PROBING THE INFLUENCE OF SYSTEM VARIABLES ON THE LIQUID CHROMATOGRAPHIC ESTIMATION OF CURCUMIN FROM BULK DRUG AND PHARMACEUTICAL DOSAGE FORM USING QUALITY BY DESIGN (QBD) APPROACH

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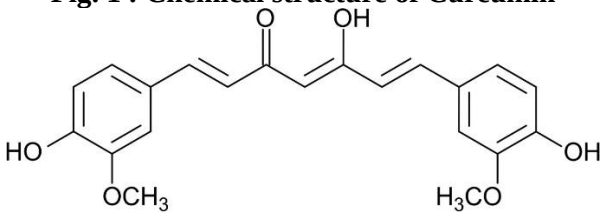
Background:

Curcumin is a natural plant polyphenol compound derived from the rhizome of the herb *Curcuma longa*. It is well known for its antioxidant, anti-cancer, and anti-inflammatory activities [3]. Pre-clinical studies have shown that curcumin can inhibit cancer genesis in a variety of cell lines, including breast, prostate, colon, cervical, gastric, hepatic, ovarian, pancreatic, and leukemia. Despite its excellent potential benefits, researchers are still facing challenges associated with its inherent poor aqueous solubility, chemical instability, rapid metabolism and low oral bioavailability[4] Chemically curcumin is 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (Figure 1).

Fig. 1 : Chemical structure of Curcumin



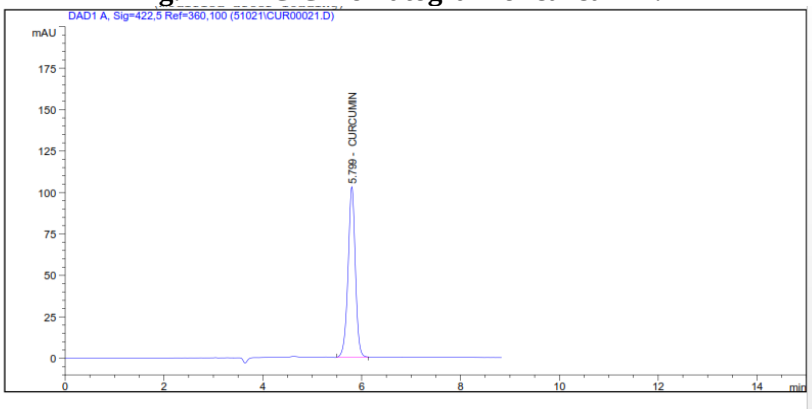
Thus far various analytical tools such as IR, TLC, HPLC, UV, LCMS have been used for the analysis of curcumin. Among all, HPLC is the method of choice owing to its good separation among the curcuminoids. The parameter like flow rate, mobile phase, column temperature influenced by HPLC separation are 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 curcumin by employing central composite design.

Separation of curcumin by design of experiments is the simple method used for optimisation to get better separation results of curcumin. In this work, we used Central composite design (CCD) for optimisation of curcumin 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 curcumin.

The Central Composite designs (Box and Wilson designs) are constituted of a full[1], factorial[2] or fractional[3] design. The points at the center 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 center 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 experimnts.

Fig. 2 HPLC Chromatogram of curcumin.



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 (P_A):

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,

$N = 16 (t_R /W)^2$ (**Equation** 1)

Where t_R 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:

3.1Materials

Standard Curcumin was procured from Loba chieme Mumbai, India and has been claimed to contain 99.980 percent(w/w). 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	%	79	82
Flow rate	ml/min	0.6	1
Wavelength	nm	417.5	425

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 3^2 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 design

Independent variables	Level used	actual (coded)	
	(− 1)	(0)	(+1)
X1=Flow rate (ml/min)	79	80	82
X2= wavelength (nm)	0.6	0.8	1
X3=Methanol (%)	417.5	422	425

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 intra-day 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 by the actual sample concentration and % RSD 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	Response 4
Std	Run	A:Flow rate	B:Methanol	C:Wavelength	RT	PA	TP	TF
		ml/min	%	Nm	min	AUC		
3	1	0.7	82	420	5.58	4007.51	9710	0.95
5	2	0.7	80	425	6.098	4112.29	9155	1.01
7	3	0.7	82	425	5.58	4119.34	9703	0.95
11	4	0.8	79	422.5	5.535	3558.69	8261	1.06
13	5	0.8	81	417.5	4.99	3328.52	8605	1.02
10	6	1	81	422.5	3.99	2872.19	7950	1.01
9	7	0.6	81	422.5	6.669	4591.76	9917	1
8	8	0.9	82	425	4.289	3242.5	8737	0.97
6	9	0.9	80	425	4.663	3222	8364	1.03
12	10	0.8	83	422.5	4.624	3591.77	9224	0.96
1	11	0.7	80	420	5.947	3886.58	9318	1.06
14	12	0.8	81	427.5	5.017	3763.13	8923	1.06
2	13	0.9	80	420	4.635	3228.95	8042	1.04
4	14	0.9	82	420	4.286	3132.56	8513	0.99

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 3^2 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.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2{}^2 + ... + \beta_k X^k \qquad 1$$

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]

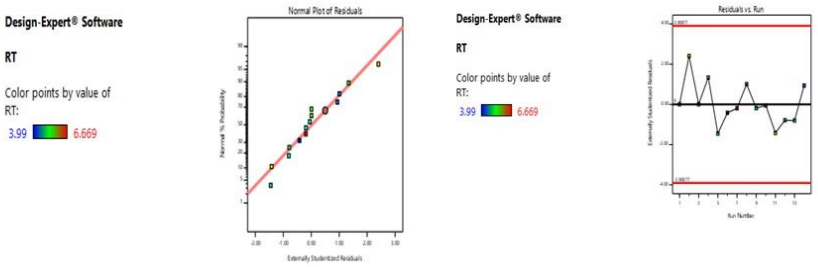


fig. 2 Plot a showing true versus predicted values, with residual plot b for response Y1

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) = 5.05 – 0.6681X1 – 0.2144X3 + 0.0709X1X3 + 0.0972X12 (2)

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.

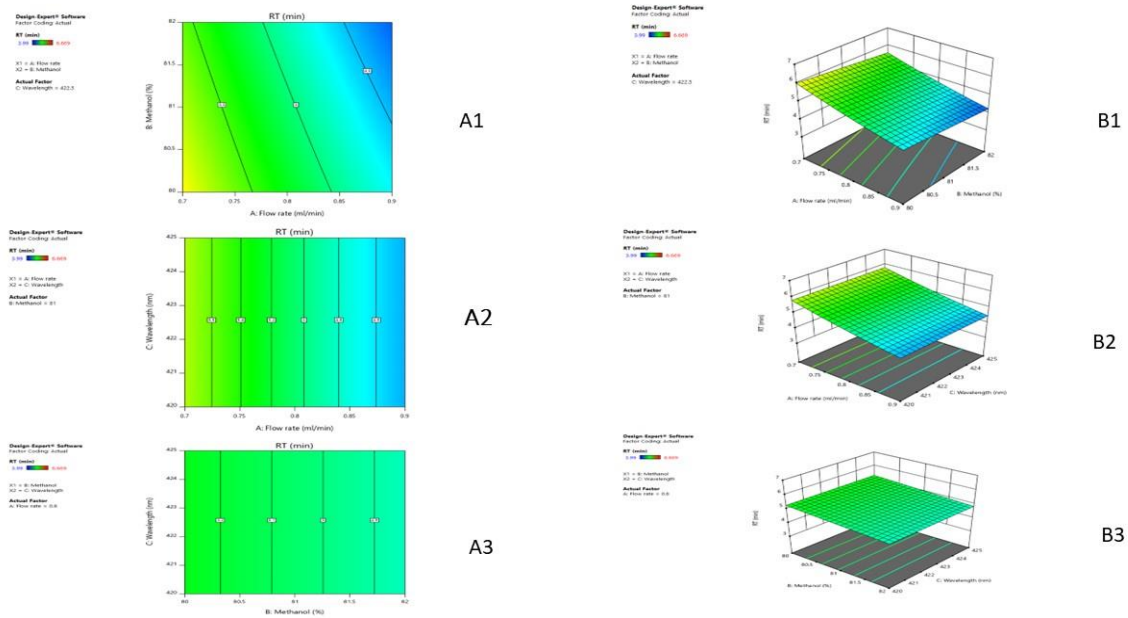


Fig. 3 Relation plots (A1-A3; B1-B3) between true and measure values for Y1

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) = 3618.41− 421.18X1 – 0.2283X2 + 0.2233X1X3 + 0.5558 X2X3 + 81.86X12 (3)

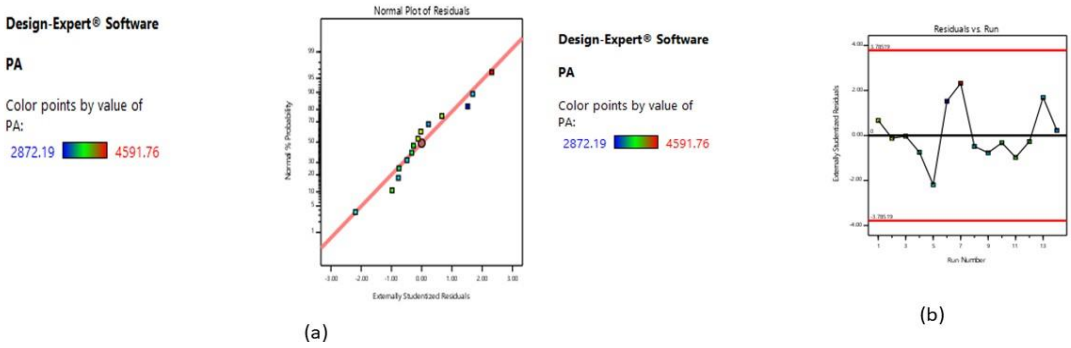


Fig. 4 Plot a showing values true verses predicted with the residual plot b for the response Y2

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.

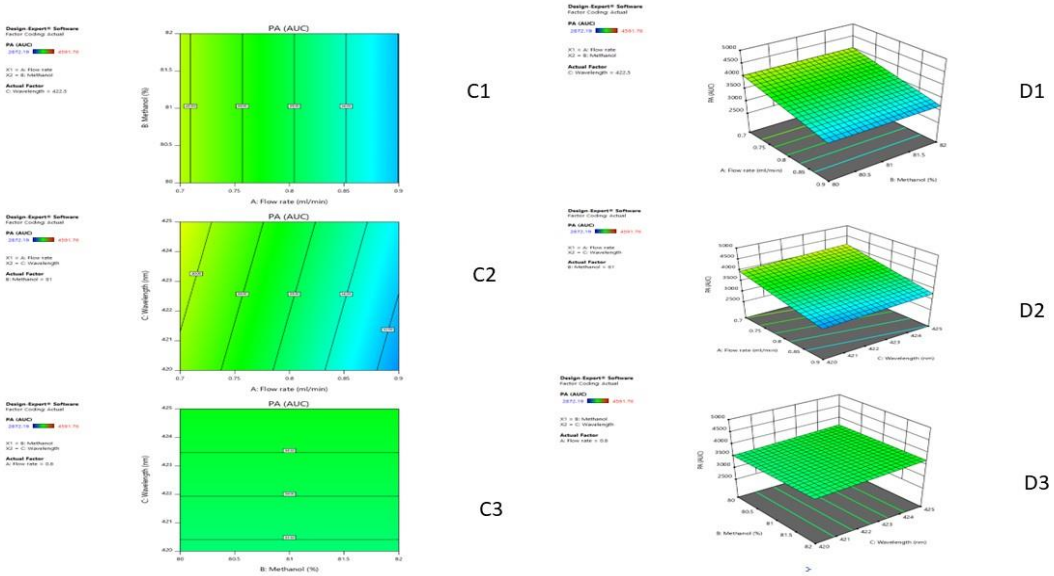


Fig. 5 Relation plot (C1-C3; D1-D3) between true with predicted values and residual plot for Y2

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:

$$Y\ 3\ (TF) = 0.823 + 0.0102X1 - 0.0023X2 \quad (4)$$

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.

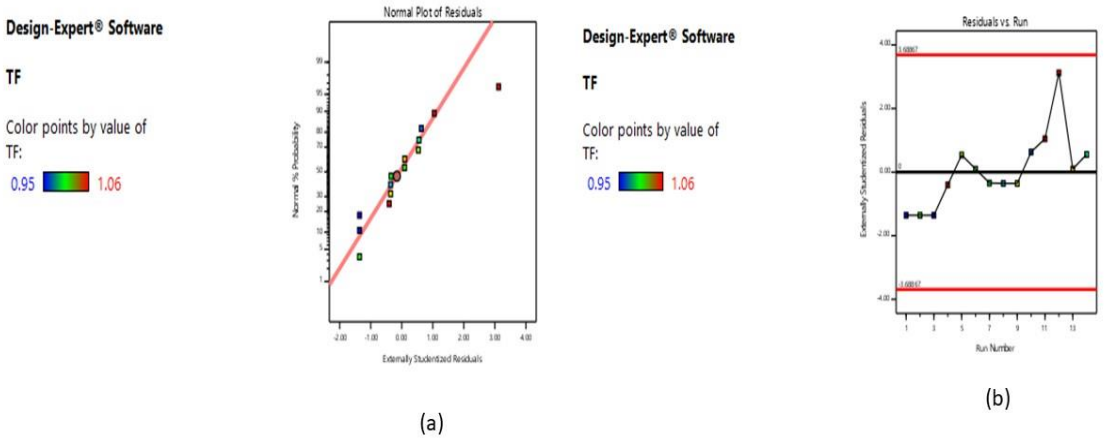


Fig. 6 Plot a showing true verses predicted values and residual plot b for the response Y3

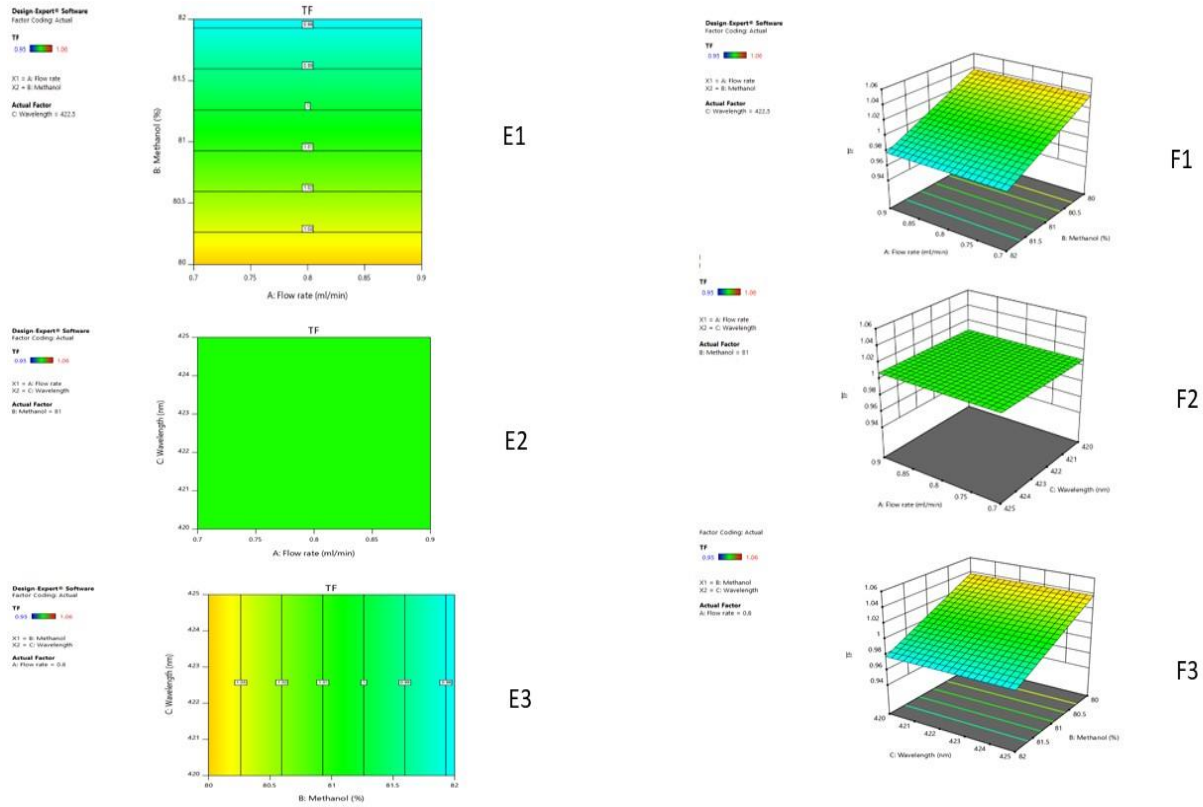
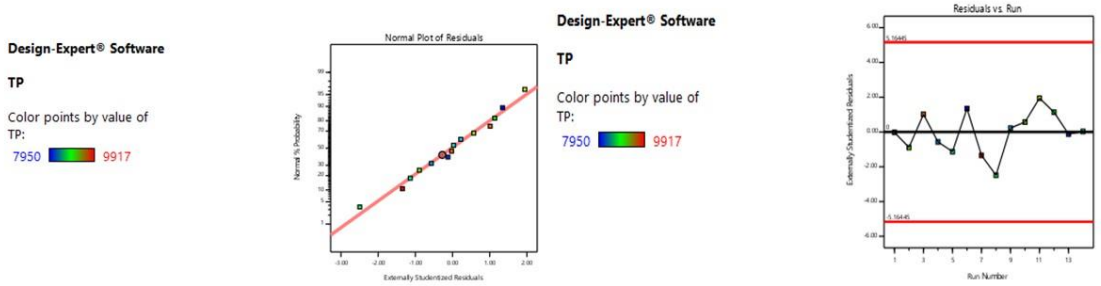


Fig. 7 Relation plot (E1-E3; F1-F3) between actual with predicted values and residual plot for Y3

Variables effect on theoretical plate: This equation expressed to predict values of response for coded variables. The response on theoretical plate is affected by flow rate (X1), temperature of column (X2), and methanol concentration in mobile phase (X3). Figures 8 and 9 (G1-G3 and H1-H3) indicate linear correlation plot for the response Y4 among true as well as measured values and the corresponding graphs.

Final equation in terms of coded factors

$$Y4(TP)=9331- 510.25X1 + 231.87X2 + 63.25X3 – 141.75X12 \quad (5)$$



(a) (b)

Fig. 8 Plot a between true and measured values with residual plot b for the response Y4

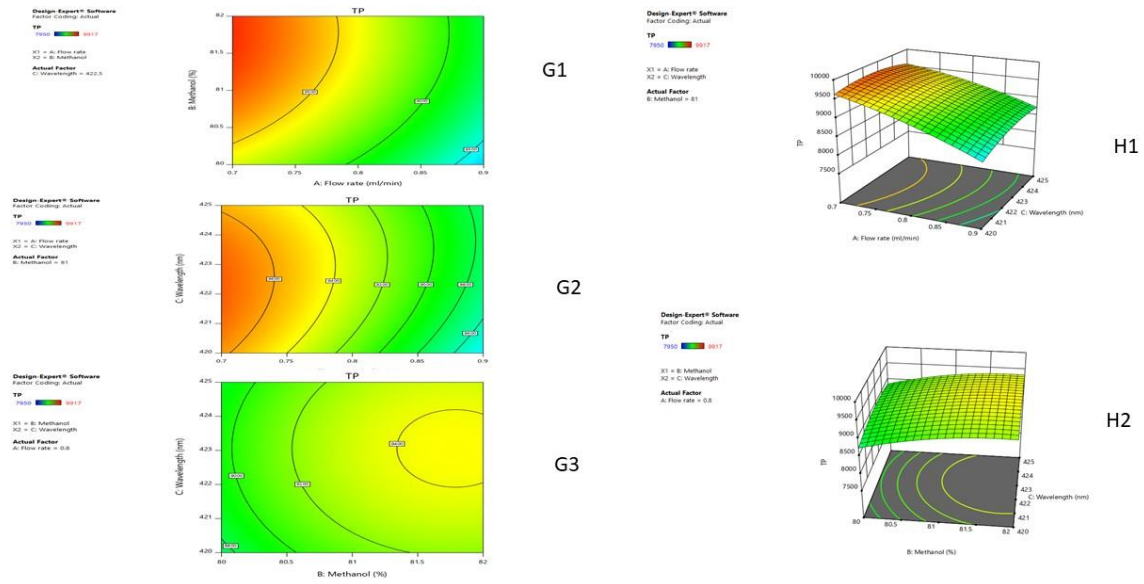


Fig. 9 Relation plot (G1-G3; H1-H2) between actual with predicted values and residual plot for Y4

Statistics of response

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

Table 4 Statistics for the model of response.

Responses	**Source**	**Std. Dev.**	**R^2**	**Adjusted R^2**	**Predicted R^2**	**PRESS**	
Retention time	Linear	0.1146	0.9836	0.9787	0.9650	0.2805	
	2FI	0.1322	0.9847	0.9717	0.9642	0.2866	
	Quadratic	**0.0384**	**0.9993**	**0.9976**	**0.9910**	**0.0719**	**Suggested**
	Cubic					*	Aliased
Peak Area	**Linear**	**84.56**	**0.9763**	**0.9692**	**0.9515**	**1.465E+05**	**Suggested**
	2FI	92.14	0.9803	0.9634	0.9344	1.979E+05	
	Quadratic	69.59	0.9936	0.9791	0.9221	2.350E+05	
	Cubic						Aliased
Tailing Factor	**Linear**	**0.0043**	0.6913	**0.6324**	**0.4316**	0.0010	**Suggested**
	2FI	0.8124	0.5621	0.5378	-0.0517	0.0022	
	Quadratic	0.3767	0.5834	0.5984	-0.5585	0.0016	
	Cubic						Aliased

Theoretical plate	Linear	136.34	0.9648	0.9542	0.9304	3.673E+05	
	2FI	131.06	0.9772	0.9577	0.9122	4.630E+05	
	Quadratic	**68.97**	**0.9964**	**0.9883**	**0.9571**	**2.264E+05**	**Suggested**
	Cubic					*	Aliased

The R2 value close to 1 shows suggested model for all bold in the said Table

Analysis of variance for the responses (Y1-Y4)

Tables 5, 6,7 and 8 show the ANOVA tables for various responses (RT, PA, TF, and TP).

Table 5 ANOVA results for Response 1: RT

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	7.99	3	2.66	1038.16	< 0.0001	significant
A-Flow rate	7.14	1	7.14	2785.21	< 0.0001	
B-Methanol	0.7353	1	0.7353	286.74	< 0.0001	
A^2	0.1091	1	0.1091	42.54	< 0.0001	
Residual	0.0256	10	0.0026			
Cor Total	8.01	13				

Table 6 ANOVA results for Response 2: PA

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.945E+06	2	1.473E+06	223.82	< 0.0001	significant
A-Flow rate	2.838E+06	1	2.838E+06	431.35	< 0.0001	
C-Wavwlength	1.072E+05	1	1.072E+05	16.29	0.0020	
Residual	72380.05	11	6580.00			
Cor Total	3.018E+06	13				

Table 7 ANOVA results for Response 3: TF

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0144	1	0.0144	27.71	0.0002	significant
B-Methanol	0.0144	1	0.0144	27.71	0.0002	
Residual	0.0062	12	0.0005			
Cor Total	0.0206	13				

Table 8 ANOVA results Response 4: TP

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	5.255E+06	7	7.507E+05	218.68	< 0.0001	significant
A-Flow rate	4.166E+06	1	4.166E+06	1213.41	< 0.0001	
B-Methanol	8.603E+05	1	8.603E+05	250.58	< 0.0001	
C-Wavelength	64009.00	1	64009.00	18.64	0.0050	
AC	64082.00	1	64082.00	18.67	0.0050	
A^2	31601.25	1	31601.25	9.21	0.0230	
B^2	69266.45	1	69266.45	20.18	0.0041	
C^2	64297.80	1	64297.80	18.73	0.0049	
Residual	20598.25	6	3433.04			
Cor Total	5.276E+06	13				

Graphical and numerical optimization

Figure 10 shows the overlay plots showing relationship of factor and responses for graphical optimization.

Calibration curves

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. 10).

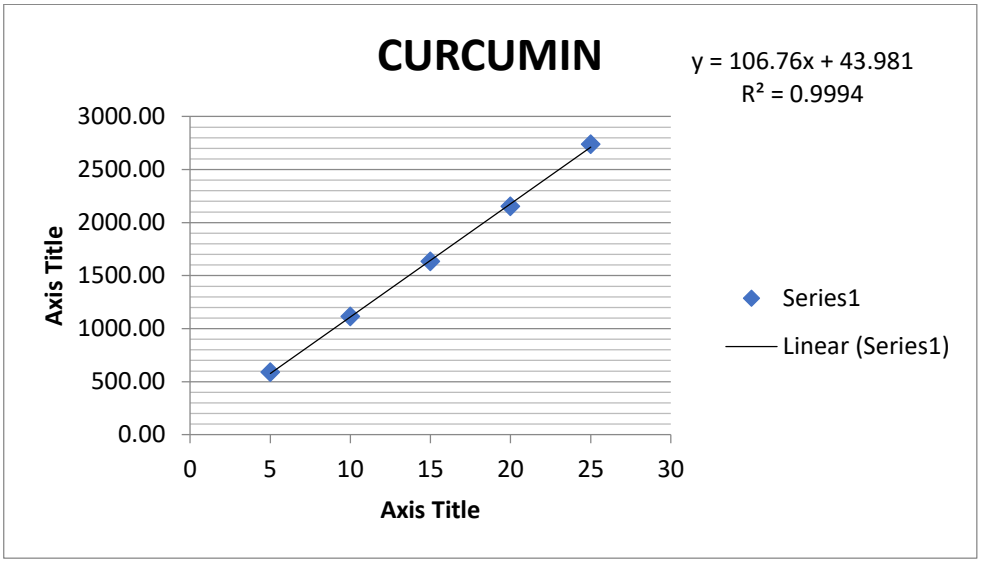


Fig. 10 Calibration curve for Curcumin

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).

precision and Intraday precision(n=3).

Table 9 precision (n=3)

PRECESION

Conc	Area	II	Mean	Amt Found	AM	% Amt Fnd	SD	%RSD
5	572.8653	572.3905	572.63	4.95	0.990905	99.09	0.34	0.06
15	1635.526	1638.196	1636.86	14.93	0.995239	99.52	1.89	0.12
25	2765.773	2769.987	2767.88	25.53	1.021143	102.11	2.98	0.11

INTERADAY PRECESION

Conc	Area	II	Mean	Amt Found	AM	% Amt Fnd	SD	%RSD
5	569.343	568.9688	569.16	4.92	0.984397	98.44	0.26	0.05
15	1622.256	1623.608	1622.93	14.80	0.986537	98.65	0.96	0.06
25	2724.104	2731.918	2728.01	25.15	1.006197	100.62	5.53	0.20

Table 10 inter-day precision (n=6)

This method is used to determine the precision values of % RSD was found 0.20 for intra-day. The outcome (Table 9) has almost no effect on the parameters due to any little variation.

Repeatability

Repeatability study was conducted and % RSD was found 0.05.

Robustness

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 11).

Table 11 Robustness study

	Change flow							
FLOW RATE-0.7						**0.9 ml**		
Sr No.	Conc	**µgm/ml**	Area		Sr No.		**µgm/ml**	Area
1		20	2418.881		1	Conc	20	1919.708
2		20	2418.468		2		20	1919.55
		Mean	2418.67				Mean	1919.63
		SD	0.29				SD	0.11
		%RSD	0.01				%RSD	0.01
MP			**buffer 18+82 MEOH**		**MP**	**80+20 MEOH**		
	Sr No.	Conc	**µgm/ml**	Area	Sr No.		**µgm/ml**	Area
	1		20	2127.458	1		20	2125.055
	2		20	1784.84	2		20	2122.429
			Mean	1956.1			Mean	2123.74
			SD	242.27			SD	1.86
			%RSD	12.38			%RSD	0.09
WAVE LENGTH CHANGE			**421**				**423**	
	Sr No.	Conc	**µgm/ml**	Area	Sr No.		**µgm/ml**	Area
	1		20	2131.032	1		20	2170.75
	2		20	1878.66	2		20	1973.92
			Mean	2004.8			Mean	2072.34
			SD	178.45			SD	139.18
			%RSD	8.90			%RSD	6.72

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 12).

Table 12 Regression data for the calibration curve. (n=3)

Parameter	Result
Linearity range	5–25 µg/ml
r 2±%RSD	0.9997±0.30
Slope±%RSD	106.7±0.30
LOD	0.224744
LOQ	0.681044

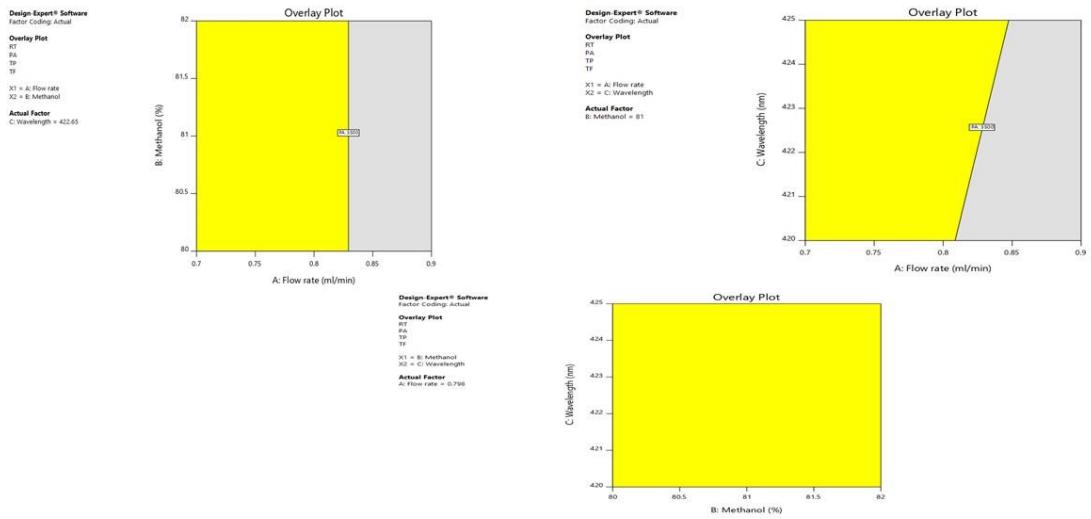


Fig. 10 Overlay plots showing relationship of factors and responses

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 curcumin. The DoE software optimization design aids in optimizing the precise circumstances needed to build a most accurate and precise analytical method for curcumin.

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