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Original Article

QbD-Driven RP-HPLC Method Development and Validation for Trigonelline Quantification in Fenugreek Seeds and Phytosomal Formulation

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ABSTRACT

Background: Trigonelline is alkaloid known for its exceptional biological activities. The aim of the present work was to develop and validate Quality by Design based RP-HPLC method for estimation of trigonelline. **Materials and Methods:** The optimization process involves the design of experiments, utilizing response surface plots, contour plots, and overlay plots. The effective implementation of this method leads to a reduced retention time of 3 min, utilizing a reversed-phase C-18 column (5 mm, 4.6 mm, 250 mm, ZORBAX column). The mobile phase consists of methanol and water (40:60 v/v), with a flow rate of 1 mL/min at 265 nm. **Results:** The analytical technique has shown a linear response at 5-25 µg/mL, with R^2=0.9994. The limits of detection and quantification were determined to be 0.425 µg/mL and 1.288 µg/mL, respectively, highlighting the sensitivity. Both precision assays, robustness and ruggedness demonstrated %RSD less than 2, indicating a reliable method. The accuracy was found to be 98.56% -99.76%, indicating the broad applicability. The established method was successfully employed to estimate the trigonelline content in fenugreek seeds and phytosomes with results showing 0.48% and 86.74%, respectively. **Conclusion:** This technique can be used to estimate trigonelline, for ensuring quality in phytopharmaceuticals.

Keywords: Fenugreek, Optimization, Phytosomes, Quality by Design, Trigonelline.

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INTRODUCTION

Ancient civilizations used herbal remedies for ailments, and their application has increased due to enhanced therapeutic properties, making medicinal plants essential in alternative healthcare systems.[1,2] The herbal drug market has grown significantly, reflecting a global interest in traditional and alternative medical systems and the economic importance of these plants.[3,4] Trigonelline is a naturally occurring alkaloid found in various plant species and has been recognized as a promising therapeutic candidate.[5] It demonstrates a range of therapeutic benefits for various pathological conditions linked to chronic metabolic diseases and age related disorders. Its diverse impacts include neuroprotection from neurodegenerative disorders and diabetic peripheral neuropathy, neuromodulation, reduction of cardiovascular diseases, treatment of skin disorders, management of diabetes mellitus, and protection against liver and kidney injuries, along with antipathogen and antitumor activities.[6] The therapeutic claims associated with phytochemicals are restricted because of their lower solubility and metabolic limitations. These issues can be resolved by adopting a targeted nanocarrier system.[7] The application of nanotechnology in drug delivery systems provides a feasible strategy to tackle these issues while improving the properties and biological functions of phytochemicals. By employing nano drug delivery systems, it is possible to increase the solubility, bioavailability, and pharmacokinetics of these substances.[8] The therapeutic action of trigonelline is influenced by critical factors, including a moderate absorption rate, rapid elimination, low bioavailability, and poor stability. These challenges can be effectively managed through the application of innovative drug delivery system solutions.[9] Recently, multiple advanced drug delivery systems have been introduced, designed to provide more convenient, controlled, and targeted methods of delivering pharmaceuticals.[10,11] Phytosomes are innovative vesicular structures akin to liposomes, designed to encapsulate active phytoconstituents within a lipid core. This lipid core

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significantly enhances the permeability of these structures across biological barriers, thereby augmenting the bioavailability and effectiveness of the enclosed plant materials.[12,13] A significant issue for researchers is the need to develop a credible and appropriate analytical methodology for evaluating the quality of new drug delivery systems. This underscores the need for a simple, rapid, precise, and efficient analytical technique.[14] The exact quantification of drugs embedded within the nanoformulation can establish a connection to the expected therapeutic benefits.[15,16] Analytical Quality by Design (AQbD) has become a fundamental framework in analytical chemistry, transforming the methodologies employed in method development and validation. It is extensively applied in the pharmaceutical sector as a component of risk management throughout the method development process.[17] AQbD is implemented to confirm that an analytical procedure is suitable for its designated purpose throughout its entire lifecycle, leading to a method that is comprehensively understood and purpose-driven. It governs each stage of the analytical process lifecycle by defining the Analytical Target Profile (ATP), recognizing Critical Method Parameters (CMPs), and choosing Critical Method Attributes (CMAs).[18] In summary, it offers a model for a highly effective and cost-conscious strategy.[19] The previous two decades have seen a marked enhancement in the use of Quality by Design principles in the pharmaceutical field, a change significantly driven by the efforts of the FDA and ICH.[20] Based on our thorough literature review, it is evident that only a few RP-HPLC methods have been reported for the estimation of trigonelline. The existing literature presents several limitations, including low sensitivity, use of complicated mobile phases, high costs, and limited applicability. Additionally, the application of quality by design principles to RP-HPLC methods for trigonelline analysis remains unexplored. There is also a significant need for an analytical method to estimate trigonelline in nanoformulations. In this context, a modest attempt has been made to develop and validate a quality by design-assisted RP-HPLC method for estimating trigonelline in fenugreek seeds and phytosomes.

MATERIALS AND METHODS

Chemicals and reagents

Analytical grade trigonelline was procured from Yucca enterprises, Mumbai, India. The authentic seeds of Fenugreek (*Trigonella foenum-graecum* L.) were obtained from Shri BM Kankanawadi Ayurveda Mahavidyalaya, Postgraduate Studies and Research Centre, Belagavi, Karnataka, India. All chemicals, including methanol (HPLC grade), are of analytical grade and are obtained from KLE College of Pharmacy, KAHER, Belagavi, Karnataka, India.

Instrumentation

The chromatographic analysis was performed using the Agilent 1220 Infinity II LC system, which includes a system controller, a low-pressure gradient pump, a solvent delivery module, an online degasser, and a manual sample injector with injection volumes 5-20 µL. Agilent OpenLab software was utilized for instrument control and data processing. A reversed-phase C-18 column (5 mm, 4.6 mm, 250 mm, ZORBAX) was utilized for the chromatographic separation. The system was operated at a flow rate of 1 mL/min, with a detection wavelength set at 265 nm. The mobile phase composed of methanol and water in a ratio of 40:60 (v/v). The total duration of the analysis was 5 min.

Method Development and Validation

Preparation of standard solution

For the preparation of standard solution, accurate 10 mg of trigonelline was dissolved in methanol and series of dilutions were prepared (5-25 µg/mL).

Selection of wavelength

The analysis involved the use of a UV spectrophotometer to scan trigonelline at 400-200 nm range, with the maximum absorbance detected at 265 nm.

Quality by Design approach

Selection of quality target product profile

The Quality Target Product Profile (QTPP) is crucial for determining the variables that influence its parameters. In the proposed method, retention time and theoretical plates have been recognized as key QTPP elements.[21-25]

Determine critical quality attributes

The Critical Quality Attributes (CQAs) represent the method parameters that have a direct impact on the Quality Target Product Profile (QTPP). Among these, the composition of the mobile phase and the flow rate are two essential parameters that must be regulated to ensure the QTPP remains within an acceptable response range.

Factorial design and method optimization using Design of Experiments (DoE)

After the identification of the Quality Target Product Profile (QTPP) and Critical Quality Attributes (CQAs), a central composite experimental design was implemented to optimize and select two fundamental components: the mobile phase and the flow rate in the HPLC method. The study focused on examining the various interaction effects and quadratic effects of the mobile phase composition and flow rate on retention time and theoretical plates, utilizing a central composite statistical screening design. A 3^2 full factorial design with two factors, each at two levels, was utilized to ascertain the optimized HPLC method, which involved conducting nine experimental runs. The experimental design was carried using Design-Expert software version 13.0, by Stat-Ease Inc. in Minneapolis, MN, USA.

Method Validation

The optimized run for estimating trigonelline has been validated in compliance with ICH Q2 (R1) guidelines, evaluating parameters including system suitability, specificity, precision, accuracy, linearity, range, Limit of Detection (LOD), Limit of Quantification (LOQ), and robustness.[26-30]

System Suitability

The system suitability was conducted as a method to ensure the dependability of the validation outcomes and to observe any potential variations arising from alterations in the chromatographic conditions. This test involved five runs using the same standard solution of trigonelline at a specified concentration. Key parameters such as peak area, retention time, theoretical plate, and tailing factor were monitored.

Specificity and Selectivity

Specificity denotes the ability to accurately and precisely detect the analyte in the presence of other components that may be found within the sample matrix. It acts as a measure of the degree of interference from various elements, such as other active pharmaceutical ingredients, excipients, impurities, and degradation products. This ensures that the detected peak response is exclusively linked to a single component, thus confirming the absence of coelutions. The specificity and selectivity of the method developed were demonstrated by comparing a blank run with a sample run, utilizing a standard solution of trigonelline at a concentration of 5 µg/mL for assessment.

Linearity and Range

Linearity refers to the capability of an analytical method to produce results that are directly proportional to the concentration of the analyte within a designated range. This was determined by assessing the peak area in relation to the analyte concentrations. The method's range defines the limits between the lowest and highest concentrations of the analyte at which precision, accuracy, and linearity are maintained. A calibration curve was developed at concentrations of 5 to 25 µg/mL, and linearity was established through linear regression analysis.

Precision

The concept of precision is defined as the degree to which a set of measurements is consistent or varies when derived from repeated sampling of a standardized sample under controlled conditions. The method's precision was investigated through two key aspects: repeatability (intra-day) and intermediate precision (inter-day). Repeatability was established by injecting six replicates of the sample solutions on the same day, while intermediate precision was assessed by analysing a set of sample solutions on different days.

Accuracy

The accuracy of an analytical technique is determined by how closely the obtained measurements match the true values. An accuracy evaluation was carried out by assessing absolute recovery at three specified levels-80%, 100%, and 120% of the mid-point concentration of the calibration curve-utilizing triplicate sample injections.

Sensitivity (LOD and LOQ)

The Limit of Detection (LOD) is defined as the smallest quantity of an analyte present in a sample that can be identified, although it may not be quantified with precision. Conversely, the Limit of Quantification (LOQ) represents the minimum concentration of an analyte that can be measured with a level of precision and accuracy. The LOQ is especially critical in quantitative analyses concerning impurities or degradation products. The values for LOD and LOQ were determined in accordance with ICH guidelines using standard formulas:

$$LOD=3.3\ \sigma/S$$

$$LOQ=10\ \sigma/S$$

Where, σ = standard deviation of the response and S = slope of calibration curve.

Robustness

The robustness of an analytical procedure serves as an indicator of its ability to remain stable despite minor, intentional alterations in method parameters, thereby reflecting its reliability under standard operating conditions. The robustness of the method was assessed through a limited range of conditions, which included variations in wavelength, flow rate and the composition of the mobile phase.

Ruggedness

The ruggedness of a method is indicated by the reproducibility of test results when analysing the same samples in varying circumstances, including different laboratories, analysts, or instruments. The ruggedness of the method was analysed by change in analysts.

Preparation of phytosomes

Phytosomes were formulated via the thin film hydration technique, utilizing soya lecithin and cholesterol. An accurately weighed quantity of both soya lecithin (25 mg) and cholesterol (50 mg) was dissolved in chloroform and evaporated with a rotary evaporator at 180 RPM. Once the evaporation was complete, the flask was refrigerated for 24 hr. The rehydration process involved the addition of water and alcohol, along with a precisely weighed amount of trigonelline (10 mg). The prepared phytosomes

were then sonicated and stored in an amber glass container for subsequent use.[31-33]

Application of developed RP-HPLC method

Estimation of trigonelline in fenugreek seeds

Accurately weighed quantity of dried fenugreek seeds are converted into coarse powder. The resultant powder was dissolved in methanol and subjected for sonication. Further dilutions were made (10 µg/mL), filtered through membrane filter and analysed using developed method.[34]

Estimation of trigonelline in phytosome

An accurately measured quantity of the phytosome sample was dissolved in methanol and then subjected to sonication. Further, same methodology was used for estimation of trigonelline in phytosomes as described for fenugreek seeds.

Comparison with previously published literatures

A detailed literature survey has been executed to analyze the available reports on HPLC methods for trigonelline. Based on the data collected, a comparative analysis has been made.[35-40]

RESULTS

This study has successfully developed a Quality by Design oriented RP-HPLC technique for the analysis of trigonelline in fenugreek seeds and phytosomes using methanol and water (40:60 v/v) and detection wavelength at 265 nm.

Quality by Design approach

Selection of quality target product profile and critical quality attributes

In the proposed method, retention time and theoretical plates have been recognized as key QTPP elements and composition of the mobile phase and the flow rate are two essential parameters that must be regulated to ensure the QTPP remains within an acceptable response range.

Factorial design and method optimization using Design of Experiments (DoE)

Central composite experimental design was implemented to optimize the method. A 3^2 full factorial design with two factors, each at two levels, was utilized to ascertain the optimized HPLC method, which involved conducting nine experimental runs. In the present research work, mobile phase composition and flow rate were selected as critical quality attributes whereas retention time and theoretical plates were regarded as critical analytical attribute. Table 1 summarizes the factorial design for the optimization of method.

Optimization

In this study, a response surface methodology was adopted, featuring a Central Composite Design (CCD) and a quadratic design model with a total of nine runs. The proposed CCD experimental design was applied, which enabled the analysis of mobile phase composition and flow rate against two key responses: retention time and theoretical plates. Tables 2A and 2B represents predicted combination factors as per central composite design for response 1 (Retention time) and response 2 (Theoretical plates) respectively. Based on the results, both the responses revealed significant model for the analysis of trigonelline. Figure 1 represent design space for optimized run by HPLC method. Based on the results, mobile phase composition of methanol and water (40:60 v/v) with flow rate of 1 mL/min was optimized by design of expert approach.

Method validation

The proposed approach was extensively validated in accordance with the ICH Q2 (R1) guidelines, for system suitability, specificity and selectivity, linearity, precision, accuracy, sensitivity (LOD and LOQ), along with robustness and ruggedness.

System Suitability

The parameters such as peak area (69,727), retention time (3.093 min), tailing factor (1.48), and theoretical plates (4565), are all

Table 1: Factorial design.

Sl. No.	Std	Run	Factor 1: Mobile phase (Methanol)	Factor 2: Flow Rate (mL/min)	Response 1: Retention time (Min)	Response 2: Plate count
1.	2	1	40	0.9	3.437	4557
2.	8	2	35	0.9	3.43	4686
3.	4	3	45	0.9	3.447	4838
4.	9	4	45	0.8	3.87	4203
5.	6	5	40	0.8	4.37	4182
6.	3	6	40	1	3.087	4892
7.	7	7	35	1	3.073	4810
8.	5	8	45	1	3.097	4834
9.	1	9	35	0.8	3.87	4201

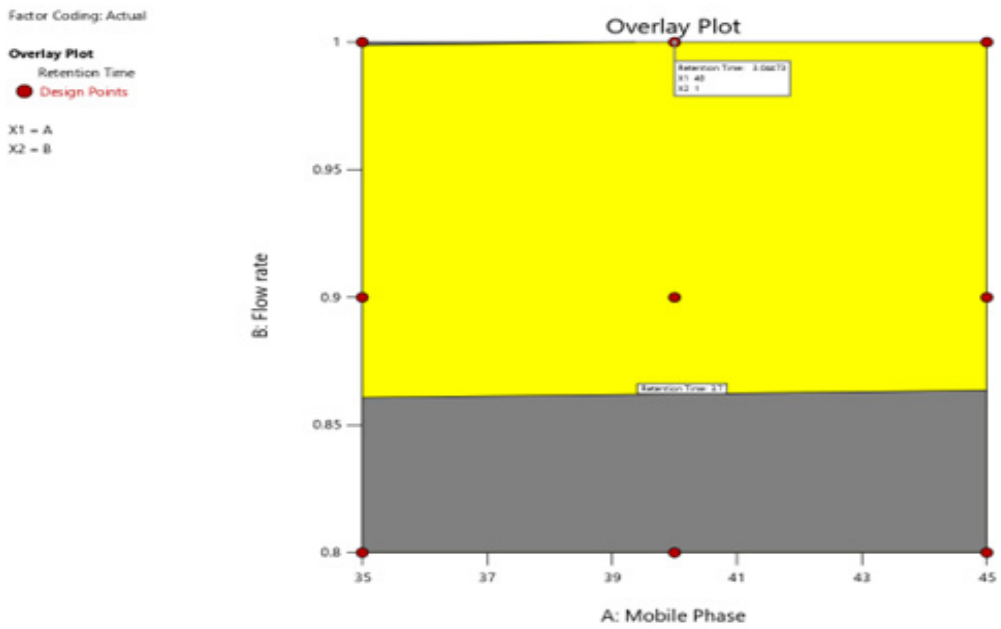

Figure 1: Design space-overlay plot.

within acceptable thresholds. The system suitability test plays a vital role in method development, as it validates the repeatability and resolution of the proposed HPLC method.

Specificity and Selectivity

The specificity and selectivity of the developed RP-HPLC method were evaluated by chromatogram of standard trigonelline. The results confirmed the method's specificity, as the chromatogram for standard exhibited a clear correspondence with the peak observed in UV spectrum of standard trigonelline with highest absorbance at 265 nm. Figure 2 represent HPLC chromatogram of trigonelline at retention time of 3.093 min with flow rate of 1 mL/min at 265 nm.

Linearity

The linear relationship between peak areas and corresponding concentrations is essential for analyzing the linearity of the developed method. The results from the regression analysis reveal that the proposed method shows linearity within a concentration range of 5-25 µg/mL at 265 nm, with a Correlation Coefficient (R^2) of over 0.9994, indicating a robust linearity. The results pertaining to linearity and regression are detailed in Table 3 and Figure 3.

Precision

The precision of an analytical method is indicated by the degree of consistency observed among a series of measurements obtained from several samplings of the same sample under comparable analytical conditions. In both precision assessments, the percent RSD values were below 2%, satisfying the acceptance criteria and showcasing the method's high precision. Table 4 represents results of intraday and interday analysis of trigonelline.

Accuracy

The degree of similarity between the experimental value and the true or reference value reflects the precision of the analytical procedure. Accuracy and precision are closely linked to the method's repeatability, which was determined through recovery trials. A pre-analyzed sample was supplemented with known quantities of trigonelline at different concentrations (80%, 100%, and 120%). The recovery rates for the three samples tested, which ranged from 98.56% to 99.76 %, clearly demonstrate that the method is considerably more accurate and appropriate for large-scale analysis.

Sensitivity

The Limits of Detection (LOD) and Quantification (LOQ) are significant parameters that reflect the sensitivity of the analytical method utilized. According to the analysis, the LOD and LOQ for trigonelline were found to be 0.425 µg/mL and 1.288 µg/mL, respectively, illustrating the high sensitivity of the proposed method. A lower LOD suggests the method's ability to detect minute concentrations of the analyte, which is essential for its application in pharmaceuticals.

Robustness and Ruggedness

Robustness implies that small changes in experimental conditions that do not significantly alter the results, while ruggedness signifies the method's consistency across various analysts. In this study, robustness was carried out by making slight changes in wavelength, mobile phase composition, and flow rate whereas for ruggedness, analyst is considered and results revealed to be % RSD below 2, thereby confirming robust and rugged characteristics of method. The results for robustness and ruggedness are detailed in Tables 5 and 6 respectively.

Table 2: **Predicted combination factors as per central composite design.**

A: Response 1 (Retention time).

Source	Sum of squares	d_f	Mean square	F- value	*p*-value	
Model	1.36	2	0.6784	20.65	0.0020	Significant
A-Mobile phase	0.0003	1	0.0003	0.0085	0.9294	
B- Flow rate	1.36	1	1.36	41.28	0.0007	
Residual	0.1972	6	0.0329			
Cor Total	1.55	8				

B: Response 2 (Theoretical plate).

Source	Sum of squares	d_f	Mean square	F- value	*p*-value	
Model	6.390E+05	2	3.195E+05	19.52	0.0024	Significant
A-Mobile phase	5280.67	1	5280.67	0.3226	0.5906	
B- Flow rate	6.338E+05	1	6.338E+05	38.72	0.0008	
Residual	98200.22	6	16366.70			
Cor Total	7.372E+05	8				

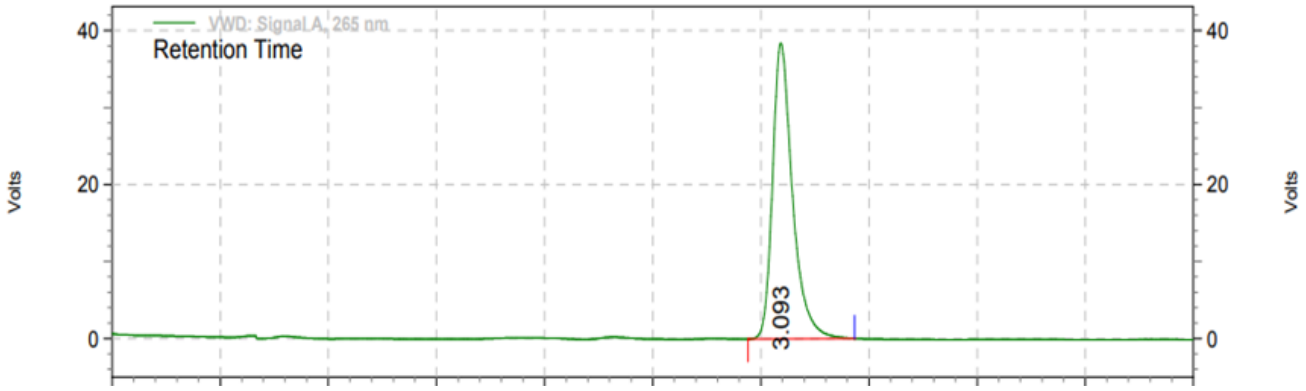

Figure 2: Chromatogram of standard trigonelline.

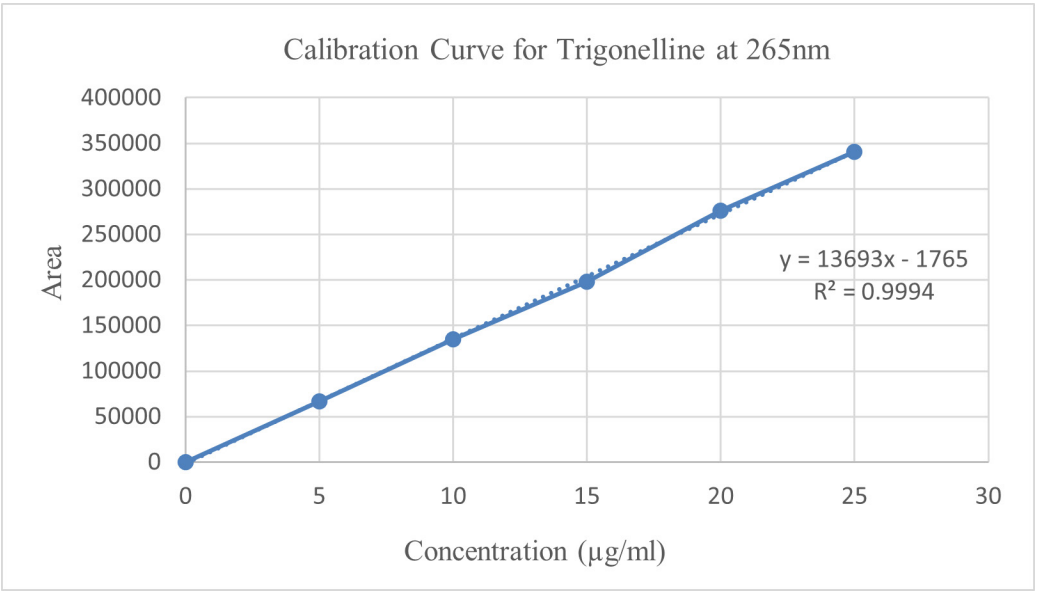

Figure 3: Calibration curve of trigonelline.

Application of developed RP-HPLC method

Estimation of trigonelline in fenugreek seeds

The trigonelline content in fenugreek seeds were estimated by developed method. The results revealed the concentration of trigonelline as 0.48% in fenugreek sample. Figure 4A represents chromatogram of trigonelline in fenugreek seeds.

Estimation of trigonelline in phytosome

Phytosomes were successfully prepared and trigonelline content was estimated using developed method. The trigonelline content was found to be 86.74%. The resulting phytosomes displayed a particle size of 146 nm, a polydispersity index of 0.3, and a zeta potential of -45.42, suggesting that they possess excellent characteristics. Figure 4B represents chromatogram of trigonelline in phytosomes.

Table 3: Linearity for trigonelline analysis.

Sl. No.	Concentration (µg/mL)	Area
1.	5	66349
2.	10	135127
3.	15	197982
4.	20	276129
5.	25	340832
R^2		0.9994
Regression equation		y = 13693x - 1765
LOD (µg/mL)		0.425
LOQ (µg/mL)		1.288

Comparison with previously published literatures

A detailed literature survey has been executed to analyze the available reports on HPLC methods for trigonelline. Based on the data collected, a comparative analysis has been conducted focusing on mobile phase composition, retention time, the columns employed, flow rate, applications, and their limitations. The results of this comparison are summarized in Table 7.

DISCUSSION

A Quality by Design (QbD) oriented RP-HPLC approach was successfully developed for the analysis of trigonelline in fenugreek seeds and phytosomes. The optimized method utilized a mobile phase of methanol and water in a 40:60 v/v ratio, with a flow rate of 1 mL/min and detection wavelength set at 265 nm. In this study, the mobile phase composition and flow rate were determined to be critical quality attributes, whereas retention time and theoretical plates were considered critical analytical attributes. These parameters were optimized through a 3^2 factorial design utilizing a central composite design approach. The validation of the method, in accordance with ICH Q2 (R1) guidelines, confirmed its system suitability, specificity, and selectivity, with trigonelline being distinctly identified at

Table 4: Precision results trigonelline.

Intraday Precision			
Concentration (µg/mL)	1st hr	4th hr	8th hr
	%RSD		
5	0.70	0.80	0.78
15	0.29	0.12	0.12
25	1.23	0.48	1.11
Interday Precision			
Concentration (µg/mL)	Day 1	Day 2	Day 3
	%RSD		
5	1.20	1.54	1.24
15	0.44	0.34	0.78
25	1.31	0.33	1.05

Table 5: Robustness results of Trigonelline.

Trigonelline	Change in Wavelength (nm)			Change in mobile phase (Percent)			Change in flow rate (mL/min)		
	263	265	267	(42:58)	(40:60)	(38:62)	0.9	1	1.1
Conc (µg/mL)	%RSD								
5	0.41	0.94	0.82	0.45	0.71	0.70	0.67	0.81	0.48
15	0.39	0.35	1.52	0.53	0.22	0.85	0.76	0.19	0.63
25	1.08	0.38	1.69	0.89	1.03	1.21	0.93	0.41	0.81

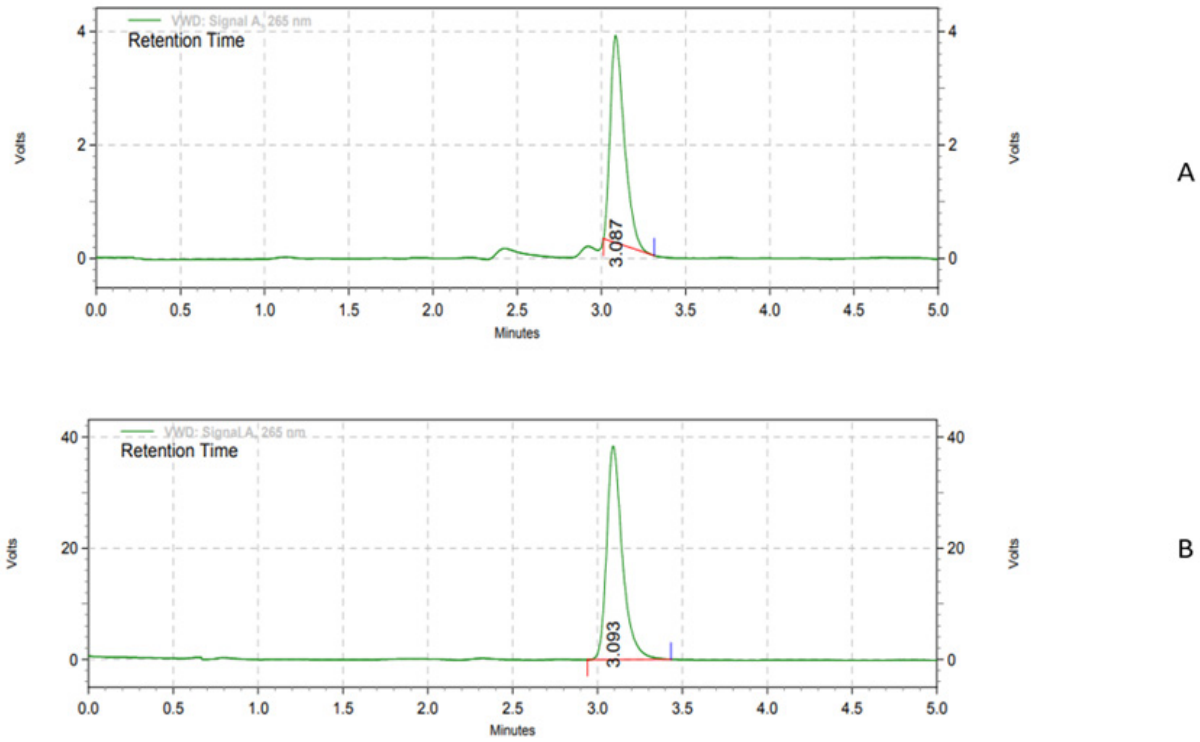

Figure 4: Estimation of trigonelline in (A) Fenugreek seeds and (B) Phytosomes.

Table 6: Ruggedness results of Trigonelline.

Trigonelline	Change in Analyst		Change in instrument	
	1	2	1	2
Conc (µg/mL)	%RSD			
5	0.85	1.20	0.86	1.22
15	0.99	1.79	1.06	0.97
25	1.55	1.58	1.41	1.18

Table 7: Comparison with previously published HPLC methods for trigonelline analysis.

Mobile phase	Column	Flow rate mL/min	Detection wavelength (nm)	Retention time (min)	Limitation
Water (adjusted to pH 3.5 with HCl): Methanol (70:30, v/v).[35]	Phenomenex Luna column (250 mm x 4.6 mm, 5 µm)	0.5	264	5.317	Limited applications
20 mM ammonium acetate buffer (pH 4.5) and methanol.[36]	GraceSmart RP C18 (150 mm x 4.6 mm, 5 µm) column	1.0	265	2.56	Less sensitive, complex mobile phase
Water (0.01% HCl): Methanol (70:30 v/v).[37]	ZORBAX C18 (250 mm x 4.6 mm, 5 µ) column	1.0	263	2.877	Limited applications
Phosphate buffer (pH 4.0): Methanol.[38]	Spherisorb ODs2 (5 pm, particle size; 25.0 x 0.46 cm)	1.0	268	3.34	Complex mobile phase, limited applications
Acetonitrile-Water (90:10, v/v).[39]	TC-C18 column	1.0	210	6.530	Expensive method, Lack of validation
Methanol: distilled water (95:5, v/v), adjusted to pH 3.5 with hydrochloric acid.[40]	Cosmosil CN-MS (250 mm × 4.6 mm) column	1.0	267	6.48	Less sensitive

3.093 min. The reliability of the method was affirmed through linearity (R^2>0.9994), precision, and accuracy, which ranged from 98.56% to 99.76% recovery. Low Limits of Detection (LOD) at 0.425 µg/mL and Limits of Quantification (LOQ) at 1.288 µg/mL underscored the method's sensitivity. Furthermore, robustness and ruggedness evaluations indicated stability, as evidenced by %RSD of less than 2%. The approach effectively quantified trigonelline content in fenugreek seeds (0.48%) and in phytosomes (86.74%), demonstrating superior sensitivity, retention time, and analytical accuracy compared to previous methods. A detailed comparison with existing literature has revealed that many previously developed HPLC techniques are subject to considerable limitations, such as the usage of complex or costly mobile phases, lack of QbD integration, limited validation parameters, and extended retention times, which restrict their routine applicability. In contrast, this study offers a simplified and cost-effective method that complies with regulatory expectations through its QbD-focused development. As a result, this study provides a methodologically enhanced and practically applicable option for trigonelline quantification, bridging an important gap in existing analytical frameworks.

CONCLUSION

In the present work, our research investigation achieved the successful development of a Quality by Design (QbD) focused RP-HPLC method for the quantification of trigonelline in fenugreek seeds and phytosomes. The mobile phase composed of methanol and water in a 40:60 (v/v) ratio, with a detection wavelength of 265 nm. The method underwent optimization via a factorial design approach. Key parameters, including retention time and theoretical plates, were determined to be critical quality attributes and were optimized using a central composite design. The method was validated in line with ICH guidelines, ensuring its accuracy, precision, specificity, and sensitivity. System suitability tests affirmed the method's reliability, while robustness and ruggedness evaluations demonstrated its stability across various conditions. This method was successfully implemented to estimate trigonelline content in fenugreek seeds and phytosomes, yielding consistent and reliable results. A comparison with the literature currently available emphasized the effectiveness of the newly developed method, highlighting its merits in terms of accuracy, efficiency, and reproducibility. Ultimately, this investigation delivers a validated and optimized HPLC method for the precise quantification of trigonelline, which holds potential for future applications in the pharmaceutical and nutraceutical fields. Further, this investigation can be strengthened by applying the technique to other herbal matrices, carrying out pharmacokinetic research, and stability-indicating study to promote wider pharmaceutical and regulatory applications.

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ABBREVIATIONS

QbD: Quality by Design; **RP-HPLC:** Reverse Phase High Performance Liquid Chromatography; **mm:** Millimetre; **v/v:** Volume by Volume; **nm:** Nanometre; **µg:** Microgram; **RSD:** Relative Standard Deviation; **AQbD:** Analytical Quality by Design; **ATP:** Analytical Target Profile; **CMP:** Critical Method Parameters; **CMA:** Critical Method Attributes; **FDA:** Food and Drug Administration; **ICH:** International Council for Harmonisation; **UV:** Ultraviolet; **QTPP:** Quality Target Product Profile; **DoE:** Design of experiments; **LOD:** Limit of detection; **LOQ:** Limit of quantification; **RPM:** Revolutions per minute; **CCD:** Central Composite Design.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

This study successfully developed and validated a Quality by Design (QbD) based RP-HPLC method for quantifying trigonelline in fenugreek seeds and phytosomes. The method utilized methanol and water (40:60 v/v) as the mobile phase with a detection wavelength of 265 nm. Mobile phase composition and flow rate were determined to be critical quality attributes, whereas retention time and theoretical plates were considered critical analytical attributes. The method was optimized using a central composite design. The optimized method was validated in compliance with ICH Q2 (R1) guidelines for system suitability, specificity, precision, accuracy, sensitivity, robustness, and ruggedness. The developed method efficiently quantified trigonelline content in fenugreek seeds (0.48%) and phytosomes (86.74%). A comparison with previous methods highlighted its improved accuracy, efficiency, and reproducibility, making it suitable for pharmaceutical and nutraceutical applications.

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