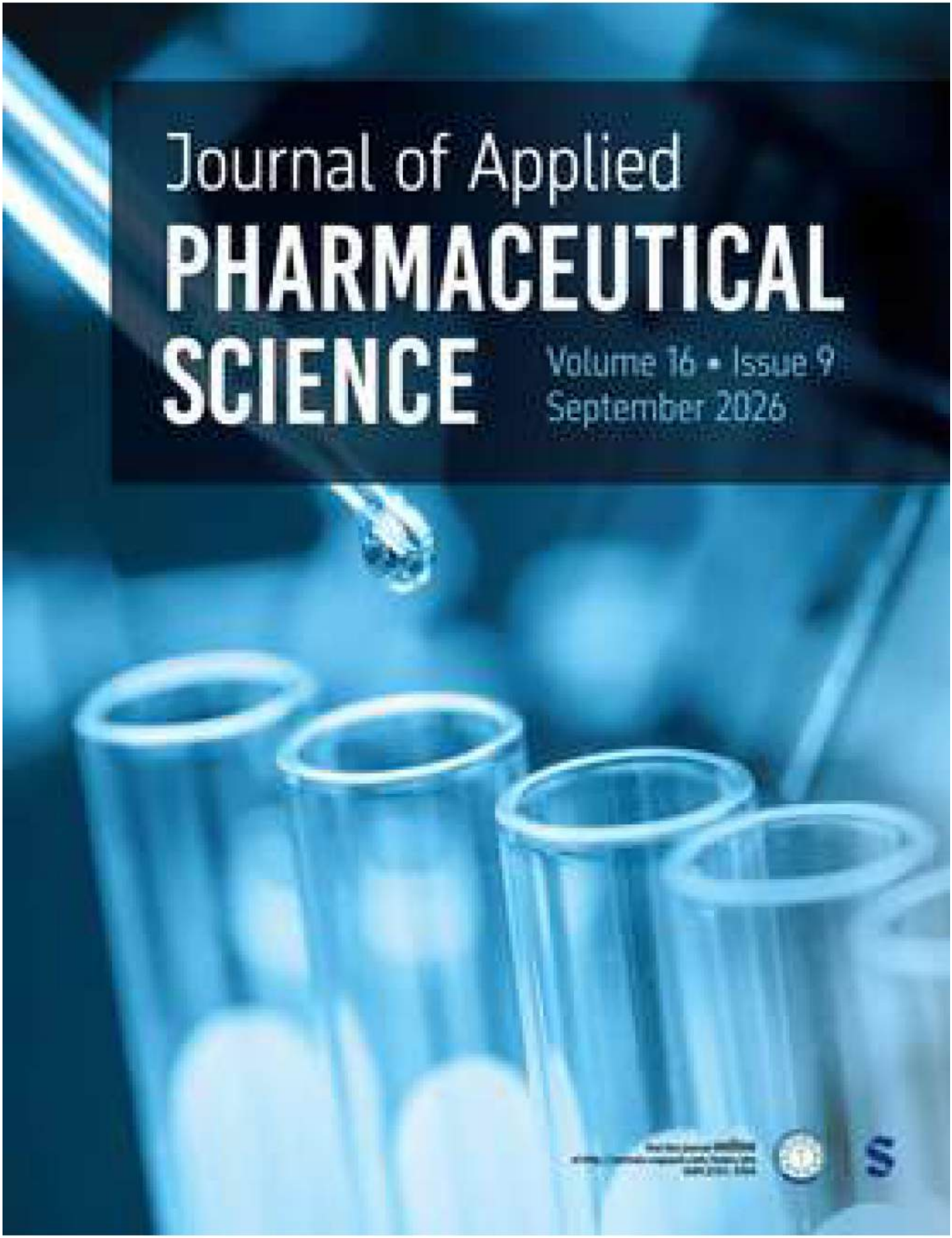
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*Original Article*

# Box–Behnken Assisted Development and Validation of an RP-HPLC Method for Determining Caffeic Acid in Robusta Coffee Brews

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**Titus Marcel Kusraynaldi[1], Theresia Niken Larasati[1], Angel Yemima Srininta Br Sembiring[1], Florentinus Dika Octa Riswanto[1] and Enade Perdana Istyastono[2,3]**

## Abstract

Indonesia remains one of the world's top coffee producers, with Robusta coffee (*Coffea canephora*) being the variety favored by most consumers. Owing to its prominence, numerous studies have highlighted caffeic acid, a polyphenolic compound in coffee extract that has been shown to demonstrate antidiabetic properties. The primary objective of this research is to measure the caffeic acid levels in Robusta coffee brews. To achieve the optimal conditions, a Box–Behnken Design was used to determine the most suitable setting for the independent variables, including acetonitrile, formic acid, and flow rate. The analytical method validation parameters assessed were linearity and range, selectivity, accuracy, and precision, limit of detection (LOD), and limit of quantification (LOQ). The optimized reversed-phase high-performance liquid chromatography system was methanol:acetonitrile:aqueous formic acid pH 2.9 (10:15:75 v/v/v), flow rate of 1.0 mL/min, column temperature of 27 °C, 10 µL injection volume, and wavelength detection at 326 nm. The method showed good specificity and selectivity (Rs ≥ 1.5), with linearity from 5.39 to 32.36 µg/mL ($R^2$ = 0.9987). LOD and LOQ were found to be 0.08032 and 0.2434 µg/mL, respectively. The recovery percentage for the intra-day assay was 92.26%–102.94%, while the inter-day percentage yield achieved 94.89% to 102.96% (90%–107%). All of the intra-day and inter-day assays met the required relative standard deviation (RSD) ≤ 5.3%. The average caffeic acid in Robusta coffee brews was 0.772 ± 0.008 mg/g (RSD = 1.066%, $n$ = 6).

## Keywords

Caffeic acid, BBD, response surface methodology, validation, determination of content

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## Introduction

Coffee has become a popular beverage in Indonesia.[1] Indonesia is not only a nation that consumes a significant amount of coffee but also plays an instrumental role as the second-largest producer of coffee in Asia.[2] According to data from the USDA Foreign Agricultural Service annual report, during the 2023–2024 period, Robusta coffee production in Indonesia reached 8.4 million 60-kg bags, contributing to an estimated total national coffee production of around 9.7 million bags. Eventually, domestic coffee consumption also showed an upward trend.[3]

Due to the increased global consumption of coffee, extensive research has been done to examine its components and their potential health benefits. Coffee has been reported to contain numerous bioactive compounds that are associated with its biological activities, including alkaloids (caffeine and trigonelline), diterpenes (cafestol and kahweol), phenolic

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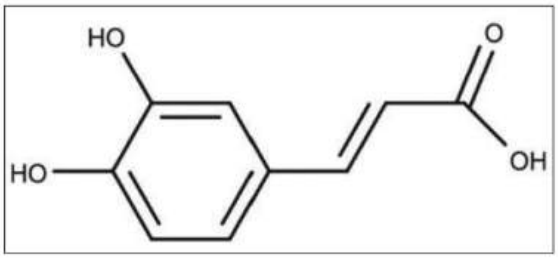


**Figure 1.** Chemical Structure of Caffeic Acid (Originally Illustrated by the Authors).

compounds (chlorogenic acid and caffeic acid), and other secondary metabolites.[4,5] Findings suggest that moderate coffee intake has been shown to reduce the risk of developing various degenerative diseases, including diabetes.[6]

Caffeic acid (Figure 1) is a natural phenolic compound of the hydroxycinnamic acid derivative.[7] A previous study reported that caffeic acid showed antidiabetic effects in a rat model of type 2 diabetes.[8] Caffeic acid has been investigated as a potential dipeptidyl peptidase-4 and matrix metalloproteinase-9 inhibitor according to the in vitro enzyme assays and molecular simulation study.[9,10]

Reversed-phase high-performance liquid chromatography (RP-HPLC) was implemented to determine the caffeic acid content in Robusta coffee brews. This method allows effective separation of caffeic acid from complex mixtures and quantifies analytes with accurate and high sensitivity.[11] Throughout the optimization phase, several RP-HPLC parameters were identified using a response surface methodology (RSM) based design of experiments that statistically evaluates multiple variables and their interactions.[12] Box–Behnken Design (BBD), an RSM-based design, was used for optimization.[13–15] The experimental design was constructed using *R* software (v4.3.3).

A study by Gani and Istyastono (2021) found that Robusta spent coffee grounds by RP-HPLC with a UV detector yielded a value of 0.17% ± 0.006% w/w caffeic acid (relative standard deviation [RSD] = 3.63%).[7] As studies on caffeic acid in Robusta coffee brews remain limited, it is necessary to establish an optimized and validated analytical method that ensures accuracy, precision, and reproducibility.

This research focused on performing RSM-based optimization of RP-HPLC methods and validating the RP-HPLC analytical method for the quantification of caffeic acid in Robusta coffee brews. Acetonitrile percentage, formic acid percentage, and flow rate were selected as independent variables, while the dependent variables were resolution, retention time, tailing factor, and theoretical plate number.

## Materials and Methods

### Material

Caffeic acid reference standard was purchased from Sigma-Aldrich; its purity was certified to be 98%. Methanol (LC grade), formic acid (98%–100%), and anhydrous sodium sulfate were acquired from Merck Millipore. Acetonitrile (LC grade) and ethyl acetate were supplied by SmartLab. Filter paper was obtained from CV. General Labora, Indonesia, and distilled water were prepared in the Analytical and Instrument Chemistry Laboratory, Faculty of Pharmacy, Sanata Dharma University. Roasted Robusta coffee ground (Excelso Robusta Gold, Santos Jaya Abadi, Sidoarjo, Indonesia) was purchased from a local store in Sleman, Yogyakarta, Indonesia.

### Instruments and Application

The analysis was performed using a Shimadzu®LC-2010CHT system (No. C21254706757) equipped with a UV/Vis detector and operated with Lab Solution software. A $C_{18}$ column (Hibar® RT 250 mm × 4.6 mm; manufacturer's part number 1.51456.0001) with Purospher® STAR RP-18 endcapped (5 µm) as the stationary phase, coffee machine (Kris® 125 mL) equipped with a specific nylon filter for Kris® 125 mL, Ohaus® semi-micro analytical balance (PAJ1003 series max. 120 g; min. 0.001 g), RADWAG® ultra-micro analytical balance (UYA 2 series. 3Y series; readability: 0.1 µg), 250 mL separatory funnel (Pyrex®, Fortuna®), HPLC vials, vacuum rotary evaporator (Buchi Rotavapor® R-300), inorganic solvent membrane filter (Whatman®, pore size 0.45 µm mixed cellulose membrane, diameter 47 mm), syringe filter (Millipore®, pore size 0.20 µm hydrophilic PTFE membrane, diameter 25 mm), and a set of micropipettes (Socorex®), glassware for analysis (Pyrex®, IWAKI®), *R* software (v4.3.3), and RStudio (v2024.09.01 Build 394) with the "rsm" package.[16]

### Preparation of Caffeic Acid Standard Solution

An accurately weighed amount of 1.0 mg of caffeic acid was transferred into a 10 mL volumetric flask, followed by dilution with methanol to the calibrated mark to obtain a 100 µg/mL stock solution. The stock solution was diluted to obtain a 5 µg/mL standard solution. The solutions were filtered through a sterile membrane syringe filter (0.45 µm) before being injected into the RP-HPLC system.

### Robusta Coffee Brews Preparation

Ten grams of roasted Robusta coffee grounds were weighed, then set on the specific nylon filter for the Kris® 125 mL coffee machine. One hundred milliliters of distilled water was poured into the water reservoir of the coffee machine, then heated to 85°C. The heated water then flowed through the coffee grounds on the nylon filter. The spent coffee grounds of Robusta coffee were removed, whereas Robusta coffee brews were collected for the sample extraction.

## Extraction of the Samples

Robusta coffee brews were transferred into a 250 mL separatory funnel for liquid–liquid extraction with 50 mL of ethyl acetate until two distinct phases were formed. This extraction was repeated three times. The ethyl acetate phase was then collected and transferred to a glass beaker, followed by the addition of anhydrous sodium sulfate. The mixture underwent filtration through qualitative filter paper obtained from CV. General Labora, Indonesia, to obtain a yellowish solution. The yellowish solution was then evaporated using a rotary evaporator until all residue was fully dried, yielding a dried residue containing caffeic acid. This residue was diluted with methanol to a volume of 10.0 mL. The final solution obtained was labeled as the extracted sample solution for subsequent analysis.

## Response Surface Methodology

In the BBD-aided optimization, three different levels of independent variables: Acetonitrile percentage (X1), formic acid percentage (X2), and flow rate (X3) were investigated (Table 1). Retention time (Y1), resolution (Y2), tailing factor (Y3), and theoretical plate count (Y4) were selected as dependent variables. Using *R*, a 16-run BBD was created. The analysis was performed at a wavelength of 326 nm with an injection volume of 10 µL.

## Multiple Response Optimization

Data from 16 experimental runs were used for multiple response optimization. *R* software was utilized to perform analysis of variance to identify significant variations between the independent variables, generating eigenvalues, stationary points, and perspective plots for each dependent variable, thereby providing a visual RSM model. The desirability function was applied to analyze all responses.[16]

## System Suitability Test

An accurately weighed amount of 1.0 mg of caffeic acid was transferred into a 10 mL volumetric flask, followed by dilution with methanol to the calibrated mark to obtain a 100 µg/mL stock solution. A standard addition method was prepared by transferring 250 µL of the extracted sample solution into a 5 mL volumetric flask. Subsequently, the solution was spiked with 500 µL of the caffeic acid stock solution, and methanol was added to the calibrated mark. This resulted in an addition standard concentration of 10 µg/mL. This result represents the concentration of added standard and does not include the actual analyte present in the extracted sample solution. Six repetitions of reference standard and coffee extracted sample solutions were injected into the RP-HPLC system at 10 µL.

## Analytical Method Validation

### Selectivity

A 100 µg/mL stock solution was prepared. Then, a 16 µg/mL standard solution of caffeic acid was prepared by transferring 0.8 mL of a stock solution into a 5 mL volumetric flask and diluting with methanol. Afterwards, 1 mL of the extracted sample solution was transferred into a 5 mL volumetric flask and added with 0.75 mL of the stock solution, then diluted with methanol. Each solution, approximately 1.5 mL, was individually filtered through a membrane filter into separate HPLC vials. Chromatographic peak profiles of the standard solution and the spiked sample were compared to assess selectivity. The method was considered selective if the resolution value (Rs) was greater than 1.5.

### Linearity and Range

A series of standard solutions (5.39–32.26 µg/mL) were assessed for linearity. Peak areas versus concentration were plotted to construct the calibration curve. Slope, intercept, and coefficient of determination ($R^2$) were calculated using linear regression.

### Accuracy and Precision

Standard addition method was carried out at concentration levels of 5, 10, and 15 µg/mL with three replicates. One milliliter of the extracted sample solution was transferred into a separate 5 mL volumetric flask and diluted with methanol. Analyses were conducted on intraday and interday. Accuracy was represented as recovery, while precision was calculated by RSD.

### Sensitivity

Sensitivity was assessed through the calculation of the limit of detection (LOD) and the limit of quantification (LOQ) using the standard deviation (SD) approach according to Miller and Miller.[17]

**Table 1.** Parameter and Optimization Levels.[22]

| Variables | Low (−1) | Medium (0) | High (+1) |
|---|---|---|---|
| X1: Acetonitrile (%) | 5 | 10 | 15 |
| X2: Aqueous phase, pH 2.9 (%) | 75 | 80 | 85 |
| X3: Flowrate (mL/min) | 0.7 | 0.9 | 1.1 |

### Content Determination of Caffeic Acid

Two milliliters of the extracted sample solution were transferred into a 5 mL volumetric flask, resulting in a dilution factor of 2.5. Before being injected into the RP-HPLC system, the sample was filtered through a membrane filter (0.45 μm).

## Results and Discussion

### Response Surface Methodology

*R* statistical software was used to construct the BBD model. BBD was selected to optimize RP-HPLC separation conditions due to its efficiency and cost-effectiveness in model development.[13,14] Sixteen runs were examined using the BBD, testing various combinations of the independent variables X1, X2, and X3. Four replicates at the central point were incorporated to support a second-order response surface model. Response data for the dependent variables Y1, Y2, Y3, and Y4 were collected. To ensure complete elution of all compounds, each BBD experiment was conducted with a run time of 40 min. Table 2 displays the results of the 16 experiments, followed by an RSM statistical analysis to examine the effects of the independent variables on the measured responses.

Table 3 presents the RSM analytical findings, including *p* value, multiple $R^2$, adjusted $R^2$, lack of fit, stationary points, and eigenvalues. In RSM statistical analysis, a predictive model was considered to have a statistically significant effect and reject the null hypothesis ($H_0$) if the *p* value was <.05.[18] The significance of factors (independent variables) in influencing the response (dependent variable) was deemed significant when the model exhibited a multiple $R^2 \geq 0.8$, adjusted $R^2 \geq 0.8$, and a difference between multiple $R^2$ and adjusted $R^2 \leq 0.2$.[12,18] The lack of fit value of the obtained model must be ≥0.05 to indicate that the regression model is appropriate and does not exhibit a significant lack of fit.[18]

In the RSM statistical analysis of all responses, all equation models generated met the acceptable *p* value

**Table 2.** Experimental Results Based on BBD Design.

| | Factors | | | | Responses | | | |
|---|---|---|---|---|---|---|---|---|
| Run | MeOH (%)* | ACN (%) | Aqueous phase, pH 2.9 (%) | Flowrate (mL/min) | $t_R$ | Rs | Tf | *N* |
| 1 | 15 | 10 | 75 | 0.7 | 14.137 | 14.657 | 1.100 | 10,468.943 |
| 2 | 15 | 5 | 80 | 1.1 | 16.829 | 31.772 | 1.057 | 10,523.179 |
| 3 | 10 | 10 | 80 | 0.9 | 14.336 | 17.134 | 1.076 | 11,178.952 |
| 4 | 5 | 10 | 85 | 1.1 | 15.266 | 26.076 | 1.065 | 11,549.982 |
| 5 | 10 | 10 | 80 | 0.9 | 14.358 | 17.209 | 1.076 | 11,147.022 |
| 6 | 15 | 5 | 80 | 0.7 | 26.565 | 37.611 | 1.051 | 12,877.376 |
| 7 | 10 | 10 | 80 | 0.9 | 14.280 | 17.253 | 1.078 | 11,139.853 |
| 8 | 5 | 10 | 85 | 0.7 | 24.552 | 21.889 | 1.059 | 14,288.066 |
| 9 | 15 | 10 | 75 | 1.1 | 9.012 | 17.377 | 1.096 | 8,698.548 |
| 10 | 10 | 5 | 85 | 0.9 | 29.962 | 41.587 | 1.063 | 13,578.445 |
| 11 | 5 | 15 | 80 | 0.7 | 12.274 | 15.674 | 1.113 | 12,001.278 |
| 12 | 5 | 15 | 80 | 1.1 | 8.475 | 13.444 | 1.115 | 9,730.352 |
| 13 | 10 | 15 | 75 | 0.9 | 8.257 | 10.485 | 1.130 | 9,233.773 |
| 14 | 10 | 10 | 80 | 0.9 | 14.259 | 17.320 | 1.077 | 11,159.831 |
| 15 | 20 | 5 | 75 | 0.9 | 14.890 | 14.403 | 1.052 | 10,216.621 |
| 16 | 0 | 15 | 85 | 0.9 | 11.552 | 5.157 | 1.068 | 13,995.954 |

**Note:** *Not included for generating the RSM model, $t_R$: Retention time, Rs: Resolution, Tf: Tailing factor, *N*: Theoretical plate number.

**Table 3.** RSM Analysis of Retention Time, Resolution, Tailing Factor, and Theoretical Plate Number.

| | | | | | Stationary Points and Eigenvalues | | |
|---|---|---|---|---|---|---|---|
| Responses | *p* Value | Multiple $R^2$ | Adjusted $R^2$ | Lack of Fit | ACN (%) | Aqueous phase, pH 2.9 (%) | Flowrate (mL/min) |
| Retention time | $7.985 \times 10^{-7}$ | 0.9969 | 0.9922 | $1.672 \times 10^{-4}$ | 13.194<br>(19.222) | 75.185<br>(0.625) | 1.137<br>(−0.023) |
| Resolution | $6.933 \times 10^{-3}$ | 0.9323 | 0.8308 | $5.665 \times 10^{-6}$ | 15.690<br>(82.853) | 80.494<br>(0.210) | 0.872<br>(−0.071) |
| Tailing factor | $1.418 \times 10^{-5}$ | 0.9919 | 0.9796 | $1.161 \times 10^{-2}$ | −3.223<br>(0.028) | 0.033<br>(0.0003) | 4.520<br>(0.000) |
| Theoretical plate number | $6.776 \times 10^{-7}$ | 0.9971 | 0.9926 | $1.412 \times 10^{-3}$ | 12.610<br>(1148.714) | 66.625<br>(3.265) | 1.964<br>(−10.898) |

(<.05), multiple $R^2$ and adjusted $R^2$ (≥0.8), and the difference between multiple $R^2$ and adjusted $R^2 < 0.2$. The lack of fit observed in the four responses implies that the regression model is statistically inadequate (<0.05).[18] Nevertheless, it must be noted that the sufficiency of the model as a prediction equation has not been guaranteed. The multiple $R^2$ and adjusted $R^2$ values for all responses approached one, suggesting strong model fit and explanatory power. The small difference between them (<0.2) also indicates that the models were not overfitted.[12,18] Although the lack-of-fit tests were statistically significant, the desirability function was used to identify candidate optimum conditions. The final optimized conditions were subsequently confirmed through experimental validation and evaluated against the predefined system suitability criteria.[18]

The stationary point represents the model's estimated optimum condition. For the retention-time response, the estimated stationary point was 13.194% acetonitrile, 75.185% formic-acid phase, and a flow rate of 1.137 mL/min. The estimated flow rate was slightly outside the investigated range of 0.7–1.1 mL/min. Furthermore, the eigenvalues had mixed signs, indicating that the stationary point was a saddle point rather than a practical optimum.[12,14] The eigenvalues indicate the response surface's stationary points. A downward-curved plot indicates the maximum point (negative eigenvalues), while an upward-curved one shows the minimum point (positive eigenvalues). A saddle point, resulting from mixed values, lacks both maximum, and minimum criteria.[12,14] Three-dimensional response surface plots were generated using *R* software, with the vertical axis representing predicted responses (retention time, resolution, tailing factor, theoretical plate number) and the horizontal axes for coded levels of two factors (acetonitrile vs. formic acid, formic acid vs. flowrate, acetonitrile vs. flowrate), as shown in Figure 2. It can be observed that the

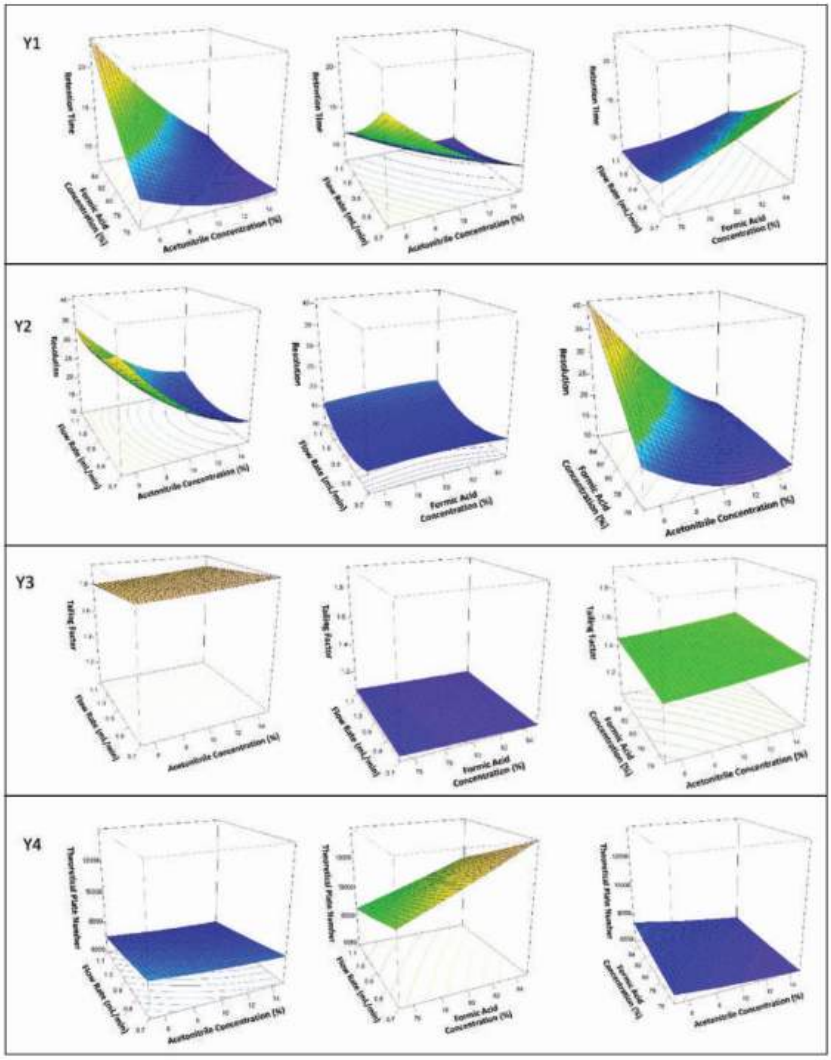


**Figure 2.** Perspective Plot.

**Note:** Retention time: Y1, Resolution: Y2, Tailing factor: Y3, Theoretical plate number: Y4.

responses of retention times (Y1), resolution (Y2), tailing factor (Y3), and theoretical plate number (Y4) for caffeic acid separations were influenced by independent variables.

Caffeic acid can be separated using RP-HPLC with a $C_{18}$ column. The $C_{18}$ stationary phase has the capacity to interact with caffeic acid through van der Waals forces, while the methanol and acetonitrile could interact with caffeic acid through hydrogen interactions, where caffeic acid can act as a hydrogen donor or acceptor. These interactions enhanced the retention and separation of caffeic acid during the chromatography process. Therefore, optimizing the mobile phase composition can significantly improve the resolution and efficiency of the separation.

## Multiple Response Optimization

The four responses used to build a prediction model for caffeic acid separation in RP-HPLC were retention time, resolution, tailing factor, and theoretical plate number. The purpose of the retention time response is to obtain the lowest possible value; the purpose of the tailing factor response is to achieve a target value (Tf = 1), and the purpose of the theoretical plate number and resolution response is to attain the highest possible value.[19]

The desirability function was employed to compare the various objectives and thereby produce optimal values for the four responses.[12] Each objective is transformed into an individual desirability function value ($d_i$) that ranges from $0 \leq d_i \leq 1$, where a value of $d_i = 0$ denotes a response outside acceptable limits and $d_i = 1$ indicates the best value.[14] Using *R* software, the desirability function was calculated based on the target criteria outlined in Table 4.

The desirability function shown in Table 4 was calculated based on four responses, including retention time, resolution, tailing factor, and theoretical plates. The optimization goal for retention time was minimum to enhance time efficiency, while the goal for resolution and theoretical plates was maximum to ensure good separation of analytes and column efficiency. The tailing factor was targeted to be as close to 1.0 as possible to indicate a symmetrical peak. The desirability function has a value from 0 to 1. The desirability value of 0 indicates the most unwanted response, whereas a value of 1 indicates the most desirable response due to its closest to the target value.[20,21] The developed model suggested that the optimum chromatographic conditions for caffeic acid analysis using RP-HPLC comprised a mobile phase of methanol:acetonitrile:aqueous formic acid pH 2.9 (10:15:75 v/v/v) with a flow rate of 1 mL/min, achieving a desirability value of 0.8107. The system suitability test was executed under these optimum conditions.

## System Suitability Test

To verify that the chromatographic system maintains adequate resolution and precision for reliable analysis, system suitability tests were carried out.[22] The system suitability is assessed by comparing six replicate injections of both the reference standard and the standard addition. Parameters, including retention times, resolution, tailing factor, and theoretical plate number, were evaluated. System suitability test results are outlined in Table 5, and chromatogram profiles in Figure 3.

For the reference standard and the standard addition, all parameters met the acceptance criteria, such as retention time <10 min, tailing factor ≤2, resolution >2, number of theoretical plates ($N > 2{,}000$), and RSD ≤ 2%.[22]

## Analytical Method Validation

### Selectivity

Selectivity refers to a method's ability to respond to multiple components in a mixture, as observed in the separation of analyte chromatogram peaks, which is expressed by the

**Table 4.** Multiple Response Optimization for Caffeic Acid with Desirability Function.

| | Optimization Parameters | | | |
|---|---|---|---|---|
| Responses | Lower | Target | Upper | Goals |
| Retention time | – | 5 | 15 | Minimum |
| Resolution | 1.5 | 15 | – | Maximum |
| Tailing factor | 0.8 | 1 | 1.2 | Target |
| Theoretical plate number | 2,000 | 10,000 | – | Maximum |

**Table 5.** Test Results of the System Suitability Test.

| | $t_R$ | | Rs | | Tf | | *N* | | AUC | |
|---|---|---|---|---|---|---|---|---|---|---|
| Analytes | Mean | RSD (%) | Mean | RSD (%) | Mean | RSD (%) | Mean | RSD (%) | Mean | RSD (%) |
| Caffeic acid | 7.671 | 0.566 | 9.632 | 1.720 | 1.031 | 1.916 | 7,903.997 | 1.755 | 321,766.167 | 1.919 |
| Coffee sample | 7.681 | 1.149 | 3.878 | 1.696 | 1.115 | 0.944 | 8,504.008 | 1.465 | 533,554.33 | 1.885 |

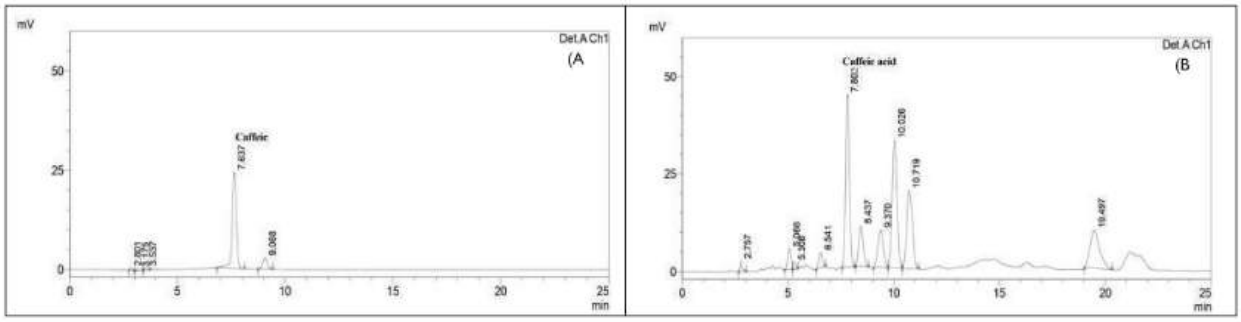


**Figure 3.** HPLC Chromatogram of Reference Standard (A) and Standard Addition Coffee Sample (B). Note: Mobile phase, methanol:acetonitrile:aqueous formic acid pH 2.9 (10:15:75 v/v/v), flowrate 1 mL/min, $C_{18}$ column of Hibar® RT 250 mm × 4.6 mm Purospher® STAR RP-18 Endcapped (5 µm), column temperature of 27 °C, 10 µL injection volume, and wavelength detection at 326 nm.

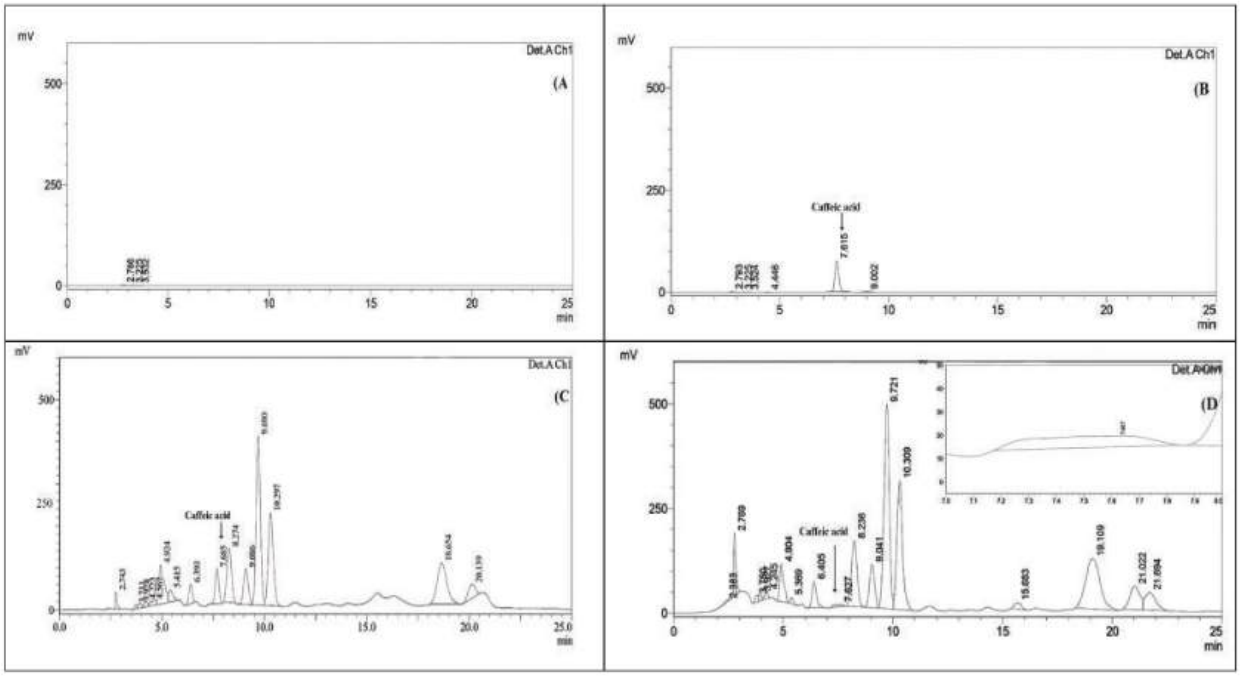


**Figure 4.** HPLC Chromatogram of Methanol as Solvent (A), Reference Standard (B), Standard Addition Coffee Sample (C), and Coffee Sample (D).

**Note:** Mobile phase, methanol:acetonitrile:aqueous formic acid pH 2.9 (10:15:75 v/v/v), flowrate 1 mL/min, $C_{18}$ column of Hibar® RT 250 mm × 4.6 mm Purospher® STAR RP-18 Endcapped (5 µm), column temperature of 27 °C, 10 µL injection volume, and wavelength detection at 326 nm.

resolution value (Rs). Analytes are said to be separated selectively if the Rs value is ≥1.5.[22] Chromatogram peaks from the methanol blank, reference standard, and the addition standard sample were evaluated. The standard peak (16 µg/mL) was used as a reference for identifying the caffeic acid peak in the sample, while the blank ensured that the solvent did not interfere with the analysis of the desired compound. The selectivity chromatogram profiles are shown in Figure 4.

## *Linearity and Range*

Caffeic acid showed good linearity from 5.36 to 32.36 µg/mL, and the calibration curve equation was $y = 61868x - 86530$, with a determination coefficient ($R^2$) of 0.9987, conforming to a linear response to caffeic acid concentrations within the specified range. The $R^2$ value met the acceptance criteria of $R^2 \geq 0.99$ and showed good linearity in the tested concentration range.[22]

**Table 6.** Results of Accuracy and Precision ($n$ = 3).

| Added Amount (µg/mL) | Found Amount (µg/mL) | Recovery (%) | RSD (%) |
|---|---|---|---|
| Intraday | | | |
| 5.15 | 4.75 | 92.26 | 1.26 |
| 10.31 | 10.23 | 99.21 | 0.71 |
| 15.46 | 15.91 | 102.94 | 1.94 |
| Interday | | | |
| 5.22 | 4.95 | 94.89 | 3.14 |
| 10.44 | 10.30 | 98.65 | 1.27 |
| 15.66 | 16.13 | 102.96 | 2.03 |

**Table 7.** The Results of Content Determination of Caffeic Acid in Roasted Robusta Coffee Brew.

| Replication | Sample weight (g) | Measured concentration (µg/ml) | Sample volume (mL) | Dilution factor | Sample concentration (% w/w) |
|---|---|---|---|---|---|
| 1 | 10.01 | 3.079 | | | 0.770 |
| 2 | 10.01 | 3.086 | | | 0.771 |
| 3 | 10.00 | 3.153 | | | 0.788 |
| 4 | 10.00 | 3.072 | 10 | 2.5 | 0.768 |
| 5 | 10.01 | 3.069 | | | 0.767 |
| 6 | 10.00 | 3.067 | | | 0.767 |
| Mean | | | | | 0.772 |
| Standard deviation | | | | | 0.008 |
| Relative standard deviation | | | | | 1.066 |

### *Accuracy and Precision*

Accuracy and precision were evaluated intraday and interday based on the standard addition method. For the addition, 1 mL of the extracted sample solution (obtained after evaporation of the extraction solvent and reconstitution to 10 mL with methanol) was transferred into three separate 5 mL volumetric flasks and spiked with caffeic acid standard solution concentrations of 5, 10, and 15 µg/mL, respectively, followed by dilution with methanol. The percent recovery and RSD were calculated from each concentration level, with each level replicated three times.[23] The acceptance criteria for recovery percentage and RSD for standard solutions in the range of 5–15 µg/mL were 90%–107% and ≤5.3%, respectively.[24,25] The results presented in Table 6 confirm that the method produced precise and accurate caffeic acid determination results across all concentration levels.

### *Sensitivity*

SD approach was used to calculate the LOD and LOQ using the equations LOD = 3.3 × $S_{(y/x)}$/b and LOQ = 10 × $S_{(y/x)}$/$b$, where $S_{(y/x)}$ represents the residual SD of the regression and $b$ is the slope of the calibration curve. The SD calculation approach according to the guidelines.[17,22] The calibration curve was constructed using five concentration levels (1.29–5.36 µg/mL) of caffeic acid. The LOD was 0.08032 µg/mL, whereas the LOQ was 0.2434 µg/mL. Levels of caffeic acid (1.29–5.36 µg/mL) with the regression equation $y = 40098x + 27139$. The residual SD of the regression, $S_{(y/x)}$ = 976.0162, and the slope of $b$ = 40,098 were implemented for the calculation.

### *Content Determination of Caffeic Acid*

Caffeic acid content in Robusta coffee brews was quantified using an optimized and validated RP-HPLC system (Table 7). Based on six replicates, the caffeic acid content was 0.772 ± 0.008 mg/g, with an RSD of 1.066%. This RSD value satisfied the acceptance criteria of less than 2%. It can be estimated that a single serving of brewed coffee (100 ml) with 10 g of coffee grounds contains approximately 7.72 mg of caffeic acid.

The level of caffeic acid detected was found to be higher than in the previous study. Gani and Istyastono reported the level of caffeic acid in Robusta spent coffee grounds found to be 0.17% ± 0.006% w/w.[7]

## Conclusion

This study successfully developed and optimized an RP-HPLC method by employing the RSM model for

the determination of caffeic acid in Robusta coffee brews. The optimized condition was implemented during the validation stage of the analytical method. It was found that the optimized analysis condition provided satisfactory separation of analytes, allowing the analysis of the target compound. Additionally, this method demonstrated a good linearity within the tested concentration range, with acceptable recovery percentage and low RSD, indicating high accuracy and precision. These results demonstrate that the developed RP-HPLC method is suitable for determining the caffeic acid content in Robusta coffee brews. However, the measured caffeic acid concentrations (3.07–3.15 μg/mL) were below the validated linear range of 5.36–32.36 μg/mL. This represents a limitation of the present study, and future work will extend the validated linear range to include lower concentration levels. The sample preparation method is relatively simple and time-efficient, supporting its applicability in analytical laboratories. The developed RP-HPLC method also provides high accuracy, sensitivity, and reproducibility, which proves its suitability for routine quality control analysis in the pharmaceutical industry, involving caffeic acid analysis in coffee or other related samples.

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## Authors' Contribution

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be authors as per the International Committee of Medical Journal Editors requirements/guidelines.

## Consent to Participate

Not applicable.

## Consent for Publication

Not applicable.

## Data Availability Statement

All the data are available with the authors and shall be provided upon request.

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The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Ethical Approval

This study does not involve experiments on animals or human subjects.

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## Informed Consent

Not applicable.

## Use of Artificial Intelligence-assisted Tools

The authors declare that they have not used artificial intelligence (AI) tools for writing and editing of the manuscript, and no images were manipulated using AI.

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