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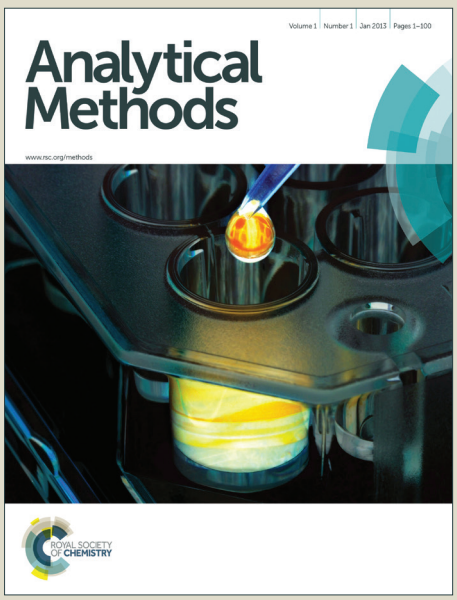

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Fingerprint Analysis of *Oxytropis Falcate* Using Ultra-performance Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry (UPLC-ESI-MS)

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Abstract: An efficient, accurate and reliable chromatographic fingerprint based on ultra-performance liquid chromatography coupled with photodiode array detection and electrospray ionization mass spectrometry (UPLC-PDA-ESI-MS) was developed for the identification and quality control of *Oxytropis falcate* Bunge. The method was validated for precision, repeatability, stability, and specificity. The RSD values of retention times and peak areas of the common peaks were 0-0.7% and 1.0-3.6% for within-day precision, while 0-0.5% and 1.5-6.1% for between-day precision. For repeatability, the values were lower than 0.5% and 2.6%, respectively, and for stability (24 h), the values were lower than 0.9% and 3.3%, respectively. Twenty-one batches of *O. falcate* obtained from different regions were analyzed by employing the developed method. Several multivariate statistical analysis including similarity analysis (SA), hierarchical clustering analysis (HCA) and principal component analysis (PCA) were applied to cluster and evaluate the *O. falcate* samples according to their collection sites. The results showed that the quality of *O. falcate* did not merely depend on the geographic origin. Thirty common peaks were found in the chromatographic fingerprints of *O. falcate* and eleven of them were

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identified by comparing the retention time, the ultraviolet spectrum and the molecular ion peaks (obtained from selective ion recording mode) with the reference compounds. This method with high precision, reliable stability and good repeatability can provide a reference for the quality evaluation of *O. falcate*.

Keywords: UPLC-PDA-ESI-MS; *Oxytropis falcate*; quality control; fingerprint

1. Introduction

Traditional Chinese medicines (TCMs) have been widely used in China for thousands of years and affirmed by the modern clinical medicine in many countries for its complementary therapeutic effects and rich resources. The internal quality of TCMs is directly related to the clinical efficacy and safety. Therefore, to establish an efficient quality control system is one of the key scientific issues for the modernization and globalization of TCMs. Quality control of TCMs has experienced a quite long historical period. During the last decades, several modern analytical techniques, such as thin layer chromatography (TLC),[1, 2] high performance liquid chromatography (HPLC),[3, 4] gas chromatography (GC),[5] capillary electrophoresis (CE),[6] and molecular biology (MB),[7] have been used in quality control of TCMs, and have improved the identification system of TCMs gradually. Among them, TLC, GC and HPLC are recommended analytical techniques for quality control in the *Chinese Pharmacopoeia*. Although the methods and strategies for controlling and evaluating the quality of TCMs have been greatly developed, their further development was restricted by the complexity of chemical constituents contained in TCMs. Fortunately, chromatographic fingerprint technology could provide integrated morphology and chemical information of a complex system and identify the authenticity of medicinal material. This strategy has gained increasing attention and has been gradually applied for the quality control of medicinal products. For example, European Agency for the Evaluation of Medical Products, the US Food and Drug Administration, and the World Health Organization (WHO) have introduced chromatographic fingerprint technology as a

strategy for assessing herbal medicines or botanical preparations successively. Likewise, chromatographic fingerprint technology has been adopted by the Chinese State Food and Drug Administration (SFDA) to control the Chinese medicine injections in China.

In recent years, ultra-performance liquid chromatography (UPLC) applying sub-2 μm particles in a short column has been employed to build fingerprint of TCMs by virtue of its excellent separation efficiency, large peak capacity, fast analytical speed, little solvent consumption and high sensitivity. Several kinds of TCMs have been successfully validated by ultra-performance liquid chromatographic (UPLC) fingerprint.[8-10] In some cases, the chemical compositions presented as chromatographic peaks in a fingerprint could be identified by UPLC coupled with photodiode array detection and mass spectrometry (UPLC-PDA-MS). UPLC-PDA-MS has been widely accepted and attracted increasing attention in fingerprint analysis owing to its effectiveness in the separation and identification of TCMs. As a soft ionization technique, electrospray ionization mass spectrometric detection (ESI-MS) could sought out the quasi-molecular ion peaks easily.[11] Thus, UPLC-PDA-ESI-MS could provide on-line ultraviolet spectrum and mass spectrum besides chromatographic profile. A certain number of chromatographic peaks could be identified by comparing their ultraviolet spectrum and mass spectrum information with literature data and reference compounds.

Oxytropis falcate Bunge, belonging to the family Leguminous, is mainly distributed in the Qinghai-Tibet plateau in China. It was first recorded in the *Chinese Pharmacopoeia* as an official herbal drug in 1977 and has been wildly used as a TCM for a long time. In addition, this medicinal plant, known as 'Edaxia' and 'the King of Drugs",[12] has been widely applied in many Tibetan and Mongolian medicine prescriptions for its therapeutic effects on influenza, hyperpyrexia, pain, wounds, haemorrhage, inflammation, anthrax, etc.[13,14-15] At present, some progress have been gained owing to the sustained studies on the phytochemistry[16-22] and pharmacological actions[23-25, 29] of *O. falcate*. It is indicated that flavonoids are the main constituents of *O. falcate* and possess anti-inflammatory, analgesic,

antioxidant, antibacterial, anti-tumor, cytotoxic activities.[26-31] Although it has been listed in the *Chinese Pharmacopoeia*, the information on the quality control of this medicinal plant, however, is still rather limited. In the present study, the UPLC fingerprint of *O. falcate* collected from various sources have been established and investigated by several multivariate statistical analysis including similarity analysis (SA), hierarchical clustering analysis (HCA) and principal component analysis (PCA). Thirty common peaks were obtained in UPLC fingerprints, and eleven of them were identified by the comparison of the retention time, ultraviolet spectrum and molecular weight with the reference substance. With the help of these statistical analyses, the quality of *O. falcate* could be differentiated efficiently.

2. Experimental

2.1. Apparatus

UPLC apparatus was a Waters ACQUITY UPLC™ system (Waters, Milford, MA, USA) equipped with a binary solvent delivery pump, an autosampler, a thermostated column compartment, and a photodiode array eλ detector. The identifications were performed on a Waters ACQUITY tandem triple quadrupole mass spectrometer (TQD-MS) equipped with a Z-Spray multi-mode ESI/APCI ion source operating in both positive ion mode and negative mode. Data acquisition and processing were performed by a Masslynx 4.1 workstation.

2.2. Reagents and materials

Chromatographic-grade acetonitrile was purchased from Merck Co. (Darmstadt, Germany). Other chemicals of analytical grade were purchased from Tianjin Chemical Reagent Co. (Tianjin, China). Ultra-pure water was purified using an OKP purification system from Shanghai Laikie Instrument Co. Ltd. (Shanghai, China).

21 batches of *O. falcate* (No. S1-S21) were collected from Gansu province, Qinghai province and Tibet Autonomous Region of China (details shown in Table 1), respectively, and all of them were identified by Dr. Huan-Yang Qi in our laboratory. Reference compounds of (3R,10S)-10, 2′-dihydroxy-3′,

4′-dimethoxyisoflav-8-ene-7-one (peak 16), cinnamic acid (peak 17), 5, 7, 2'-trihydroxy-4'-methoxyisoflavone (peak 22), 7-hydroxylflavonone (peak 23), isomucronulatol (peak 24), 5, 7-dihydroxyflavanone (peak 25), 3-phenyl-*N*-(2-phenylethyl)-2-propenamide (peak 26), 2′, 4'-dihydroxydihydrochalcon (peak 27), 2', 4'-dihydroxychalcone (peak 28), 7-methoxyflavanone (peak 29), 5-hydroxy-7-methoxyflavanone (peak 30) were isolated from *O. falcate* and identified by ^{1}H NMR, ^{13}C NMR and MS in our own laboratory.[20-22,32-38] It is worth mentioning that compound 3-phenyl-*N*-(2-phenylethyl)-2-propenamide (peak 26) has two configurations: trans- and cis-forms. Their chemical structures are shown in Figure 1.

Table 1. The sources of 21 batches of *O. falcate*

No.	Collection site	No.	Collection site	No.	Collection site
S1	Qinghai Province	S8	Qinghai Province	S15	Qinghai Province
S2	Gansu Province	S9	Qinghai Province	S16	Qinghai Province
S3	Qinghai Province	S10	Tibet Autonomous Region	S17	Qinghai Province
S4	Gansu Province	S11	Qinghai Province	S18	Tibet Autonomous Region
S5	Qinghai Province	S12	Qinghai Province	S19	Gansu Province
S6	Qinghai Province	S13	Qinghai Province	S20	Gansu Province
S7	Qinghai Province	S14	Qinghai Province	S21	Gansu Province

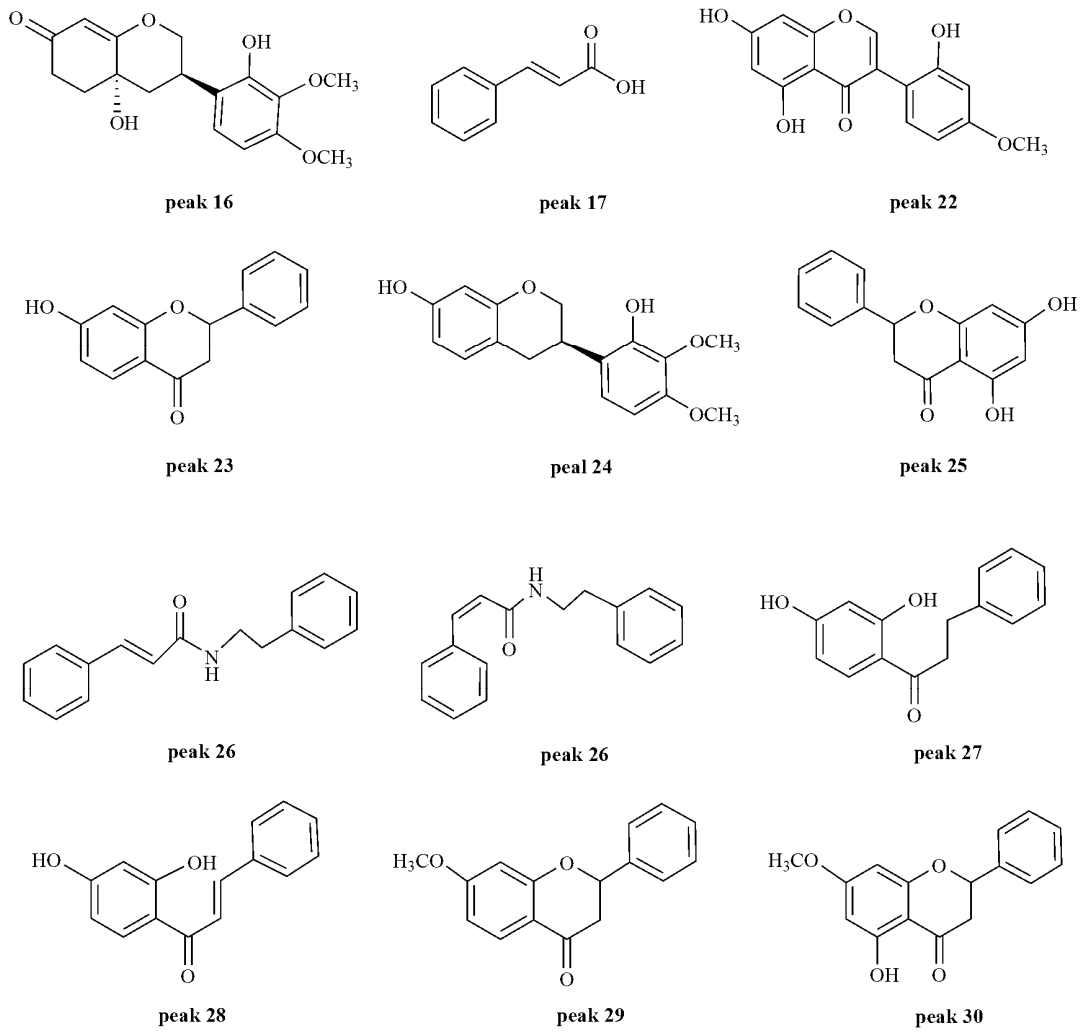

Figure 1. Chemical structures of the reference compounds (peak 16, 17, 22-30).

2.3. UPLC and MS conditions

The UPLC fingerprint analysis was conducted on a Waters ACQUITY UPLC BEH C_{18} column (50×2.1mm i.d., 1.7 μm particle), using a linear gradient elution of (A) 0.5% aqueous acetic acid and (B) acetonitrile at a flow rate of 0.3 mL min^{-1}. The gradient elution program was as follows: (a) 0-0.5min, B 2%; (b) 0.5-2.5min, B 2-10%; (c) 2.5-7.5min, B 10-22%; (d) 7.5-12.0min, B 22-25%; (e) 12.0-16.0min, B 25-38%; (f) 16.0-20.0min, B 38%; (g) 20.0-20.5min, B 38-45%; (h) 20.5-23min, B 45-50%; (i) 23.0-25.5 min, B 50-60%; (j) 25.5-28.0 min, B 60%; (k) 28.0-28.5 min, B 60-90%. The system was re-equilibrated with 2% B for 2 min before the next sample run. The temperatures of column and sample manager room were maintained 30°C and 18°C, respectively. The injection volume was 2 μL, and partial loop with needle overfill was applied for sample injection. The absorption spectra of

the compounds were recorded in the range of 190-400 nm, and the detection wavelength was set at 270 nm for fingerprint establishment.

Full-scan MS analysis and selected ion recording (SIR) in positive ion mode were employed to perform the analysis. The effluent from the PDA cell outlet was directed into the ESI probe via a capillary. The MS parameters were optimized to obtain the maximal sensitivity. The source temperature and the desolvation temperature were maintained at 110°C and 350°C, respectively. The capillary voltage and cone voltage were fixed at 3.0 kV and 25 V, respectively. The flow rates of desolvation gas and cone gas (nitrogen was used) were 600 L/h and 50 L/h, respectively. For full-scan MS analysis, the mass spectra were recorded in the range of m/z 50-800 in positive mode.

2.4. Preparation of standard solutions

The standard solutions of 11 reference compounds were separately prepared in 80% methanol, and filtered through 0.2 μm membrane before injecting into the UPLC system. The solutions prepared above were stored at 0-4^{o}C refrigerator prior to analysis.

2.5. Preparation of sample solutions

After being air-dried and crushed into powder, 2.00 g of the whole plant of *O. falcate* was accurately weighed and introduced into a conical flask with a stopper, then extracted with 50 mL of 80% methanol for 45 min in an ultrasonic bath at moderate frequency (70 kHz). After cooling down to room temperature and making up the lost weight, the extract was successively filtered through a qualitative filter paper and a 0.2 μm membrane for UPLC analysis.

2.6. Data analysis

Data analysis was performed on SPSS 16.0 software, SIMCA-P 12.0 software and the Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004 A) software.

3. Result and Discussion

3.1. Optimization of UPLC conditions

Optimization of chromatographic parameters was performed through investigating the influence of mobile phase, detection wavelength (190-400 nm), column temperature and injection volume. With the purpose of obtaining a large amount of detectable peaks on the UPLC chromatogram, the UV spectra of the analytes between 190 and 400 nm were acquired. Considering the resolution, symmetry and number of characteristic peaks on the chromatogram, 270 nm was selected as the detection wavelength for fingerprint establishment.

Selection of the mobile phase was guided by the need to obtain good resolution of adjacent peaks within a relatively short analysis time. Several different mobile phases consisting of methanol (or acetonitrile) and water with some modifiers including acetic acid, formic acid, and phosphoric acid were investigated separately. Preliminary studies indicated that acetonitrile could provide a lower column pressure and more stable baseline than methanol. In addition, more peaks would be presented by using acetonitrile and acetic acid solution. To gain more comprehensive chemical profile, different concentrations of acetic acid solution (0.1%, 0.3%, 0.5%, 0.7%, and 1.0%) were further evaluated. The results suggested that when using 0.1% acetic acid solution, the baseline was not stable. Moreover, the peak heights in the chromatogram obtained by using 0.1% and 0.3% acetic acid solution were lower than those of the others, and with continuously increasing acetic acid concentration, no visible difference was found among the other three concentrations. It seemed that the concentration of acetic acid (less than 0.3%) was not high enough to guarantee the stability of the components. Gradient, gradient time, gradient shape and initial composition of the mobile phase were also taken into consideration in this work. After trying several types of gradient elution, the optimum mobile phase was achieved with 0.5% acetic acid solution (solvent A) and acetonitrile (solvent B) in a linear gradient mode described above.

The effects of column temperature in the range of 25-45°C on the separation efficiency were investigated. The results showed that higher column temperature could bring shorter analysis time, but could not provide more efficient separation. For this reason, 30°C was a suitable temperature for measurement in this work.

Furthermore, the optimization of injection volume was necessary. The resolution and symmetry of characteristic peaks in chromatogram and the baseline of chromatogram could be influenced by excessive injection volume, while the number of characteristic peaks could be influenced by inadequate injection volume. In the test, different injection volumes (1 μL, 2 μL, 3 μL, 4 μL and 5μL) was examined, and 2 μL was a suitable volume selected.

3.2. Optimization of sample preparation conditions

The effects of extraction method, solvent, time and sample weight on the extraction efficiency was investigated, and the number of peaks and peak areas were employed to evaluate the extraction efficiency. Reflux and ultrasonication were separately used to extract the samples of *O. falcate* with methanol. Extraction efficiency for ultrasonication was equal to that for reflux. So, ultrasonication was selected owing to its simplicity. Methanol, ethanol and aqueous methanol (80%, 60%, 50%, 40%, and 30%) and aqueous ethanol (80%, 60%, 50%, 40%, and 30%) were separately evaluated as the extraction solvents. The numbers of peaks in the chromatograms and the peaks areas of most of the compounds obtained by using 80% methanol as extraction solvent were higher than those extracted by the other solvents. So, 80% methanol was chosen as the extraction solvent in the subsequent study. In addition, different extraction time (15, 30, 45, and 60 min) and sample weight (0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 g) were investigated and compared by UPLC. The results showed that 45 min was enough to extract the desired compound completely, and an optimal of 2.0 g was selected for the chromatography analysis, since the detection of all compounds in quantities appropriate for identification.

3.3. Method validation

3.3.1. Precision

The within-day and between-day precision of the developed method were evaluated by injecting a sample solution repeatedly. Within-day precision was measured for six continuous injections during the same day whereas between-day precision was measured on six consecutive days. As shown in Table 2, the relative standard deviation (RSD) values of retention times and peak areas of the common

peaks were 0-0.7% and 1.0–3.6% for within-day precision, while 0-0.5% and 1.5–6.1% for between-day precision, which is far below than the national standard of 10%.

3.3.2. Repeatability and stability

Repeatability was examined by the injections of six different sample solutions which were prepared in parallel by the same sample preparation procedure. The similarities were all above 0.99 and the RSD values of retention times and peak areas of the common peaks were 0-0.5% and 1.3-2.6%, respectively. For the stability test, the same sample solutions were analyzed at 4 h intervals during storage for 24 h at room temperature. The sample solution can be regarded as stable within 24 h because the similarities were all above 0.99 and the RSD values of retention times and the peak areas were 0-0.9% and 1.1-3.3%. The specific data are listed in Table 2.

Table 2. The RSD values for precision, repeatability and stability (n=6)

Peak Number	Within-day precision		Between-day precision		Repeatability		Stability	
	RT	PA	RT	PA	RT	PA	RT	PA
1	0.0	2.8	0.0	3.1	0.0	2.3	0.0	2.2
2	0.0	3.0	0.0	4.2	0.0	2.4	0.0	1.7
3	0.0	3.5	0.0	2.1	0.0	1.6	0.9	1.7
4	0.7	2.2	0.5	5.6	0.5	2.0	0.4	1.5
5	0.3	2.5	0.0	4.4	0.0	1.7	0.3	2.3
6	0.0	3.0	0.3	2.3	0.0	2.0	0.2	2.2
7	0.0	2.7	0.4	3.3	0.0	2.5	0.2	1.9
8	0.0	3.6	0.0	1.5	0.0	1.8	0.0	2.1
9	0.2	2.7	0.0	2.2	0.0	2.6	0.0	2.2
10	0.2	2.1	0.0	4.6	0.1	2.0	0.1	2.1
11	0.1	3.3	0.2	3.1	0.0	1.3	0.1	1.1
12	0.2	3.6	0.2	4.5	0.1	2.0	0.1	3.3
13	0.2	3.4	0.1	5.1	0.1	1.9	0.1	2.1
14	0.1	2.7	0.2	5.3	0.0	1.6	0.1	1.9
15	0.1	3.0	0.1	4.8	0.1	1.5	0.1	3.1
16	0.1	2.9	0.1	5.4	0.1	2.1	0.1	1.5
17	0.1	3.2	0.2	3.0	0.0	1.8	0.1	1.4
18	0.2	3.4	0.3	6.1	0.1	1.8	0.1	2.3
19	0.2	1.0	0.3	3.7	0.1	1.9	0.1	1.7
20	0.2	2.1	0.0	4.2	0.1	1.6	0.1	2.5

21	0.2	3.2	0.0	1.7	0.1	2.3	0.1	2.5
22	0.1	2.8	0.1	5.2	0.0	2.1	0.0	1.9
23	0.1	3.4	0.4	3.1	0.0	1.7	0.0	1.6
24	0.1	2.2	0.1	2.7	0.0	2.2	0.0	1.3
25	0.1	1.5	0.5	4.3	0.0	1.9	0.0	1.5
26	0.1	2.1	0.2	5.9	0.0	2.0	0.0	2.2
27	0.1	3.2	0.2	4.3	0.0	2.0	0.0	2.2
28	0.1	2.3	0.2	5.4	0.0	2.0	0.0	2.1
29	0.1	3.4	0.0	6.0	0.0	1.4	0.0	2.1
30	0.1	2.9	0.0	3.9	0.0	1.3	0.0	2.0

RT: Retention time

PA: Peak area

3.3.3. Specificity

Medicinal herbs, *Oxytropis kansuensis* Bunge and *Oxytropis melanocalyx* Bunge which belong to the same genus as *O. falcate* were analyzed under the optimum conditions to investigate the specificity of the developed method. As shown in Figure 2, the chromatographic profiles of *O. kansuensis* and *O. melanocalyx* were different from that of *O. falcate* significantly. This indicated that the developed method used for the fingerprint analysis of *O. falcate* was specific.

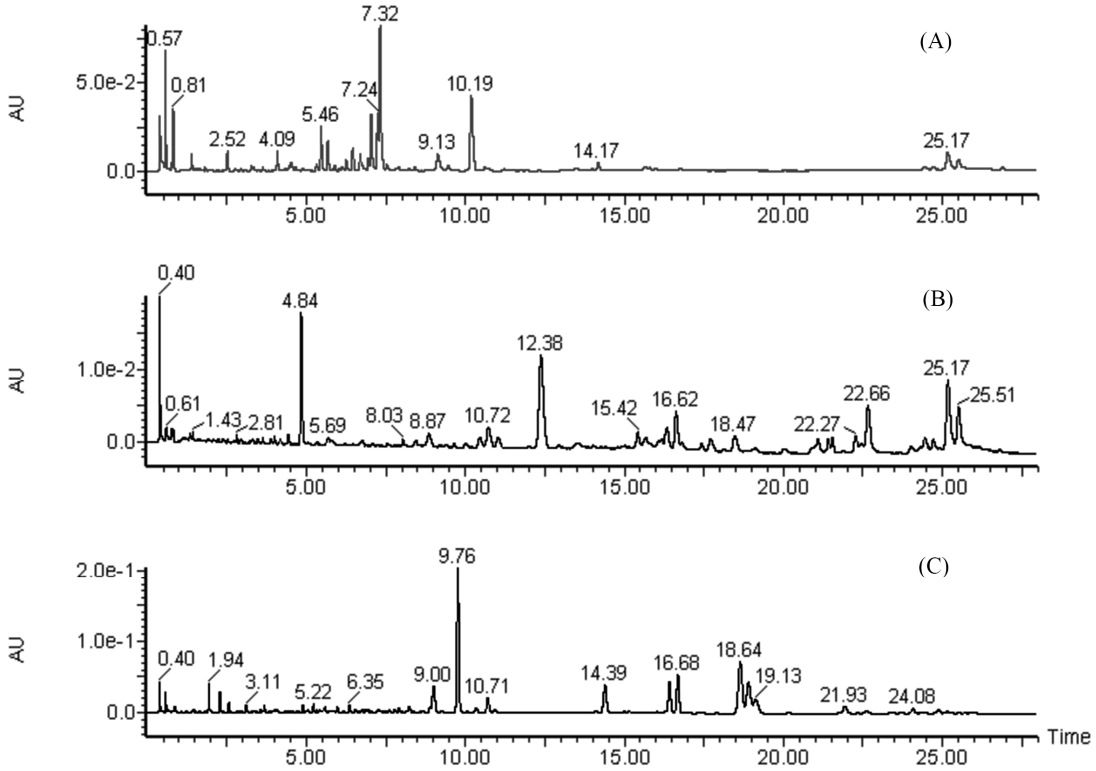

Figure 2. Chromatograms of *O. Kansuensis* (A), *O. melanocalyx* (B) and *O. falcate* (C).

3.4. Establishment of UPLC fingerprint and similarity analysis (SA)

Chromatographic fingerprint of TCMs contains large amount of information and could express the chemical characteristics of samples integrally. It can provide not only the reference for classification and quality control of medicinal materials, but also the basis for expressing the quality and stability of medicinal materials. The 21 batches of samples collected from Gansu province, Qinghai province and Tibet Autonomous Region of China were separately prepared and analyzed by UPLC under the optimum conditions. The respective chromatographic fingerprints of the samples are shown in Figure 3. It is observed that the numbers of chromatographic peaks of samples S5 and S10 are less than those of the others, and the chromatographic fingerprint profile of sample S21 is obvious different from the others.

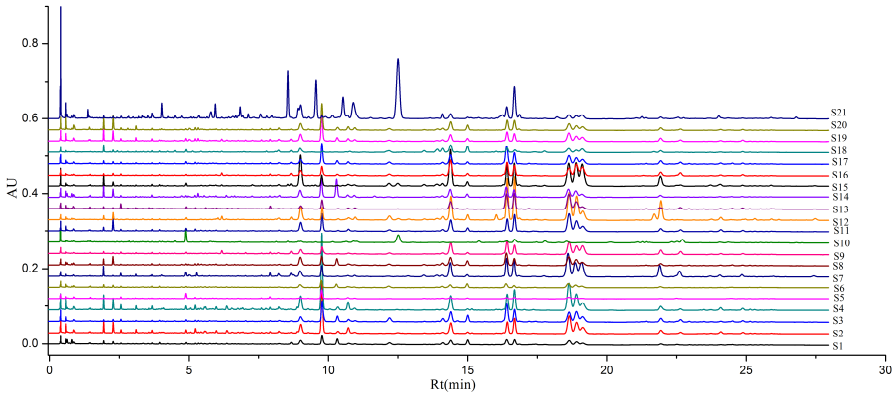

Figure 3. UPLC fingerprints for 21 batches of *O. falcate*.

A professional software, the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004 A), which is recommended by the Chinese State of Food and Drug Administration (SFDA), was applied for similarity analysis of chromatographic fingerprint. The similarities of different chromatograms were evaluated by calculating the correlative coefficient and the cosine value of vectorial angle. When the correlative coefficients between each chromatographic profile of samples were higher than 0.9, it was considered that the samples were highly related and could be classified as a group. The standard chromatographic fingerprint (shown in Figure 4) for samples was computed by the software. The information for the matched peaks was obtained

from the similarity evaluation software and the common peaks were chosen according to the matched information based on the following three principles. Firstly, the selected peaks should be present in more than 90% of samples. Secondly, the selected chromatographic peaks representing the components are pure, and the peaks which could interfere with the judgment had better not be selected. Thirdly, the peaks should be distributed evenly and stably. The similarities of 21 sets of samples are listed in Table 3.

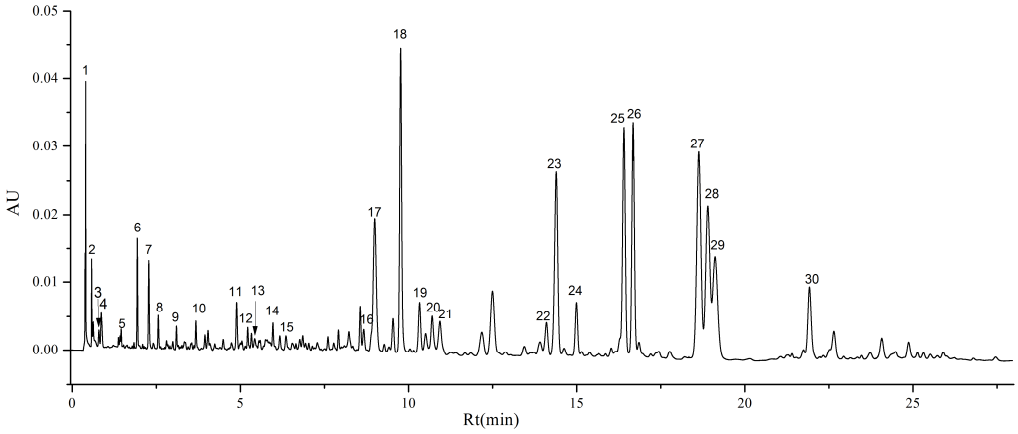

Figure 4. The standard chromatographic fingerprint.

Table 3. The similarity of chromatographic fingerprints of 21 samples

No.	Similarity	No.	Similarity	No.	Similarity
S1	0.944	S8	0.972	S15	0.953
S2	0.984	S9	0.965	S16	0.941
S3	0.981	S10	0.425	S17	0.981
S4	0.973	S11	0.984	S18	0.865
S5	0.679	S12	0.966	S19	0.974
S6	0.934	S13	0.935	S20	0.947
S7	0.952	S14	0.976	S21	0.699

From the chromatographic profiles, the general characteristics of the chromatographic peaks of samples from different regions had a good similarity except for a slight difference in the ratio of chemical component. As seen in Table 3, the similarities values of all samples are above 0.900 except for sample S5 (0.679), S10 (0.425), S18 (0.865) and S21 (0.699), and these samples obtained from Qinghai

and Gansu province could be classified as a group and referred to as 'Qualified material'. The samples S10 and S18 obtained from Tibet Autonomous Region, the sample S5 obtained from Qinghai province and the sample S21 obtained from Gansu province were 'unqualified'. It leads to the conclusion that the chemical compositions of samples from different collected sites shows small difference, while the samples of the same source may be considerable difference. Chemical constituents of different samples could not be influenced highly by sources, which may be related with collecting time or growing environment. The similarities do not reflect the distribution of herbs enough. Chromatographic fingerprint emphasizes the similarity of the same medicine group, and the similarity is reflected by the integrity and fuzziness of chromatogram. To sum up, the similarity was not appropriate enough to analyze different samples, thus HCA and PCA would be needed in this work.

3.5. Hierarchical cluster analysis (HCA)

HCA is a chemometrics analysis method to cluster samples based on the similar chemical profiles, which is a comprehensive clustering method with supreme frequency among the methods of cluster analysis. Because the classification of samples is based on the similarities of their chemical properties, the samples possessing similar characteristics will be sorted into a new cluster to provide a dendrogram with a visual form for analysts. In our work, HCA was performed using SPSS 16.0 software based on the Ward's method and the squared Euclidean distance as pattern similarity measure.

As shown in Figure 5, the dendrogram indicates that 21 batches of samples are divided into three groups. Group II consists of two samples (S2, S4) collected from Gansu province, while group III consists of two samples (S12, S15) collected from Qinghai province and the rest samples collected from Tibet Autonomous Region, and partly from Qinghai and Gansu province belong to group I. The cluster analysis results suggest that the distance among the three groups is short. In other words, the samples collected from Qinghai province have a close relationship with the other two groups in the quality of samples. Qinghai province is adjacent to Gansu

province and Tibet Autonomous Region, and the samples are collected from the areas at an altitude of 2700-4300 m above sea level which belongs to the Qinghai-Tibet plateau in China，which might be the reason why the samples cluster are not corresponding to geographically distribution. In group III, those samples collected from Qinghai province are spread out. This might be accounted of some potential reasons, such as different growing environment, growing years, and harvest season. The obtained results implied that the quality of *O. falcate* is related not only to the geographic origin, but also to the growing environment, growing years, harvest season and other factors.

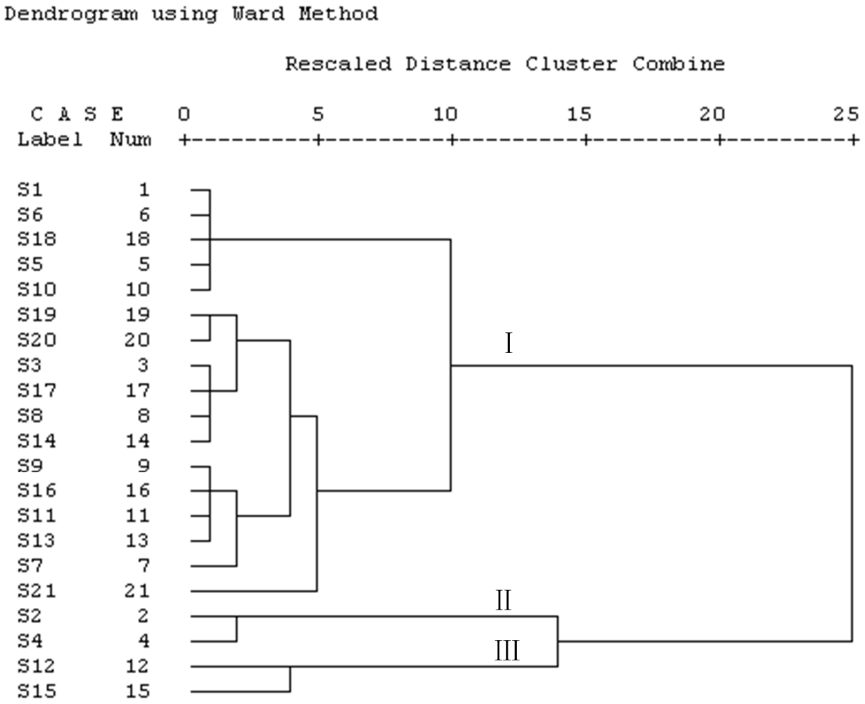

Figure 5. Cluster dendrogram from the UPLC peak area data of 21 batches *O. falcate* samples.

3.6. Principal component analysis (PCA)

PCA is an unsupervised chemometric tool widely used for information extraction and dimensionality reduction, and it simplifies the analysis and calculation simultaneously. Under the premise of lossless or small losses of the original information, many originally related indicators are translated into the comprehensive indexes by PCA analysis. Fingerprints possess large number of chromatographic peaks, characterized by some correlations amongst the peaks. For

multi-index comprehensive evaluation, the practical problem is that data calculation is extremely tedious. Fortunately, such a procedure is simplified by applying PCA, which is characterized by reducing the multidimensional data sets.

In our work, applying SIMCA-P 12.0 software, the peak areas of thirty common chromatographic peaks in fingerprints were analyzed as thirty variables. The 21 batches of samples were further analyzed and classified by PCA. So, a total of 21×30 data matrix was formed. The scatter plot of PCA is shown in Figure 6, which is provided by the aid of the first two principal components PC1 and PC2. The result showed that the samples obtained from different origins could be classified into three groups, i.e., group I (Qinghai, Gansu province and Tibet Autonomous Region), group II (Gansu province) and group III (Qinghai province). Score scatter plot suggested that the samples obtained from the three origins had the similar quality. The result of PCA was in correspondence with that obtained from HCA. It also suggested that the quality of the 21 batches of *O. falcate* were similar and the results of PCA and HCA could be validated by each other and could provide more valuable references for the quality evaluation of *O. falcate* objectively.

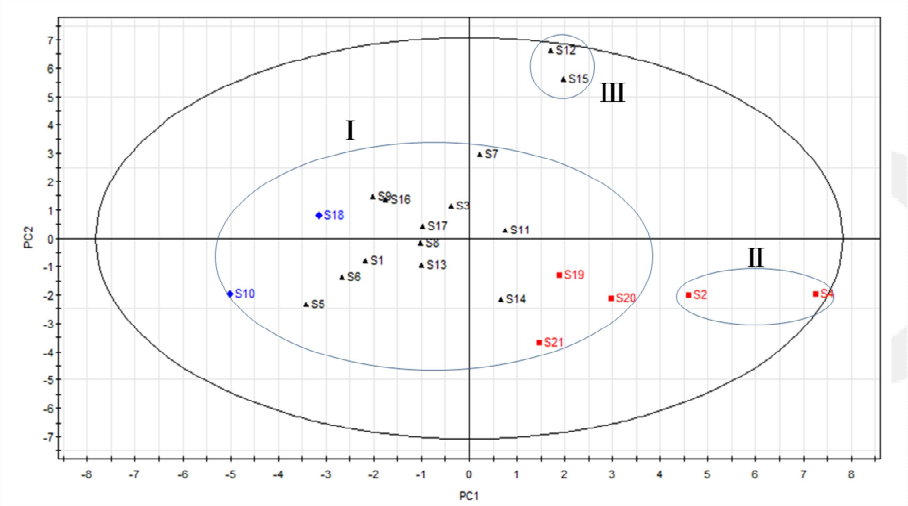

Figure 6. Score scatter plot obtained from the PCA of the 21 batches of *O. falcate*.

3.7. MS analysis of *O. falcate*

Eleven reference compounds isolated from *O. falcate* and identified in previous work were applied to optimize the MS parameters, and to identify the corresponding compounds presented in this medicinal plant. The effects of

desolvation temperature, source temperature, desolvation gas flow, capillary voltage, cone voltage, and cone gas flow were examined. The parameters were varied as follows: desolvation temperature (200, 300, 350, 400, and 450^{o}C), source temperature (100, 105, 110, 115, 120, 130, and 135^{o}C), desolvation gas (nitrogen) flow (400, 500, 600, 700, 800, 900, and 1000 L/h), capillary voltage (positive ion mode: 2.8, 2.9, 3.0, 3.1, 3.2, and 3.3 kV; negative ion mode: 2.5, 2.6, 2.7, 2.8, 2.9, and 3.0 kV), cone voltage (10, 15, 25, 30, 35, 40, 45, 50, 55, 60, and 65 V), and cone gas (nitrogen) flow (40, 45, 50, 55, and 60 L/h). The trials showed that positive ion mode fit the detection for the eleven reference compounds better than negative ion mode does, and cone voltage influenced the ionization significantly. The optimum UPLC-ESI-MS conditions were obtained after several trials.

The UPLC-ESI-MS chromatogram exhibited a good agreement with the UPLC-PDA chromatogram (Figure 7). In the ESI positive ion mode, $[M+H]^+$ ions were observed, and molecular weight were determined based on the information of $[M+H]^+$ ions. Herein, 11 common peaks in chromatographic profile were identified by comparing the retention time, ultraviolet spectrum and mass data with those of the reference compounds (Table 4). The MS/MS spectra are also very important for identification of unknown compound. In this work, the MS/MS data are shown in Table 4. The mass spectra of cinnamic acid (peak 17) and 3-phenyl-N-(2-phenylethyl)-2-propenamide (peak 26) are shown in Figure 8.

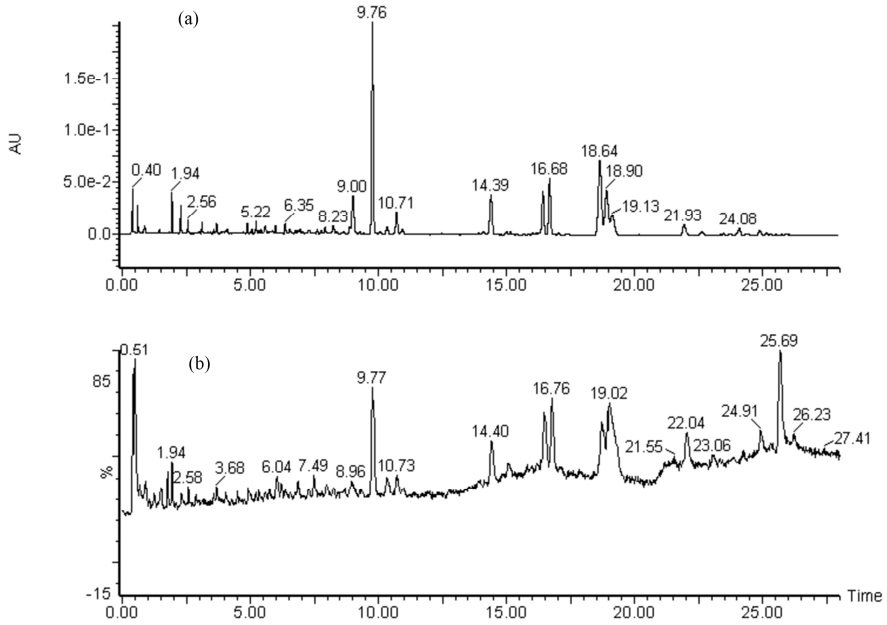

Figure 7. UPLC-PDA chromatogram at 270 nm (a), and total ion chromatogram of *O. falcate* (S4) in positive ion mode (b).

Table 4. Characterization of 11 common peaks in chromatographic profile by UPLC-PDA-ESI-MS

Peak Number	Identified Compounds	Rt (min)	λmax (nm)	$[M+H]^+$ *m/z*	MS/MS *m/z*	Molecular Formula
16	(3*R*,10*S*)-10, 2′-dihydroxy-3′,4′-dimethoxy-isoflav-8-ene-7-one	8.668	260	321	192, 167	$C_{17}H_{20}O_6$
17	Cinnamic acid	8.995	278	149	131, 103	$C_9H_8O_2$
22	5, 7, 2'-trihydroxy -4'-methoxyisoflavone	14.102	259	301	167	$C_{16}H_{12}O_6$
23	7-hydroxylflavonone	14.393	239, 277, 312	241	140, 99	$C_{15}H_{12}O_3$
24	Isomucronulatol	14.994	243, 281, 310	303	167, 123	$C_{17}H_{18}O_5$
25	5, 7-dihydroxyflavanone	16.406	235, 290	257	211, 151	$C_{15}H_{12}O_4$
26	3-phenyl-*N*-(2-phenylethyl)-2-propenamide	16.679	275	252	131, 105	$C_{17}H_{17}NO$
27	2′, 4′-dihydroxydihydrochalcon	18.633	277, 313	243	133, 105, 91	$C_{15}H_{14}O_3$

28	2', 4'-dihydroxychalcone	18.901	249, 345	241	159, 99	$C_{15}H_{12}O_3$
29	7-methoxyflavanone	19.116	241, 276, 312	255	209, 151, 131	$C_{16}H_{14}O_3$
30	5-hydroxy-7-methoxyflavanone	21.922	289	271	167, 131	$C_{16}H_{14}O_4$

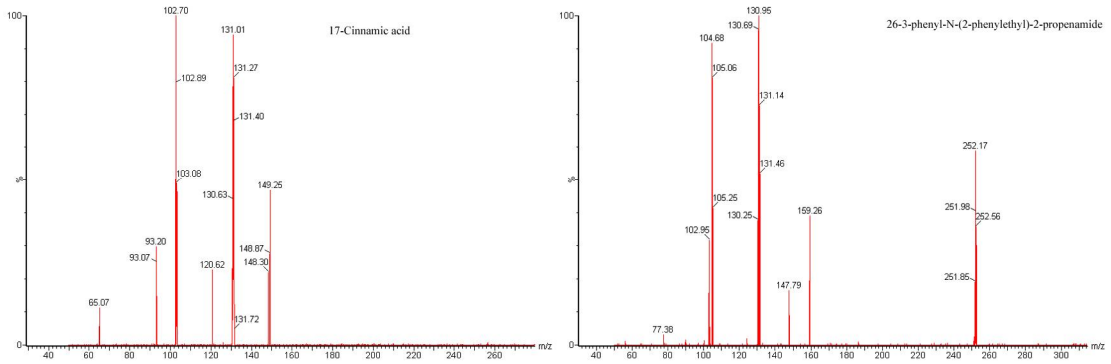

Figure 8. The mass spectra of cinnamic acid (peak 17) and -phenyl-N-(2-phenylethyl)-2-propenamide (peak 26).

4. Conclusion

In this work, a UPLC fingerprint technique with high precision, repeatability, stability, and specificity was developed to identify and evaluate the quality of the twenty-one batches of *O. falcate* combined with the chemometrics method. A representative standard fingerprint chromatogram was obtained using professional software, in which 30 common peaks were marked. The UPLC profiles imply that the quality of *O. falcate* are related to various factors such as the geographical original, growing environment, growing years, harvest season, and etc. UPLC-ESI-MS method involved the use of $[M+H]^+$ ions in the positive ion mode with SIR. Eleven common peaks in chromatographic profile were authenticated based on the comparison of retention time, ultraviolet spectrum and molecular weight with the reference substance. The method developed in this study would provide an important reference for the effective quality control of *O. falcate*.

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