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Journal of Chromatography B, 792 (2003) 147–152
JOURNAL OF CHROMATOGRAPHY B
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Fingerprint analysis of *Flos Carthami* by capillary electrophoresis

Yi Sun[a], Tao Guo[b], Yin Sui[b], Famei Li[a,*]

[a]*Department of Analytical Chemistry, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China*
[b]*Department of Pharmacy, The General Hospital of Shenyang Military Region, Shenyang 110016, China*

Received 2 September 2002; received in revised form 10 March 2003; accepted 17 March 2003

Abstract

Capillary electrophoresis (CE) was employed in fingerprint analysis of *Flos Carthami*. A standardized procedure was used to develop the CE fingerprint. An electrophoretic profile of genuine *Flos Carthami* from Fengqiu, He'nan, China, was first established as the characteristic fingerprint. This profile was then used to identify and assess the consistency of the herb. A study with a limited number of samples from nine sources showed a fair consistency in their CE fingerprints with that of the genuine sample. *Flos Carthami* was well distinguished from *Stigma Croci*, a possible substitute in traditional Chinese medicine, and *Flos Hemerocallis*, a commercial adulterant, by comparing the fingerprints of each herb.

Keywords: Fingerprint analysis; *Flos Carthami*

1. Introduction

Fingerprint analysis has been introduced and accepted by WHO as a strategy for the assessment of herbal medicines [1]. Recently, fingerprint is also required by the Drug Administration Bureau of China to standardize injections made from traditional Chinese medicines and their raw materials [2]. The use of fingerprinting in herbs tends to focus on identifying and assessing the stability of the plants [3]. High-performance liquid chromatography (HPLC) fingerprint analysis has been reported as being used on some botanical medicines and their raw materials for similar purposes [3–5].

Flos Carthami, the dried flower of *Carthamus tinctorius* L., is a herbal medicine commonly used in China for the treatment of uterine congestion, cardiovascular diseases, thrombosis and high cholesterol [6]. The constituents in *Flos Carthami* include carthamone, carthamin, safflower yellow-pigment, safflower red-pigment, adenosine, rutin and quercetin [6]. Some of these constituents are known to exhibit pharmacological and biological activities. For example, rutin and quercetin were found to inhibit the mucosal content of platelet activating factor (PAF), and exhibit anti-inflammatory, anti-allergic, anti-cancer activities and a protective effect on cardiovascular diseases [7,8]. They are also potent, non-toxic ITH deiodinase inhibitors in microsomal membranes and intact hepatocytes [9]. Thin layer chromatography (TLC) identification of raw herbs and UV measurement of safflower yellow- and red-pigments are required for *Flos Carthami* in the Chinese Pharmacopoeia [10]. The determinations of safflower yellow- and red-pigments, adenosine, rutin and quercetin have been accomplished by using UV, CE and

*Corresponding author. Tel.: +86-24-2384-3711; fax: +86-24-8389-0024.
E-mail address: fameili@163.com (F. Li).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00255-1

HPLC [11–15]. This herb is grown and used in areas all over China, though *Flos Carthami* from Fengqiu and Yanjin, both in He'nan, China, is considered the original and genuine herbal medicine [16]. *Stigma Croci* is a possible substitute for *Flos Carthami* while dyed *Flos Hemerocallis* has been found as an adulterant in the market.

The aim of this study is to develop a characteristic fingerprint of *Flos Carthami* by using CE for identifying the raw herb. This fingerprint can help distinguish the substitute or adulterant, and further assess the differences of *Flos Carthami* grown in various areas of China.

2. Experimental

2.1. Instrumentation

CE separation was carried out on a HP^{3D} G1600A capillary electrophoresis system (Hewlett-Packard, USA), equipped with a photodiode array detector, a HP Chemstation and an automatic injector.

2.2. Reagents and materials

Adenosine, rutin and quercetin were provided by the National Institute for the Control of Pharmaceuticals and Biological Products, Beijing, China. Chromatographic grade methanol and other analytical grade chemicals were used. Genuine *Flos Carthami* was collected from Fengqiu, He'nan, China. Other *Flos Carthami* samples from different sources, *Stigma Croci* and *Flos Hemerocallis* were purchased from Shenyang Herbal Medicine Company. They were verified by professor Qishi Sun of Shenyang Pharmaceutical University of China as the dried flowers of *Carthamus tinctorius* L., the dried stigmas of *Crocus sativus* L. and the dried flowers of *Hemerocallis citrina* L., respectively.

2.3. Sample preparation

Three methods were used to prepare the extracts from *Flos Carthami*.

In method I, a 2.0-g sample of the dried flower was extracted with 24 ml ethanol–water solution (6:4, v/v) in an ultrasonic water bath for 30 min. This extraction was repeated three times. The extracted solution was combined and centrifuged at 1500 *g* for 10 min. The supernatant was concentrated by evaporation. A rifampicin solution of 1 ml (2.5 mg ml^{-1}) was added into the extract and diluted to 10 ml with methanol–water (6:4, v/v). This solution was then filtered through a 0.45-μm membrane filter and injected directly into the CE system.

In method II, a 2.0-g sample was extracted with 120 ml ethanol–water solution (6:4, v/v) using a Soxhlet apparatus for 1.5 h. The extract was then processed as in method I.

In method III, a 2.0-g sample was boiled using 20 ml distilled water for 30 min and this process was repeated three times. The combined extract was further concentrated to about 8 ml. A rifampicin solution of 1 ml (2.5 mg ml^{-1}) was added into the extract. The final solution was then diluted to 10 ml with redistilled water.

2.4. CE separation

The separation was performed using a 66.5 cm (58 cm to detector)×50 μm I.D. fused-silica capillary (Ruifeng Chromatography Instrument, Hebei, China). The sample was injected at a pressure of 50 mbar for 5 s. A 24 kV (positive to negative) voltage was applied and the detection was at 210 nm. The capillary temperature during electrophoresis was maintained at 20 °C. The running buffer was composed of 50 mmol l^{-1} borax solution (pH 9.7) containing 18% methanol. The capillary was washed with the running buffer for 8 min between runs.

3. Results and discussion

3.1. Identification of constituents

Three active constituents of *Flos Carthami* with available reference standards, adenosine, rutin and quercetin were used as marker compounds in the fingerprint analysis. A standard solution consisting of adenosine, rutin and quercetin was analysed under the same CE conditions as the samples. The resulting electropherogram is shown in Fig. 1a. The peaks of the samples were identified based on their UV spectra, migration times and standard addition. Peak 12 was identified as adenosine, 16 as rutin and 19 as quercetin (Fig. 1b).

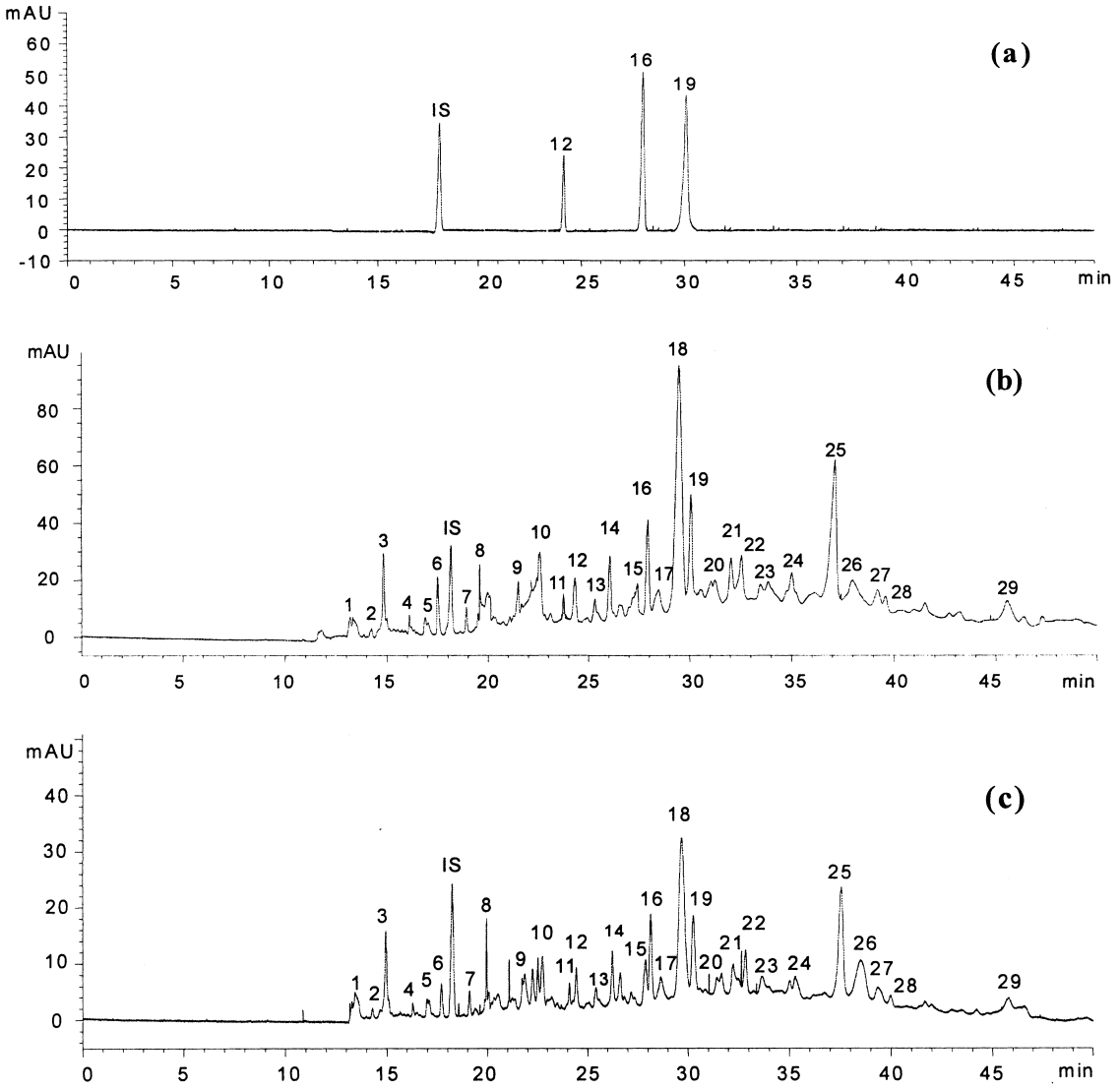

Fig. 1. Electropherogram of standard substance (a) and fingerprint of 60% ethanolic extract of *Flos Carthami* from Fengqiu (b) and Sichuan (c). Peaks represent 12 adenosine, 16 rutin, 19 quercetin, I.S. rifampicin. CE conditions: column, uncoated fused-silica capillary (58 cm/66.5 cm, 50 μm I.D.); running buffer, 50 mmol l^{-1} borax solution (pH 9.7) containing 18% methanol; voltage, 24 kV; detection, UV at 210 nm; injection, 50 mbar, 5 s; temperature, 20 °C.

3.2. Optimization of separation conditions

The criteria of the procedure are set to include: (a) the resolution between adenosine and its neighbouring peaks, as well as the resolutions between rutin, quercetin and their neighbouring peaks, together with (b) the number of peaks in the whole electropherogram, and (c) the separation duration for the process. Optimization of the parameters was done through investigating the influence of the buffer system, buffer concentration, pH, applied voltage, modifier, methanol concentration and temperature of capillary on the separation efficiency.

Methanol concentration indicated the greatest influence on the separation. Without methanol in the running buffer, adenosine and quercetin remained unseparated from their neighbouring peaks. Increasing the methanol concentration improved the res-

olution with compromise in analysis speed. When the methanol content of 18% was in the buffer system (pH 9.7), the separation process was completed within 50 min. The resolutions between adenosine, rutin, quercetin and their neighbouring peaks were 1.5, 1.2 and 1.3, respectively.

Phosphate, borax and Tris buffers of 10–100 mmol l^{-1} were also tested for their use as the running buffer in this separation. It was found that phosphate and borax provided higher separation efficiency than Tris. Good resolutions between peaks were obtained with buffers at high concentration (100 mmol l^{-1}) but required a separation time of 100 min for each run. The migration behaviour of constituents in the *Flos Carthami* and its dependency on the pH of the buffer was investigated with (a) a 50 mmol l^{-1} phosphate buffer at pH from 3 to 8.5; and (b) a 50 mmol l^{-1} borax buffer at pH from 8.5 to 10.5. The former buffer system proved unsatisfactory, while the latter buffer system achieved satisfactory separations at pH from 9 to 10, with an optimum at pH 9.7. Adding modifiers such as SDS and sodium deoxycholate deteriorated the column efficiency and gave no improvement to the separation. Finally, 50 mmol l^{-1} borax solution (pH 9.7) containing 18% methanol was chosen as the running buffer.

The influence of an applied voltage (20–30 kV) and the temperature of the capillary (15–25 °C) was examined. The electropherogram exhibited 29 peaks at an applied voltage of 24 kV compared with 27 peaks at 20 kV. Nevertheless, a reduction in resolution was observed when the applied voltage was at 30 kV due to the resulting Joule heat. A voltage of 24 kV was hence applied. A fingerprinting profile of *Flos Carthami* from Fengqiu under optimized conditions is shown in Fig. 1b. There are 29 peaks in the electropherogram, and most of them are well separated from each other.

3.3. Standardization of fingerprint

To develop characteristic fingerprints, the experimental procedure must be standardized. The sample preparation methods were evaluated for the extraction efficiency of marker compounds: adenosine, rutin and quercetin; and for the number of peaks in the electropherogram. It was found that both ultrasonic and reflux yielded 29 peaks while decoction produced 25 peaks only. Furthermore, both ultrasonic and reflux had an extraction recovery of over 95% for adenosine, rutin and quercetin measured with the standard addition. Due to its simplicity, ultrasonic extraction was selected as the proposed method. The method validation of fingerprint analysis was performed based on the relative migration time (the ratio of peak migration time of sample constituents to the internal standard) and the relative peak area (the ratio of peak area of sample constituents to the internal standard).

The sample solution was successively injected into the CE system and analysed five times. The intra-day precisions not exceeding 0.8% and 5.8% were obtained for relative migration times and relative peak areas of all peaks, respectively. The inter-day precisions of the proposed method, on the basis of analysing five replicate samples on separate days, were below 1.8% for relative migration times and within 6.2% for relative peak areas. The stability test was performed with sample solutions extracted from Fengqiu *Flos Carthami* for 24 h. The RSDs of the relative migration times and the relative peak areas were less than 8.0%. This indicated that the sample solution was stable for 24 h.

To standardize the fingerprint, 10 batches of *Flos Carthami* samples from Fengqiu were analysed with the developed procedure. The average electropherogram from the 10 batches was regarded as the standardized characteristic fingerprint of *Flos Carthami*. Peaks that existed in all 10 electropherograms were assigned as ‘‘common peaks’’ for *Flos Carthami*. There are 29 ‘‘common peaks’’ in the fingerprint (Fig. 1b). The whole electrophoretic profile, the peaks and their online UV spectra, together with the three marker compounds could provide a useful means of identifying and assessing *Flos Carthami*.

3.4. Consistency assessment of samples from various sources

The primary use of fingerprinting analysis may be in assessing the consistency of raw herbs from different sources. In this study, raw herb samples from nine sources in China were investigated. Materials were processed and CE profiles were determined by using the standardized procedure. The

''common peaks'' existed in all samples though the relative peak areas of a few showed variations to some extent. The largest variation was found in peaks 6 and 8, with both having an RSD of 12% in relative peak areas for the nine samples. An electropherogram of the sample from Sichuan exhibiting the largest variation is presented in Fig. 1c. From this study with a limited number of samples, it could be found that *Flos Carthami* samples from the nine sources exhibited a fair consistency in the constituents and their contents. A larger set of samples, however, is certainly needed for a solid conclusion. The result of this study is different from that observed for *Radix Angelicae* which showed a significant variation in the fingerprint with the origins (data not shown here).

3.5. Differentiation of raw herbs

The developed CE fingerprinting method was used to distinguish possible substitutes or adulterants from *Flos Carthami*. Samples of *Stigma Croci*, a herb of similar use in traditional Chinese medicine, that also has a similar Chinese name, and *Flos Hemerocallis*, an adulterant, were prepared by using the same procedure as described above to determine their fingerprints. The CE electropherograms (Fig. 2) of these two herbs are very different from the profile of *Flos Carthami* in the number of peaks and migration times of the peaks. In the electropherogram (Fig. 2a) of *Stigma Croci*, the first peak appeared much earlier than that of *Flos Carthami*, at about 9 min, and showed much fewer constituents than in *Flos Carthami* at 210 nm. The UV spectra of peaks were compared with those at similar migration times in *Flos Carthami*, and only quercetin could be identified (as marked with an asterisk in Fig. 2a). The results indicate that although *Stigma Croci* may be used as a substitute of *Flos Carthami* in some cases, their constituent patterns are vastly different. In the case of *Flos Hemerocallis*, the difference in fingerprint profile (Fig. 2b) is more significant when

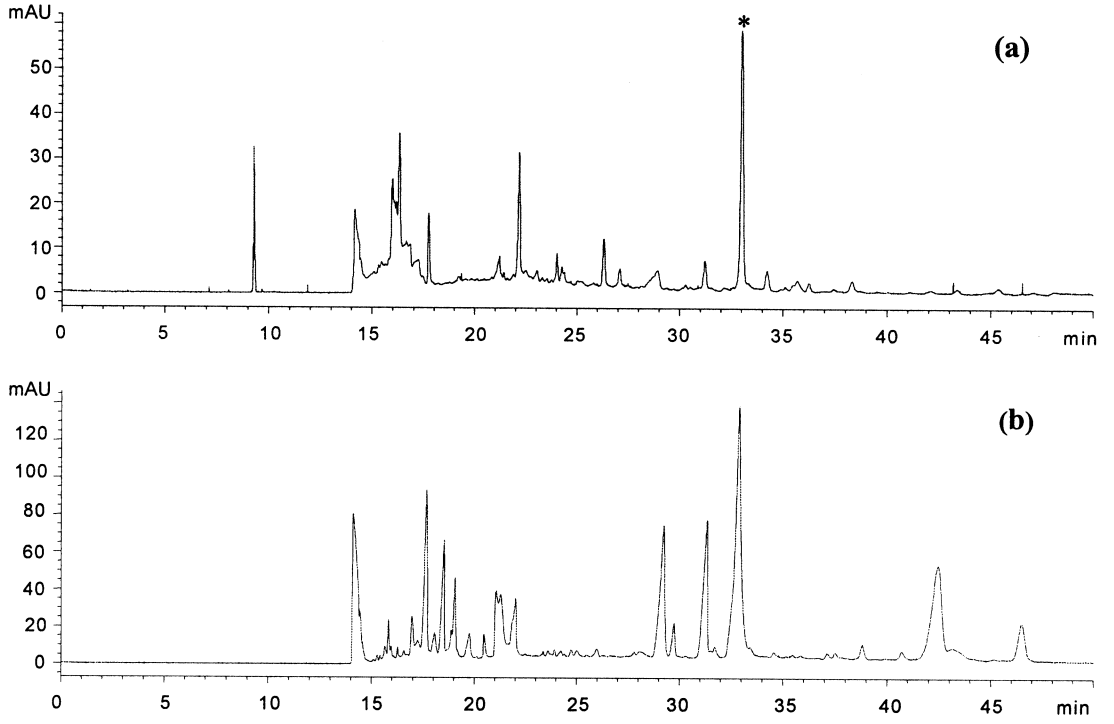

Fig. 2. Fingerprint of 60% ethanolic extract of *Stigma Croci* (a) and *Flos Hemerocallis* (b). Peak marked with an asterisk represents quercetin. The CE conditions are the same as in Fig. 1.

compared with that of *Flos Carthami*. No UV spectrum match was found for any peaks in the electropherograms. Fingerprinting analysis should be able to distinguish the adulterant from *Flos Carthami*.

Studying the species identification of the *Carthamus* family would be of great importance in demonstrating the potential of CE fingerprinting. Unfortunately, only this one species, *Carthamus tinctorius* L. is grown in China and available to us.

4. Conclusion

A CE method was developed for fingerprint analysis of *Flos Carthami*. The fingerprints of 10 batches of *Flos Carthami* from Fengqiu were obtained with a standardized procedure. The fingerprint of the genuine herb showing 29 ''common peaks'' represents the characteristics of this herb's constituents. The primary application in qualitative identification and consistency assessment of *Flos Carthami* was investigated with a limited number of samples.

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