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Validation Methods for Phenolic Components with RP-HPLC-UV in Various Bee Products

Çeşitli Arı Ürünlerinde RP-HPLC-UV ile Fenolik Bileşenlerin Validasyon Metotları

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Abstract

In this study, a simple and a fast method were developed using RP-HPLC-UV for the separation and quantitative determination of phenolic compounds in various bee products. Fourteen phenolic compounds were identified using 70% acetonitrile in water, and 2% acetic acid in water as a mobile phase with a gradient elution mode. The validation method exhibited linearity (R^2>0.994), limits of detection (0.022-0.062 mg/L) and quantification (0.030-0.187mg/L). Under the optimized conditions, 14 commercially available phenolic compounds were analyzed in less than 50 min. This method was successfully employed to study the phenolic profiles of bee products as well as other natural samples.

Keywords: RP-HPLC-UV, Phenolic Compounds, Bee Products, Quantification, Validation.

Özet

Bu çalışmada, basit ve hızlı bir yöntem olan RP-HPLC-UV kullanılarak çeşitli arı ürünlerinde fenolik bileşenlerinin ayrımı ve kantitatif tayini geliştirilmiştir. On dört fenolik bileşen için gradient %70 aetonitril-su ve %2 asetik asit elüsyonu kullanılarak ayrım gerçekleştirildi. Validasyon metodunun mevcut line eğriliği (R2> 0.994), limit dedeksiyon (0.022-0.062 mg / L) ve miktar tespiti (0.030-0.187 mg / L) belirlenmiştir. Optimize edilmiş koşullar altında, ticari olarak alınan 14 fenolik bileşik 50 dk'dan daha kısa sürede analiz edildi. Bu yöntem arı ürünlerinin fenolik profillerinin yanı sıra diğer doğal numunelerin incelenmesi için başarıyla kullanılmıştır.

Anahtar kelimeler: RP-HPLC-UV, Fenolik Bileşikler, Arı Ürünleri, Miktar, Validasyon.

Abbreviations: RP-HPLC-UV, reversed phase high performance liquid chromatographic ultra viole visible detector; DAD, diode array detection; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standart deviation.

1. INTRODUCTION

Phenolic compounds are widely distributed secondary metabolites of plants with important functions (Gómez-Caravaca et al., 2006; Escarpa & González, 2000). There has recently been an increase in studies of phenolic compounds in many natural products. These are because these compounds play an important role in growth and reproduction and provide protection against many oxidants and pathogenic predators (Balasundram et al., 2006; Bravo, 1998). Phenolic compounds have been thoroughly scrutinized in different studies because of their physico–chemical properties as well as their anti-bacterial, anti-viral,

anti-tumoral anti-carcinogenic, anti-thrombotic and highly antioxidant effects (Mariucci & Bankova, 1999; Kolayli et al., 2016). Phenolic analyses have also been performed in many pharmaceutical studies. Techniques such as LC-MS/MS, GC-MS and high performance liquid chromatography (HPLC) have been frequently used for the analysis of phenolic compounds in the literature (Seraglio et al., 2016). However, RP-HPLC-UV and RP-HPLC-DAD are the most practical methods for determining phenolic profiles of natural products. HPLC is commonly used as an analytical tool for developing and validating assay methods for phenolic compounds. Method validation provides documented evidence and a high degree of assurance that an analytical method employed for a specific test is suitable for its intended use. Validation involves procedures that demonstrate the reliability of the method. Validated results provide credibility, accuracy and precision. In deciding which parameters should be included in a validation process, linearity, accuracy, repeatability, selectivity, limit of quantification (LOQ), and limit of detection (LOD) should be considered (Fernando Mauro, 2009; Maria et al., 2012). Linearity indicates the ability to provide results directly proportional to the concentration of analyte in a sample within a given concentration range (Fernando Mauro, 2009; Maria et al., 2012; Cassiano et al., 2009). Due to the significance of phenolics in food stuffs, this study reviews the determination and quantification of phenolic compounds in different substances derived from bee products.

The purpose of this study was to focus on and develop a simple and rapid method capable of separating, quantifying and validating the analytical methodology used for phenolic compounds in different bee products. The validation processes provided objective evidence that the system and methods are suitable for their intended use.

2. MATERIALS AND METHODS

2.1. Chemicals snd Biological Materials

Acetonitrile HPLC gradient was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA), and methanol HPLC gradient from Merck KGaA, (Darmstandt, Germany). Ultrapure water was produced in an HLP5 system (arium 611 UV, Sartorius Germany). Phenolic standards, gallic acid, protocatechuic, p-OH benzoic, vanillic acid, catechin, syringic acid, ferulic acid, t-cinnamic acid, rutin and luteolin, were obtained from Sigma-Aldrich (Munich, Germany).

2.2. Preparation of Standard Solutions for Phenolic

The content of the phenolic compounds was determined using a calibration curve established with seven dilutions of each standard at concentrations of 0.312, 1.25, 2.5, 5, 10, 20 and 40 µg/mL. Following dilutions, the samples were analyzed using HPLC-UV with three repeats.

2.3. Sample Preparation for Phenolic Contents

Approximately 5 g of sample was placed into a falcon tube (100 mL), to which 50 mL 99% methanol was then added. The mixture was continuously stirred with a shaker (HeidolphPromax 2020, Schwabach, Germany) at room temperature for 24 hours, and then sonicated for 4 hours with an ultrasonicator (ultrasonic Elma Scmidbauer GmbH) Germany. Particles were removed using Whatman filter paper and concentrated in a rotary evaporator (IKA-Werke, Staufen, Germany) at 40°C. The residue was redissolved in methanol to a known final concentration and kept at 4°C until used for phenolic compound analysis.

2.4. Sample Preparation For RP-HPLC Analysis of Phenolic Compounds

The methanolic extract was evaporated until dryness with a rotary evaporator at 40° C. The residue was dissolved in 15 mL acidified distilled water (pH 2). Liquid–liquid extraction was carried out with 5×3 mL diethyl ether and 5× 3 mL ethyl acetate, consecutively (Kader et al., 1996; Kim et al., 2006). Both diethyl ether and ethyl acetate phases were pooled and dried by rotary evaporation (IKA-Werke, Staufen, Germany) at 40° C. The pellet was resuspended in 2 mL methanol, filtered with syringe filters (RC-membrane, 0.45 µm), and injected to HPLC.

2.5. HPLC-UV Detector Determination of Phenolic Compounds

HPLC analyses of the phenolic compounds were carried out on Elite LaChrom Hitachi, Japan HPLC with a UV-Vis detector. Gradient elution was used for HPLC analyses, modifying the

method previously developed by de Villiers (2004). The mobile phase consisted of (A) 2% acetic acid in water and (B) acetonitrile: water (70: 30). The sample injection volume was 20 µL, the column temperature 30° C and the flow rate 0.75mL/ min. The programmed solvent used began with a linear gradient held at 95% A for 3 min, decreasing to 80% A at 10 min, 60% A at 20 min, 20% A at 30 min and finally 95% A at 50 min. Three injections were performed for each sample (Table 1).

Table 1. RP-HPLC-UV gradient program

	A	B
Time (min)	**% 2 acetic acid (ultra pure water)**	**% 70-30 acetonitrile ultra pure water**
0.01	95.00	5.00
3.00	95.00	5.00
8.00	85.00	15.00
10.00	80.00	20.00
12.00	75.00	25.00
20.00	60.00	40.00
30.00	20.00	80.00
35.00	95.00	5.00
50.00	95.00	5.00

2.6. Analytical Method Validation

The method described in this study was validated according to the method described by Ribani (2007). The validation characteristics evaluated were selectivity, accuracy, precision, linearity, robustness and limits of detection and quantification. Standard solutions of the phenolics were prepared for calibration of the device. The standard solutions were filtered at 0.45 µm and collected in vials. These standard solutions were read in the UV on an HPLC device, and calibration graphs were generated according to their arrival times. Linearity was determined by calculation of the regression plots using the least squares method and was expressed as the determination coefficient (R^2). Concentrations of all compounds in the bee product samples were calculated based on peak area ratios.

Calibration generated at the linear measurement range was calculated from the equation data from the graph and using the formulas given below for limit of detection (LOD) and limit of quantification (LOQ). Limit of detection (LOD) was calculated using the formula $3{,}3 \times (\frac{SD}{m})$ where SD is the standard deviation of the response and m is the slope of the calibration curve [14]. Limit of quantification (LOQ) was establishedusing the formula $10 \times (\frac{SD}{m})$ LOD and LOQ were experimentally verified by injection of phenolic compounds at the LOD and LOQ concentrations.

Limit of detection (LOD) = $3{,}3 \times (\frac{SD}{m})$

Limit of quantitative measurement (LOQ)= $10 \times (\frac{SD}{m})$

SD = Standard deviation at the lowest level of the calibration curve

m= Slope of the calibration curve

Precision was estimated by evaluating intrabatch precision (repeatability) and interbatch precision (preparation process, repeatability). The precision of the method (within–day variations of replicate determinations) was checked by injecting reference compounds six times at the LOD and LOQ levels. The area values were recorded, and RSD% was calculated (Table 2).

3. RESULTS AND DISCUSSION

Bee products have been used as a food and for apitherapeutic purposes since the earliest times. In recent years, bee products have been used to treat various diseases due to their numerous bioactive properties. Phenolic compounds play a very important role in the biological activities exhibited by bee products. This study describes the first analytical method for quantifying phenolic compounds in various bee products. Fourteen phenolic compounds were identified at RP-HPLC-UV in a numbers of bee products (Table 2 and Figure 1).

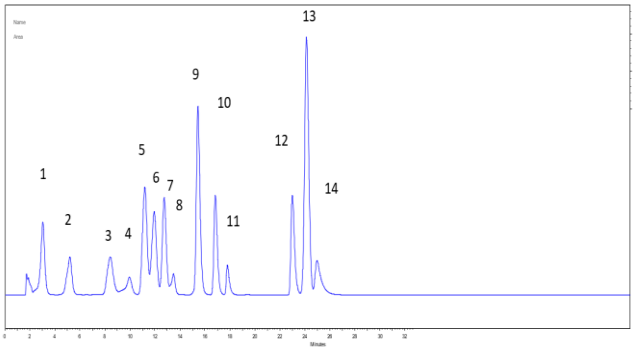

Figure 1. HPLC-UV chromatograms of phenolic standards 1.Gallic acid. 2. Protocatequic acid. 3. *p*-OH benzoic acid. 4. Catechin. 5. Vanilic acid. 6. Caffeic acid. 7. Syringic acid. 8. Epicatechin. 9. *p*-Cumaric acid. 10. Ferulic acid. 11. Rutin. 12. Daizein 13. *t*-cinnamic acid.14. Luteoli

Table 2. Phenolic profiles of some bee products extract

Results µg phenolic compound /g sample	**Sample**					
	P1	**P2**	**H1**	**H2**	**PO1**	**PO2**
Gallicacid	37.04±0.11	n.d.	0.57±0.01	n.d.	n.d.	n.d.
Protocatequic acid	3.68±0.01	n.d.	2.68±0.03	2.28±0.01	n.d.	n.d.
***p*-OH benzoic acid**	n.d.	n.d.	n.d.	0.17±0.03	n.d.	1.73±0.03
Catechin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Vanillic acid	52.70±0.01	90.36±0.01	n.d.	n.d.	n.d.	n.d.
Caffeic acid	2603.10±0.21	5344.70±0.23	2.80±0.04	4.06±0.16	n.d.	n.d.
Syringic acid	3.19±0.10	n.d.	n.d.	n.d.	n.d.	n.d.
Epicatechin	2200.61±0.46	2343.76±0.41	n.d.	n.d.	n.d.	n.d.
***p*-coumaric acid**	422.87±0.61	703.31±0.71	1.27±0.31	2.89±0.02	9.800±0.02	16.70±1.23
Ferulic acid	114.03±1.21	36.63±1.33	1.01±0.01	8.33±0.01	8.33±0.01	6.36±0.02
Rutin	1430.85±3.65	4362.30±2.36	3.04±0.01	1.13±0.06	17.63±0.08	110.38±2.26
Daidzein	n.d.	187.22±0.01	n.d.	n.d.	10.857±0.21	12.598±0.21
***t*-cinnamic acid**	346.00±6.391	753.82±7.91	0.12±0.01	0.17±0.03	11.90±0.03	13.70±0.03
Luteolin	374.86±7.38	673.87±82.11	n.d.	n.d.	237.00±7.38	344.00±8.11

*n.d: not detected.

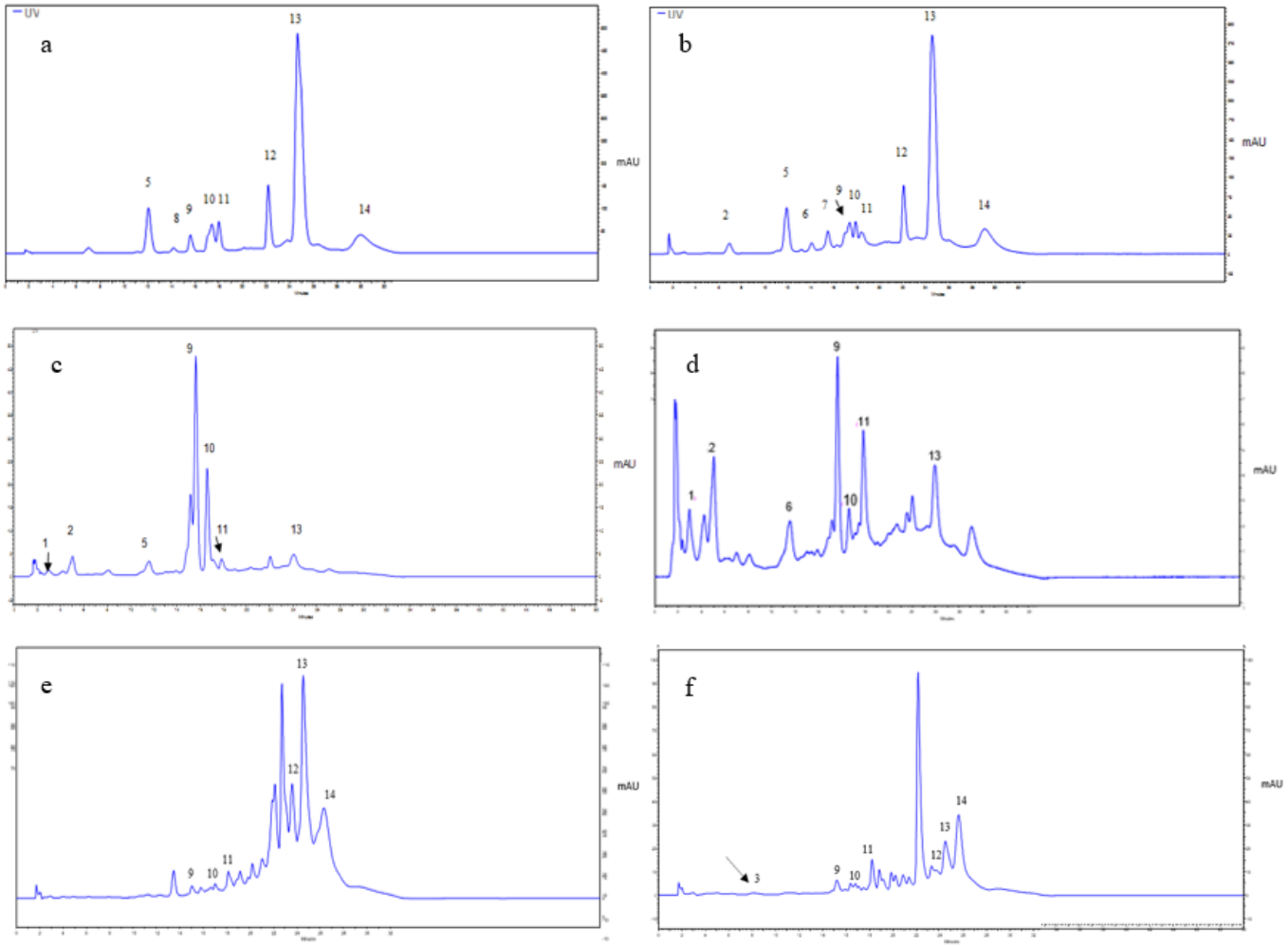

Figure 2. HPLC-UV chromatograms of samples (a) phenolic propolis 1, (b) phenolic propolis 2, (c) phenolic honey 1, (d) phenolic honey 2 figure 5, (e) phenolic pollen 1, (f) phenolic pollen 2.

Caffeic acid, syringic acid, epicatechin, p-coumaric acid, ferulic acid, rutin, t-cinnamic acid, luteolin were determined in propolis extracts (Figure 2a, 2b), protocatechuic acid, caffeic acid, p-coumaric acid, ferulic acid, rutin and t-cinnamic acid in honey samples (Figure 2c, 2d), and p-coumaric acid, ferulic acid, rutin, t-cinnamic acid and luteolin in pollen samples (Figures 3e, 3f). The quantities of these phenolic compounds were also measured in the bee products.

The development of a single liquid gradient for the analysis of phenolic compounds in bee products may constitute an interesting and fast solution to the study of phenolic composition and complete characterization. The gradient described might be used for the separation of both available and unavailable phenolics, as well as those present in real samples. Standard mixtures of 14 representative commercially available phenolic compounds were prepared. This chromatographic method was validated for the determination of the linearity, LOD, LOQ, precision, and accuracy of each compound. Linearity, LOD, and LOQ for 14 phenolic compounds were investigated, and the results are presented in (Table 3). This shows that good linearity was achieved in the range 0.994- 1.0 for all the compounds used as standards. The correlation coefficients (R2) were higher than 0.997, with the exception of luteolin (0.994). The quantification of all phenolic acid standards proved to be simple, sensitive and reproducible. The precision of the method, based on measurement repeatability, was obtained from the relative standard deviation (RSD%) by replicate injections (no. = 6) of a standard mixture of the standard components, taking into account the concentration and retention time of each compound.

Table 3. RP-HPLC-UV validation parameters of phenolic compounds

No	Compounds	R^2	%RSD (Retention Time)	%RSD (Area)	LOD[a]	LOQ[a]
1	Gallic Acid	0.999	0.210	1.941	0.022	0.067
2	Protocatequic Acid	0.999	0.871	1.920	0.042	0.128
3	*p*-OH Benzoic Acid	0.998	0.351	3.055	0.036	0.109
4	Catechin	0.998	0.492	4.279	0.040	0.121
5	Vanillic Acid	1.000	0.828	2.066	0.025	0.075
6	Caffeic Acid	0.998	0.179	4.039	0.062	0.187
7	Syringic Acid	1.000	0.550	0.848	0.009	0.027
8	Epicatechin	0.999	0.429	3.819	0.030	0.090
9	*p*-Coumaric Acid	0.999	0.204	1.562	0.010	0.030
10	Ferulic Acid	0.999	0.222	1.301	0.011	0.033
11	Rutin	1.000	0.234	3.139	0.041	0.123
12	Daidzein	0.998	0.174	1.545	0.018	0.054
13	*t*-Cinnamic Acid	1.000	0.262	1.071	0.014	0.042
14	Luteolin	0.994	0.229	5.833	0.043	0.130

a: Values are expressed as mg/L.

LOD and LOQ values for the 14 phenolic compounds studied ranged from 0.022 to 0.062 mg/L and 0.030-0.187mg/L, respectively (Table 3). The detection limit is the lowest amount of analyte in a sample that can be detected, albeit not necessarily quantified. The LOQ is defined as the lowest concentration that can be determined with acceptable accuracy and precision. The terms LOD and LOQ are used to demonstrate the ability to quantify/qualify low concentrations of a substance (Bertil, 2014). The LOD and LOQ values obtained in the validation of this method are considered suitable for the intended purpose.

Analyses of phenolic compounds in bee products are usually carried out using HPLC with diode-array detection (DAD) or a UV–Vis detector (Can et al., 2015; Escriche et al., 2014) although gas chromatography (Daher & Gülaçar, 2008) and capillary electrophoresis (Arráez-Román et al., 2006) have also been used in some instances. One previous study reported that the phenolic components of Chilean propolis were identified using liquid chromatography coupled with mass spectrometry (HPLC–MS), and that gradient elution was carried out with a binary system consisting of [A] 0.1% aqueous formic acid and [B] 0.1% formic acid in acetonitrile (Castro et al., 2014). The gradient solution used in that research is different from that in the present study. Another previous study reported extracting phenolic compounds in Algerian honeys using RP-HPLC according to a previously developed method

(Tomás Barberán et al., 2000). Our study is similar to that research in terms of extract preparation.

4. CONCLUSIONS

The analytical methodology in this study used a reverse phase HPLC that permitted rapid detection and quantification of phenolic compounds. The phenolic method comprised a mobile phase consisting of (2%) acetic acid in water with (70%) acetonitrile in water and UV detection at 280 nm for the various compounds.

The proposed analytical method for the detection and quantification of phenolic compounds in various bee products proved satisfactory in terms of linearity, precision, accuracy and stability in the range of interest. Overall, we think that the findings of this study can make an important contribution to the analytical field.

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