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Optimization of Extraction Conditions for Active Compounds of Herbal Medicinal Formula, DF, by Response Surface Methodology

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Abstract – DF formula is comprised of three traditional herbs, *Ephedra intermedia*, *Rheum palmatum* and *Lithospermum erythrorhizon*, and locally used for treating of the metabolic diseases, such as obesity and diabetes in Korea. We tried to optimize the extraction conditions of two major components, (−)-ephedrine and (+)-pseudoephedrine, in DF formula using response surface methodology with Box-Behnken design (BBD). The experimental conditions with 70% for EtOH concentrations, 4.8 hour for extraction hours and 8.7 times for the solvent to material ratio were suggested for the optimized extraction of DF formula with the highest amounts of (−)-ephedrine and (+)-pseudoephedrine in the designed model.
Keywords – Response surface methodology, *Ephedra intermedia*, (−)-ephedrine, (+)-pseudoephedrine, HPLC

Introduction

Traditional medicinal herbs have been modernized with the scientific guidelines, such as regulatory guidelines, good-manufacturing-practice guidelines and pharmaco-vigilance guideline.[1] Although many medicinal herbs are processed to increase their efficacy or reduce the toxicity with the state-of-art technologies and Westernized scientific scope, the complexity of multiple components existed in a single herb or the herbal combinations still remains the main hurdle for the quality control of the herbal products. The extraction conditions, such as temperature, extraction time, the types of the extraction solvents, play a role in the proportional changes of the bioactive components in the manufacturing process.[2]

DF formula is an herbal formulation composed of three medicinal herbs, *Ephedra intermedia* Schrenk C. A. Meyer, *Rheum palmatum* Linne, and *Lithospermum erythrorhizon* Siebold et Zuccarini. It is locally used for the treatment of diabetes, obesity and the metabolic diseases in Korea. *E. intermedia* (Ma Huang) which is native to Northern China or Inner Mongolia is one of *Ephedra* species and its aerial parts are using for the treatment of asthma, bronchial spasms and coughing.[3] Its bioactive compounds are adrenergic alkaloids, such as (−)-ephedrine, (+)-pseudoephedrine, (−)-norephedrine and (+)-norpseudoephedrine, which are pharmacologically sympathomimetic agonists acting on both α- and β-adrenergic receptors.[4] Recently, *E. intermedia* herb has received considerable attention as the therapeutic agents for the treatment of obesity due to its CNS stimulatory action.[5-6] However the overuse of *E. intermedia* preparations may give rise to severe adverse effects, such as cardiovascular symptoms, glaucoma and hyperthyroidism.[7-8] Thus, products containing of *E. intermedia* were rigorously regulated according to the amounts of ephedra alkaloids. *R. palmatum* (Da Huang) is one of Rheum species and is widely used for the treatment of constipation for a long time in Asian countries. Its cathartic effect is caused by several anthraquinones, such as emodin, chrysophanol, rhein, aloe-emodin, and physcion.[9] Also those compounds have been reported to exhibit a wide range of pharmacological effects including of anti-inflammatory, anticancer and antimicrobial activities. Besides, the improper use of *R. palmatum* may cause

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some adverse impacts on liver and kidney disorders.[10] *L. erythrorhizon* is an herb native to East Asia and has been used as a natural mordant for fabric and food dyeing. It has traditionally been used as the therapeutic agent on inflammation, burns and wounds. Shikonin is known as one of bioactive naphthoquinones exhibiting the pharmacological activities of *L. erythrorhizon*.[11] Other naphthoquinone-derivative compounds, acetylshikonin and isobutylshikonin, were changed into shikonin by the biodegradation of the hepatic enzyme.[12]

Response surface analysis (RSA) is a useful method for optimizing the experimental conditions in the laboratory or the manufacturing process for products in the industrial fields.[2,13] The polynomial model for the desired experimental conditions was fitted by the least squares method obtained through performing of a small number of well-designed experiments. It is an effective statistical method for determining the effects of experimental parameters for the chemical markers or the bioactive components in the manufacturing process for the natural products. In the present study, Box-Behnken design which is three-level incomplete factorial design for RSA was performed for the optimization of extraction parameters including EtOH concentrations, extraction hours and the solvent to material ratio for two active components, (−)-ephedrine and (+)-pseudoephedrine, in DF formula.

Experimental

Chemicals – (−)-ephedrine and (+)-pseudoephedrine were gifted from Dr. Sang Hyun Sung, a professor of College of Pharmacy, Seoul National University. Sodium dodecyl sulfate (SDS) was purchased at Sigma-Aldrich (St. Louis, MO, USA) and HPLC-grade acetonitrile at TEDIA (Fairfield, OH, USA). Water was purified with a Milli-Q system (Nihon Millipore, Tokyo, Japan).

Plant materials – The air-dried plant materials, *E. intermedia* (EI, 1 kg), *R. palmatum* (RP, 1 kg) and *L. erythrorhizon* (LE, 1 kg) and DF formula were provided from Dr. Soon Shik Shin, a professor of the Department of Korean medicine, Dong-eui University, identified by Dr. Yong Soo Kwon, a professor of College of Pharmacy, Kangwon National University. Three herbs were deposited in the Herbarium of College of Pharmacy, Kangwon National University (KNUPH-EI-1, KNUPH-RP-1 and KNUPH-LE-1).

Extraction of DF formula – DF formula was prepared with the patented technologies. Briefly, DF formula is a decoction consisting of three traditional medicinal herbs including of *E. intermedia* (EI), *R. palmatum* (RP) and *L. erythrorhizon* (LE) as described in the patent documents. Three herbs were chopped into 2 - 3 cm and proportionally combined and mixed in the different proportion. Then, the mixture was extracted using Soxhlet technique at 85 °C according to the different designed experimental conditions. The Box-Beknken design (BBD), with three parameters, the EtOH concentrations (X_1, %, v/v EtOH/water), the extraction hours (X_2, hour) and the solvent to material ratio (X_3, ml/g), at the different conditions (Table 1), was used for the optimization of the extraction condition, while the response variables were the amounts of EP and PSEP in DF decoction.

Chromatographic conditions – High performance liquid chromatography (HPLC) was carried out on Agilent 1260 Infinity system consisting of 1260 quaternary pump, autosampler and multiple wavelength detector (Agilent Technologies Mfg GmbH&Co.KG, Waldbronn, Germany) and a Hector-M C18 column (250 mm × 4.6 mm i.d.; 5 μm, RStech, Daejeon, Korea), and all chromatograms were measured at 215 nm with the mixtures of HPLC-grade H2O buffered with 25mM sodium dodecyl sulfate (solvent A) and acetonitrile (AcCN solvent B). The gradient elution condition was 60% solvent A (0 - 25 min) and 60 - 40% solvent A (25 - 35 min) and 40% solvent A (35 - 40 min) and 40 - 20% solvent A (40 - 50 min) and 20% solvent A (50 - 60 min) and 20 - 60% solvent A (60 - 60.1 min) and 60% solvent A (60 - 70 min) with the flow rate of 1.0 ml/min. Thus, the calibration curves were followed as $y = 11697.7819x + 54.081$ (linearity $R^2 = 1$) for EP and $y = 18703.4036x + 40.7734$ ($R^2 = 0.9999$) for PSEP.

Software – The response surface methodology was calculated using the Design Expert (Version 7.0.0, Stat-Ease Inc., Minneapolis) statistical software. A second-order polynomial model was used to analyze experimental data and regression coefficients as a function of the independent variables. It was fitted in the response surface analysis as following equation (1):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=1}^{3} \beta_{ij} X_i X_j \quad (1)$$

where β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i, and X_j are the independent variables. The software generated the response surfaces and contour plots while holding a variable constant in the second-order polynomial model.

Result and Discussion

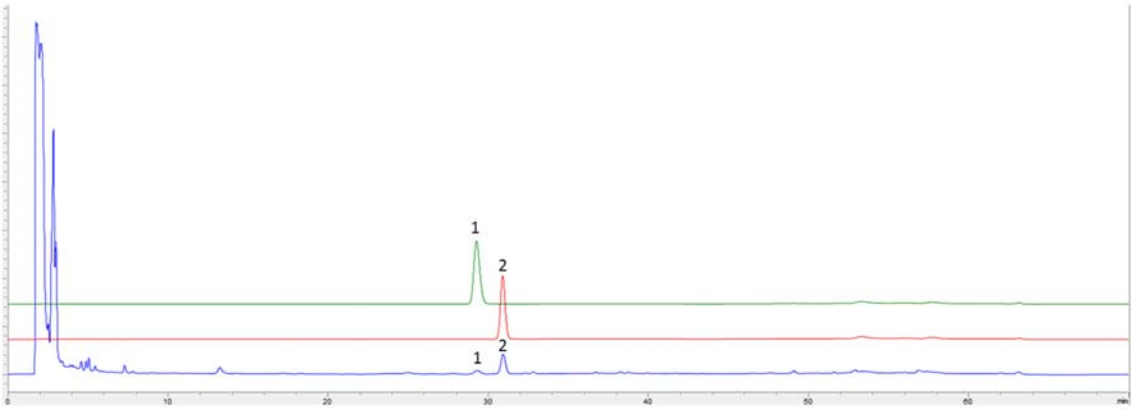

Fig. 1. HPLC chromatograms of (+)-pseudoephedrine (1), (−)-ephedrine (2) and DF formula. The detailed HPLC conditions described in Experimental section.

Table 1. Experimental design and responses of the dependent variables to extraction conditions

Std order[a]	Run order[b]	Coded variables			Independent variables			Dependent variables (responses)	
		X_1	X_2	X_3	EtOH concentration (%)	Extraction time (hour)	Solvent to material ratio (ml/g)	EP (mg/g extract)	PSEP (mg/g extract)
1	4	1	1	0	70	6.0	10	37.65	6.55
2	1	-1	-1	0	0	1.0	10	37.00	6.10
3	6	1	0	-1	70	3.5	5	37.05	6.45
4	7	-1	0	1	0	3.5	15	36.45	6.20
5	5	-1	0	-1	0	3.5	5	32.75	5.25
6	10	0	1	-1	35	6.0	5	30.80	5.15
7	16	0	0	0	35	3.5	10	35.15	5.95
8	14	0	0	0	35	3.5	10	35.40	6.05
9	13	0	0	0	35	3.5	10	35.65	6.05
10	9	0	-1	-1	35	1.0	5	32.55	5.40
11	17	0	0	0	35	3.5	10	35.05	5.95
12	3	-1	1	0	0	6.0	10	34.25	5.65
13	8	1	0	1	70	3.5	15	35.90	6.65
14	11	0	-1	1	35	1.0	15	34.10	5.85
15	15	0	0	0	35	3.5	10	35.05	5.90
16	12	0	1	1	35	6.0	15	33.60	5.70
17	2	1	-1	0	70	1.0	10	34.75	6.55

[a)]Randomized, [b)]No randomized.

To obtain the optimized extraction condition for DF formula, we firstly tried to determine the HPLC analytical condition for the chemical markers of each herb in DF formula. Because *E. intermedia* comprised the largest portion from DF formula, (−)-ephedrine (EP) and (+)-pseudoephedrine (PSEP) which are the main bioactive components in *E. intermedia* were apparently detected, but any components from *R. palmatum* and *L. erythrorhizon* were not (Fig. 1). Thus, we choose EP and PSEP as the chemical markers, which were the main biological components for DF formula due to their thermogenic properties causing the weight loss.[14] The HPLC conditions were optimized to obtain higher resolution of two components in DF formula. They are well separated from other components under the gradient solvent condition buffered with 25 mM SDS.[15]

In the preliminary study, we found that three variables,

Table 2. ANOVA for the response surface quadratic model of EP[a]

	Sum of Squares	Mean Square	F value	*p*-value
Model	48.9499	5.4389	18.3083	0.0005
X_1	3.0013	3.0013	10.1028	0.0155
X_2	0.5512	0.5512	1.8556	0.2153
X_3	6.1250	6.1250	20.6179	0.0027
$X_1 X_2$	8.1225	8.1225	27.3419	0.0012
$X_1 X_3$	5.7600	5.7600	19.3893	0.0031
$X_2 X_3$	0.3600	0.3600	1.2118	0.3074
X_1^2	12.6381	12.6381	42.5424	0.0003
X_2^2	4.7981	4.7981	16.1514	0.0051
X_3^2	8.7613	8.7613	29.4922	0.0010
Residual	2.0795	0.2971		
Lack of Fit	1.7275	0.5758	6.5436	0.0506
Pure Error	0.3520	0.0880		
R^2	0.9592			
Adj. R^2	0.9069			

[a](−)-ephedrine

Table 3. ANOVA for the response surface quadratic model of PSEP[b]

	Sum of Squares	Mean Square	F value	*p*-value
Model	3.1033	0.3448	79.7255	< 0.0001
X_1	1.0952	1.0952	253.2254	< 0.0001
X_2	0.0903	0.0903	20.8815	0.0026
X_3	0.5886	0.5886	136.0954	< 0.0001
$X_1 X_2$	0.0552	0.0552	12.7688	0.0091
$X_1 X_3$	0.1406	0.1406	32.5145	0.0007
$X_2 X_3$	0.0064	0.0064	1.4798	0.2632
X_1^2	0.7252	0.7252	167.6666	< 0.0001
X_2^2	0.1813	0.1813	41.9166	0.0003
X_3^2	0.2792	0.2792	64.5513	< 0.0001
Residual	0.0303	0.0043		
Lack of Fit	0.0159	0.0053	1.4699	0.3494
Pure Error	0.0144	0.0036		
R^2	0.9903			
Adj. R^2	0.9779			

[b](+)-pseudoephedrine

ethanol concentration (X_1, %), extraction time (X_2, hour) and solvent to material ratio (X_3, ml/g), had significantly influenced on the extraction yields of two components. They were used as the experimental factors for response surface analysis (RSA). Three process variables were applied to the Box-Bekhen design (Table 1). The response variables were the amounts of EP and PSEP.

First, the regression equation of EP in coded units was given in the following equation (2).

$$\text{EP contents} = 35.26 + 0.61X_1 - 0.26X_2 + 0.88X_3 + 1.42X_1X_2 - 1.20X_1X_3 + 0.30X_2X_3 + 1.73X_1^2 - 1.07X_2^2 - 1.44X_3^2 \quad (2)$$

As shown in Table 2 and Fig. 2., the quadratic of X_1 was the largest effect on the amount of EP during the process of the extraction, followed by the quadratic of X_3 and the interaction effect of X_1 and X_2. The total determination coefficient, $R^2 = 0.9592$, implied that the amount of EP was significantly attributable to three factors (Table 3). Also, the value of "Prob > F" was less

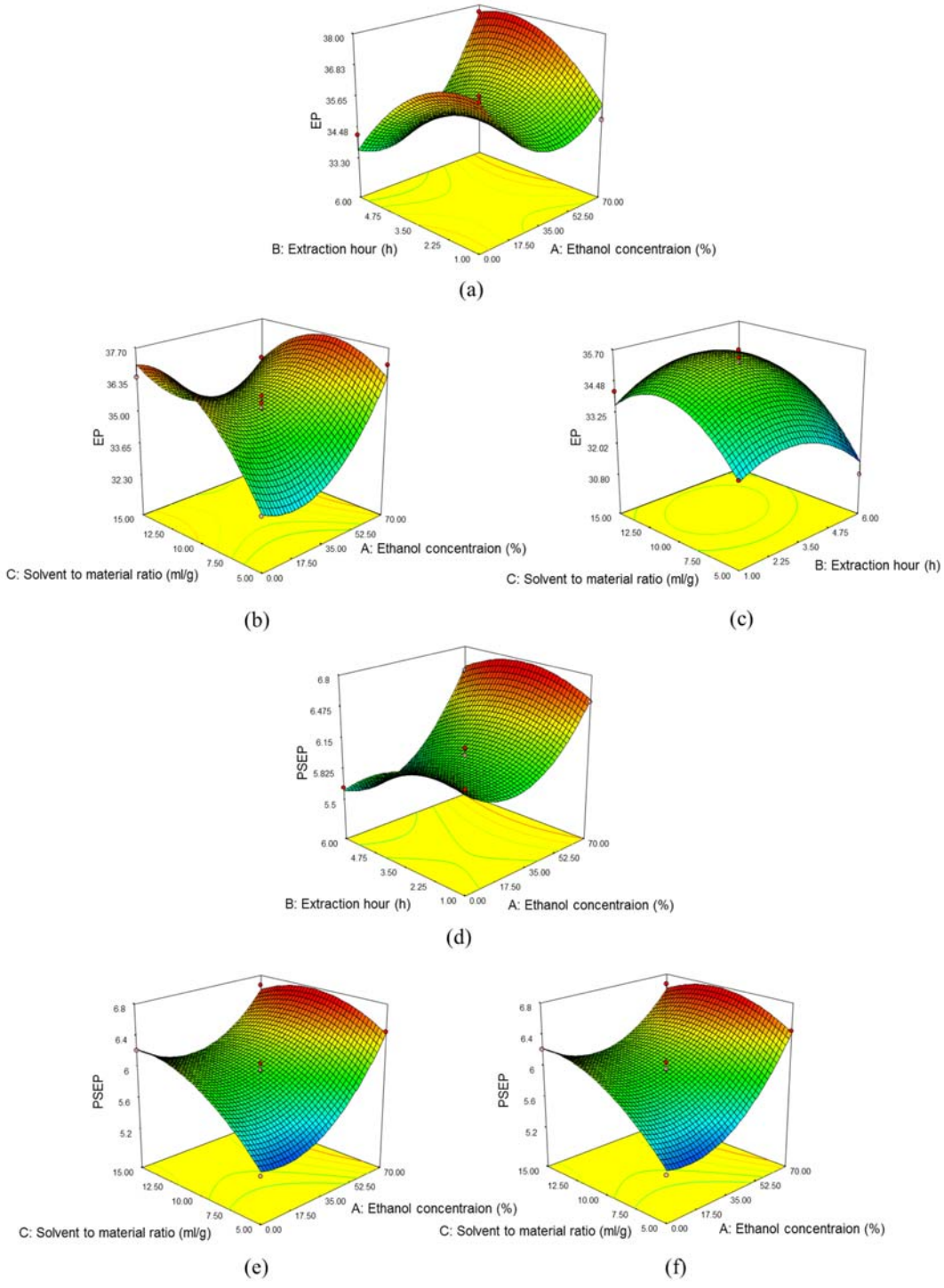

Fig. 2. 3D surface plots of the EtOH concentrations versus the extraction hours (a), the EtOH concentrations versus solvent to material ratio (b) and the extraction hours versus solvent to material ratio (c) for the yield of (−)-ephedrine (e), and the EtOH concentrations versus the extraction hours (d), the EtOH concentrations versus solvent to material ratio (e) and the extraction hours versus solvent to material ratio (f) for the yields of (+)-pseudoephedrine.

than 0.001 indicating the significance of the model. The 3D surface plots easily showed the main and interactive effects of the independent variables on the extraction of EP. Especially, the ethanol concentration significantly exerted a positive quadric effect on the amount of EP, which were least extracted in about 35% EtOH and

Table 4. The comparison between predicted and experimental values.

Parameters	Optimum values	
	Predicted values[a]	Experimental values[b]
EtOH concentration (%)	69.73	70.0
Extraction time (hour)	4.83	4.8
Solvent to material ratio (ml/g)	8.73	8.7
Ephedrine (mg/g extract)	37.7	38.4
Pseudoephedrine (mg/g extract)	6.65	7.80

highest in about 70% (Fig. 2a and b). Although the extraction time was not a significant factor on the yield of EP by p value of 0.2153, its yield was changed depending on the extraction time when the ethanol concentration factor was kept at its zero level. The conditions of extraction times and solvent to material ratio generated the contour plot in the designed range. (Fig. 2c) The higher yield of EP was extracted in the medium conditions of the extraction time of about 3.5 hour and a solvent to material ratio of 10 time.

The relationship between the extraction variables and the amount of PSEP was expressed as follows equation (3).

$$\text{PSEP contents} = 5.99 + 0.37X_1 - 0.11X_2 + 0.27X_3 + 0.12X_1X_2 - 0.19X_1X_3 + 0.04X_2X_3 + 0.41X_1^2 - 0.21X_2^2 - 0.26X_2^3 \quad (3)$$

The three most significant variables influencing on the amount of PSEP were the linear terms of X_1 and X_3, and the quadratic term of X_1 with the higher F values of 253.23, 136.10 and 167.67, respectively (Table 2). Also all their corresponding p values were significant at less than 0.0001. The coefficient of determination (R^2) was calculated to be 0.9903 for the amount of PSEP, which meant the model can well predict the amount of PSEP (Table 3). In a 3D surface plot, an EtOH concentration factor showed a linear effect on PSEP. The yield of PSEP was increased up to 70% EtOH which is the highest ratio of ethanol regardless of the extraction time and solvent to material ratio (Fig. 2d and e). The contour plot by the extraction time and solvent to material ratio demonstrated maximum at about 3 hour and 12.5 times, respectively (Fig. 2f).

The optimized extraction conditions to yield the maximum amounts of EP and PSEP were suggested based on the experimental results. The predictable variables with the highest desirability were engaged to verify the predictive capacity of the model (Table 4). With the slight modified extraction conditions (ethanol concentration of 70%, extraction time 4.8h, and solvent to material ratio 8.7:1). The extraction yields of EP and PSEP were 37.7 and 6.65 mg/g extract in the predicted values, and 38.4 and 7.80 mg/g extract in the experimental values, respectively, which indicated the modified conditions were adequate for extraction.

The Box-Behnken design was successfully employed to determine the optimum extraction conditions for yielding the maximum contents of EP and PSEP which the major components of DF formula. ANOVA analyses revealed that the EtOH concentrations, followed by the solvent to material ratio (X_3, ml/g) and the extraction hours (X_2, hour), showed the most significant factor on the yields of EP and PSEP. The experimental values generated by the optimized extraction parameters were well consistent with the predicted values. These results showed the extraction conditions optimized in the study can be useful to enhance the efficacy of a large-scale extraction system for the manufacturing of DF formula.

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