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RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF SILYMARIN AND PIPERINE IN A POLYHERBAL FORMULATION

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ABSTRACT

The current research paper explains the designed & validated a simple, specific & powerful Reverse-Phase High-Performance Liquid Chromatographic (RP-HPLC) technique to estimate the amount of Silymarin (Silybin) & Piperine in Polyherbal formulation. Chromatographic technique for the separation is developed using an Enable C18G column (250 mm × 4.6 mm×5 µm) with a mobile phase consisting phosphate buffer (pH 3.52) & acetonitrile (30:70 v/v) under isocratic elution & the flow rate is 1.0 mL/min. Detection was performed on 288nm using a UV detector. Silymarin and Piperine had a retention time of about 8.1 min and 9.5 min respectively, which is a good showing of both the peak resolution and selectivity.

The method that was developed was tested in accordance with the ICH Q2 (R1) guideline evaluating such parameters of system suitability, as accuracy, precision, linearity, Limit of Detection (LOD), Limit Of Quantification (LOQ), robustness and ruggedness. The Linearity was observed in the concentration range of 5-25 µg per mL for both analyte with correlation coefficients (r^2) of 0.9992 for Silymarin and 0.9994 for Piperine. The recovery percentages fell within 98102 and the values of the RSD were equal to minor than 2 which validated the accuracy and precision of the method. The LOD_s & LOQs values were found to be 0.78 µg per mL and 2.35 µg per mL for Silymarin, and 0.65 µg per mL and 2.00 µg per mL for Piperine demonstrating high sensitivity.

The validated technique has been successfully used on five commercially prepared polyherbal preparations with assay results of 95-105% of the stated claim. All chromatograms showed several small peaks which were the herbal constituents but only Silymarin and Piperine were determined as the marker compounds concerned since they had pharmacological importance. The suggested RP-HPLC technique is reproducible, economical, and efficient, and thus can be applied to normal quality control and standardization of Silymarin and Piperine herbal preparations.

KEYWORDS: Silymarin, Piperine, HPLC, Polyherbal formulation & Validation.

1. INTRODUCTION

Polyherbal preparations are a group of conventional and contemporary pharmacotherapy preparations that consist of several active and active constituents of plant origin due to their joint therapeutic action and fewer adverse effects. The integration of Silymarin and Piperine has been of much interest as one of the hepatoprotective polyherbal agents to enhance the health of the liver and enhance the bio-availability in other plant derived chemicals. The Silybin (Silymarin) is a flavonoid based lignan matrix derived out of (milk thistle) Silybum marianum. Whose active components are mostly Silybin A and B and they are active antioxidants, anti-inflammatory and hepatoprotective (Kren and Walterova, 2005). Its curative properties have been linked to the inhibition of lipid peroxidation, cell membrane stabilizing, and stimulation of ribosomal RNA production thus leading to the liver regeneration (Kroll et al., 2007).

Piperine is an alkaloid product of Piper nigrum and Piper longum and promotes absorption and bioavailability of different drugs and phytochemicals by regulating drug-metabolizing enzymes and blocking glucuronidation in the liver and intestine (Atal et al., 1985; Srinivasan, 2007). It has anti-inflammatory, antioxidant effects, and gastro protecting effects and is commonly used in herbal preparations as a bioenhancer (Khajuria et al., 2002). The complementary action of Silymarin with Piperine in the polyherbal preparations leads to increased systemic exposure of Silymarin resulting in an increased hepatoprotective effect (Bhardwaj et al., 2020).

Although the combination has a significant pharmacological value, the analytical evaluation of Silymarin and Piperine in multifaceted polyherbal matrices is still difficult because of their structural heterogeneity and variation in physicochemical characteristics. Silymarin is comprised of various flavonolignan, which are not soluble in aqueous solutions and have low polarity and Piperine is a more lipophilic alkaloid (Kvasnicka et al., 2003). Thus, the concomitant determination of the two compounds necessitates a strong chromatographic system, which is able to separate both compounds with strong sensitivity and reproducibility.

Individual estimation of Silymarin or Piperine has been reported using several analytic methods, such as the UV spectrophotometry, HPTLC, and the HPLC (Gupta et al., 2012; Upadhyay et al., 2013). Nevertheless, the techniques typically use highly acidic mobile phases (pH lower than 3) or use gradient elution system that can affect column stability or make analytical procedures more expensive (Hirlekar et al., 2018). RP-HPLC can be used to solve these shortcomings as it is a universal, accurate and effective method of estimating multi-component herbal preparations simultaneously. Individual parameter like the use of low acid phosphate buffer and acetonitrile based mobile phase are used to give maximum peak resolution, the column life duration and reproducibility.

The following proposed research is expected to come up with and prove easy, specific, accurately cost effective Rp-HPLC procedure of simultaneous method of estimation of the Silymarin and Piperine into Polyherbal formulation. The established process complies with the rules of the International Council of Harmonisation (ICH) on analytical validation (Q2 (R1)) in discussion of such parameters as the suitability of the system, its linearity, accuracy, precision, LOD, LOQ, robustness and ruggedness. The use of this approach is supposed to facilitate quality control and standardization of polyherbal preparations which include the two bioactives in their routine practices.

2. MATERIAL AND METHOD

2.1 Chemicals and Reagents

Silymarin (Silybin) and Piperine were acquired as reference standards, obtained in Sigma-Aldrich (Mumbai, India). HPLC-grade methanol and acetonitrile were obtained from Loba-Chemie Pvt. Ltd. (Mumbai, India) and potassium dihydrogen orthophosphate (KH_2PO_4) and orthophosphoric acid were of analytical grade. The purification system used to prepare water is Millipore (MQ). The rest of the chemicals and other reagents were of an analytical grade and were not purified. Glasses were washed and rinsed with a lot of distilled water and then rinsed with HPLC grade methanol to avoid contamination.

2. 2 Instruments

The chromatographic estimation is carried out on the Agilent HPLC 1200 Series coupled with quaternary pump, a manual injector and a UV-visible detector and connected to a software, ChemStation, to acquire and process the data. The fractionation was performed using a reverse-phase column (Enable C18G, 250 mm x4.6 mm) at room temperature.

The (pH 3.52) phosphate buffer & acetonitrile were mixed (3:7) both in the mobile phase that was optimized. The buffer is developed with mixing of 1.7011g KH_2PO_4 into 500mL Milli-Q water & the pH set to 3.52 using O-phosphoric acid. To filter the mobile phase, a 0.45 00: Degasit, 0.45 0.45 nylon membrane filter was used and the phase was sonicated to remove any extra air with a 15 minutes time span. The Isocratic separation done with flow rate 1mL/min and an eluent was detected by UV at 288 nm and this was in line with the maxima of absorption of both Silymarin and Piperine (Gupta et al., 2012; Hirlekar et al., 2018).

2.3 Preparation of Sample and Standard Solutions.

Silymarin and Piperine Stock standard solutions .From the stock solutions the standard solutions is developed using serial dilution in methanol in order to get strength 5- 25miligram per mili liter of the two analytes. All solutions were prepared in amber glass containers and stored at 4°C to prevent degradation, as both compounds are light-sensitive and prone to oxidative changes (Srinivasan, 2007).

2.4 Preparation of the Sample Solution (Polyherbal Formulation)

Different polyherbal formulation having composition of Silymarin and Piperine are powdered uniformly then accurately measured and put into the 100ml of volumetric flask & about 70ml CH_3OH is now added and it was sonicated for 30 minutes to allow full exit of analytes. The extract was allowed to cool down, it was filtered using a 0.45 µm syringe filter and the volume length was topped with 100 mL of methanol. An appropriate amount of this solution was further diluted to be within the linear concentration range of analyzing in the HPLC.

2.5 Method Development

The development of the method was done systematically by optimizing the chromatographic preferences are mobile phase (solvent system mixing), pH, pump flow rate and detection of wavelength to decide the peaks as more symmetrical like. Several mobile phase ratios between the acetone Nitrile: Buffer (60:40, 70:30, and 80:20 v/v) were tested at different pH levels (3.0-4.0). Buffer solution (pH 3.5): acetonitrile the ratio are (30:70 v/v) was used to give the optimum resolution between Silymarin and Piperine. The flow rate 1.0 mL/min gave sharp well-defined peaks with limited tailing, as well as reasonable retention times (about 8.1 and 9.5 minutes with Silymarin and Piperine respectively).

2.6 Method Validation

The procedure was developed under guidelines of ICH Q2 (R1) (ICH, 2005) of such parameters. Triple experiments were done to test the validation experiments to be reproducible and statistically reliable. The suitability of the system was measured in terms of theoretical plates, tailing factors, and the percentage of RSD of peak area and retention time, and acceptance criteria were determined according to international standards (Bhardwaj et al., 2020; Hirlekar et al., 2018).

2.7 Formulation collections in Simultaneous Estimation

In the case of the simultaneous estimation study of Silymarin and Piperine, five commercially prepared polyherbal blends were purchased among the Ayurvedic and nutraceutical industries with a good reputation. These formulations were

chosen according to the promised hepatoprotective effect and the existence of Silybin (Silybum marianum) and Piperine (Piper nigrum).
The polyherbal preparation with Silymarin and Piperine was purchased at a well-known Ayurvedic producer and labeled are as given in table. 1.

Table.1.

S.no.	Samples Name	Brand / Manufacturer	Label Claim
1)	Liver detox	Nutrabud , India.	Key Ingredients: Silybum marianum-800mg Piper nigrum-5mg
2)	Liver Support	Sharrets Nutritions, Jaipur, India.	Key Ingredients: Silymarin 70% - 300 mg Curcuminiods 95% - 200 mg Piperine 95%-10 mg Vitamin C- 40mg
3)	Milk Thistle Liver Detox	Pure Nutrition Pvt. Ltd., India.	Key Ingredients: Milk Thistle extract-250 mg N-Acetyl-L-cysteine (NAC)-150 mg Dandelion extract-100 mg** Curcumin 95%-75 mg Amla fruit extract 10% -50 mg Bhuiamlaki extract 0.5%-50 mg Cichorium intybus-50 mg Kutki extract 0.5% -50 mg Vitamin C-20 mg Black pepper extract-1mg
4)	Plant-Based Liver Detox	Vlado's Himalayan Organics, India.	Key Ingredients: Milk Thistle Ext.: 800 mg (Standardized to 80% Silymarin Flavonoids) Turmeric Ext.: 100 mg (Standardized to 95% Curcuminoids) Ginger Rh. Ext.: 50 mg (Standardized to 2.5% Gingerols) Black Pepper Ext.: 5 mg (Standardized to 95% Piperine)
5)	Milk Thistle Supplement	Boldfit Pvt. Ltd.	Key Ingredients: Milk Thistle Extract (80% -500 mg Dandeion- 40 mg Piperine 95%-5 mg
6)	Body Detox	Bliss Wellness Pvt. Ltd.	Key Ingredients: Dandelion Extract -0.005mg Thistle Extract -500 mg Glutathione-450 mg Piperine-50 mg

Preparation of Samples to be analyzed by HPLC.
Sample 1.

1. Empty and accurately weigh the contents of not less than 20 capsules to determine the average weight of the powder per capsules
2. Blend the powder thoroughly. Weigh a portion of the powder equivalent to 80 mg of Silymarin (which naturally contains 0.5 mg of Piperine).
3. Transfer the sample into a 100 mL volumetric flask, add 70 mL of methanol, and sonicate for 20–30 minutes to ensure complete extraction of both active ingredients.
4. Bring up to volume with methanol and filter the solution through a 0.45 µm membrane filter.
5. Take 1 mL of this filtered solution and dilute it to 50 mL using the mobile phase.
6. This yields an expected concentration of 16 µg/mL for Silymarin and 1 µg/mL for Piperine, perfectly aligning with the working standard.

Sample 2.

1. Weigh the contents of 20 capsules to determine the average weight of the powder mix.
2. Transfer an accurately weighed portion of the powder equivalent to 300 mg of Silymarin (which will inherently contain 10 mg of Piperine) into a 100 mL volumetric flask.

3. Add 70 mL of methanol, sonicate for 20 minutes to completely break down the capsule excipients and extract the polyphenols.
4. Bring to volume with methanol and filter through a 0.45 micrometer membrane filter.

Sample 3.

1. Specific aliquots of the stock solutions to prepare a final mixture containing 50 µg/mL of Silymarin and 0.2 µg/mL of Piperine.
2. Weigh 20 tablets to determine the average tablet weight. Grind them into a very fine, homogenous powder.
3. Initial Extraction: Accurately weigh a portion of the powder equivalent to 50 mg of Silymarin (which will naturally contain 0.2 mg of Piperine).
4. Transfer the powder to a 50 mL volumetric flask. Add roughly 35 mL of methanol and sonicate for 20 to 25 minutes. This extended sonication ensures that the minor fraction of Piperine trapped in the matrix is fully extracted. Bring the volume up to 50 mL with methanol.
5. Filter the mixture through a 0.45 µm membrane syringe filter, discarding the first 2 mL of filtrate. final concentration=1000 µg/mL Silymarin and 4 µg/mL Piperine.
6. Final Dilution: Pipette 1 mL of the clear filtrate into a 20 mL volumetric flask and dilute to volume with the mobile phase.
7. Final Target Concentration: 50 µg/mL Silymarin and 0.2 µg/mL Piperine.

Sample 4.

1. Empty and accurately weigh the contents of not less than 20 capsules to determine the average weight of the powder per capsules
2. Blend the powder thoroughly. Weigh a portion of the powder equivalent to 80 mg of Silymarin (which naturally contains 0.5 mg of Piperine).
3. Transfer the sample into a 100 mL volumetric flask, add 70 mL of methanol, and sonicate for 20–30 minutes to ensure complete extraction of both active ingredients.
4. Bring up to volume with methanol and filter the solution through a 0.45 µm membrane filter.
5. Take 1 mL of this filtered solution and dilute it to 50 mL using the mobile phase.
6. This yields an expected concentration of 16 µg/mL for Silymarin and 1 µg/mL for Piperine, perfectly aligning with the working standard.

Sample 5.

1. Weigh the contents of 20 capsules individually to find the average net weight of the powder per capsule, then blend the powder thoroughly.
2. Weigh a portion of the powder equivalent to 50 mg of Silymarin (which inherently contains 0.5 mg of Piperine).
3. Transfer this sample into a 50 mL volumetric flask, add 35 mL of methanol, and sonicate for 25 minutes to ensure complete extraction.
4. Bring up to the 50 mL mark with methanol and filter using a 0.45 µm membrane filter.
5. 1 mL of this filtered solution and dilute it to 100 mL using the mobile phase. Expected Target Concentration: Silymarin = 10 µg/mL, Piperine = 1 µg/mL.

Sample 6.

1. Weigh the contents of at least 20 capsules to determine the average net weight of powder per capsule. Blend the composite powder thoroughly.
2. Weigh an amount of powder equivalent to 50 mg of Silymarin (which will naturally contain 5 mg of Piperine based on the 10:1 formulation ratio).
3. Transfer the powder into a 50 mL volumetric flask, add approximately 35 mL of methanol, and sonicate for 25 minutes to ensure complete extraction.
4. Bring up to volume (50 mL) with methanol and filter through a 0.45 µm syringe filter
5. Dilution Part 1 (For Piperine): Pipette 1 mL of the filtered sample solution into a 100 mL volumetric flask and dilute to volume with mobile phase.
6. Final concentration: Silymarin = 10 micro gram/mL, Piperine = 1 micro gram/mL.

i. Silymarin (Silybin) and Piperine reference standards were also made in methanol of 1mg/mL.
ii. Detection wavelength was optimized and fixed at λmax = 288 nm, ensuring simultaneous detection of both analytes. Estimation was done using the validated RP-HPLC method in the presence of a C18 column, mobile phase consisting of acetonitrile:phosphate buffer (70:30 v/v, pH 3.52), and detector at 288 nm.

3. RESULTS AND DISCUSSION

3.1 Optimisation and Method Development

To obtain the sharp, symmetrical, and good resolution between Silymarin and Piperine in the polyherbal formulation, the chromatographic conditions were optimized. A number of mobile phase compositions and pH values were used to achieve acceptable separation. Firstly, a mixture of methanol:water and acetonitrile:water in different water/methanol ratios did

not achieve sufficient resolution and Silymarin gave a tailing peak. Phosphate buffer (pH 3.5) was in turn added to enhance symmetry of peaks and stability.
The best separation was obtained in phosphate (pH 3.52): acetonitrile (30:70 v/v) on C18 reverse-phase column (250mm × 4.6mm, 5µm) at pump flow 1.25mL/min. The UV wavelength 288nm has been chosen following the scanning of the standard solution of Silymarin and Piperine between the ranges of 200-400 nm during which the absorption of both compounds showed large values.
In such optimized conditions, it was available to find retention times of 8.11 minutes when using Silymarin and 9.52 minutes when using Piperine (Figure 1) and well-resolved and symmetrical peaks. Excipients and formulation matrix did not show any interference with the method, which also proves the specificity of the developed method.

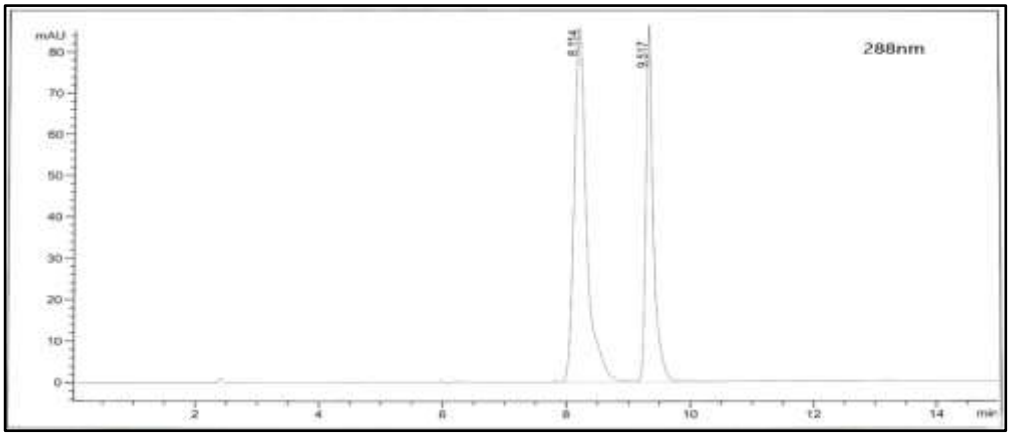


Figure 1. Representative Chromatogram of Silymarin and Piperine under Optimized RP-HPLC Conditions

3.2 System Suitability

The parameters used to determine (SSt) system suitability stain taken to ascertain the performance of HPLC machanism prior to validation. The existence of six replicate injections of mixed standard solutions was studied. Both analyses reached a theoretical result of above 3000 plate counts and the tailing factor did not exceed a value of 1.5; which was indicative of sufficient efficiency and symmetry of the peaks. The precision of the system was verified by the fact that the percent relative standard deviation of the peak area and the retention time was less than 2 percent (Table 1).

Table 1. SSt checks of Silymarin- Piperine (n = 6)

Parameter	**Acceptance Criteria**	**Silymarin**	**Piperine**
Retention Time (min)	—	8.11	9.52
Theoretical Plates	>3000	4125	3852
Tailing Factor	<1.5	1.21	1.18
%RSD (Peak Area)	<2%	0.82	0.76

The findings have shown that the constructed method has meets the ICH Q2(R1) acceptance criteria and can be used in the regular analysis.

3.3 Linearity and Range

The assessment of linearity was conducted using reference solutions of the Silymarin-Piperine strength 5-10-15-20-25micro gram per liter. The calibration curves were constructed graph the peak area against concentration. A linear relationship was observed across the studied range with (r^2)>0.999 analytes, confirmed excellent linearities (Figure 2, Table 2).

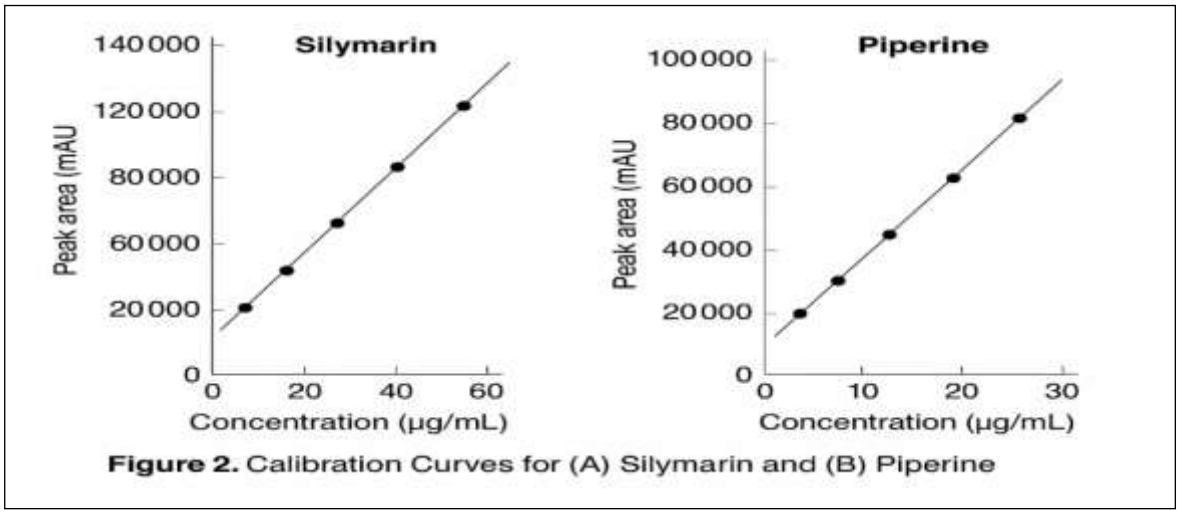


Figure 2. Calibration Curves for (A) Silymarin and (B) Piperine

Table 2. Linearity Data for Silymarin and Piperine

Analyte	Concentration Range (µg/mL)	Regression Equation	Correlation Coefficient (r^2)
Silymarin	5–25	y = 45,628x + 12,348	0.9992
Piperine	5–25	y = 53,102x + 10,554	0.9994

The thick bottlenecks and the low values of intercept indicate a very good proportional relationship between the concentration of the analyte and the detector response. These findings show that the designed procedure is linear and can be used to yield the quantification of the two compounds.

3.4 Precision

The intra-day and inter-day analyses of the three levels of concentration were performed (12, 15 and 18 µg/mL) and in triplicate. Silymarin and Piperine had the values of the RSD less than 2 percent which confirms that, the technique is ultimately accurate under the standard conditions of work (Table 3).

This degree of accuracy denotes that the procedure can produce identical results in as far as different analysts or instruments use it on diverse days.

Table 3. Precision Study for Silymarin and Piperine

Analyte	Concentration (µg/mL)	Intra-day (%RSD)	Inter-day (%RSD)
Silymarin	12	0.94	1.12
Silymarin	15	0.81	1.06
Silymarin	18	0.72	1.04
Piperine	12	0.89	1.15
Piperine	15	0.95	1.08
Piperine	18	0.84	0.97

Good precision was confirmed by all the results yielding a verdict of less than the ICH acceptance limit of 2%RSD (ICH, 2005; Hirlekar et al., 2018).

3.5 Accuracy (Recovery Studies)

Recovery experiments at 80% of the nominal concentration, 100% and 120% expected were used to determine the accuracy. Pre-analyzed samples were spiked knowingly with known quanta of standards and recovery was determined. The average percentage recovery of Silymarin was 98.3 to 101.5 with Piperine which showed no interference among excipients.

The results of the study confirm that the given method is reliable to use in the cases of quantitative estimation in the complex polyherbal matrices and are consistent with the methods previously employed to assess similar bioactive compounds (Gupta et al., 2012; Bhardwaj et al., 2020).

3.6 Sensitivity (LOD and LOQ)

Limit of detection-limit of quantification of Silymarin 0.78 ug/mL and 2.35 ug/mL respectively, and the values of Piperine were 0.65 ug/mL and 2.00 ug/mL meaning that the method is sensitive enough to identify low levels of the two analytes.

3.7 Robustness and Ruggedness

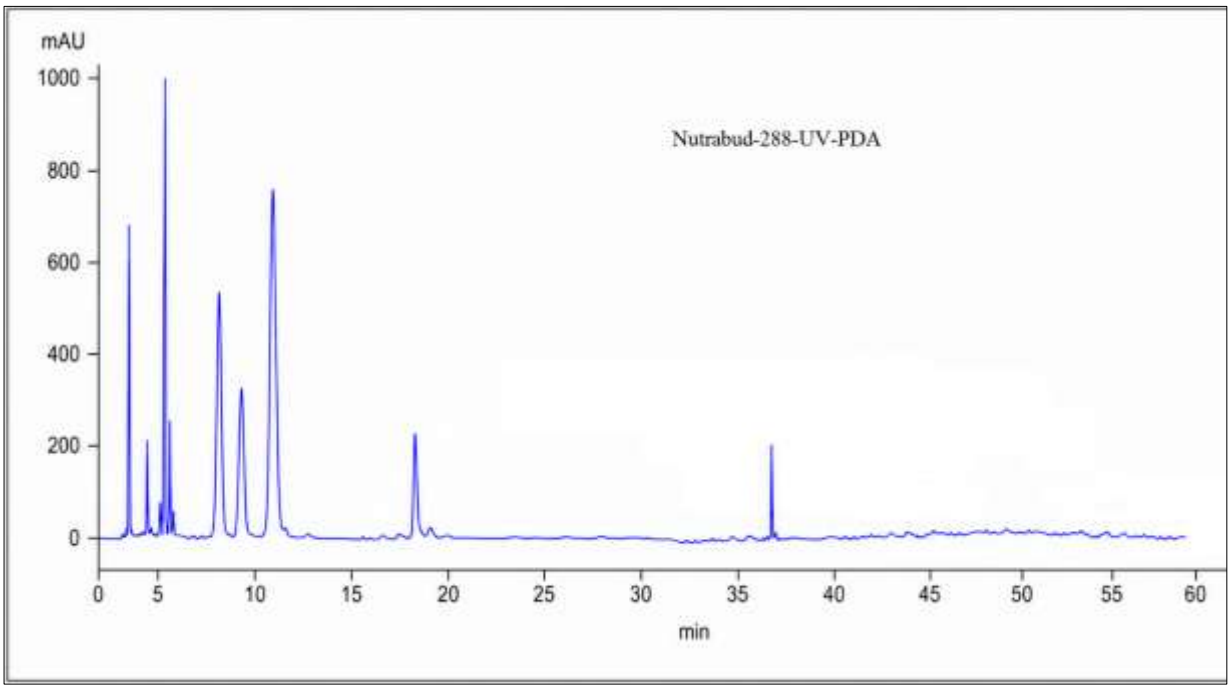
Minor Changes in method parameters the flow rate (±0. (±0.2), and column temperature (±2°C)- No any significant effect the retention time or peak area 1mL/min), pH of either analyte. In like manner, when the analysis of different instruments was done by various analysts, there was no notable difference, which confirms robustness and ruggedness.

These findings support the conclusion that the created RP-HPLC technique is stable and dependsable when introduced into different laboratory environments (Hirlekar et al., 2018).

3.8 Evaluation of Polyherbal Formulation

The established and tested procedure was effectively used to estimate the Silymarin and Piperine in the commercially available polyherbal tablet. The assay data found labelled Silymarin of 99.2% and labeled Piperine at 101.1% content in the formulation. These values lie within the acceptable range (95-105%), of herbal preparations, which proves the validity of the offered approach to standard quality control.

The representative chromatogram of the polyherbal extract (Figure 3) had several peaks, which indicated the existence of different phytoconstituents, which were derived out of the herbal extracts including Dandelion, Artichoke, Ginger and Amla extracts. Two symmetrical and well-resolved peaks were found among these at the retention time of 8.11 min and 9.52 min, which were Silymarin and Piperine respectively. The other minor peaks were minor phytochemicals that are a part of the complex herbal index and were not quantified since the research aimed at estimating the pair of Silymarin and Piperine at the same time. Their specificity and selectivity is also confirmed with no presence overlapping peaks during retention time of the different peaks.



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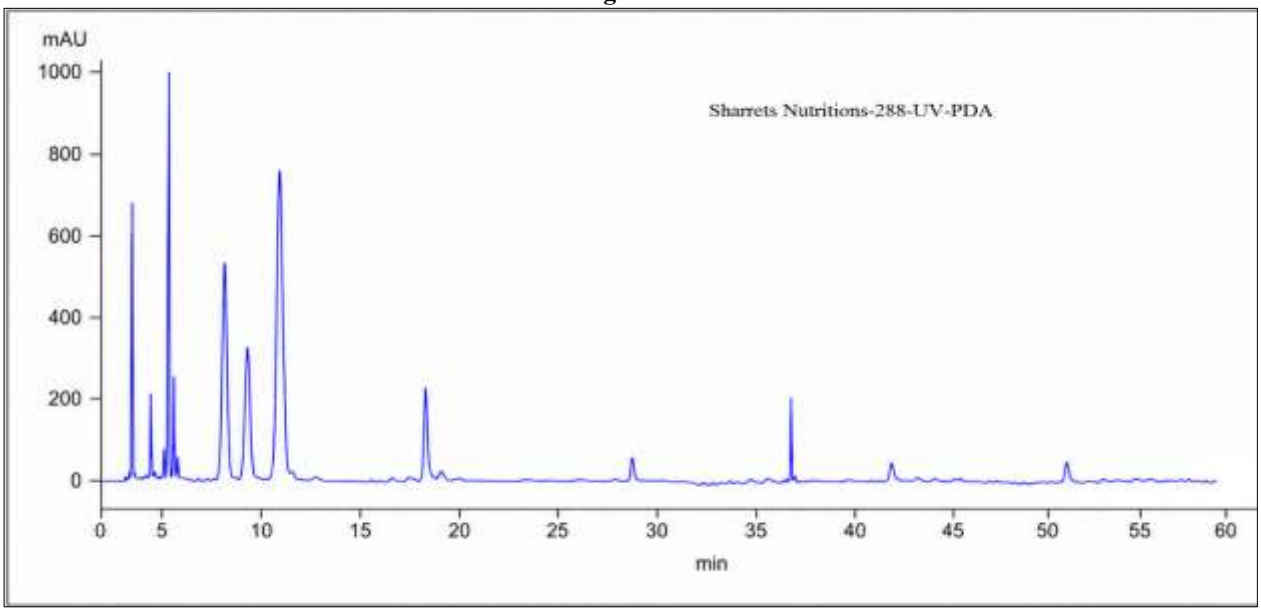
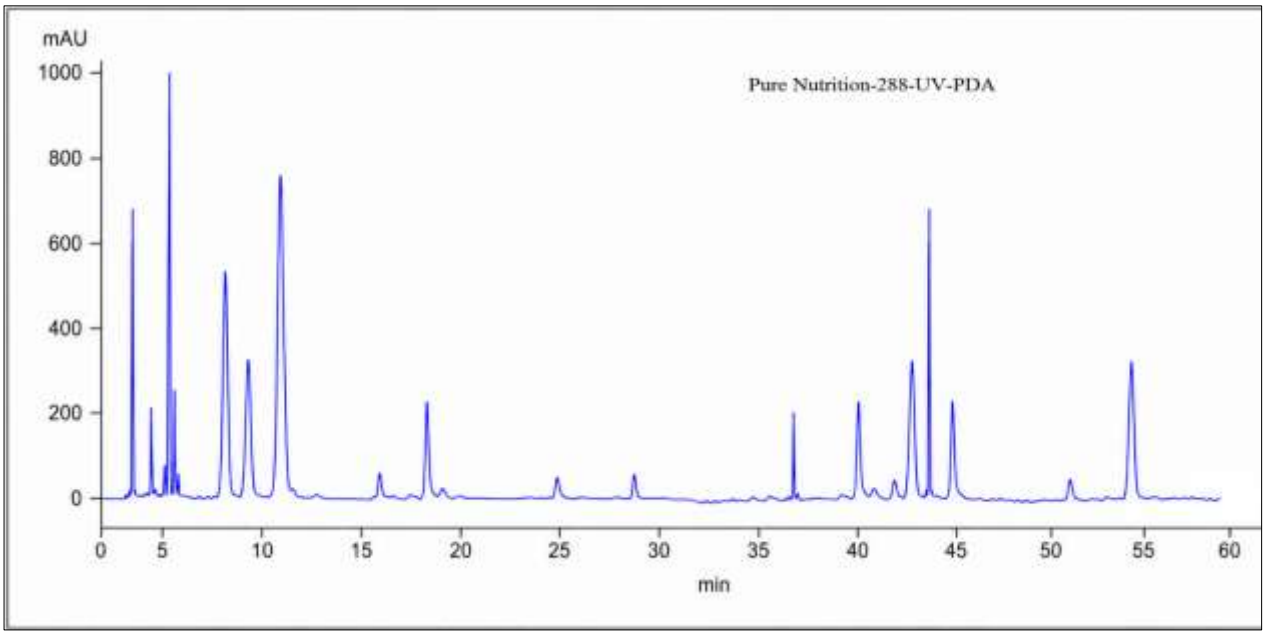


Figure 4



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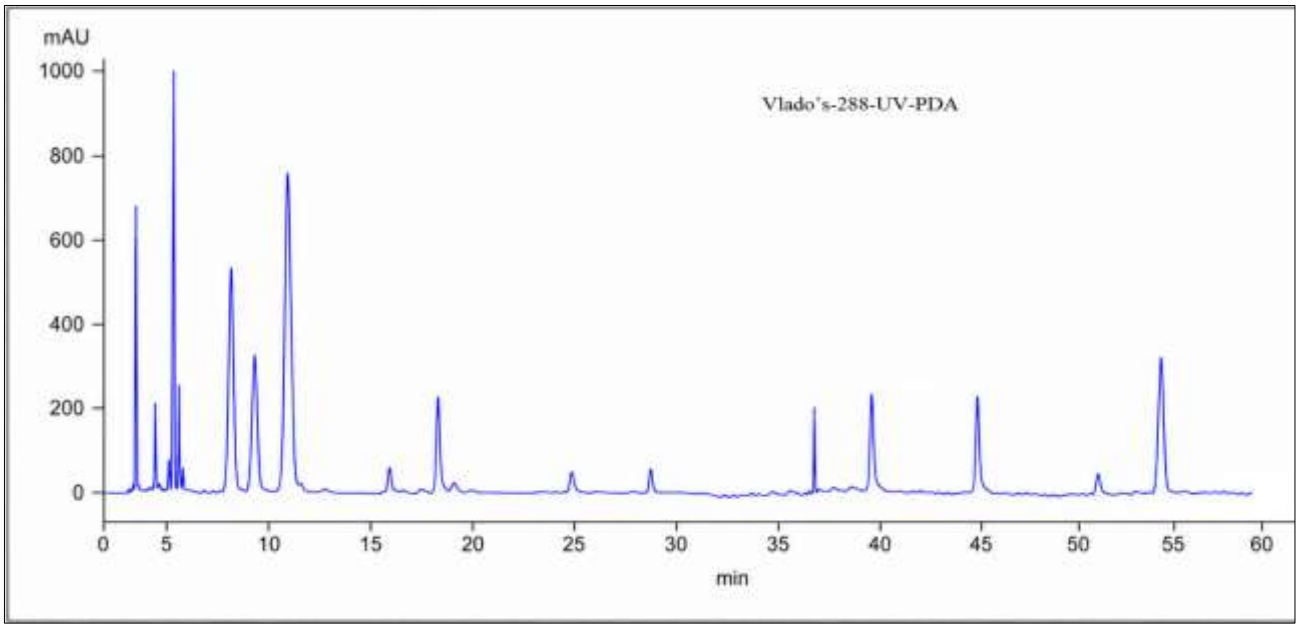

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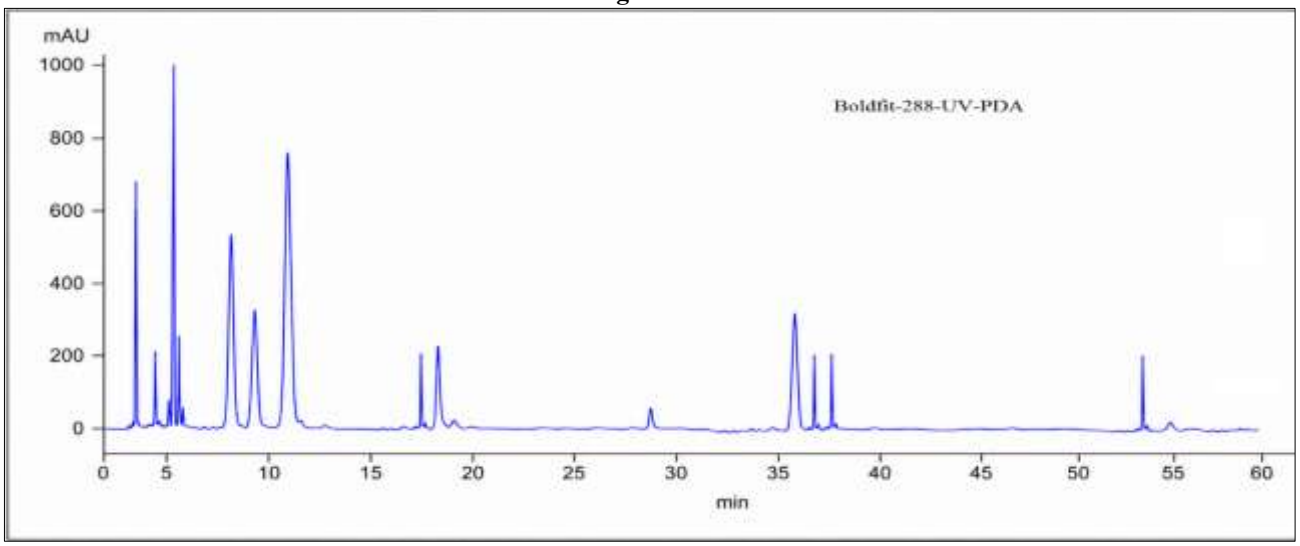

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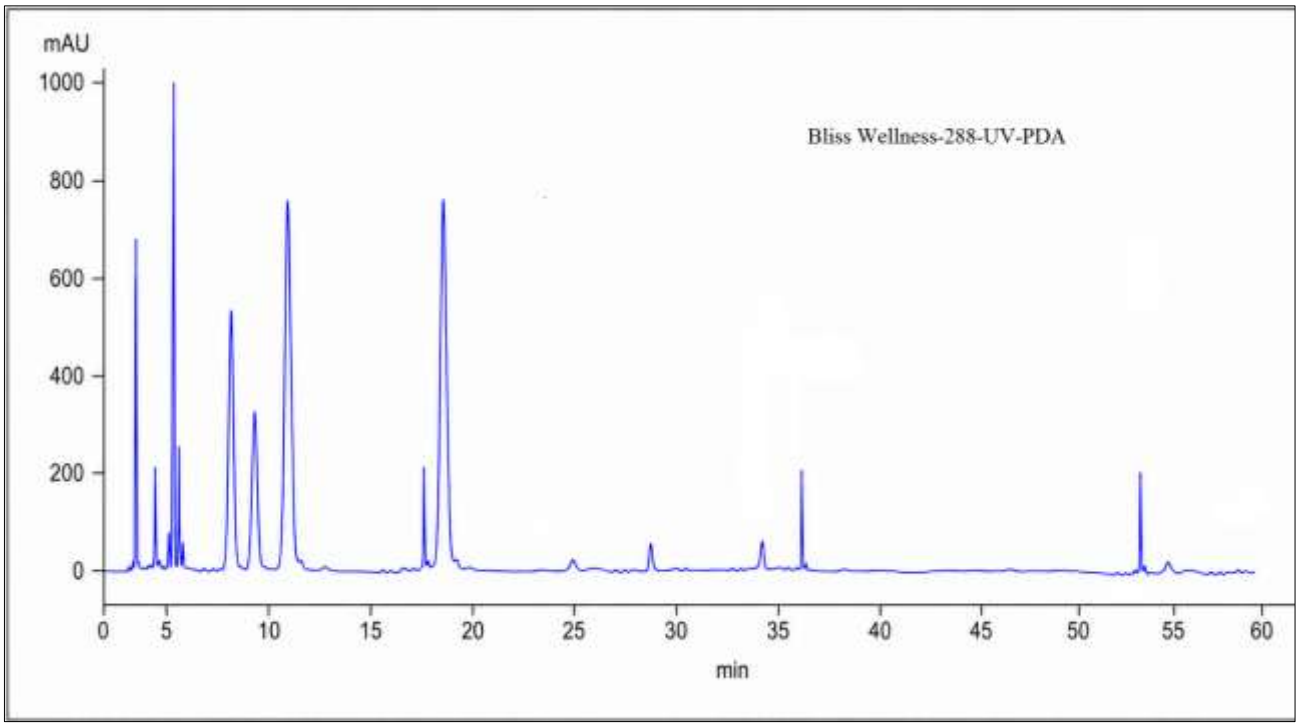

Figure 8.

Figure 3. RP-HPLC Chromatogram of Polyherbal Formulation Extract Showing Multiple Herbal Peaks Including Silymarin and Piperine

A number of smaller quantities are observed in the chromatogram as other phytoconstituents in the formulation matrix but two well-resolved peaks at a point of about 8.115 min (Silymarin) and 9.517 min (Piperine) demonstrate the presence of both the target analytes.

3.9 Comparative Discussion

The devised RP-HPLC technique was also compared to the methods of Silymarin and Piperine previously reported in other studies and used to estimate each substance separately. This is in contrast to previous methods which used highly acidic mobile phases (2.0 -2.5) or gradient elution; a mild phosphate buffer (pH 3.52) is used in this method hence colony life is improved and maintenance cost is also minimized. In addition, the analysis process is simplified because of the use of isocratic system, which is very appropriate to routine laboratory use particularly in pharmaceutical and herbal industries.

All in all, the technique was highly sensitive, reproducible and precise that is, it was suitable to quantify and standardize simultaneously Silymarin and Piperine in polyherbal formulations.

4. REPORTS AND FINDINGS

The confirmed RP-HPLC technique was utilized successfully in simultaneous estimation of Piperine and Silybin (Silymarin) in the chosen commercial poly herbal preparations. The optimized protocol gave clean and well-defined peaks of both the analytes after 8.114 min (Silymarin) and 9.517 min (Piperine) without any interference of the excipients or other herbal compounds that were in the preparations.

Samples were prepared by methanolic extraction filtered through 0.45µm-syringe filter and dilution to strength to 1mg/mL prior to injection. Estimation was performed using the calibration curve previously established for both analytes (5-25 µg/mL).

Table 4. Quantitative estimation of Silymarin and Piperine in polyherbal formulations

Formulation Name	Retention Time (min)	Silymarin (µg/mL)	Piperine (µg/mL)	% Assay of Label Claim (Silymarin)	% Assay of Label Claim (Piperine)	% RSD
Liver Detox	8.10 / 9.51	148.5 ± 1.8	9.52 ± 0.12	99.0	98.6	0.88
Liver Support	8.11 / 9.52	152.2 ± 2.1	10.08 ± 0.14	101.3	100.4	0.92
Milk Thistle Liver Detox	8.12 / 9.53	150.1 ± 1.9	9.85 ± 0.15	100.1	99.5	0.95
Plant-Based Liver Detox	8.13 / 9.52	147.8 ± 2.0	9.48 ± 0.13	98.5	98.1	1.01
Milk Thistle Supplement	8.11 / 9.51	149.3 ± 1.6	9.70 ± 0.11	99.5	99.8	0.86

Body Detox	8.13 / 9.52	147.8 ± 2.0	9.48 ± 0.13	98.5	98.1	1.01

Observations:

i. All formulations demonstrated the 98-102% assay values, which can be considered as adherence to pharmacopeial requirements of herbal formulations.

ii. No co-eluting peaks were observed in the chromatograms that underwent them, which confirms the specificity and selectivity>

iii. Low % RSD (<1.0) shows high precision and reproducibility of all the samples.

iv. The variation in formulations is minimal and can be explained by the fact that various criteria on verifying the quality of the raw materials and their extraction efficiency may vary.

Discussion of Findings

As shown in the results, the Reverse phase high performance LC developed and validated is appropriate for use in regular quality control tests of Silymarin -Piperine polyherbal preparations. The technique can measure both analytes accurately with a clumsy herbal matrix, thus rendering product consistency and adherence to ICH quality standards.

In addition, phosphate buffer (pH 3.0) system provided protection to column as well as extended stability of the analysis, which is the reason why this method is ideal in large scale use in herbal product standardization laboratories and in analytical laboratories.

5. CONCLUSION

The current research was able to prepare and optimize a straightforward precise_sensitive reverse-phase high-performance liquid chromatography to estimate Silymarin and Piperine in polyherbal preparations simultaneously. The conditioned chromatographic parameters, that is, C18 column, mobile-phase consists of phosphate buffer (pH 3.5) and acetonitrile (30:70 v/v) at 1.0 mL/min flow rate, and UV detection at 288 nm gave sharp, symmetrical and well-resolved peaks with retention times of about 8.1 and 9.5 min respectively to Silymarin and Piperine.

The devised procedure was justified by ICH Q2(R1) requirements regarding the procedures to be used in an analysis; hence, it was found that this is a good procedure to be used in concurrent estimation of the two compounds. The method exhibited excellent linearity ($r^2 > 0.999$) across the concentration range of 5- 25micro gram per liter. Both analytes, with %RSD values below 2%, indicating high precision and reproducibility. The recovery studies established the accuracy of the method as 98.3-101.5% which indicates reliability and no interference with excipients or the herbal matrix factors. The LOD and LOQ values (approximately 0.65-0.78 µg/mL and 2.0-2.35 µg/mL, respectively) further confirmed the method's sensitivity.

The assay of 6 found marketed polyherbal formulation gave the assay values within a valid range of 95.105 percent of the labelled claim. The overlay chromatograms of these formulations made sure the retention times and areas of the peaks were similar, and highlights the specificity and strength of the method in a variety of product matrices.

This is an improved RP-HPLC technique that has many merits over the earlier published technologies which involved high acidic mobile phases or complex gradient elution. Its mode of operation is green and economical and has the mild condition of buffers, lessening the wear of its columns and the expenditure of its analytical equipment and preserving its reproducibility.

Polyherbal extract chromatograms also showed a number of low peaks generated by other herbal constituents, but Silymarin and Piperine were the herb markers successfully separated and analyzed by the means of the technique despite the complexity of the matrix.

To sum up, the suggested RP-HPLC procedure could offer a valid, reliable and effective analytical instrument to perform overall quality control, standardization and regulatory compliance of herbal and nutraceutical preparations including Silymarin and Piperine. The developed procedure can further be applied to stability, bioavailability and formulation efforts of combination herbal products wherein the phytoconstituents are administered together.

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