Phytochemical Analysis
Phytochem. Anal. **19**: 499–505 (2008)
Published online 10 June 2008 in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/pca.1074

Phytochemical Analysis

Analysis of Pharmaceutical Samples of Resina Draconis by HPLC-PAD

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Received 21 July 2007; Revised 8 April 2008; Accepted 9 April 2008

Abstract:
Introduction: The quality evaluation of traditional Chinese medicine (TCM) represents a particular challenge owing to the complexity of the matrix, which renders separation and identification of the individual components extremely difficult. In recent years, fingerprinting of TCMs has played a dominant role in quality control. Resina Draconis was authorised as a new TCM in 1991, but a satisfactory HPLC fingerprint method for this preparation has not yet been published.
Objective: To develop a simple and reliable protocol for the quality control of Resina Draconis using an HPLC-PAD method.
Methodology: The TCM was extracted with methanol at room temperature. Chromatography was carried out using a Lichrospher C_{18} column eluted with a linear gradient of acetonitrile (A) and water containing 0.1% phosphoric acid (B), initially at 30:70 (A:B) and changing to 60:40 in 90 min. UV (PAD) spectra were acquired in the range 210–400 nm.
Results: Four chromatograms of samples of Resina Draconis obtained from different pharmaceutical factories showed 20 peaks in common. The average chromatogram was taken as a template from which the correlation coefficients and cosine ratios of the samples were determined. Whereas the contents of individual components in each sample were different, overall the samples were extremely similar one to another, and the products from different pharmaceutical factories were consistent.
Conclusion: A reliable and validated HPLC method has been developed for the fingerprint analysis of Resina Draconis that can be applied for the quality control of this TCM. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: HPLC-PAD; fingerprint analysis; gradient elution; Resina Draconis

INTRODUCTION

Dragon's blood is a deep red resin that has been used for diverse medical purposes for several centuries. The original source of dragon's blood resin is believed to be *Dracaena cinnabari* from Socotra in Africa (Edward *et al.*, 2001). Nowadays, several cultures have at least one indigenous resin that can be termed dragon's blood, but the botanical sources are often dissimilar. Two distinct sources from the genera *Dracaena* and *Daemonorops* have been recognised as the true dragon's blood resin. In addition, various kinds of dragon's blood resin substitutes, such as *Croton draco* (Mexico) and *Eucalyptus resinifera* (Australia), are commercially available, and there is even a powdered dark red coral from the Indian Ocean that is sold in Yemeni bazaars as dragon's blood.

Sanguis Draconis, a resin exuded from the fruit of *Daemonorops draco*. Bl. (family Palm) cultivated in Southeast Asian, is the principal source for commercially harvested dragon's blood. In China, Sanguis Draconis is recorded as dragon's blood in the official *Chinese Pharmacopoeia* (Zheng and Chen, 2007). In traditional Chinese medicine (TCM) theory, Sanguis Draconis is beneficial for promoting blood circulation and removing blood stasis, dispelling the saprophytic muscle, diminishing inflammation and easing pain, stopping bleeding and relieving congestion (Pharmacopoeia Commission of People's Repbulic of China, 2005). However, owing to its rarity, Sanguis Draconis is very expensive in the Chinese market, which limits its extensive use in clinical treatments. Following an extensive investigation, Cai and Xu (1979) have found a dragon's blood substitute in the form of a red resin from the tree-stem of *Dracaena cochichinensis* (Lour.) S. C. Chen (family Liliacea), which grows in the Yunnan and Guangxi provinces of China. The crude drug was named Resina Draconis.

Over 20 years, the chemical composition, pharmacological effect and toxicity of Resina Draconis have been investigated thoroughly in China. It contains a variety of active ingredients such as flavonoids, glycosides, stilbenes, organic acids, phenols and esters (Zhang *et al.*, 2004). It possesses haemostatic activity, some analgaesic effect, vasoactive–antithrombotic potency, antitumour, anti-bacterial and wound-healing properties, immunomodulatory activity, as well as a certain level of toxicity, including potential carcinogenicity (Zhong and Bi, 2002). It has been used to treat surgical diseases, chronic cervicitis and coronary disease clinically.

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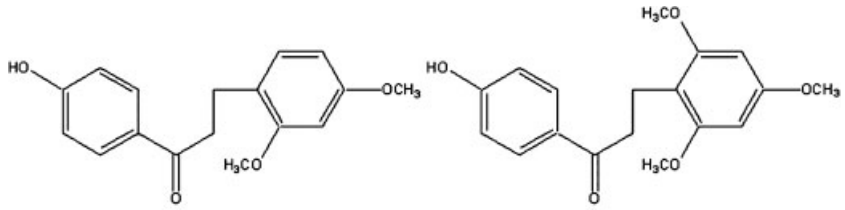
Contract/grant sponsor: National Science Foundation of China; Contract/grant number: 20775028.
Contract/grant sponsor: Jiangnan University.



Although the botanical origins of Resina Draconis and Sanguis Draconis are different, and they possess distinct chemical compositions and exhibit different pharmacology, they have been proven to have a similar therapeutic efficacy. The national standard of Resina Draconis as a new TCM was authorised in 1991 (Wen, 2001), and loureirin B was used as the marker substance for its quality control. Recently, loureirin A, together with loureirin B, were recommended as the quality markers, owing to their similar molecular structures (Sun *et al.* 2002b). Thin-layer chromatography (Sun *et al.*, 2002c), UV spectrophotometry (Sun *et al.*, 2002c) and HPLC (Sun *et al.*, 2002a; Wang *et al.*, 2006) have been widely applied to determine loureirin A and loureirin B in Resina Draconis.

However, quality control focusing on one or a few substances as the markers is not adequate for crude medicines. It is well known that a TCM usually contains numerous components that are usually responsible for the therapeutic effects. The interaction among these compounds is an important factor for safety and efficacy. Therefore, quality evaluation of a TCM is an extraordinary challenge because the matrix is so complicated that separation and identification of components is extremely difficult. In recent years, fingerprinting of TCM has played a dominant role in this aspect.

Both FDA (US Food and Drug Administration, 2000) and EMEA (European Medicines Agency, 2001) have clearly denoted that the appropriate chromatographic fingerprint should be applied to assess the consistency of a botanical drug. Fingerprint analysis has been introduced and accepted by the WHO as a strategy for the assessment of herbal medicines (World Health Organization, 1991). Fingerprint analysis is an efficient measurement for identifying and assessing the stability of crude medicines.

The TLC fingerprint of Sanguis Draconis has been reported for the identification of genuine material. Ten kinds of samples, including genuine Sanguis Draconis and Resina Draconis as well as fake materials, were analysed by IR spectroscopy and spectrofluorimetry with hierarchical cluster analysis for the classification and identification of dragon's bloods (Wang *et al.*, 2005). HPLC is regarded as a prime technique in the development of a fingerprint of a crude drug due to its precision, sensitivity and reproducibility (Chen *et al.*, 2007), and HPLC fingerprints are often applied for the quality control of TCMs. Although HPLC fingerprinting of Resina Draconis was developed in 2000 (Huang and Yong, 2001), the method described had at least two shortcomings. Firstly, the flow rate of the mobile phase was so high that all components in the samples eluted within 10 min, which was far from the standard of State Food and Drug Administration (SFDA), which requires that analytical time should be more than 60 min (Li and Wang, 2003). Secondly, owing to elution in the isocratic mode, the resolution was far from satisfactory and it was impossible to feature the chemical profile of Resina Draconis in full-scale. In the present work, a simple and reliable method by which to establish a characteristic HPLC fingerprinting of Resina Draconis for quality control is described. Moreover, in order to differentiate Sanguis Draconi from its substitute, Sanguis Draconi was also studied by HPLC under the same conditions.

EXPERIMENTAL

Standards and chemicals. Loureirin A and loureirin B were purchased from the Chinese Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade acetonitrile was provided by Hanbon (Jiangsu, China). Other chemicals were of analytical grade. Water was doubly distilled. The stock solutions of loureirin A and loureirin B (1 mg/mL each) were prepared in methanol and were stored at 5°C in the dark. Before use, all solutions were filtered through 0.25 μm nylon filters.

Drug material. Crude drugs and preparations were collected from different regions in China as shown in Table 1.

Sample preparation. All samples were stored in the desiccator until required for use. Dried material was powdered, and about 0.01 g of the accurately weighed sample was extracted exhaustively with 10 mL methanol for 20 min in an ultrasonic bath (Tongchao, Wuxi, China) at room temperature. After cooling, the supernatant was filtered through a 0.25 μm nylon filter and a 10 μL aliquot of the sample was injected into the HPLC for analysis.

HPLC analysis. The HPLC apparatus was a Waters (Milford, MA, USA) 1525 binary HPLC pump system

DOI: 10.1002.pca

Table 1 Sources of Sanguis Draconis and Resina Draconis and preparations

Sample	Brand	Pharmaceutical factory	Lot no.
Crude drugs			
Resina Draconis	Shulong	Mingsheng	001006
Sanguis Draconis	Huangguan	Huachenghang Native Produce Pte	JY20030235
Preparations			
Longxuesu caplus	Sanjin	Sanjin (Nanning, Guangxi)	Z20013230
Longxuesu caplus	Yulin	Xishuangbanna Tropical Botanical (Xishuangbanna, Yunnan)	Z53021514
Longxuesu caplus	Yunshan	Yunhe (Gejiu, Yunnan)	Z53020999

equipped with a model 2996 photodiode array detector (PAD) and a model 725i manual injector. A Lichrospher (Hanbon Science and Technology, Nanjin, Jiangsu, China) reversed-phase C_{18} column (250 × 4.6 mm i.d.; 5 μm) was employed. The mobile phase consisted of acetonitrile (A) and water containing 0.1% phosphoric acid (B). The elution was carried out in linear gradient mode in which phase A varied from 30 to 60% within 90 min and the flow rate was 0.6 mL/min. The PAD was set to monitor in the range from 210 to 400 nm.

Data analysis. Data acquisition and processing were performed using Waters Empower software. For similarity analysis of TCMs, relative standard deviation (RSD), correlative coefficient and cosine ratios were calculated using Microsoft Excel 2002 software.

The RSD (coefficient of variation) shows the precision of the analytical results. The RSD could be calculated directly according to equation (1):

$$\mathrm{RSD} = \frac{1}{\bar{X}}\sqrt{\frac{1}{n-1}\sum_{k=1}^{n}(X_i - \bar{X})^2} \tag{1}$$

The correlation coefficient (r_{ir}) measures the strength and the direction of a linear relationship between two groups of variables. The correlation coefficient is defined in equation (2):

$$r_{ir} = \frac{\sum_{k=1}^{n}(X_{ik} - \bar{X}_i)(X_{rk} - \bar{X}_r)}{\sqrt{\sum_{k=1}^{n}(X_{ik} - \bar{X}_i)^2 \sum_{k=1}^{n}(X_{rk} - \bar{X}_i)^2}} \tag{2}$$

where X_{ik} is the value of variable k in sample i; $\bar{X}_i$ is the average of all variables in sample i; X_{rk} is the value of variable k in common mode; $\bar{X}_r$ is the average of all variables in common mode. The correlation coefficient of the sample shows the extent of similarity between the sample and the common mode. Thus, in fingerprint analysis of a TCM, it indicates the similarity of degree in qualitative analysis, and helps distinguish whether the sample is genuine or false.

The cosine ratio (C_{ir}) is a vector that calculates the angle between two groups of variables in Euclidian geometry. It also displays the comparability of the samples. C_{ir} is calculated as in equation (3), and the parameters are the same as in equation (2):

$$C_{ir} = \frac{\sum_{k=1}^{n} X_{ik} \cdot X_{rk}}{\sqrt{\left(\sum_{k=1}^{n} X_{ik}^2\right)\left(\sum_{k=1}^{n} X_{rk}^2\right)}} \tag{3}$$

RESULTS AND DISCUSSION

Optimisation of the extraction method

A good extraction method for a TCM fingerprint should not only offer high extraction efficiency for active components, but also capture as many detectable components as possible. In this work, both the peak heights of loureirin A and loureirin B (commonly used as quality markers of Resina Draconis) and the number of peaks (a key factor to evaluate the integrity of a fingerprint) were used as the targets to optimise the appropriate extraction method.

TCMs are commonly employed as a decoction in water. However, most components in Resina Draconis are of low polarity and water extraction would achieve low extraction efficiency. Soxhlet, ultrasonic and microwave extraction are the other common extraction methods for TCM. In the present study, the high content of methanol used in the extracting solvent precluded the use of microwave heating.

It is readily observable that reflux extraction was somewhat more efficient than ultrasonic extraction in terms of efficiency of capture of the active ingredients by comparing the results of nine experiments shown in Table 2. However, because the Soxhlet method is very time-consuming it was not considered suitable for actual application, and so the ultrasonic method was selected as the optimum extraction method. Solvent and time for ultrasonic extraction were investigated. Methanol was chosen as extraction solvent because it

DOI: 10.1002.pca

Table 2 Efficiency of different extraction methods for the extraction of Shulong

No.	Extraction way	Extraction solvent	Extraction time	Peak height of loureirin A (au)	Peak height of loureirin B (au)	Number of peaks[a]
1	Refluxing	100% methanol	2.5 h	0.030	0.040	34
2	Refluxing	100% methanol	4 h	0.085	0.130	36
3	Refluxing	100% methanol	6 h	0.090	0.130	37
4	Refluxing	100% methanol	8 h	0.090	0.120	41
5	Ultrasonic	75% methanol	10 min	0.075	0.105	35
6	Ultrasonic	75% methanol	20 min	0.080	0.110	39
7	Ultrasonic	75% methanol	30 min	0.075	0.100	37
8	Ultrasonic	90% methanol	20 min	0.077	0.102	38
9	Ultrasonic	100% methanol	20 min	0.082	0.120	41

[a] Defined as peaks with areas > 0.5% of the total peak area: for HPLC conditions employed see Experimental section.

yielded the largest number of peaks, and the greatest peak heights for loureirin A and loureirin B. Furthermore, an appropriate extraction time was very important since a short time resulted in incomplete extraction whilst too long a time led to decomposition of active components. Experimental result showed that 20 min extraction was sufficient.

Optimisation of HPLC conditions

HPLC conditions were optimised by investigating the influence of the mobile phase, elution mode, flow rate and detection wavelength, since these parameters play key roles in determining the resolution and sensitivity. Considering the presence of flavonoids in the samples, a small amount of phosphoric acid was added to the mobile phase to reduce the ionisation and lower the polarity of these compounds. By virtue of the complexity of the TCM samples, binary solvent mobile phases in gradient elution mode were used in this work. A mixture of methanol and water containing 0.1% phosphoric acid was initially employed as the mobile phase. However, more than 10 peaks were not separated at all and the shapes of the other peaks were not acceptable. Furthermore, the velocity of elution was very slow. When methanol was replaced by acetonitrile in the mobile phase at the same flow rate in the same elution procedure, separation was improved, and the peaks were sharpened. Hence, acetonitrile (A) and water containing 0.1% phosphoric acid (B) were chosen as the mobile phase. The gradient elution procedure was also adjusted, and the optimum elution mode was that in which phase A varied in linear gradient mode from 30 to 60% within 90 min. When the flow rate was too fast, the column pressure increased unacceptably, a phenomenon attributable to the length of column used in this work, hence 0.6 mL/min was chosen as the suitable flow rate of mobile phase.

In order to obtain a large number of detectable peaks in the chromatogram, the UV spectra of all peaks in the chromatogram of Resina Draconis were recorded with PAD as shown in Fig. 1. In considering both the sensitivity of the active components and the number of detectable peaks, 280 nm was selected as the detection wavelength.

Reproducibility

Under the optimum chromatographic conditions, 10 μL aliquots of the standard mixture (0.05 mg/mL of loureirin A and loureirin B) were injected three times per day, and the same procedure was repeated on three consecutive days to evaluate the reproducibility. Intra-day RSDs of the retention time and peak height of two analytes were less than 0.8 and 3.4%, respectively ($n = 3$). Inter-day RSDs of the retention time and peak height were less than 1.5 and 2.9%, respectively ($n = 9$). The results indicate that the method for the determination of Resina Draconis fingerprint using HPLC has good stability and reproducibility.

Standardisation of fingerprint of Resina Draconis

According to the definition of fingerprinting of TCM, an HPLC fingerprint is in practice a chromatographic pattern of common kinds of pharmacologically active and characteristic components in a TCM. This chromatographic profile should feature the fundamental attributions of "similarity" and "differences" (Cao *et al.*, 2006). It is suggested that authentication and identification of a TCM can be determined accurately from its HPLC fingerprinting, even if the quantity of the chemically characteristic constituents are not exactly the same among different samples. With the HPLC method, four brands of samples of Resina Draconis from different factories in China were analysed under the optimum conditions. The average chromatogram from the four brands was regarded as the standardised characteristic fingerprint of Resina Draconis. Any peak whose area

DOI: 10.1002.pca

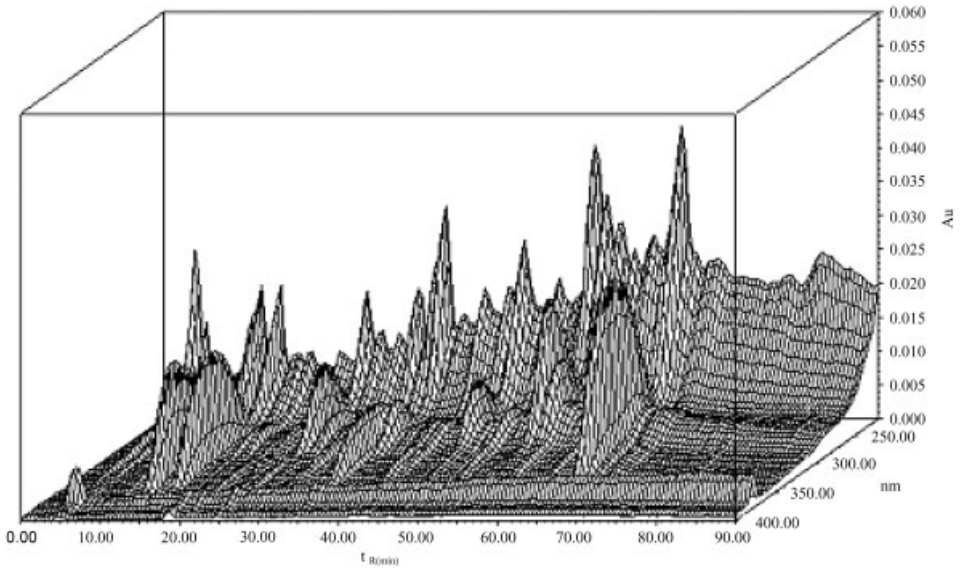


Figure 1 3D chromatogram of Resina Draconis (Sanjin). For chromatographic conditions see the Experimental section.

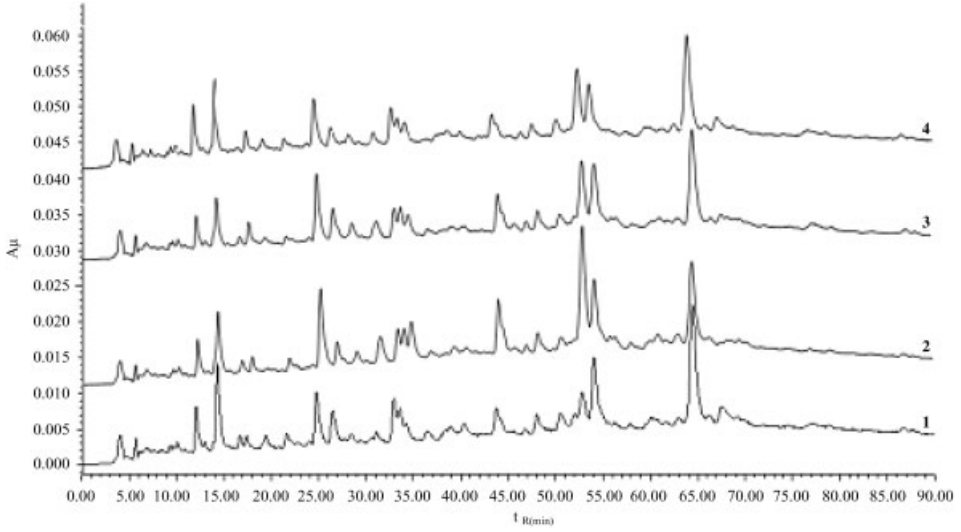


Figure 2 Superposition of chromatograms of four brands of Resina Draconis. Sample identification: 1 = shulong; 2 = sanjin; 3 = yunshan; 4 = yulin. For HPLC conditions see the Experimental section.

percentage was more than 0.5% and which was present in the chromatograms of the entire sample was assigned as a "common peak", indicating the similarity among various samples. A superposition of the chromatograms of the four brands of Resina Draconis is shown in Fig. 2. There were 27–41 peaks (peak areas larger than 0.5% of the total) within 90 min in the chromatograms of the four samples. According to the definition of common peak, 20 peaks were picked out as the common ones, as shown in Fig. 3(A). Moreover, the purity of each common peak in all of the chromatograms was confirmed from the PAD chromatograms and Empower software. Peak number 16 was taken as the reference peak due to its moderate retention time and peak height, and the relative retention times and peak heights of the common peaks were calculated with respect to this reference. The RSD values of the relative retention times of the 20 common peaks in the four samples were less than 1.5%, which means that the common peaks corresponded well in all samples. Furthermore, such low RSD values indicate that the method has high precision and stability. Hence, the peak profile of the 20 components made up the fingerprinting of Resina Draconis. The total peak area of the non-common peaks was about 5%, which is less than the national standard of 10% (State Food and Drug Administration, 2000). The similarity among the four kinds of samples was evaluated by two mathematical methods using the correlative coefficient and cosine ratio, as suggested by SFDA (Wang *et al.*, 2002, 2003; Gong *et al.*, 2003). Based on the relative peak height of the 20 common peaks in the chromatograms of the

DOI: 10.1002.pca

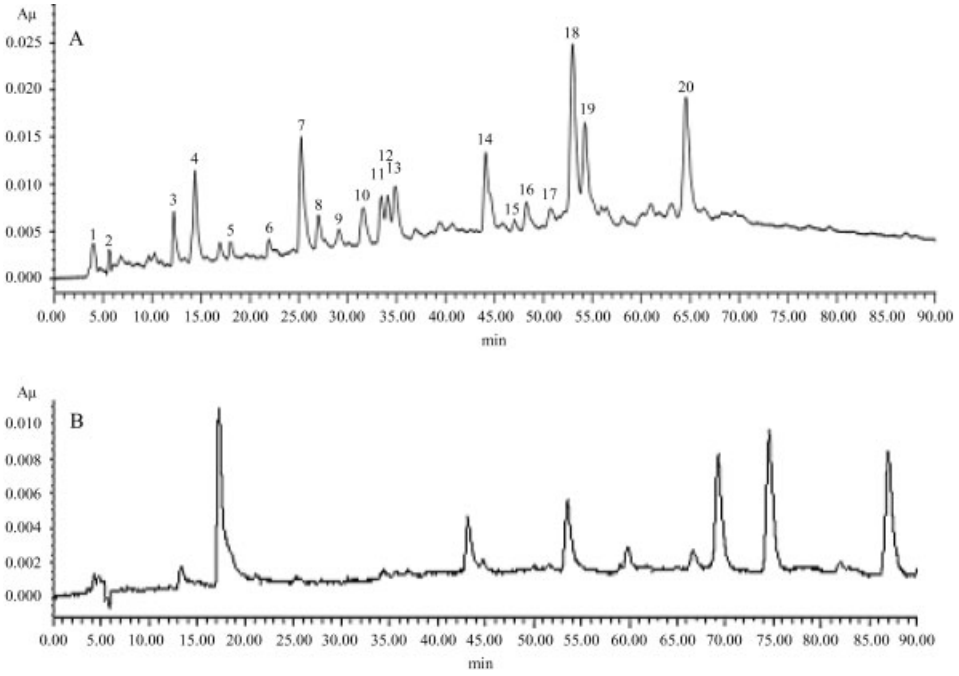


Figure 3 Chromatograms of Resina Draconis (A) (Sanjun) and Sanguis Draconis (B). Peak identify 18 = loureirin A, 19 = loureirin B. For HPLC conditions see the Experimental section.

Table 3 Relative peak heights of peaks that were common to the four brands of Resina Draconis

	Relative peak height					
Peak number	Sanjin	Yulin	Yunshan	Shulong	Average	RSD%
1	0.592757	0.964648	1.931611	1.570038	1.264764	47.4
2	0.322211	0.349035	0.684500	0.632828	0.497144	37.8
3	1.078160	0.852092	2.601437	2.121706	1.663349	50.2
4	2.449275	1.592386	4.581017	4.699142	3.330455	46.6
5	0.366863	0.573367	1.305573	0.622340	0.717036	56.9
6	0.407286	0.114418	0.779773	0.605603	0.476770	59.9
7	3.208153	2.437575	3.802091	3.251398	3.174804	17.7
8	0.767041	1.346277	0.948428	2.358345	1.355023	52.5
9	0.515414	0.750151	0.948428	0.727281	0.735319	24.0
10	1.284602	0.879285	0.872863	0.600137	0.909222	31.0
11	1.075231	0.922175	2.245416	2.251419	1.623560	44.6
12	1.142686	1.062763	1.592688	1.722787	1.380231	23.7
13	1.617546	1.033584	1.767770	1.193862	1.403190	24.7
14	2.674783	1.440031	1.586533	1.865318	1.891666	29.1
15	0.278534	0.426839	0.241482	0.265517	0.303093	27.7
16	1.000000	1.000000	1.000000	1.000000	1.000000	0.0
17	0.820741	0.866435	1.519090	1.459717	1.166496	32.1
18	6.611046	3.241146	5.871073	3.045581	4.692211	38.7
19	4.468217	3.763278	4.790255	5.930456	4.738051	19.1
20	5.464356	4.640154	10.521074	10.438477	7.766015	40.6
Correlation coefficient	0.910296	0.969711	0.981865	0.952897		
Cosine ratio	0.956999	0.985787	0.991225	0.976187		

four samples, the RSDs of the relative peak height of each common peak, the correlation coefficient and the cosine ratio were calculated using Microsoft Excel 2002 (Miao and Sun, 2003) according to equations (1)–(3), and the results are shown in Table 3. The large RSD values of the relative peak height of common peaks indicate that the contents of the common substance in the samples were very different. In this aspect, the RSD

DOI: 10.1002.pca

values displayed the "differences" among the samples, which can be easily explained since the composition of the crude product is affected by many factors, including growing conditions, harvest season, processing method and duration of storage. On the other hand, the values of the correlation coefficient and the cosine ratio were above 0.910 and 0.957, respectively, which indicates the high level of similarity between the samples. This similarity shows that the quality of Resina Draconis was stable and that products from different pharmaceutical factories were consistent.

Comparison of Resina Draconis with Sanguis Draconis

Although Sanguis Draconis and Resina Draconis possess similar colour and shape and even approximately the same curative effect, the two kinds of plants are classified in different families and genera, and their chemical compositions are absolutely dissimilar. In the medical market, Resina Draconis or a mixture of Resina Draconis and Sanguis Draconis may substitute for Sanguis Draconis because of the remarkable price gap between them, so it is necessary to identify the two medicines. The chromatogram of Sanguis Draconis, obtained under the same HPLC conditions as that of Resina Draconis, is shown in Fig. 3(B). It can readily be observed that the two chromatograms are not at all similar, showing that HPLC fingerprinting is an efficient way to differentiate samples.

Acknowledgements

The authors are grateful for the financial support provided by the National Science Foundation of China (grant no. 20775028) and Jiangnan University.

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