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REVEALING SILYMARIN: HPTLC METHOD DEVELOPMENT AND VALIDATION

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Keywords:

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ABSTRACT: Silymarin, an intricate blend of flavonolignans obtained from *Silybum marianum*, or milk thistle, has drawn a lot of interest because of its possible medical uses. This study reports on the development and validation of an analytical technique that employs high performance thin layer chromatography (HPTLC) to estimate silymarin in pharmaceutical dosage forms (tablet) and bulk drug. Chloroform: methanol (6:4 v/v) was the solvent system used in the chromatographic separation of the medication on aluminium plates pre-coated with silica gel 60 GF254, (10 cm × 10 cm with 250 µm layer thickness) as the stationary phase. At 288 nm, a densitometric analysis of the divided zones was carried out. Densitometric scanning was carried out at 288 nm using a Camag TLC scanner 3, which was controlled by Win CATS software. The radiation source utilised for the scan was a deuterium lamp with slit size of 5.00 x 0.45 mm. It was discovered that the retention factor was 0.42. In the 200–1200 ng/band range, linearity R2= 0.993 was used to evaluate the method's accuracy and repeatability. Intraday and interday Precision was found 0.307- 0.544% RSD and 0.509-0.914 % RSD and accuracy was 100.146 ± 0.537%. LOD and LOQ was found 25.355ng/band, 76.833ng/band respectively, specificity and robustness in accordance with ICH guidelines.

INTRODUCTION: Silymarin, a bioactive complex derived from the seeds of the milk thistle plant (*Silybum marianum*), has garnered significant interest in the fields of medicine, pharmacology, and natural health [1]. Hepatobiliary diseases were treated with the seed extracts as a herbal supplement [2, 3]. It's anti-inflammatory, anticarcino-genic, and hypocholesterolaemic qualities were asserted [4], along with its favourable effects on immune system illnesses [5, 6], circulatory disorders (congestion) [7], varicose veins, and menstruation discomfort [8].

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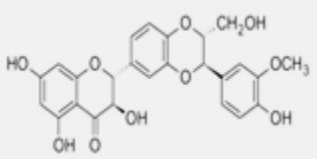
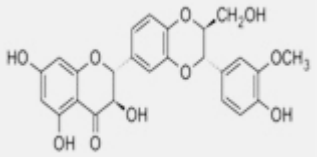
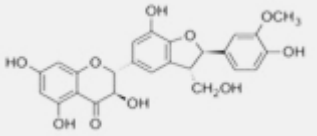
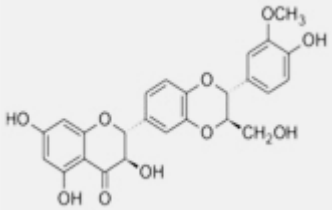
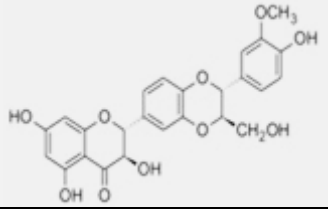
The flavonolignans known as silymarin, which are mostly composed of silibinin, isosilibinin, silicristin, and silidianin, are found in silybum marianum [9, 10]. Silymarin demonstrated strong antioxidant [11, 12] and hepatoprotective properties. When compared to other antioxidants, silymarin was said to have many times more potent antioxidant activity [13].

Its multifaceted pharmacological effects have sparked investigations into its efficacy in treating various conditions, including liver diseases such as hepatitis [14], cirrhosis, and non-alcoholic fatty liver disease (NAFLD), as well as conditions like diabetes, cardiovascular diseases, and certain types of cancer [15]. The mechanisms underlying silymarin's therapeutic actions are diverse, ranging from scavenging free radicals and inhibiting lipid peroxidation to modulating cellular signaling

pathways and promoting regeneration of hepatic cells. Additionally, silymarin has shown promise in enhancing the efficacy of conventional treatments and mitigating drug-induced toxicities, further expanding its potential clinical application [16].

TABLE 1: STRUCTURES OF CHEMICAL CONSTITUENTS OF SILYMARIN [3]

Chemical constituent of silymarin	Structure
Silybin A	
Silybin B	
Silychristin	
Silydianin	
Isosilybinin	

Various analytical methods play a crucial role in the quality assessment, standardization, and dosage form development of herbal products. The literature review reveals that several methods have been reported for the analysis (separation and quantification) of silymarin by UV-Visible Spectroscopy [17, 18]. It has also been analyzed by the UV-HPLC [19] method in bulk drugs and their pharmaceutical formulation. Methods like Using HPLC-ESI-MS [20], ultra-performance liquid chromatography (UPLC) combined with UV [21], and tandem mass spectrometry (MS/MS) detection [22, 23] were employed to analyzesilybinin in milk thistle. High-Performance Thin-Layer Chromatography (HPTLC) has emerged as a powerful analytical technique for the qualitative and quantitative determination of different chemical constituents present in herbal formulation. The advantages of HPTLC over the other chromatographic techniques includes, high sensitivity, rapid analysis, cost-effectiveness, and suitability for routine quality control purposes.

While numerous scientific techniques exist for quantifying silymarin by HPTLC [24-28] in conjunction with other compounds, but no validated HPTLC method has been established specifically for estimating silymarin alone.

Developing a method tailored to silymarin ensures that the analysis is specific to this compound, avoiding potential interference from other components that may be present in the sample. This method allowing for the detection and quantification of lower concentrations of silymarin compared to other methods that are not specifically optimized for this compound.

Therefore, we undertook the task of developing a new HPTLC method that is validated, straightforward, cost-effective, and capable of indicating stability for determining silymarin in both its bulk drug and pharmaceutical dosage form. This method was validated following the guidelines of the International Conference on Harmonization (ICH) [29, 30]. This approach not only saves time and cost but also offers an alternative to the expensive electrochemical methods typically used in quality control laboratories.

EXPERIMENTAL SECTION:

MATERIALS: Silymarin flavonolignans drug (pure) were obtained as a gift sample from Otto Chemie Pvt. Ltd. Mumbai, India and Silymarin 70 mg as pharmaceutical formulation (tablet) were procured from local market. All chemicals and reagents of analytical grade were purchased from Merck Chemicals, Mumbai, India.

Instrumentation: The samples were spotted in the form of bands of width 6 mm using Camag 100 µL sample syringe (Hamilton, Switzerland) on aluminium plates pre-coated with silica gel 60 GF_{254}, (10 cm × 10 cm with 250 µm layer thickness) using a linomat 5 applicator (Camag, Switzerland). The mobile phase used for the chromatographic separation was chloroform: methanol (6:4 v/v) with 10 mL of mobile phase per

pass through a 10 cm × 10 cm Camag twin trough glass chamber, the TLC plate was generated in a linear fashion under 15 minutes of saturation. The migratory distance was 80 mm. Densitometric scanning was done at 288 nm using a Camag TLC scanner 3 with a slit size of 5.00 x 0.45 mm with win CATS software. A deuterium lamp that continually emitted UV light with a wavelength of 190–400 nm served as the radiation source. The concentrations of the chemicals were determined by measuring the intensity of the diffused light. The assessment technique employed involved the linear regression of peak regions in relation to sample quantities, as determined by UV absorption.

Preparation of Standard Solution: To achieve a concentration of 1000 µg/mL, 10 mg of the medication was dissolved in 10 mL of methanol to create the standard solution. A working standard solution containing 100 µg/mL of silymarin was produced from the standard solution.

Preparation of Sample Stock Solution: Twenty pills (Silybon - 70; label claim: 70 mg silymarin per tablet) were weighed, an average weight was calculated, and the tablets were coarsely crushed. An amount of powder equal to 10 mg of Silymarin was put to a 10 mL volumetric flask containing 5 mL of methanol.

The mixture was ultrasonically sonicated for 10 minutes, and the resultant sample stock solution was filtered through Whatman filter paper 41 and diluted with methanol. To generate a final sample stock solution of 100 µg/mL, 1.0 mL of the solution was diluted with 10 mL of methanol.

Selection of Mobile Phase: Silymarin 100µg/mL was used as the working standard solution for chromatographic separation investigations. Initially, studies were conducted on regular TLC plates employing solvents in varying amounts to get the required Rf and shape for the drug peak. Following a few experiments, Chloroform: Methanol (6:4v/v) was chosen as the mobile phase, resulting in acceptable peak values, as given in **Fig. 1.**

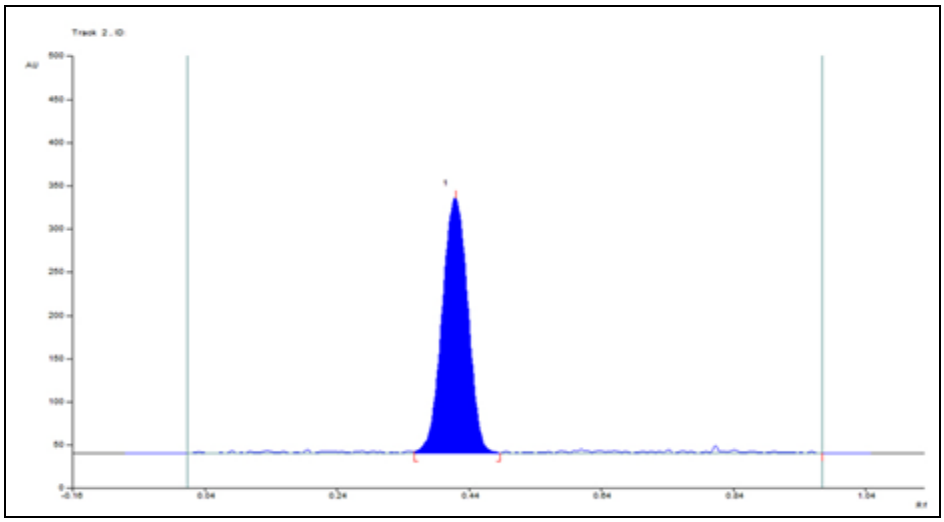


FIG. 1: DENSITOGRAM OF A STANDARD SOLUTION OF SILYMARIN (100 UG/ML)

Detection of Wavelength: To produce the spectra, dilutions of the standard solution (1000 µg/mL) were prepared with methanol and scanned between 200-400 nm. The medication had significant absorption at 288 nm.

Optimization of the HPTLC Method: The TLC approach was optimised with the goal of developing a method for silymarin. A standard solution with silymarin concentration of 100 µg/mL was produced. Using a Camag 100 µL sample syringe (Hamilton, Switzerland) and a linomat 5 applicator (Camag, Switzerland), 4 µL of the resulting solution was applied to a 6 mm wide TLC plate to achieve a concentration of 400 ng/band. The solvent system was chloroform: methanol (6:4 v/v). To lessen the neckless effect, TLC chambers were soaked for 20 minutes using saturation pads. The mobile phase was conducted up to an 8 cm distance, which takes roughly 20 minutes for full development of the TLC plate.

Validation of Developed Analytical Method by HPTLC [29, 30, 31]: Due to its simplicity, economy, accuracy, sensitivity, and reproducibility, the developed analytical method will fully validate to meet the requirements of the International Conference on Harmonisation (ICH) guidelines regarding the following parameters: accuracy, precision (repeatability, intermediate precision (intraday and inter-day study), linearity, range LOD, LOQ, specificity, robustness for HPTLC silymarin estimation, and for routine applications in quality control laboratories.

Linearity and Range: For spotting, a solution containing 100 µg/mL of silymarin was produced from the standard stock solution of silymarin (1000 µg/mL). On the plate, six duplicates of each concentration were identified. In order to create a calibration curve, six concentrations spanning the silymarin concentration range were analysed in order to ascertain the linearity, which is the connection between peak area and concentration.

Precision: Precision method Studies on repeatability variance and intra- and inter-day variations proved the method's accuracy. In the intra-day investigations, the percentage RSD was computed when three replicates of three distinct concentrations (400, 600, and 800 ng/band) were analyzed on the same day. Three concentrations were analyzed on three separate days in order to calculate the percentage RSD for the interday variation studies.

Limit of Detection and Limit of Quantification: LOD is the lowest concentration of an analyte that can be reliably detected but not necessarily quantified. It is typically determined as a signal-to-noise ratio, often set at 3:1 or higher. LOQ is the lowest concentration of an analyte that can be accurately quantified with acceptable precision and accuracy. It is usually determined as a signal-to-noise ratio, often set at 10:1 or higher. LOD and LOQ were determined by spotting smallest amount of analyte that produces a signal significantly different from the blank signal and calculating the signal-to-noise ratio for silymarin by spotting a series of solutions until the signal-to noise ratio 3 for LOD and 10 for LOQ. Serial dilutions of a standard solution of silymarin was prepared from the standard stock solution in the range of 30-270 ng/band in order to determine the LOD and LOQ. the samples was applied to a TLC plate, chromatograms were generated, and the measured signal from the samples was contrasted with that of the blank samples.

Accuracy: To check accuracy of the method, recovery studies were carried out by spiking the drug sample (silymarin and silymarin tablet) to which known amount of silymarin standard powder corresponding to 50, 100 and 150% of label claim had been added (standard addition method), mixed and analyzed by running chromatogram in optimized mobile phase.

Assay: Silybon - 70 tablet formulation analysis was carried out as mentioned under section preparation of sample solution. The process was carried out six times. After applying a 4 µL amount of sample solution, the area was measured. The sample was selected with a basic concentration of 400 ng/band from the tablet solution. using a linear equation, concentration and recovery percentage were calculated.

Robustness: By analysing the introduction of minor adjustments to the chamber saturation time, mobile phase composition, time from spotting to development, and time from development to scanning and noting the impact on the area, the robustness of the approach was ascertained.

Specificity: The specificity of the method was determined by analyzing standard drug and test sample. The spot for silymarin in the samples was confirmed by comparing the Rf and spectrum of the spot with that of a standard. The peak purity of silymarin was determined by comparing the spectrum at three different regions of the spot i.e. peak start(s), peak apex (M) and peak end (E).

RESULT AND DISCUSSION:

UV-Spectrophotometric Studies: The spectra were obtained by making subsequent dilutions using methanol from the standard stock solution (1000 µg/mL) and scanning across the 200-400 nm region. The medication was found to have significant absorbance at 288 nm. **Fig. 2** displays a representative UV spectrum of silymarin.

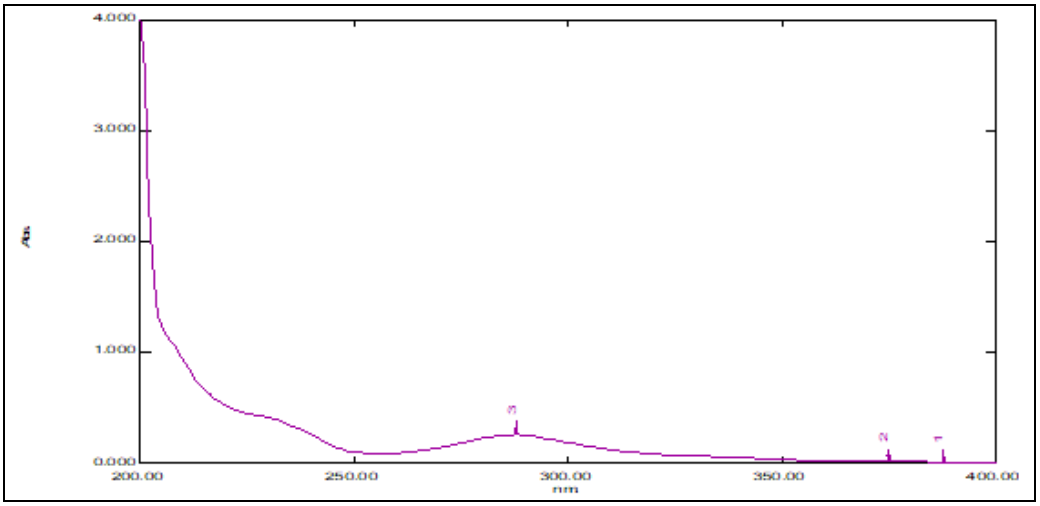


FIG. 2: THE UV SPECTRUM OF SILYMARIN (10 µG/ML)

Linearity: A solution containing 100 µg/mL of Silymarin was produced from the standard solution (1000 µg/mL). Additionally, spotting was done using this solution. For each concentration, six duplicates were observed. In order to generate a calibration curve for silymarin, six concentrations within the concentration range of 200–1200 ng/band were analysed to ascertain the linearity (connection between peak area and concentration) with a regression equation of y = 13.78 x + 2444.51 and an R2 of 0.993, the findings were determined to be linear. **Fig. 3** displays the linearity densitogram of Silymarin (200–1200 ng/band). **Table 2** displays the results of silymarin's linearity research. In **Fig. 4,** the calibration curve is displayed.

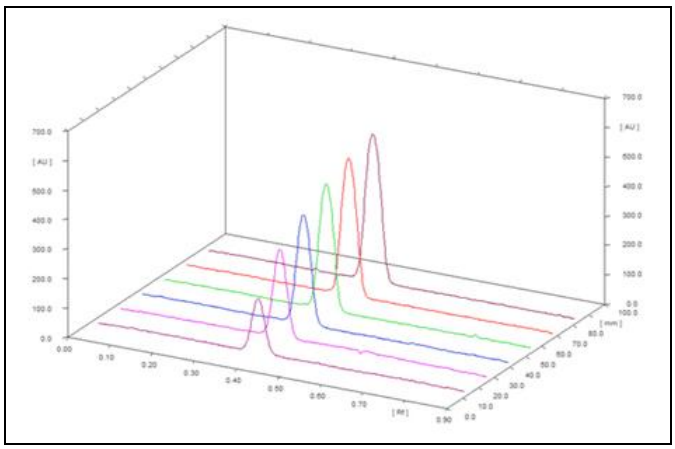


FIG. 3: 3 D OF THE HPTLC DENSITOGRAM OF CALIBRATION BAND

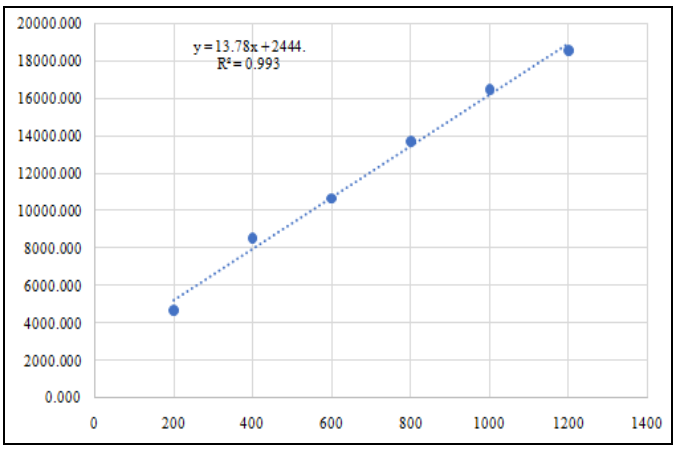


FIG. 4: CALIBRATION CURVE OF SILYMARIN (200-1200 NG/BAND) REFERENCE STANDARD

TABLE 2: LINEARITY STUDY OF SILYMARIN

Replicate	Concentrations of Silymarin (ng/band)					
	200	400	600	800	1000	1200
			Peak area			
1	4688.2	8312.8	10625.5	13595.5	16565.1	18059.5
2	4611.6	8447.7	10503.3	13980.1	16551.3	18719.9
3	4677.7	8570.7	10776.6	13513.9	16301.8	18865.2
4	4681.5	8596.6	10856.6	13977.3	16231.4	18581.1
5	4610.5	8543.5	10575	13469.7	16670.5	18564.5
6	4694.4	8572.8	10578.4	13528.8	16552.6	18594.5
Average	4660.650	8507.350	10652.567	13677.550	16478.783	18564.117
SD	38.844	58.218	135.310	236.740	171.718	272.312
% RSD	0.833	0.684	1.270	1.731	1.042	1.467

Range: Silymarin = 200-1200 ng/band.

Precision: Exactness of the technique was proven by tests of fluctuation within and between days. Three duplicates of each concentration were examined on the same day in the intraday experiments, and the percentage RSD was computed. Three concentrations were tested on three consecutive days for the interday variation investigations, and the percentage RSD was computed. The findings for both intraday and interday accuracy are displayed in **Tables 3** and **4.**

TABLE 3: INTRADAY VARIATION STUDIES DATA FOR SILYMARIN

Conc. (ng/band)	Area	% recovery	Average	SD	%RSD
400	8032.8	101.384	101.030	0.310	0.307
	8001.1	100.809			
	8005.9	100.896			
600	10618.4	98.862	99.210	0.309	0.312
	10667.3	99.453			
	10655.9	99.315			
800	13528.8	100.547	100.406	0.546	0.544
	13446.8	99.803			
	13564.2	100.868			

TABLE 4: INTERDAY VARIATION STUDIES DATA FOR SILYMARIN

Conc. (ng/band)	Area	% recovery	Average	SD	%RSD
400	8007.7	100.929	100.420	0.511	0.509
	7979.8	100.423			
	7951.4	99.907			
600	10655.5	99.310	99.730	0.911	0.914
	10776.6	100.775			
	10638.4	99.104			
800	13595.5	101.152	100.401	0.756	0.753
	13513.9	100.412			
	13428.8	99.640			

Limit of Detection (LOD) and Limit of Quantitation (LOQ): LOD and LOQ are calculated from the formula and results obtained are shown in **Table 5.**

$$LOD = 3.3*SD / S$$

$$LOQ = 10*SD / S$$

Were, S.D = S.D of the response at lowest concentration or standard deviation of Y intercept (Noise), S = Average of slope of the calibration curve (Slope).

TABLE 5: LOD AND LOQ OF SILYMARIN

Method	Avg slope	S.D	LOD (ng/band)	LOQ (ng/band)
1.Using S.D of the response at lowest concentration	13.78	38.844	9.302	28.189
2. Using S.D of y-intercept	13.78	105.876	25.355	76.833

Accuracy: Accuracy/Recovery studies were conducted by spiking the standard medication into the tablet solution at three different concentrations (50, 100, and 150%), in order to verify the correctness of the procedure. The sample's basic concentration was 400 ng/band and the linear equation was used to calculate the recovery percentage. **Table 6** displays the accuracy results that were achieved.

TABLE 6: ACCURACY STUDIES OF SILYMARIN

Level	Amount of sample taken (ng/band)	Amount of standard spiked (ng/band)	Area	% recovery	Mean % Recovery ± RSD
50%	400	200	10724.6	100.146	100.139 ± 0.139
			10735.2	100.274	
			10712.2	99.996	

100%	400	400	13508.2	100.360	100.146 ± 0.537
			13528.5	100.544	
			13417.1	99.534	
150%	400	600	16199.5	99.819	100.039 ± 0.208
			16233.6	100.066	
			16256.4	100.231	

Assay: The formulation analysis of Silybon - 70 tablets was completed as described in the section on sample solution preparation. The process was carried out six times. After applying a 4 µL amount of sample solution, the area was measured. The sample was selected with a basic concentration of 400 ng/band from the tablet solution. Concentration and recovery % were computed using a linear equation. **Table 7** displays the assay findings that were obtained. **Fig. 5** densitogram of Silymarin in sample solution.

TABLE 7: ASSAY OF MARKETED FORMULATION

Sr. no.	Peak area	Amount recovered (ng/band)	% recovery
1	7959.8	400.239	100.060
2	7947.6	399.353	99.838
3	7954.2	399.832	99.958
4	7993.8	402.706	100.677
5	7983.4	401.951	100.488
6	7985.7	402.118	100.530
Mean	7970.750	401.033	100.258
SD	19.207	1.394	0.348
%RSD	0.241	0.348	0.348

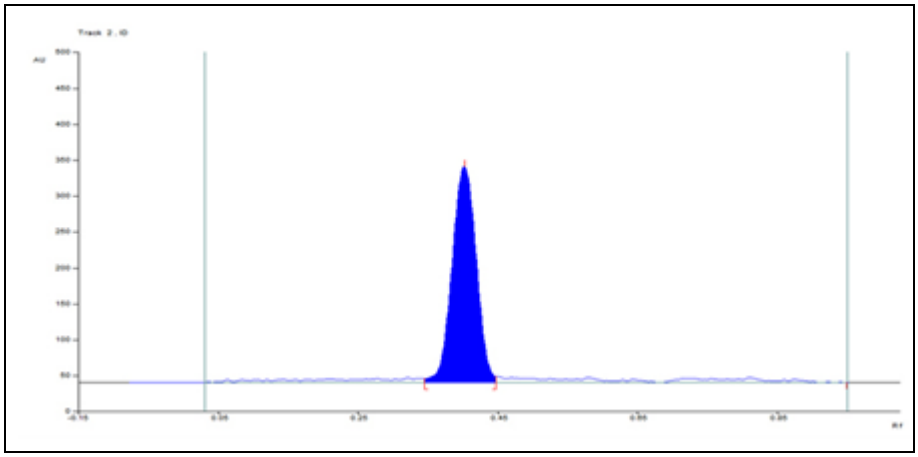


FIG. 5: DENSITOGRAM OF SAMPLE SOLUTION OF SILYMARIN (400 NG/BAND)

Robustness: The analysis was conducted under settings where the chamber saturation time, mobile phase composition, time from spotting to development, and time from development to scanning were varied, and the impact on the area was recorded. This allowed for the determination of the method's robustness. The approach was proven to be robust. **Table 8** displays the acquired results.

TABLE 8: ROBUSTNESS STUDY OF SILYMARIN

Sr. no.	Parameters	Robust condition	%RSD
1	Saturation time (15 min) ± 1 min	14 min	0663
		15 min	0.863
		16 min	0.842
2	Time from spotting to development	Immediate	0.668
		After 30 min	1.095
		After 1hr	0.486
3	Time from development to scanning	Immediate	1.472
		After 30 min	1.801
		After 1hr	1.161

Specificity: Peak purity profile tests were used to determine how specific the procedure was. The peak purity values were discovered to be more than 0.998, meaning that no other peak of the degradation product, contaminant, or matrix was interfering.

Summary of Validation Study Displayed in Table 9:

TABLE 9: SUMMARY OF VALIDATION PARAMETERS

Sr. no.	Validation parameters	Silymarin
1.	Linearity equation	y = 13.78x + 2444.51
	R^2	R^2= 0.993
	Range	200-1200 ng/band
2.	Precision	(%RSD)
	Intraday	0.307 – 0.544
	Interday	0.509 – 0.914
3.	Assay	100.258 ± 0.348
4.	Accuracy	
	50 %	100.139 ± 0.139
	100 %	100.146 ± 0.537
	150 %	100.039 ± 0.208
5.	Limit of detection	25.355ng/band
6.	Limit of quantitation	76.833ng/band
7.	Specificity	Specific
8.	Robustness	Robust

CONCLUSION: In conclusion, the High-Performance Thin-Layer Chromatography (HPTLC) method developed and validated for the analysis of silymarin offers a reliable and efficient means of quantification. Through meticulous validation procedures, including specificity, linearity, accuracy, precision, LOD, LOQ, specificity and robustness, the method demonstrates its suitability for routine analysis in pharmaceutical and herbal industries. The method's ability to provide accurate and precise results underscores its utility in quality control and assurance processes. Additionally, its simplicity, cost-effectiveness, and rapidity make it a valuable tool for researchers and practitioners alike. Moving forward, further studies could explore its application to a broader range of matrices and its potential for automation, enhancing its versatility and efficiency in pharmaceutical analysis.

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CONFLICT OF INTERESTS: It is hereby declared that there is no conflict of interest among authors.

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