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Response Surface Methodology–Based Optimization of HPLC Conditions for Quantification of Paracetamol in Indonesian Traditional Medicines (*Jamu*)

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Abstract: Indonesian traditional herbal medicines (Jamu) are prohibited from containing active pharmaceutical ingredients (APIs). Nevertheless, reports indicate that some hyperuricemia jamu products still contain such substances, including paracetamol This study aimed to optimize High-Performance Liquid Chromatography (HPLC) conditions and to validate an analytical method for the determination of paracetamol in jamu for hyperuricemia obtained from Pasar Besar, Malang City. Response Surface Methodology (RSM) combined with a Box–Behnken Design (BBD) was employed to optimize the HPLC conditions. The investigated parameters were the methanol proportion in the mobile phase (X_1, 30–90% v/v), flow rate (X_2, 0.5–1.0 mL/min), and column temperature (X_3, 20–30 °C). The evaluated response variables included peak area (Y_1), resolution (Y_2), tailing factor (Y_3), and theoretical plate number (Y_4). The optimal HPLC conditions consisted of 30% v/v methanol in aquadest, a flow rate of 1.0 mL/min, and a column temperature of 25.18 °C. Method validation demonstrated satisfactory selectivity (λmax 245 nm; paracetamol retention time ± 3.33 min), linearity (r^2 = 0.99), limit of detection (LOD) of 1.51 ppm, and limit of quantification (LOQ) of 5.03 ppm. The method also showed acceptable accuracy (101.75–104.61%) and precision (0.53–1.59%), fulfilling the

Keywords: *Jamu for hyperuricemia, paracetamol, HPLC, response surface methodology, Box-Behnken design.*

1. Introduction

Jamu is a form of Indonesian traditional herbal medicine that has been widely consumed, as its efficacy is traditionally believed to be based on hereditary knowledge and long-standing empirical use (Sumarni et al., 2019). According to the Regulation of the Minister of Health of the Republic of Indonesia No. 7 of 2012 on the registration of traditional medicines, such products are strictly prohibited from containing active pharmaceutical ingredients (APIs), defined as isolated or synthetic substances with pharmacological activity. Nevertheless, the intentional addition of APIs to traditional herbal medicines is often carried out to enhance or provide rapid therapeutic effects. A public report released by the Indonesian Food and Drug Authority (BPOM) in 2022 revealed that 95 traditional medicines and health supplements were found to contain undeclared APIs. The long-term consumption of such adulterated products may lead to serious health risks, including organ dysfunction and even death (Putri et al., 2023).

One of the APIs frequently detected in traditional herbal medicines is paracetamol. Paracetamol is a widely used analgesic and antipyretic agent. Due to its pharmacological effects, paracetamol is often illicitly added to herbal products intended to relieve pain and inflammation, including *Jamu* for hyperuricemia (Ashraful Islam et al., 2011). *Jamu* for hyperuricemia is formulated to alleviate symptoms associated with elevated uric acid levels and to reduce serum uric acid concentration. Hyperuricemia can cause various clinical manifestations, such as musculoskeletal pain, joint stiffness, and inflammatory responses in the joints (Savitri, 2017). Consequently, the presence of paracetamol in such products may provide symptomatic relief, thereby misleading consumers regarding the true efficacy of the herbal formulation. However, excessive or prolonged intake of paracetamol is associated with adverse effects, including gastrointestinal disturbances, renal impairment, cardiovascular disorders, and hepatotoxicity (Chidiac et al., 2023; McCrae et al., 2018).

Several analytical techniques have been reported for the determination of paracetamol in herbal medicines, including High-Performance Liquid Chromatography (HPLC) (Pratama et al., 2022; Wisnuwardhani et al., 2018), thin-layer chromatography (Fitrianasari et al., 2023), liquid chromatography–mass spectrometry (LC–MS) (Taupik et al., 2022), and UV–Vis spectrophotometry (Husain et al., 2023). In the present study, reverse-phase High-Performance Liquid Chromatography (RP-HPLC) was selected for paracetamol analysis. Compared with other analytical techniques, HPLC offers superior sensitivity and selectivity, particularly for quantitative analysis, enabling the detection and accurate quantification of analytes at low concentration levels. Moreover, HPLC provides efficient

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separation of complex matrices and shorter analysis times compared to thin-layer chromatography, making it more suitable for routine analysis.

RP-HPLC was chosen due to its versatility and applicability to a wide range of analytes, particularly polar and moderately ionic compounds (Hameedat et al., 2022; Gupta et al., 2022). This characteristic makes RP-HPLC especially suitable for analyzing paracetamol in *Jamu*, which contains a complex mixture of bioactive constituents. To achieve optimal chromatographic performance and comply with analytical validation requirements, RP-HPLC conditions must be carefully optimized. Therefore, this study applied Response Surface Methodology (RSM) using a Box–Behnken Design (BBD) to optimize key RP-HPLC parameters, including methanol composition, flow rate, and column temperature. The evaluated response variables were peak area, resolution, tailing factor, and the number of theoretical plates.

2. Experimental Section

Apparatus

An HPLC i-Series LC-2030C 3D Plus system (Shimadzu, Japan) was employed for method development and validation. The system was equipped with a quaternary pump, autosampler, column oven, and a photodiode array (PDA) UV detector. Chromatographic separation was performed using a C18 ODS column (150 mm × 4.6 mm i.d., 5 µm particle size) as the stationary phase.

The mobile phase consisted of methanol and water for injection (WFI) and was delivered under gradient elution conditions. The aqueous phase was filtered twice prior to use. All solutions were filtered through a 0.45 µm nylon syringe filter (13 mm diameter) before analysis. Detection was carried out at a wavelength of 245 nm, and the injection volume was set at 20 µL.

HPLC data acquisition and processing were performed using LabSolutions software (Shimadzu, Japan). Optimization and statistical analysis were conducted using Statgraphics Centurion 18 software (*Statgraphics Centurion* Inc., Virginia, USA).

Reagents and Materials

HPLC-grade methanol was purchased from Fisher Scientific (Fair Lawn, USA). Water for injection (WFI) was obtained from a local pharmacy. The crude drugs, namely *Sonchus arvensis*, *Biancaea sappan*, *Stelechocarpus burahol* L., *Curcuma xanthorrhiza*, *Curcuma longa*, and *Phyllanthus niruri*, were purchased from a local herbal store and obtained in powdered form.

Jamu samples for hyperuricemia were collected from Pasar Besar, Malang City, and labeled as AU1, AU2, and AU3. The samples originated from different commercial brands with undisclosed ingredient compositions.

Optimization of HPLC Condition

Response Surface Methodology (RSM) combined with a Box–Behnken Design (BBD) was applied to optimize the RP-HPLC conditions. The independent variables investigated were methanol proportion in the mobile phase (X_1, % v/v), flow rate (X_2, mL/min), and column temperature (X_3, °C). The evaluated response variables were peak area (Y_1), resolution (Y_2), tailing factor (Y_3), and the number of theoretical plates (Y_4).

The BBD experimental design, consisting of 15 experimental runs, was generated using Statgraphics Centurion 18 software (Statgraphics Centurion Inc., Virginia, USA). The experimental levels of methanol proportion, flow rate, and column temperature were defined according to the Box–Behnken Design, as presented in Table 1. All experiments were conducted in accordance with the designed matrix, and the response model coefficients were obtained by fitting the experimental data to a second-order polynomial equation. Analysis of variance (ANOVA) was subsequently performed to evaluate the significance and adequacy of the developed model.

Table 1. Independent Variables and Their Levels in the Box–Behnken Design

Variables Independent	Level			Unit
	-1	0	1	
Ratio of methanol (X_1)	30	60	90	% v/v
Flow rate (X_2)	0.5	0.75	1	mL/minutes
Column temperature (X_3)	20	25	30	℃

Preparation of *Jamu* for Hyperuricemia

Preparation of *Jamu* Matrix

The *Jamu* matrix for hyperuricemia was prepared by weighing powdered crude drugs consisting of 10.53% *Sonchus arvensis*, 26.32% *Biancaea sappan*, 15.79% *Stelechocarpus burahol* L., 15.79% *Curcuma xanthorrhiza*, 15.79% *Curcuma longa*, and 15.79% *Phyllanthus niruri* to obtain a total weight of 10 g. All components were thoroughly mixed and homogenized to obtain a uniform *Jamu* powder matrix.

Preparation of *Jamu* Matrix Solution

The *Jamu* matrix solution was prepared by dissolving 100 g of the control herbal powder in 100 mL of HPLC-grade methanol. The mixture was vortexed for 2 min to ensure complete homogenization.

Preparation of *Jamu* Matrix Spiked with Paracetamol (25 ppm)

A spiked *Jamu* matrix solution containing paracetamol at a concentration of 25 ppm was prepared by transferring 2.5 mL of a 100 ppm paracetamol standard solution into a 10 mL volumetric flask and diluting to volume with the *Jamu* matrix solution.

Preparation of Paracetamol Standard Solution

A paracetamol standard solution was prepared by accurately weighing 10 mg of paracetamol and dissolving it in 10 mL of HPLC-grade methanol to obtain a stock solution with a concentration of 1000 ppm. Appropriate dilutions were prepared as required.

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Sample Preparation

Each *Jamu* sample for hyperuricemia was accurately weighed (100 mg) and dissolved in 100 mL of HPLC-grade methanol. The solution was vortexed for 2 min to ensure homogeneity and subsequently filtered through a 0.45 µm membrane filter into an HPLC vial. Each sample was prepared in triplicate.

System Suitability Test (SST)

The performance of the HPLC system was verified using a system suitability test (SST) in accordance with the Indonesian Pharmacopoeia, 6th edition, which requires reproducible chromatographic performance prior to analysis. The SST was conducted using six replicate injections of paracetamol standard solutions under the optimized chromatographic conditions obtained after BBD optimization.

The evaluated SST parameters included retention time, peak area, resolution, tailing factor, and the number of theoretical plates. System suitability was considered acceptable when the relative standard deviation (RSD) for all parameters was less than 2%.

Analytical Method Validation

The HPLC method was validated by evaluating its performance characteristics according to the guidelines of the International Council for Harmonisation (ICH) and the Association of Official Analytical Chemists (AOAC).

Specificity

Specificity was assessed by injecting the *Jamu* matrix solution, *Jamu* matrix spiked with paracetamol (25 ppm), and three commercial *Jamu* samples (AU1, AU2, and AU3). Specificity was confirmed by comparing retention times, chromatographic profiles, and PDA spectral overlays between the samples and the paracetamol reference standard.

Linearity

Linearity was evaluated by constructing a calibration curve using five paracetamol concentrations (10, 20, 30, 40, and 50 ppm). The peak area was plotted against the corresponding concentration, and linear regression analysis was performed. Linearity was considered acceptable when the correlation coefficient (r) exceeded 0.99, in accordance with AOAC guidelines (AOAC International, 2013).

Limit of Detection (LOD) and Quantification (LOQ)

The LOD and LOQ were calculated using the following equations:.

$$LOD = \frac{Sy}{b} \times 3 \quad (1)$$

$$LOQ = \frac{Sy}{b} \times 10 \quad (2)$$

$$Sy = \sqrt{\frac{\Sigma(y-yi)^2}{n-2}} \quad (3)$$

where b is the slope of the calibration curve, Sy is the standard deviation of regression, y is the observed peak area, y_i is the predicted peak area from the regression equation, and n is the number of calibration points (Ihsan et al., 2022).

Accuracy and Precision

Accuracy and precision were evaluated using *Jamu* matrix solutions spiked with paracetamol at concentrations of 20, 25, and 30 ppm. Accuracy was expressed as percent recovery (% recovery), while precision was expressed as the relative standard deviation (%RSD). According to AOAC Appendix K, acceptable criteria were % recovery in the range of 92–105% and %RSD less than 2%.

Determination of Paracetamol Content in *Jamu* Samples

Quantification of paracetamol in *Jamu* samples was performed for samples that tested positive for paracetamol during the specificity assessment. The paracetamol concentration was calculated by substituting the measured peak area into the linear regression equation obtained from the calibration curve.

3. Result and Discussion

This study applied a quantitative experimental approach to optimize HPLC conditions and validate an analytical method for the determination of paracetamol in *Jamu* for hyperuricemia. The optimized chromatographic conditions fulfilled the predefined acceptance criteria, as summarized in Table 2. These criteria included adequate selectivity, demonstrated by consistent peak shape and retention time of the analyte compared with the reference standard; satisfactory peak resolution; acceptable peak symmetry, expressed as the tailing factor; and high column efficiency, indicated by the number of theoretical plates.

The results indicate that the chromatographic performance was strongly influenced by key HPLC parameters, namely the methanol proportion in the mobile phase, flow rate, and column temperature. Optimization of these parameters was therefore essential to achieve reliable separation and accurate quantification of paracetamol in a complex *Jamu* matrix. Following optimization and method validation, the developed RP-HPLC method was subsequently applied to the analysis of paracetamol in commercial *Jamu* products for hyperuricemia marketed at Pasar Besar, Malang City.

Three *Jamu* samples, coded AU1, AU2, and AU3, were selected based on predefined inclusion and exclusion criteria. The inclusion criteria comprised products with indications related to pain relief, ease of purchase in the local market, and either registered or unregistered status with the Indonesian Food and Drug Authority (BPOM). Products in liquid dosage form, expired products, and samples with damaged packaging were excluded from the analysis.

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Table 2. Acceptance Criteria for Chromatographic Responses

Responses	Acceptance Criteria
RSD of peak area (Y_1)	< 2.0 %
Resolution (Y_2)	≥ 2.0
Tailing factor (Y_3)	< 2
Theoretical plate (Y_4)	> 2000

Design for HPLC Optimization

Response Surface Methodology combined with a Box–Behnken Design (RSM–BBD) was employed to optimize the RP-HPLC conditions. This design was selected because it requires a relatively small number of experimental runs, thereby reducing analysis time and experimental cost. In addition, the Box–Behnken Design enables the evaluation of linear, quadratic, and interaction effects of independent variables on the selected responses.The independent variables investigated were the methanol proportion in the mobile phase (X_1), flow rate (X_2, mL/min), and column temperature (X_3, °C), each examined at three levels (−1, 0, +1). The evaluated response variables included peak area (Y_1), resolution (Y_2), tailing factor (Y_3), and the number of theoretical plates (Y_4). A summary of the BBD experimental design is presented in Table 3.

The BBD consisted of 15 experimental runs, including three replicates at the center point and twelve runs at factorial (edge) points. The center point replicates were used to estimate the pure experimental error, while the randomized runs minimized the influence of uncontrolled variables and potential systematic bias (Elkady et al., 2022). Based on the preliminary chromatographic evaluation, some experimental runs yielded responses in which resolution and theoretical plate values could not be determined. This result indicates inadequate peak separation and/or unsuitable peak shapes, which prevent reliable calculation of these chromatographic parameters (Barth, 2019; Dolan, 2012; Wahab et al., 2017). The experimental data were subsequently analyzed using multiple regression analysis to model the relationship between the independent variables and the measured responses. The quadratic polynomial equations describing each response are presented below.

Y_1 (Peak area) $= 2.22\times10^6 + 114062A - 9.86\times10^6 \times B + 74262.9C - 232.614A^2 - 77107.2\times AB - 110.35A\,C + 7.01\times10^6 \times B^2 + 40001.8BC - 2086.3C^2$

Y_2 (Resolution) $= -6.32 + 0.07A + 6.34B + 0.21C + 0.00A^2 - 0.11AB - 0.00AC - 0.12B^2 + 0.07BC - 0.00C^2$

Y_3 (Tailing factor) $= -2.56 - 0.04A - 0.8B + 0.29C + 0.00A^2 - 0.0343333AB + 0.0000966667AC + 2.316B^2 + 0.0BC - 0.00579C^2$

Y_4 (Theoretical plates) $= -33810.6 + 597.632A - 15448.0B + 2185.74C - 3.53718A^2 - 145.0\,AB - 1.10833AC + 16892.7B^2 - 104.4BC - 40.2583C^2$

Table 3. Box–Behnken Design Matrix and Experimental Responses

Run	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4
1	0	-1	-1	3885509	0.85	-	7028
2	1	1	0	2246871	-	0.86	3869
3	1	0	1	2993366	-	0.82	3659
4	1	-1	0	4536657	-	0.76	5777
5	0	1	-1	1945259	0.19	-	5603
6	1	0	-1	2977228	-	0.77	3732
7	-1	0	1	1409220	1.76	-	854
8	-1	0	-1	1326872	1.26	-	262
9	0	-1	1	3602825	0.98	-	7390
10	0	0	0	2429075	0.76	-	6425
11	-1	0	0	1930220	-	-	2334
12	0	0	0	2400557	0.75	-	6444
13	0	1	1	1862584	0.67	-	5443
14	-1	1	0	1953650	3.37	1.13	4776
15	0	0	0	2484912	0.81	-	6081

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Statistical Analysis

The statistical significance of the developed models was evaluated using analysis of variance (ANOVA). The ANOVA results are summarized in Table 4. A model or individual term was considered statistically significant when the corresponding p-value was less than 0.05.

Table 4. Analysis of Variance (ANOVA) Summary for the Developed Models

	P-value for each model of responses			
Source	Y1	Y2	Y3	Y4
A	0.00	0.02	0.04	0.02
B	0.00	0.27	0.16	0.30
C	0.71	0.60	0.94	0.78
AA	0.16	0.84	0.01	0.00
AB	0.01	0.06	0.11	0.05
AC	0.90	0.73	0.92	0.72
BB	0.02	0.98	0.34	0.07
BC	0.70	0.81	1	0.78
CC	0.70	0.80	0.34	0.08

A: methanol proportion in the mobile phase, B: flow rate, C: column temperature, AA: quadratic effect of methanol proportion, AB: interaction effect between methanol proportion and flow rate, AC: interaction effect between methanol proportion and column temperature, BB: quadratic effect of flow rate, BC: interaction effect between flow rate and column temperature, CC: quadratic effect of column temperature.

Optimization and Effects on the Responses

Based on the ANOVA results (Table 4), the methanol proportion exhibited *p*-values < 0.05, indicating that this variable had a statistically significant effect on all evaluated responses. According to the Pareto chart for peak area (Figure 1A), the methanol proportion showed a positive effect on peak area. In chromatographic analysis, a peak represents the presence of a specific compound in a sample, and its area is directly proportional to the concentration of the analyte (Ministry of Health of the Republic of Indonesia, 2020; Chulikhit et al., 2023). In RP-HPLC, nonpolar compounds tend to be retained longer by the stationary phase, whereas polar compounds are eluted earlier, particularly when a polar mobile phase is used (Lundanes et al., 2014). As paracetamol is a polar compound, it interacts more favorably with the polar mobile phase. Increasing the methanol proportion enhances this interaction, resulting in improved elution and higher detector response, which is reflected by an increased peak area. Flow rate also exerted a significant influence on peak area. As shown in the Pareto chart (Figure 1A), flow rate had a negative effect on peak area, indicating that higher flow rates led to a reduction in peak area. This phenomenon occurs because increasing the flow rate shortens the retention time, limiting the establishment of equilibrium between the mobile and stationary phases. Consequently, separation efficiency decreases, leading to peak broadening. Pronounced peak broadening may reduce the overall peak area detected by the system (Koszur, 2023).

Regarding resolution (Figure 1B), the methanol proportion exhibited a negative effect. In RP-HPLC, increasing the methanol content reduces the polarity of the mobile phase, thereby weakening the interaction between polar analytes and the stationary phase. As a result, polar analytes are eluted more rapidly, which reduces the retention factor (*k*) and adversely affects chromatographic resolution. The distribution of analytes between the mobile and stationary phases plays a crucial role in determining both retention behavior and resolution (Ministry of Health of the Republic of Indonesia, 2020; Meyer, 2010).

The Pareto chart for the tailing factor (Figure 1C) indicated that the quadratic effect of methanol proportion positively influenced peak symmetry. Gradual increases in the methanol proportion improved peak symmetry, resulting in lower tailing factors. Methanol, as a polar solvent, enhances analyte solubility in the mobile phase, facilitating mass transfer and reducing analyte adsorption onto the stationary phase. This solvation effect produces narrower and more symmetrical peaks. However, beyond the optimal methanol proportion, further increases resulted in tailing factor values that no longer met the acceptance criteria, indicating deteriorated chromatographic performance (Dolan, 2012; Wahab et al., 2017).

Similarly, the quadratic effect of methanol proportion had a positive influence on the number of theoretical plates (Figure 1D). This relationship followed a quadratic trend rather than a linear one. Initially, increasing the methanol proportion enhanced column efficiency, as indicated by a higher number of theoretical plates, due to improved mass transfer and peak sharpening. However, exceeding the optimal methanol proportion led to band broadening and reduced column efficiency, ultimately decreasing the number of theoretical plates (Kazmouz et al., 2022).

Based on the 15 experimental runs generated by the Box–Behnken Design, the experimental data were processed using Statgraphics Centurion 18 to develop predictive models. Three-dimensional response surface plots (Figure 2) were constructed to visualize the effects of the independent variables on the responses. The optimized RP-HPLC conditions were determined to be a methanol proportion of 30% v/v, a flow rate of 1.0 mL/min, and a column temperature of 25.18 °C. Under these conditions, all evaluated responses met the predefined acceptance criteria. The optimized method was subsequently subjected to validation prior to its application for paracetamol analysis in *Jamu* samples.

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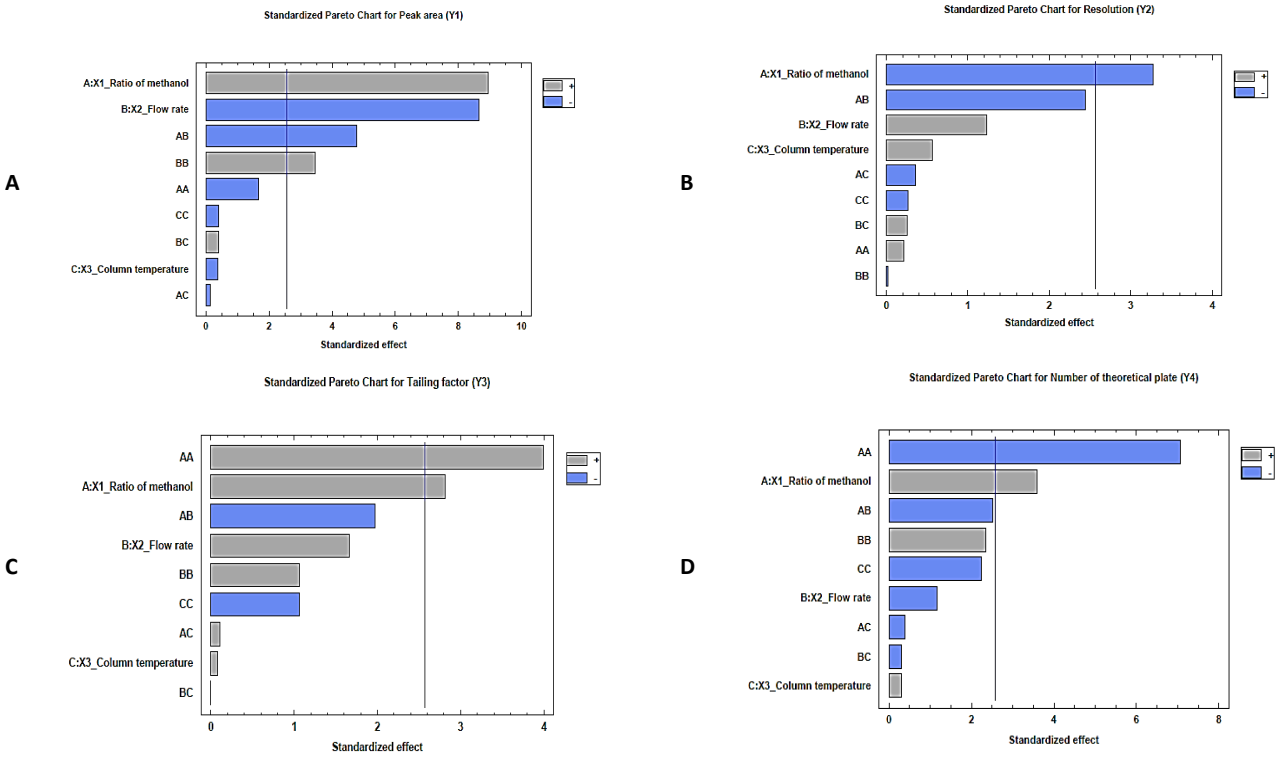
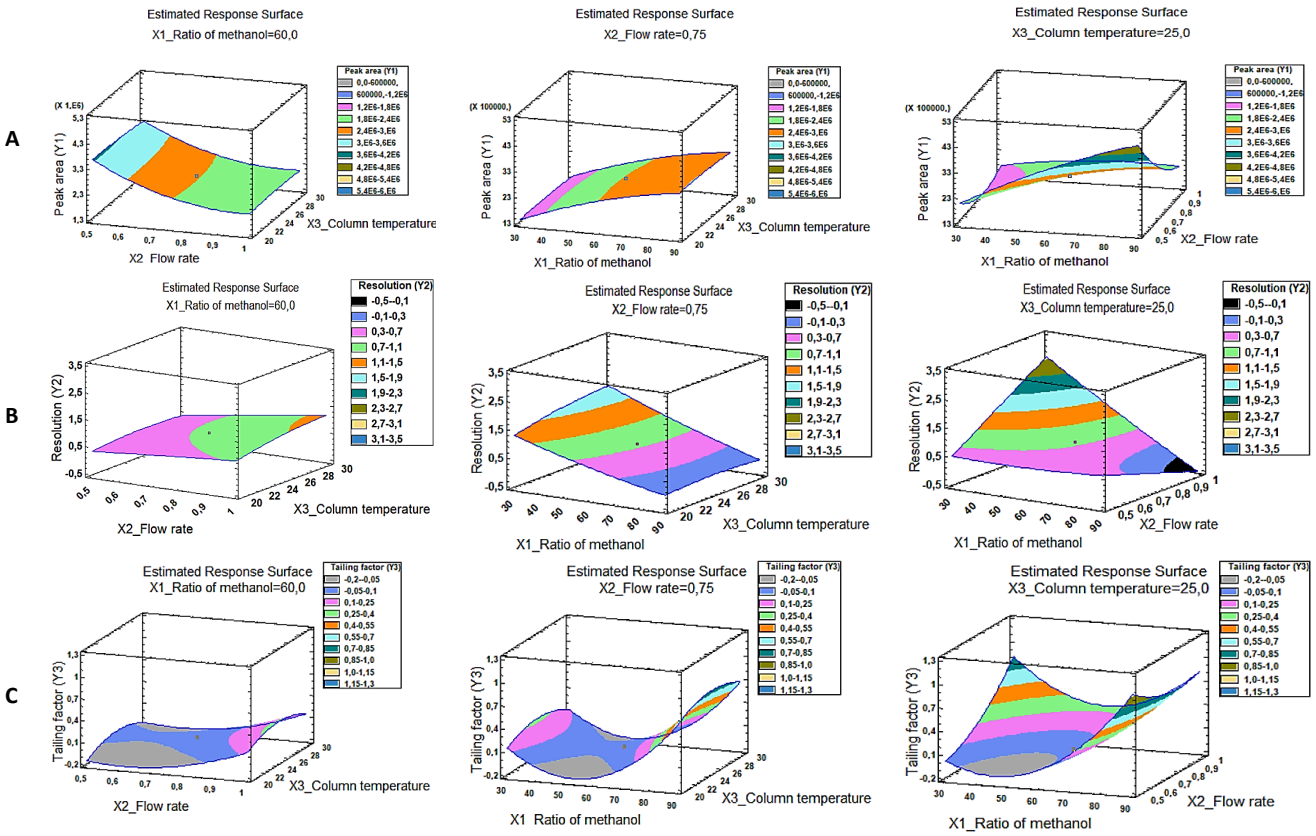


Figure 1. Pareto charts for (A) peak area, (B) resolution, (C) tailing factor, and (D) number of theoretical plates



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D

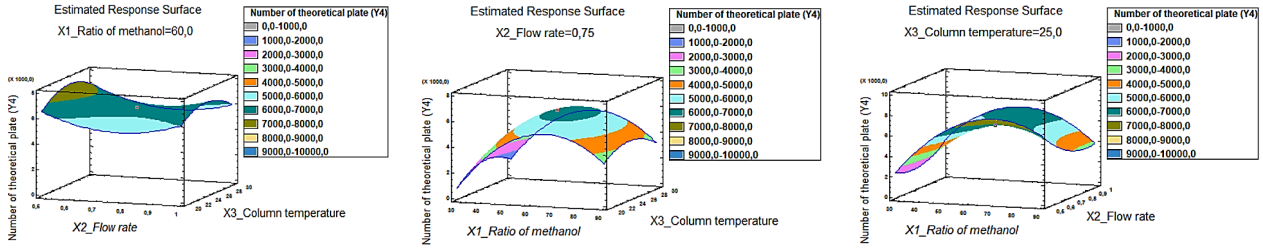

Figure 2. Three-dimensional response surface plots illustrating the effects of HPLC variables on (A) peak area, (B) resolution, (C) tailing factor, and (D) number of theoretical plates

Validation Method

System Suitability Test

The system suitability test (SST) was performed to verify the capability of the chromatographic system to provide reproducible and reliable analytical performance prior to sample analysis. According to the Indonesian Pharmacopoeia (6th edition), acceptable relative standard deviation (RSD) values for retention time, peak area, resolution, tailing factor, and number of theoretical plates should be less than 2% (Ministry of Health of the Republic of Indonesia, 2020).

Retention time consistency ensures reliable analyte identification by preventing peak position shifts. Peak area reflects the method's sensitivity and its ability to detect and quantify low analyte concentrations, which is critical for quantitative analysis. Resolution indicates the degree of peak separation, ensuring accurate quantification of individual components. The tailing factor evaluates peak symmetry, with values close to unity indicating well-shaped peaks suitable for precise integration. The number of theoretical plates represents column efficiency, with higher values indicating improved separation performance (Snow, 2021).

As summarized in Table 5, the RSD values for all evaluated SST parameters were below 2%, confirming that the chromatographic system was suitable for subsequent analysis.

Table 5. Results of the System Suitability Test (SST)

Run	Retention time	Peak Area	Resolution	Tailing Factor	Theoretical Plates
1	3.150	2207219	3.36	1.19	4557
2	3.148	2214299	3.32	1.20	4560
3	3.146	2197133	3.29	1.19	4560
4	3.149	2208261	3.31	1.20	4595
5	3.138	2196590	3.31	1.19	4551
6	3.148	2205841	3.32	1.20	4592
%RSD	0.25	0.00	0.25	0.41	0.01

Selectivity

Selectivity was evaluated using *Jamu* matrix solution for hyperuricemia, *Jamu* matrix spiked with paracetamol (25 ppm), paracetamol reference standard (30 ppm), and commercial *Jamu* samples AU1, AU2, and AU3. Selectivity was assessed by comparing chromatographic profiles, retention times, and PDA spectral overlays between the reference standard and sample solutions.

The selectivity results demonstrated comparable retention times between the paracetamol reference standard (3.33 min), paracetamol in the spiked *Jamu* matrix (3.33 min), and paracetamol detected in the AU1 sample (3.13 min). In addition, the analyte peaks were well resolved from the matrix components, and the PDA spectra of paracetamol in the spiked matrix and AU1 sample closely matched that of the reference standard, confirming the specificity of the method (Figure 3).

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a

b

c

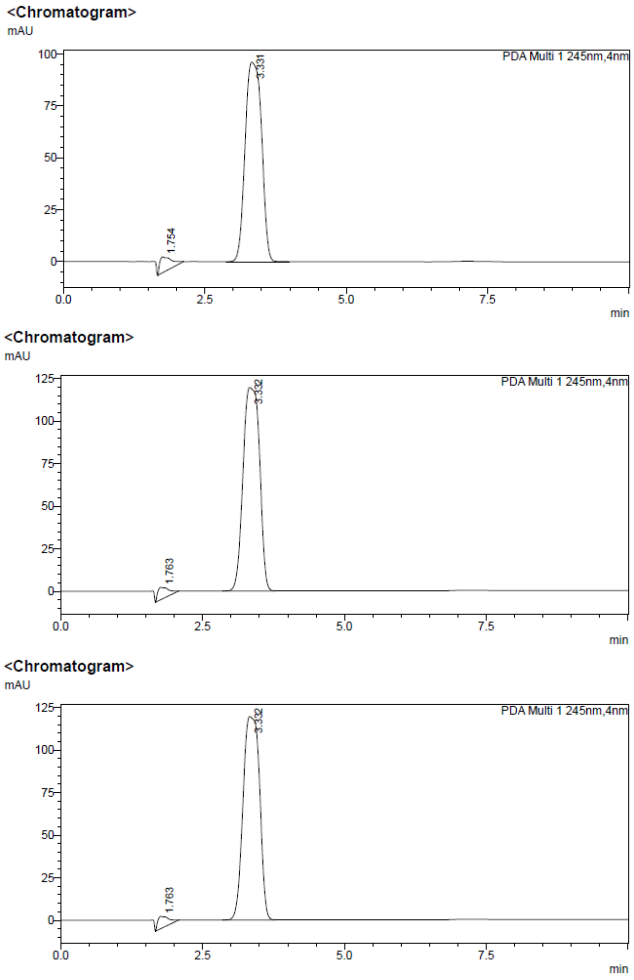

Figure 3. RP-HPLC chromatograms of (a) *Jamu* matrix solution, (b) paracetamol reference standard, and (c) *Jamu* matrix spiked with paracetamol

Linearity

Linearity was evaluated using paracetamol standard solutions at concentrations of 10, 20, 30, 40, and 50 ppm. Then data processing was carried out so that the regression equation y = 88110x - 43113, with a coefficient of determination (r^2) of 0.9992. These results indicate excellent linearity over the investigated concentration range and satisfy the acceptance criteria for quantitative analysis (Figure 4).

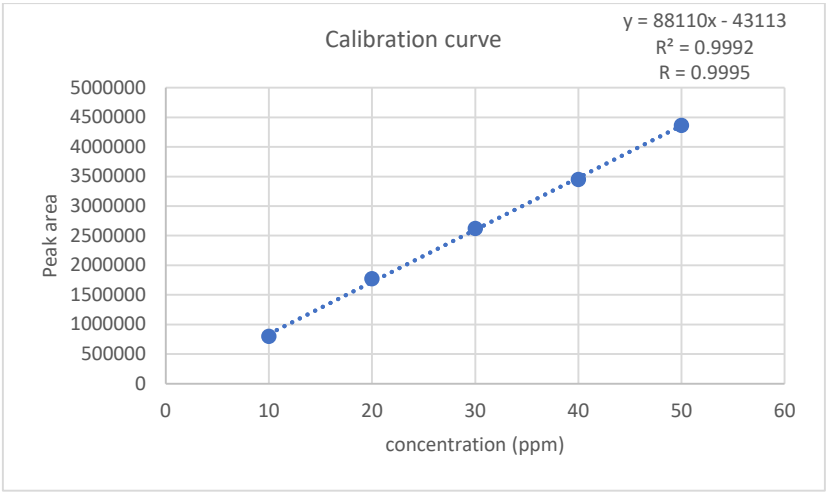

Figure 4. Calibration curve of paracetamol obtained using the optimized RP-HPLC method

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Limit of Detection and Quantification

The sensitivity of the method was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ) based on the standard deviation of the regression and the slope of the calibration curve. The LOD and LOQ values were found to be 1.51 ppm and 5.03 ppm, respectively, demonstrating the suitability of the method for detecting low levels of paracetamol in complex *Jamu* matrices.

Accuracy and Precision

Accuracy represents the closeness of agreement between the measured value and the true value, whereas precision reflects the repeatability of the analytical procedure under identical conditions. According to AOAC guidelines, acceptable accuracy is indicated by percent recovery within the range of 92–105%, while precision is considered acceptable when the %RSD is less than 2% (AOAC International, 2013). Accuracy and precision were evaluated using *Jamu* matrix solutions spiked with paracetamol at concentrations of 20, 25, and 30 ppm. The average percent recoveries ranged from 101.75% to 104.61%, while the %RSD values ranged from 0.53% to 1.59% (Table 6). These results demonstrate that the method exhibits satisfactory accuracy and precision and meets the established validation criteria.

Based on the comprehensive validation results, the developed RP-HPLC method was deemed reliable and suitable for the quantitative determination of paracetamol in *Jamu* for hyperuricemia. Accuracy is the closeness between the results obtained from the validated procedure and the correct value. The acceptance criterion for the accuracy test is % recovery around 92-105%. Precision is the closeness between individual results where the procedure is repeatedly applied to multiple sample or homogeneous samples. The acceptance criterion for the precision test is % RSD < 2% (AOAC International, 2013). Based on the calculation, the average % recovery and %RSD were 101.75 - 104.61% and 0.53 - 1.59%, respectively. Therefore, the result of accuracy and precision tests have met the acceptance requirements (Table 6).

Table 6. The result of the accuracy and precision test

Theoretical Conc. (ppm)	Measured Conc. (ppm)	% Recovery	Average % Recovery	% RSD
	20.95	104.76		
20	21.01	105.07	104.61	0.53
	20.80	103.99		
	25.80	103.20		
25	25.08	100.31	102.18	1.59
	25.76	103.04		
	30.34	101.14		
30	30.53	101.77	101.75	0.59
	30.70	102.35		

Determination of Paracetamol in Sample

The determination of paracetamol content was performed on sample AU1, as this sample tested positive for paracetamol during the selectivity assessment. Quantification was conducted in triplicate using the validated RP-HPLC method. The average paracetamol content in sample AU1 was found to be 5.08% w/w.

The presence of paracetamol at this concentration indicates that sample AU1 contains an undeclared active pharmaceutical ingredient, thereby violating the applicable regulations governing traditional medicines. Representative chromatograms of AU1, AU2, and AU3 are shown in Figure 5, While the PDA spectral overlay confirming the identity of paracetamol in AU1 is presented in Figure 6.

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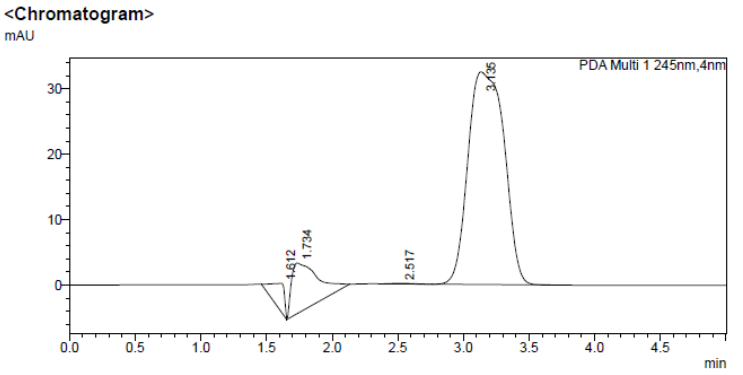
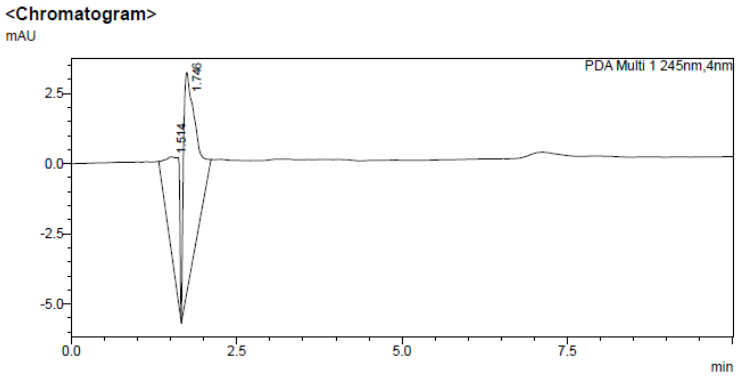
a



c



b

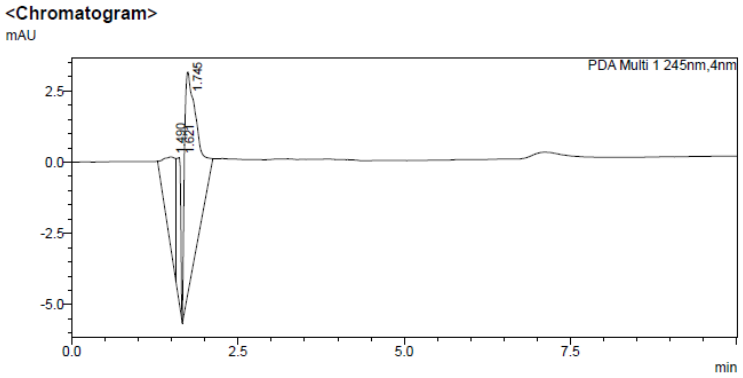


Figure 5. RP-HPLC chromatograms of (a) AU1, (b) AU2, and (c) AU3 samples

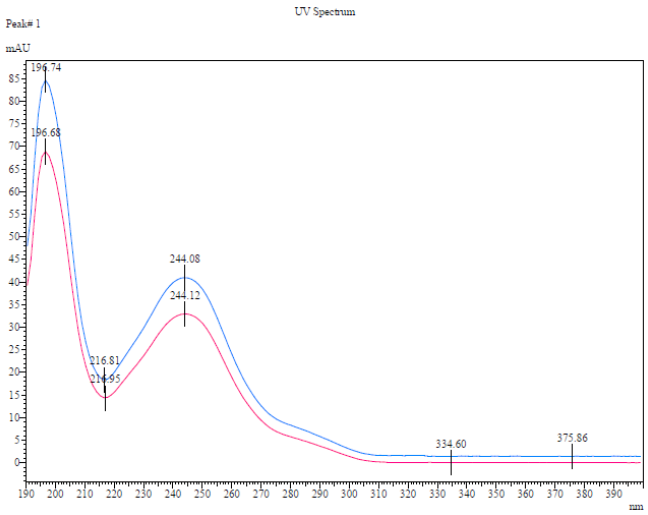


Figure 6. Overlay UV–Vis spectra of paracetamol reference standard (blue) and AU1 sample (pink)

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4. Conclusion

An RP-HPLC method was successfully optimized using Response Surface Methodology combined with a Box–Behnken Design for the analysis of paracetamol in *Jamu* for hyperuricemia. The optimized chromatographic conditions consisted of a methanol proportion of 30% v/v, a flow rate of 1.0 mL/min, and a column temperature of 25.18 °C. All validation parameters, including system suitability, selectivity, linearity, sensitivity, accuracy, and precision, met the established acceptance criteria.

Application of the validated method to commercial *Jamu* samples obtained from Pasar Besar, Malang City, revealed that sample AU1 contained paracetamol at an average concentration of 5.08% w/w. This finding indicates a violation of the Regulation of the Minister of Health of the Republic of Indonesia No. 7 of 2012 concerning the registration of traditional medicines. The developed method demonstrates strong potential for application in the routine surveillance and analysis of undeclared active pharmaceutical ingredients in traditional medicines containing analytes with physicochemical properties similar to paracetamol.

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