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Original Research Article

Response Surface Methodology-Driven Optimization of HPLC for the Determination of Caffeine, Chlorogenic Acid, and Caffeic Acid in Coffee

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ABSTRACT

Most studies on coffee bioactive compounds have focused on single targets and relied on trial-and-error optimization. This study aims to develop a high-performance liquid chromatographic (HPLC) method optimized by response surface methodology using a central composite design (RSM-CCD), and a design of experiments (DoE) approach for the simultaneous quantification of caffeine (CAF), chlorogenic acid (CGA), and caffeic acid (CA) in roasted coffee samples. Separation was performed on a Luna C18 (2) column (250 × 4.6 mm; 5 µm). Three factors – methanol ratio, flow rate, and methanol gradient were investigated and optimized through 17 CCD runs, evaluating retention time, peak area, resolution, tailing factor, and theoretical plates. The optimum condition of 31.6% methanol, 0.81 mL/min flow rate, and 1%/min gradient enabled the simultaneous elution and separation of CGA, CAF, and CA at a retention time of 6.325, 7.444, and 9.326 minutes, respectively, and over a 12-minute chromatographic runtime. Validation according to International Council for Harmonization (ICH) Q2 (R1) guidelines confirmed excellent linearity ($R^2 \geq 0.998$), LOD/LOQ (ppm) of 0.1718/0.5207 (CGA), 0.1773/0.5373 (CAF), and 0.0813/0.2464 (CA), with precision below 2% and recovery between 94.05% and 112.42%. Robusta levels (mg/g) were CGA (10.083 ± 0.229), CAF (18.838 ± 0.238), and CA (2.067 ± 0.044) while Arabica levels (mg/g) were CGA (16.641 ± 0.211), CAF (9.916 ± 0.157), and CA (2.178 ± 0.041). The RSM-driven HPLC successfully demonstrated efficient factor interaction modeling and robustness improvement. Consequently, the developed DoE-based method provides a rapid, selective, and reliable method for coffee quality evaluation and authenticity assessment.

Keywords: Coffee, Validation, Design of Experiment, Central Composite Design, Roasting, Quality Control

Introduction

Coffee's chemical composition is rich in biologically active compounds, with caffeine (CAF) and phenolic acids such as chlorogenic acid (CGA) and caffeic acid (CA) being particularly important.[1,2] CAF is a purine alkaloid that largely explains coffee's stimulant effect and also contributes to its bitterness.[3,4] CGA, an ester of caffeic and quinic acids, represents the main phenolic antioxidant in coffee, and the beverage is recognized as one of the richest dietary sources of this compound.[5] Although present in smaller amounts, CA often results from CGA degradation and is also formed during roasting,[7] contributing to both antioxidant activity and acidity.[8] Figure 1 presents the chemical structures of CAF, CGA, and CA. These compounds not only shape the flavor profile and health-related properties of coffee but also serve as important indicators of quality. Their levels vary depending on species and quality. *Coffea canephora* (Robusta) is typically richer in CAF, while *Coffea arabica* (Arabica) contains higher levels of CGA.[9] For this reason, CAF, CGA, and CA serve as key quality markers and remain central targets in research addressing the authenticity and nutritional significance of coffee.

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Various analytical techniques have been employed in coffee analysis, including advanced approaches such as liquid chromatography-mass spectrometry (LC–MS/MS)[10] and high performance liquid chromatography (HPLC) coupled with UV-Vis[11,12] or diode array detector (DAD) detection.[13–17] Among these methods, HPLC has become the predominant technique due to its high sensitivity, selectivity, and reliability.[11,18,19] Numerous studies have successfully applied it to coffee analysis, and validated assays consistently demonstrate excellent linearity with calibration coefficients close to unity ($R^2 > 0.99$), low detection limits, and strong repeatability.[18,20–22] These outcomes confirm HPLC as a robust and reliable tool for determining coffee bioactive compounds.[14,23] When properly optimized, it can resolve CAF, multiple CGA isomers such as 5-caffeoylquinic acid, and CA within complex matrices.[12,24] Hence, HPLC remains the gold standard for precise and reproducible quantification of key coffee compounds. Despite HPLC's widespread use, most coffee analysis studies still rely on conventional method development strategies, essentially a trial-and-error approach in the form of one-factor-at-a-time (OFAT) adjustments to fine-tune chromatographic conditions. In these approaches, a single parameter (such as mobile-phase composition, pH, or flow rate) is varied until acceptable retention and peak separation are achieved. This traditional strategy is straightforward but time-consuming and often suboptimal, as it neglects interactions between factors and may not achieve truly optimal conditions. Previous reports indicate that purely OFAT-based optimization struggles to deliver robust and reproducible methods, often resulting in lengthy gradients exceeding 20–40 min,[13,14,16] inefficient mobile phases (two-system setups),[15] or a narrow analytical scope that focuses only on single markers, such as CAF or CA.[11,18,19] A survey of recent coffee HPLC studies further shows that many adopted prior conditions or made incremental tweaks without systematic design,[12,24] reflecting the continued dominance of this conventional paradigm. While generally fit for purpose, the lack of statistical

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optimization underscores persistent methodological shortcomings in coffee bioactive analysis.

In coffee research, the use of the Design of Experiments (DoE) for HPLC optimization emerged recently and remains relatively limited.[18,22] Within this context, the response surface methodology (RSM) framework has been applied only to a limited extent for optimizing coffee chromatographic methods, particularly through the use of central composite design (CCD). For example, CCD-driven Analytical Quality by Design (AQbD) workflows have been employed to optimize HPLC methods for CAF quantification in coffee,[18] as well as to determine chlorogenic acid level in polyherbal formulations.[22] Multiple factors, including buffer pH and organic solvent ratio, were systematically evaluated in a factorial CCD rather than relying on trial-and-error, thereby achieving an ideal retention time, peak symmetry, and theoretical plates. The resulting methods met all validation criteria and proved both rapid and accurate. This example illustrates how RSM can efficiently explore factor interactions and define an optimum design space for HPLC conditions, reducing the number of experimental runs compared to OFAT.[20,22] Such a statistically driven optimization approach is highly relevant to coffee analysis, where multiple bioactive compounds with closely related chemical structures must be simultaneously separated and quantified. Beyond this, reports of RSM or CCD use in coffee HPLC remain rare. One recent study applied RSM to optimize extraction rather than chromatography,[20] and a few works outside the coffee domain have showcased CCD-based HPLC method development for other analytes.[22]

Within coffee analytics, however, the adoption of design-based optimization remains very limited, and only a handful of methods explicitly target CA alongside CAF and CGA. This limited uptake highlights a methodological gap. This study aims to develop and validate a RSM-CCD optimized HPLC method for the simultaneous determination of CAF, CGA, and CA in coffee. The novelty of this research lies in the integrated application of statistical design-based optimization (RSM-CCD) for multi-analyte bioactive quantification, an sapproach rarely reported in previous coffee studies. The objective of this study is to establish a robust method with proven selectivity, linearity, precision, accuracy, and sensitivity, with potential applications in coffee quality control, authenticity assessment, and functional food research.

Materials and Methods

Chemicals and reagents

CGA (purity ≥95.0%), CAF (99.8%), and CA (≥98.0%) reference standards were obtained from Sigma Aldrich (Germany). Methanol and water (analytical grade) were acquired from Thermo Fisher (Waltham, MA, USA). Potassium dihydrogen phosphate (KH_2PO_4) (Sigma Aldrich, Germany), ortho-phosphoric acid (H_3PO_4) 85% (Merck, Germany), deionized water (Brataco, Indonesia), and pro-analytical grade reagents were used for all other materials, unless stated otherwise.

Coffee sample processing

Green beans of Robusta and Arabica coffee were collected directly from local farmers in Temanggung, Central Java, Indonesia (coordinates 7.3158° S, 110.2546° E). The beans were roasted at 180 °C for 10–12 minutes using a home coffee roaster. They were then ground using an electric coffee grinder (N600, China) set to a fine setting and sieved through a 40-mesh sieve to produce particles of uniform size ahead of extraction.

Determination of maximum absorption wavelength

A mixture of standard CGA, CAF, and CA solutions (5 ppm each) was scanned using a UV–Vis spectrophotometer (Shimadzu UV-1900, Japan) across the 200-800 nm region. By overlaying the spectra, the maximum absorption wavelength (λ_{max}) at 280 nm was selected (Figure 2).

Chromatographic conditions

A HPLC system (Shimadzu, Japan), featuring a high-pressure gradient quaternary pump (LC-20AT), manual injector, UV-Vis detector (SPD-20A), and the LC-Solutions program, was employed for the analysis. A Luna® C18(2) 100 Å column (250×4.6 mm; 5 µm) (Phenomenex, USA) was used as the stationary phase, applying a gradient elution composed of phosphate buffer (KH_2PO_4, pH 2.5 adjusted using H_3PO_4) and methanol. In the preliminary stage, a buffer–methanol (60:40, v/v) isocratic mode was utilized, applying a flow rate of 1.0 mL/min. For further optimization, the methanol ratio was varied between 30-40% with a flow rate of 0.8-1.2 mL/min, and a methanol gradient of 1-3%/minute was applied. The stationary phase was maintained at 25 °C (ambient), detector was configured at a wavelength of 280 nm, with a 20 µL injection volume.

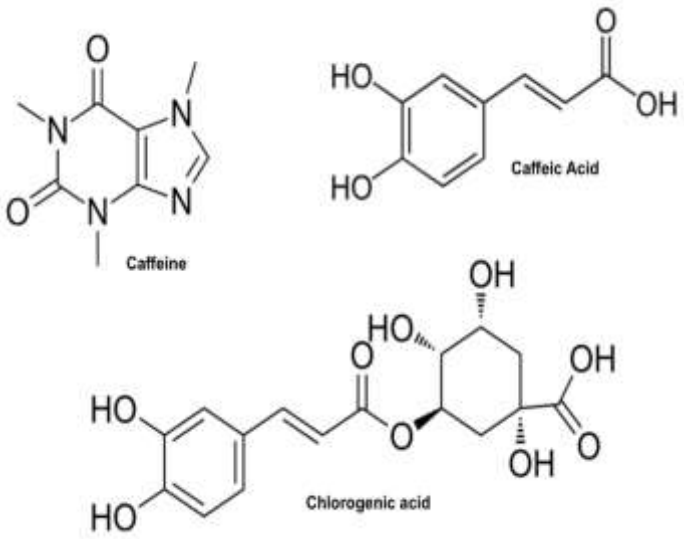

Figure 1: Chemical structures of Caffeine (CAF), Chlorogenic acid (CGA, and Caffeic acid (CA)

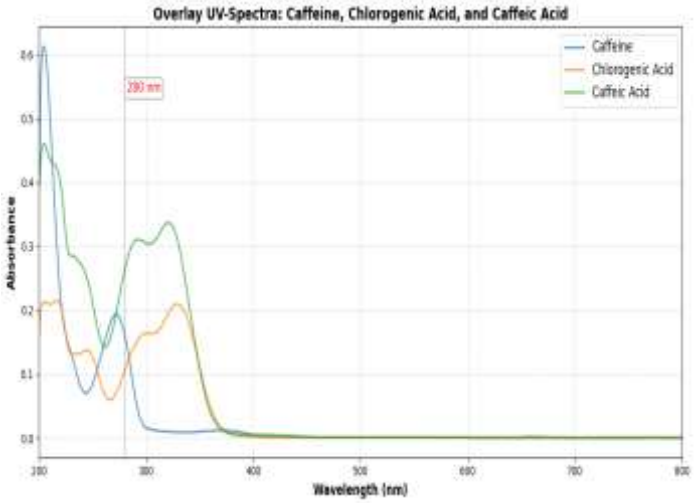

Figure 2: Overlay of UV spectra of CGA, CAF, and CA (5 ppm each) in the wavelength range of 200–800 nm.

Method optimization
This study employed a RSM–CCD optimization design incorporating a full factorial structure (block 1, with three levels per factor), an alpha level of 2, and three center-point replications. The investigated mobile-phase factors were methanol ratio (F1, % v/v), flow rate (F2, mL/min), and methanol gradient (F3, %/min), which were systematically varied (see *Chromatographic conditions*) to assess their impact on chromatographic performance and improve efficiency. The primary chromatographic responses evaluated included retention time (Rt) for each compound, peak area (AUC), and resolution (Rs), along with the tailing factor (Tf) and column efficiency, expressed as the number of theoretical plates (N). CCD was chosen for its ability to systematically evaluate factor interactions and provide response information under extreme conditions,[25] a capability that the Box–Behnken Design (BBD) lacks.[26] As a summarized in Table 1, the design matrix generated 17 experimental runs and the corresponding matrix and responses.

System suitability test
System suitability was assessed prior to the HPLC validation process by performing six consecutive injections (n = 6) of a mixed standard solution containing CGA, CAF, and CA. The evaluated chromatographic responses (as described in method optimization) were assessed for repeatability, which was assessed using percentage relative standard deviation (%RSD) with an acceptance limit of less than 2%.

Validation of the proposed method
The HPLC method, which has been optimized with RSM-CCD, was further validated in accordance with the ICH Q2 (R1) guidelines to ensure suitability for the intended analysis. The validation involved assessment of selectivity, linearity, precision, accuracy, the limit of detection (LOD) and limit of quantitation (LOQ) as measures of sensitivity.[27]

Selectivity test
To evaluate selectivity, chromatograms of standard reference solutions of CGA, CAF, and CA were compared with those of the sample extracts. Each standard and sample extract was injected once under the same chromatographic conditions. Selectivity was established when analyte peaks were adequately resolved and no solvent-related interferences were observed at the respective retention times. Further confirmation was provided by resolution values that met the acceptance criterion of ≥ 2.0.

Linearity and sensitivity
Eight concentration levels of CGA, CAF, and CA standard reference solutions (0.125–16 ppm) were prepared and analyzed. Calibration plots were constructed depicting peak area (y-axis) as a function of concentration (x-axis), and linearity was determined from the correlation coefficient (r). LOD and LOQ were calculated based on the equations 1 and 2

$$LOD = 3.3\ x\ \frac{S_y}{b} \qquad equation\ 1$$

$$LOD = 10\ x\ \frac{S_y}{b} \qquad equation\ 2$$

where Sy denotes the intercept's standard deviation and b the slope of the calibration curve.

Precision and accuracy
Method precision was determined by repeatability testing using six replicates (n = 6) injections of coffee extract analyzed across two consecutive days. Precision was reported as the %RSD of CGA, CAF, and CA concentrations. The accuracy of the method was determined through recovery assessment. In this test, standard solutions of CGA, CAF, and CA were spiked into predetermined coffee sample extracts at three concentration levels of 80%, 100%, and 120% relative to the target concentration. Each level was analysed in triplicate (n = 3), and the accuracy was calculated as the percentage recovery of each analyte.

Assay of coffee extract
Two grams of sample coffee powder were accurately weighed and extracted with demineralized water. The mixture underwent heating at 80-90 °C for 30 min with periodic gentle stirring, followed by sonication (10 min) and centrifugation (1500 rpm, 10 min) to achieve separation. The supernatant was separated and collected, adjusted to 50 mL, and passed through a 0.45 µm membrane filter. The test solutions were obtained by diluting the required quantity with methanol, followed by injecting 20 µL of each into the HPLC system. The assay for analyte determination was carried out in six replicates (n = 6).

Statistical analysis
Statistical analyses were performed using Design-Expert® software version 13 (Stat-Ease Inc., Minneapolis, USA; 2021). Analysis of variance (ANOVA) was used to evaluate the significance of the model, individual factors, and their interactions within RSM-CCD. Model adequacy was determined by the coefficient of determination (R^2), lack-of-fit test, and residual analysis.

Results and Discussion

Experimental Design of Central Composite Design
The experimental design of the CCD was carried out with 17 experimental runs designed to assess the method's robustness for the separation of the three target compounds (CGA, CAF, and CA). The best-fit model was selected and established statistically, encompassing a model that was significant ($p < 0.05$) with the lack of fit found to be non-significant ($p > 0.05$). Moreover, adjusted and predicted R^2 values exceeding 0.7, and a discrepancy between the two of less than 0.2. Data transformation was applied when necessary to meet the aforementioned criteria, as outlined in the Box–Cox analysis recommendations.[28,29]

ANOVA and Model Fit Evaluation
As shown in Table 2, the ANOVA analysis reveals that most of the responses met the required criteria, suggesting that the proposed quadratic models were adequate to represent the association linking the factors to the outcomes. The models for retention time and peak area demonstrated highly significant results ($p < 0.05$), with R^2 values exceeding 0.9, indicating strong model predictability and fit. The lack-of-fit values were non-significant ($p > 0.05$), further confirming the adequacy of the models. In contrast, the models for resolution and tailing factor exhibited acceptable performance, albeit with relatively lower R^2 values (above 0.8). Conversely, the number of theoretical plates exhibited an inadequate model fit ($R^2 < 0.5$), indicating that this parameter was not significantly influenced by the selected factors.
The obtained R^2 and adjusted R^2 values (0.90–0.99) are higher than those reported in previous CCD-based HPLC optimizations for CAF and CGA,[18,22] where R^2 typically ranged between 0.88 and 0.95. In particular, the CAF model showed an adjusted R^2 of approximately 0.9667 but a predicted R^2 near 0.7761,[18] suggesting moderate predictive capability. Meanwhile, the CCD-optimized CGA model achieved a predicted R^2 of 0.9991 with a corresponding adjusted R^2 of 0.9997,[22] indicating excellent consistency. The present RSM–CCD design likewise exhibited a minimal difference between adjusted and predicted R^2 (<0.2), confirming both strong explanatory power and predictive reliability. The adequate precision (Adeq. Prec > 16) further supports a favorable signal-to-noise ratio, reflecting the statistical robustness of the developed model. Moreover, the significant regression coefficients ($p < 0.05$) for methanol ratio and flow rate confirm these as the most influential variables affecting chromatographic performance, particularly in reducing retention time while maintaining resolution. Overall, these findings demonstrate that the present RSM–CCD approach achieved comparable or superior model fitness and predictive accuracy, highlighting its effectiveness for multi-response and multi-analyte (CGA, CAF, and CA) optimization within a unified design framework.
The regression coefficients obtained from the response surface models provided useful insights into the effects of the studied factors. In general, positive coefficients indicated a direct proportional relationship, while negative coefficients demonstrated an inverse effect between the variables and responses.[30] For retention time, both the methanol ratio (F1) and the flow rate (F2) exhibited negative coefficients, thereby confirming that an increase in these variables led to a decrease in elution time.

Table 1: Experimental design matrix of the central composite design (CCD) with factor levels and observed chromatographic responses of CGA, CAF, and CA

Std	Factor			Response													
				tR			AUC			Rs		N			Tf		
	F1	F2	F3	1	2	3	1	2	3	1-2	2-3	1	2	3	1	2	3
1	30	0.8	1	6.26	7.48	9.52	322824	582344	617839	1.92	3.14	1276	2781	2692	1.00	1.12	0.96
2	40	0.8	1	5.69	6.49	7.38	306643	555175	601194	2.44	2.55	4689	6594	5958	1.14	1.18	1.10
3	30	1.2	1	4.30	5.11	6.37	208787	381328	410909	2.09	3.07	1751	3206	3098	1.02	1.13	1.04
4	40	1.2	1	3.02	3.65	4.04	208394	369764	405098	2.20	1.26	1605	2859	2062	1.10	0.00	0.00
5	30	0.8	3	5.88	7.20	8.98	311335	568611	609048	1.87	2.82	868	2239	2980	1.01	1.11	0.92
6	40	0.8	3	4.68	5.61	6.32	300082	547288	596809	2.42	1.68	2186	3773	2766	1.11	1.18	1.02
7	30	1.2	3	3.30	4.32	5.41	204015	377182	410681	2.15	2.04	694	1503	1200	1.14	1.18	0.98
8	40	1.2	3	3.03	3.66	4.10	196143	361058	394957	2.16	1.33	1541	2732	1926	1.11	0.00	0.00
9	25	1.0	2	6.97	7.76	9.53	240394	433297	470534	1.72	4.20	2976	5584	7883	1.02	1.10	1.08
10	45	1.0	2	3.35	4.01	4.30	233036	441056	458239	2.09	0.75	1794	2453	1563	1.16	0.00	0.00
11	35	0.6	2	7.03	8.42	10.03	416366	765813	827310	2.35	2.50	1999	3709	2985	1.03	1.13	0.94
12	35	1.4	2	3.13	3.75	4.48	175317	317681	344239	2.08	2.27	1572	2782	2513	1.07	1.19	1.07
13	35	1.0	0	4.57	5.37	6.38	252772	460840	500694	2.36	2.68	2767	4227	3570	1.07	1.15	1.05
14	35	1.0	4	4.20	5.07	6.10	240952	442666	472839	2.15	2.41	1501	2878	2593	1.06	1.18	1.02
15	35	1.0	2	4.18	5.05	5.96	252344	462329	501902	2.28	2.15	1743	3115	2443	1.04	1.16	1.00
16	35	1.0	2	4.15	5.02	5.92	253731	463815	504650	2.25	2.12	1666	3032	2392	1.04	1.15	0.99
17	35	1.0	2	4.20	5.07	6.01	249649	458310	497029	2.26	2.27	1751	3151	2602	1.06	1.16	1.01

Note: F=Factor, F1 = ratio of methanol (%), F2 = flow rate (mL/min), F3 = methanol gradient (%/min); Response 1 = CGA, 2 = CAF, 3 = CA; tR = retention time, AUC = peak area, Rs = resolution, N = number of theoretical plates, Tf = tailing factor.

Table 2: ANOVA results and statistical parameters of response surface models for chromatographic responses of CGA, CAF, and CA

Response	Model (p-value)	Lack of fit (p-value)	R^2	Adj R^2	Pred R^2	Adeq Prec
tR1	1.76×10^{-7}[b]	0.0046[c]	0.9226	0.9047	0.8560	21.68
tR2	1.01×10^{-4}[b]	0.0032[c]	0.9738	0.9402	0.7886	17.60
tR3	1.46×10^{-5}[b]	0.0118[c]	0.9851	0.9658	0.8806	22.16
AUC1	3.01×10^{-9}[b]	0.3233	0.9987	0.9970	0.9909	94.32
AUC2	1.85×10^{-13}[b]	0.0611	0.9907	0.9886	(n/a)[a]	76.70
AUC3	3.63×10^{-9}[b]	0.2746	0.9986	0.9968	(n/a)[a]	91.98
Rs1–2	9.18×10^{-4}[b]	0.0359[c]	0.9498	0.8853	0.5814	16.25
Rs2–3	1.56×10^{-5}[b]	0.0426[c]	0.8448	0.809	0.7109	16.33
N1	0.0987	0.0044[c]	0.3727	0.2280	–0.1858	4.76
N2	0.1484	0.0035[c]	0.3274	0.1722	–0.2753	3.95
N3	0.0996	0.0128[c]	0.3719	0.2269	–0.1813	4.93
Tf1	0.0009[b]	0.1211	0.8601	0.7762	0.5527	10.55
Tf2	0.0387[b]	0.0001[c]	0.8630	0.6870	–0.1487	6.07
Tf3	0.0270[b]	0.0002[c]	0.4793	0.3591	0.0445	7.33

Note: Response (1 = CGA, 2 = CAF, 3 = CA); tR = retention time, AUC = peak area, Rs = resolution, N = number of theoretical plates, Tf = tailing factor.

[a] *Predicted R^2 not available.*

[b] *Model significant at $p < 0.05$*

[c] *Lack of fit significant at $p < 0.05$.*

In contrast, the methanol gradient (F3) exhibited a favorable impact on resolution, indicating that increasing the gradient improved peak separation within a shorter analysis timeframe. This finding is consistent with the fundamental principles of reversed-phase chromatography.

To illustrate, the coded regression model for the retention time of CAF is expressed as follows (equation 3):

$$tR\ (CAF) = 5.00196 - 0.76244A - 1.21031B - 0.15856C + 0.05788AB + 0.02637AC + 0.04812BC + 0.20549A^2 + 0.25524B^2 + 0.03849C^2 \qquad equation\ 3$$

In equation 3, A, B, and C represent the methanol ratio, flow rate, and methanol gradient, respectively. The negative coefficients of A and C underscore their diminishing effect on retention time. Conversely, the regression model for resolution between CAF and CA (Rs2–3) was expressed as equation 4:

$Rs\ (CAF - CA) = 2.30747 - 0.69719A - 0.18356B - 0.16819C \quad equation\ 4$

Equation 4 shows a negative coefficients. This implies that higher methanol levels, faster flow rates, or steeper gradients may compromise resolution, reflecting the trade-off between shorter analysis times and adequate chromatographic separation.

Evaluation of Factor Effects and Interactions

Perturbation plot analysis (Figure 3) provides an overview of factor sensitivity toward the studied responses. The retention time of CGA (tR1) was mainly influenced by methanol ratio (A) and flow rate (B), both with negative coefficients, confirming that increasing either parameter reduced retention time in line with reversed-phase principles. The peak area of CGA (AUC1) remained relatively stable, though slightly reduced at higher methanol ratios, suggesting decreased detection sensitivity. Resolution between CGA and CAF (Rs1–2) was strongly affected by the methanol gradient (C), where moderate increases enhanced resolution, while excessive gradients had the opposite effect. Theoretical plates of CGA (N1) were minimally influenced by factor variation, as indicated by a low R^2 value, while the tailing factor of CA (Tf3) increased at higher flow rates but approached unity under intermediate conditions.

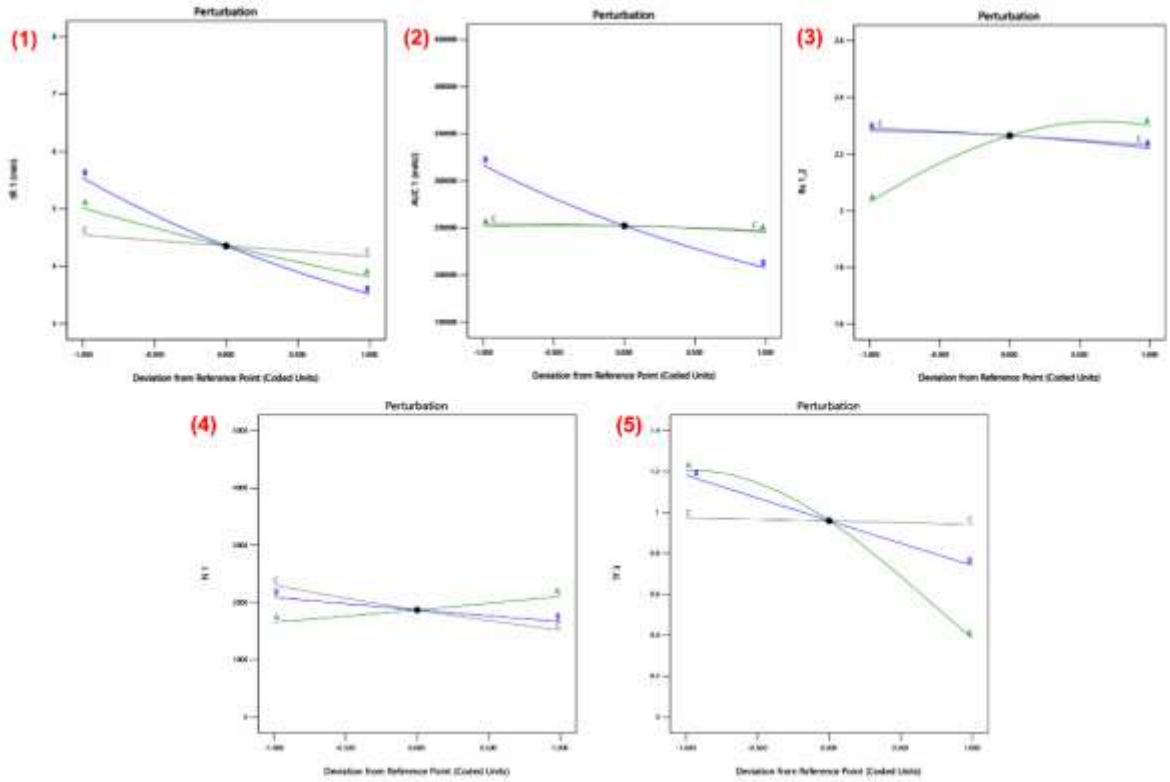

Figure 3: Representative perturbation plot of responses: (1) retention time of CGA (tR1), (2) peak area of CGA (AUC1), (3) resolution between CGA and CAF (Rs1–2), (4) number of theoretical plates of CGA (N1), and (5) tailing factor of CA (Tf3)

Interaction terms further emphasized the utility of CCD in capturing factor relationships. For example, although high methanol levels tended to reduce resolution, this effect was compensated at intermediate flow rates, producing acceptable values. Similarly, the flow rate and gradient interaction influenced peak symmetry, with moderate conditions yielding tailing factors close to unity. These interaction patterns are clearly depicted in the three-dimensional response surface plots (Figure 4), which illustrate how factor combinations affect chromatographic performance.

Optimization and Control Strategy for Chromatographic Conditions

In order to establish a control strategy within the defined design space, the desirability function was applied to select the most suitable optimization conditions.[22] This approach entailed the simultaneous consideration of all responses to identify the best compromise among the chromatographic parameters, thereby determining the optimized conditions. The optimal chromatographic condition was obtained at 31.6% methanol, with a flow rate of 0.81 mL/min and a methanol gradient of 1%/min. Under these conditions, CGA, CAF, and CA were simultaneously eluted and well separated at 6.325 ± 0.020, 7.444 ± 0.012, and 9.326 ± 0.017 minutes, respectively, all within a 12-minute chromatographic runtime.

Method Validation and HPLC Analysis

The optimized condition was selected for further validation and application in the quantitative analysis. The analytes demonstrated shortened retention times, exceeded the acceptance criterion of $Rs \geq 2.0$, tailing factors approaching 1.0, and theoretical plate numbers exceeding 2000, aligning with the performance metrics established during the system suitability test. Furthermore, the RSD values were found to be less than 2%, thereby confirming the precision and reliability of the chromatographic system. The established HPLC approach was subsequently validated in accordance with ICH Q2(R1) guidelines, covering selectivity, linearity, precision, accuracy, and sensitivity, with a summary of the results presented in Table 3.

Selectivity

The selectivity test results demonstrated that the developed HPLC method was able to clearly separate the three target analytes, CGA, CAF, and CA, without significant interference from coffee matrix components. The resulting chromatograms showed no co-elution, with resolution values between adjacent peaks exceeding 2.0, thereby fulfilling the acceptance criteria set by USP and FDA guidelines.[31] These findings confirm that the method possesses good selectivity and is therefore suitable for the quantification of bioactive compounds in coffee samples. The representative chromatogram of the selectivity test is presented in Figure 5.

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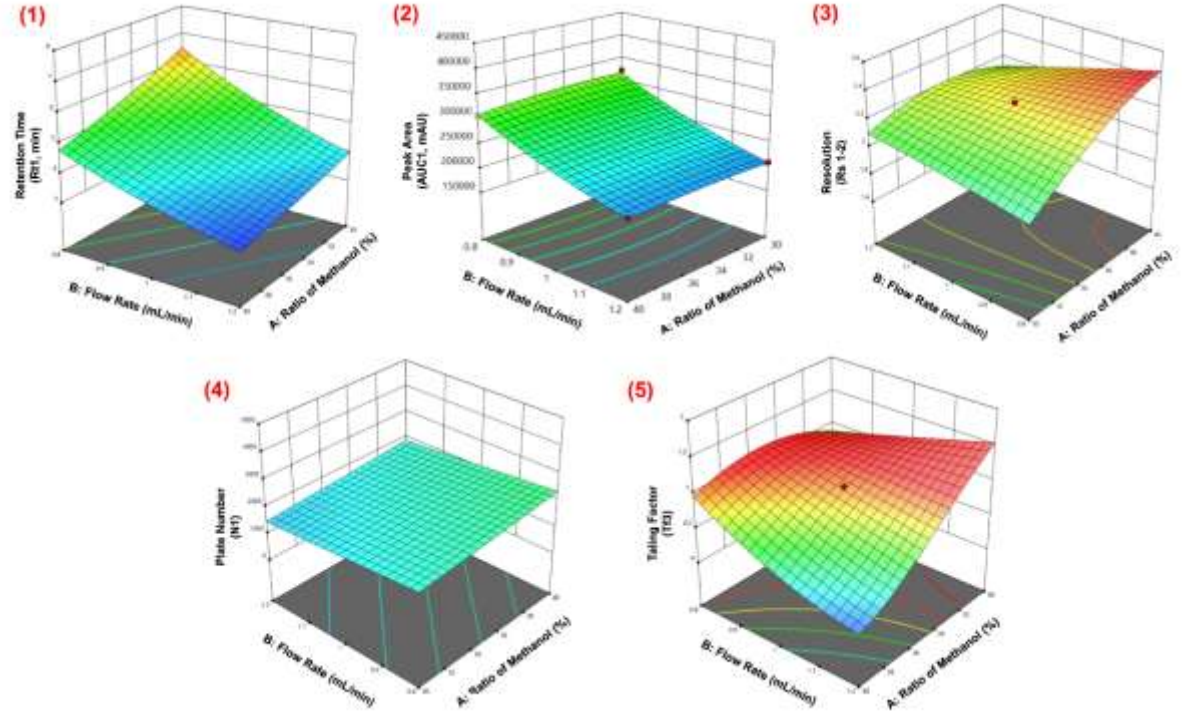

Figure 4: Representative 3D surface plots of responses: (1) retention time of CGA (tR1), (2) peak area of CGA (AUC1), (3) resolution between CGA and CAF (Rs1–2), (4) number of theoretical plates of CGA (N1), and (5) tailing factor of CA (Tf3)

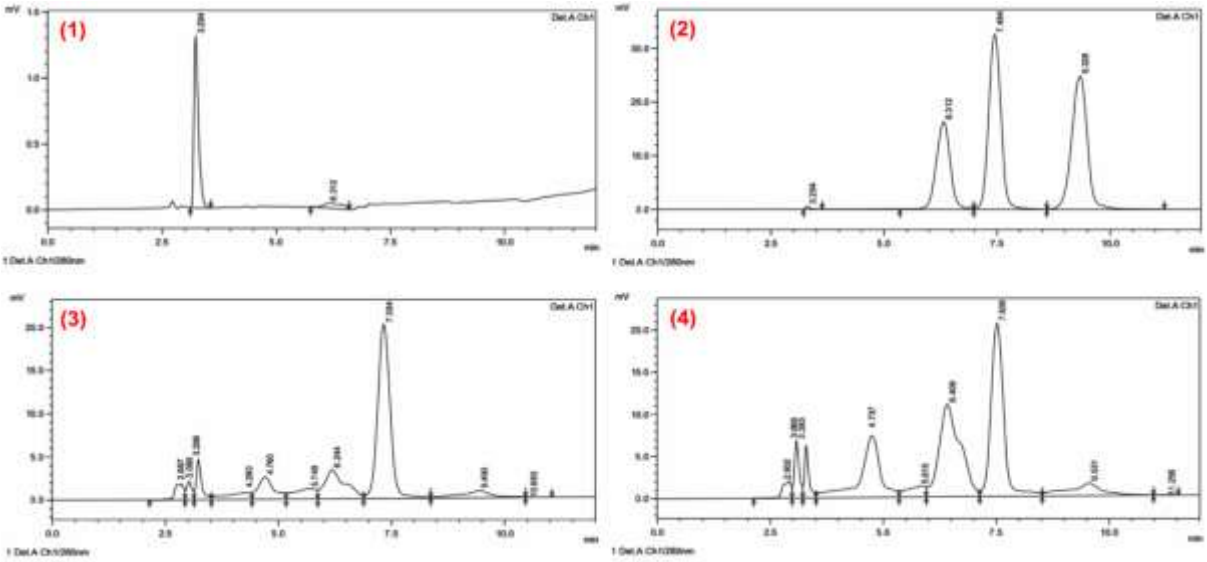

Figure 5: Optimized HPLC chromatograms showing separation of CGA, CAF, and CA at 280 nm: (1) methanol solvent; (2) mixed standard solution (10 ppm); and coffee samples from Temanggung: (3) Arabica, (4) Robusta.

Linearity, LOD, and LOQ

Linearity was evaluated across eight concentration levels: 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 ppm. The calibration curves demonstrated excellent correlation between concentration (x-axis) and peak area (y-axis), with correlation coefficients ($r > 0.999$) and coefficients of determination ($R^2 > 0.998$) for all three analytes. These values satisfied the acceptance criteria ($r \geq 0.999$ and $R^2 \geq 0.997$).[32] The obtained R^2 values (0.9984–0.9999) are comparable to or exceed those in recent validated HPLC methods for coffee bioactives, including $R^2 > 0.999$ for multi-analyte determination (CAF, CGA, CA)[21], $R^2 \geq 0.998$ in CCD-optimized CGA analysis,[22] and $R^2 > 0.99$ in RSM-CCD for CAF quantification.[18] As demonstrated in Figure 6, the regression plots confirmed strong linearity with statistically significant models ($p < 0.05$) and minimal standard error (SE), thereby substantiating the reliability of the calibration. The regression equations 5, 6 and 7 for CGA ($R^2 = 0.9995$), CAF ($R^2 = 0.9999$), and CA (($R^2 = 0.9984$), respectively were obtained as follows:

$y = 26805.76x + 1550.93 \quad equation\ 5$,

$y = 51015.16x + 1227.33 \quad equation\ 6$

$y = 51411.75x - 2272.44 \quad equation\ 7$

Sensitivity was expressed in terms of the limit of detection (LOD) and limit of quantitation (LOQ), calculated as equation 1 and 2. The calculated LOD and LOQ values were 0.1718 and 0.5207 ppm for CGA, 0.1773 and 0.5373 ppm for CAF, and 0.0813 and 0.2464 ppm for CA.

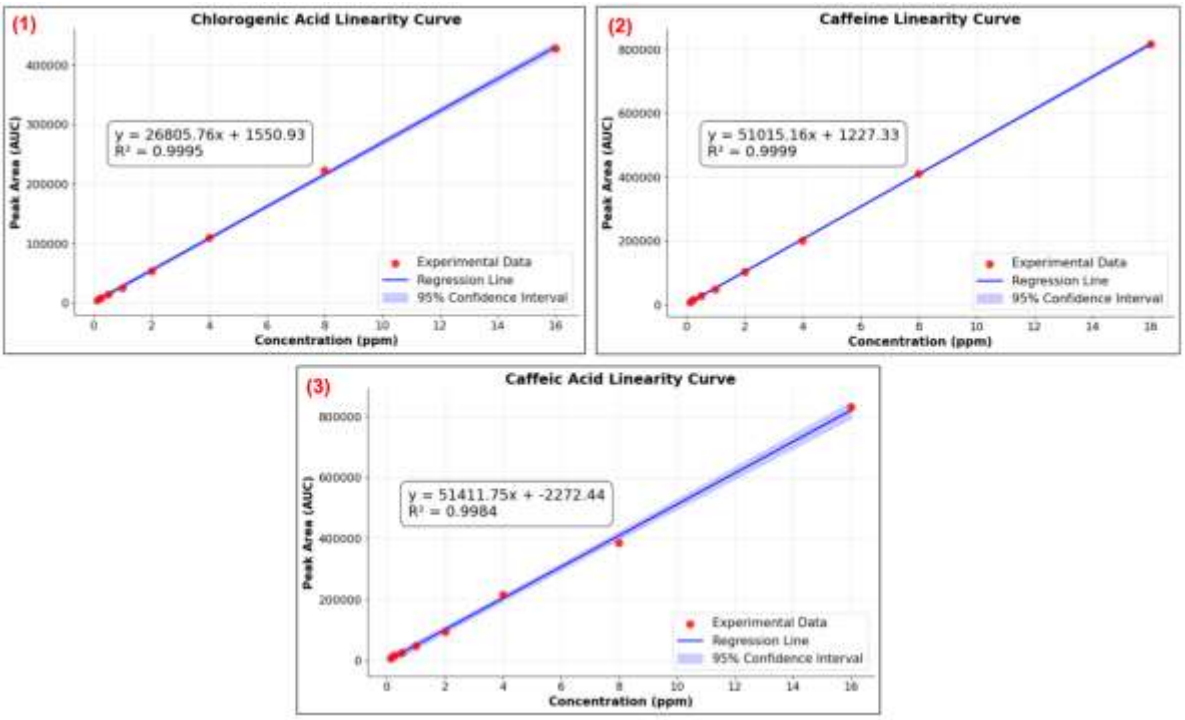

Figure 6: Linearity plots of (1) CGA, (2) CAF, and (3) CA showing the relationship between concentration (ppm) and peak area (AUC)

Table 3: Summary results of validation parameters for CGA, CAF, and CA

Parameter	CGA	CAF	CA
System suitability test			
Retention Time (% RSD)	0.3121	0.1555	0.1829
Peak area (% RSD)	1.0308	0.9271	0.8770
Resolution	-	2.140 ± 0.028	3.400 ± 0.028
Plate number (±SD)	2000 ± 42.22	3647 ± 44.96	3754 ± 57.26
Tailing Factor (±SD)	0.999 ± 0.004	1.059 ± 0.004	0.946 ± 0.006
Linearity			
Range (ppm)	0.125 – 16	0.125 – 16	0.125 – 16
Regression equation	y = 26805.76x + 1550.93	y = 51015.16x + 1227.33	y = 51411.75x – 2272.44
r, R2 value	0.9998, 0.9995	1.000, 0.9999	0.9992, 0.9984
P-value	2.87×10^{-11} [a]	1.42×10^{-13} [a]	1.37×10^{-9} [a]
LOD, LOQ (ppm)	0.1718, 0.5207	0.1773, 0.5373	0.0813, 0.2464
Precision (%RSD of Peak area)			
Intraday	2.408	1.175	2.108
Interday	1.95	0.903	2.105
Accuracy			
% Recovery	102.187 - 112.421	97.548 - 100.830	94.049 - 100.593
% RSD	6.177 - 6.669	1.697 - 8.712	1.828 - 12.656

[a] p-value < 0.05 indicates statistical significance of the regression model.

These values are lower than those reported in previous validated HPLC methods that recorded higher LOD and LOQ values of approximately 8–9 ppm (LOD) and 27–32 ppm (LOQ) for CGA and CAF,[24] as well as 0.450–0.496 ppm (LOD) and 1.0–1.503 ppm (LOQ),[18,21] confirming the proposed method's capability to detect low analyte concentrations.

Precision and Accuracy

The repeatability test of the coffee extract yielded %RSD values of 2.27% and 1.97% for CGA, 1.26% and 0.96% for CAF, and 2.11% and 2.18% for CA on day 1 and day 2, respectively. All values were below the acceptance limits recommended by Horwitz and AOAC guidelines,[33] thereby confirming good method precision. The recovery values obtained at the 80%, 100%, and 120% levels ranged from 102.19% to 112.42% for CGA, 97.55% to 100.83% for CAF, and 94.05% to 100.59% for CA, with RSD values mostly below 10%, except for CA at the 80% level (12.58%). These findings are consistent with previous reports, with recoveries ranging from 79-140% for CGA and CAF in green coffee,[24] 90.6-112.5% for CA in raw and roasted coffee with a precision below 3.6%,[21] and 103-119% (CGA), 95.9-98.0% (CAF), and 88.4-99.2% (CA) in green coffee extract with a

precision below 4%.[13] Therefore, the method can be considered to have good accuracy.

Quantification of Target Compounds in Coffee Samples Using Validated HPLC Method

The analytical HPLC method developed and optimized using RSM–CCD was successfully validated, fulfilling the required analytical parameters and proven reliable for quantifying CGA, CAF, and CA in coffee samples. In this study, two coffee variants from Temanggung, Robusta and Arabica, were tested using the validated method. In roasted Robusta coffee, the concentrations of the three compounds were consistently measured. As reported in Table 4, the average CGA content was 1.008 ± 0.023%, while CAF was present at 1.884 ± 0.024%, and caffeic acid at 0.207 ± 0.004%. These findings highlight CAF as the predominant compound in Robusta, followed by CGA, with CA present at much lower levels.

Table 4: Concentration of CGA, CAF, and CA in roasted coffee extracts using the proposed analytical method

Coffee Sample	CGA		CAF		CA	
	mg/g	%	mg/g	%	mg/g	%
Robusta	10.083 ± 0.229	1.008 ± 0.023	18.838 ± 0.238	1.884 ± 0.024	2.067 ± 0.044	0.207 ± 0.004
Arabica	16.641 ± 0.211	1.664 ± 0.021	9.916 ± 0.157	0.992 ± 0.016	2.178 ± 0.041	0.218 ± 0.004

Note: Data are reported as mean ± standard deviation (SD)

The detectable presence of CA at around 0.2% in roasted beans supports this transformation pathway and reflects the chemical complexity of the roasting process.[7] In contrast, roasted Arabica coffee demonstrated a different composition. CGA was significantly higher, with an average content of 1.669 ± 0.023%, while CAF was lower, measured at 0.983 ± 0.024%. Caffeic acid levels were comparable to those of Robusta, averaging at 0.220 ± 0.004%. These results are consistent with the general understanding that Arabica beans tend to contain higher levels of CGA, which contributes to their acidity and nuanced flavor profile, while Robusta contains higher levels of CAF, resulting in a stronger bitterness and stimulant effect.[3,8,9]

Conclusion

The present study successfully developed and optimized a HPLC method using a design of experiments approach based on RSM–CCD, and applied it for the simultaneous determination of CGA, CAF, and CA in coffee. The optimized procedure achieved reliable separation with excellent selectivity, sensitivity, precision, and accuracy. Moreover, the method was rendered more efficient for routine applications by reducing analysis time. The application of the method revealed distinct compositional differences, thereby confirming its potential as a practical analytical tool to support quality control and scientific studies related to the chemistry of coffee and its functionality. Consequently, this approach can be regarded as a reliable method for determining bioactive compounds in coffee. Future studies may extend this validated method to other coffee matrices or related phenolic compounds to further broaden its applicability.

Conflict of Interest

The authors declare no conflict of interest**.**

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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