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RESEARCH PAPER

Assessment of extraction conditions on phenolic compounds in Turkish pine honey

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Abstract

Turkiye is a major producer of pine honey, meeting approximately 92% of global demand. This honey is highly valued for its unique bioactive properties, attributed to its diverse phenolic compounds. So, accurately identifying this honey type based on its phenolic profile responsible for its bioactive properties is of utmost importance. The aim of the study was to find the best extraction conditions for each phenolic compound from the pine honey sample with the highest total phenolic content (TPC) and total flavonoid content (TFC) among five samples from Turkiye. This was attempted using a total of 81 extraction conditions by varying solvent, temperature, time and acidity parameters. Protocatechuic acid and *p*-OH benzoic acid were determined to be the major compounds in all conditions, while gallic acid, catechin, vanillic acid, caffeic acid, syringic acid, epicatechin, *p*-coumaric acid, ferulic acid, benzoic acid, and quercetin were detected in varying amounts except for a few. The optimal extraction conditions for each phenolic compound were determined by statistical analysis. According to this, the ideal condition for protocatechuic acid, the highest content in all extracts, was 3 hours of water extraction at 20 °C and enrichment at pH 7, giving a value of 524.540 ± 3.641 µg/100 g sample. Interestingly, the selected temperature options used for the extraction parameters studied did not result in a statistically significant difference in the detection of phenolic compounds. Understanding and optimizing the extraction conditions for phenolic compounds in pine honey is crucial for enhancing its potential use in functional foods, pharmaceuticals, and quality control processes, thereby supporting its scientific and industrial relevance.

Keywords

Pine Honey, Phenolic Compounds, Solvent, Heat, Time

Introduction

Scientific studies have confirmed that honey, which is a natural product, has properties with high bioactive capacity. Phenolic acids and flavonoid-type agents have a very important place in the bioactivity of honey. The amounts of these components in honey vary depending on the type of honey, the region where it is collected, collection time, plant diversity, and vegetation (Joshi et al. 2000; Küçük et al. 2007; Silici et al. 2010).

Honey is classified into two types based on origin. These are “flower honey” made from plant nectars and “honeydew honey” made from plant-sucking insects (Hemiptera) that live on plants (Ülgentürk et al. 2013). In addition to the general classification, the classification based on honey origin is critical for determining significant features of honey (Hailu and Belay 2020).

Pine honey, which is classified as honeydew honey due to its origin, varies from blossom honey in terms of organoleptic qualities. The rectal secretions of *Marchalina hellenica* that feed on phloem sap of pine trees, which has high sugar content, are the main source of pine honey, a type of secretory honey (Gounari 2006). Honey bees consume the rich, sweet material that the insects discharge as faeces after consuming the nutrients. This material is brought to the hive by honey bees, who enzymatically convert it into pine honey (Özkök et al. 2010).

With 8.2 million hives, Turkiye is one of the top honey-producing countries in the world and housed 8.7% of the total hives in the world in 2020. Turkiye ranks third with this percentage, trailing India in first and China in second place. Turkiye, which ranks third in terms of hive density, also ranks in the top three in the world in terms of honey production. However, reports indicate that the country's exports do not match production because it ranks 22nd for honey exports despite ranking third for honey production in 2020 (GEKA 2019; TEPGE 2022).

In fact, roughly one-third of all honey produced in Turkiye is pine honey, which accounts for around 90% of global pine honey output (Özkök et al. 2018; GEKA 2019). By analyzing these data, pine honey plays a prominent role in the Turkish economy. The region of Muğla and its surroundings account for the vast majority of pine honey production in Turkiye because this area is where the insects are most prevalent and concentrated (Özkök et al. 2018). Pine honey has become an appealing topic due to the necessity to expand the export potential of this product, which has significant production potential in Turkiye, and to more efficiently and precisely convey its bioactive features to the scientific literature. So, figuring out the right evaluation and analysis of phenolic components, which are the source of many of the bioactive properties of this natural product, will help promote this product in a more accurate way.

The objective of this study was to identify the phenolic components in the pine honey sample selected from among 5 samples by making methanolic, ethanolic, and aqueous extracts at various temperatures (20, 40, and 60 °C), times (1, 3, and 6 h), and then enriching them with various acidic media (pH 1, pH 2, and neutral pH (pH 7)). The findings of this study will allow us to know the conditions in which pine honey can be extracted most precisely and efficiently. This will be a positive reference for future scientific investigations, and as a result of analysis of the bioactive value of honey under more precise conditions, this product will be defined more precisely in both the scientific literature and by customers.

Material and methods

Chemicals and their purity degree

All phenolic standards used in the study were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals that were necessary for the current assays were of analytical grade, used without any further purification, and supplied by Sigma-Aldrich (St. Louis, MO, USA).

Main sample selection for advanced analysis

Within the scope of the investigation, 5 pine honeys were obtained from Muğla, Türkiye, each from a unique producer. To identify 1 honey sample with the highest bioactive value among these samples, total phenolic compound (TPC) and total flavonoid compound (TFC) analyses were performed in methanolic extracts according to the methods described by Slinkard and Singleton (1977) and Chang et al. (2002), respectively. The main sample in the project consisted of pine honey which was chosen based on the highest results, and only this honey was used in further analysis studies. So, high-performance liquid chromatography (HPLC) analysis was performed for only 1 pine honey sample using different extract conditions.

Pine honey extraction conditions for main sample

It is known that in studies focused on the extraction from honey samples, different temperature ranges are commonly employed. According to the scientific documents, honey is commonly processed at temperatures between 40 °C and 60 °C. In some cases, higher temperatures of 70 °C to 80 °C are used briefly to eliminate microbial pathogens (Islam et al. 2022; Pedisić et al. 2023). Lower temperatures, such as 20 °C or room temperature, can be used to preserve thermolabile compounds and minimize degradation during extraction. In the study by Tsavea et al. (2022), bioactivity assays were conducted on pine honeys without the application of any temperature treatment during the extraction process; however, Islam and coworkers (2022) tried different ranges of temperature (40 °C, 60 °C, and 80 °C) in a bioactivity study, which included different types of honey. In the same study conducted by Islam et al. (2022), different extraction durations (0 min, 6 h, 12 h, 24 h and 48 h) were applied to the honey samples for short-term stability study based on bioactivity assessment. A review conducted of the main techniques and extraction systems commonly used for obtaining phenolic compounds from plant materials revealed that methanol, ethanol, water, and their combinations could be used as the extraction solvents (Lama-Muñoz and Contreras 2022). In consideration of the aforementioned scientific evidence, the extraction conditions utilized in the study were established. For the methanolic, ethanolic, and aqueous extracts, approximately 10 grams of the pine honey selected as the main sample were weighed. Using a condenser, equal concentrations of methanol, ethanol, and aqueous extracts were prepared at three different temperatures (20, 40, and 60 °C) and three different time parameters (1, 3, and 6 h). The primary extracts obtained from the combination of solvent, temperature, and time were filtered with paper filters, and thus homogeneity was achieved. Solvents in methanolic and ethanolic extracts were evaporated to dryness at 40 °C with the help of a rotary evaporator. For the aqueous extracts, the solvent content was evaporated to dryness using a lyophilizer. The phenolic compounds were enhanced prior to HPLC analysis, which is known as a secondary extraction process in the obtained residues. For this, methanolic, ethanolic, and aqueous extract residues were dissolved in 10 ml of distilled water with three different pH values (pH 1, pH 2, and neutral

pH (pH 7)) and fractionated with organic solvents of diethyl ether and ethyl acetate (3 × 5 ml), respectively (Akyuz et al. 2014). The organic fraction of each extract was placed into evaporator balloons, and the solvents were removed in a rotary evaporator at 40 °C. The content of the balloon was dissolved with 1 ml of LC-grade methanol, and HPLC analysis was carried out.

Pine honey extract coding

Coding used solvent type initial letter (methanol, ethanol, water), extraction time (in hours; 1, 3, and 6 h), temperature value (20, 40, and 60 °C), and acidity value used in the enrichment process (pH 1, pH 2, neutral pH (pH 7)), e.g., W-3h-20-N.

Phenolic compound analysis by HPLC

In the study, qualitative and quantitative separation of eighteen phenolic components was performed using HPLC (Thermo Scientific Dionex Ultimate; 3000 system, Thermo Scientific, Germany). For the analyses, a reverse-phase C_{18} column (150 mm × 4.6 mm × 5 µm; Fortis) was used. A mobile phase gradient program was applied, containing 2–98% acetic acid–distilled water in reservoir A and 50–50% acetonitrile–distilled water containing 0.5% acetic acid in reservoir B (Sahin et al. 2019). In addition, working optimization was achieved by adjusting the injection volume of samples and standards to 20 µl, the mobile phase flow rate to 0.7 ml/min, and the column temperature to 25 °C in the column furnace (Sahin et al. 2019). Eleven phenolic acids (gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, ferulic acid, *p*-coumaric acid, rosmarinic acid, and benzoic acid), four flavonoids (catechin, epicatechin, rutin, and quercetin), and three phenolic aldehydes (protocatechuic aldehydes, syringaldehyde, and vanillin) were comparatively detected at 280 and 315 nm, respectively. Based on the calibration curves drawn for each phenolic compound, the quantitative data were calculated. For qualitative chromatographic detection and to check possible peak shift of all analyzed compounds, a spike solution containing all of the phenolic compounds was prepared. After repeated analyses, this solution was added to each sample, and a final chromatographic run was carried out for the last time to compare and interpret the previously obtained data.

Validation of HPLC method

The HPLC method employed to determine the phenolic components in pine honey was tested for linearity, R^2, limit of detection (LOD), limit of quantification (LOQ), and % recovery. LOD and LOQ were calculated for each phenolic standard according to the signal-to-noise (S/N) levels of 3 and 9, respectively. R^2 data for all standards were also obtained using a calibration curve with a linear measurement range of 1.5625 to 50 ppm. Moreover, to figure out the recovery percentage of this method, the values in 50 ppm and 12.5 ppm solutions were analyzed. Data (except for quercetin) for R^2, LOD, and LOQ were also previously used in the study prepared by Karaçelik and Şahin (2021). All validation data are presented in Table 1.

Table 1. HPLC validation parameters.

No	Compound	Linear range (ppm)	$R^{2,a}$	LOD[a,b]	LOQ[a,b]	Recovery (%)	
						12.5 ppm	50 ppm
1	Gallic Acid	1.563–50	0.997	0.032	0.096	98.541	101.370
2	Protocatequic Acid	1.563–50	0.997	0.010	0.029	99.832	101.091
3	Protocatequic Aldehyde	1.563–50	0.996	0.107	0.324	101.023	100.482
4	*p*-OH Benzoic Acid	1.563–50	0.997	0.016	0.047	99.673	101.030
5	Catechin	1.563–50	0.997	0.002	0.006	93.542	99.970
6	Chlorogenic Acid	1.563–50	0.996	0.008	0.024	98.774	101.551
7	Vanillic Acid	1.563–50	0.996	0.015	0.046	100.971	102.984
8	Caffeic Acid	1.563–50	0.999	0.043	0.129	99.082	101.244
9	Syringic Acid	1.563–50	0.997	0.039	0.119	98.632	100.962
10	Epicatechin	1.563–50	0.997	0.002	0.007	93.982	99.934
11	Vanillin	1.563–50	0.996	0.078	0.236	100.213	101.491
12	*p*-Coumaric Acid	1.563–50	0.999	0.096	0.291	99.584	102.590
13	Syringaldehyde	1.563–50	0.999	0.012	0.035	98.091	100.101
14	Rutin	1.563–50	0.992	0.007	0.020	97.030	99.550
15	Ferulic Acid	1.563–50	0.995	0.031	0.093	98.330	101.012
16	Benzoic Acid	1.563–50	0.998	0.001	0.004	96.011	100.192
17	Rosmarinic Acid	1.563–50	0.997	0.236	0.716	99.901	100.672
18	Quercetin	1.563–50	0.997	0.002	0.008	98.522	99.763

a: The study that Karaçelik and Şahin (2021) published previously also used some of the relevant data [15]. b: The values were given in µg/ml.

Statistical analysis

All data obtained regarding the results of the applied analysis method are given in triplicate. Means ± standard deviations of three replicates are presented to support the quantitative analysis of the analyzed data. Statistical analyses were performed using the program SPSS Version 17.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance was performed to determine statistical differences. The significance of the difference between the means was demonstrated by One-way ANOVA with post-hoc Tukey with a probability of 0.05. Since the number of dependent and independent variables is more than one in analyzing the data in accordance with the objectives, multi-way ANOVA analysis should be applied. However, since the assumptions of multivariate normal distribution, the assumption of equality of covariance matrices and the assumption of equality of error variances were not met when the data set was examined; multi-way ANOVA tests were applied separately for each dependent variable. When normality assumptions were not met in multi-way ANOVA tests, normality assumptions were checked again by switching to one-way ANOVA. When normality was not met for one-way ANOVA, Kruskal Wallis H (KW) test was applied. The significance value results obtained from the application are given in Table 2 together with the test type. According to the results obtained in Table 2, Mann Whitney U test was applied in multiple comparisons for KW tests and Tukey test was applied for ANOVA tests since homogeneous variance was detected in multiple comparisons. The results obtained are shown in groups with letters and given in Table 3.

Results

Method validation results

The calibration curve for each phenolic compound was created with six points in triplicate. These six points (1.563–50 ppm) consist of common values for each component and are important for defining the linear measurement range. In addition to the data for the linear measurement range, Table 1 shows the R^2 values of the calibration curves constructed from the data of the measurement ranges of the standards. Calibration curves of all compounds were determined to be linear with $R^2 > 0.992$ (Karaçelik and Şahin 2021). The two critical criteria used to validate analytical procedures are LOD and LOQ. The LOD values for protocatequic aldehyde, vanillin, *p*-coumaric acid, and

Table 2. Significance results obtained for ANOVA or Kruskal Wallis H (KW) tests.

Variable	Solvent	Analysis Type	Time	Analysis Type	Heating	Analysis Type	pH	Analysis Type
Gallic Acid	<0.01	KW	>0.01	KW	>0.01	KW	<0.01	KW
Protocatechuic Acid	<0.01	KW	>0.01	KW	>0.01	KW	<0.01	KW
p-OH Benzoic Acid	<0.01	KW	<0.01	KW	<0.01	KW	<0.01	KW
Catechin	<0.01	KW	>0.01	KW	>0.01	KW	<0.01	KW
Vanillic Acid	>0.01	KW	<0.01	ANOVA	>0.01	KW	<0.01	KW
Caffeic Acid	<0.01	KW	<0.01	KW	>0.01	KW	<0.01	KW
Syringic acid	<0.01	KW	<0.01	KW	>0.01	KW	<0.01	KW
Epicatechin	<0.01	KW	<0.01	KW	>0.01	KW	<0.01	KW
p-Coumaric Acid	<0.01	KW	<0.01	ANOVA	>0.01	ANOVA	>0.01	ANOVA
Ferulic Acid	<0.01	KW	<0.01	KW	>0.01	KW	<0.01	KW
Benzoic Acid	>0.01	KW	<0.01	KW	>0.01	KW	>0.01	KW
Quercetin	<0.01	KW	<0.01	KW	>0.01	KW	>0.01	KW

Table 3. Mann Whitney U test or Tukey test results for multiple comparisons.

Fixed Factor	Solvent			Time			Heating			pH		
Dependent Variable	Methanol	Ethanol	Water	1h	3h	6h	20 °C	40 °C	60 °C	N	pH 1	pH 2
Gallic Acid	A	B	C	D	D	D	G	G	G	J	K	K
Protocatechuic Acid	A	B	C	D	D	D	G	G	G	J	K	L
p-OH Benzoic Acid	A	B	B	D	E	F	G	G	G	J	K	J
Catechin	A	B	C	D	D	D	G	G	G	J	J	K
Vanillic Acid	A	A	A	D	E	E	G	G	G	J	K	J
Caffeic Acid	A	B	C	D	E	F	G	G	G	J	K	J
Syringic acid	A	A	B	D	E	E	G	G	G	J	K	J
Epicatechin	A	B	C	D	E	D	G	G	G	J	K	L
p-Coumaric Acid	A	B	B	D	E	F	G	G	G	J	J	J
Ferulic Acid	A	B	A	D	E	F	G	G	G	J	J	K
Benzoic Acid	A	A	A	D	D	E	G	G	G	J	J	J
Quercetin	A	A	B	D	E	F	G	G	G	J	J	J

The same letters in each line were not significantly different at $p < 0.01$ (Mann Whitney U test or Tukey).

rosmarinic acid were found to be greater than 0.05 µg/ml, whereas the LOD values for all other standards were found to be less than 0.05 µg/ml (Table 1) (Karaçelik and Şahin 2021). The LOQ values for caffeic acid, syringic acid, vanillin, *p*-coumaric acid, and rosmarinic acid were above 0.1 µg/ml, whereas the LOQ values for the remaining standards were below 0.1 µg/ml (Table 1) (Karaçelik and Şahin 2021). When the values in 12.5 and 50 ppm solutions were examined to calculate the recovery percentage of the method used, the value for catechin and epicatechin at 12.5 ppm had the lowest recovery values (93%). Moreover, the deviations in all recovery values at 50 ppm were seen to decrease as the concentration increased and reached more acceptable levels (Table 1).

Pine honey main sample selection

In this study, TPC and TFC methods were chosen as marker assays for main sample selection. According to the obtained results, the range of TPC was found to be 49.296–60.611 mg GAE/100 g, and the TFC range was 1.813–3.601 mg QE/100 g. The pine honey tagged as sample 3 with 60.611 ± 0.248 mg GAE/100 g for TPC and 3.601 ± 0.058 mg QE/100 g for TFC was chosen as the main sample (Table 4).

Table 4. The results of total phenolic content (TPC) and total flavonoid content (TFC) of pine honeys studied for the main sample selection.

Sample	TPC (mg GAE/100 g)	TFC (mg QE/100g)
1	57.123 ± 0.154	2.544 ± 0.031
2	49.296 ± 0.102	1.909 ± 0.015
3	*60.611 ± 0.248*	*3.601 ± 0.058 Selected main sample*
4	47.335 ± 0.129	1.813 ± 0.016
5	52.268 ± 0.137	2.337 ± 0.017

HPLC results of prepared extract types

In the comprehensive examination of the selected pine honey sample, protocatechuic acid and *p*-OH benzoic acid were identified as the main components under all conditions. Except for a few of the 81 extract conditions, twelve phenolic compounds (gallic acid, protocatechuic acid, *p*-OH benzoic acid, catechin, vanillic acid, caffeic acid, syringic acid, epicatechin, *p*-coumaric acid, ferulic acid, benzoic acid, and quercetin) were detected in all extract types. Additionally, four phenolic components (protocatechuic aldehyde, chlorogenic acid, vanillin, syringaldehyde, rutin, and rosmarinic acid) were not detected in any extract types.

When a detailed evaluation of the solvent types is performed, the methanolic extracts were subjected to temperatures of 20, 40, and 60 °C and times of 1, 3, and 6 h, followed by enrichment with diethyl ether and ethyl acetate under neutral pH (pH 7), pH 1, and pH 2. The highest value was observed for protocatechuic acid in 21 of 27 different extract types (Fig. 1). In 6 extract conditions, *p*-OH benzoic acid was observed as the dominant phenolic compound (Fig. 1). This situation was mostly detected in the pH 1 data group. Caffeic acid was detected at moderate percentage in all extract types in this data group (Fig. 1). Protocatechuic acid was observed to be the most dominant phenolic in 17 of 27 different ethanolic extracts produced under the same conditions as methanolic extracts (Fig. 2). There were also 10 extract conditions in which *p*-OH benzoic acid was detected as the most dominant component in this solvent group, and it was mostly detected in pH 1 extracts (Fig. 2). Also, moderate percentages of caffeic acid and benzoic acid were detected in all extract types, except E-3h-40-pH 1 (Fig. 2). Other than the W-1h-60-pH 1 coded extract, protocatechuic acid was found to be the most important component in 26 other aqueous extracts (Fig. 3).

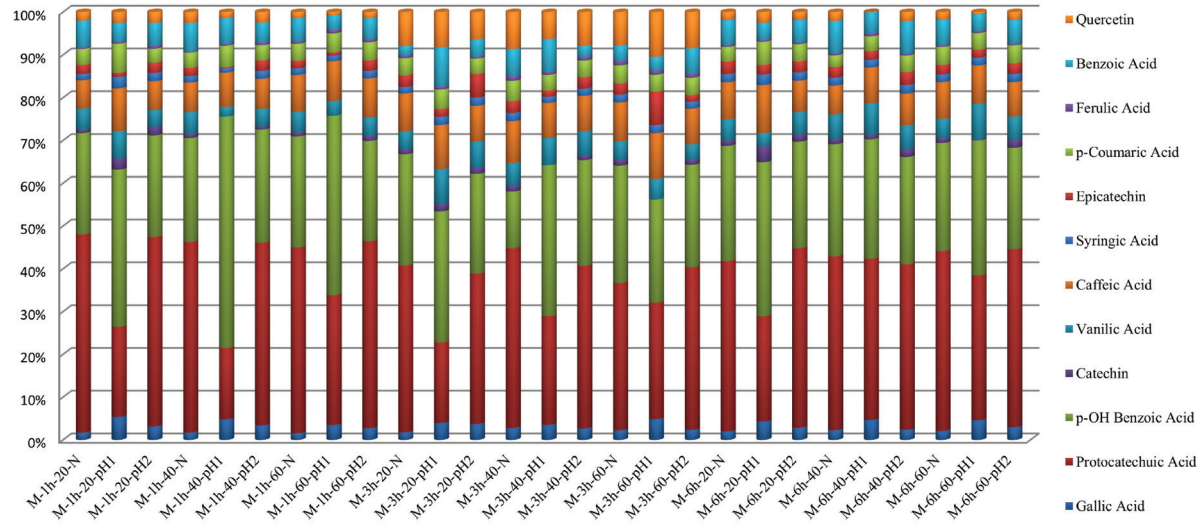

Figure 1. Percentage slice HPLC analysis results of pine honey extracts subjected to different pH values after methanolic extraction at different times and temperatures.

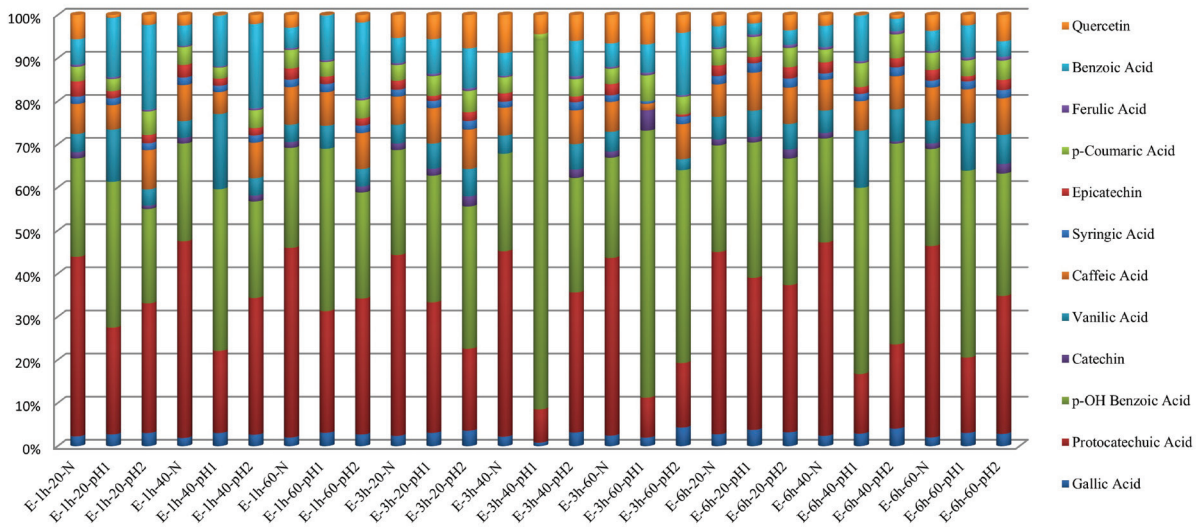

Figure 2. Percentage slice HPLC analysis results of pine honey extracts subjected to different pH values after ethanolic extraction at different times and temperatures.

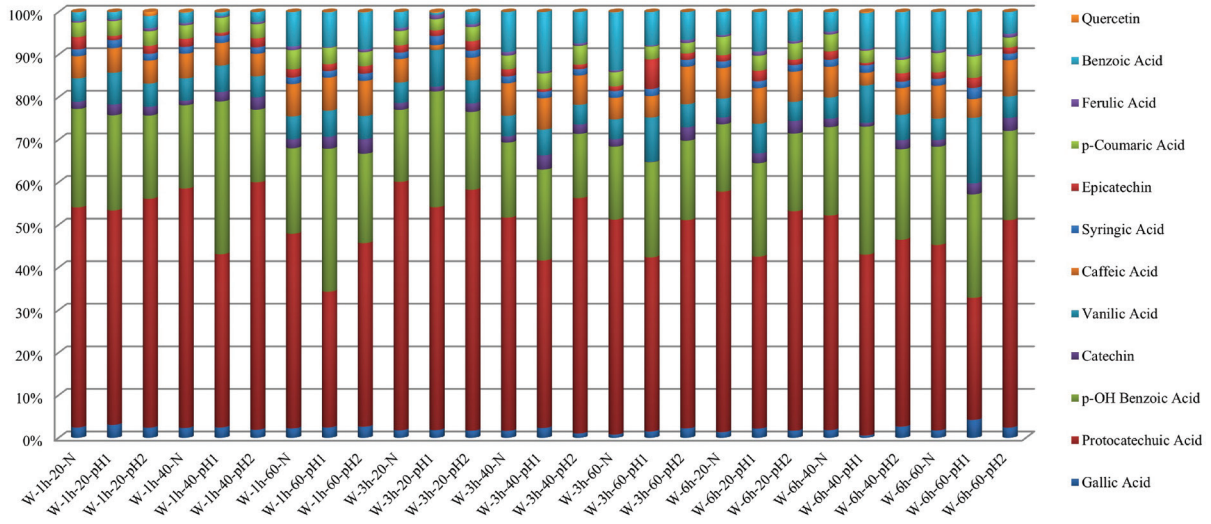

Figure 3. Percentage slice HPLC analysis results of pine honey extracts subjected to different pH values after water extraction at different times and temperatures.

Due to the large amount of data, it was not possible to provide all of the data in a single table, only protocatechuic acid, which was found to be the first major component in the study, is presented in Table 5; and *p*-OH benzoic acid, which was the second component, is shown in Table 6. All additional phenolic compounds analyzed are listed in the Suppl. materials 1–10. The primary reason for creating separate tables was that the optimal conditions for each investigated phenolic component were observed to involve different extraction types. For example, as seen in Table 4, the optimal conditions for protocatechuic acid with each type of solvent were M-6h-40-pH 2, E-3h-60-pH 2, and W-3h-20-N ($p < 0.05$). Moreover, when the highest values for each solvent were statistically analyzed, the conditions for the "W-3h-20-N" sample were significantly different from other conditions ($p < 0.05$) (Table 6). The acidity formation, which is the last step of the enrichment stage for phenolic components within the extraction conditions, was disadvantageous for protocatechuic acid because the lowest results were found in conditions with pH_1 extraction medium (Table 5). The use of neutral pH (pH 7) in the enrichment stage made the detection of the relevant component more probable. As a result, with a value of 524.540 ± 3.641 µg phenolic/100 g sample, water-3 hour-20 °C-neutral pH (W-3h-20-N) was considered the optimal condition for this component.

A comprehensive examination of the extraction parameters revealed that the HPLC analysis results for protocatechuic acid were significantly influenced by alterations in the solvent and pH levels, as evidenced by the *p*-value less

Table 5. The amount of protocatechuic acid (µg phenolic/100 g sample) in the extracts obtained under different conditions.

Protocatechuic Acid	**M-1h-20-N**	275.362 ± 1.104^{bc}	**E-1h-20-N**	140.889 ± 1.086^{j}	**W-1h-20-N**	354.396 ± 2.916^{h}
	M-1h-20-pH 1	77.756 ± 0.831^{r}	**E-1h-20-pH 1**	107.224 ± 1.127^{lm}	**W-1h-20-pH 1**	271.286 ± 1.229^{l}
	M-1h-20-pH 2	248.570 ± 1.587^{g}	**E-1h-20-pH 2**	104.160 ± 1.753^{m}	**W-1h-20-pH 2**	365.282 ± 2.138^{f}
	M-1h-40-N	257.417 ± 1.307^{f}	**E-1h-40-N**	154.482 ± 1.402^{i}	**W-1h-40-N**	357.963 ± 1.755^{gh}
	M-1h-40-pH 1	77.721 ± 0.638^{r}	**E-1h-40-pH 1**	48.036 ± 0.852^{u}	**W-1h-40-pH 1**	289.025 ± 1.244^{k}
	M-1h-40-pH 2	232.591 ± 0.500^{i}	**E-1h-40-pH 2**	115.025 ± 1.997^{k}	**W-1h-40-pH 2**	402.413 ± 2.145^{e}
	M-1h-60-N	237.951 ± 0.323^{h}	**E-1h-60-N**	156.521 ± 1.652i	**W-1h-60-N**	285.672 ± 1.570^{k}
	M-1h-60-pH 1	166.207 ± 1.226^{m}	**E-1h-60-pH 1**	89.644 ± 0.780^{p}	**W-1h-60-pH 1**	218.149 ± 1.049^{o}
	M-1h-60-pH 2	251.467 ± 1.002^{g}	**E-1h-60-pH 2**	110.682 ± 0.407^{l}	**W-1h-60-pH 2**	252.178 ± 1.367^{m}
	M-3h-20-N	260.636 ± 1.067^{ef}	**E-3h-20-N**	290.181 ± 0.479^{c}	**W-3h-20-N**	**524.540 ± 3.641^{a,1}**
	M-3h-20- pH 1	108.076 ± 1.200^{o}	**E-3h-20-pH 1**	214.990 ± 1.070^{e}	**W-3h-20-pH 1**	324.270 ± 2.150^{i}
	M-3h-20-pH 2	198.959 ± 1.385^{j}	**E-3h-20-pH 2**	93.418 ± 0.561^{o}	**W-3h-20-pH 2**	362.091 ± 1.499^{fg}
	M-3h-40-N	260.916 ± 1.303^{ef}	**E-3h-40-N**	298.479 ± 1.089^{b}	**W-3h-40-N**	362.999 ± 2.366^{fg}
	M-3h-40-pH 1	193.965 ± 1.400^{k}	**E-3h-40-pH 1**	30.365 ± 0.751^{v}	**W-3h-40-pH 1**	245.286 ± 0.721^{n}
	M-3h-40-pH 2	265.712 ± 1.645^{d}	**E-3h-40-pH 2**	197.186 ± 0.886^{f}	**W-3h-40-pH 2**	438.398 ± 0.289^{c}
	M-3h-60-N	258.586 ± 1.795^{f}	**E-3h-60-N**	**317.611 ± 0.732^{a,2}**	**W-3h-60-N**	479.700 ± 2.533^{b}
	M-3h-60-pH 1	140.251 ± 1.633^{n}	**E-3h-60-pH 1**	58.297 ± 1.512^{t}	**W-3h-60-pH 1**	265.439 ± 1.847^{l}
	M-3h-60-pH 2	276.987 ± 1.976^{b}	**E-3h-60-pH 2**	84.497 ± 0.239^{r}	**W-3h-60-pH 2**	299.792 ± 1.387^{j}
	M-6h-20-N	239.680 ± 1.698^{h}	**E-6h-20-N**	244.786 ± 1.573^{d}	**W-6h-20-N**	480.003 ± 3.901^{b}
	M-6h-20-pH 1	93.877 ± 0.814p	**E-6h-20-pH 1**	172.264 ± 1.939^{g}	**W-6h-20-pH 1**	249.483 ± 1.096^{mn}
	M-6h-20-pH 2	277.361 ± 1.658^{b}	**E-6h-20-pH 2**	160.440 ± 0.190^{h}	**W-6h-20-pH 2**	426.493 ± 0.993^{d}
	M-6h-40-N	275.221 ± 1.467^{bc}	**E-6h-40-N**	156.534 ± 1.157^{i}	**W-6h-40-N**	398.047 ± 3.906^{e}
	M-6h-40-pH 1	179.807 ± 1.915^{l}	**E-6h-40-pH 1**	49.661 ± 1.564^{u}	**W-6h-40-pH 1**	267.631 ± 2.636^{l}
	M-6h-40-pH 2	**316.653 ± 1.898^{a,2}**	**E-6h-40-pH 2**	56.055 ± 1.492^{t}	**W-6h-40-pH 2**	304.459 ± 0.391^{j}
	M-6h-60-N	263.830 ± 1.830^{de}	**E-6h-60-N**	143.525 ± 0.463^{j}	**W-6h-60-N**	301.064 ± 0.469^{j}
	M-6h-60-pH 1	194.767 ± 1.620^{jk}	**E-6h-60-pH 1**	62.733 ± 1.452^{s}	**W-6h-60-pH 1**	114.979 ± 0.295^{p}
	M-6h-60-pH 2	272.268 ± 1.594^{c}	**E-6h-60-pH 2**	97.669 ± 0.329^{n}	**W-6h-60-pH 2**	301.329 ± 0.430^{j}

The first statistical evaluation was achieved for each solvent type separately. The same letters in each column for each solvent type were not significantly different at $p < 0.05$ (One-way ANOVA with post-hoc Tukey). The second statistical evaluation was achieved for each max value obtained from methanolic, ethanolic, and water extracts. The number in each column was not significantly different at $p < 0.05$ (One-way ANOVA with post-hoc Tukey). In each column, the value written in bold indicates the ideal extract condition of the phenolic compound in the relevant solvent.

than 0.01 (Table 2). The results of the methanol, ethanol and aqueous extract, which were preferred as the solvent, and the pH 1, pH 2 and neutral (pH 7) extract, which were preferred as the pH value, also exhibited statistical differences in comparison with each other ($p < 0.01$; Table 3). However, the results of this compound were not affected by the time and heating variation options at the level of statistical significance ($p > 0.01$; Table 2). Furthermore, an analysis was conducted on the sub-options of the extracts themselves, and the changes of time and heating parameters did not reveal statistical differences ($p > 0.01$; Table 3).

In the study, *p*-OH benzoic acid was detected as the second-highest phenolic component after protocatechuic acid. Without exception, *p*-OH benzoic acid was one of the components found in all extract types (Table 6). In fact, it was found at higher amounts than protocatechuic acid in some types of ethanolic extracts (Table 6). In addition, in terms of detection amount, the highest (E-3h-60-pH 1: 389.155 ± 1.248 µg phenolic/100 g sample) and the lowest (E-6h-60-N: 72.737 ± 0.836 µg phenolic/100 g sample) detection values were also found in ethanolic extract conditions. *p*-OH benzoic acid data peaked in the methanolic and water extracts of M-3h-40-pH 1 and W-1h-40-pH 1, respectively. There were statistically significant differences ($p < 0.05$) between these extract conditions. The triple evaluation between the highest values for the three extract types indicated that the most ideal extract condition for the relevant component was "ethanolic-3-hour-60 °C-pH 1" abbreviated as E-3h-60-pH 1 ($p < 0.05$).

The quantitative HPLC value of *p*-OH benzoic acid was found to be statistically affected by solvent, time, heating and pH changes at the $p < 0.01$ significance level (Table 2). This exceptional situation was not observed only in *p*-OH benzoic acid among the 12 phenolic compounds analyzed (Table 2). Although the results for *p*-OH benzoic acid demonstrated alterations in each of the primary conditions, it was observed that the comparison of the results for the sub-conditions of the heating change parameters, which were selected as 20–40–60 °C, did not exhibit statistically significant changes ($p > 0.01$; Table 3).

According to the Suppl. materials 1–10, the optimal conditions for each of the analyzed phenolic compounds were checked statistically. The values with a significant difference at $p < 0.05$ were accepted as the optimal condition(s) for the relevant compounds. They were as follows: M-3h-40-pH 1 and M-6h-60-pH 1 for gallic acid; E-3h-60-pH 1 for catechin; W-3h-60-pH 1 for vanillic acid; M-3h-60-N for caffeic acid; M-6h-40-pH 2 for syringic

Table 6. The amount of *p*-OH benzoic acid (µg phenolic/100 g sample) in the extracts obtained under different conditions.

p-OH Benzoic Acid	**M-1h-20-N**	140.739 ± 1.277^{lm}	**E-1h-20-N**	77.215 ± 0.998^{p}	**W-1h-20-N**	158.443 ± 1.281^{f}
	M-1h-20-pH 1	135.761 ± 0.372^{no}	**E-1h-20-pH 1**	146.165 ± 1.555^{i}	**W-1h-20-pH 1**	120.395 ± 0.215^{op}
	M-1h-20-pH 2	133.376 ± 1.378^{op}	**E-1h-20-pH 2**	75.755 ± 0.556^{pr}	**W-1h-20-pH 2**	132.885 ± 0.179^{jk}
	M-1h-40-N	140.310 ± 0.735^{lm}	**E-1h-40-N**	76.937 ± 1.903^{p}	**W-1h-40-N**	124.429 ± 0.954^{mn}
	M-1h-40-pH 1	251.942 ± 0.033^{b}	**E-1h-40-pH 1**	94.597 ± 0.911^{m}	**W-1h-40-pH 1**	**254.803 ± 1.796^{a,3}**
	M-1h-40-pH 2	144.227 ± 1.614^{k}	**E-1h-40-pH 2**	80.931 ± 0.994^{o}	**W-1h-40-pH 2**	118.360 ± 1.226^{p}
	M-1h-60-N	141.952 ± 0.377^{kl}	**E-1h-60-N**	82.532 ± 0.442^{o}	**W-1h-60-N**	125.463 ± 0.018l^{mn}
	M-1h-60-pH 1	229.511 ± 1.129^{c}	**E-1h-60-pH 1**	119.529 ± 1.149^{l}	**W-1h-60-pH 1**	229.825 ± 1.788^{b}
	M-1h-60-pH 2	135.088 ± 1.720^{nop}	**E-1h-60-pH 2**	86.310 ± 0.839^{n}	**W-1h-60-pH 2**	122.565 ± 1.435^{no}
	M-3h-20-N	173.936 ± 0.250^{g}	**E-3h-20-N**	168.441 ± 0.992^{f}	**W-3h-20-N**	152.115 ± 1.816^{g}
	M-3h-20-pH 1	176.184 ± 1.167^{fg}	**E-3h-20-pH 1**	208.463 ± 1.038^{d}	**W-3h-20-pH 1**	168.359 ± 1.591^{d}
	M-3h-20-pH 2	131.833 ± 1.492^{p}	**E-3h-20-pH 2**	161.939 ± 1.485^{g}	**W-3h-20-pH 2**	116.887 ± 1.310^{pr}
	M-3h-40-N	82.199 ± 1.574^{s}	**E-3h-40-N**	156.706 ± 1.388^{h}	**W-3h-40-N**	127.739 ± 0.854^{lm}
	M-3h-40-pH 1	**269.188 ± 1.840^{a,2}**	**E-3h-40-pH 1**	337.035 ± 2.074^{b}	**W-3h-40-pH 1**	132.921 ± 1.796^{jk}
	M-3h-40-pH 2	173.138 ± 0.859^{g}	**E-3h-40-pH 2**	161.071 ± 0.493^{g}	**W-3h-40-pH 2**	119.544 ± 1.293^{op}
	M-3h-60-N	206.181 ± 0.920^{d}	**E-3h-60-N**	178.905 ± 0.104^{e}	**W-3h-60-N**	162.355 ± 0.506^{ef}
	M-3h-60-pH 1	124.105 ± 1.209^{r}	**E-3h-60-pH 1**	**389.155 ± 1.248^{a,1}**	**W-3h-60-pH 1**	144.724 ± 2.031^{i}
	M-3h-60-pH 2	174.166 ± 0.143^{g}	**E-3h-60-pH 2**	252.141 ± 1.531^{c}	**W-3h-60-pH 2**	114.055 ± 0.925^{r}
	M-6h-20-N	162.048 ± 0.382^{hi}	**E-6h-20-N**	142.890 ± 0.864^{i}	**W-6h-20-N**	134.645 ± 0.543^{j}
	M-6h-20-pH 1	137.634 ± 0.309^{mn}	**E-6h-20-pH 1**	153.163 ± 1.009^{h}	**W-6h-20-pH 1**	135.435 ± 0.473^{j}
	M-6h-20-pH 2	164.362 ± 0.990^{h}	**E-6h-20-pH 2**	137.662 ± 0.631^{j}	**W-6h-20-pH 2**	150.774 ± 1.076^{gh}
	M-6h-40-N	177.714 ± 0.806^{f}	**E-6h-40-N**	84.072 ± 0.782^{no}	**W-6h-40-N**	164.119 ± 2.189^{e}
	M-6h-40-pH 1	133.306 ± 1.591^{op}	**E-6h-40-pH 1**	154.932 ± 1.129^{h}	**W-6h-40-pH 1**	189.012 ± 2.255^{c}
	M-6h-40-pH 2	204.997 ± 0.687^{d}	**E-6h-40-pH 2**	133.732 ± 0.367^{k}	**W-6h-40-pH 2**	146.784 ± 0.044h^{i}
	M-6h-60-N	158.739 ± 0.648^{i}	**E-6h-60-N**	72.737 ± 0.836^{r}	**W-6h-60-N**	159.534 ± 0.373^{f}
	M-6h-60-pH 1	181.130 ± 0.538^{e}	**E-6h-60-pH 1**	155.289 ± 1.222^{h}	**W-6h-60-pH 1**	97.463 ± 0.545^{s}
	M-6h-60-pH 2	154.794 ± 0.806^{j}	**E-6h-60-pH 2**	86.628 ± 1.106^{n}	**W-6h-60-pH 2**	129.177 ± 0.506^{kl}

The first statistical evaluation was achieved for each solvent type separately. The same letters in each column for each solvent type were not significantly different at $p < 0.05$ (One-way ANOVA with post-hoc Tukey). The second statistical evaluation was achieved for each max value obtained from methanolic, ethanolic, and water extracts. The number in each column was not significantly different at $p < 0.05$ (One-way ANOVA with post-hoc Tukey). In each column, the value written in bold indicates the ideal extract condition of the phenolic compound in the relevant solvent.

acid; W-3h-60-pH 1 for epicatechin; E-3h-60-pH 1 and W-6h-20-N for *p*-coumaric acid; M-3h-40-N, M-3h-60-N, and M-3h-60-pH 2 for ferulic acid; W-3h-60-N for benzoic acid; and finally M-3h-60-pH 2 and E-3h-40-N for quercetin, respectively (Suppl. materials 1–10).

A detailed examination of all phenolic components, as presented in Table 2, revealed that 10 out of 12 phenolic compounds detected in all types of extracts exhibited statistically significant differences in response to solvent changes ($p < 0.01$). Additionally, 9 compounds demonstrated statistically significant variations in response to time and pH changes, respectively ($p < 0.01$). Furthermore, as indicated in Table 3, which presents the sub-options of each main change condition, the quantitative determination value of protocatechuic acid, catechin, and epicatechin differed from solvent change ($p < 0.01$). Similarly, *p*-OH benzoic acid, caffeic acid, *p*-coumaric acid, and quercetin differed from time change, while protocatechuic acid and epicatechin differed from pH change. It is noteworthy that, according to Table 2, which compares the total results of solvent, time, heating, and pH changes, only the heating application to the extracts statistically altered the result of *p*-OH benzoic acid. However, when all statistical analyses were evaluated, including 20–40–60 °C, which was selected as a sub-option of heating, it was observed that heating did not statistically change the level of any phenolic compound detected ($p > 0.01$; Table 3). The evaluation of the heating sub-option revealed that heating did not result in a statistically significant change in the levels of any detected phenolic compounds ($p > 0.01$; Table 3).

Discussion

The TPC and TFC analysis results for the main sample were in accordance with the literature. Ekici et al. (2014) reported that the total phenolic content of 20 pine honey samples was 62.01–68.78 mg GAE/100 g. Based on the results of other bioactivity tests in this related study, the methanolic extract of pine honey samples had a very significant effect (Ekici et al. 2014).

The bioactive content of honey varies according to the floral characteristics of the region where it is harvested, its geographical situation, climate, and seasonal conditions (Alvarez-Suarez et al. 2014). The fact that honeys with the same origin have different bioactive values in different seasons and years reveals how flexible this variation is. According to Akbulut et al. (2009), this change was indirectly exemplified in their study. The TPC of 15 pine honey samples from Muğla was discovered to be in the range of 234.9–394 mg GAE/100 g in that study, making it extremely effective. The findings indicate that pine honey, obtained from

the Muğla province, is a significant product in terms of its bioactive properties. (Akbulut et al. 2009). The study conducted by Özkök et al. (2010) is regarded as a comprehensive reference study for TFC. In the study of 78 pine honey samples, the range for TFC was found to be 4.80–54.78 mg QE/kg (Özkök et al. 2010). The lower limit of this value is very close to our study results. The main reason for our low value might be climatic change, particularly on a yearly basis. In the study by Sahin et al. (2022), the samples were very similar to those in the current study in terms of both year and location. The researchers determined the mean TFC of 17 pine honeys was 1.944 mg QE/100 g sample (Sahin et al. 2022). The data are in good agreement with our current study.

There are examples in the literature that are closely related to the phenolic component analysis results in the current study. Also, it was demonstrated analytically that protocatechuic acid is abundant in pine honey and this compound could potentially act as a marker for Turkish pine honey (Sahin et al. 2022). It is thought that the presence of protocatechuic acid as a major compound is due to the botanical origin of the honey. The literature supports this idea and emphasizes that the presence of phenolic compounds in honey varies depending on botanical origin. The literature also highlights that the detection of these compounds can be used to identify the type of honey (Becerril-Sánchez et al. 2021; Sahin et al. 2022). However, in scientific studies, a common and consistent procedure for the extraction conditions of the sample is not present. In Haroun et al. (2012), phenolic content analysis of honeys was performed by HPLC in a study where 7 of the investigated honeys were defined as pine honey and a total of 27 honeys were investigated. Amberlite resin and distilled water adjusted to pH 2 with HCl were used in the extraction stage of this study. Bio components retained on the resin were eluted with methanol, and then the solvent was evaporated at 40 °C. After the residue was dissolved in pure water, only diethyl ether was used for the enrichment process. According to the results obtained for pine honey, 10 of the 15 phenolic components analyzed were detected at varying quantities; the highest value was observed for protocatechuic acid in all pine honey samples (Haroun et al. 2012). The detection of the major components that emerged as a result of the study appears to correlate with the current study.

In another study by Can et al. (2015), the phenolic components were determined in different types of honey samples, including four pine honey samples. In that study, in which 18 phenolic standards were used, protocatechuic acid (81.19 µg /g) was determined to be the major component in terms of mean value for all pine honey samples. *p*-hydroxybenzoic acid, catechin, and vanillic acid were also identified as other abundant components (Can et al. 2015). The results obtained were close to our current study data, but in the relevant study, 24-hour direct methanolic extraction was performed without any temperature application during the extraction progress. The organic solvents of the obtained extracts were evaporated, and the study was carried out by applying the selective extraction process to a single condition (Can et al. 2015). In a different study conducted by Sahin et al. (2022), 17 pine honey samples were first subjected to methanolic extraction; the obtained extracts were evaporated, and then dissolved in distilled water. The selective extraction process was applied with ethyl acetate and diethyl ether for phenolic compounds. In the study, protocatechuic acid was determined to be the major component, and the value range was found to be 197–780 µg phenolic/100 g sample (Sahin et al. 2022). This value is very close to the value of protocatechuic acid, which was determined to be the major component, in the current study.

There are other studies in literature by differentiate the extract preparation procedures for secretory honeys. For example, in a study from Poland, in which *p*-coumaric and gallic acids were the major components for phenolic acids and narigenin for flavonoids, the sub-origin was not defined and only emphasized as honeydew honey, and ethyl acetate was used in the extraction procedure (Socha et al. 2011). In another study conducted by Silici et al. (2013), in which there was no emphasis on the sub-origin of honey and honeydew honeys were stated in general terminology, the phenolic components of 10 honeydew honeys were analysed in 25 samples. Nine phenolic compounds were found in these honeys, and catechin and *p*-coumaric acid were predominantly observed. In this study, samples for HPLC analysis were prepared with ethyl acetate, and the solid phase extraction method was chosen for the advanced enrichment process (Silici et al. 2013).

Essentially, the preparation of ethanolic extracts was relatively more difficult than methanolic and aqueous extracts in this study. The reason for this might be that ethanol dissolves carbohydrate groups—which are the main components of honey—more slowly than water and methanol. Alavi et al. (2014) investigated the solubility capabilities of fructose in water-ethanol and water-methanol mixtures and revealed that the methanol-water mixture dissolves fructose better than the ethanol-water mixture. Researchers attributed this to the fact that the hydrogen bond between fructose and methanol is stronger than the hydrogen bond between fructose and ethanol (Alavi et al. 2014). As a result, fructose and glucose, two of the most abundant carbohydrates in honey, precipitate in ethanolic extraction, thus complicating the extraction process. The better dissolution of sugar groups by water and methanol solvents results in the inclusion of phenolic contents in the extract with a higher yield.

It is known that phenolic components contain polar groups, and solvents with a higher polarity index highlight the extraction efficiency of phenolic components. Pure water has a polarity index of 9 and methanol has a valence of 5.6 (Mailoa et al. 2013). Despite the fact that ethanol is a polar protic solvent and hydroxyl ion supplier with a polarity index of 5.2 (Musa et al. 2011), this can be evidence that its interaction with the aforementioned sugar groups may inhibit this solubility. This situation also supported the declaration by Alavi et al. (2014).

Based on the findings discussed, this study can provide a foundation for improving industrial-scale applications in honey processing and phenolic compound extraction. Optimizing extraction conditions not only ensures

maximum recovery of phenolic compounds, which are pivotal for functional foods and pharmaceuticals, but also contributes to cost reduction by identifying efficient parameters such as solvent use, time, and temperature. These insights can also help to enhance product standardization and to expand the market potential of pine honey, particularly for Turkiye, a global leader in its production.

However, this study has limitations that should be acknowledged. Only one honey sample, selected for its high TPC and TFC values, was subjected to detailed analysis, which limits the generalizability of the results. Future studies should explore multiple samples across different regions and seasons to account for potential variations in phenolic profiles due to environmental and botanical factors. Additionally, investigating advanced extraction methods, such as ultrasound or microwave-assisted techniques, could further improve efficiency and scalability for industrial purposes.

Conclusions

Pine honey, classified as honeydew honey, is notable for its exceptional bioactive properties, which are primarily attributed to its rich and diverse phenolic composition. Turkiye is a dominant global supplier, fulfilling approximately 92% of the international demand for pine honey. Ensuring the accurate characterization and recognition of this honey type is essential for supporting its authenticity and quality. Bioactivity is an important criterion for recognition of honey samples, as it is in other natural products, and it is associated with some bio-components, such as phenolics. However, the stability of these substances *in vitro* varies greatly. The differentiation of the extraction conditions also caused a difference in the qualitative and quantitative detection of possible phenolic components in samples. The data revealed the most suitable extraction conditions among the working conditions for Turkish pine honey, which is important and valuable for Turkish exports. Essentially, given the information in the literature, it is understood that there is no single type of extraction that is standardized for pine honey samples. In this study, only one pure pine honey was used as sample, and only Muğla, Turkiye, was selected as a supply location because it has the most potential for production. The obtained data contribute to the scientific literature in terms of finding the best and most efficient way to extract phenolic compounds from Turkish pine honey. However, after evaluating the 81 extract conditions for 1 pine honey sample selected from among 5 samples, it was not possible to conclude that a single extract type stood out for all phenolic components. For this reason, the data for protocatechuic acid, which was supported by the literature and determined to be the major component, are important results in our study. In the study, the extract condition in which protocatechuic acid was detected at the highest amount with the code W-3h-20-N (Water – 3 h- 20 °C – Neutral pH (pH 7)) is suggested to be an idealized condition for pine honey extraction. Moreover, the values for *p*-OH benzoic acid were found to be higher than those of protocatechuic acid, particularly in some extraction types where pH 1 enrichment was performed. It is thought that this is due to a limitation of HPLC known as peak tailing and peak slipping. This situation was mostly observed in pH 1 extraction conditions, thereby suppressing protocatechuic acid. Therefore, the necessity of choosing neutral pH (pH 7) for acidity in extraction was once again confirmed. This study may provide a basis for possible future scientific studies in terms of this type of honey.

Author contributions

H.S.: Topic conceptualization, Investigation, Formal analysis, Methodology, Supervision of the Study Progress, Resources, Drafting, and Interpretation of the Manuscript, Review and Editing. A.A.K.: Topic conceptualization, Formal analysis, Review and Editing. K.K.: Investigation, Formal analysis, Resources, Drafting, and Interpretation of the Manuscript, Review and Editing. E.B.: Formal analysis, Methodology, and Review. E.E.: Formal analysis, Methodology, and Review.

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Compliance with ethics requirements

The current article does not contain any studies with human or animal subjects.

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Supplementary material

Supplementary material 1
Quantity of gallic acid (µg phenolic/100 g sample) in extracts obtained under different extraction conditions (.docx)
Link: https://doi.org/10.3897/ejfa.2025.141957.suppl1

Supplementary material 2
Quantity of catechin (µg phenolic/100 g sample) in extracts obtained under different extraction conditions (.docx)
Link: https://doi.org/10.3897/ejfa.2025.141957.suppl2

Supplementary material 3
Quantity of vanillic acid (µg phenolic/100 g sample) in extracts obtained under different extraction conditions (.docx)
Link: https://doi.org/10.3897/ejfa.2025.141957.suppl3

Supplementary material 4
Quantity of caffeic acid (µg phenolic/100 g sample) in extracts obtained under different extraction conditions (.docx)
Link: https://doi.org/10.3897/ejfa.2025.141957.suppl4

Supplementary material 5
Quantity of syringic acid (µg phenolic/100 g sample) in extracts obtained under different extraction conditions (.docx)
Link: https://doi.org/10.3897/ejfa.2025.141957.suppl5

Supplementary material 6
Quantity of epicatechin (µg phenolic/100 g sample) in extracts obtained under different extraction conditions (.docx)
Link: https://doi.org/10.3897/ejfa.2025.141957.suppl6

Supplementary material 7
Quantity of *p*-coumaric acid (µg phenolic/100 g sample) in extracts obtained under different extraction conditions (.docx)
Link: https://doi.org/10.3897/ejfa.2025.141957.suppl7

Supplementary material 8
Quantity of ferulic acid (µg phenolic/100 g sample) in extracts obtained under different extraction conditions (.docx)
Link: https://doi.org/10.3897/ejfa.2025.141957.suppl8

Supplementary material 9
Quantity of benzoic acid (µg phenolic/100 g sample) in extracts obtained under different extraction conditions (.docx)
Link: https://doi.org/10.3897/ejfa.2025.141957.suppl9

Supplementary material 10
Quantity of quercetin (µg phenolic/100 g sample) in extracts obtained under different extraction conditions (.docx)
Link: https://doi.org/10.3897/ejfa.2025.141957.suppl10