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Original Article

Development and Validation of Novel Separation Technique for Syringic Acid and Chlorogenic Acid using AQbD Approach

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ABSTRACT

Background: Syringic Acid and Chlorogenic Acid being natural phytochemicals**,** there is a need to ensure the quality and purity of these phytoconstituents which has become increasingly crucial nowadays. **Objectives:** Thus, the study is designed to employ AQbD approach along with RP-HPLC to develop a marker-based analytical method via green separation technique. **Materials and Methods:** A DOE assisted RP-HPLC method for Syringic acid and Chlorogenic acid. The optimized mobile phase conditions were methanol:0.1% ortho phosphoric acid (60:40) and methanol:0.1% Triethylamine with flow rates of 1 mL/min and 0.5 mL/min, achieving retention time of 3.54 min and 3.32 min, using Chemsil column. **Results:** The newly developed method exhibited good linearity (R^2 of 0.999) over a concentration of 2-10 µg/mL, with system suitability and precision meeting acceptable criteria. The Robustness and Ruggedness assessments demonstrated minimal variations in mobile phase flow and its composition. The limit of detection and limit of quantitation parameters were determined to be 0.51 µg/mL and 1.57 µg/mL for syringic acid and 0.17 µg/mL and 0.52 µg/mL for chlorogenic acid, respectively, adhering to ICH guidelines. **Conclusion:** The AQbD approach, using Design Expert 13.0, was employed for a more precise and accurate eco-friendly separation method for analysing Syringic Acid (SA) and Chlorogenic Acid (CA). The optimization of the experimental design was validated using ANOVA-based statistical analysis, which ensures that the proposed method is simple, sensitive, and highly robust which can be employed for future applications.

Keywords: Analytical Quality by design, Chlorogenic acid, Correlation coefficient, Reverse Phase-High-Performance Liquid Chromatography, Syringic acid, Triethylamine.

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INTRODUCTION

Polyphenolic compounds are a diverse class of secondary metabolites obtained from plants which are known for their diverse medicinal properties. Their remarkable bioactivity has piqued significant interest in food science and pharmaceutical research. However, the precise quantification of these compounds necessitates sophisticated and reliable analytical methodologies to uphold accuracy, consistency, and reproducibility.[1–5]

In Isocratic HPLC study, a liquid mobile phase is propelled under high pressure, driving the analyte through a stationary phase-packed column. This process fosters selective interactions, resulting in distinct elution rates and retention times that serve as unique identifiers.[6] Using high-pressure pumps, HPLC separates compounds based on their varying affinities to the adsorbent material. The factors for separation include mobile phase parameters like polarity, flow rate, pH, and composition, as well as sample matrix and stationary phase which validates the method under ICH guidelines.[7–12]

AQbD methodology advocates for assessing analytical process quality during its development phase rather than solely relying on testing final results. AQbD entails a systematic approach to development, commencing with established goals and prioritizing a comprehensive understanding of both product and process through scientific principles and effective risk management strategies.[13,14]

Analytical Quality by Design (AQbD) defines the Analytical Target Profile and Critical Method Attributes (CMA) like retention time and peak area. Using the Design of Experiments, it is possible to establish the Method Operable Design Region for consistent analytical performance.[15–17]

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Several RP-HPLC methods have been reported for the determination of syringic acid and chlorogenic acid in plant extracts, herbal formulations, coffee, wine, biological samples, and selected pharmaceutical formulations.[1,3,4,8,10,16,20] Although these methods have shown satisfactory analytical performance, they were developed under chromatographic conditions specific to individual analytes and sample matrices, limiting their broader applicability. Furthermore, most reported methods were optimized using conventional trial-and-error approaches rather than systematic optimization.[11,12] The Analytical Quality by Design (AQbD) approach offers a risk-based strategy for developing robust and reliable analytical methods through the systematic evaluation of critical method parameters.[7,12–15] However, reports on the application of AQbD for the RP-HPLC analysis of syringic acid and chlorogenic acid in pharmaceutical formulations remain limited. Therefore, the present study aimed to develop and validate two independent AQbD-based RP-HPLC methods for the determination of syringic acid and chlorogenic acid in accordance with ICH Q2 (R^1) guidelines.[21]

This study fortifies analytical method reliability while mitigating variability through the strategic application of the AQbD approach, optimizing and validating the method to ensure superior precision, and robustness. This is accomplished through the integration of rigorous statistical and experimental design principles.[18–20]

MATERIALS AND METHODS

Materials

Chemicals

SA 98% and CA 99% were procured [Sigma-Aldrich Chemicals Private Limited Bangalore, India]. Methanol (HPLC grade), (LR grade) Orthophosphoric acid and Triethylamine were obtained [Merck Mumbai Pvt. Ltd.].

Instrument

The chromatographic method was designed using the Waters Alliance 2695 separation module, outfitted with a PDA detector and Chemsil ODS-C18 Column (4.6 mm×250 mm, 5 µ).

Ethical statement

As this study does not involve any animals or humans, no ethical committee permission was obtained.

METHODS

Chromatographic conditions

Chromatographic analysis of SA used a Chemsil ODS-C18 (4.6× 250 mm, 5 µ) column with methanol:[0.1%] Orthophosphoric acid [60:40 v/v], flow rate [1.0 mL/min], injection quantity [10 µL], detection at 220 nm; CA analysis used the same column with methanol:0.1% TEA in water [80:20 v/v], flow rate [0.5 mL/min], injection quantity10 µL, detection at 320 nm.

Preparation of 0.1% orthophosphoric acid

OPA (1.0 mL) was pipetted out in 1000 mL Mili-pore water followed by sonication then filtered using a membrane filter.

Preparation of 0.1% Triethylamine

TEA (1.0 mL) was pipetted out in 1000 mL Mili-pore water, sonicated, and filtered through a membrane filter.

Sample preparation

A standard stock was prepared by weighing 10 mg SA and CA each, dissolved in 10 mL of methanol, and then the sub-stock of concentration 100 µg/mL was prepared and filtered using a membrane filter, continued with the preparation of (2-10 µg/mL).

Selection of wavelength

SA and CA at 10 µg/mL were scanned for the 200-400 nm range, and the maximum wavelengths of 220nm and 320nm was selected as detection wavelengths.

Identification ATP and CQP

The ATP outlines measures detailing what needs to be assessed and the performance criteria of the method. Critical Quality Parameters (CQP) are those defining product qualities crucial to maintaining within specified limits for achieving satisfactory shelf life. In the CQP for analytical procedures, both method properties and parameters are encompassed.

Risk assessment

The quality risk management with pharmaceutical quality systems is incorporated into ICH guidelines [Q9 and Q10]. Risk assessment under ICH Q9 provides a systematic approach to identify, control, and monitor quality risks throughout the product lifecycle. Key attributes influencing analytical methods are identified, incorporated into method design, and assessed for their impact on method performance.

Method optimization by DOE

Design of Experiments (DoE) establishes mathematical relationships between dependent and independent variables using regression analysis. In this study, a Central Composite Design (CCD) with 11 runs optimized analytical conditions at two levels, improving efficiency with fewer trials. Analysis involved two-way ANOVA, regression, 3D response surface plots (Figures 1-6), and Method Operable Design Region (MODR).

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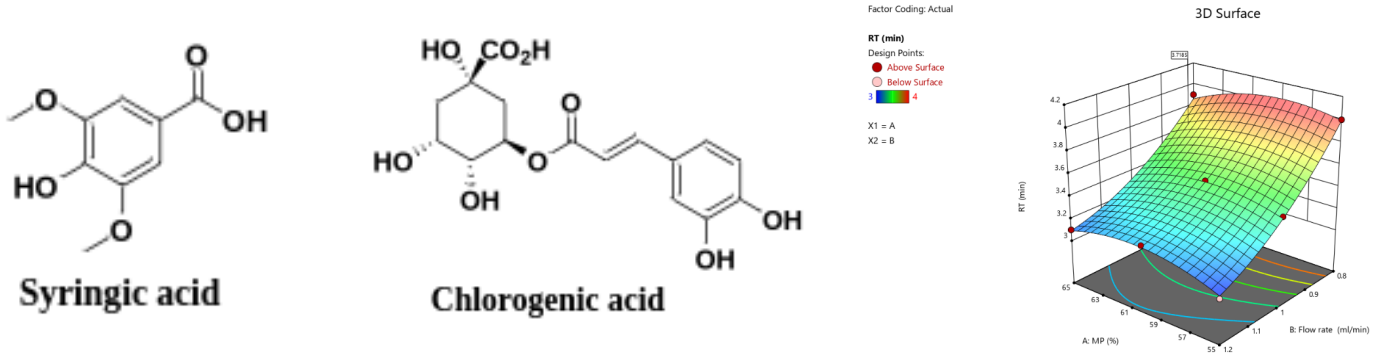


Figure 1: Response surface graph for studying the combined impact of the independent variable on the dependent variable retention time for syringic acid.

Table 1: Design matrix as per central composite design for optimization of liquid chromatographic analytical method of Syringic acid.

Run	Factor 1 A: MP (%)	Factor 2 B: Flowrate (mL/min)	Response 1 RT (min)	Response 2 TF
1	55:45	1	3.4	0.9
2	60:40	1	3.5	1.1
3	65:35	1	3.3	1
4	60:40	0.8	4	1.2
5	55:45	0.8	4	0.8
6	65:35	0.8	3.9	1.2
7	65:35	1.2	3.1	1.1
8	60:40	1	3.5	1.1
9	60:40	1	3.5	1
10	55:45	1.2	3	1.2
11	60:40	1.2	3.2	1.3

Validation

The chromatographic method was validated, as per ICH parameters [such as specificity, system suitability, linearity, precision, robustness, and ruggedness]. This process was carried out adhering to the guidelines provided by the ICH [Q2] for validating analytical method.[21]

System suitability

System suitability testing was conducted on the chromatographic system before each validation stage. Six consecutive injections of a standard preparation were administered into the system to assess retention time, tailing factor, and the theoretical plates.

Linearity

Standard solutions (0.2 to 1 mg/mL) were injected in triplicate to assess method linearity. A calibration curve plotting SA and CA concentrations against peak area was created, and linear regression analysis determined the regression equations and R^2 values to confirm proportionality

LOD and LOQ

The minimum drug concentration accurately distinguishable from the background is termed the Detection Limit (LOD), while the lesser quantifiable concentration is known as the Quantification Limit (LOQ).

Precision

The intraday (Thrice a day) and interday (three consecutive days) precision were studied on 2, 6, and 10 µg/mL solutions. The precision of the chromatographic method was determined by calculating the relative standard deviation values with acceptance criteria of less than 2%.

Robustness and Ruggedness

Robustness measures stability against minor changes in parameters like organic content, pH, buffer concentration, temperature, and injection volume. Ruggedness evaluates variability from different analysts, with both criteria requiring the Relative Standard Deviation (RSD) to stay below 2%.

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Table 2: Design matrix as per central composite design for optimization of liquid chromatographic analytical method of Chlorogenic acid.

Run	Factor 1 A: MP (%)	Factor 2 B: Flow rate (mL/min)	Response 1 RT (min)	Response 2 TF
1	80:20	0.4	3.6	1
2	80:20	0.6	3	0.9
3	85:15	0.5	3.2	1.1
4	85:15	0.4	3.6	1.3
5	85:15	0.6	3	0.8
6	75:25	0.5	3.2	1.1
7	80:20	0.5	3.3	1.2
8	75:25	0.4	3.5	0.9
9	80:20	0.5	3.3	1.3
10	80:20	0.5	3.3	1.3
11	75:25	0.6	3.1	1

Table 3: Calibration curve of SA and CA.

Concentration (µg/mL)	Peak area of SA	Peak area of CA
2	111532	93234
4	275864	194825
6	408127	307595
8	541962	411867
10	679828	519170

RESULTS

In this current chromatographic method, optimal conditions, including flow rate and retention time, were determined, guiding initial experiments involving mobile phase compositions and finally, a mobile phase comprising of methanol and [0.1%] OPA was selected, along with relevant flow speed and retention time.

Identification of ATP and CQP

The ATP that was opted for optimization of HPLC chromatographic conditions were retention time (time of elution) and theoretical plate (effectiveness of separation process). Critical quality parameters were identified on the basis of chromatographic profile, such as flow rate and mobile phase ratio.

Method optimization by DOE

A suitable design of experiment consisting of 11 trials, as outlined in Tables 1 and 2, was carried out to investigate the influence of two independent factors: the percentage of organic component [Methanol] in the mobile phase (X1) and its flow rate (X2), upon two dependent response factors such as retention time (R^1) and tailing factor (R^2). The data underwent analysis applying the TWO-WAY ANOVA test for both quadratic and linear models.

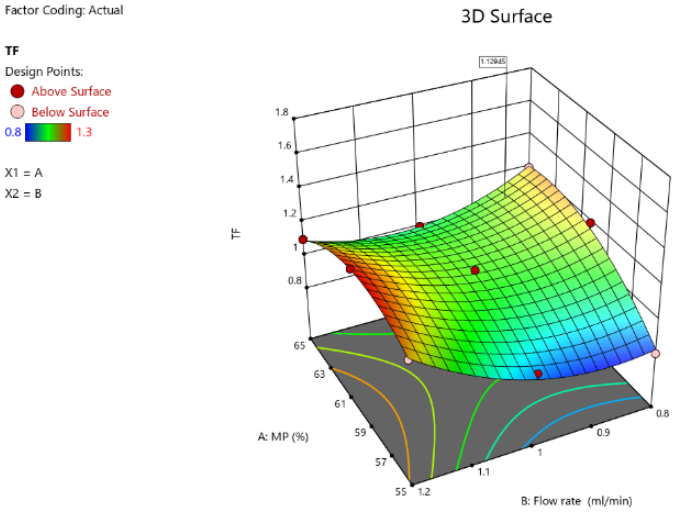


Figure 2: Response surface graph for studying the combined impact of independent variables on dependent variable tailing factor for syringic acid.

Method Operable Design Region

Validation

The Chromatographic method was validated as per ICH [Q2/R^1] guidelines, confirming its suitability through RSD calculations for various parameters [peak area, retention time, and tailing factor] within standard limits.

System suitability

System suitability was evaluated using 6 replicate injections of 10 µg/mL each, yielding %RSD values of 1.7% for SA and 0.39% for CA, with retention times of 3.54 and 3.21 min, respectively. Theoretical plates were 5468 (SA) and 3386 (CA), with tailing factors of 1.08 and 1.17.

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Table 4: Precision of SA and CA.

Precision	Concentration (µg/mL)	SA		CA	
		Peak area *n*=6	% RSD	Peak area *n*=6	% RSD
Intraday Precision	2	109905	0.92	211719	0.25
	6	417213	0.57	285748	0.40
	10	695651	0.61	358258	0.43
Interday Precision	2	116603	1.05	221629	1.37
	6	431051	1.23	294562	0.99
	10	755359	1.08	364121	0.95

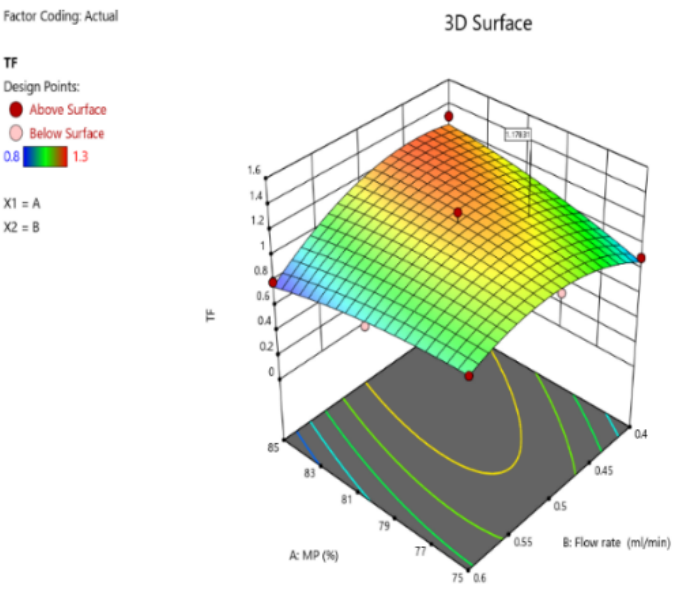


Figure 3: Response surface graph for study related to the combined impact of independent variable on dependent variable tailing factor for chlorogenic acid.

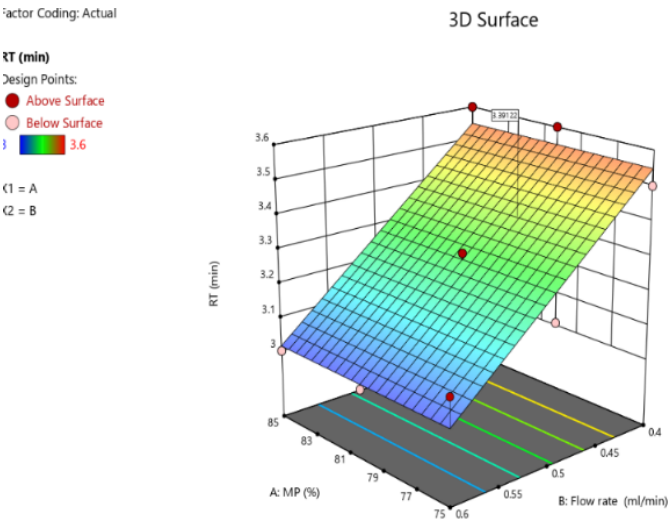


Figure 4: Response surface graph for studying the combined impact of independent variables on dependent variable retention time for chlorogenic acid.

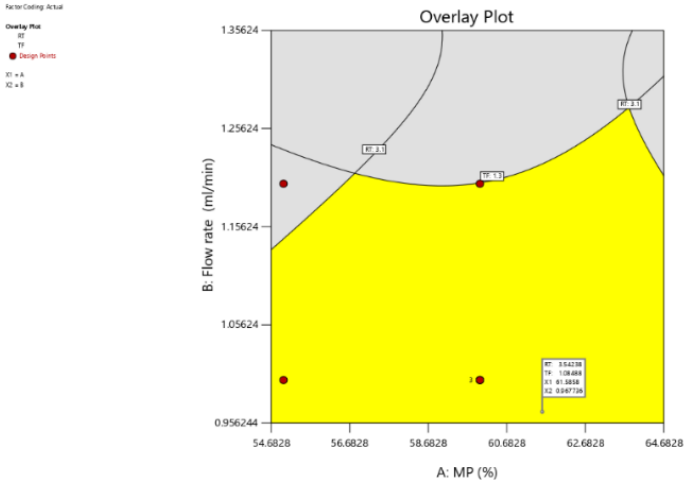


Figure 5: Overlay plot representing MODR of syringic acid.

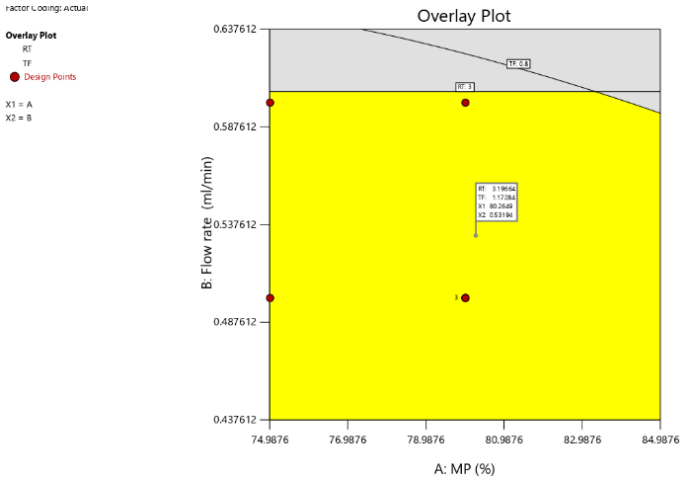


Figure 6: Overlay plot representing MODR of chlorogenic acid.

Linearity

The calibration curve for SA and CA showed linearity between 2-10 µg/mL, with regression equations y = 140269x + 17344 (R^2= 0.998) and y = 53446x - 15336 (R^2=0.999), respectively, plotting peak area against concentration Table 3 and Figures 7- 10.

Precision

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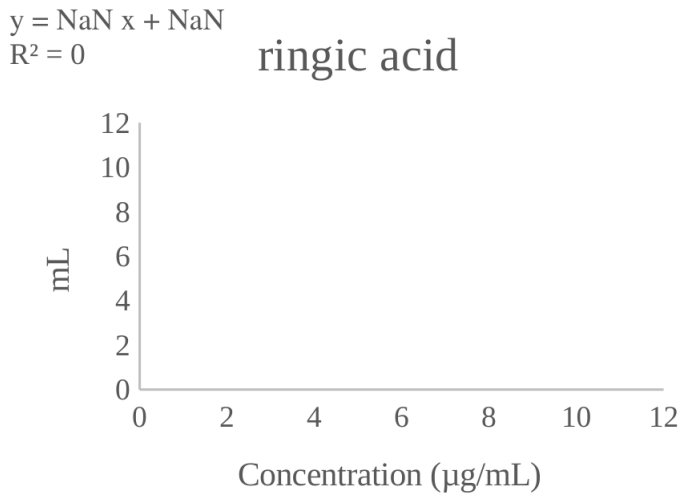


Figure 7: Calibration curve of syringic acid.

The repeatability % RSD for SA and CA, determined by six replicates of the same concentration (100 µg/mL), was below 2% Table 4.

Robustness and ruggedness

Robustness was evaluated by altering method parameters such as mobile phase and flow rate, while ruggedness assessed analyst variability as an external factor. Despite these changes, %RSD values for peak area consistently remained below 2%.

Limit of Detection and Limit of Quantification

The LOD and LOQ were ascertained with the standard deviation of slope and intercept, resulting in values of 0.51 µg/mL and 1.57 µg/mL for SA and 0.172 µg/mL and 0.52 µg/mL for CA, respectively.

DISCUSSION

AQbD for RP-HPLC technique is gaining prominence for natural products as this helps to establish the quality and purity of the pharmaceutical compounds under analysis. Hence in the current study, RP-HPLC method was developed for analysing SA and CA by integrating Analytical Quality-by-Design (QbD) principles and validated as per ICH (Q2/R[1]) guidelines. Parameters such as retention time, theoretical plates, and tailing factor were considered, and mobile phase composition and buffer pH were identified as vital quality attributes influencing these parameters of central composite design, with two factors using Design-Expert Software Version 13.0 ATP. As illustrated in Table 1, 2 consistency in chromatographic separation was ensured by controlling column selection, instrument configuration, and injection volume. Robustness was evaluated for factors such as mobile phase pH, flow rate, and column temperature.

The HPLC method for SA and CA was designed by applying the AQbD approach. It has involved a Chemsil ODS-C18 (4.6 mm×250 mm 5 µ) column and a mobile phase ratio of Methanol and 0.1% OPA in water (60:40 v/v) and Methanol: 0.1% TEA in water and SA and CA have exhibited a retention time of 3.5 and 3.2 mins respectively, with linearity observed within the range of 2-100 µg/mL (R^2: 0.998 and 0.999) indicating degree of chromatographic separation Figures 7-10. The validation parameters showed % RSD values below 2%, indicating the developed method is precise and accurate. The LOD and LOQ were found to be 0.51 µg/mL and 1.57 µg/mL for SA and 0.172 µg/mL and 0.52 µg/mL for CA, respectively.

Thus, the Study underscores the effectiveness of the Design of Experiments approach in optimizing the RP-HPLC method, illustrating an enhanced and more precise method over conventional methods.

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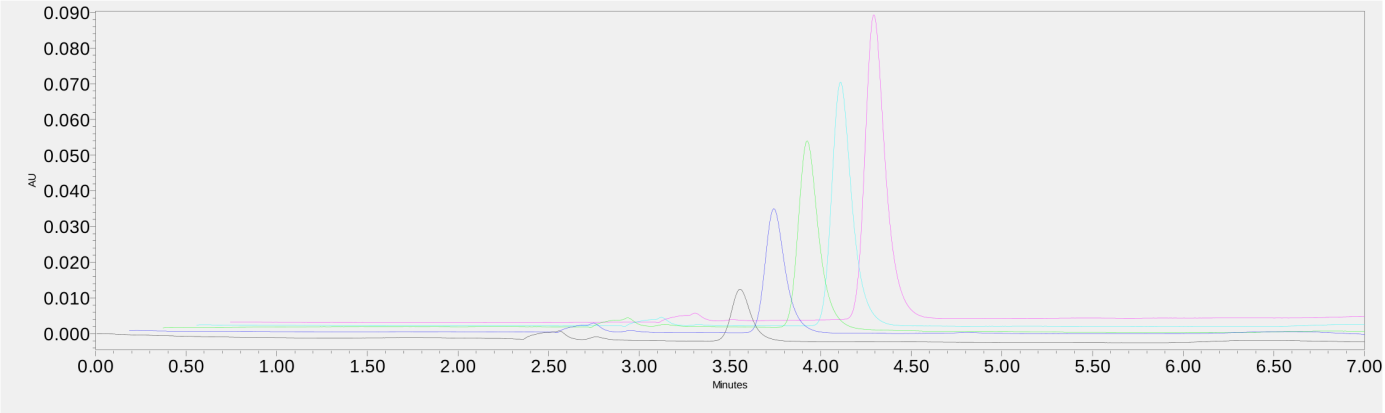


Figure 8: chromatogram of syringic acid (linearity).

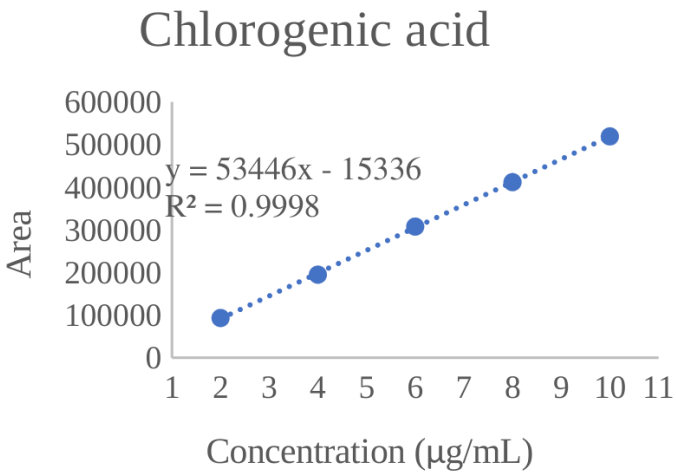


Figure 9: Calibration curve of chlorogenic acid.

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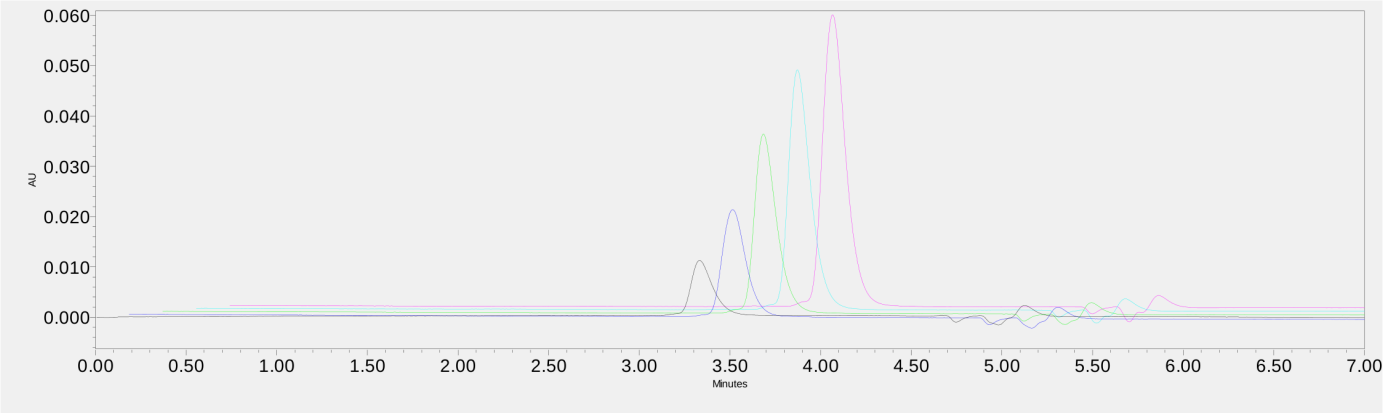


Figure 10: Chromatogram of chlorogenic acid (linearity).

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CONCLUSION

Unlike previous techniques, which often relied on trial and error, this method systematically defines critical parameters to enhance precision, robustness, and reproducibility. The use of the cost-effective solvent system with methanol, OPA, and TEA provides an economical alternative to Acetonitrile systems also emphasizes the green approach. This established chromatographic method demonstrated linearity, precision, robustness, and ruggedness, but it has yet to undergo routine analysis, a step not feasible in this current study due to the unavailability of SA and CA in the commercial dosage forms.

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ABBREVIATIONS

AQbD: Analytical Quality by Design; **RP-HPLC:** Reverse-Phase High-Performance Liquid Chromatography; **HPLC:** High-Performance Liquid Chromatography; **OPA:** Orthophosphoric Acid; **TEA:** Triethylamine; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **SA:** Syringic Acid; **CA:** Chlorogenic Acid; **CC:** Correlation Coefficient; **ATP:** Analytical Target Profile; **CQP:** Critical Quality Parameters; **CCD:** Central Composite Design; **MODR:** Method Operable Design Region; **DOE:** Design of Experiment; **RSD:** Relative Standard Deviation.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTIONS

All the authors have contributed to this research either directly or indirectly. Ms. Amruta Kamate has carried out the entire project, Dr. Basavaraj M Dinnimath has mentored the project. Mr. Nikhil Gawas has coordinated the work and Ms. Raksha Shet was associated in the project as well as in preparation of the manuscript.

SUMMARY

The project was aimed at developing an analytical method supported by the AQbD approach for natural products as they lack such data. Hence two phytochemicals-Syringic acid and Chlorogenic acid were selected for the study. Analytical method was developed using HPLC and validated as per ICH guidelines, which has produced significant results related to their quality which shall be a milestone in Quality control operations. Further study can be taken up in future to establish bio-analytical data as well for the two phytochemicals.

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