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### Optimization for Decocting Later of Menthae Herba in Eungyo-San, a Herbal Formula, Using Response Surface Methodology with Gas Chromatography/Mass Spectrometry

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#### ABSTRACT

Background: "Decocting later" is important procedure for the extraction of herbal medicines containing volatile compounds. Objective: This study was performed to investigate optimal conditions for "Decocting later" of Menthae herba in Eungyo-san (EGS) and correlation between extraction variables and the yields of d/l-menthol, a marker compound of Menthae herba. Materials and Methods: The decocting temperature, total decocting time, and decocting later time were chosen as individual variables, and the yield of d/l-menthol was set as the response value which were calculated by using a Box-Behnken design (BBD). The amount of d/l-menthol was quantified using gas chromatography/mass spectrometry. Results: Response surface methodology (RSM) was used to predict optimal conditions for decocting later of Menthae herba into the formula. Optimal conditions for "Decocting later" from RSM were as follows: 100.63°C of decocting temperature; 82.95 min of total decocting time; 19.11 min of decocting later time. Both decocting temperature and total decocting time showed significant correlation with the yield of d/l-menthol. Conclusions: These results suggest that the decocting temperature and total decocting time were influential factors, and RSM can be applied for optimizing the conditions of "Decocting later" of Menthae herba in EGS.

**Key words:** Decocting later, Eungyo-san, gas chromatography/mass spectrometry, menthae herba, response surface methodology

#### **SUMMARY**

- Gas chromatography/mass spectrometry method developed was applied to quantify the d/l-menthol, a volatile compound in Menthae Herba, in Eungyosan decoction (EGS)
- d/l-Menthol was extracted in the chloroform layer of the partition between EGS decoction and chloroform
- A Box-Behnken design produced the predicted response values (yield of d/lmenthol in EGS) from the actual response values with individual variables including decocting temperature, total decocting time, and decocting later time
- Optimal conditions for "Decocting later" of Menthae Herba in EGS obtained from the response surface methodology were 100.63°C of decocting

temperature, 82.95 min of total decocting time, and 19.11 min of decocting later time.



Abbreviations used: KM: Korean medicine; EGS: Eungyo-san; GC/MS: Gas chromatography/mass spectrometry; RSM: Response surface methodology; SIM: Selected ion monitoring; LOD: Limits of determination; LOQ: Limits of quantification; RSD: Relative standard deviation; ANOVA: Analysis of variance; BBD: Box-Behnken design.

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#### **INTRODUCTION**

Decoction, the extraction method that has been widely used for oral administration, is prepared by boiling plant materials using water. In traditional, Korean Medicine (KM), some herbal medicines containing the compounds which are easily evaporated at higher temperature are added generally 5–10 min before the end of decoction, so called as "Decocting later," to prevent losing much of volatile characteristics.<sup>[1-5]</sup> The extraction efficiency of bioactive compounds from herbal medicines is usually influenced by the extraction factors, such as extraction time, temperature, and material to solvent ratio. In addition, the extraction efficiency of volatile compounds in decoction is affected by decocting later time.<sup>[6,7]</sup> Chemical interactions between the components in KM decoction varied the extraction efficiency of herbal medicines, which consequently changed the extraction yields of volatile compounds in

KM decoction.<sup>[8]</sup> Hence, it is necessary to optimize the conditions of decocting later, such as decocting temperature, decocting later time, and total decocting time, to guarantee high efficiency of volatile compounds in herbal medicines.

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Eungyo-san (EGS) is a KM formula composed of nine herbal medicines: Forsythiae Fructus, Lonicerae Flos, Platycodonis Radix, Menthae Herba, Phyllostachys Folium, Glycyrrhizae Radix et Rhizome, Schizonepetae Spica, Glycine Semen Preparatum, and Arctii Fructus. EGS has been used to treat the symptoms caused by common cold, including headache, fever, sore throat, chills, and dipsia; and those caused by influenza infection or other viral infections, including hand-foot-mouth disease, esophagitis, pneumonia, acute tonsillitis, and mumps.<sup>[9-12]</sup> Menthae herba, one of the herbal medicines consisting EGS, contains characteristic volatile compound, d/l-menthol, which can be easily vaporized when decocted together with other herbal medicines at initial decocting time.<sup>[13]</sup> Therefore, Menthae herba is recommended to be added separately at nearly end of decoction, which can assure high quality of decoction by preventing it from losing bioactive volatile compounds.

In the present study, various types of EGS decoctions were prepared by differing the extraction variables such as decocting temperature (80, 90, and 100°C), total decocting time (90, 120, and 150 min), and decocting later time (5, 10, and 15 min). d/l-Menthol, the volatile compound in EGS decoction, was quantified by gas chromatography-mass spectrometry (GC/MS), a widely used analytical tool for volatile compounds.<sup>[14,15]</sup> Optimum extraction conditions for d/l-menthol in EGS decoction and the correlations between extraction yield of d/l-menthol and extraction variables were investigated using response surface methodology (RSM), which enables researcher to find optimal conditions for the extraction and evaluate the interaction of individual extraction variables.<sup>[16,17]</sup>

#### **MATERIALS AND METHODS**

#### Reagents and herbal materials

HPLC-grade methanol and chloroform were purchased from J. T. Baker Inc. (Center Valley, PA, USA). D/l-menthol ( $\geq$ 95%) was purchased from ChemFace (Wuhan, Hubei, China). Nine herbal medicines of EGS, Forsythiae Fructus (3.0 g), Lonicerae Flos (3.0 g), Menthae Herba (1.8 g), Arctii Fructus (1.8 g), Platycodonis Radix (1.8 g), Glycine Semen Preparatum (1.5 g), Glycyrrhizae Radix et Rhizome (1.5 g), Phyllostachys Folium (1.2 g), and Schizonepetae Spica (1.2 g), were purchased from a herbal company (Kwangmyungdang Medicinal Herbs; Ulsan, Korea).

#### Preparation of standard solution

The standard compound was accurately weighed and dissolved in methanol to make stock solution at concentration of 1,000  $\mu$ g/mL. The stock solution was diluted to produce the working solution for constructing the calibration curve.

#### Preparation of Eungyo-san decoction and samples

Nine herbal medicines of EGS were grinded up and accurately weighed. The mixture of herbal medicines, except for Menthae Herba which was prepared to be added before the end of decoction, were decocted with 300 mL of distilled water and they were boiled using reflux extracting system with temperature-controllable heating mantle (MS-DM, MTops, Korea). EGS decoction was filtered through a testing sieve (500  $\mu$ m, Chunggye, Gyeonggi-do, Korea). The filtered decoction was made up to 250 mL and centrifuged at 3500 rpm in 5 min to separate pellet from the EGS decoction. Supernatant was partitioned with chloroform at 1:1 ratio. The chloroform layer was filtered through 0.2  $\mu$ m syringe filter (BioFact, Daejeon, Korea) and analyzed by GC/MS to quantify d/l-menthol.

## Gas chromatography-mass spectrometry conditions

The GC/MS system consisted of Agilent 7890 GC (Agilent Technologies, CA, USA) equipped with 5975C inert MSD triple-axis

detector (Agilent Technologies, CA, USA), autosampler. DB-1 capillary column (50 m × 250  $\mu$ m, 0.25  $\mu$ m, Agilent Technologies, CA, USA) was used to separate the compounds. Separation was achieved with the temperature program of Table 1. N<sub>2</sub> was used as the carrier gas at a constant flow rate of 0.3 mL/min. Samples were injected at a split ratio of 1:20, and the injection temperature was held at 250°C. The mass spectrometer was operated in scan mode (50–170 m/z) and SIM mode.

#### Method validation

The stock solution was diluted at six levels to construct calibration curves in which the x-axis was the concentration of marker compound, and the y-axis was the area of the marker compound. The linearity was determined by coefficient of determination  $(r^2)$ . The limits of determination (LOD) and limits of quantification (LOQ) were evaluated as follows: LOD =  $3.3 \times$  standard deviation (SD/)S and LOQ =  $10 \times$  SD/S, where SD is the SD of the response and S is the slope of the calibration curve. The precisions were measured at three concentrations of the standard compounds (low, medium, and high levels) during a day (intra-day) and three successive days (interday), which was represented by the value of the relative SD (RSD). The recovery test was used to evaluate the accuracy of the method. Two known amounts of marker compound (low and high) were added to the samples, and the recovery was evaluated as follows: Recovery (%) = ([detected concentration – initial concentration/spiked concentration] ×100). The repeatability was determined by RSD value of the retention times and the absolute areas of marker compound (n = 6).

#### Experimental design and statistical analysis

To determine the optimum conditions for extraction of d/l-menthol in ESG, preliminary ranges of the extraction variables, decocting temperature, total decocting time, and decocting later time, were investigated using a single-factor test. A three-level-three-factor BBD was employed to determine the optimal conditions for the extraction. The experimental data obtained from the BBD were fitted to a secondorder polynomial model, and the regression coefficients were obtained. The equation is as follows:

$$Y = \beta o + \sum_{j=1}^{k} \beta_{j} X_{j} + \sum_{j=1}^{k} \beta_{jj} X_{j}^{2} + \sum_{i < j} \beta_{ij} X_{i} Y_{j}$$

Where Y is the estimated response,  $\beta_0$ ,  $\beta_j$ ,  $\beta_{jj}$ , and  $\beta_{ij}$  are the regression coefficients for intercept, linearity, square, and interaction terms, respectively.  $X_i$  and  $X_j$  are the independent variables, which were coded.

The fitness of the second-order polynomial model was expressed by the lack of fit and coefficient of determination ( $r^2$ ). The value of *t*-test and *P* value resulting from the analysis of variance (ANOVA) were calculated to confirm the significance of the regression coefficients, which was determined at *P* < 0.05, 0.01, or 0.001. The interaction and influence of the three variables on the yield of d/l-menthol were represented as three-dimensional (3D) response surface plots and 2D contour plots, on which the optimum extraction condition was obtained. The open-source software R (version 3.1.1; The R Foundation for Statistical Computing) was used to generate the experimental design, statistical analysis, and regression model.

Table 1: Temperature program of gas chromatography analysis

Rate (°C/min)	Value (°C)	Hold time (min)	Runtime (min)
0	50	1	1
15	200	1	12
10	250	1	18

#### **RESULTS AND DISCUSSION**

#### Method validation

d/l-Menthol in chloroform fraction partitioned from EGS decoction was reasonably separated and detected on total ion chromatogram [Figure 1]. Regression equation of calibration curve was y = 117,476.973x-105,112.000 with concentration range from 3.16 µg/mL to 50.00 µg/mL. The linearity d/l-menthol represented as the correlation coefficient ( $r^2$ ) was 0.9985, and LOD and LOQ were 1.08 and 3.27 µg/mL, respectively. The intra- and inter-day precisions of d/l-menthol which were represented as RSD value were 0.55%–4.37% for intra-day precision and 0.86%–6.11% for inter-day precision [Table 2]. The recoveries of d/l-menthol at low and high concentrations were 84.06% and 80.51% with RSD values 15.04% and 1.81%, respectively [Table 3]. The repeatability was 0.01% for retention time and 4.61% for absolute peak area, respectively.

#### Model fitting

The range of decocting temperature ( $X_1$ , 80°C-100°C), total decocting time ( $X_2$ , 90–150 min), and decocting later time ( $X_3$ , 5–15 min) were determined by preliminary experiments using single-factor tests (Data not shown). In this study, a three-level-three-factor BBD composed of 15 experiments was employed, and three replicates

Table 2: Intra-day and inter-day precisions of d/l-menthol

(run order 2, 5, and 8) were measured for calculating the pure error sum of squares [Table 4]. The yield of d/l-menthol was set as the response. Three levels of each variable were coded as -1, 0, and 1.

Multiple regression analysis on the experimental data was expressed by second-order polynomial equation using coded variables as follows:

 $Y = 12.987696 + 1.28109X_1 + 0.69903X_2 - 0.050757X_3 - 0.23248X_1X_2$ 

 $-0.5333488X_1X_2 + 0.449382X_1X_2 - 0.17086X_1X_1 +$ 

 $2.704081X_2X_2 + 0.198502X_3X_3$ 

Where Y is the yield of d/l-menthol ( $\mu$ g/mL), and the coded variables X<sub>1</sub>, X<sub>2</sub>, and X<sub>3</sub> represent extraction variables, including decocting temperatures, total decocting time, and decocting later time.

Table 5 shows the regression coefficients of the predicted quadratic polynomial model of d/l-menthol in EGS decoction. The significance of each coefficient was estimated using Student'S *t*-test and the *P* value, which larger *t*-value and smaller *P* value represent significant corresponding variables.<sup>[18]</sup> It was observed that decocting temperature (X<sub>1</sub>) was significant in linear term (*P* < 0.05) and total decocting time (X<sub>2</sub>) was significant in quadratic term (*P* < 0.01). Coefficients of the other terms (X<sub>2</sub>, X<sub>3</sub>, X<sub>1</sub>:X<sub>2</sub>, X<sub>1</sub>:X<sub>3</sub>, X<sub>2</sub>:X<sub>3</sub>, X<sub>1</sub>:X<sub>1</sub>, and X<sub>3</sub>:X<sub>3</sub>) were not significantly influential on the model (*P* > 0.05). It was observed that decocting temperature (X<sub>1</sub>) showed significant influence to the extraction yield of d/l-menthol in linear term (*P* < 0.05) while total decocting time

Test concentration (µg/mL)	Intra-day ( <i>n</i> =3)			Inter-day ( <i>n</i> =3)		
	Observed concentration (µg/mL)	Precision (%)	Accuracy (%)	Observed concentration (µg/mL)	Precision (%)	Accuracy (%)
50	48.18	4.37	96.35	47.09	6.11	94.19
100	96.66	2.28	96.66	94.49	4.37	94.49
200	201.69	0.55	100.85	202.78	0.86	101.39



Figure 1: Total ion chromatograms of d/l-menthol standard solution (a), the chloroform fraction of Eungyo-san decoction (b), and mass spectrum of d/l-menthol (c)

 $(X_2)$  showed significant influence in quadratic term (P < 0.01) from the regression coefficients.

The ANOVA was performed to evaluate the fitness of quadratic polynomial model for the extraction of d/l-menthol [Table 6]. Optimization of the

Table 3: Recovery of d/l-menthol (n=3)

Original amount (μg/mL)	Spiked amount (µg/mL)	Detected amount (µg/mL)	Recovery (%)	RSD (%)
9.27	2.00	1.68	84.06	15.04
	4.00	3.22	80.51	1.81

RSD (%) = (SD/mean) × 100. RSD: Relative standard deviation; SD: Standard deviation

 Table 4: Box-Behnken design and the response values for yields of d/l-menthol in Eungyo-san

Run	Coded variables levels			Yield (µg/mL)		
order	<b>Х</b> <sub>1</sub> (°С)	X <sub>2</sub> (min)	X₃ (min)	Actual value	Predicted value	
1	-1(80)	-1(90)	0 (10)	13.98	13.31	
2	0 (90)	0 (120)	0 (10)	11.84	12.99	
3	0 (90)	1 (150)	-1(5)	17.17	16.19	
4	0 (90)	-1(90)	1 (5)	13.71	14.69	
5	0 (90)	0 (120)	0 (10)	13.93	12.99	
6	-1(80)	1 (150)	0 (10)	14.98	15.17	
7	1 (100)	-1(90)	0 (10)	16.53	16.34	
8	0 (90)	0 (120)	0 (10)	13.20	12.99	
9	0 (90)	1 (150)	1 (15)	16.87	16.99	
10	0 (90)	-1(90)	-1(5)	15.81	15.69	
11	-1(80)	0 (120)	1 (15)	12.53	12.22	
12	1 (100)	1 (150)	0 (10)	16.60	17.27	
13	-1(80)	0 (120)	-1(5)	10.46	11.25	
14	1 (100)	0 (120)	-1(5)	14.57	14.88	
15	1 (100)	0 (150)	1 (15)	14.50	13.71	

X<sub>1</sub>: Decocting temperature (°C); X<sub>2</sub>: Total decocting time (min); X<sub>3</sub>: Decocting later time (min)

 
 Table 5: Regression coefficients of the predicted quadratic polynomial model of d/l-menthol in Eungyo-san

Variable	Estimate	SE	t	Р
Intercept	12.987696	0.662919	19.5917	0.000006***
X,	1.281090	0.405953	3.1558	0.025215*
X <sub>2</sub>	0.699030	0.405953	1.7219	0.145696
X <sub>3</sub>	-0.050757	0.405953	-0.1250	0.905370
X1::X2	-0.232480	0.574105	-0.4049	0.702263
X1:X3	-0.533488	0.574105	-0.9293	0.395409
X,:X,	0.449382	0.574105	0.7828	0.469205
X,:X	-0.170860	0.597547	-0.2859	0.786395
X,:X,	2.704081	0.597547	4.5253	0.006252**
X_3:X_3	0.198502	0.597547	0.3322	0.753216

Significant codes: \*\*\*P<0.001; \*\*P<0.01; \*P<0.05. X<sub>1</sub>: Decocting temperature (°C); X<sub>2</sub>: Total decocting time (min); X<sub>3</sub>: Decocting later time (min); SE: Standard error

 Table 6: Analysis of variance for the fitted quadratic polynomial model for the extraction of d/l-menthol in Eungyo-san

	df	SS	MS	F	<i>P</i> <b>r</b> > <b>F</b>
FO $(X_1, X_2, X_3)$	3	17.0593	5.6864	4.3132	0.07472
$TWI(X_1, X_2, X_3)$	3	2.1624	0.7208	0.5467	0.67161
$PQ(X_1, X_2, X_3)$	3	27.5326	9.1775	6.9612	0.03101
Residual	5	6.5919	1.3184	-	-
Lack of fit	3	4.3402	1.4467	1.2850	0.46575
Pure error	2	2.2517	1.1259	-	-

df: Degree of freedom; SS: Sum of square; MS: Mean square; X<sub>1</sub>: Decocting temperature (°C); X<sub>2</sub>: Total decocting time (min); X<sub>2</sub>: Decocting later time (min)

fitted response surface can be obtained from an adequately fitted model which approach to the true system.<sup>[18]</sup> The coefficient of determination ( $r^2$ ) was 0.8764 with no significant lack of fit at P > 0.05, which indicates that the predicted model could explain 87.64% of the results.

#### Analysis of response surface

The predicted yield of d/l-menthol calculated from second-order polynomial equation was visualized through 3-D response plots and 2-D contour plots by the levels of two independent variables with other variable set at the zero level.

As shown in Figure 2, the yield of d/l-menthol increased with increasing decocting temperature  $(X_1)$ . However, a significant correlation between decocting temperature  $(X_1)$  and total decocting time  $(X_2)$  was not observed. Increase of decocting temperature  $(X_1)$  also increased the yield of d/l-menthol, however, the interaction between decocting temperature  $(X_1)$  and decocting later time  $(X_3)$  was not significant [Figure 3]. The yield of d/l-menthol during total decoction time  $(X_2)$  decreased from 90 min to about 120 min while increased from 120 min to 150 min. However, it was not observed that significant correlation between total decocting time  $(X_2)$  and decocting later time  $(X_3)$  [Figure 4].

These results demonstrate that the extraction of d/l-menthol from EGS decoction was influenced by extraction conditions of decocting later significantly or nonsignificantly as complementary and neutralizing action among the compounds in herbal formula commonly exists during decoction.<sup>[8]</sup> Furthermore, the correlation between the extraction efficiency of volatile compounds and decocting later time indicates that herbal medicines containing volatile compounds are required to be decocted later.<sup>[6,7]</sup>

# Optimization and verification of extraction by response surface methodology

Optimized conditions for decocting later of Menthae Herba in EGS decoction were obtained through RSM: 100.63°C of decocting temperature; 82.95 min of total decocting time; and 19.11 min of decocting later time. The optimized extraction yield of d/l-menthol was predicted to be 13.54  $\mu$ g/mL. The modified conditions were 100°C of decocting temperature, 80 min of total decocting time, and 20 min of decocting later time and were applied. The extraction yield of d/l-menthol from modified conditions was 12.23 ± 0.16  $\mu$ g/mL, which is slightly different from those calculated from optimized extraction conditions [Table 7].

#### **CONCLUSIONS**

In the present study, the optimal conditions of decocting later were investigated: 100.63°C of decocting temperature; 82.95 min of total decocting time; 19.11 min of decocting later time. In addition, the interactions between the extraction variables, such as decocting



**Figure 2:** Response surface plot and contour plot of decocting temperature ( $^{\circ}C$ , X<sub>1</sub>) and total decocting time (min, X<sub>2</sub>)

Table 7: The predicted value of responses at the optimum conditions and the experimental value of responses at the modified conditions

	Decocting temperature (°C)	Total decocting time (min)	Decocting later time (min)	Yield of d/l-menthol (µg/mL)
Optimum conditions	100.63	82.95	19.11	13.54 (predicted)
Modified conditions	100	80	20	12.23±0.16 (actual)



**Figure 3:** Response surface plot and contour plot of decocting temperature ( $^{\circ}C$ , X<sub>1</sub>) and decocting later time (min, X<sub>2</sub>)



**Figure 4:** Response surface plot and contour plot of total decocting time (min,  $X_3$ ) and decocting later time (min,  $X_3$ )

temperature, total decoction time, and decocting later time, on the yield of menthol in EGS was also observed.

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#### Conflicts of interest

There are no conflicts of interest.

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