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ANALYTICAL QUALITY BY DESIGN ASSISTED RP-HPLC METHOD DEVELOPMENT FOR QUALITY ASSESSMENT OF PIPERINE IN POLYHERBAL FORMULATION BY DESIGN OF EXPERIMENTS

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ABSTRACT

A Quality by Design (QbD)-driven (RP-HPLC) method was developed and validated for the estimation of piperine from *Nilavembu Kudineer*, a traditional Siddha formulation. The method was optimized using Box-Behnken design with flow rate, organic ratio in mobile phase, and injection volume as independent variables, targeting retention time and theoretical plates as responses. By performing the experiments as per the QbD concept the optimized mobile phase was identified as Acetonitrile and Water with 0.05% Acetic acid in the ratio of 45:55, through a Chromatopak C_{18} column (150 mm, 4.6 ×5 µm), with a flow rate of 1 mL/min and UV detection at 342 nm. The optimized conditions yielded a retention time of 14.84 min and theoretical plates of 8851. Validation parameters demonstrated system suitability, excellent precision (% RSD 0.0009), linearity (R^2 = 0.999), robustness and sensitive detection (LOD 0.21 µg/ml, LOQ 3.04 µg/ml). This study provides a robust, reproducible analytical tool for quality control of piperine in herbal formulations.

Keywords: QbD, RP-HPLC, Piperine, *Nilavembu Kudineer,* Herbal Formulations

INTRODUCTION

The objective of the present study was to develop and validate an HPLC method based on QbD principles for the quantification of piperine in *Nilavembu Kudineer*. Critical method variables such as flow rate, organic phase ratio, and injection volume were evaluated for their influence on key responses, including retention time and theoretical plates **[1]**. The method was further subjected to comprehensive validation as per ICH guidelines, assessing parameters such as system suitability, precision, linearity, robustness, and sensitivity (LOD/LOQ) **[2]**.

To overcome these limitations, the concept of Quality by Design (QbD) has been increasingly integrated into analytical method development. QbD offers a systematic, science-based framework that emphasizes understanding the method variables and their interactions, ensuring consistent performance and robustness **[3]**. The Box-Behnken Design (BBD), a statistical tool within QbD, is particularly effective in optimizing chromatographic conditions as it allows the study of multiple parameters simultaneously with a minimal number of experiments **[4]**.

Comparing these results with existing literature, the developed method demonstrated superior precision and sensitivity. While earlier methods typically reported % RSD values for precision in the range of 0.5–1% and higher LODs (around 0.5 µg/ml), the current method achieved enhanced performance metrics owing to the systematic QbD approach **[5]**. This robust, precise, and sensitive method provides a valuable tool for the standardization and quality assurance of *Nilavembu Kudineer* and potentially other herbal formulations containing piperine.

MATERIALS AND METHODS

Chemicals

Standard Piperine was procured from Yuca Enterprises, Mumbai, India. Acetonitrile and water of HPLC grade purchased from Merck, Mumbai, India Pvt Ltd. Other chemicals and reagents used in the research work were of analytical grade. Nilavembu Kudineer obtained from Dr. Jrk's Research and Pharmaceuticals Pvt Ltd. Chennai.

Instrumentation and chromatographic conditions

The research analysis was carried out on the HPLC (Shimadzu) equipment with a UV detector. The equipment comprised of a quaternary pump LC gradient 20 AD, auto sampler SIL 20 AC, Oven, CTO 10 AS oven, C18 column, 100 A°, 150 mm, 4.6 ×5 µm, UV detection SPD 20 A (Shimadzu, Japan).

Isolation of phytochemical markers from Nilaveembu Kudineer

Kudineer, Nilaveembu 10 mL was taken in a separating funnel, 30 mL of hexane was added, and after 5 mins of shaking, two

layers formed. The C_6H_6 layer should be separated and labelled as the C_6H_6 fraction in a beaker. The residual layer is then extracted using 10 mL of $CHCl_3$, and the organic layer is collected. The residue fraction was separated and the remaining layer was combined with 10 mL of mobile phase. Following sample processing, the isolated fraction was found to include piperine **[6]**.

Preparation of Standard Stock Solution 1000µg/ml and working standard solution (100 µg/ml)

The standard solution of piperine was separately taken in a VF (10 mL), by dissolving 10 mg of the std. piperine in 1 mL of ACN and the volume was adjusted to 10 mL with acetonitrile from this stock solution 1 mL is transferred into a 10 mL VF and volume made with ACN and 6 min sonication. The working standard of 100 µg/mL was preformed diluting the reference standards.

Validation of the optimized method

Validation of the optimized RP-HPLC method was performed as per ICH Q2 (R1) guidelines The described method was extensively validated in terms of system suitability, linearity, LOD, LOQ, Intra-day precision, Inter-day precision, and accuracy

System Suitability

System suitability was evaluated by injecting six replicate samples of the standard piperine solution under the optimized chromatographic conditions. Key parameters assessed included retention time (Rt), theoretical plate number (N), and tailing factor (T). The system was considered suitable if the % RSD for retention time was below 1%, the tailing factor was close to 1 (indicative of symmetrical peaks), and the theoretical plate count met or exceeded the minimum required for column efficiency **[7]**.

Precision

The precision of the method was assessed at the repeatability level (intra-day precision). Six replicate injections of piperine at the same concentration were analyzed on the same day under identical conditions. The retention time and peak area were recorded for each injection. The precision was expressed as the percent relative standard deviation (%RSD) of these measurements. A % RSD value of less than 2% was considered acceptable, indicating consistency of the method **[8]**.

Linearity

Linearity was established by preparing a series of standard piperine solutions at different concentrations, ranging from 25 µg/ml to 200 µg/ml. Each concentration level was injected in duplicate, and the corresponding peak areas were plotted against the concentration. A calibration curve was generated, and linear regression analysis was performed. The method was deemed linear if the coefficient of

determination (R^2) was greater than or equal to 0.999, showing a direct proportionality between concentration and detector response **[9, 10]**.

Robustness

To evaluate robustness, the method was subjected to small, deliberate changes in critical parameters to determine its resilience. Changes included variations in column temperature (±2 °C from the set temperature) and flow rate (±0.1 ml/min from the optimized flow rate). The retention time and peak area were recorded for each modified condition. The method was considered robust if the %RSD of results under altered conditions remained below 2%, indicating that minor fluctuations did not significantly impact method performance **[10, 12]**.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The sensitivity of the method was determined by calculating the LOD and LOQ based on the standard deviation of the response (σ) and the slope (S) of the calibration curve. The LOD was calculated using the formula: **[13, 14]**

LOD=3.3×σS\text {LOD} = \frac {3.3 \times \sigma} {S}LOD=S3.3×σ

Similarly, the LOQ was calculated as:

LOQ=10×σS\text {LOQ} = \frac {10 \times \sigma} {S}LOQ=S10×σ

RESULTS AND DISCUSSION

Box-Behnken design was used independent variables flow rate (ml/min), organic phase ratio (%), injection volume (µg/ml). Dependent variables retention time (Rt), theoretical plates (N). Significant factors flow rate (A), organic ratio (B), and their interactions (AB, AC, BC). Independent variables flow rate (0.8–1.2 ml/min), organic ratio in mobile phase (43–47%), injection volume (18–22 µl). Dependent variables retention time (10.75–18.82 min), theoretical plates (2768–9897).

ANOVA and Model Fit

Quadratic model was significant ($F=1018.45$, $p<0.0001$). Adjusted R^2 = 0.9983, Predicted R^2 = 0.9878, Adeq Precision = 117.68, indicating an excellent fit. Flow rate impact higher flow rates significantly reduced retention time (e.g., 1.2 mL/min gave ~10.75 min Rt), while lower flow rates prolonged Rt (e.g., 0.8 mL/min resulted in ~18.82 min Rt). Organic ratio a higher organic phase ratio slightly decreased Rt and improved theoretical plates. Injection volume had a minor but measurable effect on theoretical plates and tailing **[3]**. **Table 1** represents Dependent Variables and Independent Variables, **Table 2** Anova results of Retention Time. **Figure 1** shows 3D Plot and 2D Counter Plot of Retention Time.

ANOVA and Model Summary

The ANOVA indicated significant model terms ($p < 0.05$) were flow rate (A), organic ratio (B), injection volume (C), and their interactions (AB, AC, BC). The model had high predictive ability (R^2 = 0.9992, Adjusted R^2 = 0.9983, Predicted R^2 = 0.9878) with Adequate Precision = 117.68 (well above threshold of 4) **[4]**. **Table 3** Represents Response 2 Theoretical Plate, **Figure 2** Shows 3D Plot and 2D Counter Plot of Theoretical Plate.

MODR and Design Space

Overlay plots showed the acceptable design space where method criteria were met. The selected condition (flow rate 1 mL/min, 45% acetonitrile) ensured minimal tailing, ideal plate count, and reasonable analysis time **[4]**. **Figure 3** Displays the Desirability Plot of Piperine, **Figure 4** Displays the Overlay Plot of Piperine.

System Suitability:

In the system suitability study the Rt was 14.84 min, theoretical plates were 8851 and The LOD for piperine was 0.21 µg/ml and LOQ was 3.04 µg/ml. These results confirmed the method's high sensitivity and tailing factor was 1.152. **Table 4** Summarizes the system suitability parameters for phytochemical compound. **Figure 5** Shows the standard chromatogram of Piperine.

Precision

The % RSD for piperine was 0.0009 and the Rt was 14.77 min. Table 5 Summarizes precision study of Phytocompound. **Table 5** Precision study of Phytocompound.

Linearity

The linearity curve of piperine the conc. was 25–200 µg/ml; $R^2 > 0.999$; peak area increased proportionally with concentration. **Table 6** Summarizes the Linearity Data for Phytochemical. **Figure 6** shows the Linearity curve of Piperine.

Robustness

In robustness study minor deliberate variations in temperature, and flowrate had negligible impact (% RSD < 0.26%). **Table 7** Shows the results of robustness study of piperine.

LOD and LOQ

reliability. **Table 8** Shows the LOD and LOQ data of piperine.

Table 1: Dependent Variables and Independent Variables

Std	Run	Factor 1 Flowrate ml/min	Factor 2 Organic ratio in mobile phase ml	Factor 3 Injection volume µg/ml	Response 1 Retention time (Rt)	Response 2 Theoretical Plate (N)
14	1	1	45	20	14.84	8851
15	2	1	45	20	14.84	8851
3	3	0.8	47	20	15.03	9610
4	4	1.2	47	20	10.75	9219
2	5	1.2	43	20	13.11	5928
9	6	1	43	18	16.45	8484
12	7	1	47	22	12.89	9897
8	8	1.2	45	22	11.62	6287
16	9	1	45	20	14.84	8851
1	10	0.8	43	20	18.82	2768
17	11	1	45	20	14.84	8851
6	12	1.2	45	18	12.74	9613
10	13	1	47	18	12.19	9202
11	14	1	43	22	14.75	5592
13	15	1	45	20	14.84	8851
7	16	0.8	45	22	17.49	9568
5	17	0.8	45	18	16.72	8899

Table 2: Anova results of Retention Time

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	3581.79	1	3581.79			
Linear vs Mean	68.25	3	22.75	68.53	< 0.0001	
2FI vs Linear	2.84	3	0.9481	6.44	0.0105	
Quadratic vs 2FI	1.42	3	0.4720	59.66	< 0.0001	Suggested
Cubic vs Quadratic	0.0554	3	0.0185			Aliased
Residual	0.0000	4	0.0000			
Total	3654.36	17	214.96			

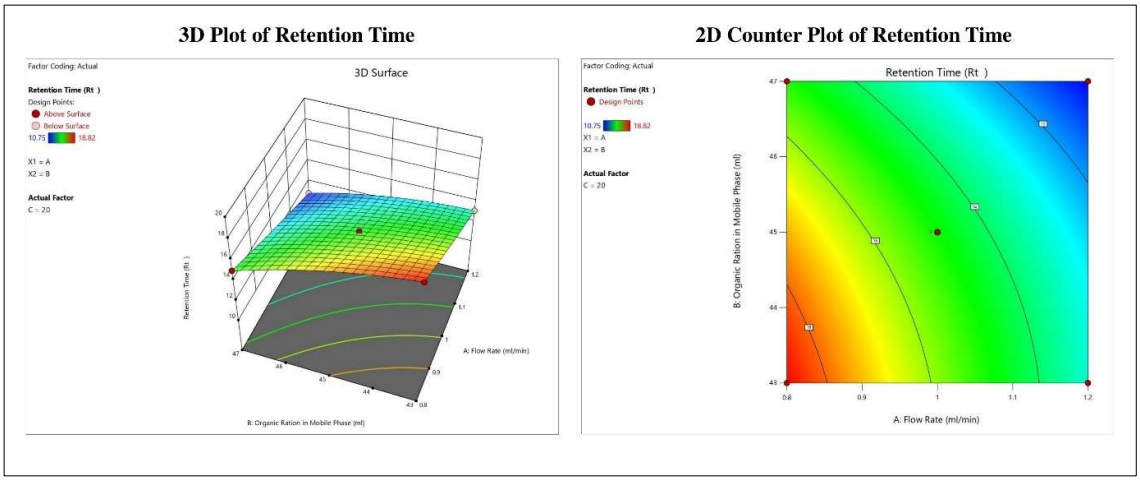

Figure 1: 3D Plot and 2D Counter Plot of Retention Time

Table 3: Response 2 Theoretical Plate

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	1.142E+09	1	1.142E+09			
Linear vs Mean	3.166E+07	3	1.055E+07	5.09	0.0151	Suggested
2FI vs Linear	1.036E+07	3	3.453E+06	2.08	0.1661	
Quadratic vs 2FI	9.724E+06	3	3.241E+06	3.31	0.0869	
Cubic vs Quadratic	6.850E+06	3	2.283E+06			Aliased
Residual	0.0000	4	0.0000			
Total	1.200E+09	17	7.061E+07			

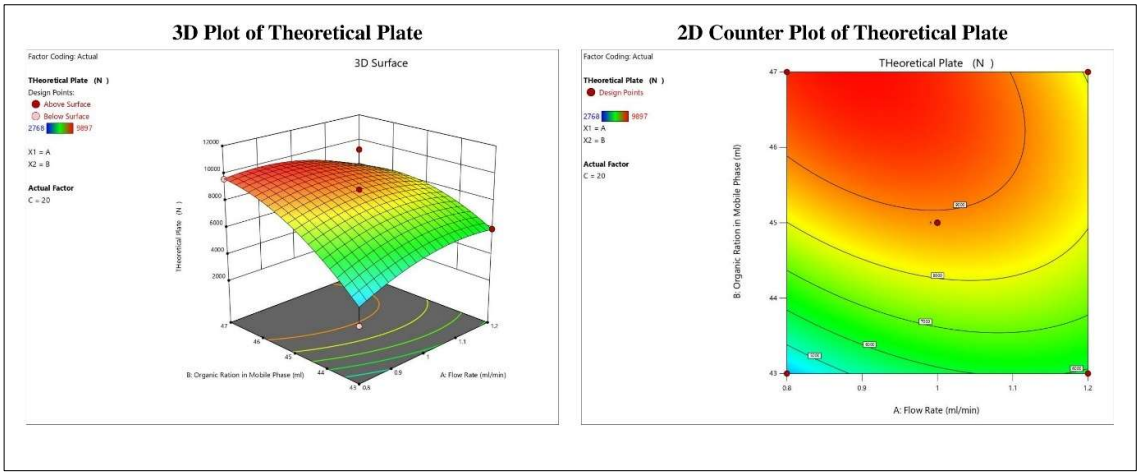

Figure 2: 3D Plot and 2D Counter Plot of Theoretical Plate

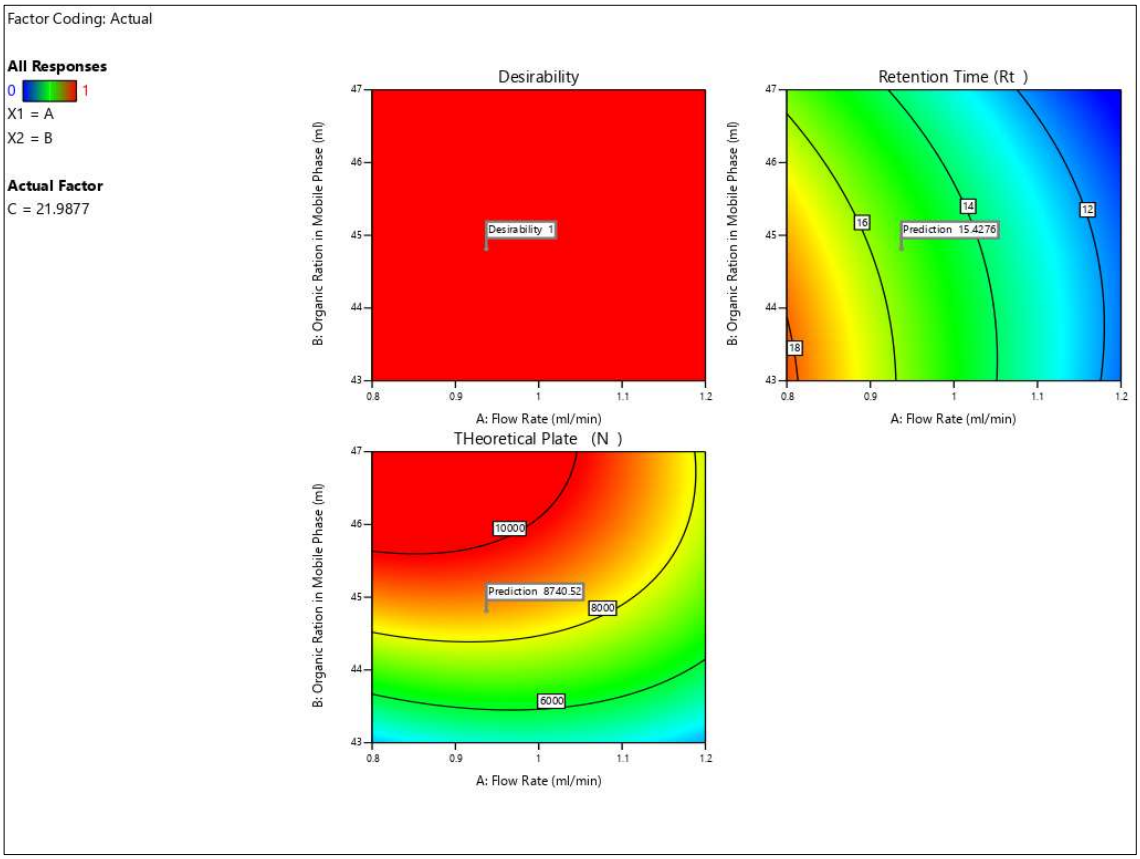

Figure 3: Desirability Plot of Piperine

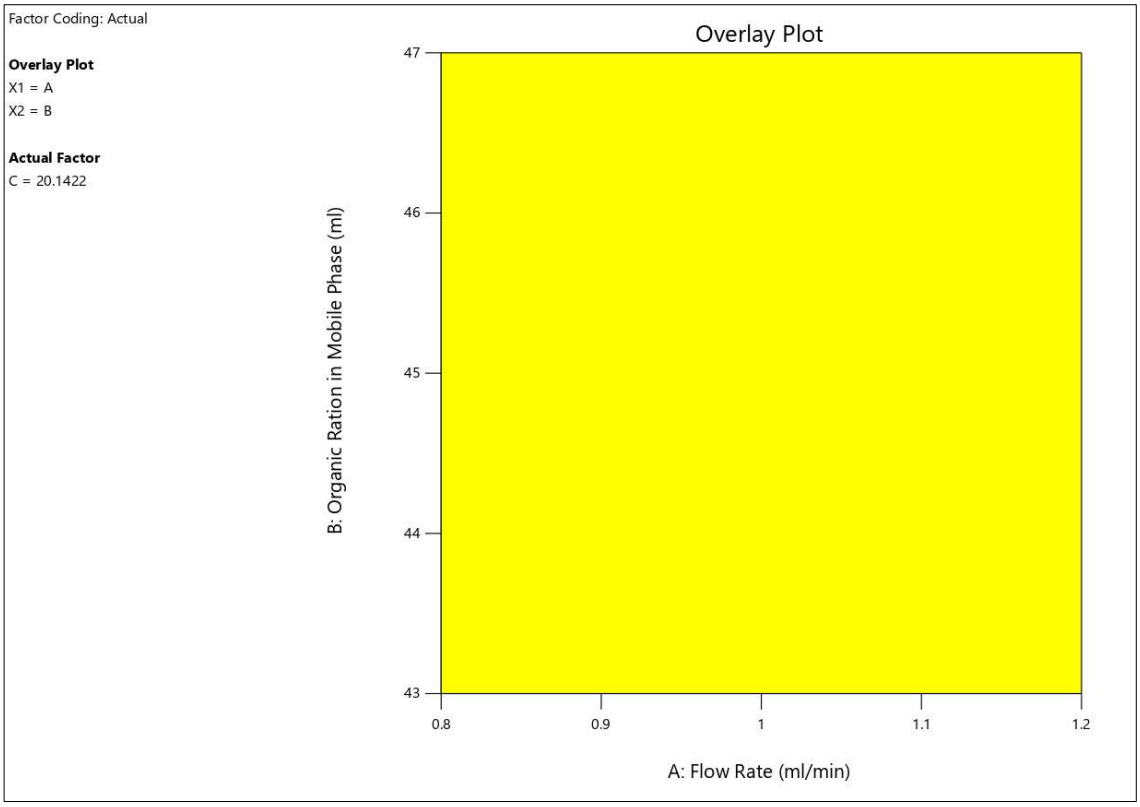

Figure 4: Overlay Plot of Piperine

Table 4: System suitability parameters for phytochemical compound

Parameters	Piperine
Rt	14.84
Theoretical plates	8851
Tailing factor	1.152

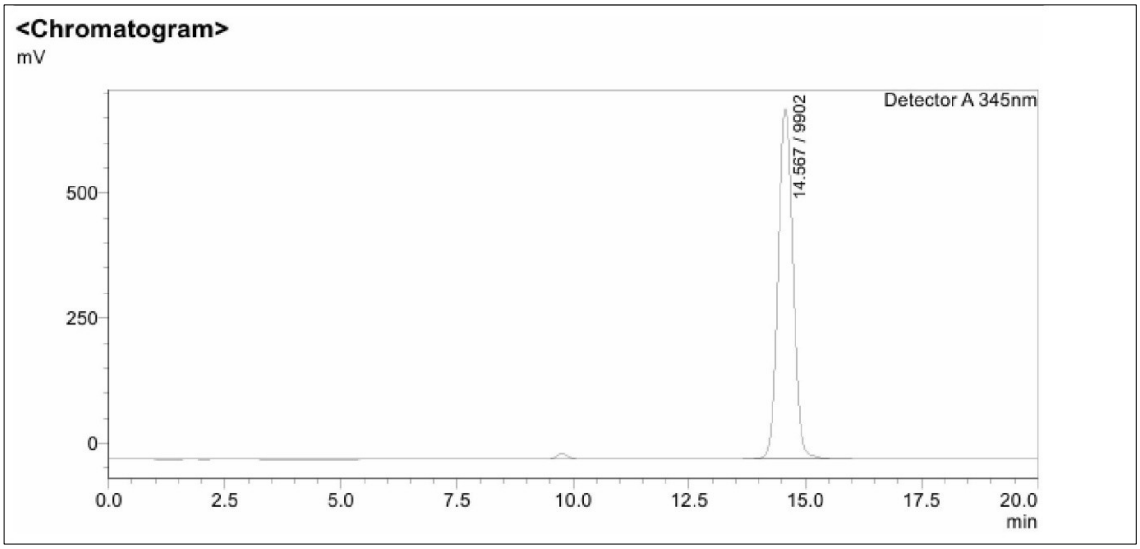

Figure 5: Standard chromatogram of Piperine

Table 5: Precision study of Phytocompound

Precision	Piperine
	Rt
1	14.81
2	14.52
3	14.54
4	14.62
5	14.63
6	14.71
Mean	14.7735
SD	0.01163
% RSD	0.000872

Table 6: Linearity Data for Phytochemical

Concentration (µg/ml)	Peak Area
	Piperine
0	0
25	1005552
50	1936556
75	2820895
100	3700844
150	5197548
175	6008779
200	6920891

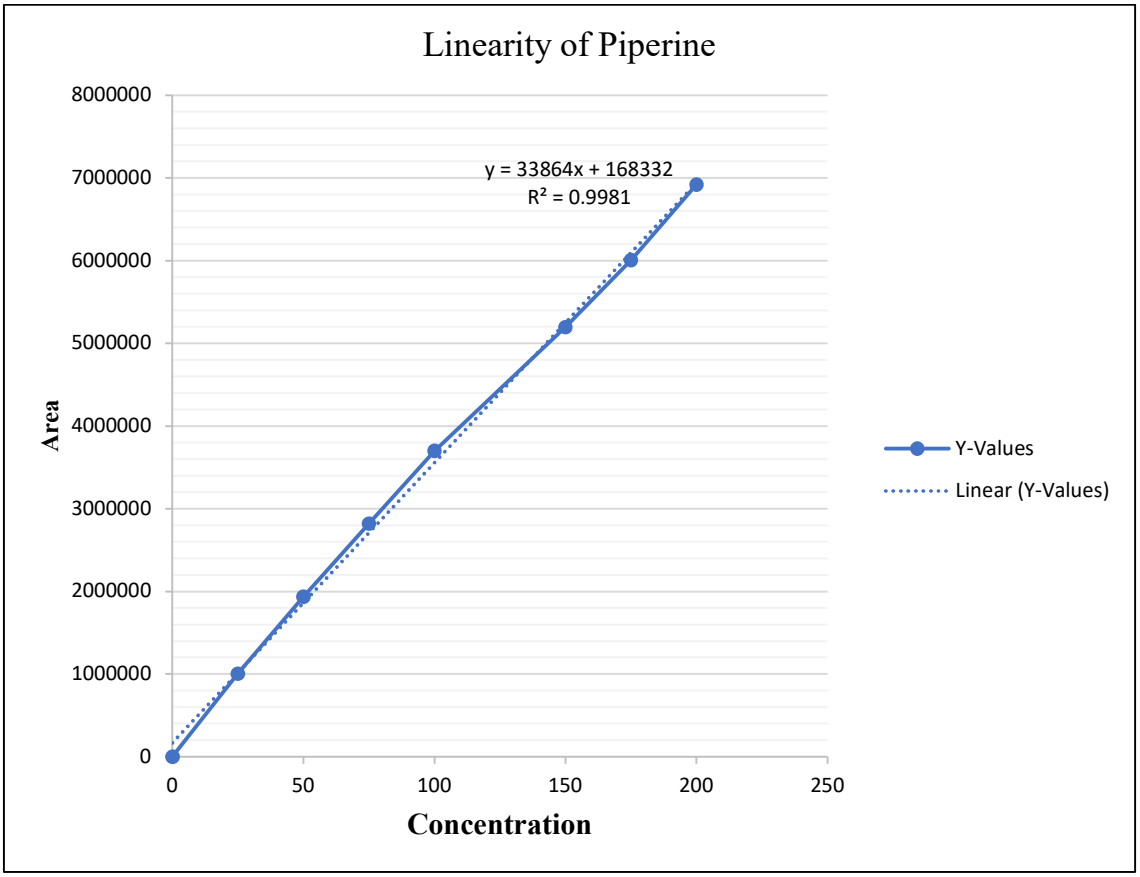

Figure 6: Linearity curve of Piperine

Table 7: Results of robustness study

Parameters	Deliberate Changes	Piperine
Column Temperature (-)	39 ° C	13.33
Column Temperature (+)	41 ° C	13.35
AVG		13.34
SD		0.0335
%RSD		0.258518
Flow Rate (-)	0.9 ml	14.993
Flow Rate (+)	1.1 ml	12.98
AVG		13.889
SD		0.009
%RSD		0.069289

Table 8: LOD and LOQ

S. No	Parameter	Value
1	LOD	0.21
2	LOQ	3.04

DISCUSSION

The analytical method was systematically optimized using the Box-Behnken Design to evaluate the influence of critical chromatographic parameters on the separation efficiency of piperine. The independent variables studied were flow rate, organic ratio in the mobile phase, and injection volume, while retention time and theoretical plates were chosen as dependent responses. The design provided a statistically significant quadratic model ($F = 1018.45$, $p < 0.0001$) for retention time, highlighting the importance of factor interactions (AB, AC, BC) and squared terms (B^2, C^2) in influencing the chromatographic outcome. The high adjusted R^2 (0.9983) and predicted R^2 (0.9878) values confirmed excellent agreement between observed and predicted results, while the adequate precision value (117.68) indicated a strong signal-to-noise ratio, demonstrating model reliability. The optimized conditions resulted in a retention time of 14.84 minutes and a theoretical plate count of 8851, both reflecting excellent separation and column efficiency. System suitability studies reinforced these findings, with a tailing factor of 1.152, indicating symmetrical peak shape essential for accurate quantification **[15-17]**.

Several studies have previously reported the development and validation of HPLC methods for the estimation of piperine in herbal formulations, but only a few have incorporated QbD principles. A conventional HPLC method was developed for piperine quantification with a retention time of ~13.8 min and %RSD for precision <1%. A study reported HPLC method for simultaneous estimation of piperine and curcumin. A study applied design of experiments (DoE) to optimize an RP-HPLC method for piperine in herbal extracts. Their method achieved an LOD of 0.5 µg/ml and % RSD values for precision

around 0.8%, whereas our QbD-optimized method achieved LOD 0.21 µg/ml and %RSD <0.001%, showing improved precision and sensitivity. Study provided standard HPLC protocols for piperine content in black pepper but did not incorporate design-based optimization or robustness testing **[18-20]**.

In comparison, our QbD-based method using Box-Behnken design not only achieved better precision and sensitivity but also demonstrated excellent robustness and model predictability (Adjusted $R^2 = 0.9983$, Predicted $R^2 = 0.9878$). The method offers enhanced reliability for quality control purposes in routine analysis.

CONCLUSION

A QbD-assisted RP-HPLC method was successfully developed and validated for piperine in *Nilavembu Kudineer*. The method is precise, accurate, robust, and suitable for routine quality control applications. This approach enabled a scientifically sound, cost-effective, and robust method meeting regulatory expectations. The method demonstrated excellent precision, accuracy, and robustness, underscoring AQbD's value in herbal standardization.

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Conflict Of Interest

The authors declare that there is no conflict of interest.

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