Journal of Pharmacopuncture

ISSN 2093-6966 [Print], **ISSN 2234-6856** [Online]
Journal of Pharmacopuncture 2014;17[4]:036-049
DOI: http://dx.doi.org/10.3831/KPI.2014.17.035

Original article

HPLC Method for Simultaneous Quantitative Detection of Quercetin and Curcuminoids in Traditional Chinese Medicines

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Key Words
curcuminoid, high-performance liquid chromatography (HPLC), international conference harmonisation (ICH), quercetin

Abstract

Objectives: Quercetin and curcuminoids are important bioactive compounds found in many herbs. Previously reported high performance liquid chromatography ultraviolet (HPLC-UV) methods for the detection of quercetin and curcuminoids have several disadvantages, including unsatisfactory separation times and lack of validation according the standard guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

Methods: A rapid, specific, reversed phase, HPLC-UV method with an isocratic elution of acetonitrile and 2% v/v acetic acid (40% : 60% v/v) (pH 2.6) at a flow rate of 1.3 mL/minutes, a column temperature of 35°C, and ultraviolet (UV) detection at 370 nm was developed. The method was validated and applied to the quantification of different types of market available Chinese medicine extracts, pills and tablets.

Results: The method allowed simultaneous determination of quercetin, bisdemethoxycurcumin, demethoxycurcumin and curcumin in the concentration ranges of 0.00488 — 200 μg/mL, 0.625 — 320 μg/mL, 0.07813 — 320 μg/mL and 0.03906 — 320 μg/mL, respectively. The limits of detection and quantification, respectively, were 0.00488 and 0.03906 μg/mL for quercetin, 0.62500 and 2.50000 μg/mL for bisdemethoxycurcumin, 0.07813 and 0.31250 μg/mL for demethoxycurcumin, and 0.03906 and 0.07813 μg/mL for curcumin. The percent relative intra day standard deviation (% RSD) values were 0.432 — 0.806 μg/mL, 0.576 — 0.723 μg/mL, 0.635 — 0.752 μg/mL and 0.655 — 0.732 μg/mL for quercetin, bisdemethoxycurcumin, demethoxycurcumin and curcumin, respectively, and those for intra day precision were 0.323 — 0.968 μg/mL, 0.805 — 0.854 μg/mL, 0.078 — 0.844 μg/mL and 0.275 — 0.829 μg/mL, respectively. The intra day accuracies were 99.589% — 100.821%, 98.588% — 101.084%, 9.289% — 100.88%, and 98.292% — 101.022% for quercetin, bisdemethoxycurcumin, demethoxycurcumin and curcumin, respectively, and the inter day accuracy were 99.665% — 103.06%, 97.669% — 103.513%, 99.569% — 103.617%, and 97.929% — 103.606%, respectively.

Conclusion: The method was found to be simple, accurate and precise and is recommended for routine quality control analysis of commercial Chinese medicine products containing the flour flavonoids as their principle components in the extracts.

Received: Sep 12, 2014 **Accepted:** Oct 16, 2014

1. Introduction

Quercetin is a category in the class of flavonoids, and

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a sub class of flavonol. Flavonoids are plant polyphenolics found as pigments in fruits, vegetables, seeds, nuts, flowers, barks and leaves. It is also found in medicinal botanicals, such as *Ginkgo biloba*, *Hypericum perforatum* (St. John's Wort), and *Sambucum canadensis* (El-der) [1]. The International Union of Pure and Applied Chemistry's (IUPAC's) name for quercetin is 3, 3′, 4′, 5, 7-pentahydroxyflavone (or its synonym 3, 3′, 4′, 5, 7-pentahydroxy-2-phenylchromen-4-one). Fig.1 shows the chemical structure of quercetin. The hydroxyl (-OH) groups attached at positions 3, 5, 7, 3′, and 4′ and the catechol B-ring produce the antioxidant properties of quercetin [2, 3]. The antioxidant and the free radical scavenging properties of quercetin have been reported to contribute to anti carcinogenic and anti inflammatory effects, and haves been extensively studied by researchers around the world [2].

Extensive amounts of *in vitro* and *in vivo* animal research on quercetin's pharmacological activities have been carried out, suggesting that quercetin might be used as a new therapeutic approach to decrease blood pressure [4], to inhibit fibronectin production by keloid derived fibroblasts [5], to inhibit neointimal hyperplasia in the abdominal aorta of rats [6], to treat gout [7], to inhibit asthmatic syndrome [8] and to promote dermal wound healing [9].

Curcumin, commercially available in a mixture of curcumins (curcuminoids), contains — 77% pure curcumin, — 17% demethoxycurcumin and — 3% bisdemethoxycurcumin [10] (Fig. 1). Curcuminoids are derived from Curcuma longa Linn, one of the most popular medicinal herbs, and are a polyphenolic. These compounds are yellow pigments and have been, commonly used as a dietary spices, natural coloring agents in foods, household medicines and insect repellents in South and Southeast Asia for thousands of years [11]. Curcumin and its synthetic derivatives (curcuminoids) show an array of pharmacological properties, such as antibacterial [12-14], antioxidant [13, 15-16], anti inflammatory [17, 18], anti tumor [19, 20] and anti proliferation [18, 21] properties. Curcumin/curcuminoids also possess potency as medicines for the treatment of diseases, including Alzheimer's disease [22, 23], cancer [24, 25, 26], diabetes, gastric ulcers [27], malaria [28, 29] and for the treatment of wounds [30-32].

A variety of methods for quantitatively detecting curcumin and quercetin contents have been reported. Among these, spectrophotometric methods are the most commonly used [33-36]. Thin layer chromatography (TLC) or column chromatography was usually used for separation of curcuminoids [37-39]. High performance liquid chromatography (HPLC) [40-45] and, high performance thin layer chromatography (HPTLC) [39, 46, 47] are the commonly used methods for quantitatively detecting the quercetin and curcuminoids contents. Some advanced methods have been developed for the analysis of curcuminoids contents, namely, ultra performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-qTOF-MS) [48], ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS) [49], high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) [50] and electrochemical-HPLC [51].

For the above techniques, spectrophotometric methods are not available to quantify the individual curcuminoids due to the curcumin derivative's being also absorbed at the same wavelength. Furthermore, LC-MS and/or qTOF are complicated and need expensive instrumentation. Even though HPTLC and TLC are widely used to study the fingerprints of plants, these methods are not suitable for analyzing compounds in combinations of herbs products like Chinese medicinal materials (because such products normally contain more than one herb). For simultaneous determination of quercetin and curcuminoids, HPLC method is the recommended technique because it uses separation, identification and quantification of the analytes from plant extracts, foods, pharmaceutical products, and body fluids.

In the present study, a simple isocratic reversed phase HPLC method was developed according to international conference harmonisation (ICH) guidelines [52] for the simultaneous quantitative detection of quercetin and curcuminoids. The method was also validated by using market available traditional Chinese medicine materials such as granules, pills and tablets.

2. Materials and Methods

Curcumin (mixture of curcumin, demethoxycurcumin, and bisdemethoxycurcumin) was obtained from Acros Organics, USA. Quercetin anhydrous was obtained from Sigma, USA. The HPLC grade acetonitrile and methanol were purchased from J.T. Baker, USA. Analytical grade acetic acid was obtained from QRëC, Malaysia. Nylon membrane filters 0.45 μm were purchased from Whatman, England.

HPLC analysis was performed using a Shimadzu-LC system (Shimadzu, Japan) equipped with an CBM-20A controller, LC-20AT pump, DGU-20A5 prominence degasser, SIL-20A auto sampler, SPD-20AV detector and CTO-10ASvp column oven.

Chromatographic separations were achieved using a Thermo Hypersil Gold column (250 mm × 4.6 mm I.D.: 5 μm). A security guard column (Zorbax Eclipse Plus) packed with a replaceable C-18 cartridge (12.5 mm × 4.6 mm ID.: 5 mm) was used to protect the analytical column. A reverse phase HPLC assay was carried out using an isocratic elution with a flow rate of 1.3 mL/minutes, a column temperature of 35°C, a mobile phase of acetonitrile and 2% v/v acetic acid (pH 2.60) (40% : 60% v/v) and a detection wavelength of 370 nm. The injection volume was 20 μL of each solutions. The total run time was 18.5 minutes for each injection. Data were acquired and processed with LC-Solution Software. Solvents and distilled water were prior filtered through a 0.45-μm nylon membrane by using a set of glass bottles with the aid of a vacuum pump (Fisherbrand FB 70155, Fisher Scientific, UK).

Twenty mg of a mixture of curcumin (containing mainly curcumin, demethoxycurcumin and bisdemethoxycurcumin) and 20 mg of quercetin were accurately weighed using a microbalance (Sartorius, MC5, Germany) and dissolved in 20 mL of HPLC grade methanol in a 20 mL volumetric flask. The mixtures were diluted to 320 μg/mL with HPLC grade methanol; and were then serially doubling diluted to 1.22 ng/mL. These solutions were used as calibration standards for the quantitative determinations of the

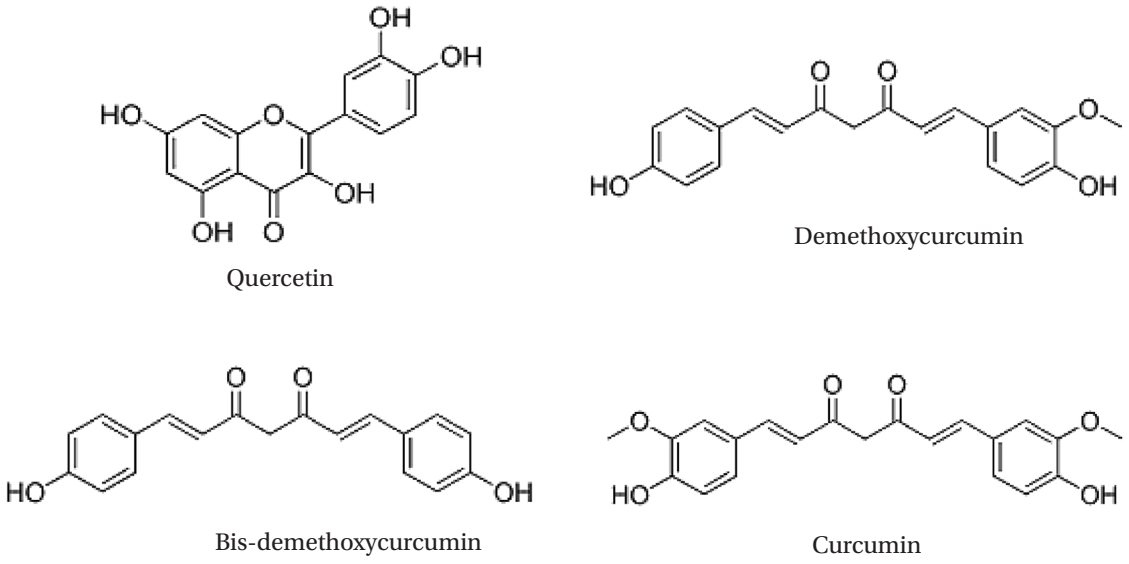

Figure 1 Chemical structures of quercetin, and the curcuminoids: curcumin, demethoxycurcumin and bisdemethoxycurcumin.

limit of detection (LOD), the limit of quantification (LOQ) and yhe limit of linearity (LOL), and for the linear range analysis. Three quality control (QC) samples at concentrations of 3.75 μg/mL, 100 μg/mL and 160 μg/mL, respectively, were prepared from the stock solution. All solutions were stored in tightened screw cap bottles to avoid evaporation and were protected from light, and were kept in a refrigerator (4°C) for not more than two weeks.

Standard solutions with concentrations in the range from of 1.22 ng/mL to 320 μg/mL were injected in duplicate into the HPLC unit. The LOD and LOQ of quercetin (QUE), bisdemethoxycurcumin (BDMC), demethoxycurcumin (DMC) and curcumin (CUR) were determined in a at the lower concentration range based on the signal to-noise ratio. According to The United Sates Pharmacopeia (USP), the LOD and the LOQ are in terms of 2 or 3 times, and 10 times the noise level respectively. The LOL was determined by plotting a calibration curve (mean value of the peak areas against the concentrations) beginnings with the LOQ concentration and proceeding to the data point that deviated from the regression line. The coefficient of determination ($R^2 \geq 0.999$) was used as a guideline to evaluate the model fit of a regression equation.

Linear ranges for quercetin, bisdemethoxycurcumin, demethoxycurcumin and curcumin included concentrations of 1.25, 5, 20, 40, 80, 140 and 200 μg/mL. Separate calibration curves were constructed for quercetin, bisdemethoxycurcumin demethoxycurcumin and curcumin by plotting the peak areas against the concentrations, and the methods were evaluated by determining the coefficient of determination (R^2). Unknown assay samples were quantified by referencing them to these calibration curves.

QC samples (3.75, 100 and 160 μg/mL) were used to validate intra day and inter day accuracies and precisions. Intra day precisions and accuracies were determined by using a replicate analysis (n = 6) of the QC samples on the same day under the same analytical conditions. Inter day precisions and accuracies were tested by using a replicate analysis (n = 3) of the same QC samples on six consecutive days. The precision is calculated from the mean of the accuracy and the relative standard deviation (RSD). Accuracy is a measure of how close the experimental value to the true value, and is expressed as a percent. The experimental value was calculated from the calibration curve by using the linear regression equation, $y = mx + c$. The constant m is the slope of the curve. The constant c is the y intercept and can be determined by extrapolating the straight line to the y axis.

Four variation parameters of robustness were studied: change in organic composition by ± 2.0% (Table 4a), change in acetic acid concentration by ± 1.0% v/v (effect of buffer pH) (Table 4b), change in the flow rate of ± 0.1 mL/min (Table 4c) and change in the column temperature of ± 5.0°C (Table 4d). The retention time, peak area, resolution, tailing factor, theoretical plate number and capacity factor values obtained from the variation parameters were compared to those obtained for the normal method conditions. The differences were analyzed by using SPSS version 20, and a one way analysis of variance (ANOVA), followed by Tukey's test. *P*-values < 0.05 were considered significant.

The system suitability parameters were assessed by using six replicate analysis of the QC sample at 160 μg/mL. The acceptance criteria were in accordance with the guidelines of the Centre for Drug Evaluation and Research [53].

The method developed in this study was used to quantitatively determination the quercetin and the curcuminoid contents of extracts, pills and tablets made from Chinese medicinal plants.

3. Results

The LOD and the LOQ were determined based on the signal to noise (S/N) ratio, with the S/N > 3 and the S/N

> 10 for the LOD and the LOQ, respectively. The LODs of quercetin, bisdemethoxycurcumin, demethoxycurcumin and curcumin were 0.00488, 0.62500, 0.07813 and 0.03906 μg/mL, respectively. The LOQs of quercetin, bisdemethoxycurcumin, demethoxycurcumin and curcumin were 0.03906, 2.5000, 0.31250 and 0.07813 μg/mL, respectively (Table 1). The linearity for detecting quercetin, bisdemethoxycurcumin, demethoxycurcumin and curcumin was tested against a mixture of calibration standards with concentration ranging from 1.22 ng/mL to 320 μg/mL. The LOL of each compound was determined from a separate calibration curve. Quercetin was linear up to 200 μg/mL, while bisdemethoxycurcumin, demethoxycurcumin and curcumin were linear up to 320 μg/mL.

Linear calibration curves in the range from 1.25 to 200 μg/mL were constructed for each compound by plotting the peak area against the concentration. The retention times and the peak areas are tabulated in Table 2. The values of R^2, the y-intercept and the slope for each compound's calibration plot are shown in Table 1. A regression analysis of the data showed a linear relationship for quercetin, bisdemethoxycurcumin, demethoxycurcumin and curcumin, with excellent R^2 values of 0.99993, 0.99984, 0.99985 and 0.99993 μg/mL, respectively.

The peaks of quercetin, bisdemethoxycurcumin, demethoxycurcumin and curcumin were well separated at different retention times with resolutions of 32.195, 2.887 and 2.830 for quercetin-bisdemethoxycurcumin, bisdemethoxycurcumin-demethoxycurcumin and demethoxycurcumin-curcumin, respectively. No interferences or excipient peaks co eluted with the analytes were observed, indicating the method is selective and specific in relation to the medium and excipients used in this study (Fig. 2, Table 2).

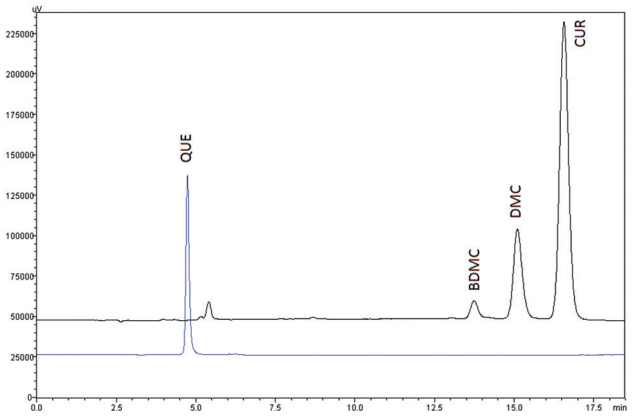

Figure 2 Chromatograms of quercetin and curcuminoids. QUE, quercetin; BDMC, bisdemethoxycurcumin; DMC, demethoxycurcumin; CUR, curcumin.

Precision and accuracy data for the intraday and the inter-day variations for the three QC samples are summarized in Table 3. The RSD values for the intraday and the inter day precisions were < 1%. For the accuracy test, the intraday and the inter day accuracies ranges from 98.292% to 103.617%, confirming the accuracy of the method.

Robustness is a measure of the method's capability to remain unaffected by small, but deliberate, variations in the method parameters [52]. The robustness parameters tested were the mobile phase's composition, the concentration of acetic acid (pH effect), the flow rate and the column temperature. The results are tabulated in Table 4(a-d). The retention times for all four compounds due to variations in the parameters were significantly different compared to those for the normal parameters. The peak area for curcumin was not significantly different after changing the acetic acid concentration from 2% to 3%, but was significantly different after changing the concentration from 2% to 1%. Quercetin, bisdemethoxycurcumin and demethoxycurcumin were shown to have significant differences in their peak area when the concentration of acetic acid was changed. Changes in the acetonitrile's composition and temperature were shown not to cause significant differences in quecetin's peak areas, however significant differences were seen in curcumin, bisdemethoxycurcumin and demethoxycurcumin peak areas. Increasing or decreasing the flow rate by 0.1 mL/min from normal conditions significantly raised or reduced the values of the peak areas of quercetin, bisdemethoxycurcumin, demethoxycurcumin and curcumin. Although changes in experimental conditions changed the retention time, the peak area and the values of the system's suitability parameters, the four analyzed peaks were still well resolved from each other and from additional small peaks and showed good resolution in the tested parameters (Fig. 3).

The system suitability criteria were in accordance with the Centre for Drug Evaluation and Research (CDER) guidelines [53] and are summarized in Table 5. The mean values of the six replicate injections of 160 μg/mL QC standards

Table 1 LOD, LOQ, LOL and linear regression analysis parameters for QUE, BDMC, DMC and CUR

Compounds	LOD (μg/mL)	LOQ (μg/mL)	LOL (μg/mL)	Regression analysis (1.25 — 200 μg/mL)		
				slope	y-intercept	Coefficient of determination (R^2)
QUE	0.00488	0.03906	200	70055.85913	1521.41433	0.99993
BDMC	0.62500	2.50000	320	1807.72930	— 440.28180	0.99984
DMC	0.07813	0.31250	320	10011.55795	40.13501	0.99985
CUR	0.03906	0.07813	320	34176.44088	3645.08890	0.99993

LOD, limit of detection; LOQ, limit of quantification, LOL, limit of linearity; QUE, quercetin; BDMC, bisdemethoxycurcumin; DMC, demethoxycurcumin; CUR, curcumin.

Table 2 Retention times and responses data of calibration standards of QUE, BDMC, DMC, and CUR

Concentration (μg/mL)	Retention time (n = 5)		Peak area (n = 5)	
	Mean (min)	RSD (%)	Mean (min)	RSD (%)
QUE				
1.25	3.970	0.117	94937	0.676
5	3.972	0.066	367965	0.739
20	3.972	0.041	1438240	0.624
40	3.973	0.055	2781685	0.508
80	3.972	0.029	5582929	0.437
140	3.972	0.048	9735618	0.866
200	3.972	0.053	14073938	0.368
BDMC				
1.25	13.823	0.308	1859	1.611
5	13.840	0.095	8843	1.181
20	13.842	0.093	37086	1.089
40	13.843	0.087	71560	1.044
80	13.846	0.117	143659	1.073
140	13.846	0.134	249462	1.835
200	13.849	0.060	363457	0.850
DMC				
1.25	15.214	0.227	14705	0.273
5	15.229	0.096	52692	0.540
20	15.230	0.074	204602	0.665
40	15.232	0.073	398446	0.436
80	15.237	0.099	798153	0.867
140	15.236	0.120	1384220	1.416
200	15.242	0.039	2015583	0.158
CUR				
1.25	16.708	0.199	46645	0.856
5	16.718	0.077	182515	0.901
20	16.719	0.061	701982	0.700
40	16.720	0.064	1358591	0.299
80	16.725	0.096	2737751	0.423
140	16.725	0.108	4749355	0.897
200	16.734	0.067	6866971	0.313

RSD, relative standard deviation; QUE, quercetin; BDMC, bisdemethoxycurcumin; DMC, demethoxycurcumin; CUR, curcumin.

were used to evaluate the retention time, the peak area, the resolutions for the analyte peaks, the tailing factor, the number of theoretical plates and the capacity factor. The results for the system suitability parameters are shown in Table 6. The RSD values for the tested parameters were < 1%, indicating the precision of the method. The tested parameters passed the criteria under the CDER guidelines except for the capacity factor value for quercetin (< 2) [53]. This is because the retention time of quercetin was quite fast and just 1 minute behind the solvent peak. However, the quercetin peak was well resolved from the solvent peak and from the front additional small peak.

The proposed method was applied to quantitatively detect the quercetin and curcuminoids in Chinese medicines

Table 3 Precisions and accuracies for intraday and interday repetitions for the quantitative detection of QUE, BDMC, DMC and CUR

Concentration (μg/mL)	Intra day*			Inter day†		
	Peak Response	Precision (RSD, %)	Accuracy (%)	Peak Response	Precision (RSD, %)	Accuracy (%)
QUE						
3.75	263151	0.432	99.589	263350	0.323	99.665
100	7064599	0.717	100.821	7221470	0.646	103.060
160	11221611	0.806	100.010	11218287	0.968	100.070
BDMC						
3.75	6243	0.576	98.588	6181	0.854	97.669
100	182293	0.723	101.084	186683	0.878	103.513
160	286851	0.654	99.32746	288040	0.805	99.738
DMC						
3.75	37700	0.635	100.310	37687	0.466	100.276
100	1010004	0.752	100.880	1037410	0.078	103.617
160	1590498	0.651	99.289	1594989	0.844	99.569
CUR						
3.75	129618	0.655	98.292	129152	0.297	97.929
100	3456218	0.732	101.022	3544535	0.275	103.606
160	5448675	0.711	99.576	5454012	0.829	99.673

*Intra day repetitions for each concentration were analyzed on the same day. †Inter day repetitions for each concentration, were analyzse on six consecutive days. RSD, relative standard deviation; QUE, quercetin; BDMC, bisdemethoxycurcumin; DMC, demethoxycurcumin; CUR, curcumin.

Table 4(a) Robustness – change in organic composition

System suitability	Com-pound	Change in the normal organic composition of acetonitrile: 2% acetic acid					
		(A) Normal condition		(B) 38% : 62% v/v		(C) 42% : 58% v/v	
		Mean (n = 6)	RSD (%)	Mean (n = 6)	RSD (%)	Mean (n = 6)	RSD (%)
Retention time, t_R (minutes)	QUE	3.993	0.690	4.251	0.155	3.761	0.040
	BDMC	13.951	0.342	17.645	0.374	11.280	0.084
	DMC	15.340	0.291	19.543	0.285	12.330	0.084
	CUR	16.829	0.245	21.617	0.296	13.464	0.082
Peak area	QUE	6853044	0.433	6836934	0.441	6867445	0.117
	BDMC	167417	0.647	161504	0.801	146484	0.578
	DMC	940836	0.404	903191	0.781	965307	0.071
	CUR	3302593	0.236	3206134	0.555	3367309	0.114
Resolution, R	QUE	-	-	-	-	-	-
	BDMC	32.498	0.379	36.449	0.471	29.120	0.063
	DMC	2.908	0.208	3.272	2.369	2.736	0.181
	CUR	2.850	0.237	3.243	1.648	2.666	0.124
Tailing factor, T_f	QUE	1.371	0.254	1.347	0.115	1.392	0.074
	BDMC	1.533	0.364	1.283	2.314	1.080	0.200
	DMC	1.160	0.484	1.083	0.151	1.431	0.082
	CUR	1.094	0.094	1.076	0.284	1.114	0.037

(Continued)

System suitability	Compound	Change in the normal organic composition of acetonitrile: 2% acetic acid					
		(A) Normal condition		(B) 38% : 62% v/v		(C) 42% : 58% v/v	
		Mean (n = 6)	RSD (%)	Mean (n = 6)	RSD (%)	Mean (n = 6)	RSD (%)
Theoretical plate, N	QUE	8752.133	1.463	8857.791	0.312	8520.171	0.238
	BDMC	15931.889	1.147	16311.011	0.058	16303.130	0.103
	DMC	14298.287	1.761	16569.474	1.029	14210.321	0.233
	CUR	16008.049	1.202	16543.754	0.535	15157.508	0.340
Capacity factor, k'	QUE	0.680	0.344	0.777	0.906	0.601	0.327
	BDMC	4.878	0.202	3.800	0.209	3.800	0.209
	DMC	5.463	0.232	7.214	1.592	4.247	0.206
	CUR	6.097	0.253	8.038	0.481	4.729	0.209

Table 4(b) Robustness – change in acetic acid concentration

System suitability	Compound	Change in the acetic acid concentration (% v/v)					
		(A) Normal condition		(B) 1.0% (pH 2.73)		(C) 3.0% (pH 2.48)	
		Mean (n = 6)	RSD (%)	Mean (n = 6)	RSD (%)	Mean (n = 6)	RSD (%)
Retention time, t_R (minutes)	QUE	3.972	0.175	4.054	0.064	3.893	0.071
	BDMC	13.868	0.310	14.549	0.086	13.177	0.167
	DMC	15.255	0.265	16.017	0.085	14.542	0.153
	CUR	16.743	0.213	17.590	0.084	16.028	0.141
Peak area	QUE	7039483	0.562	6966950	0.525	6952833	0.630
	BDMC	180475	0.541	176885	0.575	152439	0.895
	DMC	1000716	0.736	987128	0.551	956266	0.670
	CUR	3433379	0.754	3428762	0.533	3428762	0.558
Resolution, R	QUE	-	-	-	-	-	-
	BDMC	32.327	0.172	33.254	0.244	31.950	0.268
	DMC	2.900	0.370	2.974	0.303	3.033	0.527
	CUR	2.840	0.429	2.904	0.339	2.966	0.608
Tailing factor, T_f	QUE	1.366	0.077	1.364	0.215	1.370	0.110
	BDMC	1.493	1.377	1.463	0.331	1.060	0.139
	DMC	1.160	1.075	1.137	0.103	1.325	0.823
	CUR	1.085	0.148	1.092	0.050	1.083	0.108
Theoretical plate, N	QUE	8711.993	0.267	8877.546	0.460	8548.948	0.269
	BDMC	15740.557	0.397	16067.808	0.689	16308.146	0.664
	DMC	14041.181	0.701	14691.580	0.675	14241.082	1.031
	CUR	15793.019	0.472	16098.239	0.701	15531.342	0.811
Capacity factor, k'	QUE	0.656	1.783	0.680	1.484	0.610	0.803
	BDMC	4.798	1.202	5.036	0.658	4.449	0.511
	DMC	5.333	0.988	5.637	0.698	5.014	0.478
	CUR	6.016	1.416	6.295	0.628	5.629	0.443

(Continued)

Table 4(c) Robustness – change in flow rate

System suitability	Compound	Change in flow rate					
		(A) Normal condition		(B) 1.2 mL/minutes		(C) 1.4 mL/minutes	
		Mean (n = 6)	RSD (%)	Mean (n = 6)	RSD (%)	Mean (n = 6)	RSD (%)
Retention time, t_R (minutes)	QUE	3.972	0.175	4.291	0.105	3.696	0.130
	BDMC	13.868	0.310	14.953	0.321	12.909	0.333
	DMC	15.255	0.265	16.442	0.284	14.235	0.279
	CUR	16.743	0.213	18.038	0.262	15.668	0.298
Peak area	QUE	7039483	0.562	7606272	0.662	6530571	0.497
	BDMC	180475	0.541	194216	0.753	167111	1.593
	DMC	1000716	0.736	1078076	0.714	928707	1.345
	CUR	3433379	0.754	3700134	0.690	3185325	1.198
Resolution, R	QUE	-	-	-	-	-	-
	BDMC	32.327	0.172	32.779	0.199	32.047	0.928
	DMC	2.900	0.370	2.921	0.608	2.936	2.014
	CUR	2.840	0.429	2.864	0.723	2.868	1.647
Tailing factor, T_f	QUE	1.366	0.077	1.360	0.183	1.371	0.287
	BDMC	1.493	1.377	1.490	1.891	1.539	1.614
	DMC	1.160	1.075	1.157	1.447	1.181	2.364
	CUR	1.085	0.148	1.081	0.101	1.087	0.207
Theoretical plate, N	QUE	8711.993	0.267	9148.347	0.429	8249.430	0.420
	BDMC	15740.557	0.397	16035.103	1.342	15696.046	2.851
	DMC	14041.181	0.701	14374.944	1.036	13420.220	0.844
	CUR	15793.019	0.472	16216.013	1.854	15379.165	2.364
Capacity factor, k'	QUE	0.656	1.783	0.661	0.832	0.627	0.762
	BDMC	4.798	1.202	4.780	0.942	4.750	3.067
	DMC	5.351	0.661	5.355	0.497	5.350	2.846
	CUR	5.966	0.632	5.985	0.500	5.862	0.427

Table 4(d) Robustness – change in column temperature

System suitability	Compound	Change in column temperature					
		(A) Normal condition		(B) 30°C		(C) 40°C	
		Mean (n = 6)	RSD (%)	Mean (n = 6)	RSD (%)	Mean (n = 6)	RSD (%)
Retention time, t_R (minutes)	QUE	3.956	0.031	4.063	0.074	3.861	0.162
	BDMC	13.673	0.070	14.647	0.172	12.810	0.268
	DMC	15.037	0.064	15.980	0.153	14.167	0.236
	CUR	16.502	0.064	17.423	0.143	15.657	0.196
Peak area	QUE	7628483	0.252	7620525	0.254	7633341	0.259
	BDMC	196493	0.261	202870	0.253	172397	0.136
	DMC	1091099	0.300	1124567	0.281	1058404	0.205
	CUR	3738544	0.244	3836306	0.285	3643910	0.196
Resolution, R	QUE	-	-	-	-	-	-
	BDMC	31.946	1.437	32.560	0.233	31.471	0.267
	DMC	2.872	1.359	2.698	0.334	3.155	0.481
	CUR	2.829	0.575	2.718	0.305	3.106	0.559

(Continued)

System suitability	Compound	Change in column temperature					
		(A) Normal condition		(B) 30°C		(C) 40°C	
		Mean (n = 6)	RSD (%)	Mean (n = 6)	RSD (%)	Mean (n = 6)	RSD (%)
Tailing factor, T_f	QUE	1.343	0.056	1.329	0.267	1.351	0.124
	BDMC	1.551	0.421	1.233	0.525	1.089	0.077
	DMC	1.186	0.241	1.098	0.268	1.491	1.051
	CUR	1.097	0.082	1.094	0.107	1.098	0.129
Theoretical plate, N	QUE	8734.237	0.300	8837.810	0.448	8619.473	0.295
	BDMC	15779.175	0.595	15065.104	0.600	16276.545	1.203
	DMC	13866.206	1.175	15742.765	0.475	15286.704	0.311
	CUR	15846.706	0.791	15917.987	0.394	15793.349	0.183
Capacity factor, k'	QUE	0.698	1.598	0.717	1.945	0.616	0.692
	BDMC	4.855	0.115	5.190	0.989	4.366	0.214
	DMC	5.449	0.349	5.752	0.966	4.962	1.315
	CUR	6.058	0.201	6.362	0.942	5.581	1.015

The normal conditions of HPLC are a mobile phase of acetonitrile: 2% acetic acid (pH 2.60) = 40% : 60 % v/v, flow rate 1.3 mL/min at UV wavelength of 370 nm and column temperature at 35°C. RSD, relative standard deviation; QUE, quercetin; BDMC, bisdemethoxycurcumin; DMC, demethoxycurcumin; CUR, curcumin.

Table 5 System suitability parameters, calculation formula and recommendations

Parameter	Formula	Recommendation
Precision	$RSD = S/\bar{x}^*100$	RSD ≤ 1% for n ≥ 5
Resolution, R	$R = (t_{R2} - t_{R1})/(1/2)(t_{w1} - t_{w2})$	> 2
Tailing factor, T_f	$Tf = Wx/2f$	≤ 2
Theoretical plates, N	$N = 16(t_R/t_w)^2$	Column efficiency ≥ 2000
Capacity factor, k	$k' = (t_R - t_0)/t_0$	> 2

S, standard deviation; $\bar{x}$, mean of the data; t_R, retention time of analyte 1; tw, peak width measured to the baseline of the extrapolated straight sides to baseline; W_x, width of the peak determined at either 5% (0.05) or 10% (0.10) from the baseline of the peak height; f, distance between peak maximum and peak front at W_x; t_0, elution time of the void volume or non retained components.

Table 6 System suitability testing

Parameter	QUE		BDMC		DMC		CUR	
	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)
Retention time, t_R	3.970	0.021	13.840	0.027	15.230	0.025	16.723	0.021
Peak area	11221611	0.806	286851	0.654	1590498	0.651	5448675	0.711
Resolution, R	-	-	32.195	0.321	2.887	0.364	2.830	0.370
Tailing factor, T_f	1.369	0.108	1.501	0.261	1.165	0.144	1.081	0.051
Theoretical plate, N	8803.785	0.359	15552.398	0.865	13763.145	0.646	15568.252	0.910
Capacity factor, k'	0.684	0.846	4.870	0.415	5.460	0.406	6.093	0.391

RSD, relative standard deviation; QUE, quercetin; BDMC, bisdemethoxycurcumin; DMC, demethoxycurcumin; CUR, curcumin. N, number of theoretical plates; k', capacity factor; Mean of six replicate injections of quality control (QC) standard of 160 μg/mL.

such as plant granule extracts, tablets and pills. The results of 19 samples are summarized in Table 7. In the tested samples, BDMC had the highest concentration compared to the other two curcuminoids tested (DMC and CUR), and was found in the formulations of granule extracts, tablets and pills (such as samples 12, 13, 15, 16, 18 and 19) (Table 7). The preference of BDMC over CUR in the medicine might be due to its strong biological properties, which

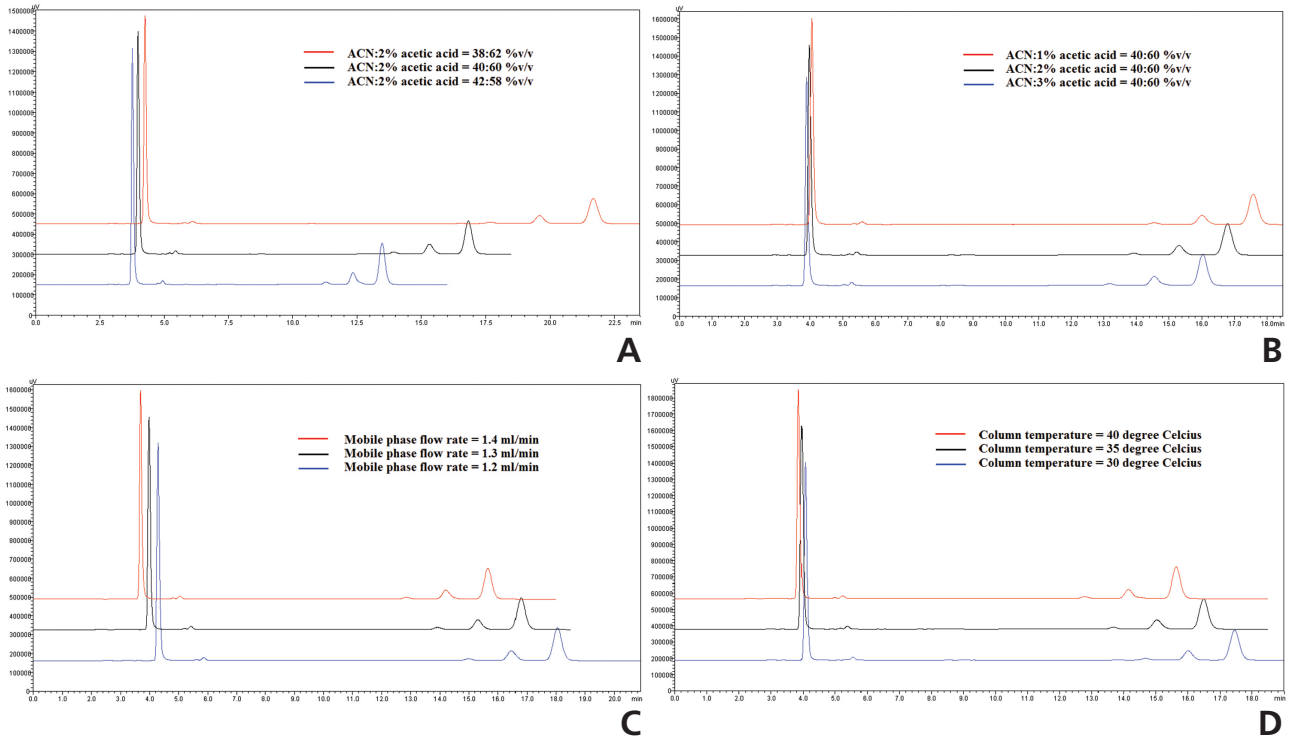

Figure 3 Combined chromatograms of quercetin (QUE), bisdemethoxycurcumin (BMDC), demethoxycurcumin (DMC), curcumin (CUR) analyzed at different conditions: (a) acetonitrile: 2% acetic acid at a flow rate of 1.3 mL/minutes, 35°C (b) acetonitrile: different acetic acid concentrations (40% : 60% v/v) at a flow rate of 1.3 mL/min, 35°C (c) acetonitrile: 2% acetic acid (40% : 60% v/v) at different flow rates, 35°C (d) acetonitrile: 2% acetic acid (40% : 60% v/v) at a flow rate of 1.3 mL/min at different temperatures.

its use as a cure for diseases or as a supplement for certain purposes. Quercetin was found in most of the tested samples, indicating that this compound is common and useful for treatment. Fig. 4 shows the chromatograms for the quercetin and the curcuminoids found in the tested samples.

4. Discussion

The HPLC method was developed by optimization of the mobile phase conditions so that quercetin, bisdemethoxycurcumin, demethoxycurcumin and curcumin peaks could be simultaneously detected by using the same solvent system and an isocratic method. The flow rate, acetic acid concentration and column temperature were varied to determine the chromatographic conditions giving the best separation and the shortest analysis time. UV visible sperctrophotometry in the wavelength from 200 to 500 nm was used for the detection of quercetin and curcuminoids; 370 nm was chosen as appropriate wavelength for the analysis of quercetin and curcumin derivatives.

The retention times for quercetin (3.97 minutes), bisdemethoxycurcumin (13.84 minutes), demethoxycurcumin (15.23 minutes) and curcumin (16.72 minutes) were reasonable because the method is simple and general. The chromatograph peaks for mixtures of curcumin were identified based on their percentages in the mixtures. Most of the commercially available curcumin/turmeric products contain mixtures of curcumin, demethoxycurcumin and bisdemethoxycurcumin. Among these, curcumin (46% — 72%) is the major compound, followed by demethoxycurcumin (11% — 28%) and bisdemethoxycurcumin (3% — 14%). All four analyte peaks were well separated from each other and from small additional peaks.

The linear ranges of quercetin (0.039 — 200 μg/mL), bisdemethoxycurcumin (2.500 — 320 μg/mL), demethoxycurcumin (0.313 — 320 μg/mL) and curcumin (0.078 — 320 μg/mL) are suitable for the analysis of most the pharmaceutical products, containing the compounds and for the analysis of crude herbs. The low LOD and LOQ values indicate that the method provides adequate sensitivity. The R^2 values > 0.999 for the regression model for the calibration curves confirm the good linearity of the method.

The accuracies ranged from 98.292% — 103.617%, and the precisions were less than 1% which indicate that the proposed method is well validated and suitable for quantitatively detecting curcuminoids and quercetin simultaneously in pharmaceutical products, herb materials and various turmeric and quercetin containing products.

System suitability testing is important to ensure the performance of the system before and during the analysis. As defined in the United States Pharmacopeia/National Formulary (USP/NF) [54] system suitability parameters were established as a direct result of the ruggedness and the robustness of the experiments. The system suitability testing proved that the proposed method will allow the separation of all four anaytes and will produce satisfactory peak shapes.

Table 7 Concentration of QUE, CUR, DMS and BDMC in Chinese medicines

No	Chinese medicine	Type	Concentration (mean ± S.D*) (μg/100 mg)			
			QUE	BDMC	DMC	CUR
1	Gao liang jiang (高良姜)	Single plant granule extract	0.7532	N.D	134.8739	0.5270
2	Jin qian cao (金钱草)	Single plant granule extract	4.0618	N.D	N.D	0.8263
3	Yu jin (郁金)	Single plant granule extract	0.3195	69.1060	27.2286	27.1020
4	E su (莪术)	Single plant granule extract	0.5983	79.5922	42.6982	8.6812
5	Jiang huang (姜黄)	Single plant granule extract	3.6523	N.D	933.8122	796.0621
6	Yu xing cao (鱼腥草)	Single plant granule extract	1.7930	N.D	N.D	1.3424
7	Ting li zi (葶苈子)	Single plant granule extract	1.3604	N.D	N.D	N.D
8	Tu si zi (菟丝子)	Single plant granule extract	3.9300	N.D	N.D	N.D
9	Di yu (地榆)	Single plant granule extract	0.8962	N.D	N.D	N.D
10	Kui hua (愧花)	Single plant granule extract	311.0307	N.D	N.D	N.D
11	Sang ju yin (桑菊饮)	Formulation granule extract	0.7402	N.D	0.3558	0.2537
12	Chai hu su gan san (柴胡疏肝散)	Formulation granule extract	0.2029	126.8843	48.3408	1.6417
13	Xiao yao san (逍遥散)	Formulation granule extract	0.4991	97.9203	2.5534	0.4301
14	Long dan xie gan tang (龙胆泄肝汤)	Formulation granule extract	11.1482	5.2111	1.2817	0.1236
15	Sang ju gan mao pian (桑菊感冒片)	Tablet	17.3489	173.6155	2.8579	N.D
16	Dan zhi xiao yao pian (丹栀逍遥片)	Tablet	7.8101	135.1892	1.0883	0.2624
17	Long dan xie gan pian (龙胆泄肝片)	Tablet	N.D	5.5352	6.7428	0.2378
18	Bu zhong yi qi (补中益气)	Tablet	0.9052	623.1338	5.9485	0.5964
19	Xiao yao wan (逍遥丸)	Pill	12.015	79.7951	11.7471	1.1516

*n = 3; N.D, not detected; QUE, quercetin; BDMC, bisdemethoxycurcumin; DMC, demethoxycurcumin; CUR, curcumin.

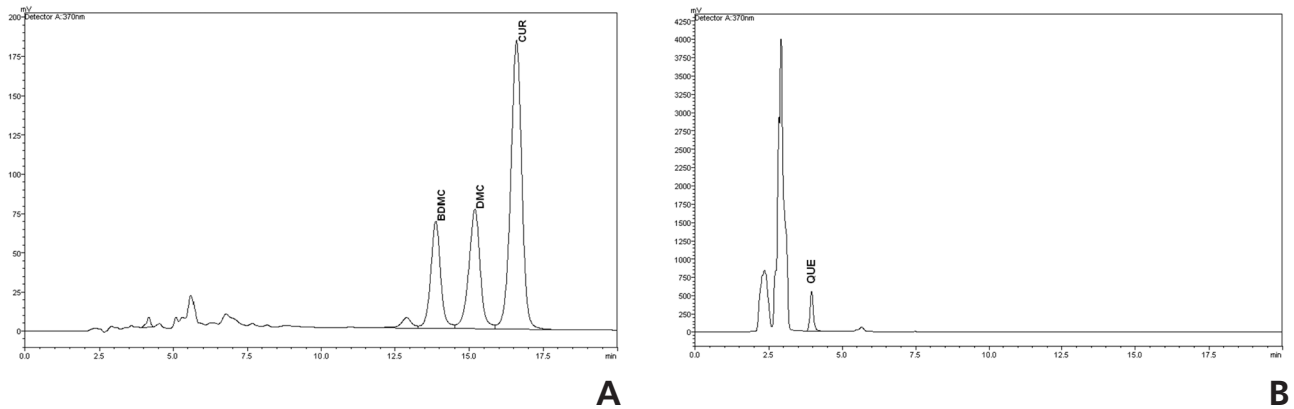

Figure 4 Chromatograms for Chinese medicinal plant extracts (a) containing quercetin and (b) containing curcuminoids. QUE, quercetin; BDMC, bisdemethoxycurcumin; DMC, demethoxycurcumin; CUR, curcumin.

5. Conclusions

A simple isocratic RP-HPLC method with UV detection has been developed for simultaneous detection of quercetin, curcumin, demethoxycurcumin and bisdemethoxycurcumin. The analytes were well separated and detected

within 19 minutes. This method was validated for specificity, linearity, precision, accuracy and robustness as per ICH guidelines. The data showed good selectivity and sensitivity, a wide linear range, precision and accuracy. The method was sensitive to HPLC conditions; that is, changes in the mobile phase's composition, the pH, the column temperature and the flow rate affected the retention time and response, but did not affected the separation of the compounds. In addition, each parameter showed good repeatability of the retention time and response. In conclusion, the proposed method is simple, easy and cost effective, no specific solvent is involved and it utilizes common HPLC instruments with UV detectors. Hence, this UV-HPLC method is suitable for routine analysis of quercetin and curcuminoid formulations or products.

Conflict of interest

The authors declare that there are no conflict of interest.

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