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Quality Analysis of Raw and Processed *Schisandra Chinensis Fructus* by Simultaneous Determination of Eleven Bioactive Lignans Using RP-HPLC Method

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ABSTRACT

A reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed for the simultaneous determination of eleven bioactive lignans, namely schisandrin, gomisin J, schisandrol B, angeloylgomisin H, gomisin G, schisantherin A, schisantherin B, deoxyschisandrin, γ-schisandrin, schisandrin B and schisandrin C in *Schisandra chinensis* with UV detection. An Elite ODS C18 column (250 mm × 4.6 mm, 5 μm) was used for chromatographic separation. The mobile phase consisted of acetonitrile and water under gradient elution. All the calibration curves of the eleven bioactive lignans showed excellent linearity ($r \geq 0.9995$) within the ranges of 25.02-150.1 μg/mL for schisandrin, 5.20-31.20 μg/mL for gomisin J, 10.99-65.94 μg/mL for schisandrol B, 14.10-84.60 μg/mL for angeloylgomisin H, 2.55-15.30 μg/mL for gomisin G, 3.79-22.74 μg/mL for schisantherin A, 8.32-49.92 μg/mL for schisantherin B, 5.16-30.96 μg/mL for deoxyschisandrin, 9.10-54.60 μg/mL for γ-schisandrin, 20.70-124.2 μg/mL for schisandrin B and 4.56-27.36 μg/mL for schisandrin C. The average recoveries ranged from 97.74 to 102.71%. This analytical method was also validated with respect to precision, repeatability and accuracy; and it was proven to be sensitive and accurate to simultaneously determine the eleven lignans in *S. chinensis*. The developed method was further applied to quantify the contents of the eleven lignans in raw and processed *S. chinensis*. The results revealed that the contents of the eleven lignans increased after processing with vinegar and wine. The lignans profiles obtained by this newly established method provided valuable information for the differentiation of crude and processed *S. chinensis* and the different effects. These content ratio differences could provide a scientific basis for the selection of origins and clinical usage.

Key words: *Schisandra chinensis*, lignans, processed, high-performance liquid chromatography, quality control

INTRODUCTION

Traditional Chinese medicines (TCM) are gaining more popularity worldwide for medical treatments in recent years. Thousands of years of history of herbal practice proved that the curative effects of herbs could be enhanced after proper processing with parching or steaming, with or without adjuvant materials such as wine, vinegar and salt[1,2]. *Fructus Schisandra* derived from the dry ripe fruits of *Schisandra chinensis* (Turcz.) Baill. The traditional theory holds that wine-processing can enhance its tonic role in the liver and kidney and the vinegar-processing can enhance its role in convergence and astringent[3,4]. The crude material is often used for the treatment of dyspnea, cough, mouth dryness, spontaneous diaphoresis, nocturnal diaphoresis, nocturnal emission, dysentery, amnesia, etc.[5,6]. Pharmacological studies on the crude material showed that it had good biological activities, including anti-hepatotoxic, anti-HIV[7], anti-cancer[8], antioxidant and anti-tumor activities[9,10], platelet activating factor antagonistic[11], and central nervous system protecting activities[12]; and these activities were obviously enhanced after proper processing of the crude material[1-4].

In the last decades, *S. chinensis* had been extensively investigated in phytochemistry. The results indicated that lignans were its main active components[13-15]. Therefore, it is important to establish a simple and valid method to control the content of lignans in *S. chinensis*. According to the Chinese Pharmacopoeia, schisandrin is recommended as a quality control marker of raw *S. chinensis*. Many HPLC methods had been developed to quantify the content of lignans in *S. chinensis*, most of which were able to determine only three or five lignans[16-21]. Other researchers have developed sophisticated methods to simultaneously quantify as many lignans as possible in their studies[22,23]. However, analytical studies on vinegar-processed and wine-processed *S. chinensis* samples were rarely reported.

In this study, a sensitive and accurate RP-HPLC method was first developed for the simultaneous determination

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of eleven major bioactive lignans in raw and processed *S. chinensis* samples collected from different regions of China, including schisandrin, gomisin J, schisandrol B, angeloylgomisin H, gomisin G, schisantherin A, schisantherin B, deoxyschisandrin, γ-schisandrin, schisandrin B and schisandrin C. Based on the determination of various batches of samples, changes in content, and correlations of the eleven individual lignans in raw and processed *S. chinensis*, the differences were compared and discussed. We believe that these findings should be considered as quality control markers to be implemented into the Pharmacopoeia.

MATERIALS AND METHODS

I. *Chemicals and Reagents*

Reference standards of schisandrin, schisandrol B, schisantherin A, deoxyschisandrin and schisandrin B (> 99% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Nanjing, China). Schisandrin C (> 98% purity) was provided by Shanghai Sunny Biotech Co. Ltd. Gomisin J, angeloylgomisin H, gomisin G, schisantherin B and γ-schisandrin (> 98%

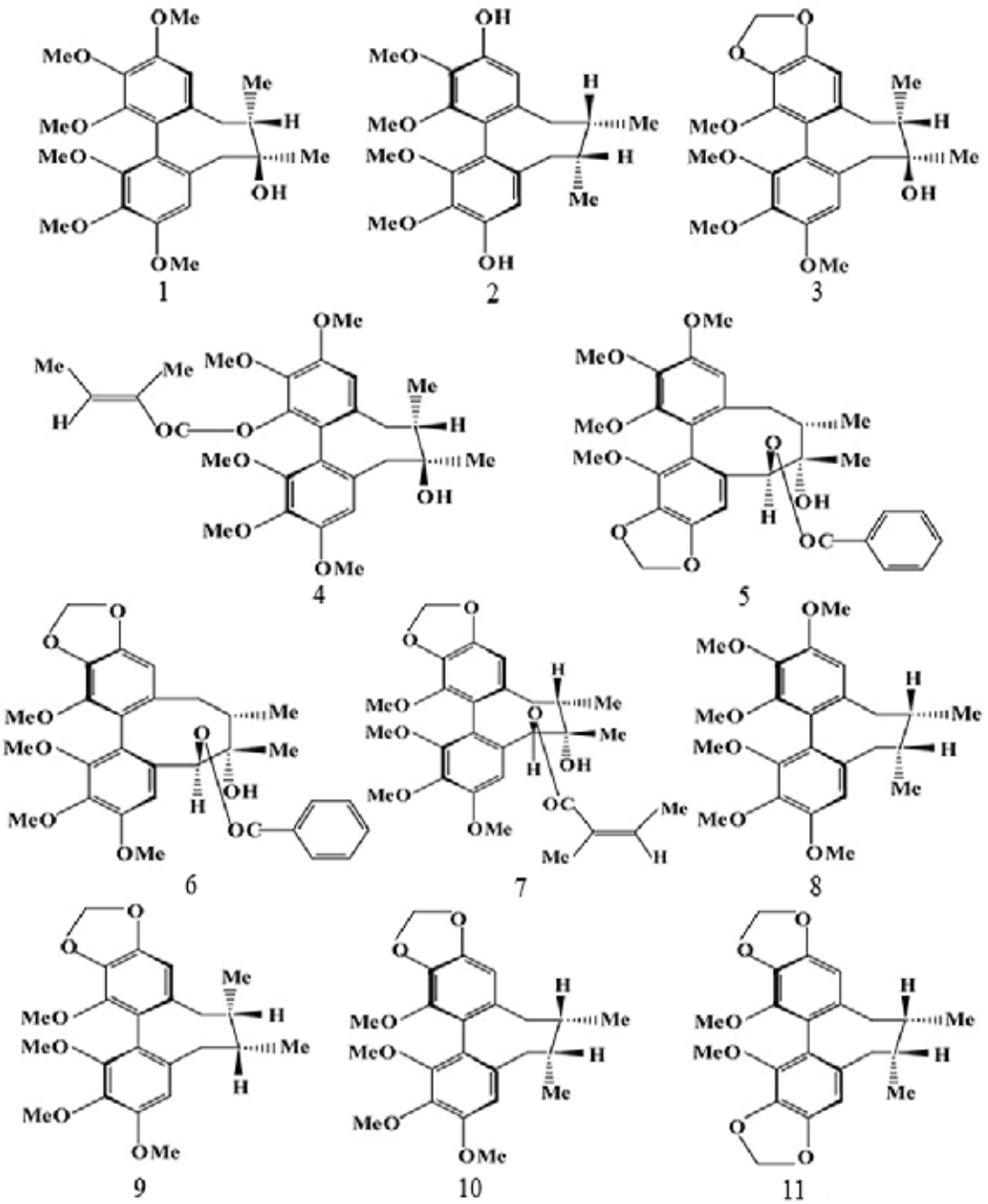

Figure 1. Chemical structures of eleven lignans in *S. chinensis*: schisandrin (1), gomisin J (2), schisandrol B (3), angeloylgomisin H (4), gomisin G (5), schisantherin A (6), schisantherin B (7), deoxyschisandrin (8), γ-schisandrin (9), schisandrin B (10) and schisandrin C (11).

purity) were provided by Shanghai Tauto Biotech Co. Ltd. Their chemical structures are shown in Figure 1. HPLC grade acetonitrile was purchased from Tedia Company (USA). Analytical grade methanol was purchased from Shandong Yuwang Industrial Co. Ltd (Shandong, China). Water was purified by Milli-Q system (Millipore, Bedford, MA, USA).

II. *Apparatus and Chromatographic Conditions*

The high-performance liquid chromatography system consists of an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA), equipped with a quaternary pump, an auto-sampler, a vacuum degasser, an automatic thermostatic column compartment and a UV detector. An Elite ODS C18 column (250 mm × 4.6 mm, 5 μm) was used for chromatographic separation at a column temperature of 30°C. The mobile phase consisted of (A) acetonitrile and (B) water (v/v) using gradient elution (see Table 1) with a flow rate of 1.0 mL/min. The injection volume was 10 μL and the detection wavelength was set at 217 nm.

III. *Preparation of Plant Materials and Processed Samples*

The raw fruits of *S. chinensis* were gathered from three northeastern provinces of China. Processing with vinegar and wine were carried out by the authors. The procedures of vinegar-processing (A) and wine-processing (B) were as follows:

(A) The dried fruits of *S. chinensis* (100 g) were mixed with vinegar (20 g). When the vinegar was completely absorbed, the mixture was steamed until the color of the crude surface was purplish black or dark brown. The final product obtained was dried at 50°C[1,2,3,24].

(B) The dried fruits of *S. chinensis* (100 g) were mixed with wine (20 g). When the wine was completely absorbed, the mixture was steamed until the color of the crude surface was purplish black or dark brown. The final product obtained was dried at 50°C[1,2,3,24].

IV. *Preparation of Sample Solutions*

The crude material and two processed products were pulverized and the powder was screened through a 60-mesh sieve. The fine powders of the samples were accurately weighed (0.3 g), and transferred into 25-mL volumetric flasks. They were extracted with 25 mL of methanol in an ultrasonic bath for 20 min at room temperature. Additional methanol was added to make up to the volume. The supernatants were centrifuged for 10 min at 14,000 g prior to injection into the HPLC system.

Table 1. Time program of the gradient elution

Time (min)	Flow (mL/min)	CH_3CN (%)	Water (%)
0	1.0	50	50
17	1.0	50	50
25	1.0	55	45
30	1.0	75	25
35	1.0	75	25
40	1.0	65	35
45	1.0	50	50
50	1.0	50	50

V. *Preparation of Standard Solutions*

Mixed standard stock solution of schisandrin (250.2 μg/mL), gomisin J (52.00 μg/mL), schisandrol B (109.9 μg/mL), angeloylgomisin H (141.0 μg/mL), gomisin G (25.50 μg/mL), schisantherin A (37.90 μg/mL), schisantherin B (83.20 μg/mL), deoxyschisandrin (51.60 μg/mL), γ-schisandrin (91.00 μg/mL), schisandrin B (207.0 μg/mL) and schisandrin C (45.60 μg/mL) were prepared in methanol and kept at -20°C. Working solutions were prepared by diluting the mixed standard solution with methanol and storing at 4°C. The solutions were centrifuged for 10 min at 14,000 g prior to injection into the HPLC system.

RESULTS AND DISCUSSION

I. *Optimization of Chromatographic Conditions*

An acetonitrile-water system was adopted to separate the eleven lignans due to its good resolution. In the pre-test, different compositions of mobile phase were tried to obtain chromatograms with good resolution of adjacent peaks. Various mixtures of water and methanol were used as the mobile phase, but separation was not satisfactory. When methanol was replaced by acetonitrile, the situation improved greatly and satisfactory resolution was obtained. In addition, other chromatographic variables were optimized, including the type of analytical column (Elite ODS C18 and Kromasil C18), column temperature (25 and 30°C) and flow rate (0.8 and 1.0 mL/min). Eventually, optimal separation was achieved on an Elite ODS C18 column (250 mm × 4.6 mm, 5 μm) at a column temperature of 30°C with a flow rate of 1.0 mL/min. The compounds were identified by comparing their retention times with the standards.

II. *Optimization of Sample Preparation*

Various extraction methods, solvents and extraction times were evaluated in an effort to optimize the extraction procedures. The results revealed that ultrasonic extraction was better than reflux, so further experiments were carried out with ultrasonic extraction. Various solvents including 20% methanol, 50% methanol, 80% methanol and pure methanol were screened with ultrasonic extraction to evaluate the efficiency of the solvent extraction. The results showed that pure methanol was the most suitable extraction solvent, as it allowed the extraction of all the major constituents with high yields. The influence of the extraction time (10, 20, 30 and

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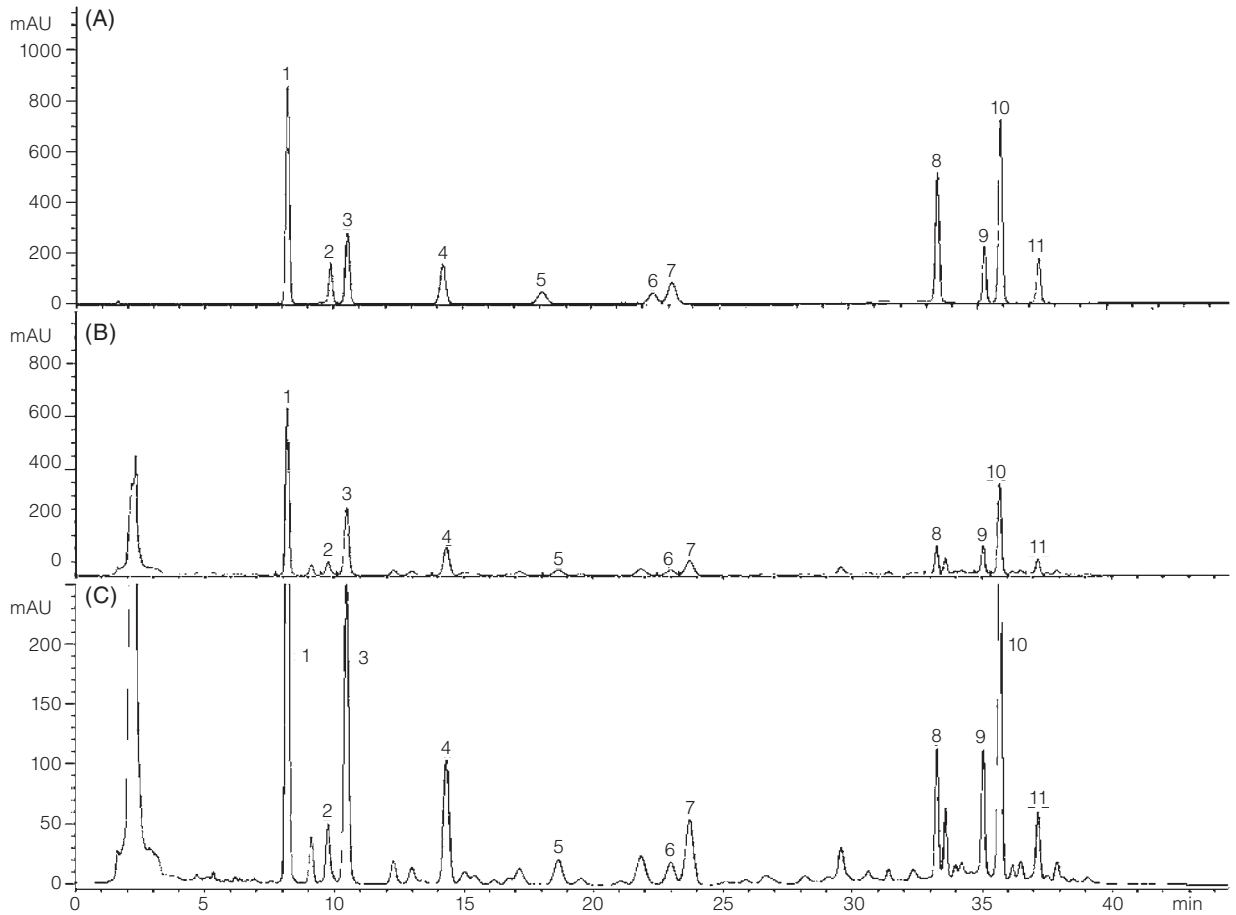

Figure 2. HPLC Chromatograms of (A) mixed standards: 1: schisandrin (125.1 μg/mL), 2: gomisin J (26.00 μg/mL), 3: schisandrol B (54.95 μg/mL), 4: angeloylgomisin H (70.50 μg/mL), 5: gomisin G (12.75 μg/mL), 6: schisantherin A (18.95 μg/mL), 7: schisantherin B (41.60 μg/mL), 8: doxyschisandrin (25.80 μg/mL), 9: γ-schisandrin (45.50 μg/mL), 10: schisandrin B (103.5 μg/mL) and 11: schisandrin C (22.80 μg/mL); (B) S. chinensis; (C) amplified (B), respectively.

60 min) on the extraction efficiency was also investigated. It was found that ultrasonic extraction for 20 min obtained optimal results and there was no obvious difference between 20 and 30 min. Finally, the following extraction conditions were selected: ultrasonic extraction of the samples in pure methanol for 20 min.

III. *Validation of the Analytical Method*

(I) *Specificity*

Validation of specificity was conducted on a mixture of the eleven standard solution, *S. chinensis* sample solution, and the mobile phase (as a blank). The HPLC chromatograms (Figure 2), indicated that the blank solutions did not interfere with the separation. Good separation for the eleven lignans in *S. chinensis* was obtained under the optimized chromatographic conditions.

(II) *Linearity, LOD and LOQ*

Standard stock solutions containing the eleven analytes were prepared and diluted to appropriate concentrations for plotting the calibration curves. At least six concentrations of the eleven analytes solution were analyzed in triplicate, and the calibration curves were then constructed by plotting the peak areas versus the concentration of each analyte. LOD (limit of detection) and LOQ (limit of quantification) were separately determined at a signal-to-noise ratio (S/N) of three and ten, respectively. Linear regressions, LOD and LOQ values were estimated with the external standard method and are reported in Table 2. Linear regression analysis of each component had a wide linear concentration range and the correlation coefficient was greater than 0.9995. The LOD and LOQ values suggested that the developed HPLC method was sufficiently sensitive for the determination of the eleven lignans in *S. chinensis*.

(III) *Precision*

The instrument precision was validated by performing the intra- and inter-day assays on the mixed standard solutions. The intra-day precision assay was carried out with six replicate injections in a single day, while the inter-day precision

Table 2. Calibration curves of the eleven lignans in *S. chinensis* (n = 3, mean value)

Components[a]	Regression equation[b]	r	Linear range (μg/mL)	Limit of detection (LOD, μg/mL)	Limit of quantification (LOQ, μg/mL)
1	y = 63.452x + 45.907	0.9999	25.02 - 150.12	0.09	0.32
2	y = 58.715x – 3.56	0.9995	5.20 - 31.20	0.08	0.28
3	y = 57.319x + 10.547	0.9997	10.99 - 65.94	0.03	0.13
4	y = 24.94x + 2.403	0.9998	14.10 - 84.60	0.04	0.16
5	y = 41.925x + 8.45	0.9999	2.55 - 15.30	0.13	0.41
6	y = 48.913x + 1.8467	0.9996	3.79 - 22.74	0.11	0.35
7	y = 41.85x + 0.13	0.9998	8.32 - 49.92	0.10	0.32
8	y = 72.707x + 3.12	0.9997	5.16 - 30.96	0.06	0.20
9	y = 38.298x + 13.052	0.9997	9.10 - 54.60	0.05	0.17
10	y = 67.394x + 22.127	0.9998	20.70 - 124.20	0.04	0.15
11	y = 65.479x + 3.0667	0.9998	4.56 - 27.36	0.03	0.11

[a] The notation for analyte refers to Figure 1.
[b] y is the peak area and x is the concentration (μg/mL) of the component.

Table 3. Intra- and inter-day precision data for concentrations of the eleven lignans in *S. chinensis*

Components[a]	Intra-day precision (n = 6, mean ± SD)						Inter-day precision (n = 18, mean ± SD)	
	Day 1		Day 2		Day 3			
	Concentration (μg/mL)	RSD (%)	Concentration (μg/mL)	RSD (%)	Concentration (μg/mL)	RSD (%)	Concentration (μg/mL)	RSD (%)
1	100.05 ± 0.14	0.14	100.07 ± 0.22	0.22	100.02 ± 0.19	0.19	100.05 ± 0.17	0.17
2	20.81 ± 0.08	0.39	20.85 ± 0.13	0.62	20.82 ± 0.06	0.29	20.83 ± 0.09	0.43
3	43.96 ± 0.15	0.34	43.97 ± 0.15	0.33	43.94 ± 0.11	0.25	43.96 ± 0.13	0.29
4	56.33 ± 0.22	0.39	56.42 ± 0.20	0.36	56.41 ± 0.16	0.28	56.39 ± 0.19	0.33
5	10.05 ± 0.04	0.38	10.09 ± 0.05	0.52	10.06 ± 0.07	0.68	10.06 ± 0.05	0.53
6	15.15 ± 0.06	0.43	15.16 ± 0.08	0.52	15.12 ± 0.05	0.36	15.14 ± 0.07	0.43
7	33.26 ± 0.08	0.24	33.22 ± 0.09	0.28	33.23 ± 0.06	0.19	33.24 ± 0.08	0.23
8	20.63 ± 0.03	0.16	20.65 ± 0.04	0.18	20.63 ± 0.05	0.23	20.64 ± 0.04	0.19
9	36.37 ± 0.12	0.33	36.36 ± 0.10	0.27	36.36 ± 0.11	0.31	36.36 ± 0.10	0.29
10	76.64 ± 0.08	0.11	76.53 ± 0.22	0.29	76.60 ± 0.12	0.16	76.59 ± 0.15	0.20
11	18.26 ± 0.05	0.30	18.21 ± 0.14	0.79	18.24 ± 0.10	0.54	18.23 ± 0.10	0.55

[a] The notation for analyte refers to Figure 1.

was analyzed everyday in duplicate for three consecutive days.

The results are summarized in Table 3. The RSD values at each concentration were less than 0.79% for both intra- and inter-day precision assays, indicating the high precision of the chromatographic system.

(IV) *Reproducibility and Sample Stability*

The reproducibility was examined by analyzing six sample solutions from the same batch under identical preparation conditions. The stability of the sample at room temperature was confirmed by analyzing one sample solution at 0, 6, 12, 24 and 72 h, respectively. The RSD values from the reproducibility experiments were less than 1.08%, which revealed that the analytical method had good reproducibility.

In the stability validation, neither appreciable change of the eleven lignans nor degradation products was detected in the chromatograms. The RSD values of concentrations were below 1.89%. The *S. chinensis* sample solution was stable within 72 h at room temperature after preparation.

(V) *Accuracy*

For accuracy validation, standard solutions at low, medium and high levels (80, 100 and 120% of the original contents) were spiked individually to a *S. chinensis* sample with known contents of the eleven lignans. The samples with the spiked standard solutions were then extracted and analyzed according to the procedure developed in this study.

Table 4. Recovery of the eleven lignans in *S. chinensis* (n = 3, mean value)

Components[a]	Original (mg)	Spiked (mg)	Determined (mg)	Recovery (%)	RSD (%)
1	1.2055	0.9664	2.1771	100.20	0.18
		1.2080	2.4132	99.46	0.22
		1.4496	2.6503	99.57	0.16
2	0.0691	0.0560	0.1255	100.38	2.60
		0.0700	0.1404	101.35	1.99
		0.0840	0.1535	101.86	1.08
3	0.2291	0.1840	0.4164	101.49	0.81
		0.2300	0.4593	99.58	1.34
		0.2760	0.5062	100.30	1.60
4	0.5488	0.4400	1.0011	102.71	2.04
		0.5500	1.0898	98.06	0.40
		0.6600	1.1934	97.74	0.91
5	0.0512	0.0416	0.0929	100.05	1.38
		0.0520	0.1031	99.33	1.40
		0.0624	0.1138	100.24	0.88
6	0.0707	0.0560	0.1268	99.89	2.39
		0.0700	0.1412	100.31	1.31
		0.0840	0.1551	100.48	0.86
7	0.2800	0.2240	0.5072	101.07	0.46
		0.2800	0.5608	99.78	0.78
		0.3360	0.6171	100.21	1.11
8	0.5155	0.4160	0.9333	100.09	1.41
		0.5200	1.0359	99.56	0.28
		0.6240	1.1450	100.79	0.84
9	0.3197	0.2560	0.5765	99.96	1.41
		0.3200	0.6460	101.46	0.87
		0.3840	0.7047	100.16	0.96
10	0.5630	0.4480	1.0111	99.67	1.24
		0.5600	1.1289	100.54	1.01
		0.6720	1.2466	101.63	0.90
11	0.0625	0.0496	0.1127	100.82	1.40
		0.0620	0.1255	101.06	0.79
		0.0744	0.1385	102.05	0.65

[a] Refer to Figure 1 for the notation of the analytes.

The results are reported in Table 4. The average recoveries ranged from 97.74 to 102.71%, with the RSD values ranging from 0.16 to 2.60%, indicating that the method was accurate.

IV. *Quantification of S. Chinensis Samples*

The validated HPLC method was applied to simultaneously determine schisandrin, gomisin J, schisandrol B, angeloylgomisin H, gomisin G, schisantherin A, schisantherin B, deoxyschisandrin, γ-schisandrin, schisandrin B and schisandrin C in raw and processed *S. chinensis*. The contents of the components are summarized in Table 5.

In order to determine the variation of *S. chinensis*, different samples of herbs were collected from three northeastern provinces of China. It was found that the content of each compound in the crude drug, wine-processed and

Table 5. Contents of the eleven lignans in *S. chinensis* (mean ± SD, n = 3, mg/g)

Components[a]	Contents (mean ± SD, n = 3, mg/g)								
	Heilongjiang			Jilin			Liaoning		
	Crude	Wine-processed	Vinegar-processed	Crude	Wine-processed	Vinegar-processed	Crude	Wine-processed	Vinegar-processed
1	7.29 ± 0.02	7.77 ± 0.01	7.67 ± 0.02	6.54 ± 0.01	7.08 ± 0.01	6.57 ± 0.01	7.33 ± 0.01	7.28 ± 0.00	6.83 ± 0.01
2	0.83 ± 0.01	0.81 ± 0.00	0.88 ± 0.00	0.61 ± 0.01	0.67 ± 0.00	0.65 ± 0.01	0.41 ± 0.01	0.48 ± 0.01	0.52 ± 0.00
3	3.37 ± 0.01	4.46 ± 0.01	3.71 ± 0.01	3.08 ± 0.00	3.27 ± 0.01	2.96 ± 0.01	2.15 ± 0.00	2.50 ± 0.01	2.37 ± 0.00
4	4.69 ± 0.01	5.34 ± 0.00	4.76 ± 0.01	3.93 ± 0.02	4.25 ± 0.01	3.97 ± 0.01	3.67 ± 0.00	3.73 ± 0.00	3.60 ± 0.01
5	0.74 ± 0.00	0.88 ± 0.00	0.79 ± 0.00	0.33 ± 0.01	0.37 ± 0.00	0.39 ± 0.00	0.52 ± 0.00	0.66 ± 0.00	0.62 ± 0.00
6	0.23 ± 0.00	0.54 ± 0.00	0.29 ± 0.00	-[b]	0.21 ± 0.00	0.20 ± 0.00	0.30 ± 0.00	0.37 ± 0.00	0.36 ± 0.00
7	1.33 ± 0.00	2.14 ± 0.01	1.34 ± 0.00	0.89 ± 0.01	0.96 ± 0.01	1.02 ± 0.01	1.66 ± 0.02	1.73 ± 0.00	1.82 ± 0.00
8	1.27 ± 0.00	0.96 ± 0.02	1.21 ± 0.01	0.90 ± 0.01	0.96 ± 0.01	0.96 ± 0.00	1.07 ± 0.02	1.06 ± 0.02	1.09 ± 0.01
9	2.20 ± 0.02	2.19 ± 0.01	2.63 ± 0.01	1.77 ± 0.01	2.07 ± 0.02	2.01 ± 0.01	1.39 ± 0.00	1.53 ± 0.01	1.46 ± 0.00
10	5.25 ± 0.01	4.24 ± 0.01	5.25 ± 0.02	4.35 ± 0.00	4.98 ± 0.00	4.80 ± 0.00	3.56 ± 0.01	3.94 ± 0.00	3.97 ± 0.00
11	1.32 ± 0.01	0.81 ± 0.00	1.30 ± 0.01	0.98 ± 0.00	1.08 ± 0.01	1.08 ± 0.01	0.50 ± 0.01	0.44 ± 0.00	0.43 ± 0.00
Total	28.53 ± 0.04	30.13 ± 0.01	29.83 ± 0.05	23.38 ± 0.04	25.90 ± 0.01	24.61 ± 0.05	22.56 ± 0.03	23.72 ± 0.02	23.08 ± 0.02

[a] Refer to Figure 1 for the notation of the analytes.
[b] Below LOD.

vinegar-processed products varied significantly. The content of schisandrin was much higher than the other lignans. This provided a scientific basis for the China Pharmacopoeias, which only stipulates that the content of schisandrin cannot be below 4 mg/g. Among the eleven analyzed lignans, the *S. chinensis* from Heilongjiang was higher than that from other origins and the processed products were all higher than the crude material. Moreover, the wine-processed product was the highest in every group. The possible reasons were as follows: the tissue of *S. chinensis* was destroyed in the steaming process, which helped to improve the rate of active ingredients fried; and the wine and vinegar helped to increase the dissolution of lignans. However, there were no significant differences in the sum of the eleven lignans. The clinical usages proved that the processed products of *S. chinensis* can obviously enhance its effectiveness. It may be related to the changes in the composition of lignans. Additionally, the changes of other constituents in *S. chinensis* can also influence its effectiveness during the steaming process. The specific reasons need to be further explored.

CONCLUSIONS

An RP-HPLC analytical method has been developed for the simultaneous determination of schisandrin, gomisin J, schisandrol B, angeloylgomisin H, gomisin G, schisantherin A, schisantherin B, deoxyschisandrin, γ-schisandrin, schisandrin B and schisandrin C in *S. chinensis* and it is applied to monitor raw and processed *S. chinensis*. The complete validation results showed that the developed method as a reliable and sensitive quality control for *S. chinensis* materials. The proposed method has potential in improving the quality control of *S. chinensis* and its processed products. The results would be helpful for further discussion of the different processing products of *S. chinensis*. Moreover, based on this multi-component assay method, further studies on phytochemistry, pharmacodynamics and statistics are expected to follow.

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