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Rapid Method for Simultaneous Determination of 20 Components in *Isodon nervosa* by High-performance Liquid Chromatography–Electrospray Ionisation Tandem Mass Spectrometry

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ABSTRACT:
Introduction – *Isodon nervosa* is a commonly used traditional Chinese medicine including diterpenoids, phenolic acids, triterpenoids and volatile oil. Qualitative and quantitative analysis of multi-components is important for its quality control.
Objective – To establish a liquid chromatography–electrospray ionisation–mass spectrometry method for simultaneous analysis of 20 bioactive constituents of *Isodon nervosa* in different places of China and different parts of this herb.
Methodology – The optimal chromatographic conditions were achieved on a C_{18} column (250 × 4.6 mm, 5 μm) with with linear gradient elution with 0.1% aqueous formic acid : methanol containing 0.1% formic acid at a flow-rate of 0.7 mL/min in 15 min. The identification and quantification of those analytes were achieved on a hybrid quadrupole linear ion trap mass spectrometer. Multiple-reaction monitoring scanning was employed for quantification with switching electrospray ion source polarity between positive and negative modes in a single run. Full validation of the method was carried out (linearity, precision, accuracy, limit of detection and limit of quantification).
Results – The results indicated that the method was simple, rapid, specific and reliable. The proposed method was successfully applied for the qualitative and quantitative analysis of 20 chemical compositions in *Isodon nervosa* samples.
Conclusion – Twenty chemical compositions in 21 batches of wild and cultivated *Isodon nervosa* samples from different sources had great variation in the contents.

Keywords: *Isodon nervosa*; HPLC-ESI-MS; qualitative; quantification; MRM

Introduction

Isodon nervosa (Hemsl.) C. Y. Wu et H. W. Li (Labiatae) is a perennial herb native to most parts of China. *Isodon nervosa* (formally named *Rabdosia nervosa*) is the dried entire plant of plant *R. nervosa* (Hemsl) Hara (Sun *et al.*, 2001). It is a well-known traditional Chinese medicine (TCM) called 'Da Ye She Zong Guan' in Chinese. The herb has long been used as a folk remedy for acute infectious hepatitis, snakebite, chuang du, eczema, skin itching amongst others (Song *et al.*, 2001).

Phytochemical studies on *Isodon nervosa* revealed that it contains diterpenoids, phenolic acids, triterpenoids, volatile oil, etc. (Song *et al.*, 2001). Among these, modern pharmacological and clinical studies have also shown that the first two types of compounds possess anti-tumour, anti-microbial, anti-inflammatory, antimutagenic and anti-oxidation effects (Qu *et al.*, 2002; Xu *et al.*, 2009). In recent years, more and more diterpenoids have been isolated from *Isodon nervosa*, which have a variety of bioactivities (Wang *et al.*, 1994; Gao *et al.*, 1994a, b, 1996, 1999, 2000).

Quantification of those compounds in *Isodon nervosa* would be of great importance for the quality evaluation of the herb. Because of different cultivation areas and climatic conditions, its chemical constituents may vary significantly (Sun *et al.*, 2001). Simple quantitative analysis of one or two active components in the herb could not represent its integral quality. Consequently, simultaneous quantitative analysis of active components is the most direct and important method for quality control of TCM.

Until now, studies on quantitative determination of chemical constituents in *Isodon nervosa* have been very few, for example, only focusing on reverse-phase high-performance liquid chromatography coupled with ultraviolet detection (HPLC-UV) (Cui *et al.*, 2006; Gao *et al.*, 2008) and GC-MS(Gao *et al.*, 2001). The constituents in *Isodon nervosa* are complex, some of them show

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low contente and most of diterpenoids have weak or no UV absorption (Sun *et al.*, 2001). It is particularly difficult to simultaneously determine much more active constituents using conventional means for quality control of TCMs, such as HPLC–UV. MS is a sensitive and selective detection method that allows detection of trace amounts of constituents. In addition, all the peaks of target compounds can be identified by comparison of retention times and parent and product ions with standards. Hence, HPLC coupled with mass spectrometry (HPLC-MS) is a powerful approach to solving the problems mentioned above, as well as having the advantages of saving time and solvent. Therefore it is very suitable for the analysis of TCM and Chinese herbal prescriptions, in particular for low abundance compounds in complex compounds which are difficult to obtain by conventional isolation means.

In present study, we first developed and validated a simple and accurate HPLC-ESI-MS method for simultaneous determination of 20 major components in *Isodon nervosa*, including 16 diterpenoids (effusanin A, enmein, lasiodonin, oridonin, epinodosinol, nervosanin B, isodonoiol, sodoponin, rabdosinate, epinodosin, nodosin, ponicidin, rabdoternin A, enmenol, hebeirubesensin K and lasiokaurin) and four phenolic acids (protocatechuic aldehyde, salicylic acid, caffeic acid and rosmarinic acid). During method development, multiple-reaction monitoring (MRM) was employed and an electrospray ionisation source was operated in positive and negative mode at the same time. Furthermore, an information-dependent acquisition (IDA) method was employed to trigger product ion scans above the MRM signal threshold so that the 20 major compounds could be identified through enhanced product ion (EPI) scans. In addition, 21 batches of *Isodon nervosa* from different sources were compared using the developed method. To the best of our knowledge, the new method presented here provides the best sensitivity and specificity for determination of *Isodon nervosa* so far.

Experimental

Chemicals, reagents and materials

Effusanin A, enmein, lasiodonin, oridonin, epinodosinol, nervosanin B, isodonoiol, sodoponin, rabdosinate, epinodosin, nodosin, ponicidin, rabdoternin A, enmenol, hebeirubesensin K and lasiokaurin isolated from different *Isodon* plants and identified based on IR, UV, NMR spectroscopy analyses in comparison with literature data (Zhang *et al.*, 2003, 2009; Yan *et al.*, 2007, 2008; Chen *et al.*, 2008) were generously provided by Professor Jixia Zhang, Henan Xinxiang Medical University. The purities of the above ingredients were more than 98% according to HPLC analysis. Protocatechuic aldehyde, salicylic acid, caffeic acid and rosmarinic acid were purchased from the China Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Their structures are shown in Fig. 1.

HPLC-grade methanol (Fisher, USA) was used for HPLC analysis. Redistilled water was prepared in our own laboratory by Heal Force-PWVF Reagent Water System (Shanghai CanRex Analyses Instrument Corporation Limited, China). Analytical-grade methanol (Tianjin Chemical Corporation, China) was used for sample preparation. Formic acid was HPLC grade purchased from Diamond Technology Incorporation (Dikma Technology Corporation, USA).

Twenty-one batches of the raw material samples of *Isodon nervosa* were collected from different provinces in China (Table 1). All the voucher specimens identified by Professor Zengke Kong were deposited in the herbarium of School of Pharmacy, Hebei Medical University.

Apparatus and conditions of measurement

Determination was performed using a 3200 QTRAP™ system from Applied Biosystems/MDS Sciex (Applied Biosystems, Foster City, CA, USA), a hybrid triple quadrupole linear ion trap mass spectrometer equipped with Turbo V sources and Turbolonspray interface. The instrument was operated using electrospray ionisation source in positive and negative mode simultaneously. The ion spray voltage was set to 5500 and −4500 kV, respectively. The turbo spray temperature was maintained at 600°C. Nebuliser gas (gas 1) and heater gas (gas 2) were set at 40 and 50 arbitrary units, respectively. The curtain gas was kept at 25 arbitrary units

Table 1. The list of *Isodon nervosa* samples

Sample no.	Code	Type and collected time	Collection region
1	Henan-1	Wild, October 2008	Henan-dabieshan
2	Henan-2	Wild, October 2008	Henan-dabieshan
3	Henan-3	Wild, October 2008	Henan-laojunshan
4	Henan-4	Wild, October 2008	Henan-laojunshan
5	Henan-5	Wild, October 2008	Henan-laojunshan
6	Henan-6	Cultivated, October 2008	Henan-jiyuan
7	Henan-7	Cultivated, October 2008	Henan-jiyuan
8	Henan-8	Cultivated, October 2008	Henan-jiyuan
9	Hebei-1	Wild—leaf, October 2008	Hebei-shexian
10	Hebei-2	Wild—stem, October 2008	Hebei-shexian
11	Hebei-3	Wild, October 2008	Hebei-shexian
12	Hebei-4	Cultivated, October 2008	Hebei-anguo
13	Hebei-5	Cultivated, October 2008	Hebei-anguo
14	Hebei-6	Cultivated, October 2008	Hebei-anguo
15	Jiangxi-1	Cultivated, October 2008	Jiangxi-yifeng
16	Jiangxi-2	Cultivated, October 2008	Jiangxi-yifeng
17	Jiangxi-3	Cultivated, October 2008	Jiangxi-yifeng
18	Anhui-1	Cultivated, October 2008	Anhui-bozhou
19	Anhui-2	Cultivated, October 2008	Anhui-bozhou
20	Hubei-1	Cultivated, October 2008	Hubei-jingzhou
21	Hubei-1	Cultivated, October 2008	Hubei-jingzhou

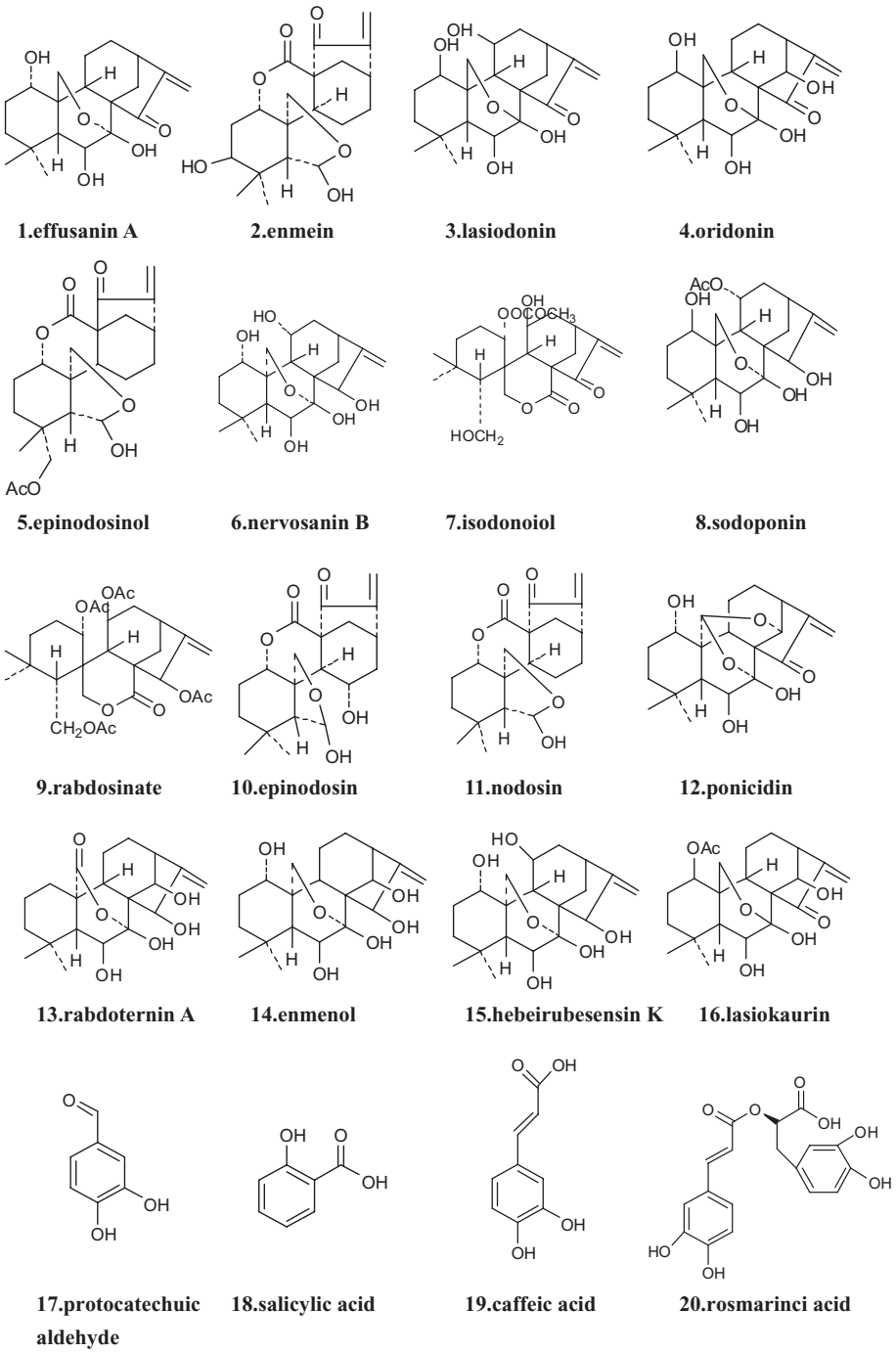

Figure 1. Chemical structures of the 20 compounds in *Isodon nervosa*.

and the interface heater was on. Entrance potential (EP) was set at 10/−10 V and collision cell exit potential (CXP) was set at 5/−5 V. Nitrogen was used in all cases. Multiple-reaction monitoring mode was employed for quantification. The precursor-to-product ion pair, declustering potential (DP) and collision energy (CE) for each analyte are described in Table 2. The dwell time of each ion pair was 40 ms. In IDA criteria, the former target ions were excluded for 15 s and the three most intense fragment ions of each analyte were selected to perform the product ion scan. All instrumentations were controlled and synchronised by Analyst software (versions 1.4.2) from Applied Biosystems/MDS Sciex.

Quantitative analysis was performed using HPLC system Agilent 1200 (Agilent Technologies, Palo Alto, CA, USA), equipped with a quaternary solvent delivery system, an auto-sampler and a column compartment. The chromatographic separation was performed on a Diamonsil C_{18} column (250 × 4.6 mm, 5 μm), and the column temperature was set at 25°C. A linear gradient elution of eluents A (methanol containing 0.1% formic acid) and B (0.1%, v/v aqueous formic acid) was used for the separation. The elution programmer was optimised and conducted as follows: a linear gradient of 55–80% A during 0.0–5.0 min; a linear gradient of 80–95% A during 5.0–10.0 min; holding this mobile phase ratio for 5 min;

Table 2. The retention time, MS/MS fragment ions, declustering potential (DP) and collision energy (CE) of the 20 active compounds in *Isodon nervosa*

Compound no.	Compounds	MW	Retention times (min)	MS^1 (*m/z*)	MS^2 (*m/z*)	DP (V)	CE (eV)
1	Effusanin A	348	10.38	349.3[a]	331.3[a]	36	16
2	Enmein	362	5.96	363.3[a]	327.3	42	21
					281.2[a]		
3	Lasiodonin	364	8.75	365.3[a]	347.3[a]	17	17
4	Oridonin	364	9.47	365.3[a]	347.3[a]	17	17
5	Epinodosinol	364	8.19	382.3[a]	347.3[a]	9	16
					329.2		
6	Nervosanin B	366	7.82	384.4[a]	349.3	10	19
					331.3[a]		
					283.4		
7	Isodonoiol	406	8.77	407.4[a]	389.3[a]	53	19
					331.4		
8	Sodoponin	408	8.88	426.3[a]	331.2[a]	15	20
9	Rabdosinate	534	10.78	535.4[a]	373.4	38	28
					295.3[a]		
10	Epinodosin	362	7.15	361.2[a]	331.3	–42	–26
					287.1[a]		
11	Nodosin	362	8.43	361.1[a]	257.1[a]	–50	–31
12	Ponicidin	362	10.13	361.2[a]	299.1[a]	–44	–21
13	Rabdoternin A	364	11.62	363.2[a]	327.1	–52	–32
					283.1[a]		
					255.1		
14	Enmenol	366	7.82	365.2[a]	347.2[a]	–45	–25
					299.1		
					267.2		
15	Hebeirubesensin K	366	7.77	365.2[a]	317.2[a]	–53	–26
16	Lasiokaurin	406	10.96	405.2[a]	387.3	–74	–65
					327.0		
					58.9[a]		
17	Protocatechuic aldehyde	138	5.42	136.9[a]	107.9[a]	–53	–31
18	Salicylic acid	138	11.06	136.9[a]	92.9[a]	–26	22
19	Caffeic acid	180	5.68	179.0[a]	135.0[a]	–30	–25
20	Rosmarinic acid	360	7.22	359.0[a]	160.9[a]	–29	–23

[a] Monitored MRM transitions.

a linear gradient of 95–55% A during 15.0–15.1 min. This was followed by 8 min equilibration period prior to the injection of each sample. The solvent flow rate was kept at 0.7 mL/min.

Preparation of solutions

Standard solutions. The appropriate amounts of standards were accurately weighed and dissolved in methanol to make 20 kinds of stock solutions. All solutions were stored in a refrigerator at 4°C for analysis.

Sample preparation. The dry plant samples were ground to a fine powder using a pulveriser, and 1.0 g of powder was placed in a 50 mL capped conical flask. All crude was added and extracted with 25.0 mL of methanol in an ultrasonic ice-water bath for 1 h. The extracted solution was adjusted to the original weight by adding methanol, and then the aliquot of the supernatant was filtered through a 0.45 μm microporous membrane before HPLC injection of 10 μL.

Peak identification

Exact identification of each analyte is a key to successful quantification. In structural identification experiment, the IDA method was employed to trigger the EPI scans by analysing MRM signals. All the peaks of target compounds in *Isodon nervosa* samples solution were unambiguously identified by comparison of retention time, parent and product ions with standards in MRM-IDA-EPI spectra. The retention times and characteristic MS/MS fragment ion data of each constituent are listed in Table 2.

Validation procedure

Linearity, limit of detection and limit of quantification. The method was calibrated using external standardisation based on seven point calibration curves. The peak area values were the average values of three replicate injections. All calibration curves were constructed from peak areas of the reference compounds vs. their concentrations and all the analytes showed satisfactory linearity within their test ranges. Limit of detection (LOD) and limit of quantification (LOQ) were determined by

Table 3. Linear regression data, LOD and LOQ of the 20 active components in *Isodon nervosa*

Analytes	Linear regression data		Linear range (μg/mL)	LOD (ng)	LOQ (ng)
	Regression equation	r^2			
Effusanin A	$y = 3.35e^3x - 4.89$	0.9996	0.0137–0.412	0.0501	0.112
Enmein	$y = 5.84e^4x + 2.82e^3$	0.9923	0.117–3.520	0.0321	0.109
Lasiodonin	$y = 4.47e^3x - 1.34e^3$	0.9936	0.346–10.401	0.295	0.614
Oridonin	$y = 4.75e^3x - 534.58$	0.9997	0.968–29.125	0.123	0.335
Epinodosinol	$y = 4.25e^4x - 22.10$	0.9987	2.168–65.200	0.0159	0.0312
Nervosanin B	$y = 4.07e^4x - 4.26e^3$	0.9981	0.0104–0.312	0.0341	0.0714
Isodonoiol	$y = 1.31e^5x + 4.89e^3$	0.9977	0.0417–1.250	0.0156	0.0337
Sodoponin	$y = 2.14e^3x + 26.5$	0.9992	0.0607–1.820	0.056	0.207
Rabdosinate	$y = 5.81e^3x + 199.3$	0.9990	0.0519–1.560	0.0261	0.0672
Epinodosin	$Y = 1.96e^5x - 5.42e^4$	0.9934	0.782–23.512	0.564	0.181
Nodosin	$y = 2.85e^5x + 273.33$	0.9947	0.00417–0.125	0.0167	0.0433
Ponicidin	$Y = 5.12e^3x + 384.55$	0.9979	0.710–21.300	0.0342	0.0744
Rabdoternin A	$y = 1.02e^6x + 1.38e^3$	0.9970	0.0337–1.012	0.0181	0.0302
Enmenol	$y = 2.79e^5x + 666.67$	0.9964	0.0271–0.812	0.0121	0.0420
Hebeirubesensin K	$y = 30.732x + 5.5417$	0.9973	0.135–4.056	0.320	0.588
Lasiokaurin	$y = 2.19e^4x - 1354.2$	0.9992	0.0743–2.230	0.0142	0.0587
Protocatechuic aldehyde	$Y = 9.22e^5x + 1.33e^4$	0.9959	0.0407–1.220	0.0129	0.0312
Salicylic acid	$Y = 3.02e^6x + 8.99e^3$	0.9985	0.0340–1.020	0.0371	0.0715
Caffeic acid	$y = 3.68e^5x + 3992.5$	0.9989	0.0586–1.761	0.0135	0.0337
Rosmarinic acid	$Y = 6.71e^4x - 1.82\ e^3$	0.9979	2.078–62.500	0.142	0.427

In the regression equation $y = ax + b$, x refers to the sample injection amount, y the peak area, and r^2 is the correlation coefficient of the equation. LOD, limit of detection; LOQ, limit of quantification.

measuring the signal-to-noise ratio (S/N ratio) for each compound. Typically, the LOD and LOQ were determined by serial dilution of standard solution to the S/N ratio 3 for LOD and 10 for LOQ. The results are given in Table 3 for each compound.

Precision. The instrument precision was evaluated by analysing the mixture standard solution in six replicate injections under the optimal conditions. Intra- and inter-day variability were utilised to determine the precision of the method. The intra-day precision was performed six replications prepared from the *Isodon nervosa* sample (Henan-1) within one day, while the inter-day precision was performed over three consecutive days. The quantitations of 20 investigated ingredients were determined from corresponding calibration curve. The relative standard deviation (RSD) was taken as a measure of precision (Table 4).

Accuracy and stability. To further evaluate the accuracy of the method, a recovery test was carried out by spiking three concentration levels (high, middle and low) of the mixture standard solution to known amounts of *Isodon nervosa* samples (Henan-1). Triplicate samples at each level were extracted and analysed with the method described above. The recovery was calculated with the value of detected vs. added amounts. The recovery was calculated with the value of detected vs. added amounts. The concentration levels and the detailed results are summarised in Table 5. In order to investigate the stability of the samples, the same *Isodon nervosa* sample solution was stored at 4°C and analysed every 12 h over 2 days.

Quantification of 20 constituents in *Isodon nervosa*

The optimised LC-ESI-MS method was used to evaluate the quality of 21 *Isodon nervosa* samples and prepared as described above. An aliquot (10 μL) of each filtrate was injected directly to HPLC system. The content of each compound analysed was calculated from the corresponding calibration curve.

Results and Discussion

Optimisation of extraction method

Four related extraction conditions were designed and evaluated, which involved the following factors and corresponding levels: extraction method (ultrasonication, reflux), methanol concentration (50, 70 and 100%, v/v), solvent volume (10, 25 and 30 mL) and extraction time (30 min, 1 and 2 h). By comparing the sum numbers and areas of characteristic peaks in each chromatogram of different factors, the optimal condition for extraction of *Isodon nervosa* was selected as 1.0 g powder of each dried sample was extracted with 25.0 mL of 100% methanol in ultrasonic ice-water bath for 1 h.

Chromatographic conditions optimisation

Selection of LC columns. Four LC columns with different types, lengths and particle sizes, i.e. (1) Diamonsil C_{18} column (250 × 4.6 mm, 5 μm), (2) Thermo C_{18} column (250 × 4.6 mm, 5 μm), (3) Agilent Zorbax SB-C_{18} (250 × 4.6 mm, 5 μm) and (4) Sepax GP-C_{18} PN (150 × 4.6 mm, 5 μm) were tested for the peak shape, signal intensity and separation efficiency of 20 constituents. The sample solution was used for this optimisation test. Lasiodonin and oridonin had the same parent and daughter ions. Figure 2 shows the extract ions chromatograms (XIC) of MRM chromatograms of lasiodonin and oridonin, which were obtained by performing the same sample solution with different columns. The column efficiency of Diamonsil C_{18} columns (250 × 4.6 mm, 5 μm) were

Table 4. Precision of the 20 ingredients for quantitative analysis

Compounds	Instrument precision ($n = 6$)		Intra-day precision ($n = 6$)		Inter-day precision ($n = 3$)	
	Mean ± SD (μg/mL)	RSD[a] (%)	Mean ± SD (μg/g)	RSD (%)	Mean ± SD (μg/g)	RSD (%)
1	2.862 ± 0.951	0.88	2.156 ± 0.862	0.49	2.231 ± 0.619	0.65
2	59.432 ± 5.360	0.69	63.862 ± 3.261	0.46	64.623 ± 4.524	0.42
3	101.621 ± 8.021	1.03	110.234 ± 6.123	0.48	109.811 ± 7.226	0.53
4	432.312 ± 6.311	0.54	453.628 ± 8.625	1.11	452.897 ± 10.251	2.05
5	1563.363 ± 16.231	0.50	1532.163 ± 18.312	0.68	1538.148 ± 20.151	0.85
6	1.125 ± 0.123	0.82	1.056 ± 0.213	1.26	1.078 ± 0.221	1.27
7	2.316 ± 0.612	0.89	2.204 ± 0.842	1.83	2.215 ± 0.615	2.29
8	3.561 ± 0.925	1.25	3.241 ± 0.651	0.37	3.311 ± 0.712	1.21
9	26.215 ± 2.154	0.48	25.143 ± 4.158	1.29	24.958 ± 2.631	1.55
10	531.216 ± 8.471	0.57	525.624 ± 10.302	1.07	525.451 ± 12.462	1.14
11	1.416 ± 0.203	0.62	1.311 ± 0.854	0.70	1.256 ± 0.894	1.16
12	34.311 ± 2.551	0.48	35.315 ± 2.814	0.73	34.687 ± 1.852	0.75
13	1.106 ± 0.461	1.26	1.315 ± 0.526	0.87	1.318 ± 0.864	1.06
14	1.115 ± 0.554	0.83	1.028 ± 0.551	0.26	1.045 ± 0.423	0.87
15	91.112 ± 3.594	0.61	90.514 ± 4.345	0.67	90.564 ± 3.285	0.60
16	31.815 ± 3.260	1.16	32.816 ± 2.645	0.76	32.315 ± 6.031	2.75
17	25.216 ± 2.716	0.67	24.657 ± 3.452	0.72	24.067 ± 1.314	1.44
18	4.916 ± 0.951	0.68	4.530 ± 0.723	0.80	4.516 ± 0.782	0.71
19	4.567 ± 0.551	0.53	4.336 ± 0.953	1.51	4.326 ± 0.916	1.96
20	136.154 ± 6.312	0.57	135.311 ± 10.312	1.07	134.263 ± 8.125	0.96

[a] RSD (%) = (SD/mean) × 100.

obviously better than Thermo C_{18} column (250 × 4.6 mm, 5 μm) and Agilent Zorbax SB-C_{18} (250 × 4.6 mm, 5 μm). In general, the separation efficiency of the C_{18} column (250 × 4.6 mm, 5 μm) was better than the C_{18} column (150 × 4.6 mm, 5 μm), so the longer column was fit for the separation of complex chemical system. Therefore, the Diamonsil C_{18} column was found to be more suitable and gave good peak separation and sharp peaks.

Selection of mobile phase and buffers. With the view of achieving higher peak responses and shorter analysis time of target compounds in chromatograms, the effect of different mobile phase compositions on chromatographic separation was compared and it was found that there was no obvious distinction between methanol–water and acetonitrile–water. Considering the high toxicity and price of acetonitrile, methanol–water was chosen. Several mobile phase additives such as ammonium acetate (0.2, 1 and 2 mmol/L), formic acid (0.05, 0.1 and 0.2%) and acetic acid (0.05, 0.1 and 0.2%) were used to achieve the high sensitivity. It was also found that an acidic eluent [A (methanol containing 0.1% formic acid) and B (0.1%, v/v aqueous formic acid)] was beneficial for enhancing the ionisation of compounds detected in positive electrospray interface mode and could guarantee sharp peak shape and reproducible retention time for phenolic acids. Although ionisation of compounds detected in negative electrospray ionisation mode was suppressed owing to the presence of formic acid in mobile phase, their quantification was not influenced, which could be proved by good sensitivity and the accuracy of analysis. Because of the long retention time of some of the late-eluting peaks in isocratic runs, gradient elution was employed in HPLC analysis. Satisfactory separation was achieved in 15.0 min by gradient elution using the HPLC conditions as described earlier.

Optimisation of MS parameters

The optimisation of the mass spectrometry conditions was achieved by direct infusion of each analyte separately at a flow rate of 10 μL/min. Full-scan and collision-activated dissociation (CAD) tests were operated to set up an appropriate MRM method. The electrospray interface was used and good sensitivity and fragmentation were obtained. It was also found that all the analytes could be ionised under positive and negative electrospray ionisation conditions. An atmospheric pressure ionisation interface was tested, but no obvious improvement was observed. In this study, the mass spectral conditions were optimised in both positive- and negative-ion modes. According to sensitivity and reproducibility of dominated ions in full-scan mass spectra, positive mode was finally selected for the detection of compounds **1–9**, and negative mode for compounds **10–19**. In the full-scan mass spectra, the 16 diterpenoids exhibited their quasi-molecular ions and fragment ions, and most of them were in good agreement with the literature (Han *et al.*, 2005; Zhou *et al.*, 2008, 2009). At the same time, the protonated molecular ions $[M + H]^+$, $[M + NH_4]^+$ and deprotonated molecular ions $[M - H]^-$ were considered stable and higher abundance, thus $[M + H]^+$, $[M + NH_4]^+$ and $[M - H]^-$ were chosen as only the precursor ions for MS/MS fragmentation analysis of compounds **1**, **2**, **3**, **4**, **7**, **9**, compounds **5**, **6**, **8**, **9** and compounds **10–20**, respectively. DP is one of the most important mass spectrometer parameters impacting ion response. Therefore DP was optimised in order to obtain the maximum sensitivity. In MS/MS analysis, only the precursor ion was isolated and then dissociated into product ions. Several fragment ions of the analytes were observed in the product ion spectra and the predominant fragment ions were chosen in MRM acquisition for quantification. The most suitable collision energy was also determined by observing the maximum

Table 5. Recoveries of the 20 constituents in *Isodon nervosa* sample ($n = 3$)

Compounds	Amount added (μg)	Amount found (μg)[a]	Recovery (%)[b]	RSD (%)[c]
1	0.414	0.409 ± 0.00820	98.8	2.29
	1.658	1.652 ± 0.0331	99.6	3.15
	3.315	3.311 ± 0.0665	99.9	2.13
2	0.289	0.285 ± 0.00495	98.6	2.19
	1.154	1.152 ± 0.0230	99.8	1.49
	2.308	2.299 ± 0.0451	99.6	1.22
3	0.851	0.849 ± 0.0162	99.8	1.93
	3.405	3.398 ± 0.0675	99.8	1.93
	6.810	6.782 ± 0.0673	99.6	2.23
4	2.782	2.768 ± 0.0543	99.5	3.03
	11.128	10.894 ± 0.205	97.9	2.59
	22.256	21.651 ± 0.431	97.3	2.52
5	6.520	6.491 ± 0.115	99.6	1.84
	26.080	26.012 ± 0.514	99.7	1.92
	52.160	52.110 ± 1.06	99.9	1.29
6	0.0310	0.0298 ± 0.000569	96.1	1.64
	0.124	0.119 ± 0.00231	96.0	2.29
	0.248	0.244 ± 0.00481	98.4	1.45
7	0.127	0.125 ± 0.00152	98.4	1.45
	0.506	0.498 ± 0.00991	98.4	2.54
	1.012	1.018 ± 0.0205	100.6	1.44
8	0.189	0.185 ± 0.00371	97.9	2.63
	0.756	0.752 ± 0.0151	99.5	3.28
	1.512	1.416 ± 0.0263	93.7	2.56
9	0.152	0.142 ± 0.00281	93.4	2.04
	0.608	0.558 ± 0.0121	91.8	1.63
	1.215	1.211 ± 0.0338	99.7	2.30
10	2.330	2.259 ± 0.0457	97.0	1.62
	9.325	9.311 ± 0.192	99.8	2.65
	18.650	18.564 ± 0.376	99.5	2.16
11	0.0128	0.0124 ± 0.000235	96.9	1.39
	0.0510	0.0505 ± 0.00102	99.0	1.93
	0.102	0.096 ± 0.00197	94.1	2.55
12	2.006	1.998 ± 0.0395	99.6	2.30
	8.023	8.015 ± 0.163	99.9	1.54
	16.045	16.041 ± 0.321	100.0	2.33
13	0.101	0.0990 ± 0.00194	98.0	1.64
	0.406	0.398 ± 0.00793	98.0	1.92
	0.811	0.792 ± 0.0162	97.7	2.17
14	0.0814	0.0808 ± 0.00151	99.3	2.77
	0.326	0.315 ± 0.00521	96.6	1.66
	0.651	0.646 ± 0.0131	99.2	2.19
15	0.408	0.398 ± 0.00768	97.5	2.23
	1.632	1.626 ± 0.0353	99.6	1.82
	3.264	3.261 ± 0.0612	99.9	2.01
16	0.223	0.221 ± 0.00442	99.1	2.51
	0.891	0.885 ± 0.0175	99.3	1.82
	1.782	1.780 ± 0.0361	99.9	2.04
17	0.120	0.121 ± 0.00242	100.8	1.96
	0.481	0.479 ± 0.00956	99.6	1.92
	0.962	0.961 ± 0.0196	99.9	2.21
18	0.101	0.0998 ± 0.00197	98.8	2.21
	0.406	0.368 ± 0.00721	90.6	1.53
	0.811	0.795 ± 0.0152	98.0	1.73
19	0.176	0.172 ± 0.00344	97.7	2.06
	0.706	0.698 ± 0.0134	98.9	2.35
	1.411	1.405 ± 0.0282	99.6	1.31
20	6.264	6.259 ± 0.125	99.9	1.68
	25.055	25.031 ± 0.510	99.9	3.03
	50.110	50.064 ± 1.021	99.9	2.08

[a] Calculated by subtracting the total amount after spiking from the amount in the *Isodon nervosa* sample before spiking. Data are means ± standard deviations.
[b] Recovery (%) = amount found/amount added × 100.
[c] RSD (%) = (SD/mean) × 100.

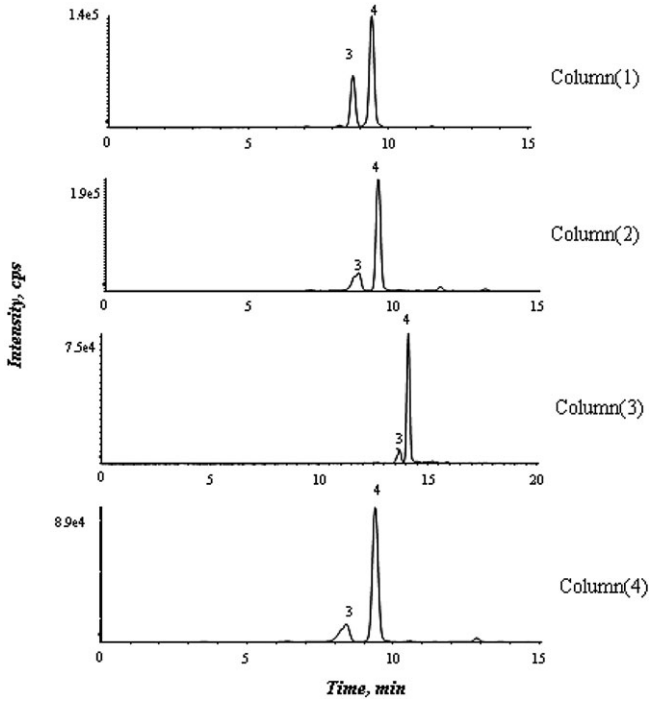

Figure 2. The extracted ion chromatograms of multiple-reaction monitoring chromatograms of lasiodonin and oridonin obtained by performing the same sample solution with different columns: (1) Diamonsil C_{18} column (250 × 4.6 mm, 5 μm); (2) Thermo C_{18} column (250 × 4.6 mm, 5 μm); (3) Agilent Zorbax SB-C_{18} (250 × 4.6 mm, 5 μm); (4) Sepax GP-C_{18} PN (150 × 4.6 mm, 5 μm).

response for the MS/MS monitoring fragment ion. Typical XIC of MRM chromatograms of standards and sample (Henan-1) are shown in Fig. 3.

Method validation

All the marker substances showed good linearity ($r^2 > 0.9923$) in the test ranges, with the LODs and LOQs for the compounds ranging from 0.0121 to 0.0501 ng and from 0.0302 to 0.614 ng, respectively. The instrument, intra- and inter-day precisions (RSD) of the investigated components were less than 1.26, 1.83 and 2.75%, respectively. The recovery was in the range of 90.6–100.8%, with RSD less than 3.28%. The results indicate that the method is accurate and reproducible. Furthmore, the solution was found to be rather stable (RSD values of the peak area were lower than 2.21%). Therefore, the method is precise, accurate and sensitive enough for simultaneous quantitative evaluation of the 20 compounds in *Isodon nervosa*.

Sample analysis

The proposed HPLC method was applied to the analysis of 20 analytes in 21 batches of *Isodon nervosa*. The analysis time was reduced to 15 min by switching the ion source polarity between positive and negative modes in a single chromatographic run. Moreover, MRM scanning mode offered good sensitivity for it significantly decreased levels of noise and accordingly enhanced the response of analytes. Thus, some minor constituents in *Isodon nervosa* also could be accurately measured. The target compounds were identified on the basis of comparison of retention time, parent and product obtained from LC-MS/MS analysis of the standard compounds and further identified on the base of fragment ions produced from the MRM-IDA-EPI mode. The quantitative analyses were performed by means of the external standard methods. The data are summarised in Table 6. The results demonstrated a successful application of this proposed method for the quantification of the major compounds in different samples of *Isodon nervosa* (Fig. 3).

As seen from the data of samples **1–21**, the contents of 20 compounds, especially nervosanin B, nodosin, rabdoternin A and enmenol, varied considerably. This can be seen clearly in Table 6, where the amounts of the 20 marker compounds in each batch of *Isodon nervosa* samples are recorded. The results demonstrate that the amounts of the 20 marker compounds within the plant material are very variable and this may be due to a number of causes including plant origin, growth conditions and the parts of the plant used to prepare the medication. Firstly, the amounts of diterpenoids were richer in natural than cultured *Isodon nervosa*. Secondly, the leaves containing much larger amounts of diterpenoids than the stems. In contrast, phenolic content was significantly higher in the stems. The standardised production plant in Jiyuan, Henan Province was significantly more abundant in total amounts than those from the other places of origin. These results may prompt suggestions for Chinese medicine clinical use of *Isodon nervosa*.

The results indicate that it is important to consider the impact of place of origin, cultivation methods and the plant parts incorporated into the medicine on product quality These all suggest that the quality of *Isodon nervosa* could be assured if above impact factors can be standardised.

The proposed method of operating both negative and positive scanning modes in a single analysis process was developed and validated to simultaneously determine and identify 20 constituents in *Isodon nervosa*. Validation of the assay showed appropriate accuracy and repeatability, and was successfully utilised to analyse 21 batches of *Isodon nervosa* samples from different sources. The satisfactory results demonstrated that the HPLC-MS/MS method offered a good alternative for routine analysis due to its rapidity, sensitivity and specificity and could be applied as a reliable quality evaluation method for *Isodon nervosa*. In the future, the HPLC-MS/MS method will become more popular in analysing herbal medicines.

Acknowledgement

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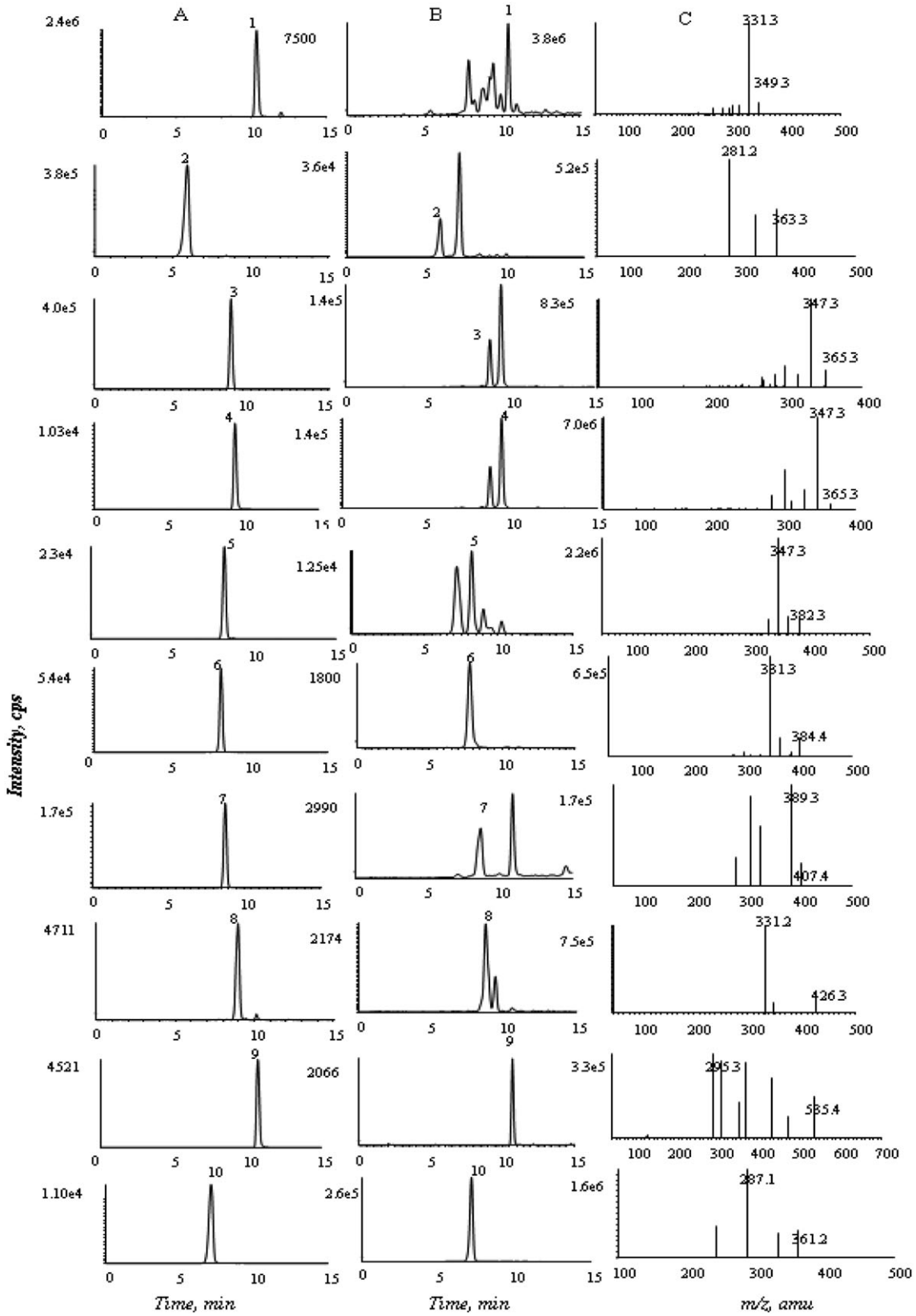

Figure 3. Representative extracted ion chromatograms of multiple-reaction monitoring chromatograms of effusanin A, enmein, lasiodonin, oridonin, epinodosinol, nervosanin B, isodonoiol, sodoponin, rabdosinate, epinodosin, nodosin, ponicidin, rabdoternin A, enmenol, hebeirubesensin K and lasiokaurin, and four phenolic acids (protocatechuic aldehyde, salicylic acid, caffeic acid and rosmarinci acid): (A) standards; (B) *Isodon nervosa* sample (Henan-1); and (C) product ion scan spectra of 20 standards.

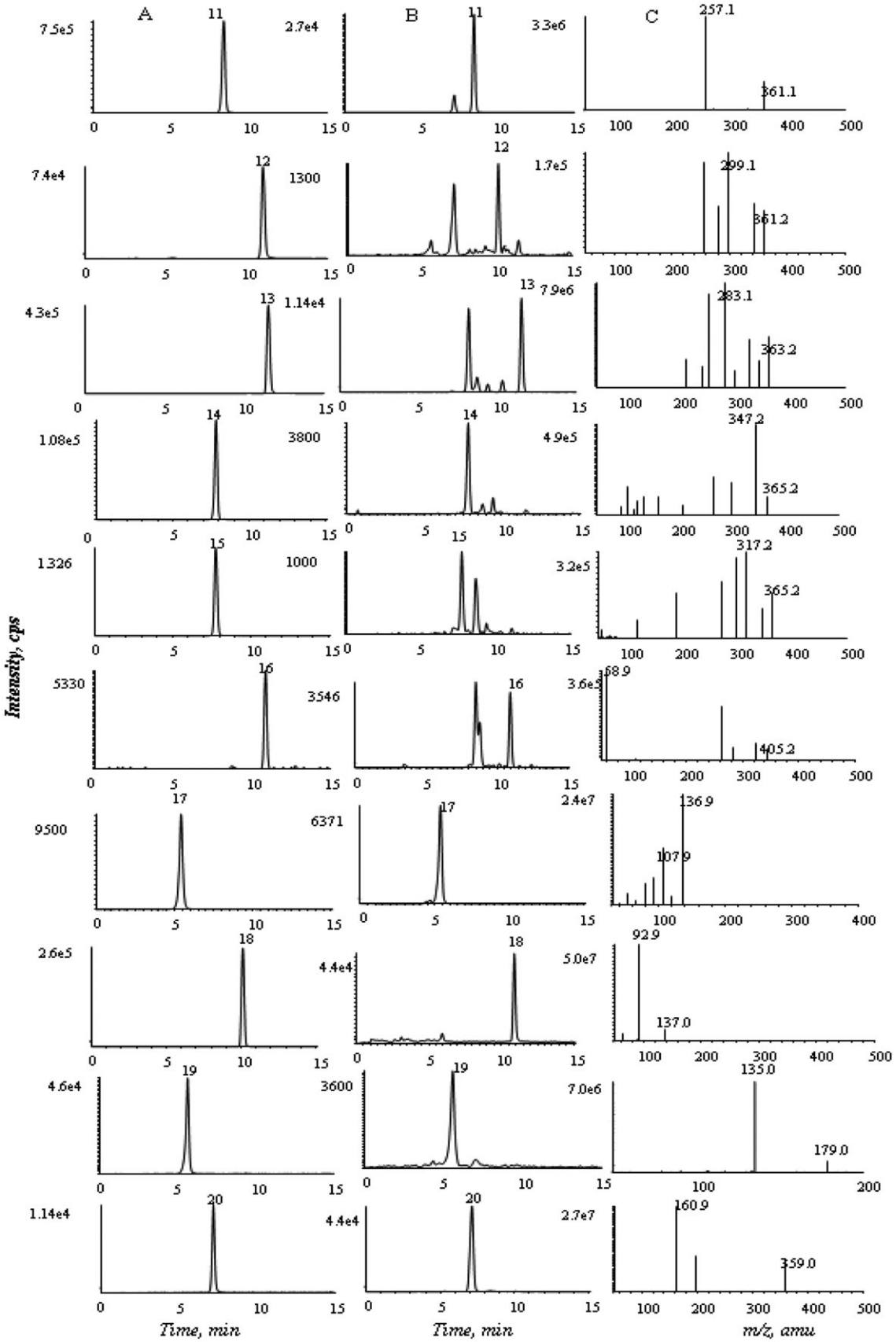

Figure 3. Continued.

Table 6. Content of the 20 active components in *Isodon nervosa*

Sample	Content[b] (μg/g)																				
	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	SUM
Henan-1	2.151	64.235	112.465	453.628	1532.163	0.985	2.134	3.142	25.143	524.335	1.322	35.215	1.232	1.025	90.124	32.549	24.157	4.513	4.235	134.067	3048.820
Henan-2	2.522	65.497	123.278	386.752	1230.521	1.254	2.315	4.126	20.134	586.213	—	15.026	0.864	2.031	82.12	30.261	23.052	3.861	3.854	142.568	2726.249
Henan-3	3.110	71.235	125.467	415.615	1326.589	1.321	2.475	3.516	19.264	512.231	—	21.36	0.961	1.625	86.511	34.518	26.011	4.263	4.135	155.305	2815.512
Henan-4	2.305	63.529	134.027	384.206	1235.785	1.204	2.316	3.687	21.345	597.205	—	32.061	1.054	1.302	85.152	31.045	27.02	3.52	3.956	144.62	2775.339
Henan-5	4.154	79.205	105.264	302.514	1126.782	—	2.531	4.321	23.105	584.356	—	28.152	—	1.415	89.205	33.245	23.061	4.035	3.562	139.578	2550.331
Henan-6	3.641	65.467	132.649	366.341	1362.051	1.26	1.876	3.625	29.135	597.261	—	29.641	—	0.642	92.045	29.645	23.516	3.861	4.035	126.315	2873.006
Henan-7	3.267	70.263	134.621	401.32	1529.742	—	2.015	3.259	26.016	542.305	—	33.024	—	0.305	88.205	30.415	29.262	5.125	3.675	133.024	3035.843
Henan-8	4.067	80.364	138.754	463.789	1052.523	1.025	2.306	4.162	24.013	538.624	—	34.21	—	1.025	90.124	26.548	25.031	3.12	4.037	135.602	2629.324
Hebei-1	6.241	92.364	162.487	723.548	1562.572	1.845	4.152	3.841	39.126	826.204	—	52.321	2.153	6.035	156.302[e]	52.415	13.064	1.023	3.695	145.784	3698.870
Hebei-2	—[c]	15.264	35.233	80.235	1571.228	—	±	3.564	3.024	123.001	—	21.205	—	—	23.064	6.231	42.306	9.231	6.356	149.315	2089.257
Hebei-3	3.216	56.026	98.762	285.315	1364.758	—	1.236	3.128	26.152	614.307	±	24.526	—	0.892	56.302	34.261	15.264	3.856	4.205	162.051	2754.257
Hebei-4	2.514	58.763	92.053	296.358	1263.425	—	1.305	3.421	19.452	526.320	±	26.351	—	—	33.104	28.051	20.548	4.025	4.062	155.054	2534.806
Hebei-5	2.361	45.127	85.214	324.062	1349.782	±[d]	—	6.321	23.415	532.021	—	28.264	—	±	51.024	29.054	23.012	4.32	3.621	120.415	2628.013
Hebei-6	2.349	52.161	86.345	315.286	1286.257	—	—	5.244	25.105	602.305	—	26.534	—	±	62.031	26.345	26.062	4.615	4.032	126.261	2650.932
Jiangxi-1	2.631	55.231	95.273	288.743	1364.326	0.951	—	5.242	28.305	587.321	0.361	26.294	±	—	59.048	28.063	24.395	3.875	3.204	130.249	2703.512
Jiangxi-2	3.241	75.125	52.365	293.251	1256.145	0.926	—	4.261	25.021	485.309	0.987	28.166	±	—	51.302	29.032	31.078	3.624	3.645	136.452	2479.930
Jiangxi-3	2.846	70.142	78.354	304.267	1396.245	0.781	—	5.321	26.074	430.261	—	26.302	—	0.865	53.041	28.315	26.302	5.234	3.875	128.561	2586.786
Anhui-1	2.516	76.546	112.458	311.256	1425.264	0.251	±	5.634	22.031	459.621	—	29.041	—	0.923	56.015	31.051	26.563	3.615	4.235	136.264	2703.284
Anhui-2	2.351	78.012	102.453	316.282	1362.152	—	—	5.026	26.518	481.25	—	30.051	—	1.047	62.243	29.051	34.026	4.124	3.646	145.021	2683.253
Hubei-1	2.437	64.267	98.325	314.205	1421.576	—	—	4.219	24.035	501.261	±	28.162	—	0.921	58.215	26.015	31.045	3.955	4.356	131.052	2714.046
Hubei-2	2.151	64.235	112.465	453.628	1332.163	0.985	2.134	3.142	25.143	429.335	1.426	35.215	1.232	1.025	90.124	32.549	24.157	4.513	4.235	134.067	2753.924

[a] The compounds are the same as Fig. 1.
[b] Average of duplicates.
[c] Undetected.
[d] Not available for quantification.
[e] Determined after appropriate dilution.

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