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ARTICLE

Characterization of Anthocyanins in Caucasian Blueberries (*Vaccinium arctostaphylos* L.) Native to Turkey

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High-performance liquid chromatography (HPLC) combined with diode array (DAD) and electrospray ionization mass spectrometric (ESI-MS) detections were used to characterize anthocyanins in the berries of *Vaccinium arctostaphylos* L. The dark purple−black berries were collected from five Caucasian blueberry populations in northeastern Turkey. The HPLC-DAD profile consisted of 19 anthocyanin peaks, but HPLC-ESI-MS revealed fragment ion patterns of 26 anthocyanins. Delphinidin, cyanidin, petunidin, peonidin, and malvidin were all glycosylated with four different monosaccharide moieties (galactose, glucose, arabinose, and xylose) with the first two also conjugated with rhamnose. Furthermore, anthocyanidin disaccharides, tentatively identified as anthocyanidin sambubiosides, were characteristic for these berries. The mean content of the total anthocyanins was 1420 mg/100 g dry weight. The most predominant anthocyanidins were delphinidin (41%), petunidin (19%), and malvidin (19%). Glucose was the most typical (61%) sugar moiety. This study revealed that wild Caucasian blueberries contain an abundance of bioactive anthocyanins and thus are ideal for various functional food purposes.

KEYWORDS: ***Vaccinium arctostaphylos*; Caucasian blueberry; anthocyanins; HPLC-DAD; ESI-MS; berry; authenticity**

INTRODUCTION

Bilberries and blueberries (*Vaccinium* spp.) have been shown to contain high levels and a wide variety of anthocyanins (*1*, *2*) that provide the red, blue, purple, and black colors of these berries. In addition to acting as pigments, the anthocyanin compounds exhibit a wide range of biological activities, e.g., antioxidant (*3*, *4*) and anti-inflammatory effects (*5*). Thus, they are assumed to promote health by protecting one from various degenerative diseases and diabetes as well as enhancing visual function (*6*) and slowing the progression of neurological disorders (*7*).

Anthocyanins are water-soluble pigments composed of an aglycone named an anthocyanidin and a sugar moiety mainly attached at the 3-position on the C-ring (**Figure 1**) (*8*). The most common anthocyanidins are delphinidin, cyanidin, petunidin, peonidin, pelargonidin, and malvidin (*8*), all of them being found in *Vaccinium* berries (*1*, *2*, *9*−*11*). Galactose, glucose, arabinose, xylose, and rhamnose are the most common sugars that are bonded to anthocyanidins in mono-, di-, or trisaccharide forms (*8*). Anthocyanins exist primarily as a stable flavylium cation at acidic pH (*8*). They can be characterized by their retention order and UV/vis spectra (*12*, *13*) in commonly used RP-HPLC-DAD systems and further tentatively identified by HPLC-ESI-MS in the positive ion mode as flavylium cations (*13*−*15*).

Many studies have been carried out examining the contents and the composition of anthocyanins in the berries of *Vaccinium* species (*1*, *2*, *9*−*11*). However, to our knowledge, though three major anthocyanins of *Vaccinium arctostaphylos* L. berries were previously identified (*16*), a detailed study on the anthocyanin composition of this berry species is still lacking. *V. arctostaphylos*, which is also known as the Caucasian blueberry, is a deciduous shrub with purple−black to black berries growing mainly along the Black Sea from southwestern Bulgaria through European and Asiatic Turkey to the Caucasus (*17*, *18*). The berries of this species are harvested for household consumption and commercial sale (*17*). Traditionally, it has also been used in folk medicine as an antidiabetic and antihypertensive agent (*19*). The aim of the current work was to examine the content and composition of anthocyanins in *V. arctostaphylos* berries native to Turkey by combining data obtained from DAD and ESI-MS after separation by RP-HPLC.

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MATERIALS AND METHODS

Chemicals. Cyanidin 3-glucoside was purchased from Extrasynthese (Genay, France). HPLC-grade methanol and acetonitrile were acquired from Lab-Scan (Dublin, Ireland) and J. T. Baker (Deventer, Holland), respectively. Formic acid (analytical grade) was acquired from Riedel-deHaen (Seelze, Germany).

Berry Samples. Five Caucasian blueberry (*V. arctostaphylos*) populations were selected from northeastern Anatolia, Turkey (**Figure 2**). The ripe berries (8.8 ± 0.7 mm in diameter) were collected as bulk population samples in their native habitats at altitudes of 600−1250 m (a. s. l.) around the cities of Artvin, Rize, Trabzon, Gumuşhane, and Ordu in August, 2007 and 2008. The berry samples were maintained below 5 °C before their

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lyophilization to a constant dry weight. The lyophilized samples were stored in nylon boxes containing silica gel in a freezer (−20 °C) until analyzed. Lyophilized bilberries (*V. myrtillus* L.) were used as reference samples.

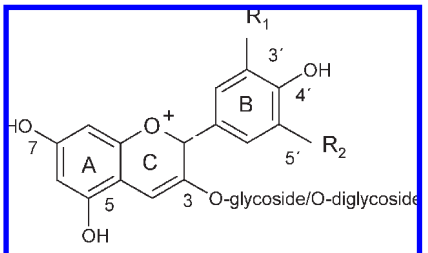
Extraction of Anthocyanins. Freeze-dried berries were ground into a powder and weighed (0.2700 g). The duplicate extractions and injections (20 μL) were performed according to the previously described method (*2*).

HPLC-DAD. The anthocyanins of the samples were separated, identified, and quantified by RP-HPLC similarly, as described in our previous study (*2*). HPLC was performed on an HP-1090 module system (Hewlett-Packard, Waldbronn Analytical Division, Germany) equipped with a quaternary pump, an autosampler, and a DAD (HP 1040M). The column used was a 150 mm × 4.6 mm i.d., 5 μm, Gemini C-18 (Phenomenex, Torrance, CA, USA) fitted with a 4 mm × 3 mm i.d. C-18 guard column. The gradient program was composed of two mobile phases, MeCN/MeOH (85:15 v/v) (A) and aqueous 8.5% HCOOH (B). A low pH (below 2) was used to stabilize anthocyanins in the form of flavylium cations, which resulted in sharp peaks in the DAD chromatogram (*14*). The anthocyanins were detected at 520 nm. The characterization of anthocyanins was performed by using the spectroscopic and/or the retention properties of the aglycone and/or sugar(s) moieties (*12*, *13*).

HPLC-ESI-MS. The tentative identification of the HPLC-peaks was further confirmed according to ESI-MS and the literature (*2*, *10*, *12*, *13*). Tandem MS (MS/MS) was used to characterize individual compounds in the separate ionization and fragmentation steps. MS^3 spectra were acquired by fragmenting the two major target ions observed in the MS^2 spectra.

The HPLC-ESI-MS system consisted of a Finnigan Surveyor HPLC and a Finnigan LTQ ion trap mass spectrometer (Thermo, San Jose, CA, USA). The column and organic mobile phase were the same as those in HPLC-DAD analyses, but in the case of the aqueous phase, 8.5% HCOOH was replaced by 1% HCOOH to achieve the lowest possible detection limit in the positive ion mode (*20*). According to our previous studies, the retention order of anthocyanins is constant and not dependent on the strength of formic acid in the mobile phase (*1*, *15*, *21*). The gradient program was as follows: 0−12 min, 89% B; 12−19 min, from 89% to 88% B; 19−35 min, from 88% to 87% B; 35−37 min, from 87% to 86% B; 37−40 min, from 86% to 84% B; 40−46 min, from 84% to 80% B; 46−55 min, from 80% to 70% B; 55−57 min, from 70% to 20% B; 57−59 min 20% B; 59−62 min from 20% to 89% B; 62−65 min 89% B.

Conditions for the initial ionization in the positive ionization mode included capillary voltages at +4.5 kV and a temperature of 225 °C. Full scan mass spectra were measured from *m*/*z* 250 to 700. Tandem MS (MS-MS) was performed using helium as the collision gas, and the collision energy was set at 35%. MS revealed the positive molecular ions, and MS-MS broke down the most abundant ions with dependent collision-induced dissociation (CID). The data was analyzed with Finnigan Xcalibur 1.4 SR1 software (Thermo Fischer Scientific Inc., Waltham, MA, USA).



R_1	R_2	Anthocyanidin
OH	OH	Delphinidin
OH	H	Cyanidin
OCH_3	OH	Petunidin
OCH_3	H	Peonidin
OCH_3	OCH_3	Malvidin

Figure 1. Chemical structures of anthocyanidins found in the berries of *V. arctostaphylos*.

RESULTS AND DISCUSSION

Identification of Anthocyanins. As far as we are aware, this is the first time that the anthocyanins in the berries of *V. arctostaphylos* have been separated and tentatively identified by HPLC-DAD and HPLC-ESI-MS systems. The HPLC-DAD profile with 19 anthocyanin peaks at 520 nm is shown in **Figure 3**. The determined profile was compared to the previously studied Finnish bilberry (*V. myrtillus*) as the reference sample by running the combined extracts (1:1) of these two berries together in HPLC-DAD (*2*, *21*). Caucasian blueberries contained 15 anthocyanins found in bilberry; these consisted of five aglycones (delphinidin, cyanidin, petunidin, peonidin, and malvidin) glycosylated with galactose, glucose, and arabinose. The remaining four peaks (2, 11, 18, and 19) with typical UV/vis spectra of anthocyanins were absent in the reference sample used. Individual anthocyanins detected and quantified with HPLC-DAD are designated with numbers (1−19), and those that were found in further HPLC-ESI-MS analyses are coded with letters (A−H).

At first, the peaks were grouped by studying their spectroscopic properties at 510−540 nm. The anthocyanidins/anthocyanins with a hydroxyl- or methoxy-group in carbon R_2 have absorption maxima at longer wavelengths than those with the hydrogen in carbon R_2 (**Figure 1**) (*13*). Thus, the difference of 8 nm in the absorption maxima between the glycosides of delphinidin/petunidin/malvidin (526 nm) and those of cyanidin/peonidin (518 nm) enabled the differentiation of the resolved peaks into two groups (**Table 1**; **Figure 3**). The peaks with the following numbers 1−3, 5, 7, 9, 11−12, 14, 16−18 belonged to the former group and the others to the latter group, except for the last peak (peak 19).

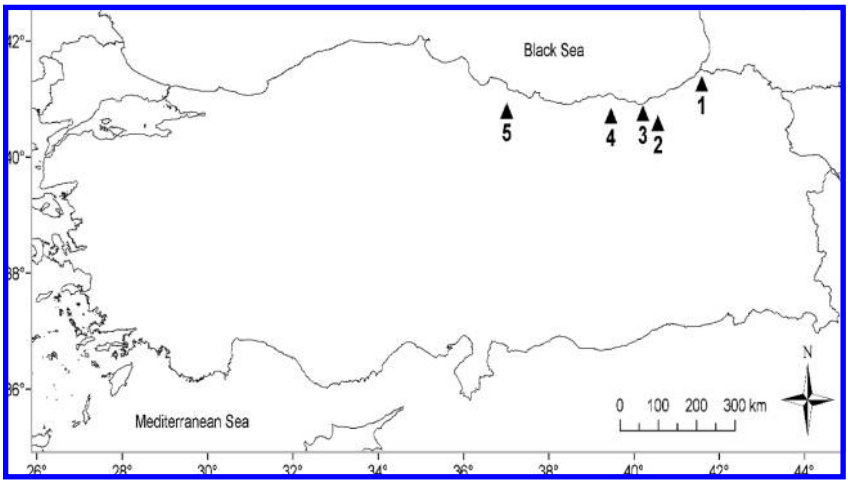


Figure 2. Collecting locations of Caucasian blueberry (*V. arctostaphylos*) samples in Turkey: Artvin (1), Rize (2), Trabzon (3), Gumuşhane (4), and Ordu (5).

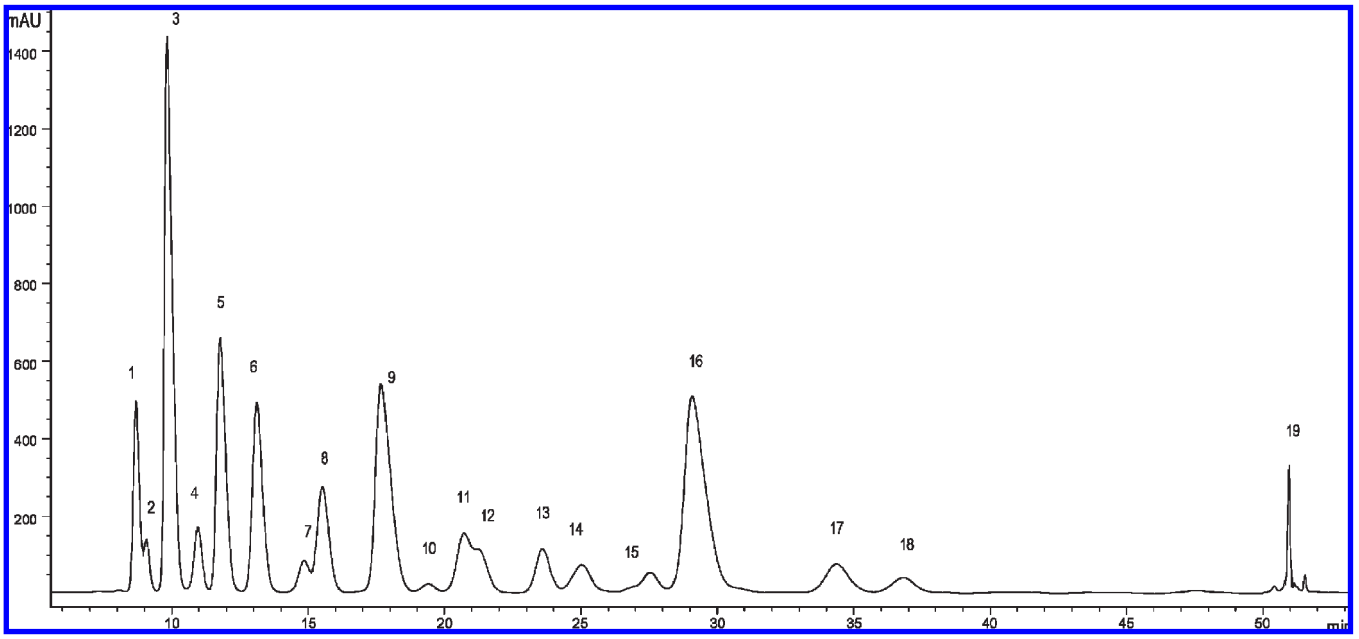


Figure 3. HPLC-DAD profile of the anthocyanins in Caucasian blueberries (*V. arctostaphylos*) monitored at 520 nm. See **Table 1** for identification of the peaks.

Table 1. Characterization of Caucasian Blueberry (*V. arctostaphylos*) Anthocyanins by HPLC-DAD and ESI-MS Detection Using Positive Ionization Mode

	Tentative Identification					
HPLC peak	maximum absorption (nm) at visible region[a]	M+	MS^2	MS^3	detected anthocyanins	literature
1	526	465	303 (100)	257 (100), 303 (56), 229 (24), 247 (8)	delphinidin galactoside	
2	526	597	303 (100)	257 (100), 303 (62), 229 (26), 247 (10)	delphinidin hexose-pentoside	delphinidin sambubioside[d]
3	526	465	303 (100)	257 (100), 303 (64), 229 (26), 247 (10)	delphinidin glucoside	
4	518	449	287 (100)	287 (100), 213 (18), 231 (12), 259 (12)	cyanidin galactoside	
5	526	435	303 (100)	257 (100), 303 (60), 229 (26), 247 (8)	delphinidin arabinoside	
6	518	449	287 (100)	287 (100), 213 (18), 231 (14), 259 (10)	cyanidin glucoside	
A	ND[b]	581	287 (100)	287 (100), 213 (18), 231 (14), 259 (10)	cyanidin hexose-pentoside	cyanidin sambubioside[d]
7	526	479	317 (100)	302 (100), 274 (6)	petunidin galactoside	
8	518	419	287 (100)	287 (100), 213 (20), 231 (14), 259 (10)	cyanidin arabinoside	
B	ND	611			petunidin hexose-pentoside	petunidin sambubioside[d]
C	ND	449	303 (100)	257 (100), 303 (56), 229 (24), 247 (8)	delphinidin deoxyhexoside	delphinidin rhamnoside[e]
9	526	479	317 (100)	302 (100), 274 (6)	petunidin glucoside	
10	ND	463			peonidin galactoside	peonidin galactoside[f]
11	526	435	303 (100)	257 (100), 303 (56), 229 (28), 247 (10)	delphinidin pentoside	delphinidin xyloside[g]
12	526	449	317 (100)	302 (100), 274 (6)	petunidin arabinoside	
13	518	463	301 (100)	286 (100)	peonidin glucoside	
D	ND	595			peonidin hexose-pentoside	peonidin sambubioside[d]
E	ND	433	287 (100)	287 (100), 213 (18)	cyanidin deoxyhexoside	cyanidin rhamnoside[e]
14	526	493	331 (100)	299 (100), 315 (94), 287 (68), 270 (56), 179 (16)	malvidin galactoside	
F	ND	419	287 (100)	287 (100), 213 (18)	cyanidin pentoside	cyanidin xyloside[g]
15	518	433	301 (100)	286 (100)	peonidin arabinoside	
16	526	493	331 (100)	299 (100), 315 (98), 287 (66), 270 (56), 179 (16)	malvidin glucoside	
17	526	463	331 (100)	299 (100), 315 (96), 287 (66), 270 (52), 179 (16)	malvidin arabinoside	
18	526	449	317 (100)	302 (100)	petunidin pentoside	petunidin xyloside[g]
19	530	433	301 (100)	286 (100)	peonidin pentoside (G) or	peonidin xyloside[g]
		463	331 (100)	299 (100), 315 (94), 287 (64), 270 (52), 179 (14)	malvidin pentoside (H)[c]	malvidin xyloside[g]

[a] The on-line UV/Vis spectra of DAD was monitored at 200−600 nm, but the visible wavelengths (510−540 nm) were particularly useful for differentiation of delphinidin- and cyanidin-based anthocyanins. [b] ND, not detected by HPLC-DAD; The table shows also detected ions (*m*/*z*) with their relative intensities in parentheses. The 15 well known bilberry anthocyanins (*2*), found both in Caucasian blueberries and the reference samples (*V. myrtillus* L.) are directly written in their monosaccharide forms. The quantified compounds are numbered. The minor compounds are marked with capital letters and also tentatively identified. [c] See Figure **4**. [d] See ref (*10*). [e] Rhamnose is the predominant deoxyhexose found in berries (*8*, *30*). [f] See refs (*2*) and (*24*). [g] If arabinose conjugate of the same aglycone has already been identified, the xylose one is the suggested pentoside (*8*, *30*).

The peaks were further identified with HPLC-ESI-MS (**Figure 4**). The characteristic molecular weight cations (M^+) and diagnostic fragmentation of sugar moieties confirmed the identification of the 15 HPLC-DAD-peaks found in bilberry and enabled the characterization of four unknown peaks (**Table 1**). Peak 2 was characterized by the loss of 294 amu from the molecular ion *m*/*z* 597 resulting in a fragment ion of delphinidin aglycone (303). On the basis of the literature (*10*), the 294 amu was indicative of the anthocyanidin disaccharide composed of one glucose (162) and one xylose (132) unit. Thus, it was

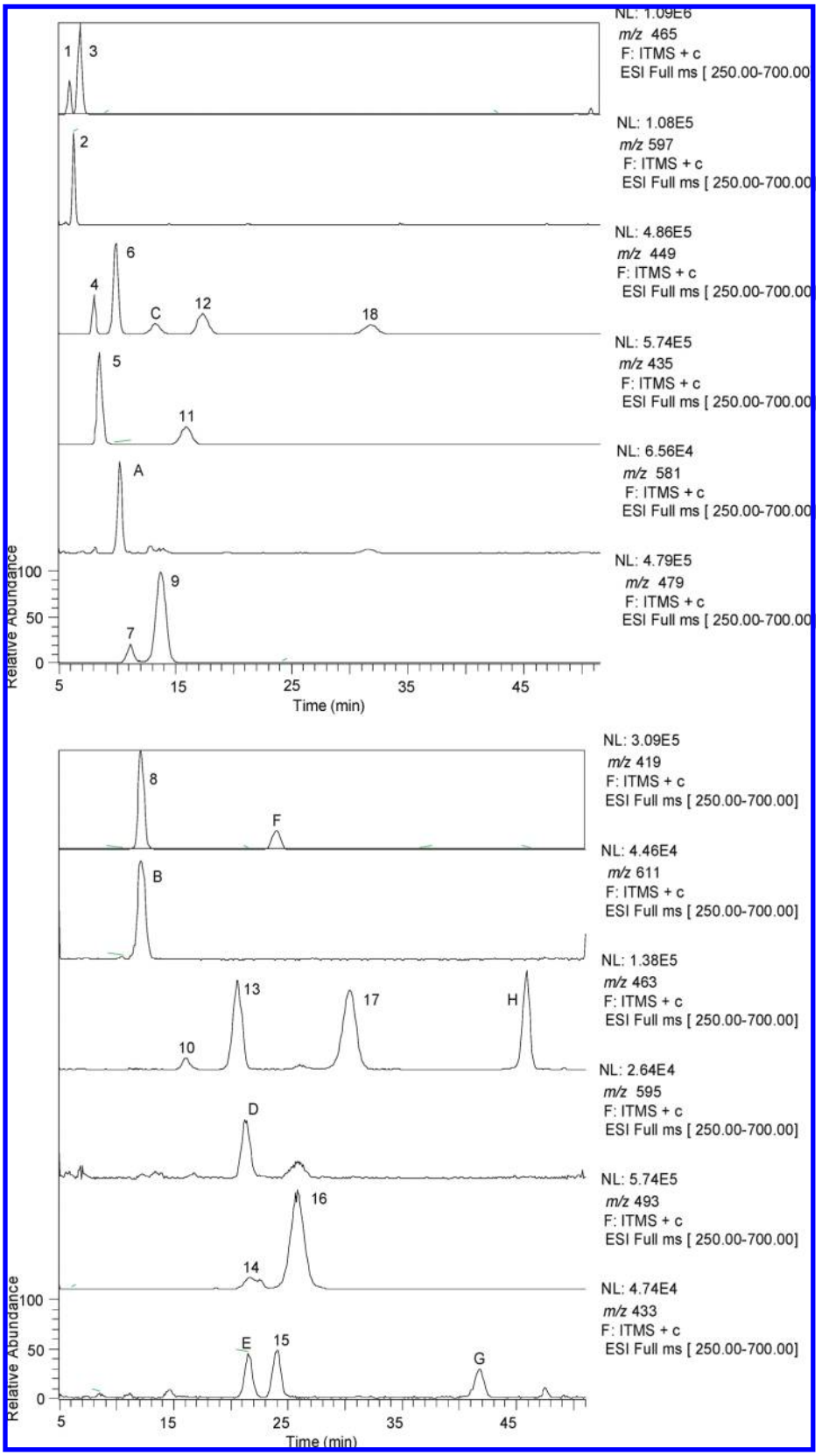


Figure 4. Extracted ion chromatograms of the full scan mass spectra (*m*/*z* 250−700) for the anthocyanins in Caucasian blueberries (*V. arctostaphylos*). See **Table 1** for the anthocyanins.

tentatively identified as delphinidin sambubioside. In addition, sambubioside conjugates of cyanidin, petunidin, and peonidin were found in ESI-MS but not in the HPLC-DAD data (peaks A, B, and D) (**Table 1**; **Figure 4**). Peaks 11 and 18 showed the spectroscopic characteristics of delphinidin-derived anthocyanins, and this was further confirmed with the molecular ion masses and the losses of the sugar mass of 132 amu indicative of delphinidin and petunidin pentosides, respectively. Anthocyanins (peaks A−H) found in HPLC-MS were suspected to be minor ones in the quantitative analyses by HPLC-DAD since they neither affected

Table 2. Contents [mg/100g Dry Weight (DW) ± Standard Deviation (SD)] of the Anthocyanins in *V. arctostaphylos* Berries from Five Populations Native to Turkey[a]

		mg/100 g DW						
HPLC peak	anthocyanins	Artvin	Rize	Trabzon	Gumuşhane	Ordu	mean	SD
1	delphinidin galactoside	79	72	71	78	102	80	13
2	delphinidin sambubioside	10	4	14	10	8	9	4
3	delphinidin glucoside	297	275	288	286	292	288	8
4	cyanidin galactoside	26	19	29	31	79	37	24
5	delphinidin arabinoside	153	167	148	172	140	156	13
6	cyanidin glucoside	103	71	123	104	187	117	43
7	petunidin galactoside	17	16	16	14	28	18	5
8	cyanidin arabinoside	68	54	78	82	100	76	17
9	petunidin glucoside	200	191	206	189	209	199	9
10	peonidin galactoside	4	2	3	3	11	5	3
11	delphinidin xyloside	56	55	49	55	37	50	8
12	petunidin arabinoside	34	37	35	23	29	32	6
13	peonidin glucoside	27	19	37	21	54	31	15
14	malvidin galactoside	25	19	29	13	28	23	6
15	peonidin arabinoside	14	12	20	13	17	15	3
16	malvidin glucoside	253	230	274	193	207	231	33
17	malvidin arabinoside	31	29	35	15	20	26	8
18	petunidin xyloside	18	16	15	13	9	14	4
19	peonidin/malvidin xyloside (G, H)[b]	15	14	16	7	7	12	4
	total	1431	1299	1486	1322	1562	1420	111

[a] See **Table 1** for the tentative identification of the peaks. [b] See **Figure 4**.

the spectroscopic characteristic of the detected peaks by DAD nor were they visible in areas between the peaks. The ESI-MS data suggested that peak 19 was either peonidin (ion fragments 433/301/132) or malvidin pentoside (ion fragments 463/331/132).

Composition of Anthocyanins. The contents of the major anthocyanins in the Caucasian blueberries originating from Turkish populations are seen in **Table 2**. The average ± SD (standard deviation, $n = 5$) content of the delphinidin was 583 ± 13 mg/100 g dry weight (DW). This is in the same range as the respective content of the bilberries from the southwestern Finnish populations (Turku) ($n = 3$) (481−541 mg/100 g DW) but lower than in the bilberries from the northern populations in Finland (*2*). The average content of delphinidin was 107 ± 1 mg/100 g as calculated on a fresh weight (FW) basis. This is higher than the respective content of the blue−purple dark-colored berries of a number of other *Vaccinium* species (*V. angustifolium* Ait. × *V. corymbosum* L.; *V. corymbosum*; *V. ovalifolium* Sm.; *V. ovatum* Pursh; *V. uliginosum* L.) (*22*). It is difficult to make a more exact comparison to other studies because of analytical, genetic, and climatic differences.

Delphinidin, the predominant anthocyanidin of Caucasian blueberries, is absent in many other blue, dark-blue, purple, or black berries or fruits such as Saskatoon berries (*Amelanchier* sp.), chokeberries (*Aronia* spp.), honeysuckle berries (*Lonicera* spp.), dark cherries and plums (*Prunus* spp.), and elderberries (*Sambucus* sp.) (*1*, *23*, *24*). The average ($n = 5$) contents of cyanidin, petunidin, and malvidin varied between 231−280 mg/100 g DW, which is equivalent to 42−52 mg/100 g FW, respectively. In particular, the contents of petunidin are noteworthy since very few berries and fruits except for *Vaccinium* species biosynthesize it. Petunidin has been detected in crowberries (*Empetrum* sp.) and grapes (*Vitis* sp.) (*24*). Peonidin constituted the minor group of anthocyanidins. The average content was 51 ± 20 mg/100 g DW, which equals to 9 ± 4 mg/100 g FW.

Total Contents of Anthocyanins. The total average ($n = 5$) anthocyanin content in the berries of *V. arctostaphylos* (**Table 2**) was 43% lower than that in the berries of *V. myrtillus* from southern Finnish populations ($n = 7$) (*2*), even lower compared to the central and northern populations analyzed in our previous study (*2*). The total anthocyanin content of *V. arctostaphylos* (261 ± 21 mg/100 g as calculated on a FW basis) was higher than that in the berries from populations of *V. ovalifolium* ($n = 3$), *V. deliciosum* Piper ($n = 2$), and *V. membranaceum* Dougl. *ex* Torr. ($n = 3$) in the section *Myrtillus* (*22*). Their anthocyanin level was even higher than that found in the berries of *V. angustifolium* × *corymbosum* ($n = 3$), *V. corymbosum* ($n = 3$) in section *Cyanococcus*, and *V. uliginosum* ($n = 2$) in section *Vaccinium* (*22*). With respect to section *Pyxothamnus*, the berries of *V. ovatum* from two populations contained less anthocyanins (*22*) than those of *V. arctostaphylos*.

Proportions of Anthocyanins. The three major anthocyanidins were delphinidin (41 ± 4%), petunidin (19 ± 1%), and malvidin (20 ± 3%) in accordance with the literature (*16*). The proportion of delphinidin was 9% greater than that in the berries of *V. padifolium* Sm. from the same section (*10*) and up to 28% greater than that in the berries of *V. ovatum* (section *Pyxothamnus*) (*11*, *25*). The proportion of the methylated form of the former, petunidin, was at least 14% higher than the corresponding value in the berries of *V. ovatum* and at least 6% higher than that in the species of *V. myrtillus*, *V. membranaceum*, and *V. ovalifolium* from section *Myrtillus* (*2*, *11*, *22*). The proportion of malvidin was 10% lower than that in the berries of *V. padifolium* but at least 9% higher than that in the berries from the populations of *V. ovatum*, *V. myrtillus*, and *V. ovalifolium* (*2*, *11*, *22*, *25*).

The proportion of cyanidin was 16 ± 5%, which is 24−39% lower than that in the berries of *V. ovatum*, *V. membranaceum*, and *V. ovalifolium* (*11*, *22*, *26*). Compared to the Finnish bilberries (*V. myrtillus*) at the population level ($n = 20$) (*2*), the Caucasian blueberries had at least a 10% lower proportion of cyanidin. The peonidin in the berries of *V. arctostaphylos* accounted for only 3 ± 1% of the total. The rather low proportion (≤14%) of peonidin is a common feature of most bilberries and blueberries in sections *Hemimyrtillus*, *Myrtillus*, *Cyanococcus*, and *Vaccinium* (*2*, *10*, *11*, *22*, *26*).

Proportions of Glycosides. The average proportions of sugar moieties were 11 ± 2%, 61 ± 7%, 22 ± 4%, and 5 ± 1% for galactosides, glucosides, arabinosides, and xylosides, respectively. The predominance of the glucoside conjugates is supported

by the previous study of Nickavar and Amin (*16*). Furthermore, the diglycosides are typical for *V. arctostaphylos*, although they accounted for only 1%. The proportion of glucosides was over 50% higher, but that of the galactosides was 39−62% lower than that in the berries of *V. ovatum* (section *Pyxothamnus*) (*11*, *25*) and *V. membranaceum* (section *Myrtillus*) (*11*, *26*). Previously, anthocyanidin sambubiosides have been found in the berries of *V. padifolium* (*10*) from the same section of *Hemimyrtillus* (*18*) and in one bilberry (*V. myrtillus*) extract (*27*). However, the possibility of adulteration cannot be excluded in the case of commercial bilberry products (*28*). With respect to the berries of other genera, sambubiosides have been found in elderberries and red currants but only as cyanidin conjugates (*15*, *29*). The characteristic sugar proportions and especially the presence of anthocyanidin sambubiosides can be utilized in authenticity studies. The occurrence of sambubiosides enables the differentiation of berries of this species from *Vaccinium* berries from other sections.

In conclusion, this study shows for the first time, that the berries of *V. arctostaphylos* are rich sources of anthocyanins and can be differentiated from other blue−black bilberries or blueberries (*Vaccinium* spp.) by their unique anthocyanin pattern, e.g., by the high proportions of both delphinidin (41%) and glucoside conjugates (61%) in addition to the diglycosides, which were tentatively identified as anthocyanidin sambubiosides.

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