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Green Reverse-Phase High-Performance Liquid Chromatography Method Development for Resveratrol in Liposomal Formulation: A Quality-by-Design Approach

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Abstract

Aim: A robust and environmentally sustainable reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and optimized for the quantification of resveratrol using a quality-by-design approach. **Materials and Methods:** Box–Behnken design was employed to investigate the influence of three critical chromatographic parameters, organic phase percentage, flow rate, and column temperature on retention time, tailing factor, and theoretical plates. A polynomial regression model and analysis of variance were used to analyze interactions and derive optimal conditions, which were validated by residual and perturbation plots, 3D response surface analysis, and overlay plots. **Results and Discussion:** The optimized method demonstrated high reproducibility and precision, with linearity ($R^2 > 0.999$) over the 2–10 µg/mL range, and limit of detection and limit of quantification values of 0.25 µg/mL and 0.81 µg/mL, respectively. Comprehensive method validation was performed by ICH Q2 (R1 & R2) guidelines. Parameters, including system suitability, accuracy (% recovery of 97.8–100.2%), precision (%RSD < 2%), and robustness, confirmed the method's reliability. The method was effectively applied to quantify resveratrol in liposomal formulations, which exhibited excellent encapsulation efficiency (87.03%) and recovery rates (96.35–99.5%). Greenness and whiteness of the method were evaluated using the Complex Green Analytical Procedure Index, Analytical GREEnness Metric Approach (AGREE), AGREE prep, Analytical Eco-Scale, and blue applicability grade index (BAGI). All tools collectively indicated excellent environmental and operational compatibility, with AGREE and BAGI scores of 0.74 and 77.5, respectively. **Conclusion:** This verifies the method's environmental friendliness and practical usefulness, making it a suitable contender for high-throughput pharmaceutical analysis and sustainable laboratory procedures.

Key words: Green analytical chemistry, liposomes, quality-by-design, resveratrol, RP-HPLC

INTRODUCTION

Neurodegenerative disorders are a group of progressive, debilitating ailments characterized by the slow degeneration of neuronal structure and function, eventually leading to cell death.[1] Among these, cognitive and extrapyramidal disorders, Alzheimer's disease (AD) is the most prevalent, particularly affecting the aging population. Clinically, AD is manifested by a spectrum of cognitive and behavioral impairments, including memory loss, disorientation, impaired judgment, and personality changes.[2,3] At the pathological level, Alzheimer's disease is characterized by the formation of amyloid beta plaques and neurofibrillary tangles made of hyperphosphorylated tau protein. These aberrant protein aggregates disrupt synaptic communication, trigger neuroinflammation, and induce widespread neuronal loss, particularly in the hippocampus,

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a brain region critical for learning and memory. As the disease progresses, neurodegeneration extends to additional cortical and subcortical areas, leading to profound cognitive and functional decline. Despite extensive research, the etiology of AD remains multifactorial and incompletely understood, involving complex interactions between genetic predisposition (e.g., APOE4 allele), environmental exposures, and lifestyle factors. Current pharmacological interventions provide only symptomatic relief, lacking the ability to halt or reverse disease progression. Thus, there is a pressing need for novel therapeutic agents and strategies aimed at early diagnosis, neuroprotection, and disease modification.[4,5]

Resveratrol, a naturally occurring polyphenolic compound found in grapes, red wine, berries, and peanuts, has garnered significant interest in the context of neurodegenerative diseases, including Alzheimer's.[6] Owing to its potent antioxidant, anti-inflammatory, and anti-apoptotic properties, resveratrol has shown promise in mitigating neurodegenerative processes in pre-clinical models. Mechanistically, it modulates several molecular targets, including the activation of sirtuin 1 (SIRT1), inhibition of pro-inflammatory cytokines, and scavenging of reactive oxygen species. These multifaceted mechanisms contribute to its neuroprotective potential. However, the clinical translation of resveratrol has been hindered by its poor aqueous solubility, rapid metabolism, and low systemic bioavailability. Consequently, innovative formulation strategies are necessary to enhance its pharmacokinetic profile and therapeutic efficacy.[7,8]

Liposomes, spherical vesicular systems composed of phospholipid bilayers, have emerged as promising nanocarriers for the targeted delivery of bioactive compounds.[9] Their unique ability to encapsulate both hydrophilic and lipophilic molecules, combined with their biocompatibility and ability to enhance drug solubility and bioavailability, makes them suitable for resveratrol delivery. Liposomes can be engineered for site-specific delivery and controlled release, thereby minimizing systemic toxicity and maximizing therapeutic outcomes. Furthermore, surface modifications, such as ligand attachment or PEGylation, can improve circulation time and targeting efficiency. Despite these advantages, challenges such as formulation stability, batch reproducibility, and cost-effectiveness persist, necessitating continued optimization and standardization in liposomal drug delivery systems.[10,11]

To address this issue, researchers are increasingly exploring alternative solvents and methodologies that minimize environmental impact while maintaining analytical precision. Innovations such as solvent-free extraction techniques and the use of biodegradable solvents are gaining traction in the pursuit of more sustainable high-performance liquid chromatography (HPLC) practices.[12-17] In addition, the economic and environmental burdens associated with conventional analytical protocols have raised concerns regarding their long-term sustainability. To bridge this gap, the present study aims to develop and validate an environmentally benign and cost-effective reverse-phase HPLC (RP-HPLC) method for the quantification of resveratrol in both bulk and liposomal formulations.

The method development was systematically guided by the principles of quality-by-design (QbD) to ensure robustness, reliability, and regulatory compliance.[18] Furthermore, the ecological impact of the analytical procedure was comprehensively evaluated using an array of green analytical metrics, including the Complex Green Analytical Procedure Index (ComplexGAPI), Analytical GREEnness Metric Approach (AGREE), AGREEprep, and the Analytical Eco-Scale (ESA). To holistically assess the method's practical applicability, the blue applicability grade index (BAGI), a key component of the white analytical chemistry (WAC) framework, was employed, balancing analytical performance, sustainability, and operational feasibility.[19] In summary, this study presents a scientifically robust and environmentally conscious HPLC method for the estimation of resveratrol in liposomal formulations. The developed method not only aligns with global sustainability goals but also demonstrates broad utility in pharmacokinetic, toxicokinetic, and clinical research contexts, offering a versatile tool for future drug development and therapeutic monitoring initiatives.

MATERIALS AND METHODS

Chemicals and reagents

Resveratrol (purity >99%) was generously provided by the Chemical Division of Sigma-Aldrich (Mumbai, India) and used as the reference standard throughout the study. HPLC-grade methanol, formic acid, and other analytical-grade reagents were obtained from Merck Life Science Pvt. Ltd. (Mumbai, India). HPLC-grade water was consistently supplied by a Milli-Q water purification system (Millipore, USA) and was used in all chromatographic technique procedures, along with other solvents and chemicals used in the study, confirmed to pharmaceutical or analytical grade standards, ensuring high purity and minimizing analytical variability or potential contamination.

HPLC instrumentational attributes and analytical setup

Chromatographic analysis was performed using an Agilent 1220 Infinity II HPLC system (LC-20AD, Japan), equipped with a quaternary pump with integrated degasser (G7111A), an autosampler (G7129A), and a photodiode array (PDA) detector (G7115A). Data acquisition and processing were conducted using OpenLab CDS software (Agilent Technologies), ensuring robust system control and precise analytical interpretation. Resveratrol was separated using a Phenomenex Luna C18(2) analytical column (150 mm × 4.6 mm, 5 µm particle size) supplied by Phenomenex Inc. (Canada, USA). The column provided high resolution and reproducibility for the analyte of interest. This preparation method eliminated potential contaminants, resulting in clearer baseline readings and enhanced peak resolution. Furthermore, the application of high-quality reagents and meticulous method development significantly improved the reliability of the analytical results produced by the HPLC system to eliminate air bubbles and particulate matter that might interfere with chromatographic performance. A fixed injection volume of 10 µL was used for all analyses. Detection of resveratrol was performed at a wavelength of 319 nm, based on its maximum absorbance, ensuring sensitive and specific quantification across all test samples.

Preparation of standard solutions

A primary stock solution of resveratrol was prepared by accurately weighing 10 mg of the compound and dissolving it in methanol in a 10 mL volumetric flask, yielding a concentration of 1000 µg/mL. Working standard solutions were subsequently prepared by appropriate dilution of the stock solution with the optimized mobile phase (methanol:0.1% formic acid, 30:70, v/v) to achieve a final concentration of 10 µg/mL. Serial dilutions of this working solution were then performed to obtain calibration standards ranging from 2 to 10 µg/mL. Before HPLC analysis, all solutions were filtered through a 0.22 µm syringe filter (Millipore, USA) to ensure removal of particulates and to protect the integrity of the chromatographic system.

Preliminary method development studies

Given the poor aqueous solubility of resveratrol, preliminary solubility assessments necessitated the use of organic solvents such as methanol and acetonitrile. Methanol was selected as the primary solvent due to its superior ability to completely dissolve resveratrol, making it a suitable candidate for both sample preparation and as a component of the mobile phase. Initial chromatographic trials using methanol and water as the mobile phase failed to yield detectable peaks, even after an extended runtime of 20 min, indicating inadequate elution and possibly poor interaction with the stationary phase. To enhance chromatographic performance, water was subsequently substituted with 0.1% formic acid, which facilitated the appearance of distinguishable peaks. However, a 50:50 (v/v) methanol: formic acid composition resulted in suboptimal chromatographic parameters, including a low number of theoretical plates (TP) and minor peak tailing, indicating compromised resolution and column efficiency. To overcome these limitations and achieve optimal separation, a systematic method optimization was undertaken using a design of experiments (DoE) approach within a QbD framework. This strategy enabled a comprehensive evaluation of critical method variables and their interactions, ultimately leading to the establishment of robust and reproducible chromatographic conditions for the accurate quantification of resveratrol.[20]

DoE-based method optimization

To achieve robust and efficient chromatographic performance, method optimization was carried out using response surface methodology (RSM) within the framework of DoE.[21-23] Specifically, the Box–Behnken design (BBD) was employed by DoE software version 13.0 (Stat-Ease Inc., USA). This statistical approach enabled the systematic evaluation of multiple experimental variables and their interactive effects on key chromatographic responses. Three critical independent variables were selected for optimization: The percentage of organic solvent in the mobile phase (factor A), flow rate (factor B), and column temperature (factor C). Each factor was studied at three levels: 25%, 30%, and 35% for organic solvent composition; 0.9, 1.0, and 1.1 mL/min for flow rate; and 25°C, 30°C, and 35°C for column temperature. The corresponding dependent variables (responses) monitored were retention time (RT) (Y_1), tailing factor (TF) (Y_2), and TP count (Y_3), which are critical indicators of chromatographic resolution, efficiency, and peak symmetry. Second-order polynomial models were developed to quantitatively elaborate on the relationship between the independent variables and the responses. The adequacy and significance of the model were validated through analysis of variance (ANOVA), and model terms were assessed for statistical relevance ($P < 0.05$). The regression equations and 3D response surface plots generated enabled visualization of response trends and identification of optimal experimental conditions. This DoE-guided approach facilitated the fine-tuning of chromatographic parameters to achieve minimal RT, improved peak shape, and a high number of TP, ensuring analytical precision and robustness. The optimized method was further validated in accordance with ICH guidelines, confirming its reliability for routine quantitative analysis of resveratrol in both bulk and liposomal formulations.

Analytical method validation

The developed HPLC method was validated in accordance with the ICH guidelines (ICH Q2 [R1 & R2]) to make sure of the accuracy, reliability, and reproducibility of the analytical results. The validation parameters evaluated included linearity and range, limits of detection (LOD) and quantification (LOQ), system suitability, precision, accuracy (% recovery), and robustness.[24-26]

Linearity and range

Linearity was assessed by preparing a series of resveratrol standard solutions at concentrations ranging from 2 µg/mL to 10 µg/mL using the optimized mobile phase (methanol: 0.1% formic acid, 30:70, v/v). Each concentration was analyzed in six replicates ($n = 6$). The peak area for each concentration was plotted against the corresponding analyte concentration, and a calibration curve was constructed using the least squares linear regression method. Linearity was evaluated based on the regression coefficient (R^2), slope, and y-intercept of the calibration curve. The method demonstrated excellent linearity over the tested range, confirming its suitability for quantitative analysis of resveratrol within this concentration interval.

LOD and LOQ

The LOD and LOQ of the developed HPLC method were determined based on the standard deviation of the response and the slope of the calibration curve, as recommended by ICH guidelines. These parameters were calculated by the following equations:

$$\mathrm{LOD} = 3.3\mathrm{X}(\mathrm{SD}/\mathrm{S}) \quad \text{(EQ1)}$$

$$\mathrm{LOQ} = 10\mathrm{X}(\mathrm{SD}/\mathrm{S}) \quad \text{(EQ2)}$$

System suitability

System suitability testing was performed to make sure the chromatographic system's adequacy for routine analysis and to verify the reliability and reproducibility of the HPLC method. Key parameters evaluated included RT, chromatogram peak area, TP number (N), and TF for resveratrol. Six replicate injections ($n = 6$) of a standard resveratrol solution (10 µg/mL) were analyzed under optimized chromatographic conditions.

Precision

The precision of the developed HPLC method was evaluated in terms of intra-day and inter-day repeatability using three different concentrations of resveratrol: 2, 6, and 10 µg/mL. For intra-day precision, each concentration was analyzed in triplicate at multiple time points within the same day. Inter-day precision was assessed by repeating the same procedure on the following day under identical experimental conditions. The results were expressed as mean ± standard deviation (SD), and the %RSD was calculated for each concentration. A % RSD value below 2% was considered acceptable, in accordance with ICH guidelines.

Accuracy

The accuracy of the developed HPLC method was evaluated through recovery studies, designed to ascertain the method's ability to measure resveratrol without interference from formulation excipients. The analysis was performed by spiking placebo matrices with known quantities of resveratrol at three concentration levels 50%, 100%, and 150%corresponding to the lower, middle, and upper ranges of the validated linearity interval. Each spiked level was analyzed in triplicate, and the % recovery was calculated by comparing the experimentally measured concentration to the theoretical amount added. The mean recovery values, along with their respective standard deviations and % RSD, were determined.

Robustness

The assessment of robustness of the developed HPLC method was evaluated to determine its reliability under small, deliberate variations in analytical conditions. This assessment ensures that the method remains unaffected by minor changes, thereby confirming its stability during routine use. Robustness was tested by introducing slight modifications to critical chromatographic parameters, including the mobile phase composition (adjusted to 28:72 and 32:68, methanol:0.1% formic acid, v/v), flow rate (altered to 0.9 and 1.1 mL/min), and detection wavelength (shifted to 317 and 321 nm). Standard resveratrol solutions (10 µg/mL) were analyzed under each modified condition. The effects of these variations were examined by monitoring changes in RT and peak area. Results were expressed as the %RSD, and values within the acceptable threshold (<2%) indicated that the method retained its analytical performance.

Method application

Preparation of liposomes

Liposome was prepared using the thin-film hydration technique, a widely employed method for encapsulating hydrophobic bioactives such as resveratrol. Key formulation parameters, namely, the amounts of soya lecithin and cholesterol, as well as vortexing time, were systematically varied to assess their impact on the physicochemical properties of the liposomes. A total of nine formulations were developed by adjusting lecithin (410 and 820 mg), cholesterol (190 and 380 mg), and vortexing time (5 and 15 min) based on a factorial design approach. Briefly, specified amounts of soya lecithin and cholesterol were dissolved in a chloroform–methanol mixture (1:1, v/v), after which 20 mg of resveratrol was added. A thin lipid film was formed on the inner wall of a round-bottom flask after the resulting solution was subjected to rotary evaporation under reduced pressure. The lipid film was subsequently hydrated with phosphate buffer (pH 7.4), and the suspension was vortexed for 30 min to facilitate bilayer swelling. To achieve nanoscale vesicle size and improve uniformity, the dispersion was further sonicated for 1 h using a probe sonicator. This method enabled efficient encapsulation of resveratrol into liposomal vesicles, suitable for subsequent physicochemical characterization and analytical evaluation. The resulting resveratrol-loaded liposomes were characterized by their particle size, entrapment efficiency, zeta potential, and polydispersity index.[27]

Sample processing for resveratrol-loaded liposomes

To quantify the resveratrol content in liposomal formulations, an accurately measured 20 mL of the liposomal dispersion, corresponding to 20 mg of resveratrol, was transferred into a volumetric flask. The sample was initially dispersed in methanol and then diluted with the optimized mobile phase (methanol:

0.1% formic acid, 30:70 v/v) to prepare a 100 mL stock solution. To ensure complete disruption of liposomal vesicles and release of the encapsulated drug, the mixture was subjected to sonication for 15 min. Subsequently, a 2 mL aliquot of the stock solution was withdrawn and further diluted with the mobile phase to obtain a suitable concentration for chromatographic analysis. The resulting solution was filtered through a 0.22 µml syringe filter to remove any particulate matter before injection into the HPLC system. Quantitative analysis of resveratrol was performed using the validated method, which enabled accurate assessment of drug loading within the liposomal formulation.

Greenness and whiteness assessment of the proposed method

The environmental impact and practical applicability of the developed HPLC method were comprehensively evaluated through an integrated suite of green and WAC assessment tools. This multidimensional evaluation provided a holistic understanding of the method's ecological footprint, analytical efficiency, and operational viability in routine laboratory settings. The greenness of the method was appraised by the ComplexGAPI, Analytical GREEnness Metric (AGREE), AGREEprep, and ESA. These tools assessed key parameters such as solvent consumption, toxicity of reagents, energy usage, and waste generation, as well as sample preparation complexity. Each metric offered unique insights into the method's alignment with the principles of green analytical chemistry (GAC).[28,29]

To evaluate the practical and operational feasibility, the method was further analyzed using the BAGI, a core component of the WAC paradigm. BAGI integrates aspects of performance, environmental impact, and usability to determine the overall "whiteness" of the analytical procedure. Collectively, these assessment tools revealed that the developed method possesses a high degree of greenness, coupled with strong analytical robustness and practical adaptability. This positions the method as a sustainable and efficient option for high-throughput quality control and analytical applications, aligning with global efforts to promote environmentally responsible and operationally feasible analytical practices.[30,31]

RESULTS AND DISCUSSION

DoE-based method optimization

A BBD was employed to systematically optimize the chromatographic conditions by evaluating three critical independent variables: percentage of the organic phase (A), flow rate (B), and column temperature (C). The corresponding response variables included RT (Y_1), TF (Y_2), and TP (Y_3), which collectively represent chromatographic performance metrics. The experimental design was implemented using DoE software (Version 13.0), generating 15 experimental runs, including three center points to ensure model reliability and reproducibility. The experimental layout is summarized in Table 1. ANOVA was applied to interpret the experimental data, allowing for the identification of statistically significant factors and their interactions. This robust statistical evaluation enabled the precise delineation of variables with the greatest influence on each response, thereby facilitating the optimization of

Table 1: Execution of the Box–Behnken experimental design and corresponding outcomes for the proposed HPLC Method

Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
	A: Organic phase	B: Flow rate	C: Column temperature	RT	TF	TP
	%		mL/min		Min	
1	25	1.1	30	4.786	1.374	5895
2	25	1	25	4.912	1.305	5610
3	35	0.9	30	3.865	1.623	5896
4	30	1.1	25	4.023	1.569	6231
5	25	1	35	4.962	1.324	5765
6	30	1.1	35	4.012	1.598	6210
7	25	0.9	30	5.098	1.289	5463
8	35	1	25	3.653	1.689	5541
9	30	0.9	35	4.412	1.405	6301
10	30	1	30	4.321	1.452	6542
11	35	1.1	30	3.235	1.726	5236
12	30	1	30	4.301	1.426	6532
13	30	1	30	4.315	1.438	6510
14	35	1	35	3.612	1.693	5468
15	30	0.9	25	4.468	1.412	6352

HPLC: High-performance liquid chromatography, RT: Retention time, TF: Tailing factor, TP: Theoretical plates method parameters. The detailed ANOVA results are presented in Tables 2-4. A second-order polynomial regression model was derived to describe the quantitative relationships between the independent variables and the respective responses. Positive regression coefficients indicate synergistic effects, whereas negative coefficients reflect antagonistic interactions. The generated models demonstrated strong correlations (R^2 values) between predicted and observed responses, confirming the validity and predictive power of the optimized chromatographic conditions. The final regression equations for RT, TF, and TP are provided as Eqs (3)-(5), respectively.

$$RT = +4.31 - 0.6741 * A - 0.2234 * B - 0.0073 * C - 0.0795 * AB - 0.0227 * AC + 0.0112 * BC - 0.0052 * A^2 - 0.0612 * B^2 - 0.0224 * C^2 \qquad \text{(EQ3)}$$

$$TF = +1.44 + 0.1799 * A + 0.0672 * B + 0.0056 * C + 0.0045 * AB - 0.0038*AC + 0.0090 * BC + 0.0355*A^2 + 0.0288 * B^2 + 0.0285 * C^2 \qquad \text{(EQ4)}$$

$$TP = +6528.00 - 74.00 * A - 55.00 * B + 1.25 * C - 273.00 * AB - 57.00 * AC + 7.50 * BC - 791.50 * A^2 - 114.00 * B^2 - 140.50 * C^2 \qquad \text{(EQ5)}$$

Response surface analysis

To systematically evaluate the effects of individual independent variables on chromatographic responses, several diagnostic and visualization tools were utilized, including residual versus predicted plots, perturbation plots, and three-dimensional

Table 2: ANOVA for response surface analysis by DoE-based Box–Behnken design (Y_1=RT)

Source	Sum of squares	df	Mean square	F-value	*P*-value	Result
Model	4.08	9	0.4531	381.60	<0.0001	Significant
A-Organic phase	3.64	1	3.64	3061.82	<0.0001	
B-Flow rate	0.3992	1	0.3992	336.18	<0.0001	
C-Column temperature	0.0004	1	0.0004	0.3541	0.5777	
AB	0.0253	1	0.0253	21.29	0.0058	
AC	0.0021	1	0.0021	1.74	0.2439	
BC	0.0005	1	0.0005	0.4264	0.5426	
A^2	0.0001	1	0.0001	0.0830	0.7848	
B^2	0.0138	1	0.0138	11.63	0.0190	
C^2	0.0019	1	0.0019	1.56	0.2666	
Residual	0.0059	5	0.0012			
Lack of Fit	0.0057	3	0.0019	18.12	0.0528	Not significant
Pure error	0.0002	2	0.0001			
Cor total	4.08	14				

ANOVA: Analysis of variance, RT: Retention time

Table 3: ANOVA for response surface analysis by DoE-based Box–Behnken design (Y_2=TF).

Source	Sum of squares	df	Mean square	F-value	*P*-value	Result
Model	0.3051	9	0.0339	40.54	0.0004	Significant
A-Organic phase	0.2588	1	0.2588	309.59	<0.0001	
B-Flow rate	0.0362	1	0.0362	43.27	0.0012	
C-Column temperature	0.0003	1	0.0003	0.3028	0.6058	
AB	0.0001	1	0.0001	0.0969	0.7682	
AC	0.0001	1	0.0001	0.0673	0.8057	
BC	0.0003	1	0.0003	0.3875	0.5609	
A^2	0.0047	1	0.0047	5.58	0.0646	
B^2	0.0031	1	0.0031	3.66	0.1139	
C^2	0.0030	1	0.0030	3.60	0.1164	
Residual	0.0042	5	0.0008			
Lack of Fit	0.0038	3	0.0013	7.56	0.1190	Not significant
Pure error	0.0003	2	0.0002			
Cor total	0.3092	14				

ANOVA: Analysis of variance, TF: Tailing factor

(3D) response surface contour plots. These graphical tools are integral components of RSM, offering both qualitative and quantitative assessments of model performance and system dynamics. Residual versus predicted plots serve as key diagnostics to validate the regression models. These plots display the distribution of residuals against their corresponding predicted values, enabling the identification of non-random patterns that may signify model inadequacies such as non-linearity, heteroscedasticity, or influential outliers. Ideally, residuals should be randomly and symmetrically scattered around the zero line, indicating that the model assumptions are met and the fitted equations are statistically sound. The residual versus predicted plots generated for all three response variables (RT, TF, and TP) demonstrated such random scatter patterns, confirming the robustness and reliability of the polynomial regression models [Figure 1].

Perturbation plots were employed to evaluate the sensitivity of each chromatographic response – RT, TF, and TP to variations in individual experimental factors, while maintaining all other variables at their central (reference) levels. These plots provide a model-based visualization that highlights the relative influence of each independent variable on the measured outcomes. In these plots, the response is plotted against each factor individually, with the steepness and curvature of the resulting lines serving as qualitative indicators of factor sensitivity. A steeper slope or pronounced curvature signifies a stronger influence of the corresponding factor on the response variable, whereas flatter regions indicate minimal impact. As depicted in Figure 2, substantial gradients were observed for specific variables, underscoring their dominant contribution to method performance. This analysis not only elucidates the hierarchy of factor effects but also aids in identifying critical process parameters for method control. The insights gained from perturbation plots are essential for informed decision-making during method refinement and optimization, thereby enhancing the robustness and predictive reliability of the developed HPLC method.

Table 4: ANOVA for response surface analysis by DoE-based Box–Behnken design (Y_3=TP)

Source	Sum of squares	df	Mean square	F-value	*P*-value	Result
Model	2.724E+06	9	3.027E+05	253.00	<0.0001	Significant
A-Organic phase	43808.00	1	43808.00	36.61	0.0018	
B-Flow rate	24200.00	1	24200.00	20.23	0.0064	
C-Column temperature	12.50	1	12.50	0.0104	0.9226	
AB	2.981E+05	1	2.981E+05	249.16	<0.0001	
AC	12996.00	1	12996.00	10.86	0.0216	
BC	225.00	1	225.00	0.1880	0.6826	
A^2	2.313E+06	1	2.313E+06	1933.25	<0.0001	
B^2	47985.23	1	47985.23	40.10	0.0014	
C^2	72887.08	1	72887.08	60.92	0.0006	
Residual	5982.50	5	1196.50			
Lack of Fit	5446.50	3	1815.50	6.77	0.1313	Not significant
Pure error	536.00	2	268.00			
Cor total	2.730E+06	14				

ANOVA: Analysis of variance, TP: theoretical plates

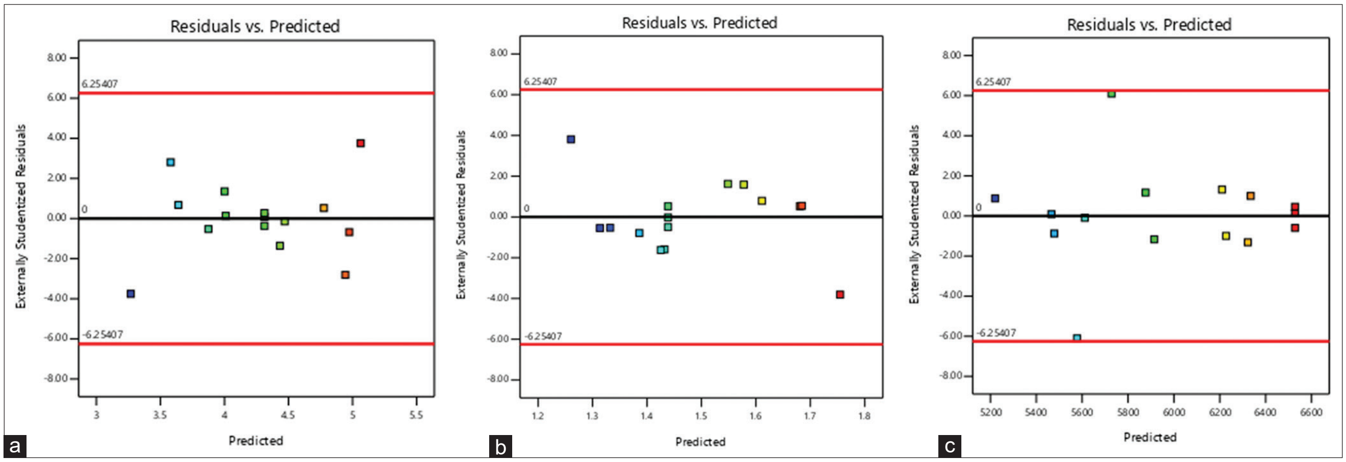

Figure 1: Residual versus predicted graphs of (a) retention time, (b) tailing factor, and (c) theoretical plates of resveratrol

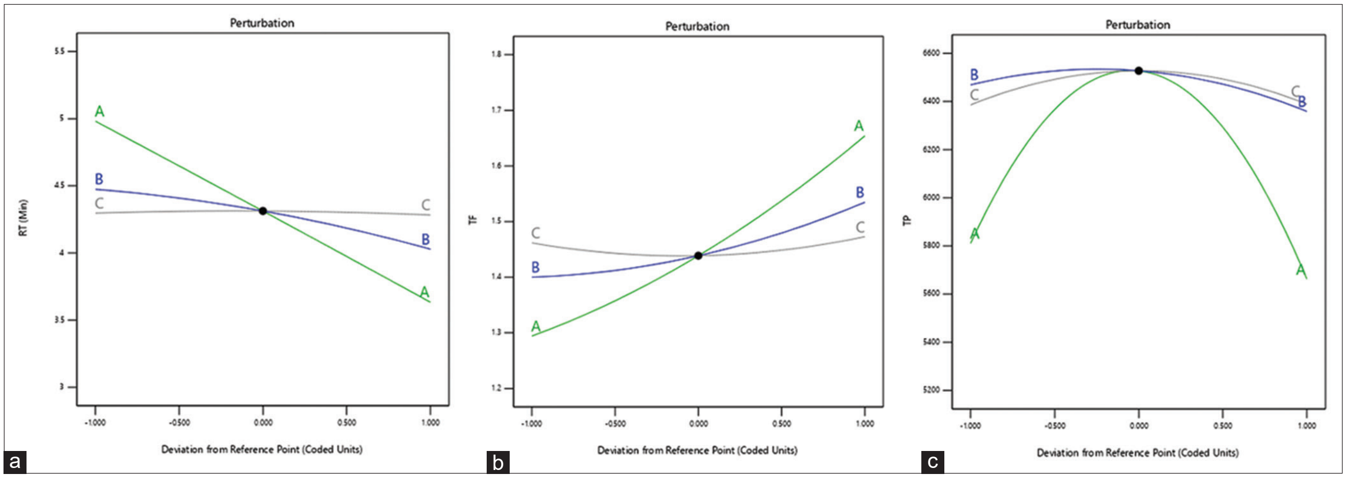

Figure 2: Perturbation graph showing the effect of each factor, A, B, and C, on the (a) retention time, (b) tailing factor, and (c) theoretical plates of resveratrol

The 3D response surface plots offered valuable insights into the interplay between key chromatographic parameters and their influence on critical analytical responses. For RT, the response surface plot [Figure 3a] revealed a clear inverse relationship with both the percentage of organic phase and the flow rate. As these variables increased, RT decreased significantly, underscoring their dominant roles in modulating analyte elution. In contrast, column temperature exhibited only a marginal effect on RT, resulting in a slight decline at elevated temperatures – suggesting a relatively weak influence on retention dynamics.

The plot for TP [Figure 3b] showed that increasing both the flow rate and organic phase concentration initially led to improved column efficiency. However, beyond an optimal threshold, further increases in these parameters resulted in a decline in TP, indicating a parabolic relationship. This trend emphasizes the importance of maintaining these variables within a defined operational window to maximize separation efficiency. Regarding the TF [Figure 3c], both the organic phase concentration and flow rate exhibited a direct positive correlation with TF values. Elevated levels of either factor resulted in increased peak tailing, signaling the need for careful modulation to preserve peak symmetry. Similar to its influence on RT, column temperature had a minimal effect on TF, confirming its limited impact on peak shape under the tested conditions. In summary, the response surface plots demonstrated that flow rate and organic phase proportion are critical variables that must be finely tuned to optimize chromatographic performance. Conversely, the relatively minor role of column temperature allows greater flexibility during method development, enabling focus on the parameters that exert the most substantial impact on analytical outcomes.

Optimized method conditions

The DoE analysis, performed using Stat-Ease software (version 13.0), confirmed that all response variables, RT, TF, and TP, met the predefined acceptance criteria. Optimization strategies were evaluated based on their composite desirability scores, with priority given to conditions yielding a desirability value of 1 or values approaching unity. The corresponding overlay plot, depicted as a yellow-shaded design space [Figure 4], defined the optimal operating region.

The final optimized chromatographic parameters were established as follows: organic phase concentration of 30% v/v**,** column temperature of 30°C, and flow rate of 1.0 mL/min**.** To verify these conditions, six replicate injections (*n* = 6) of a 10 µg/mL resveratrol standard were analyzed. The experimentally obtained results deviated by less than 5% from the predicted values, thereby validating the accuracy and robustness of the optimized method. As illustrated in Figure 5, the chromatographic separation of resveratrol under these optimized conditions demonstrated high resolution, sharp peak symmetry, and reliable reproducibility.

Analytical method validation

To confirm the reliability and applicability of the optimized RP-HPLC method, a comprehensive validation was conducted in accordance with the ICH Q2 (R1 & R2) guidelines. The validation parameters and outcomes are summarized in Table 5, all of which adhered to the established regulatory acceptance criteria. Linearity was evaluated across the concentration range of 2–10 µg/mL by plotting the average peak area against concentration. The method exhibited excellent linearity, with a correlation coefficient (R^2) greater than 0.999 and a well-defined regression equation. Sensitivity was determined via the standard deviation method, yielding a LOD of 0.25 µg/mL and a LOQ of 0.81 µg/mL, signifying high sensitivity suitable for trace-level quantification. System suitability parameters, including RT, peak area, TP count, and TF, were assessed over six replicate injections. The relative standard deviation (%RSD) for all parameters

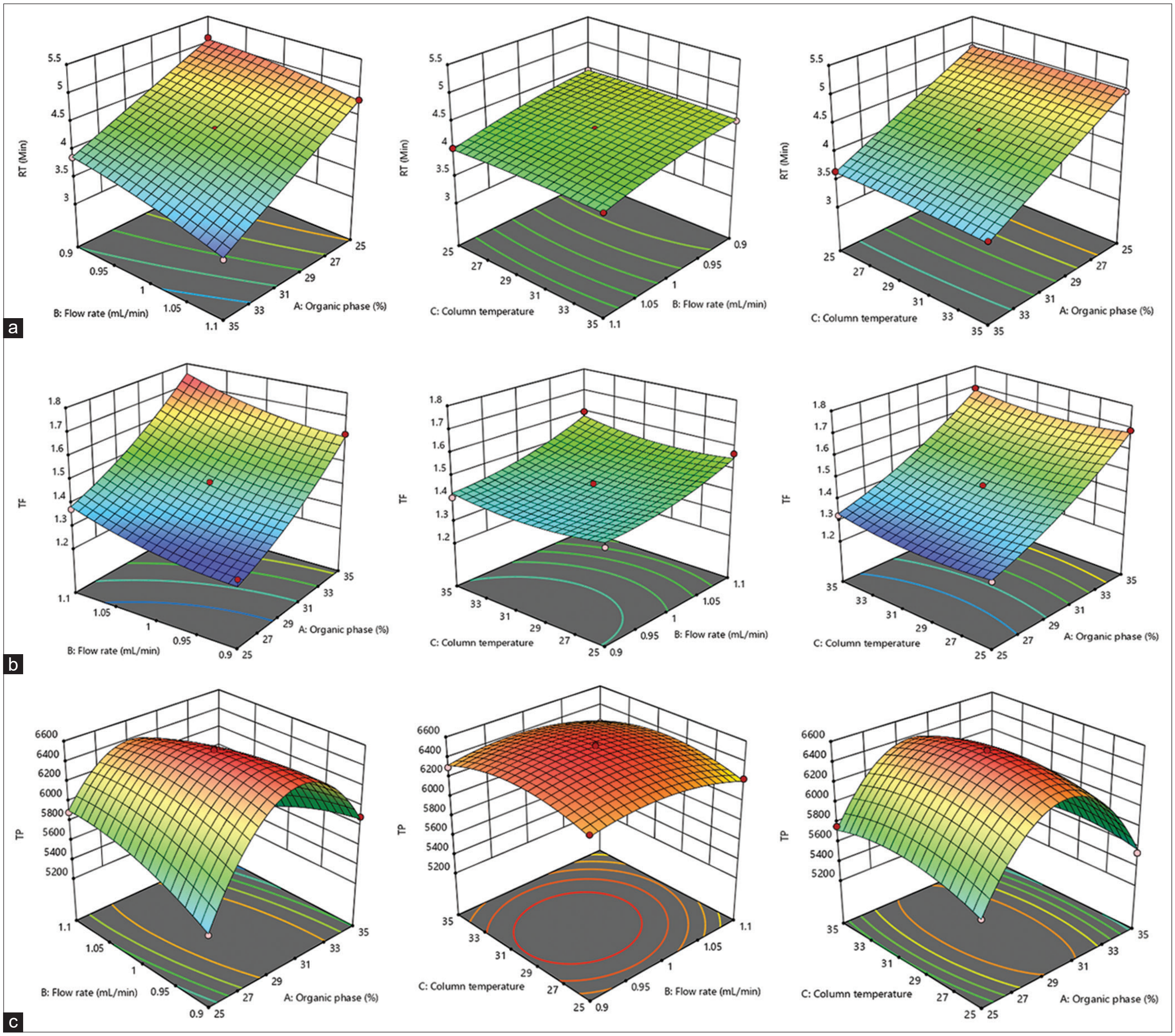

Figure 3: Response surface plot showing the effect of % organic phase (X_1), flow rate (X_2), and column temperature (X_3) on (a) retention time, (b) tailing factor, and (c) theoretical plates of resveratrol

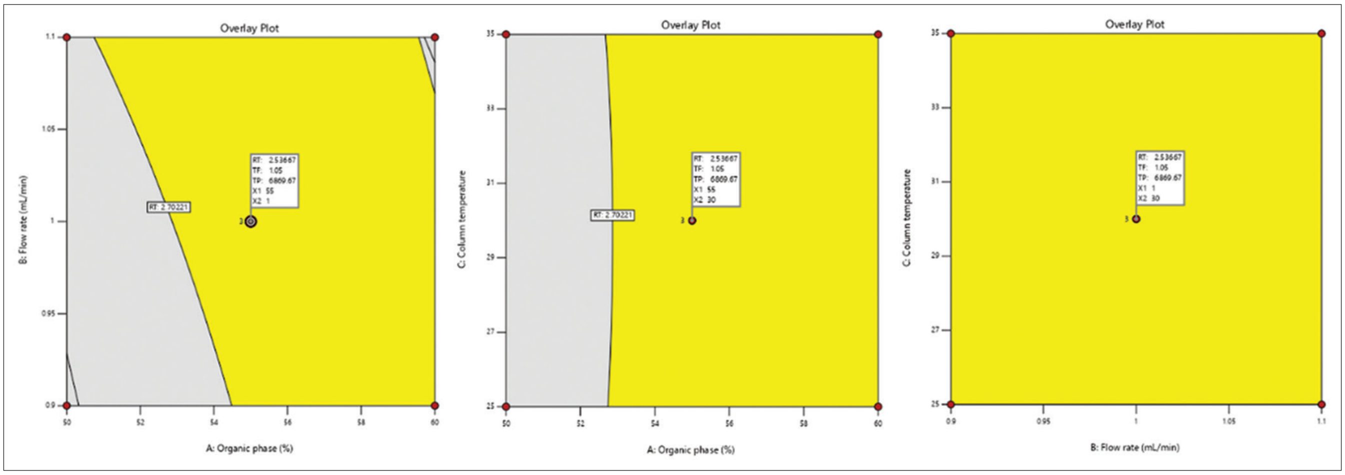

Figure 4: Design space overlay plot showing optimized chromatographic conditions

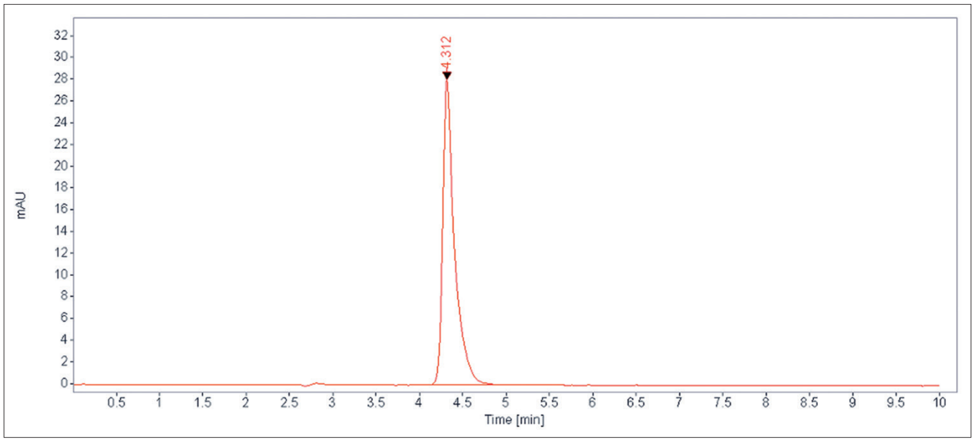

Figure 5: Optimized reverse-phase high-performance liquid chromatography chromatogram of resveratrol (10 µg/mL)

Table 5: Summary of validation parameters

S. No.	Validation parameters	Resveratrol
1	Linearity	
	Linearity range (µg/mL)	2–10
	Graph equation	y=1245.8±0.71x+ 136.07±0.74
	Correlation-coefficient	<0.9991
2	LOD (µg/mL)	0.25
	LOQ (µg/mL)	0.81
3	System suitability	
	Peak area (%RSD)	0.041
	Retention time (%RSD)	0.058
	Tailing factor (%RSD)	0.023
	Theoretical plates (%RSD)	0.124
4	Precision	
	Intra-day (%RSD)	0.087
	Inter-day (%RSD)	0.695
5	Robustness	
	Mobile phase ratio (%RSD)	0.412
	Flow rate (%RSD)	0.074
	Detection wavelength (%RSD)	0.215
6	Accuracy	
	50% recovery	97.8±0.31
	100% recovery	99.2±0.21
	150% recovery	100.2±0.28

LOD: Limit of detection, LOQ: Limit of quantification

was consistently <2%, confirming the system's stability and precision.

Robustness of the method was evaluated by introducing minor deliberate variations in chromatographic conditions, such as changes in detection wavelength (±2 nm), flow rate (±0.1 mL/min), and mobile phase composition (±2%). These variations did not significantly impact the RT or peak area, with all %RSD values remaining within the acceptable range (≤2%). Precision studies, including intra-day and inter-day assessments at three different concentrations (2, 6, and 10 µg/mL), demonstrated excellent repeatability with reproducibility, with low standard deviations and %RSD values well within regulatory limits. Accuracy was confirmed via recovery studies conducted at three spiked levels 50%, 100%, and 150% of the target concentration. The percentage recovery ranged from 97.8 ± 0.31% to 100.2 ± 0.28%, demonstrating the method's ability to recover exactly the analyte from the matrix across varying concentrations. Overall, the method displayed excellent linearity, sensitivity, precision, accuracy, robustness, and system suitability, confirming its reliability for the routine quantification of resveratrol in pharmaceutical or nutraceutical formulations.

Method application

Characterization of prepared liposomes

Resveratrol-loaded liposomes were successfully developed using the thin-film hydration method. The resulting formulation exhibited a mean particle size of 156.11 ± 0.65 nm, falling well within the nanometric range, which is advantageous for enhanced cellular uptake and bioavailability. The liposomes demonstrated a narrow particle size distribution, as indicated by a pre-delivery inspection (PDI) of 0.325 ± 0.35, reflecting good uniformity and formulation consistency (PDI < 0.9). The zeta potential was determined to be −18.2 mV, indicative of moderate electrostatic repulsion among particles, which contributes to colloidal stability by minimizing aggregation over time. %EE was evaluated by quantifying both the total and unencapsulated resveratrol content. The formulation exhibited a high %EE of 87.03 ± 0.24%, confirming the successful incorporation of resveratrol into the liposomal bilayers with minimal drug loss during processing. Collectively, the particle size, PDI, zeta potential, and encapsulation efficiency parameters confirm that the developed liposomal system is well-suited for efficient drug delivery and provides a stable nanoscale carrier for resveratrol.

Table 6: Recovery assay of resveratrol in prepared liposomes

Sample	Additional drug added to analytes (%)	Spiked (μg/mL)	Found (μg/mL)	Recovery (%)
Resveratrol	50	9	8.12	96.35±0.62
	100	12	11.01	98.24±0.36
	150	15	14.75	99.50±0.62

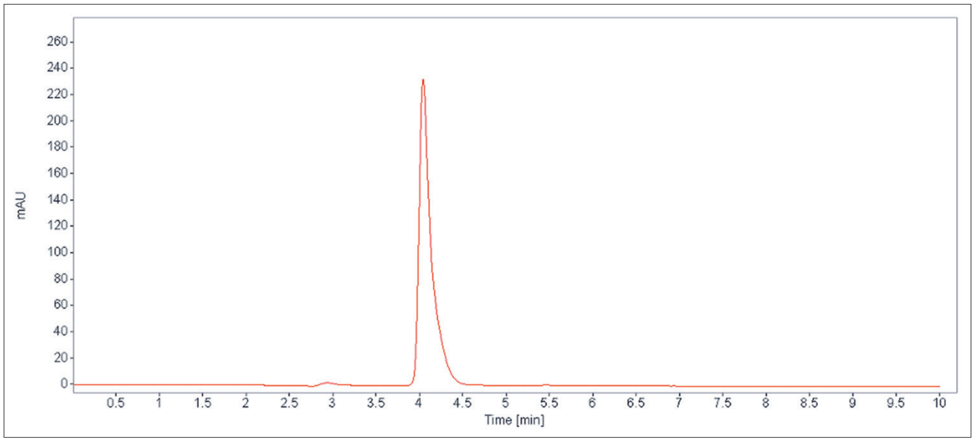

Figure 6: Optimized reverse-phase high-performance liquid chromatography chromatogram of resveratrol in prepared liposome nanoparticles

Recovery assay in prepared liposomes

The validated RP-HPLC method was effectively applied for the quantification of resveratrol encapsulated within the liposomal formulation. Quantitative assessment was conducted in six replicates to ensure analytical reliability and reproducibility. As summarized in Table 6, the method exhibited excellent recovery values ranging from 96.35 ± 0.62% to 99.50 ± 0.62%, confirming the method's accuracy and robustness in analyzing complex nanoformulations. The chromatographic analysis yielded well-resolved, sharp peaks for resveratrol, indicating the method's high sensitivity with selectivity, even in the presence of excipients. These results substantiate the method's suitability for routine quality control of liposomal resveratrol formulations. The consistency of recovery across replicates and concentrations affirms the method's reliability for quantitative analysis, as further illustrated in Figure 6.

Greenness and whiteness assessment of the proposed method

The environmental and practical sustainability of the developed RP-HPLC method was comprehensively assessed by multiple green and WAC (GAC and WAC) evaluation tools. These tools provided a holistic understanding of the method's ecological impact and operational feasibility. The ComplexGAPI tool, which offers a color-coded visual representation via pentagram and hexagonal pictograms, evaluated aspects such as sample preparation, solvent and reagent use, instrumentation, and pre-treatment steps.[32] The analysis revealed nine green, five yellow, and one red indicator, suggesting an overall low environmental burden and high sustainability, as shown in Figure 7a.

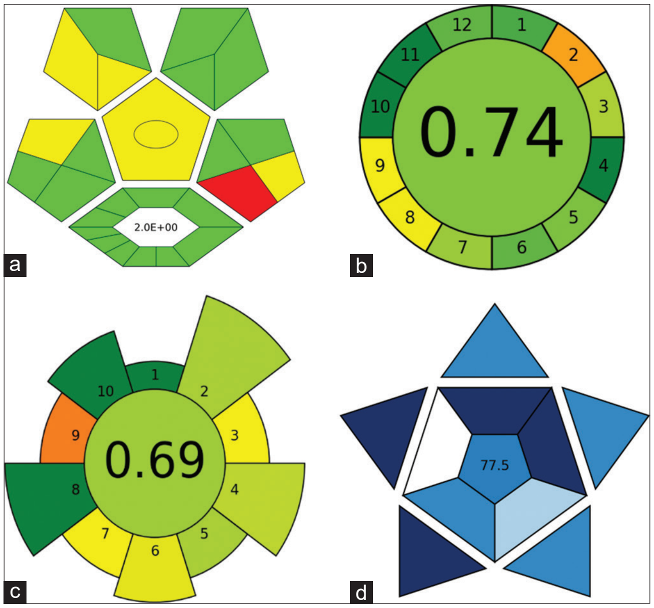

Figure 7: Evaluating the "greenness and whiteness" of the suggested technique, using (a) Complex Green Analytical Procedure Index, (b) Analytical GREEnness Metric Approach, (c) AGREEprep, and (d) blue applicability grade index

To further corroborate this, the AGREE tool – based on the 12 principles of GAC produced a predominantly green pictogram with a central score of 0.74, indicating strong compliance with GAC principles [Figure 7b]. Complementing this, the AGREEprep tool, which specifically assesses sample preparation, yielded a score of 0.69, reinforcing the eco-friendliness of the preparative steps[33] [Figure 7c]. In addition, the ESA – a semi-quantitative metric assigning penalty points for hazardous reagents, energy consumption, and waste generation – assigned a score of 77, categorizing the method as an "excellent green analysis." For WAC assessment, the BAGI was applied to evaluate the balance between environmental sustainability and method applicability. The method achieved a BAGI score of 77.5, indicating a well-optimized balance between ecological performance and analytical robustness [Figure 7d]. Collectively, these integrated evaluations confirm that the proposed RP-HPLC method not only adheres to green chemistry principles but also meets high standards of operational efficiency and analytical quality, making it highly suitable for routine and sustainable use in analytical laboratories**.**

CONCLUSION

The study successfully demonstrated the development, optimization, and validation of a green and white RP-HPLC method for the quantification of resveratrol, leveraging the principles of QbD. A systematic RSM approach using BBD enabled the identification of critical method parameters and their optimal levels, leading to enhanced chromatographic performance. The model was statistically validated through ANOVA and visually supported by response surface and perturbation plots, confirming a strong correlation between factors and responses. The developed method exhibited excellent linearity, sensitivity, and robustness, and met all ICH Q2 (R1 & R2) validation criteria. The method's applicability was confirmed through its successful implementation in quantifying resveratrol from liposomal formulations, which demonstrated favorable nano-characteristics, including high encapsulation efficiency and desirable particle size distribution. A comprehensive greenness and whiteness assessment using ComplexGAPI, AGREE, AGREEprep, ESA, and BAGI tools further validated the method's low ecological impact and high operational practicality. The high scores across these tools underscore the method's alignment with GAC principles and its suitability for routine use in sustainable laboratories. In conclusion, the proposed RP-HPLC method not only ensures high analytical performance and reproducibility but also embodies environmental responsibility and technical versatility. Its successful application in nanoformulation analysis and favorable green metrics highlight its potential as a sustainable and reliable tool for pharmaceutical quality control and bioanalytical investigations.

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AUTHOR'S CONTRIBUTIONS

PRB in the substantial contribution to conception and design, analysis and interpretation of data, drafting the article, critically revising, and manuscript review. NAK approved the article for important intellectual content and final approval of the version to be published.

DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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