EVALUATION AND QUANTIFICATION OF PIPERINE IN SHRINGYADI CHURNA -AN AYURVEDIC FORMULATION USING RP-HPLC

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Abstract

Objective: The project was aimed at developing an analytical method to quantify piperine in shringyadi churna- an Ayurvedic formulation by RP-HPLC technique.

Methods: The RP-HPLC technique was carried to work out Various parameters like, loss on drying, extractive values, powder properties like angle of repose, bulk density, Hausner's ratio, compressibility index etc. and analytical method and inspecting consistency of the method by performing the different validation parameters like system suitability, specificity, linearity, accuracy, precision, LOD, LOQ, Robustness and assay. The column used was Inertsil ODS Column C_{18} (4.6×250mm) 5µm of Shimadzu. The HPLC was Shimadzu make with PDA detector and model 20AD.

Results: In the course of stream lining the analytical method, we have used the mobile phase Methanol and 0.1% formic acid (0.1ml of formic acid dissolve in 100ml 0f water) in the ratio of 80:20. The drug was detected at 344 nm on UV-Visible spectrophotometer. The retention time was at 5.69 min with the run time of 8 min. The linearity range of piperine was from 0.5 µg/ml to 10µg/ml and the Regression coefficient calculated to be (R^2) 0.997. The corresponding recognition limits (LOD and LOQ) of the Avanafil was 2µg/ml and 10µg/ml respectively. Precision studies were carried out and the RSD values were found to be less than two. The degradation studies were successfully conducted.

Conclusion: The significant advantages were reduction of retention time, the lower limit in linearity being at least 10 times less and the mobile phase used was quite cheaper than the reported methods. The method is also sensitive, reproducible, quick and economical.

Key Words: Piperine, Shringyadi Churna, angle of repose, Hausner's ratio, Validation, ICH Guidelines and RP-HPLC.

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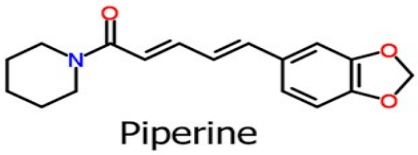
1. Introduction:

Piperine is chemically 1-[5-(1,3-Benzodioxol-5-yl)-1-oxo-2,4-pentadienyl] piperidine, Alkaloid, White or Light-yellow crystalline powder, Soluble in chloroform, alcohol, and ether, Pka-1.98. Used as Antimicrobial, Antidepressant, Antifungal, Antiepileptic, Antioxidant Anti-inflammatory, Anti mutagenic, Antiparasitic, Antipyretic, Analgesic, Antitumor, Antiproliferative agent. It is not advised to use if you have gastritis, ulcers, haemorrhoids, hypertension or gastroesophageal reflux disease. Inhibition of CYP3A4 and CYP2C9, carbamazepine and diclofenac plasma concentrations may be increased.



Piperine

Fig-1 Structure of piperine

2. MATERIALS AND METHODS

2.1 Materials:

Table No: 1 List of Chemicals / Reagents

S.NO	CHEMICALS	MANUFACTURE
1	Piperine	Sigma
2	Shringyadi churna	Lab formulation
3	HPLC Water	Merck Chemicals
4	Methanol (HPLC grade)	Merck Chemicals
5	Formic Acid	SDFCL

2.2 Instruments

Table No:2. List of Instruments

S.NO	Instrument	Make	Model. No	Batch. No
1	HPLC	Shimadzu	LC-20AD	-
2	UV -Visible spectroscopy	Shimadzu	UV 1800ENG240V, SOFT	A1163558126
3	Rota Evaporator	IKA	RV 10 D S96	-
4	UV Cabinet	Mon quartz	-	-
5	Vacuum Filtration pump	Borosil	-	-
6	Electronic balance	Shimadzu	ATY 224	D307534161
7	Digital Ultra Sonicleaner	Spincho Tech PVT.LTD	SONICA 2200 MH	2020016535

2.3: EXPERIMENTAL METHODS

The present section deals with the detailed description of various methods and techniques employed for carrying out different studies, categorized into following headings.

2.3.1 Preparation of Shringyadi churna

Shringyadi churna consists of 3 ingredients. All the ingredients are pharmacopeial quality. All ingredients are properly cleaned and powdered individually. All the powders are passed through the sieve no.80. Each ingredient was weighed separately and mixed thoroughly.

Table.No.3: Composition of Shringyadi churna

S.NO	DRUG	BOTANICAL NAME	PART USED	QUANTITY
1	Ativisa	Aconitum heterophyllum	Root	1 part
2	KarkatakaShringi	Pistacia integerrima	Galls	1 part
3	Pippali	Piper longum	Fruit	1 part

2.3.2. Proximate Analysis

Proximate analysis of the Shringyadi churna was carried out using reported methods by subjecting the formulation to various determinations

a) Determination of Moisture content:

About 5gm of the Shringyadi churna was weighed into a previously dried and tared flat weighing bottle and dried in an oven at 105°C. Continue heating & weighing after 30min intervals until constant weight has been reached, so that successive weightings should not differ by 0. 01g.Calculate the weight loss by subtracting the final weight to the initial weight & calculate the percentage and report it (WHO, 1998).

b) Determination of Ash value

Place about 5gm of ground air dried material, accurately weighed in silica crucible. Spread the materials as an even layer. Ignite the material in a muffle furnace by gradually increasing the heat for 450 degrees temperature until the material becomes white/nearly white indicating the absence of carbon. Remove the crucible from muffle furnace. Cool to the room temperature then weigh it. Calculate the ash value and report in terms of percentage. (WHO 1998).

c) Determination of Alcohol soluble extractive:
This method determines the number of active constituents extracted with solvents from a given amount of the powder material.
Take 5gm of Shringyadi churna was macerated with 100 ml of 95% ethanol in a glass stoppered conical flask for 24 h shaking frequently during first 6 h and the allowing to stand for 18 h. The solution was filtered rapidly taking care not to lose any solvent and evaporated 25 ml of the solvent in a tarred flat-bottomed dish on water bath. The residue was dried at 105°C for 6 h, cooled in a desiccator for 30 min and weighed. The content of extractable matter was calculated on the basis of air-dried material (WHO, 1998).

d) Determination of Water-soluble extractive value:
Take 5gm of Shringyadi churna was macerated with 100 ml of distilled water in a glass stoppered conical flask for 24 h shaking frequently during first 6 h and the allowing to stand for in a 18 h. The solution was filtered rapidly taking care not to lose any solvent and evaporated 25 ml of the solvent in a tarred flat-bottomed dish on water bath. The residue was dried at 105°C for 6 h, cooled in a desiccator for 30 min and weighed. The content of extractable matter was calculated on the basis of air-dried material (WHO, 1998).

Determination of powder parameters:
a) Angle of repose:
Angle of Repose has been used as indirect method of quantifying powder flow ability because of its relationship with inter particle cohesion. Angle of repose is defined as the maximum angle possible between the surface of the pile of powder & the horizontal plane.

The fixed funnel and the free-standing cone method that is secured with its tip at given height (h), above the glass paper that is placed on horizontal surface. Powder or granules were carefully poured through the funnel, until the apex of the conical pile (CVS Subrahmanyam et al, 2006)
Tan α = h/r
α = $\tan^{-1}$ (h/r)
Where h=height, r =radius

b) Bulk density determination:
Bulk density denotes the total density of the material as it exists. 5 g of the powder was taken and poured into the measuring cylinder. The initial volume is noted. Then the cylinder is dropped on to the hard wooden surface 3 times from height of 1 inch at 2 seconds intervals. The cylinder is tapped for about 500 times. This procedure is repeated for 3 times. The bulk density is calculated using the formula
Bulk density =weight of the powder/bulk volume of the powder (CVS Subrahmanyam et al, 2006)

c) Compressibility index:
Carr and Neumann developed a simple test to evaluate flow ability of a powder by comparing the poured density and tapped density of a powder and the rate at which it packed by a useful empirical guide and is given by the Carr's Compressibility Index.

Carr's Index (%) =Tapped Density – Poured Density / Tapped Density x 100.
The type of flow based on the compressibility was represented below (Table.no:3)

Table. No:4 Compressibility Index

Carr's index	Type of flow
5-15	Excellent
12-16	Good
18-21	Fair
23-25	Poor
33-38	Very poor
>40	Extremely poor

d) Hausner's ratio: It is calculated by dividing the Tapped density with poured density.
Hausner'sratio= Tapped Density / Poured Density

2.3.3 Identification of Piperine in Shringyadi churna:
One gram of lab formulation was refluxed with 20 ml of methanol for one hour. The extract was cooled, filtered and concentrated to residue using Rota evaporator.
Concentrated extract was partitioned with mixture of chloroform and water in (3:7) ratio. Chloroform layer is separated, concentrated and used for identification of piperine with the help of standard piperine by using the following parameters

- Stationary phase: Silica gel G F254
- Mobile phase : Toluene: Ethyl acetate 3.5: 1.5.
- Technique : one-way ascending.
- Sample solution: Chloroform extract of formulation
- Standard : Standard piperine in chloroform
- Developing distance : 90%
- Detection: Detection under UV 254 nm

Identification of piperine was observed from the TLC study for the test samples by comparison with the standard Rf value.

2.3.4 Method development by HPLC

2.3.4.1 Preparation of mobile phase, standard stock and sample stock solutions

Methanol and 0.1% formic acid (0.1ml of formic acid dissolve in 100ml 0f water) were used as mobile phase in the ratio of 80:20

Preparation of sample stock solution: Individually weighed 1gm of substances are subjected for reflux with 20ml of methanol (60-80º C) for 30 min. the extract was cooled and filtered then concentrated using Rota evaporator. The concentrated extracts partitioned with the mixture of chloroform & water in (3:7) ratio in a separating funnel. Chloroform layer was separated & solvent was removed to get the residue which was dissolved in 1ml of methanol. The 1ml of methanolic extract was diluted with mobile phase up to 10 ml.

Preparation of standard stock solution:

10mg of piperine was accurately weighed & transferred into 10ml volumetric flask sonicated it for dissolving and made up to mark with mobile phase(1000mg/ml), from this pipette out 1ml into 10ml volumetric flask & made up to the mark with mobile phase(100mg/ml).

2.3.4.2 Determination of Analytical wavelength:

The standard piperine (10 mg/ml) was prepared in methanol. 1ml of this solution was diluted to 10 ml using mobile phase and subjected for scanning in UV spectrophotometer between 200 to 400 nm.

2.3.5 Method Optimization:

Optimization of method was done by conducting preliminary trials under varied chromatographic conditions. The consolidated data of all the trails was shown in results.

2.3.6 Method validation

2.3.6.1 System suitability:

In system suitability, six replicates of concentrations were prepared from standard stock solution, filtered, injected. The peak areas, Retention time, tailing factor, theoretical plates of all the prepared concentrations were noted and compared with limits.

2.3.6.2Linearity:

Linearity was performed over the concentration range of 5 - 25 µg/ml by withdrawing 0.05ml to 0.25 ml from standard stock solution. The prepared dilutions were filtered and injected into HPLC. Peak responses were noted, the calibration curve was plotted across concentration on X-axis and peak area on Y- axis.

2.3.6.3 Precision:

Intra -day precision:

Intra- day precision was performed with in a day at different time intervals. The Optimized concentration 15µg/ml was injected into HPLC system and six replicates of peak responses were noted.

Inter- day precision:

Inter-day precision was performed on different days. The optimized concentration was performed from the stock solution ad six replicates were injected into HPLC on 3 days consecutively.

2.3.6.4 Accuracy:

Accuracy was carried by standard addition method. the optimized standard solution spiked to sample solution at three different concentration levels of 50%,100% and 150% respectively. The prepared sample solution was injected into HPLC system and mean recovery of the sample was calculated.

2.3.6.5 Limit of detection &Quantification:

The solutions for limit of detection & quantification was prepared based on the signal to noise ratio (S/N) obtained from standard deviation and slope, filtered and injected.

LOD= 3.3 x standard deviation/ slope

LOQ= 10 x standard deviation/ slope

2.3.6.6 Robustness:

A slight change from the optimized chromatographic conditions like flow rate ± 0.2 ml/min (1.2 ml/min), wavelength ± 2nm (343 nm) and temperature ± 5°C (30°C).

Optimized concentration 15µg/ml was prepared and injected into HPLC system.

2.3.7 Assay

From the sample stock solution, 2ml was drawn & transferred into 10ml volumetric flask and made up to the mark with mobile phase, filtered, injected into HPLC in triplicate, and the chromatograms were recorded. The peak areas were determined and the amount of Piperine was calculated. The values obtained for Assay were mentioned in results.

3. Results and Discussion:

3.1 Authentication of Raw Materials:

All the three ingredients are procured from local market. Piper longum was authenticated at Captain Srinivasa Murthy Central Ayurveda Research

Institute, Channai. Aconitum heterophyllum and Pistacia integerrima are authenticated at Raw materials herbarium and museum, National Institute of Science Communication and Policy Research, CSIR, New Delhi.

The formulation was prepared as per the procedure mentioned in Ayurvedic pharmacopoeia of India.

3.2. Proximate analysis:

Table 5: Proximate analysis of Shringadi churna

S. No.	Name of Parameters	Formulation Results
1	Loss on drying(%w/w)	5.0
2	Alcoholsoluble extractive(%w/v)	7.6
3	Water soluble extractive(%w/v)	5.5
4	Total ash (%w/w)	4.5
5	Bulk density (gm/cc)	0.42
6	Angle of repose(º)	32.45
7	Hausner ratio	1.21
8	Compressibility index	17.0

Values are indicative of Average ±SD

3.3: Identification of piperine by using TLC:

The chloroform fraction of the methanolic extract of the formulation was subjected for TLC using Toluene: Ethyl acetate (3.5: 1.5), using Silicagel GF254 as a stationary phase. The chromatogram after development was observed under UV 254 nm.

Th Rf values of standard piperine in standard and sample solutions was found to be 2.3. When observed under UV 254 nm showed fluorescence quenching.

Fig:2 Thin layer chromatogram of piperine

Table 6: The Rf values of the separated compounds in standard piperine solution and sample solutions.

Sample formulation	Standard formulation
0.4	
2.3	2.3
2.6	
3.1	

3.4 Method development by HPLC

3.4.1 Determination of Analytical wavelength:

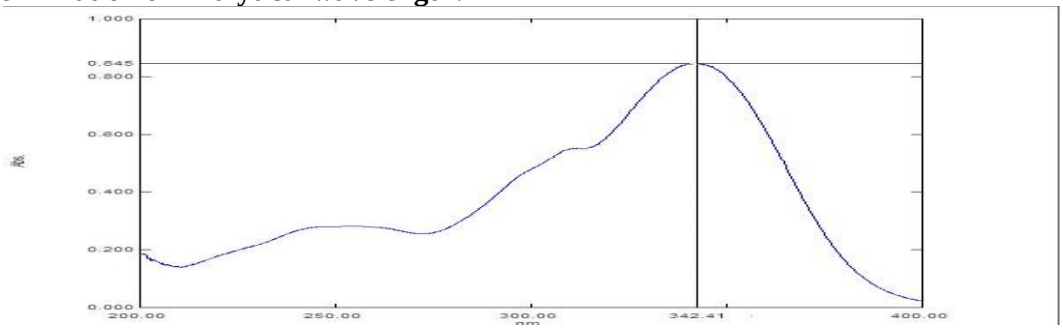


Fig:3 Determination of wavelength

The piperine solution has shown the maximum response at 343 nm in uv region.

3.4.2 Method Optimization

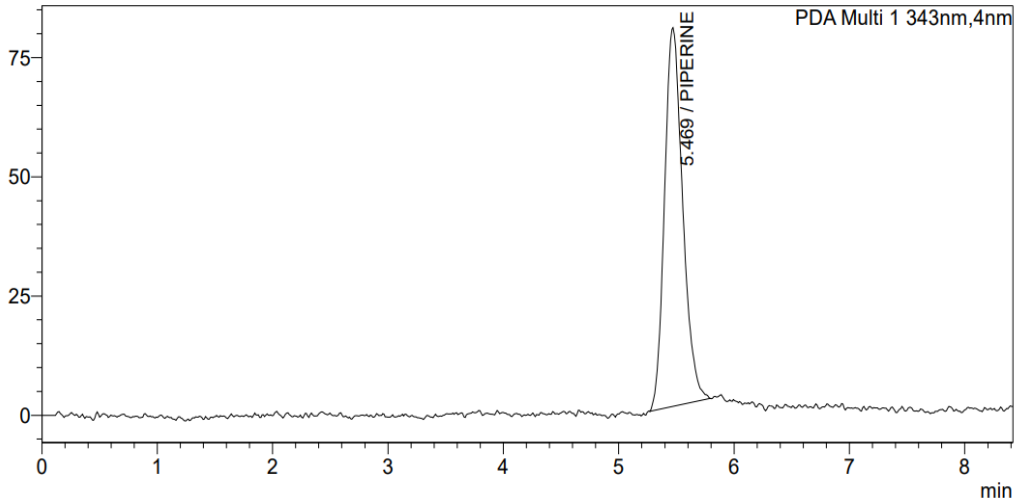


Fig:4 chromatogram of trail -3

Remarks: All the parameters were satisfied as per guidelines, and hence this trial was considered as optimized chromatograph conditions.

3.5 Analytical method validation:

Validation parameters and their acceptance criteria were mentioned in Tableno.12.

3.5.1 System Suitability:

Table. No.7 System Suitability results:

S.NO	PEAK AREA	RET.TIME	PLATE COUNT	PEAK HEIGHT	TAILINGFACTOR
1	2812532	5.547	5347	256726	1.212
2	2839167	5.542	5372	258180	1.217
3	2853518	5.532	5351	262003	1.235
4	2856858	5.551	5410	261607	1.197
5	2888840	5.554	5234	257903	1.212
6	2889362	5.535	5280	261890	1.202
Average	2856713.00	5.54	5332.33	259718.17	1.26
STDEV	29557.22	0.01	64.15	2371.45	0.01
%RSD	1.03	o.16	1.20	0.91	1.05
Theoritical plates	-	-	>2000	-	<2.0
Tailing factor	≤2.0	≤2.0	≤2.0	≤2.0	≤2.0
%RSD	<2.0	<2.0	<2.0	<2.0	<2.0

The chromatograms of system suitability were represented below**.**

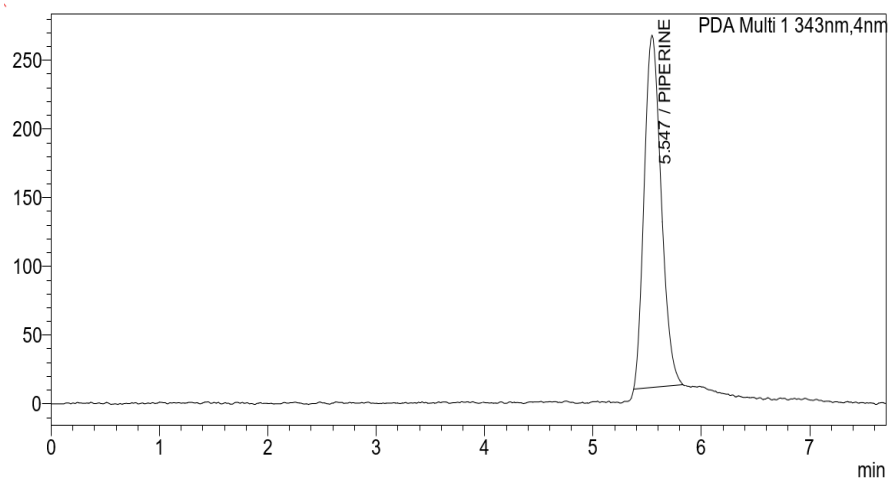


Fig-5 Chromatograms of System Suitability

Remarks: Standard deviation and % RSD were calculated and the values were found to be within the limits.

3.5.2 Linearity:

Table. No:8 Linearity results of Piperine:

S.NO	Concentration (µg/mL)	Peak area
1	5	631671
2	10	1789797
3	15	2812662
4	20	4011506
5	25	4999461

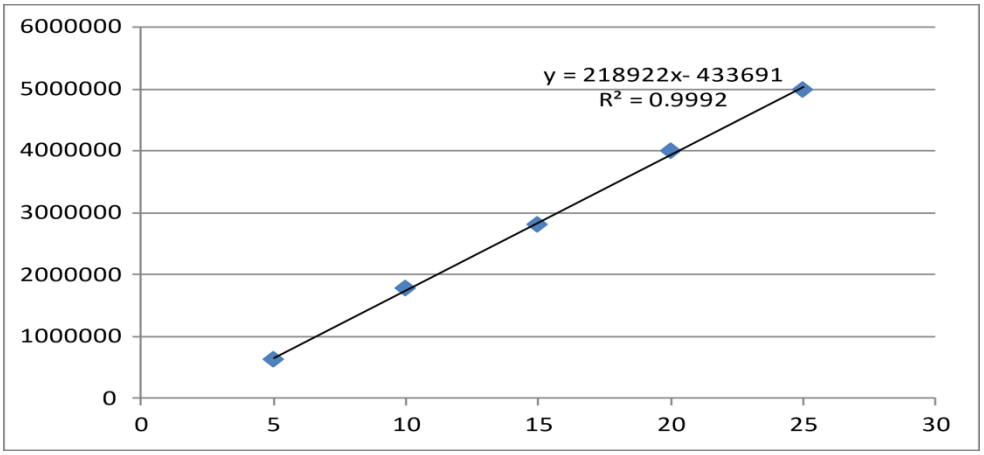


Fig.6 Calibration curve of Linearity

The Chromatogram of linearity concentrations from 5-25 µg/mL were represented below.

3.5.3 Precision:

3.5.3.1 Inter day Precision

The values obtained for inter day precision were mentioned below in Table.no.9.

Table. No.9 Results of Inter day Precision

S.NO	Peak area		
	Day1	Day 2	Day 3
1	2963647	2924629	2862364
2	2962364	2880481	2973003
3	2839167	2921559	2957019
4	2962935	2812532	2897000
5	2927431	2927431	2894925
6	2994421	2853519	2889362
Average	2941660.83	2886691.83	2912278.83
STDEV	54508.19	46804.53	42989.25
%RSD	1.85	1.62	1.48
Limits	%RSD <2.0	%RSD <2.0	%RSD <2.0

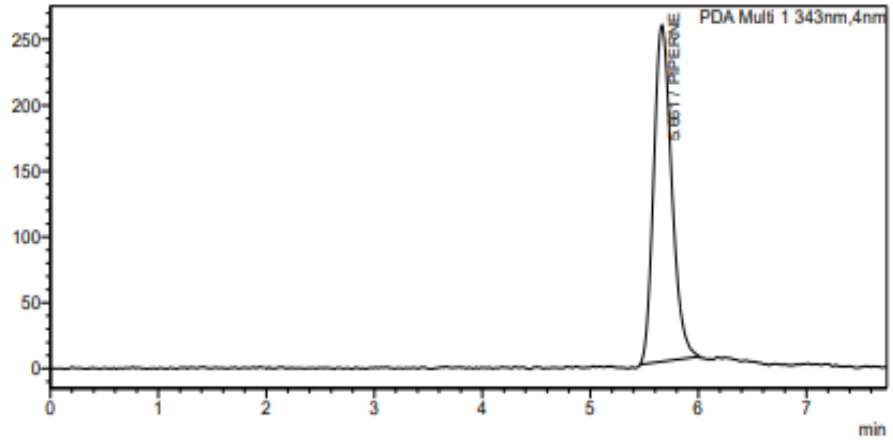


Fig:7 Chromatograms of Inter-day precision

Remarks: the % RSD values were 1.48 to 1.85 and these values are within the limits.

3.5.3.2 Intra-Day Precision

The values obtained for intraday precision were mentioned below in Table.no.10

Table.no :10 Results of Intraday precision

S.NO	Peak area		
	9:00	**1:00**	**5:00**
1	2980076	2981992	2958513
2	2888840	2856856	2880481
3	2910210	2894925	2839169
4	2812662	2889362	2984602
5	2864958	2982871	2897255
6	2853591	2980193	2949412
Average	2885056.17	2931033.50	2918238.67
STDEV	57115.16	56996.31	54983.38
%RSD	1.98	1.94	1.88
Limits	%RSD<2.0	%RSD<2.0	%RSD<2.0

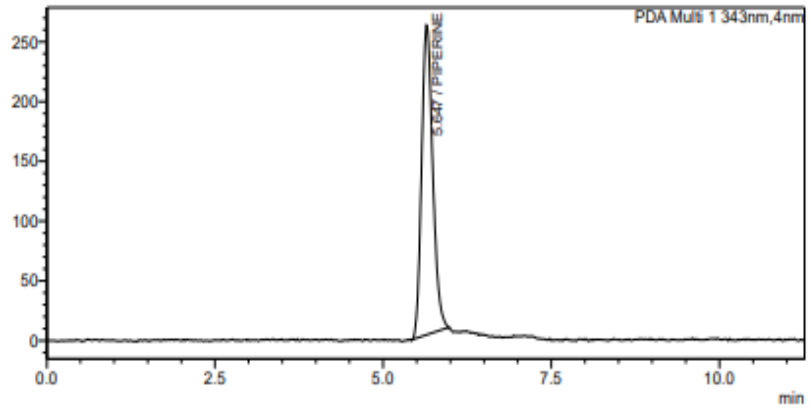


Fig-8 Chromatograms of Intra-day precision

Remarks: the % RSD values were 1.88 to 1.98 and these values are within the limits.

3.5.4 Accuracy

The accuracy was performed at 3 levels the values obtained for Accuracy were mentioned below

Table No. 11 Results of accuracy of Piperine in formation

Accuracy level	**ConcentrationTaken (µg/ml)**	**Concentration Added(µg/ml)**	**Peakarea**	**Concentration Found (µg/ml)**	**Concentration recovered (µg/ml)**	**%Recovery**	**Mean %Recovery**
50%	15	7.5	5350880	22.4609	7.4609	99.4789	101.11
	15	7.5	5318390	22.3125	7..3125	97.5001	
	15	7.5	5463700	22.9763	7.9763	106.3501	
100%	15	15	6914288	29.6023	14.6023	97.3487	98.06
	15	15	6983931	29.9204	14.9204	99.4695	
	15	15	6915103	29.6060	14.6060	97.3736	
150%	15	22.5	8542796	37.0411	22.0411	97.9603	98.38
	15	22.5	8583667	37.1380	22.1380	98.3913	
	15	22.5	8564024	37.2278	22.2278	98.7901	

3.5.5 ROBUSTNESS

Change in wavelength:

The values obtained for Robustness, when wavelength was changed ±2nm were mentioned below in Table.no.12.

Table No.12 Results of Wavelength variation

S. No.	Parameter	condition	Peak area	RT	Standard deviation	%RSD
1	Wavelength	341	2809161	5.572	15335.97	0.544%
2		343	2864924	5.546	17168.79	0.598%
3		345	2830461	5.558	12519.26	0.444%

Change in Flow rate:

The values obtained for Robustness, when flowrate was changed ±0.2 ml. was mentioned below in Table.no.19

Table No.: 13 Results of Flow rate variation

S. No.	Parameter	condition	Peak area	RT	Standard deviation	%RSD
1	Flow rate	1.0ml	3185781	6.597	55571.90	1.731%
2		1.2ml	2813532	5.547	23284.54	0.815%
3		1.4ml	2427452	4.915	11214.19	0.464%

Table No.: 14 Results of temperature variation

S. No.	Parameter	condition	Peak area	RT	Standard deviation	%RSD
1	Temperature	25ºc	2866856	5.661	37930.15	1.346%
2		30ºc	2883767	5.542	12479.26	0.443%
3		35ºc	2810210	5.481	31203.47	1.096%

Remarks: The % RSD was calculated from different conditions and the values were within the limit, so robustness was passed

3.5 .6: LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

Table. No:15: Concentrations of LOD&LOQ

LOD concentration	LOQ concentration
0.060 µg/ml	0.180 µg/ml

Remarks: The concentration of LOD and LOQ were calculated by using standard deviation and slope.

3.6 Assay of Piperine in Formulation:

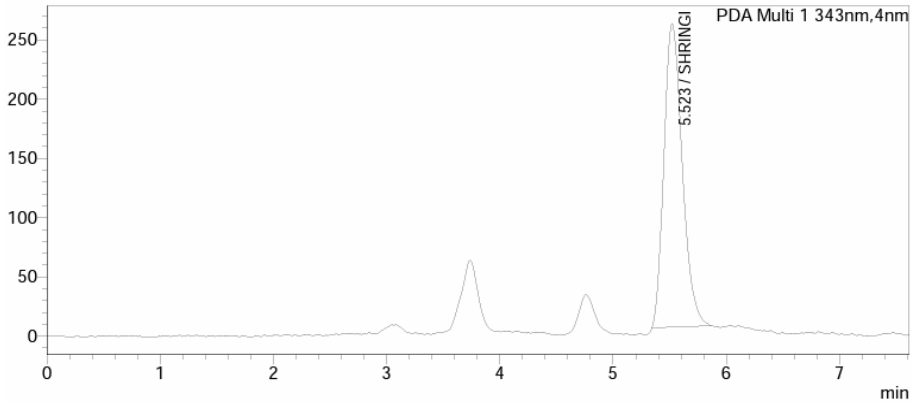


Fig:9 Chromatogram of Quantification

4. Summary:

Tableno:16-Summaryofdevelopedandvalidatedmethodof Piperine

Detection Wavelength(nm)	343nm
Solvent phase (organic phase: aqueousphase)	Methanol:0.1% Formic acid acid(80:20)
Calibrationrange(µg/ml)	5-25(µg/ml)
Regression equation(Y^*)	Y=218922x-433691
Flowrate	1.2ml/min
Retention time	5.46min
Slope(m)	218922
Correlation coefficient(R^2)	0.999
LOD(µg/ml)	0.060(µg/ml)
LOQ(µg/ml)	0.180(µg/ml)
Recovery(%) lab formulation	99.38
System suitability	<2.0
Intraday Precision	1.88 to 1.98

Intermediate precision	1.48 to 1.85
Robustness–flowrate	0.815%
Robustness-wavelength change	0.598%
Robustness-Temperature change	0.443%
Accuracy A	98%-101%

All the results obtained were evaluated

5. Conclusion:

Ayurvedic medicine Shringyadi churna has been standardized by intervention of modern specific quality control parameters in the traditional Ayurvedic preparation & individual ingredients of the formulation were authenticated and standardized as per WHO guidelines and Ayurvedic pharmacopoeia of India. Morphology as well as various pharmacogenetic aspects of the sample was studied. Shringyadi churna was prepared in the laboratory and subjected for standardization using various organoleptic, physical and chemical parameters including marker compound analysis. The results are compared with the two marketed formulations. A Rapid, simple, economical and sensitive method has been developed and validated for the quantification of Piperine in samples by RP-HPLC. The method was suitable for use in pharmaceutical industry and academic for its regular estimation in quality control and research area. On the whole, these findings off era standardization data for the formulation with regard to preparation and use of the formulation in alternative system of medicine.

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