INFLUENCE OF NON-PHENOLIC COMPOUNDS OF HONEY ON ANTIOXIDANT CAPACITY

Justinas Kretavičius[1], Bogumila Kurtinaitienė[2], Vilija Buckiūnienė[1]

[1]Lithuanian University of Health Sciences, Veterinary Academy
Tilžės str. 18, LT-47181 Kaunas, Lithuania, tel/fax: +370 37 363505, e-mail: jkretas@gmail.com
[2]Vilnius University Institute of Biochemistry Sauletekio av. 9, LT-10222 Vilnius, Lithuania

Abstract. Honey is a natural product consisting of multiple components which determine its dietary and medicinal properties. Honey of different botanical origin were analyzed for *in vitro* antioxidant capacity and physico-chemical properties. The aim of this study was to establish relationship between non-phenolic compounds (5-hydroxymethylfurfural (HMF) and glucose oxidase (GOD)) and antioxidant capacity considering botanical origin. Antioxidant capacity was measured by the scavenging of 2.2 azino-bis-3-3 ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cations. The buckwheat honey showed the highest values of antioxidant scavenging activity, the rape honey – the lowest. HMF contribution to the antioxidant capacity was tested. It had doubtful influence on the antioxidant capacity. Bee secreted compounds (enzymes, proteins, etc.) in honey also might increase antioxidant capacity: it increased strongly in light-coloured honey, and slightly – in dark-coloured honey.

Keywords: ABTS, antioxidant capacity, glucose oxidase, HMF, honey

Introduction

Despite the relevant importance of polyphenolic compounds, which are recognized as the major constituents and responsible for the health-promoting properties of honey (Meda Lamien etal., 2005; Habib et al., 2014), their identification and quantification are of great interest for understanding their contributions to the overall bioactivity of honey (Manzanares et al., 2014). Free radicals are molecules with unpaired electrons or an open shell configuration. These unpaired electrons cause radicals to be capable of reacting with other molecules, cell membrane and functional units of DNA. This results in cellular damage. The main reasons for the formation of free radicals in the living organism are the reaction of oxidation/reduction or metabolism processes. Consequently, air pollution, too intense fitness, radiation, inadequate nutrition, stress or cigarette smoke may contribute to an increased formation of free radicals. Fortunately, there are the compounds that neutralize free radicals, antioxidants. Vitamins A, C, E, phenolic and polyphenolic compounds found in fruits and vegetables are attributed to natural antioxidants. Many foods are source of antioxidants, among them – natural honey (Alvarez-Suarez et al., 2012). Honey is used for various purposes: human nutrition and remedy. It was reported that the antioxidant capacity of honey depends on the floral source used to collect nectar; seasonal, environmental and other factors also may effect on antioxidant activity. Therefore, it is important to know what type of honey neutralizes free radicals efficiently (Alvarez-Suarez et al., 2009).

Activity of enzymes (diastase or invertase), honey acidity, carbohydrates content, humidity and hydroxymethylfurfural (HMF) content are analyzed before supplying honey to the market. In addition to these physico-chemical parameters, the consumers draw attention to the colour and consistency of honey.

Fructose in honey is converted into HMF by heat, light or storage action. HMF is toxic and carcinogenic substance (Janzowski et al., 2000; Sanz et al., 2003). In fresh unheated honey HMF content is about 5-30 mg kg^{-1}. In accordance with Honey Quality and International Regulatory Standard it should not exceed 40 mg kg^{-1} (Bogdanov et al., 1997). In addition, HMF has a heterocyclic structure similar to phenolic compounds, and some authors consider that HMF may have an impact on antioxidant capacity of the honey (Gheldof et al., 2002).

Proteins found in honey also may possess antioxidant capacity. Free oxygen is consumed in liquid nectar by the enzymatic action of glucose oxidase, and it can reduce the oxidation of honey compounds (Bogdanov et al., 2008; Alvarez-Suarez et al., 2010).

Recently data on the antioxidant capacity of the different type honey and correlation between various physico-chemical properties are published a lot. Phenolic and polyphenolic compounds significantly contribute to the antioxidant capacity. On the other hand, more and more non-phenolic compounds that exhibit antioxidant activity were discovered. Some authors presented the presence of alpha-tocopherol, carotenoides, proteins and melanoidins (Brudzynski, Miotto, 2011a). Recent studies on honey indicated that the biological actions of honey can be ascribed to its polyphenolic content, which are elucidated by its antioxidant,anti-inflammatory, antiproliferative and antimicrobial actions (Alvarez-Suarez et al., 2013).

The purpose of this paper is to evaluate the antioxidant activity of honey in different botanical origin (spring, rape and buckwheat) and to clear up the influence of non-phenolic compounds (HMF and glucose oxidase) on antioxidant capacity. The paucity of papers in this area clearly indicates a need for investigation of the contribution of HMF and glucose oxidase on antioxidant activity in honey.

Materials and methods

Materials. HMF (5-hydroxymethylfurfural).

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-2-carboxylic acid) and ABTS (2,2-azino-bis-3-3ethylbenzothiazoline-6-sulfonic acid) were purchased from Sigma (Germany). Phadebas amylase test was purchased from Magle (Sweden). NaOH, HCl were purchased from Avsista (Lithuania). Sodium persulphate, sodium acetate, inorganic saline and methanol were purchased from Lachema (Czech Republic). HMF was high performance liquid chromatography (HPLC) grade. All other reagents and solutions were of analytical grade. PMMA cuvettes (1.5 ml volume, 1 cm optical path) were purchased from Brand GmbH (Germany). Spectrophotometer UV-300 was purchased from ThermoSpectronic (United Kingdom).

Honey samples. Honey samples were collected from various Lithuania districts. Polyfloral spring, monofloral rape and buckwheat honeys were selected for the research. *Sugar syrup* was produced in this way: new wax honeycomb was set in moderate honeybee colony centre in 2011 September. Bees were fed by 70% w/v aqueous sugar solution. Honey and sugar syrup was extracted separately when comb was fully capped with wax. In addition, 80% w/v *artificial syrup* was chosen as a reference. Thus, 5 honey samples and 1 bees fed sugar syrup were collected for this research. Fresh honey samples were packed in 1l plastic tare and stored at +5°C in the dark until analysis. Analyses of honey collected were performed after 14 and 2 months after honey extraction, respectively. Therefore all sample analyses were carried out simultaneosly. Aqueous honey solution (1:1 w/v) was prepared by diluting 1g of honey with 1ml of distilled water. Such solution was used in antioxidant capacity determination, glucose oxidase analysis and colour intensity measurement.

Determination of botanical origin. Botanical origin of the honey was determined using method of melissopalynology. Frequency occurrence of pollen was calculated as a percentage of total of pollen of plants. Honey is considered to be monofloral if the dominant pollen frequency of that plant was found over 45% of the total pollen amount, and polyfloral if there was not enough pollen (45%) from specific monofloral species (Louveaux et al., 1970).

Determination of total antioxidant capacity. Antioxidant capacity of honey samples in the reaction with stable $ABTS^+$ radical cations was determined according to the modified methods of Re et al. (1999) and Van den Berg et al. (1999). An ABTS solution (2mM) was prepared by mixing ABTS in phosphate buffer (pH 7.4). Sodium persulphate solution (70 mM) was prepared by mixing sodium persulphate in phosphate buffer (pH 7.4). 200 ml of prepared ABTS solution (2 mM) was added to 0.2 ml of sodium persulphate solution (70 mM).This mixture was allowed to stand in the dark at room temperature for 15 hours until use. The reaction of ABTS and sodium persulphate will result in cation radical formation. The ABTS solution was diluted with phosphate buffer (pH 7.4) to an absorbance of 2 at 736 nm. Aqueous honey solution (1:1 w/v) was diluted in 4ml of 100 mM phosphate buffer (pH 7.4) solution. For measuring antioxidant capacity 20 µl of the honey solution was mixed with 980 µl of the radical solution. The absorbance was monitored 30 minutes after this mixing. The decrease in absorption at 736 nm is proportional to the antioxidant capacity of honey. A trolox aliquot was used to develop 0-30 µmol l^{-1} standard calibration curve. Standard curve was prepared using a series of known trolox concentrations in methanol. The calculated slope of the Trolox calibration curve was 0.03 AU mM^{-1} (R^2=0.998). All data expressed as micromoles of Trolox equivalents per gram of honey (TE µmol g^{-1}). In order to evaluate the potential influence of 5-hydroxymethylfurfural (HMF) on antioxidant capacity, HMF-supplemented honey and sugar syrup was prepared. Aqueous HMF solution was prepared by diluting 80 mg of synthetic HMF with 1 l of distilled water. Aqueous honey solution (1:1 w/v) was rapidly mixed with aqueous HMF solution (80 mg l^{-1}) to make a final concentration of 40 mg kg^{-1} of honey. 20 µl of the HMF-supplemented honey solution was mixed with 980 µl of the ABTS radical solution, and antioxidant activity was measured.

Evaluation of physico-chemical parameters. HMF content and carbohydrate composition analysis were carried out by the HPLC. Glucose-oxidase activity was measured using amperometric method according to Kretavičius et al. (2010). The results were expressed in µmol $min \cdot g^{-1}$ of honey. Moisture content was determined by refractometry using VHNI model refractometer (AOAC, 1990). The pH was assessed in a 10% (w/v) aqueous solution of honey. The lactones acidity corresponds to the combined acidity which is not directly titrable. The acidity of the lactones was determined as follows: excess of 0.05 M NaOH (10 ml) was immediately added to 10% honey solution and without delay back-titrated with 0.05 M hydrochloric acid to the equilibrium point. The lactones acidity was expressed in meq kg^{-1} (Bogdanov, 2002). Colour intensity was determined by spectrophotometric measurement as described by Beretta et al. (2005), where the net absorbance of a 50% honey solution (w/v) was defined as the difference between the absorbance at 450 and 720 nm. The colour intensity was expressed in absorbance units (AU).

Statistical analysis of the results was carried out with the SPSS statistical programme package. All the analyses were carried out in triplicate. If the error between the repetitions was higher than 5%, an additional testing was made, and the average of the closest values was calculated. All values were expressed as the mean ± standard deviation. Correlation coefficients (r) between colour, antioxidant capacity, enzyme activity and physico-chemical parameters were estimated at significance level of $P < 0.05$.

Results and discussion

Characterization of the honey samples. The floral sources and detailed characterization of honey samples are summarized in Figure 1. The examined honey samples showed high variability in many parameters depending on the botanical composition.

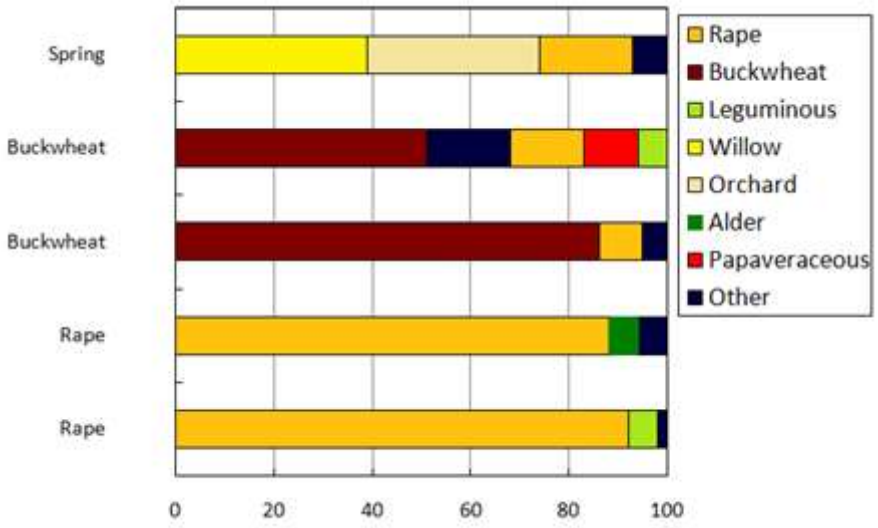

Figure 1. **Botanical composition (pollen content, %) of the examined honey samples**

Investigated parameters of honey are presented in Table 1. Humidity (a parameter related to the maturity degree) is between 15.9% and 18.4%. The values obtained are below 20% that is maximum limit allowed by the European Community Directive. All honeys are acidic having a pH in the range of 3.5-4.5. The acidity of honey is due to the presence of organic acids particularly the gluconic acid.

Table 1. **Physico-chemical parameters of honey**

Sample	Glucose oxidase activity µmol min·g^{-1}	Lactones acidity meq kg^{-1}	HMF* amount mg kg^{-1}	pH	Humidity %
Honey harvested in 2010					
Buckwheat	1.14±0.024	29.2±1.42	8.2±0.90	3.6±0.04	18.4±0.04
Rape	0.55±0.119	8.4±1.08	2.8±1.12	3.7±0.03	16.3±0.04
Spring	1.03±0.060	16.4±1.01	1.4±0.34	4.5±0.03	17.2±0.06
Honey harvested in 2011					
Buckwheat	1.09±0.120	36.1±1.68	9.6±0.87	3.6±0.05	17.7±0.04
Rape	0.34±0.073	10.0±1.08	3.1±1.03	3.5±0.04	18.0±0.04
Sugar syrup	0.13±0.050	12.0±1.17	-	3.8±0.03	15.9±0.05

*-5-hydroxymethylfurfural

Glucose oxidase activity, lactones acidity and HMF amount were found to be the highest in buckwheat honey (Table 1). The lactones acidity of rape honey was 1.95 times lower ($P<0.05$) than those of spring honey, and 3.5 times lower ($P<0.01$) than those of buckwheat honey (Table 1). This could be explained by a different glucose oxidase (GOD) activity and distinct time of action. In addition, glucose oxidase of the rape honey is 2.07 times lower than in the buckwheat honey. It has been reported that on glucose oxidase action, gluconic acid (gluconolactone) and hydrogen peroxide is produced. In the end of the process of converting nectar to honey, the glucose oxidase stops acting, but the gluconolactone remains in honey and is in equilibrium with gluconic acid. Thus, gluconolactone provides the major contribution to the lactone acidity (White et al., 1963). It was proved by our established relationship between glucose oxidase activity and lactones acidity $r = 0.504$ ($P < 0.05$, data not shown). Perhaps, sugar concentration in nectar has an influence on glucose oxidase activity. Honey ripens longer time when sugar content in nectar is lower. Therefore, the longer time of manipulating nectar caused the larger secreted enzyme content from bee hypopharyngeal glands. The sugar concentration is influenced not only by the botanical origin but also by the meteorological conditions (Bonvehi, Torrendo, 2000). Normally, buckwheat nectar ripens longer than spring or rape honey because buckwheat blooms later, in the end of summer. In August-September, the weather is wetter and chilly. Such meteorological conditions could result in lower carbohydrate content in nectar. Therefore, glucose oxidase activity in buckwheat honey is apparently higher than in rape or spring honey.

The main monosacharides (glucose, fructose) and dissacharides (sucrose, maltose) composition of examined honey is presented in Table 2. Glucose was a major sugar quantitatively in rape and spring honeys. In buckwheat honey the concentration of glucose (36.8%) was lower than that of fructose (39.2%).

Table 2. **Carbohydrate composition (%) of honey of different botanical origin**

Sample	Glucose	Fructose	Sucrose	Maltose
Honey harvested in 2010				
Buckwheat	36.80	39.20	0.86	3.35
Rape	39.52	38.80	1.24	1.26
Spring	36.50	35.49	1.38	2.49
Honey harvested in 2011				
Buckwheat	34.20	37.11	1.52	2.78
Rape	40.77	35.73	2.41	1.39
Sugar syrup	25.90	34.80	11.57	2.63

The colour intensity was very variable and ranged from 0.12 to 1.5 AU of rape and buckwheat honey, respectively (Table 3). Literature indicates that light-coloured honey is characterized by low colour intensity (approximately 0.1 AU), whereas colour intensity of dark-coloured is more than 3 AU (Berreta et al., 2005). Our studied honey samples support these data. It was found that colour intensity of rape honey is only two times greater than that of sugar syrup. Rape honey is very light whereas sugar syrup is extra-light, i.e. almost transparent.

Table 3. **Colour intensity and antioxidant capacity of honey of different botanical origin**

Sample	Colour intensity AU	Antioxidant capacity TE μmol g^{-1} of honey	HMF contribution to total antioxidant capacity ΔTE μmol g^{-1}
Honey harvested in 2010			
Buckwheat	1.55±0.077	9.81±0.412	0.24±0.063
Rape	0.19±0.025	1.15±0.289	0.33±0.058
Spring	0.32±0.010	2.53±0.374	0.38±0.071
Honey harvested in 2011			
Buckwheat	0.76±0.034	9.65±0.394	0.31±0.078
Rape	0.12±0.017	1.15±0.281	0.25±0.056
Sugar syrup	0.04±0.012	0.93±0.137	0.31±0.068

Antioxidant activity of honey samples is presented in Table 3. Antioxidant capacity of buckwheat honey noticeably distinguished from the other honey samples. It has been reported that buckwheat honey is characterized by the highest antioxidant capacity meanwhile red clover, rape, acacia and citrus honey – by the lowest (Bogdanov et al., 2008; Brudzynski, Miotto, 2011b). The values of AC of our studied buckwheat honey are similar to those found in literature.

The highest HMF amount was found in buckwheat honey (Table 1). HMF is formed from the dehydratation of fructose. It was noted that fructose content of buckwheat honey was found to be the highest (Table 2).

Evaluation of the antioxidant activity properties. In general, the antioxidant properties of different honey samples depend on the structures of their phenolic compounds, as a consequence of the ability of these compounds to donate the hydrogen ion or electrons for the free radicals (Gašić et al., 2014). Antioxidant capacity and related parameters of honeys of different botanical origin are shown in Table 3. It appears that AC of buckwheat honey is the highest, and it is 8.6 times greater than rape honey, as well as 3.8 times greater than spring honey. Antioxidant capacity is strongly correlated with colour intensity (Table 4). Correlation coefficient *r* is 0.905 between separate honey samples. Similar relationship between colour intensity and antioxidant capacity is described by several authors (Wang et al., 2004; Berreta et al., 2005; Turkmen et al., 2006). They stated that colour of honey partly reflects the content of pigments with antioxidant properties (carotenoids, flavanoids, etc.).

Correlation matrix (Table 4) shows a correlation between several parameters of honey. It was found strong correlation between antioxidant capacity and other parameters (colour intensity, lactones acidity, HMF amount and GOD activity). Data found in literature are contradictory to ours: correlation coefficient between AC and HMF is 0.144, between AC and lactones content is 0.28 (Gheldof et al., 2002). It is difficult to explain such a variance. Probably, the fact that all the parameters were investigated using different methods has an influence.

Table 4. Correlation matrix

	Colour intensity	HMF	Glucose oxidase activity	Lactones acidity
Colour intensity	1.00	0.953	0.483	0.943
Antioxidant capacity	0.905	0.944	0.801	0.964

* significance level of $P<0.05$

In general, HMF is toxic compound but some authors suggested that HMF could be developed as a novel antioxidant (Gheldof et al., 2002). On the one hand, our established correlation coefficient between antioxidant capacity and HMF amount (r=0.944, P<0.05) strongly supports this affirmation (Table 4), but on the other hand more detailed analysis of potential HMF impact is required. To examine the prevailing opinion about HMF influence on antioxidant capacity, honey samples and sugar syrup were supplemented with synthetic HMF (up to 40 mg kg^{-1} in all samples). Antioxidant capacity of HMF-supplemented honey and syrup was estimated, and corresponding correlation coefficient between antioxidant activity and added HMF amount in separate samples was calculated additionally (r=0.078, P<0.01, data not shown). Antioxidant activity of the samples with added HMF increases by approximately 0.31 TE (Table 3), but low correlation coefficient (r=0.078) indicates that HMF has not considerable contribution to the antioxidant capacity of honey.

It was examined the contribution of bees secreted compounds for the antioxidant capacity. For this purpose sugar syrup ability to neutralize free radicals was tested as a reference. This sugar syrup was used for the determination of AC of bees` secreted compounds in honey. Bees were fed with sugar syrup in September when there were no blooming plants in Lithuania. Therefore it was assured that only bees secreted compounds were found. GOD activity of sugar syrup was established to be 0.13±0.05 μmol min g^{1} of honey (Table 1). It has appeared that the AC of sugar syrup and rape honey has no statistically significant difference P>0.05 (Table 3). Colour intensity of rape honey and sugar syrup also has no large difference (Table 3). Meanwhile colour intensity of buckwheat and rape honey differs a lot as well as AC. Dark-coloured honey contain much phenols and present high AC. Rape honey usually is light-coloured, even white, and contain less phenols as well as AC (Berreta et al., 2005; Brudzynski, Miotto, 2011b). Therefore it is reasonable to consider that bees secreted compounds (enzymes, amino acids, etc.) could strongly influence AC of honey containing poor phenols content. Bogdanov et al. (2008) also supposed that proteins originating from honeybees can influence antioxidant capacity.

Conclusions

It was clarified that HMF could not influence antioxidant capacity. Bees secreted compounds (glucose oxidase, etc.) could significantly contribute to AC of light-coloured honey, and slightly increase AC of dark-coloured honey. Dark-coloured honey showed higher AC that the light-coloured honey.

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