JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Correlation between Species-Specific Metabolite Profiles and Bioactivities of Blueberries (*Vaccinium* spp.)

Sarah Lee,[†] Eun Sung Jung,[†] Seon-Gil Do,[‡] Ga-young Jung,[‡] Gwanpil Song,[§] Jung-min Song,[§] and Choong Hwan Lee*[,†]

[†]Division of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea

[‡]Wellness R & D Center, Univera, Inc, Seoul, 133-120, Republic of Korea

[§]Jeju Environment Research Institute Co., Ltd., JeJu 695-791, Republic of Korea

Supporting Information

ABSTRACT: Metabolite profiling of three blueberry species (*Vaccinium bracteatum* Thunb., *V. oldhamii* Miquel., and *V. corymbosum* L.) was performed using gas chromatography−time-of-flight-mass spectrometry (GC-TOF-MS) and ultra-performance liquid chromatography−quadrupole-time-of-flight-mass spectrometry (UPLC-Q-TOF-MS) combined multivariate analysis. Partial least-squares discriminant analysis clearly showed metabolic differences among species. GC-TOF-MS analysis revealed significant differences in amino acids, organic acids, fatty acids, sugars, and phenolic acids among the three blueberry species. UPLC-Q-TOF-MS analysis indicated that anthocyanins were the major metabolites distinguishing *V. bracteatum* from *V. oldhamii*. The contents of anthocyanins such as glycosides of cyanidin were high in *V. bracteatum*, while glycosides of delphinidin, petunidin, and malvidin were high in *V. oldhamii*. Antioxidant activities assessed using ABTS and DPPH assays showed the greatest activity in *V. oldhamii* and revealed the highest correlation with total phenolic, total flavonoid, and total anthocyanin contents and their metabolites.

KEYWORDS: *blueberries, metabolite profiling, antioxidant activity, correlation, GC-TOF-MS, UPLC-Q-TOF-MS*

INTRODUCTION

The genus *Vaccinium* (family Ericaceae) comprises more than 130 species, of which some produce edible berries; these include blueberry, cranberry, lingonberry, sparkleberry, huckleberry, whortleberry, and bilberry.[1,2] Among these species, blueberries are the major commercial crops; three main species of blueberries, highbush (*V. corymbosum* L.), lowbush (*V. angustifolium* Ait.), and rabbiteye (*V. ashei* Reade),[3] are grown commercially. A number of anthocyanins, flavonols, and phenolic acids have been identified in different blueberries, and these compounds are generally recognized as potential sources of antioxidants.[4−10] *V. oldhamii* Miquel. and *V. bracteatum* Thunb., which are wild blueberries, have been used in Korea and China as folk medicines to treat inflammation, gonorrhea, vomiting, diarrhea, and skin eruptions.[11,12] Higher contents of polyphenols, anthocyanins, and *β*-carotene and greater antioxidant activity have been found in Korean wild blueberries than in the northern highbush blueberry (*V. corymbosum*).[13]

A metabolomic approach has increasingly been used to characterize natural variance and to compare the comprehensive metabolic composition of plants.[13] This technique can provide a diagnostic tool for a better understanding of biological systems and has been successfully used for diverse plant species, such as *Arabidopsis*,[14] strawberry,[15] bilberry,[16] and tomato.[17] Several instruments have been used for nontargeted metabolite profiling in plants. Separation-based mass spectrometry approaches, such as gas chromatography−mass spectrometry (GC-MS) and liquid chromatography−mass spectrometry (LC-MS),[18,19] and a combination of these spectroscopic platforms have been used to detect broader classes of metabolites and thus provide a powerful analytical tool for metabolomics in plants.[20]

Currently, blueberries are widely promoted as rich sources of antioxidants. In this study, we focused on further characterization of wild berries as possible sources of phenolics for functional food applications. We compared differences in the metabolite composition and antioxidant activities of two Korean native wild species of *Vaccinium* (*V. oldhamii* and *V. bracteatum*) and one commercial blueberry cultivar (*V. corymbosum*). Combined MS techniques and multivariate analysis were used to characterize metabolic differences and identify the major antioxidant compounds of these three species.

MATERIALS AND METHODS

Chemicals and Reagents. Methanol, acetonitrile, chloroform, hexane, and water were purchased from Fisher Scientific (Pittsburgh, PA, USA). Methoxyamine hydrochloride, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), diethylene glycol, gallic acid, naringin, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), hydrochloric acid, potassium persulfate, 2,2″-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), formic acid, pyridine, and standard compounds were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Received: November 21, 2013
Revised: January 22, 2014
Accepted: February 2, 2014
Published: February 2, 2014

dx.doi.org/10.1021/jf405272b | J. Agric. Food Chem. 2014, 62, 2126−2133

Plant Materials. Three blueberry species, *V. bracteatum* Thunb. (VB), *V. oldhamii* Miquel. (VO), and *V. corymbosum* L. (VC), were investigated in this study. VB and VO samples were collected from Sanghyo-dong, Seogwipo-si, Jeju Island, South Korea; annual precipitation in this area is approximately 2000 mm, and they are approximately 300 and 500 m above sea level, respectively. VC, which is a common highbush blueberry, was harvested from Sagye-ri, Andeok-myeon, Seogwipo-si, Jeju Island, South Korea. This region is 100 m above sea level and receives 1400 mm of rainfall annually. The blueberry samples were freeze-dried for 5 days and stored at below −70 °C before extraction.

Sample Preparation. Dried samples were homogenized using a mortar and pestle. Each powdered sample (50 mg) was extracted with 1 mL of solvent mixture (methanol/water/chloroform = 2.5:1:1) by using a Retsch MM400 mixer mill (Retsch GmbH & Co, Haan, Germany) at 30 Hz/s for 10 min and centrifuged at 4 °C and 1200 rpm for 5 min. The supernatant (100 μL) was completely dried using a speed vacuum concentrator (Biotron, Seoul, Korea). For gas chromatography−time-of-flight-mass spectrometry (GC-TOF-MS) analysis, two derivatization steps were performed. First, oximation was conducted by dissolving the dried extracts in 50 μL of methoxyamine hydrochloride (20 mg/mL in pyridine) and reacting at 30 °C for 90 min. The samples were then silylated with 50 μL of MSTFA at 37 °C for 30 min. For ultraperformance liquid chromatography−quadrupole-time-of-flight-mass spectrometry (UPLC-Q-TOF-MS) analysis, dried extracts of supernatant (100 μL) were resolved with methanol and filtered through a 0.2 μm PTFE filter. Five biological and two analytical replications were performed using both GC-TOF-MS and UPLC-Q-TOF-MS analyses.

GC-TOF-MS Analysis. An Agilent 7890A GC system (Palo Alto, CA) equipped with an Agilent 7693 autosampler was attached to a TOF Pegasus III mass spectrometer (Leco, St. Joseph, MI, USA), operating in electron ionization (EI) mode (70 eV). A DB-5MS column (30 m length × 0.25 mm i.d. × 0.25 m film thickness, J & W Scientific, Folsom, CA, USA) was used with helium as a carrier gas at a constant flow of 1.5 mL/min. Then, 1 μL of the derivatized sample was injected in a splitless mode. The oven temperature was maintained at 75 °C for 2 min, increased to 300 °C at the rate of 15 °C/min, and then held at 300 °C for 3 min. The acquisition rate was set to 20 scans/s with a mass scan range of m/z 45−1000. The injector and ion source temperatures were 250 and 230 °C, respectively.

UPLC-Q-TOF-MS Analysis. A Waters ACQUITY UPLC system (Waters Corp., Milford, MA, USA) equipped with a binary solvent delivery system, an autosampler, and a UV detector was combined with a Waters Q-TOF Premier MS (Micromass MS Technologies, Manchester, UK) system. Aliquots (5 μL) of each sample were then injected into an ACQUITY BEH C_{18} column (100 mm × 2.1 mm i.d. × 1.7 μm particle size) at a flow rate of 0.3 mL/min. The elution was performed with an acetonitrile/water gradient containing 0.1% formic acid. The gradient was linearly increased from 5% to 100% acetonitrile over 10 min and then decreased to 5% over 2 min. The total run time, including re-equilibration of the column to the initial conditions, was 12 min. The mass spectrometer was operated in both negative and positive ion modes with an m/z range 100−1000. The desolvation gas (nitrogen) was set to 650 L/h at a temperature of 300 °C. The cone gas (nitrogen) was set to 50 L/h, and the source temperature was 80 °C. The capillary and cone voltages were set to 2.3 kV and 30 V, respectively. Data were collected in the centroid mode, with a scan accumulation time of 0.2 s. All analyses were performed using an independent reference spray via the LockSpray interference to ensure accuracy and reproducibility; leucine enkephalin ions were used as the lock mass (m/z 554.2615 [−] and 556.2771 [+]) at a flow rate of 10 μL/min. Accurate mass and elemental composition were calculated using the MassLynx software (Waters Corp.) incorporated in the instrument.

The LC-IT-MS/MS analysis was performed using a 212-LC binary solvent delivery system equipped with a Prostar 410 autosampler and a Prostar 335 photodiode array detector (PDA), which was coupled with a Varian 500-MS ion-trap mass spectrometer equipped with an electrospray interface (Varian Tech., Palo Alto, CA, USA). Aliquots (10 μL) of each sample were then injected into a PurSuit XRs C18 column (100 mm × 2.0 mm i.d., 3 μm; Varian, Lake Forest, CA, USA) with a MetaGuard 2.0 PurSuit XRs C18 guard column (Varian, Lake Forest, CA, USA). The column temperature was set at 40 °C, and the flow rate was 0.2 mL/min. The mobile phase consisted of 0.1% v/v formic acid in water (solvent A) and 0.1% v/v formic acid in acetonitrile (solvent B). The gradient applied was as follows: 10% B from 0 to 2 min, 40% B from 2 to 10 min, 70% B from 10 to 20 min, 90% B from 20 to 30 min, 10% B from 30 to 40 min, and held at 10% B before the next sample injection. Mass spectra were performed in both negative and positive modes through a range of m/z 100−1000. The parameters were as follows: drying temperature, 300 °C; capillary voltage, 70 V; needle voltage, 5 kV; drying gas pressure (nitrogen), 20 psi; and nebulizer gas pressure (air), 40 psi. MS^n analysis was performed using Turbo data-dependent scanning (DDS) under the same conditions used for full scanning. The PDA was set at an absorbance from 200 to 600 nm and managed by Polyview 2000 (version 6.9) (Varian, Walnut Creek, CA).

Data Processing and Statistical Analysis. GC-TOF-MS raw data were converted to netCDF format with ChromaTOF software (LECO). UPLC-Q-TOF-MS raw data were acquired with MassLynx software and converted into netCDF format with Waters DataBridge version 2.1 software. After conversion, the MS data were processed using the Metalign software package (http://www.metalign.nl). The resulting data were exported to Excel (Microsoft, Redmond, WA, USA), and a statistical analysis was performed using SIMCA-P+ 12.0 software (Umetrics, Umea, Sweden). Principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were used to compare the three blueberry species and to identify the major metabolites in each. The data sets were unit variance scaled in a column-wise manner prior to PCA and PLS-DA modeling. Metabolites with a variable importance projection (VIP) value greater than 0.7 and a p value less than 0.05 were selected as potential metabolites related to different species. Differences in total phenolic content (TP), total flavonoid content (TF), total anthocyanin content (TA), DPPH, and ABTS radical scavenging activities were tested by analysis of variance and Duncan's multiple range test using SPSS version 12.0 software (SPSS Inc., Chicago, IL, USA). Relative contents of significantly different metabolites and antioxidant activities were represented by Pearson's correlation coefficient.

Metabolite Identification and Visualization. After the multivariate statistical analysis, major metabolites were positively identified using standard compounds by comparing both the mass spectra and retention times. When standard compounds were not available, a tentative identification was made based on databases from the NIST05 MS Library (NIST, 2005), published references, and an in-house library. In UPLC-Q-TOF-MS analysis, accurate masses and elemental compositions were calculated using MassLynx software (Waters Corp.). MS^n fragmentation patterns and UV spectra were also used to identify major metabolites with MS workstation software (version 6.9, Varian, USA) in LC-IT-MS/MS. Metabolites were quantified by their m/z peak areas calculated using the software provided with the instrument and by data processing. This quantification aided in making relative comparisons of the metabolites in the three blueberry species. To visualize metabolite profiles, heatmaps were generated using MultiExperiment Viewer software version 4.8 (http://www.tm4.org/), and correlation networks between metabolites and antioxidant activities were constructed with Cytoscape software (http://www.cytoscape.org/).

Determination of Antioxidant Activities by ABTS and DPPH Free Radical Scavenging Activity. For the ABTS assay, we followed the method of Re et al.[21] with some modifications. The stock solutions included 7 mM ABTS solution and 2.45 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 1 day at room temperature in the dark. The solution was then diluted until the absorbance reached 0.7 ± 0.02 at 734 nm by using a spectrophotometer (Spectronic Genesys 6, Thermo Electron, Madison, WI, USA). The dried extracts of supernatant were resolved in a solvent mixture and diluted in the ratio of 1:4. Each diluted sample

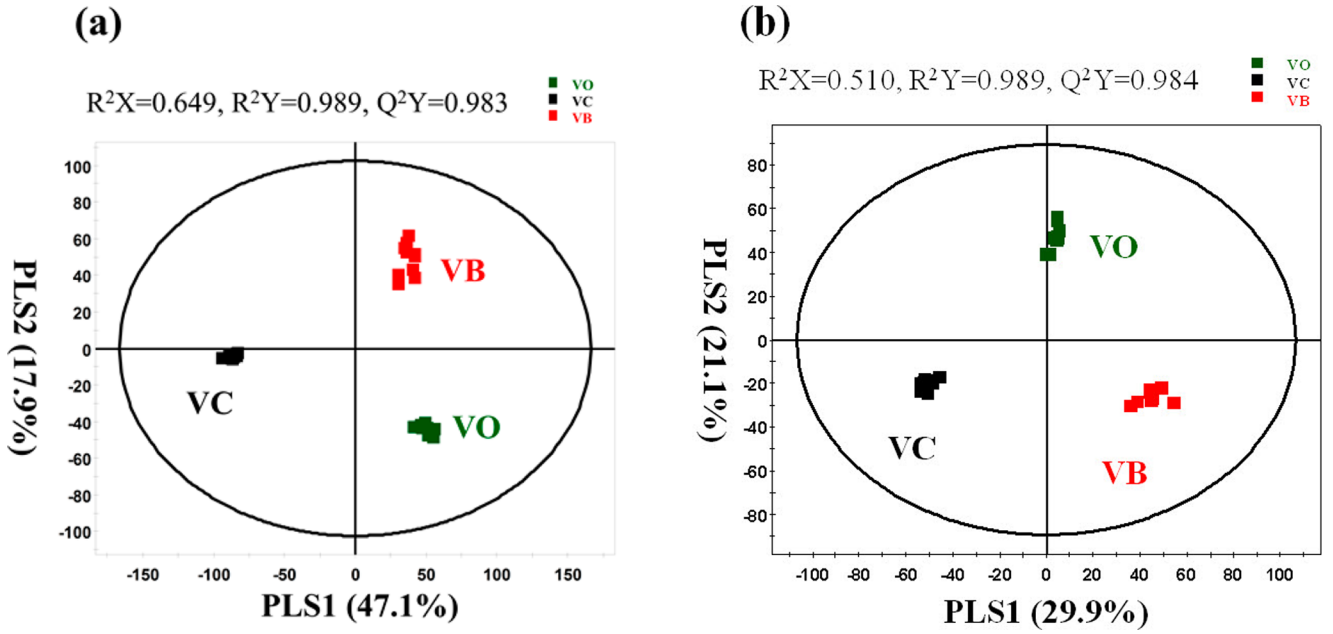

Figure 1. PLS-DA score plots of metabolite profiles in three blueberry species (*Vaccinium bracteatum* Thunb., VB; *V. oldhamii* Miquel., VO; and *V. corymbosum* L., VC). The data sets were obtained using (a) GC-TOF-MS and (b) UPLC-Q-TOF-MS.

of blueberry extract (10 μL) was reacted with 190 μL of the diluted ABTS solution for 7 min in the dark. Absorbance was then measured at 734 nm using a spectrophotometer. The standard curve was linear between 0.0625 and 1 mM Trolox equivalents (TE). Results are expressed in millimolar TE per milligram of dry weight of extract (ext). The DPPH assay was conducted according to the method described by Dietz et al.[22] with some modifications. Diluted samples of blueberry extracts (20 μL) were reacted with 180 μM DPPH solution for 20 min at room temperature. Absorbance was then measured at 515 nm. The standard curve was linear between 0.0625 and 1 mM TE. Experiments were conducted in triplicate.

Total Phenolic Content and Total Flavonoid Content. Total phenolic content (TP) in blueberry samples was determined according to the Folin−Ciocalteu colorimetric method, as modified by Yildirim et al.[23] Briefly, 100 μL of 0.2 N Folin−Ciocalteu's phenol reagent was added to 20 μL of each sample placed in 96-well plates, followed by incubation in the dark for 5 min. After that, 80 μL of 7.5% sodium carbonate solution was added to the mixture and measured at 750 nm using a microplate reader (Thermo Electron, Spectronic Genesys 6, Madison, WI, USA). TP was calculated on the basis of a standard curve with gallic acid (GA) equivalent concentration (ppm). The standard solution concentration curve ranged from 31.25 to 500 ppm. All experiments were conducted in triplicate.

Total flavonoid content (TF) was measured, and 180 μL of 90% diethylene glycol, 20 μL of 1 N NaOH, and 20 μL of each sample were then mixed and incubated for 60 min at room temperature in the dark. Absorbance was measured at 405 nm using a microplate reader (Thermo Electron, Spectronic Genesys 6, Madison, WI, USA). The results are presented as naringin (NG) equivalent concentration (ppm). The standard solution concentration curve ranged from 15.625 to 2000 ppm. All experiments were conducted in triplicate.

Total Anthocyanin Content. Total anthocyanin content (TA) of the blueberry samples was estimated using a microplate reader (Thermo Electron, Spectronic Genesys 6, Madison, WI, USA) by the pH-differential method using two buffer systems, potassium chloride buffer, pH 1.0, and sodium acetate buffer, pH 4.5, as modified by Khanal et al.[24] A diluted sample of 20 μL was mixed with 180 μL of corresponding buffer and read against a blank at 515 and 750 nm. Absorbance was calculated by the following formula: $A = (A_{515\ nm} - A_{750\ nm})_{pH1.0} - (A_{515\ nm} - A_{750\ nm})_{pH4.5}$. Monomeric anthocyanin pigment concentration was expressed as milligrams of cyanidin-3-glucoside: monomeric anthocyanin pigment (mg/L) = $A \times M_r \times$ DF $\times 1000/(\varepsilon \times 1)$, where A is the absorbance, M_r is the molecular weight (449.2), DF is the dilution factor, and ε is the molar absorptivity (26 900). The final concentration of anthocyanins (mg/100 g) was calculated based on the total volume of extract and the weight of the sample.

RESULTS AND DISCUSSION

Multivariate Analysis of *Vaccinium* spp. Using GC-TOF-MS and UPLC-Q-TOF-MS Data. Species-specific metabolite profiling of blueberries (*Vaccinium* spp.) was performed using GC-TOF-MS and UPLC-Q-TOF-MS combined multivariate analysis. Multivariate analysis was used to separate species and to identify species-specific metabolites in three blueberry species. The analysis was performed by both unsupervised PCA and by supervised PLS-DA to identify differences among the three species. Species-specific separation can be observed in the PLS-DA score plots (Figure 1). Similar patterns were found in the PCA analysis (Supporting Information, Figure 1S). As shown in Figure 1a, metabolite profiles of the three species (VO, VB, and VC) were clearly separated by PLS1 and PLS2, and the score plot of GC-TOF-MS data explained 65.0% of the total variability (PLS1: 47.1%; PLS2: 17.9%). VC was separated from VB and VO along PLS1, whereas VB was separated from VO and VC along PLS2. The satisfaction values of X and Y variables were 0.649 (R^2X) and 0.989 (R^2Y), respectively, and prediction accuracy was 0.984 (Q^2Y). PCA and PLS-DA models of metabolites analyzed by UPLC-Q-TOF-MS showed similar patterns as those obtained by GC-TOF-MS analysis (Figure 1b, Supporting Information, Figure 1S). In the score plot of PLS-DA (using positive ion mode data), VC was clearly discriminated by PLS1 (29.9%), and VO was separated by PLS2 (21.1%), with quality parameters of 0.510 (R^2X), 0.989 (R^2Y), and 0.984 (Q^2Y). By using negative ion mode data, VB was separated from VC by PLS1 (27.7%), and VO was separated by PLS2 (15.5%) (Supporting Information, Figure 2S).

Species-Specific Metabolite Profiling of *Vaccinium* spp. Using GC-TOF-MS and UPLC-Q-TOF-MS Analyses.

dx.doi.org/10.1021/jf405272b | J. Agric. Food Chem. 2014, 62, 2126−2133

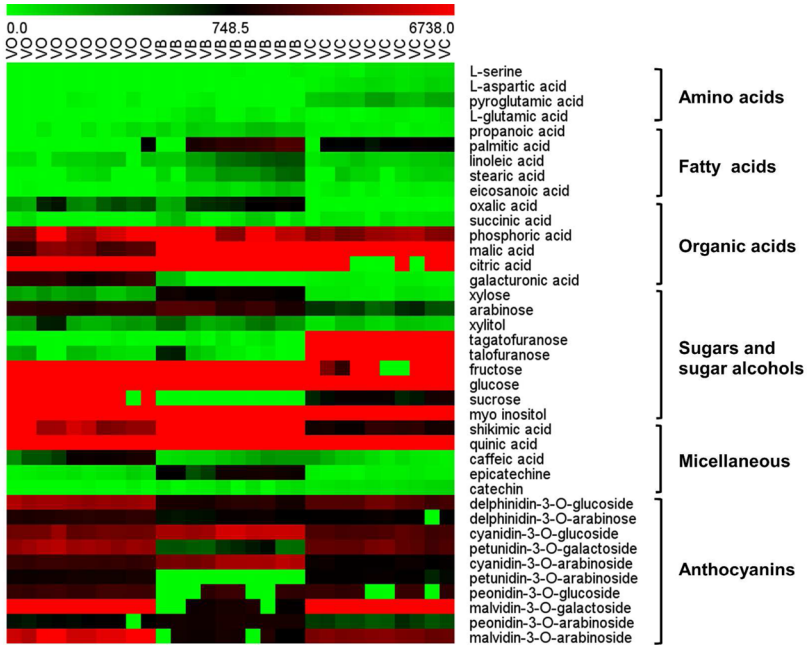

Figure 2. Key metabolites identified using PLS-DA showed a differential pattern in different blueberry species (*Vaccinium bracteatum* Thunb., VB; *V. oldhamii* Miquel., VO; and *V. corymbosum* L., VC). Metabolites (n = 39) that show statistically significant changes among samples (VIP > 0.7 and p < 0.05) are represented using a heatmap, with relative intensities indicated by the heat scale.

Table 1. Differential Metabolites Analyzed Using GC-TOF-MS in Three Species of Blueberry (*Vaccinium* spp.)

VarID	t_R (min)[a]	identified ion (m/z)[b]	VIP value	derivatized[c]	tentative metabolite[d]	ID[e]
4139	5.65	147	1.18	$(TMS)_2$	oxalic acid	MS/STD
7296	7.05	299	0.72	$(TMS)_3$	phosphoric acid	MS/STD
4142	7.39	147	0.92	$(TMS)_2$	succinic acid	MS/STD
5607	7.86	204	1.12	$(TMS)_2$	L-serine	MS/STD
4147	8.98	147	0.82	$(TMS)_3$	malic acid	MS/STD
6369	9.25	232	1.32	$(TMS)_3$	L-aspartic acid	MS/STD
4517	9.30	156	1.39	$(TMS)_2$	pyroglutamic acid	MS/STD
6609	10.03	246	1.01	$(TMS)_3$	L-glutamic acid	MS/STD
7406	10.39	307	0.75	$(TMS)_4$	xylose	MS/STD
7407	10.44	307	1.01	$(TMS)_4$	arabinose	MS/STD
4165	10.87	147	1.01	$(TMS)_5$	xylitol	MS/STD
5613	11.44	204	0.99	$(TMS)_4$	shikimic acid	MS/STD
5970	11.50	217	1.44	$(TMS)_4$	tagatofuranose	MS
6987	11.56	273	1.21	$(TMS)_4$	citric acid	MS/STD
5973	11.75	217	1.42	$(TMS)_4$	talofuranose	MS
7862	11.90	345	1.39	$(TMS)_5$	quinic acid	MS/STD
7414	11.99	307	1.36	MeOX $(TMS)_5$	fructose	MS/STD
7569	12.18	319	1.32	MeOX $(TMS)_5$	glucose	MS/STD
7759	12.48	333	0.81	$(TMS)_4$	galacturonic acid	MS/STD
3173	12.95	117	1.26	TMS	palmitic acid	MS/STD
7384	13.41	305	1.63	$(TMS)_6$	*myo*-inositol	MS/STD
6119	13.57	219	0.79	$(TMS)_4$	caffeic acid	MS/STD
3520	13.98	129	1.30	TMS	linoleic acid	MS/STD
3190	14.13	117	1.25	TMS	stearic acid	MS/STD
3196	15.22	117	1.34	TMS	eicosanoic acid	MS
8018	16.47	361	1.22	$(TMS)_8$	sucrose	MS/STD
8106	17.43	368	1.48	$(TMS)_5$	epicatechine	MS/STD
8107	17.54	368	0.96	$(TMS)_5$	catechin	MS/STD

[a] t_R is the retention time. [b] m/z are the selected ion(s) for identification and quantification of individual derivatized metabolites. [c] MEOX, methyloxime; TMS, trimethylsilyl. [d] Identified metabolites based on variable importance projection (VIP) analysis with a cutoff value of 0.7 and a p value < 0.05. [e] Identification: MS mass spectrum was consistent with those of NIST and in-house libraries; STD mass spectrum was consistent with those of standard compounds.

dx.doi.org/10.1021/jf405272b | J. Agric. Food Chem. 2014, 62, 2126−2133

Table 2. Differential Metabolites Identified Using UPLC-Q-TOF-MS in Three Species of Blueberry (*Vaccinium* spp.)

			UPLC-Q-TOF-MS				LC-IT-MS/MS				
no.	t_R[a]	VIP value	Experimental mass $[M + H]^+$	formula	Δppm	iFit[b]	$[M + H]^+$	MS^n fragment ions (m/z)	UV (nm)	tentative metabolite[c]	ID[d]
1	2.86	1.48	465.1031	$C_{21}H_{21}O_{12}$	−0.4	0.9	465	465 > 303 > 257	275, 524	delphinidin-3-*O*-glucoside	ref
2	3.00	1.39	435.0901	$C_{20}H_{19}O_{11}$	−0.6	0.3	435	435 > 303	300, 522	delphinidin-3-*O*-arabinose	ref
3	3.04	1.24	449.1088	$C_{21}H_{21}O_{11}$	0.9	0.0	449	449 > 287 > 213	297, 523	cyanidin-3-*O*-glucoside	std
4	3.11	1.45	479.1195	$C_{22}H_{23}O_{12}$	1.0	0.0	479	479 > 317 > 302	299, 521	petunidin-3-*O*-galactoside	ref
5	3.20	1.27	419.0982	$C_{20}H_{19}O_{10}$	1.0	0.0	419	419 > 287 > 213	299, 520	cyanidin-3-*O*-arabinoside	ref
6	3.23	1.44	449.1057	$C_{21}H_{21}O_{11}$	−6.0	0.0	449	449 > 317 > 302	302, 527	petunidin-3-*O*-arabinoside	ref
7	3.29	0.79	463.1132	$C_{22}H_{23}O_{11}$	−1.5	0.0	463	463 > 301	307, 522	peonidin-3-*O*-glucoside	std
8	3.31	1.36	493.1337	$C_{23}H_{25}O_{12}$	−1.8	0.0	493	493 > 331 > 299	313, 527	malvidin-3-*O*-galactoside	std
9	3.45	1.2	433.1105	$C_{21}H_{21}O_{10}$	−6.9	0.0	433	433 > 301	307, 522	peonidin-3-*O*-arabinoside	ref
10	3.47	1.46	463.1225	$C_{22}H_{23}O_{11}$	3.2	0.1	463	463 > 331 > 299	312, 534	malvidin-3-*O*-arabinoside	ref

[a] t_R is the retention time. [b] iFit is a measure of how well the observed isotope pattern matches the predicted isotope pattern for the formula on that line. A lower score indicates a better fit. [c] Identified metabolites based on variable importance projection (VIP) analysis with a cutoff value of 0.7 and a p value < 0.05. [d] Identification: std, standard compound; ref, references.

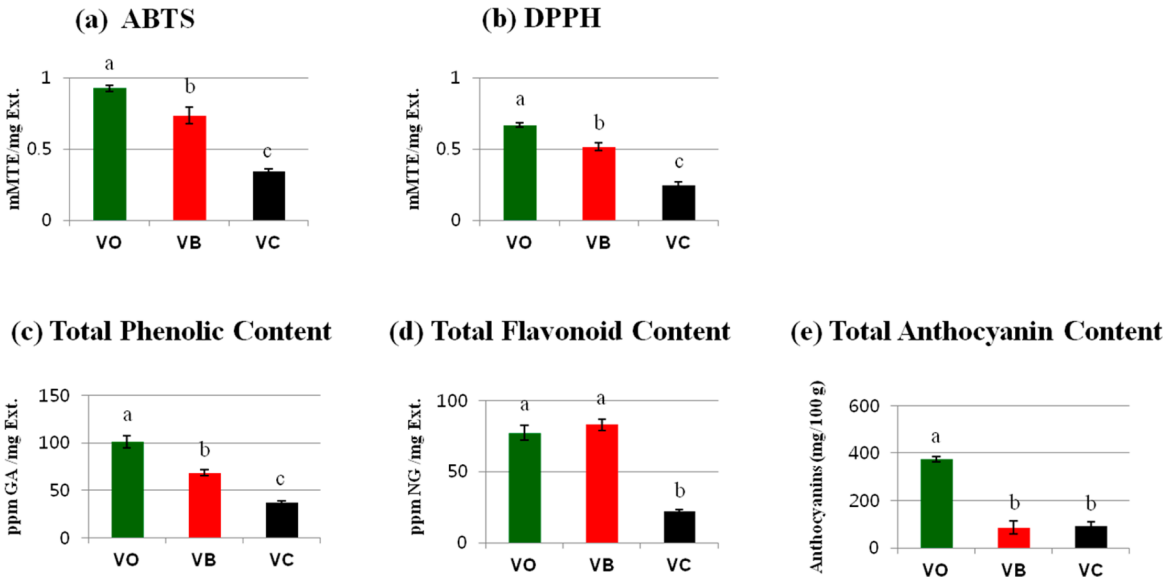

Figure 3. Antioxidant activity tests (a) ABTS and (b) DPPH and (c) total phenolic content, (d) total flavonoid content, and (e) total anthocyanin content of blueberries (*Vaccinium* spp.). Values are the averages of triplicates (n = 3). The same letters are not significantly different by Duncan's multiple range test at the 5% level.

Species-specific separation of *Vaccinium* spp. was observed in the PLS-DA score plots (Figure 1), and their related metabolites were selected based on VIP values (VIP > 0.7) and p values (p < 0.05). The relative differential distribution of selected metabolites is represented by a heatmap (Figure 2). In the GC-TOF-MS analysis, metabolite profiles of the three species were clearly separated by PLS1 and PLS2. In Table 1, 28 metabolites, including amino acids, organic acids, fatty acids, sugar and sugar alcohols, and phenolic acids, were selected as differential variables using VIP values and p values. On the basis of the separation of the three species according to PLS1, we identified oxalic acid, phosphoric acid, xylose, arabinose, xylitol, shikimic acid, citric acid, quinic acid, fructose, and glucose as major metabolites in both VO and VB, while serine, aspartic acid, tagatofuranose, talofuranose, and catechin were associated with VC. In particular, VB contained much higher levels of fatty acids, that is, palmitic acid, linoleic acid, stearic acid, and eicosanoic acid (Figure 2 and Supporting Information, Table 1S). In contrast, citric acid, quinic acid, fructose, glucose, galacturonic acid, caffeic acid, and sucrose were the main metabolites in VO. Organic acids such as oxalic acid, phosphoric acid, and citric acid were associated with both VO and VB, while succinic acid was associated with VB. Malic acid and citric acid are the predominant organic acids related to fruit ripening, and citric acid is the major compound that contributes to the acidity of fruits.[25] Citric acid was most abundant in VO (Figure 2 and Supporting Information, Table 1S). Levels of amino acids such as serine, aspartic acid, and

dx.doi.org/10.1021/jf405272b | J. Agric. Food Chem. 2014, 62, 2126−2133

pyroglutamic acid were higher in VC, while phenolic acids such as quinic acid and caffeic acid, which are related to antioxidant activity, were main components of VO.

In the UPLC-Q-TOF-MS positive ion mode analysis, the three species were separated by PLS-DA, and their related metabolites are listed in Table 2. In total, 10 metabolites were identified as highly differential metabolites contributing to species-specific separation based on VIP values (VIP > 0.7) and p values ($p < 0.05$). Anthocyanins were identified as major metabolites separating the three species. Anthocyanins and their derivatives were identified based on specific MS fragments and UV−vis absorbance (Table 2). Anthocyanins were represented by their specific aglycone cations and showed particular UV absorbance at 520−534 nm. The MS^n fragments of these compounds had characteristic ions at m/z 303, 287, 317, 301, and 331, corresponding to the $[M - sugar]^+$ product ions of delphinidin, cyanidin, petunidin, peonidin, and malvidin, respectively. The 3-glucoside(s) and 3-galactoside(s) of delphinidin, malvidin, petunidin, cyanidin, and peonidin are the primary anthocyanins that have been identified in blueberries,[1,26] and they were confirmed in this study. Glucoside and galactoside of delphinidin, petunidin, peonidin, and malvidin were mainly detected in VO and VC; in particular, the levels of delphinidin derivatives (delphinidin-3-*O*-glucoside and delphinidin-3-*O*-arabinoside) were high in VO (Figure 2 and Supporting Information, Table 2S). Cyanidin derivatives (cyanidin-3-*O*-glucoside and cyanidin-3-*O*-arabinoside) were mostly identified in VB, and their relative contents were high in this species. In the UPLC-Q-TOF-MS negative ion mode analysis, 11 metabolites, including chlorogenic acid, quercetin-3-*O*-glucoside, and quercetin-3-*O*-rutinoside, were identified as the major metabolites separating the three species (Supporting Information, Figure 2S and Table 3S). Chlorogenic acid is considered a prominent compound in highbush blueberry (*Vaccinium corymbosum* L.),[5] and levels of this compound were also high in VO as well as VC in our study (Supporting Information, Table 3S). Quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside are also known compounds in berries.[27] These metabolites showed high levels in VO and VC, respectively.

Correlations between Metabolites and Antioxidant Activities in Different Blueberries. Antioxidant activities (ABTS and DPPH), TP, TF, and TA were measured in the three species. As shown in Figure 3, the wild species (VO and VB) demonstrated significantly higher scavenging activities than VC, which is a commonly cultivated species. TP and TF of VO and VB were also higher than those of VC. In particular, VO showed the highest TP (100.69 ppm GA/mg ext), TA (374.06 mg/100 g ext), and antioxidant activities (ABTS, 0.93 mM TE/mg ext; DPPH, 0.67 mM TE/mg ext) among the three species.

Correlation networks between metabolites and antioxidant activities are illustrated in Figure 4. Pearson's correlation coefficients among all metabolites identified in GC/LC-MS, TP, TF, TA, and antioxidant activity tests (ABTS and DPPH) were calculated (Supporting Information, Table 3S). Selected metabolites, TP, TF, and TA showed positive or negative correlations with antioxidant activities. Correlation coefficients of TP and TF with antioxidant activities were higher than that of TA. In particular, TP was much more strongly correlated with antioxidant activities (DPPH, 0.986; ABTS, 0.978) than was TA (DPPH, 0.766; ABTS, 0.737). Similar results were reported by Koca et al., who found a high correlation between

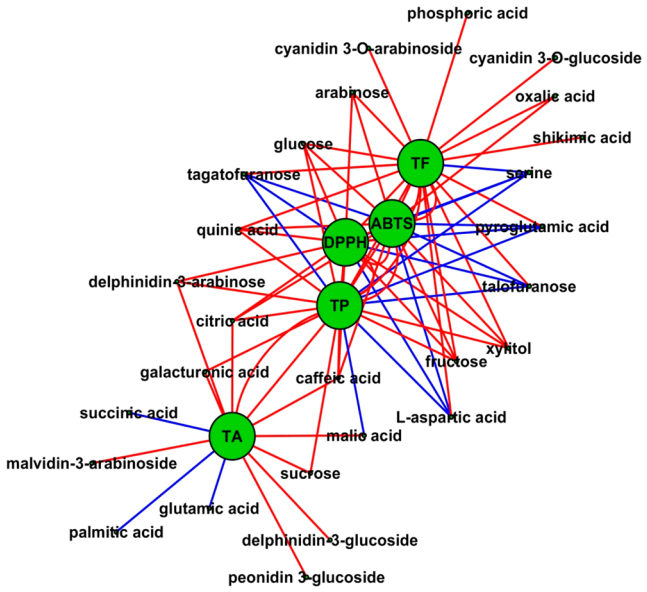

Figure 4. Visualization of the correlation network among metabolite total phenolic content (TP), total flavonoid content (TF), total anthocyanin content (TA), and antioxidant activities (ABTS, DPPH) of blueberries (*Vaccinium* spp.), according to Pearson's correlation coefficient (> ± 0.8). Positive or negative correlations are indicated by connecting red and blue lines, respectively.

total polyphenol content and antioxidant activity in *V. arctostaphylos* (a lowbush species) and *V. coymbosum*.[28]

To construct a correlation network, Pearson's correlation coefficients greater than ±0.8 were selected. TP, TF, and TA were significantly positively correlated with antioxidant activities (ABTS and DPPH). Previous research on antioxidant activity in blueberries has indicated that antioxidant activities are influenced by TP, TF, and TA.[7,29] In Figure 4, nine metabolites (oxalic acid, citric acid, arabinose, xylitol, fructose, glucose, quinic acid, caffeic acid, and delphinidin-3-*O*-arabinose) were highly positively correlated with antioxidant activities, whereas five metabolites (serine, aspartic acid, pyroglutamic acid, tagatofuranose, and talofuranose) showed a negative correlation with antioxidant activities. Some phenolic compounds may be associated with antioxidant activities, and their relative amounts in different blueberry species affect different antioxidant activities. In this study, VO and VB demonstrated significantly higher scavenging activities than VC and had higher levels of quinic acid, caffeic acid, delphinidin-3-*O*-arabinoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-arabinoside. In particular, VO, which showed the highest antioxidant activities, exhibited high contents of quinic acid, caffeic acid, delphinidin-3-*O*-glucoside, and delphinidin-3-*O*-arabinoside. Phenolic acids such as caffeic acid, quinic acid, gallic acid, hydroxybenzoic acid, and chlorogenic acid are the important antioxidants rich in blueberries.[9,30] The antioxidant activities of these phenolic acids are associated to some extent with the number of hydroxyl groups in their molecular structure.[31] In particular, caffeic acid, which exhibited high levels in VO in our study, is an antioxidant found in all plants as a key intermediate in the biosynthesis of lignin.[32] Anthocyanins are a major class of flavonoid phenolics that cause fruit pigmentation, and they are strong antioxidants that contribute to the health-beneficial properties of blueberries.[33] Monoglucosides of delphinidin, malvidin, petunidin, cyanidin, and peonidin are the primary

dx.doi.org/10.1021/jf405272b | J. Agric. Food Chem. 2014, 62, 2126−2133

anthocyanins that have been identified in blueberries.[5−8] Among these, delphinidin and cyanidin, which have more hydroxyl groups, are more active as antioxidants than those with only one hydroxyl substitute, such as pelargonidin, malvidin, and peonidin.[34,35] In particular, VO contained high levels of delphinidin-3-*O*-arabinose, which had a high positive correlation coefficient in the antioxidant activity test results. Although they are not represented in our correlation network (Figure 4), chlorogenic acid and flavonol glycoside were also related to antioxidant activities. Chlorogenic acid, the major nonflavonoid phenolic acid in blueberry,[5] was abundant in both VO and VC. Quercetin derivatives are also well-known antioxidants in plants.[36] Quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside levels were high in VO and VC, respectively.

Overall, our results suggest that antioxidant activities are correlated not only with various antioxidants such as phenolic compounds but also with the relative contents of these compounds. *Vaccinium* species rich in phenolic compounds, including phenolic acids, flavonol glycosides, and anthocyanins, represent antioxidant sources that may have potential beneficial effects on human health.

■ ASSOCIATED CONTENT

Supporting Information

Score plots; tables of significantly different metabolites and their relative contents analyzed by GC-TOF-MS and UPLC-Q-TOF-MS; and Pearson's correlation coefficients between identified metabolites and antioxidant activities of different blueberry species. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +82-2-2049-6177; fax: +82-2-455-4291; e-mail: chlee123@konkuk.ac.kr.

Funding

This research was supported by a grant from the Eco-Innovation project of the Ministry of Environment (grant no. 416-111-006), Republic of Korea.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables, and Grain*; CRC Press: Boca Raton, FL, 1993; p 362.

(2) Johns, L.; Stevenson, V. *Fruit for the Home and Garden*; Angus & Robertson: Sydney, Australia, 1990; p 252.

(3) Kotecha, P. M.; Madhavi, D. L. *Handbook of Fruit Science and Technology: Production, Composition, Storage, and Processing*. Marcel Dekker, Inc.: New York, 1995; p 315.

(4) Ayaz, F. A.; Hayirlioglu-Ayaz, S.; Gruz, J.; Novak, O.; Strnad, M. Separation, characterization, and quantitation of phenolic acids in a little-known blueberry (*Vaccinium arctostaphylos* L.) fruit by HPLC-MS. *J. Agric. Food Chem.* **2005**, *53*, 8116−8122.

(5) Kalt, W.; Lawand, C.; Ryan, D. A. J.; McDonald, J. E.; Donner, H.; Forney, C. F. Oxygen radical absorbing capacity, anthocyanin and phenolic content of highbush blueberries (*Vaccinium corymbosum* L.) during ripening and storage. *J. Am. Soc. Hortic. Sci.* **2003**, *128*, 917−923.

(6) Kalt, W.; McDonald, J. E.; Donner, H. Anthocyanins, phenolics, and antioxidant capacity of processed lowbush blueberry products. *J. Food Sci.* **2000**, *65*, 390−393.

(7) Prior, R. L.; Cao, G.; Martin, A.; Sofic, E.; McEwen, J.; O'Brien, C.; Lischner, N.; Ehlenfeldt, 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.

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

(9) Sellappan, S.; Akoh, C. C.; Krewer, G. Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *J. Agric. Food Chem.* **2002**, *50*, 2432−2438.

(10) Smith, J. L.; Marley, K. A.; Seigler, D.; Singletary, K. W.; Meline, B. Bioactive properties of wild blueberry fruits. *J. Food Sci.* **2000**, *65*, 352−356.

(11) Song, J. T. *The Sauras of Korean Resources Plants II*; II-heung: Seoul, South Korea, 1989; pp 48−49.

(12) Kim, T. J. *Korean Resources Plants III*; Seoul National University: Seoul, South Korea, 1996; p 230.

(13) Cho, H.-S.; Cho, Y.-S.; Cho, J.-A. Eco-physiological and horticultural characteristics of two Korean wild Vacciniums. *Acta Hortic.* **2012**, *926*, 149−156.

(14) Schauer, N.; Fernie, A. R. Plant metabolomics: Towards biological function and mechanism. *Trends Plant Sci.* **2006**, *11*, 508−516.

(15) Kim, J. K.; Bamba, T.; Harada, K.; Fukusaki, E.; Kobayashi, A. Time-course metabolic processing in *Arabidopsis thaliana* cell cultures after salt stress treatment. *J. Exp. Bot.* **2007**, *58*, 415−424.

(16) Zhang, J.; Wang, X.; Yu, O.; Tang, J.; Gu, X.; Wan, X.; Fang, C. Metabolic profiling of strawberry (*Fragaria*×*ananassa* Duch.) during fruit development and maturation. *J. Exp. Bot.* **2011**, *62*, 1103−1118.

(17) Ma, C.; Dastmalchi, K.; Flores, G.; Wu, S. B.; Pedraza-Peñalosa, P.; Long, C.; Kennelly, E. J. Antioxidant and metabolite profiling of North American and neotropical blueberries using LC-TOF-MS and multivariate analyses. *J. Agric. Food Chem.* **2013**, *61*, 3548−3559.

(18) t'Kindt, R.; Morreel, K.; Deforce, D.; Boerjan, W.; Bocxlaer, J. V. Joint GC−MS and LC−MS platforms for comprehensive plant metabolomics: Repeatability and sample pre-treatment. *J. Chromatogr. A.* **2009**, *877*, 3572−3580.

(19) Arbona, V.; Iglesias, D. J.; Talón, M.; Gómez-Cadenas, A. Plant phenotype demarcation using nontargeted LC-MS and GC-MS metabolite profiling. *J. Agric. Food Chem.* **2009**, *57*, 7338−7347.

(20) Wahyuni, Y.; Ballester, A.-R.; Tikunov, Y.; de Vos, R. C. H.; Pelgrom, K.T. B.; Maharijaya, A.; Sudarmonowati, E.; Bino, R. J.; Bovy, A. G. Metabolomics and molecular marker analysis to explore pepper (*Capsicum* sp.) biodiversity. *Metabolomics* **2013**, *9*, 130−144.

(21) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.

(22) Dietz, B. M.; Kang, Y. H.; Liu, G.; Eggler, A. L.; Yao, P.; Chadwick, L. R.; Pauli, G. F.; Farnsworth, N. R.; Mesecar, A. D.; van Breemen, R. B.; Bolton, J. L. Xanthohumol isolated from *Humulus lupulus* inhibits menadione-induced DNA damage through induction of quinone reductase. *Chem. Res. Toxicol.* **2005**, *18*, 1296−1305.

(23) Yildirim, A.; Mavi, A.; Kara, A. A. Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. *J. Agric. Food Chem.* **2001**, *9*, 4083−4089.

(24) Khanal, R. C.; Howard, L. R.; Brownmiller, C. R.; Prior, R. L. Influence of extrusion processing on procyanidin composition and total anthocyanin contents of blueberry pomace. *J. Food Sci.* **2009**, *74*, H52−H58.

(25) Kafkas, E.; Kosar, M.; Turemis, N.; Baser, K. H. C. Analysis of sugars, organic acids and vitamin C contents of blackberry genotypes from Turkey. *Food Chem.* **2006**, *97*, 732−736.

(26) Gao, L.; Mazza, G. Quantitation and distribution of simple and acylated anthocyanins and other phenolics in blueberries. *J. Food Sci.* **1994**, *59*, 1057−1059.

(27) Häkkinen, S.; Auriola, S. High-performance liquid chromatography with electrospray ionization mass spectrometry and diode array

ultra violet detection in the identification of flavonol aglycones and glycosides in berries. *J. Chromatogr. A* **1998**, *829*, 91−100.
(28) Koca, I.; Karadeniz, B. Antioxidant properties of blackberry and blueberry fruits grown in the Black Sea Region of Turkey. *Sci. Hortic.* **2009**, *121*, 447−450.
(29) Howard, L. R.; Clark, J. R.; Brownmiller, C. Antioxidant capacity and phenolic content in blueberries as affected by genotype and growing season. *J. Sci. Food Agric.* **2003**, *83*, 1238−1247.
(30) Castrejón, A. D. R.; Eichholz, I.; Rohn, S.; Kroh, L. W.; Huyskens-Keil, S. Phenolic profile and antioxidant activity of highbush blueberry (*Vaccinium corymbosum* L.) during fruit maturation and ripening. *Food Chem.* **2008**, *109*, 564−572.
(31) Rice-Evans, C. A.; Miller, N. J. Structure-antioxidant activity relationships of flavonoids and isoflavonoids. In *Flavonoids in Health and Disease*; Rice-Evans, C. A., Packer, C., Eds.; Marcel Dekker: New York, 1998; pp 199−219.
(32) Boerjan, W.; Ralph, J.; Baucher, M. Lignin biosynthesis. *Annu. Rev. Plant Biol.* **2003**, *54*, 519−546.
(33) Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds − Nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 1094−1117.
(34) Wang, H.; Cao, G.; Prior, R. L. Oxygen radical absorbing capacity of anthocyanins. *J. Agric. Food Chem.* **1997**, *45*, 304−309.
(35) Rahman, M. M.; Ichiyanagi, T.; Komiyama, T.; Hatano, Y.; Konishi, T. Superoxide radical- and peroxynitrite-scavenging activity of anthocyanins; structure-activity relationship and their synergism. *Free Radical Res.* **2006**, *40*, 993−1002.
(36) Rice-Evans, C. A.; Miller, N. J.; Bolwell, P. G.; Pridham, J. B. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Res.* **1995**, *22*, 375−383.