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QbD-Driven Optimization and Validation of an HPLC Approach for Precise Limonene Quantification in Bergamot Oil

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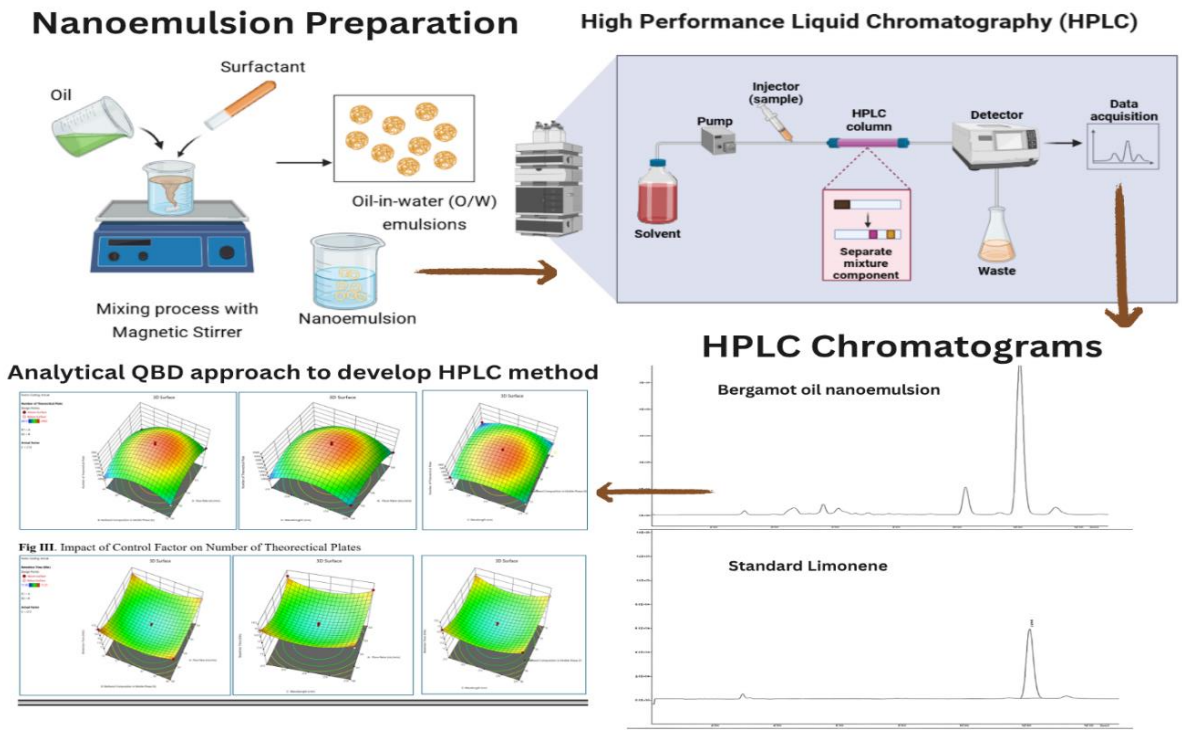
Abstract

Quality by Design (QbD) and Analytical Quality by Design (AQbD) are modern, risk-based approaches that enhance the development and validation of pharmaceutical analytical methods. This study applies QbD principles to the development and optimization of a robust reverse-phase high-performance liquid chromatography (RP-HPLC) method for the estimation of limonene in a bulk drug and nano formulation. Key method Parameters including flow rate, mobile phase composition, and detection wavelength were systematically optimized utilizing a Design of Experiments (DoE) methodology, particularly a Box-Behnken design (BBD). Chromatographic separation was performed on a C18 column using a methanol-water mobile phase (80:20 v/v) at a flow rate of 0.6 mL/min, with UV detection set at 213 nm. The method underwent validation in accordance with ICH Q2(R1) guidelines, demonstrating outstanding linearity, accuracy, precision, specificity, robustness, and system suitability. Forced degradation testing indicated that the method may demonstrate stability. The optimized method was effective for evaluating limonene in nano formulations, indicating its suitability for routine quality assessment. The technique is more dependable, adheres to regulations, and optimizes longevity through the application of AQbD principles. This study demonstrates that AQbD is effective for establishing analytical techniques that consistently perform optimally and comply with current regulatory criteria.

Keywords: Bergamot essential oil, Limonene, RP-HPLC, Design of Experiments, QbD

Graphical abstract:



Abbreviations

Abbreviation	Full Form
QbD	Quality by Design
AQbD	Analytical Quality by Design
RP-HPLC	Reverse-Phase High-Performance Liquid Chromatography
UHPLC	Ultra High-Performance Liquid Chromatography
DoE	Design of Experiments
ICH	International Council for Harmonization
CMP	Critical Method Parameters
TQP	Target Quality Profile
BBD	Box-Behnken Design
LOD	Limit of Detection
LOQ	Limits of Quantification
RSD	Relative Standard Deviation

Introduction

Limonene (C10H16) is a prevalent cyclic monoterpene hydrocarbon existing in two enantiomeric forms. The (R)- (+)-limonene (d-limonene) is the predominant type found in citrus essential oils. It is a significant volatile compound that imparts a distinctive aroma to citrus fruits and is extensively utilised in the culinary, pharmaceutical, cosmetic, and fragrance industries due to its exceptional sensory and functional properties [1]. The bergamot orange (*Citrus bergamia Linn*), predominantly cultivated in southern Italy, is a notable botanical source of limonene. Bergamot essential oil typically has limonene concentrations ranging from 30% to 45%, contingent upon the specific variety of bergamot, its origin, extraction method, and storage conditions [2]. Limonene has demonstrated several pharmacological activities, including antioxidant, anti-inflammatory, anticancer, analgesic, anxiolytic, gastroprotective, cardioprotective, immunomodulatory, and anticonvulsant effects [3,4]. Limonene has emerged as a significant indicator for assessing the quality of citrus essential oils due to its distinctive characteristics [5]. Nonetheless, the variability in essential oil quantities among batches necessitates the development of precise, reliable, and established methods for quantifying limonene content [6].

Bergamot essential oil is made up of a lot of different chemicals, both volatile and non-volatile. Limonene, linalyl acetate, and linalool are the main ones [7]. Limonene not only gives the oil its citrus smell, but it also plays a big role in its pharmacological profile, which includes activities that fight inflammation, pain, microbes, and cancer [8]. Limonene's bioactivities have established it as a crucial quality indicator for citrus essential oils, requiring accurate and proven analytical methods for its quantification, especially due to the inherent heterogeneity in essential oil content among different batches and sources [9,10].

Although gas chromatography (GC) and ultra-high performance liquid chromatography (UHPLC) are extensively employed for analysing limonene in diverse citrus oils, there is a significant lack of research specifically addressing the quantification of limonene in bergamot oil utilising HPLC, especially within a QbD framework [11]. The QbD paradigm offers a systematic, science-driven approach to analytical method development, focussing on the identification and management of critical method parameters through comprehensive risk assessment and experimental design to ensure method robustness and reliability [12–14]. This method uses tools like DoE and risk assessment to find the best critical method parameters (CMPs), which leads to consistent and reliable analytical performance [15,16].

This study discusses the development and testing of a novel HPLC method for quantifying limonene in bergamot essential oil nanoemulsion, employing a QbD methodology. This approach integrates risk assessment and experimental design to identify optimal technique parameters, which are subsequently validated in accordance with ICH standards. This methodology is designed to be a robust, reproducible, and compliant method for ensuring the quality of bergamot oil in herbal and medicinal goods.

Methods

Material And Reagents

Limonene (LNO) was obtained from Yucca Enterprises, located in Mumbai, India. Polysorbate 80 (Tween 80®) [Extra pure grade] was purchased from Loba Chemie Pvt. Ltd., headquartered in Mumbai, India. HPLC-grade methanol was supplied by Supelco® (Sigma-Aldrich, marketed by Merck Life Science Pvt. Ltd.), also based in Mumbai, India. HPLC-grade water was obtained from Qualigens® (Thermo Fisher Scientific India Pvt. Ltd.), headquartered in Mumbai, India. citrous bergamot oil essential oil, containing 69.93% limonene, was purchased from Yucca Enterprises, Mumbai, India.

Chromatographic conditions

RP-HPLC analysis was carried out by a Jasco HPLC 4000 system equipped with a UV detector. Separation was attained on a Inertsil ODS-4 (150 mm × 2.1 mm, 3 µm), maintained at an ambient temperature mentioned in Table 1. The mobile phase, composed of methanol and water in an 80:20 (v/v) ratio, along with a flow rate of 0.6 mL/min, was optimized using Design of Experiments based on a Box-Behnken design to ensure optimal chromatographic performance. Methanol was used as the diluent to maintain compatibility with the mobile phase. The detection wavelength was set at 213 nm, and a sample injection volume of 10 µL was used. The total run time for each run was 15 minutes, ensuring effective separation and elution of the analyte.

Table 1. Chromatographic Conditions for detection of Limonene by RP-HPLC

Parameter	**Condition**
RP-HPLC	Jasco HPLC 4000 separation module with UV detector
Mobile phase	Methanol: Water (80:20%v/v)
Column	Inertsil ODS-4 (150mm X 2.1 mm, 3µ)
Column Temperature	Ambient
Wavelength	213nm
Diluent	Methanol
Injector volume	20µl
Flow rate	0.6 ml/min
Runtime	15min

Sample Preparation

Nanoemulsion were prepared using a two-step mechanical emulsification approach. The primary emulsification involved processing the mixture through a high-pressure homogenizer adapting the methodology from Asmawati et al. [17]. The formulation incorporated 5% (w/v) bergamot orange peel essential oil as the active component. For extraction analysis, a stock solution (0.5 mg/mL) was prepared by diluting the nano emulsion in methanol: Water (50:50) (25mL volumetric flask) and subjecting it to ultrasonication for 20 min. An intermediate concentration (6 µg/mL) was then made by taking a small amount of the stock solution, adding methanol in a 10 mL flask, and using ultrasonication for another 15 minutes. The final sample was filtered through a 0.22 µm cellulose membrane prior to analysis [18].

Standard Solution

For method validation, a standard stock solution with a concentration of 0.594 ppm prepared dissolving 14.84 µL of limonene in 24.985 mL methanol. This stock solution then serially diluted with methanol to produce a range of concentrations (2, 4, 6, 8, 10, and 12 µg/mL) for the linearity assessment. A separate aliquot of the stock solution was subjected to ultrasonication for 20 minutes. Before injection into the HPLC system, all prepared solutions were filtered through a 0.22 µm membrane filter. Each sample was analysed in triplicate (n = 3) to ensure reproducibility.

Risk Assessment Study

The goal of this study was to find and understand the different things that affect the target quality profile (TQP) of limonene when it is analysed using HPLC. Before doing a risk assessment, the most important analytical characteristics were looked at to find significant links between the method's performance indicators and the quality outcomes that were wanted. This study aimed to find the root causes of possible problems, inconsistencies, or failures in the process, while also stressing the most important variables needed to achieve the desired TQP. We put the things that potentially affect the desired outcome into three groups: low, medium, and high potential risk. At first, seven things were looked at, and they were put in order to make the screening process easier by doing a systematic risk assessment. This made it possible to choose the most important factors with more focus. Finally, three important parameters were found and chosen for further improvement utilising experimental design methods, as shown in Table 2. We figured out the TQP in this study by looking at the peak area and the number of theoretical plates, which were based on the three independent variables we were looking at. In addition, a fishbone diagram (Fig 1) was made to show the key sources of variability that

affect the TQP. This helped us better understand the possible hazards and how they can affect the method's dependability and effectiveness.

Table 2: RP-HPLC analytical method risk assessment for Limonene

Independent Variable	Level		
	+1	0	-1
Flow rate mL/min (±0.2mL)	0.4	0.6	0.8
Composition of methanol in mobile phase (%) (±5mL)	75	80	85
Detector Wavelength (nm) (±2nm)	211	213	215

Optimization

The influence of diverse circumstances on reactions was analysed with the AQbD methodology. Analysis of the results revealed that the BBD method was the most efficient for optimisation, since it yielded high-quality compositions with fewer experimental trials compared to other strategies [19]. This enabled the team to utilise a two-level, three-factor BBD, which proved to be a more efficient approach to improving the analytical process. The researchers examined the influence of detector wavelength (nm), flow rate (ml/min), and methanol concentration (%) on several responses, including peak area (y1) and the number of theoretical plates (y2). Seventeen distinct experimental trials with diverse compositions were performed to ascertain the best one, as detailed in Table 3. To ascertain the most suitable kinetic model, we analysed the experimental data utilising various models, including linear, second-order, quadratic, and cubic. We analysed the impact of independent variables on the replies utilising polynomial equations, three-dimensional representations, and contour plots. Analysis of variance for retention time and theoretical plates given in Table 4 and Table 5. Statistical tests, including ANOVA and regression analysis, were utilised to ascertain the significance of the models and identify the optimal model.

Table 3. Optimization of parameters using BBD design

		Factor 1	Factor 2	Factor 3	Response 1	Response 2
Std	Run	A: Flow Rate	B: Methanol Composition in Mobile Phase	C: Wavelength	Retention Time	Number of Theoretical Plate
		mL/min	%	nm	Min	
6	1	0.8	80	211	12.95	2950
10	2	0.6	85	211	12.87	2870
4	3	0.8	85	213	13.12	2865
17	4	0.6	80	213	11.84	3480
2	5	0.8	75	213	13.25	3060
5	6	0.4	80	211	13.11	2975
1	7	0.4	75	213	12.91	2968
16	8	0.6	80	213	11.76	3455
11	9	0.6	75	215	12.89	2972
13	10	0.6	80	213	11.97	3400
12	11	0.6	85	215	13.04	2815
8	12	0.8	80	215	12.93	2830
3	13	0.4	85	213	12.81	2821

14	14	0.6	80	213	11.99	3405
15	15	0.6	80	213	11.06	3435
9	16	0.6	75	211	12.85	2910
7	17	0.4	80	215	13.02	2825

Method Validation

The method was checked using the International Conference on Harmonization's rules for validating analytical methods. Specificity, linearity, limits of quantification and detection, intermediate precision and repeatability, robustness, and precision were all used to check the method's validity [20].

Specificity

Assessed specificity, which is an important part of making sure that a chromatographic method works, to make sure that limonene could be measured accurately without being affected by other parts of essential oils or nanoemulsion. We compared chromatograms of bergamot oil, nanoemulsion, and regular limonene to look at this. There was 30 µg/mL of bergamot oil in the nanoemulsion. International standards require peak purity analysis, which confirmed the method's specificity even more.

Linearity, detection and quantification limits

Tested the method's linearity by making standard solutions from a 0.593 mg/mL stock solution at six different concentrations: 2, 4, 6, 8, 10, and 12 µg/mL. We made three calibration curves and used least squares regression to get the slope, y-intercept, and correlation coefficient ($p < 0.05$). We used the calibration curve to find the limits of quantification (LOQ) and detection (LOD). LOD was the slope ($3.3\sigma/S$) times the standard deviation of the intercept, and LOQ was the slope ($10\sigma/S$) times the standard deviation of the slope.

Precision

Method precision was evaluated through both intermediate precision (inter-day) and repeatability (intra-day). For intermediate precision, samples at 6 µg/mL were analyzed over three consecutive days. Repeatability was determined by injecting six replicate solutions of 6 µg/mL on the same day. Precision results were reported as relative standard deviation (RSD%).

Accuracy

Method accuracy was assessed in triplicate using nanoemulsion containing Sicilian lemon and red mandarin essential oils, each prepared at 10 µg/mL and spiked with limonene standard at three levels: 50%, 100%, and 150%, resulting in final concentrations of 15, 20, and 25 µg/mL. Recovery was calculated by comparing the measured concentrations of spiked and unspiked samples, with results expressed as percent recovery and relative RSD% for the triplicates.

Robustness

Robustness was assessed using 10 µg/mL nanoemulsion containing either Sicilian lemon or red mandarin essential oil. Method parameters were varied, including flow rate (0.5 and 0.7 mL/min), mobile phase composition [methanol: water (90:10, v/v) and (70:30, v/v)], and detection wavelength (211 and 214 nm). Each condition was tested in triplicate (n=3).

Result and Discussion

Impact of Control Variables on Theoretical Plates.

The number of theoretical plates, an essential indicator of column efficiency in chromatographic separations, was significantly influenced by the independent variables under investigation which are (a) flow rate, (b) methanol, (c) detection wavelength. The reaction was represented by a quadratic equation.

Number of theoretical plates = $3435 + 14.5a – 67.375b – 32.875c – 12ab + 7.5ac – 29.25bc – 251.625a^2 – 254.875b^2 – 288.375c^2$.

Based on the Anova findings, the model was deemed highly significant, with an f-value of 41.04 and a p-value validating its ability to predict outcomes. Notably, the quadratic terms of all three variables (a^2, b^2, c^2) had exceptionally high f-values (>90) and very low p-values ((b) showed a significant impact ($f = 12.83$, $p = 0.009$), while flow rate (a) and wavelength (c) did not show a statistically significant individual effect. The interaction terms ab, ac, and bc were not statistically significant, indicating that there was little to no synergistic influence among these variables.

Experimentally, higher theoretical plate numbers were observed in runs where moderate levels of methanol (80%) and flow rate (0.6 ml/min) were used, such as in run 4 (3480 plates), run 8 (3455 plates), and run 15 (3435 plates). This supports the idea that quadratic terms have a greater impact on linear interactions. The findings suggest that extreme levels of any factor can decrease column efficiency, whereas achieving optimal mid-range values for methanol and flow rate leads to the highest plate count (Fig 2). Wavelength had the least noticeable effect, but it still played a role in shaping the model's quadratic curvature. These findings demonstrate that the theoretical plate count is most strongly affected by the curvature of flow rate, methanol content, and wavelength, underscoring the importance of using a quadratic model for chromatographic optimization.

Impact of control factors on retention time.

The crucial response in chromatographic performance, known as retention time, was modelled using a second-order polynomial equation:

Retention time = $11.724 + 0.05a – 0.0075b + 0.0125c – 0.0075ab + 0.0175ac + 0.0325bc + 0.69425a^2 + 0.60425b^2 + 0.58425c^2$.

The Anova analysis showed that the model was statistically significant ($f = 6.06$, $p = 0.0134$), with the primary contributions to the model arising from the quadratic terms. Among the individual variables, none of the linear or interaction terms showed statistical significance ($p > 0.6$ for all), except for the squared terms: flow rate (a^2), methanol composition (b^2), and wavelength (c^2), which all had p-values effects.

The experimental results corroborated the model's prediction. The longest retention time was observed in run 5 (13.25 min) at high methanol content (75%) and flow rate (0.8 ml/min), while the shortest retention was in run 15 (11.06 min) at mid-range flow and methanol. Runs with high methanol (e.g., 85%) and high flow rate (e.g., 0.8 ml/min) generally exhibited reduced retention times. This indicates that increasing flow rate tends to reduce retention time, although in the given model, the direct linear effect of flow rate was minimal and instead dominated by its quadratic effect (Fig 3). Methanol, despite its low linear f-value, also showed significant influence through its squared term, suggesting that both very low and very high methanol concentrations lead to suboptimal retention.

In summary, the retention time is mainly influenced by the nonlinear contributions of all three variables, with flow rate and methanol concentration playing significant roles. This indicates that a second-order model is more suitable than a linear one to account for the curvature and enhance retention behaviour under different chromatographic conditions.

Table 4. Analysis of variance for retention time

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	5.62	9	0.6239	6.06	0.0134	significant
A- Flow Rate	0.02	1	0.02	0.1942	0.6727	
B-Methanol Composition in Mobile Phase	0.0005	1	0.0005	0.0044	0.9491	
C-Wavelength	0.0013	1	0.0013	0.0121	0.9154	
AB	0.0002	1	0.0002	0.0022	0.964	
AC	0.0012	1	0.0012	0.0119	0.9162	
BC	0.0042	1	0.0042	0.041	0.8453	
A^2	2.03	1	2.03	19.7	0.003	
B^2	1.54	1	1.54	14.93	0.0062	
C^2	1.44	1	1.44	13.95	0.0073	
Residual	0.721	7	0.103			
Lack of Fit	0.134	3	0.0447	0.3045	0.8219	not significant
Pure Error	0.5869	4	0.1467			
Cor Total	6.34	16				

Retention Time = 11.724 + 0.05 * A -0.0075 * B + 0.0125 * C -0.0075 * AB + 0.0175 * AC + 0.0325 * BC + 0.69425 * A^2 + 0.60425 * B^2 + 0.58425 * C^2

Table 5. Analysis of variance for number of theoretical plates

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.05E+06	9	1.16E+05	41.04	< 0.0001	Significant
A- Flow Rate	1682	1	1682	0.5943	0.466	
B-Methanol Composition in Mobile Phase	36315.12	1	36315.12	12.83	0.009	
C-Wavelength	8646.13	1	8646.13	3.05	0.124	
AB	576	1	576	0.2035	0.6655	
AC	225	1	225	0.0795	0.7861	
BC	3422.25	1	3422.25	1.21	0.3079	
A^2	2.67E+05	1	2.67E+05	94.2	< 0.0001	
B^2	2.74E+05	1	2.74E+05	96.64	< 0.0001	
C^2	3.50E+05	1	3.50E+05	123.72	< 0.0001	
Residual	19811.25	7	2830.18			
Lack of Fit	15261.25	3	5087.08	4.47	0.091	Not significant
Pure Error	4550	4	1137.5			
Cor Total	1.07E+06	16				

Number of Theoretical Plate = 3435 + 14.5 * A + -67.375 * B -32.875 * C -12 * AB + 7.5 * AC -29.25 * BC -251.625 * A^2 -254.875 * B^2 -288.375 * C^2

Optimized HPLC Condition is as follows:
Column: Inertsil ODS-4 (2.1 × 150 mm, 3 µm)
Mobile phase: Methanol: Water (80:20 %v/v)
Flow rate: 0.60 mL/min
Detector wavelength: 213 nm
Column temperature: Ambient
Injection volume: 10 µL

The standard solutions of the mobile phase were employed to enhance the criteria for method development and validation following ICH guidelines. Method specificity was assessed by injecting nanoemulsion prepared with bergamot oil and blank nanoemulsion without the active compound. Only samples containing bergamot oil, which is rich in limonene, showed a detectable response, confirming specificity. Representative chromatograms are shown in Fig 4. a, Fig 4. b, Fig 4. c [21].

Three independent calibration curves for limonene were constructed over the concentration range of 2–12 µg/mL, demonstrating the method's sensitivity across all tested levels. The mean correlation coefficient was 0.9995, meeting the linearity requirements specified by Brazilian regulations for active compound quantification. Statistical analysis confirmed a linear relationship with no significant deviation from linearity ($p < 0.05$). The average calibration curve is presented below. The calculated LOD and LOQ were 0.366 µg/mL and 1.10 µg/mL, respectively, indicating high sensitivity for limonene detection in bergamot oil nanoemulsion. Precision, assessed as both intermediate (inter-day) and repeatability (intra-day), was reported as relative standard deviation (RSD%) and is summarized in Table 6 (a), Table 6 (b).

Table 6 a) Results obtained in repeatability test

No.	Peak Name	R_t (min)	Area[µV.sec]	Height [µV]	NTP	Symmetry Factor
1	Injection-1	12.21	440399	283155	3279	1.029
2	Injection-2	12.24	440245	283142	3346	1.074
3	Injection-3	12.25	440354	283132	3319	1.039
4	Injection-4	12.21	440392	283145	3356	1.020
5	Injection-5	12.26	440426	283165	3453	1.055
6	Injection-6	12.28	440420	283155	3368	1.040
%RSD	-	0.228	0.015	0.04	1.73	1.84

Table 6 b) Results obtained in intermediate Precision

Inter-day	Conc.	Area	Mean ± SD	%RSD
Day-1	6µg/ml	3018200	3018440±224.5	0.007
		3018645		
		3018475		
	8 µg/ml	4040349	4040406±91.04	0.002
		4040511		
		4040358		
	10 µg/ml	5140399	5140777±699.04	0.014
		5141584		
		5140349		
Day-2	6µg/ml	3018546	3018815 ±233	0.008
		3018954		
		3018945		
	8µg/ml	4040234	4040422±367.97	0.009
		4040176		
		4040856		
	10µg/ml	5140846	5140743±218.29	0.004
		5140892		
		5140493		
Day-3	6µg/ml	3018956	301868±245.8	0.008
		3018476		
		3018624		
	8µg/ml	4040561	4040417±128	0.003
		4040312		
		4040379		
	10µg/ml	5140951	5141313±334	0.006
		5141609		
		5141380		
Intra-day	Conc.	Area	Mean ±SD	%RSD
Morning	6µg/ml	3018453	3018527 141	0.005
		3018439		
		3018691		
	8 µg/ml	4040289	4041013 627	0.016
		4041354		
		4041398		
	10 µg/ml	5140955	5140717 418	0.008
		5140963		
		5140234		
Afternoon	6µg/ml	3018452	3018343 191	0.006
		3018456		
		3018123		
	8µg/ml	4040951	4041188 211	0.005
		4041357		
		4041258		
	10µg/ml	5141653	5141532 461	0.009
		5141921		
		5141022		

Evening	6µg/ml	3018963	3018852 111	0.004
		3018852		
		3018741		
	8µg/ml	4041326	4041281 396	0.01
		4040865		
		4041654		
	10µg/ml	5140694	5140528 347	0.007
		5140129		
		5140761		

As shown in Table 6, both intermediate precision and repeatability, expressed as relative standard deviation, were below 2%, confirming the method's precision for quantifying limonene in citrus essential oils, particularly bergamot oil. The developed HPLC method yielded standard deviations of 1.1%, 0.006%, and 0.0077% for repeatability and intermediate precision, which are consistent with previously reported values.
Robustness was evaluated by altering the mobile phase composition (methanol: water at 90:10 and 70:30 v/v), flow rate (0.5 and 0.7 mL/min), and detection wavelength (211 and 214 nm). As shown in Table 7, the limonene content remained within 95–105% under all tested conditions, demonstrating the robustness of the developed method.

Table 7. Evaluation of the robustness of the limonene quantification method in bergamot essential oil nanoemulsion.

Conditions	Limonene content %	%RSD
Mobile Phase		
Methanol: Water (85:15)	98.52	0.21
Methanol: Water (75:25)	99.61	1.14
Flow Rate		
0.4mL/min	99.64	1.54
0.8mL/min	99.49	0.98
(nm)		
211nm	101.49	0.14
214nm	99.90	0.19

The developed method proved to be suitable for the quantification of limonene present in nano emulsified bergamot oil.
Method accuracy was reported as percent recovery (%), with results presented in Table 8.

Table 8. Assessment of method accuracy for limonene quantification in bergamot essential oil nanoemulsion.

Know Sample (µg/mL)	Added Sample (µg/mL)	Found (µg/mL)	Recovery (%)	Relative Standard Deviation (%)
10	5	14.820	98.82	0.21
10	10	20.107	100.53	0.34
10	15	24.963	99.85	0.19

The accuracy results, presented in Table 8, demonstrated recovery rates above 95% for limonene, confirming that the developed method effectively recovered the tested concentrations.

Conclusion

In order to quantify the quantity of limonene in bergamot oil nanoemulsion, we implemented a Quality by Design (QbD) methodology to develop a scientifically valid and validated reverse-phase high-performance liquid chromatography (RP-HPLC) method. To ensure the analysis was reliable and effective, the method optimisation was meticulously conducted using a Box-Behnken Design, with a particular emphasis on critical factors such as methanol concentration, flow rate, and detector wavelength. The quadratic model was the most effective model for predicting critical chromatographic outcomes, as determined by statistical analysis. The retention time and column efficiency were most significantly influenced by the methanol concentration. The approach was subjected to a rigorous evaluation and demonstrated a high degree of linearity, sensitivity, and precision, as evidenced by its low relative standard deviation. This is consistent with the internationally recognised standards. Accuracy was demonstrated by recovery rates that were typically near 100%. Specificity and robustness persisted, even when chromatographic conditions were intentionally altered. For regulatory purposes and to monitor quality, the RP-HPLC method offers a repeatable, dependable, and precise method of testing limonene in citrus essential oil-based products. It functions exceedingly well in the medicinal and nutraceutical sectors when combined with bergamot oil nanoemulsion.

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Figures:

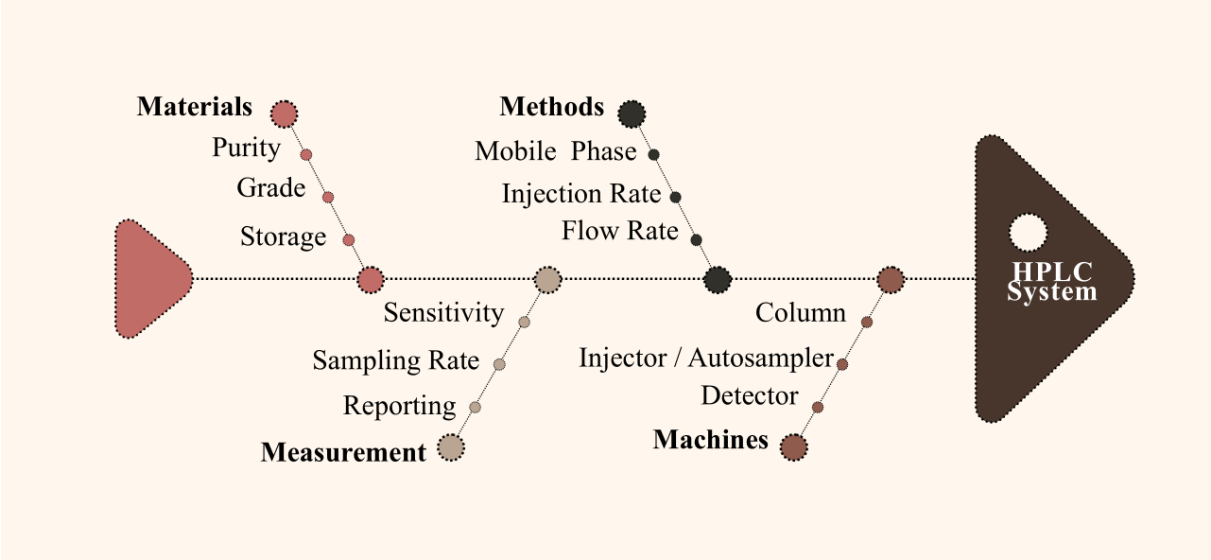

Fig 1. Risk Assessment Diagram for HPLC System: A Fishbone Model Illustrating Key Factors Influencing Chromatographic Performance.

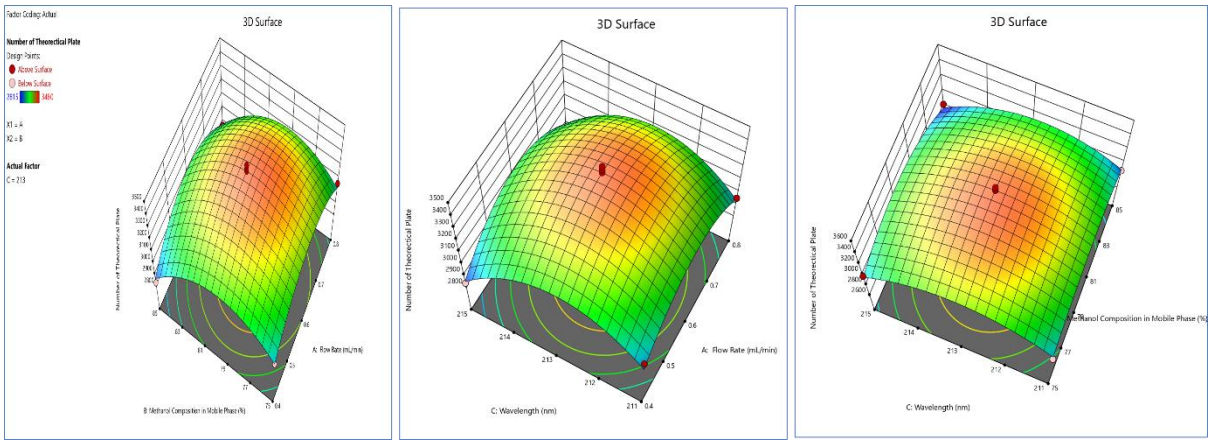

Fig 2. Impact of Control Factor on Number of Theorectical Plates

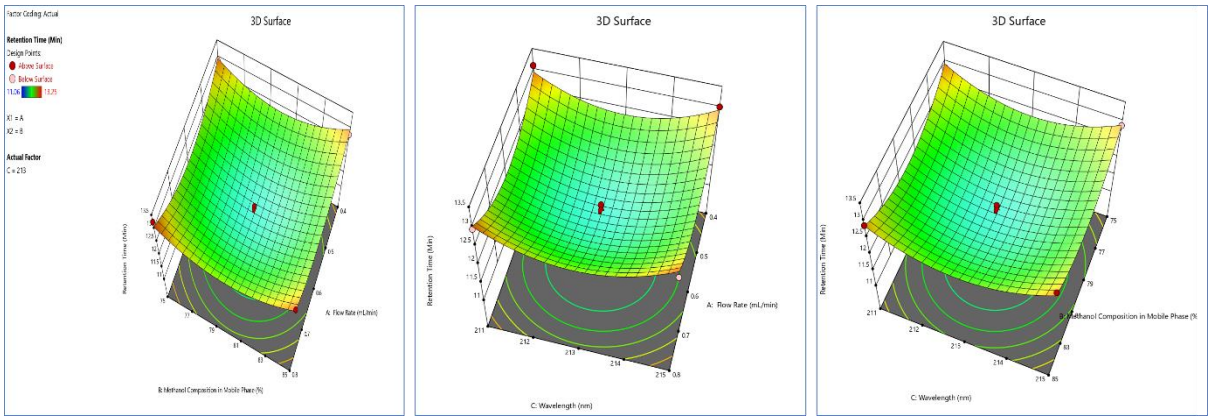

Fig 3. Impact of Control Factor on Retention Time.

Fig 4. a) chromatogram of blank

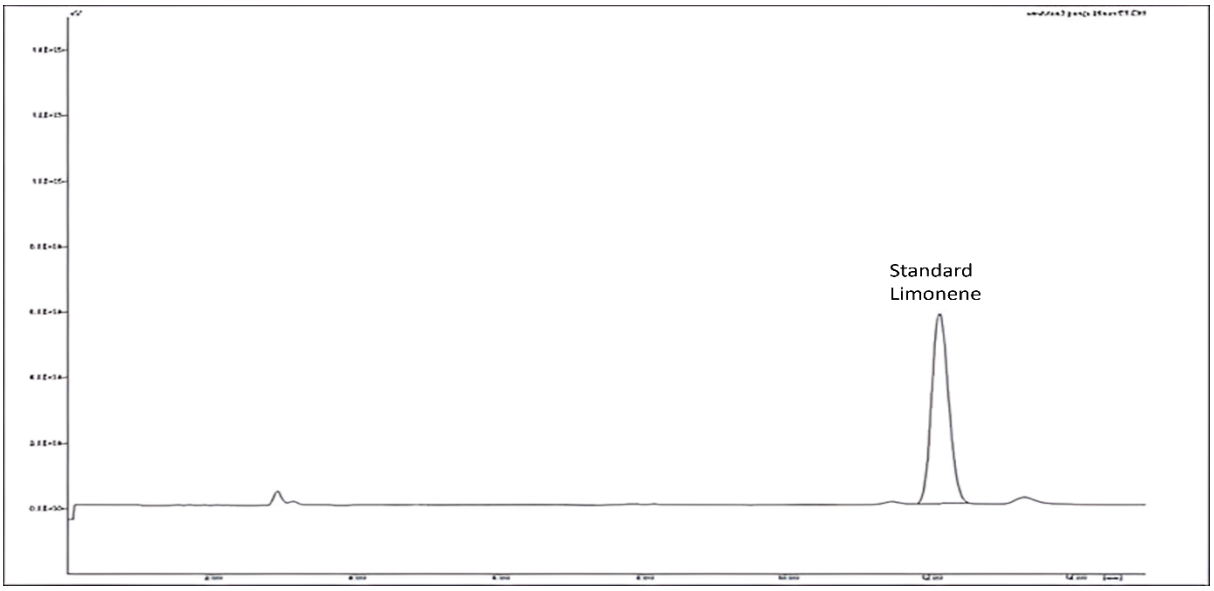

Fig 4. b) chromatogram of standard limonene

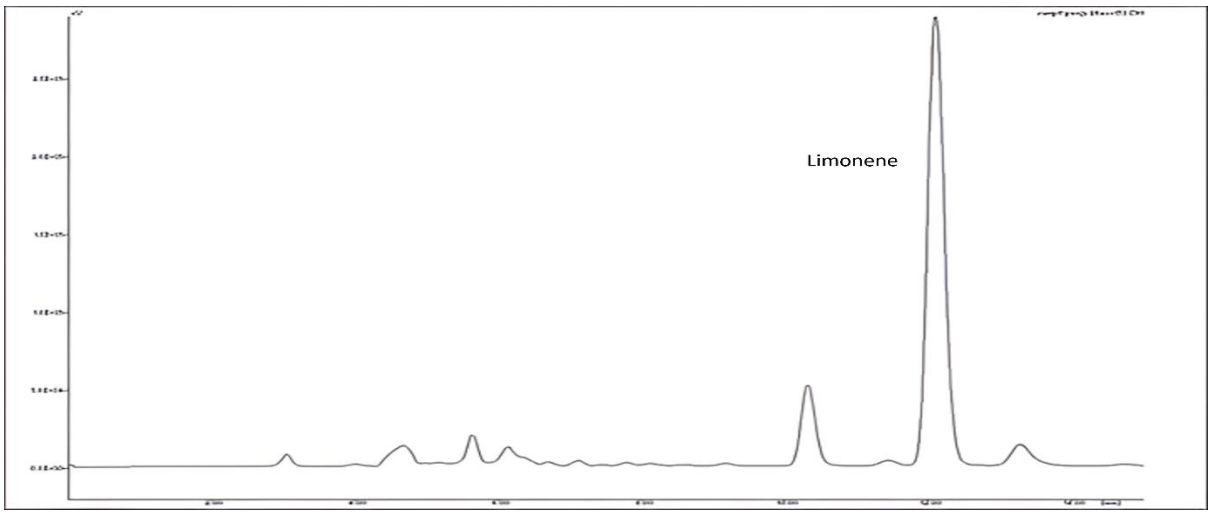

Fig 4. c) chromatogram of Emulsion containing bergamot oil

Statements & Declarations

We confirm that this work is original and has not been published elsewhere (partly or in full).

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Competing interest

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Pooja Phadtare, Tejas Ghadge, Nikhil More, Dyandevi Mathure, Malati Salunke. The first draft of the manuscript was written by Pooja Phadtare and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.