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Chemical Composition of Two Different Extracts of Berries Harvested in Serbia

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Supporting Information

ABSTRACT: Total phenolic content (TPC), total anthocyanin content (TAC), free and total ellagic acid content, sugars, minerals, and radical-scavenging activity were determined in nine berries harvested in Serbia. More than 30 phenolic compounds were identified; among them, 11 polyphenols and *cis*,*trans*-abscisic acid were quantified using UHPLC coupled with an LTQ-Orbitrap XL mass analyzer. For the first time chrysin, naringenin, pinocembrin, and galangin were quantified in some of the investigated berry species. The extraction efficiency of the two extraction systems, methanol and acetone, was investigated. It was found that acetone is a better extracting solvent for TPC, whereas more TAC was extracted by methanol. TPC in acetone extracts ranged from 177.51 to 459.71 mg gallic acid equiv/100 g frozen weight. TAC ranged from 5.39 to 96.94 mg cyanidin-3-glucoside/100 g frozen weight in methanol extracts. The amounts of both free and total ellagic acid were found to be higher in the acetone extract in comparison to the methanol extract.

KEYWORDS: *berries, polyphenols, anthocyanins, ellagic acid, radical-scavenging activity*

INTRODUCTION

Berry fruits are often valued as significant sources of different phytochemicals in human nutrition. One of the most important export products from Serbia is wild or cultivated berries.[1] The unique climate and soil of western Serbia make berry fruits distinctive worldwide especially for their quality, unique taste, color, and flavor. Almost 90% of produced berries are being exported in the frozen state to Western markets.

Numerous methods, both chemical and physical, may be used in laboratories for food research and control, mainly to evaluate the quality of the product, authenticity/adulteration, and traceability in the production and marketing chain. Besides demand for quality parameters, a very important issue is traceability of some chemical markers of food related to its origin and nutritional quality.

Plant secondary metabolites are currently the subject of much research interest. For plant-derived food, the secondary metabolites are markers of biological or geographical authenticity, as well for adulteration and traceability studies.[2] Polyphenols are secondary metabolites synthesized by plants during normal development and in response to stress conditions. Both wild and cultivated berries are known to contain many bioactive components, especially polyphenolic compounds with interesting health-protective actions.[3]

Literature concerning investigations of berry-like fruits reports on usual tests, such as determination of total phenolic contents, total anthocyanin contents, and antioxidant capacity.[4−6] Although these data provide crucial information about the quality of berries, advanced methods, such as LC-MS,[7] are needed nowadays to characterize the most relevant biomarkers (especially polyphenolic derivatives). Previously, we have reported a similar procedure for the analysis of polyphenolics in honey, which was proven to be reliable for the unambiguous detection of compounds on the basis of their molecular masses and fragmentation pattern.[8]

Comparison of chemical composition of different berries from literature data is a difficult task due to the various solvents and procedures used for sample extraction.[9−12] In this work the extraction efficiency of two different solvents and results of total phenolic content (TPC), total anthocyanin content (TAC), and antioxidant capacity are compared. Ellagitannins and ellagic acid constitute a large part of total phenolics present in berries. The aim of this work was to investigate the content of free and total ellagic acid using HPLC with UV detection. Contents of individual sugars and mineral content were also determined.

MATERIALS AND METHODS

Chemicals and Materials. 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) and standards of sugars (glucose, fructose, and sucrose) were purchased from Fluka AG (Buch, Switzerland), whereas standards of phenolic compounds used for UHPLC-MS/MS analysis, gallic acid, and ellagic acid were purchased from Sigma-Aldrich (Steinheim, Germany). For ICP-OES calibration, Alfa Aesar (Germany) standards were used. Methanol, acetone, acetonitrile (all of HPLC grade), formic acid, trifluoroacetic acid, ethyl acetate, and Folin−Ciocalteu reagent were purchased from Merck (Darmstadt,

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Germany). Ultrapure water (TKA Germany MicroPure water purification system, 0.055 μS/cm) was used to prepare standard solutions and dilutions. All other reagents were of analytical grade. Syringe filters (13 mm, PTFE membrane = 0.45 μm) were purchased from Supelco (Bellefonte, PA, USA).

Fruit Samples. Raspberry fruits (*Rubus idaeus* L.) of four cultivars (Willamette, Tulameen, Meeker, and Yellow Meeker), blackberry *Rubus fructicosus* L. (Čačanska bestrna), mulberry (*Morus nigra* L.), and strawberry *Fragaria* × *ananassa* (Clery) were collected from different commercial plantations in a few regions of Serbia. All raspberry and blackberry samples were collected from commercial plantations in Arilje, except two Willamette raspberries, which were harvested in Zlatibor and Valjevo. Mulberry was selected from a natural population in the vicinity of Belgrade, whereas the strawberry sample originated from Šabac. Samples were harvested during June−August 2011. The amount of each berry cultivar collected for the analysis was about 200 g. Before analysis, the samples were stored in a freezer at −18 °C. Aside from the fact that berries to consumers mainly come in frozen form, freezing is a good preservative process maintaining almost unchanged the content of phenolic compounds and radical-scavenging activity of berries.[13,14]

Preparation of Sample Extracts. Samples were prepared according to the slightly modified method proposed by Bobinaite et al.[15] Two different solvents, methanol and acetone, were used for extraction. A mortar and pestle was used for sample homogenization. One gram of homogenized fruit was mixed with 10 mL of methanol containing 1% HCl on ultrasonic bath for 1 h at room temperature. The extract was placed in the dark at 4 °C for 24 h and filtered, and the clear supernatant was collected. The fractions from three times repeated extractions were collected and evaporated to dryness by rotary evaporation under reduced pressure at 40 °C. Ultrapure water was added to ca. 10 mL, and these solutions were used for further analysis. The same procedure was followed with acetone.

Determination of Total Phenolic Content. The amount of total phenolics in extracts was determined according to the Folin−Ciocalteu procedure,[16] with some modification. Briefly, 0.1 mL of the sample extracts and 6 mL of ultrapure water were mixed with 0.5 mL of Folin−Ciocalteu reagent, and the solution was incubated for 6 min at room temperature. Next, 3 mL of 20% sodium carbonate was added. After 30 min at 40 °C, absorbance was measured at 765 nm using a GBC Cintra 6 UV−visible spectrophotometer. Gallic acid was used as standard at a concentration of 100−500 ppm. A mixture of water and reagent was used as a blank. The results were expressed as milligram gallic acid equivalent (GAE) per gram of frozen sample.

Determination of Total Anthocyanin Content. The total anthocyanin content was determined by using the pH-differential method.[17] Methanol and acetone extracts were diluted with buffers of pH 1.0 (hydrochloric acid−potassium chloride, 0.025 M) and pH 4.5 (acetic acid−sodium acetate, 0.4 M). Absorbencies of the extracts were measured at 510 and 700 nm against blank. Anthocyanin concentration (TAC) was calculated and expressed as milligram cyanidin 3-glucoside equivalents per 100 g of frozen weight using the formulas

$$\mathrm{TAC} = A_{\mathrm{total}} \times \mathrm{MW} \times \mathrm{DF} \times 1000/(\varepsilon \times 1) \quad (1)$$

$$A_{\mathrm{total}} = (A_{510} - A_{700})_{\mathrm{pH1.0}} - (A_{510} - A_{700})_{\mathrm{pH4.5}} \quad (2)$$

where A_{total} is absorbance calculated by eq 2, MW is molecular weight (MW = 449.2 g/mol for cyanidin 3-glucoside), DF is dilution factor, 1 is cuvette path length in cm, and ε is molar absorptivity (ε = 26900 L/mol·cm for cyanidin 3-glucoside).

Determination of the Radical-Scavenging Activity (RSA). Scavenging activity of berry extracts was evaluated using DPPH$^{\bullet}$ by a slightly modified literature method.[18] An amount of 0.2 mL of extracts (previously 10 times diluted) was mixed with 4 mL of methanol solution of DPPH$^{\bullet}$ (71 mM). The mixture was left to stand for 60 min in the dark (until stable absorption values were obtained). The reduction of the DPPH$^{\bullet}$ radical was measured by monitoring continuously the decrease of absorption at 515 nm. The RSA was calculated as a percentage of DPPH$^{\bullet}$ discoloration using the equation

$$\mathrm{RSA}\,(\%) = \frac{(A_{\mathrm{DPPH}} - A_{\mathrm{sample}})}{A_{\mathrm{DPPH}}} \times 100 \quad (3)$$

where A_{DPPH} is the absorbance of the methanol solution of DPPH$^{\bullet}$ radical and A_{sample} is the absorbance in the presence of berry extract. The assays were carried out in triplicate, and the results were expressed as mean values.

UHPLC-MS/MS Orbitrap Analysis. To investigate the main markers specific to each berry species, a UHPLC method coupled with a hybrid mass spectrometer, which combines the linear trap quadrupole (LTQ) and OrbiTrap mass analyzer, was developed in this research. Methanol extracts were used for this analysis. All experiments were performed using a Thermo Scientific liquid chromatography system constituted of a quaternary Accela 600 pump and an Accela Autosampler, connected to a linear ion trap-Orbitrap hybrid mass spectrometer (LTQ-Orbitrap XL, Thermo Fisher Scientific, Bremen, Germany), with electrosprey ionization (ESI).

Separations were performed on a Hypersil Gold C18 column (50 × 2.1 mm, 1.9 μm) from Thermo Fisher Scientific. The mobile phase consisted of (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. A linear gradient program at a flow rate of 0.400 mL/min was used: 0−5 min, from 5 to 95% (B); 5−6 min, 95% (B); then 5% (B) for 3 min. All standards used in UHPLC-MS/MS Orbitrap analysis were dissolved in methanol/water (3:2, v/v).

The mass spectrometer was operated in negative selected ion monitoring (SIM) mode. ESI source parameters were as follows: source voltage, 4 kV; capillary voltage, −47 V; tube lens voltage, −159.11; capillary temperature, 275 °C; sheath and auxiliary gas flow (N_2), 25 and 8 (arbitrary units). MS spectra were acquired by full range acquisition covering m/z 100−1000. For fragmentation study, a data-dependent scan was performed by deploying collision-induced dissociation (CID). The normalized collision energy of the CID cell was set at 35 eV.

Phenolics were identified and quantified in berry samples according to the corresponding spectral characteristics: mass spectra, exact mass, characteristic fragmentation, and characteristic retention time. Xcalibur software (version 2.1) was used for instrument control, data acquisition, and data analysis.

Determination of Free and Total Ellagic Acid Content. Methanol and acetone extracts were used for determination of free ellagic acid content. For preparation of extract for total ellagic acid determination an acid hydrolysis was used.[19] Hydrochloric acid (8.3 mL of 37% HCl, final HCl concentration = 4 M) was added in an aliquot (10 mL) of the extract, and the mixture was diluted to 25 mL with methanol. The mixture was refluxed for 6 h at 85 °C. After hydrolysis, the sample was brought to the initial volume. An aliquot was adjusted to pH 2.5 with 5 M NaOH and diluted to 10 mL with methanol. Before HPLC analysis, all extracts were filtered through a 0.45 μm PTFE membrane syringe filter and stored at −20 °C.

A Waters HPLC system consisted of a 1525 binary pump and a UV−vis Waters 2487 dual detector. Separations were performed with a Waters Symmetry C18 column, 4.5 × 150 mm size, with 5 μm particle diameter. The mobile phase was (A) 0.1% trifluoroacetic acid in acetonitrile and (B) 0.1% trifluoroacetic acid in aqueous solution. The samples were analyzed using isocratic elution with 20% A and 80% B. The flow rate was 1.2 mL/min, and the injection volume was 10 μm. Ellagic acid was detected and quantified at 254 nm. Quantification of ellagic acid was performed using the calibration curve of the ellagic acid standard, concentration range of 10−100 ppm. Ellagic acid was dissolved in methanol/water (3:2, v/v).

Determination of Individual Sugars. The content of sugars (glucose, fructose, and sucrose) was determined according to a literature method,[20] with minor modification. Frozen berries (1 g) were diluted to 100 mL with ultrapure water and homogenized on an ultrasonic bath for 30 min at 40 °C. The extract sample was centrifuged, and an aliquot (1.0 mL) was diluted to 50 mL with ultrapure water. After that, the extract was filtered through a 0.45 μm syringe filter.

Table 1. Total Phenolic Content, Total Anthocyanin Content, and Radical-Scavenging Activity in Nine Samples of Berries[a]

	TPC[b]		TAC[c]		RSA[d]	
	acetone	methanol	acetone	methanol	acetone	methanol
raspberry						
Meeker	334.63 ± 4.92	244.34 ± 11.28	20.63 ± 1.72	28.54 ± 2.17	66.45 ± 1.78	62.91 ± 1.24
Yellow Meeker	367.58 ± 3.61	317.95 ± 1.69	1.74 ± 0.29	5.39 ± 0.73	36.96 ± 3.51	31.87 ± 1.15
Tulameen	245.01 ± 1.83	243.47 ± 5.17	40.26 ± 1.53	56.18 ± 2.45	58.02 ± 0.66	53.51 ± 4.52
Willamette (Valjevo)	281.41 ± 1.26	278.92 ± 1.72	29.50 ± 0.33	89.34 ± 0.76	64.85 ± 4.60	57.16 ± 0.87
Willamette (Arilje)	324.87 ± 0.02	270.82 ± 4.53	46.52 ± 3.40	85.61 ± 1.03	62.81 ± 2.45	61.99 ± 0.60
Willamette (Zlatibor)	290.49 ± 6.03	272.42 ± 8.21	27.12 ± 2.82	50.50 ± 5.04	61.36 ± 4.21	65.12 ± 0.99
blackberry						
Čačanska bestrna	459.71 ± 15.70	375.03 ± 1.16	41.56 ± 9.38	96.94 ± 1.98	67.99 ± 0.27	66.10 ± 3.90
mulberry						
Morus nigra	177.51 ± 9.83	135.30 ± 1.18	31.90 ± 4.05	44.92 ± 4.80	59.44 ± 3.12	53.97 ± 1.03
strawberry						
Clery	281.46 ± 1.82	247.96 ± 1.05	14.19 ± 3.32	29.69 ± 1.50	36.04 ± 0.96	31.18 ± 0.82

[a]The values shown are the mean ± standard deviation of three replications. [b]Total phenolic contents are expressed as mg GAE/100 g frozen weight. [c]Total anthocyanin contents are expressed as mg cyn-3-glu/100 g frozen weight. [d]Radical-scavenging activity is presented as percent inhibition of the DPPH• radical. Two paired *t* stat values: 3.908, 3.478, and 3.007 for TPC, TAC, and RSA, respectively (*t* critical two-tail = 2.306, $P < 0.05$).

Separation and quantification of sugars in the extracts were performed using Dionex ICS 3000 equipment containing a dual gradient pump (DP). Separation of carbohydrates was carried out on a CarboPac PA-100 anion-exchange column (4 × 250 mm) with a CarboPac PA-100 guard column (4 × 50 mm). The flow rate was 0.7 mL/min, and carbohydrates were detected by electrochemical detector with a gold working electrode and Ag/AgCl reference electrode. Running time was 30 min. Carbohydrates were eluted by a gradient prepared from 600 mM sodium hydroxide (eluent A), 500 mM sodium acetate (eluent B), and ultrapure water (eluent C). Eluent A was constant (15%) during 20 min and increased to 20% at 20 min, eluent B changed from 0 to 20%, and eluent C changed from 85 to 60%. During chromatography, the eluents were kept under a blanket of He, and the mobile phase was purged with He to minimize carbonate contamination, which would affect the retention times and separation selectivity of the sugars.

Determination of Minerals. Samples of frozen fruits were prepared by microwave digestion using an Ethos 1 microwave system (Advanced Microwave Digestion System, Milestone, Italy). One gram of berry sample, 1.0 mL of 30% H_2O_2, and 7.0 mL of concentrated ultrapure HNO_3 were mixed and transferred by pouring into the microwave digestion vessel. After the effervescence had subsided, the sample was cooled for 5 min, transferred into a clean volumetric flask, and diluted to 25 mL with ultrapure H_2O. A blank was prepared in the same way. All analyses were performed in triplicate on a Thermo Scientific iCAP 6500 Duo ICP (Thermo Fisher Scientific, Cambridge, UK).

Statistical Analysis. Data of all measurements done in triplicate are expressed as the mean ± standard deviation (SD). Statistical analyses were performed with the statistical program MS Excel (Microsoft Office 2007 Professional).

RESULTS AND DISCUSSION

Determination of TPC, TAC, and RSA. Total phenolic contents, total anthocyanin contents, and radical-scavenging activity were determined in nine berry samples in acetone and methanol extracts. A difference for the two extraction systems is evident from the presented results (Table 1). Statistical significance was confirmed by using a paired *t* test with the following *t* stat values: 3.908, 3.478, and 3.007 for TPC, TAC, and RSA, respectively (*t* critical two-tail = 2.306, $P < 0.05$).

Acetone was a better extracting solvent for TPC when two extraction solvents were compared. Considering all TPC values obtained for acetone extracts (Table 1), it is evident that the lowest content of total phenolics was found in mulberry (171.51 mg GAE/100 g frozen weight), whereas the acetone extract of blackberry had the highest content of total phenolics (459.71 mg GAE/100 g frozen weight). The total phenolics of the raspberry cultivars ranged from 245.01 mg GAE/100 g frozen weight (Tulameen) to 367.58 mg GAE/100 g frozen weight (Yellow Meeker). Although dissimilarity in the TPC results of the acetone extract of Willamette cultivar collected from three different regions (Valjevo, Arilje, and Zlatibor) was noticeable, a larger number of samples is needed to reach a conclusion on the impact of geographical origin on the total phenolic content.

An investigation reported by Kafkas et al.[21] referred to the cultivars of three raspberries (Meeker, Willamette, Tulameen). A similar extraction procedure of samples allowed us to make comparison with the results presented herein. The highest content of the polyphenols was found in Meeker, similarly to the mentioned paper. However, a somewhat higher content was found in our samples when TPC values were compared.

When compared to other publications, strawberry and blackberry samples contained similar amounts of total phenolics.[6,22] TPC found in our mulberry sample was significantly lower when compared with the results of phenolic content data from mulberry grown in different regions of the world.[23]

Methanol extracted more total anthocynins than acetone. The TAC in methanol extracts ranged from 5.39 mg cyn-3-glu/100 g frozen weight in Yellow Mekeer to 96.94 mg cyn-3-glu/100 g frozen weight in blackberry (Čačanska bestrna). In raspberries, the amount of total anthocyanins is in agreement with results reported by Kafkas et al.:[21] the highest TAC was measured in Willamette (Valjevo), whereas acetone extracts of Meeker contained the lowest amounts of anthocyanin. TAC values found in blackberry and strawberry samples are of similar values when compared to other cultivars described in the literature.[6,22] However, the TAC determined in mulberry is lower than literature data.[24]

The RSA values were calculated by eq 3, and results are presented as percent inhibition of the DPPH• radical (Table 1). All investigated berry extracts exhibited potent radical-scavenging activities. Similarly to TPC, higher RSA values were obtained when acetone was used as the extraction solvent. Blackberry extracts showed the highest RSA, which is consistent

Table 2. Characterization of Phenolic Compounds and *cis,trans*-Abscisic Acid in Methanol Extract of Berries Using UHPLC-MS/MS Orbitrap

	compound name	t_R	$[M-H]^-$ calcd[a]	$[M-H]^-$ exptl[b]	fragments	species[e]
1	gallic acid[c]	0.72	169.0142	169.0136	125.02	all
2	*p*-hydroxybenzoyl-hexoside[d]	0.94	299.0771	299.0773	137.02, 179.03, 239.09	M, YM, T, W-V, W-A, W-Z, B, S
3	protocatechuic acid[c]	1.13	153.0192	153.0188	109.03	all
4	quercetin-3-*O*-hexoside[d]	1.43	463.0882	463.0884	179.03	all
5	*p*-hydroxybenzoyl-hexoside[d]	1.51	299.0771	299.0773	137.02, 179.03, 239.09	M, YM, T, W-V, W-A, W-Z, B, S
6	caffeoyl-hexoside[d]	1.52	341.0874	341.0877	179.03	all
7	chlorogenic acid[c]	1.54	353.0878	353.0876	179.03, 191.02	W-Z, B, MU
8	coumaroyl-hexoside[d]	1.62	325.0929	325.0930	163.04	M, YM, T, W-V, W-A, W-Z, B, S
9	caffeic acid[c]	1.67	179.0350	179.0351	161.04	all
10	caffeoyl-hexoside[d]	1.77	341.0874	341.0877	179.03	M, T, W-V, W-A, W-Z
11	galloyl-bis-HHDP-glucose[d]	1.88	935.0783	935.0782	301.00, 433.04, 783.07	M, YM, W-V, W-A, W-Z, B, S
12	ellagic acid pentoside[d]	1.94	433.0408	433.0414	301.00	M, YM, T, W-V, W-A, W-Z, B, S
13	*p*-coumaric acid[c]	2.01	163.0401	163.0404	119.00	M, YM, T, W-V, W-A, W-Z, B, S
14	rutin[c]	2.04	609.1461	609.1459	301.03	M, YM, W-V, B, MU, S
15	coumaroyl-hexoside[d]	2.04	325.0929	325.0930	163.04	M, YM, T, W-V, W-A, W-Z, S
16	ellagic acid[c]	2.06	300.9984	300.9992	257.01	M, YM, W-V, W-A, B, S
17	quercetin-3-*O*-glucuronide[d]	2.10	477.0674	477.0678	301.03	M, YM, T, W-V, W-A, W-Z, B, S
18	quercetin-3-*O*-hexoside[d]	2.11	463.0882	463.0884	179.03	all
19	quercetin-3-*O*-xylopyranoside[d]	2.13	433.0772	433.0772	301.03	T, W-V, W-A, W-Z, B, S
20	kaempferol-hexoside[d]	2.14	447.0928	447.0928	285.04	YM, T, W-V, W-A, W-Z, B, MU, S
21	quercetin-3-*O*-rhamnoside[d]	2.15	447.0928	447.0932	301.03	YM, T, W-V, W-A, W-Z, B, MU, S
22	feruloyl-hexoside[d]	2.18	355.1030	355.1030	193.05	M, YM, T, W-V, W-A, W-Z, B
23	ellagic acid deoxyhexoside[d]	2.19	447.0564	447.0572	301.00	M, YM, T, W-V, W-A, W-Z, B, S
24	sinapoyl-hexoside[d]	2.20	385.1136	385.1136	223.05	M, YM, T, W-V, W-A, W-Z, B, S
25	kaempferol-hexoside[d]	2.25	447.0928	447.0928	285.04	YM, T, W-A, W-Z, B, MU, S
26	kaempferol-3-malonylhexoside[d]	2.35	533.0928	533.0928	285.04, 489.10	MU, S
27	kaempferol-3-coumaroylhexoside[d]	2.69	593.1296	593.1304	285.04, 447.09	YM, S
28	luteolin[c]	2.71	285.0405	285.0406	133.01, 213.02	W-V, W-A, B, S
29	*cis,trans*-abscisic acid[c]	2.74	263.1289	263.1293	201.02, 219.01	all
30	kaempferol-3-coumaroylhexoside[d]	2.75	593.1296	593.1304	285.04, 447.09	YM, B, S
31	naringenin[c]	2.76	271.0612	271.0601	177.04	M, YM, T, W-A, B, MU, S
32	apigenin[c]	2.97	269.0455	269.0459	149.05, 183.00	M, T, W-V, W-A, W-Z, B, S
33	kaempferol[c]	3.03	285.0405	285.0407	161.05	W-V, W-A, B, MU, S
34	chrysin[c]	3.40	253.0501	253.0495	181.06, 209.06	M, YM, T, W-V, W-A, W-Z, B, S
35	pinocembrin[c]	3.68	255.0663	255.0666	211.00, 213.01	M, T, W-V, B, MU, S
36	galangin[c]	3.71	269.0455	269.0459	183.00, 197.02, 227.06	YM, T, W-V, W-Z, B, MU, S

[a]Calculated mass of the parent ion using free chemical database, ChemSpider. [b]Experimental mass of the parent ion. [c]Confirmed by standard. [d]Confirmed by reference. [e]M, Meeker; YM, Yellow Meeker; T, Tulameen; W-V, Willamette from Valjevo; W-A, Willamette from Arilje; W-Z, Willamette from Zlatibor; B, blackberry (Čačanska bestrna); MU, mulberry (red mulberry); S, strawberry (Clery).

with the highest amount of total phenolics found in blackberry. However, no evident correlation was found between the TPC and RSA when all values were compared.

UHPLC-MS/MS Orbitrap Analysis. Using this technique, 35 polyphenolic compounds and *cis,trans*-abscisic acid were identified in 9 berry samples. Thirteen phenolics (gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, rutin, kaempferol, chrysin, naringenin, apigenin, luteolin, pinocembrin, and galangin) and *cis,trans*-abscisic acid were quantified using the available standards. In the absence of standards, the identification of the corresponding compound was based on the search for the $[M-H]^-$ deprotonated molecule together with the interpretation of its fragmentations. Retention times (t_R), calculated mass, experimental mass, and MS/MS fragments for each of the identified compound and their distribution in berries are summarized in Table 2.

All investigated samples contained *cis,trans*-abscisic acid. Polyphenols in berries generally occur as conjugates of sugars, usually *O*-glycosides.[25] As can be seen from Table 2, gallic acid, protocatechuic acid, quercetin-3-*O*-hexoside, caffeoyl-hexoside, and caffeic acid were indentified in all investigated samples. Ellagic acid derivates (ellagic acid-pentoside and ellagic acid-deoxyhexoside) and chrysin were not identified only in mulberry. Generally, mulberry was found to be the poorest in polyphenols. A total of nine polyphenols were identified in strawberry extract: except chlorogenic acid, caffeoyl-hexoside, and feruloyl-hexoside, all of the other polyphenols presented in Table 2 were found in strawberry extract.

The quantitative data on the contents of the phenolic compounds and *cis,trans*-abscisic acid in berry extracts are given in Table 3. Caffeic acid was found in all samples in the concentration range from 0.245 to 1.065 mg caffeic acid/kg fw. Also, a significant amount of *p*-coumaric acid was found in all samples (see Table 3). The contents of caffeic acid and *p*-coumaric acid are in agreement with the results published in the literature.[26,27]

Ellagic Acid Content. Here, focus was on the determination of the amount of free and total ellagic acid as it is

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Table 3. Contents of Phenolic Compounds and *cis,trans*-Abscisic Acid in Methanolic Extracts of Berries[a]

	raspberry						blackberry	mulberry	strawberry
compound name	M	YM	T	W-V	W-A	W-Z	B	MU	S
gallic acid	0.047	0.045	0.045	0.041	0.031	0.052	0.105	0.026	0.041
protocatechuic acid	0.108	0.072	0.176	0.223	0.129	0.124	0.253	0.261	0.079
chlorogenic acid	0.000	0.000	0.000	0.000	0.000	0.018	0.769	2.260	0.000
caffeic acid	0.438	0.882	1.065	0.739	1.037	0.245	0.616	0.899	0.773
p-coumaric acid	0.913	1.792	0.532	0.350	0.579	0.326	0.048	0.000	1.730
rutin	0.114	0.364	0.000	0.050	0.000	0.000	2.732	7.353	0.092
luteolin	0.000	0.000	0.000	0.100	0.010	0.000	0.010	0.000	0.025
cis,trans-abscisic acid	0.076	0.240	0.029	0.070	0.091	0.045	0.106	0.047	0.050
naringenin	0.399	0.096	0.181	0.000	0.102	0.000	0.010	0.047	2.209
apigenin	0.019	0.000	0.010	0.010	0.020	0.009	0.038	0.000	0.017
kaempferol	0.000	0.000	0.000	0.020	0.112	0.000	0.029	0.170	0.067
chrysin	0.162	0.094	0.125	0.106	0.188	0.085	0.065	0.000	0.106
pinocembrin	0.019	0.000	0.010	0.010	0.000	0.000	0.010	0.009	0.210
galangin	0.000	0.029	0.019	0.010	0.000	0.009	0.000	0.009	0.076

[a]Results are expressed as mg/100 g frozen weight.

Table 4. Content of Free and Total Ellagic Acid in Berries[a]

	free ellagic acid[b]		total ellagic acid[b]	
	methanol extract	acetone extract	methanol extract	acetone extract
raspberry				
Meeker	0.83 ± 0.02	1.72 ± 0.06	41.77 ± 0.53	181.74 ± 1.92
Yellow Meeker	1.52 ± 0.04	3.19 ± 0.16	99.86 ± 1.14	145.92 ± 1.61
Tulameen	nd	nd	12.86 ± 0.14	39.62 ± 1.38
Willamette (Valjevo)	2.11 ± 0.12	1.90 ± 0.02	39.03 ± 0.27	84.96 ± 0.58
Willamette (Arilje)	0.54 ± 0.01	1.34 ± 0.05	33.90 ± 0.30	67.71 ± 0.29
Willamette (Zlatibor)	nd	nd	22.18 ± 0.24	26.54 ± 0.54
blackberry				
Čačanska bestrna	4.43 ± 0.07	1.84 ± 0.06	60.29 ± 0.21	118.57 ± 1.71
mulberry				
Morus nigra	nd	nd	nd	nd
strawberry				
Clery	3.60 ± 0.25	5.60 ± 0.10	21.76 ± 0.14	54.90 ± 0.72

[a]The values shown are the mean ± standard deviation of three replications. [b]Results are expressed as mg/100 g frozen weight. Two paired *t* stat values: 0.639 for free ellagic acid and 3.158 for total ellagic acid (*t* critical two-tail = 2.306, $P < 0.05$).

Table 5. Content of Individual and Total Sugars in Berries[a]

	glucose[b]	fructose[b]	sucrose[b]	total sugars[b]
raspberry				
Meeker	42.87 ± 0.26	47.91 ± 0.37	9.18 ± 0.09	99.96 ± 0.46
Yellow Meeker	42.52 ± 0.53	46.72 ± 0.19	6.25 ± 0.11	95.49 ± 0.57
Tulameen	22.71 ± 0.16	27.81 ± 0.27	5.71 ± 0.06	56.23 ± 0.32
Willamette (Valjevo)	23.32 ± 0.24	32.56 ± 0.34	6.02 ± 0.13	61.90 ± 0.44
Willamette (Arilje)	31.59 ± 0.15	37.54 ± 0.45	18.96 ± 0.21	88.09 ± 0.52
Willamette (Zlatibor)	20.94 ± 0.20	25.27 ± 0.13	9.27 ± 0.12	55.48 ± 0.27
blackberry				
Čačanska bestrna	23.93 ± 0.27	25.94 ± 0.20	nd	49.87 ± 0.34
mulberry				
Morus nigra	25.54 ± 0.10	27.45 ± 0.17	nd	52.99 ± 0.20
strawberry				
Clery	30.33 ± 0.10	35.04 ± 0.35	1.07 ± 0.07	66.44 ± 0.37

[a]The values shown are the mean ± standard deviation of three replications. [b]All results are expressed as mg sugar/g frozen weight.

recognized as the main phenolic compound in raspberry and strawberry.[28] The amounts of free and total ellagic acid are given in Table 4. A difference between free and total ellagic acid content in all berry samples is apparent, and these results are in agreement with results found in the literature.[29] Furthermore, our investigation clearly showed that there is a significant difference between two applied extraction systems for total ellagic acid (*t* stat = 3.158, *t* critical two-tail = 2.306). In all cases, the ellagic acid content in acetone was higher in comparison to the methanol extract. The substantially lower

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yield obtained with methanol may be ascribed to the lower solubility of ellagitannin in this solvent. In the acetone extract, the amount of free ellagic acid ranged from 1.34 mg/100 g fw (in Willamette, Arilje) to 5.60 mg/100 g fw (in strawberry, Clery), and these results are comparable to the results reported in the literature.[7] The highest total ellagic acid content was found in the acetone extract of Meeker (181.74 mg/100 g fw), followed by Yellow Meeker (145.92 mg/100 g fw) and blackberry (118.57 mg/100 g fw), whereas ellagic acid was not determined in mulberry sample. Comparing these values with the values published for the total ellagic acid found in raspberry, blackberry, and strawberry from Croatia, one can observe a somewhat higher content of total ellagic acid in our samples.[30]

Determination of Sugars. The amounts of glucose, fructose, sucrose, and total sugars in berry fruits are presented in Table 5. It is evident that the main sugars were fructose and glucose in all berry fruits. The presence of fructose was predominant in all samples, followed by glucose, contributing about 25.27−47.91 and 20.94 −42.87 mg/g, respectively. The highest levels of fructose and glucose were recorded in raspberry cultivar Meeker. Sucrose is present in low amount due to the fact that it may be converted to inverted forms during the ripening process, ranging from 1.07 mg/g (in strawberry, Clery) to 18.96 mg/g (in raspberry, Willamette, Arilje). In blackberry and mulberry sucrose was not found. With regard to total sugars, the amounts varied among different cultivars, reaching the maximum in Meeker (99.96 mg/g). These results are in agreement with the contents of individual and total sugars reported in the literature.[21]

Determination of Minerals. The content of minerals tested in berry fruits are presented as Supporting Information (Table S1). Generally, the amounts of minerals are in good agreement with literature data.[26] Toxic elements (mercury, lead, and cadmium) in the tested extracts were found in small amounts (allowable concentration), the only exception being the content of lead in mulberry extract (the allowable level is 0.2 mg/kg fruit,[31] and the found content was 0.541 mg/kg frozen fruit). Such a result can be explained by the fact that the tested mulberry was harvested in the center of the city. Arsenic was not found in any of the samples tested. The most common element in all berries was found to be potassium, with content ranging from 746.336 mg/kg fw (in blackberry, Čačanska bestrna) to 1702.325 mg/kg fw (in mulberry, *Morus nigra*). Generally, the contents of calcium and magnesium in all investigated samples were found to be significant.

The general conclusion is that results reported here are in agreement with literature data.[6,7,21−23] This investigation has shown that berries from Serbia are a rich source of phenolics, most notably, ellagitannins and anthocyanins. To our knowledge, flavonoids (e.g., chrysin, naringenin, pinocembrin, and galangin) were found in berry extracts for the first time and reported in the current paper. An important note on the differences between two extraction systems is given in this paper. Findings presented herein show that acetone should be considered as the extraction solvent of choice for total phenolic content, ellagic acid, and ellagitannins. However, methanol extracted more anthocyanins than acetone. In the end, unambiguous detection of a large number of compounds using an LTQ OrbiTrap enabled us to determine polyphenolic profiles of the investigated berries.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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