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Procyanidin, Anthocyanin, and Chlorogenic Acid Contents of Highbush and Lowbush Blueberries

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ABSTRACT: The health benefits of blueberry consumption on the vascular system and brain are mediated in part by their flavonoid content. In light of this, six cultivated highbush blueberry varieties (*Vaccinium corymbosum* L.) and one lowbush or wild blueberry (*Vaccinium angustifolium* L.) were analyzed for their anthocyanin, flavanol oligomer, and chlorogenic acid contents. The highbush varieties Bluecrop, O'Neal, Bluejay, and Brigitta had significantly greater levels of anthocyanidins compared to the other varieties, whereas Bluejay and Brigitta organic had the highest amount of flavanol oligomers. The organically grown highbush blueberry had the highest flavanol oligomer and chlorogenic acid contents but a lower anthocyanidin content than its conventionally grown counterpart. The lowbush variety contained the highest chlorogenic acid concentration. Delphinidin and malvidin were the predominant anthocyanidins in the varieties tested, with concentrations ranging between 45.0 and 74.9 mg/100 g FW for delphinidin and between 37.1 and 62.2 mg/100 g FW for malvidin. Flavanol dimers were the most abundant flavanols, with a mean percentage of 24 ± 1.5% of the total, with flavanol monomers representing 11 ± 0.7%.

KEYWORDS: *blueberry, highbush, lowbush, lowbush, anthocyanins, procyanidins, chlorogenic acid, organic*

INTRODUCTION

Epidemiological studies have provided good evidence that foods rich in flavonoids are capable of reducing cardiovascular disease (CVD) incidence.[1] A number of human intervention studies have corroborated these observational studies by indicating that flavonoid-rich foods, such as cocoa, tea, and grape juice, may induce improvements in cardiovascular health.[2] Blueberries are a rich source of flavonoids, notably anthocyanins, flavanols, and hydroxycinnamates,[3−7] and thus may also be capable of exerting beneficial vascular effects. The consumption of blueberries has been reported to induce improvements in cognitive performance,[8] to prevent oxidative stress and inhibit inflammation,[9] and to improve vascular health.[10] These beneficial effects have been attributed to their relatively high flavonoid content, in particular, anthocyanins. Prospective studies indicate that the intake of anthocyanin-rich foods is associated with a reduced risk of CVD related mortality and that regular consumption of anthocyanins (predominately from blueberries and strawberries) leads to an 8% reduction in hypertension.[11]

In plants, anthocyanidins (aglycone form) are predominately attached to one or more sugars, conjugated via the C3 hydroxyl group in ring C (anthocyanin form).[12] At least 27 structurally different anthocyanins have been identified in blueberry, including 3-glucosides, 3-galactosides, and 3-arabinosides of five anthocyanidins: delphinidin, malvidin, petunidin, cyanidin, and peonidin.[13−15] Blueberries are also a rich source of flavanol oligomers, which are present in both the monomeric and oligomeric (procyanidin) forms.[5,6,16] The latter are polymers of (−)-epicatechin and (+)-catechin and are classified according to their degree of polymerization (DP), with DP = 1 indicating a monomer and those with DP = 2−10 and DP > 10 being referred to as oligomers and polymers, respectively.[5] The polymeric nature of the flavanols present in blueberry and other foods has made it difficult to accurately assess the total flavanol content, and as such the precise flavanol content of blueberry remains unclear. Blueberry is also rich in phenolic acids, in particular, esters, glycosides, and amides of hydroxycinnamic and hydroxybenzoic acids,[17] most prominently chlorogenic acid.[7,18−20] However, as with other flavonoid-containing foods, the flavonoid content of blueberry depends on several factors, including variety, environmental growing conditions, harvest conditions, and storage.[21] It has been reported that lowbush blueberry has a higher total flavonoid content than highbush blueberry,[18] although this is disputed.[22] One reason for this discrepancy relates to the fact that few studies have assessed the flavonoid content of both low- and highbush varieties using the same analytical platform, thus making it difficult to compare individual data sets. In addition, previous studies have focused primarily on the accurate assessment of anthocyanins in blueberry and have paid less attention to the flavanol and polyphenol contents generally. To address this discrepancy, we analyzed the major flavonoids (flavanols, anthocyanins) and chlorogenic acid content of various commercial highbush varieties and one lowbush variety. We also investigated the polyphenol content of an organically grown variety versus a conventionally grown blueberry variety.

MATERIALS AND METHODS

Chemicals and Reagents. Pure standards of chlorogenic acid (5-*O*-caffeoyl quinic acid) and (−)-epicatechin were purchased from Sigma Chemical Co. (Poole, U.K.), whereas malvidin chloride,

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Table 1. Linearity, LOD, LOQ, and Validation Parameters of the Methods Used for the Quantification of Anthocyanidins, Chlorogenic Acid, and Flavanol Oligomers in Different Blueberry Varieties

					RSD (%)	
compound	calibration range	RSQ	LOD	LOQ	repeatability	intermediate precision
			Flavanols[a]			
monomers	0.012−3	0.999	0.01	0.02	1.8	7.4
dimers	0.012−3	0.999	0.01	0.02	8.8	13.0
trimers	0.012−3	0.999	0.02	0.06	8.9	13.4
tetramers	0.012−3	0.996	0.13	0.44	3.3	10.2
pentamers	0.012−3	0.998	0.10	0.33	9.7	8.2
hexamers	0.012−3	0.998	0.05	0.16	2.0	10.9
heptamers	0.012−3	0.998	0.07	0.24	9.2	11.9
octamers	0.012−3	0.997	0.10	0.35	5.3	10.5
nonamers	0.012−3	0.997	0.15	0.50	8.4	12.2
decamers	0.012−3	0.996	0.18	0.60	8.5	15.4
			Anthocyanidins[b]			
delphinidin	0.25−100	0.999	0.05	0.16	2.6	12.1
cyanidin	0.25−100	0.999	0.05	0.18	2.6	12.2
petunidin	0.25−100	0.999	0.06	0.21	2.9	13.4
peonidin	0.25−100	0.999	0.06	0.21	5.5	8.4
malvidin	0.25−100	0.999	0.06	0.19	3.2	9.7
			Phenolic Acids[b]			
chlorogenic acid	6.25−100	0.999	0.05	0.16	3.0	3.7

[a]In units of mg/mL. [b]In units of μmol/L.

peonidin chloride, petunidin chloride, cyanidin chloride, delphinidin chloride, pelargonidin chloride, malvidin-3-*O*-glucoside chloride, peonidin-3-*O*-glucoside chloride, cyanidin-3-*O*-glucoside chloride, delphinidin-3-*O*-glucoside chloride, and pelargonidin-3-*O*-glucoside chloride were purchased from Extrasynthese (Genay, France). Flavanol oligomer standards (DP1−DP10) isolated from cocoa were obtained from Mars Inc. (Hackettstown, NJ). HPLC-grade hexane, acetone, acetic acid glacial, acetonitrile, methanol, water, and hydrochloric acid were purchased from Fischer Scientific (Loughborough, U.K.). Solid phase extraction (SPE) cartridges were obtained from Phenomenex (Torrance, CA).

Blueberries. Six highbush blueberry (*Vaccinium corymbosum* L.) varieties (Agropaine, Brigitta organic, Brigitta, Bluejay, O'Neal, and Bluecrop) were purchased from supermarkets in Reading (Berkshire, U.K.). Blueberries were frozen at −20 °C, freeze-dried (−25 °C; 6 mbar vacuum) in an Edwards MFD 01 freeze-dryer (Edwards, Crawley, U.K.), and ground to a fine powder using an Apex comminuting mill (Apex Processing Technology, Dorset, U.K.). The lowbush blueberry variety (*Vaccinium angustifolium* L.) was obtained from the Wild Blueberry Association of North America as a freeze-dried powder prepared under similar conditions. All freeze-dried material was stored at −20 °C for 1−3 months prior to analysis.

Extraction of Anthocyanins and Chlorogenic Acid. Freeze-dried blueberry powder (0.2 g) was extracted three times with 5 mL of acidified methanol (0.1% HCl in MeOH). Pelargonidin-3-glucoside was added to the samples as a recovery standard (300 μL, 1 mM). Samples were vortexed for 5 min, sonicated for 5 min in an ultrasonic bath (Fisher Scientific), and centrifuged at room temperature for 15 min at 1800*g*. The supernatants were combined (final volume = 15 mL). A 50 μL aliquot of the combined supernatants was filtered and injected into the HPLC for chlorogenic acid analysis, and 3 mL was mixed with 3 mL of 5 M HCl and heated at 90 °C for 1 h for anthocyanin hydrolysis in order to cleave all anthocyanins to anthocyanidins. A 50 μL aliquot of filtered sample was injected into the HPLC for analysis. All samples were filtered through a 0.22 μm filter prior to transfer to HPLC amber vials. Blueberry samples from all varieties were also extracted and analyzed without recovery standard to ensure that no pelargonidin was present originally in the different varieties. Anthocyanidin standards were also injected to ensure the hydrolysis was complete.

HPLC-DAD Analysis of Anthocyanidins and Chlorogenic Acid. Samples were analyzed for anthocyanidin and chlorogenic acid contents using an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector. The column was a Nova-Pak C_{18} column (250 × 4.6 mm; 4 μm particle size; 30 °C; Waters Ltd., Elstree, U.K.). The mobile phase consisted of (A) methanol/water/5 M HCl (5:94.9:0.1, v/v/v) and (B) acetonitrile/water/5 M HCl (50:49.9:0.1, v/v/v) and was pumped through the system using the following gradient profile (min/% mobile phase B): 0/5, 5/5, 40/50, 55/100, 59.9/100, and 60/5, with a flow rate of 0.7 mL/min. The eluent was monitored by photodiode array detection at 320 nm (chlorogenic acid) and 520 nm (anthocyanidins) with spectra of products obtained between 220 and 600 nm. Calibration curves were obtained using authentic standards.

Extraction of Flavanols. The extraction of flavanol oligomers was performed as previously described with minor modification.[23,24] Freeze-dried blueberry powder (0.5 g) was extracted with 5 mL of acetone/water/acetic acid (70:29.5:0.5, v/v/v). Tubes were vortexed for 2 min, sonicated at 50 °C for 5 min, and vortexed again for 1 min. Samples were then centrifuged for 5 min at 2567*g* at room temperature, and the supernatant was retained and passed through a Strata SCX SPE cartridge (55 μm, 70 Å, 500 mg/3 mL; Phenomenex). Conditioning of the SPE packing bed was performed using 5 mL of deionized water on a vacuum (0.2 bar) manifold (24-position, Phenomenex). After conditioning, 2 mL of the supernatant was loaded into the SPE cartridge, and approximately half of the loaded sample was discharged from the cartridge to ensure that the sample was not diluted with the water used to condition the cartridge. A 0.45 μm PTFE filter was placed at the end of the cartridge, and approximately 1 mL was filtered and transferred to an HPLC vial. Five microliters was injected in the HPLC.

HPLC-FLD Analysis of Flavanols. The separation of flavanol oligomers was performed as previously described[23] using an Agilent 1100 series HPLC system (Agilent Technologies) equipped with photodiode array and fluorescence detectors.[23,24] A Develosil Diol 100 column (250 × 4.6 mm; 100 Å pore size; 5 μm particle size; 35 °C) together with a Cyano guard column (4 × 3 mm) (Phenomenex) was used for separation. The mobile phase consisted of (A) acetonitrile/acetic acid (98:2, v/v) and (B) methanol/water/acetic acid (95:3:2, v/v/v), and the following gradient system was used (min/% mobile

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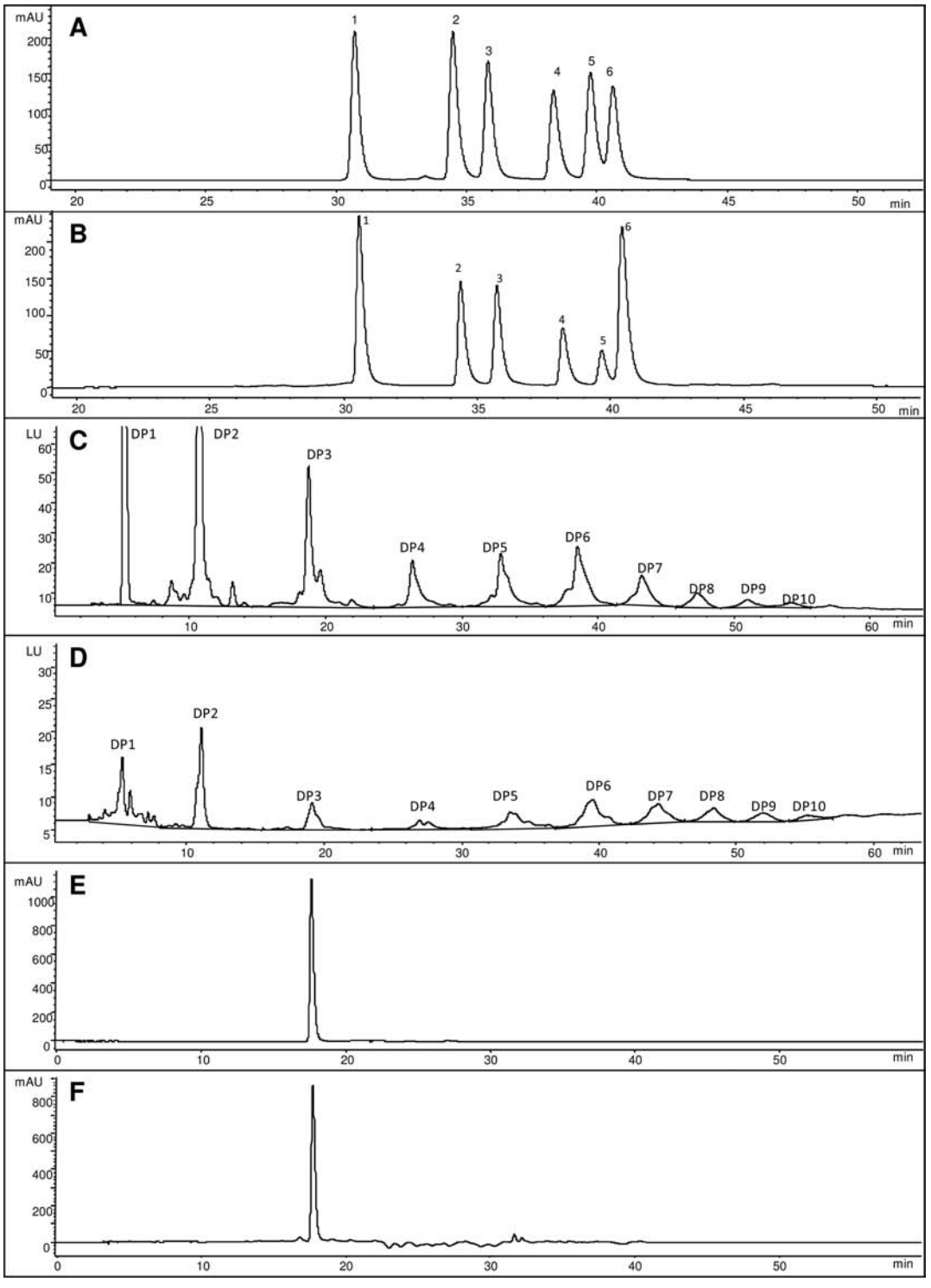

Figure 1. HPLC profiles of (A) anthocyanidin standards, (B) blueberry powder anthocyanidins after extraction with acidified methanol and acid hydrolysis, (C) flavanol oligomers standards, (D) blueberry powder flavanol oligomers, (E) chlorogenic acid standard, and (F) blueberry powder chlorogenic acid. The peaks in profiles A and B represent (1) delphinidin, (2) cyanidin, (3) petunidin, (4) pelargonidin (recovery standard, not present in blueberry), (5) peonidin, and (6) malvidin. The peaks in profiles C and D represent different degrees of polymerization (DP), from monomers (DP1) to decamers (DP10). The HPLC-UV detection wavelength was set at 520 nm for profiles A and B and at 320 nm for profiles E and F. The HPLC-FLD detection wavelength was 230 and 321 for excitation and emission, respectively, for profiles C and D.

phase B): 0/7, 3/7, 60/37.6, 63/100, 70/100, and 76/7, with a flow rate of 1 mL/min. UV data were collected at 280 nm, and fluorescence detection was conducted with an excitation wavelength of 230 nm and emission at 321 nm. Procyanidins were quantified using external calibration curves obtained using a mix of flavanol oligomers standards (DP1−DP10) isolated from cocoa (Mars, Inc.).[23,24] Flavanol polymers (DP > 10) were not quantified.

Method Validation. For each analyte, standard curves were constructed using peak area data, and linear least-squares regression was utilized to calculate the slope, intercept, and correlation coefficient (r^2). Limits of detection (LODs) and limits of quantification (LOQs)

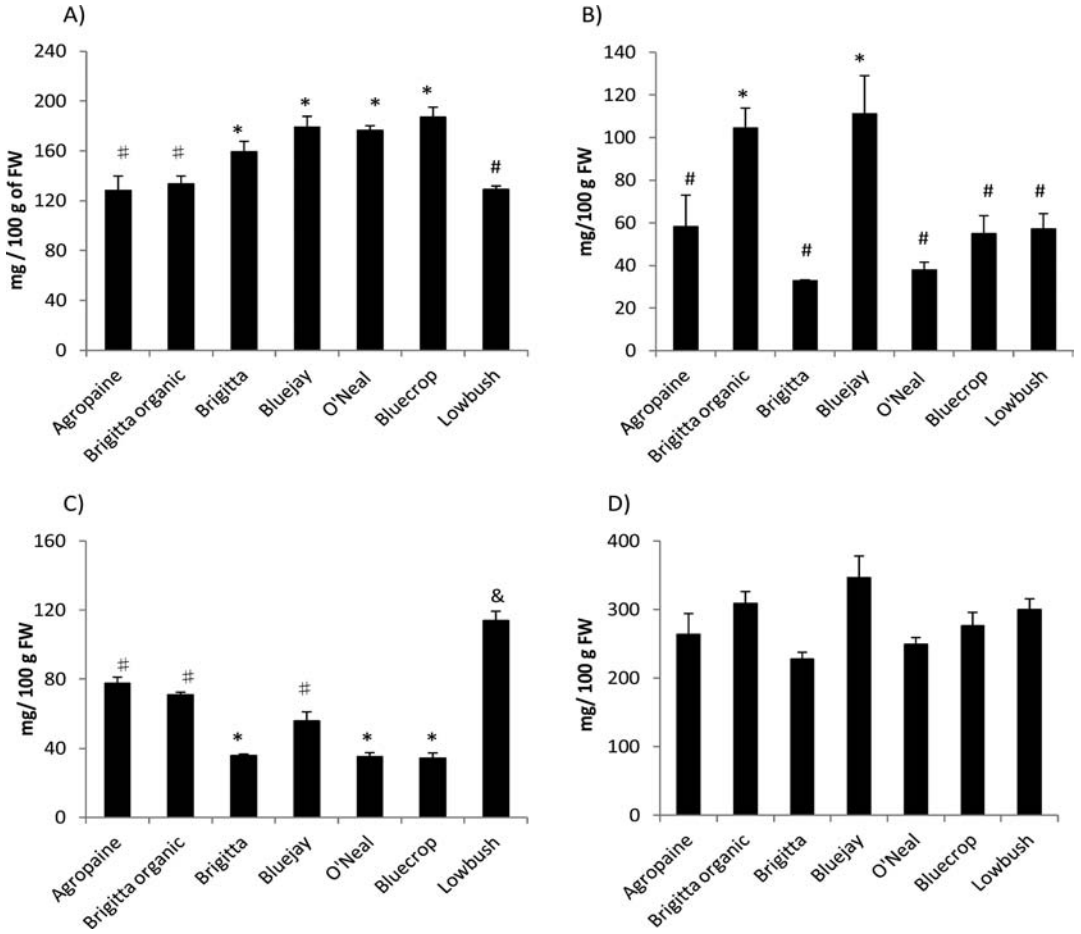

Figure 2. Comparison of flavonoid content determined by HPLC for six highbush and one lowbush blueberry varieties: (A) total anthocyanidin content; (B) flavanol oligomers content (from monomers to decamers); (C) chlorogenic acid; (D) total amount of anthocyanidins, procyanidins, and chlorogenic acid. Data are expressed as the mean ± SEM (n = 3). Symbols *, #, and & indicate significant difference (p < 0.05).

were estimated by obtaining the average height at the appropriate retention time for each compound on blank runs (n = 6), and the average height was converted to a concentration (employing calibration curves). LODs and LOQs were determined by multiplying the concentration by 3 and 10, respectively. Precision studies were carried out from the evaluation of the retention times and within-day repeatability and intermediate precision.

Statistical Analysis. All results are expressed as the mean ± SD of three replicates. The statistical evaluation of the results was performed by one-way analysis of variance (ANOVA) followed by Tukey's posthoc test using SPSS version 18 (SPSS Inc., Chicago, IL). Significance was defined as p < 0.05.

RESULTS AND DISCUSSION

Prior to sample analysis, we undertook a full validation of our analytical platform. Linearity, LOD, LOQ, and precision were calculated, and validation parameters are summarized in Table 1. Detection limits ranged from 0.01 to 0.18 mg/mL for flavanol oligomers and around 0.05 μM for anthocyanidins and chlorogenic acid. The quantification limits ranged from 0.02 to 0.6 mg/mL for flavanol oligomers and from 0.16 to 0.21 μM for anthocyanidins and chlorogenic acid. Standard curve linearity was found to be excellent (r^2 > 0.996 for flavanol oligomers and r^2 > 0.999 for anthocyanidins and chlorogenic acid). The relative standard deviation (RSD) values obtained for the retention times were <3% for all compounds, and intra- and interday precision values were lower than 9.7 and 15.4%, respectively (Table 1). Recoveries, calculated on the basis of the recovery of pelargonidin-3-glucoside, were found to be between 75 and 93% (mean = 81.52 ± 3.80%). Assessment of acid hydrolysis with authentic standards resulted in a yield of anthocyanin cleavage of between 97 and 98% (data not shown). This is in agreement with previous studies in which the acid hydrolysis of anthocyanins had been performed in similar conditions (2.5 M HCl and 90 min).[25,26] In the case of flavanol oligomers, no recovery experiments were performed as previously reported recoveries with this method were found to be between 91 and 93%.[23]

The measurement of anthocyanins in foods and biological fluids is affected by their relative instability at neutral pH and by the fact that commercially available anthocyanin standards are lacking. To resolve this, we used acid hydrolysis both to cleave the anthocyanins present into their six corresponding anthocyanidins and to stabilize the resulting compounds. Following hydrolysis, five anthocyanidins (delphinidin, RT (min) = 30.6; cyanidin RT = 34.4; peonidin RT = 39.7; petunidin RT = 35.7; and malvidin RT = 40.4) were identified via retention time and spectral comparison with authentic standards (Figure 1A,B). Anthocyanidin levels ranged between 128.1 and 187.3 mg total anthocyanidins/100 g of FW (Figure 2A), which are in range of those previously reported in the USDA database for the flavonoid content of selected foods.[27] The highbush varieties Bluecrop, Bluejay, O'Neal, and Brigitta had a significantly higher concentration of total anthocyanidins than Brigitta organic, Agropaine, and lowbush (p < 0.05)

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Table 2. Comparison of the Percentages of Anthocyanidins and Flavanol Oligomers Present in the Different Blueberry Varieties Analyzed: Agropaine, Brigitta, Brigitta Organic, Bluejay, O'Neal, Bluecrop, and Lowbush Blueberry

	Agropaine	Brigitta organic	Brigitta	Bluejay	O'Neal	Bluecrop	av highbush	lowbush	av all
				Anthocyanidins (%)					
delphinidin	35.1	39.3	43.1	37.4	42.4	38.8	39.3	36.4	38.9
cyanidin	15.8	9.8	7.6	6.8	7.7	9.0	9.5	13.1	10.0
petunidin	16.7	18.6	19.6	19.4	19.5	19.4	18.9	19.8	19.0
peonidin	3.4	1.5	1.3	1.5	1.3	2.0	1.8	4.7	2.2
malvidin	29.0	30.8	28.3	34.9	29.1	30.7	30.5	25.9	29.8
				Flavanols (%)					
monomers	10.8	11.9	10.3	8.7	8.4	10.6	10.1	17.5	11.2
dimers	22.4	32.1	18.0	28.9	20.1	24.1	24.3	22.6	24.0
trimers	8.9	9.7	7.6	9.8	11.2	10.4	9.6	13.6	10.2
tetramers	2.8	3.4	2.1	2.2	2.6	2.7	2.6	12.5	4.0
pentamers	8.4	5.1	4.4	5.5	7.4	8.4	6.5	8.4	6.8
hexamers	14.8	11.4	10.5	13.6	15.2	15.8	13.5	6.9	12.6
heptamers	11.1	9.1	16.6	11.2	12.3	11.1	11.9	5.9	11.0
octamers	7.9	5.9	10.7	8.3	7.9	6.9	7.9	5.3	7.6
nonamers	5.5	5.5	10.7	5.8	6.9	4.8	6.5	4.8	6.3
decamers	7.3	5.9	9.1	5.8	8.2	5.2	6.9	3.7	6.5

(Figure 2A). The profile of anthocyanins measured was also in agreement with previous papers,[13] with delphinidin being the most predominant anthocyanidin in all varieties (45.0 and 74.9 mg/100 g of FW), followed by malvidin (33.5−62.6 mg/100 g FW), petunidin (21.3−36.4 mg/100 g FW), cyanidin (12.1−20.3 mg/100 g FW), and peonidin (2.1−6.1 mg/100 g FW) (Figure 2A; Table 2). Varietal differences in anthocyanidin/anthocyanin levels will also depend on a number of factors in addition to genotype, including environmental factors, growing conditions, ripeness,[21] and even extraction and analytical methods used to analyze them.[21,28] For example, Bluecrop has previously been reported to contain between 42 and 184 mg anthocyanidins/100 g FW,[3,5,13,18−20,29,30] whereas in the present study we report an anthocyanidin content of 187.3/100 g FW. Furthermore, such variance may result in mixed conclusions regarding whether highbush or lowbush blueberries contain the highest levels of flavonoids. Prior et al. reported a lower anthocyanin content in lowbush blueberry relative to highbush blueberries,[22] although this is at odds with another study indicating the opposite.[18] In this work, the lowbush variety studied had levels similar to those of two highbush varieties and levels lower than those of four highbush varieties, so we can conclude only that the anthocyanin levels are similar between the highbush and lowbush varieties studied here.

All blueberry varieties were found to contain significant amounts of flavanol oligomers (Figures 1C,D and 2B). A considerable variation was observed among varieties with respect to the total flavanol content, ranging between 32.9 and 111.2 mg/100 g of FW (Figure 2B), with the Bluejay and Brigitta organic varieties containing the highest amounts (111.2 ± 17.8 and 104.4 ± 9.4 mg/100 g FW, respectively) and the conventionally grown Brigitta variety containing significantly lower levels of flavanol oligomers than its organic counterpart (p = 0.046) (Figure 2B). These data, including the flavanol oligomer levels of the lowbush blueberry (57.1 mg/100 g FW), were within the range of previously reported values.[28,31−33] Flavanol dimers represented the predominant flavanols present across all varieties, representing around 24% of the total flavanols measured (Table 2), with flavanol hexamers, monomers, and heptamers next with average percentages of 12.6, 11.2, and 11.0%, respectively (Table 2). Brigitta organic, lowbush, and Bluejay had the highest concentrations of flavanol monomers (12.4 ± 0.5, 10.0 ± 10.2, and 9.7 ± 1.6 mg/100 g FW, respectively), in line with previous resultss.[3,20,28,31,32] From these data we can conclude that flavanol oligomer levels in blueberry are similar in the highbush and lowbush varieties studied here, as the lowbush variety had total flavanol levels similar to those of four highbush varieties and levels lower than those of two highbush varieties.

In addition to the flavonoids, we also measured significant amounts of chlorogenic acid in all varieties, a hydroxycinnamate reported to be the most predominant phenolic acid in blueberry.[3,18,20] Chlorogenic acid has been avidly investigated for its health potential, with epidemiological studies indicating a correlation between chlorogenic acid-rich coffee consumption and reductions in Parkinson's and Alzheimer's diseases and diabetes.[34] A representative chromatogram of chlorogenic acid standard and of chlorogenic acid present in the blueberry extract is shown in Figure 1E,F. The levels of chlorogenic acid ranged between 34.3 and 113.8 mg/100 g FW (Figure 2C), indicating that blueberry is unlikely to deliver as much of this hydroxycinnamate as coffee, thought to provide 0.5−1 g of chlorogenic acid per day in regular coffee drinkers.[35] Our data are in agreement with previous studies, which also report significant variation in chlorogenic acid levels, notably even within the same variety, with Bluecrop ranging between 27.4 and 74 mg/100 g FW[3,19,20] and other highbush varieties ranging from 36.3 to 126.1 mg/100 g FW[20,33,36,37] and lowbush blueberry ranging between 58.9 and 109.96 mg/100 g FW.[18] As with flavanol oligomer levels, the organic Brigitta variety had a significantly higher level of chlorogenic acid compared to its conventionally grown equivalent, which agrees with reports that organic apples and strawberries contain higher levels of chlorogenic acid content than conventionally grown fruit.[38−40]

With regard to the total levels of polyphenols (flavonoids and chlorogenic acid), no significant differences were observed between varieties (Figure 2D). The lowbush blueberry had a total of 300.2 mg/100 g FW, compared to an average of 278.8 mg/100 g FW for highbush. Furthermore, organic practices appeared to influence both flavanol oligomers and chlorogenic acid content, although this was not the case for anthocyanins. There are limited and variable data regarding the impact of

organic versus integrated cultivation on the polyphenol content of fruits and vegetables, with some authors suggesting that levels are higher in organically grown products [39,41] and others reporting the opposite.[42,43] Polyphenols such as flavonoids and hydroxycinnamates are known to be involved in defense against pests, pathogens, and disease in plants, and as such it has been hypothesized that organic fruits may have higher phenolic content due to their greater exposure to these attacks in the absence of pesticides.[44] There is now excellent evidence to suggest that polyphenol-rich foods may exert beneficial health effects in humans. However, further randomized, controlled clinical trials are required before firm conclusions can be drawn with regard to their health benefits. For such clinical studies to be effective, it is essential that intervention diets be well characterized for both their polyphenol content and profile. In this study we provide further information with regard to the profile and overall content of polyphenols in blueberry, which we expect to better inform human clinical studies aimed at understanding the health benefits of this flavonoid-rich food.

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