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Journal of Pharmaceutical and Biomedical Analysis 45 (2007) 38–46
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Chromatographic fingerprint analysis of *Cephalotaxus sinensis* from various sources by high-performance liquid chromatography–diodearray detection–electrospray ionization-tandem mass spectrometry

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Received 9 March 2007; received in revised form 16 May 2007; accepted 21 May 2007
Available online 25 May 2007

Abstract

Selective and efficient analytical methods are required not only for quality assurance but also for authentication of Chinese herbal medicine. A simple, rapid and valid fingerprint method has been first carried out for the quality control of *Cephalotaxus sinensis* by using high-performance liquid chromatography (HPLC) coupled with photodiode array detection (DAD) and electrospray ionisation-tandem mass spectrometry (ESI-MS). The characteristic analytical fingerprints of this plant extract showed 18 common peaks, and out of these, 10 compounds involving 2 new compounds were identified by comparing the retention time, UV and ESI-MS/MS spectrum of each standard with those of each peak separated by on-line HPLC–DAD–MS/MS. Moreover, the effects of collecting locations, harvesting time, storage time, drying methods, and medicinal portions on herbal chromatographic fingerprints were examined by similarity analysis and principal component analysis (PCA) along with markers. Using the reference fingerprint along with markers, the best harvesting time, cultivation location and medicinal part were determined. The results obtained suggest that the chromatographic fingerprint combining similarity evaluation and PCA along with markers or pharmacologically active constituents can efficiently identify raw herb of *Cephalotaxus sinensis* from different sources, which provide helpful clues to the study of plant's secondary metabolites and benefit quality control.

Keywords: Fingerprint analysis; HPLC–DAD–MS/MS; *Cephalotaxus sinensis*; Similarity analysis; PCA

1. Introduction

The identification of traditional Chinese medicine (TCM) from various sources is crucial in order to ensure authenticity, quality, safety and efficacy. But the traditional quality control of TCM encounter more and more challenges because one or two markers or pharmacologically active components in the herbal medicine employed for evaluating the quality and authenticity of herbal medicine cannot give a complete picture of a herbal product. The therapeutic effect of the TCM is often based on the synergic effect of its mass constituents, and the content of naturally occurring active constituents and even the concentration proportion of mass constituents in the TCM may influence the therapeutic effect. Fingerprint has gained more and more attention and been internationally accepted as a feasible means for the quality control of TCM [1–6] due to its ability to identify a particular herb and, moreover, to distinguish it from closely related species [7]. Moreover, this technique emphasizes on the systemic characterization of compositions of samples and focuses on the identification and assessment of the stability of the components [7]. The identity, consistency and authenticity of samples can be determined by comparison of their chromatographic fingerprints (chromatograms representing the chemical characteristics of TCM) using similarity analysis and chemometrics methods. It is well known that hyphenated chromatographic approaches such as GC–MS, HPLC–MS, HPLC–DAD–MS

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doi:10.1016/j.jpba.2007.05.027

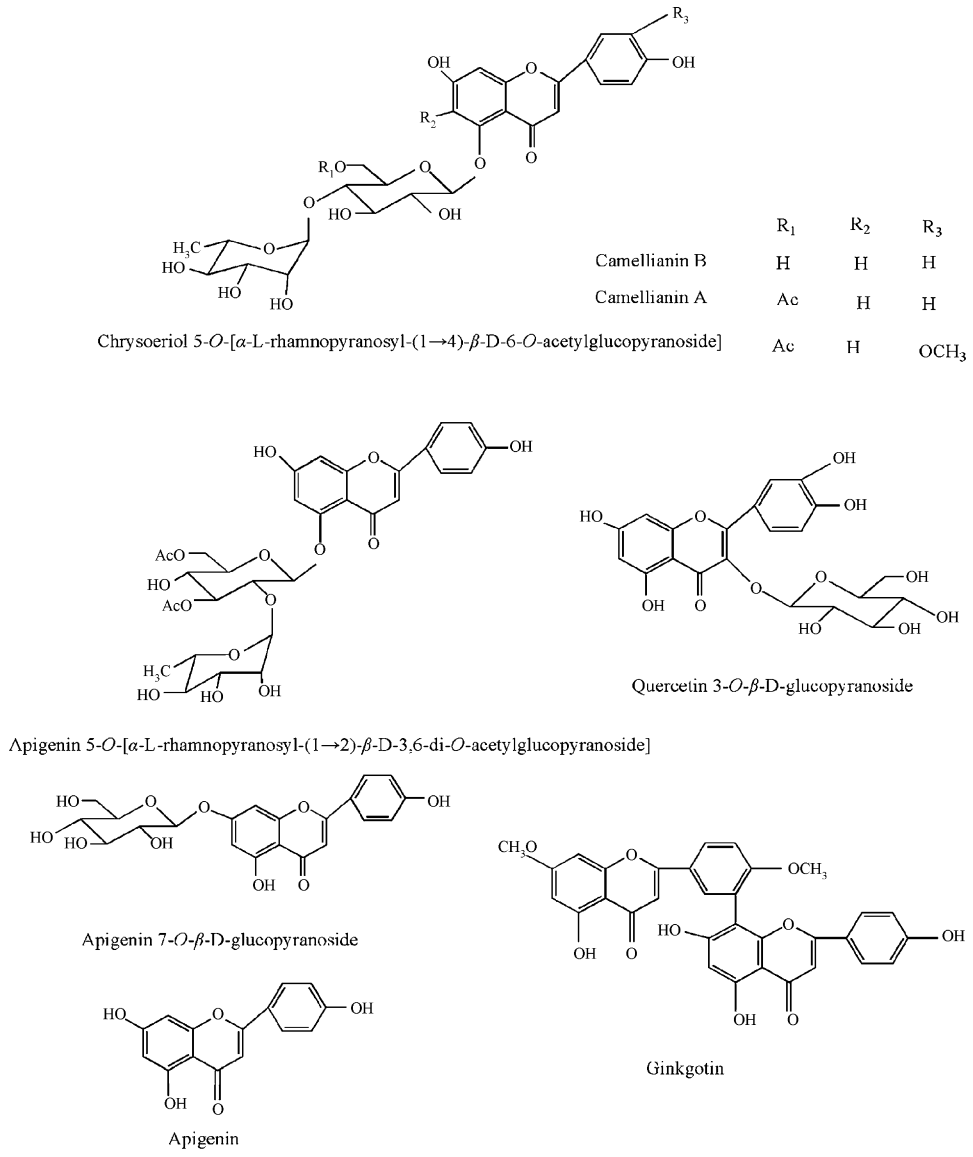

Fig. 1. Structures of the constituents identified from *C. sinensis*.

and CE–MS could show greatly improved performances in terms of correction of retention time shift, selectivity, chromatographic separation abilities and measurement precision [8]. The chemical fingerprint obtained by hyphenated chromatography will become the primary tool for quality control of TCM [9–12].

Cephalotaxus sinensis (Rehd et Wile) Li, a TCM and widely distributed in southern China, has been used against dyspepsia, ascariasis, inflammation and cough [13]. The phytochemical and toxicological studies of Kuo et al. [14], Wang et al. [15] and Politi et al. [16] have suggested that flavonoids, biflavonoids, alkaloids, and diterpenes are the likely active components of *Cephalotaxus* species. Recently, osteoblast differentiation stimulating activity of biflavonoids from *Cephalotaxus koreana* Nakai (Cephalotaxaceae) has been reported [17]. In the screening of anti-diabetic natural substances in our laboratory, it was found that the leaves of *Cephalotaxus sinensis*, collected from Dongzhi, Anhui province, PR China, possessed hypoglycemic effect and our recent studies also revealed that the activity was associated with flavonoids in the herb. Eight flavonoids have been isolated from *C. sinensis* in our laboratory (Fig. 1), including 2 new compounds: Chrysoeriol 5-*O*-[α-L-rhamnopyranosyl-(1 → 4)-β-D-6-*O*-acetylglucopyranoside], Apigenin 5-*O*-[α-L-rhamnopyranosyl-(1 → 2)-β-D-3,6-di-*O*-acetylglucopyranoside], and 3 compounds isolated for the first time from the genus: Camellianin B, Quercetin 3-*O*-β-D-glucopyranoside, Camellianin A. The phytochemical isolation and purification procedures and spectrometric identification will be reported in another paper. As is known, the composition of the various active compounds in the herb varies significantly due to difference in geographic origin, climate condition, environmental or other factors [18,19]. Due to the existence of such differences, the quality of *C. sinensis* and derived medicine preparations also varied greatly. And till now, the analysis of *C. sinensis* by HPLC has not been much explored and no report about fingerprint of *C. sinensis* has been found yet.

The present paper describes a comprehensive HPLC–DAD–MS/MS analysis of *C. sinensis* from various sources. This HPLC–DAD–ESI-MS/MS method enabled the simultaneous identification of the major bioactive constituents present in the leaves of *C. sinensis*, and can form the basis for the successful quality control of this medicinal herb. The correlation coefficients/angle cosines of the entire chromatographic profiles were analyzed among tested samples, and the PCA was performed. The effects of cultivation locations, harvesting time, storage time, drying methods, medicinal portions on chromatographic fingerprints were examined and the major chemical constituents of this plant were identified. Furthermore, by using the fingerprint along with markers, the best harvesting time, cultivation location and medicinal part of this medicinal plant were determined.

2. Experimental

2.1. Apparatus and conditions

An Elite P230 series HPLC–DAD system consisting of a binary pump and a DAD detector (DAD230, Dalian Elite, PR China) was used for acquiring chromatograms and UV spectra. A Kromasil® C18 analytical column (250 mm × 4.6 mm i.d., 5 μm, AKZO Nobel Corporation, Sweden) coupled with a C18 guard column (8 mm × 10 mm i.d., 5 μm, Tianhe Corporation, PR China) was used at room temperature. Data acquisition and processing were performed by WorkDad workstation software (Dalian Elite, PR China). LC–MS was performed with an Agilent 1100 Series HPLC and an Agilent 1100 SL series mass spectrometer (Agilent Technologies Inc., USA). The electrospray interface was set in alternating ionization mode with the capillary voltage at 3500 V and a source of temperature of 325 °C in full scan spectra (100–1000 Da). Nitrogen was used as a drying (7 L/min) and nebulizing gas (25.0 psi). Software versions were 4.0 LC/MSD trap control 4.2 and Data Analysis 2.2 (Agilent Technologies Corporation). The HPLC conditions were the same as above. An ultrasonic cleaner made in Kunshan ultrasonic Instrumentation Factory (Kunshan, Jiangsu, PR China) was used for extraction.

2.2. Reagents and solutions

Acetic acid (analytical grade) was from Beijing Chemical Reagent Company (Beijing, PR China). Methanol and acetonitrile (HPLC grade) were purchased from Fisher Scientific (USA). Deionized water was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA).

All the standard reference chemical compounds were extracted, isolated and purified from *C. sinensis* in our laboratory. Their purities were more than 96% by HPLC analysis, and their structures were elucidated by comparison of their spectroscopic data (ESI-MS/MS, ^{1}H- and ^{13}C NMR, HMBC, HMQC and H–H COSY) with references. Before use, all solutions were filtered through 0.22 μm nylon filters.

2.3. Plant materials

Samples of *C. sinensis* were collected from three cultivation locations (Liangtian, Gegong and Makeng) in Dongzhi, Anhui province, PR China. All the samples were authenticated by Dr. Sheng-yuan Xiao (School of Life Science and Technology, Beijing Institute of Technology, Beijing, PR China) and Associate Researcher Bin Wen (Xishuanbanna Tropical Botanical Garden, Chinese Academy of Sciences, PR China). These included different cultivation locations, different medicinal parts or harvesting time and so on (Table 1).

2.4. Sample preparation

0.5 g fine powder was accurately weighed and extracted with 50 mL of anhydrous methanol in ultrasonic cleaner for 30 min and filtered. The original solvent weight was restored. The extract was filtered through a 0.22 μm membrane filter. An aliquot of 10 μL solution was injected for HPLC analysis.

2.5. Data analysis

The correlation coefficients/angle cosines of entire chromatographic patterns among samples were calculated, and the simulative mean chromatogram was calculated and generated. The similarities of the entire chromatographic profiles were analyzed among tested samples. The principal component analysis (PCA) of Samples 1–27 was performed. All above were imple-

Table 1
Raw herbs used in this work

Sample	Cultivation area	Sample part	Harvesting time	Description
A1	Gegong, Anhui	Leaf	May 2003	Dried
A2	Gegong, Anhui	Leaf	May 2003	Dried
B1	Makeng, Anhui	Leaf	May 2003	Dried
B2	Makeng, Anhui	Leaf	May 2003	Dried
B3	Makeng, Anhui	Leaf	May 2003	Dried
C1	Liangtian, Anhui	Leaf	May 2003	Dried
C2	Liangtian, Anhui	Leaf	May 2003	Dried
C3	Liangtian, Anhui	Leaf	May 2003	Dried
D1	Liangtian, Anhui	Leaf	March 2003	Dried
D2	Liangtian, Anhui	Leaf	March 2003	Dried
D3	Liangtian, Anhui	Leaf	March 2003	Dried
D4	Liangtian, Anhui	Leaf	March 2003	Dried
D5	Liangtian, Anhui	Leaf	March 2003	Dried
E1	Liangtian, Anhui	Twig	May 2003	Dried
E2	Liangtian, Anhui	Twig	May 2003	Dried
F	Liangtian, Anhui	Leaf	March 2005	Fresh
G1	Liangtian, Anhui	Leaf	March 2004	Dried
G2	Liangtian, Anhui	Leaf	March 2004	Dried
H	Liangtian, Anhui	Leaf	March 2005	Dried
I	Liangtian, Anhui	Leaf	April 2005	Dried
J	Liangtian, Anhui	Leaf	May 2005	Dried
K	Liangtian, Anhui	Leaf	June 2005	Dried
L	Liangtian, Anhui	Leaf	July 2005	Dried
M	Liangtian, Anhui	Leaf	August 2005	Dried
N	Liangtian, Anhui	Leaf	September 2005	Dried
O	Liangtian, Anhui	Leaf	October 2005	Dried
P	Liangtian, Anhui	Leaf	November 2005	Dried

A1, A2 denote two bathes of sample A. Others are likewise.

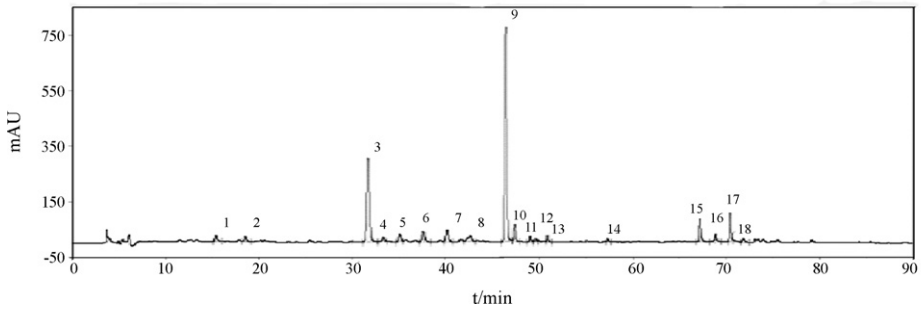

Fig. 2. Representative HPLC fingerprints of *C. sinensis*.

mented by using SPSS software (SPSS for Windows 13.0, SPSS Corporation, USA). Eighteen characteristic peaks in the chromatograms were selected and the peak at retention time 46.3 min was used as a reference. The relative retention and relative peak area (RPA) of each characteristic peak to reference peak were also calculated in the chromatograms.

3. Results and discussion

3.1. Optimization of the extraction method

A good extraction method not only requires complete isolation of active components from the matrix, but also gains comprehensive chemical profile, i.e. the more and the larger the peaks in the chromatograms, the better the extraction method. In this work, the content of Peak 9 (reference peak) as shown in Fig. 2, and the number of peaks were determined by HPLC to evaluate the extraction efficiency. Firstly, refluxing, soaking, and ultrasonic were compared. The result indicated that the highest content of Peak 9 in *C. sinensis* extract had been obtained with ultrasonic method, which also had the most peak number. Moreover, ultrasonic extraction was convenient and rapid. So it was selected as the optimum extraction method in this experiment. Subsequently, the extraction solvent, extraction periods, and solvent volume were studied. The results showed that the content of Peak 9 had highest value and the peak number was the most when the sample was extracted with 50 mL of anhydrous methanol in ultrasonic processor for 30 min. So the optimization of the extraction method was completed.

3.2. Optimization of the chromatographic conditions

Optimization of parameters in HPLC was done through investigating the influence of the mobile phase and detection wavelength, because these two parameters play a key role on resolution and sensitivity.

In this work, a mixture of methanol–acetonitrile (1:1) and water was chosen as the mobile phase. Considering the presence of flavonoids in the herbal extraction, a little amount of acetic acid was added to the mobile phase to reduce the ionization and lower the polarity of these compounds. The optimum mobile phase was achieved with solvent A (H_2O + 0.1% acetic acid) and solvent B (methanol–acetonitrile (1:1)) in the gradient mode as shown in Table 2. The flow-rate was 0.7 mL/min. In order to obtain a large amount of detectable and stronger peaks in the HPLC chromatogram, the spectra of all peaks in the chromatogram of sample C were investigated with photodiode array detection. The programmed wavelength gave the best abundance for target compounds within the chromatographic windows: 0–30 min, 238 nm; 30–55 min, 330 nm; 55–90 min, 238 nm.

Method precision was based on replicated analysis of samples, with reported relative standard deviations (RSDs) of 1.34 and 3.31% for relative retention and RPA of all peaks, respectively. The method reproducibility was studied through five-replicated sample solutions extracted from a single batch of *C. sinensis*. The corresponding RSDs of relative retention and RPA were reported less than 5% over 1 week of investigation. The stability test was performed with a sample solution over 3 days of standing period. The RSDs of the relative retention and RPA were found less than 2.07 and 4.80%, respectively. The result indicated that the developed method was validated and applicable for sample analysis.

3.3. HPLC fingerprints of C. sinensis

According to the definition of fingerprint of TCM, a chromatographic fingerprint is in practice a chromatographic pattern of some common kinds of pharmacologically active and chemically characteristic components in the TCM [20,21]. With the help of chromatographic fingerprints, the authentication and identification of TCM can be accurately conducted. The chromatographic fingerprints can demonstrate both the "sameness" and "differences" between various samples successfully [10,22].

Table 2
Solvent gradients

Time (min)	*A* (%)	*B* (%)
0	88	12
15	75	25
20	75	25
30	70	30
40	65	35
60	30	70
65	10	90
80	0	100
90	0	100

Altogether 27 samples collected from a variety of sources were analyzed. These included different cultivation locations, different medicinal parts or harvesting time and so on.

It is well known that the efficacy of TCM has a characteristic of a complex mixture of chemical compounds present in the herb. The chemical fingerprints should be closely related to the efficacy of herbs. In our general screening for antidiabetic natural substances, samples C collected from Liangtian in May 2003 were found to possess hypoglycemic effect and the activity was associated with the flavonoids in *C. sinensis*. So the simulative mean chromatogram of the tested samples C could be applied as a standard HPLC fingerprint of *C. sinensis.* In general, the raw herbs of *C. sinensis* could be easily identified and assessed by using the reference fingerprint [23]. Among the acquired chromatograms, representative peaks existing in samples C were assigned as characteristic common peaks for *C. sinensis*. As shown in Fig. 2, altogether there were 18 common peaks in the fingerprint. Most of them were flavonoids by HPLC–DAD–ESI-MS/MS analysis. To calculate the relative retention and RPA, a reference substance should be chosen [24]. Peak 9 had a considerably high content of about 9% in our preliminary quantitative analysis, and it indicated the highest content among others. Therefore, it was chosen as the reference peak, and the relative retention, RPA of all common peaks with respect to this reference compound were obtained. The results indicated that chromatographic patterns of 27 samples were generally consistent although the absorption intensities of some peaks were different.

3.4. Similarity of fingerprints

SFDA suggested that all of herbal chromatograms should be evaluated by their similarity, which came from the calculation on the correlative coefficient and/or angle cosine values of original data [25–27]. And the relationship within a set of chromatographic fingerprints can be currently analyzed through comparison in terms of similarity or dissimilarity of the objects with the reference fingerprint. With different mathematic methods including correlation coefficient, angle cosine calculated with the software of SPSS, the data of fingerprints of these samples were processed to analyze similarity among these samples.

The data indicated that the correlation coefficient or angle cosine of each chromatogram to themselves simulative mean chromatogram was about 1.000, and the S.D. value was very low. So the chromatograms of different batches of samples resembled to each other. Based on the similarities of all herbal simulative mean chromatograms to the simulative mean chromatogram of samples C, all the samples could fell into three groups: Group A, Group B and Group C. The similarities of Groups A, B, C were 0.400–0.750, 0.750–0.940 and 0.940–1.000, respectively. Group A consisted of samples K, L, M, N, P and Group B consisted of B, D, E, G, H, I, O. Samples A, C, J, F fell into Group C. However, the chromatogram of E showed drastic differences from that of other Group B samples by direct visual inspection. So in order to distinguish these samples of Group B, the similarity between simulative mean chromatograms were compared.

Table 3
The correlation coefficients among mean chromatograms of E, B, D, G, H, I, O

Sample	B	D	G	H	I	O
E	0.598	0.594	0.577	0.527	0.557	0.657
B		0.763	0.779	0.721	0.720	0.879
D			0.998	0.990	0.984	0.940
G				0.991	0.981	0.948
H					0.979	0.925
I						0.927

Table 4
The angle cosines among mean chromatograms of E, B, D, G, H, I, O

Sample	B	D	G	H	I	O
E	0.730	0.711	0.702	0.654	0.697	0.763
B		0.825	0.837	0.788	0.799	0.913
D			0.999	0.992	0.988	0.955
G				0.992	0.986	0.962
H					0.981	0.941
I						0.947

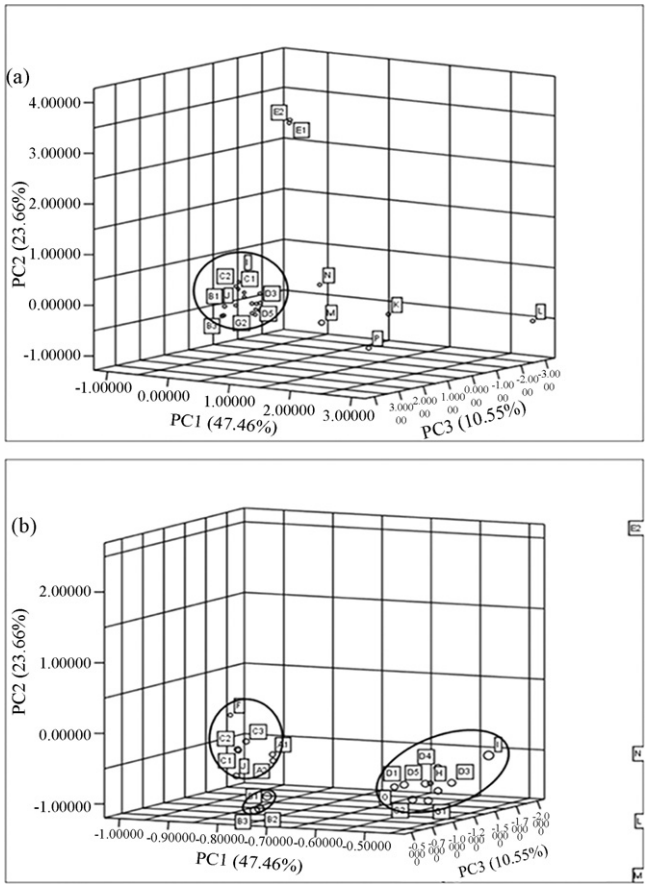

Fig. 3. (a) 3D-projection plots of principal component analysis (PCA) of three principal components for the 27 samples. PC1, PC2 and PC3 are first three principal components using entire chromatographic profile as input data; (b) the amplified 3D-projection plots of principal component analysis (PCA) of three principal components for the 20 samples. PC1, PC2 and PC3 are first three principal components using entire chromatographic profile as input data.

As shown in Tables 3–4, sample E was different from B, D, G, H, I, O. Among the similarities of E mean chromatogram to mean chromatograms of B, D, G, H, I, O, the highest was just 0.657(correlation coefficient)/0.763(angle cosine) to O. The similarity of B mean chromatogram to mean chromatograms of D, G, H, I, O were further analyzed. The highest similarity was just 0.879(correlation coefficient)/ 0.913(angle cosine) to O, so B was also different from D, G, H, O, I. But samples D, G, H, O, I resembled well to each other, and the correlation coefficients/angle cosines between simulative mean chromatograms of D, G, H, I, O were very close and high. So they could fell into one group, though D, G, H were more similar (>0.990).

3.5. PCA

From the projected dots of the 27 chromatograms in the 3D-projection plot of PCA (Fig. 3a), the chromatograms of samples outside E, K, L, M, N, P localized in a confined cluster could be seen. It indicated that the samples E, K, L, M, N, P were different from those in the confined cluster. The samples localized in the confined cluster were further analyzed. It could be visualized in Fig. 3b that three grades of 20 samples were divided: samples A, C, J, F built up one subcluster, and D, G, H, I, O were projected together; samples B were farther apart from the former two subclusters. The samples classified into one cluster or subcluster were associated with similar chemical properties/components. What is more, these results were correspondence with the similarity analysis.

Comparing the similarity analysis with the PCA, PCA could give a more visual comparison of the chromatograms, and the similarity analysis could afford a more quantitative comparison of the samples. In our study, their results were consistent.

3.6. Identification of chemical compounds in fingerprint chromatograms

HPLC–DAD–ESI-MS/MS has been employed to the components analysis in *C. sinensis* raw herbs. In the ESI-MS experiment, the molecular weight of each peak could be obtained. From MS/MS spectra, information on the sequence of sugars and on the aglycone could be provided. In our condition, the results showed that ESI in positive mode was particularly sensitive to the flavonoids, and most of the *m/z* data are $[M+H]^+$. In this ionization mode, flavonoids combined with glucopyranosyl or rhamnopyronosyl would lose the group and indicate characteristic *m/z* data as $[M+H-162]^+$, $[M+H-146]^+$, respectively, from which we can deduce whether the substance has glucopyranosyl or rhamnopyronosyl unit. Moreover, flavonoids combined acetylglucopyranosyl unit, so there would be $[M+H-162-42]^+$ fragments. By comparing the retention time, UV and characteristic ESI-MS/MS spectrum of each standard with those of each peak separated by on-line HPLC–ESI/MS, 8 peaks in *C. sinensis* were unambiguously identified, and 2 peaks were tentatively identified as isomers (Table 5). Otherwise the Peaks 2, 4, 8, 12, 15, 17, 18 were identified as flavonoids because their UV spectra exhibited characteristic UV maximum absorbance of flavonoids, namely, two maximum absorbance bands: band I (300–400 nm), band II (220–280 nm) [28,29].

3.7. Distinguishing C. sinensis from different harvesting time

The quality of herbal medicine is closely related to the concentrations of their chemical constituents. As a result of the growth cycle of herbs, the concentrations of their chemical constituents may change. Therefore, the harvesting time always

Table 5
The on-line detected chromatographic and spectrometric data of 18 characteristic common peaks extracted with methanol in the HPLC fingerprints

Peak	t_R (min)	$[M+H]^+$ (*m/z*)	Other positive ions (*m/z*)	λ_{max} (nm)	Identification
1	15.5	316.9	284.1	280.2	nd
2	18.7	594.9	432.9, 286.9	271.9, 301.8(sh), 326.5	Flavonoid
3	31.7	578.9	432.9, 270.9	262.7, 331.2	Camellianin B
4	33.3	608.8	462.8, 300.9	240.0, 264.3(sh), 337.9	Flavonoid
5	35.0	465.1	302.9	256.8, 286.9(sh), 355.4	Quercetin 3-*O*-β-D-glucopyranoside
6	37.5	433.0	270.9	261.8, 335.4	Apigenin 7-*O*-β-D-glucopyranoside
7	40.1	620.8	474.8, 270.9	263.5, 331.2	Isomer of peak 9
8	42.5	679.0	532.8, 286.9	266.9, 339.4	Flavonoid
9	46.3	620.8	474.8, 270.9	262.7, 331.2	Camellianin A
10	47.3	650.9	504.9, 301.0	240.0, 265.0(sh), 339.8	Chrysoeriol 5-*O*-[α-L-rhamnopyranosyl-(1 → 4)-β-D-6-*O*-acetylglucopyranoside]
11	48.9	663.0	516.8, 271.0	261.8, 329.6	Apigenin 5-*O*-[α-L-rhamnopyranosyl-(1 → 2)-β-D-3,6-di-*O*-acetylglucopyranoside]
12	49.5	475.2	271.0	261.0, 336.2	Flavonoid
13	50.8	663.0	516.8, 270.9	263.5, 331.3	Isomer of peak 11
14	57.2	271.6	270.9	269.4, 338.7	Apigenin
15	67.2	553.3	417.2, 391.2	269.4, 333.8	Flavonoid
16	68.9	567.4	567.2	269.4, 333.8	Ginkgotin
17	70.4	569.3	415.1, 363.2, 181.0	274.4, 333.8	Flavonoid
18	71.9	554.6	–	271.0, 333.7	Flavonoid

nd: unidentified. sh: shoulder peak.

plays an important impact on the pharmacological activities of herbs. Raw herbs of *C. sinensis* harvested in March, April, May, June, July, August, September, October, November 2005, at the same cultivation location were analyzed. The harvesting details and similarities of the chromatograms to samples C mean chromatogram are shown in Fig. 4a. The results showed that the similarities were distributed almost symmetrically and revealed two growth cycles. The highest similarity sample was those harvested in May, so it could be decided that the best harvesting time was May. It was very interesting that the sample in May also had the highest content of Peak 9 as shown in Fig. 4b.

3.8. Analyzing fingerprints of C. sinensis from various cultivation locations

The chemical constituents of herbs may vary depending on cultivation location. The HPLC chromatograms of *C. sinensis* from various cultivation locations are shown in Fig. 5a. The similarites of chromatograms of *C. sinensis* from Gegong, Makeng to liangtian mean chromatogram were 0.996, 0.847 (correlation coefficient)/0.997, 0.880(angle cosine), respectively, so the samples collected from Liangtian, Gegong had very high similarity between chromatograms. The PCA also gave the same results. Moreover, the content (peak area/sample weight, mAUs/mg) of Peak 9 (reference peak) in sample A (Gegong, May 2003), C (liangtian, May 2003) were almost identical. So the cultivation location could be determined to be Liangtian or Gegong.

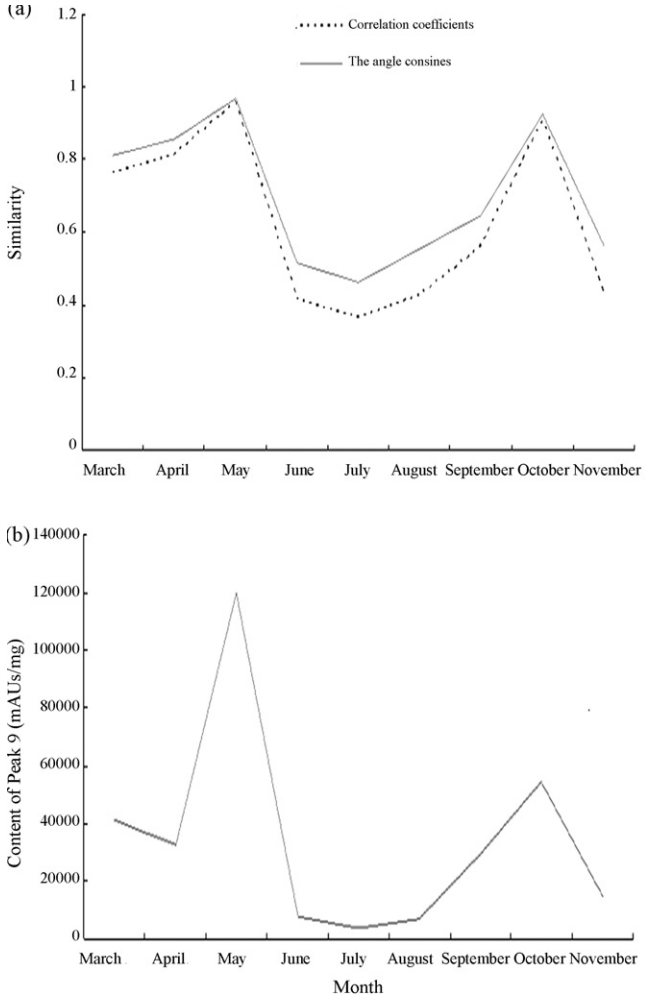

Fig. 4. The similarities of *C. sinensis* harvested in different time. (a) Correlation coefficients and the angle cosines of samples harvested in different time to the simulative mean chromatogram of the tested C samples; (b) the content of compound 9 in different harvesting time.

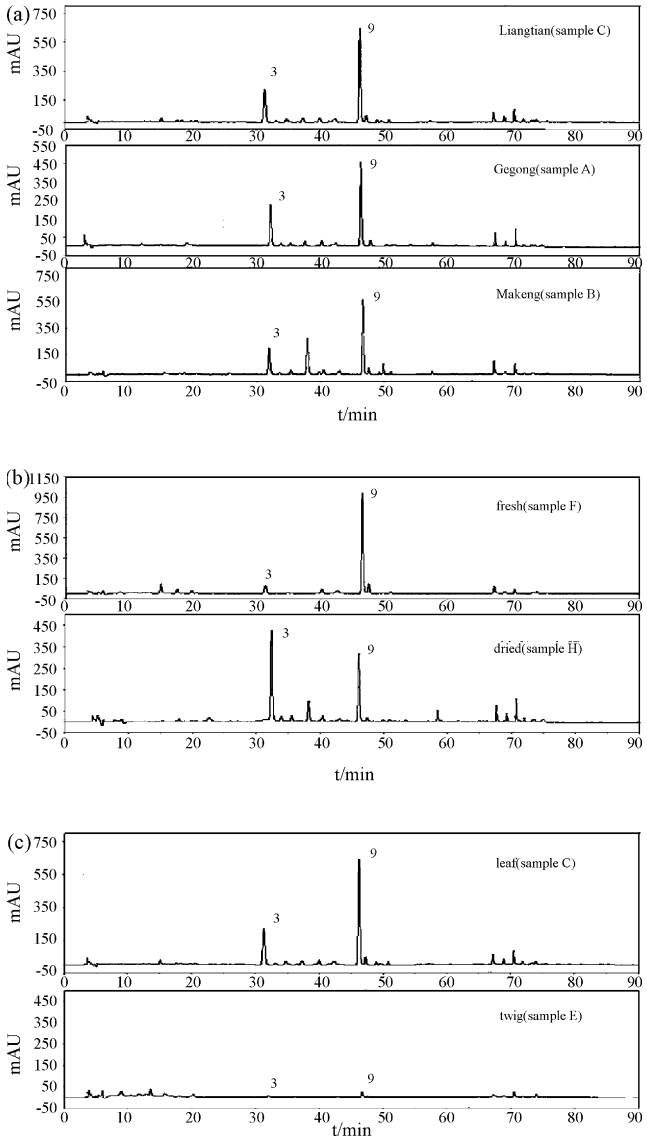

Fig. 5. Representative HPLC chromatograms of (a) *C. sinensis* leaves from various cultivation locations; (b) fresh and dried *C. sinensis* leaves; (c) various medicinal parts of *C. sinensis*.

3.9. Distinguish between fresh and dried C. sinensis

In general, dried raw herbs are preferentially used as medicinal materials because they are convenient for storage and use. However, the process of dryness can easily influence the components and their proportions. The fresh and dried samples of *C. sinensis* collected from the same cultivation location and in the same harvesting time were analyzed. The results revealed that the components obviously changed after dryness as shown in

Fig. 5b. The content (mAUs/mg) of Peak 3 increased by almost four fold, while Peak 9 decreased by about 75%. It was very obvious to visually differentiate the chromatograms. And the similarities between fresh and dried samples were 0.574 (correlation coefficient)/0.637 (angle cosine). In pharmacological experiment, samples C were found to possess biological activity, which were dried. So the influence of dryness on biological activity is needed to further research.

The variety in chromatogram similarities of herbs with the difference of harvesting time, cultivation locations and dryness provided a picture that the herbal chemical components were affected by other factors. The identification of compounds revealed that maybe there were dynamic transformations among the compounds, because Peaks 3, 9 had the same nucleus moiety (apigenin, i.e. Peak 14).

3.10. Comparative fingerprints among various medicinal parts of C. sinensis

It has been well known that the different medicinal parts of raw herbs usually are used for different curative purpose. It is therefore important to learn about any difference in composition among various medicinal parts of *C. sinensis*. The similarities between the chromatograms of leaf and twig showed drastic differences: 0.782(correlation coefficient)/0.826 (angle cosine). In the PCA score plot, samples E (twigs) were far apart from other samples (leaves). Although the chromatogram of twig had the similar peaks as that of leaf, the intensities of the peaks were generally very low, and the peaks after 65 min were relatively intense than other peaks as shown in Fig. 5c. The content (mAUs/mg) of Peaks 3 and 9 in twig sample were less than those in leaf sample by about 20-fold. The data indicated that the content of chemical constituents in twig were lower than those in leaf, so the leaf was better for the medicinal part of *C. sinensis* than twig.

3.11. The effects on herbs of storage time

The similarities among samples D, G, H, collected from the same cultivation location but in March 2003, 2004, 2005, respectively, were very close. PCA also gave the same result. Moreover, the content (mAUs/mg) of Peak 9 in these samples were almost identical. These samples were stored for 3, 2, 1 year, respectively. It proved that the raw materials could be stored stably for 3 years in our circumstances.

3.12. The correlation between the chromatographic fingerprints and efficacy of the herbal medicines

In order to develop the herbal medicine product of *C. sinensis,* the manufacture process was constructed. The HPLC chromatograms of raw herb and its medicinal preparation were shown in Fig. 6. The main common peaks of 13–60 min in medicinal preparation fingerprint could be found in its raw herbal fingerprint. Nevertheless, the common peaks 15, 16, 17 and 18 (after 65 min) in raw herbal fingerprint were not found in the preparation fingerprint. But the herbal medicinal preparation possessed the hypoglycemic effect in STZ-induced diabetic rats

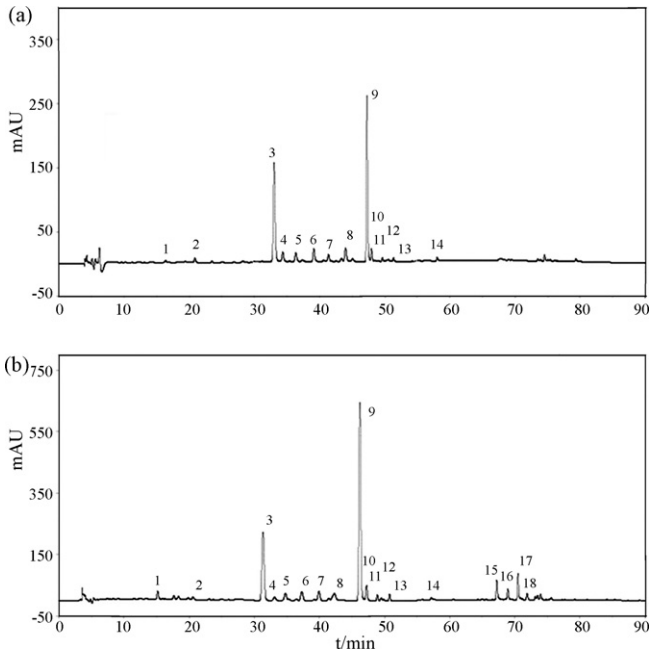

Fig. 6. HPLC chromatograms of medicinal preparation and raw herb of *C. sinensis*: (a) medicinal preparation; (b) raw herb.

like the raw herb in pharmacological experiments. So it was conjectured that maybe the common peaks of 13–60 min were hypoglycemic active constituents, although further investigation is required.

4. Conclusion

The developed chromatographic fingerprint by the similarity analysis or PCA along with markers could differentiate *C. sinensis* from various sources. This investigation also revealed that the secondary metabolites of *C. sinensis* were affected by harvesting time, cultivation location, drying methods, medicinal parts, and in order to get the consistent raw materials of *C. sinensis*, the factors above should be fixed. Using the reference fingerprint along with markers, the harvesting time, cultivation location and medicinal part of raw materials of *C. sinensis* were determined to be May, Liangtian/Gegong, and leaf, respectively. Otherwise, in our study, the similarity analysis and PCA gave the same results. But the similarity analysis could afford a more quantitative comparison of the samples, while the PCA could give a more visual comparison of the chromatograms.

The main common peaks in *C. sinensis* preparation fingerprint could be found in its raw herbal fingerprint, showing there is a sufficient correlation between them. Of course, the further investigation on the relationship between the chromatographic fingerprint and efficacy of the preparation is required. But the fingerprint actually can afford us a quality control method of herbs or herbal preparations which can ensure the consistency of them. Ten compounds involving 2 new compounds were for the first time identified in *C. sinensis* by using HPLC–DAD–ESI-MS/MS techniques. The analysis indicated that the major principles contained in *C. sinensis* were flavonoids and formed the basis for the successful quality control of this

medicinal herb. The results demonstrate that this developed method is feasible for comprehensive quality evaluation of *C. sinensis.*

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