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QBD ASSISTED GREEN AND WHITE ANALYTICAL UPLC METHOD FOR QUANTIFICATION OF PHARMACOPEIA IMPURITIES OF PRAVASTATIN IN BULK DRUG AND PHARMACEUTICAL FORMULATIONS

N. Usha Rani[1*], P. T. S. R. K. Prasad Rao[2], K. Ramanjaneyulu[3]

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ABSTRACT

Background: A QbD-assisted green and white UPLC method was optimized for the simultaneous quantification of pravastatin and its EP impurities B, C, and D. **Methods:** A Design of Experiments (DoE) approach was used to evaluate the influence of critical method parameters (CMPs) on key chromatographic responses. **Results and discussion:** The statistical modeling using quadratic response surface methodology yields excellent regression coefficients (R^2 = 0.9399 for Pravastatin–impurity C and R^2 = 0.9758 for impurities C-D) and significant F-values ($p < 0.0001$), confirming robust model performance. The optimized chromatographic conditions were ethanol and 0.1% formic acid (65:35, v/v) at 0.3 mL/min as the mobile phase, with detection at 239 nm and a Waters ACQUITY BEH C18 column (100 × 2.1 mm, 1.7 µm). These conditions provide sharp, symmetrical peaks at retention times of 0.31, 0.89, 1.16, and 1.43 min for impurity B, pravastatin, impurity C, and D, respectively. Method was validated in accordance with ICH Q2(R2) guidelines, and results demonstrate excellent linearity (r^2 > 0.999), precision (%RSD < 1%), recovery (98.17–101.09%), and sensitivity (LOD = 0.015 µg/mL; LOQ = 0.05 µg/mL). The AGREE metric yields the greenness score of 0.81, and the GAPI pictogram confirms the minimum environmental impact (E-factor = 7.0×10^{-2}). Furthermore, RGB 12 whiteness assessment provides a whiteness brilliance (MB) score of 80.6%, indicating an optimal balance among analytical performance, eco-friendliness, and operational efficiency. **Conclusion:** The proposed method was robust, rapid, environmentally sustainable, and practically efficient for routine impurity profiling and quality control of pravastatin formulations.

INTRODUCTION

Pravastatin sodium is a lipid-lowering agent that belongs to the statin class of medical drugs prescribed in the treatment of cardiovascular disease and dyslipidemia [1]. It acts as a selective and competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, a rate-limiting enzyme in cholesterol biosynthesis [2,3]. It effectively reduces intracellular cholesterol synthesis, leading to upregulation of LDL (low-density

[1]*Department of FED, PVP Siddhartha Institute of Technology, Vijayawada, Andhra Pradesh, PIN: 520007, India.*
[2]*Department of Chemistry, P B Siddhartha College of Arts & Science, Vijayawada-520 010, Andhra Pradesh, India.*
[3]*Department of ECE, NRI Institute of Technology, Agiripalli, Vijayawada-521212, Andhra Pradesh, India.*

For Correspondence: **nannapaneniusharani73@gmail.com**

lipoprotein) receptors and enhancing the clearance of circulating LDL cholesterol [4]. It exhibits potent hypolipidemic activity, an improved pharmacokinetic profile, and a relatively low potential for drug–drug interactions, making it a key therapeutic agent for the treatment of hypercholesterolemia, atherosclerosis, and cardiovascular diseases [5]. Pravastatin can generate process-related impurities and degradation products during synthesis, purification, and storage, as with other statin-class drugs. These impurities at trace levels can also influence the efficacy, safety, and stability of pravastatin. Hence, impurity profiling was considered an essential aspect in pharmaceutical quality assurance [6]. The conventional impurity profiling analytical methods predominantly rely on HPLC with acetonitrile-based mobile phases. These methods produce adequate resolution and sensitivity, but they suffer from long run times, higher solvent consumption, toxic solvent use, and poor environmental sustainability.

In light of the increasing regulatory emphasis on green analytical chemistry (GAC), there is a growing need to design methods that minimize environmental impact without compromising analytical performance [7]. The implementation of GAC principles in analytical methods can eliminate hazardous substances from analytical workflows by promoting the use of safer solvents and minimizing waste generation. The greenness concept evaluates the environmental impact of analytical methods but does not fully capture other key aspects, such as cost-efficiency, operator safety, and overall method performance.

The concept of White Analytical Chemistry (WAC) emerged to address these limitations. The WAC integrates the "green" (environmental) and "red" (analytical efficiency and quality) aspects of the method, providing a more holistic evaluation of method sustainability [8]. Further, the application of the Quality by Design (QbD) paradigm in analytical development has transformed the traditional trial-and-error approach into a systematic, science-based, and risk-managed process [9].

QbD focuses on identifying Critical Quality Attributes (CQAs) and Critical Method Parameters (CMPs) that influence method performance. This approach optimizes the method using multivariate statistical tools such as Design of Experiments (DoE). This systematic framework ensures the robust method performance across a defined Design Space with enhanced reliability, reproducibility, and regulatory compliance [10].

Table 1: Representative literature on pravastatin analytical methods

S No	Study	Method Description	Key Features	Gap identified	Ref.
1	HPLC Formulation assay	Teknokroma C8 (250 mm) column with methanol, ammonium acetate (10mM), and triethylamine in 60:40:0.17 (v/v) at 1.0 mL/min and 239 nm wavelength	Retention time: 2.15 min, Linearity: 0.4-1000 µg/mL LOD: 12 ng/mL LOQ: 0.4 µg/mL	Method not suitable for impurity analysis, green/white analytical assessment absent	11
2	HPLC for hydrolytic degradation study	C18 Bondapak (150 mm) column with phosphate buffer (30 mM) at pH 2 and acetonitrile in 72: 28 (v/v) at 1 mL/min and 239 nm wavelength	Identifies degradation behavior; a method developed for stability-indicating purposes	Does not focus on systematic impurity profiling, QbD, or green frameworks; conventional solvents were utilized, and a longer runtime.	12
3	HPLC formulation assay of Aspirin and Pravastatin	Phenomex C18 column (250 mm) Acetonitrile, water, and acetic acid in 59: 40:01 at 1.5 mL/min	10-30 µg/mL as linearity with LOD:0.077 µg/mL LOQ:0.256 µg/mL	Not suitable for impurity analysis, and the green approach was not addressed	13

The literature survey on the reported analytical methods for the analysis of pravastatin, along with the identified gap, was tabulated in Table 1. The reported analytical procedures don't address the integration of QbD-based method optimization with green and white analytical assessment. Therefore, the present study aims to develop a QbD-assisted, green, and white analytical UPLC method for the simultaneous quantification of EP impurities of pravastatin in bulk drug and pharmaceutical formulations. This study aimed to replace toxic organic solvents to achieve maximum resolution and sensitivity while minimizing the environmental footprint. Furthermore, methodological greenness and whiteness were assessed using

GAPI, AGREE, and WAC metrics. The pravastatin contains several listed related substances, and among these, impurities B, C, and D were considered the most significant from a stability & process perspective. Impurity B is well-recognized as a degradation product of pravastatin, whereas impurities C and D are structurally related process impurities often present due to variations in the synthetic route.

These impurities pose considerable chromatographic challenges because of their close structural similarity to the parent drug. Therefore, these three EP-specified impurities were selected in the present study for systematic QbD-assisted method development and validation in accordance with green and white analytical chemistry principles. The structure of pravastatin and its impurity were presented in Figure 1.

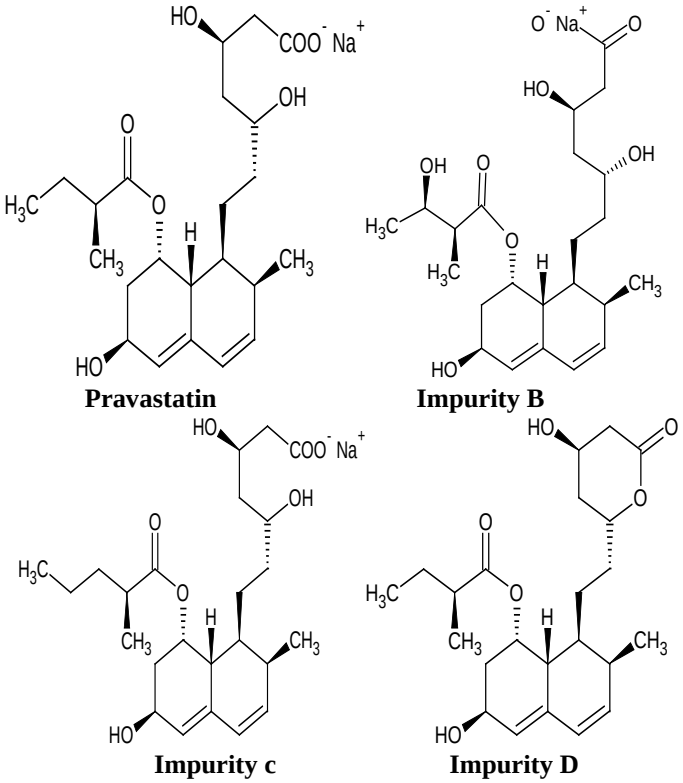


Figure 1: Structure of pravastatin and its EP impurities in the study

MATERIALS AND METHODS

Materials

The active pharmaceutical ingredient of pravastatin sodium, with 98.75% purity, along with its EP impurity B, C, and D, was kindly supplied by Emcure Pharmaceuticals Ltd, Pune, Maharashtra, India. The commercial tablet formulation of pravastatin (Pravator® 20 mg) was purchased from a local pharmacy and used for analysis without further modification. The UPLC-grade ethanol (LiChropur™) and formic acid were obtained from Merck Chemicals, Mumbai, India.

The ultrapure water used throughout the study was prepared through the Millipore Direct-Q 3UV water purification system (Merck Millipore, Darmstadt, Germany). All solvents and reagents were of analytical or chromatographic grade and were filtered through 0.22 µm membrane filters prior to use.

Instrumentation and Chromatographic Conditions

The chromatographic analysis of pravastatin and its EP impurities was performed on the ACQUITY™ UPLC (Waters Corporation, Milford, MA, USA) system. The instrument was equipped with a Binary Solvent Manager (BSM) for solvent delivery, a Sample Manager–FTN (Flow Through Needle) for sample injection into the chromatographic system, a column component, and a Photodiode Array (PDA) detector.

Data acquisition, peak integration, and system control were performed using Empower™ 3 software (Waters, USA). The system was operated in a temperature-controlled laboratory environment (25 ± 2 °C) throughout the analysis. The separation of pravastatin and its EP impurities was achieved on an ACQUITY BEH C18 (100 mm × 2.1 mm i.d., 1.7 µm particle size) column (Waters, USA). The mobile phase consists of ethanol and 0.1% (v/v) aqueous formic acid in a 65:35 (v/v) ratio, with isocratic flow at 0.3 mL/min. The sample, injected at 2 µL, was analyzed, and the eluents were detected at 239 nm.

Preparation of Standard and Calibration Solutions

The stock solutions of pravastatin and its EP impurities were individually prepared at a concentration of 1000 µg/mL. During preparation, 10 mg of each reference standard was accurately weighed and dissolved in 10 mL of diluent (equal volumes of ethanol and water). An appropriate dilution of this standard stock solution was prepared to achieve a 20 µg/mL concentration, and this solution was designated as the 100% level for quantification. The working standard solution dilutions of pravastatin were freshly prepared from the stock at a concentration of 5-30 µg/mL (25% to 150% of the nominal concentration).

The working solutions of the impurities were also prepared in a similar way to yield concentrations corresponding to 1% (0.20 µg/mL) relative to the pravastatin test concentration. Equal volumes of each concentration level of pravastatin and its impurities were mixed separately to achieve combined

calibration dilutions. Each prepared combined calibration level solution was injected in triplicate (n = 3) under the optimized UPLC chromatographic conditions.

Preparation of test solutions

Twenty Pravator® tablets that contain 20 mg of pravastatin sodium were accurately weighed and finely powdered through a clean mortar and pestle. The table powder portion equivalent to one tablet (20 mg pravastatin) was transferred into a 100 mL volumetric flask. The flask was filled with approximately 70 mL of diluent and sonicated for 2 min to ensure complete drug dissolution.

The volume in the flask was made up to the mark with the same diluent, and then the solution was filtered through a 0.22 µm membrane filter. Then the solution was diluted to obtain a test solution equivalent to 100% (20 µg/mL). Simultaneously, the impurity-spiked formulation sample solution was prepared by adding 1% concentrations of each known impurity solution to the 100% level impurity solution. The unspiked and spiked solutions were analyzed under the optimized chromatographic conditions to assess the resolution, recovery, and impurity-quantification capability of the proposed method.

Method development

The initial phase of method development involves the preliminary investigation of solubility and UV absorbance characteristics of pravastatin sodium and its EP impurities. The UV spectral scan was recorded in the 200-400 nm range to determine the optimal detection wavelength at which pravastatin and its impurities exhibit maximum absorbance. The Acquity BEH C18 columns of 100 and 50mm dimensions under varied column temperatures (25–35 °C) were tested for adequate resolution, peak symmetry, and efficiency.

The mobile phase composition was systematically optimized using ethanol as the organic modifier, in accordance with GAC principles. The aqueous phase, consisting of 0.1% formic acid or ammonium formate buffer across a wide pH range (3.0–6.5), was tested to produce sharp, symmetrical peaks with excellent baseline resolution for pravastatin and its impurities.

Method optimization by the QbD approach

The resolution and accurate, robust quantification of pravastatin and its EP impurities were considered key objectives for the QbD-driven method development study. This study aimed to achieve baseline separation (Rs > 2) of analytes with a short analysis time and minimal solvent consumption under green analytical conditions. Retention time, peak resolution, and symmetry were considered critical method attributes (CMAs), whereas organic solvent composition, buffer strength, flow rate, and column temperature were identified as critical method parameters (CMPs).

The central composite design (CCD) within the Design of Experiments (DoE) framework was used to screen CMPs. The influence of key variables, such as mobile phase ratio and column temperature (25–40°C), was studied using CCD to optimize conditions for the resolution of pravastatin and its EP impurities. The CCD was selected for response surface optimization because it effectively models quadratic effects and curvature within the experimental domain.

The inclusion of axial (star) points in CCD enables exploration beyond the factorial levels, yielding more accurate prediction of response surfaces and ensuring robust estimation of second-order interactions, thereby improving model predictability within the QbD framework.

Method validation

The developed method was validated in accordance with ICH Q2(R2) guidelines to confirm its reliability for routine quality control applications. The chromatographic results obtained after analyzing the 100% level standard solution of pravastatin and its impurities under the optimized chromatographic conditions demonstrate the method's specificity and selectivity. The linearity was evaluated across 25–150% of the target level with its corresponding concentration range of 5-30 µg/mL for pravastatin and 0.05-0.30 µg/mL for its impurities. The calibration curves were constructed individually for pravastatin and its EP impurities by plotting peak area versus analyte concentration.

The correlation coefficient (r^2) value of not less than 0.999 was considered to be acceptable. The system precision, method precision (intra-day), and intermediate precision (inter-day) were evaluated by analyzing the 100 % level solution of pravastatin and its impurities. The %RSD for peak area and retention time for the resultant chromatograms was ≤ 2.0% and was considered acceptable.

Accuracy was determined from recovery studies conducted at 50%, 100%, and 150% of the target concentration, using a

pravastatin tablet matrix spiked with known quantities of impurities. Recovery values in the range of 98.0–102.0% of the nominal value were considered accurate. The signal-to-noise (S/N) ratio approach was utilized to establish the limit of detection (LOD) and limit of quantification (LOQ) for pravastatin and its impurities in the proposed method.

The S/N of ≥ 3 was concluded as LOD, and the S/N of ≥ 10 was confirmed as LOQ of the proposed method. The introduction of deliberate small variations in critical method parameters, such as flow rate (±0.02 mL/min), detection wavelength (±2 nm), and mobile phase composition (±2%), demonstrates the method's robustness. The % peak-area response change for each condition, along with system suitability conditions, was tabulated to assess the method's robustness. The 100% level solution of pravastatin and its impurities was stored at room temperature (25 ± 2 °C) and at refrigerated conditions (2–8 °C) for 24 hours. The chromatographic results for these solutions demonstrate the stability of the analytes under the proposed method.

Assessment of Method Greenness and Whiteness

The environmental sustainability of the proposed method was comprehensively assessed through the Analytical Greenness (AGREE) metric and the Green Analytical Procedure Index (GAPI) tools. The AGREE assesses the method's compliance with 12 GAC principles and provides a quantitative greenness score ranging from 0.00 (poor) to 1.00 (excellent).

The GAPI tool qualitatively assesses the various stages of the analytical workflow, such as sample preparation, solvent and reagent use, instrument energy consumption, waste generation, and overall ecological footprint. The GAPI evaluation produces a color-coded pentagonal pictogram with green, yellow, and red regions that correspond to low, moderate, and high environmental impact, respectively.

Further, the overall sustainability of the method was evaluated using the RGB 12 algorithm. This algorithm integrates the three fundamental dimensions: analytical performance (red), environmental impact (green), and practical and economic efficiency (blue). This approach assigns scores to various method parameters, including method simplicity, speed, solvent safety, energy use, waste production, cost, and analytical reliability. The combined RGB assessment produces a single "whiteness" score that reflects the method's balance between analytical efficiency, environmental safety, and practical feasibility. The summary of AGREE, GAPI, and RGB 12 analysis results proves the compliance of the proposed method with GAC principles.

RESULTS AND DISCUSSION

The primary objective of this study was to establish a robust, rapid, and environmentally sustainable UPLC method for the simultaneous quantification of pravastatin sodium and its EP impurities in bulk drug and pharmaceutical formulations.

Since no comprehensive green analytical method has been reported for the resolution and quantification of EP impurities in pravastatin, this work was planned to develop a method that uses eco-friendly solvents, short analysis times, and superior chromatographic efficiency, in compliance with GAC principles. During method optimization, several chromatographic conditions were systematically evaluated to achieve optimal resolution, peak symmetry & detection sensitivity (Table 2; Figure 2).

Qbd aided method optimization

The QbD approach was used to systematically optimize a robust, reliable analytical method for the quantification of pravastatin and EP impurities. The DoE-guided method optimization enables evaluation of the influence of CMPs (organic solvent composition, flow rate, and detection wavelength) on key chromatographic responses (resolution and peak symmetry).

A D-optimal experimental design was used to identify the most influential factors affecting the separation between the analytes. The results from twenty experimental runs (Table 3) reveal that the organic solvent composition displays the most significant effect on the resolution between pravastatin and impurity C, as well as between impurities C and D.

The resolution between pravastatin and impurity C was selected as Response 1 in the DoE model to evaluate the effect of critical chromatographic parameters on separation efficiency.

The impact of CMPs on the resolution between pravastatin and impurity C was assessed statistically through a quadratic model generated through RSM. The model exhibits a high R^2 of 0.9399, indicating that approximately 93.99% of the variability in resolution is explained by the selected model.

Table 2: Summary of method optimization experiments performed for the UPLC analysis of pravastatin & its EP impurities

S No	Method condition	Result observed	Method status
1	Ethanol & 5 mM ammonium formate in 75:25 (v/v) at 0.3 mL/min; ACQUITY BEH C18 (150 mm) column; 239 nm	No proper separation was noticed. Only a single broad peak was observed at 1.9 min with a clear baseline (Figure 2A).	Rejected
2	Ethanol and 10 mM ammonium formate in 60:40 (v/v) at 0.3 mL/min; ACQUITY BEH C18 (100 mm) column; 239 nm	No proper separation was noticed. Three broad peaks were detected in the chromatogram. The resolution was very poor, suggesting that the composition was unsuitable for resolving the analytes (Figure 2B).	Rejected
3	Ethanol and 10 mM ammonium formate with formic acid in 50:50 (v/v) at 0.4 mL/min; ACQUITY BEH C18 (100 mm) column; 239 nm	The use of formic acid improves analyte separation. Peaks correspond to pravastatin, and its impurities were detected, but the peak symmetry, resolution, and area response were very poor (Figure 2C)	Rejected
4	Ethanol and 0.1% formic acid in 95:05 (v/v) at 0.4 mL/min; ACQUITY BEH C18 (100 mm) column; 239 nm	The removal of ammonium formate enhances the resolution of analytes. Peaks correspond to pravastatin and its impurities. The resolution between impurities C and D was very poor (Figure 2D).	Rejected
5	Ethanol and 0.1% formic acid in 85:15 (v/v) at 0.35 mL/min; ACQUITY BEH C18 (100 mm) column; 239 nm	The increase in the composition of formic acid enhances the resolution between impurity C and D. The symmetry and peak area response were also noticed to be enhanced in this condition (Figure 2E).	Rejected
6	Ethanol and 0.1% formic acid in 75:25 (v/v) at 0.35 mL/min; ACQUITY BEH C18 (100 mm) column; 239 nm	Well-resolved peaks were observed for pravastatin and its EP impurities, with acceptable system suitability and high peak responses	Accepted and utilized for QbD studies

The adjusted & predicted R^2 values were observed to be 0.9279 & 0.8771, respectively. These values were in good agreement, demonstrating the model's strong predictive power and minimal overfitting. The model F-value of 78.16 and the associated p-value of <0.0001 indicate that the model is highly significant. The experimental design matrix (Table 3) shows the resolution between pravastatin and impurity C was in the range of 2.6 to 3.5. The maximum separation was reported at a moderate organic composition of 60-65% at a flow rate of 0.3 mL/min. As illustrated in the 3D surface plot and contour diagrams (Figure 3), the resolution was improved gradually with the increase of organic solvent content from 60% to 65%. This increase in resolution was due to enhanced elution strength and optimized partitioning between the stationary and mobile phases. However, further increases in organic solvent beyond 65% cause a marginal decline in resolution, which may be due to reduced interaction time and excessive peak coalescence. Similarly, flow rate exhibits a strong inverse relationship with resolution. Lower flow rates (0.25 mL/min) improve analyte interaction with the stationary phase but cause peak broadening.

A higher flow rate (0.35 mL/min) can reduce retention and compromise separation efficiency. The optimized flow rate of 0.3 mL/min balances analysis time and peak sharpness, yielding a resolution of 3.1 for pravastatin and impurity C. The detection wavelength has minimal effect on the study. The response surface and contour plots clearly show a slightly curved 3D surface, confirming a nonlinear relationship between organic phase percentage and flow rate with respect to resolution.

The resolution between impurities C and D was identified as Response 2 in the DoE model to evaluate the influence of critical chromatographic parameters on their separation. The statistical analysis demonstrates the excellent model fit with an R^2 of 0.9758. The high R^2 value indicates that approximately 97.6% of the variation in resolution could be explained by the selected variables. The adjusted R^2 was 0.9516, with a predicted R^2 of 0.7923. These results are in agreement and confirm the high predictive accuracy and minimal model bias. The model F-value (40.36) and the associated p-value (<0.0001) indicate that the model is highly significant and that the studied factors exert a pronounced influence on the separation of impurities C and D. The experimental result (Table 3) shows that the resolution between impurities C and D was 3.7-4.8. The 3D response surface and contour plots (Figure 4) reveal a clear nonlinear relationship between the two critical factors: organic solvent

percentage and flow rate. As the ethanol composition increases from 60% to 65%, a remarkable improvement in resolution was observed, with a maximum of 4.6 at 65% ethanol at a flow rate of 0.3 mL/min. The model graphs observed in the DoE evaluation of CMPs for the resolution between impurities C and D are presented in Figure 4.

The tailing factor was considered a critical chromatographic parameter that reflects peak symmetry and column performance. This factor directly influences the quantitative accuracy and method robustness. Hence, the QbD-based optimization, Response 3 (tailing factor of pravastatin) and Response 4 (tailing factor of Impurity C) were evaluated to understand the effects of organic solvent composition, flow rate, and wavelength on peak shape characteristics. The quadratic model in response 3 yields an R^2 value of 0.8993, indicating that approximately 89.9% of the variation in peak tailing was explained in this model. The adjusted R^2 value (0.7986) demonstrates a reasonable model fit, and the predicted R^2 (–0.0182) suggests the limited predictive capability due to variability in experimental observations. The model F-value (8.93) and p-value (0.0016) prove that the model was statistically significant. This indicates that at least one of the experimental factors significantly influenced the peak symmetry of pravastatin. The experimental results (Table 3) show that pravastatin exhibits a nearly ideal Gaussian peak shape across all design points, with tailing factor values ranging from 1.02 to 1.10. The response surface plots (Figure 5) indicated that moderate ethanol concentration (≈65%) and a flow rate of 0.3 mL/min produce the most symmetrical peaks.

The model demonstrates stronger statistical performance for response 4 with an R^2 value of 0.9471. The adjusted and predicted R^2 values were 0.8943 and 0.4443, respectively, indicating a good overall fit and moderate predictive reliability. The F-value (17.92) and p-value (<0.0001) confirm that the model is highly significant, and that the selected parameters substantially affect the peak shape of impurity C. The observed tailing factors ranged from 0.94 to 0.98, suggesting excellent column performance and minimal peak distortion. The 3D surface, contour, and cube plots (Figure 5) reveal that both organic solvent composition and flow rate exert a mild but noticeable effect on symmetry.

Table 3: Experimental design and chromatographic responses observed during the QbD-based method optimization for the analysis of pravastatin and EP impurities.

Run	Factor 1: Organic Solvent(%)	Factor 2: Flow Rate (mL/min)	Factor 3: Wavelength (nm)	Response 1: Resolution (Pravastatin Imp C)	Response 2: Resolution (Imp C–Imp D)	Response 3: Tailing Factor (Pravastatin)	Response 4: Tailing Factor (Imp C)
1	70	0.25	234	2.9	4.8	1.05	0.96
2	70	0.35	234	2.7	4.5	1.04	0.98
3	65	0.3	239	3.1	4.6	1.03	0.97
4	60	0.35	244	3.3	4.1	1.06	0.95
5	60	0.25	244	3.4	4.0	1.08	0.94
6	70	0.25	244	2.8	4.4	1.05	0.97
7	60	0.35	234	3.2	4.0	1.07	0.95
8	65	0.3	239	3.1	4.6	1.03	0.97
9	60	0.25	234	3.4	3.9	1.08	0.94
10	65	0.3	239	3.1	4.6	1.03	0.97
11	70	0.35	244	2.6	4.3	1.04	0.98
12	65	0.3	239	3.1	4.6	1.03	0.97
13	56.6	0.3	239	3.5	3.7	1.10	0.93
14	65	0.3	239	3.1	4.6	1.03	0.97
15	65	0.3	239	3.1	4.6	1.03	0.97
16	73.4	0.3	239	2.8	4.5	1.05	0.96
17	65	0.3	230	3.0	4.5	1.04	0.97
18	65	0.3	247	3.1	4.5	1.04	0.96
19	65	0.4	239	2.9	4.4	1.05	0.97
20	65	0.2	239	3.3	4.7	1.02	0.96

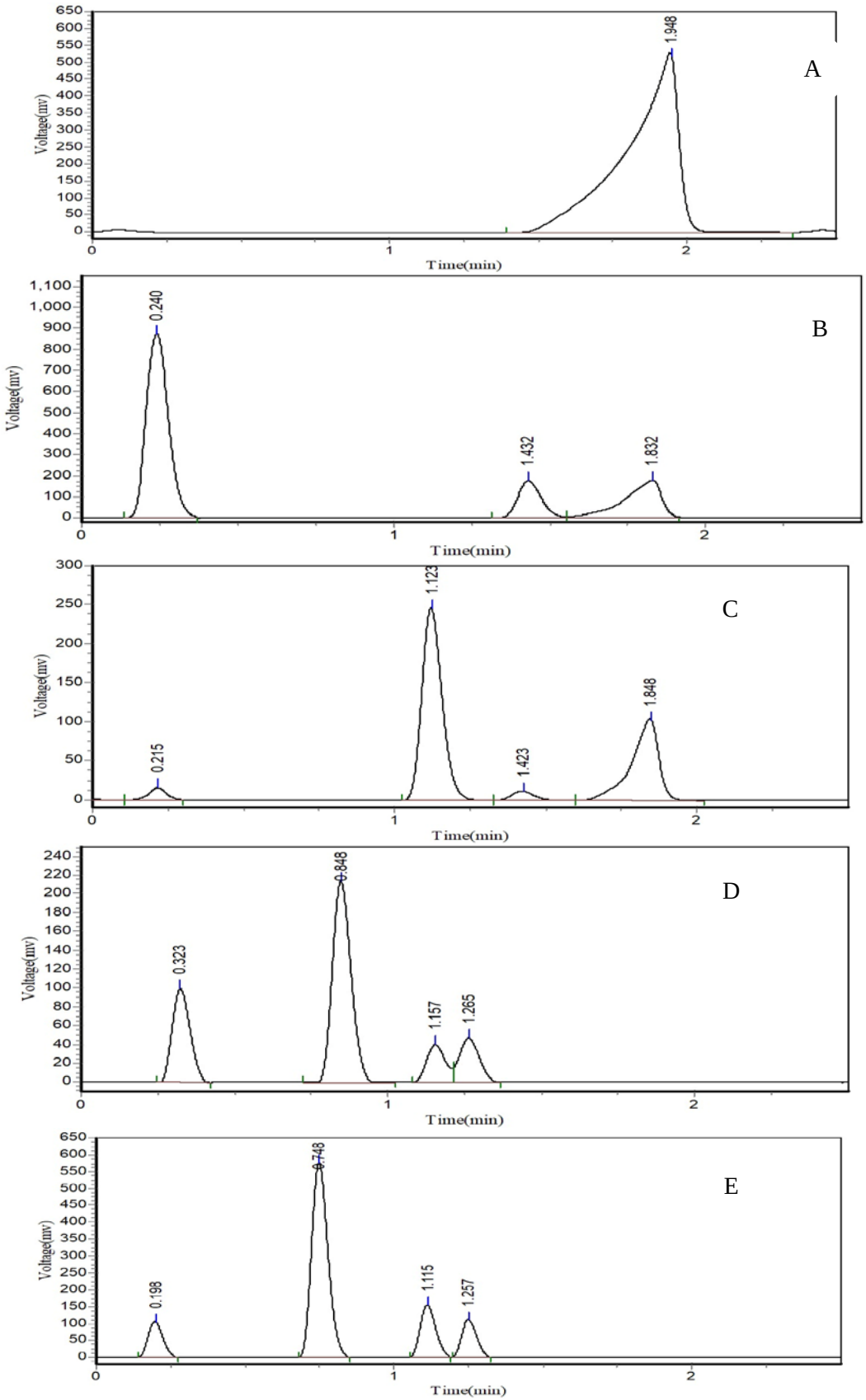
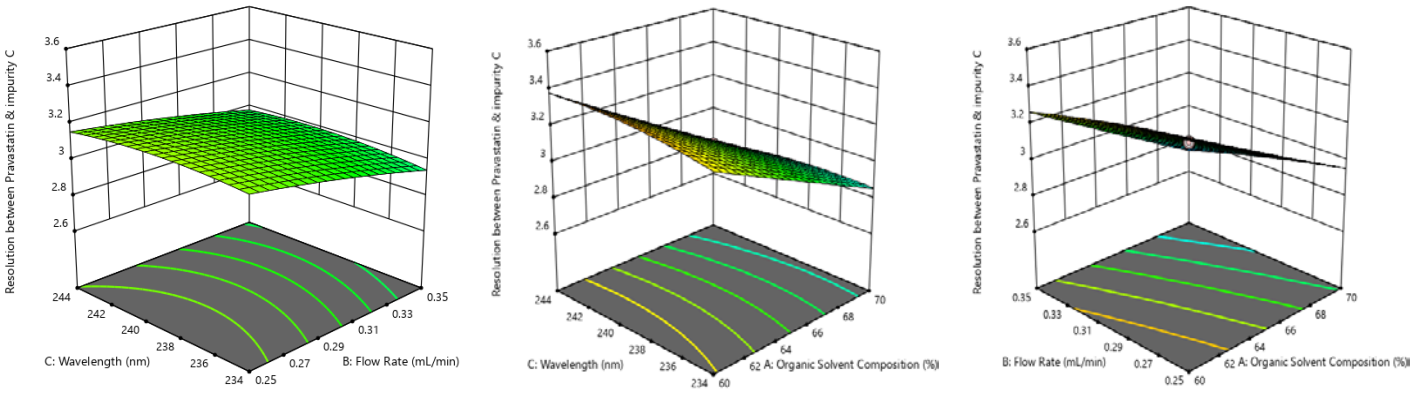
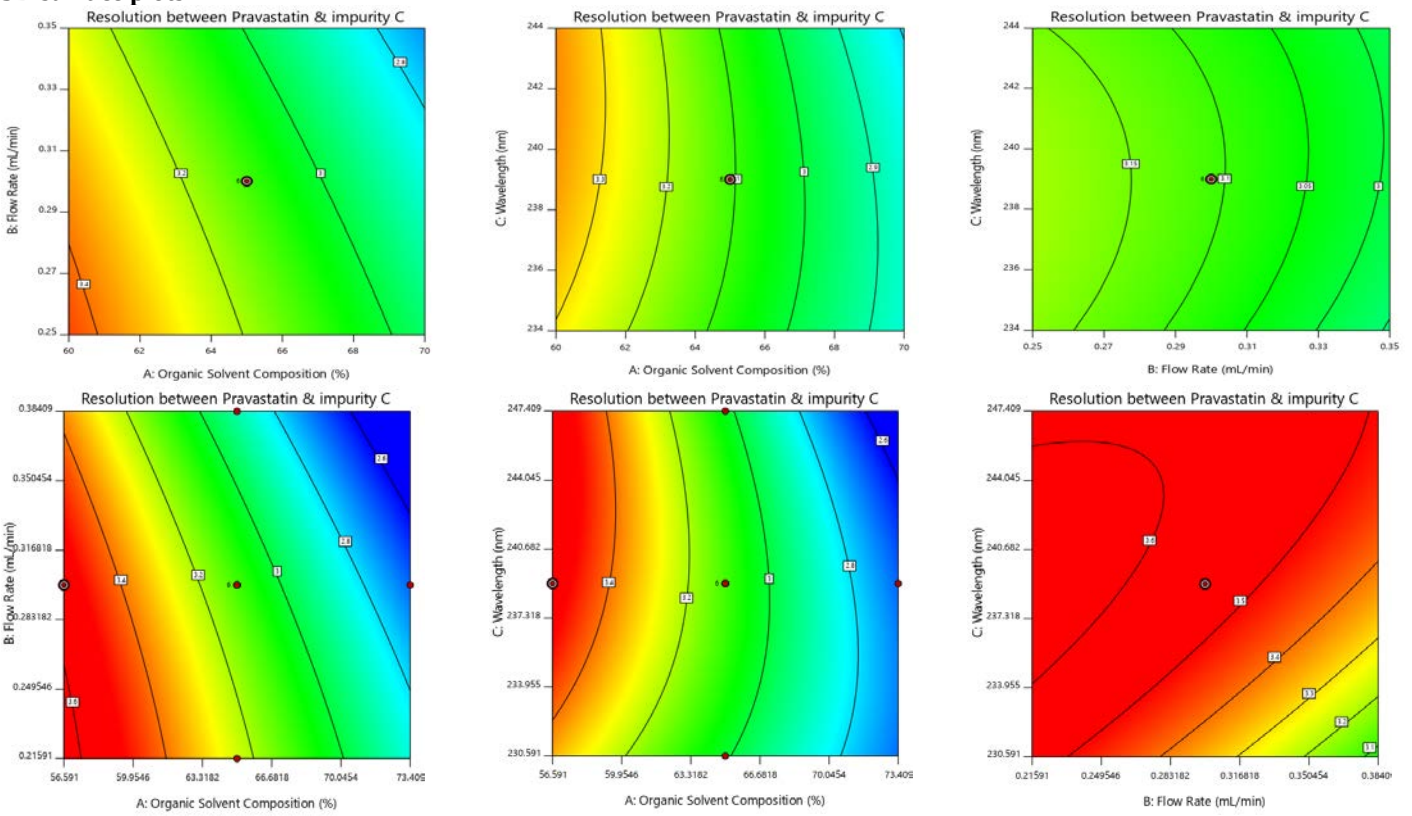


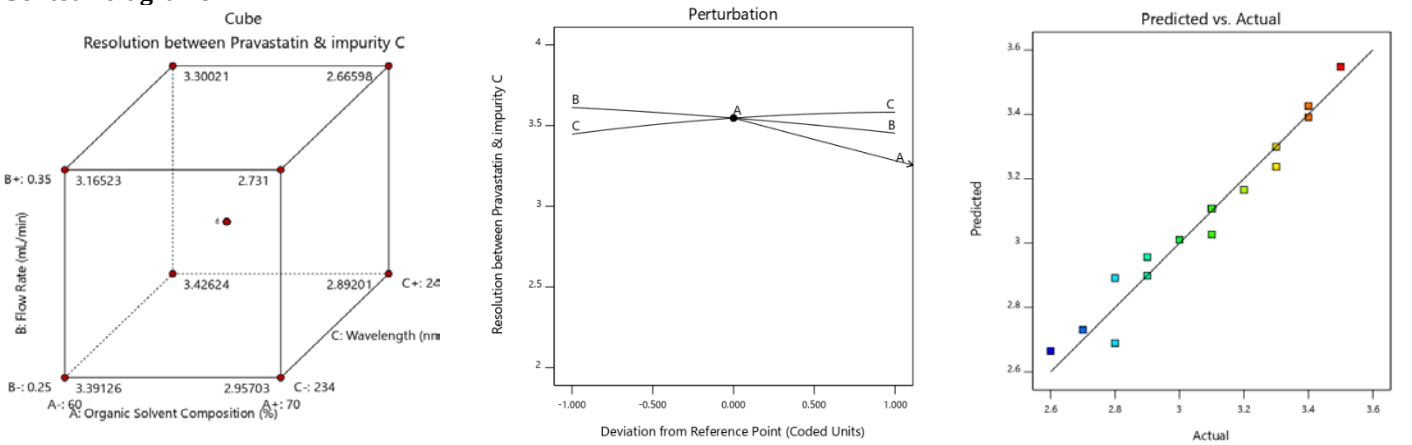
Figure 2: Representative chromatograms obtained during the method optimization studies for UPLC separation of pravastatin sodium & its impurities that illustrate the effect of varying chromatographic parameters on resolution & peak symmetry



3D surface plots

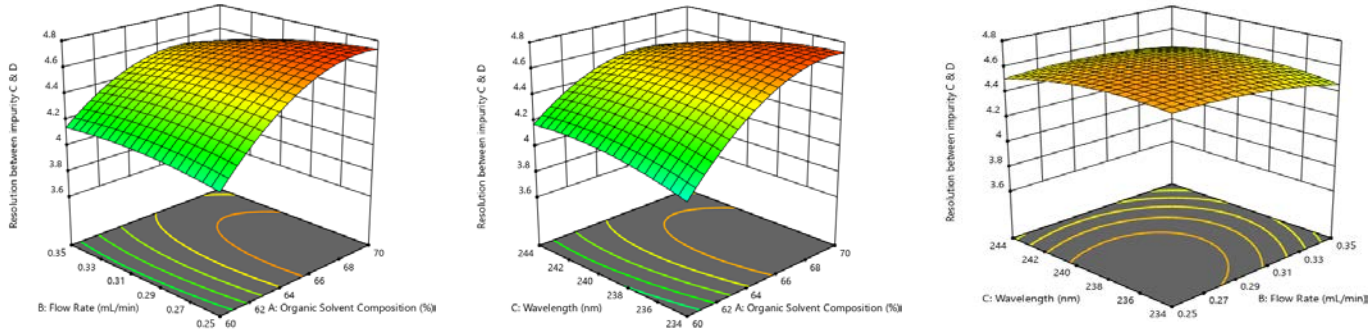


Contour diagrams

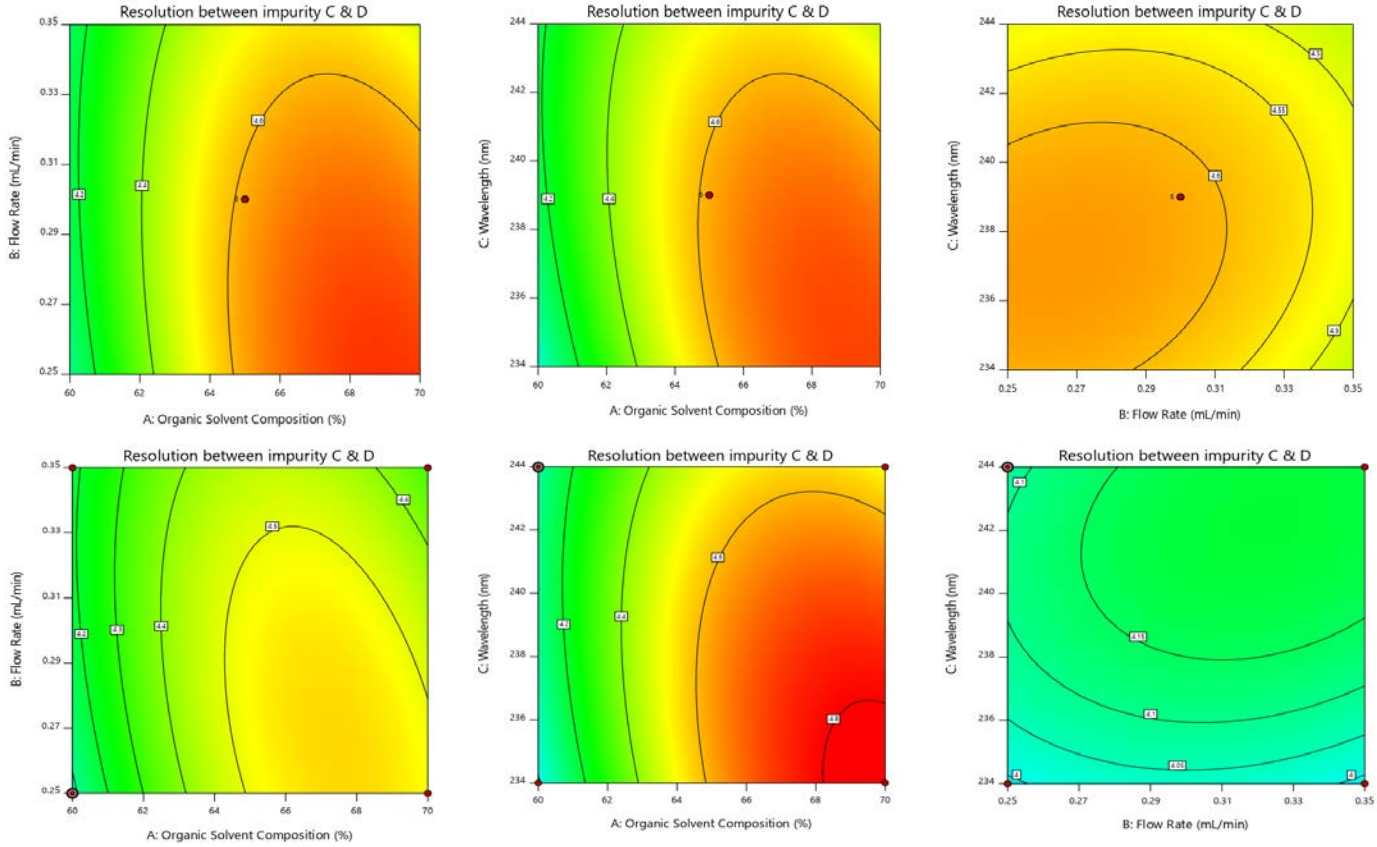


Cube plot **Perturbation plot** **Predicted vs actual**

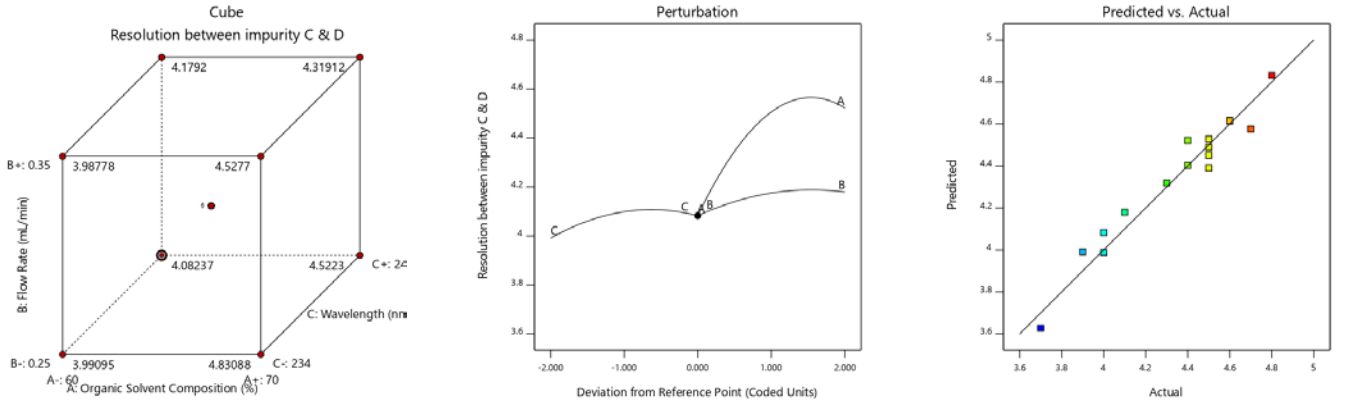
Figure 3: QbD-DoE model graphs observed during the assessment of the impact of organic solvent composition and flow rate on the resolution between pravastatin and impurity C



3D surface plots



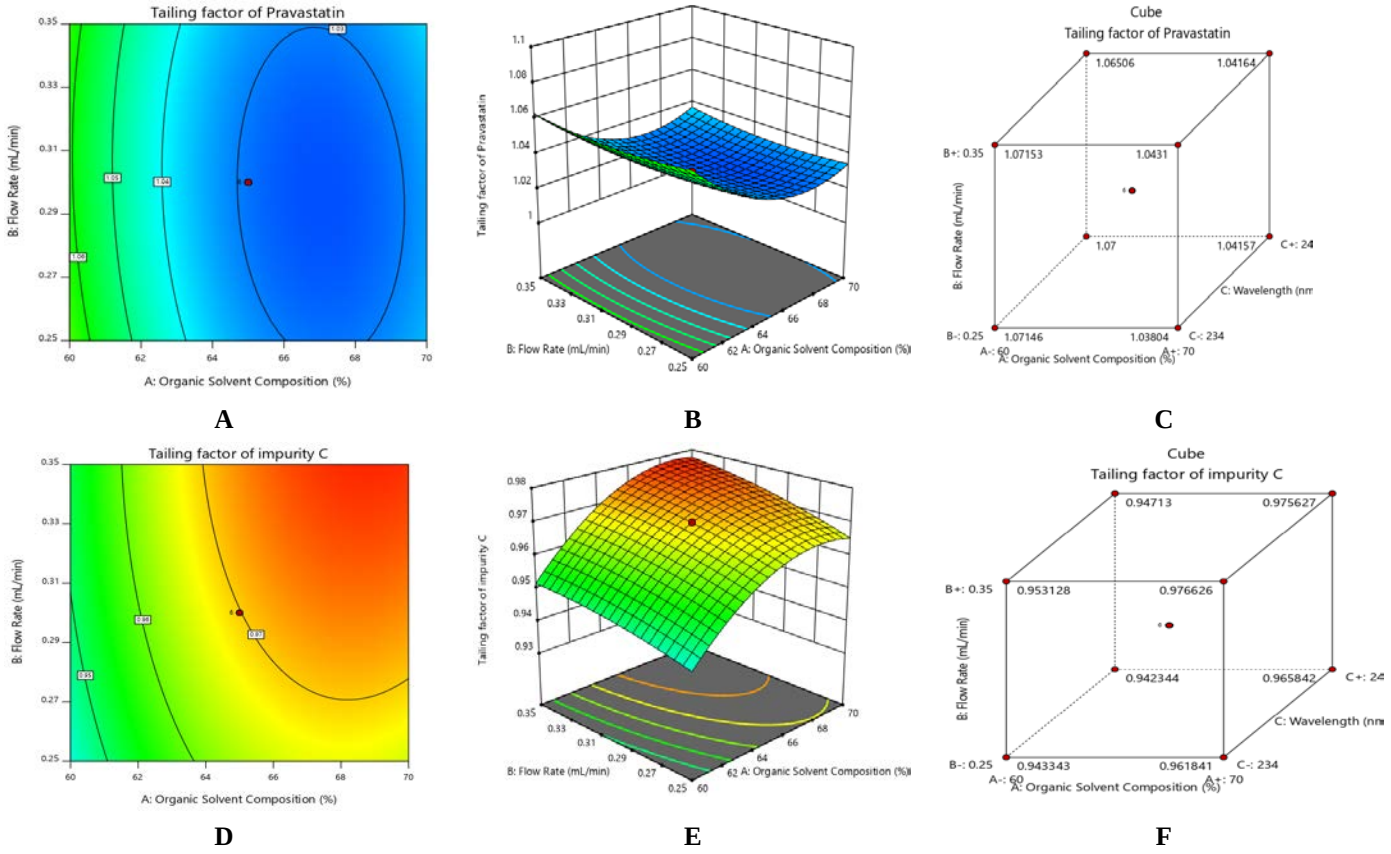
Contour diagrams



Cube plot **Perturbation plot** **Predicted vs actual**

Figure 4: QbD-DoE model graphs observed during the assessment of the impact of organic solvent composition and flow rate on the resolution between impurity C and D.

The model demonstrates stronger statistical performance for response 4 with an R^2 value of 0.9471. The adjusted and predicted R^2 values were 0.8943 and 0.4443, respectively, indicating a good overall fit and moderate predictive reliability. The F-value (17.92) and p-value (<0.0001) confirm that the model is highly significant, and that the selected parameters substantially affect the peak shape of impurity C. The observed tailing factors ranged from 0.94 to 0.98, suggesting excellent column performance and minimal peak distortion. The 3D surface, contour, and cube plots (Figure 5) reveal that both organic solvent composition and flow rate exert a mild but noticeable effect on symmetry.



The contour (A), 3D surface (B), and cube (C) plots observed for the tailing factor of pravastatin; The contour (D), 3D surface (E), and cube (F) plots observed for the tailing factor of impurity C

Figure 5: QbD-DoE model graphs observed during the assessment of the impact of organic solvent composition and flow rate on the tail factor of pravastatin and impurity C

The summary of the QbD analysis reveals that organic solvent composition was the most influential factor, and a 65% organic solvent composition was optimal for analyte resolution. The flow rate significantly affects peak symmetry and separation efficiency, and a flow rate of 0.3 mL/min yielded superior results. The desirability function analysis yields the final optimized chromatographic conditions: ethanol and 0.1% formic acid (65:35, v/v) at 0.3 mL/min, with detection at 239 nm using a Waters ACQUITY BEH C18 column (100 × 2.1 mm, 1.7 µm). Under these optimized green conditions, pravastatin and its impurities were well resolved with sharp, symmetrical peaks with a short analysis time. These finalized optimized chromatographic conditions produce sharp, symmetrical, and well-resolved peaks for pravastatin and its EP impurities in the study. The retention times were 0.31 min for impurity B, 0.89 min for pravastatin, 1.16 min for impurity C, and 1.43 min for impurity D (Figure 6). The analysis was completed within 2.5 minutes, demonstrating the rapidity and high-throughput nature of the proposed method. The system suitability parameters were within acceptable limits, with a resolution (Rs) of 3.1 between pravastatin and impurity C, and 4.6 between impurities C and D, ensuring adequate separation of all analytes. The tailing factors were found to be within the acceptable level, indicating excellent peak symmetry and column efficiency. The theoretical plates exceed 4000 for all analytes, confirming high column performance and minimal band broadening. The analysis of

blank, placebo, individual impurity standards, and spiked sample solutions proves the method specificity. No interference was observed at the retention times of pravastatin or its impurities, confirming that the peaks were well resolved and free from excipient interference. The combination of short run time, baseline resolution, and reproducible retention under environmentally benign conditions highlights the efficiency, robustness, and greenness of the proposed analytical method.

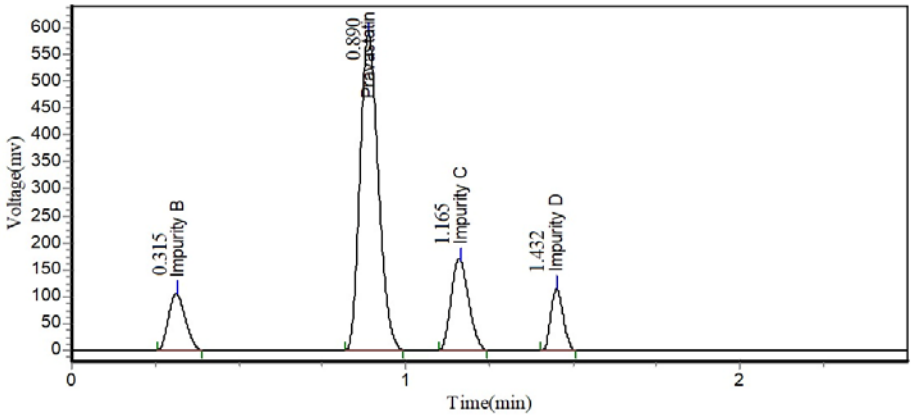


Figure 6: System suitability chromatogram illustrates the baseline separation of pravastatin and its EP impurities under optimized UPLC conditions

Method validation

The developed method for the analysis of pravastatin and its impurities was validated in accordance with the ICH Q2(R2) guidelines. These study results confirm the reliability, precision, accuracy, and sensitivity of the proposed method for the routine analysis of pravastatin and its impurities in bulk drug and pharmaceutical formulations. The method displays excellent linearity for pravastatin and its impurities within 5–30 µg/mL for pravastatin and 0.05-0.30 µg/mL for its impurities. The r^2 value was 0.9998 for pravastatin and more than 0.999 for its impurities. The linearity range for correlation and regression data demonstrates a strong linear relationship between concentration and detector response. The intra-day (repeatability), inter-day (intermediate precision), and ruggedness studies were conducted to assess the precise nature of the proposed method. The intra-day precision (%RSD) values were observed to be within 0.26-0.85%, while the inter-day precision values were in the range of 0.31–0.83%, and 0.39–0.89% in the ruggedness test. The results were within the 2% acceptance limit, confirming that the method is highly reproducible and robust.

The recovery studies at 50%, 100%, and 150% spiking levels were conducted to evaluate the accuracy of the proposed method. The mean recovery values were noticed to be within 98.17%–101.09% for pravastatin and its impurities. These results, which meet the acceptance criteria of 98–102%, confirm the accuracy and reliability of the proposed method for quantitative estimation of impurities. The method also demonstrates high sensitivity, with LOD & LOQ values of 0.015 µg/mL & 0.05 µg/ mL, respectively, for impurities. These low LOD & LOQ values indicate that the method can detect & quantify trace levels of impurities with high precision & reliability. The summary of validation data is presented in Table 4. The robustness of the proposed method was evaluated by introducing small, deliberate variations in key chromatographic parameters, including mobile phase composition (±3%), flow rate (±0.02 mL/min), detection wavelength (±3 nm), and column temperature (±3 °C). As shown in Table 4, none of these minor adjustments produces a significant impact on peak area or resolution values, and the results remain within the acceptable limits (Rs > 2.0 for all critical pairs). These results confirm that the optimized chromatographic conditions were robust and reproducible enough. These conditions produce consistent performance and reliability for routine quantification of pravastatin and its EP impurities.

Greenness assessment

The greenness and environmental sustainability of the proposed method were quantitatively assessed through the AGREE metric and the GAPI tool. The AGREE evaluation (Figure 7) yielded a composite green score of 0.81, indicating that the method was highly compliant with the 12 principles of GAC. The radar diagram, which is predominantly green, indicates that the method meets most environmental sustainability criteria. The use of a green solvent (ethanol), minimal sample preparation, low solvent consumption (0.75 mL per run), and short analytical runtime (2.5 min) were the main factors contributing to the

method's high greenness score. The high AGREE score (>0.75) confirms that the method falls within the "highly green" classification, reflecting both analytical efficiency & ecological responsibility. The few yellow & orange sectors correspond to minor limitations associated with the flammability of ethanol & the mild corrosivity of formic acid, which are manageable under standard laboratory safety protocols. The GAPI pictogram (Figure 7) further supports the method's environmental compatibility.

Most of the pentagonal zones display green shades, and very few are yellow; no red shades were observed. This result indicates that the analytical procedure imposes minimal environmental burden. The low E-factor value (7.0×10^{-2}) was observed, suggesting that the method produces minimal lab waste and environmental pollution. The results clearly demonstrate that the developed UPLC method was eco-efficient, operator-safe, and suitable for sustainable analytical practices in alignment with GAC principles.

Table 4: Method validation results of pravastatin & its EP impurities under optimized conditions as per ICH Q2(R2) criteria

S No	Test	Parameter	Results observed			
			Pravastatin	**Impurity B**	**Impurity C**	**Impurity D**
1	**Linearity**	Range (µg/mL)	5-30	0.05-0.30	0.05-0.30	0.05-0.30
2		Slope	83744	408120	495728	365758
3		Intercept	15775	2837.1	3576.9	2507.2
4		Correlation coefficient	0.9998	0.9995	0.9995	0.9992
5	**Repeatability (% RSD)$**	Intra-day precision	0.42	0.59	0.26	0.85
6	**Intermediate Precision (% RSD)$**	Inter-day precision	0.63	0.83	0.31	0.79
7		Ruggedness	0.57	0.89	0.39	0.47
8	**Recovery/ Accuracy (% recovery)$$**	50 % spiked level	98.52±0.028	99.08±0.015	98.40±0.036	99.13±0.045
9		100 % spiked level	100.26±0.09	99.37±0.013	100.88±0.01	98.17±0.017
10		150 % spiked level	98.79±0.047	100.36±0.02	101.09±0.07	100.85±0.03
11	**Sensitivity (µg/mL)**	LOD	-	0.015	0.015	0.015
12		LOQ	-	0.05	0.05	0.05

% RSD values for n = 3 ($); average±SD for n=6

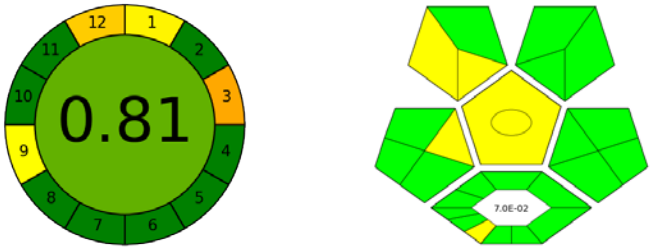
Table 5: Effect of minor deliberate changes in chromatographic parameters under optimized UPLC conditions and results demonstrate the method robustness

S No	Parameter	Change	% change in peak area of pravastatin	Resolution between pravastatin & imp. C	Resolution between pravastatin & imp. D
1	Mobile phase	+3% change in organic solvent	0.84	2.9	4.4
2		-3% change in organic solvent	0.73	3.3	4.8
3	Wavelength	+3 nm change	0.58	3.1	4.6
4		+-3 nm change	0.41	3.0	4.5
5	Flow rate	+0.02 mL/min	0.66	2.8	4.3
6		-0.02 mL/min	0.72	3.4	4.7
7	Column temperature	+3 °C	0.55	3.0	4.6
8		-3 °C	0.48	3.2	4.5

Assessment of method whiteness

The cumulative sustainability and efficiency of the proposed method were comprehensively evaluated using the RGB 12 model. This model integrates the analytical performance (redness), environmental impact (greenness), and practical efficiency (blueness). This model quantifies the balance between method reliability, eco-friendliness, and operational productivity and generates the final "whiteness" score. This score reflects the overall methodological excellence of the proposed method. As shown in Figure 8, the developed method produces a whiteness brilliance (MB) score of 80.6%, indicating high overall sustainability and analytical quality. The individual color scores were achieved as 76.5% for redness, 80.0% for greenness, and 85.4% for blueness. These individual color scores exceeded the minimum threshold, confirming the acceptable white analytical procedure. The high redness score indicates excellent analytical performance, with a low %RSD (<1%) and strong linearity (r^2 = 0.9998). The greenness score indicates that the proposed method uses minimal solvent (0.75 mL per run) and poses low occupational hazards due to the use of ethanol and dilute formic acid. The blueness score further highlights the superior practical efficiency of the proposed method, characterized by a low cost

per analysis, a short run time (2.5 min), and minimal sample consumption (0.2 µL). The balanced contribution of all three-color components classifies the developed UPLC method as a "white analytical method". This high whiteness index demonstrates that the method not only adheres to the principles of GAC but also ensures practical sustainability suitable for routine pharmaceutical quality control.



A **B**

Figure 7: greenness assessment of the developed method for pravastatin and its EP impurities using the AGREE (A) and GAPI (B) tools.

		w=3			w=3			w=3			
REDNESS (analytical performance)	W=3	Precision (RSD)			Accuracy (RE)			Linearity			
CS: 76.5%	LAV=33.3	2.0%			2.0%			0.999			
	LSV=66.6	0.5%			0.5%			1.0000			
	Result	0.42%			0.39%			0.9998			
	Score (0-100)	79	79	79	85	85	85	67	67	67	
		w=3			w=3			w=3			
GREENNESS (safety and eco-friendliness)	W=3	Reagent consumption			Waste amount			Other occupational hazards			
CS: 80.0%	LAV=33.3	800 mL			1500 mL			4 hazards			
	LSV=66.6	200 mL			400 mL			2 hazards			
	Result	160 mL			290 mL			0 hazards			
	Score (0-100)	83	83	83	92	92	92	67	67	67	
		w=3			w=3			w=3			
BLUENESS (productivity / practical effectiveness)	W=3	Cost of analysis			Time of analysis			Sample consumption			
CS: 85.4%	LAV=33.3	250			400 min / 20 runs			acceptable			
	LSV=66.6	125			70 min / 20 runs			satisfactory			
	Result	83			50 min / 20 runs			satisfactory			
	Score (0-100)	100	100	100	93	93	93	67	67	67	
FINAL COLOR:		REDNESS		GREENNESS		BLUENESS		BRILLIANCE (MB):		80.6%	
WHITE		≥33.3%	≥66.6%	≥33.3%	≥66.6%	≥33.3%	≥66.6%				
		yes	yes	yes	yes	yes	yes				
Short annotation: 80.6white		Long annotation: 80.6white(76.5/3red-80.0/3green-85.4/3blue)									

Figure 8: RGB 12-based whiteness assessment of the developed QbD-assisted green UPLC method for pravastatin and its EP impurities

CONCLUSION

A QbD-assisted, green-and-white UPLC method was successfully developed, optimized, and validated for the simultaneous quantification of pravastatin sodium and its EP impurities. The DoE-based optimization establishes that organic solvent composition & flow rate are the most influential parameters affecting chromatographic resolution and peak symmetry. This leads to the selection of ethanol and 0.1% formic acid (65:35, v/v) as a sustainable mobile phase at a flow rate of 0.3 mL/min and a detector wavelength of 239 nm. Under these optimized conditions, complete baseline separation of all analytes was observed, with excellent system suitability, sharp peak symmetry, and minimal solvent consumption within a 2.5 min runtime. Method validation as per ICH Q2(R2) guidelines confirms the method produces outstanding linearity ($r^2 > 0.999$), precision (%RSD < 1%), accuracy (recoveries 98.17–101.09%), and robustness. These outstanding results establish the method's reliability for routine impurity profiling and quality control of pravastatin formulations. The integration of greenness and whiteness evaluations confirms the environmental and sustainability performance of the proposed method. The AGREE metric score of 0.81 and GAPI E-factor of 7.0×10^{-2}

demonstrate strong compliance with GAC principles, and the RGB 12 whiteness brilliance score of 80.6% further highlights the optimal balance among analytical performance, ecological safety, and operational efficiency. Despite its strong green and white analytical credentials, ethanol's high flammability requires appropriate safety measures for large-scale routine use. The future work may focus on extending this QbD–green–white analytical framework to other statins or related lipid-lowering agents to establish a universal eco-analytical platform for impurity profiling. In summary, this study introduces a scientifically rigorous, environmentally responsible, and practically sustainable UPLC method that represents a step toward integrating QbD principles with green and white analytical chemistry for comprehensive impurity profiling of pravastatin in pharmaceutical quality control.

FINANCIAL ASSISTANCE
NIL

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTION
N. Usha Rani and P. T. S. R. K. Prasad Rao collected data and performed experiments. N. Usha Rani and K. Ramanjaneyulu conducted the analysis. N. Usha Rani wrote the first draft of the manuscript, and all authors reviewed and revised previous versions. All authors contributed to the study's conception and design and gave final approval.

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