Characterization, Quantitation, and Distribution of Anthocyanins and Colorless Phenolics in Sweet Cherries

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The anthocyanins and colorless phenolics in 11 cultivars and hybrids of sweet cherries were characterized and quantified by high-performance liquid chromatography and gas chromatography. All of the dark-colored cherry genotypes were found to contain 3-rutinoside and 3-glucoside of cyanidin as the major anthocyanins and the same glycosides of peonidin as minor anthocyanins. Another minor anthocyanin, pelargonidin 3-rutinoside, was characterized in sweet cherries for the first time. The major colorless phenolics were characterized as neochlorogenic acid and *p*-coumaroylquinic acid. The total anthocyanin content ranged from 82 to 297 mg /100 g of pitted cherry for the dark cherries and from 2 to 41 mg/100 g of pitted fruit for the light-colored cherries. The neochlorogenic acid ranged from 24 to 128 mg/100 g of pitted cherry and *p*-coumaroylquinic acid from 23 to 131 mg/100 g, respectively. The relative amounts of the two phenolic acids varied widely across the cherry cultivars examined in this study.

Keywords: *Flavonoids; phenolic acids; HPLC; GC; plant pigments; Prunus avium*

INTRODUCTION

The sweet cherry (*Prunus avium* L.) is important commercially as a table fruit and as an ingredient in fruit cocktails and maraschino cherries (Mazza and Miniati, 1993). Color is the most important indicator of maturity and quality for both fresh and processed cherries. In cherries for processing, color has a direct influence on quality of the finished products (Drake et al., 1982). Development of red color in dark sweet cherries is an index of maturity and can be used to predict processing grades. Light-colored cultivars of sweet cherries develop a red blush, but intensity of red color is not a good index of maturity.

That sweet cherries contain anthocyanins has been known since the beginning of the 20th century. Willstätter and Zolinger (1916), and later Robinson and Robinson (1931), identified the 3-rutinoside and 3-glucoside of cyanidins in ripe sweet cherries. The occurrence of these anthocyanins was confirmed by Li and Wagenknecht (1958), Lynn and Luh (1964), and Okombi (1979). Lynn and Luh (1964) also reported the presence of peonidin and two of its glycosidic derivatives in cv. Bing cherries, and Okombi (1979) identified peonidin 3-rutinoside as the main pigment of cv. Bigarreau Napolèon cherries. However, Harborne and Hall (1964) and Olden and Nybom (1968) found only cyanidin derivatives and no peonidin glycosides in cultivars of *P. avium*. Similarly, Fouassin (1956) reported the presence of the two monoglycosides of cyanidin in different cultivars of sweet cherries analyzed by paper chromatography.

Casoli et al. (1967), using two-dimensional paper chromatography, separated nine pigments of which they identified a 3-monoglycoside of cyanidin, a 3-diglycoside of peonidin, and a diglycoside of cyanidin which appeared to be acylated with coumaric acid. Tanchev et al. (1971) and Tanchev (1975) identified cyanidin 3-glucoside, cyanidin 3-rutinoside, peonidin 3-rutinoside, and cyanidin 3-sophoroside in cv. Lambert, Helmsdorf, Somaya, Kozerskia, and Bing cherries.

In practically all of the above cited studies, however, the anthocyanins of sweet cherries were extracted with methanol containing hydrochloric acid, which can decompose labile pigments, and characterized by paper and/or thin layer chromatography, UV–vis or fluorescence spectroscopy, and chemical methods involving alkaline degradation, controlled demethylation, and reductive cleavage. High-performance liquid chromatography (HPLC) of anthocyanins, pioneered during the 1970s, has not been used for isolation, identification, and quantitative analysis of the anthocyanins and other phenolics in sweet cherries.

This investigation was undertaken to rectify this situation and to determine the qualitative and quantitative composition of anthocyanins and colorless phenolics in cultivars and hybrid lines of sweet cherries from the Okanagan Valley of British Columbia, Canada.

MATERIALS AND METHODS

Cherry Samples. Samples of seven cultivars and four hybrid lines of sweet cherry (*P. avium*) at commercial maturity were obtained during the month of July 1993 from the orchard of Agriculture Canada, Research Centre, Summerland, British Columbia. The harvested fruit were immediately packed in plastic bags, frozen within 1 h of harvest, and kept at −38 °C for 3–6 weeks before analysis.

Standards. Cyanidin 3-glucoside, cyanidin 3-rutinoside, and pelargonidin chloride were purchased from Sarsynthèse (Merignac, France), and chlorogenic, caffeic, *p*-coumaric, and quinic acids, glucose, galactose, xylose, rhamnose, and arabinose, from Sigma Chemical Co. (St. Louis, MO).

Pelargonidin 3-rutinoside was extracted from strawberries (Bakker et al., 1994) and peonidin 3-glucoside from Cabernet Sauvignon grape (Wulf and Nagel, 1978).

Extraction of Pigments. The extraction of anthocyanins and colorless phenolic compounds was carried out as described previously (Gao and Mazza, 1994a). Pitted cherries (30 g) were homogenized in 50 mL of MeOH/formic acid/water (70/2/28) in a thermostated (15 ± 1 °C) Waring Blendor for 6 min. The homogenate was filtered through a 0.45 μm hydrophilic Durapore filter (Millipore Corp., Bedford, MA), and the filtrate was injected into the HPLC for separation and quantitation of anthocyanins and other phenolic compounds. For characterization of the minor and major anthocyanins, an aqueous extract of Bing cherries was concentrated by open column chromatography on Amberlite CG-50 resin, as described below.

Concentration and Purification of Anthocyanins. Pigments were extracted by homogenizing 60 g of pitted Bing cherries (with 1 mL of formic acid to lower the pH). The

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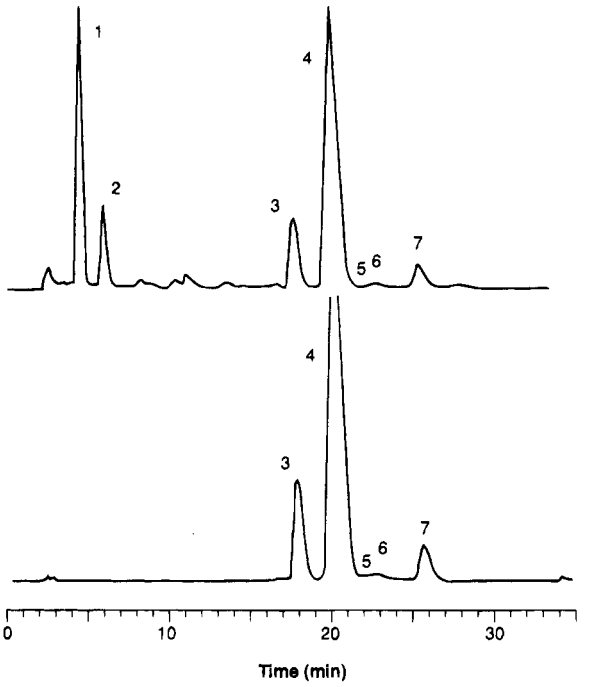

Figure 1. HPLC separation of anthocyanins in a methanolic extract of cv. Bing cherries, monitored at 280 (top) and 525 nm (bottom). Peaks: 1, neochlorogenic acid; 2, *p*-coumaroylquinic acid; 3, cyanidin 3-glucoside; 4, cyanidin 3-rutinoside; 5, peonidin 3-glucoside; 6, pelargonidin 3-rutinoside; 7, peonidin 3-rutinoside.

Table 1. HPLC Characterization of Anthocyanidins and Phenolic Acids in a MeOH/HCl Extract of Cv. Bing Sweet Cherry

anthocyanin/ standard	aglycons t_R (min)	phenolic acid t_R (min)[c]
peak 1[a]	–[b]	8.9
peak 2	–	13.5
peak 3	28.0	–[b]
peak 4	28.0	–
peaks 5 and 6	34.4/32.1	–
peak 7	34.2	–
delphinidin	23.8	
cyanidin	28.2	
petunidin	31.4	
pelargonidin	31.9	
peonidin	34.2	
malvidin	35.5	
caffeic acid		8.9
p-coumaric acid		13.6

[a] Peak numbers refer to Figure 1. [b] – denotes not detected. [c] Compounds found after alkaline hydrolysis of peaks 1 and 2.

homogenized material was filtered under vacuum, through two layers of Whatman No. 4 filter paper. Twenty of the 25 mL of filtrate was applied to an Amberlite CG-50 column (2 × 46 cm) which had been washed with 95% ethanol containing 1% formic acid and equilibrated with 1% formic acid in water. The loaded column was washed with 1000 mL of 1% formic acid in water, which eluted most of the major anthocyanins. The minor anthocyanins and some of the remaining major pigments were eluted by washing the column with 1% formic acid in ethanol. The pigments that eluted as a dark band (total 7 mL) were concentrated by flushing the eluate with nitrogen to remove about half of the ethanol. The resulting solution was diluted with an equal volume of water, filtered through a 0.45 μm hydrophilic Durapore filter, and injected on the RP C_{18} HPLC column for separation of minor and major anthocyanins. The fractions collected from the HPLC of both the raw extract (for colorless phenolics) and the anthocyanin concentrate from the open column chromatography were dried by a stream of nitrogen and used for further characterization.

Table 2. GC Identification of Sugars and Acids from Anthocyanins and Other Phenolics in Sweet Cherry

anthocyanin/ standard	sugars, TMS-derivatized (min)	acid, methylated (min)
peak 1[a]	–[b]	10.56
peak 2	–	10.56
peak 3	11.03/12.74	–[b]
peak 4	11.02/12.73; 6.93/8.10	–
peaks 5 and 6	11.04/12.76; 6.94/8.12	–
peak 7	11.05/12.76; 6.94/8.12	–
glucose	11.04/12.75	
galactose	10.32/11.54	
rhamnose	6.94/8.12	
xylose	8.35/9.03	
arabinose	6.70/6.95/7.46/7.69	
quinic acid		10.56
succinic acid		10.19

[a] Peak numbers refer to Figure 1. [b] – denotes not detected.

Spectral and Compositional Analysis. Spectral characteristics of individual anthocyanins and colorless phenolics were determined as described by Markham (1982), and the composition of individual anthocyanins and other phenolics was determined by RP-HPLC and capillary GLC as described by Gao and Mazza (1994b).

HPLC and Calibration Curves. The HPLC was a Waters 990 system (Waters Chromatography, Milford, MA) equipped with a photodiode array detector and a SuperPac Pep-S column (5 μm, 4 × 250 mm; guard column, 4 × 10 mm; Pharmacia, Uppsala, Sweden), which was thermostated (26.0 ± 0.1 °C) in a temperature-controlled system. The following solvent system and elution profiles were used: solvent A, formic acid in water (5:95 v/v), solvent B, methanol; elution profile 30% B (initial), 35% B (8 min), 40% B (8.5 min), 46% B (20 min), 60% B (30 min), 85% B (30.5–34.5 min), and 30% B (35 min). The solvent flow rate was 1.0 mL/min, and all gradients were linear.

The concentration of anthocyanins and colorless phenolics was determined from standard curves prepared by injecting different volumes of stock solutions of standards. Cyanidin 3-glucoside and chlorogenic acid were used as standard compounds.

GLC of Sugars and Quinic Acid from Collected Fractions. The anthocyanin and the two major UV-absorbing peaks (Figure 1) collected from HPLC of Bing cherries were analyzed for sugars and acids by capillary gas–liquid chromatography (GLC) after derivatization, as described elsewhere (Gao and Mazza, 1994b).

RESULTS AND DISCUSSION

HPLC analyses of a methanolic extract of Bing cherries (Figure 1) revealed the presence of five anthocyanin peaks, two major ones, eluting at about 18 and 20 min, and three minor ones, eluting from 22 to 26 min (Figure 1, bottom). The other cultivars showed the same anthocyanin profile, and the variation in the proportion of anthocyanin peak areas was relatively small.

Characterization of Colorless Phenolics. The peak areas of the two colorless phenolics (peaks 1 and 2 in Figure 1) varied greatly among the cherry genotypes examined. These compounds were considered important for their possible contribution to the visual color of the cherries through copigmentation (Asen et al., 1972; Mazza and Brouillard, 1990) with anthocyanins.

The spectral data showed that peaks 1 and 2 (the colorless phenolic compounds) were similar to caffeic and *p*-coumaric acids, but their HPLC retention times were different from those of the standards. However, upon alkaline hydrolysis (Table 1), peak 1 yielded caffeic acid (t_R = 8.9) and another two relatively minor peaks (t_R = 8.1 and 10.5 min, respectively). Both of the two

Table 3. Spectral Characteristics of Anthocyanins and Associated Phenolics from Cv. Bing Sweet Cherry

anthocyanin/phenolic acid	t_R (min)	peak area (%) 280 nm	peak area (%) 525 nm	λ_{max} (nm)	$E_{440nm}/E_{vis\ max}$ (%)	$E_{uv\ max}/E_{vis\ max}$ (%)
peak 1[a]	4.7	20.3		325, 305sh[c]		
peak 2	6.3	6.1		312, 300sh		
peak 3	18.1	9.2	14.0	518, 280	31.4	67.5
peak 4	20.7	52.7	79.7	519, 280	31.8	68.1
peak 5	22.5	tr[d]	0.3	517, 280	35.5	80.1
peak 6	23.2	1.2	1.2	508, 280	41.2	77.6
peak 7	25.8	2.8	4.2	519, 280	31.3	68.9
cyanidin 3-glu[b]	18.0			518, 280	31.8	68.7
petunidin 3-glu	20.7			527, 278	29.1	61.3
peonidin 3-glu	22.4			517, 280	33.0	70.5
pelargonidin 3-rut[b]	23.3			506, 278	44.2	79.9
malvidin 3-glu	24.9			528, 279	27.4	62.2
delphinidin 3-glu	31.3			528, 280	27.5	59.9
caffeic acid	8.9			324, 305sh		
chlorogenic acid	10.5			325, 305sh		
p-coumaric acid	13.6			310, 300sh		

[a] Peak numbers refer to the peaks in Figure 1. [b] Abbreviations: glu, glucoside; rut, rutinoside. [c] sh denotes a shoulder of the major peak. [d] Trace.

Table 4. Contents[a] (Milligrams per 100 g of Flesh Weight) of Colorless Phenolic Compounds and Anthocyanins in 11 Cultivars and Hybrid Lines of Sweet Cherries

	phenolic acids		anthocyanins				
cultivar	neochlorogenic acid	*p*-coumarylquinic acid	cyanidin 3-glucoside	cyanidin 3-rutinoside	peonidin 3-glucoside	pelargonidin 3-rutinoside	peonidin 3-rutinoside
Bing	128.16 ± 1.25	42.67 ± 3.22	31.06 ± 0.49	180.57 ± 0.24	0.76 ± 0.08	3.11 ± 0.21	9.17 ± 0.18
Lambert	118.89 ± 1.35	46.99 ± 2.23	44.10 ± 0.52	150.50 ± 1.25	0.69 ± 0.03	0.71 ± 0.09	2.41 ± 0.03
Sam	38.40 ± 2.04	131.45 ± 6.01	24.82 ± 0.22	193.33 ± 0.42	0.56 ± 0.05	1.87 ± 0.02	6.46 ± 0.30
Stella	92.92 ± 1.03	32.54 ± 2.02	18.25 ± 0.81	128.70 ± 2.30	0.30 ± 0.01	0.67 ± 0.01	6.27 ± 0.06
Summit	28.96 ± 0.49	84.00 ± 0.72	6.32 ± 0.12	72.16 ± 2.79	0.25 ± 0.01	0.59 ± 0.00	2.63 ± 0.22
Sylvia	34.21 ± 0.29	76.34 ± 2.35	15.30 ± 1.06	211.40 ± 2.09	0.57 ± 0.01	2.14 ± 0.10	15.8 ± 0.17
Van	87.54 ± 1.14	23.02 ± 0.32	11.95 ± 0.60	130.25 ± 3.25	0.50 ± 0.05	0.69 ± 0.13	7.37 ± 0.22
2C-61-22	23.84 ± 0.58	64.83 ± 2.73	42.73 ± 0.11	174.76 ± 1.16	0.59 ± 0.01	1.64 ± 0.06	12.20 ± 0.30
13N-7-70	66.86 ± 1.30	126.08 ± 0.44	tr[b]	1.57 ± 0.02	ND[c]	ND	ND
13S-10-40	31.75 ± 3.84	108.44 ± 4.02	31.92 ± 0.57	243.90 ± 3.67	1.13 ± 0.07	3.91 ± 0.04	16.60 ± 0.65
13S-39-51	49.53 ± 1.48	94.24 ± 0.67	4.22 ± 0.07	34.49 ± 0.50	ND	0.27 ± 0.01	1.74 ± 0.10

[a] Mean and standard deviation of five replicates. [b] Trace. [c] Not detected.

minor peaks had UV spectra identical to that of the original peak, and one of these peaks had a retention time corresponding to that of chlorogenic acid. It has been shown that hydroxycinnamoylquinic acid can undergo isomerization reactions under alkaline conditions and the caffeoylquinic acid isomers elute on reversed-phase columns (C_{18}) in the order of 3′-, 4′-, and 5′-quinoylcaffeic acids (Nagels et al., 1980). Therefore, peak 1 is likely neochlorogenic acid.

Analysis by gas chromatography revealed that the colorless phenolic compounds both contained quinic acid, and no sugars were found in any of the compounds (Table 2). The two phenolic compounds are therefore neochlorogenic acid and *p*-coumaroylquinic acid, respectively. Möller and Herrmann (1983) examined three cultivars of sweet cherries and found that the major hydroxycinnamoylquinic acids were neochlorogenic acid and 3′-*p*-coumaroylquinic acid. The coumaroylquinic acid identified in this study is, therefore, likely 3′-*p*-coumaroylquinic acid.

Characterization of Anthocyanins. The anthocyanins represented by peaks 3, 4, and 7 were collected as separate fractions from the HPLC column, hydrolyzed in methanol/2 N HCl (1:1 v/v), and subjected to HPLC/GC analyses. The anthocyanins represented by peaks 5 and 6 did not resolve properly and were collected as one fraction and subjected to hydrolysis and HPLC/GC analysis.

In Table 1 are the HPLC retention times for the aglycons produced upon acid hydrolysis of the collected anthocyanin fractions and the retention times for the phenolic acids from the alkaline hydrolysis of the colorless phenolic compounds. It can be seen that the first two eluting anthocyanins contained cyanidin as the aglycon, and peaks 5 and 7 contained peonidin as the aglycon. On the basis of retention time, the aglycon of peak 6 is likely pelargonidin. One of the two aglycons from the fraction containing peaks 5 and 6 had a UV–vis spectrum identical to that of pelargonidin standard and the other to peonidin. Peak 6 probably is a pelargonidin-based anthocyanin. None of the anthocyanins were acylated because the spectra (data not shown) displayed no shoulder at 300–320 nm, which is an indication of phenolic acid acylation. Furthermore, alkaline hydrolysis caused no change in the retention time of the anthocyanins, and no phenolic acids (Table 1) or aliphatic acids (Table 2) were detected in the hydrolysates.

Further analysis by gas chromatography showed that the anthocyanin represented by peak 3 contained glucose as the only sugar and peaks 4–7 contained glucose and rhamnose as the sugars (Table 2). Spectral data (Table 3) showed that all of the anthocyanins were 3-glycosides as they all possessed a pronounced shoulder near 440 nm of the UV–vis spectrum, an indication of 3-glycoside structures (Harborne, 1967). Recently, Bakker et al. (1994) showed that pelargonidin 3-rutinoside is a common anthocyanin in strawberry but pelargonidin 3-glucoside is the major anthocyanin. The spectral and chromatographic data obtained from the analyses

of a strawberry extract showed that pelargonidin 3-rutinoside corresponded to peak 6 (Table 3). Pelargonidin 3-glucoside had a significantly shorter retention time (17.64 min) on the HPLC column which suggests that peak 6 is pelargonidin with rhamnose in its sugar moiety. The anthocyanin peaks 3–7 are, therefore, identified as cyanidin 3-glucoside, cyanidin 3-rutinoside, peonidin 3-glucoside, pelargonidin 3-rutinoside, and peonidin 3-rutinoside.

Our results are generally consistent with the results reported in the literature in that the sweet cherry has an anthocyanin composition of 3-glucoside and 3-rutinoside of cyanidin as the major anthocyanins and the same glycosides of peonidin as the minor anthocyanins (Lynn and Luh, 1964; Mazza and Miniati, 1993). However, this is the first report on the occurrence of pelargonidin 3-rutinoside in sweet cherries.

Since pelargonidin-based anthocyanins have a different hue as compared to cyanidin-based pigments, the presence of this anthocyanin may offer a possibility to modify the appearance of cherries and their products by changing the anthocyanin composition through breeding.

Quantitation and Distribution of Anthocyanins and Colorless Phenolic Compounds. The contents of all major and minor anthocyanins and the neochlorogenic and 3′-*p*-coumaroylquinic acid in the 11 genotypes of sweet cherries analyzed are presented in Table 4. The cherries used in this study were of comparable commercial maturity, and the significant differences in the levels of phenolics among cultivars are assumed to be due to factors other than maturity.

It is evident that the contents of the anthocyanins are relatively constant among the cultivars, and cyanidin 3-rutinoside is always the predominant anthocyanin in any given cultivar. However, the total contents of the pigments in the cherries varied widely from 82 to 298 mg/100 g for the dark-colored cherries and from a few to 41 mg/100 g for the light-colored cherries.

The contents of the two colorless compounds also varied widely. The ratio of neochlorogenic acid to 3′-coumaroylquinic acid was 3.8 in Van cherries, whereas it was only 0.29 in Sam, which represents a 13-fold difference. The lowest and highest levels of the two colorless phenolic compounds were found in two of the hybrid lines (88.7 and 193 mg/100 g of fleshy tissue in 2C-61-22 and 13N-7-70, respectively). Bing cherries also contained a high level (171 mg/100 g of flesh) of the two colorless phenolics while the cultivars Sylvia and Van contained a relatively low level (111 mg/100 g of flesh for both) of the compounds.

With the trend away from synthetic colors, natural pigments are actively sought to meet the demand of the consumers. Sweet cherries, especially the dark-colored genotypes examined in this study, are a rich source of anthocyanins and may represent a potential source of natural food colors.

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