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Usability of the phenolic profile analysis method developed in RP-HPLC-PDA in natural products

RP-HPLC-PDA'da geliştirilen fenolik profil analiz yönteminin doğal ürünlerde kullanılabilirliği

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Abstract

The predominant majority of bioactive compounds in natural products are polyphenols. Reverse Phase High Performance Liquid Chromatography (HPLC) is the most employed analytical method for determining the polyphenol profiles of natural products. Analyses are conducted based on methods validated according to the number and type of phenolic standards used. In this study, it was aimed to determine 26 phenolic compound standards with HPLC-fotodiot array (PDA) detector, which is preferred for the separation of secondary metabolites commonly found in natural products. The analysis was carried out utilizing a C18 column (250 mm x 4.6 mm, 5 µm; GL Sciences) with a gradient program. The HPLC method was developed, determining the limit of detection within the range of 0.019-0.072 µg/mL, and the limit of quantification within the range of 0.063-0.239 µg/mL. All calibration curves exhibited linear corelations with R^2 values exceeding 0.994 across the specified range. The developed method has been optimized and validated by assessing detection and quantification limits, accuracy, repeatability, and recovery data suitable for phenolic analysis. It has been concluded that the optimized method allows for the rapid and reliable evaluation of the phenolic content of natural products and their quantitative determination.

Keywords: Phenolic content, Bee products, Plant, HPLC

Özet

Doğal ürünlerdeki biyoaktif bileşenlerin büyük çoğunluğunu polifenoller oluşturur. Polifenol kompozisyonlarının belirlemesinde en sık kullanılan analitik yöntemlerden biri Ters-Faz Yüksek Performanslı Sıvı Kromatografisidir. Analizler, kullanılan fenolik standartların sayısına ve türüne göre validasyonu yapılmış yöntemlere uygulanır. Bu çalışmada doğal ürünlerde yaygın olarak bulunan sekonder metabolitlerin ayrımında tercih edilen HPLC-fotodiyot dizisi (PDA) dedektörü ile 26 tane fenolik bileşen standardının belirlenmesi amaçlandı. Analiz C18 (250 mm x 4.6 mm, 5 µm; GL Sciences) kolonda gradient program kullanılarak yapıldı. HPLC yöntemi, tespit sınırını 0,019-0,072 µg/mL aralığında ve miktar belirleme sınırını 0,063-0,239 µg/mL aralığında belirleyecek şekilde geliştirildi. Tüm kalibrasyon eğrileri, belirtilen aralıkta R^2>0.994 değerleri ile doğrusal ilişkiler sergiledi. Oluşturulan metot optimize edilmiş, tespit ve nicelik sınırları, doğruluk, tekrarlanabilirlik ve fenolik analiz için uygun geri kazanım verileri değerlendirilerek doğrulanmıştır. Optimize edilmiş metot ile doğal ürünlerin fenolik içeriklerinin değerlendirilmesi ve kantitatif tayininin hızlı ve güvenilir bir şekilde yapılabileceği sonucuna varılmıştır.

Anahtar Kelimeler: Fenolik içerik, Arı ürünleri, Bitki, HPLC

Abbreviations: RP-HPLC, Reverse Phase High Performance Liquid Chromatography; PDA, Photodiode array; LOD, Limit of detection; LOQ, Limit of quantification

1. INTRODUCTION

Plants tend to protect themselves against harmful effects with secondary metabolites such as phenolic acids and polyphenols in their structures (Fang et al., 2007). These phenolic compounds, found in plants and transferable to animal products (bee products etc.), rank prominently among bioactive compounds (Rahsmi & Negi, 2020). Studies on phenolic compounds are increasing each day, primarily due to their bioactivities, including antioxidant properties as well as anti-inflammatory and antitumoral activities. Experimental and epidemiological studies also reveal the impact of phenolic compounds against degenerative diseases (Kumar et al., 2019).

Rhubarb (*Rheum ribes*), belonging to the Polygonaceae family, is a perennial herbaceous plant that grows at high altitudes and produces yellowish flowers in May and June. It has been reported that rhubarb, which contains various bioactive components, has metabolic benefits (Oktay et al., 2007). Knowing its phenolic content may contribute to elucidating these benefits. Among bee products, propolis and honey are two basic natural products, and various *in vitro* and in vivo studies are carried out on their phenolic contents (Ali & Kunugi, 2021; Ożarowski & Karpiński, 2023).

Various extraction methods are employed to obtain phenolic compounds from plant and bee products. Different solvents or solvent mixtures can be utilized depending on the selectivity of the bioactive compound to be extracted and the efficiency of the extraction method (Kumar & Goel, 2019). After extraction, meaningful content analyses can be achieved by identifying and quantifying the bioactive compounds obtained. However, despite numerous studies on the quantitative and qualitative analyses of bioactive compounds, it is an undeniable fact that the entire spectrum of these compounds, which has a wide range, has not been fully elucidated (Ignat et al., 2011; Kara et al., 2022). In this context, a broad network of research is aimed at developing quantitative and qualitative analysis methods for determining phenolic content (Liu et al., 2008).

While there are relatively simple spectroscopic methods available for the bulk quantification of phenolic compounds (such as the Folin-Ciocalteu reagent for total phenolic content determination), high-performance liquid chromatography (HPLC) enables both quantitative and qualitative analysis of individual phenolic compounds (Santos & MagalhÃes, 2020). In addition to addressing the diversity of phenolic compounds to be analyzed, HPLC methods are developed and utilized to gain advantages in terms of time and efficiency, enabling the determination of many analytes (Bae et al., 2015; Madrera & Valles, 2020; Michalaki et al., 2023). In methods developed for HPLC analysis, often two or more solvents are employed to determine the mobile phase for achieving the best separation. Generally, a gradient program is established to create optimal conditions for the mobile phase that will be used in the analysis. However, another crucial aspect of high analysis efficiency is the selection of a detector with high sensitivity, selectivity, and a broad analysis spectrum. In the literature, it is observed that detectors such as ultraviolet (UV), photodiode array (PDA), and tandem mass spectrometry (MS/MS) are preferred for phenolic content analyses (Michalaki et al., 2023; Kara et al., 2022; Zhang et al., 2023). Especially with the preference for PDA detectors, it is possible to operate within a wavelength range, and the spectra of analytes can be visualized. Thus, the PDA detector provides the opportunity to work with methods that offer high accuracy and sensitivity.

In the case of a research-based analytical method, it is essential to conduct reliability, repeatability, and applicability studies for its validation. In this context, the method's measurement range, quantitative and qualitative measurement limits, as well as accuracy and precision values, can be investigated (Can et al., 2015; Chaudhary et al., 2023; Sobral et al., 2017). Accuracy and precision assessment can involve the calculation of relative error and recovery percentage, along with the examination of various parameters. This study aims to

demonstrate the applicability of a chromatographic method for the analysis of phenolic content in plants and certain animal products (propolis, honey, etc.).

2. MATERIALS and METHODS

2.1 Extraction of Samples

In the context of our study, the rhubarb (*Rheum ribes*) was sourced from the Hakkari region, propolis from the Trabzon region, and honey from beekeepers in the Balıkesir region of Türkiye (Figure1). The samples were extracted with a solvent at a ratio of 1:10 (g/mL) for 24 hours, room tempruture at 200 rpm (Kara et al., 2022). The propolis sample was extracted with 70% EtOH, while the honey samples and rhubarb (*Rheum ribes*) were extracted with MeOH. Following extraction, a filtration process was conducted to remove solid particles, and the samples were divided into two parts for antioxidant analysis and phenolic content analysis. For phenolic analysis, the samples were subjected to the sample preparation procedure reported by Kara et al. (2022), and phenolic content analysis was conducted using the method developed and validated within the scope of this study. All antioxidant analyzes were performed in triplicate.

Figure 1. A: Propolis, B: Honey, C: Rhubarb (*Rheum ribes*)

2.2. Validation of the Phenolic Content Method

The validation of the developed method for phenolic content analysis included assessments of accuracy, precision, recovery, and analytical measurement limits. Accuracy, which expresses the closeness of the obtained value to the true value, can be examined through both absolute and relative error values. Relative error is calculated by dividing the absolute error by the true value. The closeness of the values obtained in the analysis is expressed as precision. The method's accuracy can be further examined through recovery and repeatability analyses.

Within the scope of the developed phenolic analysis method, each standard was studied in three repetitions at six different concentrations (38-1.188 ppm). Using the obtained

standard calibration curves, detection (LOD) and measurement (LOQ) limits were determined. The standard deviation at the lowest concentration and the slope of the curve were used to calculate these values. The ratio of standard deviation to slope was calculated by multiplying by 3.3 for the LOD value and by 10 for the LOQ value (Ribani et al., 2006).

2.3. Analysis Conditions of the Phenolic Content Method

The HPLC system used for phenolic content analysis consists of an LC-20AT liquid chromatograph (Shimadzu), SIL-20AC HT autosampler (Shimadzu), SPD-M20A diode array detector (Shimadzu), and an InertSustain C18 column (5 µm, 4.6 mm, 250 mm; GL Sciences). In the developed method, 70% acetonitrile (ACN)-ultrapure water (reservoir A) and 2% acetic acid (AcH)-ultrapure water (reservoir B) were used for the mobile phase. In the gradient program, a 50-minute analysis time was applied with the following composition: 0. min 82%(B), 5. min 81%(B), 10. min 73%(B), 14. min 62%(B), 25. min 35%(B), 40. min 10%(B), 40.01. min 82%(B), and 50. min 82%(B). For phenolic content analysis, the applied flow rate was 1 mL/min, column temperature was set at 30 °C, injection volume was 20 µL, and autosampler cell temperature was maintained at 20 °C. Within the scope of the analysis, four different wavelengths (250, 280, 320, 360) were utilized for the standards. The created phenolic content method involved the analysis of standards at specific wavelengths: protocatechuic acid, *p*-OH benzoic acid, vanillic acid, rutin, ellagic acid, daidzein at 250 nm; gallic acid, catechin hydrate, epicatechin, syringic acid, *t*-cinnamic acid, naringenin, hesperetin, chrysin, pinocembrin at 280 nm; chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, apigenin, caffeic acid phenethyl ester (CAPE) at 320 nm; and myricetin, luteolin, quercetin, rhamnetin, galangin at 360 nm. Standards were prepared by first dissolving them with a small amount of 100% MeOH and then making up their volume with 50% MeOH.

2.4. Antioxidant Capacity and Activity Analyzes

As part of the antioxidant capacity analyses, the total phenolic content (Slinkard & Singleton, 1977) and total flavonoid content (Fukumoto & Mazza, 2000) were determined. The Folin-Ciocalteu method was used for total phenolic content (TP). For this, 400 µL of a 1:10 diluted Folin-Ciocalteu reagent was mixed with 20 µL of the sample, 680 µL of distilled water, and 400 µL of 10% Na_2CO_3. After incubation for 120 minutes, the absorbance was measured at 760 nm wavelength. Gallic acid was used for the standard calibration curve, and the results were expressed as mg of gallic acid equivalent per gram of the sample. Regarding total flavonoid content (TF), 250 µL of the sample was mixed with 2150 µL of MeOH, 50 µL of 10% $Al(NO_3)_3$, and 50 µL of 1 M NH_4CH_3COO solution. After a 40 minute incubation, the absorbance was

measured at 415 nm wavelength. Quercetin served as the standard, and the results were reported as mg of quercetin equivalent per gram of the sample. In the assessment of antioxidant activity, we conducted analyses using the Fe(III) Reducing Antioxidant Power method (Benzie & Strain, 1999) and 2,2-diphenyl-1-picrylhydrazyl radical scavenging activities (Molyneux, 2000). For Fe(III) Reducing Antioxidant Power analysis, 1500 µL of freshly prepared FRAP reagent (Kara et al., 2022) was mixed with 50 µL of the sample. After 4 minutes of incubation, the absorbance was measured at 593 nm wavelength. $FeSO_4.7H_2O$ was used as the standard, and the results were expressed as µmol of $FeSO_4.7H_2O$ equivalent per gram of the sample. A solution of 100 µM 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) was prepared for DPPH· radical scavenging activity. The solution was mixed in a 1:1 (v/v) ratio with varying concentrations of the sample and incubated for 50 minutes. Subsequently, the absorbance was measured at 517 nm wavelength. The sample concentration corresponding to half of the concentration of the initial DPPH· radical in the medium (SC_{50}) was calculated, and the results were expressed in mg/mL. Trolox was used as standard.

3. RESULTS and DISCUSSION

For the validation of the method developed for the phenolic content analysis in plants and bee products, standards were tested in the range of 1,188-38,000 ppm, and the linearity of calibration curves, relative error, recovery of the method, LOD, and LOQ values were calculated (Table 1). When examining the recovery values at the standard concentration of 38 ppm, it is observed to be in the range of 97.803% to 103.948%. The low relative error of this method, both quantitatively and qualitatively applicable, suggests high precision and accuracy. When examining the LOD values of each standard, it was found that caffeic acid had the highest value at 0.072 µg/mL, while catechin hydrate had the lowest value at 0.019 µg/mL. The LOQ values are observed to be in the range of 0.071-0.239 µg/mL. Cayan et al. (2020) reported LOD and LOQ values of their method, established with 16 phenolic standards, in the ranges of 0.001–0.970 and 0.001–2.940 µg/L, respectively. Švecová et al. (2015) stated that the LOD and LOQ values of their method, developed with 12 phenolic standards, were in the ranges of 1.17-5.35 µg/L and 3.89-17.80 µg/L, respectively. In another study, the LOD and LOQ values for the HPLC analysis of 14 phenolic standards were found to be in the ranges of 0.009-0.192 µg/mL and 0.027-0.582 µg/mL, respectively (Seal, 2016). Skendi et al. (2017) determined the LOD and LOQ values as 0.005-0.16 µg/mL and 0.01-0.48 µg/mL, respectively, in the method they developed for the analysis of 24 phenolic contents in plant samples. In a study by Aktaş

Karaçelik and Şahin (2021), LOD and LOQ values for RP-HPLC-DAD analysis of 18 phenolic standards were found to be in the range of 0.019-0.2363 mg/L and 0.039-0.7162 mg/L, respectively. These valuable findings indicate that low amounts of phenolic content can be detected with RP-HPLC, but the limits may vary depending on the condition of the equipment.

Tablo 1. R^2, LOD, LOQ, relative error and recovery values of standards

Standards	**R^2**	**Limit of Detection (LOD) (µg/mL)**	**Limit of Quantification (LOQ) (µg/mL)**	**Relative Error**	**Recovery (%)**
Gallic acid	0.999	0.043	0.142	0.007	99.296
Protocatechuic acid	0.999	0.062	0.205	0.008	100.838
Chlorogenic acid	0.999	0.034	0.115	0.018	101.757
Catechin hydrate	0.999	0.019	0.063	0.011	98.917
P-OH benzoic acid	0.999	0.044	0.148	0.028	102.816
Epicatechin	0.997	0.033	0.109	0.028	102.843
Caffeic acid	0.999	0.072	0.239	0.027	102.716
Vanillic acid	0.998	0.043	0.143	0.022	97.803
Syringic acid	0.999	0.021	0.071	0.013	98.725
P-coumaric acid	0.999	0.064	0.214	0.028	102.830
Rutin	0.999	0.047	0.156	0.004	100.417
Ellagic acid	0.999	0.062	0.208	0.004	99.554
Ferulic acid	0.995	0.026	0.088	0.031	103.073
Myricetin	0.998	0.032	0.108	0.013	101.348
Daidzein	0.999	0.050	0.166	0.007	100.719
Luteolin	0.994	0.032	0.106	0.034	103.351
Quercetin	0.999	0.029	0.098	0.009	99.101
t-Cinnamic acid	0.999	0.021	0.071	0.008	99.242
Naringenin	0.996	0.026	0.087	0.008	99.181
Apigenin	0.999	0.037	0.123	0.040	103.948
Hesperetin	0.999	0.023	0.075	0.013	101.289
Rhamnetin	0.996	0.028	0.094	0.014	101.445
Chrysin	0.999	0.027	0.091	0.009	99.146
Pinocembrin	0.999	0.037	0.123	0.007	99.303
CAPE	0.999	0.025	0.083	0.035	103.456
Galangin	0.999	0.023	0.077	0.036	103.647

The chromatograms of the standards used in the developed phenolic analysis method in the scope of the study are presented in Figure 2.

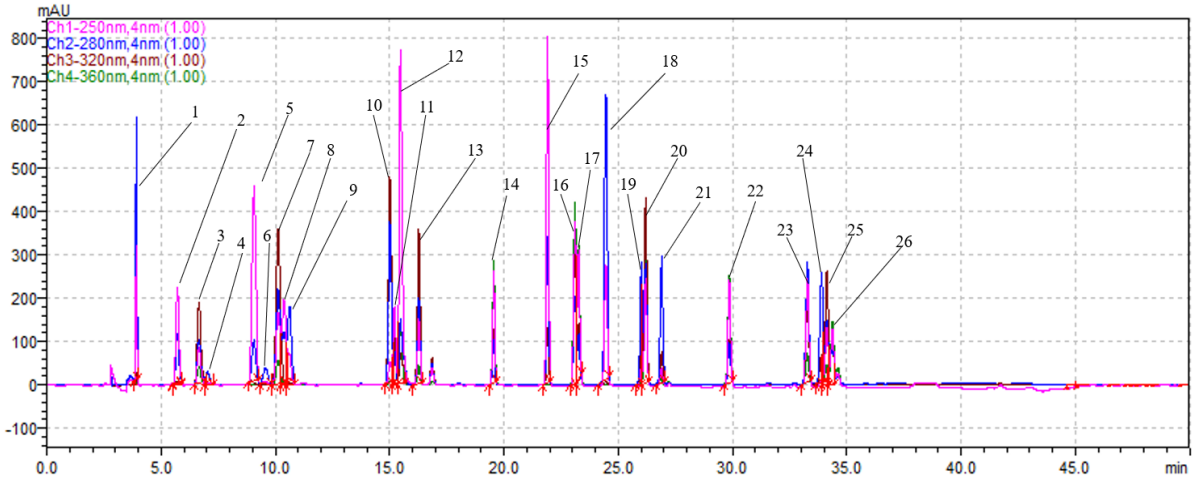

Figure 2. Phenolic Standard Chromatogram. 1. Gallic acid, 2. Protocatechuic acid, 3. Chlorogenic acid, 4. Catechin hydrate, 5. *p*-OH Benzoic acid, 6. Epicatechin, 7. Caffeic acid, 8. Vanillic acid, 9. Syringic acid, 10. *p*-Coumaric acid, 11. Rutin, 12. Ellagic acid, 13. Ferulic acid, 14. Myricetin, 15. Daidzein, 16. Luteolin, 17. Quercetin, 18. *t*-Cinnamic acid, 19. Naringenin, 20. Apigenin, 21. Hesperetin, 22. Rhamnetin, 23. Chrysin, 24. Pinocembrin, 25. CAPE, 26. Galangin

The developed phenolic content method has been applied to plant extracts and bee products, and the results are provided in Table 2. Quercetin has been reported as a significant component in the sample of rhubarb. (Mısır et al., 2023). Keser et al. (2020) reported that caffeic acid and ferulic acid were major components in the phenolic content analysis of the rhubarb sample (302.45 mg/g and 269.25 mg/g, respectively). Another study indicated the presence of 284.00 µg/mL gallic acid and 218.26 µg/mL rutin in the ethanolic rhubarb extract. (Abdulla et al., 2014). Meral (2017) reported that the phenolic content of rhubarb samples subjected to different drying conditions varied. The sample dried at 80 °C exhibited higher phenolic content (gallic acid: 353.5±2.4 mg/100g, rutin: 68.8±0.4 mg/100g, caffeic acid: 40.7±0.6 mg/100g). In the rhubarb sample used in our study, significant levels of ellagic acid (20.573 µg/g), ferulic acid (34.205 µg/g), and gallic acid (17.433 µg/g) were observed.

Kumazawa et al. (2004) conducted phenolic analysis on propolis samples collected from 16 different countries and reported that the samples contained high levels of chrysin and pinocembrin. In another study, propolis samples collected from 11 different regions of Türkiye were reported to be rich in chrysin, CAPE, pinocembrin, and rutin (Can et al., 2022). Halagarda et al. (2020), in their study on Polish honeys, reported that honey samples contained chrysin, caffeic acid, *p*-coumaric acid, pinocembrin, kaempferol, galangin, and apigenin. The phenolic contents of the propolis (chrysin, pinocembrin, CAPE, caffeic acid) and honey (ellagic acid, *p*-OH benzoic acid) samples used in our study have been elucidated with the new method.

Table 2. Phenolic content of rhubarb (*Rheum ribes*), propolis and honey samples

		Rhubarb (*Rheum ribes*)	**Propolis**	**Honey**
µg phenolic content /g sample	Gallic acid	17.433	-	-
	Protocatechuic acid	4.358	-	-
	Chlorogenic acid	-	-	-
	Catechin hydrate	-	-	-
	p-OH Benzoic acid	1.777	-	5.150
	Epicatechin	-	-	-
	Caffeic acid	-	1477.234	-
	Vanillic acid	-	-	-
	Syringic acid	1.762	-	-
	p-Coumaric acid	7.093	406.450	1.639
	Rutin	2.425	-	-
	Ellagic acid	20.573	-	17.903
	Ferulic acid	34.205	349.937	-
	Myricetin	-	-	-
	Daidzein	-	-	-
	Luteolin	-	-	-
	Quercetin	3.205	304.848	-
	t-Cinnamic acid	0.590	1128.909	0.563
	Naringenin	-	-	-
	Apigenin	-	361.103	-
	Hesperidin	-	-	-
	Rhamnetin	-	-	-
	Chrysin	1.300	6924.761	0.669
	Pinocembrin	-	9996.916	0.698
	CAPE	-	2652.344	-
	Galangin	-	-	-

* - : not detected

Antioxidant activity in various parts of the rhubarb collected from the Erzurum region of Türkiye was investigated using different solvents, and it was reported to exhibit antioxidant properties (Oktay et al., 2007). Ceylan et al. (2019) conducted a study where they examined the TP, TF, FRAP, and DPPH values per dry sample of rhubarb collected from the Erzurum region of Türkiye. They reported values of 112.82 ± 11.68 mg GAE/g, 2.50 ± 0.31 mg QUE/g, 42.50 ± 2.44 µmol Fe/g, and 0.11 mg/mL, respectively. Ozturk et al. (2007) extracted the stem part with aqueous methanol and expressed the TP and TF values as 35.71 ± 1.23 µg pyrocatechol equivalents/mg extract and 13.66 ± 0.75 µg QUE/mg extract, respectively. They stated that the extract at 100 µg/mL concentration had a radical scavenging activity of 87.07 ± 0.54%. The TP and TF values of the rhubarb sample collected from the Iğdır (Türkiye) region were found to be 18.644 mg GAE/g and 1.427 mg QUE/g, respectively (Mısır et al., 2023).

Studies on propolis have stated that the TP and TF values of propolis samples from different regions vary between 31.2±0.7-299±0.5 mg/g and 2.5±0.8-176±1.7 mg/g, respectively

(Kumazawa et al., 2004). In honey, the TP, TF, FRAP, and DPPH analysis results have been shown to vary in the ranges of 16.02 ± 2.70 to 120.04 ± 18.56 mg GAE/100g, 0.65 ± 0.42 to 8.10 ± 2.56 mg QUE/100g, 0.59 ± 0.21 to 4.30 ± 0.13 µmol $FeSO_4.7H_2O$/g, and 12.56 ± 2.50 to 152.40 ± 6.20 mg/mL, respectively (Can et al., 2015). Antioxidant analyses were performed on the rhubarb (*Rheum ribes*), propolis, and honey samples, and the obtained results are presented in Table 3.

Table 3. Antioxidant results of Rhubarb (*Rheum ribes*), propolis and honey samples

	Rhubarb (*Rheum ribes*)	**Propolis**	**Honey**	**Trolox**
TP (mg GAE/ g)	1.742±0.078	124.847±0.152	0.392±0.005	
TF (mg QUE/ g)	0.343±0.012	31.566±0.052	0.029±0.007	
FRAP (µmol $FeSO_4.7H_2O$/g)	17.494±0.107	5193.974±13.244	3.242±0.022	
DPPH SC_{50} (mg/mL)	6.968±0.098	0.033±0.004	83.256±0.126	0.006 ± 0.000

4. CONCLUSIONS

With this study, a method has been developed, validated, and applied for the determination of the phenolic content in plant and some animal samples (bee products, etc.). In our work, a goal was set to develop a method capable of analyzing 26 standards in a short period, as compared to methods created with different devices and different phenolic standards in the literature. When selecting standards, examples from the literature were reviewed, and commonly used phenolic standards were attempted to be identified. For the application of the developed method, rhubarb (*Rheum ribes*) was chosen as a plant sample, and two important bee products, propolis, and honey, were selected as animal products. With this study, a method was created for the phenolic content analysis of samples taken from different sources in a short time, less costly and with high sensitivity. Considering that natural products have rich phenolic content, studies can be carried out to develop easily applicable methods that can detect more phenolics.

DECLARATIONS

The authors declare that they have no conflicts of interest.

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