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

V. Paramita[1,✉], M.E. Yulianto[1], I. Hartati[2], E. Yohana[3], D. Rohdiana[4], S. Shabri[4], D. Ariwibowo[1], T. Sutrisno[1] and B. Wijayanto[5]

[1]Department of Technology Industry, Diponegoro University, 50275, Semarang, Indonesia
[2]Department of Chemical Engineering, Wahid Hasyim University, 50232, Semarang, Indonesia
[3]Department of Mechanical Engineering, Diponegoro University, 50275, Semarang, Indonesia
[4]Research Institute for Tea and Cinchona, 40010, Bandung, Indonesia
[5]Metal 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 hydro peroxidase 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.

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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 ($3CL^{Pro}$) 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 ($3CL^{Pro}$) 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 ($3CL^{Pro}$) has proteolytic activity with a cysteine thiol group acting as a nucleophile at the catalytic $3CL^{Pro}$ 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 $3CL^{Pro}$ crystal structure, thereby forming a direct interaction between the active ingredient and the catalytic dyad distances.[8,9] EGCG is effective in counteracting 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–Ciocâlteu 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.[15]

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).[10]

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.[16,17]

Determination of Physical Characteristics of Extracted-Blanched Fresh Tea

Density determination was carried out based on calculating data[18], specifically:

$$\rho_{tea} = \frac{m_b - m_a}{v_c} \quad (1)$$

Where, ρ_{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[19] were determined by the relationship between the flowing time and the density, specifically:

$$\frac{\eta_1}{\eta_2} = \frac{\rho_1 . t_1}{\rho_2 . t_2} \quad (2)$$

Where, η_1 = the viscosity value of the extract sample (poise), η_2 = the viscosity value of distilled water (poise), ρ_1 = the density of the extract sample (g/ml), ρ_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 - α, -1, 0, +1, and + α, 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

Independent Variables	Coded Variables Levels				
	-α	-1	0	+1	+α
Hot Water Temperature (°C)	74.90	80.00	87.70	95.00	100.1
Leave-to-water ratio (-)	0.0200	0.0357	0.0500	0.0710	0.3570
Time (min)	2.6	4.0	6.0	8.0	9.3

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

Run	Experimental design matrix				Responses		
	Blocks	X_1	X_2	X_3	Y_1	Y_2	Y_3
	Condition (-)	Dipping temperature (°C)	Leave-to-water ratio (gr/ml)	Dipping time (min)	Caffeine content (mg/ml)	Total catechin content (mg/ml)	Total phenolic content (mg gallic acid equivalent/g dry material)
1	Hot Water	80.00	0.0357	4.0	0.027	17.62	180.30
2	Hot Water	80.00	0.0357	8.0	0.034	20.69	194.55
3	Hot Water	80.00	0.0710	4.0	0.031	23.02	476.81
4	Hot Water	80.00	0.0710	8.0	0.064	33.91	178.70
5	Hot Water	95.00	0.0357	4.0	0.106	33.73	321.81
6	Hot Water	95.00	0.0357	8.0	0.113	34.69	335.40
7	Hot Water	95.00	0.0710	4.0	0.109	35.96	236.34
8	Hot Water	95.00	0.0710	8.0	0.117	36.20	195.77
9	Hot Water	87.50	0.0500	6.0	0.067	30.95	216.62
10	Cold Water	74.90	0.0500	6.0	0.003	7.03	176.62
11	Cold Water	100.1	0.0500	6.0	0.001	3.84	576.59
12	Cold Water	87.50	0.0200	6.0	0.068	2.34	170.58
13	Cold Water	87.50	0.3570	6.0	0.000	37.63	2485.07
14	Cold Water	87.50	0.0500	2.6	0.000	1.58	410.30
15	Cold Water	87.50	0.0500	9.3	0.002	3.95	264.83
16	Cold Water	87.50	0.0500	6.0	0.002	5.43	376.62

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[20] and the inhibition of catechin aerobic oxidation catalysis.[21] The subsequent immersing of cold water is aimed at prohibiting further heating, which also leads to catechin epimerization[22] due to thermal degradation.[23,24]

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.[25,26] 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,[27,28] caffeine,[29,30] and phenolic contents[31,32] 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-3: Analysis of Variance Results for Caffeine, Total Catechin, and Phenolic Contents versus Temperature and Time Dipping, as Well as Leave-To-Water

Source	DF	Caffeine content		Total catechin content		Total phenolic content	
		Adj SS	P-Value	Adj SS	P-Value	Adj SS	P-Value
Model	10	0.024498	0.266	2733.06	0.083	3088371	0.571
Blocks	1	0.014710	0.022	1617.34	0.006	605175	0.235
Linear	3	0.006790	0.287	627.79	0.148	1163692	0.409
Temp	1	0.006157	0.086	120.87	0.259	38032	0.749
L/W	1	0.000370	0.624	479.72	0.052	1103104	0.128
Time	1	0.000263	0.678	27.19	0.573	22556	0.805
Square	3	0.001549	0.771	345.46	0.313	1306126	0.368
Temp ×Temp	1	0.000004	0.957	10.68	0.721	26212	0.790
L/W × L/W	1	0.001078	0.413	159.52	0.204	769656	0.188
Time ×Time	1	0.000011	0.932	38.22	0.506	41841	0.737
2-Way Interaction	3	0.000258	0.977	54.27	0.864	57013	0.980
Temp × L/W	1	0.000085	0.812	27.64	0.570	31973	0.769
Temp × Time	1	0.000082	0.816	20.32	0.624	8249	0.881
L/W × Time	1	0.000091	0.806	6.30	0.783	16791	0.831
Error	5	0.006769		373.57		1659679	
Total	15	0.031266		3106.62		4748050	

DF = degree of freedom; Adj SS = adjusted sums of squares

Table-4: Response of the Quadratic Polynomial Model Equations

Response	Quadratic Polynomial Model Equations	R-square (%)
Caffein content (TF)	$-0.41 + 0.0077\ X_1 - 2.57\ X_2 + 0.0191\ X_3 - 0.000013\ X_1^2 + 36.2\ X_2^2 - 0.00028\ X_3^2 - 0.0247\ X_1X_2 - 0.000213\ X_1X_3 + 0.096\ X_2X_3$	78.35
Total catechin content (TC)	$-285 + 5.28\ X_1 - 67\ X_2 + 15.0\ X_3 - 0.0199\ X_1^2 + 13.916\ X_2^2 - 0.531\ X_3^2 - 14.0\ X_1X_2 - 0.106\ X_1X_3 + 25.1\ X_2X_3$	87.98
Total phenolic content (PC)	$-8031 + 193\ X_1 - 37270\ X_2 + 72\ X_3 - 0.99\ X_1^2 + 966646\ X_2^2 - 17.6\ X_3^2 - 478\ X_1X_2 + 2.1\ X_1X_3 - 1298\ X_2X_3$	65.05

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.

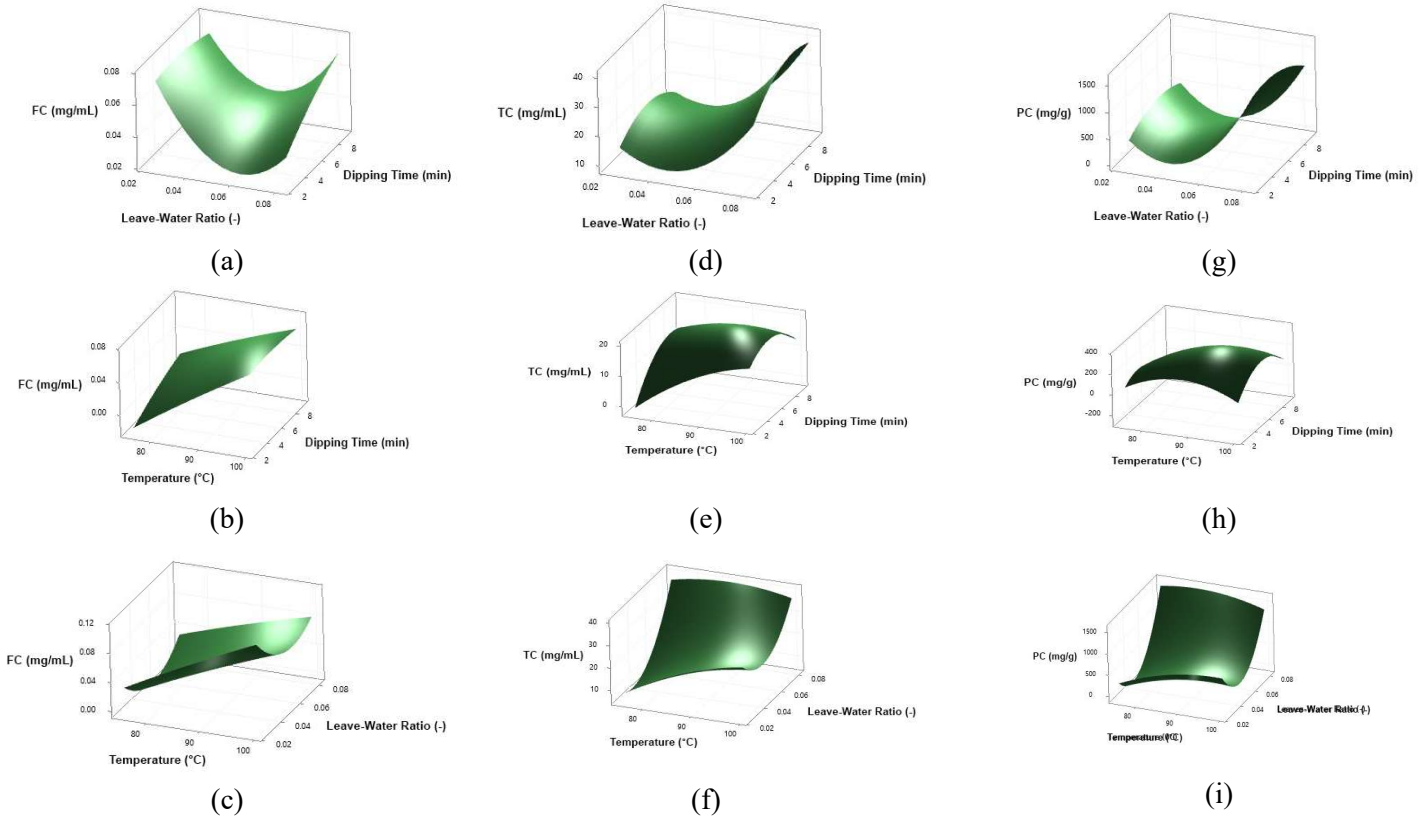

Fig.-1: Response Surface and Profile of Caffeine Content (a; b; c), Total Catechin Content (d; e; f), and Total Phenolic Content (g; h; i) Regarding Temperature-L/W Ratio at 6 min of Dipping Time (a; d; g), Temperature-Time at L/W Ratio 0.05335 (b; e; h), L/W Ratio-Time at 87.5 °C (c; f; i)

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.[33,34] This means that increasing the temperature to 95 °C leads to a decrease in the total catechins content. This led to the reason behind the thermal degradation and epimerization of catechin.[34,35] Furthermore, the temperature was increased to over 90 °C, which led to a decrease in the total phenolic content.[36]

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.

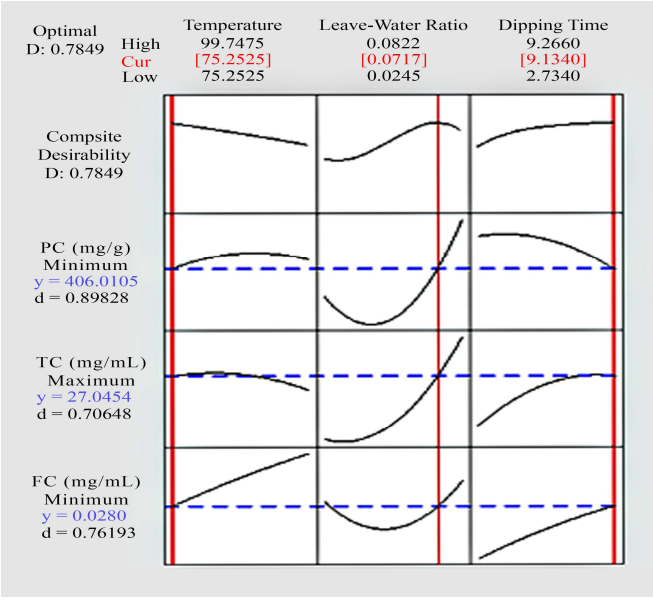

Fig.-2: Multiple Response Prediction on A Minimum Value of Total Phenolic and Caffeine Contents Regarding Maximum Value of Total Catechin Content. D = Composite Desirability; d = Individual Desirability; PI = Prediction Interval; PC = Total Phenolic Content; TC = Total Catechin Content; FC = Caffeine 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.[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.[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 °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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