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Optimization of Ultrasound Assisted Extraction of Phenolic Compounds and Antioxidants from Grape Peel through Response Surface Methodology

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Functional components from Campbell Early grape peel were extracted by ultrasound-assisted extraction technology. The experiments were carried out according to a five level, three variable central composite rotatable design. The best possible combinations of extraction variables were obtained for the maximum phenolic compounds and antioxidant activities of grape peel extracts using response surface methodology. The optimal conditions include 53.14% ethanol, 46.03°C temperature and 24.03 min time for the maximum total phenolic compounds (6.70 mg GAE/ 100 mL) and 53.06% ethanol, 50.65°C temperature and 25.58 min time for the maximum antioxidant activity (555.90 mg/L). Under these conditions, the experimental total phenolics were 6.67 mg GAE/100 mL and antioxidant activity was 554.84 mg/L of the grape peel extract, which is well matched with the predicted values.

Key words: *antioxidant activity, grape peel, response surface methodology, total phenols, ultrasound assisted extraction*

Grapes (*Vitis vinifera*) are among the most widely consumed fruits and the demand for grapes and grape products is increasing because of the associated health benefits [Ghafoor *et al*., 2008]. Grapes are rich in phenolic compounds [Sanchez-Alonso *et al*., 2008] with approximately 75% of grape polyphenols existing in the skin and seeds. Grape skin phenols may be classified as cell-wall phenols, which are bound to polysaccharides by hydrophobic interactions and hydrogen bonds, non-cell-wall phenols, encompassing phenols confined in the vacuoles of plant cells, and phenols associated with the cell nucleus [Pinelo *et al*., 2006]. The positive physiological effects associated with the consumption of grape and grape derivatives are currently believed to be mainly due to the antiradical and antioxidant properties of the occurring phenolic species [Lurton, 2003]. Phenolic compounds can be used in different therapeutic procedures with the purpose of free radical neutralization in biological systems [Yilmaz and Toledo, 2004] and oxidation of human low-density lipoproteins [Meyer *et al*., 1997].

Extraction is a very important stage in the isolation, identification and use of phenolic compounds [Lapornik *et al*., 2005]. The recovery of these components is commonly performed through a solvent-extraction procedure and the concentration of solvent, time and temperature are important parameters to be optimized for maximum recovery of the targeted compounds [Spigno *et al*., 2007]. Response surface methodology (RSM) has been applied in the industrial processes for optimization of response of interest [Wang *et al*., 2008]. Ultrasound-assisted extraction is an inexpensive, simple and efficient alternative to conventional extraction techniques. The enhancement in extraction obtained by using ultrasound is mainly attributed to the effect of acoustic cavitations produced in the solvent by the passage of an ultrasound wave [Wang *et al*., 2008]. Ultrasound also offers a mechanical effect allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between the solid and liquid phase, as a result the solute quickly diffuses from the solid phase to the solvent [Rostagno *et al*., 2003]. It has been reported that application of ultrasound-assisted extraction in grape peel can enhance the recovery of functional compounds up to 30% as compared to conventional solvent extraction [Cho *et al*., 2006]. In addition the use of ultrasound-assisted extraction also prevents the possible chemical degradation of targeted compounds [Wang and Weller, 2006].

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Abbreviations: GAE, gallic acid equivalent; RSM, response surface methodology; SD, standard deviation

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In this study, ultrasound-assisted extraction parameters such as the solvent concentration, extraction temperature and extraction time were optimized using RSM, by employing a five level, three variable central composite rotatable design, in order to obtain the optimal conditions for the extraction of functional components from the peel of Campbell Early grapes.

Materials and Methods

Materials. Grapes were purchased from a local farm in Kyungbuk, Province of Korea and the grape cultivar was identified as 'Campbell Early'. All the chemicals used were of analytical grade and they were purchased either from Sigma Chemical Co. (St. Louis, MO) or Duksan Pure Chemical Co. (Ansan, Korea).

Ultrasonic-assisted extraction from grape peel. Grapes were excised from the stems and washed. Grapes were manually cut into halves and the grape peels were separated with a knife. Grape peel was oven dried at 50°C until the moisture level was constant. Dried grape peel was ground to a powdered form using an electrical grinder. A sample of 2 g powdered grape peel was kept in a glass flask and the volume was made up to100 mL with the extraction solvent. Contents were dissolved by using a magnetic stirrer (KMC 130SH; Vision Scientific Co., Ltd., Daegu, Korea) for 5 min. Ultrasonic assisted extraction was performed in a sonication water bath (JAC Ultrasonic 2010P; Jinwoo Engineering Co., Ltd., Hwasung, Korea) with a useful volume of 10 L. The working frequency was fixed at 40 KHz and the temperature and time of extraction was controlled from the panel. After extraction the flask was cooled to room temperature using cool water. The extract was filtered through filter paper No 5A under vacuum and the solution was collected in a volumetric flask. It was then used for the determination of total phenolics compounds, antioxidants and anthocyanin contents. All the measurements were carried out in triplicates and the data reported were means±SD.

Experimental design. A five level, three variable central composite rotatable design [Cochran and Cox, 1992] was applied to determine the best combination of extraction variables for the extraction of total phenolic compounds and antioxidants from grape peel. Three independent variables selected for this study were the concentration of solvent, the extraction temperature and the extraction time. The factorial design consisted of eight factorial points, six axial points (two axial points on the axis of each design variable at a distance of 1.68 from the design center) and four center points leading to 18 sets of experiments. Regression analysis was performed on the data of response variables such as total phenols (Y_1) and antioxidant activity (Y_2) obtained by triplicate observations as effected by the extraction conditions and was fitted into an empiric second order polynomial model as shown in the following equation:

$$Y_n=b_0+b_1X_1+b_2X_2+b_3X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2+b_{12}X_1X_2 +b_{13}X_1X_3+b_{23}X_2X_3 \quad (1)$$

where Y_n is the response variable, X_1, X_2 and X_3 correspond to the independent variables namely ethanol concentration, extraction temperature and extraction time, respectively. The b_n values represent corresponding regression coefficients.

Analysis for total phenolic compounds. The total phenolic compounds were analyzed using the Folin Ciocalteu method with some modification [Singleton and Rossi, 1965]. A 200 μL properly diluted sample or a standard solution of varying concentrations were mixed with 400 μL Folin Ciocalteu reagent. The deionized water was used for dilution and control. The solution was diluted to a total volume of 4.6 mL using deionized water then thoroughly mixed. After incubation for 10 min at room temperature, 1 mL of 20% Na_2CO_3 solution was added then immediately mixed and incubated for 2 h. The absorbance was read at 765 nm on a spectrophotometer (TU-1800; Human Corporation, Seoul, Korea). Measurements were recorded in triplicates. Gallic acid of 1 mg/mL was used as the standard and the total phenolic compounds of the samples were expressed in milligram gallic acid equivalent (GAE) per 100 mL (mg GAE/100 mL).

Determination of antioxidant activity. The antioxidant activity of the grape peel extracts was evaluated by the phosphomolybdenum complex method [Prieto *et al*., 1999]. In brief, 0.4 mL of sample solution (100 mL of grape seed extract dissolved in 1 mL of methanol) was combined with 4 mL of reagent solution containing 0.6 M sulphuric aicd, 2 mM sodium phosphate and 4 mM ammonium molybdate. The blank solution contained 4 mL of reagent solution and the 1 mL of methanol. Test tubes were caped and placed in hot water for 90 min at 95°C. After samples were cooled to room temperature absorbance was measured at 695 nm against a blank. Antioxidant activity was expressed relative to that of ascorbic acid.

Statistical analysis. All the analysis was carried out in triplicates and the experimental results obtained were expressed as means±SD. The responses obtained from the experimental design set were subjected to multiple nonlinear regression analysis to obtain the coefficients of the second polynomial model. The quality of the fit of polynomial model was expressed by the coefficient of determination R^2, and its statistical significance was

checked using an *F*-test. The optimal extraction conditions were estimated through three dimensional response surface analyses of the three independent variables and each dependent variable. Statistical analysis was performed by using the Statistical Analysis System (SAS, version 9.1). Data was analyzed by analysis of variance and the mean values were considered significantly different at $p<0.05$.

Results and Discussion

Modeling of the extraction process from grape peel. In order to optimize the extraction process with reference to the extraction of phenolic and antioxidant components from grape peel under sonication a central composite design was developed as represented in Table 1. Table 1 also presents the experimental values of total phenols and antioxidant activities of grape peel extracts at various experimental conditions. The results of analysis of variance, goodness of fit and the adequacy of the models are summarized in Table 2. The data showed a good fit with the Eq. (1) which were statistically acceptable at $p<0.05$ and adequate with satisfactory R^2 values. The full model filled Eq. (1) was used for three dimensional plots to predict the relationships between independent variables and the dependent variables.

Table 2. Regression coefficients and analysis of the model for three response variables

Coefficient	Coefficients estimated	
	Total phenols	Antioxidants
b_0	-3.143	-309.130
b_1	0.144b	14.447b
b_2	0.113b	7.774c
b_3	0.278b	22.225b
b_{11}	-0.001b	-0.120b
b_{22}	-0.001b	-0.098b
b_{33}	-0.006a	-0.538a
b_{12}	-0.000	-0.022
b_{13}	-0.001	-0.023
b_{23}	0.001	0.129
Probability of *F* value	<0.001	<0.001
Probability of lack of fit	0.06	0.09

'a' means $p<0.001$, 'b' means $p<0.01$, 'c' means $p<0.05$

Effect of process variables on the total phenolics compounds. Solid-liquid extraction is a mass transport phenomenon in which solids contained in a matrix migrate into solvent brought into contact with the matrix. This mass transport phenomenon can be enhanced with

Table 1. Experimental design of five-level, three-variable central composite design and total phenols and antioxidant activities of ultrasonic assisted grape peel extracts

Test set	Extraction conditions and Analytical results[a]				
	X_1, Ethanol concentration (%)	X_2, Extraction temperature (C)	X_3, Extraction time (Min)	Total phenols (mg GAE/100 mL)	Antioxidant activity (mg/L)
1	40 (-1)	30 (-1)	15 (-1)	5.74	455.23
2	40 (-1)	30 (-1)	25 (+1)	6.12	493.80
3	40 (-1)	50 (+1)	15 (-1)	5.98	476.49
4	40 (-1)	50 (+1)	25 (+1)	6.47	526.99
5	60 (+1)	30 (-1)	15 (-1)	6.10	484.47
6	60 (+1)	30 (-1)	25 (+1)	6.29	504.56
7	60 (+1)	50 (+1)	15 (-1)	6.09	483.03
8	60 (+1)	50 (+1)	25 (+1)	6.55	542.86
9	33 (-1.68)	40 (0)	20 (0)	5.95	475.23
10	67 (+1.68)	40 (0)	20 (0)	6.49	527.71
11	50 (0)	23 (-1.68)	20 (0)	5.88	476.49
12	50 (0)	57 (-1.68)	20 (0)	6.46	539.37
13	50 (0)	40 (0)	11 (-1.68)	5.51	444.28
14	50 (0)	40 (0)	29 (-1.68)	6.56	540.26
15	50 (0)	40 (0)	20 (0)	6.51	531.83
16	50 (0)	40 (0)	20 (0)	6.53	527.71
17	50 (0)	40 (0)	20 (0)	6.58	533.98
18	50 (0)	40 (0)	20 (0)	6.52	539.55

[a]Analytical results are means± SD (n=3).

changes in diffusion coefficients induced by ultrasounds and extraction temperature [Corrales *et al*., 2009]. Solvent concentration and extraction time also play a significant role in extraction of phenolic compounds from plant materials [Wang *et al*., 2008]. The use of ultrasonication process was due to the fact that ultrasonic waves break the cells of the vegetal matrix and the cells' contents are released into the extraction medium [Vinatoru *et al*., 1997]. In our experiments, the total phenols of grape peel extracts obtained by ultrasound-assisted extraction based on the central composite design are shown in Table 1. Multiple regression analysis was performed on the experimental data and the coefficients of model were evaluated for significance. The effect of extraction time was highly significant ($p<0.001$) on the extraction of phenolics and it was consistent with the findings of Revilla *et al*. [1998] who obtained higher yields of phenolics from grape skins when extraction was done for a longer time. The values of the coefficients as presented in Table 2 were used for final predictive equation neglecting the non-significant cross terms as given below:

$$Y_1 = -3.143 + 0.144X_1 + 0.113X_2 + 0.278X_3 - 0.001X_1^2 - 0.001X_2^2 - 0.006X_3^2 \quad (2)$$

To determine the optimal levels of variables for the ultrasound-assisted extraction of total phenols from grape peel, three-dimensional surface plots (Fig. 1) were constructed according to the Eq. (2). Extraction process variables significantly effected ($p<0.05$) the extraction of total phenols from peel of Campbell Early grapes. Fig. 1A shows the effect of ethanol concentration and extraction time on the content of the total phenolic compounds. The total phenolic contents increased slowly with the increase of ethanol concentration at a fixed extraction temperature and nearly reached a peak at the highest ethanol concentration tested. Similarly, the increase in extraction temperature at a fixed ethanol concentration led to a gradual increase in the total phenolic content, and reached a maximum at the highest extraction temperature tested. The plot of total phenols as affected by ethanol concentration and extraction time (Fig. 1B) demonstrates a marked increase in phenolic contents with the increase of ethanol concentration at a fixed extraction time while an increase in extraction time at a fixed ethanol concentration also led to marked increase in total phenol contents. A similar linear increase in total phenolic contents with the increase of extraction temperature at a fixed extraction time, while an obvious quadratic effect of extraction time were both observed (Fig. 1C).

Effect of process variables on the antioxidant activity. The mean experimental data showing the extraction of antioxidant components from grape peel at various extraction conditions are presented in Table 1. The highest contents of antioxidants (542.86 mg/L) were observed in experimental run 8 with 60% ethanol concentration, 50°C extraction temperature and 25 min extraction time. The lowest yield of antioxidants (444.28 mg/L) was observed in experimental run 13. Statistical analysis revealed that most relevant variable with $p<0.001$ was extraction time. The extraction time has this kind of effect on the antioxidant activity because increasing the contact time of the solvent with solids may

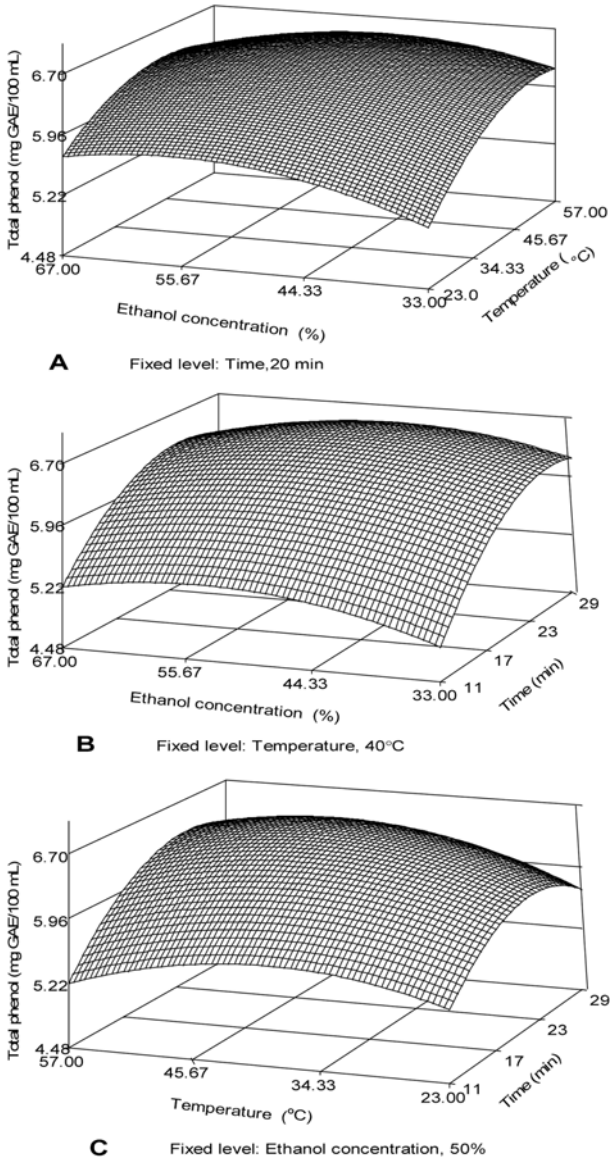

Fig. 1. The response surface plots of the total phenolic contents of grape peel extract as affected by ethanol concentration, temperature, and extraction time in ultrasonic-assisted extraction. Where (A) is ethanol concentration and temperature (time 20 min); (B) is ethanol concentration and time (temperature 40°C); (C) is temperature and time (ethanol concentration 50%).

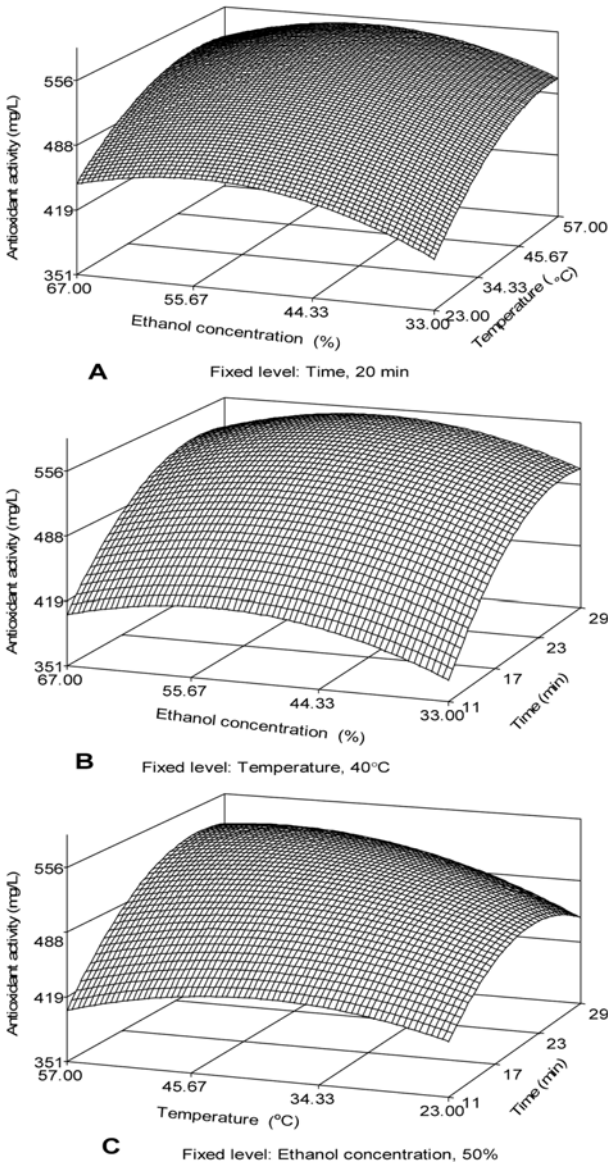

Fig. 2. The response surface plots of the antioxidant activity of grape peel extract as affected by ethanol concentration, temperature and extraction time in ultrasonic assisted extraction. Where (A) is ethanol concentration and temperature (time 20 min); (B) is ethanol concentration and time (temperature 40°C); (C) is temperature and time (ethanol concentration 50%).

improve the diffusion of the compounds (Corrales *et al*., 2009). The results of multiple regression analysis showed that the antioxidant contents of grape peel were significantly ($p<0.05$) affected by the linear and quadratic terms of ethanol concentration, extraction temperature and extraction time (Table 2). The final predictive equation for antioxidant activity of grape peel extract by using significant terms is as follows:

$$Y_2 = -309.130+14.447X_1+7.774X_2+22.225X_3-0.120X_1^2 -0.098X_2^2-0.538X_3^2 \quad (3)$$

As presented in the three dimensional plots for antioxidant contents (Fig. 2), the extraction process variables effected the extraction of antioxidants in a similar way as in the case of total phenolic compounds. This is due to the fact that antioxidant activities of grapes are closely associated with the phenolic compounds [Lu and Foo, 2001]. Antioxidant activity increased with the increase of ethanol concentration at a fixed temperature and also increased significantly ($p<0.05$) with the increase of extraction temperature at a fixed ethanol concentration (Fig. 2A). Similarly antioxidant value increased with the increase of ethanol concentration at a fixed time and it rapidly increased with the increase of extraction time at a fixed ethanol concentration as represented in Fig. 2B. Fig. 2C shows a linear increase in antioxidant activity with the increase of extraction temperature at fixed time and a similar increase in antioxidant activity with the increase of extraction time at a constant extraction temperature.

Optimization. The estimated levels of optimum extraction conditions for maximum response of total phenols and antioxidant activities of grape peel extracts obtained by ultrasonic-assisted extracts are summarized in Table 3. The predicted ultrasonic-assisted extraction conditions were 53.14% ethanol concentration, 46.03°C extraction temperature and 24.03 min extraction time for the maximum total phenols (6.70 mg GAE/100 mL) and 53.06% ethanol, 50.65°C temperature and 25.58 min time for maximum antioxidant activity (555.90 mg/L) The R^2, adjusted R^2 values of the model are 0.959, 0.936 and 0.948, 0.920 for total phenols and antioxidants respectively which represents that the model had adequately represented the real relation between the parameters chosen. To compare the predicted results with experimental values, experimental rechecking was performed for each response using the optimum extraction conditions. Mean values of 6.67 mg GAE/100 mL total phenols and 554.84 mg/L antioxidants obtained from real experiment validated the RSM model. The good correlation between these results confirmed that the response model was adequate in reflecting the expected optimization (Table 3).

This study indicated that the Campbell Early grape peel is a good source of phenolic compounds and the use of ultrasonication for the extraction of total phenols and antioxidants was an effective method because it could greatly decrease the extraction time compared with other extraction methods. Lapornik *et al*. [2005] found that increasing the time from 12 to 24 h during conventional solvent extraction was found to have statistically significant effects on the yields of total phenols and antioxidant activities from red grape pressed marc. The efficiency of ultrasonication could be explained by the fact that sonication simultaneously enhanced the hydration and

Table 3. Estimated optimum conditions, predicted and experimental values of responses under these conditions

Response variables	R^2	R^2-adjust	F-value	p-value	Optimum extraction conditions			Maximum value	
					Ethanol (%)	Temp (°C)	Time (min)	Estimated	Experimental[a]
Total phenols (mg GAE/100 mL)	0.959	0.936	20.85	0.0001	53.14	46.03	24.03	6.70	6.67±0.03
Antioxidant activities (mg/L)	0.948	0.920	20.94	0.0001	53.06	50.65	25.58	555.90	554.84±0.56

[a]Means±standard deviation (n=3).

fragmentation process while facilitating mass transfer of solutes to the extraction solvent (Toma *at al*., 2001).

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