https://doi.org/10.48047/AFJBS.6.Si4.2024.1644-1656

African Journal of
BiologicalSciences

Probing the influence of system variables on the liquid chromatographic estimation of Naringin from bulk drug and pharmaceutical dosage form using Quality by design (QbD) approach

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Article History
Volume 6, Issue Si4, 2024
Received: 01 June 2024
Accepted : 30 June 2024
Doi:
10.48047/AFJBS.6.Si4.2024.1644-1656

Background:

Naringin (4, 5, 7-trihydroxy flavanone-7-rhamnoglucoside) is a bitter flavonoid present in several citrus fruits, such as grapefruits (Citrus paradise) and citrus herbs including fruits of C. aurantium, C. grandis var. tomentosa and C. maxima. It exhibits various pharmacological activities including antimicrobial, antioxidant, antitumor, anti-inflammatory, and anti-allergic [8]. Here, it is necessary to realize that naringin is hydrolyzed to naringenin by gut flora prior to being absorbed.Regretfully, the clinical relevance of naringenin is limited by its low solubility and minimal bioavailability owing to its largely hydrophobic ring structure.

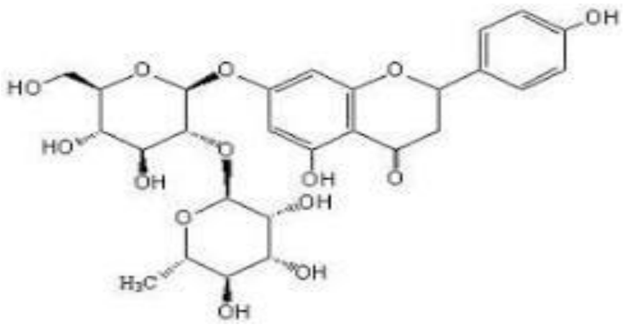


Fig. 1 : Chemical structure of naringin

Thus far various analytical tools such as IR, TLC, HPLC, UV, LCMS have been used for the analysis of naringin. Among all, HPLC is the method of choice owing to its good separation among the naringenin. The parameter like flow rate, mobile phase, column temperature influenced by HPLC separation are responsible for better separation hence optimization of these parameters are important [2]. Thus the key objective of present work was to develop validated stability indicating HPLC method for the determination of naringin by employing central composite design. Separation of naringin by design of experiments is the simple method used for optimisation to get better separation results of naringin. In this work, we used Central composite design (CCD) for optimisation of naringin using three independent variables flow rate, mobile phase and wavelength. To the best of our knowledge there is no reports regarding the use of central composite design for HPLC optimisation in analysis of naringin.

The Central Composite designs (Box and Wilson designs) are constituted of a full[1] , factorial [2] or fractional [3] design. The points at the centre of the experimental domain and the “star” points outside this domain make it possible to estimate the curvature of the response surface. A response surface is a geometrical representation of a response variable plotted as a function of the independent variables. This experiment provides more information about a dependent or response variable than a two-level factorial or fractional- factorial design. A three-level factorial design has a centre point included for each independent variable along with the high and low points, requiring three experiments for each independent variable. This is called a three-level factorial design because of the third factor level. Inclusion of the third factor greatly increases the number of experiments.

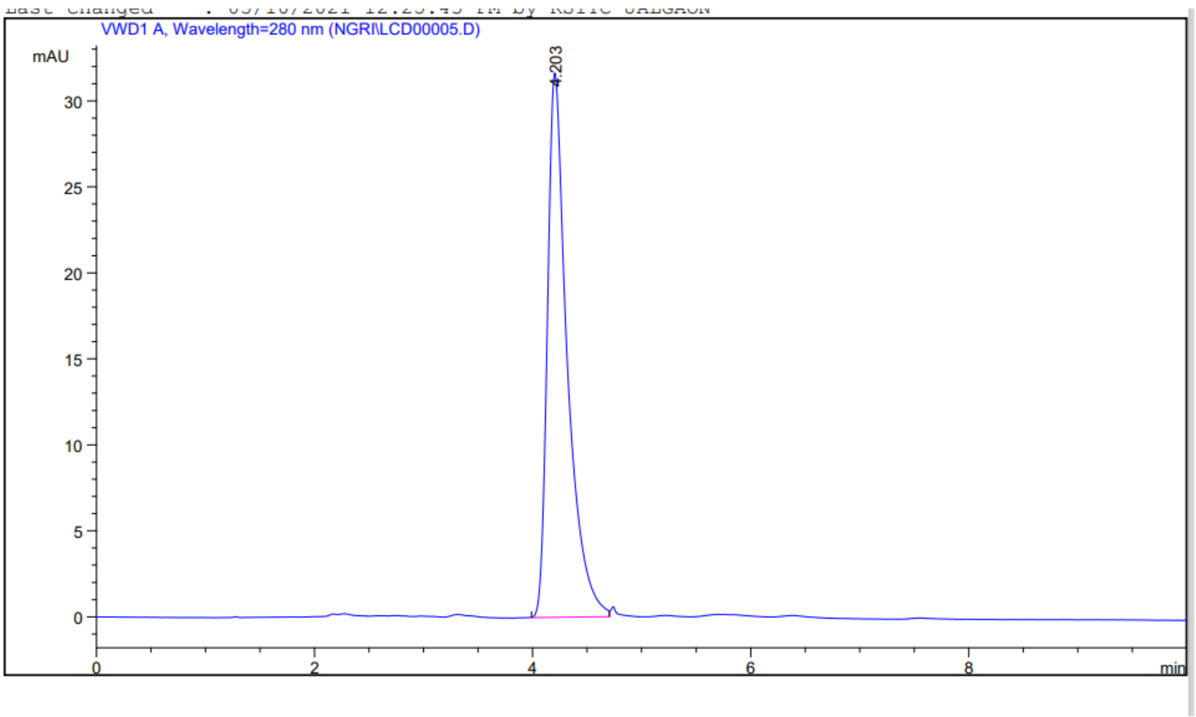


Fig. 2 HPLC Chromatogram of Naringin.

2.Theory:

2.1 Retention Time: It is the time between the point of sample injection and the analyte reaching the detector. It is shown as tR. Retention time of analyte is strongly influenced by polarity of mobile phase. The column temperature mostly important point of consideration for selecting a column and also it is strong determinant of retention time. An precision of retention time is achieved at 30-50 0 c while at temperature > 60 0 c thermal degradation of analyte takes place. [5,6]

2.2 Peak Area (PA): The peak area is directly proportional to the concentration of the sample. Peak area is generally useful for most quantitative determination of amount of particular component present in sample. It is less susceptible for flow variations. Peak area is useful for calculating the reproducibility and system linearity while retention time gives the data of pump repeatability. Hence in HPLC Method, the relationship of sample concentration and detector response is used to make determinations. [5,6]

2.3 Theoretical Plates(N): These are used for column efficiency and also useful for determining the number of peaks that can located per unit run time of chromatograph. It is calculated by, N = 16 (tR /W)2 (Equation 1) Where tR is the retention time and W is the peak width. Peak width is based on the baseline intercepts of the tangents lines of the Gaussian peak, which is equivalent to the peak width at 13.4% of the peak height. Flow rate of the mobile phase and column temperature will be affect on number of theoretical plates. [6,7]

Materials and methods:

Material:

Naringenin ≥ 95% was procured from Sigma Aldrich (St. Louis, MO, USA). Methanol and ortho phosphoric acid HPLC analytical grade methanol was procured from Merck life science pvt. Ltd ., Mumbai. HPLC grade water was acquired from Ranchem , India. The HPLC system used was an Agilent 1220 Infinity LS system with an autosampler. The column used was Nucleosil C-18 segment (4.6 mm I.D. × 250 mm) with UV detector.

3.2 Methods

3.2.1 Design of experiment (DOE): "The design of experiments" is a well-structured and well-organized process strategy for identifying the association between elements, having an impact on a process and its output. DoE is a fantastic approach that allows pharmaceutical person to modify parameters in a systematic manner according to a pre-determined design to obtain best suited results [08-10].

3.2.1.1 Screening of design variables: The critical method variables have a impact on absolute recoveries, retention time, theoretical plates, peak area and tailing factors are considered in a HPLC method and they are considered for screening analysis by using fractional factorial design. The dependent variables included flow rate, methanol and a wavelength, and studied at two levels low (-1) and high (+1) as summarized in Table 1. The Design-Expert® Software version 11 recommended a total of 14 experiments. All the 16 experiments were carried out to identify CMVs that have a considerable impact on the ARs of the HPLC method.

Table 1 List of independent factors and their levels used for screening design

Name	Units	Level Low (− 1)	Level High (+1)
Conc. Of Methanol	%	49	53
Flow rate	ml/min	0.85	1.05
Wavelength	nm	279	283

3.2.1.2 Optimization design: According to the screening investigation, parameters that have a significant impact on the responses were chosen, and further study was carried out using 32 full factorial designs. MeOH concentration, flow rate, and wavelength for some instances were proved to affect the Critical method variables. For this, there are two types of optimizations; one is graphical and other is numerical optimization if there are more than three responses. The STATISTICA program was used to plot the response surface. The obtained data were subjected into various models, but the final model was chosen for future experiments based on the highest F-value, P-value, and R2, and highest desirability found was selected as optimized batch (Tables 2 & 3).

Table 2 Screening variables and their levels (in coded and actual) used for 3^2 factorial designs

Independent variables	Level used	actual (coded)	
X1=Flow rate (ml/min)	(− 1) 0.85	(0) 0.95	(+ 1) 1.05
X2= wavelength (nm)	279	281	289
X3=Methanol (%)	49	51	53

Development of method and validation process: The optimized chromatographic process for the determination of FBP was validated as per the ICH guidelines Q2 (R1) for linearity, accuracy, intra-day and inter-day precision, limit of quantification and limit of detection, repeatability, robustness, and assay study. Both intra-day precision and inter-day precision were performed at six replicates of concentration levels. The % RSD measured for inter-day compared to intraday accuracy is high due to the high-end stability of the solvent. Assay of Curcumin (20 µgm/ml) was performed. The accuracy has been determined was calculated. In standard graph of calibration, the value of drug content was calculated through regression equations. The robustness is the capacity to remain unaffected where any small change in process parameters do not lead to any change in its reliability during the day-today usage. The method was investigated by intentionally altering the process, such as by changing the mobile phase's flow rate, its percentage of organic content, or its wavelength [11].

Table 3: Full factorial design matrix (in coded level) along with optimized formulation and experimental data

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
Std	Run	A:Flow rate	B:Methanol	C:Wavelength	RT	PA	TP
		ml/min	%	nm	min	AUC	
2	1	1	50	280	4.473	440.377	6394
10	2	1.05	51	281	4.114	429.468	6370
14	3	0.95	51	283	4.508	489.125	6662
12	4	0.95	53	281	4.112	474.765	6653
8	5	1	52	282	4.032	458.78	6397
6	6	1	50	282	4.401	458.051	6515
9	7	0.85	51	281	4.861	526.433	7182
5	8	0.9	50	282	4.789	507.393	6972
1	9	0.9	50	280	4.865	488.291	6853
13	10	0.95	51	279	4.371	449.477	6775
11	11	0.95	49	281	4.867	474.827	6538
4	12	1	52	280	3.944	441.213	6553
7	13	0.9	52	282	4.376	510.901	6898
3	14	0.9	52	280	4.37	489.768	6880

System suitability: The system suitability test verifies that the HPLC is sufficiently precise, most specific, and repeatable for the analytical estimations. The tests were carried out by injecting any sample six times in a row. Peak area, theoretical plate, retention time, and tailing factor are the system suitability parameters and are represented as an %RSD.

Response surface methodology analysis and optimization model validation: An aggregate of fourteen runs for factor optimization were done by employing 32 full factorial design, and the impact of three independent variables was analyzed using factorial structure based on the dependent variables (responses, Table 3). The following equation is standard equation showing the correlation of critical factors and the analytical responses.

$Y = \beta0 + \beta1X1 + \beta2X2\ 2 + ... + \beta kXk\ 1$

Y is the expected outcome value for the polynomial model and β represents the regression coefficients 1 to k for each degree and β0 is the Y intercept. The model is only a general linear regression model with k predictors raised to the power of I where i=1 to k. A quadratic expression follows second-order (k=2) polynomial forms. X1, X2, X3 are the critical factors which are depending on the correlation of factors and responses [12-13]

Variables effect on retention time (Y1): Figures 2 and 3 (A1-A3 and B1-B3) graph shows the effect of independent factors (X1, X2 and X3) over dependent response retention time (Y1). Final Equation in Terms of Coded Factors Y1 (RT, min) = 4.43 – 0.1903X1 – –0.2073X3 + 0.0564X1X3 + 0.0865X12 (2)

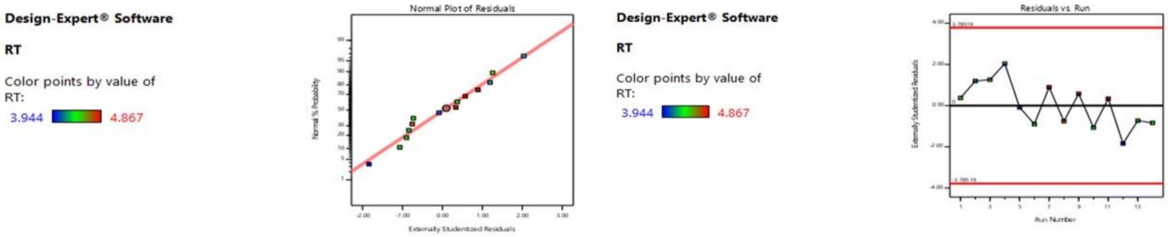


Fig 2 : plot showing true verses predicted value,with residual plot b for response Y1

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

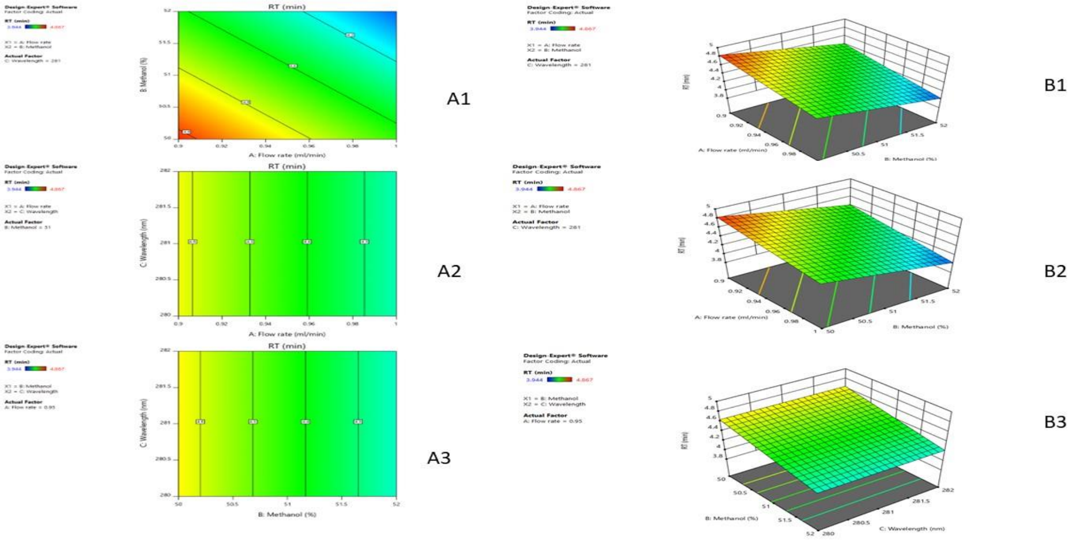


Fig 3: Relation plots (A1-A3, B1-B3) between true and measure values for Y1

Variables effect on peak area (Y2): Figures 4 and 5 (C1-C3 and D1-D3) show linear correlation plot for the response Y2 among true as well as measured values and the corresponding remaining graphs. Increase in flow rate makes the pinnacle zone (Area under curve) rise. Likewise, the peak area is additionally increased by increment in column temperature and amount of methanol in the mobile phase. Equation 3 shows impact of flow rate, column temperature and methanol concentration on peak area.

$Y2$ (Peak area) = $2792.84 - 121.18X1 - 32.41X2 + 10.12\ X1X3 + 0.4358\ X2X3 + 41.86X12$ (3)

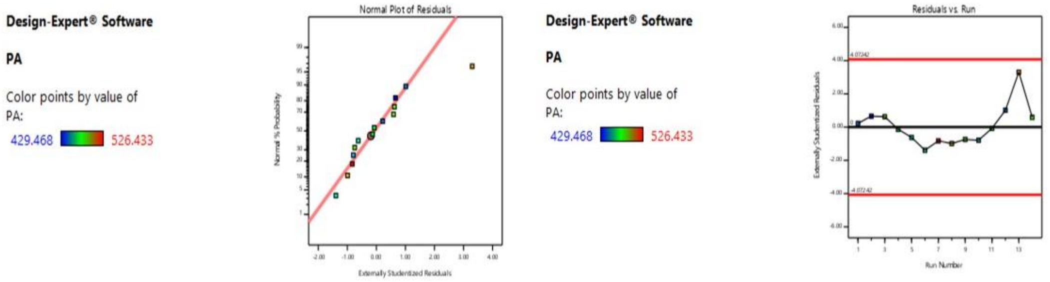
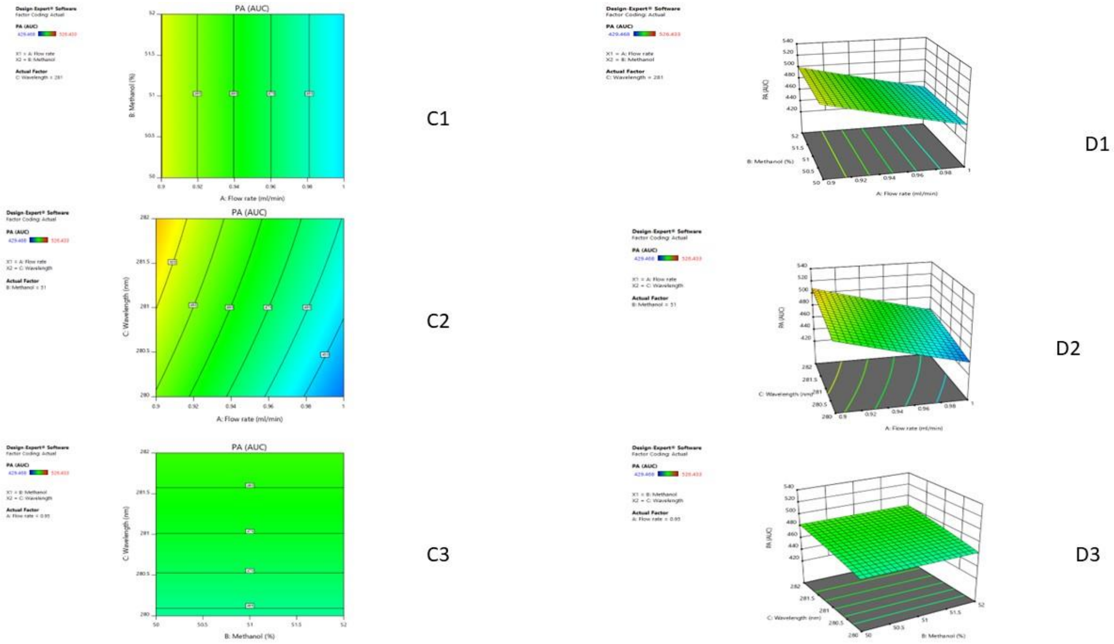


Fig 4: plot showing true verses predicted values with the residual plot for response Y2

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.



C1

D1

C2

D2

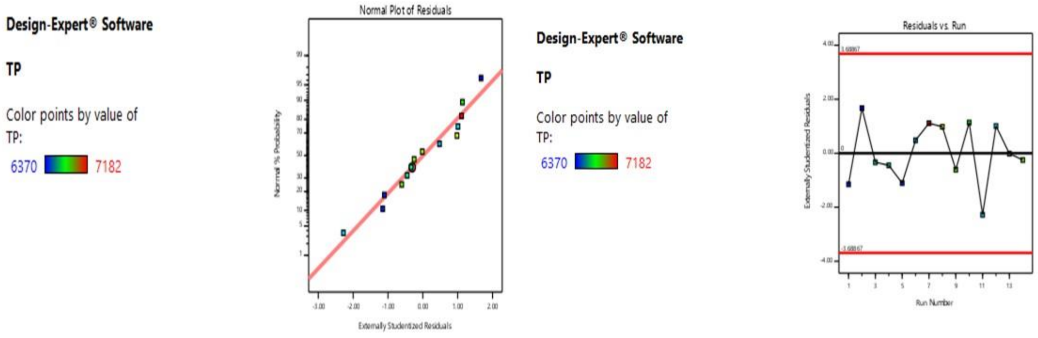
C3

D3

Fig 5: Relation plots (C1-C3, D1-D3) between true and measure values for Y2

Variables effect on tailing factor: Figures 6 and 7 (E1-E3 and F1-F3) show linear correlation plot for the response Y3 among true as well as measured values and the corresponding graphs. Final Equation in Terms of Coded Factors: Y 3 (TF) = 0.8148 + 0.079302X1 − 0.0041X2 (4).

This equation shows the positive impact of flow rate (X1) so whenever increase in flow rate there will be elevation of TF value and negative impact of column temperature (X2). The increase in temperature value decreases the tailing factor.



a

b

Fig 6: Plot (a) showing true verses predicted values and residual plot(b) for response Y3

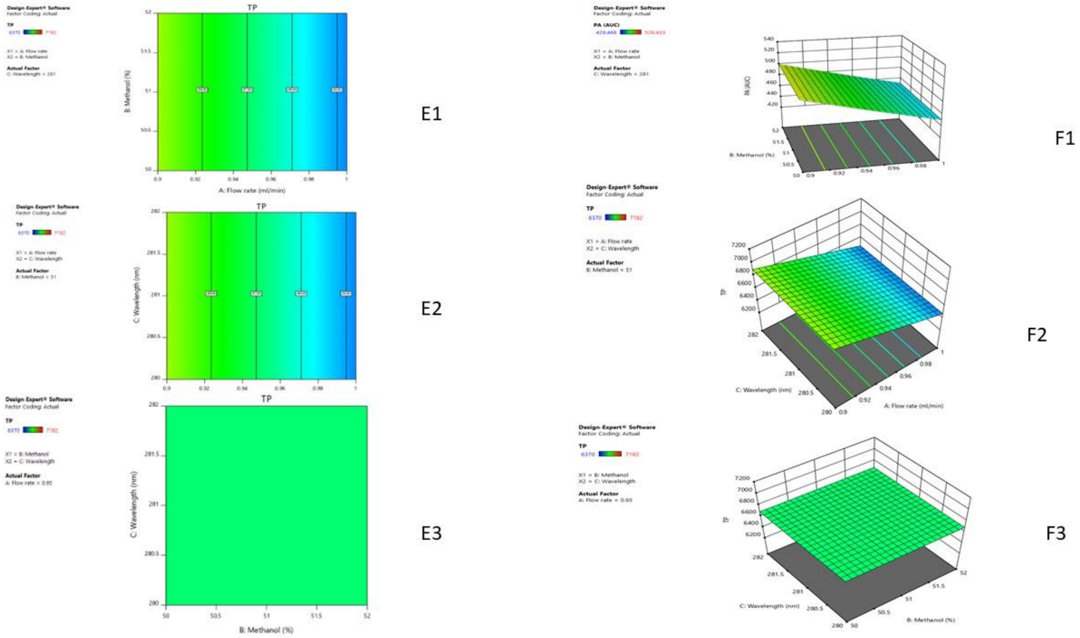


Fig 7: Relation plots (E1-E3, F1-F3) between true and measure values for Y3

Statistics of response All the dependent critical analytical responses are analysed statistically, i.e., RT, PA and TP. (Table 4)

Table 4 Statistics for the model of response.

Responses	Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	
Retention time	Linear	**0.0629**	**0.9698**	**0.9608**	**0.9402**	**Suggested**
	2FI	0.0668	0.9761	0.9556	0.8942	
	Quadratic	0.0574	0.9899	0.9673	0.8698	
	Cubic					Aliased

Peak Area	**Linear**	0.087	0.9899	0.9833	**0.9515**	**Suggested**
	2FI	0.092	0.9863	0.9826	0.9344	
	Quadratic	0.065	**0.9985**	**0.9940**	0.9221	
	Cubic					Aliased
Theoretical plate	**Linear**	**0.0043**	0.8913	**0.8805**	**0.8148**	**Suggested**
	2FI	0.8124	0.8621	0.8826	0.7973	
	Quadratic	0.3767	0.9834	0.9380	0.7640	
	Cubic					Aliased

Analysis of variance for the responses (Y1-Y3) Tables 5, 6, and 7show the ANOVA tables for various responses (RT, PA and TP).

Table 5 ANOVA results for Response 1: RT

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	1.27	2	0.6332	163.72	< 0.0001	significant
A-Flow rate	0.5791	1	0.5791	149.74	< 0.0001	
B-Methanol	0.6872	1	0.6872	177.70	< 0.0001	
Residual	0.0425	11	0.0039			
Cor Total	1.31	13				

Table 6 ANOVA results for Response 2: PA

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	11171.36	4	2792.84	1941.10	< 0.0001	significant
A-Flow rate	9597.25	1	9597.25	6670.36	< 0.0001	
C-Wavelength	1497.16	1	1497.16	1040.57	< 0.0001	
A^2	10.12	1	10.12	7.03	0.0264	
C^2	32.41	1	32.41	22.53	0.0010	
Residual	12.95	9	1.44			
Cor Total	11184.31	13				

Table 7 ANOVA results for Response 3: TP

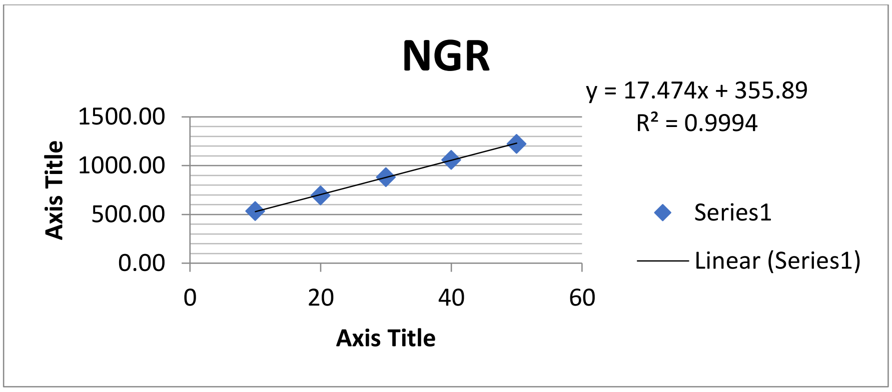
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	7.090E+05	1	7.090E+05	111.54	< 0.0001	significant
A-Flow rate	7.090E+05	1	7.090E+05	111.54	< 0.0001	
Residual	76274.86	12	6356.24			
Cor Total	7.852E+05	13				

Graphical and numerical optimization

Figure 8 shows the overlay plots showing relationship of factor and responses for graphical optimization.

Calibration curve:

The span of the linearity can be analysed by the standard solution of 10-50 μg/ml (r 2=0.9994, slope=106.76) (Fig. 8).



Validation

During validation all the graphs were clear, sharp, and very well without any impurities. Results for precision, the RSD percentage were less than 2. A recovery study is well utilized to determine the accuracy and the response of the peak area.

The ICH limit decides different parameters of linearity with the system variables. In FLP linearity test was executed at 5 separate levels. The suggested approach shows a great linearity span of 10, 20, 30, 40, 50µg/ml (r 2=0.9997).

LINEARITY

Table 8 Linearity study

Conc	Area I	II	III	IV	V	VI	Mean	SD	%RSD
10	535.6043	532.9675	355.8	17.47	178.49	10.22	534.29	1.86	0.35
20	695.6066	695.6010	355.8	17.47	339.80	19.45	695.60	0.00	0.0006
30	884.6823	883.7592	355.8	17.47	528.42	30.25	884.22	0.65	0.07
40	1061.049	1061.4126	355.8	17.47	705.43	40.38	1061.23	0.26	0.02
50	1224.913	1225.4146	355.8	17.47	869.36	49.76	1225.16	0.35	0.03
							Avrg SD	0.63	

PRECISION

Table 8 precision study

Conc	Area	II	III	IV	Mean	Amt Found	% Amt Fnd	SD	%RSD
20	698.8974	355.8	17.47	343.57	699.37	19.67	98.33	0.66	0.09
30	889.8872	355.8	17.47	534.10	889.90	30.57	101.91	0.02	0.00
40	1072.018	355.8	17.47	716.75	1072.55	41.03	102.57	0.75	0.07

INTRADAY PRECISION

Table 9 Intradayprecision study

Conc	Area	II	III	IV	Mean	Amt Found	% Amt Fnd	SD	%RSD
20	704.2919	355.8	17.47	346.08	701.88	19.81	99.05	3.41	0.49
30	893.9696	355.8	17.47	538.61	894.41	30.83	102.77	0.62	0.07

40	1059.553	**355.8**	**17.47**	**703.80**	1059.60	40.29	100.71	0.06	0.01

This method is used to determine the precision values of % RSD was found 0.49 for intra-day. The outcome has almost no effect on the parameters due to any little variation.

REPEATABILITY

Repeatability study was conducted and % RSD was found 0.05.

ASSAY

	Area						
Conc	I	C	M	CM	Amt Found	LC	% Label Claim
40.00	2158.265	**355.8**	**17.47**	**1802.47**	103.1749	2.579372	257.94
40.00	2148.279	**355.8**	**17.47**	**1792.48**	102.6033	2.565082	256.51
Mean	2153.27				102.89		257.22
SD	7.061				0.404		1.010
%RSD	0.328				0.393		0.393

ROBUSTNESS STUDY: The process parameters were checked for robustness study; it is found acceptable % RSD value less than 2 percent within the limits. The fact that there were no obvious alterations in the chromatograms suggested that the HPLC procedures that have been developed are robust (Table 10).

	Change flow							
FLOW RATE-1.5						**0.85 ml**		
Sr No.	Conc	**µgm/ ml**	Area		Sr No.		**µgm/ ml**	Area
1		40	774.7588		1	Conc	40	1107.736
2		40	778.1074		2		40	1101.448
		Mean	776.43				Mean	1104.59
		SD	2.37				SD	4.45
		%RSD	0.30				%RSD	0.40
MP			**50 meoh+50**		**MP**	**52 MEOH+48**		
	Sr No.	Conc	**µgm/ml**	Area	Sr No.		**µgm/ ml**	Area
	1		40	989.7109	1		40	987.6986
	2		40	988.2397	2		40	986.0645
			Mean	989.0			Mean	986.88
			SD	1.04			SD	1.16
			%RSD	0.11			%RSD	0.12

WAVE LENGTH CHANGE			282				280	
	Sr No.	Conc	µgm/ml	Area	Sr No.		µgm/ml	Area
	1		40	1003.291	1		40	959.95
	2		40	1003.082	2		40	959.4817
			Mean	1003.2			Mean	959.72
			SD	0.15			SD	0.33
			%RSD	0.01			%RSD	0.03

Limit of quantification (LOQ), limit of detection (LOD)

LOD and LOQ values determine the sensitivity of method. The lowest concentration can be detected by system is LOD, whereas LOQ is lowest concentration in analytes in stated sample determined under acceptable precision values. To obtain LOQ & LOD, actual drug concentration in linear range and calibration curve were used for 6 repetition assessments. LOD and LOQ values were 0.14µg / ml and 0.42µg / ml. (Table 11).

Table 11 Regression data for the calibration curve. (n=3)

Parameter	Result
Linearity range	10-50 µg/ml
r 2±%RSD	0.999±0.30
Slope±%RSD	17.47±0.30
LOD	0.1183
LOQ	0.3587

Conclusions:

Based on the study, it can be concluded that screening and optimization of analytical-dependant and independent factors and responses by using statistical designs and screened best factors affecting the process of optimization, developing, and validation of method. A new precise, reliable, quick, simple, analytical method can be developed and validated for determination of the curcumin. The use of the DoE approach for parameter screening aids in identifying crucial parameters that influence ARs of HPLC method for Naringin. The DoE software optimization design aids in optimizing the precise circumstances needed to build a most accurate and precise analytical method for Naringin.

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