Fingerprint Analysis of *Flos Carthami* by Pressurized CEC and LC
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**Abstract:**
A fingerprint analysis of *Flos Carthami* was performed using a standardized capillary electrochromatography (CEC) procedure. This procedure was first used to establish the electrochromatographic profile of genuine *Flos Carthami* from Tacheng, Xinjiang, China. This *Flos Carthami* fingerprint was then used to identify and assess the consistency of raw herbs from different sources in China. The study of a limited number of samples from ten different sources demonstrated a reasonable consistency among their CEC fingerprints relative to that of the genuine sample. Using this technique, we can readily distinguish the fingerprint of *Flos Carthami* from that of *Stigma Croci*, a possible substitute in traditional Chinese medicine, and *Flos Hemerocallis*, a commercial adulterant. A method based on high performance liquid chromatography (HPLC) is described to establish fingerprints of *Flos Carthami* simultaneously. The fingerprints obtained with HPLC consist of 21 common peaks within 65 min while 43 common peaks obtained with CEC. CEC showed better performance on fingerprinting of hydroxysaffloryellow A and its neighboring peaks, which contained more chemical information than that of HPLC. It was proven that CEC could be a feasible and effective method for development of fingerprint of TCM based on the comparison with HPLC.

**Article:**
**INTRODUCTION**
Fingerprint analysis has been introduced and accepted by the US Food and Drug Administration (FDA) as one of the requirements for botanical preparations [1] and by the European Agency for the Evaluation of Medicinal Products for herbal preparations [2]. Furthermore, fingerprint analysis has been introduced and accepted by the World Health Organization (WHO) as a strategy for assessing herbal medicines [3]. Recently, fingerprinting has also been required by the State Food and Drug Administration Bureau of China for the standardization of injections made from TCMs or their raw materials [4]. For herbs, fingerprinting is primarily used for identification and assessment of the stability of the plants [5]. High-performance liquid chromatography (HPLC) fingerprint analysis has reportedly been used for similar purposes with some botanical medicines and their raw materials [5, 6].
Flos Carthami, the dried flower of Carthamus tinctorius L., has long been used as a food colorant, dye, and flavoring agent [7] in oriental countries. Now, it is being used as an herbal medicine in China for the treatment of uterine congestion, cardiovascular diseases, thrombosis, and high cholesterol [8]. Some of its constituents are known to exhibit pharmacological and biological activities. For example, hydroxysaffloryellow has been shown to restrain the conglomeration of platelet, promote blood circulation, remove blood stasis, anti-oxidation, and promote metabolism [9]. According to the Chinese Pharmacopoeia, TLC is required for the identification of raw herbs and the UV measurement of safflower yellow- and red-pigments in Flos Carthami [10]. The quantitation of safflower yellow- and red-pigments, adenosine, rutin, and quercetin in Flos Carthami using UV, CE, and HPLC has been reported previously [11–17]. Stigma Croci (also known as Fan Honghua, Saffron) is known to be a possible substitute for Flos Carthami, and Flos Hemerocallis has been found as a common adulterant in the market.

The application of capillary electrophoresis (CE) in fingerprint analysis was limited in China; although, the CE fingerprint of ethanol–water extracts of Flos Carthami were reported [18]. There were only 15 peaks observed in the HPLC fingerprint of water extracts from Flos Carthami been previously reported [19]. HPLC has high precision, sensitivity and reproducibility for fingerprinting TCM, but HPLC is not suitable for analysis of some highly viscous samples. While CE has high speed, efficiency, ultra small sample volume, and minimal consumption of solvent, but the reproducibility and the selectivity are not as good as in HPLC. Capillary electrochromatography (CEC) is a hybrid technique that combines the selectivity of HPLC and the separation efficiency of CE. CEC can provide more chemical information of the raw herbs and which will be more powerful in differentiating geographical origins. Now CEC has become an attractive technique for pharmaceutical analysis because it provides highly efficient and rapid separations. So we intend to develop a characteristic fingerprint of Flos Carthami by CEC that can be used to identify and control the quality of the raw herbs. This fingerprint can help to distinguish between Flos Carthami and its substitutes or adulterants and can be used to assess variations in Flos Carthami that are grown in the different areas of China.

EXPERIMENTAL

Apparatus

CEC separation was carried out using a Trisep™-2100 capillary electrochromatography system (Trisep™-2100, Unimicro Technologies Inc.) consist of a Unimicro binary CEC pump system equipped with a UV detector and a Unimicro Trisep™ workstation 2003. A 50 cm (packed to 30 cm) × 150 μm I.D. reversed-phase column (EP-150-30/50-5-C18, Unimicro Technologies Inc.) was used. Detection windows (~2 mm long) were burned into the column walls, approximately 2 mm downstream of the outlet frit. A voltage of −5 kV was applied across the capillary to produce EOF. The data was collected directly from the UV detector (using a sample wavelength of 275 nm) and analyzed using the Unimicro Trisep™ workstation 2003. The final CEC mobile phase used water (0.02% TFA) (A) and 95% aqueous methanol (0.02%TFA) (B) that was filtered using 0.2 μm HPLC filters. The separation was performed using the following gradient: 0–10 min, 2–15% B; 10–40 min, 15–40% B; 40–65 min, 45–100% B. Prior to use, the samples and CEC mobile phases were sonicated in an ultrasonic bath for 10 min at room temperature to remove any air bubbles.
An Agilent 1100 liquid chromatography (Agilent, USA) equipped with quaternary gradient pump and UV detection system was used. A HPLC method was developed using a reversed-phase column (Elite Symmetry C18, 250 × 4.6 mm I.D., 5 μm). The binary gradient elution system consisted of A (water + 0.5% H3PO4) and B (acetonitrile) and separation was achieved using the following gradient: 0–55 min, 0–25% B; 55–65 min, 25–0% B. The column temperature was kept constant at 25 °C. The flow-rate was 1 mL min⁻¹ and the injection volume was 10 μL. The UV detection wavelength was set at 275 nm.

Reagents and Materials
Hydroxysaffloryellow A was provided by the National Institute for the Control of Pharmaceutics and Biological Products, Beijing, China. Chromatographic grade methanol, acetonitrile and other analytical grade chemicals were used. HPLC water was prepared with the Millipore Milli-Q SP water purification system (18.2 MΩ, Milipore, Bedford, MA, USA). All aqueous solutions were prepared with this HPLC water. Genuine Flos Carthami was collected from Tacheng, Xinjiang, China. Other Flos Carthami samples from different sources and Stigma Croci were purchased from Shanghai Huayu Chinese Herbs Co. Ltd. Flos Hemerocallis was purchased from Shanghai Herbal Medicine Company. A professor of Shanghai Jiao Tong University of China verified that the samples were indeed the dried flowers of C. tinctorius L., the dried stigmas of Crocus sativus L. and the dried flowers of Hemerocallis citrine L., respectively.

Sample Preparation
Samples were pulverized, and the resulting powder was screened through 180 μm sieves. Fine powder (1.0 g) was weighed, and 10 mL of water was added. Then, the powder was extracted in a water bath at 50 °C for 120 min. This extraction was repeated three times. After cooling, the solution was filtered and concentrated. The residue was then dissolved in water by ultrasonication and transferred to a 25-mL volumetric flask. This solution was filtered through a syringe filter (0.45 μm) and injected directly into the CEC or HPLC system.

Standard Sample Preparation
Standard sample of hydroxysaffloryellow A (0.18 mg mL⁻¹) was prepared with ultra pure water.

RESULTS AND DISCUSSION
Choice of Extract Solvent
Saffloryellow pigment is the mixture of flavone compounds. These compounds are polar substances easily dissolved in water and ethanol. To determine the optimal extract solvent, we tested extractions in water, 30% ethanol, 70% ethanol and 95% ethanol, and the amount and content of hydroxysaffloryellow A was used as the index. We found that the maximum amount of hydroxysaffloryellow A was detected when water was used as the extraction solvent. Additionally, when water was used as the extract solvent, there was no excess work or difficulties with nonpolar compounds, as they are hardly extracted with water. Therefore, we selected water as our extract solvent for the subsequent studies.

Identification of Hydroxysaffloryellow A with CEC
The main constituents of the Flos Carthami water extracts were saffloryellow pigments. Hydroxysaffloryellow A is a compound that is isolated from saffloryellow pigments [20]. Therefore, the active constituent hydroxysaffloryellow A was used as a marker compound in the
Flos Carthami fingerprint analysis. A standard solution of hydroxysaffloryellow A was analyzed under the same CEC conditions as the samples. The resulting electrochromatogram is shown in Fig. 1a. The peaks of the samples were identified based on their UV spectra, migration times, and the standard hydroxysaffloryellow A. Peak 27 was identified as hydroxysaffloryellow A (Fig. 1b).

![Figure 1: Chromatogram of marker compound hydroxysaffloryellow A (a), fingerprint of water extract of Flos Carthami (b), Tacheng (c), and solvent (d). Peak 27 was the peak of hydroxysaffloryellow A. CEC conditions: column, EP-150-30/50-5-C18, Unimicro Technologies Inc. (30 cm/50 cm, 150 μm I.D.); voltage, −5 kV; detection, UV at 275 nm; mobile phase, water (0.02% TFA) (A) and 95% aqueous methanol (0.02%TFA) (B)](image)

Optimization of Separation Conditions of CEC
The criteria of the separation procedure included the following: (a) the peak shape of hydroxysaffloryellow A and the resolution between it and its neighboring peaks, as well as the resolutions between all of the peaks, (b) the number of peaks in the whole electrochromatogram, and (c) the duration of the separation process. Several parameters were examined to achieve good separation, including the mobile phase composition, the buffer system, and the applied voltage.

The binary gradient elution system consisted of water (0.02% TFA) (A)–95% aqueous acetonitrile (0.02 TFA) (B), 2% aqueous acetonitrile (0.02% TFA)–90% aqueous acetonitrile (0.02%TFA) (B), and water (0.02% TFA) (A)–95% methanol (0.02%TFA) (B) were tested during the separation. Phosphate and trifluoroacetate of which the current generated were also tested as potential running buffers in this separation scheme. From the results we found that good resolutions between peaks were obtained with mobile phase consisting of water (0.02% TFA) (A) and 95% aqueous methanol (0.02%TFA) (B) when using the following gradient: 0–10 min, 2–15% B; 10–40 min, 15–40% B; 40–65 min, 45–100% B. Under these conditions, the
resolution between the peak of hydroxysaffloryellow A and its neighboring peak was 1.47. From
the examination of the effect of an applied voltage (−10 to +10 kV) we found that the resolution
between peaks were good and no bubbles appeared when applying a voltage of −5 kV. The peaks
eluted with negative and positive voltage were the same on the whole. A fingerprinting profile of
*Flos Carthami* from Tacheng performed under these optimized conditions is shown in Fig. 1c.
There are 43 well-separated peaks in the electrochromatogram.

**Standardization of Fingerprint of the Raw Herb by CEC**
To develop characteristic fingerprints of compounds, all of the experimental procedures,
including the extraction and analytical conditions and methods, must be standardized. The
determination of the analytical conditions and methods were described earlier. Methods for
extracting raw herbs were evaluated by examining the efficiency of extracting the marker
compound, hydroxysaffloryellow A, and the number of peaks in the electrochromatogram. A set
of orthogonal tests based on the proportions of the flowers and the water, extraction temperature
(°C), time (min), and extraction times were used. We found that the maximum amount of
hydroxysaffloryellow A was detected when a 1.0 g sample of dried flower powder was extracted
with water (10 mL × 3) in a 50 °C water bath for 120 min. 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 reference peak of hydroxysaffloryellow A) and the relative peak area
(the ratio of peak area of sample constituents to the reference peak of hydroxysaffloryellow A).

The sample solution was successively injected into the CEC system and analyzed five times. The
precisions not exceeding 1.5 and 5.8% were obtained for the relative migration times and the
relative peak areas of all peaks, respectively. The inter-day precisions of the proposed method
(the five replicate samples were analyzed on separate days) were below 1.8% for the relative
migration times and within 6.2% for the relative peak areas. Stability tests were performed with
sample solutions extracted from Fengqiu *Flos Carthami* for 24 h. The relative standard
deviations (RSDs) of the relative migration times and the relative peak areas were less than
5.8%. This result indicates that the sample solution is stable for 24 h and is suitable for this
analysis. A reproducibility test was performed on results from the analysis of five samples
prepared from the same batch of the dried flowers. The findings demonstrated that the RSDs of
the relative retention times and the relative peak areas were less than 5.0%.

To standardize the characteristic fingerprint of the raw herb, ten batches of 1.0 g of Tacheng *Flos
Carthami* samples were mixed homogeneously. A 1.0 g sample was taken from that mixture and
analyzed by the above-described procedure. The average electrochromatogram from the ten
batches was regarded as the standardized characteristic fingerprint of *Flos Carthami*. Peaks
present in all ten of the electrochromatograms were labeled as *Flos Carthami* “common peaks”.
We observed 43 “common peaks” in the *Flos Carthami* fingerprint (Fig. 1b). The entire
electrochromatographic profile, which consists of all the peaks together with the marker
compound hydroxysaffloryellow A, can be a useful tool for the identification and assessment of
*Flos Carthami*.

**Fingerprint Development of Flos Carthami by HPLC**
HPLC has been applied to develop the fingerprint of *Flos Carthami* in our study [21]. In order to
obtain good resolution and a large number of peaks, optimization of separation conditions in
HPLC was done through investigating the influence of the mobile phase, the detection wavelength, and the gradient mode. The optimum mobile phase was A (water + 0.5% H₃PO₄) and B (acetonitrile) in the gradient mode as follows: 0–55 min, 0–25% B; 55–65 min, 25–0% B. The column temperature was kept constant at 25 °C. The flow-rate was 1 mL min⁻¹ and the injection volume was 10 μL. There are about 40 peaks within 65 min in the chromatogram. Among these, 21 common peaks (shown in Fig. 2) were found in the standard fingerprint of *Flos Carthami*. Twenty-one common peaks, which appeared in the fingerprint of the genuine herb, represented the characteristic of the herb’s constituents, and the result of the relative values of the ten samples, the peaks, together with the index compound hydroxysafflor yellow A, could provide a useful means of identifying and assessing *Flos Carthami*.

![HPLC chromatogram of Flos Carthami](image)

**Figure 2**: HPLC chromatogram of *Flos Carthami*. Peak 11 was the peak of hydroxysafflor yellow A. HPLC conditions: Elite Symmetry C₁₈ column (250 × 4.6 mm I.D., 5 μm); mobile phase was solvent A (water + 0.5% H₃PO₄) and B (acetonitrile) in the gradient mode as follows: 0–55 min, 0–25% B; 55–65 min, 25–0% B; detection, UV at 275 nm.

**Comparison of CEC and HPLC Fingerprinting**
Both CEC and HPLC could display the whole concentration distribution of different kinds of components, which is the most important character of fingerprint. Characteristics of CEC and HPLC methods used to develop TCM fingerprints are summarized in Table 1.

<table>
<thead>
<tr>
<th>Content of comparison</th>
<th>CEC</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Separation column</td>
<td>50 cm (30 cm was packed) × 150 μm (I.D.)</td>
<td>250 mm × 4.6 mm (I.D.)</td>
</tr>
<tr>
<td>Sample size</td>
<td>ng</td>
<td>μg</td>
</tr>
<tr>
<td>Volume of crude sample</td>
<td>10 nL</td>
<td>10 μL</td>
</tr>
<tr>
<td>Temperature</td>
<td>Non-controlled</td>
<td>25 °C</td>
</tr>
<tr>
<td>Analysis time</td>
<td>65 min</td>
<td>65 min</td>
</tr>
<tr>
<td>Total peak number</td>
<td>50–55</td>
<td>37–45</td>
</tr>
<tr>
<td>Common peaks</td>
<td>43</td>
<td>21</td>
</tr>
<tr>
<td>Non-common peak area</td>
<td>&lt;1.5%</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>Average of RSD of retention time (%)</td>
<td>3.7</td>
<td>2.7</td>
</tr>
</tbody>
</table>

The numbers of common peaks, non-common peak area, reproducibility and analysis time were key factors. The sample loaded on CEC was very small (10 nL), which led to relatively low stability. Anyway precisions of CEC and HPLC were preferable for fingerprinting. As shown in
Table 1, HPLC had advantages in precision and stability, and CEC had advantages in the number of common peaks and non-common peak area. The fingerprints from CEC and HPLC both meet the Chinese National Standards, and are suitable to identify *Flos Carthami*, to distinguish it from closely related species and for quality control. The CEC fingerprints of *Flos Carthami* which contain more characteristic peaks are obviously more powerful in differentiating geographical origins.

**Application of Standardized Characteristic Fingerprints of the Raw Herbs**

Fingerprinting analysis can be used to assess the consistency of raw herbs that come from different sources. For example, using a standardized procedure for the generation of CEC fingerprints, we can compare the electrochromatograms of raw herbs with those of known, characteristic compounds. By examining the number of “common peaks”, the relative retention time of each peak, and the relative peak area, we can determine whether a raw herb is genuine. But the most important application of fingerprints is that it can be used to distinguish between *Flos Carthami* and its possible substitutes or shams. To demonstrate this application, we prepared samples of *Stigma Croci*, a Chinese herb (also known as Fan Honghua, Saffron) with uses similar to *Flos Carthami* in traditional Chinese medicine, and *Flos Hemerocallis*, a *Flos Carthami* adulterant. These samples were prepared using the same procedures described above and were used to determine their fingerprints. The CEC electrochromatograms of these two herbs (Fig. 3) are very different from that of *Flos Carthami* in both the number of peaks and the migration times of the peaks. For example, in the electrochromatogram of *Stigma Croci* (Fig. 3a), the areas and heights of the peaks from 0 to 30 min are smaller than those of the *Flos Carthami* peaks from the same time frame. However, after 30 min, the two herbs showed similar peak structure at 275 nm. These results demonstrate that although *Stigma Croci* may be used as a substitute of *Flos Carthami* in the treatment of some diseases, their fingerprint profiles are vastly different. The fingerprint of *Flos Hemerocallis*, the *Flos Carthami* adulterant, (Fig. 3b) is very different from that of *Flos Carthami*. Less UV spectrum match was detected between the electrochromatograms. Therefore, *Flos Carthami* can be distinguished from its coordinal species by examination of fingerprint profiles, and thus be determined to be genuine or not.

**CONCLUSION**

A CEC and an HPLC method were developed for fingerprint analysis of *Flos Carthami*. The fingerprints of ten batches of Tacheng *Flos Carthami* were determined using a standardized
procedure. The fingerprint of the genuine *Flos Carthami* herb displays 43 “common peaks” that represent the characteristics of this herb’s constituents while the HPLC chromatogram consist of 21 common peaks. CEC showed better performance on fingerprinting of hydroxysaffloryellow A and its compounds, and contained more chemical information than that of HPLC. It was proven that CEC could be a feasible and effective method for development of fingerprints of TCM based on the comparison with HPLC. We used this CEC fingerprinting method to distinguish between *Flos Carthami* from different sources as well as between *Flos Carthami* and its substitutes and shams. Therefore, this technique can be used for the qualitative identification and consistency assessment of *Flos Carthami*.

CEC, as a new method in fingerprinting, should be further studied in following aspects: accuracy, repeatability, detection limit and other factors.

REFERENCES