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Original Article

Analytical Quality by Design Enabled HPTLC Method for Estimation of Phytoconstituents Andrographolide, Piperine and Quercetin in Nilavembu Kudineer

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ABSTRACT

Background: This work outlines a sensitive, cost-effective, reliable and ecofriendly novel AQbD approach based HPTLC for the simultaneous determination of standard samples of Andrographolide (A), Piperine (P) and Quercetin (Q) and in Nilavembu Kudineer. The dependent variable retardation factor was considered as critical method response for each of the three phytomarkers whereas the independent variables, methanol %, saturation time and development distance were regarded as critical method parameters. **Materials and Methods:** The chromatographic separation was performed on aluminium sheets Merck TLC of silica gel 60 F 254 using a mixture of Toluene, Ethyl Acetate, Methanol, and Formic Acid (6:2:1.5:0.5) as the mobile phase. The developed plates were analysed using densitometric scanner at 254 nm. **Results:** The markers were suitably resolved under optimized conditions, with R_f values of 0.58, 0.78, and 0.63 for Andrographolide, Piperine, and Quercetin. The linearity was observed in the range of 100-500 ng/band for A, P, and Q with accuracy of 98.9-99.8 %, and precision of %RSD <2. This QbD-based HPTLC method developed and validated was successfully applied to quantify andrographolide, piperine and quercetin, in Nilavembu kudineer powder and liquid formulations. **Conclusion:** A green chemistry approach based HPTLC method was found to be cost-effective, reliable, time-efficient and for the simultaneous quantification of A, P, and Q in NK formulation.

Keywords: Analytical QbD, Andrographolide, Piperine, Quercetin, Nilaveembu Kudineer, HPTLC.

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INTRODUCTION

Nilavembu Kudineer (NK) is an herbal preparation containing 9 medicinal plants is an immune modulator and used for the treatment of SARS-COVID 2, anti-inflammatory, antioxidant and hepatoprotective. NK contains phytoconstituents such as alkaloids, terpenoids, flavonoids and phenolic acids.[1,2] NK comprises a rich tapestry of botanical resources, each contributing its unique attributes to this remarkable polyherbal formulation. *Andrographis paniculata*, known as Nilavembu, *Plectranthus vettiveroides*, or Vilamichamver, *Zingiber officinale*, referred to as Sukku, *Cyperus rotundus* (Koraikizhangu), while *Trichosanthes dioica* (Pei pudal). *Vetiveria zizanioides*, commonly known as Vetiver, *Piper nigrum* (Milagu) lends its distinctive character, and *Mollugo cerviana* (Parpadagam), and the wood of *Santalum album*, also known as Sandanam, creating a harmonious blend of healing elements in NK. QbD is used for emphasizing product and process understanding, uses risk management and scientific understanding to establish objectives and guide the analytical process lifecycle. It helps to create robust, reliable, and cost-efficient analytical procedures.[3,4]

In Quality by Design (QbD), the ATP is a concept that describes the performance of an analytical method and is used to guide the design and development of the method. The ATP is a key part of the analytical lifecycle model and is used to ensure that the approach is utilized for purpose throughout the product's life cycle. It provides a clear and comprehensive description of the method's intended purpose, accuracy, precision, selectivity, sensitivity, and other critical attributes.[5,6]

Box Behnken Design (BBD) is a type of RSM, which uses statistical techniques and mathematical models to optimize processes by analyzing the relationship between input factors and output responses. BBD helps identify the optimal conditions for HPTLC method development by systematically exploring the experimental space and finding the combination of factors that yield the desired results. Compared to full factorial designs, BBD

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requires fewer experimental runs to explore the factor space, making it efficient for optimizing HPTLC methods. By analyzing the results of the BBD experiments, researchers can identify the optimal conditions for HPTLC analysis, leading to improved resolution, sensitivity, and accuracy.

HPTLC is a green chemistry approach for the simultaneous analysis of multiple phytoconstituents present in herbs/herbal products using small volume of mobile phase thereby minimising environmental pollution. The experimental design approach has exhibited to be useful for verifying HPTLC procedures since it may assess multiple features that change simultaneously.[7,8] In this work, an established approach that helps evaluate the amount of markers by comparing their Rf values to those of the phytomarkers is optimized using the Box Behnken design of BBD.[9,10] To effectively confirm the purity, quality and identity of the Nilavembu Kudineer, the study's goal is to quantify the possible phytomarkers through optimization studies based on the DoE-based HPTLC method.[11,12]

The methodical process of developing and validating analytical processes is known as a QbD.[13,14] It provides improved comprehension and management of the pharmaceutical process in order to produce better products.[15,16] A range of approaches are available for screening and optimizing process variables that may affect responses.[17,18] A QbD methodology is a straightforward, quick, cost-effective, and reliable method development strategy.[19,20] A Quality by Design (QbD) approach has been shown to be useful by numerous researchers for the development of HPTLC methods and the quantification of analytes in a variety of dosage forms and bioanalytical samples.[21] As far as we are aware, the analysis of A, P, and Q has not yet been done using this approach.

Therefore, the current work marks the first attempts to use the design of experiment technique to establish a straightforward, sensitive, accurate, and robust HPTLC method to analyze A, P, and Q in herbal formulation (in house).

MATERIALS AND METHODS

Instruments

Analysis was conducted using the CAMAG HPTLC system, which included a Wensor ultrasonic sonicator, a Win CATS software version 1.3.0, a 100 µL Hamilton syringe, and an automated sample applicator (CAMAG), twin trough glass chambers (CAMAG, Switzerland) were used for the establishment and analysis of the HPTLC approach utilizing ascending mode Stat–Ease Design Expert® software version 13.0.11.0.

Materials and reagents

All the three phytomarkers andrographolide, piperine and quercetin were procured from Yucca Enterprises Mumbai. Nilavembu Kudineer kashayam was procured from Ayurveda Pharmacy, Tirupati. Silica gel 60 GF 254 TLC plates layer thickness, (0.2 mm) Served as the stationary phase. Analytical grade chemicals and reagents were procured from (Merck Pharmaceuticals, India).

Preparation of Standard solution

The standard samples of phytomarkers of andrographolide, quercetin and piperine were individually prepared in a volumetric flask (10 mL), accurately weigh 10 mg of the standard andrographolide, quercetin and piperine by dissolving in 1 mL (CH_3OH) and the final solution volume was raised to 10 mL with CH_3OH, from this stock solution 1 mL is measured and poured into a 10 mL volumetric flask and volume made with CH_3OH and was sonicated for 6 min. The working standard stock concentration was 100 µg/mL.

Fractionation of Nilavembu Kudineer

10 mL of NK was transferred into a separating funnel and extracted using 30 mL of hexane by shaking and keeping it aside for 5-10 min, two layers were formed. Collect the hexane layer separately in a beaker. Aqueous layer is further extracted with 10 mL of $CHCl_3$ and the remaining layer was extracted with mobile phase. All the fractions were concentrated separately for the identification and estimation of andrographolide, quercetin and piperine and was subjected for sample preparation.

Preparation of sample solution

The sample formulation 10 mL was precisely measured by dissolving in 1 mL CH_3OH and the final solution volume was raised to 10 mL with CH_3OH, from this stock solution 1 mL is measured and transferred into a 10 mL volumetric flask and volume made with CH_3OH and was sonicated for 6 min. The working sample stock concentration was 100 µg/mL.

Development of Method by QbD approach

The Analytical Target Profile (ATP) was established for the development of the A, P, and Q methods. Saturation time, development distance, and methanol content were chosen as Critical Method Parameters (CMPs). CAA was found to be the R_f. Three-level, three-factor (BBD) was used for the optimization. The selection of methanol percentage in the mobile phase depends on the specific compound being analyzed and the desired separation, for good separation and resolution. The chamber saturation time refers to the duration needed for the HPTLC chamber to equilibrate with the mobile phase, typically ranging from 15 to 30 min, crucial for optimal separation and reproducible results. The optimum developing distance was selected for achieving effective separation and resolution. Seventeen runs were carried out using Stat–Ease Design Expert® software version 13.0.11.0. To determine the relationship between the factors and responses, response surface approach was employed. The R2 value, (SD), adequacy precision, and coefficient of variation (% CV) were

taken into account while estimating the model's applicability. ANOVA was used to evaluate the model's significance.

To assess the significance of the model, the *p* value, Model F value, and Lack of Fit Test were calculated. The polynomial equation was evaluated if the model coefficient of statistical significance was less than 0.05. In order to choose dependent and independent variables, exploratory experiments were conducted. Table 1 shows the Variables and Factor levels selected in Box Behnken design. Three-dimensional response surface plots were taken into consideration for response surface analysis. Dependent and independent variables selected in BBD design for three markers is explained in Table 2. The approach was validated according to ICH recommendations.[22]

Simultaneous Quantification of potential phytomarkers by HPTLC

To simultaneously measure the concentrations of andrographolide, piperine and quercetin in the methanolic extract of a Nilavembu Kudineer, a HPTLC method was initiated. In a twin trough chamber, the plates were saturated. The slit's measurements were set at 4 mm for length and 0.3 mm for width. The monochromator's scanning rate was 20 mm/s, and its bandwidth was 20 nm. The data resolution was 100 µm/step, and the spraying rate was 10 s/µL. A 254 nm-wavelength filter and a deuterium source were employed in the absorption mode. The stationary phase used was Silica Gel 60 F254 (0.25 mm), served as the chromatographic plates for this investigation. Following a 20-min saturation period, a mobile phase comprising of T, E.A, M, and F.A (in a volumetric ratio of 6:2:1.5:0.5) was applied to the plates.[11] The ICH standards were followed in the validation of the optimized procedure.

Software and statistics

Using Stat Ease Design Expert software version 13, the RSM was evaluated using Analysis of Variance (ANOVA) through Box Behnken Design (BBD). In addition, the desirability function D was identified and 3D plots were produced.[23] The ICH regulations were adhered to in order to validate the approach. The following parameters were examined: recovery, robustness, linearity, precision, LOD and LOQ.

Calibration curve of phytomarkers

To prepare a stock solution (100 µg/mL), andrographolide, piperine, and quercetin were dissolved in CH_3OH. The concentration used was 0.1 to 0.5 µg/spot of andrographolide, piperine, and quercetin, the stock solution was plotted in triplicate on TLC plates in various volumes, specifically 1 to 5 µL. Linear Least Square Regression was used to plot and analyze the peak area versus drug concentration graph.

Linearity

A range of standard concentrations, from 100 to 500 ng/band, were used to analyze the linearity of the devised method for estimating andrographolide, piperine, and quercetin. Plotting the results as peak area versus band concentration was carried out.

Precision

By repeating the sample analysis at various intervals within the day, the precision study was illustrated, and the % RSD was calculated.

Accuracy

To verify accuracy, the substance was recovered at three distinct levels (50, 100, and 150% addition). After the sample was spiked with a required quantity of phytomarker, it was examined and the % RSD was determined.

LOD and LOQ

The least amount of analyte detected, which is not always precisely quantifiable, is known as LOD. The following formula was used to determine it: LOD is equal to 3.3 times the response/calibration curve slope's Standard Deviation (SD).

The least amount of substance that can be evaluated with relevant precision and accuracy is known as LOQ. LOQ = 10 x (SD) of response/ slope of calibration curve is an equation that can be used to estimate it.

Robustness

It was established to evaluate the method's reliability. Small adjustments were purposefully made to the mobile phase composition and saturation time to test the method's resilience. The impact of these modifications on the R_f was evaluated.

RESULTS

Quantification of three phytomarkers by HPTLC studies

Using a HPTLC technique based on the AQbD-BBD approach, several combinations of $C_6H_5CH_3$, $CH_3CO_2CH_2CH_3$, CH_3OH, and HCOOH were employed as the MP on the stationary phase. Following testing, it was found that a combined ratio of (6:2:1.5:0.5) v/v produced a resolution that was acceptable. The data's visual representation is depicted in Figure 1. Andrographolide, piperine, and quercetin were found to have respective R_f values of 0.58, 0.78, and 0.63. The peak areas of the extract and the phytomarkers were compared. This comparison revealed that the extract's estimated concentrations of andrographolide, piperine, and quercetin were 0.36%, 2.41%, and 1.29% w/w, respectively. For the standard concentration range of 100–500 mcg per mL, the correlation coefficient was observed to be between 0.9936 and 0.09996.[24]

Table 1: Factors and responses selected in BBD design for three markers.

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
Std	Run	A: Methanol percentage	B: Saturation time	C: Developing distance	R_f (Andrographolide)	R_f (Piperine)	R_f (Quercetin)
		% v/v	min	cm			
15	1	1.5	20	90	0.58	0.78	0.63
6	2	2	20	85	0.62	0.82	0.72
1	3	1	15	90	0.53	0.73	0.57
13	4	1.5	20	90	0.57	0.77	0.64
14	5	1.5	20	90	0.56	0.76	0.62
4	6	2	25	90	0.62	0.82	0.72
8	7	2	20	95	0.54	0.74	0.58
16	8	1.5	20	90	0.59	0.75	0.61
10	9	1.5	25	85	0.59	0.79	0.64
9	10	1.5	15	85	0.6	0.8	0.65
5	11	1	20	85	0.54	0.74	0.64
7	12	1	20	95	0.39	0.59	0.4
17	13	1.5	20	90	0.53	0.72	0.64
12	14	1.5	25	95	0.35	0.55	0.34
11	15	1.5	15	95	0.48	0.68	0.53
3	16	1	25	90	0.59	0.78	0.63
2	17	2	15	90	0.51	0.71	0.57

Table 2: Linearity regression data for estimation of Andrographolide, Piperine and Quercetin.

Parameter	Phytoconstituents		
	Andrographolide	Piperine	Quercetin
Linearity range ng/spot	100-500	100-500	100-500
Slope	784x	1126.6x	7.58x
Intercept	14541	18069	15541
Correlation Coefficient (r^2)	R^2 = 0.9956	R^2 = 0.9965	R^2 = 0.994

The variables and factor levels chosen in the Box Behnken design are displayed in Table 2, and the factors and responses chosen in the BBD design for three markers for seventeen runs are displayed in Table 2. Figure 1, shows the TLC of standards and sample under UV 254 nm (a) Andrographolide (b) Piperine (c) Quercetin. Figure 2 shows the HPTLC Chromatograms of Andrographolide and Piperine standards, Figure 3 displays the HPTLC Chromatograms of Quercetin Standard and Sample Extract.

The R_f value was positively impacted by the methanol concentration (factor A) and saturation time (factor B), whereas the development distance (factor C) had a negative impact.[15] The approach optimized for three markers-quercetin, piperine, and andrographolide-had a desirability of 0.969. Figure 4 displays the 3D Overlay Chromatograms of Sample and Standards at Lambda 254 nm Figure 5 displays 3D response surface plots of Andrographolide and Piperine, Figure 6 displays 3D response surface plots of Quercetin and Desirability plot.

Validation of proposed Method

Andrographolide, Piperine, and Quercetin were effectively resolved at R_f values 0.58, 0.78, and 0.63 by the DOE's optimized chromatography conditions, which included a MP proportion of T, E.A, M, and F.A. (in a volumetric ratio of 6:2:1.5:0.5), development distance of 80 mm, band width of 6 mm, and chamber saturation time of 20 min. Peak area and applied amount, which varies from 100 to 500 ng/band, showed a good linear correlation in the HPTLC method established for estimating A, P, and Q.[25]

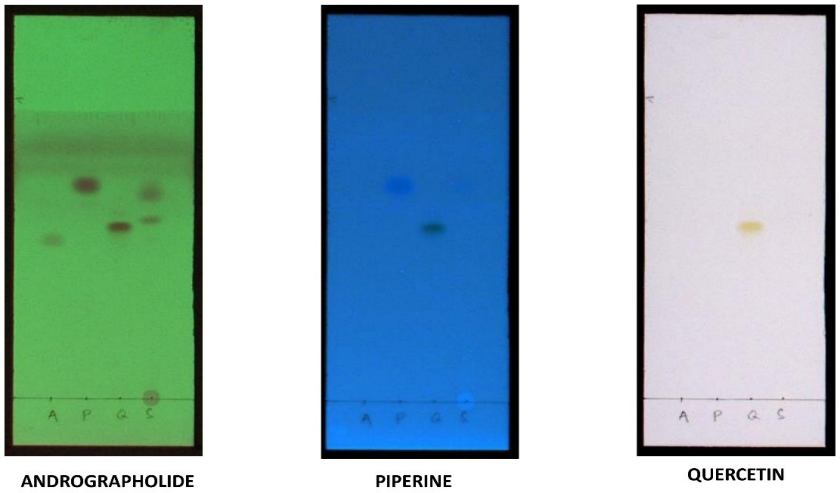
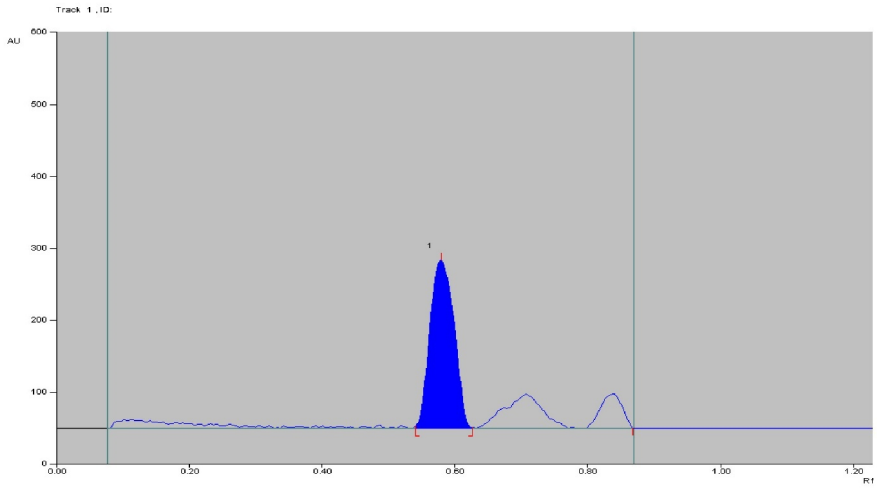
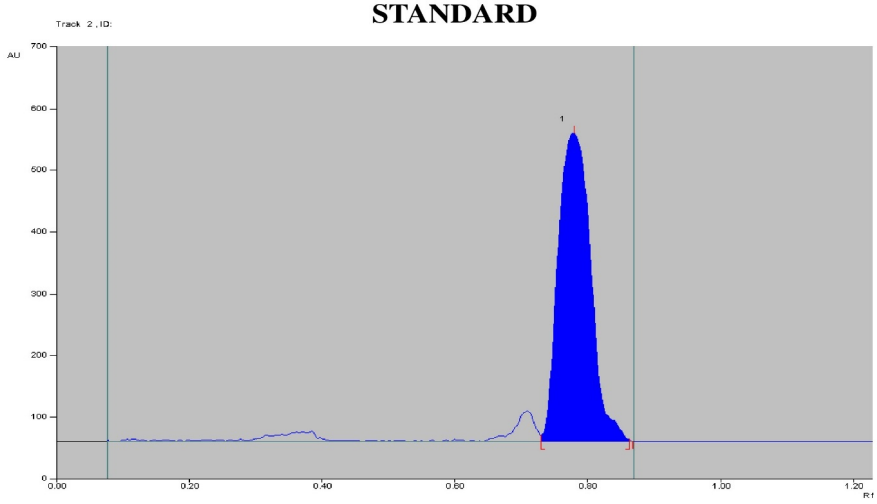


Figure 1: TLC of standards and sample under UV 254 nm (a) Andrographolide (b) Piperine (c) Quercetin.

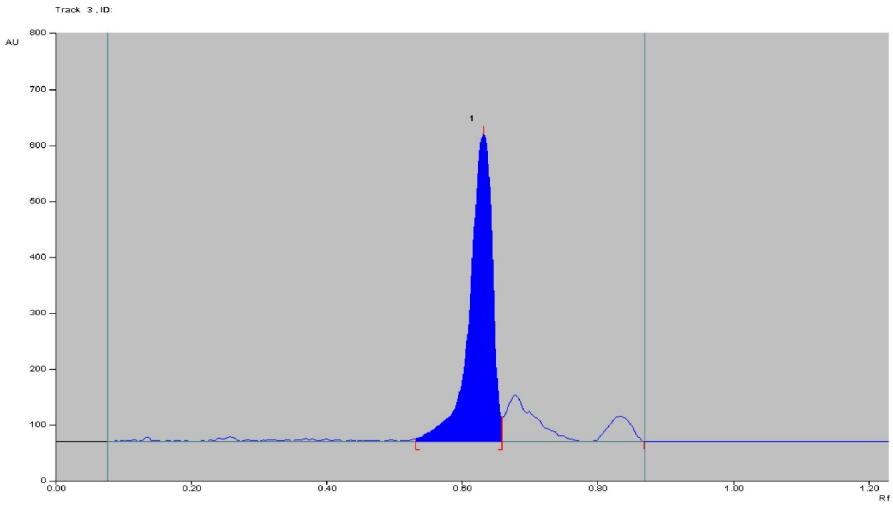


HPTLC CHROMATOGRAM OF ANDROGRAPHOLIDE STANDARD

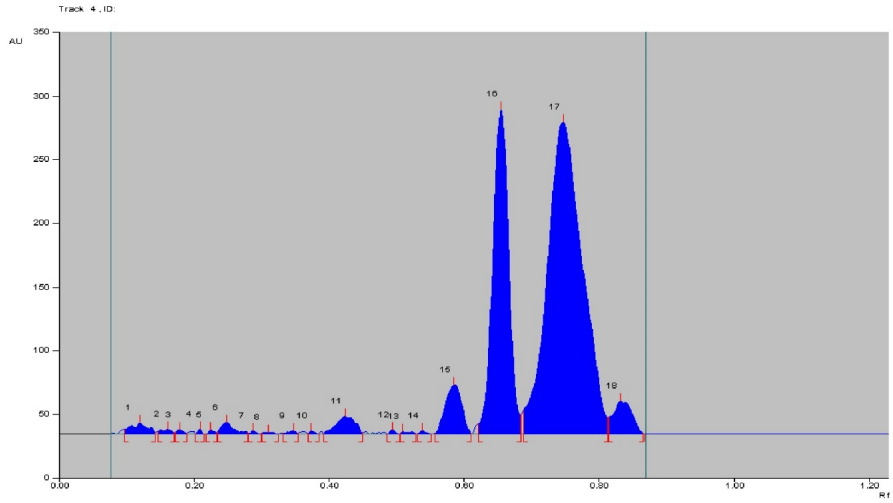


HPTLC CHROMATOGRAM OF PIPERINE STANDARD

Figure 2: HPTLC Chromatograms of Andrographolide and Piperine Standards.



HPTLC CHROMATOGRAM OF QUERCETIN STANDARD



HPTLC CHROMATOGRAM OF SAMPLE EXTRACT

Figure 3: HPTLC Chromatograms of Quercetin Standard and Sample Extract.

Table 3: Results of Accuracy study of Andrographolide, Piperine and Quercetin.

% Recovery	% Recovery (A)	% Recovery (Q)	% Recovery (P)
50	99.72	98.92	98.81
100	100.29	100.12	100.33
150	101.50	101.73	101.51

Table 4: Results of precision study of Andrographolide, Piperine and Quercetin.

Phytoconstituents	Mean Peak Area	SD	% RSD
Andrographolide	15493	233.2	1.50
Piperine	19448.4	219.9	1.11
Quercetin	19739.2	226.6	1.16

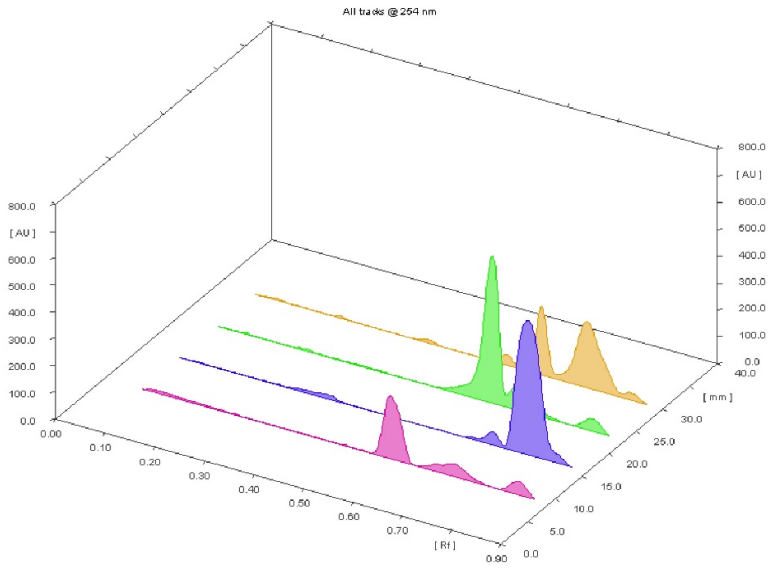


Figure 4: 3D Overlay Chromatograms of Sample and Standards at Lambda 254 nm.

Table 5: LOD and LOQ data for Andrographolide, Piperine and Quercetin.

Phytoconstituents	LOD (ng/spot)	LOQ (ng/spot)
Andrographolide	0.93	2.84
Piperine	0.08	2.55
Quercetin	2.16	6.55

Specificity

Glass plates coated with silica gel and containing toluene, ethyl acetate, methanol, and formic acid in a volumetric ratio of 6:2:1.5:0.5 produced well-developed spots of A, P, and Q that were appropriate for densitometric analysis. The R_f value and the spectra of the sample and the standard spot were compared in order to verify the method's specificity. The R_f values of the pharmaceutical sample and the standard exhibit a good degree of agreement. There were no additional peaks seen on the obtained densitogram (sample) besides the peak originating from the active compounds, A, P, and Q. In order to determine A, P, and Q in the tested herbal formulation, the developed HPTLC-method is specific and appropriate.

Linearity

For A, P, and Q, the correlation coefficient values were found to be 0.9956, 0.9965, and 0.994, respectively. The regression equations Y= 784x + 14541, Y= 1126.6x + 18069, and Y= 7.58x + 15541 were used to determine that the peak area (y) was proportional to the concentration of A, P, and Q as reported in Table 2.

Accuracy

This method's accuracy was assessed using the recovery study (% R) using the standard addition procedure at three concentration levels. For 50%, 100%, and 150% of the content in the tested drug sample, known quantities of pure standards of A, P, and Q were introduced. Each 5 µL sample was examined in triplicate using the chosen chromatographic parameters. A measurement of the peak area was made at every concentration. The recovery was found to be between 98.8 and 101.7% for pure standards of A, P, and Q added at 50%, 100%, and 150%. The label claim of the formulation under study and the ICH guidelines both state that the recovery outcomes fall within the acceptable range. The recovery of the phytomarkers was described and the values were reported in Table 3.

Precision

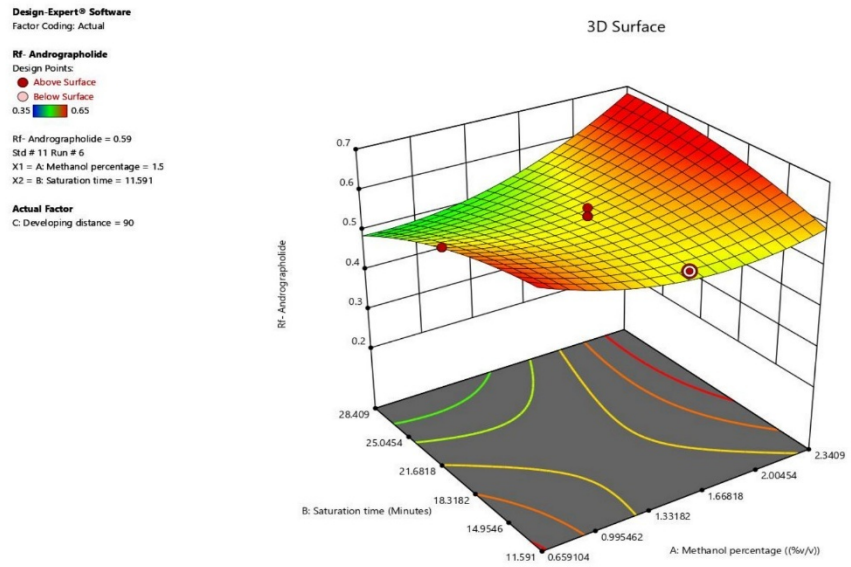
The precision study describes the % RSD the values were observed to be within the described limits. The results are displayed in Table 4.

LOD and LOQ

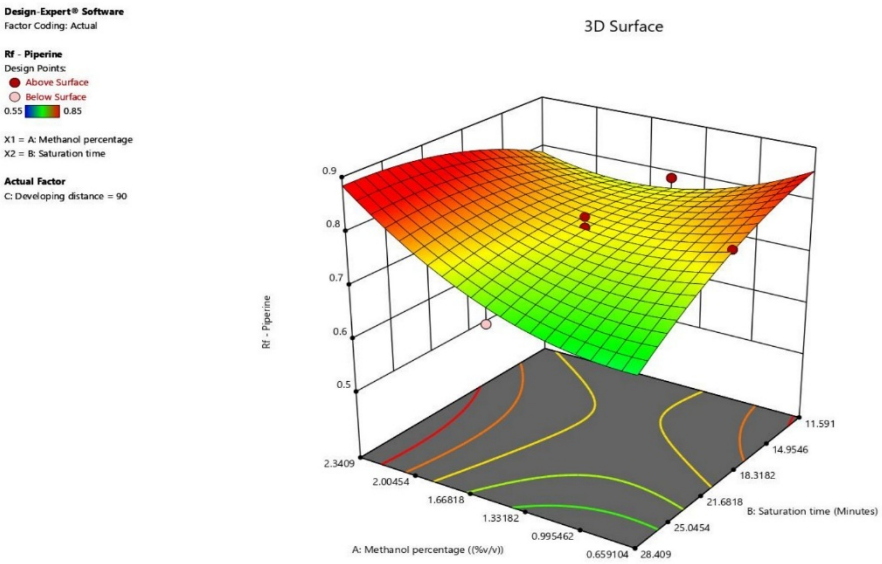
The mean slope of calibration curve and RSD were used to characterize LOD and LOQ. The anticipated method's sensitivity was demonstrated by the findings shown in Table 5.

Robustness

The approach was deemed robust as there were only minor variations in R_f values and no changes reported in peak resolution. Based on the findings shown in Table 6.



3D RESPONSE SURFACE GRAPH OF ANDROGRAPHOLIDE



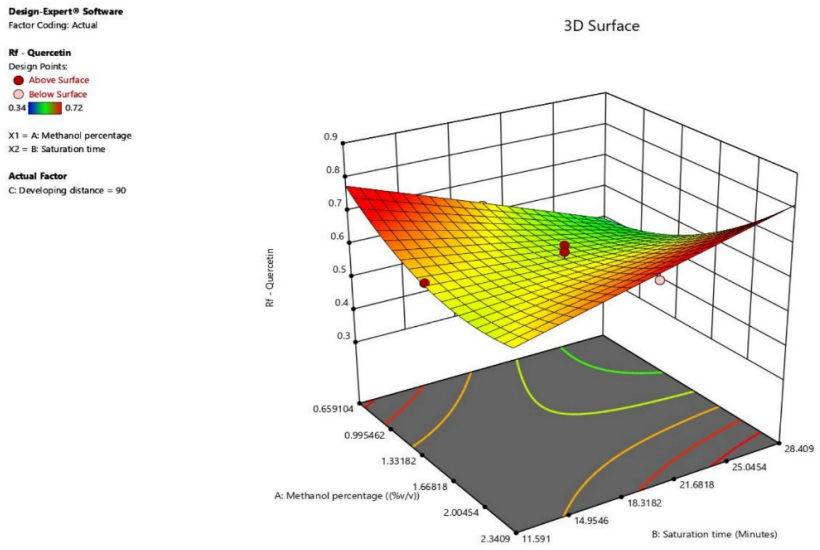
3D RESPONSE SURFACE GRAPH OF PIPERINE

Figure 5: 3D response surface plots of Andrographolide and Piperine.

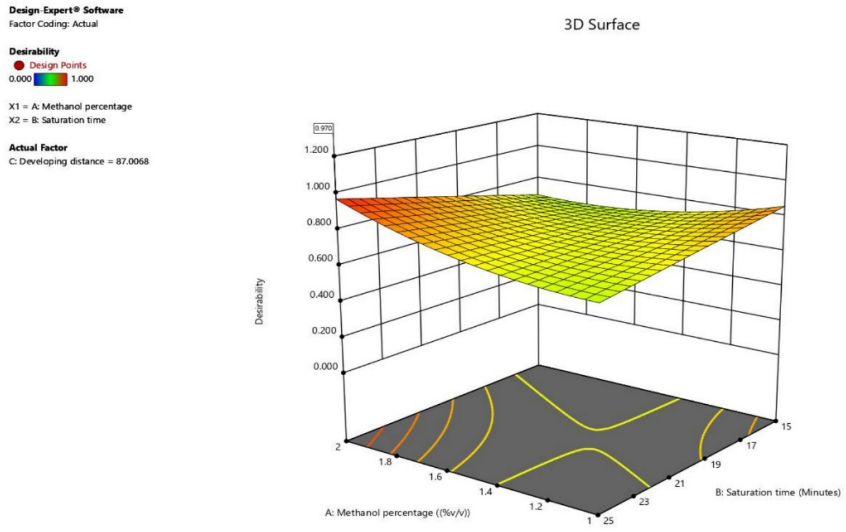
DISCUSSION

Since the probability value was less than 0.0001, the model was considerably significant. The plots displayed changes in the response at the reference point when all factors were constant; the curvature of the line indicates sensitivity to a particular factor. When the toluene concentration increased and the development distance decreased, the R_f value increased as well. The R_f value declined significantly as the chamber saturation time increased. A numerical optimization technique based on the desirability approach was implemented to attain optimal chromatographic performance. The desirability function ranges from a completely desirable response (1) to an undesirable response (0). Global optimization of a selected combination of many criteria is determined by a desirability value close to 1.[26]

Regression analysis showed that the model was highly adequate, with low standard deviation values of 0.0346 for A, 0.0372 for P, and 0.0489 for Q, and higher R square values of 0.9966 for Andrographolide, 0.9968 for Piperine, and 0.9946 for Quercetin.



3D RESPONSE SURFACE GRAPH OF QUERCETIN



3D RESPONSE SURFACE PLOT OF DESIRABILITY FUNCTION

Figure 6: 3D response surface plots of Quercetin and Desirability plot.

With a coefficient of variation (% CV) of 5.96 for andrographolide, 4.75 for piperine, and 7.63 for quercetin, the model's predicted repeatability was below 10%. The signal to noise ratio depends on sufficient precision. It must be more than four. Fit statistics data indicated that the signal's adequacy was 9.5366 for A, 9.6340 for P, and 9.4426 for Q. Thus, this approach may help with navigate the design. In order to reduce bias, 17 design runs were carried out randomly.[27]

The chemical fingerprint of andrographolide NK churnam in methanol extract was determined by an HPTLC analysis. Chloroform: methanol (9:1) was employed as the mobile phase, and the R_f value was 0.6.[26] As the mobile phase, ethyl acetate: n-hexane (8.5:1.5 v/v) was used in another study to measure andrographolide in NK was determined to be the reference standard andrographolide R_f value 0.55.[27] An instrumental Thin-Layer Chromatographic (TLC) approach based on QbD was

Table 6: Results for Robustness experiments of Andrographolide, Piperine and Quercetin.

Parameter	Altered Conditions	Effect on R_f values		
		Andrographolide	Piperine	Quercetin
Saturation Time				
	15 min	0.51	0.73	0.61
Optimized	20 min	0.58	0.78	0.63
	25 min	0.59	0.80	0.67
Mobile Phase composition				
	T: EA:M: FA (5:3:1.5:0.5)	0.61	0.82	0.71
Optimized	T: EA:M: FA (6:2:1.5:0.5)	0.58	0.78	0.63
	T: EA:M: FA (7:1:1.5:0.5)	0.49	0.67	0.52

designed to measure Andrographolide (AG), Gallic Acid (GA), and Quercetin (QR) from polyherbal tablets simultaneously. With densitometric scanning at 247 nm, a chromatographic separation was performed using a mobile phase consisting of toluene, ethyl acetate, methanol, and formic acid (4.3:3:1:0.05, V/V). Under ideal circumstances, the biomarkers were appropriately resolved, with R_f values for AG, GA, and QR being 0.38, 0.24, and 0.50, respectively.[13] NK extract was shown to contain large amounts of terpenoids and flavonoids using Thin-Layer Chromatography (HPTLC).[27] In order to screen for active principles, the extract of Nilavembu Kudineer Chooranam is analyzed using High Performance Thin-Layer Chromatography (HPTLC). The mobile phase consists of 3:4:3 ratio of Methanol, Formic acid and Toluene in ethyl acetate extract. Such phytochemicals, which are widely known for their therapeutic qualities, were confirmed to be present by the HPTLC study.[28]

CONCLUSION

A novel AQBD enabled HPTLC method was developed for simultaneous quantification of phytomarkers andrographolide, piperine and quercetin is suitable for their estimation in NK formulation also. This method was observed to be highly reliable, accurate, precise, and linear. This can be a desirable method for the concurrent determination of andrographolide, piperine and quercetin phytomarkers in NK formulation.

ABBREVIATIONS

AQBD: Analytical Quality by Design; **BBD:** Box Behnken Design; **NK:** Nilavembu Kudineer; **CAA:** Critical Analytical Attribute; **CMA:** Critical Method Attributes; **DOE:** Design of Experiment; **HPLC:** High Performance Liquid Chromatography; **TLC:** Thin Layer Chromatography; **HPTLC:** High Performance Thin Layer Chromatography; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **ICH:** International Council for Harmonization; **SD:** Standard Deviation; **RSD:** Relative Standard Deviation; **T:** Toluene; **E.A:** Ethyl Acetate; **M:** Methanol; **F.A:** Formic Acid.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTIONS

Conception and Design: Sivagami B, Drafting the Manuscript: Chandrasekar R, Critical Revision and Supervision: Sailaja B.

SUMMARY

This work describes a sensitive, cost-effective and reliable HPTLC approach based on QbD for the simultaneous measurement of andrographolide, piperine and quercetin in NK. The methanol percentage, saturation time and development distance were regarded as factors the retardation factor was considered as response for each of the three phytomarkers. Using a mixture of Toluene, Ethyl Acetate, Methanol, and Formic Acid (in a volumetric ratio of 6:2:1.5:0.5) as the mobile phase, the plates were developed using densitometric scanning at 254 nm. The markers were suitably resolved under optimal conditions, with R_f values of 0.58, 0.78, and 0.63 for A, P, and Q. They exhibited linearity in the range of 100-500 ng/band for A, P, and Q high accuracy of 98.9-99.8%, and precision of %RSD <2%. The optimized technique was found to be cost-effective, reliable, and time-efficient for the simultaneous estimation of Andrographolide, Piperine, and Quercetin in herbal extracts and herbal formulations. This QbD-developed validated HPTLC method can be effectively used to quantify quercetin, piperine, and andrographolide in plant extracts.

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