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Characterization and Quantification of Anthocyanins and Polyphenolics in Blue Honeysuckle (*Lonicera caerulea* L.)

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Anthocyanins and phenolics of 10 blue honeysuckle (*Lonicera caerulea* L.) genotypes were characterized and quantified by HPLC-DAD. Peak assignments were confirmed by low-resolution electrospray mass spectrometry. Six anthocyanins were detected with the major peak identified as cyanidin 3-glucoside. Five additional anthocyanins were characterized as cyanidin 3,5-diglucoside, cyanidin 3-rutinoside, pelargonidin 3-glucoside, peonidin 3-glucoside, and peonidin 3-rutinoside. Four polyphenolics were identified as chlorogenic acid, neochlorogenic acid, quercetin 3-rutinoside, and quercetin 3-glucoside. Two additional unidentified phenolics were characterized as flavonol and hydroxycinnamic derivatives based on UV−vis spectra. Hydroxycinnamate levels ranged from 30.4 to 156.2 mg/100 g, whereas the flavonol content ranged from 12.6 to 32.8 mg/100 g. The *L. caerulea* subspecies *boczkarnikovae* contained the highest amounts of hydroxycinnamic derivatives and flavonols.

KEYWORDS: Blue honeysuckle; *Lonicera caerulea*; anthocyanins; phenolics; hydroxycinnamic acid derivatives; flavonols; HPLC-DAD; ESMS

INTRODUCTION

Blue honeysuckle or sweet berry honeysuckle (*Lonicera caerulea* L.) fruits are widely harvested in Russia, China, and Japan. Fruit shapes are oval to long and dark navy blue to purple in color. Their flavor is similar to that of bilberries, black currants, and blueberries. Although blue honeysuckle is commercially produced in Russia and Japan, this species is unknown as an edible berry in North America. Oregon State University has established an experimental program to evaluate the potential of blue honeysuckle fruits as a commercial crop in Oregon (*1*). Blue honeysuckle grown in Oregon contains levels of anthocyanins, total phenolics, and antioxidant activity comparable to those of blueberry, blackberry, and black currant (*1*, *2*). The beneficial effects of anthocyanins and polyphenolics on several chronic diseases such as cancer, heart disease, and aging are believed to be associated with their antioxidant properties (*3*−*9*).

Terahara et al. (*10*) and Oszmianski et al. (*11*) reported the presence of cyanidin 3-glucoside, cyanidin 3,5-diglucoside, and cyanidin 3-rutinoside in *L. caerulea* L. and *Lonicera kamtschatica.* The major anthocyanin was identified as cyanidin 3-glucoside. In addition, cyanidin 3-gentiobioside was detected in *L. caerulea* L., whereas malvidin 3-glucoside was found in *L. kamtschatica*. Plekhanov et al. (*12*) characterized the polyphenolics in *L. caerulea* as caffeic acid, chlorogenic acid, *p*-coumaroylquinic acids, quercetin 3-rutinoside, quercetin 3-glucoside, quercetin 3-rhamnoside, 7-*O*-luteolin rutinoside, 7-*O*-luteolin glycoside, diosmin, procyanidins, and catechin.

The purposes of this study were to characterize and quantify the anthocyanin and polyphenolic compositions in blue honeysuckle fruits grown in Oregon.

MATERIALS AND METHODS

Standards. Phenolic standards (caffeic acid, chlorogenic acid, catechin, epicatechin, and quercetin 3-rutinoside) were purchased from Sigma Chemical Co. (St. Louis, MO). Quercetin 3-glucoside and quercetin 3-rhamnoside were purchased from Extrasynthese (Genay, France). Concord grape juice (Welch Foods Inc., Concord, MA) was purchased from a local supermarket. Cranberry and strawberry juice concentrates were provided by Kerr Concentrates Inc. (Salem, OR). Sweet and sour cherries were provided by the Oregon State University (OSU) Department of Horticulture, Corvallis, OR.

Fruits. Fruits from 10 blue honeysuckle genotypes were harvested at the OSU Lewis Brown horticultural farm, OSU Department of Horticulture, Corvallis, OR, during May 2001. Upon arrival at the Department of Food Science and Technology, the samples were frozen and then stored at −23 °C until further analyses.

Polyphenolic Extraction. Samples were powdered in liquid nitrogen using a stainless steel Waring blender. A sample (∼5 g) was blended with 20 mL of acetone, sonicated by an ultrasonic cleaner (Branson Cleaning Equipment Corp., Shelton, CT) for 10 min, and then filtered on a Büchner funnel using Whatman no. 1 paper. The filter cake was re-extracted two times with 10 mL of 70% aqueous acetone. Filtrates were combined and mixed with 80 mL of chloroform and then

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centrifuged at 170*g* for 20 min by a model UV IEC centrifuge, (International Equipment Co., Boston, MA). The aqueous fraction was collected and evaporated in vacuo at 40 °C for ∼15 min until the acetone was removed. The extract was made up to 25 mL with acidified water and stored at −70 °C until further analyzed. Sample extractions were replicated twice.

Anthocyanin Purification. Anthocyanins were purified following the procedure described by Rodriguez-Saona and Wrolstad (*13*). The aqueous extract (.∼2 mL) was filtered through a C-18 Sep-Pak cartridge (sorbent mass = 360 mg) (Waters Corp., Milford, MA), previously activated with methanol followed by 0.01% aqueous HCl.

Anthocyanins and polyphenolics were adsorbed onto the Sep-Pak column while sugars, acids, and other water-soluble compounds were removed by washing the minicolumn with 10 mL of 0.01% aqueous HCl. The minicolumn was dried with nitrogen gas. Polyphenolics were subsequently eluted with 5 mL of ethyl acetate. Anthocyanins were eluted with 5 mL of methanol containing 0.01% HCl. The acidified methanol fraction was concentrated by using a rotary evaporator at 40 °C until the methanol was evaporated. The anthocyanins were then redissolved in acidified water. This purified extract was stored at −70 °C until further analyses by acid hydrolysis and saponification.

Acid Hydrolysis of Anthocyanins. The acid hydrolysis was performed according to the procedure described by Durst and Wrolstad (*14*). Five milliliters of 2 N HCl was added to purified anthocyanins or purified phenolics (∼1 mL) in a screw-cap test tube, flushed with nitrogen, and capped. The purified compound was hydrolyzed for 30 min at 100 °C and then immediately cooled in an ice bath. The hydrolysate was purified by solid-phase extraction using a C-18 Sep-Pak cartridge (Waters Associates) as described above.

Alkaline Hydrolysis of Anthocyanins. The saponification was performed according to the procedure described by Durst and Wrolstad (*14*). Purified anthocyanins (∼1 mL) were saponified in a screw-cap test tube with 5 mL of 10% KOH for 8 min in the dark at room temperature. Then, 5 mL of 2 N HCl was added to neutralize the solution. The hydrolysate was purified by using a C-18 Sep-Pak cartridge (Waters Corp.) as previously described.

High-Performance Liquid Chromatography (HPLC) Analytical System. *Characterization of Anthocyanins.* A Perkin-Elmer series 400 high-performance liquid chromatograph, equipped with a Hewlett-Packard 1040A photodiode array detector and Hewlett-Packard HPLC^{2D} Chemstation software, was used with simultaneous detection at 520 nm for anthocyanins, saponified anthocyanins, and anthocyanidins. Samples were injected by an Agilent 1100 series autosampler (Agilent Technologies, Wilmington, DE). Injection volume for anthocyanin analysis was 50 μL. The flow rate was 1 mL/min.

Characterization and Quantification of Polyphenolics. A Varian 5000 high-performance liquid chromatograph, equipped with a Hewlett-Packard 1040A photodiode array detector and Hewlett-Packard HPLC^{2D} Chemstation software, was used with simultaneous detection at 280 and 370 nm for flavanols, 320 nm for hydroxycinnamic acids, and 520 nm for anthocyanins. Samples were injected by an Agilent 1100 series autosampler (Agilent Technologies). Injection volume of phenolics analysis was 20 μL. The flow rate was 1 mL/min.

HPLC Separation of Anthocyanins and Saponified Anthocyanins. Anthocyanins were separated using a 250 × 4.6 mm i.d., 5 μm, Prodigy ODS-3 column (Phenomenex, Torrance, CA), fitted with a 10 × 4.6 mm i.d. Allsphere ODS-2 guard column (Alltech, Deerfield, IL). Acetonitrile (100% HPLC grade; solvent A) and a solution of 1% phosphoric acid/10% acetic acid (glacial)/5% acetonitrile (1:1:1, v/v/v) in water (solvent B) were used. Separations were effected as follows: isocratic at 0% A for 5 min, a linear gradient from 0 to 20% A for 15 min, and a linear gradient from 20 to 40% in 5 min. Peak identification was performed by matching the UV−vis spectra and retention times with juice standards and confirming molecular weight by electrospray mass spectrometry (ESMS).

HPLC Separation of Anthocyanidins. Anthocyanidin separation utilized the same column and solvent system as for anthocyanins. The program was a linear gradient from 10 to 30% A in 20 min. Identification was made from matching the UV−vis spectra and retention times with six anthocyanidins obtained from acid hydrolysis of grape and strawberry juice concentrates.

Table 1. Peak Assignments for Blue Honeysuckle Anthocyanins Analyzed by HPLC-DAD and ESMS

anthocyanin	λ_{max} (nm)	HPLC peak	ESMS mass/charge ratio (*m*/*z*)
cyanidin 3,5-diglucoside	515	1	611
cyanidin 3-glucoside	517	2	449
cyanidin 3-rutinoside	519	3	595
pelargonidin 3-glucoside	505	4	433
peonidin 3-glucoside	519	5	463
peonidin 3-rutinoside	519	6	609

HPLC Separation and Quantification of Polyphenolics. Polyphenolics were separated using a 250 × 4.6 mm i.d., 4 μm, Synergi Hydro-RP column (Phenomenex), fitted with a 10 × 4.6 mm i.d. Allsphere ODS-2 guard column (Alltech). Solvent A was 100% HPLC grade acetonitrile. Solvent B was 1% formic acid in deionized water. The program followed a linear gradient from 10 to 25% A for 30 min, followed a linear gradient from 25 to 50% for 3 min, and was isocratic at 50% for 5 min. Samples were filtered through a 0.45 μm Millipore filter (type HA) before HPLC injection.

Identification was performed by matching the UV−vis spectra and retention time with authentic standards (when available) and confirming the molecular weight by ESMS. Hydroxycinnamic derivatives were quantified by comparisons with the external standard of chlorogenic acid at 320 nm and flavonols as quercetin 3-rutinoside (rutin) at 260 nm. Concentration was expressed as milligrams per 100 g of fresh weight (fw).

Electrospray Mass Spectrometry. Low-resolution MS was acquired using ESMS. The instrument was a Perkin-Elmer SCIEX API III bimolecular mass analyzer (Ontario, Canada) equipped with an ion spray interface (ISV, 4000; orifice voltage, 50). The mass spectrometer was operated in the positive ion mode. The purified anthocyanins or the purified phenolics were dissolved in acidified water and injected directly into the system.

Statistical Analyses. Mean and standard deviation of hydroxycinnamic derivatives and flavonol glycoside were analyzed by using S-Plus 4.5 (MathSoft, Seattle, WA). The Pearson correlation matrix was used to determine the relationship among antioxidant activities, hydroxycinnamates, and flavonols.

RESULTS AND DISCUSSION

Characterization of Anthocyanins. Six different blue honeysuckle anthocyanins were separated by reverse phase HPLC (**Figure 1**). Peak assignments are based on matching UV−vis spectra and identical HPLC retention time with known anthocyanins from fruit juices (*15*): cyanidin 3,5-diglucoside from sour cherries; cyanidin 3-glucoside, cyanidin 3-rutinoside, and peonidin 3-rutinoside from sweet cherries; pelargonidin 3-glucoside from strawberries; and peonidin 3-glucoside from cranberries. HPLC of acid hydrolysis products showed cyanidin to be the major anthocyanidin with trace amounts of peonidin and pelargonidin. Saponification indicated the absence of acylation with cinnamic or aliphatic acids because it caused no change in the pigment profile.

The major anthocyanin in blue honeysuckle is cyanidin 3-glucoside (79−88%) with five minor anthocyanins. The variation in the proportion of minor anthocyanins was found to be as follows: cyanidin 3-rutinoside (1−11%), cyanidin 3,5-diglucoside (2.2−6.4%), peonidin 3-glucoside (2.8−4.5%), peonidin 3-rutinoside (0.3−1.3%), and pelargonidin 3-glucoside (0.2−1.0%). The identities were confirmed by ESMS (**Table 1**). In addition, ESMS also detected the presence of compounds with MW of 531 and 545, which do not correspond to glycosides of cyanidin, peonidin, and pelargonidin. The presence of cyanidin 3-glucoside, cyanidin 3,5-diglucoside, and cyanidin 3-rutinoside confirms the earlier reports of Terahara et al. (*10*)

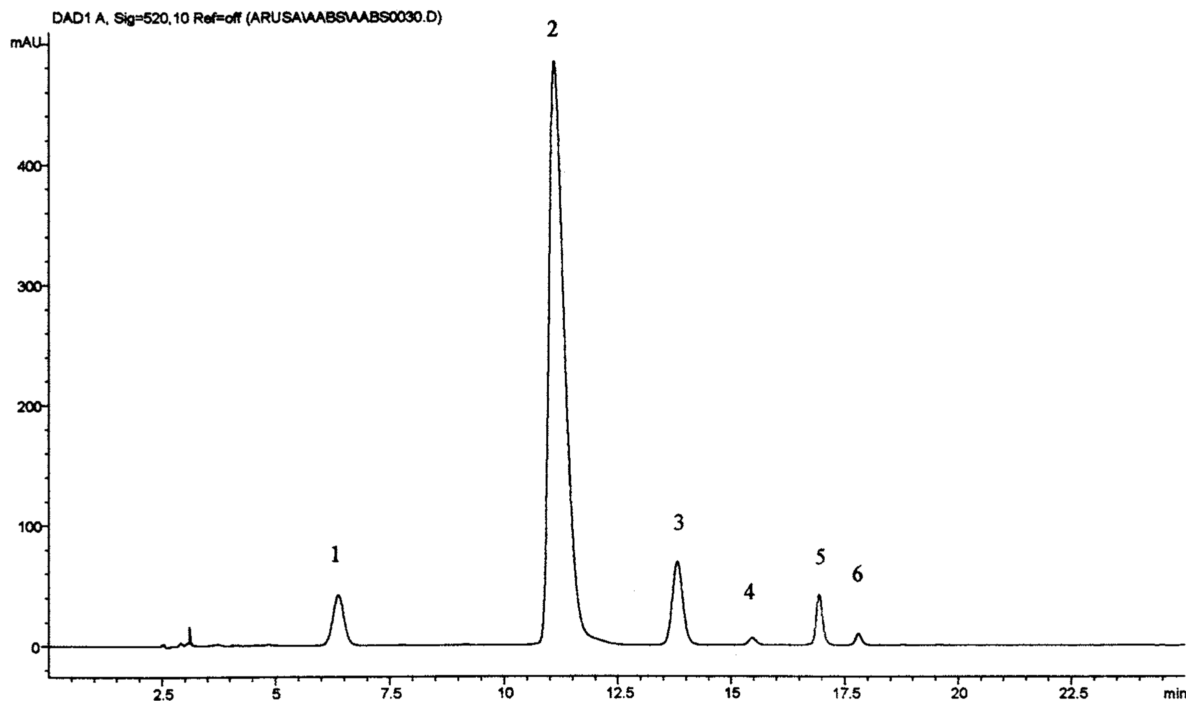

Figure 1. HPLC anthocyanin profile of blue honeysuckle: 1. cyanidin 3,5-diglucoside; 2, cyanidin 3-glucoside; 3, cyanidin 3-rutinoside; 4, pelargonidin 3-glucoside; 5, peonidin 3-glucoside; 6, peonidin 3-rutinoside.

and Oszmiansi et al. (*11*). In addition, they reported the presence of malvidin 3-glucoside and cyanidin 3-gentiobioside. In our study, malvidin 3-glucoside was not detected by either HPLC-DAD or ESMS. The possible presence of cyanidin 3-gentiobioside is inconclusive because the mass of cyanidin 3-gentiobioside is equal to that of cyanidin 3,5-diglucoside and no cyanidin 3-gentiobioside standard was available. This paper is the first report of peonidin and pelargonidin glycosides in blue honeysuckle fruits.

Characterization of Polyphenolics. The HPLC-DAD blue honeysuckle phenolic profile was monitored at 320 nm for hydroxycinnamic derivatives, at 260 and 370 nm for flavonols, and at 520 nm for anthocyanins and is illustrated in **Figure 2**. All blue honeysuckle fruits presented similar HPLC profiles composed of at least five identified and two unidentified phenolic compounds. Peak 2 accounted for >70% of total peak area of non-anthocyanin polyphenolics at highest maximum wavelength.

Identifications were made by comparison of their retention times and UV−vis spectra with available standards and by confirming molecular weight by ESMS. The major polyphenolic, peak 2, was identified as chlorogenic acid. Peak 1 had the same mass, *m*/*z* 355, and its UV−vis spectrum and retention behavior are consistent with neochlorogenic acid, which was previously identified in cherries in our laboratory (*16*). Peak 3 was identified as cyanidin 3-glucoside with *m*/*z* 449. Peaks 4 and 5 were identified as quercetin 3-rutinoside, *m*/*z* 609, and quercetin 3-glucoside, *m*/*z* 463, respectively. The peak eluting at 22.5 min had UV−vis spectroscopic characteristics similar to those of flavonols and was assigned as an unidentified flavonol. The peak eluting at 30.5 min has UV−vis characteristics similar to those of caffeic acid or ferulic acid and was characterized as a hydroxycinnamic derivative. These identifications and peak assignments are in agreement with Plekhanov et al. (*12*). Caffeic acid and quercetin 3-rhamnoside were not detected in our samples.

Quantification of Blue Honeysuckle Polyphenolics. Quantification of blue honeysuckle hydroxycinnamates and flavonols is shown in **Table 2**. For comparative purposes, the anthocyanin content, total phenolics, and antioxidant properties that were reported in a previous study (*1*) are also listed. The 10 blue honeysuckle genotypes contained wide ranges of anthocyanins, total phenolics, hydroxycinnamic derivatives, flavonols, oxygen radical absorbing capacity (ORAC), and ferric reducing antioxidant power (FRAP). The *boczkarnikovae* subspecies contained the highest anthocyanin, total phenolics, hydroxycinnamates, flavonols, ORAC, and FRAP. ORAC and FRAP were highly correlated with hydroxycinnamic acid derivatives (R = 0.828 and 0.796) and flavonols (R = 0.83 and 0.82). However, those correlations were lower than the correlation of ORAC and FRAP with anthocyanins (R = 0.93 and 0.95) and total phenolic (R = 0.95 and 0.97) (*9*, *16*). Antioxidant activities are

Table 2. Hydroxycinnamate and Flavonol Content of 10 Genotypes of Blue Honeysuckle[a]

cultivar	hydroxycinnamate[b]	flavonol[c]
boczkarnikovae	156 ± 8.2	32.8 ± 0.78
edulis		
Zarnitsa	121 ± 1.14	26.4 ± 0.16
stenantha	79.0 ± 3.16	15.7 ± 0.53
pallassi	92.8 ± 1.70	18.1 ± 0.52
kamtschatica		
Selec. 2-32	40.8 ± 0.45	12.6 ± 0.09
Selec. F1-9-58	30.4 ± 1.11	18.1 ± 0.37
Bluebird	69.2 ± 2.91	14.0 ± 0.37
Berry Blue	61.1 ± 0.09	16.1 ± 0.24
Magadan	77.0 ± 0.84	17.9 ± 0.18
Selec. 8-18	68.7 ± 2.15	14.2 ± 0.08

[a] All are subspecies of *Lonicera caerulea*. All values are presented as mean ± SD, $n = 2$. Values for total anthocyanin pigments, total phenolics, and antioxidant properties ORAC and FRAP for these same samples are reported in Thompson and Chaovanalikit (*1*). Anthocyanins ranged from 116 to 593 mg/100 g; total phenolics from 427 to 1140 mg of gallic acid equivalents/100 g; ORAC from 1840 to 10370 μmol of Trolox equivalents/100 g, and FRAP from 3810 to 9380 μmol of Trolox equivalent/100 g. [b] Concentration based on chlorogenic acid as standard (mg/100 g of fresh weight). [c] Concentration based on quercetin 3-rutinoside as standard (mg/100 g of fresh weight),

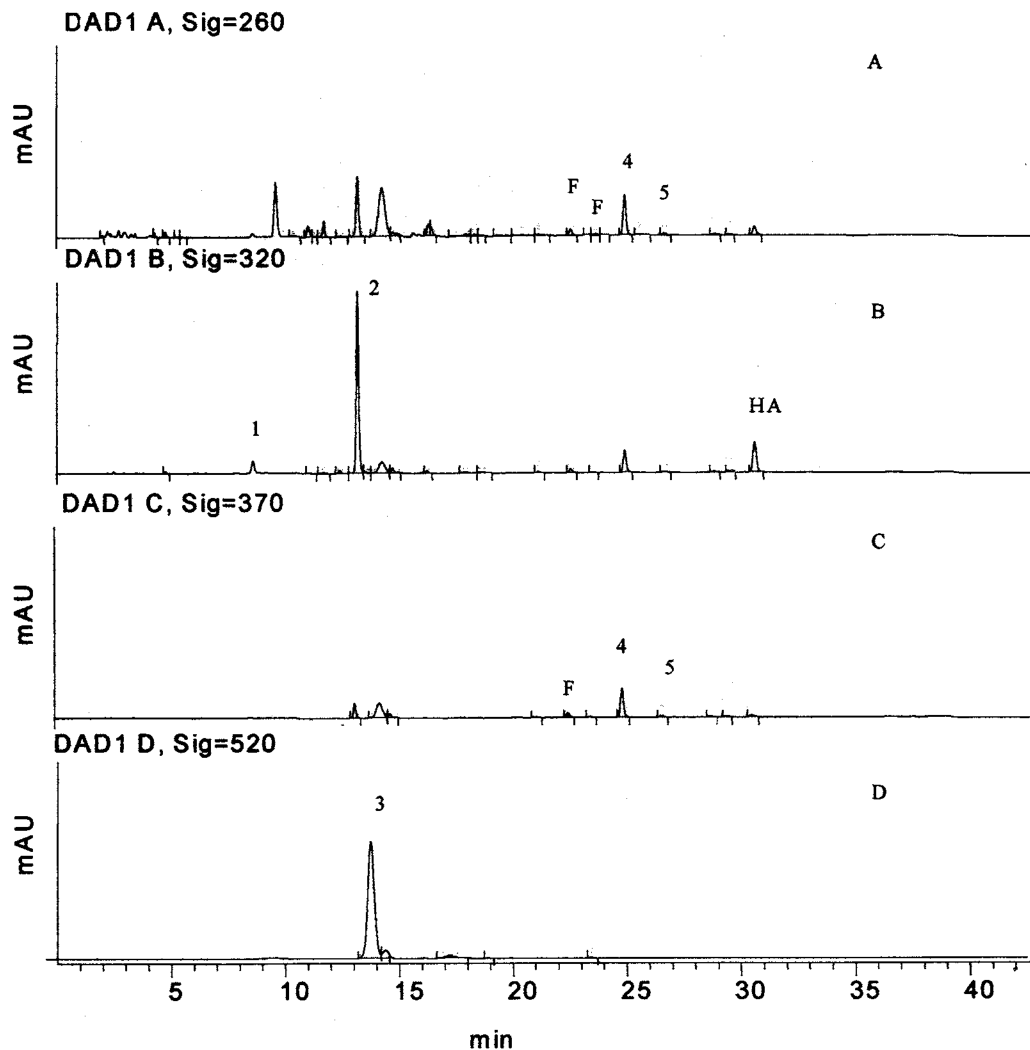

Figure 2. HPLC polyphenolics in blue honeysuckle, monitored at 260, 320, 370, and 520 nm: 1, neochlorogenic acid; 2, chlorogenic acid; 3, cyanidin 3-glucoside; 4, quercetin 3-rutinoside; 5, quercetin 3-glucoside; HA, unidentified hydroxycinnamic acid derivatives; F, unidentified flavonols.

more associated with the total phenolics than with a single class of compounds.

Hydroxycinnamates and flavonols are widely distributed in fruits (*17*, *18*). Blue honeysuckle fruits contain an amount of hydroxycinnamic acid derivatives (30.4−156.2 mg/100 g) comparable to those of blueberries (*17*) (114.9 mg/100 g) and black currants (*18*) (58−93 mg/100 g) but less than that of bilberries (*18*) (113−231 mg/100 g). The amount of flavonols in blue honeysuckle fruits (12.6−32.8 mg/100 g) was lower than that in bilberries (54−130 mg/100 g), black currants (72−74 mg/100 g), and blueberries (60.9 mg/100 g).

Although the content of hydroxycinnamic acid derivatives and flavonols of blue honeysuckle was somewhat different from that of other berries, blue honeysuckle provided comparable ORAC (1840−10370 μmol of Trolox equivalents/100 g) to berries in several genera: *Vaccinium* such as blueberries (1900−13100 μmol of Trolox equivalents/100 g), *Rubus* such as blackberries (1300−14600 μmol of Trolox equivalents/100 g), and *Ribes* such as black currants (1700−11600 μmol of Trolox equivalents/100 g) (*9*, *16*).

In conclusion, blue honeysuckle berries are an excellent source of dietary phytochemicals such as anthocyanins and polyphenolics, being comparable to *Vaccinium*, *Rubus*, and *Ribes* fruits. The use of blue honeysuckle as natural antioxidants, natural colorants, and an ingredient of functional foods seems to be promising.

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