Optimization of the Separation and Determination of Nitidine and Chelerythrine in *Zanthoxylum nitidum* by High-Performance Liquid Chromatography with Fluorescence Detection

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A new ion-pair high-performance liquid chromatography method with fluorescence detection was developed for the determination of nitidine and chelerythrine in *Zanthoxylum nitidum*. To optimize the separation of the two compounds in reversed-phase liquid chromatography, a response surface method (Box–Behnken designs) was used. Three important factors: concentration of ion pair agent, mobile phase composition and buffer pH, were studied for their contribution to the analytes’ response, leading to a total of 17 experiments performed on a Kromasil C18 column. The experimental responses were fitted into a second-order polynomial to predict the best conditions. The optimal mobile phase conditions were predicted to be acetonitrile–sodium dodecyl sulphate (17.8 mM, 20 mM citric acid, pH 2.98, 57:43, v/v). The proposed method was validated according to International Conference on Harmonization guidelines and it is suggested to be appropriate for the routine quality control analysis of nitidine and chelerythrine in *Zanthoxylum nitidum*.

Introduction

*Zanthoxylum nitidum* DC (Rutaceae), known locally as Liangmianzhen, is found in the southeastern part of China, India and Australia (1, 2). It has been used as a remedy against tumors (3–6), bacteria (7, 8) and pain (9), etc (10). The plant contains a series of alkaloids (9, 11–13), coumarins (14) and lignins, among which alkaloids are regarded as primary bioactive components. Consequently, the content of alkaloids is a chief index to evaluate the quality of *Zanthoxylum nitidum*. However, some reference standards of alkaloids are not commercially available; thus, nitidine and chelerythrine were selected as research objectives.

A literature survey indicates that many analytical methods have been developed for the separation and determination of nitidine and chelerythrine, alone or separate, including high-performance liquid chromatography–ultraviolet (HPLC–UV) (15, 16), capillary electrophoresis (CE) (17, 18), HPLC–electrospray ionization tandem mass spectrometry (ESI-MS) (1, 19–21). However, LC–MS is expensive and its availability is limited. In addition, the reported HPLC–UV methods lack analytical selectivity and sensitivity. Therefore, the development of simpler and more reliable methods for the determination of alkaloids is necessary. The two compounds are known to possess natural fluorescence (22); however, no HPLC–fluorescence detection (FLD) method has been reported for the analysis of nitidine.

Because a variety of chromatographic factors have to be considered in the development process for HPLC, such as concentration of organic modifier, pH and temperature, it is a complicated and time-consuming process to optimize them. Hence, chemometric tools, like response surface methodology, can be used.

Response surface methodology (RSM) is a collection of mathematical and statistical techniques that quantifies the functional relationship between many measured response variables and several explanatory factors to acquire the best response by using a sequence of tests (23, 24). The major advantage of RSM is that it conducts a smaller number of experiments than full factorial design; it has been widely applied to optimize chromatographic methods (25–27). Box–Behnken design (BBD) is a popular form of RSM that is more effective than other response surface designs, such as central composite designs (28). Thus, it was employed in the study.

In summary, the objective of this study was to develop an HPLC–FLD method for the determination of alkaloids in *Zanthoxylum nitidum* with the help of the BBD methodology.

Experimental

**Standards, samples and chemicals**

The reference standards of nitidine and chelerythrine (≥98% purity) were purchased from Mansite (Chendu, China). The chemical structures of the two alkaloids are shown in Figure 1.

Seven batches of *Zanthoxylum nitidum* were purchased in China from Hunan Province Solita Chinese Medicine Slices Co. (Lots 20070801 and 110607), Anhui Jingwan Chinese Herbal Pieces Co. (Lot 100901), Guangdong Province Chinese Herbal Pieces Co. (Lot 20110701), Anhui Xiehecheng Chinese Herbal Pieces Co. (Lot 20110506), Bozhou Chinese Herbal Co. (Lot 20100417) and Anguo Lenbei Chinese Herbal Co. (Lot 20101011).

HPLC-grade methanol and acetonitrile were purchased from Hanbon (Jiangsu, China) and Lingfeng (Shanghai, China). Sodium dodecyl sulfate (≥98.5% purity) was purchased from Sigma-Aldrich (St. Louis, MO). Other reagents were of analytical grade and distilled water was used throughout the experiment.
Preparation of samples
After collection, the plant samples were pulverized and dried; approximately 1 g of each sample was exactly weighed and sonicated with 20 mL of 70% methanol for 30 min and filtered (2). The extraction was repeated once. The extracts were combined and the solution was completed to 50 mL with 70% methanol to obtain a concentration of 20 mg/mL. The solution was diluted 10 times with the mobile phase and then centrifuged at 17,000 × g for 10 min. A volume of 10 µL of the supernatant was injected into the HPLC column. The compounds were identified by retention time matching and determined by means of the external standard method.

Software
The experimental design and response surface optimization were conducted by Design-Expert, version 7.1.6 (Stat-Ease Inc., Minneapolis, MO). Microsoft Excel 2003 (Microsoft, Redmond, WA) was employed for desirability function calculations.

Method validation
The optimized chromatographic conditions were validated according to International Conference on Harmonization (ICH) guidelines, such as selectivity, linearity, accuracy, precision, limit of detection, limit of quantification, stability and robustness.

Results and Discussion
Fluorescence spectra
The fluorescence spectra of the analytes were registered in the mobile phase to determine the optimal excitation and emission wavelengths. Because the different positions of the substituted groups, the excitation and emission spectra between the two alkaloids were different. Figure 2 shows the excitation and emission spectra of nitidine and chelerythrine. The extract of the plant was analyzed at excitation wavelengths of 265 and 325 nm. The results revealed that the former produced a more complex chromatographic profile than the latter. Hence, the excitation wavelength at 325 nm and emission wavelength at 540 nm were selected as the compromise wavelengths.

Optimization
Because of the weak retention of nitidine and chelerythrine on reversed-phase columns in acidic pH, and nitidine is not stable in alkaline range (8.0), ion-pair chromatography was the best choice. The combination of protonated sample ions with a sulfonate or sulfate group in acidic pH would increase their retention on chromatography. Several ion pair agents were investigated, such as sodium heptane sulfonate, sodium decane sulfonate and sodium dodecyl sulphate (SDS), and SDS was chosen for its affordable price yet provided satisfactory separation.

Previous experiments have shown that the pH of the mobile phase has an effect on separation. Moreover, the percentage of organic mobile phase (%)B was known as one of the greatest factors impacting the retention of analytes. Therefore, both buffer pH and percentage of organic phase were considered.
Preliminary experiments were performed to study the effect of concentration of SDS, buffer pH and percentage of organic phase on the responses of the analytes. The results indicated that they all had remarkable effects on resolution, peak shape and analysis time of the compounds (data not shown). Therefore, SDS concentration, pH and percentage of mobile phase B were the key factors selected for the optimization process. The fluorescence excitation and emission wavelengths for optimization were set at 330 and 550 nm, based on prior knowledge.

According to BBDs, the three independent variables were investigated at three different levels (−1, 0, 1). A total of 17 tests containing five replicates at the center point were conducted in random order (Table I). In each experiment, the resolution between nitidine and its adjacent peak was used as the response.

According to the results of significance evaluation of different equations (linear and quadratic) fitted to the response, a quadratic model was selected and the evolution phenomenon is described as follows [Eq. (1)]:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i<j}^{3} b_{ij} X_i X_j$$

(1)

where $Y$ represents the predicted response; $b_0$, $b_i$, $b_{ii}$ and $b_{ij}$ are regression coefficients; $X_i$ and $X_j$ represent independent variables.

In this study, the predicted second-order model was as follows [Eq. (2)]:

$$R = 1.51 + 0.31X_1 - 0.12X_2 - 0.48X_3 + 0.045X_1X_2 - 0.033X_1^2 - 0.072X_2^2 - 0.95X_3^2 - 0.022X_1^3 + 0.093X_3^2$$

(2)

where $R$ represents the resolution between nitidine and its adjacent peak. The adjusted $R^2$ for the model was 0.99945, which suggested that the experimental results adequately fitted the quadratic equation.

The analysis of variance (ANOVA) was conducted for the quadratic model. The $p$-value for the model is smaller than 0.0001, which reveals that the model is significant. A $p$-value less than 0.05 suggests that the terms of the model are significant. The lack of fit $p$-value of 0.3028 indicates that the quadratic model is adequate. In this case, seven coefficients are important, the most important of which are SDS concentration, pH and percentage of acetonitrile. Also, pH, percentage of acetonitrile, SDS concentration and pH interaction are important.

To better comprehend the predicted models, three-dimensional graphs were mapped by plotting the response versus two of the factors and the third was fixed at its central level (figures not shown). As shown in the three-dimensional plots, the optimal conditions were: SDS at 17.8 mM, pH at 2.98 and percentage of acetonitrile (ACN) at 57. Under the predicted conditions, a satisfactory separation of nitidine and chelerythrine peaks was obtained in 14 min.

### Method validation

**Linearity, detection limit and quantification limit**

Under the proposed conditions, calibration curves were constructed by analyzing the reference compounds at six levels of concentration (50, 100, 200, 500, 750 and 1,000 ng/mL). Each solution was determined in triplicate. A satisfactory linear relationship ($R^2 = 0.9999$) was obtained between the concentration of the analyte and corresponding peak area. The limit of detection (LOD) and limit of quantification (LOQ) of the two compounds were defined as the concentrations leading to signal-to-noise ($S/N$) ratios of 3 and 10, respectively. Table II lists the detailed information regarding linear ranges, slopes, intercepts, correlation coefficients ($r$), LOD and LOQ for the HPLC–FLD method.

### Precision and accuracy

Intra-day precision was investigated by determining three different concentrations of standard solution on the same day; inter-day precision was evaluated by testing the assays on each of the three consecutive days. The results indicated that both

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Table I

**BBD Arrangement and Experimental Response**

Figure 2. Excitation (left) and emission (right) fluorescence spectra of nitidine (dotted line) and chelerythrine (solid line) in the mobile phase.
intra-day and inter-day precision of the two compounds was smaller than 1.9%.

The precision of the extraction process was studied by repeating the extraction procedure of the sample of *Zanthoxylum nitidum* in Guangdong. An aliquot of each extract was determined and the data show that both intra-day and inter-day precision of the two compounds was smaller than 2%.

The accuracy was studied by spiking nitidine and chelerythrine at three different amounts (approximately 0.5, 1.0 and 1.5 times of the content of the matrix), respectively, into *Zanthoxylum nitidum* samples (Lot 20110701). The fortified samples were subsequently extracted and determined by using the presented method. The mean recoveries of the two compounds ranged between 93.9 and 103.8%, with relative standard deviation (RSD) values less than 8.0% (*n* = 3).

**Stability**

The stability was studied with stock solutions of reference standard and extract of *Zanthoxylum nitidum* in Guangdong. An aliquot of each extract was determined and the data show that both intra-day and inter-day precision of the two compounds was smaller than 2%.

**Robustness**

The selection of factors for robustness was based on preliminary experiments and prior knowledge. The robustness of the proposed method was evaluated with respect to minor changes in the concentration of ACN (57 ± 0.5%), pH (3.0 ± 0.2) and column temperature (25 ± 2°C). The results indicated no significant changes (less than 5%) in retention times and resolutions of the two compounds.

**Application to the quantitation of nitidine and chelerythrine in *Zanthoxylum nitidum***

The two compounds in *Zanthoxylum nitidum* were simultaneously analyzed by the developed HPLC–FLD method. Typical chromatograms for nitidine and chelerythrine from *Zanthoxylum nitidum* are given in Figure 3. The contents of the two alkaloids in seven batches of the samples were subsequently extracted and determined by using the presented method. The results revealed that the two alkaloids were stable under these conditions.

**Conclusion**

A rapid and sensitive ion-pair HPLC method with fluorescence detection was developed for the simultaneous analysis of nitidine and chelerythrine in herbal samples. The RSM (BBD) was successfully employed to optimize the significant parameters of HPLC. When applied to the determination of the two analytes in *Zanthoxylum nitidum*, the method was found to be applicable and revealed vigorous potential for routine quality assurance of alkaloids from *Zanthoxylum nitidum*.

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**References**


