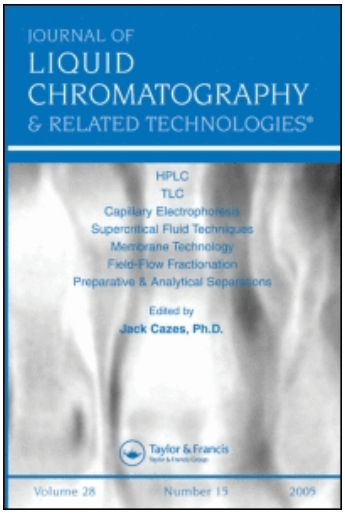

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IDENTIFICATION AND DETERMINATION OF THE MAJOR CONSTITUENTS IN AN ANTIANGIOGENESIS HERBAL FORMULA, QING-LUO-FANG, BY HPLC—DAD—ESI/MS

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□ *A high-performance liquid chromatography—diode array UV detection—electrospray ionization tandem mass spectrometry (HPLC—DAD—ESI-MS) method was established for the simultaneous qualitative and quantitative analysis of the major bioactive constituents in Qing-Luo-Fang (QLF), an anti-angiogenesis herbal formula. The separation was performed on a Phenomenex Gemini C_{18} column using gradient elution. The mobile phase A was ammonium acetate (10 mM) in water; and mobile phase B was methanol-acetonitrile (1:3, v/v) containing 10 mM ammonium acetate and 0.2% ammonia. Twelve compounds were identified by online ESI-MS, and eight of which were quantitated by HPLC—DAD. The eight compounds are major alkaloid compounds present in QLF, including magnoflorine, oxymatrine, oxysophocarpine, sinomenine, sophocarpine, matrine, palmatine, and berberine. The method was validated and the linearities ($r^2 > 0.999$) and recoveries (ranged from 95.24 to105.50%) were acceptable. The limits of detection (LOD) and the limits of quantitation (LOQ) of these alkaloids ranged from 0.63 to 6.70 ng/mL and from 2.01 to 20.69 ng/mL, respectively. The intra- and inter-day precision was less than 3% and the accuracy ranged from 97.4% to 102.7%. These results prove that this established method can be used to identify the structure of active constituents responsible for the pharmacological effects of QLF and to control the quality of QLF.*

Keywords alkaloids, HPLC—DAD—ESI-MS, qing-luo-fang, simultaneous analysis

INTRODUCTION

Traditional Chinese medicine (TCM), most of which are formulae, has been attracting greater attention because of its complementary therapeutic effects with western medicine and few or no side effects.[1,2] However, although many TCM have been proven effective by modern pharmacological

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studies and clinical trials, their bioactive constituents and the remedial mechanisms are still not well understood. So far, it is widely accepted that multiple constituents are responsible for the therapeutic effect of TCM.[1] This situation makes the quality control of TCM products very difficult.

In recent years, the strategy, based on chromatographic fingerprint technology, has been gradually applied to the quality control standards of more TCM products in China. Nevertheless, this strategy is a "blind analysis," which lacks the chemical information about the constituents of TCM products; therefore, it does not reflect the pharmaceutical activity of the TCM products. To solve this problem, chemical studies on the major constituents of TCM products are essential as the complements to chromatographic fingerprint technology.

The Chinese herbal formula Qing-Luo-Fang (QLF) is an example of a prescription, that is effective for treating patients suffering from rheumatoid arthritis,[3] because of its anti-angiogenesis activity.[4] QLF, which is considered to be a representative TCM for anti-angiogenesis-related diseases,[5] is prepared from three herbs: *Sophora flavescens*, *Sinomenium acutum*, and *Phellodendron amurense*. It is known that many plant alkaloids have antiangiogenic activity.[6] Recent pharmacological research[7–10] indicate that alkaloids in QLF may contribute significantly to the antiangiogenesis activity of this prescription. However, a detailed study on the profile of constituents of QLF formula has not yet been reported. To date, there is only one report on the quantitative analysis of the five active alkaloids in QLF.[11] In this study, the five control standards did not reflect comprehensive analysis of the active constituents of QLF. To more rigorously control the quality and batch-to-batch consistency of QLF products, the quality control standard based on the chemical identification of its major constituents and chromatographic fingerprint technology is required. Therefore, the chemical identification of the major constituents is necessary. Thus, a reliable method for the qualitative and quantitative analysis of the major constituents in QLF is essential.

HPLC coupled with additional techniques, such as DAD and mass spectrometry methods, has proven to be a powerful approach for the rapid identification of constituents in botanic extracts and TCM.[12–15]

In this paper, a HPLC—DAD—ESI-MS method for the qualitative and quantitative analysis of the major bioactive constituents in QLF formula will be described.

EXPERIMENTAL

Chemicals and Materials

Methanol and acetonitrile for HPLC analysis was of HPLC grade from Burdick & Jackson (SK chemicals, Korea); ammonium acetate and

ammonia water were of AR grade from Beijing Chemical Factory (Beijing, China); and water for HPLC analysis was purified by a Milli-Q academic water purification system (Milford, MA, USA).

The reference compounds magnoflorine, oxymatrine, oxysophocarpine, sinomenine, sophocarpine, matrine, palmatine, and berberine were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Magnoflorine was purchased from Tianjin mark company (Tianjin, China). The purities of all the standards were at least 98%.

Sophora flavescens, *Sinomenium acutum*, and *Phellodendron amurense* were purchased from Beijing Tong-ren-tang company (Beijing, China) and were identified by Professor Shi Yue. The voucher specimens were deposited in our laboratory.

Standard Solution and Sample Preparation

Each standard was accurately weighed, then dissolved in 50% methanol, and serially diluted to produce the calibration curves, check linearity, and determine limits of detection (LOD) as well as limits of quantitation (LOQ). All the standard solutions were stored in the refrigerator at 4°C prior to analysis.

The three herbs required for the preparation of QLF were carefully weighed to obtain the equivalent of a single dose of QLF. The materials were crushed into small pieces and were extracted with 2000 mL 75% ethanol for 1 hr. The procedure was repeated three times. Total extracts were combined and solvents were removed at 60°C under vacuum by Rotavapor. 8.8 mg of residue was then dissolved in 10 mL 50% methanol to obtain the QLF extract for quantitative analysis. The same procedure was used to prepare each plant extract of the three individual herbs: *Sophora flavescens*, *Sinomenium acutum*, and *Phellodendron amurense*. All of the samples were filtered through a 0.45 μm filter before HPLC analysis.

HPLC-UV Analysis

A Waters-2695 Alliance HPLC instrument (Waters Corporation, Milford, MA, USA), equipped with an on-line degasser, an auto-sampler, and a 2996 photo-diode array detector, was used. The separation was carried out on a Phenomenex Gemini C_{18} column (250 mm × 4.6 mm, 5 μm). The mobile phase A was ammonium acetate (10 mM) in water; and mobile phase B was methanol-acetonitrile (1:3, v/v) containing 10 mM ammonium acetate and 0.2% ammonia. The gradient program is presented in Table 1. The detection wavelength was 215 nm, the solvent flow rate was 1.0 mL/min, the column temperature was set at 35°C and the injection volume was 10 μL.

TABLE 1 Solvent Gradient Program of HPLC Analysis

Time (min)	A (%)	B (%)
0	95	5
35	83	17
60	73	27
70	70	30
90	68	32
105	60	40
110	60	40
120	5	95

HPLC—DAD—ESI-MS^2 Analysis

For HPLC—DAD—ESI-MS analysis, an API 3000 triple quadrupole ion trap mass spectrometer (MDS Sciex/Applied Biosystems, CA, USA) was connected on an Agilent 1100 HPLC instrument (Angilent, USA) coupled with a quaternary solvent delivery system, an autosampler and a diode array detector via an ESI interface. The chromatographic conditions were as described previously. The ESI conditions were as follows: capillary voltage, 5.5 kV; source temperature, 500°C; nebulizer gas, 8 Ll/min; curtain gas, 12 L/min; declustering potential, 80 V; focusing potential, 400 V; entrance potential, 10 V; collision energy, 52 V. For full scan ESI-MS analysis, the spectra were recorded in the range of 100–1000 in positive ion mode. MS^2 data were acquired in the automatic data-dependent mode.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

The selection of mobile phase was a critical factor in achieving good chromatographic behavior (peak shape and resolution). Because the ingredients in the sample could not be separated with isocratic HPLC elution, gradient elution was carried out. A comparison of the modifiers acetic acid and ammonia, alone or in combination, in different concentrations was performed. Better peak shape was obtained when 10 mM ammonium acetate was dissolved in the aqueous phase. The baseline drifted until the same amount of ammonium acetate was added into the organic eluent. The optimal mobile phase, consisting of solvent A (10 mM ammonium acetate) and solvent B (methanol-acetonitrile 1:3 (v/v) containing 10 mM ammonium acetate and 0.2% ammonia), was subsequently employed leading to good resolution and satisfactory peak shape. The representative chromatogram is shown in Figure 1. Baseline separation of the major constituents was obtained.

DAD detection was employed at the wavelength range of 200–400 nm and the UV spectra of sample were investigated. It was found that the

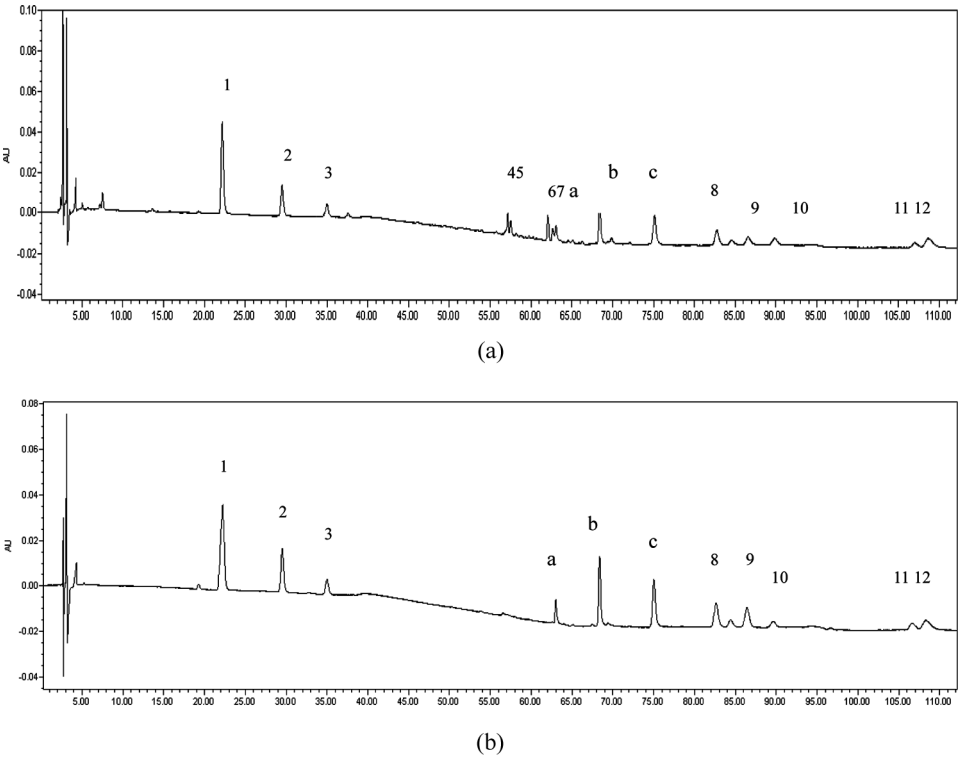

FIGURE 1 HPLC-UV chromatograms of (a) QLF and (b) 8 mixed bioactive markers with the detection at 215 nm: (1) magnoflorine; (2) oxymatrine; (3) oxysophocarpine; (8) sinomenine; (9) sophocarpine; (10) matirine; (11) palmatine; and (12) berberine. a, b, and c: impurities in the solvent.

chromatogram at 215 nm could properly represent the profile of the constituents in QLF. Therefore, the UV wavelength was set at 215 nm, where all the marker compounds had adequate absorption. In addition, it was found that the separation was better when the column temperature was kept at 35°C rather than 25, 30, or 40°C.

By comparing the chromatogram of QLF with those of its composition plant's extracts, the plant derivation of each peak was confirmed in terms of the retention time and UV spectra achieved from the DAD detection. It was found that all peaks were attributed to *Sophora flavescens*, *Sinomenium acutum*, and *Phellodendron amurense*.

Identification of the Bioactive Markers in QLF

In order to identify the structures of the main constituents in QLF, the sample was analyzed by HPLC-MS techniques. ESI in both negative and positive ion mode was tried. The results showed that ESI in positive ion

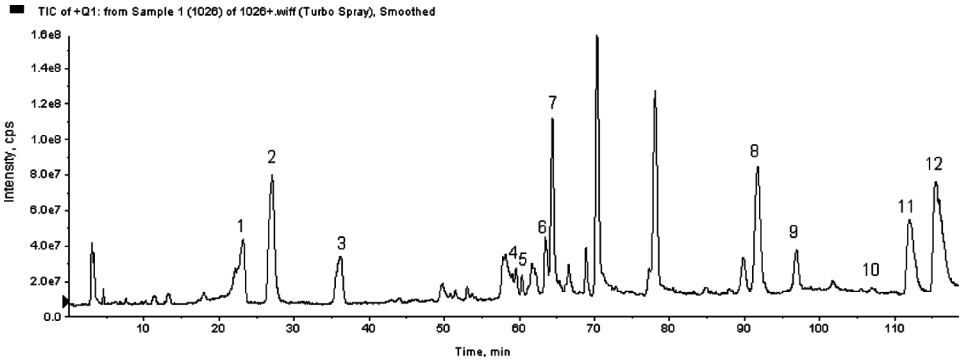

FIGURE 2 HPLC-MS TIC chromatogram of QLF in positive ion mode.

mode was sensitive to alkaloids. The major constituents in QLF were well detected (shown in Figure 2), most of the investigated compounds exhibited quasi-molecular ions $[M+H]^+$ or adducted ions $[M+Na]^+$ in positive ion mode. Based on the m/z value, UV spectra and the comparison with standard compounds, eight peaks were unambiguously identified as magnoflorine (1), oxymatrine (2), oxysophocarpine (3), sinomenine (8), sophocarpine (9), matirine (10), palmatine (11), and berberine (12). Another four peaks were tentatively identified as menisporphine (4), stepharanine (5), menisperine (6), and lupanine (7) by comparing their m/z value and UV spectra with the literature data. In addition, peaks, eluted at 63.05, 68.34, 75.07, and 84.76 min, respectively, in Figure 1, could be assigned as impurities from mobile phase by means of blank analysis and LC-MS identification. The results are listed in Table 2

TABLE 2 HPLC-ESI-MS/MS Data and Identification of Compounds[a]

Peak no.	Retention Time (min)	MS (m/z)	MS^2 (m/z)	Crude Drug	Identification
1	22.17	342 $[M]^+$	297, 237, 58	(2), (3)	magnoflorine
2	29.50	265 $[M+H]^+$	247, 206, 162	(1)	oxymatrine
3	35.00	263 $[M+H]^+$	246, 204, 178, 161, 151, 137	(1)	oxysophocarpine
4	57.15	322 $[M+H]^+$	265, 247	(2)	menisporphine
5	57.50	324 $[M]^+$	286, 269	(2)	stepharanine
6	62.64	356 $[M]^+$	236, 192, 58	(2)	menisperine
7	63.47	249 $[M+H]^+$	150, 110, 98, 97, 84	(1)	lupanine
8	82.76	330 $[M+H]^+$	223, 192, 181, 107	(2)	sinomenine
9	86.59	247 $[M+H]^+$	176, 148, 122	(1)	sophocarpine
10	89.85	249 $[M+H]^+$	177, 176, 150, 148, 98	(1)	matrine
11	106.94	352 $[M]^+$	336, 294	(3)	palmatine
12	108.65	336 $[M]^+$	320, 292, 278	(3)	berberine

[a]The number in crude drug item means which crude drug the ingredient belongs to: (1) *Sophora flavescens*, (2) *Sinomenium acutum*, and (3) *Phellodendron amurense*.

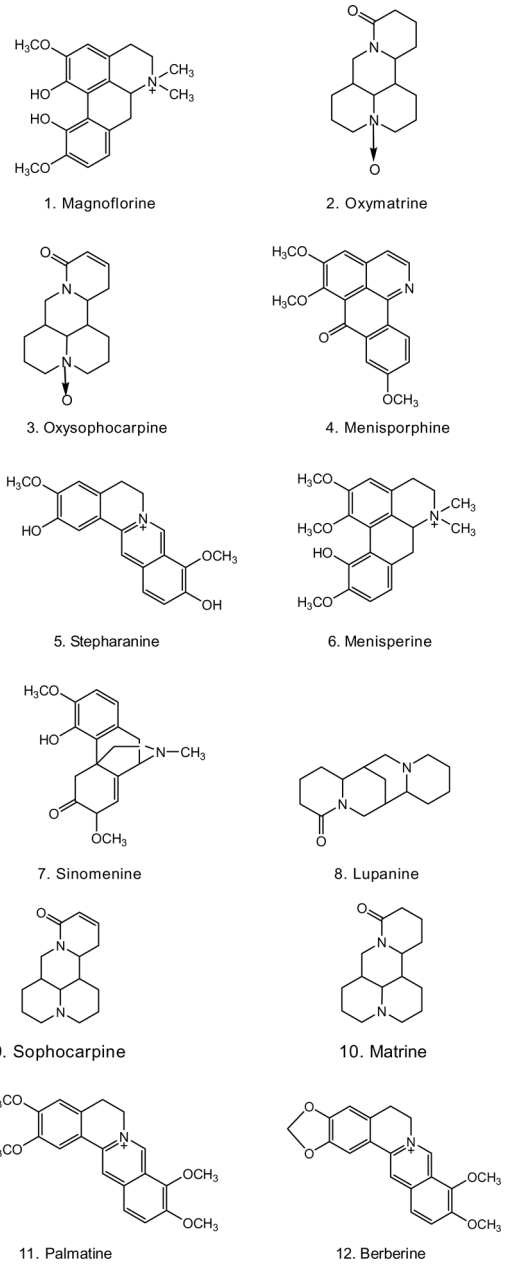

FIGURE 3 Structures of the constituents identified from QLF.

and the structures of these compounds are shown in Figure 3. It was shown that the main types of the bioactive constituents in QLF detected in this assay were alkaloids.

Validation of the Quantitative Analysis

Linearity, Limits of Detection, and Limits of Quantification

The linear calibration curves were constructed by at least six different concentrations of chemical markers. Each concentration was analyzed in triplicate. The limits of detection (LOD) and limits of quantification (LOQ) were measured on the basis of the signal-to-noise ratio of 3 and 10 as criteria, respectively. Good linear correlation and high sensitivity at these chromatographic conditions were confirmed by the correlation coefficients ($R^2 > 0.999$) for LOD (0.63–6.70 ng/mL) and LOQ (2.01–20.69 ng/mL) (Table 3).

Accuracy, Precision, and Repeatability

The mixture standard solution was analyzed under the optimal conditions five times in 1 day for intra-day variation and on 3 successive days for inter-day variation to evaluate the precision and accuracy. The intra- and inter-day precisions were within 2.74% and 2.82%, respectively, with accuracy from 97.4% to 102.7%. In order to check the repeatability, five different solutions were made from the same sample (S1). The R.S.D. of repeatability was less than 2.33%. These results indicate that the developed method has acceptable precision, accuracy, and repeatability (Table 4).

Recoveries

In order to evaluate the recovery of the method, three different concentration levels (approximately equivalent to 0.8, 1.0, and 1.2 times the concentration of the matrix) of the reference standards were added into

TABLE 3 Linear Regression Equation Analysis in the Determination of the Eight Compounds

Peak no.	Compounds	Regression Equation	Linear Range ($\times 10^3$ ng)	r^2	LOD (ng/mL)	LOQ (ng/mL)
1	magnoflorine	$y = 3.46 \times 10^6 x - 5.56 \times 10^4$	0.0222–2.59	0.9991	0.63	2.01
2	oxymatrine	$y = 1.05 \times 10^6 x - 9.39 \times 10^4$	0.0316–3.68	0.9991	2.60	8.34
3	oxysophocarpine	$y = 1.25 \times 10^6 x - 2.12 \times 10^4$	0.0115–1.07	0.9992	3.03	9.53
8	sinomenine	$y = 1.45 \times 10^6 x - 9.89 \times 10^4$	0.0168–1.96	0.9997	2.03	6.10
9	sophocarpine	$y = 1.47 \times 10^6 x - 3.11 \times 10^3$	0.00576–0.538	0.9998	1.51	4.80
10	matrine	$y = 1.14 \times 10^6 x - 6.18 \times 10^2$	0.0188–2.19	0.9992	6.70	20.69
11	palmatine	$y = 1.42 \times 10^6 x - 3.08 \times 10^3$	0.0493–0.575	0.9993	1.00	2.74
12	berberine	$y = 1.12 \times 10^6 x + 1.38 \times 10^4$	0.00482–0.563	0.9997	0.96	2.68

TABLE 4 Statistical Results of Precision, Accuracy, and Repeatability of the Eight Components

Peak no.	Compounds	Intra-day		Inter-day		Repeatability R.S.D. (%) (n = 6)
		Accuracy R.E. (%)	Precision R.S.D. (%)	Accuracy R.E. (%)	Precision R.S.D. (%)	
1	magnoflorine	98.9	2.460	100.8	2.69	1.23
2	oxymatrine	100.6	0.945	100.9	2.82	0.90
3	oxysophocarpine	101.3	1.290	100.1	2.59	2.33
8	sinomenine	98.6	1.440	98.9	2.17	0.73
9	sophocarpine	102.6	2.530	97.4	2.67	1.32
10	matrine	99.1	2.740	102.7	1.76	2.11
11	palmatine	102.4	2.380	98.8	2.47	1.06
12	berberine	97.6	1.210	102.3	1.20	0.87

the S1 sample in triplicate. The solutions were extracted and quantified as described previously. The results show that the assay is satisfactory with a mean recovery from 95.24% to 105.50% with R.S.D. less than 4.84% for the eight components (Table 5).

TABLE 5 Recovery of the Eight Components in QLF (n = 3)

Peak no.	Compounds	Added ($\times 10^3$ g)	Detected ($\times 10^3$ g)	Recovery[a] (%)	R.S.D.[b] (%)
1	magnoflorine	0.1100	0.113 ± 5.14	102.73	4.55
		0.1380	0.133 ± 5.84	96.38	4.39
		0.1650	0.171 ± 5.84	103.64	3.42
2	oxymatrine	0.1380	0.138 ± 2.40	100.00	1.74
		0.1730	0.176 ± 2.90	101.73	1.65
		0.2070	0.200 ± 2.44	96.62	1.22
3	oxysophocarpine	0.0423	0.0439 ± 2.00	103.78	4.56
		0.0528	0.0536 ± 1.50	101.52	2.80
		0.0634	0.0658 ± 6.43	103.79	0.98
8	sinomenine	0.0805	0.0836 ± 1.80	103.85	2.15
		0.1010	0.102 ± 1.73	100.99	1.70
		0.1210	0.116 ± 1.78	95.87	1.53
9	sophocarpine	0.0484	0.0485 ± 1.08	99.79	2.23
		0.0606	0.0616 ± 1.36	101.65	2.21
		0.0727	0.0711 ± 0.69	97.80	0.97
10	matrine	0.0403	0.0388 ± 1.44	96.28	3.71
		0.0504	00480 ± 0.87	95.24	1.81
		0.0605	0.0581 ± 1.23	96.03	2.12
11	palmatine	0.0241	0.0252 ± 1.20	104.56	4.76
		0.0301	0.0314 ± 1.38	104.32	4.39
		0.0362	0.0376 ± 1.71	103.87	4.55
12	berberine	0.0729	0.0746 ± 3.61	102.33	4.84
		0.0911	0.0951 ± 3.79	104.39	3.99
		0.1090	0.115 ± 4.23	105.50	3.68

[a][(Mean of measured concentration-spiked concentration)/spiked concentration] × 100.
[b](S.D./mean) × 100.

TABLE 6 Contents of the Eight Components in QLF (n = 3)

	Contents of the Compounds (mg/g)							
Sample no.	1	2	3	8	9	10	11	12
1	2.378 ± 0.037	2.999 ± 0.044	0.900 ± 0.009	1.756 ± 0.031	1.040 ± 0.017	0.746 ± 0.022	0.554 ± 0.018	1.776 ± 0.082
2	2.307 ± 0.019	2.880 ± 0.019	0.945 ± 0.025	1.781 ± 0.057	1.108 ± 0.035	0.771 ± 0.026	0.606 ± 0.027	1.924 ± 0.045
3	2.289 ± 0.038	2.936 ± 0.044	0.873 ± 0.036	1.699 ± 0.035	1.023 ± 0.026	0.776 ± 0.019	0.573 ± 0.036	1.817 ± 0.052

Sample Analysis

The established analytical method was successfully applied simultaneously to determine the contents of the eight constituents in three batches of QLF. The results are shown in Table 6. It is clear that these alkaloids make up considerable percentage of QLF and possess a number of pharmacological activities. Hence, the contents of the alkaloid constituents play a vital role in the quality evaluation of QLF products.

CONCLUSIONS

An HPLC—DAD—ESI-MS method has been established, which can be used to simultaneously separate, identify, and determine major constituents in an antiangiogenesis herbal formula, QLF. Twelve compounds were identified. Eight of these compounds were quantitated, with a method that presented acceptable linearity, accuracy, precision, repeatability, and recovery. The method can be applied as a convenient, effective technique to control the quality of QLF products. This study provides an approach to developing a bioactive chromatographic profile of major constituents to ensure the quality of QLF products. Since multiple constituents are responsible for the therapeutic effects of TCM and its preparations, the ingredients and their contents in TCM may have an extreme affect on its therapeutic effect. The experimental results strongly show that it is important to systematically control the content of bioactive compounds in TCM and its preparations so as to insure its therapeutic effects in clinical applications.

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