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Optimization of Bioactive Compound Extraction from Propolis by Reflux, Maceration and Ultrasound-assisted Methods and Characterization of the Extracts

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ABSTRACT
The region, botanical origin, and bee species influence the raw propolis content and its bioactive properties. Additionally, the extraction methods, solvents, and various process parameters significantly affect the bioactive properties of propolis extract, which is consumed as a food supplement or pharmaceutical product. In this study, propolis with a chestnut botanical origin, obtained from the Black Sea region in Turkey, was used as the raw material. The process parameters of three basic extraction methods—maceration (M), reflux (R), and ultrasound-assisted (UA)—were optimised using response surface methodology. Antioxidant activity (AA) and total phenolic content (TPC) were used as response parameters. The optimised levels for M were 78.46% ethanol concentration and 71.05 hours for extraction time; for R, 80.64% ethanol concentration, 117.44 minutes for extraction time, and 38.38°C for temperature; and for UA, 82.49% ethanol concentration, 59.12 minutes for extraction time, and 40.53°C for temperature. The results were statistically validated using the t-test. The AA, TPC, and phenolic, volatile, and mineral contents were compared among the optimised chestnut propolis extracts. Chrysin, a flavone, and pinocembrin, a flavanone, along with ferulic and ellagic acid, among the phenolic acids, were identified as the most abundant phenolic compounds. Among the 11 elements, the highest macro elements were Na, K, and Ca, while the trace elements were Fe and Zn. The phenolic, volatile, and mineral compositions of the optimised propolis extracts exhibited heterogeneous distributions. However, fatty acids (e.g., 18:0, 18:1) were present at relatively high levels only in R; phenolic compounds were obtained in relatively high amounts via M extraction. Some minor volatiles were detected only by UA extraction. Following the characterisation of the optimised extracts, it was determined that each extraction method has its own unique advantages. The results indicate that all three methods should be optimised and used together to achieve the highest component composition and bioactivity.

Keywords: Food supplement, Bee products, Response surface methodology, Bioactivity

1. Introduction

Propolis is a bee product with resinous material collected from the exudates and buds of plants mixed with pollen, wax, and bee enzymes. It is known to be used in complementary medicine because of its anti-inflammatory, antimicrobial, immunomodulatory, antioxidant, and immunostimulating activities from ancient times (Mele 2023; Bankova et al. 2021; Asem et al. 2019). The content of propolis, which is rich in bioactive components, varies depending on its botanical and geographical origin and harvest time (Kasote et al. 2022). The chemical composition of propolis varies depending on the plant source of the resin and balsam collected by bees (Al Dreini et al. 2023). Propolis is basically composed of 50% vegetable balsam and resin, 30% wax, 10% aromatic and essential oils, 5% pollen, and some other organic compounds, such as polyphenols and terpenoids, in nature (Rocha et al. 2023). Overall, more than 300 different bioactive compounds, such as phenolic aldehydes, ketones and polyphenols (phenolic acids, flavonoids, and esters) have been identified in propolis. The flavonoid group included chrysin, pinocembrin, apigenin, galangin, kaempferol, quercetin, tectocrisin, and pinostrobin, among others (El-Guendouz et al. 2019). The flavonoids that had the highest concentrations were pinosembrin (~4.7%), pinobencin (~3.1%), galangin (~2.2%), and chrysin (~2.1%). Another important group of identified bioactive compounds comprises phenolic acids, which also exhibit aromatic properties; these compounds include ferulic, cinnamic, caffeic, benzoic, salicylic, and p-coumaric acids (Do Nascimento Araújo et al. 2020).

Since the plant flora in which the hives are located determines the dominant primary, secondary, and minor pollen contents of bee products, the product characteristics and bioactive properties differ. For example, since the dominant pollen content of bee products such as honey, propolis, and pollen obtained from beekeeping activities carried out in locations where chestnut trees are dense in the Black Sea region of Turkey is chestnut, these products are called as "chestnut propolis, chestnut honey and

chestnut pollen". The color, composition, and related bioactive properties of propolis vary among countries worldwide and of different botanical origins (Özdal et al. 2023).

Raw propolis cannot be consumed directly due to its wax, resin, herbal balsam content, and bioactive compounds; therefore, it must be extracted appropriately. There are different extraction methods for the preparation of propolis extracts. The maceration method is the most basic method for extracting a sample by reducing it to the appropriate size and then maintaining it at room temperature for a certain period of time in a suitable solvent and a closed container (Silici & Kutluca 2005). In reflux extraction, heat is applied to the prepared solution during extraction, and the solution is kept in a water bath at a specific temperature and time (Margeretha et al. 2012). Ultrasound-assisted extraction aims to increase extraction efficiency with the help of sound waves during extraction processes. Many studies on extraction methods, solvent types, extraction times, and temperatures are available in the literature. Several of these studies examined the extraction yield, and several examined the extraction efficiency via total phenolic (TPC) and/or flavonoid (TFC) contents. Some studies include evaluations of the extraction of specific components (Özdal et al. 2023). Increasing the number of studies in which different solvents, temperatures, times, extraction methods, and their combinations are optimized will benefit both scientific and industrial users in eliminating deficiencies in this field. This study aimed to optimize the process parameters of three basic extraction methods (maceration, reflux, and ultrasound-assisted) for producing chestnut propolis ethanolic extracts. For this purpose, the response surface methodology was used, and the bioactive qualities of the optimized chestnut propolis extracts obtained were compared.

2. Material and Methods

2.1. Materials

Chestnut propolis samples were obtained from the apiaries of the Azdavay, Bozkurt, Cide, Doğanyurt, İnebolu and Küre districts, which include the borders of the Kastamonu chestnut forest region, during the 2021 harvest period, through the Kastamonu Beekeepers Association. The raw propolis samples were divided into small pieces and milled into powder then kept in a deep freezer at -18 °C until extraction.

2.2. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH) and Folin–Ciocalteu's reagent were obtained from Sigma-Aldrich Co. (Munich, Germany). Acetonitrile, sodium carbonate, ethanol and methanol were supplied by Merck KGaA (Darmstadt Germany). All phenolic standards as pure HPLC grade were purchased Sigma-Aldrich Co. (Munich, Germany).

This study consisted of 3 stages: optimization of the extraction conditions according to the experimental design, validation of the optimization results and characterization of the extracts which were produced at optimum extraction conditions.

2.3. Optimization of the extraction conditions

Experimental design: The ethanol concentration, extraction time, and temperature parameters were chosen as three independent variables. Antioxidant Activity (AA) (inhibition %) and TPC (mg GAE/100 mL) were two dependent variables. Response surface methodology (RSM) – The central composite rotatable design (CCRD) desirability function was used to evaluate the effect of the three independent factors on the two responses (Design Expert 7.0.0, Stat-Ease, Inc., Minneapolis, MN, USA). The experimental design involved 13 design points for maceration, 20 for reflux, and 20 for ultrasound-assisted extraction, including three replicates of the central point. Probability values (P) at a 95% confidence level were used to determine the significance and effectiveness of the response. Lack of fit test and Analysis of variance (ANOVA) was carried out using the software. For reflux extraction, a temperature of 70 °C and a duration of 2 hours have been recommended (Cottica et al. 2011; Abduh et al. 2023). Studies in the literature show that for maceration, a wide range from 3 hours to 7 days has been used. For ultrasound-assisted extraction, an average duration of 30 minutes has been suggested. While the generally accepted ethanol concentration for propolis extraction is 70% absolute ethanol (Pobiega et al. 2019), studies in the literature report concentrations ranging from 50% to 98% (Kim et al. 2009; Margeretha et al. 2012; Kara et al. 2022). The selection of minimum and maximum variable ranges for the extraction parameters of all three methods in this study was based on data from the literature. The experimental levels of independent variables were given in Table 1.

According to the experimental design of each extraction method, the ethanol concentration was applied. The mixture of ethanol and propolis was homogenized (Ultra-Turrax IKA T25, Staufenim Breisgau, Germany) at 10000 rpm for 30 s. Then, the extraction time and temperature parameters were applied according to the design. After centrifugation (Nüve, Germany) at 10000 rpm for 5 min, the extracts were filtered and stored at + 4 °C.

Maceration extraction: Propolis (5 g) was extracted with 50 mL of ethanol for 6-72 hours (at 25 ºC) at concentrations (50-90% v/v) specified in the experimental design, with periodic mixing at room temperature.

Reflux extraction: Propolis (5 g) was extracted with 50 mL of ethanol for 10-160 min at various temperatures (30-90 °C) and concentrations (50-90% v/v) specified in the experimental design.

Ultrasound-assisted extraction: Propolis (5 g) was extracted in an ultrasonic bath (Mikrotest, MUB12, Türkiye) (Operating frequence 28 KHz; dimension: 255*310*400 mm) with 50 mL of ethanol for 5-60 min at various temperatures (30-70 °C) and concentrations (50-90% v/v), as specified in the experimental design.

Table 1- The experimental levels of independent variables

Independent variables	*Levels*				
	-1.414	-1	0	+1	+1.414
Ethanol conc. (%)	**50**	55.86	**70**	84.14	**90**
Extraction time (hour)	**6**	15.67	**39**	62.33	**72**

a

Independent variables	*Levels*				
	-1.681	-1	0	+1	-1.681
Ethanol conc. (%)	**50**	58.11	**70**	81.89	**90**
Extraction time (min)	**10**	40.40	**85**	129.60	**160**
Temperature (°C)	**30**	42.16	**60**	77.84	**90**

b

Independent variables	*Levels*				
	-1.681	-1	0	+1	+1.681
Ethanol conc. (%)	**50**	58.11	**70**	81.89	**90**
Extraction time (min)	**5**	16.15	**32.50**	48.85	**60**
Temperature (°C)	**30**	38.11	**50**	61.89	**70**

c

** a: Maceration extraction, b: Reflux extraction, c: Ultrasound-assisted extraction methods*

2.4. Validation of the optimization results

Validation was performed by triplicate extraction under optimum conditions for each method. The average values of the responses were calculated. The estimated values from the model and averages were compared by using one-sample *t*-test. The lack of a statistically significant difference ($P>0.05$) between the results obtained from the validation test indicated that the model obtained via optimization was experimentally successful.

2.5. Analysis methods

2.5.1. Total phenolic content (TPC)

TPC of the propolis extracts was determined according to the Folin–Ciocalteu assay (Shahidi & Naczk 1995). Briefly, 0.5 mL of Folin–Ciocalteu's reagent (0.2 N) was added to 0.1 mL of extract (100 times diluted with ethanol). Then mixing the tube using a vortex, 0.4 mL of Na_2CO_3 solution and 4 mL of distilled water were added to the reaction mixture.

The absorbance readings were taken at 760 nm after incubation at room temperature for 1 hour using a UV–VIS spectrophotometer (Shimadzu Corporation, Japan) against the blank prepared using ethanol instead of extracts. Results were

expressed as gallic acid equivalent (mg GAE/100 mL) using the calibration curve [concentration = (Abs + 0.041)/0.002] obtained using gallic acid standard solutions.

2.5.2. Antioxidant activity

The antioxidant activity (inhibition %) of the propolis extracts was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging procedure. 1500 µL of DPPH solution was added to 75 µL of diluted (100 times) extracts, and the mixture was vortexed. After the mixture was incubated for 30 min, the absorbance readings were taken at 517 nm using a UV–VIS spectrophotometer. Inhibition % value was calculated with the following equation:

$$\text{Inhibition \%} = [\ 1 - (\frac{Abs\ Sample}{Abs\ Control})]*100 \quad (1)$$

2.5.3. Phenolic compounds

Phenolic content analysis was performed on a reversed-phase (RP) column. HPLC-PDA was carried out according to a validated method using 25 polyphenols (Kara et al. 2022). The ethanolic propolis sample was concentrated using the diethyl ether/ethyl acetate liquid–liquid extraction technique and subsequently passed through a C18 column (20 µL). For HPLC analysis (Shimadzu Corporation LC 20AT), a gradient phase was applied using 70–30% acetonitrile-ultrapure water and 2% acetic acid-ultrapure water mobile phases. The column oven temperature was 30 °C, and the mobile flow rate was 1.0 mL/min. Results were expressed as µg standard phenolic substance per mL extract sample.

2.5.4. Volatile compounds

Head-space solid-phase microextraction (SPME) method was used to determine the volatile compounds. The homogenized propolis extract was placed in a 125 mL flask with a magnetic stirring bar to extract the volatile compounds. The flask was then sealed with silicone septa and immersed in a water bath at 50 °C. After 5 min of equilibration, a 65 mm DCP (divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber was exposed to the sample headspace for 15 min. The sample was stirred continuously using a magnetic stir bar throughout the extraction process. After extraction, the fiber was placed into the injector port of a gas chromatograph for 5 minutes to desorb volatiles thermally. The volatile compounds were identified and quantified with a gas chromatograph coupled to a quadrupole mass detector (Shimadzu GCMS QP 2010 Ultra, Japan). Volatile compounds were separated on an RXİ-5MS capillary column (30 m; 0.25 mm; 0.25 μm). Helium was used as the carrier gas with a flow rate of 1 mL/min (pressure: 100 kPa). The injector temperature was set at 250 °C. The oven temperature program was held for 5 min at 50 °C, after which the temperature was increased by 5 °C/min to 270 °C. The temperature was maintained at 270 °C for 5 minutes, resulting in a total runtime of 54 minutes. The mass detector operated at 250 °C in scan mode with electron impact ionization (ion source temperature: 200 °C).

2.5.5. Mineral contents

The mineral content of the samples was assessed using a microwave-assisted nitric acid digestion procedure (CEM MARS6, USA) followed by analysis with inductively coupled plasma–optical emission spectrometry (ICP–OES) (Spectro Blue, Germany). Approximately 1 mL of each sample was mixed with 1 mL of H_2O_2 (30% v/v) and 10 mL of HNO_3 (67% v/v) in PTFE flasks. The digestion program included heating to 200 °C for 15 min, followed by a 15-min hold at 200 °C. After cooling to room temperature, the solutions were transferred to 50 mL polyethylene flasks and diluted with ultrapure water. The digested samples were filtered through microfilters and analyzed by ICP–OES (Al Khalifa & Ahmad 2010). Calibration standards were prepared using a multi-element standard stock solution (Merck, Germany). All measurements were performed in triplicate.

2.5.6. Statistical analysis

Analysis of variance (ANOVA) (IBM SPSS 1.0.0.781) was used to statistical evaluation of the results. After optimization, the obtained results were validated experimentally. For comparison ($P<0.05$) of the results, one-sample *t*-test (SPSS 17.0.1, Chicago, IL, USA) was used. The data were given as the mean ± standard deviation.

3. Results

3.1. Optimization of extraction parameters

Based on the experimental design, the software subsequently analyzed the results of the AA and TPC values. “Lack of fit” and “Sequential model sum of squares” tests were carried out (Table 2) for AA and TPC. R-squared (R^2), adjusted R^2, and the standard deviation were calculated for each function. After a comparison of the values subsequently, the suggested functions were ascertained.

Table 2- Statistical parameters of optimization; P values for model selection and lack of fit tests; model and independent variable factors; variance analysis results of functions (a); Selected solutions determined by desirability function and comparison of the results obtained from optimum point verification tests with the estimated values from model (b)

Statistical parameters		*Maceration extraction*		*Ultrasound-assisted extraction*		*Reflux extraction*	
		TPC	***AA***	***TPC***	***AA***	***TPC***	***AA***
Model selection and lack of fit test	Quadratic	**0.0277**	**0.0006**	**0.0104**	**0.0073**	**0.0037**	**0.0002**
	Lineer	0.1147	0.0075	0.0216	0.0199	0.2144	0.0501
	Cubic	0.1658	0.3557	0.3172	0.4388	0.1861	0.0876
	Lack of fit	0.1497	0.6065	0.3738	0.3976	0.1093	0.0676
Model and independent variable factors	Model	**0.0294**	**0.0001**	**0.0077**	**0.0055**	**0.0098**	**0.0002**
	A-Ethanol conc.	**0.0130**	**<0.0001**	**0.0084**	**0.0011**	**0.0496**	**0.0007**
	B-Extraction time	0.6552	0.3173	**0.0106**	**0.0207**	0.5489	0.1657
	C-Temperature	-	-	**0.0465**	0.6566	**0.0199**	**0.0006**
	AB	0.3297	0.1202	0.1725	0.2570	0.2064	0.6488
	AC	-	-	0.5199	0.4636	0.2239	0.0067
	BC	-	-	0.7049	0.4530	0.1997	**0.0311**
	A^2	**0.0316**	**0.0019**	**0.0054**	0.0022	**0.0008**	**<0.0001**
	B^2	0.0933	**0.0023**	0.1035	0.0366	0.3890	0.6390
	C^2	-	-	**0.0243**	0.7046	0.1104	**0.0309**
R^2		0.78	**0.96**	0.83	0.84	0.82	**0.92**
Adjusted R^2		0.62	0.93	0.67	0.70	0.65	0.86
Variation coefficient (%)		11.81	3.98	10.17	8.82	12.11	7.04

**TPC: Total phenolic content, AA: Antioxidant activity; ** The values P<0.05 are statistically significant*

(a)

Extraction type	*Independent factors*			*AA (%)*	*TPC (mg/100 mL)*
	A (%)	*B (time)*	*C (℃)*		
Maceration	78.46	71.05 (h)	-	84.16	980.39
Reflux	80.64	117.44 (min)	38.38	79.76	788.55
Ultrasound-assisted	82.49	59.12 (min)	40.53	80.68	695.91

(b)

Extraction type	*Response*	*Estimated value*	*Average experimental result**	*Difference*	*P-value**
Maceration	AA	84.16	88.61 ± 2.05^{A}	-4.45	0.275
	TPC	980.39	977.5 ± 4.11^{a}	-2.89	0.610
Reflux	AA	79.76	79.50 ± 1.95^{AB}	+0.26	0.916
	TPC	788.56	917.5 ± 11.02^{b}	+128.94	0.054
Ultrasound-assisted	AA	80.68	75.22 ± 2.17^{B}	-5.46	0.241
	TPC	695.91	812.5 ± 10.18^{c}	+116.59	0.055

** Average ± standard deviation; Values P<0.05 indicate statistical significance*
AA: Antioxidant activity; TPC: Total phenolic content; A: Ethanol concentration; B: Extraction time; C: Temperature

The quadratic function was accepted for AA and TPC for all extraction methods (P<0.05). 'Lack of fit' was not significant (P>0.05) for both properties. The influence of the ethanol concentration, which is one of the independent factors on the TPC and AA, was statistically significant (P<0.05) for all extraction types. However, the influence of extraction time was statistically significant (P<0.05) for only ultrasound-assisted extraction. The temperature factor had a statistically significant effect (P<0.05) on the TPC for reflux and ultrasound-assisted extractions and AA for only ultrasound-assisted extraction. The model was approved to be statistically significant (P<0.05) for all the extraction types and responses. The results of the ANOVA for the quadratic function were presented in Table 2. Final equations were coded with the following factors:

For maceration extraction;

$TPC= +768+104.07*A+14.66*B+46.59*AB-90.32*A^2 + 65.49B^2$
$AA= +67.84+9.59*A+1.03*B+2.38*AB-4.92*A^2 + 4.76B^2$

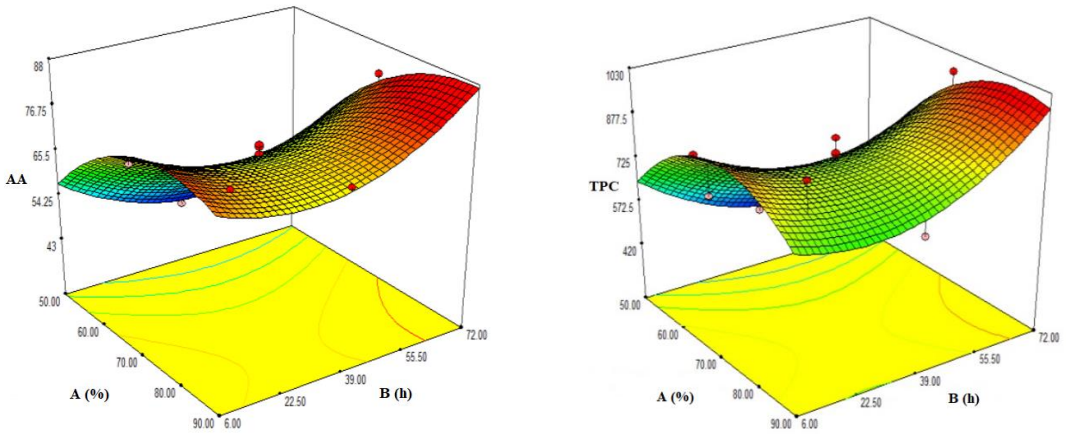
For ultrasound-assisted extraction;

$TPC=+621.74+48.87*A+46.85*B-33.92*C+28.67*AB-13.01*AC-7.61*BC-51.44*A^2-26.04*B^2 - 38.53*C^2$
$AA=+74.51+7.31*A+4.45*B-0.74*C+2.55*AB+1.61*AC-1.65*BC-6.44*A^2-3.80B^2 +0.62*C^2$

For reflux extraction;

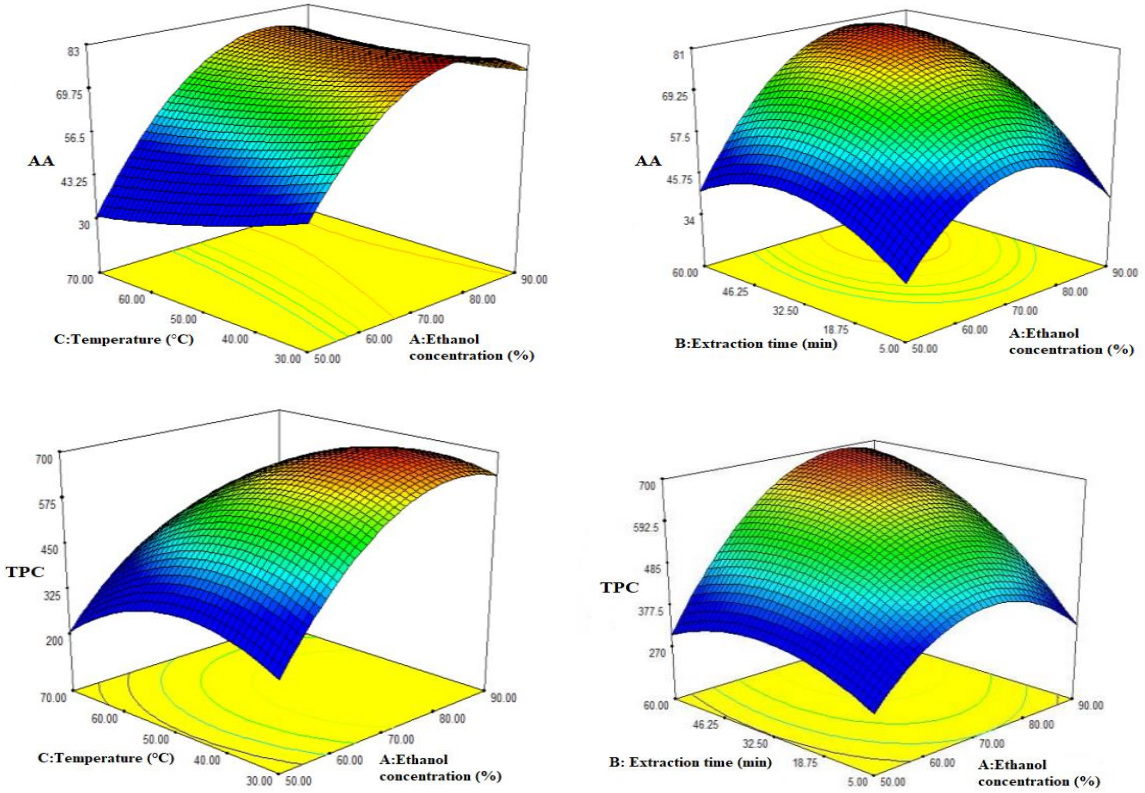
$TPC=+710.94+46.38*A-12.89*B-57.50*C+36.68*AB-35.19*AC-37.27*BC-95.80*A^2-18.21*B^2-35.42*C^2$
$AA=+70.32+5.69*A-1.78*B-5.88*C+0.73*AB-5.29*AC-3.89*BC-8.21*A^2-0.56*B^2-2.90*C^2$

Figure 1a shows the response of the interaction effect of the ethanol concentration and extraction time on the AA concentration (a) and on the TPC (b) for maceration extraction. AA and TPC increased with increasing ethanol concentration. However, both responses tended to decrease after the central point with increasing ethanol concentration. The opposite was true for the extraction time.



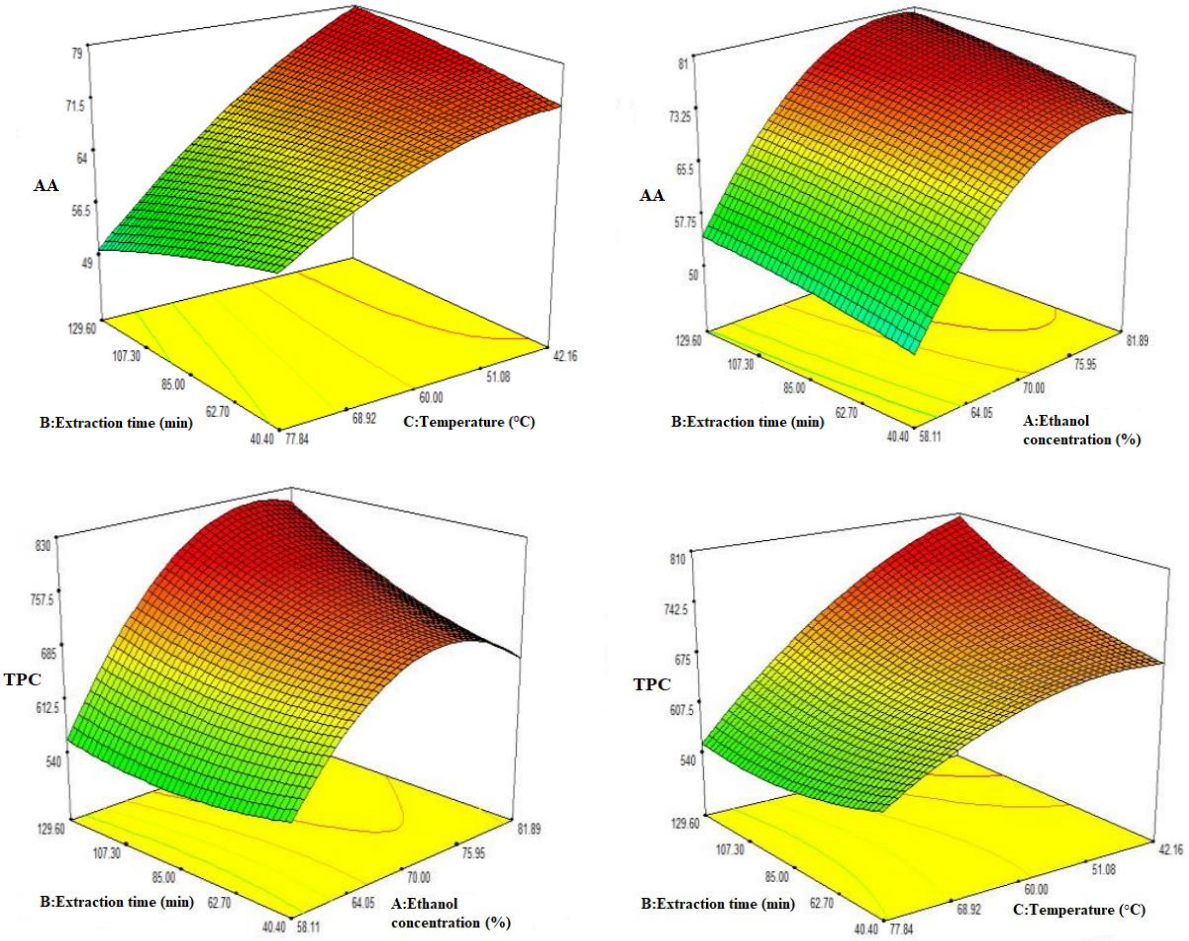
(a)

The TPC and AA exhibited quadratic functions for ultrasound-assisted extraction. Figure 1b shows the response of the interaction effect on the ethanol concentration (A), extraction time (B), and temperature (C) on AA (a) and the TPC (b) for ultrasound-assisted extraction. AA and TPC increased with ethanol concentration, extraction time, and temperature. However, both responses tended to decrease after the central point with increasing independent factors (except for temperature for AA).



(b)

The TPC and AA exhibited a quadratic function for reflux extraction. Figure 1c shows the response of the interaction effect of ethanol concentration (A), extraction time (B), and temperature (C) on the AA concentration (a) and on the TPC (b) for reflux extraction. AA and TPC increased with increasing ethanol concentration and temperature. However, both responses tended to decrease after the central point with the increase in both independent factors. The opposite trend was observed for the extraction time.



(c)

Figure 1- Response surface plots showing the mutual effects of the ethanol concentration (A), extraction time (B) and temperature (C) on the AA concentration and TPC for maceration (a), ultrasound-assisted extraction (b) and reflux extraction (c)

The AA and TPC were evaluated according to the desirability function which was based on the idea that the ‘‘quality’’ of a product or process has multiple quality characteristics. The first solution had a desirability value (100%) from the suggested solutions by the software. This solution was selected as the optimum point and applied in the study (Table 2b).

3.2. Experimental validation of the optimization results

The optimized levels given in Table 2b were used for the preparation of the extracts in triplicate. The AA and TPC contents of the optimized extracts were analyzed for all extraction types, and the average values of the results were determined. Whether there was a statistically significant ($P<0.05$) difference between the average and estimated values from the model by applying the one-sample *t*-test, was evaluated. One-sample *t*-test results for each response were given in Table 2b. There was not detected any statistical significance ($P>0.05$) between the results obtained from the validation test. This result indicates that the model obtained via optimization was experimentally successful.

3.3. Characterization of optimized chestnut propolis extracts

The propolis extracts produced by the optimized extraction models were compared in terms of bioactive properties, volatile compounds, and mineral contents.

3.3.1. Bioactive properties

TPC and AA values of the chestnut propolis extracts obtained by maceration, reflux and ultrasound-assisted extraction optimization are shown in Table 2b. The highest TPC and AA were achieved by maceration extraction ($P<0.05$).

In this study, the phenolic compounds in optimized chestnut propolis extracts obtained by three extraction methods were characterized via RP-HPLC-PDA. Chromatograms were presented in the Supplementary Material file, and the results were given in Table 3.

Among 25 phenolic substances, chlorogenic acid, protocatechuic acid, epicatechin, m-OH benzoic acid, syringic acid, routine, myricetin, resveratrol, daidzein, luteolin, rhamnetin, and curcumin could not be detected in any chestnut propolis extract in the present study. Gallic acid was detected only in samples prepared by reflux and ultrasound-assisted extraction. Chrysin, a flavone, was determined to be the most abundant compound, followed by pinocembrin. In addition, chestnut propolis extracts were rich in other phenolics, such as ferulic acid, ellagic acid, and caffeic acid phenyl ester (CAPE). The extract obtained by maceration had a higher content of polyphenols, especially CAPE.

Table 3-Phenolic compounds in chestnut propolis extracts identified via HPLC

	Maceration extraction		*Reflux extraction*		*Ultrasound-assisted extraction*	
	µg phenolic/mL	*Retention time*	*µg phenolic/mL*	*Retention time*	*µg phenolic/mL*	*Retention time*
Phenolic acids (%)	51.62		52.19		51.21	
Gallic acid	-		4.47	7.24	4.67	7.10
Protocatechuic acid	-		-		-	
Chlorogenic acid	-		-		-	
p-OH benzoic acid	6.63	16.13	7.15	15.57	6.23	15.45
m-OH benzoic acid	-		-		-	
Ellagic acid	432.40	19.70	416.04	19.27	400.67	19.22
p-coumaric acid	194.36	19.98	174.40	19.51	216.03	19.43
Ferulic acid	453.41	20.74	412.56	20.29	424.05	20.23
CAPE	292.89	43.08	263.70	42.18	283.46	42.44
Caffeic acid	210.72	17.17	190.69	16.64	208.94	16.52
Syringic acid	-		-		-	
t-cinnamic acid	117.87	28.02	106.72	27.41	111.90	27.35
Flavonoids (%)	48.38		47.81		48.79	
Flavonol						
Rhamnetin	-		-		-	
Quercetin	32.03	26.43	31.87	25.83	31.94	25.74
Rutin	-		-		-	
Myricetin	-		-		-	
Flavan–3–ols						
Epicatechin	-		-		-	
Flavones						
Chrysin	763.85	41.76	668.45	40.83	764.67	41.07
Daidzein	-		-		-	
Apigenin	41.11	30.17	38.40	29.43	38.69	29.41
Luteolin	-		-		-	
Flavanones						
Pinocembrin	740.35	42.60	687.05	41.66	726.41	41.9
Hesperetin	23.96	30.76	17.88	30.05	15.87	30.02
Nonflavonoid polyphenols						
Resveratrol	-		-		-	
Curcumin	-		-		-	

3.3.2. Identification of volatile compounds

The volatile compounds in the optimized chestnut propolis extracts identified via the SPME-GC/MS method were listed in Table 4.

Table 4- Volatile compounds identified in optimized chestnut propolis extracts

Compound type	*Compound*	*Maceration extraction (%)*	*Reflux extraction (%)*	*Ultrasound-assisted extraction (%)*
Benzene derivative	Styrene	-	-	0.20
Monoterpene	Trisiklen	0.23	-	0.24
Monoterpene	cis-Ocimene	47.40	36.02	-
Monoterpene	.Δ.3-Carene	-	-	60.94
Monoterpene	Kampen	2.56	0.83	1.42
Monoterpene	Verbenene	-	-	0.48
Aromatic aldehyde	Benzaldehyde	0.25	0.42	0.47
Monoterpene	Pinen	5.27	3.4	5.67
Hydrocarbon	Decane	4.73	3.47	4.43
Aliphatic hydrocarbon	Limonen	7.69	4.05	4.27
Aromatic alcohol	Benzyl alcohol	-	1.12	-
Other	p-Cresol	0.48	0.64	0.18
Alcohol	Farnesol	0.29	-	-
Aromatic hydrocarbon	Undecane, 5-methyl-	0.36	-	-
Carboxylic acid and esters	Benzoic acid	0.91	0.42	0.62
Aromatic hydrocarbon	Dodecane	2.47	1.17	2.21
Other	Bornyl acetate	-	-	0.22
Phenolic monoterpenoid	Carvacrol	4.58	2.21	2.23
Phenol	Eugenol	2.79	1.61	2.07
Sesquiterpenoid	Ylangene	-	-	1.03
Sesquiterpene	α-Cubebene	1.04	0.48	0.75
Sesquiterpene	α-Copaene	2.37	1.01	1.91
Sesquiterpene	Longifolene	0.44	-	0.44
Sesquiterpene	Caryophyllene	1.17	1.14	1.74
Sesquiterpene	Germacrene	0.47	0.38	0.51
Phenylpropene	trans-Isoeugenol	-	-	0.19
Sesquiterpene	α--Humulene	-	-	0.40
Phenol	Phenol, 2-methoxy-3-(2-propenyl)-	1.04	-	-
Sesquiterpene	Amorphene	1.89	0.64	0.94
Sesquiterpene	β.-Selinene	-	-	0.14
Sesquiterpene	α--Guaiene	1.32	-	-
Sesquiterpene	Muurolene	-	-	0.54
Sesquiterpene	Bisabolene	0.37	-	0.30
Sesquiterpene	Cadinene	0.83	0.62	0.51
Fatty acid	Myristic acid	-	3.17	-
Fatty acid	Pentadecylic acid	-	2.43	-
Ketone	Cyclopentadecanone, 2-hydroxy-	-	9.44	-
Fatty acid	Palmitic acid	-	7.69	-
Fatty acid	Oleic acid	-	3.79	-
Fatty acid	Stearic acid	-	0.48	-
Fatty acid	Arachidic acid	-	2.89	-
Other	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	1.10	-	1.24
Other	Alloaromadendrene oxide-(1)	0.19	-	0.22
Sterol	Amyl acetate	0.43	-	-
Triterpene	Squalene	6.00	5.86	3.49
Other	Cyclooctasiloxane, hexadecamethyl-	0.43	-	-
Sterol	Stigmast-5-en-3-ol, (3.beta.)- (CAS)	0.86	-	-

The GC-MS analysis of propolis extracts revealed approximately 40 peaks, suggesting a variety of compounds. According to Table 4, these compounds are categorized into several classes: hydrocarbons, terpenoids, diterpenoids, triterpenoids, and phenols. Terpenoids were the most prevalent class among them. Among these compounds, cis-ocimene was dominant in the maceration (44.05%) and reflux (36.02%) extraction samples, while Δ-3-carene (56.60%) was predominant in the ultrasound-assisted propolis extract. A total of 47 compounds were detected in the SPME extracts isolated from the samples. Based on the results of this study, various volatile aromatic compounds, ranging from 26 to 31 in number, were determined in each extracted propolis sample.

3.3.3. Mineral composition

This study determined the concentrations of 13 individual elements (Pb, Cd, Zn, Cu, Ni, Fe, Mn, Cr, Al, P, Na, Ca, and K) in chestnut propolis extracts using ICP–OES. Heavy metals and other elements demonstrated a heterogeneous distribution in propolis extracts (Table 5).

Table 5- Mineral contents of optimized chestnut propolis extracts

	Trace elements (ppm)								
	Al	***Cr***	***Mn***	***Fe***	***Ni***	***Cu***	***Zn***	***Cd***	***Pb***
M	-	0.055±6.631^{A}	0.148±3.354^{A}	0.676±10.929^{B}	0.048±10.479^{B}	-	0.150±4.779^{B}	-	0.806±5.326^{B}
R	0.203±8.520	-	0.147±2.601^{A}	2.710±9.481^{A}	-	-	0.569±9.979^{A}	-	1.361±7.447^{A}
U	-	0.070±2.713^{B}	0.093±0.432^{B}	0.422±2.259^{C}	0.071±2.647^{A}	-	0.167±2.426^{B}	-	0.941±2.284^{B}

(a)

Macro elements (ppm)			
P	***Na***	***Ca***	***K***
7.386±33.971^{A}	12.750±0.141^{A}	10.750±0.070^{B}	78.550±417^{A}
7.998±36.452^{A}	12.625±0.368^{A}	49.250±0.043^{A}	-
6.160±60.032^{B}	11.400±0.062^{B}	7.000±0.071^{C}	56.800±0.317^{B}

(b)

*-: Non-determined; * Different letters (A-C) in the same column indicate the statistical significance (P<0.05) of the difference between extraction methods*
**M: Maceration extraction, R: Reflux extraction, U: Ultrasound-assisted extraction

Generally, the element levels detected in reflux extraction were the highest. While the K content was found to be highest after maceration (78.55 ppm) and ultrasound-assisted extraction (56.80 ppm), K could not be detected after reflux extraction. Conversely, Ca was found at the highest concentration (49.25 ppm) by the reflux method, whereas it was found at lower and similar concentrations (10.75-7.00 ppm) during maceration and ultrasound-assisted extraction. P and Na were detected via all the extraction methods. P and Na were found at similar concentrations (P>0.05) via maceration and reflux extraction and at lower concentrations (P<0.05) via ultrasound-assisted extraction. Cu and Cd could not be detected by any of the three methods. While the Al content was determined via reflux extraction, Ni and Cr could not be detected. Mn was found at similar concentrations (0.148-0.147 ppm) in chestnut propolis extracted by maceration and reflux extraction methods but at a concentration of 0.093 ppm for ultrasound-assisted extraction. Fe, Zn, and Pb were detected via all three methods, with concentrations being higher for the reflux extraction method compared to the other methods.

4. Discussion

Phenolic compounds, which are secondary metabolites of plant origin and are found in many natural products, are agents responsible for AA. In this study, the highest TPC and AA were achieved by maceration extraction. Maceration is one of the most commonly used traditional methods for extracting the active components of propolis. A study comparing maceration, soxhlet, and ultrasound-assisted extraction methods revealed that the extracts obtained with the 5-day maceration method provided the highest TPC (Zin et al. 2018). Conditions such as solvent type and extraction time also affect the extraction efficiency and final product properties. In a study conducted by Mokhtar et al. (2019), have been reported that the use of ethanol as a solvent in maceration extraction resulted in higher phenolic and flavonoid contents than did the use of water. The chemical composition and bioactivity of propolis vary according to seasonality, the flora of the region where hives are located, dominant botanical origin, bee species, and extraction types and parameters (Calegari et al. 2020).

At least 300 different propolis compounds have been identified; their biological activities are attributed mainly to phenolic components, such as flavonoids (flavonols, flavones, flavonones, dihydroflavonols, and chalcones); aromatic aldehydes; terpenes; alcohols; and beta-steroids. Phenolic compounds have biological activities, such as antioxidant potential, because they have hydroxyl groups and aromatic compounds in their chemical structures (Calegari et al. 2020). Chromatographic analyses are the most frequently used methods for determining phenolic substances in propolis. In this study, the phenolic compounds in optimized chestnut propolis extracts obtained by three extraction methods were characterized via RP-HPLC-PDA. In this study, 12 of the 25 phenolic substances were not detected in any extract.

According to different studies, the main phenolic compounds identified in propolis are hydroxycinnamic acids, hydroxybenzoic acid, chrysin, galangin, pinocembrin, quercetin, apigenin, ferulic acid, luteolin, cinnamic acid, benzoic acid, flavones, flavonols, and flavanones (Calegari et al. 2020; Woźniak et al.2020). Several studies have shown that the ethanol ratio in the solvent affects the phenolic composition of propolis extracts. While the phenolic acid content is high in solvents with high water content, no significant change is observed in solvents containing 70% or more ethyl alcohol (Kara et al. 2022). The ethanol rates (%) determined due to the optimization of all three extraction methods were very close in this study. Accordingly, it was determined that there was no significant difference in terms of the distribution of phenolic compounds. Additionally, gallic acid was found only in samples prepared by reflux and ultrasound-assisted extraction in the study. Kara et al. (2022) reported that the ethanol content is an effective parameter for determining the gallic acid content during propolis extraction. They detected gallic acid and protocatechuic acid in all extraction methods for solvents containing only 0% and 20% ethanol. When the general phenolic constituents of the optimized chestnut propolis extracts were evaluated, chrysin, a flavone, was determined to be the most abundant compound, followed by pinocembrin. A study comparing the antioxidant activities and phenolic compositions of propolis samples extracted under different extraction conditions and ethanol concentrations revealed caffeic acid as the main component in samples prepared with solvents containing 0-40% ethanol. Chrysin and pinocembrin were identified as the main components in extracts containing 60% ethanol or higher (Kara et al. 2022). In another study comparing lactic acid and ethanol solutions for propolis extraction, it was found that the Chrysin and naringenin were found as the most abundant phenolic compounds in the initial samples (Atayoglu et al. 2023)

In addition, chestnut propolis extracts were rich in other phenolics, such as ferulic acid, ellagic acid, and caffeic acid phenyl ester (CAPE). Propolis samples containing CAPE were reported to exhibit stronger radical scavenging activity (Sulaiman et al. 2014). In this study, the extract obtained by maceration had a higher content of polyphenols, especially CAPE. It was also determined to be the sample with the highest antioxidant activity (inhibition %).

The beneficial biological activities of propolis, such as antimicrobial, anti-inflammatory, anti-ulcer, and anti-cancer properties, are closely related to its bioactive compounds. Flavonoids are effective against various bacteria and protect against ulcers (Ruiz-Hurtado et al. 2021). Chrysin, which was detected in the highest amount in the chestnut propolis extracts in this study, is known to have anti-inflammatory and antineoplastic effects and functions as an important antioxidant and hepatoprotective agent (liver protector) (Shahbaz et al. 2023).

According to the Turkish Food Codex Bee Products Communiqué (Anonymous 2021), published as a draft by the Ministry of Agriculture and Forestry, "propolis extracts must contain at least 8 of the 20 phenolic compounds (ferulic acid, pinobanksin 3-acetate, chrysin, cinnamic acid, galangin, pinocembrin, kaempferol, caffeic acid, p-coumaric acid, benzoic acid, quercetin, artepilin C, rutin, catechol, naringenin, myricetin, hesperidin, apigenin, chlorogenic acid and CAPE) and at least 1 mg/L or 1 mg/kg." In this study, chestnut propolis extract prepared by maceration as a result of optimization contained 11 phenolic components specified in the draft communique, and extracts prepared by refluxing and ultrasound-assisted extraction methods contained 12 components. The optimized extracts met the requirements of draft communique limits.

The volatile compounds of propolis strongly depend on the bee species, the botanical flora at the collection site, and the solvents used in the extraction of propolis. The compounds identified in the propolis volatile fraction represent one of the primary botanical sources of propolis. Due to their flavour and biological activity, volatile compounds in propolis are beneficial for chemical characterization. The compounds detected in the extracts were categorized as hydrocarbons, terpenoids, diterpenoids, triterpenoids, and phenols in Table 4. Among these, terpenoids are the most prevalent due to their nature as volatile organic compounds. Therefore, GC–MS is the best method for their detection. Hydrocarbons are also volatile, but most of those found in propolis can be detected at moderate levels because they are long chains. In this study, the extraction method affected the detection of fatty acids (such as oleic, stearic, and palmitic acids), which were determined mainly via the reflux method. The flavonoid and phenol classes of compounds were the least common (Table 4).

Floral volatile compounds can vary greatly in their prevalence, ranging from widespread to quite rare. Many floral scents are frequently found across various plant families. These volatile compounds are highly effective at attracting pollinators. The most frequently encountered floral volatiles include benzaldehyde, limonene, β-ocimene, and linalool, which are common and dominant components in the floral aromas of numerous species (Farré-Armengol et al. 2017). Among them, ocimene is particularly notable for its significant ecological function due to its prevalence and abundance in floral scents.

β-Ocimene (3,7-dimethyl-1,3,6-octatriene), the chemical formula $C_{10}H_{16}$, is a monoterpenoid with two stereoisomers, cis- and trans-β-ocimene. The trans isomer is more common than the cis isomer. Cis-β-ocimene is less abundant than trans-β-ocimene in floral scents (Farré-Armengol et al. 2017). Since the propolis used in the present study was of chestnut origin, it is thought that the cis-isomer of ocimene is dominant (Table 4). While the percentage of Cis-Ocimen was 44.05% in maceration and 36.02% in reflux extraction samples, it could not be detected in ultrasound-assisted extraction samples.

While styrene was detected with ultrasound-assisted extraction, other methods could not obtain this compound. Stytrene is a benzene derivative and occurs naturally in small amounts in some plants and foods (cinnamon, coffee beans, balsam trees, and peanuts). Δ-3-carene, which constitutes 56.60% of the ultrasound-assisted propolis extract, is a bicyclic monoterpene found in hemp and other plants. Δ-3-carene is a natural component of turpentine. It is found in citrus, cypress, and pine trees and has a sweet, pungent, herbal, and earthy scent. Basil, rosemary, some peppers, and pine and cedar trees also naturally produce Δ-3-carene. Δ-3-carene is found in many plants essential oils and is a popular ingredient in cosmetics, fragrances, and food flavouring agents. Its presence in turpentine also makes it a powerful agent for use as an industrial-strength insect repellent. Δ-3-caren is known to have anti-inflammatory and anti-Alzheimer effects. It is effective against fungal infections and has the potential to improve bone health through increased calcium absorption into bones (Anonymous 2024). Another compound found in optimized propolis extracts obtained by all three methods is benzaldehyde, an important aromatic aldehyde. Its aroma was likened to bitter almond oil. A study on benzaldehyde indicated that it has anticancer effects and potential as an antitumor compound (Saitoh & Saya 2016). It was reported that carvacrol is a phenolic monoterpenoid found in the essential oils of some plants, especially thyme (*Thymus vulgaris*), oregano (*Origanum vulgare*), wild bergamot (*Citrus aurantium bergamia*) and pepperwort (*Lepidium flavum*). Additionally, carvacrol has a wide range of bioactivities, such as anticancer, antimicrobial, and antioxidant activities. The antimicrobial activity of carvacrol is greater than that of other volatile compounds and essential oils due to a phenol moiety, a free hydroxyl group, and hydrophobicity (Sharifi-Rad et al. 2018). Eugenol, primarily derived from clove oil, is a phenolic aromatic compound. Its well-known antibacterial, antiviral, antifungal, anticancer, anti-inflammatory, and antioxidant properties have made it valuable for many years in sectors like cosmetics, medicine, and pharmacology. Additionally, it is recognized as a natural mosquito repellent (Ulanowska & Olas 2021).

Mineral diversity is transferred to the composition of propolis by transferring the mineral composition of the soil to the plants from which propolis is obtained. Therefore, plant sources strongly influence the elemental composition of propolis (Lovakovic et al. 2018). The basic propolis content is used to develop reliable traceability methods and distinctive features of the geographical areas where it is produced to indicate environmental pollution (Golubkina et al. 2016). Propolis is an important food supplement for human nutrition because of its antibacterial, antifungal, antiviral, and antioxidant activities. Therefore, it is crucial to determine propolis's content in terms of essential minerals and heavy metals. In this study, the concentrations of 13 individual elements (Pb, Cd, Zn, Cu, Ni, Fe, Mn, Cr, Al, P, Na, Ca, and K) and heavy metals in chestnut propolis extracts demonstrated a heterogeneous distribution (Table 5). Generally, the element levels detected in reflux extraction were the highest. Parameters such as time, temperature, and applications in propolis extraction impact the elemental distribution and concentrations of the extracts.

A study conducted by Bayram (2020) revealed that K, Ca, Fe, Mg, and Na are the main elements in propolis obtained from Turkey, China, Brazil, and Ethiopia. Similarly, our study found K, Ca, Na, and P as key elements. Acun & Gül (2021) reported the Fe content of chestnut propolis to be 1.53 ppm, which aligns with our findings (0.422-2.71 ppm). While their study found the K content to be 3.11 ppm, our study found it to be in the range of 56.80-78.55 ppm. Another study examined (Arda, 2022) the mineral composition of three different commercial propolis drop products (brands A, B, and C) using the ICP-OES method. It found Ca levels of 9.28-5.53 ppm in samples B and C and Zn levels of 2.09-0.12 ppm in the same samples. Additionally, Cu was detected at 0.79 ppm in sample A but was not found in samples B or C. These findings indicate that the elemental distribution can vary widely in propolis extracts used as food supplements.

5. Conclusions

Various factors, such as region, botanical origin, and bee species, affect the raw propolis content and bioactive properties. However, propolis is not consumed in its raw form; it is consumed in its extract form as an important food supplement due to its bioactive features, such as anti-inflammatory, antimicrobial, and antioxidant properties. For this reason, extraction methods, parameters, and process parameters such as solvents and even contaminants significantly impact the bioactive properties and qualities of the final product.

In this study, three basic extraction methods, namely, maceration, reflux, and ultrasound-assisted methods were optimized, and the bioactive qualities of the obtained extracts were compared. As a result of optimization, similar values were suggested for the ethanol concentration for all three methods (min. 78.46%-max.82.49%). In this case, there was no significant difference between the predicted and validated AA and TPC values. However, considering the recommended times for maceration, reflux, and ultrasound-assisted extraction methods (71.05 h, 117.44 min, and 59.12 min, respectively), ultrasound-assisted extraction may be preferred because of its production process speed and high capacity. The reflux method may be preferred when the time factor is ignored because of the investment cost. On the other hand, the phenolic, volatile, and mineral compositions of the optimized propolis extracts showed varied distributions. Fatty acids were found to be more abundant in extracts obtained via the

reflux method, while phenolic compounds were more prevalent in the maceration method. Based on the results of this study, it was determined that different characteristics of the propolis extract emerged as a result of optimizing the parameters for the three extraction methods. In light of this conclusion, it is recommended to conduct a study where all three extraction methods are used in combination, with their parameters optimized collectively.

Abbreviations	**Full name**
Biological, Chemical and Microbiological	
AA	Antioxidant activity
ANOVA	Analysis of variance
CAPE	Caffeic acid phenyl ester
CCRD	The central composite rotatable design
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DVB/CAR/PDMS	Divinylbenzene/carboxen/polydimethylsiloxane
GAE	Gallic acid equivalent
RSM	Response surface methodology
TPC	Total phenolic content
Instrumental techniques	
GC-MS	Gas chromatography/Mass spectrometry
HPLC	High pressure liquid chromatography
HS/SPME	Head-space/solid-phase microextraction
ICP–OES	Inductively coupled plasma–optical emission spectrometry
UV	Ultraviolet

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