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Original Article

Stability-Indicating HPTLC Method for Concurrent Estimation of Triacontane and Beta-Sitosterol in *Ailanthus excelsa* Roxb.: A DoE Approach

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ABSTRACT

Background: Triacontane and beta-sitosterol, though promising in various applications, face challenges due to low solubility and susceptibility to photosensitivity. Enhancing their effectiveness requires overcoming these limitations. Design of Experiment (DoE) coupled with High-Performance Thin Layer Chromatography (HPTLC) offers a potent approach to address these issues. **Materials and Methods:** The HPTLC method was optimized using a Box-Behnken Design (BBD), considering factors like saturation time, migration distance and application length. Response Surface Methodology (RSM) was employed to analyze critical risk factors' effects on the method. Validation parameters, including linearity, accuracy, precision, robustness, solution stability and forced degradation studies, were assessed according to ICH guidelines. Additionally, a different fractions of *Ailanthus excelsa* Roxb. plant extracts was analyzed. **Results:** Triacontane and beta-sitosterol displayed distinct R_f values of 0.94±0.010 and 0.75±0.02 for triacontane and beta-sitosterol, respectively. The method demonstrated excellent linearity across the concentration range of 2-10 mg/band, with correlation coefficients of 0.999 triacontane and 0.9989 beta-sitosterol, respectively. Percentage recovery for both found within the range of 98.84-102.92%. This validated method offers specificity and robustness, ideal for routine quality control testing in pharmaceutical applications. Solution stability and forced degradation studies further supported the method's stability-indicating nature. **Conclusion:** The stability-indicating HPTLC method, developed and validated using DoE approach, proved suitable for simultaneous estimation of triacontane and beta-sitosterol in *Ailanthus excelsa* Roxb. plant extracts. Compliance with ICH guidelines, coupled with robustness and specificity, positions it as a valuable tool for quality evaluation and standardization of herbal formulations.

Keywords: Beta-sitosterol, Design of Expert, High performance thin-layer chromatography, Stability study, Triacontane.

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INTRODUCTION

Plants have a significant impact on illness prevention and treatment and they can even mitigate the negative consequences of conventional treatments. They may be a source of chemical compounds that are significant in biology and pharmacology. Plants have historically provided effective medications and they will continue to be important for the screening of novel lead chemicals.[1] Due to its likeness to the neem tree, the Simaroubaceae plant *Ailanthus excelsa* Roxb. is frequently referred to as "Mahanimba" (*Azadirachta indica*).[2,3] One of the species in the Moluccas is known by the name ailanto, which translates to "Tree of Heaven," while the title "*Ailanthus*" comes from the Latin word *excelsa*, which means "tall".[4,5]

Triacontane Figure 1 (a) and beta-sitosterol Figure 1 (b), were selected for this study among the large number of secondary metabolites present in this plant. Numerous plants, including *Ailanthus excelsa* Roxb., have been shown to contain triacontane, according to reports. Additionally, it has been claimed that triacontane has been examined for biological activities, including antibacterial, antidiabetic and anticancer action and beta-sitosterol is a main phytosterol found in many plants. According to reports, it possesses anti-inflammatory, anti-cancer, antipyretic and immunomodulating properties.[6,7]

A lot of work has been put into developing appropriate and trustworthy analytical procedures that confirm the quality of herbal products in addition to enhancing and increasing their quality over the past several decades.[8-10] Chromatographic and

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spectroscopic fingerprinting are now the most widely used techniques for assessing and keeping track of the quality of diverse herbal products. For the individual estimation of triacontane and beta sitosterol, many analytical techniques are available but none have been reported simultaneous determination of both the markers by applying stability indicating HPTLC method with the help of Design of Experiment (DoE) approach in *Ailanthus excelsa* Roxb. plant samples.[11,12]

For quality evaluation and the development of standards for herbal medications, the use of innovative quality methodologies is crucial, along with the deployment of marker-based techniques. Recently, DoE has become a core paradigm for pharmaceutical quality adopted by pharmaceutical companies.[13] Utilizing the DoE approach to analytics is one way to shorten the trial period and lower the cost of drug analysis. The DoE method advises assessing the analytical process' quality from the very beginning of the development phase. The scientific understanding of method variables and their interactions are explored by analytical DoE, which ultimately provides a region for a highly reliable and efficient technique.[14,15]

In the present research work, an attempt has been made to develop and validate DoE assisted stability indicating HPTLC technique for estimation of triacontane and beta sitosterol in *ailanthus excelsa* Roxb. plant samples in compliance with ICH criteria. Several stress or forced degradation study methodologies, including alkaline, acidic, thermal, oxidative and photolytic were used to verify the appropriate of the developed method.

MATERIALS AND METHODS

Materials

Triacontane (TC) and Beta-sitosterol (BS) were procured from Sigma Aldrich, Germany, ensuring high-quality standards. Toluene, Ethyl acetate and formic acid were sourced from Molychem, Mumbai, India. Methanol and acetonitrile were obtained from M/s Merck Ltd., Mumbai, India. The *Ailanthus excelsa* Roxb. plant was responsibly collected from Hidakal dam, Belagavi, India, under appropriate guidelines and ethical considerations.

Instrumentation

In this study, various equipment and instruments were utilized, including the Linomat 5 sample applicator, Twin trough chamber measuring 20×10 cm, UV chamber and Hamilton microliter syringe (Linomat syringe), all sourced from Camag, Switzerland. Additionally, pre-coated silica gel aluminum plates 60 F254 measuring 20×10 cm with a thickness of 1 mm were obtained from E. Merck, Darmstadt, Germany. The analysis was completed using a TLC scanner 4 from Camag, Switzerland, with data processing facilitated by vision CATS version 3 software.

Preparation of standard solution

Individual stock solutions of Triacontane and Beta-sitosterol were meticulously prepared by accurately weighing 10 mg of each bioactive compound into separate 10 mL volumetric flasks. The compounds were then carefully diluted up to the mark in the volumetric flasks with solvent, resulting in a final concentration of 1000 µg/mL for each solution. Subsequently, to prepare working standard solutions at a concentration of 500 ng/bands, the individual stock solutions of both compounds were diluted with methanol.

Collection of *Ailanthus excelsa* Roxb. plant

The bark of *Ailanthus excelsa* Roxb. plant was collected from Hidakal dam, Belagavi, in June and authenticated by Dr. Harsha V. Hegde (Scientist E) of ICMR NITM, Belagavi with Authentication no: RMRC-1715. After manual removal, the bark was air-dried on muslin cloth for 10-15 days. Subsequently, it underwent grinding for further processing.

Sample application

On precoated HPTLC plates, both the standards and extract samples were applied as thin bands, each measuring 6 mm in length. The bands were positioned with 10 mm of space from the bottom and left margins and 9 mm between each band. Subsequently, the samples were subjected to continuous nitrogen gas drying at a rate of 150 nL/s.[16]

Response Surface Methodology (RSM) based Box-Behnken Design (BBD)

Following the screening design analysis, it became evident that only three risk factors namely, saturation time, migration distance and application length were deemed crucial for the development of the HPTLC method. To delve deeper into these critical risk factors and understand their association with the R_f value of the drugs, a Design of Experiments (DoE) based box-behnken design was employed.[17,18] The optimization of the HPTLC method involved the consideration of factors such as chromatographic chamber saturation time (X1), migration distance (X2) and application length. The selected responses for optimization were the R_f values of triacontane and beta-sitosterol, as outlined in Table 1.

In terms of optimization, the chamber saturation time (X1) was varied within the range of 15, 20 and 25 min. Concurrently, the migration distance (X2) was adjusted to levels of 50, 70 and 90 mm, while the application length (X3) was set at levels of 6, 8 and 10 mm, respectively. This DoE approach facilitated a systematic exploration of the relationships between the R_f value of the drugs and the identified critical risk parameters. In a controlled laboratory environment, the responses of the peaks corresponding to the two drugs were meticulously evaluated through a series of experimental runs prescribed by the Design-Expert software.[19]

Utilizing the software, responses were assigned to each experimental run, enabling a comprehensive analysis of the box-behnken design. The data generated from these experimental runs were then subjected to Analysis of Variance (ANOVA) to discern significant effects and interactions. Additionally, response surface plots were employed to visualize the relationships between the critical risk factors and the responses, aiding in the interpretation of the experimental outcomes. Through this methodical approach, a deeper understanding of the influence of the identified risk factors on the HPTLC method development was attained, facilitating informed decision-making and optimization strategies.[20]

Validation of the optimized method

Validating an analytical method is essential to guarantee reliable chromatographic separation. The method underwent comprehensive validation to assess its linearity, Limits of Detection (LOD), Limit of Quantification (LOQ), precision, accuracy (% recovery), robustness, solution stability and forced degradation studies.[21,22]

Solution stability study

Drug solutions (500 ng/bands each) were prepared and stored at room temperature, protected from light, for 24 hr. Subsequently, they were periodically examined at intervals of 0, 6 and 12 hr to detect any presence of bands differing from those of the standard drugs.[23]

Forced degradation studies

Forced drug degradation tests were performed to subject drug samples to stress-induced conditions including acid, base, oxidation and photolytic, along with the interference of degraded products. These tests serve to assess the intrinsic stability of the active ingredients in the medicinal product and identify their potential breakdown products.[24,25]

Estimation of Triacontane and Beta-sitosterol in *Ailanthus excelsa* Roxb. plant extract

Different fractions of *Ailanthus excelsa* Roxb. plant extract was obtained by utilizing various solvents such as hydroalcoholic, ethyl acetate, chloroform, petroleum ether and n-hexane. Each

Table 1: Chromatographic Factors for Box-Behnken Design.

Factors	Lower level (-1)	Intermediate (0)	Higher level (+1)
Saturation time	15 min	20 min	25 min
Migration Distance	50 mm	70 mm	90 mm
Application length	6 mm	8 mm	10 mm
Responses	$Y_{1=}$ R_f value of Triacontane and $Y_{2=}$ R_f value of Sitosterol		

Table 2: Design metrics for response surface method by Box-Behnken design.

	Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run	A: Saturation time	B: Migration Distance	C: Application length	R_f Triacontane	R_f Beta-Sitosterol
	min	mm	mm		
1	25	70	6	0.89	0.67
2	25	90	8	0.87	0.68
3	15	90	8	0.56	0.48
4	20	50	10	0.78	0.61
5	20	70	8	0.94	0.75
6	20	70	8	0.93	0.75
7	20	90	6	0.98	0.81
8	20	70	8	0.94	0.76
9	25	70	10	0.82	0.58
10	15	70	10	0.55	0.45
11	25	50	8	0.85	0.63
12	20	50	6	0.75	0.62
13	20	90	10	0.91	0.72
14	15	50	8	0.52	0.4
15	15	70	6	0.54	0.42

fraction, weighing 100 mg, was meticulously transferred into individual 100 mL volumetric flasks. methanol was added to each flask in precise quantities, ensuring a final concentration of 1000 µg/mL. Following this, the mixtures underwent a thorough process of sonication, lasting 15 min, to facilitate proper dissolution. Subsequently, the resulting solutions were further diluted with methanol, meticulously adjusted to achieve a concentration of 500 ng/band.[26]

RESULTS

Method optimization by Box-Behnken Design

Utilizing the adopted Box-Behnken design, three independent variables were subjected to variation across three distinct levels, each coded for low, medium and high (-1, 0 and +1 respectively). These variables included saturation time, migration distance and application length. The response variables chosen were the R_f values of triacontane (R1) and beta-sitosterol (R2), serving as indicators of the experimental outcomes. Employing the Design of Experiments (DoE) program facilitated a deeper understanding of the critical factors influencing the desired outcomes. Through systematic experimentation, the program aimed to unveil the optimal conditions necessary to achieve the desired results. The chromatographic trials yielded valuable data, with the R_f values of triacontane (Y1) and beta-sitosterol (Y2) being extracted and compiled for analysis. Table 2 presents a comprehensive summary of these R_f values obtained from each trial, providing insights into the effects of the varied independent variables on the dependent responses.

Furthermore, the analytical approach was statistically optimized using Design Expert® Software, Version 13. This involved a meticulous comparison of various statistical factors to ensure robustness and accuracy in the experimental design. The Analysis of Variance (ANOVA) results, crucial for evaluating the effectiveness of the applied design, are succinctly presented in Tables 3 and 4. These tables encapsulate the statistical data essential for assessing the significance of the experimental variables and their interactions in driving the observed outcomes.

The mathematical statement in the form of polynomial equations was examined in order to determine the relationship between the dependent variables. An influence on the response that is synergistic is shown by a positive coefficient sign, whereas an antagonistic effect is indicated by a negative coefficient sign. The independent variable has a stronger impact on the response if the coefficient is greater equations 1 and 2.

$$\text{Rf Triacontane} = +0.9367 + 0.1575*A + 0.0525*B - 0.0125*C - 0.0050*AB - 0.0200*AC - 0.0250*BC - 0.1958*A2 - 0.0408*B2 - 0.0408*C2$$

$$\text{Rf Beta} - \text{Sitosterol} = +0.7533 + 0.1012*A + 0.0537*B - 0.0200*C - 0.0075*AB - 0.0300*AC - 0.0200*BC - 0.1829*A2 - 0.0229*B2 - 0.0404*C2$$

Graphical representations, including response surface plots and perturbation plots, were generated to analyze the impact of each factor on the responses. Specifically, 3D response surface plots and perturbation plots were created to visualize the interactions among saturation time, migration distance and application length concerning the R_f values of triacontane and beta-sitosterol (Figures 2 and 3). Using the Design-Expert software by Stat-Ease, Inc. (version 13.0), it was determined that all responses met satisfactory criteria. The design space, represented by a shaded zone with yellow coloring in the overlay plot (Figure 4), indicated

Table 3: ANOVA for response 1: R_f value of (Triacontane) by Box-Behnken design.

Source	Sum of Squares	d_f	Mean Square	F-value	*p*-value	
Model	0.3722	9	0.0414	18.11	0.0026	Significant
A-Saturation time	0.1984	1	0.1984	86.91	0.0002	
B-Migration Distance	0.0220	1	0.0220	9.66	0.0266	
C-Application length	0.0013	1	0.0013	0.5474	0.4926	
AB	0.0001	1	0.0001	0.0438	0.8425	
AC	0.0016	1	0.0016	0.7007	0.4407	
BC	0.0025	1	0.0025	1.09	0.3433	
A^2	0.1416	1	0.1416	62.02	0.0005	
B^2	0.0062	1	0.0062	2.70	0.1615	
C^2	0.0062	1	0.0062	2.70	0.1615	
Residual	0.0114	5	0.0023			
Lack of Fit	0.0114	3	0.0038	113.50	0.0087	Not significant
Pure Error	0.0001	2	0.0000			
Cor Total	0.3836	14				

the successful operating ranges. Within this space, optimal values for the independent parameters, saturation time (20 min), migration distance (20 mm) and application length (8 mm) were identified under specific conditions. The model's ability to accurately predict response variable values was underscored by the remarkably low prediction error (5%) observed between predicted and actual observations. These optimal conditions facilitated a remarkable chromatographic separation of both the drugs, as visually depicted in Figure 5.

Method validation

To ensure that the optimized HPTLC method is appropriate for the intended use as outlined in ICH Q2 (R1) standards, it has undergone validation. Table 5 provides a summary of the proposed HPTLC method's validation parameters, which were determined to be within the ICH Guidelines' standard limits.

The developed HPTLC method exhibited excellent linearity over the range of 100-500 ng/band for both triacontane and beta-sitosterol, with correlation coefficients of 0.999 and 0.998, respectively (Figure 6). Additionally, the method demonstrated

Table 4: ANOVA for response 2: R_f value of (Beta-sitosterol) by Box-Behnken design.

Source	Sum of Squares	d_f	Mean Square	F-value	*p*-value	
Model	0.2400	9	0.0267	31.82	0.0007	Significant
A-Saturation time	0.0820	1	0.0820	97.83	0.0002	
B-Migration Distance	0.0231	1	0.0231	27.57	0.0033	
C-Application length	0.0032	1	0.0032	3.82	0.1082	
AB	0.0002	1	0.0002	0.2684	0.6265	
AC	0.0036	1	0.0036	4.29	0.0930	
BC	0.0016	1	0.0016	1.91	0.2257	
A^2	0.1235	1	0.1235	147.36	< 0.0001	
B^2	0.0019	1	0.0019	2.31	0.1888	
C^2	0.0060	1	0.0060	7.19	0.0437	
Residual	0.0042	5	0.0008			
Lack of Fit	0.0041	3	0.0014	41.25	0.0238	Not significant
Pure Error	0.0001	2	0.0000			
Cor Total	0.2442	14				

Table 5: Summary of validation parameters.

Sl. No.	Validation parameters	Triacontane	Beta-sitosterol
1	Linearity		
	Linearity range (ng/band)	100-500	100-500
	Correlation-coefficient	0.9992	0.9986
2	LOD (ng/band)	8. 22	10.49
	LOQ (ng/band)	24.99	31.79
3	Precision		
	Intra-day (%RSD)	0.719±0.08	0.5±0.0021
	Inter-day (%RSD)	1.24±0.032	0.44±0.00001
4	Robustness		
	Mobile phase volume (%RSD)	0.616±0.047	0.88±0.041
	Migration distance (%RSD)	0.653±0.087	0.53±0.012
	Duration of saturation (%RSD)	0.517±0.014	0.69±0.14
5	Accuracy		
	50% recovery	99.75±0.04	100.31±0.042
	100% recovery	100.04±0.27	100.28±0.61
	150% recovery	99.91±0.32	99.71±0.25

Table 6: Solution stability study.

Compound	Time (hr)	Area	% RSD
Triacontane	0	0.01841	1.24±0.0124
	6	0.01873	
	12	0.01832	
	24	0.01881	
Beta-sitosterol	0	0.00603	0.56±0.0567
	6	0.00598	
	12	0.00597	
	24	0.00595	

Table 7: Forced degradation study.

Stress condition	Temperature °C	Time (hr)	% Degradation	
			Triacontane	Beta-sitosterol
Acidic (1M HCl)	80°C	2	32	15
Basic (1M NaOH)	80°C	2	18	11
Oxidative (30% H_2O_2)	80°C	2	13	9
Photolytic	Sun light	6	12	13

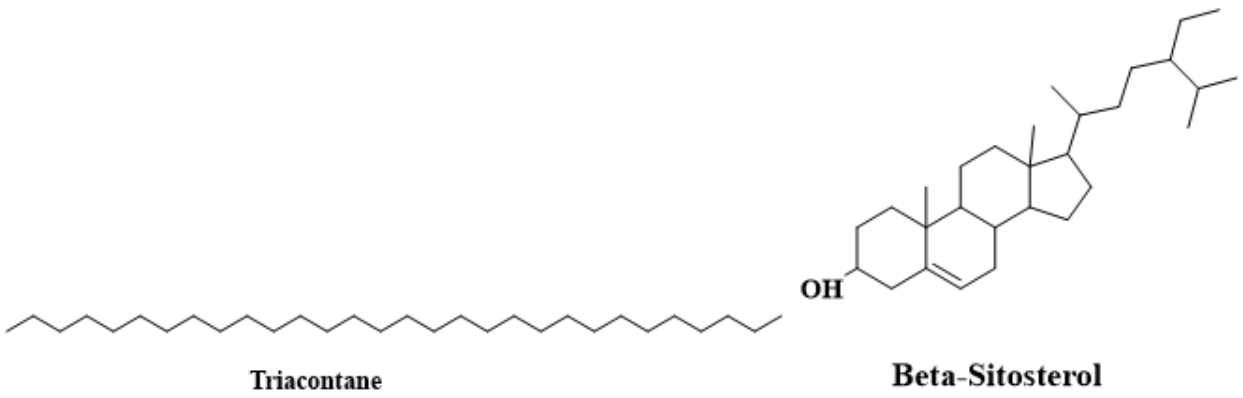

Figure 1: Chemical structures of Triacontane and Beta-Sitosterol.

satisfactory Limits of Detection (LOD) and Quantification (LOQ), with triacontane LOD and LOQ at 8.22 and 24.99 ng/bands and beta-sitosterol LOD and LOQ at 10.49 and 31.79 ng/band, respectively. Evaluation of accuracy through recovery studies at three different levels (50%, 100% and 150%) revealed close agreement between observed and expected values, with mean percentage recoveries falling within the range of 99.7% to 100.31% for both compounds in ethyl acetate and hydroalcoholic fractions. Precision analysis, including intra-day and inter-day variability, demonstrated %RSD values meeting the criterion of <2%, indicating excellent reproducibility and reliability of the method for both triacontane and beta-sitosterol. Robustness assessment, exploring variation in chromatographic parameters under slight experimental changes, yielded %RSD values <2%, affirming the method's robustness with minimal variability despite small variations in experimental conditions.

Solution stability study

The results of the solution stability study were compared with freshly prepared standards solutions of the same concentration in the form of changes in the response's % RSD. In the simultaneous peak area of both the drugs, there is no significant change were observed and results of solution stability study were summarized in Table 6.

Forced degradation study

Table 7 shows the results of a study on forced degradation. This study provides evidence for the relevancy of the developed

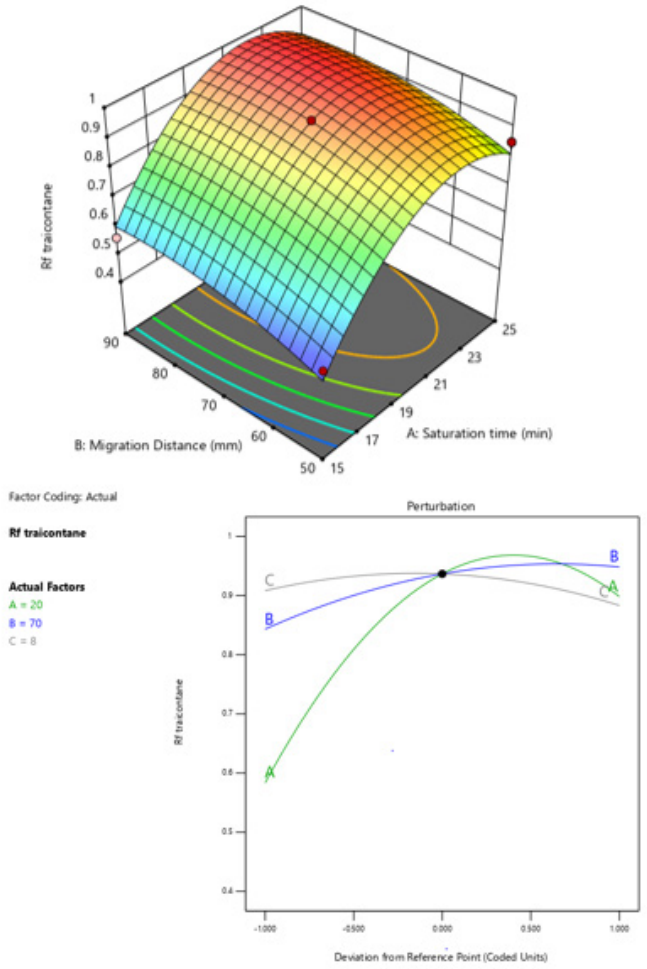

Figure 2: Response surface and perturbation plots of response Y1 (R_f Triacontane).

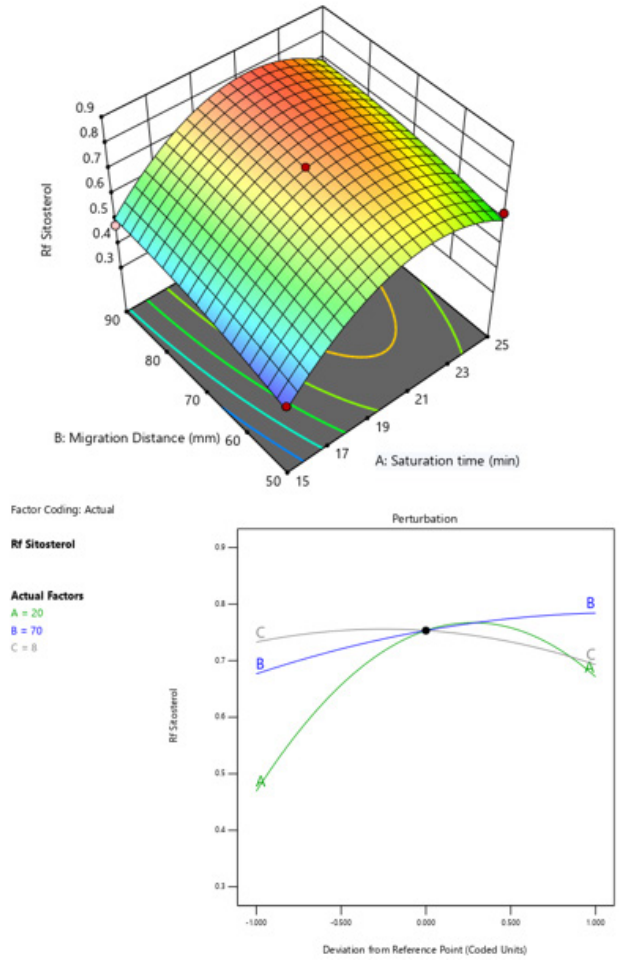

Figure 3: Response surface and perturbation plots of response Y2 (R_f Beta-sitosterol).

method so there was sufficient separation between drug peak and their degradation product peak observation (Figure 7).

Estimation of Triacontane and Beta-sitosterol in *Ailanthus excelsa* Roxb. plant extract

HPTLC analysis unveiled the presence of triacontane and beta-sitosterol within the hydroalcoholic and ethyl acetate fractions of the *Ailanthus excelsa* Roxb. plant extract. However, the chloroform and n-hexane as well as petroleum ether fractions did not exhibit the presence of either compound. Chromatograms depicting the profiles of both the ethyl acetate and hydroalcoholic fraction samples are depicted in Figure 8, contrasting with the chromatograms of the other fractions.

DISCUSSION

The primary objective of this study was to develop and validate a stability-indicating HPTLC method for the simultaneous quantification of triacontane and beta-sitosterol in *Ailanthus excelsa* Roxb. plant extract, in accordance with ICH guidelines. The method optimization was carried out using a Design of Experiments (DoE) approach, specifically employing a

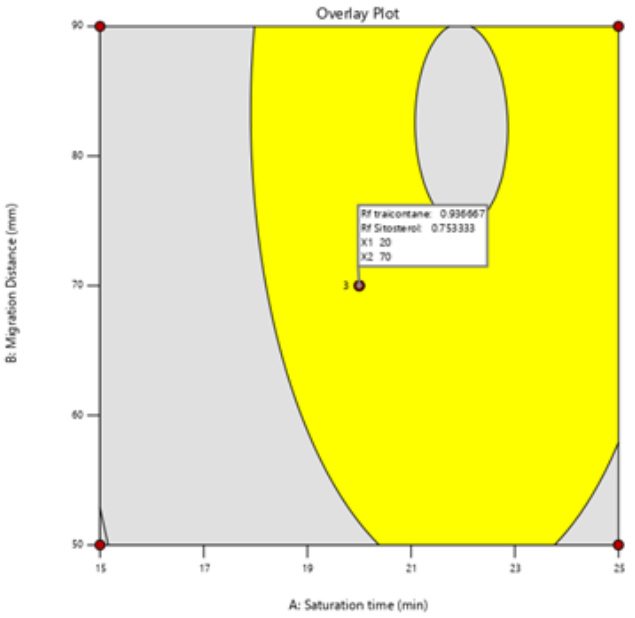

Figure 4: Design space overlay plot.

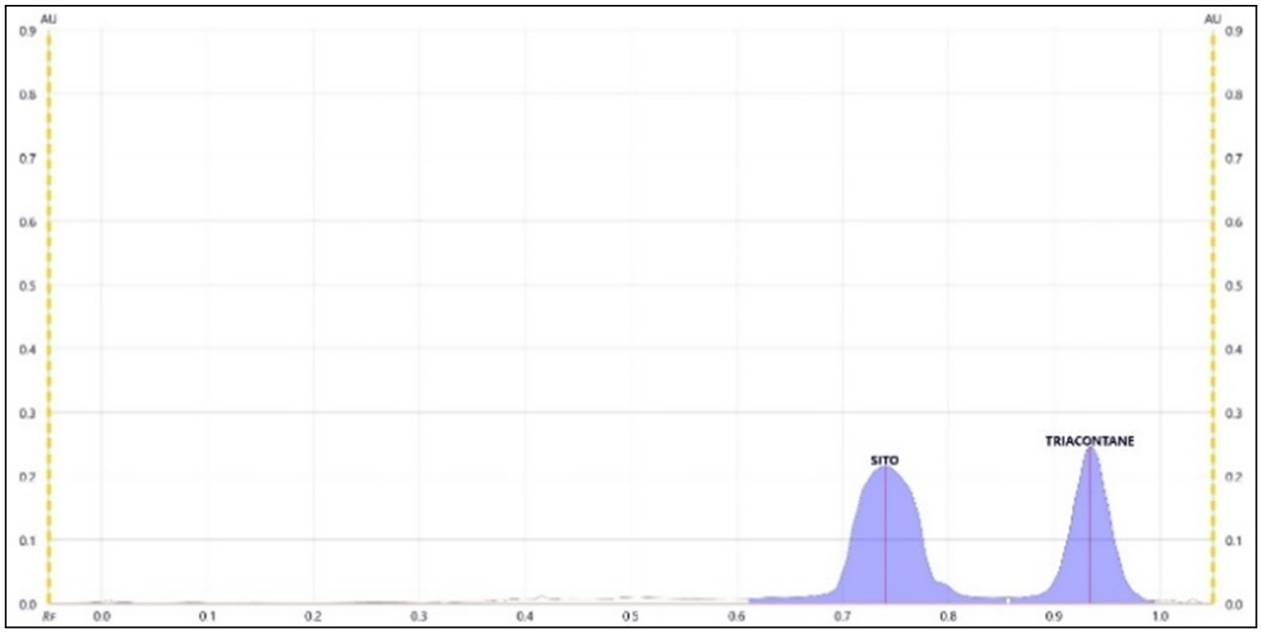

Figure 5: Simultaneous HPTLC Chromatogram of Beta-Sitosterol and Triacontane.

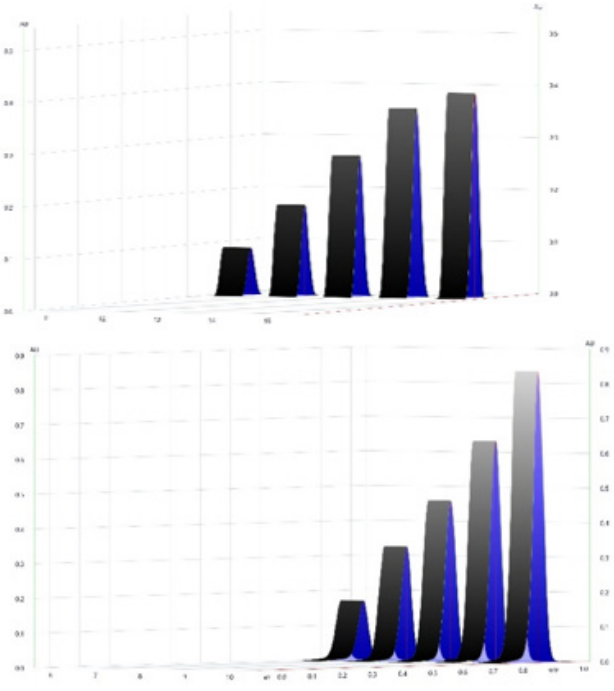

Figure 6: Linearity Curve of Beta-Sitosterol and Triacontane.

Box-Behnken Design (BBD). Subsequently, the method underwent comprehensive validation for various parameters including linearity, accuracy, precision, robustness, solution stability and forced degradation studies, utilizing toluene: ethyl acetate: formic acid (5:5:0.5 V/V) as the mobile phase. Overall, the study demonstrates the successful development of a reliable and efficient analytical method for the quantitative determination of triacontane and beta-sitosterol in herbal medications.

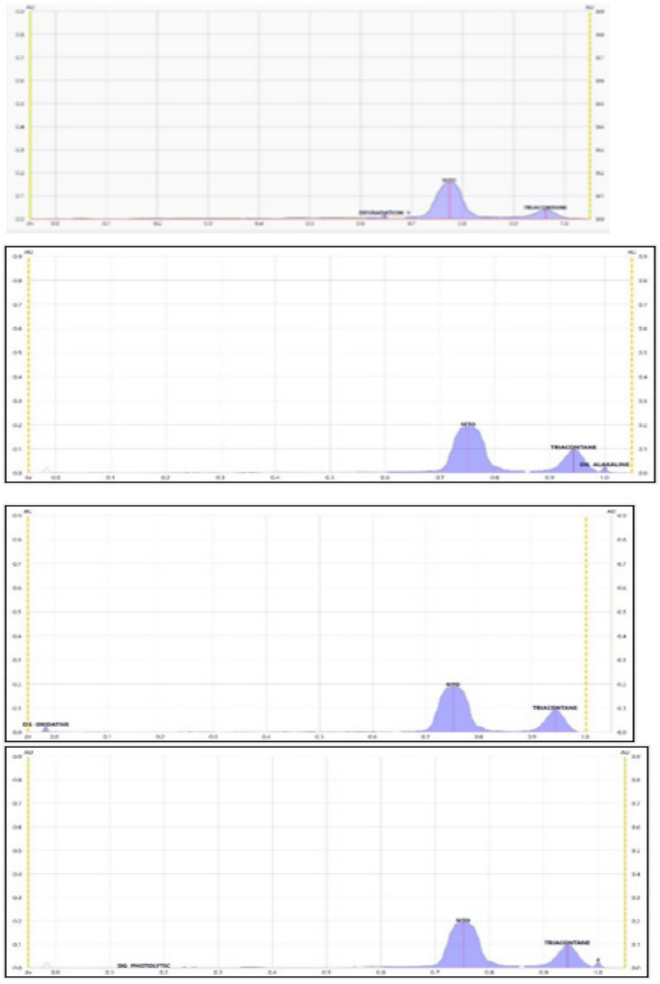

Figure 7: Simultaneous HPTLC Chromatograms of triacontane and beta-sitosterol under different stress conditions (a) Acid, (b) Base, (c) Oxidative and (d) Photolytic conditions.

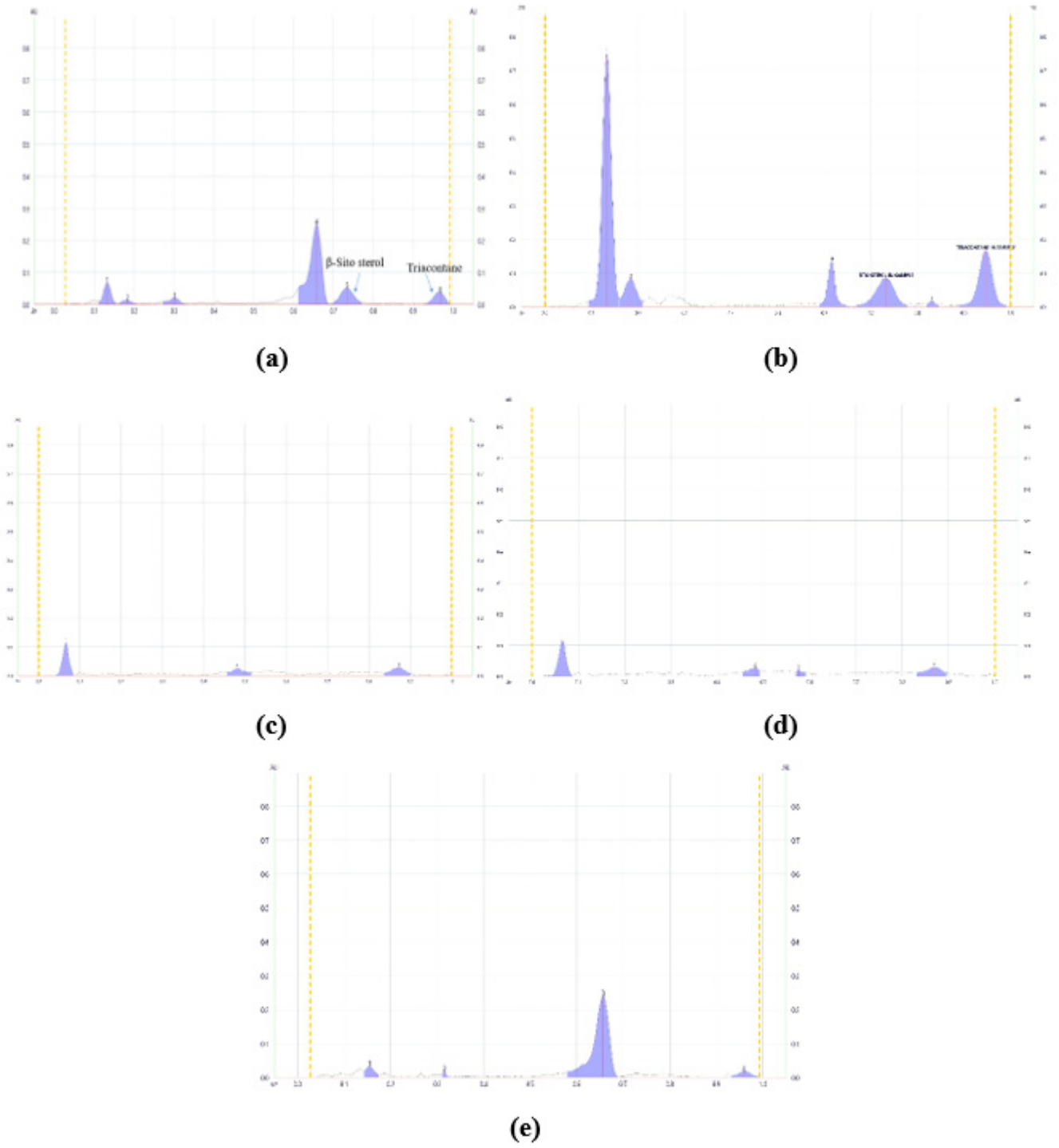

Figure 8: Simultaneous HPTLC chromatograms of Triacontane and Beta- sitosterol in different fractions of *Ailanthus excelsa Roxb* plant extract, (a) ethyl acetate, (b) hydroalcoholic, (c) chloroform, (d) petroleum ether and (e) n-hexane.

The linearity of the method was confirmed over a concentration range of 100-500 ng/band for both compounds, with correlation coefficients exceeding 0.99, indicating excellent linearity. The Limits of Detection (LOD) and Quantification (LOQ) were determined to be suitable for the intended purpose, ensuring the method's sensitivity. Specifically, the LOD and LOQ for triacontane were found to be 8.22 and 24.99 ng/band, respectively, while for beta-sitosterol, the corresponding values were 10.49 and 31.79 ng/bands.

Accuracy assessment through recovery studies at three different levels (50%, 100% and 150%) demonstrated close agreement between the observed and expected values, indicating good accuracy across the specified concentration range. The mean percentage recoveries of triacontane and beta-sitosterol from the

ethyl acetate and hydroalcoholic fractions fell within the range of 99.7% to 100.31%, further affirming the method's accuracy.

Precision, evaluated in terms of intra-day and inter-day variability, yielded %RSD values within acceptable limits (<2%) for both compounds, indicating excellent reproducibility and reliability of the method. Robustness studies indicated minimal variability in chromatographic parameters under slight changes in experimental conditions, further supporting the method's robustness and suitability for routine analysis. Solution stability studies demonstrated the stability of the drug solutions over a 24-hr period, with no significant changes observed in the chromatographic profiles, indicating the method's suitability for practical applications. Forced degradation studies provided valuable insights into the stability of triacontane and beta-sitosterol under various stress conditions, highlighting the method's ability to separate the drugs from their degradation products and confirming its stability-indicating nature.

Moreover, the developed method was successfully applied to estimate triacontane and beta-sitosterol in different fractions of *Ailanthus excelsa* Roxb. plant extract. Chromatographic analysis revealed the presence of both compounds in hydroalcoholic and ethyl acetate fractions, demonstrating the method's specificity and selectivity for the targeted analytes in complex herbal matrices.

In summary, the study demonstrates the effectiveness of the stability-indicating HPTLC method for the quantitative analysis of triacontane and beta-sitosterol in herbal medications. Its compliance with ICH guidelines, coupled with its robustness, accuracy and specificity, positions it as a valuable tool for quality evaluation and standardization of herbal formulations. The application of Quality by Design (QbD) principles in method development underscores its scientific rigor and regulatory compliance, paving the way for its widespread adoption in pharmaceutical quality control laboratories. Ultimately, the method holds significant potential for enhancing the quality, safety and efficacy of herbal medications, thereby contributing to the overall advancement of pharmaceutical product quality and patient care.

CONCLUSION

Consequently, a novel stability-indicating HPTLC approach has been formulated to concurrently determine triacontane and beta-sitosterol in various fractions of *Ailanthus excelsa* Roxb. plant extract, a method not previously documented in literature. Advances in analytical techniques, particularly through the implementation of DoE-based Quality by Design (QbD), are becoming increasingly essential in accordance with ICH recommendations Q8 and Q9. In alignment with regulatory standards, the current HPTLC technique was developed utilizing the Quality by Design methodology. The method underwent rigorous validation and demonstrated specificity, accuracy, precision and robustness in estimating triacontane and beta-sitosterol in *Ailanthus excelsa* Roxb. plant extract. Furthermore, it exhibited well-defined peaks, facilitating precise estimation of both compounds. A forced degradation study revealed the resilience of triacontane and beta-sitosterol to alkaline, oxidative and thermal conditions, with only acidic conditions affecting both compounds. This study highlights the stability-indicating nature of the developed method. The established method holds significant promise for utilization in quality control and analysis of both drugs in various herbal formulations, thereby contributing to the enhancement of pharmaceutical product quality and safety.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DOE: Design of Expert; **TLC:** Thin layer chromatography; **ICH:** International Conference on Harmonization; **ANOVA:** Analysis of variance; **HPTLC:** High Performance Thin Layer Chromatography; **TLC:** Thin Layer Chromatography; **ICH:** International Conference on Harmonisation; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **R_f:** Retention factor; **RSD:** Percent Relative Standard Deviation.

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