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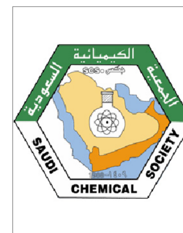

ORIGINAL ARTICLE

Phenolic and free amino acid profiles of bee bread and bee pollen with the same botanical origin – similarities and differences

Nesrin Ecem Bayram [a], Yusuf Can Gercek [b,c,*], Saffet Çelik [d], Nazlı Mayda [e], Aleksandar Ž. Kostić [f,*], Aleksandra M. Dramićanin [g], Aslı Özkök [e,h]

[a] *Department of Food Processing, Aydıntepe Vocational College, Bayburt University, Bayburt, Turkey*
[b] *Department of Biology, Faculty of Science, Istanbul University, Istanbul, Turkey*
[c] *Centre for Plant and Herbal Products Research-Development, Istanbul, Turkey*
[d] *Technology Research and Development Application and Research Center, Trakya University, Edirne, Turkey*
[e] *Department of Biology, Faculty of Sciences, Hacettepe University, Ankara, Turkey*
[f] *University of Belgrade, Faculty of Agriculture, Chair of Chemistry and Biochemistry, Nemanjina 6, 11080 Belgrade, Serbia*
[g] *University of Belgrade, Faculty of Chemistry, Chair of Analytical Chemistry, Studentski Trg 12-16, 11000 Belgrade, Serbia*
[h] *Hacettepe University, Bee and Bee Products Application and Research Center, Ankara, Turkey*

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Abstract In this study, the chemical profile of bee pollen (BP) and bee bread (BB) samples collected from the same beehive were analyzed by LC–MS/MS (liquid chromatography technique coupled with tandem mass spectrometry), providing the identification of 23 phenolic compounds and 42 free amino acids (FAAs). Rutin was the phenolic compound with the highest rate of occurrence in both BP and BB samples. However, concentrations of protocatechuic acid, 2,5-dihydroxybenzoic acid and kaempferol compounds were significantly higher in BB samples than in BP samples from the same hive probably as result of microbial activity and glycosides degradation. The obtained data revealed that the phenolic profiles of the samples differ not only by the type of a product but also by region. Among FAAs proline was the predominant compound in all the analyzed BP and BB samples followed by L-asparagine (BP samples) and L-aspartic acid (BP and BB samples). A high content of proline can be used as a parameter of sample freshness. Also, Principal Component Analysis (PCA) and Cluster analysis proved the possibility of using phlorizin as a chemotaxonomic marker for Rosaceae (*Malus* or *Prunus* genus) pollen presence in BP1 sample. In addition,

* Corresponding authors at: Department of Biology, Faculty of Science, Istanbul University, Istanbul, Turkey (Y.C. Gercek), University of Belgrade, Faculty of Agriculture, Chair of Chemistry and Biochemistry (A.Ž. Kostić).
E-mail addresses: yusuf.gercek@istanbul.edu.tr (Y.C. Gercek), akostic@agrif.bg.ac.rs (A.Ž. Kostić).
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amino acid profile had higher impact on BP and BB sample differentiation due to lower FAAs content in BB samples probably caused by microbial activity. To the best of our knowledge, this study is the first to compare the individual phenolic compounds and free amino acids of bee pollen and bee bread samples with the same botanical origin (predominantly originated from plants belonging to the following families: Asteraceae, Fabaceae, Plantaginaceae and Rosaceae).

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1. Introduction

Bee pollen (BP) containing the plant's male gametophytes is located in the anthers of flowering plants. When bees visit flowers, their bodies are covered with pollen powder (Bogdanov, 2011). This powder is transported back to hive in a specialized pollen basket on their hind legs (Krell, 1996). After the foragers collect the pollen, it is packed into the cells of the brood comb by bees and a small amount of honey is added to the pollen to forestall spoilage and maintain its quality. Stored pollen, which has been exposed to chemical processes and changes, is called bee bread (BB) (Gilliam et al., 1989; Bogdanov, 2011). During this storage period, it is believed that a two-week natural lactic acid fermentation process occurs, caused by the intervention of different microorganisms (Vasquez and Olofsson, 2009).

An adequate supply of pollen is obligatory to continue productivity of a colony and to ensure its long-term survival (Bogdanov, 2011). Pollen is the bees' principal source of crucial nutrients such as proteins (10–40 g/100 g dry weight), lipids (1–13 g/100 g dry weight), total carbohydrates (13–55 g /100 g dry weight), dietary fiber and pectin (0,3–20 g/100 g dry weight), ash (2–6 g/100 g dry weight), minerals (Fe, Mg, Cu, Mn, Ca, K, P, Zn) and vitamins (β-carotene, B1, B2, B3, B5, B6, C, biotin, folic acid, tocopherol) (Kieliszek et al., 2018). Moreover, BP is consumed as a dietary supplement by humans and it is recognized as an excellent functional food ingredient (Kostić et al., 2020). Pollen contains a high percentage of phenolics (especially flavonoids and phenolic acids). The amount of flavonoids varies between 0.2 and 2.5% in pollen (Kieliszek et al., 2018). These compounds affect the bioactive characteristics (such as antimicrobial, anti-radiation, antioxidant, antifungal, hepatoprotective, chemoprotective, and/or anti-inflammatory effects) of pollen, as well as physicochemical properties such as color, taste and odor (Gibriel et al., 2016; Kieliszek et al., 2018). Compared to pollen BB has higher nutritional value mostly due to higher bioavailability of nutrients caused by activity of lactic acid bacteria presented in bee's digestive system (Vasquez and Olofsson, 2009). It is also characterized by significantly lower quantity of starch i.e. higher quantity of vitamin K (Vasquez and Olofsson, 2009). Free amino acids are also one of the most important components in both BP and BB (Kieliszek et al., 2018) where, along with 20 usual protein amino acids, a significant quantity of non-protein amino acids also can be found. Free amino acids (FAAs) are important as part of nectar taste (Nicolson and Thornburg, 2007) as an attractant to pollinators (especially bees) i.e. repellent to herbivores so it is possible to influence the pleasant smell of pollen also. In many studies, it was revealed that the individual composition, as well as the antioxidant activity of pollen samples collected from different locations were different. The chemical contexture of bee pollen depends on factors such as botanical and genetical sources, soil type, beekeeper activities, and climatic conditions (Pascoal et al., 2014; Kostić et al., 2019). In case of bee bread, pollen fermentation processes as well as presence of nectar determine the final composition (Vasquez and Olofsson, 2009; Malihah Mohammad et al., 2020).

Moreover, it has been reported that changes to bee pollen during storage do not ensure obvious benefits for honeybees (Nicolson et al., 2018). Its bioactive characteristics such as antibacterial, antioxidant and antitumor, as well as its flavonoid content, have been correlated with the floral origin (Sobral et al., 2017). BB has been reported to have antimicrobial, anti-atherosclerotic, anti-aging and anti-proliferative properties and can act as a liver protector (Kieliszek et al., 2018).

Although there are chemically important differences between the first product (BP) and the final one (BB) in the literature which are consistently reported, there is not enough comparative data on this topic especially regarding the samples with the same origin. There are very few scientific studies that reveal the phenolic and amino acid composition of bee bread. Therefore, in this study, we evaluated the phenolic and free amino acid profiles of bee pollen and bee bread samples (obtained from the same hives), both qualitatively and quantitatively. For this LC MS-MS analysis of BP and BB was performed followed by PCA and cluster statistical analysis in order to determine if the same botanical origin affects sample differentiation.

2. 2. Experimental

2.1. Reagents

All the chemical solvents and standards were of analytical grade. The standards used in the study were obtained from Sigma-Aldrich and Cayman Chemical (USA), methanol, acetonitrile, acetic acid and formic acid from Merck (Darmstadt, Germany). Standard stock solutions were prepared in methanol and diluted with extraction solvent (water, methanol, formic acid, v:v:v, 79:20:1) and were stored at −20 °C.

2.2. Collection of bee pollen and bee bread

Fresh bee pollen (BP) and bee bread (BB) samples were collected from five different beehives in Kırklareli/Çağlayık (BP1 and BB1), Bursa/Cumalıkızık (BP2 and BB2), Ankara/Beytepe (BP3 and BB3), Ankara/Kahramankazan (BP4 and BB4) and Rize/Hala (BP5 and BB5) localities in Turkey (Fig. 1). The details about sample collections, plant sources, total phenolic-flavonoid content, antioxidant activity, fatty

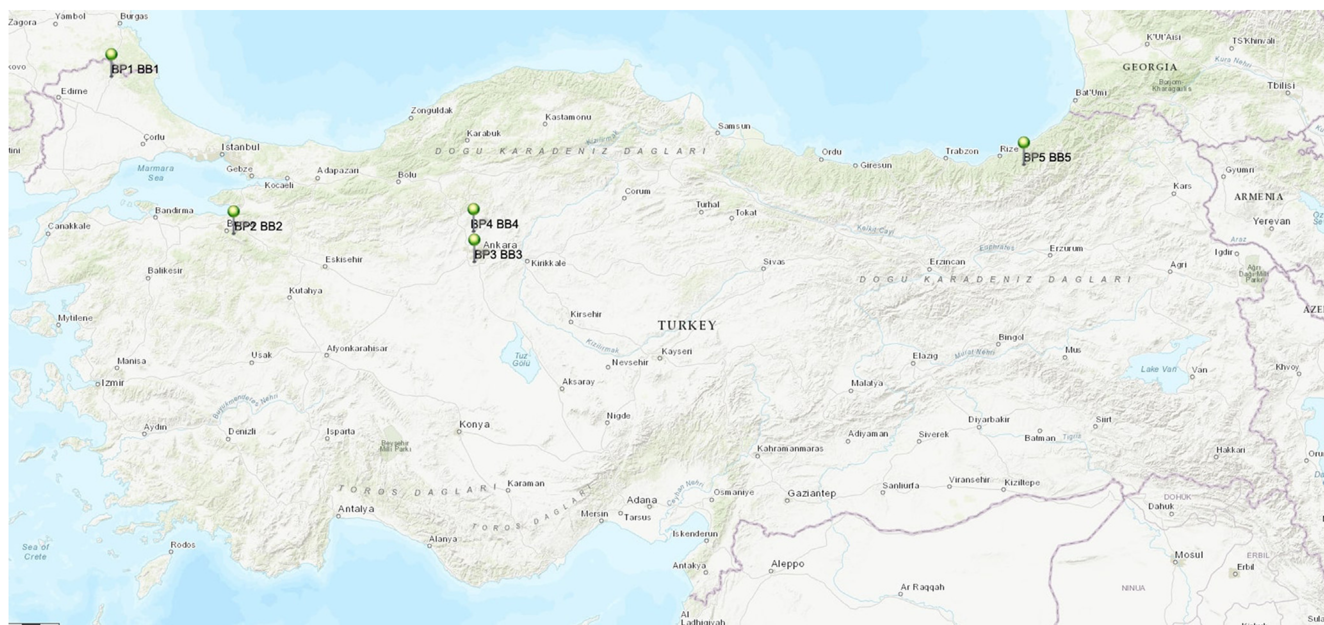

Fig. 1 Location map of sample sites.

acid and element profiles of these samples are given in the previously published article (Mayda et al., 2020).

2.3. Analysis of phenolic compounds of bee pollen and bee bread samples

2.3.1. Preparation of extracts

Extracts were prepared according to Zhou et al. (2015) with some modification. The BP/BB samples were pulverized using a grinder. After that, 1.5 g sample was dissolved in 10 mL ethanol (95%) followed by ultrasonic assisted extraction in an ultrasonic cleaning bath for 60 min at 40 °C. This mixture was centrifuged at 5000 rpm for 30 min at +4 °C and the supernatant was collected into a volumetric flask. Extraction procedure was repeated twice.

5 mL ethanol was added to the sample again and ultrasonic was performed at 40 °C for 30 min and centrifuged. Finally, the supernatants were combined into a 25 mL volumetric flask and the volume was made up to the mark with ethanol (95%). 100 μL of sample was mixed with 900 μL extraction solution (water, methanol, formic acid: v:v:v, 79:20:1), and samples were vortexed for 30 s. After that, the mixture was homogenized using sonicator at 45 °C 10 min. Samples were centrifuged at 13,500 rpm for 5 min and supernatant injected into the LC–MS/MS system for quantitative analysis.

2.3.2. Calibration curve and quantification in liquid chromatography-tandem mass spectrometry (LC–MS/MS)

LC was performed using an Agilent 6460 (Agilent Technologies, Waldbronn, Germany) LC system. Ion pairs are presented in Table 1**.** Data acquisition and processing were accomplished using MassHunter, the Agilent LC-MS software (Fischer et al., 2011; Ecem Bayram et al., 2020). All parameters are presented in the **Table S1.** The concentration of phenolic acids in each sample was calculated using the calibration curve prepared on the same day and analyzed in the same analytical run. All calibration curves were prepared with the following concentrations: blank (water, methanol, formic acid: v:v:v, 79:20:1), 5, 10, 25, 50, 100 ng/mL and injected all points three times. The linearity of all the phenolic acids was $R^2 \geq 0{,}995$. These samples were analyzed according to the procedure described for sample preparation. LOD and LOQ values of the phenolic acids (calculated over S/N ratio) are presented in Table 1**.**

2.3.3. Method specifications

There are several important things to note when using this analytical method. All phenolic acids were analyzed to a sensitivity of 1 ng/mL and some of the isomer acids were separated based on chromatography conditions. Three pairs of isomers were separated, qualified and quantified via this method.

2.4. Amino acid analysis by liquid chromatography-tandem mass spectrometry (LC–MS/MS)

Amino acid analysis was performed by using an LC system (Agilent Technologies, Waldbronn, Germany). MS/MS analyses were conducted on an Agilent 6460 triple quadruple LC-MS equipped with an electrospray ionization interface. 1 g sample was taken into falcon and added 10 mL extra-pure water. The solution was vortexed for 1 min and sonicated for 15 min at 45 °C. BB and BP samples were centrifuged for 5 min at 13,500 rpm. Then, 50 μL clear supernatant was mixed with 50 μL internal standard and 900 μL extraction solution (mobile phase A, methanol, acetonitrile: v:v:v, 5:15:15), and the sample was injected to LC-MSMS system. All the details of the method (calibration curve and quantification) applied for amino acid analysis are given in previous research (Çelik et al., 2020). All parameters are presented in the **Table S1.**

Table 1 The calibration parameters of phenolic compound standards.

Compound	RT (Retention time, min.)	RSD % (RT)	$[M-H]^-$ *m/z*	Ion pair	R^2 (Linearity)	LOD (ng/mL)	LOQ (ng/mL)
2,5-Dihydroxybenzoic Acid	2.176	0.482	152.9	152.9/107.9; 152.9/53.1	0.9985	0.14	0.47
2-Hydroxycinnamic Acid	4.158	0.477	162.9	162.9/119; 162.9/92.8	0.9968	0.48	1.58
Caffeic Acid	3.750	0.488	179	179/135.1; 179/117.3	0.9974	0.05	0.17
Catechin + Epicatechin	3.890	1.155	288.9	288.9/245; 288.9/205	0.9953	0.22	0.75
Chlorogenic Acid	3.737	0.488	352.9	352.9/191; 352.9/82	0.9969	0.09	0.29
Ethyl gallate	4.114	0.492	197	197/169; 197/124	0.9984	0.09	0.28
Gallic Acid	1.702	0.482	168.9	168.9/125; 168.9/78.8	0.9993	0.31	1.02
Isorhamnetin	4.459	0.489	314.9	314.9/299.9; 314.9/151	0.9990	0.06	0.18
Kaempferol	4.443	0.956	284.9	284.9/226.9; 284.9/93	0.9969	0.31	1.08
Luteolin	4.351	0.981	284.9	284.9/150.9; 284.9/133	0.9977	0.04	0.14
Myricetin	4.204	0.058	317	317/178.8; 317/150.9	0.9966	0.05	0.18
Naringin	4.041	0.491	579.1	579.1/458.9; 579.1/271	0.9975	0.07	0.24
p-Coumaric Acid	4.022	0.551	163.1	163.1/118.9; 163.1/93	0.9984	0.62	2.05
Phlorizin	4.127	0.540	434.1	434.8/272.9; 434.8/167	0.9984	0.02	0.08
Propyl gallate	4.250	0.539	211	211/124.1; 211/78	0.9972	0.07	0.24
Protocatechuic Acid	1.831	0.482	153.1	153.1/109.1; 153.1/90.8	0.9990	0.04	0.15
Quercetin	4.316	0.473	301	301/178.9; 301/150.9	0.9974	1.01	3.35
Resveratrol	4.215	0.491	226.9	226.9/184.9; 226.9/142.8	0.9977	0.27	0.89
Rutin	3.964	0.494	609	609/299.9; 609/270.9	0.9964	0.04	0.13
Salicylic Acid	3.784	0.577	136.8	136.8/93.1; 136.8/65	0.9967	0.08	0.26
Sinapic Acid	4.054	0.417	222.9	222.9/208; 222.9/120.9	0.9956	0.91	2.97
Syringic Acid	3.774	0.489	196.9	196.9/182.1; 196.9/121.1	0.9979	1.01	3.34
Trans Ferulic Acid	4.083	0.442	193	193/177.9: 193/134.1	0.9975	0.21	0.64

2.5. Statistical analysis

The results of phenolic and amino acid analysis were expressed as a mean ± standard deviation (SD) of the mean of replications for each analyzed extract. Principal component (PCA) and hierarchical cluster (HCA) analyses were performed in the software package PLS ToolBox, v.6.2.1 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 of hierarchical cluster analysis (HCA) are presented as a dendrogram where steps in the hierarchical clustering solution and values of the distances between clusters (Euclidean distance) are represented. The statistical analysis was performed with Minitab 17 statistics program. The statistical significance of the results was determined by using a one-way ANOVA analysis then the ranking of significance was determined using the Tukey post-hoc test. The results were given as means ± SD. Significance levels were defined $p \leq 0.05$.

3. Results and discussion

3.1. Phenolics profile

The phenolic compounds of BP and BB were detected by LC-MS/MS using twenty-three phenolic standards. In LC-MS/MS analysis**,** the calibration parameters of standards are listed in Table 1. Of the 23 phenolic compounds investigated (Table 2) 18 were quantified in the samples. The ratio of the following compounds was higher in BB samples than BP samples from at least three regions - protocatechuic acid (nd-166.61 μg/100 g), *p*-coumaric acid (28.70–142.44 μg/100 g), quercetin (381.1 0–3918.12 μg/100 g), 2,5-dihydroxybenzoic acid (2.69–35.09 μ g/100 g), kaempferol (112.94–2681.20 μg/100 g), gallic acid (34.65–347.37 μg/100 g), chlorogenic acid (3.89–36.09 μg/100 g), salicylic acid (19.27–65.20 μg/100 g), luteolin (21.91–3490. 83 μg/100 g) and isorhamnetin (nd-1227.93 μg/100 g). In contrast, the concentrations of caffeic acid (in samples from three regions), rutin (in samples from three regions), ethyl gallate (in samples from four regions), *trans*-ferulic acid (in samples from three regions) and myricetin (in samples from three regions) were higher in BP samples. The observed differences and diminished quantity of some polyphenols like ethyl gallate in BB can be provoked with bacterial digestion in BB. Namely, bacteria can hydrolyze esters and glycosides to aglycone forms (Viskupičova et al., 2008; Tarko et al., 2013).

Some compounds were detected in all the samples under study, whereas other compounds were specific to a particular sample type (BP or BB) from any region. For example, gallic acid, 2,5-dihydroxybenzoic acid, protocatechuic acid, caffeic acid, salicylic acid, chlorogenic acid, catechin, rutin, *p*-coumaric acid, ethyl gallate, *trans*-ferulic acid, myricetin, luteolin, quercetin, isorhamnetin, and kaempferol were present in varying concentrations in all the BP and BB samples while syringic acid, 2-hydroxy-*trans*-cinnamic acid, naringin, sinapic acid and propyl gallate were not detected in any BB and BP samples. In terms of concentration, rutin (1225.54–12613.49 μ g/100 g) was the main component in BP and BB samples from all the regions compared to other compounds. According to the literature data rutin is the most common glycoside observed in pollen samples (Rzepecka-Stojko et al., 2015). However, despite the fact that rutin is glycoside it remains the main BB component due to its high content as well as the presence of sugar moiety that is different from glucose (Tarko et al., 2013). Namely, rutin's saccharide component is actually disaccharide rutinose instead of glucose or some other

Table 2 Phenolic composition of bee pollen and bee bread extracts (μg/100 g).

COMPOUNDS	BP1	BB1	BP2	BB2	BP3	BB3	BP4	BB4	BP5	BB5
2,5-dihydroxybenzoic acid	9.84 ± 0.29^{ijD}	35.09 ± 0.70^{fghA}	6.68 ± 0.40^{ijE}	12.00 ± 0.84^{hC}	2.69 ± 0.13^{eF}	8.73 ± 0.70^{fD}	9.85 ± 0.99^{ghD}	10.14 ± 0.20^{fD}	3.57 ± 0.29^{ghiF}	21.61 ± 0.65^{fgB}
2-hydroxytranscinnamic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Caffeic acid	40.19 ± 0.40^{giC}	17.86 ± 0.18^{hEF}	56.17 ± 1.69^{ghiB}	102.09 ± 5.10^{efghA}	26.46 ± 0.79^{eD}	23.08 ± 1.15^{fDE}	23.22 ± 1.86^{ghDE}	11.49 ± 1.15^{fG}	12.46 ± 0.25^{ghiFG}	21.82 ± 1.09^{fgDE}
Catechin	nd	nd	nd	nd	nd	nd	nd	nd	76.73 ± 3.07^{ef}	nd
Chlorogenic acid	36.09 ± 1.08^{hijA}	3.89 ± 0.08^{hD}	4.37 ± 0.26^{ijD}	23.31 ± 1.63^{hB}	10.77 ± 0.54^{eCD}	7.75 ± 0.62^{fD}	8.40 ± 0.84^{hD}	16.82 ± 0.34^{fBC}	8.63 ± 0.69^{ghiD}	19.18 ± 0.58^{fgB}
Ethyl gallate	3.14 ± 0.06^{ijA}	0.71 ± 0.02^{hBC}	2.89 ± 0.14^{jA}	0.24 ± 0.01^{hDE}	0.59 ± 0.02^{eBCD}	0.10 ± 0.01^{fE}	0.78 ± 0.07^{hB}	0.18 ± 0.01^{fE}	0.04 ± 0.00^{iE}	0.37 ± 0.01^{gCDE}
Gallic acid	155.29 ± 1.38^{eC}	329.92 ± 3.30 ^{dB}	157.84 ± 4.74^{fC}	65.73 ± 3.29^{fghD}	35.31 ± 1.06^{eE}	34.65 ± 1.73^{fE}	39.24 ± 3.14^{fghE}	56.43 ± 5.64^{fD}	53.83 ± 1.08^{fgD}	347.37 ± 17.37^{cA}
İsorhamnetin	337.76 ± 6.75^{cE}	310.44 ± 9.31^{dE}	274.65 ± 13.73^{eF}	820.09 ± 49.21 ^{dB}	238.42 ± 9.54^{dG}	484.12 ± 29.05^{eD}	249.80 ± 22.48^{eFG}	1227.93 ± 98.23^{dA}	775.16 ± 31.01^{bC}	nd
Kaempferol	309.11 ± 2.99^{cFG}	380.02 ± 3.80^{dFG}	768.04 ± 23.04^{dE}	1589.37 ± 79.47^{cB}	279.94 ± 8.40^{dGH}	1216.04 ± 60.80^{cC}	476.07 ± 38.09^{cF}	2681.20 ± 160.12^{bA}	112.94 ± 2.26^{deH}	1020.18 ± 51.01^{bD}
Luteolin	21.91 ± 0.21^{hijI}	121.29 ± 1.21^{efgH}	3325.73 ± 99.77^{cB}	217.01 ± 10.85^{eG}	797.76 ± 23.93^{bE}	3490.83 ± 174.54^{bA}	1225.84 ± 98.07^{bC}	393.38 ± 39.34^{eF}	52.79 ± 1.0^{fghI}	1019.15 ± 50.96^{bD}
Myricetin	244.78 ± 7.34 ^{dB}	702.42 ± 14.05^{cA}	172.19 ± 10.33^{fC}	31.22 ± 2.19 g^{hE}	20.46 ± 1.02^{eE}	15.32 ± 1.23^{fE}	194.60 ± 19.46^{eC}	13.96 ± 0.28^{fE}	140.09 ± 11.21^{dD}	266.01 ± 7.98 ^{dB}
Naringin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
p-coumaric acid	78.47 ± 1.56^{fgCD}	123.15 ± 3.69^{efB}	56.95 ± 2.85^{ghiE}	142.44 ± 8.55^{efgA}	60.49 ± 2.42^{eDE}	73.47 ± 4.41^{fCDE}	83.67 ± 7.53 ^{fC}	28.70 ± 2.30^{fF}	36.28 ± 1.45^{fghiF}	81.24 ± 1.62^{eC}
Phlorizin	78.6 ± 0.78^{fgA}	nd	10.98 ± 0.33^{hijB}	nd	nd	nd	nd	nd	nd	nd
Propyl gallate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Protocatechuic acid	61.3 ± 1.226^{ghD}	166.61 ± 5.00^{eA}	59.79 ± 2.99^{ghD}	151.81 ± 9.11^{fB}	nd	77.77 ± 4.67^{fC}	50.00 ± 4.50^{fghD}	82.35 ± 6.59^{fC}	1.11 ± 0.04^{hiE}	54.97 ± 1.10^{efD}
Quercetin	617.53 ± 18.52^{bF}	2622.03 ± 52.44^{bD}	3631.93 ± 217.92^{bB}	3033.31 ± 212.33^{bC}	409.72 ± 20.49^{cG}	1027.72 ± 82.22^{dE}	414.73 ± 41.47^{dG}	3918.12 ± 78.36^{aA}	381.10 ± 30.49^{cG}	971.09 ± 29.13^{bE}
Resveratrol	nd	nd	nd	nd	nd	nd	26.75 ± 2.41^{ghC}	nd	122.81 ± 4.91^{deA}	74.82 ± 1.50^{eB}
Rutin	3445.54 ± 103.36^{aF}	3803.28 ± 76.07^{aE}	5845.77 ± 350.75^{aB}	3678.63 ± 257.50^{aE}	5597.08 ± 279.85^{aC}	12613.49 ± 309.08^{aA}	4227.46 ± 422.75^{aD}	2330.54 ± 46.61^{cG}	1728.76 ± 138.30^{aH}	1225.54 ± 36.77^{aI}
Salicylic acid	19.27 ± 0.19^{ijF}	23.37 ± 0.23^{ghDE}	26.02 ± 0.78^{hijCD}	20.86 ± 1.04^{hEF}	24.04 ± 0.72^{eDE}	25.20 ± 1.26^{fCD}	65.20 ± 5.22^{fgA}	28.35 ± 2.84^{fC}	34.96 ± 0.70^{fgiB}	35.95 ± 1.80^{efgB}
Sinapic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Syringic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Trans ferulic acid	107.86 ± 3.23^{fA}	14.92 ± 0.30^{hG}	82.06 ± 4.92^{gC}	96.10 ± 6.73^{fghB}	47.57 ± 2.38^{eE}	56.73 ± 4.54^{fD}	40.79 ± 4.08^{fghE}	21.25 ± 0.43^{fFG}	23.23 ± 1.86^{ghiF}	2.47 ± 0.07^{gH}
Total	5566.68	8655	14482.03	9984.21	7551.3	19,155	7136.4	10820.84	3564.49	5161.77

* All results are expressed as mean ± standard deviation (n = 3). nd stands for not detected. In each column, difference (a-j) between compounds according to Tuckey's test ($p < 0.05$). In each row, difference (A-J) between BB and BP samples according to Tuckey's test ($p < 0.05$).

monosaccharides. It is determined that glucose replacement as sugar moiety in glycosides, with some other saccharides, additionally slowing down breaking of glycoside's bond during digestion process (Tarko et al., 2013).

It should be pointed out that compound phlorizin (pholiridzin) was quantified only from two bee pollen samples (BP1 and BP2). This glycoside belongs to dihydrochalcones subclass and almost exclusively it can be found among plants which belong to Rosaceae or Ericaceae families (Gosh et al., 2010). In addition, only *Malus* genus (especially apple tree i.e. *Malus domestica*) is considered as a significant source of it (Gosh et al., 2010). Since previously published palynological analysis of these pollen samples (Mayda et al., 2020) confirmed the presence of pollen of plants from Rosaceae family (3.3–5.3%), it can be assumed that this compound can be used as botanical marker for these two pollen samples. To the best of our knowledge, this is the first report about the presence of this glycoside in pollen samples. Previously, there were only two reports about the phloretin, (aglycone form of phlorizin) in Serbian sunflower bee-collected pollen (Kostić et al., 2019) and floral pollen collected from sour cherry tree (Fotirić-Akšić et al., 2019). In the first case, presence of this compound is related to the fact that sunflower bee-collected pollen contained about 15% of Rosaceae plants pollen as accompanying one, while cherry tree is the member of Rosaceae family. In addition to the importance of being a potential chemotaxonomic marker, phlorizin is useful as a potential functional food ingredient since it is well documented that it can cause renal glycosuria and decrease intestinal absorption of glucose which can be important for patients with diabetes (Ehrenkranz et al., 2005). Interestingly, phlorizin was not detected in bee bread samples (BB1 and BB2) with same geographical and botanical origin. This divergence may be explained with increased activity of enzymes during fermentation process presented in bee bread. Namely, it is well known that glycosides of flavonoids are more susceptible to interactions with digestive enzymes compared to aglycone form or some phenolic acids (Martinez-Gonzalez et al., 2017). It is probably caused by the presence of sugar moiety in the molecule of glycosides. As it was mentioned above the most susceptible glycosides for bacterial degradation are those which contain glucose as sugar component. And this is exactly the case with phlorizin. Similarly, one more interesting compound that has been detected in selected samples of pollen (BP4 and BP5) is resveratrol. However, unlike phlorizin, which was completely absent from appropriate bee bread samples, resveratrol was quantified from BB5 but not from BB4 sample. This probably can be explained by significantly higher quantity of resveratrol in BB5 sample compared to BB4 which led to incompletely degradation in BB5 sample and preservation of one part. Since human *in vitro* model digestion system caused decreased bioaccessibility of resveratrol (Lee et al., 2020) it can be assumed that fermentation process in bee bread also affected this compound. There is only one report about the presence of resveratrol in natural bee pollen samples originated from Spain (Ares et al., 2015). But sometimes it can be found in pollen due to its application in beehives against *Nosema* infection or in order to improve longevity of honeybees (Costa et al., 2010). Resveratrol is important since there is an increased interest for this compound due to its expressed bioactivity as a pharmaceutical (Salehi et al., 2018) or functional food ingredient (Tian and Liu, 2020).

Similar to our study, the existence of isorhamnetin, quercetin, luteolin, myricetin, kaempferol (Freire et al., 2012), caffeic acid, *trans*-ferulic acid, chlorogenic acid, *p*-coumaric acid, gallic acid and protocatechuic acid was previously reported in bee pollen (Ulusoy and Kolayli, 2014). Rutin was the compound with the highest concentration of occurrence in both BP and BB samples. Rutin has been used in medicine due to its several pharmacological activities such as antiviral, antibacterial, cytoprotective, neuroprotective, anti-inflammatory, vasoactive, antitumor, antioxidant, cardioprotective, antispasmodic and anticarcinogenic ((Yang et al., 2008). The availability of rutin in bee pollen indicates its biological and nutritional quality owing to its high antioxidant activity (Carpes et al. 2013; Kostić et al., 2019). Related to BB phenolics Tavdidishvili et al. (2014) investigated the flavonoid content in Georgian bee bread and identified rutin, naringin and quercetin in high amounts (20% of the total flavonoid content). Similarly, rutin, *p*-coumaric acid and ferulic acid have been previously reported as primary phenolic compounds present in high amounts in all pollens (Ulusoy and Kolayli, 2014). Although these investigators have indicated that these compounds may be markers for Anzer pollen, the obtained results indicate that they are not specific to pollen samples from a particular region. As these compounds described in the phenolic composition of pollen have been found in high rates in pollen extracts in many different studies (Almeida et al., 2017; Mohdaly et al., 2015), including our study, they may be considered as general markers for bee pollen and bee breads. Metabolic activities during the processing of pollen and bee bread by bees may cause the ratio of these compounds to be more dominant than other components. However, qualitative and quantitative determinations of these compounds can play a crucial role in the formation of national or international standards for bee bread and pollen.

Isidorov et al. (2009) detected ferulic acid, caffeic acid, *p*-coumaric acid, kaempferol and isorhamnetin in bee bread samples. Sobral et al. (2017) reported that bee bread samples contained flavonol derivatives, isorhamnetin, quercetin, myricetin, kaempferol, and herbacetin glycoside derivatives. In addition, catechin was present in BP1, BP2 and BP5 pollen samples, but not in any BB sample. This suggests that there is a link between the fermentation process and the formation/degradation mechanism of phenolic compounds in the formation phase of bee bread. This association may occur in several ways, perhaps involving the ability of lactic acid bacteria to break down phenolic compounds or the positive-negative effects of phenolic compounds on bacterial growth (Gözde et al., 2011). According to literature polyphenols are subjected to the degradation process in digestive system leading to the formation of different phenolic acids. At the end, all metabolites obtained from polyphenols during digestion, are transformed into benzoic acid (Tarko et al., 2013). Additionally, after loss of sugar moiety, aglycone form of flavonoids is also metabolized by forming sulphate and glucuronate conjugates which are the main forms in plasma and urine (Viskupičova et al., 2008). The compound catechin has not been previously reported in pollen samples from any region of Turkey, indicating that geographic differences and hence floral differences affect the variety of chemical compositions of bee products. The similarities and differences of bee pollen samples from different regions may vary depending on many factors, such as

differences in regional flora, climatic conditions, altitude and storage conditions of pollen.

As a result of the phenolic scans, the values of protocatechuic acid, 2,5-dihydroxybenzoic acid and kaempferol content for samples from all regions were higher in bee bread samples compared to pollen samples. Therefore, bee bread can be presented as an alternative to bee pollen as a source of these phenolics. These phenolic compounds could be isolated for use as a functional food ingredient as a source of antioxidants. However, as far as other phenolic compounds are concerned, since the individual phenolic compound content of these two product varieties vary widely depending on the region, it would be more beneficial to use the product containing specific phenolic compounds for the desired purpose.

3.2. Free amino acids profile

A significant part of amino acids is located in pollen's outer membrane- exine (Paramás Gonzáles et al., 2006). However, for liberation of amino acids from proteins some hydrolysis process must be applied. As the main food source for bees pollen must contain at least ten amino acids which are essential for them: isoleucine, leucine, lysine, methionine, histidine, arginine, phenylalanine, threonine, tryptophan and valine (deGoot, 1953). Besides, the presence of proline is important since it is an amino acid used by bees as phagostimulatory compound and as a source of energy for their flights (deGrandi-Hoffman et al., 2013). The current research revealed that the content of total FAAs (Table 3) was a quite uniform in pollen samples ranging from 48.8 to 64.2 mg/g while in case of bee bread higher variations were observed: 23.1–61.1 mg/g. The obtained results for bee pollen are higher compared to values obtained for Spanish bee-collected pollen ranging from 23 mg/g to 37.6 mg/g (Serra-Bonvehí and Escolá Jordá, 1997). This can be caused by fact that authors monitor a significantly lower number of amino acids (18 amino acids) compared to our study (42 amino acids). According to results presented in Table 3, the main FAAs in pollen samples were L-proline (8384.22–16670.79 μg/g) and L-asparagine (4851.27–15336.14 μg/g) followed by L-aspartic acid (3669.36–5260.56 μg/g). Interestingly, a high content of gamma-aminobutyric acid (GABA) (2329.81–5079.35 μg/g) as non-protein amino acid is also detected. Compared to them bee bread samples contained a significantly lower content of L-asparagine (2475.48–5891.12 μg/g) while L-proline (4939.23–22212.82 μg/g), L-aspartic acid (2833.36–5207.37 μg/g) as well as GABA (2703.27–4588.44 μg/g) remained the main amino acids in bee bread samples. In addition, significant quantities of L-phenylalanine were recorded in BP and BB samples-1298.99–3353.80 μg/g and 1308.44–3345.67 μg/g respectively. The presence and quantity of L-proline is strongly influenced by two parameters: adequate storage and floral pollen origin without almost any influence of bees (Serra-Bonvehí and Escolá Jordá, 1997). Also, the ratio of proline content and total amino acids can be used as a parameter of freshness. With the value of this parameter lower than 0.65 it can be assumed that samples were adequately dried and stored (Serra-Bonvehí and Escolá Jordá, 1997). Since the values for all the samples proline/total amino acids were between 0.13 and 0.36 (Table 3) it can be concluded that beekeepers successfully handled and stored pollen and bee bread samples. The results for methionine, threonine, glycine, alanine, isoleucine and lysine content in pollen samples were in line with the results of Serra-Bonvehí and Escolá Jordá (1997). Contrary, proline content was significantly higher (average value: 19,670 μg/g) while results for L-aspartic acid (average value: 330 μg/g), L-glutamic acid (average value: 260 μg/g), and L-serine (average value: 440 μg/g) were significantly lower compared to the current study. Quite the opposite, given results for proline are in line with results for several Chinese bee-collected pollen samples (7400 μg/g – 20,010 μg/g) (Yang et al., 2013). Since proline content in bee pollen is almost exclusively dependent on floral origin (Serra-Bonvehí and Escolá Jordá, 1997) the observed differences/similarities can be provoked by botanical origin of samples. The observed decrease of L-asparagine content in bee bread could be caused by fermentation process and acidic conditions which will trigger deamination of this amino acid. In addition, for most of the BB samples lower content of the main amino acids is recorded compared to adequate BP samples. This observation can also be explained by microbial activity during BB production (deGrandi-Hoffman et al., 2013). However, for some amino acids BP1 and BB1 (GABA) as well as BP2 and BB2 samples (L-proline, L-aspartic acid, GABA) were an exception. In case of L-proline and L-aspartic acid it is possible that the microbial activity causes the liberation of some part of amino acids from proteins which leads to increased content in BB sample (deGrandi-Hoffman et al., 2013) while GABA can be synthetized *in situ* by different microbes like bacteria or fungi (deGrandi-Hoffman et al., 2013; Ramos-Ruiz et al., 2018). The presence of GABA in both bee pollen and bee bread samples is important since this bioactive compound began to gain a significant attention among scientists due to positive effects on human health such as cancer cells development prevention, improvement of immune system status, hypotensive effect and relaxation, etc. (Ramos-Ruiz et al., 2018). Interestingly, the absence of free S-containing amino acids is observed during current research since only L-methionine was quantified in all the samples (BP: 108.11–427.21 μg/g; BB: 98.79–384.79 μg/g) while L-cysteine, D,L-homocysteine and L-cystathionine were not detected. However, it is worth mentioning that the presence of a significant quantity of taurine (BP: 45.35–487.40 μg/g; BB: 83.70–180.41 μg/g) was recorded in all samples. This unusual S-containing non protein amino acid is important in animals and humans since it participates in the regulation of several physiological processes such as conjugation of bile acids, osmoregulation and Ca^{2+} ion metabolism, regulation of retinal function, etc. (McCusker et al., 2013). The taurine content in BP and BB samples was significantly higher compared to amounts found in 20 other plant materials and similar to some marine macro algae or insects (*Eisenia arborea*, *Macrocystis* spp., *Pelvetropsis limitata*, *Lessoniopsis littoralis*, Black soldier fly larva) presented in literature (McCusker et al., 2013). It makes bee pollen and bee bread a good source of this compound for humans or pets (essential for cats) whose diet demands significant quantity of this amino acid (McCusker et al., 2013).

3.3. PCA and cluster analysis

In order to get a detailed insight into the data structure and identify similarities and specificities of object groupings,

Table 3 Free amino acids composition of bee pollen and bee bread samples (μg/g), total free amino acids (TAAs) (mg/g) and proline/ total amino acids ratio.

	BP1	BB1	BP2	BB2	BP3	BB3	BP4	BB4	BP5	BB5
L-Tryptophan	2478.87 ± 92.62^{efB}	377.12 ± 8.27^{klG}	2307.98 ± 51.73^{eC}	1421.98 ± 20.90 ^{gD}	924.30 ± 28.05 ^{ijkE}	564.22 ± 1.04^{jkF}	1310.77 ± 45.51^{hijD}	366.41 ± 17.45^{lmG}	2829.06 ± 59.25^{defgA}	488.85 ± 13.82^{jklFG}
Taurine	45.35 ± 2.79^{pH}	92.28 ± 1.72^{mD}	77.41 ± 2.15^{oEF}	144.50 ± 4.34 ^{kC}	487.40 ± 12.24 ^{mnopA}	180.41 ± 4.30^{lmnoB}	64.99 ± 1.43^{qrFG}	83.70 ± 0.44^{nopDE}	59.66 ± 1.41^{kG}	85.37 ± 3.11^{nDE}
L-Tyrosine	939.59 ± 24.03^{hijCD}	656.02 ± 30.86^{ghE}	846.17 ± 3.05^{ijCDE}	1644.91 ± 178.63 ^{gA}	1076.70 ± 60.00 ^{hijBC}	991.14 ± 4.69^{gCD}	1318.53 ± 68.33^{hijB}	776.71 ± 9.54^{hDE}	1628.17 ± 169.92^{ghijkA}	1047.69 ± 21.84^{fgC}
L-Phenylalanine	2602.69 ± 155.06^{eBCD}	2134.91 ± 151.35^{eD}	1298.99 ± 102.74^{gE}	2157.72 ± 229.23 ^{eD}	3353.80 ± 251.55 ^{eA}	2973.27 ± 182.93^{dAB}	2849.84 ± 260.59 ^{dABC}	3345.67 ± 41.18^{cA}	2423.89 ± 73.0^{fghiCD}	1308.44 ± 200.41^{deE}
L-isoleucine	608.87 ± 7.65^{lmC}	437.53 ± 5.28^{ijklDE}	542.03 ± 26.22^{klC}	747.09 ± 49.12 ^{hiB}	573.44 ± 27.23 ^{lmnoC}	529.41 ± 13.05^{jkCD}	917.87 ± 30.38^{lmA}	357.61 ± 32.36^{lmE}	918.07 ± 50.49^{hijkA}	625.15 ± 61.06^{ijkC}
L-Leucine	1126.54 ± 170.29^{hDE}	59.44 ± 8.19^{mG}	1012.40 ± 203.85^{hiDE}	1590.24 ± 233.39 ^{gBC}	1358.88 ± 233.13 ^{ghCD}	877.87 ± 11.62^{ghiEF}	2120.40 ± 64.15^{fA}	488.21 ± 9.96^{jkF}	1881.13 ± 122.88^{fghijAB}	1073.83 ± 115.62^{efgDE}
Gamma-aminobutyric acid	2329.81 ± 173.56^{fE}	3132.16 ± 106.91^{bD}	4386.46 ± 229.61^{cB}	4588.44 ± 230.19 ^{cAB}	4389.38 ± 130.53 ^{dB}	3652.92 ± 294.40^{cC}	4851.08 ± 165.29^{bAB}	2946.68 ± 89.83^{dD}	5079.35 ± 23.10^{cA}	2703.27 ± 152.98^{cDE}
3-Amino isobutyric acid	1070.08 ± 82.44^{hiE}	1438.68 ± 82.4^{fCD}	1999.93 ± 15.33^{fB}	2090.03 ± 96.22 ^{efAB}	2020.06 ± 61.81 ^{fB}	1668.00 ± 99.75^{eC}	2226.27 ± 69.29^{fAB}	1374.88 ± 81.97^{fCDE}	2335.83 ± 232.49^{fghiA}	1255.45 ± 101.07^{defDE}
L-Methionine	315.41 ± 3.16^{noB}	98.79 ± 2.46 ^{mE}	112.58 ± 13.67^{oE}	213.92 ± 27.67^{jkC}	115.75 ± 10.83^{qrsE}	165.62 ± 10.63^{mnoD}	427.21 ± 29.04 ^{opA}	108.11 ± 7.41^{nopE}	390.44 ± 17.77^{jkA}	384.79 ± 6.58^{klmA}
L-2-Aminoadipic acid	52.45 ± 1.19^{pDEF}	48.71 ± 2.46^{mEF}	51.72 ± 2.47 ^{oDEF}	68.43 ± 2.82^{kC}	71.00 ± 1.44^{qrsC}	58.80 ± 2.65^{noD}	54.36 ± 3.85 ^{qrDE}	45.96 ± 2.70^{opF}	460.85 ± 1.40^{jkA}	113.48 ± 2.77^{nB}
Beta-Alanine	801.58 ± 5.33^{jklA}	360.29 ± 4.92^{klBCD}	368.96 ± 12.71^{lmnBC}	320.75 ± 10.54 ^{jkCDE}	327.15 ± 70.94^{nopqCDE}	398.34 ± 10.13 ^{klB}	268.33 ± 11.88 ^{pqE}	168.74 ± 0.47^{nF}	296.75 ± 8.41^{jkDE}	171.54 ± 6.43^{mnF}
L-Aspartic acid	5260.56 ± 95.31^{cA}	2833.36 ± 62.85^{cD}	3669.36 ± 87.20^{dC}	4584.11 ± 41.10^{cB}	5144.53 ± 180.72^{cA}	4311.40 ± 96.19 ^{bB}	4521.46 ± 65.45 ^{cB}	3828.10 ± 42.60^{bC}	4338.01 ± 83.77^{cdeB}	5207.37 ± 282.32^{aA}
L-Glutamic acid	4143.17 ± 117.89^{dA}	457.05 ± 11.78^{ijkF}	2465.55 ± 60.48^{eB}	828.09 ± 28.16^{hiE}	1108.26 ± 82.80^{hiD}	285.72 ± 9.47^{lmnF}	1342.62 ± 113.66 ^{ghiC}	374.38 ± 9.15^{klmF}	2626.95 ± 126.13^{efghB}	1019.16 ± 12.09^{fgDE}
L-Valine	853.85 ± 11.16^{ijkCD}	530.52 ± 11.69^{hiEF}	913.15 ± 7.92^{ijCD}	994.26 ± 8.05^{hC}	807.19 ± 8.96^{jklD}	662.85 ± 6.70^{ijE}	1469.50 ± 112.63 ^{ghB}	399.58 ± 12.67^{klmF}	1850.50 ± 99.75^{fghijA}	843.88 ± 18.41^{ghiD}
L-2-aminobutyric acid	19.27 ± 2.89^{pA}	10.03 ± 0.23^{mCDE}	15.23 ± 2.83^{oAB}	7.74 ± 0.27^{kEF}	12.43 ± 1.37 ^{sBC}	12.32 ± 0.74^{oBCD}	8.04 ± 0.97^{rDEF}	5.04 ± 1.01^{pF}	11.69 ± 0.13^{kBCDE}	10.26 ± 1.21^{nCDE}
Ethanolamine	928.79 ± 8.06^{hijC}	305.56 ± 7.35^{lF}	1014.10 ± 11.89^{hiC}	563.01 ± 6.20^{ijE}	1180.62 ± 23.79^{hiB}	745.53 ± 7.30^{hijD}	1574.57 ± 71.92^{gA}	143.53 ± 5.28^{noG}	1275.92 ± 69.03^{ghijkB}	248.18 ± 8.97^{lmnF}
L-Alanine	1130.97 ± 25.28^{hE}	505.07 ± 11.2 ^{ijH}	1222.24 ± 6.01^{ghD}	1730.49 ± 28.76^{fgA}	1470.92 ± 11.94^{gB}	921.98 ± 26.80^{ghF}	1429.86 ± 66.42^{ghiB}	764.37 ± 9.52^{hG}	1316.65 ± 38.00^{cdC}	896.46 ± 20.83^{ghF}
L-Threonine	213.07 ± 11.51^{opCD}	74.89 ± 5.31^{mG}	198.32 ± 9.07^{mnoDE}	230.78 ± 9.75^{jkC}	217.51 ± 12.95^{pqrsCD}	185.15 ± 11.21^{lmnoEF}	441.80 ± 3.55 ^{opB}	62.08 ± 3.17^{nopG}	503.59 ± 12.69^{jkA}	163.42 ± 8.00^{mnF}
L-Serine	2278.50 ± 21.48^{fB}	707.41 ± 4.88^{gFG}	1812.69 ± 33.44^{fC}	1538.94 ± 50.97^{gD}	1971.89 ± 13.32^{fC}	1322.29 ± 26.04^{fE}	2596.66 ± 109.41^{eA}	625.51 ± 60.24^{iG}	2625.31 ± 133.05^{efghA}	876.73 ± 10.95^{ghF}
L-Glycin	610.22 ± 9.16^{klmF}	534.14 ± 5.31^{hiG}	735.89 ± 7.38^{jkC}	774.42 ± 28.17^{hiC}	664.64 ± 5.29^{klmDE}	635.13 ± 8.01^{jEF}	1053.58 ± 4.83 ^{klA}	472.35 ± 4.10^{jklH}	986.85 ± 17.99^{hijkB}	677.51 ± 19.26^{hijD}
L-asparagine	12476.10 ± 89.85^{bB}	2475.48 ± 73.39^{dG}	7018.12 ± 198.13^{bC}	5891.12 ± 342.87^{bD}	6248.01 ± 10.02^{bD}	3076.98 ± 81.31^{dF}	4851.27 ± 61.58^{bE}	2585.82 ± 15.21^{eG}	15336.14 ± 79.88^{aA}	4839.80 ± 80.45^{bE}
Trans-4-hydroxy L-proline	1415.71 ± 17.46^{gD}	nd	2371.37 ± 84.39^{eC}	4183.65 ± 193.96^{dA}	1131.72 ± 23.72^{hiE}	725.92 ± 10.10^{hijF}	1073.06 ± 4.25^{jklE}	1069.59 ± 22.74^{gE}	3410.42 ± 14.34^{cdefB}	1383.96 ± 72.11^{dD}
L-Glutamine	206.09 ± 2.83^{opD}	36.23 ± 4.18 ^{mE}	433.06 ± 4.80^{lmC}	342.21 ± 9.04^{jkC}	1175.21 ± 68.63^{hiA}	142.17 ± 5.92^{noDE}	1197.23 ± 94.01^{ijkA}	nd	796.26 ± 10.01^{ijkB}	nd
L-Proline	16670.79 ± 214.79^{aB}	5047.80 ± 44.52^{aG}	14025.02 ± 38.77^{aC}	22212.82 ± 206.57^{aA}	14267.23 ± 211.01^{aC}	6929.35 ± 103.10^{aF}	9109.33 ± 101.08^{aD}	8893.73 ± 137.48^{aD}	8384.22 ± 95.97^{bE}	4939.23 ± 47.15^{bG}
Sarcosine	4.89 ± 0.93^{pD}	21.52 ± 1.24 ^{mA}	3.87 ± 0.61^{oD}	5.28 ± 0.07^{kD}	5.27 ± 0.29^{sD}	14.30 ± 1.95^{oB}	3.66 ± 0.02^{rD}	11.24 ± 0.06^{pC}	3.25 ± 0.27^{kD}	16.29 ± 0.33^{nB}
L-Homocitrulline	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-Citrulline	6.65 ± 0.40^{pC}	1.74 ± 0.49^{mEF}	13.16 ± 0.19^{oA}	4.78 ± 0.71^{kD}	12.84 ± 0.84^{sA}	2.95 ± 0.09^{oE}	10.07 ± 0.79^{rB}	0.91 ± 0.26^{pF}	9.18 ± 0.17^{kB}	4.84 ± 0.56^{nD}
DL-Homocystine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
O-Phosphoryl Ethanolamine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Argininosuccinic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-Arginine	427.95 ± 0.26 ^{mnoD}	115.36 ± 3.90^{mI}	186.70 ± 10.55^{noG}	1044.25 ± 18.99^{hA}	305.84 ± 1.59^{opqrF}	157.94 ± 0.96^{mnoH}	375.09 ± 6.88^{pE}	119.43 ± 12.56^{nopI}	744.51 ± 6.30^{ijkB}	651.83 ± 5.42^{hijC}
L-Cystathionine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-Cystine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-Histidine	962.79 ± 5.52^{hijB}	87.82 ± 5.55^{mH}	981.76 ± 3.17^{hijB}	254.58 ± 10.42^{jkG}	1263.70 ± 14.79^{ghA}	377.80 ± 10.23^{klmE}	632.38 ± 16.06^{noD}	314.68 ± 9.41^{mF}	752.59 ± 6.50^{ijkC}	116.05 ± 8.79^{nH}
L-ornithine	25.77 ± 2.41^{pA}	2.01 ± 0.51 ^{mDE}	5.33 ± 0.35^{oBC}	7.44 ± 0.35^{kB}	4.36 ± 0.29^{sCD}	5.67 ± 0.95^{oBC}	5.20 ± 0.48^{rBC}	1.61 ± 0.05^{pE}	4.74 ± 0.76^{kC}	3.30 ± 0.02^{nCDE}
L-Carnosine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-Lysine	518.92 ± 7.33^{mnF}	547.61 ± 11.36^{hiF}	543.81 ± 15.60^{mnF}	787.43 ± 40.45^{hiB}	602.50 ± 6.29 ^{lmnE}	694.65 ± 7.44^{hijCD}	720.43 ± 16.22^{mnC}	544.18 ± 7.36^{ijF}	852.02 ± 5.75^{ijkA}	652.78 ± 13.12^{hijD}
O-Phospho-L-Serine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
DL-5-Hydroxy lysine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
3-Methyl-L-Histidine	22.85 ± 3.29^{pB}	3.33 ± 0.27^{mF}	8.45 ± 0.44^{pDE}	35.13 ± 0.87^{kA}	10.76 ± 1.49^{sD}	3.79 ± 0.78^{oF}	8.70 ± 0.04^{rDE}	2.63 ± 0.15^{pF}	16.55 ± 2.34^{kC}	5.11 ± 0.05$^{n\ EF}$
1-Methyl-L-Histidine	46.42 ± 2.24^{pC}	9.46 ± 0.38^{mF}	25.52 ± 0.46^{oD}	61.47 ± 2.21^{kA}	29.12 ± 0.85^{rsD}	16.33 ± 0.37^{oE}	55.55 ± 2.39^{qrB}	6.85 ± 2.84^{pF}	62.04 ± 0.92^{kA}	25.58 ± 1.32^{nD}
L-Anserine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Proline/ TAAs ratio	**0.27**	**0.22**	**0.28**	**0.36**	**0.27**	**0.21**	**0.19**	**0.29**	**0.13**	**0.15**
Total free amino acids (TAAs) (mg/g)	**60.59459**	**23.1423**	**50.66732**	**61.07002**	**52.33244**	**33.29021**	**48.88972**	**30.2883**	**64.21059**	**31.83963**

* All results are expressed as mean ± standard deviation (n = 3). nd stands for not detected. In each column, difference (a-j) between compounds according to Tuckey's test ($p < 0.05$). In each row, difference (A-J) between BB and BP samples according to Tuckey's test ($p < 0.05$).

principal component analysis (PCA) and hierarchical cluster analysis (HCA) were conducted. Principal component analysis (PCA) based on the content of phenolic compounds (Table 2) and amino acids (Table 3) in various samples of bee pollen and bee bread extracts results in six-component model explaining 89.26% of the total variance among data. The results obtained by analyzing the first two principal components are shown in score and loading plots **(**Fig. 2**a, b).**

The score plot (Fig. 2a) shows the separation of two groups of objects. Samples of bee bread (BB) (group I) are separated from samples of bee pollen (BP) (group II) (Fig. 2a, Table 2). Only the sample BB2 is an exception and belongs to group II. The reason for that is higher content of several amino acids (1, 3, 5–8, 14, 17–24, 27, 29–33, see Table 3) and phenolic compounds (II, IV, XI, XVIII, see Table 2). It can be observed that proline content (amino acid no. 24) is the highest in BB2 sample compared to all the examined samples. In addition to microbial activity this can be related to possible a higher degree of nectar in this bee bread sample since the bees mixed nectar with pollen during bee bread production (Vasquez and Olofsson, 2009; Malihah Mohammad et al., 2020). It is well known that high the content of proline in nectar can be linked not only to the plant abiotic stress but also to an increased proline content in plants' reproductive system (Mattioli et al., 2009). Amino acids (1, 3, 5–24, 26–33) and phenolic compounds (II-V, XII, XV, XVII, and XVIII) have the strongest positive influence along the PC1 axis on the separation of samples of bee pollen, which is in accordance with the fact that concentrations of these amino acids and phenolic compounds are the highest in this sample (Fig. 2, see Tables 2 and 3), while amino acids (2, 4 and 25) and phenolic compounds (I, VI-XI, XIII, XIV, XVI, and XIX), have a negative influence in the separation of this sample along the PC1 axis. It should be emphasized that pholorizin (XII) had strong influence on separation of BP1 sample which confirms previously mentioned possibility (subsection 3.1.) to use this phenolic as chemotaxonomic marker for this sample. The highest content of beta-alanine, L-2-aminobutyric acid, and L-ornithine, as well as ethyl gallate, phlorizin, and *trans*-ferulic acid in the sample BP2, and L-glutamic acid, L-proline, 3-methyl-L-histidine, and chlorogenic acid in sample BP1, have the strongest positive effect along the PC2 axis on the separation of these samples. Furthermore, it can be noted that the content of amino acids (2, 4, and 25) and phenolic compounds (I, VI-XI, XIII, XIV, XVI, and XIX) is higher in the samples of bee bread and more characteristic for this bee product. The positive correlation of taurine (amino acid no. 2) content with the bee bread samples can be connected with its possible presence of nectar of some plant species originated from Mediterranean region (Nepi et al., 2012; Nocentini et al., 2012) as well as to its important functions for insects' nervous system such as bees (Nepi et al., 2012). Phenylalanine (amino acid no. 4) in bee bread can also originate from nectar since it is an essential amino acid for bees, it belongs to the group of amino acids which accelerate synthesis of sugars in the cell and in that way stimulate bees chemosensors (Nicolson and Thornburg, 2007) and it is usually presented in nectar of Mediterranean plants (Nepi et al., 2012). It is reported that sarcosine (amino acid no. 25) can be dominant non-proteogenic amino acid in nectar (Brzosko and Bajguz, 2019) which can be the reason for its elevated content in bee bread. Additionally, sarcosine has been found in bee larvae as well (Kageyama et al., 2018).

Above presented results have also been confirmed by the Hierarchical Cluster Analysis (HCA) and are shown in a dendrogram (Fig. 3). At a distance 15, HCA results in the separation of samples into two clusters. The samples of bee bread belong to the first cluster, while samples of bee pollen create the second cluster. By comparing the results of principal

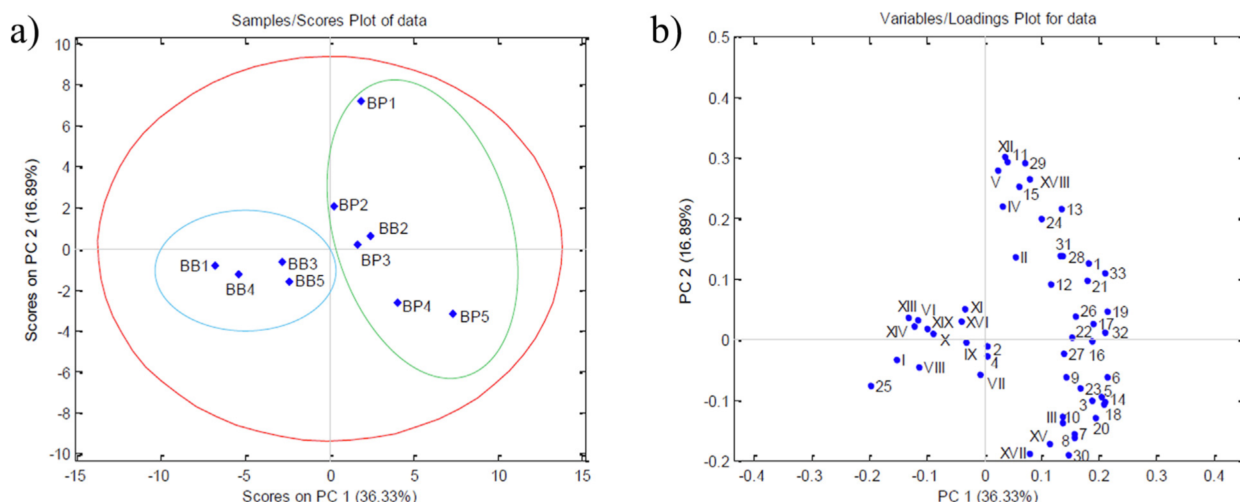

Fig. 2 Score (a) and loading (b) plot. Abbreviations and labels: BP- bee pollen, BB- bee bread; 1 – L-Tryptophan, 2 – Taurine, 3 – L-Tyrosine, 4 – L-Phenylalanine, 5 – L-Isoleucine, 6 – L-Leucine, 7 – Gamma-aminobutyric acid, 8 – 3-Amino isobutyric acid, 9 – L-Methionine, 10 – L-2-Aminoadipic acid, 11 – Beta-Alanine, 12 – L-Aspartic acid, 13 – L-Glutamic acid, 14 – L-Valine, 15 – L-2-Aminobutyric acid, 16 – Ethanolamine, 17 – L-Alanine, 18 – L-Threonine, 19 – L-Serine, 20 – L-Glycin, 21 – L-Asparagine, 22 – *trans*-4-hydroxy-L-proline, 23 – L-Glutamine, 24 – L-Proline, 25 – Sarcosine, 26 – L-Citrulline, 27 – L-Arginine, 28 – L-Histidine, 29 – L-Ornithine, 30 – L-Lysine, 31 – 3-Methyl-L-Histidine, 32 – 1-Methyl-L-Histidine, 33 – Total free amino acids; I – 2,5-Dihydroxybenzoic Acid, II – Caffeic acid, III – Catechin, IV – Chlorogenic acid, V – Ethyl gallate, VI – Gallic acid, VII – Isorhamnetin, VIII – Kaempferol, IX – Luteolin, X – Myricetin, XI – *p*-Coumaric acid, XII – Phlorizin, XIII – Protocatechuic acid, XIV – Quercetin, XV – Resveratrol, XVI – Rutin, XVII – Salicylic acid, XVIII – *trans*- Ferulic acid.

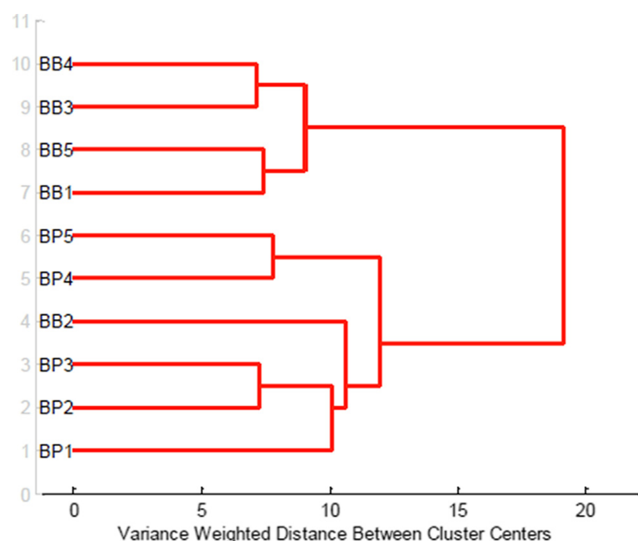

Fig. 3 Dendrogram. Abbreviations: BP-bee pollen, BB-bee bread.

component analysis and hierarchical cluster analysis (HCA) (Fig. 3) it can be concluded that there is a similarity between samples of bee bread, as well as between samples of bee pollen. The dendrogram also shows that the BB2 sample differs from other bee bread samples and belongs to the second cluster, i.e. associated with samples of bee pollen. In general, statistical analysis revealed that amino acids are a more important parameter for distinguishing BP and BB samples than phenolics.

At the end it should be pointed out that, due to limited number of samples available for analysis, these statistical results should be consider as preliminary and as indication of possible trends. Further investigation with higher number of samples should be performed.

4. Conclusion

Bee products naturally contain many components that are necessary to carry out basic life functions. Especially in recent years, human population growth and subsequent increases in the need for food resources, as well as increasing environmental awareness, have led to growing interest in many biologically active natural organic products, including bee products. This in turn has led to growth in the number of such natural products and product combinations. It is important to investigate the chemical properties of such products marketed as healthy and useful. For this reason, it is crucial to investigate the chemical properties of such products and to contribute to the standardization studies. In this study, we presented detailed comparative data on the phenolic and amino acid profile of BP and BB. The results revealed that BP and BB samples possess a great diversity of phenolics and amino acids. We observed that regional differences, in which the phenolic and amino acid profile of the samples do not depend solely on the product type, had the great impact on the phenolic and amino acid composition of the products monitored through determination of both quantity and quality of these bioactive compounds. BP and BB can be preferred as a good source of amino acids for humans. However, when the samples taken from the same region are evaluated, it can be said that bee bread can be presented as an alternative to bee pollen as the source of protocatechuic acid, 2,5-dihydroxybenzoic acid and kaempferol phenolics. In addition, this study shows that chemometric analysis such as PCA and HCA, appear to be potential tools for classification and discrimination of BP and BB using the profile of amino acid and phenolic. However, chemometric analysis revealed that amino acids are a more important parameter for distinguishing BP and BB samples than phenolics.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

All authors contributed to the idea, experimental planning and writing of the manuscript. All authors read and approved the final version of the manuscript.

Appendix A. Supplementary material

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