Establishment of HPLC-DAD-MS Fingerprint of Fresh *Houttuynia cordata*

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A HPLC-DAD-MS fingerprint method of fresh *Houttuynia cordata* THUNB. was developed basing on the consistent chromatographic features among 11 batches of authentic samples. Major chemical components including phenolic compounds, flavones and alkaloids were simultaneously analyzed. Eleven common peaks in the fingerprint were chosen and identified by comparing their UV and ESI-MS data with the authentic compounds. The unique properties of this HPLC-DAD-MS fingerprint were successfully applied to analyze and differentiate samples from different geographical origins, processing methods and various medicinal parts of *H. cordata*. The results showed that these variations will give rise to differences in identities and/or abundance of chemical compounds, indicating that a comprehensive quality evaluation of those major ingredients in *H. cordata* is critical to assess and represent its overall quality.

**Key words** fingerprint; *Houttuynia cordata*; HPLC-DAD-MS; quality control

Chromatographic fingerprinting technique is a useful and facile tool for the quality evaluation of herbal medicine and their related products. This technique has been introduced and accepted by WHO as a strategy for the quality assessment of herbal medicines in early 1990s. Among those commonly used methods, HPLC-DAD-MS technique has been used widely for the simultaneous qualitative and quantitative analysis of herbal medicine and their related products, with on-line UV and mass spectrometric information available for each individual peak in the chromatogram. In this regard, HPLC-DAD-MS fingerprint analysis has recently been considered as a more comprehensive and powerful technique for establishment of chromatographic fingerprinting.

Fresh *Houttuynia cordata* THUNB. has been a well known traditional Chinese medicinal material widely used in China, Japan and also listed in the Chinese Pharmacopoeia (2005 edition). It possesses a variety of pharmacological activities including anti-platelet aggregation, antibacterial, antitumor, antimicrobial, anti-inflammatory, antileukemic, immunomodulatory and recently, demonstrated its efficacy in anti-SARS. In general, the chemical components of *H. cordata* comprised of three major types, namely: essential oil, phenols and alkaloid components.

How, however, most of the previous related studies were mainly focused on the chemistry of essential oil, which has been considered responsible for the claimed clinical efficacy. Recent pharmacological studies also revealed that the flavonoid components in *H. cordata* possess antineoplastic, antioxidant, antimutagenic and free radical scavenging capacity. Similarly, the alkaloid components demonstrated significant potent anti-platelet and cytotoxic activities, whereas chlorogenic acid had significant antiplatelet and antibiotic properties. Although a variety of pharmacological activities associated with these groups of chemical in *H. cordata* was demonstrated, the common used quality standard of *H. cordata* is still relied merely on the content of undecanone which served as a marker compound for the quality evaluation standard as stipulated in the Chinese Pharmacopoeia (2005 edition). This phenomenon stimulated our thought to develop a chromatographic fingerprinting technique to associate various pharmacological activities with the chemical profile and thus the quality of *H. cordata* and its related products. Moreover, as the crude herbs are generally cultivated for use over China, a vast variety of external factors can therefore affect the chemical composition, and thus the pharmacological activity of that particular herbal medicine. These factors include geographical origin for cultivation, drying methods used, different medicinal portions, etc. In this regard, a facile and comprehensive means for evaluating the overall quality of *H. cordata* is urgently needed.

In literature, related analyses on *H. cordata* species are particularly focused on TLC, GC, GC-MS and a few of them on HPLC. Recently, a method using GC-MS fingerprinting for quality evaluation was established by our research group. In that part of work, GC-MS was employed to analyze for the volatile components in *H. cordata* but not yet covered with other phenolic and alkaloid-typed components. In fact, there still exists an analytical void for simultaneous detection of alkaloids, phenols and other major chemical compounds in *H. cordata* for comprehensive quality evaluation purpose. Among the various chromatographic methods available, HPLC-DAD-MS is proven particularly useful for the purpose.

The present study aims at developing a HPLC-DAD-MS fingerprint of *H. cordata* and characterized the major active chemical constituents of *H. cordata*. The developed fingerprinting comprises of all the major chemical components of *H. cordata* and useful in reflecting the quality and associated with the clinical efficacy of *H. cordata*. The developed HPLC-DAD-MS fingerprint was then applied to differentiate *H. cordata* among various cultivation areas, different drying methods and also demonstrated applicable to distinguish among different medicinal portions.

**Experimental**

**Instrumentation** An Agilent 1100 series HPLC system was equipped with a photodiode array detector (DAD). An Alltima C18 analytical column (150 mm×4.6 mm, 5 μm, Alltech Associates, Inc., U.S.A.) coupled with a C18 guard column (7.5 mm×4.6 mm, 5 μm, Alltech Associates, Inc., U.S.A.)
was used at room temperature. The mobile phase consisted of 0.2% acetic acid in water (A) and acetonitrile (B) using a gradient program of 20% (B) in 0—30 min, 20—21% (B) in 30—50 min, 21—70% (B) in 50—80 min. The flow rate was 0.6 ml/min. DAD detector was set at 280 nm for acquiring chromatograms. The injection volume was 10 μl. The data were obtained and processed with the software of Chemstation for LC3D (Hewlett Packard, U.S.A.).

The mass spectrometer was equipped with an ESI source. The interface and MS parameters were as follows: dry gas, N₂ (7 l/min); dry gas temperature, 400 °C; for positive ion mode: sprayer voltage, 5000 V; orifice voltage: 101 V; focusing ring voltage: 380 V; for negative ion mode: S.V.: ~4000 V; O.V.: ~101, F.R.V.: ~380 V; scan range, m/z 130—800. All data acquired were processed by MacC Chrom 1.1 software (Applied Biosystem, CA, U.S.A.).

Solvents and Chemicals Methanol (Analytical grade), glacial acetic acid (analytical grade) and acetonitrile (HPLC grade) were purchased from E. Merck (Darmstadt, Germany). Deionized water was obtained from a Milli-Q water system (Millipore, Bedford, MA, U.S.A.).

All the standard reference chemical compounds were extracted, isolated and purified from fresh *H. cordata* in our laboratory. Their purities were shown to be higher than 96% by HPLC analysis, and their structures were elucidated by comparison their spectroscopic data (ESI-MS, 1H- and 13C-NMR, HMBC, HMOC and H- COSY) with references.15,28—36 The detailed procedures for isolation and spectrometric identification of these compounds will be reported in another paper.

Plant Materials Samples of fresh *H. cordata* were collected from the cultivation areas in Sichuan, Guangdong provinces and retailers in Hong Kong of China. All the samples were authenticated by one of the authors, Prof. Zhong-Zhen ZHAO. Freeze-dried processes were as follows: soon after collection, samples were placed in a ~20 °C freezer. After pre-cooling for at least 12 h, samples were placed in the freeze-drying system (Model 77530, LABCONCO Co., Switzerland) for further 48 h, and finally sealed in air-tight containers.

Sample Preparation 0.5 g fine powder was accurately weighed and extracted with 50 ml of 50% methanol in ultrasonic processor (1875HTAG, about 4 °C before use.

Results and Discussion

Optimization of HPLC Systems Photodiode array detector (DAD) was used in the current study. The detection wavelength at 280 nm gave the best abundance for target compounds within the chromatographic windows.

Method precision was based on replicated analyzing of samples, with reported relative standard deviations (RSD) of 0.27% and 0.11% for relative retention time (RRT) and relative peak area (RPA) of all peaks, respectively. The method reproducibility was studied through six replicated sample solutions extracted from a single batch of *H. cordata*. The corresponding RSD of RRT and RPA were reported less than 3% over 2 d of investigation. The stability test was performed with a sample solution over 24 h of standing period. The RSD of the RRT and RPA were found less than 0.25% and 2.95%, respectively. The result indicated that the developed method was validated and applicable for sample analysis.

HPLC Fingerprint of Fresh *H. cordata* To establish a representative chromatographic fingerprint for fresh *H. cordata*, 11 authentic batches of fresh *H. cordata* acquired from EMei (major production area in Sichuan province, P. R. China) (EM-1 to EM-11) were analyzed using the established HPLC method. Among the acquired chromatograms, representative peaks existing in all batches of samples were assigned as common peaks for *H. cordata*. Altogether there were eleven common peaks and compounds identified in the fingerprint (Figs. 1, 2). Peak 7 (Quercitin) indicated the highest content among others, with good resolution and pharmacological activities in other studies.26,37 Therefore, it has been chosen as the reference peak. The RRT and RPA of all common peaks with respect to this reference compound were obtained. The entire HPLC profiles with the information on RRT and RPA of the common peaks will be used to identify and assess the quality of *H. cordata*.

HPLC-MS Analysis for Components Identification In order to identify structures of the major chemical compounds in *H. cordata*, the sample was analyzed by HPLC-DAD-MS techniques. ESI in both negative and positive modes were performed. The results showed that ESI in negative mode was particularly sensitive to the flavones while ESI in positive mode was useful to detect the alkaloids. By studying on the characteristic mass spectra of these peaks and comparing with the UV and ESI-MS spectra with the respective authentic compounds, 11 common peaks in *H. cordata* were designated and identified (Table 1). Among the 11 chemical compounds identified, Compounds 2 and 3 are the first time observed in *H. cordata* and successfully isolated. Compound 2 has a considerably high content of about 4% in our preliminary quantitative analysis. In literature, this compound has already reported antioxidant and DPPH radical-scavenging activities. The results of isolation will be reported separately.

Analyzing Fingerprinting of *H. cordata* from Various Cultivation Locations The concept of “phytoequivalence” is a very important task in herbal fingerprints analysis.39 The pharmacological activities of a herbal medicine always vary with its cultivation origin. These differences can be associated with and retrieved in the chemical profiles of a fingerprint pattern. Therefore, it is essential to perform analysis of
similarity and difference of the overall fingerprint pattern. In this part, we have compared the samples from four different locations: Emei (EM-1 to EM-11), Yaan (YA-1 to YA-11), GuangDong (GD-1 to GD-5) and Hong Kong (HK-1 to HK-5) of P. R. China. Their total ion chromatograms are shown in Fig. 3. In general, the HPLC profiles among samples from the four locations are similar to each other. Comparing the chemical components among various cultivation areas (Table 2), compound 1 was found highest in content in YA and GD, whilst compound 7 was the highest content in HK and EM. The RP A of compound 1 in YA and GD were 4 times higher than that of HK, about 2 times higher than that of EM. On the other hand, the RP A of compound 8 in HK was generally doubled of the others. Thus, fresh H. cordata of different production areas can be distinguished by assessing the RP A of compound 1, which differentiates YA and GD from HK and EM. The highest content of compound 8 in HK among others helped to distinguish itself from EM. Likewise, YA and GD can differentiate from each other by considering the level of compound 2.

**Comparative Fingerprint among Various Medicinal Parts of Fresh H. cordata** It has been well known that different medicinal portions always possess variety in pharmacological activities.39) In some circumstances, Chinese medicine practitioner may use different medicinal portions of the herb for the same curative purpose. It is therefore important to learn about any difference in composition among various medicinal portions of H. cordata.

The total ion chromatograms for different medicinal portions of H. cordata are given in Fig. 4. It is obvious that the overall chemical profile among these fingerprints vary considerably for different medicinal portions. Considering the chemical profile for each medicinal part (Table 3), compound 3 was not available in leaf portion whilst compounds 10 and 11 were absent in root. These unique features help to identify themselves from the others. Although the chemical profiles

Table 1. The On-Line Detected Chromatographic and MS Data of the Eleven Identified Compounds in the HPLC Fingerprints

<table>
<thead>
<tr>
<th>Peak/Compound no.</th>
<th>tR (min)</th>
<th>[M−H]− (m/z)</th>
<th>[M+H]⁺ (m/z)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.73</td>
<td>353</td>
<td>355</td>
<td>Chlorogenic acid</td>
</tr>
<tr>
<td>2</td>
<td>20.62</td>
<td>627</td>
<td>627</td>
<td>Quercetin-3-O-β-D-galactopyranosyl-7-O-β-D-glucopyranoside</td>
</tr>
<tr>
<td>3</td>
<td>24.68</td>
<td>609</td>
<td>611</td>
<td>Quercetin-3-O-α-L-rhamnopyranosyl-7-O-β-D-glucopyranoside</td>
</tr>
<tr>
<td>4</td>
<td>34.93</td>
<td>609</td>
<td>611</td>
<td>Rutin</td>
</tr>
<tr>
<td>5</td>
<td>36.87</td>
<td>463</td>
<td>465</td>
<td>Hyperin</td>
</tr>
<tr>
<td>6</td>
<td>37.55</td>
<td>463</td>
<td>465</td>
<td>Isoquercitrin</td>
</tr>
<tr>
<td>7</td>
<td>45.05</td>
<td>447</td>
<td>449</td>
<td>Quercetin</td>
</tr>
<tr>
<td>8</td>
<td>56.95</td>
<td>429</td>
<td>433</td>
<td>Asetin</td>
</tr>
<tr>
<td>9</td>
<td>63.32</td>
<td>301</td>
<td>303</td>
<td>Quercetin</td>
</tr>
<tr>
<td>10</td>
<td>70.20</td>
<td>264</td>
<td>266</td>
<td>Piperolactam A</td>
</tr>
<tr>
<td>11</td>
<td>76.02</td>
<td>278</td>
<td>280</td>
<td>Aristolactam B</td>
</tr>
</tbody>
</table>
of entire herb and stem are similar to one another, the distinctively high contents of compounds 1 and 2 in stem region differentiated itself from the entire herb. The RPA for each medicinal portion is given in Table 3. The corresponding data for entire herb is shown in Table 2 (EM).


distinguish between Fresh and Dried H. cordata In general, dried H. cordata was preferentially used as a medicinal material and the practice was recorded in previous editions of Chinese pharmacopoeia. However, the fresh counterpart has been added in the latest 2005 edition. Our recent pharmacological studies showed that a methanolic extract of fresh herb gave better activities than its dried counterpart in term of anti-inflammatory activity, relieving cough and antibiosis (The result will be reported in another paper). In this regard, the corresponding fingerprint between these two types of processed herbs with different processing methods was compared. 11 fresh (EM-1 to EM-11) and 5 dried (EM-27 to EM-31) samples were compared in terms of their fingerprinting profiles. Comparing the chemical components

<table>
<thead>
<tr>
<th>Peak/</th>
<th>YA (n=11)</th>
<th>GD (n=5)</th>
<th>HK (n=5)</th>
<th>EM (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RRT</td>
<td>RPA</td>
<td>RRT</td>
<td>RPA</td>
</tr>
<tr>
<td>1</td>
<td>0.29±0.004</td>
<td>1.31±0.101</td>
<td>0.29±0.006</td>
<td>1.35±0.368</td>
</tr>
<tr>
<td>2</td>
<td>0.40±0.002</td>
<td>0.16±0.041</td>
<td>0.39±0.010</td>
<td>0.60±0.529</td>
</tr>
<tr>
<td>3</td>
<td>0.55±0.002</td>
<td>0.09±0.028</td>
<td>0.55±0.003</td>
<td>0.04±0.033</td>
</tr>
<tr>
<td>4</td>
<td>0.76±0.004</td>
<td>0.27±0.013</td>
<td>0.76±0.002</td>
<td>0.19±0.076</td>
</tr>
<tr>
<td>5</td>
<td>0.80±0.003</td>
<td>0.96±0.101</td>
<td>0.80±0.002</td>
<td>0.75±0.085</td>
</tr>
<tr>
<td>6</td>
<td>0.81±0.009</td>
<td>0.29±0.026</td>
<td>0.82±0.001</td>
<td>0.22±0.042</td>
</tr>
<tr>
<td>7</td>
<td>1.19±0.011</td>
<td>0.07±0.005</td>
<td>1.20±0.006</td>
<td>0.08±0.034</td>
</tr>
<tr>
<td>8</td>
<td>1.30±0.016</td>
<td>0.05±0.002</td>
<td>1.29±0.018</td>
<td>0.02±0.030</td>
</tr>
<tr>
<td>9</td>
<td>1.47±0.016</td>
<td>0.02±0.001</td>
<td>0.72±0.018</td>
<td>0.02±0.030</td>
</tr>
<tr>
<td>10</td>
<td>1.59±0.010</td>
<td>0.03±0.001</td>
<td>1.56±0.010</td>
<td>0.04±0.044</td>
</tr>
</tbody>
</table>

RRT and RPA are the ratio of retention time and peak area of each peak with respect to peak 7, respectively. The value is mean±S.D.
between the fresh and dried herbs (Tables 2, 3), most of the contents in the fresh herb are generally higher than that of the dried counterpart (Fig. 5). Probably the differences were attributed to the lost or decomposition during the drying process.\(^{40,41}\) This observation also helps to interpret their differences in pharmacological activities.

**Comparison between GC/MS and HPLC-DAD-MS Fingerprints of H. cordata**

The results and conclusion generated from the present developed direct HPLC-DAD-MS fingerprint analysis are generally comparable and consistent with our previously studies using direct GC-MS fingerprint method on *H. cordata*.\(^{23}\) However, the analysis in previously established GC-MS fingerprint only focused on those volatile components. These volatile compounds are always unstable and easily decomposed during the course of sample preparation and analysis. With the aims to streamline the analysis and more comprehensively representing the quality of *H. cordata*, a new HPLC-DAD-MS fingerprinting method has been developed. The method gives an overall picture about the various major types of chemical components present in *H. cordata* and more comprehensively reflecting its quality. The validated method has been proven simple, sensitive and selective with good accuracy and reproducibility. In other words, the HPLC-DAD-MS method can be readily utilized as a comprehensive quality evaluation tool for simultaneous determination of phenols, flavones and alkaloid components in *H. cordata*. Furthermore, the method can further be extended its application to authenticate and assess the quality of related medicinal products for *H. cordata*.

**Conclusion**

A HPLC-DAD-MS fingerprint method has been developed for the analysis of *H. cordata*. The simulative mean chromatogram of 11 batches of samples from Emei of Sichuan was obtained using a standardized procedure. The eleven common peaks were identified by the HPLC-DAD-MS with respect to the authentic chemical reference compounds. The entire HPLC profiles with 11 common peaks were successfully used to authenticate *H. cordata* and differentiate among different cultivation areas and different portions of herb. The results showing that the developed method is a straightforward, sensitive and selective tool with good accuracy and reproducibility. This HPLC-DAD-MS fingerprint method can be readily utilized as a suitable quality control method for quality evaluation of *H. cordata* and its related products.

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**References and Notes**


