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Short communication

Development of an in-line HPLC fingerprint ion-trap mass spectrometric method for identification and quality control of *Radix Scrophulariae*

Jing Jing[a,c], Chi-on Chan[a], Lijia Xu[b], Dengping Jin[a], Xinwei Cao[a], Daniel K.W. Mok[a,*], H.S. Parekh[c], Sibao Chen[a,b,**]

[a] State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Shenzhen 518057, China
[b] The Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100193, China
[c] School of Pharmacy, The University of Queensland, Brisbane, QLD 4072, Australia

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A B S T R A C T

Chromatographic fingerprinting has been widely accepted as a crucial method for qualitative and quantitative analyses of bioactives within traditional Chinese medicine. A fingerprint provides detailed information, specific for any given herb, thus facilitating the quality control measures of a given traditional Chinese medicine. In this article, quality assessment of *Radix Scrophulariae* was achieved by using high performance liquid chromatography combining diode-array detection and electrospray ionization mass spectrometry (HPLC–DAD–ESI/MS). Eight batches of sample obtained from different origins in China were used to establish the fingerprint and quantitative analyses. By comparing the retention times, UV and MS spectral data with reference standards, four characteristic peaks in the chromatograms were confirmed as corresponding to acetoside, angoroside C, cinnamic acid, and harpagoside. In addition, other two characteristic peaks were tentatively identified, following the literature interpretation of HPLC–ESI-MS and LC–MS/MS (affording structural information) to be sibirioside A and scrophuloside B_4, respectively. The results indicated that the newly developed HPLC–DAD–MS fingerprint method would be suitable for quality control of *Radix Scrophulariae*.

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1. Introduction

Radix Scrophulariae, the root of *Scrophularia ningpoensis* Hemsl., is a widely used component within traditional Chinese medicine (TCM), also being listed in the 2010 edition of the Chinese Pharmacopoeia. It has been successfully used for the clinical treatments of rheumatism, pharyngalgia, arthritis, constipation and conjunctival congestion in China [1]. More recent pharmacological study indicated that the extract of *Radix Scrophulariae* possesses anti-angiogenesis activity [2]. The efficacy of this herb results from the synergetic action of the various constituents, and these primarily involve iridoid- & phenylpropanoid glycosides as well as cinnamic acid [3]. The aforementioned constituents are present in defined proportions and this is ultimately responsible for delivering constituent-driven effects.

A few analytical methods were reported to analyze the chemical compositions by using high-performance liquid chromatography (HPLC) with different detection methods like diode array detection, evaporative light scattering detection and mass electrospray ionization time-of-flight mass spectrometry and micellar electrokinetic capillary chromatography [4–7]. Although lots of iridoid and phenylpropanoid glycosides were tentatively identified and quantitated, it is also of great significance to develop a simple, rapid and reliable analytical method to characterize as much as chemical compositions from the view of quality control. Nowadays, it is promising that high-performance liquid chromatography coupled with mass spectrometry has been proved to be a very useful tool applied to the characterization of constituents in medicinal herbs.

In the present study, three iridoid glycosides (acteoside, angoroside C, harpagoside), two phenylpropanoid glycosides (sibirioside A and scrophuloside B_4) and one organic acid (cinnamic acid) in *Radix Scrophulariae* were analyzed by ion-trap electrospray ionization mass spectrometry (ESI-MS) in positive ion mode to obtain semi-molecular ions or adduct ions under a single HPLC run. The MSn spectra were obtained by low-energy collision-induced dissociation and analyzed to propose possible fragmentation pathways for each compound. Using these techniques, the eight batches of *Radix Scrophulariae* samples collected from different provinces in China

* Corresponding author.
** Corresponding author at: State Key Laboratory of Chinese Medicine and Molecular Pharmacology, The Hong Kong Polytechnic University, Shenzhen 518057, China. Tel.: +86 755 26737182; fax: +86 755 26972852.
E-mail addresses: bcdaniel@inet.polyu.edu.hk (D.K.W. Mok), bcsbchen@inet.polyu.edu.hk (S. Chen).

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were analyzed. It is believed that the obtained HPLC–DAD–ESI-MS fingerprint would provide a more comprehensive strategy for quality evaluation of *Radix Scrophulariae* and its prescription products in the future.

2. Experimental

2.1. Chemicals and materials

The reference standards of acteoside, angoroside C, harpagoside and cinnamic acid (purity > 98%) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purities of all the standards (Fig. 1) were ≥98%. Eight batches of *Radix Scrophulariae* authenticated samples were collected from different provinces of China including Anhui, Zhejiang and Hubei.

HPLC-grade methanol, formic acid and analytical grade ethanol were purchased from Thermo Fisher Scientific Inc., Tedia (USA) or Guangdong Guanghua Chemical Factory Company Ltd. (China). Purified water was obtained from Hangzhou Wahaha Inc. (China). All solvents and sample solutions were filtered through 0.45 μm membrane filters prior to performing HPLC analysis.

2.2. Standard solutions and sample preparation

Each accurately weighed standard of four markers, acteoside, angoroside C, harpagoside and cinnamic acid were dissolved in methanol and diluted to provide a series of standard solutions for constructing the calibration curve. The solutions were stored at 4 °C.

The herbal samples were blended into a powder and passed through a mesh no. 100 sieve (150 μm). 2.0 g sample, accurately weighted and placed into a 50 mL centrifuge tube, was sonicated at room temperature with 20 mL 50% ethanol for 30 min. Then, the mixture was centrifuged at ca. 3000 × *g* for 5 min and then filtrated through a 0.45 μm filter. Repeat the extraction again. Finally, the sample solution was marked up to 50 mL before HPLC analysis.

2.3. Analytical method

Chromatographic analysis was carried out on a Brava BDS C_{18} analytical column (5 μm, 4.6 mm × 250 mm, Alltech Co., USA) at 25 °C using an Agilent 1200 liquid chromatography system, equipped with a quaternary solvent delivery system, an autosampler and a DAD detector. The detection wavelength of interest was 280 nm. The gradient elution of the mobile phase consisting of (A) 0.05%(aq) formic acid and (B) methanol with a gradient program as follows: gradient program: 0–5 min, linear gradient 10–15% B; 5–10 min, linear gradient 15–35% B; 10–15 min, 35–48% B; 15–30 min, 48–70% B; 30–36 min, 70–88% B; 36–42 min, isocratic 88% at flow rate of 1.0 mL/min. In order to adapt to the flow rate to requirements of the ESI-MS, the flow rate was reduced by a split valve at that ratio of 1:4. Aliquots of 10 μL were injected into the HPLC–DAD–ESI/MS.

The ESI/MS system used was a Bruker Daltonics Inc. HCT electrospray ionization-ion trap mass spectrometer (Bremen, Germany), equipped with an orthogonal ESI interface. The ionization parameters were set as follows: positive ion mode; capillary voltage 4 kV, end plate voltage 3.5 kV; nebulizing gas of nitrogen at 20.0 psi; drying gas of 10.0 L/min at 300 °C. The in-line mass analyzer scanned from 100 to 900 amu. Collision induced dissociation (CID) spectra were obtained with a fragmentation amplitude of 0.8 V (MS/MS) using helium as the collision gas.

2.4. Data analysis of chromatograms

Data analysis of chromatograms was performed with the professional software ‘Analysis of Traditional Chinese Medicine (chromatograms) and Data Management Systems’ [4]. In this study,

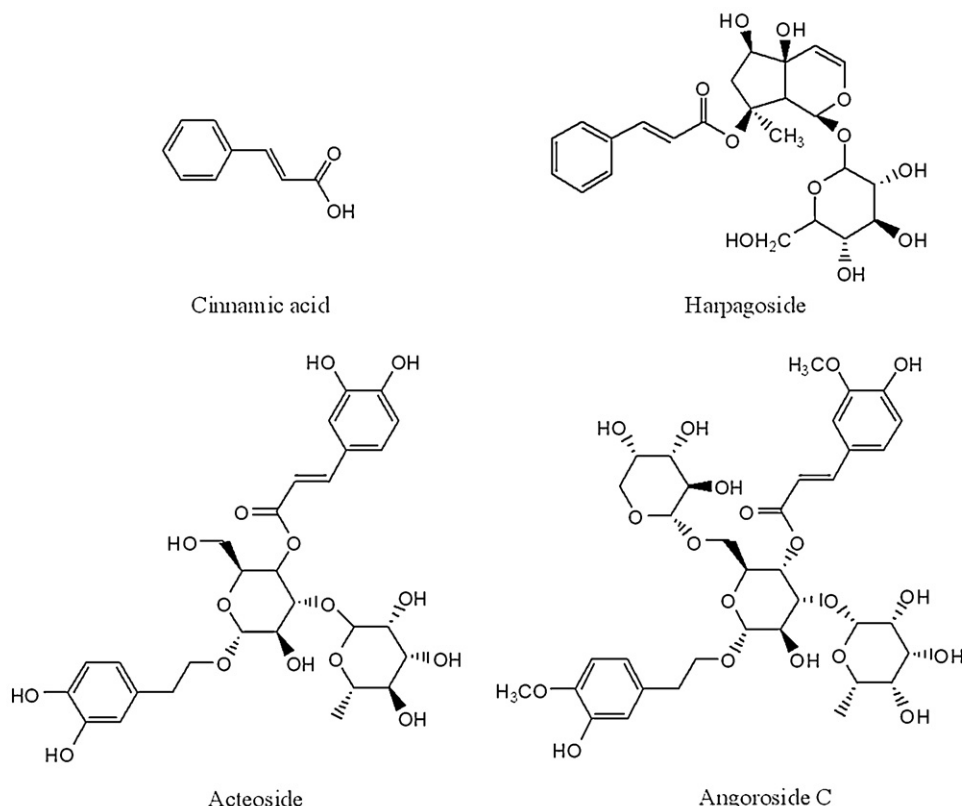

Fig. 1. Chemical structure of cinnamic acid, acteoside, harpagide and angoroside C.

the software was employed to synchronize and do quantitative comparison among different samples. Then the correlation coefficient of similarity between each chromatographic profile of *Radix Scrophulariae* samples and the simulative mean chromatogram could be calculated. This approach is via the calculation of the correlative coefficient of original data, based on the relative peak areas of each major constituent. Furthermore, the relative retention time (RRT) and relative peak area (RPA) of each characteristic peak related to the reference peak were calculated for quantitative expression of the chemical properties in the chromatographic pattern of herbs [5].

3. Results and discussion

3.1. Optimization of extraction procedures and chromatographic conditions

To optimize the extraction procedure, 2.0 g of sample was extracted using different concentrations of ethanol solution (30%, 50%, 70% and 95%). By comparing the peak areas of the HPLC chromatograms obtained, 20 mL of 50% ethanol was found to be optimal extraction medium. Hence, ultrasonication and heat-reflux with 60 min were examined and the results shown that ultrasonication extracted a little more than that of heat-reflux method. Investigating the effects on the duration of ultrasonication (15, 20, 30 and 40 min), it was found that the six compounds were almost completely extracted when 2 × 30 min extraction cycles were used. The yield following a third extraction cycle of further 24 h yielded negligible increase in sample. To sum up, ultrasonication with 50% ethanol for 60 min using two extraction cycles was the optimal extracting condition for chemical components in *Radix Scrophulariae* samples.

Monitoring the chromatograms at 280 nm was found to provide absorption maximum and a steady baseline. Two different acids (acetic acid and formic acid) with different ratios (0.00%, 0.05%, 0.10 and 0.15%) were added into the mobile phase system and compared in an attempt to improve peak shape, reduce the tailing effect as well as promote the ionization of analytes within the samples. Six key constituents could be well separated when 0.05% formic acid was employed. According to the sequence of retention times, these peaks were numbered 1–6 (Fig. 2a). In addition, different types of HPLC column (Agilent XDB Eclipse C_{18}, Zorbax SB C_{18} and Alltech Brava BDS C_{18}) were studied. It was found that Brava BDS column provide the best separation efficiency among the peaks.

3.2. Identification of the bioactive markers in fingerprint chromatograms

3.2.1. LC-MS full scan

In the HPLC–ESI/MS spectra most of the compounds being investigated exhibited their adduct & quasi-molecular ions $[M+Na]^+$ and/or $[M+NH_4]^+$, in positive ion mode (Table 1). Quasi-molecular ions of cinnamic acid, the main bioactive constituent within

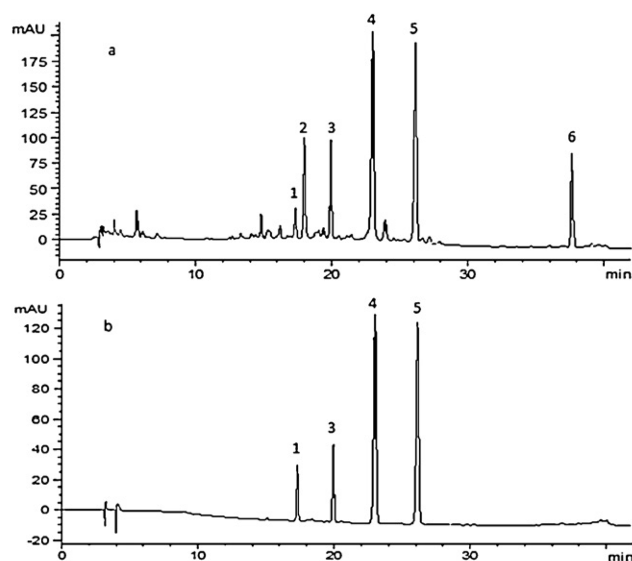

Fig. 2. HPLC–DAD chromatogram of the (a) extract of *Radix Scrophulariae* and (b) standards solution.

Radix Scrophulariae, were not observed most probably due to ion-suppression resulting from the use of formic acid in the mobile phase [6]. However, the cinnamoyl derivative, present as cinnamoyl cations (m/z = 131) were observed in the ESI/MS chromatogram. Following careful comparison of peak retention times, UV data and ESI/MS spectra with reference to the literature profiles and/or reference standard compounds (Fig. 2b), peaks 1, 3, 4 and 5 were identified as acetoside, angoroside C, cinnamic acid, and harpagoside respectively, while peaks 2 and 6 were, after further detailed interpretation unambiguously identified as being sibirioside A [7] and scrophuloside B_4, respectively [8].

3.2.2. LC-MS/MS and proposed fragmentation pathway

The presence of sibirioside A and scrophuloside B_4 was confirmed by MS/MS fragmentation patterns using an ion-trap mass spectrometer in the ESI positive ion mode. The fragment ions of 495 $[M+Na]^+$ were observed at m/z 333 and m/z 185 (Fig. 3a), which correspond to the sodium adduct ion minus 162 amu (a terminal glucose residue) and a sodium adduct ion of a terminal glucose residue, respectively. Fig. 3b shows the proposed fragmentation pathways for the m/z ion 863 $[M+Na]^+$. Structurally informative radical ions of scrophuloside B_4 were present at m/z 479, due to loss of a catalpol moiety. Protonated molecular ions at m/z 663 (loss of 177 amu) and m/z 679 (loss of 162 amu) were consistent with the loss of methoxycinnamic acid and terminal glucose residue, respectively. In addition, fragmentation ions were observed, which could all be rationalized on the basis of the proposed structure.

Table 1
Compounds identified in *Radix Scrophulariae* by LC-MS.

Peak no.	t_R (min)	Proposal ions (m/z)	Molecular weight	Chemical formula	Compound identity
1	17.3	647 $[M+Na]^+$, 642$[M+NH_4]^+$	624	$C_{19}H_{36}O_{15}$	Acteoside[a]
2	18.0	495 $[M+Na]^+$, 490$[M+NH_4]^+$	472	$C_{21}H_{28}O_{12}$	Sibirioside A[b]
3	20.0	807 $[M+Na]^+$, 802 $[M+NH_4]^+$	784	$C_{36}H_{48}O_{19}$	Angoroside C[a]
4	23.0	131 $[M–OH]^+$	148	$C_9H_8O_2$	Cinnamic acid[a]
5	26.1	517 $[M+Na]^+$	494	$C_{24}H_{30}O_{11}$	Harpagoside[a]
6	37.6	863 $[M+Na]^+$	840	$C_{42}H_{48}O_{18}$	Scrophuloside B_4[c]

[a] Further confirmation in comparison with authentic standards.
[b] Reported in the literature [9].
[c] Reported in the literature [10].

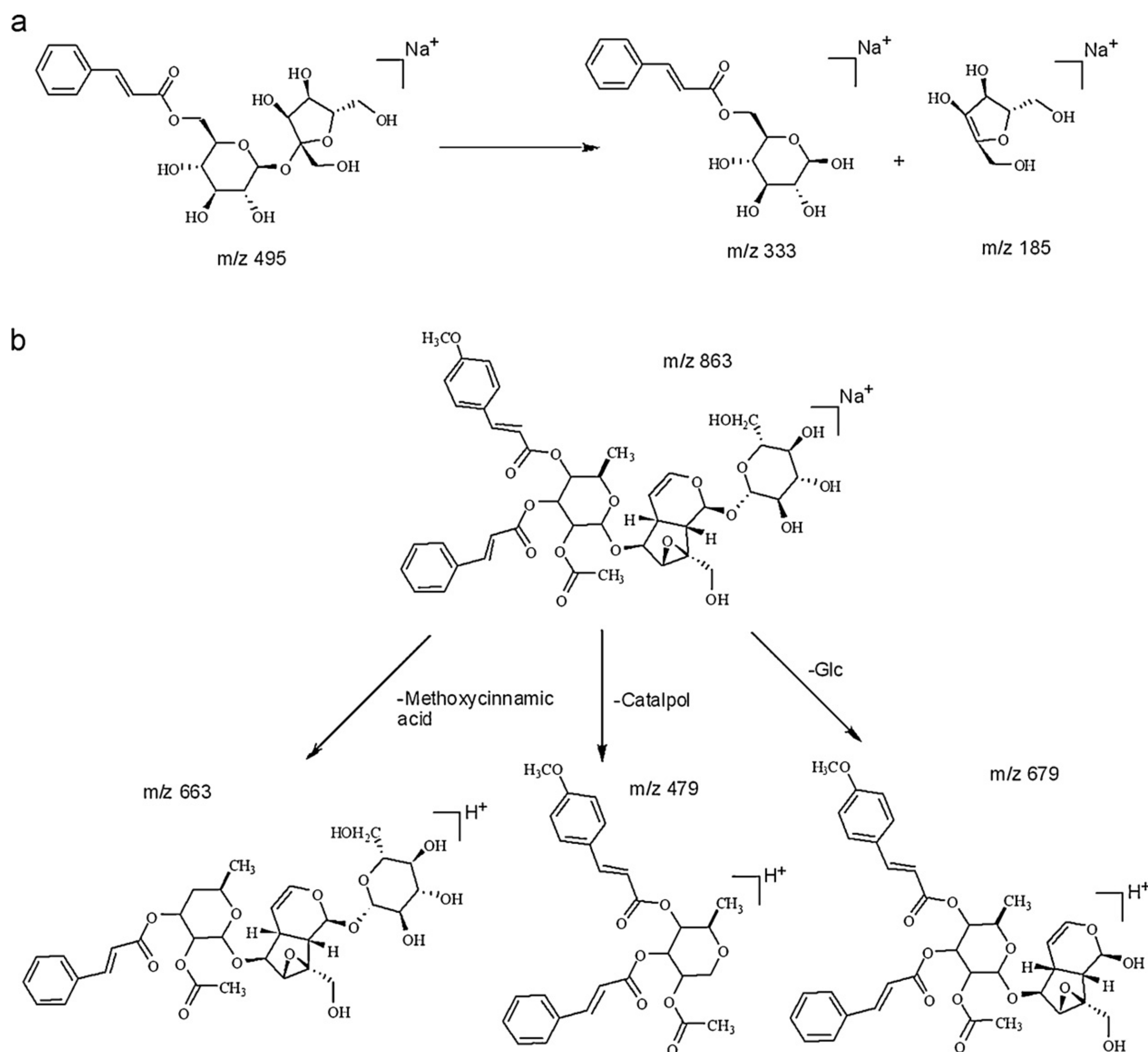

Fig. 3. Proposed fragmentation pathways of sibirioside A (a) and scrophuloside B_4 (b).

3.3. Validation of quantitative analysis method

3.3.1. Linearity (r^2), limit of detection (LOD) and limit of quantification (LOQ)

The linear calibration curves were constructed with at least six different concentrations of chemical markers, in triplicate. The LOD and LOQ values were measured on the basis of the signal-to-noise ratio (S/N) of 3 and 10 as criteria, respectively. Good linear correlation and high sensitivity at these chromatographic conditions were confirmed by the correlation coefficients ($r^2 > 0.999$), LOD (0.1–0.5 μg), and LOQ (0.2–5 μg) (Table 2).

3.3.2. Precision, accuracy and recoveries

The intra-day and inter-day variabilities of four markers (low, medium and high) in herbal extracts were examined by five replicate injections in one day and over three consecutive days, respectively. The relative standard deviation (RSD) was taken as a measure of precision. Meanwhile, recovery tests were performed to examine the efficiency of the extraction method. Accurate amounts of the four markers (low, medium and high) were added to 2.0 g of sample prior to extraction, and then analyzed with the HPLC method outlined earlier. Triplicate experiments were performed at each level to determine the percentage recoveries. The intra- and inter-day precision values were within 1.0% and 2.0%, respectively, with accuracy ranging from 94% to 107% ($n = 3$) for all four markers within the *Radix Scrophulariae* sample (Table 3).

3.4. Regional sample analysis

Eight sample batches of *Radix Scrophulariae* from the provinces of Anhui, Zhejiang and Hubei in China were analyzed by the

Table 2
Linear regression data, LOD and LOQ of the investigated compounds ($n = 6$).

Peak no.	Analytes	Regression equation	r^2	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
1	Acteoside	$y = 9.377x + 14.37$	0.999	5.0–200	0.5	5
3	Angoroside C	$y = 14.90x - 3.970$	0.999	10–400	0.1	1
4	Cinnamic acid	$y = 178.3x + 134.9$	1.000	5.0–200	0.1	0.2
5	Harpagoside	$y = 54.56x + 60.37$	1.000	1.0–400	0.3	0.5

Table 3
Results of precision and accuracy of the investigated compounds.

Amount used/spiked		Precision		Accuracy	
Compound	(μg/mL)	Intra-day	Inter-day	Recovery[b] (%)	RSD[a] (%)
		RSD[a] (%)	RSD[a] (%)		
Acteoside	5	1.63	0.89	107	0.63
	25	0.69	0.24	98	0.69
	100.0	0.18	0.28	104	0.18
Angoroside	10	0.49	0.75	101	0.49
C	50	0.38	0.15	100	0.38
	200	0.21	0.08	100	0.21
Cinnamic	5	0.24	0.06	104	0.24
Acid	25	0.79	0.06	104	0.79
	100	0.13	0.02	102	0.13
Harpagoside	10	0.74	0.35	94	0.74
	50	0.86	0.10	101	0.86
	200	0.24	0.04	101	0.24

[a] RSD (%) = (SD of amount detected/mean of amount detected) × 100.
[b] Recovery (%) = (Detected amount/spiked amount) × 100, data was means of three experiment.

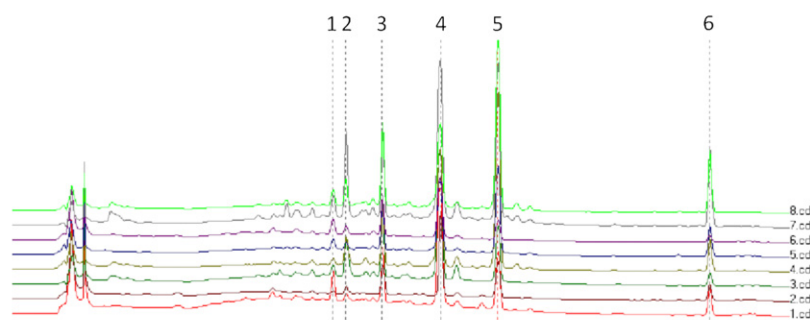

Fig. 4. HPLC–DAD chromatograms of eight batches samples of *Radix Scrophulariae*.

Table 4
The relative retention time (RRT) and relative peak area (RPA) of the six characteristic peaks, and the average content of 4 investigated components in 8 batches of *Radix Scrophulariae* samples.

Peak no.	RRT	RPA	Content (mg/g)
1	0.661 ± 0.001	0.181 ± 0.200	0.693 ± 0.419
2	0.689 ± 0.001	0.227 ± 0.164	–
3	0.763 ± 0.001	0.566 ± 0.361	1.573 ± 0.535
4	0.880 ± 0.001	1.774 ± 1.065	0.386 ± 0.134
5 (Marker, harpagoside)	1.000	1.000	1.084 ± 0.755
6	1.440 ± 0.003	0.285 ± 0.093	–

–, not undergoing quantitative analysis for lack of standards.

HPLC–DAD method. Six characteristic peaks were identified from each of the 8 batches tested, with reasonable peak heights and good resolution being obtained and could be further used for authentication of this herb (Fig. 4). Harpagoside (peak 5), considered one of the most important bioactive constituents of *Radix Scrophulariae* was chosen to calculate the relative retention time of the other seven characteristic peaks (Table 4). Four investigated compounds were quantified in the 8 samples using the quantitative analysis method described above and the contents of these compounds in the 8 batch samples were summarized in Table 4.

3.5. Similarity analysis

Given that regional differences are known to affect the constituent of any given herb, it was deemed necessary to compare the similarity of chromatographic fingerprints of *Radix Scrophulariae* obtained from the various regions. The correlation coefficient between each chromatogram of *Radix Scrophulariae* samples and the simulative mean chromatogram was found to be 0.954, 0.943, 0.937, 0.967, 0.986, 0.899, 0.987, and 0.930 respectively. The closer the cosine values are to 1, the greater the two similarities between respective chromatograms. Our results indicated that the samples shared different correlation coefficients of similarities, showing that the internal quality of these samples was indeed different. Thus, all samples collected were in the acceptable range, in which 0.85 was widely accepted as an appropriate threshold.

4. Conclusions

Fingerprinting techniques are considered as an effective method of controlling TCM quality, namely because it reveals multiple qualitative and quantitative characteristic features of a given herb. It provides a chromatographic pattern of those common chemical constituents of pharmacological interest. Mass spectrometry is nothing but one facet of 'fingerprinting', allowing reliable confirmation of the identity of the detected compounds. Determination of the active or principal chemical constituents in TCMs, as well as potentially toxic compounds will ensure that batch-to-batch variations of primary active constituents within TCMs are curbed, while the quality control of TCMs is maintained. By adopting tech-

niques with encompassing qualitative and quantitative analyses it will ensure that future TCM-based treatments are assessed along similar stringent guidelines.

As we have demonstrated with the method, combining HPLC with MS affords a very powerful tool in the comprehensive identification and determination of key constituents in *Radix Scrophulariae*. This paves the way toward universally acceptable quality control measures that effectively address issues of safety and efficacy of TCM in treating human diseases.

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