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ORIGINAL ARTICLE

Method Development on Reverse-Phase High-Performance Liquid Chromatography for the Improvement of Routine Analysis of Flavonoids on Agricultural and Food Products

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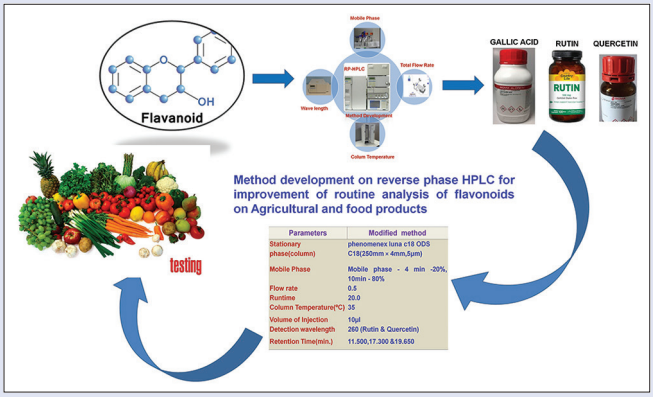
ABSTRACT

Objectives: The objective of this work was to investigate the effect of reversed-phase high-performance liquid chromatography conditions for the determination of flavonoids in the plant extract. **Methods:** The analytical conditions (total flow rate, mobile phase, colum temperature, and detection wavelength) optimized for gallic acid, rutin, and quercetin. The optimized method was compared with the control method and evaluated with the use of reference compounds. **Results:** The result shows that the maximum flavonoids recovery was in observed on modified chromatographic conditions, Colum: Octadecyl-silica C_{18} column (250 mm × 4 mm, 5 µm) Mobile Phase solution water-acetic acid (25:1) and methanol. Mobile Phase solution B was a Mobile phase – 4th min - 20%, 10th min - 80%, and a flow rate of 0.5 mL/min. Colum temperature 35°C and detection wavelength was 260 nm. **Conclusion:** In this work, the proposed method will increase the analysis time but minimize the mobile solvents and have the potential of being useful for the routine analysis of flavonoids.

Key words: Agriculture and food products, flavonoids, flow rate, gallic acid, high-performance liquid chromatography, method development

SUMMARY

- The current work has been performed to study the effect of chromatographic conditions for the estimation of flavonoids in the plant extract. The ultra-high-performance liquid chromatography conditions such as flow rate, mobile phase, and colum temperature exposure wavelength was optimized for the easy identification of flavonoids (gallic acid, rutin, and quercetin). The modified conditions were compared with the reference method and evaluated. The highest recovery of flavonoids observed in optimized chromatographic conditions and this method will minimize the utilization of high-performance liquid chromatography grade solvents and have the possibility of being useful for the regular study of phenolic compounds.



Abbreviations used: RP-HPLC: Reverse-Phase High-performance liquid chromatography; ODS: Octadecyl-silica; GA: Gallic acid; RU: Rutin, QU: Quercetin; C: Control method; M: Modified method.

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INTRODUCTION

High-performance liquid chromatography (HPLC) has been the most widely used chromatographic technique in the flavonoid analysis. It has added a new aspect to the examination of flavonoids in food and plant extracts and gives high resolution and sensitivity.[1-5] Gallic acid, rutin, and quercetin are phenolic compounds, and they have been proved to have potential preventive and beneficial effects in many diseases, where the oxidative stress has been concerned, as well as cardiovascular diseases, cancer, and neurodegenerative disorders.[6-10] The reverse phase HPLC with UV detector is a significant investigative method with well-built chromophores that attract light in the wavelength area from 200 to 800 nm.[11] The aim of the present work was to investigate the effect of reversed-phase (RP) HPLC conditions for the determination of flavonoids and improve the HPLC conditions for increasing the recovery. In this article described to develop and validate a liquid chromatographic method that can be used for the usual analysis of gallic acid, rutin, and quercetin on agricultural and food products.

MATERIALS AND METHODS

Instrumentation

HPLC system consisted of a Shimadzu Corporation (Make), Japan, LC-10ATvp (Model) connected to SPD-10A vp UV detector with dual wavelength and column oven was used in this study. The LC solution classvp software was used for the data analysis and extracts the

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chromatograms. A Phenomenex Luna C_{18} column (250 mm·4.6 mm id, 5 lm,) was used to perform the separation, with a C_{18} Phenomenex, 4 3.0 mm Id, security guard column. The mobile phase was filtered through the millipore filter assembly attached with a vacuum pump. The mobile phase was sonicated with ultrasonic cleaner. The gradient elution of mobile phase a (water-acetic acid [25:1 v/v]) and solvent B (methanol) with gradient proportions, solvent B was improved to 50% in 4 min, and then increased to 80% in 10 min at a flow rate of 1.0 mL/min. The detection wavelength was 280 nm.[12,13]

Modification of reverse phase high-performance liquid chromatography conditions

In this research, the packed HPLC C_{18} column (octadecyl silica [ODS]) was used for flavonoids purification. In arrange to avoid pressure increasing of column, acetonitrile was used as inorganic reagent because of its low viscosity. The flow rate, column temperature, mobile phase variations, and wavelength take part in an important responsibility in the reverse-phase liquid chromatographic method given in Table 1. In this investigation, these experiments were standardizing for the determination of flavonoids.

Method validation

The chromatographic condition was validated using the ICH guidelines[14] Q2(R1) with linearity, precision, accuracy, detection limit, and quantification limit.

RESULTS AND DISCUSSION

In this study, different HPLC conditions were investigated, and the modified analytical technique was developed to RP HPLC with UV detector, including detection wavelength, mobile phase, total flow rate, and colum temperature. The chromatographic conditions were optimized on the Phenomenex ODS C_{18} column (250 mm × 4 mm,) 5 µm column. The optimized method is summarized on Table 2, and validation and system suitability studies results are given in Table 3.

Effect the Flow rate on retention time

The total flow rate of the mobile phase has an important effect on retention time and peak area and little effect on separation for gallic acid, rutin, and quercetin. The result shows the gradient scaling of flow rates from 0.5 to 1.50 mL/min using RP-HPLC and well-resolved peaks were observed for GA, RU, and QU. The elution order and the retention times for control method gallic acid, rutin, and quercetin were 35.742, 10.525, and 12.41, and modified method, gallic acid, rutin, and quercetin were 11.500, 17.300, and 19.650. The 0.5 ml/min flow rate produced high peak area percent gallic acid (76.06%) compares to control flow rate 1.0 ml/min [Figures 1a and 2].

The flow rate may influence the detection sensitivity of flavonoids**.** The increasing of flow rates and volumes of solvent also increased by Beer's law, adsorption decreases. Low flow rates lead to the usage of small volumes of solvent and adsorption and sensitivity increase. The major reason that narrow-bore HPLC columns enhance detection sensitivity is because they are run at low flow rates and flavonoids are eluted in small volumes of solvent.

Effect the colum temperature

The result shows gradient scaling of colum temperature from 25°C to 45°C with reverse phase C_{18} colum (250–4.6 mm id, 5 lm) and well-resolved peaks were observed for GA, RU, and QU. The separation of flavonoids on the reverse-phase chromatographic conditions and linearity of column temperature versus peak area were obtained in the temperature range of 25°C–45°C. The colum oven temperature 35°C was produced high-peak area percent gallic acid (74.45%), rutin (25.54%), and quercetin (70.41%) compare to control colum temperature 40°C, gallic acid (53.60%), rutin (17.23%), and quercetin (29.16%). Previous literature reported that the optimum temperature is 35°C for the extraction of flavonoids.[15] The chromatogram efficiency reached a maximum at the temperatures in 35°C [Figure 1b]. This maximum of peak ability shows that the decrease in peak area was smaller than the decline in peak width at the short temperature range.

Variations in the mobile phase

One important factor which influences on selectivity is mobile phase compositions. The exact mobile phase compositions will give suitable polarity with respected compounds separated. The result shows that the mobile phase concentrations of control and modified methods no variations and quercetin shows 29.16 percent of high peak area. Increasing the methanol composition can improve the resolution

Table 1: Experimental design

Factors	Wavelength (nm)	Flow rate (mL/min)	Concentration of mobile phase	Colum temperature
Effect of colum temperature	260	0.5	Mobile phase - 4 min -20%, 10 min - 80%	25°C, 30°C, 35°C, 40°C, 45°C
Effect of flow rate (ml/min)	260	0.5, 0.75, 1.0, 1.25, 1.50	Mobile phase - 4 min -20%, 10 min - 80%	35°C
Effect of concentration of the mobile phase on separation	260	0.5	4 min (20%)-10 min (80%), 4 min (40%)-10 min (80%) 4 min (30%)-10 min (80%), 4 min (600%)-10 min (80%)	35°C
Effect of wavelength of detection (nm)	260, 270, 280, 290, 300	0.5	Mobile phase - 4 min -20%, 10 min - 80%	35°C

Table 2: Optimized chromatographic conditions

Parameters	Control method[12]	Modified method
Stationary phase (column)	Phenomenex luna C_{18} ODS C_{18} (250 mm × 4 mm, 5 µm)	Phenomenex luna C_{18} ODS C_{18} (250 mm × 4 mm, 5 µm)
Mobile phase	Mobile phase - 4 min -50%, 10 min - 80%	Mobile phase - 4 min -20%, 10 min - 80%
Flow rate	1.0	0.5
Runtime	13.0	20.0
Column temperature (°C)	40	35
Volume of injection	10 µl	10 µl
Detection wavelength	280	260 (rutin and quercetin)
Retention time (min)	5.742, 10.525, and 12.41	11.500, 17.300, and 19.650

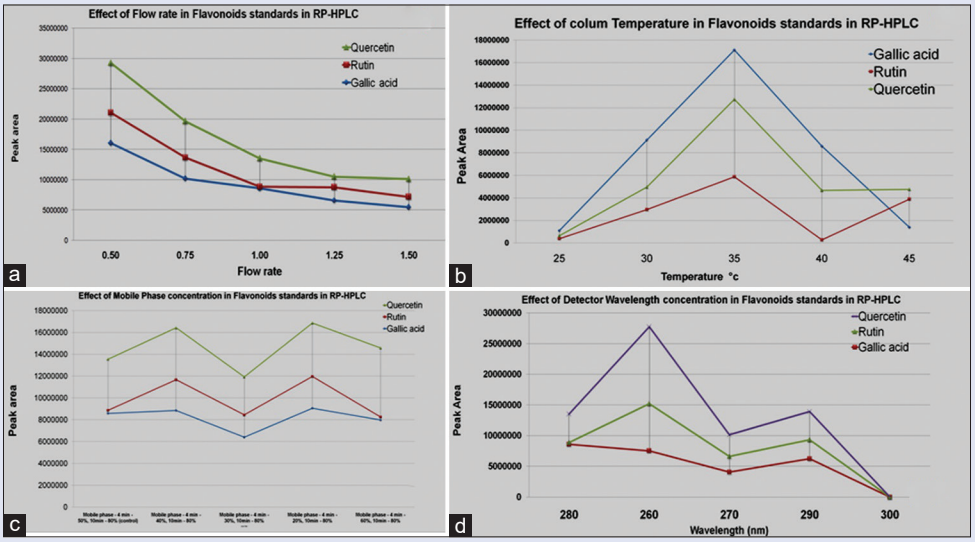

Figure 1: (a) Effect of flow rate (b) Effect of colum temperature (c) Effect of Mobile phase solvent proportions (d) Effect of Detection wavelenth for investigation of flavonoids

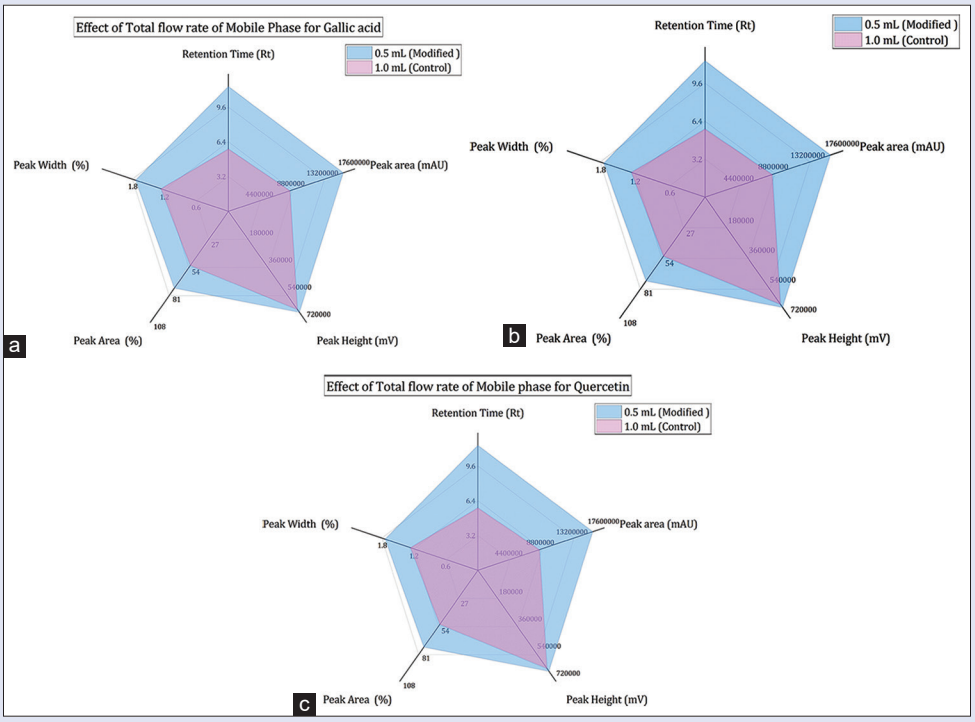

Figure 2: (a) Effect of total flow rate of the mobile phase on reversed-phase high-performance liquid chromatography conditions for gallic acid. (b) Effect of total flow rate of the mobile phase on reversed-phase high-performance liquid chromatography conditions for rutin. (c) Effect of total flow rate of the mobile phase on reversed-phase high-performance liquid chromatography conditions for quercetin

for the determination of flavonoids.[16] Polarity and strength elution of mobile phase were more appropriate at the methanol level <80% [Figure 1c].

Variations in detection wavelength mobile phase concentration at 0.5 ml flow rate and 35°C

The wavelength of the HPLC UV detector has an important effect on the detection of peak area for gallic acid, rutin, and quercetin. The result shows the level of detection wavelength from 260 nm to 300 nm using the UV detector RP-HPLC-and highest peaks area were observed for quercetin (45.08%) and rutin (27.52%) compare to control detection wavelength [Figure 1d].

Calibration details of the improved method

The calibration curve was prepared by plotting the peak area using a modified method [Tables 2 and 3] and was linear in the range of 5–50 mg/Kg. The observed data were focused to linear regression analysis to calculate the calibration equation and correlation coefficients.

The regression equation was found as gallic acid (r^2 = 0.9915, n = 5), rutin (r^2 = 0.9847, n = 5), and quercetin (r^2 = 0.9971, n = 5). The goodness-of-fit (r^2) was found to be 0.99, representing a linear connection between the concentration and peak area of the flavonoids. The HPLC standard chromatogram and validation data of gallic acid, rutin, and quercetin are presented in Table 4.

Table 3: Validation and system suitability studies on modified conditions

Parameter	Gallic acid	Rutin	Quercetin
Linearity (mg/kg)	5-50	5-50	5-50
Correlation coefficient (r^2)	0.9915	0.9847	0.9971
RSD (%)	0.589	0.565	0.517
LOD (mg/kg)	0.054	0.063	0.051
LOQ (mg/kg)	0.5-1.00	0.5-1.00	0.5-1.00
Rt	11.500	17.300	19.650
Robustness	Robust	Robust	Robust

Rt: Retention time

Standard accuracy of the method

The different concentration (2–100 mg/kg) mixed flavonoids of certified reference materials was analyzed using optimized HPLC conditions. The standard accuracy percentage calculated by compared with injected standard value and obtained instrument obtained values. The results show that the injected standard concentration, instrument reading value, and recovery percentage given in Figure 4.

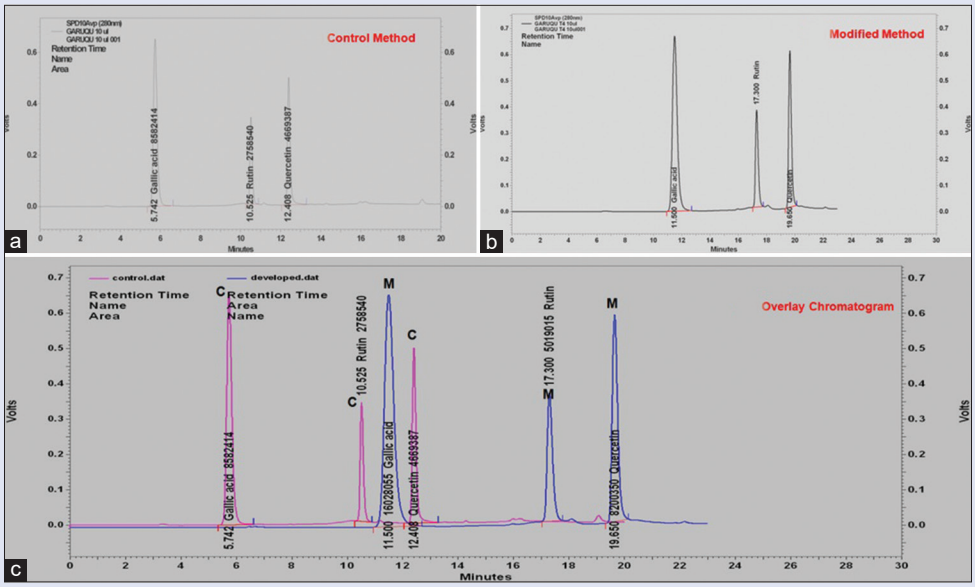

Figure 3: (a) control method (b)modified method (c) overlay HPLC chromatogram of flavonoid standards

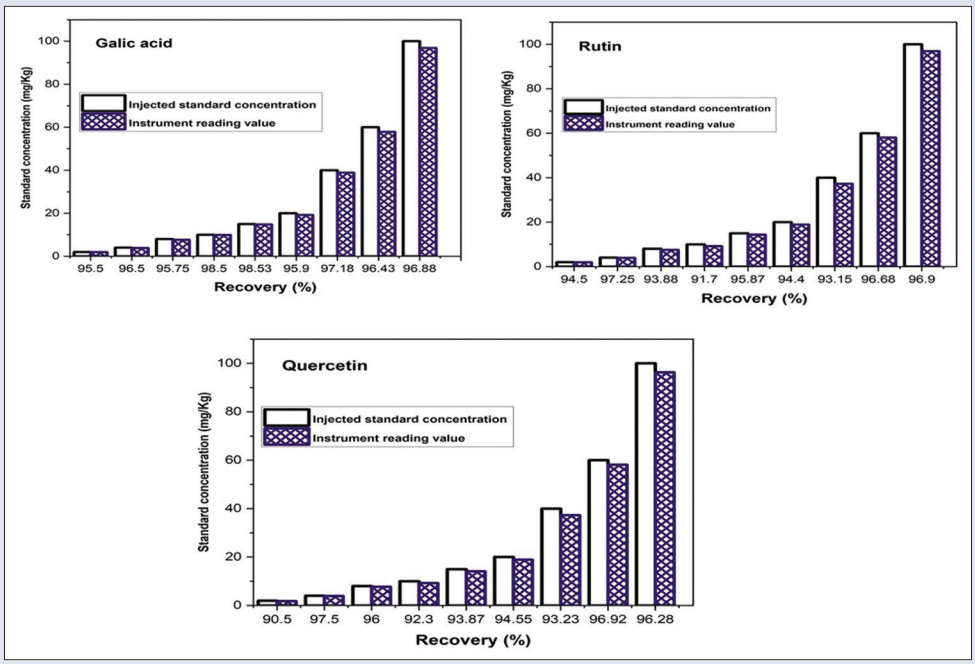

Figure 4: (a) Gallic acid (b) Rutin (c) Quercetin Standard accuracy percentage of certified reference materials of flavonoids in the optimized method

Table 4: Validation data for control and modified method of flavonoids standard

Flavonoids	Retention time		Standard peak area		Standard peak height		Injected value (mg/kg)		Instrument obtained value (mg/kg)	
	C	M	C	M	C	M	C	M	C	M
Gallic acid	5.742	11.5	2758540	16028055	650869	669342	5	5	4.610	4.980
Rutin	10.525	17.3	4669387	5019015	336553	370354	5	5	4.430	4.690
Quercetin	12.408	19.65	8582414	8200350	496081	597660	5	5	4.712	4.910

C: Control method, M-Modified method. Values represent mean±SD, *n*=6. SD: Standard deviation

Table 5: Repeatability studies

Number of individual injections	Flavonoids standard					
	Gallic acid		Rutin		Quercetin	
	Rt	Peak area	Rt	Peak area	Rt	Peak area
1	11.500	16428055	17.300	5019015	19.650	8200350
2	11.415	16434764	17.347	5018946	19.601	8210432
3	11.493	16434871	17.291	5020147	19.598	8212567
4	11.520	16425278	17.314	5018945	19.661	8207894
5	11.494	16447984	17.322	5012471	19.627	8209679
6	11.587	16433950	17.375	5014987	19.587	8210258
7	11.697	16443521	17.415	5021243	19.587	8220543
8	11.604	16444358	17.078	5013765	19.874	8225697
9	11.514	16496745	17.284	5015478	19.478	8216749
Average	11.5360	16443281	17.3029	5017222	19.6292	8212685
STDEV	0.0817	21412.64	0.0944	3091.105	0.1058	7438.906
%RSD	0.7085	0.130221	0.5456	0.06161	0.5390	0.090578

RSD: Relative standard deviation; Rt: Retention time; STDEV: Standard deviation

Table 6: Recovery percentage of flavonoids in plant extracts samples on optimized method

Flavonoids	Replicates	Spiked concentration (mg/kg)	Control plant extract sample (mg/kg)	Spiked plant extract sample (mg/kg)	Percentage of recovery	Percentage of RSD
Gallic acid	6	10	4.126	15.542	114.16	1.41
Rutin	6	10	2.698	13.98	112.82	1.39
Quercetin	6	10	8.289	17.86	95.71	1.46

RSD: Relative standard deviation; Rt: Retention time

The accuracy of an optimized HPLC conditions was expressed the nearness of obtained outcome by modified RP-HPLC conditions to the accurate value. The repeatability results produced good RDS values in the range of 0.06 %–0.70% as given in Table 6. The standard accuracy results showed in the range of 95.71%–114.16% and percentage of RDS values were in the range of 1.39%–1.46%, as given in Table 6. The results exhibits that the recovery percentage and RSD% were within the accepted limits, and it indicates the applicability of the method for phytochemical evaluation in plants.

CONCLUSION

The importance of the work is the improvement the HPLC conditions designed for the simultaneous analysis of flavonoids in plant extracts, and it will be applied for short-type HPLC column. The mobile solvent economy uniqueness of HPLC method is very advantageous, compared to the most widely used control HPLC technique. The modified reverse phase HPLC method was competent in quantifying phytochemicals in commercial agriculture and food products.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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