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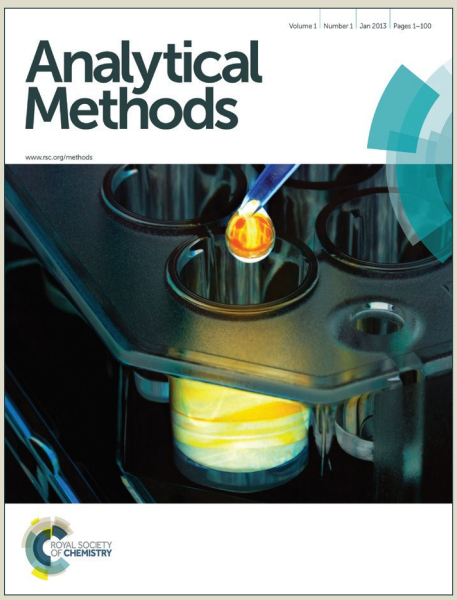


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# A hierarchical cluster analysis of ten index constituents based on microwave-assisted extraction by UHPLC-MS/MS for the evaluation and quality control of Cortex Juglandis Mandshuricae

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## ABSTRACT:

A novel and rapid microwave-assisted extraction (MAE) was optimized and compared with that of ultrasound-assisted extraction and heat reflux extraction for the quality control of ten index constituents in Cortex Juglandis Mandshuricae (CJM) based on a developed ultra-high performance liquid chromatography-tandem mass spectrometry method. The operation of MAE optimized through orthogonal array design experiments was performed at 70°C for 8 min with ethanol-water (70:30, *v/v*) as the extracting solvent. The chromatographic separation was completed on a Waters ACQUITY UPLC® BEH Phenyl (50 mm×2.1 mm, 1.7 μm) with a gradient elution of acetonitrile and 0.1% (*v/v*) aqueous formic acid at a flow rate of 0.2 mL/min. The method developed was validated with acceptable linearity ($r > 0.999$), intra- and inter-day precision, reproducibility, and extraction recoveries, which was successfully applied to analyzing 15 batches of CJM obtained from different regions of Northern China. The results was differentiated and classified by hierarchical cluster analysis (HCA) which indicated that the influence of CJM cultivation regions on the contents of index constituents was very obvious. The developed procedure is a promising analytical tool for the overall quality control of CJM.


**Keywords:** Cortex Juglandis Mandshuricae; Microwave-assisted extraction; Quality control; UHPLC-MS/MS; HCA

## 1. Introduction

Cortex Juglandis Mandshuricae (CJM) is the bark of *Juglans Mandshurica* Maxim. (JMM), which belongs to the Juglandaceae family and is widely distributed in the northern part of China.[1] For hundreds of years, CJM have been used as a folk medicine for the treatment of cancer, diarrhea and dysentery.[2,3] Modern phytochemical investigations have revealed that quinones, flavonoids, phenolics are the major bioactive constituents in CJM.[4-9] Due to the presence of these compounds, CJM have been reported to possess various pharmacological activities, such as anti-oxidant,[10-12] anti-tumor,[13] anti-inflammatory,[14] anti-HIV,[15] and anti-parasitic actions.[16] The above listed constituents are believed to be the active components in CJM, and could be considered as the 'marker compounds' for the chemical evaluation or standardization of CJM. So far, there are very few reports regarding the quantification of the active components in CJM for quality control, including determination of juglone by ultraviolet spectrophotometry (UV)[17] and assay of two or three ingredients by high performance liquid chromatography-ultraviolet (HPLC-UV) method.[18-19] It was well known that the content of a single or a few marker compounds might not accurately reflect the intrinsic quality and response for the overall pharmacological activities of the complex herbal products. Thus, a comprehensive and efficient quality control approach based on the bioactive compounds of CJM is urgently needed to ensure the efficacy and safety of this herbal medicine.

As known, effective extraction is one of the key steps in the investigation and utilization of target compounds from botanical materials. Currently, soxhlet extraction (SE), heat reflux extraction (HRE) and ultrasound-assisted extraction (UAE)[20] were applied for the extraction of active constituents from CJM. However, they are often time-consuming, requiring bulk volume of extraction solvent, which result in lower extraction yield, increase cost, and generate environmental pollution.[21] Therefore, an innovative extraction technique that could

solve some of the above mentioned problems is imperative. As an alternative, microwave-assisted extraction (MAE) has been proved to reduce the volume of the extraction solvent, shorten the extraction time, improve the reproducibility and recovery of the analytes and increase sample throughput in comparison to conventional techniques mentioned above.[22-25] On the other hand, a variety of constituents in CJM exists in an extremely complicated matrix and some bioactive components have weak or no ultraviolet response, which makes it impossible to simultaneously determine multi-components in CJM by using common HPLC with ultraviolet light detector or evaporative light scattering detector. The emergence of ultra-high chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) allows us to solute above mentioned difficulties with shorter analysis time, greater resolution, higher peak capacity, less solvent consumption and extremely high sensitivity.

In the present work, a simple and rapid method based on MAE combined with UHPLC-MS/MS technique was selected and developed for the simultaneous determination of ten bioactive components in the CJM, including two quinones (juglone, 5,8-dihydroxy-1,4-naphthoquinone), seven flavonoids (myricitrin, quercitrin, taxifolin, myricetin, quercetin, naringenin and kaempferol) and one phenolic (gallic acid), which are the representative compounds of three types in CJM. Their structures are listed in Fig.1. The MAE extraction conditions were optimized through orthogonal array design experiments. Fast UHPLC separation with sensitive detection by ESI-MS/MS using a triple quadrupole instrument in MRM mode was employed and excellent sensitivity and selectivity were obtained. Fifteen batches of CJM from different sources were analyzed by hierarchical cluster analysis (HCA) for the quality control of CJM. As such, this approach provides promising for use in the quality control and for references in the pharmacological and pharmacokinetic study of Cortex Juglandis Mandshuricae.

<Fig. 1>

## 2. Materials and methods

### *2.1. Materials and samples*

Standards of gallic acid, myricetrin, quercetrin, taxifolin, myricetin, quercetin, kaempferol, naringenin and chloromycetin (used as Internal Standard, IS) were purchased from Chengdu Must Bio-technology Co., Ltd. (Sichuan, China). 5,8-dihydroxy-1,4-naphthoquinone was obtained from Johnson Matthey Company (Royston, England). Juglone was purchased from Sigma-Aldrich (Colorado, USA). The purity of standard compounds was higher than 97% by peak area normalization method detected by HPLC-DAD. Acetonitrile and formic acid of HPLC grade were obtained from Fisher Scientific (Pittsburgh, PA, USA). Methanol of HPLC grade was purchased from Yuwang Industrial Co., Ltd. (Shandong, China). HPLC-grade water was purified using a Milli-Q Reagent Water system (Millipore, Bedford, MA). Other chemicals and solvents were all of analytical grade.

Fifteen samples of CJM were collected from different regions in the north part of China and were identified by Professor Ying Jia, Department of Traditional Chinese Medicine, Shenyang Pharmaceutical University (Shenyang, China), by means of the morphological characteristics. The sample labeled as S1 was used for the optimization of the extraction conditions.

### *2.2. Preparation of standard solutions and internal standard solution*

Stock solutions were separately prepared by dissolving the accurately weighed ten standard reference compounds with methanol. A mixed stock solution was obtained by mixing all the ten stock solutions above, and giving a final concentration of 28.6 μg/mL for gallic acid, 20.8 μg/mL for myricitrin, 21.6 μg/mL for quercitrin, 28.8 μg/mL for taxifolin, 10.4 μg/mL for myricetin, 2.3 μg/mL for quercetin, 31.0 μg/mL for naringenin, 2.02 μg/mL for kaempferol,

22.72 μg/mL for 5,8-dihydroxy-1,4-naphthoquinone, 421.6 μg/mL for juglone, respectively. A series of working standard solutions were prepared by the successive dilution of the mixed stock solution with acetonitrile–water (30:70, *v/v*). The internal standard solution of chloromycetin was prepared at a final concentration of 1.25 μg/mL in methanol. All the solutions were stored away from light at 4°C. The solutions were brought to room temperature and filtered through 0.22 μm membrane filters before analysis.

*2.3. Preparation of sample solutions*

The dried CJM samples were cleaned manually to remove all foreign materials then powdered into a homogeneous size by a disintegrator, passed through a stainless steel sieve (40 mesh). Two conventional extraction techniques as given below were used for comparison with MAE.

*2.3.1. Microwave-assisted extraction (MAE)*

In the present study, a MAE apparatus (MDS-8G, Shanghai Sineo Microwave Chemistry Technology Co., Ltd., China) was applied for sample extraction. Accurately weighed drug powder (0.5 g) was transferred into a 100 mL Teflon-lined extraction vessel and 20 mL 70% ethanol was added in it. The vessel was then transferred into the chamber of the microwave extraction apparatus. The extraction was carried out at 70°C for 8 min for one cycle under the microwave power of 400 W. After the process of extraction, the vessel was spontaneously cooled to the room temperature before being opened.

Next, the solution was filtered and evaporated to dryness on a rotary vacuum evaporator (40°C). The residue was diluted to 25 mL with acetonitrile–water (30:70, *v/v*) in 25 mL volumetric flask after adding 0.5 mL internal standard solution and filtered through 0.22 μm filter before sample injection. The sample solutions whose concentrations exceeded the upper quantification scope were accurately diluted with acetonitrile–water (30:70, *v/v*) to an appropriate concentration within the linear scope.

*2.3.2. Ultrasound-assisted extraction (UAE)*

The materials (0.5 g) were weighed and put into a conical flask. Then 20 mL of 70% ethanol solution was added to the flask, and extracted in an ultrasonic bath at 70°C for 30 min with ultrasonic power of 150 W.

*2.3.3. Heat reflux extraction (HRE)*

An accurately weighed sample (0.5 g) of the CJM powder was extracted at 80°C for 3 h under reflux with 20 mL of 70% ethanol in a 50-mL round bottom flask heated in a water bath.

*2.4. Chromatographic and mass spectrometric conditions*

Based on our previous investigation by Du et al. and Sun et al.,[19,26] some developments have been improved for separation of the ten index constituents. Briefly, Chromatographic separation was carried out on a Waters ACQUITY UHPLC® BEH Phenyl (50 mm×2.1 mm, 1.7 μm) with an in-line filter in front of the column. The mobile phase was composed of aqueous formic acid (0.1%, *v/v*) (A) and acetonitrile (B), with a gradient elution as follows: 20% B at 0-2 min, 20–40% B at 2–3 min, 40% B at 3–6 min, 40–80% B at 6-8 min and the re-equilibration time was 3 min. The column temperature was set at 20□ and the flow rate was set at 0.2 mL/min. The injection volume was 20 μL.

Triple-quadrupole tandem mass spectrometric detection was equipped with an electrospray ionization (ESI) interface in negative ionization mode. The parameters in the source were used as the following conditions: capillary voltage was set at 3.0 kV, source temperature was maintained at 110°C, cone gas flow was 450 L/h and desolvation gas flow was 450 L/h. The pressure was set at $2.85\times10^{-3}$ mbar. Argon was used as the collision gas in all cases. The MS parameters were individually optimized for each target compound and are listed in Table1.

<Table 1>

### *2.5. Method validation*

#### *2.5.1. Linearity, LOD and LOQ*

The linearity of the method was investigated by injecting a series of working standard solutions at six concentration levels. Calibration curves were constructed by least squares linear regression analysis in term of the peak area ratio ($y$) of the analyte and the internal standard versus the concentration ($x$). For each analyte, the limit of detection (LOD) and the limit of quantification (LOQ) were separately determined by the serial dilution of working standard solutions at signal-to-noise ratios (S/N) of 3 and 10 under the chromatographic conditions, respectively.

#### *2.5.2. Precision, repeatability, stability and accuracy*

The mixed working standard solutions were analyzed for six replicates within one day for intra-day precision test and examined in duplicates for consecutive three days for inter-day precision test. RSDs of the precision were evaluated in term of the peak area ratio of the target analyte and the internal standard. In the repeatability experiment, six replicates of the same sample (S1) were extracted and analyzed followed the mentioned procedure. The concentrations of each analyte were used to calculate the RSD value to judge the method repeatability. The stability of the target analytes in the final extraction solution stored at room temperature was tested by replicate assays of a freshly prepared sample solution (S1) at 0, 2, 4, 6, 8, 12 and 24 h.

The accuracy of this method was determined by application of the standard addition method. In the accuracy experiment, a certain amount of sample (0.25 g, S1) spiked with known amounts of the standards at low (80% of the known amounts), medium (100% of the known amounts), and high (120% of the known amounts) levels were extracted and analyzed in triplicate by the above-established method. The average recovery percentage was obtained

using the following equation.

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

*2.7. Data statistics and analysis*

The extraction method was optimised by Orthogonal array design (OAD) and analysis of variance (ANOVA). (SPSS for Windows 13.0, SPSS Inc., USA).

The hierarchical clustering analysis of samples was also performed using SPSS software (SPSS for Windows 13.0, SPSS Inc., USA). A method called "average linkage between groups" was applied, and the Pearson correlation was selected as the measurement.

## 3. Results and discussion

*3.1. Optimization of MAE conditions*

Initially, we utilised the mono-variate investigation method, wherein one factor is examined while all other factors remained constant. However, this method was not scientific or logical because only certain valuable conditions might have already been drawn from the mono-variate investigation. Investigating all possible combinations of these factors would make it possible to find the optimum operating conditions experimentally. In order to obtain an efficient extraction of active components in CJM, an orthogonal array design (OAD) was used to generate useful information on the key variables.

All parameters (A–D) were tested in a wider range prior to OAD optimization, which narrowed down the ranges of the parameters tested. According to the literature[20] and our experience, a OAD L9($3^4$) was selected. The four variables were optimized in three levels as follows: factor A, ethanol concentration (60, 70 and 80%, *v/v*); factor B, extraction time (5, 8 and 10 min); factor C, extraction temperature (60, 70, 80°C); factor D, solid to solvent ratio (1:20, 1:30 and 1:40, *w/v*). The sensitivity index for the evaluated method was the total

concentration of flavonoids (TFC) in CJM. The run order was provided in Table 2.

<Table 2>

Observation of the statistical analysis shown in Table 2 allowed us to know that the largest range of the three levels was 3.956 for factor A and the smallest was 0.664 for factor C, which suggested that factor A (ethanol concentration) was the primary variable in the extraction conditions of flavonoids in CJM. $k_1$, $k_2$ and $k_3$ represent the average measured contents of TFC for each factor at each level. Based on the largest donating rule, the largest value refers to the optimal condition. Thus, the optimum experimental conditions obtained were $A_2B_2C_2D_3$.

To verify whether the effect of individual factors on MAE efficiency is statistically significant, an analysis of variance (ANOVA) was used to interpret the experimental data obtained from the OAD optimization. The significance of each factor was evaluated by calculating the *F* value and the results were summarized in Table 3. As can be seen, the influence by the parameters on the mean extraction yields of TFC decreased in the order of A (ethanol concentration) > B (extraction time) > D (solid-to-solvent ratio) > C (extraction temperature) according to the *F* values. The ethanol concentration has statistical significance at $p < 0.05$. The ANOVA result was in good accordance with what was observed in Table 2. Combing these analysis results and other considerations such as the cost of energy and the feasibility of experiment, the optimum conditions of extraction were therefore determined as follows: extraction solvent 70% ethanol, extraction time 8 min, extraction temperature 70°C and solvent to solid ratio 1:40. The extraction yield of total flavonoids from CJM under the optimum conditions was 39.03 mg/g.

<Table 3>

To evaluate the extraction efficiency of MAE, CJM was also extracted using HRE and

UAE method. In terms of yield of total flavonoids, the best results were obtained by MAE (39.03 mg/g), which gave significantly higher values. The extraction yields with traditional extraction methods for total flavonoids were 25.01 with HRE and 27.13 mg/g with UAE. On extraction time, MAE was also the fastest method with only 8 min of extraction time, the highest yield for UAE and HRE was obtained after 30 min and 3 h, respectively. MAE gave higher extraction yields and in a shorter extraction time, indicating that MAE was an efficient sample preparation technique.

*3.2. Optimization of liquid chromatographic and mass spectrometric conditions*

The chromatographic conditions were optimized systematically to improve the separation of the analytes. Different mobile phase compositions (including methanol-water, acetonitrile-water, methanol-formic acid solution, and acetonitrile-formic acid solution) and flow rate (0.2, 0.3 and 0.4 mL/min) as well as column temperature (20, 30 and 35℃) were examined and compared. As a result, acetonitrile-0.1% formic acid solution by gradient elution at a flow rate of 0.2 mL/min with the column temperature of 20℃ resulted in a satisfactory separation within a short analysis time (only 11 min). Chloromycetin was chosen as the internal standard due to its similarity in the retention and ESI ionization to those of the analytes.

For MS condition, the standards of the target analytes were analyzed by direct flow injection to optimize the ESI-MS/MS conditions. Full-scan mass spectra and MS/MS spectra were acquired to obtain the available transition for each compound. Identification of the precursor ions and optimum ionization conditions were performed in the full scan mode by recording mass spectra from *m/z* 100 to 1000. Further identification of the most abundant fragment ions and selection of the optimum collision energy (CE) for each analyte were carried out in the product ion scan mode. The ionization mode was optimized in both positive-

and negative-ion modes. Based on the sensitivity and reproducibility of the dominant ions in the full scan mass spectra, the negative mode was selected for the detection. The ions used for quantitative analysis were selected based on the highest peak intensity. Table 1 shows the MS/MS transitions selected for quantification, together with the optimized parameters for all the compounds studied.

### *3.3. Method validation*

#### *3.3.1. Linearity, LOD and LOQ*

Full calibration curves of the ten analytes calculated by least squares regression and the performance characteristics are presented in Table 4. The satisfactory correlation coefficients and the *F*-values and *t*-values ($\alpha = 0.05$, $p < 0.001$) of the analysis of variance (ANOVA) confirmed that ten analytes responses were linear over the studied range. The LOD and LOQ ranged from 0.02 to 3.25 ng/mL and 0.05 to 6.25 ng/mL for the analytes, respectively, which demonstrate that the proposed UHPLC-MS/MS method has a good sensitivity for the determination of the active constituents of CJM.

<Table 4>

#### *3.3.2. Precision, repeatability, stability and accuracy*

The present method was found to have an acceptable level of precision, with the intra-day precision RSD values between 1.20% and 2.74%, and the inter-day precision RSD values between 1.64% and 2.95%. RSD values of the analysis repeatability ranged from 1.92% to 3.49%, which give rise to an acceptable repeatability. The sample solution was found to be stable from 0 to 24 h and their RSD values were lower than 3.86%. The recovery of the method was in the range of 95.8–102.4% along with the RSD less than 2.57%, strongly indicating that the established method was accurate for the determination of the ten active compounds in CJM. The results are shown in Table 5.

<Table 5>

*3.4. Sample analysis*

The developed analytical method was subsequently applied to analysis of ten bioactive components in 15 batches of CJM samples collected from different main producing areas in northern China (Table 6). The typical MRM chromatograms are shown in Fig. 2. The contents of ten analytes were calculated with internal standard methods based on the respective calibration curves and each sample was extracted and analyzed in triplicate to determine the mean content (μg/g) (Table 6). The results showed that there were remarkable content differences of the ten components in the selected fifteen batch samples. For example, juglone is one of the important constituents in CJM, while its contents varied from 6.44 to 12.49 mg/g; myricetin has the lowest content than other flavonoids in CJM, while its contents changed in the range of 0.98-6.23 μg/g. The total contents of each type of composition were also calculated and are listed in Table 6. It is obvious that quinones (including juglone and 5,8-dihydroxy-1,4-naphthoquinone) with the total content range of 6.48-12.59 mg/g are the most abundant constituents among the analytes. The concentrations of flavonoids to be the second abundant constituents fall into the range of 227.99-2108.16 μg/g. Gallic acid was the least ranging from 16.99-1497.70μg/g. Generally, the content variations might be ascribe to both of some intrinsic factors such as genetic variation and plant origin and some extrinsic factors such as climate or geography (soil or minerals), harvest time, storage and processing of the CJM.

< Fig. 2>

<Table 6>

*3.5. HCA of the samples*

Hierarchical cluster analysis (HCA) was used on the standardized data to investigate the similarities between different samples. To classify the quality of CJM, HCA was performed

on ten different components from 15 samples using a method called ‘‘average linkage between groups’’ by SPSS 16.0 software. The obtained results are shown as a dendrogram in Fig. 3, in which two clusters are visible. Sample 1 to 8 was observed in cluster A and the other samples were in cluster II. The results pointed out that the samples collected from the same or close cultivation regions were mostly classified in one cluster, such as samples1-8 collected from Liaoning province, which implies that the influence of CJM cultivation regions on the contents of the ten analytes is very obvious.

< Fig. 3>

## 4. Concluding remarks

A novel, rapid and reliable method based on MAE combined with UHPLC–MS/MS technique was developed and validated for the simultaneous determination of two quinones, seven flavonoids and one phenolic in CJM for the first time. High extraction efficiency was achieved by an optimized MAE procedure, along with shorter extraction time (8 min) and less solvent consumption than HRE and UAE methods. The established UHPLC–ESI-MS/MS method demonstrated superiority in terms of time savings （just 11 min）and sensitivity for quantitative analysis. The developed method was successfully applied to determine ten bioactive components of CJM in 15 batches of CJM obtained from different regions of northern China. The achieved determination of the pharmacologically active constituents in CJM is essential for the quality control of CJM, which also provide us some possibilities to discover the pharmacological activity and useful guidance in the clinical use of CJM.

## Acknowledgments

This work was supported by the S&T plan projects of Liaoning Provincial Education

Department (L2012355); Research Promotion Grants-in-Aid for Graduates of Kochi University of Technology Special Scholarship Program (2013); and College students' innovative training program in Liaoning province (2013).

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## Figure Captions

**Fig. 1.** Product spectra and fragmentation reaction of the target analytes and IS in negative electrospray

ionization mode.

**Fig. 2.** Representative extract ions chromatograms obtained using the multiple reaction monitoring (MRM) mode. (A) Mixed working standards: gallic acid (1) (1.43 μg/mL), myricitrin (2) (1.04 μg/mL), quercitrin (3) (1.08 μg/mL), taxifolin (4) (1.44 μg/mL), juglone (5) (21.08 μg/mL), myricetin (6) (0.528 μg/mL), quercetin (7) (0.115 μg/mL), 5,8-dihydroxy-1,4-naphthoquinone (8) (1.136 μg/mL), kaempferol (9) (0.101 μg/mL), naringenin (10) (1.55 μg/mL)，chloromycetin, IS (11) (0.025μg/mL); (B) Liaozhong sample (S1).

**Fig. 3.** Dendrograms illustrating the hierarchical clustering of the 15 CJM samples using the ‘‘average linkage between groups’’ method.

**Table 1** Mass spectrometric parameters in negative ion mode of ten analytes and IS.

| No. | Analyte | Retention time (min) | MW (Da) | MS1 (m/z) | MS2 (m/z) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|---|---|---|
| 1 | Gallic acid | 1.16 | 170.1 | 168.9 | 125.0 | 20 | 15 |
| 2 | Myricitrin | 2.11 | 464.3 | 463.0 | 315.9 | 35 | 25 |
| 3 | Quercitrin | 3.12 | 448.3 | 447.4 | 301.2 | 40 | 25 |
| 4 | Taxifolin | 3.2 | 304.2 | 303.3 | 125.1 | 25 | 15 |
| 5 | Juglone | 3.62 | 174.1 | 173.0 | 144.9 | 30 | 20 |
| 6 | Myricetin | 3.83 | 318.2 | 317.2 | 151.1 | 35 | 25 |
| 7 | Quercetin | 4.35 | 302.0 | 300.9 | 150.9 | 30 | 23 |
| 8 | 5,8-dihydroxy-1,4-naphthoquinone | 4.64 | 190.1 | 189.1 | 160.8 | 25 | 20 |
| 9 | kaempferol | 4.85 | 286.2 | 285.3 | 92.8 | 35 | 25 |
| 10 | Naringenin | 4.92 | 272.2 | 271.0 | 150.9 | 30 | 20 |
| 11 | Chloromycetin(IS) | 4.15 | 322.3 | 321.0 | 151.9 | 30 | 18 |

**Table 2** Factors in the orthogonal design for the optimization of MAE extraction conditions.

| Run no. | A: concentration of ethanol (%) | B: extraction time (min) | C: extraction temperature (°C) | D: solvent to solid ratio (mL/g) | Total content of flavonoids (mg/g) |
|---|---|---|---|---|---|
| 1 | 60 | 5 | 60 | 20 | 32.86 |
| 2 | 60 | 8 | 70 | 30 | 36.06 |
| 3 | 60 | 10 | 80 | 40 | 34.87 |
| 4 | 70 | 5 | 70 | 40 | 38.63 |
| 5 | 70 | 8 | 80 | 20 | 38.97 |
| 6 | 70 | 10 | 60 | 30 | 38.06 |
| 7 | 80 | 5 | 80 | 30 | 36.15 |
| 8 | 80 | 8 | 60 | 40 | 37.96 |
| 9 | 80 | 10 | 70 | 20 | 36.18 |
| $k_1$[a)] | 34.59 | 35.88 | 36.29 | 36.003 | -- |
| $k_2$ | 38.55 | 37.66 | 36.95 | 36.757 | -- |
| $k_3$ | 36.76 | 36.37 | 36.66 | 37.153 | -- |
| Range | 3.956 | 1.783 | 0.664 | 1.15 | -- |
| Optimized scheme | $A_2$ | $B_2$ | $C_2$ | $D_3$ | -- |

a) k represents the average values of the same level of the same factor.

**Table 3** F values obtained from ANOVA results.

| Factors | Sum of squares | $F_{0.05}$ | $F$ value |
|---|---|---|---|
| ethanol concentration | 23.554 | 19.00 | 35.526* |
| extraction time | 5.093 | 19.00 | 19.682 |
| extraction temperature | 0.663 | 19.00 | 1.000 |
| solid-to-solvent | 2.047 | 19.00 | 3.087 |

*$p < 0.05$.

**Table 4** Calibration curves, LODs and LOQs for the target analytes (n = 6)

| Analyte | Regression equation | *r* | *F* | *t* | Linear range (μg/mL) | LOD (ng/mL) | LOQ (ng/mL) |
|---|---|---|---|---|---|---|---|
| 1[a)] | $Y = 4.5058X + 0.7846$ | 0.9994 | 2963* | 54.433* | 0.143-14.3 | 0.15 | 0.50 |
| 2 | $Y = 4.7563X - 0.2721$ | 0.9994 | 3699* | 60.822* | 0.104-10.4 | 0.85 | 2.50 |
| 3 | $Y = 7.9989X - 1.3122$ | 0.9991 | 2736* | 52.310* | 0.108-10.8 | 0.12 | 0.50 |
| 4 | $Y = 1.3869X - 0.0879$ | 0.9996 | 2035* | 45.108* | 0.144-14.4 | 0.63 | 1.56 |
| 5 | $Y = 0.0032X + 0.0013$ | 0.9991 | 2570* | 50.698* | 2.108-210.8 | 3.25 | 6.25 |
| 6 | $Y = 12.597X + 0.1311$ | 0.9998 | 9720* | 98.588* | 0.052-5.2 | 0.05 | 0.20 |
| 7 | $Y = 27.163X - 0.3565$ | 0.9992 | 3037* | 55.111* | 0.0115-1.15 | 0.25 | 1.25 |
| 8 | $Y = 1.2799X - 0.1521$ | 0.9993 | 2615* | 51.135* | 0.1136-11.36 | 1.25 | 5.00 |
| 9 | $Y = 2.7926X - 0.0350$ | 0.9993 | 3347* | 57.853* | 0.0101-1.01 | 1.56 | 6.25 |
| 10 | $Y = 43.106X + 11.516$ | 0.9997 | 9559* | 97.768* | 0.155-15.5 | 0.02 | 0.05 |

a) The analyte numbers are the same as in Table 1.

* significant coefficient ($p < 0.001$).

**Table 5** Precision, repeatability, stability and accuracy for the target analytes.

| Analyte | Precision | | Repeatability | Stability | Accuracy | |
|---|---|---|---|---|---|---|
| | Intra-day (RSD%, $n$ = 6) | Inter-day (RSD%, $n$ = 3) | (RSD%, $n$ = 6) | (RSD%, $n$ = 6) | Mean (%) | (RSD%, $n$ = 3) |
| 1 [a)] | 1.72 | 1.99 | 2.04 | 1.57 | 98.9 | 1.58 |
| 2 | 2.09 | 2.64 | 2.93 | 2.67 | 96.5 | 2.33 |
| 3 | 2.74 | 2.78 | 2.96 | 2.21 | 101.2 | 2.42 |
| 4 | 1.44 | 1.79 | 1.92 | 2.54 | 102.4 | 1.96 |
| 5 | 1.20 | 1.64 | 2.75 | 2.92 | 100.9 | 1.21 |
| 6 | 1.93 | 2.01 | 2.91 | 3.13 | 97.3 | 1.97 |
| 7 | 1.58 | 2.60 | 2.88 | 1.96 | 96.4 | 1.99 |
| 8 | 2.59 | 2.95 | 3.49 | 3.86 | 98.5 | 2.01 |
| 9 | 2.01 | 2.61 | 3.29 | 3.54 | 100.8 | 1.96 |
| 10 | 1.45 | 1.87 | 1.95 | 2.28 | 95.8 | 2.57 |

a) The analyte numbers are the same as in Table 1.

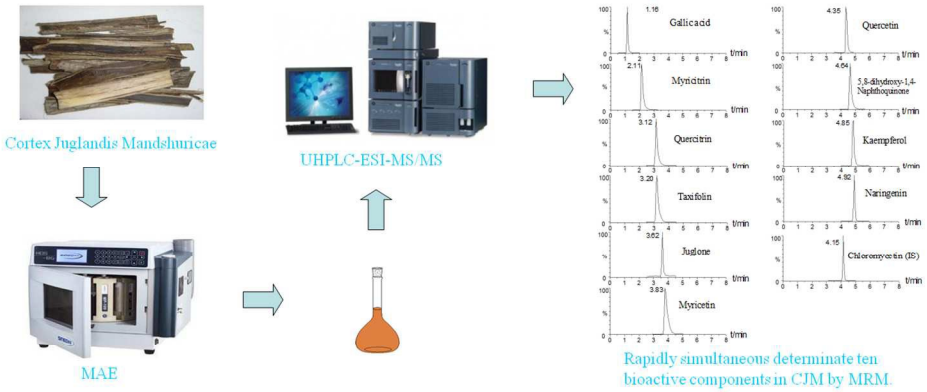
**Table 6** Quantitative analytical results for the target analytes in CJM from different sources ($n$ = 3, μg/g).

| Sample | Sources | Gallic acid | Flavonoids | | | | | | | | Quinones | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 [a)] | 2 | 3 | 4 | 6 | 7 | 9 | 10 | Total | 5 | 8 | Total |
| S1 | Liaozhong, LN[b)] | 247.79 | 94.366 | 54.138 | 207.89 | 1.4947 | 3.1506 | 1.3274 | 16.945 | 379.31 | 12240 | 82.176 | 12323 |
| S2 | Benxin, LN | 255.66 | 97.916 | 56.088 | 214.03 | 1.5493 | 3.1774 | 1.2888 | 18.064 | 392.11 | 12496 | 90.242 | 12587 |
| S3 | Jinzhou, LN | 117.18 | 89.553 | 73.054 | 58.049 | 1.4757 | 1.7337 | 2.7859 | 1.3476 | 228.00 | 6510.5 | 33.288 | 6543.8 |
| S4 | Huairen, LN | 194.63 | 119.77 | 74.840 | 79.381 | 2.1119 | 1.6342 | 1.9678 | 7.3253 | 287.03 | 10111 | 50.695 | 10162 |
| S5 | Dandong, LN | 91.598 | 118.83 | 108.82 | 65.095 | 1.7960 | 1.3941 | 3.3731 | 1.7879 | 301.09 | 11304 | 78.252 | 11382 |
| S6 | Liaoyang, LN | 137.14 | 114.57 | 109.77 | 86.028 | 2.4574 | 1.6086 | 3.3383 | 5.1237 | 322.89 | 12410 | 92.784 | 12503 |
| S7 | Fushun, LN | 478.07 | 109.45 | 160.05 | 386.84 | 6.2332 | 8.6652 | 18.031 | 90.669 | 779.94 | 9763.6 | 35.043 | 9798.6 |
| S8 | Anshan, LN | 299.94 | 123.16 | 27.589 | 358.17 | 4.9772 | 6.7559 | 15.917 | 86.261 | 622.83 | 8968.1 | 34.512 | 9002.6 |
| S9 | Tangshan, HB[c)] | 122.22 | 67.306 | 373.83 | 109.22 | 1.4278 | 27.553 | 12.593 | 1.7562 | 593.69 | 6828.7 | 40.165 | 6868.9 |
| S10 | Handan, HB | 165.85 | 73.237 | 422.58 | 116.68 | 2.1831 | 34.029 | 13.574 | 4.7463 | 667.03 | 7070.8 | 41.446 | 7112.3 |
| S11 | Xingtai, HB | 16.995 | 484.73 | 595.25 | 181.69 | 1.5577 | 74.884 | 9.8727 | 3.4555 | 1351.4 | 7278.9 | 57.615 | 7336.6 |
| S12 | Hengshui, HB | 54.409 | 120.47 | 514.48 | 190.98 | 1.5296 | 19.453 | 5.2462 | 0.93657 | 853.09 | 6687.0 | 59.570 | 6746.6 |
| S13 | Beijing | 1497.7 | 81.561 | 552.41 | 78.361 | 0.97594 | 166.02 | 14.446 | 5.6852 | 899.45 | 6441.6 | 36.663 | 6478.2 |
| S14 | Yixian, HB | 81.089 | 214.04 | 310.49 | 296.63 | 3.2098 | 8.0970 | 12.365 | 2.2691 | 847.10 | 8618.3 | 43.353 | 8661.7 |
| S15 | Shijiazhuang, HB | 99.670 | 824.11 | 621.27 | 564.21 | 1.7931 | 83.366 | 7.4473 | 5.9696 | 2108.2 | 7144.1 | 59.884 | 7204.0 |

a)The compound numbers are the same as in Table 1.

b)LN is the abbreviation of Liaoning province.

c)HB is the abbreviation of Hebei province.




Graphical Abstrac
379x169mm (96 x 96 DPI)

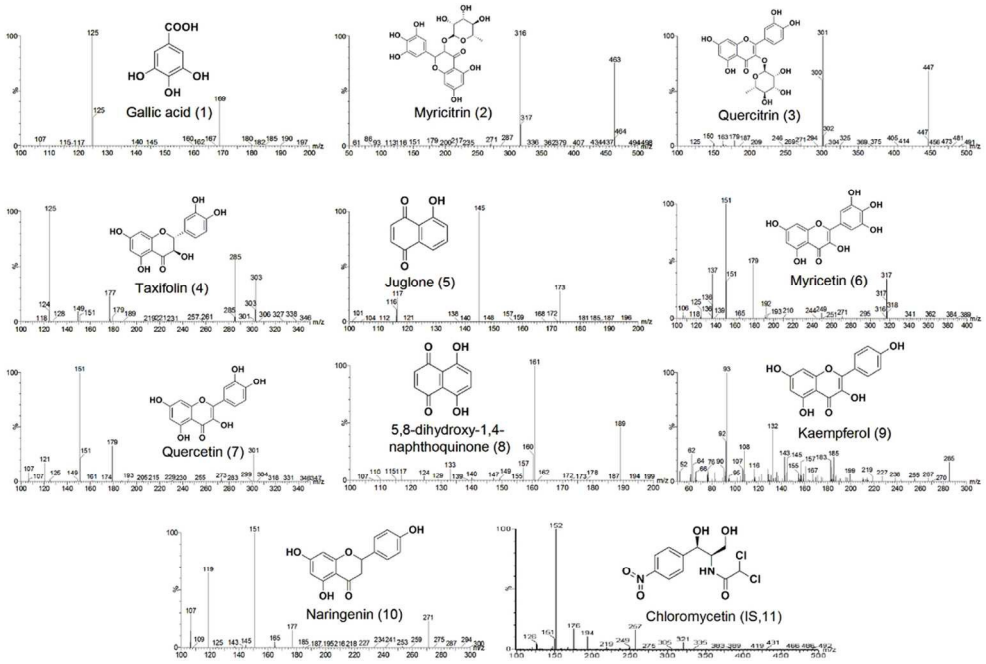



Fig. 1. Product spectra and fragmentation reaction of the target analytes and IS in negative electrospray ionization mode.
52x34mm (600 x 600 DPI)

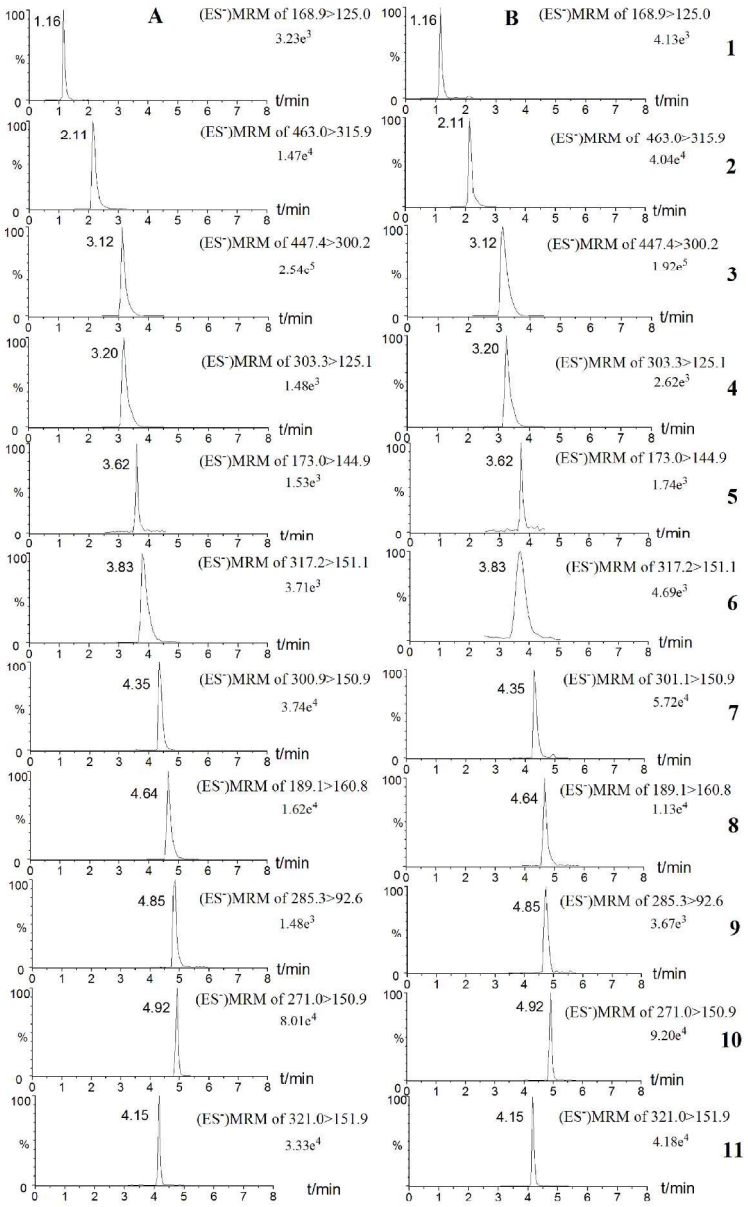



Fig. 2. Representative extract ions chromatograms obtained using the multiple reaction monitoring (MRM) mode. (A) Mixed working standards: gallic acid (1) (1.43 μg/mL), myricitrin (2) (1.04 μg/mL), quercitrin (3) (1.08 μg/mL), taxifolin (4) (1.44 μg/mL), juglone (5) (21.08 μg/mL), myricetin (6) (0.528 μg/mL), quercetin (7) (0.115 μg/mL), 5,8-dihydroxy-1,4-naphthoquinone (8) (1.136 μg/mL), kaempferol (9) (0.101 μg/mL), naringenin (10) (1.55 μg/mL), chloromycetin, IS (11) (0.025μg/mL); (B) Liaozhong sample (S1).
128x206mm (600 x 600 DPI)

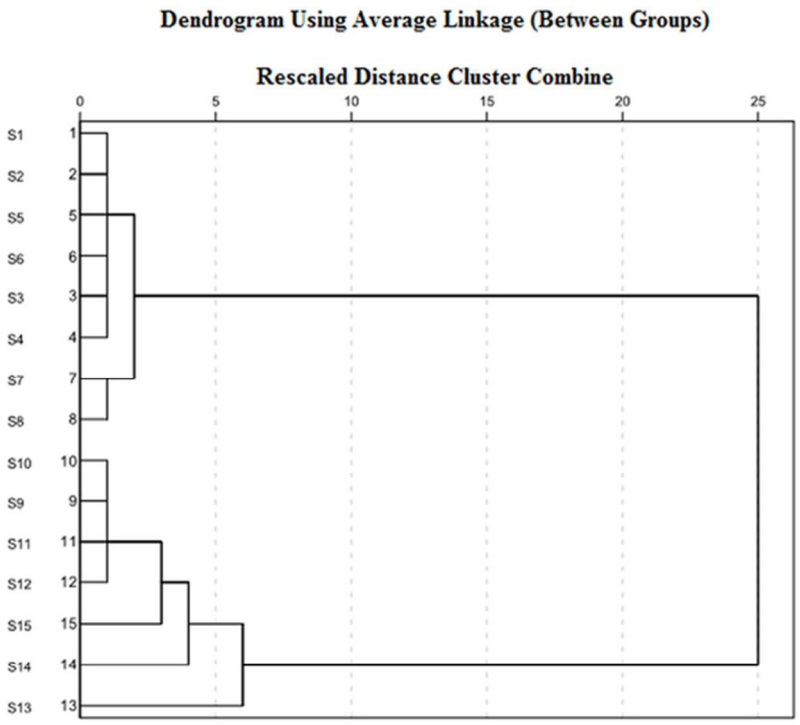



Fig. 3. Dendrograms illustrating the hierarchical clustering of the 15 CJM samples using the ‘‘average linkage between groups’’ method.
60x46mm (300 x 300 DPI)