



Effect of Extraction Conditions of Green Tea on Antioxidant Activity and EGCG Content: Optimization using Response Surface Methodology

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Abstract – Green tea, the leaves of *Camellia sinsneis* (Theaceae), is generally acknowledged as the most consumed beverage with multiple pharmacological functions including antioxidant activity. This study was performed to analyze the effect of extraction conditions of green tea on its antioxidant effects using DPPH assay. Three extraction factors such as extraction solvent (EtOH, 0 - 100%), extraction time (3 - 15 min) and extraction temperature (10 - 70 °C) were analyzed and optimized extraction condition for antioxidant activity of green tea extract (GTE) was determined using response surface methodology with three-level-three-factor Box-Behnken design (BBD). Regression analysis showed a good fit of data and the optimal conditions of extraction were found to be 57.7% EtOH, 15 min and 70 °C. Under this condition, antioxidant activity of experimental data was 88.4% which was almost fit to the ideal value of 88.6%. As epigallocatechin gallate (EGCG) is known for the major ingredient for antioxidant activity of green tea, we investigated the effect of EGCG on antioxidant activity of GTE. EGCG showed antioxidant activity with the IC₅₀ value of 4.2 µg/ml and a positive correlation was observed between EGCG content and the antioxidant activity of GTE with R² = 0.7134. Interestingly, however, GTE with 50 - 70% antioxidant activity contain less than 1.0 µg/ml of EGCG, which is much lower than IC₅₀ value of EGCG. Therefore, we suppose that EGCG together with other constituents contribute to antioxidant activity of GTE. Taken together, these results suggest that green tea is more beneficial than EGCG alone for antioxidant ability and optimal extraction condition of green tea will be useful for the development of food and pharmaceutical applications

Key words – Green tea, Optimization, Response surface methodology, Antioxidant activity, Epigallocatechin gallate

Introduction

The leaves of *Camellia sinsneis* (Theaceae), commonly called as green tea, is one of the most popular beverage for a long time. Green tea is generally used as a beverage to help digesting, shortening their sleep, clearing the head and eyes and detoxifying the body. Diverse beneficial effects to prevent cancer, high cholesterol, allergy and diabetes are also reported.¹⁻⁴ Epigallocatechin gallate (EGCG) is a characteristic constituent of green tea and is known to exert diverse effect of green tea.⁵⁻⁷

Oxidative stress is derived from the imbalance between the production of reactive oxygen species and antioxidant defenses and contributes to age-related symptoms and pathogenesis of many diseases such as cancer, diabetes, atherosclerosis, neurodegenerative diseases and osteoporosis.⁸⁻⁹ Therefore, consumption of antioxidant-rich fruits or botanical

extract are thought to enhance health and minimize the senescence and chronic diseases.¹⁰⁻¹² Green tea also has been reported to exert beneficial effect against various diseases, in parts, via antioxidant effects.¹³⁻¹⁴

For use of green tea as a beverage or development as functional products, extraction procedure is required. Many factors during extraction procedure such as extraction solvent, extraction time, extraction temperature and solid-liquid ratios determine efficacy of extract.¹⁵⁻¹⁶ Therefore, optimization of extraction condition is required for maximum efficacy. Response surface methodology (RSM) is a useful statistical tool that can derive optimal condition by taking into several factors simultaneously. It consists of mathematical and statistical methods and derives optimal condition based on experimental data obtained from rationally designed experiment.¹⁷⁻¹⁹ Therefore, RSM is effective method for optimization of extraction condition, especially in case of several variables.

In the present study, we investigated the effect of extraction conditions of green tea on antioxidant activity using RSM. As EGCG is known for the major ingredient

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for antioxidant activity of green tea, we investigated the effect of EGCG content of green tea on antioxidant activity. Ultimately, optimized extraction condition for maximum antioxidant activity was suggested using RSM.

Experimental

Plant material – Dried green tea, the leaves of *Camellia sinensis*, was purchased from the local herbal market, Chungbuk, Korea in March 2015. They were identified by the herbarium of College of Pharmacy at Chungbuk National University, where a voucher specimen was deposited (CBNU201503-GT). EGCG was purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA).

Experimental design for response surface methodology – A Box-Behnken design (BBD) with three variables and three levels using Minitab17 software (Eretec Inc.) was chosen to optimize the extraction conditions for GTE. Extraction solvent (X_1), extraction time (X_2) and extraction temperature (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%), and extraction time (X_2 , 3, 9 and 15 min) extraction temperature (X_3 , 10, 40, and 70 °C) 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 antioxidant activity was selected as the

dependent responses.

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

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

Y is the response, β_0 is the constant coefficient, β_i are the linear coefficients, β_{ii} are the quadratic coefficients and β_{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.

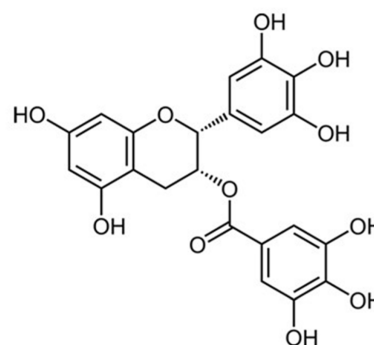


Fig. 1. Chemical structure of EGCG.

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 (°C)	Extraction time (min)	Antioxidant activity (%)	EGCG content (ig/mg extract)
1	-1	0	-1	0	40	3	46.5	16.9
2	1	0	-1	100	40	3	69.3	13.1
3	-1	0	1	0	40	15	52.0	31.7
4	1	0	1	100	40	15	64.8	17.5
5	0	0	0	50	40	9	85.2	115.1
6	0	-1	1	50	10	15	57.9	10.9
7	-1	-1	0	0	10	9	51.2	40.0
8	1	1	0	100	70	9	71.9	29.2
9	0	-1	-1	50	10	3	82.3	105.4
10	0	-1	1	50	10	15	82.1	107.3
11	0	1	-1	50	70	3	83.4	98.2
12	0	1	1	50	70	15	86.3	111.6
13	0	0	0	50	40	9	81.7	107.1
14	0	0	0	50	40	9	83.6	109.9
15	1	0	-1	100	40	3	44.9	26.5

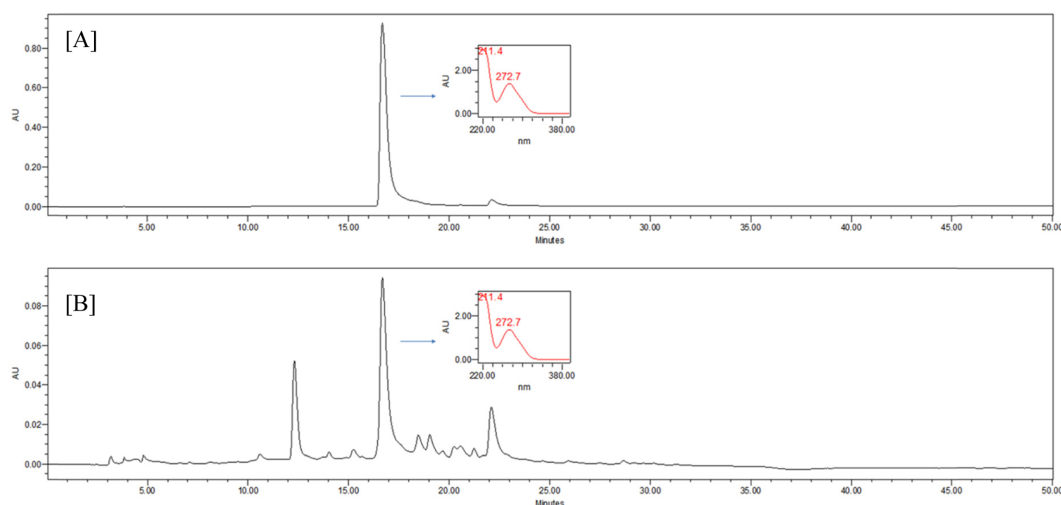


Fig. 2. (A) HPLC chromatogram of EGCG and (B) HPLC chromatogram of GTE.

Antioxidant activity – The antioxidant activity of GTE was evaluated by measuring the free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) by measuring the absorbance at 517 nm.²⁰ The relative radical scavenging activity (%) was calculated as $[1 - \text{observed value of solution with sample and DPPH} / \text{observed value of solution with DPPH}] \times 100$.

HPLC conditions for quantitation of EGCG – 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 5 μ C18 110A (150 \times 10.0 mm) for quantitation. Chromatographic separation was accomplished using acetonitrile with water (10:90 to 50:50) at a flow rate of 2.0 ml/min. The wavelength for detection was set at 291 nm.

Stock standard solution of EGCG 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 EGCG was achieved with a correlation coefficient of 0.9983.

For the preparation of GTE, 1 g of the dried powdered green tea were weighed accurately and extracted with 10 ml extraction solvent as indicated.

Result and Discussion

To maximize the antioxidant effect of green tea, the effects of three extraction variables, such as extraction solvent, extraction temperature and extraction time were

tested in this study. The ranges of these variables were selected through a preliminary single factor experiment as extraction solvent (X_1 , ethanol ratio as 0 - 100%), extraction time (X_2 , 3 - 15 min) and extraction temperature (X_3 , 40 - 70 °C). Antioxidant effect of GTE was analyzed 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:

$$\begin{aligned} \text{Antioxidant activity (\% of control)} \\ = 55.409 - 1.245X_1 + 0.116X_2 - 0.076X_3 + 0.011X_1^2 - \\ 0.024X_2^2 + 0.001X_3^2 + 0.008X_1X_2 - 0.001X_2X_3 - 0.004X_1X_3 \end{aligned}$$

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 the quadratic term of ethanol concentration (X_1^2) showed great importance for the antioxidant effect of green tea. However, other factors did not show any significant effects. Determination of the suitability was confirmed by an *F*-value of 52.4 and a *p*-value of < 0.001. The values of coefficient determination (R^2) and the adjusted coefficient determination (adj. R^2) of the predicted model in this response were 0.990 and 0.971, respectively, which suggested a high degree of correlation between observed and predicted values (Table 3). The relationship between every two variables in the

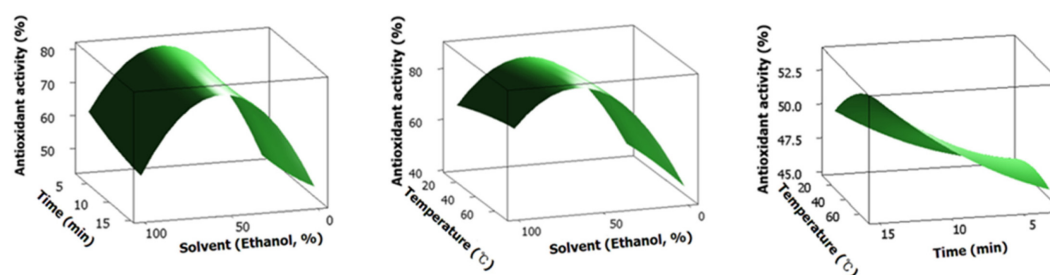
Table 2. Regression coefficients and their significances in the second-order polynomial regression equation for antioxidant activity of GTE

	Coefficient	Standard error coefficient	<i>t</i> value	<i>p</i> value
Intercept	55.409	5.370	10.317	<0.001
X_1	-1.245	0.079	-15.818	<0.001
X_2	0.116	0.795	0.146	0.889
X_3	-0.076	0.149	-0.509	0.632
X_1^2	0.011	0.001	19.047	<0.001
X_2^2	-0.024	0.038	-0.637	0.552
X_3^2	0.001	0.002	0.588	0.582
X_1X_2	0.008	0.004	1.882	0.119
X_1X_3	-0.001	0.001	-1.457	0.205
X_1X_2	-0.004	0.007	-0.577	0.589

Table 3. ANOVA for response surface regression equation

	Sum of square	Degree of freedom	Mean square	<i>F</i> value	<i>p</i> value
Regression	3297.48	9	366.387	52.4	<0.001
Residual error	34.96	5	6.992	2.0	
Lack-of-fit	28.71	3	9.569	3.06	0.256
Pure error	6.25	2	3.126		
Total	3332.44	14			

$R^2 = 0.990$, adjusted $R^2 = 0.971$

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

antioxidant effect was shown in three-dimensional response surface plots (Fig. 3).

Based on these results, the optimal extraction conditions for maximum antioxidant effect were suggested to be an extraction solvent (EtOH concentration) of 57.6%; an extraction temperature of 70°C; and an extraction time of 15 min, which predicted 88.6% activity. GTE prepared under these conditions gave 88.4% activity, which was well-matched with predicted values (Table 4).

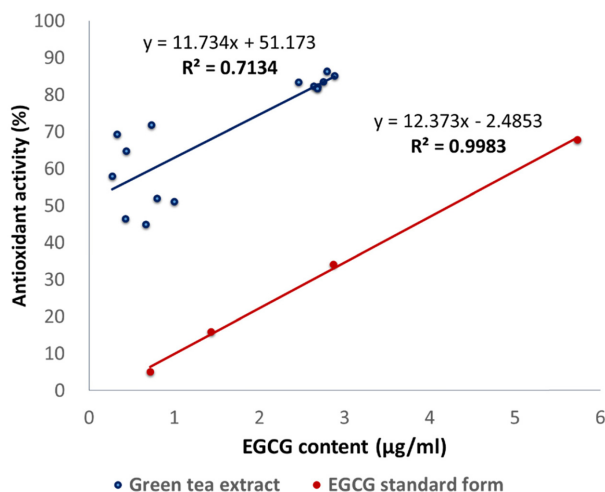
As EGCG is known for the major ingredient for antioxidant activity of green tea, we investigated the effect of EGCG content of GTE on antioxidant activity. EGCG showed antioxidant activity with the IC_{50} value of 4.2 $\mu\text{g/ml}$ in our assay system. The EGCG content in GTE was greatly varied depending on extraction conditions

(Table 1). Therefore, correlations between antioxidant activity and EGCG content was analyzed. High correlation was observed between antioxidant activity and EGCG content, as demonstrated by the value of R^2 was 0.7134 (Fig. 4). Interestingly, however, GTE with 50–70% antioxidant activity contains less than 1.0 $\mu\text{g/ml}$ of EGCG, which is much lower than IC_{50} value of EGCG.

Green tea characteristically contains high contents of catechin derivatives. Catechin belongs to flavonoids and has the flavan skeleton with a hydroxyl group at C-3-ols. It is well known for its diverse biological activities of green tea. In addition, it is converted to tannin by polymerization. Green tea contains diverse catechin derivatives and (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG) and EGCG are known as

Table 4. Predicted and observed values of antioxidant activity yield under optimized conditions

Extraction conditions			Antioxidant activity (%)	
Extraction solvent (ethanol, %)	Extraction time (min)	Extraction temperature (°C)	Predicted	Observed
57.6	15.0	70.0	88.6	88.4

**Fig. 4.** Correlation of EGCG or EGCG content in GTE and with antioxidant activity.

major constituents of green tea.²¹ All catechin derivatives of green tea are known for strong antioxidant activity in vitro and in vivo system.²¹⁻²² Among them, EGCG is the most abundant catechin of green tea and the most potent activity, which is followed by EGC > ECG > EC.²³ Therefore, we suppose that EGCG is a major active constituents of GTE and other constituents of GTE also contribute to antioxidant activity of GTE.

Taken together, these results suggest that green tea has antioxidant ability and optimal extraction conditions of green tea will be useful for the development of food and pharmaceutical applications

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