamonti. 2012. Bilberry and blueberry anthocyanins act as powerful intracellular antioxidants in mammalian cells. *Food Chem.* 134:1878-1884.
- Cho, M.J., L.R. Howard, R.L. Prior, and J.R. Clark. 2004. Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* 84:1771-1782.
- Cuevas-Rodríguez, E.O., V.P. Dia, G.G. Yousef, P.A. García-Saucedo, J. López-Medina, O. Paredes-López, E.G. De Mejia, and M.A. Lila. 2010. Inhibition of pro-inflammatory responses and antioxidant capacity of Mexican blackberry (*Rubus* spp.) extracts. *J. Agric. Food Chem.* 58:9542-9548.
- Fan-Chiang, H.J. and R.E. Wrolstad. 2005. Anthocyanin pigment composition of blackberries. *J. Food Sci.* 70:C198-C202.
- Hager, T.J., L.R. Howard, and R.L. Prior. 2008. Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed blackberry products. *J. Agric. Food Chem.* 56:689-695.
- Harkins, R.H., B.C. Strik, and D.R. Bryla. 2013. Weed management practices for organic production of trailing blackberry: I. Plant growth and early fruit production. *HortScience*

48:1139-1144.

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

Jennings, D.L., 1988. *Raspberries and blackberries: Their breeding, diseases and growth.* Academic Press, San Diego.

Kader, A.A., 2002. *Postharvest technology of horticultural crops*, 3rd Ed. Division of Agriculture and Natural Resources, University of California, Oakland.

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

Krüger, E., H. Dietrich, E. Schöpplein, S. Rasim, and P. Kürbel. 2011. Cultivar, storage conditions and ripening effects on physical and chemical qualities of red raspberry fruit. *Postharvest Biol. Technol.* 60:31-37.

Lowe, J.D., K.W. Pomper, S.B. Crabtree, J.R. Clark, and J.G. Strang. 2012. Yield characteristics of thorny primocane-fruiting blackberries from the University of Arkansas breeding program grown under organic growing conditions in Kentucky. *J. Amer. Pomolog. Soc.* 66:2-8.

Nunes, M.C.N., J.K. Brecht, A. Morais, and S.A. Sargent. 2005. Possible influences of water loss and polyphenol oxidase activity on anthocyanin content and discoloration in fresh ripe strawberry (cv. Oso Grande) during storage at 1 degrees C. *J. Food Sci.* 70:S79-S84.

Perkins-Veazie, P. and J.K. Collins. 2002. Quality of erect-type blackberry fruit after short intervals of controlled atmosphere storage. *Postharvest Biol. Technol.* 25:235-239.

- Perkins-Veazie, P., J.K. Collins, and J.R. Clark. 1996. Cultivar and maturity affect postharvest quality of fruit from erect blackberries. *HortScience* 31:258-261.
- Perkins-Veazie, P., J.K. Collins, and J.R. Clark. 1999. Shelf-life and quality of 'Navaho' and 'Shawnee' blackberry fruit stored under retail storage conditions. *J. Food Qual.* 22:535-544.
- Pineli, L.D.D., C.L. Moretti, M.S. dos Santos, A.B. Campos, A.V. Brasileiro, A.C. Córdova, and M.D. Chiarello. 2011. Antioxidants and other chemical and physical characteristics of two strawberry cultivars at different ripeness stages. *J. Food Compos. Anal.* 24:11-16.
- Prior, R.L., X.L. Wu, and K. Schaich. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* 53:4290-4302.
- Seeram, N.P., L.S. Adams, Y.J. Zhang, R. Lee, D. Sand, H.S. Scheuller, and D. Heber. 2006. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* 54:9329-9339.
- Strik, B.C., J.R. Clark, C.E. Finn, and M.P. Bañados. 2007. Worldwide blackberry production. *HortTechnology* 17:205-213.
- Strik, B.C., J.R. Clark, C.E. Finn, and M.P. Bañados. 2008. Worldwide production of blackberries. *Acta Hort.*:209-217.
- USDA. 2013a. Organic production. Nov. 5 2013. 2013. <http://www.ers.usda.gov/data-products/organic-production.aspx#.UnklP7D3RM>s
- USDA. 2013b. USDA National nutrient database for standard reference, release 26. Nov. 5.

2013. <http://www.ars.usda.gov/nutrientdata>

- Wang, S.Y. and H.S. Lin. 2000. Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *J. Agric. Food Chem.* 48:140-146.
- Wu, R.Y., B. Frei, J.A. Kennedy, and Y.Y. Zhao. 2010. Effects of refrigerated storage and processing technologies on the bioactive compounds and antioxidant capacities of 'Marion' and 'Evergreen' blackberries. *LWT-Food Sci. Technol.* 43:1253-1264.
- You, Q., F. Chen, X. Wang, Y.M. Jiang, and S.Y. Lin. 2012. Anti-diabetic activities of phenolic compounds in muscadine against alpha-glucosidase and pancreatic lipase. *LWT-Food Sci. Technol.* 46:164-168.
- Zhao, X., E.E. Carey, W.Q. Wang, and C.B. Rajashekar. 2006. Does organic production enhance phytochemical content of fruit and vegetables? Current knowledge and prospects for research. *HortTechnology* 16:449-456.

Table 2-1. Subjective ratings of organically grown shiny black and dull black ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries after storage at two temperature regimes, CL and RT, for 15 days.

Cultivars	Subjective ratings				
	Leakage	Decay	Red drupelets	Softness	Overall rating
Shiny black					
CL ^y					
Natchez	41.03a ^z	3.47b [*]	18.31a [*]	21.59a	77.97b [*]
Ouachita	35.21a [*]	9.85a [*]	5.93b [*]	23.98a [*]	76.99b [*]
Navaho	20.64b [*]	3.26b [*]	3.57b	23.19a	84.30a [*]
RT					
Natchez	77.24a [*]	47.18a [*]	21.69a [*]	23.26a	50.77b [*]
Ouachita	61.94b [*]	51.72a [*]	10.00b [*]	28.36a [*]	52.66b [*]
Navaho	44.11c [*]	30.16b [*]	2.10b	26.23a	66.50a [*]
Dull black					
CL					
Natchez	76.45a [*]	9.18b [*]	21.35a	41.78a [*]	57.53b [*]
Ouachita	63.98a [*]	25.11a [*]	9.42b	39.31a [*]	57.20b [*]
Navaho	48.17b [*]	12.05b [*]	2.40c [*]	37.40a [*]	67.46a [*]
RT					
Natchez	95.27a [*]	67.96a [*]	24.54a	53.44a [*]	27.77b [*]
Ouachita	83.32b [*]	62.31ab [*]	12.50b	53.01a [*]	33.79ab [*]
Navaho	76.15b [*]	52.08b [*]	4.73b [*]	48.88a [*]	40.97a [*]

Table 2-1 Continued

Significance					
Cultivar (C)	**	*	*	NS	**
Ripeness stage (R)	**	**	NS	**	**
Storage (S)	**	**	NS	**	**
Year (Y)	NS	NS	*	**	**
C x R	NS	NS	NS	NS	NS
C x S	NS	NS	NS	NS	NS
C x Y	NS	NS	*	NS	NS
R x S	NS	NS	NS	NS	NS
C x R x S	NS	NS	NS	NS	NS

^zMean separation within column in each ripeness stage and storage condition by HSD at $p < 0.05$.

Asterisk (*) indicates difference between storage conditions within each cultivar and ripeness stage by LSD at $p < 0.05$.

^yCL, constant low (1°C for 15 days); RT, retail (1°C for 13 days + 20°C for 2 days).

Table 2-2. Soluble solids content, titratable acidity, and pH of organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries after storage for 0 and 15 days at constant low (CL) and retail temperature (RT).

Storage condition ^y	Soluble solids content (°Brix)			Titratable acidity (%citric acid equivalent)			pH		
	Natchez	Ouachita	Navaho	Natchez	Ouachita	Navaho	Natchez	Ouachita	Navaho
Shiny black									
Day 0	10.36a	10.57a	10.47a	1.02a	1.04a	1.05a	3.67c	3.67c	3.65b
CL	9.26b	10.01b	10.13ab	0.95b	0.99b	1.05a	3.75b	3.75b	3.62b
RT	8.60c	9.31c	9.60b	0.85c	0.82c	0.90b	3.84a	3.92a	3.77a
Dull black									
Day 0	10.62a	10.54a	10.61a	0.95a	0.91a	0.89b	3.73b	3.77b	3.73a
CL	9.82b	10.08b	10.36a	0.92a	0.94a	0.98a	3.74b	3.75c	3.65b
RT	9.13c	9.35c	9.57b	0.86b	0.74b	0.90b	3.83a	3.96a	3.73a

Table 2-2 Continued

Significance			
Cultivar (C)	**	**	**
Ripeness stage (R)	**	**	**
Storage (S)	**	**	**
Year (Y)	*	**	**
C x R	*	**	**
C x S	**	**	**
C x Y	**	NS	**
R x S	NS	**	**
C x R x S	NS	*	*

^zMean separation within column in each ripeness stage and storage condition by HSD at $p < 0.05$.

^yDay 0, unstored; CL, constant low (1°C for 15 days); RT, retail (1°C for 13 days + 20°C for 2 days).

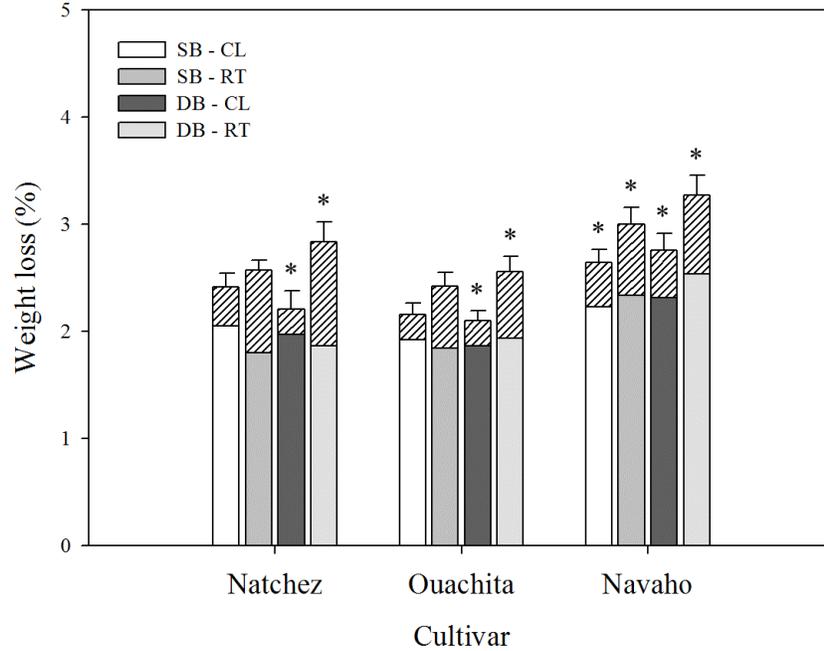


Fig. 2-1. Weight loss during storage. SB, shiny black ripeness stage; DB, dull black ripeness stage; CL, constant low (15 days at 1°C); RT, retail (13 days at 1°C + 2 days at 20°C); Non-patterned bars represent the weight loss after 13 days of storage and patterned bars represent the weight loss between day 13 and day 15. Asterisk (*) shows the significance between storage conditions for weight loss during 15 days of storage in each cultivar and ripeness stage separated by Fisher's LSD at $p < 0.05$. Weight loss during the last two days of storage was significantly higher in DB berries or berries held at 20°C.

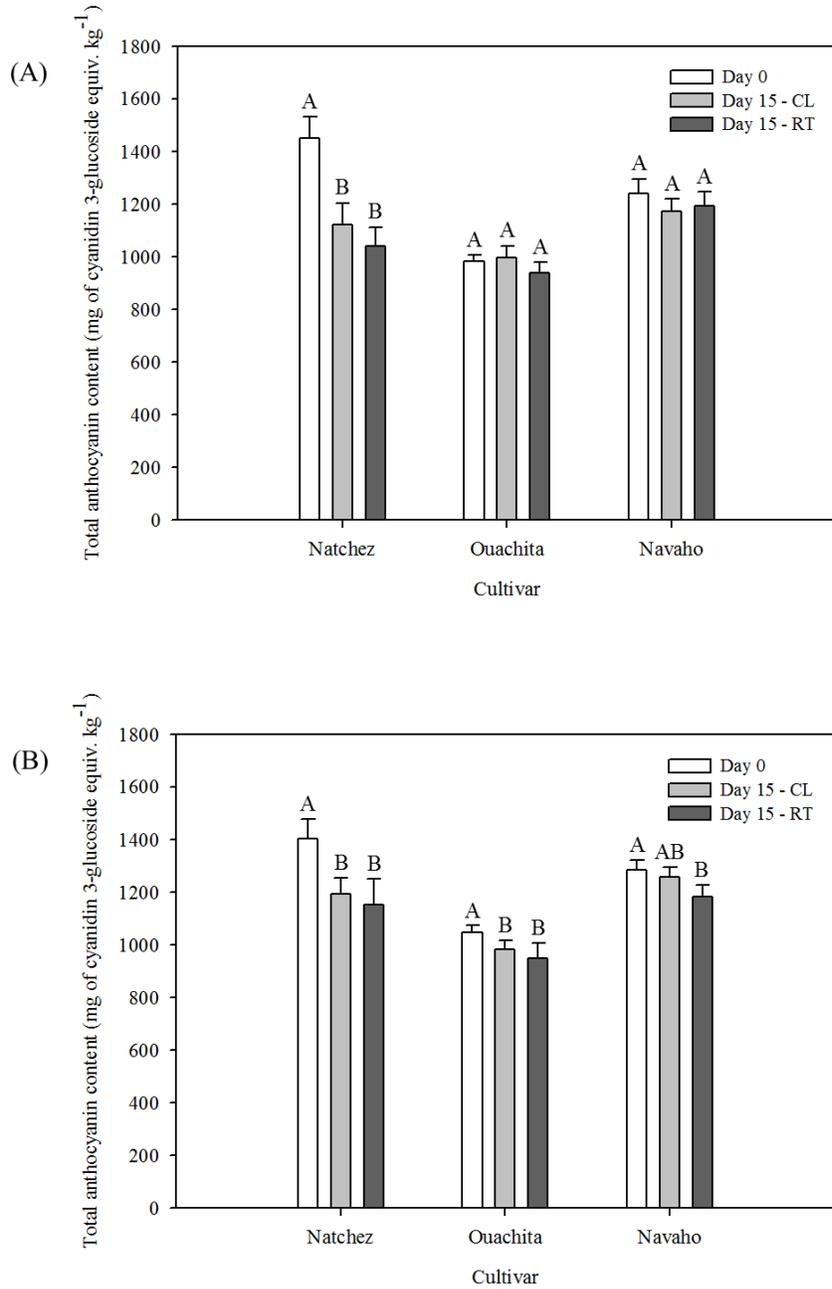


Fig. 2-2. Total anthocyanin content in organically grown blackberries. (A), shiny black ripeness stage; (B), dull black ripeness stage; Day 0, not stored; CL, constant low (15 days at 1°C); RT, retail (13 days at 1°C + 2 days at 20°C).

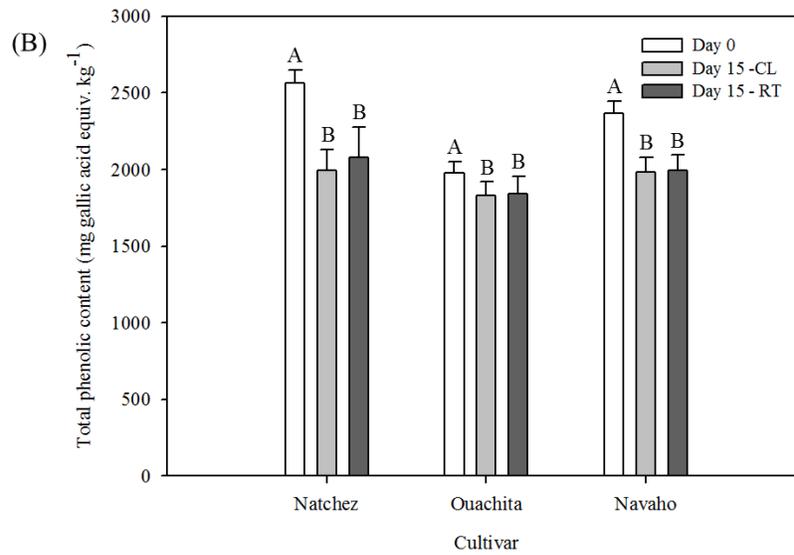
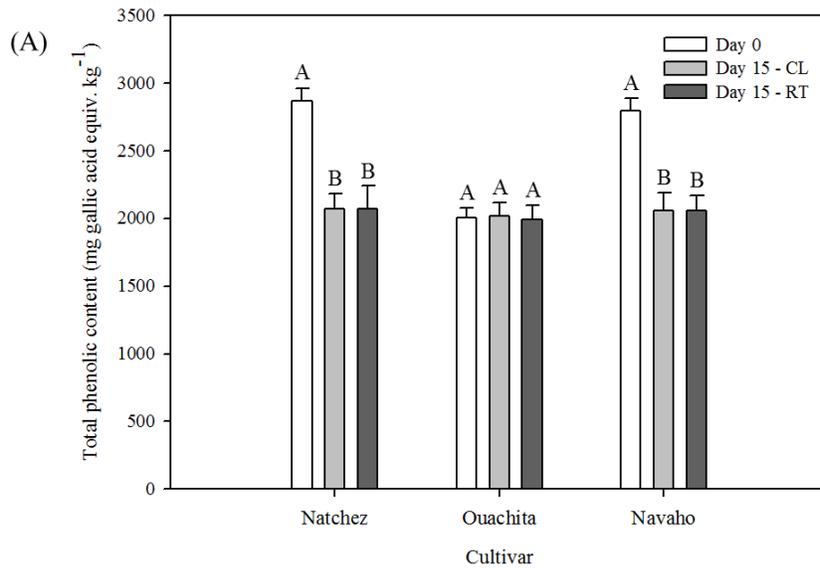


Fig. 2-3. Total phenolic content in organically grown blackberries. (A), shiny black ripeness stage; (B), dull black ripeness stage; Day 0, not stored; CL, constant low (15 days at 1°C); RT, retail (13 days at 1°C + 2 days at 20°C).

CHAPTER 3

Phenolic profile of organically grown blackberry cultivars at varying ripeness stages, and storage conditions

Abstract

Organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries at two stages of commercial ripeness were used to determine storage effects on anthocyanin and non-anthocyanin phenolic composition. Berries were stored at 1°C for 15 days or at 1°C for 13 days followed by two days at 20°C and composition was determined using HPLC. The anthocyanin pigments identified were cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-xyloside, and pelargonidin 3-glucoside, and cyanidin 3-glucoside was the predominant anthocyanin in all three blackberry cultivars at both ripeness stages and before and after storage. Gallic acid, vanillic acid, quercetin 3-galactoside, and quercetin 3-glucoside were identified as non-anthocyanin phenolic compounds and hydroxybenzoic acids were the major group of phenolic compounds. The content and proportion of cyanidin 3-xyloside and vanillic acid in ‘Natchez’ were significantly lower than in the other cultivars. Anthocyanin and phenolic contents generally increased after storage, especially in berries stored at 20°C for two days. Both anthocyanin and non-anthocyanin phenolics decreased in the riper dull black fruit compared to shiny black fruit. The results of this study indicate that phenolic composition was slightly affected by genotype in these fresh market cultivars. Some differences in relative amounts, but not profiles, were found after storage and effects were dependent on genotype.

Introduction

Demand for organically produced food continues to increase, and a recent study found that 81% of American families buy organically produced foods and growth is expected to continue at a rate of 14% for the next four years (Food Navigator USA, 2014). The Organic Trade Association (OTA) (2011) reported that the US organic industry was valued at about \$29 billion in 2010, and fruits and vegetables represented 39.7% of total organic food values. In 2011, the US organic market was \$31.5 billion in sales (OTA, 2012). According to the U.S. Department of Agriculture (USDA), organic berry production acreage has increased from 6,111 acres (2,546 ha) in 2006 to 12,796 acres (5,332 ha) in 2011 (USDA, 2013) in response to the expanding demand for organic foods (Zhao et al., 2006).

Domesticated blackberries (*Rubus* sp.) are grown worldwide with concentrated production in Europe and North America (about 15,000 ha) and new production in Asia and Central and South America (about 1,550 and 3,200 ha, respectively) (Strik et al., 2008). Blackberry production has increased steadily over last three decades (Strik et al., 2007). Oregon is the leading state of blackberry production in the US and over 90% of Oregon-produced blackberries is processed (Strik et al., 2007). North Carolina was the 9th largest blackberry producer in the US in 2005 (Strik et al., 2007) and it is in top 5 states for fresh market blackberry production these days (G. Fernandez, personal communication). Production of blackberries using organic systems in the US is of interest, with several studies focusing on yield (Harkins et al., 2013; Lowe et al., 2012).

Blackberries are consumed either fresh or as an ingredient of processed products (Türkben et al., 2010). Fresh market blackberry storage life is influenced by several factors such as storage temperature and cultivar. Blackberries are very perishable and shelf life is at best three weeks at temperatures below 5°C (Perkins-Veazie et al., 1999). It is recommended to store blackberries at 0-0.5°C and 90-95% relative humidity (Kader, 2002). Controlled atmosphere storage with 15 kPa CO₂ and 10 kPa O₂ for 7 days was helpful to reduce decay without off-flavor (Perkins-Veazie and Collins, 2002). Fresh market blackberries decrease in organic acids and sugar contents during storage, and the decrease is accelerated at warmer storage temperatures (Perkins-Veazie et al., 1996; Perkins-Veazie et al., 1999). Since the majority of blackberries are harvested and then shipped for commercial distribution, the consumer often eat fruit after 7-18 days in cold storage (P. Perkins-Veazie, personal communication). Once fruit arrives at the market, it is often put on display for 1-2 days and then sold, thus the cold chain is broken and fruit is stored on the shelf at conditions less than optimal.

Berry fruits, including blackberries, are rich in phenolic compounds such as anthocyanins and flavonols (Benvenuti et al., 2004; Moyer et al., 2002; Pantelidis et al., 2007). Phenolic compounds in several species of berries have been studied for their potential health benefits in cell and animal models. Phenolic extracts of black raspberries, blackberries, blueberries, cranberries, red raspberries, and strawberries have shown anticancer activity as measured by inhibition of cell proliferation or by induced apoptosis of cancer cells (Jeong et al., 2010; Seeram et al., 2006; Wang et al., 2008). Anti-inflammatory activity at the cell level was demonstrated by inhibition of pro-inflammatory responses

(Cuevas-Rodríguez et al., 2010; Jeong et al., 2010; Pergola et al., 2006; Wang and Mazza, 2002). The pigment cyanidin 3-glucoside extracted from blackberries was found to have chemopreventive and chemotherapeutic activities *in vitro* and *in vivo* by scavenging the hydroxyl radicals and superoxide anions induced by ultraviolet B or by inhibiting cell proliferation (Ding et al., 2006). Blackberries have also shown their protective effects against age-related change in cognitive function in an animal model using 19-21 month-old rats (Shukitt-Hale et al., 2009). Other small fruits, such as blueberries, are being tested in US human clinical trials or animal models for protective effect in cognitive function, and in various cancer and cardiovascular researches (Erlund et al., 2008; Joseph et al., 2003).

Total anthocyanin and phenolic contents are often used as general markers of the phenolic composition of fruit because they are simple and inexpensive assays. However, these tests do not provide information on the specific phenolic compounds present, or of the classes of phenolic compounds. When determined using HPLC or mass spectrometer, these profiles have been found to differ considerably among berry species and genotypes.

Total phenolic and anthocyanin content of berries can be affected by crop and storage conditions (Jin et al., 2011; Krüger et al., 2011; Türkben et al., 2010). Most of these studies were done on frozen processing types rather than commercially refrigerated fresh fruits. Poiana et al. (2010) stored blackberries (*R. fruticosus*) at -18°C for 10 months and reported decreased phenolic and anthocyanin contents during storage. It was reported that individual phenolic compounds, including ellagic acid, ferulic acid, caffeic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, and quercetin, differed among four blackberry cultivars (Türkben et al.,

2010). They also reported that phenolic contents except quercetin in just frozen (at -35°C for 5 h) or frozen (at -22°C for 6 months) samples were lower than in fresh berries and the effect was different among cultivars.

The objectives of this study were to determine the phenolic composition of three organically grown fresh market blackberry cultivars: 1) before and following cold storage (0 and 15 days), 2) at two cold storage regimes (at 1°C for 15 days or at 1°C for 13 days followed by two days at 20°C), and 3) of berries harvested at two ripeness stages, shiny black (SB) and dull black (DB).

Materials and Methods

Chemicals

Anthocyanins and phenolic standards were purchased from ChromaDex (Irvine, CA). HPLC grade methanol and formic acid were purchased from Fisher Scientific (Waltham, MA) and Sigma (St. Louis, MO), respectively.

Plant material and storage conditions

Freshly harvested organically grown fruit of ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries were purchased from a certified organic farm in Bunn, NC (USDA hardiness zone 7a) on three dates representing early, mid, and late season fruit, in June and July, 2012. The soil type was Vance sandy loam and Varina loamy sand. Plants were planted in 2008 and

organically grown following the US National Organic Standards. Fruit were harvested at the fresh market commercial ripe stage consisting of firm fruit of SB or DB appearance, and held at 5°C during transport to the laboratory in Kannapolis, NC (about 3 h). Berries were sorted into SB and DB ripeness stages, and samples representing day 0 (no storage) were immediately frozen at -20°C and then held at -80°C until analysis. Remaining berries were divided into two groups for storage, maintaining ripeness stage and cultivar integrity. One group was stored at 1°C for 15 days and the other group was stored at 1°C for 13 days and at 20°C for the next two days in 250 g vented plastic boxes (clamshells) (No. 1560, Southern Containers, Wilson, NC). The latter scenario represents the typical handling of fruit after it is harvested and sent to markets to be sold off the shelf.

Extraction for HPLC analysis

All samples were held at -80°C then freeze-dried using a VirTis LyoTroll (SP Scientific, Warminster, PA, USA). Freeze-dried blackberries were ground using a mortar and pestle, and pyrenes (seeds) were removed and a uniform particulate size was obtained by filtering powder through mesh sieves. Blackberry powders were sealed in plastic bags and kept at -80°C until analysis.

Blackberry powder was extracted following the method of Bradish et al. (2012) with slight modification. Briefly, 20 mg of blackberry powder were extracted with 1.5 mL of acidified methanol (formic acid:methanol:DH₂O, 1:60:39, v/v/v). Samples were vortexed and centrifuged at 10,600 g for 20 min at 4°C. All samples were re-extracted, as preliminary trials

indicated that 95-98% of phenolics were recovered with double extraction. Supernatants were combined and filtered through a 0.2 μm syringe filter into HPLC vials, and headspace was flushed with nitrogen gas to inhibit oxidation. All samples were kept at -80°C until analysis.

HPLC condition and quantitation

A high performance liquid chromatography system (Elite LaChrom, Hitachi Ltd., Tokyo, Japan) equipped with autosampler, diode array detector, and binary solvent delivery manager was used to analyze anthocyanins and phenolic compounds. D-2000 Elite software (Hitachi Ltd., Tokyo, Japan) was used. A reversed phase C18 column (Synergi 4 μ Hydro-RP 80 \AA , 250 X 4.6 mm Phenomenex, Torrance, CA, USA) was used with the column temperature of 30°C . Five percent formic acid (solvent A) and 100% methanol (solvent B) were used as mobile phases with the flow rate of 1 mL min^{-1} following gradient system: 0-5 min, 90% A; 5-15min, 85% A; 15-20 min, 80% A; 20-25 min, 75% A; 25-45 min, 70% A; 45-47 min, 40% A, 47-60 min, 90% A. Twenty microliter of sample was injected in duplicate. Anthocyanins were detected at 520 nm, and hydroxybenzoic acids, hydroxycinnamic acids, and flavonols were detected at 280, 320, and 360 nm, respectively. Cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-xyloside, and pelargonidin 3-glucoside were quantified based on external standards, and unknown anthocyanins were quantified as cyanidin 3-glucoside equivalent. Among non-anthocyanin phenolic compounds, standard curves of gallic acid, vanillic acid, quercetin 3-galactoside, and quercetin 3-glucoside were used. Unknown hydroxybenzoic acids, hydroxycinnamic acids, and flavonols

were quantified as gallic acid, chlorogenic acid, and quercetin 3-glucoside equivalents, respectively. Sum of individual anthocyanins or phenolic compounds were calculated to obtain total anthocyanin and phenolic contents.

Statistical analysis

SAS (SAS 9.2, SAS institute, Cary, NC, USA) statistical program was used. Univariate analysis of variance (ANOVA) was carried out, and the means were separated using Tukey's honestly significant difference test (HSD) or Fisher's least significant difference (LSD) at 95% significance level.

Results and Discussion

Anthocyanins

A total of eight peaks were detected at 520 nm and four of these were identified as cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-xyloside and pelargonidin 3-glucoside (Fig. 3-1). Cyanidin 3-glucoside was the predominant anthocyanin in the three blackberry cultivars used in this study, representing 87 to 95% of the total anthocyanin content (Table 3-1 and 3-2). These results are in agreement with previous studies that showed cyanidin 3-glucoside was the most abundant anthocyanin in blackberries (Cho et al., 2004; Fan-Chiang and Wrolstad, 2005; Koca and Karadeniz, 2009; Wang et al., 2008). However, 'Marion' and 'Siskiyou' blackberries showed lower percentage of cyanidin 3-glucoside and

higher percentage of cyanidin 3-rutinoside compared to our results (Fan-Chiang and Wrolstad, 2005). Cyanidin 3-glucoside and cyanidin 3-rutinoside proportion in ‘Marion’ and ‘Siskiyou’ blackberries were 68-70 and 26-30%, respectively, based on the ratio of each peak to the total peak area. In contrast, the percentage of cyanidin 3-glucoside and cyanidin 3-rutinoside in ‘Navaho’ blackberries was 87.5 and 0.8%, respectively, in agreement with our results. ‘Marion’ and ‘Siskiyou’ blackberries are from the Pacific Northwest breeding programs and have distinctly different genetic background from the three cultivars used in this study (Finn et al., 1999). It was also reported that a large portion of anthocyanins (94%) in blackberries (genotype unspecified) are present in nonacylated form, and 90% of anthocyanins are monoglycosides while 10% are diglycosides (Wu et al., 2006).

Anthocyanin content and composition of small fruits are known to be affected by genetic background (Connor et al., 2005; Mertz et al., 2007). In our study, total anthocyanin content and cyanidin 3-glucoside, cyanidin 3-rutinoside, and pelargonidin 3-glucoside contents were generally higher in ‘Natchez’ than in ‘Ouachita’ blackberries (Table 3-1). However, cyanidin 3-xyloside content and percentage was higher in ‘Navaho’ and ‘Ouachita’ than in ‘Natchez’ blackberries (Table 3-1 and 3-2). Our results for ‘Navaho’ are similar to those reported by Cho et al. (2004) and Fan-Chiang and Wrolstad (2005) while the anthocyanin composition of ‘Ouachita’ and ‘Natchez’ has not been previously reported.

Cyanidin 3-glucoside, cyanidin 3-rutinoside, and total anthocyanin contents were higher in SB compared to DB berries of ‘Natchez’ and ‘Ouachita’ but did not differ significantly between ripeness stages in ‘Navaho’ berries (Table 3-3). It has been shown that berry fruit

accumulate anthocyanins as fruit ripens (Acosta-Montoya et al., 2010; Pineli et al., 2011; Wang and Lin, 2000). Increase in total anthocyanin content was reported in ‘Marion’ blackberries from ripe to overripe stages (Siriwoharn et al., 2004) and in tropical highland blackberries (*Rubus adenotrichus*) from purple to dark bluish purple stage (Acosta-Montoya et al., 2010). However, anthocyanin content decreased or did not change during ripening from SB and DB in the blackberries investigated in this study. It is not clear why our blackberries showed different trend in anthocyanin content, but it might be related to genetic difference, environmental conditions during fruit production, and enzyme activities.

It was reported that anthocyanin content in ‘Kent’ and ‘Earliglow’ strawberries was highest in the fruit grown under 30/22°C (day/night) compared to other temperatures (18/12, 25/12, and 25/22°C) (Wang and Zheng, 2001). The primary enzymes involved in synthesis of anthocyanins are phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) as chalcones are subsequently converted to flavonoids (Fig. 3-2 and 3-3). However, PAL activity and CHS gene expression in ‘Darkridge’ grapes were higher in the berries grown under 30/15°C than under 30/30°C (Mori et al., 2005). Cheng and Breen (1991) found that PAL activity sharply increased from 23 days after anthesis with anthocyanin accumulation and reached the maximum at 27 days after anthesis (red stage) in ‘Tillikum’ strawberries. Then PAL activity rapidly decreased and was almost undetectable at 30 days after anthesis. These studies show that anthocyanin synthesis is a complicated process affected by various factors such as temperature and involved enzymes such as PAL and CHS. However, PAL and CHS are involved in the early stage of anthocyanin biosynthesis, and there are many other enzymes, such as dihydroflavonol 4-reductase (DFR) or anthocyanidin synthase, in

latter stages (Fig. 3-2 and 3-3). Therefore, studies on those enzymes in response to fruit ripening and environmental conditions are needed to determine how anthocyanin synthesis is regulated during ripening.

Anthocyanin content was significantly affected by cultivar and storage condition (Table 3-1). A significant interaction between cultivar and storage treatment was found for all anthocyanins and for total anthocyanin content. In contrast, cultivar x ripeness was significant only for cyanidin 3-rutinoside while the interaction of ripeness and storage condition was significant for all but cyanidin 3-rutinoside pigments. These results suggest that ripeness stage had a similar effect across cultivars while storage treatment response was dependent on ripeness stage and cultivar.

Anthocyanin content in response to storage treatment differed among cultivars. Levels of total anthocyanin content in 'Natchez' increased only when held at room temperature for two days, while both 'Ouachita' and 'Navaho' increased in total anthocyanin in both storage conditions (Table 3-1). Cyanidin 3-glucoside content generally increased after storage in all cultivars, especially when berries were stored at 20°C for two days. However, cyanidin 3-xyloside and pelargonidin 3-glucoside contents changed differently in 'Natchez' compared to 'Ouachita' and 'Navaho' (Table 3-1). The content of these compounds decreased after storage in 'Natchez' but increased in the other cultivars. Moreover, the proportion of cyanidin 3-rutinoside did not change and pelargonidin 3-glucoside decreased after storage in 'Natchez', but cyanidin 3-rutinoside decreased and pelargonidin 3-glucoside increased after storage in 'Ouachita' and 'Navaho' (Table 3-2). This may be due to their genetic

background. 'Navaho' is a parent of 'Ouachita', and 'Natchez' has a different genetic background (Clark and Moore, 2005; Clark and Moore, 2008).

Many studies with fresh small fruits analyzed total anthocyanin content rather than individual anthocyanins during storage. Total anthocyanins have been reported to increase in strawberries, raspberries, and blueberries after storage at 10, 20, and 30°C for 8 days (Kalt et al., 1999), and in strawberries after 7 days at 0, 5, and 10°C (Jin et al., 2011). In contrast, Wu et al. (2010) reported both fluctuated and decreased total anthocyanin content in 'Evergreen' and 'Marion' blackberries after 7 or 9 days at 2°C. These blackberries are processing cultivars with a complex genetic background of blackberry and raspberry species and are very soft after harvest, which may have led to juice loss and subsequent variation of anthocyanin content. In contrast, the blackberries used in our study are firm fresh market cultivars and have different genetic background. In addition, the anthocyanin composition of 'Evergreen' and 'Marion' blackberries were different from that of 'Navaho' (Fan-Chiang and Wrolstad, 2005). In particular, the percentage of cyanidin 3-rutinoside was higher in 'Evergreen' and 'Marion' blackberries compared to 'Navaho'.

The increase in phenolic compounds during storage, including anthocyanins, can be affected by several factors. Kalt et al. (1999) reported that titratable acidity of various small fruits decrease during storage and that these organic acids may provide carbon skeletons for biosynthesis of anthocyanins and non-anthocyanin phenolics. In this study, increased anthocyanin contents was found with storage (Table 3-1) and decreased titratable acidity was

also found (Chapter 2). The increase in anthocyanin contents during storage exceeded the relative decrease in titratable acidity in this study.

The increase in anthocyanin during storage may be related with expression of enzymes involved in anthocyanin biosynthesis. In 'Cardinal' grapes, total anthocyanin content increased during storage at 0°C and mRNA accumulation of PAL and CHS, enzymes that are involved in early stages of phenolic biosynthesis, also increased (Romero et al., 2008; Sanchez-Ballesta et al., 2007). Total anthocyanin content in red oranges increased during storage at 4°C (Crifò et al., 2012; Lo Piero et al., 2005). In addition to PAL and CHS, gene expression of DFR and UDP-glucose flavonoid glucosyl transferase, enzymes that catalyze formation of flavanols and glycosylation of anthocyanidins, respectively, also increased during storage. However, Crifò et al. (2012) observed that the enzyme expression increase did not occur at the same time with anthocyanin increase. Therefore, there might be other factors affecting anthocyanin increase during refrigerated storage. There are environmental/temperature studies with raspberries that indicated some anthocyanins are malleable to specific temperatures, such as cyanidin 3-glucoside and cyanidin 3-rutinoside tended to increase with the average hours of exposure to temperatures above 29°C while high temperature had negative effect on cyanidin 3-sophoroside in 'Caroline' raspberries (Bradish et al., 2012). It might explain that cyanidin 3-glucoside and cyanidin 3-rutinoside in the three cultivars used in this study increased during storage, with greater increase in berries exposed to 20°C for two days, except for cyanidin 3-rutinoside content in 'Natchez' blackberries.

Phenolic compounds

Although not all phenolic peaks found could be identified to specific phenolic compounds, relative types could be grouped based on spectrum. Based on external standards, gallic acid, vanillic acid, quercetin 3-galactoside, and quercetin 3-glucoside were identified among 3, 7, and 6 peaks for hydroxybenzoic acids and derivatives, hydroxycinnamic acids and derivatives, and flavonols, respectively (Fig. 3-1). Hydroxybenzoic acids were the major phenolic compounds in the three cultivars (Table 3-4). Cho et al. (2004; 2005) reported that quercetin 3-galactoside or quercetin 3-glucoside was the predominant flavonol in blackberries, depending on cultivar. Zadernowski et al. (2005) reported the presence of gallic acid, vanillic acid, salicylic acid, caffeic acid, *m*- and *p*-coumaric acids, and hydroxycaffeic acid in blackberries (*R. plicatus*) using GC-MS. Most of the phenolic compounds were present as esters or glycosides rather than in free form. Ellagic acid and flavan 3-ols including (+)-catechin and (-)-epicatechin have also been reported in *R. fruticosus* blackberries (Jakobek et al., 2009); we were unable to detect ellagic acid using our system, possibly because we did not include pyrene (seed) tissue which is a major source of this phenolic compound in blackberry fruit (Siriwoharn and Wrolstad, 2004).

Total phenolic content was generally higher in ‘Natchez’ than in the other cultivars (Table 3-4). In ‘Natchez’ and ‘Ouachita’, total hydroxybenzoic acids and flavonols represented 45-56 and 28-39% of the total phenolic content, respectively (Table 3-5). In ‘Navaho’, the relative ratio of hydroxybenzoic acids as a portion of total phenolics was 61-71%, compared to the flavonols of 20-27%. Quercetin 3-galactoside and quercetin 3-

glucoside represented 6-11% of the total phenolic content in the three cultivars (Table 3-5). Cho et al. (2004) analyzed flavonoids in 'Apache', 'APF-12', 'Arapaho', 'Chickasaw', 'Kiowa', and 'Navaho' blackberries and found only cyanidin and quercetin derivatives. They suggested that this simple and unique flavonoid profile showed that these blackberries may lack the enzyme flavonoid-3',5'-hydroxylase, which converts dihydrokaempferol to dihydromyricetin. Dihydromyricetin is further converted to delphinidin and petunidin (He et al., 2010). Among hydroxybenzoic acids, content and percentage of vanillic acid were significantly lower in 'Natchez' (4-6%) than in 'Ouachita' and 'Navaho' (16-29%). In addition, quercetin 3-glucoside and quercetin 3-galactoside contents were highest in 'Natchez' while gallic acid content was highest in 'Navaho'. Zadernowski et al. (2005) reported that hydroxycinnamic acid content was higher than hydroxybenzoic acids contents in *R. plicatus* blackberries, with differences due to different genetic background and environmental conditions during cultivation. Phenolic contents were generally higher in SB than in DB berries (Table 3-6). However, gallic acid in 'Natchez' and 'Ouachita' blackberries and unknown 1 in 'Navaho' blackberry were not different between ripeness stages. Unknown 1 in 'Ouachita' was higher in DB compared to SB berries. Phenolic contents in berries during maturation have been reported and showed variation among studies (Krüger et al., 2011; Wang and Lin, 2000). Decreased phenolic content was found in the investigated cultivars, and the difference among studies may be due to genetic difference, analytical protocol, and fruit composition.

In general, total phenolic content increased after storage especially in the fruit stored at 20°C for two days (Table 3-4). Wu et al. (2010) observed fluctuated or decreased total

phenolic content in 'Marion' and 'Evergreen' blackberries during 7-9 days of storage at 2°C, in contrast to our results. This could be due to cultivar differences discussed above or to the different production environment. In strawberries, total phenolic content increased after 7 days of storage at 0, 5, and 10°C, with largest increases in fruit held at 10°C (Jin et al., 2011). Contents of gallic acid, total hydroxybenzoic acids, total hydroxycinnamic acids, quercetin 3-galactoside, and total flavonols increased after storage in all cultivars especially when the fruit were held at 20°C for two days (Table 3-4). Vanillic acid increased only in 'Navaho' berries after storage and quercetin 3-glucoside increased in 'Natchez' and 'Navaho' but not in 'Ouachita'. Unknown 1, a hydroxycinnamic acid, also changed differently among cultivars. It increased in 'Natchez' when held at 20°C for two days, did not change in 'Ouachita', and decreased in 'Navaho'. In particular, it was not detected in 'Navaho' berries when stored at 20°C for two days. Several studies have looked at quercetin content among species, genotype, and frozen storage. Genetic effects on relative amounts of quercetin following frozen storage for six months have been found among four blackberry cultivars (Türkben et al., 2010). Odriozola-Serrano et al. (2010) reported that quercetin content in fresh-cut 'Camarosa' strawberries increased as the pressure of O₂ increased and CO₂ pressure decreased during modified atmosphere storage at 4°C for 21 days. These studies showed that phenolic content can be affected by storage as well as genetic background, in agreement with our results.

Increase in non-anthocyanin phenolic compounds during refrigerated storage has not been studied extensively. However, titratable acidity reduction might play a role in biosynthesis of phenolic compounds as discussed above (Kalt et al., 1999). Increased

expression levels of PAL and CHS in table grapes and red oranges during storage at 4°C was also reported (Crifò et al., 2012; Lo Piero et al., 2005; Romero et al., 2008). Although these studies focused on anthocyanins and non-anthocyanin phenolic compounds were not analyzed, those enzymes are involved in the early stages of phenolic biosynthesis (Fig. 3-2 and 3-3). Biosynthesis of phenolic compounds and their change during storage seem to be complicated process involving various factors such as genetic background, storage condition, fruit composition, and related enzymes.

Correlation among phenolic compounds

Correlations of anthocyanins and non-anthocyanin phenolic compounds were done (Table 3-3). As expected, individual anthocyanins were positively correlated with total anthocyanin content. However, cyanidin 3-xyloside was negatively correlated with cyanidin 3-rutinoside. This result may be related with the sugar specificity of glycosyltransferase. *O*-Glycosyltransferase isolated from apple skin glycosylated anthocyanidins and flavonols and showed different activity among sugar donors including UDP-glucose, UDP-galactose, and UDP-xylose (Lister et al., 1997). Relative activity was the highest with galactose, followed by glucose and xylose. Although the authors did not use UDP-rhamnose, which is needed to form rutinoside, this study showed that the enzyme glycosyltransferase has sugar specificity among sugar donors when transferring sugar moieties to anthocyanidins or flavonols and may explain the negative correlation between cyanidin 3-rutinoside and cyanidin 3-xyloside of our results.

Cyanidin 3-glucoside and cyanidin 3-rutinoside were positively correlated with total hydroxycinnamic acids, quercetin 3-glucoside, and total flavonols while cyanidin 3-xyloside was positively correlated with vanillic acid. As anthocyanins and non-anthocyanin phenolic compounds share early stages of the biosynthetic pathway (Tsao, 2010; Vogt, 2010), an increase in synthesis of phenolic compounds would likely increase all classes of phenolics. Both anthocyanins and flavonols are flavonoids, and this may explain high correlation coefficients between total flavonol contents and anthocyanins contents. Since cyanidin 3-rutinoside was negatively correlated with cyanidin 3-xyloside and vanillic acid and all differed in content and percentage among cultivars, these phenolics may be useful as determinants of cultivar identification. This is demonstrated by the fact that content and percentage of cyanidin 3-xyloside and vanillic acid were very similar between ‘Ouachita’ and ‘Navaho’, which are genetically very close, but differed from ‘Natchez’, which is not genetically close.

Conclusion

Organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries varied in their phenolic compounds and phenolic content was significantly affected by storage treatment. Fruit stored at 1°C for 15 days or at 1°C for 13 days and at 20°C for the next two days had higher levels of anthocyanins and non-anthocyanin phenolic compounds compared to fruit that were not stored. Anthocyanin and phenolic contents decreased or maintained during fruit changes from SB to DB. Cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-xyloside, and pelargonidin 3-glucoside were identified and cyanidin 3-glucoside was the predominant

anthocyanin in the blackberries used in this study. Among non-anthocyanin phenolic compounds, gallic acid, vanillic acid, quercetin 3-galactoside, and quercetin 3-glucoside were identified and hydroxybenzoic acids were the major phenolic compounds in the samples. Phenolic composition differed among cultivars in cyanidin 3-xyloside and vanillic acid. Total anthocyanins and phenolic contents generally increased with storage, with the greater increase in the berries stored at 20°C for two days, but individual compounds changed differently among cultivars. The results of this study suggest that phenolic profile differs among cultivars while storing blackberries at either constant low temperature or for a brief period at a higher temperature will increase rather than decrease phenolic contents.

Literature Cited

- Acosta-Montoya, O., F. Vaillant, S. Cozzano, C. Mertz, A.M. Pérez, and M.V. Castro. 2010. Phenolic content and antioxidant capacity of tropical highland blackberry (*Rubus adenotrichus* Schltdl.) during three edible maturity stages. *Food Chem.* 119:1497-1501.
- Benvenuti, S., F. Pellati, M. Melegari, and D. Bertelli. 2004. Polyphenols, anthocyanins, ascorbic acid, and radical scavenging activity of *Rubus*, *Ribes*, and *Aronia*. *J. Food Sci.* 69:C164-C169.
- Bradish, C.M., P. Perkins-Veazie, G.E. Fernandez, G.X. Xie, and W. Jia. 2012. Comparison of flavonoid composition of red raspberries (*Rubus idaeus* L.) grown in the Southern United States. *J. Agric. Food Chem.* 60:5779-5786.
- Cheng, G.W. and P.J. Breen. 1991. Activity of phenylalanine ammonia-lyase (PAL) and concentrations of anthocyanins and phenolics in developing strawberry fruit. *J. Am. Soc. Hortic. Sci.* 116:865-869.
- Cho, M.J., L.R. Howard, R.L. Prior, and J.R. Clark. 2004. Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* 84:1771-1782.
- Cho, M.J., L.R. Howard, R.L. Prior, and J.R. Clark. 2005. Flavonol glycosides and antioxidant capacity of various blackberry and blueberry genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* 85:2149-2158.
- Clark, J.R. and J.N. Moore. 2005. 'Ouachita' thornless blackberry. *HortScience* 40:258-260.

- Clark, J.R. and J.N. Moore. 2008. 'Natchez' thornless blackberry. *HortScience* 43:1897-1899.
- Connor, A.M., C.E. Finn, T.K. McGhie, and P.A. Alspach. 2005. Genetic and environmental variation in anthocyanins and their relationship to antioxidant activity in blackberry and hybridberry cultivars. *J. Am. Soc. Hortic. Sci.* 130:680-687.
- Crifò, T., G. Petrone, L. Lo Cicero, and A.R. Lo Piero. 2012. Short cold storage enhances the anthocyanin contents and level of transcripts related to their biosynthesis in blood oranges. *J. Agric. Food Chem.* 60:476-481.
- Cuevas-Rodríguez, E.O., V.P. Dia, G.G. Yousef, P.A. García-Saucedo, J. López-Medina, O. Paredes-López, E.G. De Mejia, and M.A. Lila. 2010. Inhibition of pro-inflammatory responses and antioxidant capacity of Mexican blackberry (*Rubus* spp.) extracts. *J. Agric. Food Chem.* 58:9542-9548.
- Ding, M., R.T. Feng, S.Y. Wang, L. Bowman, Y.J. Lu, Y. Qian, V. Castranova, B.H. Jiang, and X.L. Shi. 2006. Cyanidin-3-glucoside, a natural product derived from blackberry, exhibits chemopreventive and chemotherapeutic activity. *J. Biol. Chem.* 281:17359-17368.
- Erlund, I., R. Koli, G. Alfthan, J. Marniemi, P. Puukka, P. Mustonen, P. Mattila, and A. Jula. 2008. Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol. *Am. J. Clin. Nutr.* 87:323-331.
- Fan-Chiang, H.J. and R.E. Wrolstad. 2005. Anthocyanin pigment composition of blackberries. *J. Food Sci.* 70:C198-C202.
- Finn, C.E., F.J. Lawrence, B.C. Strik, B. Yorgey, and J. DeFrancesco. 1999. 'Siskiyou' trailing blackberry. *HortScience* 34:1288-1290.

- Food Navigator USA. 2014. US organic food market to grow 14% from 2013-18.
<http://www.foodnavigator-usa.com/Markets/US-organic-food-market-to-grow-14-from-2013-18>
- Harkins, R.H., B.C. Strik, and D.R. Bryla. 2013. Weed management practices for organic production of trailing blackberry: I. Plant growth and early fruit production. *HortScience* 48:1139-1144.
- He, F., L. Mu, G.L. Yan, N.N. Liang, Q.H. Pan, J. Wang, M.J. Reeves, and C.Q. Duan. 2010. Biosynthesis of anthocyanins and their regulation in colored grapes. *Molecules* 15:9057-9091.
- Jakobek, L., M. Šeruga, B. Šeruga, I. Novak, and M. Medvidović-Kosanović. 2009. Phenolic compound composition and antioxidant activity of fruits of *Rubus* and *Prunus* species from Croatia. *Int. J. Food Sci. Technol.* 44:860-868.
- Jeong, J.H., H. Jung, S.R. Lee, H.J. Lee, K.T. Hwang, and T.Y. Kim. 2010. Anti-oxidant, anti-proliferative and anti-inflammatory activities of the extracts from black raspberry fruits and wine. *Food Chem.* 123:338-344.
- Jin, P., S.Y. Wang, C.Y. Wang, and Y.H. Zheng. 2011. Effect of cultural system and storage temperature on antioxidant capacity and phenolic compounds in strawberries. *Food Chem.* 124:262-270.
- Joseph, J.A., N.A. Denisova, G. Arendash, M. Gordon, D. Diamond, B. Shukitt-Hale, and D. Morgan. 2003. Blueberry supplementation enhances signaling and prevents behavioral deficits in an Alzheimer disease model. *Nutr. Neurosci.* 6:153-162.

- Kader, A.A., 2002. Postharvest technology of horticultural crops, 3rd Ed. Division of Agriculture and Natural Resources, University of California, Oakland.
- Kalt, W., C.F. Forney, A. Martin, and R.L. Prior. 1999. Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. *J. Agric. Food Chem.* 47:4638-4644.
- Koca, I. and B. Karadeniz. 2009. Antioxidant properties of blackberry and blueberry fruits grown in the Black Sea Region of Turkey. *Sci. Hortic.* 121:447-450.
- Krüger, E., H. Dietrich, E. Schöppllein, S. Rasim, and P. Kürbel. 2011. Cultivar, storage conditions and ripening effects on physical and chemical qualities of red raspberry fruit. *Postharvest Biol. Technol.* 60:31-37.
- Lister, C.E., Lancaster, J.E., Sutton, K.H., Walker, J.R.L., 1997. Aglycone and glycoside specificity of apple skin flavonoid glycosyltransferase. *J. Sci. Food Agric.* 75, 378-382.
- Lo Piero, A.R., I. Puglisi, P. Rapisarda, and G. Petrone. 2005. Anthocyanins accumulation and related gene expression in red orange fruit induced by low temperature storage. *J. Agric. Food Chem.* 53:9083-9088.
- Lowe, J.D., K.W. Pomper, S.B. Crabtree, J.R. Clark, and J.G. Strang. 2012. Yield characteristics of thorny primocane-fruiting blackberries from the University of Arkansas breeding program grown under organic growing conditions in Kentucky. *J. Amer. Pomolog. Soc.* 66:2-8.
- Mertz, C., V. Cheynier, Z. Günata, and P. Brat. 2007. Analysis of phenolic compounds in two blackberry species (*Rubus glaucus* and *Rubus adenotrichus*) by high-performance liquid

- chromatography with diode array detection and electrospray ion trap mass spectrometry. *J. Agric. Food Chem.* 55:8616-8624.
- Mori, K., S. Sugaya, and H. Gemma. 2005. Decreased anthocyanin biosynthesis in grape berries grown under elevated night temperature condition. *Sci. Hortic.* 105:319-330.
- 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.
- Odriozola-Serrano, I., R. Soliva-Fortuny, and O. Martín-Belloso. 2010. Changes in bioactive composition of fresh-cut strawberries stored under superatmospheric oxygen, low-oxygen or passive atmospheres. *J. Food Compos. Anal.* 23:37-43.
- OTA. 2011. U.S. organic industry valued at nearly \$29 billion in 2010. Mar. 30. 2013. http://www.organicnewsroom.com/2011/04/us_organic_industry_valued_at.html
- OTA. 2012. Consumer-driven U.S. organic market surpasses \$31 billion in 2011. Nov.5. 2013. http://www.organicnewsroom.com/2012/04/us_consumerdriven_organic_mark.html
- Pantelidis, G.E., M. Vasilakakis, G.A. Manganaris, and G. Diamantidis. 2007. Antioxidant capacity, phenol, anthocyanin and ascorbic acid contents in raspberries, blackberries, red currants, gooseberries and Cornelian cherries. *Food Chem.* 102:777-783.
- Pergola, C., A. Rossi, P. Dugo, S. Cuzzocrea, and L. Sautebin. 2006. Inhibition of nitric oxide biosynthesis by anthocyanin fraction of blackberry extract. *Nitric Oxide-Biol. Chem.* 15:30-39.

- Perkins-Veazie, P. and J.K. Collins. 2002. Quality of erect-type blackberry fruit after short intervals of controlled atmosphere storage. *Postharvest Biol. Technol.* 25:235-239.
- Perkins-Veazie, P., J.K. Collins, and J.R. Clark. 1996. Cultivar and maturity affect postharvest quality of fruit from erect blackberries. *HortScience* 31:258-261.
- Perkins-Veazie, P., J.K. Collins, and J.R. Clark. 1999. Shelf-life and quality of 'Navaho' and 'Shawnee' blackberry fruit stored under retail storage conditions. *J. Food Qual.* 22:535-544.
- Pineli, L.D.D., C.L. Moretti, M.S. dos Santos, A.B. Campos, A.V. Brasileiro, A.C. Córdova, and M.D. Chiarello. 2011. Antioxidants and other chemical and physical characteristics of two strawberry cultivars at different ripeness stages. *J. Food Compos. Anal.* 24:11-16.
- Poiana, M.A., D. Moigradean, D. Raba, L.M. Alda, and M. Popa. 2010. The effect of long-term frozen storage on the nutraceutical compounds, antioxidant properties and color indices of different kinds of berries. *J. Food Agric. Environ.* 8:54-58.
- Romero, I., M.T. Sanchez-Ballesta, R. Maldonado, M.I. Escribano, and C. Merodio. 2008. Anthocyanin, antioxidant activity and stress-induced gene expression in high CO₂-treated table grapes stored at low temperature. *J. Plant Physiol.* 165:522-530.
- Sanchez-Ballesta, M.T., I. Romero, J.B. Jiménez, J.M. Orea, A. González-Ureña, M.I. Escribano, and C. Merodio. 2007. Involvement of the phenylpropanoid pathway in the response of table grapes to low temperature and high CO₂ levels. *Postharvest Biol. Technol.* 46:29-35.
- Seeram, N.P., L.S. Adams, Y.J. Zhang, R. Lee, D. Sand, H.S. Scheuller, and D. Heber. 2006. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts

- inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* 54:9329-9339.
- Shukitt-Hale, B., V. Cheng, and J.A. Joseph. 2009. Effects of blackberries on motor and cognitive function in aged rats. *Nutr. Neurosci.* 12:135-140.
- Siriwoharn, T. and R.E. Wrolstad. 2004. Polyphenolic composition of Marion and Evergreen blackberries. *J. Food Sci.* 69:C233-C240.
- Siriwoharn, T., R.E. Wrolstad, C.E. Finn, and C.B. Pereira. 2004. Influence of cultivar, maturity, and sampling on blackberry (*Rubus* L. hybrids) anthocyanins, polyphenolics, and antioxidant properties. *J. Agric. Food Chem.* 52:8021-8030.
- Springob, K., J. Nakajima, M. Yamazaki, and K. Saito. 2003. Recent advances in the biosynthesis and accumulation of anthocyanins. *Nat. Prod. Rep.* 20:288-303.
- Strik, B.C., J.R. Clark, C.E. Finn, and M.P. Bañados. 2007. Worldwide blackberry production. *HortTechnology* 17:205-213.
- Strik, B.C., J.R. Clark, C.E. Finn, and M.P. Bañados. 2008. Worldwide production of blackberries. *Acta Hort.*:209-217.
- Türkben, C., E. Sariburun, C. Demir, and V. Uylaşer. 2010. Effect of freezing and frozen storage on phenolic compounds of raspberry and blackberry cultivars. *Food Anal. Meth.* 3:144-153.
- Tsao, R. 2010. Chemistry and biochemistry of dietary polyphenols. *Nutrients* 2:1231-1246.
- USDA. 2013. Organic production. Nov. 5 2013. 2013. <http://www.ers.usda.gov/data-products/organic-production.aspx#.UnklP7D3RMs>
- Vogt, T. 2010. Phenylpropanoid Biosynthesis. *Mol. Plant.* 3:2-20.

- Wang, J. and G. Mazza. 2002. Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN- γ -activated RAW 264.7 macrophages. *J. Agric. Food Chem.* 50:850-857.
- Wang, S.Y., L. Bowman, and M. Ding. 2008. Methyl jasmonate enhances antioxidant activity and flavonoid content in blackberries (*Rubus* sp.) and promotes antiproliferation of human cancer cells. *Food Chem.* 107:1261-1269.
- Wang, S.Y. and H.S. Lin. 2000. Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *J. Agric. Food Chem.* 48:140-146.
- Wang, S.Y. and W. Zheng. 2001. Effect of plant growth temperature on antioxidant capacity in strawberry. *J. Agric. Food Chem.* 49:4977-4982.
- Wu, R.Y., B. Frei, J.A. Kennedy, and Y.Y. Zhao. 2010. Effects of refrigerated storage and processing technologies on the bioactive compounds and antioxidant capacities of 'Marion' and 'Evergreen' blackberries. *LWT-Food Sci. Technol.* 43:1253-1264.
- Wu, X.L., G.R. Beecher, J.M. Holden, D.B. Haytowitz, S.E. Gebhardt, and R.L. Prior. 2006. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *J. Agric. Food Chem.* 54:4069-4075.
- Zadernowski, R., M. Naczki, and J. Nesterowicz. 2005. Phenolic acid profiles in some small berries. *J. Agric. Food Chem.* 53:2118-2124.
- Zhao, X., E.E. Carey, W.Q. Wang, and C.B. Rajashekar. 2006. Does organic production enhance phytochemical content of fruit and vegetables? Current knowledge and prospects for research. *HortTechnology* 16:449-456.

Table 3-1. Anthocyanin contents (mg g⁻¹ DW) in ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries 0 and 15 days after storage.

Cultivar	Storage condition ^y	Anthocyanins ^x				
		C3G	C3R	C3X	P3G	Total
Natchez	Day 0	25.821b ^z	0.389b	0.036a	0.263a	28.106b
	CL	25.231b	0.414b	0.001b	0.174b	27.668b
	RT	29.055a	0.477a	0.007b	0.184b	31.887a
Ouachita	Day 0	15.879b	0.100a	0.766b	0.058b	17.742b
	CL	20.692a	0.095a	1.009a	0.134a	23.283a
	RT	21.569a	0.098a	1.036a	0.147a	24.218a
Navaho	Day 0	17.832c	0.135b	1.304c	0.088c	20.644c
	CL	24.251b	0.140b	1.589b	0.174b	27.951b
	RT	27.560a	0.153a	1.791a	0.217a	31.724a

Table 3-1 Continued

Significance ^w					
Cultivar (C)	**	**	**	**	**
Ripeness stage (R)	**	**	NS	NS	**
Storage condition (S)	**	**	**	**	**
C X R	NS	**	NS	NS	NS
C X S	**	**	**	**	**
R X S	**	NS	**	**	**
C X R X S	**	NS	**	NS	**

^zMean separation within column in each cultivar by HSD at $p < 0.05$.

^yDay0, 0 day after storage; CL, constant low (1°C for 15 days); RT, retail (1°C for 13 days + 20°C for 2 days).

^xC3G, cyanidin 3-glucoside; C3R, cyanidin 3-rutinoside; C3X, cyanidin 3-xyloside; P3G, pelargonidin 3-glucoside.

^wNS, non-significant; **, significant at $p < 0.01$.

Table 3-2. Anthocyanin composition (as a relative ration of each compound to the total anthocyanin content, %) in in organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries.

Storage condition ^y	Anthocyanins (% of total) ^x			
	C3G	C3R	C3X	P3G
Natchez				
Day 0	94.93a ^z	1.43a	0.13a	0.99a
CL	91.92b	1.36a	0.01b	0.74b
RT	91.58b	1.40a	0.03b	0.59b
Ouachita				
Day 0	91.40a	0.58a	4.45ab	0.34b
CL	89.13b	0.42b	4.52a	0.59a
RT	89.20b	0.41b	4.28b	0.61a
Navaho				
Day 0	89.55a	0.61a	6.53a	0.53b
CL	87.28b	0.50b	5.54b	0.59ab
RT	87.31b	0.47c	5.46b	0.64a

^zMean separation within column in each cultivar by HSD at $p < 0.05$.

^yDay0, 0 day after storage; CL, constant low (1°C for 15 days); RT, retail (1°C for 13 days + 20°C for 2 days).

^xC3G, cyanidin 3-glucoside; C3R, cyanidin 3-rutinoside; C3X, cyanidin 3-xyloside; P3G, pelargonidin 3-glucoside.

Table 3-3. Anthocyanin contents (mg g⁻¹ DW) in ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries at SB and DB ripeness stages.

	Anthocyanins ^y				
	C3G	C3R	C3X	P3G	Total
Natchez					
SB ^x	27.33a	0.45a	0.02a	0.20a	29.99a
DB	25.40b	0.39b	0.01a	0.21a	27.71b
Ouachita					
SB	20.42a	0.10a	0.98a	0.12a	22.94a
DB	18.23b	0.09b	0.90b	0.11a	20.45b
Navaho					
SB	23.69a	0.15a	1.58a	0.16a	27.38a
DB	22.35a	0.14a	1.53a	0.16a	25.74a

^zMean separation within column in each cultivar by LSD at $p < 0.05$.

^yC3G, cyanidin 3-glucoside; C3R, cyanidin 3-rutinoside; C3X, cyanidin 3-xyloside; P3G, pelargonidin 3-glucoside.

^xSB, shiny black; DB, dull black.

Table 3-4. Phenolic contents (mg 100 g⁻¹ DW) in ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries 0 and 15 days after storage.

Cultivar	Storage conditions ^y	Phenolic compounds ^x								
		HBA			HCA		Flavonols			Total
		GA	VA	T HBA	Unk 1	T HCA	Q-gal	Q-glu	T Fla	
Natchez	Day 0	10.166ab	21.100a	202.170b	7.157b	63.560b	22.687b	19.081b	157.434b	423.164b
	CL	6.939b	23.046a	245.476b	7.276b	64.340b	28.573b	22.290ab	164.562b	474.378b
	RT	15.611a	25.641a	350.987a	9.666a	80.077a	37.775a	28.586a	209.513a	640.578a
Ouachita	Day 0	9.937a	76.609a	154.200b	3.806a	43.417b	11.294b	5.990a	76.83b	274.450b
	CL	6.170b	75.549a	163.726b	4.667a	52.672a	16.805ab	7.232a	97.992a	314.390b
	RT	6.785ab	83.855a	224.460a	3.327a	56.285a	20.972a	8.368a	111.844a	392.588a
Navaho	Day 0	10.844b	85.800b	227.103b	3.156a	43.300b	18.385b	7.374b	93.196b	363.598b
	CL	15.559ab	97.687ab	402.368a	0.363b	50.071ab	28.231a	10.211a	119.773a	572.211a
	RT	24.219a	104.092a	433.101a	NDb	52.514a	31.108a	11.377a	129.643a	615.257a

Table 3-4 Continued

Significance ^w									
Cultivar (C)	**	**	**	**	**	**	**	**	**
Ripeness stage (R)	**	**	**	NS	**	**	**	**	**
Storage condition (S)	**	**	**	**	**	**	**	**	**
C X R	NS	**	NS	**	**	NS	**	**	NS
C X S	**	NS	**	**	**	NS	NS	NS	**
R X S	NS								
C X R X S	NS	NS	NS	**	**	NS	NS	NS	NS

^zMean separation within column in each cultivar by HSD at $p < 0.05$.

^yDay0, 0 day after storage; CL, constant low (1°C for 15 days); RT, retail (1°C for 13 days + 20°C for 2 days).

^xGA, gallic acid; VA, vanillic acid; T HBA, total hydroxybenzoic acids; Unk 1, unknown 1; T HCA, total hydroxycinnamic acids; Q-gal, quercetin 3-galactoside; Q-glu, quercetin 3-glucoside; T Fla, total flavonols.

^wNS, non-significant; **, significant at $p < 0.01$.

Table 3-5. Composition of phenolic compounds (as a relative ration of each compound to the total anthocyanin content, %) in ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries.

Cultivar	Storage conditions ^y	Phenolic compounds (% of total) ^x							
		HBA			HCA			Flavonols	
		GA	VA	T HBA	Unk 1	T HCA	Q-gal	Q-glu	T Fla
Natchez	Day 0	2.30a ^z	5.06a	45.71b	1.79a	15.59a	5.57a	4.64a	38.69a
	CL	1.48a	4.91a	50.54ab	2.72a	14.09ab	6.01a	4.68a	35.37a
	RT	2.48a	4.09a	54.72a	1.47a	12.49b	5.89a	4.48a	32.79a
Ouachita	Day 0	3.48a	28.90a	55.49a	1.39ab	16.09ab	4.15b	2.21a	28.43b
	CL	2.30ab	26.90a	47.85b	1.81a	18.15a	5.74a	2.58a	34.00a
	RT	1.51b	24.50a	52.65ab	1.09b	15.70b	5.73a	2.40a	31.65ab
Navaho	Day 0	2.98b	24.31a	61.65b	0.87a	12.27a	5.11a	2.05a	26.08a
	CL	2.61b	17.03b	70.09a	0.04a	8.96b	4.81a	1.73a	20.95b
	RT	4.10a	16.97b	69.91a	0a	8.86b	4.95a	1.79a	21.24b

Table 3-5 Continued

^zMean separation within column in each cultivar by HSD at $p < 0.05$.

^yDay0, 0 day after storage; CL, constant low (1°C for 15 days); RT, retail (1°C for 13 days + 20°C for 2 days).

^xGA, gallic acid; VA, vanillic acid; T HBA, total hydroxybenzoic acids; Unk 1, unknown 1; T HCA, total hydroxycinnamic acids; Q-gal, quercetin 3-galactoside; Q-glu, quercetin 3-glucoside; T Fla, total flavonols.

Table. 3-6. Phenolic content (mg 100 g⁻¹ DW) in ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries at SB and DB ripeness stages.

Cultivar	Phenolic compounds ^x								
	HBA			HCA		Flavonols		Total	
	GA	VA	T HBA	Unk 1	T HCA	Q-gal	Q-glu	T Fla	
Natchez									
SB	12.28a	26.13a	305.37a	26.14a	76.08a	35.11a	27.91a	199.32a	580.77a
DB	7.86a	19.57b	207.88b	22.71b	59.09b	22.28b	17.14b	144.80b	411.77b
Ouachita									
SB	7.73a	88.76a	228.80a	11.72b	53.58a	18.83a	8.34a	104.34a	386.73a
DB	7.17a	65.00b	111.82b	17.70a	47.24b	12.86b	5.68b	83.40b	242.47b
Navaho									
SB	19.65a	106.61a	407.06a	19.35a	52.70a	30.69a	11.59a	129.18a	588.94a
DB	13.29b	83.60b	290.78b	17.78a	43.92b	20.33b	7.42b	96.79b	431.49b

^zMean separation within column in each cultivar by LSD at $p < 0.05$.

^yDay0, 0 day after storage; CL, constant low (1°C for 15 days); RT, retail (1°C for 13 days + 20°C for 2 days).

^xGA, gallic acid; VA, vanillic acid; T HBA, total hydroxybenzoic acids; Unk 1, unknown 1; T HCA, total hydroxycinnamic acids; Q-gal, quercetin 3-galactoside; Q-glu, quercetin 3-glucoside; T Fla, total flavonols.

Table 3-7. Pearson's correlation coefficients for anthocyanins and non-anthocyanin phenolic compounds in organically grown 'Natchez', 'Ouachita', and 'Navaho' blackberries.

	C3G	C3R	P3G	C3X	T-ACY	GA	VA	T-HBA	T-HCA	Q-gal	Q-glu	T-Fla	T-PC
C3G ^z													
C3R	0.57**												
P3G	0.65**	0.30**											
C3X	-0.02	-0.73**	-0.02										
T-	0.99**	0.50**	0.63**	0.09									
GA	0.37**	0.02	0.25**	0.34**	0.42**								
VA	-0.15	-0.69**	-0.23**	0.80**	-0.06	0.30**							
T-BA	0.57**	0.13	0.26**	0.39**	0.63**	0.61**	0.46**						
T-CA	0.71**	0.60**	0.44**	-0.28**	0.68**	0.25**	-0.25**	0.46**					
Q-gal	0.60**	0.44**	0.22*	-0.02	0.63**	0.40**	0.19*	0.66**	0.57**				
Q-glu	0.53**	0.82**	0.13	-0.55**	0.49**	0.11	-0.34**	0.30**	0.60**	0.79**			
T-Fla	0.72**	0.75**	0.35**	-0.37**	0.70**	0.28**	-0.22*	0.52**	0.83**	0.87**	0.90**		
T-PC	0.70**	0.35**	0.33**	0.18*	0.74**	0.57**	0.28**	0.96**	0.67**	0.79**	0.52**	0.74**	

Table 3-7 Continued

^zC3G, cyanidin 3-glucoside; C3R, cyanidin 3-rutinoside; P3G, pelargonidin 3-glucoside; C3X, cyanidin 3-xyloside; T-ACY, total anthocyanin; GA, gallic acid; VA, vanillic acid; T-HBA, total hydroxybenzoic acid; T-HCA, total hydroxycinnamic acid; Q-gal, quercetin 3-galactoside; Q-glu, quercetin 3-glucoside; T-Fla, total flavonol; T-PC, total phenolic.

^yAsterisks * and ** shows significance at $p < 0.05$ and $p < 0.01$, respectively; others are non-significant.

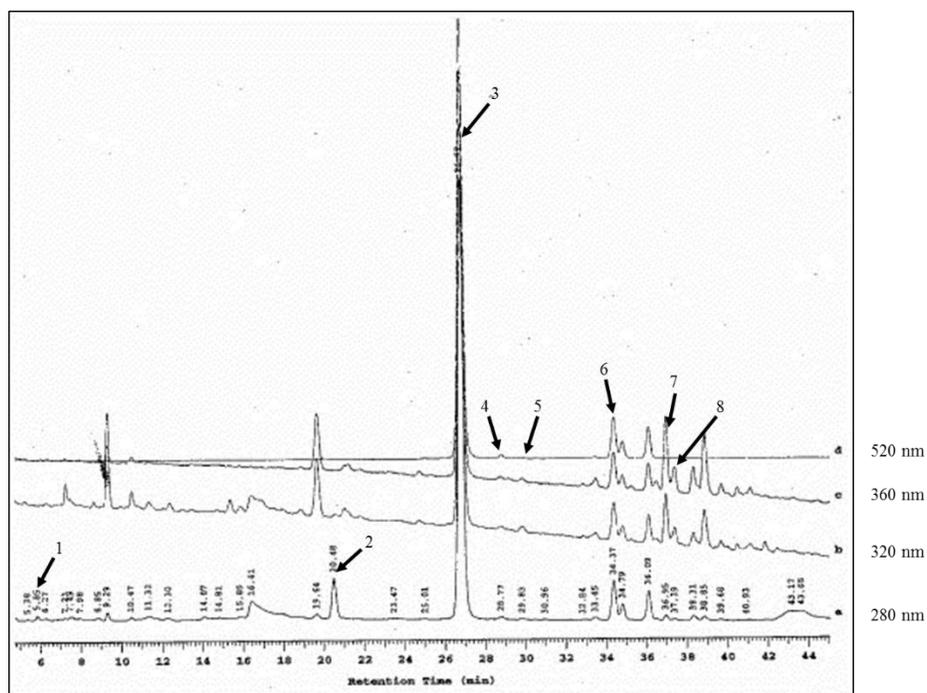


Fig. 3-1. HPLC chromatogram of 'Navaho' blackberries at SB ripeness stage (day 0).

Hydroxybenzoic acids, hydroxycinnamic acids, flavonols, and anthocyanins were detected at 280, 320, 360, and 520 nm, respectively. 1, gallic acid; 2, vanillic acid; 3, cyanidin 3-glucoside; 4, cyanidin 3-rutinoside; 5, pelargonidin 3-glucoside; 6, cyanidin 3-xyloside; 7, quercetin 3-galactoside; and 8, quercetin 3-glucoside.

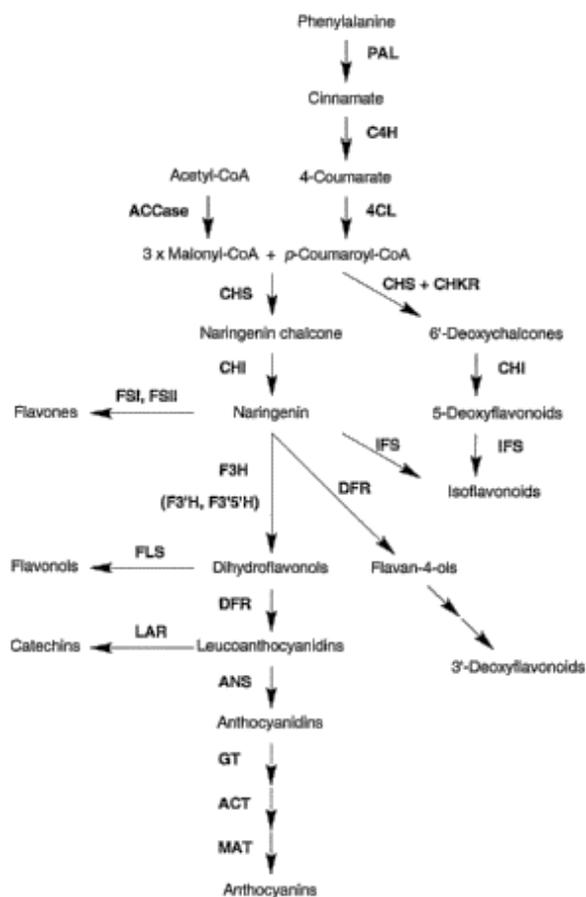
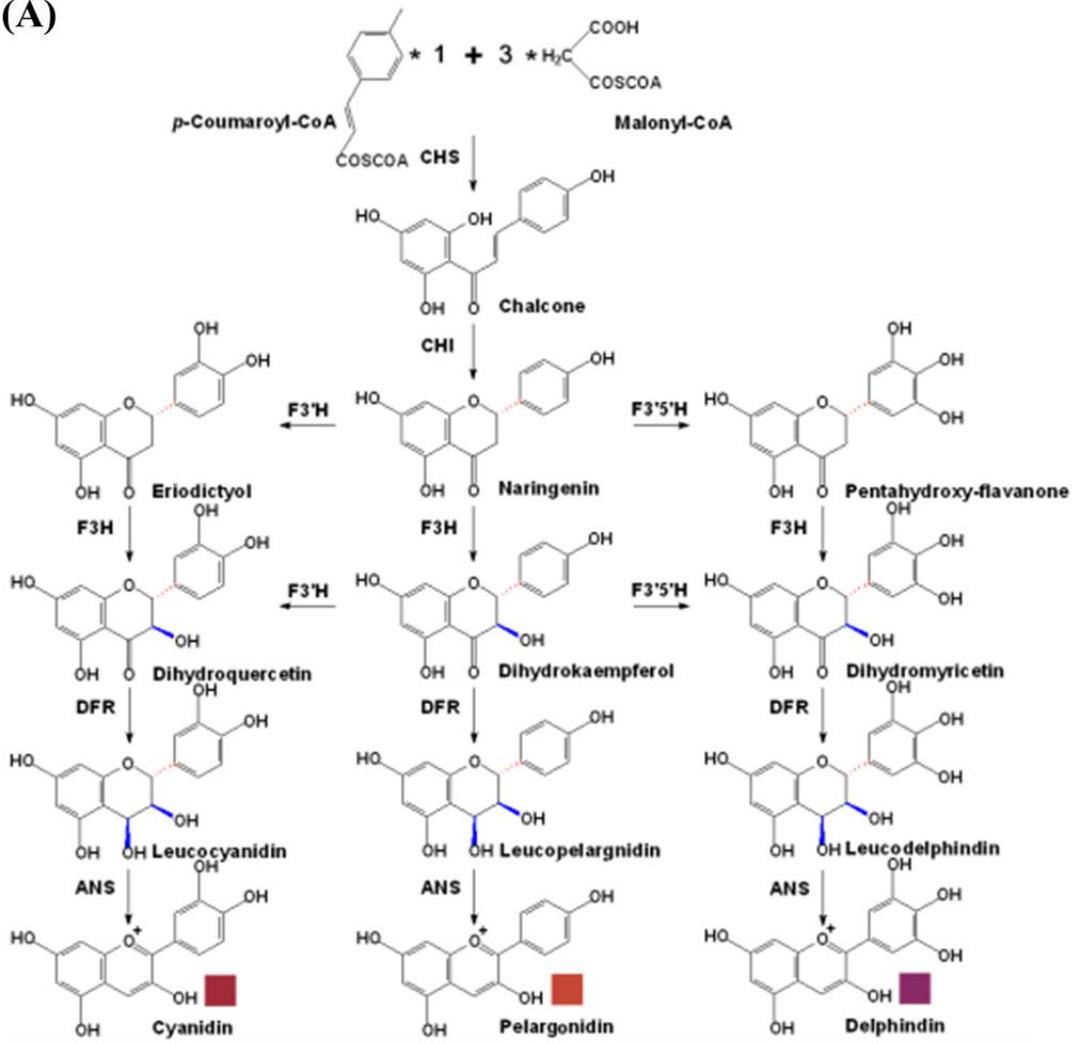


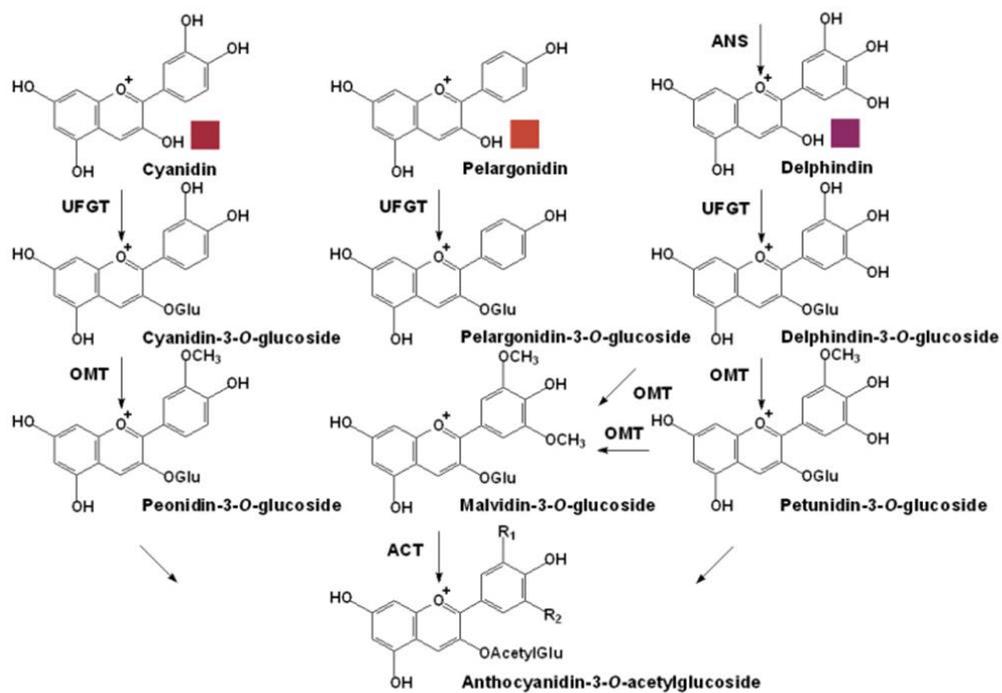
Fig. 3-2. Scheme of the flavonoid biosynthetic pathway. ACCase, acetyl-CoA carboxylase; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHKR, chalcone ketide reductase; CHI, chalcone isomerase; FS, flavone synthase; IFS, isoflavone synthase; F3H, flavanone 3 β -hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin reductase; ANS, anthocyanidin synthase; GT, glucosyltransferase; ACT, anthocyanin acyltransferase; MAT, malonyltransferase (From Springob et al., 2003).

Fig. 3-3. Schematic pathway of anthocyanins biosynthesis in grapes. (A) The basic upstream flavonoid pathway leading to the biosynthesis of colored anthocyanidins in grapes. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3 β -hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase. (B) The specific pathway for the anthocyanin modification of free anthocyanidins in grapes. UFGT, flavonoid glucosyltransferase; OMT, *O*-methyltransferase; ACT, anthocyanin acyltransferase (From He et al., 2010).

(A)



(B)



CHAPTER 4

Anti-inflammatory activities of organically grown blackberries as affected by storage

Abstract

Berries have been reported to have anti-inflammatory effects, but the effects of storage on these properties have not been thoroughly studied. Organically grown blackberries were analyzed for anti-inflammatory activity in RAW 264.7 macrophages. Berries were stored at 1°C for 15 days to determine the effects of storage on anti-inflammatory activity. After storage, blackberries were acetone-extracted. Blackberry extracts or cyanidin 3-glucoside standards were applied to RAW 264.7 cells prior to lipopolysaccharide (LPS) stimulation. Blackberry extracts significantly inhibited nitric oxide and prostaglandin E₂ (PGE₂) production and COX-2 expression in LPS-stimulated cells, and the inhibitory effect was concentration-dependent. The inhibitory effects of 'Ouachita' blackberries on nitric oxide production and COX-2 expression decreased after storage. Cyanidin 3-glucoside showed no inhibitory effects on PGE₂ and COX-2 but significantly reduced nitric oxide at low concentration (50 µg mL⁻¹). The results indicate that organically grown 'Natchez' and 'Ouachita' blackberries can inhibit inflammation by reducing nitric oxide and PGE₂ production and COX-2 expression and the inhibitory effects can decrease after storage. Results also indicate that phenolic compounds in addition to cyanidin 3-glucoside that are present in blackberries are responsible for COX-2 and PGE₂ inhibitory effects.

Introduction

Inflammation is the immunological mechanism of the body in response to cell damage and vascularized tissue (Kim et al., 2012; Yoon and Baek, 2005), and it is generally accepted that the chronic inflammation can result in various diseases such as rheumatoid arthritis, diabetes, atherosclerosis, and cancers (Wang et al., 2008). Inflammation is a complicated process that involves various cytokines, enzymes, and other metabolites (Kim et al., 2004). Nitric oxide is an important signaling molecule and a free radical produced from L-arginine by nitric oxide synthase (NOS) (Vuong et al., 2006; Wang and Mazza, 2002). Three isoforms of NOS have been identified, and the inducible form (iNOS) is an important enzyme in inflammatory processes and is induced by cytokines or other inflammation-stimulating agents such as lipopolysaccharide (LPS) (Kim et al., 2004; Vuong et al., 2006). It was suggested that nitric oxide can be either a pro- or anti-inflammatory agent (Coleman, 2001). But because it is found in the site of inflammation in various cell types and increases blood flow and vascular permeability (Weinberg, 2000), nitric oxide is believed to have an important role in inflammatory responses.

Cyclooxygenase-2 (COX-2) is another very important enzyme in the inflammatory process. COX-2 catalyzes arachidonic acid released from cell membranes and produces prostaglandin H₂ (PGH₂) (Vane et al., 1998). PGH₂ can be converted to various forms such as prostaglandin E₂ (PGE₂), prostaglandin F_{2α} (PGF_{2α}), prostaglandin D₂ (PGD₂), prostaglandin I₂ (PGI₂), and thromboxane A₂ (TXA₂). Prostaglandins can cause symptoms of vasodilation, enhanced blood flow, erythema, fluid exudation from the vasculature, and pain during

inflammation (Simon, 1999; Weinberg, 2000). PGE₂ is known to be important in inflammatory process, in particular hyperalgesia, increased sensitivity to pain (Zhang et al., 1997). It was suggested that treatments that suppress both iNOS and COX-2 can provide the most effective anti-inflammatory activity (Weinberg, 2000).

Berry fruit including blackberries contain high levels of phenolic compounds such as anthocyanins and flavonols (Benvenuti et al., 2004; Moyer et al., 2002; Pantelidis et al., 2007). Phenolic compounds have demonstrated health benefits against oxidation, cancers, inflammation, and age-related neurodegeneration (Bornsek et al., 2012; Cuevas-Rodríguez et al., 2010; Seeram et al., 2006; Shukitt-Hale et al., 2009). Phenolic content in berry fruit can be affected by genetic difference, cultural conditions and practices, and storage (Jin et al., 2011; Kalt et al., 1999; Krüger et al., 2011; Türkben et al., 2010). Total anthocyanin and phenolic contents varied within species and cultivar (Cho et al., 2004; Moyer et al., 2002; Pantelidis et al., 2007), and total anthocyanin content in ‘Earliglow’ and ‘Allstar’ strawberries increased after storage at 0, 5, 10°C for 7 days (Jin et al., 2011) or in ‘Kent’ strawberries during 8 days of storage at 0, 10, 20, and 30°C (Kalt et al., 1999) with greater increase at warmer temperature. However, total phenolic and anthocyanin contents in ‘Marion’ and ‘Evergreen’ blackberries fluctuated or decreased during storage at 2°C for 7 or 9 days (Wu et al., 2010). Although there are a number of studies on berry phenolics and how phenolic content change during storage, there is no study focusing on how anti-inflammatory activity of berries may be affected by storage. The objectives of this study were to investigate the anti-inflammatory activities of organically grown blackberries and to determine the storage effects on anti-inflammatory activities.

Materials and Methods

Plant materials

Fruit of 'Natchez' and 'Ouachita' blackberries were purchased from a certified organic farm in Bunn, NC. Plants were organically grown following US National Organic Standard, and the berries were hand-harvested at commercial ripe stage and transported to the laboratory in Kannapolis, NC, kept at 5-10°C during transportation. One group was frozen immediately at -20°C overnight and kept at -80°C to represent unstored berries, and the other group was stored at 1°C for 15 days. After storage, berries were rated for leakage and decay incidence, and berries without these characteristics were used. Berries were freeze-dried using a VirTis LyoTroll (SP Scientific, Warminster, PA, USA) and then ground using a mortar and pestle. Seeds were removed by using mesh sieves. Freeze-dried blackberry powder was kept at -80°C until used for phenolic content determination and cell culture.

Phenolic composition analysis

Freeze-dried blackberry powder was extracted to analyze anthocyanin and non-anthocyanin phenolic compounds following the method of Bradish et al. (2012) with slight modification. Blackberry powder (20 mg) was extracted with 1.5 mL of acidified methanol (formic acid:methanol:DH₂O, 1:60:39, v/v/v), vortexed, and centrifuged at 10,600 g for 20 min at 4°C. Samples were re-extracted, as 95-98% of recovery of phenolic compounds was found with double extraction in the preliminary experiment. Supernatants were combined and filtered through a 0.2 µm syringe filter, and headspace was flushed with nitrogen gas to

inhibit oxidation. All samples were kept at -80°C until analyzed.

Anthocyanins and non-anthocyanin phenolic compounds were analyzed using a high performance liquid chromatography system (Elite LaChrom, Hitachi Ltd., Tokyo, Japan) equipped with autosampler, diode array detector, and binary solvent delivery manager. D-2000 Elite software (Hitachi Ltd., Tokyo, Japan) was used. A reversed phase C18 column (Synergi 4 μ Hydro-RP 80Å, 4.6 x 250 mm Phenomenex, Torrance, CA, USA) was used at the column temperature of 30°C to separate phenolic compounds. Five percent formic acid (solvent A) and 100% methanol (solvent B) were used as mobile phases with the flow rate of 1 mL min⁻¹ and following gradient system was used: 0-5 min, 90% A; 5-15min, 85% A; 15-20 min, 80% A; 20-25 min, 75% A; 25-45 min, 70% A; 45-47 min, 40% A, 47-60 min, 90% A. Twenty microliter of sample was injected in duplicate. Anthocyanins were detected at 520 nm and standards curves of cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-xyloside, and pelargonidin 3-glucoside were used for quantification. Hydroxybenzoic acids, hydroxycinnamic acids, and flavonols were detected at 280, 320, and 360 nm, respectively, and gallic acid, vanillic acid, quercetin 3-galactoside, and quercetin 3-glucoside standards were used for identification and quantitation. Unknown anthocyanins were quantified as cyanidin 3-glucoside equivalent, and gallic acid, chlorogenic acid, and quercetin 3-glucoside were used for quantifying unknown hydroxybenzoic acids, hydroxycinnamic acids, and flavonols, respectively. Total contents were obtained as a sum of individual anthocyanins or phenolic compounds.

Total phenolic content determination

Eight gram of freeze-dried blackberry powder was extracted with 80 mL of acidified acetone (acetone:DH₂O:acetic acid, 70:68:2, v/v/v). Samples were vortexed, centrifuged at 4,000 g for 15 min, and then re-extracted. Supernatants were collected and concentrated using evaporator (TurboVap II, Biotage AB, Uppsala, Sweden) under N₂ pressure of 20 psi. Concentrated extracts were kept at -80°C until used.

Total phenolic content of extracts was determined by using a microplate reader (Enspire 2300 Multilabel Reader, PerkinElmer, Waltham, MA). Samples or gallic acid standard of 50 µL was added to a 96-well microplate in triplicate and 50 µL of 1 N Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO) was added. After 5 min of incubation at room temperature, 100 µL of 20% Na₂CO₃ was added followed by 10 min of incubation. Phenolic compounds were detected at 690 nm, and total phenolic content was calculated based on a standard curve of gallic acid.

Cell culture

RAW 264.7 cells (ATCC, Manassas, VA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO) containing 5% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO) at 37°C and 5% CO₂. Cells were plated at a concentration of 1.5×10^5 cells/well in 96-well plates for MTT assay and nitric oxide analysis, and 7.5×10^5 cells/well in 12-well plates to analyze PGE₂ production and COX-2 expression. After incubating for 10

h at 37°C and 5% CO₂, cells were treated with acetone extracts of 'Natchez' and 'Ouachita' blackberries at final concentrations of 0.01, 0.015, and 0.02 mg gallic acid equivalents mL⁻¹ or with cyanidin 3-glucoside at concentrations of 50 and 100 µg mL⁻¹. Cells were incubated for 2 h, then LPS (Sigma-Aldrich, St. Louis, MO) was added at a final concentration of 2 µg mL⁻¹. Plates were incubated for another 12 h. Then MTT solution was treated for cell viability test or cell media was immediately analyzed for nitric oxide determination. Other media aliquots and lysates were harvested and stored at -80°C until PGE₂ analysis and Western blotting COX-2, respectively.

Cell viability

MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay was employed to test the cell viability. Cells at a density of 1.5×10^5 cells/well were plated in 96-well plates and treated with blackberry extract or cyanidin 3-glucoside standard and LPS as described above. Cells in each well were treated with 16 µL of MTT solution (7.8 mg mL⁻¹) (Sigma-Aldrich, St. Louis, MO) and incubated for 3 h at 37°C and 5% CO₂. After incubation, media was carefully removed not to disrupt formed crystals. Crystals were dissolved in 200 µL of acidified isopropanol (hydrochloric acid:isopropanol, 1.66:50, v/v). Then 200 µL was transferred to 96-well plates to measure absorbance at 620 nm using microplate reader (Enspire 2300 Multilabel Reader, PerkinElmer, Waltham, MA). Cell viability was calculated as a relative ratio of each sample to the negative control.

Nitric oxide determination

Nitric oxide production was measured by analyzing nitrite, which is a stable metabolite of nitric oxide. To determine nitrite, 96-well plates with cells were prepared as described above and a Griess reagent (Promega, Madison, WI) was used following manufacturer's protocol. In brief, 50 μ L of sample media or sodium nitrite standard was added to a 96-well plate in triplicate, and 50 μ L of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) was added. After 10 min of incubation at room temperature in the dark, 50 μ L of NED solution (0.1% N-1-naphthylethylenediamine dihydrochloride in water) was added. Plates were incubated in the dark and at room temperature for 10 min before measuring absorbance at 540 nm. Samples containing anthocyanins were treated with 100 μ L of 2.5% phosphoric acid to obtain the net absorbance of nitrite produced. Standard curve of sodium nitrite was used to quantify nitrite concentration in the samples.

Determination of total protein content

Total protein content was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo Scientific) prior to the Western blot. To analyze total protein content, 25 μ L of cell lysate or bovine serum albumin (BSA) standard was added to a 96-well microplate in triplicate, and 200 μ L of working reagent was added. Plates were covered and incubated at 37°C for 30 min. Absorbance was read at 562 nm using a microplate reader (Enspire 2300 Multilabel Reader, PerkinElmer, Waltham, MA). Standard curve of BSA was used to quantify

total protein content.

Western blot of COX-2

COX-2 expression was analyzed following the method of Jordan (2013) with slight modification. Cell lysates were thawed and aliquot was mixed with sample buffer based on the BCA assay results to make total protein content the same in all samples, and then heated at 100°C for 10 min before loading. Pre-stained protein marker and protein samples were loaded onto a 12% Tris-Glycine precast gel (Life Technologies, Carlsbad, CA) and separated by electrophoresis at 125 mV for 1.5 h. Proteins were transferred to PVDF membrane using iBlot® Dry Blotting System (Life Technologies, Carlsbad, CA) for 7 min at room temperature. The membranes were blocked using 5% BSA in tris buffered saline (TBS) for non-specific binding at room temperature for 4 h on a shaker (Orbital Lab Shaker, Labnet Orbit LS, Edison, NJ). After blocking, membranes were incubated with the primary antibody buffer (COX-2, mouse polyclonal, Cayman Chemical Company, Ann Arbor, MI) overnight at 4°C on a rocker (Mini BlotBoy™, Benchmark Scientific Inc., Edison, NJ). The membranes were washed 3 x 10 min with TBS and then incubated with the secondary antibody buffer (Anti-rabbit IgG, HRP-linked Antibody, Cell Signaling Technology, Danvers, MA) for 1.5 h at room temperature on a shaker (Orbital Lab Shaker, Labnet Orbit LS, Edison, NJ). After incubation, membranes were washed 3 x 10 min with TBS-T (1% Tween-20 in TBS) and then incubated with a chemiluminescent substrate (Clarity™ Western ECL Substrate, Bio-Rad, Hercules, CA) for 5 min at room temperature prior to exposure to X-ray film

(BioExpress, Kaysville, UT).

After COX-2 blotting, membranes were stripped and re-probed to blot β -actin as an internal control. Membranes were re-hydrated in 100% methanol for 15 sec, rinsed with deionized water, and then incubated in stripping buffer at room temperature for 1 h on a shaker (Orbital Lab Shaker, Labnet Orbit LS, Edison, NJ). Stripped membranes were rinsed 3 x 5 min with TBS and blocked using 5% BSA at room temperature for 4 h on a shaker (Orbital Lab Shaker, Labnet Orbit LS, Edison, NJ). Membranes were incubated with the primary antibody (β -Actin (13E5) Rabbit mAb, Cell Signaling Technology, Danvers, MA) and secondary antibody (Anti-rabbit IgG, HRP-linked Antibody, Cell Signaling Technology, Danvers, MA) buffers, and washed as described above, and the same chemiluminescent detection procedures were used.

PGE₂ analysis

PGE₂ production was analyzed using PGE₂ monoclonal EIA kit (Cayman Chemical Company, Ann Arbor, MI) following the manufacturer's protocol. Cell media was analyzed at two dilutions. In brief, 50 μ L of DMEM was added to non-specific binding (NSB) and maximum binding (B₀), and 50 μ L of EIA buffer was added to NSB. Then 50 μ L of PGE₂ standard or sample was added followed by 50 μ L of tracer to all wells except for total activity (TA) and blank. Antibody of 50 μ L was added to standard, samples, and B₀. Plates were covered and incubated for 18 h at 4°C. After incubation, buffers were discarded and all wells were rinsed 5 times with wash buffer. Two hundred microliter of Ellman's reagent was added

followed by 5 μ L of tracer to TA. Plates were covered and incubated for 60-90 min on a shaker (Orbital Lab Shaker, Labnet Orbit LS, Edison, NJ) at room temperature. Absorbance was read at 420 nm using microplate reader (Enspire 2300 Multilabel Reader, PerkinElmer, Waltham, MA), and PGE₂ level was calculated using PGE₂ standard curve.

Results and Discussion

Phenolic composition

Anthocyanin and non-anthocyanin phenolic composition was analyzed (Table 4-1). In both cultivars, cyanidin 3-glucoside, cyanidin 3-rutinoside, pelargonidin 3-glucoside, and cyanidin 3-xyloside were identified, and cyanidin 3-glucoside was the predominant anthocyanin. These results are in agreement with previous studies. Cyanidin 3-glucoside was found to be the predominant anthocyanin in blackberries whereas other anthocyanins such as cyanidin 3-rutinoside, cyanidin 3-xyloside, cyanidin 3-malonylglucoside, and cyanidin 3-dioxalylglucoside were also present (Cho et al., 2004; Fan-Chiang and Wrolstad, 2005). Anthocyanin composition in 'Natchez' and 'Ouachita' blackberries differed in cyanidin 3-xyloside. Proportion of cyanidin 3-xyloside in 'Natchez' blackberries was below 0.2% of the total anthocyanin content while that in 'Ouachita' blackberries was about 4.5% (data not shown). Gallic acid, vanillic acid, quercetin 3-galactoside, and quercetin 3-glucoside were found as non-anthocyanin phenolic compounds in 'Natchez' and 'Ouachita' blackberries at both storage regimes. Content and proportion of vanillic acid differed between cultivars. Proportion of vanillic acid of the total phenolic content in 'Natchez' and 'Ouachita'

blackberries was 5 and 26-29%, respectively (data not shown). In *Rubus plicatus* blackberries, gallic acid, vanillic acid, salicylic acid, caffeic acid, *m*- and *p*-coumaric acids, and hydroxycaffeic acid were found (Zadernowski et al., 2005), and quercetin 3-galactoside and quercetin 3-glucoside were reported as the major flavonols in blackberries depending on cultivar (Cho et al., 2004; Cho et al., 2005). Jakobek et al. (2009) reported ellagic acid and flavan 3-ols of (+)-catechin and (-)-epicatechin in *R. fruticosus* blackberries. However, those compounds were not detected in this study, and this is possibly because pyrenes (seeds), which is a major source of ellagic acid and flavan 3-ols in blackberry fruit (Siriwoharn and Wrolstad, 2004), were not used for analyzing phenolic compounds.

Pelargonidin 3-glucoside and cyanidin 3-xyloside contents in ‘Natchez’ blackberries decreased after storage but other compounds did not change (Table 4-1). In contrast, cyanidin 3-glucoside, pelargonidin 3-glucoside, cyanidin 3-xyloside, and quercetin 3-galactoside contents increased after storage, and these changes resulted in increased total anthocyanin and phenolic contents in ‘Ouachita’ blackberries. However, cyanidin 3-rutinoside and gallic acid contents decreased in ‘Ouachita’ blackberries after storage. Phenolic contents in berry fruit have been reported to change during storage. Cyanidin 3-sophoroside, cyanidin 3-rutinoside, and cyanidin 3-glucoside content in ‘Tulameen’ raspberries increased when stored for one day at 20°C or for three days at 2-4°C followed by one day at 20°C (Krüger et al., 2011). However, cyanidin 3-glucosylrutinoside content did not change. Türkben et al. (2010) reported quercetin content in ‘Chester’ blackberries increased in just frozen (5 h at -35°C) and frozen (6 months at -22°C after freezing at -35°C for 5 h) while that of ‘Bursa 2’ blackberries decreased in both freezing treatments. As shown in these studies, phenolic

contents can change during storage and the storage effects vary with species, cultivar, or storage condition.

Total phenolic content

Total phenolic content in 'Natchez' and 'Ouachita' blackberries ranged from 14.3 to 17.4 mg of gallic acid equivalent g^{-1} DW and did not change after storage in both cultivars (Fig. 4-1). It is generally accepted that total phenolic content can vary with genotype or storage (Jin et al., 2011; Kalt et al., 1999; Wu et al., 2010) but the total phenolic content of blackberries used in this study was not affected by storage regime. Storage effects on the total phenolic content in berries differ among studies. Wu et al. (2010) reported decreased total phenolic content after 7 or 9 days at 2°C in 'Evergreen' and 'Marion' blackberries, respectively. Total phenolic content of organically grown 'Earliglow' and 'Allstar' strawberries increased after 7 days of storage at 0, 5, and 10°C and 5 and 10°C, respectively (Jin et al., 2011). Ayala-Zavala et al. (2004) reported that total phenolic content in 'Chandler' strawberries increased during storage at 5 and 10°C but did not change when stored at 0°C. These difference might be related with genetic difference, phenolic composition, and enzyme activity. In particular, Folin-Ciocalteu reagent is not specific to phenolic compounds. It measures the reducing capacity of the samples, so the results can reflect other compounds that can reduce the Folin-Ciocalteu reagent such as ascorbic acid and sugars (Huang et al., 2005; Prior et al., 2005). However, the Folin-Ciocalteu method is a simple and inexpensive way to estimate the total phenolic content in samples and therefore it has been widely used for total phenolic content

analysis.

Cell viability

Cell viability was assayed to determine if blackberry extracts and cyanidin 3-glucoside had cytotoxicity. The effect of blackberry extracts on cell viability varied with cultivar, concentration, and storage (Fig. 4-2). Relative to the negative control, low concentrations (0.01 mg mL⁻¹) of 'Natchez' and 'Ouachita' extracts did not significantly lower viability. However, all of the medium and high blackberry extract concentrations (0.015 and 0.02 mg mL⁻¹) significantly lowered cell viability. The highest extract concentrations lowered viability to the greatest degree. For the 'Natchez' extract, cell viability was significantly lower in medium and high concentrations for cells treated with extracts from blackberries stored for 15 days compared to those treated with extracts from berries that were not stored. Storage did not significantly change the effects of concentration in the 'Ouachita' extracts. Cyanidin 3-glucoside showed no cytotoxicity at either treated concentrations. The three concentrations of blackberry extracts were based on gallic acid equivalents, and cyanidin 3-glucoside content in blackberry extracts was lower than in standard treatments (50 and 100 µg mL⁻¹), ranging from 7.1 to 32.5 µg mL⁻¹ depending on treatment (data not shown). These results indicate that non-anthocyanin phenolic compounds were responsible for the decreased viability at treated concentrations. Since blackberry extract significantly affected cell viability, reported values for nitric oxide and PGE₂ were adjusted to account for this.

Decreasing cell viability in response to increasing concentration of berry extracts or

phenolic standards has been reported (Poulose et al., 2012; Wang and Mazza, 2002). It was reported that flavonoids can be antioxidant or pro-oxidant depending on concentration, cell type, and cultural condition, and cytotoxicity of flavonoids is possibly due to pro-oxidant properties (Matsuo et al., 2005).

Nitric oxide production

Nitric oxide concentration was measured as nitrite in the culture media, and nitrite inhibition was calculated by subtracting a relative ratio of the nitrite level in each sample to that in the positive control from 100 (Fig. 4-3). Due to cytotoxicity of blackberry extracts, all nitrite levels were adjusted for cell viability. LPS significantly increased nitrite level in the cell media (data not shown) and blackberry extracts inhibited nitrite production in a concentration-dependent manner. The cyanidin 3-glucoside standard had a significant inhibitory effect at 50 $\mu\text{g mL}^{-1}$ while that at 100 $\mu\text{g mL}^{-1}$ showed less effect on nitrite inhibition. Even after adjusting nitrite inhibition for cell viability, the inhibitory effect of blackberry extracts was the highest at high concentrations (0.02 mg mL^{-1}). At this concentration, blackberry extracts reduced nitrite production 72-82% depending on cultivar and storage treatment.

Inhibitory effect of extracts from both blackberry cultivars did not change after storage except for 'Ouachita' blackberry extract at 0.01 mg mL^{-1} . Phenolic contents analyzed by HPLC increased after storage in 'Natchez' and 'Ouachita' blackberries although the increase in 'Natchez' blackberries was not significant (Table 4-1). These results indicate that phenolic

composition is important as well as total content in inhibiting nitric oxide production. It was reported that inhibition of nitrite differed among tested phenolic compounds (Wang and Mazza, 2002).

Dose-dependent inhibitory effects of berry extracts and phenolic compounds against nitric oxide production have been reported (Cuevas-Rodríguez et al., 2010; Pergola et al., 2006; Schreckinger et al., 2010; Wang and Mazza, 2002). Cuevas-Rodríguez et al. (2010) investigated the inhibitory activity of six genotypes of blackberries grown in Mexico by applying extracts (polyphenol, anthocyanin, and proanthocyanidin fractions) to RAW 264.7 cells and observed over 60% of inhibition from polyphenol and anthocyanin fractions at 50 μM in all genotypes. However, using a 5 μM extract yielded about 20% inhibition. Significant and dose-dependent inhibition of blackberry extracts was reported in J774 macrophages with the inhibition of 23, 34, 60, and 70% at 11, 22, 45, and 90 $\mu\text{g mL}^{-1}$, respectively (Pergola et al., 2006). Concentrated extracts of Saskatoon berries, blueberries, blackberries, and black currants showed significant inhibition of nitrite production that was comparable with flavonol standards while crude extracts hardly inhibited nitrite production in RAW 264.7 cells stimulated with LPS and interferon- γ (IFN- γ) (Wang and Mazza, 2002). The inhibitory effect slightly differed among berry types, possibly due to different phenolic content and composition. Proanthocyanidin-enriched extracts of *Aristotelia chilensis* (maqui berry) and *Vaccinium floribundum* (mortiño) also showed significant inhibition of nitrite production in LPS-stimulated RAW 264.7 cells (Schreckinger et al., 2010). However, the inhibitory activity of berry extracts was lower than that of quercetin. Wang and Mazza (2002)

reported that phenolic standards suppressed nitrite production in LPS/IFN- γ -stimulated RAW 264.7 cells, and the inhibitory effect differed among compounds. Kaempferol and quercetin inhibited nitrite production at 16 μ M but the inhibitory effect decreased with glycosylation. Cyanidin, malvidin, pelargonidin, delphinidin, and peonidin showed concentration-dependent inhibition in RAW 264.7 cells stimulated with LPS/IFN- γ , and glycosylation also reduced the inhibitory effect of anthocyanins. It was suggested that penetrating cell membranes of flavonoid glycosides may be inhibited due to their hydrophilicity (Kim et al., 1999). In addition, inhibitory effect varied with type of compound, indicating that structural difference such as number of hydroxyl groups or degree of methylation may affect inhibitory activity of anthocyanins (Wang and Mazza, 2002).

COX-2 expression

LPS-treated cells showed significantly higher COX-2 expression while COX-2 was not detected in untreated cells (a negative control) (Fig. 4-4). Cyanidin 3-glucoside did not have inhibitory activity but blackberry extracts suppressed the expression of COX-2 at all treated concentrations in a concentration-dependent manner. Blackberry extracts at medium and high concentrations (0.015 and 0.02 mg mL⁻¹) lowered cell viability (Fig. 4-2). But extracts of both 'Natchez' and 'Ouachita' blackberries at a concentration of 0.01 mg mL⁻¹ showed significant inhibitory effects on COX-2 expression without affecting cell viability. Moreover, total protein amount for the Western blot was the same in all treatments, therefore the suppression of COX-2 expression can be due to the treatment effect, not just cytotoxicity.

Storage did not affect the inhibitory effect of extracts from 'Natchez' blackberries while extracts from stored 'Ouachita' showed lower inhibitory activity at 0.015 and 0.02 mg mL⁻¹ compared to those from the berries that were not stored. Again, the inhibitory effects seem to be closely related with phenolic composition, not just total content.

In general, our results were similar to those of Cuevas-Rodríguez et al. (2010) who reported dose-dependent inhibition of COX-2 expression by applying extracts of Mexican blackberries. Açai berry extract also suppressed COX-2 expression in BV-2 murine microglial cells and the inhibitory effect varied among different fractions of extracts (Poulose et al., 2012). Ethanol and methanol fractions showed the greatest inhibition of COX-2 and contained high level of phenolic compounds, in particular anthocyanins and ferulic acid, compared to the acetone and ethyl acetate fractions. Ethanol and methanol fractions contained higher levels of total phenolic content and some phenolic compounds such as cyanidin glucoside and delphinidin glucoside, which were not detected or detected in low level in ethyl acetate and acetone fractions. It was suggested that there might be a synergistic effects of these phenolic compounds on tumor necrosis factor- α , an inflammatory cytokine. The results of this study also showed that blackberry extracts, which contain various phenolic compounds such as anthocyanins and flavonols, had greater inhibitory effect on COX-2 expression compared to a single compound cyanidin 3-glucoside.

PGE₂ production

LPS treatment significantly increased PGE₂ level while the negative control showed a

detectable amount of PGE₂ (Fig. 4-5). Blackberry extracts reduced the PGE₂ level at all treated concentrations, in contrast to no inhibitory effect of cyanidin 3-glucoside at both concentrations. Although blackberry extracts at medium and high concentrations (0.015 and 0.02 mg mL⁻¹) showed cytotoxicity in both cultivars (Fig. 4-2), adjusted PGE₂ levels were significantly low compared to the positive control. The inhibitory effect of blackberry extracts was not different among concentrations in extracts of 'Natchez' blackberries while extracts of 'Ouachita' blackberries after 15 days of storage showed greater inhibitory activity at 0.02 mg mL⁻¹ compared to the concentration of 0.01 mg mL⁻¹. Storage did not significantly affect inhibitory activity of blackberry extracts from both cultivars. Content of individual phenolic compounds changed in 'Ouachita' blackberries in response to storage and total content increased (Table 4-1), indicating that the inhibitory activity can be affected by the change of composition of phenolic compounds, not just total content. Nitric oxide and PGE₂ are important mediators in inflammation but they are produced via different pathways (Coleman, 2002; Yoon and Baek, 2005), and this might explain why the inhibitory effects of blackberries and cyanidin 3-glucoside on nitric oxide and PGE₂ slightly differed in the present study. In the present study, the effects of blackberry extracts on COX-2 expression showed a similar trend to that for PGE₂ production. This can be due to the biosynthetic pathway of PGE₂. COX-2 catalyzes arachidonic acid to PGH₂, which can subsequently be converted to PGE₂ (Vane et al., 1998). Therefore, suppressed COX-2 could result in inhibited PGE₂ production.

Berry extracts and phenolic compounds have been studied for anti-inflammatory activity against PGE₂ production in cell models. Polyphenol, anthocyanin, and proanthocyanidin

fraction of four wild Mexican blackberries (*Rubus adenotrichus*, *R. corifolius*, and *R. glaucus*), a commercial cultivar ‘Tupy’, and a breeding line inhibited PGE₂ production in LPS-stimulated RAW 264.7 cells, and proanthocyanidin-enriched extracts had a slightly higher inhibitory effect (Cuevas-Rodríguez et al., 2010). Extracts of *A. chilensis* and *V. floribundum* (Schreckinger et al., 2010) and *R. coreanus* black raspberry extracts (Kim et al., 2013) also reduced PGE₂ production in LPS-stimulated RAW 264.7 cells. Hämäläinen et al. (2011) tested 26 phenolic standards for anti-inflammatory activity in LPS-stimulated J774 cells and reported significant inhibition of PGE₂ by 16 compounds. However, cyanidin showed no effect on PGE₂ production. This result was in agreement with the results of this study where no inhibitory activity of cyanidin 3-glucoside was found in LPS-stimulated RAW 264.7 cells. In contrast, other phenolic compounds such as gallic acid, quercetin, and pelargonidin showed significant inhibitory effect on PGE₂ production (Hämäläinen et al., 2011; Wang and Mazza, 2002) and may contribute to high inhibitory activity of blackberries used in this study.

Conclusion

In the present study, extracts from organically grown ‘Natchez’ and ‘Ouachita’ blackberries held for 0 and 15 days at 1°C were investigated for their anti-inflammatory activity using RAW 264.7 macrophage cells. Although blackberry extracts lowered cell viability at 0.015 and 0.02 mg mL⁻¹, cell viability adjusted results showed that blackberry extracts inhibited nitric oxide production in a concentration-dependent manner. The cyanidin

3-glucoside standard showed the greater inhibitory effect at 50 $\mu\text{g mL}^{-1}$ than at 100 $\mu\text{g mL}^{-1}$. COX-2 expression and PGE₂ production were significantly suppressed by blackberry extracts while cyanidin 3-glucoside did not show inhibitory activity. The results of this study suggest that organically grown 'Natchez' and 'Ouachita' blackberries can inhibit inflammation by reducing nitric oxide and PGE₂ production and suppressing COX-2 expression at cell levels. Given that cyanidin 3-glucoside level in blackberry extracts was lower than in cyanidin 3-glucoside standard treatment, the mixture of various phenolic compounds may have greater effects compared to cyanidin 3-glucoside on inhibiting COX-2 expression and PGE₂ production. Storage did not affect the inhibitory activity of extracts from 'Natchez' blackberries while the extracts of stored 'Ouachita' showed lower inhibitory activity. The results also suggest that composition of those phenolic compounds, not just total content, can have great impact on inhibitory effects on PGE₂ and COX-2.

Literature Cited

- Ayala-Zavala, J.F., S.Y. Wang, C.Y. Wang, and G.A. González-Aguilar. 2004. Effect of storage temperatures on antioxidant capacity and aroma compounds in strawberry fruit. *LWT-Food Sci. Technol.* 37:687-695.
- Benvenuti, S., F. Pellati, M. Melegari, and D. Bertelli. 2004. Polyphenols, anthocyanins, ascorbic acid, and radical scavenging activity of *Rubus*, *Ribes*, and *Aronia*. *J. Food Sci.* 69:C164-C169.
- Bornsek, S.M., L. Ziberna, T. Polak, A. Vanzo, N.P. Ulrih, V. Abram, F. Tramer, and S. Passamonti. 2012. Bilberry and blueberry anthocyanins act as powerful intracellular antioxidants in mammalian cells. *Food Chem.* 134:1878-1884.
- Bradish, C.M., P. Perkins-Veazie, G.E. Fernandez, G.X. Xie, and W. Jia. 2012. Comparison of flavonoid composition of red raspberries (*Rubus idaeus* L.) grown in the Southern United States. *J. Agric. Food Chem.* 60:5779-5786.
- Cho, M.J., L.R. Howard, R.L. Prior, and J.R. Clark. 2004. Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* 84:1771-1782.
- Cho, M.J., L.R. Howard, R.L. Prior, and J.R. Clark. 2005. Flavonol glycosides and antioxidant capacity of various blackberry and blueberry genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* 85:2149-2158.

- Coleman, J.W. 2001. Nitric oxide in immunity and inflammation. *Int. Immunopharmacol.* 1:1397-1406.
- Coleman, J.W. 2002. Nitric oxide: A regulator of mast cell activation and mast cell-mediated inflammation. *Clin. Exp. Immunol.* 129:4-10.
- Cuevas-Rodríguez, E.O., V.P. Dia, G.G. Yousef, P.A. García-Saucedo, J. López-Medina, O. Paredes-López, E.G. De Mejia, and M.A. Lila. 2010. Inhibition of pro-inflammatory responses and antioxidant capacity of Mexican blackberry (*Rubus* spp.) extracts. *J. Agric. Food Chem.* 58:9542-9548.
- Fan-Chiang, H.J. and R.E. Wrolstad. 2005. Anthocyanin pigment composition of blackberries. *J. Food Sci.* 70:C198-C202.
- Hämäläinen, M., R. Nieminen, M.Z. Asmawi, P. Vuorela, H. Vapaatalo, and E. Moilanen. 2011. Effects of flavonoids on prostaglandin E₂ production and on COX-2 and mPGES-1 expressions in activated macrophages. *Planta Medica* 77:1504-1511.
- Huang, D.J., B.X. Ou, and R.L. Prior. 2005. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* 53:1841-1856.
- Jakobek, L., M. Šeruga, B. Šeruga, I. Novak, and M. Medvidović-Kosanović. 2009. Phenolic compound composition and antioxidant activity of fruits of *Rubus* and *Prunus* species from Croatia. *Int. J. Food Sci. Technol.* 44:860-868.
- Jin, P., S.Y. Wang, C.Y. Wang, and Y.H. Zheng. 2011. Effect of cultural system and storage temperature on antioxidant capacity and phenolic compounds in strawberries. *Food Chem.* 124:262-270.
- Jordan, C., 2013. The effects of aromatic alcohols on LPS and IFN- γ induced inflammation

in RAW 264.7 macrophage cells. North Carolina State University, Raleigh, Master Thesis.

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

Kim, E.O., K.J. Min, T.K. Kwon, B.H. Um, R.A. Moreau, and S.W. Choi. 2012. Anti-inflammatory activity of hydroxycinnamic acid derivatives isolated from corn bran in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Food Chem. Toxicol.* 50:1309-1316.

Kim, H.K., B.S. Cheon, Y.H. Kim, S.Y. Kim, and H.P. Kim. 1999. Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. *Biochem. Pharmacol.* 58:759-765.

Kim, H.P., K.H. Son, H.W. Chang, and S.S. Kang. 2004. Anti-inflammatory plant flavonoids and cellular action mechanisms. *J. Pharmacol. Sci.* 96:229-245.

Kim, S.K., H. Kim, S.A. Kim, H.K. Park, and W. Kim. 2013. Anti-Inflammatory and anti-superbacterial activity of polyphenols isolated from black raspberry. *Korean J. Physiol. Pharmacol.* 17:73-79.

Krüger, E., H. Dietrich, E. Schöpplein, S. Rasim, and P. Kürbel. 2011. Cultivar, storage conditions and ripening effects on physical and chemical qualities of red raspberry fruit. *Postharvest Biol. Technol.* 60:31-37.

Matsuo, M., N. Sasaki, K. Saga, and T. Kaneko. 2005. Cytotoxicity of flavonoids toward cultured normal human cells. *Biol. Pharm. Bull.* 28:253-259.

- 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.
- Pantelidis, G.E., M. Vasilakakis, G.A. Manganaris, and G. Diamantidis. 2007. Antioxidant capacity, phenol, anthocyanin and ascorbic acid contents in raspberries, blackberries, red currants, gooseberries and Cornelian cherries. *Food Chem.* 102:777-783.
- Pergola, C., A. Rossi, P. Dugo, S. Cuzzocrea, and L. Sautebin. 2006. Inhibition of nitric oxide biosynthesis by anthocyanin fraction of blackberry extract. *Nitric Oxide-Biol. Chem.* 15:30-39.
- Poulose, S.M., D.R. Fisher, J. Larson, D.F. Bielinski, A.M. Rimando, A.N. Carey, A.G. Schauss, and B. Shukitt-Hale. 2012. Anthocyanin-rich açai (*Euterpe oleracea* Mart.) fruit pulp fractions attenuate inflammatory stress signaling in mouse brain BV-2 microglial cells. *J. Agric. Food Chem.* 60:1084-1093.
- Prior, R.L., X.L. Wu, and K. Schaich. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* 53:4290-4302.
- Schreckinger, M.E., J.Z. Wang, G. Yousef, M.A. Lila, and E.G. de Mejia. 2010. Antioxidant capacity and in vitro Inhibition of adipogenesis and inflammation by phenolic extracts of *Vaccinium floribundum* and *Aristotelia chilensis*. *J. Agric. Food Chem.* 58:8966-8976.
- Seeram, N.P., L.S. Adams, Y.J. Zhang, R. Lee, D. Sand, H.S. Scheuller, and D. Heber. 2006. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food*

Chem. 54:9329-9339.

Shukitt-Hale, B., V. Cheng, and J.A. Joseph. 2009. Effects of blackberries on motor and cognitive function in aged rats. *Nutr. Neurosci.* 12:135-140.

Simon, L.S. 1999. Role and regulation of cyclooxygenase-2 during inflammation. *Amer. J. Med.* 106:37S-42S.

Siriwoharn, T. and R.E. Wrolstad. 2004. Polyphenolic composition of Marion and Evergreen blackberries. *J. Food Sci.* 69:C233-C240.

Türkben, C., E. Sariburun, C. Demir, and V. Uylaşer. 2010. Effect of freezing and frozen storage on phenolic compounds of raspberry and blackberry cultivars. *Food Anal. Meth.* 3:144-153.

Vane, J.R., Y.S. Bakhle, and R.M. Botting. 1998. Cyclooxygenases 1 and 2. *Annu. Rev. Pharmacol. Toxicol.* 38:97-120.

Vuong, T., L. Martin, and C. Matar. 2006. Antioxidant activity of fermented berry juices and their effects on nitric oxide and tumor necrosis factor-alpha production in macrophages 264.7 gamma NO(-) cell line. *J. Food Biochem.* 30:249-268.

Wang, J. and G. Mazza. 2002. Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN- γ -activated RAW 264.7 macrophages. *J. Agric. Food Chem.* 50:850-857.

Wang, Q., M. Xia, C. Liu, H.H. Guo, Q.Y. Ye, Y. Hu, Y.H. Zhang, M.J. Hou, H.L. Zhu, J. Ma, and W.H. Ling. 2008. Cyanidin-3-O- β -glucoside inhibits NOS and COX-2 expression by inducing liver X receptor alpha activation in THP-1 macrophages. *Life Sci.* 83:176-184.

Weinberg, J.B. 2000. Nitric oxide synthase 2 and cyclooxygenase 2 interactions in

inflammation. *Immunol. Res.* 22:319-341.

Wu, R.Y., B. Frei, J.A. Kennedy, and Y.Y. Zhao. 2010. Effects of refrigerated storage and processing technologies on the bioactive compounds and antioxidant capacities of 'Marion' and 'Evergreen' blackberries. *LWT-Food Sci. Technol.* 43:1253-1264.

Yoon, J.H. and S.J. Baek. 2005. Molecular targets of dietary polyphenols with anti-inflammatory properties. *Yonsei Med. J.* 46:585-596.

Zadernowski, R., M. Naczek, and J. Nesterowicz. 2005. Phenolic acid profiles in some small berries. *J. Agric. Food Chem.* 53:2118-2124.

Zhang, Y., A. Shaffer, J. Portanova, K. Seibert, and P.C. Isakson. 1997. Inhibition of cyclooxygenase-2 rapidly reverses inflammatory hyperalgesia and prostaglandin E₂ production. *J. Pharmacol. Expt. Ther.* 283:1069-1075.

Table 4-1. Anthocyanin and non-anthocyanin phenolic compounds in organically grown ‘Natchez’ and ‘Ouachita’ blackberries at 0 and 15 days after storage.

Cultivar	Storage condition ^x	Anthocyanins (mg g ⁻¹ DW) ^z				
		C3G	C3R	P3G	C3X	Total ^w
Natchez	Day 0	25.82 ^y	0.39	0.26*	0.04*	28.11
	Day 15	25.23	0.41	0.17*	0.001*	27.67
Ouachita	Day 0	15.88*	0.10*	0.06*	0.77*	17.74*
	Day 15	20.69*	0.09*	0.13*	1.01*	23.28*
		Non-anthocyanin phenolics (mg 100 g ⁻¹ DW) ^z				
		GA	VA	Q-gal	Q-glu	Total
Natchez	Day 0	10.17	21.10	22.67	19.08	423.16
	Day 15	6.94	23.05	28.57	22.29	474.38
Ouachita	Day 0	9.94*	76.61	11.29*	5.99	274.45*
	Day 15	6.17*	75.55	16.81*	7.23	314.39*

^zC3G, cyanidin 3-glucoside; C3R, cyanidin 3-rutinoside; P3G, pelargonidin 3-glucoside; C3X, cyanidin 3-xyloside; GA, gallic acid; VA, vanillic acid; Q-gal, quercetin 3-galactoside; Q-glu, quercetin 3-glucoside.

^yAsterisks (*) shows significance between storage treatment in each cultivar by Fisher’s LSD at $p < 0.05$.

^xDay 0, not stored berries; Day 15, berries stored at 1°C for 15 days.

^wTotal content represents the sum of content of individual compounds including unidentified compounds.

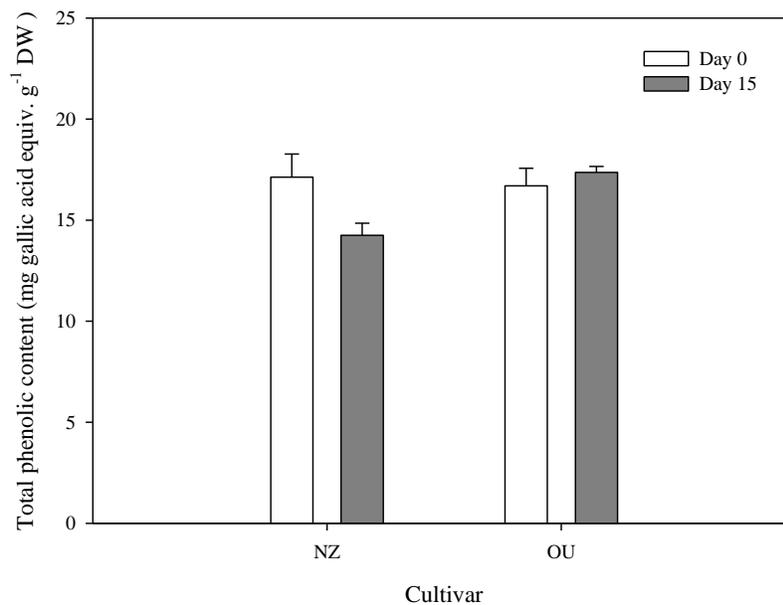


Fig. 4-1. Total phenolic content in organically grown ‘Natchez’ and ‘Ouachita’ blackberries at 0 and 15 days after storage. No significant difference was found among treatments, between cultivar or storage date by Fisher’s LSD at $p < 0.05$. NZ, Natchez; OU, Ouachita; Day 0, unstored; Day15, 15 days of storage at 1°C.

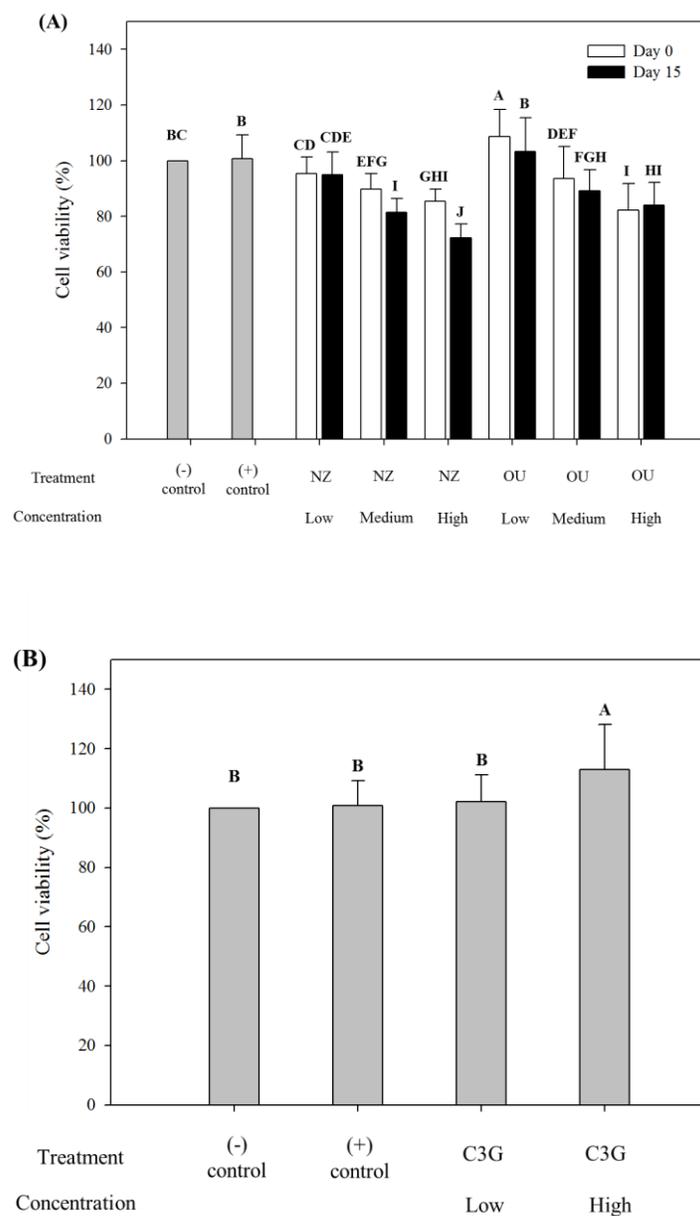
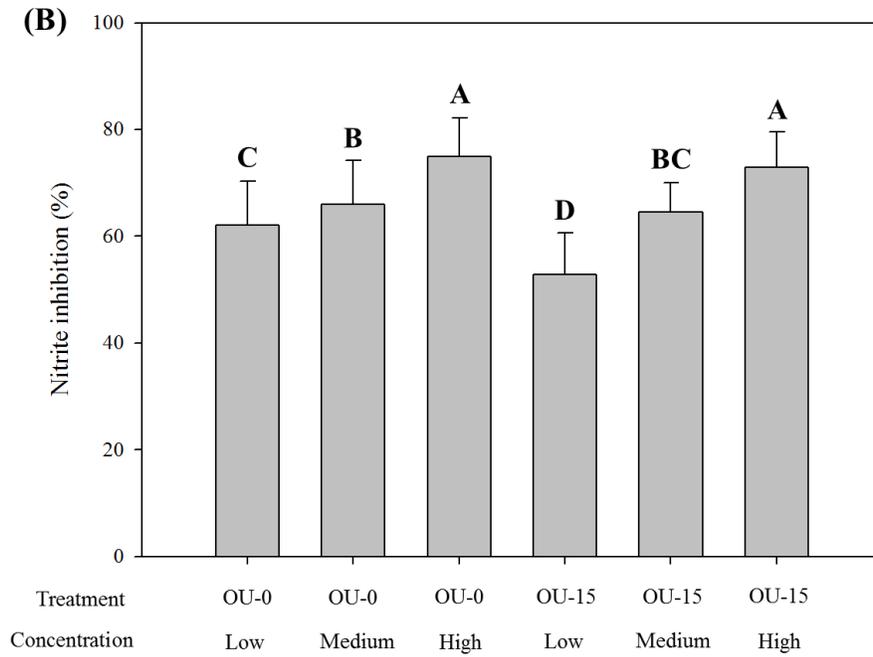
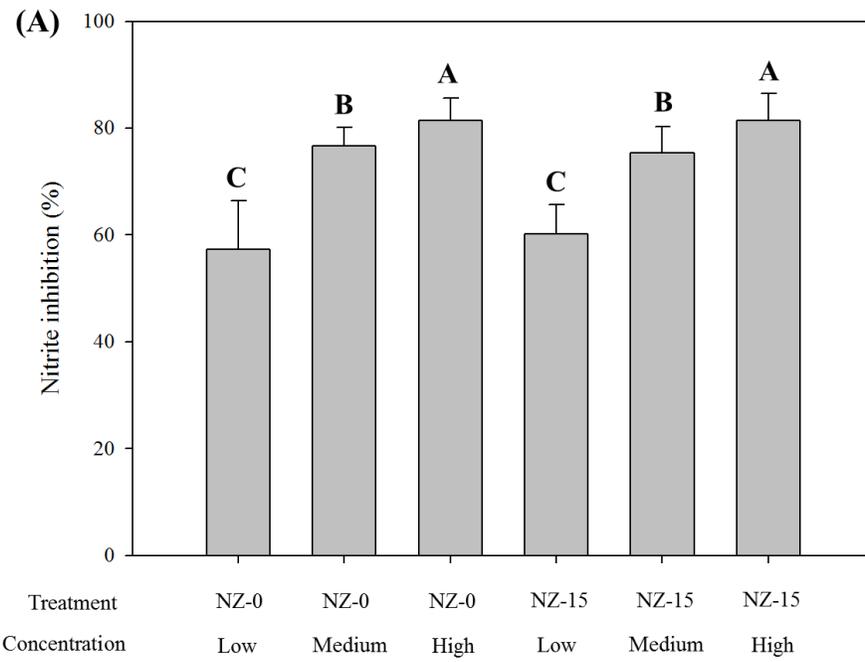


Fig. 4-2. Viability of RAW 264.7 cells treated with (A) blackberry extracts or (B) cyanidin 3-glucoside followed by LPS treatment. Cell viability was calculated as a percentage of the negative control. NZ, Natchez; OU, Ouachita; Day 0, unstored; Day 15, 15 days of storage at 1°C; C3G, cyanidin 3-glucoside. Means were separated by Fisher's LSD at $p < 0.05$.

Fig. 4-3. Inhibition of nitrite production by (A) 'Natchez' and (B) 'Ouachita' blackberry extracts or (C) cyanidin 3-glucoside treatment. Nitrite levels were adjusted for cell viability and nitrite inhibition was calculated by subtracting a relative ratio of the nitrite level in each sample to that in the positive control from 100. NZ-0, 'Natchez' at 0 day after storage (not stored); NZ-15, 'Natchez' at 15 days after storage; OU-0, 'Ouachita' at 0 day after storage (not stored); OU-15, 'Ouachita' at 15 days after storage; C3G, cyanidin 3-glucoside. Means were separated by Fisher's LSD at $p < 0.05$.



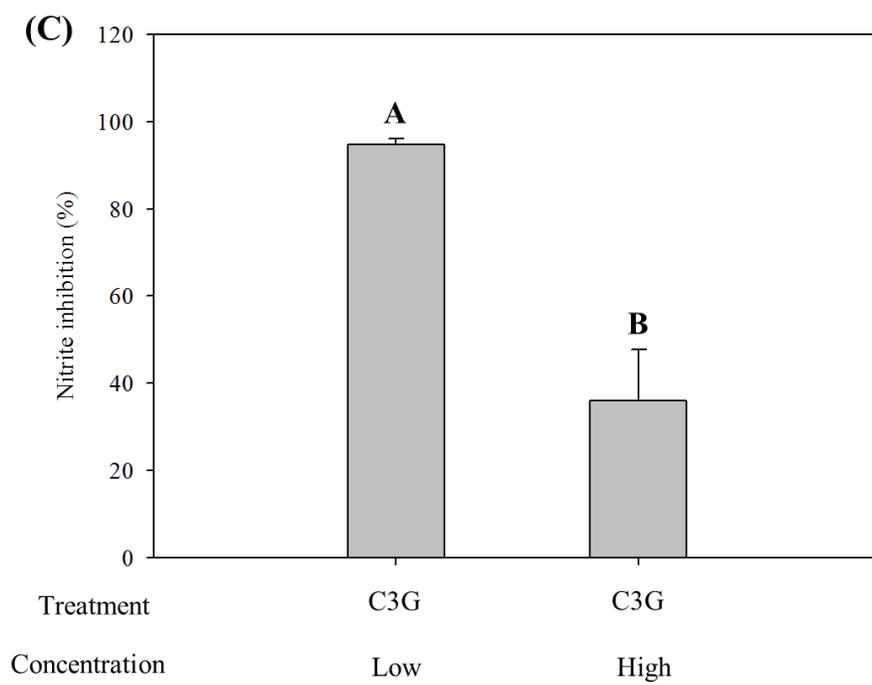
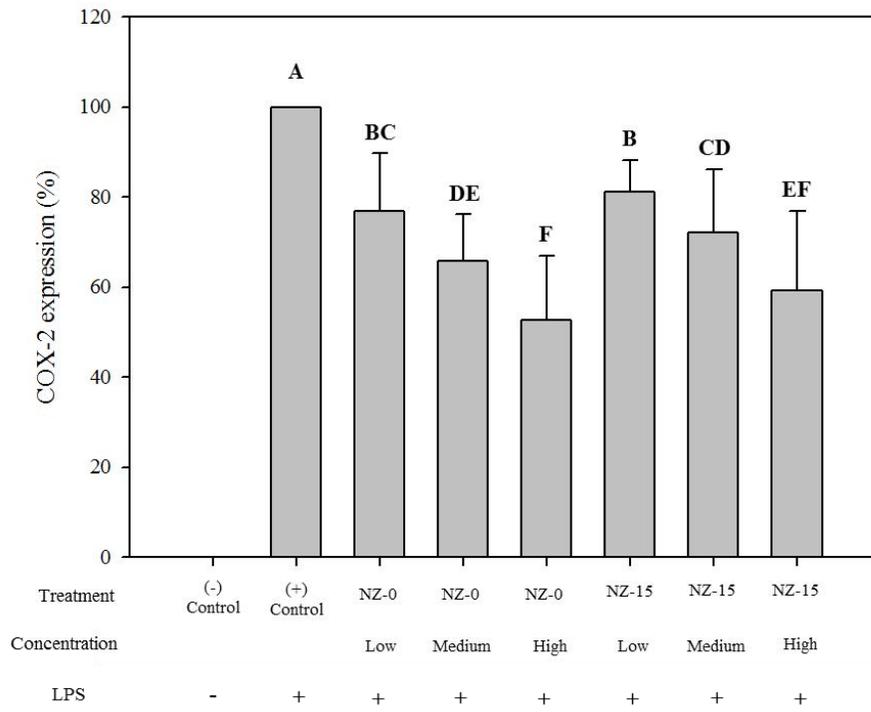
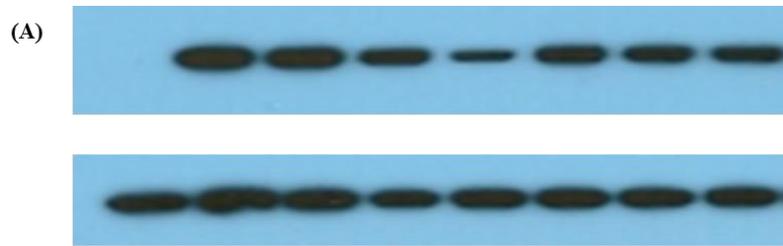
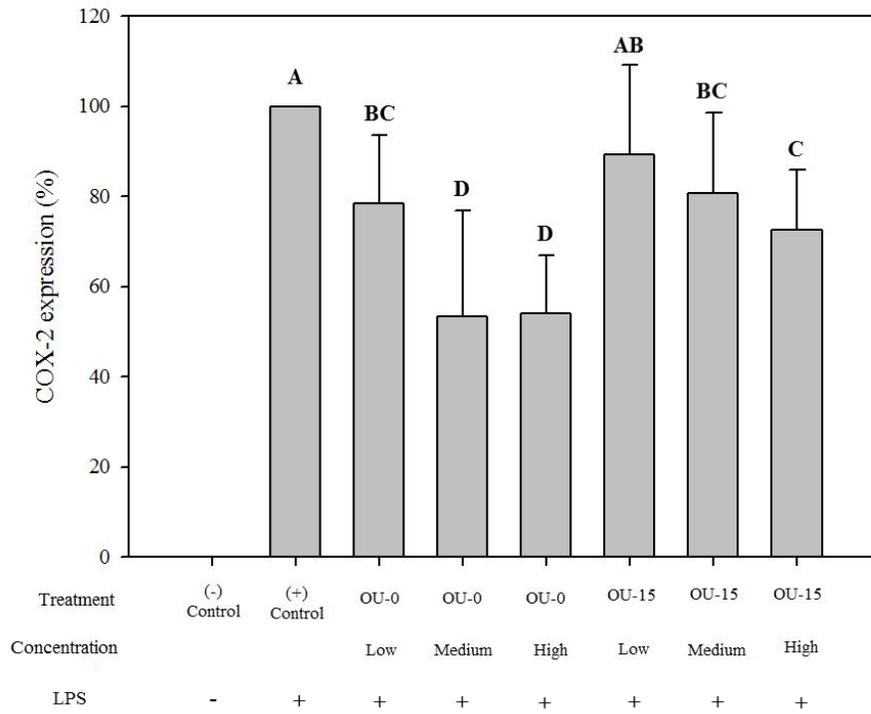
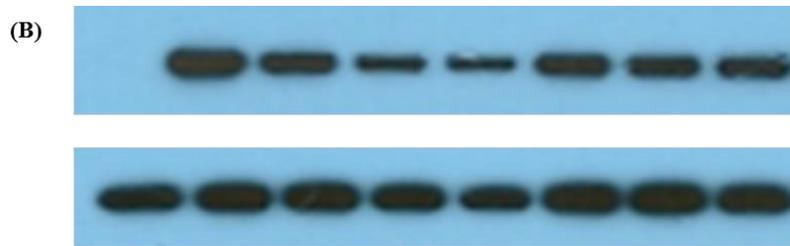


Fig. 4-4. Effects of (A) 'Natchez' and (B) 'Ouachita' blackberry extracts or (C) cyanidin 3-glucoside in COX-2 expression in LPS-stimulated RAW 264.7 cells. COX-2 expression of each treatment was calculated as a percentage of the positive control, and β -actin was blotted as an internal control. NZ-0, 'Natchez' at 0 day after storage (not stored); NZ-15, 'Natchez' at 15 days after storage; OU-0, 'Ouachita' at 0 day after storage (not stored); OU-15, 'Ouachita' at 15 days after storage; C3G, cyanidin 3-glucoside. Means were separated by Fisher's LSD at $p < 0.05$.





(C)

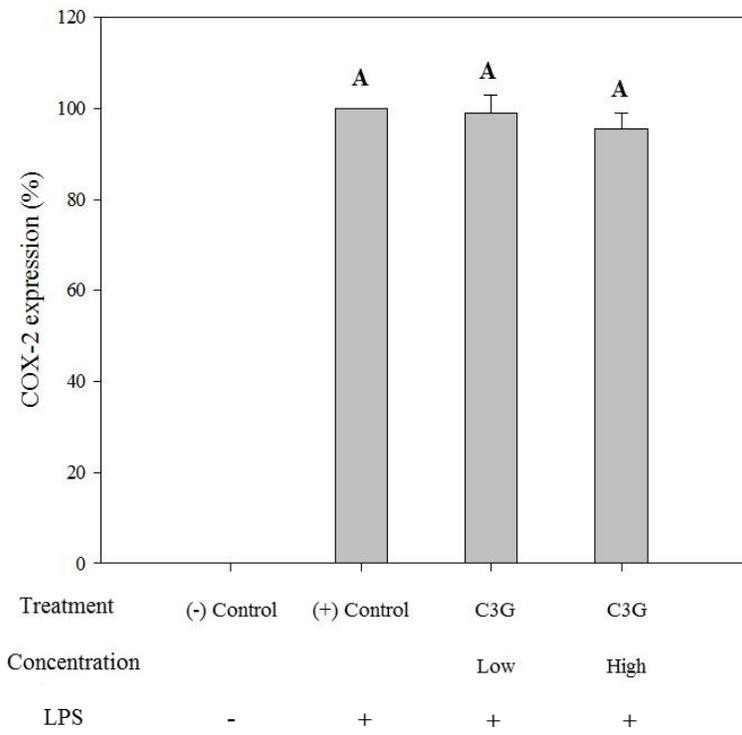
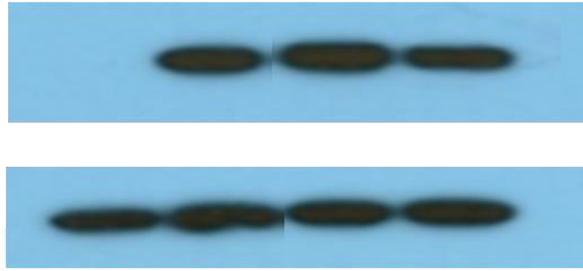
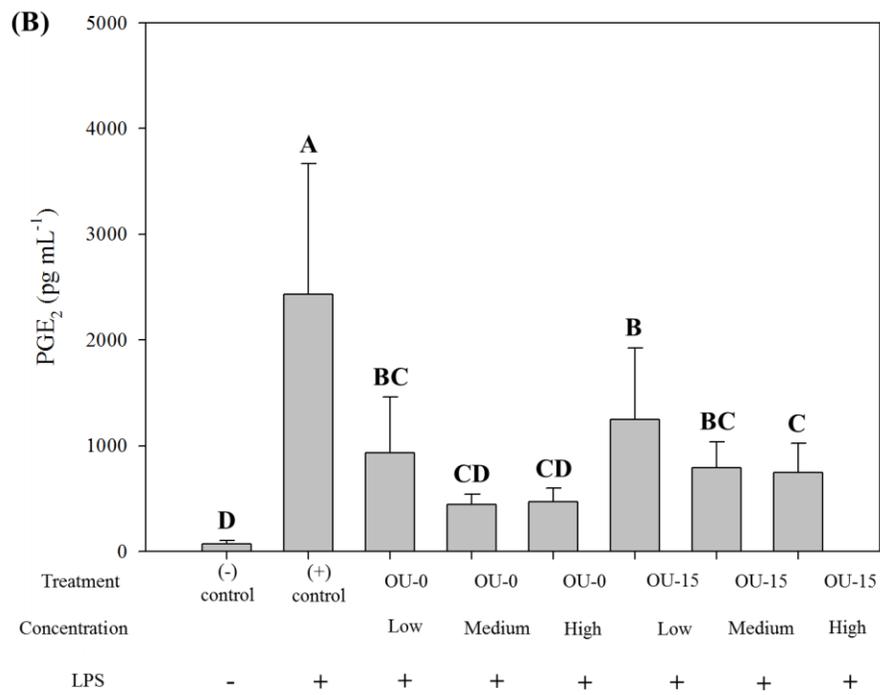
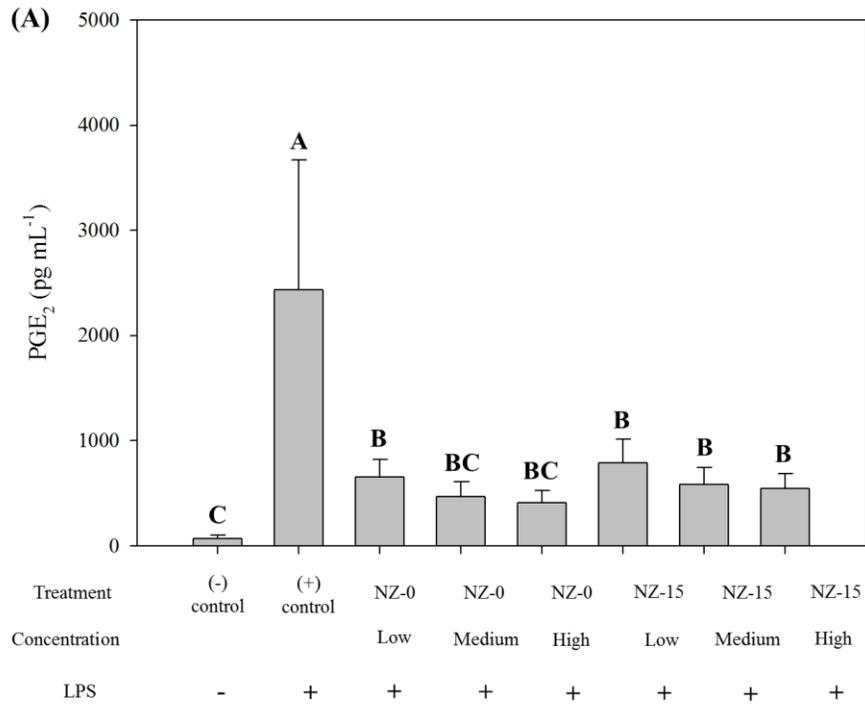
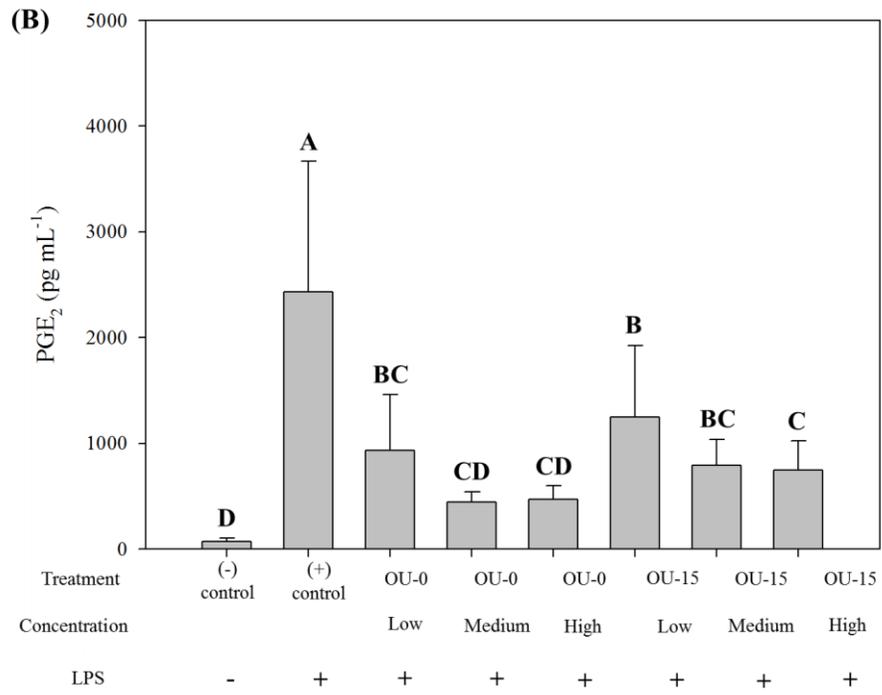


Fig. 4-5. PGE₂ production in LPS-stimulated RAW 264.7 cells. Cells were treated with (A) 'Natchez' and (B) 'Ouachita' blackberry extracts or (C) cyanidin 3-glucoside prior to LPS application. PGE₂ levels were adjusted for cell viability. NZ-0, 'Natchez' at 0 day after storage (not stored); NZ-15, 'Natchez' at 15 days after storage; OU-0, 'Ouachita' at 0 day after storage (not stored); OU-15, 'Ouachita' at 15 days after storage; C3G, cyanidin 3-glucoside. Means were separated by Fisher's LSD at $p < 0.05$.





APPENDICES

A. Antioxidant capacity of organically grown blackberries

The term “antioxidant” has been commonly used to for synthetic or natural substances that can prevent or delay action of oxygen and resulted deterioration, or enzymes or other organic substances that can counteract the oxidation damage in tissues (Huang et al., 2005). Antioxidant capacity can be measured using chemical reactions under specific conditions, and there are two types of assays: hydrogen atom transfer reaction based assays and single electron transfer reaction based assays. Of various assays, ferric ion reducing antioxidant power (FRAP) assay was conducted to analyze antioxidant capacity of organically grown blackberries in this study. This assay is based on electron transfer reaction and measures intense blue color developed by the reduction of ferric ion to ferrous ion under low pH (Benzie and Strain, 1996; Huang et al., 2005).

The antioxidant capacity analyzed by FRAP assay was the highest in ‘Natchez’ and the lowest in ‘Ouachita’ (Table A-1). Storage reduced antioxidant capacity in all cultivars and at both ripeness stages. However, ripeness stage had less effects on antioxidant capacity. It only affected unstored ‘Natchez’ and ‘Navaho’ berries. No change and slight increase of FRAP value was reported in ‘Marion’ and ‘Evergreen’ blackberries stored at 2°C for 9 or 7 days (Wu et al., 2010). Different response to storage might be due to phenolic composition. Cyanidin 3-glucoside represents 69-84% of the total anthocyanin content in ‘Evergreen’ and ‘Marion’ blackberries (Fan-Chiang and Wrolstad, 2005) while that in the cultivars used in this study was 87-95%. In addition, cyanidin 3-rutinoside content in ‘Evergreen’ and

'Marion' blackberries is higher than 'Natchez', 'Ouachita', and 'Navaho', representing 3-27% compared to 0.4-1.5% in the three cultivars used in this study.

B. Phenolic profile of organically grown blackberries

This section provides unidentified anthocyanins and non-anthocyanin phenolic compounds that were not included in Chapter 3. Unidentified anthocyanins were quantified as cyanidin 3-glucoside equivalent, and unidentified hydroxybenzoic acids, hydroxycinnamic acids, and flavonols were quantified using standard curves of gallic acid, chlorogenic acid, and quercetin 3-glucoside, respectively.

1. Anthocyanins

In general, anthocyanin content was slightly higher in ‘Natchez’ than in the other cultivars and increased with storage except for unknown 4 in ‘Natchez’ and ‘Ouachita’ (Table A-2). Storage significantly affected all four anthocyanin contents while cultivar and ripeness stage affected three anthocyanins. There was no significant interaction between cultivar and ripeness stage but interaction between cultivar and storage was significant for three anthocyanins. Among four unidentified anthocyanins, percentage of unknown 2 and 3 was higher than the other compounds and higher in SB than in DB berries in ‘Natchez’ and ‘Navaho’ (Table A-3).

2. Non-anthocyanin phenolic compounds

Hydroxybenzoic acids (HBAs) were the most abundant phenolic compounds in ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries (Table A-4). Unlike most identified phenolic compounds increased during storage, some unidentified phenolic compounds

decreased with storage. HBA, hydroxycinnamic acid (HCA) 3, HCA 6, and flavonol (Flav) 2 tended to increase during storage in all cultivars. However, the other compounds did not change or slightly decreased depending on cultivar. HBA, all HCAs, Flav 1, Flav 2, and Flav 3 were significantly affected by cultivar and storage but ripeness stage did not affect Flav 4 and HCA 6. Significant interaction between cultivar and storage was found for all HCAs but not Flav 2 and Flav 3 (Table A-5). The results showed that storage had great impact on phenolic contents while ripeness stage less affected phenolic contents. Proportion of HBA in 'Natchez' and 'Navaho' was above 40% of the total phenolic content while that in 'Ouachita' was much lower (Table A-6). HBA content was significantly different between ripeness stages in 'Ouachita' in contrast to no difference in the other cultivars. Content and proportion of Flav 3 was higher in 'Natchez' than in the other cultivars, and percentage of HCA 6 was higher in DB berries of all three cultivars. The results suggest that composition of non-anthocyanin phenolic compounds were more complicated than anthocyanins in organically grown 'Natchez', 'Ouachita', and 'Navaho' blackberries.

C. Phenolic composition in blackberry seeds

Seeds of berry fruit are a source of phenolic compounds. Blackberry seeds have been reported to contain anthocyanins, epicatechin, ellagic acid, and ellagitannins (Hager et al., 2008; Siriwoharn and Wrolstad, 2004). In this study, phenolic composition in unstored 'Natchez', 'Ouachita', and 'Navaho' blackberries were analyzed using HPLC (Elite Lachrome, Hitachi Ltd., Tokyo, Japan).

Freeze-dried blackberry seeds were ground using a commercial coffee grinder, and 0.04 g of freeze-dried blackberry seeds powder was extracted with 1.5 mL of extraction solvent (60:37:3, methanol: DH₂O: formic acid, v/v/v). Extracts were centrifuged at 15,294 g for 20 min at 4°C. Supernatants were filtered through 0.2 µm syringe filter and packed with N₂ to inhibit oxidation. HPLC condition is described in Chapter 3 and 20 µL of samples was injected. Anthocyanins, flavonols, and flavan 3-ols and phenolic acids were detected at 520, 360, and 280 nm, and quantified using standard curves of cyanidin 3-glucoside, quercetin 3-glucoside, and gallic acid, respectively.

Anthocyanins and flavonols contents were higher in 'Natchez' than in 'Ouachita' while flavan-3-ols and phenolic acids contents were not different among cultivars (Table A-7). Although small amount compared to flesh, anthocyanins were found in blackberry seeds. Anthocyanin in 'Marion' and 'Evergreen' blackberry seeds was reported by Siriwoharn and Wrolstad (2004), however, they did not find flavonols in contrast to the results of this study. Blackberry seeds are a good source of ellagitannins (Hager et al., 2008). 'Apache' blackberries were reported to contain ellagitannins in seeds, torus, and flesh, and some

ellagitannins such as castalagin/vescalagin isomer, lambertianin C isomer, and ellagic acid were only found in seeds. However, Siriwoharn and Wrolstad (2004) reported 13-15 mg 100 g⁻¹ of ellagic acid in 'Marion' and 'Evergreen' blackberry seeds. They also found procyanidins, which were not detected in the berries without seeds. Procyanidins are condensed tannin composed of epicatechin, and epicatechin content was also higher in seeds compared to whole berries or berries without seeds. The results of this study also indicate that 'Natchez', 'Ouachita', and 'Navaho' blackberries contain high level of flavan 3-ols and phenolic acids.

D. Cell culture

The laminar flow hood (1300 Series A2, Class II, Type A2 Biological Safety Cabinet, Thermo Scientific, Waltham, MA) was turned on and kept with UV light for 30 minutes. After the UV light was turned off, inside the hood was sanitized and all supplies used in the hood were cleaned using 70% ethanol before they were placed in the hood. Dulbecco's modified eagle medium (DMEM) containing 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin was used as media.

1. Growing RAW 264.7 cells

A vial containing RAW 264.7 cells were taken out from the liquid nitrogen dewar and the cells were thawed in a water bath at 37°C for about 2 minutes. The vial was removed from the water bath and dried using paper towel. Seventy percent ethanol was used to clean the vial. The vial was gently shaken and then the content was transferred into a 75 cm² cell culture flask. Fifteen milliliter of media was added and the flask was kept at 37°C and 5% CO₂.

2. Passaging, counting and plating RAW 264.7 cells

To passage, the old media in each flask was discarded and 3 mL of fresh media was added. The cells were carefully scraped and the suspensions were transferred to one flask. Cell suspension was mixed well and aliquoted into each flask based on the dilution factor.

The cells were typically diluted 1 to 6 in this study. Total volume was adjusted to 15 mL with fresh media. Cells were passaged every 2-3 days.

Number of cells were measured to plate cells with the specific cell concentration in all wells. To count cells, cells were scraped and transferred in one flask as described above. Next, 10 μ L of cell suspension was mixed with 90 μ L of trypan blue. Ten microliter of the mixture was pipetted to each side of the hemocytometer, and live and dead cells in the quadrants were counted. Cell concentration was determined:

$$\begin{aligned}\text{Live cell concentration (cells/mL)} &= (\text{total live cells}) \times (\text{dilution factor}) \times 2500 \\ &= (\text{total live cells}) \times \frac{(\text{100 mL of trypan blue+cell suspension})}{(\text{10 mL of cell suspension})} \times 2500 \\ &= (\text{total live cells}) \times (\text{dilution factor of 10}) \times 2500\end{aligned}$$

Live cell concentration and desired cell concentration were used to determine the dilution factor. Using the dilution factor and total volume needed for plating, cell suspension was diluted with fresh media. Diluted cell suspension was mixed well, and 100 μ L or 1.5 mL was pipetted to a 96-well or 12-well plate, respectively.

3. Harvesting

Prior to harvest, microcentrifuge tubes were labeled for prostaglandin E₂ (PGE₂), BCA (an assay to measure total protein content), and Western blot. Cell media of 100 μ L was directly transferred to the tubes, and immediately frozen and kept at -80°C until used.

Remaining cell media was carefully discarded, and wells were rinsed three times with cold phosphate buffered saline. The cells were lysed by adding 200 μL of M-PER containing 1% halt protease inhibitor cocktail and gentle scraping. About 100 μL of cell lysate was transferred to the tubes both for BCA and Western blot. Tubes were kept at -80°C until analyzing total protein content and Western blotting.

E. Western blot

Western blot is an analytical technique for detection of specific protein. Proteins are separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to adsorbent membrane (Kurien and Scofield, 2006; Kurien and Scofield, 2009). Nitrocellulose and polyvinylidene difluoride (PVDF) are widely used membranes and PVDF membranes were used in the present study. This section provides the recipes of all buffers and developing procedure that were not included in Chapter 4.

Buffers needed:

1. 5X Sample buffer

- 1.0 M Tris HCl, pH 6.8 (2.25 mL)
- Glycerol (5 mL)
- Dithiothreitol (1.54 g)
- SDS (0.5 g)
- Bromophenol blue (5 mg)

Ingredients were dissolved in deionized (DI) water with the final volume of 10 mL.

NOTE: This concentrated buffer (5X) was not diluted prior to use. The buffer was diluted when added to cell lysates and M-PER/protease inhibitor cocktail during sample preparation.

2. 10X Running buffer

- Tris base (29 g)
- Glycine (144 g)
- SDS (10 g)
- DI water up to a liter

NOTE: The buffer was diluted 10 times before use.

3. 10X Tris buffer, pH 7.5 (TBS)

- Tris base (24.2 g)
- NaCl (90 g)

Tris base and NaCl were dissolved in about 800 mL of DI water, and HCl (6 N) was added to adjust the pH to 7.5. DI water was added for the final volume of 1 L.

NOTE: The buffer was diluted 10 times before use.

4. 1X Tris buffer with Tween-20 (TBS-T)

- 10X TBS (100 mL)
- DI water (900 mL)
- Tween-20 (1 mL)

5. Blocking buffer (5% Bovine serum albumin (BSA))

- 2.5 g of BSA in 1X TBS (final volume 50 mL)

6. Primary antibody buffer

- BSA (1.5 g)
- TBS-T (30 mL)

In this study, 1:2000 and 1:3000 dilution were used for COX-2 and β -actin, respectively.

7. Secondary antibody buffer

- BSA (2.0 g)
- TBS-T (30 mL)

In this study, the secondary antibody was diluted at 1:3000 and used for both COX-2 and β -actin blotting.

NOTE: The blocking buffer and antibody buffers were stored at -20°C when not used.

The blocking buffer and primary antibody buffer were used 3-4 times and secondary antibody buffer was used 6-8 times.

8. Stripping buffer, pH 2.2

- Glycine (0.3 g)
- SDS (0.02 g)
- Tween-20 (0.2 mL)

Ingredients were dissolved in about 15 mL of DI water and HCl (6 N) was added to get the pH of 2.2. The final volume was 20 mL.

NOTE: There are various stripping buffer recipe and procedure available. Stripping can result in losing proteins and give more background compared to the first image. In this study, three buffers were tested and the buffer above was selected.

Sample buffer, running buffer, TBS, TBS-T, and stripping buffer were stored at 4°C when not in use. Sample buffer needs to be warmed to room temperature to use because it's thick and viscous. It is hard to pipet sample buffer when the buffer is cold.

Developing images

In this study, membranes were exposed to Clarity™ Western ECL Substrate (Bio-Rad, Hercules, CA) for 5 min at room temperature. The excessive liquid was removed by touching the edge of membranes to Kimwipe. The membranes were placed between parafilms and bubbles were removed. Membranes were put in a film cartridge and taken to the dark room with x-ray films, developer, fixer, paper towels, and timer.

In the dark room, trays with developer, fixer, and tap water were set, and light was turned off except red light. Under the dark condition, x-ray film was place on the membranes and the cartridge was shut. Film was exposed about 10 sec and immersed in the developer for 1 min followed by gentle rinse with tap water and immersing in the fixer for 1 min. Film was washed with tap water again, and light was turned on. Overexposed film shows black membranes with clear surroundings so exposing time needs to be reduced. If film is blank or the band signal is too weak, longer exposure is needed. Developed films were rinsed well

with water and dried to prevent sticking to counter, papers, or other films. Every time touching undeveloped films, hands and gloves were totally dried.

Well dried films were put on the membrane again and the location of membranes in the cartridge was marked on the films. Date, protein type, antibody dilution were also recorded on the membranes.

NOTE: Developer and fixer are corrosive and developer stains plastics, clothes, and papers very quickly. Lab coat, gloves, and carefulness are necessary.

Literature cited

- Benzie, I.F.F. and J.J. Strain. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* 239:70-76.
- Fan-Chiang, H.J. and R.E. Wrolstad. 2005. Anthocyanin pigment composition of blackberries. *J. Food Sci.* 70:C198-C202.
- Hager, T.J., L.R. Howard, R. Liyanage, J.O. Lay, and R.L. Prior. 2008. Ellagitannin composition of blackberry as determined by HPLC-ESI-MS and MALDI-TOF-MS. *J. Agric. Food Chem.* 56:661-669.
- Huang, D.J., B.X. Ou, and R.L. Prior. 2005. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* 53:1841-1856.
- Kurien, B.T. and R.H. Scofield. 2006. Western blotting. *Methods* 38:283-293.
- Kurien, B.T. and R.H. Scofield, 2009. Protein blotting and detection. Oklahoma Medical Research Foundation, Oklahoma City.
- Siriwoharn, T. and R.E. Wrolstad. 2004. Polyphenolic composition of Marion and Evergreen blackberries. *J. Food Sci.* 69:C233-C240.
- Wu, R.Y., B. Frei, J.A. Kennedy, and Y.Y. Zhao. 2010. Effects of refrigerated storage and processing technologies on the bioactive compounds and antioxidant capacities of 'Marion' and 'Evergreen' blackberries. *LWT-Food Sci. Technol.* 43:1253-1264.

Table A-1. Antioxidant capacity (mmol Fe²⁺ equiv. kg⁻¹) of organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries.

Storage condition ^y	Natchez ^z		Ouachita		Navaho	
	SB ^x	DB	SB	DB	SB	DB
Day 0	71.38a ^{*z}	64.02a [*]	45.26a	43.94a	64.91a [*]	52.78a [*]
CL	45.61b	42.31b	37.17b	35.58b	42.65b	42.37b
RT	44.10b	43.83b	36.18b	36.91b	42.35b	41.19b

^zMeans separated within storage conditions in each cultivar and ripeness stage by Tukey’s HSD at $p < 0.05$. Asterisks (*) shows significant difference between ripeness stages in each cultivar and storage condition by LSD at $p < 0.05$.

^yDay 0, unstored; CL, constant low, 15 days at 1°C; RT, retail, 13 days at 1°C + 2 days at 20°C.

^xSB, shiny black; DB, dull black.

Table A-2. Anthocyanin content (mg g⁻¹ DW) in organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries at 0 and 15 days after storage.

Cultivar	Storage condition ^y	Anthocyanins			
		Unknown 1	Unknown 2	Unknown 3	Unknown 4
Natchez	Day 0	0.024b ^z	0.620b	0.927c	0.026a
	CL	0.021b	0.654b	1.155b	0.017b
	RT	0.030a	0.778a	1.330a	0.026a
Ouachita	Day 0	0.008c	0.541b	0.383b	0.005a
	CL	0.022b	0.684a	0.623a	0.022a
	RT	0.027a	0.683a	0.633a	0.027a
Navaho	Day 0	0.010c	0.518c	0.745b	0.013b
	CL	0.016b	0.669b	1.092a	0.021a
	RT	0.028a	0.778a	1.172a	0.025a
Significance					
Cultivar (C)		**	*	**	NS
Ripeness stage (R)		NS	**	**	**
Storage(S)		**	**	**	*
C x R		NS	NS	NS	NS
C x S		**	**	NS	**
R x S		*	NS	NS	NS
C x R x S		**	NS	NS	NS

^zMeans separated within storage conditions in each cultivar by Tukey’s HSD at $p < 0.05$.

^yDay 0, unstored; CL, constant low, 15 days at 1°C; RT, retail, 13 days at 1°C + 2 days at 20°C.

Table A-3. Anthocyanin composition of organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries at SB and DB ripeness stages.

Anthocyanins	Natchez		Ouachita		Navaho	
	SB ^y	DB	SB	DB	SB	DB
Unknown 1	0.09 ^z	0.08	0.08	0.09	0.06	0.06
Unknown 2	2.40*	2.27*	3.02	2.99	2.59*	2.31*
Unknown 3	4.10*	3.74*	2.61	2.35	4.05*	3.43*
Unknown 4	0.10*	0.05*	0.10*	0.04*	0.10*	0.05*

^zAsterisks (*) shows significant difference between ripeness stages in each cultivar by Fisher’s LSD at $p < 0.05$.

^ySB, shiny black; DB, dull black.

Table A-4. Phenolic content (mg 100 g⁻¹ DW) in organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries at 0 and 15 days after storage.

Phenolic compound ^x	Natchez			Ouachita			Navaho		
	Day 0 ^y	CL	RT	Day 0	CL	RT	Day 0	CL	RT
HBA	170.90b ^z	215.49b	309.74a	67.66b	82.01b	133.82a	130.46b	289.12a	304.79a
HCA 1	7.59a	4.60b	6.21ab	11.24a	8.83b	9.74ab	4.62a	4.53a	5.38a
HCA 2	6.86b	7.69b	9.21a	3.98a	2.81b	1.85b	12.76b	17.08a	18.22a
HCA 3	3.75b	4.66ab	6.03a	1.68b	2.79a	2.44a	2.89b	4.39a	4.23a
HCA 4	3.58b	4.26ab	4.97a	3.52a	4.07a	4.10a	2.09a	2.64a	2.31a
HCA 5	12.50a	12.46a	14.81a	5.43c	14.40b	20.66a	3.55a	1.31c	0.17b
HCA 6	22.11c	23.39b	29.17a	13.77b	15.11a	14.17ab	14.25b	19.75a	22.20a
Flav 1	12.71a	8.36b	12.50a	3.75a	3.13a	4.10a	5.72a	4.64a	5.31a
Flav 2	31.88b	32.66b	40.21a	11.39b	16.70a	20.03a	16.31b	23.12a	25.55a
Flav 3	7.51b	8.33ab	10.36a	0.99a	1.46a	2.09a	2.11b	3.50a	3.79a
Flav 4	0.031a	0.030b	0.030b	0.031a	0.030a	0.029a	0.040a	0.030b	0.030b

^zMeans separated within storage conditions in each cultivar by Tukey’s HSD at $p < 0.05$.

^yDay 0, unstored; CL, constant low, 15 days at 1°C; RT, retail, 13 days at 1°C + 2 days at 20°C.

^xHBA, hydroxybenzoic acid; HCA, hydroxycinnamic acid; Flav, flavonol.

Table A-5. Main effects and interaction on non-anthocyanin phenolic content in organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries.

	Phenolic compounds ^z					
	HBA	Flav 1	Flav 2	Flav 3	Flav 4	
Significance						
Cultivar (C)	**	**	**	**	NS	
Ripeness stage (R)	**	**	**	**	NS	
Storage(S)	**	**	**	**	**	
C x R	NS	NS	NS	**	NS	
C x S	**	**	NS	NS	*	
R x S	NS	**	NS	NS	NS	
C x R x S	NS	NS	NS	NS	NS	
	HCA 1	HCA 2	HCA 3	HCA 4	HCA 5	HCA 6
Significance						
Cultivar (C)	**	**	**	**	**	**
Ripeness stage (R)	**	**	**	**	**	NS
Storage(S)	**	**	**	**	**	**
C x R	NS	**	NS	NS	**	**
C x S	*	**	**	*	**	**
R x S	NS	NS	*	NS	**	NS
C x R x S	NS	NS	NS	NS	**	**

^zHBA, hydroxybenzoic acid; HCA, hydroxycinnamic acid; Flav, flavonol.

Table A-6. Composition of non-anthocyanin phenolic compounds in organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries at SB and DB ripeness stage.

Phenolic compound ^x	Natchez		Ouachita		Navaho	
	SB ^y	DB	SB	DB	SB	DB
HBA	44.57 ^z	42.22	27.75*	15.01*	45.79	42.87
HCA 1	1.18	1.27	3.34*	4.01*	1.01	1.05
HCA 2	1.61	1.58	0.57*	1.65*	2.90*	3.48*
HCA 3	0.93	1.01	0.74	0.75	0.78	0.74
HCA 4	0.83*	0.97*	1.33	1.46	0.51	0.48
HCA 5	2.69	2.69	4.31*	1.80*	0.42	0.43
HCA 6	4.60*	5.78*	3.46*	7.73*	3.41*	4.37*
Flav 1	2.11	2.3	1.01	1.29	1.21*	0.91*
Flav 2	6.77	7.57	5.42	5.58	4.10	4.48
Flav 3	1.81	1.68	0.49	0.39	0.64*	0.50*
Flav 4	0.008*	0.006*	0.011	0.010	0.009*	0.007*

^zAsterisks (*) shows significant difference between ripeness stages in each cultivar by Fisher’s LSD at $p < 0.05$.

^ySB, shiny black; DB, dull black.

^xHBA, hydroxybenzoic acid; HCA, hydroxycinnamic acid; Flav, flavonol.

Table A-7. Phenolic content (mg 100g⁻¹ DW) in the seeds of organically grown ‘Natchez’, ‘Ouachita’, and ‘Navaho’ blackberries.

Cultivar	Phenolic compounds ^y		
	Anthocyanin	Flavonols	Flavan 3-ols and phenolic acids
Natchez	317.98a ^z	69.18a	1310.51a
Ouachita	185.61b	47.69b	1000.72a
Navaho	244.08ab	67.16a	1223.49a

^zMeans separated within column by Tukey’s HSD at $p < 0.05$.

^yAnthocyanins and flavonols were quantified as cyanidin 3-glucoside and quercetin 3-glucoside equivalent, respectively, and flavan 3-ols and phenolic acids were quantified as gallic acid equivalent.