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Original Article

Sustainability Assessment Analytical Quality by Design (AQbD)-Based Reverse-Phase High- Performance Liquid Chromatography (RP-HPLC) Method Development for Eriocitrin Using Box- Behnken Approach

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ABSTRACT

Background: Eriocitrin is a citrus flavonone, and has potential as a phytopharmaceutical compound. A robust and reliable analytical method is required for its routine quality control and quantification. **Objectives:** To establish a Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) technique guided by Analytical Quality by Design (AQbD) for estimating Eriocitrin by Box-Behnken Design (BBD) and assessing the analytical sustainability. **Materials and Methods:** Optimization of methanol concentration, rate of flow and injected volume was performed using BBD. The method was validated based on the International Council for Harmonisation (ICH) Q2 (R^2) protocols. Sustainability indices were measured, including AGREE (Analytical GREEnness Metric Approach), GAPI (Green Analytical Procedure Index), Analytical Eco-Scale, and BAGI (Blue Applicability Grade Index). **Results:** Good separation was obtained using the optimized chromatographic conditions, and exhibited excellent linearity within the concentration range (10-60 µg/mL, R^2=0.9997). Present approach demonstrated acceptable accuracy, precision, specificity, reliability and sensitivity and thus it could be considered for routine quantitative analysis. Sustainability assessment showed that there was an acceptable judgement between analytical performance, practicability and environmental impact. **Conclusion:** The developed method was simple, robust, reliable and environment friendly. The steps and standards of the analytical design space were established, ensuring the performance of the method, which could be applicable for quantitative determination and quality control of Eriocitrin in a routine application.

Keywords: Analytical Quality by Design, Box-Behnken Design, Eriocitrin, RP-HPLC, Sustainability assessment, Validation.

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INTRODUCTION

Eriocitrin (eriodictyol-7-O-rutinoside) is a naturally occurring flavanone glycoside that is predominantly present in citrus fruits, particularly lemons. Due to its various therapeutic activities, including hepatoprotective, cardioprotective, and antidiabetic properties, it has attracted increasing attention as a valuable natural compound for pharmaceutical, nutraceutical, and functional food applications.[1] As interest in Eriocitrin based products continues to grow, the availability of reliable analytical methods capable of accurately quantifying this flavonoid has ensured product quality, consistency, and its adherence to regulations. Among the several methodical approaches employed for flavonoid estimation, RP-HPLC is commonly known to produce excellent sensitivity, selectivity, reproducibility, and appropriateness for regular quantitative evaluation of phytochemicals.[2,3]

Conventional chromatographic method development generally relies on sequential adjustment of one experimental variable at a time. Although this empirical strategy is simple to perform, it provides limited information regarding factor interactions and often results in methods with reduced robustness. Consequently, regulatory agencies encourage analytical methods using Quality by design principles.[4] Although straightforward, this approach often overlooks potential interactions and provides only a limited understanding of the factors governing method performance. As a result, methods developed through empirical optimization may exhibit poor robustness and require repeated adjustments during validation or routine application.[5] To address these challenges, regulatory agencies and scientific communities have increasingly

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encouraged the implementation of concept of Qbd for method development. This paradigm promotes an efficient and knowledge driven strategy that combines risk assessment, statistical experimentation, and scientific understanding to achieve reliable and reproducible analytical performance throughout the method lifecycle.[6] Recent studies have demonstrated that QbD-assisted RP-HPLC approaches integrated with response surface methodology enhance chromatographic understanding, robustness, and reliability through systematic evaluation of critical method parameters, supports the sustainable and greener analytical methods.[7]

Despite growing relevance of Eriocitrin, analytical methods reported for its quantification remain relatively limited. An LC-MS/MS technique for assessing Eriocitrin in bio-samples was published by Li *et al*. However, the method was designed primarily for pharmacokinetic investigations and relies on sophisticated instrumentation that may not be practical for regular quality control testing.[8] Earlier work by Miyake *et al*. utilized RP-HPLC analysis to determine phenolic glycosides in citrus matrices, demonstrating the applicability of chromatographic techniques for Eriocitrin analysis.[9] Nevertheless, these methods were developed using conventional optimization strategies and did not systematically investigate the combined influence of critical chromatographic variables. Furthermore, no published study has reported analysis of Eriocitrin by AQbD framework or comprehensively evaluated method sustainability using recognized green and white analytical chemistry assessment tools.

AQbD has emerged as an effective framework approach to understand method and its robustness. The framework contains various parameters like Analytical Target Profile (ATP), Critical Quality Attributes (CQAs) and Critical Method Parameters (CMPs). Statistical experimental designs are subsequently employed to find out the effect and association of these parameters on analytical performance. The Box-Behnken Design (BBD) is particularly attractive because it enables efficient optimization while requiring fewer experimental runs than many alternative designs.[10,11] This strategy provides an Analytical Design Space (ADS), where predetermined chromatographic performance criteria can be achieved, thus enhancing reliability and minimizing analytical variability. In recent years, analytical method development has increasingly focused not only on performance but also on sustainability. Green Analytical Chemistry (GAC) and White Analytical Chemistry (WAC) methods has encouraged researchers to minimize solvent consumption, reduce environmental impact, enhance operator safety, and improve overall method efficiency. Consequently, integrating sustainability considerations into analytical development has become an important aspect of modern quality-control practices.[12] Also, applications of QbD-driven strategies in flavonoid analysis have further confirmed their effectiveness in improving chromatographic optimization and method performance.[13]

Despite the reported pharmacological significance of Eriocitrin, a systematic AQbD-based RP-HPLC method integrating experimental design, comprehensive method validation, and analytical sustainability assessment has not been reported. Therefore, there is a need of reliable method to analyze and ensure consistent performance while supporting environmentally responsible analytical practices. Therefore, the purpose of present exploration is finding out an effective and rugged AQbD based RP-HPLC technique which will determine quantification of Eriocitrin. The method was optimized by BBD to find out the result of critical chromatographic parameters and to define an ADS. Furthermore, a rigorous approach assessment was performed using the ICH Q2 (R^2) protocols, also assessing the greenness of the framework.[14,15]

MATERIALS AND METHODS

Materials

Eriocitrin sample was offered from Aaisland Chemical Company, Paithan, Aurangabad, Maharashtra, India. For chromatographic analysis analytical grade methanol and acetonitrile were used and orthophosphoric acid (analytical grade) was the acidic phase in the mobile phase. Ultrapure water, purified using Milli-Q water, is utilized for all solution preparations. Solvents and chemicals used were chromatographic grade, and were used in their raw state without extra purification. Filtering of the solutions is performed using a filter aid (0.45 µm membrane filter) to clear it from any foreign elements, then degassed a 5.0L 150H ultrasonic bath (PCI Analytics, Mumbai, India).

Instrumentation

HPLC Analysis was performed with the help of Jasco LC-4000 system using an MD-4010 Photodiode Array (PDA) detector under the control of a ChromNAV software package (Version 2.0). The software was used to control the instrument, acquire and transfer of chromatograms, peak integration and analysis of the system suitability parameters. YMC C18 analytical column of YMC Co., Ltd., Kyoto, Japan, (Dimension: 150 mm × 4.6 mm) with the size of particle as 5 µm is used for Chromatographic separation. Weighing the samples was performed by an analytical balance, AUW-220D (Shimadzu Corporation, Kyoto, Japan).

Analytical Separation

A YMC C18 (Dimension: 150 mm × 4.6 mm) with the size of particle as 5 µm column is utilized for analysis. Separating process is performed with isocratic conditions and the mobile phase of acetonitrile, methanol and 0.4% orthophosphoric acid in the ratio of 10:20:70 (v/v/v) at 1.0 mL/min. For equilibration of the chromatographic system with the mobile phase, a period of about 30 min was used prior to analysis, while a re-equilibration

time of 5 min between injections was used to keep the retention times in the system stable throughout the analysis and ensure a reproducible performance of the chromatographic system. The main organic solvent used was chosen to be methanol, as it has suitable elution characteristics, being reasonably polar, with Eriocitrin having a polar structure of flavanone glycoside. To increase chromatographic efficiency, lower the viscosity, improve mass transfer, and obtain sharp, symmetrical peaks, a certain fraction of acetonitrile was introduced. Earlier it has been reported on getting similar insights about the complementary roles of methanol and acetonitrile in reverse-phase liquid chromatography.[16,17]

The OPA 0.4% served to keep the mobile-phase pH at about 2.2. Predicted pKa values for the most acidic phenolic proton of Eriocitrin range from approximately 7.15 to 9.1 across different computational estimation tools, reflecting typical variability among *in silico* pKa predictors for polyhydroxylated flavonoids. Irrespective of which estimate is used, the operating mobile-phase pH (2.2) lies at least 5-7 pH units below the pKa. By the Henderson-Hasselbalch relationship, this corresponds to more than 99.999% of the analyte remaining in its non-ionized, molecular form throughout the chromatographic run. Thus, under the selected chromatographic conditions, Eriocitrin is expected to remain predominantly in its protonated, non-ionized form throughout the chromatographic run. This assessment describes the ionization state of Eriocitrin under the analytical conditions and should not be interpreted as direct evidence of chemical stability. Formal solution-stability and forced-degradation studies would be required to establish chemical stability under these conditions. Throughout the analyses, 25±2°C temperature was regulated and PDA detector with a wavelength of 283 nm was used. The injection loop was rinsed after each injection into the chromatographic system and the whole run was finished in 12 min. To avoid carryover, the injection loop was flushed after every injection. The mobile phase was freshly prepared. The injection volume, flow rate, and methanol concentration was considered as CMPs for BBD optimization.

Standard Stock Solution

A 500 µg/mL Eriocitrin stock solution was prepared in 100 mL of methanol using 50 mg of Eriocitrin. The stock solution was stored at 4°C and checked for stability before use. Working sub-stock solutions were freshly prepared before each analysis to minimize potential changes in analyte concentration during storage.

Selection of Analytical Wavelength

Analysis of Eriocitrin (50 µg/mL) was performed in the wavelength range of 200-400 nm, with a maximum absorbance (λ_{max}) at 283 nm (Figure 1), so this was the wavelength chosen for detection. The selection was based on maximum absorbance and minimal baseline noise to ensure optimal sensitivity.

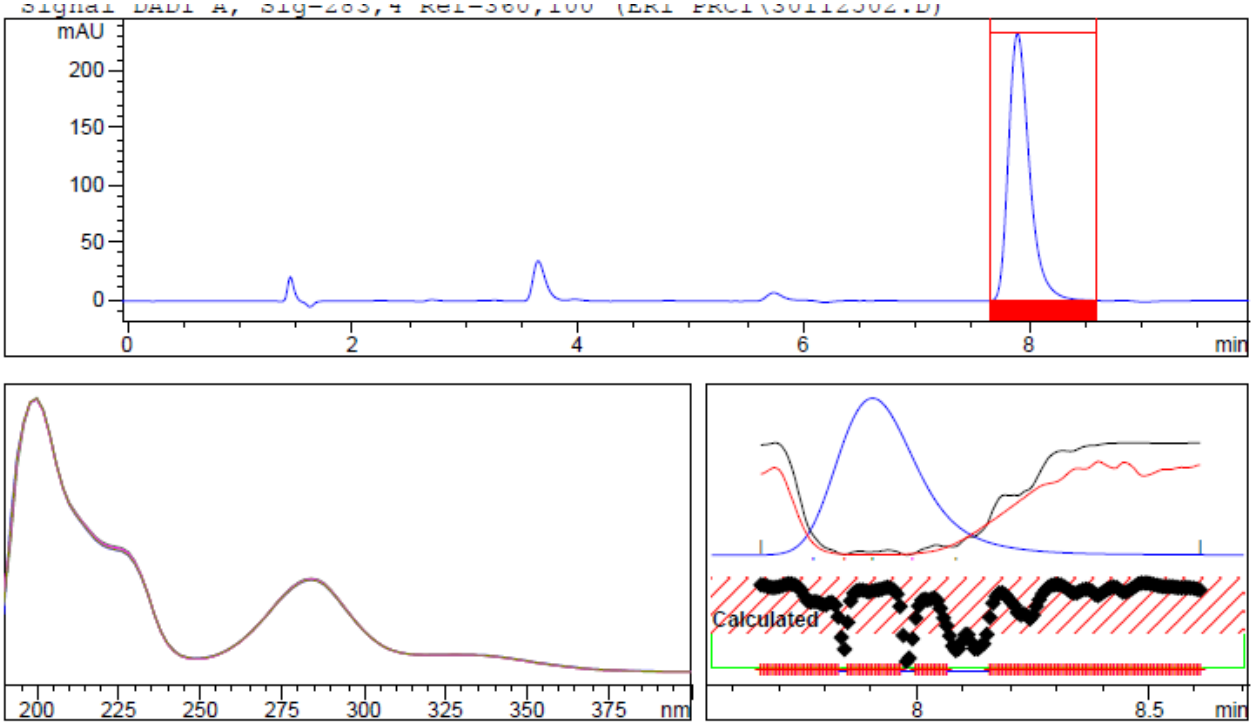


Figure 1: Selection of analytical wavelength for Eriocitrin.

Table 1: BBD experiment design matrix containing the three most important design parameters of the procedure i.e. methanol concentration, flow rate and injection volume, which are fixed at three coded levels of (-1, 0, +1) with Factors (F1, F2, F3) and Responses (R^1, R^2, R^3).

Std	Run	F1	F 2	F 3	R^1	R^2	R^3
		A: Methanol Conc. (%)	B: Flow rate (mL/min)	C: Injection Volume (µL)	Theoretical Plate Number	Tailing Factor	Retention Time
1	12	15	0.9	40	8646	0.71	11.555
2	13	25	0.9	40	9136	0.72	11.014
3	2	15	1.1	40	8806	0.74	11.098
4	7	25	1.1	40	9800	0.7	5.881
5	11	15	1	35	8991	0.73	12.628
6	1	25	1	35	6657	0.68	6.283
7	3	15	1	45	9596	0.75	13.179
8	8	25	1	45	8612	0.66	6.308
9	14	20	0.9	35	9372	0.66	8.66
10	9	20	1.1	35	9727	0.71	7.021
11	5	20	0.9	45	9038	0.7	8.684
12	4	20	1.1	45	9184	0.71	7.023
13	15	20	1	40	9247	0.71	7.761
14	6	20	1	40	9578	0.71	7.769
15	10	20	1	40	9327	0.71	7.808

Note: While conventional RP-HPLC methods typically use 10-20 µL injection volumes, the higher volume employed here was justified by the relatively low molar absorptivity of Eriocitrin at 283 nm and the need to achieve acceptable signal-to-noise ratios for quantification at lower concentrations.

Method Development

Quality Target Product Profile (QTPP)

For RP-HPLC method development, the criteria of satisfactory chromatographic performance determined by the key performance parameters were set by the QTPP.

Determine CQAs

CQAs (Critical quality attributes) are measurable responses related to the impact on the QTPP. These were identified as theoretical plate number, retention time and peak asymmetry (tailing factor) of the chromatography method which has direct correlation with the chromatographic efficiency, the resolution and the performance of the method. The acceptance criteria used were fixed as retention time of less than 8 min, tailing factor <1.5 and the number of theoretical plates > 4000. ChromNAV software generated chromatographic symmetry/tailing response was used to assess peak symmetry and used consistently as a system suitability indicator throughout the study. Subsequent risk assessment conducted with an Ishikawa diagram (Figure S1). Risk Priority Number analysis (RPN) was used to choose the 3 CMPs to go to the BBD. A RPN filtering approach (C-N-X logic: Controlled, Noise, experimental) was used to select the high-risk variables for further study in the DoE. Methanol concentration (Factor A), flow rate (Factor B) and injection volume (Factor C) were determined to be risk factors that need to be systematically studied. These factors were then dichotomised as a high, center, or low level (-1, 0, +1).

Factorial Design

The effect of three CMPs on identified CQAs was done using a BBD. These features enabled the study of interaction and quadratic effects of these variable on some important responses like retention time, theoretical plates, peak symmetry etc. A 3 x 3 factorial design with BBD approach was applied and an effective second-order polynomial model was obtained using Design Expert® software (13.0, Stat-Ease Inc., Minneapolis, USA) to analyze quadratic response surface so as to investigate and to optimize the process response. The relation of factors and responses was expressed as a polynomial equation of degree 2. Independent variables were represented as 3 levels (+1, 0, -1) for high, center, and low values, respectively. Therefore, QbD provided a clear connection between CMP and CQAs and allowed systematic optimization of the chromatographic performance in a range of design space. Clearly differentiating between CQAs and CMPs facilitated the identification of cause-and-effect relationships and enhanced understanding of the process and allowed for the optimization of the process, which was achieved through science and not in a trial and error manner. Model adequacy was judged with the use of coefficient of determination (R^2), ANOVA, lack-of-fit test.[18]

Selecting Method Conditions

Systemic evaluation of CMPs was done using the BBD methodology. Initially, the effect on time of retention, peak asymmetry and number of theoretical plates was assessed, leading to the identification of optimized conditions specific to Eriocitrin. Acceptable operational ranges were established within robust regions, where intentional changes in variables do not compromise method performance, ensuring reliability during subsequent validation. If the desired responses were not achieved through initial modeling, parameter levels were further refined until acceptable outcomes were obtained. The final optimized conditions were selected using Design Expert® software.

Risk Assessment

The optimized method was ultimately chosen for its consistent compliance with established analytical performance requirements, supporting dependable and efficient operation. To further evaluate its robustness and reliability, a risk-based QbD framework was applied in-line with ICH Q8 (R^2), ICH Q9 guidelines.[19,20] The evaluation involved systematic testing of method performance under varied conditions, such as varying labs, instruments, analysts, reagents, and days. Such variability studies support the robustness of the analytical procedure under evaluated conditions.[21] A semi-quantitative risk ranking was performed based on severity, occurrence, and detectability to prioritize critical factors.

Control Strategy Implementation

Following method development, it was crucial to implement an appropriate control strategy to ensure consistent method performance. Control limits for CMPs were a) Methanol concentration: 20±1% v/v; b) Acetonitrile concentration: 10±1% v/v; c) Flow rate: 1.0±0.05 mL/min; d) Detection wavelength: 283±2 nm; e) Column temperature: 25±2°C; and f) Injection volume: 40±5 µL. The composition of mobile phase was determined to behave the highest influence retention behavior and peak symmetry. Therefore, solvent preparation procedures, volumetric verification, and routine calibration of flow delivery systems were identified as key control measures.

Ongoing performance verification was performed through routine system suitability monitoring, periodic trend analysis of retention time and tailing factor, and change management procedures for column replacement, instrument transfer, and reagent source changes. Future lifecycle activities may include method transfer studies, forced degradation investigations, and extension to alternative matrices.

Greenness and Whiteness Assessment

Optimized HPLC has been assessed for environmental and practice-based sustainability using Green Analytical Chemistry (GAC) tools and one of the whiteness metrics in line with current good reporting practice in analytical chemistry.

Analytical GREEnness Calculator (AGREE)

AGREE tool was used for evaluating how environmentally friendly an analytical procedure is. This is done regarding 12 Principles of GAC, the tool was developed to compare an analytical method with these principles, and to calculate a score between 0 and 1, with a higher indicating higher "greenness". The score for agreement was computed according to rules of Pena-Pereira *et al.*[22] Standard software and scoring criteria from literature were used to all evaluations.

Green Analytical Procedure Index (GAPI)

The two tails on the GAPI are used to highlight the different stages of an analytical assessment and indicate how the method is likely to impact the environment. To show the potentially environmental impact of a method on all stages from sample preparation to measurement, the main body of the GAPI is divided into five sections with the two tails representing two different stages. Color coding was used to represent levels of impact on each of the sections spanning green, yellow, and red.[23,24]

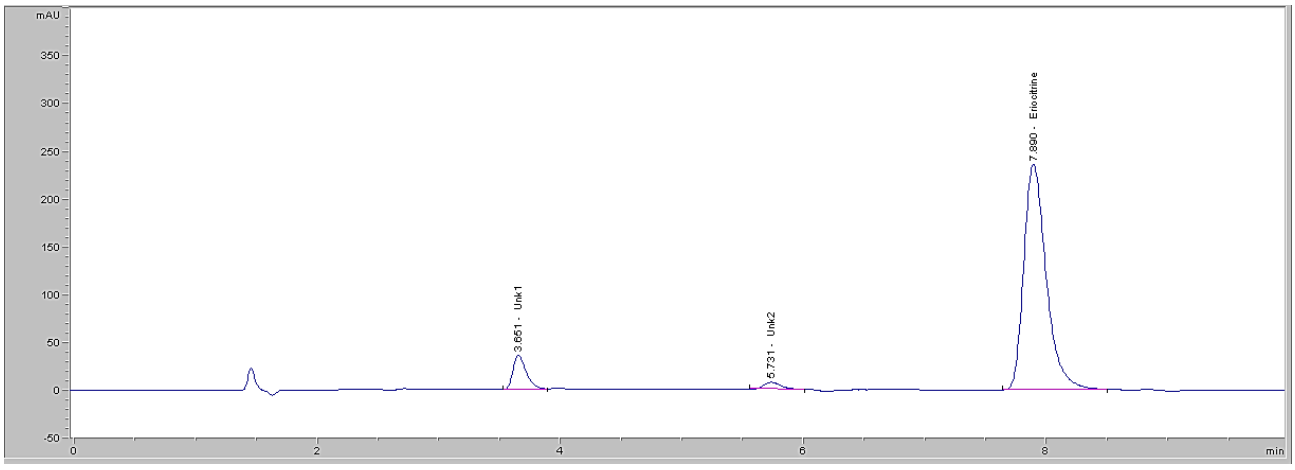


Figure 2: Optimized chromatogram of Eriocitrin (X-axis: Time of retention (minutes); Y-axis: Absorbance (mAU)).

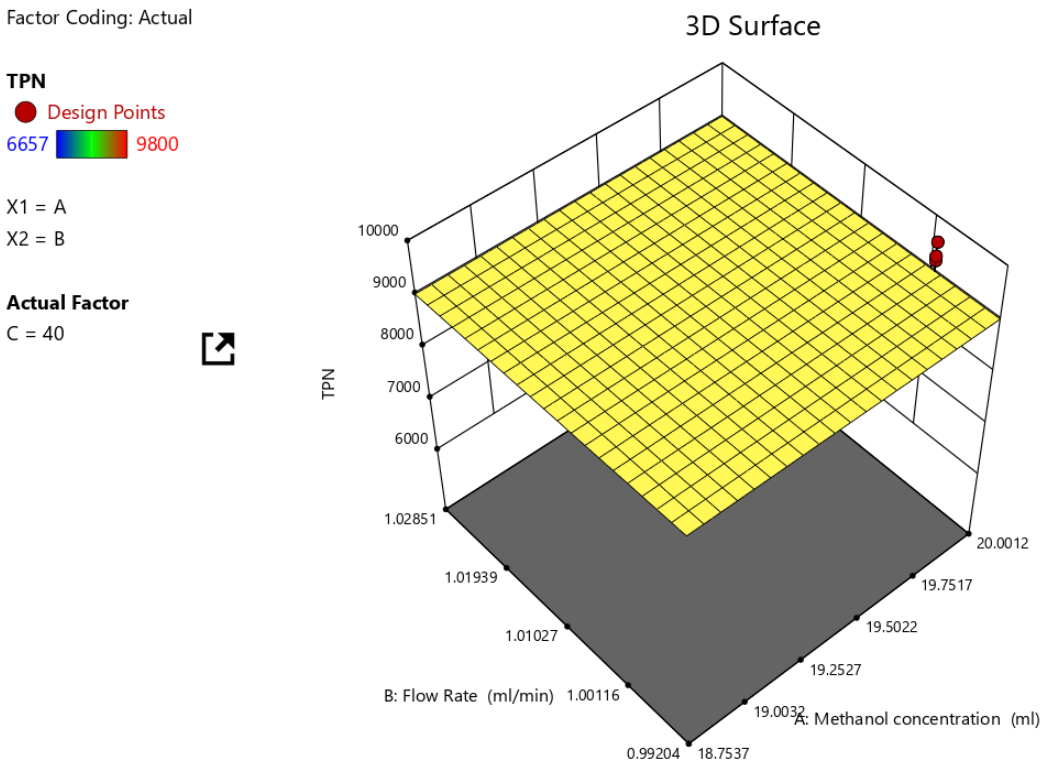


Figure 3: Plot of combined effects of factors on TPN of Eriocitrin.

Analytical Eco-Scale

The Analytical Eco-Scale evaluates how environmentally friendly a procedure is by penalizing the method for factors like using unsafe chemicals, amount of waste produced, energy utilized for instruments, risks to worker safety, and the need for waste management. The penalty points are deducted from a the final value 100. Final scores were interpreted as follows: values >75 represent excellent green performance, 50-74 indicate acceptable greenness, and < 50 reflect poor environmental suitability.[19,29]

Blue Applicability Grade Index (BAGI)

The practical and economic (blue) dimension is assessed in BAGI in tandem with the White Analytical Chemistry (WAC) framework.[25] BAGI assesses ten criteria related to type of analysis, analytes used, throughput, level of automation, accessing reagent and sample preparation complexity, yielding a score between 0 and 100on a visual pictogram. This tool, introduced by Manousi *et al*. (2023),[20,26-28] complements the green metrics.

Analytical Method Validation

Analytical Method validation is crucial to establish reliability and accuracy of an analytical method to make sure the appropriateness of required objective. The developed method for the quantification of Eriocitrin underwent validation as per ICH Q2 (R^2) guidelines. Optimized chromatography conditions were validated to demonstrate the ability of chromatograms to consistently fulfill ATPs over intended operating ranges to be suitable for routine QC applications.

Specificity

It is found out with the help of comparison between blank and standard Eriocitrin chromatograms. Interfering peak was not observed at the retention time of Eriocitrin. Since no marketed formulation was evaluated in the present study, placebo/sample chromatograms were not applicable. Further formulation-specific interference studies were considered during extension of the method to commercial dosage forms.

Linearity

Six levels of concentrations (10- 60 µg/mL) were utilized to check the method linearity of Eriocitrin. On y-axis peak area is taken and x-axis concentration is taken for graph formation. The regression equation and Correlation Coefficient (r^2) was used to evaluate the linear association. The amount of linearity was deemed acceptable if $r^2 \geq 0.999$.

Precision

Repeatability was done by preparing six parallel samples of Eriocitrin at 50 µg/mL. There was intraday variation in the samples were analyzed at 25, 50, and 75 µg/mL, at three different time intervals in a given day to assess intraday variation, and the same was repeated with three consecutive days of samples to

assess interday variation. The criteria for precision was set at ≤ 2% RSD according to ICH guidelines.

Accuracy

Spiked standard samples were prepared using a blank mixture and the test concentration of 50%, 100% and 150% for performing recovery studies. The amount of Eriocitrin standard added to the blank mixture was the test concentration and the % recovery was calculated. The accuracy range of 98-102% has been established as the acceptance limits.

Limit of Detection and Limit of Quantification

LOD is the detection threshold of an analyte detectable under specific experimental conditions. At this amount, the analyte cannot be quantified precisely. LOQ is the lowest concentration measurable using an analytical method with accuracy and precision.

The LOD and LOQ were found out using formula:

$$LOD = 3.3 \times \frac{\sigma}{S}$$

$$LOQ = 10 \times \frac{\sigma}{S}$$

σ: standard deviation, S: slope.

Robustness and Ruggedness Studies

By deliberately introducing moderate changes into the key parameters, robustness was assessed. These variations were flow rate ±0.05 mL/min (0.95 and 1.05 mL/min); mobile phase composition ±2% methanol (18% and 22%); and detection wavelength ±2 nm (281 nm and 285 nm). The method was considered robust with all %RSD values less than or equal to 1.8% for area of the peaks. Ruggedness was evaluated as low as %RSD < 2% from the analysis carried out by two different analysts on two different days. This lack of significant change in chromatographic responses with each deliberate change in parameters further demonstrated the inherent robustness of this method and its ability to perform with the desired change parameters within the ADS defined.

Statistical Analysis

The analyses of experimental data and method development was performed with the help of Design-Expert® software (13.0, Stat-Ease Inc., Minneapolis, USA). To optimize the chromatographic conditions, a BBD was used, with three factors and three levels with center points replicated three times. A p-value <0.05 was found to be statistically significant.

Ethics Statement

No human participants, human samples, or experimental animals were used in this study. Therefore, no ethical approval was required.

RESULTS

The initial mobile phase was composed of 0.4% OPA with methanol and acetonitrile (70:15:15 (v/v/v)); however, this composition had an extremely long retention time for the analyte. Upon modifying the ratio to 0.4% OPA (75%) with methanol (15%) and acetonitrile (10%), no well-defined chromatographic peak was observed, suggesting inadequate elution and poor resolution. A further adjustment to 0.4% OPA (70%) with methanol (25%) and acetonitrile (5%) was also investigated, but the chromatographic performance remained unsatisfactory. The observed variations in retention behavior can be attributed to changes in mobile phase polarity and elution strength, where increasing organic content reduced analyte-stationary phase interactions in reverse-phase chromatography.

Following systematic optimization, a mobile phase constituting 0.4% OPA (70%) with methanol (20%) and acetonitrile (10%) v/v produced sharp peaks within acceptable retention time and met all system suitability requirements (Figure S2). To further optimize and establish method robustness within the ADS, BBD was utilized to produce consistent and reproducible chromatographic method, that confirms balanced ratio of methanol and acetonitrile was essential to achieve optimal resolution and peak symmetry for polyhydroxylated flavonoids such as Eriocitrin.

Factorial Design

The optimization results indicated that the chromatographic method was optimized with mobile phase. The optimum experimental factors are determined as 1.0 mL/min flow rate and 40 µL of injection volume to achieve good separation in the chromatograms (Table 1 and Table S1).

Mathematical Models and Polynomial Equations

The relationship between the CMPs and chromatographic responses was evaluated. Based on model fit statistics and ANOVA results, linear models were selected for retention time

Table 2: Comparison of predicted and observed chromatographic responses for the optimized method, along with percentage prediction error.

Sl. No.	Batch	Theoretical Plate Number	Tailing Factor	Retention Time
1.	Predicted	9047.8	0.615	8.397
2.	Observed	9471	0.62	7.682
3.	Prediction error (%)	4.68%	0.81%	8.51%

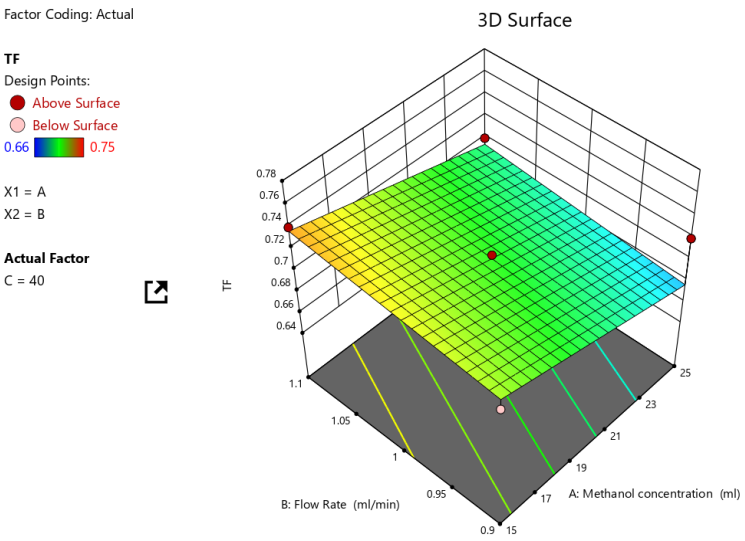


Figure 4: Plot of interactive effects of selected factors on peak tailing factor of Eriocitrin.

Table 3: Linearity data for the RP-HPLC determination of Eriocitrin over the concentration range of 10-60 µg/mL (*n*=6), including regression parameters, Limits of Detection (LOD) and Quantification (LOQ).

Concentration(µg/mL)	Peak Area at 283 nm
10	559.45
20	1039.66
30	1618.49
40	2135.71
50	2672.53
60	3191.88
Slope	53.08
Intercept	11.82
Correlation Coefficient (R^2)	0.9997
SE of Intercept	27.04
SD of Intercept	66.24
LOD (µg/mL)	4.12
LOQ (µg/mL)	12.48

and tailing factor, whereas a mean model was considered most appropriate for theoretical plate number.

The equations of regression expressed w.r.t coded factors:

RT=8.84 − 2.37A − 1.11B + 0.0752C

TF=0.7067 − 0.0213A + 0.0087B + 0.0050C

TPN=9047.80

Where A represents methanol concentration, B represents flow rate, and C represents injection volume.

For retention time, methanol concentration exhibited the largest negative coefficient (-2.37), indicating that increasing methanol concentration significantly reduced analyte retention. Flow rate exhibited a declining effect on time of retention, while injection volume had a negligible influence.

Similarly, methanol concentration was the most influential factor affecting tailing factor, showing a negative coefficient (-0.0213), which suggests improved peak symmetry at higher methanol levels. Injection volume and flow rate exerted comparatively minor effects on peak asymmetry within the investigated ADS.

For theoretical plate number, none of the investigated factors demonstrated a statistically significant effect. Consequently, the response was best described by the overall mean value (9047.80), indicating that column efficiency remained essentially constant across the evaluated experimental region. The consistently high theoretical plate counts obtained in all experimental runs confirmed satisfactory chromatographic performance throughout the ADS.

The experimental results demonstrated variation in chromatographic responses across different factor combinations, indicating the influence of method parameters on system performance. Notably, retention time varied significantly with changes in methanol concentration and flow rate, while the tailing factor remained relatively stable across the ADS.

Design Space

The quadratic model for theoretical plate number showed a greater coefficient of determination (R^2=0.9477), which indicated an apparently good fit of the model. However, model exhibited a significant lack-of-fit (F-value=22.15, *p*-value=0.0440), indicating that the variability in this response was not well-described by the quadratic polynomial within the studied ranges. Rather than indicating model failure, this finding supported the conclusion that theoretical plate number was primarily governed by intrinsic column properties (particle size, packing quality) and was relatively

insensitive to the three CMPs evaluated. This interpretation was consistent with the narrow range of observed values (mean ~9047 plates; all runs exceeded the ATP criterion of >4000 plates) and was supported by the Adequate Precision analysis. The model was retained for descriptive purposes, but theoretical plate number was re-classified as a monitoring parameter (non-critical) within the ADS, rather than a critical response for optimization. This interpretation was also consistent with ICH Q8 (R^2) guidance, which allows for non-critical attributes to be monitored rather than actively optimized.

The linear model was identified as the most suitable model for tailing factor optimization (p=0.0496). The model showed acceptable goodness of fit (R^2=0.4953; adjusted R^2=0.3577). The predicted R^2 was negative (-0.0771). Nonetheless, the model retains utility for understanding the directional effect of methanol concentration, even with limited absolute predictive power. The Adequate Precision value of 5.739 (>4) confirms an acceptable signal-to-noise ratio for ADS navigation.

Methanol concentration (A) was a statistically significant factor (p=0.0128) influencing tailing factor, with a negative coefficient, indicating improved peak symmetry at higher methanol levels. Flow rate (B) and injection volume (C) did not show significant effects (p>0.05). The reduction in tailing factor with increased methanol concentration may be attributed to improved mass transfer and reduced secondary interactions between analyte and stationary phase. The absence of a significant lack-of-fit further supported model adequacy. Overall, the QbD results indicated that tailing factor was moderately driven by mobile phase composition, and was minimally affected by flow rate and injection volume within the studied ADS. The relatively low R^2 value suggests moderate model predictability, indicating that additional factors such as pH or column temperature may also influence peak symmetry.

The linear model was identified as the most appropriate model for retention time optimization (p=0.0055). The model shown a good fit (R^2=0.6689; adjusted R^2=0.5785). The predicted R^2 (0.3320) indicated acceptable predictive capability. The appropriate precise value of 8.579 was well above the recommended minimum value 4, shows satisfactory signal-to-noise ratio, supporting the suitability of the model for navigating ADS.

Methanol concentration (A) had significant effect on retention time (p=0.0013), with a negative coefficient, indicating a decrease in retention time at higher methanol levels. Flow rate (B) showed a borderline effect (p=0.0709), suggesting a moderate influence on RT, whereas injection volume (C) did not significantly affect retention time (p=0.8948). This is consistent with reverse-phase chromatographic principles, where increased organic solvent content enhances elution strength and decreases analyte retention. Although a significant lack-of-fit was seen, the model provided useful insights into the dominant role of mobile phase composition on analyte retention. Despite this, the model provides meaningful insight into factor effects and is adequate for method optimization within the studied ADS. Overall, the QbD analysis confirmed that retention time was primarily governed by methanol concentration, making it a critical method parameter for achieving desired chromatographic performance. Although theoretical plate number was initially included as a response variable, DoE analysis indicated that it was not significantly affected by the studied CMPs within the evaluated ADS. Therefore, it was considered a monitoring parameter rather than a critical optimization attribute.

The ADS was established by overlaying the contour plots of the CQAs (Figure S3). The Operable Design Region (ODR) was identified where all CQAs met the ATP criteria (retention time<8 min, tailing factor <1.5, plates > 4000). A verification run performed at the optimized conditions (Coordinates: Methanol 20%, Flow 1.0 mL/min, injection Vol 40 µL) yielded results

Table 4: Intraday and interday precision data for the RP-HPLC method for Eriocitrin at a concentration of 50 µg/mL (n=6).

Concentration (µg/mL)	Intraday		Interday	
	Peak Area	Retention time (min)	Peak Area	Retention time (min)
50	3040.81	7.90	3047.89	7.89
50	3032.12	7.91	3047.18	7.86
50	3033.70	7.87	3030.50	7.94
50	3033.43	7.87	3046.60	7.95
50	3028.50	7.92	3049.46	7.83
50	3030.22	7.87	3034.47	7.84
AVER	3033.13	7.89	3042.68	7.88
SD	4.25	0.02	8.05	0.05
RSD	0.14	0.3	0.3	0.6
Limit	NMT 2.0%	NMT 1.0%	NMT 2.0%	NMT 1.0%

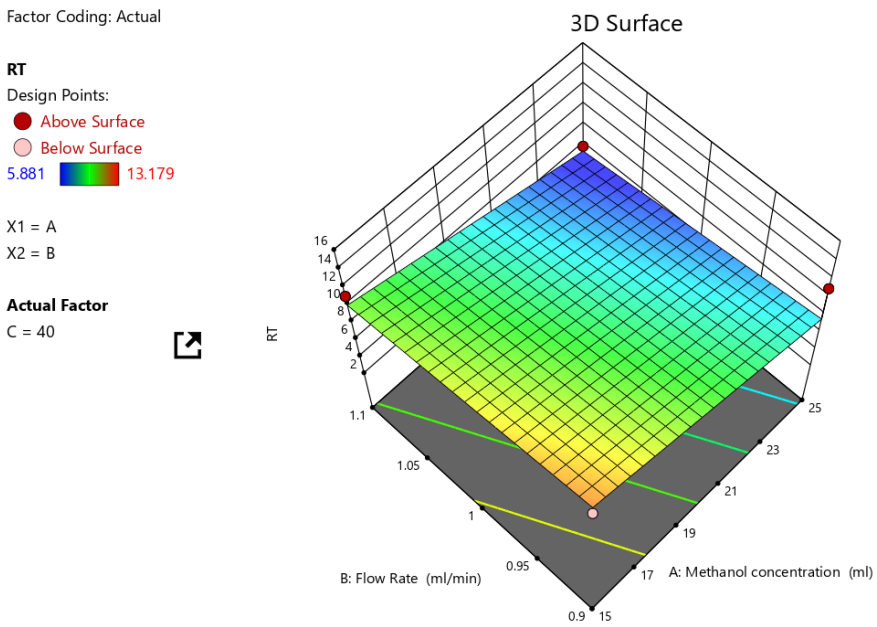


Figure 5: Plot of combined factors on time of retention of Eriocitrin.

within the 95% prediction interval, validating the design space. In comparison with traditional trial-and-error approaches, the DoE-based optimization provided a systematic understanding of factor interactions and enabled identification of a robust design space, minimizing the risk of method failure during routine application.

Optimized Conditions

The optimal parameters were selected by considering all the responses measured under different experimental conditions and analyzing them with the software Design Expert 13.0. Table 2, Figure 2 outlines the optimization conditions finally obtained with HPLC and their expected results for their predictions. The experimental response values were obtained by the measurement of HPLC chromatograms under definite conditions of the mobile phase composition, injection volume and flow rate. Two minor additional peaks were observed at approximately 3.6 and 5.7 min (Figure 2), designated as Unk1 and Unk2, respectively. These peaks were well separated from the Eriocitrin peak at approximately 7.68 min and did not interfere with baseline integration, chromatographic resolution, or quantitative determination of Eriocitrin. Based on their relatively low and fixed-level responses, these peaks were considered unlikely to represent concentration-dependent impurities or degradation products of Eriocitrin and may be associated with minor UV-active constituents from the diluent/mobile-phase system.

A minor baseline disturbance was also observed in the chromatogram. This feature was considered a chromatographic artifact that may arise from a minor refractive-index or UV-absorbance mismatch between the injected diluent and the mobile phase when the injection plug enters the flow path. The baseline returned to a stable and flat signal before the subsequent chromatographic peaks eluted and did not affect peak integration, resolution, or quantitative determination of Eriocitrin.

The low prediction error (<12%) between observed and predicted values confirm the reliability and predictive capability of the developed models.

Method Validation

Control Strategy

Based on the QbD outcomes, a control strategy was implemented for consistent routine chromatographic performance. The methanol content in the mobile phase was controlled within ±1% of the optimized concentration (20%, v/v) to maintain method robustness. The flow rate was maintained at 1.0±0.05 mL/min to ensure reproducible time of retention, chromatographic performance. System suitability variables, especially peak asymmetry/tailing factor (<1.5), were monitored before analysis to confirm acceptable system performance.

System Suitability

A representative chromatogram was used to assess the system suitability by evaluating the essential chromatographic parameters. The retention time was recorded as 7.68 min, theoretical plate number was 9471, and the peak asymmetry was 0.62. Additionally, the %RSD for six repeated injections was determined to be 0.14. All system suitability parameters were within acceptable limits, confirming adequate system performance prior to analysis. The plot illustrating the suitability for the optimized formulation is presented in Figures 3-5.

Specificity

The blank chromatogram did not exhibit any peak at the retention time corresponding to Eriocitrin, while the standard chromatogram showed a well-resolved and symmetrical peak at approximately 7.68 min. These observations indicate that the diluent and chromatographic system did not interfere with the detection of Eriocitrin under the optimized chromatographic conditions. Representative chromatograms are provided in Figure S4.

Carry-over was evaluated by injecting a blank (diluent) immediately following injection of the highest-concentration standard (60 µg/mL). No peak was observed at the Eriocitrin retention time in the resulting blank chromatogram, confirming absence of carry-over under the optimized chromatographic conditions.

Linearity

The calibration curve constructed for Eriocitrin was linear for concentration rage of 10-60 µg/mL (Figure S2, Figure 6, and Table 3). The regression equation was obtained when the peak area of each concentration was plotted against that concentration, to give high linearity (r=0.9997). The obtained n-value of 0.997 is a good confirmation of the strong linear dependence of the concentration on detector response within the investigated range.

Precision

The repeatability of Eriocitrin was assessed by analyzing the same concentration (50 µg/mL) six times, yielding a %RSD of less than 0.2. Outcomes of both interday and intraday precision were presented in Table 4. Since the % RSD values were below 2, the method can be considered precise. The low %RSD values indicate minimal variability and high repeatability of the analytical method.

Accuracy

The percentage recovery results obtained using the proposed HPLC method were presented in Table 5. 98-102% values prove that the developed approach match accuracy criteria specified in ICH Q2 (R^2) protocols. The recovery results demonstrated absence of matrix interference under the studied analytical conditions.

Robustness and Ruggedness Studies

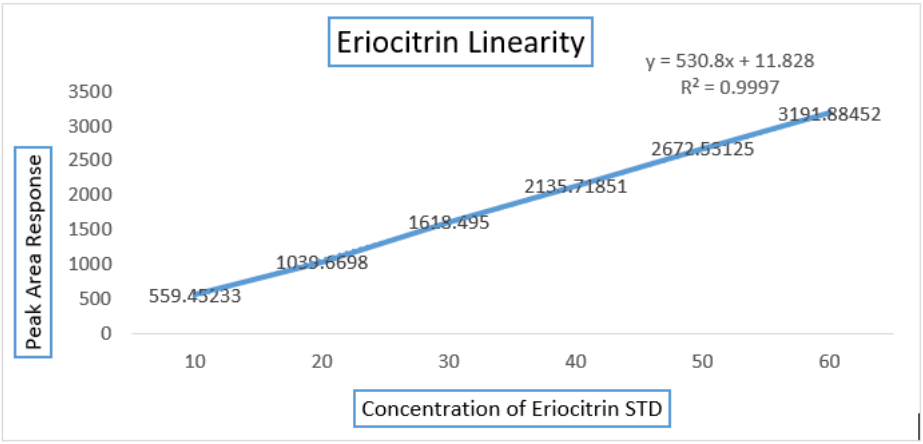
A 50 µg/mL solution of Eriocitrin was utilized to evaluate both robustness and ruggedness. Robustness is with the help of little, intentional changes in key method variables, including flow

Table 5: Accuracy assessment by recovery studies for the RP-HPLC method for Eriocitrin (*n*=3).

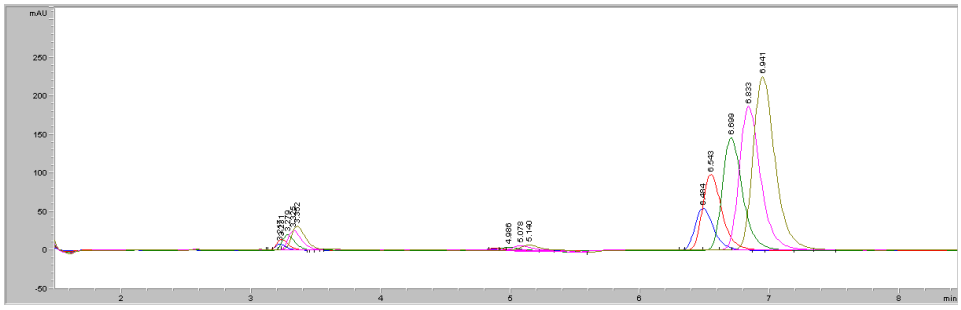
Level of Recovery in %	% Recovery	Average	SD	% RSD
Level-1 (50%)	99.1	99.1	0.75	0.8
	99.9			
	98.4			
Level-2 (100%)	100.0	100.4	0.71	0.7
	100.0			
	101.3			
Level-3 (150%)	99.6	100.2	0.86	0.9
	99.8			
	101.2			

Table 6: Analytical Eco-Scale penalty point calculation with RP-HPLC method for Eriocitrin.

Factors	Benchmarks	Base Penalty	Multiplier	Adjusted Points
Acetonitrile (toxic, flammable)	<10 mL	2	×2	4
Methanol (flammable, toxic)	10-100 mL/run	6	×2	12
Orthophosphoric acid (corrosive)	1-10 mL	2	×2	4
Energy consumption (HPLC + PDA)	~1.5 kWh/run	1	×1	1
Waste generation	1-10 mL/run organic	3	×1	3
Waste treatment	Collected, no on-site treatment	3	×1	3
Occupational hazard	Moderate (fume hood required)	1	×1	1
Sample preparation	Simple dissolution, no extraction	0	×1	0
Total Penalty Value				28
Eco-Scale Result			100-28 =	72



(a)



(b)

Figure 6: Linearity evaluation of Eriocitrin using AQbD-based RP-HPLC approach : (a) calibration curve of Eriocitrin; (b) Overlay chromatograms of Eriocitrin standard solutions.

rate, wavelength, mobile phase composition. Ruggedness was determined by considering variations due to different analysts as an external factor. In all cases %RSD of peak areas remained below 2%. These findings confirm that small changes in method parameters have no major impact on analytical performance.

LOD and LOQ

The results are found out as4.12µg/mL, 12.48 µg/mL respectively. Li *et al.* (2020) found out LOQ as 3.125 ng/mL using HPLC-MS/MS a technique with inherently superior sensitivity compared to UV-PDA detection[8]. For UV-based HPLC methods of flavonoid glycosides, LOD values in the µg/mL range were appropriate[27].

Greenness and Whiteness Assessment

The AGREE score was calculated as 0.52, reflecting the adoption of conventional organic solvents (acetonitrile, methanol) and the generation of approximately 4 mL of organic waste per run. This score is comparable to conventional HPLC methods, indicating moderate environmental impact (Table S2, Figure 7). The score indicates moderate greenness. The primary detractors were the use of acetonitrile (a relatively toxic, energy-intensive solvent classified under Tier 2 of CHEM21 solvent selection guide) and the absence of online waste treatment. The positive contributors to the AGREE score were the relatively short run time (7.7 min), the small sample volume (40 µL injection), the isocratic elution mode (energy-efficient vs. gradient), and the use of UV detection without derivatization.

The GAPI pentagram showed three green, one yellow, and one red sector. The red sector corresponds to using acetonitrile in the mobile phase, which is classified as hazardous. The yellow sector corresponded to waste generation (> 1 mL per run). The green sectors reflected: (i) absence of sample pre-treatment or extraction steps; (ii) use of UV detection (non-destructive, no chemical reagents); and (iii) short analysis time with isocratic elution (Figure 8). Overall, the GAPI assessment categorized the method as moderately green, with improvement achievable through partial replacement of acetonitrile with ethanol or through flow rate reduction.

The Eco-Scale penalty point calculation was summarized in Table 6. The total penalty point deduction was 28, yielding resulted value 72 suggesting the approach as green method (score 50-74). The largest penalty contributions were methanol usage and OPA corrosivity. The absence of sample extraction or derivatization steps was beneficial, avoiding additional penalty points that are common in methods requiring solid-phase extraction or pre-column derivatization.

The BAGI score was 72.5 out of 100 (Table S3), indicating good practical applicability. The criteria contributing positively to the BAGI score included use of a routine HPLC platform with commercially available columns, single-analyte determination with a well-established UV-PDA detector, straightforward sample preparation (dissolution only, no solid-phase extraction), reagents available from standard laboratory suppliers, and an established isocratic method that is easy to implement in any standard HPLC laboratory. Negative contributors included the absence of automation, moderate throughput (~7-8 min per run without considering column equilibration), and the need for OPA buffer preparation. The composite WAC assessment, combining the green (AGREE: 0.52), red (validation performance: excellent linearity, precision, and accuracy), and blue (BAGI: 72.5) dimensions, confirmed that the method achieves a balanced and acceptable WAC profile.[28] Overall, the method represented

Table 7: Comparison of previously reported chromatographic methods for Eriocitrin determination with the proposed QbD-based RP-HPLC method.

Parameter	Miyake *et al*. (2001)	Li *et al*. (2020)	Proposed QbD-Based RP-HPLC Method
Analytical technique	RP-HPLC with UV detection for phenolic glucoside analysis in citrus juices	LC-MS/MS for Eriocitrin quantification in rat plasma	RP-HPLC-PDA developed using QbD and Box-Behnken Design
Purpose of analysis	Identification and quantification of phenolic glucosides in citrus juices	Pharmacokinetic finding of Eriocitrin in rat plasma	Routine quantitative analysis and quality control of Eriocitrin in pharmaceutical dosage forms
Analyte matrix	Citrus fruit juices	Rat plasma	Eriocitrin analytical sample / quality-control application
Column	YMC-ODS, 4.6 × 250 mm, 5 µm	C18 column, 150 × 4.6 mm, 5 µm	YMC C18, 150 × 4.6 mm, 5 µm
Mobile phase	Methanol-water (5% acetic acid with gradient elution)	0.1% aqueous solution of formic acid-acetonitrile (gradient)	0.4% OPA: methanol: acetonitrile (70:20:10, v/v/v), isocratic
Flow rate	1.0 mL/min	0.8 mL/min	1.0 mL/min
Detection wavelength / mode	UV, 330 nm	MS/MS (MRM mode)	PDA, 283 nm
Robustness assessment	Not reported	Bioanalytical validation performed	Systematically evaluated through deliberate parameter variations; %RSD < 2%
QbD/DoE optimization	No	No	Yes
Analytical Design Space established	No	No	Yes
Risk assessment and control strategy	No	No	Yes
ICH-guided validation for routine QC	Not reported.	FDA bioanalytical validation for plasma analysis.	Yes, validated according to ICH Q2(R^2).
Sustainability assessment	Not reported.	Not reported.	AGREE=0.52; Eco-Scale=72; BAGI=72.5; GAPI evaluated.
Practical applicability	Research-oriented analysis of citrus constituents.	Pharmacokinetic/bioanalytical application requiring MS instrumentation.	Routine pharmaceutical quality control using conventional HPLC instrumentation.
Major advantage	Useful for characterization of citrus phenolic.	High sensitivity for biological samples.	Robust AQbD-driven method with defined design space, validated performance, and quantified sustainability profile.

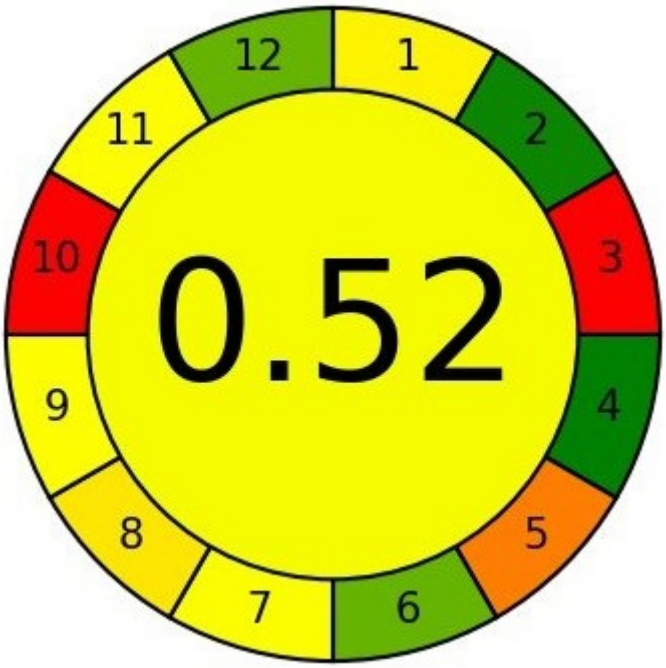


Figure 7: Greenness evaluation using the AGREE metric.

a harmony of analytical performance and environmental sustainability, aligning with modern green analytical chemistry principles.

DISCUSSION

This study reports on reliable RP-HPLC process established for Eriocitrin (eriodictyol-7-O-rutinoside) quantification. Using a mobile phase consisting of 0.4% OPA, 70% methanol, and 10% acetonitrile at a flow rate of 1 mL/min with UV detection at 283 nm, the method demonstrated excellent chromatographic performance. Validation results for linearity, LOD, LOQ, accuracy, and precision, complied with ICH Q2 (R^2) guidelines.[14,15]

Initial method development through trial-and-error highlighted the challenges associated with flavonoid chromatography. Early mobile phase combinations produced prolonged retention times and poor peak symmetry, reflecting the strong effect of mobile phase acidity and organic composition on the chromatographic behavior of polyhydroxylated flavonoids. Similar observations have been reported by Miyake *et al.* (2001), who emphasized the need for carefully balanced acidic aqueous phases and organic modifiers for Eriocitrin analysis.[9] The optimized acidic environment maintained Eriocitrin predominantly in its non-ionized form, reducing interactions with residual silanol groups and improving peak symmetry. Increasing organic solvent content enhanced elution strength and reduced retention through decreased hydrophobic interactions with the stationary phase. These findings were consistent with established reverse-phase chromatographic principles and explain the improved resolution obtained after optimization.

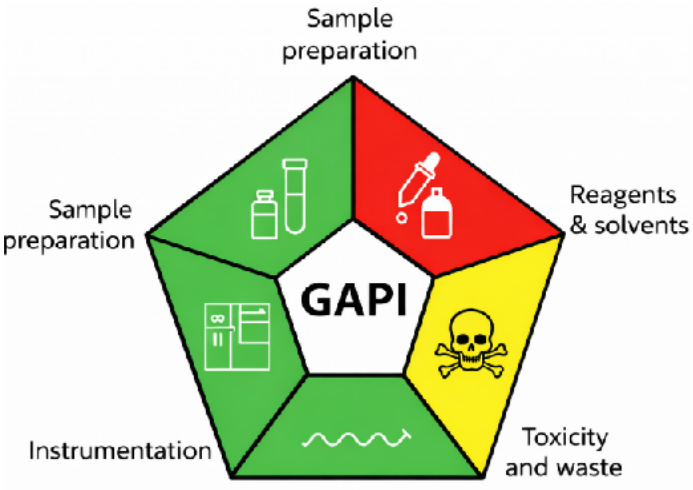


Figure 8: GAPI pentagram for the developed method for Eriocitrin.

The process exhibited very good linearity over the concentration interval 10-60 µg/mL with a correlation coefficient of 0.9997, exceeding the minimum ICH requirement. Recovery values between 98% and 102% and repeatability with %RSD below 0.2% demonstrated high accuracy and precision. Robustness and ruggedness studies further confirmed that little changes in the method seldom affect its performance, supporting its potential application for routine laboratory use.

The AGREE score 0.52 shows acceptable greenness, primarily penalized by the use of acetonitrile and the absence of online waste treatment. In comparison, published QbD-based RP-HPLC methods for flavonoids report higher AGREE scores of 0.6-0.79.[29,30] The Eco-Scale score of 72 was consistent with conventional isocratic RP-HPLC methods using standard UV detection.[31] The BAGI score of 72.5 reflects good practical applicability, consistent with single-analyte isocratic UV methods reported in the literature.[32] Factors such as the use of methanol/acetonitrile, solvent waste generation, and the absence of miniaturization or in-line analysis inherently reduce greenness scores, even when analytical performance is excellent. Nevertheless, the relatively short run time, isocratic elution, simple sample preparation, and lack of derivatization contributed positively to the sustainability profile of the developed method. Future optimizations could improve sustainability by exploring ethanol-water systems or UHPLC adaptation (reducing solvent consumption by ~70%).

Eriocitrin has attracted considerable attention due to its reported antioxidative, anti-inflammatory, antidiabetic, antihyperlipidemic, and neuroprotective activities.[1] Experimental and clinical studies suggested its potential role in reducing oxidative stress, modulating inflammatory pathways, and improving cardiometabolic parameters.[33,34] These therapeutic prospects underscored the importance of reliable analytical methods for accurate quantification in pharmaceutical and nutraceutical products, as well as for supporting clinical and pharmacological investigations. The method employs a standard isocratic RP-HPLC platform, without requiring specialized

equipment such as mass spectrometers or advanced sample preparation systems. The total analysis time of approximately 12 min per sample supports a high daily throughput suitable for batch release testing.

The operating mobile-phase pH (2.2) lies close to the lower boundary of the manufacturer-recommended pH range for conventional silica-based C18 columns (typically pH 2.0-7.5). This was a deliberate choice to suppress ionization of the analyte's phenolic groups and residual stationary-phase silanols. However, prolonged, repeated exposure to low-pH mobile phase in routine use could accelerate stationary-phase degradation and shorten column lifetime. No evidence of column performance drift (retention time, tailing factor, or plate count) was observed during the present validation period (interday RT %RSD=0.6%), but periodic system-suitability trending is recommended to monitor this risk during long-term routine use. Future refinements could evaluate hybrid-silica or trifunctionally end-capped C18 phases (e.g., YMC-Triart C18, rated pH 1-12) to extend the column-lifetime margin while preserving the low-pH selectivity advantage.

A notable strength of this research was the incorporation of the BBD within the QbD framework. Unlike traditional one-factor-at-a-time optimization, BBD enabled systematic evaluation of interaction and quadratic effects among method variables (Table 7). The statistical models showed strong agreement between predicted and observed responses. Methanol concentration emerged as the most influential CMP affecting both retention time and peak symmetry, consistent with its role in controlling mobile phase polarity and analyte-stationary phase interactions. The QbD approach also facilitated the establishment of a ADS in which the method consistently performs within predefined quality criteria. This provided greater assurance of method robustness and lifecycle reliability compared with empirically optimized methods. In addition, the developed method offered advantages over previously reported HPLC methods for Eriocitrin, including reduced analysis time, optimized solvent use, and systematic robustness optimization.

Despite its strengths, certain constraints to the present approach. Lack-of-fit in the theoretical plate model, while interpreted as evidence of a non-critical parameter within the evaluated design space, could also suggest that there were hidden variables not accounted for in the design, such as column temperature or stationary phase age. Temperature was a critical factor because it influences the viscosity of the mobile phase[35] and the rate of mass transfer. As suggested by Monks *et al.* (2012), including temperature as a factor in the BBD might have resolved the lack-of-fit and provided a better understanding of the design space.[36] This approach was validated for general quantification of Eriocitrin in dosage forms; however, its applicability to stability-indicating analysis has not yet been established because forced degradation studies were not performed. In addition, the method has not been evaluated for biological matrices or complex herbal extracts containing structurally related flavonoids, where higher selectivity and sensitivity may be required. Although a PDA detector was used for chromatographic detection, a formal peak purity assessment based on spectral homogeneity analysis was not performed in the present study. Under the investigated chromatographic conditions, no interfering peak was observed in the blank chromatogram. Therefore, acceptable selectivity toward blank matrix interference was demonstrated. The method was not assessed for specificity, and cannot be classified as a stability-indicating method. Future investigations involving PDA peak purity analysis and forced degradation studies will be undertaken to further establish analyte peak homogeneity and stability-indicating capability. However, a comprehensive specificity assessment according to ICH Q2(R^1), including placebo interference studies, PDA-based peak purity evaluation, and forced degradation investigations, was beyond the scope of this study. The successful application of the developed method to Eriocitrin samples confirmed its suitability for routine quantitative analytical quality assessment. Future research should assess the method in biological matrices, evaluating its suitability for pharmacokinetic applications, incorporating stability-indicating capabilities, and adapting the method to UHPLC platforms for improved efficiency and lower environmental impact.

CONCLUSION

A robust QbD-based RP-HPLC method for the quantification of Eriocitrin was appropriately validated. As per ICH protocols, the approach showed resilience, accuracy, and precision. Statistical evaluation confirmed methanol concentration as the critical method parameter influencing retention behavior and peak symmetry. The integration of greenness and whiteness assessment further demonstrates the method's balance between analytical performance and environmental sustainability. Present approach provided reliable, validated, and environmentally attentive analytical tool for quantitative evaluation of Eriocitrin. The method demonstrated satisfactory chromatographic performance and can serve as a useful platform for routine analytical applications involving Eriocitrin. The QbD-based framework, with its documented design space and control strategy, positions this method for regulatory submission in ICH Q2(R^2) and ICH Q14 guidelines for regulatory filing. The established design space and operable design region allow for science-based post-approval method adjustments. The method demonstrated robustness across analysts, days, and minor parameter variations supporting its suitability for multi-site transfer. Future studies should focus on extending the method to biological matrices and pharmacokinetic applications, incorporating stability-indicating capabilities through forced degradation studies, and adapting the platform to UHPLC for improved throughput and further reduction of solvent consumption.

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ABBREVIATIONS

ADS: Analytical Design Space; **AGREE:** Analytical GREEnness Metric; **AQbD:** Analytical Quality by Design; **ATP:** Analytical Target Profile; **BAGI:** Blue Applicability Grade Index; **CMP:** Critical Method Parameter; **CQA:** Critical Quality Attribute; **DoE:** Design of Experiments; **GAC:** Green Analytical Chemistry; **GAPI:** Green Analytical Procedure Index; **ODR:** Operable Design Region; **QbD:** Quality by Design; **RPN:** Risk Priority Number; **WAC:** White Analytical Chemistry; **ACN:** Acetonitrile; **ANOVA:** Analysis of Variance; **BBD:** Box-Behnken Design; **C18:** Octadecylsilane; **HPLC:** High-Performance Liquid Chromatography; **ICH:** International Council for Harmonisation; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **OPA:** Orthophosphoric Acid; **PDA:** Photodiode Array Detector; **QC:** Quality Control; **RP-HPLC:** Reverse-Phase High-Performance Liquid Chromatography; **RSD:** Relative Standard Deviation; **RSM:** Response Surface Methodology; **RT:** Retention Time; **SD:** Standard Deviation; **TF:** Tailing Factor; **TPN:** Theoretical Plate Number; **UHPLC:** Ultra-High-Performance Liquid Chromatography; **UV:** Ultraviolet.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

Vishin Ashish Patil: Conceptualization, Methodology, Investigation, Formal Analysis, Data Curation, Validation, Writing-Original Draft Preparation, Visualization. **Ashwin J. Mali:** Supervision, Review and Editing of the Manuscript, Project Administration.

SUMMARY

An AQbD-based RP-HPLC method was successfully established, optimized, validated for quantitative determination of Eriocitrin with help of Box-Behnken Design. The optimized method complied with ICH Q2(R^2) validation requirements, demonstrating satisfactory sensitivity, resilience, accuracy, precision, linearity, and selectivity toward blank/diluent interference. Analytical sustainability was further established using multiple greenness and whiteness assessment tools. Defined analytical design space provided consistent chromatographic performance, confirming the applicability of the approach for the quantitative determination and quality control of Eriocitrin in analysis.

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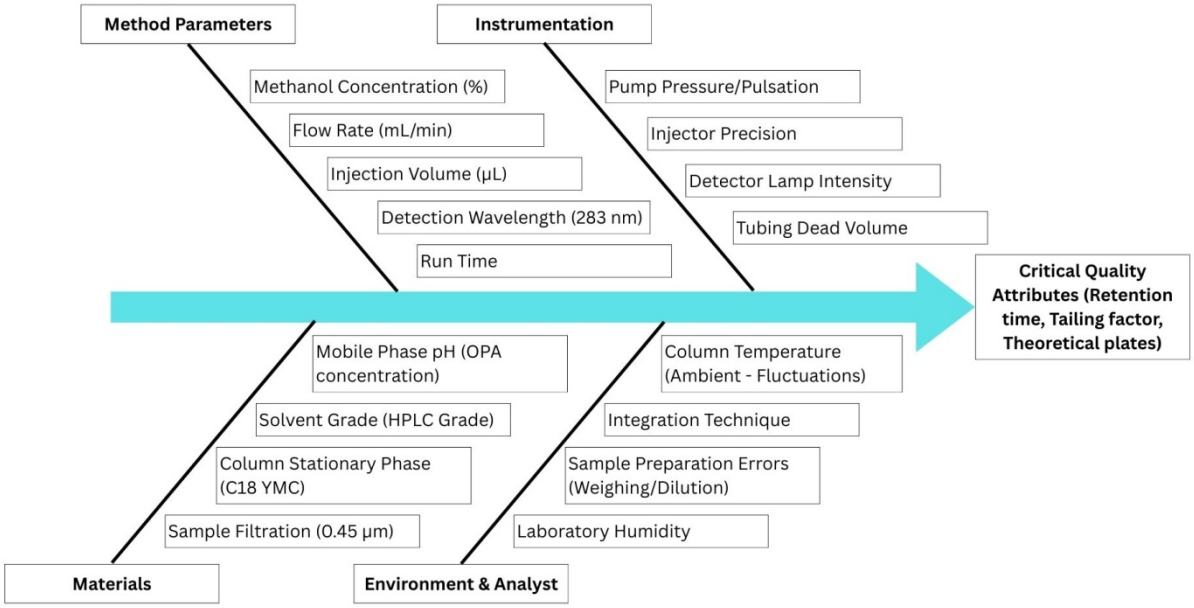


Figure S1: Ishikawa Diagram.

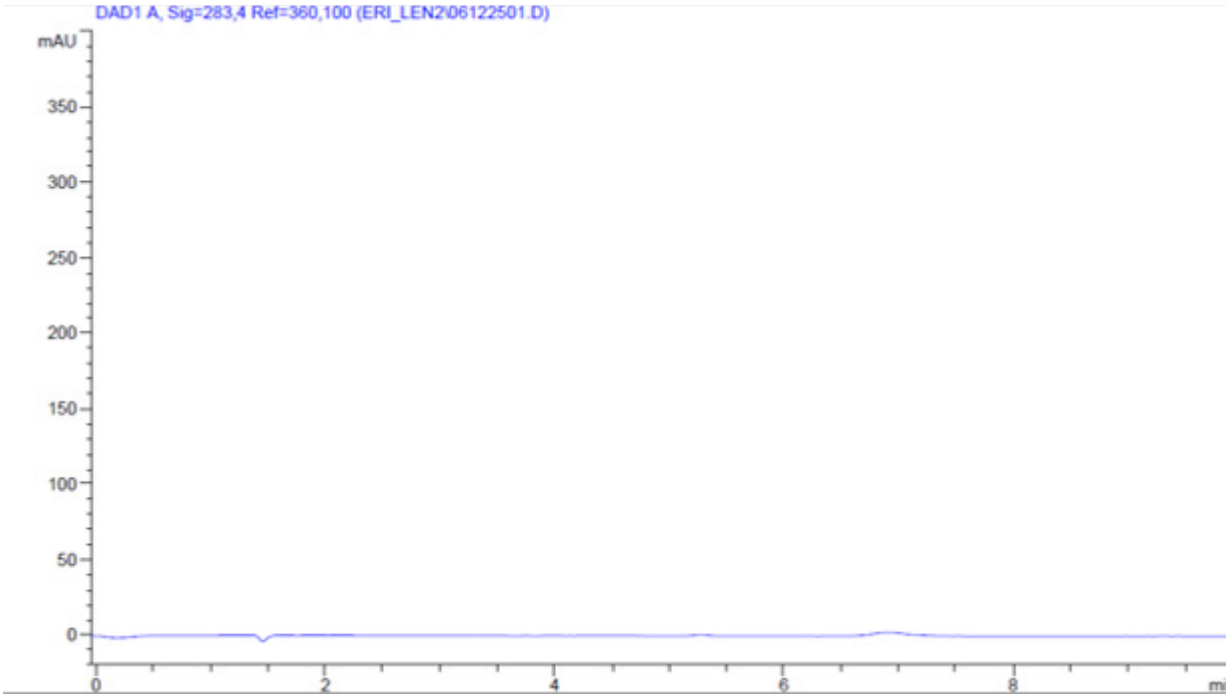
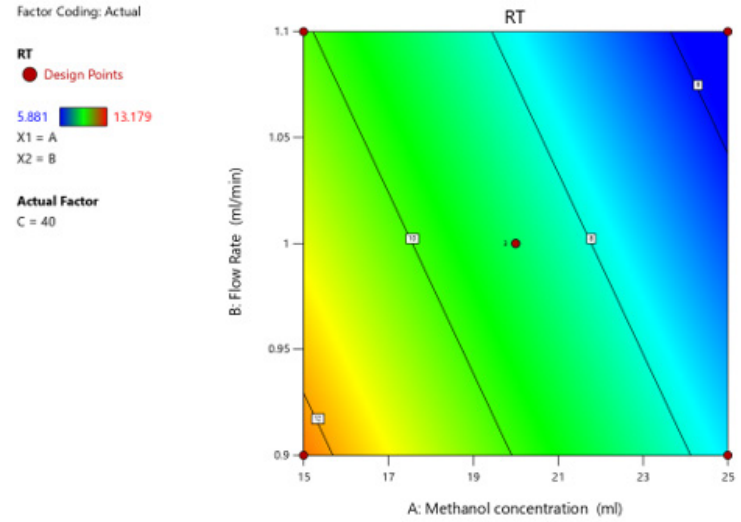
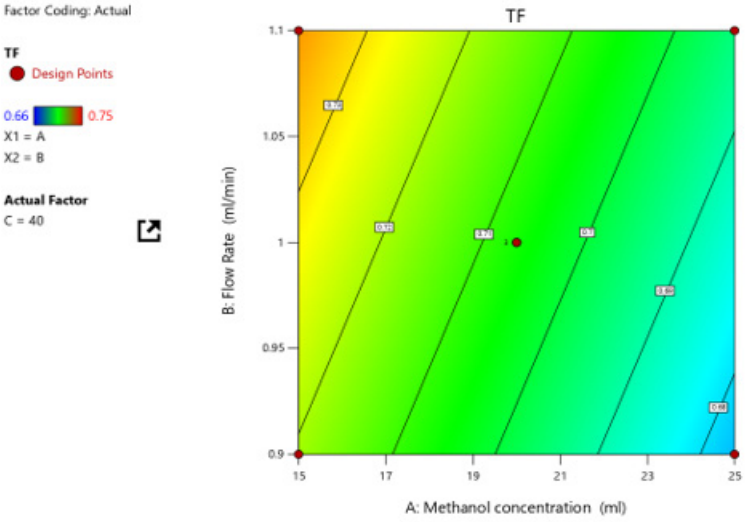


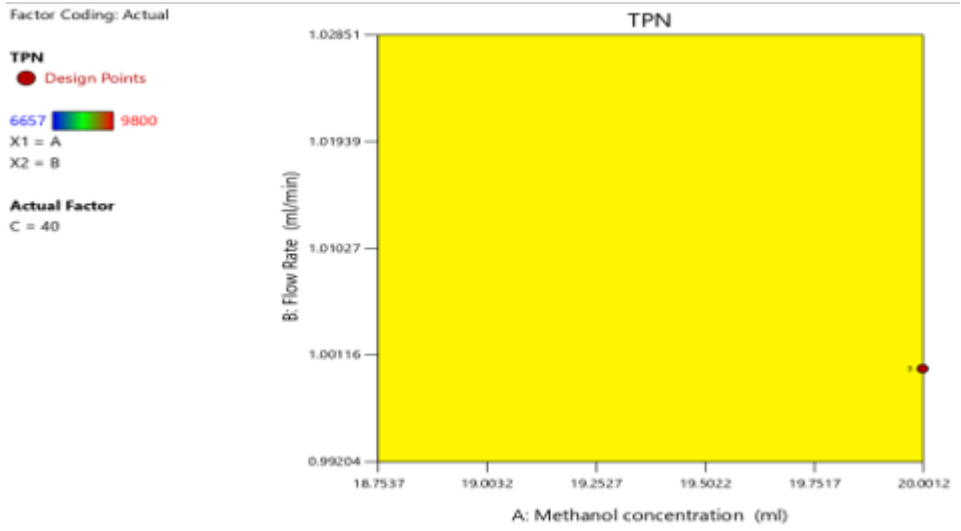
Figure S2: Chromatogram for Blank.



a)

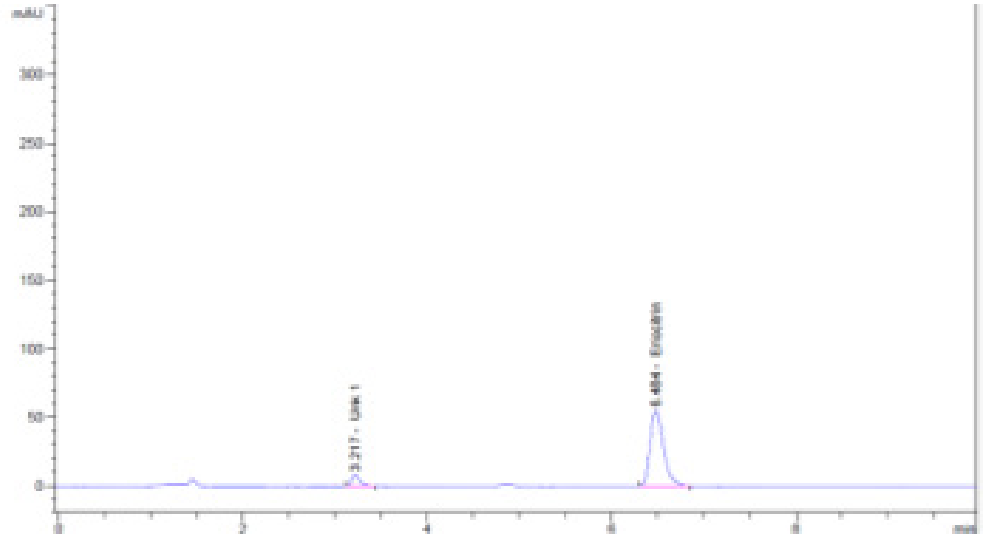


b)

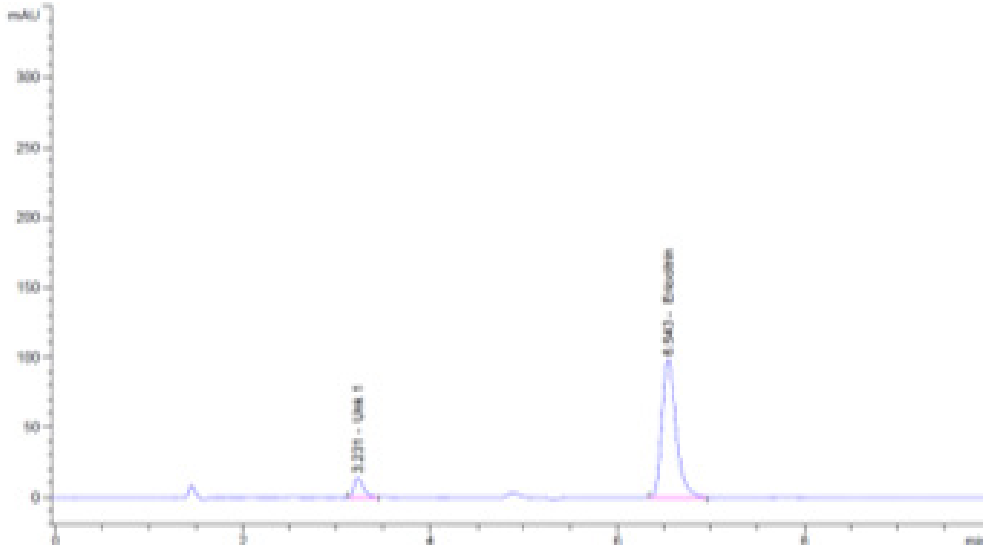


c)

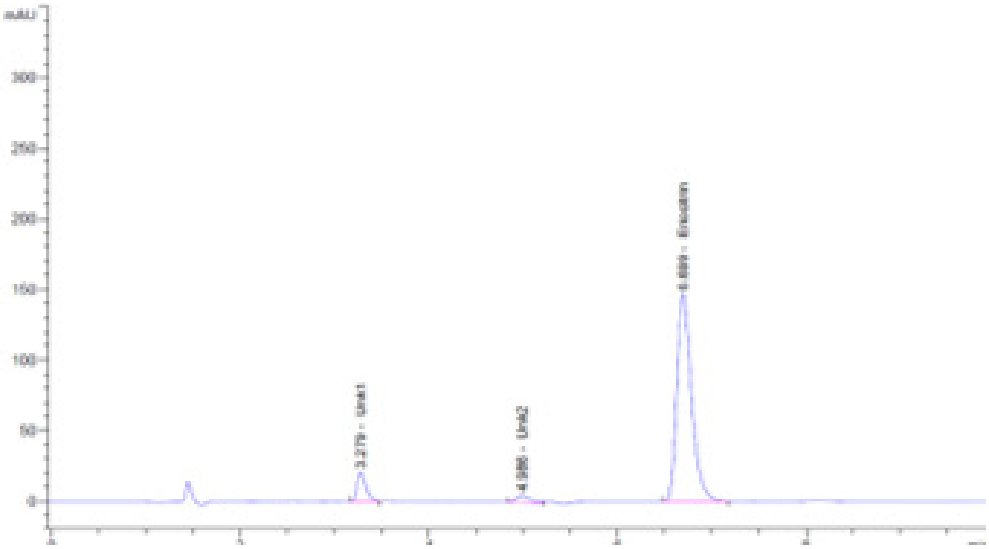
Figure S3: Contour plots. A) Retention time; b) Tailing factor; c) Theoretical plate number.



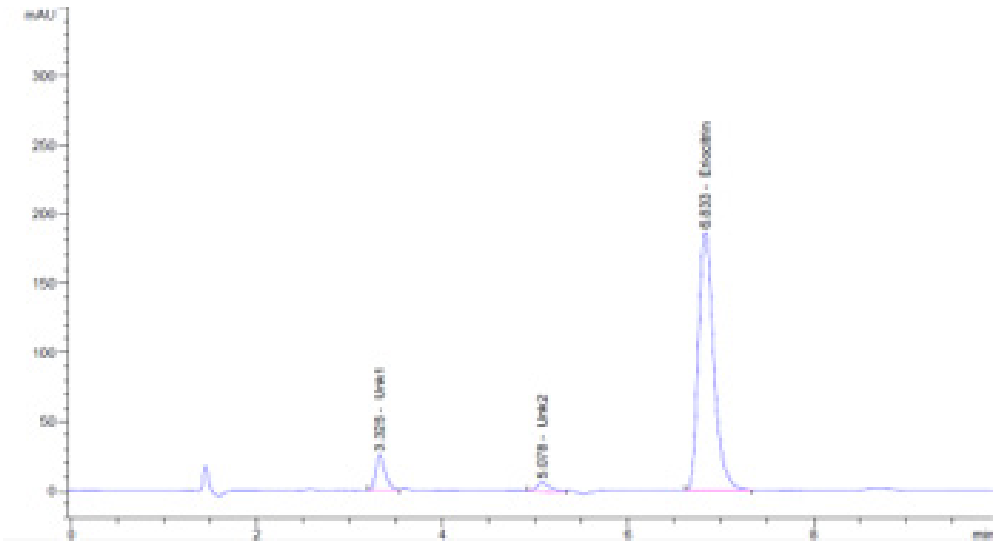
a)



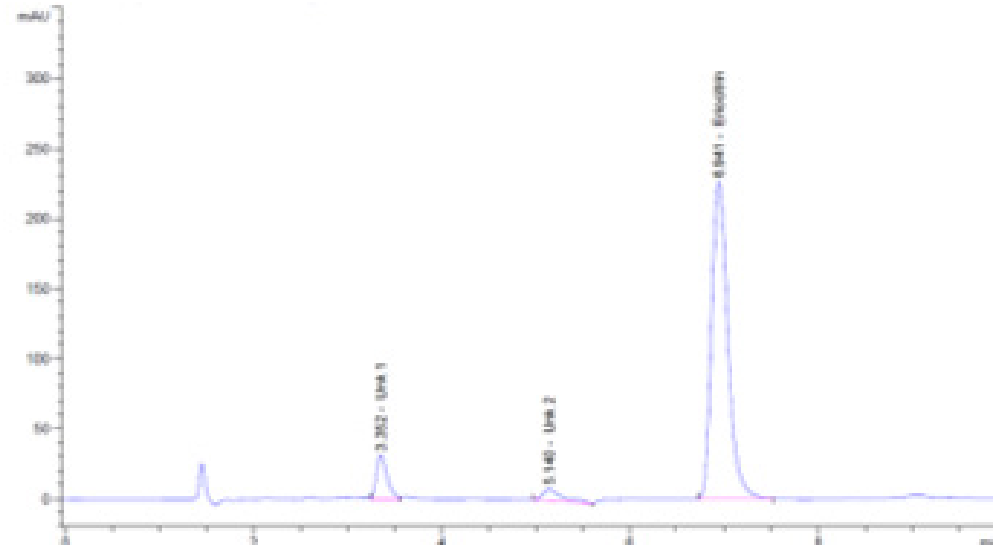
b)



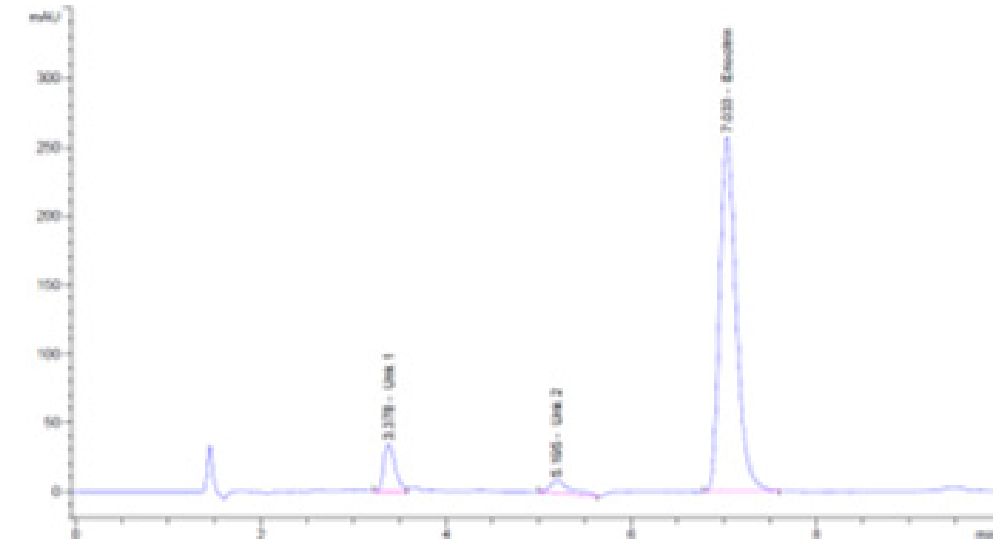
c)



d)



e)



f)

Figure S4: Representative standard Eriocitrin chromatograms at different concentration levels (10–60 µg/mL).

Table S1: ANOVA for Linear model.

Retention time						
Source	**Sum of Squares**	**d_f**	**Mean Square**	**F-value**	***p*-value**	
Model	54.93	3	18.31	7.41	0.0055	significant
A-Methanol concentration	45.00	1	45.00	18.20	0.0013	
B-Flow Rate	9.88	1	9.88	4.00	0.0709	
C-Injection Volume	0.0453	1	0.0453	0.0183	0.8948	
Residual	27.19	11	2.47			
Lack of Fit	27.19	9	3.02	4778.19	0.0002	significant
Pure Error	0.0013	2	0.0006			
Cor Total	82.12	14				
Tailing Factor						
Source	**Sum of Squares**	**d_f**	**Mean Square**	**F-value**	***p*-value**	
Model	0.0044	3	0.0015	3.60	0.0496	significant
A-Methanol concentration	0.0036	1	0.0036	8.81	0.0128	
B-Flow Rate	0.0006	1	0.0006	1.49	0.2471	
C-Injection Volume	0.0002	1	0.0002	0.4880	0.4993	
Residual	0.0045	11	0.0004			
Lack of Fit	0.0045	9	0.0005			
Pure Error	0.0000	2	0.0000			
Cor Total	0.0089	14				
Theoretical Plate Numbers						
Source	**Sum of Squares**	**d_f**	**Mean Square**	**F-value**	***p*-value**	
Model	0.0000	0				
Residual	7.987E+06	14	5.705E+05			
Lack of Fit	7.927E+06	12	6.606E+05	22.15	0.0440	significant
Pure Error	59654.00	2	29827.00			
Cor Total	7.987E+06	14				

Table S2: AGREE scoring.

Principle	Assessment	Score
Direct analytical technique	Sample dissolution required	0.3
Minimal sample size	40 µL injection	0.8
In-situ measurements	No	0
Reduced sample preparation	Simple dissolution only	0.8
Automation	Conventional HPLC	0.5
Reduced derivatization	None used	1.0
Reduced waste	~7.7 mL/run mobile phase	0.4
Multi-analyte capability	Single analyte	0.3
Energy efficiency	HPLC-PDA	0.5
Renewable reagents	No	0.2
Safer solvents	Methanol + ACN	0.2
Operator safety	Moderate	0.4

Table S3: BAGI scoring.

Criterion	Score
Instrument availability	10
Reagent availability	10
Sample preparation simplicity	9
Throughput	7
Cost effectiveness	7
Automation	5
Safety	6
Ease of implementation	8
Number of analytes	5
Waste management	5.5
Total	72.5