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**Research Article**

# Box Behnken Design Assisted RP-HPLC Method Development and Validation of Phytomarker Zingerone in Polyherbal Formulation

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## Abstract

A robust, precise, and accurate reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the quantification of zingerone using an Analytical Quality by Design (AQbD) approach. The Box-Behnken Design (BBD) was employed to optimize three critical method parameters: mobile phase composition, flow rate, and injection volume. The dependent variables Retention time, Theoretical plate and tailing factor were considered as critical method response. The chromatographic separation was achieved using a C18 column (150mm x 4.5mm x 5µm) with a mobile phase consisting of Acetonitrile: Ortho phosphoric acid 0.1% pH 3 (28:72), UV detection wavelength optimized at 282 nm. The developed method was validated according to ICH Q2 (R2) guidelines and demonstrated excellent analytical performance. The method showed good linearity over the concentration range of 50–150 µg/mL with a correlation coefficient ($R^2$) of 0.9968. Precision studies showed % RSD less than 2%, indicating good repeatability and reproducibility. Accuracy was confirmed through recovery studies, with mean recovery of 100.83%. The method also showed good robustness under small deliberate variations in chromatographic conditions. The limits of detection and quantification were found to be 0.532 µg/mL and 1.613 µg/mL respectively, indicating high sensitivity. This study confirms that the developed method is suitable for routine analysis of zingerone in herbal formulations.

**Keywords:** AQbD, Box-Behnken Design, Method Validation, Nilavembu Kudineer, RP-HPLC, Zingerone

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## Introduction

Zingerone is a bioactive phenolic compound derived from ginger (*Zingiber officinale* Roscoe), formed during the cooking or drying of gingerols. It has the IUPAC name 4-(4-Hydroxy-3-methoxyphenyl)-2-butanone, (Figure 1) a molecular formula of $C_{11}H_{14}O_3$, and a molecular weight of 194.23 g/mol. Known for its antioxidant, anti-inflammatory, antidiarrheal, and antimicrobial properties, zingerone plays a key role in traditional and modern medicine. Due to its pharmacological significance, accurate quantification is essential. High-Performance Liquid Chromatography (HPLC), particularly reverse-phase HPLC, is widely used for its precise, sensitive, and reliable analysis in herbal and pharmaceutical formulations. (1,2)

The growing utilization of zingerone in pharmaceutical and nutraceutical products necessitates a reliable and reproducible method for its quantification. (3) High-Performance Liquid Chromatography (HPLC) has emerged as the preferred analytical technique owing to its high sensitivity, specificity, and precision. In particular, reverse-phase HPLC (RP-HPLC) methods using C18 columns and UV detection at wavelengths around 282 nm have been commonly employed for zingerone analysis. (4) These methods typically utilize mobile phases consisting of acetonitrile and acidified water (e.g., with orthophosphoric acid), optimized for peak resolution, retention time, and symmetry. (5)

Moreover, the application of Analytical Quality by Design (AQbD) in HPLC method development has advanced the robustness and regulatory compliance of such analytical methods. (6) By using statistical tools like Box-Behnken Design (BBD),

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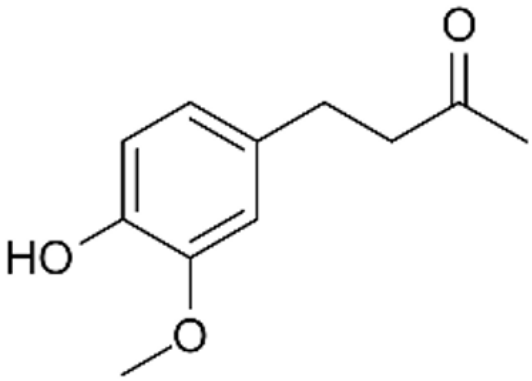
critical method parameters such as mobile phase composition, flow rate, and injection volume can be systematically optimized to ensure method reproducibility across different conditions and laboratories. (7)

In summary, zingerone's chemical versatility and therapeutic potential have made it an important marker compound in herbal research and quality control. Developing and validating efficient HPLC methods for its quantification is essential for ensuring the consistency, safety, and efficacy of zingerone-containing formulations.

**Figure 1 Chemical structure of Zingerone**




## Materials and Methods

Zingerone was procured from Yucca Enterprises Mumbai, India, all the solvents Acetonitrile and Ortho phosphoric acid HPLC Grade were procured from Merck

**Instruments used**

The instrument specification and model used for the study include, Shimadzu LC 2050 Quaternary Pump, UV Detector.

**Preparation of Standard and sample solution**

Zingerone 1 mg was precisely measured and taken in a VF of 10 ml and the contents were solubilized in mobile phase consisting of Acetonitrile: Ortho phosphoric acid. The volume was increased with diluent solution after sonification for 5 mins to bring the conc. of 100 µg/ml.

**Fractionation**

10 mL of NK was transferred into a separating funnel and extracted using 30 mL of hexane by shaking and keeping it aside for 5-10 min, two layers were formed. Collect the hexane layer separately in a beaker. Aqueous layer is further extracted with 10 mL of $CHCl_3$ and the remaining layer was extracted with ethyl acetate, collect the ethyl acetate fraction and concentrate it. All the fractions were concentrated separately for the identification and estimation of zingerone and was subjected for sample preparation.

**Preparation of sample solution**

Sample solution was established by taking 1 mg of residual fraction and mobile phase Acetonitrile: Ortho phosphoric acid.

**System suitability**

System suitability testing was conducted prior to sample analysis to verify the performance of the chromatographic system. A standard solution of Zingerone was prepared at a known concentration and injected six times consecutively. Parameters such as retention time, peak area, theoretical plates (N), resolution, and tailing factor were recorded. Acceptance criteria were set as follows: RSD of peak area ≤ 2%, tailing factor ≤ 2.0, and theoretical plates ≥ 2000. The system was considered suitable only if all parameters met the predefined criteria.

**Linearity**

The linearity of the method was evaluated by preparing a series of standard solutions of Zingerone at different concentrations, typically ranging from 50 to 150 µg/ml of the target concentration. Each concentration was injected in triplicate into the HPLC system, and the peak areas were recorded. A calibration curve was plotted between peak area and concentration, and the correlation coefficient ($R^2$) was calculated. A value of $R^2 \geq 0.999$ was considered acceptable, indicating a strong linear relationship.

**Precision**

**Repeatability (Intra-day precision):** A standard solution of Zingerone at 100% concentration was prepared and injected six times within the same day under identical conditions. The relative standard deviation (RSD) of peak areas was calculated.

**Accuracy**

The accuracy of the method was evaluated using a recovery study. Known amounts of Zingerone standard were spiked into a pre-analysed sample matrix at three concentration levels 50%, 100%, and 150% of the target level. Each level was analysed in triplicate. The percentage recovery was calculated. An average recovery in the range of 98–102% was considered acceptable.

**Robustness**

Robustness was evaluated by deliberately varying critical chromatographic parameters within a small range to assess the method's reliability under minor changes. The parameters altered included, flow rate: ±0.1 mL/min, mobile phase composition, ±2%, column temperature, ±2°C and detection wavelength: ±2 nm. Standard solution of Zingerone was analysed under each modified condition, and the system suitability parameters were monitored. The method was considered robust if the changes did not significantly affect the retention time, resolution, or peak symmetry.

**Limit of Detection & Limit of Quantification**

LOD and LOQ were determined using the standard deviation of the response (σ) and the slope (S) of the calibration curve, The standard deviation was obtained from the y-intercepts of the regression lines of multiple calibration curves. The calculated LOD and LOQ values were verified by injecting solutions at the respective concentrations and ensuring signal-to-noise ratios of approximately 3:1 and 10:1, respectively.

## Results and Discussion

**Optimization Table (Design of Experiments - DoE)**

This table summarizes the impact of three critical parameters, A: Mobile Phase Composition (%), B: Flow Rate (mL/min), C: Injection Volume (µL) on three analytical outcomes. Response 1 Retention Time (RT) – the time at which the peak for Zingerone appears, Response 2 Theoretical Plates – a measure of column efficiency and Response 3 Tailing Factor – indicates peak symmetry; values closer to 1 are ideal. By altering combinations of these factors across 17 experimental runs, the study evaluates how each parameter (and their interactions) affects the quality of the chromatographic method. The results are shown in Table 1.

**Table 1: Factors and responses selected in BBD design for Zingerone**

| Std | Run | Independent Variables | | | Dependent Variables | | |
|---|---|---|---|---|---|---|---|
| | | A: Mobile phase (%v/v) | B: Flow rate (mL/ min) | C: Injection volume (µL) | R1 Retention time (min) | R2 Theoretical plates | R3 Tailing factor |
| | | | | | | | |
| 10 | 1 | 72 | 1.2 | 18 | 5.475 | 15156 | 1.202 |
| 9 | 2 | 72 | 0.8 | 18 | 7.817 | 21210 | 1.115 |
| 6 | 3 | 74 | 1 | 18 | 6.85 | 16079 | 1.172 |
| 1 | 4 | 70 | 0.8 | 20 | 7.817 | 20210 | 1.202 |
| 8 | 5 | 74 | 1 | 22 | 6.733 | 16327 | 1.146 |
| 2 | 6 | 74 | 0.8 | 20 | 7.458 | 21540 | 1.159 |
| 14 | 7 | 72 | 1 | 20 | 6.817 | 16210 | 1.144 |
| 5 | 8 | 70 | 1 | 18 | 6.767 | 16906 | 1.192 |
| 4 | 9 | 74 | 1.2 | 20 | 5.693 | 15427 | 1.202 |
| 7 | 10 | 70 | 1 | 22 | 6.167 | 16754 | 1.195 |
| 13 | 11 | 72 | 1 | 20 | 6.908 | 16493 | 1.135 |
| 15 | 12 | 72 | 1 | 20 | 6.817 | 16210 | 1.102 |
| 17 | 13 | 72 | 1 | 20 | 6.267 | 16494 | 1.123 |
| 11 | 14 | 72 | 0.8 | 22 | 5.708 | 21755 | 1.152 |
| 12 | 15 | 72 | 1.2 | 22 | 5.917 | 15345 | 1.213 |
| 16 | 16 | 72 | 1 | 20 | 6.817 | 15210 | 1.143 |
| 3 | 17 | 70 | 1.2 | 20 | 5.7 | 15521 | 1.202 |

**The effect of Retention Time on Zingerone**

The model is statistically significant ($p = 0.0104$), suggesting that the selected factors significantly affect retention time. Flow rate (B) has the strongest influence on retention time ($p = 0.0006$), indicating that faster flow rates reduce RT. The interaction BC (Flow rate × Injection volume) is also significant ($p = 0.0091$). The lack of fit is not significant ($p = 0.1517$), which confirms that the model fits the data well. The results are shown in Table 2 Figure 2 represents 3D Plot, Counter plot and Perturbation plot of Zingerone (Retention Time).

**Table 2: ANOVA table for Retention time of Zingerone**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | Significance |
|---|---|---|---|---|---|---|
| **Model** | 7.60 | 9 | 0.8440 | 6.62 | 0.0104 | Significant |
| A-Mobile phase | 0.0100 | 1 | 0.0100 | 0.0785 | 0.7874 | |
| B-Flow rate | 4.52 | 1 | 4.52 | 35.47 | 0.0006 | |
| C-Injection volume | 0.7104 | 1 | 0.7104 | 5.57 | 0.0503 | |
| AB | 0.0310 | 1 | 0.0310 | 0.2429 | 0.6372 | |
| AC | 0.0583 | 1 | 0.0583 | 0.4574 | 0.5206 | |
| BC | 1.63 | 1 | 1.63 | 12.76 | 0.0091 | |
| $A^2$ | 0.1230 | 1 | 0.1230 | 0.9644 | 0.3588 | |
| $B^2$ | 0.2210 | 1 | 0.2210 | 1.73 | 0.2295 | |
| $C^2$ | 0.2998 | 1 | 0.2998 | 2.35 | 0.1690 | |
| **Residual** | 0.8926 | 7 | 0.1275 | | | |
| Lack of Fit | 0.6240 | 3 | 0.2080 | 3.10 | 0.1517 | Not significant |
| Pure Error | 0.2686 | 4 | 0.0672 | | | |
| **Cor Total** | 8.49 | 16 | | | | |

**The effect of Theoretical Plates on Zingerone**

This model is highly significant ($p < 0.0001$), with flow rate (B) again being the most influential factor ($p < 0.0001$). The quadratic term $B^2$ is also significant ($p = 0.0002$), indicating a non-linear relationship between flow rate and plate number. Other factors have minimal or no significant impact. Non-significant lack of fit ($p = 0.3675$) supports the model adequacy. The results are shown in Table 3, Figure 3 represents the 3D Plot, Counter plot and Perturbation plot of Zingerone (Theoretical plate).

**Table 3: ANOVA table for Theoretical plates of Zingerone**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 8.522E+07 | 9 | 9.469E+06 | 28.89 | 0.0001 | significant |
| A-Mobile phase | 40.50 | 1 | 40.50 | 0.0001 | 0.9914 | |
| B-Flow rate | 6.766E+07 | 1 | 6.766E+07 | 206.43 | < 0.0001 | |
| C-Injection volume | 86112.50 | 1 | 86112.50 | 0.2627 | 0.6240 | |
| AB | 5.069E+05 | 1 | 5.069E+05 | 1.55 | 0.2537 | |
| AC | 40000.00 | 1 | 40000.00 | 0.1220 | 0.7371 | |
| BC | 31684.00 | 1 | 31684.00 | 0.0967 | 0.7649 | |
| $A^2$ | 42569.69 | 1 | 42569.69 | 0.1299 | 0.7292 | |
| $B^2$ | 1.602E+07 | 1 | 1.602E+07 | 48.87 | 0.0002 | |
| $C^2$ | 3.604E+05 | 1 | 3.604E+05 | 1.10 | 0.3292 | |
| **Residual** | 2.294E+06 | 7 | 3.278E+05 | | | |
| Lack of Fit | 1.171E+06 | 3 | 3.904E+05 | 1.39 | 0.3675 | not significant |
| Pure Error | 1.123E+06 | 4 | 2.808E+05 | | | |
| **Cor Total** | 8.752E+07 | 16 | | | | |

**Figure 2: 3D Plot, Counter plot and Perturbation plot of Zingerone (Retention Time)**

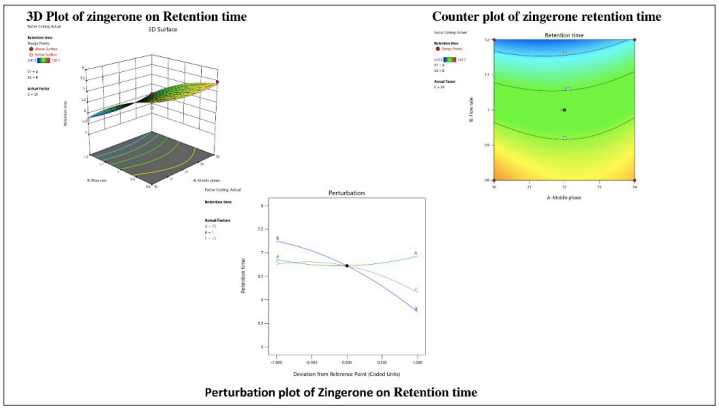
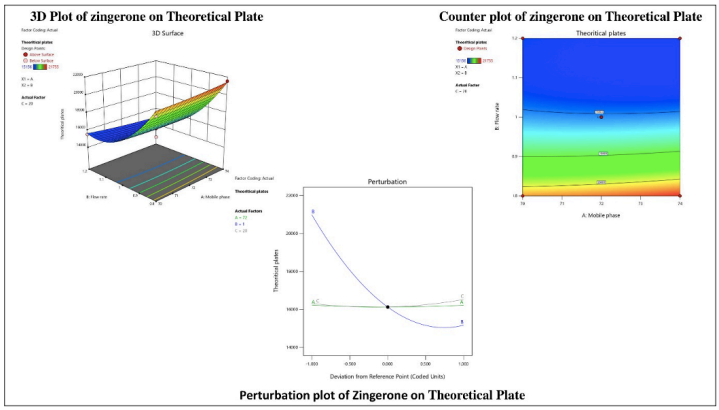



**Figure 3: 3D Plot, Counter plot and Perturbation plot of Zingerone (Theoretical plate)**




**The effect of Tailing Factor on Zingerone**

The model is significant (p = 0.0423), with flow rate (B) being the only linear term significantly affecting tailing (p = 0.0172). The quadratic terms $A^2$ and $B^2$ are significant (p = 0.0153 and 0.0332 respectively), suggesting curvature in the relationship. Other terms, including injection volume and interaction terms, show minimal influence. The model has a non-significant lack of fit (p = 0.2208), confirming its suitability. The results are shown in Table 4, Figure 4 represents 3D Plot, Counter plot and Perturbation plot of Zingerone (Plate Count). Figure 5 represents the Desirability plot of zingerone, Figure 6 Represents the Overlay plot of zingerone.

**Table 4: ANOVA table for Tailing factor of Zingerone**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| **Model** | 0.0168 | 9 | 0.0019 | 3.93 | 0.0423 | significant |
| A-Mobile phase | 0.0016 | 1 | 0.0016 | 3.31 | 0.1116 | |
| B-Flow rate | 0.0046 | 1 | 0.0046 | 9.63 | 0.0172 | |
| C-Injection volume | 0.0001 | 1 | 0.0001 | 0.1650 | 0.6967 | |
| AB | 0.0005 | 1 | 0.0005 | 0.9764 | 0.3560 | |
| AC | 0.0002 | 1 | 0.0002 | 0.4441 | 0.5265 | |
| BC | 0.0002 | 1 | 0.0002 | 0.3570 | 0.5690 | |
| $A^2$ | 0.0048 | 1 | 0.0048 | 10.16 | 0.0153 | |
| $B^2$ | 0.0033 | 1 | 0.0033 | 7.00 | 0.0332 | |
| $C^2$ | 0.0007 | 1 | 0.0007 | 1.51 | 0.2582 | |
| **Residual** | 0.0033 | 7 | 0.0005 | | | |
| Lack of Fit | 0.0021 | 3 | 0.0007 | 2.28 | 0.2208 | not significant |
| Pure Error | 0.0012 | 4 | 0.0003 | | | |
| **Cor Total** | 0.0201 | 16 | | | | |

**Figure 4: 3D Plot, Counter plot and Perturbation plot of Zingerone (Plate Count)**

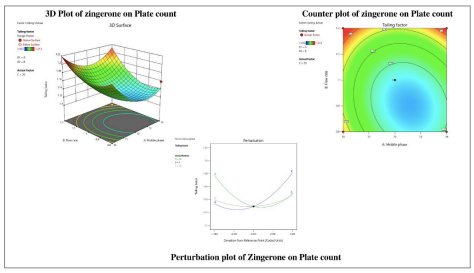



**Figure 5: Desirability plot of zingerone**

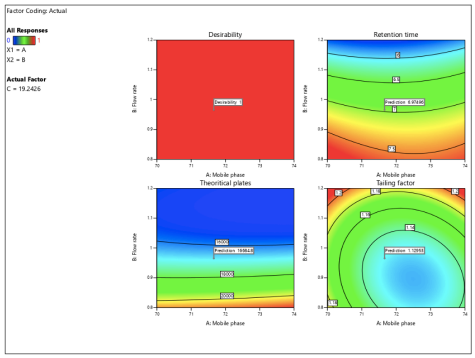
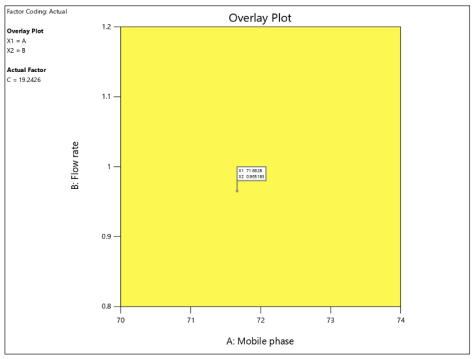



**Figure 6: Overlay plot of zingerone**




## Precision

Precision was assessed using six replicate injections of the standard solution. The %RSD (Relative Standard Deviation) was 1.06%, well below the acceptable limit of 2%, confirming method reproducibility. The results are displayed in Table 5, Figure 7 Shows the Standard Chromatogram of Zingerone, Figure 8 shows the Sample Chromatogram of Zingerone.

**Table 5: Precision values of Zingerone**

| Injection | Area of Zingerone |
|---|---|
| Injection 1 | 6621367 |
| Injection 2 | 6467713 |
| Injection 3 | 6444776 |
| Injection 4 | 6453147 |
| Injection 5 | 6453994 |
| Injection 6 | 6449581 |
| Average | 6481763 |
| Standard deviation | 68819.17 |
| Relative standard deviation | 1.06 |

## Linearity

The method showed excellent linearity over the concentration range of 50–150 µg/mL. The correlation coefficient ($R^2$) was 0.9968, indicating strong proportionality between concentration and peak area. The results are displayed in Table 6, figure 9 represents the Linearity plot of zingerone.

**Table 6: Linearity results of Zingerone**

| Zingerone | | |
|---|---|---|
| **S.No** | **Concentration (µg/ml)** | **Area** |
| 1 | 50 | 4225641 |
| 2 | 75 | 5548621 |
| 3 | 100 | 6453447 |
| 4 | 125 | 7554667 |
| 5 | 150 | 8556278 |
| | Slope (m) | 42669 |
| | STD | 68819.17 |
| | INTERCEPT | 2200803 |
| | Correlation coefficient (R2) | 0.9968 |

**Figure 7: Standard Chromatogram of Zingerone**

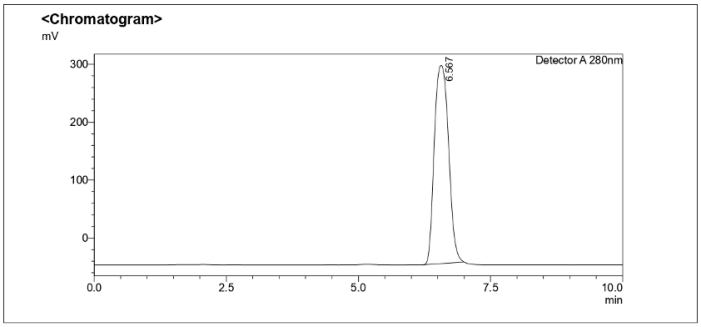



**Figure 8: Sample Chromatogram of Zingerone**

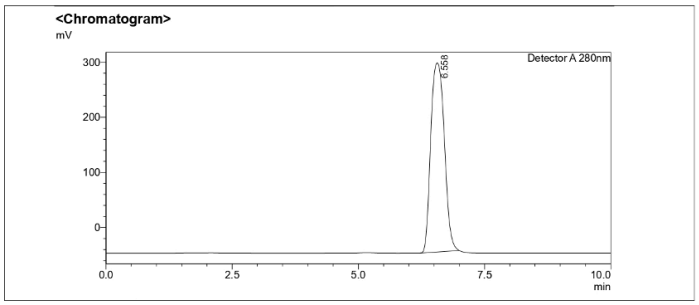
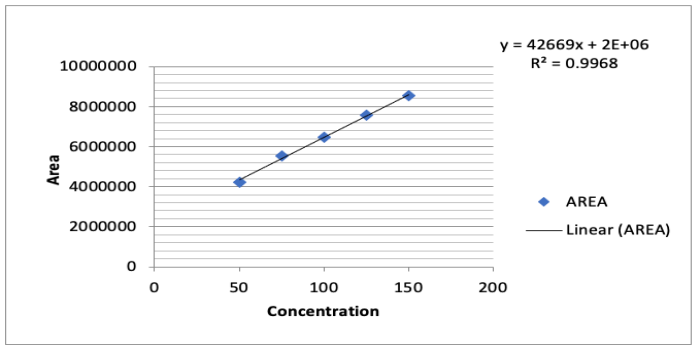



**Figure 9: Linearity plot of zingerone**




## Limit of Detection & Limit of Quantification

The Limit of Detection (LOD) was 0.532 µg/mL, and the Limit of Quantification (LOQ) was 1.613 µg/mL. These values reflect high sensitivity, allowing detection and quantification of low levels of Zingerone. The results are displayed in Table 8

**Table 8: LoD & LoQ**

| Parameter | Value |
|---|---|
| LOD | 0.532173 |
| LOQ | 1.612646 |

**Accuracy**

Recovery studies were conducted at three levels: 50%, 100%, and 150%. The mean recovery was 100.83%, indicating that the method is accurate and shows minimal deviation from the true concentration. The results are displayed in Table 9.

**Table 9: Accuracy Results for Zingerone**

| % | Area | % Recovery | Mean |
|---|---|---|---|
| 50% | 4225641 | 100.95 | 100.83 |
| 100% | 6453447 | 100.83 | |
| 150% | 8556278 | 99.87 | |

**Robustness**

Evaluated by deliberate variations in column temperature and flow rate. Under all conditions tested (± variations), the %RSD remained below 1.25%, demonstrating that the method remains reliable under small changes. The results are displayed in Table 10.

**Table 10: Robustness values for Zingerone**

| Parameters | Deliberate Changes | Zingerone | Parameters | Deliberate Changes | Zingerone |
|---|---|---|---|---|---|
| Column Temperature (-) | 39$^0$c | 6761446 | Column Flow Rate (-) | 0.9ml | 7390325 |
| | | 6911004 | | | 7382611 |
| | | 6908148 | | | 7385673 |
| Average | | 6860199 | Average | | 7386203 |
| Standard deviation | | 85534.82 | Standard deviation | | 3884.215 |
| %RSD | | 1.25 | %RSD | | 0.05 |
| Column Temperature (+) | 41$^0$c | 6507462 | Column Flow Rate (+) | 1.1ml | 6097196 |
| | | 6586388 | | | 6098300 |
| | | 6585655 | | | 6096110 |
| Average | | 6559835 | Average | | 6097202 |
| Standard deviation | | 45357.83 | Standard deviation | | 1095.012 |
| %RSD | | 0.69 | %RSD | | 0.02 |

## Discussion

The application of the Analytical Quality by Design (AQbD) approach, aided by Box-Behnken Design (BBD), enabled a comprehensive understanding of the effects of critical method parameters on chromatographic performance in the quantification of zingerone. Three independent variables—mobile phase composition (A), flow rate (B), and injection volume (C)—were systematically varied, and their impact was studied on three dependent responses: retention time (R1), theoretical plates (R2), and tailing factor (R3). Among these variables, flow rate emerged as the most influential factor, significantly affecting all three responses. For retention time, flow rate exhibited a highly significant inverse relationship ($p = 0.0006$), with increased flow rates resulting in reduced retention times. Additionally, the interaction between flow rate and injection volume (BC) was statistically significant ($p = 0.0091$), indicating that the combined effect of these variables also impacts the elution profile. (8)

For theoretical plates, which reflect column efficiency, the model was highly significant ($p < 0.0001$), with flow rate again being the dominant contributor ($F = 206.43$). A non-linear (quadratic) influence of flow rate was also observed ($p = 0.0002$), suggesting that optimal efficiency lies within a moderate flow rate range. In contrast, mobile phase and injection volume had negligible effects on column efficiency. The tailing factor, representing peak symmetry, was also significantly influenced by flow rate ($p = 0.0172$), along with the quadratic terms of mobile phase and flow rate ($p = 0.0153$ and $p = 0.0332$, respectively). These findings highlight the importance of precise control of the flow rate to ensure ideal peak shape and resolution. (9)

Validation of the optimized method confirmed its reliability. Precision studies showed a %RSD of 1.06%, indicating excellent reproducibility. The method displayed good linearity over the tested concentration range (50–150 µg/mL) with an $R^2$ value of 0.9968. Accuracy was verified through recovery studies, yielding an average recovery of 100.83%, within acceptable ICH Q2(R2) limits. Robustness testing under slight variations in column temperature and flow rate demonstrated the method's resilience, with %RSD values consistently below 1.25%. Sensitivity, as determined by LOD and LOQ, was also satisfactory at 0.532 µg/mL and 1.613 µg/mL, respectively. (10)

Overall, the AQbD-based method development process facilitated the establishment of a robust design space and highlighted flow rate as the most critical parameter influencing analytical performance. The statistically validated model and compliance with ICH guidelines confirm that the developed RP-HPLC method is precise, accurate, robust, and suitable for routine analysis of zingerone in herbal formulations.

## Conclusion

A reverse-phase HPLC method for the quantification of zingerone was successfully developed and validated using an Analytical Quality by Design approach. The method demonstrated strong performance in terms of precision, accuracy, robustness, linearity, and sensitivity. Statistical analysis confirmed that flow rate is the most critical variable influencing chromatographic quality. The validated method complies with ICH guidelines and is well-suited for the routine analysis of zingerone in herbal extracts and Herbal formulations.

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**Conflict of Interest:** The authors declare that there is no conflict of interest.

**Funding:** Not applicable.

**Ethical approval and consent to participate:** Not applicable.

**Availability of data and materials:** Data and material are available upon request.

**Abbreviations**

**AQBD:** Analytical Quality by Design; **ATP:** Analytical Target Profile; **BBD:** Box Behnken Design; **CAA:** Critical Analytical Attribute; **CMA:** Critical Method Attributes; **DOE:** Design of Experiment; **HPLC:** High Performance Liquid Chromatography; **TLC:** Thin Layer Chromatography; **HPTLC:** High Performance Thin Layer Chromatography; **ICH:** International Council for Harmonization; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **NK:** Nilavembu Kudineer; **SD:** Standard Deviation; **RSD:** Relative Standard Deviation;

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