Application of randomly methylated cyclodextrin in extraction of antioxidant-like compounds from bee bread

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Summary
The paper presents data on cyclodextrin-assisted water-ethanol extraction of bioactive compounds from bee bread. Such reactive extraction is a novel technique used for enhancing the process. Experiments were designed according to Box-Behnken composition design using randomly methylated cyclodextrin (RMCD, 0–15 mmol·l^{-1}) and aqueous ethanol solutions (0–97 %, v/v). Extraction was carried out in a temperature range 30–60 °C for 1–24 h. Antioxidant capacity of obtained extracts was determined as 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH$^{\bullet}$) scavenging ability, total phenolic content (expressed as grams of gallic acid equivalents per kilogram of bee bread) and ferric reducing antioxidant power. It was stated that ethanol concentration is the crucial parameter for the most effective extraction of bioactive compounds. Additionally, some interactions resulting in higher yields of bioactive compounds were revealed between RMCD concentration and temperature and/or time of extraction, which further confirmed the efficiency of the reactive extraction method.

Keywords
bee bread; antioxidant properties; cyclodextrin; extraction

"Bee bread" is defined as a bee product obtained from pollen, to which bees add honey and digestive enzymes, and then deposit the raw product in the cells of honeycomb. Additionally, a small amount of beeswax is added to this mixture [1, 2]. Prepared in this way, the final product is highly acidic (pH of about 4) and contains 40–50 % monosaccharides [3, 4]. It is considered that, during bee bread maturation, some complicated chemical transformations take place as a result of the action of enzymes and microorganisms including lactobacilli, which increase the digestibility and bioavailability of nutrients for bees. On the other hand, ANDERSON et al. [5] stated that bacteria do not participate, or their influence is negligible, in the transformation of pollen into bee bread and in improvement of pollen nutrition values. The greatest impact on the functional features of this product has the addition of honey, nectar and bee secretions as well as its own properties [6].

Principally, in more economically developed countries, some part of society is increasingly interested in the application of a healthy diet and especially in usage of bee products and apitherapy. Apiarists, through proper management actions, can promote bee bread gathering. This allows for increased availability of bee bread for consumers as a product recently considered a dietary supplement due to its content of a wide range of bioactive compounds. Some components including proteins, vitamins and especially phenolic compounds, which are natural antioxidants, contribute to a high nutritional and functional value of bee bread [2]. Application of bee bread in food formulations can also help to control the level of some food pathogens [6], which may have influence on applicability of this product.

It is believed that bee bread usage will grow extensive due to its functional features, including antioxidant properties. Its ability to participate in reactive oxygen species quenching has already been used in prevention and treatment of various diseases like cancer, diabetes, hypertension and cardiovascular diseases [1].

One of the techniques used to extract biologically active compounds is reactive extraction. It

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is a process in which mass exchange is intensified through a mechanism involving a reversible reaction between the extracted chemical species and a chemical species constituting, or present in, the extractant [7]. One of those reactions may be a complexation process that employs cyclodextrins (CDs) as host molecules [8]. Complexation at such supramolecular level is a technique commonly used to increase solubility of food or drug ingredients that are poorly soluble in water. Application of CDs in those cases is adavantageous due to, among other properties, their safety and ability to complex a number of organic compounds of low molecular weight [9]. Polarity differences are one of the driving forces in the complex forming and the polar structure of the outer part is responsible for solubility of the complexes in water. As a result, non-polar constituents in the form of a complex may enter the aqueous solution, increasing the bioavailability of the complexed compound [10].

The aim of this work was examination and optimization of extraction of bioactive compounds from bee bread using reactive extraction technique. For this purpose, randomly methylated cyclodextrin (RMCD) was used in water, ethanol as well as in water-ethanol mixtures. RMCD was used on the basis of its well known high solubility both in water and in ethanol solution.

MATERIALS AND METHODS

Materials

Bee bread was purchased from beekeeping cooperatives (Bartnik Sadecki, Nowy Sacz, Poland). 2,4,6-Tripyridyl-*s*-triazine (TPTZ), 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH$^{\bullet}$), gallic acid and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Methanol and ethanol (analytical grade) were purchased from Chempur (Piekary Slaskie, Poland). All other chemicals were purchased from Avantor Performance Materials (Gliwice, Poland), except RMCD, which was obtained from Wacker Chemie, Munich, Germany.

Methods

Three different experiments concerning extraction of biologically active substances from bee bread were conducted according to Box-Behnken Design (BBD). In the first step, the effect of four independent factors (RMCD concentration, ethanol concentration, temperature, time) on extraction of bioactive compounds from bee bread was investigated. In total, 27 experiments were carried out in randomized order, according to the design (Tab. 1). The 3-level design included a subset of the runs in the full three-level factorial and 3 center points per block to estimate the experimental error. Experimental ranges for factors were: RMCD from 0 mmol·l^{-1} to 15 mmol·l^{-1}; ethanol from 0 % to 97 % (v/v); temperature (T) from 30 °C to 60 °C, time (t) from 1 h to 24 h. Experimental data from the experiment were fitted to an empirical second-order polynomial model according to Eq. 1 given in Tab. 2.

In the next experiments, the same concentration of RMCD as well as the same time and temperature were used, but extraction was carried out in pure water or in 97 % (v/v) ethanol (Tab. 3). Experiments in step 2 and 3 were performed in random run sequence using BBD as well. In all cases, experimental data from BBD 2 and BBD 3 were fitted to an empirical second-order polynomial modelaccording to Eq. 2 given in Tab. 2.

In all experiments, 2 g of bee bread and 20 ml of solvent system were used.

Total phenolic content

Total phenolic content (*TPC*) was estimated using Folin-Ciocalteu reagent according to the method of Meda et al. [11]. Bee bread extracts were diluted (1 : 2) with a proper solvent (the same as the type of extractant used in the experiment) and filtered through a membrane filter (pore size 0.45 μm). A volume of 0.5 ml of filtrate was mixed with 2.5 ml of Folin-Ciocalteu reagent solution (10%, v/v) and then mixed with 2.5 ml of sodium carbonate solution (75 g·l^{-1}). After 1 h incubation at room temperature in the absence of light, absorbance was measured spectrophotometrically (Spectro UV-VIS Dual Beam UVS-2800; Labomed, Los Angeles, California, USA) at a wavelength of 760 nm. A standard curve was made for gallic acid in the range from 6.6 mg·l^{-1} to 60.6 mg·l^{-1} (R^2 = 0.9990) with limits of detection and quantification of 3.18 mg·l^{-1} and 9.64 mg·l^{-1}, respectively. Results were expressed as grams of gallic acid equivalent (GAE) per kilogram of bee bread. Measurements were done in duplicate.

DPPH free radical-scavenging ability

Antioxidant activity against DPPH free radical was determined on the basis of the method used by Turkmen et al. [12]. Bee bread extracts were diluted (fourfold and eightfold in case of water and ethanol extraction, respectively) in the same manner as described above, and then filtered. A volume of 0.75 ml of extract was mixed with 2.25 ml of 0.1 mmol·l^{-1} DPPH$^{\bullet}$ (methanol so-

lution). In a blank sample, bee bread extract was replaced with distilled water. After 1 h incubation in the dark at room temperature, absorbance was measured spectrophotometrically (Spectro UV-VIS Dual Beam UVS-2800) at a wavelength of 517 nm. A standard curve was made for Trolox in the range from 7.7 mg·l^{-1} to 28.7 mg·l^{-1} (R^2 = 0.9980) with limits of detection and quantification of 1.35 mg·l^{-1} and 4.09 mg·l^{-1}, respectively. Results were expressed as grams of Trolox equivalent per kilogram of bee bread. Measurements were done in duplicate.

Ferric reducing antioxidant power

Ability to reduce ferric ions was established according to Benzie and Strain [13]. Bee bread

Tab. 1. Experiment design and results of bee bread extraction with ternary water-ethanol-randomly methylated cyclodextrin.

Extraction conditions from Box-Behnken experimental design								Results obtained in experiment		
Natural				Coded						
RMCD [mmol·l^{-1}]	*T* [°C]	*t* [h]	Ethanol [%]	RMCD	*T*	*t*	Ethanol	*DPPH* [g·kg^{-1}]	*FRAP* [mmol·kg^{-1}]	*TPC* [g·kg^{-1}]
0	30	12.5	48.5	–1	–1	0	0	31.06 ± 1.66	194.48 ± 1.96	14.96 ± 0.51
0	45	1	48.5	–1	0	–1	0	23.32 ± 1.10	212.43 ± 6.46	15.52 ± 0.71
0	45	12.5	0	–1	0	0	–1	2.99 ± 0.03	39.71 ± 1.99	6.15 ± 0.13
0	45	12.5	97	–1	0	0	+1	21.92 ± 0.89	209.19 ± 1.54	14.51 ± 0.45
0	45	24	48.5	–1	0	+1	0	33.01 ± 1.38	198.20 ± 10.09	14.64 ± 0.47
0	60	12.5	48.5	–1	+1	0	0	31.55 ± 2.33	213.16 ± 3.80	15.91 ± 0.82
7.5	30	1	48.5	0	–1	–1	0	28.98 ± 1.91	236.62 ± 7.36	17.43 ± 0.65
7.5	30	12.5	0	0	–1	0	–1	3.88 ± 0.16	132.00 ± 0.70	12.28 ± 0.09
7.5	30	12.5	97	0	–1	0	+1	25.77 ± 0.63	216.08 ± 10.06	15.83 ± 0.61
7.5	30	24	48.5	0	–1	+1	0	32.88 ± 1.50	234.59 ± 2.22	16.89 ± 0.57
7.5	45	1	0	0	0	–1	–1	4.29 ± 0.00	52.39 ± 3.04	7.72 ± 0.13
7.5	45	1	97	0	0	–1	+1	22.90 ± 1.76	200.71 ± 2.68	14.81 ± 0.27
7.5	45	12.5	48.5	0	0	0	0	31.12 ± 1.34	241.81 ± 4.66	18.01 ± 0.36
7.5	45	12.5	48.5	0	0	0	0	32.23 ± 0.58	227.13 ± 7.07	16.86 ± 0.78
7.5	45	12.5	48.5	0	0	0	0	28.87 ± 2.59	218.61 ± 4.51	16.23 ± 0.66
7.5	45	24	0	0	0	+1	–1	3.91 ± 0.21	45.08 ± 0.45	8.08 ± 0.06
7.5	45	24	97	0	0	+1	+1	29.36 ± 2.12	241.78 ± 12.19	16.87 ± 0.66
7.5	60	1	48.5	0	+1	–1	0	35.47 ± 1.86	282.73 ± 21.63	17.46 ± 0.76
7.5	60	12.5	0	0	+1	0	–1	4.28 ± 0.36	44.79 ± 0.22	8.17 ± 0.01
7.5	60	12.5	97	0	+1	0	+1	29.63 ± 1.09	221.56 ± 5.34	17.10 ± 0.51
7.5	60	24	48.5	0	+1	+1	0	30.43 ± 1.37	207.93 ± 2.91	16.54 ± 0.54
15	30	12.5	48.5	+1	–1	0	0	33.47 ± 2.17	230.55 ± 7.14	17.43 ± 0.49
15	45	1	48.5	+1	0	–1	0	27.59 ± 1.96	221.72 ± 4.86	16.49 ± 0.54
15	45	12.5	0	+1	0	0	–1	8.72 ± 0.15	90.70 ± 0.23	12.58 ± 0.13
15	45	12.5	97	+1	0	0	+1	28.46 ± 1.76	244.54 ± 0.62	17.21 ± 0.68
15	45	24	48.5	+1	0	+1	0	6.35 ± 7.10	256.09 ± 8.27	27.80 ± 9.67
15	60	12.5	48.5	+1	+1	0	0	39.23 ± 6.10	245.87 ± 12.26	18.65 ± 0.82

Results are expressed as mean ± standard deviation of three experiments.
RMCD – randomly methylated cyclodextrin, *T* – temperature, *t* – time, *DPPH* – 1,1-diphenyl-2-picrylhydrazyl free radical-scavenging ability (expressed as grams of Trolox equivalents), *FRAP* – ferric reducing antioxidant power (expressed as millimoles of Fe^{2+}), *TPC* – total phenolic content (expressed as grams of gallic acid equivalents).

Tab. 2. Equations of second-order polynomial models.

Eq. 1	$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_{ii}^2 + \sum_{i<j=1}^{4} \beta_{ij} X_i X_j + \sum_{ii<jj=1}^{4} \beta_{iijj} X_{ii}^2 X_{jj}^2 + \sum_{i<jj=1}^{4} \beta_{ijj} X_i X_{jj}^2 + \sum_{ii<j=1}^{4} \beta_{iij} X_{ii}^2 X_j$
Eq. 2	$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_{ii}^2 + \sum_{i<j=1}^{3} \beta_{ij} X_i X_j + \sum_{ii<jj=1}^{3} \beta_{iijj} X_{ii}^2 X_{jj}^2 + \sum_{i<jj=1}^{3} \beta_{ijj} X_i X_{jj}^2 + \sum_{ii<j=1}^{3} \beta_{iij} X_{ii}^2 X_j$

Y – dependent variable; β_0 – constant; β_i, β_{ii}, β_{ij} – coefficients estimated by the model (linear, quadratic and coefficient for the interaction effect); X_i, X_j – independent variables affecting the response. Coding of independent variables was performed at three levels (–1, 0, +1).

Tab. 3. Experiment design and results of bee bread extraction with binary water-randomly methylated cyclodextrin and ethanol-randomly methylated cyclodextrin solution.

Extraction conditions from Box-Behnken experimental design						Results					
Natural			Coded			Experiment with water as solvent			Experiment with ethanol as solvent		
RMCD [mmol·l^{-1}]	*T* [°C]	*t* [h]	RMCD	*T*	*t*	*DPPH* [g·kg^{-1}]	*FRAP* [mmol·kg^{-1}]	*TPC* [g·kg^{-1}]	*DPPH* [g·kg^{-1}]	*FRAP* [mmol·kg^{-1}]	*TPC* [g·kg^{-1}]
0	30	12.5	–1	–1	0	4.64 ± 0.02	32.78 ± 0.48	5.86 ± 0.06	16.03 ± 0.66	102.64 ± 6.14	14.33 ± 0.49
0	45	1	–1	0	–1	3.98 ± 0.18	32.99 ± 0.18	6.32 ± 0.12	15.74 ± 0.70	95.72 ± 2.48	13.82 ± 0.52
0	45	24	–1	0	+1	4.04 ± 0.18	27.12 ± 0.47	6.13 ± 0.06	17.94 ± 0.97	123.29 ± 6.14	15.53 ± 0.47
0	60	12.5	–1	+1	0	4.19 ± 0.08	32.13 ± 1.12	6.17 ± 0.12	19.10 ± 1.21	130.40 ± 7.33	16.50 ± 0.43
7.5	30	1	0	–1	–1	5.17 ± 0.11	44.32 ± 0.17	8.08 ± 0.15	13.47 ± 0.66	85.52 ± 5.12	12.15 ± 0.53
7.5	30	24	0	–1	+1	4.77 ± 0.12	36.93 ± 0.00	8.00 ± 0.13	16.72 ± 0.96	107.30 ± 3.05	15.88 ± 0.56
7.5	45	12.5	0	0	0	4.75 ± 0.13	36.13 ± 0.35	8.01 ± 0.12	17.17 ± 0.64	113.65 ± 1.84	16.01 ± 0.58
7.5	45	12.5	0	0	0	4.64 ± 0.08	37.12 ± 0.41	8.11 ± 0.18	18.02 ± 0.96	118.96 ± 9.31	16.90 ± 0.57
7.5	45	12.5	0	0	0	5.02 ± 0.13	40.79 ± 0.91	8.39 ± 0.19	17.74 ± 0.69	114.64 ± 4.92	16.58 ± 0.53
7.5	60	1	0	+1	–1	5.25 ± 0.13	45.84 ± 0.06	8.46 ± 0.23	17.24 ± 0.61	110.28 ± 5.82	16.38 ± 0.69
7.5	60	24	0	+1	+1	5.46 ± 0.09	44.74 ± 0.60	9.13 ± 0.11	17.91 ± 1.20	115.04 ± 5.64	17.49 ± 0.71
15	30	12.5	+1	–1	0	6.89 ± 0.15	58.26 ± 0.21	11.25 ± 0.25	18.34 ± 0.43	120.35 ± 2.82	17.48 ± 0.70
15	45	1	+1	0	–1	5.24 ± 0.04	49.59 ± 0.11	10.58 ± 0.24	14.50 ± 0.80	103.12 ± 4.18	13.80 ± 0.51
15	45	24	+1	0	+1	6.03 ± 0.08	47.66 ± 0.76	9.88 ± 0.18	17.70 ± 1.13	114.67 ± 7.78	17.50 ± 0.63
15	60	12.5	+1	+1	0	6.51 ± 0.08	51.46 ± 0.47	10.23 ± 0.22	17.34 ± 0.68	113.05 ± 4.36	18.10 ± 0.64

Results are expressed as mean ± standard deviation of three experiments.
RMCD – randomly methylated cyclodextrin, *T* – temperature, *t* – time, *DPPH* – 1,1-diphenyl-2-picrylhydrazyl free radical-scavenging ability (expressed as grams of Trolox equivalents), *FRAP* – ferric reducing antioxidant power (expressed as millimoles of Fe^{2+}), *TPC* – total phenolic content (expressed as grams of gallic acid equivalents).

extracts were diluted (twelve and fifty fold in case of water and ethanol extraction, respectively) and filtered. A volume of 5.0 ml of filtrate was mixed with 1.5 ml of working solution and incubated at 37 °C for 30 min. Subsequently, absorbance was measured spectrophotometrically (Spectro UV-VIS Dual Beam UVS-2800) at a wavelength of 593 nm. Working solution was obtained by mixing acetate buffer (pH 3.6) with TPTZ (10 mmol·l^{-1} in 40 mmol·l^{-1}HCl) and $FeCl_3$ (20 mmol·l^{-1} in water) in 10 : 1 : 1 proportions. A standard curve was made for $FeSO_4$ in the range from 0.01 mmol·l^{-1} to 0.08 mmol·l^{-1} (R^2 = 0.9995) with limits of detection and quantification of 0.02 mmol·l^{-1} and 0.06 mmol·l^{-1}, respectively. Results were expressed as millimoles of Fe^{2+} per kilogram of bee bread. Measurements were done in duplicate.

Statistical analysis

Box-Behnken experiment design (BBD) as well as response surface analysis were carried out using Statistica 12.0 (StatSoft, Tulsa, Oklahoma, USA) at $p < 0.05$.

RESULTS AND DISCUSSION

In order to investigate and optimize the mutual influence of proposed factors (RMCD concentration, extraction temperature, time and ethanol concentration) on the response (*DPPH, FRAP, TPC*), a set of 27 experiments with random combinations of factors were performed. The obtained results are presented in Tab. 1. On the basis of results obtained in this experiment, an analysis of the response surface was performed.

The data obtained were analysed by multiple regression analysis and fitted to various models (linear, interactive and quadratic). The results of calcula-

tions clearly showed that quadratic model following Eq. 1 exhibited higher R^2, adjusted R^2, and also low *p*-values, when compared with other models. According to that, a quadratic model incorporating linear, interactive and quadratic terms was chosen to fit the experimental data into developing of an empirical model. Variable coefficient as well as statistical parameters of second-order polynomial model are presented in Tab. 4. Based on the data, it can be stated that the strongest impact on extraction efficiency had alcohol concentration, as well as duration of extraction (quadratic coefficient) (Fig. 1). The influence of the alcohol concentration was mainly due to its lower polarity (relative dielectric constant for ethanol is almost four times lower than for water, 24.5 vs 80 [14]). The lower polarity allowed to extract the non-polar components of bee bread more efficiently than in case of water or water-CD systems. The differences in extraction into less polar solvent and complexation in low polar CD cavity may result in dramatic concentration differences of those two compounds in the system. On the other hand. the influence of time obviously follows the classical Fick's law of diffusion. Based on the obtained results, it can be stated that ethanol in concentration of about 60 % is the most effective extractant for transferring radical-scavenging agents (determined by DPPH method) from bee bread into the liquid phase. It was also found that RMCD had statistically significant influence only on the extraction yield of phenolic compounds (*TPC*).

Tab. 4. Second-order polynomial models obtained by response surface methodology.

Coefficient	*DPPH*		*FRAP*		*TPC*	
	[g·kg^{-1}]	*p*	[mmol·kg^{-1}]	*p*	[g·kg^{-1}]	*p*
Aqueous ethanolic solution						
β_0	18.050 ± 0.873	0.0023	162.929 ± 5.975	0.0013	14.641 ± 0.461	0.0010
β_1	–	–	–	–	4.917 ± 0.906	0.0323
β_{33}	5.793 ± 1.060	0.0319	–	–	–	–
β_4	21.295 ± 1.715	0.0064	137.221 ± 11.735	0.0072	5.843 ± 0.906	0.0232
β_{44}	15.839 ± 0.892	0.0032	90.525 ± 6.107	0.0045	4.448 ± 0.472	0.0111
β_{13}	–15.465 ± 1.715	0.0121	–	–	6.103 ± 0.906	0.0213
β_{133}	8.665 ± 1.213	0.0190	–	–	–	–
β_{1133}	–4.504 ± 0.857	0.0344	–	–	–	–
R^2	0.9984		0.9979		0.9965	
R^2 adjusted	0.9787		0.9732		0.9545	
Aqueous solutions						
β_0	5.182 ± 0.057	0.0001	41.985 ± 0.709	0.0003	8.154 ± 0.015	< 0.0001
β_1	2.066 ± 0.146	0.0050	21.124 ± 1.830	0.0074	3.906 ± 0.038	0.0001
β_2	–	–	–	–	0.445 ± 0.038	0.0074
β_{22}	–0.547 ± 0.102	0.0331	–	–	–	–
β_{33}	–	–	–	–	–0.132 ± 0.027	0.0389
β_{112}	–	–	–	–	0.449 ± 0.037	0.0065
β_{113}	–	–	–	–	0.471 ± 0.037	0.0060
β_{23}	–	–	–	–	0.546 ± 0.052	0.0088
R^2	0.9924		0.9883		0.9998	
R^2 adjusted	0.9466		0.9182		0.9988	
Ethanolic solutions						
β_0	16.836 ± 0.125	0.0001	110.116 ± 0.814	0.0001	15.748 ± 0.130	0.0001
β_1	–	–	–	–	1.907 ± 0.335	0.0295
β_2	1.516 ± 0.323	0.0424	12.235 ± 2.102	0.0283	1.900 ± 0.335	0.0297
β_3	2.454 ± 0.323	0.0169	17.460 ± 2.102	0.0142	2.609 ± 0.335	0.0161
β_{33}	1.269 ± 0.225	0.0301	9.313 ± 1.468	0.0240	1.232 ± 0.234	0.0342
β_{12}	–2.039 ± 0.433	0.0423	–17.526 ± 2.820	0.0249	–	–
R^2	0.9881		0.9910		0.9899	
R^2 adjusted	0.9168		0.9367		0.9291	

Results are expressed as mean ± standard deviation of three experiments.
RMCD – randomly methylated cyclodextrin, *T* – temperature, *t* – time, *DPPH* – 1,1-diphenyl-2-picrylhydrazyl free radical-scavenging ability (expressed as grams of Trolox equivalents), *FRAP* – ferric reducing antioxidant power (expressed as millimoles of Fe^{2+}), *TPC* – total phenolic content (expressed as grams of gallic acid equivalents).

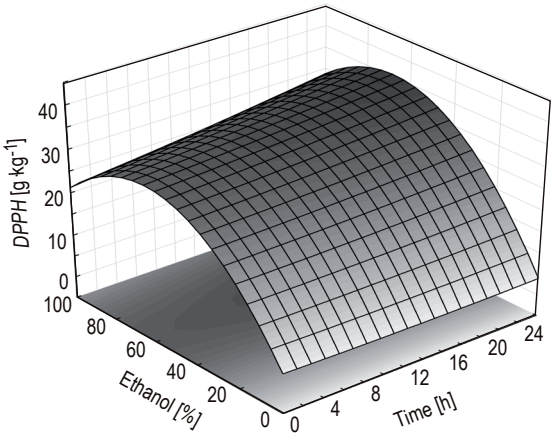

Fig. 1. Influence of ethanol and time on DPPH-scavenging activity in water-ethanol-randomly methylated cyclodextrin system.

DPPH – 1,1-diphenyl-2-picrylhydrazyl free radical-scavenging ability (expressed as grams of Trolox equivalents).

However, it is worth to point out that interaction between RMCD concentration and duration of extraction was detected in case of scavenging of DPPH free radicals as well as in case of *TPC* (Fig. 2, 3). When analysing Fig. 2 and Fig. 3, it can be concluded that although *TPC* reached the highest value when the highest RMCD concentration was applied and after prolonged extraction time, ability of such extracts to scavenge free radicals decreased (Fig. 3). The phenomenon can be explained on the basis of geometry of CD-bioactive compounds complex. It is possible that phenolic compounds, which give *TPC* response in analysis, are not accessible for free radicals scavenging. In the system, some kind of equilibrium is reached. It results in lowering of *TPC* response but protecting the active molecules by RMCD takes place, which will prolong their antioxidant activity. This is in concordance with Lukasiewicz et al. [10], who studied antioxidant properties of quercetin complexed by β-cyclodextrin. The authors postulated that although the presence of cyclodextrins increased the solubility of quercetin, the obtained complex might be less active due to hindering of the functional groups by covering them by CD moieties.

In case of *FRAP* analysis, the only factor that influenced the extraction efficiency was ethanol solution. Regarding this, both linear as well as quadratic coefficient were statistically significant at $p < 0.01$ (Tab. 4). It was also found that the most effective ethanol concentration for *FRAP*-sensitive compounds was between 65 % and 75 % (v/v).

In a further stage of research, extraction of bioactive compounds from bee bread with aqueous RMCD solutions (water-RMCD) as well as ethanolic RMCD solutions (ethanol-RMCD) was conducted. Experimental design and output values are reported in Tab. 3. In case of water-RMCD solutions used as extractant, RMCD concentration had a key influence on extraction yield monitored by means of *TPC*, *FRAP* and *DPPH* methods. It was clearly shown that, in the absence of low po-

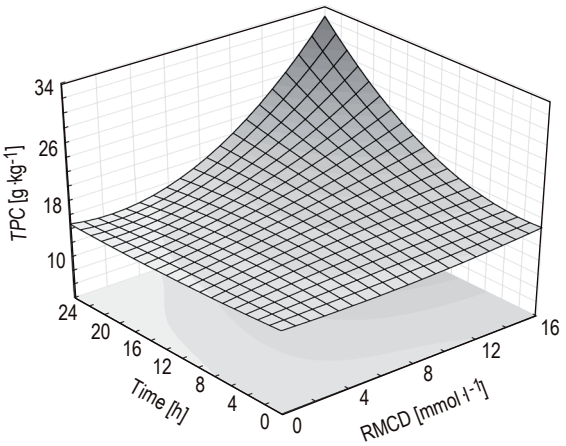

Fig. 2. Influence of time and randomly methylated cyclodextrin concentration on total phenolic content in water-ethanol-randomly methylated cyclodextrin system.

TPC – total phenolic content (expressed as grams of gallic acid equivalents), RMCD – randomly methylated cyclodextrin.

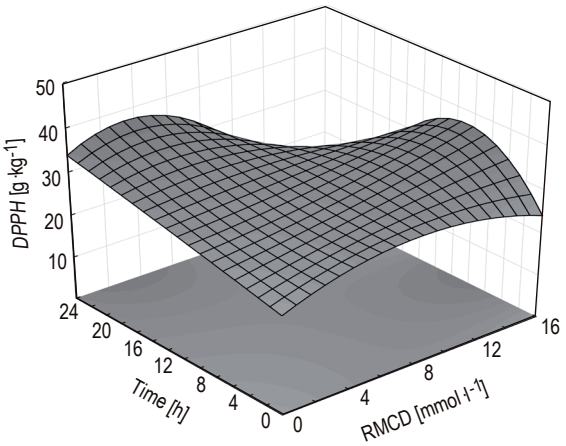

Fig. 3. Influence of time and randomly methylated cyclodextrin concentration on DPPH-scavenging activity in water-ethanol-randomly methylated cyclodextrin system.

DPPH – 1,1-diphenyl-2-picrylhydrazyl free radical-scavenging ability (expressed as grams of Trolox equivalents), RMCD – randomly methylated cyclodextrin.

larity solvent, "host-guest" complexation using cyclodextrins was a driving force of mass transfer of organic (low polarity) substances from bee bread into solution. Polynomial model fitted to experimental data indicated interactions between RMCD concentration (coefficient in the second power) and both duration and temperature of extraction. Joined effect of time and temperature was also observed (Fig. 4). It was the result of the influence of temperature on complex formation and/or dissociation, same as in case of any equilibrium process. According to the obtained results, it is worth to state that application of reactive extraction with RMCD may be a useful tool if organic solvents should not be used. The effect of ethanol seemed to be the strongest one, mainly due to its concentration in the extraction system, which was described above. The action of RMCD was less effective but also facilitated extraction of antioxidant-like substances from bee bread. Additionally, RMCD may play a protective role for antioxidant compounds by "covering" the host molecule. Ethanol does not work in this manner.

In case of ethanol-RMCD solutions used as extractant, time of extraction as well as process temperature had crucial impact on extraction process. Only in case of *TPC* analysis, RMCD concentration was found to be statistically significant. Scavenging ability of obtained extracts measured against DPPH free radicals (Fig. 5) as well as their capability for reduction of ferric ions (Fig. 6) followed a similar trend as in the first experiment (Fig. 3). Additionally, influence of interaction coefficient of RMCD and temperature was found in case of both *FRAP*- and *DPPH*-sensitive compounds extracted from bee bread. The values of coefficients in this case were negative, which might indicate the lower extraction efficiency as a result of a relatively high dissociation constant of the complex. Explanation of the phenomena needs further study but some preliminary theory may be hypothesized that the large amount of ethanol molecules present in the system was responsible for blocking the RMCD cavity against complex-

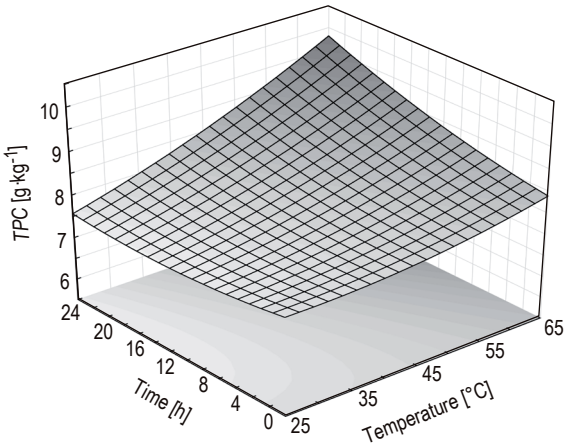

Fig. 4. Influence of time and temperature on total phenolic content in water-randomly methylated cyclodextrin system.

TPC – total phenolic content (expressed as grams of gallic acid equivalents).

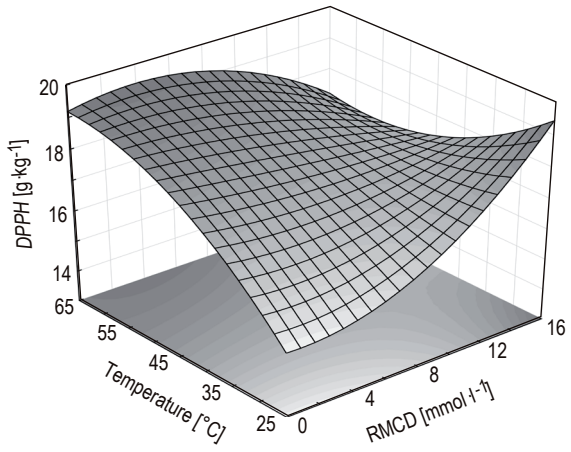

Fig. 5. Influence of temperature and randomly methylated cyclodextrin concentration on DPPH-scavenging activity in ethanol-randomly methylated cyclodextrin system.

DPPH – 1,1-diphenyl-2-picrylhydrazyl free radical-scavenging ability (expressed as grams of Trolox equivalents), RMCD – randomly methylated cyclodextrin.

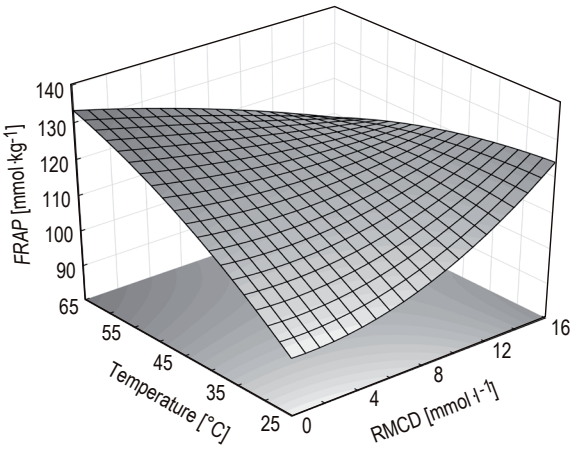

Fig. 6. Influence of temperature and randomly methylated cyclodextrin concentration on ferric reducing activity power in ethanol-randomly methylated cyclodextrin system.

FRAP – ferric reducing antioxidant power (expressed as millimoles of Fe^{2+}), RMCD – randomly methylated cyclodextrin.

ation with active compounds from bee bread. As it is known for basic β-cyclodextrin, ethanol has a quite strong affinity to form CD-ethanol complexes with the apparent formation constant of 0.93 $l \cdot mol^{-1}$ [15].

CONCLUSIONS

Extraction of antioxidant-like compounds from bee bread was possible both using an ethanol-based system as well as using reactive extraction employing RMCD as a complexing agent. The greatest influence on the extraction of biologically active compounds from bee bread had the concentration of ethanol. Additionally it was also found that the use of RMCD-assisted extraction process can provide extracts with a higher antioxidant activity. RMCD is a useful extraction enhancer especially in case of aqueous solutions and allows to omit the application of organic solvent (ethanol) in some cases. Reactive extraction of antioxidant-like components from bee bread using RMCD can be treated as a novel and original technique to increase the yield of the process.

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