JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Phenolics in Slovenian Bilberries (*Vaccinium myrtillus* L.) and Blueberries (*Vaccinium corymbosum* L.)

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ABSTRACT: Phenolics from bilberries (*Vaccinium myrtillus* L.) sampled from seven different locations and highbush blueberries (*Vaccinium corymbosum* L.) from one location in Slovenia were analyzed. In samples of both species 15 anthocyanins were identified by LC-MS/MS. Their contents were expressed as cyanidin 3-glucoside equivalents (C3GE); bilberries contained 1210.3 ± 111.5 mg C3GE/100 g fw and blueberries 212.4 ± 14.1 mg C3GE/100 g fw. Glycosides of delphinidin and cyanidin were predominant (488.5 vs 363.6 mg C3GE/100 g fw) in the bilberries and glycosides of malvidin (108.0 vs 100.8 mg C3GE/100 g fw) in the blueberries, whereas the contents of peonidin were lowest (74.5 vs 4.8 mg C3GE/100 g fw) in both berries. The contents of flavanols, flavonols, phenolic acids, and stilbenes were determined by LC-MS. For the first time, rutin was identified (bilberries, 0.2 ± 0.0 mg/100 g fw; blueberries, 3.1 ± 0.1 mg/100 g fw). Chlorogenic acid (as 3-caffeoylquinic acid) was the most abundant among the phenolic acids (23.1 ± 1.0 mg/100 g fw in bilberries and 70.0 ± 3.4 mg/100 g fw in blueberries). Statistical analysis shows that the content of 27 individual flavonoids, phenolic acids, and stilbenes can be used to identify the picking region of these Slovenian bilberries.

KEYWORDS: bilberries, blueberries, anthocyanins, flavanols, flavonols, phenolic acids, rutin, *trans*-resveratrol, LC-MS/MS, LC-MS, multivariate analysis

INTRODUCTION

The enrichment of the diet with fruits and vegetables is generally accepted as beneficial to human health. Bilberries (*Vaccinium myrtillus* L.) are native to Europe, and highbush blueberries (*Vaccinium corymbosum* L.) originate from North America. These berries are considered to be two of the most important sources of various phenolic compounds. They contain flavonoids, phenolic acids, and stilbenes.[1−3] It is known that both of these berries are especially rich in anthocyanins, with these compounds responsible for their blue color. Moreover, in bilberries, anthocyanins have been estimated to represent nearly 90% of the total phenolics.[4]

All berries, including bilberries and blueberries, have been shown to have high antioxidant potential due to their richness in phenolics. The antioxidant activities of bilberries and blueberries have been demonstrated using several in vitro antioxidant assays, such as radical-scavenging capacity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), ferric-reducing antioxidant activity (FRAP), oxygen radical absorbance activity (ORAC), inhibition test for lipid peroxidation,[3,5] and a cell antioxidant activity assay that can simulate in vivo conditions.[6] Due to their high levels of phenolics, bilberries and blueberries have been reported to have multiple biological effects, including anticarcinogenic,[7] anti-inflammatory, and antimicrobial activities.[8] Beneficial effects have also been established in cardiovascular diseases and diabetes,[9] as well as in improved vision[10] and neurodegenerative functions.[11]

Phenolics in bilberries and blueberries have been studied in many countries in Europe, for example, Italy,[3] Finland,[12] Sweden,[13] Lithuania,[14] and Estonia,[2] although, to date, never in Slovenia. However, bilberries are traditionally collected during the main season in June and July in Slovenian woods, whereas blueberries are cultivated in plantations in Slovenia. Both of these berries are consumed as fresh fruits; furthermore, bilberries are processed into various foods, such as jams, syrups, alcoholic and nonalcoholic beverages, teas, and sweets. Consequently, in Slovenia, bilberries and blueberries might represent an important source of phenolics, and particularly anthocyanins, in the everyday human diet. According to the literature, bilberries (ca. 90% of total phenolics) were estimated to be a richer source of anthocyanins in comparison to other berries such as cranberries (ca. 40%) or strawberries (ca. 48%).[4]

We determined the contents of individual anthocyanins, flavanols, flavonols, phenolic acids, and stilbenes using liquid chromatography−tandem mass spectrometry (LC-MS/MS) and LC-MS. The phenolic profiles of both berries were then compared and statistical analyses used to examine potential correlations with the locations of the bilberry sampling.

MATERIALS AND METHODS

Chemicals and Reagents. Methanol and formic acid were from Merck (Darmstadt, Germany). Acetonitrile was from J. T. Baker (Deventer, The Netherlands). All of the solvents used were of HPLC purity. The standard cyanidin 3-glucoside was from Polyphenols

Received: February 24, 2011
Revised: May 10, 2011
Accepted: May 16, 2011
Published: May 16, 2011

Figure 1. Map of Slovenia with the picking locations of bilberries. Bilberries (*Vaccinium myrtillus* L.) were sampled in woods from seven different locations in Pokljuka (n = 3), Celje (n = 4), Goricko (n = 5), Skofja Loka (n = 6), Ljubljana (n = 6), Pohorje (n = 6), and Kranj (n = 6), where n means the number of microlocations. The blueberries were picked from a plantation in Ljubljana. All samples were collected at the full ripe stage in the latter part of June 2007.

Laboratories AS (Sandnes, Norway), and standards chlorogenic (3-caffeoylquinic acid), caffeic, ferulic, *p*-coumaric, ellagic, and gallic acids, catechin, myricetin, quercetin, rutin, *trans*-resveratrol, and ammonium formate were from Sigma-Aldrich (Steinheim, Germany). Aqueous solutions were prepared using Milli-Q water (Millipore, Bedford, MA).

Extraction. Bilberries (*V. myrtillus* L.) were sampled in woods from seven different locations in Slovenia: Pokljuka (n = 3), Celje (n = 4), Goricko (n = 5), Skofja Loka (n = 6), Ljubljana (n = 6), Pohorje (n = 6), and Kranj (n = 6), where n means the number of microlocations (Figure 1). All samples were collected at the full ripe stage in the latter part of June 2007. Locations were chosen on the basis of the distance among autochthonous growing regions representative for Slovenia and pedoclimatic differences among them (unpublished results). Blueberries (*V. corymbosum* L.) were sampled from a plantation on the Ljubljansko barje in Slovenia. The ripe bilberries and blueberries were stored at −20 °C for 3 months, when extracts were prepared. The extraction method was as previously published[15] and is described only briefly here. Frozen samples (50 g) were homogenized in 150 mL of ice-cold deoxygenated methanol that had previously been flushed for a few minutes with nitrogen. The homogenates were extracted for 3 h by shaking (Shaker EV403, Tehtnica, Zelezniki, Slovenia) in the dark at room temperature. The extracts were centrifuged at 3472*g* for 5 min, and the supernatants were stored at −20 °C. The sediments were extracted again in 100 mL of deoxygenated methanol for 2 h in the dark at room temperature, and the suspensions were centrifuged as before. Finally, the paired supernatant samples were pooled, flushed with nitrogen for a few minutes, and then stored at −20 °C for 1 year before analysis.

HPLC Analysis. Individual anthocyanins of berry extracts were determined by LC-MS/MS, whereas flavanols, flavonols, phenolic acids, and stilbenes were determined by LC-MS. For both methods, the HPLC system consisted of an Agilent 1100 binary pump (G1312A) and autosampler (G1330B) coupled to a Micromass Quattro Micro mass spectrometer equipped with an electrospray ionizer source (ESI) (Waters, Milford, MA). Reversed-phase HPLC separations were carried out using a Gemini C18 column (150 × 2.00 mm, 3 μm), protected by a Gemini C18 Security Guard cartridge (4.0 × 2.0 mm) (Phenomenex, Torrance, CA).

Sample Cleanup for LC-MS/MS. Individual anthocyanins were analyzed according to the optimized method of Lätti et al.[12] The berry extracts were cleaned up as follows: 500 μL samples of the berry extracts were dried using a rotavapor, and the dried residues were resuspended in 1 mL of 3% formic acid. The samples were then loaded onto 1 g Sep-Pak C18 cartridges (Waters) that had been previously activated with 3 mL of pure methanol and 5 mL of 3% formic acid. After the cartridges had been washed with 6 mL of 3% formic acid, the anthocyanins were eluted with 5 mL of pure methanol. The eluates were evaporated to dryness and resuspended in 5 mL of HPLC injection phase: 7% mobile phase A (3% formic acid) and 93% mobile phase B (acetonitrile/methanol, 85:15, v/v).

LC-MS/MS. The anthocyanins were separated at 40 °C with the following gradient: 0−2 min, 7−9% B; 2−4 min, 9−11% B; 4−12 min, 11−12% B; 12−13 min, 12% B; 13−25 min, 12−13% B; 25−40 min, 13−100% B. The flow rate of the mobile phase was 0.250 mL/min from 0 to 4 min, 0.225 mL/min from 4 to 13 min, and 0.200 mL/min from 13 to 40 min and offered better separation according to Lätti et al.[12] and our own experience. The injection volume was 10 μL. The mass spectrometer was operated in positive-ion mode (ESI^+) with the following operating parameters: capillary voltage, 3.0 kV; cone voltage, 20 V; extractor, 5 V. The source temperature was 100 °C and the desolvation temperature was 350 °C. The cone gas flow was set at 40 L/h, the desolvation gas flow at 400 L/h, and the collision energy at 30 V. The anthocyanins were identified on the basis of their retention times (t_R), MS spectra, and molecular ion identification. The quantification of individual anthocyanins was determined from the calibration curve of 10 points with spiked bilberry samples that covered the range from 0.5 to 45 mg/L and calculated relative to cyanidin 3-glucoside (the external standard).

Samples Cleanup for LC-MS. The flavanols, flavonols, phenolic acids, and stilbenes were analyzed according to the method of Bertoncelj et al.[16] as follows. For clean-up of the berry extracts, 500 μL of berry extract was supplemented with 500 μL of Milli-Q water and 3 mL of 20 mmol/L ammonium formate (pH 2.4). These samples were loaded onto 60 mg Strata-X cartridges (Phenomenex) previously conditioned with 2 mL of pure methanol, and 2 mL of 20 mmol/L ammonium formate (pH 2.4). After these cartridges had been washed with 2 mL of 15% methanol in 20 mmol/L ammonium formate (pH 2.4), the retained phenolic fraction was eluted with 2 mL of pure methanol. The eluates were evaporated, and then the dried samples were resuspended in 500 μL of 1% formic acid in 50% methanol.

LC-MS. The mobile phase components were 1% formic acid (A) and acetonitrile (B). The mobile phase gradient used was 0−5 min, 10% B; 5−50 min, 10−60% B; 50−52 min, 60−80% B; 52−60 min, 80% B; 60−70 min, 80−10% B; 70−80 min, 10% B. The injection volume was 20 μL, and the column temperature was 25 °C. The flow rate of the mobile phase was 0.200 mL/min. The mass spectrometer operated in negative ion mode (ESI^-) with the following parameters: capillary voltage, 3.0 kV; cone voltage, 25 V; extractor, 5 V. The source temperature was 100 °C, the desolvation temperature was 350 °C, the cone gas flow was 50 L/h, and the desolvation gas flow was 400 L/h. The flavanols, flavonols, phenolic acids, and stilbenes were identified on the basis of their t_R, MS spectra, and molecular ion identification. Quantification of the compounds was calculated relative to the corresponding external standards from the calibration curves of 10 points with spiked bilberry samples that covered the range from 0.1 to 40 mg/L. The flavanols were quantified according to catechin, whereas other compounds were quantified according to their corresponding standards: (flavonols) myricetin, quercetin, and rutin; (phenolic acids) chlorogenic acid as 3-caffeoylquinic acid, caffeic acid, ferulic acid, *p*-coumaric acid, ellagic acid, and gallic acid; and (stilbene) *trans*-resveratrol.

Multivariate Analysis. Statistical analysis was performed using SPSS for Windows, version 15.0, as the evaluation version (SPSS Inc., Chicago, IL). Relations between the main components were assessed by Pearson correlation coefficients. The multivariate analyses included principal component analysis (PCA) and linear discriminant analysis

(LDA). Previously, the normality of the data was tested using the Shapiro−Wilk test.

RESULTS AND DISCUSSION

Anthocyanins. The individual anthocyanin contents were determined by LC-MS/MS according to the linear calibration curve (correlation coefficient = 0.9983) from spiked samples and expressed as C3GE equivalents (mg/100 g berry fresh weight (fw)). The limit of detection (LOD) and the limit of quantification (LOQ) were determined from signal-to-noise ratios at the beginning of our experiments, and they are given together with repeatability (CV) of the standards in Table 1.

The contents of the individual anthocyanins in bilberries and blueberries are given in Table 2. The variations of the anthocyanin profile and content observed in bilberries might occur because of genetic and/or pedoclimatic diversity. In our investigations (unpublished data) no genetic diversity was detected among the bilberries sampled in the growing regions in question. With regard to climate, Pokljuka and Pohorje belong to the mountainous growing region; Celje, Goricko, and Skofja Loka belong to the hilly growing region; and Ljubljana and Kranj belong to the lowland growing region (Figure 1). In all growing regions bilberries are grown on acidic soil rich with organic matter. The sum of the individual anthocyanins (1210.3 ± 111.5 mg/100 g fw) in the bilberries was about 6-fold compared to that in the blueberries (212.4 ± 14.1) (Table 2). These data are in good agreement with published data of the spectrophotometrically determined total anthocyanins of the same samples of bilberries and blueberries.[15] However, the total anthocyanins in these Slovenian bilberries were about 3-fold compared to previously reported data for bilberries from Finland (350−525 mg/100 g fw).[12,17] The higher anthocyanin levels in bilberries than in blueberries are due to anthocyanins found in both the skin and pulp of bilberries, whereas in blueberries they are only in the skin.[18] Also, red anthocyanins are mainly found in the skin of red grapes and black currants,[19] but the entire fruits of strawberries, blackberries, raspberries, and elderberries are colored by anthocyanins.[20−22] Bilberries are richer in anthocyanins than other berries such as black currants, cranberries, lingonberries, raspberries, and strawberries,[4] whereas blueberries have a content of anthocyanins similar to that of black currant,[17] but lower than that of blackberries,[1] cranberries,[22] and rowanberries.[23]

In Slovenian bilberries and the blueberries planted in Slovenia, we identified 15 anthocyanins (see Table 2) of 5 glycoside classes of delphinidin, cyanidin, petunidin, peonidin, and malvidin. The identification of the anthocyanidins was performed by mass spectrometry according to the m/z of their positive ions.

Table 1. LC-MS/MS and LC-MS Characteristics for Quantification of Standards

standard	LOQ[a] (10^{-6} g/kg)	LOD[a] (10^{-6} g/kg)	CV[a] (%)
cyanidin 3-glucoside	0.9	0.3	2.8
catechin	12.8	3.9	1.1
quercetin	1.1	0.3	0.8
myricetin	13.5	4.1	2.8
rutin	0.4	0.1	1.5
chlorogenic acid	9.6	2.9	1.2
caffeic acid	6.0	1.8	1.7
ferulic acid	22.5	6.8	2.3
p-coumaric acid	9.6	2.9	2.6
ellagic acid	5.6	1.7	1.3
gallic acid	6.0	0.2	1.8
trans-resveratrol	3.2	0.1	2.5

[a] LOD and LOQ were determined from signal-to-noise ratio by our experiments. LOQ, limit of quantification with signal-to-noise ratio ≥10; LOD, limit of detection with signal-to-noise ratio ≥3; CV, repeatability of standards.

Table 2. Individual Anthocyanins in the Slovenian Bilberries and Blueberries[a]

anthocyanin	M_w	MS/MS (m/z)	t_R (min)	bilberries[b] (mg/100 g fw)	blueberries[b] (mg/100 g fw)
delphinidin 3-galactoside	465	303	10.50 ± 0.01	167.1 ± 6.5	23.4 ± 1.4
delphinidin 3-glucoside	465	303	11.51 ± 0.01	169.1 ± 6.6	15.4 ± 1.4
cyanidin 3-galactoside	449	287	12.15 ± 0.01	122.6 ± 5.7	4.2 ± 0.2
delphinidin 3-arabinoside	435	303	12.70 ± 0.01	152.3 ± 6.1	24.6 ± 1.7
cyanidin 3-glucoside	449	287	13.69 ± 0.02	130.4 ± 6.2	2.6 ± 0.3
petunidin 3-galactoside	479	317	14.76 ± 0.02	50.0 ± 2.6	11.7 ± 0.8
cyanidin 3-arabinoside	419	287	15.15 ± 0.02	110.6 ± 4.9	3.5 ± 0.3
petunidin 3-glucoside	479	317	16.72 ± 0.02	101.9 ± 6.2	12.4 ± 0.8
peonidin 3-galactoside	463	301	17.82 ± 0.03	13.3 ± 1.2	1.8 ± 0.1
petunidin 3-arabinoside	449	317	18.98 ± 0.03	23.9 ± 1.5	9.3 ± 0.6
peonidin 3-glucoside	463	301	20.75 ± 0.03	56.7 ± 5.1	2.1 ± 0.1
malvidin 3-galactoside	493	331	21.66 ± 0.03	27.5 ± 3.3	34.9 ± 3.1
peonidin 3-arabinoside	433	301	22.99 ± 0.04	4.5 ± 0.4	1.0 ± 0.1
malvidin 3-glucoside	493	331	24.89 ± 0.05	67.7 ± 5.9	31.2 ± 3.4
malvidin 3-arabinoside	463	331	28.24 ± 0.05	12.8 ± 1.3	34.7 ± 3.7
total				1210.3 ± 111.5	212.4 ± 14.1

[a] The bilberries (*Vaccinium myrtillus* L.) were sampled in woods from seven different locations in Slovenia: Pokljuka (n = 3), Celje (n = 4), Goricko (n = 5), Skofja Loka (n = 6), Ljubljana (n = 6), Pohorje (n = 6), and Kranj (n = 6), where n means a number of microlocations. The blueberries were picked from a plantation in Ljubljana. All samples were collected at the full ripe stage in the latter part of June 2007. M_w, molecular weight; t_R, retention time. [b] Anthocyanins quantified as mg of cyanidin-3-glucoside (C3GE) equivalents/100 g berries fresh weight (fw). Data expressed as the mean ± SEM. Number of independent measurements: bilberries n = 36, blueberries n = 5.

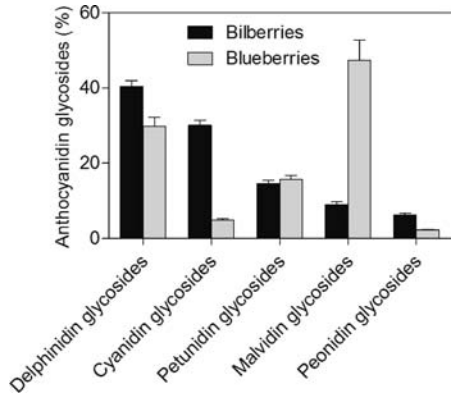

Figure 2. Anthocyanidin glycosides in Slovenian bilberries and blueberries. The bilberries (*Vaccinium myrtillus* L.) were sampled in woods from seven different locations in Slovenia: Pokljuka (n = 3), Celje (n = 4), Goricko (n = 5), Skofja Loka (n = 6), Ljubljana (n = 6), Pohorje (n = 6), and Kranj (n = 6), where n means a number of microlocations. The blueberries were picked from a plantation in Ljubljana. All samples were collected at the full ripe stage in the latter part of June 2007. Data are expressed as the mean (% of total anthocyanidin glycosides) ± SEM of glycoside types indicated. Number of independent measurements: bilberries n = 36; blueberries, n = 5.

Their sugar component and the position of glycosidic bonds in the anthocyanins according to the t_R of the corresponding standards were also determined. The t_R values here were as follows: delphinidin 3-galactoside < delphinidin 3-glucoside < delphinidin 3-arabinoside. The pattern was the same for other anthocyanins identified here. Only one molecule of a monosaccharide (glucose, galactose, or arabinose) was bound to an anthocyanidin position 3 of the C-ring, as has been seen previously.[3,12,18]

The contents of each individual anthocyanin in the berries (Table 2) were calculated according to the five anthocyanidin classes found (delphinidin, cyanidin, petunidin, malvidin, peonidin) and expressed as percentages of the total anthocyanins (Figure 2). In the Slovenian bilberries, the delphinidin and cyanidin glycosides were predominant (>70%), as has also been reported recently for Finnish[12] and Swedish bilberries,[13] whereas glycosides of malvidin were predominant in blueberries planted in Slovenia (Figure 2; ca. 47%). Some published data agree with those reported here,[5,14] whereas others contradict. In blueberries from Canada and the United States, for instance, only the delphinidin glycosides were predominant.[24] However, in both Slovenian berries, the peonidin glycosides content was the lowest among the anthocyanidin glycosides (Figure 2). The same was true for blueberries grown in Finland.[12] The pattern of anthocyanins in the berries of genus *Vaccinium* could be used for chemotaxonomic classification. Furthermore, the diversity of anthocyanins in bilberries might affect their bioavailability and bioactivity as antioxidants in mammals. It was shown that after a bilberry extract was orally administered to rats, the plasma level of anthocyanidin glycosides after 15 min was galactoside > glucoside > arabinoside and that the arabinoside disappeared more rapidly than glucoside and galactoside. On the other hand, when anthocyanins carrying the same sugar moiety were compared, the half-disappearance time of plasma anthocyanins was in the following order: delphinidin > cyanidin > petunidin = peonidin > malvidin. After anthocyanins appear in blood, a number of biochemical events might affect their bioavailability and bioactivity. In a short time window (<30 min), the major anthocyanins are rapidly distributed from blood to liver and kidneys, where they are *O*-methylated at the B ring.[25,26]

Table 3. Flavanols, Flavonols, Phenolic Acids, and Stilbenes in Bilberries and Blueberries[a]

phenolics	M_w	t_R (min)	bilberries[b] (mg/100 g fw)	blueberries[b] (mg/100 g fw)
flavanols				
catechin	290	9.70 ± 0.05	0.2 ± 0.0	1.8 ± 0.1
epicatechin	290	15.94 ± 0.01	2.0 ± 0.2	0.5 ± 0.01
flavonols				
quercetin	302	32.79 ± 0.01	0.8 ± 0.0	0.1 ± 0.0
myricetin	318	32.85 ± 0.01	0.4 ± 0.0	nd
rutin	610	23.65 ± 0.04	0.2 ± 0.0	3.1 ± 0.1
phenolic acids				
chlorogenic acid	354	10.45 ± 0.01	23.1 ± 1.0	70.0 ± 3.4
caffeic acid	180	13.24 ± 0.04	0.3 ± 0.0	0.2 ± 0.0
ferulic acid	194	26.24 ± 0.01	0.4 ± 0.0	2.2 ± 0.2
p-coumaric acid	164	19.73 ± 0.02	0.3 ± 0.0	id
ellagic acid	302	24.13 ± 0.01	1.2 ± 0.0	0.1 ± 0.0
gallic acid	170	3.67 ± 0.01	6.2 ± 0.3	1.8 ± 0.2
stilbene				
trans-resveratrol	228	29.28 ± 0.01	0.2 ± 0.0	0.4 ± 0.0
total			35.3 ± 0.84	80.2 ± 1.93

[a] The bilberries (*Vaccinium myrtillus* L.) were sampled in woods from seven different locations in Slovenia: Pokljuka (n = 3), Celje (n = 4), Goricko (n = 5), Skofja Loka (n = 6), Ljubljana (n = 6), Pohorje (n = 6), and Kranj (n = 6), where n means a number of microlocations. The blueberries were picked from a plantation in Ljubljana. All samples were collected at the full ripe stage in the latter part of June 2007. Data expressed as the mean ± SEM. Number of independent measurements: bilberries n = 36, blueberries n = 5. M_w, molecular weight; t_R, retention time; id, identified, but under the LOQ; nd, not detected. [b] Compounds quantified as mg equivalents/100 g berries fresh weight (fw).

Flavanols, Flavonols, Phenolic Acids, and Stilbenes. The flavanols, flavonols, phenolic acids, and stilbenes in the bilberries and blueberries were determined by LC-MS. The values obtained were expressed according to the corresponding standard equivalents in mg/100 g berry fw. The calibration curves of the spiked standards were linear, with correlation coefficients of ≥0.99. Their LODs, LOQs, and CVs are also given in Table 1.

The data set for the contents of these flavanols, flavonols, phenolic acids, and the stilbene in the bilberries and blueberries is given in Table 3. These compounds and the anthocyanins can act as antioxidants and may be important components of functional foods. The two flavanols were found in both types of berries, the bilberries being a better source of epicatechin (2.0 mg/100 g fw), and, on the other hand, the blueberries for catechin (1.8 mg/100 g fw). Both of these flavanols have already been detected in both types of berries in previous studies[24] as well as in other berries, such as blackberries[1] and cranberries.[24] The three flavonols determined in the present study were quercetin, myricetin, and rutin. Quercetin was found in both types of berries, whereas myricetin was present only in the bilberries (Table 3). According to already published data, these berries showed less quercetin than described previously.[18] Rutin, which has been reported in high amounts in buckwheat,[27] was found in both types of berries under

Table 4. Quantitative Analysis of Individual Anthocyanins, Flavanols, Flavonols, Phenolic Acids, and Stilbenes Identified in the Slovenian Bilberries from Seven Different Locations[a]

	region						
parameter	Pokljuka (n = 3)	Celje (n = 4)	Goricko (n = 5)	Ljubljana (n = 5)	Kranj (n = 5)	Skofja Loka (n = 5)	Pohorje (n = 5)
delphinidin 3-galactoside	158.5 ± 26.7	134.1 ± 17.6	177.7 ± 47.9	164.8 ± 35.6	141.2 ± 26.1	219.3 ± 17.6	160.7 ± 31.2
delphinidin 3-glucoside	161.2 ± 21.1	145.8 ± 29.1	166.2 ± 57.0	171.0 ± 35.9	140.5 ± 23.4	220.4 ± 17.8	166.5 ± 30.4
cyanidin 3-galactoside	93.7 ± 6.6	94.9 ± 34.5	144.9 ± 52.3	110.4 ± 20.0	120.8 ± 29.0	150.6 ± 26.0	122.6 ± 26.1
delphinidin 3-arabinoside	156.4 ± 12.9	121.1 ± 18.5	140.6 ± 42.3	155.8 ± 30.5	131.6 ± 24.1	207.1 ± 10.7	143.0 ± 28.2
cyanidin 3-glucoside	107.0 ± 2.6	119.6 ± 53.2	145.8 ± 63.2	120.0 ± 28.2	130.7 ± 28.9	154.6 ± 29.3	122.4 ± 27.4
petunidin 3-galactoside	39.9 ± 2.6	44.4 ± 1.9	66.2 ± 23.8	48.6 ± 10.3	44.8 ± 13.6	61.5 ± 14.7	40.4 ± 6.9
cyanidin 3-arabinoside	97.2 ± 1.1	93.6 ± 23.1	121.8 ± 51.6	106.1 ± 18.5	118.2 ± 24.5	133.3 ± 17.0	93.7 ± 27.8
petunidin 3-glucoside	78.2 ± 22.2	91.3 ± 29.7	119.0 ± 52.3	81.2 ± 37.3	90.7 ± 21.0	148.0 ± 19.1	92.2 ± 19.4
peonidin 3-galactoside	4.5 ± 1.7	15.3 ± 14.0	19.2 ± 4.6	12.3 ± 5.8	10.7 ± 2.5	17.3 ± 5.9	11.1 ± 3.0
petunidin 3-arabinoside	16.9 ± 5.9	17.4 ± 5.0	20.2 ± 4.1	23.3 ± 11.7	22.1 ± 6.6	35.3 ± 5.9	25.8 ± 5.0
peonidin 3-glucoside	23.2 ± 3.1	60.6 ± 62.5	68.9 ± 33.5	57.4 ± 30.9	56.8 ± 25.5	69.3 ± 15.2	47.2 ± 14.4
malvidin 3-galactoside	10.8 ± 1.8	17.1 ± 5.3	48.4 ± 42.2	19.5 ± 10.3	22.0 ± 8.5	39.1 ± 11.8	27.3 ± 4.8
peonidin 3-arabinoside	1.9 ± 1.0	3.7 ± 2.7	5.3 ± 4.1	3.2 ± 0.8	4.5 ± 1.6	6.7 ± 1.8	4.8 ± 1.7
malvidin 3-glucoside	26.7 ± 4.4	53.8 ± 29.5	60.9 ± 46.3	63.5 ± 27.3	44.3 ± 17.7	108.3 ± 28.3	90.0 ± 17.8
malvidin 3-arabinoside	5.6 ± 1.3	9.8 ± 3.6	12.8 ± 10.9	9.3 ± 2.0	7.9 ± 3.0	22.2 ± 9.8	17.4 ± 3.5
catechin	0.3 ± 0.2	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2
epicatechin	2.7 ± 2.3	1.3 ± 0.4	1.2 ± 0.8	2.0 ± 1.2	2.1 ± 0.7	1.7 ± 0.4	3.1 ± 1.6
quercetin	1.2 ± 0.6	0.7 ± 0.2	0.8 ± 0.2	0.73 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
myricetin	0.4 ± 0.2	0.4 ± 0.1	0.5 ± 0.2	0.5 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
rutin	0.2 ± 0.1	0.4 ± 0.7	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
chlorogenic acid	29.7 ± 5.2	21.0 ± 5.0	22.8 ± 2.7	22.2 ± 9.2	23.4 ± 4.6	24.6 ± 4.7	20.1 ± 7.2
caffeic acid	0.4 ± 0.2	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.5 ± 0.2
ferulic acid	0.8 ± 0.1	0.5 ± 0.1	0.4 ± 0.2	0.5 ± 0.4	0.2 ± 0.2	0.4 ± 0.2	0.5 ± 0.3
p-coumaric acid	0.2 ± 0.1	0.3 ± 0.3	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.0
ellagic acid	0.8 ± 0.1	0.9 ± 0.3	1.2 ± 0.3	1.2 ± 0.2	1.4 ± 0.2	1.3 ± 0.3	1.1 ± 0.1
gallic acid	8.5 ± 2.4	5.2 ± 0.3	6.0 ± 1.5	7.5 ± 1.7	5.4 ± 0.6	6.2 ± 0.4	5.4 ± 1.1
trans-resveratrol	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1
total phenolics determined[b]	1027 ± 62 b	1054 ± 224 b	1352 ± 409 ab	1182 ± 247 b	1121 ± 211 b	1629 ± 120 a	1198 ± 209 b

[a] n means a number of microlocations. The blueberries were picked from a plantation in Ljubljana. All samples were collected at the full ripe stage in the latter part of June 2007. Compounds quantified as mg equivalents/100 g berries fresh weight (fw), according to the corresponding standard. Data are the mean ± SEM. [b] Different letters indicate significant differerence at $p < 0.05$.

investigation, the blueberries containing ca. 16-fold more rutin than the bilberries (3.1 vs 0.2 mg/100 g fw, respectively). To our best knowledge, there have not been any studies to report the presence of rutin in bilberries or blueberries. Although this determined content of rutin in the bilberries is low, it is still 20000 times above its limit of detection, which is 0.1×10^{-6} g/kg (Table 1).

However, phenolic acids were present in high amounts. The predominant phenolic acid in both types of berries was chlorogenic acid (determined as 3-caffeoylquinic acid), its content being 3 times higher in blueberries than in bilberries (23.1 vs 70.0 mg/100 g fw). Caffeic, ferulic, ellagic, and gallic acids were all detected in both types of berries, as well as *p*-coumaric acid, but the latter was quantifiable only in the bilberries. The presence of phenolic acids in bilberries and blueberries has been reported in a number of previous studies.[1,2,5,23] Stilbenes have also been detected in bilberries and blueberries, but only *trans*-resveratrol was found in both of the berries in our samples and in similar quantities. There are not many reports of *trans*-resveratrol in bilberries and blueberries. However, previous data for *trans*-resveratrol content in bilberries[2] agree with ours, but it was determined to be lower for blueberries.[5] It was also found in other berries such as cowberries, cranberries, strawberries, and red currant[2] and in high levels in red grapes and red wine (as one of the compounds behind the French paradox).

In this study, the contents of 15 anthocyanins, 2 flavanols, 3 flavonols, 6 phenolic acids, and the stilbene *trans*-resveratrol were determined in bilberries and blueberries to provide chemotaxonomic information as well nutritional information on sources of phenolic antioxidant compounds in fresh fruits. Due to their high content of phenolics, bilberries and blueberries are already considered functional foods. Which phenolic compound, either alone or in synergism with others, contributes the most to biological effects of berries is still an open question. On the one hand, anthocyanins are considered to be the most effective antioxidants[4] among all phenolic compounds in the berries. The reason for that is their high contents in *Vaccinium* species and their transport across membranes of different mammalian cells, among them also the blood−brain barrier. However, to understand the mechanisms of positive health effects after

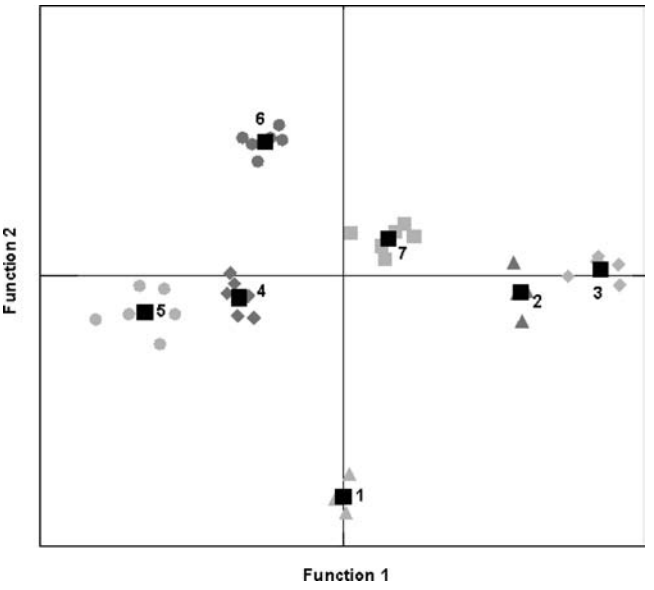

Figure 3. Linear discriminant analysis (LDA) performed using the levels of individual anthocyanins, flavanols, flavonols, phenolic acids, and stilbenes from 36 bilberry samples from seven different regions in Slovenia: 1, gray triangle, Pokljuka; ▲2, Celje; 3, gray diamond, Goricko; ◆4, Ljubljana; 5, gray circle, Kranj; ●6, Skofja Loka; 7, gray square, Pohorje; ■, group centroid.

consumption of bilberries and blueberries even better, there are still questions to be answered about bioavailability, metabolism, molecular targets, and bioactivity of phenolics in the human body.

Multivariate Analysis. PCA and LDA were performed to classify the Slovenian bilberries according to the relative compositions of their anthocyanins, flavanols, flavonols, phenolic acids, and stilbenes. The 15 anthocyanins (see Table 2) and 12 phenolic compounds (see Table 3) were included. Their contents were above the detection limits in all of the bilberry samples analyzed (Table 4). PCA was performed to provide a data structure study over a reduced dimension, covering the maximum amounts from the information present in the basic data set. The seven principal components thus accounted for 82.97% of the variation among the bilberry samples analyzed (PC1, 30.93%; PC2, 15.81%; PC3, 12.94%; PC4, 7.54%; PC5, 6.54%; PC6, 5.19%; and PC7, 4.02%). Therefore, all of the parameters were included in the LDA.

Using LDA, six parameters were selected as the most discriminating variables: cyanidin 3-arabinoside, ellagic acid, gallic acid, *p*-coumaric acid, quercetin, and cyanidin 3-glucoside. The other four parameters (catechin, chlorogenic acid as 3-caffeoylquinic acid, cyanidin 3-galactoside, and delphinidin 3-galactoside) also contributed significantly to the better separation of the bilberries. When the LDA was applied to the data set (36 samples, 27 variables), two discriminant functions were obtained. Function 1 explains 49.1% of the total variance, and function 2 explains 20.7%. The scores of the samples for these two functions are plotted in Figure 3. As can be seen, the bilberry samples were well separated according to the picking regions. They were sampled in seven different locations. The statistical analysis showed that 27 parameters (anthocyanins, flavanols, flavonols, phenolic acids, and stilbene profiles) can be used for picking-region determination of these Slovenian bilberries. This conclusion is in agreement with the recently published results of a Finnish study, in which obvious differences among the various regions in Finland were observed.[12]

It is generally recognized that content of phenolic compounds in plants depends on cultivar used, temperature, light, amount of rainfall, agro-technical conditions, and food processing. In Table 4 the content of total phenolics determined was calculated and statistically analyzed. It can be concluded that the Goricko and Skofja Loka picking regions differ significantly ($p < 0.05$) from the other five locations. Also, when picking regions were correlated to climate, the total phenolics determined in the bilberries from the lowland growing region (Ljubljana, Kranj) and from the mountainous region (Pokljuka, Pohorje) are comparable (1182 vs 1121 or 1027 vs 1198 mg equiv/100 g fw). As mentioned before, the pedoclimatic and the autochthonous growing region differences among picking locations in Slovenia are the only reasons for the different phenolics composition because no genetic variability in bilberries was noted (unpublished results). Similarly, there have been other recognized and published differences found for other fruits and vegetables that have been considered to be due to different cultivars[28] and/or agronomic and climate conditions.[29] These conditions can result in complex combinations of bioactive compounds that will generally be related to specific characteristics of the genotype and of its interaction with the environment, potentially leading to increased total phenolic content and to higher total antioxidant capacity.[30] In other words, the positive influences of these conditions can be used toward producing fruits with more phenolic compounds and with higher nutritional quality.

The classification of the data obtained using LDA is shown in Table 4. It can be seen that all of the picking locations of the bilberries were 100% correctly classified. Overall, the accuracy of the placement of each sample into its corresponding group (location) was also 100%, with none of the 36 samples misplaced. Similar statistical analysis was performed for flavonoids and other components in different types of Slovenian honey that were collected from different locations in Slovenia that also allowed the successful differentiation of their botanical origin.[16]

In the present study, we identified and for the first time quantitatively determined 15 anthocyanidins, 2 flavanols, 3 flavonols (including rutin), 6 phenolic acids, and the stilbene *trans*-resveratrol in Slovenian bilberries (*V. myrtillus* L.) and blueberries (*V. corymbosum* L.) planted in Slovenia. We can conclude that the predominant phenolics in both types of berries were anthocyanins, although the bilberries are obviously a richer source than the blueberries. The statistical analysis showed that profiles including 27 parameters (anthocyanins, flavanols, flavonols, phenolic acids, and stilbenes) can be used to distinguish among the picking regions of these Slovenian bilberries. Moreover, due to various phenolics that are commonly recognized as good antioxidants, fresh bilberries and fresh blueberries can be classified as functional foods. Other questions that remain to be answered are if and how does the processing of the berries before consumption effect their phenolic content and if and how these compounds contribute to human health; the latter includes questions about their bioavailability in the first place.

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Funding Sources

The Young Research Fellow (S.M.) and authors V.A. and N.P.U. express gratitude for the financial support through doctoral degree studies and for financing of the P4-0121 research program to the Slovenian Research Agency. A.V. and D.K. acknowledge financial support from the Ministry of Agriculture, Forestry and Food in Slovenia through project V4-0321.

REFERENCES

(1) 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.

(2) Ehala, S.; Vaher, M.; Kaljurand, M. Characterization of phenolic profiles of northern European berries by capillary electrophoresis and determination of their antioxidant activity. *J. Agric. Food Chem.* **2005**, *53*, 6484–6490.

(3) Giovanelli, G.; Buratti, S. Comparison of polyphenolic composition and antioxidant activity of wild Italian blueberries and some cultivated varieties. *Food Chem.* **2009**, *112*, 903–908.

(4) Heinonen, M. Antioxidant activity and antimicrobial effect of berry phenolics — a Finnish perspective. *Mol. Nutr. Food Res.* **2007**, *51*, 684–691.

(5) Wang, S. Y.; Chen, C.; Sciarappa, W.; Wang, C. Y.; Camp, M. J. Fruit quality, antioxidant capacity, and flavonoid content of organically and conventionally grown blueberries. *J. Agric. Food Chem.* **2008**, *56*, 5788–5794.

(6) Wolfe, K. L.; Kang, X.; He, X.; Dong, M.; Zhang, Q.; Liu, R. H. Cellular antioxidant activity of common fruits. *J. Agric. Food Chem.* **2008**, *56*, 8418–8426.

(7) Ziberna, L.; Lunder, M.; Moze, S.; Vanzo, A.; Tramer, F.; Passamonti, S.; Drevensek, G. Acute cardioprotective and cardiotoxic effects of bilberry anthocyanins in ischemia-reperfusion injury: beyond concentration-dependent antioxidant activity. *Cardiovasc. Toxicol.* **2010**, *10*, 283–294.

(8) Chatterjee, A.; Yasmin, T.; Bagchi, D.; Stohs, S. J. Inhibition of *Helicobacter pylori in vitro* by various berry extracts, with enhanced susceptibility to clarithromycin. *Mol. Cell. Biochem.* **2004**, *265*, 19–26.

(9) Burns Kraft, T. F.; Dey, M.; Rogers, R. B.; Ribnicky, D. M.; Gipp, D. M.; Cefalu, W. T. Phytochemical composition and metabolite performance enhancing activity of dietary berries traditionally used by Native North Americans. *J. Agric. Food Chem.* **2008**, *56*, 654–660.

(10) Kowluru, R. A.; Tang, J.; Kern, T. S. Abnormalities of retinal metabolism in diabetes and experimental galactosemia. VII. Effect of long-term administration of antioxidants on the development of retinopathy. *Diabetes* **2001**, *50*, 1938–1942.

(11) Galli, R. L.; Bielinski, D. F.; Szprengiel, A.; Shukitt-Hale, B.; Joseph, J. A. Blueberry supplemented diet reverses age-related decline in hippocampal Hsp70 neuroprotection. *Neurobiol. Aging* **2006**, *27*, 344–350.

(12) Lätti, A. K.; Riihinen, K. R.; Kainulainen, P. S. Analysis of anthocyanin variation in wild populations of bilberry (*Vaccinium myrtillus* L.) in Finland. *J. Agric. Food Chem.* **2008**, *56*, 190–196.

(13) Åkerström, A.; Forsum, Å.; Rumpunen, K.; Jäderlund, A.; Bång, U. Effects of sampling time and nitrogen fertilization on anthocyanidin levels in *Vaccinium myrtillus* fruits. *J. Agric. Food Chem.* **2009**, *57*, 3340–3345.

(14) Burdulis, D.; Sarkinas, A.; Jasutiené, I.; Stackevicené, E.; Nikolajevas, L.; Janulis, V. Comparative study of anthocyanin composition, antimicrobial and antioxidant activity in bilberry (*Vaccinium myrtillus* L.) and blueberry (*Vaccinium corymbosum* L.) fruits. *Acta Pol. Pharm.* **2009**, *66*, 399–408.

(15) Može, S.; Koron, D.; Vanzo, A. Antocianini v slovenskih gozdnih borovnicah (*Vaccinium myrtillus*) [Anthocyanins in bilberries from Slovenia]. In *Zbornik povzetkov referatov s posvetovanja*; Glavič, P., Brodnjak-Vončina, D., Eds.; Slovenski kemijski dnevi, Sept 2008; University of Maribor: Maribor, Slovenia, 2008; pp 137—145.

(16) Bertoncelj, J.; Polak, T.; Kropf, U.; Korošec, M.; Golob, T. LC-DAD-ESI/MS analysis of flavonoids and abscisic acid with chemometric approach for the classification of Slovenian honey. *Food Chem.* **2011**, *127*, 296—302.

(17) Kähkönen, M. P; Heinämäki, J.; Ollilainen, V.; Heinonen, M. Berry anthocyanins: isolation, identification and antioxidant activities. *J. Sci. Food Agric.* **2003**, *83*, 1403–1441.

(18) Riihinen, K.; Jaakola, L.; Kärenlampi, S.; Hohtola, A. Organ-specific distribution of phenolic compounds in bilberry (*Vaccinium myrtillus*) and 'Northblue' blueberry (*Vaccinium corymbosum* × *V. angustifolium*). *Food Chem.* **2008**, *110*, 156–160.

(19) Bakker, J.; Timberlake, C. F. The distribution of anthocyanins on grape skin extracts of port wine cultivars as determined by high performance liquid chromatography. *J. Sci. Food Agric.* **1985**, *36*, 1315–1324.

(20) Mullen, W.; Stewart, A. J.; Lean, M. E. J.; Gardner, P.; Duthie, G. G.; Crozier, A. Effect of freezing and storage on the phenolics, ellagitannins, flavonoids, and antioxidant capacity of red raspberries. *J. Agric. Food Chem.* **2002**, *50*, 5197–5201.

(21) Hartmann, A.; Patz, C.; Andlauer, W.; Dietrich, H.; Ludwig, M. Influence of processing on quality parameters of strawberries. *J. Agric. Food Chem.* **2008**, *56*, 9484–9489.

(22) Cuevas-Rodríguez, E. O.; Yousef, G. G.; García-Saucedo, P. A.; López-Medina, J.; Paredes-López, O.; Lila, M. A. Characterization of anthocyanins and proanthocyanidins in wild and domesticated Mexican blackberries (*Rubus* spp.). *J. Agric. Food Chem.* **2010**, *58*, 7458–7464.

(23) Hukkanen, A. T.; Pölönen, S. S.; Kärenlampi, S. O.; Kokko, H. I. Antioxidant capacity and phenolic content of sweet rowanberries. *J. Agric. Food Chem.* **2006**, *54*, 112–119.

(24) Taruscio, T. G.; Barney, D. L.; Exon, J. Content and profile of flavanoid and phenolic acid compounds in conjunction with the antioxidant capacity for a variety of northwest *Vaccinium* berries. *J. Agric. Food Chem.* **2004**, *52*, 3169–3176.

(25) Ichiyanagi, T.; Shida, Y.; Mamunur Rahman, M.; Hatano, Y.; Konishi, T. Bioavailability and tissue distribution of anthocyanins in bilberry (*Vaccinum myrtillus* L.) extract in rats. *J. Agric. Food Chem.* **2006**, *54*, 6578–6587.

(26) Vanzo, A.; Terdoslavich, M.; Brandoni, A.; Torres, A. M.; Vrhovsek, U.; Passamonti, S. Uptake of grape anthocyanins into the rat kidney and the involvement of bilitranslocase. *Mol. Nutr. Food Res.* **2008**, *52* (10), 1106–1116.

(27) Quettier-Eleu, C.; Gressier, B.; Vasseur, J.; Dine, T.; Brunet, C.; Luyckx, M.; Cazin, M.; Cazin, J.-C.; Bailleul, F.; Trotin, F. Phenolic compounds and antioxidant activities of buckwheat (*Fagoppyrumesculentum Moench*) hulls and flour. *J. Ethnopharmacol.* **2000**, *72*, 35–42.

(28) Usenik, V.; Stampar, F.; Veberic, R. Anthocyanins and fruit colour in plums (*Prunus domestica L.*) during ripening. *Food Chem.* **2009**, *114* (2), 529–534.

(29) Kacjan-Maršić, N.; Gašperlin, L.; Abram, V.; Budič, M.; Vidrih, R. Quality parameters and total phenolic content in tomato fruits regarding cultivar and microclimatic conditions. *Turk. J. Agric. For.* **2010**, *34*, 1–10.

(30) Capocasa, F.; Diamanti, J.; Tulipani, S.; Battino, M.; Mezzetti, B. Breeding strawberry (Fragaria × ananassa Duch) to increase fruit nutritional quality. *Biofactors* **2008**, *34*, 67–72.