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Research Article

Ultrasonic extraction conditions using response surface methodology: total phenolic content of bee pollen

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ABSTRACT

Bee pollen plays a significant role in bee nutrition, bee population sustainability, pollination processes, and its health and nutritional benefits for humans. It contains protein, vitamins, minerals, and antioxidants, offering valuable nutritional properties. The total phenolic content (TPC) is an important parameter in determining the nutritional and health value of pollen. The presence of high levels of phenolic compounds in pollen enhances their health benefits and can provide protective effects against diseases by combating oxidative stress. In the study, ultrasonic extraction conditions for pollen were optimized using the Response Surface Method to maximize TPC. The experimental study was designed according to Box-Behnken design: 30–70% ethanol ratio, 5–15 min of extraction time, and 10–20% ultrasonic amplitude modulation (AM). The TPC of the obtained extracts were determined using the Folin-Ciocalteu procedure. The optimal extraction conditions were predicted as 60.012% ethanol ratio, 11.054 min, and 19.160% AM for reaching 9.572 mg/GAE g extract.

Keywords: Optimization, bee pollen, response surface method, total phenolic content.

1. INTRODUCTION

Apitherapy refers to the therapeutic application of beekeeping products, which have been utilized in human nutrition for an extended period owing to their advantageous effects on health.[1, 2] Apitherapy uses bee products like honey, beeswax, pollen, royal jell, bee bread, and propolis to treat and prevent diseases.[3-6] In recent times, there has been an observable increase in the market demand for bee pollen, as a growing number of individuals are becoming aware of the importance of apitherapy and the increasing trend of consuming natural products.[7]

Bee pollen, a bee-derived substance, is created through the amalgamation of flower pollen with honey, nectar, beeswax, enzymes, and secretions produced by bees. This composite mixture is retained within the hive and serves as a nutritional resource for all stages of growth among the bee colony.[8] Bee pollen is regarded as a very nutritious and beneficial bioactive food source for human consumption due to its notable protein content and comprehensive provision of key amino acids necessary for bodily functions.[2] The chemical makeup of pollen varies depending on its geographical origin and the plant species from which it is derived. On average, pollen typically consists of approximately 55% carbohydrates, 20% proteins, 5% lipids, and 10% fiber. Additionally, it contains various fatty acids, sterols, vitamins, and minerals. Notwithstanding the fact that the chemical composition of pollen varies based on the geographical location of the plant's growth.[9]

The pollen produced by bees is a plentiful reservoir of diverse primary and secondary metabolites that exhibit antioxidant qualities and other advantageous characteristics for human well-being.[10, 11] Oxidative stress occurs as a result of an increase in the levels of

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reactive oxygen species (ROS) in the human body. Increased concentrations of reactive oxygen species have been associated with various pathological conditions, encompassing cardiovascular illness, metabolic disorders such as diabetes, and degenerative ailments such as Alzheimer's disease, Parkinson's disease, and arthritis. The antioxidative effect means inactivation of oxygen radicals so that antioxidant sources play an important role for a healthy body. Pollen's antioxidative qualities may be attributed to antioxidant enzymes included in its structure, as well as phenolic compounds, vitamins E and C, carotenoids, and elements like glutathione.[12]

The amount of bee pollen can be impacted by various factors, such as bee species, geographical characteristics, climate conditions, botanical sources, and methods employed for collecting, storing, and extracting bee pollen. [13-16] In addition, it is of the greatest significance to optimize the extraction conditions. Furthermore, it is necessary to improve the extraction conditions. The influence of extraction conditions on the composition and antioxidant properties of bee products is a key factor to consider. The optimization of extraction parameters, including solvent type, extraction duration, temperature, and material ratio, can be employed to effectively increase the quantity of bioactive components and increase the antioxidant capacity of bee pollen.[17]

One of the next-generation extraction methods is the ultrasonic extraction technique. These reasons demonstrate the importance of ultrasonic extraction as a significant method. Higher extraction efficiency, faster processes, reduced solvent usage, and improved compound separation capabilities make it a preferred option in many industries. The practice of ultrasonic extraction is increasingly being utilized in a variety of industries, including those dealing with food, medicines, cosmetics, biotechnology, and the environment. It can be effectively utilized for obtaining plant extracts, extracting aroma compounds, isolating active pharmaceutical ingredients, and more.[18,19] The advantages of ultrasonic extraction make it a valuable technique in these industries.[20]

The utilization of the response surface method (RSM) is a statistical technique employed to optimize experimental designs and analyze the response surface. The application of this technique aids in the assessment of the impacts and interactions of independent variables inside a given model. The Box-Behnken design and the Central Composite Design (CCD) are two kinds of design matrices commonly employed in this methodology. The utilization of this technique simplifies the examination of empirical data and aids in the performance of optimization procedures.[21]

Response surface analysis is a valuable methodology employed to optimize the factors that show an influence on the response or output of a given system. It aids in identifying the ideal conditions required to achieve desired objectives. The objective of this work is to optimize the ultrasonic extraction methods of bee pollen through the implementation of the RSM.

2. MATERIALS AND METHODS

2.1. Bee pollen

The used extract in the research was obtained from a dried pollen sample bought from Bee &You (Bee'O®) (SBS Scientific Bio Solutions Inc., Istanbul, Turkey) in the year 2022.

2.2. Experimental design

Experimental design is a systematic process that involves the planning, implementation, and evaluation of an experiment. This approach aims to enhance the reliability of obtained results by determining cause-and-effect relationships and ensuring controllability. A robust experimental design facilitates efficient use of limited resources, while testing hypotheses and the ability to generalize findings contribute to the expansion of scientific understanding. From scientific research to product development, this process is utilized across diverse fields, enabling the attainment of solid results and the enhancement of knowledge accumulation.

In the study, independent parameters were chosen as ethanol ratio of solvent, extraction time and Amplitude Modulation and the dependent parameters was total phenolic content of bee pollen extract. The study employed a Box-Behnken design with three levels and three factors to effectively estimate the total phenolic content of the bee pollen samples. The actual and coded values of the experimental design were shown in Table 1. A value of -1 represents the minimum level within the range of the independent variable, a value of 0 represents the mean level of the range, and a value of 1 represents the maximum level of the range.

Table 1. Actual and coded values of experimental design

Independent Variables	Coded values		
	-1	0	1
Ethanol ratio (%)-X_1	30	50	70
Time (min) - X_2	5	10	15
AM (%) - X_3	10	15	20

2.3. Total phenolic content

Different methods can be used to determine the total phenolic content (TPC) in samples, and one of these methods is the Folin method. The Folin-Ciocalteu [22] method was employed to determine the TPC of each pollen extract. The Folin method is a spectrophotometric technique commonly used to determine the amount of phenolic compounds.[23] It's particularly suitable for

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measuring the TPC in plant samples. After sample preparation, Folin-Ciocalteu reagent is added, followed by the addition of sodium carbonate solution. After a specific incubation period, the solution is measured with a spectrophotometer, and the total phenolic content is determined using a standard curve. The TPC was quantified and reported in milligrams of gallic acid equivalent (GAE) per gram of sample.

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2.4. Response surface methodology

The optimization process was carried out using Design Expert 13 software with a second-order polynominal response as:

$$Y_k = \beta_{k0} + \sum_{i=1}^{n} \beta_{ki} x_i + \sum_{i=1}^{n} \beta_{kii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{kij} x_i x_j$$

where Y_k was response variable (Y_i was total phenolic content of bee pollen extract); x_i was coded process variables (x_1 was ethanol ratio of solvent, x_2 was extraction time, and x_3 was Amplitude Modulation) and β_{k0} is the value of fitted response at the design center point, respectively.

2.5. Statistical analysis

Three different analyses were performed in order to determine the total phenolic content of the extracts. The data analysis was conducted using the Statistical Package for the Social Sciences (SPSS) version 23.0. The results were presented in the form of means together with each of their standard deviations. The analysis of variance (ANOVA) was employed to examine the means of the experimental results, taking into consideration statistically significant differences at a significance level of p<0.05. Following that, Duncan's multiple range tests were carried out to determine the significance of the differences that were observed.

3. RESULTS AND DISCUSSION

Antioxidative capabilities of bee pollen can be attributed to many components present in its composition. Some of the variables involved in antioxidant activity include antioxidant enzymes, phenolic compounds, carotenoids, vitamins C and E, and elements such as glutathione. Collectively, these components effectively neutralize the deleterious impact of oxygen radicals, hence inhibiting oxidative harm to cellular structures and tissues in the body. The overall antioxidant capacity of pollen is affected by both antioxidant enzymes and other chemicals, as stated in previous studies [24,25.] references. In this investigation, a total of 15 distinct extracts of bee pollen were acquired, and the total phenolic content (TPC) values of these obtained extracts were provided in Table 2.

Table 2. TPC values of obtained bee pollen extracts.

Extract number	Ethanol ratio (%)	Time (min)	AM (%)	TPC (mg GAE/g sample)
1	50	15	10	8.497±0.102^{ef*}
2	30	15	15	6.406±0.000^{b}
3	50	10	15	9.508±0.036^{j}
4	30	10	20	7.113±0.000^{c}
5	70	15	15	8.793±0.087^{gh}
6	50	5	20	9.065±0.071^{hi}
7	50	15	20	8.486±0.124^{ef}
8	70	10	20	9.305±0.376^{ij}
9	50	10	15	8.872±0.036^{gh}
10	30	10	10	6.133±0.131^{ab}
11	30	5	15	6.105±0.056^{a}
12	70	10	10	8.612±0.121^{fg}
13	70	5	15	7.824±0.324^{d}
14	50	10	15	9.181±0.258^{i}
15	50	5	10	8.277±0.062^{e}

* Means followed by different letter(s) differ significantly at p<0.05 (Duncan's multiple range test)

The places in which bee products (propolis, pollen honey, royal jelly, etc.) are collected affect how much phenolic content they contain.[26] In the study, the highest TPC was found in the extraction condition with an ethanol concentration of 50%, an extraction duration of 10 minutes, and an AM percentage of 15. In a different investigation, TPC concentrations of 16 bee pollen samples from three different Croatian locations were reported to range from 4.00 to 15.80 mg GAE/g.[27] According to a study, bee pollen obtained from various Turkish towns had TPC values ranging from 26.69 to 43.42 mg GAE/g.[28] A different study reported the total phenolic component content to be 21.30 mg GAE/g [29], while another investigation found it to be 23.3 mg GAE/g. [30] The study results presented show similarities to the findings obtained in our research. In addition, Duncan's multiple range test demonstrated a statistically important difference (p<0.05) in the total phenolic content (TPC) of bee pollen.

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The data presented in Table 2. The observed differences in the results can be attributed to the diversity in the extraction methodology, the choice of solvent, and the geographical source of the pollen samples. Linear, cubic and two-factor interaction models were examined in the Box-Behnken design, which was applied for experimental studies in which the total phenol content of bee pollen was determined. As a result of the examination, it was determined that the model that best explains the extraction conditions is the quadratic model. Fit summary was presented at Table 3.

The variance analysis (ANOVA) findings for the quadratic model is presented in Table 4. Based on the obtained p-value of the model being less than 0.05 and the lack of significant fit, it may be inferred that the quadratic model aligns satisfactorily with the experimental data. In the present investigation, the p-value was calculated to be 0.0041, indicating statistical significance. Furthermore, the lack of fit was found to be non-significant.

Table 3. Fit summary.

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.0264	0.1031	0.4314	0.2246	
2FI	0.9606	0.0724	0.2451	-0.5347	
Quadratic	**0.0039**	**0.4144**	**0.9004**	**0.5775**	**Suggested**
Cubic	0.4144		0.9253		Aliased

Table 4. ANOVA results for Quadratic model.

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	
Model	18.27	9	2.03	15.06	0.0041	significant
X_1	9.63	1	9.63	71.41	0.0004	
X_2	0.1037	1	0.1037	0.7693	0.4206	
X_3	0.7503	1	0.7503	5.56	0.0648	
X_1X_2	0.1116	1	0.1116	0.8272	0.4048	
X_1X_3	0.0206	1	0.0206	0.1527	0.7121	
X_2X_3	0.1596	1	0.1596	1.18	0.3263	
X_1^2	6.71	1	6.71	49.73	0.0009	
X_2^2	1.15	1	1.15	8.50	0.0332	
X_3^2	0.0087	1	0.0087	0.0644	0.8098	
Residual	0.6743	5	0.1349			
Lack of Fit	0.4720	3	0.1573	1.56	0.4144	not significant
Pure Error	0.2023	2	0.1012			
Cor Total	18.95	14				

The process of Response Surface Methodology (RSM) includes several key steps. These stages include the evaluation of the impacts exerted by independent variables on dependent variables, the representation of these impacts using mathematical models, and the subsequent optimization of the independent variables. A p-value below 0.05 indicates that the model terms are statistically significant. In the present scenario, the model parameters X_1, X_1^2, and X_2^2 show statistical significance. The statistical analysis revealed that the ethanol ratio of the solvent, among the several extraction parameters investigated, had a significant impact on the overall phenolic content of bee pollen. The graphical representation of the overall phenolic content of bee pollen was depicted in Figure 1. The total phenolic content of bee pollen was significantly influenced by the ethanol ratio of the solvent. In contrast to the ethanol ratio of the solvent, the total phenolic content of bee pollen exhibited minor variations with respect to extraction time and amplitude modulation. The quadratic polynomial equation created as a result of the multiple regression analysis to determine the total amount of phenolic substances in bee pollen is shown below.

$$TPC = 1.097\,X_1 + 0.113\,X_2 + 0.306\,X_3 + 0.167\,X_1X_2 - 0.071\,X_1X_3 - 0.199\,X_2X_3 - 1.347\,X_1^2 - 0.557\,X_2^2 - 0.048\,X_3^2$$

Optimization results were given at Table 5. Optimal extraction conditions of bee pollen for maximum total phenolic content were determined ethanol of 60.012 %, time of 11.054 min, 19.160 AM %. In addition, the phenolic content was predicted as 9.572 mgGAE/g sample at suggested extraction conditions. Extraction optimization is crucial in chemistry studies because this process enables the efficient separation of a desired component from a mixture. Extraction optimization ensures the extraction of the target compound with the highest possible efficiency. This allows for more effective utilization of materials and reduces waste. An

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optimized extraction process minimizes time and energy costs, which is important in both laboratory studies and industrial production. Extraction optimization allows for the minimal extraction of unwanted components, ensuring the targeted compound is obtained in a pure form. This facilitates analysis and characterization processes. A well-established extraction protocol ensures the reproducibility of results. This makes it easier for others to verify your work or obtain similar results under the same conditions. An optimized extraction process often reduces material and labor costs, thereby lowering overall expenses. More effective extraction processes help minimize waste and by-products, contributing to the reduction of environmental impact.

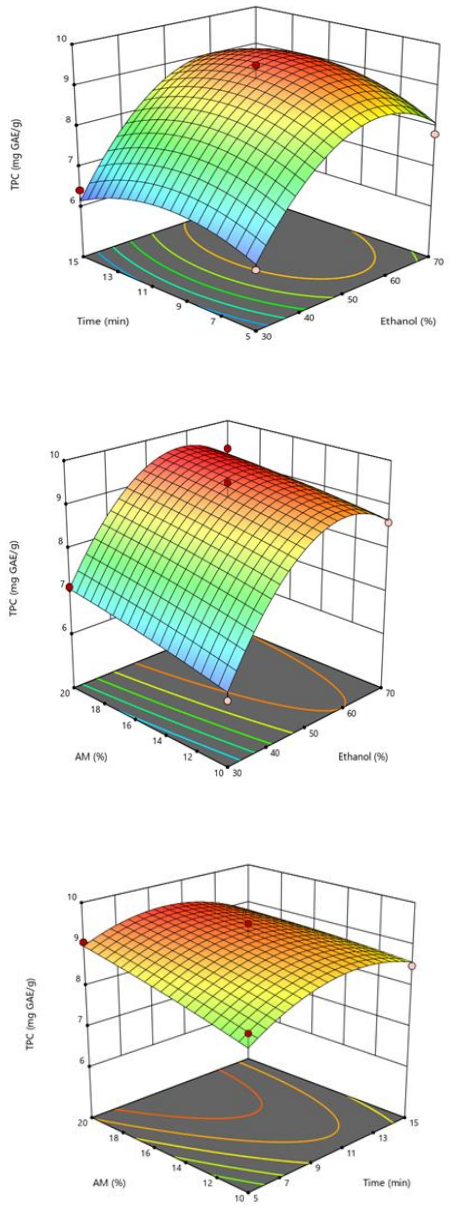

Figure 1. Response surface plots of total phenolic content of bee pollen.

Table 5. Optimization results

Ethanol (%)	Time (min)	AM (%)	TPC (mgGAE/g sample)
0.012	11.054	19.160	9.572

The objective of a previous investigation was to enhance the antioxidant activity and tyrosinase-inhibitory capabilities of bee pollen by the optimization of its extraction methods. The RSM was employed to optimize many factors, such as temperature, time, and extraction solvent. Regression analysis showed a reasonable correspond, revealing that the optimal parameters for the extraction process were determined to be a concentration of 69.6% ethyl acetate (EtOAc) in methanol (MeOH), a temperature of 10.0°C, and an extraction period of 24.2 hours.[30]

A study was conducted to examine the impact of deep eutectic solvents (DESs) and ultrasonic extraction on the extraction efficiency of bioactive compounds from bee pollen. An experimental study was conducted to examine the impact of several process parameters on the result of the experiment. These parameters included the molar ratio of DES (1, 1.5, and 2), the duration of sonication (15, 30, and 45 minutes), and the level of ultrasonic power (90, 135, and 180 W). The investigation employed response surface methodology (RSM) to analyze the data and draw conclusions. The experimental results revealed that the most favorable conditions included a molar ratio of 2, sonication for a duration of 45 minutes, and an ultrasonic power of 180 W. [31] The study's findings indicate that the optimization process is dependent on the extraction method utilized and the exact parameters applied within that procedure.

Extraction optimization is a significant area of research and development in chemistry to enhance efficiency, reduce costs, and minimize environmental impacts. The objective of this work was to optimize the extraction conditions of bee pollen using ultrasonic techniques to enhance the total phenolic content (TPC). The TPC is an essential metric for evaluating the nutritional and medical benefits of pollen samples.

The optimization of the extraction conditions was conducted using the RSM, while the experimental design followed to the principles of Box-Behnken design. In brief, this investigation aimed to enhance the ultrasonic extraction parameters for bee pollen to maximize the total phenolic content (TPC), hence enhancing the nutritional and health-promoting properties of the pollen. The results of this study offer significant contributions to the optimization of bee pollen use and its potential efficacy in disease prevention, owing to its abundant phenolic components and their capacity to counteract oxidative stress.

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Conflict of interests

I declare that there is no a conflict of interest with any person, institute, company, etc.

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