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DESIGN OF EXPERIMENT (DOE) APPROACH FOR A SIMPLER HPLC TECHNIQUE ESTIMATING THYMOQUINONE FROM *NIGELLA SATIVA* SEEDS, COMMERCIAL FORMULATION, POLYMERIC NANOPARTICLES, AND ITS STABILITY INDICATION

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Keywords:

Thymoquinone, Black seed, Design experiment, Stability-indicating, ICH, Analysis of variance, Applicability, Quality by design, Polymeric nanoparticle

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ABSTRACT: To the best of our knowledge, the factorial design methodology has never been used to estimate Thymoquinone (TQ) using the reverse phase High-Performance Liquid Chromatography (RP-HPLC) method, making our work unique. The study aimed to create, verify and validate a simple stability-indicating analytical technique for quantitative detection of TQ in Ayurvedic formulations, black seed and polymeric nanoformulation using Design-experiment® (DOE) version 12. The technique was developed utilizing a Luna C18 HPLC column with a mobile phase of water: methanol (25:75, v/v), administered isocratic ally at 1 ml/min, at 253 nm. The validation parameters were carried out in compliance with ICH criteria using the Quality by Design (QbD) approach. The devised technique was shown to be specific, linear (R2 > 0.999) across the specified concentration range of 0.5 to 16 µg/ml, with detection and quantification limits of 0.02 and 0.08 µg/ml, respectively. The applicability of the new approach was evaluated using the TQ entrapped polymeric nanoparticle. TQ entrapment efficiency for prepared nanoformulation was found to be 82.87 percent. The experimental model was significant (P 0.0001), as evidenced by purposeful adjustments in the approach examined using analysis of variance. The approach was used to calculate the amount of TQ in black seed, and commercially available formulations were discovered to be equivalent to the labeled concentration. A deeper understanding of the parameters that drive chromatographic separation is achieved using the QbD approach. The proposed approach proved fast, cheap and exact for quantifying TQ in bulk and nanoparticulate systems.

INTRODUCTION: *Nigella sativa* Linn. (Ranunculaceae), known as black seed, is a herbaceous plant native to the Middle East, Western Asia has been used for over 2000 years to prevent and treat a variety of diseases.

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The black seed oil has been utilized as herbal medicine to treat arthritis, lung ailments and hyper-cholesterolemia. TQ **Fig. 1** is a valuable major Nigella sativa seeds' bioactive ingredient that has demonstrated significant therapeutic effects.

In experimental studies, Nigella sativa has been proven to have anti-neoplastic and antioxidant effects *in-vivo* animal models. TQ is a monocyclic monoterpene with the chemical formula $C_{10}H_{12}O_2$. TQ's anticancer effect is dependent on three regulatory pathways: cell cycle arrest, induction of

apoptosis and suppression of nuclear factor kappa-B (NF-B) activation. TQ has significant antioxidant action via modulating oxidative indicators [1].

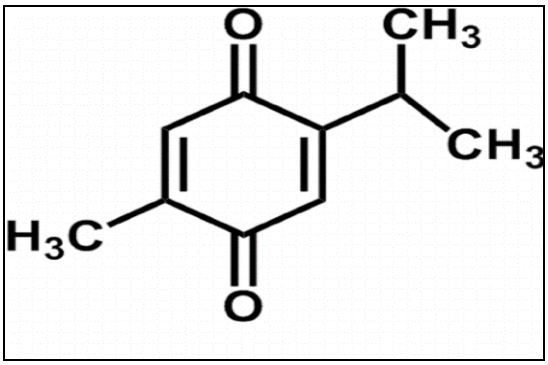


FIG. 1: THYMOQUINONE STRUCTURE

Since, humans recognized the use of herbal medicines and handmade treatments, they have been extensively employed to treat many ailments, and there has lately been an increase in the demand for herbal formulations. The rising demand for herbal remedies has increased the difficulty of obtaining and sustaining their quality. Since, then, a greater focus has been placed on the quality control of herbal-related or ayurvedic goods. The current work attempted to examine TQ-loaded polymeric nanoparticles, emphasizing quantitative quantification of TQ in commercially available black seed oil. Many techniques for determining TQ have been documented in the literature. However, the described HPLC approaches have several limitations, including high flow rates, high costs, poorer sensitivity, various wavelengths, and a lack of stability studies. As a result, the current study sought to develop an HPLC technique for accurately estimating TQ from Ayurvedic marketed formulations. As a result, a technique based on lipid-based nanoformulation has been studied to circumvent these restrictions. The current study also focuses on the formulation of TQ-loaded polymeric nanoparticles. Following that, stress degradation research was carried out to verify the existing RP-HPLC and formulation design and analysis [2]. The current work discusses developing and validating a risk-based HPLC technique for TQ in the pharmaceutical dosage form.

MATERIALS: Sigma Aldrich, United States, provided free samples of TQ (99%) and Pluronic F-127 (PF-127). The following ingredients were obtained at the Local Ayurvedic Pharmacy in Belagavi, India: Black seed oil, Black seed churna. Merck, Mumbai, India, provided HPLC-grade H_2O and methanol. The study employed deionized water used for formulation purposes collected after filtering by Millipore Direct-Q®-3 equipment (Molsheim, France).

Instrumentation: The chromatographic process was designed using a Shimadzu Prominence HPLC-20 AT system (Shimadzu, Kyoto, Japan) with LC Solution software and binary pumps (LC-20AD) with a degasser (DGU20A5), UV–Vis detector, auto-sampler (SIL 20AC HT) and column oven (CTO-10AS). The data was interpreted and analyzed using Shimadzu LC solution software (version 1.25). Chromatographic separation and analysis were carried out using the analytical column Luna C18 (250 4.60 mm). 0.22-micron membrane filters were employed. An Ultrasonic Bath Sonicator was used for degassing the mobile phase (Bransonic ultrasonic corporation, USA). Milli-Q water is used in a direct-Q3 Millipore company water purification system (Millex HV®, Millipore, USA). Design-Expert® software, version 13, was used to validate the experiment (Stat-Ease, Inc., Minneapolis, USA).

Calibration Standards Preparation and Method Development: A stock solution in methanol (1 mg/mL) of TQ was created by diluting the stock with mobile phase and used for calibration standard preparations of TQ in concentrations ranging from 0.5 to 16 µg/ml. Further solutions were maintained in light-resistant flasks to avoid photo-isomerization. H_2O and MeOH mixtures in various ratios such as 50:50 percent v/v, 60:40 percent v/v, 70:30 percent v/v, 65:35 percent v/v and 25:75 percent v/v were tested. Various mobile phase compositions were utilized to enhance the drug's retention time (Rt) and theoretical plate count (N) while decreasing the tailing factor (Tf). TQ's peak area, Rt, N and Tf were calculated to identify the optimal approach. The technique for analysis was developed by experimenting with various mobile phase ratios, flow rates and column oven temperature [2, 3].

Assay for Stress Degradation: All accelerated degradation investigations were carried out for 3 h with 1 mg of TQ. In the acidic degradation investigation, the drug solution was treated with 0.1 N HCl for the period specified (1 h).

Neutralized (NaOH) before analysis. 0.1N NaOH was added to the drug solution for the base degradation study and the solution was neutralized with hydrochloric acid after 1 h. 3% hydrogen peroxide solution was used for oxidative degradation. While in thermal degradation, methanol (2ml) was used to treat the drug solution. The flasks containing oxidative and heat degradation samples were sealed and then heated for 2 h (80 °C). For photo deterioration, drug samples were subjected to UV light. In all of the preceding investigations, materials were diluted with an adequate amount of mobile phase before being exposed to HPLC analysis [3-5].

Validation of the Created Technique and Statistical Analysis: Validation and stress studies were performed in triplicate, and data was presented as mean SD using Microsoft Excel. The DOE® tool verified the proposed approach using ICH criteria for system suitability, linearity, precision, accuracy, and stability. ANOVA analysis was also carried out using the same [6-9].

Linearity and Range: Seven concentration levels were tested to assess the method's linearity (0.5-16µg/ml). Every concentration was made three times. The linearity was determined by graphing the peak area at 254 nm against the respective drug concentrations.

System Suitability: The appropriateness of the instruments, analyzers, and columns was evaluated by injecting a 1µg/ml solution of TQ analyte (n = 6) to determine the system suitability parameters**.**

LOD and LOQ: Calibration curves were also employed to assess the method's sensitivity. LOD and LOQ were calculated using the following formulas: standard deviation of responses (r) and calibration curves (s) slopes.

$$LOD = 3.3 (\sigma /S) \text{ and } LOQ = 10 (\sigma /S)$$

Precision: These metrics are concerned with a method's repeatability. Low (1µg/ml), intermediate (4 µg/ml) and high (8 µg/ml) concentrations were evaluated on intra-day precision and inter-day precision.

Accuracy: A known quantity of TQ samples was spiked in triplicate injections at 50, 100 and 150 levels of a defined concentration to previously examined TQ sample solutions (5g/ml) and assessed using a devised technique.

Ruggedness and Robustness Study: These investigations were conducted to determine the method's applicability in modest changes. Small adjustments were applied to several technical parameters, such as mobile phase composition. Some instrument conditions, such as changing the detector from UV to PDA and the column from Synergi 4l Hydro-RP 80A to Luna 5l 100A, were also altered. Using Design-Expert software, process parameters such as column oven temperature, injection volume, mobile phase flow rate, and organic phase % were all purposefully changed.

Application of the Established Approach for Quantifying TQ in Nanoformulation, Black Seed Powder, and Marketed Formulations:

TQ Nanoformulation: A double emulsion solvent evaporation process was used to create TQ-loaded PLGA nanoparticles. Aqueous TQ solution was emulsified in 6 mL of ethyl acetate containing PLGA by homogenizing in an ice bath at a speed of 13500 rpm.

The original emulsion was further emulsified with 2 percent (w/v) PVA by homogenizing at 6000 rpm for 10 min in an ice bath. The resultant double emulsion was spun for 4 hours at 25 °C at 600 rpm. The nanoparticle suspension was then chilled overnight. Ultracentrifugation was used to recover the NPs at 13,500 rpm (15 min, 4 °C). After that, the nanoparticle sediments were rinsed three times with water and lyophilized overnight [10, 11].

Black Seed and Marketed Ayurvedic Preparations: TQ content was estimated using a developed HPLC technique employing black seeds and ayurvedic marketed goods containing TQ (Black seeds oil and black seed churna). To create a fine powder, a black seed churna was ground using a mortar and pestle.

The weighed amount of black seed powder and other commercial preparations were placed in a muslin cloth and maintained in a beaker with enough methanol for 72 h. Following that, the methanolic extracts were filtered using Whatman paper no. 42 and the filtrates are kept in a sealed amber-colored glass jar at 4°C [11, 12].

RESULTS:

HPLC Technique Development and Optimization: the Influence of Mobile Phase Composition: To make the procedure more practical and cost-effective, water and methanol were selected as mobile phases. The UV spectra of TQ (10 mg/ml in methanol) were obtained with TQ absorption peaking at 253 as λ_{max} **Fig. 2**, which was confirmed by PDA detection at several wavelengths ranging from 200 to 400 nm. At a flow rate of 1ml, the mobile phase with an H_2O and MeOH v/v ratio of 25:75 was determined to be the most reliable and the best HPLC chromatogram was achieved **Table 1, Fig. 3**.

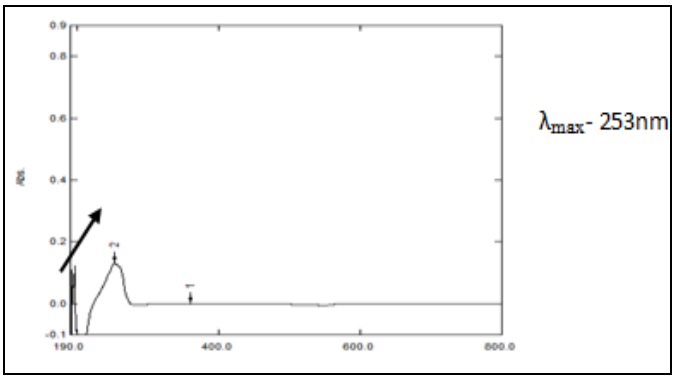


FIG. 2: UV–VIS ABSORPTION SPECTRA OF TQ

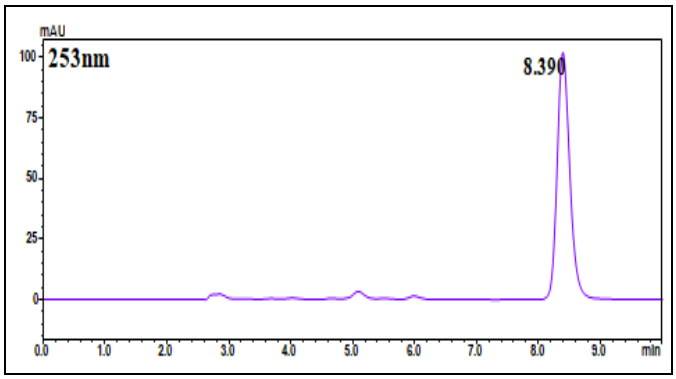


FIG. 3: HPLC CHROMATOGRAM OF TQ

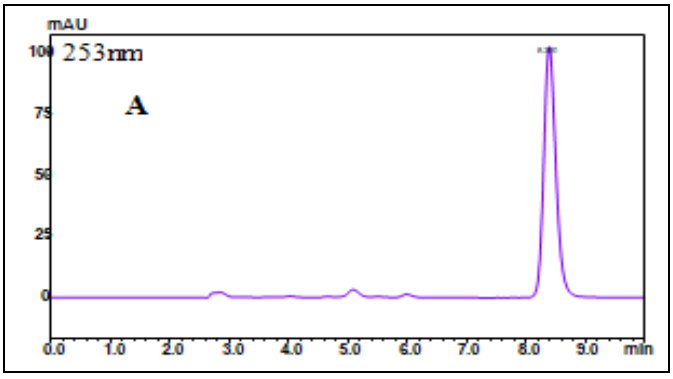
TABLE 1: METHOD DEVELOPMENT PARAMETERS AND CHROMATOGRAPHIC CONDITIONS

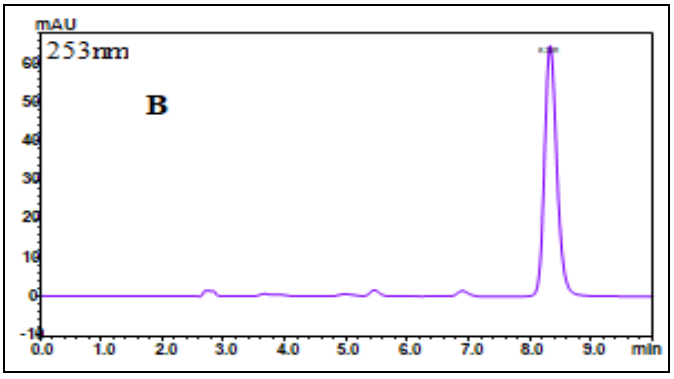
Parameter	Chromatographic conditions
Mobile phase	Water : MeoH (25:75)
Flow rate	1 ml /min.
wavelength	253nm
Run time	10 min
Column pressure	160 kgf
Temperature	Ambient temperature (30˚C)
The volume of the Injection loop	20 µl
Retention time (Rt)	8.33 min

Assay for Stress Degradation: Table 2 depicts the findings of the force degradation investigations. Significant TQ deterioration was detected under photolytic degradation. The mean percent recovery of TQ in acidic circumstances, moist heat, and photolytic conditions were 98.16±0.14, 98.85±0.02, 97.86 ± 0.55, 96.51 ± 0.57 and 83.32 ± 0.71, respectively. However, TM mild deterioration was in thermal and oxidative conditions, with 2.36 ± 0.05and 3.14±0.00 % degradation respectively. **Fig. 4** depicts chromatograms of deteriorated samples.

TABLE 2: THYMOQUINONE FORCED DEGRADATION STUDY

Exposed stress degradation conditions for TQ		
Stress type	**Assay (%) observed for TQ**	**% Degradation observed for TQ**
1 N HCl; 80°C, 1 h	98.16±0.14	1.73±0.02
I N NaOH; 80°C 1h	98.85±0.02	0.03±0.02
Thermal; 80°C, 1h	97.86±0.55	2.36±0.05
30% H_20_2; 80°C, 1h	96.51±0.57	3.14±0.00
Sunlight, 30 min	83.32±0.71	15.88±0.58





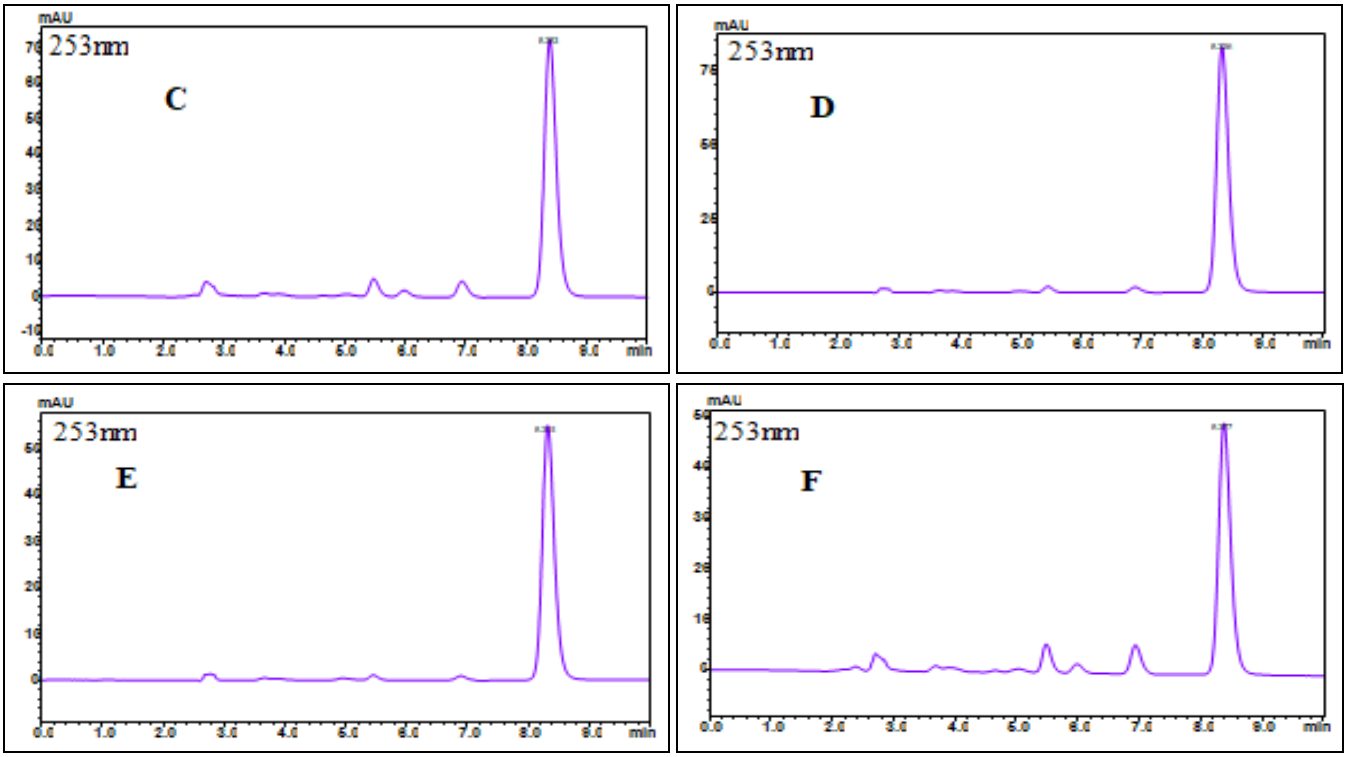


FIG. 4: HPLC CHROMATOGRAMS OF TQ INFLUENCED UNDER NORMAL (A), ACID DEGRADATION (B), ALKALI DEGRADATION (C), HEAT DEGRADATION (D), OXIDATIVE DEGRADATION (E), AND PHOTOLYTIC DEGRADATION (F)

Validation of the Optimized Method: The devised approach was validated for different parameters with the International Committee on Harmonization (ICH) principles.

Linearity: With a correlation coefficient R^2 > 0.9999, regression analysis revealed a strong linear relationship between peak area and concentration **Table 3**.

System Suitability: As illustrated in Fig. 3, drug peaks were found to be crisp and well resolved. Data on system appropriateness was presented as % RSD of Rt, peak area, N, and Tf. All of the parameters satisfied the approval requirements **Table 4A**.

LOD and LOQ: The limits of detection and quantification were found to be 0.02 and 0.08 µg/ml, respectively.

Precision: Percent RSD was computed and found to be less than 2% in all cases, indicating the method's outstanding repeatability **Table 4B**.

Accuracy: The mean percent recovery of TQ was determined to be between 100.13%-104.9%. **Table 4C** shows the results.

Ruggedness and Robustness Study: Analytes concentrations of 1 µg/ml were tested, and the findings demonstrated that minor fluctuations could not impair the method's applicability **Table 4D**. As a result, the approach was deemed feasible for normal use in any laboratory.

Optimized procedure parameters were purposefully changed to determine if the created strategy gives unaltered results.

A complete factorial experimental model was used to calculate RT, peak area, N, and Tf (with two levels and four factors).

DoE program examined 16 runs with two levels of -1 and +1 to detect chromatographic reactions Table 5. To investigate the impact of independent variables, the analysis of variance (ANOVA) model was utilized in **Table 6**.

TABLE 3: DATA FROM LINEAR REGRESSION AND THE SENSITIVITY PARAMETERS OF THE DERIVED METHOD

Linear regression data and sensitivity parameters						
Analyte	**Concentration range (µg/ml)**	**Slope**	**intercept**	R^2	**LOD (ug/ml)**	**LOQ (ug/ml)**
TQ	0.5-16	111391	10152	0.9998	0.02	0.08

TABLE 4: RESULTS OF METHOD VALIDATION WITH ACCEPTANCE CRITERIA ACCORDING TO ICH GUIDELINES

A. System suitability study parameters			
Parameters	**TQ**		**Acceptance criteria**
	Mean ± SD	**% RSD**	
Retention time (Rt)	8.33±0.02	0.35	%RSD < 2
Peak area	146885±23766	1.61	%RSD < 2
Theoretical plates	8020±28.87	0.36	>2000
Tailing Factor	1.22 ±0.004	0.33	Tf < 2

B. The precision of TQ Intraday and Interday					
Active Content (µg/ml)	**Intraday (n=3)**		**Interday (n=3)**		
	Found ±SD (%) (µg/ml)	**RSD (%)**	**1st day Found ±SD RSD (µg/ml) (%)**	**2nd day Found ±SD RSD (µg/ml) (%)**	**3rd day Found ±SD RSD (µg/ml) (%)**
1	0.99±0.005	0.57	0.99±0.005	0.58	0.98±0.005
4	4.01±0.004	0.49	4.00±0.01	0.38	4.02±0.06
8	7.96±0.06	0.76	8±0.01	0.12	7.99±0.005

C. Recovery studies						**Acceptance Criteria**
Active content (µg/ml)	**Level (%)**	**Spiked quantity (µg/ml)**	**Recovered quantity**	**Recovery (%)**	**RSD (%)**	
10	50	7.57 ±0.00	7.64 ±0.21	100.13	1.29	90-110%
10	100	10.02 ±0.01	10.03 ±0.05	100.09	0.43	
10	150	12.38 ±0.12	12.99 ±0.01	104.9	0.94	

D. Robustness and Roughness study			
Parameters	**Changes made**	**TQ Retention time ± Sd**	**RSD (%)**
composition of mobile phase WATER: MeOH	25:75	8.37±0.02	0.27
	27:73	8.33±0.01	0.13
	23:77	8.37±0.02	0.34
Flow rate	1	8.34±0.04	0.56
	0.8	8.39±0.01	0.13
	1.2	8.36±0.04	0.55
Detection wavelength	253	8.39±0.01	0.13
	255	8.37±0.02	0.34
	251	8.34±0.04	0.49
Column oven temperature	35	8.32±0.01	0.18
	30	8.37±0.03	0.41

TABLE 5: FULL FACTORIAL EXPERIMENTAL DESIGN FOR TQ ROBUSTNESS ANALYSIS

Run	Factor 1	Factor 2	Factor 3	Factor 4	Response1	Response 2	Response 3	Response 4
1	-1	-1	-1	+1	8.04	123654	8134	1.41
2	+1	+1	+1	+1	7.25	142457	8035	1.91
3	+1	+1	+1	-1	8.23	152361	8546	1.20
4	+1	-1	-1	-1	8.30	150256	8432	1.21
5	-1	+1	+1	+1	8.35	142547	7421	1.21
6	+1	-1	+1	+1	8.30	132456	7000	1.93
7	-1	-1	-1	-1	8.70	132456	7489	1.24
8	-1	+1	-1	-1	8.31	145786	7898	1.87
9	-1	-1	+1	-1	7.14	145236	8033	1.21
10	+1	+1	-1	-1	8.12	152369	8745	1.26
11	+1	-1	+1	-1	8.04	145326	8012	1.20
12	+1	-1	-1	+1	8.31	124569	8452	1.20
13	-1	-1	+1	+1	8.31	139239	8137	1.28
14	-1	+1	-1	+1	8.39	139586	8015	1.20
15	-1	+1	+1	-1	8.40	150003	8310	1.21
16	+1	+1	-1	+1	8.42	149813	8455	1.11

TABLE 6: ANOVA ROBUSTNESS STUDY RESULTS FOR TQ

TQ	P value	R- square	Equation in terms of coded factors	Predicted value	Observed value	Relative Error (%)
Retention time	<0.0001	0.9794	7.71-0.0256A+0.2131B+0.1031D -0.3369AB	7.71±0.01	8.33±0.03	1.83
Peak area	<0.0001	0.9878	138214E+055955.75A+ 10074.75B+5970.75D +6402.25AB+5530.275AD	138214± 32466	146885± 23766	1.71
Theoretical plates	<0.0001	0.9170	7397.50+36.63A+511.12B+ 171.88C+37.62D+128.50AB	7397.50± 57	8020±28.87	1.96
Tailing factor	<0.0001	0.9123	1.32-0.0319A-0.2044B+0.0244C +0.14690D+0.0131AB	1.32±0.05	1.14±0.008	0.74

An Examination of Black Seed and Ayurvedic-Marketed Items: TQ content in black seeds and commercially available Ayurvedic marketed items was determined using the recognized analytical approach. The TQ concentration of black seeds, black seed churna, and black seed oil was determined to be 99.00, 98.89, and 99.29 percent, respectively, which was within the permitted range as stated on the label. The lack of interfering peaks in the HPLC chromatograms of black seed and other marketed formulations showed non-interference of other chemicals used, with the parent TQ peak, proving the suitability of this new analytical approach for routine TQ assessment quality control areas **Fig. 5**.

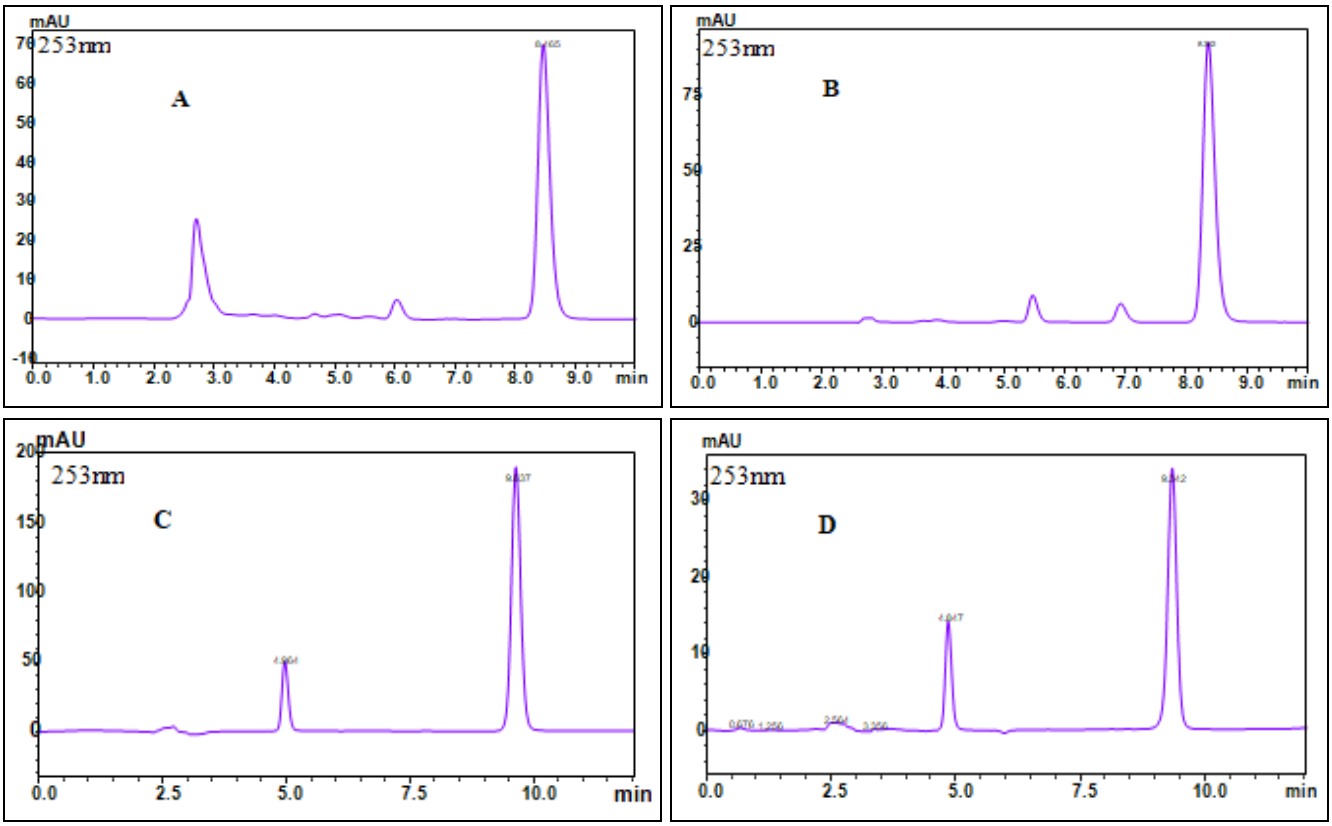


FIG. 5: HPLC CHROMATOGRAM OF TQ OBTAINED IN POLYMERIC NANOFORMULATION (A), BLACK SEEDS (B), BLACK SEED OIL(C), AND BLACK SEED CHURNA (D)

TQ Quantification in Polymeric Nano-formulation: As indicated in **Table 7**, the mean percentage recoveries of TQ from PLGA nanoparticles was determined to be in the 91-98 percent ranges, which were within the recognized limits (90–110 percent). The EE was 82.87 percent, with a loading content of 1.57 percent. TQ-loaded nanoparticles were measured at 117nm. The results also demonstrated that the formulation was homogeneous, with low polydispersity values **Table 8**.

TABLE 7: PRECISION AND ACCURACY DATA FOR DRUG ESTIMATION IN POLYMERIC NANOPARTICLES

Drug	Spike level (%)	Average area n=3	% RSD	Acceptance criteria	Drug amount (µg/ml) Recovered	Added	Mean Recovery (%)	Observed	Acceptance criteria
TQ	50	416220	0.014	<2.0	5	4.78	95.6		90-110%
	100	807410	0.007		10	9.87	98.7		
	150	1190489	0.049		15	13.71	91.93		

TABLE 8: CHARACTERIZATION FOR POLYMERIC NANOFORMULATION

Polymeric nanoformulation	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Drug content (%)	Entrapment Efficiency (%)
TQ loaded PLGA NPs	117.1±2.4	0.14±0.23	-15±3.12	1.57±0.82	82.87±0.21

DISCUSSION: Several experiments were carried out using various solvent systems containing methanol, water, and Acetonitrile. The majority of them yielded reasonable results, with methanol: water producing high resolution but widened peaks with weak separation and MeOH: H_2O producing characteristic peaks with little resolution.

As a result, several H_2O and MeOH ratios were tested to produce improved resolution and well separation. The resulting chromatogram for TQ was found to be fairly crisp and well separated under a ratio 25:75, with a retention duration of 8.33minutes.

Table 1 shows the technique development parameters and chromatographic conditions. The specificity of the presented approach was validated using a system appropriateness analysis. In all of the ICH-required conditions, such as stress study, the investigation proved the method's applicability since TQ may endure degradation to a large extent.

According to the degradation investigations, TQ could tolerate acidic and alkaline environments, albeit mild degradation peaks were identified for the thermal and oxidative environment making it fairly stable.

The photolytic environment showed a significant effect on TQ with 15.88% degradation. Under optimal chromatographic settings, analyte retention times remained consistent and peaks kept their integrity in all circumstances.

The enhanced chromatographic technique was validated in compliance with ICH standards. The proposed method has a high level of precision and accuracy. The system suitability test findings showed that the approach was appropriate for the intended application. It was also sensitive, precise, and accurate while determining TQ at the same time. The equation expressed in terms of coded components can be used to predict responses for different values of each ingredient.

According to the perturbation chart, a change in injection volume (B) significantly influences retention time for TQ.

Results showed that increasing B increased in Rt but was unaffected by column oven temperature (A) and Methanol content (D). Changes in A, B, and D all significantly influence the peak area, but only B has a bigger impact on the peak area.

The Peak area increased when the B and D were increased, whereas a decrease in peak area was seen when the A was raised. Increases in B resulted in a drop in N, unaffected by changes in A and D. B had a substantial impact on the Tf but remained unchanged with A and mobile phase flow rate (C). Tf decreased as the injection volume (B) increased but showed a positive effect when D was raised.

Fig. 6 and 7depict all of the results 3D contour plots were plotted to optimize chromatographic conditions **Fig.8**. DoE software was used to identify statistically significant quadratic effects of factors and responses in robustness experiments.

The relationship between a response variable and predictor factors was investigated using 3D Surface Plot **Fig. 9**.

By comparing a simulation-based data set to actual data from fielded products, overlay plots proved data reliability **Fig. 10**.

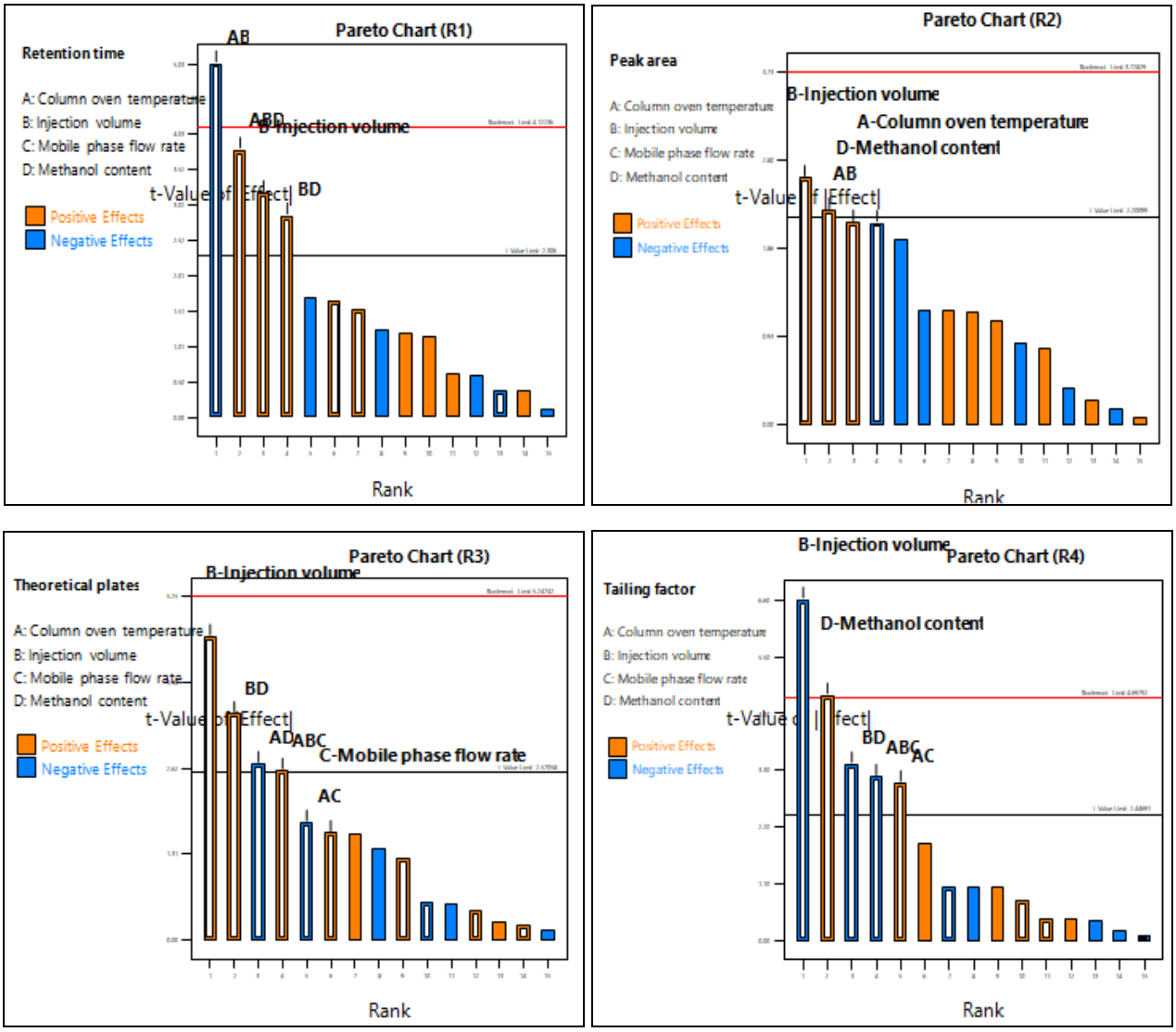
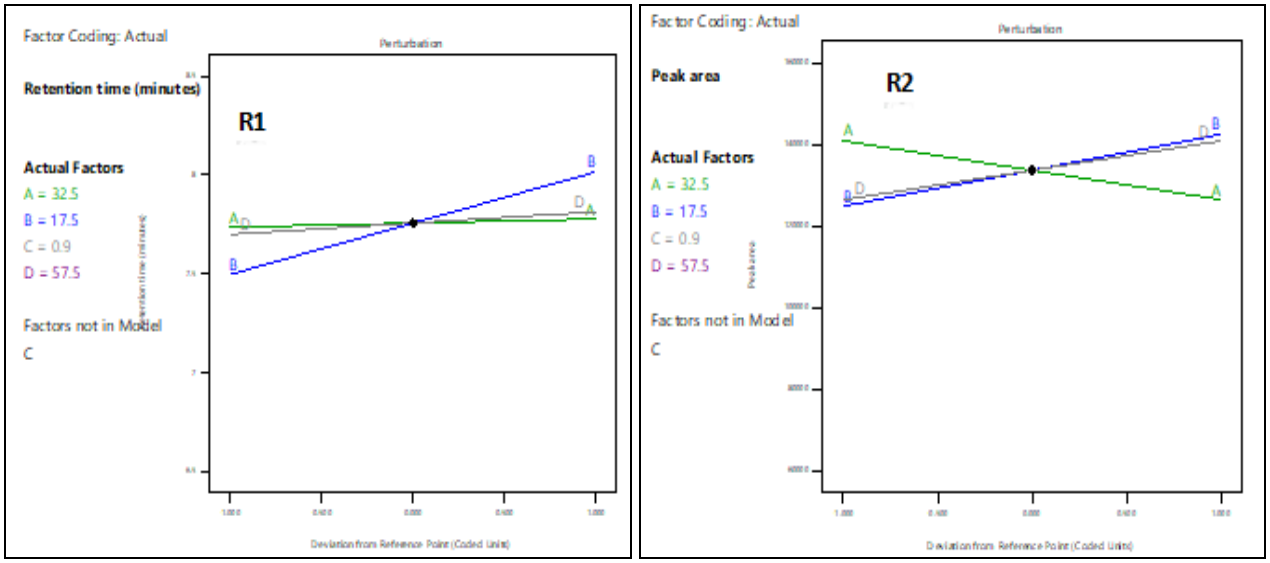


FIG. 6: A PARETO DIAGRAM DEMONSTRATING THE IMPACT OF INDEPENDENT FACTORS ON TQ. THE COLUMN OVEN TEMPERATURE (A), INJECTION VOLUME (L) (B), MOBILE PHASE FLOW RATE (ML/MIN) (C), ACETONITRILE CONTENT (PERCENT) (D), AND INTERACTION EFFECTS ON RETENTION TIME (R1), PEAK AREA (R2), THEORETICAL PLATE NUMBER (R3), AND TAILING FACTOR (R4)



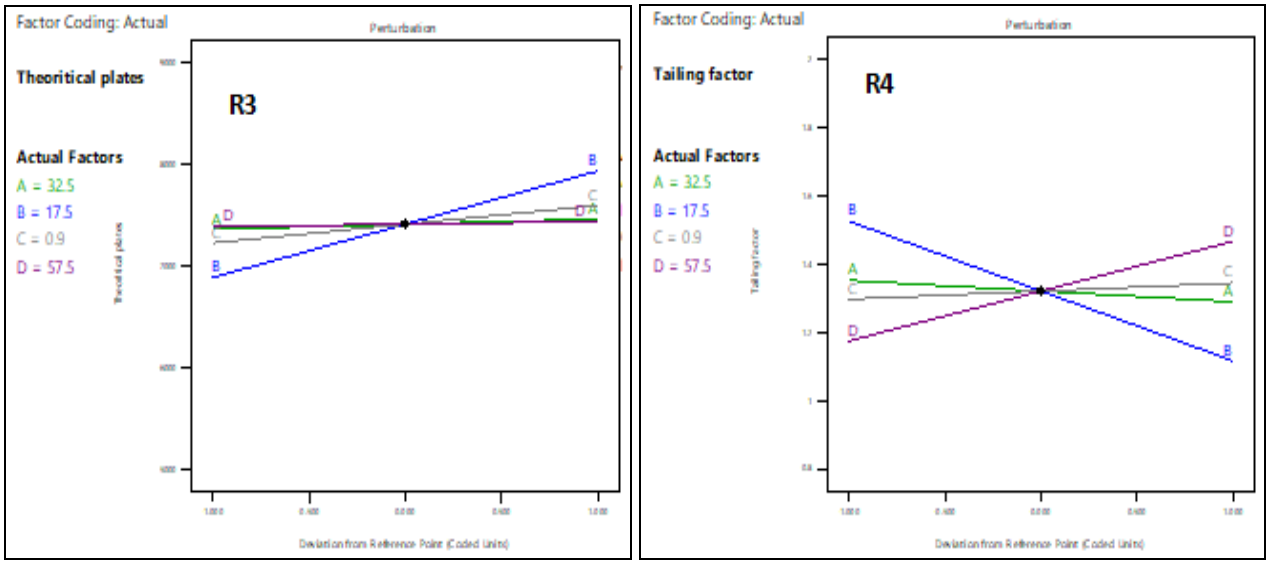


FIG. 7: A PERBUTATION PLOT DEMONSTRATING THE IMPACT OF INDEPENDENT FACTORS ON TQ. THE COLUMN OVEN TEMPERATURE (A), INJECTION VOLUME (L) (B), MOBILE PHASE FLOW RATE (ML/MIN) (C), ACETONITRILE CONTENT (PERCENT) (D), AND INTERACTION EFFECTS ON RETENTION TIME (R1), PEAK AREA (R2), THEORETICAL PLATE NUMBER (R3), AND TAILING FACTOR (R4)

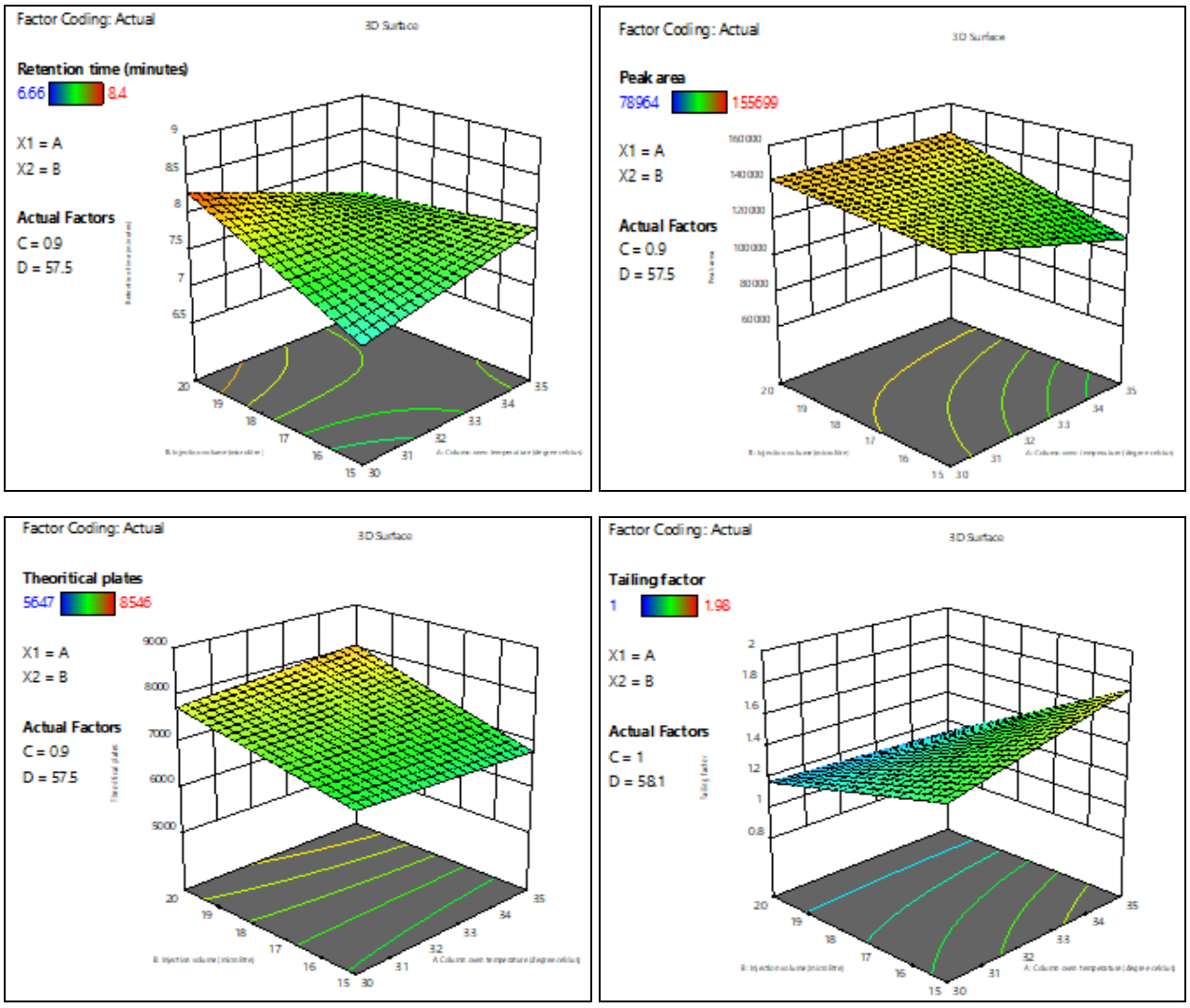


FIG. 8: THE TQ 3D SURFACE GRAPH DEPICTS THE INTERACTION OF COLUMN OVEN TEMPERATURE (A), INJECTION VOLUME (B), MOBILE PHASE FLOW RATE (C), AND PERCENT ACN (D) ON RETENTION TIME (R1), PEAK AREA (R2), THEORETICAL PLATES (R3), AND TAILING FACTOR (R4)

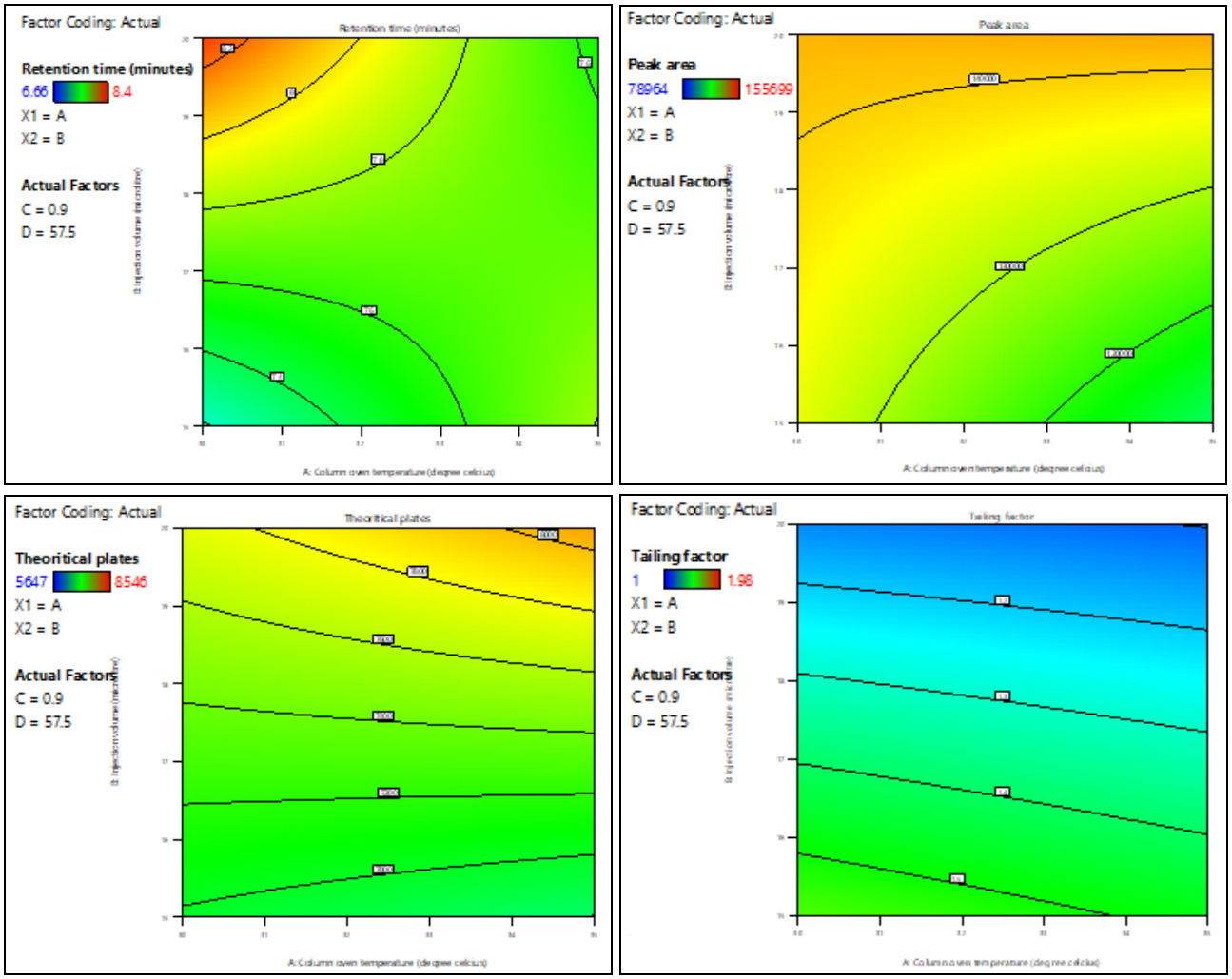


FIG. 9: 3D CONTOUR PLOTS FOR CHROMATOGRAPHIC CONDITION OPTIMIZATION

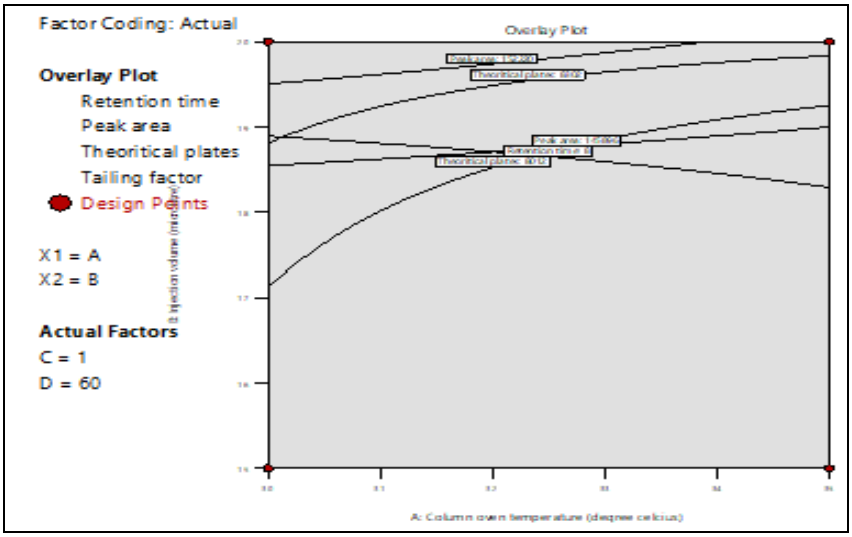


FIG. 10: OVERLAY PLOTS FOR TQ

Comparison to Prior Reported HPLC Techniques: Based on mobile phase ratios, mobile phase flow rate, wavelength, column, stability studies, drawbacks and application of the HPLC techniques, a comparative assessment of previously published methods and the present devised HPLC method was undertaken **Table 9**. There is currently no single HPLC technique available that can be utilized for numerous studies such as estimating TQ in polymeric nanoparticles, black seeds and its

marketed goods and evaluating TQ degradation behavior utilizing the exact parameters of the established HPLC method. No HPLC approach has been described for estimating TQ using a complete factorial design (DOE-version 12). Compared to previously established techniques, the current approach with the mobile phase composition of H2O: MeOH (25:75)1ml/min of flow rate and 253 nm as detecting wavelength is shown to be more sensitive and economical stable. The created approach exhibits accurate and simple measurement of TQ in polymeric nanoformulation, suggesting the method's dependability and sensitivity. With the use of the Design Expert 12.0 version, the 2^4 full factorials experimental design explains the interrelationships between the mobile phase and column oven temperature at four distinct levels, and the responses to be examined were retention time, theoretical plates and peak asymmetry, and tailing factor. The QbD technique for analytical method development was adopted to understand method variables with varied levels better.

TABLE 9: COMPARATIVE DATA BETWEEN PREVIOUSLY REPORTED HPLC TECHNIQUES

S. no.	Column	Wavelength (nm)	Mobile phase a flow rate	Limitations	Applications	Ref.
1	C_{18} (inerto) ODS 3V,	254	0.1%aq. Formic acid and Methanol (40:60) 1.5 ml/min	High flow rate Less sensitive Non-robust	Distinct separation and identification of TQ	7
2	Econosphere CN	295	Hexane and propanol (99:1) 1ml/min	Lack of stability Study Expensive Non-ecofriendly Non-robust	Sensitive with low limit of 5nmoles/ml	8
3	C_{18} Eurosph er	254	Methanol with 0.1% TFA and water 12ml/min	High flow rate Less sensitive Lack of stability Study Non-robust	Method effective for preparation and purification of TQ	9
4	C_{18} ODS	254	Water and methanol (40:60) 1.5ml/min	Lack of stability Study Non-robust	Simple method for estimation of TQ extraction	10
5	C_{18} ODS	254	ACN and methanol (30:70) 1.5ml/min	Lack of stability Study Expensive Non-sensitive Non-robust	Yield is enhanced by Employed extraction method	13

CONCLUSION: The current study aimed to develop and optimize an RP-HPLC-based analytical technique for the quantitative measurement of TQ, using a 24 factorial design. ANOVA was used to examine the influence of independent variables and the result was documented in perturbation plots and Pareto charts form. According to ICH requirements, the devised approach was determined to be simple, quick, extremely sensitive, exact, and efficient. We effectively used the notion of analyzing numerous compounds with varied λ_{max} making the procedure more reliable and cost-effective. Forced degradation experiments demonstrated that the TQ could tolerate acidic, alkaline and high-temperature environments but were susceptible to oxidative and photolytic deterioration. Furthermore, there was no interference from excipients or degradation products in the analytes' peaks in the nanoparticles. As a result, the approach design is suited for fast analysis of TQ in formulation development and is cost-effective. Because of the emphasis on risk assessment and management, a quality-by-design approach to method development resulted in a more robust/rugged technique than a typical or conventional approach. Understanding dependent variables, various factors, and their interaction effects by a desired set of experiments on the answers were studied efficiently as an important component of QbD.

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CONFLICTS OF INTEREST: None

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