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Development and Validation of HPTLC and RP-UHPLC Methods for Estimation of Silymarin in Herbal Formulation

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Abstract

Background: Silymarin, a flavonolignan complex isolated from *Silybum marianum* L. (milk thistle), is widely incorporated into herbal formulations because of its hepatoprotective, antioxidant, and anti-inflammatory properties. Reliable and validated analytical methods are essential to ensure the quality, safety, and consistency of Silymarin-containing herbal products. **Aim:** The present study aimed to develop and validate simple, rapid, accurate, and reliable High-Performance Thin-Layer Chromatography (HPTLC) and Reverse Phase-Ultra High-Performance Liquid Chromatography (RP-UHPLC) methods for the quantitative estimation of Silymarin in herbal formulations. **Methods:** The identity of Silymarin was confirmed by FTIR spectroscopy. HPTLC analysis was performed using silica gel 60 F254 plates with hexane:ethyl acetate:ortho-phosphoric acid (6:3.5:0.5 v/v/v) as the mobile phase and detection at 287 nm. RP-UHPLC analysis was carried out on a C18 column using methanol:0.1% glacial acetic acid (85:15 v/v) at a flow rate of 1.0 mL/min with UV detection at 287 nm. Both methods were validated according to ICH Q2(R2) guidelines for linearity, accuracy, precision, specificity, sensitivity, ruggedness, and robustness. **Results:** The HPTLC method produced a well-resolved peak with an R_f value of 0.52 and showed excellent linearity over 500–3000 ng/spot (r^2 = 0.9992). The RP-UHPLC method exhibited a retention time of 2.6 min with linearity over 20–120 µg/mL (r^2 = 0.9991). Both methods demonstrated recoveries ranging from 99–101%, precision with %RSD below 2%, satisfactory Limit of Detection (LOD) and Limit of Quantification (LOQ) values, and excellent specificity without interference from formulation excipients. Analysis of commercial herbal formulations showed Silymarin content within 98–103% of the labelled claim. **Conclusion:** The developed HPTLC and RP-UHPLC methods are simple, precise, accurate, sensitive, robust, and suitable for the routine quality control, standardisation, and quantitative estimation of Silymarin in herbal formulations.

Major Findings: This study developed and validated simple HPTLC and RP-UHPLC methods for the estimation of Silymarin in herbal formulations. Both methods demonstrated excellent accuracy, precision, and specificity, making them suitable for routine quality control.

Keywords: Herbal Formulation, High-Performance Thin-Layer Chromatography, ICH Guidelines, Method Validation, Reverse Phase-Ultra High-Performance Liquid Chromatography, Silymarin

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1. Introduction

Herbal products and plant-based formulations are widely utilised for diverse therapeutic and health-promoting purposes[1]. It is estimated that more than 80% of the global population depends on herbal medicines for maintaining health and well-being. Plant-derived materials and their preparations are extensively manufactured and marketed in the pharmaceutical sector owing to their advantages such as affordability, therapeutic efficacy, better patient tolerance, effectiveness in managing chronic conditions, easy availability, safety, minimal adverse effects, and sustainable nature[2]. However, poor quality of such medicinal products can result in reduced consumer confidence and may also pose serious health risks, including potentially life-threatening outcomes[3].

High-performance liquid chromatography is a widely recognised and robust method for the isolation, separation, identification, and quantification of components within natural products, making it a primary choice for fingerprinting investigations in quality control of herbal drugs[4,5]. This technique is particularly valuable given the documented variability in the quality of herbal products available on the market, necessitating rigorous analytical methods to ensure consistency and efficacy[6]. The development and validation of novel, accurate, and precise chromatographic methods, such as RP-UHPLC and HPTLC, are therefore crucial for the simultaneous estimation of active markers in complex herbal formulations[7]. Specifically, HPTLC offers distinct advantages in stability studies and the quantitative analysis of marker compounds in raw plant materials and finished formulations, demonstrating rapidness, reliability, and accuracy[8-10]. Moreover, HPTLC enables simultaneous analysis of multiple samples with minimal mobile phase consumption, facilitating comprehensive quality control and authentication of diverse plant species[11,12]. This advanced form of planar chromatography is extensively employed for fingerprinting medicinal plants and their derived products, as well as for screening lichen substances and quantifying active ingredients[13,14]. This method is especially beneficial for evaluating the quality of herbal drugs, as it minimises exposure to toxic solvents and reduces analysis time while offering high separation efficiencies[15]. Furthermore, the ability of HPTLC to analyse multiple samples concurrently, coupled with its advanced detection limits, enhances its utility in the comprehensive quality control of herbal medicines[16].

Silymarin, a complex mixture of flavonolignans derived from the milk thistle (*Silybum marianum* L.) plant, serves as a crucial example of a multi-component active pharmaceutical ingredient whose accurate quantification in herbal formulations necessitates advanced chromatographic techniques[17,18]. In this context, the development of both HPTLC and RP-UHPLC methods becomes imperative for the precise estimation of Silymarin, ensuring compliance with regulatory guidelines and pharmaceutical good manufacturing practices[19]. These sophisticated analytical approaches are essential for addressing the challenges posed by the inherent chemical complexity and potential adulteration of herbal products, thereby upholding product safety and therapeutic efficacy[5,20]. HPTLC, in particular, offers a low-cost, high-resolution solution for initial screening, qualitative, and quantitative examination of plant materials and herbal formulations, even allowing for visual detection of analytes at microliter concentrations[21-23]. Although several HPTLC and HPLC/UHPLC methods have been reported for the estimation of Silymarin in bulk drugs, plant extracts, or individual dosage forms, no study has comprehensively developed and validated both HPTLC and RP-UHPLC methods under ICH Q2(R2) guidelines for the same herbal formulation and compared their analytical performance. The present work provides a direct comparison of these complementary techniques, allowing selection of either a rapid high-throughput RP-UHPLC method or an economical HPTLC method for routine quality control of commercial herbal formulations.

2. Materials and Methods

2.1 Materials

Standard Silymarin was procured from Sigma-Aldrich, Mumbai. The herbal formulation (Milk Thistle extract capsules) was obtained from a commercial source. All solvents, including methanol, ethanol, ethyl acetate, toluene, dichloromethane, and acids, were of HPLC grade and used without further purification.

2.2 Confirmation of Marker Compound

The identity of Silymarin was confirmed using FTIR spectroscopy. Characteristic absorption peaks corresponding to hydroxyl, carbonyl, aromatic, and phenolic functional groups were observed, confirming its structural integrity.

2.3 HPTLC Method Development and Validation

2.3.1 Chromatographic Conditions

HPTLC analysis was performed using pre-coated silica gel 60 F254 aluminium-backed TLC plates (20 × 10 cm) as the stationary phase. Sample application was carried out using an automatic sample applicator equipped with a 100 µL Hamilton syringe. Chromatographic development was performed in a twin-trough glass chamber pre-saturated with the mobile phase for 15 min. The optimised mobile phase consisted of hexane:ethyl acetate:ortho-phosphoric acid (6:3.5:0.5 v/v/v). The plates were developed to a migration distance of 80 mm, air-dried, and scanned densitometrically at 287 nm using a TLC scanner. Data acquisition and processing were performed using the instrument software[24,25].

2.3.2 Preparation of Standard and Sample Solutions

Accurately weighed 10 mg of Silymarin was transferred to a 10 mL volumetric flask and dissolved in methanol to obtain a stock solution of 1000 µg/mL. Appropriate dilutions were made to obtain the desired working concentrations.

For sample preparation, the contents of twenty herbal capsules were mixed thoroughly. An accurately weighed quantity of powder equivalent to 10 mg of Silymarin was transferred to a 10 mL volumetric flask, extracted with methanol by sonication for 30 min, filtered through Whatman filter paper, and diluted appropriately with methanol. The resulting solution was used for HPTLC analysis.

2.3.3 Method Validation

The developed HPTLC method was validated according to ICH Q2(R2) guidelines. Linearity was evaluated over the concentration range of 500–3000 ng/spot by plotting peak area against concentration. Accuracy was determined by the standard addition method at 50%, 100%, and 150% recovery levels. Precision was assessed by repeatability, intra-day precision, and inter-day precision. The sensitivity of the method was determined by calculating the LOD and LOQ. Ruggedness was evaluated by different analysts under similar operating conditions, while robustness was assessed by introducing deliberate variations in chromatographic parameters[26].

2.4 RP-UHPLC Method Development and Validation

2.4.1 Chromatographic Conditions

RP-UHPLC analysis was performed using a C18 column (250 × 4.6 mm, 5 µm) maintained under ambient laboratory conditions. The mobile phase consisted of methanol and 0.1% glacial acetic acid (85:15 v/v), which was filtered through a 0.45 µm membrane filter and degassed by sonication before use. The mobile phase was delivered at a flow rate of 1.0 mL/min, and detection was carried out at 287 nm using a UV detector. The injection volume was 10 µL, and the analyte exhibited a retention time of approximately 2.6 minutes under the optimised chromatographic conditions[27].

2.4.2 Preparation of Standard and Sample Solutions

Accurately weighed 10 mg of Silymarin was transferred to a 10 mL volumetric flask and dissolved in methanol to obtain a stock solution of 1000 µg/mL. Appropriate dilutions were made to obtain the desired working concentrations.

For sample preparation, twenty herbal capsules were weighed, and their contents were powdered uniformly. An accurately weighed quantity of powder equivalent to 10 mg of Silymarin was transferred into a 10 mL volumetric flask, extracted with the solvent mixture, filtered through a 0.45 µm membrane filter, and diluted to the required concentration with the mobile phase. The prepared sample solution was injected into the RP-UHPLC system for analysis.

2.4.3 Validation Parameters

The optimised RP-UHPLC method was subsequently validated in accordance with ICH Q2(R2)

recommendations. Linearity was evaluated over the concentration range of 20–120 µg/mL. Accuracy was assessed by the standard addition method at 50%, 100%, and 150% recovery levels. Precision was determined by repeatability, intra-day precision, and inter-day precision. The LOD and LOQ were calculated to evaluate method sensitivity. Ruggedness was assessed by different analysts under identical experimental conditions, while robustness was evaluated by introducing deliberate changes in chromatographic parameters, including mobile phase pH and flow rate[28].

3. Results

3.1 FTIR Analysis

The FTIR spectrum of Silymarin was recorded to confirm the identity of the marker compound. The spectrum exhibited characteristic absorption bands corresponding to the functional groups present in Silymarin. A broad absorption band was observed in the region of 3500–3200 cm^{-1}, corresponding to O–H stretching vibrations. The bands between 3000–2850 cm^{-1} were assigned to aromatic and aliphatic C–H stretching vibrations, while a strong absorption band in the region of 1720–1680 cm^{-1} corresponded to C=O stretching vibrations. The absorption bands observed between 1600–1500 cm^{-1} were attributed to aromatic C=C stretching, whereas the bands in the region of 1300–1000 cm^{-1} corresponded to C–O stretching of phenolic groups. Peaks between 900–700 cm^{-1} were assigned to aromatic C–H bending vibrations.

3.2 HPTLC Method Development and Validation for Estimation of Silymarin in Herbal Formulation

3.2.1 HPTLC Method Development

Different mobile phase compositions were evaluated for the chromatographic separation of Silymarin. The initial mobile phase systems did not produce a suitable R_f value or satisfactory spot resolution. Among the mobile phases investigated, the combination of Hexane:Ethyl acetate:Ortho-phosphoric acid (6:3.5:0.5, v/v) produced a well-resolved, sharp, and symmetrical spot with an R_f value of 0.52.

3.2.2 HPTLC Method Validation

The developed HPTLC method was validated in accordance with the ICH Q2(R2) guidelines.

3.2.2.1 Linearity Study

The linearity of the developed HPTLC method was evaluated over the concentration range of 500–3000 ng/spot of Silymarin. As presented in Table 1, the peak area increased proportionally with increasing concentration, indicating a linear analytical response. The calibration curve (Figure 1) showed excellent linearity with the regression equation Y = 1.1107x + 34.822 and a correlation coefficient (r^2) of 0.9992. The %RSD values ranged from 0.1469% to 0.5146%, demonstrating good repeatability of the analytical response across the studied concentration range.

3.2.2.2 Accuracy

The accuracy of the developed HPTLC method was evaluated by the standard addition method at 50%, 100%, and 150% recovery levels. The percentage recovery of Silymarin ranged from 99.89% to 99.99%, with %RSD values ranging from 0.0449% to 0.0735%. The corresponding amounts recovered ranged from 1499.21 ± 0.7351 ng to 2497.38 ± 1.1229 ng. The results of the recovery study are presented in Table 2.

3.2.2.3 Precision (Intra-day and Inter-day Precision)

The precision of the developed HPTLC method was evaluated by intra-day and inter-day studies at Silymarin concentrations of 1500, 2000, and 2500 ng/spot. The inter-day mean peak areas ranged from 1700.33 ± 1.2472 to 2810.00 ± 1.6329, with %RSD values ranging from 0.0553% to 0.0733%. The corresponding amount found ranged from 1499.52 ± 1.1229 to 2498.58 ± 1.4702 ng/spot, with percentage recoveries of 99.94% to 99.96%. Similarly, the intra-day precision results showed mean peak areas ranging from 1699.33 ± 1.2472 to 2808.33 ± 1.6996, with %RSD values between 0.0552% and

Table 1. Linearity study of Silymarin

Sr. No.	Concentration of Silymarin (ng/spot)	Peak area means ± S.D. (n = 3)	% RSD
1	500	634.66 ± 3.2659	0.5146
2	1000	1106 ± 2.4944	0.2255
3	1500	1688.33 ± 3.8586	0.2285
4	2000	2246.33 ± 3.2998	0.1469
5	2500	2804.67 ± 5.3124	0.1894
6	3000	3391.33 ± 5.3124	0.1566

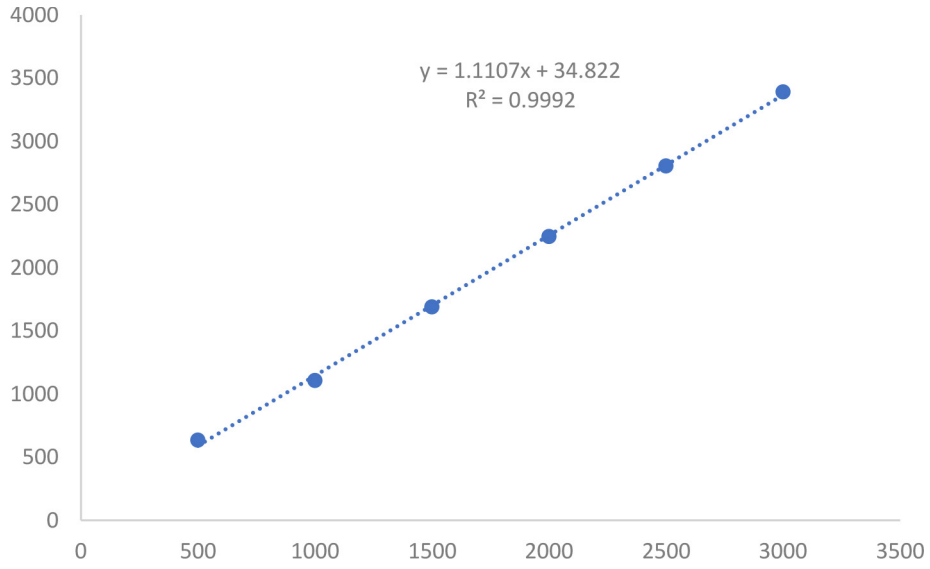


Figure 1. Calibration curve for Silymarin.

Table 2. Results of recovery studies for Silymarin

Drug	Initial Amount (ng)	Amount Added (ng)	Amount Recovered ± S.D.	% Recovered	% R.S.D.
Silymarin	1000	50 %	1499.21 ± 0.7351	99.94	0.049
	1000	100 %	1999.80 ± 1.4702	99.99	0.0735
	1000	150 %	2497.38 ± 1.1229	99.89	0.0449

0.0733%. The amount found ranged from 1498.61 ± 1.1229 to 2497.08 ± 1.5302 ng/spot, corresponding to percentage recoveries of 99.88% to 100.02%.

3.2.2.4 Repeatability

The repeatability of the developed HPTLC method was evaluated by six replicate analyses of 1500 ng/spot of Silymarin. The peak area values ranged from 1699 to 1705, with a mean peak area of 1701.83 and a standard deviation of 2.6087. The %RSD was found to be 0.1532%.

3.2.2.5 Sensitivity

The sensitivity of the developed HPTLC method was evaluated by determining the LOD and LOQ for Silymarin. The peak area increased with increasing application volume over the range of 500–3000 ng/spot, and the %RSD values ranged from 0.1469% to 0.5146%. The linearity equation was y = 0.47x + 81.756. The LOD and LOQ for Silymarin were found to be 225.72 ng and 684.01 ng, respectively. The results of the sensitivity study are presented in Table 3.

Table 3. Results of sensitivity studies of Silymarin

Sr. No.	Application Volume (ng/spot)	Area of Silymarin Mean ± SD	% RSD
1	500	634.66 ± 3.2659	0.5146
2	1000	1106 ± 2.4944	0.2255
3	1500	1688.33 ± 3.8586	0.2285
4	2000	2246.33 ± 3.2998	0.1469
5	2500	2804.67 ± 5.3124	0.1894
6	3000	3391.33 ± 5.3124	0.1566
LOD		225.72	
LOQ		684.01	

3.2.2.6 Ruggedness

The ruggedness of the developed HPTLC method was evaluated by two different analysts under the same experimental and environmental conditions using a Silymarin concentration of 1500 ng/band. Each analyst performed the analysis in triplicate. Analyst I obtained a mean assay value of 100.06 ± 0.1428% with a %RSD

of 0.1427, while Analyst II obtained a mean assay value of 100.08 ± 0.2182% with a %RSD of 0.218. The results obtained by both analysts are summarised in Table 4.

3.2.2.7 Robustness

The robustness study was carried out by introducing deliberate variations in the chromatographic conditions, including mobile phase composition, mobile phase volume, development distance, relative humidity, chamber saturation time, and activation time of prewashed TLC plates. A standard solution of 1000 ng/band of Silymarin was analysed by varying one parameter at a time. The standard deviation of the peak area ranged from 4.36 to 14.58, while the %RSD values ranged from 1.5% to 9.1% under the different experimental conditions. The results obtained under each variation are summarised in Table 5.

3.2.2.8 Analysis of Herbal Formulation

The developed HPTLC method was successfully applied for the quantitative estimation of Silymarin in the marketed herbal capsule formulation. As shown in Table 6, the amount of Silymarin found ranged from 1497.41 to 1502.81 ng/band, corresponding to an assay of 99.82% to 100.18%. The mean amount of Silymarin was 1499.81 ± 2.0574 ng/band, with a mean assay value of 99.98 ± 0.1371%. The %RSD was 0.1371%, indicating excellent precision and confirming the suitability of the developed HPTLC method for routine analysis of Silymarin in herbal formulations.

3.2.2.9 Specificity and Selectivity

Specificity refers to the ability of an analytical method to quantitatively determine the analyte in the presence of components that may be expected in the sample matrix, whereas selectivity refers to its ability to qualitatively distinguish the analyte from other matrix components. The HPTLC chromatograms of the standard Silymarin and the herbal formulation exhibited well-resolved peaks with no interference from excipients or other matrix components. The baseline was free from significant noise, and no interfering peaks were observed at the R_f value corresponding to Silymarin, demonstrating the high specificity and selectivity of the developed HPTLC method. These results confirm the suitability of the method for routine qualitative and quantitative analysis.

Table 4. Results of Ruggedness of Silymarin

Analyst	Amount of Silymarin Found (%)	%RSD (n = 3)
I	100.06 ± 0.1428	0.1427
II	100.08 ± 0.2182	0.218

3.3 RP-UHPLC Method Validation for Estimation of Silymarin in Herbal Formulation

3.3.1 Method Development

The RP-UHPLC method for the quantitative estimation of Silymarin in herbal formulations was developed

Table 5. Results of Robustness Studies for Silymarin

Parameters	Silymarin	
	SD of Peak Area	% RSD
Mobile Phase Composition		
a Chloroform:Ethyl acetate:Methanol:ortho-phosphoric acid (6:2:1.5:0.5)	4.36	1.5
b Chloroform:Ethyl acetate:Methanol:ortho-phosphoric acid (4.5:3:1.5:0.5)	7.5	3.9
Mobile Phase Volume (mL)		
5	12.11	9.1
8	04.45	7.1
Development Distance (mm)		
70	05.31	1.9
75	11.47	1.9
80	05.60	2.4
Relative Humidity (%)		
55	10.42	4.2
65	11.64	3.3
Duration of Saturation (min)		
20	14.58	3.5
25	06.14	3.89
30	07.39	1.64
Activation of Prewashed TLC Plates (min)		
08	7.19	2.17
10	6.16	2.49
12	8.31	2.46

Table 6. Analysis of formulation by HPTLC

Drug	Amount Taken (ng/band)	Amount Found (ng)	Amount Found %
Formulation	1500	1499.21	99.94
	1500	1497.41	99.82
	1500	1501.91	100.12
	1500	1502.81	100.18
	1500	1500.11	100.00
	1500	1497.41	99.82
Mean ± SD		1499.81 ±2.0574	99.98 ±0.1371
%RSD		0.1371	0.1371

by systematically optimising the chromatographic conditions. Different mobile phase compositions comprising acetonitrile, methanol, water, phosphate buffer, and acid modifiers were investigated to achieve adequate peak resolution, acceptable retention time, and symmetrical peak shape. Initial trials using acetonitrile–methanol, acetonitrile–water, and acetonitrile–phosphate buffer resulted in poor peak symmetry and noticeable peak tailing. Further optimisation was performed by modifying the aqueous phase with glacial acetic acid, which significantly improved chromatographic performance. The optimised mobile phase consisting of methanol:0.1% glacial acetic acid (85:15, v/v) provided a sharp, well-resolved, and symmetrical peak for Silymarin with a retention time of approximately 2.6 min. Chromatographic separation was achieved on a Zodic C18 RP-UHPLC column (250 × 4.6 mm, 5 µm) using isocratic elution at a flow rate of 1.0 mL/min, with UV detection at 287 nm and an injection volume of 10 µL. The optimised chromatographic conditions exhibited excellent peak shape, reproducibility, and sensitivity and were selected for subsequent validation of the developed RP-UHPLC method in accordance with ICH Q2(R2) guidelines.

3.3.2 RP-UHPLC Method Validation

The developed RP-UHPLC method was validated in accordance with the ICH Q2(R2)guidelines.

3.3.2.1 Linearity Study

The linearity of the developed RP-UHPLC method was evaluated over the concentration range of 20–120 µg/mL

Table 7. Linearity study of Silymarin (RP-UHPLC)

Sr. No.	Concentration of Silymarin (µg/mL)	Peak Area (mAU*min) (Mean ± SD; n = 3)	% RSD
1	20	14.4 ±0.0055	0.0386
2	40	28.27 ±0.0047	0.0167
3	60	40.91 ±0.005	0.0123
4	80	56.45 ±0.004	0.0071
5	100	67.91 ±0.004	0.0069
6	120	81.72 ±0.004	0.0049

of Silymarin. The mean peak area increased from 14.40 ± 0.0055 to 81.72 ± 0.004 mAU·min with increasing concentration. The %RSD values ranged from 0.0049% to 0.0386% (Table 7). The calibration curve (Figure 2) was linear over the studied concentration range and was described by the regression equation Y = 0.673x + 1.1745 with a correlation coefficient (R^2) of 0.9991.

3.3.2.2 Accuracy

The accuracy of the developed RP-UHPLC method was evaluated by the standard addition method at 50%, 100%, and 150% recovery levels. The percentage recovery of Silymarin ranged from 99.16% to 101.60%, with %RSD values ranging from 0.4404% to 0.4605%. The corresponding recovered amounts ranged from 30.48 ± 0.1342 µg/mL to 49.58 ± 0.2283 µg/mL. The results of the accuracy study are shown in Table 8.

3.3.2.3 Precision

Precision of the developed RP-UHPLC method was assessed by intra-day, inter-day, and repeatability studies. The method exhibited low %RSD values for both intra-day (0.006–0.0321%) and inter-day (0.1647–0.3102%) analyses, indicating good precision. Repeatability evaluation also demonstrated satisfactory reproducibility, with a mean amount found of 60.30 µg/mL and a %RSD of 0.5544.

3.3.2.4 Ruggedness

The ruggedness of the developed RP-UHPLC method was evaluated by analysing a 60 µg/mL Silymarin solution independently by two analysts. The method demonstrated good reproducibility between analysts, with Analyst I obtaining a mean amount of 60.30 ± 0.6066 µg/mL, corresponding to a recovery of 100.51 ± 1.0114% and a %RSD of 1.0059, while Analyst II obtained a mean amount

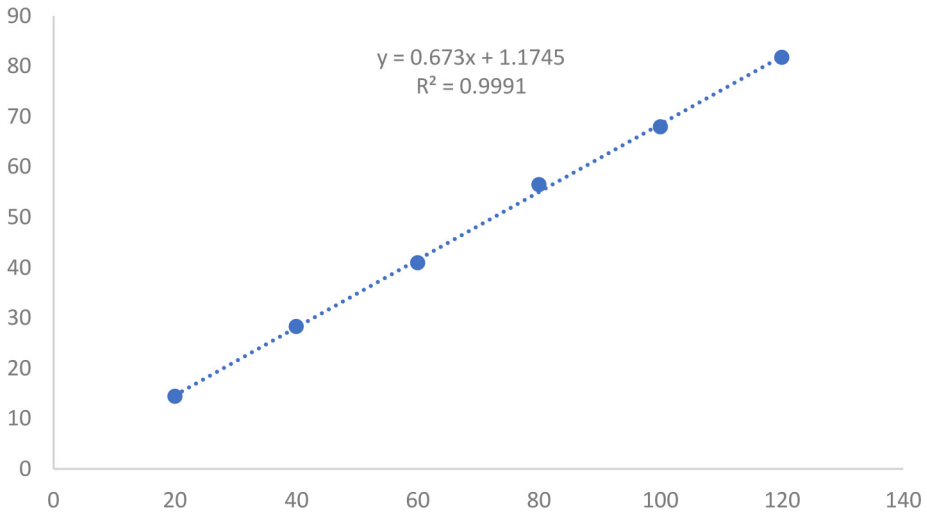


Figure 2. Calibration curve of Silymarin.

Table 8. Results of recovery studies for Silymarin

Drug	Initial Amount (µg/mL)	Excess Drug Added to the Analyte (%)	Amount Recovered ± S.D (µg/mL)	Recovery (%)	%RSD (n = 3)
Silymarin	20	50	30.48 ±0.1342	101.6	0.4404
	20	100	40.33 ±0.1811	100.84	0.4490
	20	150	49.58 ±0.2283	99.16	0.4605

Table 9. Results of ruggedness study of Silymarin

Drug	Amount in µg/ml	Amount Found (n = 6) Mean ± SD		% Amount Found (n = 6)		%RSD	
		Analyst I	Analyst II	Analyst I	Analyst II	Analyst I	Analyst II
Silymarin	60	60.30 ± 0.6066	60.15 ± 0.3686	100.51 ± 1.0114	100.26 ± 0.6143	1.0059	0.6127

of 60.15 ± 0.3686 µg/mL, corresponding to a recovery of 100.26 ± 0.6143% with a %RSD of 0.6127 (Table 9).

3.3.2.5 Robustness

The robustness of the developed RP-UHPLC method was evaluated by varying the pH of the mobile phase and the flow rate. The retention time (R_t) of Silymarin ranged from 3.1 to 6.2 min with changes in pH and from 2.2 to 3.9 min with changes in flow rate. The results of the robustness study are summarised in Table 10.

3.3.2.6 Sensitivity

The sensitivity of the developed RP-UHPLC method was evaluated by determining the LOD and LOQ for Silymarin. The LOD and LOQ were found to be 9.5699 µg and 28.999 µg, respectively.

Table 10. Results of robustness studies for Silymarin

Parameters	Silymarin R_t
Change in pH	
5.5	3.1
6.0	4.5
7.0	6.2
Change in Flow Rate	
0.8 ml/min	2.2
1.2 ml/min	3.3
1.8 ml/min	3.9

3.3.2.7 Analysis of Herbal Formulation

The developed RP-UHPLC method was applied for the analysis of a marketed herbal capsule formulation

Table 11. Analysis of formulation by RP-UHPLC

Drug	Amount Taken (µg/mL)	Amount Found (µg/mL)	Amount Found %
Silymarin	20	20.34	101.72
	20	20.30	101.53
	20	20.25	101.28
	20	19.76	98.80
	20	20.53	102.69
	20	20.73	103.68
	Mean ± SD	20.32 ±0.2993	101.62 ±1.4968
	%RSD	1.4729	1.4729

containing Silymarin. As shown in Table 11, the amount of Silymarin found ranged from 19.76 to 20.73 µg/mL, corresponding to an assay of 98.80% to 103.68%. The mean amount found was 20.32 ± 0.2993 µg/mL, with a mean assay value of 101.62 ± 1.4968% and a %RSD of 1.4729%.

4. Discussion

Silymarin is one of the most extensively investigated phytopharmaceutical compounds because of its hepatoprotective, antioxidant, anti-inflammatory, and membrane-stabilising properties. Since herbal formulations contain multiple phytoconstituents and excipients, the development of reliable analytical methods for the accurate estimation of marker compounds is essential to ensure product quality, efficacy, and regulatory compliance[29]. The present study successfully developed and validated HPTLC and RP-UHPLC methods for the quantitative estimation of Silymarin in herbal formulations according to ICH Q2(R2)validation guidelines. Both methods exhibited excellent analytical performance with respect to linearity, accuracy, precision, specificity, sensitivity, ruggedness, and robustness, demonstrating their suitability for routine quality control analysis.

The developed HPTLC method provided a well-resolved chromatographic peak with an R_f value of 0.52 using the optimised mobile phase consisting of hexane:ethyl acetate:ortho-phosphoric acid (6:3.5:0.5 v/v/v). The method showed excellent linearity over the concentration range of 500–3000 ng/spot with a correlation coefficient (r^2 = 0.9992), indicating a strong linear relationship between concentration and peak area. Similarly, the RP-UHPLC method achieved efficient chromatographic separation using methanol:0.1% glacial acetic acid (85:15 v/v) with a short retention time of 2.6 min and excellent linearity over the concentration range of 20–120 µg/mL (r^2 = 0.9991). These findings are consistent with previous reports demonstrating that optimised HPTLC and RP-UHPLC methods provide rapid and reliable quantification of herbal marker compounds while minimising analysis time and solvent consumption[30,31].

Accuracy studies performed using the standard addition technique demonstrated percentage recoveries ranging from 99.89–99.99% for the HPTLC method and 99.16–101.60% for the RP-UHPLC method, confirming that both analytical procedures are free from matrix interference and possess excellent trueness. Furthermore, the intra-day, inter-day, and repeatability studies produced %RSD values well below the acceptance criterion of 2%, indicating high precision and reproducibility. Similar validation characteristics have been reported for HPTLC and chromatographic methods developed for herbal formulations, where low %RSD values and recovery close to 100% are considered indicators of method reliability for routine pharmaceutical analysis[32].

Sensitivity is an important validation parameter, particularly when herbal formulations contain variable concentrations of bioactive constituents. The low LOD and LOQ values obtained for both methods demonstrate their capability to detect and quantify Silymarin even at low concentrations. In addition, specificity studies confirmed that no interfering peaks originating from formulation excipients co-eluted with the analyte, indicating excellent selectivity of both chromatographic methods. The robustness studies further demonstrated that small deliberate variations

in chromatographic conditions did not significantly affect analytical performance, confirming the stability and reliability of the developed methods during routine laboratory operation. These observations agree with previous investigations highlighting that optimised chromatographic methods should maintain consistent analytical performance despite minor experimental variations[33].

Application of the validated methods to commercially available herbal formulations revealed Silymarin content within 98–103% of the labelled claim, confirming acceptable product quality and demonstrating the practical applicability of both analytical procedures for routine quality control. The rapid analysis time achieved by RP-UHPLC offers an additional advantage for high-throughput pharmaceutical laboratories, whereas HPTLC provides a cost-effective alternative with the capability of simultaneous analysis of multiple samples using minimal solvent. Therefore, the complementary use of both methods provides flexibility according to laboratory infrastructure, analytical workload, and economic considerations. Similar conclusions have been reported by recent studies emphasising that HPTLC serves as an economical yet reliable tool for herbal standardisation, whereas UHPLC-based methods provide superior sensitivity and shorter analysis time for quantitative analysis[34].

Overall, the findings of the present investigation demonstrate that both HPTLC and RP-UHPLC methods satisfy all ICH Q2(R2) validation requirements and can be confidently employed for the routine estimation of Silymarin in herbal formulations. These validated methods may also serve as useful analytical tools for quality assurance, batch-to-batch consistency evaluation, stability studies, and regulatory assessment of Silymarin-containing pharmaceutical and nutraceutical products.

5. Conclusion

The present study successfully developed and validated HPTLC and RP-UHPLC methods for the quantitative estimation of Silymarin in herbal formulations in accordance with ICH Q2(R2) guidelines. Both methods demonstrated excellent linearity, accuracy, precision, specificity, sensitivity, robustness, and ruggedness, confirming their reliability for routine analysis. The RP-UHPLC method offered rapid analysis with a retention time of 2.6 min, while the HPTLC method provided a simple and cost-effective alternative for simultaneous sample analysis. Analysis of commercial herbal formulations showed Silymarin content within acceptable limits (98–103%) without interference from formulation excipients. Therefore, both validated methods are suitable for routine quality control, standardisation, and regulatory evaluation of Silymarin-containing herbal formulations.

6. References

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Author Contribution

Vaibhav Jagtap: Investigation, Methodology, Data curation, Writing–original draft; Vinod Wagh: Conceptualisation, Supervision; Deepak Somavanshi: Methodology, Validation; Sujit Jadhav: Formal analysis, Investigation; Chandrashekhar Patil: Data interpretation, Writing–review and editing; Ganesh Sonawane: Conceptualisation, Supervision, Writing–review and editing, Correspondence; Sandip Ahire: Resources, Validation; Durgesh Gautam: Visualisation, Review and editing. All authors have read and agreed to the published version of the manuscript.