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Applicability of Phenolic Profile Analysis Method Developed with RP-HPLC-PDA to some Bee Product

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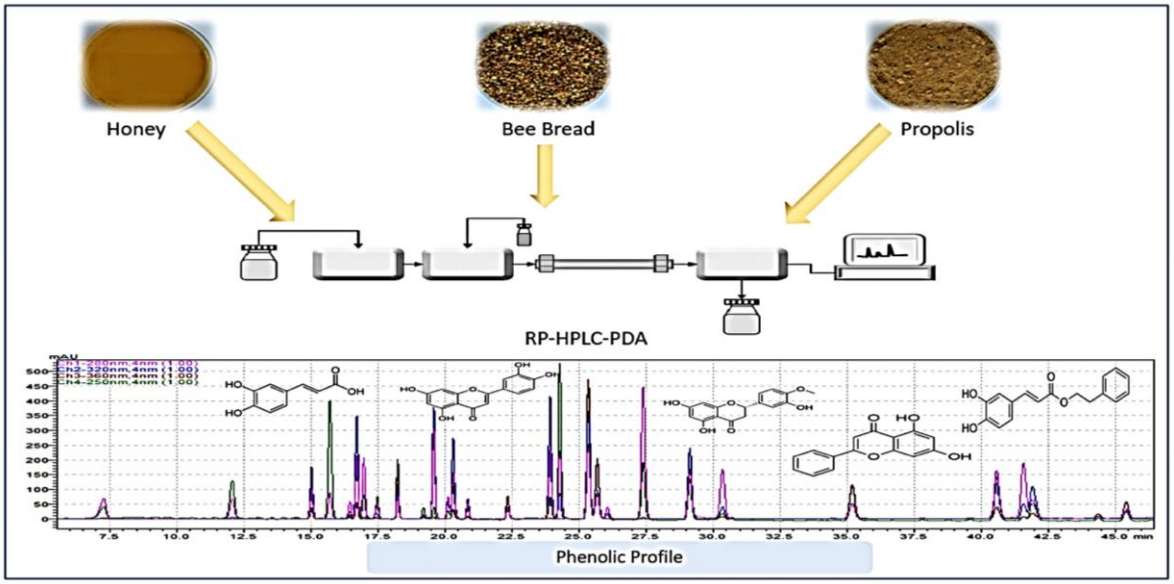
HIGHLIGHTS

- RP-HPLC-PDA method was developed for the simultaneous determination of 25 phenolic compounds.
- The reliability of the developed method was confirmed by the validation test.
- An HPLC method has been developed to quantify phenolic acid and flavonoids in some bee products.
- The method showed good results in a run time of only 60 min.

Abstract: In this work, a reverse phase high-performance liquid chromatographic method (RP-HPLC) with photo diode array (PDA) at 4 different wavelengths (250, 280, 320 and 360) for the determination of some polyphenols in some bee products (honey, pollen, and propolis) is developed. The analyses were carried out on C18 column (250 mm x 4.6 mm, 5 µm; GL Sciences), and the gradient program was used with a mobile phase A reservoir with 70% acetonitrile and the B reservoir with 2% acetic acid. The HPLC method was founded that limit of detection was in the range 0.022-0.0908 µg/mL; the limit of quantification was in the range 0.074-0.3027 µg/mL, all calibration curves were linear R^2>0.995 within the range, the recovery range was 91.43- 111.37% for 10 ppm and 98.44-101.68% for 40 ppm and relative error levels 0.0330-0.0290 respectively. The developed method was applied to some bee products available on the Turkey market. The study aimed at phenolic profiles of the bee products extracts were revealed by using 25 phenolic standards. The proposed method was optimized, quickly, and simple validated by evaluating the linear range, the limits of detection and quantification, the accuracy, the precious, the repeatability, and recoveries suitable for the phenolic analysis. It is concluded that evaluated and the quantitative determination of the bee products can be made quickly and reliably with the optimized method.

Keywords: Validation; optimization; HPLC-PDA; phenolic; flavonoids; bee products.

GRAPHICAL ABSTRACT



INTRODUCTION

The most important technique used in the separation, identification, and determination of organic and inorganic compounds in biological, pharmacological, nutritional, environmental and industrial samples is high performance liquid chromatography (HPLC) [1]. A standard HPLC device consists of a mobile phase reservoir, pump, injector, column, detector, and recorder [2].

The type and percentage of the solvent system to be used as the mobile phase should be optimally adjusted to increase the efficiency of the analysis. If two or more solvent systems are used for analysis, programs with gradients are prepared for optimum analysis. The flow rate to be applied is determined according to the inner diameter of the column, and the lower the inner diameter of the column, the lower the flow rate.

HPLC systems generally work on the principle of dispersion chromatography. In dispersion chromatography, the polarities of mobile and stationary phases constitute the basic principle in analysis. The basic principle in normal phase and reverse phase chromatography is since the substance close to the polarity of the stationary phase spends too much time in the column, and the substances close to the polarity of the mobile phase leave the column. Reverse phase liquid chromatography is the most widely used method [3-5].

In chromatography, features such as high sensitivity, low noise level, high selectivity and wide sample analysis spectrum are sought in the ideal detector. The most used detectors in the HPLC system are UV detector and photo diode array (PDA) detector [6]. Organic molecules with various functional groups absorb electromagnetic energy at 190-800 nm wavelength in the UV detector. A part of the beam sent to the sample is absorbed by the sample and the qualitative and quantitative analysis of the analyzed compound is made from the inverse ratio between the decrease in the density of the beam and the concentration of the substance [7].

Information about the substance is provided from the absorbance value measured as a function of time in UV detectors. In UV detectors, measurement can be taken at one wavelength or two wavelengths. As a result of the development of UV detectors, photodiode array detectors that can simultaneously scan in different wavelengths and work on the same principle as the UV detector have been developed [6]. One of the important advantages of PDA detector is that it allows to work in wavelength range, not a single wavelength. As light source in the PDA detector deuterium and tungsten lamps are used. In addition to the high performance of HPLC, HPLC-PDA systems also offer high accuracy and precision analysis by obtaining their spectra.

The applicability, reproducibility and reliability of a developed chromatographic method should be demonstrated by the validation of the method. For the validation of a method, the linear measurement range, precision, accuracy, limit of quantification and limit of determination of the method are calculated. At the same time, R^2 values are calculated, which is an indicator of the linearity of the calibration curve. Repeatability and

recovery tests are carried out to determine the accuracy and precision of the method developed. In addition, the calibration equation of the standards, the standard deviation of the peak areas, relative standard deviation, absolute error, and relative error are calculated [8].

Plants have the ability to produce a limitless number of compounds. Polyphenols are the secondary metabolites of plants and are responsible for the biological, physiological activity and sensory properties of the plant [9,10]. Honey, pollen and propolis are valuable bee products and rich in polyphenols. Phenolic composition of the bee products is very important and is mostly determined by chromatographic analysis techniques. HPLC-UV and HPLC-PDA are the most used practical techniques, preparation of the bee products for analysis and validation of phenolic components are important analytical studies [11-13]. The aim this study was to obtain a quick, reliable, and simplified methodology for the detection and quantification of phenolic acids and flavonoids in some bee products.

MATERIAL AND METHODS

Method validation

The RP-HPLC-PAD method used to detect in natural bee products the phenolic compounds were the amount of quantification validated for linearity, precision, recovery, and accuracy (absolute recovery study).

Linearity, Accuracy and limits of detection (LOD) and of quantification (LOQ)

Precision

Precision is the closeness of data obtained under the same conditions in an analysis. The precision of a method must be supported by its repeatability and recovery values. Repeatability is expressed in standard deviation and relative standard deviation. Relative standard deviation is obtained by proportioning the sample standard deviation to the mean. In order to determine the recovery values of the standards, three replicate analyzes were performed at two different concentrations and the recovery values were calculated as percentages.

Accuracy

The closeness of the result obtained in the analysis to the real value is expressed with accuracy. The accuracy of a method is indicated by absolute error and relative error values. The deviation of the measured value from the actual value is absolute error, the relative error is calculated by proportioning the absolute error to the real value.

Linearity, Limit of detection (LOD) and limit of quantification (LOQ)

In order to determine the linearity of the developed method, triplicates were performed from the solutions of each standard with six different concentrations. Calibration graphics of the standards were obtained by serial dilution in the range of 40-1.25 ppm. A calibration curve for each standard phenolic was created by plotting the peak area (y-axis) versus concentration (x-axis).

Both limits of detection (LOD) and quantification (LOQ) of the developed method was evaluated. The LOD and LOQ were calculated according to the standard deviation of the sample at the lowest concentration and the slope of the calibration curve. Parameters were calculated using the following equations, respectively.

LOD = 3.3 × SD / m and LOQ = 10 × SD / m, where m is the slope, and SD is the standard deviation at the lowest level of the calibration curves [14].

RP-HPLC-PDA Analysis Conditions

The HPLC analyses were conducted on a Shimadzu liquid Corporation LC 20AT HPLC system equipped with a PDA detector, C18 column (250 mm × 4.6 mm, 5 µm; GL Sciences). The elution was performed using mobile phase A (10% Acetonitrile-ultra pure water solution), and mobile phase B (2% acetic acid in water). The flow rate was 1 ml/min, injection volume of 20 µl, a column at 30 ºC, were used and the detection range from 250, 280, 320 and 360 nm. The gradient program changed according to the following conditions: 95% A and 5% B as initial conditions,15% A and 85% for 8 min, 21%A and 79%B for 10 min, 52% A and 48% B for 20 min, 67% A and 33% B for 35 min, 90% A and 10%B for 50, 5%A and 95% B for 50.1 and finally 5% A and 95% B for 60 min. Confirmation of phenolic compounds of samples and determination of their

concentrations are based on comparison of retention times with actual standards and ultraviolet absorption spectrum data. Before all the prepared samples were given to the device, they were injected filtered through 0.45 µm membranes.

Standard Phenolics

25 phenolic standards including gallic acid, protocatechuic acid, *p*-OH benzoic acid, *m*-OH benzoic acid, chlorogenic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, epicatechin, rutin, myricetin, quercetin, apigenin, resveratrol, daidzein, *t*-cinnamic acid, hesperetin, luteolin, rhamnetin, chrysin, pinocembrin, CAPE, curcumin and ellagic acid can be analyzed simultaneously. The standard calibration graph was obtained using absorbances at 250 nm wavelength for protocatechuic acid, *p*-OH benzoic acid, *m*-OH benzoic, rutin, ellagic acid and daidzein, at 280 nm for gallic acid, epicatechin, syringic acid, *t*-cinnamic acid, hesperetin, chrysin, and pinocembrin, at 320 nm for chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, resveratrol, apigenin and CAPE, and at 360 nm for myricetin, luteolin, quercetin, rhamnetin, and curcumin. Gallic acid, protocatechuic acid, *p*-OH benzoic acid, *m*-OH benzoic, chlorogenic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, and ellagic acid was dissolved in 50-50% methanol-pure water. Epicatechin, rutin, myricetin, quercetin, apigenin, resveratrol, daidzein, *t*-cinnamic acid, hesperetin, luteolin, rhamnetin, chrysin, pinocembrin, CAPE, curcumin was dissolved in 100% methanol.

Samples and Extraction

The developed method for the phenolic profile analyses in this study was applied to three different bee products. The samples used in the study were obtained from experienced beekeepers from Anatolia, Turkey. The study is selected commonly named eryngo (Eryngium Campestre) as unifloral honey, chestnut (*Castanea Sativa L.*) pollen as pollen, and mixed Anatolian propolis as propolis (Figure 1). The preparation and extraction of the bee products for the phenolic component analysis is schematized in Figure 2.

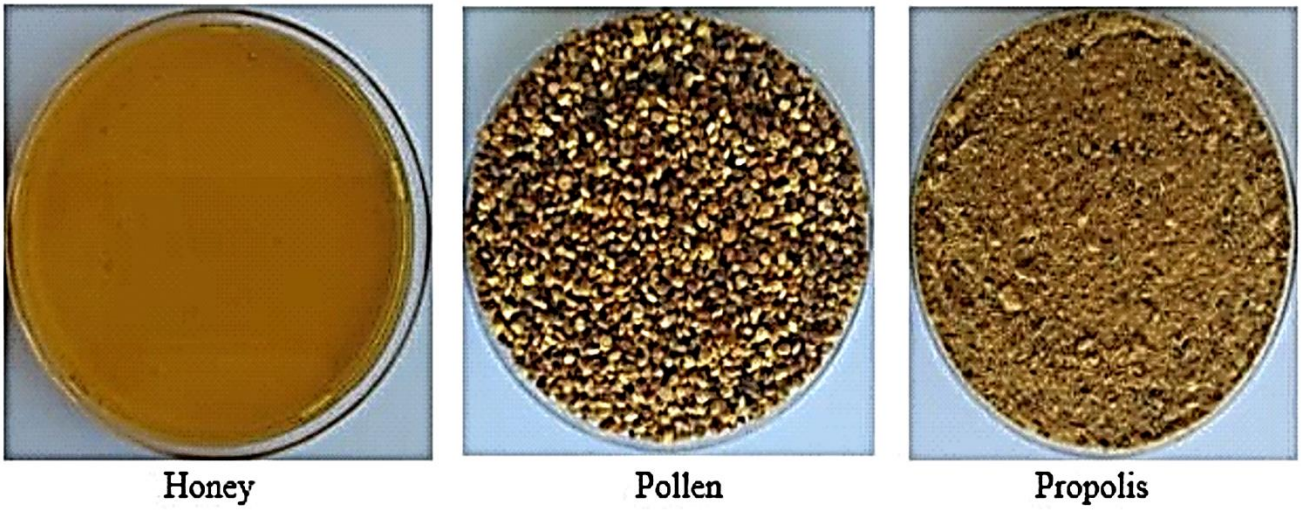


Figure 1. Pictures of samples.

Sample preparation scheme for bee products

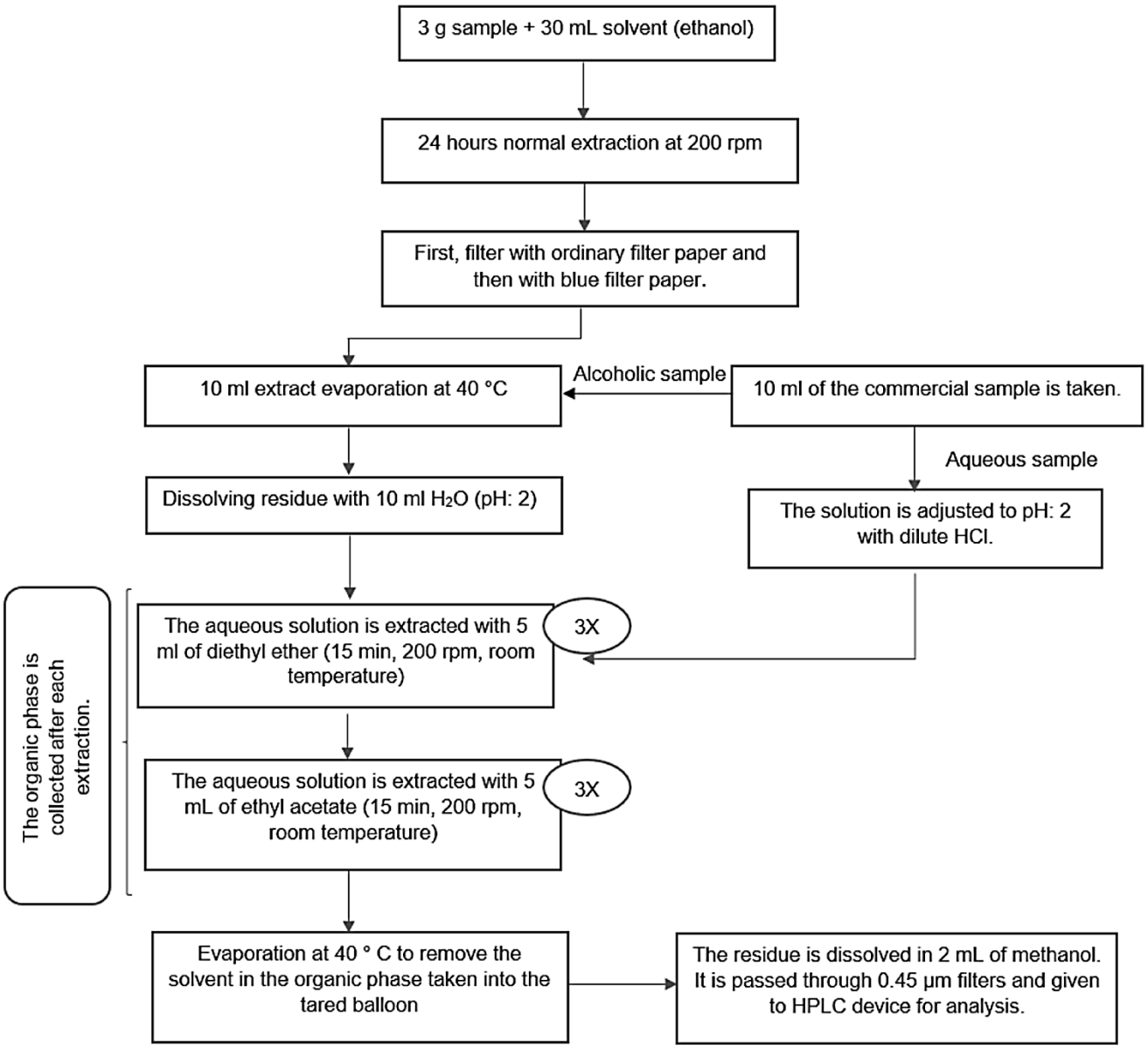


Figure 2. Sample preparation scheme for bee products.

RESULTS AND DISCUSSION

Precision

Repeatability is expressed in standard deviation and relative standard deviation. Relative standard deviation is obtained by proportioning the standard deviation of the samples to the mean. To determine the recovery values of the standards, three replicate analyzes were performed at two different concentrations (40 ppm and 10 ppm) and the results were expressed as % recovery. In the analysis, when the recovery test was applied for the standard of 40 ppm, it was founded that there was a recovery rate of 98.44% and 101.68%. The precision results of the developed method are as in Table 1. Relative standard deviations for epicatechin, myricetin and ellagic acid were found as 0.28855, 0.2712 and 0.26261, respectively. Relative standard deviation values of other standard compounds are lower it has been observed. According to the results of the study, the relative standard deviation values of the standards show that their reproducibility is high. When the values in 40 and 10 ppm solutions are examined to calculate the recovery percentage of the method used, there are very few deviations from 100% recovery, the recovery percentage of the majority

was found to be acceptable (Table 1). It is seen that the deviations in the percentage of recovery increase as the concentration decreases but are still at acceptable levels.

Table 1. Peak area, precision, relative error, and recovery (%) of standards

Standards	Peak Area	Precision (%RSD)	Relative Error	Recovery (%)	
				10 ppm	40ppm
Gallic acid	2402.240	0.05429	0.0122	91.43	101.22
Protocatechuic acid	10693.820	0.16203	0.0117	97.13	101.17
Chlorogenic acid	4258.514	0.08881	0.0168	93.13	101.68
p-OH Benzoic acid	8449.922	0.05913	0.0117	99.47	101.17
Epicatechin	3729.323	0.28855	0.0092	94.80	100.93
Caffeic acid	4829.501	0.05327	0.0104	97.68	101.04
Syringic acid	2886.402	0.05351	0.0126	96.03	101.26
m-OH Benzoic acid	4612.176	0.18167	0.0037	102.83	100.37
Rutin	5345.815	0.12367	0.0075	106.00	99.25
Ellagic acid	2299.031	0.26261	0.0017	98.03	100.17
p-Coumaric acid	4387.275	0.04304	0.0153	97.13	101.53
Ferulic acid	6692.479	0.08876	0.0140	95.43	101.40
Myricetin	4366.155	0.2712	0.0137	95.90	101.37
Resveratrol	3333.882	0.02816	0.0290	100.13	100.29
Daidzein	3872.142	0.02484	0.0068	101.53	100.68
Luteolin	4470,869	0.02898	0.0019	101.00	100.19
Quercetin	1358.870	0.02003	0.0012	102.50	99.88
t-Cinnamic acid	7084.483	0.04027	0.0147	99.00	101.47
Apigenin	2800.038	0.02880	0.0049	101.20	100.49
Hesperidin	1503.143	0.01902	0.0060	100.50	100.60
Rhamnetin	3065.129	0.05390	0.0128	111.37	98.73
Chrysin	4433.44	0.04999	0.0049	101.80	100.49
Pinocembrin	3940,919	0.04018	0.0033	100.70	100.33
Cape	1385.744	0.02199	0.0034	103.53	99.66
Curcumin	1732.539	0.06444	0.0156	107.90	98.44

Accuracy

The method, and purely for quantitative and qualitative purposes, also was carefully evaluated for precision and accuracy. The accuracy of a method is indicated by absolute error and relative error values. The deviation of the measured value from the actual value is the absolute error, and the relative error is calculated by proportioning the absolute error to the actual value. Accuracy was evaluated from recovery studies of phenolic compound standards. Results are given in Table 1. The relative standards of the method

developed in our study error levels were observed below 0.02 except for resveratrol. The low level of relative error indicates the accuracy of the calibration chart and sample analysis results.

RP-HPLC-PDA Method Validation Parameters

The calibration curves were constructed with six points, in triplicate, for each phenolic compound. The equation and R^2 values of the calibration curve found based on the data obtained to find the measurement ranges of the standards are given in Table 2. The calibration curves of all compounds were determined linearly $R^2 > 0.995$.

Linear measurement range, precision, accuracy, observability, and detection limits were examined for the validation of the method developed in the study. In evaluating the validation parameters, standard deviation and relative standard deviations were calculated by drawing calibration curves for each standard. The limits of detection (LOD) and the limits of quantification (LOQ) were determined based on the LOD = 3.3 × SD / m and LOQ = 10 × SD / m, where m is the slope, and SD is the standard deviation at the lowest level of the calibration curves [14]. The LOD values of epicatechin, caffeic acid, rutin, ellagic acid, myricetin, pinocembrin, and curcumin standards were above 0.05, and the LOD values of other standards were found below 0.05 Table 2. Caffeic acid, rutin, ellagic acid, myricetin, pinocembrin, and curcumin LOQ values of the standards were above 0.2, and the other standards were below 0.20 (Table 2).

Table 2. Linear range, R^2, LOD and LOQ values of standards.

Standards	Linear range ppm	R^2	Limit of Detection (LOD) (µg/ml)	Limit of Quantification (LOQ) (µg/ml)
Gallic acid	1.25-40	0.9984	0.0099	0.0331
Protocatechuic acid	1.25-40	0.9986	0.0042	0.0139
Chlorogenic acid	1.25-40	0.9975	0.0199	0.0662
p-OH benzoic acid	1.25-40	0.9988	0.0309	0.1031
Epicatechin	1.25-40	0.9991	0.0569	0.1896
Caffeic acid	1.25-40	0.9991	0.0859	0.2865
Syringic acid	1.25-40	0.9986	0.0203	0.0676
m-OH benzoic acid	1.25-40	0.9997	0.0074	0.0247
Rutin	1.25-40	0.9991	0.0838	0.2793
Ellagic acid	1.25-40	0.9998	0.0896	0.2988
p-coumaric acid	1.25-40	0.9981	0.0333	0.1108
Ferulic acid	1.25-40	0.9982	0.0196	0.0653
Myricetin	1.25-40	0.9980	0.0868	0.2895
Resveratrol	1.25-40	0.9999	0.0336	0.1120
Daidzein	1.25-40	0.9995	0.0230	0.0768
Luteolin	1.25-40	0.9999	0.0254	0.0847
Quercetin	1.25-40	0.9999	0.0022	0.0074
t-Cinnamic acid	1.25-40	0.9982	0.0286	0.0954

Cont. Table 2

Apigenin	1.25-40	0.9997	0.0439	0.1463
Hesperidin	1.25-40	0.9997	0.0035	0.0117
Rhamnetin	1.25-40	0.9978	0.0165	0.0546
Chrysin	1.25-40	0.9997	0.0206	0,0687
Pinocembrin	1.25-40	0.9999	0.0852	0.2841
CAPE	1.25-40	0.9998	0.0037	0.0124
Curcumin	1.25-40	0.9952	0.0908	0.3027

Chromatographic RP-HPLC-PDA Determination of the Phenolic Compounds of Some Bee Products

To analyze phenolic composition of bee products, the HPLC method must be validated. In this study, twenty-five phenolic compounds of some bee products were determined by using different gradient mobile phases and RP-HPLC-PDA detector using acetic acids and acetonitrile solutions to obtain the separation conditions of twenty-five standards. Chromatograms of the standards at 4 different wavelengths are given in Figure 3.

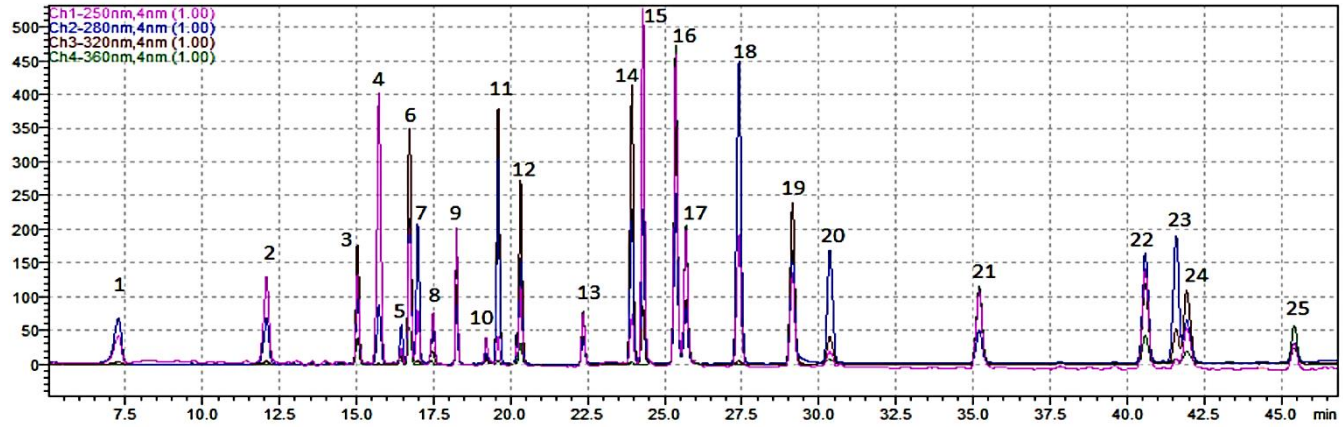


Figure 3. Chromatograms of the standards at four different wavelengths, (1)Gallic acid, (2)Protocatechuic acid, (3)Chlorogenic acid, (4)*p*-OH Benzoic acid, (5)Epicatechin, (6)Caffeic acid, (7)Syringic acid, (8)*m*-OH Benzoic acid, (9)Rutin, (10)Ellagic acid, (11)*p*-Coumaric acid, (12)Ferulic acid, (13)Myricetin, (14)Resveratrol, (15)Daidzein, (16)Luteolin, (17)Quercetin, (18)*t*-Cinnamic acid, (19)Apigenin, (20)Hesperetin, (21)Rhamnetin, (22)Chrysin, (23)Pinocembrin, (24)CAPE, (25)Curcumin.

After the application and validation of the twenty-five phenolics, three bee products were analyzed. The chromatograms of all three bee products are given in Figure 4 and the results is given as quantitatively in Table 3. Nine phenolic compounds were detected in the honey and pollen samples and twelve in the propolis at different amounts. Although gallic acid was detected only in the honey sample, protocatechuic acid was detected only in the pollen. Rutin is a flavonoid glycoside is only detected in the pollen at high level. Chrysin, pinocembrin, flavones and caffeic acid phenethyl ester were detected in all bee products, but higher quantity in the propolis. Generally, these phenolic compounds were found in the propolis, followed by the pollen and the honey. Caffeic acid phenethyl ester (CAPE) and pinocembrin were among the major compounds in the studied propolis sample.

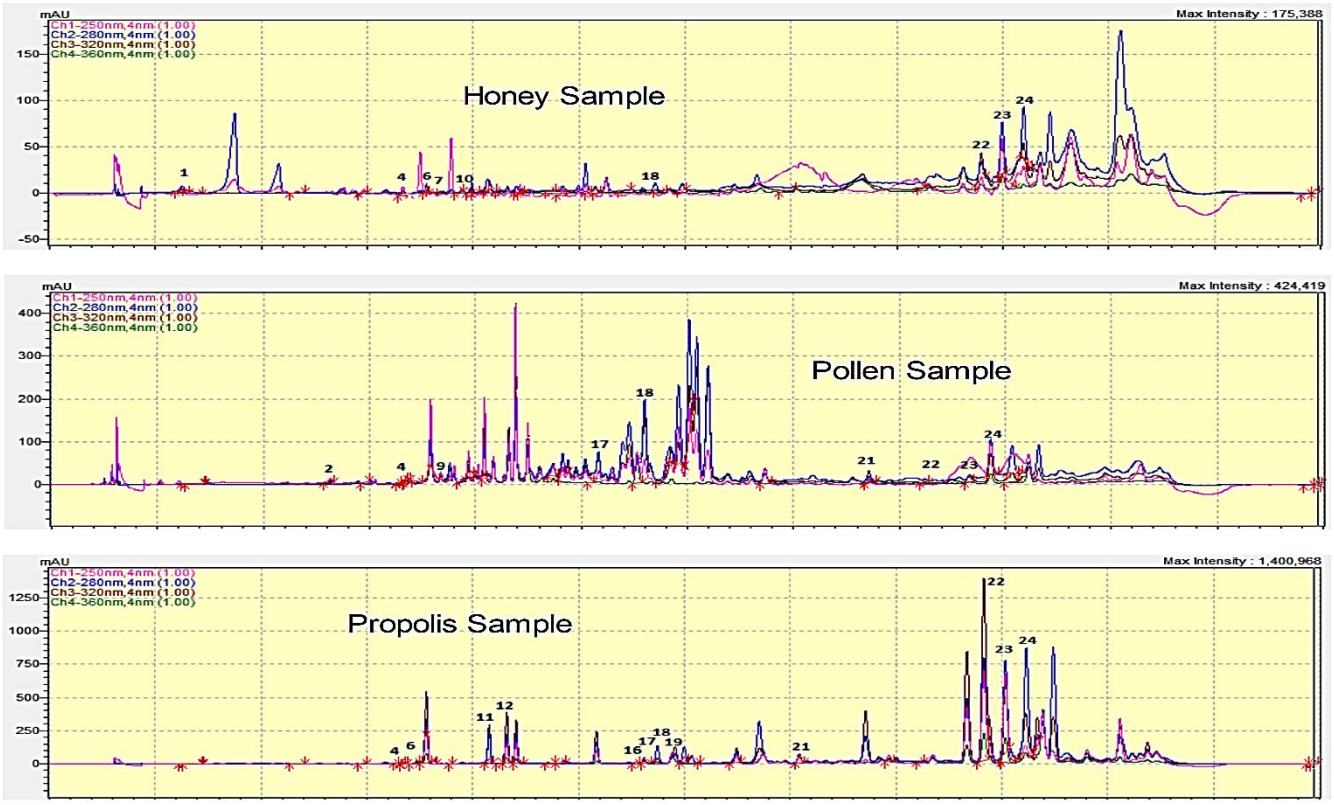


Figure 4. Phenolic content analysis chromatograms of samples.

Table 3. Phenolic content analysis result of samples.

Standards	Honey (mg/ 100 g)	Pollen (mg / 100 g)	Propolis (mg / 100 g)
Gallic acid	91.929	-	-
Protocatechuic acid	-	5.557	-
Chlorogenic acid	-	-	-
p-OH Benzoic Acid	0.926	1.249	0.852
Epicatechin	-	-	-
Caffeic acid	0.159	-	0.512
Syringic acid	0.332	-	-
m-OH Benzoic Acid	-	-	-
Rutin	-	5.472	-
Ellagic acid	0.909	-	-
p-Coumaric acid	-	-	23.880
Ferulic acid	-	-	39.700
Myricetin	-	-	-
Resveratrol	-	-	-
Daidzein	-	-	-
Luteolin	-	-	0.749
Quercetin	-	2.095	6.292
t-Cinnamic Acid	0.084	2.741	6.876

Cont Table 3

Apigenin	-	-	14.180
Hesperidin	-	-	-
Rhamnetin	-	1.629	9.718
Chrysin	0.920	2.568	106.720
Pinocembrin	0.734	1.859	113.080
CAPE	0.644	0.723	307.400
Curcumin	-	-	-

(-): not detection

Studies on the bioactive components of natural products have become very popular in recent years. In general, natural products can be considered healthier than many synthetic products. Some natural products (bee products, some medicinal plants, etc.) have been used for treatment in complementary and traditional medicine for many years and it is very important to be able to identify the bioactive origin of these products [15]. Honey, pollen and propolis are very valuable bee products, possess some widely biological active properties such as antioxidant, antibacterial, antiviral, anticarcinogenic, anti-inflammatory, anti-atherogenic, anti-diabetic, antithrombotic, immune-modulating, and analgesic activities, etc. [16-17].

In a study that included the analysis of twenty-three standards in eighty minutes, two different wavelengths (256 and 280 nm) were studied and propolis samples obtained from different regions were analyzed [18]. In another study, ten standards were analyzed in a single wavelength at hundred-five minutes [19]. There are studies in the literature analyzing bioactive flavonoids using different chromatographic methods [20]. In addition to these studies, a method has been developed in our study where twenty-five different phenolic components can be analyzed using four different wavelengths (250, 280, 320 and 360) in a shorter time.

In a study, 16 phenolic compounds were analyzed the LOD range was 0.001-0.970 µg/L and the LOQ range 0.001-2.949 µg / L was reported [21]. Compared our results of the study, LOD, LOQ values were found to be significantly lower.

Studies in the literature reveal that propolis is a rich mixture of polyphenols, like our study [22-24]. It was reported that kaempferol, apigenin, and derivatives of caffeic acid were detected in Algerian propolis [25]. A dispersive liquid-liquid microextraction (DLLME) with UHPLC–UV detection was studied in honey and syringic acid content ranged from 10-20 µg/100 g [26]. Many studies show that the phenolic composition of bee products changes depending on the plant flora and the climate characteristics of the area where it is collected. However, it shows that bee products rich in polyphenols have high antioxidant, antimicrobial and anti-inflammatory properties. For this reason, it is important to use an effective method to determine the phenolic components in bee products.

CONCLUSION

According to the determination of phenolic components with RP-HPLC-PDA, precision, accuracy, linearity, and the limits of detection and quantification were determined a runtime in 60 minutes. The chromatographic and extraction conditions applied, allowed 25 phenolic compounds to be characterized. The parameters evaluated by this method such as precision, accuracy, and LOD, LOQ gave satisfactory results, as well as good, could allow its use for the phenolic analysis of natural bee products.

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