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# Physicochemical analysis and phenolic profile of polyfloral and honeydew honey from Montenegro†

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The research subject of this paper was a detail physicochemical analysis of 28 honey samples from the northern part of Montenegro. The honey from Montenegro has not been previously studied in such detail. Differentiation between samples, such as honeydew honey and polyfloral honey, was based on electrical conductivity, which was higher than 0.8 mS cm<sup>-1</sup> for honeydew honey, as was expected. Other investigated physicochemical parameters (water content, free acids, diastase activity, hydroxymethylfurfural (HMF) content and sugar content) have shown great similarity for all honey samples. The main interest of this study was the identification and quantification of phenolic compounds using ultra-high performance liquid chromatography (UHPLC) with mass spectrometry detection. The results show that honey samples are very rich in phenolic compounds, especially quercetin. Among the 31 quantified phenolic compounds, the most dominant were phenolic acids. The highlight was based on *p*-hydroxybenzoic acid, *p*-coumaric acid, caffeic acid and ferulic acid. Considering polyphenolic compounds and sugar content, a high nutritional value can be observed in all samples, with an emphasis on polyfloral honeys, as was confirmed with principal component analysis (PCA). In addition, all honey samples were tested for total phenolic content (TPC) and radical scavenging activity (RSA). The results indicate the higher antioxidant ability of honeys from Montenegro in comparison to some honey samples from other countries in the region.

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## Introduction

Honeybee products are very popular and highly valued food products with a widespread use in nutrition and medicine. They have a potential role in contributing to human health. Honey is a highly energetic sweet food. It is an easily accessible source of energy. European Legislation<sup>1</sup> has provided a definition of honey. Based on that, bees produce a floral honey from the collected nectar, and honeydew honey from collected sugar rich excretion of plants or excrement of insects. Which honey they produce primarily depends on their location and geographical area that will affect the availability of food source. Polyfloral honey originates from flowers of blossoming plants. Honeydew honey, also called forest honey, made from honeydew, is mainly found on plants such as conifers (fir, pine, spruce), but also oak,

beech, *etc.* Nectar and honeydew may contain different phenolic compounds and sugars, which leads to the diverse characteristics of each type of honey. Some studies have shown appreciably higher content of bioactive compounds for honeydew honey that causes its higher antioxidant activity.<sup>2–5</sup> These higher values point to more therapeutic properties of honeydew honey, which give a rise to its commercial interest. The demand for honeydew honey is increasing, especially in certain regions of Europe where consumers enjoy its remarkable flavor.<sup>6,7</sup> Honeydew honey usually has lower content of monosaccharides and higher content of di- and trisaccharides, such as melezitose, maltotriose or raffinose, which is a good indicator for the recognition of honeydew type of honey.<sup>6</sup>

Montenegro is a country in the southwestern part of the Balkan. For a relatively small area, it has rich biodiversity due to its geographic location and nearness of the sea. Northern part of Montenegro is the area of mountains and valleys. Water wealth and numerous meadows and pastures give contribute to the abundance of significant honey plants. The areas of the mountains include diverse forest vegetation through which permeates silicate rocks and limestone. Mostly dominant forests in high mountains areas are spruce forests, in the lower there are also fir trees, and in the valleys of river oak forests. In the basin of Bijelo Polje and Berane, there are also beech forests. This contributes to its rich flora and excellent natural

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conditions for beekeeping. The distribution of vegetation at different altitudes is important due to flowering and enables extension of the bee pasture. Thanks to the floral diversity and successive bloom of good melliferous plants, bees use meadow pasture for a long-period time. However, at the end of summer and in early autumn, the contribution of forest bee pasture becomes important because of the honeydew.

Until now, different techniques of honey characterization are well known developed in the literature, and a selection of the most appreciated, such as liquid chromatography (LC), is presented in this study.

This study presents a physicochemical analysis of honey samples from northern part of Montenegro. The main goal was a detailed characterization of these honey samples, which had not been thoroughly examined before. This study is focused on three issues: (1) physicochemical analysis, (2) phenolic profile analysis and evaluating of its influence on antioxidant activity, and (3) an attempt to group the obtained results in order to distinguish two types of honeys. The first topic present the physicochemical characterization of honey samples, which relays on determination of water content, electrochemical conductivity, free acidity, HMF content, and sugar content, as a factors of general classification and descriptions of honey.<sup>8</sup> A very important issue of potential establishing sample differentiation was given to the measurement of electrochemical conductivity. The sugar profile was determined by using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Particular topic include identification and quantification of polyphenolic compounds, using the UHPLC system coupled with linear trap quadrupole and Orbitrap mass analyzer (UHPLC-LTQ Orbitrap MS) and UHPLC with a diode array detector and a triple quadrupole mass spectrometer (UHPLC-DAD-MS/MS). Spectrophotometric test, such as TPC and RSA was also observed. Chemometric technique PCA, which was performed on all determined parameters, was used to distinguish obtained honey samples according to their origin. In addition, a summary outlining of the obtained results was compared with the results of other authors that are found in the literature.

## Experimental

### Honey samples

“The Association of the Beekeeping Organizations of Montenegro” provided twenty-eight honey samples, marked as a polyfloral honey. Honey samples were collected during August–September 2017 from three locations: Berane (42.84°N 19.86°E), Bijelo Polje (43.04°N 19.75°E), and Pljevlja (43.36°N 19.36°E), which are in the northern part of Montenegro.

### Reagents, standards and materials

All chemicals (sodium hydroxide, starch, acetate buffer, sodium chloride, 5-hydroxymethyl-furan-2-carbaldehyde, Folin-Ciocalteu's reagent, sodium carbonate, gallic acid, methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrochloric acid, acetone-trile) were of analytical grade. Stock solutions, diluted solutions

and blank samples were prepared with ultrapure water 18 MΩcm (0.055 μS cm<sup>-1</sup>) resistivity obtained from a TKA Micro-Pure water purification system (Thermo Fisher, TKA, Germany). Sugar standards (glucose, fructose, sucrose, maltose, isomaltose, trehalose, turanose, melibiose and melezitose) were purchased from Tokyo Chemical Industry, Europe (Zwijndrecht, Belgium). Standards of phenolic compounds were purchased from Sigma-Aldrich (Steinheim, Germany). Syringe filters (25 mm, nylon membrane, 0.45 μm) were from Supelco (Bellefonte, PA). Cartridges for solid phase extraction (SPE) used for pre-concentration of samples were Strata C18-E (500 mg/3 mL) obtained from Phenomenex (Torrance, USA).

Physicochemical parameters were determined according to the method described by Bogdanov *et al.*<sup>8</sup> Water content was determined by measuring the refractive index of honey samples at the temperature of 20 °C using an Abbe-type refractometer (Atago RX refractometer, Japan) and Chataway's table correction. Electrical conductivity of a 20% (w/v) aqueous solution of honey was measured using a conductivity meter (WTW Golden lab & engineering, Cond 7110, Inolab) equipped with conductivity cell with the constant of 0.108 cm<sup>-1</sup>. Free acidity was determined by titration of the honey solutions (5 g of honey were dissolved in 40 mL of ultrapure water) with sodium hydroxide (0.05 M) to pH 8.5, followed by pH-metry. Determination of diastase activity was based on hydrolysis of 1% starch solution by an enzyme from honey at a temperature of 40 °C, as was described by Bogdanov *et al.*<sup>8</sup> The absorbance of the honey solutions was measured at different time intervals on a wavelength of 660 nm using the UV/Vis spectrophotometer (GBC UV/Visible Cintra 6, Australia). Results were expressed in Schade units per gram of honey and termed as diastase number (DN). Content of HMF was determined using the HPLC system with UV detection (Waters 1525 HPLC dual pump system).<sup>8</sup> The preparation of honey samples and measurement of sugar content was carried out by using a HPAEC/PAD method as exactly as described by Gašić *et al.*<sup>9</sup>

### Phenolic profile

The extraction of phenolic compounds from honey samples was carried out by using as exactly as described by Gašić *et al.*<sup>10</sup>

Qualitative analysis of phenolic compounds was performed by UHPLC system with a quaternary Accela 600 pump and Accela autosampler (Thermo Fisher Scientific, Bremen, Germany) connected to LTQ Orbitrap MS with heated electrospray ionization probe (HESI-II, Thermo Fisher Scientific, Bremen, Germany). Analytical column Syncronis C18 column (100 × 2.1 mm, 1.7 μm particle size; Thermo Fisher Scientific) was used for separation of phenolic compounds. The chromatographic conditions were previously described by Gašić *et al.*<sup>10</sup> For qualitative analysis of phenolic compounds six honey samples were chosen, one honeydew honey and one polyfloral honey sample from each area.

UHPLC equipped with a diode array detector (DAD) connected to TSQ Quantum Access Max triple-quadrupole mass spectrometer (Thermo Fisher Scientific, Basel, Switzerland) was used for quantitative analysis of phenolic compounds. The



separation of phenolic compounds was achieved by using a Syncronis C18 column (100 × 2.1 mm, 1.7 μm). Chromatographic conditions and mass spectrometry parameters for quantification of phenolic compounds were previously described by Gašić *et al.*<sup>9</sup>

### Determination of total phenolic content (TPC) and radical scavenging activity (RSA)

For TPC and RSA determination, honey samples were prepared according to method proposed by Gašić *et al.*<sup>10</sup>

TPC was evaluated by method described by Meda *et al.*<sup>11</sup> The absorbance was measured at 765 nm by UV/Vis spectrophotometer (GBC UV/Visible Cintra6, Australia).

RSA was determined by method published by Chua *et al.*,<sup>12</sup> with some modifications. Prepared honey extracts (0.1 g mL<sup>-1</sup>) in 4 mL of DPPH methanol solution were left in the dark for 60 minutes for incubation.

### Data analysis

Descriptive statistics and Mann–Whitney test were performed by SPSS statistical software (IBM SPSS Statistics 20, IBM Corporation). Principal component analysis has been carried out by PLS ToolBox, v.6.2.1, for MATLAB 7.12.0 (R2011a). All data were autoscaled prior to any multivariate analysis. PCA was carried out by using a singular value decomposition algorithm and a 0.95 confidence level for Q and T2 Hotelling limits for outliers.

## Results and discussion

### Honey classification

Obtained honey samples were specified as polyfloral, only by beekeepers from the “The Association of the Beekeeping Organizations of Montenegro”. Through analysis, the honey samples, have been differently identified as polyfloral and honeydew honeys. Based on the electrical conductivity of the 28 honey samples, eight samples belong to the group of honeydew honey, marked as No. 1, 3, 4, 7, 8, 16, 25, 26 (Fig. 1). This classification is a consequence of their higher values of electrical conductivity, above 0.8 mS cm<sup>-1</sup>, which is the limit value

for honeydew honey.<sup>1</sup> There are studies that also reported this way of honey classification.<sup>3,6,13,14</sup>

### Physicochemical analysis

The results of descriptive analysis for physicochemical parameters of 28 honey samples, their main values, standard deviations and ranges are presented in Table 1, while the results obtained for each sample are shown in Table S1.† The determined values of water content are below than 17.15% (Table 1) and they are in accordance to international requirement with levels lower than 20%.<sup>1</sup> These values point to an appropriate storage and beekeepers handling of the honey. Similar values of low water content in polyfloral and/or honeydew honey samples were also reported by other authors.<sup>14–17</sup> The electrical conductivity of these samples is the cause of the classification between them. The obtained values for honeydew honey samples varied between 0.94–1.13 mS cm<sup>-1</sup> (Table 1). These results are similar with reported values for some honeydew honey samples from other geographical origins such as Romania,<sup>16</sup> Serbia,<sup>18</sup> Bulgaria,<sup>19</sup> Croatia,<sup>20</sup> Brazil,<sup>21</sup> or with fir honeydew honey from Greece.<sup>22</sup> Range of electrical conductivity for polyfloral honey goes from 0.27 to 0.78 mS cm<sup>-1</sup>, very closely to the range 0.40–0.75 reported by Nascimento *et al.*<sup>23</sup> The mean value for polyfloral honey is 0.59 mS cm<sup>-1</sup>, similar to the results of Popek *et al.*<sup>14</sup> Results of free acidity is in compliance with recommended value of not more than 50 meq. kg<sup>-1</sup> of honey.<sup>1</sup> Mean value for free acidity in honeydew honey are higher than in polyfloral honey (Table 1). The diastase activity of tested honey samples is higher than 8 Schade units, which is the minimal value recommended by the EU Directive 2014/63.<sup>1</sup> Results of the content of HMF in honey samples are mostly below the detection limit, <5 mg kg<sup>-1</sup>, except in six samples (Table S1†), but still in permitted range, up to 40 mg kg<sup>-1</sup>.<sup>1</sup> Higher content of HMF was noticed in polyfloral honey samples (Table 1).

The results of descriptive analysis of sugar content in honey samples are presented in Table 1. Content of monosaccharides is 65.84 g/100 g for honeydew honey and 66.77 g/100 g for polyfloral honey. The determined reducing monosaccharides, glucose and fructose, which are the major constituents of honey, are in agreement with the proposed value by European Legislation.<sup>1</sup> There is a similarity in determined range of fructose and glucose content for both types (Table 1). Salonen *et al.*<sup>24</sup> also reported no significant differences of monosaccharides between polyfloral and honeydew honey samples. The content of sucrose in all samples is less than the maximum allowed concentration of 5 g/100 g.<sup>1</sup> The trisaccharide melezitose was also evidenced in all samples (Fig. S1†) containing higher main value in honeydew honey (0.27 g/100 g) than in polyfloral honey samples (0.15 g/100 g) (Table 1). Melezitose is recognized as a good indicator in differentiation honeydew honey from polyfloral honey.<sup>6</sup>

According to the results of the physicochemical parameters, all samples are within the established limits and in agreement with international quality regulations, suggesting good conditions of honey production. As other studies shown,<sup>6,14,21</sup> physicochemical variables were successfully used to differentiate

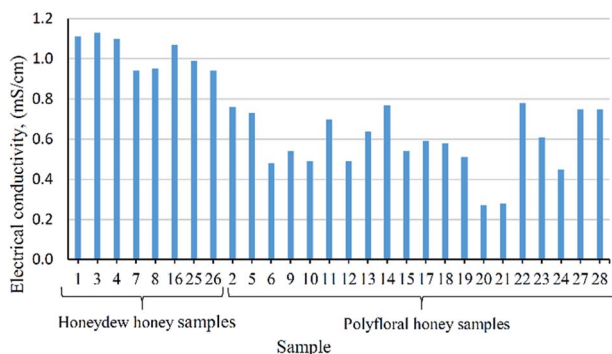


Fig. 1 Distribution of electrical conductivity (mS cm<sup>-1</sup>) in honey samples.



Table 1 Descriptive analysis of all physicochemical parameters of honey according to their botanical origin ( $p \leq 0.05$ )<sup>a</sup>

Variable	Honeydew honey				Polyfloral honey			
	Mean	SD	Median	Min–max	Mean	SD	Median	Min–max
Water content (%)	15.96	0.77	16.28	14.93–16.88	16.08	0.67	15.98	14.85–17.15
Electrical conductivity (mS cm <sup>-1</sup> )	1.03	0.08	1.03	0.94–1.13	0.59	0.15	0.59	0.27–0.78
Free acidity (meq. kg <sup>-1</sup> )	29.84	3.66	29.05	25.81–36.63	27.18	5.08	28.38	17.41–34.37
Diastase activity (DN)	31.64	2.34	31.85	27.93–34.81	34.14	6.14	33.58	19.77–46.11
HMF (mg kg <sup>-1</sup> )	0.69	1.94	0	0–5.50	2.33	4.24	0	0–10.95
Glucose (g/100 g)	29.48	2.83	29.59	24.98–34.19	30.56	2.87	30.37	26.41–36.08
Fructose (g/100 g)	36.35	2.72	36.77	32.58–41.41	36.21	2.89	35.67	31.45–41.27
Sucrose (g/100 g)	1.62	0.94	1.33	0.43–3.01	1.43	0.78	1.09	0.51–3.35
Maltose (g/100 g)	0.74	0.18	0.74	0.54–1.00	1.09	0.59	0.86	0.30–2.57
Isomaltose (g/100 g)	0.49	0.19	0.53	0.22–0.79	0.68	0.34	0.59	0.24–1.47
Trehalose (g/100 g)	0.52	0.35	0.43	0.15–1.08	0.29	0.21	0.27	0.02–0.72
Turanose (g/100 g)	0.80	0.16	0.79	0.60–1.07	0.65	0.55	0.48	0.08–2.14
Melibiose (g/100 g)	0.05	0.03	0.06	0–0.08	0.06	0.07	0.03	0–0.27
Melezitose (g/100 g)	0.27	0.24	0.22	0.03–0.59	0.15	0.14	0.11	0.001–0.53
Sum of sugars (g/100 g)	70.33	6.45	70.69	62.64–79.91	71.13	7.49	69.30	62.19–85.03

<sup>a</sup> Mean – mean value, SD – standard deviation, median – median value, min–max – concentration range.

honeydew honey from floral honey, but in our study, specific electrical conductivity appears to be the most effective in distinguishing these two types of honey. Moreover, botanical origin of honey could be successfully determined by electrical conductivity, among the other physicochemical parameters.<sup>3,13</sup> Considering the results of sugar content, only negligible extent content of melezitose indicate differences between samples, while other studies suggested considerable lower levels of monosaccharides,<sup>13</sup> and higher levels of oligosaccharides<sup>6,7</sup> in honeydew honey. Great similarity of physicochemical parameters for two types of honey types originating from a small geographical region indicates that honeydew honeys and polyfloral honeys represent a complex mixture of honeydew and nectar from different botanical species.

### Phenolic profile

**Qualitative analysis of phenolic compounds.** The results of the 32 identified phenolic compounds: 12 flavonoids, nine phenolic acids, and their derivatives, are shown in Table 2. Notable number of phenolic compounds (24 of them) was detected in six chosen samples. There are several differences in appearing of phenolic compounds, without consistency. The highlights are on 3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid, present only in one polyfloral sample (No. 6), and dihydroxybenzoic acid methyl ether presents only in one honeydew honey sample (No. 4) which is the only one sample where was no presence of kaempferol 7-*O*-rhamnoside (Table 2). Some of major identified phenolic compounds (from 14 to 20 of them) were also reported by Gašić *et al.*<sup>10</sup> and Combarros-Fuertes *et al.*,<sup>25</sup> for polyfloral samples, by Vasić *et al.*<sup>20</sup> for honeydew honey samples, and by Ciucure *et al.*<sup>5</sup> and Holouška *et al.*<sup>26</sup> for both types of honey with more observed differences between them.

**Quantitative analysis of phenolic compounds.** With UHPLC-DAD MS/MS it was quantified 32 phenolic compounds, 14

flavonoids, 9 phenolic acids and nine of their derivatives (in form of glycosides). Among 14 quantified flavonoids, seven were present in all studied samples, quercetin, kaempferol, galangin, kaempferide, luteolin, pinocembrin, naringenin (Fig. S2†). In Table 3 is given a descriptive analysis of each phenolic compound and their sums, given as mean value, standard deviation, median and range (from minimum to maximum value). Polyphenolic compounds are involved in a large number of physiological functions, and they are substantially responsible for the antioxidant activity of honey.<sup>6,11,23,27</sup> They occurs in a wide variety of vegetables, fruits, herbs, and plant-based beverages. In this study, among all quantified phenolic compounds, the highest content was found for quercetin, 9.865 mg kg<sup>-1</sup> for polyfloral and 4.947 mg kg<sup>-1</sup> for honeydew honey samples (Fig. S2†). Quercetin is one of the most widespread flavonoids in food, and its bioavailability can highly variable, so it is important to ingesting it by the food or supplements, such as honey.<sup>25,28</sup> The obtained average value for quercetin are in same order of magnitude as Oroian *et al.*<sup>16</sup> got. Comparing the obtained results for quercetin to polyfloral honey from Serbia<sup>10</sup> and polyfloral and honeydew honey from Romania<sup>5</sup> and Slovenia<sup>29</sup> it can be seen the superiority of the results in this study. The next dominant content of flavonoids in studied samples (Fig. S2†) have flavonoids that originate from propolis, such as chrysin, pinocembrin and galangin,<sup>30</sup> (2.166 mg kg<sup>-1</sup> 1.448 mg kg<sup>-1</sup> 0.858 mg kg<sup>-1</sup>, sequently, in polyfloral honey and 1.609 mg kg<sup>-1</sup>, 1.289 mg kg<sup>-1</sup>, 0.747 mg kg<sup>-1</sup>, sequently, in honeydew honey) (Table 3). Flavones are absorbed systemically, and they are found as both *O*- and *C*-glycosides. Apigenin is well known flavone because of its nutritional and organoleptic characteristics that also contribute to beneficial health properties.<sup>31</sup> It was absent in one polyfloral sample (Fig. S2†), while in other samples it was present in considerable amount with average content of 0.939 mg kg<sup>-1</sup> for polyfloral and 0.710 mg kg<sup>-1</sup> for honeydew honey samples (Table 3). Salonen *et al.*<sup>24</sup> reported lower content of apigenin in



Table 2 High-resolution MS data and negative ion MS<sup>2</sup> and MS<sup>3</sup> fragmentation of phenolic compounds identified in six honey samples (H-honeydew, P-polyfloral)

No	Compound name	$t_{R}$ min	Molecular formula [M - H] <sup>-</sup>	Calculated mass, [M - H] <sup>-</sup>	Exact mass, [M - H] <sup>-</sup>	$\Delta$ ppm	MS <sup>2</sup> fragments, (% base peak)	Berane						MS <sup>3</sup> fragments, (% base peak)			
								H4	P6	H16	P9	H25	P20				
<b>Phenolic acids and derivatives</b>																	
1	Protocatechuic acid <sup>a</sup>	4.53	C <sub>7</sub> H <sub>5</sub> O <sub>4</sub> <sup>-</sup>	153.0193	153.0191	1.57	109(100), 95(75), 79(20), 59(10)	+	+	+	+	+	+	81(100), 68(25), 65(15)	+	+	+
2	3-O-Caffeoylquinic acid	5.37	C <sub>16</sub> H <sub>17</sub> O <sub>9</sub> <sup>-</sup>	353.0878	353.0872	1.76	191(100), 179(30), 135(10)	-	+	-	-	-	-	173(75), 127(100), 111(40), 93(60), 85(90)	-	-	-
3	<i>p</i> -Hydroxybenzoic acid <sup>a</sup>	5.54	C <sub>7</sub> H <sub>5</sub> O <sub>3</sub> <sup>-</sup>	137.0244	137.0242	1.39	109(10), 93(100)	+	+	+	+	+	+	93(100)	+	+	+
4	5-O-Caffeoylquinic acid <sup>a</sup>	5.86	C <sub>16</sub> H <sub>17</sub> O <sub>9</sub> <sup>-</sup>	353.0878	353.0875	0.82	191(100), 179(5)	-	+	-	-	-	-	173(75), 127(100), 111(40), 93(60), 85(90)	-	-	-
5	Caffeic acid <sup>a</sup>	5.92	C <sub>9</sub> H <sub>7</sub> O <sub>4</sub> <sup>-</sup>	179.0350	179.0345	2.85	135(100)	+	+	+	+	+	+	135(60), 117(15), 107(100), 91(55), 79(15)	+	+	+
6	<i>p</i> -Coumaric acid <sup>a</sup>	6.81	C <sub>9</sub> H <sub>7</sub> O <sub>3</sub> <sup>-</sup>	163.0401	163.0398	1.41	119(100)	+	+	+	+	+	+	119(60), 101(20), 93(25), 91(100), 72(10)	+	+	+
7	Ellagic acid	6.83	C <sub>14</sub> H <sub>5</sub> O <sub>8</sub> <sup>-</sup>	300.9990	300.9996	-2.13	284(40), 271(60), 257(100), 229(85), 185(40)	+	+	-	+	+	+	229(100), 213(20), 185(85)	+	+	-
8	Dihydroxybenzoic acid methyl ether	6.97	C <sub>8</sub> H <sub>7</sub> O <sub>4</sub> <sup>-</sup>	167.0350	167.0346	2.51	152(100), 108(20)	+	-	-	-	-	-	108(100)	+	-	-
9	Benzyl caffeate	11.56	C <sub>16</sub> H <sub>13</sub> O <sub>4</sub> <sup>-</sup>	269.0819	269.0815	1.45	225(10), 179(10), 178(70), 161(10), 134(100)	+	+	+	+	+	+	121(90), 111(40), 106(100)	+	+	+
<b>Flavonoids and derivatives</b>																	
10	Tricetin	6.58	C <sub>15</sub> H <sub>9</sub> O <sub>7</sub> <sup>-</sup>	301.0354	301.0353	0.10	283(100), 273(50), 255(15), 245(20), 227(15)	-	-	+	+	+	+	255(100), 239(70), 227(70), 211(40), 199(20)	-	-	+
11	Quercetin 3-O-rhamnoside <sup>a</sup>	7.30	C <sub>21</sub> H <sub>19</sub> O <sub>11</sub> <sup>-</sup>	447.0933	447.0927	1.36	301(100), 300(35), 284(20)	+	+	+	+	+	+	273(25), 257(20), 179(100), 151(75)	+	+	+
12	Kaempferol 3-O-rhamnoside	7.77	C <sub>21</sub> H <sub>19</sub> O <sub>10</sub> <sup>-</sup>	431.0984	431.0974	2.37	327(5), 285(100), 284(70), 255(10)	+	+	-	+	+	+	267(50), 257(100), 241(40), 229(60), 213(30)	+	+	+
13	Kaempferol 7-O-rhamnoside	8.56	C <sub>21</sub> H <sub>19</sub> O <sub>10</sub> <sup>-</sup>	431.0984	431.0972	2.78	286(10), 285(100), 284(20), 257(5), 151(5)	+	+	+	+	+	+	257(40), 241(30), 213(10), 151(100), 107(10)	+	+	+
14	Luteolin <sup>a</sup>	8.80	C <sub>15</sub> H <sub>9</sub> O <sub>6</sub> <sup>-</sup>	285.0405	285.0403	0.63	257(40), 241(100), 217(50), 199(70), 175(70)	+	+	+	+	+	+	255(50), 227(100), 211(75), 197(35), 183(85)	+	+	+
15	Quercetin <sup>a</sup>	8.87	C <sub>15</sub> H <sub>9</sub> O <sub>7</sub> <sup>-</sup>	301.0354	301.0345	3.06	283(15), 271(60), 257(25), 179(100), 151(80)	+	+	+	+	+	+	151(100)	+	+	+
16	Pinobanksin 5-methyl ether	9.18	C <sub>16</sub> H <sub>13</sub> O <sub>5</sub> <sup>-</sup>	285.0771	285.0761	1.30	267(100), 252(20), 239(30), 179(5), 165(5)	+	+	+	+	+	+	252(100), 239(5), 224(10)	+	+	-
17	Naringenin <sup>a</sup>	9.66	C <sub>15</sub> H <sub>11</sub> O <sub>5</sub> <sup>-</sup>	271.0612	271.0599	4.76	225(5), 177(10), 151(100)	+	+	+	+	+	+	107(100)	+	+	+
18	Apigenin <sup>a</sup>	9.67	C <sub>15</sub> H <sub>9</sub> O <sub>5</sub> <sup>-</sup>	269.0455	269.0456	-0.30	225(5), 177(15), 151(100)	+	+	+	+	+	+	65(100)	+	+	+
19	Kaempferol <sup>a</sup>	9.83	C <sub>15</sub> H <sub>9</sub> O <sub>6</sub> <sup>-</sup>	285.0405	285.0398	2.35	255(100), 227(10)	+	+	+	+	+	+	211(100), 195(5), 167(15)	+	+	+
20	Methoxy kaempferol	9.84	C <sub>16</sub> H <sub>11</sub> O <sub>7</sub> <sup>-</sup>	315.0510	315.0496	4.57	301(20), 300(100)	+	+	+	+	+	+	272(100), 256(60), 216(20), 202(20), 166(35)	+	+	+
21	Pinobanksin	9.98	C <sub>15</sub> H <sub>11</sub> O <sub>5</sub> <sup>-</sup>	271.0612	271.0600	4.43	253(100), 225(29), 269(21), 215(16), 197(15)	+	+	+	+	+	+	197(100), 225(85), 209(51), 185(23)	+	+	+

Table 2 (Contd.)

No	Compound name	$t_R$ , min	Molecular formula, $[M - H]^-$	Calculated mass, $[M - H]^-$	Exact mass, $[M - H]^-$	$\Delta$ ppm	MS <sup>2</sup> fragments, (% base peak)	MS <sup>3</sup> fragments, (% base peak)	Berane			Bijelo Polje			Pjervija		
									H4	P6	H16	P9	H16	P9	H25	P20	
22	Isohamnetin	10.06	$C_{16}H_{11}O_7^-$	315.0510	315.0506	1.27	301(20), 300(100)	283(30), 271(100), 255(50), 227(52), 151(90)	+	+	+	+	+	+	+	+	
23	Chrysoeriol	10.16	$C_{16}H_{11}O_6^-$	299.0561	299.0555	1.91	285(10), 284(100)	255(100), 227(100)	+	+	+	+	+	+	+	+	
24	Quercetin dimethyl ether isomer 1	10.33	$C_{17}H_{13}O_7^-$	329.0667	329.0672	-1.67	314(100)	299(100), 271(10)	+	+	+	+	+	+	+	+	
25	Rhamnetin	10.85	$C_{16}H_{11}O_7^-$	315.0510	315.0512	-0.57	300(40), 207(10), 193(40), 165(100), 121(10)	150(50), 121(100), 97(60), 91(15), 65(20)	+	+	+	+	+	+	+	+	+
26	Quercetin dimethyl ether isomer 2	11.22	$C_{17}H_{13}O_7^-$	329.0667	329.067	-0.94	314(100)	299(100), 271(10)	+	+	+	+	+	+	+	+	+
27	Chrysin <sup>a</sup>	11.76	$C_{15}H_9O_4^-$	253.0506	253.050	3.56	253(30), 209(100), 181(20), 165(15), 151(15)	181(100), 165(30), 153(20), 141(10)	+	+	+	+	+	+	+	+	+
28	Sakuranetin	11.81	$C_{16}H_{13}O_5^-$	285.0769	285.076	2.07	270(40), 268(100), 243(50), 164(50), 151(10)	240(100), 239(40), 223(30), 207(20), 193(10)	+	+	+	+	+	+	+	+	+
29	Pinoembrin <sup>a</sup>	11.89	$C_{15}H_{11}O_4^-$	255.0663	255.066	2.86	213(100), 187(15), 151(30), 145(10), 107(5)	185(100), 169(20), 145(20)	+	+	+	+	+	+	+	+	+
30	Galangin <sup>a</sup>	12.01	$C_{15}H_9O_5^-$	269.0455	269.045	0.93	241(40), 227(80), 213(100), 197(90), 169(50)	211(10), 198(20), 185(40), 169(100), 143(25)	+	+	+	+	+	+	+	+	+
31	Kaempferide <sup>a</sup>	12.05	$C_{16}H_{11}O_6^-$	299.0561	299.056	0.47	284(100), 271(20), 255(5), 240(5), 165(20)	255(25), 240(25), 227(20), 164(40), 151(100)	+	+	+	+	+	+	+	+	+
32	Acacetin <sup>a</sup>	12.41	$C_{16}H_{11}O_5^-$	283.0612	283.061	-0.53	268(100)	268(100), 240(30)	+	+	+	+	+	+	+	+	+

<sup>a</sup> Confirmed using available standards.

Table 3 Descriptive analysis of content of phenolic compounds (mg kg<sup>-1</sup>), TPC (mg GAE/100 g) and RSA (%) in honey samples, ( $p \leq 0.05$ )<sup>a</sup>

Phenolic compounds	Honeydew				Polyfloral			
	Mean	SD	Median	Min-max	Mean	SD	Median	Min-max
<b>Flavonoids</b>								
Quercetin	2.709	1.305	2.795	1.137–4.947	3.038	2.348	2.398	0.469–9.865
Kaempferol	0.901	0.253	0.836	0.573–1.322	0.961	0.396	0.974	0.196–1.886
Galangin	0.747	0.646	0.519	0.130–2.059	0.858	0.599	0.702	0.056–1.820
Kaempferide	0.044	0.038	0.031	0.007–0.121	0.042	0.028	0.036	0.008–0.133
Apigenin	0.710	0.336	0.739	0.280–1.323	0.939	0.43	1.038	0.138–1.676
Chrysin	1.609	1.031	1.323	0.395–3.440	2.166	1.289	1.974	0.252–4.515
Acacetin	0.043	0.015	0.045	0.017–0.059	0.050	0.017	0.050	0.011–0.082
Luteolin	0.096	0.030	0.092	0.058–0.142	0.172	0.12	0.149	0.015–0.551
Genkwanin	0.054	0.020	0.058	0.021–0.078	0.068	0.027	0.065	0.014–0.121
Pinocembrin	1.289	1.181	0.915	0.314–3.954	1.448	1.123	1.206	0.055–3.843
Naringenin	0.234	0.166	0.244	0.017–0.475	0.203	0.169	0.157	0.033–0.692
Eriodictyol	0.018	0.030	0.009	0–0.088	0.022	0.044	0.018	0–0.201
Taxifolin	0.061	0.061	0.058	0–0.148	0.043	0.050	0.014	0–0.154
Daidzein	0.016	0.024	0.003	0–0.064	0.027	0.053	0.007	0–0.200
Sum of flavonoids	8.533	3.857	7.113	5.047–15.371	9.872	4.911	8.663	1.637–21.286
<b>Phenolic acids</b>								
<i>p</i> -Hydroxybenzoic acid	2.182	1.081	1.667	1.374–4.475	2.318	0.821	2.315	0.679–3.621
Protocatechuic acid	1.008	0.338	0.990	0.618–1.529	0.721	0.527	0.705	0.046–1.858
Vanillic acid	1.319	0.347	1.318	0.871–1.892	1.093	0.275	1.052	0.509–1.841
<i>p</i> -Hydroxyphenylacetic acid	0.946	0.318	0.868	0.670–1.693	0.895	0.454	0.788	0.461–2.306
Caffeic acid	2.125	0.956	2.066	1.021–4.220	2.697	1.575	2.078	0.748–6.707
5- <i>O</i> -Caffeoylquinic acid	0	0	0	0	1.154	0.511	0	0–2.229
<i>p</i> -Coumaric acid	2.802	1.194	2.480	1.671–4.699	2.949	1.167	3.020	0.895–5.416
Ferulic acid	2.077	0.983	1.903	0.777–3.411	2.554	1.221	2.445	0.703–4.929
Sinapic acid	0.033	0.066	0	0–0.177	0.022	0.047	0	0–0.148
Sum of phenolic acids	12.327	4.206	11.276	7.022–18.566	13.402	3.823	12.759	6.153–20.338
<b>Glycosides</b>								
Apigenin-7- <i>O</i> -glucoside (apigetrin)	0.001	0.002	0	0–0.005	0.004	0.007	0	0–0.021
Apigenin-8- <i>C</i> -glucoside (vitexin)	0.001	0.002	0	0–0.007	0.002	0.004	0	0–0.012
Kaempferol-7- <i>O</i> -glucoside	0.023	0.026	0.021	0–0.078	0.056	0.044	0.045	0–0.171
Isorhamnetin-3- <i>O</i> -glucoside	0.004	0.011	0	0–0.031	0.003	0.127	0	0–0.057
Aesculetin-6- $\beta$ - <i>D</i> -glucoside (aesculin)	0.005	0.015	0	0–0.041	0.008	0.018	0	0–0.056
Naringenin-7- <i>O</i> -glucoside (naringin)	0.121	0.042	0.109	0.086–0.208	0.205	0.183	0.170	0–0.805
Quercetin-3- <i>O</i> -rutinoside (rutin)	0.050	0.045	0.049	0–0.142	0.069	0.064	0.074	0–0.201
Quercetin-3- <i>O</i> -galactoside (hyperoside)	0.016	0.007	0.014	0.006–0.026	0.026	0.012	0.024	0–0.052
Quercetin-3- <i>O</i> -rhamnoside	0.148	0.129	0.111	0–0.373	0.133	0.130	0.099	0–0.586
Sum of glycosides	0.369	0.145	0.362	0.210–0.622	0.505	0.241	0.485	0.246–1.206
Sum of phenolic compounds	21.229	6.875	20.260	13.091–33.798	23.780	7.911	23.651	8.046–38.363
TPC	72.884	9.106	71.673	60.448–92.083	70.019	22.491	63.828	39.163–110.645
RSA	10.008	2.803	8.898	8.151–16.521	8.155	4.826	7.459	2.361–21.273

<sup>a</sup> Mean – mean value, SD – standard deviation, median – median value, min-max – concentration range.

polyfloral (0.09 mg kg<sup>-1</sup>) and no content in honeydew honey samples. Presence of eriodictyol, daidzein and taxifolin was variable by samples as it can be seen from Fig. S2.†

The main health benefits of polyphenols include mechanisms of anti-oxidative and anti-free radical effects. Phenolic acids, as free radical scavengers, have pronounced antioxidant activity. In our study, phenolic acids have the highest proportion in total phenolic compounds. Among nine quantified phenolic acids, six phenolic acids are present in all studied samples. Four of them (*p*-coumaric acid, caffeic acid, *p*-hydroxybenzoic acid, and ferulic acid) contain higher mean values in polyfloral samples, and two acids (protocatechuic acid

and *p*-hydroxyphenylacetic acid) have higher mean values in honeydew honey samples (Table 3). After the quercetin, *p*-coumaric acid has the next dominant proportion in total phenolic compounds, followed by caffeic acid, ferulic acid, and *p*-hydroxybenzoic acid. Maximum value of *p*-coumaric acid for polyfloral samples was 5.416 mg kg<sup>-1</sup>, and for honeydew honey samples 4.699 mg kg<sup>-1</sup> (Table 3). In contrary, Gašić *et al.*<sup>10</sup> reported higher results such as maximum value of 9.97 mg kg<sup>-1</sup> for polyfloral sample from East Serbia, and Oroian *et al.*<sup>16</sup> reported 9.1 mg kg<sup>-1</sup> for polyfloral and 8.4 mg kg<sup>-1</sup> for honeydew honey samples from East Romania. One of the most common phenolic acids is caffeic acid. It has two hydroxyl groups bonded



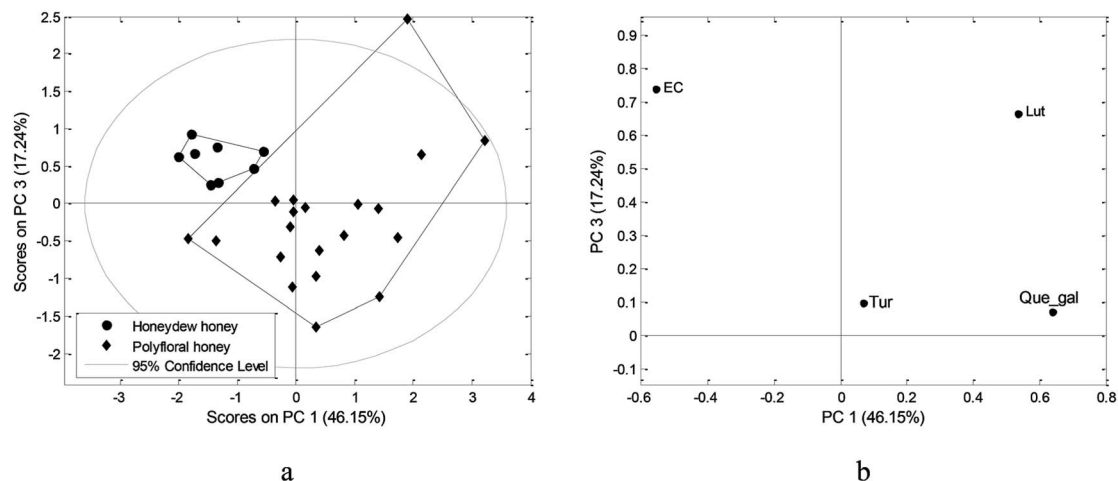


Fig. 2 PCA applied on physiochemical parameters, sugar and phenolic compounds content: (a) score plot, (b) loading plot.

to benzene ring, which directly influence on its antioxidant activity.<sup>28</sup> Its range in honey samples goes from 0.748 to 6.707 mg kg<sup>-1</sup> (Table 3), which is similar to results of Holouzka *et al.*,<sup>26</sup> but one size higher than Ciucure *et al.*<sup>5</sup> and Socha *et al.*<sup>28</sup> were reported. No occurrence of 5-*O*-caffeoylquinic acid in honeydew honey (Fig. S2†) is in contrast to results published by Vasić *et al.*<sup>20</sup> who showed its presences in all honeydew honey samples from Croatia in range from 0.004 to 0.082 mg kg<sup>-1</sup>. Hydroxybenzoic acid and its derivatives have ability to modify cellular signalling processes that introduces a multiplier effect on enhancement of multiple anti-oxidant mechanisms.<sup>31</sup> In this study, the highest value of *p*-hydroxybenzoic acid is 4.475 mg kg<sup>-1</sup> in honeydew honey sample. In flavonoids native forms, they are found as both *O*- and *C*-glycosides, with *O*-glycosides being more common and usually better absorbed. Occurrence of glycosides considerably fluctuated going through the samples (Fig. S2†). Nine flavonoid-glycosides were identified. Naringenin-7-*O*-glucoside (naringin) and quercetin-3-*O*-rhamnoside are dominant, and then quercetin-3-*O*-rutinoside (rutin). Apigenin-7-*O*-glucoside, apigenin-8-*C*-glucoside and aesculetin-6-β-*D*-glucoside were present only in one honeydew honey sample, while isorhamnetin-3-*O*-glucoside is present only in one polyfloral and one honeydew honey sample.

It can be noticed that in polyfloral honey samples, is present higher average values of sums of flavonoids, phenolic acids and glycosides (9.87 mg kg<sup>-1</sup>, 13.40 mg kg<sup>-1</sup>, and 0.51 mg kg<sup>-1</sup>, respectively) than in honeydew honey samples (8.53 mg kg<sup>-1</sup>, 12.33 mg kg<sup>-1</sup>, and 0.37 mg kg<sup>-1</sup>, respectively) (Table 3). Opposite to these values, Ciucure *et al.*<sup>5</sup> reported lower average content of phenolic acids, as they quantified 2.54 mg kg<sup>-1</sup> for polyfloral and 1.88 mg kg<sup>-1</sup> for honeydew honey samples. Salonen *et al.*<sup>24</sup> reported the same order of magnitude as in this study, for Nordic honey. Comparing obtained results for individual phenolic compounds with the results of some other authors<sup>10,20,24,26</sup> a sufficient number of phenolic compounds with similar values can be noticed (Table S2†).

In general, as it was for sugar content, it could be noticed a higher content of phenolic compounds for polyfloral honey

samples. Although, the levels of quantified phenolic compounds are lower than in another foods,<sup>31</sup> it is important to considered their sums in honey samples and ability of polyphenols for synergism. In addition, the obtained quantified content for individual phenolic compounds of the present study with the finding results from countries in region, such as honey from Romania,<sup>5</sup> Serbia,<sup>10</sup> Croatia<sup>20</sup> and Slovenia<sup>29</sup> suggest that Montenegrin honey contains higher amounts of phenolic compounds.

#### Antioxidant activity

Descriptive analysis of obtained results of TPC and RSA in honey samples is shown in Table 3. Average value of TPC for honeydew honey is 72.88 mg GAE/100 g and for polyfloral samples is 70.02 mg GAE/100 g. Obtained results are similar to the other reported results for polyfloral samples.<sup>10,23</sup> For honeydew honey, there are some studies of higher,<sup>5,11,26</sup> and lower reported average values.<sup>2,28</sup> Average value of RSA for polyfloral honey is 8.16% (8.80 μmol/100 g) and for honeydew honey samples is 10.01% (10.86 μmol/100 g) (Table 3). Similar variability between samples, but with higher average value (15.83%) was reported for Croatian honeydew honey.<sup>32</sup> Larger variability was reported for Romanian honeydew honey samples<sup>5</sup> with their higher average value of 23.3%. Similar obtained values of TPC and RSA for polyfloral honey were reported by Ciucure *et al.*<sup>5</sup> and Gašić *et al.*<sup>10</sup>

Besides the variations within the range of TPC and RSA that are more noticeable for polyfloral samples, there is high similarity between polyfloral and honeydew honey (Fig. S3†). This is contrary to other obtained conclusions stating that the highest antioxidant activity is in honeydew honey, compared to polyfloral honey.<sup>2</sup>

By determining the Pearson coefficient, a correlation regression analysis was used for comparison of TPC, RSA (spectrophotometric results) and sum of phenolic compounds (chromatographically obtained results) (Table S3†). As a measure of linear correlation between the two variables, high values of Pearson coefficient were noticed for TPC and RSA,



0.907 for honeydew honey and 0.928 for polyfloral honey (Table S3†). Considering it as a satisfactory regression coefficient, it indicates higher total phenolic compounds content and consequently higher antioxidant capacity. It can be assumed that the antioxidant activity is possibly based on the content of phenolic compounds as other suggested.<sup>10,23</sup> Differently, there is unsatisfactory Pearson coefficient for sum of phenolic compounds. The assumption for negative correlation ( $-0.305$ ,  $-0.012$ ) (Table S3†) can be seen in the numeric obtained values for some samples. Expressed values (high or low) have a significant influence on correlation, especially for a small number of variables. Although one sample should not distort the assessment of correlations, however, when there is a total of eight samples, it is very important. Low correlations (0.061, 0.018, Table S3†) can indicate that TPC and RSA were not changed in the same way as sum of phenolic compounds. It gives rise to independent change of chromatographically determined value in relation to spectrophotometrically.

The results suggested that the values of the individual phenolic compounds in the honey samples were insufficient to have a significant antioxidant effect. However, antioxidant activity can be noticed if the results of the sums of phenolic compounds and results of TPC and RSA values are observed (Table 3). As phenolic compounds show synergism, their biological activity depends on many factors, as well as the ratio of pure components in the mixture, also on other present compounds in honey such as peptides, organic acids, enzymes, Maillard's reaction products, in which combined activities and the interaction can be expose antioxidantivity.<sup>12,25</sup>

### Statistical analysis

In order to mark the factors which could be used for differentiation of certain source of bees production, nectar or honeydew, data analysis which includes Mann–Whitney *U*-test and PCA, was performed on physicochemical parameters, sugar and phenolic compounds content, and TPC and RSA values.

Mann–Whitney *U*-test, used to compare the medians of 49 parameters of honeydew and polyfloral honey, revealed that only electrical conductivity among physicochemical parameters, turanose among sugars and luteolin and quercetin-3-*O*-galactoside among phenolic compounds, have *p* values below 0.05. These parameters therefore indicate statistically significant difference between honeydew and polyfloral honey and could be selected as the most discriminating factors. It must be emphasized that only 4 out of 49 parameters determined in analyzed samples is as small number of discriminating factors but also expected number taking into account a fact that honeydew honey and polyfloral honey represent a complex mixture of honeydew and nectar from different botanical species in a relatively small geographical area. Number of parameters which could be used for differentiation between monofloral honey and honeydew honey is much higher due to the domination of nectar of certain, particular plant in monofloral honey.<sup>5,15,16,29</sup>

For the task of differentiation and classification of honey it is better to combine the several groups of parameters than to observe only one class of compounds, such as sugars, or

phenolic compounds, or elements, *etc.* However, physicochemical parameters mainly gave a good classification results when applied alone, while all other analytical parameters should be combined to evaluate better results.<sup>33</sup> In order to evaluate whether physicochemical parameters, on one side, and combining analytical parameters, on the other, could differentiate honeydew honey from polyfloral honey, the data was subjected to pattern recognition analysis.

PCA performed on five physicochemical parameters, water content, electrical conductivity, free acidity, diastase activity and HMF, resulted in three component model which explained 79.64% of the total variance. Mutual projection of factor scores (Fig. S4†) revealed two groups of objects which correspond to honeydew and polyfloral honey. Grouping of honeydew honey was imposed by higher values of electrical conductivity and free acidity; while polyfloral honey was separated according to higher water and HMF content (Fig. S4†).

PCA derived on the initial data matrix, which included all determined parameters, resulted in a model with low percent of variability in the first few principal components. However, observing only those variables showed significant influence on the first PCA model and were additionally matched with the results of Mann–Whitney *U*-test, new, improved model was obtained. Three-component model explaining 92.62% of variability revealed grouping of samples according to botanical origin (Fig. 2a). The obtained model pointed out that only electrical conductivity could be marked as important factor, which discriminates and characterizes honeydew honey, while turanose, luteolin and quercetin-3-*O*-galactoside are more abundant in polyfloral honey (Fig. 2b). These results also revealed a higher nutritive value of polyfloral honey compared to honeydew honey, as far as honey from Montenegro is concerned.

## Conclusions

This research represents a detail physicochemical analysis of the Montenegrin honey. In the present study, among 28 analyzed honey samples, eight of them were honeydew honey and twenty were polyfloral honey samples, differentiated by electrical conductivity.

Based on the obtained results, it can be seen that all investigated honey samples possess considerable nutrition value containing a notable number of phenolic compounds. Generally, there are great similarity between examined honeydew honey and polyfloral honey samples.

Comparing the obtained results of phenolic analysis with the results from countries in the region, such as Serbia, Slovenia, Croatia, Bulgaria, a higher antioxidant potential of honey from Montenegro can be noticed.

It can be concluded that honeydew honey and polyfloral honey from small geographical areas such as northern part of Montenegro, represent a complex mixture of honeydew and nectar from different botanical species, which can cause of similarity of samples.



## Conflicts of interest

There are no conflicts to declare.

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