AN ABSTRACT OF THE DISSERTATION OF

Thanyaporn Siriwoharn for the degree of Doctor of Philosophy in Food Science and Technology presented on April 22, 2004.

Title: Influence of Cultivar and Maturity on Blackberry Polyphenolics and Investigation of Sediment Formation in Blackberry Juice.

Abstract approved:

Ronald E. Wrolstad

The influence of cultivar and maturity on polyphenolic composition and antioxidant activity was investigated by UV-Visible spectrophotometry and analytical high performance liquid chromatography (HPLC) in commercially important blackberry cultivars and selections. The anthocyanin pigments changed tremendously with ripening. Total anthocyanins increased from 74.7-317 mg/100g fw from underripe to over ripe for 'Marion', and from 69.9-164 mg/100g fw for 'Evergreen'. Total phenolics did not show marked change with maturity with values slightly decreasing from underripe to ripe. Antioxidant activities, while increasing with ripening, did not show the noticeable change that total anthocyanins exhibited. The total phenolic and total anthocyanin contents varied greatly among 11 cultivars. Total anthocyanins ranged from 131-256 mg/100g fw

(mean = 198), total phenolics from 682-1056 mg GAE/100g fw (mean = 900), ORAC from 37.6-75.5 μmolTE/g fw (mean = 50.2), and FRAP ranged from 63.5-91.5 μmolTE/g fw (mean = 77.5). Four blackberry cultivars were found to be higher in total anthocyanins and total phenolics than 'Marion' and 'Evergreen', the predominant commercial cultivars in the Pacific Northwest. The results from these studies confirmed that blackberries are a good source of natural antioxidants and colorant, and demonstrated the potential for obtaining new cultivars with high pigment/phenolic content through classical plant breeding.

The investigation on the incidence of haze and sediment formation revealed that the sediment in the commercial reconstituted 'Evergreen' blackberry (CRE) juice was composed of ellagic acid, protein, and unidentified compounds. The qualitative tannin and protein-tannin haze test indicated that the sediment was predominantly tannin or protein-tannin complexes. Nitrogen determination showed the sediment to be 6.69 ± 2.21% (w/w) protein on a dry weight basis. Almost all of the extractable material was identified as ellagic acid by HPLC and LC-MS. The ellagic acid content of the wet sediment was 0.05 g/100g while it was 7.41 g/100g in freeze-dried sediment. Tannase enzyme did not significantly decrease the concentration of ellagitannins in 'Marion' blackberry juice in this study.

Influence of Cultivar and Maturity on Blackberry Polyphenolics and Investigation of Sediment Formation in Blackberry Juice

by

Thanyaporn Siriwoharn

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented April 22, 2004

Commencement June 2004

Doctor of Philosophy dissertation of Thanyaporn Siriwoharn
presented on April 22, 2004.

APPROVED:

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Thanyaporn Siriwoharn, Author

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my advisor Dr. Ronald E. Wrolstad for his guidance, invaluable support and encouragement during my studies at Oregon State University. His enthusiasm, kindness, and remarkable ways of thinking have truly inspired me to be all that I can be.

I am grateful to Robert W. Durst for his unbelievable patience, constant encouragement and technical advice; and especially for his valuable friendship throughout these years. He gave me opportunities to learn from my mistakes and think for myself, and along the way he helped me become a good scientist.

I would like to express sincere appreciation to my committee members: Dr. Vincent T. Remcho, Dr. James A. Kennedy, Dr. Clifford B. Pereira, and Dr. Florian L. Cerklewski for their valuable advice and wonderful support.

I wish to thank Dr. Chad E. Finn, USDA-ARS, Department of Horticulture, Brian A. Yorgey, Department of Food Science and Technology (FST), Connie Pace, the North Willamette Research and Extension Center (Aurora, OR), as well as Eric Johnson, Milne Fruit Products, Inc. (Prosser, WA) for providing studied materials.

I would also like to extend my appreciation to the FST faculties and staffs, my fellow students, and all my Thai friends, for their friendship and for making my life in Oregon both enjoyable and memorable. Special thanks to Chat Udompijitkul, Chantalak Tiyayon, Duanghathai Katwibun, Arusa Chaovanalikit, Kannapon

Lopetcharat, and Doolalai Sethajintanin, for their sincerity and encouragement all these years.

Finally, my deepest gratitude and love goes to my family, Suphornchai, Nitaya, and Pasuda Siriwoharn, and to my grandmother, Srinual Panyaruing, for their love and incredible support.

CONTRIBUTION OF AUTHORS

Dr. Ronald E. Wrolstad was involved in the experimental design, interpretation of the data, and writing of each manuscript. Dr. Clifford B. Pereira assisted with the designs and in the interpretation of the data.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

INFLUENCE OF CULTIVAR AND MATURITY ON BLACKBERRY POLYPHENOLICS AND INVESTIGATION OF SEDIMENT FORMATION IN BLACKBERRY JUICE

CHAPTER I. INTRODUCTION

Interest in blackberry phenolics has increased owing to their roles as antioxidants and the possible beneficial implications in human health, such as in the treatment and prevention of cancer, cardiovascular disease, and other pathologies (10,48). Blackberries are a rich source of anthocyanins and other polyphenolic antioxidants (85,135,142). Their extract has showed better anti-inflammatory activity when compared to aspirin (73).

Besides variations from sample preparation and analytical methodology, it is known that the levels of phenolics and the antioxidant capacity in blackberries can be greatly affected by maturity (94,95,142) and cultivar (56,112). Unfortunately, a complete investigation of these environmental effects and analytical methodology, especially in blackberries, has never been reported. Thus, the first part of this research was aimed at the evaluation of the influence of cultivar and maturity on the available blackberry polyphenolics and antioxidant activity, and also to determine the variations caused by sample, location, and analytical procedures. This knowledge will offer a number of opportunities including being able to select the appropriate variety and maturity stage to achieve the desired products.

Most blackberries produced in the United States are processed, including 99% of the Pacific coast crop (121). The Pacific Northwest is a major producer of blackberries and a significant portion of the crop is processed into juice concentrate. Berry juices are ideal for blending with other juices such as clarified apple or pear juice because of their intense flavor and color (110). However, the importance of blackberry juice in the United States is very minor (121) due to its excessive haze and sediment formation during storage (109). Local processors have indicated that the propensity for haze and sediment formation is a major quality defect limiting the usage of blackberry juice in blended juice beverages and other products, and that 'Evergreen' is more problematic than 'Marion'. It is of significant interest to the industry dealing with blackberry juices that the formation of haze and sediment is eliminated or prevented. Since there is little information in the literature on the formation and composition of the sediment in blackberry juices, the second part of the study was dedicated to determine the composition of the 'Evergreen' blackberry juice sediment. This knowledge may possibly lead to an understanding of the cause of the problem and suggest preventative measures.

CHAPTER II. LITERATURE REVIEW

BLACKBERRY

Origin

Blackberries are in the large and very diverse Rose family (*Rosaceae*), the same family that includes many fruit and flowering plants, such as apples, peaches, plums, strawberries, raspberries, and roses. Blackberries belong in the genus *Rubus*, subgenus *Eubatus*. Blackberry species are native, and usually ruderal, in many parts of the world, but little domestication and commercial use has been made of them except in North America (72). There is evidence that they were domesticated in the seventeenth century in Europe and during the nineteenth century in North America (73,91).

Most species of blackberries produce biennial canes, which vary from erect to procumbent in growth habit and are usually armed with sharp prickles. The blackberry is an aggregate fruit, composed of drupelets arranged on a column of receptacle tissue. The present blackberry cultivars originated from the interbreeding of many genetically heterogeneous and morphologically variable species, causing great differences in fruit and plant habit (73). Although growing blackberries is relatively easy, according to Jennings et al. (48) and McPheeters and Shirvin (70) the Pacific Northwest (western Oregon, western Washington and southwestern

British Columbia), parts of coastal California, Texas, Missouri, and Arkansas, and New Zealand are the only areas where blackberries are of major importance.

Cultivated types

There are three types of blackberries commercially grown in the United States: 1: erect, 2) semi-erect (and/or semi-trailing), and 3) trailing. Both trailing and semi-erect types require some kind of support to grow, while erect plants can stand alone. Erect and semi-erect types predominate in the eastern and southern regions of the United States. The Pacific Northwest (California, Oregon, and Washington, USA, and British Columbia, Canada) is the principal producing area for trailing blackberries (55,114). The most important blackberry cultivars of the Pacific Northwest are characterized in Table 2.1.

In general, the erect thorny types of blackberries have the highest yields and the largest fruit with excellent fruit quality, generally much better than semi-erect thornless varieties (57,91). Erect blackberries are recommended for commercial production because they require less labor than trellised brambles (133). However, their thorns are objectionable to many growers. For that reason special emphasis has been placed on the development of thornless varieties with erect growth that could make hand harvesting easier while eliminating the need for trellising (57). The western trailing types are high yielding, but the fruit bruises easily, and they are less cold hardy than the eastern erect blackberries. The lower dessert quality of the semi-erect thornless blackberries may be excused on the basis of their

incredible productivity. A yield of 30 pounds per plant is not uncommon with an average semi-erect thornless blackberry variety (91).

Table 2.1. Trailing, erect, and semi-erect blackberry cultivars in the Pacific Northwest. Cultivars in italics were those most commonly planted in 1990.

Location	Cultivars and % of Hectarage
Trailing	
British Columbia	Thornless Evergreen (30); Kotata (30); Silvan (30)
California	Boysen (66); Olallie (33); Silvan (1)
Oregon	*Marion* (50); Thornless Evergreen (29); *Boysen* (17); Logan (2); *Kotata* (1); *Waldo* (1)
Washington	Thornless Evergreen (85); *Boysen* (10); *Marion* (4); Kotata (1)
Erect	
British Columbia	---
California	*Shawnee* (30); *Comanche* (20); *Cherokee* (20); *Cheyenne* (15)
Oregon	*Cherokee* (48); Shawnee (43); Choctaw (9)
Washington	---
Semi-Erect	
British Columbia	*Loch Ness* (60); Black Satin (30); Hull Thornless (2)
California	*Hull Thornless* (50); *Chester* (40); Black Satin (5)
Oregon	*Chester* (67); Hull Thornless (33)
Washington	Chester (100)

Adapted from Strik (114).

Ripening time, ease of cultivation and harvest, and winter hardiness are important factors that affect growers' selection of cultivars. Thornless canes are always preferred, as the presence of thorny canes interferes with cultivation operations and harvest, and thorns are a contaminant in processed fruit (70).

'Thornless' blackberries

There are two main sources for thornless blackberry varieties: mutations and genetic. The earliest thornless blackberry varieties resulted from chance mutations. The thornless character of the mutant cane is present only in the outer epidermal layer of stem tissue, through which thorns arise. The interior portion of the cane remains genetically thorny. Such an arrangement of two genetically different tissues is called a periclinal chimera. As long as this periclinal arrangement is maintained, the plant remains thornless. Thornless mutants or sports of blackberries may be found naturally in wild species or varieties, especially Boysen (52). Over the past 100 years many thornless mutant varieties have been introduced, but few have remained. Many mutants are unstable and gradually lose the thornless character, or the mutation is associated with other undesirable characteristics such as poor set, loss of vigor or more disease susceptibility (52). However, 'Thornless Logan' and 'Thornless Evergreen' are exceptions; both have remained stable over a long period and the fruit and yields are similar to the thorny type.

Thornless cultivars have only become widely available recently through the identification of specific genes for the thornlessness and advances in tissue culture (82). The breakthrough discoveries on the source of genetic thornlessness initially came in the 1950s from the European cultivar 'Merton Thornless' and in 1984 from the cultivar 'Austin Thornless'. These afterward resulted in manipulation of plants to be completely thornless and capable of transferring this trait through breeding.

In addition, Pelczar (82) reported that crossing thornless with thorny selections gives several desirable qualities of thornless, such as hardiness, erect habit, and large, flavorful fruit.

'Marion' berries

'Marion' is the standard for growers in the Willamette valley and it became the most widely planted blackberry cultivar in the world by the early 1980s (20). It was selected from a cross of 'Chehalem' x 'Olallie' in 1948 and released in 1956 by the cooperative breeding program of the U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS) and the Oregon Agricultural Experiment Station. Strik in 1992 (114) reported that 'Marion' accounts for more than 50% of the hectarage in the Pacific Northwest and continues to be widely planted. 'Marion' is valued for its flavor, shape, and its processing quality. It also perceived as being "less seedy" than 'Thornless Evergreen', eastern U.S. erect, and semi-erect blackberry cultivars (20). However, 'Marion' is thorny, lacks winter hardiness, and has fruit that are too soft for easy handling (19). The fruit yield is also not particularly high when compared to 'Thornless Evergreen' and 'Chester Thornless' (20). Nonetheless, the outstanding fruit quality, and particularly flavor, of 'Marion' has been the reason it has risen to such dominance in the worldwide market.

'Thornless Evergreen' blackberries

'Thornless Evergreen' blackberry, discovered in 1926, is the second most widely planted cultivar in the Pacific Northwest. It accounts for 85% of the trailing blackberry cultivars grown in Oregon and around 30% of the trailing cultivars grown in British Columbia and California (114). 'Thornless Evergreen' is a periclinal chimera. The plants are vigorous, drought-resistant, and late bearing. Fruits are large and firm, with large seeds. Waldo (126) in 1938 reported that 'Thornless Evergreen', in limited acreages, appeared to be equivalent in fruit yield, and quality to the thorny 'Evergreen' blackberry. The success of this selection, aside from its thornlessness, is due to it adaptability to western Oregon climatic conditions and to its high yields of acceptable fruit for processing purposes (127). Processors have found a ready market for the fruit in all parts of the United States.

'Chester Thornless' blackberries

'Chester' is a thornless, semi-erect variety released by the USDA in 1985. It was originated from the cross of SIUS 47 x 'Thornfree' made in 1968 by USDA-ARS geneticist J. W. Hull, who selected 'Chester Thornless' at Carbondale, Illinois in 1972 (26). 'Chester' makes up nearly 70% of the semi-erect blackberry cultivars grown in Oregon and was the only major semi-erect cultivar grown in Washington in 1992 (114). Its outstanding characteristics are high yields of large fruit of good quality (firm and sweet), plant hardiness and disease resistance. These durable handing qualities combined with the relatively long shelf life of the fruit have been

instrumental in gaining a shopping market for 'Chester' across the United States (40,91,114). In the Pacific Northwest, 'Chester' is primarily grown for the fresh market, but in other parts of the world it is used for processing. Productive plants can bear over a five- to six-week season (82).

'Silvan' blackberries

'Silvan' is a trailing blackberry, selected in Victoria, Australia from the progeny of a cross between US-Oregon 742 (a Boysenberry hybrid) and US-Oregon 928 (subsequently was released as 'Marion') (10,46,69). Fruit are borne on thorny peduncles arising from leaf axils along the entire length of the cane and extend beyond the foliage. Fruit size is normally 20-25 mm diameter, 40 mm long, with a mean weight of 6-8 g. The most outstanding features of 'Silvan' are its high yield, good fruit quality and disease tolerance. 'Silvan", accounting for one third of the trailing blackberry cultivars grown in British Columbia, has gained favor among consumers and processors because of its excellent flavor, which when fully ripe, is sweeter and less acid than 'Boysen', 'Marion', or 'Young' (69,114).

'Waldo' blackberries

Breeders from Oregon State University introduced 'Waldo', a first genetically thornless trailing blackberry cultivar released, in 1988 (82). Its canes are less vigorous than those of 'Marion' or 'Kotata'. Their characteristics include high-yielding, late-bearing plants, and flavorful firm fruits with small seeds.

However, 'Waldo' is not widely planted because the plants lack sufficient vigor making management more difficult. In addition, the fruit lack the 'Marion' flavor.

'NZ' selections

'NZ9128R-1' and 'NZ9351-4' thornless blackberries are crosses made in New Zealand by Harvey Hall, but grown and selected at the North Willamette Research and Extension Center, Aurora, OR. The NZ designation refers to the origin of blackberry seeds, which is New Zealand. The fruit of 'NZ9128R-1' has similar appearance to 'Marion' and good flavor, but the size is larger than 'Marion'. Growers favor it. The fruit of 'NZ9351-4' is a small medium fruit size and the plant is somewhat upright growth like raspberry. Both are processing berries.

'ORUS' selections

ORUS- blackberries are crosses made and selected at the North Willamette Research and Extension Center, Aurora, OR. The ORUS designation is an indicative of the USDA-Oregon State collaboration. 'ORUS1489-1' and 'ORUS1380-1' are thornless cultivars. The 'ORUS-1380-1' fruit has good flavor, and similar berry size and yield to those of 'Marion'. It is used as a processed berry. Some of the 'ORUS' selections also are thorny cultivars, for example, 'ORUS1369-3' blackberry. The characteristics of 'ORUS1369-3' include early ripening, large fruit, excellent flavor, and vigorous plant. It is used for fresh market.

RIPENING PHYSIOLOGY

Development and ripening

Blackberries follow the pattern of other drupeaceous fruits in showing three distinct phases of development. Following pollination there is an initial phase of rapid growth and enlargement due to cell division; this is followed by a maturation phase of slow growth during which the embryo develops and the endocarp becomes hardened, and finally there is a period of very rapid growth due to cell enlargement (46,61). Redgwell et al. (93) reported a pronounced cell wall swelling in blackberry as the fruit ripens. Progress in ripening is also associated with increasing ease of abscission and the action of cellulase to release pectins from the cell walls of ripening fruit (1).

Ripening itself is defined by a set of physico-chemical changes usually involving: transitions in color arising from the degradation of existing pigments and the synthesis of new and often intensely colored pigments, texture changes resulting in tissue softening and sometimes liquefaction, and the production of highly distinctive flavors and aromas (61). Changes involving senescence occur throughout development and are most obvious in the later stages as the fleshy tissues disintegrate, leaving the seeds to survive. Ripening is thus part of a continuous developmental process in which several physiological phases may overlap.

The ripening time of blackberries varies from 40 to 70 days, depending on genotype and temperature. Walsh et al. (128) reported that, in general, blackberry cultivars that evolve less ethylene are known for having a longer shelf life.

Composition and antioxidant activity

Sugars and acids

Both sugars and organic acids are important flavor components and sugar/acid ratios are often used as an index of consumer acceptability and quality in fruits. In general, blackberries grown in areas that have warm dry summer, have a higher content of sugars and are more aromatic and more highly colored than fruit grown in more humid and milder regions (46).

It has not been possible to obtain from the literature a consistent picture of the chemical composition of blackberries. Some groups have reported that blackberries contain only glucose and fructose but no sucrose (137) whereas others have found sucrose level are highly variable, for example it can be up to 5 (83), 10 (89) and 13% (18) of the total sugars by weight. Nonetheless, the glucose:fructose ratio of blackberries is about 1.0 by weight of the total sugars (18,83,89). Wrolstad et al. (137,138) reported that blackberries have glucose and fructose as the principal sugars and contain no sorbitol. Thus, presence of sorbitol is a very useful indicator of adulteration. In addition, xylose is the predominant cell wall neutral sugar in blackberry (61).

A number of acids (phosphoric, malic, isocitric, quinic, lactoisocitric, shikimic, citric) have been reported in blackberries. Fan-Chiang (18) also reported a presence of fumaric and succinic acids in some authentic blackberry samples. Two distinct patterns of non-volatile acids in blackberry are identified; one consisting of isocitric acid, lactoisocitric, and malic acid but with a smaller proportion of citric acid, and the other dominated by citric acid with malic, lactoisocitric and isocitric acids present at lower concentrations (18,89). Fan-Chiang (18) reported that lactoisocitric is a marker compound in blackberry and all 52 blackberry samples contain lactoisocitric in substantial quantities (3.4-32.6% of total acids). However, Wrolstad et al. (138) found lactoisocitric being absent in six of the fifteen samples. Table 2.2 shows the differences in the soluble solids, pH, and titratable acidity of blackberries.

Table 2.2. Soluble solids, pH, and titratable acidity of blackberries

Reference	n	Soluble solids	pH	Titratable acidity
Jennings and Carmichael (47)	3	9.13-9.20	3.12-3.26 (mean = 3.18)	1.24-1.53 [a] (mean = 1.36)
Sapers et al. (103)	40	7.7-13.9 (mean = 10.8)	3.2-4.1 (mean = 3.6)	0.4-1.3 [b] (mean = 0.8)
Polesello et al. (90)	5	10.0-13.4 (mean = 12.0)	3.55-4.65 (mean = 3.82)	4.10-12.9 [d] (mean = 10.9)
Perkins-Veazie and Collins (87)	4	6.8-8.5 (mean = 7.75)	-	1.1-1.4 [c] (mean = 1.25)
Perkins-Veazie et al. (84)	4	9.0-12.8 (mean = 10.1)	-	1.19-1.37 [c] (mean = 1.29)
Fan-Chiang (18)	52	6.88-16.8 (mean = 10.8)	2.65-3.61 (mean = 3.19)	0.52-2.24 [a] (mean = 1.35)

[a] Data were calculated as citric acid, g/100mL juice; [b] data were calculated as citric acid, g/100g fruit; [c] data were calculated as percent citric acid; [d] data were calculated as meq./100g; N = number of sample.

As ripening progresses, blackberry weight and total soluble solids increase while titratable acidity decrease (86-88,93,103,128). There is a positive correlation between soluble solids content and weekly mean temperatures. The titratable acidity decreased considerably during the whole ripening period and higher temperatures also accelerate it for all varieties (79). Although few studies have been made on the changes in acid content in soft fruits during ripening, there is evidence that the proportion of the different acids changes with maturity (46) (61). Walsh et al. (128) reported that the sugar-to-acid ratios of several 'Thornless' blackberries change more than 5-fold during berry maturation and ripening.

Perkins-Veazie et al. (84) reported that sucrose and fructose decreased and glucose increase slightly during storage at 2°C after 21 days. For the fresh market, low acidity and high sugar content are preferred. These demands can easily be fulfilled when preharvest temperatures are rather high, but during cool weather it takes more time until the lowest acidity and the highest soluble solids are obtained.

Anthocyanins

Anthocyanins, the most common group of water-soluble pigments in plants, are glycosides of an anthocyanidin C_6-C_3-C_6 skeleton. In plant tissues, the anthocyanins, which are largely responsible for the attractive color of many flowers, leaves, fruits, and vegetables, produce blue, purple, red, and intermediate hues. Several chemical factors influence the color of anthocyanin solutions, but pH

is probably the most important of them, as their hue and structure are dependent on pH value and the presence of co-pigments (14)

Many studies have been done on blackberry anthocyanins (Table 2.3) and their identity has been well characterized as solely cyanidin-based compounds (18,43,68,90,100,104,113,124). Total anthocyanin content of blackberries is generally intermediate between red and black raspberries (118). In general, five anthocyanins are detected in blackberries and they are identified as cyanidin 3-glucoside (major anthocyanin), cyanidin 3-rutinoside, xylose-cyanidin derivative, and two acylated cyanidin derivatives (104). The two acylated cyanidin derivatives have been more recently identified as malonic acid acylated cyanidin 3-glucoside (18) and cyanidin 3-dioxalyl-glucoside (113). Wada and Ou (124) reported the presence of cyanidin 3-(6'-*p*-coumaryl)-glucoside in 'Marion' berries and cyanidin 3-arabinoside in 'Evergreen' blackberries, and Dugo et al. (17) reported the presence of cyanidin 3-galactoside, cyanidin 3-arabinoside, pelargonidin 3-glucoside and malvidin 3-glucoside in a commercial Italian blackberry. However, no other investigators have found these pigments in blackberries.

Table 2.3. Total anthocyanin content (mg/100g) of blackberries

Reference	n	Method	Quantified as	Total anthocyanins
Sapers et al. (103)	13	pH differential	-	57-144 [c] (mean = 103)
Polesello et al. (90)	5	Swain & Willis method [b]	Cyanidin 3-glucoside	209-302 (mean = 253)
Wilska-Jeszka et al. (136)	1	pH differential	-	115
Heinonen et al. (37)	1	HPLC	Malvidin	765
Fan-Chiang (18)	52	pH differential	Cyanidin 3-glucoside	70-201 (mean = 137)
Fukumoto and Mazza (24)	3	Glories' method[a]	Malvidin 3-glucoside	149
Wang and Lin (131)	3	pH differential	Cyanidin 3-glucoside	134-172 (mean = 153)
Moyer et al. (77)	32	pH differential	Cyanidin 3-glucoside	80-230 (mean = 145)
Sellappan et al. (107)	2	pH differential	Cyanidin 3-glucoside	110-123 (mean = 117)
Siriwoharn and Wrolstad (110)	2	pH differential	Cyanidin 3-glucoside	154-225 (mean = 190)

[a] The modified Glories' method (66); [b] the method of Swain & Willis (115); [c] unit is absorbance units per gram (A.U./g); N = number of sample.

As the fruit ripens, the amount and composition of anthocyanin increase (7,86,87,90,93,103,131). Sapers et al. (104) reported that during ripening, cyanidin 3-glucoside increases to a greater extent (10 to 16 times) than the other pigments, and the accumulation of individual anthocyanins is linearly related to the total anthocyanin content and to the soluble solids: acidity ratio. However, cultivar differences in anthocyanin pattern and changes in individual anthocyanin in fruit approaching full ripeness are insufficient to influence fruit color (104).

The rate of decrease of anthocyanin contents is also accelerated by an increase of temperatures. Considering this the intervals between pickings should not exceed four or five days, because otherwise the percentage of very ripe fruits would increase and the average anthocyanin contents and acidity would decrease.

Other polyphenolics

A number of studies and reviews on blackberries phenolics have been published (4,7,15,24,37,38,42,60,67,76,77,94,105,107,110,124,125,131,132, 135,136). They have shown that blackberries contain a complex mixture of phenolic acids (hydroxybenzoic acid, cinnamic acid, and their derivatives), anthocyanins, flavonols, flavanols, and ellagitannins. Table 2.4 shows the total phenolic content of blackberries as reported in literature.

The hydroxybenzoic acid derivatives are phenolic metabolites with a general structure of C_6-C_1. They are generally present as conjugates, though their free acids can be detected in some fruits or after being released during processing. In blackberries, the β-D-glucosides of, *p*-hydroxybenzoic and protocatechuic acids, and ellagic acid occur regularly (16,42,105,107,132). Rommel (97) reported that ellagic acid exists in nature mainly in the form of ellagitannins, esterified with glucose; the glucose molecule is often further esterified with gallic acid. Ellagitannins are reported to be present in various plants and nut species. Sanguiin H-6 **2** is the main ellagitannins present in blackberry fruits alongside casuarictin **1**, potentillin **2**, and pedunculagin **3** (15).

Table 2.4. Total phenolic content (mg/100g) of blackberries

Reference	n	Method	Total phenolics (mg/100g) [b]
Wilska-Jeszka et al. (136)	1	Folin-Ciocalteu	448
Heinonen et al. (37)	1	Folin-Ciocalteu	435
Fukumoto and Mazza (24)	3	Glories' method [a]	383 [c]
Wang and Lin (131)	3	Folin-Ciocalteu	204-248 (mean = 226)
Moyer et al. (77)	32	Folin-Ciocalteu	275-678 (mean = 478)
Sellappan et al. (107)	2	Folin-Ciocalteu	418-555 (mean = 486)
Wada and Ou (124)	2	Folin-Ciocalteu	495-583 (mean = 539)
Siriwoharn and Wrolstad (110)	2	Folin-Ciocalteu	822-844 (mean = 833)

[a] The modified Glories' method (66); [b] quantified as gallic acid equivalent; [c] quantified as chlorogenic acid equivalent; N = number of sample.

Cinnamic acids are a series of *trans*-phenyl-3-propenoic acids (C_6-C_3 skeleton) differing in their ring substitution. These compounds are also widely distributed as conjugates in plant material and rarely found as free acids in unprocessed plant material. The common acids in blackberries are *p*-coumaric caffeic, ferulic, chlorogenic, and quinic acids, and glucose esters of hydroxycinnamic acids (76,105).

Flavonols and flavanols are both subclasses of flavonoids. Flavonols are characterized by an unsaturated 3-C chain with a double bond between C-2 and C-3, and by the presence of a hydroxy group in the 3-position (60). They are usually found in plants bound to sugars as *O*-glycosides. Major flavonols in blackberries are exclusively glycosides of quercetin and kaempferol (7,38,134). Since their

formation normally depends on light, flavonols are mainly concentrated in the outer tissues (39). Flavanols' structure is similar to that of flavonols, except for the lack of an oxygen group at the 4-position of the heterocyclic C-ring and the absence of a double bond at the 2-3 positions. They exist in free form not as glycosides. Flavanols are important constituents of fruits in oligomeric or polymeric forms as proanthocyanidins or condensed tannins (96). Flavanols are referred to as flavan-3-ol monomers, such as (+)-catechin and (-)-epicatechin, while procyanidins are usually referred to flavan-3-ol and flavan-3,4-ol oligomers and polymers (97). (-)-Epicatechin is a predominate flavanol in blackberries, followed by (+)-catechin (4,76). However, Wilska-Jeszka et al. (136) reported that blackberry is a rather poor source of monomeric and oligomeric flavanols.

The effect of maturity on blackberry polyphenolics, except for anthocyanins and total phenolic content, has received very little attention. Bilyk and Sapers (7) reported a positive correlation between flavonol content and blackberry maturity.

Antioxidant activity

Various phytochemical components, including carotenoids, flavonoids, phenylpropanoids, and phenolic acids are known to be responsible for antioxidant capacity in fruits and vegetables (95). In a study on LDL oxidation, extracts of blackberries show a higher scavenging activity than those of red raspberries, sweet cherries, and blueberries (37). Wang and Jiao (129) also reported that blackberry juices have the highest antioxidant capacity inhibition of $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$,

compared to those of blueberries, cranberries, raspberries, and strawberries. Moreover, blackberries contain oxygen scavenging enzymes (49) and high amounts of ellagic acid, which has potent antimutagenic and anticarcinogenic properties (15,16,42,132). Blackberries thus contribute a significant source of antioxidants that may have potential health effects. Table 2.5 shows the antioxidant activity of blackberries as reported in literature.

Table 2.5. ORAC and FRAP contents of blackberries

Reference	n	ORAC (μmoleTE/g)	FRAP (μmoleTE/g)
Jiao and Wang (49)	6	14.8-22.6	-
Wang and Lin (131)	3	20.3-24.6 (mean = 22.4)	-
Moyer et al. (77)	32	26.7-78.8 (mean = 47.0)	40.6-106 (mean = 71.4)
Wada and Ou (124)	2	27.5-28.0 (mean = 27.8)	-
Siriwoharn and Wrolstad (110)	2	34.3-35.5 (mean = 34.9)	74.2-79.1 (mean = 76.6)

N = number of sample; TE = Trolox equivalent.

Nonetheless, the antioxidant activity varies considerably in different types of berry crops and also with different levels of maturity and different plant parts. Wang and Lin (131) reported that blackberries have the highest ORAC values during the green stages and showed a linear correlation between total phenolic content and ORAC activity. However, as the fruit ripens, the activities of oxygen-scavenging enzymes are decreasing (130).

Factors influencing berry composition

Factors such as cultivar, maturity, harvesting method, and mold contamination, play an important role in berry composition and antioxidant activity. According to Parr and Bolwell (81), mineral nutrition can have a major effect on phenolic accumulation and a limited nitrogen supply is typically associated with higher levels of phenolics in the plant. Alleyne and Clark (2) observed that increasing nitrogen (N) rates by applied ammonium nitrate (56 or 112 kg•ha-1) boost fruit N and pH of 'Thornless Evergreen' blackberries, but does not affect the soluble solids concentration, titratable acidity, sugar-acid ratio, and total solids. Due to the fact that the energy of UV light is sufficient to induce photochemical degradation of many plant components, terrestrial plants have evolved barrier to absorb this UV radiation before damage is done. It appeared that flavonoids, and in particular the flavones and flavonols, are major components of this screen (81). Thus, they tend to occur in highest levels in plant tissues exposed to strong light, being prominent components of flowers and leaves where their screening potential will be greatest. As well as UV light inducing enhanced flavonoid accumulation, temperature and water availability also influence phenolic metabolism. Lower temperatures and water deficit tend to favor increased anthocyanin production, and impair phenolic accumulation, respectively (81).

It has long been known that the levels of phenolics in blackberries can be influenced by maturity (7,86,87,90,93,103,131). According to Jiao and Wang (49), the antioxidant capacity in fruit juice of blackberry varied greatly among various

cultivars. Rommel and Wrolstad (98) also reported great differences in amount of total flavonols among red raspberry cultivars and different geographic origins. Considerable variation is found in hand-picked vs. machine-harvested blackberries (73,74). In line with Rommel and Wrolstad (98), mold appeared to only affect the glycosidic bond of flavonol glycosides, but left the ester bonds of ellagic acid derivatives untouched

In addition to environmental factors, genetic factors within crop populations can have important effects on the phenolic composition in fruits (81,96). Prior and Cao (92) suggested that antioxidant capacity of fruits and vegetables may be influenced by genetics as well as environmental factors.

Differences in methodology used for extraction and analysis, fruit tissues (pulp, flesh, skin, and so on), and the way in which the quantitative results are expressed (on a dry or fresh weight basis) are vital reasons for great variation in the phenolic content of fruits and vegetables (117). These multiple sources of variability make comparison difficult and seriously limit the precision of any generalized estimates of burden. Reliable analytical methods, as well as simple, controlled methods for the extraction of phenolics from fruits are essential, when fruit extract with high antioxidant is the target. A number of phenolic isolation and identification methods have been reviewed (59,60,62-64,96).

Possible health benefits

Man has used blackberries for a very long time. Their juice was used medicinally and recommended for infections of the mouth and eye (67). Interest in blackberry phenolics has increased owing to their roles as antioxidants, anticarcinogens, antimutagens, and scavengers of free radicals and their implication in the enhancement of visual acuity and prevention of pathologies such as cancer and cardiovascular disease. There is convincing evidence that suggests the role of oxidative stress, active oxygen species and free radicals in a variety of diseases, such as heart disease, cataract, atherosclerosis, vascular dysfunction, inflammatory-immune injuries, neurodegenerative disorders, and cancer (21,22). Epidemiological evidence shows that increased levels of fruit and vegetables in the diet reduce the risk of cancer and heart disease, as well as cataracts, brain and immune dysfunction, and stroke (81,112).

Blackberries are a rich source of anthocyanins and other polyphenolic antioxidants (49,77,107,110,124,131), which function as terminators of free radicals and chelators of metal ions that are capable of catalyzing lipid peroxidation. These antioxidants also interfere with the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals (9). Various berry juice antioxidants are variably absorbed and are active as antioxidants *in vivo* (80). Marquina et al. (65) reported that aqueous extract of blackberry fruits inhibits the activity of hyaluronidase enzyme and shows better anti-inflammatory activity when compared with aspirin.

Impact of polyphenolics

Most flavonoids are effective radical scavengers. Epidemiological evidence shows that the consumption of flavonoid-rich foods, especially those containing flavonols and flavones, is inversely associated with coronary heart and artery diseases (28,44) in both a prospective cohort study and in a cross-cultural study (41). The antioxidant or free-radical-scavenging property of flavonoids has been proposed to contribute to this chemopreventive effect (28). Moreover, they may reduce peroxidative tendencies and retard the several interactions involved in atherogenesis and thrombosis (50). A significant correlation is found between the radical-scavenging capacity and the anthocyanin and total phenolic contents in blackberry fruits, but no correlation is found between this parameter and the ellagic acid and vitamin C contents (29). Vinson et al. (121) observed that many polyphenols are stronger antioxidants than the vitamin antioxidants using the LDL + VLDL oxidation model.

Flavonoids and phenolic acids are believed to exert cardioprotective effects in humans via their ability to inhibit oxidation of low-density lipoprotein (LDL). Ishikawa et al. (44) reported that LDL exposed to tea flavonoids *in vitro* or *in vivo* reduces oxidizing ability. This finding is in agreement with those of Vinson et al. (122,123), which found flavonoids in fruit juices and teas enriching LDLs and increase their oxidative resistance after *ex vivo* spiking in human plasma. The order of antioxidant activity is catechin > cyanidin ≈ caffeic acid > quercetin > ellagic acid (71). The efficiency of polyphenols as antioxidant compounds greatly depends

on their chemical structure. Ellagic acid is a putative inhibitor of certain chemically induced cancers (132). Quercetin 3-*O*-β-D-glucuronide, which is one of the quercetin metabolites in the blood after intake of quercetin-rich food, significantly inhibits lipid peroxidation (108). The principal site of scavenging reactive oxygen species in quercetin is the *O*-dihydroxyl substituent in the B-ring, as well as the C-ring loefinic linkage (51).

Possible health benefits of proanthocyanidins (102) and ellagic acid and ellagitannins (15) have been reviewed. Yamakoshi et al. (140) suggested that procyanidins and their antioxidative metabolites prevent the progression of cataract formation by their antioxidative action. Previously we have shown that blackberry seeds are a rich source of procyanidins, which are about 4 times higher than that of whole berries (110). Thus, they can be used in foods and nutritional supplement formulations.

HARVESTING AND STORAGE

Blackberry season

According to Moore and Skirvin (73), blackberries ripen from May in the southeastern U.S. production regions to July in the Pacific Northwest . The fruit period of individual cultivars ranges from 4 to 7 weeks at a given location. Among the three types, the trailing blackberries are usually the earliest to ripen, and the

erect blackberries can begin ripening a week or so later, depending on variety. Semi-erect thornless blackberries usually do not begin ripening until mid-summer (91).

In southern Victoria, Australia, 'Silvan' is harvested from the first week of December to the first week of January, two weeks earlier than 'Marion' (69). In the Pacific Northwest, the 'Marion' crop typically begins to ripen at the end of June with commercial harvest beginning the first week of July and finishing in late July (20). On contrary, 'Evergreen' blackberry season is normally July to September.

Harvesting

Blackberry fruit is considered to be one of the most perishable commodities. It requires frequent picking and prompt marketing. The fruits must be harvested, packed, stored, and marketed with utmost care if they are to remain in good condition. The best index of maturity is the ease of separation of the fruit from the pedicel. Fruit color is a poor indicator since many varieties turns black before they are ripe (13,45,73,82,106). Most cultivars need a couple more days to mature to their best flavor. With these, there is a tendency of most pickers to harvest fruit that is still under ripe and sour or sometimes bitter. They must be more discriminated in waiting for the fully mature fruit. However, for distant markets this cannot be wholly avoided, as the fruit must be picked while still firm, even at the expense of quality (13).

The best time of day to pick is in the cool of morning, but berries should never be picked when wet in dew or rain, since wet berries mold very quickly (73). Harvested fruits also should be protected at all times from the sun, since exposure for even a few minutes will cause them to turn reddish and the flavor become bitter, and at the same time reduce the length of time that they will stand up in transit (13,73,106). Removing field heat by rapid cooling will greatly extend the shelf life of the fruit.

According to Moore and Skirvin (73), partial crop of fruit may be expected the second summer after planting blackberries. By the third harvest season, full production is reached. A properly managed blackberry planting should yield 6,000 to 8,000 lb/acre (6,700 to 9,000 kg/ha), and even higher yields are possible (73). Since the crowns and roots of blackberries are perennial, a well-cared-for planting should remain productive for many years.

Hand- vs. mechanical harvester

Recently, mechanical harvesters have been developed to rapidly harvest large acreages of blackberries for processing. Mechanization of the blackberry industry has been necessary due to the increasing scarcity and expense of hand-labor, which threatened to eliminate blackberries as a processing crop (74). For example, some blackberries have long harvest duration (5 to 6 weeks) and the ripe berries must be harvested at two- to four-day intervals, the cost of multiple

harvesting is the single largest annual expenditure and can require up to 900-man-h/ha for hand harvest (33).

Generally, the machines selectively harvest higher-quality fruit than hand pickers (73,74), since the machine utilizes a shaking principle that removes fully ripe fruit, while immature fruit remains on the plant for later harvest. Morris et al. (75) reported that under commercial conditions at harvest, mechanically harvested blackberries had raw and processed quality comparable to hand-picked fruits regardless of berry temperature. Morris (74) reported that machine-harvested berries are larger and have a higher percentage of total soluble solids, lower acidity, and superior color than hand-harvested berries. Moreover, processed berries that have been machine-harvested have been rated superior to hand-harvested berries for wholeness, flavor, and color. Development of mechanical harvesters that can distinguish ripe from unripe fruit and make several harvests a year without destroying plants has made it feasible to grow blackberries on large acreages (57).

Some disadvantages of the mechanical harvesting are: 1) the fruit is often severely damaged during machine harvest; therefore quick processing is critical (33,74), and 2) many existing cultivars of blackberry do not have adequate fruit firmness to be machine harvested for fresh market use. In those situations, where a fresh-market quality product is essential, hand picking has been required. In the absence of a machine harvester, only allowing a long picking interval, which must inevitably be a compromise to avoid deterioration of the fully ripe fruit, can attain

adequate fruit maturity (47). The optimum-picking interval is influenced by weather at harvest time, and may be relatively long in a northern environment.

Over 85% of the trailing blackberry Hectarage in Oregon is machine harvested whereas only 10% is machine harvested in California (114). All of the erect and semi-erect blackberries are handpicked in the Northwest. Fruit for the IQF (individually quick frozen) market and the very limited fresh market is usually harvested by hand since the lack of fruit firmness is a major limitation, especially for 'Marion' (20).

Storage and shelf life

Freshly picked blackberries have the shortest shelf life of any small fruit (91). The rapid loss of blackberry fruit quality following harvest and perishability are severe marketing limitations. The berries can be over grown with fungus within 24-48 hours of harvest if proper storage conditions are not provided (111). The longer or excellent shelf life of blackberries indicates the more suitable the fruit for long distance shipment. Morris et al. (75) reported that use of high CO_2 storage atmospheres with fruit held at 20°C partially offset the need for refrigeration to reduce postharvest quality loss. Basiouny (5) found 'Chester' fruit harvested early during the season, stored better than fruits of later harvest. Though berries machine-harvested at high temperature (36°C) deteriorate more rapidly than handpicked berries at the same temperature, storage of machine-harvested fruit in 20% and 40% CO_2 at 20°C for up to 48 hours has been found to maintain their raw

and processed quality (75). Basiouny (6) reported that blackberry fruit stored under UV-B radiation are better than similar fruits stored under the same conditions without UV-B radiation.

Sugars and acids, the dominant chemical entities in fruit juices, are less affected by processing and storage than are constituents such as pigments and flavors (138). Perkins-Veazie et al. (88) found that during storage for seven days at fresh market shelf life condition (2°C, 95% RH), soluble solids, titratable acidity, and skin resistance do not change. Although there is an agreement that soluble solids are unchanged during storage, some studies found the titratable acidity to decline (up to 40-50%) during storage of several blackberry cultivars (83,84). In addition, Perkins-Veazie and Collins (83) reported that sucrose and glucose decrease, while fructose slightly increases during storage.

The anthocyanin content of blackberries stored under retail storage condition has been found to increase from 20-100% depending on cultivar, storage temperature and storage intervals (83). Moreover, Sapers et al. (103) observed that rapid thawing of frozen fruit results in less anthocyanin loss than does slow thawing. However, the ORAC of five blackberry cultivars is not significantly increase at the latter stages of ripeness or by fruit storage at 2°C (85). Mullen et al. (78) reported that the antioxidant capacity of the fresh fruit and the levels of vitamin C and phenolics are not affected by freezing.

COMMERCIAL IMPORTANCE

Most blackberries produced in the United States are processed, including 99% of the Pacific coast crop (111). Blackberry cultivars grown for processing uses vary in popularity according to the flavor, color, acidity, sugar content, fruit and seed size, and whether the berry is cooked or otherwise processed before consumed. No cultivar has universal popularity, and even in Oregon the higher flavored cultivar 'Marion' is often mixed with 'Thornless Evergreen' for reasons of dependability and cost of production (32).

Blackberries are processed into many products including preserves, jams, juices, and yogurt, as well as canned and fresh-frozen berries. The fruit should be processed when fully ripe, as the flavor is best at this stage and the seeds are less noticeable (12). Quantity and price of blackberry products vary annually, primarily due to total production and competition from other fruit crops with similar uses (e.g., blueberry) (111).

Fresh and frozen berries

The blackberry is primarily a table fruit for immediate consumption (13). Most blackberries produced outside of the major production regions are sold fresh-picked. Fruit firmness is critical for shelf life for fresh market. Most of the trailing blackberries, though having a better fruit flavor than erect or semi-erect types, have softer fruit than the erect types. Thus, erect types contribute to most of the fresh market fruit, except fruit produced in California (114). Pick-your-own sales of

berry fruit have been common across all the popular berry species for many decades, but on most properties in the past this outlet has been secondary to the much more important wholesale fresh and processing markets (23). However, the pick-your-own concept of direct marketing has greatly stimulated an interest in blackberry culture in many areas of the United States (73) and is especially desirable as a marketing solution to the high harvest labor input required (45).

Nonetheless, blackberries, as many other soft fruits, do not store well. They are susceptible to molds and yeasts, and fruit held outside a comparatively short harvest season is usually stored deep-frozen (116). Blackberries are well suited to preservation by freezing (25). However, the quality of the product may vary after thawing (103). One of the principal factors affecting frozen blackberry quality is the cultivar selection. The most desirable blackberry cultivar characteristics for freezing include: tender skin, small seeds, solid black color, plumpness, bright appearance, and distinctive flavor (25). A significant portion of the blackberry crop, about 50% is frozen, rapidly replacing the canned product for this purpose (56). About 18% of Oregon's production is sold retail as individual quick-frozen (IQF) berries (111).

Blackberry juice and beverages

Berry juices are usually highly flavored and colored and as such are ideal for blending with other juices such as clarified apple or pear juice (101). Importance must be placed on retaining these characteristics during processing into

juice. Preservations of the juice include thermal processing, freezing and freeze-concentration, in which the latter two are used when flavor is important. Combinations of ultrafiltration and reverse osmosis have been used to concentrate fruit juices without heating (101).

Berry juice concentrates have a higher economic value than apple, pear, or grape concentrates because of the higher cost of raw berries and lower processing yields (137). Fruit juice concentrates have become an important ingredient in the manufacture of many foods and beverages (43). The high economic value (about $70/gal in 1999) and increasing demand for blackberry juice concentrate in blended juices and drinks has made it a likely target for adulteration (18). Wrolstad et al. (137) reported that adulteration in blackberry juice concentrates and wines with juice of sorbitol-containing fruits can be detected by high performance liquid chromatography (HPLC), gas-liquid chromatography (GC), and thin layer chromatography (TLC). Products derived from fruit juice include fruit juice drink, fruit nectars, and carbonated beverages (101).

Haze and sediment formation

Some blackberries are used for juice production; however, the importance of blackberry juice in the United States is very minor (111). The reason that production of blackberry juice and wine (100), especially from the cultivar 'Evergreen', has not been commercially successful is due to its excessive haze and sediment formation along with color loss and browning in the wines during storage.

The similar problem has been reported earlier in apple juice (34-36,120,139), and muscadine grape juice and wine (8,27,30,53,54). However, the literature contains little information of the formation and composition of hazes and sediment in blackberry juices. Rommel et al. (100) observed that haze development and sediment formation occurred in blackberry wine over 6 months of storage at 20°C.

(1) Cause of haze and sediment. Hazes or sediment in fruit juices may result from several factors, such as microorganisms, starches and dextrins, protein-tannin complexes, tannins, gums, and metal ions. These haze precursors can be derived from the fruit, although some are introduced in the processing line (e.g., the addition of enzymes and gelatin at the clarification stage) (120). The most frequent cause of haze in beer, wine, and clear fruit juices results from protein-polyphenol interaction, and these clear beverages are typically stabilized to delay the onset of protein-polyphenol haze formation (109). In apple juice and apple wine, the problem occurs after bottling (or post-bottling haze) and the causes are due to oxidized polymerized phenolics and phenolic-protein complexes (36), pectin (35), starch (34), tannins, or proteins (139), either alone or in combination (120).

Unlike apple juice, the sediment in muscadine grape juice is identified through various chemical and Spectrophotometric means as ellagic acid (8,27,54). Ellagic acid is a dimer of two molecules of gallic acid, found in many foods, including blackberries, and nuts. Though levels of ellagic acid in muscadine grape juice are very low and do not correlate well with amount of sediment formed (53),

Garrido et al. (27) monitored ellagic acid sedimentation and found that high storage temperatures (40°C) greatly accelerate sediment formation and sediment increased substantially after juice was hydrolyzed (121°C and pH 2 for 10 min).

It has been reported that processing techniques influence initial concentrations of ellagitannins and ellagic acid and the extent of sediment formation (53). Especially, ellagic acid content in fruits is influenced by cultivar (58), processing and storage conditions (30,99). Maas et al. (58) and Rommel and Wrolstad (99) reported large differences in ellagic acid content among cultivars for strawberry (n = 35) and red raspberry (n = 10), respectively. Zafrilla et al. (141) observed that the content of free ellagic acid of raspberry jam increased 3-fold during the storage period, and 2-fold with processing and suggested that the increase in ellagic acid is due to a release of ellagic acid from ellagitannins with the thermal treatment. In addition, Ancos de et al. (3) reported a significant decrease of 14-21% in ellagic acid of raspberries at the end of long-term frozen storage (12 months). Häkkinen et al. (30) also showed that the content of ellagic acid in strawberries and red raspberries was reduced by 40% and 30%, respectively, during the 9 months of storage at –20°C.

(2) Possible prevention. Van Buren (120) reviewed several procedures for dealing with after-bottling hazes in apple juice. In general, there are two approaches to this problem: attempting to prevent haze formation or removing haze after it has formed in stored juice. The first approach focus on removing as much of the potential haze-

forming materials from the juice as possible with corrective procedures (such as fining) before bottling. Lin and Vine (54) and Garrido et al. (27) showed that treatment of juice with polyvinylpolypyrrolidone (PVPP), egg albumen, or gelatin resulted in a significant reduction of juice phenolics and sediment formation. Presumably, the polyvinylpolypyrrolidone bound to polyphenols, which in turn are complexed with protein (139). PVPP works very effectively in apple juice, but less so in beer (109). Bentonite, and in particular silica-sol, are useful fining agents when used in conjunction with gelatin for the prevention and removal of hazes containing polyphenolics, proteins and cation complexes (36,109). Heatherbell (35) reported that the maximal removal of starch and pectin is of prime importance for the prevention of haze and sediment formation in clarified apple juice and wine. Starch and pectin that cannot be removed by physical processes such as fining, centrifugation and filtration can be removed by thorough enzymatic hydrolysis (34). HTST-pasteurizing also retards polymerization and also minimizes haze and sediment formation in 'Evergreen' blackberry wine (100).

The second approach involves reworking on juice to give a clear product free of haze and haze-forming materials. The particular techniques used depend on the composition of the haze present. One novel way to obtaining top-grade juices is to separate, through crossflow microfiltration, suspended solids from the serum, pasteurize the retentate, then re-introduce it into the 'sterile' clarified juice (27,119).

Other products

Preserves, jams, jellies, and pastry fillings

The most important use for blackberries is production of preserves, jams, and jellies (111). Waldo (127) estimated in 1977 that 70% of Oregon's crop was processed into jelly, with the remainder used for bakery products. Very few large commercial concerns now produce jams and marmalades from fresh fruit during the harvesting season; most use fruit that has been preserved one way or another (11). Three types of preservation are used: freezing, canning, and sulfiting. Halat et al. (31) reported that jams made from hand-harvested blackberries are less seedy and have more intense blackberry flavor than machine-harvested fruit (n = 6) and jam of 'Marion', 'Black Satin', and 'Chester' fruit had less off-flavor than the imported fruit blend and Thornfree jams.

Canned blackberries

Canned blackberries are no longer a major product due to the high price of an individual can and the increased use of frozen blackberries (111). The latter are less expensive and easy to process. Ordinary tin is said to discolor the fruit when it comes in contact with it (13). Nonetheless, canners are able to use large quantities of wild and other low-grade fruit, which might otherwise go to waste. The process (12) starts with the berries are tipped onto an inspection belt where any leaves, calices, and other foreign material are removed. They are then washed in a flood washer and sprayed with clean water as they emerge on an inclined belt. Following

this they are filled into cans, hot syrup is added, and the cans are seamed and pasteurized.

Dried blackberries

Blackberries are sometimes grown for this purpose, but the shrinkage is so great with them that they are not nearly so profitable as the firmer fleshed and more seedy black raspberries (106). Dried blackberries are nearly always quoted in market, the supply coming almost wholly from the South, where the wild berries are gathered and are dried in the sun (13). They are usually poor in quality, and quoted at a price which would render it unprofitable to dry them if there were a market for fresh fruit.

Ice cream and yogurt

About 5% of Oregon's blackberry crop is used in ice cream and yogurt (111). The rapid expansion of the flavored yogurt industry is seen as potentially large new outlet for blackberries. Blackberries are used commonly as flavoring for "mixed berries" yogurt, which usually contains strawberries and blueberries as well as blackberries (111).

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CHAPTER III

INFLUENCE OF CULTIVAR AND MATURITY ON BLACKBERRY ANTHOCYANINS, POLYPHENOLICS, AND ANTIOXIDANT PROPERTIES

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ABSTRACT

The influence of ripening on anthocyanin pigments, polyphenolics and antioxidant properties of 'Marion' and 'Evergreen' blackberries was investigated. The anthocyanin pigments changed tremendously with total anthocyanins increasing from 74.7-317 mg/100g fw from underripe to over ripe for 'Marion', and from 69.9-164 mg/100g fw for 'Evergreen'. Total phenolics did not show marked change with maturity with values slightly decreasing from underripe to ripe. Antioxidant activities while increasing with ripening also did not show the marked change that total anthocyanins exhibited. The impact of variation due to plots, sub-sampling, sample preparation, and measurement on 'Marion' composition was examined in detail. Plot-to-plot and sample differences were the major contributor to variation, with sample preparation accounting for variation for some parameters. Measurement variation was generally a relatively small component of the total variation. The anthocyanins and polyphenolic composition of ripe fruits from 11 blackberry cultivars were determined along with their antioxidant properties. Total anthocyanins for the 11 cultivars ranged from 131-256 mg/100g fw (mean = 198), total phenolics from 682-1056 mg GAE/100g fw (mean = 900), ORAC from 37.6-75.5 μmolTE/g fw (mean = 50.2), and FRAP ranged from 63.5-91.5 μmolTE/g fw (mean = 77.5). Four blackberry cultivars were found to be higher in total anthocyanins and total phenolics than 'Marion' and 'Evergreen', the predominant commercial cultivars in the Pacific Northwest. The results from these studies confirm that blackberries are a good source of natural

antioxidants and pigments, and demonstrate the potential for obtaining new cultivars with high pigment/phenolic content through classical plant breeding.

Keywords: blackberries, composition, anthocyanins, polyphenolics, antioxidant activity, cultivar, sample variation, and maturity.

INTRODUCTION

Polyphenolics include several classes of phenolic compounds that are secondary plant metabolites and an integral part of both human and animal diets. While phenolics historically were considered in some instances to be anti-nutrients, interest in food phenolics has increased greatly because of their antioxidant capacity (free radical scavenging and metal chelating activities) and possible beneficial roles in human health, such as reducing the risk of cancer, cardiovascular disease, and other pathologies (4,14).

Blackberries are a rich source of anthocyanins and other polyphenolic antioxidants (18,34,36). Extensive studies have been done on blackberry anthocyanins and their identity has been well characterized as being solely cyanidin-based compounds (8,31,34). In general, five anthocyanins are detected in blackberries and they are identified as cyanidin 3-glucoside (major anthocyanin) (8,25,33,34), cyanidin 3-rutinoside (8,33), malonic acid acylated cyanidin 3-glucoside (8,34), xylose-cyanidin derivative (8,25) and cyanidin 3-dioxalyl-

glucoside (31). Wada and Ou (34) also reported the presence of cyanidin 3-(6'-*p*-coumaryl)-glucoside in 'Marion' berries and cyanidin 3-arabinoside in 'Evergreen' blackberries, and Dugo et al. (6) reported the presence of cyanidin 3-galactoside, cyanidin 3-arabinoside, pelargonidin 3-glucoside and malvidin 3-glucoside in a commercial Italian blackberry. However, no other investigators have found these pigments in blackberries.

We recently investigated the polyphenolics of blackberries and found that flavonols (primarily quercetin glycosides) were the major phenolics in the berries whereas procyanidins (catechin and epicatechin-based) and ellagic acid derivatives predominated in seeds (29). The presence of ellagic acid derivatives, quercetin and kaempferol glycosides, catechin and epicatechin confirmed identifications of previous workers (1,12,17,26,27,35,37).

Genetic and environmental factors, such as cultivar, maturity, and harvesting method, play an important role in berry composition (19). It is well known that levels of phenolics and the antioxidant capacity of blackberries are influenced by maturity (36) and that there is pronounced variation among cultivars (15). Small differences in ripeness can have an effect on sample-to-sample variation, however, the effects of plot-to-plot differences, sample preparation (milling, extraction, isolation) and measurement have not been systematically investigated. Differences in methodology used for extraction and analysis, and the way in which the quantitative results are expressed (on a dry weight or fresh weight

basis), are reasons for the great variation in phenolic content of fruits and vegetables as reported in the literature (32).

The objectives of this study were to investigate the anthocyanins, polyphenolics, and antioxidant properties of blackberries, to evaluate the influence of cultivar and maturity, and to determine the variation caused by plot difference, sub-sampling, sample preparation, and analytical measurement.

MATERIALS AND METHODS

Reagents and standards

Folin-Ciocalteau reagent was purchased from Sigma Chemical Co. (St. Louis, MO) as were citric acid and the following phenolic standards: gallic acid, (+)-catechin, ellagic acid, and quercetin 3-rutinoside (rutin). Acetone, chloroform, sodium carbonate, HPCL grade methanol, HPLC grade ethyl acetate, formic acid, phosphoric acid, were purchased from EM SCIENCE (A division of EM Industries, Inc., Gibbstown, NJ). Acetic acid (glacial) and hydrochloric acid were obtained from Allied Chemical (General Chemical Division, Morristown, NJ) and J. T. Baker Inc. (Phillipsburg, NJ), respectively. Cranberry and blackberry juice concentrates were provided by Kerr Concentrates Inc. (Salem, OR).

Plant materials

All blackberries were harvested at the Oregon State University (OSU) North Willamette Research and Extension Center, Aurora, OR, from late-June to mid-August, 2002.

'Marion' and 'Evergreen' blackberries, the two major commercial varieties in the Pacific Northwest, were chosen for investigation of three maturity stages (part-red-part-black, ripe, and over ripe). Berries were handpicked by the senior author from three plots of 'Marion' (kept separately) and from one plot of 'Evergreen' at three maturity stages: underripe (part-red-part-black), full ripe, and over ripe. Determination was based on visual appearance of the fruits. Berries were transported (ca. 1 hr) in insulated cooled containers to the OSU Food Science and Technology pilot plant where they were immediately frozen and stored at -23°C.

Ripe berries from eleven blackberry cultivars ('Marion', 'Waldo', 'Evergreen', 'Chester', 'Silvan', 'NZ 9128R-1', 'NZ 9351-4', 'ORUS 1843-3', 'ORUS 1380-1', 'ORUS 1489-1', 'ORUS 1369-3') were selected for their genetic diversity and commercial importance, and used as materials for the cultivar study. Berries were grown at the North Willamette Research and Extension Center, Aurora, OR, and provided by C. E. Finn, USDA-ARS, Corvallis, OR. 'Evergreen' and 'Chester' berries were harvested from a single plot while berries of the other nine cultivars were harvested from three different plots and combined. Samples were stored at -23°C. Figure 3.1 illustrates the sampling design for the maturity and cultivar studies.

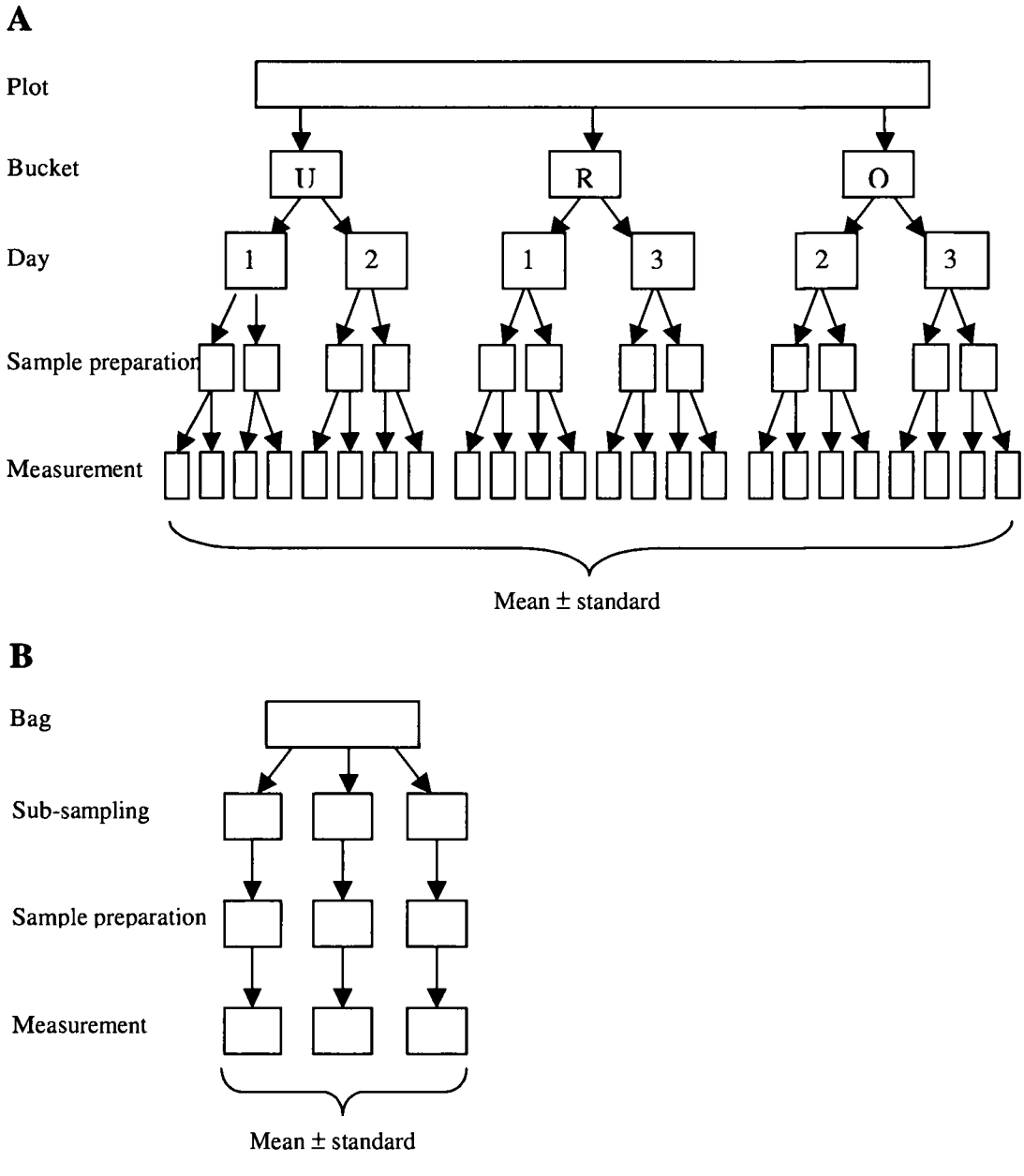

Figure 3.1. Sampling design for: (A) maturity study, (B) cultivar study. Each flowchart represents an experimental plan for one plot. U, underripe berries; R, ripe berries; O, over ripe berries.

Extraction of anthocyanins and polyphenolics

Berries were cryogenically milled with liquid nitrogen using a stainless steel Waring Blender as previously described (23). The powder (ca. 5 g) was mixed 1:1 (w/v) with acetone, sonicated with an ultrasonic cleaner (Branson Cleaning Equipment Co., Shelton, CT) for 3 min, and centrifuged. The remnant was re-extracted with 70% (v/v) aqueous acetone twice. The filtrates were combined and gently mixed with chloroform (1:2, v/v). After centrifugation at 3000 rpm for 30 min on an IEC International Centrifuge (Model UV, International equipment Co., Boston, MA), the aqueous phase (top portion) was collected and placed on a Büchi rotary evaporator (Brinkmann Instruments, Westbury, NY) at 40 °C under vacuum to remove residual acetone. The aqueous extract was then made up to a known volume with de-ionized water and stored at –70 °C until analyzed. Extractions were done in triplicate for each sample.

Total soluble solids and titratable acidity

Total soluble solids was measured using an Auto Abbe refractometer 10500 (Reichert-Jung, Leica Inc., Buffalo, NY). The instrument was set to measure % total soluble solids with the temperature compensated mode. A Metrohm titration unit (Brinkmann, Metrohm Herisau, Switzerland) equipped with a TT A80 Titration Assembly (Radiometer A/S Copenhagen, Denmark) was used for total titratable acidity determination. Results were calculated as grams of citric

acid/100g fresh weight (fw). Analyses were replicated twice for the maturity study with no replication for the cultivar study.

Total phenolics

Total phenolic content was determined with Folin-Ciocalteau reagent (Sigma Chemical Co.) by the method modified from Singleton and Rossi (28) using gallic acid as a standard. A series of seven test tubes each containing 7.5 mL de-ionized water and 0.5 mL reagent was prepared. To each test tube one of the following solutions was added, 0.5 mL of sample (diluted as necessary), 0.5 mL of 40, 120, 200 ppm gallic acid dilution, or 0.5 mL de-ionized water as a blank. All solutions were well mixed using VWR Vortexer (model G-560, Scientific Industries, Inc., Bohemia, NY), held at room temperature for 10 minutes. Then, to each test tube 3 mL of 20% Na_2CO_3 solution was added, mixed well, and placed in a heat block (VWR International, West Chester, PA) at 40 °C for 20 minutes. After the reaction, test tubes were immediately cooled in an ice bath for 3 minutes. The absorbance of the samples and standards were measured at 755 nm, using a Shimadzu 300 UV spectrophotometer and 1 cm pathlength disposable cells. Results were calculated as mg of gallic acid equivalent (GAE) per 100 g fw. Analyses were replicated twice for the maturity study with no replication for the cultivar study.

Total monomeric anthocyanins

Total anthocyanin content was determined using a pH differential method (10). A Shimadzu 300 UV spectrophotometer and 1 cm pathlength disposable cells were used for spectral measurements at 510 and 700 nm. Pigment content was calculated and expressed as Cyanidin-3-glucoside (Cyd-3-glu)/ 100 g fw, using an extinction coefficient (ε) of 26,900 $Lcm^{-1}mol^{-1}$ and molecular weight of 449.2 $gmol^{-1}$. Analyses were replicated twice for the maturity study with no replication for the cultivar study.

Antioxidant activities

Antioxidant properties were determined by the oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays at the Linus Pauling Institute, Oregon State University. The ORAC assay was performed as described by Cao et al. (5) and adapted for use in a 96-well microplate fluorometer (model Cytofluor 4000, PerSeptive Biosystems, Framingham, MA). β-Phycoerythrin acted as target for the peroxyl radicals generated by 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH). ORAC values, derived from triplicate analyses, were expressed as μmole Trolox equivalent (TE) per gram fw. The FRAP assay (2) was adapted for use in a 96-well microplate spectrophotometer (ThermoMax, Molecular Devices, Foster City, CA). FRAP values, derived from duplicate analyses, were expressed as μmole TE per gram fw. Both assays were

performed on one randomly selected replicate.

Anthocyanin and polyphenolic purification

The method described by Skrede et al. (30) for separation of blueberry anthocyanins from other phenolics was followed. The extract (1 mL) was applied to C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA), which had been previously activated with 5 mL ethyl acetate, 5 mL acidified (0.01% HCl) methanol, and 5 mL acidified (0.01% HCl) water, respectively. The cartridge with the absorbed extract was then washed with 10 mL acidified (0.01% HCl) water, after which the cartridge was dried with a current of nitrogen for 3 minutes. Ethyl acetate (10 mL) eluted polyphenolics free from anthocyanins. Anthocyanins were eluted with 10 mL of acidified (0.01% HCl) methanol. Both eluents were evaporated to near dryness on a Büchi rotary evaporator (40 °C under vacuum), and taken up in de-ionized water. Samples were filtered through a 0.45 μM Millipore filter; type HA (Millipore Corp., Bedford, MA) before being injected onto the HPLC system.

High Performance Liquid Chromatography (HPLC)

Analytical HPLC for anthocyanins

(1) Apparatus. A Hewlett-Packard 1090 Liquid Chromatograph (Agilent Technologies, Palo Alto, CA), equipped with photodiode array detector and

Gateway 2000 P5-90 computer with Hewlett-Packard HPLC^{2D} Chemstation software was used.

(2) Column, mobile phases, and HPLC condition. Chromatographic analysis was done according to the method of Durst and Wrolstad (7) using a Prodigy ODS-3 column (5 μm), 250 × 4.60 mm i.d. (Phenomenex, Torrance, CA), fitted with an Allsphere 10 × 4.6 mm i.d. ODS-2 guard column (Alltech, Deerfield, IL). Mobile phase A consisted of 100% HPLC grade acetonitrile, and mobile phase B was a mixture of 1% phosphoric acid and 10% acetic acid (glacial) (v/v) in de-ionized water. Solvents and samples were filtered through a 0.45 μM Millipore filter type HA for aqueous or HV for organic solvent (Millipore Corp., Bedford, MA).

The program was: (a) 0 minute 2% A; (b) 0 – 25 minutes linear gradient from 2 to 20% A; (c) 25 – 30 minutes linear gradient from 20 to 40% A; (d) 30 – 34 minutes linear gradient from 40 to 2% A; 5 minutes post time and gradient repeated. Simultaneous monitoring was performed at 280, 320, and 520 nm at a flow rate of 1 mL/min and injection volume of 20 μL. Identification was made from matching UV-visible spectra and retention times with known anthocyanins from well-characterized cranberry and blackberry juices.

Analytical HPLC for other polyphenolics

(1) Apparatus. A Varian 5000 Liquid Chromatograph (Varian Instrument Group, Sunnyvale, CA) equipped with a Hewlett-Packard 1040A photodiode array detector

and Gateway 2000 P5-90 computer with Hewlett-Packard HPLC^{2D} Chemstation software was used.

(2) Column, mobile phases, and HPLC condition. Chromatographic analysis was done using a Synergi Hydro-RP column (4 μm), 250 × 4.60 mm i.d. (Phenomenex, Torrance, CA), fitted with an Allsphere 10 × 4.6 mm i.d. ODS-2 guard column (Alltech, Deerfield, IL). Mobile phase A consisted of 100% HPLC grade methanol, and mobile phase B was 1% formic acid in de-ionized water. Solvents and samples were filtered through a 0.45 μM Millipore filter type HA for aqueous or HV for organic solvent.

The program was: (a) 0 minute 10% A; (b) 0 – 50 minutes linear gradient from 10 to 35% A; (c) 50 – 55 minutes linear gradient from 35 to 70% A; (d) 55 – 60 minutes isocratic at 70% A; (e) 60 – 66 minutes linear gradient from 70 to 10% A; 5 minutes post time and gradient repeated. Simultaneous monitoring was performed at 255, 280, 320, 360, and 520 nm at a flow rate of 1 mL/min and injection volume of 20 μL. Identification was made from matching UV-visible spectra and retention times with authentic standards (when available). Quantitation of individual polyphenolic peaks was done by the external standard method. Catechin and procyanidins were determined as catechin at 280 nm; ellagitannins as ellagic acid at 255 nm; flavonols as rutin at 360 nm; ellagic acid and ellagic acid derivatives as ellagic acid at 255 nm.

Electrospray Mass Spectrometry (ESMS)

Low-resolution MS was obtained using ESMS. The instrument was a Perkin Elmer SCIEX API III bimolecular mass analyzer (Ontario, Canada) equipped with an ion spray interface (ISV = 5500, orifice voltage = 50) and loop injection. The mass spectrometer was operated in the positive-mode. Purified anthocyanin fraction was bled into the system by a 100-μL-glass syringe connected with the infusion pump at a flow rate of 5 μL/min.

Tandem Mass Spectrometry (MS/MS)

Collision-induced dissociation (CID) of the purified anthocyanin fraction was carried out using argon as the target gas. The mass of the parent ion was scanned in the first quadrupole (Q1), *m/z* selected and collisionally activated in Q2, and the daughter ions were analyzed in the third quadrupole (Q3). MS/MS was set in the multiple reaction monitoring (MRM) mode and performed using a collision energy set of +30 eV.

Statistical analyses

Variance components for different levels in the sampling design (plots, sub-sampling, sample preparation, and measurements) of 'Marion' data were estimated by ANOVA method of components using the MIXED procedure in SAS version 8.1 (SAS® Software, SAS Institute Inc., Cary, NC). Means and standard deviations of both cultivars were reported.

In the case of 'Marion' data, for each response maturity groups were compared at the plot level by (1) calculating the mean for each plot-by-maturity combination (3 X 3) and then (2) analyzing the 9 observations using an ANOVA model with plots (2 d.f.), maturity level (2 d.f.) and residual (4 d.f.) The residual measures the consistency of the maturity differences across plots. Tukey's-b procedure was used for the pairwise comparisons. Analyses were conducted in SPSS 12.0 Software (SPSS Inc., Chicago, IL) and the significance level was 0.05 unless otherwise indicated.

For the cultivar study, means and standard deviations of the samples were reported. The variability that was observed was a combination of measurement, sample preparation, and sub-sampling variability. Due to the lack of plot replication and the fact that cultivar is confounded with plots, the 11 blackberry cultivars were not compared statistically. With regards to the ORAC and FRAP measurements, since there was only one replicate, measures of variability were not obtained except at the measurement level. No statistical analysis was done due to lack of replication.

RESULTS AND DISCUSSION

Influence of maturity on composition

The total soluble solids (TSS) increased considerably (9.46 to 16.0 °Brix for 'Marion', 11.0 to 18.0 °Brix for 'Evergreen') during the course of ripening (Table 3.1 and figure 3.2). The standard deviation for TSS was relatively high indicating considerable sample variation despite the careful attention given in sample collection. Titratable acidity (TA) decreased (2.32 to 1.29 g/100g fw for 'Marion', 2.38 to 0.47 g/100g fw for 'Evergreen') during ripening as expected. The loss of acids was more rapid in 'Evergreen' berries than those of 'Marion'. The °Brix:acid ratio of 'Evergreen' blackberries also changed almost 10-fold during ripening. Perkins-Veazie et al. (21,22) observed similar trends in blackberries changing from mottled (50% black) to Shiny black, and dull black stages, respectively (5.85 to 7.92 °Brix, 2.45 to 0.98 g, citric acid /100g; n = 4).

Total anthocyanin content increased from 74.7 to 317 mg/100g fw from underripe to overripe for 'Marions', and from 69.9 to 164 mg/100g fw for 'Evergreens' (Table 3.1 and Figure 3.2). The total anthocyanin content of 'Marion' berries, which significantly increased with ripening, was about the same as that of 'Evergreen' berries in underripe stage, but were almost twice as much in ripe and over ripe stages. Since the data were taken at a single point in time (rather than following groups of berries through time), we are comparing ripe earlier-ripening

berries to unripe later-ripening berries. Therefore we cannot conclude that the differences are solely due to ripening (since time of ripening is also different).

Total phenolics did not show as pronounced change with ripening. The change in total phenolics for 'Marion' from underripe to ripe stages (975 to 903 mg/100g fw) was not significant while the increase from ripe to over-ripe stages (903 to 1541 mg/100g fw) was. 'Evergreen' berries showed an apparent decrease from underripe to ripe (1190 to 960 mg/100g) and an apparent increase from ripe to overripe (960 to 1035 mg/100g). The total phenolic contents of 'Evergreen' berries were slightly higher than those of 'Marions' during underripe and ripe stages, but it was the reverse in over ripe stage. In general, total phenolics did not show marked change with maturity as total anthocyanins did.

Table 3.1. Total soluble solids (TSS), titratable acidity, maturity index, polyphenolic composition, and antioxidant properties of 'Marion' and 'Evergreen' blackberries at three maturity stages [a].

Cultivar	TSS (°Brix)	Titratable acidity (Citric acid, g/100g)	Maturity index (°Brix:acid)	Total phenolics (mg GAE/100g)	Total anthocyanins (mg Cyd-3-glu/100g)	ORAC** (μmoleTE/g)	FRAP** (μmoleTE/g)
Marion							
Underripe	9.46 ± 1.34a	2.32 ± 0.09b	4.10 ± 0.66a	975 ± 144a	74.7 ± 11.2a	43.0 ± 2.30	87.6 ± 1.86
Ripe	13.5 ± 1.67b	1.28 ± 0.12a	10.8 ± 2.08b	903 ± 145a	221 ± 36.6b	60.9 ± 20.6	78.2 ± 1.15
Over ripe	16.0 ± 1.48c	1.29 ± 0.20a	12.6 ± 2.41b	1541 ± 376b	317 ± 35.3c	62.7 ± 12.3	102 ± 12.4
Evergreen*							
Underripe	11.0 ± 0.91	2.38 ± 0.15	4.62 ± 0.66	1090 ± 77.3	69.9 ± 10.6	46.1 ± 4.68	80.9 ± 8.21
Ripe	15.7 ± 1.71	1.12 ± 0.20	14.0 ± 3.89	960 ± 101	131 ± 9.62	58.5 ± 2.23	94.8 ± 5.31
Over ripe	18.0 ± 0.75	0.47 ± 0.09	38.3 ± 9.07	1035 ± 46.5	164 ± 11.7	64.4 ± 2.06	91.1 ± 8.94

[a] Different letters in the same column for 'Marion' indicate significant differences ($p \leq 0.05$); data expressed as means ± standard deviations (n = 3 plots) on fresh weight basis; *data expressed as means ± standard deviations for two sub-samples within one plot (n =1 plot); **data expressed as means ± standard deviations for 2 measurements, for one sample from one plot.

Wang and Lin (36) and Perkins-Veazie et al. (20) observed the same trends, from green to ripe (0.93 to 153 mg/100g fw) and from green to dull-black stage, respectively, on total anthocyanin contents of several thornless blackberry cultivars. Bilyk and Sapers (3) also observed a positive correlation between total anthocyanin content and maturity in several thornless blackberry cultivars (79.3 to 112 mg/100g; from red to black). Moreover, Wang and Lin (36) reported the reduction in total phenolic content from green to ripe stages (295 to 226 mg/100g fw; n = 3).

In both 'Marion' and 'Evergreen' berries, the underripe stage had the lowest ORAC values (Table 3.1). The ORAC values increased from 43.0 to 62.7 μmole TE/g fw for 'Marions' and from 46.1 to 64.4 μmole TE/g fw for 'Evergreens'. Changes in the FRAP values with maturity for the two varieties were inconsistent with the lowest values in ripe berries for 'Marion' (78.2 μmole TE/g fw) and in underripe berries for 'Evergreen' (80.9 μmole TE/g fw). The ORAC value correlated with the total anthocyanin content (r = 0.80) and the FRAP value (r = 0.69), and slightly correlated with the total phenolic content (r = 0.28). Our ORAC values (mean = 44.6 to 59.7 μmole TE/g fw; from underripe to ripe stages) were about 2-3 times higher than the range reported by Wang and Lin (36) (15.6 to 22.4 μmole TE/g fw; from pink to ripe stages, n = 3).

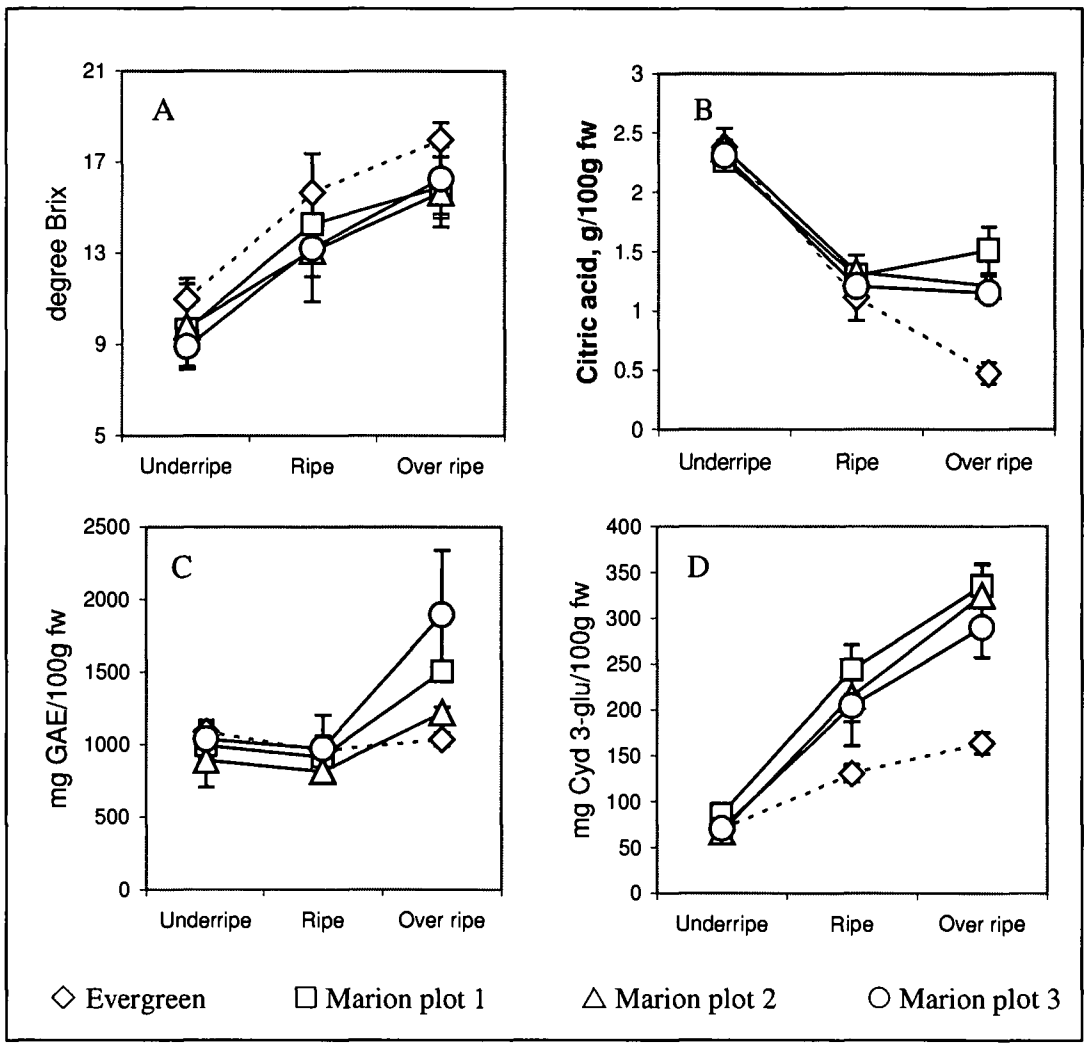

Figure 3.2. Comparison between blackberry compositions and cultivar by maturity stage and plot: (A) total soluble solids, (B) total titratable acidity, (C) total phenolic content, (D) total anthocyanin content.

Variance component estimation

An advantage to having plot replication for 'Marion' was accessibility to the estimates of the components of variance at different levels: plots, sub-sampling, sample preparation, and measurement (Table 3.2). In this and subsequent variance component tables, the entries are estimates of the variance contribution from each level after accounting for the variation at the lower (nested) levels. The estimation method was based on the expectations of the mean squares in nested ANOVA, except that negative estimates were set to zero when in the ANOVA higher level mean squares were slightly smaller than lower level mean squares (16). In each column the plot variance component is based on just two plots, so the precision is low. The number of observations doubles moving down each level in the study design, so the precision for estimating variance components increases until it is greatest for measurement error.

One common trait we found in the variance components of TSS and TA is that there was almost no variability at the measurement level (Table 3.2). This indicates the consistency of measurement devices, which in this case were the Auto Abbe refractometer and the Metrohm titration unit, respectively. Most of the TSS variation came from sample-to-sample difference (77.78% for ripe) and caused by sample preparation (ranged from 22.21% to 99.98%).

On the other hand, most of the variation for total phenolics was from sample-to-sample difference (ranged from 7.50% to 80.05%) and sample

preparation (ranged from 17.38% to 73.14%). The variation from measurement or the analysis was low (0.43% to 2.57%) when compared to the above.

For total anthocyanin content, underripe and over ripe berries were more susceptible to plot difference (31.50% to 46.21%) than that of ripe berries (~0%). Considerable variations were found at sample preparation (35.78% for underripe, 51.60% for ripe) and analysis (ranged from 17.35% to 57.95%) levels.

Table 3.2. Variance component estimates for blackberry composition at different sampling levels for 'Marion' blackberries.

Analysis	Under-ripe	% of total variance	Ripe	% of total variance	Over ripe	% of total variance
Total soluble solids						
Plot	~0	~0%	~0	~0%	~0	~0%
Sub-sampling	~0	~0%	2.4343	77.78%	~0	~0%
Sample preparation	1.8753	99.96%	0.6951	22.21%	2.2766	99.98%
Measurement	0.0007	0.04%	0.0003	0.01%	0.0004	0.02%
Total variance	1.8760	100.00%	3.1297	100.00%	2.2770	100.00%
CV (%) [a]	*14.48*	-	*13.07*	-	*9.46*	-
Titratable acidity						
Plot	~0	~0%	~0	~0%	0.0233	46.23%
Sub-sampling	0.0066	78.57%	0.0137	91.95%	0.0266	52.78%
Sample preparation	0.0017	20.24%	0.0012	8.05%	0.0004	0.79%
Measurement	0.0001	1.19%	~0	~0%	0.0001	0.20%
Total variance	0.0084	100.00%	0.0149	100.00%	0.0504	100.00%
CV (%)	*3.96*	-	*9.56*	-	*17.36*	-
Total phenolics						
Plot	~0	~0%	~0	~0%	91751	52.81%
Sub-sampling	18678	80.05%	18346	26.43%	13034	7.50%
Sample preparation	4055.1	17.38%	5076.9	73.14%	67837	39.04%
Measurement	601.08	2.57%	297.40	0.43%	1119.6	0.65%
Total variance	23334	100.00%	69412	100.00%	173741	100.00%
CV (%)	*15.66*	-	*29.19*	-	*27.05*	-
Total anthocyanins						
Plot	71.968	46.21%	~0	~0%	436.10	31.50%
Sub-sampling	55.718	35.78%	742.57	51.60%	~0	~0%
Sample preparation	1.0329	0.66%	~0	~0%	146.13	10.55%
Measurement	27.014	17.35%	696.54	48.40%	802.28	57.95%
Total variance	155.73	100.00%	1439.1	100.00%	1384.5	100.00%
CV (%)	*16.70*	-	*17.15*	-	*11.75*	-

[a] CV (%), percent coefficient of variance was 100 × (square root of total variance divided by overall mean response).

Influence of maturity on anthocyanin composition

HPLC chromatographic profiles of 'Marion' and 'Evergreen' at different maturity stages (Figure 3.3 and 3.4) were qualitatively the same, but their proportions were different (Table 3.3). Five anthocyanins were identified, using LC-MS/MS, as cyanidin 3-glucoside (*m/z* 449.0), cyanidin 3-rutinoside (*m/z* 595.1), cyanidin-containing xylose (*m/z* 419.0), cyanidin 3-glucoside acylated with malonic acid (*m/z* 535.1), and cyanidin 3-dioxalylglucoside (*m/z* 593.0). The primary anthocyanins in 'Marion' berries were cyanidin 3-glucoside (57 to 73%) and cyanidin 3-rutinoside (24 to 37%). On the other hand, the anthocyanin in 'Evergreen' blackberries was predominantly cyanidin 3-glucoside (70 to 85%).

Table 3.3. Anthocyanins distribution (% total peak area at 520 nm) of 'Marion' and 'Evergreen' blackberries at different maturity stages [a].

Cultivar	Cyanidin 3-glucoside	Cyanidin 3-rutinoside	Cyanidin-containing xylose	Cyanidin 3-glucoside acylated with malonic acid	Cyanidin 3-dioxalyl-glucoside	Total anthocyanins (mg Cyd-3-glu/100g)
Marion						
Underripe	57.0 ± 4.17a	37.0 ± 3.90b	0.01 ± 0.05a	1.61 ± 0.13b	4.39 ± 0.50b	74.7 ± 11.2a
Ripe	72.6 ± 1.49b	24.2 ± 1.42a	0.18 ± 0.03b	1.12 ± 0.08a	1.91 ± 0.17a	221 ± 36.6b
Over ripe	71.0 ± 1.17b	25.8 ± 1.10a	0.23 ± 0.02c	1.06 ± 0.07a	1.92 ± 0.14a	317 ± 35.3c
Evergreen *						
Underripe	70.4 ± 2.29	6.89 ± 0.93	4.87 ± 0.40	7.22 ± 0.48	10.6 ± 1.27	69.9 ± 10.6
Ripe	81.2 ± 0.37	2.71 ± 0.04	6.30 ± 0.15	4.72 ± 0.05	5.07 ± 0.42	131 ± 9.62
Over ripe	84.3 ± 0.16	1.91 ± 0.05	7.53 ± 0.18	3.36 ± 0.04	2.86 ± 0.12	164 ± 11.7

[a] Different letters in the same column for Marion indicate significant differences ($p \leq 0.05$); data expressed as means ± standard deviations (n = 3 plots) on fresh weight basis; *data expressed as means ± standard deviations for two sub-samples within one plot (n =1 plot).

The anthocyanin profiles for 'Marion' and 'Evergreen' are distinctive with Marion's containing substantially more cyanidin-3-rutinoside (Table 3.3). Nevertheless, they show similar trends with ripening (Figure 3.5), with proportions of cyanidin 3-glucoside increasing (57.0 to 71.0% for 'Marion', 70.4 to 84.3% for 'Evergreen'), increasing cyanidin-containing xylose (0.01 to 0.23% for 'Marion', 4.87 to 7.53% for 'Evergreen'), decreasing for cyanidin 3-rutinoside (37.0 to 25.8% for 'Marion', 6.89 to 1.91% for 'Evergreen'), decreasing cyanidin 3-glucoside acylated with malonic acid (1.61 to 1.06% for 'Marion', 7.22 to 3.36% for 'Evergreen'), and marked decrease in cyanidin 3- dioxalylglucoside with ripening (4.39 to 1.92 % for 'Marion', 10.6 to 2.86% for 'Evergreen'). The standard deviation for cyanidin-containing xylose was relatively high compared to the other anthocyanins.

Sapers et al. (25) reported similar trends in two thornless blackberry cultivars (from pink to black stages) with cyanidin 3-glucoside (37.5 to 73.8%) and cyanidin derivative containing xylose (1.3 to 10.2%) increasing, and acid-acylated derivative of cyanidin 3-glucoside (6.6 to 3.3%) and dicarboxylic derivative of cyanidin 3-glucoside (45.8 to 10.4%) decreasing with increasing ripeness. However, Sapers et al. (25) reported that dicarboxylic derivative of cyanidin 3-glucoside (subsequently identified as cyanidin-3-dioxalyl-glucoside) to be about 4 to 6-fold higher than our results (mean = 7.50 to 2.39%; underripe to over ripe). Their investigation was on 'Black Satin' and 'Hull Thornless' blackberries.

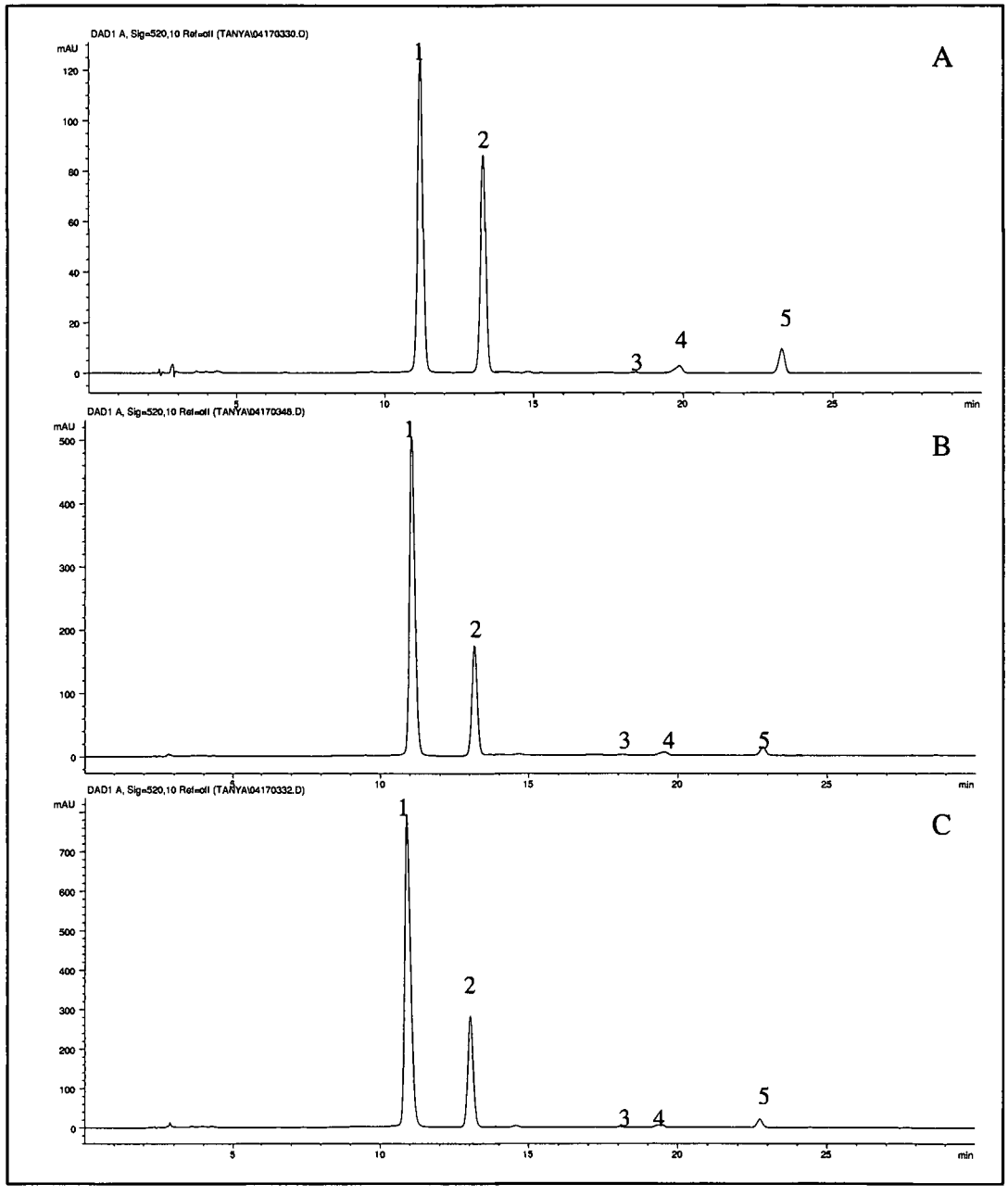

Figure 3.3. HPLC anthocyanin profiles of 'Marion' blackberries at three maturity stages: (A) underripe, (B) ripe, (C) over ripe, at 520 nm. Peak assignment: 1. cyanidin 3-glucoside, 2. cyanidin 3-rutinoside, 3. cyanidin-containing xylose, 4. cyanidin 3-glucoside acylated with malonic acid, 5. cyanidin 3-dioxalylglucoside.

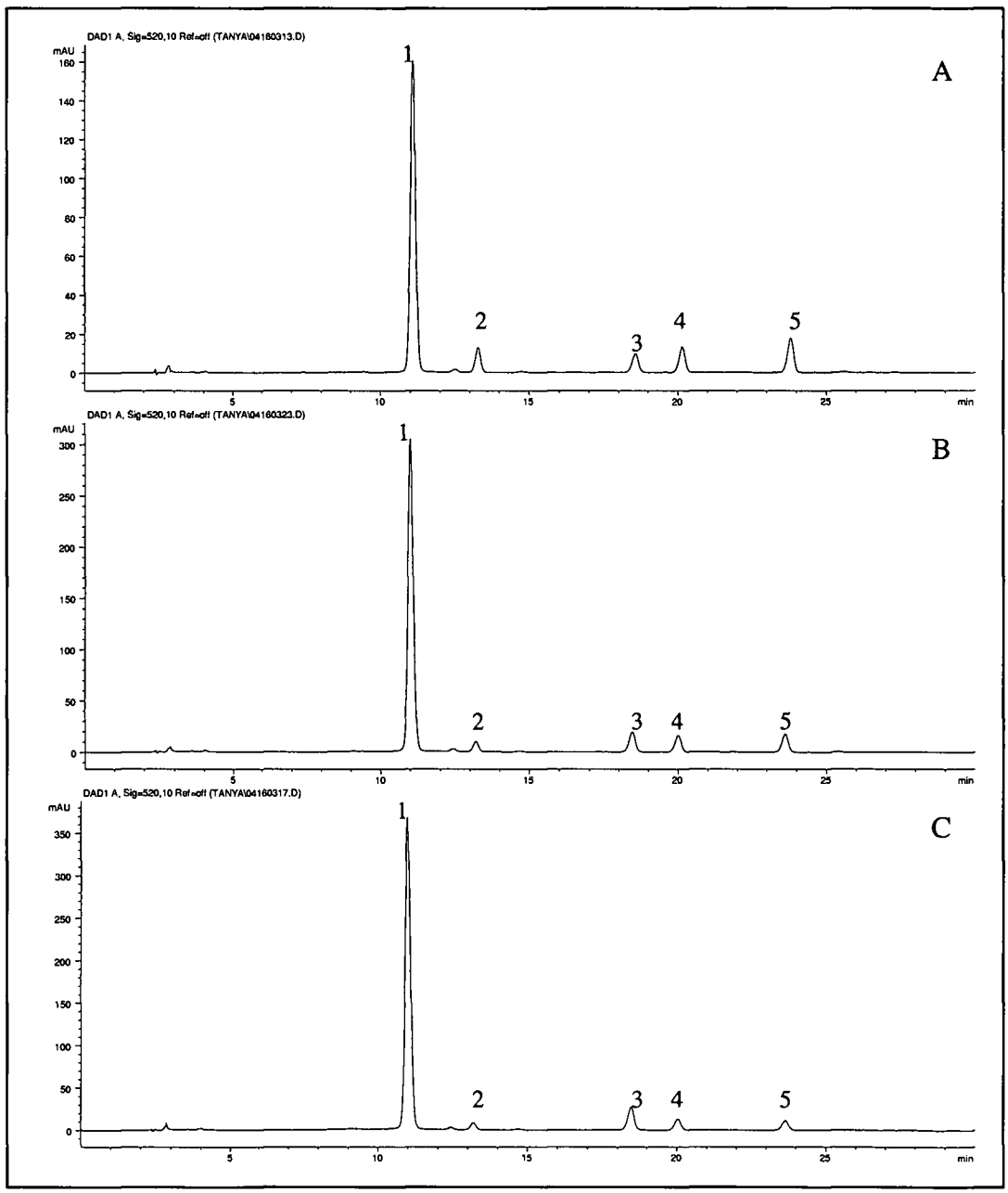

Figure 3.4. HPLC anthocyanin profiles of 'Evergreen' blackberries at three maturity stages: (A) underripe, (B) ripe, (C) over ripe, at 520 nm. Peak assignment: 1. cyanidin 3-glucoside, 2. cyanidin 3-rutinoside, 3. cyanidin-containing xylose, 4. cyanidin 3-glucoside acylated with malonic acid, 5. cyanidin 3-dioxalylglucoside.

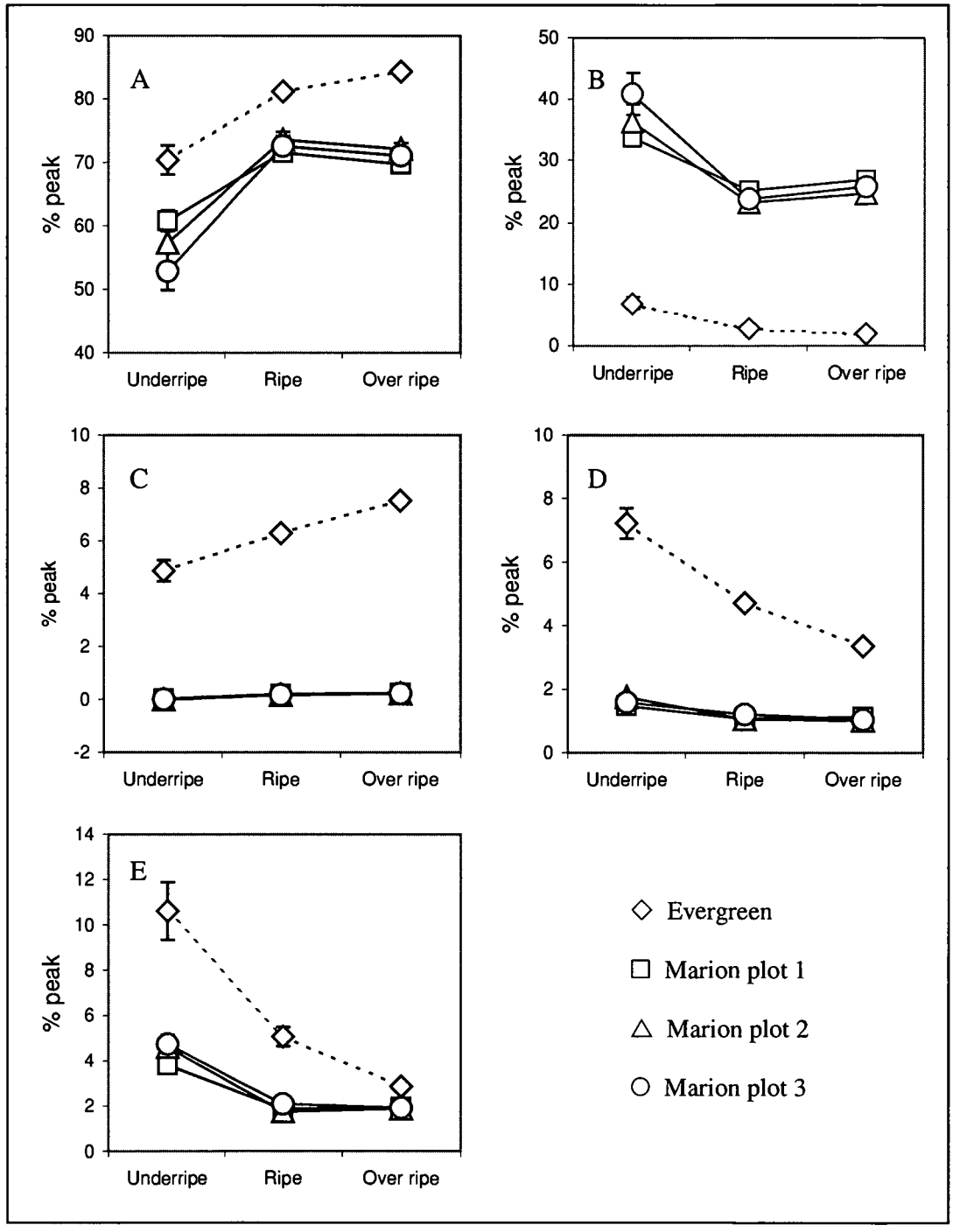

Figure 3.5. Comparison between relative anthocyanin composition and cultivar by maturity stage and plot: (A) cyanidin-3-glucoside, (B) cyanidin-3-rutinoside, (C) cyanidin-containing xylose, (D) malonic acid acylated cyanidin-3-glucoside, (E) cyanidin 3-dioxalylglucoside.

Variance component estimation

For the distribution of anthocyanins, the largest source of variation was the generally the sample-to-sample (sub-sampling) component (Table 3.4).

Table 3.4. Variance component estimates for blackberry anthocyanin distribution at different sampling levels for 'Marion' blackberries.

Anthocyanins	Under-ripe	% of total variance	Ripe	% of total variance	Over ripe	% of total variance
Cyanidin 3-glucoside						
Plot	9.7381	44.39%	~0	~0%	1.0892	61.08%
Sub-sampling	12.081	55.07%	2.4991	98.33%	0.6609	37.06%
Sample preparation	0.1116	0.51%	0.0217	0.86%	0.0251	1.41%
Measurement	0.0061	0.03%	0.0207	0.81%	0.0080	0.45%
Total variance	21.936	100.00%	2.5415	100.00%	1.7832	100.00%
CV (%) [a]	*8.22*	-	*2.20*	-	*1.88*	-
Cyanidin 3-rutinoside						
Plot	6.2735	33.50%	~0	~0%	0.8752	55.72%
Sub-sampling	12.110	64.67%	2.2701	98.19%	0.6687	42.57%
Sample preparation	0.3388	1.81%	0.0297	1.29%	0.0203	1.29%
Measurement	0.0034	0.02%	0.0122	0.53%	0.0066	0.42%
Total variance	18.726	100.00%	2.3120	100.00%	1.5708	100.00%
CV (%)	*11.70*	-	*6.29*	-	*4.86*	-
Cyanidin-containing xylose						
Plot	~0	~0%	0.0001	8.32%	0.0001	26.02%
Sub-sampling	~0	~0%	0.0006	66.70%	~0	12.57%
Sample preparation	~0	~0%	~0	~0%	0.0001	40.06%
Measurement	0.0024	100.00%	0.0002	24.97%	0.0001	21.35%
Total variance	0.0024	100.00%	0.0009	100.00%	0.0003	100.00%
CV (%)	*489.89*	-	*16.21*	-	*8.03*	-

Table 3.4. (Continued)

Malonic acid acylated cyanidin 3-glucoside						
Plot	0.0178	80.96%	0.0058	75.20%	0.0007	13.29%
Sub-sampling	~0	~0%	0.0016	20.95%	0.0036	72.89%
Sample preparation	0.0039	17.87%	~0	0.26%	0.0005	10.53%
Measurement	0.0002	1.17%	0.0003	3.59%	0.0002	3.30%
Total variance	0.0220	100.00%	0.0077	100.00%	0.0050	100.00%
CV (%)	*9.20*	-	*7.87*	-	*6.64*	-
Cyanidin 3-dioxalylglucoside						
Plot	0.1936	59.96%	0.0146	39.06%	~0	~0%
Sub-sampling	0.0881	27.28%	0.0220	58.81%	0.0233	99.52%
Sample preparation	0.0398	12.31%	0.0006	1.61%	0.0001	0.28%
Measurement	0.0014	0.45%	0.0002	0.52%	~0	0.20%
Total variance	0.3229	100.00%	0.0374	100.00%	0.0234	100.00%
CV (%)	*12.95*	-	*10.11*	-	*7.98*	-

[a] CV (%), percent coefficient of variance was 100 × (square root of total variance divided by overall mean response).

We observed that the major anthocyanins, such as cyanidin 3-glucoside and cyanidin 3-rutinoside are most susceptible to sample-to-sample difference in underripe (55.07 to 64.67%) and ripe (98.19% to 98.33%) berries, and to plot difference in over ripe berries (55.72% to 61.08%). Overall, there were lesser variations at sample preparation and analysis levels. Cyanidin-containing xylose was particularly low in underripe berries, which resulted in a very large coefficient of variation and relatively large variation between measurements on the same sample preparation.

Influence of maturity on polyphenolic composition

HPLC chromatographic profiles of 'Marion' and 'Evergreen' polyphenolics at different maturity stages (Figure 3.6 and 3.7) were qualitatively similar, yet quantitatively very different (Table 3.5). Both varieties contained two major ellagitannin peaks (60 to 80%), and had flavonols and ellagic acid derivatives as minor compounds. 'Evergreen' blackberries had more complex profiles of flavonols and ellagic acid derivatives than did 'Marion' berries.

Table 3.5. Concentrations of polyphenolics (mg/100g fw) in 'Marion' and 'Evergreen' blackberries at different maturity stages [a].

Cultivar	Procyanidins (as catechin)	Ellagitannins (as ellagic acid)	Flavonols (as rutin)	Ellagic acid derivatives (as ellagic acid)
Marion				
Underripe	0.60 ± 2.95	34.8 ± 5.92b	5.39 ± 1.54a	0.96 ± 0.23a
Ripe	nd [b]	20.7 ± 3.09a	6.84 ± 1.20b	0.66 ± 0.14a
Over ripe	13.8 ± 9.36	32.3 ± 5.66b	12.4 ± 1.38c	1.39 ± 0.42b
Evergreen *				
Underripe	38.9 ± 5.61	34.6 ± 1.98	14.1 ± 0.99	2.17 ± 0.34
Ripe	27.1 ± 13.4	25.4 ± 3.32	16.0 ± 1.56	1.29 ± 0.27
Over ripe	19.5 ± 9.12	17.6 ± 2.12	15.0 ± 1.65	2.61 ± 0.52

[a] Different letters in the same column for Marion indicate significant differences ($p \leq 0.05$); [b] nd, not detected; data expressed as means ± standard deviations (n = 3 plots) on fresh weight basis; *data expressed as means ± standard deviations for two sub-samples within one plot (n =1 plot).

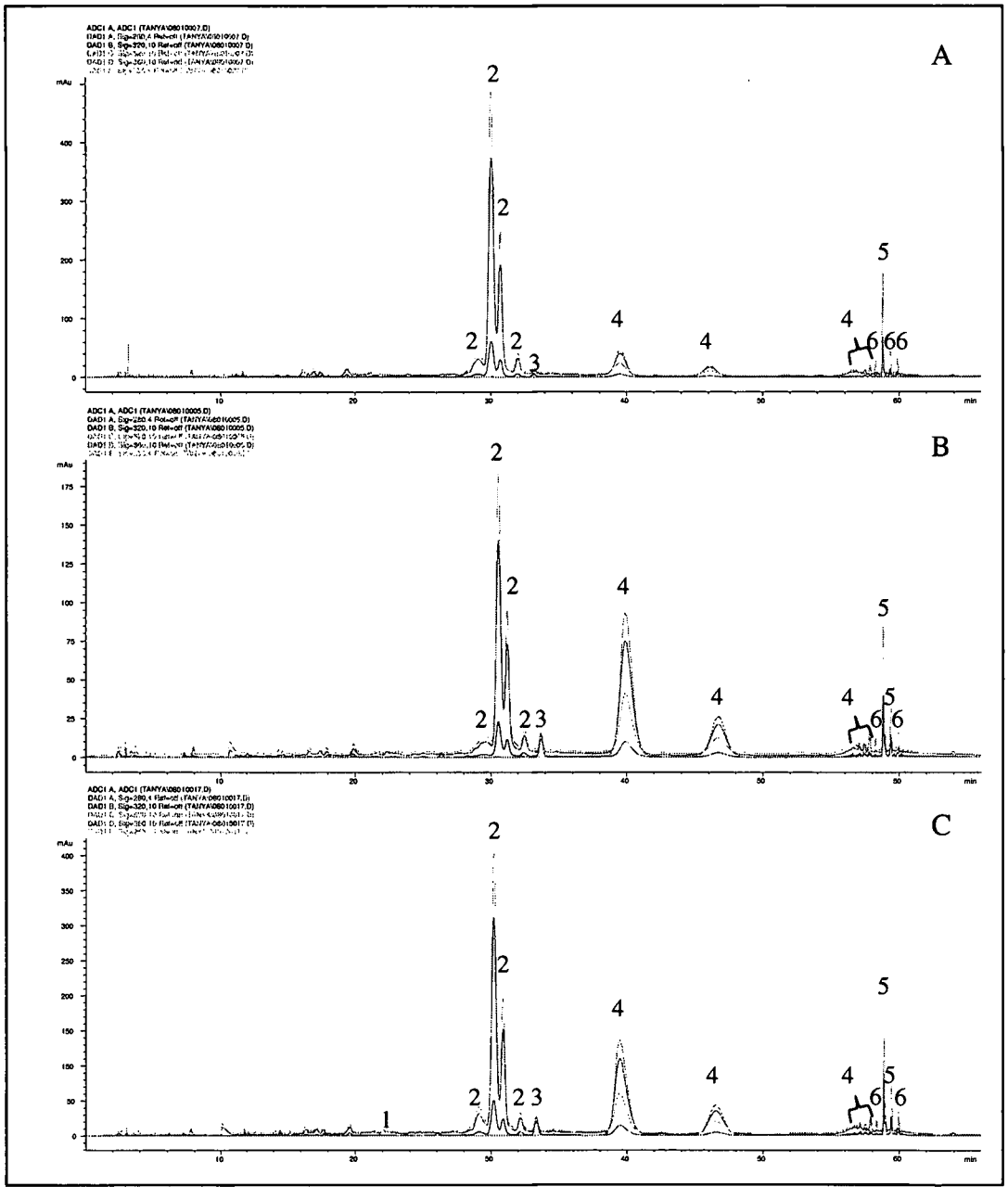

Figure 3.6. HPLC polyphenolic profiles of 'Marion' blackberries at three maturity stages: (A) underripe, (B) ripe, (C) over ripe, at 255 nm, 280 nm, 320 nm, 360 nm, and 520 nm. Peak assignment: 1. procyanidins, 2. ellagitannins, 3. unidentified polyphenolics, 4. anthocyanins, 5. flavonols, 6. ellagic acid derivatives.

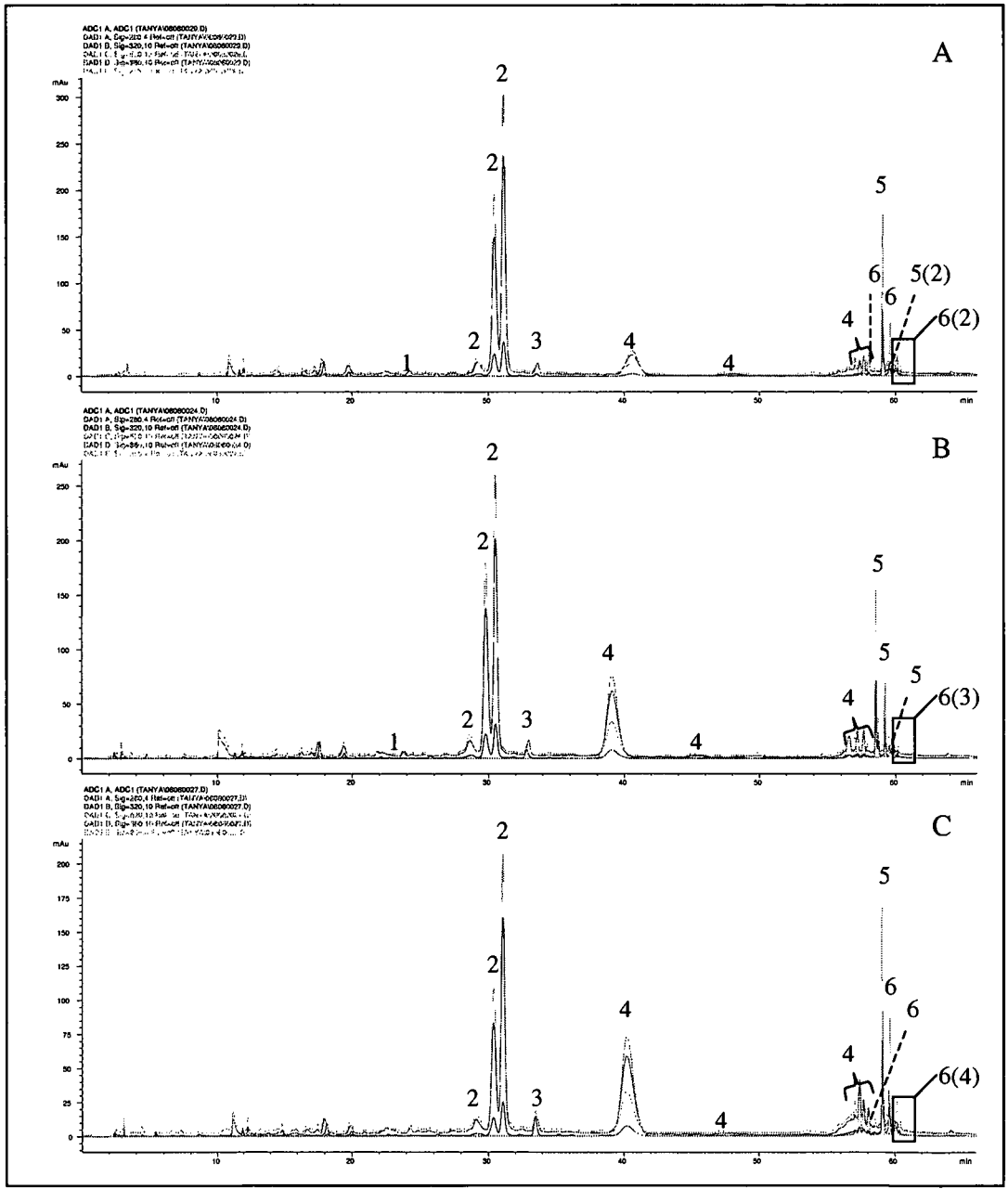

Figure 3.7. HPLC polyphenolic profiles of 'Evergreen' blackberries at three maturity stages: (A) underripe, (B) ripe, (C) over ripe, at 255 nm, 280 nm, 320 nm, 360 nm, and 520 nm. Peak assignment: 1. procyanidins, 2. ellagitannins, 3. unidentified polyphenolics, 4. anthocyanins, 5. flavonols, 6. ellagic acid derivatives.

The changes in the amounts of the different polyphenolic classes with ripening were substantially different between 'Marions' and 'Evergreens' (Table 3.5 and Figure 3.8). In general, 'Evergreen' berries contained higher levels of procyanidins (5.9-fold), flavonols (1.8-fold), and ellagic acid derivatives (2-fold), but lower level of ellagitannins (1.6-fold) than those of 'Marion'. In 'Marion' berries, the trends for procyanidins and ellagitannins were similar being lower at the ripe stage (not detected for procyanidins, 20.7 mg/100g fw for ellagitannins) compared to underripe (0.60 mg/100g fw for procyanidins, 34.8 mg/100g fw for ellagitannins) and over ripe (13.8 mg/100g fw for procyanidins, 32.3 mg/g for ellagitannins) stages. On the other hand, the concentrations of procyanidins and ellagitannins of 'Evergreen' blackberries continuously decreased with maturity (38.9 to 19.5 mg/100g fw for procyanidins, 34.6 to 17.6 mg/100g fw for ellagitannins). The concentrations of flavonols increased with ripening (5.39 to 12.4 mg/100g fw) for 'Marion' berries, while those of 'Evergreen' berries increased from underripe to ripe (14.1 to 16.0 mg/100g fw), before slightly decreasing in over ripe (15.0 mg/100g fw). The only similarity between these two varieties was the changes in the concentration of ellagic acid derivatives, which the lowest were those of ripe berries (0.66 mg/100g fw for 'Marion', 1.29 mg/100g fw for 'Evergreen'). The standard deviation for procyanidins, ellagitannins, flavonols, and ellagic acid derivatives was relatively high indicating considerable sample variation.

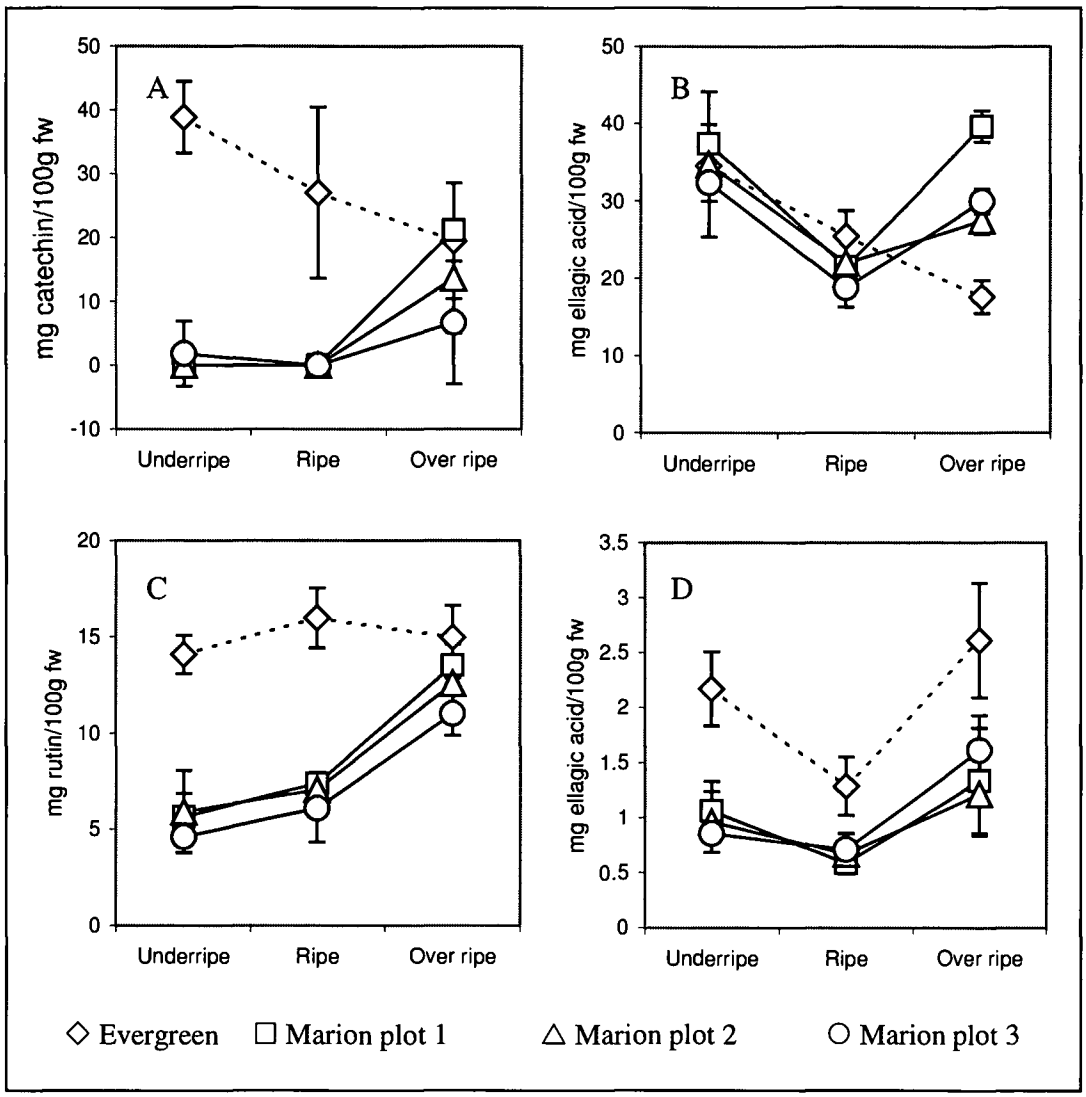

Figure 3.8. Comparison between polyphenolic concentrations and cultivar by maturity stage and plot: (A) procyanidins, (B) ellagitannins, (C) flavonols, (D) ellagic acid derivatives.

Bilyk and Sapers (3) reported a positive correlation between flavonol contents and blackberry maturity (9.01 to 15.8 mg/100g fw for quercetin content, 0.7 to 1.74 mg/100g for kaempferol content; from red to black).

Variance component estimation

In general, only over ripe 'Marion' berries had variations in the polyphenolic concentrations, except for ellagic acid derivatives, at the plot level (ranged from 37.88% to 88.79%) compared with other maturity stages (Table 3.6). Variability of ellagitannins and flavonols mainly came from sample-to-sample difference in underripe (97.15% for ellagitannins, 94.43% for flavonols) and ripe berries (72.14% for ellagitannins, 91.99% for flavonols), while those of procyanidins were from analysis level for underripe (100.00%) and over ripe (42.36%) fruit. Nonetheless, concentrations of ellagic acid derivatives were most affected by sample preparation in ripe (54.53%) and over ripe (53.76%) berries, and by analysis in underripe berries (65.99%). Procyanidins were particularly low in underripe berries, which resulted in a very large coefficient of variation and relatively large variation between measurements on the same sample preparation. Procyanidins in ripe berries could not be detected.

Table 3.6. Variance component estimates for blackberry polyphenolic composition at different sampling levels for 'Marion' blackberries.

Analysis	Under-ripe	% of total variance	Ripe	% of total variance	Over ripe	% of total variance
Procyanidins						
Plot	~0	~0%	-	-	38.423	37.88%
Sub-sampling	~0	~0%	-	-	14.229	14.03%
Sample preparation	~0	~0%	-	-	5.8096	5.73%
Measurement	8.6791	100.00%	-	-	42.970	42.36%
Total variance	8.6791	100.00%	-	-	101.43	100.00%
CV (%) [a]	*489.90*	-	-	-	*72.74*	-
Ellagitannins						
Plot	~0	~0%	~0	~0%	39.563	88.79%
Sub-sampling	39.065	97.15%	7.7003	72.14%	3.6496	8.19%
Sample preparation	0.8910	2.22%	2.6656	24.97%	1.2009	2.70%
Measurement	0.2548	0.63%	0.3075	2.88%	0.1420	0.32%
Total variance	40.211	100.00%	10.673	100.00%	44.5551	100.00%
CV (%)	*18.21*	-	*15.75*	-	*20.64*	-
Flavonols						
Plot	~0	~0%	~0	~0%	1.0622	45.19%
Sub-sampling	2.5493	94.43%	1.5086	91.99%	0.9213	39.19%
Sample preparation	0.0511	1.89%	0.0400	2.44%	0.2764	11.76%
Measurement	0.0994	3.68%	0.0914	5.57%	0.0909	3.87%
Total variance	2.6997	100.00%	1.6400	100.00%	2.3508	100.00%
CV (%)	*30.47*	-	*18.72*	-	*12.38*	-
Ellagic acid derivatives						
Plot	~0	~0%	~0	~0%	~0	~0%
Sub-sampling	0.0195	34.01%	0.0050	23.04%	0.0295	16.29%
Sample preparation	~0	~0%	0.0119	54.53%	0.0973	53.76%
Measurement	0.0378	65.99%	0.0049	22.43%	0.0542	29.95%
Total variance	0.0573	100.00%	0.0218	100.00%	0.1810	100.00%
CV (%)	*24.95*	-	*22.51*	-	*30.65*	-

[a] CV (%), percent coefficient of variance was 100 × (square root of total variance divided by overall mean response).

Influence of cultivar on composition

Table 3.7 summarizes the composition and antioxidant properties of 11 blackberry cultivars. The TSS of the blackberries ranged from 8.20 to 13.6 °Brix with a mean of 11.5. 'Waldo' fruit (13.6 °Brix) had the highest TSS of the cultivars compared; NZ 9351-4 (12.4 °Brix) and ORUS 1369-3 (11.4 °Brix) were the highest of their selections. The TA ranged from 0.70 to 2.12 g/100g fw (mean = 1.27). 'Chester' fruit (0.84 g/100g fw) were the least acidic of the cultivars compared; NZ 9128R-1 (1.13 g/100g fw) and ORUS 1369-3 (1.30 g/100g fw) were the least acidic selections. Our results of TSS and TA were similar to the ranges of Sapers et al. (24) (7.7-13.9 °Brix, 0.4-1.3 g/100g fw; n = 37) and the TSS of Fan-Chiang (8) (6.88-16.8 °Brix; n = 52). The TSS of 'Chester' berries was very close to those reported by Himelrick and Nesbitt (13) (8.6 and 8.2 °Brix; year 2000 and 2001).

Our data showed that the total phenolic and total anthocyanin contents varied greatly among cultivars. The total phenolic content of the samples ranged from 682 to 1056 mg/100g fw (mean = 900). The ORUS-selections (954-1056 mg/100g fw) and 'Marion' (1005 mg/100g fw) had considerably higher total phenolics content. Total anthocyanins for the 11 cultivars ranged from 131-256 mg/100g fw (mean = 198). The ORUS selections (197-256 mg/100g fw) and 'Marion' (245 mg/100g fw) were among the cultivars containing the highest total anthocyanin pigment. Some of the experimental ORUS selections were higher in total anthocyanins and total phenolics than the common commercial varieties

Table 3.7. Total soluble solids (TSS), titratable acidity, maturity index, polyphenolic composition, and antioxidant properties of selected blackberry cultivars [a].

Cultivar	TSS (°Brix)	Titratable acidity (Citric acid, g/100g)	Maturity index (°Brix:acid)	Total phenolics (mg GAE/100g)	Total anthocyanins (mg Cyd-3-glu/100g)	ORAC* (μmoleTE/g)	FRAP* (μmoleTE/g)
Marion	12.9 ± 5.49	1.26 ± 0.12	10.2 ± 5.24	1005 ± 54.2	245 ± 33.9	56.2 ± 4.64	91.7 ± 0.47
Waldo	13.6 ± 0.67	1.83 ± 0.13	7.43 ± 0.85	940 ± 26.1	156 ± 30.1	54.1 ± 1.36	81.7 ± 7.15
Evergreen [b]	11.5 ± 2.32	0.91 ± 0.06	12.6 ± 3.28	959 ± 27.0	131 ± 6.25	75.5 ± 20.7	91.5 ± 3.61
Chester [b]	8.20 ± 1.05	0.84 ± 0.06	9.76 ± 1.88	697 ± 94.5	192 ± 30.6	48.9 ± 3.91	63.2 ± 0.14
Silvan	12.8 ± 1.44	1.13 ± 0.15	11.3 ± 2.90	779 ± 14.2	161 ± 8.22	37.6 ± 2.81	67.4 ± 1.19
NZ 9128R-1	12.1 ± 1.15	1.13 ± 0.04	10.7 ± 1.40	758 ± 46.6	176 ± 11.4	39.0 ± 1.58	63.5 ± 1.87
NZ 9351-4	12.4 ± 0.89	1.93 ± 0.13	6.42 ± 0.86	682 ± 14.6	186 ± 6.66	45.3 ± 1.13	57.8 ± 1.01
ORUS 1843-3	9.64 ± 0.76	2.09 ± 0.07	4.61 ± 0.49	954 ± 40.7	256 ± 35.2	46.4 ± 1.65	84.8 ± 0.26
ORUS 1380-1	11.1 ± 1.52	1.38 ± 0.41	8.04 ± 3.27	1026 ± 104	197 ± 63.2	42.8 ± 1.21	75.2 ± 0.40
ORUS 1489-1	11.1 ± 0.06	2.62 ± 0.28	4.24 ± 0.48	1056 ± 31.5	235 ± 47.2	54.4 ± 6.80	97.3 ± 1.59
ORUS 1369-3	11.4 ± 0.52	1.30 ± 0.16	8.77 ± 1.44	1040 ± 252	246 ± 31.1	51.8 ± 3.00	77.9 ± 7.02
Mean ± s.d.	11.5 ± 2.24	1.49 ± 0.56	8.56 ± 3.37	900 ± 157	198 ± 48.8	50.2 ± 11.7	77.5 ± 13.1

[a] single combined sample from 3 plots; [b] sample from 1 plot; data expressed as means ± standard deviations (n = 3 sub-samples) on fresh weight basis; *data expressed as means ± standard deviations (n = 1 sub-sample).

(Figure 3.9) indicating the potential for obtaining new cultivars with high pigment/phenolic content through classical plant breeding.

In this study, blackberries had a similar range in total phenolics to our earlier investigation (29) (822-844 mg/100g, mean = 833; n = 2), however, it was approximately twice that reported by Wilska-Jeszka et al. (37) (448 mg/100g), Heinonen et al. (11) (435 mg/100g), Moyer et al. (18) (275-678 mg/100g, mean = 478; n = 32), Sellappan et al. (27) (418-555 mg/100g, mean = 486; n = 2), and Wada and Ou (34) (495-583 mg/100g, mean = 539; n = 2), and 4 times higher than that of Wang and Lin (36) (204-248 mg/100g, mean = 226; n = 3). Our total anthocyanins were similar to reports by Fan-Chiang (8) (70-201 mg/100g, mean = 137; n = 52), Wang and Lin (36) (134-172 mg/110, mean = 153; n = 3), Moyer et al. (18) (80-230 mg/100g, mean = 145; n = 32), Sellappan et al. (27) (110-123 mg/100g, mean = 117; n = 2), and Siriwoharn and Wrolstad (29) (154-225 mg/100g, mean = 190; n = 2), but was slightly higher than the results of Wilska-Jeszka et al. (37) (115 mg/100g).

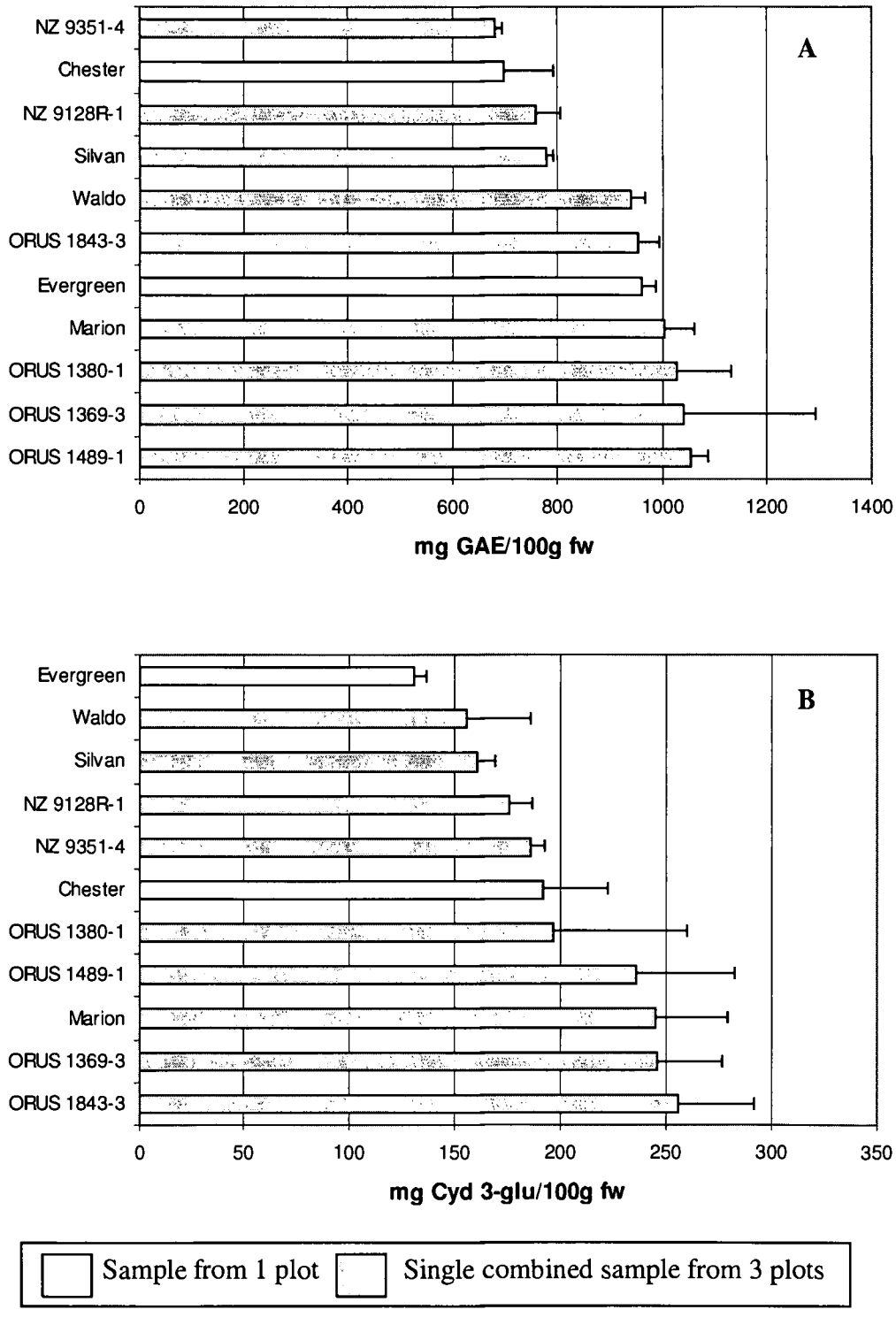

Figure 3.9. Comparison of blackberry composition between selected blackberry cultivars: (A) total phenolics, (B) total anthocyanins.

The ORAC values of blackberries ranged from 37.6-75.5 μmoleTE/g with a mean of 50.2. While 'Evergreen' fruit (75.5 μmoleTE/g) had the highest ORAC values of the cultivars compared, it was not highest in either total phenolics or total anthocyanins. There tends to be high correlations between total phenolics, total anthocyanins, and antioxidant properties. In this study, the ORAC value positively correlated with the FRAP value (r = 0.67) and the total phenolic content (r = 0.36), however slightly negative correlated with the total anthocyanin content (r = -0.06). FRAP values ranged from 57.8 to 97.3 μmoleTE/g (mean = 77.5). 'ORUS 1489-1' (97.3 μmoleTE/g), 'Marion' (91.7 μmoleTE/g), and 'Evergreen' (91.5 μmoleTE/g) were varieties containing the highest FRAP values. Figure 3.10 gives a graphic comparison of blackberry antioxidant properties.

Our ORAC results were higher than that reported by Jiao and Wang (15) (14.8-22.6 μmoleTE/g; n = 6), Wang and Lin (36) (20.3-24.6 μmoleTE/g; n = 3), Wada and Ou (34) (27.5-28 μmolTE/g; n = 2), and Siriwoharn and Wrolstad (29) (34.3-35.5 μmoleTE/g; n = 2), but fell within in the range of Moyer et al. (18) (26.7-78.8 μmoleTE/g; n = 32). The FRAP values of blackberries were within the ranges of Moyer et al. (18) (40.6-106 μmoleTE/g; n = 32) and similar to that of Siriwoharn and Wrolstad (29) (74.2-79.1 μmoleTE/g; n = 2).

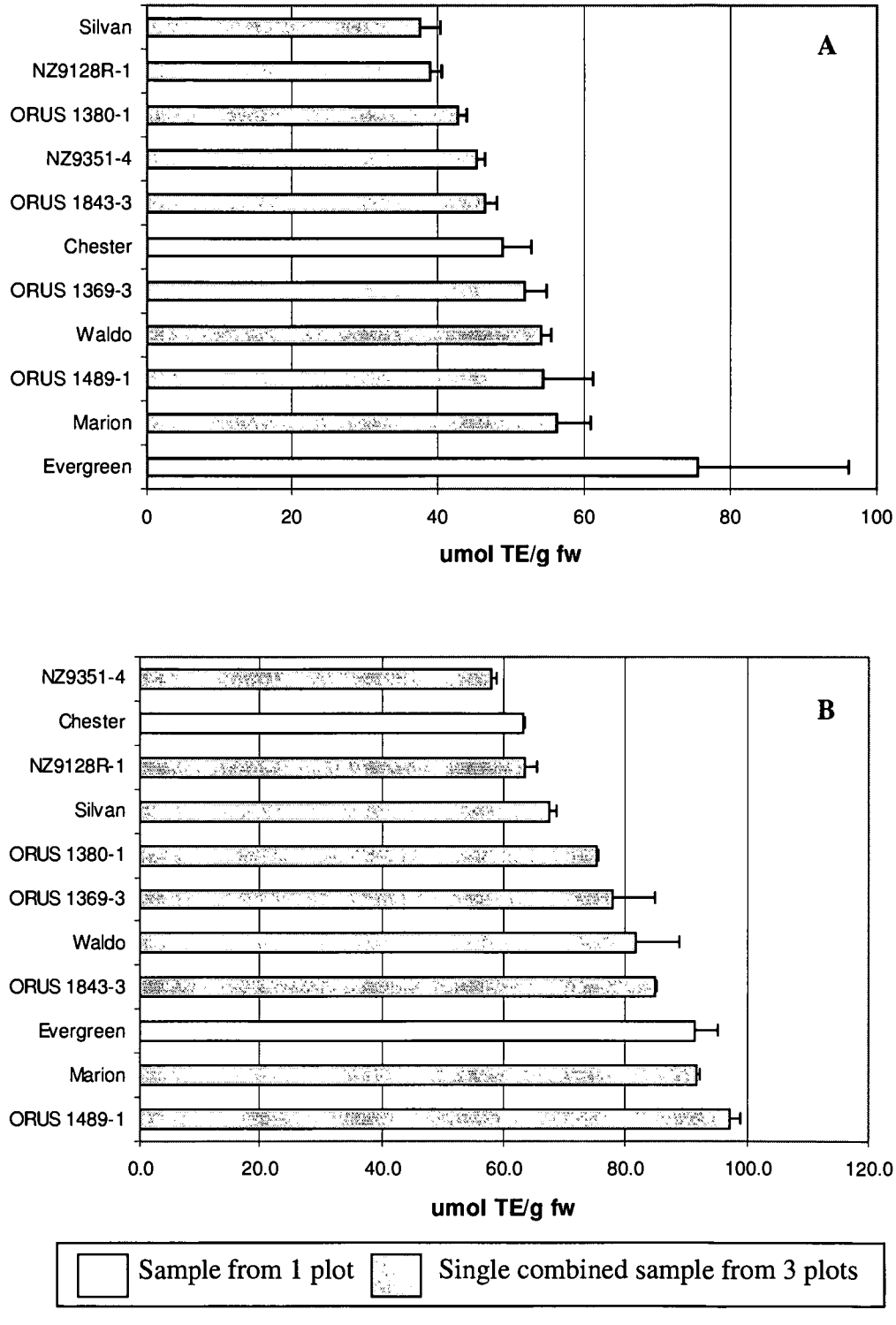

Figure 3.10. Comparison of antioxidant activities between selected blackberry cultivars: (A) ORAC, (B) FRAP.

Influence of cultivar on anthocyanin composition

Table 3.8 summarizes the anthocyanin patterns for the 11 blackberry cultivars. We found that the proportions of anthocyanins varied greatly among cultivars. Nonetheless, three anthocyanin patterns were observed (Figure 3.11). The first pattern (I) was found in 8 of the 11 studied cultivars. It consisted of cyanidin 3-glucoside as the major pigment, a second major pigment of cyanidin 3-rutinoside, traces of cyanidin-containing xylose, cyanidin 3-glucoside acylated with malonic acid, and/or presence of cyanidin 3-dioxalylglucoside. The following cultivars all exhibit the pattern: 'Marion', 'Waldo', 'Silvan', 'NZ 9128R-1', 'NZ 9351-4', 'ORUS 1843-3', 'ORUS 1380-1', and 'ORUS 1369-3'. The second pattern (II) was similar to pattern (I), but with much smaller amounts of cyanidin 3-rutinoside which was of the same order as cyanidin 3-glucoside acylated with malonic acid and cyanidin 3-dioxalylglucoside. It contained a larger proportion of cyanidin-containing xylose than those obtained in other patterns. It was shown by 'Evergreen'. The last pattern (III) resembles pattern (I), but with a larger proportion of cyanidin 3-dioxalylglucoside and an absence of cyanidin 3-rutinoside. Both 'Chester' and 'ORUS 1489-1' exhibited that profile. These patterns are consistent with previous investigations in our laboratory (8,31). Sapers et al. (25) observed similar patterns in several thornless blackberry varieties.

Table 3.8. Anthocyanins distribution (% total peak area at 520 nm) of selected blackberry cultivars [a].

Cultivar	Cyanidin 3-glucoside	Cyanidin 3-rutinoside	Cyanidin-containing xylose	Cyanidin 3-glucoside acylated with malonic acid	Cyanidin 3-dioxalyl-glucoside	Monomeric anthocyanins (mg of Cyd-3-glu/100g)[d]
Marion	73.8 ± 1.07	22.8 ± 1.19	0.22 ± 0.01	1.12 ± 0.07	1.97 ± 0.07	245 ± 33.9
Waldo	87.4 ± 1.58	9.81 ± 1.18	nd	0.76 ± 0.08	2.02 ± 0.35	156 ± 30.1
Evergreen [b]	83.4 ± 0.67	2.59 ± 0.14	6.59 ± 0.43	3.92 ± 0.12	3.53 ± 0.26	131 ± 6.25
Chester [b]	89.2 ± 0.69	nd [c]	3.49 ± 0.61	2.43 ± 0.21	4.87 ± 0.30	192 ± 30.6
Silvan	69.8 ± 2.22	29.9 ± 2.32	0.13 ± 0.12	0.22 ± 0.02	nd	161 ± 8.22
NZ 9128R-1	80.7 ± 0.86	17.9 ± 0.14	0.56 ± 0.97	0.78 ± 0.11	nd	176 ± 11.4
NZ 9351-4	91.0 ± 0.68	7.95 ± 0.66	0.36 ± 0.03	0.72 ± 0.09	nd	186 ± 6.66
ORUS 1843-3	93.9 ± 0.46	5.77 ± 0.44	0.13 ± 0.01	0.15 ± 0.01	nd	256 ± 35.2
ORUS 1380-1	75.3 ± 4.36	21.4 ± 3.78	0.13 ± 0.11	0.69 ± 0.10	2.48 ± 0.59	197 ± 63.2
ORUS 1489-1	92.3 ± 0.91	nd	0.39 ± 0.03	1.40 ± 0.11	5.91 ± 0.81	235 ± 47.2
ORUS 1369-3	74.6 ± 1.85	24.5 ± 1.82	0.12 ± 0.01	0.79 ± 0.04	nd	246 ± 31.1
Mean ± s.d.	82.9 ± 8.36	13.0 ± 10.4	1.10 ± 2.03	1.18 ± 1.07	1.89 ± 2.09	198 ± 48.8

[a] single combined sample from 3 plots; [b] sample from 1 plot; [c] nd, not detected; [d] on fresh weight basis; data expressed as means ± standard deviations (n = 3 sub-samples).

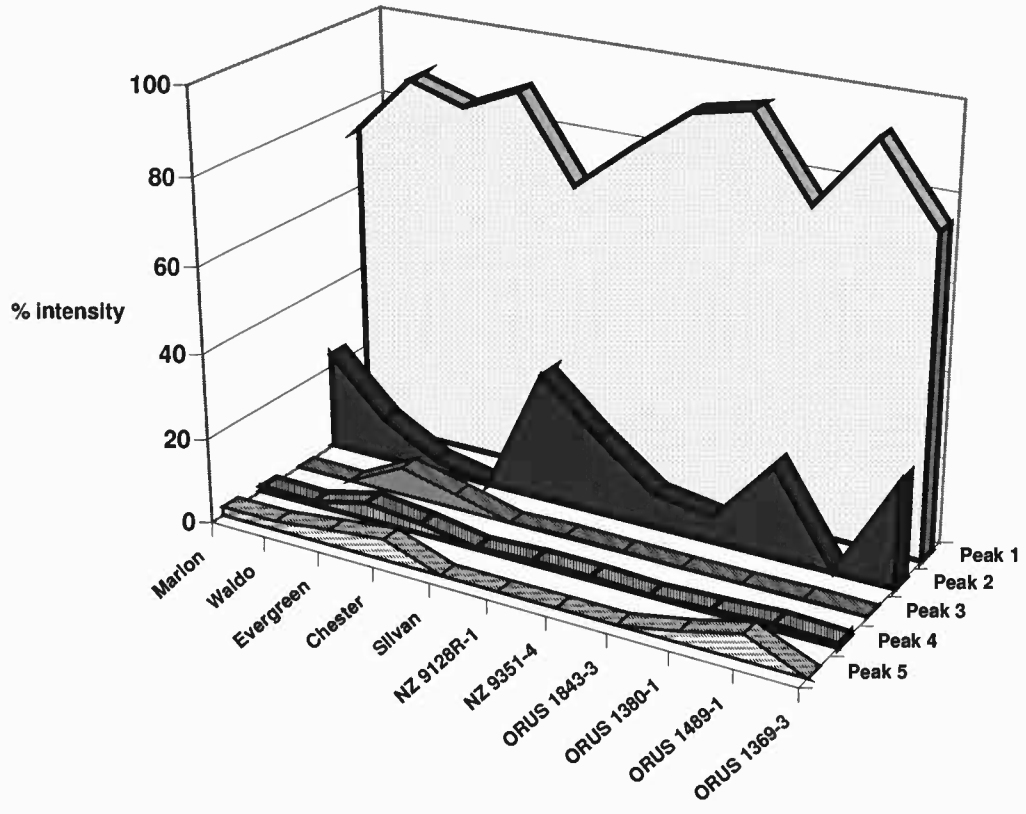

Figure 3.11. Comparison of anthocyanin composition (% peak area) between selected blackberry cultivars. Peak 1, cyanidin 3-glucoside; Peak 2, cyanidin 3-rutinoside; Peak 3, cyanidin-containing xylose; Peak 4, Cyanidin 3-glucoside acylated with malonic acid; Peak 5, cyanidin 3-dioxalylglucoside.

There are considerable differences with respect to the proportions of individual anthocyanins among the different cultivars (Table 3.3). Blackberries contained cyanidin 3-glucoside ranging from 69.8 to 93.9% (mean = 82.9), cyanidin 3-glucoside acylated with malonic acid from 0.15 to 3.92% (mean = 1.18), and cyanidin 3-rutinoside, cyanidin-containing xylose, and cyanidin 3-dioxalylglucoside all ranging from not detected to 29.9% (mean = 13.0), 6.59% (mean = 1.10), and 5.91% (mean = 1.89), respectively. Our data fell within the range reported by Fan-Chiang (8) and matched that of Stintzing et al. (31) for 'Evergreen' blackberry. However, Sapers et al. (25) reported almost 6- and 3-fold higher in cyanidin-containing xylose (mean = 6.4%; n = 8) and cyanidin 3-dioxalylglucoside (mean = 5.6; n = 8), respectively, and a 9-fold lower in cyanidin 3-rutinoside (mean = 1.5; n = 8).

We discovered negative correlation between total anthocyanins and cyanidin-containing xylose (r = -0.42), cyanidin 3-glucoside acylated with malonic acid (r = -0.35), and cyanidin 3-dioxalylglucoside (r = -0.06), a slight positive correlation with cyanidin 3-glucoside (r = 0.10) and cyanidin 3-rutinoside (r = 0.05).

Influence of cultivar on polyphenolic composition

The qualitative composition of blackberry polyphenolics was similar for all cultivars, yet quantitatively very different (Table 3.9). The procyanidin concentration ranged from 3.29 to 27.2 mg/100g fw (mean = 14.1), ellagitannins

from 7.77 to 27.2 mg/100g fw (mean = 18.8), flavonols from 4.06 to 11.9 mg/100g fw (mean = 7.02), and ellagic acid derivatives from 0.46 to 1.63 mg/100g fw (mean = 0.94). 'Evergreen' and 'Waldo' berries had the highest flavonol (11.9 mg/100g fw) and procyanidin (27.2 mg/100g fw) concentrations, respectively (Figure 3.12 and 3.13), whereas, the ORUS-selections were highest in both ellagitannins (27.2 mg/100g for 'ORUS-1489-1') and ellagic acid derivatives (1.63 mg/100g for 'ORUS-1843-3').

Table 3.9. Concentrations of polyphenolics (mg/100g fw) in selected blackberry cultivars [a].

Cultivar	Procyanidins (as catechin)	Ellagitannins (as ellagic acid)	Flavonols (as rutin)	Ellagic acid derivatives (as ellagic acid)
Marion	3.29 ± 5.70	21.8 ± 2.19	8.63 ± 2.83	0.84 ± 0.33
Waldo	27.2 ± 6.17	23.6 ± 2.34	8.73 ± 2.46	0.81 ± 0.32
Evergreen [b]	23.8 ± 5.32	21.9 ± 1.67	11.9 ± 1.84	1.38 ± 0.47
Chester [b]	8.49 ± 8.10	7.77 ± 0.38	4.21 ± 0.69	0.46 ± 0.30
Silvan	22.1 ± 19.1	14.9 ± 2.18	5.46 ± 0.85	0.61 ± 0.28
NZ 9128R-1	7.33 ± 6.49	16.1 ± 1.43	6.19 ± 0.56	0.88 ± 0.17
NZ 9351-4	10.7 ± 9.32	8.78 ± 1.10	4.06 ± 1.50	1.04 ± 0.25
ORUS 1843-3	15.2 ± 6.04	21.3 ± 0.51	8.76 ± 1.47	1.63 ± 0.58
ORUS 1380-1	13.5 ± 11.7	25.1 ± 0.48	7.14 ± 0.99	1.10 ± 0.42
ORUS 1489-1	8.42 ± 7.34	27.2 ± 4.18	6.96 ± 0.83	0.91 ± 0.54
ORUS 1369-3	15.5 ± 6.81	18.7 ± 3.28	5.18 ± 0.48	0.71 ± 0.15
Mean ± s.d.	14.1 ± 10.6	18.8 ± 6.40	7.02 ± 2.61	0.94 ± 0.45

[a] single combined sample from 3 plots; [b] sample from 1 plot; data expressed as means ± standard deviations (n = 3 sub-samples) on fresh weight basis.

The procyanidin and flavonol concentrations were quite similar to those reported by Heinonen et al. (11) (10.8 mg/100g for procyanidins, 8.3 mg/100g for flavonols). The flavonol concentration was 4 times higher than those reported by

Bilyk and Sapers (3) (mean = 1.55 mg/100g; n = 9), approximately half that of Siriwoharn and Wrolstad (29) (11.6-17.8 mg/100g, mean = 14.7; n = 2), and one-third that reported by Fukumoto and Mazza (9) (mean = 24 mg/100g; n = 3).

Moreover, the flavonol concentrations of 'Marion' and 'Evergreen' were considerably lower than that reported by Wada and Ou (34) (11 mg/100g, Marion; 24 mg/100g, Evergreen). There may well be flavonols that were not separated with this analytical procedure since this particular gradient was mainly modified for separation of ellagitannins. These values are considerably lower than our previous investigation (29) where we reported a 3 times higher concentration of ellagic acid derivatives (1.64-3.62 mg/100g, mean = 2.63; n = 2). Since we used the same analytical methods as our previous investigations, we believe these to be due to seasonal or plot-to-plot variation.

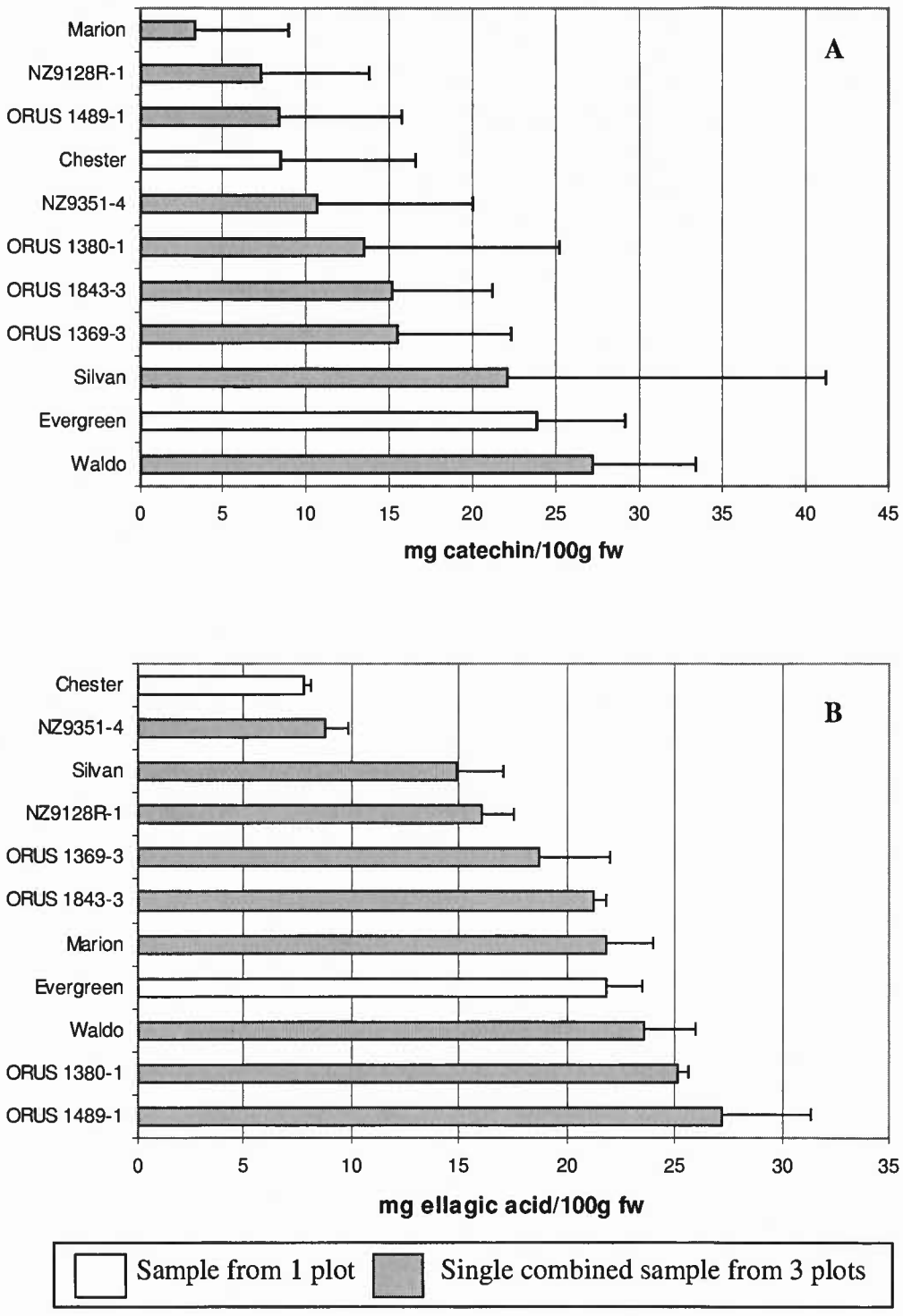

Figure 3.12. Comparison of polyphenolic concentrations between selected blackberry cultivars: (A) procyanidins, (B) ellagitannins.

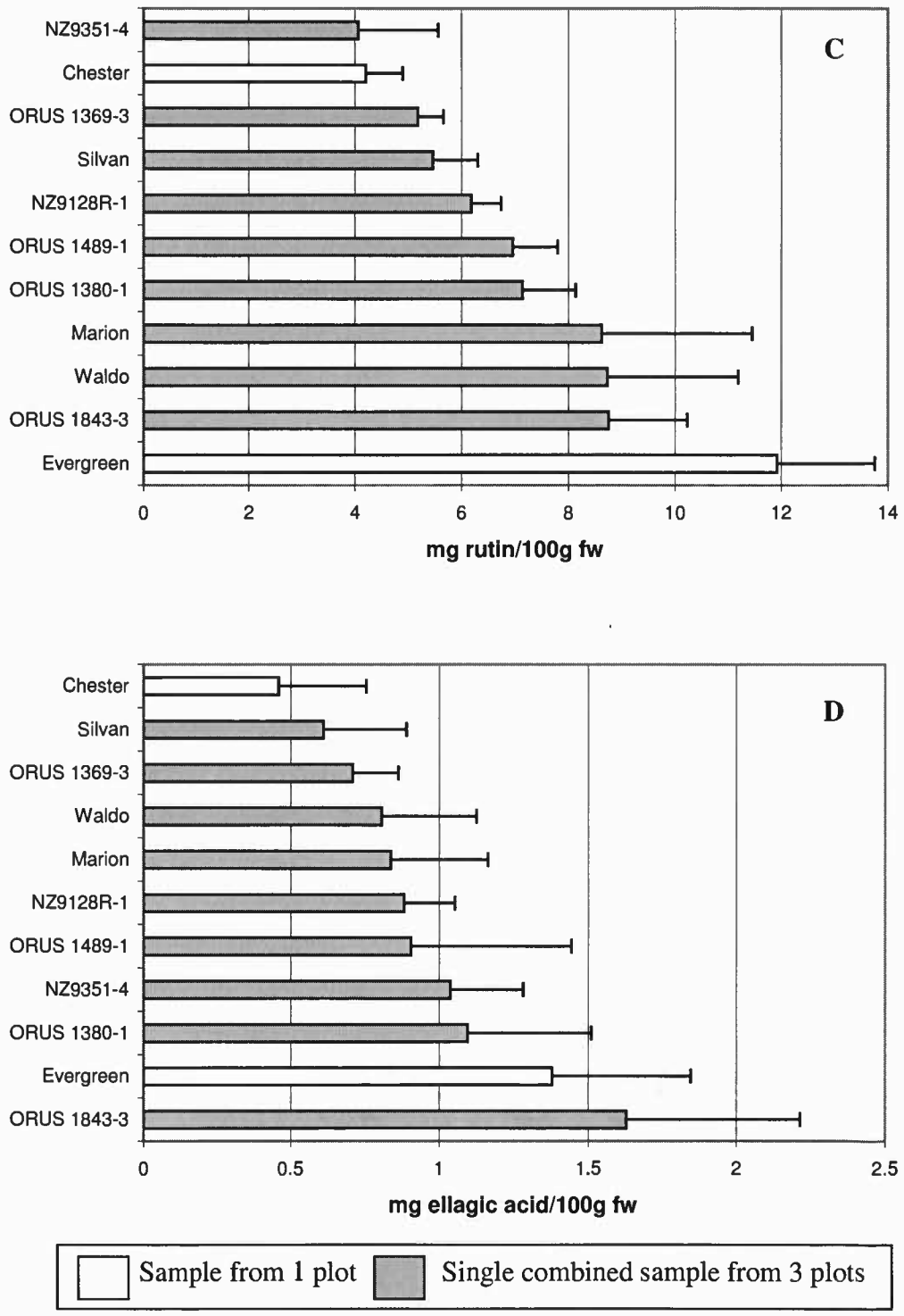

Figure 3.13. Comparison of polyphenolic concentrations between selected blackberry cultivars: (C) flavonols, (D) ellagic acid derivatives.

CONCLUSION

In 'Marion' and 'Evergreen' blackberries, the total anthocyanin content increased considerably during the course of ripening, while total phenolic content and antioxidant properties did not show such pronounced changed. Identities of five anthocyanins were confirmed in both berries. The anthocyanin profiles of 'Marion' and 'Evergreen' were qualitatively the same, but their proportions were different at different maturity stages with proportions of cyanidin 3-glucoside and cyanidin-containing xylose increasing, and decreasing for cyanidin 3-rutinoside, cyanidin 3-glucoside acylated with malonic acid, and cyanidin 3-dioxalylglucoside. The changes in the amounts of the different polyphenolic classes with ripening were also substantially different between 'Marions' and 'Evergreens'.

Effect of sub-sampling, sample preparation, and analytical measurement on compositional variation in 'Marion' berries was determined. Sample-to-sample difference and sample preparation were major contributors to variation in total phenolic and total anthocyanins contents. For total anthocyanin content, ripe and over-ripe berries were more susceptible to sample-to-sample difference, sample preparation, and analytical measurement than those of underripe. Most variation in the distribution of anthocyanins was from sample-to-sample difference, especially in the less ripe fruit, with cyanidin 3-glucoside and cyanidin 3-rutinoside being more susceptible than other anthocyanins. Variability of ellagitannins and flavonols mainly came from sample-to-sample difference, while that of procyanidins was from analytical measurement. Ellagic acid derivative content was almost unaffected

by sample-to-sample difference, sample preparation, or analytical measurement. It should be noted that samples used in this study were collected from one location with the same growing conditions and fertilization. Still substantial variations were found. Thus, even more variation would be expected in materials obtained from different farms or fields, such as that in commercial sector. Plot-to-plot difference also greatly influenced anthocyanin composition and over ripe blackberry polyphenolic composition. As a result this factor should be taken into account especially for one-sample studies.

Total phenolic and total anthocyanin contents varied greatly among cultivars. The ORUS-selections and 'Marion' contained the highest total anthocyanin and total phenolic contents among the 11 cultivars studied. 'ORUS 1489-1', 'Marion', and 'Evergreen' were varieties containing the highest ORAC and FRAP values. The proportion of anthocyanins also varied greatly among cultivars. Nonetheless, three anthocyanin patterns were observed. 'Evergreen' and 'Waldo' berries had the highest flavonol and procyanidin concentrations, respectively, whereas, the ORUS-selections were highest in both ellagitannins and ellagic acid derivatives. Although 'Marion' and 'Evergreen' blackberries were still an excellence source for dietary antioxidants and anthocyanin pigments, the results showed the potential for obtaining new cultivars with higher pigment and phenolic content through classical plant breeding.

ACKNOWLEDGEMENT

This research project was supported by a grant from the Northwest Center for Small Fruit Research, the Oregon Agricultural Experiment Station, and the Fruit Juice Quality Advisory Committee. We are grateful to Robert W. Durst, Department of Food Science and Technology, for guidance in the HPLC and LC-MS/MS analyses, to Chad E. Finn, USDA-ARS, Department of Horticulture, as well as to Brian M. Yorgey, Department of Food Science and Technology, Oregon State University (Corvallis, OR) for providing the study materials.

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CHAPTER IV

IDENTIFICATION OF ELLAGIC ACID IN BLACKBERRY JUICE SEDIMENT

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ABSTRACT

The sediment in a commercial reconstituted 'Evergreen' blackberry (CRE) juice concentrate was found to be composed of ellagic acid, protein, and other unidentified compounds. The qualitative tannin and protein-tannin haze test indicated that the sediment was predominantly tannin or protein-tannin complexes. Nitrogen determination showed the sediment to be 6.69 ± 2.21% protein on a dry weight basis. Almost all of the extractable material was identified as ellagic acid by HPLC and LC-MS. The ellagic acid content of the wet sediment was 0.05 g/100g while it was 7.41 g/100g in freeze-dried sediment. Tannase enzyme did not significantly decrease the concentration of ellagitannins in 'Marion' blackberry juice in this study.

Keywords: blackberry, juice, ellagic acid, sediment and haze.

INTRODUCTION

Fruit juice concentrates are an important ingredient in the manufacture of many foods and beverages (6). Recently, a large variety of new products based on clarified fruit juice have appeared on the market (14). Berry juices, in particular, are ideal for blending with other juices such as clarified apple or pear juice because of their intense flavor and color (11). However, the propensity for haze and sediment formation is a serious quality defect in clarified blackberry juice. Rommel et al. (10) reported that the production of 'Evergreen' blackberry wine had not been commercially successful because of excessive haze and sediment formation along with color loss and browning in wines during storage. This problem limits the utilization of blackberry juice in blended beverages and other products

A similar problem occurs with apple juice (15,16), and with muscadine grape juice and wine (1,3,7,8). Heatherbell (5) reported that a major problem in apple juice and apple wine is post-bottling haze, which is most commonly due to oxidized polymerized phenolics and phenolic-protein complexes. Boyle and Hsu (1) reported that muscadine grape juice occasionally produces sediment of yellowish to red crystals upon standing for up to six months. Lin and Vine (8) also reported a presence of precipitate during storage of clarified and pasteurized 'Magnolia' and 'Carlos' muscadine juices. Workers (1,8) analyzed the methanol solutions of muscadine juice sediment and identified them as ellagic acid.

There is little information in the literature on the formation and composition of haze and sediment in blackberry juices. Rommel et al. (10) observed that haze

and sediment formation occurred in blackberry wine over six months of storage at 20°C. We originally suspected that this sediment may be the product of hydrolyzable ellagitannins, which are major polyphenolic in blackberries, since after hydrolysis these compounds produce water-insoluble ellagic acid (12). In addition, our early investigation found that ellagitannins and ellagic acid derivatives predominate in blackberry seeds (12), thus, with an aid of processing conditions and enzymes, seed ellagitannins can be extracted into the juice as well. Garrido et al. (3) also reported that heat processing and high storage temperatures accelerated sediment formation in a muscadine juice. Moreover, adding a commercial pectinase to muscadine grapes increased sediment formation in the juice (3). It is of interest to the industry using blackberry juices that the formation of haze and sediment in blackberry juices be eliminated or somehow prevented.

Our objectives were to determine the composition of blackberry juice sediment, identify the cause of the haze and sediment formation, and create preventative measures for its occurrence.

MATERIALS AND METHODS

Reagents and standards

Ellagic acid standard was purchased from Sigma Chemical Co. (St. Louis, MO). Dimethylformamide was obtained from J. T. Baker Chemical Co.

(Phillipsburg, NJ). Enzyme DP-519-Tannase from *Aspergillus oryzae* was obtained from Valley Research (South Bend, IN). Solvents used in this study were HPLC grade

Materials

A commercial 'Evergreen' blackberry concentrate with a high level of sediment and haze was reconstituted by Milne Fruit Products, Inc. (Prosser, WA) and sent to us frozen. The juice was stored at -23°C upon arrival.

Ripe 'Marion' and 'Evergreen' blackberries, the two major commercial varieties in the Pacific Northwest, were used for enzyme experiments. Berries were handpicked by the senior author from three plots of 'Marion' (kept separately) and from one plot of 'Evergreen'. Determination was based on visual appearance of the fruits. Berries were transported (ca. 1 hr) in insulated cooled containers to the OSU Food Science and Technology pilot plant where they were immediately frozen and stored at -23°C.

Extraction of polyphenolics

Berries were cryogenically milled with liquid nitrogen using a stainless steel Waring Blender as previously described (9). The powder (ca. 5 g) was mixed 1:1 (w/v) with acetone, sonicated with an ultrasonic cleaner (Branson Cleaning Equipment Co., Shelton, CT) for 3 min, and centrifuged. The remnant was re-extracted with 70% (v/v) aqueous acetone twice. The filtrates were combined and

gently mixed with chloroform (1:2, v/v). After centrifugation at 3000 rpm for 30 min on an IEC International Centrifuge (Model UV, International equipment Co., Boston, MA), the aqueous phase (top portion) was collected and placed on a Büchi rotary evaporator (Brinkmann Instruments, Westbury, NY) at 40 °C under vacuum to remove residual acetone. The aqueous extract was then made up to a known volume with de-ionized water and stored at –70 °C until analyzed.

Sediment preparation

The commercial reconstituted juice was subjected to two preparation procedures. First, it was centrifuged to separate the juice and sediment, which were kept separately. The sediment was then thoroughly mixed with de-ionized water and centrifuged. The supernatant was discarded. This step was repeated until the supernatant solution contained no color or no further visual dilution in color was observed. Then the washed sediment (or wet sediment) was subjected to three different experiments.

(1) Dried in an oven and used for protein analysis.

(2) Dissolved in methanol, called "wet sediment extract".

(3) Frozen in a beaker and place in a Freeze-Drier model Consol 4.5 (The Virtis Company, Inc. Gardiner, NY) to remove all water from the washed sediment. The freeze-dried sediment was kept in tight container until analysis. The moisture content was calculated. The sediment was dissolved in methanol before subjected to the HPLC analysis.

Solutions of the latter two were filtered through a 0.45 μM Millipore filter; type HV (Millipore Corp., Bedford, MA) before being injected onto the HPLC system.

Tannin and protein-tannin haze test

The method described by Van Buren (15) was followed. Three volumes of dimethyl formamide was added to one volume of hazy juice, mixed well, and the clarity of the mixture was observed after five minutes. A control was prepared by adding three volumes of distilled water to one volume of the hazy juice. The treated sample was compared with the control. Hazes consisting largely of tannins or protein-tannin complexes greatly diminish when suspended in 75% dimethyl formamide, while starch, dextrin, microbiological, and inorganic hazes should be unaffected under this condition (15).

Total nitrogen content

Total nitrogen (%) was determined by automated combustion method at the Central Analytical Laboratory, Oregon State University. The analysis was performed as described by Gavlak et al. (4), and modified for use of LECO model CNS 2000 equipment (LECO Corporation, St. Joseph, MI). Protein content was calculated using total nitrogen (%) values (2), and expressed as % protein or g/100g dry weight (dw) with the mean value reported.

Polyphenolic purification

The method described by Skrede et al. (13) for separation of blueberry anthocyanins from other phenolics was modified to achieve a better recovery for blackberry ellagitannins. The sample solution (1 mL) was applied to C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA), which had been previously activated with 5 mL ethyl acetate, 5 mL methanol, and 5 mL water, respectively. The cartridge with the absorbed extract was then washed with 10 mL water, after which the cartridge was dried with a current of nitrogen for 3 minutes. Ethyl acetate (10 mL) eluted polyphenolics free from anthocyanins. The eluent was evaporated to near dryness on a Büchi rotary evaporator (40 °C under vacuum), and taken up in de-ionized water. Samples were filtered through a 0.45 μM Millipore filter; type HA (Millipore Corp., Bedford, MA) before being injected onto the HPLC system.

Acid hydrolysis of phenolics

Fifteen mL of 2 N HCL was added to approximately 1 mL of sediment in a screw-cap test tube, flushed with nitrogen and capped. The mixture was hydrolyzed for 45 minutes at 100°C, then cooled in an ice bath as described by Hong and Wrolstad . The hydrolysate was purified using a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA) and re-dissolved in MeOH. The hydrolysate was filtered through a 0.45 μM Millipore filter; type HA (Millipore Corp., Bedford, MA) before being injected onto the HPLC system.

Enzyme experiment

Experiments were carried out in 0.01 M (pH 5.0) acetate buffer. The buffer was made by mixing 14.8 mL of 0.1 M acetic acid and 35.2 mL of 0.1 M sodium acetate solution and adjusting the final volume to 100 mL with deionized water. The final pH was adjusted to 5.0 using 1 N NaOH. Enzyme solution contained at least 15 units of enzyme per 2 mL of acetate buffer.

Three sets of seven solutions were prepared. In the first set, they were composed of 2 mL of acetate buffer and 0.5 mL of one of the following solutions: three 'Marion' berry extracts (one for each plot) and four 'Evergreen' berry extracts (all from one plot). The second and third set were the same as the first set except using enzyme solution and boiled enzyme solution (in 100°C boiling water, 10 min), respectively, instead of acetate buffer. All mixture solutions were left at room temperature for one hour. Then they were purified using a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA), as described earlier, filtered through a 0.45 μM Millipore filter; type HA (Millipore Corp., Bedford, MA) before being injected onto the HPLC system.

High Performance Liquid Chromatography (HPLC)

Analytical HPLC for polyphenolics

(1) Apparatus.

a. A Varian 5000 Liquid Chromatograph (Varian Instrument Group, Sunnyvale, CA) equipped with a Hewlett-Packard 1040A photodiode array

detector and Gateway 2000 P5-90 computer with Hewlett-Packard HPLC^{2D} Chemstation software was used for preliminary work.

b. A Hewlett-Packard 1090 Liquid Chromatograph (Agilent Technologies, Palo Alto, CA), equipped with photodiode array detector and Gateway 2000 P5-90 computer with Hewlett-Packard HPLC^{2D} Chemstation software was used for identification and the enzyme experiment.

(2) Column, mobile phases, and HPLC condition. Chromatographic analysis was done using a Synergi Hydro-RP column (4 μm), 250 × 4.60 mm i.d. (Phenomenex, Torrance, CA), fitted with an Allsphere 10 × 4.6 mm i.d. ODS-2 guard column (Alltech, Deerfield, IL). Mobile phase A consisted of 100% HPLC grade methanol, and mobile phase B was 1% formic acid in de-ionized water. Solvents and samples were filtered through a 0.45 μM Millipore filter type HA for aqueous or HV for organic solvent.

The program was: (a) 0 minute 10% A; (b) 0 – 50 minutes linear gradient from 10 to 35% A; (c) 50 – 55 minutes linear gradient from 35 to 70% A; (d) 55 – 60 minutes isocratic at 70% A; (e) 60 – 66 minutes linear gradient from 70 to 10% A; 5 minutes post time and gradient repeated. Simultaneous monitoring was performed at 255, 280, 320, 360, and 520 nm at a flow rate of 1 mL/min and Injection volume of 20 μL. Identification was made from matching UV-visible spectra and retention times with authentic standards (when available). Quantitation of individual polyphenolic peaks was done by the external standard method.

Ellagitannins, ellagic acid, and ellagic acid derivatives were determined as ellagic acid at 255 nm.

Analytical HPLC for LC-MS/MS

(1) Apparatus. A Hewlett-Packard 1090 Liquid Chromatograph (Agilent Technologies, Palo Alto, CA), equipped with photodiode array detector and Gateway 2000 P5-90 computer with Hewlett-Packard HPLC^{2D} Chemstation software.

(2) Column, mobile phases, and HPLC condition. Chromatographic analysis was done using a Synergi Hydro-RP column (4 μm), 250 × 2.00 mm i.d. (Phenomenex, Torrance, CA), fitted with an Allsphere 10 × 4.6 mm i.d. ODS-2 guard column (Alltech, Deerfield, IL). Mobile phase A consisted of 100% HPLC grade methanol, and mobile phase B was 1% formic acid in de-ionized water. Solvents and samples were filtered through a 0.45 μM Millipore filter type HA for aqueous or HV for organic solvent.

The program was: (a) 0 minute 10% A; (b) 0 – 10 minutes linear gradient from 10 to 30% A; (c) 10 – 25 minutes isocratic at 30% A; (d) 25 – 35 minutes linear gradient from 30 to 70% A; (e) 35 – 40 minutes isocratic at 70% A; (f) 40 – 46 minutes linear gradient from 70 to 10% A; 5 minutes post time and gradient repeated. Simultaneous monitoring was performed at 280, 255, 320, 360, and 520 nm at a flow rate of 0.2 mL/min and injection volume of 20 μL. Identification was made from matching UV-visible spectra and retention times with authentic

standards (when available). Quantitation of individual polyphenolic peaks was done by the external standard method. Ellagitannins, ellagic acid, and ellagic acid derivatives were determined as ellagic acid at 255 nm.

Electrospray Mass Spectrometry (ESMS)

Low-resolution MS was obtained using ESMS. The instrument was a Perkin Elmer SCIEX API III bimolecular mass analyzer (Ontario, Canada) equipped with an ion spray interface (ISV = 5500, orifice voltage = 50) and loop injection. The mass spectrometer was operated in the negative-mode. Purified polyphenolic fraction was bled into the system by a 100-µL-glass syringe connected with the infusion pump at a flow rate of 5 µL/min.

Tandem Mass Spectrometry (MS/MS)

Collision-induced dissociation (CID) of the purified polyphenolic fraction was carried out using argon as the target gas. The mass of the parent ion was scanned in the first quadrupole (Q1), *m/z* selected and collisionally activated in Q2, and the daughter ions were analyzed in the third quadrupole (Q3). MS/MS was performed using a collision energy set of +30 eV.

Statistical analysis

For the enzyme experiment, differences in ellagitannin and ellagic acid contents of 'Marion' blackberries among treatment means were determined using

Tukey's-b procedure, SPSS 12.0 Software (SPSS Inc., Chicago, IL). Significance level was 0.05 unless otherwise indicated.

RESULTS AND DISCUSSION

Characteristics of sediment

We requested from Milne Fruit Products, Inc. (Prosser, WA) a commercial reconstituted 'Evergreen' blackberry (CRE) juice, heavy in haze, for the investigation of blackberry juice sediment. The hazy juice was first subjected to the tannin and protein-tannin haze test and found to consist largely of tannins or protein-tannins complexes. Next, the juice sediment was separated from the juice by centrifugal method and washed thoroughly with de-ionized water to eliminate all water-soluble components. The sediment consisted of tiny granulated, purple black particles. The wet sediment contained 97.5% moisture content. After freeze-drying, the dried sediment (freeze-dried sediment) was weighed and found to account for 0.10% of the starting weight of the hazy CRE juice. The nitrogen analysis estimated 6.69 ± 2.21% of protein present in the dried sediment.

Qualitative and quantitative ellagic acid in sediment

The polyphenolic identification was done using the methanol extracts of wet and freeze-dried sediment. The sample preparation included dissolving a

known amount of sediment in methanol and sonicating for 15 minutes. The filtered extracts contained no color and were injected directly onto the HPLC system. HPLC chromatographic profiles of wet sediment extract and freeze-dried sediment extract (Figure 4.1) were qualitatively similar. The major phenolic compound in both sediment extracts was identified, by comparing to and spiked with external standard, and LC-MS/MS (*m/z* 301.0, Figure 4.2), as ellagic acid (Figure 4.3). The ellagic acid content was measured to be 0.05 g/100g fw for wet sediment extract and 7.41 g/100g dw for freeze-dried sediment extract. Acid hydrolysis of the freeze-dried sediment resulted in increased recovery of ellagic acid (10.8 g/100g dw). Trace amounts of anthocyanin and ellagic acid derivatives were detected in the extract of freeze-dried sediment (Figure 4.1B), even though the sediment had been previously washed. The fact that anthocyanin could be recovered with methanol extraction suggests that it was associated with the sediment.

Nonetheless, methanol extraction was unable to solubilize a substantial proportion of the sediment, which may consist of polymeric tannin or unidentified compounds, perhaps similar to the insoluble material described in muscadine grape juice sediment (7). Change in physical property of the sediment during sample preparation (i.e. freeze-drying) may also contribute to this difficulty.

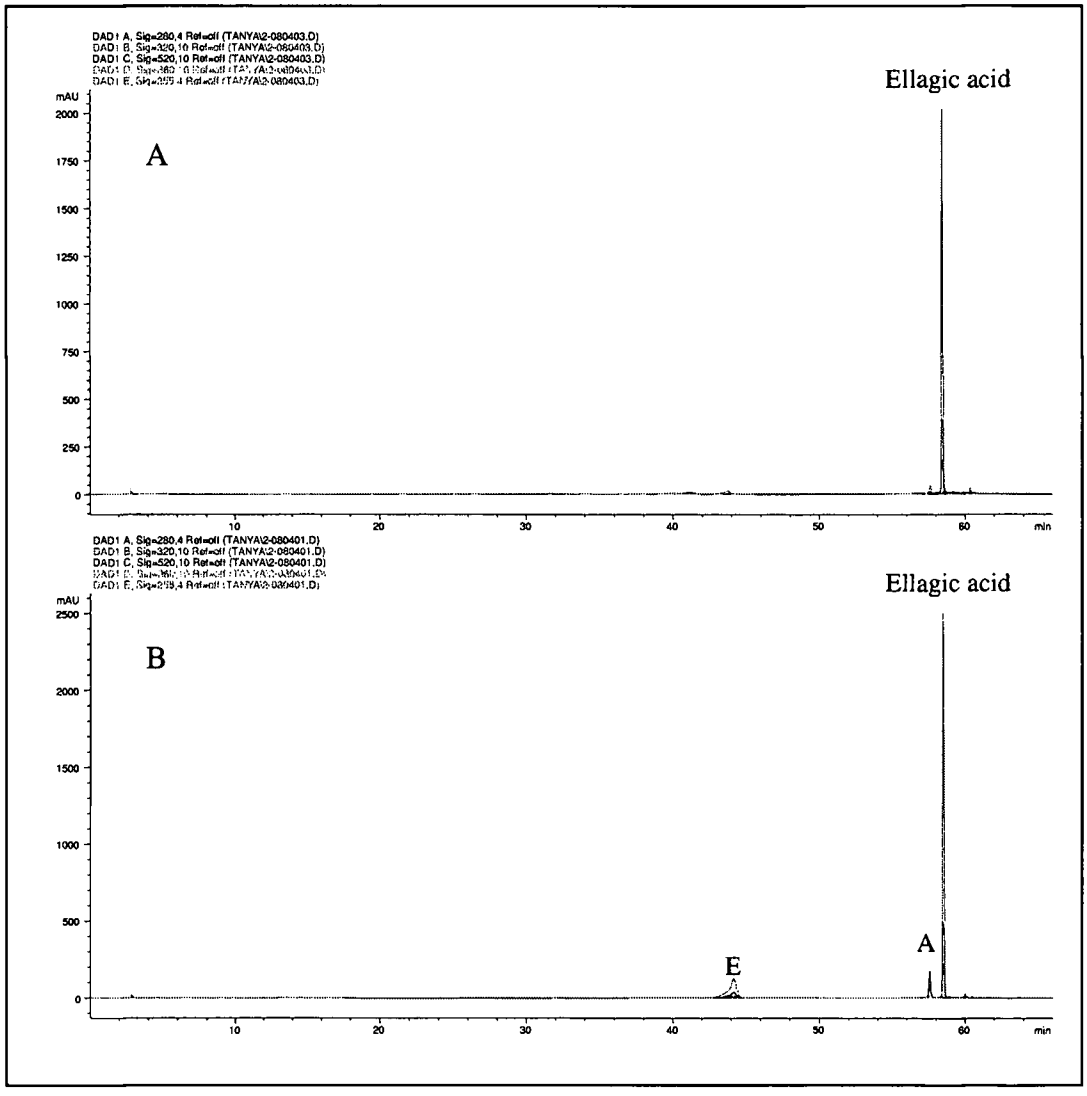

Figure 4.1. HPLC polyphenolic profiles of: (A) wet sediment extract, (B) freeze-dried sediment extract, at 255 nm, 280 nm, 320 nm, 360 nm, and 520 nm. (A, anthocyanins; E, ellagic acid derivatives).

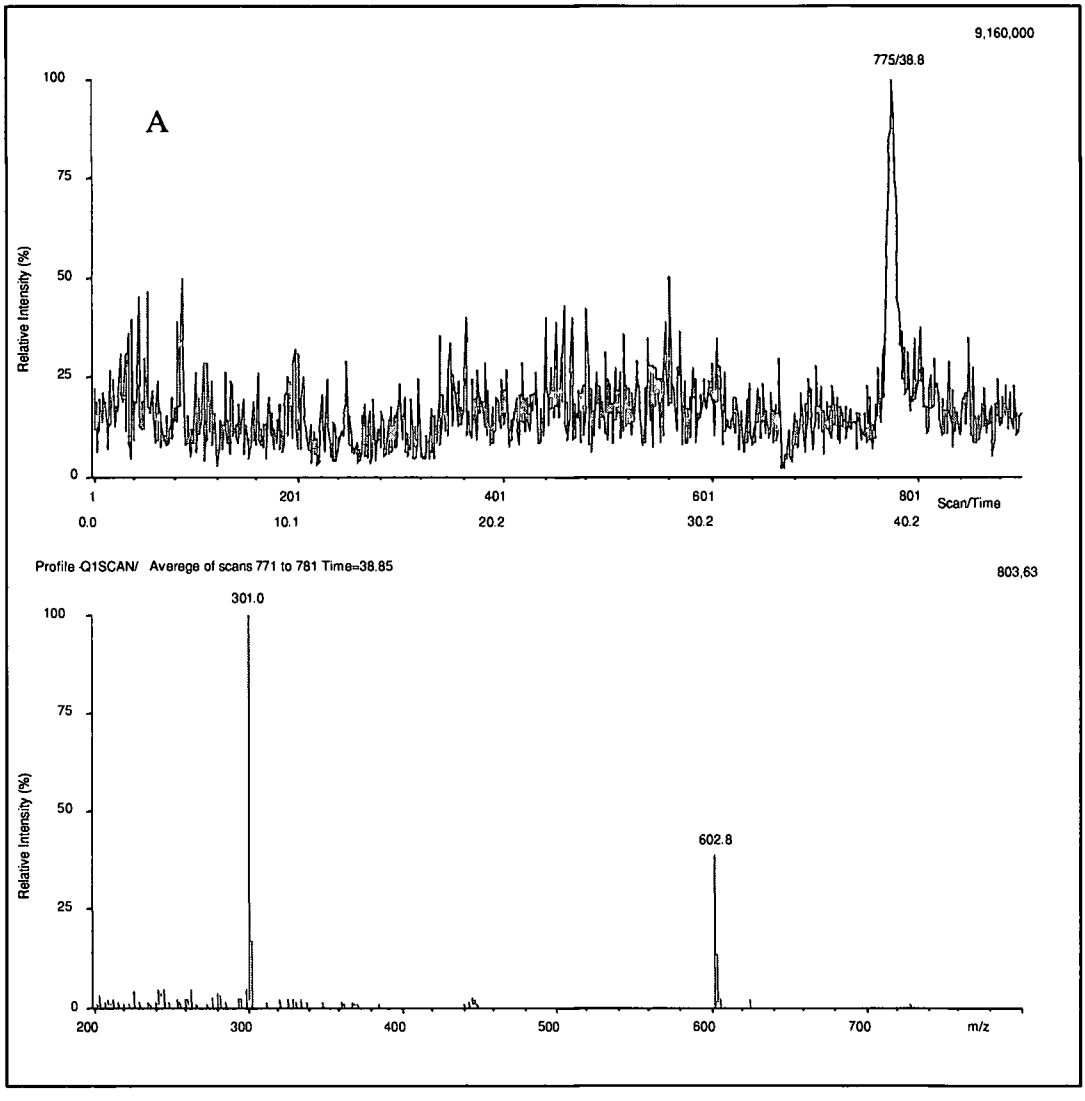

Figure 4.2. (A) Mass scan of ellagic acid standard, (B) mass spectrum of (A).

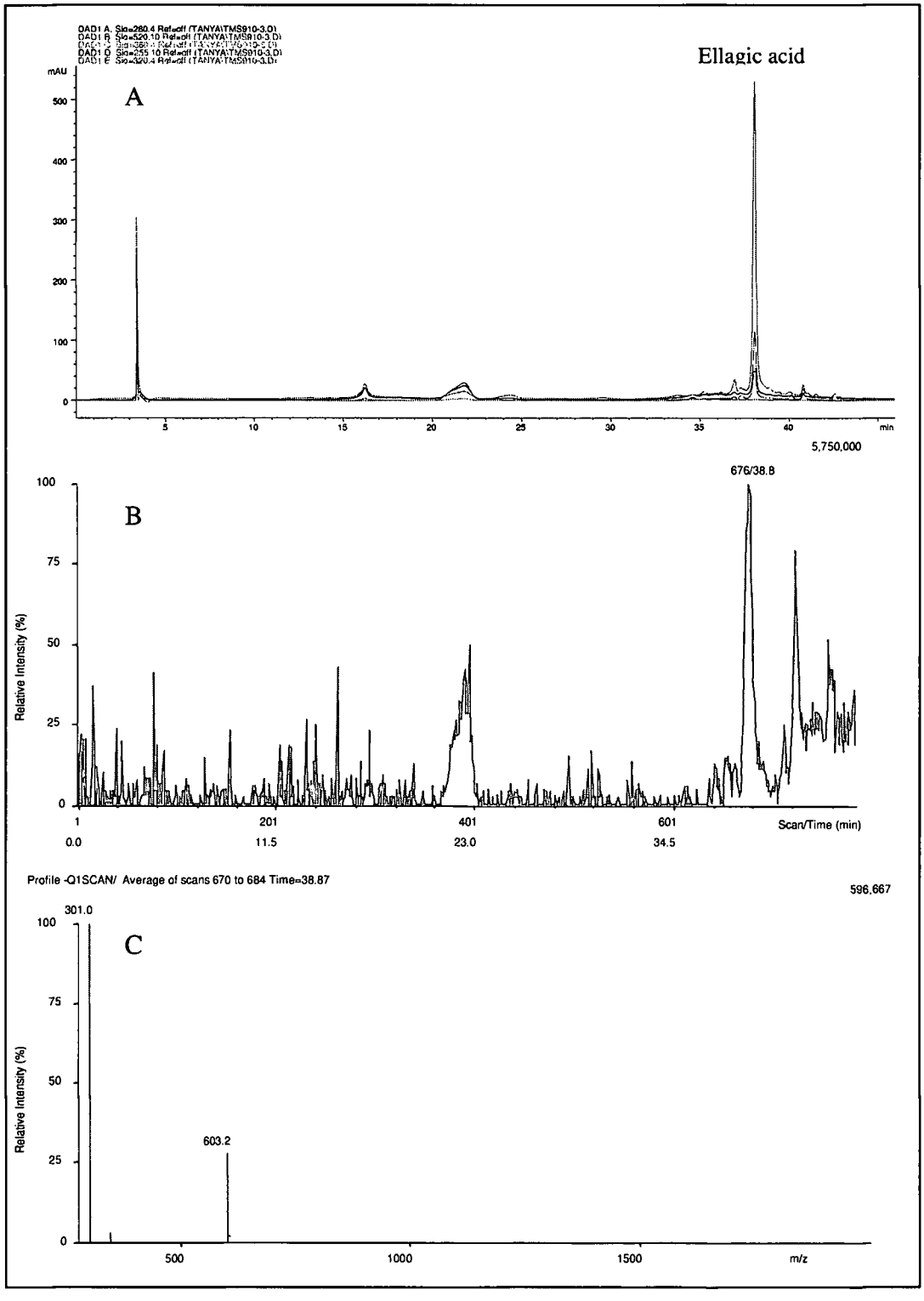

Figure 4.3. (A) HPLC polyphenolic profile of wet sediment extract, (B) mass scan of (A), (C) mass spectrum of ellagic acid peak.

Enzyme experiment

Our original hypothesis was that ellagitannins are extracted from the seeds into the juice and subsequently undergo hydrolysis during processing and storage, releasing ellagic acid that precipitates. This preliminary study using tannase enzyme was an attempt to accelerate the reaction so more stable blackberry juice could be obtained. Also, it was to evaluate whether treating blackberry juice with tannase would produce insoluble ellagic acid from ellagitannins that could be recovered.

HPLC chromatographic profiles of 'Marion' and 'Evergreen' juice at different treatments were qualitatively and quantitatively similar (Table 4.1, Figure 4.4 and 4.5). The effect of tannase relative to the control can be estimated by subtracting the boiled tannase (positive control) mean from the tannase mean then dividing the difference by the control mean. For example, for ellagitannin form #1 the effect of tannase is to increase the concentration by 9.90% = 100 × (5.73-5.24)/ 4.95. In both cultivars, the concentration of ellagitannin form #1 (T1) increased (9.90% for 'Marion', 34.6% for 'Evergreen') when treated with tannase, whereas other ellagitannins reduced: ellagitannin form #2 (T2) (7.79% for 'Marion', 7.91% for 'Evergreen'), ellagitannin form #3 (14.4% for 'Marion', 14.7% for 'Evergreen'), and ellagic form #4 (T4) (78.0% for 'Marion', 26.9% for 'Evergreen'). In 'Marion' berry extract, the concentration of ellagic acid derivatives (E) increased with enzyme treatment (66.4% for E form #1, 8.20% for E form #2).

On the other hand, the concentration of ellagic acid derivatives decreased from that of 'Evergreen' (6.90%).

Under these conditions, there was no statistical difference among the treatments for 'Marion' berry extracts ($p > 0.05$). This might be due to insufficiency of the treatment conditions used in this study (one hour at room temperature) for enzyme to react. Thus, the optimum conditions for the tannase enzyme should be further explored in future study.

Table 4.1. Impact of enzyme treatments on the concentrations of polyphenolics (mg/100g fw) in 'Marion' and 'Evergreen' berry extracts [a].

Compound	Control	Tannase	Boiled tannase
Marion [b]			
Ellagitannin			
Form #1	4.95 ± 0.81a	5.73 ± 0.40a	5.24 ± 0.76a
Form #2	32.1 ± 5.36a	31.3 ± 2.62a	33.8 ± 4.14a
Form #3	11.8 ± 1.65a	10.6 ± 1.03a	12.3 ± 1.18a
Form #4	2.27 ± 1.11a	0.41 ± 0.04a	2.18 ± 0.95a
Ellagic acid derivatives			
Form #1	2.38 ± 0.93a	4.19 ± 0.85a	2.61 ± 1.04a
Form #2	0.61 ± 0.08a	0.71 ± 0.22a	0.66 ± 0.12a
Evergreen*			
Ellagitannin			
Form #1	5.20 ± 0.68	7.45 ± 0.97	5.65 ± 0.77
Form #2	27.8 ± 2.40	25.3 ± 3.54	27.5 ± 2.44
Form #3	34.7 ± 2.39	29.7 ± 3.57	34.8 ± 2.75
Form #4	0.52 ± 0.09	0.36 ± 0.15	0.50 ± 0.10
Ellagic acid derivatives	1.16 ± 0.06	1.13 ± 0.13	1.21 ± 0.08

[a] Values were mg/100g on fresh weight basis; [b] Different letters in the same row for Marion indicate significant differences ($p \leq 0.05$); data expressed as means ± standard deviations (n = 3 plots, 1 replicate each); *data expressed as means ± standard deviations (n = 1 plot, 4 sub-samples).

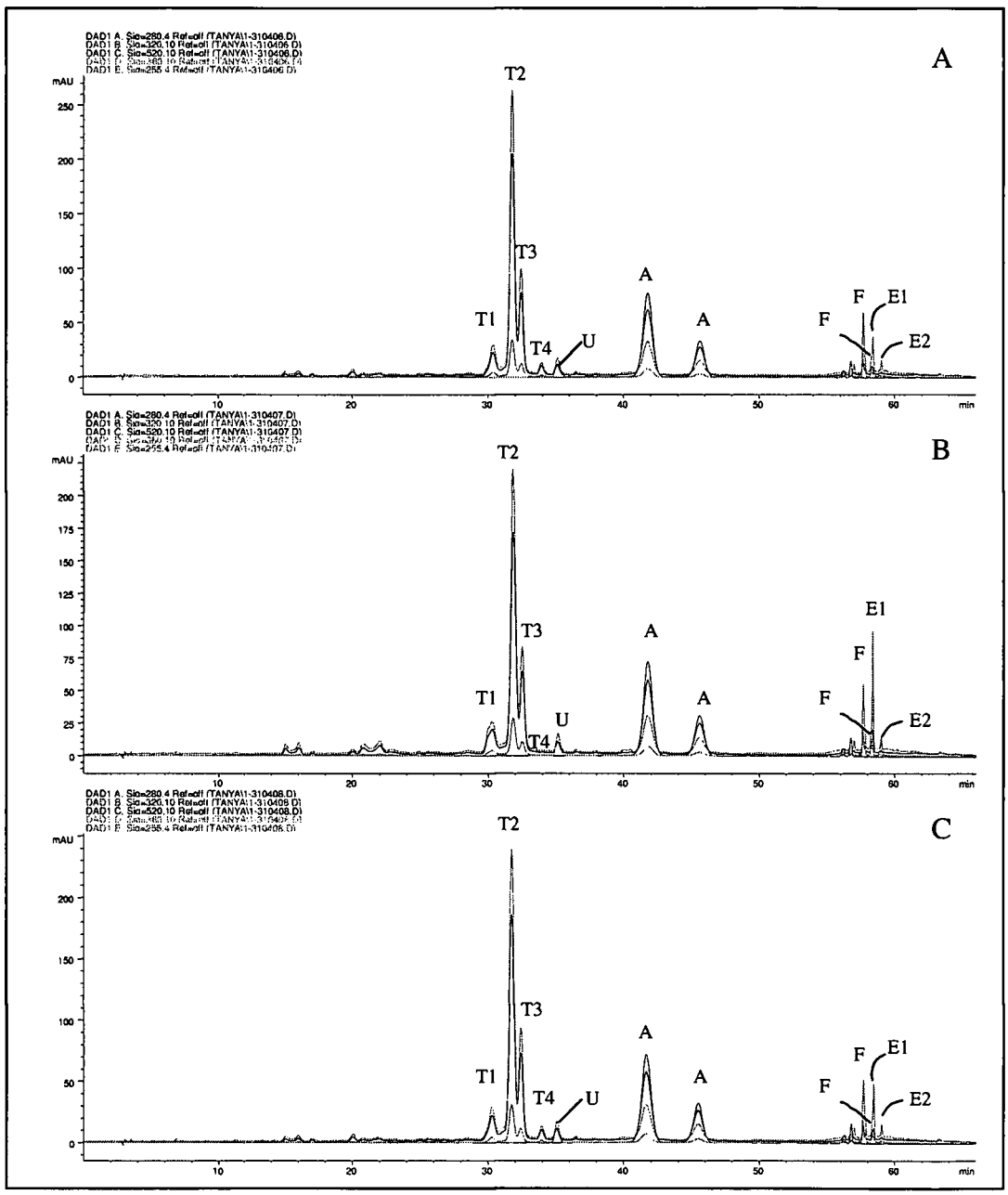

Figure 4.4. HPLC polyphenolic profiles from enzyme experiment of 'Marion' blackberries: (A) control, (B) tannase, (C) boiled tannase, at 255 nm, 280 nm, 320 nm, 360 nm, and 520 nm. (T, ellagitannins; U, unidentified polyphenolics; A, anthocyanins; F, flavonols; E, ellagic acid derivatives).

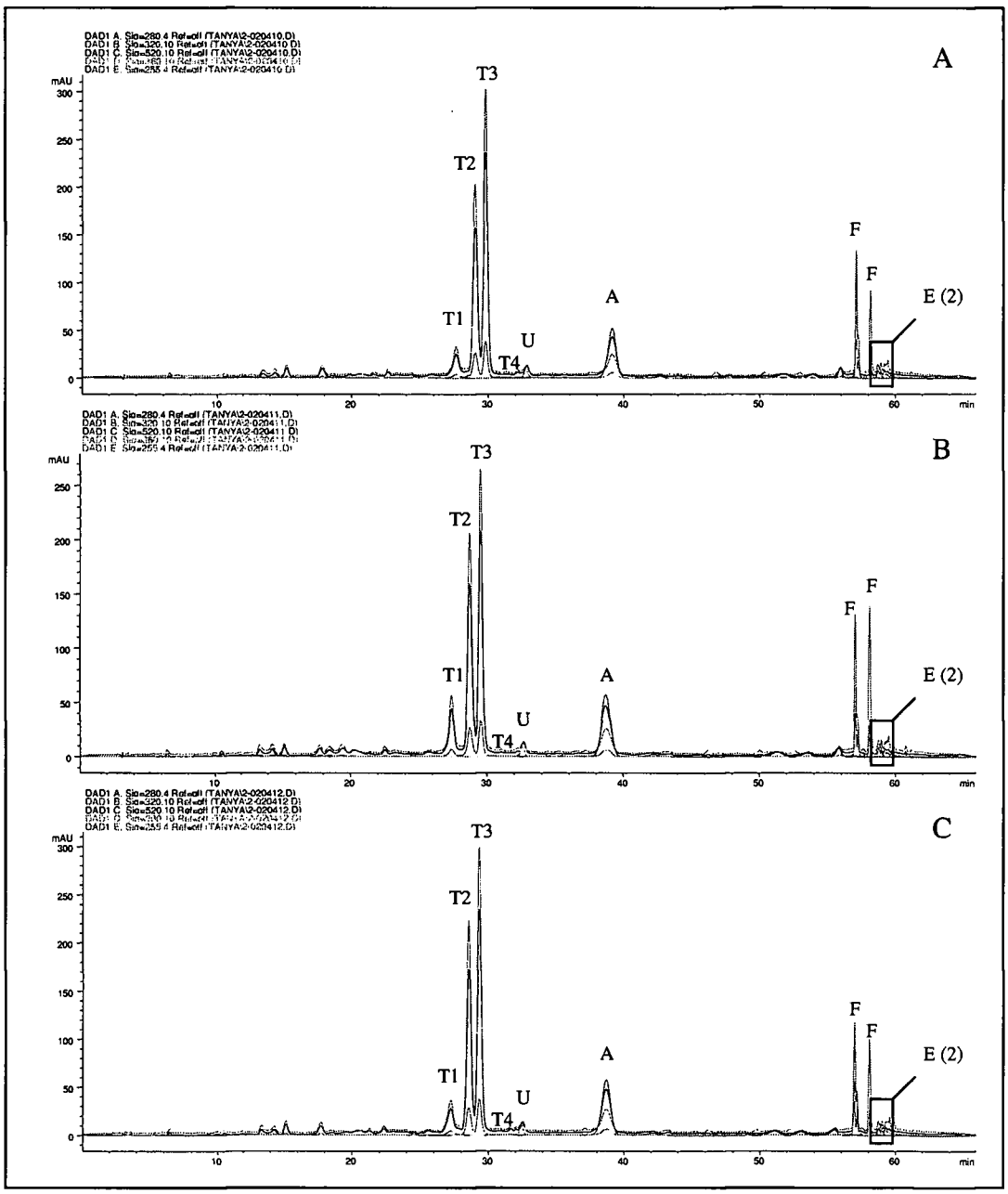

Figure 4.5. HPLC polyphenolic profiles from enzyme experiment of 'Evergreen' blackberries: (A) control, (B) tannase, (C) boiled tannase, at 255 nm, 280 nm, 320 nm, 360 nm, and 520 nm. (T, ellagitannins; U, unidentified polyphenolics; A, anthocyanins; F, flavonols; E, ellagic acid derivatives).

Discussion

Substantial amount of ellagic acid and protein were found in the CRE juice sediment. Even though the recovery of ellagic acid and protein did not add up to the original weight of the sediment, that did not mean ellagic acid and protein were not major contributors to the problem. A possible explanation could be that not all sediment was dissolved in methanol and the remainder sediment may consist of more conjugated forms of ellagic acid, protein, or unidentified compounds. Lee and Talcott (7) also reported a similar result in muscadine juice and wine that no more than 12% of free ellagic acid is actually present in the collected sediment with the remainder consisting of either unidentified compounds or conjugated forms of ellagic acid.

Figure 4.6 demonstrates an impact of processing on phenolic composition of 'Evergreen' blackberry juice with ellagitannins markedly decreasing in the processed juice (Figure 4.6B). This was similar to report by Lee and Talcott (7) that processing techniques influence initial concentrations of ellagitannins and ellagic acid and the extent of sediment formation in muscadine juice and wine. Since ellagitannins, when hydrolyzed, produces water-insoluble ellagic acid, these findings supported our original hypothesis that ellagic acid plays a major role in sediment formation in blackberry juice.

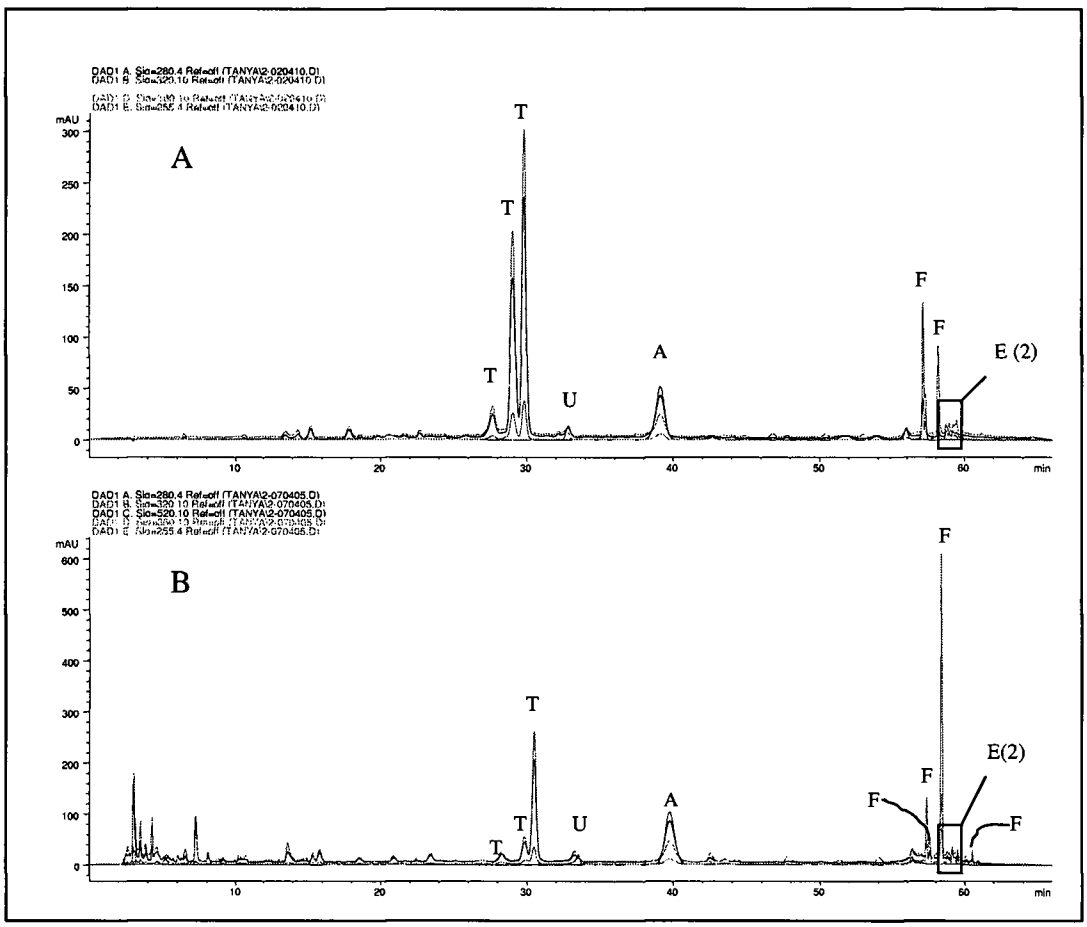

Figure 4.6. HPLC polyphenolic profiles of: (A) 'Evergreen' blackberries, (B) commercial reconstituted 'Evergreen' blackberry juice, at 255 nm, 280 nm, 320 nm, 360 nm, and 520 nm. (T, ellagitannins; U, unidentified polyphenolics; A, anthocyanins; F, flavonols; E, ellagic acid derivatives).

According to this hypothesis, to avoid sediment formation in blackberry juice the following recommendations could be made: 1.) Completely eliminating all ellagitannins in the juice via thermal processing (3) or hydrolysis, followed by ellagic acid filtration before bottling. Rommel et al. (10) reported that HTST-pasteurizing retards polymerization and also minimizes haze and sediment formation in 'Evergreen' blackberry wine. 2.) Removing blackberry seeds before processing to reduce the chance that additional ellagitannins from the seeds will leak into the juice; therefore reduces the degree of sedimentation. 3.) Choosing blackberry cultivars with lower concentration of ellagitannins for juice processing may help reduce the severity of the problem. For example, in this study 'Evergreen' blackberries (68.2 mg/100g) contained higher amount of ellagitannins than that of 'Marion berries (51.1 mg/100g), thus the juice made from 'Evergreen' cultivar may as well be more susceptible to sedimentation than that made from 'Marion' variety.

CONCLUSION

The CRE juice sediment consisted of ellagic acid, protein, and unidentified compounds were found in the CRE juice sediment. Acid hydrolysis aided the recovery of ellagic acid from the sediment. Hydrolysis of ellagitannins during processing and storage to form the less water-soluble ellagic acid is a plausible explanation for the formation of sediment and haze problem in blackberries. A possible strategy for reducing the incidence of haze and sediment formation in

blackberry juice would be to promote ellagitannin hydrolysis through enzymatic treatment or modifying processing operation conditions. In this study, tannase enzyme did not significantly decrease the concentration of ellagitannins in 'Marion' blackberry extracts. Further research on complete identification of the sediment is essential, as it will provide vital insight on the formation of haze and sediment in blackberry juices and possible preventative measures. The results will expand currently limited use of blackberry juice in commercial products. In addition, ellagic acid, which has been shown to have anti-carcinogen properties, could be recovered by centrifugation/filtration and utilized as an added-value by-product.

ACKNOWLEDGEMENT

This research project was supported by a grant from the Northwest Center for Small Fruit Research, Oregon Agricultural Experiment Station, and Fruit Juice Quality Advisory Committee. We are grateful to Robert W. Durst, Department of Food Science and Technology, for guidance in the HPLC and LC-MS/MS analyses, to Chad E. Finn, USDA-ARS, Department of Horticulture, Oregon State University, as well as to Eric Johnson, Milne Fruit Products, Inc. (Prosser, WA) for providing the study materials.

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CHAPTER V. SUMMARY

Blackberry polyphenolic composition and antioxidant activity were clearly affected by maturity. The anthocyanin pigments increased tremendously with ripening, while the total phenolics and antioxidant activity were less affected. Similar results were found with the polyphenolic concentration and anthocyanin distribution.

Plot-to-plot and sample differences were the major contributor to variation, with sample preparation accounting for variation for some parameters. Measurement variation was a relatively small component of the total variation. Substantial variations were found, even though samples were collected from the same growing location and conditions. Thus, even more variation would be expected in materials obtained from different farms or fields, such as that in commercial sector.

Blackberry polyphenolic composition and antioxidant activity varied greatly among 11 cultivars. Although 'Marion' and 'Evergreen' blackberries were still an excellence source for dietary antioxidants and anthocyanin pigments, the results showed the potential for obtaining new cultivars with higher pigment and phenolic content through classical plant breeding.

The investigation on haze and sediment formation in a commercial reconstituted 'Evergreen' blackberry juice identified the sediment to consist of ellagic acid, protein, and unidentified compounds. Hydrolysis of ellagitannins

during processing and storage to form the less water-soluble ellagic acid is a plausible explanation for the problem. A possible strategy for reducing the ellagic acid sediment formation in blackberry juice would be to promote ellagitannin hydrolysis through enzymatic treatment or modifying processing operation conditions. In this study, tannase enzyme did not significantly decrease the concentration of ellagitannins in 'Marion' blackberry extracts. Further research on the sediment composition is still needed to gain more understanding on how these haze and sediment are formed and can be prevented.

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