Multi-component analysis in sun-dried and sulfur-fumigated *Angelicae Sinensis Radix* by single marker quantitation and chemometric discrimination

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ABSTRACT

Background: A new method has been developed for the simultaneous determination of ferulic acid, senkyunolide A, and Z-ligustilide in *Angelicae Sinensis Radix* before and after sulfur-fumigation using quantitative analysis of multi-components by a single marker (QAMS). **Materials and Methods:** The feasibility and accuracy of QAMS were checked by the external standard method, and various high-performance liquid chromatographic instruments and chromatographic conditions were investigated to verify its applicability. Using ferulic acid as the internal reference substance, and the contents of senkyunolide A and Z-ligustilide were calculated according to relative correction factors by high-performance liquid chromatography. Meanwhile, the influence of sulfur-fumigation on these chemical components in *Angelicae Sinensis Radix* were evaluated and discriminated by chromatographic fingerprint and chemometrics. **Results:** There was no significant difference observed between the QAMS method and the external standard method. Furthermore, sulfur-fumigation reduced the contents of ferulic acid, senkyunolide A, and Z-ligustilide in *Angelicae Sinensis Radix* by some degree, and the sun-drying and sulfur-fumigation processing could be easily discriminated by chromatographic fingerprint and chemometrics. **Conclusion:** QAMS is a convenient and accurate approach to analyzing multi-component when reference substances are unavailable, simultaneously, chemometrics is an effective way to discriminate sun-dried and sulfur-fumigated *Angelicae Sinensis Radix*.

Key words: *Angelicae Sinensis Radix*, chemometrics, quantitative analysis of multi-components by a single marker, relative correction factor, sulfur-fumigation

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INTRODUCTION

A characteristic of traditional Chinese medicines (TCMs) is the inclusion of a multitude of herbal ingredients, such that quality is evaluated using multiple components and indices. However, separating and analyzing individual chemical markers in TCMs is difficult owing to the instability of monomers and the high cost. Quantitative analysis of multi-components by a single marker (QAMS) is a new methodological approach based on the principle that the effective components in TCMs should maintain their internal function and proportional relations.[1] Therefore, determining the composition of one component (a reference substance) will allow simultaneous monitoring for the remaining ingredients. This has led to the development of innovative multi-component analysis techniques that can address the shortcomings of existing chemical marker analysis approaches for quality control in TCMs. In recent years, researchers have successfully applied the QAMS method to analyze the contents of different medicinal herbs.[2-7] In addition, the QAMS standard of *Rhizoma Coptidis* has been adopted by Chinese Pharmacopoeia (2010 eds.).[8] Nevertheless, analysis using QAMS in *Angelicae Sinensis Radix*, one of the oldest and

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most frequently used herbs in TCMs, has never been reported in the literature.

The theory of QAMS: Within a certain linear range, the quantity and concentration of chemical component is proportional to the response of the detector. In the multi-index valuation model (s, a, b…, i…,), taking a typical active ingredient of herbs (or medicines) as the internal reference substance (IS) and developing the relative correction factor (RCF, f_{sa}, f_{sb}, f_{sc}, …) between the IS with other components, is calculated as follows:

$$f_{si} = \frac{f_s}{f_i} = \frac{A_s / C_s}{A_i / C_i} \quad (1)$$

As is the peak area of IS, C_s is the concentration of IS, *Ai* is the peak area of the sample *i*, and *Ci* is the concentration of the sample *i*. From formula (1) we can export formula (2):

$$C_i = f_{si} \times C_s \times \frac{A_i}{A_s} \quad (2)$$

We can calculate the concentration of samples using formula (2).[9] In this study, ferulic acid was used as the IS and was measured using the external standard method. This reference was then used to calculate the quantity and concentration of senkyunolide A and Z-ligustilide by RCFs. Finally the accuracy of the method was assessed through the external standard method.

In addition to QAMS, fingerprint analysis methods, such as high-performance liquid chromatography (HPLC), has also been widely introduced as a useful multi-component approach for quality control of medicinal herbs as well as herbal products.[10] With regard to the ability to identify a particular herb from related species, fingerprint analysis methods have gained more attention than QAMS. Chemical pattern recognition is acknowledged as a more objective and effective quality evaluation method compared with determination of single or multiple markers in medicinal herbs. Chemometric analyses, especially similarity analysis (SA), hierarchical cluster analysis (HCA), and principal components analysis (PCA) are widely used for chemical classification and chromatographic profile aligning. In this study, the simultaneous determination of three compounds in *Angelicae Sinensis Radix*, before and after sulfur-fumigation, was achieved using QAMS to assess the feasibility and accuracy of which it can be applied in the quality control of *Angelicae Sinensis Radix*. Furthermore, chromatographic fingerprint and chemometrics methods were employed to assess chemical alterations in *Angelicae Sinensis Radix* before and after sulfur-fumigation.

Angelicae Sinensis Radix is derived from the root of *Angelica sinensis* (*Oliv.*) *Diels*. Pharmacological and clinical studies have demonstrated that *Angelicae Sinensis Radix* possesses various bioactivities, including tonifying blood, promoting blood circulation, regulating menstruation, alleviating pain, lubricating the bowels to relieve constipation, and has preventative effects on cardiovascular disease, chronic obstructive pulmonary disease, and bronchial asthma.[11,12] The major active components of *Angelicae Sinensis Radix* are volatile oils, organic acids, polysaccharides, and amino acids. In particular, volatile oils and organic acids are the most important components of *Angelicae Sinensis Radix* and are critical for its therapeutic effect in TCMs. Consequently, we chose three chemical components for the simultaneous multi-component analysis comprising an organic acid (ferulic acid) and two volatile oils (senkyunolide A, Z-ligustilide).

Sulfur-fumigation processing provides many benefits for storage and curing of medicinal materials, such as mold resistance, pest control, curtailing the drying duration, and maintaining a better appearance.[13] However, this process leaves large amount residues of sulfur dioxide and harmful heavy metal residues in the medicinal materials, which are harmful to human health, and reduces its curative effects and medicinal quality.[14] In preliminary studies, we found that sulfur-fumigation led to changes in the quality and quantity of main active ingredients in both crude and prepared drugs.[15-18] Moreover, previous studies have also found that the sulfur-fumigation process severely damages chemical components in *Fritillaria thunbergii Miq*. (Zhebeimu) and *White Ginseng*.[19,20] Thus, after the simultaneous determination by QAMS, we also adopted chromatographic fingerprint and chemometrics methods to assess the influence of sulfur-fumigation on chemical components in *Angelicae Sinensis Radix*, which can supply scientific evidence for quality control.

MATERIALS AND METHODS

Materials and reagents

The reference substance, ferulic acid, was purchased from the National Institute for the Control of Pharmaceutical and Biological Products. Senkyunolide A and Z-ligustilide were purchased from Sichuan Weikeqi Biological Technology Co., Ltd. The chemical structures of these three compounds were confirmed by ^{1}H, ^{13}C nuclear magnetic resonance (NMR) spectroscopy and mass spectrum, and purities, assessed using HPLC analysis, were all over 98%. The chemical structures of these three components are shown in [Figure 1]. HPLC-grade methanol was purchased from Tedia (Ohio, USA). Analytical-grade formic acid and phosphoric acid were purchased from Nanjing Chemical Reagent Co., Ltd. Purified water was purchased from Wahaha (Hangzhou Wahaha Group).

A total of thirty-three *Angelicae Sinensis Radix* was acquired from different regions and pharmacies in

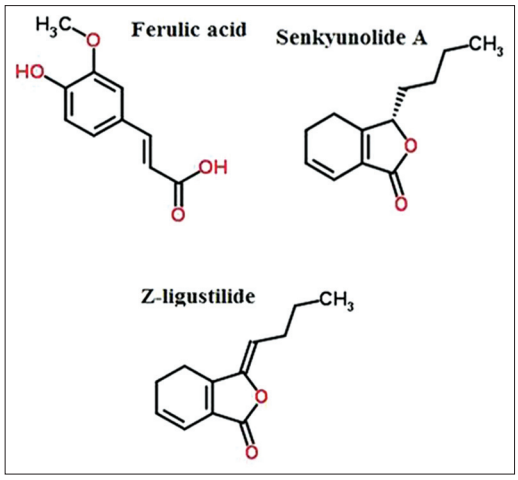

Figure 1: Chemical structures of ferulic acid, senkyunolide A, and Z-ligustilide

China. All samples were tested using acid distillation iodine titration method (GB/T 5009.34) for presence of sulfur-fumigation processing. Thirteen of them were found to be sun-dried (samples 1-13), twenty of them were found to be sulfur-fumigated (samples 14-33). Sample number 34 was prepared in the laboratory from sample number 2 by sulfur-fumigation as comparison. All samples were authenticated by an expert in the field.

Instruments and HPLC conditions

Analyses were performed using HPLC system Varian 920-LC, including Prostar 240 quatpump, Prostar 410 automatic sampler, Prostar 335 DAD, Galaxie Chemstation station (USA Varian); Agilent 1100, including G1312A Binary Pump, G1322A Degasser, G1367A WPLS, G1315B DAD (USA Agilent); Waters 515-2487, including Waters 515 HPLC Pump, 717 Plus Autosampler, Waters 2487 Dual λ Absorbance Detector, Empower station (USA Waters). Columns used included Kromasil C_{18} (250 mm × 4.6 mm, 5 μm), Symmetry C_{18} (250 mm × 4. 6 mm, 5 μm), Eclipse Plus C_{18} (250 mm × 4. 6 mm, 5 μm), and Hypersil ODS C_{18} (250 mm × 4. 6 mm, 5 μm). The flow rate of each column was set at 1.0 mL/min. The injection volume was 20 μL and the column temperature was maintained at 35°C. Mobile phase was composed of: (A) 0.05% phosphoric acid in water and (B) methanol using a gradient elution of 70%→40% A at 0 ~ 20 min, 40%→15% A at 20 ~ 50 min, and 15%→0% A at 50 ~ 55 min. Detection wavelength was set at 270 nm.

Preparation of sample solutions

Powdered *Angelicae Sinensis Radix* were precisely weighed (0.5 g), and transferred into dark brown calibrated flasks. They were extracted with 25 mL of methanol: formic acid (95:5) in an ultrasonic bath for 40 min. Additional methanol was added to compensate for any loss during this process. The supernatants were filtered through a 0.45-μm membrane prior to injection.

Preparation of standard solutions

The standard stock solutions of ferulic acid (0.2 mg/mL), senkyunolide A (0.3 mg/mL), and Z-ligustilide (1.1 mg/mL) were prepared in methanol and stored at 4°C. The calibration curves were prepared at seven different concentration levels, and the diluting factors were 1, 2, 4, 8, 16, and 32 times, respectively.

Data analysis

Chromatographic fingerprint analysis and similarity analysis (SA) were performed using professional computer software recommended by the State Food and Drug Administration (SFDA), entitled the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine. This software was used in the SA of chromatographic and spectral patterns, and also to calculate the similarities of fingerprint profiles. The 3D model in [Figure 2] was produced by Origin 8.0. The HCA and PCA were performed on the characteristic chromatographic peaks in the HPLC fingerprints using SPSS 16.0 (IBM). In HCA, a statistical method called average linkage between groups was applied and the squared Euclidean distance was used as the metric.

RESULTS AND DISCUSSION

Optimization of extraction conditions

To obtain optimal chromatograms, effects on extraction rate of three components in *Angelicae Sinensis Radix* with methanol, 70% methanol, and methanol: formic acid (95:5) were investigated. The results indicated that the contents of ferulic acid in methanol and 70% methanol were higher in accordance with the report in literature.[21] Coniferyl ferulate in methanol solution was easily decomposed and a small quantity was converted into ferulic acid. As a result, this led to an inaccurate calculation of ferulic acid and coniferyl ferulate quantities. This may be the reason why the quantity of ferulic acid was higher in these two solutions. However, when they were examined in the methanol-formic acid, these three components were more stable and possessed a higher extraction rate. Furthermore, in comparison with ultrasonic method and reflux method, the result indicated that ultrasonic method had a higher extraction rate and operated easily. Extraction time was also investigated. We tested at 20 min, 40 min, and 60 min, and found that the highest extraction rate occurred at 40 min. Therefore, methanol: formic acid (95:5) in an ultrasonic bath for 40 min was determined to be optimal parameters to extract sample solutions.

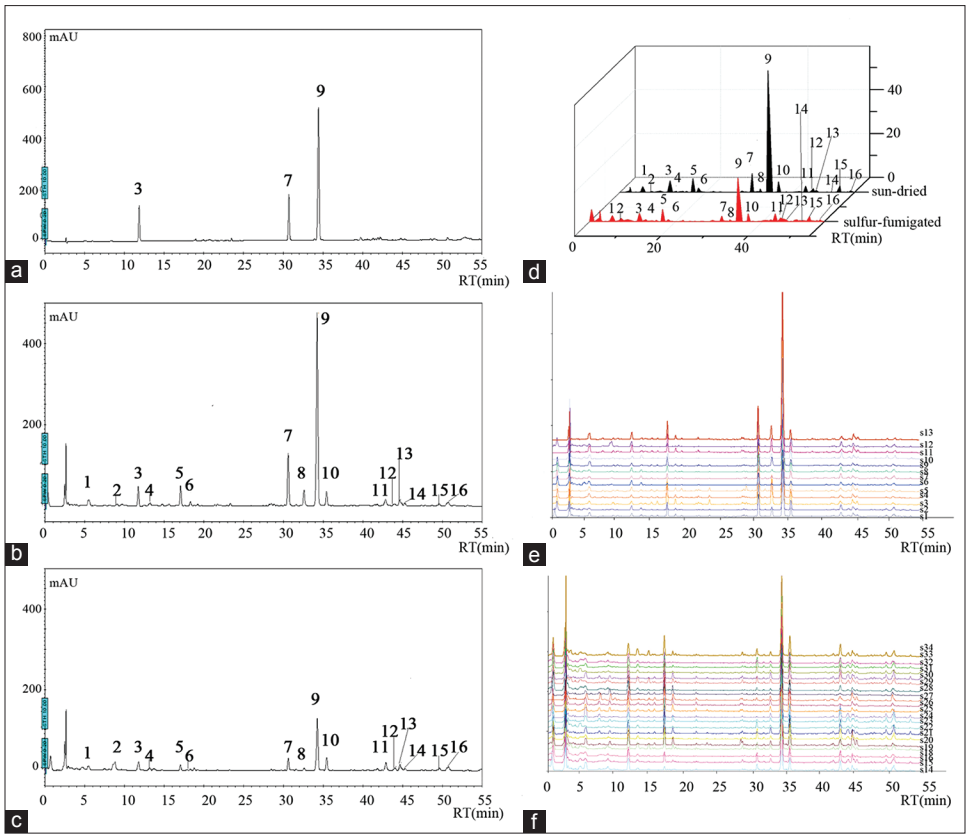

Figure 2: Representative chromatograms and chromatographic fingerprints. (a) HPLC chromatogram of reference substances. (b) HPLC chromatogram of sun-dried *Angelicae Sinensis Radix*. (c) HPLC chromatogram of sulfur-fumigated *Angelicae Sinensis Radix*. Ferulic acid (3), senkyunolide A (7), Z-ligustilide (9). (d) 3D representation of sun-dried and sulfur-fumigated samples. (e) The chromatographic fingerprint of sun-dried *Angelicae Sinensis Radix*. (f) The chromatographic fingerprint of sulfur-fumigated *Angelicae Sinensis Radix*

HPLC method validation

Calibration curves

The injection volume of the mixed reference standard solutions No. 1 - No. 7 was 20 μL precisely. Every concentration was repeated three times, and an average value was calculated. Calibration curves were then drawn and the regression equations were calculated via partial least squares. The results showed that ferulic acid, senkyunolide A, and Z-ligustilide in *Angelicae Sinensis Radix* had good linear relationships in the ranges of 0.003125 - 0.2 mg/mL, 0.0046874 - 0.3 mg/mL, 0.0171875 - 1.1 mg/mL, respectively. The regression equations were $Y = 67.11\ X - 82.56$, $Y = 91.7\ X + 4.6381$, $Y = 1076.40\ X + 32.58$, respectively, with the correlation coefficients (r^2) of 0.9998, 0.9995, 0.9999, respectively.

Precision

The precision test was conducted by replicating the injection of the standard solution six times, following the above procedure. Peak areas were measured and relative standard deviations (RSDs) were calculated. The RSDs of relative peak areas of ferulic acid, senkyunolide A, and Z-ligustilide were 1.20%, 1.11%, and 1.35%, respectively. Given that these values did not exceed 5%, the instrument had high precision.

Repeatability

Repeatability was tested by injecting six independently prepared samples. Samples were prepared using the method outlined in section "Preparation of sample solutions." The RSDs of relative peak areas of ferulic acid, senkyunolide A, and Z-ligustilide were 2.78%, 3.21%, and 2.56%, respectively, which indicated that the experiment had high repeatability.

Stability

Stability was determined with one sample solution that was analyzed at 0 h, 2 h, 4 h, 8 h, 10 h, 12 h, and 24 h. The RSDs of relative peak areas of ferulic acid, senkyunolide A, and Z-ligustilide were 2.79%, 3.36%, and 2.50%, respectively. Therefore, samples were stable within 1 day.

Average recovery

Recovery tests were carried out to investigate the accuracy of the method by spiking known amounts of the mixed reference standard solutions to approximately 0.25 g of the testing sample. The resulting recoveries of ferulic acid, senkyunolide A, and Z-ligustilide were 101.1%, 93.26%, and 100.91%, respectively, and the RSDs were 2.33%, 3.01%, and 1.78%, respectively.

Selection of internal reference substances

Ferulic acid is readily accessible and inexpensive; moreover, it is one of the most pharmacologically active components, showing anti-platelet aggregation and inhibitory effects on platelet serotonin release. Owing to its widespread application and cost-effectiveness, it is the most commonly used substance for determining standard of quality in *Angelicae Sinensis Radix* (Chinese Pharmacopoeia 2010 eds.). In our previous investigation, it was also found that the retention time error of ferulic acid was minimal in diverse instruments and chromatographic columns. Therefore, ferulic acid was chosen as the IS for our study.

Calculation of relative calibration factor

RCF was calculated according to formula (1) outlined in section of "INTRODUCTION." The IS used was ferulic acid. Relative calibration factors of senkyunolide A and Z-ligustilide are shown in [Table 1]. From [Table 1], we can see that there were no significant differences between these seven concentration samples.

Comparison of the external standard method and QAMS

The HPLC method was applied to the simultaneous determination of ferulic acid, senkyunolide A, and Z-ligustilide compounds in *Angelicae Sinensis Radix*. Under these conditions, well-separated and reproducible chromatograms were produced [Figure 2]. Ferulic acid, contained in samples of *Angelicae Sinensis Radix*, before and after sulfur-fumigation were determined by the external standard method. Senkyunolide A and Z-ligustilide were calculated according to their RCFs. The contents of these three components, in samples, were determined with the external standard method to verify the applicability and accuracy of QAMS. There was no significant difference between these two methods, and RSDs were below 5% [Table 2]. Therefore, QAMS is a technique both feasible and accurate in the simultaneous determination of chemical compounds in *Angelicae Sinensis Radix*.

Table 1: Relative correction factors of ferulic acid, senkyunolide A, and Z-ligustilide in *Angelicae Sinensis Radix*

No	RCF	
	$f_{\text{senkyunolide A/ferulic acid}}$	$f_{\text{Z-ligustilide/ferulic acid}}$
1	1.125	1.649
2	1.099	1.585
3	1.112	1.630
4	1.087	1.622
5	1.113	1.636
6	1.100	1.550
7	1.088	1.613
Mean	1.103	1.612
RSD (%)	1.27	2.11

RCF: Relative correction factor; RSD: Relative standard deviation

Evaluation of durability and system suitability of QAMS

Effects of different instruments, different column temperature, and different flow rates on RCFs

The experiment was carried out in different labs. A Kromasil C_{18} (250 mm × 4. 6 mm, 5 μm) was used. The results showed that three different HPLC instruments, including Varian 920-LC, Agilent 1100, and Waters 515-2487, had little influence on RCFs. The effects of different column temperatures and different flow rates on RCFs were analyzed using Varian 920-LC and Kromasil C_{18}. The RSDs were all below 5% [Table 3].

Effects of different chromatographic columns on RCFs and position of analyte peaks

Agilent 1100 and Varian 920-LC were used to investigate effects of four chromatographic columns on RCFs. As there were no other reference substances except for the IS, we only judged correct analyte peak positions by relative retention value, that is, knowing the IS together with retention time and peaks shape. The results indicated that the RSDs were all below 5% [Table 4].

To assess the accuracy, validity, and scientific value of QAMS, we compared the measured value via the external standard method with the calculated value using QAMS. By examining the impact of RCFs for various chromatographic columns and HPLC systems, the results demonstrated that there was no significant difference between these two methods (RSDs <5%). The accuracy of RCFs were also relatively high. Therefore, QAMS is suitable for quantifying the multi-component in *Angelicae Sinensis Radix*, when authenticated standard substances are unavailable.

Comparison of ferulic acid, senkyunolide A, and Z-ligustilide contents in sun-dried and sulfur-fumigated Angelicae Sinensis Radix

From [Figure 2], we can see that, in the same coordinate, the peak height of sulfur-fumigated *Angelicae Sinensis Radix* was distinctly lower than that received from the sun-drying processing. Analysis of ferulic acid, senkyunolide A, and Z-ligustilide content using the external standard method and QAMS both found reduced levels in all three components after sulfur-fumigation. One-way analysis of variance (ANOVA) analysis, comparing sun-dried to sulfur-fumigated samples, found the differences were significantly different ($P < 0.05$).

The contents of senkyunolide A and Z-ligustilide decreased more than ferulic acid following sulfur-fumigation. This

Table 2: Comparing average determination of ferulic acid, senkyunolide A, and Z-ligustilide by the external standard method and QAMS (*n*=3) as well as the similarities in chromatograms of 34 samples. Values indicate sample mean and standard deviation from the mean and the similarities in chromatograms of 34 samples

Origin	Ferulic acid	Senkyunolide A			Z-Ligustilide			Similarity
	External standard method	External standard method	QAMS	RSD (%)[a]	External standard method	QAMS	RSD (%)[b]	
Gansu, 101125	0.0465±0.002	0.108±0.004	0.107±0.002	0.66	0.516±0.001	0.512±0.001	1.10	0.954
Qijiazhuang Minxian, Gansu	0.0679±0.004	0.110±0.004	0.108±0.003	1.30	1.032±0.001	1.030±0.002	1.51	0.972
Lanzhou, Gansu	0.0578±0.002	0.0881±0.002	0.0879±0.001	0.16	0.516±0.002	0.513±0.001	0.97	0.941
Dinxi, Gansu	0.0621±0.001	0.138±0.001	0.138±0.002	0.00	0.537±0.003	0.536±0.002	1.06	0.910
Qinxuxiang (A), Gansu	0.0893±0.003	0.0114±0.001	0.0113±0.001	0.11	0.456±0.002	0.455±0.002	0.62	0.939
Qinxuxiang (B), Gansu	0.095±0.005	0.0978±0.005	0.0973±0.003	0.36	0.772±0.003	0.770±0.003	0.83	0.946
Bozhou, 110930	0.0352±0.003	0.142±0.005	0.141±0.003	0.50	0.848±0.004	0.847±0.002	1.34	0.953
Gansu	0.0682±0.001	0.128±0.006	0.127±0.004	0.55	0.613±0.005	0.611±0.003	1.87	0.922
Minxian, Gansu	0.0509±0.004	0.197±0.004	0.195±0.002	0.72	0.841±0.003	0.840±0.003	1.18	0.919
Minyangzhen, Gansu	0.0602±0.006	0.165±0.004	0.164±0.003	0.43	1.000±0.002	1.000±0.002	0.57	0.979
Shilixiang, Gansu	0.0416±0.004	0.208±0.003	0.206±0.003	0.68	1.000±0.001	0.998±0.002	1.07	0.965
Gansu, 110106	0.0563±0.002	0.135±0.003	0.134±0.002	0.53	0.882±0.006	0.881±0.004	1.70	0.914
Xinhuajie, Gansu	0.0453±0.005	0.109±0.004	0.108±0.003	0.65	1.033±0.004	1.032±0.004	1.45	0.935
Gansu, 100801	0.0443±0.003	0.0443±0.002	0.0439±0.002	0.64	0.405±0.003	0.403±0.002	1.06	0.811
Gansu, 101206	0.0375±0.003	0.0363±0.001	0.036±0.001	0.59	0.417±0.003	0.415±0.003	1.37	0.829
Gansu, 01101600	0.0528±0.001	0.041±0.004	0.0407±0.002	0.52	0.394±0.002	0.393±0.001	0.90	0.733
Gansu, 20101201	0.0542±0.002	0.0289±0.002	0.0287±0.001	0.49	0.393±0.002	0.391±0.001	1.27	0.825
Gansu, 20101203	0.0499±0.004	0.0298±0.003	0.0296±0.003	0.47	0.431±0.001	0.429±0.001	0.49	0.833
Gansu, 101202	0.0439±0.007	0.012±0.005	0.012±0.003	0.00	0.356±0.002	0.355±0.002	1.60	0.775
Gansu, 100722	0.0399±0.003	0.0222±0.002	0.0221±0.001	0.16	0.414±0.003	0.414±0.002	1.03	0.647
Gansu, 110117	0.0560±0.005	0.0615±0.006	0.0611±0.004	0.46	0.449±0.003	0.445±0.003	1.43	0.831
Qinyang, Gansu	0.0390±0.006	0.0374±0.005	0.0371±0.004	0.57	0.437±0.004	0.436±0.002	1.47	0.786
Wangjiaxiang, Gansu	0.0434±0.005	0.00669±0.002	0.00664±0.001	0.38	0.548±0.002	0.548±0.001	1.57	0.747
Tianshui, Gansu	0.0340±0.003	0.022±0.003	0.0219±0.002	0.31	0.389±0.004	0.388±0.003	0.73	0.814
Changhongcun, Gansu	0.0380±0.002	0.00722±0.003	0.00717±0.002	0.48	0.444±0.003	0.442±0.003	0.80	0.769
Gansu, 101018	0.0445±0.002	0.00486±0.001	0.00482±0.001	0.32	0.520±0.002	0.519±0.002	1.51	0.801
Zhangpincun, Gansu	0.0746±0.001	0.0311±0.001	0.0309±0.001	0.45	0.362±0.001	0.361±0.001	1.18	0.738
Gansu, 111018	0.0419±0.004	0.0534±0.003	0.0530±0.004	0.53	0.390±0.001	0.388±0.001	0.55	0.669
Gansu, 111101	0.0261±0.001	0.0706±0.003	0.0699±0.003	0.70	0.482±0.002	0.481±0.002	0.89	0.689
Gansu, 09102003	0.0375±0.005	0.0971±0.005	0.0964±0.004	0.51	0.453±0.003	0.452±0.002	0.78	0.633
Gansu, 101026	0.0207±0.004	0.028±0.002	0.027±0.001	2.60	0.540±0.002	0.539±0.001	1.19	0.777
Gansu, 100801	0.0277±0.002	0.106±0.002	0.105±0.002	0.67	0.453±0.002	0.452±0.001	1.10	0.632
Gansu, 100901	0.0163±0.004	0.128±0.001	0.126±0.002	1.11	0.535±0.003	0.533±0.002	0.80	0.539
Qijiazhuang Minxian, Gansu	0.0198±0.003	0.050±0.003	0.048±0.002	2.89	0.494±0.002	0.493±0.002	0.86	0.800

[a]is the RSD value of Senkyunolide A contents obtained by external standard method and QAMS; [b]is the RSD value of Z-Ligustilide contents obtained by external standard method and QAMS

Table 3: Effects of different instruments, different column temperatures and different flow rates on relative correction factors

Instrument	RCF		Column temperature (°C)	RCF		Flow rate (mL/min)	RCF	
	$f_{\text{senkyunolide A/ ferulic acid}}$	$f_{\text{Z-ligustilide/ ferulic acid}}$		$f_{\text{senkyunolide A/ ferulic acid}}$	$f_{\text{Z-ligustilide/ ferulic acid}}$		$f_{\text{senkyunolide A/ ferulic acid}}$	$f_{\text{Z-ligustilide/ ferulic acid}}$
Waters 515-2487	1.101	1.712		-	-		-	-
Agilent 1100	1.067	1.803		-	-		-	-
Varian 920-LC	1.125	1.649	30	1.088	1.575	0.9	1.106	1.612
			35	1.125	1.649	1.0	1.125	1.649
			40	1.101	1.686	1.1	1.074	1.570
RSD (%)	2.65	4.49		1.70	3.45		2.34	2.45

RCF: Relative correction factor; RSD: Relative standard deviation

Table 4: Effects on four chromatographic columns for relative correction factors using Agilent 1100 and Varian 920-LC and the relative retention values of different instruments and chromatographic columns

Instrument	Chromatographic column	RCF		r_{as}	
		$f_{\text{senkyunolide A/ferulic acid}}$	$f_{\text{Z-ligustilide/ferulic acid}}$	$t_{R\ \text{senkyunolide A}}/t_{R\ \text{ferulic acid}}$	$t_{R\ \text{Z-ligustilide}}/t_{R\ \text{ferulic acid}}$
Agilent 1100	Symmetry C_{18}	1.184	1.682	2.653	3.074
	Kromasil C_{18}	1.067	1.803	2.630	3.045
	Eclipse Plus C_{18}	1.106	1.771	2.533	2.792
	Hypersil ODS C_{18}	1.109	1.618	2.520	2.924
Varian 920-LC	Symmetry C_{18}	1.089	1.577	2.457	2.923
	Kromasil C_{18}	1.125	1.649	2.501	2.881
	Eclipse Plus C_{18}	1.166	1.621	2.480	2.858
	Hypersil ODS C_{18}	1.103	1.700	2.412	2.781
Waters 515-2487	Symmetry C_{18}	-	-	2.778	3.124
	Kromasil C_{18}	-	-	2.622	3.015
	Eclipse Plus C_{18}	-	-	2.528	2.908
	Hypersil ODS C_{18}	-	-	2.546	2.756
RSD (%)		3.48	4.52	3.91	4.09

RCF: Relative correction factor; RSD: Relative standard deviation

is due to the sulfur-fumigation process changing the condition of these two components from being lactone to acidic. During sulfur-fumigation, the lactonic ring is opened and reacts with oxygen. As a result, the double bond in the structure is oxidized, which brings about a reduction in content.

Ferulic acid, senkyunolide A, and Z-ligustilide provide important functions, such as platelet aggregation as well as antithrombotic and tracheal smooth muscle effects. These three substances are the main medicinal components in *Angelicae Sinensis Radix*. Our results indicated that sulfur-fumigation had a negative impact on the effective chemical components in *Angelicae Sinensis Radix*, leading to a decline in quality. This brings to question whether the practice of sulfur-fumigation should replace sun-drying processing, at least in the preparation of medicinal herbs.

HPLC fingerprint of Angelicae Sinensis Radix and similarity analysis

The thirty-four samples studies were processed by analytical instrument association (AIA) style and imported into the Similarity Evaluation System for Chromatographic Fingerprint of TCM software. Analyses confirmed the presence of 16 common peaks and identified the three chemical compounds through the reference substances. Peaks 3, 7, and 9 were identified as ferulic acid, senkyunolide A, and Z-ligustilide, respectively. The chromatographic fingerprint profiles showed abundant diversity of chemical constituents in sun-dried and sulfur-fumigated *Angelicae Sinensis Radix* [Figure 2]. Similarities of each chromatogram from the thirty-four samples were calculated with the reference fingerprint, which was the median of all chromatograms. Similarities of sun-dried samples (1-13) varied from 0.914 to 0.979, while chromatograms of sulfur-fumigated samples (14-34) ranged from 0.539 to 0.833 [Table 2]. These results implied that the chemical constituents of sun-dried and sulfur-fumigated *Angelicae Sinensis Radix* differ greatly.

Quality assessment by hierarchical clustering analysis

Hierarchical clustering analysis is a multivariate analysis method that is used to evaluate the resemblance and differences in these two groups of samples. The areas of the 16 common peaks of samples were calculated; the areas of the 16 constituents of samples 1-34 formed a matrix of 34 × 16. Clustering analysis, using average linkage (between groups) and square euclidean distance, was performed to differentiate and classify the thirty-four samples. The results of the hierarchical clustering analysis showed that samples could be divided into two main groups (I and II) [Figure 3a]. Samples 1-13 (sun-dried) were in cluster I and the remaining samples 14-34 (sulfur-fumigated) were in cluster II, indicating that the two processing methods result in significant differences in quality and illustrating that the sulfur-fumigation processing of *Angelicae Sinensis Radix* significantly alters chemical components than the sun-drying processing.

Discrimination of Angelicae Sinensis Radix using principal components analysis

To analyze further differences in *Angelicae Sinensis Radix* before and after sulfur-fumigation, PCA was used to independently discriminate each chemical component. A 34 × 16 data matrix was created. The first two principal components (PC 1 and PC 2) accounted for more than 80% of variance, 56.785% and 23.491%, respectively [Figure 3b]. Similar to HCA, samples 1-13 (sun-dried) were categorized differently from samples 14-34 (sulfur-fumigated), further illustrating that alterations in chemical components occur as a result of different post-harvest processing methods.

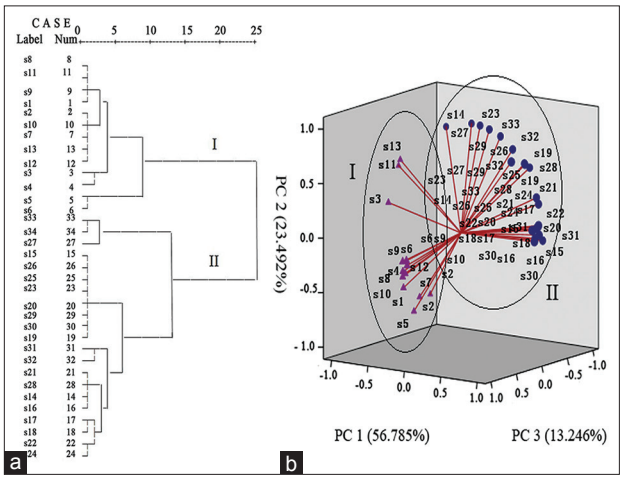

Figure 3: Chemometric figures of sun-dried and sulfur-fumigated *Angelicae Sinensis Radix*. (a) Result of hierarchical clustering analysis. (b) Loading plot obtained by principal components analysis

CONCLUSIONS

In this study, we successfully applied QAMS for the simultaneous determination of ferulic acid, senkyunolide A, and Z-ligustilide and chemometrics for the discrimination in sun-dried and sulfur-fumigated *Angelicae Sinensis Radix*. Our findings indicated that through the validation of the methodology and the verification of durability and system suitability, QAMS possesses high accuracy and feasibility. Furthermore, QAMS analyses showed that sulfur-fumigation processing had an obvious impact on the quantity of three crucial chemical compounds in *Angelicae Sinensis Radix*. The results of decreased quantity of the main effective components in sulfur-fumigated *Angelicae Sinensis Radix* tested by QAMS and chemometric discrimination in sun-dried and suflur-fumigated *Angelicae Sinensis Radix* concluded that sulfur-fumigation could significantly change the quality of *Angelicae Sinensis Radix*. The method developed in this study will be useful for providing a more efficient and feasible quality assessment of *Angelicae Sinensis Radix*, as well as application in evaluating other medicinal herbs.

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