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Development and Validation of an HPLC-DAD Method for the Simultaneous Analysis of Phenolic Compounds

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Abstract Phenolic compounds are natural substances that exhibit different functional bioactivities and provide health-protective actions against chronic illnesses. The vast potential of these compounds in health and other sectors demands the establishment of analytical procedures for their immediate and simultaneous analysis. In this study, a high-performance liquid chromatography with diode-array detection (HPLC-DAD) method was developed and validated for the simultaneous analysis of gallic acid, catechin, epicatechin, rutin hydrate, caffeic acid, syringic acid, ellagic acid, p-coumaric acid, trans-ferulic acid, myricetin, resveratrol, and quercetin. The chromatographic separation of the selected polyphenols was carried out in a reversed-phase Inertsil ODS-3 column (250mm x 4.5mm x 5μm) at a flow rate of 0.8 mL/min, injection volume of 20 μL, and column temperature of 30°C. The detection and quantification of phenolic compounds were done at specific wavelengths (254, 275, 305, and 325 nm) using gradient elution for 40 minutes, with acidified water and acetonitrile solution as mobile phase. Validation of the established analytical procedure showed that the coefficient of determination (R^2 > 0.99), limit of detection (0.01 to 0.35 μg/mL), limit of quantitation (0.03 to 1.07 μg/mL), recovery values (98.33 to 101.12%), and repeatability (RSD < 5%) respectively indicated a linear, sensitive, accurate, and precise analytical method for the simultaneous chromatographic analysis of the 12 phenolic compounds. Overall, the developed HPLC-DAD procedure can offer adequate confidence for the identification and quantification of specific polyphenols and can be modified or updated for future analysis of phenolic compounds in different plant extracts.

Keywords: HPLC, diode-array detection method development, method validation, phenolic compound.

Introduction

The interest in the study of phenolic compounds has increased continually during the past years. These natural compounds have gained special emphasis as they have been reported to exhibit different functional bioactivities including antioxidant, antimutagenic, antitumor, anticarcinogenic, antimicrobial, anti-inflammatory, antihepatotoxic, and antiviral properties [1-5]. Several epidemiological studies have revealed that regular consumption of plant phenolic compounds provides health-protective and disease-preventive actions against chronic illnesses such as cancer, diabetes, cardiovascular diseases, and chronic respiratory diseases [6-11].

Phenolic compounds are bioactive substances that are naturally found in plants and are the second most abundant group of organic compounds in the plant kingdom [12]. It encompasses a vast array of molecules that have either a single phenol or polyphenol structure (i.e. aromatic ring with one or several hydroxyl groups) and more than 8000 of which have been already characterized in different plant species [13].

In plants, phenolic compounds have been associated with numerous functionalities, especially during growth and development, and exposure to biotic (microorganisms, parasites) and abiotic (UV radiation, wounds, oxygen level instability) stressors [3]. In humans, it provides a wide array of benefits in terms of health promotion and illness reduction or prevention. These bioactive substances are of immense importance in the food, nutrition, and health sectors due to their high potential as functional food ingredients [14].

The plant phenolic compounds arise from two main biochemical pathways, the shikimate, and acetate pathways [15]. In the shikimate pathway, they are derived from a common intermediate like phenylalanine, tyrosine, and shikimic acid. Meanwhile, the acetate pathway utilizes the Krebs cycle to provide malonyl-coenzyme A for the elongation of a certain compound. Phenolic compounds are grouped according to the carbon atom number, general structure, number of phenol rings, and the substituents of the ring structure. The predominant phenolic compounds in different horticultural crops, particularly fruits and vegetables, include phenolic acids, flavonoids, stilbenes, lignans, coumarins, and proanthocyanidins or tannins [16]. Three major groups (phenolic acids, flavonoids, stilbenes) out of the mentioned classes of phenolic compounds are used in the present study.

Phenolic compounds have been analyzed by different analytical procedures including thin-layer chromatography, gas chromatography, electrophoresis, and high-performance liquid chromatography (HPLC). However, among these methods, HPLC is the most used technique for the separation, detection, and quantification of phenolic compounds [17] The HPLC system utilizes a pump to enhance the elution of a sample dissolved in a solvent into the column packed with specific packing material (e.g. C18 and amino column). The eluted compounds exit the HPLC column and are detected through a certain detector apparatus (e.g. electrochemical, refractive index, or UV-Vis detectors), which generate signals that correspond to the amount of the eluted analyte. The obtained signals are then transferred, recorded, and analyzed in a readout system like a computer with software connected to the HPLC system capable of data acquisition, storage, and post-run analysis [18].

The HPLC method is a convenient and flexible technique with several advantages including high selectivity, sensitivity, resolution, and precision [19]. The basic principle of HPLC generally revolves around the separation of analytes from a complex matrix based on their solubility, polarity, and interaction between the stationary phase and mobile phase [20]. According to Ignat *et al.* [21], the typical chromatographic conditions of the HPLC analysis of phenolic compounds in plants include the use of a reversed-phase C18 column, UV–Vis diode-array detector (DAD), a low-pressure gradient elution (LPGE) pump, and a binary solvent system containing acidified water (solvent A) and a polar organic solvent (solvent B). By emulating or modifying these chromatographic settings, it is possible to conduct structural, functional, purification, and characterization of various compounds over a short period.

The identification and quantification of phenolic compounds in plants are essential in assessing their different functionalities. However, associated with the growing recognition of these compounds' bioactivities is the increase in the demand for a highly precise, accurate, and validated analytical method for their analysis. Although there have been several approaches to the analysis of phenolic compounds, the simultaneous determination of the different polyphenolic groups in various samples (e.g. plant material) remains a challenge [22]. Moreover, some of these approaches are mainly on the total estimation of polyphenol content and not on the individual analysis or quantification of the compounds. Other related concerns include the absence of a validated analytical method that is necessary for having higher confidence in results [23].

Since no analytical procedures based on the employed method in this research have been reported, this study aimed to develop and validate an HPLC-DAD method for the simultaneous analysis of gallic acid, catechin, epicatechin, rutin hydrate, caffeic acid, syringic acid, ellagic acid, p-coumaric acid, trans-ferulic acid, myricetin, resveratrol, and quercetin. The developed method will be useful in the identification and quantification of phenolic compounds from various samples, particularly plant extracts. This can also serve as a basis for the development of other methods for analysis of similar compounds.

Materials and Methods

Chemicals

The chemicals and standards used in this study were analytical grade and HPLC grade reagents. Phenolic compound standards like gallic acid, catechin, epicatechin, rutin hydrate, caffeic acid, syringic acid, ellagic acid, p-coumaric acid, trans-ferulic acid, myricetin, resveratrol, and quercetin (GA, C, E, RH, CA, SA, EA, pCA, tFA, M, R, and Q) were purchased from Sigma-Aldrich Corporation (Singapore). HPLC

water (H_2O), acetonitrile (ACN), and methanol were acquired from Duksan Pure Chemicals Co., Ltd (South Korea). Sodium hydroxide and glacial acetic acid (HOAc) were obtained from RCl Labscan (Bangkok, Thailand).

Preparation of Phenolic Compound Standards

A standard stock solution (500 µg/mL) for each phenolic compound was prepared by dissolving 2.5 mg of each standard in 5 mL methanol. A mixed standard stock solution with a final concentration of 80 µg/mL for each phenolic compound standard was made by combining and diluting aliquots of the standard stock solutions in methanol. Standard calibration plots were established through serial dilution of the mixed standard stock solution with methanol to obtain 5-7 calibration points that were within the concentration range of 0.05-80 µg/mL. The standard solutions were stored at 4°C, in clean containers protected from light, until use. All standard solutions were filtered using a 0.22 µm polyvinylidene fluoride (PVDF) syringe filter prior to HPLC analysis.

High-Performance Liquid Chromatography (HPLC) System

The HPLC analysis for the determination of phenolic compounds was performed using a Shimadzu Prominence (Tokyo, Japan) chromatographic system equipped with an LC-20 AD pump, DGU-20A5R degasser, LPGE unit, Sil-20AHT UFLC autosampler, and SPD-M20A DAD as detector. The separation of phenolic compounds was carried out using an Inertsil ODS-3 (250 mm x 4.5 mm x 5 µm) reversed-phase column protected with an Inertsil ODS-3 (4.0 mm x 10 mm x 5 µm) guard column. The temperature for the entire chromatographic analysis was controlled using a CTO-10ASVP column oven. Data acquisition, post-run analysis, and peak characterization were performed with LabSolution software (version 6.87 SP1).

Chromatographic Conditions

About 500 µL of the filtered standards was transferred into an HPLC vial and placed in the autosampler for the chromatographic analysis of phenolic compounds. The employed mobile phase consisted of ACN:H_2O (2:98, v/v; Solvent A) and ACN:H_2O (98:2, v/v; Solvent B) both acidified with 1% (HOAc). The chromatographic system followed the specific gradient elution program: 0 min, 10% B; 3 min, 10% B; 6 min, 30% B; 8 min, 30% B; 12 min, 50% B; 17 min; 50% B; 25 min, 90% B; 35 min 10% B; and 40 min, 10% B. A constant flow rate of 0.8 mL/min, injection volume of 20 µL, and column temperature of 30°C was maintained throughout the whole duration of the analysis. A baseline check was also conducted at the beginning of each analysis. The detection and quantification of the phenolic compound were done at their respective absorption maxima (254 nm: RH, CA, EA, M, Q; 275: GA, C, E, SA; 305 nm: pCA, R; 325 nm: tFA).

Peak Identification and Quantification

The post-run analysis such as peak integration and generation of calibration graphs for quantification was done after the HPLC data acquisition of the analytes. The individual chromatogram of each phenolic compound standard was first obtained to determine its specific identity in the generated chromatogram of the mixed phenolic standard solution. The peak characteristics that were acquired from the generated chromatograms include the retention time (RT) and the peak area (PA). Another important parameter that was considered was the absorption maxima of each phenolic compound. In this study, the retention time, and the absorption maxima of each phenolic standard were used for the identification of phenolic compounds, whereas the peak area was utilized for the quantification of phenolic compounds through standard calibration graphs.

Method Validation

The performance characteristics of the developed method were evaluated by using phenolic compound standard solutions. The figure of merits and method parameters that were established to validate the analytical method includes linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and precision [24]. The linearity of the method was assessed by identifying the coefficient of determination (R^2) from the established calibration graph (plot of concentration versus peak area) of the series of phenolic standard solutions (0.05–80 µg/mL). The LOD and LOQ of each phenolic standard compound were derived based on the ratio of the standard deviation of the y-intercepts (SDy) and the slope (m) of the regression line multiplied by 3.3 and 10, respectively. The accuracy was evaluated through the percent recovery of analytes. An unknown matrix was spiked with 5 µg/mL and 10 µg/mL of mixed standard phenolic compound. The percent recovery of each phenolic compound was calculated based on the ratio of the recovered amount and the injected amount multiplied by one hundred. The precision or repeatability of the analytical method was evaluated by determining the relative standard deviation (RSD) of the analytical readings (RT and PA) coming from the replicated (n = 6) analyses of the analytes at three different concentrations (6, 12, and 24 µg/mL) of the mixed phenolic standard solution.

Statistical Analysis

Statistical calculations were mainly performed using Microsoft Excel for Microsoft 365 MSO (Version 2301 Build 16.0.16026.20002) 64-bit.

Results and Discussion

Method Development

Presented in Table 1 is the summary of the selected chromatographic parameters employed in the analysis of phenolic compounds. Like the stationary phase used by Jorjong *et al.* [25], Nour *et al.* [5], and Seal *et al.* [26], the current research also conducted the chromatographic separation of phenolic compounds in a reverse phase C-18 column (250mm x 4.5mm x 5µm, Inertsil ODS-3). Reversed-phase chromatography particularly involves a nonpolar stationary phase and a polar mobile phase. It is the most common HPLC separation technique that is used to easily elute polar substances such as most phenolic compounds [27]. Also, based on the adapted and modified organic solvents from Chen *et al.* [28], Sanches *et al.* [29], and Svedström *et al.* [30], the use of ACN with HOAc as the acid modifier was observed to provide adequate sensitivity and acceptable peak shape of the overall chromatographic results. Acetonitrile is typically selected as the organic component due to its low UV cutoff (190 nm) and lower viscosity which provide better detection performance and narrower chromatographic peaks, respectively [31]. The addition of an acid modifier in the mobile phase is usually done to reduce the degree of adsorption and tailing of basic compounds by keeping the residual silanol in the column packing to be in its undissociated state [32].

Regarding the selection of flow rate, better separation but a longer elution time of the analytes were observed when 0.8 mL/min flow rate was used - lower than the usual HPLC analysis (1.0 - 2.0 mL/min). Moreover, further lowering of the flow rate to 0.6mL/min achieved greater separation but in consequence, resulted in notable peak broadening and significantly extended duration of the analysis. A similar observation was also experienced by Tasioula-Margari and Tsabolatidou [33], wherein they acquired a good analyte separation at a lower flow rate (0.5 ml/min), but also achieved an associated widening of the peaks and prolonged HPLC runtime. Hence, a 0.8 mL/min flow rate was selected in this study as a compromise between separation, and peak widening or extended analysis period.

Table 1. HPLC operating settings for the analysis of phenolic compounds

Settings	Description
Column Temperature	30°C
Injection Volume	20µL
Flow Rate	0.8mL/min
Mobile Phase A	2ACN:98H_2O + 1% HOAc
Mobile Phase B	98ACN:2H_2O + 1% HOAc
Column	RP C-18 Inertsil ODS-3 (250mm x 4.5mm x 5µm)

Gradient Setting		
Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	90	10
3	90	10
6	70	30
8	70	30
12	50	50
17	50	50
25	10	90
35	90	10
40	90	10

Concerning the injection volume, a smaller sample volume (10 µL) introduced to the HPLC system resulted in lower sensitivity, while a larger volume (30 µL) injected into the chromatographic analysis

enhanced band spreading. This observation was also consistent with the findings of Boonen *et al.* [34] They reported that higher injection volumes (25-100 µL) generally improved the sensitivity of the analytes, but also observed an accompanying loss of peak performance. Thus, a 20 µL injection volume was adopted in this research to deal with the issues of sensitivity and band widening linked with injection volumes.

In the selection of column temperature, it was observed that operating the column oven above ambient temperature (T > 25°C) generally provided better chromatographic performance. In a study conducted by Dembek and Bocian [35], it was found that doing the HPLC analysis at an elevated temperature resulted in lower viscosity, faster analysis time, and better peak shape. However, they also emphasized that HPLC analysis at higher temperatures compromises the integrity of the thermally labile compounds and the lifetime of the column. Therefore, an average temperature of 30°C was selected in the current study to balance the positive and negative outcomes associated with analyzing either lower or higher temperatures.

In the initial isocratic run of the analytes, inadequate separation of the eluted compounds was observed resulting in an overall poor chromatographic output. The obtained results confirmed that the broad polarity spectrum of phenolic compounds makes the analysis through the isocratic mode not feasible. Hence, to achieve better HPLC separation, a gradient elution program was developed in this study. Several gradient programs were used as references [21, 22, 36-40] until an adequate chromatographic separation was achieved. During the initial test run, phenolic compounds eluted at the 13-16 minute mark did not properly separate. However, after a few adjustments in the gradient settings near the mid-runtime, a decent separation was obtained. The final gradient setting is summarized in Table 1. In addition, small amounts of the opposite solvent (i.e. ACN in mobile phase A and H2O in mobile phase B) were added respectively to each component of the mobile phase to assist the gradient mixing and avoid problems related to pumps and back pressure [41].

The individual chromatographic characteristics of each phenolic compound standard were initially obtained using the established HPLC method. This was done to determine the specific identity of each standard in the mixed solution. A typical HPLC chromatogram of the phenolic compound standard mixture measured at 254 nm is presented in Figure 1.
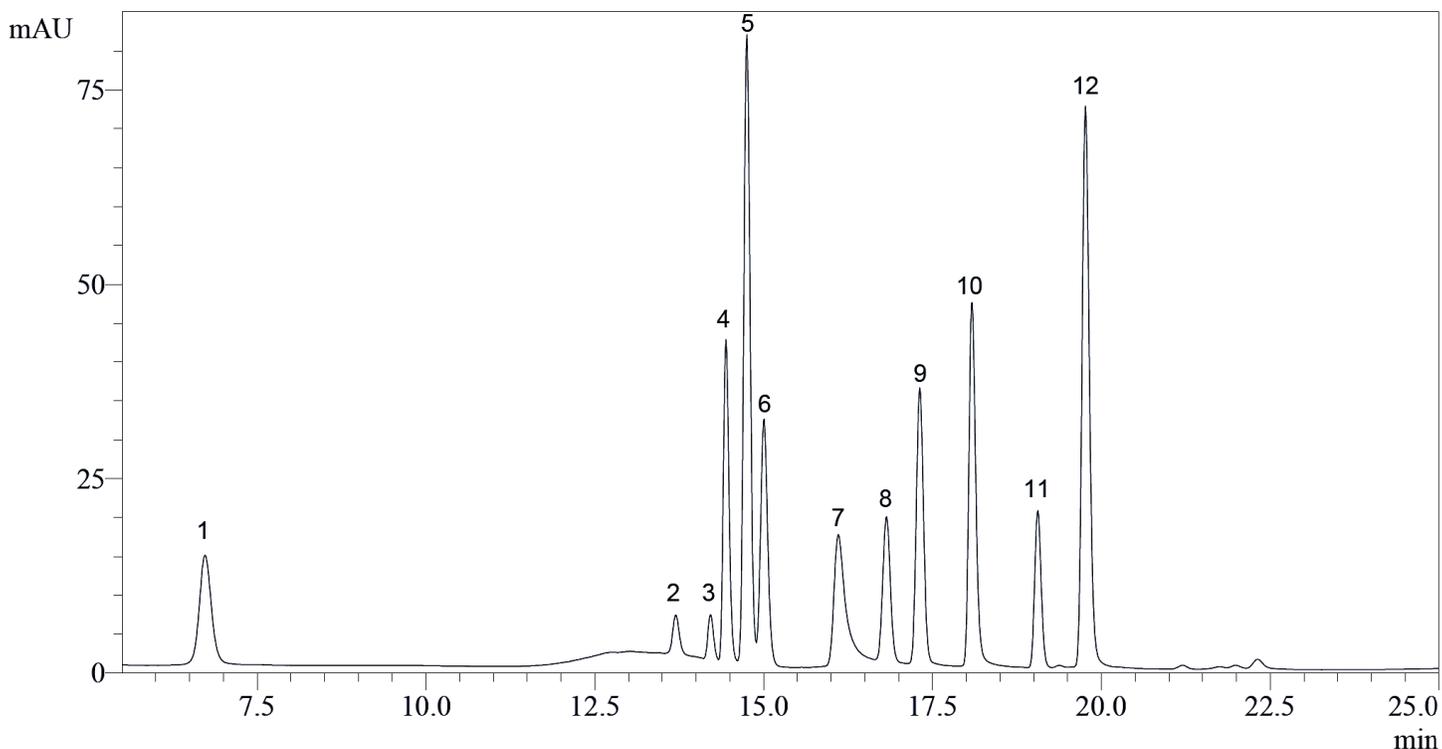

Figure 1. HPLC chromatogram of the standard phenolic compound solution (λ= 254 nm) using the developed HPLC-DAD method

As seen in the chromatogram, the established HPLC method provided adequate separation and appreciable peak characteristics for the analyzed phenolic compound standards. It was also depicted in the figure that individual analyte identification was possible at 254 nm, however, the quantification of each phenolic compound was done at their respective absorption maxima (Table 2). The chromatographic separation of the mixed phenolic standards was possible after 25 minutes, but for the overall analysis, it took 40 minutes to achieve the complete reequilibration of the gradient program back to the initial gradient setting. The developed gradient setting in the current study was generally observed to be more efficient with respect to time and solvent utilization than the method employed by Butkhup and Samappito [36], Jorjong *et al.* [25], Natividade *et al.* [39], and Seal [40].

Method Validation

The performance characteristics of the established HPLC method were validated according to the parameters or figure of merits indicated by Magnusson and Örnemark [24]. The linearity, LOD, LOQ, precision, and accuracy of the established HPLC procedures were evaluated during method validation. Summarized in Table 2 are the results of the method evaluation parameters together with the elution order, retention time, and selected wavelength of the analyzed standard phenolic compounds.

Linearity

Calibration graphs for each phenolic compound standard were created using 5-7 data points ranging from 0.05 µg/mL to 80 µg/mL. Triplicate injections were done for the standard solutions. The retention time and peak area were recorded and evaluated at the corresponding wavelengths of each standard. Calibration curves were made by plotting the selected range of concentration against the obtained peak area. The linearity of the established HPLC method was assessed by acquiring the coefficient of determination (R^2) from the established calibration graph of each standard. Strong linear relationships ($R^2 > 0.99$) for all analyzed phenolic compounds were achieved between the peak area and the concentration range (0.05 µg/mL – 80 µg/mL) of the established standard calibration graph.

Table 2. Peak order, retention time, wavelength of detection, and HPLC method validation parameters (regression, LOD, LOQ, precision, and accuracy) of the developed analytical method

Peak Number	Phenolic Compound	λ (nm)	RT (min)	Regression R²	Regression m	Regression b	LOD (µg/mL)	LOQ (µg/mL)	Precision (Mean %RSD) RT	Precision (Mean %RSD) PA	Accuracy (Mean %Recovery)
1	GA	275	6.563	0.9901	46274	-63355	0.35	1.07	0.71	2.68	98.97
2	C	275	13.685	0.9999	18545	-1412	0.07	0.21	0.09	2.19	101.04
3	E	275	14.180	0.9999	19076	-9796	0.17	0.51	0.09	2.48	98.96
4	RH	254	14.438	0.9999	54111	-3235	0.13	0.39	0.05	2.47	101.12
5	CA	254	14.737	0.9996	119909	-8906	0.01	0.04	0.08	1.08	98.33
6	SA	275	14.994	0.9998	82031	-18728	0.12	0.36	0.05	2.44	98.87
7	EA	254	15.832	0.9945	77220	-91866	0.28	0.86	0.48	1.66	101.11
8	pCA	305	16.787	0.9999	193845	-2842	0.01	0.03	0.13	1.47	99.59
9	tFA	325	17.303	0.9997	139480	-3999	0.03	0.09	0.05	2.45	101.11
10	M	254	18.077	0.9987	56276	-4308	0.03	0.10	0.05	3.09	99.22
11	R	305	19.051	0.9999	192806	-5517	0.02	0.06	0.05	2.36	101.12
12	Q	254	19.691	0.9997	103747	-4357	0.02	0.06	0.20	2.87	99.70

λ – wavelength; R^2 – coefficient of determination; m – slope; b - y-intercept; LOD - limit of detection; LOQ - limit of quantification; RT - retention time; PA - peak area; RSD - relative standard deviation; GA - gallic acid; C - catechin, E – epicatechin; RH - rutin hydrate; CA - caffeic acid; SA - syringic acid; EA - ellagic acid; pCA - p-coumaric acid; tFA - trans-ferulic acid; M – myricetin; R - resveratrol; Q - quercetin.

Linearity is one of the most important validation parameters that must be defined during the establishment of an analytical method [39]. Urbstaite *et al.* [42] demonstrated linearity ($R^2 > 0.99$) over the concentration range of 0.7 to 200 µg/mL for the UPLC analysis of nine phenolic compounds. A linear HPLC method with R^2 values greater than 0.99 at a concentration range of 2 to 50 µg/mL was also attained by Gonçalves *et al.* [43] for the analysis of nine polyphenols. Moreover, good linearity with R^2 values exceeding 0.99 in the concentration range of 0.02 to 0.24 µg/mL was obtained by Sellappan *et al.* [44] for the analysis of eleven phenolic compounds.

LOD and LOQ

The LOD and LOQ values of each standard compound were derived by using the information generated from the previous linear regression analysis. In this study, LOD and LOQ values were obtained by multiplying the ratio of the standard deviation of the y-intercepts and the slope of the regression line by 3.3 and 10, respectively. The respective LOD and LOQ values for the analyzed phenolic compounds range from 0.01 to 0.35 µg/mL and 0.03 to 1.07 µg/mL. The acquired LOD and LOQ values, which are less than or near 1.00 µg/mL, indicated the capability of the established method to analyze phenolic compounds at low concentration levels.

The determination of LOD and LOQ values is crucial because it is associated with the establishment of valid concentration limits for the detection and quantification of analytes analyzed in the developed analytical method [45]. In general, the established LOD and LOQ values in the current study were lower than the respective values obtained by Nour *et al.* [5] - 0.030 to 0.905 µg/mL; 0.095 to 3.019 µg/mL, Park *et al.* [46] - 0.100 to 0.500 µg/mL; 0.500 to 2.000 µg/mL, and Urbstaite *et al.* [42] - 0.380 µg/mL to 1.010 µg/mL; 0.540 µg/mL to 3.060 µg/mL.

Accuracy

The accuracy of the developed chromatographic method was evaluated by determining the percent recovery of the analytes in a given matrix. Two different mixed standard concentrations (5 µg/mL and 10 µg/mL) were tested in the current study. The obtained test results revealed mean percent recovery values that are within the 100 ± 5% range (98.33 - 101.12%). This demonstrated the consistency of the results regarding the employed measures in the established analytical method.

The measure of accuracy as recovery pertains to the amount of the analyte of known concentration retrieved after its incorporation into a given matrix [47]. Assessment of accuracy through the recovery method by several studies also attained values that are within the 100 ± 5 % range. Ciric *et al.* [48], Tang *et al.* [49], and Seal *et al.* [26] achieved percent recovery values ranging from 98.57 –100.27%, 95.00% - 104%, and 98.13 - 99.20%, respectively.

Precision

The precision of the established HPLC method was evaluated by measuring the repeatability of the analytical readings (RT and PA) coming from the replicate (6) analyses of analytes at three different mixed phenolic standard concentrations (6, 12, and 24 µg/mL). The test results were expressed as percent relative standard deviation (RSD). The obtained mean RSD for the repeatability test of RT and PA ranges from 0.05 to 0.71% and 1.08 to 3.09%, respectively. According to Waters Corporation [50], there is a direct relationship between RT and PA in terms of precision. They indicated that as the repeatability of RT worsens, the PA is likely to have high variability. In this regard, the low variability observed in PA can be associated with the good precision of RT obtained in this study. Overall, the low obtained RSD values (RSD < 5%) of the developed method implied the non-variability of the data with respect to the tested analytical readings.

Based on Al-Rimawi and Odeh [51], the measure of precision as repeatability is done to assess the degree of proximity between the data values tested within a short interval period and obtained using the same analytical method, similar sample, the same laboratory and equipment, and similar analyst. In a study conducted by Luaces *et al.* [52], the percent RSD values they obtained (0.017 - 3.83%) which were also less than 5% confirmed the precision of their established chromatographic method for the analysis of four phenolic compounds. In addition, Park *et al.* [46] obtained satisfactory RSD values of less than 5% (0.036 - 3.91%) which validated the precision of their HPLC method for the quantitative analysis of four polyphenols.

In general, the obtained figure of merits for the present analytical technique indicated a linear, sensitive, accurate, and precise chromatographic method for the simultaneous HPLC-DAD analysis of the selected phenolic compounds.

Conclusions

The study on phenolic compounds has been gaining much interest due to their multiple positive health benefits to humans. However, the growing popularity of these natural compounds demands a comprehensive and validated analytical technique for their analysis. In this study, a simple and reliable HPLC-DAD method was developed for the simultaneous analysis of gallic acid, catechin, epicatechin, rutin hydrate, caffeic acid, syringic acid, ellagic acid, p-coumaric acid, trans-ferulic acid, myricetin, resveratrol, and quercetin. Validation studies revealed that the established chromatographic method is linear ($R^2 > 0.99$), sensitive (LOD: 0.01 - 0.035 µg/mL; LOQ: 0.03 - 1.07 µg/mL), accurate (98.33 - 101.12%), and precise (RSD < 5%) for the assessment of the 12 selected phenolic compounds. Thus, the current HPLC-DAD method can be employed for the identification and quantification of specific polyphenols in various plant extracts. Furthermore, it is suggested to conduct additional stability testing to enhance the reliability of the desired results.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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