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Full Length Research Paper

Simultaneous quantification of four saponins, three alkaloids and three fatty acids in *Solanum nigrum* Linn. by HPLC-ELSD

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A new high-performance liquid chromatography (HPLC) coupled with evaporative light scattering detection (ELSD) method was developed, and the quantity of four major saponins, three alkaloids and three fatty acids were determined simultaneously in *Solanum nigrum* Linn., a commonly used traditional Chinese medicine (TCM) herb. The simultaneous separation of four saponins, three alkaloids and three fatty acids was achieved on a C_{18} analytical column. The mobile phase consisted of 0.3‰ triethylamine in acetonitrile and 0.3‰ triethylamine water-methanol (80: 20, v/v) performed on a gradient program in 80 min. The flow rate was 1.0 ml min^{-1} and the column temperature was maintained at 30°C. During ELSD, the drift tube temperature was 40°C and the nebulizer nitrogen gas flow-rate was controlled at 3.5 bars. All calibration curves showed good linear regression (r^2 > 0.9981) within test ranges. This method showed good reproducibility for the quantification of four saponins, three alkaloids and three fatty acids in *S. nigrum* with intra- and inter-day variations of less than 5.0%. The validated method was successful in quantifying the four saponins, three alkaloids and three fatty acids in ten sources of *S. nigrum*, and provided a new basis for the overall assessment of *S. nigrum* quality.

Key words: HPLC-ELSD, *Solanum nigrum* Linn., saponin, alkaloid, fatty acid.

INTRODUCTION

Traditional Chinese medicine (TCM), which aims to maintain the dynamic balance of the whole body to achieve harmony between human and nature, has been highlighted by many researchers in modern biomedicine. Over the last decade, there has been a growing and sustaining interest in TCM worldwide, largely based on natural plants. Longkui, the whole herb of *Solanum nigrum* Linn. (Solanaceae) has long been used for many years as a remedy to treat trachitis, cancer, hepatic damage, mastitis and aerodontalgia (Ikeda et al., 2003; Raju et al., 2003; Hebbar et al., 2004; Meng, 1954).

S. nigrum was used mostly as an herbal ingredient in TCM prescriptions for cancers, including liver cancer, breast cancer, uterine cervix cancer gastric cancer and other cancers (Sammon, 1998; Son et al., 2003; Yen et al., 2001). It has attracted attention in the exploitation for anticancer Chinese's medicines, recently. There were plenty of secondary metabolites such as solasonine, solasnine, oleic acid, linoleic acid, and saponins in it (Saijo et al., 1982; Sultana et al., 1995; Tsuyoshi et al., 2000), which showed high pharmacological and biological activities (Jimoh et al., 2010; Li et al., 2010; Yang et al., 2010). However, in the process of further research and application, we found the herb quality were variable, which influenced its use. This herb grew wildly in many provinces of China. Due to the complicated terrains and diverse climates in China, the secondary metabolites often varied greatly depending on the different environmental parameters. Recently, the application of *S. nigrum* highly increased; thereby, making it necessary to develop a method of controlling its quality.

Even though several chemical and pharmacological studies on *S. nigrum* have been reported, no available

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evaluation method for the quality control of *S. nigrum* has been reported until now. Because of the chemical property, such as high polarity, weak UV absorbance and low content in herb, etc, HPLC-UV analysis of the aforenamed compounds result in a high level of baseline noise, low sensitivity, thus, limiting the choice of mobile-phase. With high sensitivity and selectivity, LC-mass spectrometry is a good choice for its quantification, but the expensive running costs do not permit its application for routine analysis. The advantages of HPLC-ELSD include high sensitivity, strong specificity, good repeatability, low cost, etc. based on these chemical features of HPLC-ELSD, it could be used to determine the chemical contents of herbs.

By using HPLC and ELSD, the procedure for simultaneously separating four saponins, including 5α-pregn-16-en-3β-ol-20-one lycotetraoside (1), nigrumnin I (8), degalactotigonin (9), tigogenin3-O-β-D-glucopyranosyl-(1→2)-O-[β-D-glucopyranosyl-(1→3)]-O-β-D-glucopyranosyl-(1→4)-O-β- D- galactopyranoside (10); three alkaloids, including solasonine (5), solamargine (6) and β2-solamargine (7); as well as three fatty acids, linoleic acid (2), palmitic acid (3) and oleic acid (4) from this species was elucidated in the present work, which was the first report to quantify them at the same time with a HPLC method.

MATERIALS AND METHODS

Samples, chemicals and reagents

Ten raw samples of *S. nigrum* were collected from nine provinces in China (Table 7). All of these samples were identified by Prof. Qianhai Chen (Guizhou University, China). Voucher specimens and the samples were stored at the Institute of Biology, Guizhou Academy of Sciences.

Ten reference compounds: 5α-pregn-16-en-3β-ol-20-one lycotetraoside (1), linoleic acid (2), palmitic acid (3), oleic acid (4), solasonine (5), solamargine (6), β_2-solamargine (7), nigrumnin I (8), degalactotigonin (9), and tigogenin 3O- β- D-glucopyranosy l-(1→2)- O-[β- D- glucopyranosyl -(1→3)]-O-β-D-glucopyranosyl -(1→4) - O -β-D-galactopyranoside (10), were isolated previously from the dried herb of *S. nigrum* by repeated silica gel, Sephadex LH-20 and Rp-18 silica gel column chromatography, and their structures were elucidated by comparing their spectral data with acknowledged standards, respectively (UV, IR, MS, ^{1}HNMR, ^{13}CNMR, HMBC and COSY). Their purity was all determined to be more than 98% by normalization of the peak areas as detected by HPLC-ELSD, and in such experiments, all compounds were very stable in methanol solution. Methanol, acetonitrile, and water were HPLC grade while ethanol was analytical grade.

Instrumentation and chromatographic conditions

The chromatographic analysis was carried out on a Shimadzu LC-10ADvp series HPLC system (Shimadzu Corporation, Japan) with a vacuum degasser, binary pump, auto injector, thermostated column compartment and a Sedex 75 ELSD (Seder Corporation, France). A Phenomenex RP column (C_{18}, 5 µm, 250 × 4.6 mm) was used. An SK8200H ultrasonic cleaner (KUDOS Company, China) was used for extraction. The vacuum concentrator system consisted of a rotary evaporator, a digital bath, and a cool ace. Isolation was performed by LC-8A prep. A Phenomenex C_{18} column (5 µm, 250 × 4.6 mm) was used for these procedures. The mobile phase consisted of 0.3‰ triethylamine in acetonitrile (A) and 0.3‰ triethylamine water-methanol (80: 20, v/v) (B) performing a gradient program of 10 to 15% of A for 10 min, 15 to 30% of A for 15 min, 30 to 34% of A for 30 min, 34 to 50% of A for 52 min, 50 to 55% of A for 55 min, and 55% of A for 80 min. The flow rate was 1.0 ml min^{-1} and the column temperature was maintained at 40°C. During ELSD, the drift tube temperature was 40°C, and the nebulizer nitrogen gas flow-rate was kept at 3.5 bars.

Calibration curves

Methanol stock solutions containing the ten analytes were prepared and diluted to appropriate concentrations. Six concentrations of the ten analyte solutions were injected in triplicate, respectively, and the calibration curves were then constructed by plotting the peak areas versus the concentration of each analyte.

Limits of detection and quantification

Stock solutions containing the ten reference compounds were diluted with methanol to a series of appropriate concentrations, and were then injected into the HPLC system for analysis, respectively. The limits of detection (LOD) and quantification (LOQ) in the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

Precision and accuracy

Intra- and inter-day variations were chosen to determine the precision of the developed assay. Approximately, 2.0 g pulverized *S. nigrum* were weighed, extracted and analyzed as described in ”Instrumentation and chromatographic conditions”. For intra-day variability, the samples were analyzed in triplicate three times within one day, while for inter-day variability; the samples were examined in triplicate on three consecutive days. Variations were expressed using relative standard deviations. Recovery tests were used to evaluate the accuracy of this method. Accurate amounts of the ten reference compounds were added to approximately 1.0 g of *S. nigrum*, and the samples were then extracted and analyzed as described in ”Instrumentation and chromatographic conditions”. The average recoveries were calculated using the formula:

Recovery (%) = (amount found-original amount)/amount spiked × 100%, and RSD (%) = (SD/mean) × 100%.

Sample preparation

A total of 2.0 g of powdered dried herb was extracted with 20.0 ml ethanol-water (6: 4, v/v) solution in an ultrasonic water bath for 20 min. The procedure was repeated twice. The extracted solution was blended and filtered using analytical filter paper. The filtered solution was evaporated at 50°C via vacuum. The dried extract was dissolved in 25.0 ml ethanol-water (6: 4, v/v) and then filtered through a 0.45 µm membrane filter.

RESULTS AND DISCUSSION

Validation of the method

It has been generally observed that the detector respose,

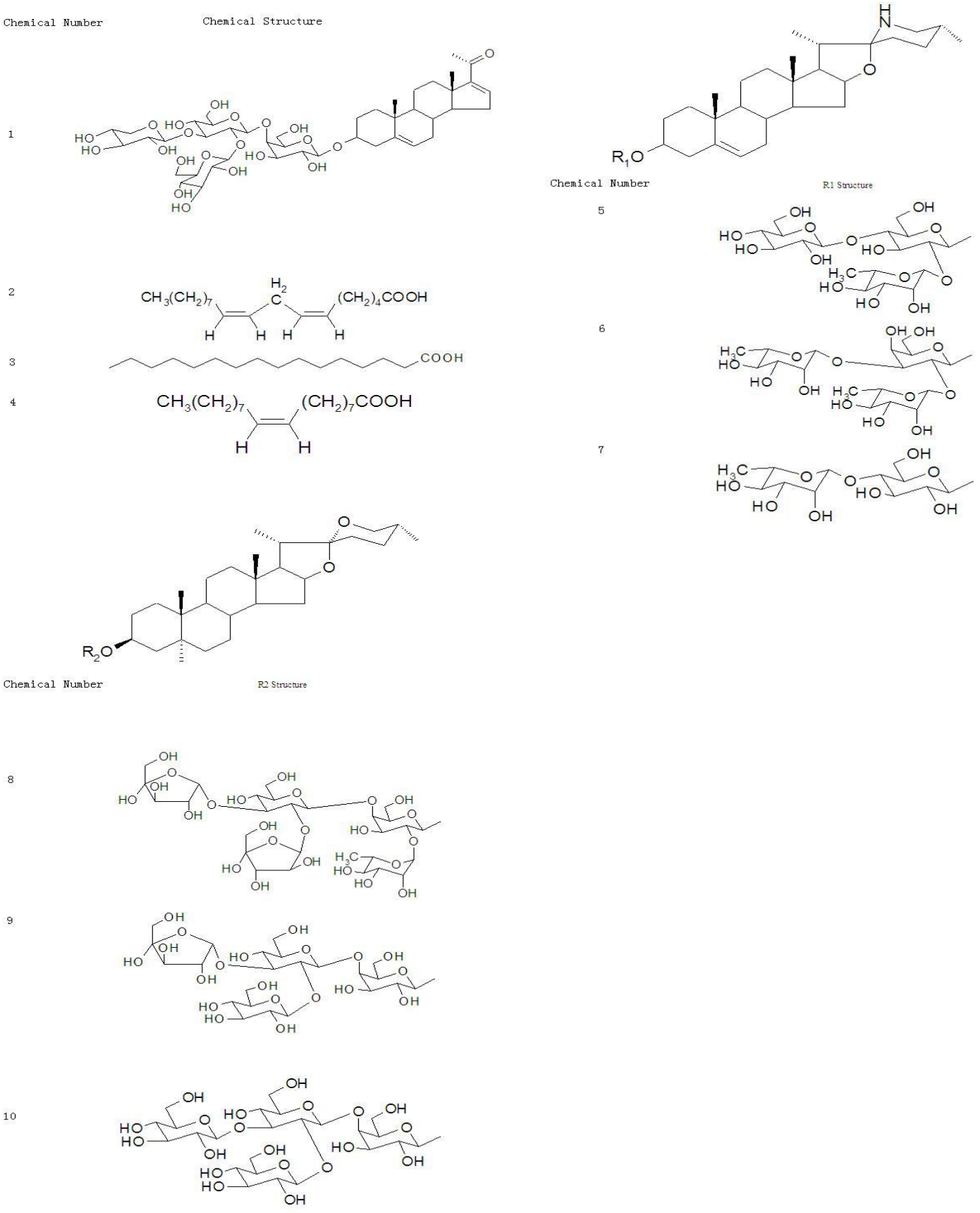

Figure 1. Chemical structures of analytes used in the study.

Table 1. Calibration curves for four saponins, three alkaloids and three fatty acids.

Compound[a]	Calibration curve [b]	r^2	Test range (μg)	LOD (μg)	LOQ (μg)
1	y = 0.6875x + 13.29	0.9982	1.500-12.00	0.18	0.54
2	y = 1.0307x+13.418	0.9998	14.13-70.65	0.28	0.94
3	y =1.2011x + 11.664	0.9983	6.48-29.16	0.21	0.69
4	y= 1.3826x + 5.1426	0.9991	6.28-25.12	0.19	0.71
5	y= 1.1621x + 7.587	0.9989	1.31-10.48	0.12	0.47
6	y= 1.0109x + 11.629	0.9995	1.80-15.00	0.21	0.74
7	y= 1.1173x + 8.176	0.9992	1.42-14.20	0.17	0.67
8	y= 0.5769x + 10.371	0.9987	1.07-8.56	0.20	0.69
9	y= 1.4294x + 11.722	0.9991	16.4-80.21	0.22	0.87
10	y = 0.7168x + 9.867	0.9981	1.11-8.88	0.19	0.67

[a] (1) 5α-pregn-16-en-3β-ol-20-one lycotetraoside, (2) linoleic acid, (3) palmitic acid, (4) oleic acid, (5) solasonine, (6) solamargine, (7) β_2-solamargine, (8) nigrumnin I, (9) degalactotigonin, (10) tigogenin 3-O-β-D-glucopyranosyl - (1→2)-O-[β-D- glucopyranosyl-(1→3)]-O-β-D- glucopyranosyl-(1→4)-O-β-D-galactopyranoside. [b] y = lgA; x = lg C (A:peak area; C: concentration,μg; lg: logarithm).

Table 2. Intra- and inter-day variations in the HPLC method for determination of ten analytes.

Compound	Intra-day precision		Inter-day precision	
	Content (mg g^{-1})	RSD (%)	Content (mgg^{-1})	RSD (%)
1	0.206 ± 0.004	1.78	0.211 ± 0.0045	2.12
2	1.142 ± 0.022	1.95	1.161 ± 0.022	1.87
3	0.451 ±0.009	2.10	0.457 ± 0.009	1.99
4	0.582 ± 0.011	1.89	0.578 ± 0.009	1.63
5	0.384 ± 0.010	2.58	0.401 ± 0.008	2.03
6	0.325 ± 0.002	2.11	0.337 ± 0.009	2.81
7	0.301 ± 0.009	2.97	0.297 ± 0.009	3.01
8	0.176 ± 0.005	3.01	0.178 ± 0.006	3.41
9	0.762 ± 0.014	1.81	0.748 ± 0.016	2.12
10	0.137 ± 0.004	3.01	0.141 ± 0.005	3.29

RSD (%) = (SD/mean) ×100%.

as measured by the peak area, varies exponentially depending on the concentration of analyte, which may be mathematically expressed as a logarithmic equation. The data showed that the logarithm of the peak area for each standard was linearly correlated to the logarithm of the injected concentration within a specific range. All calibrations curve significantly showed linear regression (r^2>0.9987) within test ranges; the LOD (S/N = 3) and the LOQ (S/N = 10) were more than 0.28 and 0.94 μg, respectively (Table 1). Validation studies also suggested that this method was repeatable. As given in Table 2, the overall intra- and inter-day variations were less than 4% for all ten analytes, respectively. As mentioned in Table 3, the developed analytical method had good accuracy with an overall recovery more than 95%. Therefore, the HPLC-ELSD method was precise, accurate and sensitive enough to evaluate major analytes in *S. nigrum* quantitatively.

Optimization of extraction conditions

Concerning the fact that *S. nigrum* contains a variety of compounds with relatively high polarity, ethanol was chosen as the preferred extraction solvent herein. Multiple extraction conditions were designed and employed, which included the following ethanol concentrations and extraction methods: ethanol 50, 60 or 70%, v/v and extraction by ultrasonication, reflux or soxhlet.

In this study, the herbs were extracted in an ultrasonic bath with 60% ethanol, which was more accurate than the other methods. Orthogonal experiments were also carried out, which involved three factors: (A) extraction time (10, 15 or 20 min), (B) ultrasonication times (1, 2 or 3 times) and (C) solvent volume (20, 16, or 12 mL) (Table 4). The orthogonal designs L9 (3^4) are presented in Table 5. Comparing the sum of all the characteristic peak

Table 3. Accuracy of the HPLC method for determination of ten analytes.

Compound	Recovery (%)	Mean (%)	RSD (%)
	95.16		
1	100.00	99.18	3.71
	102.39		
	98.24		
2	98.37	99.24	1.64
	101.12		
	98.50		
3	95.72	97.49	1.57
	98.24		
	99.05		
4	98.25	99.32	1.21
	100.66		
	98.23		
5	95.63	97.27	1.46
	97.93		
	101.11		
6	99.39	100.33	0.87
	100.48		
	95.03		
7	99.71	99.06	3.79
	102.46		
	95.28		
8	94.00	95.31	1.38
	96.64		
	104.56		
9	97.94	101.56	3.29
	102.13		
	95.12		
10	96.15	96.73	2.03
	98.92		

Recovery (%) = (Observed amount − original amount)/spiked amount×100%, RSD (%) = (SD/mean) ×100%.

Table 4. Factors and levels for the optimization of extraction conditions.

Factors	Levels		
	1	2	3
A: sonication time (min)	10	15	20
B: extraction times	1	2	3
C: solvent volume (mL)	20	16	12

numbers and areas in each chromatogram for the different factors, the overall extraction of the herb was quantified, and the recovery was used as a criterion to optimize conditions.

Optimal conditions for *S. nigrum* extraction were then selected and presented in Table 5.

Optimization of HPLC conditions

An optimized strategy for HPLC conditions was performed to develop a validated analytical method for *S. nigrum*. Various HPLC parameters, including mobile phase (acetonitrile-water, acetonitrile-triethylamine-water,

Table 5. The results and analysis of orthogonal design.

Run no	Factors			A_{12p}[a]
	A	B	C	
	Levels			
1	1	1	1	21772386
2	1	2	2	19755385
3	1	3	3	16353330
4	2	1	2	19742457
5	2	2	3	21904465
6	2	3	1	16789500
7	3	1	3	21784353
8	3	2	1	23364612
9	3	3	2	16347143
K1	19293700	16496658	20642166	
K2	19478807	21674821	18614995	
K3	20498703	21099732	20014049	
R	1205002	5178163	2027171	
SS	2.52648E+12	4.83324E+13	6.46131E+12	
Optimized scheme	A3	B2	C1	
Primary and secondary order	3	1	2	

[a] A_{12p} represents the area sum of 12 peaks. Ps. Factors and levels are as Table 4 described

Table 6. Mobile phases in optimization of HPLC conditions.

Systems		Gradients (%) in min
A	B	
Acetonitrile	Water	40% A in 90
Acetonitrile	Water	20 - 50% A in 60
Acetonitrile (0.3‰ triethylamine)	Water (0.3‰ triethylamine)	(1) 20 - 30%A in 10 (2) 30 - 36%A in 28 (3) 36 - 50%A in 52 (4) 50%A in 80
Acetonitrile (0.3‰ triethylamine)	Water : Methanol (8:2) (0.3‰ triethylamine)	(1) 10 - 15%A in 10

or acetonitrile-triethylamine-methanol-water) (Table 6), category of column (Phenomenex RP C_{18} column 250 × 4.6 mm I.D. 5 µm, Ultimate^{TM} C_{18} column 250 × 4.6 mm I.D. 5 µm, SHIM-PACK vp-ODS C_{18} column 250 × 4.6 mm I.D. 5 µm) and column temperature (30 or 40°C), were examined. The optimized HPLC conditions were obtained based on comprehensively comparing HPLC resolution, base line, elution time, peak area and number of characteristic peaks. The main parameters in the optimization of the ELSD response were the flow rate of nebulizer gas (pressure) and drift tube temperature (Cardenas et al., 1999). (When chromatographic conditions were fixed, these two parameters were evaluated by injecting the fatty acid linoleic acid and the saponin degalactotigonin, to optimize ELSD conditions at different detector temperatures ranging from 40 to 80°C and different pressures ranging from 2.0 to 3.5 bars, respectively). In this study, a drift tube temperature of 40°C and a gas pressure of 3.0 bars were performed. These optimized parameters resulted in complete solvent evaporation and negligible base line noise during the experiment.

Table 7. Contents of ten analytes in samples of *S. nigrum* (mg g^{-1}).

Sample habitat	1 (mg g^{-1})	2 (mgg^{-1})	3 (mgg^{-1})	4 (mgg^{-1})	5 (mgg^{-1})	6 (mgg^{-1})	7 (mgg^{-1})	8 (mg g^{-1})	9 (mgg^{-1})	10 (mgg^{-1})	Sum total (mgg^{-1})
Taian, Shandong	tr[a]	0.313	0.151	nd[b]	0.147	0.112	nd[b]	tr[a]	0.592	tr[a]	1.315
Changchun, Jilin	0.178	0.879	0.412	0.451	0.248	0.316	0.264	tr[a]	0.734	tr[a]	3.304
Shenyang, Liaoning	0.206	1.142	0.451	0.582	0.384	0.325	0.301	0.176	0.762	0.137	4.26
Shijiazhuang, Hebei	0.137	1.014	0.351	0.612	0.401	0.287	0.307	0.157	0.624	0.145	3.898
Siping, Jilin	0.124	1.781	0.677	0.715	0.141	0.164	nd[b]	0.243	0.571	tr[a]	4.292
Chifeng, Inner Mongolia	nd[b]	1.451	0.437	0.449	0.214	tr[a]	nd[b]	tr[a]	0.276	tr[a]	2.827
Pingdingshan, Henan	tr[a]	0.351	0.323	tr[a]	nd[b]	0.154	0.231	nd[b]	0.477	tr[a]	1.536
Yuncheng, Shanxi	tr[a]	0.313	0.151	nd[b]	0.147	0.112	nd[b]	tr[a]	0.592	tr[a]	1.315
Xian, Shanxi	tr[a]	0.455	0.321	nd[b]	0.332	0.316	nd[b]	tr[a]	0.734	tr[a]	2.158
Shunyi, Beijing	0.137	2.157	tr[a]	0.319	nd[b]	0.227	0.215	tr[a]	0.213	tr[a]	3.131

[a] Not detected. [b] Trace.

Quantitative determination of *S. nigrum*

The newly established HPLC-ELSD method was applied to analyze ten components of *S. nigrum* collected from different regions (nine provinces) in China. More than 3.0 mgg^{-1} (Table 7) of analytes were detected in the samples from the main producing areas such as Jilin, Liaoning, Hebei and Beijing. The total concentration of ten analytes ranged from 1.315 to 4.296 mgg^{-1}. Remarkable differences in the concentrations of the ten analytes were investigated (Table 7). Analytes 2 and 9 were found to be abundant in most samples. However, the amounts of these analytes varied greatly among the tested samples. Considering analytes content variable, we should adopt the method as earlier mentioned to control herb quality before the further pharmacodynamic experiment.

Conclusions

This is the first report on a validated analytical method for the qualification and quantification of analytes in *S. nigrum*.

This newly established HPLC-ELSD method was used to simultaneously quantify four saponins, three alkaloids and three fatty acids from this herb, including 5α-pregn-16-en-3β-ol-20-one lycotetraoside (a), linoleic acid (b), palmitic acid (c), oleic acid (d), solasonine (e), solamargine (f), β_2-solamargine (g), nigrumnin I (h), degalactotigonin (i), and tigogenin 3- O- β- D-glucopyranosyl- (1 →2)- O-[β- D- glucopyranosyl-(1→ 3)]- O- β- D- glucopyranosyl-(1→4)- O- β-D- galactopyranoside (j). This new analytical method was proving to be precise, accurate and sensitive. Considering the analytes activity, content, and chromatographic results, we could draw a conclusion that the four saponins, three alkaloids and three fatty acids could represent most of the constituents in the samples, and be used as a biochemical marker to assess the quality of *S. nigrum*. The method could also be an analytical tool for the rapid determination of *S. nigrum*. Ten batches of herb were analyzed in our study. Experimental data on the ten herb samples also suggested that the quality of *S. nigrum* was correlated from the habitat.

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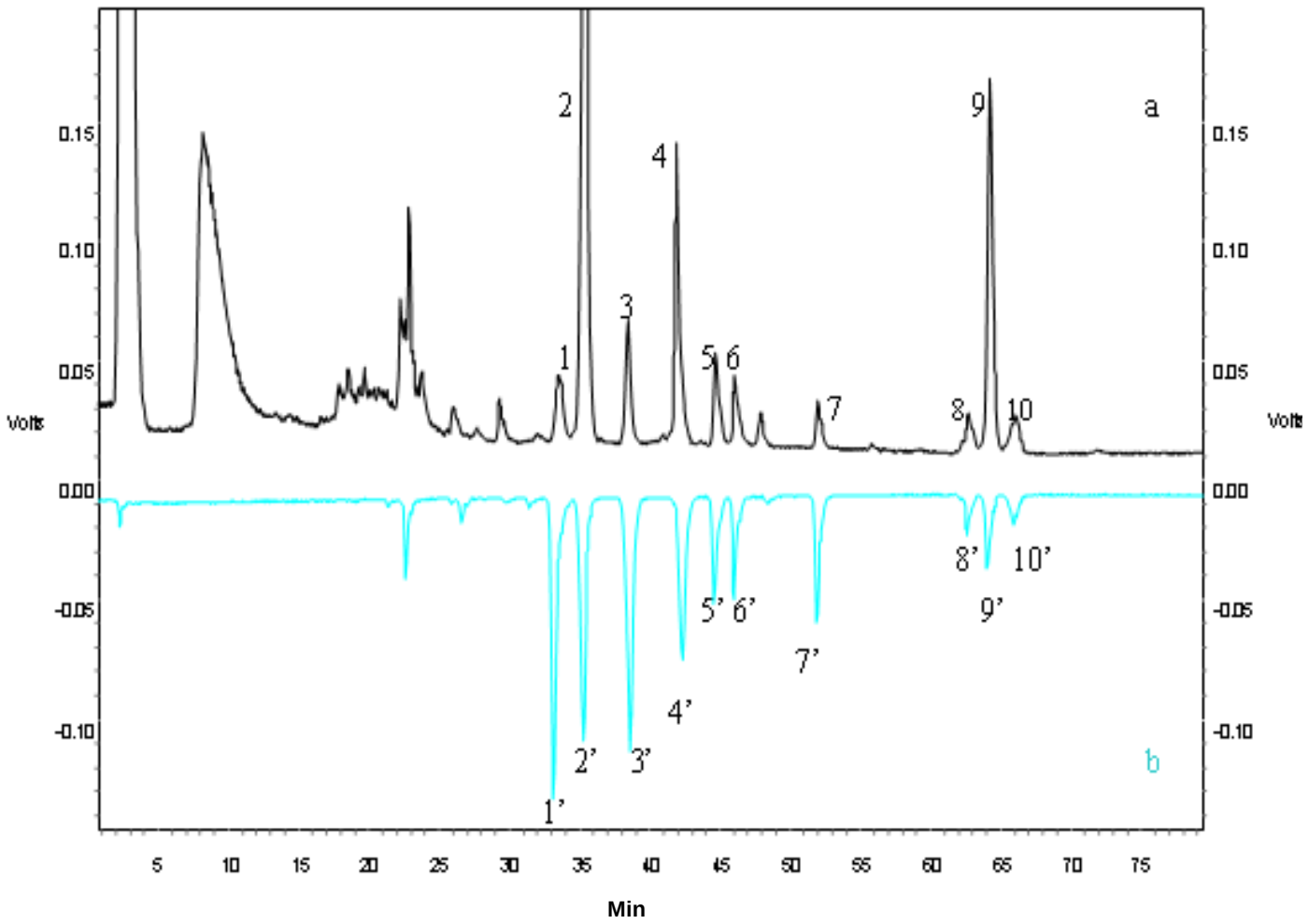

Figure 2. HPLC-ELSD chromatograms Comparison between representative herb and mixed standards (a) representative herb chromatogram; (b) mixed standards chromatogram. 1':5α-pregn-16-en-3β-ol-20-one lycotetraoside,mg/ml; 2': linoleic acid, mg/ml; 3': palmitic acid, mg/ml; 4': oleic acid, mg/ml; 5': solasonine, mg/ml; 6': solamargine, mg/ml; 7': β2-solamargine, mg/ml; 8': nigrumnin I, mg/ml; 9': degalactotigonin, mg/ml; 10': tigogenin3-O-β-D- glucopyranosyl-(1→2)-O-[β-D-glucopyranosyl-(1→3)] -O-β-D-glucopyranosyl-(1→4)- O-β- D- galactopyranoside.

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