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Antioxidant Effect of Natural Honeys Affected by Their Source and Origin

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In purpose to examine the antioxidant activity of 15 natural honeys of different origin ABTS method was used, total phenol content and dry matter content of honey samples were determined. Honeys were collected from different locations of Slovakia, Poland and Serbia and were represented as monofloral and multifloral samples (10) which originated from Poland and Slovakia, forest samples (4) originated from Serbia and honeydew honey. Average values of antioxidant activity observed in samples of honeys ranged from 0.62 to 4.63 mmol/kg. The highest antioxidant activity was detected in buckwheat honey and the lowest was shown in acacia honey. By observing the impact of individual honey samples on antioxidant activity it was found that the sample had a highly statistically significant effect. 10 homogeneous groups which varied in the antioxidant activity among each other were established by all 15 samples. Antioxidant activity of honeys could be a positive influence factor in terms of honey differentiation, especially in the case of the forest honeys collected from different places. Monofloral and multifloral honeys (10) established 5 homogenous groups, but in the case of several multifloral honeys which originated from different places of Poland and Slovakia no statistically significant differences were found.

INTRODUCTION

By the Codex Alimentarius honey is the natural sweet substance, produced by honeybees from the nectar of plants or from secretions of living parts of plants, or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature. The colour, aroma and consistency of honey all depend upon which flowers the bees have been foraging. The consumption of honey is increasing because of its beneficial biological properties, including antioxidant and antibacterial activities [Montenegro & Mejias, 2013]. A large number of *in vitro* and limited clinical studies have confirmed the broad-spectrum antibacterial, antifungal, antiviral, and antimycobacterial properties of honey, to immune modulating and anti-inflammatory properties of honey [Israili, 2014]. In our previous study, we tested potential antimicrobial activity of selected honeys against four species of bacteria (*Escherichia coli* CCM 3988, *Pseudomonas aeroginosa* CCM 1960, *Staphylococcus epidermis* CCM 4418, *Bacillus cereus* CCM 2010) and two species of yeasts (*Saccharomyces cerevisiae* CCM 8191, *Candida albicans* CCM 8216). The strongest antimicrobial activity was shown in honey samples of 50% concentration against *Escherichia coli, Pseudomonas aeroginosa* and *Staphylococcus epidermis* [Fikselova *et al*., 2014].

Antioxidant activities were demonstrated in commercial Indian honeys, which may have therapeutic potential, though honey has a significant amount of phenols [Saxena *et al*., 2010]. Monofloral Malaysian honeys were analysed by Hussein *et al*. [2011] to determine their antioxidant activities and total phenolic and flavonoid contents, with and without gamma irradiation. Honey can scavenge free radicals and exhibit high antioxidant-reducing power, in good correlation with its phenolic content [Hussein *et al*., 2011]. Honey provides sugars and other nutrients, such as mineral elements, proteins, and antioxidant active compounds. The presence of several minor components and the antioxidant activity in honey is related to the botanical origins of the product [Escuredo *et al*., 2013]. It is a rich source of antioxidant and antiseptic compounds including Maillard reaction products, vitamins, carotenoids and polyphenols [O'Sullivan *et al*., 2013]. Carotenoids were the predominant floral pigments in several tested honeys, while xanthophylls and anthocyanins were the least predominant ones [Alqarni *et al*., 2012], total chlorophylls were also found.

By the European Parliament resolution there are Calls on the Commission to respond to the requests for example by improving statistical data in relation to production forecasts, including the application of the same quality requirements for honey, Calls to consider, in the framework of the legislative proposal on agricultural quality policy, changing the rules on origin labelling of honey in order to avoid misleading information to consumers, especially in the case of a blend of honeys originating from EU and non-EU. Therefore quality parameters of honey are studied in order to confirm the au-

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thenticity, the food safety and also to provide the nutritional values of regional honeys. Physicochemical properties, main mineral content and antioxidant activity were determined in Portuguese honeys, statistical analysis demonstrated that antioxidant parameter and main mineral content had a positive influence on honeys differentiation [Alves *et al*., 2013]. New criteria based on regional characteristics of Saudi honeys including antioxidants, micro-constituents were suggested by Alqarni *et al*. [2012].

Therefore the purpose of this study was to determine the antioxidant activity of 15 natural honeys in samples originated from different sources and areas, also in order to test the influence of honey origin on its antioxidant activity. Antioxidant methods were used to support the determination of the total phenolic content in the analysed honeys.

MATERIALS AND METHODS

Honey samples (Table 1) were collected from different locations of Slovakia (6 samples), Poland (5 samples) and Serbia (4 samples) and were obtained directly from local beekeepers. Honeys were represented as monofloral and multifloral samples (10) which originated from Poland and Slovakia (Table 1), forest samples (4) originated from Serbia and honeydew honey (1) from Slovakia.

TABLE 1. Investigated honey samples.

No	Sample	Origin
1	forest	Serbia (Milevici), altitude 800 m
2	forest	Serbia (Babine), altitude 1250 m
3	forest	Serbia (Jabuka), altitude 1250 m
4	(multi)floral	Poland
5	heather	Poland
6	(multi)floral	Poland
7	floral (rape)	Slovakia (Nitra)
8	acacia	Slovakia (Nitra)
9	(multi)floral	Slovakia (Michalovce)
10	forest raspberry	Slovakia (Bystrá)
11	forest	Serbia, altitude 1000 m
12	buckwheat	Poland
13	(multi)floral	Poland
14	(multi)floral	Slovakia (Stupava)
15	honeydew	Slovakia (Relov)

Antioxidant activity determination

Samples of honey (2 g) were mixed with 10 mL of distilled water. The prepared mixture was centrifuged for 5 min at 10,000 rpm and a temperature of 20°C. Extracts prepared were stored during the experiments in a refrigerator at 4–6°C. Experiments were performed either directly, or samples were further diluted before measurement as needed.

ABTS test of honeys

$ABTS^{\bullet+}$ solution was prepared as described Re *et al*. [1999]. Before use of 1 mL of this solution, $ABTS^{\bullet+}$ was diluted with 50 mL of distilled water. Concentration of the solution prepared was around $c = 1 \times 10^{-4}$ mol/L.

Aqueous solution of $ABTS^{\bullet+}$ (1×10^{-4} mol/L) was put into a syringe (1 mL volume) and into identical second syringe there was put 1 mL of aqueous solution of honey. Both syringes were connected with a micro-mixing chamber, connected into an EPR cell (internal volume 400 μL), during the measurement permanently placed in the cavity of the EPR spectrometer (Bruker, Germany). This arrangement allowed to start the measurement at the moment of mixing the sample and $ABTS^{\bullet+}$. The time evolution of 15 spectra for 22.5 min of mixing $ABTS^{\bullet+}$ with a solution of honey was observed. Each spectrum represents the average of 30 individual scans. Distilled water was used as a reference sample. All measurements were performed in duplicates and EPR spectrometer was used for determination.

EPR spectrometer parameters were as follows: Central field: 350 mT, Sweep width: 9 mT, Modulation amplitude: 0.052 mT, Receiver gain: $4.48.10^3$ G, Source performance: 6 mW, Frequency of microwave radiation: 9.81 GHz.

The measured EPR spectra were processed in programs WINEPR (Bruker ®) and ORIGIN (MicroCalc ®). Results were expressed as Trolox equivalent values ($TEAC_{ABTS\bullet+}$) as follows :

$$TEAC_{ABTS^{\bullet+}} = \frac{(c_{0(ABTS^{\bullet+})} - c_{t(ABTS^{\bullet+})}) \cdot V_{(ABTS^{\bullet+})}}{V_{(vzorky)}} \cdot \nu \cdot Z$$

whereas: $c_{0\ (ABTS\bullet+)}$, $c_{t\ (ABTS\bullet+)}$ is concentration of $ABTS^{\bullet+}$ in time t=0, resp. t=10.5 min.; $V_{(ABTS\bullet+)}$ is volume of $ABTS^{\bullet+}$ added; $V_{(vzorky)}$ is volume of sample added; ν is stoichiometric coefficient of the reaction ABTS and TROLOX, in this case ν=1/2; and Z is the dilution factor.

Determination of total phenol content (TPC) in honey

Total polyphenol content was determined according to the modified method using the Folin-Ciocalteau reagent [Singleton *et al*., 1999]. Exactly 200 mL sample of the aqueous solution of honey was mixed with 15.8 mL of distilled water and with 1 mL of Folin-Ciocalteau reagent. After 10 min, 3 mL of a 20% sodium carbonate solution was added and the mixture was stirred well. After 60 min, absorbance of each solution was measured at 765 nm. The results were expressed as gallic acid equivalent (GAE mg/kg). Calibration curve was prepared using standards of gallic acid in the range of 0–1000 mg/L. All measurements were performed in duplicate. Determination was performed with the use of UV-VIS-NIR Shimadzu spectrometer (UV - 3600 with accessories).

Determination of dry matter content

Dry matter content of honeys was determined refractometrically by digital refractometer [Kačániová *et al.,* 2009].

Statistical analysis

The mean values and standard deviations were calculated. Data were elaborated with the analysis of variance (ANOVA),

Fisher's least significant difference (LSD), and Pearson's correlation coefficient were determined. Analysis of the results was performed using the statistical software Statistica 8.0 [Statsoft, Inc. 2008].

RESULTS AND DISCUSSION

Antioxidant effect of honeys determined by the ABTS method

Mean values of the antioxidant activity in the analysed samples of honeys determined with the ABTS method ranged from 0.62 to 4.63 mmol/kg. By monitoring the impact of individual honey samples on the antioxidant activity it was found that the sample had a highly statistically significant effect (Table 2). By further testing with the use of the Fisher test there were monitored differences among groups of samples of honey in terms of the antioxidant activity. Ten homogeneous groups were established by 15 samples of honeys that varied in the antioxidant activity among each other (Table 3).

Results of ABTS assay showed that the mean antioxidant activity in all multifloral honeys ranged from 0.64 to 1.47 mmol/kg, including from 0.9 to 1.42 mmol/kg in multifloral honeys originating from Poland and from 0.64 to 1.47 mmol/kg in Sloval multifloral honeys. All analysed monofloral and multifloral honeys (10) established 5 homogenous groups (Table 3). In the same homogenous groups (b,d), there were found multifloral honeys originating from Slovakia and from Poland as well, so there were no statistical differences among them regarding antioxidant activity.

The lowest antioxidant activity was evaluated in a sample of acacia honey (0.62 mmol/kg). In Slovenia, with using the FRAP and the DPPH methods, results showed that the antioxidant activity varies depending on the type of honey. Similarly, the honey from Acacia belonged to the antioxidatively weakest sample [Bertoncelj *et al*., 2007; Fikselová *et al*., 2014]. Floral honeys were observed to be low at antioxidant effect by DPPH method as well as at antimicrobial activity [Fikselová *et al*., 2014].

Forest honeys originating from Serbia were proved to have more effective antioxidant activity ranging from 1.58 to 2.71 mmol/kg. Dark honeys were shown to be antioxidative the best samples in several studies [Bertoncelj *et al*., 2007; Wilczyńska 2010], they are often a rich source of vitamins and minerals, but their variation in the content of the various honeys is large [Bradbear *et al*., 2009]. Four forest samples monitored in our study (No. 1,2,3,11) created four homogenous groups, which shows that their origin had a significant effect on their antioxidant effect. Even forest samples (no. 2 and 3) from the same altitude (1250 m) but originating from different places of Serbia created different homogenous groups (i, fg).

The ABTS assay showed good antioxidant effect of honeydew honey (2.12 mmol/kg). Honeydew and chestnut honeys produced in a European Atlantic area had the highest mineral, protein, and flavonoid contents, as well as the highest antioxidant activities [Escuredo *et al*., 2013]. The highest antioxidant activity in our study showed buckwheat honey (4.63 mmol/kg), which was confirmed also in our previous research by DPPH method [Fikselová *et al*., 2014]. High antioxidative effect is mainly due to flavonoids content, which represent a major group of natural antioxidants in buckwheat. Following our previous results [Ivanišová & Fikselová, 2010], the highest antiradical activity measured by DPPH method was shown for the sample of buckwheat extract as well.

TABLE 2. Analysis of variance (ANOVA) for ABTS (mmol/kg) by sample.

Source of variability	Sum of squares	Df	Mean square	F-Ratio
Between groups	30.03	14	2.15	678.98**
Within groups	0.05	15	000	–
Total	30.08	29	–	–

** statistically significant at $\alpha<0.01$; df - degree of freedom; n=30.

TABLE 3. The average values of ABTS method determined in honeys and homogeneous groups based on Fisher's test.

No. of sample[b]	ABTS (mmol/kg)	Conf. intervals	
	Mean [a]	-95 %	+95 %
8	0.62 [a]	0.54	0.71
7	0.64 [a]	0.56	0.73
14	0.85 [b]	0.77	0.94
13	0.90 [b]	0.81	0.98
4	1.11 [c]	1.03	1.20
6	1.42 [d]	1.33	1.50
9	1.47 [de]	1.38	1.55
5	1.49 [de]	1.40	1.57
11	1.58 [e]	1.49	1.66
15	2.12 [f]	2.04	2.21
3	2.19 [fg]	2.11	2.28
1	2.25 [g]	2.16	2.33
10	2.45 [h]	2.36	2.53
2	2.71 [i]	2.62	2.79
12	4.63 [j]	4.55	4.72

[a] Values in the same column with different letters are significantly different ($\alpha<0.05$).

[b] **1** forest (Serbia), **2** forest (Serbia), **3** forest (Serbia), **4** floral (Poland), **5** heather (Poland), **6** floral (Poland), **7** floral, rape (Slovakia), **8** acacia (Slovakia), **9** floral (Slovakia), **10** forest raspberry (Slovakia), **11** forest (Serbia), **12** buckwheat (Poland), **13** floral (Poland), **14** floral (Slovakia), **15** honeydew (Slovakia).

Phenolic compounds variation in samples of honeys

A number of studies assumed that eating plant foods containing phenolic compounds may contribute significantly to health improvement [Naczk & Shahidi, 2004]. Honey is a rich source of phenolic acids and flavonoids [Farooqui *et al*., 2011], polyphenolic compounds of honey act as natural antioxidants and are becoming increasingly popular because of their

TABLE 4. The average levels of polyphenols (TPC) expressed as gallic acid equivalent and homogeneous groups based on Fisher's test.

No. of sample	TPC (mg/kg)	Conf. interval	
	Mean	-95 %	+95 %
13	611.20[a]	593.35	629.04
8	635.98[a]	618.14	653.82
7	703.72[b]	685.88	721.57
14	704.20[b]	686.36	722.04
11	769.50[c]	751.66	787.34
1	804.64[d]	786.80	822.48
15	899.72[e]	881.87	917.56
2	909.17[e]	891.33	927.01
6	961.19[f]	943.35	979.03
4	990.01[g]	972.17	1007.85
3	1052.09[h]	1034.25	1069.93
5	1124.73[i]	1106.89	1142.57
10	1244.80[j]	1226.95	1262.64
9	1257.12[j]	1239.28	1274.96
12	2962.24[k]	2944.40	2980.08

[a] Values in the same column with different letters are significantly different ($\alpha<0.05$).
[2] **1** forest (Serbia), **2** forest (Serbia), **3** forest (Serbia), **4** floral (Poland), **5** heather (Poland), **6** floral (Poland), **7** floral, rape (Slovakia), **8** acacia (Slovakia), **9** floral (Slovakia), **10** forest raspberry (Slovakia), **11** forest (Serbia), **12** buckwheat (Poland), **13** floral (Poland), **14** floral (Slovakia), **15** honeydew (Slovakia).

TABLE 5. Analysis of variance (ANOVA) for TPC (mg/kg) by sample.

Source of variability	Sum of squares	Df	Mean square	F-Ratio
Between groups	9,076,740.00	14	648,339.00	4626.15**
Within groups	2102.20	15	140.15	–
Total	9,078,840.00	29	–	–

** statistically significant at $\alpha<0.01$; df - degree of freedom; n=30.

potential role in the protection of human health. These substances can also be used as an indicator of geographical origin and source of honey [Tulipani *et al*., 2009]. Correlation analysis related the contents of the polyphenolic compounds with the antioxidant activities of the honeys, indicated that the flavonoids had a great influence on this activity [Escuredo *et al*., 2013]. Thirty two samples of different types of Polish honeys were investigated by Wilczyńska [2010] in order to assess their total phenolic content and potential antioxidant activity. Results of the study showed that the total phenolic content and antioxidant activity differed widely among different honey types.

Mean TPC in our samples expressed as gallic acid equivalent ranged from 611.20 to 2962.24 (mg/kg). By monitoring the impact of individual samples on TPC it was found that the sample had a highly statistically significant effect. Samples formed 11 homogeneous groups which differed among each other in their TPC (Table 4 and 5). Four forest samples established similarly 4 homogenous groups as in the case of the antioxidant activity .

TPC in multifloral honeys ranged from 611.00 to 1257.12 mg/kg, within them in honeys originating from Poland it ranged from 611 to 990 mg/kg, and Slovak multifloral honeys it ranged from 703.7 to 1257.12 mg/kg.

Forest honeys originating from Serbia were found to have similar TPC, which ranged from 769 to 1052 mg/kg. Although the forest honeys were found to be more effective antioxidants in general compared to floral ones, in TPC they were similar, therefore it can be seen that not only phenolic substances are involved in the antioxidant effectiveness of forest honeys.

The highest TPC (2962.24 mg/kg) was found in a sample of buckwheat honey, which is in accordance with our antioxidative results of buckwheat honey. Phenolic content of the honey samples is partially responsible for their antioxidant activity, which supports the relevance of this type of honey being an important dietary source of antioxidant compounds and its traditional use as a medicinal product [Silva *et al*., 2013].

Total polyphenols of honeydew honeys originating from Romania, Bulgaria, Croatia, Greece and Turkey were evaluated by Bobis *et al*. [2011]. Polyphenols, expressed as mg GAE/100 g, ranged from 53.91 to 196.0 mg GAE in honey from Romania; from 118.46 to 133.12 mg GAE in honey from Bulgaria; from 94.58 to 133.92 mg GAE in honey from Croatia *etc*. Honeydew honey from our study originating from Slovakia showed a high content of polyphenolic compounds in the amount of 899.72 mg/kg GAE.

Dry matter content variation in samples of honeys

The last parameter observed was dry matter content of honeys. The mean content of the dry matter in honey samples (Figure 1) ranged from 78.2 to 83.77%. By monitor-

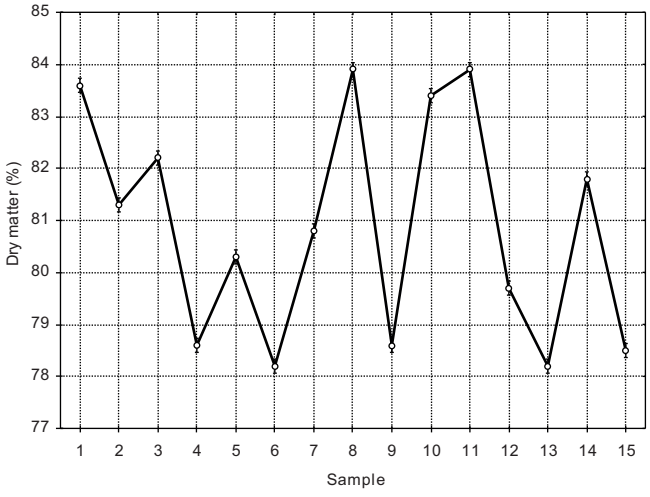

FIGURE 1. Variability of dry matter content (%) in honeys. Explanations: **1** forest (Serbia), **2** forest (Serbia), **3** forest (Serbia), **4** floral (Poland), **5** heather (Poland), **6** floral (Poland), **7** floral, rape (Slovakia), **8** acacia (Slovakia), **9** floral (Slovakia), **10** forest raspberry (Slovakia), **11** forest (Serbia), **12** buckwheat (Poland), **13** floral (Poland), **14** floral (Slovakia), **15** honeydew (Slovakia).

ing the impact of individual honey samples on the dry matter content of honey we confirmed that the sample has a highly statistically significant effect (not shown). Further testing by the Fisher test showed relative differences among samples of honey in dry matter content, 11 homogenous groups were formed (not shown).

SUMMARY AND CONCLUSIONS

Antioxidant activity of honeys could be a positive influence factor in terms of honey differentiation, especially in the case of the forest honeys collected from different places. The analysed monofloral and multifloral honeys (10) established 5 homogenous groups, but in the case of several multifloral honeys which originated from Poland and Slovakia no statistical significant differences were found.

Forest honeys were proved to have more effective antioxidant effect compared to floral ones, although their phenolic content was similar to floral honeys. The ABTS assay showed good antioxidant activity of honeydew honey, the highest antioxidant activity had buckwheat honey, while the antioxidative weakest sample was the sample of acacia honey. By observing the impact of individual honey samples on the antioxidant activity it was statistically confirmed that the sample has a highly statistically significant effect and similar trend was determined in the case of total phenol and dry matter content of honeys.

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