Analysis of Fructus Arctii from Different Regions of China by HPLC Coupled with Chemometrics Methods

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Summary. To evaluate the quality of Fructus Arctii, an accurate and reliable method of high-performance liquid chromatography/diode array detection–electrospray ionization–mass spectrometry (HPLC/DAD–ESI–MS) was developed. Nine compounds, including chlorogenic acid, caffeic acid, trans-p-hydroxycinnamic acid, arctiin, arctignan A, ethyl caffeate, matairesinol, arctigenin, and lappaol B, were determined simultaneously in 19 batches of Fructus Arctii samples collected from different localities. Nineteen common peaks were identified or tentatively assigned by comparing their mass spectrometric data with reference compounds, self-established compound library, and published literatures. Also, the 19 common peaks were selected as characteristic peaks to assess the similarity of chromatographic fingerprinting of these samples. Moreover, hierarchical clustering analysis (HCA) and principal components analysis (PCA) were successfully applied to demonstrate the variability of samples. The results indicated the content of nine compounds that varied greatly among the samples, and 19 samples collected from different localities could be discriminated. Furthermore, chlorogenic acid, arctin, and arctigenin were found to be chemical markers for evaluating the quality of Fructus Arctii.

Key Words: Fructus Arctii, fingerprint analysis, chemometric analysis

Introduction

Fructus Arctii (Niubangzi in Chinese), the dry fruit of Arctium lappa L., is one of the most popular Traditional Chinese Medicine (TCM) [1], which has been used as a kind of vegetable and medicinal plant in China for centuries. The major constituents in Fructus Arctii are lignans and phenolic compounds [2, 3]. Up to now, a lot of lignans and phenolic compounds in Fructus Arctii have been separated and identified, including matairesinol; lappaol A, E, F, and H [4]; arctignan A, G, and H; neoarctin A [5]; 4,5-dicaffeoylquinic acid; 3,5-dicaffeoylquinic acid; 3,4-dicaffeoylquinic acid [6]; chlorogenic acid; caffeic acid [3], etc. They possess many kinds of bioactivi-
ties and a number of important pharmacological properties including anti-inflammatory [7–9], antioxidant [10], antiviral [11], demutagenic, cytotoxic, antiproliferative, platelet activating factor (PAF) antagonist [12], calcium antagonist, and anticarcinogenesis activities [13]. With the increased usage in food, it becomes more important to control and evaluate its quality.

In the literature, few studies on HPLC analysis of Fructus Arctii are reported [14–16]. An accurate and reliable high-performance liquid chromatography/diode array detection–electrospray ionization–mass spectrometry (HPLC/DAD–ESI–MS) of multiple compounds determination in combination with chromatographic fingerprint analysis was developed for the quality evaluation of Fructus Arctii. Nine compounds, including chlorogenic acid, caffeic acid, trans-p-hydroxycinnamic acid, arctiin, arctignan A, ethyl caffeate, matairesinol, arctigenin, and lappaol B, were developed simultaneously, and 19 common peaks were unequivocally identified or tentatively assigned by comparing their mass spectrometric data with reference compounds, self-established compound library, and published literatures. Meanwhile, 19 common peaks were selected as characteristic peaks to assess the similarity of 19 batches of Fructus Arctii samples in chromatographic fingerprinting analysis.

Hierarchical clustering analysis (HCA) and principal components analysis (PCA) are the multivariate analysis technique that are used to sort samples into groups [17]. The two methods are well-known and have been applied for fingerprint analysis, because they are nonparametric data interpretation methods and simple to use [18–19]. Moreover, HCA and PCA were successfully applied to demonstrate the variability of the chromatographic fingerprinting analysis in 19 batches of Fructus Arctii samples.

**Experimental**

**Materials and Chemicals**

Chlorogenic acid and arctiin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPB, Beijing, China). Caffeic acid, trans-p-hydroxycinnamic acid, arctignan A, ethyl caffeate, matairesinol, arctigenin, and lappaol B were isolated by our laboratory. Their structures were elucidated by comparison of spectral data (1H-NMR and 13C-NMR) and the purities were greater than 98%.

HPLC grade acetonitrile and phosphoric acid were purchased from Te-dia (Fairfield, USA). Deionized water for the HPLC analysis was purified from Hangzhou Wahaha Group Co., Ltd. Analysis grade methanol was purchased from Nanjing Chemical Company (Nanjing, China).
Nineteen batches of Fructus Arctii were collected from different origins in China (Table I). All of the samples were identified by Professor Jianwei Chen from Nanjing University of Chinese Medicine. The air-dried samples were pulverized into power, passed through a 65-mesh sieve, and stored at room temperature.

Table I. Origins of 19 batches of Fructus Arctii samples collected

| No. | Origin    | Batch No. | No. | Origin    | Batch No. |
|-----|-----------|-----------|-----|-----------|-----------|
| 1   | Gansu     | 121108    | 11  | Jilin     | 120701    |
| 2   | Hangzhou  | 121104    | 12  | Sichuan   | 120501    |
| 3   | Gansu     | 121101    | 13  | Helongjiang| 121105    |
| 4   | Anhui     | 120801    | 14  | Zhejiang  | 121101    |
| 5   | Liaoning  | 120102    | 15  | Dongbei   | 121101    |
| 6   | Shandong  | 121105    | 16  | Hubei     | 120901    |
| 7   | Guizhou   | 121109    | 17  | Neimenggu | 120928    |
| 8   | Shandong  | 121119    | 18  | Shanxi    | 121101    |
| 9   | Shanxi    | 121106    | 19  | Anhui     | 121101    |
| 10  | Henan     | 120101    |     |           |           |

HPLC–DAD–MS Analysis

The HPLC system consisted of a Shimadzu LC-20AD solvent delivery pump, a DGU-20A3 degasser, a CTO-20A column oven, an SPD-M20A photodiode array detector, and a SIL-20A autosampler (Shimadzu, Kyoto, Japan). The mass spectrometer was a Shimadzu LCMS-2020 single quadrupole equipped with an ESI source interface. At the end of data collection, the chromatograms were processed with the software LCMS Solution (Shimadzu, Kyoto, Japan).

For chromatographic analysis, a YMC-ODS C18 column (250 mm×4.6 mm, 5 μm) was used. HPLC separation was performed using a linear gradient and a flow rate of 1.0 mL min⁻¹. The column temperature was 35 °C. The mobile phase consisted of acetonitrile (A) and water containing 0.1% formic acid (B) using the elution gradient 5–25% A at 0–35 min, 25–35% A at 35–65 min, 35–50% A at 65–80 min, 50–70% A at 80–90 min, 70–5% A at 90–95 min, and 5% A at 95–100 min. Detection wavelength was set at 254 nm. The ESI-MS spectra were acquired in both negative and positive modes scanning from 100 to 800. The typical ion source parameters were as follows: ESI probe temperature, 350 °C; CDL temperature, 280 °C; heat block temperature, 320 °C; ESI probe voltage, 4.5 kV; detector voltage, 1.5 kV; and nebulizing gas flow, 1.5 L min⁻¹.
Standard Preparation

Nine reference compounds were accurately weighed and dissolved in methanol and then diluted to appropriate concentration ranges for the establishment of calibration curves. All stock and working standard solutions were stored at 4 °C.

Sample Preparation

Powers of the samples (1.0 g) were accurately weighted and placed in a sealed vessel by adding 20 mL of the methanol solvent. The weight of vessel was recorded, and the sealed vessel was placed into the ultrasonic cleaner for ultrasonic extraction for 30 min at room temperature (25 °C). After cooling to the room temperature, the original solvent weight was restored. The sample solution was filtered through a 0.45-μm membrane filter prior to analysis.

Statistical Analysis

The chromatographic profiles of all extracts were performed by professional software named Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (TCM) (Version 2004 A), which is recommended by the China Food and Drug Administration (CFDA) for evaluating similarities of chromatographic profiles of TCM (15). PCA and HCA were performed using SPSS software (SPSS 19.0 for Windows 7™, SPSS Inc., Chicago, IL, USA).

Results and Discussion

Identity Confirmation by HPLC–DAD–MS

HPLC–DAD–MS was employed to analyze the extract solution of Fructus Arctii samples, and 19 peaks were detected. Most of the peaks were tentatively assigned as lignans according to their maximal ultraviolet (UV) absorption wavelength and mass spectrometric data. By comparing the retention time, UV absorption and mass spectrometric data with reference compounds, nine peaks were designed as chlorogenic acid, caffeic acid, trans-p-hydroxycinnamic acid, arctiin, arctignan A, ethyl caffeate, matairesinol, arctigenin, and lappaol B. Also, other peaks frequently exhibited their quasi-molecular ions [M+H]^+, [M+Na]^+, [M–H]^−, and [M+HCOO]^−,
which were tentatively assigned by comparing their mass spectrometric data with established compound library and published literatures [20–21] (Table II).

Table II. Retention time, molecular weights (MW), and MS spectra data for constitutes from Fructus Arctii

| Peak No. | Retention time (min) | Molecular weight | Proposed structure | Molecular formula | In positive mode | In negative mode | Peak area* |
|----------|----------------------|------------------|--------------------|------------------|----------------|----------------|------------|
|          |                      |                  |                    |                  | [M+H]+ | [M+Na]+ | [M−H]− | [M+HCOO]− |          |
| 1        | 16.1                 | 354              | Chlorogenic acid   | C_{16}H_{18}O_{9} | 354.9  | 352.8   |          |          | 541,908   |
| 2        | 19.5                 | 180              | Caffeic acid       | C_{9}H_{8}O_{4}  | 181.0  | 179.1   |          |          | 156,676   |
| 3        | 24.7                 | 164              | trans-p-Hydroxycinnamic acid | C_{9}H_{8}O_{3} | 165.1  | 163.0   |          |          | 49,861    |
| 4        | 33.3                 | 516              | Dicaffeoylquinic acid | C_{25}H_{24}O_{12} | 516.9  | 514.8   |          |          | 317,382   |
| 5        | 38.4                 | 750              | Lappaol H          | C_{27}H_{34}O_{11} | 534    | 532.8   |          |          | 26,644    |
| 6        | 44.2                 | 534              | Arctiin            | C_{18}H_{18}O_{8} | 556.9  | 554.9   |          |          | 746,618   |
| 7        | 45.4                 | 554              | Arctignan A        | C_{9}H_{10}O_{10} | 577.0  | 575.0   |          |          | 41,535    |
| 8        | 46.5                 | 208              | Ethyl caffeate     | C_{11}H_{12}O_{4} | 209.5  | 207.9   |          |          | 107,327   |
| 9        | 51.5                 | 732              | Arctignan E        | C_{30}H_{42}O_{12} | 755.0  | 753.0   |          |          | 6503      |
| 10       | 54.0                 | 358              | Matairesinol       | C_{20}H_{32}O_{8} | 358.9  | 356.9   |          |          | 31,814    |
| 11       | 61.2                 | 536              | Lappaol A          | C_{30}H_{32}O_{8} | 559.0  | 557.0   |          |          | 33,641    |
| 12       | 65.9                 | 520              | Matairesinoside    | C_{25}H_{20}O_{14} | 520.9  | 518.9   |          |          | 41,051    |
| 13       | 67.6                 | 372              | Arctigenin         | C_{21}H_{20}O_{8} | 372.9  | 370.9   |          |          | 58,174    |
| 14       | 66.0                 | 550              | Lappaol B          | C_{31}H_{32}O_{8} | 573.0  | 571.0   |          |          | 31,536    |
| 15       | 86.0                 | 742              | Diarctigenin       | C_{25}H_{20}O_{12} | 742.9  | 740.8   |          |          | 6253      |
| 16       | 90.0                 | 314              | Methyl arctate-b   | C_{18}H_{18}O_{8} | 337.2  | 335.2   |          |          |           |

Peak S1, peak S2, and peak S3 (Fig. 1A) gave the base peaks at m/z 506.95 [M+H]+, 632.82 [M+H]+, and 642.65 [M+H]+ in positive mode, respectively. In the negative mode, peak S1, peak S2, and peak S3 showed the abundant ion at m/z 505.05 [M−H]−, 630.95 [M−H]−, and 641.50 [M−H]− in their MS spectra. Therefore, 506, 632, and 642 were considered as their molecular weight, respectively. However, we did not identify them through published data, which indicated that they are unknown compounds in Fructus Arctii. Also, we are trying to gain and identify the three important compounds by a traditional chemical isolation method, which is still in progress in our laboratory.
Fig. 1. Typical chromatograms for chemical analysis. (A) Total wavelength chromatogram of DAD spectral data from Fructus Arctii. (B) Mixture of the nine compounds (1: chlorogenic acid; 2: caffeic acid; 3: trans-p-hydroxycinnamic acid; 6: arctiin; 7: arctignan A; 8: ethyl caffeate; 10: matairesinol; 13: arctigenin; 14: lappaol B). (C) Total chromatograms of MS.
Table III. Regression equation, linear range, detection limits of the developed method, data of precision, repeatability, stability, and recovery of the nine compounds

| Compound               | Regression equation<sup>a</sup> | <sup>R</sup><sup>b</sup> | Linearity range (μg mL<sup>−1</sup>) | LOD (μg mL<sup>−1</sup>) | LOQ (μg mL<sup>−1</sup>) | Precision RSD (%)<sup>c</sup> (<i>n</i> = 6) | Repeatability RSD (%)<sup>c</sup> (<i>n</i> = 6) | Stability RSD (%)<sup>c</sup> (<i>n</i> = 6) | Recovery (%) mean ± RSD (%) (<i>n</i> = 6) |
|------------------------|---------------------------------|------------------------|------------------------------------|------------------------|------------------------|------------------------------------------|-------------------------------------------|------------------------------------------|------------------------------------------|
| Chlorogenic acid       | <i>Y</i> = 16,169<i>x</i> + 29,562 | 0.9998                 | 16.80–430.0                       | 2.909                  | 8.814                  | 0.3900                                    | 2.400                                      | 0.2900                                    | 103.9 ± 1.450                            |
| Caffeic acid           | <i>Y</i> = 48,750<i>x</i> + 2179.8 | 1.000                  | 1.014–12.68                       | 0.1780                 | 0.5380                 | 0.3300                                    | 3.000                                      | 0.9700                                    | 99.33 ± 2.350                            |
| trans-p-Hydroxycinnamic acid | <i>Y</i> = 110,000<i>x</i> + 70,214 | 0.9997                 | 0.0375–0.9375                     | 0.0110                 | 0.0350                 | 1.100                                     | 2.900                                      | 2.000                                     | 97.21 ± 2.510                            |
| Arctiin                | <i>Y</i> = 4714.8<i>x</i> + 39,0000 | 0.9997                 | 239.7–7191                        | 0.7898                 | 2.393                  | 0.2400                                    | 2.900                                      | 0.1700                                    | 101.1 ± 2.850                            |
| Arctignan A            | <i>Y</i> = 5052.7<i>x</i> − 1695.2 | 0.9999                 | 16.14–134.5                       | 3.193                  | 9.676                  | 0.4500                                    | 2.200                                      | 1.200                                     | 101.8 ± 2.710                            |
| Ethyl caffeate         | <i>Y</i> = 26,475<i>x</i> − 910.96 | 0.9999                 | 0.5470–11.40                      | 0.1570                 | 0.4780                 | 1.100                                     | 2.300                                      | 2.000                                     | 96.60 ± 1.130                            |
| Matairesinol           | <i>Y</i> = 10,227<i>x</i> + 1056.2 | 0.9999                 | 0.9380–23.45                      | 0.1200                 | 0.3620                 | 0.6700                                    | 2.200                                      | 0.3900                                    | 104.6 ± 2.090                            |
| Arctigenin             | <i>Y</i> = 9029.7<i>x</i> + 1982.9 | 1.0000                 | 6.480–162.0                       | 1.480                  | 4.490                  | 0.3400                                    | 2.800                                      | 0.4400                                    | 98.85 ± 2.640                            |
| Lappaol B              | <i>Y</i> = 6544.7<i>x</i> − 594.55 | 0.9998                 | 0.8640–18.00                      | 0.2700                 | 0.8200                 | 1.100                                     | 2.400                                      | 1.700                                     | 102.4 ± 2.610                            |

<sup>a</sup>Y, peak area; x, concentration of compound (μg mL<sup>−1</sup>).

<sup>b</sup><i>R</i> = correlation coefficient (<i>n</i> = 8).
Method Validation

Linearity and linear ranges of nine compounds were determined by using the developed method. Their correlation coefficient values ($R^2 > 0.9997$) indicated appropriate correlations between concentrations of the investigated compound and their peak areas within the test ranges. The developed method had good repeatability and stability with RSD <3%, and a good accuracy with the recoveries in the range of 96.60%–104.61% with RSD <3% (Table III).

Quantitative Analysis

The newly developed method was subsequently applied to quantitative analysis of nine compounds in 19 batches of Fructus Arctii samples. Each sample was analyzed two times to determine the mean content (mg g$^{-1}$), and the results were shown in Table IV. These results indicated that the con-

| Sample | Content of investigated components |
|--------|-----------------------------------|
|        | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| S1     | 2.76 | 0.06 | 0.009 | 69.14 | 3.96 | 0.08 | 0.87 | 1.74 | 0.13 |
| S2     | 2.14 | 0.07 | 0.01 | 73.63 | 2.92 | 0.08 | 0.61 | 1.41 | 0.10 |
| S3     | 2.56 | 0.04 | 0.006 | 54.37 | 3.73 | 0.07 | 0.75 | 1.48 | 0.20 |
| S4     | 2.24 | 0.04 | 0.009 | 64.80 | 3.40 | 0.07 | 0.75 | 1.62 | 0.13 |
| S5     | 3.29 | 0.05 | 0.007 | 70.23 | 4.87 | 0.10 | 1.20 | 2.89 | 0.22 |
| S6     | 2.27 | 0.05 | 0.009 | 68.67 | 3.39 | 0.08 | 0.79 | 2.45 | 0.18 |
| S7     | 1.14 | 0.03 | 0.003 | 26.48 | 1.96 | 0.03 | 0.45 | 2.54 | 0.07 |
| S8     | 2.14 | 0.04 | 0.008 | 62.28 | 4.59 | 0.08 | 1.02 | 7.99 | 0.18 |
| S9     | 2.70 | 0.04 | 0.006 | 56.70 | 4.65 | 0.08 | 1.02 | 1.30 | 0.15 |
| S10    | 1.32 | 0.03 | 0.005 | 47.71 | 4.24 | 0.07 | 0.85 | 4.52 | 0.15 |
| S11    | 1.30 | 0.03 | 0.005 | 44.58 | 3.20 | 0.05 | 0.66 | 3.06 | 0.13 |
| S12    | 1.63 | 0.03 | 0.006 | 46.38 | 2.81 | 0.06 | 0.58 | 1.90 | 0.11 |
| S13    | 0.13 | 0.002 | 0.009 | 3.62 | 1.21 | 0.02 | 0.61 | 1.40 | 0.62 |
| S14    | 0.43 | 0.01 | 0.002 | 11.85 | 1.17 | 0.01 | 0.28 | 1.73 | 0.07 |
| S15    | 2.16 | 0.04 | 0.003 | 42.66 | 3.02 | 0.05 | 0.73 | 2.15 | 0.16 |
| S16    | 0.31 | 0.005 | 0.009 | 11.18 | 1.91 | 0.02 | 0.74 | 8.84 | 0.68 |
| S17    | 3.64 | 0.04 | 0.008 | 67.19 | 5.58 | 0.10 | 1.36 | 1.52 | 0.23 |
| S18    | 2.79 | 0.03 | 0.005 | 52.04 | 3.95 | 0.09 | 1.00 | 2.04 | 0.21 |
| S19    | 1.87 | 0.04 | 0.007 | 64.59 | 5.69 | 0.09 | 1.04 | 4.49 | 0.19 |

*The date was present at average of duplicates: 1: chlorogenic acid; 2: caffeic acid; 3: trans-p-hydroxycinnamic acid; 4: arctin; 5: arctignan A; 6: ethyl caffeate; 7: matairesinol; 8: arctigenin; and 9: lappaol B.
tent of nine compounds varied greatly among the samples collected from different localities, and the content of arctiin was higher in the samples collected from Gansu province and Liaoning province than those collected from other localities in China. The total content of arctiin and arctigenin in the samples S13 and S16 was much lower than in the other samples. The results further revealed that arctiin and arctigenin were the main chemical constituents of Fructus Arctii, which were of great importance to establish a better determination method for its quality control.

**Similarity Analysis**

In order to evaluate the similarity and differences in these samples, Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004A) was performed based on their HPLC profiles. Chromatograms of these samples are shown in Fig. 2. Nineteen peaks that existed in all 19 batches of Fructus Arctii samples with reasonable heights and good resolution were assigned as “characteristic peaks”. The similarities of the chromatograms of 19 batches of Fructus Arctii samples were compared to the reference fingerprint “R”. The closer the cosine values approached 1, the more similar the two chromatograms were. If a similarity value over a certain value was regarded as the threshold value for qualification, it was easy to identify the qualified sample based on the chromatographic fingerprint. The similarity values of 19 samples were more than 0.97, except for S13 and S16. These meant that 19 batches of Fructus Arctii samples showed good similarity with chemical constituents.

![Fig. 2. HPLC fingerprint graphics of 19 batches of Fructus Arctii](image-url)
Hierarchical Cluster Analysis

Hierarchical cluster analysis (HCA) was performed based on 19 peaks in the HPLC profiles. The results of HCA are shown in Fig. 3. The samples were divided into two clusters obviously. Cluster II was performed by the samples S13 and S16. Cluster II consisted of the remaining samples. The total contents of arctiin and arctigenin of samples in Cluster II were much lower than those of samples in Cluster I. S13 and S16 collected from Heilongjiang and Hubei had lower content of arctiin, and the content of arctiin was one of the important distinguishing factors. These results indicated that HCA was helpful to distinguish the origin information of samples and evaluate the quality of Fructus Arctii.

![Dendrogram using Average Linkage (Between Groups)](image)

Fig. 3. Results of HCA of 19 batches of Fructus Arctii

Principle Component Analysis

To identify the differences among 19 batches of Fructus Arctii samples, principle component analysis (PCA) was performed on the 19 peaks in their HPLC profiles. To display the points on three principal components, PC1, PC2, and
PC3 were chosen to represent the information, and their scores were more than 90%. As shown in Fig. 4, PCA displayed the results that 19 batches of Fructus Arctii samples were classified into two groups, which were very similar to the results of HCA. Group 2 was formed by the samples S13 and S16, and group 1 was formed by the remaining samples. Moreover, the results of the PCA indicated that the nine compounds might have more influence on the discrimination of the samples from the different localities than other compounds. The results showed that arctiin, arctigenin, and chlorogenic acid could be chosen as the chemical markers for evaluating the quality of Fructus Arctii.

![Fig. 4. 3D projection plots of PCA of chlorogenic acid, arctiin, and arctigenin for the 19 batches of Fructus Arctii](image)

**Conclusion**

A reliable method for comprehensive chemical analysis of Fructus Arctii by HPLC–DAD–MS combined with chemometrics was developed for discriminating the origin information and evaluating the quality of Fructus Arctii. Nineteen peaks in the extract solution of Fructus Arctii were unequivocally identified or assigned, and nine compounds were analyzed quantitatively in 19 batches of Fructus Arctii samples. Chemometrics were successfully applied to comprehensive chemical analysis of Fructus Arctii samples. The results indicated the content of nine compounds that varied greatly among the samples, which could be discriminated according to the results of chemometrics.
Moreover, arctiin and arctigenin were found to be chemical markers for evaluating the quality of Fructus Arctii. The results of chemometrics analysis of various Fructus Arctii make it necessary to compare the pharmacological activities of different batches in some pharmacological models and adapt the application and development of new Schisandra-containing formulas accordingly.

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