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HPLC fingerprinting and LC–TOF-MS analysis of the extract of *Pseudostellaria heterophylla* (Miq.) Pax root

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Abstract

High-performance liquid chromatographic (HPLC) was developed for fingerprint analysis of *Pseudostellaria heterophylla* (Miq.) Pax. Liquid chromatography–electrospray ionization-time-of-flight mass spectrometry (LC–TOF-MS) technique was first employed to identify the components of the fingerprint. Twelve major peaks in chromatographic fingerprint were analyzed by on-line LC–TOF-MS analysis; one cyclic peptide was unequivocally identified and five cyclic peptides were tentatively assigned based on their MS data. These cyclic peptides served as the marker peaks in the HPLC fingerprints. The chromatographic fingerprints have been analyzed by similarity index calculations and hierarchical clustering analysis (HCA). The result showed that the HPLC fingerprints could be used to determine the optimal harvest time for *P. heterophylla* (Miq.) Pax and to authenticate the species of the herb.

Keywords: Fingerprint chromatogram; *Pseudostellaria heterophylla* (Miq.) Pax; HPLC–TOF-MS; Hierarchical clustering analysis; Similarity analysis; Cyclic peptides

1. Introduction

Recently, chromatographic fingerprinting technique, as an approach to control the quality of herbal samples, has been attracting more and more attention because of its effectiveness and convenience in real world applications [1–6]. In 2000, SFDA (State Food and Drug Administration of China) has promulgated the regulation requiring all the injections made from herbal medicines or related materials to be standardized by chromatographic fingerprint [7]. However, despite these intense research efforts in recent years, there is still no general agreement on how to standardize the fingerprinting procedures. When chromatograms from the same batch of samples are compared for reproducibility evaluation, effective analytical techniq1ues are lacking to quantify the similarities among the complex chromatograms generally observed for herbal medicines. SFDA suggested that such similarity matching should be based on calculations of correlation coefficient and/or cosine value of the vectorial angle of the chromatograms after proper reduction of the dimensions of the original data [8,9]. The validity of the methods remains to be established, and our study was initiated because of this consideration.

Pseudostellaria heterophylla (Miq.) Pax or Taizishen (TZS), one of the most popular Traditional Chinese Medicine (TCM), is distributed widely in Fujian, Zhejiang, Jiangsu, Shandong and Anhui provinces. TZS is commonly used in China for the treatment of various diseases associated with the lung, and as a spleen tonic [10]. Because of its widespread use in Chinese medicinal practices, it is of importance to have a valid method for quality control.

In standardizing herbal medicines, the ideal method is to quantify directly those components responsible for bioactivities. However, this is often difficult to do because the active ingredients in herbal products are poorly understood in many cases. Take TZS as an example, the specified quality control technique in Chinese Pharmacopoeia (2005) is by TLC assay. However, the compositional information provided by the assay is too limited to reflect the true quality of the herb. Based on these reason, a

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new and reliable HPLC–TOF-MS method for the identification and quality evaluation of the extract from TZS was established at the same time.

Liquid chromatography–electrospray ionization-time-of-flight mass spectrometry (LC–TOF-MS) has grown into one of most powerful analytical technique currently available [11,12]. Among its advantages, LC–TOF-MS has provided a high level of sensitivity and resolution. It is widely used in the analysis of complex mixtures. In this work, LC–TOF-MS was first utilized to corroborate the structure of the main constituent in the TZS.

2. Experimental

2.1. Instrumentation and reagents

An Agilent Technology 1100 Series HPLC system equipped with a quaternary pump, a degasser, a thermostatic auto-sampler and a photodiode array detector (DAD), was used for analysis. A SK3200LH ultrasonic cleaning instrument (Shanghai Kudos Ultrasonic Instrument Co., Shanghai, China) was used for extraction. The vacuum concentrator system consisted of a rotary evaporator and a digital bath (EYELA, Japan). Acetonitrile is of chromatographically grade and purchased from Merck (Germany). The water used was treated with a Milli-Q water purification system (Millipore, Molsheim, France). The MS instrument used to perform the studies was a G1969A TOF-MS from Agilent. Pseudostellarin B (Fig. 1) standard was purified and identified in our laboratory [13].

2.2. Materials

Two sets of samples labeled respectively as Set 1 and Set 2 were collected for analysis. The two sets were collected from different sub-species of TZS. A total of 21 samples were collected—10 samples in Set 1 and 11 samples in Set 2. They were sampled during the growing period of the herb at a 5-day interval in May, June, and July (25/05/2005, 30/05/2005, 05/06/2005, 10/06/2005, 15/06/2005, 20/06/2005, 25/06/2005, 30/06/2005, 05/07/2005, 10/07/2005 for Set 1 samples; 20/05/2005, 25/05/2005, 30/05/2005, 05/06/2005, 10/06/2005, 15/06/2005, 20/06/2005, 25/06/2005, 30/06/2005, 05/07/2005, 10/07/2005 for Set 2 samples). The herb species were authenticated and prepared by Mr. Wenshen Wang (B&C Technology Inc., Xiamen, China) according to morphological characteristics.

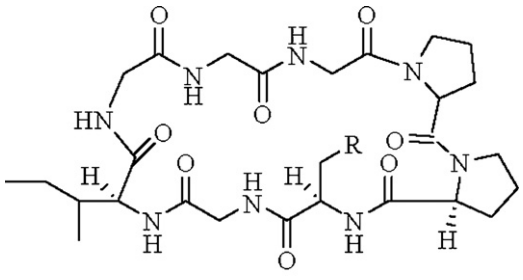

Fig. 1. The structure of Pseudostellarin B.

2.3. Sample preparation

0.5 g sample of the fine-grinded powder was accurately weighted and extracted with 50 ml of methanol in ultrasonic bath for 30 min and filtered. This extraction was repeated two additional times. The combined filtrate was evaporated to dryness in vacuo. The residue was then dissolved and diluted to 10 ml volumetric flask and filtered through a 0.45 μm filter membrane before analysis. Twenty microliters of the sample solution was injected to HPLC column and separated under below chromatographic conditions.

2.4. HPLC procedures

Chromatographic separations were carried out on a C_{18} analytical column (SinoChrom ODS-BP 4.6 mm × 200 mm, 5 μm) supplied by Dalian Elite Analytical Instruments, Dalian, China. The mobile phase consisted of water–acetic acid (A; 100:0.1, v/v) –acetonitrile (B); A:B was as follows: 0 min, 98:2; 10 min, 90:10; 30 min, 55:45; 40 min, 45:55; 60 min, 10:90; 65 min, 0:100; 75 min, 0:100; the flow-rate was 1.0 ml/min and the column temperature was maintained at 30 °C. All solvents were filtered through a 0.45 μm filter and were then degassed by sonication in an ultrasonic bath before use.

2.5. TOF-MS parameter

This HPLC system was interfaced to a time-of-flight mass spectrometer Agilent G1969A TOF-MS (Agilent Technologies) equipped with an electrospray interface operating in positive ion mode, using the following operation parameters: Capillary voltage: 3500 V; nebulizer pressure: 40 psi; drying gas: 12.0 l/min; gas temperature: 350 °C; fragmentor voltage: 200 V; skimmer voltage: 60 V. LC–TOF-MS accurate mass spectra were recorded across the range from 100 to 2000 *m/z*. The data recorded was processed with the Applied Biosystems/MDS-SCIEX Analyst QS software (Frankfurt, Germany) with accurate mass application specific additions from Agilent MSD TOF software. The mass axis was calibrated using the mixture provided by the manufacturer over the *m/z* 100–3000 range. A second orthogonal sprayer with a reference solution was used as a continuous calibration using the following reference masses: 121.0509 and 922.0098 *m/z* (resolution: 9500 ± 500 at 922.0098 *m/z*). Spectra were acquired over the *m/z* 100–2000 range at a scan rate of one second per spectrum.

2.6. Data analysis

Similarity analysis was performed by a professional software named Similarity Evaluation System for Chromatographic Fingerprint, which was recommended by SFDA of China. The software quantifies the similarity indexes among different chromatograms by calculating the correlative coefficient and/or

cosine value of vectorial angle [8–9]. In this article, all of the results were calculated by these two calculated indexes.

The hierarchical clustering analysis (HCA) of samples was performed using SPSS software (SPSS for Windows 11.5, SPSS Inc., USA). A method called average linkage between groups was applied and Euclidean Distance was selected as the measurement [14].

3. Results and discussion

3.1. Optimization of HPLC conditions

Photodiode array detector (DAD) was used in HPLC analysis, and full scan runs were made initially to select the optimum wavelength that provided the best result in chromatographic fingerprinting analysis. Chromatogram at 203 nm showed the most abundant components information than at other wavelength. The gradient was applied in order to ensure the good repeatability without reducing their resolutions. Satisfactory results are obtained within 75 min for the HPLC separation.

The effect of the composition of mobile phase on the chromatographic separation of the extracts was investigated in this study. The results indicated that a mobile phase composed of water–acetic acid (100:0.1, v/v) –acetonitrile was suitable for the separation of the extracts.

3.2. Standardization of fingerprint

The chromatographic peaks in different samples with the same relative retention time were defined as the common peaks. 54 peaks are identified as the common peaks in the Set 1 samples and 65 peaks are identified in the Set 2 samples. The peaks which are too close to the solvent peak (retention time 5 min) are excluded from the list of common peaks, leaving only 42 and 52 peaks, respectively, as the chromatographic fingerprints in the Set 1 and Set 2 samples. Among the common peaks, peak at retention time of 39.8 min is designated as the reference peak for relative retention time calculation because it is an intense peak situated in the middle of the chromatogram [15]. The area sum of all the common peaks in both sample sets account for more than 90% of the total area of all the observed peaks in the chromatograms. The relative retention times of all the common peaks are illustrated in Figs. 2 and 3. In Figs. 2 and 3, the points

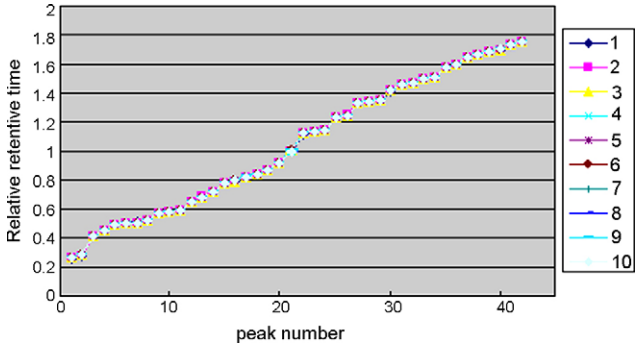

Fig. 2. Results of peaks matching (The Set 1 raw materials).

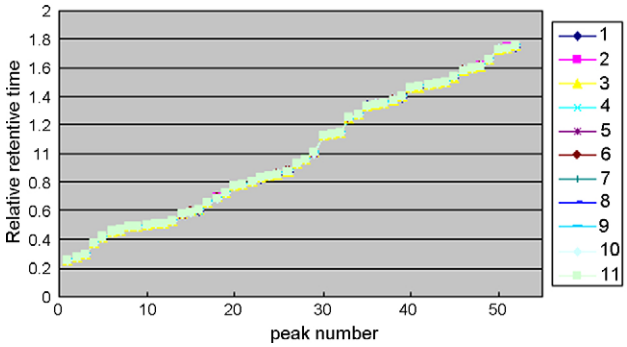

Fig. 3. Results of peaks matching (The Set 2 raw materials).

of the relative retention time's superposition is good and there is no point out of the curve and the results of peaks matching are good [16].

3.3. Similarity analysis

In this study, a similarity analysis based on matching against the mean of the fusion vectors of all the samples within the sample set was performed for both sets of samples. The objective is to examine whether samples in the same sample set can indeed be grouped together based on their similarity in HPLC fingerprints. Both correlation coefficient and cosine angle methods were used in similarity calculations. The result shows that the similarity indexes of the samples within their respective sample sets calculated by either method are good. In both sample sets, the calculated similarities for sample 01 are relatively poorer. This is not surprising since sample 01 was harvested at the early stage of the growing cycle in both sample sets, and their compositional fingerprints are expected to be quite different from the rests of the samples.

The harvesting details and similarities of the chromatograms were shown in Figs. 4 and 5. The results were very interesting that the similarities were distributed symmetrically and the time between two troughs may be the growth cycle of TZS. In Fig. 4 (Set 1 samples) and Fig. 5 (Set 2 samples), the results clearly showed two and three growth cycles respectively. From Fig. 4, the best herbs of TZS (Set 1 samples) were harvested on 5 June

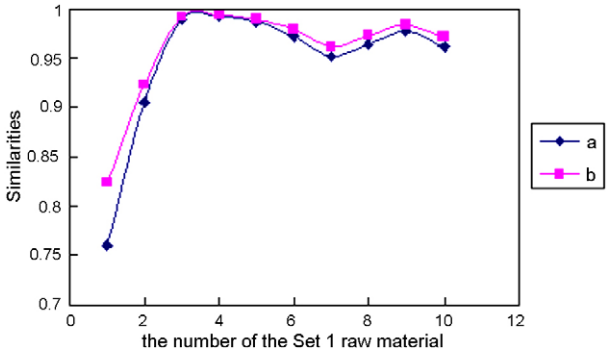

Fig. 4. The similarities of 10 chromatogrrarns (The Set 1 raw materials) Similarities[a]: calculated by the correlative coefficient Similarities[b]: calculated by the cosine value ofvectonal angle.

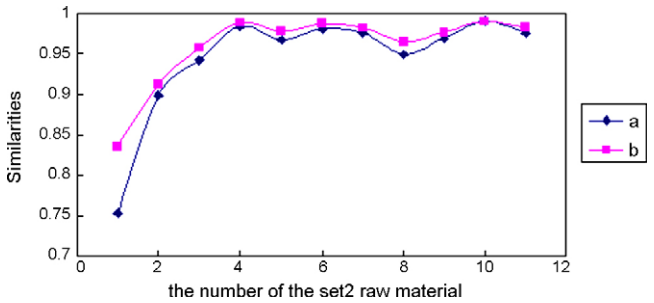

Fig. 5. The similarities of 11 chornatograrns (The Set 2 raw materials) Similarities[a]: calculated by the correlative coefficient Similarities[b]: calculated by the cosine value ofvectonal angle.

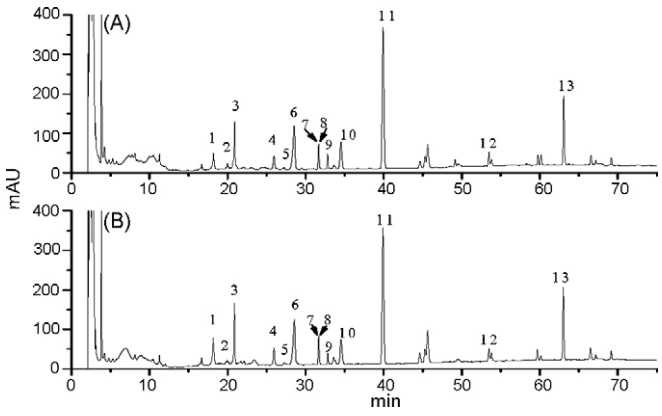

Fig. 6. Fingerprint chromatograms of (A) harvesting on 5 June and (B) harvesting on 5 July (the Set lraw materials).

and 5 July. From Fig. 5, the best herbs of TZS (Set 2 samples) were harvested on 5 June, 20 June and 5 July [17].

Results from fingerprint analysis show that the characteristic features of the two chromatograms (Figs. 6 and 7) are closely similar. This suggested the major components of the herb collected in the Set 1 samples and the Set 2 samples are also closely similar. However, the relative abundances of some of the components in the two groups are significantly different, e.g., peak 11 and peak 13. In the extract of Set 1 samples, the concentration of peak 11 is higher than that of peak 13 (Fig. 6). Contrary to this, the concentration of the peak 13 fraction is higher than that of the peak 11 fraction in the extracts from the Set 2 samples

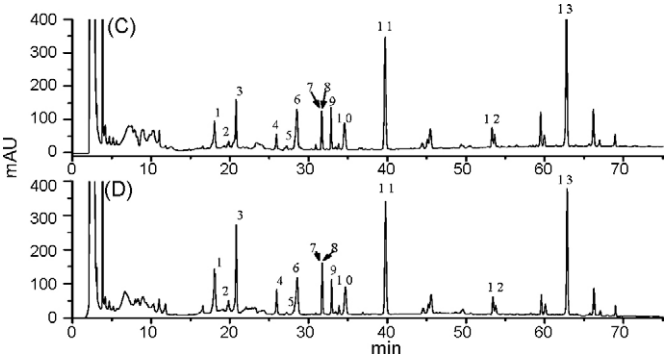

Fig. 7. Fingerprint chromatograms of (A) harvesting on 5 June and (B) harvesting on 5 July (the Set 2 raw materials).

(Fig. 7). Further study is needed to investigate the significance of the differences between the two groups.

3.4. Hierarchical clustering analysis

As shown by the information listed in section 2.2, the individual TZS samples studied here were collected from different sub-species. It would therefore be of interest to see if the sample set can be further divided into subgroups based on hierarchical clustering analysis (HCA). Based on the common peaks observed in the two sample sets, 31 common peaks were selected. Thirty-two characteristic peaks were selected and the relative retention times of these constituents were calculated with respect to the reference peak at retention time 39.8 min. The relative areas of the 31 characteristic peaks were calculated based on their ratios to the reference peak. Relative areas of the 31 peaks of samples 1–21 formed a 21×31 matrix. Distances among the 21 samples were calculated using the SPSS software. The results of HCA are shown in (Fig. 8). It is clear that the samples could be divided into three clusters: The nine samples of Set 1 except sample 01 sample can be grouped as Cluster one; the ten samples of Set 2 except sample 01 sample are grouped into Cluster two. The two 01 samples are grouped together separated from the rests of the samples. Hierarchical clustering analysis thus shows clearly that the samples can be separated into three distinct groups based on their compositional fingerprints.

3.5. HPLC–TOF-MS analysis

HPLC–TOF-MS was employed to analyze the components separated by HPLC. In ESI-TOF-MS experiment, accurate molecular mass of the components can be obtained. Compar-

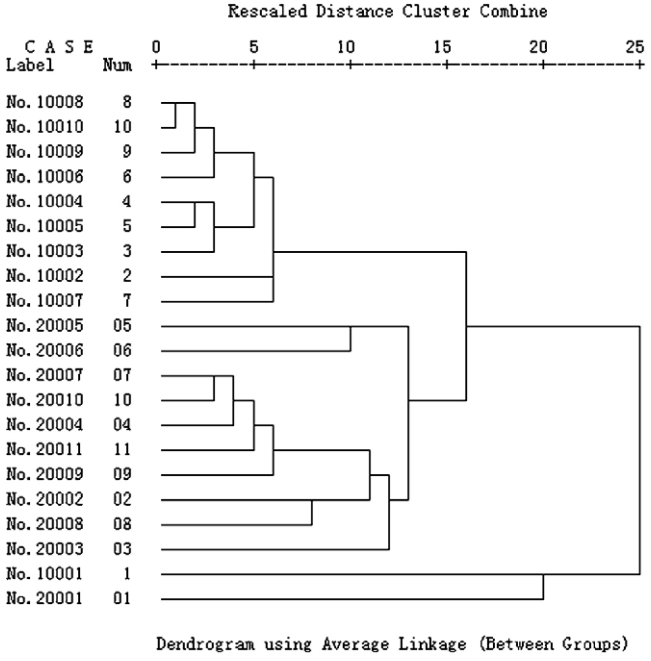

Fig. 8. Results of hierarchical clustering analysis of Set 1 and Set 2 samples.

Table 1
The mass data and compound names of the 12 peaks

Peak no.	Observed mass	Calculated mass	Assignment	Error (ppm)
1	343.1604	–	Unknown substance 1	
2	384.1204 $[M+H]^+$ 406.1028 $[M+Na]^+$	–	Unknown substance 2	
3	502.2669 $[M+H]^+$ 524.2484 $[M+Na]^+$	502.2665 $[M+H]^+$	Pseudostellarin A ($C_{25}H_{34}N_5O_6$)	0.68
4	728.4348 $[M+H]^+$ 750.4164 $[M+Na]^+$	–	Unknown substance 3	
5	314.1528	–	Unknown substance 4	
6	683.3519 $[M+H]^+$ 705.3331 $[M+Na]^+$	683.3518 $[M+H]^+$	Pseudostellarin B ($C_{33}H_{46}N_8O_8$)	−0.31
7	813.4504 $[M+H]^+$ 835.4485 $[M+Na]^+$	813.4510 $[M+H]^+$	Pseudostellarin C ($C_{40}H_{60}N_8O_{10}$)	−0.14
8	714.4189 $[M+H]^+$ 736.3998 $[M+Na]^+$	714.4190 $[M+H]^+$	Pseudostellarin D ($C_{36}H_{55}N_7O_8$)	0.43
9	817.4237 $[M+H]^+$ 839.4069 $[M+Na]^+$	817.4248 $[M+H]^+$	Pseudostellarin G ($C_{42}H_{56}N_8O_9$)	−0.85
10	878.5138 $[M+H]^+$ 900.4956 $[M+Na]^+$	878.5140 $[M+H]^+$	Pseudostellarin E ($C_{45}H_{67}N_9O_9$)	0.50
11	342.3221 $[M+H]^+$ 364.1596 $[M+Na]^+$	–	Unknown substance 5	
12	279.1610 $[M+H]^+$ 301.1427 $[M+Na]^+$	–	Unknown substance 6	

ing MS results with the Pseudostellarin B standard, and those in the literature [18–20], we have deduced 6 possible structures (cyclic peptides) in the HPLC peaks, one compound (Pseudostellarin B) was unequivocally identified and five compounds were tentatively assigned based on their MS date. For the first time, these cyclic peptides were considered as the marker components in TZS. Since positive ionization ESI mode was used, most of the *m/z* data are $[M+H]^+$, $[M+Na]^+$. The mass data and the tentatively identified compound names of the peaks are given in Table 1, the TIC TOF-MS mass spectrum of the Set 1 samples and Set 2 samples are shown in Fig. 9 (a–b) and the TOF-MS mass spectrum of peak numbers 1–12 are shown in Fig. 10 (a–k).

3.6. Six cyclic peptides deduced from TOF-MS data

In ESI (+)-TOF-MS data, $[M+H]^+$is 502.2669 while $[M+Na]^+$ is 524.2484, ($[M+H]^+$, Calculated for $C_{25}H_{35}N_5O_6$ 502.2665, Found 502.2669), so peak 3 was deduced as Pseudostellarin A [18]. $[M+H]^+$is 683.3519 while $[M+Na]^+$ is 705.3331, the ($[M+H]^+$ is therefore assigned as $C_{33}H_{47}N_8O_8$ 683.3518, Found 683.3519), comparing the mass spectrum

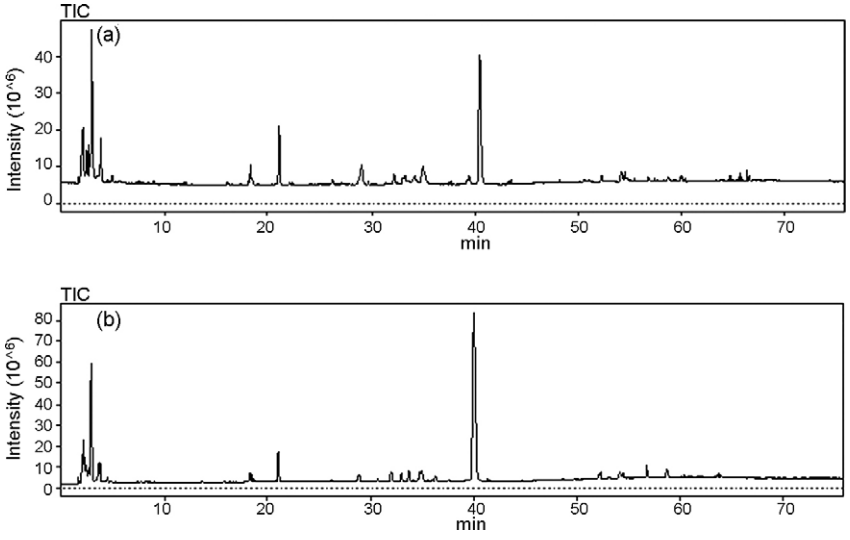

Fig. 9. TIC TOF-MS mass spectrum of the representative samples of Set 1 samples (a) and Set 2 samples (b).

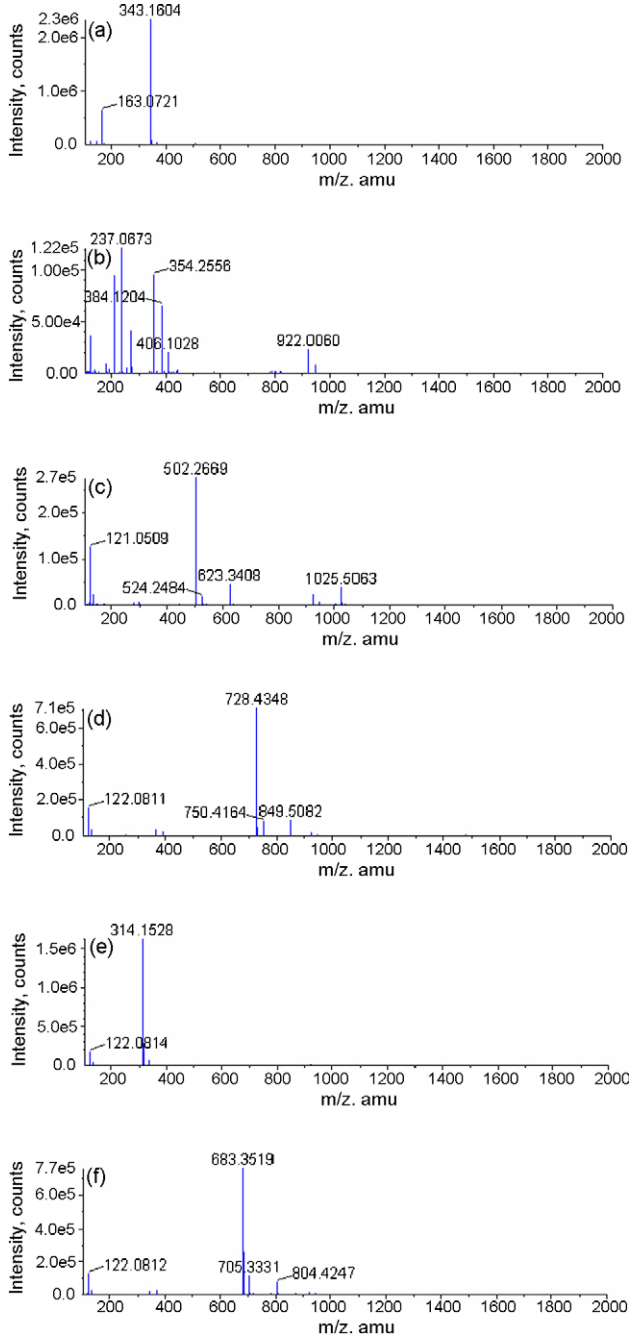

Fig. 10. TOF-MS mass spectrum of peaks (peak l–12).

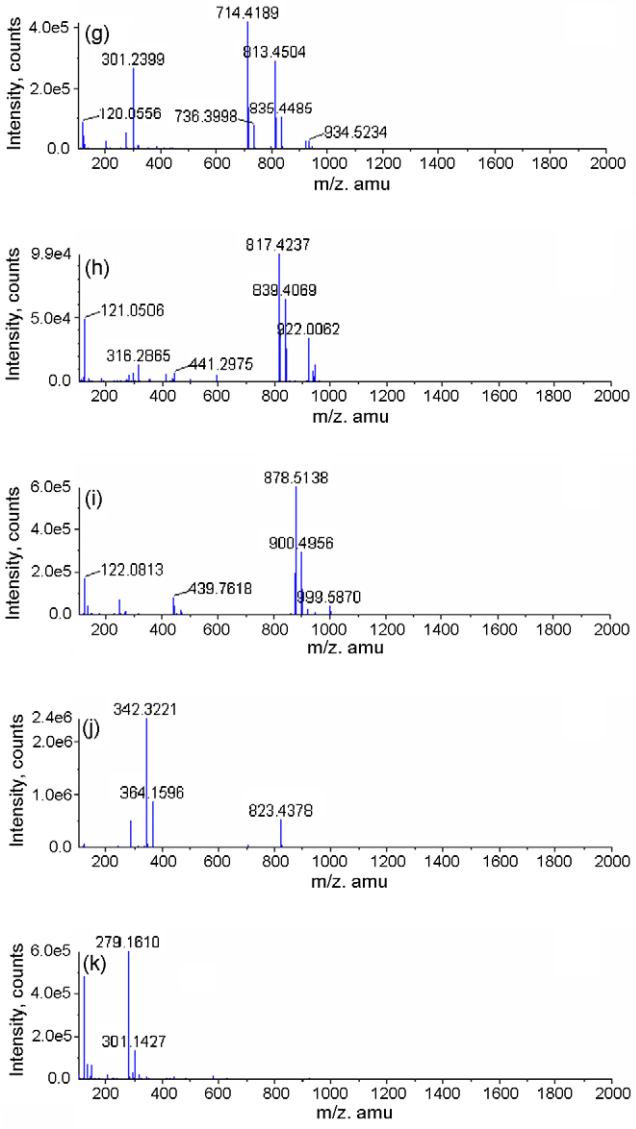

Fig. 10. (*Continued*)

and LC spectrum with Pseudostellarin B standard, peak 6 was positively identified as Pseudostellarin B [18]. $[M+H]^+$is 813.4504 while $[M+Na]^+$ is 835.4485, ($[M+H]^+$, Calculated for $C_{40}H_{61}N_8O_{10}$ 813.4510, Found 813.4504), so peak 7 was deduced as Pseudostellarin C [18]. $[M+H]^+$is 714.4189 while $[M+Na]^+$ is 736.3998, ($[M+H]^+$, Calculated for $C_{36}H_{55}N_7O_8$ 714.4190, Found 714.4189), so peak 8 was deduced as Pseudostellarin D [19]. So the peak 7 and peak 8 was the mixture of Pseudostellarin C and D and not separated on C_{18} analytical column. $[M+H]^+$is 817.4237 while $[M+Na]^+$ is 839.4069, ($[M+H]^+$, Calculated for $C_{42}H_{57}N_8O_9$ 817.4248, Found 817.4237), so peak 9 was deduced as Pseudostellarin G [20]. $[M+H]^+$is 878.5138 while $[M+Na]^+$ is 900.4956, ($[M+H]^+$, Calculated for $C_{45}H_{68}N_9O_9$ 878.5140, Found 878.5138), so peak 10 was deduced as Pseudostellarin E [20]. Peaks 1, 2, 4, 5, 11 and 12 have not been reported in reference articles, so we could not deduce them at present. Unfortunately, peak 13 is not ionizable under the chosen MS conditions.

4. Conclusion

A HPLC method has been developed for the fingerprint of TZS collected from different sub-species of TZS. The standardized HPLC fingerprints show high stability and reproducibility, and thus can be used effectively for the screening analysis or quality assessment of the herb or its derived products. Similarity index calculations based on cosine angle values or correlation methods have been performed on the HPLC fingerprints. For same sub-species of TZS, the fingerprints of the TZS samples studied are highly correlated with closely similar fingerprints.

Between the different sub-species of TZS, the samples can be further divided into subgroups based on hierarchical clustering analysis (HCA). A series of cyclic peptides compounds have been tentatively identified by HPLC–TOF-MS technique. These species constitute the HPLC fingerprints of TZS. Although the sample set is too small to draw a definite conclusion, the preliminary results here do show that the fingerprints might be used to differentiate samples originated from different sub-species of TZS. It can also determine the similarity between samples. Therefore, it is a potentially useful tool in ensuring the quality and safety of herbal products.

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