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Rapid and easy method for simultaneous measurement of widespread 27 compounds in natural products and foods

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Abstract: Phenolic compounds are dietary bioactive compounds that are available in foods and beverages. They are produced by the plant and/or by the mushrooms, naturally. Some of the natural phenolic compounds were also used in dietary supplements in the pharmaceutical or food industries. For simultaneous analyses of phenolic compounds in one injection, a rapid and sensitive method was developed for twenty-seven natural compounds using reverse-phase high-performance liquid chromatography (RP-HPLC) coupled with diode array detector (DAD). The linear gradient elution systems of 0.1% acetic acid-methanol were used, and the analyzing time was 61 minutes. The method was validated with linearity, relative error, reproducibility, LOD values. Relative standard deviation between (n = 7) within and between days for reproducibility is less than 10%, and relative error (or recovery) is less than 5%. The method exhibited excellent linearity (R^2>0.999), good precision (RSD<6.1%), recovery (97.6–104.1%) and limits of detection (0.23–28.81 µg/m1) and quantification (1.62– 87.29 µg/m1). The detection of compounds was performed at 220 nm, 280 nm, and 330 nm. The developed method for the rapid determination of phenolic compounds using RP-HPLC can be used to identify the availability of these phenolic compounds in natural and commercial products.

Keywords: RP-HPLC; phenolic compounds, food; dietary supplements; natural products.

1. Introduction

The plants produce phenolic compounds to protect themselves against ultraviolet radiation or pathogenic aggression. Phenolic compounds are considered as secondary metabolites except for some of them. Plants contain bioactive phenolic compounds. Previous studies revealed that phenolic compounds possess antioxidant, anticancer, antimicrobial, anticholinesterase, anti-urease, and anti-tyrosinase activities. They are also known as antipyretic, biogenic, analgesic, antibiotic, and sedative properties [1-3]. Therefore, the plants containing bioactive phenolic compounds are used to prepare food supplements. Moreover, mainly they exhibit antioxidant activity. Therefore, to screen them in foods and medicinal plants, many studies have been conducted.

Many different methods have been developed for determining the phenolic and organic components in natural products [4]. For this purpose, the best instrument is HPLC (high-performance liquid chromatography), which can be successfully applied for analyzing chemical compounds of natural

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products [5]. Thirteen phenolic compounds were also used to develop the HPLC method [6]. In another developed method, nineteen phenolic and flavonoid derivatives were simultaneously analyzed using HPLC-DAD and fluorescence detector using a fused-core type column in 10 minutes [7]. The phenolics of Manzanilla olives collected from various regions in Spain were analyzed using this method, where ODS was the column for separation in the extracts obtained by the different extraction techniques [8]. There is another study to analyze twenty-five phenolic compounds using HPLC-DAD was developed [9]. Herein, beside the retention times, three different wavelengths (254, 280 and 330 nm) were used to accurate analyses. The method was used to identify the phenolic compounds of grapes. Such an analysis was also developed by [10] using different columns, which is faster and having high resolution. In this HPLC assay beside retention times 220, 280, 320, and 520 nm wavelengths were used to determine accurately. Thirty phenolic compounds using a phenyl-hexyl-fixed phase, and two detectors were separated in a developed method [11]. The separation took place in 64 minutes, and the resolution and the accuracy were accepted. In another study, 13 phenolic compounds were investigated in the water extracts of *Dianthus carmelitarum* using reverse-phase column and acetonitrile-water gradient elution system. The analyses took 36 minutes [12].

The literature survey indicated that to analyze the phenolic compounds, abundantly, Diode Array Detector (DAD) was employed. Beside, Florescence, PDA, ECD, and Refractive Index detectors were also used. For the separation of phenolic compounds, in general, the ODS (C_{18}) column in (4.6 mm x 250 mm, ID, 5 µm) was used. The mobile phase mainly consists of 0.5-3 % organic or inorganic acid, such as acetic acid or phosphoric acid in water and/or methanol/acetonitrile [11, 13-22].

Almost in many laboratories, there is an HPLC coupled with DAD or UV detectors and a standard ODS column. Therefore, by changing some parameters, 27 phenolic compounds mostly available in plant extracts were used to develop a fast and reliable method, simultaneously using HPLC as an instrument. This study aimed to develop an HPLC method that could be easily adapted related studies. The fumaric acid, gallic acid, *trans*-aconitic acid, *p*-benzoquinone, pyrocatechol, protocatechuic acid, 2-(4-hydroxyphenyl) ethanol, 4-hydroxybenzoic acid, catechin, methyl-*p*-benzoquinone, rosmarinic acid, quercetin, naringenin, *trans*-cinnamic acid, ellagic acid, rutin, *trans*-2-hydroxycinnamic acid, coumarin, ferulic acid, *p*-coumaric acid, chlorogenic acid, 2,4-dihydroxybenzoic acid, vanillin, caffeic acid, 6,7-dihydroxy coumarin, vanillic acid, chrysin which are available in plant samples were used for separation in one injection. This method was validated concerning linearity, reproducibility, and accuracy.

2. Experimental

2.1. Chemicals

Methanol (HPLC, analytical grade) and glacial acetic acid were supplied from Merck (Sternheim, Germany). Fumaric acid (≥99.0%), gallic acid (≥99.0%), *trans*-aconitic acid (≥98.0%), *p*-benzoquinone (≥98.0%), pyrocatechol (≥99.5%), protocatechuic acid (≥99.9%), 2-(4-hydroxyphenyl) ethanol (≥98.0%), 4-hydroxybenzoic acid (≥99.0%), (+)-catechin hydrate (≥98.0%), methyl-*p*-benzoquinone (≥98.0%), rosmarinic acid (≥98.0%), quercetin (≥95.0%), naringenin (≥98.0%), *trans*-cinnamic acid (≥99.0%), ellagic acid (≥99.0%), rutin hydrate (≥94.0%), *trans*-2-hydroxycinnamic acid (≥97.0%), coumarin (≥99.0%), ferulic acid (≥99.0%), *p*-coumaric acid (≥98.0%), chlorogenic acid (≥99.0%), 2,4-dihydroxybenzoic acid (≥97.0%), vanillin (≥99.0%), caffeic acid (≥98.0%), 6,7-dihydroxy coumarin (≥98.0%), vanillic acid (≥97.0%), chrysin (≥97.0%) were purchased from Sigma-Aldrich GmbH (Sternheim, Germany). The structures of reference compounds were given in supporting information.

2.2. Preparation of Stock Solution of Standard Compounds and Mixture

To detect the exact retention times, 1.0 mg of every compound dissolved in 2 mL methanol, were injected into the HPLC, individually. To prepare the mix of reference compounds, 10.0 mg fumaric acid, 2.0 mg gallic acid, 8.0 mg *trans*-aconitic acid, 2.0 mg *p*-benzoquinone, 10.0 mg pyrocatechol, 2.0 mg protocatechuic acid, 13.66 mg 2-phenyl ethanol, 0.50 mg 4-hydroxybenzoic acid, 5.0 mg (+)-catechin hydrate, 1.0 mg methyl-*p*-benzoquinone, 2.0 mg 6,7-dihydroxy coumarin, 1.0 mg vanillic acid, 1.2 mg

caffeic acid, 1.0 mg vanillin, 1.0 mg 2,4-dihydroxybenzoic acid, 1.0 mg chlorogenic acid, 4.0 mg *p*-coumaric acid, 3.0 mg ferulic acid, 1.0 mg coumarin, 4.0 mg *trans*-2-hydroxycinnamic acid, 2.0 rutin hydrate, 2.0 mg ellagic acid, 0.50 mg *trans*-cinnamic acid, 6.2 mg naringenin, 1.0 mg quercetin, 1.2 mg rosmarinic acid, 1.0 mg chrysin were weighted using a balance (Sartorius CPA225D, Gottingen, Germany). Then all compounds weighted accurately were mixed and dissolved in a 10.0 mL flask with methanol. The mixture was stored at 4 °C for a maximum period of 2 weeks. From the mixture of 27 compounds stock solutions, eight dilutions were prepared, and each was injected into the HPLC-DAD to prepare the calibration curves of each compound.

2.3. HPLC Analysis Conditions

The analyses of 27 compounds were carried out using a Shimadzu high-performance liquid chromatography (Shimadzu Cooperation, Japan) system that consists of a Shimadzu model LC-20AT solvent delivery unit and a Shimadzu model SPD-M20A diode array detection system. All were controlled by LC-solution software (CBM-20A System Controller Shimadzu). The column temperature was set at 35 ºC. The chromatographic separation was performed on an Inertsil ODS-3 (4 µm, 4.0 mm x 150 mm) column and Inertsil ODS-3 guard column. The mobile phases were 0.1% acetic acid in water (A) and 0.1% acetic acid in methanol (B). The elution profile was as follows: 2% B in 3 min, 2–5% B in 3 min, 5–6% B in 2 min, 6–10% B in 4 min, 10% B in 1 min, 10–25% B in 5 min, 25–30% B in 7 min, 30–40% B in 5 min, 40–42% B in 6 min, 42–54% B in 5 min, 54–55% B in 1 min, 55–56% B in 10 min, 56–65% B in 4 min, 65–75% B in 3 min, 75–85% B in 2 min, 85–95% B in 5 min, 95% B in 2 min, 95–100% B in 1 min, 100% B in 5 min, 100–80% B in 2 min, 80–50% B in 2 min, 50–2% B in 3 min (Table 1). The flow rate was 1.0 mL/min. The injected volume was 20 µl. Detection was carried out a diode array detector (DAD) using 200-600 nm wavelengths. All the samples and standards were filtered using an Agilent 0.45 µm PTFE filter.

Table 1. The HPLC pump program

Time (Minute)	Solvent B	Minute	Solvent B
0-3 (3 min)	2%	43-52 (10 min)	56%
3-6 (3 min)	5%	53-56 (4 min)	65%
7-8 (2 min)	6%	57-59 (3 min)	75%
9-12 (4 min)	10%	60-61 (2 min)	85%
13 (1 min)	10%	62-66 (5 min)	95%
14-18 (5 min)	25%	67-68 (2 min)	95%
19-25 (7 min)	30%	69 (1 min)	100%
26-30 (5 min)	40%	70-74 (5 min)	100%
31-36 (6 min)	42%	75-76 (2 min)	80%
37-41 (5 min)	54%	77-78 (2 min)	50%
42 (1 min)	55%	79-81 (3 min)	2%

2.4. Method Validation

The linearity, accuracy, and precision, along with the limit of detection (LOD), the limit of quantitation (LOQ), and effectiveness were considered to validate the reproducibility of the developed method. Various proportions of Solvent A and Solvent B was used to achieve the best resolution. 0.1% acetic acid in acetonitrile and pure acetonitrile solvents were also used for separation. However, the resolution was unsuccessful for all compounds. The best separation was achieved by using 0.1% acetic acid in water as a solvent A and 100% methanol as a solvent B. On the other hand, the general column ODS-2 (5 µm, 4.6 mm x 250 mm) was also used for separation. However, the time was longer, and the peak shapes are a bit broad. Thus, the ODS-3 (4 µm, 4.0 mm x 150 mm) was better to separate the compounds in excellent resolution.

The stock solution containing 27 standards was diluted to nine different concentrations. All diluted concentrations were injected to HPLC in triplicate. The concentration versus peak area for each standard in the mixture was plotted to obtain the calibration curve. Except for some of the compounds, the square of the correlation coefficient at 254 nm was more than R^2>0.99, which indicates the linearity. At both 280 and 330 nm, however, the square of correlation coefficients was more than R^2>0.999.

$$Recovery\ \% = \frac{Measured\ conncentration - endegeneus\ concentration}{spiked\ concentration} x100$$

The recovery rates were in the range of 97–104% for each standard at 254 nm while 96–102% for each standard at 280 and 330 nm wavelengths. The recovery values indicate the efficacy and consistency of the method. The detection and quantification limits were calculated according to the general formula:

LOD : 3.3 ($\sigma\sigma$) / SS

LOQ : 10 ($\sigma\sigma$) / SS

Where ($\sigma\sigma$) is standard deviation of peak area, while *SS* is the slope of the calibration curve [15].

The percentage relative standard deviation (RSD%) indicating the degree of proximity of precision of the retention time, and the peak area was calculated. A known concentration (20 µg/mL) of the solution of mixed standards was injected into the HPLC system to check the repeatability of the retention time and peak areas (Pa). Then RSD of peak areas of each retention time were calculated using seven replicate determinations.

2.5. Calibration curves

Standards compounds were dissolved in methanol to prepare stock solutions. Then retention times were detected by injecting each standard item individually. The mixture of 27 standard phenolic compounds was prepared in methanol and then injected into the HPLC-DAD. For quantitative analysis, six different concentrations of the twenty-seven phenolic compounds mixtures were injected triplicate. Then the calibration curves were obtained by plotting the concentration versus the peak areas of every compound. R^2 values were greater than 0.992 except *p*-benzoquinone, R^2=0.983; caffeic acid, R^2=0.988; ellagic acid R^2=0.988 (Table 2).

3. Results and Discussion

3.1. Optimization of Chromatographic Condition

In order to determine optimum chromatographic conditions, experiments were carried out under different mobile phase conditions. Acetonitrile, methanol, acetonitrile-acetic acid, and methanol acetic-acid mobile phases by changing the acetic acid ratio were used to find the best resolution. Finally, the best mobile phase for separation was under the conditions of acetic-acid 0.1% in water as a solvent A and methanol as a solvent B. In many studies, acetonitrile use was suggested as one of the mobile phases [5,7,20-23]. Acetonitrile was also used for separation in this study. However, only 14 compounds, namely, gallic acid, protocatechuic acid, pyrocatechol, 2,4-dihydroxy phenyl ethanol, 4-hydroxy benzoic acid, vanillic acid, vanillin, *p*-coumaric acid, ferulic acid, *trans*-2-hydroxycinnamic acid, coumarin, cinnamic acid, naringenin, and chrysin could be separated. Therefore, it was aimed to change the solvent system to analyze more phenolic components at the same time. For this purpose, Inertsil ODS-3 (4µm, 4.0 mm x 150 mm) analytical column was used to see sharp peaks and to reduce the elution time. The column temperature was kept at 35 °C. With this optimized method, 27 phenolic components were analyzed with high accuracy and absolutely within 61 minutes.

3.2. Method Validation

In order to obtain separated peaks and optimum chromatograms with peak intensities close to each other, 500 µg/mL solutions were prepared from each standard and injected into the HPLC-DAD

system. The location and peak intensities of each phenolic compound from the symmetrical and sharp peaks were determined. Then, concentrations of the compounds to be included in the mixture were determined (Figure 1). In order to completely separate the peaks, a gradient program was done. The linear gradient elution was provided by increasing the methanol ratio from 2% to 100%. The phenolic mixture was prepared in methanol with amounts of standard compounds to test the linearity of the method.

The prepared mixture was diluted as a stock solution. From the stock solution, six standard dilutions were prepared, and each standard dilution was injected three times into HPLC-DAD under the same conditions (Figure 2). Calibration curves were created by determining the mean peak areas against the concentration of each dilution concentrations obtained from the chromatograms. Each standard was analyzed three times to increase the accuracy of the method, and the average values were calculated. Equations and R^2 values obtained for linear calibration curves are given in Table 2a, 2b, 2c. Table 2a contains the validation parameters of 27 components at 254 nm wavelength. As seen Table 2a, R^2 values are above 0.992 except *p*-benzoquinone, (R^2 = 0.983), caffeic acid (R^2 = 0.988), and ellagic acid (R^2 = 0.988). Table 2b shows the calculated validation parameters of 19 phenolic compounds at 280 nm wavelength. All R^2 values are above 0.999. In Table 2c, the validation parameters of 11 compounds were calculated at 330 nm wavelength (R^2> 0.999).

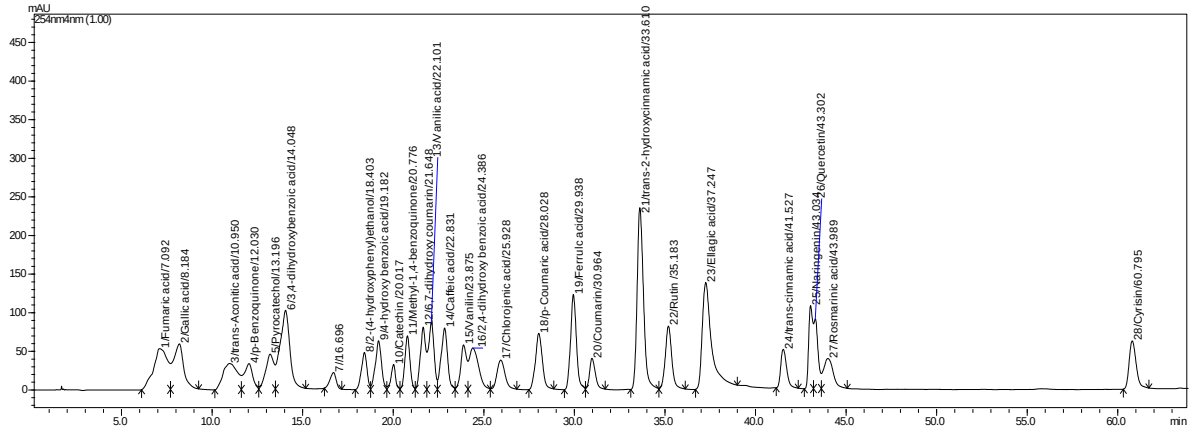

Figure 1. HPLC–DAD chromatograms of 27 phenolic standards at 254 nm: (**1**) fumaric acid , (**2**) gallic acid, (**3**) *trans*-aconitic acid, (**4**) *p*-benzoquinone, (**5**) pyrocatechol, (**6**), protocatechuic acid, (**7**) unknown, (**8**) 2-(4-hydroxyphenyl) ethanol, (**9**) 4-hydroxybenzoic acid, (**10**) (+)-catechin, (**11**) methyl-*p*-benzoquinone, (**12**) 6,7-dihydroxy coumarin, (**13**) vanillic acid, (**14**) caffeic acid, (**15**) vanillin, (**16**) 2,4-dihydroxybenzoic acid, (**17**) chlorogenic acid, (**18**) *p*-coumaric acid, (**19**) ferulic acid, (**20**) coumarin, (**21**) *trans*-2-hydroxycinnamic acid, (**22**) rutin, (**23**) ellagic acid, (**24**) *trans*-cinnamic acid, (**25**) naringenin, (**26**) quercetin, (**27**) rosmarinic acid, (**28**) chrysin. Inertsil ODS-3 column (4 µm, 4 mm x 150 mm). Mobile phase 0.1% acetic acid-methanol (gradient elution). Flow rate 1mL/min. Diode array detection 254 nm.

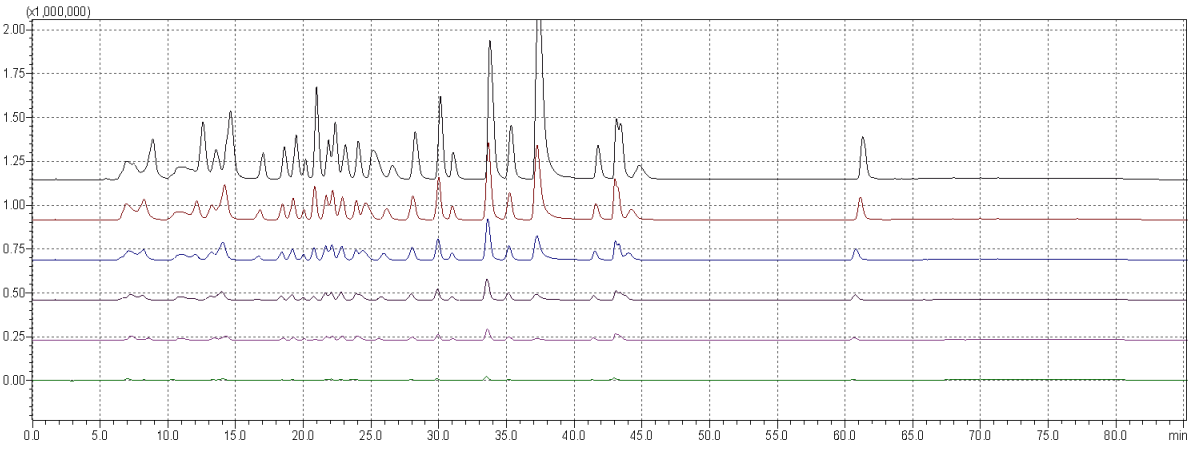

Figure 2. HPLC-DAD chromatograms of calibration standards

Table 2a. Retention time, calibration curves, regression coefficient (R^2), linearity ranges, LODs and recoveries of phenolic standards at 254 nm.

No	Compounds	RT [a] (min)	Calibration equation	R^2 [b]	Linear range (μg/mL)	λ_{max}, nm	LOD [c] (μg/mL)	LOQ [c] (μg/mL)	Recovery (%)	RSD [d] within day (n= 7)	RSD between days (n=7)
1	Fumaric acid	6.81	y=11584x-45691	0.9970	7.5-125	254	12.91	39.13	102.45±6.96	4.96	2.20
2	Gallic acid	8.39	y=45439x-137402	0.9950	6.25-200	254	2.23	6.77	102.46±4.46	3.50	1.87
3	*trans*-Aconitic acid	10.95	y=8314.5x+57114	0.9920	25.0-400	254	6.12	18.54	101.16±8.11	4.50	2.69
4	*p*-Benzoquinone	12.62	y=39428x-759865	0.9970	50.0-200	254	28.81	87.29	100.96±2.97	4.88	2.17
5	Pyrocatechol	13.35	y=5269.6x+40422	0.9950	31.3-500	254	4.78	14.48	103.07±5.46	5.44	1.40
6	Protocatechuic acid	14.10	y=76181x-88801	0.9995	3.13-100	254	3.42	10.35	102.35±4.21	3.19	1.22
7	2-(4-Hydroxyphenyl)-ethanol	18.40	y=2907.6x-7629.4	0.9995	21.3-1366	254	2.77	8.39	102.32±3.57	3.39	1.27
8	4-Hydroxybenzoic acid	19.50	y=111102x+21691	0.9993	1.56-50.0	254	1.58	4.79	100.82±3.89	4.00	2.41
9	(+)-Catechin	20.00	y=3865.1x+32660	0.9980	15.6-500	254	3.29	9.96	102.11±4.08	3.78	2.87
10	Methyl-*p*-benzoquinone	20.83	y=81195x-420112	0.9830	3.13-50.0	254	5.37	16.27	103.03±4.23	4.86	5.29
11	6,7-Dihydroxycoumarin	21.99	y=34377x-32740	0.9940	5.00-50	254	3.98	12.07	104.11±5.06	4.94	3.72
12	Vanillic acid	22.37	y=74653x-9634.1	0.9998	1.56-100	254	1.56	4.68	103.58±4.43	5.06	3.88
13	Caffeic acid	22.94	y=67972x-32965	0.9880	3.00-30.0	254	4.54	13.75	102.67±4.92	4.01	5.87
14	Vanillin	24.02	y=45495x+313074	0.9920	3.13-100	254	1.21	3.67	100.59±4.84	1.76	3.76
15	2,4-Dihydroxybenzoic acid	24.78	y=70870x+258749	0.9920	1.56-100	254	4.17	12.63	100.35±4.75	5.10	5.81
16	Chlorogenic acid	26.13	y=39264x+66133	0.9920	6.25-50.0	254	6.25	18.75	101.23±4.98	5.20	3.53
17	*p*-Coumaric acid	28.43	y=18300x+6153.3	0.9998	6.25-400	254	5.46	16.56	101.60±2.36	3.14	0.44
18	Ferulic acid	29.93	y=35737x+12977	0.9999	2.34-300	254	3.96	11.99	100.99±3.54	3.20	0.51
19	Coumarin	31.10	y=36021x-23215	0.9999	3.13-100	254	2.21	6.69	101.74±4.83	3.59	1.08
20	*trans*-2-Hydroxycinnamic acid	33.65	y=53843x+124308	0.9996	3.13-400	254	3.09	9.27	99.75±3.75	2.85	0.75
21	Rutin	35.02	y=40347x-30437	0.9999	3.13-200	254	2.49	7.56	101.99±3.45	3.01	2.53
22	Ellagic acid	37.61	y=165729x-3000000	0.9880	12.5-100	254	2.68	8.13	100.70±5.43	7.34	6.56
23	*trans*-Cinnamic acid	41.54	y=87505+4540.2	0.9999	1.25-50.0	254	0.58	1.74	100.85±1.58	5.78	5.66
24	Naringenin	43.07	y=9895.8x+159212	0.9950	4.84-620	254	2.75	8.34	100.00±4.91	2.88	2.39
25	Quercetin	43.49	y=68024x+7902.3	0.9999	1.56-100	254	1.01	3.06	98.57±3.84	1.87	5.04
26	Rosmarinic acid	44.20	y=20734x+853142	0.9950	30.0-120	254	6.38	19.33	97.92±4.36	6.12	4.97
27	Chrysin	60.45	y=66794x-17157	0.9999	3.13-100	254	1.96	5.93	101.35±3.17	3.64	1.11

[a] RT: Retention time of the compound in minutes, [b] R^2: linearity of the calibration graph, [c] LOD: Limit of Detection in μg/m Land LOQ: Limit of Quantification in μg/mL, [d] RSD: Percentage relative standard deviation

Table 2b. Retention time, calibration curves, regression coefficient (R^2), linearity ranges, LODs and recoveries of phenolic standards at 280 nm.

No	Phenolic Compounds	RT [a] (min)	Calibration equation	R^2 [b]	Linear range (μg/mL)	λ_{max}, nm	LOD [c] (μg/mL)	LOQ [c] (μg/mL)	Recovery (%)	RSD [d] within days (n= 7)	RSD between days (n=7)
1	Pyrocatechol	13.35	y = 20632x + 53090	0.9999	7.8-250	280	4.72	14.32	100.35±2.07	3.51	3.20
2	Protocatechuic acid	14.10	y = 33573x + 21157	0.9995	6.25-200	280	6.25	18.75	100.12±3.05	2.41	6.10
3	2-(4-Hydroxyphenyl) ethanol	18.40	y = 11284x + 95007	0.9999	10.6-341	280	5.60	16.2	98.99±2.25	2.03	4.15
4	4-hydroxybenzoic acid	19.50	y = 30844x + 18883	0.9998	1.25-50	280	1.33	4.03	99.07±3.97	4.43	4.38
5	(+)-Catechin	20.00	y = 14456x + 37858	0.9999	3.9-500	280	0.95	2.86	101.25±2.16	4.00	3.82
6	Methyl-*p*-benzoquinone	20.83	y = 2566.8x + 14266	0.9998	3.13-50	280	1.45	4.40	100.11±1.42	25.8	4.90
7	6,7-Dihydroxycoumarin	21.99	y = 21346x + 25710	0.9992	1.56-200	280	0.92	2.76	104.06±4.08	7.40	6.11
8	Vanillic acid	22.37	y = 36710x + 2454.3	0.9990	0.78-100	280	0.54	1.62	102.13±4.64	3.59	4.55
9	Caffeic acid	22.94	y = 46945x + 12850	0.9995	0.94-120	280	0.23	0.69	101.36±5.19	2.80	4.83
10	Vanillin	24.02	y = 215103x + 89945	0.9997	0.78-25	280	0.75	2.28	101.80±3.81	6.82	7.84
11	*p*-Coumaric acid	28.43	y = 96659x + 175014	0.9999	3.13-400	280	3.08	9.24	98.48±7.84	1.59	3.19
12	Ferulic acid	29.93	y = 63874x - 3785.8	0.9997	2.34-300	280	1.62	4.92	102.29±4.96	0.88	3.65
13	Coumarin	31.10	y = 45026x + 56387	0.9995	0.78-25	280	0.93	2.82	99.25±5.13	1.81	3.21
14	*trans*-2-Hydroxycinnamic acid	33.65	y = 123059x + 435116	0.9996	6.25-200	280	6.25	18.95	97.47±4.30	1.62	3.00
15	Rutin	35.02	y = 15485x - 919.6	0.9998	1.56-200	280	4.00	12.00	101.28±3.35	4.79	3.58
16	*trans*-Cinnamic acid	41.54	y = 174388x + 79400	0.9997	1.25-50	280	1.43	4.35	97.62±4.49	4.12	3.27
17	Naringenin	43.07	y = 72633x + 837946	0.9996	4.84-155	280	5.01	15.03	100.58±4.12	1.87	4.25
18	Quercetin	43.49	y = 420803x + 735567	0.9998	3.13-100	280	2.83	8.58	101.90±3.92	2.60	6.28
19	Chrysin	60.45	y = 63792x + 41075	0.9999	1.56-100	280	1.51	4.59	98.61±4.93	2.47	3.00

[a] RT: Retention time of the compound in minutes
[b] R^2: linearity of the calibration graph
[c] LOD: Limit of Detection in μg/m Land LOQ: Limit of Quantification in μg/mL
[d] RSD: Percentage relative standard deviation

Table 2c. Retention time, calibration curves, regression coefficient (R^2), linearity ranges, LODs and recoveries of phenolic standards at 330 nm.

No	Phenolic Compounds	RT [a] (min)	Calibration equation	R^2 [b]	Linear range (μg/mL)	λ_{max}, nm	LOD [c] (μg/mL)	LOQ [c] (μg/mL)	Recovery (%)	RSD [d] within days (n= 7)	RSD between days (n=7)
1	6,7-Dihydroxycoumarin	21.99	y = 54384x - 77273	0.9998	1.56-200	330	3.89	11.81	102.77±4.70	2.90	4.56
2	Caffeic acid	22.94	y = 75745x - 36431	0.9993	0.94-60	330	2.76	8.36	101.66±5.78	5.53	3.36
3	Vanillin	24.02	y = 74432x + 43718	0.9998	1.56-100	330	2.06	6.02	99.41±3.67	29.89	4.31
4	*p*-Coumaric acid	28.43	y = 286585x + 162918	0.9998	1.56-100	330	2.14	6.47	96.90±10.27	1.53	3.52
5	Ferulic acid	29.93	y = 104472x + 105130	0.9999	4.70-300	330	3.99	12.11	99.79±4.51	1.06	3.77
6	Coumarin	31.10	y = 29083x + 1992.1	0.9999	1.56-100	330	1.44	4.36	99.64±5.76	2.19	3.20
7	*trans*-2-Hydroxycinnamic acid	33.65	y = 67576x + 162187	1.000	3.13-400	330	2.99	9.06	99.42±1.73	1.58	3.08
8	Rutin	35.02	y = 25414x + 35748	0.9997	1.56-200	330	5.76	17.44	97.82±4.53	2.16	4.52
9	Ellagic acid	37.61	y = 18964x - 109643	0.9998	6.25-200	330	5.60	16.97	101.27±4.60	6.05	8.43
10	*trans*-Cinnamic acid	41.54	y = 129885x + 78457	1.0000	1.56-100	330	0.98	2.97	99.93±4.08	4.24	5.65
11	Chrysin	60.45	y = 42937x + 1034.7	0.9998	1.56-100	330	1.60	4.80	100.39±4.27	2.75	3.11

[a] RT: Retention time of the compound in minutes
[b] R^2: linearity of the calibration graph
[c] LOD: Limit of Detection in μg/m Land LOQ: Limit of Quantification in μg/mL
[d] RSD: Percentage relative standard deviation

Repeatability of the method throughout a day and between days of a standard solution mixture consist of fumaric acid (125 µg/mL), gallic acid (25 µg/mL), *trans*-aconitic acid (100 µg/mL), *p*-benzoquinone (25 µg/mL), pyrocatechol (125 µg/mL), protocatechuic acid (25 µg/mL, 2-(4-hydroxyphenyl) ethanol (170 µg/mL), 4-hydroxybenzoic acid (6.25 µg/mL), (+)-catechin (62.5 µg/mL), methyl-*p*-benzoquinone (12.5 µg/mL), 6,7-dihydroxy coumarin (25 µg/mL), vanillic acid (12.5 µg/mL), caffeic acid (15.0 µg/mL), vanillin (12.5 µg/mL), 2,4-dihydroxybenzoic acid (12.5 µg/mL), chlorogenic acid (12.5 µg/mL), *p*-coumaric acid (50 µg/mL), ferulic acid (37.5 µg/mL), coumarin (12.5 µg/mL), *trans*-2-hydroxycinnamic acid (50 µg/mL), rutin (25 µg/mL), ellagic acid (25 µg/mL), *trans*-cinnamic acid (6.25 µg/mL), naringenin (77.5 µg/mL), quercetin (12.5 µg/mL), rosmarinic acid (15 µg/mL), chrysin (12.5 µg/mL, verified by injecting into HPLC-DAD seven times. Relative standard deviation values were calculated from the data obtained by seven times repeating during the day and between days. These were used to determine the repeatability. As seen in Table 2a, the repeatability of the method is 1.76-7.34 RSD during the day and 0.44-6.56 RSD between the days. However, except for ellagic acid and rosmarinic acid during the day, the repeatability of other compounds is below 6%. Repeatability is higher for compounds rather than ellagic acid between days. RSD was between 0.88-4.79% (excluding methyl-*p*-benzoquinone, vanillin, and 6,7-dihydroxy coumarin), as given in Table 2b and RSD 1.06-5.53 (excluding ellagic acid, vanillin) as given in Table 2c. The RSD values were coherent with those of studies in the literature [9-10].

The precision of the method was also calculated using the equations obtained from the calibration curve as a result of six repeated analyses. The above mentioned standard mixture of compounds was injected six times. Then the calibration curve and the concentrations of the compounds were calculated using the peak areas obtained from the chromatograms. The obtained concentration values were compared with actual values to determine the recovery and, therefore, the precision of the method. At 254 nm, the recovery of the method is in the range of 97.92 ± 4.36-103.07 ± 5.46 as given in Table 2a, while at 280 nm 97.47 ± 4.30 - 104.06 ± 4.08 in Table 2b. At 330 nm, however, it is 96.90 ± 10.27 - 102.77 ± 4.70, as given in Table 2c. Therefore, it is possible to say that the accuracy of the optimized method is quite high. Besides, LOD values of the method were determined and given in Tables 2a, 2b, and 2c. The obtained values provide accuracy and low detection of the compounds using HPLC in a cheaper and faster way.

4. Conclusions

A practical, fast rapid, and accurate RP-HPLC-DAD method was developed to analyze twenty-seven natural compounds simultaneously in one injection. The total analyzing time was 61 minutes. Developed method validation was performed using linearity, relative error, reproducibility, LOD, and LOQ values. The method exhibited excellent linearity, good precision, and recovery. Various wavelengths, such as 220 nm, 280 nm, and 330 nm, were used to detect the compounds for high accuracy. Since the synthetic drugs have many side effects, the researchers start to investigate the natural products to find effective drugs. Some of the natural products studied are the potential drugs since possessing adequate *in vitro* and *in vivo* activities. However, it is tough to be approved of any effective natural product as a drug. Therefore, entrepreneurs or companies get permission to be able to sell these products as dietary supplements. If the productions of food supplements are produced by the following their production procedure, there is no problem. However, some of the food traders and malicious people who want to make more money can adulterate food supplements, then which will be dangerous for human health. Therefore, commercial food supplements are necessary to be analyzed for their ingredients. The developed method can be used for any natural product analyses and any commercial food supplement related to twenty-seven compounds.

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Supporting Information

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