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Establishment of Characteristic Fingerprint Chromatogram for the Identification of Chinese Herbal Medicines

SIU-KAY WONG [1]*, SHU-KI TSUI[1], SIK-YIU KWAN[1], XIAO-LI SU[2], RUI-CHAO LIN[2], LI-MING TANG[3] AND JIAN-XING CHEN[3]

[1.] *Government Laboratory, 88 Chung Hau St., Homantin, Hong Kong SAR, China*

[2.] *National Institute for the Control of Pharmaceutical and Biological Products, Beijing 100050, China*

[3.] *Shanghai Institute for Drug Control, Shanghai 200233, China*

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ABSTRACT

Despite the fact that chromatographic fingerprint analysis has been used increasingly for the identification of Chinese herbal medicines, there is no recognized procedure for the establishment of characteristic fingerprint chromatogram. In this paper, a chromatographic response function (CRF) was introduced to quantitatively compare chromatograms obtained under different experimental conditions. As a result, the optimum experimental conditions for the establishment of characteristic fingerprint chromatogram could be identified. Moreover, since the proposed function involved data that were directly available from the workstation after each chromatographic analysis, calculation could be automated. In this paper, the function was successfully applied to obtain characteristic fingerprint chromatogram for Chinese herb Langdu.

Key words: characteristic fingerprint chromatogram, optimization, chromatographic conditions, Chinese herbal medicines, Langdu

INTRODUCTION

Chinese medicines are receiving an increasing interest for their role as alternative medicines and health supplements, as evidenced by the growing global markets and increasing number of rigorous scientific studies conducted. In particular, a number of herbal medicines have been proven to be an efficacious alternative to synthetic drug substance in preventing and treating various chronic and mild diseases, provided they are of adequate quality and properly used.

Chromatographic fingerprint analysis, which enables a particular herb to be identified and distinguished from closely-related species, is now widely used for the quality control of herbal medicines and their proprietary products. For example, the U.S. Food and Drug Administration (FDA) proposed to accept chromatographic fingerprinting technique for the identification of herbal materials[(1)]. Also, the same technique was suggested by the World Health Organization (WHO) to ensure consistent quality of the plant materials and finished products[(2)]. Moreover, in China, the State Drug Administration (SDA) stipulated that registration of injections made of Chinese medicines requires chromatographic fingerprints characteristic of the products.

Despite the widespread use of the chromatographic fingerprint analysis, no recognized procedures are available for the establishment of characteristic fingerprint chromatogram in the identification of herbal medicines. This was perhaps due to the lack of objective criterion to compare chromatograms obtained under different experimental conditions. In view of this problem, a new chromatographic response function was proposed in this paper and was successfully applied to obtain characteristic fingerprint chromatograms for the identification of Chinese herb Langdu.

Langdu has been used for the treatment of hydrothorax, ascites, carbuncles, sores and scrofula. It is identified as the root of species *Stellera chamaejasme* L., *Euphorbia ebracteolata* Hayata or *Euphorbia fischeriana* Steud[(3,4)]. However, quite a number of other species are habitually used as Langdu in some areas in China. For example, *Alocasia macrorrhiza* (L.) Schott is used as Langdu in Guangdong[(5)] though it belongs to a different genus and has different medicinal uses. In addition, there are several other herbs belonging to the same genus of the orthodox Langdu species and having similar medicinal uses. For example, the root of *Euphorbia kansui* Liou is used for the treatment of anasarca, hydrothorax, ascites with dyspnea, constipation and oliguria. Hence, there is a need to differentiate the orthodox Langdu species from the unconventional Langdu and other taxonomically close species.

MATERIALS AND METHODS

I. *Reagents and Standards*

Methanol was of HPLC grade (Lab-Scan, Bangkok, Thailand). Diethyl ether and acetic acid were of analytical grade (Merck, Darmstadt, Germany). Jolkinolide B, neochamaejasmin and ebracteoloata compound B were obtained from Herbstandard (Chesterfield, MO, U.S.A.).

* Author for correspondence. Tel: 852-2762-3866;
Fax: 852-2714-4083; E-mail: skwong@govtlab.gov.hk

Journal of Food and Drug Analysis, Vol. 12, No. 2, 2004

II. *Plant Materials*

Reference herbal materials for *Euphorbia ebracteolata* Hayata, *Euphorbia fischeriana* Steud *and Stellera chamaejasme* L. were provided by the National Institute for the Control of Pharmaceutical and Biological Products. Samples of *Alocasia macrorrhiza* (L.) Schott *and Euphorbia kansui* Liou were purchased from local medicinal herb shops and their identity were verified by the National Institute for the Control of Pharmaceutical and Biological Products. Further, 12 certified Langdu samples obtained from different localities in China, including three *Euphorbia ebracteolata* Hayata, one *Euphorbia fischeriana* Steud and eight *Stellera chamaejasme* L., were procured from the Beijing Institute of Chinese Materia Medica.

III. *Equipment*

A Hewlett-Packard HPLC Model HP-1100 equipped with a multi-wavelength detector and diode-array detector was used. The chromatographic separation was carried out with a Hypersil BDS (250 × 2.1 mm; i.d., 5 μm) column. The mobile phase was methanol/water with composition changing from 40/60 to 100/0 in 50 min and was kept at the final composition for 20 min. The detection wavelength was set at 230 nm.

IV. *Sample Preparation*

Pulverized plant sample (0.5 g) was mixed with 20 mL of diethyl ether in a capped 50-mL centrifuge tube. The mixture was sonicated for 45 min and then heated in a water bath at 40˚C for 3 hr. After cooling at the room temperature, the mixture was centrifuged and the supernatant was transferred into another tube. The solvent was evaporated and the residue was reconstituted in 2 mL of methanol. The solution was filtered through 0.45 μm membrane filter prior to HPLC analysis.

RESULTS AND DISCUSSION

The basic requirements for fingerprint analysis include the establishment of a characteristic chromatogram for the substance of interest. Also, at least one marker compound has to be identified in the fingerprint chromatogram. However, it cannot determine whether a chromatogram obtained under specific experimental conditions is good enough to be a characteristic fingerprint chromatogram for the purpose of identification. Conceivably, a characteristic fingerprint chromatogram should display as many well-resolved and large peaks as possible to ensure a characteristic and distinctive chromatographic pattern. However, some kinds of objective criteria are required for quantitative comparison. Otherwise, the characteristic fingerprint chromatogram could only be identified solely by visual comparison of chromatograms obtained from different experiment conditions.

To start with, CRFs used in the HPLC optimization were referred. Table 1 shows a list of some commonly used CRFs summarized in a review by Berridge[6]. However, these functions were found inapplicable to the present study for the following reasons. It was observed that the number of components present in the mixtures was known in advance in these cases of HPLC optimization. Hence, the objective of these CRFs was solely to improve the peak separation by optimizing parameters such as mobile phase composition and gradient setting. Besides, effects due to different extraction procedure and detection wavelength were not considered and parameters affecting the chromatographic pattern such as peak area ratios were not included in these functions. Lastly, in the case of fingerprint chromatogram optimization, the number of peaks expected (M) and the expected retention times for the last peaks (t_m) were usually unknown and might vary with different experimental conditions.

In this paper, a new CRF named as fingerprint index Y was proposed as follows:

$$Y = \sqrt{(\log\ P)^2 + (\log\ R)^2}$$

$$P = \sum_{i}^{n} P_i / P_m \text{ and } R = \sum_{i}^{n-1} R_i$$

P_i / P_m represents the relative intensity of the *i*th peak with P_i as the peak area of the *i*th peak and P_m the peak area of the most intense peak other than the solvent peak. *R* represents the summation of R_i, the resolution of the *i*th pair of peaks, for the n-1 pairs. As a resolution value of 2 represents a base line separation between two peaks, R_i would be counted as 2 if its calculated value is larger than 2. Hence, a large P value implied that the chromatogram was composed of more large peaks, whereas a large R value implied that the

Table 1. Examples of chromatographic response functions used in HPLC optimization

Function	Variables
$P_{inf} = \sum^2 \log S_i$	Binary mobile phase
$F_{obj} = \sum [10(1.5-R_i)]^2$	Ternary mobile phase
$F_{obj} = \sum 100e^{1.5-Ri}+(t_m-t_n)^3$	Ternary mobile phase
$CRF = \sum \ln(P_i/P_d)+a(t_m-t_n)$	Gradient parameters and flow rate
$CRF = \sum \ln(fi/gi)-100(M-n)$	Concentration of organic modifier, pH
$CRF = \sum R_i+n^a-b\|t_m-t_n\|-c(t_0-t_1)$	Composition of ternary mobile phase, flow rate, temperature, pH
$CRF = \sum r_i+n-(t_m-t_n)$ (for $t_m-t_n > 1$)	Composition of ternary mobile phase
Y = p/M	Gradient parameters

Note: P_{inf} = informing power; S_i = peak overlap;
R_i, P_i = peak resolution; P_d = desired peak resolution;
t_n = retention time of last peak; t_m = desired retention time;
t_0 = void time; t_1 = retention time of the first eluted peak;
f, g = peak separation factors; M = no. of peaks expected;
n = no. of peaks detected;
p = no. of peaks separated with a given resolution;
CRF = chromatographic response function;
Y = the extent of separation;
a, b and c = selectable weightings.

chromatogram was composed of more well resolved peaks. To combine the contributions from these two factors, the root mean square of their sum was recommended. Considering that their values might involve a large range of values, the logarithm of the values was taken. As peak areas and peak resolution values were directly available from the instrument workstation after each chromatographic analysis, the calculation of Y values could be easily automated through the execution of an Excel macro programme.

As the Chinese herb Langdu includes three different species, the tactic used in the study was to establish the optimum experimental conditions for one of the species first and then apply the optimum conditions to the other two species and other related herbs. The species chosen for the optimization was *Euphorbia ebracteolata* Hayata. In the process to identify the optimum experimental conditions, different experimental conditions were used and the calculated Y values of the chromatograms obtained were compared. The choices of experimental conditions came from different combinations of nature of extraction solvent, extraction method, mobile phase composition, and gradient of mobile phase. Water, ethanol, methanol and petroleum ether were the extraction solvents used in the study. Samples were extracted by leaching, fast speed homogenization or ultra-sonication. Organic/aqueous and organic/buffer were the two major types of mobile phase used for HPLC separation. To increase the variety, different gradient settings for the mobile phase composition and the wavelength for the UV detection were chosen. By comparison of the Y values for chromatograms obtained from different experiment conditions, the optimized experimental conditions, for the *Euphorbia ebracteolata* Hayata extracts were identified. For example, the chromatogram and Y value obtained from a non-optimized experimental condition are shown in Figure 1 and Table 2 respectively. In the repeatability and reproducibility test for the determi-

Table 2. Comparison of the P, R and Y values obtained from two different experiment conditions for *Euphorbia ebracteolata* Hayata

	P value	R value	Y value
Non-optimized condition[a]	2.0	12.0	1.12
Optimized condition[b]	12.2	29.4	1.83

[a]Ethanol extraction followed by HPLC analysis using acetonitrile/potassium phosphate buffer (pH2.0) as the mobile phase system at 230nm as the detection wavelength.
[b]Condition as stated in MATERIALS AND METHODS.

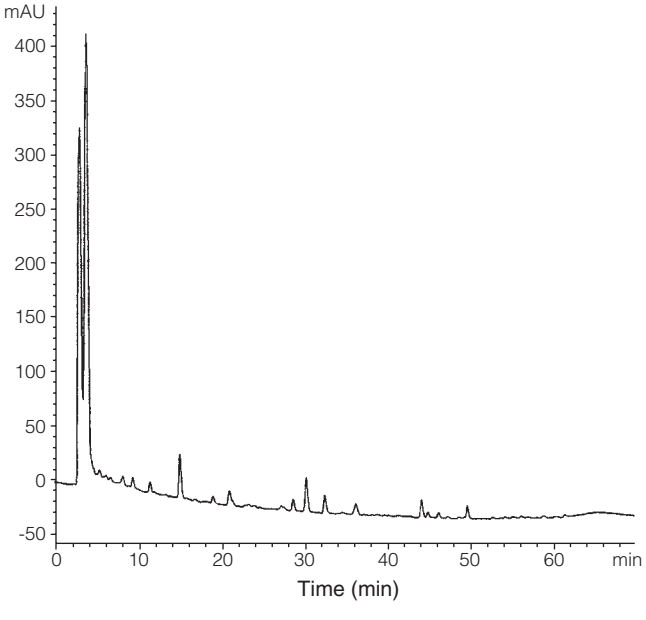

Figure 1. Fingerprint chromatogram of *Euphorbia ebracteolata* Hayata obtained from an non-optimized condition (ethanol extraction followed by HPLC analysis using acetonitrile/potassium phosphate buffer (pH 2.0) as the mobile phase system at 230nm as the detection wavelength)

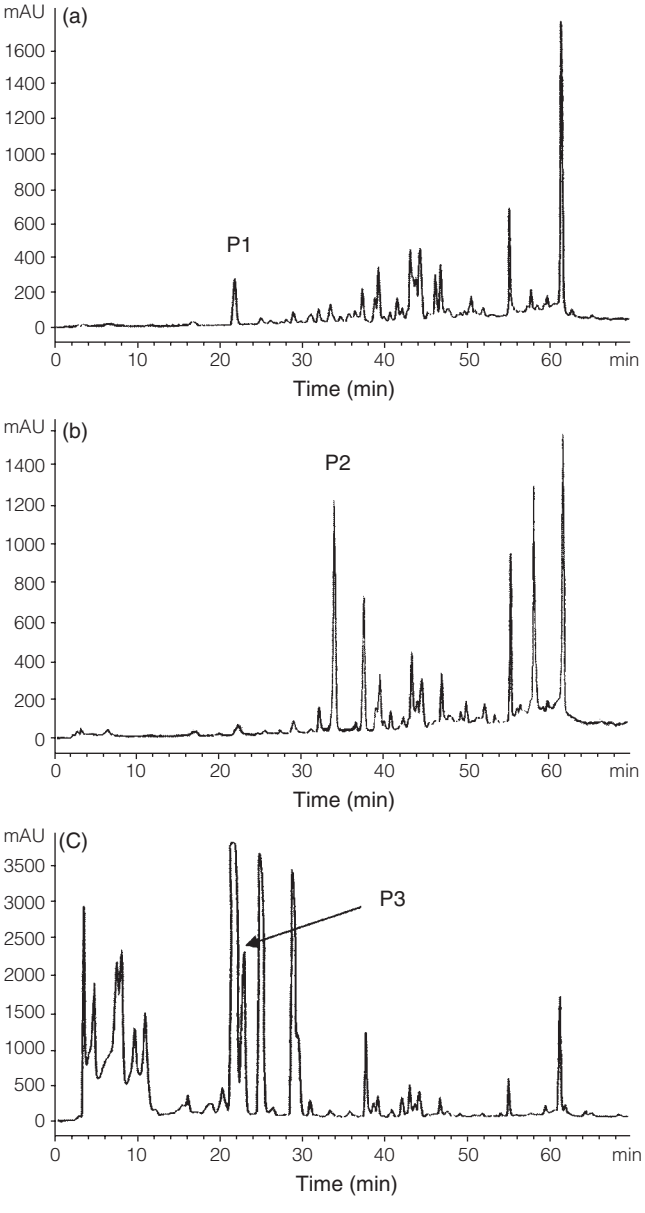

Figure 2. Fingerprint chromatograms of (a) *Euphorbia ebracteolata* Hayata, (B) *Euphorbia fischeriana* Steud, and (c) *Stellera chamaejasme* L. (Note: P1 = Ebracteolata compound B, P2 = Jolkinolide B and P3 = Neochanmaejasmin)

Journal of Food and Drug Analysis, Vol. 12, No. 2, 2004

nation of the Y value, the RSD value was found to be 1.7% and 3.2% respectively. Using the optimized experimental conditions obtained for *Euphorbia ebracteolata Hayata*, fingerprint chromatograms of other two species of Langdu i.e. *Euphorbia fischeriana* Steud and *Stellera chamaejasme* L., were obtained (Figure 2). It was observed that due to the different chromatographic patterns, these three species of Langdu could be easily differentiated.

In addition to characteristic pattern, common fingerprint analysis methods usually require identification of marker compounds of the herb in the fingerprint chromatogram. This is the other reason why we need a CRF to optimize the experimental conditions under which the number of intense peaks and their resolution in the chromatogram are maximized. To meet this requirement, peak tracking method using diode-array detector was employed to find out if the marker compounds of the herbal medicine could be identified in the fingerprint chromatograms. According to the literature[7-10], Ebracteolata compound b, Jolkinolide B and Neochamaejasmin were recognized as the characteristic marker compounds for *Euphorbia ebracteolata* Hayata, *Euphorbia fischeriana* Steud and *Stellera chamaejasme* L. respectively. These marker compounds were identified according to their distinctive UV spectra in their characteristic fingerprint chromatograms (Figures 2 and 3).

To test applicability of this method, 12 certified Langdu samples from different localities in China, including three *Euphorbia ebracteolata* Hayata, one *Euphorbia fischeriana* Steud and eight *Stellera chamaejasme* L., were analyzed using the optimized experimental conditions. It was found that identifications deduced from the fingerprint chromatograms matched exactly with the certified species identity of the individual Langdu samples. Furthermore, fingerprint chromatogram of two closely related herbs, namely *Euphorbia kansui* Liou and *Alocasia macrorrhiza* (L.) Schott, were also obtained under the established experimental conditions (Figures 4 and 5). The former is under the same genus as *Euphorbia ebracteolata* Hayata but has different medicinal uses, whereas the latter is named as Langdu by inhabitants of the Guangdong area. Again, great differences in the chromatographic patterns between these two chromatograms and those of the Langdu

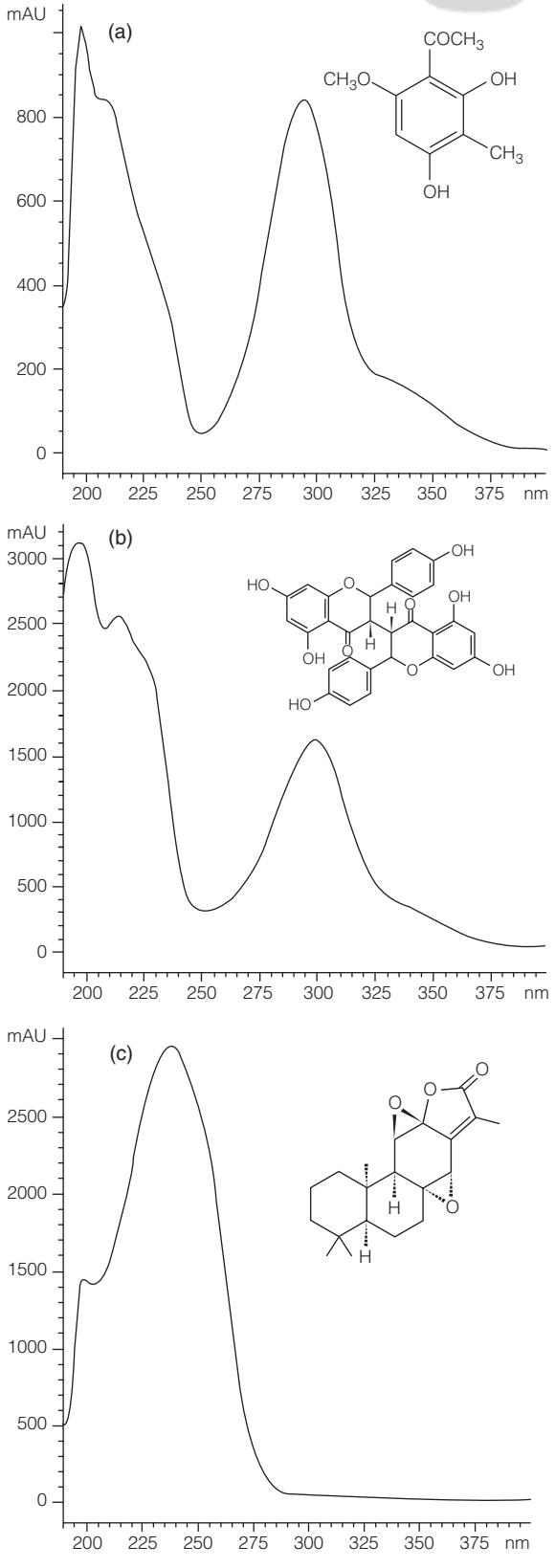

Figure 3. Chemical structure and UV spectrum of the marker compounds (a) Ebracteolata compound B, (b) Neochamaejasmin and (c) Jolkinolide B.

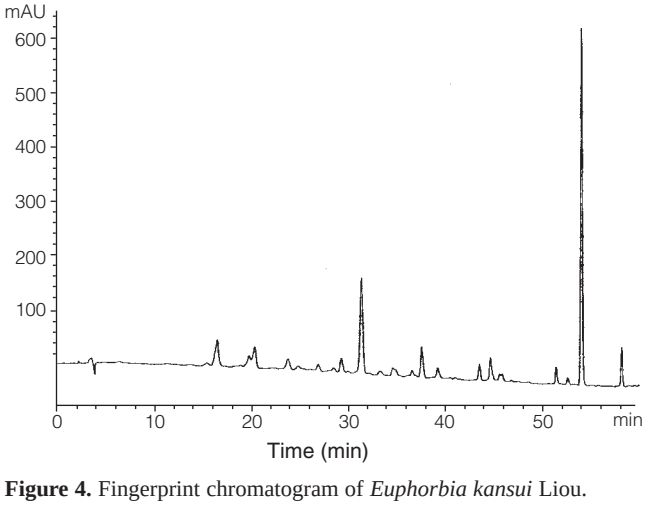

Figure 4. Fingerprint chromatogram of *Euphorbia kansui* Liou.

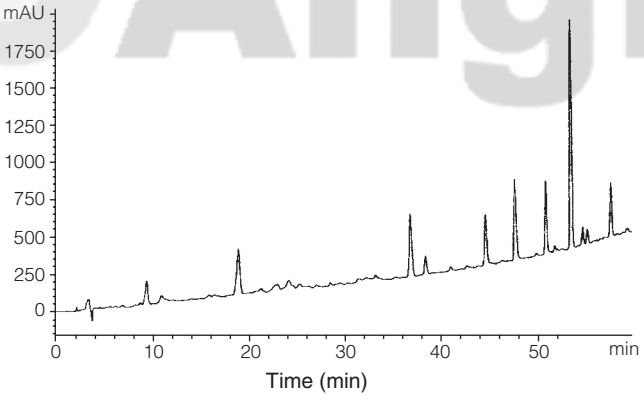

Figure 5. Fingerprint chromatogram of *Alocasia macrorrhiza* (L.) Schott.

species were observed. However, none of the marker compounds of the three Langdu species could be identified in these two fingerprint chromatograms.

CONCLUSION

A new chromatographic response function was introduced to identify the optimum experimental conditions for the establishment of characteristic fingerprint chromatogram. This paper demonstrated how this function could successfully applied to establish the fingerprint chromatogram for the differentiation of various Langdu species and two closely related species based on the fingerprint chromatograms. It is believed that the same analytical strategy can be carried out to obtain fingerprint chromatograms for the identification of other herbal material.

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