SUPPLEMENTARY MATERIAL

Simultaneous determination of seven bioactive components in Guizhi Fuling capsule by microwave-assisted extraction combined with ultra performance liquid chromatography tandem mass spectrometry

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ABSTRACT

A simple, rapid and reliable microwave-assisted extraction (MAE) combined with ultra performance liquid chromatography tandem mass spectrometry method (UPLC-M/MS) was developed for simultaneous determination of the seven bioactive constituents in Guizhi Fuling capsule (GFC), namely gallic acid, amygdalin, albiflorin, paeoniflorin, paeonol, cinnamic acid and pachymic acid, respectively. The operation of MAE optimized through orthogonal array design experiment was performed at

80°C for 10min with methanol-water (70:30, v/v) as the extracting solvent. The chromatographic separation was obtained by Waters ACQUITY UPLC[®] BEH C₁₈ (50 mm×2.1 mm, 1.7 μm) with a gradient elution of acetonitrile and 0.1% (v/v) formic acid at a flow rate of 0.2 mL/min. The seven analytes were simultaneously determined by a triple quadrupole tandem mass spectrometer in multiple reaction monitoring (MRM) mode under positive and negative electrospray ionization mode for the first time. The method was validated including intra- and inter-day precision, repeatability and stability, with RSD less than 3.9%, 3.3%, 4.4% and 3.1%, respectively. All analytes showed good linearity in linear ranges ($r > 0.999$), and their average recoveries varied between 98.2% and 101.2%. The results indicated that this method was simple, effective and suitable for multi-component determination quantitative analysis and quality control of GFC.

Keywords: MAE; UPLC-MS/MS; Quantitative analysis; GFC

1. Experimental

1.1 Materials

Guizhi Fuling capsule (GFC) (130713, 131231, 140309) was purchased from Kanion Pharmaceutical Co.Ltd. (Jiangsu,China). The reference standard of gallic acid (MUST-13040103), cinnamic acid (MUST-13090903) was purchased from Chengdu Must Bio-technology Co.,Ltd. (Sichuan,China). Amygdalin (Lot No.131028), albiflorin (Lot No.130518) were obtained from Chengdu Must Bio-technology

Co.,Ltd. (Sichuan,China). Paeoniflorin, paeonol, pachymic acid were isolated and purified in our laboratory; their structures were confirmed by UV, IR, MS, ^1H -NMR and ^{13}C -NMR. The purity of all these standards was determined to be over 98% by HPLC-DAD analysis.

1.2 Reagents

HPLC-grade acetonitrile, methanol were purchased from Tedia (Fair-field, OH, USA). HPLC-grade formic acid was purchased from Dikmapure (Richmond Hill, NY, USA). Water was purified by redistillation and filtered through a 0.22 μm membrane filter before use.

1.3 Apparatus

UPLC-MS/MS system consisted of a triple-quadrupole tandem mass spectrometer (Micromass[®] Quattromicro[™] API mass spectrometer, Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) source, an Acquity[™] UPLC system equipped with a cooling auto-sampler, a quaternary solvent delivery system and a column temperature controller (Waters Corp., Milford, MA, USA). All data were collected and analyzed using MassLynx[™] NT 4.1 software with a QuanLynx[™] program (Waters Corp., Milford, MA, USA). The chromatographic separation of the analytes was carried out on a Waters ACQUITY UPLC[®] BEH C₁₈ (50 mm×2.1 mm, 1.7 μm) with an on-line filter in front of the column.

1.4 Chromatographic-mass spectrometric conditions

The separation of seven compounds was performed on a linear gradient elution using a mobile phase containing 0.1% formic acid (A) and acetonitrile (B). The method

used gradient elution with a total run time of 8 min, the gradient program was as follows: 0–3.5 min, 15% B; 3.5–4.0 min, linear gradient 15–55%B; 4.0–8.0 min, linear gradient 55–90% B. After a 3 min equilibration period, the samples were used for injection. The solvent flow rate was kept at 0.2 mL/min, the autosampler temperature and the analytical column were set at 4°C and 20°C, respectively. Injection volume was set at 20µL. Representative chromatograms were shown in Fig.S1. The mass spectrometer parameters were as follows: desolvation temperature of 400°C. Nitrogen was used as the desolvation and cone gas at a flow rate of 450 and 30 L/h, respectively. Argon was used as the collision gas in all cases. The analyte detection was performed by using MRM mode at precursor→product transitions of gallic acid for 169.0→124.7, amygdalin for 456.2→323.2, albiflorin for 479.4→120.7, paeoniflorin for 479.3→449.4, cinnamic acid for 146.9→102.7, paeonol for 167.1→120.7 and pachymic for 527.3→465.6, verapamil(IS) for 455.4→165.0. Verapamil was chosen as the internal standard due to its similarity in the retention and ESI ionization to those of the analytes. The results are shown in Fig. S1.

Fig. S2. Representative MRM chromatograms of standards (a) and sample (b) .

1.5 Preparation of standard solutions

Standard stock solutions of the seven reference standards (gallic acid, amygdalin, albiflorin, paeoniflorin, paeonol, cinnamic acid, pachymic acid) were prepared by dissolving accurately weighed amount of standard compounds in methanol. They were diluted to a series of appropriate concentrations for construction of calibration curves in the ranges of gallic acid 0.5053-6.064 $\mu\text{g/mL}$, amygdalin 1.006-12.07 $\mu\text{g/mL}$, albiflorin 0.9962-11.95 $\mu\text{g/mL}$, paeoniflorin 0.5003-6.003 $\mu\text{g/mL}$, cinnamic acid 0.05015-0.6018 $\mu\text{g/mL}$, paeonol 0.5050-6.060 $\mu\text{g/mL}$, pachymic acid 0.05000-0.6000 $\mu\text{g/mL}$, respectively. Stock solutions of the internal standards were prepared by dissolving accurately weighed amount of standard compounds in methanol. All solutions were stored at 4 °C in a refrigerator before injection.

1.6 Preparation of sample

Sample preparation was performed with MAE. In brief, the powder from GFC (about 0.25g) accurately weighed was transferred into a 100 mL Teflon-lined extraction vessel and 25 mL 70% methanol was added. The extraction was performed at 80 °C for 10 min for one cycle under the microwave power of 400 W, and then the same solvent was added to compensate for the lost weight during the extraction. After the process of extraction, the vessel was cooled down to the ambient temperature before being opened. The sample solution of 5 mL was diluted to 100 mL with 70% methanol in 100 mL volumetric flask. The resultant mixture was centrifuged at 13000 rpm for 10 min, and then the supernatant was collected and filtered through a 0.22 μm filter before sample injection.

1.7 Method validation

1.7.1 Calibration curves

The standard curves were prepared under six different concentration levels of the seven compounds and two injections were made at each level. All calibration curves were constructed by plotting the peak area ratio of analyte/IS versus the analyte concentration of the corresponding working standard solution.

1.7.2 Precision

Intra- and inter-day variations were utilized to evaluate the precision of the developed method. The intra-day precision was investigated for a mixed standard solution of seven analytes using six replicates within one day, and inter-day precision was determined in duplicates over consecutive three days. Inter- and intra-day precisions for all investigated analytes were expressed by relative standard deviations (RSDs).

1.7.3 Reproducibility and stability

To evaluate the repeatability of the analysis method, six samples were extracted and analyzed followed the mentioned procedure, variations were expressed by relative standard deviations (RSDs). The stability of the sample at ambient temperature was analyzed by replicate assays of a freshly prepared sample solution at 0, 2, 4, 6, 8, 12 and 24 h. RSDs of the stability were evaluated in term of the peak area ratio of analyte/IS.

1.7.4 Accuracy

The test for recovery was performed to examine the accuracy of the method. A certain amounts (about 50% of the content) of the seven standard substances were spiked into a certain amount of sample of GFC (0.125g), which were extracted and analyzed as

mentioned above in six replicates. For comparison, an unspiked sample was prepared and analyzed simultaneously.

2. Results and Discussion

2.1 Method validation

2.1.1 Linearity

All calibration curves of the seven analytes were gained with good linear relationship and the correlation coefficients of all the calibration curves were found to be higher than 0.999. The results are shown in Table S1.

Table S1

Calibration curves for the target analytes (n=6).

Analyte	Regression equation	r	Linear range (µg/ml)
Gallic acid	$y = 1.4587x + 0.5071$	0.9993	0.5053-6.064
Amygdalin	$y = 0.2173x + 0.175$	0.9993	1.006-12.07
Albiflorin	$y = 0.4036x + 0.1619$	0.9992	0.5003-6.003
Paeoniflorin	$y = 0.1194x + 0.1398$	0.9990	0.9962-11.95
Cinnamic acid	$y = 0.4715x + 0.0104$	0.9992	0.05015-0.6018
Paeonol	$y = 0.7939x + 0.1871$	0.9992	0.5050-6.060
Pachymic acid	$y = 0.1509x + 0.0013$	0.9991	0.05000-0.6000

2.1.2 Precision, repeatability, stability and accuracy

The present method established to have an acceptable level of precision, repeatability, stability and accuracy of all analytes. The intra-day and inter-day precision RSD values of the seven active constituents were in the ranges 2.2-3.9% and 1.7-3.3%, respectively, demonstrating that the developed analytical method was sensitive enough for the quantitative evaluation of the analytes. RSD values of the analysis repeatability ranged from 2.0% to 4.4% indicated that the developed analytical method was reproducible for all analytes. The sample solution was found to be stable within 24h and RSD values were less than 3.1%. The recovery test was in the range of

98.22-101.2 and RSD values were lower than 4.8, which indicated that the established method was sufficiently reliable and accurate for the determination of all analytes.

The results are shown in Table S2.

Table S2

Precision, repeatability, stability and recoveries for the target analytes.

Analyte	Precision		Repeatability (RSD%, n=6)	Stability (RSD% , n=6)	Recovery(%)	Recovery (RSD%, n=6)
	Intra-day (RSD%, n=6)	Inter-day (RSD%, n=3)				
Gallic acid	2.9	1.9	3.0	1.6	99.82	2.0
Amygdalin	3.8	2.2	3.3	2.7	98.83	3.6
Albiflorin	3.1	3.3	2.0	2.2	101.1	3.4
Paeoniflorin	2.2	2.1	3.7	2.5	101.2	2.8
Cinnamic acid	3.9	2.4	4.4	3.1	99.32	3.9
Paeonol	3.2	2.2	4.1	2.9	100.2	3.2
Pachymic acid	2.4	1.7	3.6	1.9	98.22	4.8

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

2.2 Optimization of MAE conditions

Many factors of MAE can affect the efficiency of extraction of active ingredients of GFC. A four-factor, three-level orthogonal array design (OAD) L9 (3⁴) was selected as a chemometric method for investigating the effects of the following factors on the extraction efficiency of pachymic acid: methanol concentration (A), solid-to-solvent ratio (B), extraction time (C), and extracting temperature (D). The four most important variables affect the extraction efficiency of pachymic acid which were set as follows: factor A, methanol concentration (60, 70 and 80%, v/v); factor B, solid to solvent ratio (1:60, 1:80 and 1:100, w/v); factor C, extraction time (5, 10 and 15 min); factor D, extracting temperature (40, 60, 80°C). All parameters (A–D) were tested in a wider

range prior to OAD optimization, which narrowed down the ranges of the parameters tested. The OAD L9 (3^4) matrix used for optimization and the three levels of factor are depicted in Table S3. All samples were tested in triplicate.

Table S3

Factors in the orthogonal design for the optimization of MAE extraction conditions.

Run no.	A: concentration of methanol (%)	B: solvent to solid ratio (mL/g)	C: extraction time(min)	D: extraction temperature (°C)	Total content of Pachymic acid (mg/g)
1	60	80	5	40	0.3914
2	60	90	10	60	0.4167
3	60	100	15	80	0.4616
4	70	80	10	80	0.5832
5	70	90	15	40	0.5390
6	70	100	5	60	0.5723
7	80	80	15	60	0.4006
8	80	90	5	80	0.4374
9	80	100	10	40	0.4523
k ₁ ^a	0.423	0.458	0.467	0.461	
k ₂	0.565	0.464	0.484	0.463	
k ₃	0.430	0.495	0.467	0.494	
Range	0.142	0.037	0.017	0.033	
Optimized scheme	A ₂	B ₃	C ₂	D ₃	

^ak represents the average values of the same level of the same factor.

It was showed that the maximum range of the three levels was 0.142 for factor A and the smallest was 0.017 for factor C, which admitted that factor A (methanol concentration) was the significantly variable in the extraction conditions of pachymic acid in GFC. k_1 , k_2 and k_3 represent the average measured contents of pachymic acid for each factor at each level. Thus, the optimum experimental conditions were achieved $A_2B_3C_2D_3$.

To indicate whether the effect of individual factors on MAE efficiency is statistically significant, an analysis of variance (ANOVA) was used to explain the experimental data obtained from the OAD optimization. The significance of each factor was estimated by calculating the F value and the results were shown in Table S4. As can be seen, the impact by the parameters on the mean extraction yields of pachymic acid decreased in the sequence of A (methanol concentration) > B (solid-to-solvent ratio) = D (extraction temperature) > C (extraction time) according to the F values. The methanol concentration was considered significant at $p < 0.05$. Based on this analysis result and the feasibility of experiment, the optimum conditions of extraction were determined as follows: extraction solvent 70% methanol, extraction time 10 min, extraction temperature 80°C, solvent to solid ratio 1:100, microwave power 400 W, and extraction cycle one.

Table S4

F values obtained from ANOVA results.

Factors	Sum of squares	$F_{0.05}$	F value
methanol concentration	0.038	6.940	25.333*
solid-to-solvent	0.002	6.940	1.333
extraction time	0.001	6.940	0.667
extraction temperature	0.002	6.940	1.333

* $p < 0.05$

2.3 Optimization of chromatographic conditions

The optimization of chromatographic conditions was guided by the requirement of obtaining chromatograms with better resolution of adjacent peaks within a short analysis time, especially when isomer of the two analytes were to be analyzed. The chromatographic conditions were optimized systematically to improve the separation of the analytes. Different mobile phase compositions were assessed to increase the baseline separation and sensitivity for detection of the analytes. It was observed that the presence of acetonitrile in the mobile phase generated higher response and better peak shapes compared to methanol. Therefore acetonitrile was used as the organic phase. Meanwhile, the effect of different additives (formic acid, formic acid-ammonium acetate) and column temperature were also examined, including formic acid (0.05%, 0.1%), formic acid-ammonium acetate (1mmol, 2mmol, 3mmol, 4mmol) and column temperature (20, 30 and 40°C), respectively. It was found that the presence of acetonitrile–0.1% formic acid solution by gradient elution at a flow rate of 0.2 mL/min with the column temperature of 20°C lead to a significant improvement on the better resolution of adjacent peaks within a short analysis time (8min) of the different component in GFC.

2.4 Optimization of mass spectrometric conditions

A standard solution containing the individual analyte or IS was analyzed by direct flow injection to optimize the ESI-MS/MS conditions. For MS condition, both positive and negative ionization modes were optimized. It was observed that the signal intensity of paeonol in positive ionization mode was much better than that in

negative ionization mode, which was able to form a stable protonated ion of $[M+H]^+$ in the positive full-scan mass spectra. The other analytes was able to form a stable deprotonated ion of $[M-H]^-$ in the negative full-scan mass mode, and the signal intensity in negative ionization mode was much better than that in positive ionization mode. MRM mode was used to monitor both quasi-molecule and fragment ions, which made the detection more specific. The MS parameters such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rate of desolvation gas and cone gas were optimized to obtain the highest intensity for the analytes. In this study, cross-talk effect produced using different channels in mass system was not observed. The results are shown in Table S5.

Table S5

MS conditions for the seven analytes and IS.

Compound	Transition ions	Capillary voltage (kV)	Cone voltage(V)	Collision energy (eV)
Gallic acid	169.0→124.7	3(-)	30	15
Amygdalin	456.2→323.1	3(-)	40	15
Albiflorin	479.4→120.7	3(-)	35	16
Paeoniflorin	479.3→449.4	3(-)	30	10
Cinnamic acid	147.0→102.6	3(-)	30	13
Paeonol	167.0→120.7	1(+)	25	20
Pachymic acid	527.2→465.4	3(-)	45	50
Verapamil (IS)	455.2→165.2	1(+)	30	25

2.5 Sample analysis

The established analytical method was successfully applied to the simultaneous analysis of the seven active components (gallic acid, amygdalin, albiflorin, paeoniflorin, cinnamic acid, paeonol, pachymic acid) of GFC. The contents of the seven analytes in the samples were quantified and the results were listed in Table S6 with the mean content of three replicate analysis (n=3).

Table S6

Quantitative analytical results for the target analytes in GFC. (n = 3, mg/g).

Analytes	130713	131231	140309
Gallic acid	4.359±0.155	4.335±0.127	4.263±0.143
Amygdalin	3.151±0.090	3.170±0.072	3.254±0.056
Albiflorin	1.318±0.036	1.295±0.043	1.361±0.031
Paeoniflorin	10.460±0.065	10.403±0.044	10.447±0.049
Paeonol	6.245±0.040	6.192±0.036	6.207±0.042
Cinnamic acid	0.206±0.006	0.211±0.004	0.220±0.003
Pachymic acid	0.156±0.005	0.161±0.005	0.148±0.006