COLOR, TOTAL PHENOLS AND ANTIOXIDANT ACTIVITY OF HONEY FROM NORTHWESTERN BOSNIA AND HERZEGOVINA

ORIGINAL SCIENTIFIC PAPER

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ABSTRACT:
Thanks to the climatic and geographical conditions, the area of the Northwestern part of Bosnia and Herzegovina has a long tradition of producing honey and other bee products. However, there is little or no literature data on the physico-chemical properties and biological activity of different types of honey and other bee products from Bosnia and Herzegovina. Five different types of honey were analyzed: monofloral honey (acacia, chestnut, linden), meadow honey and forest honey. Physico-chemical parameters, sensory analysis, color of honey, antioxidant activity, and content of total phenols were analyzed in five types off collected honey samples. The analyzes performed showed that chestnut honey contains the highest and acacia honey has the lowest content of total phenolic compounds. The forest honey showed the best antioxidant activity. The color of the honey was measured according to the CIELab system and the estimated L^*, a^*, b^* parameters show that all types of honey from this area can be characterized as dark types of honey ($L^* < 50$) with the presence of a yellow color. The obtained results show that the analyzed samples of five different types of honey are rich in polyphenolic components and represent a good source of antioxidants in the human diet.

KEYWORDS: honey, physico-chemical parameters, color, antioxidant activity, total phenols

INTRODUCTION

The positive influence of honey on human health is attributed to its antibacterial, antiseptic, and antioxidant properties. The physical and chemical properties of honey depend on its botanical origin, climatic conditions, and the area where bees collect nectar and pollen [1], so we can say that no two honey samples are identical. The therapeutic potential of honey is also connected with its antioxidant capacity [2]. So far, more than 150 polyphenolic compounds in honey have been investigated, including phenolic acids and flavonoids. It has been noted that the content of polyphenols is significantly correlated with the color of honey, the content of mineral substances, and electrical conductivity, which indicates that dark-colored honey shows a higher content of phenolic compounds, which in turn indicates increased antioxidant activity [2,3]. Examinations of phenolic compounds content and flavonoids in honey have shown that there is a correlation with botanical and geographical origin on the one hand and antimicrobial activity on the other [4].

One of the important properties of honey is the color, which is primarily important for customer preference. According to research by Szabo et al. [5], by using the Minolta Chroma Meter, very detailed data on the color of honey can be obtained, and due to the stability of the circumstances, accurate and objective results can always be obtained. The most popular color distance is based on the CIELab method, where the values are L* (brightness), a* (degree of greenness/redness), and b* (degree of blueness/yellowness) [6,7]. This color system is practical because each color can be defined by a mixture of red, blue, and green. For some types of honey, chemical-physical analyzes do not provide enough characteristic values, therefore sensory analysis has become an indispensable process in assessing the quality of honey and defining the overall properties of honey [8].

The quality of honey from northwestern Bosnia and Herzegovina was confirmed in a study by Alibabić et al. [9]. However, there is a lack of data on the effect of different geographical and floral origins on the color, total phenolic content, and antioxidant activity of honey.

In this research, multivariate statistical techniques were used to evaluate the influence of botanical origin of honey, physicochemical properties and color intensity using principal component analysis (PCA).

Statistical analysis showed significant correlations between the antioxidant activity tested by the DPPH method and the total phenolic content. In addition, a sensory analysis of honey samples was performed, as an indispensable parameter in the assessment of honey quality.

MATERIAL AND METHODS

SAMPLES

In this research, a total of fifty honey samples were analyzed and classified into five groups: acacia honey (*Robinia pseudoacacia* L.), chestnut honey (*Castanea sativa* Mill.), linden honey (*Tilia* spp.), meadow honey, and forest honey. The analyzed samples were collected in 2019, and they are representative samples from the Second International Honey Evaluation in Cazin (2019), for samples from the area of Una-Sana Canton: ten samples each of chestnut, acacia, and forest honey, five samples of linden honey and fifteen samples of meadow honey. The honey samples were stored in a dark place in a glass container at +4 °C until analyses.

In order to determine the antioxidant activity, in addition to the mentioned honey samples, a sugar analogue solution was also prepared, so that the obtained values could be corrected. The sugar analogue, which consisted of 40% fructose, 30% glucose, 10% maltose and 20% water, was prepared by dissolving 4 g of fructose, 3 g of glucose, 1 g of maltose in 2 mL of distilled water using an ultrasonic bath. The sugar analogue sample was further analyzed in the same way as the honey samples.

CHEMICALS AND INSTRUMENTS

All used chemicals in this work were analitical grade: 5,5-Dithiobis(2-nitrobenzoic acid) DTNB, 2,4,6-Tris(2-pyridyl)-*s*-triazine TPTZ, glucose, fructose, sucrose, maltose (Sigma-Aldrich, GmbH, Steinheim, Germany); BHA (Butylated hydroxyanisole), BHT (Butylated hydroxytoluene), Sodium carbonate (Acros Organics, USA); Gallic acid (Carl Roth, GmbH); Folin-Ciocalteu's reagent (Darmstadt, Germany); ethanol 96%, HCl, $FeCl_3$ x $6H_2O$, $FeSO_4$ x $7H_2O$ (Kemika, Zagreb). The spectrophotometric measurements were performed on a photoLab 6600 UV-VIS spectrophotometer.
Colorimeter LCC-A11 (LABTRON, Japan) was used to determine the color parameters, equipped with a standard light source D65, the color characteristics are expressed in the CIE $L^*a^*b^*$ system.

PHYSICAL-CHEMICAL AND SENSORY ANALYSIS OF HONEY

The sensory analysis was carried out according Golob et al. 2008 [10].The honey botanical origin was determined by the beekeepers, and it was additionally confirmed by sensory analysis by 5 sensory assessors, who evaluated the appearance, smell, taste, and aroma of the honey samples. All honey samples were analyzed for water content and electrical conductivity as part of the *Second International Honey Evaluation*. Water content was determined using a refractometer at 20ºC, using official AOAC methods (1995) [11]. Electrical conductivity was measured according to the method proposed by the International Honey Commission (IHC) [12]. All samples were analyzed in triplicate.

INSTRUMENTAL DETERMINATION OF HONEY COLOR

The honey samples color was measured using a LABTRON colorimeter LCC-A11 (LABTRON, Japan). The samples were measured in Petri dishes (diameter 6.5 cm and height 1.5 cm) on a white background indicating the sum of the colors [13]. The resulting L* value refers to the brightness of the sample (0=black; 99=white), the a* value refers to the redness of the sample (in the +60 direction red, in the -60 direction green) and the b* value gives the yellowness of the sample (in the + 60 yellow, in the direction -60 blue).

ANALYSIS OF TOTAL PHENOLIC CONTENT

The total phenolic content was determined by the Folin-Ciocalte method, and the results were expressed in mg of gallic acid/kg of honey [3]. For the purposes of determining the total phenolics content in honey samples, individual samples were prepared as follows: 5 g of honey were weighed and dissolved in about 20 mL of water using an ultrasonic bath. The dissolved samples were quantitatively transferred into 50 mL volumetric flasks and topped up with distilled water up to the mark. A sample of the sugar analogue was prepared in the same way.

For the analysis, a volume of 100 μL of the sample was measured, to which 1 mL of FC reagent (diluted with distilled water in a ratio of 1:10) was added, after which the contents in the test tube were intensively mixed for 2 minutes. After holding the sample for 20 minutes at room temperature, the absorbance was measured at a wavelength of 750 nm. A sugar analogue was used as a control, which was analyzed in the same way. Measurements for each sample were made 3 times. The concentration of total phenols was read from the calibration curve of gallic acid, which was analyzed in the concentration range of 8 to 120

mg/L. The results are expressed as mg gallic acid/kg of honey.

DPPH METHOD

The antiradical activity of honey samples was determined according to the procedure of Brand-Williams et al. [14] with certain modifications made by Beretta et al. [15]. 800 μL of acetate buffer (100 mM, pH 5.5) and 1900 μL of DPPH reagent (130 μM DPPH radical solution in 96% ethanol) were added to 300 μL of honey solution (10%). A control sample (sugar analogue) was also prepared. Vitamin C, BHA and BHT (0.1%) were used as standards. Absolute alcohol was used to set the zero on the apparatus, and the absorbance of the DPPH reagent was also measured. For blank tests, acetate buffer was added instead of DPPH for each sample. The prepared samples were left in the dark for 60 minutes, after which the absorbance was read at 517 nm.

The ability of tested samples in radical scavenging was calculated using the following formula: % inhibition = $[(A_0 - A_{sample}) / A_0] \times 100$; A_0 – absorbance of the DPPH ethanol solution measured at the beginning at 517 nm; A_{sample} – absorbance of the sample measured after 60 minutes. Obtained results were expressed as a percentage of inhibition of DPPH radicals. For measurement, the spectrophotometer Perkin Elmer Lambda EZ 201 UV-VIS was used.

FRAP METHOD

The antioxidant impact of honey was described by Bertoncelj et al. [3]. The principle of this method is based on the reduction of the iron complex 2,4,6-tripyridyl-s-triazine (Fe^{3+}-TPTZ) into its colored form (Fe^{2+}-TPTZ) in the presence of antioxidants. The FRAP reagent contained 2.5 ml of a 10 mM solution of TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl, 2.5 ml of 20 mM $FeCl_3 \times 6H_2O$ and 25 ml of 0.3 M acetate buffer (300 mM, pH 3.6). The FRAP reagent was prepared immediately before use and was kept in a water bath at t 37 °C during the analysis. Aliquots of 200 μL of the sample were mixed with 1.8 ml of FRAP reagent and the absorbance of the reaction mixture was measured spectrophotometrically at 593 nm after incubation at 37°C for 10 minutes against the sugar analogue. Aqueous standard solutions of $FeSO_4 \times 7H_2O$ (0.1 to 1 mM) were used for the calibration curve, and the results were expressed as FRAP value μM Fe(II) of 10% honey solution.

STATISTICAL ANALYSIS OF THE RESULTS

The results of the analyzed samples are presented as mean value ± standard deviation. One-way analysis of variance (ANOVA) and multiple comparisons (Duncan's Post Hoc Test) were used to assess the significant difference in data at the $p<0.05$ significance level. Statistics were implemented using Microsoft Office 2014 and the demo version of the statistical package MS Office XLSTAT-Pro 2014 [16]. A principal component analysis (PCA) was also performed. Measurements for all methods used in this research were done in three repetitions for each sample.

RESULTS AND DISCUSSION

PHYSICO-CHEMICAL PARAMETERS AND SENSORY ASSESSMENT

Table 1. Physico-chemical parameters and sensory assessment of analyzed honey samples

Parameter	Statistics	Honey type				
		Acacia (n=10)	Linden (n=5)	Chestnut (n=10)	Forest (n=10)	Meadow (n=15)
Water content (%)	Mean ± SD	(16.08 ±0.98)[a]	(16.36±0.45)[a]	(15.94±1.04)[a]	(16.17±0.85)[a]	(16.67±0.69)[a]
	Range	15.10-17.06	15.90-16.80	14.89-16.98	15.31-17.02	15.98-17.36
Electrical conductivity (mS/cm)	Mean ± SD	(0.19±0.07)[e]	(0.68±0.09)[c]	(1.46±0.04)[a]	(1.06±0.02)[b]	(0.54±0.06)[d]
	Range	0.12-0.25	0.59-0.77	1.42-1.49	1.04-1.07	0.48-0.59
Sensory evaluation	Mean ± SD	(28.55±3.05)[a]	(28.02±2.45)[a]	(28.08±2.49)[a]	(27.97±1.04)[a]	(27.39±0.60)[a]
	Range	25.50-31.60	25.58-30.47	25.59-30.56	26.94-29.01	26.79-27.99

[a,b,c,d,e] – Mean values in the same row marked with different letters are statistically significantly different according to Duncan's test ($p<0.05$).

The values of physico-chemical parameters and sensory evaluations of all honey samples are given in Table 1. In general, the values for water content and electrical conductivity for all tested honey samples were within the established legal requirements [17]. The honey samples from this study did not show

statistically significant differences ($p<0.05$) in terms of water content in the examined types of honey (Table 1). Electrical conductivity values ranged from 0.19 (mS/cm) in acacia honey to 1.46 (mS/cm) in chestnut honey and statistically significant differences ($p<0.05$) were found between all honey samples.

Honey sensory analysis evaluated the appearance of the sample (purity, color, smell, taste, and aroma), which also enabled the identification of the honey's botanical origin. The values for all types of honey ranged from 25.00 to 31.60 points, while the value for acacia honey was the highest and amounted to 32.4 points. Sensory analysis of honey showed that there are no statistically significant differences between different types of honey (Table 1).

HONEY COLOR INSTRUMENTAL ANALYSIS

The color characteristics are shown in Table 2, where the mean values, standard deviations, and range of parameters L*, a*, b* are shown. The samples of acacia and linden honey have the highest average L* values, 41.78 and 39.25 (Table 2). The color of the analyzed honey determined with a colorimeter showed that there are statistically significant differences between the measured values, among the samples.

Table 2. Color characteristics of the analyzed types of honey

Parameter	Statistics	Honey type				
		Acacia (n=10)	Linden (n=5)	Chestnut (n=10)	Forest (n=10)	Meadow (n=15)
L*	Mean ± SD	(41.78±1,26)^{a}	(39.25±1.95)^{a,b}	(34.22±2.00)^{c,d}	(31.57±3.07)^{d}	(35.69±2.00)^{b,c}
	Range	40.51-43,03	37.30-41.20	32.22-36.22	28.13-34.03	33.70-37.70
a*	Mean ± SD	(-5.90±1.15)^{b}	(-4.60±1.43)^{a,b}	(-2.03±0.15)^{a}	(-1.99±0.24)^{a}	(-1.47±3.27)^{a}
	Range	-7.10 to -4.80	-35.98 to 3.12	-2.20 to -1.90	-2.23 to -1.75	-4.75 to 1.80
b*	Mean ± SD	(11.48±4.50)^{a}	(13.04±1.96)^{a}	(10.63±3.99)^{a}	(4.96±0.97)^{b}	(12.32±1.91)^{a}
	Range	7.01-16.01	11.08-15.01	6.64-14.62	3.98-5.93	10.42-14.23

^{a,b,c,d} – Mean values in the same row marked with different letters are statistically significantly different according to Duncan's test ($p<0.05$).

The obtained values of L∗ for monofloral and multifloral types of honey are lower than the values obtained in the research of Szabó et al. [5] and Bertocelj et al. [3] for the same Hungarian and Slovenian types of honey, while the values are similar to those in the study by Flanjak et al. [18] for chestnut and linden honey and honeydew from Croatia. All types of honey from the area of the Una-Sana Canton can be characterized as dark types of honey based on the obtained value of L (L∗<50). Smetanska et al. [19] reported a positive correlation between the honey color and the content of phenols, flavonoids, and carotenoids. In all the analyzed samples, except for one meadow honey sample that had a positive a∗ value, negative a∗ values were obtained, which indicates a green shade in all analyzed types of honey. In the research by Flanjak et al. [18] the negative a∗ value is explained by the greater presence of chlorophyll in the nectar. The values of the parameter b∗ in the tested types of honey ranged in a positive range from 4.96 for honeydew to 13.04 for linden honey, which indicates the presence of a yellow color in all samples that depends on the presence of carotenoids and flavonoids in honey [18]. The color of each honey is the result of the presence of pigments such as flavonoids and carotenoids, which depends on the botanical and geographical origin of the product. Storage conditions can also affect color intensity. The color of honey also depends on the content of water, pollen, and saccharides [20].

TOTAL PHENOLS AND ANTIOXIDANT CAPACITY

The results of the content of total phenolics and antioxidant activity in samples of different types of honey are shown in Table 3. Samples of different types of honey were analyzed to evaluate their antioxidant activity and to find a correlation between antioxidant activity and the content of total phenolics. The obtained results showed (Table 3) that all the analyzed samples were antioxidant active, and that the total phenolics content and antioxidant activity varies significantly among the types of honey.

The total phenolics content in honey samples ranged between 264.89-406.02 mg of gallic acid/kg of honey for chestnut honey, 210.44-353.78 mg of gallic acid/kg of honey for forest honey, meadow honey 192.67-354.89 mg of gallic acid/kg of honey, linden honey 137.11-220.44 mg of gallic acid/kg of honey and acacia honey 120.44-189.33 mg of gallic acid/kg of honey . Analysis of variance revealed that there were statistically significant differences in the total phenolics content between different types of honey (Table 3). The total phenolics content in chestnut, forest, and meadow honey was significantly higher than in other types of honey. In general, our chestnut honey samples show a higher content of total phenols

compared to Slovenian chestnut honey 199.9 mg of gallic acid/kg of honey [3] and Croatian chestnut honey 162.1 mg of gallic acid/kg of honey [18]. The obtained values for forest honey were similar to the values for forest honey from Slovenia [3] and lower than the total phenolics content for forest honey from Croatia and Serbia [18], [20]. The total phenolics content in meadow honey was higher compared to the research conducted on honey by Bertoncelj et al. [3], Beretta et al. [15], and Srećković et al. [21], except for polyfloral honey from Poland, where the obtained values were higher [2]. Also, the total phenolics content in the samples of acacia honey was higher compared to several recent studies [3], [15], [18] and [21]. The high content of phenol in the tested types of honey from the Una-Sana Canton area confirmed the good quality of the honey. Certain variations in the phenolic content of our honey samples compared to those from different countries may be due to different geographical and botanical origins of honey.

Table 3. Total phenol content and antioxidant activity of honey samples

Parameter	Statistics	Honey type				
		Acacia (n=10)	Linden (n=5)	Chestnut (n=10)	Forest (n=10)	Meadow (n=15)
Total phenols mg of Gallic acid/kg of honey	Mean ± SD	(142.29± 40.77)^{c}	(166.37± 46.88)^{b,c}	(343.03± 71.77)^{a}	(282.67± 71.67)^{a,b}	(261.96± 83.67)^{a,b,c}
	Range	120.44-189.33	137.11-220.44	264.89-406.02	210.44-353.78	192.67-354.89
FRAP µM Fe(II) of 10% solution	Mean ± SD	(304.89± 95.85)^{b}	(488.67± 113.26)^{a,b}	(551.89± 313.96)^{a,b}	(874.11± 353.3)^{a}	(338.67± 94.39)^{b}
	Range	209.04-400.74	375.41-601.92	237.93-865.85	520.81-1227.41	244.27-433.06
DPPH % inhibition of 10% solution	Mean ± SD	(12.97± 3.67)^{c}	(35.12± 7.47)^{b}	(48.38± 1.07)^{a}	(59.15± 8.80)^{a}	(49.61± 5.95)^{a}
	Range	9.29-16.65	27.65-42.59	47.31-49.46	50.35-67.96	43.66-55.56

^{a,b,c} – Mean values in the same row marked with different letters are statistically significantly different according to Duncan's test (p<0.05).

DPPH AND FRAP

DPPH and FRAP methods were chosen to measure antioxidants in honey because they are simple, precise, and accurate methods. The obtained values for the tested samples of honey from the Una-Sana Canton area using the DPPH method showed inhibition of DPPH radicals in the range of 59.15% for forest honey and 12.97% for acacia honey (Table 3). The inhibition ability decreased in the following order: forest > meadow > chestnut > linden > acacia. This order differed slightly from the results obtained for the FRAP method. The results for forest honey, chestnut honey, and linden honey showed that they differ statistically significantly compared to honey from linden and acacia (Table 3). Several types of compounds may contribute to the antioxidants in honey, including carotenoids, ascorbic acid, tocopherols, and polyphenolic compounds. The obtained values for the ability of the reduction potential of five types of honey from the Una-Sana Canton area ranged from 874.11 ± 353.3 µM Fe(II) of 10% honey solution for forest honey to 304.89 ± 95.85 µM Fe(II) of 10% honey solution for acacia honey. The reduction potential decreased as follows: forest > chestnut > linden > meadow > acacia, which is in accordance with the results for different types of honey from Slovenia [3]. Analysis of variance revealed that there were statistically significant differences (Duncan test, p<0.05) when it comes to the antioxidant activity of the samples obtained by the FRAP method. As can be seen from the following literature data, the values obtained for honey from the Una-Sana Canton area were higher than those obtained by the same method for honey from the areas of Nigeria, Malaysia, Italy, Algeria, Slovenia, and Croatia.

CORRELATION OF COLOR, TOTAL PHENOLS, AND ANTIOXIDANTS

The correlation established between color, total phenols, and antioxidant activity is presented in Fig. 1 and Fig. 2, in order to investigate possible relationships between these values. There was a

positive correlation between color and total phenols (0.76) and total phenols and antioxidant activity (0.67).

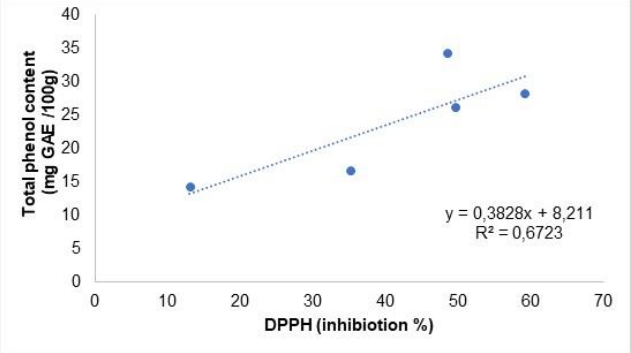

Figure 1. Correlation between total phenol content and total antiradical activity (DPPH) of honey samples

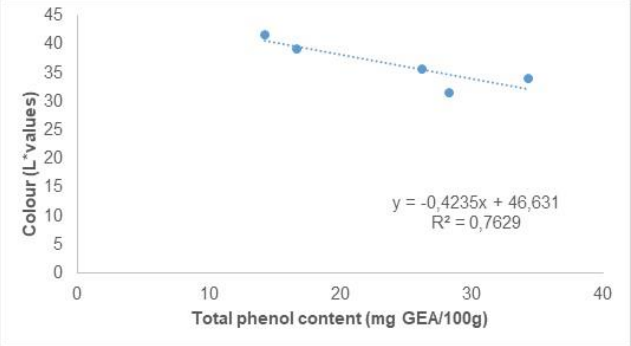

Figure 2. Correlation between total phenolic content and color (L*value) of honey samples

The positive correlation between color and total phenols (0.76) indicates that darker types of honey can be attributed to the presence of a greater amount of flavonoids and phenols that increase the number of antioxidants. For example, the average L* color value was the lowest in forest and chestnut honey (31.57 and 34.22), which indicates that it is dark honey, and has a higher content of total phenolics (forest 282.67 mg of gallic acid/kg of honey and chestnuts; 343.04 mg of gallic acid/kg of honey) and higher antioxidant activity (551.89 and 874.11 μM Fe(II) of 10% honey solution).

PRINCIPAL COMPONENT ANALYSIS

Variances Analysis of physico-chemical parameters and sensory evaluation showed that there are statistically significant differences between the tested types of honey. Principal component analysis (PCA) was performed with the aim of studying the interrelationship between different variables, which in this case are physico-chemical parameters and sensory evaluation. PCA was performed on the results obtained with different types of honey from the Una-Sana Canton area. The first principal component (PC1) included 60.76% of the total data variability, and the second principal component (PC2) was 26.18%. The value of PCA, i.e. their mutual projections for the first two components are presented in Fig. 3.

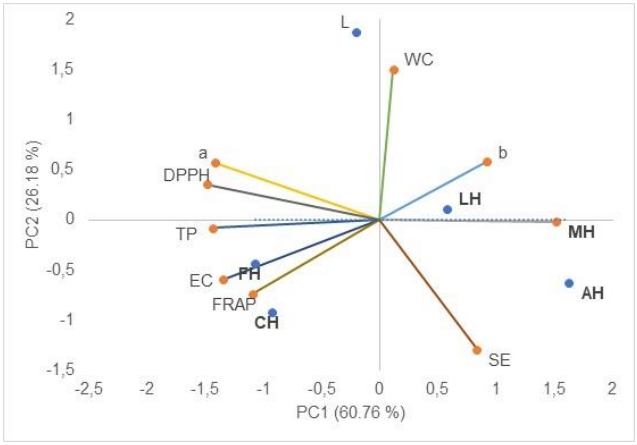

Figure 3. Principal component analysis (PCA) of the chemical composition and sensory analysis of honey samples
FH-Forest honey; CH-Chestnut honey; LH- Linden honey; MH- Meadow honey; AH Acacia honey. TP-Total phenols; EC-Electrical conductivity; WC-Water content; SE-Sensory evaluation.

According to the obtained honey samples results with the first factor PC1, the physico-chemical parameters that correlate best are the amounts of DPPH, TP, EC, FRAP, L* (forest and chestnut honey). According to PC2, the physico-chemical parameters that are positively correlated are the results of the sensory evaluation and the composition of the water (acacia and linden).

CONCLUSIONS

These are the first studies that classify the properties of color, total phenolics, and antioxidant activity of honey from the area of northwestern Bosnia and Herzegovina. The mentioned parameters varied in different types of honey: among the examined samples, forest and chestnut honey were the richest in color, total phenols, and antioxidant activity. Compared to honey from other countries, the analyzed honey from northwestern Bosnia was rich in total phenols and showed noticeable antioxidant activity, which is an important feature that should be taken into account when it comes to human health. Based on the obtained results of the physico-chemical parameters of different types of honey, the justification for using honey in human nutrition as a functional product with a high content of bioactive components, mineral elements, and significant nutritional and sensory

properties can be explained. In further research, it would be desirable to identify and quantify individual flavonoids and phenolic acids, the compounds that contribute the most to the antioxidant activity of honey.

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