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# Optimization of Extraction Condition of Hesperidin in *Citrus unshiu* Peels using Response Surface Methodology

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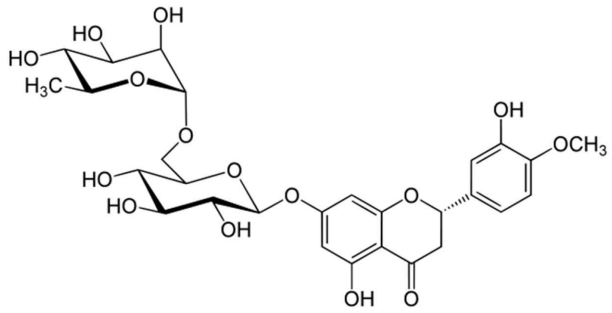
**Abstract** – Hesperidin, which is the most abundant flavonoid of *Citrus unshiu* (Rutaceae), has been reported to possess diverse activities and widely used as functional foods and cosmetics. For the development of functional products, extraction procedure is indispensable. Extraction conditions affect the composition of extract as well as its biological activity. Therefore, we tried to optimize extraction conditions such as extraction solvent, extraction time and extraction temperature for maximum yield of hesperidin using response surface methodology with three-level-three-factor Box-Behnken design (BBD). Regression analysis showed a good fit of the experimental data and the optimal condition was obtained as ethanol concentration, 59.0%; temperature 71.5 ºC and extraction time, 12.4 h. The hesperidin yield under the optimal condition was found to be 287.8 μg per 5 mg extract, which was well matched with the predicted value of 290.5 μg. These results provides optimized extraction condition for hesperidin and might be useful for the development of hesperidin as functional products like health supplements, cosmetics and medicinal products.
**Keywords** – Hesperidin, Optimization, Response surface methodology, Extraction conditions

## Introduction

*Citrus unshiu* Marcov, which belongs to the family of Rutaceae, is a seedless and easy-peeling Korean citrus fruit that accounts for 30% of the total fruits produced in Korea. Its dried peels have been widely used as a folk medicine in Asian countries to improve bronchial and asthmatic conditions or blood circulation.[1-4] Citrus peel, a by-product of the citrus juice industry, contains a large amount of pectin and flavonoids. Hesperidin (Fig. 1), a flavanone glycoside, is one of the abundant flavonoid of *C. unshiu*.[5] It has been reported to possess various biological activities such as antioxidant, anti-inflammatory, anti-allergic, anti-cancer, anti-obesity and hypolipidemic effects.[6-10] Therefore, hesperidin has been widely used as functional foods, cosmetics and medicinal products.

For development of functional products from *C. unshiu*, extraction procedure is indispensable. Many factors such as extraction solvent, extraction time and extraction temperature affect the composition of extract as well as its biological activity.[11-13] Therefore, optimization of extraction condition is essential for maximum efficacy. Response surface methodology can derive optimal condition by taking into several factors together. Thus, it is effective for optimization of extraction condition, especially in case of several variables.[14,15]

In the present study, we tried to optimize extraction conditions such as extraction solvent, extraction time and extraction temperature for maximum yield using response surface methodology with three-level-three-factor Box-Behnken design (BBD).




Hesperidin

**Fig. 1.** Chemical structure of hesperidin.

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**Table 1.** A Box-Behnken design for independent variables and their responses

| Run | Coded variables | | | Actual variables | | | Observed values |
|---|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | Extraction solvent (ethanol, %) | Extraction temperature (ºC) | Extraction time (h) | Hesperidin (μg/5 mg extract) |
| 1 | 1 | -1 | 0 | 100 | 30 | 13 | 42.2 |
| 2 | 0 | 1 | -1 | 50 | 90 | 2 | 240.9 |
| 3 | 0 | -1 | 1 | 50 | 30 | 24 | 58.5 |
| 4 | 1 | 1 | 0 | 100 | 90 | 13 | 149.0 |
| 5 | -1 | -1 | 0 | 0 | 30 | 13 | 15.7 |
| 6 | -1 | 1 | 0 | 0 | 90 | 13 | 19.4 |
| 7 | 0 | 0 | 0 | 50 | 60 | 13 | 266.8 |
| 8 | 0 | 0 | 0 | 50 | 60 | 13 | 272.1 |
| 9 | 0 | 1 | 1 | 50 | 90 | 24 | 171.4 |
| 10 | -1 | 0 | 1 | 0 | 60 | 24 | 9.5 |
| 11 | -1 | 0 | -1 | 0 | 60 | 2 | 15.4 |
| 12 | 1 | 0 | 1 | 100 | 60 | 24 | 126.5 |
| 13 | 1 | 0 | -1 | 100 | 60 | 2 | 94.6 |
| 14 | 0 | -1 | -1 | 50 | 30 | 2 | 37.8 |
| 15 | 0 | 0 | 0 | 50 | 60 | 13 | 289.0 |

## Experimental

**Plant material** – The peels of *C. unshiu* were purchased from a local herbal market in Chungbuk, Korea in November 2013. They were identified by the herbarium of College of Pharmacy at Chungbuk National University, where a voucher specimen was deposited (CBNU201311-CU). Hesperidin was purchased from Sigma-Aldrich Chemical Co.

**Experimental design for response surface methodology** –A Box-Behnken design (BBD) with three variables and three levels was used to optimize the extraction conditions for hesperidin from *C. unshiu* peels. Extraction solvent ($X_1$), extraction temperature ($X_2$) and extraction time ($X_3$) were chosen for independent variables. The ranges of these variables were determined as extraction solvent ($X_1$, ethanol ratio as 0, 50 and 100%), extraction temperature ($X_2$, 30, 60, and 90 ºC) and extraction time ($X_3$, 2, 13 and 24 h) on the basis of a preliminary single factor experiment. The variables were coded at three levels (−1, 0, and 1) and the complete design consisted of 15 experimental points including three replication of the center points (all variables were coded as zero), as shown in Table 1. The hesperidin yields per *C. unshiu* extract were selected as the dependent responses.

Regression analysis was performed according to the experimental data; the mathematical model can be explained by the following equation:

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

$Y$ is the response, $\beta_0$ is the constant coefficient, $\beta_i$ are the linear coefficients, $\beta_{ii}$ are the quadratic coefficients and $\beta_{ij}$ are the interaction coefficients. The statistical significance of the coefficients in the regression equation was checked by analysis of variance (ANOVA). The fitness of the polynomial model equation to the responses was evaluated with the coefficients of $R^2$ and the lack of fit was evaluated using $F$-test.

**HPLC conditions for quantitation of hesperidin** – Analysis was performed using a Waters HPLC system equipped with Waters 600s pumps, a 996 photodiode array detector, and Waters Empower software using Phenomenex Gemini-NX 5μ C18 110A (150 × 10.0 mm) for quantitation. Chromatographic separation was accomplished using methanol with water (40:60) at a flow rate of 2.0 ml/min. The wavelength for detection was set at 280 nm.

Stock standard solution of hesperidin was prepared in DMSO at a concentration of 1.0 mg/ml. Standard working solutions were prepared with serial dilution of 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/ml and used for a calibration curve. A good linearity of calibration curve for hesperidin was achieved with a correlation coefficient of 0.9938.

For the preparation of *C. unshiu* peel extract, 1 g of the

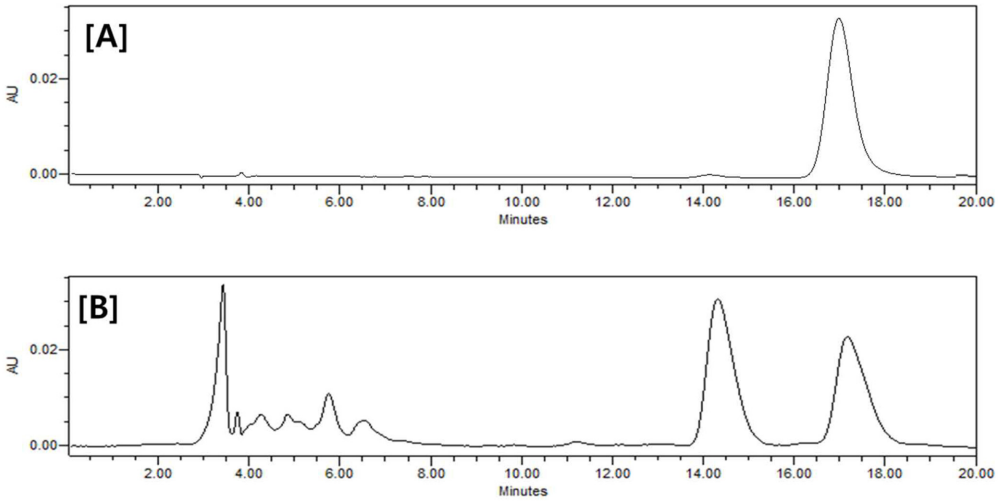


**Fig. 2.** (A) HPLC chromatogram of hesperidin and (B) HPLC chromatogram of *C. citrus* peels extract.

dried powdered peels of *C. unshiu* were weighed accurately and extracted with 10 ml extraction solvent as indicated. The hesperidin yield was expressed as μg hesperidin per 5 mg *C. unshiu* peel extract.

## Results and Discussion

To develop hesperidin as functional product ingredients, efficient production of hesperidin is required. Hesperidin is known to be a major compound of *C. citrus* peels and HPLC analysis clearly showed the presence of hesperidin in the total extract of *C. citrus* peels (Fig. 2). Therefore, the extraction conditions of *C. citrus* peels for the maximum yield of hesperidin were investigated by quantitation of hesperidin using HPLC analysis. The effects of three extraction variables, such as extraction solvent, extraction temperature and extraction time were tested in this study. Extraction solvent was chosen as ethanol because ethanol is safer than other alcohols for function foods. The ranges of these variables were selected through a preliminary single factor experiment as extraction solvent ($X_1$, ethanol ratio as 0 - 100%), extraction temperature ($X_2$, 30 - 90ºC) and extraction time ($X_3$, 2 - 24 h). Hesperidin yield was evaluated as hesperidin content per dried *C. unshiu* peel extract using a Box-Behnken design (BBD) with three-level-three-factor, as shown in Table 1.

Multiple regression analysis of the experimental data yielded this second-order polynomial regression equations as follows:

$$\text{Hesperidin yield (μg/5 mg extract)} = 275.967 + 44.029X_1 + 53.279X_2 - 142.538X_1^2 - 76.807X_2^2 - 71.928X_3^2$$

**Table 2.** Regression coefficients and their significances in the second-order polynomial regression equation for hesperidin yield

| | Coefficient | Standard error coefficient | *t* value | *p* value |
|---|---|---|---|---|
| Intercept | 275.967 | 20.70 | 13.329 | 0.000 |
| $X_1$ | 44.029 | 12.68 | 3.473 | 0.018 |
| $X_2$ | 53.279 | 12.68 | 4.202 | 0.008 |
| $X_3$ | -2.805 | 12.68 | -0.221 | 0.834 |
| $X_1^2$ | -142.538 | 18.66 | -7.638 | 0.001 |
| $X_2^2$ | -76.807 | 18.66 | -4.116 | 0.009 |
| $X_3^2$ | -71.928 | 18.66 | -3.854 | 0.012 |
| $X_1X_2$ | 25.750 | 17.93 | 1.436 | 0.210 |
| $X_1X_3$ | 9.474 | 17.93 | 0.528 | 0.620 |
| $X_1X_2$ | -22.652 | 17.93 | -1.263 | 0.262 |

The significance of each coefficient was determined using *t*-tests and *p*-values (Table 2). ANOVA analysis of the regression equation was also used for the determination of significance and suitability (Table 3). Greater *F*-values and smaller *p*-values were considered significant. The quality of the model was also determined by lack of fit.

As shown in Table 2, the linear term of ethanol concentration ($X_1$) and extraction temperature ($X_2$) and the quadratic term of ethanol concentration ($X_1^2$) and extraction temperature ($X_2^2$) showed great importance for the hesperidin yield. However, other factors such as the linear terms of extraction time ($X_3$), the quadratic terms of extraction time ($X_3^2$), and the interaction terms of variables, $X_1X_2$, $X_1X_3$ and $X_2X_3$ did not show any significant effects. Determination of the suitability of the prediction of hesperidin yield per dried extract was confirmed by an *F*-

**Table 3.** ANOVA for response surface regression equation

| | Sum of square | Degree of freedom | Mean square | *F* value | *p* value |
|---|---|---|---|---|---|
| Regression | 146282 | 9 | 16253.5 | 12.64 | 0.006 |
| Linear | 38281 | 3 | 12760.4 | 9.92 | 0.015 |
| Square | 102937 | 3 | 34312.3 | 26.68 | 0.002 |
| Interaction | 5064 | 3 | 1687.9 | 1.31 | 0.368 |
| Residual error | 6430 | 5 | 1286 | | |
| Lack-of-fit | 6162 | 3 | 2054.1 | 15.34 | 0.062 |
| Pure error | 268 | 2 | 133.9 | | |
| Total | 152712 | 14 | | | |

$R^2 = 0.958$, adjusted $R^2 = 0.882$

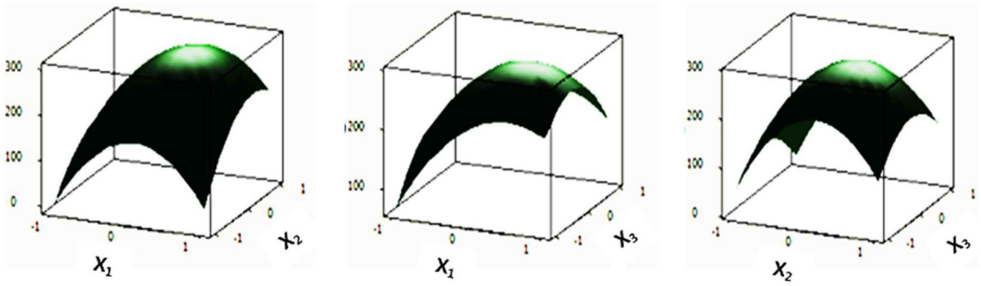

**Fig. 3.** Response surface plot analysis of extraction solvent ($X_1$), extraction temperature ($X_2$) and extraction time ($X_3$) on hesperidin yield.

**Table 4.** Predicted and observed values of hesperidin yield under optimized conditions

| Extraction conditions | | | Hesperidin (μg/5 mg extract) | |
|---|---|---|---|---|
| Extraction solvent (ethanol, %) | Extraction time (h) | Extraction time (ºC) | Predicted | Observed |
| 59.0 | 12.4 | 71.5 | 290.5 | 287.8 |

value of 12.64 and a *p*-value of 0.006. The values of coefficient determination ($R^2$) and the adjusted coefficient determination (adj. $R^2$) of the predicted model in this response were 0.958 and 0.882, respectively, which suggested a high degree of correlation between observed and predicted values (Table 3).

The relationship between every two variables in the hesperidin yield was shown in three-dimensional response surface plots based on regression equations of hesperidin yield per dried extraction (Fig. 3). Hesperidin yield per dried extract is mostly affected by the ethanol ratio ($X_1$), which followed by extraction temperature ($X_2$) and extraction time ($X_3$).

Based on these results, the optimal extraction conditions for maximum hesperidin yield were suggested to be an extraction solvent (ethanol concentration) of 59.0%; an extraction temperature of 71.5 ºC; and an extraction time of 12.4 h for maximum hesperidin, which predicted 290.5 μg hesperidin/5 mg extract. An extract of *C. citrus* peels prepared under these conditions gave 287.8 μg hesperidin/5 mg extract, which was well-matched with predicted values (Table 4).

For development of functional products using hesperidin, extraction procedure from *C. unshiu* is indispensable. In the present study, the extraction condition was optimized by taking into three factors such as extraction solvent, extraction time and extraction temperature using response surface methodology. Our present results clearly showed importance of extraction condition, especially the extraction solvent, for the maximum yield of hesperidin. Therefore, the optimized extraction condition for hesperidin might be useful for the development of hesperidin as functional products like health supplements, cosmetics and medicinal products.

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