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Fingerprint quality control of Tianjihuang by high-performance liquid chromatography–photodiode array detection

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Abstract

A novel, simple and accurate fingerprint method was developed using high-performance liquid chromatography–photodiode array detection (HPLC–DAD) for the quality control of *Hypericum japonicum* thunb (Tianjihuang), a Chinese herbal medicine used for the treatment of several bacterial diseases, infectious hepatitis, gastrointestinal disorder, internal hemorrhage and tumors. For the first time, the feasibility and advantages of employing chromatographic fingerprint were investigated and demonstrated for the evaluation of Tianjihuang by systematically comparing chromatograms with a professional analytical software recommended by State Food and Drug Administration (SFDA). Our results revealed that the chromatographic fingerprint combining similarity evaluation could efficiently identify and distinguish raw herbs of Tianjihuang from different sources. The effects resulted from collecting locations, harvesting time and storage time on herbal chromatographic fingerprints were also examined.
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Keywords: Quality control; Chromatographic fingerprint; *Hypericum japonicum* Thunb (Tianjihuang); HPLC–DAD; Herbal medicines

1. Introduction

It is well known that the therapeutic effect of the herbal medicine is based on the synergic effect of its mass constituents, which is different from that of western medicines [1–6]. However, a few markers or pharmacologically active constituents are generally employed to assess the quality and authenticity of the complex herbal medicine or preparations in traditional standards [7]. Unfortunately, those markers and/or pharmacologically active constituents are hardly demonstrated to stand for the complex herbal medicine or preparations. So the traditional quality control of herbal medicines has a number of very severe challenges. In the standardization of herbal medicines, a direct quantification of the naturally occurring active constituents is a desirable criterion. Often it would be impossible because most reference compounds are not commercially available, in addition, most of herbal medicines are so complex that the analysis during pharmaceutical quality control is rather tedious [4]. For example, EGb761, developed in the laboratories of Schwabe in Karlsruhe, was finally standardized on the basis of the presence of 24% (w/w) "*Ginkgo* flavone" glycosides and 6% (w/w) terpene lactones. In fact, it was a depressed standard contrasting the complex production of EGb761 [4,5]. The *Ginkgo* extracts from other manufacturers could easily accord with the above-mentioned standard, but they were utterly not EGb761 [4]. Xie has reported that the standard of EGb761 could not veraciously distinguish it from some preparations of *Ginkgo* extracts with the accession of rutin [6]. It is absolutely necessary to develop new analytic methods for quality control of herbal medicines [8]. Among all quality control systems, chromatographic fingerprint has gained more and more attention recently [6,8–11].

Both Food and Drug Administration (FDA) [2] and European Medicines Agency (EMEA) [3] clearly denoted that the appropriate fingerprint chromatogram should be applied to assess the consistency of the botanical drugs. In 2004,

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SFDA also required that all the injections made from herbal medicines or their raw materials should be standardized by chromatographic fingerprint [12]. Chromatographic fingerprint, as a comprehensive quantifiable identification method to show chemical information of herbal medicines with chromatogram, spectrograms and other graphs by chemically analytical techniques [6,8], could show not only naturally occurring active constituents but also the chemically characteristic ratios of them. It is very important for herbal medicines because the different concentration proportion of mass constituents may represent different therapeutic effect. So the chromatographic fingerprint would be more valid and efficient than the traditional methods in quality control of herbal medicines. However, the valid fingerprint method has not been accepted in general quality standards of herbal medicines until now. One of the main difficulties is the shortage of an analytical method of scientifically evaluating the complex chromatograms of herbal medicines. Sticher also reported it [4]. In order to resolve the problem, SFDA suggested that all of herbal chromatograms should be evaluated by their similarities, which come from the calculation on the correlative coefficient and/or cosine value of vectorial angel of original data [13–15]. Now the similarity evaluation system has been used in many academies and universities in China, which offers us an excellent ruler to study on chromatographic fingerprint.

Table 1
Raw materials used in this work

Sample no.	Origins	Harvesting time	Length (cm, approx.)
01	Guilin, Guangxi	August 2002	30
02	Guipin, Guangxi	August 2002	40
03	Wuzhou, Guangxi	July 2002	35
04	Baise, Guangxi	June 2002	30
05	Guangxi (from market)	–	30
06	Liujiang, Guangxi	September 2002	25
07	Yizhou, Guangxi	September 2002	60
08	Laibin, Guangxi	August 2002	37
09	Nanning, Guangxi	January 2003	15
10	Yulin, Guangxi	July 2002	30
11	Du'an, Guangxi	June 2002	40
12	Nanning, Guangxi	July 2002	36
13	Guangxi (from market)	–	25
14	Guangxi (from market)	–	28
15	Guangxi (from market)	–	27
16	Qinzhou, Guangxi	February 2003	20
17	Chenzhou, Hu'nan	March 2003	30
18	Kunming, Yunnan	March 2003	30
19	Mengzi, Yunnan	March 2003	15
20	Huadu. Guangdong	April 2003	15
21	Huadu. Guangdong	May 2003	20
22	Huadu. Guangdong	June 2003	25
23	Huadu. Guangdong	July 2003	25
24	Huadu. Guangdong	August 2003	25
25	Mengzi, Yunnan	July 2003	18
26	Changde, Hu'nan	July 2003	30
27	Shuangfeng, Hu'nan	July 2003	30
28	Wanan, Jiangxi	July 2003	40
29	Shaoyang, Hu'nan	July 2003	30
30	Chenzhou, Hu'nan	August 2003	28
31	Qinzhou, Guangxi	July 2002	18
32	Qinzhou, Guangxi	July 2003	22
33	Nanning, Guangxi	July 2003	28
34	Wuzhou, Guangxi	August 2003	25
35	Tengxian, Guangxi	August 2003	30
36	Dongguan, Guangdong	August 2003	30
37	Zhanjiang, Guangdong	August 2003	30
38	Shaoguan, Guangdong	August 2003	22
39	Jieyang, Guangdong	August 2003	15
40	Longyan, Fujian	August 2003	28
41	Dongguan, Guangdong	September 2003	20
42	Huadu, Guangdong	October 2003	30
43	Huizhou, Guangdong	September 2003	25
44	Wenzhou, Guangdong	September 2003	30
45	Wuzhou, Guangxi	April 2004	30
46	Wuzhou, Guangxi	May 2004	25
47	Wuzhou, Guangxi	May 2004	30
48	Wuzhou, Guangxi	June 2004	35
49	Wuzhou, Guangxi	June 2004	35
50	Wuzhou, Guangxi	July 2004	30
51	Wuzhou, Guangxi	July 2004	25
52	Wuzhou, Guangxi	August 2004	30
53	Wuzhou, Guangxi	August 2004	35
54	Wuzhou, Guangxi	September 2004	35
55	Wuzhou, Guangxi	September 2004	25
56	Wuzhou, Guangxi	October 2004	30

Table 2
The tried mobile phases in optimization of HPLC conditions

Systems	Gradients
Methanol (M) and water (W)	(1) 5–80% M and 95–20% W in 60 min
	(2) 5–80% M and 95–20% W in 80 min
	(3) 5–60% M and 95–40% W in 80 min
	(4) 4–60% M and 96–40% W in 100 min
Methanol (M) and buffer solution (B, water–KH_2PO_4–H_3PO_4, pH 3.0)	(1) 4–60% M and 96–40% B in 100 min
	(2) 4–50% M and 96–50% B in 120 min
Acetonitrile (A) and water (W)	(1) 4–60% A and 96–40% W in 100 min
	(2) 4–60% A and 96–40% W in 60 min
	(3) 4–30% A and 96–70% W in 100 min
	(4) 4–27% A and 96–73% W in 100 min
Acetonitrile (A) and buffer solution (B, water–KH_2PO_4–H_3PO_4, pH 3.0)	(1) 4–27% A and 96–73% B in 100 min
	(2) 4–35% A and 96–65% W in 80 min
	(3) 4–20% A and 96–80% W in 120 min

Table 3
Factors and levels for the optimization of extraction conditions

Factors	Levels		
	1	2	3
A: solvent volume (ml)	10	20	30
B: ethanol concentration (%)	40	60	80
C: extraction times	1	2	3
D: sonication time (min)	5	10	15

Table 4
The results and analysis of orthogonal design

Run no.	A: solvent volume (ml)	B: ethanol concentration (%)	C: extraction times (times)	D: sonication time (min)	A_{5p}[a]
1	10	40	1	5	316
2	10	60	2	10	625
3	10	80	3	15	625
4	20	40	2	15	502
5	20	60	3	5	544
6	20	80	1	10	593
7	30	40	3	10	621
8	30	60	1	15	617
9	30	80	2	5	644
K_1	1567	1439	1525	1505	
K_2	1637	1786	1774	1837	
K_3	1882	1861	1790	1744	
k_1	522	480	508	502	
k_2	546	595	591	612	
k_3	627	620	596	583	
Range	105	141	88	111	
Optimized scheme	A3	B3	C2	D2	
Primary and secondary order	3	1	4	2	

[a] A_{5p} represents the area sum of 5 peaks.

Hypericum japonicum thunb (Tianjihuang) is a Chinese herbal medicine used for the treatment of bacterial diseases, infectious hepatitis, gastrointestinal disorder, internal hemorrhage and tumors [16–20]. It is a prolific producer of secondary metablites such as phloroglucinol derivatives, flavonoids (including quercitrin, isoquercitrin and taxifolin-7-*O*-rhamnoside), lactones, xanthonoids, chromone glycosides and peptides [21]. Some of these constituents are known to exhibit pharmacological and biological activities. For example, quercitrin and isoquercitrin showed anticoagulation of activated partial thromboplastin time (APTT) reagent and taxifolin-7-*O*-rhamnoside showed promoting coagulation of APTT [20]. The herb grows wildly in the warm and humid southern China. Because of the complicated terrains and diverse climates in China, its secondary metabolites may vary greatly with the circumstances. Along with more and more extensive application of Tianjihuang, it is absolutely necessary and urgent to develop a novel quality standard to validly control its quality.

The thin-layer chromatography (TLC) was ever used in developing chromatographic fingerprint in our laboratory, but its precision was poor. In the present study, we used high-performance liquid chromatography–photodiode array detection (HPLC–DAD) to develop a simple, rapid and valid chromatographic fingerprint method for the qualitative analysis of Tianjihuang from various areas. The effects resulted from collecting locations, harvesting time and storage time on herbal chromatographic fingerprints were also examined in this study.

Table 5
The similarities of 44 chromatograms

No.	Similarities[a]	No.	Similarities[a]	No.	Similarities[a]	No.	Similarities[a]
01	0.90	15	0.97	29	0.94	43	0.66
02	0.96	16	0.92	30	0.97	44	0.92
03	0.96	17	0.97	31	0.94	45	0.94
04	0.94	18	0.95	32	0.96	46	0.84
05	0.96	19	0.25	33	0.97	47	0.94
06	0.92	20	0.83	34	0.97	48	0.96
07	0.91	21	0.64	35	0.96	49	0.90
08	0.95	22	0.71	36	0.64	50	0.91
09	0.91	23	0.66	37	0.97	51	0.85
10	0.93	24	0.55	38	0.90	52	0.89
11	0.93	25	0.44	39	0.90	53	0.94
12	0.97	26	0.97	40	0.97	54	0.95
13	0.98	27	0.94	41	0.70	55	0.84
14	0.93	28	0.94	42	0.70	56	0.88

[a] The reference fingerprint was developed with the median of all chromatograms.

2. Experimental

2.1. Instrumentation and reagents

A Dionex® HPLC systems (Dionex Corporation, USA) was used which including a quaternary pump 680, an autosampler ASI-100, an injector with a 200 μl loop, a column oven STH 585, a photodiode array detector PDA-100 and a data system (Chromeleon® version 6.40); An ultrasonic cleaner (T660/H, Elma, Germany) was used for extraction. Reverse osmosis water (18 MΩ, Simplicity 185, Millipore, France) was used for all the solutions and dilutions. The vacuum concentrator system consisted of a rotary evaporator, a cool ice and a digital bath (EYELA, Japan). Acetonitrile and methanol were HPLC grade. Standards of quercitrin and isoquercitrin were purchased from Fluka (Allentown, USA). Taxifolin-7-*O*-rhamnoside was purified and identified by Dinghong Wu in our laboratory.

2.2. Materials

Fifty-six raw herbs of Tianjihuang from six provinces of China were investigated and collected (Table 1). All of them were identified by Professor Wen-bo Liao (Sun Yat-sen University, China) according to morphological characteristics. The samples were stored in Guangzhou Quality R&D Center of Traditional Chinese Medicine, Sun Yat-sen University, China.

2.3. Sample preparation

A 2.0-g powder of dried materials was extracted with 20.0 ml ethanol–water (6:4, v/v) solution in an ultrasonic water bath for 10 min. This extraction was repeated three times. The extracted solution was mixed and filtrated through analytical filter papers. The filtered solution was evaporated at 45 °C to dryness by vacuum. The dry extract was dissolved in 10.0 ml methanol-water (50:50, v/v) and suspended particles were then filtrated through a 0.45-μm-membrane filter. Each sample was prepared with the above protocol for HPLC analysis.

2.4. HPLC procedures

The column was a reversed-phase column (LiChrospher 100 RP18e, 5 μm, 250 mm × 4.0 mm I.D. Merck, German). Separation was performed by linear gradient elution using acetonitrile (4–27% in 100 min) and buffer solution (water–KH_2PO_4–H_3PO_4, pH 3.0, 96–73% in 100 min). The flowing rate was 1.0 ml/min. The detection wavelength and column temperature were set at 300 nm and 28 °C, respectively. The loading volume was 10 μl.

2.5. Data analysis

Data analysis was performed by a professional software named Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004 A), which was recommended by SFDA, used for evaluating similarities of different chromatograms by calculating the correlative coefficient and/or cosine value of vectorial angel [13–15]. In this article, all of results were calculated by the correlative coefficient.

3. Results

3.1. Optimization of HPLC conditions

To develop a fingerprint for Tianjihuang, an optimized strategy for HPLC conditions was performed. In order to obtain good resolution, the tried mobile phase systems were shown in Table 2. Both systems with methanol had longer duration of analysis than those with acetonitrile. The acetonitrile–water system had the same analytical time as the acetonitrile–buffer solution system, but the former had a poor resolution. So the acetonitrile (4–27% in 100 min)–buffer solution (water–KH_2PO_4–H_3PO_4, pH 3.0, 96–73% in 100 min)

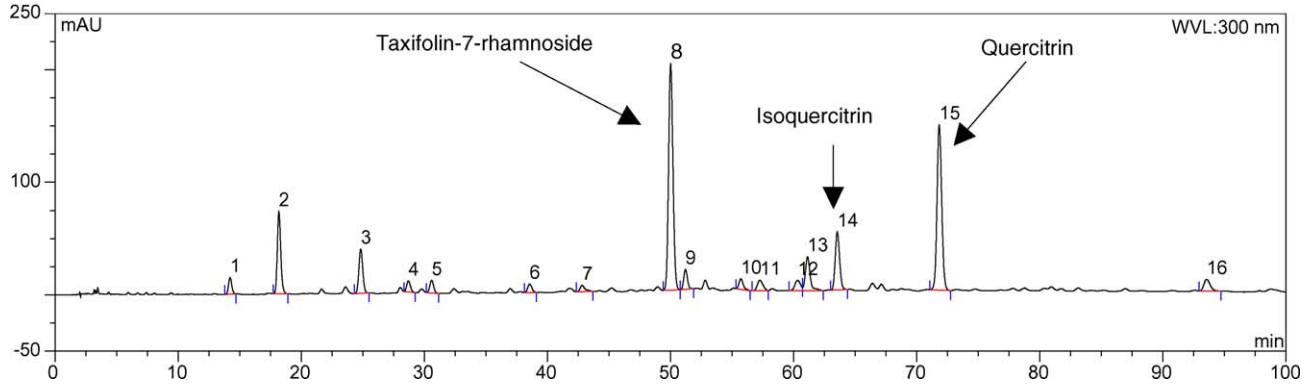

Fig. 1. The reference fingerprint of Tianjihuang. HPLC conditions: column: LiChrospher 100 RP18e, 5 μm, 250 mm × 4.0 mm I.D. Merck, Germany; the mobile phases: acetonitrile (4–27% in 100 min) and buffer solution (water–KH_2PO_4–H_3PO_4, pH 3.0, 96–73% in 100 min); flowing rate: 1.0 ml/min; detection: 300 nm; temperature: 28 °C; injection: 10 μl.

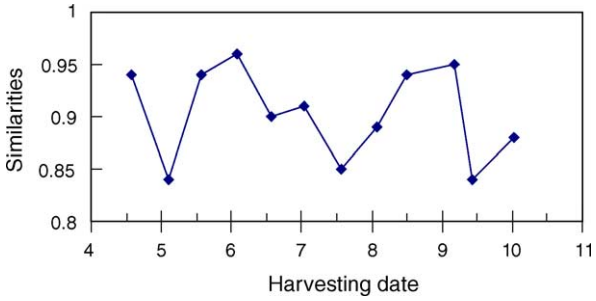

Fig. 2. The similarities of Tianjihuang harvested in different time.

system was chosen for its well baseline resolution and suitable duration for analysis. The linear gradient was applied in order to ensure the good repeatability without reducing their resolutions. In order to obtain a sufficiently large number of detectable peaks on the HPLC chromatogram, the spectra of all main peaks were investigated (data not shown) and 300 nm was selected as detection wavelength.

3.2. Optimization of extraction conditions

An orthogonal experiment was employed in order to optimize the extraction conditions. It involved four factors: (A)

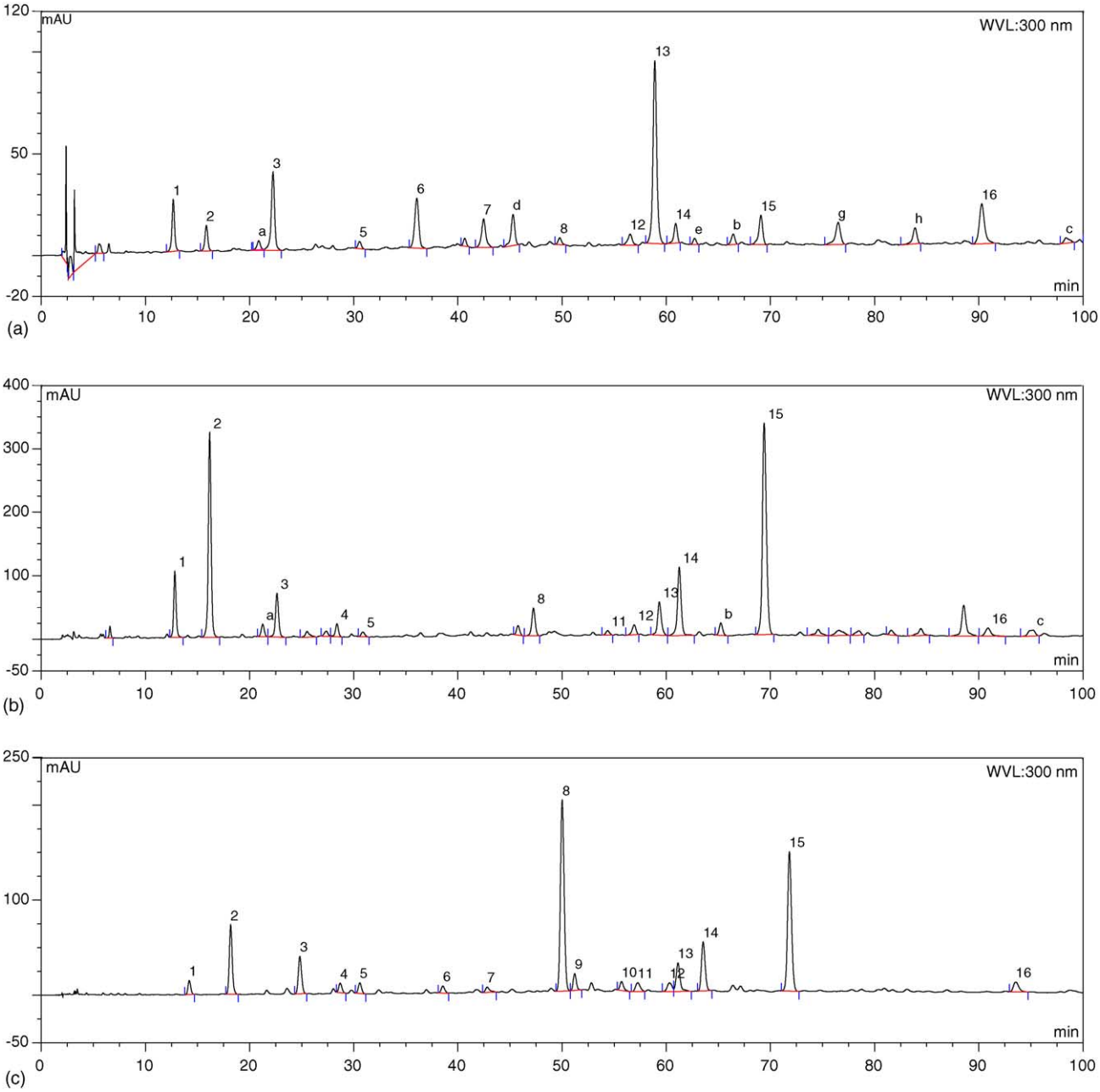

Fig. 3. Representative chromatographic fingerprints of Tianjihuang: (a) Group A; (b) Group B; (c) Group C. HPLC conditions as Fig. 1.

Table 6
The similarities of 10 samples in 1-year storage

	No.									
	02	03	04	05	06	08	10	12	18	19
Similarities										
0 Year	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.000
1 Year	0.99	0.99	1.00	1.00	1.00	1.00	1.00	1.00	0.99	0.95

solvent volume; (B) proportion between ethanol and water; (C) extraction time; (D) sonication time. The experimental factors and corresponding levels were shown in Table 3, and orthogonal designs L_9 (3^4) were presented in Table 4. The optimal condition for extraction of Tianjihuang could be obtained by intuitionistic analysis of the experimental results of the orthogonal design. In order to fully show the quality of an herb, the more relative intensities of all peaks the better in an herbal fingerprint. So the relative sum area of the five biggest peaks, which was more than 90% of the area of all peaks, was used as a criterion for the selection of the optimal sonication conditions. According to statistic analysis theory, the biggest range of the four factors was 141 of substance B; the smallest was 88 of substance C. It means that the substance B was the most important factor in the extract conditions of Tianjihuang, which changed a little, the sum area would change greatly. Optimized factors' ordering was obtained according to range analysis (Table 3). The optimal condition was presented in detail in Section 2.3.

3.3. Standardization of chromatographic fingerprint of Tianjihuang

The reference fingerprint must be representative of the authentic Tianjihuang. In the present study, 56 raw herbs from various locations, including almost all of growing sites of Tianjihuang, ensured that the reference fingerprint we had developed was representative and authentic. No. 13 was selected as the reference fingerprint, for it had the biggest similarity in 56 raw herbs (Table 5). Sixteen peaks were shown in the chromatogram of No. 13 (Fig. 1). Based on their UV spectra, migration, standard addition and LC–MS (data not shown), peak 8, peak 14 and peak 15 were identified as taxifolin-7-*O*-rhamnoside, isoquercitrin and quercitrin, respectively.

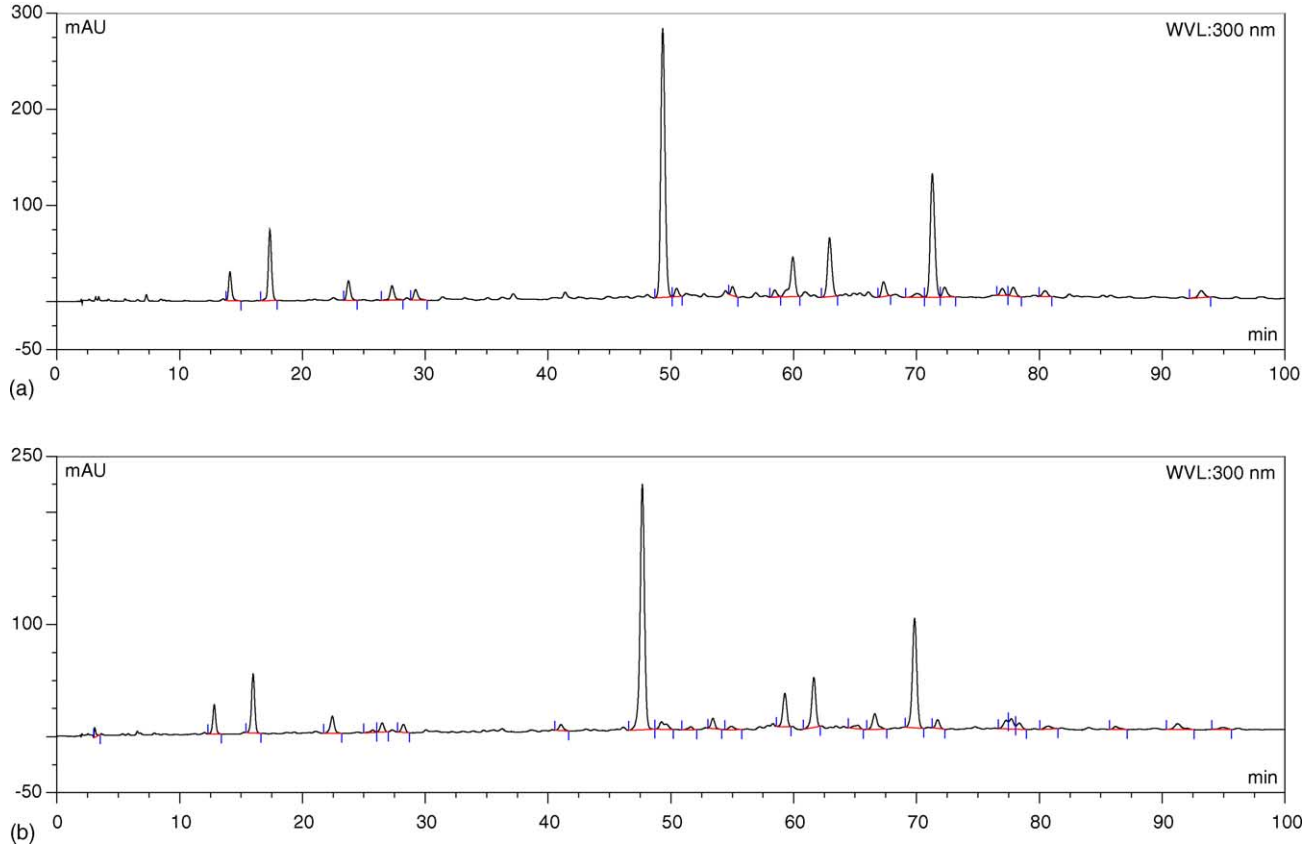

Fig. 4. Chromatographic fingerprints of Tianjihuang stored for zero year (a) and stored for a year (b). HPLC conditions as Fig. 1.

In general, using the reference fingerprint, we can easily identifying and assessing raw herbs of Tianjihuang.

3.4. The effects on herbs of harvesting time

Twelve raw herbs of Tianjihuang were harvested every 15 days in April, May, June, July, August, September, October 2004, at the same location, Wuzhou, Guangxi, China. The harvesting details and similarities of the chromatograms were shown in Fig. 2. The results were very interesting that the similarities were distributed symmetrically and the time between two troughs may be the growth cycle of Tianjihuang. The results clearly showed two growth cycles. The first growth cycle was two and a half months and the second was 2 months. The regular changes of secondary metabolites provided us guidelines for harvesting the herb. From Fig. 2, the best herbs of Tianjihuang were harvested in the last 15 days of May and the first 15 days of Jun or in the last 15 days of August.

3.5. The effects on herbs of various locations

According to Sections 2.3 and 2.4, 56 samples were extracted and the extracted solutions were injected into HPLC system. The 56 obtained chromatograms were compared with the software presented in Section 2.5. Based on the similarity values of all herbal chromatograms (Table 5), it is interesting to note that all the samples fell into three groups: Group A, Group B and Group C. The similarities of Groups A, B, C were 0.2–0.5, 0.5–0.84 and 0.84–1.0, respectively. Group A consisted of No. 19 and No. 25 collected from Mengzi, Yunnan and their representative fingerprint was shown in Fig. 3a. Group B consisted of nine herbs collected from the east of Guangdong and their representative fingerprint was shown in Fig. 3b. Group C consisted of 45 samples collected from other locations and their representative fingerprint was shown in Fig. 3c. The important finding was that the similarities of herbs were very relative to their collecting locations (Tables 1 and 5). The secondary metabolites of Tianjihuang would vary greatly in different locations. Moreover, the effects brought from collecting locations were more visible than those from harvesting time, for the 12 samples from different harvesting time had been assembled to the same Group C.

3.6. The effects on herbs of storage time

In order to assess their storage stability, 10 samples were stored for a year in a cool, dry and good ventilation place. The comparison results of the 10 samples showed that the similarities between each two samples changed slightly (Table 6). It proved that the raw materials could be stored stably for a year in the above circumstances (Fig. 4).

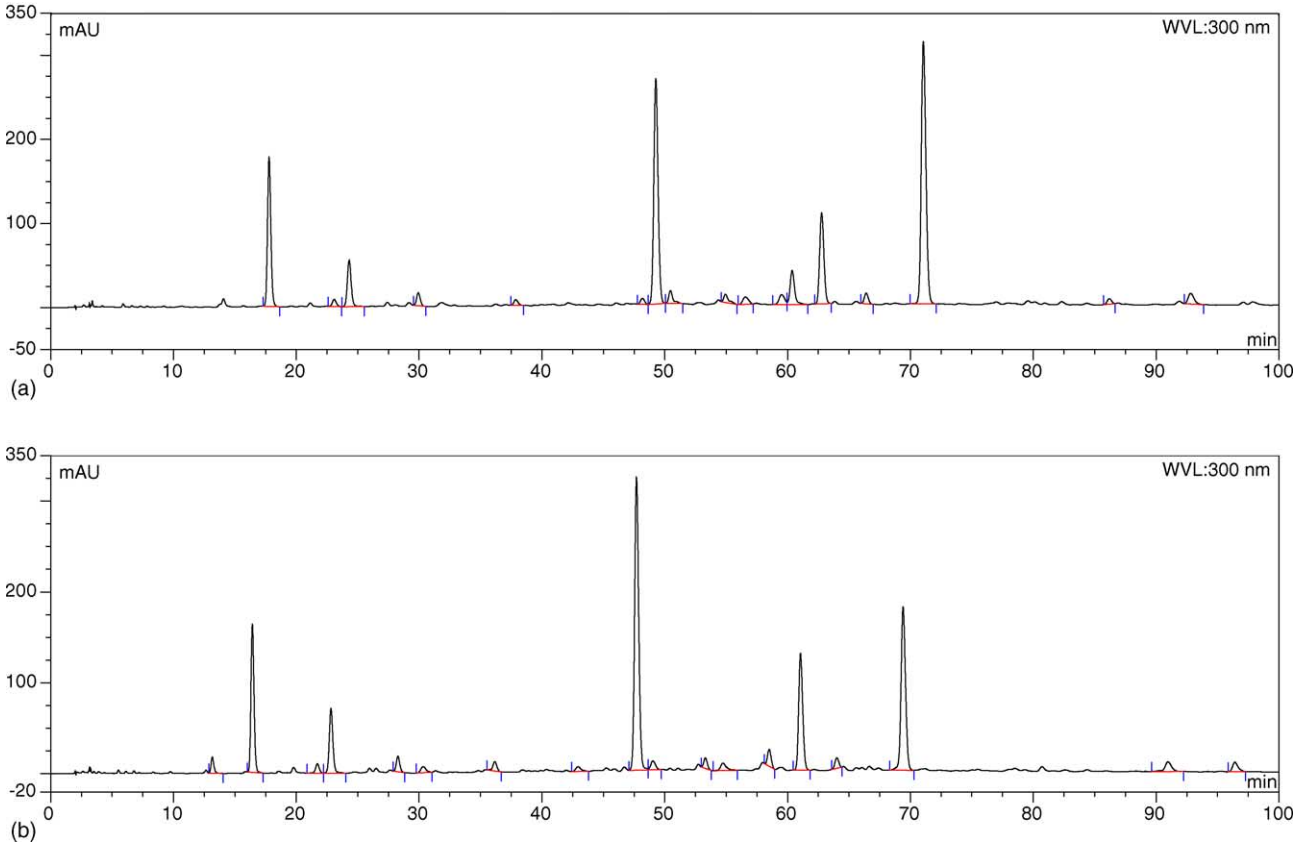

Fig. 5. Chromatographic fingerprints of Tianjihuang harvested in 2002 (a) and 2003 (b) from Qinzhou, the same site. HPLC conditions as Fig. 1.

3.7. The comparison of successively collected samples from the same site

In order to assess the quality of samples in different years from the same location, four sites were investigated, where consecutive collections of Tianjihuang were done. There was little difference between samples collected in 2003 and those in 2002 at the same site (Fig. 5). This showed that the secondary metabolites of herbs in the same site varied a little in different years.

4. Conclusion

Chromatographic fingerprint analysis in plant extracts or herbal medicines have been reported in many papers recently [8,22–25]. Unfortunately, all of the analysis methods for fingerprints in those papers were so tedious that they were impossible to be accepted in general quality standards of herbal medicines. In the present work, an impersonal, valid and rapid chromatographic method and a new fingerprint analysis method was developed and applied. Fifty-six herbs of Tianjihuang were identified and distinguished. According to their similarities, those herbs were assorted to three groups. The taxonomy based on similarities had a fair consistency with the authentic chromatographic profiles (see Fig. 3). In a word, our results have demonstrated that the chromatographic fingerprint combined with the similarity analysis may be accepted in general quality standards of herbal medicines in the recent future.

Comparing the quantification of a few markers or pharmacologically active constituents, the chromatographic fingerprint has more predominance in showing the authenticity of an herb. For example, only peak 14 (isoquercitrin) and/or peak 15 (quercitrin) were quantified, Group B and Group C would not be distinguished distinctly. The purified taxifolin-7-*O*-rhamnoside (peak 8, Fig. 3) is unstable and easily oxidized, so its quantification is very difficult. Another advantage of using chromatographic profiles for quality assessment of herbal products is that it is often unnecessary to know the individual components that make up the fingerprint [26].

The most important findings were that the most relevant factor on secondary metabolites of Tianjihuang was the collecting location and then was the harvesting time. So in order to get the consistent raw materials of Tianjihuang, the collecting location should be fixed and then the harvesting stage.

Acknowledgements

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