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Article

# Characterization of Anthocyanins Including Acetylated Glycosides from Highbush Blueberry (*Vaccinium corymbosum* L.) Cultivated in Korea Based on UPLC-DAD-QToF/MS and UPLC-Qtrap-MS/MS

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**Abstract:** In this study, anthocyanin glycosides from nine cultivars of highbush blueberries grown in Korea were characterized using UPLC-DAD-QToF/MS and UPLC-Qtrap-MS/MS. A total of twenty-two derivatives were identified, consisting of mono-glycosides with galactose, glucose, arabinose, acetyl-galactose, or acetyl-glucose moieties attached to aglycones such as cyanidin, peonidin, delphinidin, petunidin, and malvidin. Among them, seven acetylated glycosides were tentatively determined by comparing the related authentic standards and previous reports, and presented mass fragmentation in which the acetyl group remained as form attached to the sugar without deesterification in positive ionization mode. The mid-season cultivar 'New Hanover' showed the highest total anthocyanin content (1011.7 mg/100 g dry weight) with predominant malvidin and delphinidin glycosides. Particularly, the 'Patriot' (early-season) recorded the highest proportion of acetylated glycosides (19.7%). These detailed anthocyanin profiles will be supported as fundamental data for the development of superior cultivars and functional blueberry-based products as well as the further research of anthocyanin changes by cultivars, harvest time, cultivated methods, storage conditions, etc..

**Keywords:** blueberry; anthocyanin; acetylated glycoside; UPLC-DAD-QToF/MS; UPLC-Qtrap-MS/MS

## 1. Introduction

Blueberries (*Vaccinium* spp.), along with bilberries, belonging to the *Vaccinium* genus of Ericaceae family, which comprises approximately 400 species, mainly distributed in Southeast Asia. These species are classified into highbush (*V. corymbosum* L.), lowbush (*V. angustifolium* L.), and rabbiteye (*V. ashei* L.). The blueberries grown in Korea are of the highbush type, which is characterized by cold hardiness and suitability for cultivation [1]. In general, highbush blueberries can be further classified as northern and southern depending on chilling requirements [2], and early-, mid-, and late-season by harvest time [3].

Blueberries are known to be rich in phenolic compounds (anthocyanins, flavonoids), carotenoids, and vitamin C [4], and closely related to their antioxidant [5], anticancer [6], antidiabetic [7], and cardiovascular disease prevention [8]. Blueberry anthocyanins are composed of aglycones such as delphinidin, cyanidin, petunidin, peonidin, and malvidin, with sugar moieties (glucose, galactose, and arabinose) attached. Among them, malvidin (37–52%) and delphinidin (27–31%) glycosides are the most commonly identified [9,10]. Additionally, acetylated anthocyanins, which are present in small amounts in blueberries, have a structure in which an acetyl group is acylated at the

6"-OH position of glucose or galactose. These compounds are reported to contribute to the intensity, stability of pigments in foods [11] and exhibit antioxidant effects [12].

Recently, studies on the identification of individual anthocyanin have been actively conducted in blueberries using mass spectrometry (MS). A total of 25 anthocyanin glycosides were detected and fragmented through positive ionization mode of ESI-ToF-MS from southern highbush blueberries in the United States [13]. The anthocyanin composition and content in blueberries were distributed differently depending on their cultivar and cultivated condition [14,15]. Although 74 cultivars grown in China were characterized by Chai et al. [16], only total content was provided without detailed individual components for acetylated glycosides, which account for 0-42% of total anthocyanin. The structural elucidation of acetylated glycosides have been rarely performed through NMR [17] and LC-MS [18,19] analyses. Since the overall quantification of blueberry anthocyanins primarily focused on major group such as non-acetylated glycosides [20], these detailed profiles are still limited.

The objective of this study was to rapidly and precisely identify and quantify individual anthocyanins including acetylated glycosides from nine highbush blueberry cultivars (Reka, Hannah's Choice, Spartan, Draper, Suziblue, Legacy, New Hanover, Farthing, and Patriot) grown in Korea based on ultra-performance liquid chromatography-diode array detection quadrupole time-of-flight mass spectrometry (UPLC-DAD-QToF/MS) and ultra-performance liquid chromatography-quadrupole trap tandem mass spectrometry (UPLC-Qtrap-MS/MS) with internal and external quantification methods. Thus, it is considered that the anthocyanin profiles provided will be supported as fundamental data for the development of superior cultivars and functional blueberry-based products.

## 2. Materials and Methods

### 2.1. Plant Materials

Blueberries (*Vaccinium* spp.) were obtained from a farm in Anseong-si, Gyeonggi-do (latitude/longitude: 37°0'4.7"N/127°12'35.7"E), in June 2023, respectively. The samples were collected with permission of Rural Development Administration (RDA, Republic of Korea), and identified by the author (Heon-Woong Kim) of this article. Detailed information about each cultivar is provided in Table 1. Experimental research, including the collection of plant material, complied with relevant institutional, national, and international guidelines and legislation. All samples were lyophilized, crushed with a grinding machine, and sieved through a 30 mesh sieve.

**Table 1.** List of 9 highbush blueberry cultivars in Korea.

Cultivar	Harvest season	Species
Reka	Early	northern highbush
Hannah's choice	Early	northern highbush
Spartan	Early	northern highbush
Draper	Early	northern highbush
Patriot	Early	northern highbush
Legacy	Mid	northern highbush
Suziblue	Early	southern highbush
Farthing	Early	southern highbush
Newhanover	Mid	southern highbush

### 2.2. Chemicals

Cyanidin 3-O-arabinoside, cyanidin 3-O-galactoside, cyanidin 3-O-glucoside, delphinidin 3-O-glucoside, malvidin 3-O-galactoside, malvidin 3-O-glucoside, peonidin 3-O-arabinoside, peonidin 3-O-galactoside, peonidin 3-O-glucoside, petunidin 3-O-glucoside, and delphinidin 3,5-di-O-glucoside (delphin) were purchased from Extrasynthese (Genay Cedex, France). Petunidin 3-O-galactoside was

provided by Chemfaces (Wuhan, China). LC-MS-grade acetonitrile and water were obtained from Thermo Fisher Scientific (Fair Lawn, NJ, USA), and formic acid from Junsei Chemical Co. (Tokyo, Japan).

### 2.3. Extraction and Sample Preparation

For UPLC-DAD-QToF/MS and UPLC-QTrap-MS/MS analyses, powdered blueberry (0.5 g) was extracted with 10 mL of 5% formic acid in water using an orbital shaker (for 40 min at 200 rpm), and centrifuged at  $2,016\times g$  and 10 °C for 15 min (LABOGENE 1580R, Bio-Medical Science Co., Seoul, Korea). The supernatant was filtered through a 0.45  $\mu\text{m}$  syringe filter and purified using a HyperSep Retain-PEP solid-phase extraction (SPE) cartridge (Thermo Scientific, Bellefonte, PA, USA). In order to remove undesirable components and improve separation and detection in UPLC-MS, the SPE was performed and the process was as follows: The cartridge was conditioned with methanol (4 mL) and water (8 mL). The samples (0.5 mL) and internal standard solution (delphin, 100 ppm; 1 mL) were loaded into cartridge and washed with water (6 mL). Finally, the loaded samples were eluted with 1% formic acid in methanol (12 mL) and concentrated using  $\text{N}_2$  gas, then re-dissolved with 5% formic acid in water (0.5 mL).

### 2.4. Anthocyanin Identification and Quantification

The anthocyanin derivatives in different blueberry cultivars were comprehensively performed using an UPLC system coupled with a diode array detector (DAD) (ACQUITY UPLC™ system, Waters Co., Milford, MA, USA) and a QToF-MS (Xevo G2-S QToF, Waters MS Technologies, Manchester, UK). Chromatographic conditions were as follows: CORTECS UPLC T3 (2.1  $\times$  150 mm, 1.6  $\mu\text{m}$ , Waters, Wexford, Ireland) column and CORTECS UPLC T3 VanGuard™ pre-column (2.1  $\times$  50 mm, 1.6  $\mu\text{m}$ , Waters), representative detection wavelength: cyanidin and peonidin, 515 nm; delphinidin, 520 nm; petunidin and malvidin, 525 nm; flow rate, 0.3 mL/min; Column oven temperature, 30 °C; Sample injection volume, 1  $\mu\text{L}$ ; Mobile phase, 5% formic acid in water (A), 5% formic acid in water/acetonitrile (1:1, v/v) (B). Elution gradient conditions: 0 min, 10% B; 28 min, 50% B; 33-38 min, 90% B; 43-50 min, 10% B. Mass spectra were simultaneously scanned with the range of 50-1200  $m/z$  in positive ionization mode using an electrospray ionization (+ESI) source, and their parameters used were: capillary voltage, 3500 V; sampling cone voltage, 40 V; extraction cone, 4.0 V; Ion source, 120 °C; desolvation temperature, 500 °C; desolvation  $\text{N}_2$  gas flow, 1020 L/h. All standard compounds were externally quantified based on multiple reaction monitoring (MRM) mode using UPLC-QTrap-MS/MS (SCIEX QTrap 4500, SCIEX CO.), whereas other anthocyanins internally using QToF/MS (Xevo G2-S QToF, Waters MS Technologies, Manchester, UK). The optimizing MRM conditions are shown in Table 2. Internal quantification was implemented by calculating the relative peak areas of the compounds compared to an internal standard (delphin), and the external quantification was performed in MRM mode with selected 11 standards. The contents of anthocyanin derivatives were expressed in mg/100 g DW (dry weight). The triplicate results are expressed as mean  $\pm$  standard deviation. One-way ANOVA was performed with SPSS (version 28.0), SPSS institute; Chicago, IL, USA) to determine a significant difference between individual averages using Duncan's multiple range test ( $p < 0.05$ ). To quantify anthocyanins from blueberry, linearity was determined by analyzing standard solutions at concentrations of 0.01, 0.05, 0.1, 0.5, 1, 2, and 5  $\mu\text{g/mL}$  using UPLC-QTrap-MS/MS, with each concentration analyzed in triplicate. The calibration curves were constructed by plotting the peak area against the concentration of the corresponding standards using least-square linear regression (Table 2). LOD and LOQ for used standards were calculated using the equations  $\text{LOD} = 3.3 \times \text{SD}/\delta$  and  $\text{LOQ} = 10 \times \text{SD}/\delta$ , respectively, where SD represents the standard deviation of response ( $y$ -intercept), and  $\delta$  is a slope of calibration curve Intra- and Inter-day precision was evaluated by analyzing standard mixtures (0.5  $\mu\text{g/mL}$ ,  $n=6$ ) on single day and 6days. The variations were expressed as the relative standard deviation (RSD) of the replicates (Table 3).

**Table 2.** Optimized MRM conditions for quantitative analysis of blueberry anthocyanins.

No.	Compound name	Formula	RT <sup>1)</sup> (min)	Precursor ion (m/z)	Product ion (m/z)	DP (V) <sup>2)</sup>	CE (V) <sup>3)</sup>
2	Delphinidin 3-O-glucoside (mirtillin)	C <sub>25</sub> H <sub>21</sub> O <sub>12</sub> <sup>+</sup>	6.36	465	303	86	29
3	Cyanidin 3-O-galactoside (ideain)	C <sub>21</sub> H <sub>21</sub> O <sub>11</sub> <sup>+</sup>	7.09	449	287	86	33
5	Cyanidin 3-O-glucoside (asterin)	C <sub>21</sub> H <sub>21</sub> O <sub>11</sub> <sup>+</sup>	8.08	449	287	31	39
6	Petunidin 3-O-galactoside	C <sub>22</sub> H <sub>23</sub> O <sub>12</sub> <sup>+</sup>	8.62	479	317	86	35
7	Cyanidin 3-O-arabinoside	C <sub>20</sub> H <sub>19</sub> O <sub>10</sub> <sup>+</sup>	8.87	419	287	236	27
8	Petunidin 3-O-glucoside	C <sub>22</sub> H <sub>23</sub> O <sub>12</sub> <sup>+</sup>	9.57	479	317	86	31
9	Peonidin 3-O-galactoside	C <sub>22</sub> H <sub>23</sub> O <sub>11</sub> <sup>+</sup>	10.35	463	301	20	33
11	Peonidin 3-O-glucoside	C <sub>22</sub> H <sub>23</sub> O <sub>11</sub> <sup>+</sup>	11.50	463	301	20	33
12	Malvidin 3-O-galactoside (primulin)	C <sub>22</sub> H <sub>25</sub> O <sub>12</sub> <sup>+</sup>	11.79	493	331	11	35
13	Peonidin 3-O-arabinoside	C <sub>21</sub> H <sub>21</sub> O <sub>10</sub> <sup>+</sup>	12.36	433	301	104	33
14	Malvidin 3-O-glucoside (enin)	C <sub>22</sub> H <sub>25</sub> O <sub>12</sub> <sup>+</sup>	12.81	493	331	16	37

<sup>1)</sup>RT: retention time <sup>2)</sup>DP: declustering potential <sup>3)</sup>CE: collision energy.

**Table 3.** Linearity, regression equation, LOD and LOQ, intra- and inter-day precisions of 11 anthocyanin standards.

Compound name	Regression equation	Correlation coefficient <sup>1)</sup> (R <sup>2</sup> )	LOD <sup>2)</sup> ( $\mu\text{g}/\text{mL}$ )	LOQ <sup>3)</sup> ( $\mu\text{g}/\text{mL}$ )	Precision RSD (%)		
					intra- day (n = 6)	inter- day (n = 6)	
Delphinidin glucoside	3-O- $Y = 1779297.1511X + 9835.2387$	+	0.9999	0.0025	0.0076	1.83	6.54
Cyanidin galactoside	3-O- $Y = 2178374.9200X + 25655.6942$	+	1.0000	0.0026	0.0080	0.50	5.63
Cyanidin glucoside	3-O- $Y = 3597678.5489X + 27299.5857$	+	1.0000	0.0030	0.0091	0.03	5.27
Petunidin galactoside	3-O- $Y = 1184176.7790X + 8718.2247$	+	0.9998	0.0011	0.0033	5.90	4.34
Cyanidin arabinoside	3-O- $Y = 532110.7X + 5725.4070$		0.9999	0.0039	0.0117	0.50	1.96
Petunidin glucoside	3-O- $Y = 2320782.4X + 22315.0959$		0.9999	0.0017	0.0053	0.03	3.42
Peonidin galactoside	3-O- $Y = 1537207.3321X + 46.9756$		0.9999	0.0035	0.0106	5.43	3.21
Peonidin glucoside	3-O- $Y = 1639444.6549X + 6829.8984$	+	0.9999	0.0035	0.0105	0.49	4.57
Malvidin galactoside	3-O- $Y = 3097011.3147X + 49762.1926$	+	0.9998	0.0042	0.0128	0.03	3.31
Peonidin arabinoside	3-O- $Y = 817530.9732X + 15169.9675$	+	0.9998	0.0028	0.0085	6.69	3.84

Malvidin glucoside	3-O-	$Y = 3094396.6155X + 62198.3776$	0.9998	0.0017	0.0051	0.49	3.95
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<sup>1)</sup> Correlation coefficient ( $R^2$ ) of calibration curve (range; 0.01-5  $\mu\text{g/mL}$ ),  $Y$ = Peak area,  $X$ = concentration (ppm) <sup>2)</sup> LOD: limit of detection <sup>3)</sup> LOQ: limit of quantitation.

### 3. Results and Discussion

#### 3.1. Identification of 22 anthocyanin glycosides from Blueberry Cultivars

In blueberries, anthocyanins are primarily identified as glycosides of cyanidin ( $m/z$  287), peonidin ( $m/z$  301), delphinidin ( $m/z$  303), petunidin ( $m/z$  317), and malvidin ( $m/z$  331). These compounds consist of a sugar moiety (galactose, glucose, or arabinose) attached to the 3-OH position of the aglycone and acylated with acetic acid at the 6"-OH position of the sugar (Table 4, Figure 1).

A total of twenty-two derivatives were tentatively identified as galactoside (5), glucoside (5), arabinoside (5), acetyl-galactoside (2), and acetyl-glucoside (5) based on cyanidin, peonidin, delphinidin, petunidin, and malvidin aglycones and confirmed with the presence of galactose (162 Da), glucose (162 Da), arabinose (132 Da), acetyl-galactose (42 + 162 Da), and acetyl-glucose (42 + 162 Da) moieties from whole structure. Particularly, in positive ionization mode, the acetylated glycosides were fragmented in which the acetyl group remained as form attached to the sugar without deesterification [21,22]. To further characterize these compounds, the previously reported retention times ( $R_t$ , min) [23], fragmentation patterns [24], ultraviolet spectra (UV) [25], and other sources [26] supported the isolation and identification of individual anthocyanins with UPLC-DAD-QToF/MS experimental data (Table 4, Figures 2–4).

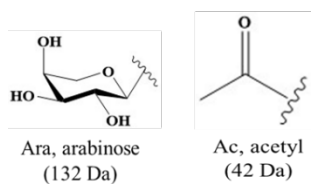
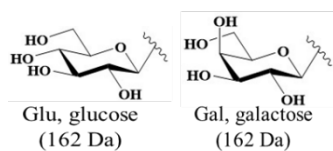
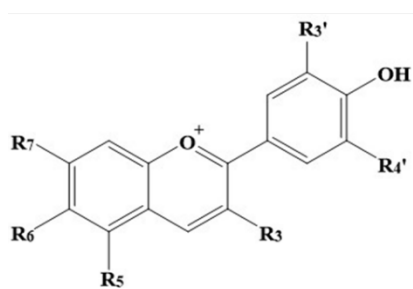
**Table 4.** 22 Anthocyanins identified from highbush blueberry cultivars.

Peak No.	RT (min)	Compound name	Formula	Experimental ion ( $m/z$ , [M] <sup>+</sup> )	Error (ppm) <sub>1)</sub>	Product ions ( $m/z$ )	Species <sup>2)</sup>
1	5.99	Delphinidin 3-O-galactoside	$C_{21}H_{21}O_1$ 2 <sup>+</sup>	465.1022	-1.2	465, 303	a, b, c, d, e, f, g, h, i
2	6.82	Delphinidin 3-O-glucoside	$C_{25}H_{21}O_1$ 2 <sup>+</sup>	465.1021	-1.4	465, 303	a, b, c, d, e, f, g, h, i
3	7.52	Cyanidin 3-O-galactoside	$C_{21}H_{21}O_1$ 1 <sup>+</sup>	449.1080	0.4	449, 287	a, b, c, d, e, f, g, h, i
4	7.79	Delphinidin 3-O-arabinoside	$C_{20}H_{19}O_1$ 1 <sup>+</sup>	435.0922	0.0	435, 303	a, b, c, d, e, f, g, h, i
5	8.56	Cyanidin 3-O-glucoside	$C_{21}H_{21}O_1$ 1 <sup>+</sup>	449.1080	0.4	449, 287	a, b, c, d, e, f, g, h, i
6	9.09	Petunidin 3-O-galactoside	$C_{22}H_{23}O_1$ 2 <sup>+</sup>	479.1184	0.0	479, 317	a, b, c, d, e, f, g, h, i
7	9.41	Cyanidin 3-O-arabinoside	$C_{20}H_{19}O_1$ 0 <sup>+</sup>	419.0975	0.5	419, 287	a, b, c, d, e, f, g, h, i
8	10.06	Petunidin 3-O-glucoside	$C_{22}H_{23}O_1$ 2 <sup>+</sup>	479.1185	0.2	479, 317	a, b, c, d, e, f, g, h, i
9	10.79	Peonidin 3-O-galactoside	$C_{22}H_{23}O_1$ 1 <sup>+</sup>	463.1270	0.5	463, 301	a, b, c, d, e, f, g, h, i
10	11.08	Petunidin 3-O-arabinoside	$C_{21}H_{21}O_1$ 1 <sup>+</sup>	449.1078	-0.1	449, 317	a, b, c, d, e, f, g, h, i
11	11.98	Peonidin 3-O-glucoside	$C_{22}H_{23}O_1$ 1 <sup>+</sup>	463.1234	-0.2	463, 301	e, g

12	12.20	Malvidin 3-O-galactoside	$C_{22}H_{25}O_1$ 2 <sup>+</sup>	493.1337	-0.7	493, 331	a, b, c, d, e, f, g, h, i
13	12.85	Peonidin 3-O-arabinoside	$C_{21}H_{21}O_1$ 0 <sup>+</sup>	433.1133	0.9	433, 301	a, b, c, d, e, f, g, h, i
14	13.25	Malvidin 3-O-glucoside	$C_{22}H_{25}O_1$ 2 <sup>+</sup>	493.1342	0.3	493, 331	a, b, c, d, e, f, g, h, i
15	14.30	Malvidin 3-O-arabinoside	$C_{22}H_{23}O_1$ 1 <sup>+</sup>	463.1235	0.0	463, 331	a, b, c, d, e, f, g, h, i
16	14.88	Delphinidin 3-O-(6''-O-acetyl)glucoside	$C_{23}H_{23}O_1$ 3 <sup>+</sup>	507.1135	0.4	507, 303	a, b, c, e, g
17	15.78	Petunidin 3-O-(6''-O-acetyl)galactoside	$C_{24}H_{25}O_1$ 3 <sup>+</sup>	521.1292	0.4	521, 317	a, b, c, e, g, i
18	17.13	Cyanidin 3-O-(6''-O-acetyl)glucoside	$C_{23}H_{23}O_1$ 2 <sup>+</sup>	491.1187	0.6	491, 287	a, b, c, e, g
19	18.39	Petunidin 3-O-(6''-O-acetyl)glucoside	$C_{24}H_{25}O_1$ 3 <sup>+</sup>	521.1292	0.4	521, 317	a, b, c, e, g
20	19.06	Malvidin 3-O-(6''-O-acetyl)galactoside	$C_{25}H_{27}O_1$ 3 <sup>+</sup>	535.1449	0.5	535, 331	a, b, c, e, g, i
21	20.69	Peonidin 3-O-(6''-O-acetyl)glucoside	$C_{24}H_{25}O_1$ 2 <sup>+</sup>	505.1343	0.4	505, 301	a, b, c, e, g
22	21.62	Malvidin 3-O-(6''-O-acetyl)glucoside	$C_{25}H_{27}O_1$ 3 <sup>+</sup>	535.1448	0.3	535, 331	a, b, c, e, g, i

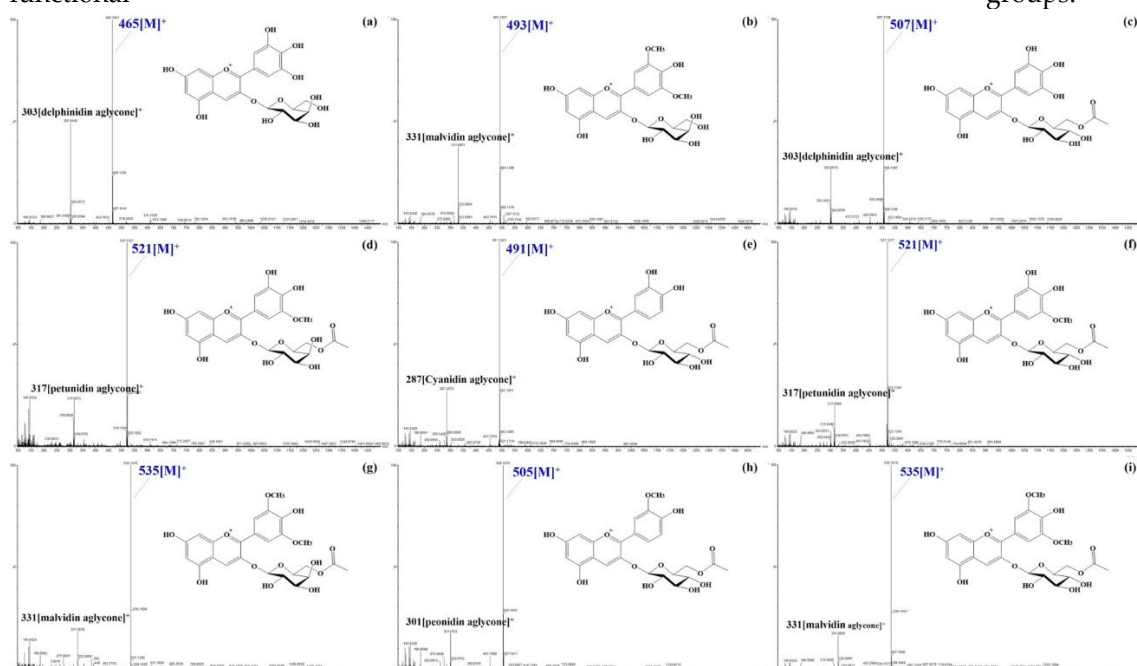
<sup>1</sup>) Error (ppm) indicates the mass accuracy of QTOF data and was calculated as [(calculated ion – observed ion) / (calculated ion)] × 10<sup>6</sup> based on *m/z* [M+H]<sup>+</sup>.

<sup>2</sup>) a, Reka; b, Hannah's choice; c, Spartan; d, Draper; e, Patriot; f, Legacy; g, Suziblu; h, Farthing; i, New Hanover.

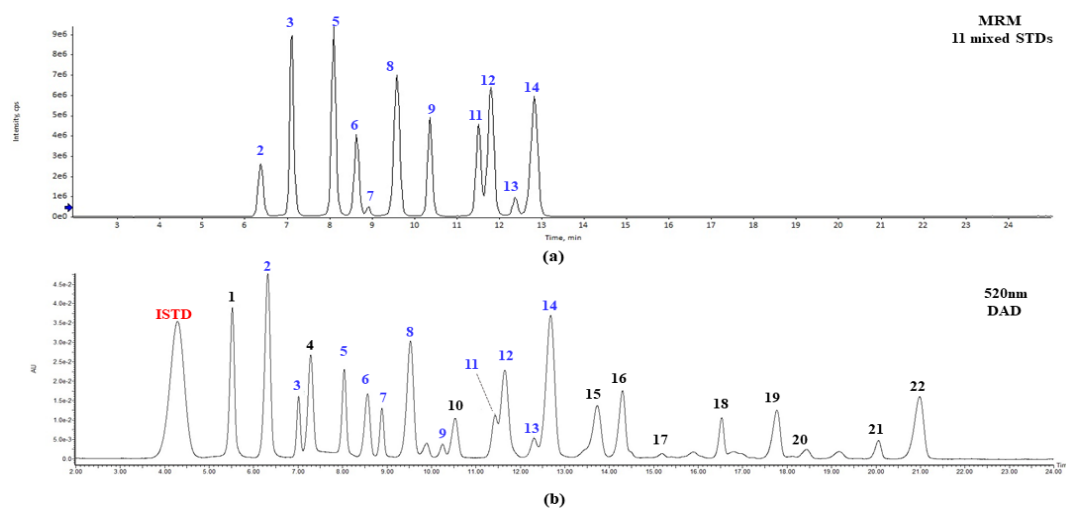


Aglycones	Peak No.	R <sub>3</sub>	R <sub>5</sub>	R <sub>6</sub>	R <sub>7</sub>	R <sub>3'</sub>	R <sub>4'</sub>
Delphinidin ( <i>m/z</i> 303)	1	<i>O</i> -gal	OH	H	OH	OH	OH
	2	<i>O</i> -glu	OH	H	OH	OH	OH
	4	<i>O</i> -ara	OH	H	OH	OH	OH
	16	<i>O</i> -Ac-glu	OH	H	OH	OH	OH
Cyanidin ( <i>m/z</i> 287)	3	<i>O</i> -gal	OH	H	OH	OH	H
	5	<i>O</i> -glu	OH	H	OH	OH	H
	7	<i>O</i> -ara	OH	H	OH	OH	H
Petunidin ( <i>m/z</i> 317)	18	<i>O</i> -Ac-glu	OH	H	OH	OH	H
	6	<i>O</i> -gal	OH	H	OH	OH	OCH <sub>3</sub>
	8	<i>O</i> -glu	OH	H	OH	OH	OCH <sub>3</sub>
	10	<i>O</i> -ara	OH	H	OH	OH	OCH <sub>3</sub>
Peonidin ( <i>m/z</i> 301)	17	<i>O</i> -Ac-gal	OH	H	OH	OH	OCH <sub>3</sub>
	19	<i>O</i> -Ac-glu	OH	H	OH	OH	OCH <sub>3</sub>
	9	<i>O</i> -gal	OH	H	OH	OCH <sub>3</sub>	H
	11	<i>O</i> -glu	OH	H	OH	OCH <sub>3</sub>	H
Malvidin ( <i>m/z</i> 331)	13	<i>O</i> -ara	OH	H	OH	OCH <sub>3</sub>	H
	21	<i>O</i> -Ac-glu	OH	H	OH	OCH <sub>3</sub>	H
	12	<i>O</i> -gal	OH	H	OH	OCH <sub>3</sub>	OCH <sub>3</sub>
	14	<i>O</i> -glu	OH	H	OH	OCH <sub>3</sub>	OCH <sub>3</sub>
	15	<i>O</i> -ara	OH	H	OH	OCH <sub>3</sub>	OCH <sub>3</sub>
	20	<i>O</i> -Ac-gal	OH	H	OH	OCH <sub>3</sub>	OCH <sub>3</sub>
22	<i>O</i> -Ac-glu	OH	H	OH	OCH <sub>3</sub>	OCH <sub>3</sub>	

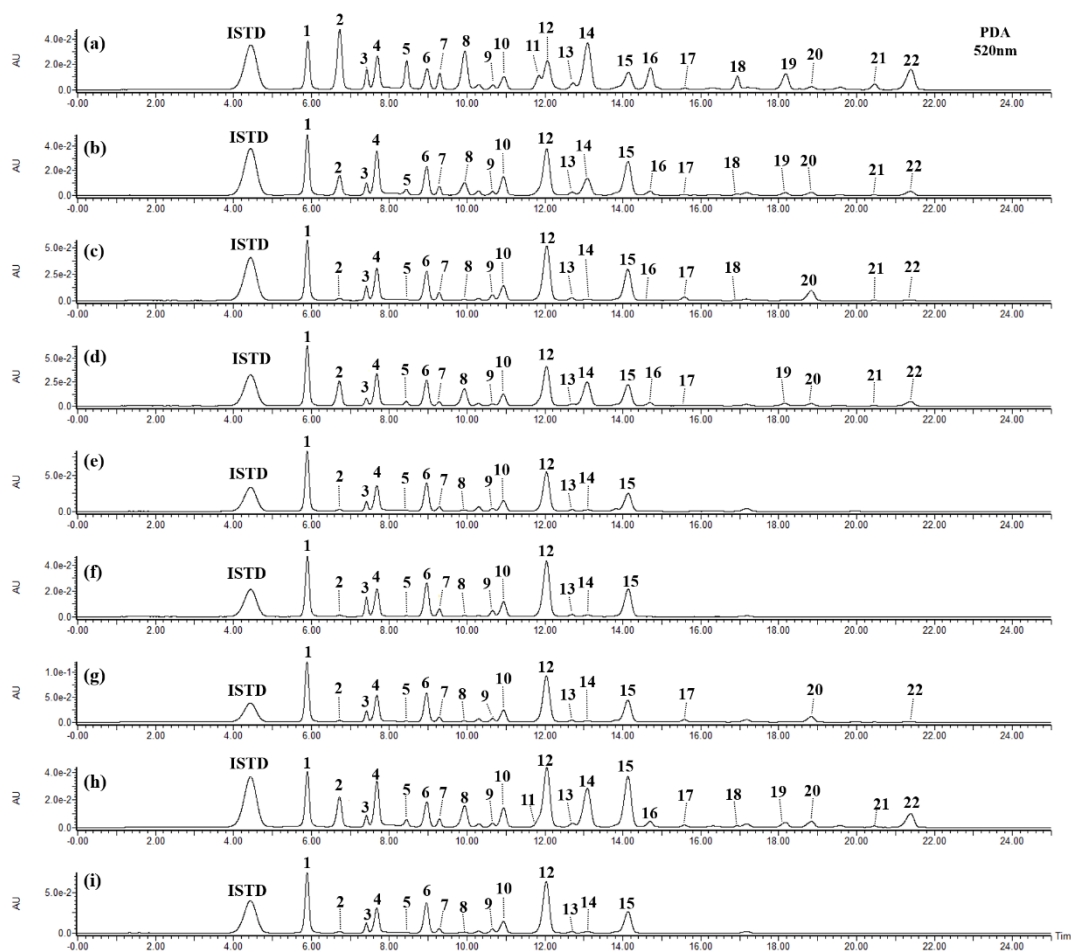
**Figure 1.** Chemical structure of 22 anthocyanin derivatives according to position of functional groups.



**Figure 2.** Fragmentation ( $m/z$ ,  $[M]^+$ ) patterns of two major and seven acetylated anthocyanins identified in highbush blueberry (a, peak 1) Delphinidin 3-*O*-galactoside, (b, peak 12) malvidin 3-*O*-galactoside, (c, peak 16) delphinidin 3-*O*-(6''-*O*-acetyl)glucoside, (d, peak 17) petunidin 3-*O*-(6''-*O*-acetyl)galactoside, (e, peak 18) cyanidin 3-*O*-(6''-*O*-acetyl)glucoside, (f, peak 19) petunidin 3-*O*-(6''-*O*-acetyl)glucoside, (g, peak 20) malvidin 3-*O*-(6''-*O*-acetyl)galactoside, (h, peak 21) peonidin 3-*O*-(6''-*O*-acetyl)glucoside, (i, peak 22) malvidin 3-*O*-(6''-*O*-acetyl)glucoside.



**Figure 3.** MRM chromatograms of 11 mixed standards (a) and UPLC-DAD chromatograms of 22 anthocyanins in Patriot (wavelength at 520 nm) (b). IS (internal standard): delphin 100 ppm.



**Figure 4.** UPLC chromatogram of 22 anthocyanins (wavelength at 520 nm) from Patriot (a), Reka (b), Hannah's choice (c), Spartan (d), Legacy (e), Draper (f), New hanover (g), Suziblue (h), and Farthing (i) highbush blueberry samples (*V. corymboums* L.) are presented according to peak number in Table 4. IS (internal standard): delphin 100 ppm.

Peaks 1 and 4 were identified as delphinidin glycosides. Peak 1 had a fragmentation pattern indicating the loss of galactose or glucose from  $m/z$  465 ( $[M]^+$ ), while peak 4 ( $R_t = 7.79$ ) showed a pattern consistent with arabinose dissociated from  $m/z$  435 ( $[M]^+$ ). Delphinidin derivatives eluted in the following order: peak 1 ( $R_t = 5.99$ ) < peak 2 (delphinidin 3-*O*-glucoside) < peak 4 ( $R_t = 7.52$ ). This elution order is consistent with the previously reported order of compounds with the same aglycone attached to sugars at the 3-OH position (galactose < glucose < arabinose) [27]. Consequently, peaks 1 and 4 were identified as delphinidin 3-*O*-galactoside and delphinidin 3-*O*-arabinoside, respectively.

Peaks 10 and 15 were found to be petunidin and malvidin glycoside, respectively. Based on the parent ion, aglycone ion, and differences in elution times corresponding to the linked sugars, they were further identified as petunidin 3-*O*-arabinoside (peak 10), and malvidin 3-*O*-arabinoside (peak 15).

Meanwhile, acetylated glycosides have been identified in blueberries with fragmented characteristics of acetyl-galactoside (42 + 162 Da) and acetyl-glucoside (42 + 162 Da), where deacetylation did not occur in positive mode [28]. A total of seven acetylated glycosides were identified. Among them, delphinidin 3-*O*-(6''-*O*-acetyl)glucoside (peak 16), cyanidin 3-*O*-(6''-*O*-acetyl)glucoside (peak 18), petunidin 3-*O*-(6''-*O*-acetyl)glucoside (peak 19), and malvidin 3-*O*-(6''-*O*-acetyl)glucoside (peak 22) have been previously reported in berries and wine [29]. Additionally, three acetylated glycosides were identified: petunidin 3-*O*-(6''-*O*-acetyl)galactoside (peak 17), malvidin 3-*O*-(6''-*O*-acetyl)galactoside (peak 20), and peonidin 3-*O*-(6''-*O*-acetyl)glucoside (peak 21). Especially, the acetylated petunidin glycosides (peaks 17 and 19) showed a parent ion at  $m/z$  521 ( $[M]^+$ ), eluted similarly to peaks 6 (petunidin 3-*O*-galactoside) and 8 (petunidin 3-*O*-glucoside), but were detected

later due to the acetyl group at the at the sugar's 6''-OH position [26]. Thus, peaks **17** and **19** were identified as petunidin 3-O-(6''-O-acetyl)galactoside and petunidin 3-O-(6''-O-acetyl)glucoside, respectively.

Peak **16** ( $m/z$  507, 303), **18** ( $m/z$  491, 287), **20** and **22** ( $m/z$  535, 331), and **21** ( $m/z$  505, 301) were assigned to delphinidin 3-O-(6''-O-acetyl)glucoside, cyanidin 3-O-(6''-O-acetyl)glucoside, malvidin 3-O-(6''-O-acetyl)galactoside and malvidin 3-O-(6''-O-acetyl)glucoside, and peonidin 3-O-(6''-O-acetyl)glucoside, respectively, based on their elution times, parent and aglycone ions, and characteristic MS spectra of acetylated glycosides.

### 3.2. Variation in Anthocyanin Contents Depending on Highbush Blueberry Cultivars

Table 5 presents the composition and content of 22 anthocyanins from nine blueberry cultivars. Total anthocyanin content ranged from 581.1 to 1011.7 mg/100 g dry weight (DW), similar to the Chinese results for variation (108.1–279.1 mg/100 g fresh weight) by highbush cultivars (Spartan, Northland, Bluecrop, and Duke) and regions (Dandong, Qingdao, and Zhuanghe) [30]. In total contents, Legacy and New Hanover as mid-season cultivars were 725.4 and 1011.7 mg/100 g, respectively, and the early-season cultivars were observed in the following order: Patriot (910.4) > Spartan (855.5) > Draper (795.3) > Suziblue (771.1) > Reka (635.9) > Farthing (599.3) > Hannah's Choice (581.1).

**Table 5.** Contents of individual anthocyanin glycosides in different highbush blueberry (*Vaccinium corymbosum* L.) cultivars.

Peak No. <sup>1)</sup>	Anthocyanin content (mg/100 g <sub>dry weight</sub> )									
	Reka	Hannah's choice	Spartan	Suziblue	Farthing	Patriot	Draper	Legacy	New Hanover	
	Early-season					Mid-season				
1	86.7 ± 1.4 <sup>d</sup>	94.7 ± 1.8 <sup>d</sup>	123.4 ± 7.7 <sup>c</sup>	71.2 ± 2.1 <sup>e</sup>	119.7 ± 2.6 <sup>c</sup>	72.8 ± 2.5 <sup>e</sup>	164.4 ± 19.4 <sup>b</sup>	165.1 ± 3.1 <sup>b</sup>	200.4 ± 16.8 <sup>a</sup>	±
2*	33.2 ± 1.9 <sup>d</sup>	4.1 ± 0.2 <sup>e</sup>	74.2 ± 2.2 <sup>b</sup>	49.0 ± 2.0 <sup>c</sup>	3.3 ± 1.3 <sup>e</sup>	110.0 ± 0.9 <sup>a</sup>	4.0 ± 0.6 <sup>e</sup>	4.8 ± 0.7 <sup>e</sup>	4.8 ± 0.4 <sup>e</sup>	±
3*	10.7 ± 0.7 <sup>fg</sup>	14.8 ± 0.5 <sup>de</sup>	8.3 ± 0.1 <sup>e</sup>	9.0 ± 0.5 <sup>ge</sup>	12.2 ± 1.4 <sup>ef</sup>	17.0 ± 1.6 <sup>c</sup>	27.7 ± 3.0 <sup>a</sup>	16.2 ± 2.2 <sup>cd</sup>	22.8 ± 2.5 <sup>b</sup>	±
4	65.7 ± 0.9 <sup>cd</sup>	51.8 ± 0.9 <sup>e</sup>	71.1 ± 2.9 <sup>bc</sup>	59.5 ± 2.7 <sup>d</sup>	52.0 ± 1.5 <sup>e</sup>	49.4 ± 2.6 <sup>e</sup>	74.3 ± 6.7 <sup>b</sup>	74.4 ± 1.5 <sup>b</sup>	91.7 ± 7.4 <sup>a</sup>	±
5*	5.4 ± 0.7 <sup>b</sup>	0.7 ± 0.2 <sup>c</sup>	6.9 ± 0.2 <sup>b</sup>	6.2 ± 0.5 <sup>b</sup>	0.4 ± 0.1 <sup>c</sup>	33.7 ± 2.3 <sup>a</sup>	0.7 ± 0.2 <sup>c</sup>	0.6 ± 0.2 <sup>c</sup>	0.6 ± 0.1 <sup>c</sup>	±
6*	84.4 ± 3.7 <sup>de</sup>	102.7 ± 5.9 <sup>d</sup>	108.0 ± 1.5 <sup>d</sup>	73.8 ± 6.0 <sup>ef</sup>	127.8 ± 10.3 <sup>c</sup>	61.9 ± 0.3 <sup>f</sup>	159.4 ± 13.4 <sup>b</sup>	157.3 ± 14.6 <sup>b</sup>	193.6 ± 18.3 <sup>a</sup>	±
7*	7.5 ± 0.8 <sup>c</sup>	7.3 ± 1.2 <sup>c</sup>	3.6 ± 0.3 <sup>f</sup>	6.0 ± 0.5 <sup>de</sup>	5.3 ± 0.4 <sup>e</sup>	13.2 ± 0.9 <sup>a</sup>	11.0 ± 0.1 <sup>b</sup>	7.0 ± 0.6 <sup>cd</sup>	10.6 ± 0.8 <sup>b</sup>	±
8*	22.5 ± 2.1 <sup>d</sup>	2.8 ± 0.0 <sup>e</sup>	56.0 ± 1.0 <sup>b</sup>	38.6 ± 2.6 <sup>c</sup>	2.7 ± 0.1 <sup>e</sup>	72.1 ± 3.9 <sup>a</sup>	3.4 ± 0.1 <sup>e</sup>	3.8 ± 0.3 <sup>e</sup>	3.8 ± 0.3 <sup>e</sup>	±
9*	5.0 ± 1.0 <sup>ef</sup>	8.6 ± 2.1 <sup>b</sup>	3.4 ± 0.1 <sup>g</sup>	4.5 ± 0.2 <sup>f</sup>	7.5 ± 1.1 <sup>bc</sup>	6.1 ± 0.3 <sup>de</sup>	11.2 ± 0.8 <sup>a</sup>	6.7 ± 0.6 <sup>cd</sup>	11.2 ± 1.0 <sup>a</sup>	±

10	35.4 ± 0.5 <sup>d</sup>	±27.1 ± 0.5 <sup>e</sup>	32.8 ± 1.8 <sup>d</sup>	32.5 ± 1.8 <sup>d</sup>	27.8 ± 1.0 <sup>e</sup>	22.7 ± 1.3 <sup>f</sup>	±42.6 ± 1.4 <sup>b</sup>	±39.4 ± 0.8 <sup>c</sup>	±47.5 ± 3.5 <sup>a</sup>
11*	4.4 ± 0.7 <sup>d</sup>	±1.0 ± 0.3 <sup>e</sup>	5.8 ± 0.6 <sup>c</sup>	7.7 ± 0.5 <sup>b</sup>	0.7 ± 0.1 <sup>e</sup>	23.1 ± 1.4 <sup>a</sup>	±0.9 ± 0.2 <sup>e</sup>	0.8 ± 0.1 <sup>e</sup>	0.9 ± 0.1 <sup>e</sup>
12*	103.5 ± 3.2 <sup>d</sup>	±133.8 ± 11.7 <sup>c</sup>	±121.8 ± 3.1 <sup>cd</sup>	±120.9 ± 7.3 <sup>cd</sup>	±157.9 ± 14.0 <sup>b</sup>	±59.9 ± 3.7 <sup>e</sup>	±177.9 ± 15.9 <sup>b</sup>	±157.7 ± 13.1 <sup>b</sup>	±238.1 ± 22.7 <sup>a</sup>
13*	0.8 ± 0.5 <sup>e</sup>	±2.2 ± 0.2 <sup>c</sup>	ND <sup>f</sup>	0.7 ± 0.1 <sup>e</sup>	1.5 ± 0.1 <sup>d</sup>	1.9 ± 0.1 <sup>c</sup>	2.2 ± 0.2 <sup>b</sup>	1.2 ± 0.1 <sup>d</sup>	2.7 ± 1.1 <sup>a</sup>
14*	49.7 ± 2.6 <sup>c</sup>	±3.9 ± 0.3 <sup>d</sup>	109.4 ± 7.9 <sup>b</sup>	±110.5 ± 6.6 <sup>b</sup>	4.0 ± 0.4 <sup>d</sup>	140.9 ± 9.0 <sup>a</sup>	±4.5 ± 0.3 <sup>d</sup>	4.7 ± 0.4 <sup>d</sup>	5.8 ± 0.4 <sup>d</sup>
15	85.1 ± 1.5 <sup>c</sup>	±87.1 ± 1.7 <sup>c</sup>	78.7 ± 3.6 <sup>d</sup>	109.7 ± 5.8 <sup>b</sup>	76.4 ± 2.0 <sup>d</sup>	46.2 ± 3.4 <sup>e</sup>	±111.1 ± 4.7 <sup>b</sup>	±85.6 ± 0.5 <sup>c</sup>	±133.2 ± 7.5 <sup>a</sup>
16	6.9 ± 0.1 <sup>c</sup>	±0.9 ± 0.3 <sup>d</sup>	9.0 ± 0.5 <sup>b</sup>	9.0 ± 0.6 <sup>b</sup>	ND <sup>d</sup>	43.9 ± 2.8 <sup>a</sup>	±ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>
17	1.4 ± 0.0 <sup>e</sup>	±6.5 ± 0.4 <sup>b</sup>	1.7 ± 0.3 <sup>de</sup>	3.2 ± 0.1 <sup>c</sup>	ND <sup>f</sup>	2.1 ± 0.2 <sup>d</sup>	ND <sup>f</sup>	ND <sup>f</sup>	10.5 ± 0.9 <sup>a</sup>
18	2.3 ± 0.5 <sup>b</sup>	±0.9 ± 0.1 <sup>de</sup>	1.3 ± 0.1 <sup>cd</sup>	2.0 ± 0.2 <sup>bc</sup>	ND <sup>e</sup>	21.0 ± 1.1 <sup>a</sup>	±ND <sup>e</sup>	ND <sup>e</sup>	ND <sup>e</sup>
19	5.8 ± 0.4 <sup>c</sup>	±ND <sup>d</sup>	9.8 ± 0.4 <sup>b</sup>	9.3 ± 0.6 <sup>b</sup>	ND <sup>d</sup>	36.0 ± 1.5 <sup>a</sup>	±ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>
20	6.7 ± 0.2 <sup>e</sup>	±26.9 ± 0.5 <sup>b</sup>	8.9 ± 0.3 <sup>d</sup>	11.8 ± 0.8 <sup>c</sup>	ND <sup>f</sup>	6.2 ± 0.3 <sup>e</sup>	ND <sup>f</sup>	ND <sup>f</sup>	31.6 ± 2.0 <sup>a</sup>
21	1.3 ± 0.0 <sup>c</sup>	±0.7 ± 0.0 <sup>d</sup>	0.9 ± 0.1 <sup>cd</sup>	2.1 ± 0.2 <sup>b</sup>	ND <sup>e</sup>	11.2 ± 0.7 <sup>a</sup>	±ND <sup>e</sup>	ND <sup>e</sup>	ND <sup>e</sup>
22	11.5 ± 0.0 <sup>d</sup>	±2.7 ± 0.1 <sup>e</sup>	20.7 ± 0.5 <sup>c</sup>	34.0 ± 1.8 <sup>b</sup>	ND <sup>f</sup>	59.2 ± 3.9 <sup>a</sup>	±ND <sup>f</sup>	ND <sup>f</sup>	1.9 ± 0.1 <sup>e</sup>
Total anthocyanin	635.9 ± 18.9 <sup>d</sup>	±581.1 ± 23.8 <sup>de</sup>	±855.5 ± 27.3 <sup>bc</sup>	±771.1 ± 32.7 <sup>c</sup>	±599.3 ± 12.5 <sup>e</sup>	±910.4 ± 36.8 <sup>ab</sup>	±795.3 ± 31.5 <sup>c</sup>	±725.4 ± 36.8 <sup>ab</sup>	±1011.7 ± 66.4 <sup>a</sup>

<sup>1)</sup> 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, petunidin 3-O-galactoside; 7, cyanidin 3-O-arabinoside; 8, petunidin 3-O-glucoside; 9, peonidin 3-O-galactoside; 10, petunidin 3-O-arabinoside; 11, peonidin 3-O-glucoside; 12, malvidin 3-O-galactoside; 13, peonidin 3-O-arabinoside; 14, malvidin 3-O-glucoside; 15, malvidin 3-O-arabinoside; 16, delphinidin 3-O-(6"-O-acetyl)glucoside; 17, petunidin 3-O-(6"-O-acetyl)galactoside; 18, cyanidin 3-O-(6"-O-acetyl)glucoside; 19, petunidin 3-O-(6"-O-acetyl)glucoside; 20, malvidin 3-O-(6"-O-acetyl)galactoside; 21, peonidin 3-O-(6"-O-acetyl)glucoside; 22, malvidin 3-O-(6"-O-acetyl)glucoside. \*External standard. <sup>a-f</sup>Different superscript letters next to mean values ( $n = 3$ ) indicate significant differences ( $p < 0.05$ ) according to Duncan's multiple range test.

In the two mid-season cultivars (Legacy and New Hanover), malvidin glycosides accounted for 34 and 41% of total content, followed by delphinidin, petunidin, cyanidin, and peonidin derivatives. The seven early-season cultivars (Reka, Hannah's Choice, Spartan, Draper, Suziblue, Farthing, and Patriot) accounting for malvidin 34–50%, delphinidin 24–32%, petunidin 20–26%, cyanidin 2–9%, and peonidin 1–5% were similar with mid-season cultivars. These results indicated that delphinidin, petunidin, and malvidin derivatives affected the total content regardless of cultivar, and also consistent with the aglycone proportions (delphinidin 27–40%, malvidin 22–33%, petunidin 19–26%, cyanidin 6–14%, peonidin 1–5%) reported by Cho et al. [29]. In particular, delphinidin, petunidin, and malvidin derivatives identified as major components are closely associated with blue and purple colors, while cyanidin, peonidin, and pelargonidin contribute to red colors [31].

The color of anthocyanins is influenced by the position and number of hydroxyl (-OH) or methoxyl (-OCH<sub>3</sub>) groups on the B-ring. Furthermore, Jung et al. [32] suggested that the biosynthetic pathway for malvidin is more dominant than that cyanidin in highbush blueberries, which plays a crucial role in the berries' blue color and antioxidant properties.

In other cultivars except for 'Patriot', delphinidin 3-*O*-galactoside, petunidin 3-*O*-galactoside, and malvidin 3-*O*-galactoside were identified as major components [9,10], and the 'Patriot' contained mainly glucose-bound anthocyanins in the present study, where galactose was the predominant glycoside in 'Patriot' blueberries provided from Italy and China [33,34]. The differences in composition and content of blueberry anthocyanins are considered to be generated by cultivars as well as various environmental factors, such as cultivated conditions [35], temperature [36], UV radiation [37], soil conditions [38], and genetic factors [39].

The seven acetylated glycosides (peaks 16–22) accounted for 0–19.7% of total anthocyanin were highest in 'Patriot' (179.5), whereas non-acetylated glycosides were only detected in Legacy, Draper, and Farthing (Table 5). Similarly, acetylated glycosides also showed significant proportions of delphinidin and malvidin derivatives relative to their total content as follows: Delphinidin 3-*O*-(6"-*O*-acetyl)glucoside (2–24%), malvidin 3-*O*-(6"-*O*-acetyl)galactoside (3–72%), malvidin 3-*O*-(6"-*O*-acetyl)glucoside (4–48%) (Table 5). These values were comparable to those reported for Canadian lowbush and highbush blueberries [40].

The synthesis of acylated anthocyanins is facilitated by acyltransferases and has been shown to improve metabolic health, including improved insulin sensitivity, reduced inflammation, and regulation of gut microbiota [41]. In particular, malvidin 3-*O*-(6"-*O*-acetyl)glucoside presented free radical scavenging activity [42] and potential anti-inflammatory effects via selective COX-2 inhibition [43]. Further research is required to investigate not only the *in vivo* metabolism and biological activities of these anthocyanins including acetylated glycosides, but also the changes of anthocyanin profile by blueberry cultivar, harvest time, cultivated methods, storage conditions, etc..

#### 4. Conclusions

A total of 22 anthocyanins from nine highbush blueberry cultivars include 4 delphinidin, 4 cyanidin, 5 petunidin, 4 peonidin, and 5 malvidin derivatives. 'New Hanover' (1011.7 mg/100 g DW) showed the highest total anthocyanin content among all cultivars, with malvidin and delphinidin glycosides accounting for over 70% of its total content. Especially, 'Patriot' (910.4), an early-season cultivar, recorded the highest proportion of acetylated anthocyanins (up to 19.7%), while non-acetylated anthocyanins were only detected in 'Legacy,' 'Draper,' and 'Farthing.' These study provided detailed chemical profiles on both major and trace components of acetylated anthocyanins from nine cultivars grown in Korea. Furthermore, it is believed that a comprehensive anthocyanin profile of blueberries will be suggested as fundamental data for breeding superior cultivars, evaluation of related products, and application in various industries.

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