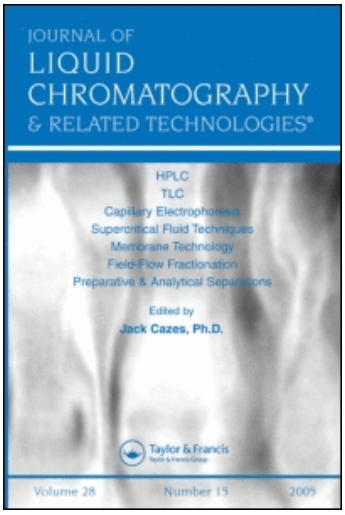


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# A Novel HPLC Method to Analyze Imperatorin and Isoimperatorin of *Angelica dahurica* Oils Obtained by Supercritical Fluid Extraction

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**Abstract:** In this paper, a novel HPLC method was developed for analyzing imperatorin and isoimperatorin in the SFE extracts of *Angelica dahurica.* The separations were effected by using a mobile phase of methanol and water. The gradient elution of the mobile phase was 80% in 0–5 min, 80–68% in 5–7 min, and 68% in 7–12 min. The detector wavelength was set at 249 nm. Analytical characteristics of the separation, such as limit of detection, limit of quantification, linear range, and reproducibility were evaluated. The developed method was applied to optimizing supercritical fluid extraction of coumarins from *Angelica dahurica.* The maximum extraction yield was obtained at 30 MPa and 50°C with a flow rate of 25 L/h and extraction time of 2 h.

**Keywords:** *Angelica dahurica*, Coumarins, HPLC analysis, Supercritical fluid extraction

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## INTRODUCTION

*Angelica dahurica* (Baizhi in Chinese) has been widely used in traditional Chinese medicine as a common acesodyne. It has strong effects on headaches and toothaches.[1] In addition, it can be used to treat cough, asthma, coryza, hypertension, vitiligo, psoriasis, acne, herpes zoster, freckle, etc.[2,3] The major effective components of this herb are coumarins, including imperatorin and isoimperatorin. Pharmacological studies and clinical practice demonstrated that they have remarkable anticancer, antibacterial, and codeine effects.[4–6] The commonly used methods to extract from *Angelica dahurica* are steam distillation and ethanol percolation, but both of them have great disadvantages. Whether steam distillation or ethanol extraction, it only focuses on the yield of coumarins, but the yield of essential oils is usually very poor, even ignored.

Moreover, after costing a lot of solvents and time, the yields of coumarins by these two methods are always unsatisfactory. Besides, other active ingredients would be spoiled in the extraction by these two methods, especially by steam distillation.[7]



Supercritical fluid extraction (SFE) is a novel and effective technique to extract the lipid-soluble and micro molecule compounds from traditional Chinese herbs. Since SFE is accomplished at low operating temperature, it can avoid the degradation of thermally labile compounds, and conserve more active ingredients.[8] As for *Angelica dahurica*, the significant advantage of SFE is that it can simultaneously extract essential oils as well as coumarins from this herb.

Different from Western medicine, the therapeutic effects of traditional Chinese medicines are usually based on multifarious essential components or the combination of them instead of only one component. Since the therapeutic effect of *Angelica dahurica* was related to them, imperatorin and isoimperatorin were regarded as the essential components of this herb.[9–11] Methods of simultaneously detecting the two coumarins to evaluate the SFE of *Angelica dahurica* have already been established.[12–14] However, their retention times in these methods reported was too long, even lasting up to 40 min. Besides the high mobile phase cost, it is unfit for a rapid analysis to evaluate SFE processes. Thus, it is necessary to find a convenient and simultaneous analysis method for the SFE product evaluation. In this article, a new HPLC method of simultaneously detecting the two coumarins (isoimperatorin and imperatorin) was presented to evaluate the SFE of *Angelica dahurica*, shortening the retention time down to 8.5 min. This would be used as the rapid analysis method for the detection of coumarins from *Angelica dahurica*.

## EXPERIMENTAL

### Materials and Reagents

Dried root of *Angelica dahurica* was purchased from Changxing Medicine Co. (Jiangsu, Zhejiang). HPLC-grade methanol was from Merck (Darmstadt, Germany). Ultrapure water was prepared by using a Milli-Q academic water purification system (Milford, MA, USA). Reference standards of imperatorin (CASRN482-44-0), isoimperatorin (CASRN482-45-1) (shown as Fig. 1) were purchased from Zhejiang Institute for Food & Drug Control. $CO_2$ with the purity of 99.5% was kindly supplied by Hangzhou Electrochemical-Gas Factory (Hangzhou, China). The other chemicals, such as ethanol were used as received.



### UV Scanning

A Jasco V-550 series (Jasco international Co., Ltd., Japan) was used; the scanning wavelength was from 200 nm to 400 nm, and the scan rate was 100 nm/s. The standard preparations of imperatorin and isoimperatorin were dissolved in methanol and adjusted to the suitable concentration before UV scanning.

### HPLC Analysis

The HPLC system consisted of an Agilent 1100 series (Agilent Technologies, Wilmington, DE) equipped with a quaternary pump, online degasser, column heater, autosampler, and a UV detector. Data collection and analysis were treated by ChemStation software (Agilent Technologies, Wilmington, DE). The chromatographic column was a Lichrospher $C_{18}$

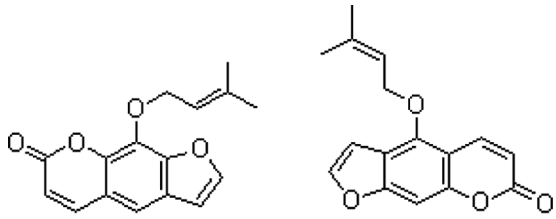


***Figure 1.*** Molecular structures of the two coumarins.

column (250 mm × 4.6 mm i.d., 5.0 μm particle size) from Hanbang Science & Technology (Jiangsu, China) coupled with an Agilent $C_{18}$ pre-column (4 mm × 5 mm). The column temperature was maintained at 25°C and the injection volume of sample was 10 μL.

## Preparation of Standard Solution

Primary standard stock solutions for the investigated compounds, i.e., imperatorin and isoimperatorin, were prepared in methanol with concentrations of 197 μg/mL, respectively. This solution was stored away from light at 4°C until used for analysis.

## Preparation of Sample Solution



The product of SFE extraction (about 0.1 g) was accurately weighted, put into a clean dry 25 mL volumetric flask and dissolved with methanol; 1 mL solution was transferred to a clean dry 10 mL volumetric flask and dissolved with methanol. The obtained solution was then centrifuged at 10,000 rpm for 5 min. After filtering through a 0.45 μm membrane filter, the supernatant was injected into the HPLC system for analysis.

## Validation of the Method

The validation of the method included linearity, limits of detection and quantification, precision, repeatability, and stability. The quantification of the chromatogram was performed using the peak area of the two investigated compounds. Six standard solutions were prepared and each of the standard solutions was analyzed by HPLC in triplicate. The peak area, together with concentration, ranging from 3.08 to 197 μg/mL, was plotted.

The standard solution containing the two reference compounds was diluted to provide appropriate concentrations to detect the limits of detection (LOD) and quantitation (LOQ). LOD and LOQ were detected when the signal-to-noise (S/N) was 3 and 10, respectively.

A standard mixture solution of the coumarins was detected under selected chromatography conditions six times in a day for intra-assay precision and once a day on three consecutive days for inter-assay precision. Six different working solutions prepared from the same product of SFE were analyzed to detect the repeatability. In evaluating the stability, the same sample solution was analyzed 3, 6, 9, 15, 24 h, respectively after being prepared, at room temperature. Each solution analysis was repeated three times under the selected chromatographic condition.

### Supercritical Fluid Extraction

The experiment was carried out on a HA220-50-06 extraction system (Hua An SFE Company, Jiangsu, China) equipped with a 1,000 mL volume extractor, two 1,000 mL separators, and a 100 DX syringe pump to step up the pressure as demanded. A pressure regulator displayed the extraction pressure of the system and the temperature was automatically adjusted by a thermostatic water bath. The plant material was loaded into the extractor, and the products were collected from the bottom of separators after extraction by supercritical $CO_2$. Then, the coumarins of the extracts were detected by the HPLC method mentioned above.[15]

### Orthogonal Design Experiments of SFE

Orthogonal design experiments of SFE were achieved. The effective factors of the experiments are listed in Table 1. In each experiment, 300 g dried roots of *Angelica dahurica* were comminuted to 40 mesh, and loaded into the extractor. After a certain time of extraction, the products were analyzed by HPLC to determine the yields of imperatorin and isoimperatorin.



## RESULTS AND DISCUSSION

### HPLC Separation Optimization

The selection of the HPLC conditions was evaluated by the ability of obtaining chromatograms with good resolution of adjacent peaks in the shortest time. A wavelength of 249 nm or 300 nm is ordinarily used for the detection of coumarins in *Angelica dahurica* preparation

***Table 1.*** Factors of orthogonal design experiments of SFE

| | Factors | | | |
|---|---|---|---|---|
| | A | B | C | D |
| Levers | Temperature (°C) | Pressure (MPa) | Extraction time (h) | Flow rate (L/h) |
| 1 | 40 | 20 | 1 | 15 |
| 2 | 50 | 25 | 2 | 20 |
| 3 | 60 | 30 | 3 | 25 |

samples.[1,12,14] However, the results of UV scanning indicate that both imperatorin and isoimperatorin show a stronger absorption in 249 nm (see Fig. 2). Although, 249 nm is not the maximum absorption wavelength, the absorption at 249 nm is already satisfactory. Besides, the detector is more sensitive in 249 nm. Therefore, we selected 249 nm as the appropriate wavelength for detection of the coumarins.

Then, the mobile phase composition was optimized using an isocratic elution system with flow rate of 0.9 mL/min. In most published articles, methanol/water (55:45) system and methanol/water (70:30) system were used for coumarin analysis.[12,13] However, using the methanol/water (55:45) system, the whole analytical process required about 60 min. So we tried to reduce the retention time by using the methanol/water (70:30) system. Though the methanol/water (70:30)



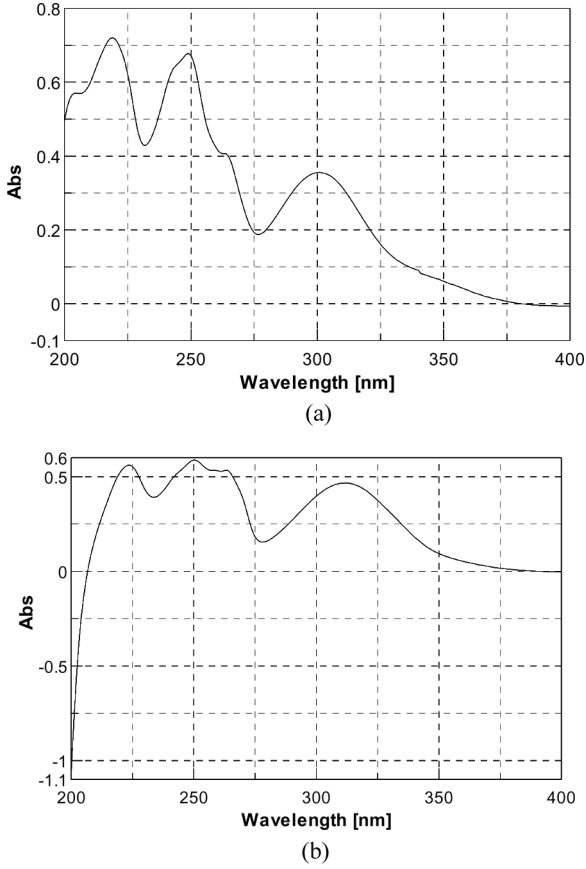


***Figure 2.*** UV scans of the two coumarins: (a) imperatorin; (b) isoimperatorin.

system reduced the retention time of coumarins, it still took about 20 min. Besides, the isolation of the peaks was not good enough. Then, we tried to use the gradient elution method and used the mobile phase composition as methanol/water (80:20), the gradient elution of mobile phase was 80% (methanol) in 0–5 min, 80–68% (methanol) in 5–7 min, 68% (methanol) in 7–12 min. Under such chromatographic conditions, we can obtain good resolution of adjacent peaks within the shortest time (Fig. 3).

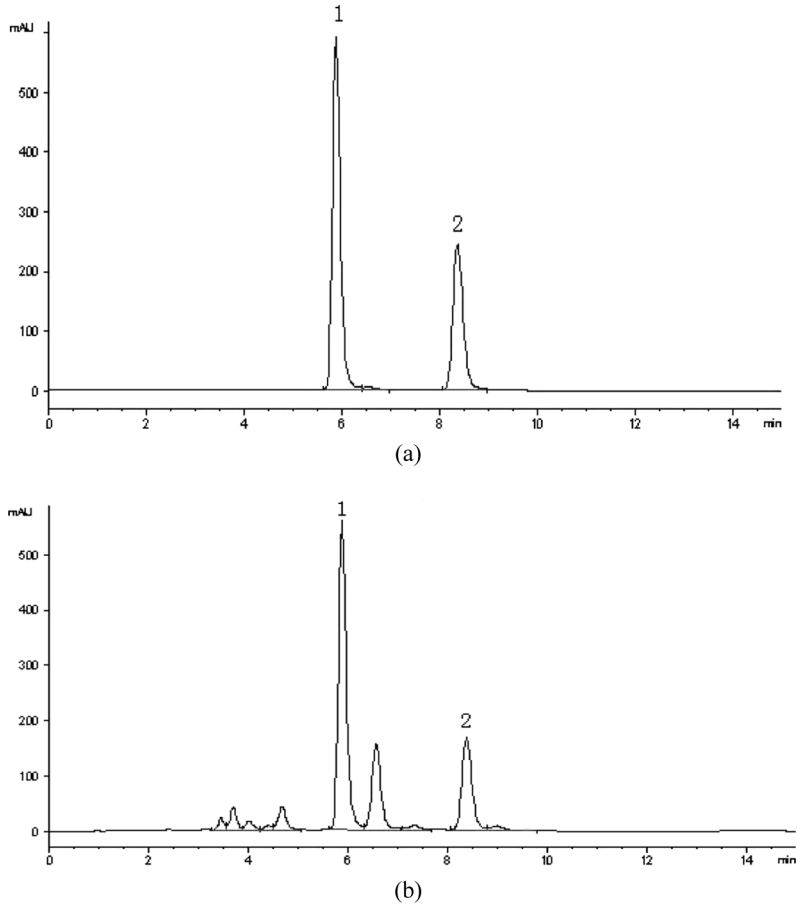


***Figure 3.*** HPLC chromatograms of standard mixture (a) and SFE products (b) of *Angelica dahurica*; 1) imperatorin; 2) isoimperatorin. The mobile phase of gradient elution was 80% (methanol) in 0–5 min, 80–68% (methanol) in 5–7 min, 68% (methanol) in 7–12 min.

***Table 2.*** Linear regression data, LODs and LOQs of the investigated coumarins

| Compounds | Linear range (μg/mL) | Regression equation[a] | $r^2$ | LOD (ng) | LOQ (ng) |
|---|---|---|---|---|---|
| Isoimperatorin | 3.08–197 | $y = 65.664x - 191.56$ | 0.9986 | 4.25 | 14.14 |
| Imperatorin | 3.08–197 | $y = 34.813x - 56.979$ | 0.9983 | 4.52 | 15.04 |

*Note*. [a]$y$: peak area; $x$: concentration (μg/mL).

## Method Validation

### Linearity, Limits of Detection, and Quantitation

The results of linear regression data are presented in Table 2. The regression equation was calculated in the form:

$$y = ax + b$$

where $y$ was the value of the peak area and $x$ was the value of concentration of the standard compounds. The $r^2$ in Table 2 is referred to the correlation coefficient of the equation. The two standard compounds (isoimperatorin and imperatorin) both showed good linearity ($r^2 >$ 0.9983) in the investigated concentration range.

LODs were 4.25 and 4.52 ng for isoimperatorin and imperatorin, respectively, while LOQs were 14.14 to 15.04 ng. The data of LOD and LOQ for the two compounds are also in Table 2.

### Precision

The results related to intra-assay and inter-assay variability obtained from the assay of the standard mixture are shown in Table 3. The intra-assay precision measured by RSDs were 0.21% (isoimperatorin) and 0.38% (imperatorin). The inter-assay precision was detected by analyzing a standard mixture at the same concentration three times a

***Table 3.*** Precision of the investigated compounds extracted from *Angelica dahurica*

| | Intra-assay (n = 6) | | Inter-assay (n = 3) | |
|---|---|---|---|---|
| Compounds | Mean (μg/mL) | RSD (%) | Mean (μg/mL) | RSD (%) |
| Isoimperatorin | 29.34 | 0.21 | 29.41 | 0.38 |
| Imperatorin | 26.68 | 0.38 | 26.93 | 1.35 |

day, and consecutively analyzed for 3 days. The RSD value was 0.38% for isoimperatorin and 1.35% for imperatorin, respectively.

Repeatability and Stability

To evaluate the repeatability, six different working solutions prepared from the same product of SFE were analyzed. The RSD values of the two investigated compounds were lower than 0.55%, suggesting that it has good repeatability (Table 4).

The data in Table 4 also show that no significant degradation of the two investigated compounds was observed at room temperature for 24 h. The RSD values of the compounds were 0.78% and 0.71% for isoimperatorin and imperatorin, respectively.



## Extraction Method Development

From analysis of variance, we recognized that the four effective factors were of different importance in the extraction process. In order to increase the yields, factors such as pressure, temperature, and extraction time should be optimized. However, flow rate of carbon dioxide ($CO_2$), which did not affect the extraction much, could be set at 25 L/h for the design experiments. Pressure, temperature, and extraction time were each divided for three levels, respectively. The yields of the three levels of each factor would show its link between the changing factor. The influence of pressure on the efficiency of extraction was first investigated. Samples were extracted under the divided pressures of 20, 25 and 30 MPa, and the products were detected. The results, as Fig. 4 shows, suggested that the yield of coumarins increased when the pressure was increased. The largest amount of coumarins was obtained at 30 MPa. Though, the yield still increased a little, if the pressure increased more, it would be worthless to do so, because high pressure is harmful to the equipment and would affect the quality of the products. Then, different temperatures (40, 50, and 60°C) were evaluated to optimize the extraction process. The experiments show that the yields of coumarins increased when the

***Table 4.*** Repeatability and stability of the investigated compounds

| | Repeatability (n = 6) | | Stability (n = 5) | |
|---|---|---|---|---|
| Compounds | Mean (μg/mL) | RSD (%) | Mean (μg/mL) | RSD (%) |
| Isoimperatorin | 97.79 | 0.55 | 97.86 | 0.78 |
| Imperatorin | 38.58 | 0.42 | 38.67 | 0.71 |

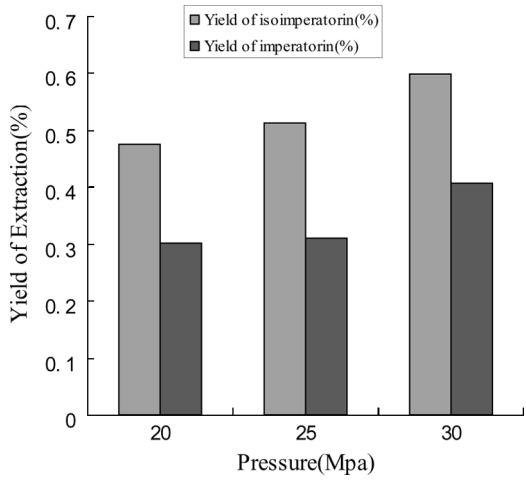


***Figure 4.*** The influence of pressure on the yields.



extraction temperature increased from 40 to 50°C. But, when the temperature was increased from 50 to 60°C, the yields of the coumarins decreased. As Fig. 5 shows, the highest extraction yield was obtained when the temperature was at 50°C. Finally, extraction time was investigated; as Fig. 6 shows, most of the coumarins were extracted in 2 h, and the yield did not increase significantly thereafter. So the optimized conditions of SFE are as follows: extraction pressure (30 MPa), temperature (50°C), and extraction time (2 h).

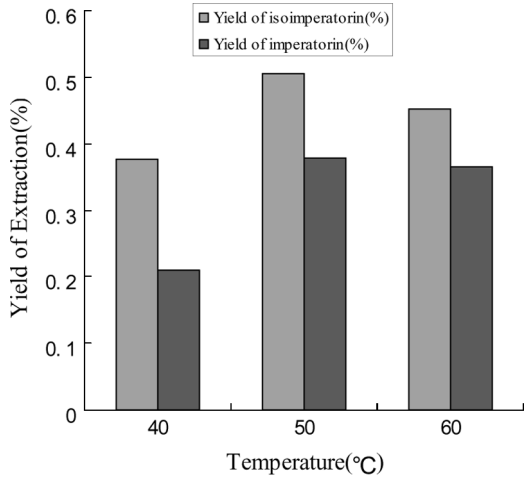


***Figure 5.*** The influence of temperature on the yields.

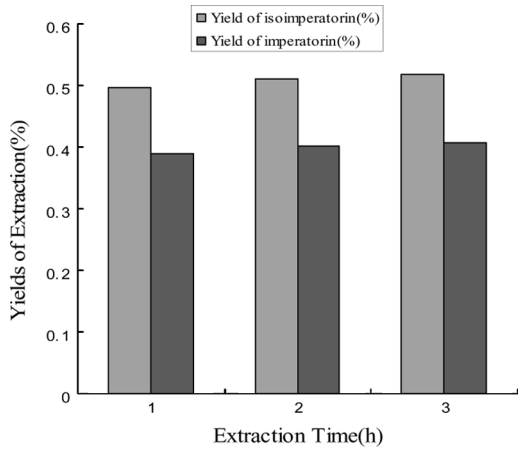


***Figure 6.*** The influence of extraction time on the yields.



## CONCLUSION

A new reverse-phase HPLC method was established and proved to be accurate, precise, and time-saving compared with the former analytical methods reported. This method could simultaneously detect the two coumarins from the SFE extraction product of *Angelica dahurica* with good sensitivity, precision, and repeatability. So this method can be applied to the rapid analysis of the SFE products of *Angelica dahurica*. Thus, it can improve the efficiency of the detection, and also promote the application of the SFE products of *Angelica dahurica* in the pharmaceutical industry.

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