OPTIMIZATION AND VALIDATION OF RP-HPLC/UV DETECTION FOR SEVERAL COMPOUNDS SIMULTANEOUSLY IN SEMI-PURIFIED EXTRACT OF WHITE TEA

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ABSTRACT

Modification of separation, detection, and quantification of several compounds simultaneously in white tea leaf extract using HPLC aims to obtain easy, fast, precise, and accurate analysis procedures. An HPLC optimization strategy was devised that allowed simultaneous determination of the effectiveness of separation and quantification of gallic acid, theobromine, theophylline, catechin, caffeine and epigallocatechin gallate. The compounds were separated and isocratically on a LiChrocart-C18 column. The optimal conditions for separation were determined using central composite design (CCD) with a solvent consisting of acetonitrile: methanol: 0.005% orthophosphoric acid (10:10:80 v/v/v) at a 0.90 mL/min flow rate. Validation procedures for modified analysis methods have been carried out following the terms and conditions. All validation points meet the requirements set by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use. The validation results were successfully applied to a white tea leaf extract. CCD is very effective for HPLC system optimization and was efficient for the simultaneous analysis of several compounds in white tea extract.

Keywords: Optimization, Central Composite Design, Validation, HPLC, White Tea, EGCG.

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INTRODUCTION

White tea is a product derived from the buds of *Camellia sinensis* L. leaves, this part which contains many polyphenol compounds. In Indonesia, white tea is widely produced on the island of Java and Sumatera. White tea leaves are processed quickly after harvest by a short drying period to avoid enzymatic oxidation of these compounds. The main polyphenols in these natural resources include epigallocatechin gallate (EGCG) and catechin (C) derivatives¹. Tea leaves also contain several purine alkaloid compounds², such as caffeine (CAF), theobromine (TB), and theophylline (TP). Phenolic and purine alkaloids in tea leaves have pharmacological effects^{3,4}, and extracts from white tea leaves have antioxidant⁵, antidiabetic^{6,7}, and neuroprotective⁸ activities. In vitro and in vivo test results indicate that both white tea and EGCG have good activity as oxidative stress inhibitors⁹.

General analytical methods have been widely reported for the quantitative analysis of phenolic, flavonoid, and alkaloids in tea samples. However, the spectroscopic techniques used previously do not allow simultaneous separation of each compound. Methods previously reported for the analysis of some compounds in tea leaves include high-performance thin layer chromatography¹⁰, liquid chromatography-mass spectrophotometry^{11,12}, high-performance liquid chromatography¹³, electrochemical detection¹⁴, ultrafast liquid chromatography^{15,16}, nuclear magnetic resonance spectroscopy¹⁰, and Fourier transform infrared spectroscopy^{17,18}. In general, chromatographic-based techniques are more appropriate for the analysis of phenolic and purine alkaloids as they also allow separation of each compound.

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Development of an effective and beneficial method for the simultaneous analysis of several compounds in white tea leaf extracts remains a research challenge. In the routine analysis, a cost-effective, simple, easy and fast method is required but provides precise and accurate results. Some of the reported chromatographic separations have several disadvantages and some reported methods provide incomplete separation of compounds of interest^{12,19}. Problems with C18 column separations include long run times, the chemical and structural complexities of phenolic and purine alkaloids, poor resolution of the compounds of interest, and the need for complex solvent mixtures for applications involving industrial extracts²⁰. The mobile phase composition and flow rate are the factors that determine the separation of compounds in the HPLC system²¹. Therefore, current research efforts are aimed at overcoming these shortcomings by optimizing the mobile phases and flow rates.

The design of experiments (DoE) approach is an application of simple statistics that can be used to obtain optimal HPLC conditions^{22,23}. Several studies have conducted relating to DoE for tea leaf analysis, but the use of the DoE approach has been limited to optimization of the tea leaf extraction process^{24,25}. One suitable design in the DoE approach for complicated process optimization is the central composite design (CCD). As far as our scientific knowledge, no study has yet examined the use of CCD to determine the optimal conditions of the HPLC system in simultaneous analysis of gallic acid (GA), TB, TP, CAF, C, and EGCG in a white tea extract. Determining the optimal condition of the HPLC system for the separation of six compounds using the CCD approach is one of the objectives of this study. An optimal separation was obtained using an isocratic system with a LiChrocart-C18 column and a solvent of acetonitrile, methanol, and 0.005% ortho-phosphoric acid at an optimum flow rate. To obtain accurate and precise separation methods, validation is required to be accepted by health authorities, industries, and laboratories.

EXPERIMENTAL

Chemicals and Samples

The standard epigallocatechin gallate, catechin, gallic acid, theophylline, and theobromine used as references acquired from Sigma-Aldrich (St. Louis, MO, USA). Caffeine and ortho-phosphoric acid were obtained from Merck (Darmstadt, Germany). The solvent acetonitrile and methanol (HPLC-grade) were achieved from Fisher Scientific (Loughborough, Leics, UK). All eluents were purified using a 0.45 µm membrane from Millipore (Bedford, USA). White tea product from *Camellia sinensis* L. acquired from the Research Institute of Tea and Cinchona (RITC) Gamboeng, Jawa Barat, and Mitra Kerinci, Sumatera Barat, Indonesia.

HPLC Instrumentation

The HPLC instrument Hitachi series 2000 (Hitachi, Japan) equipped with an injection valve pump L-2130 with a 20 µL loop volume was used. The system also included a detector of UV-Vis L-2420 (Hitachi, Japan) take action at 280 nm. The results of the separation, both qualitative and quantitative data were processed and displayed using D-2000 Elite software. The separation was carried out on a reversed-phase C18 column using LiChrocart-C18 (250 x 4.6 mm i.d.; 5 µm) with a guard column were acquired from Merck (Darmstadt, Germany).

Optimization Method Using CCD

A CCD was applied to optimize the analytical method development using HPLC. The factors (independent variables) studied for the analytical method development were: the ratio of acetonitrile (% X_1), methanol (% X_2) and flow rate (mL/min, X_3). Experimental design of analytical methods is presented in Table-1. Verification of the optimum conditions was evaluated by injecting a standard mixture on the HPLC system by six replications. The data obtained were tested statistically by comparing the optimum value of the predicted result using one sample t-test.

System Suitability Test

The mixed solutions of GA, TB, TP, CAF, C, and EGCG were prepared. The standard mixture was included in the HPLC instrumentation with six replications. The retention time (t_R), resolution (R_s), theoretical plates (N), tailing factor (T_f) and peak area are recorded and observed. The system suitability

test was measured by reference to precision parameters; the critical value in this test is based on percent relative standard deviation (RSD)²⁶. The % RSD values should not be more than 2%.

Table-1: Completely CCD and the HPLC Condition Experimental Results

Run	Factors on the System (X_n)			Response (Y_n)								
	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6	Y_7	Y_8	Y_9
1	9.00	9.00	1.00	18.74	4353	3650	2.37	6.38	1.76	7.95	1.51	547265
2	10.00	11.00	0.75	18.12	5097	3659	1.72	5.1	2.16	5.51	1.64	677763
3	9.00	13.00	1.00	13.06	3777	2976	1.68	4.02	2.52	4.36	1.6	647601
4	10.00	11.00	1.25	10.93	3806	3028	1.53	4.59	2.01	5.04	1.55	451152
5	9.00	9.00	0.50	31.43	5968	4613	2.11	7.03	1.83	8.02	1.68	1176023
6	8.00	7.00	1.25	25.57	4705	3809	3.43	8.38	1.27	10.64	1.47	379312
7	9.00	9.00	1.50	13.47	3629	2969	2.03	5.90	1.44	7.23	1.48	415548
8	10.00	7.00	0.75	26.51	5582	4228	2.44	8.07	1.17	9.37	1.62	671108
9	10.00	7.00	1.25	15.51	4311	3419	2.08	6.87	1.18	8.12	1.55	389237
10	9.00	9.00	1.00	19.16	4756	3686	2.42	6.49	1.95	7.83	1.62	548145
11	8.00	11.00	0.75	25.67	5278	3911	2.77	6.42	2.49	7.73	1.59	637998
12	9.00	5.00	1.00	31.50	4723	3958	3.29	6.59	0.64	8.33	1.58	555992
13	9.00	9.00	1.00	19.63	4410	3540	2.3	6.50	1.66	7.85	1.55	620200
14	7.00	9.00	1.00	31.14	4589	4120	3.85	7.88	1.90	10.54	1.39	477663
15	8.00	7.00	0.75	43.78	6064	4820	4.00	9.66	1.37	12.16	1.5	562638
16	9.00	9.00	1.00	18.81	4706	3721	2.43	6.57	1.86	7.99	1.52	546714
17	9.00	9.00	1.00	20.01	4373	3661	2.31	6.49	1.68	7.97	1.53	623882
18	9.00	9.00	1.00	19.32	4672	3535	2.38	6.65	1.78	7.9	1.59	570289
19	8.00	11.00	1.25	15.49	4061	3069	2.38	5.37	2.38	6.49	1.59	414440
20	11.00	9.00	1.00	13.40	4049	3237	1.41	5.04	1.71	5.42	1.62	604139

Selectivity Test

The white tea extract solution and the standard solution mixture prepared with GA, TB, TP, CAF, C, and EGCG concentrations were 2, 5, 5, 5, 10, and 25 $\mu\text{g/mL}$, respectively. White tea extract solution and standard solution mixture were analyzed by HPLC. The selectivity is good when the resolution value (R_s) ≥ 1.5 ²⁷.

Linearity and Sensitivity Test

The mixtures of GA, TB, TP, CAF, C, and EGCG solutions were prepared with seven different concentration levels. Measurements were carried out in each series of concentrations in the HPLC instrumentation that had been conditioned. Concentration data and peak obtained by calculation of intercept value (a), slope (b) and correlation coefficient (r^2). The analytical method is called good to have good linearity if the value of $r^2 \geq 0.999$ ²⁸. The standard curve parameters that include a, b, and r^2 were determined using the least square linear regression analysis with the help of Microsoft Excel software.

Limit of Detection (LoD) and Limit of Quantification (LoQ)

The LoD and LoQ parameters were evaluated based on the residual standard deviation of the regression line response (r) the measured catechin concentration series and slope of the calibration curve (S)²⁹. The LoD value follows the equation $3.3 r/S$, and LoQ is $10 r/S$.

Precision and Accuracy

The catechin standard was added to the sample (50 $\mu\text{g/mL}$) and the standard solution mixed was taken from three points on the standard curve, namely 4, 5, and 6 $\mu\text{g/mL}$. Analyses were performed in three replications. Precision analytical methods are eligible if RSD testing is nothing more than RSD Horwitz^{27,30}. The accuracy of the HPLC analysis method in this study used additional standard, by adding GA, TB, TP, CAF, C, and EGCG to the extract. The analytical method is eligible if the recovery of the determination result is in the range of acceptability.

Robustness

Robustness can be evaluated statistically from DoE²². The robustness parameter was assessed from the surface response, and all the responses from the factors on the CCD were 20 experiments³¹.

Analysis of White Tea Extract Sample

A number of purified white tea extract is weighed more or less 10 mg and put into a 10 mL flask. A total of approximately 10 mL of eluent was mixed using an ultrasonicator for 5 minutes. The sample was diluted with a mobile phase to produce an analyte mixture in a solvent of 75 µg/mL. The sample solution was purified with a 0.45 µm membrane for further analysis with HPLC. The validated calibration curve was used to calculate the content of GA, TB, TP, CAF, C, and EGCG in the sample. The final content of each compound was expressed in percent (%).

Statistical Analysis

The HPLC system optimization data were evaluated using Design-Expert software (Stat-Ease Inc, Minneapolis, USA). Statistical analysis was performed using analysis of variance (ANOVA). The data were statistically analyzed with the help SPSS software (SPSS Inc, Chicago, USA). The significance value in the data analysis determined $p < 0.05$.

RESULTS AND DISCUSSION

Optimization of HPLC Condition Using CCD Model Analysis

The CCD experimental design was performed using 20 experimental runs (Table-1). The independent variables were the acetonitrile (X_1), methanol (X_2), and the flow rate (X_3); the dependent variables were the total retention time (Y_1), the theoretical plates of C (Y_2), the theoretical plates of EGCG (Y_3), the resolution of GA–TB (Y_4), the resolution of TP–C (Y_5), the resolution of C–CAF (Y_6), the resolution of CAF–EGCG (Y_7), the tailing factor of EGCG (Y_8), and the peak area of EGCG (Y_9). An approach using DoE, the best model selection is based on several important criteria. The criteria include, the model is significant ($p < 0.05$), lack of fit has a non-significant value ($p > 0.05$), the coefficients of determination (R^2) should be above 0.7 and the difference in value between adjusted R^2 (Adj. R^2) and predicted R^2 (Pred. R^2) below 0.2^{32,33}.

The overall response (Y) provides a significant model. In theory, the lack of fit ideally gives not significant results. However, in the assessment of lack of fit, the difference from Adj. R^2 and Pred. R^2 is a special consideration. If the resulting difference is below 0.2 and R^2 above 0.7, the model obtained can be used to predict optimal conditions. Differences Predicted R^2 and Adjusted R^2 for all responses below 0.2 (Table-2). Adequate precision is used to determine the ratio of signals to noise. All model obtained have a ratio of more than 4. Based on statistical data obtained in Table-2, the overall equation model meets the requirements to be used in predicting optimal conditions.

The least squares regression is used in estimating the coefficient value on the model. A positive sign shows a favorable response. The inverse correlation between factors (X) and responses (Y) is indicated by a negative value. The model transformation was carried out at Y_1 and Y_9 to explain accurately strong interaction. The inverse model means that a high coefficient value reduces the main effect (interaction) and vice versa. The relative effect of factors is evaluated from the code equation by comparing the R^2 value. The fitting result equation model proposed for nine responses is presented in Table-3.

Factors X_1 , X_2 , and X_3 contributed significantly to the overall model equation with $p < 0.05$ (Y_1 , Y_2 , Y_3 , Y_4 , Y_5 , Y_6 , Y_7 , Y_8 , and Y_9). However, no significant status was found for the lack of fit parameters, namely in the Y_4 , Y_5 , and Y_7 responses. The actual and predicted response variable plot for the total retention time, the theoretical plates of EGCG, the resolution of C–CAF and the peak area of EGCG (Fig.-1) reveals the good closeness between the predicted and experimental results. Similarly, for response variables GA–TB, TP–C, and CAF–EGCG, although the value of lack of fit is significant. As observed, all of the actual response point variables are quite close to their predictive value, indicating that the model is strong and accurate.

It is clear from the equation that the factor of the acetonitrile, methanol and flow rate ratios have a positive effect on the total retention time factor. The 3D surface response contour plot is presented as

Fig.-2 which is very useful for studying the interaction effects of factors on the response. The relationship between the response factor and the independent factor is linear, whereas in case the Y_3 and Y_4 response factor are squared (Table-3). The ratio of acetonitrile, methanol and flow rate is optimized to obtain the separation of GA, TB, TP, CAF, C, and EGCG with good resolution results (Fig.-4a). These compounds were eluted at 3.68, 4.14, 5.70, 7.90, 8.91, and 13.18 minutes.

Table-2: Evaluation Parameters of the Response Equation Model From CCD

	Std. Dev.	Mean	%CV	Press	R^2	Adj. R^2	Pred. R^2	Adeq. Precision	Model	Lack of Fit
Y_1	0.0048	0.2255	2.14	0.0006	0.9874	0.9851	0.9791	68.5262	Significant	Not significant
Y_2	279.12	4645.45	6.01	219100000	0.8582	0.8316	0.7506	19.6593	Significant	Not significant
Y_3	107.34	3680.45	2.92	73510000	0.9766	0.9555	0.8506	23.7942	Significant	Not significant
Y_4	0.1553	2.45	6.35	1.87	0.9751	0.9526	0.8071	22.0631	Significant	Significant
Y_5	0.6474	6.50	9.96	12.87	0.8021	0.7650	0.6204	14.6015	Significant	Significant
Y_6	0.1177	1.74	6.77	0.3698	0.9476	0.9378	0.9126	37.0854	Significant	Not significant
Y_7	0.7982	7.82	10.20	19.61	0.8543	0.8270	0.7198	17.0672	Significant	Significant
Y_8	0.0462	1.56	2.97	0.0572	0.6138	0.5414	0.3541	9.2483	Significant	Not significant
Y_9	0.0002	0.002	10.20	0.0000	0.8410	0.8112	0.7328	19.6933	Significant	Not significant

Fig.-1: Actual Values and Predicted Results Calculated from the Polynomial Equation for the Total Retention Time (A), the Theoretical Plates of EGCG (B), the Resolution of C-CAF (C), and the Peak Area of EGCG (D)

Fig.-2: Response Surface Plots (3D) for the Total Retention Time (A), Theoretical Plates of Catechin (B), Theoretical Plates of EGCG (C), the Resolution of GA–TB (D), the Resolution of TP–C (E), the Resolution of C–CAF (F), the Resolution of CAF–EGCG (G), the Tailing Factor of EGCG (H), the Peak Area of EGCG (I) and Desirability (J)

Verification of Optimum Conditions and Suitability Test (SST)

The verification of optimum conditions and SST was performed before the validation process. Based on the one sample t-test, only the resolution of C–CAF with result $p < 0.05$ (0.000), others parameter $p > 0.05$. Although statistically unverified, the compounds C and CAF are well separated. Average value ($n=6$) of the resolution of C–CAF 1.83 (good resolution required more than 1.5). The total retention time ($n=6$) averaged 15.89 minutes from the optimum prediction of 15.90 minutes ($p > 0.05$). The resolution of GA–TB ($n=6$) averaged 1.74 of the 1.74 ($p > 0.05$) predictions. The theoretical plates of C ($n=6$) averaged 4702 from 4602 predictions ($p > 0.05$). The theoretical plates of EGCG ($n=6$) averaged 3458 of the predicted 3513 ($p > 0.05$). The tailings factor of EGCG ($n=6$) averaged 3458 of the 3513 predictions and the peak area of EGCG ($n=6$) averaged 23158 from 23742 predictions ($p > 0.05$). These results indicate the optimal conditions of HPLC systems acquired using DoE have been verified.

Tabel-3: Models and Regression Equations for All Responses

Response	Transform	Model	Regressed Equation
Y ₁	Inverse sqrt	Linear	$\frac{1}{\sqrt{Y_1}} = 0.23X_1 + 0.024X_2 + 0.026X_3$
Y ₂	None	Linear	$Y_2 = 4645.45 - 149.50X_1 - 269.50X_2 - 613.50X_3$
Y ₃	None	Quadratic	$Y_3 = 3650.48 - 190.06X_1 - 285.81X_2 - 411.31X_3 + 86.12X_1X_2 + 51.62X_1X_3 + 43.37X_2X_3 + 20.74X_1^2 - 32.14X_2^2 + 48.86X_3^2$
Y ₄	None	Quadratic	$Y_4 = 2.40 - 0.6056X_1 - 0.4231X_2 - 0.1044X_3 + 0.1263X_1X_2 + 0.0512X_1X_3 + 0.0437X_2X_3 + 0.0795X_1^2 + 0.0433X_2^2 + 0.0605X_3^2$
Y ₅	None	Linear	$Y_5 = 6.50 - 6.800X_1 - 1.04X_2 - 0.3937X_3$
Y ₆	None	Linear	$Y_6 = 1.74 - 0.0856X_1 + 0.4881X_2 - 0.0706X_3$
Y ₇	None	Linear	$Y_7 = 7.82 - 1.20X_1 - 1.47X_2 - 0.3787X_3$
Y ₈	None	Linear	$Y_8 = 1.56 + 0.0419X_1 + 0.0169X_2 - 0.0369X_3$
Y ₉	Inverse	Linear	$1/Y_8 = 1.847E-06 - 9.496E-08X_1 - 8.184E-08X_2 + 4.148E-07X_3$

The suitability test, RSD value is found to be less than 2% for all parameters tested (0.22–1.99; $n=6$), the system suitability test data shown in Table-4. The RSD value for all test variables is less than 2% both for retention time, theoretical plates, resolution, tailing factor and peak area. The data obtained indicate that the HPLC system applied for the separation of GA, TB, TP, CAF, C, and EGCG is appropriate.

Table-4: System Suitability Test ($n=6$; *value of % RSD)

Compounds	Retention time/ RT (min)	Theoretical plates	Resolution	Tailing factor	Peak area (mAU)
Gallic acid	3.68 ± 0.01; 0.22*	3482 ± 58; 1.67*	-	NA	120502 ± 2236; 1.86*
Theobromine	4.14 ± 0.01; 0.29*	3950 ± 26; 0.67*	1.78 ± 0.02; 0.87*	1.59 ± 0.02; 1.54*	329006 ± 5970; 1.81*
Theophylline	5.70 ± 0.02; 0.29*	4404 ± 35; 0.78*	5.14 ± 0.01; 0.29*	1.65 ± 0.02; 1.13*	328797 ± 5989; 1.82*
Catechin	7.90 ± 0.04; 0.47*	4330 ± 86; 1.99*	5.36 ± 0.05; 0.85*	1.51 ± 0.03; 1.66*	225117 ± 1478; 0.66*
Caffeine	8.91 ± 0.03; 0.33*	4464 ± 44; 0.99*	2.00 ± 0.02; 0.86*	1.63 ± 0.02; 1.31*	291510 ± 5362; 1.84*
EGCG	13.18 ± 0.11; 0.82*	3279 ± 63; 1.93*	5.88 ± 0.05; 0.78*	1.66 ± 0.01; 0.62*	686148 ± 4322; 0.63*

Validation of RP-HPLC/UV Separation and Detection Method

The method was selected because the resolution parameter has met the minimum requirements (Table-4). The value of $R_s \geq 1.5$ indicates the six compounds (GA, TB, TP, CAF, C, and EGCG) separate well and are supported by a chromatogram profile (Fig.-4a). The chromatogram profile shows no overlap at the peak between compounds. The variable used as one of the indicators for evaluating the standard curve equation is r^2 . The six equations obtained have a value of $r^2 > 0.999$ and show a good correlation between curves with the concentration of standard compounds (Table-5 and Fig.-3). The LoD found was 0.13, 0.18, 0.26, 0.17, 0.61, and 0.75 µg/mL, whereas, the LoQ was 0.40, 0.54, 0.79, 0.50, 1.82, and 2.24 µg/mL for GA, TB, TP, CAF, C, and EGCG, respectively.

Table-5: Validation Parameters on the Method of Separating Several Compounds Simultaneously Using RP-HPLC

Active Ingredient	Concentration Range (µg/mL)	Calibration Curve Regression	Correlation Coefficient (r^2)	LoD (µg/mL)	LoQ (µg/mL)
Gallic acid	0.11-9.68	$y = 64280x - 483.57$	0.9999	0.11	0.34
Theobromine	0.10-9.15	$y = 66405x + 111.68$	0.9998	0.16	0.47
Theophylline	0.01-9.49	$y = 60918x - 2079.7$	0.9996	0.26	0.79
Catechin	0.10-9.05	$y = 15911x - 1557.6$	0.9998	0.16	0.49
Caffeine	1.02-17.13	$y = 63648x - 25303$	0.9993	0.59	1.78
EGCG	0.99-36.22	$y = 26755x - 16302$	0.9998	0.56	1.69

A precision and accuracy study was also carried out; the percent of RSD and recovery are presented in Table-6. The RSD ($n=3$) for GA, TB, TP, C, CAF, and EGCG were within the range of 0.36–3.74 %. The results of the separation of the six compounds show a high value of precision and good accuracy of the analysis procedures developed. Percent recovery ($n=3$) for GA, TB, TP, C, CAF, and EGCG were in the range of 93.54 ± 2.30 – 101.99 ± 0.36 (mean $\pm$ SD). Robustness evaluation is related to the resolution of as the SST, and in this study, the value of R_s must be greater than 1.5. Therefore, acetonitrile and methanol ratios should be maintained at 10% and the flow rate of 0.90 mL/min to meet the SST criteria. Robustness can be evaluated from the predicted normal operation (factors) and the desirability value of the response according to the design. However, the analysis procedure with the developed system has a good response, namely, acetonitrile 10%, methanol $10 \pm 0.15\%$ and flow rate 0.90 ± 0.02 mL/min, according to the optimal conditions in the design space of the method.

Fig.-2: The Calibration Curve of Each Standard, Gallic Acid (A), Theobromine (B), Theophylline (C), Catechin (D), Caffeine (E), and Epigallocatechin Gallate (F)

Assay of GA, TB, TP, CAF, C, and EGCG in White Tea Extract

The separation profile of the active compounds in white tea extract samples, as presented in Fig.-4, which indicate good separation and detection. Table-7 shows the results of GA, TB, TP, CAF, C, and EGCG analysis data on samples of white tea extract obtained from several regions in Indonesia. The highest EGCG compound was found in sample 1 with a percentage of $41.27 \pm 0.60\%$. The percentages of CAF in samples 1, 2, and 3 were $16.80 \pm 0.49\%$, $18.38 \pm 0.29\%$, and $14.93 \pm 0.26\%$, respectively. During the analysis of compound C on sample 2 was not detected.

The chemical structures of GA, TB, TP, CAF, C, and EGCG include chromophores consisting of aromatic rings with conjugate and Auxo chrome systems³⁴. Therefore, some of these compounds can absorb electromagnetic radiation in the UV region at wavelengths of 210 and 280 nm. However, a chromatogram with detection set at a wavelength of 210 nm will have interference due to noise from UV absorption by organic solvents, such as methanol and acetonitrile^{12,19}. Therefore, a 280 nm wavelength was chosen in this study to detect several compounds in chromatographic separation from a sample of white tea leaf extract.

Differences in process parameters (i.e., the ratio of acetonitrile and methanol, the flow rate, etc.) lead to differences in the effectiveness and efficiency of HPLC methods.^{22,30,35} Therefore, in the present study, CCD was employed to estimate the effects of solvent composition and flow rate on chromatographic separation to obtain an optimal analytical HPLC procedure for alkaloids and phenolics in a white tea leaf extract.

Table-6: Precision and Accuracy of the HPLC-UV Separation and Detection Method

Compound	Value ($\mu\text{g/mL}$)	Intra-Day ($n=3$)			Inter-Day ($n=3$)		
		Mean $\pm$ SD ($\mu\text{g/mL}$)	Precision, RSD (%)	Accuracy $\pm$ SD (%)	Mean $\pm$ SD ($\mu\text{g/mL}$)	Precision, RSD (%)	Accuracy $\pm$ SD (%)
Gallic acid	4	4.33 $\pm$ 0.01	1.88	97.89 $\pm$ 1.84	4.27 $\pm$ 0.001	0.18	97.08 $\pm$ 0.17
	5	5.13 $\pm$ 0.01	1.36	98.34 $\pm$ 1.34	5.18 $\pm$ 0.003	0.49	97.91 $\pm$ 0.48
	6	6.07 $\pm$ 0.003	0.59	98.64 $\pm$ 0.58	6.07 $\pm$ 0.01	1.13	98.66 $\pm$ 1.11
Theobromine	4	3.86 $\pm$ 0.003	1.83	98.95 $\pm$ 1.81	3.85 $\pm$ 0.003	1.75	99.78 $\pm$ 1.74
	5	4.82 $\pm$ 0.002	1.41	98.76 $\pm$ 1.39	4.76 $\pm$ 0.002	1.40	98.30 $\pm$ 1.38
	6	5.90 $\pm$ 0.001	0.80	96.82 $\pm$ 0.78	5.87 $\pm$ 0.003	1.95	97.53 $\pm$ 1.90
Theophylline	4	3.82 $\pm$ 0.001	1.72	97.44 $\pm$ 1.67	3.80 $\pm$ 0.001	1.28	97.62 $\pm$ 1.25
	5	4.65 $\pm$ 0.001	1.44	98.41 $\pm$ 1.42	4.91 $\pm$ 0.001	1.37	96.12 $\pm$ 1.32
	6	5.75 $\pm$ 0.001	1.30	95.19 $\pm$ 1.23	5.70 $\pm$ 0.001	1.42	94.67 $\pm$ 1.34
Catechin	4	3.92 $\pm$ 0.002	0.71	96.54 $\pm$ 0.69	3.88 $\pm$ 0.003	1.22	96.91 $\pm$ 1.18
	5	4.66 $\pm$ 0.003	1.07	97.01 $\pm$ 1.04	4.75 $\pm$ 0.003	1.35	96.90 $\pm$ 1.31
	6	5.81 $\pm$ 0.01	2.01	94.71 $\pm$ 1.90	5.71 $\pm$ 0.003	1.40	94.71 $\pm$ 1.32
Caffeine	4	10.87 $\pm$ 0.05	0.63	98.06 $\pm$ 0.62	11.01 $\pm$ 0.11	1.50	99.98 $\pm$ 1.50
	5	11.73 $\pm$ 0.05	0.70	100.40 $\pm$ 0.71	11.68 $\pm$ 0.13	1.87	97.85 $\pm$ 1.83
	6	12.55 $\pm$ 0.03	0.42	97.89 $\pm$ 0.41	12.55 $\pm$ 0.06	0.84	97.95 $\pm$ 0.82
EGCG	4	22.49 $\pm$ 0.22	1.20	98.41 $\pm$ 1.18	22.59 $\pm$ 0.11	0.61	99.33 $\pm$ 0.61
	5	23.19 $\pm$ 0.15	0.80	99.78 $\pm$ 0.80	23.45 $\pm$ 0.25	1.36	99.39 $\pm$ 1.35
	6	24.53 $\pm$ 0.07	0.36	101.73 $\pm$ 0.36	24.64 $\pm$ 0.37	1.96	100.57 $\pm$ 1.97

Table-7: The Contents of Gallic Acid, Theobromine, Theophylline, Catechin, Caffeine, and EGCG in White Tea (*Camellia sinensis* L.) Leaf Extract ($n=6$)

Sample	Concentration (% b/b)					
	GA	TB	TP	C	CAF	EGCG
Sample 1	0.97 $\pm$ 0.01	0.37 $\pm$ 0.02	0.13 $\pm$ 0.002	0.49 $\pm$ 0.01	13.23 $\pm$ 0.32	30.09 $\pm$ 0.37
Sample 2	1.11 $\pm$ 0.02	0.31 $\pm$ 0.01	0.14 $\pm$ 0.003	ND	15.27 $\pm$ 0.66	22.54 $\pm$ 0.41
Sample 3	1.31 $\pm$ 0.05	0.27 $\pm$ 0.01	0.16 $\pm$ 0.01	0.74 $\pm$ 0.03	11.26 $\pm$ 0.40	28.08 $\pm$ 0.21

Fig.-4: The Typical HPLC Chromatographic Profile of Six Standards Using Optimized Condition (A) and the Chromatographic Profile of White Tea Extracts from Sample 1 (B), Sample 2 (C), and Sample 3 (D). HPLC Peaks: 1 Gallic Acid; 2 Theobromine; 3 Theophylline; 4 Catechin; 5 Caffeine; 6 Epigallocatechin Gallate

CCD is commonly applied to nonlinear responses and can be used to optimize the separation of several compounds on HPLC systems by elucidating the main factors and their interaction effects.^{21,36}

The HPLC system at optimum conditions meets the system suitability test. The validation parameters specified refer to the guidelines established by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use Q2(R1) guidelines²⁹. Determining several validation parameters, namely selectivity, linearity, the limit of detection (LoD), the limit of quantification (LoQ), precision, and accuracy. Selectivity, sensitivity, and linearity in the developed method have good results as required. This method also has strong accuracy and precision. Robustness is an indicator of the analytical method not to be affected by slight changes, for example, deliberate variations created in the parameters of the analysis method^{22,31}.

Validation results on optimal conditions of RP-HPLC/UV instrumentation were used in the separation, detection, and quantification of GA, TB, TP, CAF, C, and EGCG in the white tea extract. The three samples showed varying GA, TB, TP, C, CAF, and EGCG data due to the influence of the plant origin (e.g., climate, soil conditions, rainfall). Active ingredient separation using basic HPLC for detection and quantification of compounds in tea such as HPLC-ECD¹⁴, HPLC-MS-MS³⁷, UPLC³⁸, Nano HPLC³⁹, UFLC-MS/MS¹⁵, and UPLC-Q-TOF/MS¹⁶ were also developed. The HPLC-MS, UFLC-MS/MS or UPLC-Q-TOF/MS methods generally have high sensitivity and suitable for plasma analysis. However, for the purposes of routine analysis on extract samples, the system is expensive and requires special technical expertise^{19,20}. In this case, routine analysis methods for laboratory and industrial extracts, are less suitable. The results of modification, HPLC system optimization with UV detector and method

validation can be applied to analyze the gallic acid, theobromine, theophylline, catechin, caffeine, and EGCG simultaneously in white tea extract samples.

CONCLUSION

The HPLC method developed here is simple, easy, fast, and precise to measure gallic acid, theobromine, theophylline, catechin, caffeine, and EGCG levels from white tea extract products. The approach with DoE and CCD was effectively used for optimizing the separation of six compounds from white tea extract. The developed method is suitable for the analysis of the quality control sample extract. Simple isocratic HPLC systems, easy preparation, and quick analysis make it more suitable for the daily procedures in the food, phytopharmaceutical or extracting industries and quality control laboratories.

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