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Synchronised determination of chlorogenic acid and five flavonoids in mulberry leaves using HPLC with photodiode array detection

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RESEARCH ARTICLE

Abstract

An analytical method based on high-performance liquid chromatography separation with diode array detection was developed and validated for the synchronised determination of chlorogenic acid and five flavonoids (rutin, isoquercitrin, quercitrin, quercetin, and luteolin) in mulberry leaves (*Morus* spp.). Chromatographic separation was carried out under gradient elution conditions on a Shim Pack VP-ODS C18 (250 × 4.6 mm, particle size 5 μm) column at a temperature of 30 °C using a mobile phase consisting of 0.5% (v/v) phosphoric acid in water and acetonitrile at a flow rate of 0.9 ml/min. The column eluent process was best at 330 nm (for chlorogenic acid, quercitrin, and luteolin), 350 nm (for rutin and isoquercitrin), and 365 nm (for quercetin). Application of optimum extraction conditions led to extraction of chlorogenic acid and five flavonoids from mulberry leaves with mean recoveries of 97.78-103.24%. The developed method was validated in terms of linearity, recovery, precision, and stability. The relative standard deviation for intra-day precision (n=6) and inter-day precision (n=6) was <1.45%. The optimised protocol provides a simple, sensitive and reproducible method for quantitative analysis of chlorogenic acid and flavonoids from mulberry leaves and may further be explored for other natural products.

Keywords: flavonoids, chlorogenic acid, HPLC-PDA, mulberry leaves

1. Introduction

The mulberry (*Morus* spp.) (Gryn-Rynko *et al.*, 2016; Hu *et al.*, 2013) is extensively and widely planted throughout the warm temperate world for several benefits. It is distributed throughout Asia, Africa and Latin America. China is the primary distribution centre of the world for mulberry natural products (Hu *et al.*, 2017; Zhou *et al.*, 2014). Mulberry is not only used as edible cooked food, but its leaves, roots and bark have also long been used in Chinese medicine. The Chinese Ministry of Public Health has identified mulberry as an edible food with medicinal benefits (Chan *et al.*, 2016; Wu *et al.*, 2015).

A group of flavonoids including quercetin, kaempferol, isoquercitrin, morin hydrate, astragalin, morusin and rutin, are thought to be the main active constituents in mulberry (Gryn-Rynko *et al.*, 2016; Sugiyama *et al.*, 2016). It has been reported that mulberry leaves contain flavonoids, which can be used to treat tearing eyes, dizziness, fever, sore throat, cough, skin ailments, neurosis and thirst as well as reduce blood glucose levels and hypertension (Chan *et al.*, 2016; Gryn-Rynko *et al.*, 2016; Wilson and Islam, 2015; Zou *et al.*, 2014). It has also been reported to prevent the development of leukaemia, colon and breast cancer (Deepa *et al.*, 2012; 2013; Fallah *et al.*, 2016; Naowaratwattana *et al.*, 2010; Zhang *et al.*, 2018). These flavonoids have a great tendency to be absorbed in the body through the small intestine with no negative side effects.

Conventional methods, such as ultraviolet-visible spectroscopy, high-performance liquid chromatography

(HPLC), mass-spectrometry (MS) and fluorescence spectroscopy have been used for the determination of flavonoids from different materials (He *et al.*, 2013; Sanchez-Salcedo *et al.*, 2016; Suntornsuk *et al.*, 2003). These methods have made a significant contribution in the qualitative as well as quantitative assessment of constituents for quality control of herbs. As the content of various substances in mulberry leaves from different habitats vary, it is therefore necessary to establish a valid method for quality control of multiple active components that may help to evaluate the quality of the mulberry leaves for various substances in a single procedure. Our present study is focused on exploring and establishing an HPLC method using a photodiode array detector (PDA) for the simultaneous separation, identification and determination of chlorogenic acid as well as flavonoids in mulberry leaves.

2. Materials and methods

Material

Mulberry leaves (*Morus* spp.) in different growth districts were used in this study, which were collected from the Anhui, Zhejiang and Liaoning provinces of China. The dried mulberry leaves were crushed in an electric grinder (Xuzhong Food Machinery Co., Guangzhou, China), and then the powder was passed through a 100 mesh sieve. The milled mulberry leaf powder was stored under cool, dry conditions.

Reagents and chemicals

For standardisation, reference compounds including chlorogenic acid (PubChem CID: 1794427), rutin (PubChem CID: 5280805), isoquercitrin (PubChem CID: 5280804), quercitrin (PubChem CID: 5280459), quercetin (PubChem CID: 5280343), and luteolin (PubChem CID: 5280445) were purchased from Qiushi Bio. Sci. and Tec. Co. Ltd. (Nanjing, China). The purity of each reference compound was determined to be above 98% by HPLC analysis. HPLC grade acetonitrile and methanol were purchased from Fisher Chemical (Waltham, CT, USA). Deionised water was used for sample preparations, and the mobile phase water was provided by a Milli-Q Academic ultrapure water system (Millipore, Bedford, MA, USA). All other chemicals and solvents were of analytical grade.

Sample preparation

The standard stock solutions (approximately 1.0 mg/ml) of chlorogenic acid and flavonoids were prepared using methanol as a solvent and stored at 4 °C in the dark. Combined standard solutions containing the six reference compounds were prepared by dissolving accurately weighed portions of the standards in methanol at a stock concentration of 0.443 mg/ml for chlorogenic acid, 0.251 mg/ml for rutin, 0.302 mg/ml for isoquercitrin, 0.330 mg/ml for quercitrin, 0.288 mg/ml for quercetin, and 0.405 mg/ml for luteolin. The mixed standard solution containing all the compounds was serially diluted with HPLC grade methanol to obtain the working standard solution.

A total of 1.0 g milled mulberry leaf powder was accurately obtained and then extraction was done by using conventional heating reflux for 2 h with 25 ml pure methanol. After cooling at room temperature, the supernatant was filtered through a 0.45 µm microporous membrane into HPLC vials for future testing. Twenty microliters of the sample solution was injected for analysis.

HPLC-PDA analysis

Chromatography was performed using a Shimadzu Technologies LC series (Kyoto, Japan) system consisting of a quaternary pump, a thermostatted column compartment, a vacuum degasser, a Rheodyne injection valve with 20 µl loop, and a PDA. Chromatograms were recorded and evaluated by the station software, LC solution Lite system (Zhai *et al.*, 2014). The analyses were carried out on a Shim Pack VP-ODS C18 column (250 × 4.6 mm i.d., 5 µm; Shimadzu Technologies). The mobile phase was composed of different proportions of (A) acetonitrile and (B) 0.5% phosphoric acid in water (acidified water). The initial mobile phase composition was 80% B and 20% A, followed by a linear gradient to 70% B and 30% A in 10 min; 10-25 min, from 70 to 78% B and 30 to 22% A; 25-30 min, from 78 to 80% B and 22 to 20% A. The post-running time was 35 min. The flow rate was set at 0.9 ml/min, the column temperature was kept at 30 °C, and the sample injection volume was 20 µl. The PDA acquisitions were performed in the range 190-450 nm and the chromatograms were integrated at 330 nm (for chlorogenic acid, quercitrin, and luteolin), 350 nm (for rutin and isoquercitrin), and 365 nm (for quercetin). Three injections were done for each sample. The identity of each peak in mulberry leaf samples was confirmed by comparison of retention time and ultraviolet spectrum of each peak with that of reference compounds (Li *et al.*, 2017).

Linearity

A stock solution of six reference compounds was prepared with methanol and diluted to a series of appropriate concentrations for the construction of calibration curves. Linear regression analysis was used to calculate the intercept, slope, and the correlation coefficient of each calibration line. The calculations for limit of detection (LODs) and quantitation (LOQs) were based on the standard deviation of 'y' intercepts of regression lines (Sxy) and the slope (α), using the equations LOD = 3.3Sxy/α and LOQ = 10Sxy/α. All six compounds showed good linearity (R>0.999) in a relatively wide concentration range, which was adequate

for the analytical method. Calibration data for the standard curves of the six compounds are given in Table 1.

Recovery

The accuracy of the HPLC method was tested by using the recovery studies. The recovery was determined by spiking certain amounts of each of the six compounds with three different concentrations (10, 20, and 40 µg/ml). After HPLC analysis, the recovery of each standard was determined based on the ratio of the standard concentration after HPLC (spiked amount minus original amount) and before HPLC (spiked amount):

$$\text{Recovery (\%)} = \left(\frac{\text{spiked amount} - \text{original amount}}{\text{spiked amount}} \right) \times 100\%$$

The recovery study findings of the six compounds ranged from 97.78 to 103.24%, and the relative standard deviation (RSD) for this technique was lower than 2.4%. Results are reported in Table 2.

Precision

Intra- and inter-day variations were utilised to determine the precision of the developed assay by analysing samples with six concentrations of chlorogenic acid, rutin, isoquercitrin, quercitrin, quercetin and luteolin. The intra-day variation was determined by analysing the six replicates on the same day and inter-day variation was determined in four consecutive days. Precision was expressed as the percentage of the coefficient of variation. As the results show in Table 3, RSD values were all ≤1.45%.

Stability

For the stability studies, the same real sample was analysed after storage at room temperature for 2, 6, 12, 24, 32, and 48 h. The solution was stable when both RSD values of retention time and peak area were lower than 2.0%.

Table 1. Linear regression data, limit of quantitation (LOQ) and limit of detection (LOD) for the six compounds.[1]

Standards	Linear range (µg/ml)	Regression equation[2]	r[3]	LOQ (µg/ml)	LOD (µg/ml)
Chlorogenic acid	22.15-443.00	$Y = 5.4 \times 10^6X - 8{,}359.090$	0.9990	0.93	3.07
Rutin	12.55-251.00	$Y = 4.1 \times 10^7X - 35{,}088.010$	0.9992	0.67	2.21
Isoquercitrin	27.30-169.00	$Y = 4.2 \times 10^7X + 23.044$	0.9997	0.45	1.48
Quercitrin	3.30-166.00	$Y = 2.6 \times 10^7X + 1{,}818.016$	0.9991	0.92	3.21
Quercetin	1.44-28.80	$Y = 2.0 \times 10^6X + 60.118$	0.9992	0.21	0.71
Luteolin	0.20-4.05	$Y = 1.0 \times 10^8X - 72.061$	0.9994	0.16	0.51

[1] Six data points (n=3).
[2] X = the concentration of the compounds (µg/ml); Y = peak area.
[3] r = relevance.

Table 2. Recovery/accuracy of the six compounds (n=3).

Analytes	10 µg/ml		20 µg/ml		40 µg/ml		Mean	
	Recovery (%)	RSD[1] (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Chlorogenic acid	101.32	1.07	98.96	1.31	98.54	1.51	99.61	1.30
Rutin	97.84	2.03	98.15	1.91	102.43	1.32	99.47	1.75
Isoquercitrin	99.24	0.84	101.94	1.21	98.72	1.13	99.97	1.06
Quercitrin	100.43	1.87	98.62	1.47	97.78	1.62	98.94	1.65
Quercetin	103.24	1.24	101.82	1.33	101.43	1.27	102.16	1.28
Luteolin	102.21	1.63	98.43	2.31	100.92	0.96	100.52	1.63

[1] RSD = relative standard deviation.

Table 3. Intra-day and inter-day precision of the HPLC method.[1]

Analytes	Intra-day (n=6)				Inter-day (n=6)			
	RT (min)		Response (AU)		RT (min)		Response (AU)	
	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)
Chlorogenic acid	3.45	0.86	135.1	0.94	3.42	0.92	134.6	0.87
Rutin	11.19	1.02	89.2	0.97	11.15	1.10	89.6	1.07
Isoquercitrin	27.34	1.17	5.4	1.25	27.36	1.40	6.0	1.30
Quercitrin	12.30	0.97	351.2	1.05	12.26	1.02	352.3	1.05
Quercetin	25.83	1.20	9.8	1.23	25.83	1.37	9.5	1.20
Luteolin	14.64	0.93	132.0	1.12	14.63	0.95	132.4	0.89

[1] RT = retention time; RSD = relative standard deviation.

3. Results and discussion

Optimisation of sample pre-treatment

In order to obtain an efficient extraction of chlorogenic acid and flavonoids from mulberry leaves, multiple related extraction conditions were optimised as described (Jeong *et al.*, 2015; Wang *et al.*, 2014), which involved the following factors and corresponding levels: extraction method (reflux, ultrasonication or soxhlet); extraction solvent (ethanol and methanol); solvent concentration (50, 75 or 100%); solid-liquid ratio (1:10, 1:15 or 1:25); extraction time (30 min, 40 min, 60 min or 2 h); extraction temperature (60, 66 or 70 °C); and powder of particle size (pass over 80 mesh sieve, 100 mesh sieve or 120 mesh sieve).

The results showed that refluxing was better than ultrasonic extraction, so refluxing was used in further experiments. Pure and aqueous ethanolic and methanolic solutions were tested as the extraction solvent, and the best solvent was found to be pure methanol, which gave optimum extraction of all the six components. The amounts of chlorogenic acid and flavonoids showed no significant difference with the solid-liquid ratio, while excessive pigmentation was likely to cause column damage. Considering these observations, solid-liquid ratio was chosen as 1:25. To determine the extraction time, 1.0 g milled mulberry leaf powder was extracted with 25 ml pure methanol by refluxing for 30 min, 40 min, 60 min and 2 h, respectively. With the extension of extraction time, the extraction yield of six components may have been increased; therefore, a period of 2 h was chosen as the optimal extraction time. To investigate extraction temperature, 1.0 g milled mulberry leaf powder was extracted with 25 ml pure methanol by refluxing at 60, 66 and 70 °C. The extraction rate could also be increased by increasing the extraction temperature, and smaller powder particles could also significantly increase the extraction rate by means of increased surface area. However, the excessively small particle size of the powder could absorb excess moisture and agglomerate, causing oxidation and the loss of active ingredients. Preliminary experiments indicate that 100 mesh sieve size is optimal following powder extraction in the present study.

By comparison of the six analyte contents in our study, the final optimised extraction method for mulberry leaves was determined. The use of 25 ml pure methanol for the extraction of 1.0 g milled mulberry leaf powder for 2 h under heating reflux at 66 °C gave optimum output (0.2954 g).

Optimisation of the chromatographic conditions

The optimisation of the HPLC-PDA simultaneous separation of mulberry leaf powder extract contents was tested using standard solutions of chlorogenic acid and five flavonoids (Dugo *et al.*, 2009; Zhang *et al.*, 2014). As shown in Figure 1, the six selected marker compounds portrayed a broad range of polarity, so gradient elution was carried out to separate these components in mulberry leaves. Considering the polarity differences of six compounds, different gradient elution methods of acetonitrile-water (0, 0.2, 0.4 and 0.5% phosphoric acid) and methanol-water (0, 0.2, 0.4 and 0.5% phosphoric acid) were investigated for mulberry leaves. As the chemical structures of the compounds are very similar and the retention time was short, they could not be separated when methanol was used regardless of the conditions of the step linear gradient. When acetonitrile and methanol were respectively mixed with acidified water in the same ratio, the acetonitrile-based solution had a higher elution strength. To elute most of the compounds, we selected acetonitrile instead of methanol. By optimising the compositions of mobile phase, 0.5% (v/v) phosphoric acid (solvent B) and acetonitrile (solvent A) in the ratio of 80:20, we were finally able to utilise it, and all six compounds were eluted with baseline separation in less than 35 min (Figure 2).

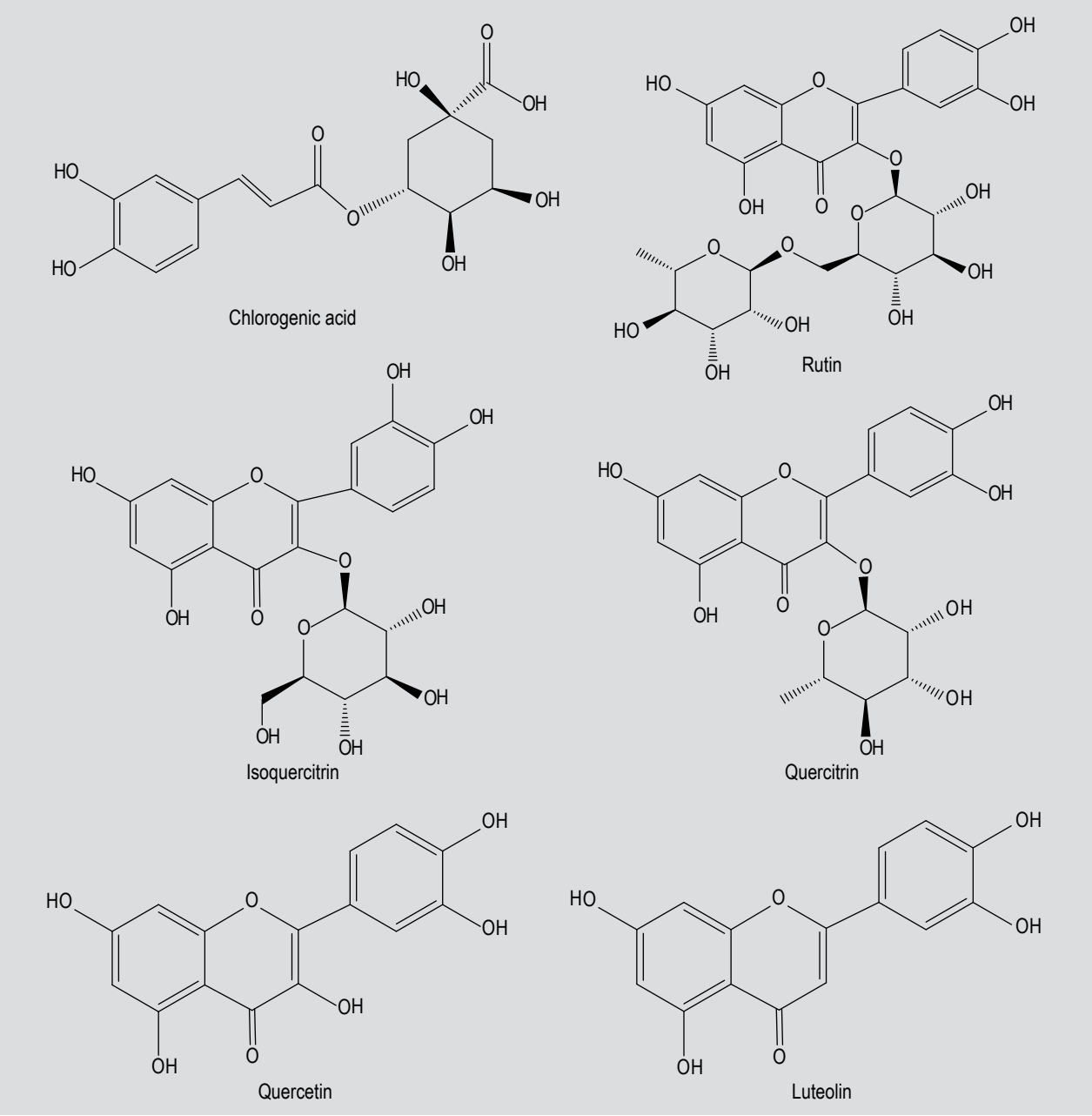

Figure 1. The chemical structures of the six selected active components of mulberry leaves.

As these compounds have different UV absorption characteristics, different detection wavelengths were chosen to monitor these components. For example, 330 nm was set for monitoring chlorogenic acid, quercitrin and luteolin, 350 nm for rutin and isoquercitrin, while a 365 nm wavelength was selected to monitor quercetin. At the selected detection wavelengths, the selectivity and sensitivity for the quantitative analysis was improved.

Increasing the flow rate from 0.8 ml/min to 0.9 ml/min led to a decrease in the retention time required for the different compounds in the separation process. We found that the optimum flow rate was 0.9 ml/min. These optimum chromatographic conditions allowed the satisfactory resolution of all analytes.

Quantitative determination of chlorogenic acid and flavonoids in mulberry leaves

The validated HPLC-PDA method was successfully applied for the simultaneous determinations of six active compounds in mulberry leaves (Dugo *et al.*, 2009; Zhang *et al.*, 2014). Each mulberry leaf sample was analysed in triplicate. HPLC chromatograms of mulberry leaves samples are shown in Figure 2A and 2B.

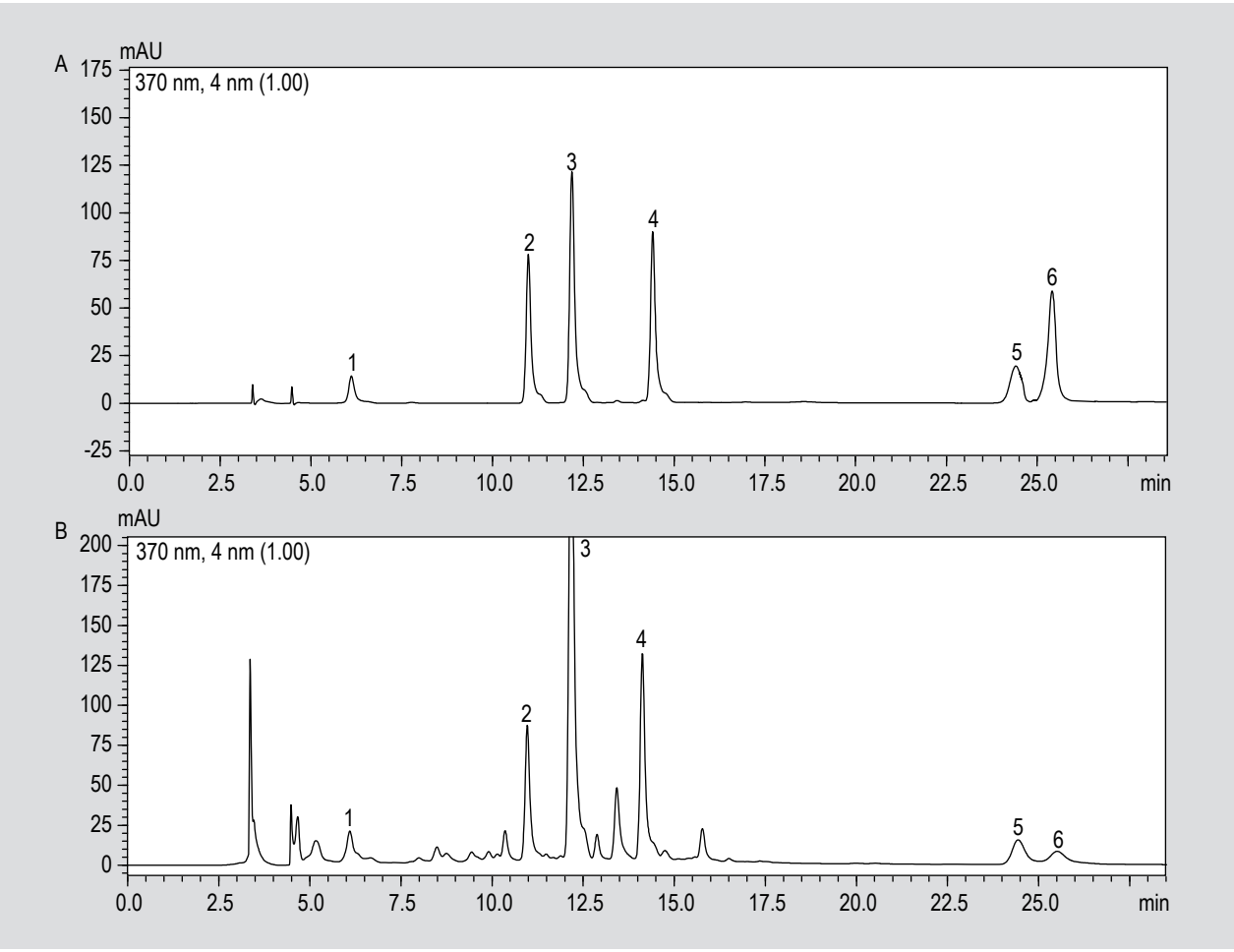

Figure 2. HPLC chromatogram of (A) standard, and (B) mulberry leaf samples. Key to peak identification: 1 = chlorogenic acid; 2 = rutin; 3 = isoquercitrin; 4 = quercitrin; 5 = quercetin; 6 = luteolin.

Identification and peak projection of chlorogenic acid and five flavonoids was based on a comparison of their retention times and UV spectra to those of standards by using PDA. The contents of individual chlorogenic acid and five flavonoids in mulberry leaves samples from different geographical regions are given in Table 4. Comparative assessment of the average amount of chlorogenic acid and flavone flavonoids in mulberry leaf from different geographical regions showed the richest content of chlorogenic acid and flavone flavonoids in the leaves obtained from the state of Bozhou. The major compounds in extracts of mulberry leaves were chlorogenic acid and

Table 4. Contents of the six compounds (μg/100 g) in samples from different regions.[1]

Source of materials	Chlorogenic acid (μg/100 g)	Rutin (μg/100 g)	Isoquercitrin (μg/100 g)	Quercitrin (μg/100 g)	Quercetin (μg/100 g)	Luteolin (μg/100 g)
Hefei	25.10±0.23	16.81±0.14	14.77±0.05	8.81±0.15	ND[2]	0.21±0.07
Bozhou I	126.43±0.98	53.14±0.26	32.70±0.13	11.56±0.23	2.01±0.10	1.16±0.11
Bozhou II	199.94±1.03	128.15±0.73	134.29±0.43	76.65±0.46	1.87±0.25	0.52±0.10
Dalian	73.97±0.44	11.79±0.09	12.19±0.07	5.01±0.14	0.95±0.23	ND
Suzhou	89.62±0.35	16.17±0.11	32.73±0.73	19.46±0.28	1.43±0.06	1.67±0.09
Hangzhou	140.70±0.98	40.66±0.28	65.32±0.22	34.55±0.53	1.03±0.15	1.42±0.14

[1] Each value is a mean of three samples (n=3).
[2] ND = not detected.

isoquercitrin followed by rutin and quercitrin; whereas, quercetin and luteolin were found in the least amounts from the extracts of mulberry leaves. Additionally, we found that chlorogenic acid, isoquercitrin, rutin and quercitrin were present in all mulberry leaf samples, whereas quercetin and luteolin were detected only in some batches of mulberry leaves. This content variation of six compounds in mulberry leaves may be mainly due to the different treatment of the raw materials. In addition, the amount of the six compounds also depends on environmental factors that affect mulberry growth, such as soil characteristics, picking seasons, and the difference between mulberry leaves on the same tree.

4. Conclusions

Our present study is the first to report the determination of six compounds using HPLC coupled with PDA, which is helpful for quality control of mulberry leaves. The proposed method can be very useful in the analysis of composite flavonoid extracts for the identification and quantification of multiple components simultaneously and as an alternate to LC-MS, using a simple HPLC-PDA methodology. The developed assay was applied successfully for the determination of the flavonoid compounds in mulberry leaves from different habitats and geographical regions. The developed method could also be a useful analytical approach for profiling the flavonoid compounds in other materials of plant origin.

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Conflict of interest

The authors have declared no conflict of interest.

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