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Homogenate extraction of isoflavones from soybean meal by orthogonal design

Xing-Yi Zhu, Hai-Min Lin, Jie Xie, Shan-Shan Chen and Ping Wang*
College of Pharmaceutical Sciences, Zhejiang University of Technology, Key Laboratory of Pharmaceutical Engineering of Ministry of Education, Hangzhou 310014, People's Republic of China

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Factors that affect yield of isoflavones extracted from soybean meal by using homogenate extraction method were studied by orthogonal design experiment [$L_9(3^4)$]. Optimum extraction conditions were found as follows: sample particle size, 60 mesh; ethanol concentration, 60%; extraction time, 50 s; ratio of material to liquid, 1 g: 20 ml; and yield of isoflavones, 0.52 ± 0.01%. Compared with traditional heat refluxing extraction method, yield of homogenate extraction was 29.3% higher, and extraction time was significantly shortened.

Keywords: Homogenate, Isoflavones, Orthogonal design, Soybean meal

Introduction

Isoflavones are a major class of flavonoids in soybean and soybean products[1]. There are 12 main isoflavones including 3 free aglycone isoflavones (daidzin, genistin and glycitin) and their respective glucosidic, malonyl and acetyl glucosidic conjugates (Table 1)[2-5]. Soybean isoflavones have many health benefits[6-9] with the prevention of human cancers, cardiovascular diseases, osteoporosis and postmenopausal symptoms, etc. Conventional methods for extraction of isoflavones from soybeans and soy products range from classical heat refluxing extraction[10-12] to modern techniques (ultrasonic-assisted extraction[13,14], microwave-assisted extraction[15] and supercritical fluid extraction[1,2,16,17]). These procedures give good extraction yields, but most of them require high temperature or long time to complete the process. Homogenate extraction is reported to be effective in extracting protein[18], farnesoic acid[19], terpenoids[20] etc. This study presents homogenate extraction of isoflavones from soybean meal using orthogonal test design.

Experimental Section

Materials

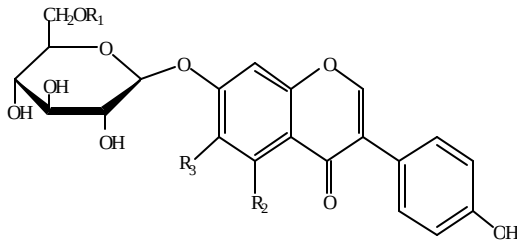
Soybean meal was purchased from Hangzhou Anyou Feed Co. Ltd (Hangzhou, China). Genistein was purchased from National Institute for control of pharmaceutical and biological products (Beijing, China). Ethanol was obtained from Tianjin Yong Da Chemical Reagent Development Center (Tianjin, China). Ultrapure water was prepared by a Milli-Q water purification system. All reagents were analytically pure. Homogenate extractor (JHBE-50A) was carried out by Golden Star Technology, Inc., Ltd (Zhengzhou, China). High speed pulverizer (DFY-500) was provided by Ding Guang Machinery Equipment Co. Ltd (Shanghai, China). Water Bath Incubator (HHS) was provided by Boxun Co., Ltd (Shanghai, China). Rotary evaporator (EYELA N-1100) was purchased from Ailang Co. Ltd (Shanghai, China). UV-Vis spectrophotometer (756PC) was carried out by Shanghai Spectrum Instruments Co. Ltd (Shanghai, China).

Establishment of Calibration Curve

Stock solution of standard genistein (0.44 mg/ml) was prepared with 95% ethanol. Stock solutions (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 ml) were transferred into calibrated volumetric flasks (10 ml each). Then 1.0 ml of 95% ethanol was added to each flask. Volume was made up to the mark with ultrapure water to obtain a series of standard solutions. Subsequently, all standard solutions were determined by UV-Vis spectrophotometer method, with a detecting wavelength of 260 nm. Each was measured in triplicate. Corresponding calibration curve was established by plotting absorbance value of

*Author for correspondence
Tel: +86 57188320867; Fax: +86 57188320867
E-mail: pharmlab@zjut.edu.cn

Table 1—Chemical structure of soybean isoflavones



S No.	Denomination	R_1	R_2	R_3
1	Daidzein	-	H	H
2	Genistein	-	H	OH
3	Glycitein	H		H
4	Dadzien	H	H	H
5	Genistien	H	H	OH
6	Glycitien	H	OCH_3	H
7	6"-O-Acetyldaidzin	$COCH_3$	H	H
8	6"-O-Acetyl genistin	$COCH_3$	H	OH
9	6"-O-Acetyl glycitin	$COCH_3$	OCH_3	H
10	6"-O-Malony daidzin	$COCH_2COOH$	H	H
11	6"-O-Malony genistin	$COCH_2COOH$	H	OH
12	6"-O-Malony glycitin	$COCH_2COOH$	OCH_3	H

each standard solution (Y-axis) against concentration (X-axis, μg/ml) and a regression equation was established.

Sample Preparation and Homogenate Extraction

Soybean meal was preliminarily ground to a certain particle size (diam, 3-5 mm) and defatted with mineral ether by heat refluxing extraction. Defatted residues were dried at 40°C for 2 h in heating oven, and stored in a refrigerator until use.

Dried and defatted soybean meal powder (20.0 g) and ethanol were put in homogenate extractor. After extraction, mixture was centrifuged for 10 min at 4700 rpm. Supernatant was collected and concentrated by rotary evaporator. In order to assure accuracy of experimental data, each experimental group was replicated three times. Standard error from replicate experiments was controlled to ± 10%.

Analysis of Isoflavones

Extracted solution (0.25 ml) was transferred into a volumetric flask (10 ml) and diluted with 95% ethanol (1.0 ml) and volume was made up to the mark with ultrapure water. UV-Vis spectrophotometer test was done in triplicate. Yield of isoflavones (%) = amount of isoflavones (g) / amount of defatted material (g) × 100%.

Statistical Analysis

Results were average of three replicates ± standard deviations. Statistical software Origin Pro 8.1 SR3 and SPSS 13.0 were used.

Results and Discussion

Linear Relationship of Calibration Curve

Regression equation of genistein was obtained by regression analysis of absorbance value of genistein solution (Y axis) against concentration (X axis, μg/ml). Linear equation (Y = 139.99 X - 0.1336) had excellent correlation coefficient, r = 0.9996 (n = 8).

Effect of Particle Size on Yield of Isoflavones

Effect of different samples (particle size, 20, 40, 60, 80, 100 mesh) on yield of isoflavones was investigated at: ethanol concentration, 60%; extraction time, 30 s; and ratio of material to liquid, 1 g: 20 ml. When sample particle size was in the range of 20-100 mesh, no significant effect on yield of isoflavones was observed. (Fig. 1a). High speed mechanical shear stress can cut material with different particle to the smallest diameter size and break cell walls to make chemical compositions dissolved. Particle size (80 mesh) was selected as optimum in subsequent experiments.

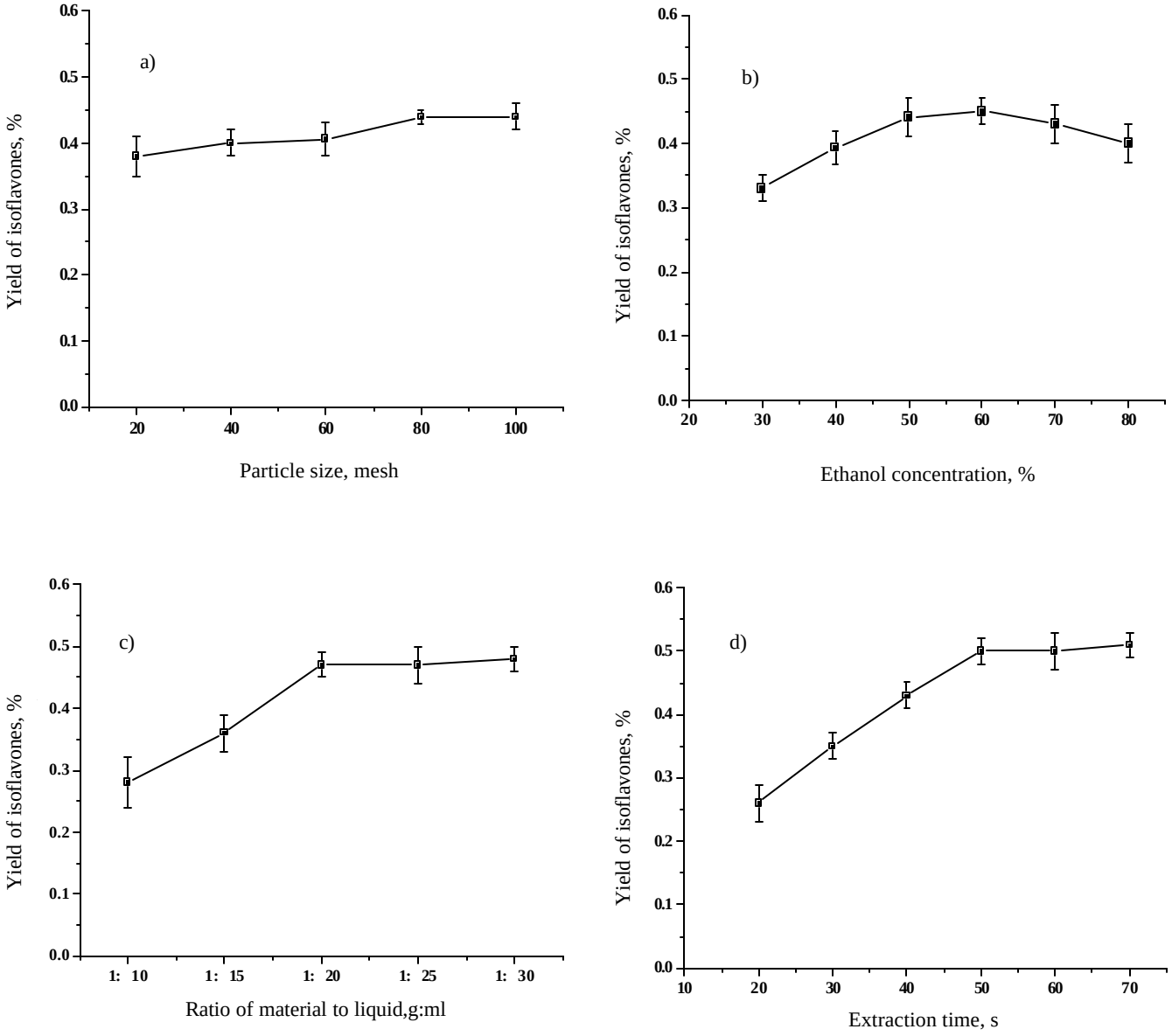


Fig.1 — Effect on yield of isoflavones of: a) particle size; b) ethanol concentration; c) ratio of material to liquid; and d) extraction time

Effect of Ethanol Concentration on Yield of Isoflavones

For effective extraction of isoflavones from soybeans, a certain amount of water to extraction solvent is necessary[15,17]. Effect of different ethanol concentrations (30, 40, 50, 60, 70 and 80%) on yield of isoflavones was studied at: extraction time, 30 s; ratio of material to liquid, 1 g: 20 ml; and particle size, 80 mesh. When ethanol concentration increased, yield of isoflavones increased and reached highest at ethanol concentration of 60% (Fig. 1b), and then decreased with higher ethanol concentration, might be due to solubility of different isoflavones are different in ethanol. Therefore, ethanol concentration (60%) was optimum.

Effect of Ratio of Material to Liquid on Yield of Isoflavones

Effect of different ratio of material to liquid on yield of isoflavones was studied at: particle size, 80 mesh; ethanol concentration, 60%; and extraction time, 50 s. Yield of isoflavones increased with increase of ratio of material to liquid (Fig. 1c). When ratio of material to liquid was lower than optimum (1 g: 20 ml), isoflavones could not be extracted completely. When using lager

Table 2—Levels and factors of orthogonal experiments

Levels	Factors			
	A Particle size mesh	B Ethanol concentration, %	C Ratio of material to liquid, g : ml	D Extraction time s
1	40	40	1 : 15	40
2	60	60	1 : 20	50
3	80	80	1 : 25	60

Table 3—Orthognal experimental program $L_9(3^4)$ and results of extraction (N = 3)

Test No.	Factors				
	A	B	C	D	Extraction yield of isoflavones (%)
1	1	1	1	1	0.37 ± 0.03[a]
2	1	2	2	2	0.48 ± 0.04
3	1	3	3	3	0.41 ± 0.04
4	2	1	2	3	0.45 ± 0.02
5	2	2	3	1	0.38 ± 0.01
6	2	3	1	2	0.41 ± 0.06
7	3	1	3	2	0.43 ± 0.02
8	3	2	1	3	0.44 ± 0.02
9	3	3	2	1	0.40 ± 0.01
K1	1.26	1.25	1.22	1.15	
K2	1.24	1.30	1.33	1.32	
K3	1.27	1.22	1.22	1.30	
R	0.03	0.08	0.11	0.17	

[a] Average of duplicate analyses ± standard deviation

material to liquid ratios than optimum (1 g: 20 ml), yield of isoflavones had no increase. Thus ratio of material to liquid (1 g: 20 ml) was selected as an economical extraction parameter.

Effect of Extraction Time on Yield of Isoflavones

Extraction time (20 - 70 s) was studied with: particle size, 80 mesh; ethanol concentration, 60%; and ratio of material to liquid, 1 g: 20 ml. Yield of isoflavones was optimum at 50 s (Fig. 1d), beyond which no enhanced yield was observed.

Orthogonal Experiment Design

Extraction requirement of soybean meal sample was further optimized by a $L_9(3^4)$ orthogonal design, in which particle size, ethanol concentration, ratio of material to liquid and extraction time were considered as orthogonal layout factors for extracting isoflavones from soybean meal. Factors and levels design of orthogonal layout (Table 2) and its experimental conditions for each experimental group (Table 3) were used for extraction of isoflavones.

Second group ($A_1B_2C_2D_2$) (Table 3) generated highest yield of isoflavones (0.48 ± 0.04%), whose levels of homologous factors involved particle size (40 mesh), ethanol concentration (60%), ratio of material to liquid (1 g: 20 ml), and extraction time (50 s), respectively. First group showed lowest yield of isoflavones with 0.37 ± 0.03%, whose corresponding factors and levels were $A_1B_1C_1D_1$ including particle size (40 mesh), ethanol concentration (40%), ratio of material to liquid (1 g: 15 ml), and extraction time (40 s), respectively. In view of orthogonal analysis, statistical software SPSS was used to calculate values of K and R. For influence of extraction factors and levels on yield of isoflavones, decreased order of effects of all factors was D > C > B > A, according to magnitude order of R value (maximum difference) (Table 3). This was also demonstrated by F and/or P value of variance analysis (Table 4). According to R value among four factors and result of analysis of variance table (Table 4), extraction time (D) was found to be markedly correlative with extraction yield of isoflavones. So maximum yield of isoflavones was obtained when particle size, ethanol concentration, ratio

Table 4—Variance analysis of orthogonal experimental results

Source	Sum of squares	Degree of freedom	Mean square	F value[b]	Significant level
Corrected Model	0.010[a]	6	0.002	20.429	0.047
Intercept	1.579	1	1.579	20304.143	0.000
B	0.001	2	0.001	7.000	0.125
C	0.003	2	0.001	17.286	0.055
D	0.006	2	0.003	37.000	0.026
Error(A)	0.000	2	0.000		
Total	1.589	9			
Corrected total	0.010	8			

[a]R squared = 0.984 (Adjusted R squared = 0.936); [b]$F_{0.05}$ (2, 2) = 19.000

Table 5—Comparison of different extraction methods

Methods	Extraction time	Ethanol Concentration, %	Ratio of material to liquid, g : ml	Extraction Yield, %
Homogenate extraction	50 s	60	1:20	0.52 ± 0.01
Heat refluxing extraction	120 min	80	1:20	0.41 ± 0.03

of material to liquid, and extraction time were $A_3B_2C_2D_2$ (80 mesh, 60%, 1 g: 20 ml, 50 s respectively). For industrialization, optimal conditions are as follows: $A_2B_2C_2D_2$ (60 mesh, 60%, 1 g: 20 ml, 50 s).

Verification Experiment

Triplicate experiments were performed under optimal conditions obtained from orthogonal experiment. Yield of isoflavones was 0.52%, 0.52%, 0.53%, respectively. This indicated a good validation for orthogonal extraction parameters.

Comparison of Different Extraction Methods

Homogenate extraction and heat refluxing extraction were compared for their yields at optimized conditions. Compared (Table 5) with heat refluxing extraction, yield of homogenate extraction was 29.3% higher, and extraction time was significantly shortened. Besides, homogenate extraction was performed at room temperature, which could avoid damage of thermosensitive components and save more energy.

Conclusions

Homogenate extraction technology was used to extract isoflavones from soybean meal. Optimal parameters were determined by $L_9(3^4)$ orthogonal design as follows: sample particle size, 60 mesh; ethanol concentration, 60%; extraction time, 50 s; and ratio of material to liquid, 1 g: 20 ml. Under these extraction conditions, extraction yield of isoflavones is higher than heat refluxing extraction method. Homogenate extraction technology also has advantages of time saving, low temperature and higher efficiency. It is a more efficient method for extracting isoflavones from soybean meal.

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