

Orthogonal test design for optimizing the extraction of total flavonoids from *Inula helenium*

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Submitted: 02-06-2012

Revised: 08-08-2012

Published: 11-06-2013

ABSTRACT

Background: *Inula helenium*, which belongs to the composite family, is an important crude drug in traditional Chinese medicine. **Materials and Methods:** The effects of ethanol concentration, liquid to solid ratio, extraction temperature, and duration of microwave irradiation on the flavonoid extraction yield were studied through a single-factor experiment. An orthogonal array ($L_9(3^4)$) was then constructed to achieve the best extraction conditions. **Results:** Variance analysis revealed that ethanol concentration significantly affected the extraction yield. The optimal conditions were as follows: ethanol concentration, 50% (v/v); liquid to solid ratio, 15:1; duration of microwave irradiation, 240 s; and extraction temperature, 60°C. **Conclusion:** Under these optimal conditions, the total flavonoid yield was 18.34 ± 0.64 mg/g. The use of a microwave-assisted process dramatically reduced the time needed for extraction of flavonoids from *I. helenium*.

Key words: *Inula helenium*, microwave-assisted extraction, orthogonal design, total flavonoids

Access this article online

Website:
www.phcog.com

DOI:
10.4103/0973-1296.113260

Quick Response Code:



INTRODUCTION

Inula helenium (elecampane), a member of the composite family, is widely distributed throughout central and southern Europe as far as the Himalayas, in Asia. In the Chinese Pharmacopoeia, *I. helenium* is described as an expectorant, diaphoretic, and antitussive that is often used to treat a variety of respiratory and digestive diseases. Recent research has demonstrated that the extracts of *I. helenium* exhibit many effects including antibacterial, antitumor, and antiproliferative.^[1-3]

Flavonoid compounds are diverse class of secondary plant metabolites that are widely distributed throughout the plant kingdom and have been reported to possess a variety of biological activities such as antioxidant, anti-atherosclerotic, anti-inflammatory, and antithrombotic; they also aid in cardiovascular disease prevention and anti-platelet aggregation.^[4-6]

Conventional flavonoid extraction methods include

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Soxhlet extraction, maceration, and heat reflux extraction. However, these methods result in decreased yield because of oxidation and hydrolysis. As a new technique, microwave-assisted extraction (MAE) has attracted a great deal of attention, especially in the food and medicinal plant research industries. Compared with conventional extraction methods, MAE has many advantages such as reduced time, less solvent consumption, higher extraction rates and greater environmental protection.^[7]

The purpose of this work is to optimize the MAE procedure for extracting flavonoids from *I. helenium*, which has not been reported before. An orthogonal array is employed to analyze the interaction among the MAE operating factors.

MATERIALS AND METHODS

Material and reagents

The roots of *I. helenium* purchased and identified from the An-Guo herbal medicine market, China by Prof. Hengcheng Zhao (College of Traditional Chinese Medicine, Hebei North University) which were chosen as the raw material for this experiment. The specimens (No.2009-10) were deposited in the Department of Pharmacy, Hebei North University. Analytical grade used to extract the total flavonoids from *I. helenium*. Rutin was obtained from the National Institutes for

Food and Drug Control. Sodium nitrite and sodium hydroxide were purchased from Tianjin Fengchuan Chemical Reagent Science and Technology Co. Ltd. (Tianjin, China) Analytical-grade aluminum nitrate was purchased from Tianjin Jinhuitaiya Chemical Reagent Co., Ltd. (Tianjin, China)

Apparatus

Total flavonoids were extracted from the *I. helenium* roots using a MAS-I microwave synthesis system (Shanghai Sineo Microwave Chemistry Technology Co., Ltd., Shanghai, China). The apparatus included a digital temperature control panel, a time regulator, mechanical and magnetic variable speed stirrers, a non-contact infrared temperature sensor, a condensation system, and a liquid crystal display monitor system. The microwave power would dynamically adjust within 0–1000 W according to the temperature inside the vessels.

Microwave-assisted extraction

The *I. helenium* roots were sliced and air-dried at room temperature. They were then milled into a powder using a DFY-800B universal high-speed grinder (Wenling Linda Machine Co., Ltd., Wenzhou, China). The powder was sifted through a 100-mesh sieve for the extraction experiments. The process of total flavonoid extraction from *I. helenium* was performed in a MAS-I microwave synthesis system. The powder (5 g) was placed in a round-bottom flask and mixed with ethanol solvent (varying ethanol concentrations of 0–100%, v/v; varying liquid to solid ratios of 5:1 to 30:1). The extraction variables were temperature (30, 40, 50, 60, and 70°C) and microwave irradiation time (30, 60, 120, 180, 240, and 300 s). The stirring speed in the extraction solution was maintained at 500 rpm for all experiments to eliminate the effects of external energy transfer and internal diffusion. The extracts were allowed to cool to room temperature before being centrifuged at 3000 rpm for 15 min, which facilitated the deposition of suspended particles. The supernatants were collected and filtered through a 0.45- μ m nylon filter before being analyzed using a spectrophotometer.

Colorimetric analysis of total flavonoids

The concentration of total flavonoids in the extracts was measured using the aluminum nitrate colorimetric method.^[8] This method is based on the reaction of chelated flavonoids with aluminum under neutral or weak alkaline conditions (in the presence of sodium nitrite). After the addition of sodium hydroxide, the color of the reaction solution was red-orange, and the wavelength of its maximum absorption was 500 nm. The total flavonoid content was calculated and expressed as rutin equivalents.

Optimization of flavonoids extraction

Optimization of the MAE conditions for *I. helenium* has yet not been reported. Other related articles used solvent concentration, microwave irradiation time, liquid to solid

ratio, and extraction temperature as parameters. In this study, an orthogonal array ($L_9(3^4)$) was constructed to evaluate the effects of the following factors: ethanol concentration (A), liquid to solid ratio (B), extraction temperature (C), and duration of microwave irradiation (D). Factors and experimental data are displayed in Tables 1 and 2, respectively. The data were analyzed using SPSS statistical software (SPSS for Windows version 16.0; SPSS Inc, Chicago, American.).

Statistical analysis

All of the experiments were performed in triplicate. The data are expressed as mean \pm standard deviation (SD) and the significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of ethanol concentration on flavonoid yield

Solvents of different polarity are known to yield different component types and quantities of extracts. Thus, the selection of a suitable solvent for extraction is an important step in obtaining an optimal extraction yield. In this study, water and different ethanol concentrations were tested as extraction solvents. Five grams of powder was extracted using 50 mL of each different solvent in a 300 mL round-bottom flask under microwave heating at 50°C for 300s. The total flavonoid yields using the different solvents are shown in Figure 1. The flavonoid yields peaked when the ethanol concentration was 50 %; at higher or lower concentrations, the yield was suppressed. The main reason for this was that, as a polar solvent with a high dielectric constant, water can absorb more microwave energy, facilitating easy heat transfer to the reaction system. The extraction efficiency increased with increasing temperature. Furthermore, the flavonoids had low polarity and the ethanol had high affinity for them. Owing to these factors, subsequent experiments were conducted using 50% ethanol.

Effect of liquid to solid ratio on flavonoid yield

The extraction yield was greatly influenced by the liquid to solid ratio. Figure 2 shows that the extraction yield increased and then decreased as the liquid to solid ratio increased. This was probably because a decrease in the liquid to solid ratio reduced the surface area of the plant tissue available to the solvent for penetration and solubilization of the target

Table 1: Orthogonal design factors and levels

Factors	Level		
	1	2	3
Ethanol concentration (A)	40%	50%	60%
Liquid to solid ratio (B)	12:1	15:1	18:1
Extraction temperature (C)	55°C	60°C	65°C
Duration of microwave irradiation (D)	210 s	240 s	270 s

components, which was not beneficial for the extraction process. On the other hand, a greater volume of solvent caused sufficient swelling of the material, but inadequate agitation of the solvent may have occurred during the irradiation step. The experimental results show that 15 mL/g produced the maximum flavonoid yield.

Effect of duration of microwave irradiation on flavonoid yield

Studies have shown that microwave irradiation is an important factor affecting active ingredient yield. To achieve the maximum flavonoid yield, different irradiation times (30, 60, 120, 180, 240, and 300 s) were investigated. The other experimental conditions included a material to solvent ratio (50% ethanol) of 1:15 and extraction temperature of 50°C. Figure 3 shows the correlation between the extraction microwave irradiation time and flavonoid yield. The result suggests, as expected, that increased microwave irradiation time increases flavonoid yield. The extraction yield increased rapidly in the initial

stage and reached equilibrium at 240 s. Longer extraction time may have negative effects of product degeneration or conversion. Therefore, 240 s was chosen as the microwave irradiation time in subsequent experiments.

Effect of extraction temperature on flavonoid yield

The effect of temperature on flavonoid yield was evaluated in the present study under the optimal conditions described above. As shown in Figure 4, the temperature increase from 30°C to 60°C improved the total flavonoid yield from 13.79 to 18.08 mg/g. The increase suggests that high temperature leads to increased extraction efficiency since it accelerates active ingredient diffusion. Furthermore, the flavonoid solubility increased as a result of the temperature improvement. Meanwhile, as temperature increased, solvent viscosity and surface tension decreased, which contributed to the sample wetting and matrix penetration. Once the extraction temperature exceeded 60°C, the extraction yield decreased slightly. Hence, the temperature of 60°C was considered appropriate to achieve peak total flavonoid yield via MAE.

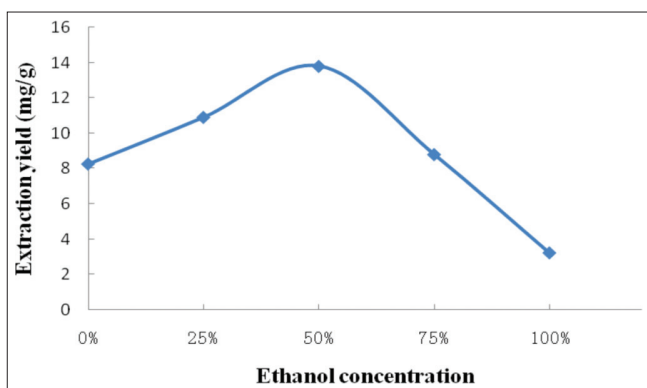


Figure 1: Effect of ethanol concentration on extraction yield (liquid to solid ratio, 10:1 [mL/g]; amount of root material, 5 g; microwave-assisted extraction for 300 s; extraction temperature, 50°C)

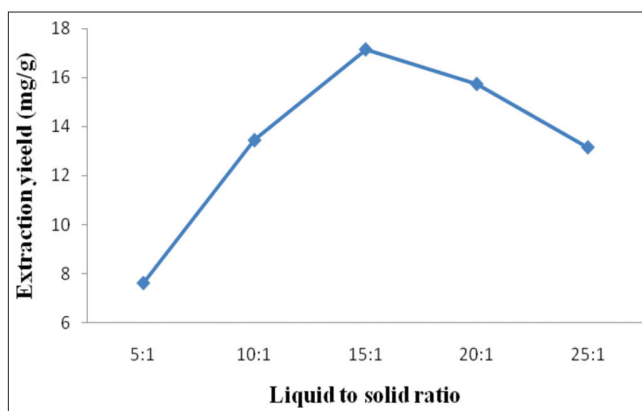


Figure 2: Effect of liquid to solid ratio on extraction yield (amount of root material, 5 g; solvent, 50% ethanol; microwave-assisted extraction for 300 s; extraction temperature, 50°C)

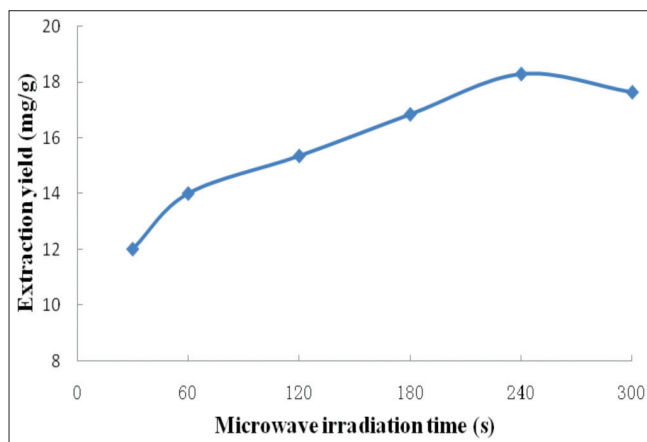


Figure 3: Effect of microwave irradiation time on extraction yield (amount of root material, 5 g; solvent, 50% ethanol; liquid to solid ratio, 15:1 (mL/g); extraction temperature, 50°C)

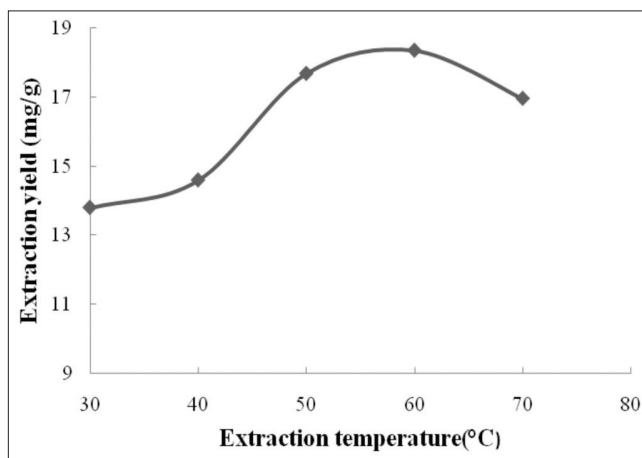


Figure 4: Effect of extraction temperature on extraction yield (amount of root material, 5 g; solvent, 50% ethanol; liquid to solid ratio, 15:1 (mL/g); microwave-assisted extraction for 240 s)

Table 2: Orthogonal design and experimental results

No.	A	B	C	D	Yield (mg/g)
1	1	1	1	1	13.34 ± 0.4
2	1	2	2	2	15.05 ± 0.4
3	1	3	3	3	12.84 ± 0.5
4	2	1	2	3	16.26 ± 0.6
5	2	2	3	1	16.18 ± 0.6
6	2	3	1	2	15.79 ± 0.4
7	3	1	3	2	13.81 ± 0.5
8	3	2	1	3	14.51 ± 0.4
9	3	3	2	1	14.13 ± 0.5
K1	13.74 ± 0.4	14.47 ± 0.5	14.55 ± 0.4	14.55 ± 0.5	
K2	16.08 ± 0.5	15.25 ± 0.5	15.15 ± 0.5	14.88 ± 0.4	
K3	14.15 ± 0.5	14.25 ± 0.5	14.28 ± 0.6	14.54 ± 0.6	
R	2.33	1.00	0.87	0.34	

Table 3: Analysis of variance of the four studied microwave-assisted extraction parameters

Source	Sum of squares	Degree of freedom	F-ratio	F _{0.05}	Significant
A	9.322	2	40.36	19.00	*
B	1.637	2	7.09	19.00	
C	1.190	2	5.512	19.00	
D	0.231	2	1.00	19.00	
Error	0.231	2			

*Significant ($P < 0.05$) by F-test

Optimizing the *I. helenium* flavonoid extraction conditions

The experimental data obtained from orthogonal design are shown in Table 2. $K1-K3$ was the average total flavonoid yield under the various investigated conditions, and the maximum value was the optimum value. In addition, according to the largest donating rule, the factor with the largest range value ($K_{max}-K_{min}$) have the greatest effect on the extraction of flavonoids. Table 2 shows that the rank order of the four influential factors is $R_A > R_B > R_C > R_D$.

To evaluate which factors had the greatest influence on flavonoid extraction yield, variance analyses (ANOVA) were applied to assess the results. The result [Table 3] indicated that ethanol concentration (factor A) had the greatest impact and that duration of microwave irradiation (factor D) had the least influence. The ANOVA result was in good agreement with what was observed in Table 2. The maximal yields were obtained when the ethanol concentration was 50 %; liquid to solid ratio was 15:1; the extraction temperature was 60°C; and the microwave irradiation time was 240 s.

CONCLUSIONS

Through this optimization study, an efficient MAE method was developed to isolate total flavonoids from *I. helenium* roots. Optimal conditions of 50% (v/v) ethanol as the solvent, liquid to solid ratio of 15:1, and duration of microwave

irradiation of 240 s at 60°C resulted in a total flavonoid yield of 18.34 ± 0.64 mg/g. MAE dramatically reduced the time needed for extraction of flavonoids from *I. helenium*.

ACKNOWLEDGEMENTS

This work was financially supported by the Hebei administration of traditional Chinese medicine project (No.2010046), the project of Hebei Education Department (No.Z2011304), and Natural Science Foundation of Hebei Province (H2012405016).

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Cite this article as: Guo C, Wang J, Hou Y, Zhao Y, Shen L, Zhang D. Orthogonal test design for optimizing the extraction of total flavonoids from *Inula helenium*. *Phcog Mag* 2013;9:192-5.

Source of Support: financially supported by the Hebei administration of traditional Chinese medicine project (No.2010046), the project of Hebei Education Department (No.Z2011304), and Natural Science Foundation of Hebei Province (H2012405016). **Conflict of Interest:** None declared.