

## SUPPLEMENTARY MATERIAL

### Functional properties of honey supplemented with bee bread and propolis

S. Kowalski<sup>1\*</sup> and M. Makarewicz<sup>2</sup>

<sup>1</sup>*Department of Carbohydrates Technology, Faculty of Food Technology, University of Agriculture in Krakow, e-mail: rrkowals@cyf-kr.edu.pl*

<sup>2</sup>*Department of Fermentation Technology and Technical Microbiology, Faculty of Food Technology, University of Agriculture in Krakow, e-mail: rrmakare@cyf-kr.edu.pl*

#### Abstract

The aim of this work was characterization of functional properties of honey enriched with propolis and beebread. In first step of experiment soft propolis extract (SPEx) was obtained by extraction of propolis with ethanol. SPEx (0.25 to 1.0% w/w) as well as beebread (5 to 15% w/w) were implemented into natural honey. Fortified honeys, were investigated in terms of total phenolic content, radical scavenging activity and ferric reducing antioxidant power, also their effects on the microorganisms growth was examined. It was found that beebread had the most significant influence on antioxidant properties. On the other hand, all tested honeys showed antibacterial activity against *Escherichia coli* but not against *Micrococcus luteus*. Honeys with 1% of propolis addition were the most effective in this case. Research has indicated that for antioxidant and antimicrobial properties of honey it is beneficial to enrich it in both beebread and propolis.

**Keywords:** bee bread, propolis, antioxidant properties, antimicrobial effect

### 3. Experimental

#### 3.1. Materials

Bee bread, propolis and lime honey were purchased from beekeeping cooperatives (Bartnik Sadecki, Poland). TPTZ, DPPH, gallic acid and Trolox were purchased from Sigma-Aldrich Inc. USA. Methanol and ethanol (analytical grade) were purchased from Chempur, Poland. All other Chemicals were purchased from Avantor Performance Materials, Poland.

## 3.2.Methods

### 3.2.1. *Preparation of honey with bee bread and propolis*

Honey fortified with beebread and propolis was prepared by mixing appropriate amounts of propolis extract, bee bread and honey. For this purpose propolis extract was prepared according to Osés et al. (2016). Briefly, 20g of grounded propolis was extracted with 75 ml of 90% food grade ethanol for 48 h at room temperature. After filtration through filter paper extract was evaporated by vacuum evaporator.

Bee bread was frozen at -20 °C and then grounded in laboratory mortar. After obtaining a free-flowing consistency, bee bread were weighed and added to the honey. Mixture of honey, propolis and beebread was blended with mechanical stirrer at 37 °C during 1h at 300 rpm.

### 3.2.2. *Total phenolic content*

Total phenolic content estimated using Folin-Ciocalteu reagent (Meda et al. 2005). Samples were prepared at 0.2 g/mL concentration. and filtered through disc filter 0.45µm. Filtrate was mixed with Folin- Ciocalteu reagent solution (10% v/v) and sodium carbonate solution (7.5% w/v) in proportion 1:25:25 and mixed. After 1 hour incubation at ambient temperature in the dark, the absorbance was measured spectrophotometrically (Spectro UV-VIS Dual Beam UVS-2800, Labomed, Inc. USA) at a wavelength of 760 nm. A standard curve was made for gallic acid in the range from 14.0 to 112.0 mg/L ( $R^2=0.9981$ ) with limits of detection and quantification of 6.38 and 19.34 mg/L respectively. Results were expressed as mg of gallic acid equivalent (GAE) per 100 g of product. Measurements were done in duplicate.

### 3.2.3. *Free radical scavenging ability*

Antioxidant activity against DPPH<sup>\*</sup> free radical was done according to Turkmen et al. (2006). Solutions for analysis were prepared at the same concentration as before and filtered. 0.75 mL of solution was mixed with 2.25 mL of 0.1 mM DPPH<sup>\*</sup> (methanol solution). In a blank sample, sample solution was replaced with distilled water. After 1 hour incubation in the dark at room temperature the absorbance was measured spectrophotometrically (Spectro UV-VIS Dual Beam UVS-2800, Labomed, Inc. USA) at a wavelength of 517 nm. A standard curve was made for Trolox in the range from 7.7 to 28.7 mg/L ( $R^2=0.9980$ ) with limits of detection and quantification of 1.35 and 4.09 mg/L respectively. Results were expressed as mg of Trolox per 100 g of product. Measurements were done in duplicate.

#### 3.2.4. Ferric ions reducing capability

Ferric ions reducing ability was established according to work Benzie and Strain (Benzie & Strain 1996). Sample solutions (0.2 g/mL) were filtered and then 5.0 mL of filtrate was mixed with 1.5 mL FRAP solution and incubated at 37 °C for 0.5 hour. Subsequently absorbance was measured spectrophotometrically (Spectro UV-VIS Dual Beam UVS-2800, Labomed, Inc. USA) at a wavelength of 593 nm. FRAP solution was obtained by mixing acetate buffer (pH 3.6) with 2,4,6-Tri(2-pyridyl)-s-triazine (10 mM in 40 mM HCl) and FeCl<sub>3</sub> (20mM aqueous solution) in 10:1:1 proportions.

A standard curve was made for FeSO<sub>4</sub> in the range from 0.01 to 0.08 mM (R<sup>2</sup>=0.9995) with limits of detection and quantification of 0.002 and 0.006 mM respectively. Results were expressed as µg of Fe<sup>2+</sup> per 1 g of the product. Measurements were done in duplicate.

#### 3.2.5. Antibacterial activity

For antibacterial activity measurements G<sup>-</sup> bacteria *Escherichia coli* (DSMZ 20030) and G<sup>+</sup> *Micrococcus luteus* (DSMZ 4261) were used. Microbial strains were purchased from the German Collection of Microorganisms and Cell Cultures in Leibniz Institute (DSMZ). Tested microorganisms were grown in nutrient broth (Biocorp PS 90) at 37°C for 24 h. Bacterial samples were enumerated by using the McFarland densitometer method (Silici et al. 2010). The final cell concentrations were in the range of 10<sup>8</sup> – 10<sup>9</sup> cfu/mL. 250 µL of bacterial suspension was added to the flask containing 25 mL sterile nutrient agar (Biocorp PS 85) at 45°C and poured into Petri dishes (9 cm diameter). The agar was allowed to solidify at 4°C for 1 h. Five equidistant holes were made in the agar using sterile cork borers (Ø = 6 mm). The 75%, 50%, 25%, 10% or 5% solutions of tested honeys were prepared in sterilized physiological saline (0.85%) and applied to the holes. Standard chloramphenicol test discs (30 mcg) (Bioanalyse Ltd.) served as a positive control, while sterilized physiological saline served as negative control (K). The plates were incubated at 37°C for 24 h. At the end of the incubation, diameters of inhibition zones present on the medium were measured in millimeters (mm).

#### 3.2.6. Statistical analysis

Response surface analysis was carried out using Statistica 12.0 (StatSoft, Poland) at p<0.05. Experimental data were fitted to an second-order polynomial model, as follows:

$$Y = \beta_0 + \sum_{i=1}^2 \beta_i X_i + \sum_{i=1}^2 \beta_{ii} X_i^2 + \sum_{i<j=1}^2 \beta_{ij} X_i X_j + \sum_{ii<jj=1}^2 \beta_{iijj} X_i^2 X_j^2 + \sum_{i<jj=1}^2 \beta_{ijj} X_i X_j^2 + \sum_{ii<j=1}^2 \beta_{ij} X_i^2 X_j$$

where Y is the dependent variable;  $\beta_0$  is a constant;  $\beta_i$ ,  $\beta_{ii}$ , and  $\beta_{ij}$  are coefficients estimated by the model (linear, quadratic and the coefficient for the interaction effect; and  $X_i$  and  $X_j$  are independent variables. Analysis of variance and the statistical significance of differences was performed using Tukey's and Duncan's test at  $\alpha=0,05$ .

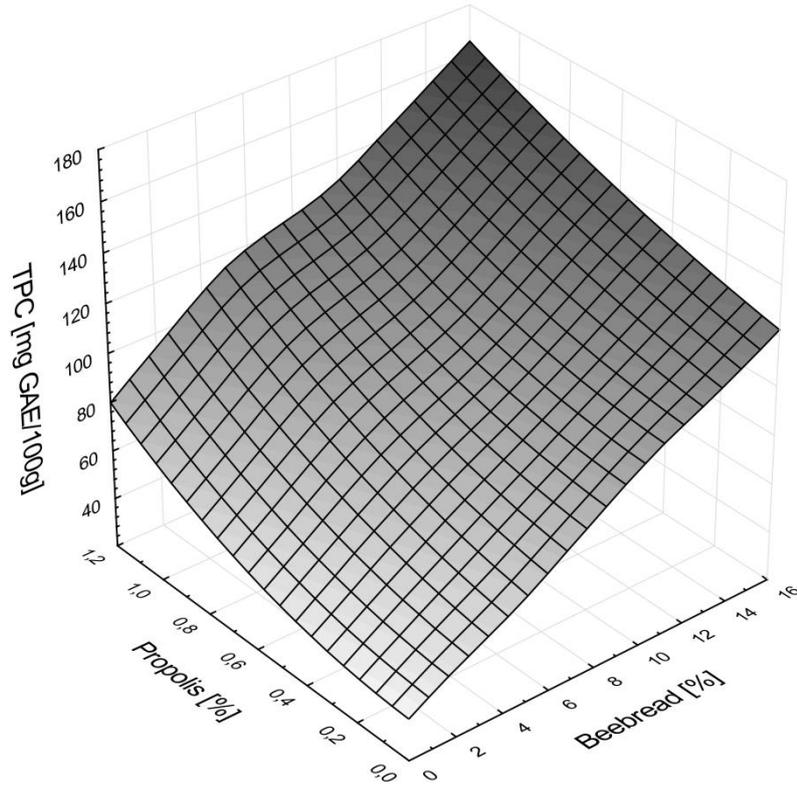


Figure S2 Total phenolic content as a function of bee bread and propolis concentration

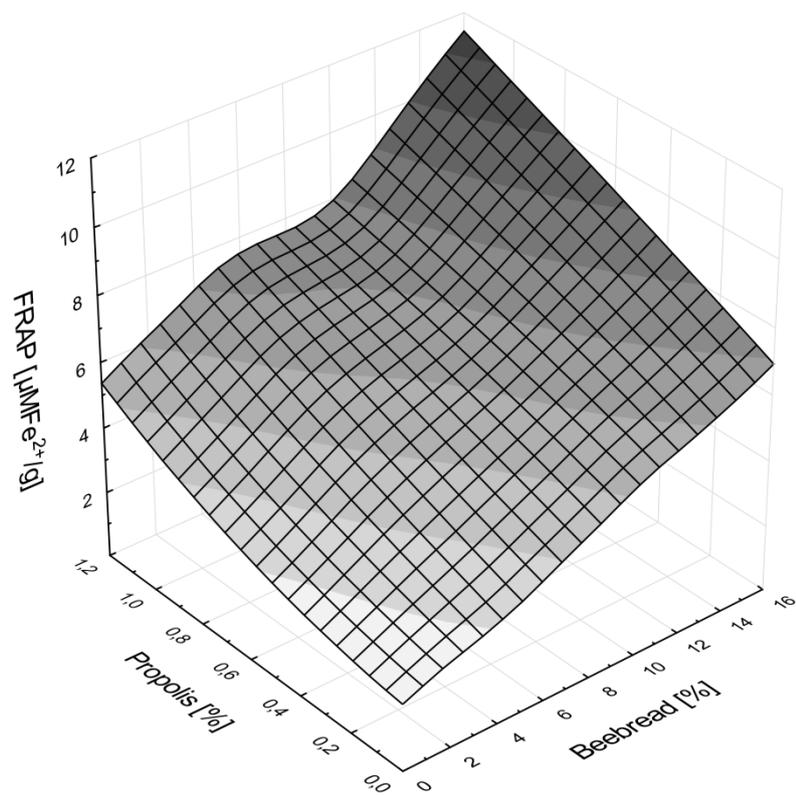


Figure S3 Ferric ions reducing as a function of bee bread and propolis concentration

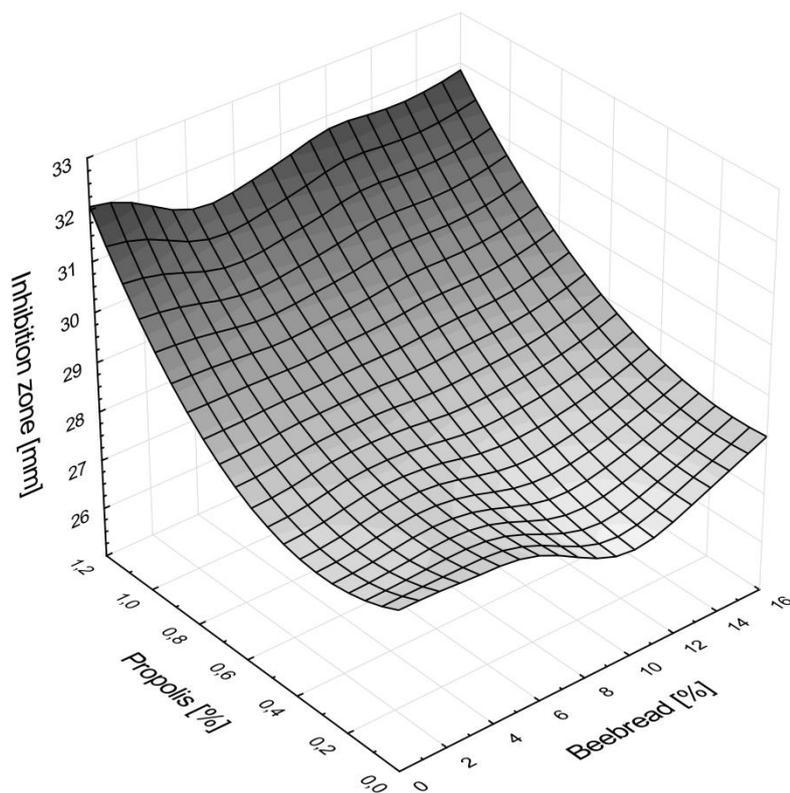


Figure S4 Antibacterial effect of 75% honey solution against *E. coli* as a function of bee bread and propolis concentration

#### Abbreviations Used

DPPH<sup>•</sup> - 1,1-diphenyl-2-picryl-hydrazyl radical,

GAE – Gallic Acid Equivalent,

TPTZ - 2,4,6-Tri(2-pyridyl)-s-triazine

FRAP - Ferric Reducing Antioxidant Power

TPC – Total Phenolic Content

K- negative control

Table S1. Total phenolic content, antioxidant properties and antibacterial activity of honey samples with propolis and bee bread (diameter of inhibition zones mm) for *Escherichia coli*

Sample		TPC [mg GAE /100g]	DPPH [mg Trolox/100g]	FRAP [ $\mu\text{MFe}^{2+}$ /g]	Sample concentration [% w/w]					
Bee bread [% w/w]	Propolis [% w/w]				75	50	25	10	5	K
0	0	36.00±0.10	7.28±0.25	1.96±0.11	28.0±2.8 ab	30.0±2.8 b	24.0±0.0 bcd	18.0±0.0 ab	0	0
5	0	42.94±1.74 a	8.95±0.52 a	2.65±0.05 a	28.0±0.0 ab	26.5±0.7 ab	24.0±0.0 bcd	18.0±0.0 ab	0	0
10	0	42.35±0.20 a	9.67±0.08 a	2.64±0.00 a	27.0±0.0 a	27.0±0.0 b	21.0±1.4	18.0±0.0 ab	0	0
15	0	67.42±2.73 c	15.52±0.60	4.53±0.14	28.0±0.0 ab	27.0±0.0 b	24.5±0.7 cd	18.0±0.0 ab	0	0
0	0.25	63.43±0.00 b	16.93±0.38 b	3.39±0.00	28.5±0.7 abc	26.0±1.4 ab	24.5±0.7 cd	18.0±2.1 a	0	0
5	0.25	64.61±0.94 bc	17.50±1.24 b	3.78±0.08	28.5±0.7 abc	27.0±0.0 b	24.5±0.7 cd	18.0±0.1ab	0	0
10	0.25	80.02±0.89	19.59±0.28	5.14±0.07 b	27.5±0.7 a	26.5±0.7 ab	24.5±0.7 cd	18.0±0.0 ab	0	0
15	0.25	102.40±1.15 d	25.85±0.44	6.67±0.09 c	28.0±0.0 ab	27.0±0.0 b	24.0±0.0 bcd	19.0±1.0 bc	0	0
0	0.5	92.12±0.11	23.43±0.18	5.00±0.01 b	27.5±2.1 a	27.0±0.0 b	24.0±0.0 bcd	18.0±0.0 ab	0	0
5	0.5	101.51±1.57 d	28.13±1.15	6.46±0.19 c	28.0±0.0 ab	25.5±0.7 ab	23.0±0.0 ab	18.0±0.0 ab	0	0
10	0.5	97.12±1.16	30.32±0.45	6.01±0.37 c	28.0±0.0 ab	25.0±0.0 a	23.0±0.0 ab	18.0±0.0 ab	0	0
15	0.5	119.62±1.56	39.32±0.99 c	7.45±0.01	28.5±0.7 abc	26.5±0.7 ab	22.5±0.7 a	19.0±1.4 bc	14.0±0.0 a	0
0	1.0	115.51±0.29	34.58±0.90	6.38±0.11	30.5±0.7 c	27.0±0.0 b	24.0±0.0 bcd	18.0±0.0 ab	12.5±0.7	0
5	1.0	126.99±1.99 e	36.30±0.00	8.06±0.11 d	30.0±0.0 b	27.0±0.0 b	23.5±0.7 abc	17.5±0.7 ab	14±1.4 a	0
10	1.0	128.30±2.78 e	39.63±0.44 c	8.06±0.16 d	30.5±0.7 c	26.5±0.7 a	24.5±0.7 cd	19.0±1.4 bc	15.0±0.0	0
15	1.0	150.51±2.84	53.31±0.80	10.27±0.38	30.5±0.7 c	27.0±0.0 b	25.0±0.0 d	20.5±0.7 c	17.5±0.7	0
<i>Positive control</i> Chloramphenicol					15.5±0.7					

Values which do not differ statistically significantly at  $P < 0.05$  were marked with the same letters.

Table S2. Parameters of second-order polynomial model

	TPC [mg GAE/100g]			DPPH [mg Trolox/100g]			FRAP [ $\mu\text{MFe}^{2+}/\text{g}$ ]			Inhibition zone [mm]		
	Variable coefficient	SE	P value	Variable coefficient	SE	P value	Variable coefficient	SE	P value	Variable coefficient	SE	P value
$\beta_0$	35.267	2.229	<0.0001	8.221	0.546	<0.0001	1.879	0.236	<0.0001	28.153	0.472	<0.0001
$\beta_i$	5.958	0.491	<0.0001	1.540	0.120	<0.0001	0.303	0.052	<0.0001	-	-	-
$\beta_j$	-	-	-	-	-	-	2.301	0.817	0.0091	-	-	-
$\beta_{ij}$	20.246	6.708	0.0056	8.041	1.644	<0.0001	-	-	-	3.500	1.422	0.0208
$\beta_i \beta_j$	-	-	-	0.789	0.088	<0.0001	-	-	-	-	-	-
$R^2$	0.9935			0.9973			0.9839			0.7941		
$R^2$ adj	0.9845			0.9935			0.9620			0.5595		

$P < 0.05$  indicates statistical significance.

## References

- Benzie IFF, Strain JJ. 1996. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of “Antioxidant Power”: The FRAP Assay. *Anal Biochem.* 239:70–76.
- Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG. 2005. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. *Food Chem.* 91:571–577.
- Osés SM, Pascual-Maté A, Fernández-Muiño MA, López-Díaz TM, Sancho MT. 2016. Bioactive properties of honey with propolis. *Food Chem.* 196:1215–1223.
- Silici S, Sagdic O, Ekici L. 2010. Total phenolic content, antiradical, antioxidant and antimicrobial activities of Rhododendron honeys. *Food Chem.* 121:238–243.
- Turkmen N, Sari F, Poyrazoglu ES, Velioglu YS. 2006. Effects of prolonged heating on antioxidant activity and colour of honey. *Food Chem.* 95:653–657.
- Vásquez A, Olofsson TC. 2009. The lactic acid bacteria involved in the production of bee pollen and bee bread. *J Apic Res.* 48:189–195.
- Viuda-Martos M, Ruiz-Navajas Y, Fernández-López J, Pérez-Álvarez JA. 2008. Functional properties of honey, propolis, and royal jelly. *J Food Sci.* 73:R117–R124.
- Weston RJ. 2000. The contribution of catalase and other natural products to the antibacterial activity of honey: a review. *Food Chem.* 71:235–239.
- Wilczynska A. 2010. Phenolic content and antioxidant activity of different types of Polish honey - A short report. *Pol J Food Nutr Sci.* 60:309–313.
- Wilczyńska A. 2014. Effect of filtration on colour, antioxidant activity and total phenolics of honey. *LWT - Food Sci Technol.* 57:767–774.
- Zuluaga CM, Serrato JC, Quicazan MC. 2015. Chemical, nutritional and bioactive characterization of Colombian bee-bread. *Chem Eng Trans.* 43:175–180.