

# Characterisation of the total phenolic, vitamins C and E content and antioxidant properties of the beebread and honey from the same batch

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**Abstract:** The aim of this study was to compare total phenolic and flavonoid content, antioxidant and reducing activity, as well as vitamin C and E content in beebread and honey. The antioxidant activity of samples was determined by photochemiluminescence (PCL) assay, while reducing potential was measured using FRAP and ORAC assays. Vitamin C was analysed by HPLC–MS–TripleTOF method and vitamin E by HPLC–DAD. Beebread was characterised by the higher level of the total phenolic (~90%), flavonoid (~97%), vitamin C (~99%) and E (~98%) as compared to the honey. The data showed that beebread had also higher values of antioxidant activity and reducing potential by ~99% and ~41–98%, respectively, than honey. Our findings indicate that beebread is valuable source of bioactive substances, with the high beneficial properties.

**Keywords:** antioxidant status; ascorbic acid; bee products; lipid-soluble antioxidants; phenolics and flavonoids; reducing capacity

Honey is a natural product formulated by bees from flowers' pollen. Numerous studies have shown that honey due to its health properties can be used in prevention of some diseases e.g. cancer, infectious diseases, and wounds (Mandal & Mandal 2011; Badolato et al. 2017; Waheed et al. 2018). As studies show, these health effects might be correlated with high antioxidant activity of different honey samples (Al-Marmay et al. 2002). Nowadays, more attention is focused on nutritional and health benefits of bee products, other than honey, such as propolis and beebread. Beebread is a mix of flower pollen with a small amount of honey and bee saliva, which are packed by bees to the honeycomb and then undergo chemical changes (Kieliszek et al. 2018). This mixture undergoes lactic fermentation under anaerobic conditions due to activity of enzymes from bee's saliva and *Lactobacillus*

(Ivanišová et al. 2015). Nagai et al. (2004) confirmed the high scavenging activity of beebread against superoxide anion and hydroxyl radicals. The high scavenging ability might be correlated with significant quantities of bioactive compounds in beebread, such as flavonoids and phenolic acids that are described as strong antioxidants. However, it is important to remember that the phenolic compounds content and their antioxidant activity might be different in case of beebread and honey depending on the type and geographical origin. For consumers the main positive aspects of beebread usage in daily intake are e.g. immune system support, regulation of digestive system function, reduction of allergic reactions, and decrease in cholesterol and total lipids level in blood (Kieliszek et al. 2018).

In our study, we presented differences between honey and beebread from the same origin and same bath

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in aspects of total phenolic and flavonoid compounds, vitamin C and E content, and functional properties, antioxidant activity and reducing potential.

## MATERIAL AND METHODS

**Chemicals.** Aluminium chloride hexahydrate; L-ascorbic acid; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); Folin-Ciocalteu's phenol reagent; 2,4,6-tris(2-pyridyl)-1,3,5-triazyn (TPTZ); iron (III) chloride hexahydrate; sodium acetate; acetic and hydrochloric acid, gallic acid and quercetin were purchased from Sigma (Sigma Chemical Co., USA). PCL kits for lipophilic (ACL) and hydrophilic antioxidants (ACW) were bought from Analytik Jena (Germany). Water was purified by a Mili-Q-system (Milipore, USA).

**Determination of total phenolics and flavonoids content (TP, TF).** Beebread and multifloral honey were obtained from Podlasie region (north-east of Poland) by professional beekeeper. The samples were collected in the year 2018. Beebread was packed in polypropylene bags and kept in refrigeration at 4 °C. Beebread and honey samples were extracted according to methodology described by Wilczyńska (2009) with ethanol. Then extracts were stored in –20 °C until the analysis.

The measurements of TP and TF contents were performed in microplates (Infinite M1000 Pro Multimode Microplate Reader; Tecan, Austria) according to the procedure described previously by Horszwald & Andlauer (2011). The results were calculated as milligram of gallic acid equivalent (GAE) g<sup>-1</sup> in TP, and as mg of quercetin equivalent (QE) g<sup>-1</sup> in TF.

**Determination of vitamin C content.** Vitamin C was determined according to Szultka et al. (2014). The extract of vitamin C (L-ascorbic acid) from the samples was obtained using 5% metaphosphoric acid (MPA). About 0.1 g of each sample was extracted with 1 mL of the solvent by vortexing for 30 s. The mixture was sonicated for 30 s (VC 750; Sonics & Materials, USA), vortexed and sonicated again, and centrifuged (Centrifuge 5415R; Eppendorf, Germany) for 10 min (13 200 × g at 4 °C). Supernatant was collected in 3 mL flask. This step was repeated 3 times.

The analysis of obtained extracts was performed by means of micro-HPLC system (LC200; Eksigent, Canada) coupled with TripleTOF 5 600+ mass spectrometer (AB SCIEX, Canada). After injecting an aliquot (5 µL) of sample solution into HPLC systems, the separation of the compounds was carried out on 3C18-AQ-120 100 × 0.5 mm column (Eksigent, Can-

ada) at 45 °C with the flow rate of 25 µL min<sup>-1</sup>. The analysis was based on scanning in negative ionization mode.

**Determination of vitamin E content.** The analysis of vitamin E (tocotrienols and tocopherols) was performed according to the methodology described by Zieliński et al. (2007). Approximately 150 mg of honey or beebread sample was extracted with 1 mL of methanol. The mixture was vortexed for 30 s, then sonicated for 30 s and centrifuged for 10 min (13 200 × g at 4 °C). The supernatants were combined and collected in a 3 mL flask, then the methanolic extract was dried under nitrogen at 30 °C. All samples with a dry residue after methanol evaporation were dissolved in 0.5 mL of 100% acetonitrile, centrifuged (20 min) and injected into the HPLC (LPG-3400M WPS-3000TSL; Dionex, USA) with fluorescent detector (FLD-3400RS; Dionex, USA).

**Determination of antioxidant activity and reducing potential.** The PCL measurement was performed using PHOTOCHEM apparatus (Analytik Jena, Germany) according to the protocols of PCL ACL and ACW developed by Zieliński et al. (2007). The reducing power was determined using ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assay according to Horszwald & Andlauer (2011). All measurements were conducted three times. The obtained results were expressed as µmol Trolox g<sup>-1</sup>.

**Statistical analysis.** All results were expressed as means ± SD of three independent experiments. Differences among samples were tested using one-way ANOVA with Tukey multiple comparisons test using the GraphPad Prism version 8 (GraphPad Software, USA).

## RESULTS AND DISCUSSION

**Total phenolic and flavonoids content.** The TP and TF content in beebread and honey are presented in Table 1. Ten times higher TP value was observed in beebread comparing to honey. Our results are in agreement with Zuluaga et al. (2015) findings, who measured TP content of beebread in a range of 2.5–13.7 mg GAE g<sup>-1</sup>. TP of Romanian beebread was estimated also at high level of 6.30–12.83 and 8.32 mg GAE g<sup>-1</sup> (Stanciu et al. 2007; Urcan et al. 2018). Furthermore, beebread from Ukraine was characterised as a rich source of phenolic compounds at the level of 12.36–18.24 mg GAE g<sup>-1</sup> (Ivanišová et al. 2015). This might be due to geographical location and difference in the year of the harvest. TP and TF content in honey was similar to results obtained by Socha et al. (2018) They determined TP in multifloral honey in a range from 0.42 to 0.56 mg GAE g<sup>-1</sup>,

Table 1. Results of TP, TF and vitamin C content in beebread and honey

	TP (mg GAE g <sup>-1</sup> )	TF (mg QE g <sup>-1</sup> )	Vitamin C (ng g <sup>-1</sup> )
Beebread	4.87 ± 0.04	59.06 ± 0.06	0.36 ± 0.01
Honey	0.49 ± 0.02	1.83 ± 0.01	0.002 ± 0.00

Data are expressed as mean ± standard deviation ( $n = 3$ )

and TF content between 0.07–0.13 mg QE g<sup>-1</sup>, however, it was almost 2-times higher than results presented by Wesołowska and Dżugan (2017), who established the level of TP for multifloral honeys from southern part of Poland in the range of 0.09–0.276 mg QE g<sup>-1</sup>. Socha et al. (2018), observed a significant increase in phenolic and flavonoid compounds in honey fortified with beebread (with 10 and 20% addition). Therefore, TP value increased up to 1.38 mg GAE g<sup>-1</sup>. This trend was similar to TF, where value increased up to 0.48 mg QE g<sup>-1</sup> in honey samples with beebread addition. This experiment demonstrated an interesting approach for the improvement of nutritional value of multifloral honeys, which usually are characterised by considerably low TP (0.37–0.46 mg GAE g<sup>-1</sup>), in comparison to dark honeys (Wilczyńska 2010).

**The content of vitamin C and E.** As shown in Table 1, the content of vitamin C was about 99.5% higher in beebread than in honey. Our data of ascorbic acid concentration are lower than level of this substance found in the Portuguese (Karabagias et al. 2018) and Tunisian (Boussaid et al. 2018) honeys. The level of vitamin C in the Brazilian bee pollen was in the range from 60.3 to 797.0 µg g<sup>-1</sup> (Sattler et al. 2015), while bee pollen harvested in Portugal was characterised by even higher content of vitamin C (46.9–214.4 mg g<sup>-1</sup>) (Estevinho et al. 2019). Moreover, the place of origin and the harvest period affect the concentration of bioactive substance (including vitamin C) (Sawicki et al. 2019), as well as the extraction and analysis method (Wang et al. 2004; Boussaid et al. 2018). Another reason for low vitamin C amount in tested samples, could be pH-unstable environment. The pH values of honey and beebread were 4.31 ± 0.01 and 3.90 ± 0.01, respectively, with significant differences ( $P < 0.01$ ) between samples.

As shown in Table 2, the total Vitamin E content was detected within the range of 0.45–32.55 ng g<sup>-1</sup>. The highest concentration of Vitamin E was characterised in the beebread (32.55 ± 1.41 ng g<sup>-1</sup>) and was over seventy-two times higher than in honey. However, in the case of T3, the content of these compounds was 0.02 for honey and 4.63 ng g<sup>-1</sup> for beebread. The predominant compound among T3 in beebread was β+γ-T3, and constituted 75.8% of total T3, next was α-T3 with approx. 20% of the total T3. On the other hand, the main compounds among T3 in honey were δ-T3 and β+γ-T3, and both accounted for 50% of the total T3. Moreover, in honey α-T3 has not been detected. The highest concentration of tocopherols was found in the beebread, similar as in the case of T3 level. The T content in beebread was sixty-five times higher than content of these compounds in honey. The main isomer of T in the beebread was α-T and constituted 86.5% of the total T (Table 2). The second isomer from the T found in beebread was β+γ-T, and represented approx. 9% of the total T. This profile was similar to the data showed by Tomás et al. (2017). The similar profile of T was characterised by honey, where the dominant compound was α-T (81% of the total T). Whereas, the isomer from T in the honey with next highest contribution was β+γ-T (12%). High α-T content was previously shown in beebread samples, and it was a predominant lipophilic antioxidant (Hryniewicka et al. 2016). Moreover, the individual contribution of β+γ-T and δ-T was higher in honey than in beebread. Furthermore, in both cases, for the level of vitamin E in honey and beebread mainly tocopherols were responsible, which constituted 95.5% and 85.8% of the vitamin E content, respectively. The results provided by this study show that the beebread is characterised by the richest profile of T and T3, which results consequently in a higher content of vitamin E comparing to honey.

**Determination of antioxidant activity and reducing potential.** Figure 1 illustrates the antioxidant activity measured by PCL method for ACL or ACW predisposition. PCL results are also expressed here as a sum of ACL and ACW measurements. According to our data beebread represents significantly higher ( $P < 0.001$ ) antioxidant activity in comparison to honey. As a sum, PCL level for beebread was estimated

Table 2. Tocotrienol (T3) and tocopherol (T) content in beebread and honey samples (ng g<sup>-1</sup>)

	δ-T3	β+γ-T3	α-T3	Δ-T	β+γ-T	α-T	Total T3	Total T	Total
Beebread	0.17 ± 0.01	3.51 ± 0.11	0.95 ± 0.08	1.26 ± 0.02	2.51 ± 0.11	24.15 ± 1.07	4.63	27.92	32.55 ± 1.41
Honey	0.01 ± 0.00	0.01 ± 0.00	ND	0.03 ± 0.00	0.05 ± 0.00	0.35 ± 0.00	0.02	0.43	0.45 ± 0.00

Data are expressed as mean ± standard deviation (SD) ( $n = 3$ ); ND – not detected

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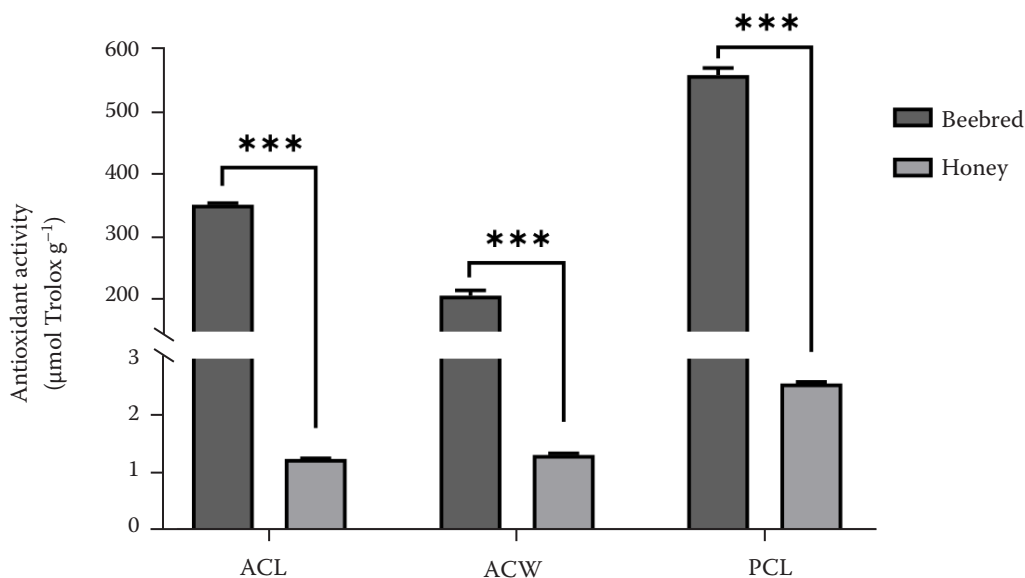


Figure 1. Antioxidant activity ( $\mu\text{mol Trolox g}^{-1}$ ) of beebread and honey provided by PCL method (ACL, ACW)

The results represent mean  $\pm$  SD of three independent determinations; significantly different values determined by one-way ANOVA analysis were marked as: \*\*\* $P < 0.001$ ; ACL – lipophilic antioxidants; ACW – hydrophilic antioxidants; PCL – photochemiluminescence

at  $557.00 \mu\text{mol g}^{-1}$ , and for honey at  $2.57 \mu\text{mol g}^{-1}$ , which gives 200-times higher antioxidant activity of beebread than honey. Our findings were similar to the results of multifloral Polish honey samples (Wesołowska & Dżugan 2017). Our results showed that beebread tended to be highly active in reaction with  $\text{O}_2^{\cdot-}$ . Moreover, the ratio of ACW/ACL was calculated in beebread and honey, and resulted in 0.59 and 1.06, respectively. This means that in honey the hydrophilic

fraction dominated, whereas in beebread-lipophilic. Higher value of ACL in beebread, might be linked to the high content of lipophilic antioxidants such as vitamin E. Furthermore, it may imply that tocopherols, and among them  $\alpha\text{-T}$ , are very active antioxidants and contribute highly in an overall antioxidant activity of beebread.

In Figure 2 reducing activity (RA) is presented. In both measurements the RA was higher for the beebread.

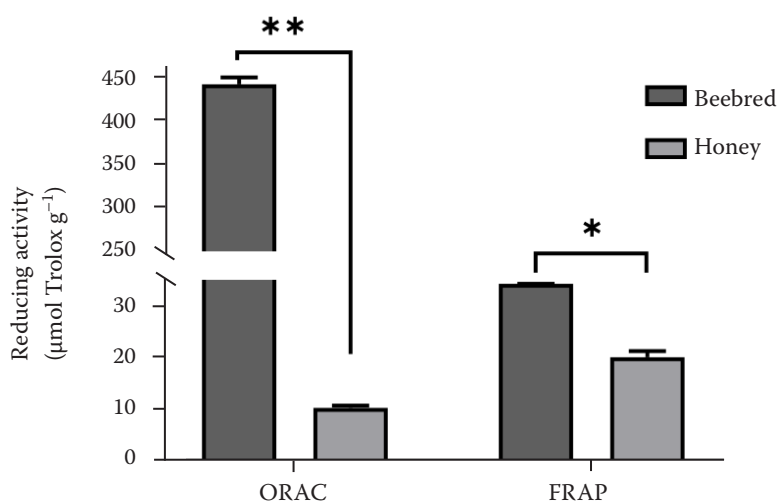


Figure 2. Reducing activity ( $\mu\text{mol Trolox g}^{-1}$ ) of beebread and honey provided by ORAC and FRAP assays

The results represent mean  $\pm$  SD of three independent determinations; significantly different values determined by one-way ANOVA analysis were marked as: \* $P < 0.01$ ; \*\* $P < 0.001$ ; ORAC – oxygen radical absorbance capacity; FRAP – ferric reducing antioxidant power

Moreover, in the FRAP assay the RA was almost 2-times higher, while in ORAC assay RA was more than 42-fold higher in beebread. Wesołowska & Dżugan 2017 showed that the multiflorous honey samples from south-eastern part of Poland were also characterised by the lower reducing activity determined by FRAP. Similar trend was observed in the case of reducing activity determined by ORAC assay. In the multiflorous honey purchased from the south Poland, the lowest reducing activity was determined using ORAC assay (Jamróz et al. 2014). It suggests that the multiflorous honey from north-eastern Poland was characterised by the higher ability to reduce  $\text{Fe}^{3+}$  (up to 35 times higher) and the ability to absorb reactive oxygen species. Note that the determination of reducing potential depends on the content of bioactive substances and also on the harvest period of honey (Wesołowska & Dżugan 2017; Dżugan et al. 2018).

## CONCLUSION

Applied methods showed that among tested samples, beebread was characterised by much higher content of bioactive compounds and higher level of vitamin C and E. High concentration of bioactive substances in beebread is associated with higher scavenging ability of superoxide anion radical as determined by PCL assay, and with higher reducing values as determined using FRAP and ORAC tests. Our research shows that beebread is a product with stronger antioxidant action than honey and confirm the conclusion of Kieliszek et al. (2018) that beebread could be administered in smaller amounts or for a short period of time for therapeutical reasons in order to get health benefits.

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