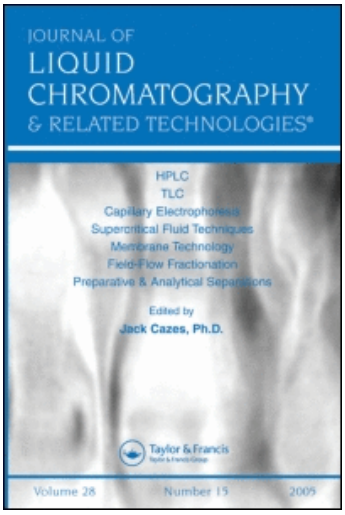

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A Simple Thin-Layer Chromatographic Fingerprint Method for Distinguishing Between Radix Paeoniae Rubra and Radix Paeoniae Alba

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Abstract: According to different processing methods and growth conditions, the dry roots of *Paeonia lactiflora* Pallas are generally classified as Radix Paeoniae Alba (Bai-shao) and Radix Paeoniae Rubra (Chi-shao), which have been officially recorded under different monographs in all editions of Chinese Pharmacopoeia. Although deriving from the same plant origin, the two herbs have individual chemical characteristics, which eventually result in their different efficacies. To better understand their clinical effects, it is important to display the similarity and differentiation of entire chemical patterns of the two drugs in detail. In the present study, the high performance thin layer chromatographic fingerprints combining digital scanning profiling were developed using two suites of mobile phases and derivatization reagents for the 'high polarity component' and 'lipophilic component' fractions contained in the herbal drugs, respectively. The unique properties of the HPTLC fingerprints were validated by analyzing twelve batches of Bai-shao and Chi-shao. Although the HPTLC fingerprint images of the two herbs were similar, significant differences including the presence or absence of characteristic bands and the respective different ratios of the chemical distribution could directly distinguish between Bai-shao and Chi-shao. Furthermore, the corresponding digital scanning profiles can be used as an easy tool for quantifiable comparison among the samples.

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Keywords: Chromatographic fingerprint, High performance thin layer chromatography, *Paeonia lactiflora*, Quality assessment, Radix Paeoniae Alba, Radix Paeoniae Rubra

INTRODUCTION

Both Radix Paeoniae Alba (Bai-shao in Chinese) and Radix Paeoniae Rubra (Chi-shao in Chinese) are popularly used in traditional Chinese medicine. According to traditional Chinese medicine theory, Radix Paeoniae Alba is usually used for the remedy of female disorders as an analgesic and anti-inflammatory agent,[1–4] but Radix Paeoniae Rubra is often employed to remove heat from blood, eliminate blood stasis, and relieve pain.[5–7] Interestingly, although the two herbs have different efficacies, *Paeonia lactiflora* Pallas is their common plant origin recorded in the Chinese pharmacopoeia 2005 edition.[8] In terms of the herbal source, the only difference consists in the processing method and growth condition of the two herbal roots. Bai-shao is the steamed and dried root of cultivated *P. lactiflora*, while Chi-shao is the dried root of wild *P. lactiflora*. That means they have not only a similar chemical pattern but also some different characteristics between the two crude drugs. The different processing methods and growth circumstances should be responsible for the different chemical characteristics of the two crude medicines and, consequently, cause their different bioactivities and efficacies. Thus, a comprehensive investigation of the chemical difference is necessary and of great importance for more reasonable quality assessment and proper clinical application of the two herbal drugs.

The active chemical constituents of *Paeoniae lactiflora* have been investigated in detail, in which monoterpene glycosides as the high polarity constituents are the most bioactive and predominant components in the two roots.[9–11] Thereof, paeoniflorin is the most representative one of these monoterpene glycosides, so it is officially designated as the only marker component to be measured for the qualitative assessment of both the herbal drugs in all Chinese pharmacopoeia editions. Besides paeoniflorin, there are many other monoterpene glycosides in the two herbs including albiflorin, oxypaeoniflorin, benzoylpaeoniflorin, benzoyoxypaeoniflorin, and so on. Furthermore, the two roots also contain some galloyl glucoses, flavonoids, and lipothilic components such as monoterpene aglycons, triterpenes, and acetophenones. These compounds possess respective bioactivities and accordingly play individual roles in clinical effects of the crude drugs. Obviously, current quality monographs of the two Chinese medicines can hardly cater for the purposes of species differentiation and quality evaluation of them.

Chromatographic fingerprint analysis has shown to be a rational and feasible approach for the quality assessment and species authentication of traditional Chinese medicine (TCM).[12–15] It utilizes chromatographic techniques to construct specific patterns of recognition for herbal drugs. The developed fingerprint pattern of components can then be used to determine not only the absence or presence of markers of interest but the ratio of all detectable analytes as well. Although high performance thin layer chromatography (HPTLC) has a few limitations, such as the limited developing distance and lower plate efficiency by comparison with HPLC and GC, it is still an effective tool for quality evaluation of herbal drugs due to its simplicity, low cost, and requirement, and it has been successfully utilized to develop the chromatographic fingerprint for botanical drugs.[16–18] Moreover, the abovementioned shortcomings can be overcome by separately developing fractions of different polarity on two or several thin layer plates. Thus, the unique feature of the picture like image of HPTLC coupled with the digital scanning profile is gradually attractive to the herbal analysts to construct the herbal chromatographic fingerprint.

In the present study, by combining the digital TLC scanning software, the HPTLC fingerprints of Bai-shao and Chi-shao were developed for the high polarity and lipophilic component using two mobile phases plus two derivatization reagents, respectively. This HPTLC image coupling with the scanning profile could provide adequate information and parameters for comprehensive identification and differentiation of the two closely related herbal medicines.

EXPERIMENTAL

Instrumentation

HPTLC was carried out with a Camag TLC system (Camag, Muttenz, Switzerland) fitted with WinCATS 1.2.3 software. Samples were applied with a Camag automatic TLC sampler 4 (ATS 4) and developed in a twin trough glass chamber (24.5 cm × 8 cm × 22.5 cm). A ReproStar 3 with VideoStore 2 documentation software (Camag, Muttenz, Switzerland) was used for the imaging and archiving of the TLC chromatograms. TLC digital scanning software used to transfer the plate image to the digital scanning plot was developed by our research group (Zhuhai Chromap Institute of Herbal Medicine Research). HPTLC precoated plates, silica gel Merck 60, 20 cm × 10 cm were used (Merck, Darmstadt, Germany, code: OB516990).

Plant Materials

Both of twelve raw herbs of Chi-shao and Bai-shao were collected from several sources within China (Table 1). All of the samples were identified by Professor Mian Zhang (China Pharmaceutical University, Nanjing, China) based on the description in the Chinese Pharmacopoeia 2005 edition, and retention samples are housed in the laboratory of Guangdong provincial hospital of traditional Chinese medicine, Guangzhou, China.

Chemicals and Reagents

Albiflorin was provided by Shanghai University of TCM, Shanghai, China. Paeoniflorin and (+)-catechin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products,

Table 1. A summary of the tested samples

No.	Latin name	Chinese name	Collection location
1	*Paeonia lactiflora* Pall (wild)	Chi-shao	Puning, Guangdong
2	*Paeonia lactiflora* Pall (wild)	Chi-shao	Nanning, Guangxi
3	*Paeonia lactiflora* Pall (wild)	Chi-shao	Taibei, Taiwan
4	*Paeonia lactiflora* Pall (wild)	Chi-shao	Nanchang, Jiangxi
5	*Paeonia lactiflora* Pall (wild)	Chi-shao	Bozhou, Anhui
6	*Paeonia lactiflora* Pall (wild)	Chi-shao	Changchun, Jilin
7	*Paeonia lactiflora* Pall (wild)	Chi-shao	Hong Kong
8	*Paeonia lactiflora* Pall (wild)	Chi-shao	Shanghai
9	*Paeonia lactiflora* Pall (wild)	Chi-shao	Guangzhou, Guangdong
10	*Paeonia lactiflora* Pall (wild)	Chi-shao	Hong Kong
11	*Paeonia lactiflora* Pall (wild)	Chi-shao	Duolun, Inner Mongolia
12	*Paeonia lactiflora* Pall (wild)	Chi-shao	Duolun, Inner Mongolia
13	*Paeonia lactiflora* Pall (cultivated)	Bai-shao	Zhongjiang, Sichuan
14	*Paeonia lactiflora* Pall (cultivated)	Bai-shao	Shanghai
15	*Paeonia lactiflora* Pall (cultivated)	Bai-shao	Puning, Guangdong
16	*Paeonia lactiflora* Pall (cultivated)	Bai-shao	Nanning, Guangxi
17	*Paeonia lactiflora* Pall (cultivated)	Bai-shao	Bozhou, Anhui
18	*Paeonia lactiflora* Pall (cultivated)	Bai-shao	Hong Kong
19	*Paeonia lactiflora* Pall (cultivated)	Bai-shao	Taibei, Taiwan
20	*Paeonia lactiflora* Pall (cultivated)	Bai-shao	Nanchang, Jiangxi
21	*Paeonia lactiflora* Pall (cultivated)	Bai-shao	Chengdu, Sichuan
22	*Paeonia lactiflora* Pall (cultivated)	Bai-shao	Hangzhou, Zhejiang
23	*Paeonia lactiflora* Pall (cultivated)	Bai-shao	Bozhou, Anhui
24	*Paeonia lactiflora* Pall (cultivated)	Bai-shao	Guangzhou, Guangdong

Tiantanxili No. 2, Beijing, China. Benzoylpaeniflorin, benzoyloxypaeniflorin, and β-sitosterol were prepared by our own lab. All chemicals and reagents were of analytical grade and were purchased from commercial sources.

Standard Solution Preparation

Albiflorin, paeoniflorin, benzoyloxypaeniflorin, benzoylpaeniflorin, (+)-catechin, and β-sitosterol reference substances were dissolved in absolute alcohol to prepare the solution containing 1 mg/mL each. This is the standard substances solution.

Sample Preparation

Each dried sample was ground to a fine powder (40 mesh) using a pulverizer. An aliquot of 2 g of each sample was accurately weighed into an appropriately sized volumetric flask, and macerated for 30 min with 50 mL of acetone. The sample was placed in an ultrasonic water bath for 30 min. The extract solution was filtered, and then the residue was rinsed with 20 mL of acetone two times. The extract and washing were combined and concentrated to dryness under vacuum. The dry residue was dissolved with 1 mL absolute alcohol and then filtrated through a 0.45-μm membrane filter.

HPTLC Procedures

HPTLC glass plates were prewashed with isopropanol and then activated at 105°C for 30 min prior to use. Two microlitres of standard and sample solutions were spotted with a constant delivery rate of 100 nL/s in the form of bands of 8 mm width at 10 mm from the lower edge and 12 mm from the left edge, and with a space of 6 mm between two bands. Then the plate was placed in a vacuum trunk containing phosphoric anhydride (P_2O_5) for 2 h. The twin trough chamber was pre-equilibrated with the mobile phase vapor for 15 min prior to analysis. The plates were developed upward for 85 mm at ambient temperature of 18°C using chloroform–ethyl acetate–methanol–formic acid (30:5:10:1, v/v; for 'high polarity component' fraction) and toluene–ethyl acetate–methanol–formic acid (20:4:2:1, v/v; for 'lipophilic component' fraction) as mobile phases. The plates were dried in a stream of cold air for 5 min. Visualization of the chromatograms was performed by spraying with freshly prepared vanillin–sulphuric acid–ethanol (1 g:5 mL:95 mL; derivatization

reagent 1) and 10% sulphuric acid in ethanol (derivatization reagent 2) for 'high polarity' and 'lipophilic component' fractions, respectively, and then heating at 105°C until the bands were clearly visible. The plates for different fractions were observed immediately under visible light and at UV (366 nm) cabinet, respectively. Then the HPTLC visible and fluorescence images were documented. The corresponding digital scanning profiles were generated with the self developed software by our research team.

RESULTS AND DISCUSSION

Establishment of the Developing Method

In general, each traditional Chinese medicine contains multiple compounds with different polarities, so it is difficult and unrealistic to isolate the complex components of herbal drugs on one plate using only a mobile phase in TLC analysis, due to the limitations of developing distance and plate efficiency. As for the two herbal drugs, monoterpene glycosides as the high polarity constituents are the dominant and are thought to be responsible for the main efficacies of the two herbs. Obviously, the qualitative and quantitative analyses of monoterpene glycosides are very important to the quality evaluation and species differentiation of the herbs. However, other components, especially the lipophilic components, could also supplement much useful information for comprehensive analysis of similarities and differences of chemical patterns between the two drugs in this study, so selection of two solvent systems as developing agents were considered to be feasible for separation of the high polarity and lower polarity fractions, respectively. As a result, two HPTLC plates with two solvent systems were very effectively for generating optimal resolution between bands and rich, informative fingerprints of the two herbs.

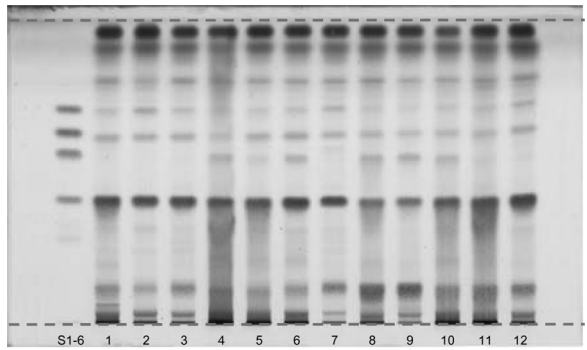
Selection of the Extraction Method and Reagent for Derivatization

Previously, attention was intensively paid to the yield of paeoniflorin during optimization of extraction methods of the two herbal drugs, regardless of other monoterpene glycosides, let alone lipophilic components, so hot water, ethanol, or methanol were usually used as the extract solvent.[19–21] In the present study, the extraction with acetone by maceration and sonication in turn could not only obtain most monoterpene glycosides but give attention to those compounds with lower polarity as well. In the same time, the extraction method could also reduce

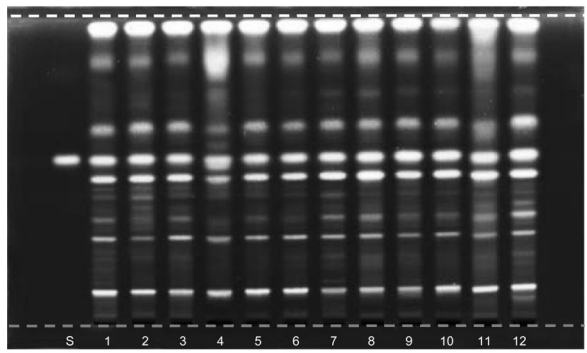
interfering substances such as polyphenolic acids and carbohydrates, which make the HPTLC fingerprint clearer and more fruitful in results. The derivatization reagent 1 is a general reagent for derivatization of terpenoid in TLC analysis, so it was used for derivatization of 'high polarity component' fraction in this study. However, the derivatization reagent 2 can generate a more informative fluorescence fingerprint of the 'lipophilic component' fraction by comparison with the derivatization reagent 1.

Construction of HPTLC Images and Digital Scanning Profile of Standard Substances and Samples

In order to get over the limitations of HPTLC and produce the high resolution images, two solvent systems were selected as mobile phase, which were solvent system 1 (chloroform–ethyl acetate–methanol–formic acid (30:5:10:1, v/v)) for the separation of the 'high polarity component' fraction and solvent system 2 (toluene–ethyl acetate–methanol–formic acid (20:4:2:1, v/v)) for the 'lipophilic component' fraction. As shown in Figures 1a and 2a, there were 8 and 12 visible bands with the R_F value from 0.1 to 0.8 in the respective HPTLC chromatograms of samples of Chi-shao and Bai-shao using solvent system 1 and derivatization reagent 1, which were regarded as the HPTLC fingerprints of 'high polarity component' fractions. However, the distribution characteristics of lower polarity components contained in the two herbs were not able to be obtained by means of solvent system 1 and derivatization reagent 1. For getting high resolution and more image information of lower polarity components, another HPTLC image was developed using the solvent system 2 and derivatization reagent 2. Figures 1b and 2b displayed 14 and 11 fluorescence bands with the R_F value from 0.1 to 0.85 in the respective HPTLC fingerprint images of Chi-shao and Bai-shao samples. Meanwhile, digital scanning profiles of the two fractions' fingerprints were generated by means of self developed digital TLC scanning software (Figure 3). Correspondingly, the peaks 5, 7, 9, 10, and 11 in the chromatogram of the 'high polarity component' fraction were identified as albiflorin, paeoniflorin, (+)-catechin, benzoyloxypaeoniflorin, and benzoylpaeoniflorin by comparison with the R_F values and visible colors of the chemical reference substances on the same plate, and the most remarkable peak 24 was assigned as β-sitosterol in the fluorescence chromatogram of the 'lipophilic component' fraction by means of chemical reference substance. All standard substances and various unknown components in different samples were clearly separated without diffuseness. Because digital scanning fingerprints were intuitively converted from HPTLC images, all the peak areas and intensities were in accordance with visible color or fluorescence bands and their brightness. As a result,



(a)



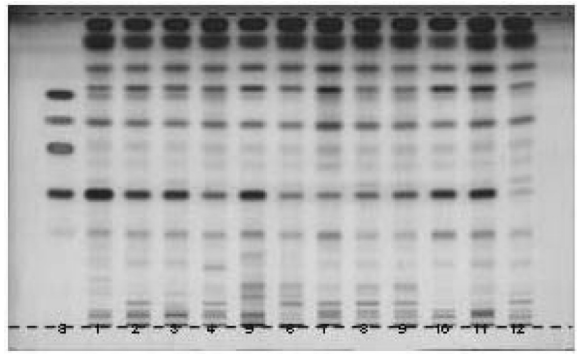
(b)

Figure 1. (a) HPTLC images under visible light of the high polarity component fraction of Chi-shao and chemical reference substances (CRS). Tracks: CRS (albiflorin, paeoniflorin, (+)-catechin, benzoyloxypaeniflorin and benzoylpaeniflorin, from bottom to front of the plate); tracks 1–12 represents Chi-shao samples. (b) HPTLC fluorescence images under the excitation wavelength 366 nm of lipophilic component fraction of Chis-hao and chemical reference substances. Tracks: CRS (β-sitosterol); tracks 1–12 represents Chi-shao samples.

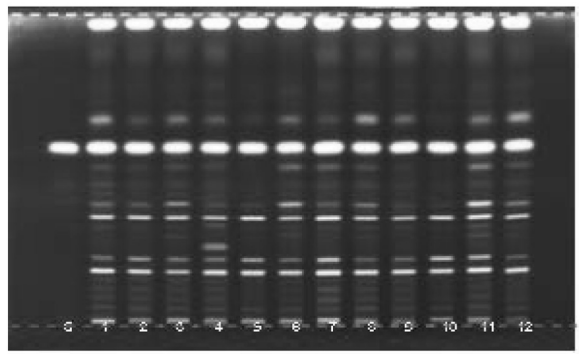
the digital scanning fingerprints could be easily used for quality evaluation and species differentiation by quantifiable comparison of the areas and intensities of peaks and peak-to-peak ratios.

Recognition the Common Characteristics and Distinguish the Two Herbal Drugs

By combining the 'high polarity component' and 'lipophilic component' HPTLC fingerprints as a whole, and comparing the entire fingerprints of



(a)



(b)

Figure 2. (a) HPTLC images under visible light of the high polarity component fraction of Bai-shao and chemical reference substances (CRS). Tracks: CRS (albiflorin, paeoniflorin, (+)-catechin, benzoyloxypaeniflorin, and benzoylpaeniflorin, from bottom to front of the plate); tracks 1–12 represents Bai-shao samples. (b) HPTLC fluorescence images under the excitation wavelength 366 nm of lipophilic component fraction of Bai-shao and chemical reference substances. Tracks: CRS (β-sitosterol); tracks 1–12 represents Bai-shao samples.

Chi-shao and Bai-shao, it was easily found to have a similar chemical pattern between the two herbal drugs. As shown in Figures 1–3, and Table 2, a total of 17 peaks, especially the main bands/peaks could be observed in their chromatograms. The common distributed constituents in Bai-shao and Chi-shao included 2, 3, 7 (paeoniflorin), 9 ((+)-catechin), 10 (benzoyloxypaeoinflorin), 11 (benzoylpaeoniflorin), 13, 14, 17–21, 23, 24 (β-sitosterol), 25, and 26. However, the fingerprint of Bai-shao displayed more bands than that of Chi-shao, and the ratios of peak-to-peak intensities were also rather different between the two roots. Moreover,

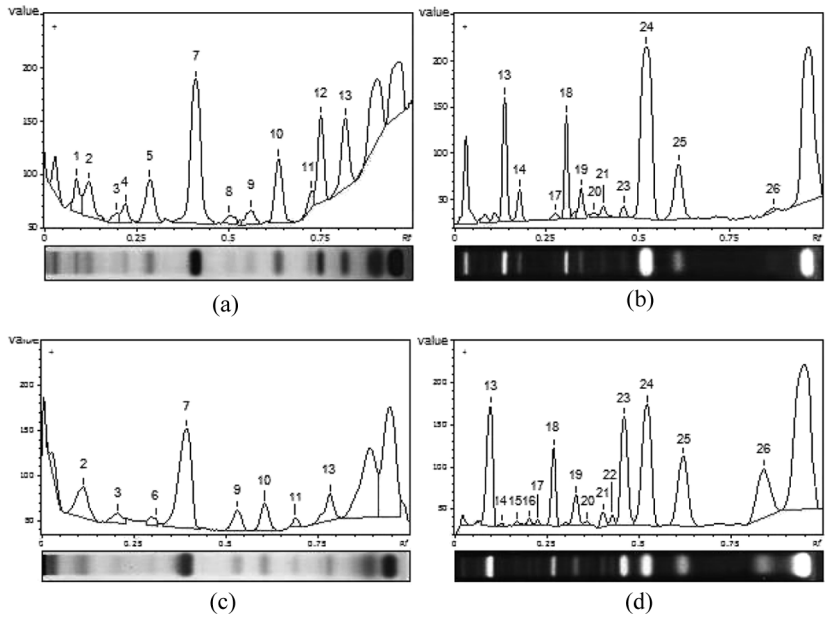


Figure 3. Typical HPTLC images and corresponding digital scanning profiles of Chi-shao and Bai-shao. (a) 'high polarity component' fraction fingerprint of Chi-shao; (b) 'lipophilic component' fraction fingerprint of Chi-shao; (c) 'high polarity component' fraction fingerprint of Bai-shao; (d) 'lipophilic component' fraction fingerprint of Bai-shao. Peak 5: albiflorin; peak 7: paeoniflorin; peak 9: (+)-catechin; peak 10: benzoyloxypaeoniflorin; peak 11: benzoylpaeoniflorin; peak 18: β-sitosterol.

Table 2. The peak distribution in the fingerprint of Chi-shao and Bai-shao

	Peak												
Sample	1	2	3	4	5[a]	6	7[a]	8	9[a]	10[a]	11[a]	12	13
Chi-shao	−	++	±	−	−	±	+++	−	+	+	+	−	++
Bai-shao	+	±	+/±	+	++	−	+++	+	+	++	+	++	++
	14	15	16	17	18	19	20	21	22	23	24[a]	25	26
Chi-shao	+/±	±	±	±	++	+	+/±	±	±	++	+++	++	++
Bai-shao	+	+/±	+/±	+/±	++	+	+/±	+/±	−	+/±	+++	+	±

+: clearly detected; ++: stronger peak/band; +++: strongest peak/band; ±: very weak; +/±: very weak or invisible in a few samples; −: invisible.
[a]Peak 5: albiflorin; peak 7: paeoniflorin; peak 9: (+)-catechin; peak 10: benzoyloxypaeoniflorin; peak 11: benzoylpaeoniflorin; peak 24: β-sitosterol.

there were 8 bands/peaks recognized as the characteristic components, which could be also used as the marks to differentiate the two herbal medicines. As shown in Table 2, the distinct peaks 1, 4, 5 (albiflorin), 8, and 12 could be only detected in the 'high polarity component' fraction fingerprints of Bai-shao samples. On the other hand, in the 'lipophilic component' fraction fingerprints, the peaks 23 and 26 of Chi-shao were relatively stronger than those of Bai-shao, while relative to Bai-shao, the peak 14 was very weak or invisible in the chromatogram of Chi-shao. By means of these main characteristics of the HPTLC fingerprint, the two herbal drugs collected from different locations could be definitely distinguished from each other.

CONCLUSIONS

By means of data analysis system and optimized experimental conditions, HPTLC is also feasible for development of chromatographic fingerprint methods to determine and identify complex herbal extracts just like HPLC and GC.[16] Furthermore, the colorful picture like HPTLC image provides extra intuitive parameters of visible color and/or fluorescence and, unlike HPLC and GC, HPTLC can simultaneously determine different samples on the same plate. In the present study, HPTLC fingerprints for both of the 'high polarity' and 'lipophic component' fractions of the two herbal medicines were constructed using two solvent systems plus two derivatization reagents. Such an approach causes the HPTLC method to maintain its innate advantage as well as get over the limitations of developing distance and plate efficiency.

In conclusion, the HPTLC fingerprints ('high polarity' and 'lipophilic component' fractions) disclosed in detail that there are enough individual characteristics to differentiate between the roots of Chi-shao and Bai-shao though fingerprint patterns of two herbs appear similar by and large. The bands corresponding to the peaks 1, 4, 5 (albiflorin), 8, 12, 14, 23, and 26 can be applied as the marks to distinguish the two roots. Consequently, the proposed HPTLC fingerprints can cater for the identification and differentiation of the two kinds of herbs.

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