Postprint: Optimization of Ferulic Acid and Ligustilide Extraction from Angelica sinensis by Response Surface Methodology

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Abstract

To optimize the extraction conditions for simultaneously extracting the main active components ferulic acid and ligustilide from the traditional Chinese medicinal material Angelica sinensis (Danggui), and to provide a reference for its effective development and utilization, this study employed ultrasound-assisted response surface methodology to optimize the extraction process of ferulic acid and ligustilide from Danggui, and used quantitative hydrogen nuclear magnetic resonance (qHNMR) spectroscopy to simultaneously determine the contents of ferulic acid and ligustilide in Danggui. In the optimization process, the comprehensive score of ferulic acid and ligustilide contents was used as the evaluation index, with three main factors selected: ethanol concentration, extraction time, and liquid-to-material ratio. Single-factor screening combined with Box-Behnken central composite design experiments were employed to optimize the extraction process parameters. Experimental results demonstrated that the optimal extraction conditions were: ethanol concentration 80.87%, liquid-to-material ratio 13.04 $mL \cdot g^{1}$, and extraction time 30.14 min, with validation test results being consistent with the predicted values. For the quantitative hydrogen nuclear magnetic resonance method, deuterated dimethyl sulfoxide was used as the test solvent and pyrazine as the internal standard, with the quantitative resonance peaks of pyrazine, ferulic acid, and ligustilide at 8.66 ppm, 6.37-6.35 ppm, and 5.55-5.53 ppm, respectively. The method exhibited good precision, stability, repeatability, and spike recovery, with low limits of quantification and detection, meeting the requirements for practical analytical testing. In summary, the use of response surface methodology to optimize the extraction process yielded accurate and reliable experimental results with good reproducibility, making it suitable for the simultaneous extraction of ferulic acid and ligustilide from Danggui; the quantitative hydrogen nuclear magnetic resonance method is simple to operate, offers rapid analysis, and possesses strong

specificity, and can be used for the simultaneous determination of ferulic acid and ligustilide in Danggui.

Full Text

Preamble

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Title: Optimization of Extraction Process for Ferulic Acid and Ligustilide from Angelica sinensis by Response Surface Methodology

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Abstract

This study aimed to optimize the simultaneous extraction of ferulic acid and ligustilide, the principal active components in Angelica sinensis, and to provide a reference for its effective development and utilization. Ultrasonic-assisted extraction was optimized using response surface methodology, with quantitative determination performed by ^{1}H-qNMR. The comprehensive score of ferulic acid and ligustilide content served as the evaluation index, while ethanol concentration, extraction time, and liquid-to-solid ratio were selected as the three main factors. Single-factor screening combined with Box-Behnken central composite design was employed to optimize extraction parameters. The optimal conditions were determined to be 80.87% ethanol concentration, 13.04 mL · g ^{1} liquid-to-solid ratio, and 30.14 min extraction time, with validation experiments confirming the predicted values. For ^{1}H-qNMR quantification, DMSO-d was used as the solvent and pyrazine as the internal standard, with quantitative resonance peaks at 8.66 ppm for pyrazine, 6.37–6.35 ppm for ferulic acid, and 5.55–5.53 ppm for ligustilide. The method demonstrated excellent precision, stability, repeatability, and spike recovery, with low limits of quantification and detection, meeting practical analytical requirements. In summary, response surface methodology provided accurate, reliable, and reproducible optimization for the simultaneous extraction of ferulic acid and ligustilide from Angelica sinensis. The ^{1}H-qNMR method offers simple operation, rapid analysis, and strong specificity, making it suitable for simultaneous determination of these two components.

Keywords: extraction process, Angelica sinensis, Box-Behnken response surface method, ^{1}H-qNMR

Angelica sinensis, the dried root of *Angelica sinensis* (Oliv.) Diels (Apiaceae), is one of the most commonly used traditional Chinese medicines in clinical practice, with functions of nourishing blood, promoting blood circulation, regulating menstruation, relieving pain, and moistening intestines (State Pharmacopoeia Commission, 2015; Dong and Chen, 2016). The main bioactive components include volatile oils, organic acids, polysaccharides, and flavonoids (Zhao et al., 2013; Zhou et al., 2012). Ferulic acid represents the organic acid fraction and is one of the earliest isolated active constituents (Zhao et al., 2013). The volatile oil content is approximately 1%, with ligustilide being the most abundant component. The 2015 edition of the Chinese Pharmacopoeia uses ferulic acid content determination alongside conventional identification for quality control of Angelica sinensis, though ligustilide content is not yet specified (State Pharmacopoeia Commission, 2015).

In recent years, response surface methodology and quantitative ^{1}H-NMR have been widely applied for extraction optimization and content determination of active components in traditional Chinese medicines (Gao et al., 2017; Lin et al., 2014; Tanaka et al., 2019; Wang et al., 2016; Hu et al., 2019; Huang, 2019; Liu et al., 2014; Sun et al., 2017; Xu et al., 2013; Wang et al., 2019; Zhao, 2016; Zhou et al., 2019). The ^{1}H-qNMR method offers extremely short testing times (2–5 min), excellent reproducibility of characteristic peak chemical shifts across different instruments, and eliminates the need for expensive reference standards, enabling quantification using inexpensive, readily available internal standards (Gao et al., 2017; Lin et al., 2014; Tanaka et al., 2019; Wang et al., 2016). Compared with conventional orthogonal experimental design, response surface methodology offers numerous advantages: fewer experimental runs, higher detection precision, more accurate predictive models, and better reflection of relationships between experimental factors and responses (Hu et al., 2019; Huang, 2019; Sun et al., 2017; Xu et al., 2013; Wang et al., 2019; Zhao, 2016; Zhou et al., 2019). This study employed the comprehensive score of ferulic acid and ligustilide content as the response value, using Design-Expert 10.0.4.0 for experimental design to optimize the extraction process, providing a reference for Angelica sinensis application and quality standard establishment.

1.1 Experimental Instruments and Materials

Instruments: Bruker Advance III 600 NMR spectrometer, AL-104 electronic analytical balance (Cixi Tiandong Weighing Apparatus Factory), KQ-500B ultrasonic cleaner (Shenzhen Dekang Technology Co., Ltd.), R-101N rotary evaporator (Zhengzhou Greatwall Scientific Industrial and Trade Co., Ltd.), constant temperature water bath (Jiangsu Zhengji Co., Ltd.).

Materials: Ferulic acid reference standard (batch No. FD170255) was pur-

chased from Saen Chemical Technology Co., Ltd.; ligustilide (>98% purity) from Beijing Kulaibo Technology Co., Ltd.; pyrazine from Shanghai Macklin Biochemical Technology Co., Ltd.; DMSO-d (99.9% deuterated purity) from Saen Chemical Technology Co., Ltd. Angelica sinensis medicinal material was purchased from Min County, Gansu Province, and identified as authentic by Associate Professor Daiyu Qiu from the Traditional Chinese Medicine Teaching and Research Section of Gansu Agricultural University.

1.2 Methods

1.2.1 ^{1}H-qNMR Test Conditions DMSO-d served as the test solvent and pyrazine as the internal standard. The quantitative resonance peaks for pyrazine, ferulic acid, and ligustilide were 8.66 ppm, 6.37–6.35 ppm, and 5.55–5.53 ppm, respectively. The zg30 pulse sequence was employed with a spectral width (SWH) of 11904.8 Hz, relaxation delay (D1) of 1 s, pulse width (P1) of 14.90 s, and 32 scans (NS). All data were processed using MestReNova software.

1.2.2 Solution Preparation Internal Standard Solution: Accurately weigh 19.70 mg of pyrazine internal standard and dissolve in 3.94 mL DMSO-d to prepare a 5.00 mg · mL ^{1} solution for ^{1}H-qNMR sample quantification. For linearity studies, accurately weigh 7.50 mg of pyrazine and dissolve in 0.75 mL DMSO-d to prepare a 10.00 mg · mL ^{1} internal standard solution.

Reference Standard Solutions: Accurately weigh 22.00 mg of ferulic acid reference standard and dissolve in 2 mL DMSO-d to prepare an 11.00 mg · mL ^{1} solution. Accurately weigh 59.00 mg of ligustilide reference standard and dissolve in 2 mL DMSO-d to prepare a 29.50 mg · mL ^{1} solution.

Angelica Extract Preparation: Place approximately 10.00 g of powdered medicinal material (passed through 40-mesh sieve) in a volumetric flask, add 100 mL of 90% ethanol, and ultrasonicate for 40 min at 40 °C. Filter and repeat the extraction twice, combining the filtrates. Concentrate under reduced pressure at 50 °C until no alcohol odor remains, extract twice with double volume of ethyl acetate, combine the ethyl acetate layers, and evaporate to dryness on a 70 °C water bath to obtain the crude extract, which is then weighed.

Test Sample Preparation: Accurately weigh 20 mg of the above extract, add 50 L of pyrazine internal standard solution (19.70 mg pyrazine dissolved in 3.94 mL DMSO-d), then add 450 L DMSO-d , sonicate to dissolve, and transfer to an NMR tube for ^{1}H-qNMR determination [Figure 1: see original paper].

Note: A. Pyrazine; B. Ferulic acid standard; C. Ligustilide standard; D. Angelica extract; 1. Quantitative peak of ferulic acid; 2. Quantitative peak of ligustilide; = 8.66 is the quantitative peak of pyrazine internal standard.

Figure 1: ^{1}H-NMR Spectra

1.2.3 Methodology Validation **1.2.3.1 Linearity Study:** Transfer 400, 200, 100, 50, 25, and 13 L of reference standard stock solution from Section 1.2.2 into NMR tubes, add 50 L of pyrazine internal standard solution (7.5 mg pyrazine dissolved in 0.75 mL DMSO-d), then sequentially add 50, 250, 350, 400, 425, and 437 L DMSO-d to obtain ferulic acid reference solutions at 8.80, 4.40, 2.20, 1.10, 0.550, and 0.286 mg · mL ^{1}, and ligustilide reference solutions at 23.6, 11.8, 5.90, 2.95, 1.48, and 0.767 mg · mL ^{1} for ^{1}H-NMR determination.

1.2.3.2 Precision Study: Using the reference standard solution from Section 1.2.2, perform five replicate determinations under the NMR conditions specified in Section 1.2.1, and calculate the RSD values for ferulic acid and ligustilide based on the ratio of reference standard peak area to internal standard peak area.

1.2.3.3 Repeatability Test: Prepare six parallel test sample solutions and determine under identical experimental conditions, calculating RSD values for ferulic acid and ligustilide based on the ratio of sample peak area to internal standard peak area.

1.2.3.4 Stability Test: Store the test sample solution at room temperature for 0, 2, 4, 8, 10, 12, and 24 h, then perform stability determination, calculating RSD values based on the ratio of sample peak area to internal standard peak area.

1.2.3.5 Spike Recovery Test: Prepare five test solutions from known-content Angelica extract samples, add ligustilide and ferulic acid reference standard DMSO-d solutions, determine under Section 1.2.1 conditions, and calculate recovery and RSD values.

1.2.4 Limits of Detection and Quantification The ^{1}H-qNMR limit of detection (LOD) and limit of quantification (LOQ) were calculated using LOD = 3.3 /S and LOQ = 10 /S, where represents the residual standard deviation of the regression line and S represents the slope of the linear regression curve with non-zero intercept.

1.2.5 Sample Content Determination Prepare two parallel test sample solutions following the method in Section 1.2.2, and perform duplicate determinations under the NMR conditions specified in Section 1.2.1, calculating the average content.

1.2.6 Data Standardization Single-factor and response surface test results were standardized using a standardization formula to obtain a comprehensive score for the two index components.

1.2.7 Single-Factor Experimental Design Using the comprehensive score of ligustilide and ferulic acid extraction content as the evaluation index, three

factors—ethanol concentration, extraction time, and liquid-to-solid ratio—were investigated for their effects on extraction efficiency.

1.2.8 Response Surface Experimental Design Based on the optimized single-factor values, Box-Behnken response surface design was implemented using Design-Expert 10.0.4.0 software to further optimize ethanol concentration, liquid-to-solid ratio, and extraction time, with replicated experiments and analysis.

2.1 Method Validation Results

2.1.1 Linearity Study Linear regression of reference standard integrated area (X) versus concentration (Y) yielded the equations: ferulic acid $Y = 7.007X + 0.016$, $R^2 = 0.999$, linear range 0.286–8.80 mg · mL ^{1}; ligustilide $Y = 7.223X + 0.211$, $R^2 = 0.999$, linear range 0.767–23.6 mg · mL ^{1}.

2.1.2 Precision Study RSD values for ferulic acid and ligustilide, calculated from the ratio of reference standard peak area to internal standard peak area, were 1.41% and 0.34%, respectively, indicating good instrument precision.

2.1.3 Repeatability Test RSD values for ferulic acid and ligustilide, calculated from the ratio of sample peak area to internal standard peak area, were 1.50% and 1.54%, respectively, demonstrating good method repeatability.

2.1.4 Stability Test RSD values for ferulic acid and ligustilide, calculated from the ratio of sample peak area to internal standard peak area after storage, were 2.21% and 1.62%, respectively, indicating that test solutions remained stable for 24 h.

2.1.5 Spike Recovery Test Recoveries for ferulic acid and ligustilide were 95.2%–102.3% (RSD = 2.91%) and 97.7%–103.7% (RSD = 2.41%), respectively, with mean recoveries of 98.6% and 100.24%, demonstrating good spike recovery.

2.2 Limits of Detection and Quantification

Calculated LOD and LOQ values were ferulic acid LOD = 21.11 g · mL ^{1}, LOQ = 63.97 g · mL ^{1}; ligustilide LOD = 58.76 g · mL ^{1}, LOQ = 178.07 g · mL ^{1}.

2.3 Single-Factor Experimental Results

TABLE:1 shows the single-factor experimental design scheme (triplicate extractions). **TABLE:2** presents the standardized data processing results. The comprehensive score initially increased with ethanol volume fraction, then decreased, reaching maximum at approximately 80% ethanol, indicating optimal solubility of both components. For liquid-to-solid ratio, the comprehensive score plateaued above 12 mL · g ^{1}, suggesting most active components were extracted

at this ratio. The comprehensive score increased gradually from 10–30 min, stabilized around 30 min, and decreased slightly after 40 min, establishing 30 min as the optimal ultrasonic extraction time.

TABLE:1 Single-factor test conditions

Factor	Changing conditions	Fixed condition
Ethanol concentration (%)	50, 60, 70, 80, 90	Liquid-to-solid ratio 12 mL · g ^{1}, extraction time 40 min, temperature 40 °C
Solid-to-liquid ratio (mL · g ^{1})	4, 8, 12, 16, 20	Ethanol concentration 90%, extraction time 40 min, temperature 40 °C
Extraction duration (min)	10, 20, 30, 40, 50	Ethanol concentration 90%, liquid-to-solid ratio 12 mL · g ^{1}, temperature 40 °C

TABLE:2 Single-factor investigation results

Factor	Level	Ferulic acid (mg · g ^{1})	Ligustilide (mg · g ^{1})	Z-score
Ethanol concentration (%)	50	0.48 (-1.43)	3.11 (-1.29)	-1.36
	60	0.64 (-0.49)	3.78 (-0.79)	-0.64
	70	0.82 (0.58)	4.59 (-0.18)	0.20
	80	0.98 (1.53)	6.64 (1.36)	1.45
	90	0.69 (-0.19)	6.04 (0.91)	0.36
Solid-to-liquid ratio (mL · g ^{1})	4	0.43 (-1.97)	4.12 (-1.99)	-1.98
	8	0.64 (0.14)	5.86 (0.33)	0.24
	12	0.69 (0.64)	6.04 (0.57)	0.61
	16	0.63 (0.29)	6.04 (0.57)	0.43
	20	0.63 (0.29)	6.01 (0.53)	0.41
Extraction duration (min)	10	0.52 (-1.96)	6.25 (0.55)	-0.71
	20	3.47 (-2.00)	6.43 (0.72)	-0.64

Factor	Level	Ferulic acid (mg • g ^{1})	Ligustilide (mg • g ^{1})	Z-score
	30	0.68 (0.31)	6.04 (0.36)	0.34
	40	0.72 (0.88)	6.02 (0.34)	0.61
	50	0.69 (0.45)	6.01 (0.53)	0.49

Note: Values in parentheses are standardized data.

2.4 Response Surface Experimental Results

2.4.1 Response Surface Experimental Design Ethanol concentration (A), liquid-to-solid ratio (B), and extraction time (C) were selected as independent variables, with the comprehensive score of ferulic acid and ligustilide content as the response value (R). Box-Behnken design was implemented, with the design matrix and results shown in **TABLE:3**.

2.4.2 Model Significance Analysis Data from **TABLE:3** were processed using Design-Expert 10.0.4.0 software, yielding the regression equation: R = 2.09 + 0.47A + 0.13B + 0.19C -0.12AB + 0.44AC -0.38BC -3.29A^2 -0.73B^2 - 0.43C^2. The adjusted coefficient was 0.9927 and determination coefficient (r^2) = 0.9968, indicating good model fit and minimal experimental error, suitable for analysis.

TABLE:4 presents the variance analysis and significance test results, demonstrating that ethanol concentration, liquid-to-solid ratio, extraction time, and the interactions between ethanol concentration and liquid-to-solid ratio, and between extraction time and liquid-to-solid ratio, significantly affected the comprehensive score. The order of factor influence was ethanol concentration (A) > liquid-to-solid ratio (C) > extraction time (B).

2.4.3 Optimal Extraction Parameter Prediction and Experimental Validation **FIGURE:2** shows that the 3D response surface plots for ethanol concentration versus liquid-to-solid ratio and ethanol concentration versus extraction time exhibit steep slopes, indicating significant interactions and substantial effects on extraction of both active components. Targeting maximum comprehensive score, software analysis predicted optimal conditions of 80.87% ethanol concentration, 13.04 liquid-to-solid ratio, and 30.14 min extraction time. For practical operation, parameters were adjusted to 81% ethanol, 13 mL • g ^{1} liquid-to-solid ratio, and 30 min extraction time, with triplicate validation experiments confirming high accuracy and stability for determining both components in Angelica sinensis.

TABLE:3 Response surface experiment design and results

Run	A: Ethanol concentration	B: Extraction duration (min)	C: Solid-to-liquid ratio (mL · g ^{1})	Ferulic acid (mg · g ^{1})	Ligustilide (mg · g ^{1})	Z-score
1	90 (1)	40 (1)	12 (0)	0.69 (-1.51)	6.04 (-0.08)	-0.80
2	80 (0)	30 (0)	12 (0)	1.03 (1.00)	6.94 (0.88)	0.94
3	80 (0)	40 (1)	16 (1)	0.97 (0.56)	6.53 (0.44)	0.50
4	90 (1)	30 (0)	16 (1)	0.75 (-1.06)	5.53 (-0.63)	-0.85
5	70 (-1)	30 (0)	16 (1)	0.98 (0.63)	6.77 (0.70)	0.67
6	80 (0)	20 (-1)	16 (1)	0.81 (-0.62)	4.41 (-1.82)	-1.22
7	80 (0)	40 (1)	8 (-1)	0.82 (-0.55)	4.59 (-1.63)	-1.09
8	90 (1)	20 (-1)	12 (0)	0.84 (-0.40)	6.59 (0.50)	0.05
9	70 (-1)	20 (-1)	12 (0)	1.05 (1.15)	6.97 (0.91)	1.03
10	70 (-1)	30 (0)	8 (-1)	0.76 (-0.99)	6.56 (0.47)	-0.26
11	90 (1)	30 (0)	8 (-1)	1.06 (1.22)	6.96 (0.90)	1.06
12	80 (0)	30 (0)	12 (0)	1.07 (1.30)	6.94 (0.88)	1.09
13	80 (0)	30 (0)	12 (0)	0.84 (-0.40)	4.76 (-1.45)	-0.93
14	80 (0)	30 (0)	12 (0)	0.79 (-0.77)	4.49 (-1.74)	-1.26
15	70 (-1)	40 (1)	12 (0)	0.68 (-1.58)	6.25 (0.14)	-0.72
16	90 (1)	30 (0)	12 (0)	1.08 (1.37)	6.93 (0.87)	1.12
17	80 (0)	20 (-1)	8 (-1)	0.98 (0.63)	6.73 (0.65)	0.64

Note: Values in parentheses are standardized data.

TABLE:4 Analysis of variance

Source of variance	Sum of squares	Freedom	F value	P value
Model	<0.0001			
Residual				
Missing				
Pure error				
Total	<0.0001			

Note: $P < 0.05$ indicates significant difference; $P < 0.01$ indicates highly significant difference.

FIGURE:2 Optimized response surface map

TABLE:5 Verification results

Index	Ferulic acid (mg · g ^{1})	Ligustilide (mg · g ^{1})	Z-score
1	1.07 (1.30)	7.01 (0.95)	1.13
2	1.04 (1.07)	6.98 (0.92)	1.00
3	1.09 (1.45)	6.96 (0.90)	1.18

Note: Values in parentheses are standardized data.

Extraction Solvent Selection: Different concentrations of ethanol and methanol were evaluated for Angelica powder extraction. Ethanol extraction yielded higher contents of both ferulic acid and ligustilide compared to methanol. Additionally, ethanol' s lower toxicity and environmental friendliness make it more suitable for industrial production, establishing it as the preferred extraction solvent.

Deuterated Solvent and Internal Standard Selection: An appropriate deuterated solvent should provide good solubility for both sample and internal standard. The internal standard should have an easily identifiable peak that does not overlap with analyte signals. Following literature review and screening experiments, pyrazine was selected as the internal standard due to its low cost, stability, non-reactivity with analytes, and non-overlapping signals, with its ^{1}H-NMR peak at 8.66 ppm. DMSO-d effectively dissolved both the test samples and internal standard without peak overlap, making it the optimal solvent choice.

Response surface methodology offers advantages including short experimental cycles, high detection precision, accurate predictive models, ability to study factor interactions, rational experimental design, and reliable results, leading to widespread application in optimizing traditional Chinese medicine extraction processes (Hu et al., 2019; Huang, 2019; Sun et al., 2017; Xu et al., 2013; Wang

et al., 2019; Zhao, 2016; Zhou et al., 2019). This study investigated ethanol concentration, liquid-to-solid ratio, and extraction time, revealing significant effects on simultaneous extraction of ferulic acid and ligustilide from Angelica sinensis. Box-Behnken central composite design optimization yielded optimal extraction conditions, providing a reference for effective development and utilization of Angelica sinensis.

Current content determination of active components in Angelica sinensis primarily employs HPLC, which suffers from complex mobile phase preparation, long detection times, and requirement for reference standards. Quantitative ^{1}H-NMR offers rapid detection, high accuracy, good reproducibility, and simultaneous quantification of multiple components using inexpensive internal standards instead of costly reference standards, with the entire detection process completed in approximately 3 minutes (Gao et al., 2017; Lin et al., 2014; Tanaka et al., 2019; Wang et al., 2016). This study successfully applied ^{1}H-qNMR for simultaneous determination of ferulic acid and ligustilide in Angelica sinensis, effectively reducing costs.

In this study, Angelica sinensis was investigated using quantitative ^{1}H-NMR to determine ferulic acid and ligustilide content in extracts. With the comprehensive score of these two components as the evaluation index, single-factor screening combined with Box-Behnken central composite design optimized extraction parameters. The findings revealed: (1) Optimal extraction conditions were 80.87% ethanol concentration, 13.04 mL·g^{-1} liquid-to-solid ratio, and 30.14 min extraction time, yielding a comprehensive extraction score of 2.14. (2) Under these optimal conditions, ferulic acid and ligustilide contents were 1.07 mg·g^{-1} and 6.98 mg·g^{-1}, respectively. This optimized process is simple, reproducible, and provides valuable reference for extraction of active components from Angelica sinensis.

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Note: Figure translations are in progress. See original paper for figures.