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Characterisation of Phenolics and other Quality Parameters of Different Types of Honey

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Abstract

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In order to provide a general picture of phytochemical characteristics of nectar honey, honeydew, and mixed honeys, an overall comparison of physicochemical parameters, and phenolic profile as well as antioxidant activity of various types of honey samples has been made. Among all samples analysed, honeydew samples possess the best quality parameters in the mean content of hydroxymethylfurfural, proline, and diastase activity. Moreover, the highest content of phenolic compounds as potential radical scavengers was found in honeydews, then in mixed and multifloral nectar honeys, while samples of monofloral honeys revealed the lowest, but still considerable amounts of natural antioxidants. The overall results of this study indicate that the quality parameters as well as the distribution of phenolic acids and flavonoids are affected by the type of honey.

Keywords: physicochemical parameters; phenolic acids; flavonoids; liquid chromatography; antioxidant activity

Honey is an important natural product that provides beneficial effects on human health. It has a complex composition consisting of a high concentration of sugars, water, minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, and enzymes. These components define both the physical properties and the nutritional and nutraceutical characteristics of the product itself. According to the present European law, there are three types of honeys with regard to their origin: (1) nectar honey – made from plant nectar that can be monofloral or multifloral, (2) honeydew – made mostly from the secretion of insects feeding on plant juices or plant secretion, and (3) mixed honey of honeydew and nectar honey (EU 2001). In each of the mentioned types of honeys we may distinguish many varieties depending on their organoleptic and physicochemical properties as well as their botanical origin (Anklam 1998).

Beside physicochemical characteristics, analysis of phenolic compounds has also been regarded as a very promising way of studying floral and geographical origins of honeys (Gomes *et al*. 2010; Bertoncelj *et al*. 2011; Manzanares *et al*. 2011; Juan-Borrás *et al*. 2014; Karabagias *et al*. 2014). For example, hesperetin has been used as a marker for citrus honey and kaempferol for rosemary honey as well as quercetin for sunflower honey (Thomas-Barberan *et al*. 2001).

The objectives of this study were to perform a complete physicochemical analysis, quantify total flavonoid and phenolic compounds by spectrophotometric methods, as well as to determine a detailed phenolic profile by the chromatographic analysis of various honeys available in the Czech Republic, and to evaluate their radical scavenging activity employing a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay.

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MATERIAL AND METHODS

Honey samples and chemicals. Honey samples were purchased in the market or from beekeepers in the Czech Republic (Table 1). According to their origin, they were divided into four groups, i.e. monofloral honeys (group a), multifloral honeys (group b), mixed honeys (group c), and honeydews (group d). Monofloral honeys were of black locust, *Robinia pseudoacacia* L. (sample 1a), rosemary, *Rosmarinus officinalis* L. (sample 2a), and linden, *Tilia cordata* L. (samples 3a and 4a). Voucher samples are deposited at the Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University Olomouc.

All applied reagents were of the highest purity available and purchased from the Sigma-Aldrich Chemical Company (Munich, Germany).

Physicochemical analysis. Physicochemical parameters were analysed using The Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC 1990), and The Harmonised Methods of the European Honey Commission (Bogdanov *et al*. 1997). Samples were analysed in triplicate and during the same time period to ensure uniform conditions and comparability.

Water content (g/kg of honey) was determined by an indirect refractometry method by comparison of the obtained refractive index with standard values from the literature. The electrical conductivity of honey was measured as electrical resistance of 20 g of honey in 100 ml of distilled water, of which the electrical conductivity is the reciprocal. Free acidity was determined by titration of 10 g of honey dissolved in 75 ml of distilled water, using phenolphthalein as indicator and 0.1 N NaOH as titrator. The results are presented as milliequivalents (meq) of acid per kg of honey as 10 times the volume of NaOH used in titration. The diastase activity was determined spectrophotometrically using a buffered solution of soluble starch and honey incubated in a thermostatic bath at 40°C. The results were expressed in Gothe degrees as the amount (ml) of 1% starch hydrolysed by the enzyme in 1 g of honey in 1 h (de Almeida-Muradian *et al*. 2013).

The content of proline was determined from the colour developed with ninhydrin at a wavelength of 510 nm according to Ough (1969). The content of hydroxymethylfurfural (HMF) was determined by two different methods, spectrophotometric one and by HPLC-UV (White 1979; Jeuring & Kuppers 1980) with absorption maximum at 285 nm. The GraceSmart RP-C18 column (250 × 4.6 mm, 5 µm) was used; water/methanol mobile phase (90 : 10, v/v) with flow rate of 1 ml/min and total analysis time of 7 minutes. Injection volume was 10 µl, and column temperature 30°C.

The analysis of monosaccharides (glucose and fructose) was performed by HPLC with the evaporative light scattering detector (Pazourek 2010), using a LiChrospher 100 (125 × 4 mm, 5 µm; Merck) column. The mobile phase was a mixture of water/acetonitrile (10 : 90 v/v) at a flow rate of 1 ml/min, and total analysis time was 25 minutes. Injection volume was 10 ml, and column temperature 31°C. Detector temperature was 40°C with nitrogen flow rate of 2 l/minute.

Total phenolic and flavonoid content. Total phenolic content was measured by the Folin-Ciocalteu spectrophotometric method (Singleton & Rossi 1965), using gallic acid for a calibration curve. The data were calculated according to the standard curve of gallic acid (1–20 µg/ml), and they were expressed as gallic acid equivalents (GAE) per kilogram of honey.

Total flavonoid content of extracts was measured by two spectrophotometric methods (Chang *et al*. 2002) using quercetin and naringenin as standards for calibration curves.

Chromatographic analysis of phenolic compounds. Chromatographic separation was performed using a Luna C18(2) analytical column (150 × 2 mm; 5 µm; Phenomenex, Torrance, USA) on Shimadzu LC-2010c. Temperature of the column thermostat was kept at 40°C, flow rate was 0.4 ml/min, injection volume for both standards and extracts was 5 ml, and detection was performed at 270 nm. The mobile phase consisted of 20 mM formic acid (A) in water and acetonitrile (B) with elution as follows: 5% B for 4 min, 40% B over 50 min, isocratic 40% B for 6 min, 5% B for 9.5 minutes. Phenolic acids and flavonoids were identified by comparison of the obtained MS/MS spectra from standards and with data from the literature. The mass spectra were obtained by electrospray ionisation in negative mode on Amazon speed ETD IT-MS (Bruker, Bremen, Germany), with the following operating parameters: capillary voltage +4500 V; cone voltage 500 V; ultrascan mode; scan 85–900 *m/z*; auto MS(2), desolvation temperature was 350°C and desolvation gas flow was set to 8 l/minutes. The MS/MS spectra of quasi-molecular ions were acquired (Table 3), and they correspond with the mass spectra of major phenolic compounds found in the literature.

Quantitation of detected phenolic compounds was done using UHPLC–MS/MS system, ACQUITY Ultra Performance LC™ system coupled with Quattro micro™ API (Waters, Manchester, UK) benchtop triple quadrupole mass spectrometer, equipped with an ESI

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source operating in negative mode. Sample solutions were injected onto a reversed phase column (BEH C8, 1.7 µm, 2.1 × 150 mm; Waters, UK), the temperature of which was maintained at 30°C. The mobile phase consisted of the following: 9.5 min sequence of linear gradients and isocratic flows of solvent B (acetonitrile) balanced with aqueous; 7.5 mM formic acid (solvent A) at a flow rate of 0.25 l/min.: 5% B for 0.8 min, 5–10% B over 0.4 min, isocratic 10% B for 0.7 min, 10–15% B over 0.5 min, isocratic 15% B for 1.3 min, 15–21% over 0.3 min, isocratic 21% B for 1.2 min, 21–27% B over 0.5 min, 27–50% B over 2.3 min, 50–100% B over 1 min, and finally 100–105% B over 0.5 minutes. At the end of this sequence the column was equilibrated under initial conditions for 2.5 minutes. The pressure ranged from 4000 to 8000 psi during the chromatographic run. The effluent was introduced into an electrospray source (source block temperature 100°C, desolvation temperature 350°C, capillary voltage 2.5 kV, cone voltage 25 V). Argon was used as collision gas (collision energy 16 eV) and nitrogen as desolvation gas (500 l/h). The retention windows used for quantification were as follows: 0.00–3.00 min; 3.00–4.25 min; 4.25–4.70 min; 4.70–5.15 min; 5.15–5.80 min; 5.80–6.30 min; 6.30–7.30 min; 7.30–7.80 min; 7.80–8.90 min; 8.90–10.00 min (Gruz *et al*. 2008).

1,1-Diphenyl-2-picrylhydrazyl radical-scavenging activity. The ability of the extracts to donate an electron and scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined by the slightly modified method of Brand-Williams (Brand-Williams *et al*. 1995).

The radical-scavenging activity of the tested samples, expressed as percentage inhibition of DPPH, was calculated according to the formula:

$$IC\ (\%) = [(A_0 - A_t)/A_0] \times 100$$

where: A_0, A_t – absorbance values of the blank sample and the test sample, at particular times

Percent inhibition after 30 min was plotted against concentration, and the equation for the line was used to obtain the IC_{50} value presented as mg of the tested sample required to reduce 50% of the used radical within 30 minutes. A lower IC_{50} value indicates greater antioxidant activity.

RESULTS AND DISCUSSION

Physicochemical analysis. The results obtained for the determined physicochemical parameters of analysed honey samples are summarised in Table 1.

Honey moisture content depends on the environmental conditions and the beekeeper manipulation in the harvest period (Acquarone *et al*. 2007). Honey having high water content is more likely to ferment, making the preservation and storage more difficult. The water content ranged from 1.54 g/kg to 1.84 g/kg in investigated honey samples, which is in the acceptable range according to the EC Directive 2001/110 (EU 2011).

Honey pH is affected by the conditions during processing and storage, which also influences texture, stability, and shelf-life. Moreover, values of pH together with conductivity help in distinction between honeydew and floral honeys. In general, honeydews tend to have higher values of pH and conductivity than floral ones (Manzanares *et al*. 2011). As it is presented in Table 1, monofloral honeys (sample group a) had the lowest pH values while honeydews (sample group d) had the highest.

The free acidity of honey may be explained by taking into account the presence of organic acids in equilibrium with their corresponding lactones, or internal esters, and some inorganic ions, such as phosphate (Gomes *et al*. 2010). High acidity can be indicative of fermentation of sugars into organic acids. The values of free acidity of the investigated samples ranged between 9.6 and 17.8 meq/kg, and they were within the limits of the European legislation (below 50 meq/kg), indicating the absence of undesirable fermentation.

Diastase is the common name of the enzyme α-amylase, naturally present in honey, which is logically weakened and destroyed by heat (White 1994). In this context, diastase activity is an indicator of the freshness and is a useful tool to detect heat-induced defects and improper storage of honey (Ramalhosa *et al*. 2011). Diastase activity measured in this study was in the range of 4.65 to 17.56 units (Table 1). Only four samples (10b, 11c, 12c, and 17d) were found to be under limitations of the European legislative (EU 2011). In general, honey samples from beekeepers showed high activity, which was in the range of 14.87–25.29 units.

The hydroxymethylfurfural (HMF) content is widely recognised as a parameter of honey sample freshness, as it is absent in fresh honeys immediately stored by bees and tends to increase during processing and/or ageing of the product (Gomes *et al*. 2010). In this study the content of HMF has been determined by two different methods, spectrophotometric and chromatographic. The comparison of these two methods

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Table 1. Physicochemical parameters of the investigated samples related to age and quality of honey

Entry	Code	pH	Water (g/kg)	EC (mS/cm)	FA (meq/kg)	HMF (mg/kg)		DA	Proline (mg/kg)	F (g/kg)	G (g/kg)	F/G
						UV-Vis	HPLC					
1a	M-MNF-H	3.82 ± 0.01	1.70	0.18	9.6 ± 0.3	17.8 ± 0.6	11.1 ± 0.2	10.8 ± 0.2	173.4 ± 5.7	4.37 ± 0.06	2.65 ± 0.04	1.65
2a	M-MNF-S	3.69 ± 0.03	1.72	0.17	14.4 ± 0.3	25.8 ± 1.1	23.3 ± 0.1	12.4 ± 0.5	313.7 ± 1.7	3.82 ± 0.69	2.99 ± 0.04	1.28
3a	M-MNF-EU	4.06 ± 0.01	1.74	0.39	14.9 ± 0.3	32.9 ± 0.9	31.1 ± 0.2	11.4 ± 0.1	430.1 ± 3.9	3.79 ± 0.11	2.62 ± 0.02	1.45
4a	BK-MNF-CR	4.31 ± 0.01	1.60	0.98	33.1 ± 1.3	2.1 ± 0.1	3.4 ± 0.1	24.5 ± 0.9	724.6 ± 29.6	3.56 ± 0.03	2.34 ± 0.01	1.52
5b	M-MLF-A	4.36 ± 0.02	1.56	0.58	12.6 ± 0.0	11.6 ± 0.5	8.9 ± 0.1	13.4 ± 0.6	327.0 ± 6.5	3.84 ± 0.11	2.66 ± 0.09	1.44
6b	M-MLF-EU	4.02 ± 0.03	1.54	0.55	11.3 ± 0.6	19.8 ± 0.6	20.1 ± 0.6	8.4 ± 0.1	241.0 ± 11.5	3.80 ± 0.07	3.19 ± 0.04	1.19
7b	BK-MLF-CR	4.39 ± 0.00	1.74	0.67	15.7 ± 0.3	2.6 ± 0.2	1.6 ± 0.1	21.1 ± 0.8	318.9 ± 11.2	3.36 ± 0.10	3.02 ± 0.03	1.11
8b	BK-MLF-CR	3.87 ± 0.01	1.70	0.27	17.8 ± 0.6	2.1 ± 0.2	1.4 ± 0.1	21.4 ± 0.3	308.8 ± 11.6	3.59 ± 0.09	3.52 ± 0.03	1.02
9b	BK-MLF-CR	4.04 ± 0.01	1.82	0.33	16.9 ± 0.3	2.6 ± 0.1	2.6 ± 0.1	14.9 ± 0.1	522.5 ± 4.3	3.49 ± 0.09	3.41 ± 0.07	1.02
10b	BK-MLF-SRB	3.37 ± 0.03	1.58	0.11	13.0 ± 0.3	264.4 ± 2.3	1132.8 ± 42.9	nd	nd	3.52 ± 0.03	3.99 ± 0.01	0.90
11c	M-MX-CR	3.72 ± 0.00	1.54	0.55	9.7 ± 0.0	43.2 ± 0.3	46.3 ± 1.5	8.0 ± 0.3	152.2 ± 2.5	3.91 ± 0.10	3.90 ± 0.09	1.00
12c	M-MX-CR	3.69 ± 0.02	1.68	0.52	9.6 ± 0.3	43.3 ± 1.5	41.3 ± 1.0	7.8 ± 0.1	118.9 ± 5.6	3.36 ± 0.06	3.32 ± 0.09	1.01
13c	BK-MX-CR	4.20 ± 0.01	1.62	0.99	41.2 ± 1.1	4.1 ± 0.2	3.3 ± 0.1	25.3 ± 1.1	666.1 ± 32.0	3.23 ± 0.07	2.74 ± 0.01	1.18
14c	BK-MX-CR	4.06 ± 0.01	1.80	0.47	23.0 ± 0.7	3.5 ± 0.1	2.4 ± 0.2	21.8 ± 0.3	385.0 ± 9.5	3.65 ± 0.03	3.27 ± 0.01	1.11
15d	BK-MX-CR	4.34 ± 0.02	1.68	1.07	38.7 ± 0.3	17.8 ± 0.1	15.6 ± 0.1	18.1 ± 0.7	368.6 ± 17.2	3.67 ± 0.07	3.21 ± 0.03	1.17
16d	M-HD-IT	3.95 ± 0.00	1.82	0.38	19.1 ± 0.6	8.0 ± 0.2	8.1 ± 0.1	17.6 ± 0.8	653.3 ± 8.9	3.62 ± 0.02	2.81 ± 0.02	1.29
17d	M-HD-EU	5.06 ± 0.02	1.50	1.59	24.6 ± 0.6	45.6 ± 0.9	42.0 ± 0.4	4.7 ± 0.2	201.9 ± 7.2	3.94 ± 0.08	2.74 ± 0.06	1.43
18d	M-HD-EU	3.87 ± 0.01	1.84	0.37	15.1 ± 0.5	31.8 ± 1.1	28.9 ± 0.1	12.7 ± 0.5	528.2 ± 12.3	3.92 ± 0.10	2.86 ± 0.06	1.37
19d	BK-HD-CR	4.19 ± 0.02	1.72	0.60	17.5 ± 0.5	3.2 ± 0.2	3.1 ± 0.1	24.9 ± 0.5	646.9 ± 29.7	3.09 ± 0.06	2.61 ± 0.11	1.19
20d	BK-HD-CR	4.36 ± 0.01	1.54	0.96	35.0 ± 0.5	2.2 ± 0.2	2.2 ± 0.1	19.0 ± 0.2	685.0 ± 32.6	3.10 ± 0.03	2.57 ± 0.07	1.20

BK – honey from beekeper; MNF – monofloral honey; MLF – multifloral honey; MX – mixed honey; M – honey from market; HD – honeydew honey; H – Hungary; S – Spain; EU – European Union; CR – Czech Republic; A – Australia; SRB – Serbia; It – Italy; EC – electrical conductivity; FA – free acidity; HMF – hydroxymethylfurfural; DA – diastase activity; F – fructose; G – glucose; nd – not detected

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indicated similar results (Table 1). Considering the sample 10b as outlier, the majority of data correlate around diagonal with correlation coefficient R^2 = 0.9827. It means that the simple and fast spectrophotometric method provides as reliable data as sophisticated, but time-consuming and expensive high performance liquid chromatography. In general, honey samples obtained from beekeepers had a significantly lower concentration of HMF, with values ranging from 1.42 mg/kg to 15.58 mg/kg (Student's *t*-test with unequal variances at α = 0.001; without sample 10b). These results are in agreement with those published earlier (Kubiš & Ingr 1998; Kalabova *et al*. 2003; Kahoun *et al*. 2008; Bartakova *et al*. 2011). Three samples of honey purchased in the market slightly exceeded the allowable limit of 40 mg/kg, i.e. samples 11c, 12c, and 17d, while sample 10b (obtained from beekeeper) exceeds 28 times the limit value of HMF. Additional information on the packaging of these purchased honeys indicated that it is a mixture of honeys from different areas of the European Union and even from areas with subtropical climate, where the allowable limit is 80 mg/kg of HMF. Regardless of the possible subtropical origin of sample 10b, HMF content is far over the allowable limit, which indicates that processing and/or storage conditions of this sample were not adequate. The results of diastase activity and HMF show that these two factors are closely related. Enzyme activities combined with low concentration of HMF are the most important factors for determining the quality of honey. For example, samples 4a and 8b had low concentrations of HMF, and high diastase activities, while sample 10b showed the highest levels of HMF, and non-detectable enzymatic activity.

Proline is the predominant free amino acid of honey and it is a measure of total amino acids (Iglesias *et al*. 2004). The proline content of honey is measured as a criterion for estimating the quality and the antioxidant activity of the honey and it may be used also for its characterisation on the basis of botanical origin (Meda *et al*. 2005). Proline originates mainly from the salivary secretions of honeybees (*Apis mellifera* L.) during the conversion of nectar into honey (Da Silva *et al*. 2016). Generally honeydews have a high content of total amino acids, unlike floral honeys (Iglesias *et al*. 2004). Presented results (Table 1) are in agreement with this fact. Among floral honeys, the lowest content of proline was found in black locust honey (sample 1a), while the highest content was found in sample 20d (685.0 ± 32.6 mg/kg), honeydew from a beekeeper.

Carbohydrates are the major constituents of honey, corresponding to 95–99% of the dry matter (Olaitan *et al*. 2007). These sugars are composed mainly of fructose, glucose, and sucrose. The sugars of honey were determined by HPLC. Presented results (Table 1) are consistent with Manzanares *et al*. (2011), who reported amounts of fructose in the range 2.9–3.8 g/kg and of glucose 3.6–4.2 g/kg in floral honeys. In this study, floral honeys contained fructose in the range of 3.49–4.37 g/kg and glucose 2.34–3.90 g/kg. Honeydews had lower values of total monosaccharides. The ratio of fructose/glucose (F/G) is used to determine the granulation of honey because glucose is less soluble than fructose (Ojeda De Rodríguez *et al*. 2004). Honeys with high fructose/glucose ratios would remain liquid for longer periods. The F/G ratio may have an impact on honey flavour, since fructose is much sweeter than glucose (White *et al*. 1962).

The proportion of fructose and glucose depends largely on nectar sources (Anklam 1998). Nozal Nalda *et al*. (2005) reported an average ratio of F/G of about 1.2, which is in agreement with the presented results. In this study, the average ratio of F/G was the lowest for mixed honeys (1.00–1.11), while monofloral honeys had an average ratio of F/G of 1.28–1.65 (Table 1).

Total phenolic and flavonoid content. Total phenolic content as well as flavonoid compounds were determined using three spectrophotometric methods: Folin-Ciocalteu method (total phenolics), the method with aluminium chloride (total flavonols and flavones), and the method with 2,4-dinitrophenylhydrazine (total flavonones and flavanonols) (Table 2). Folin-Ciocalteu spectrophotometric assay is based on a chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides. The colour development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens in which the metals have lower valence (Singleton & Rossi 1965). The principle of the aluminium chloride colorimetric method is that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminium chloride forms acid labile complexes with the *ortho*-dihydroxyl groups in the A- or B-ring of flavonoids, while the principle of another method lies in the fact that 2,4-dinitrophenylhydrazine reacts with ketones and aldehydes to form corresponding hydrazones (Chang *et al*. 2002).

Among all examined honeys, samples of honeydew origin revealed the highest amounts of total phenolics,

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Table 2. Phenolic content and antioxidant activity of honey samples

Sample No.	TPC (mg GAE/kg)	TF		DPPH IC_{50} (mg)
		(mg QE/kg)	(mg NE/kg)	
1a	238.36 ± 10.26	8.73 ± 0.00	2.37 ± 0.07	100.59 ± 2.50
2a	321.59 ± 13.43	16.90 ± 0.55	3.77 ± 0.16	56.41 ± 1.95
3a	450.37 ± 15.67	18.79 ± 0.55	1.99 ± 0.10	76.82 ± 1.71
4a	730.09 ± 16.15	35.61 ± 0.47	3.41 ± 0.09	26.05 ± 0.48
5b	723.13 ± 20.52	11.10 ± 0.47	9.87 ± 0.24	40.75 ± 1.75
6b	446.49 ± 15.67	29.17 ± 0.27	9.41 ± 0.23	56.16 ± 1.20
7b	711.99 ± 16.15	35.46 ± 0.55	3.25 ± 0.46	42.57 ± 1.24
8b	412.08 ± 11.85	17.22 ± 0.47	1.11 ± 0.18	42.01 ± 1.63
9b	679.18 ± 28.59	16.27 ± 0.82	7.58 ± 0.12	30.64 ± 0.74
10b	363.75 ± 16.15	8.41 ± 0.27	50.62 ± 1.70	75.52 ± 0.09
11c	530.52 ± 17.91	22.09 ± 0.72	10.44 ± 0.15	41.19 ± 1.45
12c	288.77 ± 13.43	8.41 ± 0.27	5.26 ± 0.27	64.19 ± 1.54
13c	836.10 ± 39.04	25.10 ± 0.55	7.21 ± 0.12	19.78 ± 0.27
14c	571.88 ± 7.76	33.10 ± 0.72	4.26 ± 0.24	35.81 ± 0.53
15d	804.57 ± 15.67	38.60 ± 0.55	8.31 ± 0.16	32.16 ± 0.11
16d	1465.16 ± 11.85	36.71 ± 0.55	14.62 ± 0.31	8.66 ± 0.28
17d	848.53 ± 2.24	25.87 ± 0.98	13.13 ± 0.15	43.77 ± 0.87
18d	324.97 ± 12.47	10.61 ± 0.47	10.58 ± 0.33	73.72 ± 0.23
19d	903.32 ± 20.52	40.02 ± 0.72	6.75 ± 0.29	23.11 ± 0.46
20d	841.27 ± 33.81	31.84 ± 0.82	4.63 ± 0.02	23.43 ± 0.87

TPC – total phenolic content; TF – total flavonoid content; GAE – gallic acid equivalents; QE – quercetin equivalents; NE – naringenin equivalents

ranging from 324.97 ± 12.47 to 1465.16 ± 11.85 mg GAE/kg, while samples of monofloral origin revealed the lowest amounts, ranging from 238.36 ± 10.26 to 730.09 ± 16.15 mg GAE/kg (Table 2). Similarly, the highest amounts of flavonols, measured by aluminium chloride method, were found in honeydew samples, ranging from 10.61 ± 0.47 to 40.02 ± 0.72 mg QE/kg, as well as the highest amounts of flavonons, measured by 2,4-dinitrophenylhydrazine method, ranging from 4.63 ± 0.02 to 14.62 ± 0.31 mg NE/kg (Table 2). Moreover, according to Piljac-Zegarac *et al.* (2009), total phenolic content measured by Folin-Ciocalteu method can be considered an important determinant for the antioxidant capacity of the examined honey samples. Presented results are in agreement with those published earlier (Lachman *et al.* 2010; Jasicka-Misiak *et al.* 2012).

It is known that the levels of phenolic compounds in honey are greatly influenced by geographical origin, climatic conditions and plant species (Gomez-Caravaca *et al.* 2006). However, it is important to note that spectrophotometric measurements of phenolic compounds have some disadvantages. The Folin-Ciocalteu reaction is based on chemical reduction of the reagent, which can be easily reduced by other compounds in honey, such as reducing sugars. Moreover, interferences of HMF with 2,4-dinitrophenylhiydrazine were noted in this study. Samples 10b, 11c, and 15d were characterised by a high content of HMF, and they also showed high values of total flavanones.

Chromatographic analysis of phenolic compounds. Honeys that contained higher amounts of phenolic compounds were subjected to a detailed chromatographic analysis of phenolic compounds. So far, different methods have been developed for determination of flavonoids in honey (da Silva *et al.* 2016). Due to the complex matrix of honey and the low concentration of these compounds, numerous authors have proposed a sample clean-up including a preconcentration step (Bertoncelj *et al.* 2011). The clean-up depends on the decision whether flavonoid aglycones or glycosides should be analysed. Then, the flavonoid aglycones are most likely extracted by the solid phase extraction on reversed

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phase materials. Since the goal of this study was to determine phenolic acids and flavonoid aglycones, the honey samples were dissolved in acidified water (pH 2) and preconcentrated on a polymeric RP SPE-cartridge with Strata-X and eluted with 100% methanol, according to Bertoncelj *et al.* 2011.

The representatives of all types of honey, with an emphasis on monofloral honeys were selected for these analyses. Using an HPLC-ESI-MS/MS technique, 25 phenolic compounds were identified in 10 samples (Table 3). Identification of detected phenolics was accomplished by matching the chromatographic behaviour and collision spectra with those of known compounds. Among all detected phenolics, only *p*-coumaric acid, ferulic and isoferulic acids were detected in all samples – Quercetin, luteolin, naringenin, rhamnetin, and kaempherol as flavonoids were found in all examined honeys. The same samples were subjected to a quantitative UHPLC-MS/MS analysis using 23 standards of phenolic acids and flavonoids (Table 4). The highest amounts of phenolics were found in honeydew samples, and the lowest in samples of monofloral honey. Protocatechuic acid (PCA), the major acid quantified, was found in high concentrations in samples of mixed honey (3.59–8.72 mg/kg) and honeydew (3.23–9.11 mg/kg). The second most abundant acid was salicylic (SaA), followed by *p*-hydroxybenzoic (pHBA), *p*-coumaric (pCoA), and caffeic acid (CaA), with concentrations in a range of 0.22–9.92 mg/kg, 0.69–4.90 mg/kg, 0.23–4.91 mg/kg, and 0.10–5.63 mg/kg, respectively. Among the examined flaovonoids, pinocembrin was found to be the most abundant (0.01–6.67 mg/kg).

On the contrary, Lachman *et al.* (2010) found that ferulic acid and the flavone chrysin are the main compounds found in Czech honeys. As it was written above, ferulic acid was detected in all examined samples, but in lower ranges than described in literature.

Table 3. Qualitative HPLC-ESI-MS analysis of phenolic compounds in the investigated honey samples

No.	Rt (min)	Compound	MRM	MS fragments	Entry									
					1a	2a	4a	5b	7b	13c	14c	16d	19d	20d
1	4.2	gallic acid	169 > 125	95, 109, 125, 169		+	+	+	+	+	+	+		+
2	13.9	*p*-hydroxybenzoic acid	137 > 93	93, 137			+					+		
3	16.4	chlorogenic acid	353 > 191	179, 191, 353								+	+	
4	18.2	vanillic acid	167 > 125	125, 127, 137, 167			+	+					+	+
5	18.5	caffeic acid	179 > 135	135, 179		+	+			+	+	+		+
6	20.5	syringic acid	197 > 182	153, 182, 197							+			
7	22.2	*p*-coumaric acid	163 > 137	119, 137, 163	+	+	+	+	+	+	+	+	+	+
8	24.6	*trans*-cinnamic acid	147 > 119	119, 147	+	+	+	+	+		+	+	+	+
9	26.6	*m*-coumaric acid	163 > 123	123, 137, 163	+	+	+	+	+	+		+		+
10	27.7	ferulic acid	193 > 149	149, 178, 193	+	+	+	+	+	+	+	+	+	+
11	28.2	sinapic acid	223 > 164	134, 164, 179, 187, 223					+			+		
12	29.3	isoferulic acid	193 > 178	134, 149, 178, 193	+	+	+	+	+	+	+	+	+	+
13	30.6	salicylic acid	137 > 93	93, 137	+	+	+		+	+		+	+	+
14	35.7	myricetin	317 > 289	181, 289, 299, 317	+	+	+		+	+			+	+
15	42.3	quercetin	301 > 179	151, 179, 257, 285	+	+	+	+	+	+	+	+	+	+
16	43.6	luteolin	285 > 267	239, 267, 285	+	+	+	+	+	+	+	+	+	+
17	47.0	naringenin	271 > 151	151, 177, 271	+	+	+	+	+	+	+	+	+	+
18	47.5	apigenin	269 > 253	151, 197, 215, 225, 253, 269	+		+	+	+	+	+	+		
19	47.6	pinobaksin	271	271	+		+	+	+	+	+	+	+	
20	48.5	rhamnetin	315 > 300	165, 300, 315	+	+	+	+	+	+	+	+	+	+
21	48.7	kaemferol	285	285	+	+	+	+	+	+	+	+	+	+
22	60.6	chrysin	253	253	+	+	+	+	+	+		+	+	+
23	62.2	pinocembrin	255	255	+	+	+	+	+	+		+	+	+
24	63.2	galangin	269 > 267	269, 267	+	+	+	+	+	+		+	+	+
25	64.8	pinobaksin-3-*O*-acetate	313 > 253	253, 313			+			+				

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Table 4. Quantitative UPLC-MS/MS analysis of phenolic compounds (mg/kg) in the investigated honey samples

No.	Compound	Entry									
		1a	2a	4a	5b	7b	13c	14c	16d	19d	20d
1	gallic acid	0.01	0.16	0.83	1.38	1.20	0.84	0.47	1.33	0.85	0.79
2	protocatechuic acid	0.03	0.43	7.74	0.69	3.41	8.72	3.59	3.23	9.11	5.75
3	gentisic acid	0.02	0.04	0.58	0.05	0.31	0.94	0.22	0.43	0.65	0.24
4	*p*-hydroxybenzoic acid	0.69	1.16	1.63	0.97	4.90	6.16	3.69	1.65	2.88	4.25
5	chlorogenic acid	0.02	0.04	0.07	0.02	2.07	0.08	0.06	0.22	0.11	0.05
6	caffeic acid	0.65	1.00	0.79	0.10	5.63	1.33	1.30	2.19	1.04	1.25
7	vanillic acid	0.09	0.01	0.31	0.19	1.50	1.28	1.10	0.36	0.43	0.53
8	syringic acid	0.01	0.03	0.10	0.19	0.10	0.39	0.42	0.14	0.16	0.25
9	mHBA	0.01	0.01	0.02	0.01	0.05	0.06	0.03	0.05	0.03	0.04
10	*p*-coumaric acid	0.38	0.35	1.39	0.23	4.91	2.39	3.07	2.00	3.20	4.78
11	sinapic acid	0.02	0.01	0.01	nd	0.02	0.01	0.03	0.01	0.02	0.02
12	ferulic acid	0.34	0.13	0.71	nd	2.28	1.75	2.34	1.87	1.85	1.51
13	*p*-coumaric acid	nd	nd	0.04	nd	nd	0.01	nd	0.01	0.03	0.01
14	isoferulic acid	0.35	0.21	0.47	0.06	1.92	0.69	1.04	0.78	0.81	0.88
15	salicylic acid	0.39	0.18	2.22	0.22	1.37	1.98	0.71	9.92	2.29	4.60
16	*trans*-cinnamic acid	0.07	0.07	0.02	0.27	0.13	0.02	0.31	0.53	0.04	0.12
17	eriodictyol	0.01	0.01	nd	nd	nd	nd	nd	tr	nd	nd
18	taxifolin	0.01	0.04	nd	nd	nd	nd	nd	0.01	nd	0.04
19	naringenin	0.03	0.02	0.01	nd	nd	nd	0.02	0.01	nd	0.03
20	pinocembrin	3.33	2.99	0.17	0.42	0.01	0.14	4.66	5.15	tr	6.67
21	apigenin	0.05	0.06	nd	nd	0.01	nd	nd	0.02	nd	nd
22	rhamnetin	0.01	0.01	nd	nd	nd	nd	nd	nd	nd	nd
23	chrysin	0.05	0.08	nd	0.06	nd	nd	0.10	nd	nd	nd

tr – traces (< 0.01); nd – not detected

1,1-Diphenyl-2-picrylhydrazyl radical-scavenging activity. The antioxidant activity of honey samples was evaluated by the DPPH radical-scavenging test. Assessed samples were able to reduce the stable violet DPPH radical to the yellow DPPH-H form reaching 50% of reduction with IC_{50} values ranging from 8.66 ± 0.28 mg (sample 16d) to 100.59 ± 2.50 mg (sample 1a) (Table 2). These values are comparable with the amounts of total phenolics measured by the Folin-Ciocalteu method, i.e. higher content of phenolics gives better antioxidant activity. Thus, a positive correlation between antioxidant activity and total phenolic content was found (R^2 = 0.686) and this result evidences that the antioxidant activity of honeys is caused mainly by the presence of phenolics. Moreover, presented results are comparable with previously published results on the antioxidant activity of honeys from the Czech Republic (Lachman *et al*. 2010) as well as for honeys of other geographical origin (Bertoncelj *et al*. 2007; Perna *et al*. 2013).

However, in order to compare the antioxidant activity of honeys with their chemical composition, several representatives of major classes of components were tested as well. Gallic acid and *trans*-cinnamic acid were chosen as representatives of hydroxybenzoic and hydroxycinnamic acids found in honeys, and their IC_{50} values were 1.72 ± 0.02 mg for gallic acid, and 73.27 ± 1.78 mg for *trans*-cinnamic acid. These results prove the fact that phenolic acids are the main carrier of antioxidant activity of natural extracts (Zheng & Wang 2001). Quercetin and naringenin, the representatives of flavonoid compounds found in honeys, showed similar antioxidant activity, when IC_{50} values of 3.63 ± 0.06 mg for quercetin and 63.19 ± 1.84 mg for naringenin were found. The IC_{50} value of proline, the main amino acid of honey, was 32.17 ± 1.06 mg, while hydroxymethylfurfural revealed IC_{50} of 3.63 ± 0.22 mg. Moreover, main sugars in honey also possess a weak antioxidant activity, much lower but comparable with the tested honey samples. Glucose revealed IC_{50} value of 162.51 ± 1.42 mg, while

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fructose showed slightly better activity with IC_{50} value of 157.41 ± 1.47 mg.

CONCLUSIONS

The results of this study indicate that the quality parameters as well as the distribution of phenolic acids and flavonoids are affected by the type of honey.

According to the obtained data, honeydews revealed the lowest levels of hydroxymethylfurfural, and the highest levels of proline. Moreover, samples of this type of honey possess the lowest pH among others that might be correlated with the content of phenolic acids. These samples are found to be the richest in phenolic compounds, as well as they gave the best results in an antioxidant assay.

In general, among all samples analysed, it might be stated that honeydew possesses the best quality parameters and the highest content of phenolic compounds as potential radical scavengers. Mixed and multifloral honeys follow, while samples of monofloral honeys revealed the lowest, but still considerable amounts of natural antioxidants.

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