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APPLICATION OF DESIGN OF EXPERIMENTS FOR ENHANCED ESTIMATION OF QUERCETIN IN *AVERRHOA CARAMBOLA* EXTRACT USING HPTLC

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ABSTRACT

The current article highlights the estimation of quercetin in Averrhoa carambola fruits by High-Performance Thin Layer Chromatography (HPTLC). Averrhoa carambola is commonly called a Starfruit. The fruits of the plant comprise a number of constituents, mainly rich in flavonoids. Quercetin, Gallic acid and Epicatechin are the flavonoids present in starfruit. Quercetin amounts to a wide range of activities which include anti-inflammatory, anti-diabetic and also in preventing cardiovascular diseases. HPTLC is an advanced sophisticated analytical technique of thin layer chromatography. It has the primary ability to determine the accuracy and specific identification of the compounds in the plants. We used aluminium back-coated silica gel of 60F 254 stationary phase and mobile phase of Toluene: Propanol: Glacial acetic acid: n-Hexane (7:3:1:1v/v/v/v) with dosing speed of 20µL/sec and 5x5mm band length and width at 254 nm detection wavelength. Quercetin was developed in ascending mode and quantified by using JustTLC software. Rf value of quercetin was found to be 0.76. To supplement the result the developed method was validated for system suitability, accuracy, linearity, precision, LOD, LOQ and robustness according to ICH guidelines. We finally conclude that the estimation of quercetin by the HPTLC method was found to be simple, precise and accurate and can be carried out for routine analysis.

Keywords: Quercetin; Averrhoa Carambola; HPTLC

INTRODUCTION

Biologically active compounds such as alkaloids, flavonoids, phenolic acids, and terpenoids are found in plants and many edible plants. Flavonoids are one of the most important groups of secondary metabolites. Among the 5000 varieties of flavonoids, quercetin (QC) is the most extensively studied dietary flavonoid obtained from green leafy edible vegetables, onions, apples, and green tea. Quercetin **[Figure 1]** is a water-soluble plant pigment consisting of three rings and five hydroxyl groups, with several health benefits, including antioxidant, anti-inflammatory, anticancer, antiviral, improvement of cardiovascular health, asthma, canker sores, neurological disease, and diabetes **[1-5]**.

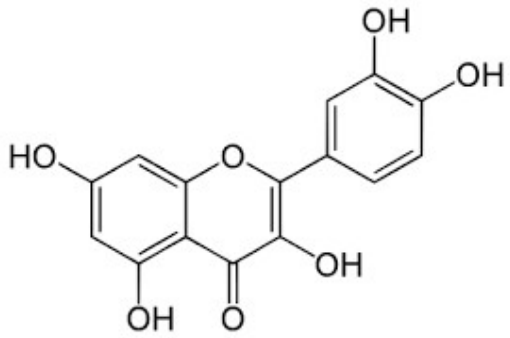


Figure 1: Structure of Quercetin

Starfruit (SF) is an edible fruit, also referred to as carambola (Averrhoa carambola and cross-sectionally resembles a star when cut. It is primarily grown in the tropical regions of the world, including Southeast Asia, the USA, and the Caribbean. It is eaten to relieve sore throats and can also be used as a mordant in dyeing. SF principally contains phytochemicals, such as quercetin, gallic acid, and epicatechin. Several flavonoids have been isolated from the fresh sweet fruit of Averrhoa carambola (AC), including some previously unreported compounds **[6]**. These flavonoids exhibit antioxidant activity, with some showing more potent ABTS radical cation scavenging activity than ascorbic acid. Additionally, some of these compounds show weak inhibitory activity against porcine pancreatic lipase **[7]**. Although quercetin is not explicitly mentioned, SF contains a variety of bioactive compounds, including flavonoids, benzoquinones, and their glycosides, which are responsible for various biological activities. These compounds contribute to antioxidant, anti-hyperglycemic, anti-inflammatory, and other beneficial properties. The presence of such a diverse range of bioactive compounds highlights the potential of Averrhoa carambola in the development of functional foods and pharmaceutical products **[8]**.

Many herbal medicines and formulations containing quercetin are commercially available. We have reviewed various scientific studies published on quality control analysis and standardization of quercetin in its isolated form, extract, or any other herbal or polyherbal preparation using various analytical techniques, such as spectroscopic, UV-VIS spectroscopy **[9-13]**, photoluminescence **[14]**, chromatographic, HPTLC **[15-33]**, HPLC **[34-56]**, UPLC **[57, 58]**, LC-MS **[59-61]**. However, a systematic approach to the analytical method designed for chromatographic quantification of QC from selected fruit extracts has not yet been reported.

Therefore, in current research, we have attempted to use of the analytical design of experiments (DOE) to quantify phytoconstituent such as QC from AC fruit extracts by HPTLC. Using the DOE approach, we developed a fast, sensitive, robust, precise, and economical HPTLC method for estimating the QC in SF extracts. This was accomplished by applying a combination of experimental design and mobile phase optimization, in which toluene, propanol, glacial acetic acid, and n-hexane were selected as variables. A reliable method was developed based on these variables at the retention time of QC **[62, 63]**.

The best experimental design approach for modelling and optimization is response surface design. In the present study, a box-bhenken (BB) was used to optimize the chromatographic conditions of the HPTLC method **[64]**. This study aimed to develop a rapid, precise, and accurate HPTLC method using a DoE approach for the quantitative analysis of QC and validate the method according to the ICH guidelines ICH Q2 (R1) **[65]**.

MATERIALS AND METHODS

Plant collection and identification: In this work, the fresh fruits of Averrhoa carambola were collected and were authenticated by Botanical Survey of India, Andhra Pradesh.

5.1. Materials

Analytically pure QC was purchased from Sigma–Aldrich (India). SF was procured from a local market. All solvents and chemicals used were of analytical grade and were purchased from Merck Specialities Pvt. Ltd., India.

Preparation of Standard solution

Standard QC (10 mg of standard QC was accurately weighed in a 10 ml volumetric flask with ethanol (1000µg/ml). Aliquots of the stock solution were appropriately diluted with ethanol to obtain a working standard of 100 µg/mL QC.

5.2. Preparation of working Standard solution

Averrhoa carambola fruits were collected, air-dried, and powdered. The Extraction process was achieved by placing the powder in a Soxhlet apparatus using ethanol for four

days. The collected solvent was evaporated using a rotary film evaporator at bath temperatures of 10ºC and 50ºC with a 100rpm rotating speed. The residue was collected and dissolved in ethanol for further analysis.

5.3. HPTLC Instrumentation

An Aetron manufactured High Performance Thin Layer Chromatography instrument was used with a sample applicator to document and quantify the compound documentation system, and TLC software was used. Sampling was performed by using a Hamilton syringe. Hamilton-Bonaduz Schweiz, Camag, Switzerland), precoated silica gel aluminium Plate 60 F254, (10 cm × 10 cm, 100µm thickness; E. Merck, India), A Soxhlet apparatus and rotary film evaporator were used to extract QC from SF fruits.

Chromatography specifications

Standard solutions of different concentrations were spotted with a micro syringe in the form of bands with a bandwidth of 6 mm on a pre-coated silica gel aluminium plate 60 F254 using a sample applicator. The linear ascending development was performed in a twin-trough glass chamber. Gradient elution was used with a mobile phase composed of toluene: propanol: glacial acetic acid: n-hexane (7:3:1:1v/v/v/v).

The injection volume was 20µL, and the dosing speed was 20µL/sec. Densitometric scanning was performed using the Just TLC scanner software. All measurements were performed in reflectance–reflectance–absorbance mode at 254 nm. The concentrations of both drugs were determined based on the intensities of diffusely reflected light, and the data were evaluated using ordinary linear regression analysis of peak areas.

Table 1: Chromatographic Conditions

Parameters	Conditions
Stationary Phase	Aluminium back-coated silica gel of 60F 254
Mobile phase	Toluene: Propanol: Glacial acetic acid: n-Hexane (7:3:1:1v/v/v/v)
UV detection wavelength	254nm
Dosing speed	20µL/sec
Band length and width	5×6mm
Runtime	10 mins

Preliminary HPTLC analysis: Initially HPTLC trials were carried out employing solvents like toluene, n-butanol, propanol, n-hexane, formic acid, and glacial acetic acid in varying proportions as mobile phase. However, the issue concerning large Rf value and overlapping's were identified. The addition of propanol resulted in the improvement of Rf value. Considering the information acquired from the trials, a mixture of toluene, propanol, glacial acetic acid and n-hexane (7:3:1:1 v/v/v/v) was

selected as mobile phase for fair estimation of quercetin. The trials suggested that there was a major influence of chromatographic method conditions on the RF value of quercetin.

Method development and Enhancement of method parameters using DoE:

Based on the initial trails, Design of experiment (DoE) approach was also used in present study, to characterise and comprehend how the method conditions affect analytical output for better comprehension of the process. The aim of this study was to define the optimum conditions for separation of the quercetin and to investigate how the method parameters influenced the identified critical characterization attribute (CA) (RF value) in accordance with the pre-defined objective.

Figure 2: Process of workflow

Factor screening studies: The Design of Experiments (DOE) approach was used to develop an HPTLC estimation method for quercetin. A Box-Behnken full factorial design was used to select lower and upper parameter values. A four-factor one-level Box-Behnken design (BBD) was applied for mobile phase condition optimization using Design-Expert 13 software (Stat-Ease Inc., Minneapolis, USA). To evaluate the influence of the independent variables on the responses, response surface analyses were performed (Table), showing 29 experiments obtained using a Box-Behnken design (BBD) with the respective observed and predicted responses. ANOVA was used to

statistically analyze the responses. A numerical optimization procedure, the desirability function, was applied to select the optimum conditions.

BBD has the advantage of optimizing experiments by using a 4k-factorial design (where k=1, 2, 3) with at least four dependent variables or factors and one response, compared to other experimental designs, such as central composite design (CCD) and fractional factorial design (FFD). The general polynomial quadratic model is expressed as follows:

$$Y = \beta0 + \beta1\ X1 + \beta2\ X2 + \beta3\ X3 + \beta12\ X1X2 + \beta13\ X1X3 + \beta23\ X2X3 + \beta11\ X1^2 + \beta22\ X2^2 + \beta33\ X3^2 + \cdots$$

The measured response, Y, was connected to each combination of factor levels in a specific manner. β0 is a fixed value, whereas β1, β2, and β3 are the linear coefficients where β12, β13, and β23 are the interaction coefficients of the four factors. β11, β22, β33, and β44 are quadratic coefficients derived from the observed experimental values of Y in the experimental runs. A, B, C, and D are the coded levels of the independent variables: high (+), low (−), and center point (0). The terms AB and A2 represent the interaction and quadratic terms, respectively.

After inputting the data into the Design Expert software, the fit summary was applied to the data, resulting in the suggestion of a "quadratic model" by the software. This model provides a polynomial equation in coded terms, which can be used to predict the response for the specified levels of each factor. The equation in terms of coded factors is beneficial for determining the relative influence of the factors by comparing the coefficients of each factor. By default, the high levels of the factors were coded as +1, and the low levels were coded as -1. The coded equation is useful for identifying the relative impact of these factors.

DOE Optimization Findings: A technique known as response surface modeling was utilized for optimization using both numerical and graphical methods. The desirability function, which ranges from zero outside the limits to one at the target, serves as the objective function. The highest value of the desirability function was determined through numerical optimization. The equation, represented by the coded factors, assists in predicting the response for specific factor levels. The high levels of the factors were designated as +1, while the low levels were designated as -1 by default. This coded equation was valuable for assessing the relative impact of each factor by analysing the coefficients.

Table 2: Optimization of HPTLC method parameters using a box-benkhen

Std	Run	Factor 1 Toluene	Factor 2 Propanol	Factor 3 Glacial acetic acid	Factor 4 n-Hexane	Response Rf
8	1	1	2	0.5	0	0.35
28	2	1	3	0.5	0.5	0.32
22	3	1	2	0.5	1	0.38
10	4	1	1	0.5	0.5	0.53
16	5	1	2	1	0.5	0.37
14	6	1	2	0	0.5	0.49
21	7	4	2	0.5	0.5	0.53
4	8	4	3	1	0.5	0.68
27	9	4	2	1	1	0.54
19	10	4	3	0	0.5	0.6
15	11	4	1	0.5	0	0.59
7	12	4	2	0.5	0.5	0.63
24	13	4	1	0	0.5	0.59
11	14	4	1	1	0.5	0.6
18	15	4	2	0	1	0.6
1	16	4	3	0.5	0	0.58
6	17	4	2	0.5	0	0.6
29	18	4	2	0.5	0.5	0.57
20	19	4	3	0.5	1	0.62
17	20	4	1	0.5	1	0.6
13	21	4	2	0.5	0.5	0.56
26	22	4	2	0.5	0.5	0.63
5	23	4	2	0	0	0.59
23	24	7	2	0.5	0	0.66
2	25	7	2	0.5	1	0.74
3	26	7	1	0.5	0.5	0.62
25	27	7	2	0	0.5	0.71
9	28	7	2	0.5	0.5	0.79
12	29	7	3	1	1	0.74

Method validation of proposed HPTLC method: The validity of the proposed analysis approach was confirmed by employing ICH guidelines for evaluation criteria such as linearity, precision, system suitability, accuracy, limit of detection (LOD), and limit of quantification (LOQ), robustness.

Linearity: Linearity refers to the capacity of a sample within a specific range to clarify the relationship between the concentration and the amount of analyte in an analytical process. The linearity of quercetin was evaluated by serially diluting the corresponding stock solutions with a suitable diluent, which produced calibration curves encompassing a concentration ranges of 100-500 µg/ml. Calibration curves were generated by plotting concentration levels against the corresponding mean peak areas.

Precision: Precision refers to the degree of scattering (or closeness of agreement) between a series of measurements obtained from multiple homogeneous samples performed under the established conditions of an analytical procedure. The percentage relative standard deviation (RSD, %) based on six replicate injections of the standard solution mixture (100% of the target concentration) applied to HPTLC plates to

obtain bands corresponding to concentration i.e. 300 µg/ml.

System suitability: This parameter determines whether the conditions for performing the analysis are acquired by determining the appropriateness of developed method. The system suitability parameters were measured to evaluate the system performance. Quercetin was included in the HPTLC system as a solution mixture. System suitability was performed by the application of six replicate injections of the solution in order to acquire the peak area. The system suitability was expressed as relative standard deviation (RSD) % of peak area.

Accuracy (%recovery): Correctness, trueness, or analytical accuracy is the degree of agreement between the measured value and accepted conventional true value or accepted reference value. The method was developed by adding 50%, 100%, and 150% standard solutions to the sample at three different levels. The recoveries and % RSD were determined by analysing the resultant mixtures. The accepted mean recovery window was set between 98% and 102%, and all the values collected from the samples were within this range, indicating adequate recovery rates.

Detection and Quantification limits: The lowest concentration of an analyte in a sample that can be identified but not quantified is the detection limit, which is calculated with the formula;

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

where, σ – Standard deviation

S – Slope of calibration curve

The lowest concentration of the analyte in a sample that can be determined with the appropriate precision and accuracy is the quantification limit, which is calculated with the formula;

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

where, σ – Standard deviation

S – Slope of calibration curve

The limits of Detection and Quantification were determined according to a method based on three times the standard deviation (SD) of the response at concentrations close to the detection and quantification limits.

Robustness: The robustness is performed for the critical factors such as mobile phase composition, dosage speed, and band width that affect the response and the analysis of the Rf and peak area were determined.

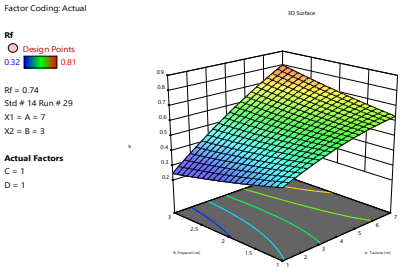


Figure 3

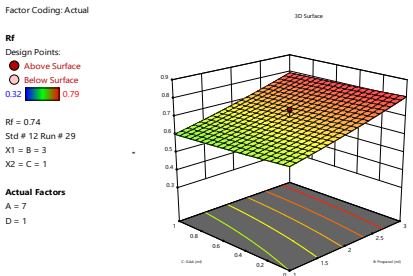


Figure 4

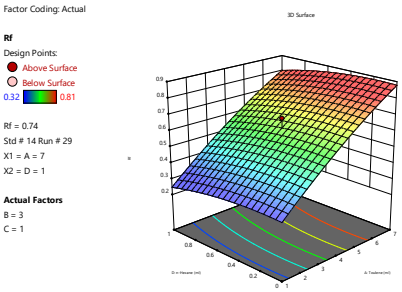


Figure 5

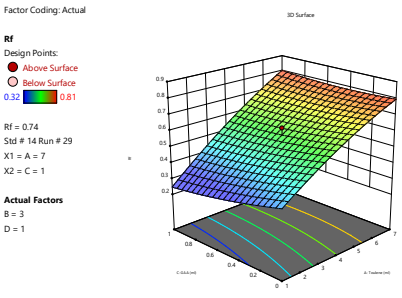


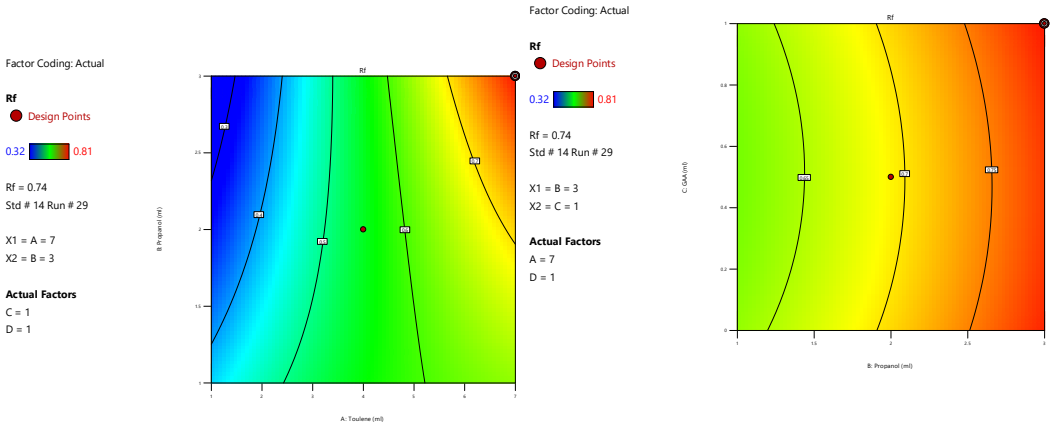
Figure 6

Figures 3,4,5,6- 3D response surfaces Effect of factor Toulene, Effect of factor Propanol, Effect of factor Glacial Acetic acid, Effect of factor n-Hexane on response Retention factor for Quercetin

Table 3: Summary analysis of ANOVA results and Final Equation in Terms of Coded Factors for Quercetin

ANOVA parameters	Retention Factor
Adjusted R2	0.7691
Predicted R2	0.4136
P value	0.0002
R2 value	0.8845
Suggested model	Quadratic
Y(Retention Factor) = 0.5840+ 0.1642- 0.0008- o.0108+ 0.0092+ 0.0775+ 0.0175+ 0.0125+ 0.0175+ 0.00175- 0.0175- 0.0208+ 0.004+ 0.0392- 0.0207	

Figures: 2D contour plot for factors affecting the retention factor of Quercetin



Factor Coding: Actual

Rf

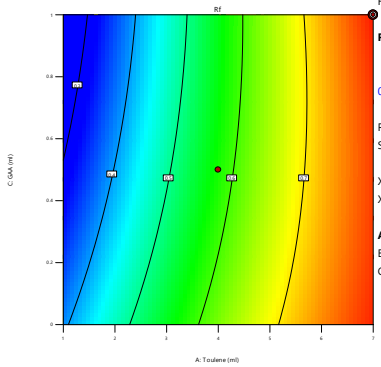
Design Points

0.32 0.81

Rf = 0.74
Std # 14 Run # 29

X1 = A = 7
X2 = C = 1

Actual Factors
B = 3
D = 1



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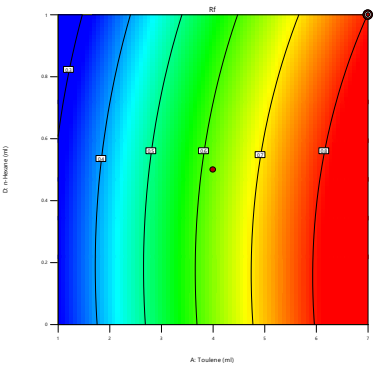


Overlay plot of Quercetin

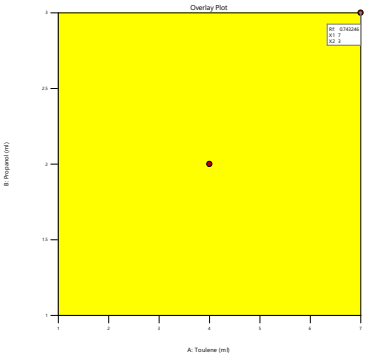
Factor Coding: Actual

Overlay Plot
Rf

Design Points

X1 = A
X2 = B

Actual Factors
C = 1
D = 1



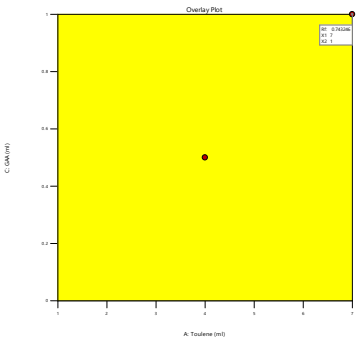
Factor Coding: Actual

Overlay Plot
Rf

Design Points

X1 = A
X2 = C

Actual Factors
B = 3
D = 1



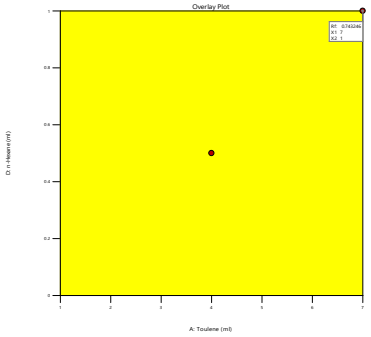
Factor Coding: Actual

Overlay Plot
Rf

Design Points

X1 = A
X2 = D

Actual Factors
B = 3
C = 1



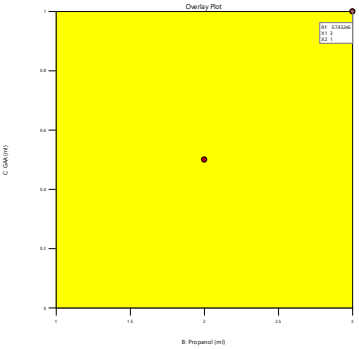
Factor Coding: Actual

Overlay Plot
Rf

Design Points

X1 = B
X2 = C

Actual Factors
A = 7
D = 1



Method validation of proposed HPTLC method

Linearity: The data for the linear regression obtained for an accurate calibration curve showed linear relationship of high standard over a wide concentration range of 100-500µg/ml for quercetin **(Table 4)**.

Table 4: Linearity Data

S. No	Concentration	Area
1	100	189
2	200	375
3	300	512
4	400	775
5	500	945

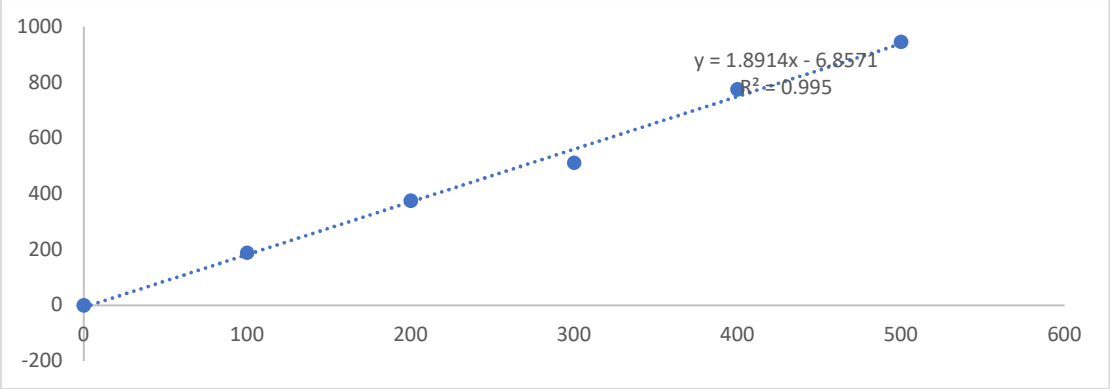


Figure 7: Linearity graph

Precision: The percentage relative standard deviation (RSD, %) was less than 2% for the analyte. This indicated that the repeatability of the process was acceptable. A summary of the precision observations for the method and system is presented in **Tables 5, 6**.

Table 5: Data of Method Precision

S. No	Concentration	Area
1	300	588
2	300	585
3	300	584
4	300	592
5	300	594
6	300	586
	Average	588.17
	SD	4.020
	%RSD	0.68

Table 6: Data of System Precision

S. No	Concentration	Area
1	300	586
2	300	589
3	300	594
4	300	583
5	300	584
6	300	583
	Average	586.50
	SD	4.326
	%RSD	0.74

Accuracy: The accuracy of the proposed HPTLC method demonstrated through recovery studies performed by spiking sample with pure drug at 50%, 100%, and 150% indicated good recovery of the quercetin with % recovery in the range of 99.83-100.20% **(Table 7)**.

Table 7: Data of Accuracy

	Std. Conc	Spiked Conc.	Std.Area	Std. Area+Spiked	Spiked Area	%Recovery	Mean %Recovery
50% Level	300	100	587	784	197	100.68	99.83
	300	100	587	781	194	99.15	
	300	100	587	782	195	99.66	
100% Level	300	300	587	1172	585	99.66	100.17
	300	300	587	1176	589	100.34	
	300	300	587	1177	580	100.51	
150% Level	300	500	587	1565	978	99.97	100.20
	300	500	587	1568	981	100.27	
	300	500	587	1569	982	100.37	

System Suitability:

As shown in **Table 8** injections resulted in peak areas within a single range. These results indicate that the proposed method is specific. The system suitability fell within the acceptance criteria limit (within the %RSD limit).

Table 8: Data of System Suitability

S. No	Concentration	Area
1	300	583
2	300	585
3	300	591
4	300	598
5	300	590
6	300	584
	Average	586.83
	SD	3.311
	%RSD	0.56

Detection and Quantitation Limits:

Because the limit of detection was estimated to be 0.58, which is acceptable (i.e.,< 3), the alternate hypothesis was accepted.

For a specific analytical procedure, the quantification limit can be defined. The quantification limit was found to be 2.07, which is within the accepted range (i.e., <10) so you can consider it.

Robustness:

Determination of quercetin in Averrhoa carambola extract

The analysis was performed on the extract of Averrhoa carambola which resulted in the obtaining og the densitogram of extract with well resolved peak at Rf 0.74 for quercetin. The PDA spectral scan of the separated band at 300 nm and the UV spectra generated exactly superimposed with the standard spectra indicating that there was no interference from other components present in the extracts.

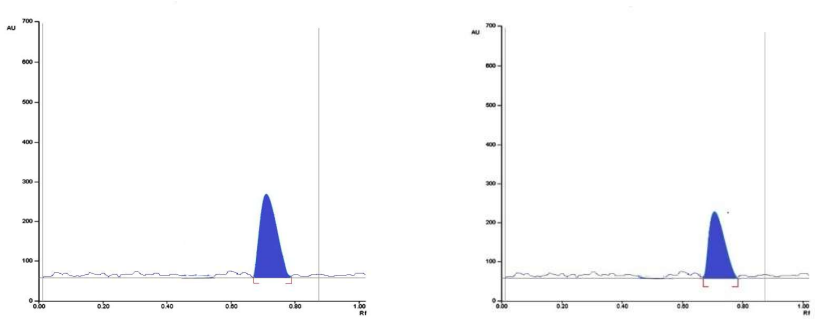


Figure 8: Densitograms of quercetin as per design expert software

RESULTS AND DISCUSSION

The 3D response surface plots and polynomial equations derived from the models indicated that the chosen factors had a notable impact on the responses of quercetin. The desirability and overlay plots employed provided appropriate solutions that were experimentally validated. Under the optimized conditions, the RF value of 0.74±0.02 for quercetin. The calibration curve exhibited a good linear correlation coefficient (r2 = 0.995). The linearity range was 100–500 µg/spot, and accuracy of 100.06%. The system and method precision with %RSD<2%. The limits of detection (LOD) and quantitation (LOQ) of quercetin were 0.58 and 0.27 µg/ml, respectively.

DOE Optimization Result:

Optimization was performed based on response surface modelling using numerical and graphical optimization methods. Desirability is an objective function that ranges from zero outside the limits to one at the goal. The numerical optimization determines the point that maximizes the desirability function.

CONCLUSION

A sensitive, accurate and robust HPTLC method has been developed for the estimation of quercetin in ethanolic extract of Averrhoa carambola using a set composition of mobile phase comprising of [(toluene: propanol: glacial acetic acid: n-Hexane solution (7:3:1:1 v/v/v/v)] and densitometric analysis at 300 nm. The chromatographic conditions were screened and then optimized by DoE approach involving use of box-benkhen method using Design Expert software. The current study describes, for the first time, a constant mobile phase composition to achieve separation of quercetin and its successful application in estimation in Averrhoa carambola extract. This approach can be applied for the determination of quercetin in other herbal extracts thus minimizing time and could serve as a cost-effective analytical tool.

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