Chromatographia
https://doi.org/10.1007/s10337-025-04439-y

ORIGINAL

Quality by Design-Guided LC–MS Method for Simultaneous Bioanalysis of Montelukast, Theophylline, and Etofylline in Synthetic Plasma

Gnanasekaran D[1] · Gandhimathi R[2] · Mohana Vamsi Nuli[3] · Pavan Kumar Jaini[4] · Ramanjaneyulu Seemaladinne[5] · Lalitha Repudi[6] · Kumaraswamy Gandla[6]

Received: 12 April 2025 / Revised: 11 August 2025 / Accepted: 3 September 2025

Abstract

A robust and environmentally sustainable LC–MS method was developed and optimized using a Box–Behnken Design (BBD) to simultaneously quantify montelukast, etofylline, and theophylline in synthetic plasma and marketed formulations. Design-Expert software (version 13.0) facilitated a systematic exploration of three critical chromatographic variables—methanol concentration, flow rate, and mobile phase pH—across three coded levels, with their effects assessed on retention time (RT) and tailing factor (TF). Response surface methodology and statistical analyses (ANOVA) confirmed model significance and predictive reliability, with second-order polynomial equations describing variable-response relationships. The optimized conditions (78.13% methanol, 1.0 mL/min flow rate, pH 4.4) yielded reproducible retention and peak symmetry, with < 5% deviation between predicted and experimental values. Mass spectrometry analysis employed a triple quadrupole instrument in positive ESI mode with SRM transitions: m/z 593.27 (montelukast), m/z 224.22 (etofylline), and m/z 180.164 (theophylline). The validated method met all regulatory criteria per EC guidelines, with excellent linearity ($R^2 > 0.999$), precision (%CV < 15%), and recovery (99–100.46%). Stability studies demonstrated analyte robustness under various storage conditions. Notably, method greenness was evaluated using Analytical GREEnness (AGREE), ComplexGAPI, Eco-Scale tools and Blue Applicability Grade Index (BAGI), confirming a low environmental footprint due to minimal solvent consumption, non-toxic reagents, and efficient runtime. In addition, the method was successfully applied to quantify analytes in commercial tablet formulation, confirming accuracy, selectivity, and absence of interference. This study not only establishes a highly sensitive, precise, and green LC–MS platform for multi-analyte quantification in biological matrices but also extends its applicability to quality control in marketed products.

Keywords Montelukast · Etofylline · Theophylline · Design of Expert · Synthetic plasma · Green analytical chemistry

✉ Kumaraswamy Gandla
drkumaraswamygandla@gmail.com

[1] Faculty of Pharmacy, Shree Balaji College of Pharmacy, SBMCH Campus, BIHER, Chromepet, Chennai 600044, Tamil Nadu, India

[2] Department of Pharmaceutical Chemistry & Analysis, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai 600117, Tamilnadu, India

[3] Raghavendra Institute of Pharmaceutical Education and Research (RIPER)-Autonomous, Ananthapuramu, Andhra Pradesh 515721, India

[4] Research Scholar, Raffles University, Neemrana, Rajasthan 301705, India

[5] Department of Chemistry, School of Applied Sciences & Humanities, Vignan's Foundation for Science, Technology and, Research (VFSTR), Vadlamudi, Guntur 522213, Andhra Pradesh, India

[6] Department of Pharmacy, Chaitanya (Deemed to Be University), Gandipet, Himayat Nagar (Vill), Moinabad (Mandal), Hyderabad 500075, Telangana, India

Published online: 11 September 2025

Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are prevalent respiratory disorders characterized by airflow limitation and inflammation, yet they differ significantly in their pathophysiology, triggers, and management [1, 2]. Asthma is a reversible, immune-mediated condition often triggered by allergens, exercise, or respiratory infections, manifesting as wheezing, dyspnea, and bronchial hyperresponsiveness [3]. In contrast, COPD, primarily caused by chronic exposure to tobacco smoke or environmental pollutants, involves irreversible airway obstruction, persistent inflammation, and progressive decline in lung function [4]. While asthma is highly responsive to bronchodilators and corticosteroids, COPD management primarily focuses on symptom control, exacerbation prevention, and slowing disease progression [5]. Despite overlapping clinical features, these conditions require distinct therapeutic strategies tailored to their underlying mechanisms [6].

Montelukast (Fig. 1a), a leukotriene receptor antagonist, is widely used for the management of asthma and allergic rhinitis by mitigating inflammation and bronchoconstriction. It improves airway function and prevents exercise-induced bronchospasm [7, 8]. Unlike corticosteroids, montelukast is administered orally and is generally well-tolerated; however, neuropsychiatric effects, including mood alterations, have been reported in some patients [9]. Theophylline (Fig. 1c) and its derivative etofylline (Fig. 1b), both methylxanthines, function as bronchodilators in respiratory diseases, such as asthma and COPD [10]. Theophylline exerts its effects by inhibiting phosphodiesterase, leading to increased intracellular cyclic AMP and subsequent relaxation of bronchial smooth muscle. Etofylline, a more water-soluble derivative, enhances bioavailability and reduces adverse effects. Despite their efficacy, both drugs require therapeutic drug monitoring due to their narrow therapeutic index and the potential for serious adverse effects, including nausea, arrhythmias, and central nervous system stimulation [11].

Given that montelukast, theophylline, and etofylline fall within the same pharmacological category, the development of an analytical method for their simultaneous quantification is imperative. Monitoring plasma concentrations of these agents is essential for optimizing therapeutic efficacy, assessing pharmacokinetic profiles, and ensuring patient safety through therapeutic drug monitoring. Currently, no validated method exists for the simultaneous estimation of these compounds. While individual analytical methods, including HPLC and UPLC, have been reported, they lack the sensitivity and selectivity required for comprehensive pharmacokinetic and bioequivalence studies. Liquid chromatography–mass spectrometry (LC–MS) offers superior analytical capabilities, integrating high chromatographic resolution with mass detection, which is particularly advantageous for the analysis of complex biological matrices such as plasma. The incorporation of photodiode array (PDA) detection further enhances analyte identification and purity assessment.

To address this analytical gap, we have developed a novel, highly selective, and analytically robust LC–MS method for the simultaneous quantification of montelukast, etofylline, and theophylline in human plasma. The method employs an optimized solid-phase extraction (SPE) protocol for sample preparation, coupled with electrospray ionization tandem mass spectrometry (ESI–MS/MS) to ensure exceptional sensitivity and selectivity. This validated approach provides a reliable tool for assessing bioavailability, bioequivalence, and drug–drug interactions, offering significant utility in both preclinical and clinical pharmacokinetic investigations.

Beyond its analytical advancements, this study integrates principles of green analytical chemistry (GAC) to enhance the environmental sustainability of pharmaceutical analysis

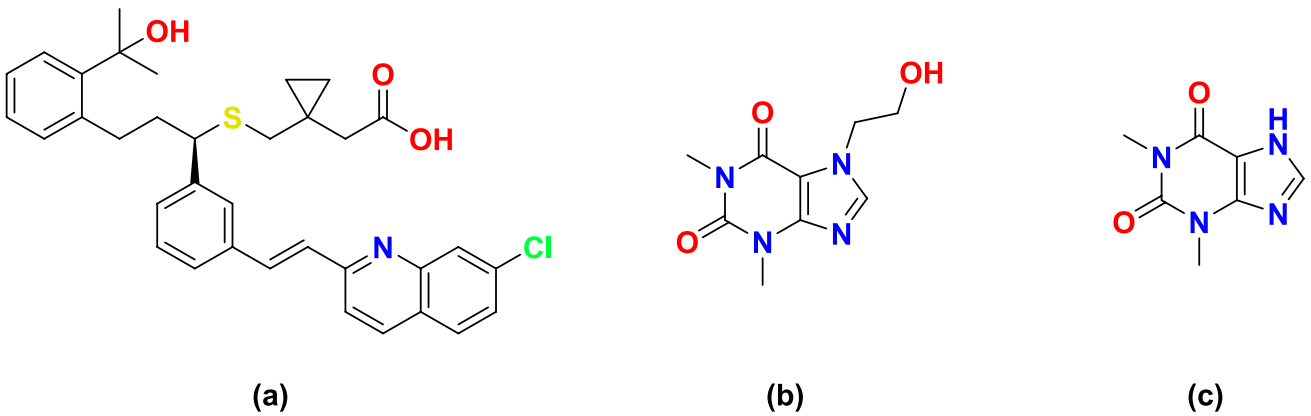

Fig. 1 Chemical structures of **a** Montelukast, **b** Etofylline, and **c** Theophylline

[12, 13]. The ecological impact of the developed LC–MS method was systematically evaluated using multiple complementary metrics, including the Complementary Green Analytical Procedure Index (ComplexGAPI), the Analytical GREEnness (AGREE) metric, and the Eco-Scale Analytical (ESA) assessment. Furthermore, the method's broader applicability and operational efficiency were assessed using the Blue Applicability Grade Index (BAGI), a framework within White Analytical Chemistry (WAC) that quantifies the "blueness" of an analytical procedure. By integrating these multi-dimensional assessments, this study ensures not only high analytical performance but also environmental compatibility, aligning with contemporary trends in sustainable analytical methodologies.

Experiment

Chemicals and Reagent

Reference standards of montelukast, etofylline, and theophylline were obtained from Glenmark Pharmaceuticals Ltd., Hyderabad. High-purity solvents, including LC–MS grade methanol and acetonitrile, were procured from Merck Chemical Division, Mumbai. Ultrapure water was consistently sourced from a Milli-Q water purification system and used throughout the study. All other solvents and reagents adhered to pharmaceutical or analytical-grade specifications to maintain methodological consistency and ensure analytical reliability.

Preparation of Phosphate Buffer Solution

A phosphate buffer solution (pH 4.4) was prepared by dissolving 6.80 g of potassium dihydrogen phosphate in 600.0 mL of distilled water with thorough mixing. The volume was adjusted to 1000 mL using distilled water. The pH was carefully adjusted to 4.4 by adding hydrochloric acid dropwise. The prepared buffer was stored at controlled room temperature and utilized within 5 days. Further dilutions were performed as required to achieve the desired concentrations.

Preparation of Mobile Phase (Methanol:Phosphate Buffer, 78.13:21.87 v/v)

The mobile phase was prepared by mixing 218.7 mL of phosphate buffer (pH 4.4) with 781.3 mL of LC–MS grade methanol, followed by degassing in an ultrasonic water bath for 5 min. The resulting solution was then filtered through a 0.45 µm pore size membrane under vacuum and transferred to a 1000 mL volumetric flask.

Preparation of Stock Solutions

Individual stock solutions of montelukast, etofylline, and theophylline were prepared by accurately weighing 10 mg of each compound into separate 100 mL volumetric flasks. Each compound was dissolved in methanol and the volume was adjusted to 100 mL, yielding stock solutions with a final concentration of 100 µg/mL.

LC–MS Conditions

Simultaneous estimation of montelukast, etofylline, and theophylline was performed using an Agilent LC-2010CHT high-performance liquid chromatography (HPLC) system (Tokyo, Japan) coupled with a photodiode array (PDA) detector (I227). The system was interfaced with a Sciex API 4000 mass spectrometer (Ontario, Canada), enabling highly sensitive and selective detection. Chromatographic separation was achieved on a Phenomenex C18 analytical column (100 × 4.6 mm, 2.6 µm particle size). The mobile phase comprised methanol and phosphate buffer (pH 4.4) in a ratio of 78.13:21.87 (v/v), delivered at a constant flow rate of 1.0 mL/min. A fixed injection volume of 10 µL was employed, with a total run time of 5 min. Detection was carried out at 272 nm, corresponding to the isosbestic point of the analytes. Under these optimised chromatographic conditions, montelukast, etofylline, and theophylline exhibited well-resolved and reproducible retention times. The mass spectrometer operated in positive electrospray ionisation (ESI) mode with the following optimised parameters: collision energy of 14 V (using nitrogen as the collision gas), ion spray voltage of 5500 V, source temperature of 550 °C, drying gas temperature ranging from 120 °C to 250 °C, drying gas flow rate of 5 L/min, declustering potential of 5 V, entrance potential of 10 V, exit potential of 7 V, and a dwell time of 1 s per transition. Data acquisition and analysis were conducted using Analyst software (AB SCIEX), ensuring accurate peak integration, quantification, and identification of the target analytes.

Design of Expert-Guided LC–MS Method Development

Method optimization was carried out using Design of Experiment (DoE) software (version 13.0), applying a Box–Behnken Design (BBD), a response surface methodology known for its efficiency in achieving optimal outcomes with a reduced number of experimental runs compared to the Central Composite Design (CCD). Critical method parameters and their operational ranges were identified through preliminary univariate screening and systematic chromatographic evaluations. The selected independent variables included the proportion of the organic phase (methanol,

73.13–83.13%), flow rate (0.9–1.1 mL/min), and buffer pH (4.1–4.7). Each experimental run was assessed using predefined response variables, specifically analyte retention time (RT) and tailing factor (TF). Model performance and statistical relevance were evaluated through analysis of variance (ANOVA), while multiple linear regression analysis (MLRA) was used to delineate the individual and interactive effects of the selected parameters. This systematic approach to method optimization enabled the identification of robust chromatographic conditions, ensuring effective separation, enhanced peak symmetry, and reproducible retention times across multiple replicates [14–16].

Extraction of Drugs from Blood Plasma Followed by Processing

Synthetic blood plasma was procured from a certified vendor and stored in a freezer as per the manufacturer's instructions. For sample preparation, 500 µL of each standard solution was added to 200 µL of plasma and mixed thoroughly. Protein precipitation was induced by the addition of 300 µL of acetonitrile, promoting effective removal of plasma proteins. The mixture was vortexed using a cyclomixer to ensure homogeneity, followed by centrifugation at 4000 rpm for 15–20 min. The resulting supernatant was carefully collected to avoid disturbing the protein pellet and subsequently reconstituted in 1 mL of the mobile phase. To ensure sample clarity and protect the chromatographic column from particulate matter, the reconstituted samples were filtered through a 0.22 µm syringe filter prior to injection [17, 18].

Analytical Method Validation

System Suitability

To ensure consistent analytical performance, a comprehensive system suitability evaluation was conducted on the LC–MS system prior to sample analysis. Critical chromatographic parameters—including retention time, peak area reproducibility, resolution, and peak symmetry—were assessed using a standard solution prepared at medium quality control (MQC) concentrations: 10 ng/mL for montelukast, 231 ng/mL for etofylline, and 69 ng/mL for theophylline. Each analyte was injected in six replicates to evaluate system stability and reproducibility. The coefficient of variation (CV) for retention time, peak area, theoretical plates, and tailing factor was calculated to determine system precision, with acceptance criteria set at $\leq 2\%$. The system was deemed suitable for analysis when all measured parameters fell within the predefined limits, confirming adequate performance for subsequent quantitative assays [19].

Linearity and Range

The linearity of the LC–MS method was evaluated to establish a direct and proportional relationship between analyte concentration and detector response over a defined range. Calibration standards were prepared at multiple concentration levels: 2.5–15 ng/mL for montelukast, 57.75–346.5 ng/mL for etofylline, and 17.25–103.5 ng/mL for theophylline. Each concentration was injected, and the resulting peak areas were plotted against nominal concentrations to construct calibration curves. Linearity was assessed by calculating the correlation coefficient (R^2), with values ≥ 0.99 indicating excellent linear response across the tested range, suitable for reliable quantification in biological matrices [20].

Sensitivity

The sensitivity of the developed LC–MS method was assessed by spiking blank plasma samples with the analytes at low concentrations, followed by sample preparation and analysis using the validated protocol. Calibration curves were generated to evaluate the method's ability to accurately detect and quantify analytes at the lower end of the concentration range. The lower limit of quantification (LLOQ) was defined as the lowest concentration that could be measured with acceptable precision (coefficient of variation $\leq 20\%$) and accuracy (within $\pm 20\%$ of the nominal value). The established LLOQ confirmed the method's suitability for quantifying trace levels of analytes in complex biological matrices, ensuring robust performance in pharmacokinetic and bioavailability studies.

Matrix Effect

To assess potential ion suppression or enhancement due to endogenous matrix components, a matrix effect study was conducted using chromatographically screened synthetic plasma. Analytes were spiked into both blank matrix samples and pure standard solutions, followed by identical sample preparation and LC–MS analysis. The analyte response in matrix-spiked samples was compared to that of pure solutions to quantify matrix-induced signal variations. The %CV was calculated for both high-quality control (HQC) and low-quality control (LQC) samples to evaluate the reproducibility of the matrix effect across the concentration range. Consistently low %CV values indicated minimal and stable matrix interference, confirming the method's robustness and reliability for accurate analyte quantification in complex biological matrices [21].

Accuracy and Precision

The precision and accuracy of the developed LC–MS method were systematically evaluated to ensure its reliability for quantitative bioanalysis. Precision was assessed by determining the reproducibility of replicate measurements under identical conditions, expressed as the %CV across four concentration levels: HQC, MQC, LQC, and LLQC. Accuracy was evaluated by comparing the mean measured concentrations to nominal values and expressed as the percentage deviation from the target concentration. Regulatory acceptance criteria require precision (%CV) to be within ± 15% for HQC, MQC, and LQC, and within ± 20% for LLQC. Similarly, accuracy must fall within ± 15% of the nominal value for HQC–LQC levels and ± 20% for LLQC. The method demonstrated acceptable precision and accuracy for all three analytes—etofylline, montelukast, and theophylline—across the specified concentration ranges: 346.5 to 5.8 ng/mL for etofylline, 15 to 0.25 ng/mL for montelukast, and 103.5 to 1.7 ng/mL for theophylline.

Robustness

The robustness of the developed LC–MS method was assessed by introducing intentional, small variations in critical chromatographic parameters to determine its reliability under minor operational deviations. The parameters evaluated included mobile phase composition (76.13:23.87 and 80.13:19.87% v/v), flow rate (0.9 and 1.1 mL min^{-1}), and pH of the aqueous phase (4.2 and 4.6). Standard solutions of all three analytes were analyzed under each modified condition. RT and other key chromatographic responses were recorded, and the impact of each variation was quantified in terms of the %RSD.

Carryover

Carryover, defined as the unintended persistence of analyte residues within the chromatographic system following sample injection, poses a risk to the integrity of subsequent analyses by introducing spurious signals. To evaluate carryover in the developed LC–MS method, a sequence comprising duplicate injections at the LLOQ was immediately followed by blank plasma samples and ULOQ standards. This approach enabled the detection and quantification of residual analyte signals potentially affecting subsequent runs. The extent of carryover was quantified by comparing the signal detected in the post-LLOQ blank sample to the mean response at the LLOQ concentration. According to the European Medicines Agency (EMA) guidelines for bioanalytical method validation, carryover was deemed acceptable if the residual response did not exceed 20% of the LLOQ [22].

Stability Assessment

Stability studies were conducted in accordance with the method reported by Gandu et al. (2025) to ensure the integrity of analytes during all phases of sample handling, processing, and storage. A series of stability assessments were performed under various experimental conditions to evaluate the robustness of the developed LC–MS method. Benchtop stability was assessed to determine analyte stability at ambient temperature, while freeze–thaw stability was evaluated across multiple cycles to simulate routine laboratory handling. Autosampler stability was examined to verify the persistence of analyte integrity during prolonged residence in the autosampler environment. In addition, wet and dry extract stability tests were conducted to ensure sample stability post-extraction, and both short-term and long-term storage stability were assessed in the biological matrix under controlled conditions. These comprehensive stability evaluations demonstrated that the analytes remained stable across all tested conditions, affirming the method's reliability and reproducibility. Such robustness is critical for ensuring accurate quantification in long-term pharmacokinetic and bioanalytical studies, especially under variable operational workflows [23].

Analysis of Deriphyllin M—A Commercial Formulation

To evaluate the content of montelukast, etofylline, and theophylline in a marketed pharmaceutical formulation, a representative sample containing 10 mg of each active ingredient was accurately weighed and dissolved in 10 mL of methanol. The solution was sonicated for 15 min to ensure complete dissolution of the analytes. Subsequently, the resulting solution was diluted with the mobile phase to achieve final concentrations of 10 ng/mL for montelukast, 231 ng/mL for etofylline, and 69 ng/mL for theophylline. The prepared sample was subjected to LC–MS analysis using the validated method. The assay enabled precise quantification of the three analytes in the commercial formulation, confirming the method's applicability for routine quality control and post-marketing surveillance [24].

Greenness and Whiteness Assessment of the Proposed Method

A comprehensive evaluation of the environmental sustainability and operational feasibility of the developed LC–MS method was conducted using a suite of complementary green and white analytical metrics. This multipronged assessment

provided a holistic understanding of the method's ecological footprint, efficiency, and practical applicability within routine laboratory workflows. The evaluation adhered to previously established protocols to ensure methodological consistency and comparability. Green metrics included the Complementary Green Analytical Procedure Index (ComplexGAPI), the Analytical Eco-Scale, and Analytical GREEnness (AGREE), each offering unique insights into factors, such as solvent usage, energy consumption, reagent toxicity, and waste generation. In parallel, the Blue Applicability Grade Index (BAGI) was employed to assess the method's white analytical chemistry attributes, including its operational simplicity, transferability, and integration into standard laboratory practices. Together, these tools offered synergistic perspectives, reinforcing the method's classification as both environmentally sustainable and analytically robust—positioning it as a suitable candidate for adoption in eco-conscious, high-throughput analytical environments [25–28].

Results and Discussion

Design of Expert-Guided LC–MS Method Development

A BBD was employed to systematically optimize chromatographic conditions for LC–MS analysis, enabling the structured investigation of three critical process variables—organic phase proportion, flow rate, and pH—at three coded levels: low (–1), medium (0), and high (+ 1). This multivariate approach facilitated a comprehensive evaluation of their individual and interactive effects on two primary response variables: RT and TF, assessed for montelukast, etofylline, and theophylline (Y_1–Y_6). The design space was generated using Design-Expert software (version 13.0), allowing for efficient exploration of the chromatographic landscape. The response surface methodology provided not only optimization but also valuable mechanistic insights into variable-response relationships. RT and TF were recorded for each analyte across all experimental runs, and the summarized data (Table 1) served as the foundation for method refinement and subsequent validation.

ANOVA was employed to evaluate the statistical significance and predictive capability of the developed models. Key parameters such as model F values, p values, lack-of-fit statistics, and regression coefficients are detailed in Tables S1–S6. These results affirmed the robustness and validity of the optimization framework, with statistically significant models supporting the reliability of predictions across the examined design space. Second-order polynomial equations were derived for each response (Y_1–Y_6) to quantitatively describe the relationship between input variables and chromatographic outcomes. Positive coefficients reflected synergistic effects, while negative coefficients indicated antagonistic influences. The magnitude of each coefficient provided insight into the strength of variable impact, highlighting dominant factors driving system behavior. The strong correlation between experimental and predicted values validated the models' accuracy, confirming their utility

Table 1 Execution of the Box–Behnken experimental design and corresponding outcomes for the proposed LC–MS method

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4	Response 5	Response 6
Run	A: Organic phase	B: Flow rate	C: pH	RT (Montelukast)	TF (Montelukast)	RT (Etofylline)	TF (Etofylline)	RT (Theophylline)	TF (Theophylline)
	%	mL/min		Min		Min		Min	
1	78.13	1.1	4.7	2.4	1.2	1.3	1.48	4.1	1.41
2	83.13	1	4.7	1.9	1.28	0.9	1.52	3.5	1.5
3	83.13	0.9	4.4	2.1	1.26	1.1	1.5	3.9	1.46
4	83.13	1.1	4.4	1.8	1.3	0.8	1.57	3.2	1.53
5	83.13	1	4.1	2	1.29	1	1.53	3.6	1.49
6	78.13	1	4.4	2.6	1.17	1.7	1.43	4.5	1.39
7	73.13	0.9	4.4	3.5	0.96	2.6	1.21	5.6	1.19
8	78.13	1	4.4	2.6	1.18	1.7	1.44	4.5	1.39
9	78.13	0.9	4.1	2.8	1.11	1.9	1.39	4.7	1.31
10	73.13	1	4.7	3.3	1	2.4	1.27	5.2	1.21
11	78.13	0.9	4.7	2.8	1.12	1.9	1.38	4.6	1.32
12	78.13	1.1	4.1	2.3	1.19	1.4	1.49	4.2	1.4
13	73.13	1.1	4.4	3.1	1.03	2.1	1.3	4.9	1.26
14	73.13	1	4.1	3.3	0.98	2.3	1.26	5.3	1.22
15	78.13	1	4.4	2.5	1.16	1.6	1.45	4.4	1.38

in guiding method optimization. Collectively, this data-driven approach ensured enhanced method robustness, precision, and reproducibility while establishing a systematic foundation for future analytical development and scale-up:

$$RT(Montelukast) = +2.57 - 0.6750 * A - 0.2000 * B + 0.0000 * C + 0.0250 * AB - 0.0250 * AC + 0.0250 * BC + 0.0542 * A2 + 0.0042 * B2 + 0.0042 * C2$$

$$TF(Montelukast) = +1.17 + 0.1450 * A + 0.0337 * B + 0.0038 * C - 0.0075 * AB - 0.0075 * AC + 0.000 * BC - 0.0250 * A2 - 0.0075 * B2 - 0.0075 * C2$$

$$RT(Etofylline) = +1.67 - 0.7000 * A - 0.2375 * B - 0.0125 * C + 0.0500 * AB - 0.0500AC - 0.0250 * AB + 0.0042 * A2 - 0.0208 * B2 - 0.0208 * C2$$

$$TF(Etofylline) = +1.44 + 0.1350 * A + 0.0450 * B - 0.0025 * C - 0.0050 * AB - 0.0050 * AC + 0.0000 * BC - 0.0425 * A2 - 0.0025 * B2 - 0.0025 * C2$$

$$RT(Theophylline) = +4.47 - 0.8500 * A - 0.3000 * B - 0.0500 * C + 0.0000 * AB + 0.0000 * AC + 0.0000 * BC - 0.0333 * A2 - 0.0333 * B2 - 0.0333 * C2$$

$$TF(Theophylline) = +1.39 + 0.1375 * A + 0.0400 * B + 0.0025 * C + 0.0000 * AB + 0.0050 * AC + 0.0000 * BC - 0.0158 * A2 - 0.0108 * B2 - 0.0158 * C2$$

Response Surface Analysis

To comprehensively assess the influence of independent variables on chromatographic responses, a series of diagnostic and interpretive plots were employed, including residual versus predicted plots, perturbation plots, and three-dimensional (3D) response surface plots. These graphical tools provided critical insights into model adequacy, factor–response relationships, and system behavior within the design space. Residual versus predicted plots serve as a fundamental diagnostic tool in the context of DoE, enabling evaluation of model fit by plotting the residuals (the difference between observed and predicted values) against predicted responses. An ideal model exhibits a random, patternless distribution of residuals, indicating homoscedasticity and linearity, which are essential assumptions for valid statistical inference. Conversely, systematic trends or heteroscedastic patterns may suggest model misspecification or the presence of non-linear effects. In this study, residual versus predicted plots for all six responses confirmed model adequacy and reliability, with random dispersion observed across all data sets (Fig. S1).

Perturbation plots were further employed to visualize the relative sensitivity of each response to changes in individual factors. These plots illustrate the deviation of a given response as one factor is varied across its range while keeping all other factors constant at a reference point. The slope and curvature of each line serve as indicators of the degree of influence exerted by the corresponding factor. Steeper gradients or pronounced curvature, as observed in the perturbation plots for montelukast, etofylline, and theophylline (Fig. 2), denote heightened sensitivity, thereby identifying critical parameters that warrant precise control. This model-based visualization offers a mechanistic understanding of factor–response dynamics, ultimately guiding rational optimization of chromatographic conditions.

The influence of three critical chromatographic parameters—methanol concentration, flow rate, and pH—was systematically investigated at low, medium, and high levels using RSM to evaluate their impact on RT and TF for montelukast, etofylline, and theophylline. The effects on RT (responses Y_1, Y_3, and Y_5) are illustrated in Fig. 3a, b, c, corresponding to montelukast, etofylline, and theophylline, respectively. The results clearly demonstrate that all three independent variables exert a significant influence on chromatographic retention. Notably, increases in methanol proportion, flow rate, and pH resulted in a marked reduction in RT across all analytes. This observation suggests a synergistic interaction among these parameters, collectively contributing to reduced analyte retention and underscoring their critical role in optimizing method throughput and efficiency.

The influence of the same variables on TF (responses Y_2, Y_4, and Y_6) is presented in Fig. 4a, b, c. The data reveal that both methanol concentration and flow rate substantially improved peak symmetry for all three analytes, as evidenced by lower TF values. In contrast, pH demonstrated a negligible effect on peak shape, indicating its limited role in modulating analyte dispersion under the tested conditions. These findings emphasize the dominant role of the organic modifier and flow dynamics in shaping chromatographic resolution and symmetry. Furthermore, the presence of diminishing returns beyond optimal ranges for methanol and flow rate suggests the importance of precise parameter control to maintain peak quality without compromising system stability.

Optimized Method Conditions

Using Stat-Ease, Inc.'s Design-Expert software (version 13.0), each response variable was analyzed to generate statistically robust models, with high predictive accuracy and model validity. The software's optimization module

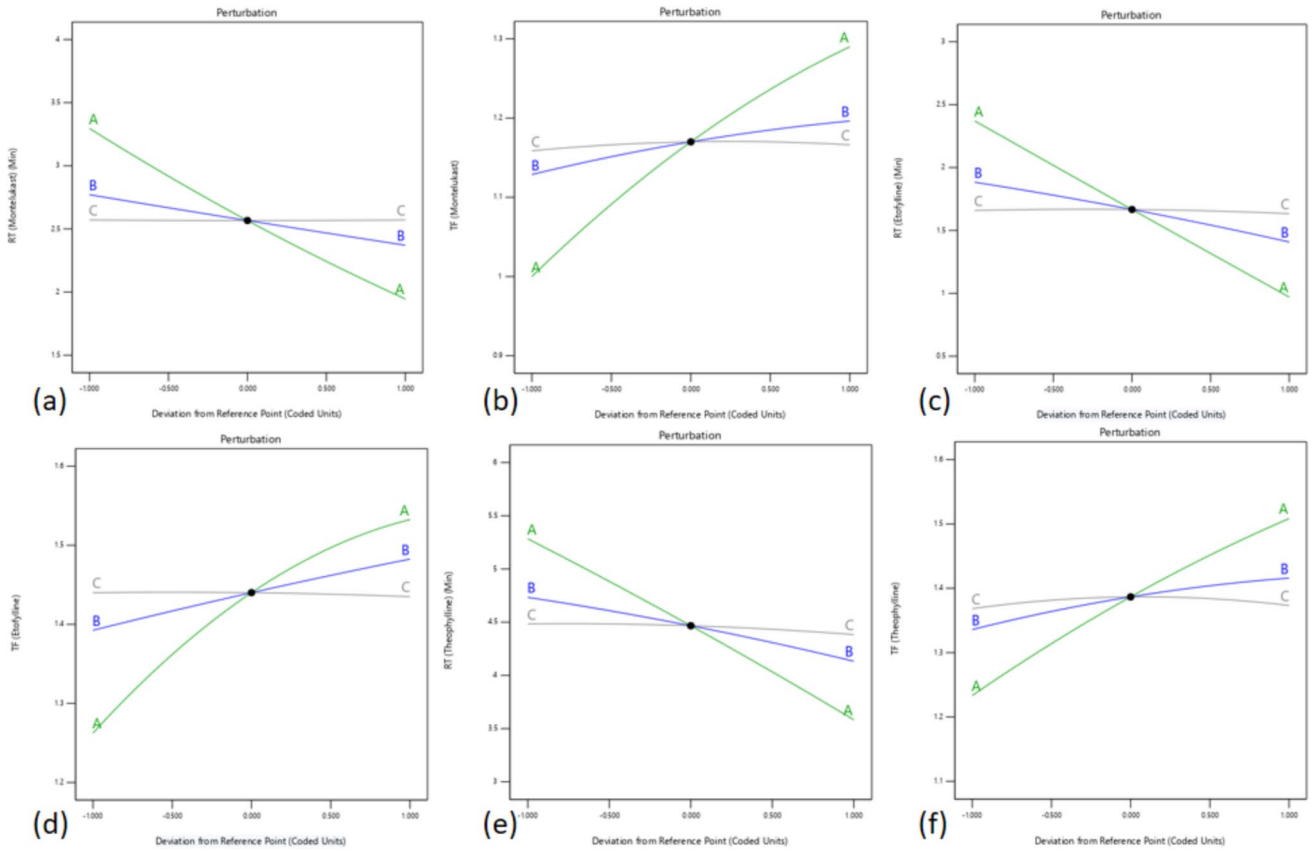

Fig. 2 Perturbation graph showing the effect of each factor, A, B, and C, on the **a** RT (Montelukast), **b** TF (Montelukast), **c** RT (Etofylline), **d** TF (Etofylline), **e** RT (Theophylline), and **f** (Theophylline)

identified experimental conditions with a desirability index of 1.0 or values approaching unity, indicating an optimal balance across all response variables. The corresponding design space, delineated by the shaded yellow region in the overlay **plot (Fig. S2),** defined the multidimensional region, where all response criteria were simultaneously satisfied. The optimal chromatographic parameters were identified as 78.13% (v/v) organic phase, a flow rate of 1.0 mL/min, and a mobile phase pH of 4.4. To experimentally validate these predicted conditions, six replicate injections of each analyte were performed. The observed retention times and tailing factors deviated by less than 5% from the model-predicted values for montelukast, etofylline, and theophylline, confirming the reliability and accuracy of the optimization process. Chromatographic resolution under these optimized conditions was efficient and reproducible, as demonstrated by the representative chromatograms presented in Fig. 5**.** The absence of interfering peaks in the blank plasma chromatogram (Fig. S3) further confirmed the method's selectivity and suitability for bioanalytical applications.

Mass Spectrometry Analysis

LC–MS analysis was carried out on a Sciex API 4000 triple quadrupole mass spectrometer (Ontario, Canada), equipped with an electrospray ionization (ESI) source operating in positive ion mode. Quantification of montelukast, etofylline, and theophylline in chromatographically screened synthetic plasma was achieved using selected reaction monitoring (SRM). The optimized ion transitions were m/z 593.27 → product ion for montelukast, m/z 224.22 → product ion for etofylline, and m/z 180.164 → product ion for theophylline. These transitions were selected based on the highest signal-to-noise ratios obtained during method development, ensuring maximal selectivity and sensitivity under fully validated analytical conditions. The corresponding product ion spectra, presented in Fig. S4, confirm the fragmentation profiles used for quantitation. This highly specific SRM approach provided consistent and reproducible detection across biological replicates, thereby establishing a robust platform for subsequent pharmacokinetic evaluations.

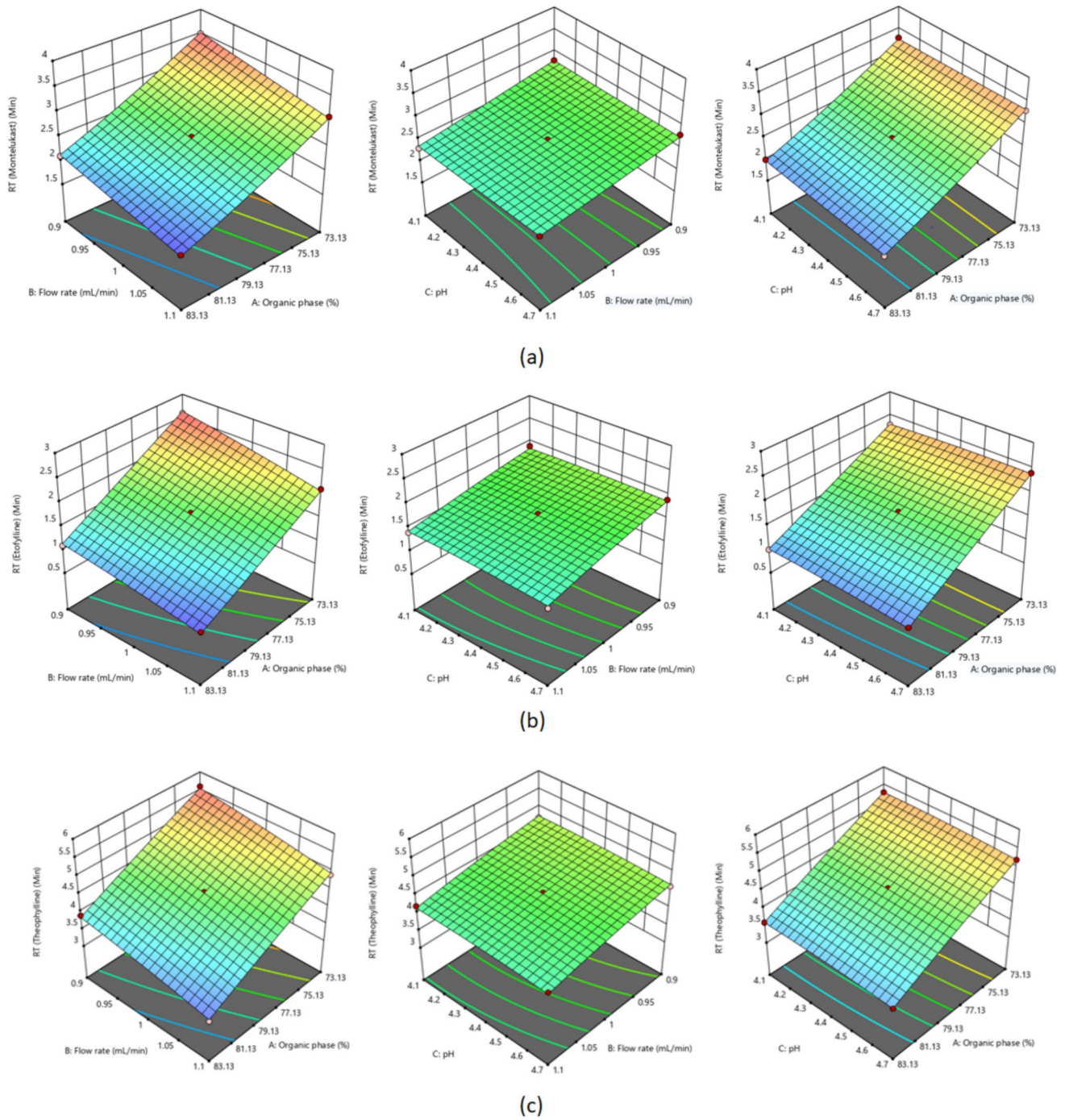

Fig. 3 Response surface plot showing the effect of % organic phase (X_1), flow rate (X_2) and pH (X_3) on RT of **a** Montelukast, **b** Etofylline, and **c** Theophylline

Analytical Method V

The analytical robustness of the optimized LC–MS method for the simultaneous quantification of montelukast, etofylline, and theophylline in synthetic plasma was rigorously validated in accordance with the performance criteria established by European Commission Regulation No. 2002/657/EC. To minimize matrix-related biases and enhance analytical accuracy, matrix-matched calibration was employed. Calibration curves were constructed via linear regression of peak area versus concentration, yielding excellent linearity across the validated ranges with coefficients of determination (R^2) > 0.999 for all analytes (Table 2)**.** Method sensitivity was characterized by determining the LOD and LOQ,

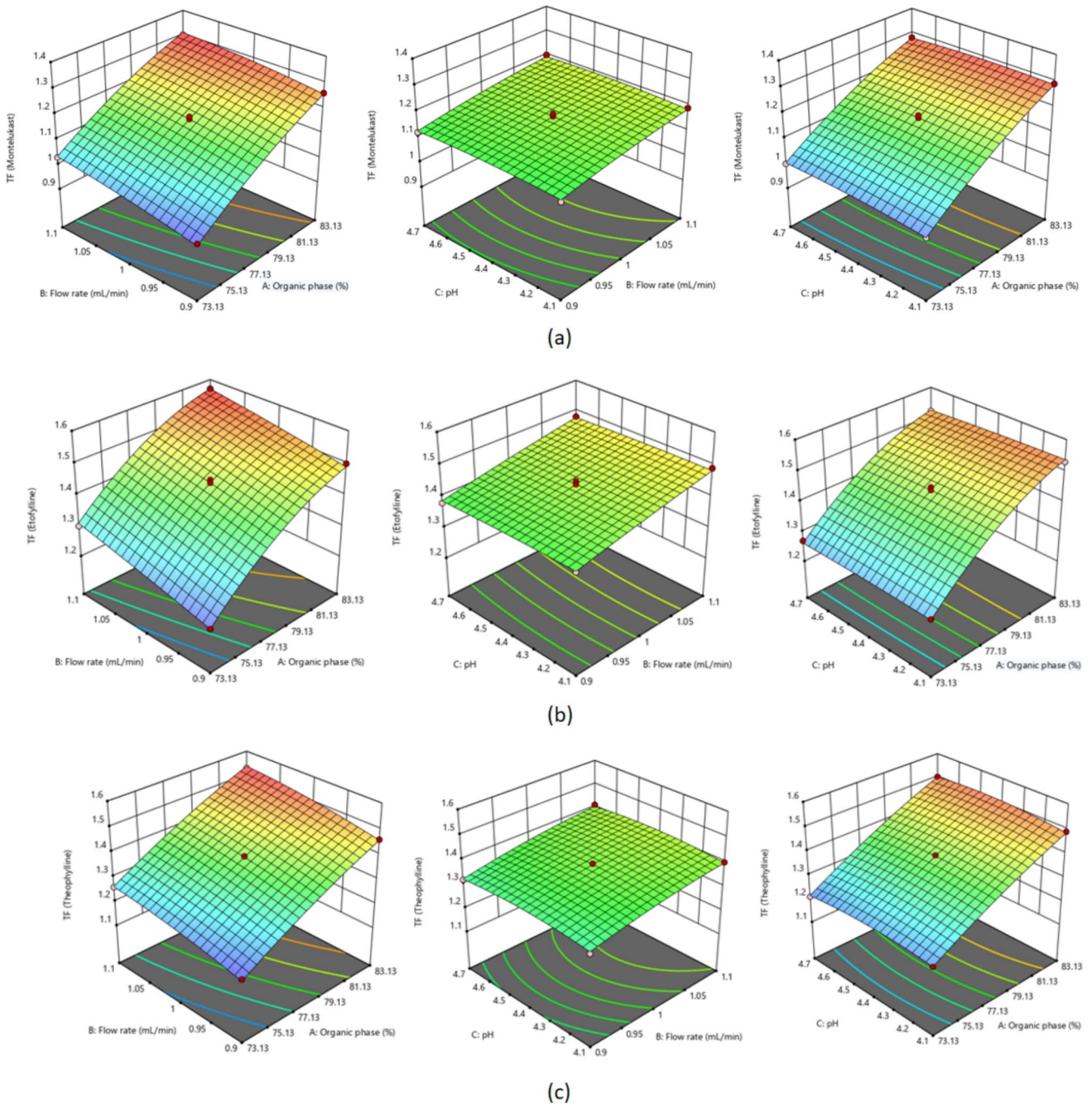

Fig. 4 Response surface plot showing the effect of % organic phase (X_1), flow rate (X_2) and pH (X_3) on TF of **a** Montelukast, **b** Etofylline, and **c** Theophylline

which were 0.094 ng/mL and 0.28 ng/mL for montelukast, 16.5 ng/mL and 50 ng/mL for etofylline, and 1.06 ng/mL and 3.2 ng/mL for theophylline, respectively. These results underscore the method's ability to detect and quantify trace concentrations with high precision.

Accuracy was evaluated using QC samples prepared at four concentration levels: LLOQ, LQC, MQC, and HQC. Percent recoveries ranged from 99.0% to 100.46%, confirming excellent method accuracy across all analytes. Precision, assessed by intra-day and inter-day variability, remained within ± 15% %CV for all QC levels (Table 3), confirming method reproducibility and robustness.

Matrix effects were evaluated using blank, chromatographically screened synthetic plasma spiked at LQC and HQC levels. Mean accuracies and %CV values for all three analytes were within regulatory acceptance criteria

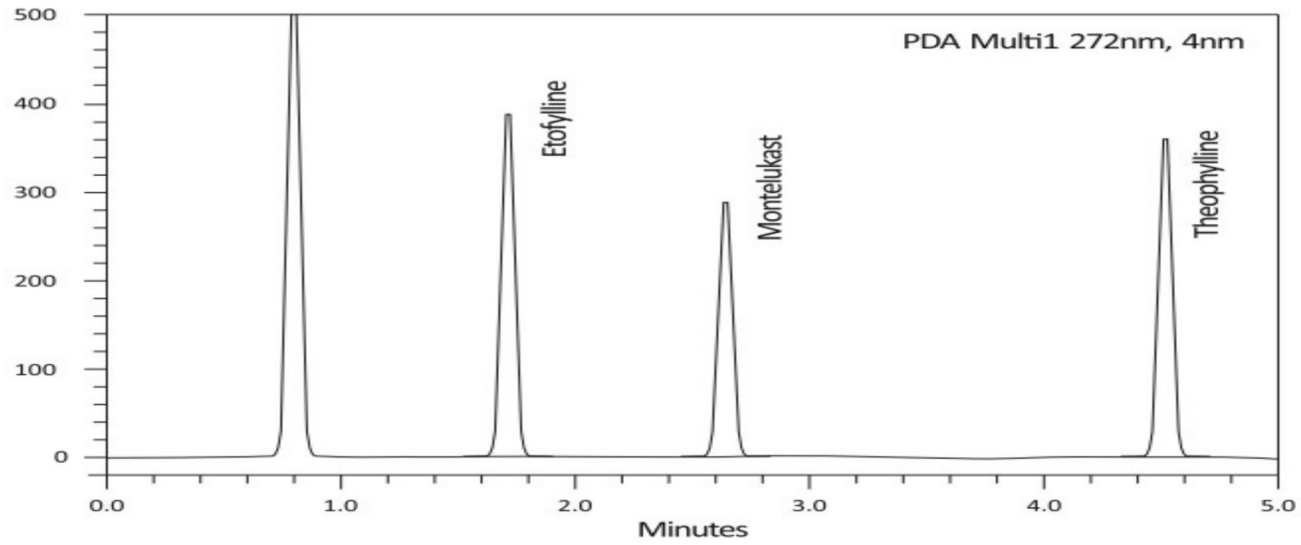

Fig. 5 Simultaneous LC chromatogram of Montelukast, Etofylline, and Theophylline in plasma sample

Table 2 Linearity data, LOD, and LOQ of the compounds

Sr. no	Validation parameters	Montelukast	Etofylline	Theophylline
1	Linearity			
	Linearity range (ng/mL)	2.5–15	57.75–346.5	17.25–103.5
	Graph equation	y = 11,575.2x + 194.43	y = 1149x -241.71	y = 3094.4x + 220.54
	Correlation coefficient	0.9999	0.9999	0.9999
2	LOD (ng/mL)	0.094	16.5	1.06
	LOQ(ng/mL)	0.28	50	3.2

Table 3 Results of average recovery (accuracy) and precision of the Montelukast, Etofylline, and Theophylline in synthetic plasma

Compound	Spiking levels (ng/mL)	Recovery (%)	Interday precision (%CV)	Intra-day precision (%CV)
Montelukast	LLQC (0.25 ng/mL)	99.0	2.54	2.32
	LQC (2.5 ng/mL)	100.12	0.87	0.54
	MQC (10 ng/mL	100.23	0.87	0.74
	HQC (15 ng/mL)	99.31	0.98	3.65
Etofylline	LLQC (5.8 ng/mL)	100.25	4.52	3.65
	LQC (57.75 ng/mL)	99.64	3.74	2.54
	MQC (173.25 ng/mL	100.64	2.51	0.87
	HQC (346.5 ng/mL)	99.35	0.87	1.45
Theophylline	LLQC (1.7 ng/mL)	99.87	0.68	0.87
	LQC (17.25 ng/mL)	99.25	0.87	0.54
	MQC (51.75 ng/mL	100.54	2.54	1.74
	HQC (103.5 ng/mL)	99.74	1.85	2.21

(Table 4), indicating negligible matrix interference. Robustness was assessed by introducing deliberate but minor variations in chromatographic conditions, including adjustments to the mobile phase composition, flow rate, and pH. These modifications had no significant impact on the method's performance, as evidenced by %RSD values consistently within the acceptable limit of < 2%. Carryover, assessed via analysis of LLOQC and ULOQC samples, was consistently low, with mean carryover values of 3.28% and 2.07% for montelukast, 2.68% and 3.47% for etofylline, and 3.61% and 3.04% for theophylline. Collectively, these findings confirm that the developed LC–MS method is precise, accurate, sensitive,

Table 4 Results of matrix effects

Parameter	Spiking levels (ng/mL)	% CV	%Accuracy
Montelukast	HQC	0.23	99.82
	LQC	1.78	98.62
Etofylline	HQC	0.69	100.12
	LQC	1.87	98.74
Theophylline	HQC	0.62	99.63
	LQC	2.41	100.74

and compliant with regulatory requirements, ensuring its applicability for reliable bioanalytical quantification in pharmacokinetic and clinical studies.

Stability Assessment

To ensure reliable quantification and minimize potential inter-analyte interactions, the stability of montelukast, etofylline, and theophylline was comprehensively evaluated under conditions mimicking routine laboratory handling and storage. All experiments were conducted in synthetic plasma, and the summarized results are presented in Table 5**.** Bench-top stability studies revealed that the analytes remained stable for up to 8 h at room temperature, with recovery values ranging from 99 to 101% and %CV well within the accepted limit of 15%. Freeze–thaw stability was assessed over five cycles, with samples stored at − 70 °C and thawed at ambient temperature. The analytes consistently exhibited recoveries between 98 and 101%, indicating excellent freeze–thaw resilience. Further assessments of autosampler, dry extract, and wet extract stability demonstrated that all three analytes maintained accuracy and precision within regulatory limits. Short-term stability data confirmed analyte integrity in rat plasma for up to 4 days at 25 °C, while long-term storage at − 70 °C preserved analyte stability for up to 28 days. These findings collectively confirm the robustness and reliability of the developed LC–MS method, ensuring accurate quantification of montelukast, etofylline, and theophylline under a broad range of storage and handling conditions typical of pharmacokinetic and clinical workflows.

Analysis of Deriphyllin M—A Commercial Formulation

The validated LC–MS method was applied to the quantitative analysis of montelukast, etofylline, and theophylline in the marketed formulation Deriphyllin M. Six replicate measurements were performed, yielding % assay values ranging from 98.1 ± 0.61 to 99.02 ± 0.87, thereby confirming the method's accuracy and precision. Chromatographic analysis revealed sharp, well-resolved peaks for all three analytes with no interference, underscoring the method's high

Table 5 Stability assessment of Montelukast, Etofylline, and Theophylline in Human plasma

Storage condition	Conc (ng/mL)	Montelukast		Etofylline		Theophylline	
		% accuracy	% CV	% accuracy	% CV	% accuracy	% CV
Bench-top (8 h.)	HQC	99.61	0.12	99.62	1.63	99.64	0.69
	MQC	101.67	0.06	100.4	3.41	99.41	2.54
	LQC	100.2	0.65	101.5	2.36	100.23	1.63
Freeze thaw (-70 °C)	HQC	99.74	0.78	99.74	4.71	99.74	1.41
	MQC	100.45	0.63	100.54	2.12	100.47	0.35
	LQC	99.87	2.75	101.71	3.68	99.63	2.45
Autosampler (24 h.)	HQC	98.62	0.98	97.01	3.01	99.63	2.84
	MQC	97.74	1.41	95.68	5.68	96.41	1.65
	LQC	96.87	2.87	96.74	5.54	95.71	0.23
Dry extract (24 h.)	HQC	98.71	0.49	98.94	6.41	96.80	0.58
	MQC	98.06	0.41	98.52	6.03	98.10	1.62
	LQC	97.51	1.63	95.07	7.41	97.63	2.41
Wet extract (24 h.)	HQC	97.28	0.74	97.63	6.63	95.02	3.65
	MQC	98.63	0.62	97.10	4.08	96.32	0.87
	LQC	95.74	1.27	94.19	7.96	97.2	1.54
Short-term (4 days)	HQC	96.63	1.46	94.52	6.41	94.71	2.65
	MQC	94.20	2.68	93.81	3.72	93.52	3.74
	LQC	91.38	3.32	91.06	4.85	92.65	4.85
Long term (28 days)	HQC	91.98	1.74	83.96	3.63	97.8	6.98
	MQC	87.33	3.69	82.74	8.78	92.6	5.41
	LQC	85.74	5.96	82.63	14.63	91.06	4.05

sensitivity and selectivity. Representative chromatograms are shown in Fig. 6**.** These results affirm the suitability of the method for routine quality control of multi-component pharmaceutical formulations.

Greenness and Whiteness Assessment of the Proposed Method

The environmental sustainability of the developed LC–MS method was critically evaluated using multiple GAC assessment tools. The ComplexGAPI tool, employing color-coded pentagram and hexagon pictograms, evaluated parameters, including sample preparation, reagent and solvent use, instrumentation, and pre-analytical operations. Out of the assessed indicators, nine appeared green (low environmental impact), five were yellow (moderate impact), and one was red (high impact), reflecting an overall eco-friendly analytical method with a single area requiring improvement (Fig. 7a**).** The red classification corresponded to the absence of recycling or waste-treatment procedures for the generated solvents/reagents. Future work will focus on implementing suitable recycling or neutralization strategies to minimize this environmental burden. The AGREE tool provided a complementary evaluation using a clock-face pictogram aligned with the 12 principles of GAC. A central score of 0.70 and a predominance of green zones confirmed the method's environmentally favorable profile (Fig. 7b**).** The Analytical Eco-Scale further validated these findings, awarding the method a score of 77, classifying it as an "excellent green method" based on minimal waste generation, low energy demands, and limited use of hazardous reagents. For the BAGI assessment, a score of 75.0 was obtained, reflecting an optimal balance between sustainability and analytical performance (Fig. 7c). In the context of instrument energy use, the demands of both LC–MS and PDA detectors were estimated using manufacturer specifications and found to be comparable to those reported for other modern green analytical methods, thus supporting their inclusion without significantly compromising the environmental profile. Collectively, these evaluations affirm the LC–MS method as

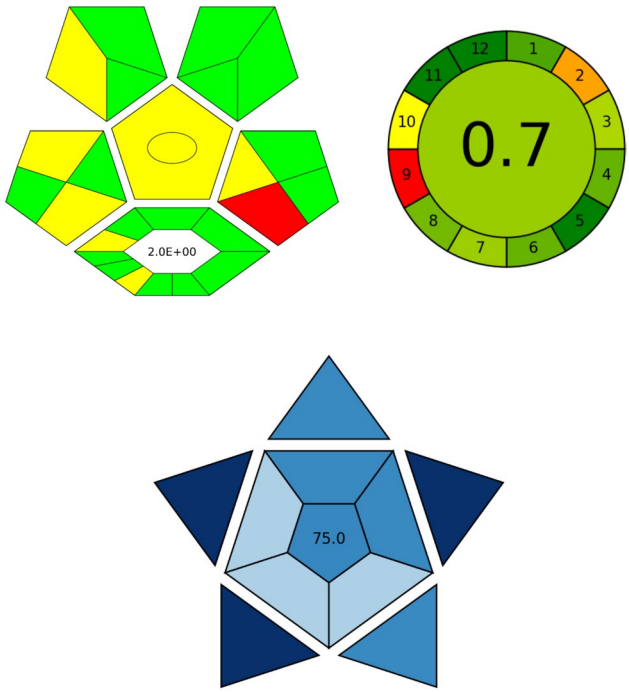

Fig. 7 Evaluating the "greenness and whiteness" of the suggested technique, using **a** ComplexGAPI, **b** AGREE, and **c** BAGI

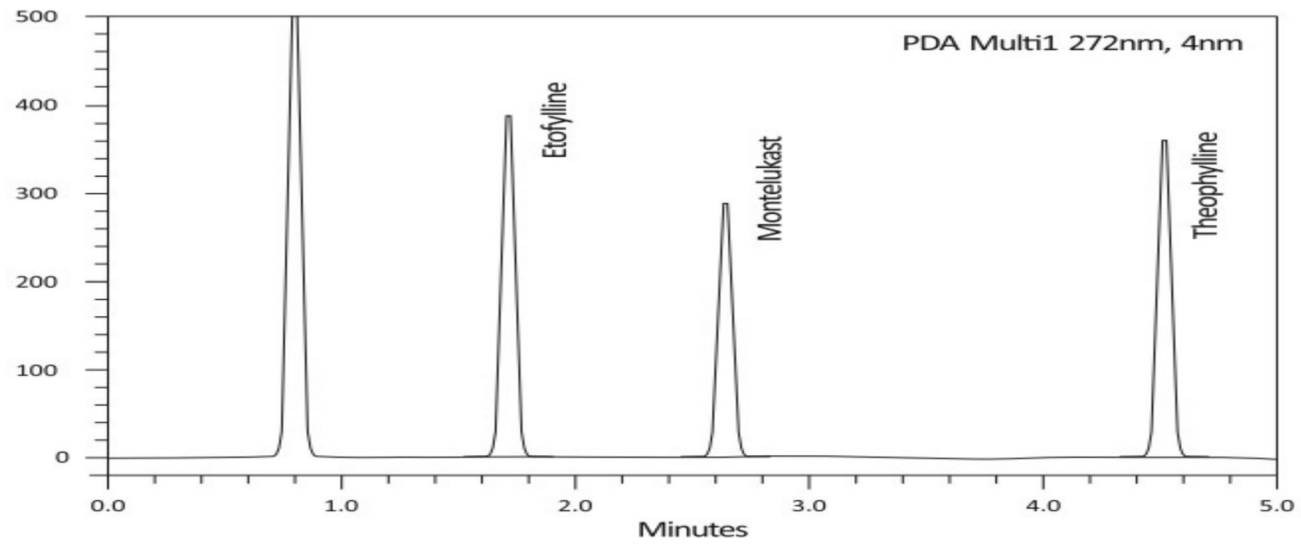

Fig. 6 Simultaneous LC chromatogram of marketed formulation (Deriphyllin M)

a robust, environmentally conscious approach suitable for routine laboratory application.

Conclusion

This study presents a comprehensive QbD-driven development of a green, robust, and validated LC–MS method for the simultaneous quantification of montelukast, etofylline, and theophylline in both synthetic plasma and pharmaceutical formulations. Utilizing a BBD enabled precise optimization of methanol content, flow rate, and mobile phase pH, ensuring excellent resolution, minimal peak tailing, and efficient runtime. The statistical strength of the model was confirmed through ANOVA, residual analysis, and second-order polynomial equations, ensuring a reliable predictive framework for chromatographic performance. The method demonstrated outstanding analytical performance with excellent sensitivity (LODs in the ng/mL range), recovery (99–100.46%), linearity ($R^2 > 0.999$), and precision (CV < 15%). The electrospray ionization-based SRM detection ensured high selectivity and reproducibility, supporting its application in complex biological matrices and marketed formulations. Importantly, the greenness of the developed method was confirmed using eco-analytical tools, including AGREE, Analytical Eco-Scale, ComplexGAPI and BAGI. The high AGREE score and minimal penalty points reflect its alignment with green analytical chemistry principles—characterized by reduced solvent usage, non-hazardous chemicals, and minimal energy demands. These attributes make it not only scientifically rigorous but also environmentally sustainable. The method's applicability was further established by accurately quantifying all three analytes in commercial oral dosage form with no matrix interference observed. The findings confirm the method's robustness, reproducibility, and environmental compatibility, providing a valuable analytical tool for routine quality control, pharmacokinetic studies, and green pharmaceutical research.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10337-025-04439-y.

Acknowledgements The authors sincerely express their gratitude to the Shree Balaji College of Pharmacy, SBMCH Campus, BIHER, Chromepet, Chennai-600044, Tamil Nadu, India, Department of Pharmaceutical Chemistry & Analysis, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai-600117, Tamilnadu, India, Raghavendra Institute of Pharmaceutical Education and Research (RIPER)-Autonomous, Ananthapuramu, Andhra Pradesh 515721, India, Raffles University, Neemrana, Rajasthan 301020, India, Department of Chemistry, School of Applied Sciences & Humanities, Vignan's Foundation for Science, Technology and, Research (VFSTR), Vadlamudi, Guntur—522213, Andhra Pradesh, India and the Department of Pharmacy, Chaitanya (Deemed to be University), Gandipet, Himayath Nagar (Village), Hyderabad, Telangana, for providing the essential research facilities that contributed to this study.

Authors' Contributions We affirm that "all authors have read and approved the manuscript." Each contributor played an equal role and actively engaged in this research project.

Funding None.

Data Availability The research work has been carried out by us, and we assure you that it can be provided to you whenever required.

Declarations

Conflict of Interests The authors declare no competing interests.

Ethics Approval and Consent to Participate No.

Consent for Publication No.

References

1. Hogg JC (2004) Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. Lancet 364:709–721
2. Cukic V, Lovre V, Dragisic D, Ustamujic A (2012) Asthma and chronic obstructive pulmonary disease (COPD) – differences and similarities. Mater Socio-Med 24:100–103
3. Naik SR, Wala SM (2013) Inflammation, allergy and asthma, complex immune origin diseases: mechanisms and therapeutic agents. Recent Pat Inflamm Allergy Drug Discov 7:62–95
4. Taylor JD (2010) COPD and the response of the lung to tobacco smoke exposure. Pulm Pharmacol Ther 23:376–383
5. Castillo JR, Peters SP, Busse WW (2017) Asthma exacerbations: pathogenesis, prevention, and treatment. J Allergy Clin Immunol Pract 5:918–927
6. Bollmeier SG, Hartmann AP (2020) Management of chronic obstructive pulmonary disease: a review focusing on exacerbations. Am J Health-Syst Pharm 77:259–268
7. Nayak A (2004) A review of montelukast in the treatment of asthma and allergic rhinitis. Expert Opin Pharmacother 5:679–686
8. Nathan RA (2003) Pharmacotherapy for allergic rhinitis: a critical review of leukotriene receptor antagonists compared with other treatments. Ann Allergy Asthma Immunol 90:182–191
9. Shao M, Sun J, Zheng Q (2025) Efficacy and safety of montelukast–levocetirizine combination therapy in combined allergic rhinitis and asthma syndrome: a systematic review and meta-analysis. J Asthma 62:376–385
10. Sankar K (2015) Comparative study of efficacy and adverse effect profile of theophylline and etofylline combination versus doxofylline in patients with COPD. Sch J App Med Sci 3(1E):310–317
11. Palai S, Chandra S, Pandey N, Singh R (2023) Theophylline: A bioactive dimethylxanthine alkaloid. In: The Essential Guide to Alkaloids, pp 205–217
12. Abd-Elhafeez M, Arafa MM, Amro FH, Youssef FS (2024) Green analytical chemistry to eco-friendly HPLC techniques in pharmaceutical analysis: a review. Egypt J Vet Sci 55:795–801
13. Kokilambigai KS, Lakshmi KS (2022) Analytical quality by design assisted RP-HPLC method for quantifying atorvastatin with green analytical chemistry perspective. J Chromatogr Open 2:100052
14. Dessai S, Mannur VS, Koli R, Dhond M, Badiger P (2024) Quality by design-engineered reversed-phase high-performance

liquid chromatography method development and validation for simultaneous estimation of neomycin sulfate and beclomethasone dipropionate in bulk and pharmaceutical dosage form. Separation Science Plus 7:2400001
15. Badiger P, Mannur VS, Koli R (2024) Dual drug-loaded cubosome nanoparticles for hepatocellular carcinoma: a design of experiment approach for optimization and in vitro evaluation. Future J Pharm Sci 10:38
16. Koli R, Mannur VS, Shetti PP (2024) Robust high-performance thin-layer chromatography (HPTLC) method for stability assessment and simultaneous quantification of epigallocatechin-3-gallate and rosmarinic acid in lipid-based nanoparticles and biological matrices. Phytochem Anal (in press)
17. Tong Q, Hou X, Fang J, Wang W, Xiong W, Liu X, Xie X, Shi C (2015) Determination of dihydromyricetin in rat plasma by LC–MS/MS and its application to a pharmacokinetic study. J Pharm Biomed Anal 114:455–461
18. Liang Y, Ma T, Li Y, Cai N (2022) A rapid and sensitive LC–MS/MS method for the determination of vanillic acid in rat plasma with application to pharmacokinetic study. Biomed Chromatogr 36:e5248
19. Rane S, Adhyapak A, Gharge S, Koli R, Singadi R, Halagali P (2022) UV and HPTLC-based approaches towards rutin determination in *Abutilon theophrasti* extract. J Pharm Sci Res 14:790–794
20. Usgaonkar P, Adhyapak A, Koli R (2024) Analytical quality by design (AQbD) principles in development and validation of stability-indicating RP-HPLC method for simultaneous estimation of diltiazem HCl and eugenol in nanoethosomes. Sep Sci Plus 7:e202400155
21. Alshogran OY, Al-Shdefat R, Hailat M (2023) Simple and rapid quantification of ribociclib in rat plasma by protein precipitation and LC-MS/MS: an application to pharmacokinetics of ribociclib nanoparticles in rats. J Mass Spectrom 58:e4984
22. Long F, Ji J, Wang X, Wang L, Chen T (2021) LC–MS/MS method for determination of seneciphylline and its metabolite, seneciphylline N-oxide in rat plasma, and its application to a rat pharmacokinetic study. Biomed Chromatogr 35:e5145
23. Gandu S, Gandla K, Repudi L (2025) Development and validation of an LC-MS/MS method for the simultaneous estimation of tadalafil and macitentan in rat plasma: greenness assessment and design of experiment approach. Green Anal Chem. https://doi.org/10.1016/j.greeac.2025.100211
24. Kumar P, Mangla B, Beg S, Afzal O, Altamimi AS, Almalki WH, Ullah SN, Aggarwal G (2022) Optimization and validation of stability indicating RP-HPLC method for the quantification of gefitinib in bulk drug and nanoformulations: an application towards in vitro and ex vivo performance evaluation. Arab J Chem 15:104333
25. Koli R, Mannur VS (2024) Green RP-HPLC method for simultaneous quantification of epigallocatechin-3-gallate and rosmarinic acid in lipid-based nanocarriers and biological fluids: quality by design-driven optimization and lean six sigma approach. Green Anal Chem 11:100153
26. Hammouda ME, Salem YA, El-Ashry SM, El-Enin MA (2023) Inclusive study for sustainable enantioseparation of racemic chlorpheniramine and caffeine by HPLC using dual cyclodextrin system as chiral mobile phase additive: assessment with AGREE and Complex-GAPI approaches. Sustain Chem Pharm 35:101201
27. Sezgin B, Soyseven M, Arli G (2024) Greenness assessment and comparison of the developed and validated green HPLC-PDA, HPLC-FLD, and HPLC-ELSD methods for the determination of melatonin in various products using analytical eco-scale, NEMI, GAPI, and AGREE greenness metric tools. Microchem J 205:111211
28. Manousi N, Wojnowski W, Płotka-Wasylka J, Samanidou V (2023) Blue applicability grade index (BAGI) and software: a new tool for the evaluation of method practicality. Green Chem 25:7598–7604