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Efficient Quantification of the Health-Relevant Anthocyanin and Phenolic Acid Profiles in Commercial Cultivars and Breeding Selections of Blueberries (*Vaccinium* spp.)

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ABSTRACT: Anthocyanins and phenolic acids are major secondary metabolites in blueberry with important implications for human health maintenance. An improved protocol was developed for the accurate, efficient, and rapid comparative screening for large blueberry sample sets. Triplicates of six commercial cultivars and four breeding selections were analyzed using the new method. The compound recoveries ranged from 94.2 to 97.5 ± 5.3% when samples were spiked with commercial standards prior to extraction. Eighteen anthocyanins and 4 phenolic acids were quantified in frozen and freeze-dried fruits. Large variations for individual and total anthocyanins, ranging from 201.4 to 402.8 mg/100 g, were assayed in frozen fruits. The total phenolic acid content ranged from 23.6 to 61.7 mg/100 g in frozen fruits. Across all genotypes, freeze-drying resulted in minor reductions in anthocyanin concentration (3.9%) compared to anthocyanins in frozen fruits. However, phenolic acids increased by an average of 1.9-fold (±0.3) in the freeze-dried fruit. Different genotypes frequently had comparable overall levels of total anthocyanins and phenolic acids, but differed dramatically in individual profiles of compounds. Three of the genotypes contained markedly higher concentrations of delphinidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside, and malvidin 3-*O*-glucoside, which have previously been implicated as bioactive principles in this fruit. The implications of these findings for human health benefits are discussed.

KEYWORDS: *anthocyanins, chlorogenic acid, blueberry, HPLC-DAD-MS, Vaccinium spp., freeze-drying, health benefits*

INTRODUCTION

Commercially grown blueberries (Vacciniaceae family) comprise three predominant species, the lowbush or wild blueberry (*Vaccinium angustifolium* Aiton.), the highbush (northern and southern) (*Vaccinium corymbosum* L.), and rabbiteye (*Vaccinium ashei* Reade). Blueberries collectively are a major fruit commodity in the United States with a total annual production of 511 million pounds valued at U.S. $860.1 million in 2011.[1] The per capita annual consumption of blueberry in the United States has steeply risen through the past decade to a new record (from 0.6 lb in 2000 to 1.5 lb in 2010). This rise was mainly due to the increased public awareness of blueberries' impact on human health maintenance and studies that have shown an inverse association between concentration of phytoactives and incidence of human chronic diseases.[2−6]

Blueberry fruit contains several classes of bioactive phytochemicals including phenolic acids, anthocyanins, proanthocyanidins, stilbenes, and organic acids.[7−10] Blueberry is one of the highest sources for five major anthocyanidins, namely, cyanidin, delphinidin, malvidin, peonidin, and petunidin (Figure 1), among vegetables and fruits.[6,9] The major health benefits of anthocyanins are due to their cellular activity as free radical scavengers and interaction with biological systems. Anthocyanins have been demonstrated to be enzyme inhibiting, antibacterial, cardiovascular protective, and protective against other diseases such as diabetes, cancer, and Alzheimer's.[5,6,11−13]

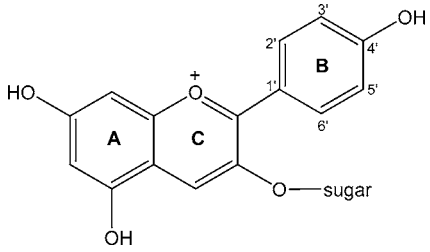

Figure 1. Molar mass and chemical structure for the anthocyanin aglycones (anthocyanidins) in blueberry fruits. Different combinations of the substituents on ring B and the conjugated sugar types (glucose, galactose, or arabinose) on ring C result in the formulation of different anthocyanin species in blueberry shown in Table 1.

Phenolic acids also contribute to these human health benefits as free radical scavengers, and chlorogenic acid, a major phenolic acid found in blueberry, was shown to slow the release of glucose into the bloodstream after meals.[14]

Human health benefits of anthocyanins are well documented; however, it is crucial to develop a method of extraction and

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accurate estimation of individual compound concentrations in berries to accurately determine their contributions to overall bioactivity. Routine handling of blueberry samples for analysis can affect the integrity of flavonoids, because they are sensitive and may be destabilized by laboratory procedures and variables such as temperature, pH, oxygen, and exposure to anthocyanin degradation enzymes.[15] To obtain a precise profile of anthocyanins that characterizes their native forms in situ, the analytical protocol must be simple, fast, gentle, accurate, and reproducible. In previous studies, depending on the extraction methods and solvents used, markedly different concentrations of anthocyanins have been reported for the same tissue type, and it was noted that prolonged extraction and/or harsh procedures resulted in significant anthocyanin degradation.[7,16]

Anthocyanin concentrations in blueberries have primarily been estimated using colorimetric methods such as pH differential, which gives only a total combined estimate. Various LC and LC-MS methods have also been reported; however, many of these methods have shown variable results with long HPLC run times of 70−90 min per sample[17−20] and have been unable to provide an accurate estimation and quantification of individual anthocyanins due to poor resolution for some of the anthocyanins. This can be attributed to anthocyanin sensitivity to acidity, long exposure during experimental procedures, and the oxidative enzymes responsible for the degradation of flavonoids in general.[16] Brownmiller et al.[21] reported a loss of monomeric anthocyanins and antioxidant capacity by up to 59% and 71%, respectively, due to enzymatic polymerization and/or degradation of anthocyanins in thermally treated berry fruits. Acid type and concentration used during analysis, for instance, can induce degradation via hydrolysis, obscuring a realistic profile of anthocyanins, and can also result in changes in individual peak response and the appearance of additional false peaks.[20]

Recently, private and public breeding programs have responded to the consumer demand for healthier fruits and vegetables and have recognized the need for development of extraction and analysis procedures that permit accurate, rapid and reliable comparative screening for a large number of samples.[22] In this work, we developed a novel extraction and analysis protocol which was simpler, faster, more efficient, and more accurate than previously described methods, which allowed us to simultaneously estimate individual anthocyanins and phenolic acids (from frozen or freeze-dried fruits) and comparatively screen large populations of different blueberry genotypes.

MATERIALS AND METHODS

Chemicals and Reagents. Commercial standards of cyanidin 3-*O*-glucoside, cyanidin 3-*O*-galactoside, cyanidin 3-*O*-arabinoside, and peonidin 3-*O*-galactoside were purchased from Chromadex (Irvine, CA, USA) and chlorogenic, caffeic, *p*-coumaric, and ferulic acids from Sigma (St. Louis, MO, USA). Glucosides of delphinidin and malvidin were purchased from Polyphenols Laboratories AS (Sandnes, Norway). Methanol, formic acid, acetic acid, and 0.1% formic acid in water or methanol (LC-MS) were purchased from Fisher Scientific (Pittsburgh, PA, USA). All solvents were of HPLC or HPLC-MS grades.

Plant Materials. Blueberry fruits of commercial cultivars and breeding selections were collected from Piedmont Research Station, Salisbury, North Carolina (NC), in the summer of 2009 when plants were 5 years of age, where each genotype was replicated three times (three individual plants). Commercial cultivars included Arlen, Legacy, Lenoir, O'Neal, Pamlico, and Sampson, and breeding selections (clones), developed by the NCSU blueberry breeding program, included NC4385, NC4900, SHF2B-1 21:3 and SHF2B-1 25:41. All blueberry plants received adequate rainfall with mulch covering the soil around plants year around. Approximately 500 g of fully ripe fruits was collected from each plant and immediately frozen on dry ice to eliminate postharvest degradation of fruit constituents. Fruits were consistently harvested at a uniform stage of maturity and from comparable locations on each plant to facilitate cross-comparisons. Frozen fruits were transported on dry ice to our adjacent laboratory in the Plants for Human Health Institute (PHHI), North Carolina Research Campus (NCRC), NC State University (NCSU), Kannapolis, NC. Frozen fruits from each plant were divided into two samples (~250 g each); the first sample was designated for direct frozen tissue extraction, whereas the second sample was freeze-dried before extraction. Samples were then stored at −80 °C until extraction as described below.

Extraction of Frozen Blueberries. Frozen fruits (20−22 g) were extracted with 30 mL of 0.3% acetic acid in MeOH using a high-speed grinder while completely frozen for 2 min. Ground contents were transferred into 50 mL tubes and vortexed for 2 s before centrifugation at 4000 rpm for 15 min at 20 °C, and then supernatants were decanted into 100 mL volumetric flasks. To the precipitated pellet was added 20 mL of 0.3% acetic acid, and the mixture was incubated for 15 min at room temperature with vortexing to mix contents every 5 min. Samples were centrifuged and supernatants were collected into flasks. The extraction was repeated for a third time collecting each substrate into the corresponding flasks, and the final volume was brought to a 100 mL with the same extraction solvent. Flasks containing supernatants were swirled briefly to mix the three extractions, and then two 1.5 mL samples were filtered into 2 mL amber HPLC vials using 0.2 μm PTFE syringe filters (Fisher Scientific). From each vial, a 10 μL volume was injected into the HPLC on the same day of extraction or within a week (stored at −20 °C) in the case of LCMS-IT-TOF mass spectrometer analysis, described under the HPLC analyses sections below.

Extraction of Freeze-Dried Blueberries. Frozen berries (−80 °C) were weighed prior to lyophilization using a VirTis 24Dx48 freeze-dryer (SP Scientific, Stone Ridge, NY, USA) with a temperature-controlled chamber for samples. The freeze-drying program started with −35 °C for 6 h, and then the sample temperature was raised every 20 h to −20 °C, −10 °C, 0 °C, 5 °C, 10 °C, and 20 °C for each temperature setting. After complete dryness, with no further moisture loss from samples, freeze-dried berries were weighed to estimate the moisture content and dry matter percent (DM%). The freeze-dried samples were then kept at −20 °C until extraction. Samples were extracted using the same extraction procedures described above for frozen tissues, except the fruit sample weight was 2.5−3.0 g and the extraction solvent was 0.3% acetic acid in MeOH/H_2O (70:30, v/v) to compensate for the water removed in the freeze-drying process and to improve extraction efficacy for anthocyanins and phenolic acids. From the three combined extracts, two 1.5 mL samples were filtered into amber HPLC vials using 0.2 μm PTFE filters, where 10 μL was injected into HPLC on the same day of extraction or within a week (stored at −20 °C) in the case of LCMS-IT-TOF mass spectrometer analysis.

Compound Recovery. To gauge the efficacy of the extraction method, 2 or 1 mg of the anthocyanin or phenolic acid commercial standards, respectively, was added to the ground tissue and solvent mix at the first extraction in both frozen and freeze-dried extraction procedures. This spiking was performed with 10 random samples, and 10 μL of each sample extract was injected into the HPLC-DAD instrument. Samples extracted without the addition of commercial standards were compared to the spiked samples, and recovery of standard compounds was estimated by examining the difference in compound concentration when spiked. For each compound, recovery formula was calculated as follows: (TCC − CC)/TCC × 100), where TCC is the total compound amount (μg) in spiked blueberry extract sample and CC is compound amount in blueberry sample extract (μg) without spiking.

HPLC-DAD Analysis. Filtered samples were injected (10 μL) into a 1200 HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a UV−vis diode array detector (DAD), controlled-temperature autosampler (4 °C), and column compartment (30 °C). Chemstation software (Agilent Technologies Inc.) was used as the system run controller and for data processing. Anthocyanin and phenolic acid separation was performed using a reversed-phase Supelcosil LC-18 column, 25 mm × 4.6 mm × 5 μm (Supelco, Bellefonte, PA, USA). The mobile phase consisted of 5% formic acid in H_2O (A) and 100% methanol (B). A step gradient of 10%, 15%, 20%, 25%, 30%, 60%, 60%, 10%, and 10% of solvent B at 0, 5, 15, 20, 25, 35, 36, 37, and 40 min, respectively, at a constant flow rate of 1 mL/min, was applied for samples and standards. Because there were a large number of compounds in blueberry fruits, and not all standards were available or were excessively expensive, the available standards were mainly used for HPLC and LC-MS identification and verification analysis. Individual and total anthocyanins in samples were quantified as cyanidin 3-*O*-glucoside equivalents. Seven concentrations of standards were prepared at 500, 250, 125, 62.5, 31.3, 15.6, and 7.8 μg/mL of 100% methanol, where 5 μL was injected before each sample set as external standards. Compounds were quantified using a standard curve calculated using peak areas at UV of 520 or 325 nm for anthocyanins or phenolic acids, respectively, and the known injected concentrations for standards.

HPLC-IT-TOF MS/MS Analysis. For compound verification, 2 μL of sample extract was injected into the HPLC MS-IT-TOF mass spectrometer (Shimadzu Scientific Institute, Columbia, MD, USA). The mass spectrometer was attached to a UFLC system (Shimadzu) equipped with photodiode array detector (PDA, flow cell 40 °C), controlled-temperature autosampler (10 °C), and column oven (40 °C). UV−vis spectra were acquired at a range of 200−600 nm. LCMS Solution software (Shimadzu) was used as a system controller for HPLC and mass spectrometer and for data analysis. Compounds were separated using a reversed-phase Shim-pack XR-ODS column, 50 mm × 3.0 mm i.d. × 2.2 μm (Shimadzu). For MS acquisition, gas flow of 1.5 L/min, detector voltage of 1.6 kV, and TOF area vacuum of 1.9e−002 Pa were applied. The mobile phase consisted of 0.1% formic acid in H_2O (A) and 0.1% formic acid in methanol (B). A step gradient of 0%, 30%, 60%, 90%, 0%, and 0% of solvent B at 0, 30, 45, 50, 51, and 60 min, respectively, at a constant flow rate of 0.35 mL/min was applied. Mass data were acquired at ranges of m/z 150 −1500 for original parent ions and m/z 100−1000 for resulting ion fragments, where mass data were further fragmented (MS/MS) to verify the parent ions for compound molecules in the blueberry samples.

Data Presentation and Statistical Analysis. Data are presented as milligrams per 100 g with the frozen and freeze-dried blueberry fruits. To investigate if the freeze-drying had any adverse effects on anthocyanin and phenolic acid profiles and concentrations, data from freeze-dried berries were converted back to obtain estimates of compounds on a frozen fruit basis. Analysis of variance (ANOVA) was performed using *PROC GLM* procedures to compare concentrations among cultivars using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA; 2009), and means were compared using the least significant difference (LSD) test at $P \leq 0.05$. Student's t test with two-tailed distribution was used to test if lyophilization had effects on individual and total compound concentrations in berries when freeze-dried ($P \leq 0.05$).

RESULTS AND DISCUSSION

Analysis Method and Compound Recovery. The extraction procedure and HPLC protocol developed in this study accomplished the simultaneous quantification of 18 anthocyanin and 4 phenolic acid compounds in blueberry fruits. A list of the anthocyanins and phenolic acids identified in frozen and freeze-dried blueberries and their average occurrences (percent) across 10 genotypes are presented in Table 1. The identification of anthocyanins and phenolic acids was verified using maximum absorption for UV−vis wavelength spectra, commercial standards when available, HPLC-ESI-MS/MS data, and comparison with published studies.[9,13,17,19,23,24] The HPLC-DAD chromatogram obtained at a wavelength of 520 nm demonstrating the separation of individual anthocyanins in blueberry frozen fruits is shown in Figure 2. A representative mass spectrum of LC-ESI-MS showing m/z for anthocyanins identified in the O'Neal cultivar is presented in Figure 3. The compound recovery for commercial standards ranged from 94.2 to 97.5 ± 5.3% when added at the first step of extraction. Retention times were validated for stability during chromatographic separation by spiking a subset of samples with known amounts of commercial standards prior to extraction. Using this extraction method, the average quantities of anthocyanin and phenolic acid compounds extracted from fruit tissues were 90.2 ± 3.3%, 8.4 ± 2.2%, and 2.7 ± 0.6% for the first, second, and third extractions, respectively. This has assured efficient and complete extraction of compounds in fruit tissues in much less time than previously reported by other investigators.[13,17,20,23,25] Compared to our previous HPLC protocols,[13,25] the compound separation in this method was shorter by 20 min per sample (from 60 min), using a standard HPLC system, which can significantly save on analysis time.

Across all genotypes, malvidin 3-*O*-galactoside (**11**), delphinidin 3-*O*-galactoside (**1**), malvidin 3-*O*-arabinoside (**13**), cyanidin 3-*O*-arabinoside (**6**), and delphinidin 3-*O*-arabinoside (**4**) constituted 69.3% of the total anthocyanins

Table 1. Anthocyanins and Phenolic Acids Identified in Frozen and Freeze-Dried Blueberries and Their Average Occurrence (Percent) in 10 Blueberry Genotypes[a]

no.[b]	compound name	t_R[c]	m/z[d]	%[e]
	anthocyanins			
1	delphinidin 3-*O*-galactoside	20.7	465/303	16.0
2	delphinidin 3-*O*-glucoside	22.4	465/303	3.4
3	cyanidin 3-*O*-galactoside	23.8	449/287	3.1
4	delphinidin 3-*O*-arabinoside	24.9	435/303	9.2
5	cyanidin 3-*O*-glucoside	25.7	449/287	1.0
6	cyanidin 3-*O*-arabinoside	26.9	419/287	9.0
7	petunidin 3-*O*-galactoside	27.7	479/317	1.3
8	petunidin 3-*O*-glucoside	28.3	479/317	3.5
9	peonidin 3-*O*-galactoside	29.4	463/301	1.5
10	petunidin 3-*O*-arabinoside	30.3	449/317	4.8
11	malvidin 3-*O*-galactoside	30.9	493/331	24.1
12	malvidin 3-*O*-glucoside	31.7	493/331	6.7
13	malvidin 3-*O*-arabinoside	32.7	463/331	11.0
14	delphenidin 3-*O*-(6″-acetyl)glucoside	33.9	507/303	1.6
15	cyanidin 3-*O*-(6″-acetyl)glucoside	34.7	491/287	0.5
16	malvidin 3-*O*-(6″-acetyl)galactoside	35.1	535/331	1.0
17	petunidin 3-*O*-(6″-acetyl)glucoside	35.7	521/317	0.4
18	malvidin 3-*O*-(6″-acetyl)glucoside	36.7	535/331	0.9
	phenolic acids			
	chlorogenic acid	11.6	355/303	96.5
	caffeic acid	12.9	181/−	1.2
	p-coumaric acid	19.2	164/−	1.4
	ferulic acid	23.6	194/−	0.9

[a]Blueberry genotypes included commercial cultivars (Arlen, Legacy, Lenoir, O'Neal, Pamlico, and Sampson) and breeding selections/clones (NC 4385, NC4900, SHF2B-1 21:3, and SHF2B-1 25:41) developed by North Carolina State University blueberry breeding program. [b]HPLC peak number identification for anthocyanins. [c]Retention time (minutes). [d]MS and MS/MS (m/z) masses recorded at positive ion mode. [e]Average ratio for individual anthocyanins and phenolic acids across all 10 genotypes.

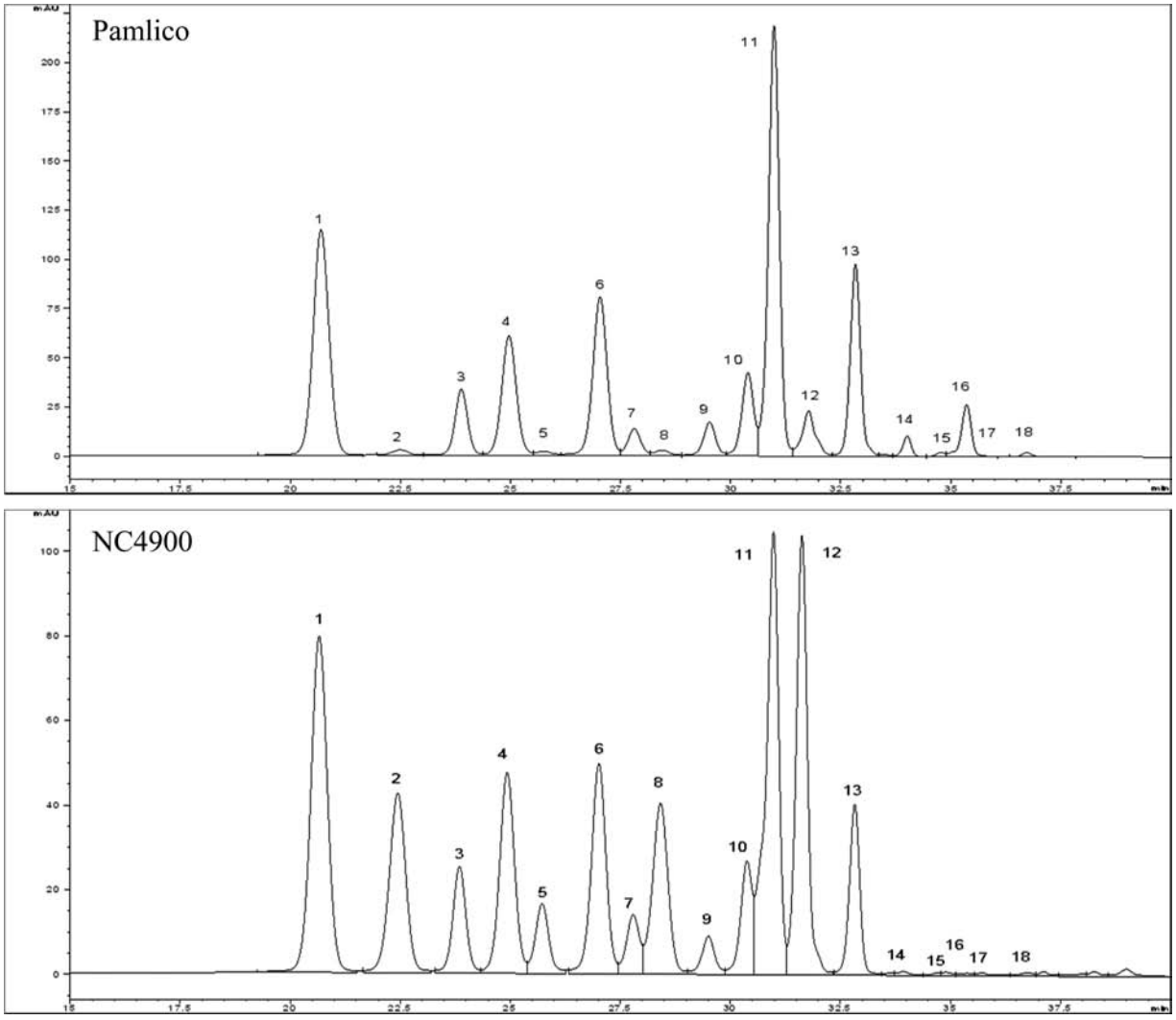

Figure 2. Representative HPLC-DAD chromatogram showing relative abundance of individual anthocyanins in the frozen fruits of Pamlico and NC4900 blueberries. Anthocyanin peak identification: 1, delphinidin 3-*O*-galactoside; 2, delphinidin 3-*O*-glucoside; 3, cyanidin 3-*O*-galactoside; 4, delphinidin 3-*O*-arabinoside; 5, cyanidin 3-*O*-glucoside; 6, cyanidin 3-*O*-arabinoside; 7, petunidin 3-*O*-galactoside; 8, petunidin 3-*O*-glucoside; 9, peonidin 3-*O*-galactoside; 10, petunidin 3-*O*-arabinoside; 11, malvidin 3-*O*-galactoside; 12, malvidin 3-*O*-glucoside; 13, malvidin 3-*O*-arabinoside; 14, delphenidin 3-*O*-(6″-acetyl)glucoside; 15, cyanidin 3-*O*-(6″-acetyl)glucoside; 16, malvidin 3-*O*-(6″-acetyl)galactoside; 17, petunidin 3-*O*-(6″-acetyl)glucoside; 18, malvidin 3-*O*-(6″-acetyl)glucoside.

where their proportions were 24.1%, 16.0% 10.9%, 9.0%, and 9.2%, respectively. These proportions were in agreement with previously published data,[17] where derivatives of malvidin and delphinidin accounted for the majority of anthocyanins in blueberry.[26] Chlorogenic acid was the predominant phenolic acid in all genotypes, constituting 96.5% of the total phenolic acid quantities found in frozen blueberry fruits. This was in agreement with previous work, which also reported that minor phenolic acids in berry constituted about 3% of the total phenolic acids.[27]

Most published work has relied on using pH differential or colorimetric methods, which can give variable results with a tendency to underestimate concentrations, and cannot discriminate between anthocyanin species.[28,29] We hypothesized that extended extraction and incubation times used in previous studies[17,19,20] were responsible for diminished anthocyanin yields. To test this hypothesis and to compare extraction efficiencies, we extracted three subsets of eight random samples at different incubation conditions including (1) extracts incubated for 2 h at room temperature, (2) extracts incubated overnight at 4 °C, and (3) extracts incubated overnight at 20 °C. Total anthocyanins were diminished by 15.3%, 18.8%, and 23.7% after extracts were incubated for 2 h, overnight at 4 °C, and overnight at 20 °C, respectively, compared to our new streamlined method (15 min incubation time). Although all possible precautions were taken while practicing this new method, a minor decrease in compound concentration can be expected due to sensitivity to extraction procedures.

Whereas previous studies have used abrasive reagents (hexane, acetone, or ethyl acetates and hydrochloric acid), only methanol and acetic acid (0.3%) were used in this study to stabilize anthocyanins during extraction. Cleaning procedures typically used in sample preparation and analysis such as fractionation and drying down were completely avoided in this streamlined method, which helped to avoid degradation due to temperature and loss of compounds during prolonged drying down. The extraction procedures in this study were simple and rapid, which eliminated excessive compound exposure to

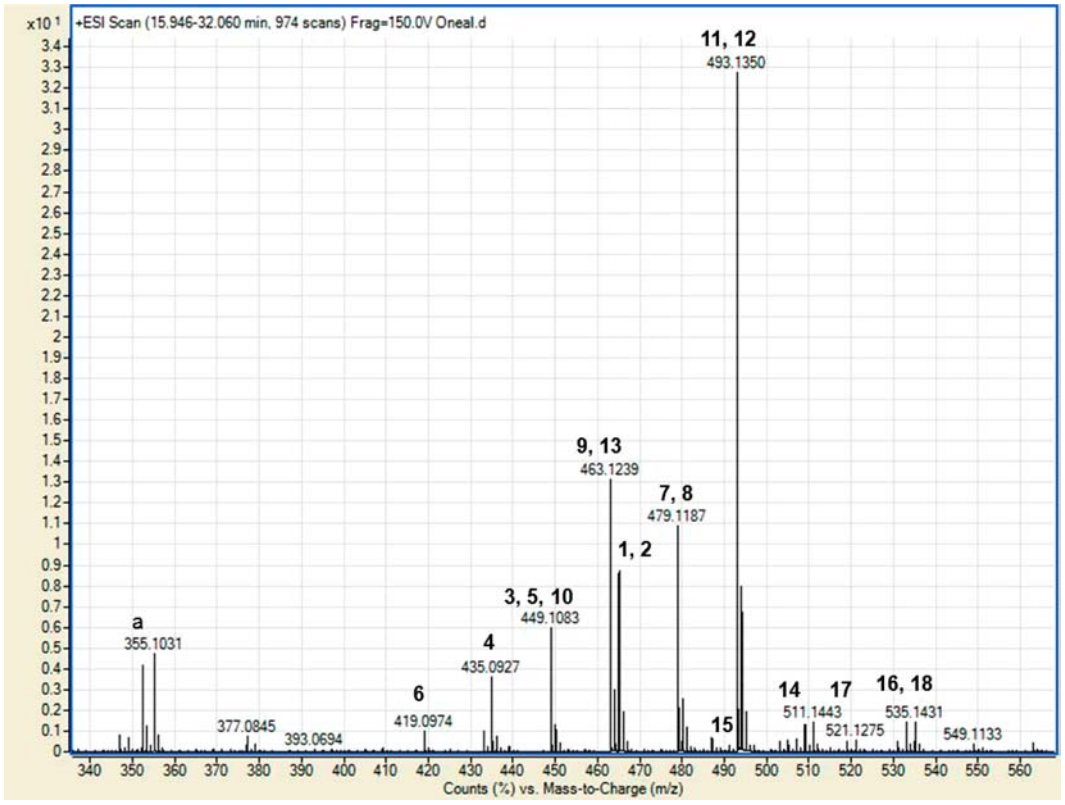

Figure 3. Representative HPLC-MS spectrum showing mass-to-charge ratio (m/z) for anthocyanins and chlorogenic acid in frozen fruits of O'Neal blueberry cultivar. Bold numbers refer to the 18 anthocyanin compounds listed in Table 1. Electrospray ionization scan (ESI) was produced by extracting total ion current (TIC) chromatogram for a range of 15−32 min, where all anthocyanins were eluted from the LC column using 6220 TOF LC/MS (Agilent Technologies, Inc.). Peak a, chlorogenic acid.

oxidative factors such as oxygen, temperature, pH, and internal oxidative enzymes.[16,30]

Variation in Anthocyanins and Phenolic Acids. The total anthocyanins in the frozen fruits of 10 genotypes ranged from 201.4 to 402.8 mg/100 g with a mean of 282.7 ± 58.3 mg/100 g (Table 2). This indicated that the highest yielding genotype for total anthocyanins (Pamlico) accumulated twice the anthocyanin concentration compared to the lowest accumulating genotype (Sampson). However, relative levels of individual anthocyanin species varied even more dramatically between genotypes. For example, quantities for delphinidin 3-*O*-glucoside (**2**), cyanidin 3-*O*-glucoside (**5**), petunidin 3-*O*-glucoside (**8**), and malvidin 3-*O*-glucoside (**12**) were up to 77-, 52-, 75-, and 33-fold higher in high-yielding genotypes compared to other genotypes. A similar trend was observed for phenolic acids estimated in this study. The total phenolic acids ranged from 23.6 to 61.7 mg/100 g, with a mean of 33.9 ± 9.2 mg/100 g in the frozen fruits (Table 2). The highest concentration of chlorogenic acid observed was 2.7-fold higher in NC4900 compared with the lowest inherent concentration (SHBF2B-1 25:41). These results agree with previous studies, where large variations in anthocyanins depending on genotype were observed.[23,24,26,32] The wide range in anthocyanin and phenolic acid concentrations observed in this study may have a direct bearing on the in vivo bioactivity and suggests a strong potential for selectively breeding blueberry for improved anthocyanin and phenolic acid content.

Individual and Total Anthocyanins and Phenolic Acids. The mean concentrations for individual and total anthocyanins estimated in the frozen fruits are presented in Table 3. The data showed significant differences among blueberry genotypes for individual and total anthocyanins in the frozen berries. Pamlico and O'Neal contained the highest concentrations of total anthocyanins among the commercial cultivars (383.9 mg/100 g and 357.0 mg/100 g, respectively), whereas Arlen, Legacy, Lenoir, and Sampson contained significantly lower concentrations (Table 3). For the breeding selections, NC4900 had a total anthocyanin concentration of 315.7 mg/100 g, significantly higher than SHF2B-1 25:41, but lower than Pamlico and O'Neal cultivars. In addition to the total anthocyanin variations, certain genotypes had significantly higher or lower concentrations of individual anthocyanin compounds. For instance, Pamlico contained the highest concentration of delphinidin 3-*O*-galactoside (**1**) (79.1 mg/100 g), whereas Lenoir had only 27.8 mg/100 g of this compound. For delphinidin 3-*O*-glucoside (**2**), one of the most variable compounds among genotypes, O'Neal had 36.8 mg/100 g followed by the breeding selections NC4900 and SHF2B-1 21:3 with 27.3 mg/100 g and 25.3 mg/100 g, respectively. Six of the genotypes accumulated low amounts of this compound, and levels were only 0.5 mg/100 g in Lenoir (Table 3). Similarly, for the rest of anthocyanins, data showed that individual anthocyanins significantly differed among genotypes (Table 3). Generally, the blueberry genotypes accumulated mostly glycosidic anthocyanins (compounds **1**−**13**, listed in Table 1), whereas the acylated anthocyanins (compounds **14**−**18**, listed in Table 1) constituted a small portion of the total anthocyanins. The SHF2B-1 21:3 breeding selection contained the highest content of the acylated anthocyanins, significantly higher than the rest of the genotypes, in particular for malvidin

Table 2. Descriptive Statistics and Ranges for Anthocyanins and Phenolic Acids in the Frozen Fruits of 10 Blueberry Genotypes

no.	compound name	mean ± SD[a]	range		CV[b]
			min	max	
	anthocyanins (mg/100 g)				
1	delphinidin 3-*O*-galactoside	45.2 ± 14.7	26.5	81.4	10.3
2	delphinidin 3-*O*-glucoside	9.7 ± 13.9	0.5	38.7	28.5
3	cyanidin 3-*O*-galactoside	8.7 ± 4.2	3.7	18.3	10.0
4	delphinidin 3-*O*-arabinoside	26.0 ± 6.1	15.8	39.2	9.1
5	cyanidin 3-*O*-glucoside	2.9 ± 3.6	0.2	10.5	24.0
6	cyanidin 3-*O*-arabinoside	27.9 ± 9.0	16.8	52.8	9.0
7	petunidin 3-*O*-galactoside	3.8 ± 2.6	1.0	8.7	22.6
8	petunidin 3-*O*-glucoside	10.0 ± 11.9	0.5	37.9	22.7
9	peonidin 3-*O*-galactoside	4.2 ± 1.6	2.7	8.8	9.5
10	petunidin 3-*O*-arabinoside	13.6 ± 2.9	9.4	21.3	7.2
11	malvidin 3-*O*-galactoside	68.1 ± 16.2	34.4	108.8	10.0
12	malvidin 3-*O*-glucoside	19.0 ± 25.6	2.6	86.1	18.5
13	malvidin 3-*O*-arabinoside	31.1 ± 9.3	14.4	47.3	9.1
14	delphenidin 3-*O*-(6″-acetyl)glucoside	4.5 ± 7.7	0.0	25.1	19.9
15	cyanidin 3-*O*-(6″-acetyl)glucoside	1.4 ± 2.1	0.0	7.5	75.9
16	malvidin 3-*O*-(6″-acetyl)galactoside	2.9 ± 3.4	0.1	9.1	8.3
17	petunidin 3-*O*-(6″-acetyl)glucoside	1.2 ± 2.4	0.0	10.2	69.7
18	malvidin 3-*O*-(6″-acetyl)glucoside	2.6 ± 5.0	0.1	21.4	64.6
phenolic acids (mg/100 g)					
	chlorogenic	32.7 ± 9.0	22.7	60.2	13.6
	caffeic	0.4 ± 0.3	0.1	1.4	45.6
	p-coumaric	0.5 ± 0.2	0.2	1.0	17.2
	ferulic	0.3 ± 0.1	0.2	0.6	28.1
fruit weight (g)[c]		1.9 ± 0.4	1.1	2.8	5.7
fruit diameter (mm)[d]		16.1 ± 1.7	12.5	19.0	8.3

[a]Mean and standard deviation (n = 10 genotypes). [b]Coefficient of variation (n = 10). [c]Average frozen fruit weight (n = 10). [d]Average frozen fruit width (n = 10), measured using a Westward digital caliper, 0 −150 mm (Fisher Scientific). Anthocyanin values are calculated as cyanidin 3-*O*-glucoside equivalents.

3-*O*-(6″-acetyl) glucoside (**18**) (16.2 mg/100 g). This was in agreement with previous studies, where acylated anthocyanins constituted a minor portion of the total anthocyanins depending on the genotypes.[7,9,19,23]

Genotypes included in this study showed variable capacity in accumulating phenolic acids (Table 4). The breeding selection NC4900 contained the highest concentration for total phenolic acids (53.2 mg/100 g). Commercial cultivars contained different but not statistically significant concentrations of chlorogenic acid, ranging from 27.6 mg/100 g (Arlen) to 34.9 mg/100 g (Sampson), whereas the breeding selection NC4900 had significantly higher concentration (51.8 mg/100 g). In addition to chlorogenic acid, all genotypes accumulated minor amounts of the caffeic, *p*-coumaric, and ferulic acids constituting 3.5% of the total phenolic acids across all 10 genotypes (Table 4). Chlorogenic acid was reported as the major phenolic acid in blueberry species.[27,32]

Anthocyanins are primarily stored in the blueberry fruit skins; therefore, fruit size (weight and diameter) was a factor in the estimation due to different fruit surface area.[26] Legacy and Arlen had the largest fruit weight (2.4 g), whereas Pamlico fruit was the smallest (1.4 g). The fruit weight was positively correlated with fruit diameter (R = 0.92) (Table 4). Significant but negative correlations between total anthocyanins and both fruit weight and diameter were observed with R = −0.70 and −0.66, respectively. This was in agreement with previous studies;[26] however, in contrast, a report by Kalt et al.[7] on a survey of 22 high- and lowbush genotypes showed no relationship between anthocyanins and fruit size. Phenolic acid concentration did not show correlation with either fruit weight or diameter (R = −0.16 and 0.09, respectively), indicating that phenolic acids are primarily accumulated in the fruit flesh rather than the outer skins.

Anthocyanin content in blueberry can vary depending on genotype, maturity, and growing environment.[7,24,31,32] In a survey of nine blueberry cultivars, Conner et al.[32] reported a range of 105−236 mg/100 g in frozen fruit, with Legacy containing 183 mg/100 g. In contrast, the streamlined methodology presented here provided higher estimates (261.4 mg/100 g), most likely because of the shorter extraction and analysis time and reduced solvent/tissue ratios (1:5 as opposed to 1:3 in previous studies), which protected the anthocyanin integrity. Also, the use of HCl, a strong acid, during extraction can induce anthocyanin degradation via hydrolysis,[20] which was avoided in this new method. In another survey by Prior et al.[31] on 15 blueberry varieties grown in different locations in 1997, total anthocyanins ranged from 61.8 mg/100 g to 235.4 mg/100 g. The low estimates can be attributed to differences in cultivars/genotypes, differences in maturity, long transportation period before analysis, and the use of pH differential assay, which tends to underestimate concentrations.[28,29] Lower estimates of anthocyanins were reported by Gavrilova et al.[33] when a one-time extraction was applied with 1:2 of tissue/solvent ratio (i.e., 56.4 mg/100 g). Overall, it is evident that the experimental protocol has a significant influence on anthocyanin estimates and can

Table 3. Mean Concentrations of Individual and Total Anthocyanins Estimated in 10 Blueberry Genotypes Harvested in the Summer of 2009 in North Carolina

no.	Arlen	Legacy	Lenoir	O'Neal	Pamlico	Sampson	NC4385	NC4900	SHF2B-1 21:3	SHF2B-1 25:41	LSD α 0.05
anthocyanin (mg/100 g)[a]											
1	48.0 bc	42.8 cd	27.8 e	28.0 e	79.1 a	41.4 cd	52.7 b	52.4 b	36.5 d	42.3 cd	7.9
2	1.1 c	0.9 c	0.5 c	36.8 a	1.7 c	0.9 c	1.2 c	27.3 b	25.3 b	0.9 c	4.7
3	7.3 cd	5.8 e	3.7 f	7.0 de	18.2 a	6.4 de	12.0 b	12.5 b	8.6 c	6.2 de	1.5
4	31.0 b	20.4 d	19.4 d	19.3 d	38.3 a	21.9 d	26.3 c	28.0 bc	28.0 bc	27.8 bc	4.1
5	0.5 d	0.3 d	1.1 d	10.0 a	1.1 d	0.3 d	0.8 d	8.0 b	6.3 c	0.3 d	1.2
6	28.5 cd	30.5 bc	19.6 e	17.7 e	49.5 a	26.8 cd	32.9 b	28.2 cd	19.8 e	26.0 d	4.3
7	2.1 d	1.9 d	1.0 d	7.2 a	5.7 b	1.5 d	1.5 d	4.8 bc	3.8 c	8.1 a	1.5
8	1.1 e	1.0 e	0.5 e	36.1 a	3.7 de	2.9 e	7.3 d	24.2 b	18.8 c	2.4 de	3.8
9	3.5 def	4.0 cd	3.2 ef	4.2 c	8.1 a	3.3 ef	5.9 b	3.7 cde	2.9 f	3.7 cde	0.7
10	15.7 b	12.8 cd	12.9 cd	9.9 e	20.1 a	12.0 cd	13.2 c	11.5 de	12.6 cd	15.0 b	1.7
11	71.6 bc	81.1 b	65.4 cd	61.9 cde	97.3 a	59.7 de	78.1 b	52.1 ef	41.9 f	71.5 bc	11.7
12	3.6 d	3.0 d	4.6 d	81.0 a	8.6 d	3.7 d	4.3 d	46.0 b	32.1 c	3.3 d	6.0
13	37.7 b	31.2 c	45.1 a	23.7 d	39.1 b	23.5 d	29.7 c	16.0 e	24.2 d	40.4 ab	4.8
14	0.5 de	25.1 a	1.4 cde	2.4 c	2.8 c	1.7 cd	0.1 e	0.3 de	10.3 b	0.2 de	1.5
15	0.2 d	0.2 d	4.2 ab	2.6 bc	1.2 cd	0.4 d	0.0 d	0.1 d	5.5 a	0.2 d	1.9
16	0.3 e	0.2 e	8.7 a	3.4 c	8.6 a	0.8 d	0.5 de	0.1 e	5.7 b	0.3 e	0.4
17	0.1 c	0.1 c	0.0 c	1.9 b	0.0 c	1.7 b	0.0 c	0.1 c	7.6 a	0.1 c	1.4
18	0.1 d	0.1 d	0.3 d	3.8 b	0.8 cd	3.3 bc	0.3 d	0.5 cd	16.2 a	0.2 d	2.8
total anthocyanins (mg/100 g)[b]	252.9 de	261.4 d	219.3 ef	357.0 a	383.9 a	212.0 f	266.6 cd	315.7 b	306.0 bc	248.9 def	40.1
total anthocyanins, converted (mg/100 g)[c]	247.1 c	254.3 c	199.1 d	331.5 b	373.3 a	203.1 d	257.8 c	300.6 b	304.1 b	246.7 c	39.0

[a]Identification of individual anthocyanins **1**–**18** as listed in Tables 1 and 2. Means with different letters within rows are significantly different at $P \leq 0.05$. [b]Total anthocyanins (sum of individuals) in frozen fruits. [c]Total anthocyanins (sum of individuals) estimated in freeze-dried fruits, but values were converted back to frozen fruit basis. Values are calculated as cyanidin 3-*O*-glucoside equivalents.

Table 4. Mean Concentrations of Individual and Total Phenolic Acids, Fruit Weight, and Size and Dry Matter Content (DM%) Estimated in 10 Blueberry Genotypes Harvested in the Summer of 2009 in North Carolina

	Arlen	Legacy	Lenoir	O'Neal	Pamlico	Sampson	NC4385	NC4900	SHF2B-1 21:3	SHF2B-1 25:41	LSD α 0.05
phenolic acids (mg/100 g)[a]											
chlorogenic	27.6 bcd	34.6 bc	29.3 bcd	35.0 bc	27.3 bcd	34.9 bc	26.6 cd	51.8 a	36.5 b	23.3 d	9.7
caffeic	0.3 bc	0.3 bc	0.9 a	0.5 b	0.3 bc	0.4 bc	0.4 bc	0.5 b	0.2 c	0.2 c	0.3
p-coumaric	0.3 de	0.7 a	0.7 a	0.2 de	0.6 b	0.5 bc	0.2 e	0.5 bc	0.9 a	0.3 cd	0.1
ferluic	0.3 ab	0.3 ab	0.2 b	0.3 ab	0.4 ab	0.3 ab	0.4 ab	0.4 ab	0.3 ab	0.3 ab	0.1
total phenolic acids (mg/100 g)[b]	28.5 bcd	35.9 bc	31.2 bcd	36.1 bc	28.5 bcd	36.0 bc	27.6 cd	53.2 a	37.8 b	24.1 d	10.0
total phenolic acids, converted (mg/100 g)[c]	48.2 ef	65.6 bcd	43.9 f	72.5 bc	54.3 def	59.7 bcd	69.7 bc	99.7 a	76.7 b	49.3 ef	14.5
fruit weight (g)[d]	2.4 a	2.4 a	1.9 bc	1.6 c	1.4 c	2.2 ab	1.6 c	1.8 bc	1.5 c	2.3 ab	0.5
fruit diameter (mm)[e]	17.2 ab	17.7 a	16.6 abcd	14.7 cde	14.5 de	16.8 abc	14.2 e	16.5 abcd	15.3 bcde	17.2 ab	2.3
DM%[f]	16.8 c	14.5 e	17.2 b	17.3 b	15.5 d	14.5 e	14.2 f	16.8 c	18.1 a	15.5 d	0.2

[a]Means with different letters within rows are significantly different at $P \leq 0.05$. [b]Total phenolic acids(sum of individuals) in frozen fruits. [c]Total phenolic acids (sum of individuals) estimated in freeze-dried fruits but values were converted back to frozen fruit basis. [d]Average frozen fruit weight ($n = 10$). [e]Average frozen fruit width ($n = 10$), measured using a Westward digital caliper, 0–150 mm (Fisher Scientific). [f]: percent of dry mater contents in fruits (dry fruit weight/frozen fruit weight x 100).

condition levels reported in various publications. This is particularly true for sample handling, extraction procedure, and method of quantification, variations in which can result in inconsistent estimates for compounds of interest. Although in previous investigations, investigators have used extended incubation times and harsh conditions in an attempt to more thoroughly extract all anthocyanins from tissue, instead we have demonstrated that such conditions actually lead to underestimation.

Freeze-Drying Effects on Anthocyanins and Phenolic Acids. To evaluate the effects of freeze-drying, anthocyanin and phenolic acid contents in freeze-dried fruits were compared with those in frozen fruits. To attain this comparison, compound concentrations in freeze-dried tissues were converted back to a frozen fruit basis. The converted values for individual anthocyanins and phenolic acids were statistically analyzed using ANOVA and showed comparable results as was observed with the frozen fruits (data not shown). The

converted total anthocyanin and phenolic acid values for the 10 freeze-dried blueberry genotypes are presented in Tables 3 and 4, respectively. Paired comparisons between anthocyanin concentrations in frozen fruits with those in freeze-dried fruits (after conversion) showed no significant differences, using the *t* test ($P \leq 0.05$) within genotypes. However, there was a small reduction in anthocyanin content (3.9%) when fruits were freeze-dried, with a range of 0.6−9.2%, depending on the genotype. In contrast, large increases in phenolic acid concentrations by an average of 1.9-fold (±0.3) in freeze-dried over frozen fruits were observed in this study and were genotype-dependent (Table 4). In the course of freeze-drying, the sample temperature was gradually increased (from −35 to 20 °C) to allow moisture removal; however, this process appeared to allow minor degradation in anthocyanins by active enzymes such as anthocyaninase, polyphenol oxidase, and peroxidase.[16,21,34] In previous studies, depending on the fruit treatment after harvest, significant reductions in anthocyanins and phenolics were reported.[35,36] Partial degradation of anthocyanins due to their sensitivity to experimental procedures was observed in several studies.[15,20,21,25,36,37] On the other hand, increases in phenolic acid concentrations when fruits were maintained at low temperatures were reported in different plant commodities including blueberries.[38−41] The increase in chlorogenic acid concentration was attributed to the accumulation of sugars, which act as substrates for the synthesis of this compound. Data from this study demonstrated that while freeze-drying resulted in minor reductions in anthocyanins, an increase in the chlorogenic acid was observed; however, the changes in phytochemical composition during freeze-drying appeared to be genotype-dependent. Whereas data showed the best results can be obtained when fresh fruits are analyzed, with a large number of samples it becomes necessary to freeze-dry samples to maintain their phytochemical content and for safe handling, particularly when samples are to be kept for an extended period of time.

Implications for Human Health Benefits. Blueberry genotypes accumulated significant anthocyanin and phenolic acid concentrations as shown in Tables 3 and 4. Because anthocyanin bioavailability depends on their basic structures and the conjugated glycosides,[42−44] anthocyanins with the same glycosidic type were grouped and are presented in Table 5. Across all genotypes, glucoside, galactoside, arabinoside, and acylated anthocyanins constituted 14.6%, 46.0%, 34.9%, and 4.6% of total anthocyanins, respectively. However, some of the genotypes accumulated roughly equal concentrations of glucoside- and galactoside-based anthocyanins (O'Neal, NC4900, and SHF2B-1 21:3) (Table 5). Although a relatively small number of blueberry genotypes were evaluated in this study, three of the genotypes displayed significantly higher concentrations for malvidin 3-*O*-glucoside (**12**) (O'Neal, NC4900, and SHF2B-1 21:3) (Table 3). These genotypes also contained higher concentrations of other glucoside-based anthocyanins (delphinidin 3-*O*-glucoside (**2**) and cyanidin 3-*O*-glucoside (**5**). In a recent study, malvidin 3-*O*-glucoside (**12**) showed significantly higher hypoglycemic activity compared to delphinidin 3-*O*-glucoside (**2**).[13] This indicated that hypoglycemic activity of blueberry extract was largely anthocyanin specific. Delphinidin 3-*O*-glucoside (**2**) showed lower absorption and transport efficiency compared to malvidin 3-*O*-glucoside (**12**), and glucoside-based anthocyanins were more readily bioavailable than galactose-based anthocyanins.[43] The absorption and antioxidant activities of acylated anthocyanins in

Table 5. Concentrations (mg/100 g) of Grouped Glucoside-, Galactoside-, and Arabinoside-Based and Acylated Anthocyanins in Frozen Fruits for 10 Blueberry Genotypes[a]

anthocyanin group	Arlen	Legacy	Lenoir	O'Neal	Pamlico	Sampson	NC4385	NC4900	SHF2B-1 21:3	SHF2B-1 25:41	mean ± SD
glucoside	6.3 (2.5)	5.2 (2.0)	6.7 (3.0)	163.9 (49.5)	15.1 (3.9)	7.8 (3.7)	13.6 (5.1)	105.5 (33.4)	82.5 (27.0)	6.9 (2.8)	41.4 ± 56.1 (14.6)
galactoside	132.5 (52.4)	135.6 (51.9)	101.1 (46.1)	108.4 (30.4)	208.4 (54.3)	112.3 (52.9)	150.2 (56.3)	125.5 (39.7)	93.7 (30.6)	131.8 (53.0)	130.0 ± 32.6 (46.0)
arabinoside	112.9 (44.6)	94.9 (36.3)	97.0 (44.2)	70.6 (19.8)	147.0 (38.3)	84.2 (39.7)	102.1 (38.3)	83.7 (26.5)	84.6 (27.6)	109.2 (43.9)	98.6 ± 21.3 (34.8)
acylated	1.2 (0.5)	25.7 (9.8)	14.5 (6.6)	14.1 (4.0)	13.4 (3.5)	7.9 (3.7)	0.9 (0.3)	1.1 (0.4)	45.3 (14.8)	1.0 (0.4)	12.5 ± 14.2 (4.6)

[a]Values in parentheses are percent of total anthocyanins within genotypes.

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humans were remarkably low compared to the glycosidic forms.[42] Except for Legacy, Lenoir, and SHF2B-1 21:3, these genotypes contained low concentrations of the acylated anthocyanins (Table 5). Whereas intact anthocyanins were detected in pig organs,[18] low anthocyanins (0.03 μmol/L) but higher chlorogenic acid (0.26 μmol/L) concentrations were observed in human plasma.[45] However, in previous studies, anthocyanin bioavailability could be underestimated due to incomplete understanding of their metabolism and the lack of assessment techniques for their in vivo formulations.[45,46]

Accurate and efficient estimation of individual anthocyanins and phenolic acids, as well as other phytochemicals in blueberry, is critical to understanding their potential impact on human health maintenance.[47,48] However, inclusive absorption, bioavailability, and metabolism assessments for individual compound(s) are needed to help explain their specific bioactivities. On the basis of the existing literature for anthocyanin bioavailability and our data presented in Tables 3−5, we concluded that genotypes with higher concentrations for certain anthocyanins and chlorogenic acid can be more influential in health interventions and potentially prioritized targets in future blueberry breeding programs.

In conclusion, the streamlined analytical protocols developed and validated in this study can provide simple and efficient analyses without sacrificing the accuracy of results. Individual and total anthocyanins and phenolic acids were estimated in frozen and freeze-dried berries, which offered insight into freeze-drying effects on metabolites in blueberry fruits. Large variability in total and individual anthocyanins and phenolic acids were observed across the evaluated genotypes. Even when genotypes contained similar concentrations of total anthocyanins, they varied significantly for the individual species of anthocyanins. Several genotypes contained uniquely higher concentrations of individual anthocyanins and phenolic acids. This paper can assist in the design of blueberry breeding programs by aiding in prioritization of genotypes with improved concentrations for the more readily bioavailable anthocyanins, which can potentially confer better human health benefits.

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Notes

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REFERENCES

(1) USDA. *Economic Research Service*, 2012; http://www.ers.usda.gov/data/foodconsumption/app/availability.aspx.

(2) Martineau, L. C.; Couture, A.; Spoor, D.; Benhaddou-Andaloussi, A.; Harris, C.; Meddah, B.; Leduc, C.; Burt, A.; Vuong, T. Anti-diabetic properties of the Canadian lowbush blueberry *Vaccinium angustifolium* Ait. *Phytomedicine* **2006**, *13*, 612−623.

(3) Blanck, H. M.; Gillespie, C.; Kimmons, J. E.; Seymour, J. D.; Serdula, M. K. Trends in fruit and vegetable consumption among U.S. men and women, 1994−2005. *Prev. Chron. Dis.* **2008**, *5* (A35), 1−10.

(4) Stull, A. J.; Cash, K. C.; Johnson, W. D.; Champagne, C. M.; Cefalu, W. Bioactives in blueberry improve insulin sensitivity in obese, insulin-resistant men and women. *J. Nutr.* **2010**, *140*, 1764−1768.

(5) Basu, A.; Du, M.; Leyva, M. J.; Sanchez, K.; Betts, N. M.; Wu, M.; Aston, C. E.; Lyons, T. J. Blueberries decrease cardiovascular risk factors in obese men and women with metabolic syndrome. *J. Nutr.* **2010**, *140*, 1582−1587.

(6) González-Gallego, J.; Garcia-Mediavilla, M. V.; Sánchez-Campos, S.; Tuñón, M. J. Fruit polyphenols, immunity and inflammation. *Br. J. Nutr.* **2010**, *104*, S15−S27.

(7) Kalt, W.; Ryan, D. A.; Duy, J.; Prior, R. L.; Ehlenfeldt, M. K.; Kloet, S. V. Interspecific variation in anthocyanins, phenolics, and antioxidant capacity among genotypes of highbush and lowbush blueberries (*Vaccinium* section *cyanococcus* spp.). *J. Agric. Food Chem.* **2001**, *49*, 4761−4767.

(8) Hakkinen, S.; Heinonen, M.; Karenlampi, S.; Mykkanen, H.; Ruuskanen, J.; Torronen, R. Screening of selected flavonoids and phenolic acids in 19 berries. *Food Res. Int.* **1999**, *32*, 345−353.

(9) Prior, R. L.; Lazarus, S. A.; Cao, G.; Muccitelli, H.; Hammerstone, J. F. Identification of proanthocyanidin and anthocyanins in blueberries and cranberries (*Vaccinium* spp.) using high-performance liquid chromatography/mass spectrometry. *J. Agric. Food Chem.* **2001**, *49*, 1270−1276.

(10) Rimando, A. M.; Kalt, W.; Magee, J. B; Dewey, J.; Ballington, J. R. Resveratrol, pterostilbene, and piceatannol in *Vaccinium* berries. *J. Agric. Food Chem.* **2004**, *52*, 4713−4719.

(11) De Groot, H.; Rauen, U. Tissue injury by reactive oxygen species and the protective effects of flavonoids. *Fundam. Clin. Pharmacol.* **1998**, *12*, 249−255.

(12) Serafini, M.; Maiani, G.; Ferro-Luzzi, A. Alcohol-free red wine enhances plasma antioxidant capacity in humans. *J. Nutr.* **1998**, *128*, 1003−1007.

(13) Grace, M.; Ribnicky, D.; Kuhn, P.; Poulev, A.; Longendra, S.; Yousef, G. G.; Raskin, I.; Lila, M. A. Hypoglycemic activity of a novel anthocyanin-rich formulation from lowbush blueberry, *Vaccinium angustifolium* Aiton. *Phytomedicine* **2009**, *16*, 406−415.

(14) Johnston, K. L.; Clifford, M. N.; Morgan, L. M. Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: glycemic effects of chlorogenic acid and caffeine. *Am. J. Clin. Nutr.* **2003**, *79*, 728−733.

(15) Buckow, R.; Kastell, A.; Terefe, N. S.; Versteeg, C. Pressure and temperature effects on degradation kinetics and storage stability of total anthocyanins in blueberry juice. *J. Agric. Food Chem.* **2010**, *58*, 10076−10084.

(16) Garcia-Viguera, C.; Zafrilla, P.; Tomas-Barberan, T. The use of acetone as an extraction solvent for anthocyanins from strawberry fruit. *Phytochem. Anal.* **1998**, *9*, 274−277.

(17) Kader, K.; Girardin, M.; Metche, M. Fractionation and identification of the phenolic compounds of highbush blueberries (*Vaccinium corymbosum*, L.). *Food Chem.* **1996**, *55*, 35−40.

(18) Kalt, W.; Blumberg, J. B; McDonald, J. E.; Vinqvist-Tymchuk, M. R.; O'Leary, J. M.; Milbury, P. E. Identification of anthocyanins in liver, eye, and brain of blueberry-fed pigs. *J. Agric. Food Chem.* **2008**, *56*, 705−712.

(19) Wu, X.; Prior, R. L. Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: fruits and berries. *J. Agric. Food Chem.* **2005**, *53*, 2589−2599.

(20) Barnes, J. S.; Nguyen, H. P.; Shen, S.; Schug, K. A. General method for extraction of blueberry anthocyanins and identification using high performance liquid chromatography-electrospray ionization-ion trap-time of flight-mass spectrometry. *J. Chromatogr., A* **2009**, *1216*, 4728−4735.

(21) Brownmiller, C.; Howard, L. R.; Prior, R. L. Processing and storage effects on monomeric anthocyanins, percent polymer color,

and antioxidant capacity of processed blueberry products. *J. Food Sci.* **2008**, *73*, 72−79.
(22) Guzman, I.; Yousef, G. G.; Brown, A. F. Simultaneous extraction and quantification of carotenoids, chlorophylls, and tocopherols in *Brassica* vegetables. *J. Agric. Food Chem.* **2012**, *60*, 7238−7244.
(23) Kalt, W.; McDonald, J. E.; Ricker, R. D.; Lu, X. Anthocyanin content and profile within and among blueberry species. *Can. J. Plant Sci.* **1999**, *79*, 617−623.
(24) Kellogg, J.; Wang, J.; Flint, C.; Ribnicky, D.; Kuhn, P.; De Mejia, E. G.; Raskin, I.; Lila, M. A. Alaskan wild berry resources and human health under the cloud of climate change. *J. Agric. Food Chem.* **2010**, *58*, 3884−3900.
(25) Gustafson, S. J.; Yousef, G. G.; Grusak, M. A.; Lila, M. A. Effect of postharvest handling on phytochemical concentrations and bioactive potential in wild blueberry fruit. *J. Berry Res.* **2012**, *2*, 215−227.
(26) Stevenson, D.; Scalzo, J. Anthocyanin composition and content of blueberries from around the world. *J. Berry Res.* **2012**, *2*, 179−189.
(27) Kalt, W.; McDonald, J. E. Chemical composition of lowbush blueberry cultivars. *J. Am. Soc. Hortic. Sci.* **1996**, *121*, 142−146.
(28) Lee, J.; Rennaker, C.; Wrolstad, R. E. Correlation of two anthocyanin quantification methods: HPLC and spectrophotometric methods. *Food Chem.* **2008**, *110*, 782−786.
(29) Grace, M. H.; Massey, A. R.; Mbeunkui, F.; Yousef, G. G.; Lila, M. A. Comparison of health-relevant flavonoids in commonly consumed cranberry products. *J. Food Sci.* **2012**, *77*, H176−H183.
(30) Jackman, R. L.; Yada, R. Y.; Tung, M. A.; Speers, R. A. Anthocyanins as food colorants − a review. *J. Food Biochem.* **1987**, *11*, 201−247.
(31) Prior, R. L.; Cao, G.; Martin, A.; Sofic, E.; McEwen, J.; O'Brien, C.; Lischner, N.; Ehlenfedt, M.; Kalt, W.; Krewer, G.; Mainland, C. M. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. *J. Agric. Food Chem.* **1998**, *46*, 2686−2693.
(32) Connor, A. M.; Luby, J. J.; Tong, C. B.; Finn, C. E.; Hancock, J. F. Genotype and environmental variation in antioxidant activity, total phenolic content, and anthocyanin content among blueberry cultivars. *J. Am. Soc. Hortic. Sci.* **2002**, *127*, 89−97.
(33) Gavrilova, V.; Kajdzanoska, M.; Gjamovski, V.; Stefova, M. Separation, characterization and quantification of phenolic compounds in blueberries and red and black currents by HPLC-DAD-ESI-MS. *J. Agric. Food Chem.* **2011**, *59*, 4009−4018.
(34) Shi, Z.; Bassa, I. A.; Gabriel, S. L.; Francis, F. J. Anthocyanin pigments of sweet potatoes − *Ipomoea batatas*. *J. Food Sci.* **1992**, *57*, 755−757.
(35) Klopotek, Y.; Otto, K.; Bohm, V. Processing strawberries to different products alters contents of vitamin C, total phenolics, total anthocyanins, and antioxidant capacity. *J. Agric. Food Chem.* **2005**, *53*, 5640−5646.
(36) Wojdylo, A.; Figiel, A.; Oszmianski, J. Effect of drying methods with the application of vacuum microwaves on the bioactive compounds, color, and antioxidant activity of strawberry fruits. *J. Agric. Food Chem.* **2009**, *57*, 1337−1343.
(37) Srivastava, A.; Akoh, C. C.; Yi, W.; Fisher, J.; Krewer, G. Effect of storage conditions on biological activity of phenolic compounds of blueberry extract packed in glass bottles. *J. Agric. Food Chem.* **2007**, *55*, 2705−2713.
(38) Hasegawa, S.; Johnson, R. M.; Gould, W. A. Changes during storage, effect of cold storage on chlorogenic acid content of potatoes. *J. Agric. Food Chem.* **1966**, *14*, 165−169.
(39) Connor, A. M.; Luby, J. J.; Hancock, J. F.; Berkheimer, S.; Hanson, E. J. Changes in fruit antioxidant activity among blueberry cultivars during cold-temperature storage. *J. Agric. Food Chem.* **2002**, *50*, 893−898.
(40) Padda, S.; Picha, D. H. Quantification of phenolic acids and antioxidant activity in sweetpotato genotypes. *Sci. Hortic.* **2008**, *119*, 17−20.
(41) Ishiguro, K.; Yahara, S.; Yoshimoto, M. Changes in polyphenolic content and radical-scavenging activity of sweetpotato (*Ipomoea batatas* L.) during storage at optimal and low temperatures. *J. Agric. Food Chem.* **2007**, *55*, 10773−10778.
(42) Prior, R. L.; Wu, X. Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. *Free Radical Res.* **2006**, *40*, 1014−1028.
(43) Yi, W.; Akoh, C. C.; Fischer, J.; Krewer, G. Absorption of anthocyanins from blueberry extracts by Caco-2 human intestinal cell monolayers. *J. Agric. Food Chem.* **2006**, *54*, 5651−5658.
(44) McGhie, T. K.; Walton, M. C. The bioavailability and absorption of anthocyanins: towards a better understanding. *Mol. Nutr. Food Res.* **2007**, *51*, 702−713.
(45) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in human. I. Review of 97 bioavailbility studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S−242S.
(46) McGhie, T. K.; Ainge, G. D.; Barnett, L. E.; Cooney, J. M.; Jensen, D. J. Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolized by both humans and rats. *J. Agric. Food Chem.* **2003**, *51*, 4539−4548.
(47) Prior, R. L.; Cao, G. Variability in Dietary antioxidant related natural product supplements: the need for methods of standardization. *J. Am. Nutraceut. Assoc.* **1999**, *2*, 46−56.
(48) Martin, C.; Butelli, E.; Petroni, K.; Tonelli, C. How can research on plants contribute to promoting human health? *Plant Cell.* **2011**, *23*, 1685−1699.