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Analytical Quality by Design Approach for Simultaneous Determination of Phytomarkers Orientin, Quercetin and Piperine in Nilaveembu Kudineer by RP-HPLC Method

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ABSTRACT

Orientin, quercetin and piperine are significant phytoconstituents present in Nilaveembu Kudineer (NK), NK is a polyherbal formulation consisting of 9 medicinal plants used in the management of dengue fever, inflammation, pyrexia, digestive and liver disorders etc. The present objective is to develop a Quality by Design (QbD)-based RP-HPLC method for standardization of orientin, quercetin and piperine phytomarkers present in NK by using Box Behnken Design. The Independent variables selected for QbD method are flow rate, acetic acid concentration, injection volume and the dependent variables selected are retention time, theoretical plate and tailing factor. Effective chromatographic separation of orientin, quercetin and piperine was established on C_{18} (150mm x 4.6mm x 5µm) column using moving fluid comprised of a combination of ACN and H_2O (pH adjusted with 0.5% v/v GAA) in gradient elution, with 1 mL/min flow rate, the temperature was set at 40°C and the injection volume was 20 µL. The R_t for orientin was found to be 4.108 min, quercetin 7.450 min and piperine 12.92 min respectively. Lambda max (λ_{max}) was 340 nm. The method was found to be specific for the concurrent analysis of orientin, quercetin and piperine in NK, with (>90%) of accuracy and less then (%RSD<2%) of precision. The novel RP-HPLC approach was effectively applied for the quantification of orientin, quercetin and piperine in NK. The developed RP-HPLC method could be further used for the simultaneous quantification of these phytoconstituents present in other herbal preparations.

Keywords: Analytical QbD, Nilaveembu kudineer, Orientin, Quercetin, Piperine, RP-HPLC.

INTRODUCTION

Herbal medicine requirements are growing globally in health care and making quality control and standardization of these products increasingly essential. Herbal drug quality control and standardization are significant tasks that has batch-to-batch uniformity of herbal products is difficult. Due to varied geography, environment condition, possible adulteration, microbial contamination and foreign materials. Therefore, it is crucial to evaluate the raw material's quality in terms of pharmacognostic and phytochemical standards to establish the identification and purity of herbal

medicines. Herbal medicine quality control can be achieved by the widely recognized technique of marker-based standardization.[1]

NK a polyherbal siddha formulation made of 9 medicinal plants containing orientin quercetin and piperine phytochemical markers in significant quantity. NK is a popular antiviral formulation also used for inflammation, pyrexia, digestive and liver disorders property. Orientin is categorized under a C-glycoside of flavonoid soluble in water, Orientin is chemically known as 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-8-[(2S,3R,4R,5S,6R)],[3–4,5-trihydroxy-6–(hydroxymethyl) oxan-2–yl] chromen-4–one.[2] Quercetin (3,3',4',5,7-pentahydroxyflavone) Quercetin. is a member of the flavonol class.[3] Pepper a popular spice, and other genus and species of Piper fruits belong to piperaceae family contain, main alkaloid piperine. The characteristic odour and spicy taste of black pepper is due to piperine. (Structure of Orientin, Quercetin and Piperine are depicted in Figure. 1)[4,5]

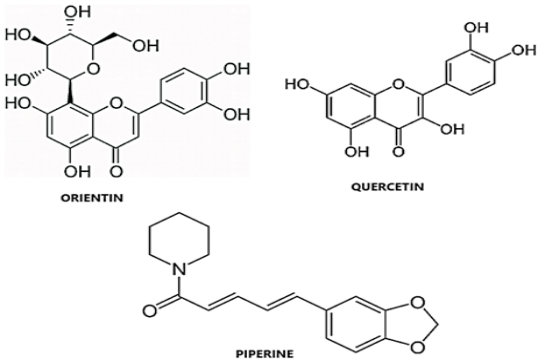


Fig. 1. Structure of Orientin, Quercetin and Piperine

AQbD application in quality control of herbal formulation

Quality, safety and efficacy are essential for herbal products. RP-HPLC method is widely used for quality assessment of herbal products to develop more robust quality control analytical technique AQbD is required. maintain the quality of products scientific techniques like process analytical technology and quality by design (QbD) are employed.[6] According to the ICH, QbD is a methodology that starts with predetermined goals and places a special emphasis on determining and developing processes and products through sound research and risk assessment.[7] The analytical process makes use of analytical QbD.[8] By taking the method factor into consideration, AQbD, which is the equivalent of QbD, aids in the establishment of a reliable and economical analytical technique that results in the establishment of a method-operable design.[9] Certain factorial experimental designs are commonly utilized including (BBD) and (CCD).[10]

BBD trial runs with very few design points and fewer parameters were utilized from the software generated data.[11] It provides statistically significant values and relates variables with responses.[12] Resolution, theoretical plate number and peak asymmetry were regarded as responses in the experimental runs whereas flow rate, acetic acid and injection volume were chosen as independent variables. The desined approach was validated in accordance to ICH Q2 R1 guidelines.[13-15]

MATERIALS AND METHODS

Chemicals and Reagents

Orientin, Quercetin and Piperine phyto-chemicals were purchased from Yucca Enterprises (Mumbai), India. ACN & H_2O LC Grade obtained from (Merck, India). OPA (85%) was obtained from SD fine chem. Milli-Q water (Merck, India).

HPLC Analytical Setup and Instrumental Attributes

The research analysis was carried out on the HPLC (Shimadzu) equipment with a UV detector. The equipment comprised of a quaternary pump LC gradient 20 AD, auto sampler SIL 20 AC, Oven, CTO 10 AS oven, C18 column, 100 A°, 150 mm, 4.6×5 µm, UV detection SPD 20 A (Shimadzu, Japan). The mobile phase for orientin comprising of a mix of ACN and water 20:80 (GAA 0.5% v/v was used to adjust the pH 2.5) for quercetin ACN (0.5% GAA) 30:70 and piperine consisting of 45:55 ACN and 0.5% GAA in gradient method. A 1.0 mL/min flow rate, injection volume was taken as 20 µL and the run time was 20 min at 40°C temperature. A membrane filter (0.45 µm Millipore) was utilized for the MP and a syringe filter (0.22 µm) was utilized for chromatographic samples. Chromatographic conditions for preliminary method development are shown in Table 1.

Table 1: Chromatographic conditions for preliminary method developed

Parameters	Value
Injection Volume	20 µL
Column Temperature	40^0C
Detection wavelength	340 nm
Flow rate	1 mL/min
Mobile Phase	Acetonitrile and 0.5% Acetic acid
Mode of Separation	Gradient
Run time	20 min

Preparation of Standard Stock Solution 1000*µ*g/ml and working standard solution (100 *µ*g/ml)

The standard solutions of orientin, quercetin and piperine were separately taken in a VF (10 mL), by dissolving 10mg of the std. orientin, quercetin and piperine in 1 mL of ACN and the volume was adjusted to 10 mL with acetonitrile from this stock solution 1 mL is transferred into a 10 mL VF and volume made with ACN and 6 min sonication. The working standard of 100 µg/mL was preformed diluting the reference standards.

Isolation of phytochemical markers from Nilaveembu Kudineer

Nilaveembu kudineer 10 mL was poured into a separating funnel, to this hexane 30 mL was mixed and shake for 5 min and two layers appeared. Separate the hexane layer in a beaker and label it as hexane fraction. Then add 10 mL of chloroform to the remaining layer extract it, and collect the organic layer. 10 mL of mobile phase was mixed to the leftover layer and the separated residual fraction was left behind. The separated fraction was found to contain orientin, quercetin and piperine and was subjected to sample preparation.

Preliminary Method Development Studies. Optimization of chromatographic conditions

Mobile phase mixtures tried were CH_3OH: H_2O/ACN:H_2O in varying ratios resulted in high R_t, unsymmetrical peak shape and unsuccessful separation. Finally, R_t was reduced with peak symmetry and resolution of phytochemical markers was improved with OPA and ACN combination.

Optimized chromatographic parameters were established to estimate single marker compounds and also for simultaneous estimation of the amount of orientin, quercetin and piperine sample solutions to estimate the phytoconstituents present along with mixture of individual reference standards (Fig. 4). Individual marker compounds used in the study, with mixture of phytochemical markers (Fig. 5 & 6) orientin, quercetin and piperine.

Assessment of the Analytical Method Using the QbD Approach

There are various steps in the AQbD methodology. An analytical target product profile is the first step in an AQbd system which guarantees what and how to measure for the intended quality process. The next method is known as the critical quality attribute (CQA) and it describes the features of the analytical method and parameters such as robustness, accuracy of the method, precision and product identification and peak separation. The performance of analytical techniques is determined by critical method attributes (CMAs) such as temp of the column, injection volume, pH of the MP. CMAs have an impact on method execution parameters such as reagent and sample grades and concentrations. A central process is found using the DoE approach, which also generates design space based on statistical significance.

RESULTS AND DISCUSSION

AQbD Methodology

The DOE was established using Stat Ease software version 12.0 (Stat-Ease Inc). This approach yields higher independent variable responses with fewer probable runs than CCD. Three independent variables (also known as factors) were used in this study the flow rate (A), acetic acid concentration (B) and the injection volume (C). Resolution (R1), theoretical plate number (R2), and peak asymmetry (R3) were the responses. The 16 tests were analyzed as indicated in Table 3. RSM of the factors and responses and analysis of variance was confirmed by (ANOVA). Table 4. The factors were chosen between 0.5 and 0.6 for the acetic acid concentration, for flow rate 0.8 to 1.2 mL/min and injection volume was in the range of 15 to 25 µL as given in Table 2 and the 3D plots for RT, TP and Tf of orientin are shown in Figures 2, 3, 4.[1]

Table 2: Variables and Factor levels selected in Box Behnken Design

Independent variables	Factor levels used		
	Low (-1)	Medium (0)	High (+1)
A: Flow rate (ml)	0.8	1	1.2
B: Acetic acid (%)	0.4	0.5	0.6
C: Injection volume (mcl)	15	20	25

Table 3: Factors and responses selected in BBD design for three markers

Std	Run	Factor 1 A:Flow rate mL	Factor 2 B:Acetic acid %	Factor 3 C:Injection volume MCL	Response 1 RT (O)	Response 2 Theoritical Plate (O)	Response 3 Tailing Factor (O)	Response 4 RT (Q)	Response 5 Theoritical Plate (Q)	Response 6 Tailing Factor (Q)	Response 7 RT (P)	Response 8 Theoritical Plate (P)	Response 9 Tailing Factor (P)
13	1	1	0.5	20	4.124	6880	1.171	7.425	11083	1.016	12.92	10181	1.112
14	2	1	0.5	20	4.128	6880	1.162	7.405	11083	1.116	12.82	10181	1.123
5	3	0.8	0.5	15	5.124	6978	1.323	8.243	12645	1.234	13.43	11987	1.345
11	4	1	0.4	25	4.198	6880	1.182	7.415	11083	1.116	12.92	10181	1.134
9	5	1	0.4	15	4.178	6880	1.115	7.435	11083	1.136	12.72	10181	1.145
15	6	1	0.5	20	4.124	6880	1.171	7.425	11083	1.016	12.92	10181	1.112
2	7	1.2	0.4	20	3.923	6723	1.923	6.243	10143	1.723	11.12	9912	1.561
3	8	0.8	0.6	20	5.145	6998	1.345	8.134	12879	1.245	13.89	11867	1.356
7	9	0.8	0.5	25	5.156	6987	1.356	8.435	12687	1.256	13.98	11675	1.367
16	10	1	0.5	20	4.128	6880	1.162	7.405	11083	1.116	12.82	10181	1.123
12	11	1	0.6	25	4.198	6880	1.182	7.415	11083	1.116	12.92	10181	1.134
1	12	0.8	0.4	20	5.167	6978	1.367	8.167	12756	1.267	13.87	11465	1.378
6	13	1.2	0.5	15	3.945	6789	1.834	6.145	10342	1.634	11.24	9934	1.545
4	14	1.2	0.6	20	3.956	6745	1.934	6.256	10421	1.745	11.45	9945	1.656
10	15	1	0.6	15	4.124	6880	1.171	7.425	11083	1.016	12.92	10181	1.112
8	16	1.2	0.5	25	3.989	6778	1.845	6.267	10532	1.656	11.89	9956	1.567

Table 4: Summary of results of ANOVA of Orientin, Piperine and Quercetin

Parameters	RT (O)	Theoritical Plate (O)	Tailing Factor (O)	RT (Q)	Theoritical Plate (Q)	Tailing Factor (Q)	RT (P)	Theoritical Plate (P)	Tailing Factor (P)
Std. Dev.	0.0160	15.96	0.0239	0.0838	110.90	0.0500	0.2153	86.79	0.0205
Mean	4.35	6876.00	1.39	7.33	11316.81	1.28	12.74	10511.81	1.30
C.V.%	0.3672	0.2322	1.72	1.14	0.9800	3.92	1.69	0.8256	1.58
R^2	0.9996	0.9711	0.9976	0.9949	0.9940	0.9853	0.9538	0.9947	0.9957
Adjusted R^2	0.9989	0.9639	0.9939	0.9874	0.9850	0.9632	0.9423	0.9867	0.9893
Predicted R^2	0.9931	0.9395	0.9619	0.9198	0.9043	0.9041	0.9087	0.9147	0.9345
Adeq Precision	97.9808	29.6903	42.2387	32.4637	28.3138	17.2764	25.2411	30.0261	31.7614
Lack of Fit (p-values)	0.0018		0.0062	0.0016		0.7080	0.0180		0.0176

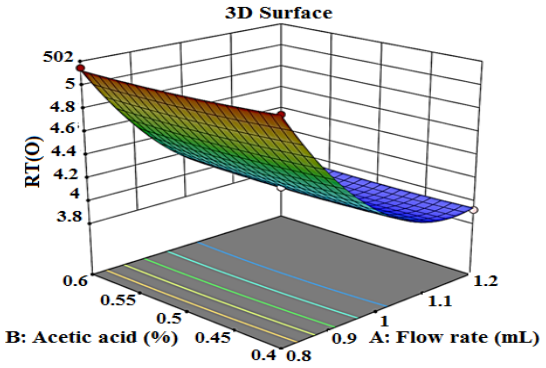


Fig. 2. 3D Response Surface plots Retention Time of Orientin

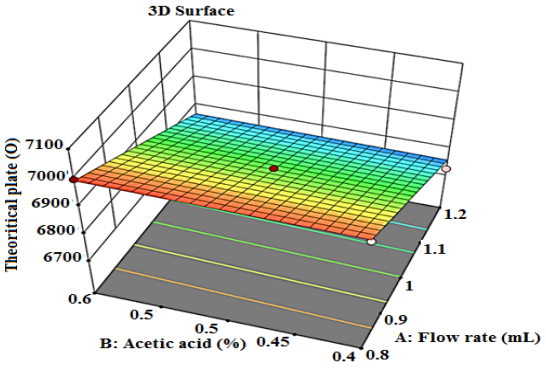


Fig. 3. 3D Response Surface plots of Theoretical Plate of Orientin

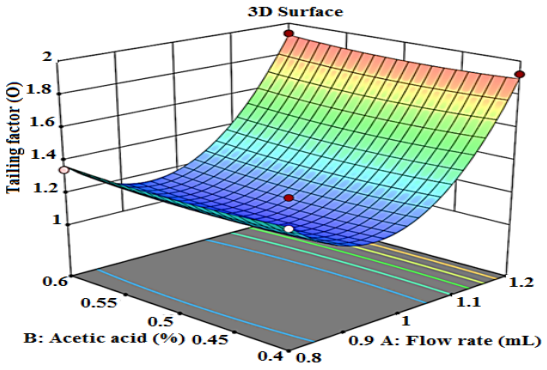


Fig. 4. 3D Response Surface plots of Tailing Factor of Orientin

The effect of Rt, N and Tf of Orientin

The model's F-value of 1527.00 for (R_t), 134.48 for theoretical plate (N), and 273.12 for peak asymmetry (Tf) suggests that the effect is substantial. The P-value was below 0.0500. Orientin's Lack of Fit F-value was 94.67, and its value of 41.16 suggests that the lack of fit is substantial. The difference is less than zero, meaning that the Predicted R^2 of 0.9931, 0.9395, and 0.9619 and the Adjusted R^2 of 0.9989, 0.9639, and 0.9939 are in reasonable agreement. The signal to noise ratio is determined by adequate precision. It should to be greater than 4. The results of adequacy precision, as determined by fit statistics, were 97.981, 29.690, and 42.239, indicating that the signal was adequate.

The effect of Rt, N and Tf of Piperine

The model's significance is indicated by the effect (Rt), theoretical plate (N), and peak asymmetry (Tf), whose respective F-values were 82.63, 124.34, and 155.16. P-values less than 0.0500. A substantial lack of fit is shown by the Lack of Fit F-values of 18.21 and 19.85. With a difference of less than 0.2, the Predicted R^2 of 0.9087, 0.9147, and 0.9345 and the Adjusted R^2 of 0.9423, 0.9867, and 0.9893 are in reasonable agreement. Adequate accuracy determines the signal-to-noise ratio. It must be more than four. Fit statistics revealed that the results of Adequate precision were 25.241, 30.026, and 31.761, indicating that the signal was adequate. The 3D plots for RT, TP and Tf of Piperine are shown in Figures 5, 6 & 7.

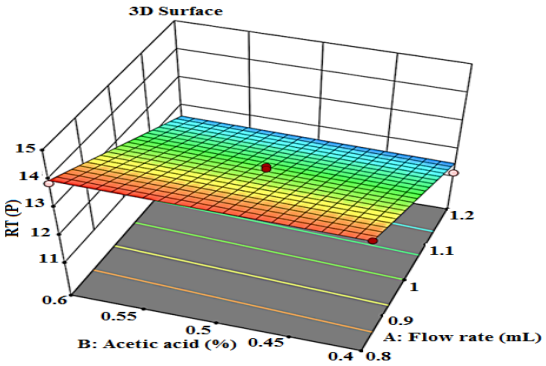
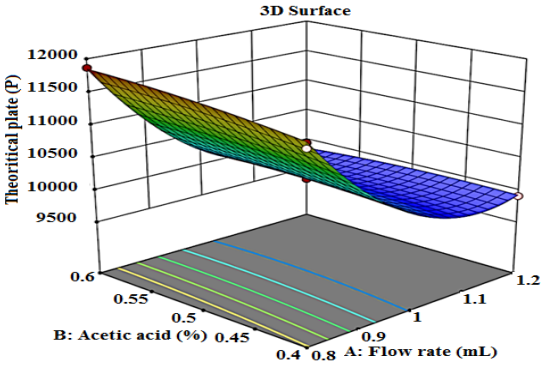


Fig. 5. 3D Response Surface plots Retention Time of Piperine



FFig. 6. 3D Response Surface plots of Theoretical Plate of Piperine

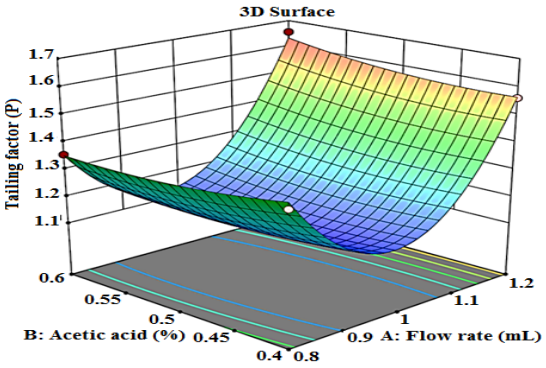


Fig. 7. 3D Response Surface plots of Tailing Factor of Piperine

The effect of Rt, N and Tf of Quercetin

The model F-values for the influence of (Rt), theoretical plate (N), and Peak asymmetry (Tf) were 131.26, 110.78, and 44.67, respectively. P-values less than 0.0500 show that the model terms are significant. 104.29 is the Lack of Fit F-value. In other words, the difference between the Adjusted R^2 of 0.9874, 0.9850, and 0.9632 and the Predicted R^2 of 0.9198, 0.9043, and 0.9041 is less than 0.2. Adequate precision was determined to be 32.464, 28.314 and 17.276 according to fit statistics, indicating a sufficient signal. The 3D plots for RT, TP and Tf of Quercetin are shown in Figures 8, 9 & 10.

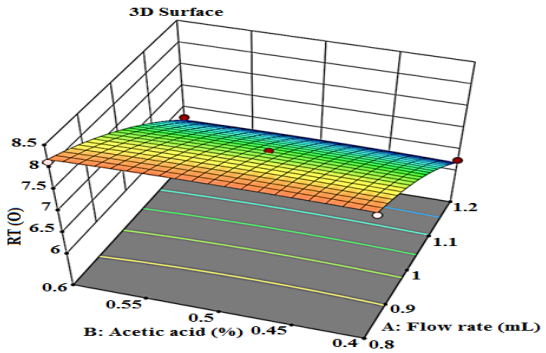


Fig. 8. 3D Response Surface plots of Retention Time of Quercetin

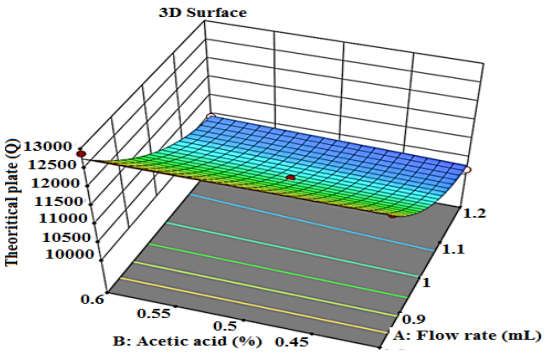


Fig. 9. 3D Response Surface plots of Theoretical Plate of Quercetin

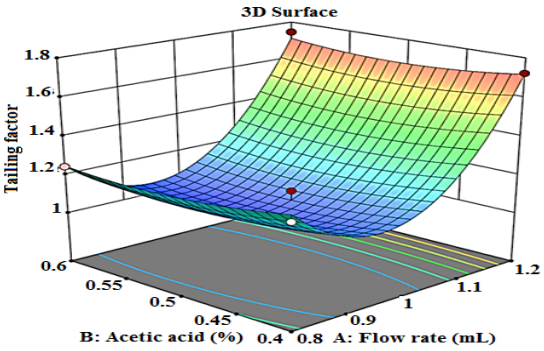


Fig. 10. 3D Response Surface plots of Tailing Factor of Quercetin

System Suitability Test

The percentage RSD of PA and RT for three phytochemical markers was determined and found to be within the range of ±2%, as shown in the Table 6. The mean±%RSD of theoretical plate number and Tf for six samples were 1.192 for orientin, 1.016 for quercetin, and 1.112 for piperine. In terms of system suitability, the N value examined for testing was higher than 2000. The Tf %RSD for three analytes was determined to be within the limits specified in ICH standards indicate that the developed HPLC technique was adequate for concurrent estimation of orientin, quercetin and piperine. The results are displayed in Table 5.

Table 5: System suitability parameters for Marker Compounds

Parameters	Orientin	Quercetin	Piperine
Rt mins	4.108	7.425	12.925
Theoretical plates	6880	11083	10181
Resolution	-	4.011	11.243
Tailing factor	1.192	1.016	1.112

Specificity

Specificity can be assessed by evaluating the peaks of orientin, quercetin, and piperine at predefined concentrations (Fig. 11-13). Injecting 20 µL of samples containing orientin, quercetin, and piperine into the HPLC resulted in RT values of 4.108 min for orientin, 7.425 for quercetin and 12.925 for piperine, implies that the suggested approach is highly specific for estimating orientin, quercetin and piperine without interference.

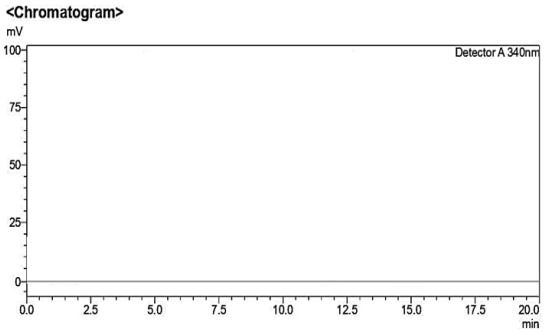


Fig. 11. Blank chromatogram

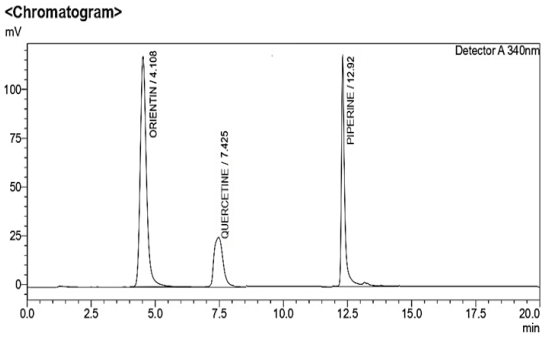


Fig. 12. Standard chromatogram of Orientin, Quercetin and Piperine

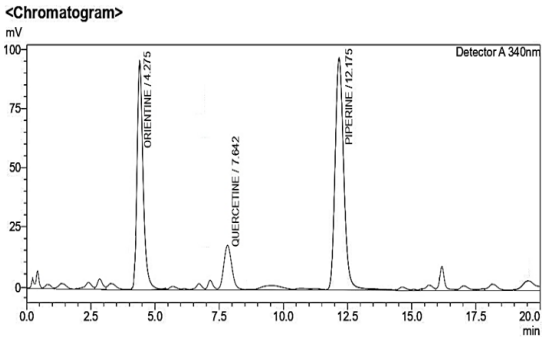


Fig. 13. Sample chromatogram of Orientin, Quercetin and Piperine

Linearity

A regression line was obtained by plotting the mean peak areas of orientin, quercetin and piperine against their determined concentrations (25–200 µg/mL). The regression equations and R^2 values for three analytes were determined (y = 15844x + 69248 R^2 = 0.9984, y = 41690x + 36889 R^2 = 0.9988, and y = 407949x + 1E+06 R^2 = 0.9993, respectively). These findings indicate a linearity of the three phytochemical markers. Representation of results obtained is given in Table 6. The linearity graphs are depicted in Fig. 14 Linearity curve of Orientin, Fig. 15 Linearity curve of Quercetin, Fig. 16 Linearity curve of Piperine.

Table 6: Linearity Data for orientin, quercetin and piperine

Concentration (µg/mL)	Peak Area Orientin	Peak Area Quercetin	Peak Area Piperine
0	0	0	0
25	510025	1170736	12476918
50	910012	2114533	22456624
75	1624533	4105464	42575593
100	2478856	6191276	62575593
150	3210025	8489737	82575593
200	510025	1170736	12476918

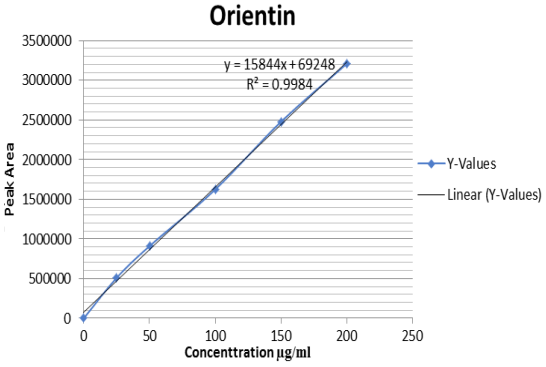


Fig. 14. Linearity Plot of Orientin

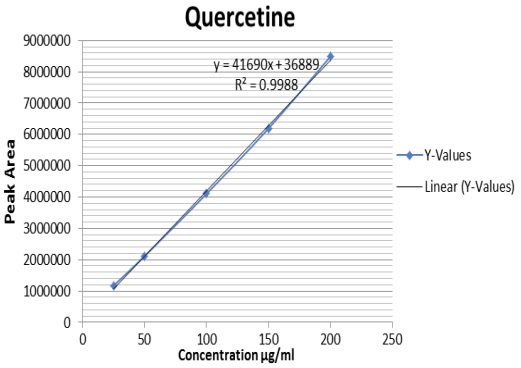


Fig. 15. Linearity Plot of Quercetin

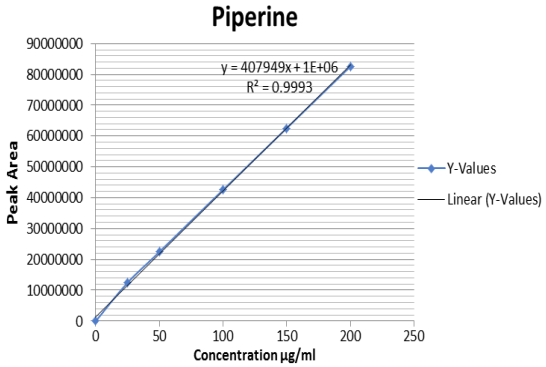


Fig. 16. Linearity Plot of Piperine

Robustness

Analytical procedure robustness was evaluated by varying the HPLC conditions, specifically the flow rate of the three analytes and the column oven temperature. The RSD of peak area for three phytochemical markers evaluated was less than 2%, which is acceptable. Similarly, in symmetry of peak and the N value had no noticeable effect. A slight alteration in the procedure demonstrated that the established approach is optimal and robust for estimating three phytochemical markers simultaneously. The results are summarized in Table 7.

Accuracy

The %recovery is analysed to be nearness to the true numbers. Table 6 shows the %recovery for orientin is 100.96-102.39%, 99.17-102.28% for quercetin, and 99.69-101.33% for piperine. These results were observed to be within the limits, indicating that the approach is accurate. The values are represented in Table 8.

Precision

The %RSD for intraday precisions was calculated at all QC levels, with values ranging from

0.03 & 0.17 for orientin, 0.16 & 0.20 for quercetin, and 0.05 & 0.26 for piperine. The findings reveal that the approach provides good repeatability, with %RSD of less than 2%, as shown in Table 9. This indicates that the methods are reproducible and precise.

LOD and LOQ

The S/N ratio were 3:1 and 10:1, significantly. The LOD and LOQ for orientin was 0.23 and 0.45 µg/mL, quercetin was 0.41 and 1.26 µg/mL and piperine was 0.21 and 3.04.

Table 7: Robustness study

Parameters	Deliberate Changes	% RSD		
		Orientin	Quercetin	Piperine
Flow Rate (-)	0.9 mL	0.09	0.21	0.12
Flow Rate (+)	1.1 mL	0.22	0.09	0.23
Column Temperature (-)	39°C	0.32	0.51	0.71
Column Temperature (+)	41°C	0.09	0.62	0.72

Table 8: Accuracy data of Marker compounds

Parameters (µg/mL)	% Recovery		
	Orientin	Quercetin	Piperine
50	100.96	99.17	99.69
100	100.83	100.67	100.76
150	102.39	102.28	101.33
Average	101.3933	100.7067	100.5933
% RSD	0.86	1.55	0.83

Table 9: Precision study of Marker compounds

Precision	% RSDOrientinPeak area	Rt	QuercetinPeak area	Rt	PiperinePeak area	Rt
1	4469695	4.108	3099074	7.451	3788524	12.983
2	4468266	4.117	3095134	7.475	3789563	12.925
3	4467239	4.125	3088534	7.475	3792518	12.908
4	4472154	4.125	3097117	7.451	3793585	12.883
5	4470072	4.125	3085545	7.442	3791988	12.901
6	4470190	4.125	3093520	7.442	3794133	12.925
Mean	4469603	4.120833	3093154	7.456	3791719	12.92083
SD	1700.392	0.007055	5177.79	0.015258	2230.537	0.034319
% RSD	0.038043	0.171193	0.1674	0.204638	0.058827	0.265607

Table 10: LOD and LOQ data for Phytochemical Markers

Parameters	Orientin	Quercetin	Piperine
LOD	0.23	0.41	0.21
LOQ	0.45	1.26	3.04

DISCUSSION

The research aims at establishing a Quality by Design (QbD)-based Reverse Phase-HPLC approach for quality assessment of orientin, quercetin and piperine phytomarkers present in NK by using Box Behnken Design. Using BBD the experimental parameters were optimized. 3D plots showing the effects of the factors and responses were acquired. There were clear differences in values in each response. Table 2 displays the expected and actual r2 values for each dependent variable. There was a fair amount of agreement between the projected and revised r2 values. The enhanced adjusted r2 value indicates an excellent correlation between the fitted data and the experimental model. The Independent variables selected for QbD method are flow rate, acetic acid concentration, injection volume and the dependant variables selected are resolution, theoretical plate number and peak asymmetry. The Rt for orientin was found to be 4.108 min, quercetin 7.450 min and piperine 12.92 min respectively. Lambda max (λ_{max}) was 340 nm. The approach was found to be specific for the concurrent analysis of orientin, piperine and quercetin in NK, with (>90%) of accuracy and less then (%RSD< 2%) of precision. The LOD and LOQ for orientin was 0.23 and 0.45 µg/mL, quercetin was 0.41 and

1.26 µg/mL and piperine was 0.21 and 3.04. The established approach can be effectively applied for the determination of orientin, quercetin and piperine in NK. The established RP-HPLC approach can be further utilized for the concurrent quantification of these phytoconstituents present in other herbal preparation. An increase in retention time was observed for orientin, quercetin and piperine when concentration of acetic acid and increase in flow rate. Theoretical plate also increases when concentration of acetic acid and increase in flow rate. Acetic acid concentration and flowrate does not have much effect on tailing factor.[16-18]

CONCLUSION

The present work provides a novel Reverse Phase-HPLC technique that was developed and evaluated for the concurrent analysis of piperine, quercetin, and orientin in Nilaveembu Kudineer. The method's performance and robustness for successfully separating and estimating the three phytochemical markers were greatly enhanced by the combination of AQbD and the BBD. For the simultaneous analysis of orientin, quercetin, and piperine in Nilaveembu Kudineer, a polyherbal siddha formulation, the devised approach was shown to be effective. The ICH Q2 R (1) requirements are met by validation and the Box Behnken Design approach reduces the number of experimental runs. The technique may also be used to measure the amounts of piperine, quercetin, and orientin in herbal formulations that include these phytochemical markers.

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; LOD: Limit of Detection; LOQ: Limit of Quantification; ICH: International Council for Harmonization; SD: Standard Deviation; RSD: Relative Standard Deviation; N: Theoretical Plate; Tf: Tailing Factor; S/N: Signal to Noise Ratio; Rs: Resolution; GAA: Glacial Acetic Acid; Rt: Retention Time; ACN: Acetonitrile.

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Conflict of interests

Authors have declared that no competing interests exist.

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