Analytical Method Development and Validation of Silymarin and Silybin in Hydroalcoholic Seed Extract of *Silybum marianum* by UV Spectroscopy and HPTLC

Poorva A. Kamble*, Vijay R. Salunkhe, Manisha M. Murgude, Aditi S. Haval, Namrata B. Ranpise.
Rajarambapu college of pharmacy, Kasegaon.
A/P. Kasegaon, Tal: Walwa, Dist: Sangli, 415404.Maharashtra, India.

Abstract

In this study, we developed UV and HPTLC methods to analyze the hydroalcoholic extract of Silybum marianum for the presence of silymarin and silybin. The stationary phase employed was 60F254 TLC plate, which is precoated silica gel. The mobile phase consisted of toluene, ethyl acetate, and formic acid in a ratio of 7:2.5:0.5. The RF values for the marker chemicals were 0.30 and 0.23, respectively, and they were identified at 254 and 366 nm. The technique that was developed for Silymarin and Silybin was tested for relevant criteria. The markers' HPTLC technique development was optimized utilizing a mobile phase comprising of toluene, ethyl acetate, and formic acid at a ratio of 7:2.5:0.5. This approach was determined to be exact, accurate, straightforward, robust, and specific based on the statistical analysis of UV and HPTLC.

Keywords: HPTLC, *Silybum marianum*, Silymarin, UV spectroscopy, Method development.

1. Introduction

Herbal medications are therefore now frequently utilized and have increased in popularity. A wide range of herbal preparations are available for purchase. Major pharmaceutical corporations worldwide have promoted herbal remedies for liver issues, which have been utilized for a long time in India. [1]

Hepatoprotective properties of the medicinal herb *Silybum marianum* have been known for a long time. In addition to its antioxidant & anti-inflammatory effects, it also regulates cell permeability and stabilizes membranes, stimulates liver regeneration, and prevents cirrhosis-causing collagen fiber deposition. [2]

This plant's dried seed extract has silymarin concentrations of up to 4%. Dihydroxysilibin, silibinin A and B, silidianin, and silicristin are the flavonoids that make up silymarin. A number of flavonolignans are present in this plant's extract and may have abilities to protect the liver. Furthermore, the plant's dried seeds may have as much as 20% oil, which is inert and has no medicinal use. [2]

Figure 1. Silybum marianum

1.1 Objectives

To procure and identify marker compound as Silymarin and Silybin
To prepare TLC profile data which shall be used for HPTLC analysis.
To develop UV and HPTLC method for Silymarin and Silybin in extract of *Silybum marianum.*
To validate developed HPTLC method.
To provide scientific based approach for herbal industry for the same.

1.2 Pharmaceutical analysis

"Pharmaceutical analysis" might mean different things to different people. Finding, analyzing, isolating, purifying, and explaining the molecular structure of a molecule is what this process is all about in the pharmaceutical industry. All sorts of things, including final goods, biological samples, pollutants, and impurities, can be analyzed in the pharmaceutical industry. A wide range of analytical methods can be employed in pharmaceutical analysis.[3]

1.2.1 Types of Pharmaceutical Analysis:

I. Qualitative analysis:

This type of pharmaceutical analysis involves identifying the chemical components of an unknown sample or molecule using a non-quantifiable approach. [3]

II. Quantitative analysis:

Quantitative analysis is sometimes called determination since its main purpose is to find the exact concentration of the substance of interest. [3]

1.3 UV – VIS Spectroscopy:

Light in the visible or ultraviolet range, with wavelengths between 200 and 800 nanometers, may be effectively measured using UV spectroscopy, an analytical tool. It is possible to use this method to assess both dyed compounds in the visible (400-800 nm) and colorless (200-400 nm) UV region. When comparing a sample to a reference or blank, ultraviolet (UV) spectroscopy is typically employed to determine which specific visible or UV light wavelengths the sample absorbs or transmits. This feature, which is made more difficult by the composition of the sample, allows one to determine the components and concentrations of the sample.[4]]

1.4 HPTLC:

It is used to discrete, identify & quantify components in mixtures, particularly in pharmaceuticals, herbal remedies, cosmetics, food products, and forensic science.

Typical steps in HPTLC: [5]

1. Sample Preparation: Extract the analyte with a suitable solvent.
2. Plate Pre-treatment: Silica gel or other stationary phase plates are utilized.
3. Application: The samples are applied in precise bands using an automated applicator.
4. Development: The plate is grown in a chamber containing a mobile phase
5. Detection - Visualized under UV light or after derivatization for color development.
6. Scanning - The densitometer examines and quantifies the separated bands.
7. Data Analysis - The results are processed with software.

2. MATERIALS AND METHODS

2.1 Evaluation of *Silybum marianum* Extract and Standard Compounds:

2.1.1 Organoleptic Properties:

The extract of *Silybum marianum* was evaluated for its colour, taste, odour, and physical appearance. Similarly, the standard compounds Silybin and Silymarin were also examined for their organoleptic characteristics.

2.1.2 Physical Properties:

a) **Solubility:**

The solubility of *Silybum marianum* extract, along with standard compounds Silymarin and Silybin, was tested in various solvents including n-Hexane, Chloroform, Ethanol, and Methanol.

b) **Melting Point:**

The melting points of Silymarin and Silybin were determined by the capillary tube method.

2.1.3 Chemical Tests: [7]

Test 1: A small piece of magnesium and concentrated HCl was added to the Silybum marianum extract.

Test 2: Lead acetate solution was mixed with the extract.

Test 3: Sodium hydroxide (NaOH) was added to the extract of Silybum marianum.

2.1.4 UV Spectroscopic Analysis: [8]

λ max Determination:

For Silymarin: 100 mg of the compound was dissolved in methanol to prepare a 100 ml solution. Then, 10 ml of this solution was diluted to 100 ml to obtain a concentration of 100 µg/ml, scanned between 200–400 nm. Results are presented in Figure 9.2. A calibration curve was plotted using concentrations of 10, 20, 30, 40, and 50 µg/ml.

For Silybin: 100 mg of the compound was dissolved in methanol to make a 100 ml solution. From this, 10 ml was diluted to 100 ml to yield 100 µg/ml solution, which was scanned over the 200–400 nm range. Results are displayed in Figure 9.3. The calibration curve was generated using concentrations of 10, 20, 30, 40, and 50 µg/ml.

2.1.5 FTIR Spectroscopy Analysis: [9]

The *Silybum marianum* extract along with standard compounds Silymarin and Silybin were analyzed using FTIR spectroscopy. The spectra were recorded using Bruker Opex 9.0 Alpha 2.

2.2 Extraction of Plant Material: [10]

The extraction was carried out using the maceration method. A total of 50 grams of *Silybum marianum* powder was placed in a sealed container, and an appropriate quantity of methanol was added. The mixture was left to stand for seven days with occasional shaking. After the extraction period, the mixture was filtered using Whatman filter paper.

2.3 TLC Profile of the Drug: [11]

2.3.1 Preparation of TLC Plate:

A silica gel slurry was prepared in a glass beaker by mixing Silica Gel-G with distilled water. The slurry was poured onto a glass TLC plate and spread evenly. Afterward, the plate was dried and activated in a hot air oven at 110°C for 30 minutes. The prepared TLC plate was then used for experimental analysis.

2.3.2 Sample Application:

A sample solution was prepared by dissolving 10 mg of Silybum marianum extract in 10 ml of methanol. This solution was applied to the TLC plate using a capillary tube.

2.3.3 Selection of Mobile Phase:

To separate the methanol-dissolved extract, solvent systems of varying polarity were explored as mobile phases. Spots were visualized under a UV cabinet & by developing the TLC plates in an iodine chamber.

2.4 HPTLC method development [12]

2.4.1 Selection of HPTLC Plate

The study employed pre-coated silica gel G 60 F254 HPTLC plates for the chromatographic separation.

2.4.2 Mobile Phase Preparation and Chamber Saturation

The mobile phase, comprising Toluene: Ethyl acetate: Formic acid (7:2.5:0.5), was freshly prepared and poured into a clean, dry twin trough chamber. The chamber was then allowed to saturate for 20 minutes before use.

2.4.3 Activation of Pre-Coated Plate

Since the silica gel G 60 F254 plates are pre-coated, no activation was required prior to sample application.

2.4.4 Application of Sample

Using the CAMAG LINOMAT 5 applicator, sample bands were applied on the pre-coated plate along designated tracks. Bands were spotted 8 mm from the bottom and measured 8 mm in length on a plate of dimensions 200 mm x 100 mm. The sample volume was adjusted based on the volatility of the solvent, and a sharp band was obtained with 5 µl of sample application.

2.4.5 Development of Spots

The plate was placed in the saturated twin trough chamber for development. After

the development process, the plate was dried and examined under UV light. The observed spots were sharp with no tailing, indicating a good separation.

2.4.6 Rf Value Calculation

The Rf value was calculated using the formula:

Rf = Distance travelled by the compound ÷ Distance travelled by the solvent front,

with both measurements taken from the point of origin.

2.4.7 Detection and Visualization

The produced plates were examined in both visible and UV light.

2.5 Method validation [12]

2.5.1 Linearity

Linearity was assessed by spotting 200–600 ng/spot of standard Silymarin and Silybin. Plates were scanned at 254 nm and peak area was recorded.

2.5.2 Precision

2.5.2.1 Repeatability

Repeatability was determined using 2 µL of 100 µg/ml Silymarin and Silybin solutions, applied six times using a CAMAG LINOMAT 5.

2.5.2.2 Intermediate precision (Inter-Day)

Inter-day precision was evaluated by applying 100 ng, 300 ng, and 500 ng of standard solutions and scanning the chromatograms on three different days.

2.5.3 Accuracy

Accuracy was assessed by spiking the extract (10 mg/ml) with standard Silymarin and Silybin at three levels (1.6 µL, 2.0 µL, 2.4 µL of 0.1 mg/ml).

2.5.4 Specificity

Specificity was demonstrated by analyzing Silybum marianum extract (1000 µg/ml), standard solutions (100 µg/ml), mobile phase, and diluent. Spotting was performed at specific volumes and plates were scanned at 254 nm using a CAMAG HPTLC Scanner V.

2.5.5 Limit of Detection and Limit of Quantitation

Limit of detection and limit of quantitation were used to determine the suggested method's sensitivity for measuring standard samples. The slope and standard deviation of the intercept were used to compute the LOD and LOQ. By entering the slope and standard deviation of the intercept values into the formula, the limit of detection and limit of quantitation were determined.

- **LOD = 3.3×σ/S**
- **LOQ = 10×σ/S**

2.5.6 Robustness

Robustness was assessed at 300 ng/band by varying the chamber saturation time by ±10%.

2.6 Chromatogram Development [13]

2.6.1 Chromatographic Layer Selection

Silica gel 60 F_{254} pre-coated HPTLC plates were used for spotting both standard and sample solutions.

2.6.2 Mobile Phase and Chamber Saturation

The mobile phase (Toluene: Ethyl acetate: Formic acid, 7:2.5:0.5) was freshly prepared and the chamber was saturated for 20 minutes prior to development.

2.6.3 Sample Application and Plate Activation

Plates were used without additional activation. Standard solutions (100 ng/µL) were applied in increasing volumes from 1 µL to 7 µL on nine tracks. Sample solutions (10,000 µg/ml) were applied in volumes of 2 µL, 3 µL, and 4 µL in duplicate. A CAMAG ATS 5 applicator was used for all spotting operations. Bands were 8 mm long and spaced uniformly on 100 mm × 100 mm plates.

2.6.4 Spot Development

The plate was developed in the saturated chamber. after development, the plate was dried and visualized. Uniform spot development without tailing was observed.

2.6.5 Chromatogram Scanning

The dried plate was scanned using a CAMAG TLC Scanner IV at 254 nm at a speed of 100 mm/s.

3. Result and Discussion

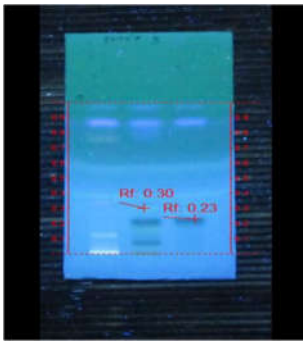

Figure 2. spots of standards

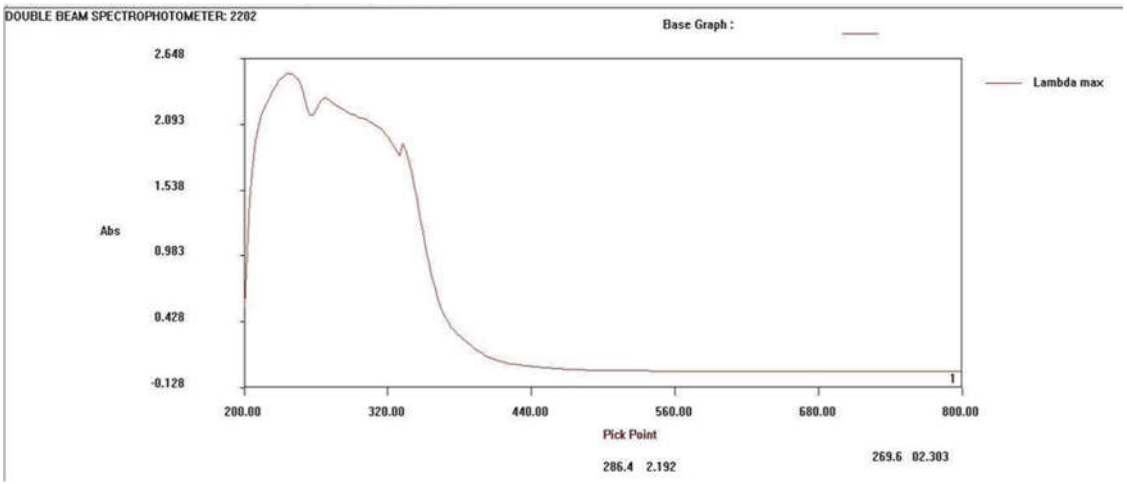

Figure 3. UV spectra of Silymarin

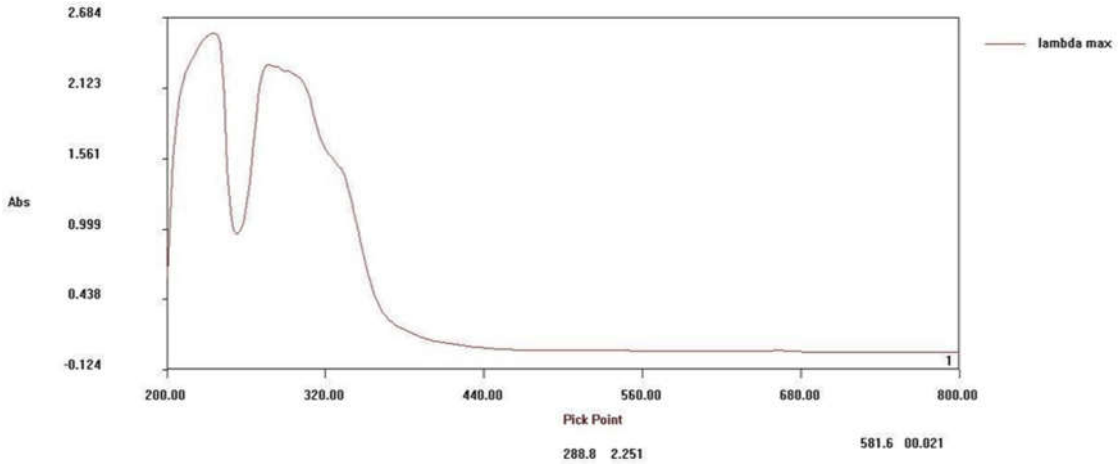

Figure 4. UV spectra of Silybin

Table I: Chemical tests for *Silybum marianum*

Sr No.	Chemical Test	Observation	Inference
1	Add a small piece of magnesium and conc. HCl to the extract.	Pink/red color	Complies
2	Add lead acetate solution to the extract.	Yellow precipitate	Complies
3	Add NaOH to the extract.	Yellow color disappears on acidification	Complies

Method Validation Summary

1. Linearity

- Range: 10–50 µg/mL
- Silymarin: y = 2E-05x + 0.0013, R^2 = 0.9877
- Silybin: y = 2E-05x + 0.0028, R^2 = 0.9752
- Shows excellent linearity.

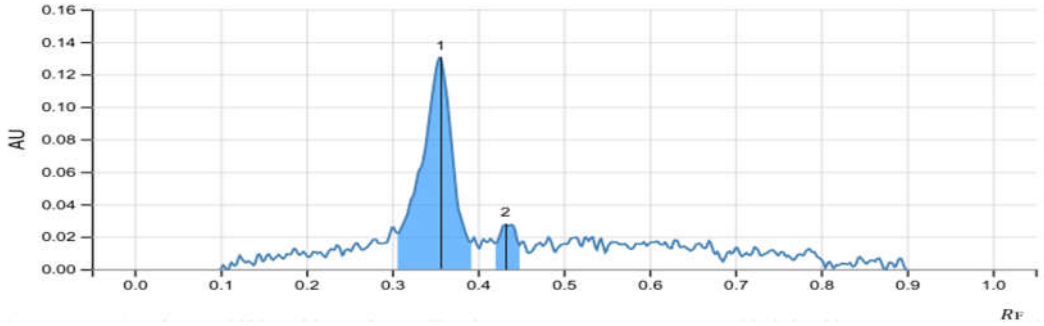

Figure 5. linearity graph

2. Precision

- Repeatability (%RSD ≤ 2%) and Interday precision (%RSD ≤ 2%): Confirmed high precision for both Silymarin and Silybin.

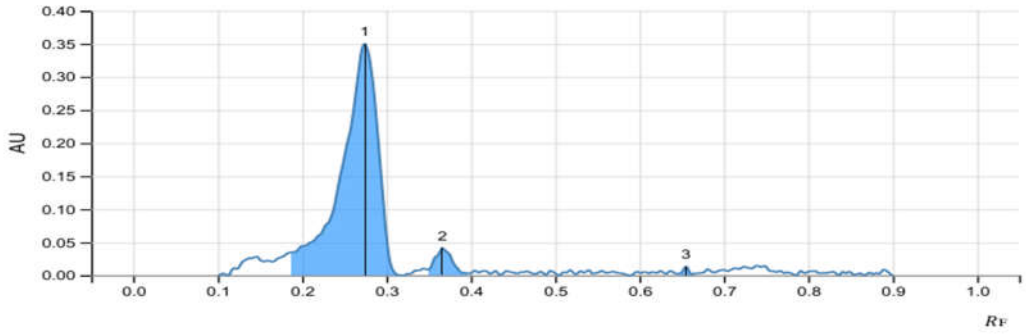

Figure 6. precision graph

3. Accuracy

- % Recovery:
  - Silymarin = 91.50%
  - Silybin = 91.37%
- Within acceptable limits, confirms accuracy.

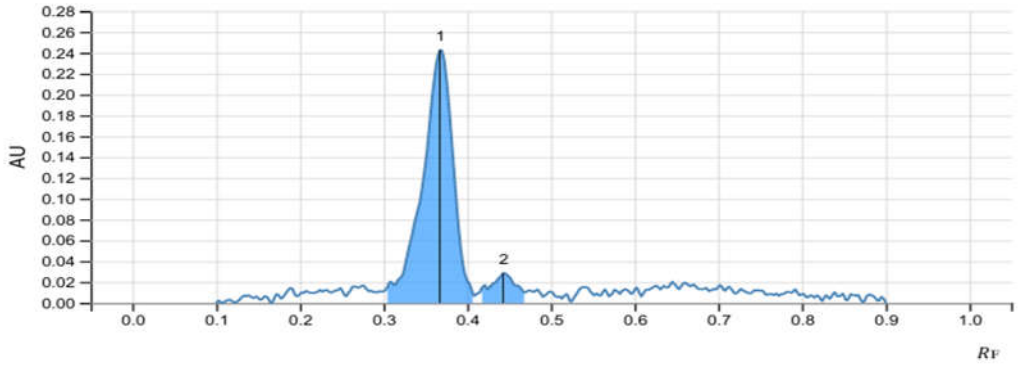

Figure 7. accuracy graph

4. Specificity

- Peaks at Rf 0.30 (Silymarin) and Rf 0.23 (Silybin) observed in standard and extract.
- No peaks for mobile phase/diluent ⇒ Method is specific.

5. Robustness

- Minor changes in saturation time showed %RSD < 0.5%, proving the method is robust.

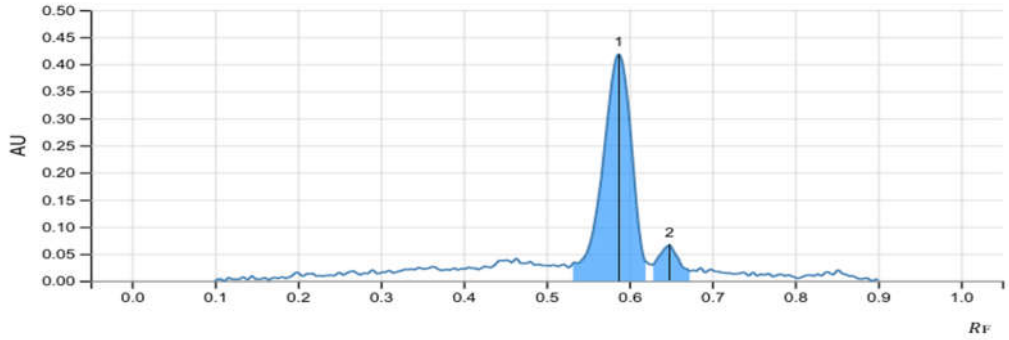

Figure 8. robustness graph

6. LOD & LOQ

(Using formula LOD = 3.3σ/S; LOQ = 10σ/S)

- Values calculated based on slope and SD of intercept (σ).
- Indicates method is sensitive.

4. Conclusion

A new HPTLC method was developed and validated as per ICH Q2(R1) guidelines for the estimation of Silymarin and Silybin in *Silybum marianum* seed extract. The method is simple, specific, accurate, precise, robust, and suitable for routine analysis. It shows good linearity in the range of 100–600 ng/band with no interference from the mobile phase or diluent. Toluene: ethyl acetate: formic acid (8:2.5:0.5) was used as the mobile phase with a 20-minute saturation time. The method proved robust under slight variations in conditions. It is suitable for quality control in pharmaceutical industries and drug testing laboratories.

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