MULTIPLE RESPONSE OPTIMIZATION OF THE GREEN TEA DE-CAFFEINATED PROCESS FOR STIMULATING THE HUMAN ANTIBODY

V. Paramita1,2, M.E. Yulianto1, I. Hartati2, E. Yohana3, D. Rohdiana4, S. Shabri4, D. Ariwibowo1, T. Sutrisno1 and B. Wijayanto5

1Department of Technology Industry, Diponegoro University, 50275, Semarang, Indonesia
2Department of Chemical Engineering, Wahid Hasyim University, 50232, Semarang, Indonesia
3Department of Mechanical Engineering, Diponegoro University, 50275, Semarang, Indonesia
4Research Institute for Tea and Cinchona, 40010, Bandung, Indonesia
5Metal and Wood Industry Centre, 50111, Semarang, Indonesia

Corresponding Author: vparamita@live.undip.ac.id

ABSTRACT

The process of separating tea leaves caffeine, as well as the enzymes inactivating the polyphenol oxidase and hydroperoxidase were carried out through a dipping process. This study aimed to de-caffeine green tea extracts and focus on maximizing the catechin content and minimizing the caffeine and phenolic contents during the dipping stage. The result showed that the provided variable was 75.25 °C, 0.072 leave-to-water ratio, and 9.13 min of dipping time. The response of total phenolic, catechin and caffeine contents are 406.02 mg/g, 27.05 mg/ml, and 0.028 mg/ml, respectively. The highest catechin indicated the minimum polyphenol oxidase performed.

Keywords: Catechins, Caffeine, Phenolic, Multiple optimizations, De-caffeinated green tea.

INTRODUCTION

Green tea polyphenols are bioactive compounds consisting of catechin, epicatechin, epigallocatechin, epicatechin-gallate, epigallocatechin-gallate, and gallic acid, which have been shown to have anti-cancer activity, able to prevent cardiovascular disease, obesity, and other degenerative diseases.1–4 An in silico study analyzed the primary protease enzyme inhibitor (3CLPro) on the SARS-CoV-2 virus that causes COVID-19 using drug design techniques.5 Liu et al stated that caffeine, (–)-epigallocatechin-gallate, epicatechin, theophylline, catechin, epicatechin-gallate, and epigallocatechin can inhibit the activity of 3C-like protease (3CLPro) which is active in the SARS-CoV-2 virus so that it can stop the replication of the SARS-CoV-2 virus in infected host cells.6 3C-like protease (3CLPro) has proteolytic activity with a cysteine thiol group acting as a nucleophile at the catalytic 3CLPro active sites C145 and H41 dyad.7 The best natural product capable of maintaining unique contact with C145 and H41 in G and d dyad were the aflavins (ΔG = -9.16 kcal/mol, d dyad = 3.75 Å) and epigallocatechin-gallate (EGCG; ΔG = -8.28 kcal/mol, d dyad = 3.50 Å). The smaller the d dyad the higher its performance with respect to the 3CLPro crystal structure, thereby forming a direct interaction between the active ingredient and the catalytic dyad distances.8,9 EGCG is effective in countering allyl peroxyl radicals, which are carried out 10 times more than vitamin C and β-carotene.10,11 Therefore, it is necessary to produce low-caffeine and high-catechin green tea through enzymatic activation using the dipping process for health and preventing diseases caused by SARS-CoV-2 virus infection. One of the pharmacological activities that support the incorporation of food industry products is the incorporation of caffeine-free green tea with the dipping process and the application of green solvents that are beneficial to health.12–14 Green tea caffeine separation can be carried out with low dipping temperature for inactivation of enzymes other than polyphenol oxidase and hydro-peroxidase. This study aimed to remove caffeine from green tea extract, and maximize the catechin and phenolic content during the dipping...
process by investigating the effect of leaves to water ratio, the hot water temperature of the dipping process, time and temperature of cold-water dipping on the major chemical (catechin, caffeine, and phenolic content) and physical (viscosity and density) properties of green tea extract.

EXPERIMENTAL

Materials
The green tea leave was obtained from PT. Rumpun Sari Medini (Ungaran, Indonesia). The chemicals were Folin–Ciocălteu reagent (FCR), carbon tetrachloride (CCl_4), chloroform (CHCl_3), and sodium carbonate (Na_2CO_3). These chemicals were purchased from Merck and Co., Inc. (New Jersey, US). Gallic acid was obtained from Sigma-Aldrich Fine Chemicals (Missouri, US).

General Procedure
Five hundred grams of fresh green tea leave were weighed and blanched in accordance with the designed variables, namely temperature, leave-to-water ratio, time, and conditions, such as varied hot water, followed by cold water immersing at 30.0±2.0°C. Response Surface Method design was used to obtain the optimal tea extraction. Furthermore, the dipping of fresh tea in the hot fluid was followed by direct immersion in cold water (room temperature).

Determination of Caffeine Content
The caffeine content was analyzed using the UV-Vis spectrophotometer (GENESYS™ 10 Series, Massachusetts, US) at 270 nm of the absorbance in a 10 mm quartz cuvette. A total of 5 ml parts of the sample was placed in a 125 ml separation funnel followed by the addition of 10 ml distilled water, 1 ml of 20% sodium carbonate (Na_2CO_3) solution, and 20 ml of carbon tetrachloride (CCl_4) pro analytic. Caffeine extraction is carried out by turning the separating funnel upside down and repeating the process three times. Subsequently, during the initial extraction, the non-polar CCl_4 layer formed was transferred to a 50 ml clean volumetric flask. Furthermore, 20 ml of the new CCl_4 was added to the inner layer of the separating funnel. The extraction procedure was repeated twice till the volume reached 50 ml of the solvent.

Determination of Total Catechin Content
Approximately 40 ml of the extracted tea leave were placed in the 125 ml separating funnel according to the previously mentioned variables. Furthermore, washing is carried out using an additional 40 ml of chloroform to remove caffeine, pigments, and other non-polar impurities. This technique was repeated four times, and the absorbance of the extract was obtained at a wavelength of 274 nm by double scanning using UV-Vis Spectrophotometry (GENESYS™ 10 Series, Massachusetts, US).

Determination of Total Phenol Content
The total phenolic content was determined by using gallic acid as the standard. A total of 1 ml of the extracted sample and standard gallic acid (100 µg/ml) were placed in a test tube. Five milliliters of distilled water and 0.5 ml of FCR were added and mixed properly for 5 min, followed by the addition of 1.5 ml of 20% sodium carbonate (Na_2CO_3) and distilled water until a total volume of 10 ml is achieved. The solution was incubated for 2 hours at room temperature, and the color turned dark blue. Subsequently, the absorbance was measured by using a UV-Vis Spectrophotometer (GENESYS™ 10 Series, Massachusetts, US) with a wavelength of 750 nm. The total phenol content data are expressed as mg gallic acid equivalent weight (GAE)/100 g dry mass.

Determination of Physical Characteristics of Extracted-Blanched Fresh Tea
Density determination was carried out based on calculating data, specifically:

\[ \rho_{tea} = \frac{m_b - m_a}{v_c} \]  \hspace{1cm} (1)

Where, \( \rho_{tea} \) = density of tea extract (g/ml), \( m_a \) = mass of empty pycnometer (g), \( m_b \) = mass of filled pycnometer (g), \( v_c \) = volume of pycnometer (ml). The viscosity values were determined by the relationship between the flowing time and the density, specifically:

\[ \frac{\eta_1}{\eta_2} = \frac{\rho_1 t_1}{\rho_2 t_2} \]  \hspace{1cm} (2)
Where, $\eta_1$ = the viscosity value of the extract sample (poise), $\eta_2$ = the viscosity value of distilled water (poise), $\rho_1$ = the density of the extract sample (g/ml), $\rho_2$ = the density of the distilled water sample (g/ml), $t_1$ = the sample extract flowing time (s), $t_2$ = distilled water sample flowing time (s).

**Multiple Response Surface Methodology**

The multiple response surface experiments are designed by applying the Central Composite Design of the alpha for orthogonality (Minitab 19 Statistical Software, Pennsylvania, US). The independent variables of the dipping process of green tea were temperature ($X_1$), leave-to-water ratio ($X_2$), and dipping time ($X_3$) considering the hot and cold water in conditions 1 and 2, respectively. Each optimized variable was coded at five levels, namely - $\alpha$, -1, 0, +1, and + $\alpha$, with the range of decaffeinated green tea, shown in Table-1. The multiple responses obtained were caffeine content (TF), total catechin content (TC), and total phenolic content (PC).

### Table-1: Central Composite Design for Optimization of Decaffeinated Green Tea

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Coded Variables Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot Water Temperature (°C)</td>
<td>-$\alpha$</td>
</tr>
<tr>
<td>Leave-to-water ratio (-)</td>
<td>0.0200</td>
</tr>
<tr>
<td>Time (min)</td>
<td>2.6</td>
</tr>
</tbody>
</table>

**RESULTS AND DISCUSSION**

**Model Fitting Parameters of Major Chemical Properties of Extracted-Blanched Fresh Tea**

### Table-2: Alpha for Orthogonality of Central Composite Design Matrix and Observed Responses

<table>
<thead>
<tr>
<th>Run</th>
<th>Experimental design matrix</th>
<th>Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blocks</td>
<td>$X_1$</td>
</tr>
<tr>
<td></td>
<td>Condition (-)</td>
<td>Dipping temperature (°C)</td>
</tr>
<tr>
<td>1</td>
<td>Hot Water</td>
<td>80.00</td>
</tr>
<tr>
<td>2</td>
<td>Hot Water</td>
<td>80.00</td>
</tr>
<tr>
<td>3</td>
<td>Hot Water</td>
<td>80.00</td>
</tr>
<tr>
<td>4</td>
<td>Hot Water</td>
<td>80.00</td>
</tr>
<tr>
<td>5</td>
<td>Hot Water</td>
<td>95.00</td>
</tr>
<tr>
<td>6</td>
<td>Hot Water</td>
<td>95.00</td>
</tr>
<tr>
<td>7</td>
<td>Hot Water</td>
<td>95.00</td>
</tr>
<tr>
<td>8</td>
<td>Hot Water</td>
<td>95.00</td>
</tr>
<tr>
<td>9</td>
<td>Hot Water</td>
<td>87.50</td>
</tr>
<tr>
<td>10</td>
<td>Cold Water</td>
<td>74.90</td>
</tr>
<tr>
<td>11</td>
<td>Cold Water</td>
<td>100.1</td>
</tr>
<tr>
<td>12</td>
<td>Cold Water</td>
<td>87.50</td>
</tr>
<tr>
<td>13</td>
<td>Cold Water</td>
<td>87.50</td>
</tr>
<tr>
<td>14</td>
<td>Cold Water</td>
<td>87.50</td>
</tr>
<tr>
<td>15</td>
<td>Cold Water</td>
<td>87.50</td>
</tr>
<tr>
<td>16</td>
<td>Cold Water</td>
<td>87.50</td>
</tr>
</tbody>
</table>
Running parameters were carried out through 16 experiments using independent variables of leave-to-water ratio, dipping temperature, and time by dipping the sample in hot and cold water sequentially (Table-2). The hot water dipping provides certain benefits, such as the inactivation of enzyme polyphenol oxidase and the inhibition of catechin aerobic oxidation catalysis. The subsequent immersing of cold water is aimed at prohibiting further heating, which also leads to catechin epimerization due to thermal degradation.

The physical characteristics data lacked significant differences, which ranged from 915 to 993 mg/ml for density and 0.786 to 1.025 mPa·s for viscosity. The experimental results show that the cold water (30.0±2.0°C) dipped poorly and affected the discharge of caffeine and total catechin contents, as well as the release of total phenolic content. Furthermore, to conceive the relevance between the response and experimental value of the independent variables for the dipping condition, a three-dimensional surface plot was developed according to the quadratic polynomial model equation, as shown in Table-4.

### Parameters Effect on Total Catechins, Caffeine, and Phenolic Contents

Several studies on the optimization of biomolecule component extraction of catechin, caffeine, and phenolic contents were carried out by applying the response surface methodology (RSM). The variance analysis of caffeine, total catechin, and phenolic contents versus blocks regarding temperature, leave-to-water, and time are shown in Table-3. The degree of freedom (DF) showed that linear, square, and 2-way interaction of sources provides 3 variables of freedom. The adjusted sum of the squares for a term quantifies the amount of variation in the response data, and this showed that caffeine provides the minimum while total phenolic content offers the maximum. The response data is explained by each term in the model. The least p-value, which was obtained as 0.006 for the total catechin, is determined by the hot water (determined as block 1) during the dipping process. It shows that hot water offered a significant effect on the total catechin content during the de-caffeinated process. The most influential independent variable in the caffeine content is the dipping temperature (0.086 of p-value), while the total catechins and phenolic contents were mostly affected by leave-to-water ratios with p-values of 0.052 and 0.128, respectively. The primary effects plot displays the means of responding to each variable. The coefficient of determination, R-square, was obtained at 0.8798, 0.7835 and 0.6505 for each content of total catechin, caffeine, and total phenolic, respectively. These values show the variability of the response described by the model. Table-4 shows the responses of the quadratic polynomial model equation.
Table-4: Response of the Quadratic Polynomial Model Equations

<table>
<thead>
<tr>
<th>Response</th>
<th>Quadratic Polynomial Model Equations</th>
<th>R-square (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffein content (TF)</td>
<td>-0.41 + 0.0077 X₁ - 2.57 X₂ + 0.0191 X₃ - 0.000013 X₁² + 36.2 X₂² - 0.00028 X₃² - 0.0247 X₁X₂ - 0.000213 X₂X₃ + 0.096 X₁X₃</td>
<td>78.35</td>
</tr>
<tr>
<td>Total catechin content (TC)</td>
<td>-285 + 5.28 X₁ - 67 X₂ + 15.0 X₃ - 0.0199 X₁² + 13.916 X₂² - 0.531 X₃² - 14.0 X₁X₂ - 0.106 X₁X₃ + 25.1 X₂X₃</td>
<td>87.98</td>
</tr>
<tr>
<td>Total phenolic content (PC)</td>
<td>-8031 + 193 X₁ - 37270 X₂ + 72 X₃ - 0.99 X₁² + 966646 X₂² - 17.6 X₃² - 478 X₁X₂ + 2.1 X₁X₃ - 1298 X₂X₃</td>
<td>65.05</td>
</tr>
</tbody>
</table>

The variables and interactions’ effect on response is shown in Fig.-1. Subsequently, when the dipping time was set at 6 min, there was a slight increase in the caffeine content and dipping temperature from 75 °C to 100 °C, thereby reaching its minimum value. The least temperature in the leave-to-water ratio remains 0.07 (Fig.-1a). Figure-1b shows the effect of the interaction between the dipping temperature and time on caffeine content at a fixed leave-to-water ratio (0.5335). The minimum caffeine content was obtained at the least dipping temperature, and this caused a slight increase of 0.08 mg/ml in the dipping temperature at a dipping time of 9.1 minutes. Figure-1c presents the interaction between the leave-to-water ratio and the dipping time on the caffeine content at a dipping temperature of 87.5 °C. The minimum caffeine content was obtained at the least leave-to-water ratio, which slightly increased at a dipping time of 2.7 minutes.

The 3D image shows the dipping temperature and the leave-to-water ratio interactive effect on the total catechin content at a dipping time of 6 min. The maximum total catechin content was obtained at the least dipping temperature and decreased at the leave-to-water ratio of 0.08 (Fig.-1d). Figure-1e shows the dipping temperature and dipping time, including the interactive effect between the total catechin content, and leave-to-water ratio, which remains at the level of 0.5335. The maximum total catechin content was also obtained.
at the minimum dipping temperature, decreasing dipping time at a dipping temperature of 75 °C. Figure-1f shows the leave-to-water ratio and dipping time, including its interactive effect on the total catechin content at a dipping temperature of 87.5 °C. The maximum total catechin content was obtained at the maximum leave-to-water ratio and decreased with the decrease in the leave-to-water ratio at a dipping time of 9.1 minutes. It was discovered that the dipping conditions in hot water (Block 1) were the most significant factor affecting the response at a level of p-value <0.05 for caffeine and total catechin levels (Table-3). Figure-1g shows that the dipping temperature's interactive effect and the leave-to-water ratio on the total phenolic content were at a fixed dipping time of 6 min. The minimum total phenolic content was obtained at the least dipping temperature, which reached a maximum value of 82 °C while the leave-to-water ratio remained at 0.08. Figure-1h shows the interactive effect between the dipping temperature and dipping time on the total phenolic content at the leave-to-water ratio at a level of 0.5335. The minimum total phenolic content was also obtained at the least dipping temperature, which reached a maximum value of 92 °C at a dipping time of 9.1 minutes. Figure-1i shows the interactive effect of the leave-to-water ratio and dipping time on the total phenolic content at a dipping temperature of 87.5 °C. The minimum total phenolic content was obtained at the least leave-to-water ratio (0.02), the maximum value was reached at the highest leave-to-water ratio of 0.08 and the dipping time was 9.1 minutes. The research showed that the higher the temperature, the greater the caffeine content in the green tea extract. This means that increasing the temperature to 95 °C leads to a decrease in the total catechins content. Furthermore, the temperature was increased to over 90 °C, which led to a decrease in the total phenolic content.

Response to Optimization of Phenolic, Catechins, and Caffeine Contents

The optimization response of green tea's main components, namely phenolic, total catechin, and caffeine contents, was determined. The observation encompasses the minimum and maximum values of phenolic, caffeine, including total catechin contents. Fig.-2 shows the prediction of the multiple responses, it was discovered that the setting variable was 75 °C, 0.072 of leave-to-water ratio, and 9.13 min of dipping time. The fit solution was 406.011 mg/g of total phenolic content, 27.0454 mg/ml of total catechin content, and 0.0279631 of caffeine content. It predicted the multiple responses on the minimum and maximum values of total phenolic and catechin contents as well as caffeine. The composite desirability of the minimum and maximum values of the total phenolic and catechin content, including caffeine, was discovered at 0.784889.

**Fig.-2: Multiple Response Prediction on A Minimum Value of Total Phenolic and Caffeine Contents Regarding Maximum Value of Total Catechin Content.** D = Composite Desirability; d = Individual Desirability; PI = Prediction Interval; PC = Total Phenolic Content; TC = Total Catechin Content; FC = Caffeine Content
The individual values of desirability were obtained at 0.89828, 0.70648, and 0.76193 in accordance with the fit at 406.01 mg/g, 27.05 mg/ml, and 0.028 mg/ml for total phenolic, catechin, and caffeine contents, respectively. The highest catechin obtained indicated the minimum polyphenol oxidase affected.\textsuperscript{12,34,35} This was supported by the previous result on EGCG in obtaining catechin, which provides low $d_{dyad}$ capable of maintaining unique contacts with H41 and C145.\textsuperscript{8,9} There is no conflict of interest of all data performed.

**CONCLUSION**

In conclusion, the proposed model presented an adequate prediction of the dipping process of decaffeinated green tea containing 0.8983, 0.7065, and 0.7619 of total phenolic, catechin, and caffeine, respectively. The process provided the setting variable of 75.25 $^\circ$C for temperature, 0.072 for leave-to-water ratio and 9.13 min for dipping time. Furthermore, the total phenolic, catechin, and caffeine contents are 406.02 mg/g, 27.05 mg/ml, and 0.028 mg/ml. The highest catechin obtained indicated the minimum polyphenol oxidase affected.

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