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Development of an HPLC-DAD–ESI-MS^n method for quantitative analysis of *Saussurea tridactyla*

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ABSTRACT

An improved HPLC-DAD–ESI-MS^n method has been developed to simultaneously quantify eight major compounds in *Saussurea tridactyla* Sch.-Bip. ex Hook. f. which has long been used as a traditional Tibetan medicine. This method was validated to be sensitive, precise and accurate with the LODs of 0.11–5.01 μg/ml, the overall intra-day and inter-day variations less than 2.70%, and the overall recovery over 98.0%, respectively. The correlation coefficients (r^2) of the calibration curves were higher than 0.991. This newly established method was successfully applied to reveal the difference in the chemical profiles and contents of these analyses in *S. tridactyla* from different localities. In addition, by comparison UV and MS spectra with those of authentic compounds and literatures, a total of fourteen peaks were identified. It can be concluded that this method was effective to ensure the safety and efficacy consistency of *S. tridactyla*, and can be applied to other traditional Tibetan medicinal plants from different resources in Tibet.

1. Introduction

Genus *Saussurea*, firstly recorded in Tibetan ancient medicinal literature (Yue Wang Yao Zhen, eighth century A.D.), has been commonly used as an herbal medicine for curing rheumatic arthritis, gynecological disorders and the common cough [1]. *Saussurea tridactyla*, a large species of genus *Saussurea*, is widely spread throughout Tibet. As a traditional Tibetan medicine, *S. tridactyla* had attracted much interest to study their chemical constituents [2–4], bioactivity and pharmacologic effect [5–7]. Although, some quantitative methods have used to study *S. tridactyla*, such as rutin in *S. tridactyla* was quantified by fluorescence [8], but it failed to estimate rich chemical compounds in the plant; an HPLC method for quantitative seven compounds in *S. tridactyla* was reported [9], but it was hard to evaluate the biodiversities of the plant in its' vast resources. Therefore, there exists a void of validated LC–MS method for the identification of major components in the plant and quality assessment of it from different localities.

Nowadays, HPLC with photodiode array detection–electrospray ionization multiple-stage mass spectrometry (HPLC-DAD–ESI-MS^n) has grown into one of the most powerful analytical techniques available for analyzing complex herbal extracts. It can simultaneously provide UV and multiple-stage mass spectra, which can be applied to identify known components by comparing on-line detected chromatograms and spectra with those of authentic compounds, and can elucidate unknown structures based on the tandem mass fragmentation pathways of known ones. Previously, there were some successful applications of HPLC-DAD–ESI-MS^n methods on analysis of major components in herbs: *Ophiopogon japonicus* [10], *Qiang huo* [11], *Gymnadenia conopsea* [12] and *Angelica dahurica* [13]. In this paper an HPLC-DAD–ESI-MS^n method was optimized and established to simultaneously quantify eight main peaks in *S. tridactyla* from seven locations of Tibet. The application of this method was employed for establishment of the chemical fingerprint of *S. tridactyla* and assessment of the quality based on the contents of the main eight components in the seven samples.

2. Experimental

2.1. Chemicals and materials

HPLC grade acetonitrile (Fisher, USA), acetic acid (China) and deionized water obtained from a Milli-Q water system (Millipore Corp., Bedford, MA, USA) were used for during sample preparation procedures and HPLC analysis. 70% Methanol–water was used for sample extraction. Standard of thirteen components: scopolin (**1**), umbelliferone (**2**), scopoletin (**3**), luteolin-7-*O*-glucosid (**4**), apigenin-7-*O*-lutinoside (**5**), apigenin-7-*O*-glucoside (**6**), luteolin

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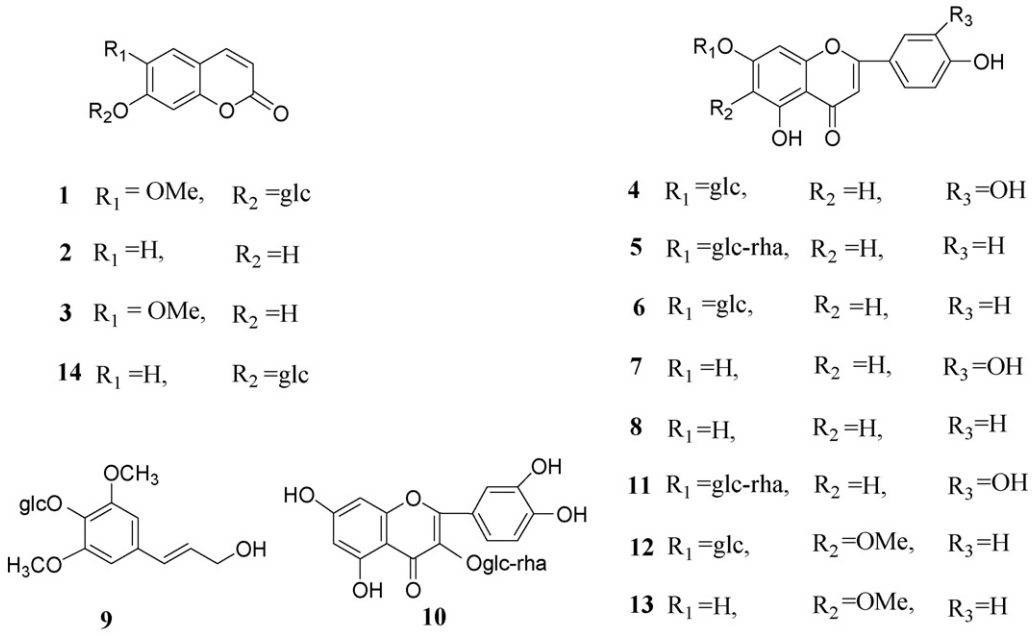

Fig. 1. The chemical structures of compounds **1**–**14**.

(**7**), apigenin (**8**), syringin (**9**), rutin (**10**), 1uteolin-7-*O*-lutinoside (**11**), homoplantaginin (**12**) and hispdulin (**13**) were isolated in our laboratory from *S. species* and their structures (Fig. 1) were elucidated by comparison of UV, MS, ^{1}H NMR and ^{13}C NMR spectral data [2–4,14–22]. The purity of each compound was determined to be higher than 95% by normalization of the peak area detected by HPLC–UV.

Samples of *S. tridactyla* were collected from southern (Sample I: Cuomei county, Sample II: Luozha county, Sample III: Langkazi county), western (Sample IV: Yadong county), central (Sample V: Dangxiong county), northern (Sample VI: Nima county) and eastern (Sample VII: Leiwuqi county) parts of Tibet Autonomous Region in 2007. All of them were identified as the genuine medicinal plants by Mr. Suolang Gesang of Tibet Autonomous Region Institute for Food and Drug Control based on the characteristics of rhizomes measuring 8–16 cm in height which covered with white or thin brown hair. Leaves distributed densely with spoon-shapes, blunt tips, 5–6 cm in length and covered with white or light brown hair on both sides. Capitulums were hemispherical without pedicel. Involucres were cylindrical and measuring 1.5 cm in length and bracts were long-circular and had sharp tips. Blue-violet flowers were about 1.3 cm in length. Pappuses were thin and brown. Achenes were about 4 mm in length. It only gave off a faint odour and the taste was little bitter.

2.2. Sample preparation

An accurately weighed sample of 0.500 g grinded powder and 100 ml 70% methanol were added to a flask, and were distilled circum-fluently for 2 h and then filtered off and repeat this extrac-

Table 1
Regression equation, correlation coefficients, linearity ranges and limit of detection (LOD) and quantitation (LOQ) for the eight markers of *S. tridactyla*

	Compounds	Regression equation	Linear range (μg/ml)	r^2	LOD (μg/ml)	LOQ (μg/ml)
1	Scopolin	Y = 4E + 06x − 1E + 07	10.03–180.11	0.9985	5.01	10.21
2	Umbelliferone	Y = 4E + 06x − 12E + 06	1.19–105.90	0.9984	0.41	1.62
3	Scopoletin	Y = 2E + 06x − 12E + 06	2.76–117.04	0.9990	0.45	3.26
4	Luteolin 7-*O*-glucoside	Y = 1E + 07x − 15E + 06	2.87–54.37	0.9995	2.13	2.99
5	Apigenin 7-*O*-lutinoside	Y = 7E + 06x − 12E + 06	0.31–98.66	0.9992	0.11	0.44
6	Apigenin 7-*O*-glucoside	Y = 5E + 06x − 15E + 06	0.60–40.50	0.9987	0.31	1.27
7	Luteolin	Y = 1E + 07x + 6E + 06	0.44–35.10	0.9975	0.18	0.99
8	Apigenin	Y = 6E + 06x − 14E + 06	0.69–53.03	0.9917	0.13	0.52

Table 2
Precision, repeatability, stability, recovery for the eight markers of *S. tridactyla*

	Compounds	Precision		Repeatability R.S.D.% (n = 6)	Stability R.S.D.% (n = 6)	Recovery (n = 3)
		Intra-day R.S.D.% (n = 6)	Inter-day R.S.D.% (n = 6)			
1	Scopolin	1.39	0.45	1.40	1.41	98.83%
2	Umbelliferone	1.65	1.30	0.96	1.98	100.04%
3	Scopoletin	2.05	0.58	0.05	2.25	99.96%
4	Luteolin 7-*O*-glucoside	2.50	1.40	2.00	2.50	99.01%
5	Apigenin 7-*O*-lutinoside	0.06	0.88	2.05	0. 60	99.14%
6	Apigenin 7-*O*-glucoside	0.99	1.44	0.22	1.99	98.38%
7	Luteolin	1.60	2.70	1.40	1.69	98.96%
8	Apigenin	1.68	1.14	0.88	1.86	98.10%

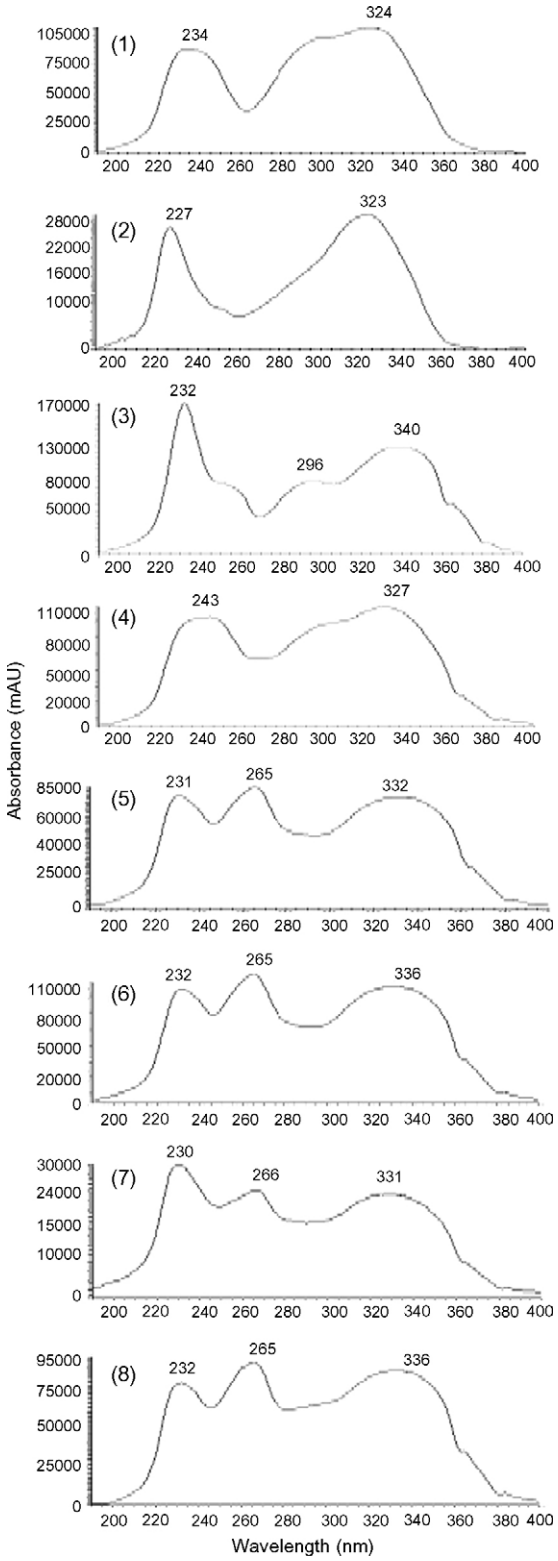

Fig. 2. The UV spectrum data (190–400 nm) of eight main compounds (**1**–**8**).

tion process one time. All extracts were combined, and evaporated under vacuum and then diluted to volume with 70% methanol in a 25 ml volumetric flask. A volume of 2 ml of the solution was then filtered through a 0.45 μm membrane filter into an HPLC vial for analysis. An aliquot of 10 μl solution was injected for HPLC–MS analysis.

2.3. Instrumentation and chromatographic condition

A HPLC system (Thermo Separation Products Inc., Riviera Beach FL, USA) was used for acquiring chromatograms and UV spectra. For chromatographic analysis, Hypersil C_{18} column (5 μm, 250 mm × 4.6 mm) with a suitable guard column was used. A linear gradient elution with acetonitrile/0.1% acetic acid solution (start with v/v: 5/95 and hold this rate for 5 min, and then increase to 35/65 within 55 min) as mobile phase was run in 60 min. The flow rate was 1.0 ml/min, the room and column temperature was at 20 °C and 30 °C, respectively. The injection volume was 10 μl. DAD detector was set to scan from 190 nm to 400 nm.

ThermoQuest Finnigan LCQ^{DECA} system equipped with an electrospray ionization source (ThermoQuest LC/MS Division, San Jose, CA, USA) was used for mass spectrometric measurements. The ESI-MS^n spectra were acquired in both the positive and negative ion modes. The mass spectrometry detector (MSD) parameters were as follows: nebulizer sheath gas: N_2 (80 arbitrary unit); nebulizer Aux gas: N_2 (20 arbitrary unit); capillary 350 °C; spray voltage: 4.5 kV; capillary voltage: −13 V in negative ESI, 25 V in positive ESI; lens voltage: 18 V in negative ESI, −16 V in positive ESI; collision energy: ranged from 35% to 50%. All data were preceded by Finnigan Xcalibur^{TM} core data system Rev. 1.2 (ThermoQuest Corporation, San Jose, CA, USA).

2.4. Validation procedure

2.4.1. Calibration curves, limits of detection and quantification

All calibration graphs were plotted based on linear regression analysis of the integrated peak areas (y) versus concentrations (x, μg/ml) of the eight markers in the standard solution at six different concentrations. The results are demonstrated in Table 1.

The dilute solution was further diluted to a series of concentrations with 70% methanol for the gain of the limits of detection (LOD) and limits of quantitation (LOQ). The LOD and LOQ under the present chromatographic conditions were defined as each compound's signal to baseline noise peak ratio high of 3 and 10, respectively. LOD and LOQ for each analysis are shown in Table 1.

2.4.2. Precision, repeatability, stability and recovery

The intra-day variation was evaluated by determining a standard mixture solution of the eight markers under the optimized condition six times within a day. For inter-day variation, the measurement was conducted two times per day for three consecutive days. The R.S.D. was taken as a measure of precision and the results shown in Table 2.

Six independently prepared solutions which extracted from sample I by the above measure with the same mount were calculated for repeatability. The sample stability test was evaluated by sample I at room temperature and analyzed at 0 h, 2 h, 4 h, 8 h, 12 h and 24 h within 1 day. The R.S.D. was taken as a measure of repeatability and stability and the results shown in Table 2.

Recovery was carried out by spiked accurate amounts of eight standards into sample I, and then extracted and analyzed under this proposed method. Each sample was analyzed in triplicate. The total amount of each analysis was calculated from the corresponding calibration curve. The average recoveries were determined by the formulas: related recovery (%) = (sample contents after adding − original contents)/contents of standard solution for adding × 100% and R.S.D.% = (S.D./mean) × 100%. The results are shown in Table 2.

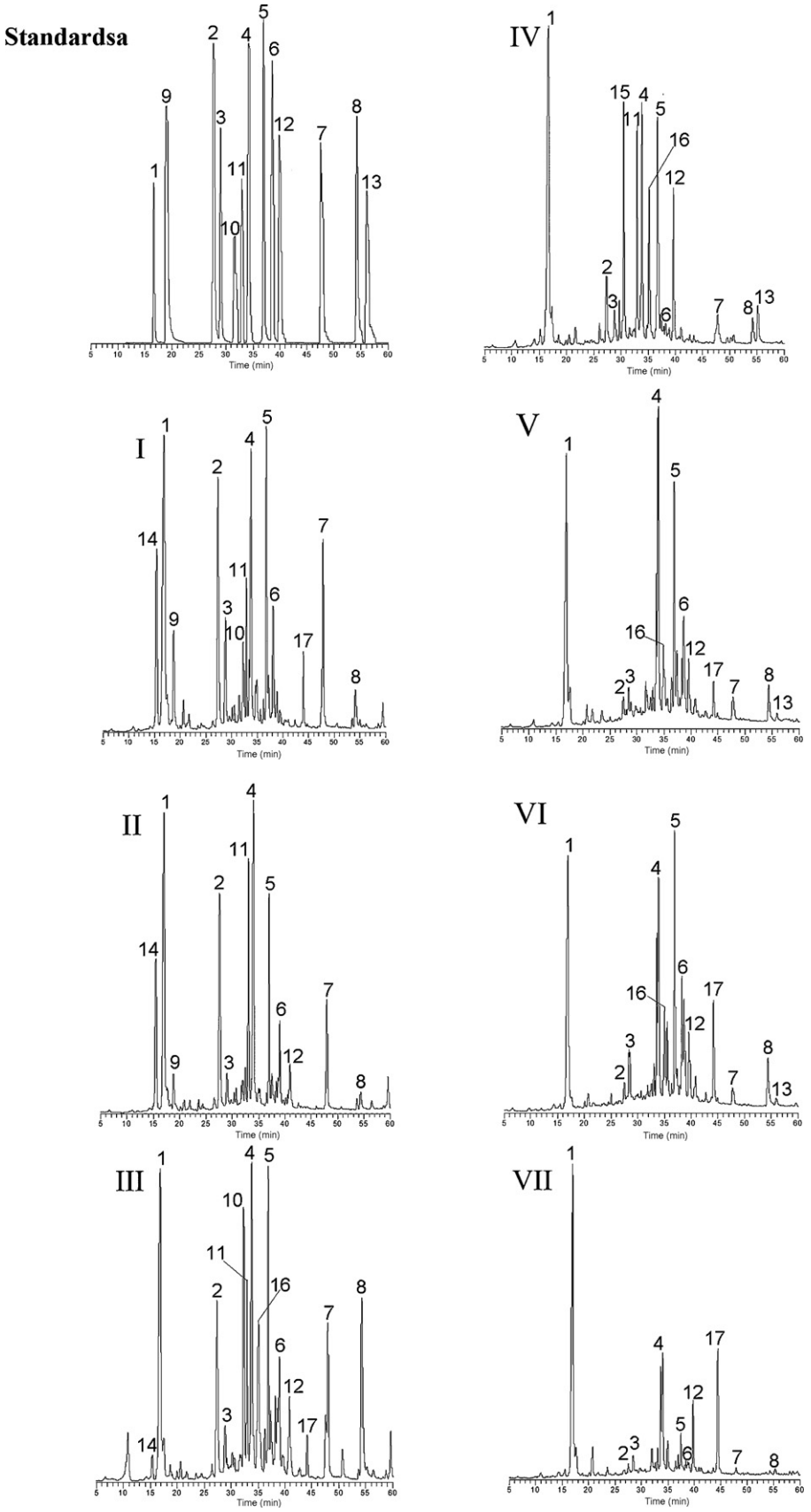

Fig. 3. The chromatograms of standard compounds (**1**–**14**) in samples (I–VII).

Table 3
The contents of eight compounds in the tested samples (μg/g)

Samples	Scopolin	Umbelliferone	Scopoletin	Luteolin 7-*O*-glucoside	Apigenin 7-*O*-lutinoside	Apigenin 7-*O*-glucosid	Luteolin	Apigenin
I	771.45	478.45	327.75	186.15	264.05	109.45	115.50	67.40
II	476.30	261.45	68.70	129.50	94.95	89.00	43.45	20.65
III	746.00	287.85	149.65	167.60	172.35	182.75	119.05	238.75
IV	598.85	78.90	94.50	89.45	135.75	28.70	10.30	25.15
V	490.95	24.45	63.70	154.95	94.00	74.45	10.85	37.05
VI	334.90	16.85	102.20	103.90	106.55	144.20	6.20	78.15
VII	322.80	16.25	21.75	20.45	12.00	18.65	0.05	5.45

3. Results and discussion

3.1. Extraction method development

In order to achieve quantitative extraction, variables factors involved in the procedure, such as solvent, extraction method and time. Considering varied polarities of these standard compounds in different solvents, various contents of methanol and ethanol solutions were tested as extraction solvents. As a result, 70% methanol was the preferred choice for most compounds which were co-extracted from *S. tridactyla* effectively. And compare with extraction methods of steeping at room temperature, ultrasonic treatment and distilling circum-fluently, distilling circum-fluently was detected to be the best extraction method with highest yield of all the eight components. For the sake of measuring extraction time, sample I extracted for half hour, 2 h and consecutive twice times of 2 h, respectively. It was tested that all eight components were almost sufficiently extracted within the last procedure.

3.2. Optimization of chromatographic conditions

DAD detector was set 190–400 nm scan ranges to test UV absorption maximum wavelength of the eight main compounds (Fig. 2). Two wavelengths at 230 nm and 330 nm were chosen but all compounds showed maximum absorption around 230 nm, thus 230 nm was chosen as the detection wavelength.

The mobile phase was optimized through comparisons of different solvents, solvent ratio and gradient profile. Compare with other solvents, acetonitrile showed the best separation, shortest analyzing time and lowest column pressure. An acidified mobile phase could minimize peak tailing and improve resolution, and facilitate ionization. Thus, acetonitrile (**A**) and 0.1% acetic acid solution (**B**) were chosen as mobile phases with a flow of 1.0 ml/min in the above gradient. Consequently, under above chromatographic conditions, a standard mixture solution of scopolin (**1**), umbelliferone (**2**), scopoletin (**3**), luteolin-7-*O*-glucosid (**4**), apigenin-7-*O*-lutinoside (**5**), apigenin-7-*O*-glucoside (**6**), luteolin (**7**), apigenin (**8**), syringin (**9**), rutin (**10**), 1uteolin-7-*O*-lutinoside (**11**), homoplantaginin (**12**) and hispdulin (**13**) obtained for the optimal and well-separating chromatogram which was shown in Fig. 3.

3.3. Validation of the method

In Table 1, all the marker standards showed a good linearity ($r^2 > 0.9900$) between the peak area and concentrations; the LOD and LOQ for eight common components ranged from 0.11 μg/ml to 5.01 μg/ml and 0.44 μg/ml to 10.21 μg/ml, respectively. Table 2 presents the results of precision test of the eight standards components. It indicates that the overall R.S.D. of the intra-day and inter-day were 0.06–2.50% and 0.45–2.70%, respectively, and the overall repeatability and stability were less than 3.00%. It is shown in Table 3 that the proposed method was accurate for quantification of the eight common bioactive components in *S. tridactyla*. Thus, the method is precise, accurate and sensitive for simultaneous quantitative evaluation of the eight components in *S. tridactyla*.

3.4. Identification of chemical compounds in S. tridactyla

HPLC-DAD–ESI-MS^n method was employed to analyze the components in the whole plant of *S. tridactyla*. By comparing the UV and

Table 4
Major ions observed in online HPLC–UV–MS/MS analysis

Peak no.	t_R (min)	UV λ_{max} (230 nm)	Positive ions in MS (m/z)	Negative ions in MS (m/z)	HPLC–ESI-MS^n	Identification	References
1	16.89	232, 325	355[M+H]$^+$ 377[M+Na]$^+$	[a]353[M−H]$^-$	191[M−H-glc]$^-$	Scopolin	[14,21]
2	27.45	226, 320		[a]161[M−H]$^-$	104[M−H-$CHCO_2$]$^-$	Umbelliferone	[2]
3	28.68	229, 297, 337	193[M+H]$^+$	[a]191[M−H]$^-$	176[M−H-CH_3]$^-$ 147[M−H-CH_3−CHO]$^-$	scopoletin	[2]
4	33.88	243, 320, 327	449[M+H]$^+$	[a]447[M−H]$^-$	285[M−H-glc]$^-$	Luteolin, 7-*O*-glucoside	[3,20]
5	36.87	229, 266, 332	579[M+H]$^+$	[a]577[M−H]$^-$	431[M−H-rha]$^-$, 269[M−H-rha-glc]$^-$	Apigenin, 7-*O*-lutinoside	[3]
6	38.51	228, 252, 336		[a]431[M−H]$^-$	269[M−H-glc]$^-$ 225[M−H-glc- CO_2]$^-$	Apigenin, 7-*O*-glucoside	[3]
7	47.84	230, 267, 330, 336	287[M+H]$^+$	[a]285[M−H]$^-$	241[M−H-CO_2]$^-$	Luteolin	[3]
8	54.34	231, 266, 337	271[M+H]$^+$	[a]269[M−H]$^-$	225[M−H-CO_2]$^-$	Apigenin	[3]
9	18.68	228, 257, 336	[a]395[M+Na]$^+$	371[M−H]$^-$	364[M+Na− CH_2OH]$^+$	Syringin	[15,16]
10	32.80	231, 326, 336	611[M+H]$^+$	[a]609[M−H]$^-$	301[M−H-rha-glc]$^-$	Rutin	[17,20]
11	33.23	231, 252, 334, 344	595[M+H]$^+$	[a]593[M−H]$^-$	285[M−H-rha-glc]$^-$	Luteolin, 7-*O*-glucoside	[3,22]
12	40.89	228, 251, 265, 341	463[M+H]$^+$	[a]461[M−H]$^-$	284[M−H- glc-CH_3]$^-$	Homoplantaginin	[18,22]
13	55.25	233, 264, 333		[a]299[M−H]$^-$	284[M−H-CH_3]$^-$	Hispdulin	[18,19]
14	15.45	226, 316	[a]325[M+H]$^+$		163[M+H−glc]$^+$	Umbelliferone, 7-*O*-glucoside	[4,21]
15	30.53	231, 251, 334, 344		[a]387[M−H]$^-$	304, 222		
16	35.34	229, 265, 331, 336	539[M+H]$^+$	[a]537M−H]$^-$	375[M−H-glc]$^-$		
17	44.33	229, 265, 336	[a]577[M+H]$^+$ 599[M+Na]$^+$		271		

[a] Parent ion for MS/MS.

ESI-MS^n spectra data and retention time of those authentic compounds, fourteen peaks were unequivocally identified. Under the optimized MS conditions, both positive and negative modes were used to identify the peaks. The on-line detected chromatographic and spectrometric data of the common (**1**–**8**) and uncommon (**9**–**17**) peaks in the HPLC chromatograms are given in Table 4.

3.5. Sample analysis

It was challenging to evaluate the quality of *S. tridactyla* from different resources due to their diverse chemical constituents. The application of this method was employed for establishment of the chemical fingerprint of *S. tridactyla*. Seven resources of *S. tridactyla* were analyzed, and a total of seventeen peaks were well separated. Among them, fourteen peaks were identified based on comparison with UV and MS spectra standards. The contents of eight marker compounds in each samples were analyzed by related regression equation which shown in Table 3 and their relative chromatogram were shown in Fig. 3.

It is shown that the contents of the main constituents in *S. tridactyla*, relatively decreasing from southern, western, central, northern to eastern parts of Tibet, have remarkable diversities. And the nearer resources of the samples collected (samples I–III) the closer contents of their main chemical constituents presented. Furthermore, there are six uncommon peaks were identified: syringin (**9**) in samples I and II; rutin (**10**) in samples I and III; 1uteolin-7-*O*-lutinoside (**11**) in samples I–IV; homoplantaginin (**12**) in samples II–VII; hispdulin (**13**) in samples IV–VI; umbelliferone-7-*O*-glucoside (**14**), the structure show in Fig. 1, in samples I–III. It indicated that the quality of the plant which collected from southern, western and central parts is better than northern and eastern parts of Tibet, which showed higher contents of the eight major compounds. Thus, the method was employed to fingerprinting *S. tridactyla*.

4. Conclusions

An HPLC-DAD–ESI-MS^n method was firstly developed to simultaneously quantify eight major compounds in *S. tridactyla* from seven localities of Tibet. The newly established method was validated to be sensitive, precise and accurate which provided references on the identification of main chemical compounds of *S. tridactyla* and simultaneously promoted comparison of their contents from different resources in the vast species of genus *Saussurea* wherein allowing for better use in broadening medicinal resources and developing traditional Tibetan and Chinese medicines.

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