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Application of Factorial Design on the Extraction of Green Tea Leaves (*Camellia sinensis* L.)

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ABSTRACT

Green tea (*Camellia sinensis* L.) contains bioactive compounds such as epigallocatechin gallate (EGCG), caffeine, and gallic acid. The study aimed to optimize the extraction condition using the experimental design of factorial design. Two variables namely water temperature (75 and 95°C) and brewing number (one and two-times) were used and objected to factorial design in order to get the optimum condition. The determination of EGCC, caffeine, and gallic acid was carried out using high-performance liquid chromatography method equipped with the UV-visible detector. The result showed that the extraction yield varied from 4.48%-7.56%. The level of EGCG and caffeine in green tea extract varied from 251.96-393.34 mg/g dry weight and 32.94-46.82 mg/g dry weight, while the level of gallic acid could not be quantified in each experiment because it was below the limit of quantification (LOQ). The predicted optimum extraction condition consisted of water temperature at 95°C with two-times brewing. Using this optimum condition, the concentrations of EGCG, caffeine and the extraction yield were of 356.43 mg/g dry weight, 38.76 mg/g dry weight, and 5.76%, respectively.

INTRODUCTION

Green tea is manufactured by steaming and drying of *Camellia sinensis* L. Green tea is rich in polyphenols, and most of them are catechins (Michele *et al.,* 2014). Catechins in green tea can be (+)-catechin (C), (-)-catechin 3-gallate (CG), (-)-epicatechin (EC), (-)-epicatechin 3-gallate (ECG), (-)-epigallocatechin (EGC), (-)-gallocatechin 3-gallate (GCG), (-)-gallocatechin (GC), and epigallocatechin 3-gallate (EGCG) (58-55% of total polyphenols) (Fang *et al.,* 2006; Michele *et al.,* 2014; Perva-Uzunalić *et al.,* 2006). EGCG is the most abundant and commonly used as biomarker compound. EGCG has been reported have antioxidant, antimutagenic, anticarcinogenic and antibacterial properties (Komes *et al.,* 2010; Michele *et al.,* 2014; Perva-Uzunalić *et al.,* 2006).

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Other components such as caffeine (2.5-3.5% of dry weight) (Michele *et al.,* 2014), fats (16%), triterpenoids, proteins, amino acids, sterols, vitamins, minerals were found in green tea (Perva-Uzunalić *et al.,* 2006). Caffeine is related to diuretic responses and influences central nervous system activity. Higher dose (>200 mg/day) of caffeine induces nervousness, headache, tremors, sleeplessness, increased blood pressure, etc. (Michele *et al.,* 2014; Yang *et al.,* 2007). Green tea also comprises a gallic acid, a yield of degradation generated from galloyled catechins (Khalaf *et al.,* 2008).

Green tea is widely known as a traditional beverage (Yang *et al.*, 2007; Venditti *et al.*, 2010; Damiani *et al.*, 2014; Michele *et al.*, 2014). The method for preparing the beverage varies around the world. Chinese brew tea leaves in hot water (70-80°C), and the brewing is done repeatedly seven-times. In Japan, green tea is prepared in hot water for two minutes and it can be used for two to three times. In Taiwan, it is brewed in cold water (4 or 25°C). Brewing tea in cold water provides lower levels of caffeine, reduces bitterness and creates more flavor (Venditti *et al.,* 2010). Different techniques for preparing the beverage product lead to varied levels of green tea compounds (Lin *et al.,* 2003).

In recent years, many green tea leaf extractions have been developed including extraction of catechin, caffeine and gallic acid from tea bag infusions using different steeping method (Yang *et al.,* 2007), extraction of major catechin and caffeine from green tea using different solvents (Perva-Uzunalić *et al.,* 2006), solvent extraction of catechin from Korean tea (Row and Jin 2006), extraction of bioactive compounds from green tea using aqueous extraction (Komes *et al.,* 2010). In addition, comparison of the hot and cold water extraction on the antioxidant activity has been reported (Venditti *et al.,* 2010). The application of factorial design to optimize the extraction condition has been performed in previous studies such as optimization of subcritical water extraction of flavanols from green tea leaves (Ko *et al.,* 2014) and optimization extraction of *Syzygium cumini* L. (Migliato *et al.,* 2011). This method was chosen because it is more simple and efficient than other methods. To our best knowledge, there is no report related to the use of the experimental design of factorial design to optimize the extraction conditions of green tea extract.

Several methods have been used to establish the level of EGCG, caffeine and gallic acid from green tea. Previous studies were done using HPLC with UV-visible detector (Li *et al.,* 2012; Row and Jin 2006; Rusak *et al.,* 2008), high-liquid chromatography using diode array detector (Perva-Uzunalić *et al.,* 2006), electrophoresis (Kotani *et al.,* 2007), thin-layer chromatography (Vovk *et al.,* 2005), fourier transform near infrared spectrometry (Chen *et al.,* 2009b; Chen *et al.,* 2009a), near infrared spectrometry (Chen *et al.,* 2006), reflectance spectroscopy, centrifugal precipitation chromatography (Baldermann *et al.,* 2009), potentiometric flow injection (Koutelidakis *et al.,* 2009; Nieh *et al.,* 2009), high-speed counter current chromatography (Kumar and Rajapaksha 2005; Yanagida *et al.,* 2006).

In this study, high-liquid chromatography was chosen to establish the levels of EGCG, caffeine and gallic acid from green tea leaf extract. This method was able to provide data both qualitatively and quantitatively precisely and meticulously than other analysis methods. However, this method requires a relatively large cost (Sugihartini *et al.,* 2014). The objective of this study was to determine the optimum extraction conditions such as water temperature and brewing number for the extraction of three primary compounds (EGCG, caffeine and gallic acid) and yields of extracts from green tea leaves.

MATERIALS AND METHOD

Materials

Green tea (*Camellia sinensis* L.) samples were obtained from Minangkabau, West Sumatera, Indonesia and they were determined by The Indonesian Institute of Science, Candi Kuning, Bali, Indonesia (No. B-450/IPH.7/AP/VI/2017). The reference standard of (-)-epigallocatechin gallate 80% (E4268), caffeine (Y0000787), gallic acid 98% (91215) were obtained from Aldrich (Sigma-Aldrich, Singapore). All the solvents utilized for analytical and extraction process were obtained from E. Merck (Darmstadt, Germany).

Instrumentation and software

HPLC system that was used consisted of Knauer HPLC Germany Smart-Line series with UV detector (Smart-Line UV detector 2500 A5140), Smart-Line pump 1000 V7603, 20 µL Rheodyn Loop A135 sample injector, Eurosphere C-18 column (250 × 4.6 mm, i.d 5 µm). Data were further analyzed using Chromgate software version 3.1. The water contents in green tea extract were determined by Ohaus Moisture MB 25. Design of Experiments (DoE) was performed using Design-Experts® software (Ver. 7.1.5: Stat-Ease Inc. Minneapolis, MN, USA).

Validation of HPLC analysis

Before the validation of HPLC, the system suitability test was performed by injecting a standard solution mixture of 20 µL with a concentration of 1 mg/mL (Martono and Martono 2013). The results of the system suitability test can be seen in Table 1. The validation of HPLC method was performed by assessing several parameters such as selectivity, linearity and range, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision (Prabaningdyah *et al.,* 2017).

Preparation of tea infusion

The preparation of green tea extract was carried in single step extraction, and multiple step extraction. In the single step extraction, green tea samples (10 g) (Demir *et al.,* 2015) were extracted in 250 mL hot water (75 and 95°C) for 20 minutes. The extract solution was cooled in cold water for 10 min and was fractionated with 100 mL ethyl acetate. The solvent was evaporated using a water bath and the yield of the extract was accurately weighed. This procedure was replicated three-times in each experiment. During the multiple step extraction, green tea samples (10 g) were extracted two-times under the same conditions as in the single step extraction in 150 mL hot water and continued in 100 mL hot water.

Sample preparation of HPLC

Green tea extracts (10 mg) were diluted in mobile phase (10 mL) and sonicated (Krisbow DSA50-GL2-2.5L) for 15 minutes. The temperature was arranged at 30°C. The mixture was then filtered using nylon membrane (0.45 µm) and injected (20 µL) into a port injector. It was replicated three times for each sample. The chromatographic separations were performed on Eurosphere C-18 column (250 × 4.6 mm, i.d 5 µm). Mobile phase used was 0.1% orthophosphoric acid: water: acetonitrile: methanol (14;7;3;1 v/v/v/v) at pH 4.00 delivered isocratically with a flow rate of 1.2 mL/min. Analytes were detected using UV-visible detector at a wavelength of 280 nm. The temperature was set at room temperature (Martono and Martono 2013; Sugihartini *et al.,* 2014). The determination of the level of the compounds was performed by plotting the Area Under Curve (AUC) of each compound (EGCG, caffeine and, gallic acid) chromatogram with the regression of standard curve (Martono and Martono 2013; Proyong *et al.,* 2007; Saito *et al.,* 2006).

Experimental design of extraction

Factorial design (2-levels, 2-factors) was applied to determine the optimal condition of water temperature and brewing number to extract the green tea compounds. Water temperature (A) and brewing number (B) were independent variables studied to optimize the response (Y) such as the yield of extract (Y_1) and levels of EGCG (Y_2), CAF (Y_3), and

GA (Y_4). The water temperatures varied at 75 and 95°C and the number of brewing at one and two-times. The factorial design (full factorial) requires an experiment number according to the following equation (Politis *et al.,* 2017; Yu *et al.,* 2014):

$$\text{Number of experiments} = \text{Levels}^{\text{factors}}$$

Table 1: System suitability test results.

Gallic acid					
Replication	**tR (min)**	**Asymetry USP**	**Width USP**	**Plates USP**	**HETP USP**
1	3.33	1.210	0.28	15042.98	3761
2	3.48	1.210	0.22	14795.00	3699
3	3.41	1.150	0.22	15280.91	3820
Mean	3.41	1.19	0.24	15039.63	3760.00
SD	0.08	0.03	0.03	242.97	60.51
%RSD	0.02	0.03	0.14	0.02	0.02
Caffeine					
Replication	**tR (min)**	**Asymetry USP**	**Width USP**	**Plates USP**	**HETP USP**
1	12.25	0.924	0.52	21900.00	5475
2	12.68	0.997	0.67	21648.00	5412
3	12.80	0.990	0.67	22431.69	5608
Mean	12.58	0.97	0.62	21993.23	5498.33
SD	0.29	0.04	0.09	400.08	100.06
%RSD	0.02	0.04	0.14	0.02	0.02
EGCG					
Replication	**tR (min)**	**Asymetry USP**	**Width USP**	**Plates USP**	**HETP USP**
1	17.70	1.082	1.05	16406.03	4102
2	17.80	1.071	1.06	16618.00	4155
3	17.90	1.065	1.07	17395.34	4349
Mean	17.80	1.07	1.06	16806.46	4202.00
SD	0.10	0.01	0.01	520.89	130.03
%RSD	0.01	0.01	0.01	0.03	0.03

For example, a full factorial of three factors requires $2^2 = 4$ experiments and when it is replicated three times, it will generate 12 experiments to run. The response was estimated by the following factorial equation (Çelik 2017):

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{12} AB + \varepsilon$$

Wherein, Y is the estimated response, A and B indicate the independent variables, β_0 is the intercept value, β_1 and β_2 are linear coefficients, while β_{11} and β_{22} are the factorial coefficient (Çelik 2017; Pramod *et al.,* 2016; Yu *et al.,* 2014). Analysis of Variance (ANOVA) was used to evaluate the effect of independent variables on the response. The optimized conditions were prepared and compared with the predicted values.

RESULTS AND DISCUSSION

Validation method

During the system suitability test, the percent of the relative standard deviation (%RSD) of the retention time was evaluated. The result showed RSD values of 0.02%, 0.02%, and 0.01% for the retention time of gallic acid, caffeine, and EGCG, indicating the suitability of HPLC system (RSD < 2%). The numbers of theoretical plates (N) and Height Equivalent of A Theoretical Plate (HETP) for the three replicate injections were found about 15039.63 and 3760 for gallic acid, 21993.23 and 5498.33 for caffeine as well as, 16806.46 and 4202 for EGCG, indicating the acceptable criteria for parameters of N (>2000) and HETP.

Selectivity

A standard solution mixture of gallic acid, caffeine, and EGCG at a concentration of 1 mg/mL, respectively was prepared by diluting into a mobile phase and 20 µL of it was injected into an HPLC system. Resolution (Rs) value obtained was 21.32 for the gallic acid and caffeine and 6.22 for the caffeine and EGCG indicating that HPLC is selective enough for analysis of gallic acid, caffeine, and EGCG (Rs > 2). The HPLC chromatogram of gallic acid, caffeine, EGCG, and green tea extract is shown in Figure 1.

Linearity and range

The linearity of gallic acid, caffeine, and EGCG was evaluated from coefficient correlation (r-value) and intercept of the linear regression describing the relationship between the concentration of analytes (x-axis) and peak area (y). The concentration ranges used were 10-60 µg/mL for gallic acid, 1-25 µg/mL for caffeine, and 5-50 µg/mL for EGCG. The results showed a good relationship with an R-value of gallic acid, caffeine, and EGCG of 0.999, 0.996, and 0.998, respectively.

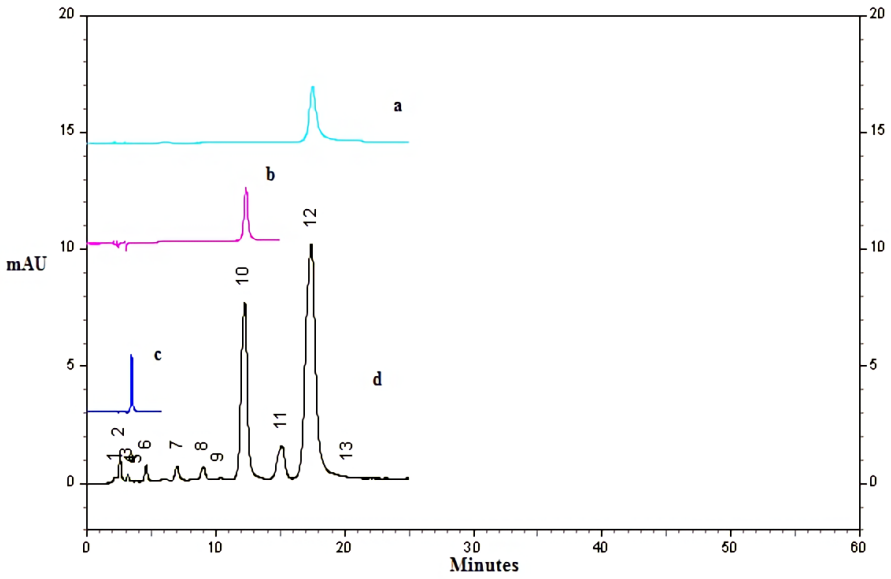

Fig. 1: HPLC chromatogram of EGCG standard (a), caffeine standard (b), gallic acid standard (c) and green tea extract (d).

Table 2: The linear regression equations and validation method of the EGCG, caffeine and gallic acid.

Compound	UV 280 nm						
	Linear range (ug/mg)	Linear regression equation	Linearity (r^2)	LOD (ug/mL)	LOQ (ug/mL)	Accuracy (% recovery)	Precision (%RSD)
EGCG	5–50	y = 21254x – 111.9	0.998	1.07	3.57	98.2–101.8	1–2
Caffeine	1–25	y = 43688x + 63740	0.996	1.72	1.72	98.5–102	1.2–1.75
Gallic acid	10–60	y = 41800x + 14517	0.999	3.8	12.8	98–102.2	1.2–2

LOD and LOQ were calculated by using the equations below:

$$LOD = \frac{3.3 \times Sy/x}{b} \quad LOQ = \frac{10 \times Sy/x}{b}, \quad Sy/x = \sqrt{\frac{\Sigma(yi\text{-}yc)2}{n\text{-}2}}$$

Where LOD and LOQ values are 3.8 µg/mL and 12.8 for gallic acid, 0.52 µg/mL and 1.72 µg/mL for caffeine, and 1.07 µg/mL and 3.57 µg/mL for EGCG.

Precision and accuracy

The precision test was determined by repeatability test (intra-day precision) by analysis of three replicates of standard solution at concentration levels of 40, 50, and 60 µg/mL for gallic acid, 15, 20, and 25 µg/mL for caffeine as well as, 30, 40, and 50 µg/mL for EGCG. The results showed the RSD values of gallic acid, caffeine, and EGCG were to be less than 2% (Table 2).

Recovery test was used to test the accuracy of the method. It was carried out at three different concentrations and repeated three times. The concentration of each standard solution was the same as that used in the precision test. The results showed that the % recovery of each compound was in the range of 98–102% (Table 2).

Optimization of experimental design

The DoE was adopted on the basis coded level from two variables (Table 3). The matrix of experimental design and response values are shown in Table 4. The selected factors such as water temperature (in °C) and brewing number were considered to have an influence on the experimental responses. The experiments obtained the levels of extraction yield varied from 4.48% to 7.56%, EGCG levels that varied from 251.96 mg/g dry weight to 393.34 mg/g dry extract, caffeine levels varied from 32.94 mg/g dry extract to 39.17 mg/g dry extract, meanwhile gallic acid level was not quantified or not detected in each experiment. Therefore, this study only used three responses for further analysis.

The effect of the extraction conditions on the yield extraction (Y_1) can be illustrated by the following equation:

$$Y_1 = 5.67 + 0.80(A) - 0.36(B) - 0.27(A)(B)$$

The statistical results showed that the adjusted coefficient of determination (Adj.R^2) generated was 0.8675 ($R^2 > 0.8$). It indicates that the equation model is the best fit using this equation (Prabaningdyah *et al.,* 2017). The relationship between equation model and observation data of extraction yield is shown in Figure 2a. The variables of A, B, and the interaction between A and B contributed significantly to the response of Y_1 (p-value < 0.05). The equation model showed that the water temperature had a positive effect on the extraction yield indicating that with an increasing water temperature, yield extraction increases. It is due to the cell wall of green tea leaves that become more permeable to the solvent and the solubility and diffusion coefficient of the constituents increase (Vuong *et al.,* 2011b; Vuong *et al.,* 2011a; Vuong *et al.,* 2010).

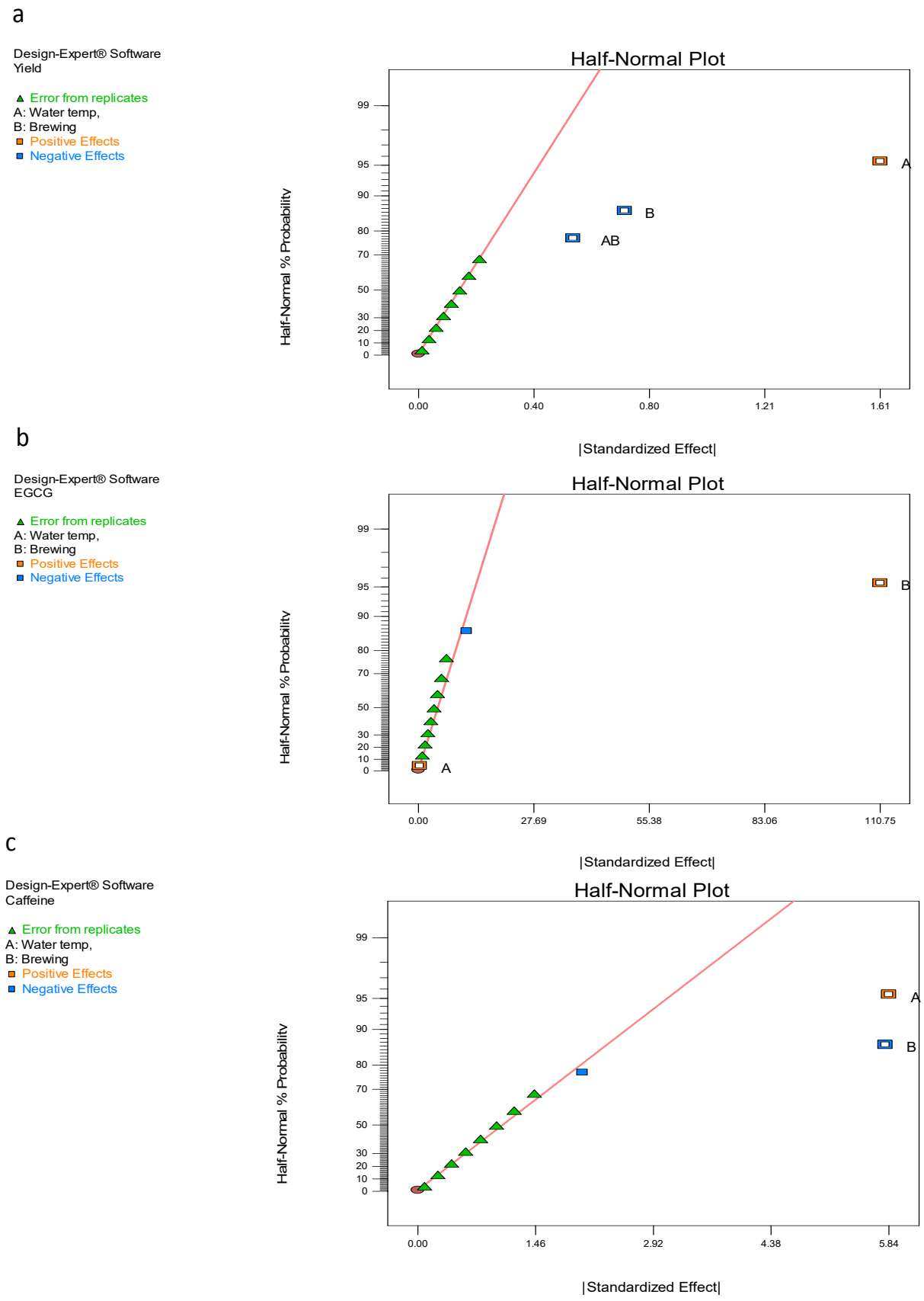

Fig. 2: The relationship between observation results of extraction yield on the selected model graph (a), the relationship between observation results of EGCG Level on the selected model (b), the relationship between observation results of caffeine level on the selected model (c).

Table 3: The codes and uncoded levels of independent variables used in the factorial design.

Independent variables	Symbol	Levels	
		Low (−1)	High (+1)
Water temperature (°C)	A	75	95
Brewing number	B	One-time	Two-times

The effect of the extraction conditions on the EGCG level (Y_2) can be illustrated by the following equation:

$$Y_2 = 314.84 + 55.38(B)$$

The statistical results showed that the adjusted coefficient of determination (Adj.R^2) generated was 0.9669 ($R^2 > 0.8$). It indicates that the equation model is the best fit using this equation. The relationship between equation model and observation data of EGCG levels is shown in Figure 2b. Only variable B contributed significantly to the response of Y_2 (p-value < 0.05). The equation model showed that only brewing number had a positive effect on the EGCG level. It indicates that with an increasing brewing number, EGCG level increases. In the first infusion, EGCG did not release entirely. They would release entirely during to the next infusion (Yang *et al.,* 2007). The water temperature contributed insignificantly to the EGCG level. In this case, that the temperature exceeding 75°C for 20 minutes may affect the stability of EGCG and it did not significantly differ (p-value of 0.9565). The extraction temperature above 80°C could induce an increased epimerization reaction.

Table 4: The experimental design of extraction conditions using 22 factorial design.

Std	Run	Factor 1 (A)	Factor 2 (B)	Response 1 (Y_1)	Response 2 (Y_2)	Response 3 (Y_3)	Response 4 (Y_4)
		Water temperature (°C)	Brewing number	Yield extraction (%)	EGCG (mg/g dry weight)	Caffeine (mg/g dry weight)	Gallic acid (mg/g dry weight)
1	9	75	1	4.64	251.96	44.07	Under LOQ
2	4	75	1	5.36	256.08	35.71	Under LOD
3	8	75	1	4.86	252.53	36.31	Under LOD
4	2	95	1	6.89	266.55	46.82	Under LOQ
5	10	95	1	6.85	265.31	46.43	Under LOD
6	6	95	1	7.56	264.37	46.48	Under LOQ
7	3	75	2	4.78	365.61	32.94	Under LOQ
8	11	75	2	4.96	368.53	36.18	Under LOQ
9	12	75	2	4.58	393.34	35.70	Under LOQ
10	5	95	2	6.36	367.80	38.13	Under LOQ
11	1	95	2	5.69	374.20	38.92	Under LOQ
12	7	95	2	5.48	351.84	39.17	Under LOQ

The effect of the extraction conditions on the caffeine level (Y_3) can be illustrated by the following equation:

$$Y_3 = 3.974 + 2.92(A) - 2.90(B)$$

The statistical results showed that the adjusted coefficient of determination (Adj.R^2) generated was 0.7115 ($R^2 < 0.8$). Although the value of adjusted coefficient determination was less than 0.8, lack of fit p-value showed insignificant value (p-value > 0.05). Lack of fit illustrates the variation of the data around the fitted model. If the model is not best to fit the data, this will be significant. The relationship between equation model and observation data of caffeine levels is shown in Figure 2c. Variables A and B significantly contributed to the response of Y_3 (p-value < 0.05). The equation model showed that water temperature had a positive effect on the caffeine level. It indicates that with an increasing water temperature, caffeine level increases. Increased caffeine level at a higher temperature is caused by an increased solubility of caffeine. In contrast to the brewing number, it had a negative effect on the caffeine level, indicating that with an increasing brewing number, caffeine level decreases. It is due to the saturation of extraction.

The optimization process was carried out by determining the criteria of each response such as the maximum level of the extraction yield, maximum level on the EGCG and minimum level on the caffeine. The assessment was made upon consideration that EGCG is the main compound contained in the extract of green tea leaf that potentially has a biological activity, whereas caffeine is not a biomarker compound that will mask the biological activity of EGCG in this case. The extraction yield was a significant factor in the preparation of pharmaceutical dosage form and it was used as raw material. The optimum extraction conditions were to use water temperature at 95°C with two-times brewing obtaining these extraction yields, EGCG and caffeine level of 5.76%, 356.43 mg/g dry weight, and 38.76 mg/g dry weight, respectively. There was no significant difference (p-value of extraction yield, EGCG, caffeine level of 0.787; 0.167; 0.077, respectively) between the observation results and prediction values. The model predicted extraction yields, EGCG and caffeine levels of 5.84%, 370.39 mg/g dry weight, and 39.76 mg/g dry weight, respectively. The model obtained a desirability value of 0.67, illustrating that the model is close to the observation results. Desirability value ranged from 0 to 1, illustrating a relationship between the observation results and the model predicted. The effect of the optimum extraction conditions on the desirability value can be seen in Figure 3.

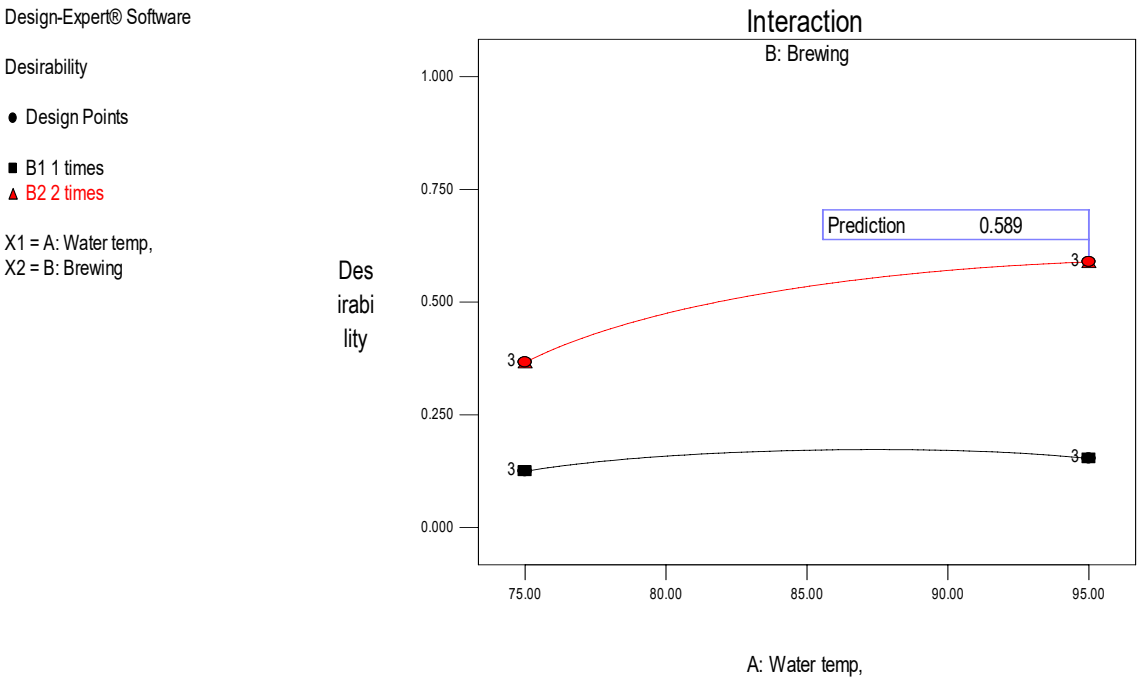

Fig. 3: The effect of optimum extraction conditions on the desirability value.

CONCLUSION

The factorial design has been used successfully to optimize the extraction condition of green tea leaves. Extraction process using water temperature at 95°C with two-times brewing is the optimum conditions to obtain the extraction yield, EGCG, and caffeine. Using this optimum condition, the concentrations of EGCG, caffeine and the extraction yield are of 356.43 mg/g dry weight, 38.76 mg/g dry weight, and 5.76%, respectively.

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CONFLICT OF INTEREST

The author declares there is no conflict of interest.

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