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Marker Based Standardisation of Sarasvata Ghrita, An Ayurvedic Polyherbal Formulation using Rapid Validated High Performance Thin Layer Chromatography Method

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ABSTRACT

Sarasvata ghrita (SG) is an ayurvedic polyherbal formulation prescribed for improvement of intelligence and memory, treatment of speech delay, speaking difficulties and better digestion in children. Literature survey revealed that there is lack of standardisation aspects of SG. Standardisation of the SG was done by newly developed high performance thin layer chromatography (HPTLC) method for determination of Bebeerine (BEB), Piperine (PIP), 6- Shogaol (SHO) and β- Asarone (ASA) in commercially available marketed and in-house prepared formulations of SG. The simultaneous estimation of these markers was carried out on silica gel precoated thin layer chromatography plates with 60F_{254} as the stationary phase and eluted using Toluene: Methanol: Triethylamine (9.2:0.5:0.3) as mobile phase. Camag TLC scanner III was used for densitometric scanning. Optimum wavelength 282 nm was selected for detection and quantification. The method was validated as per ICH guidelines. The retention factor for BEB, PIP, 6-SHO and ASA were found to be 0.11, 0.39, 0.51 and 0.81 respectively. The developed HPTLC method was found to be linear with correlation coefficient 0.999 for BEB, PIP and SHO; and 0.998 for ASA. The limits of detection and limit of quantification were determined to be 10.89 and 33.04 ng/spot, 8.12 and 24.62 ng/spot, 27.38 and82.98 ng/spot, 18.13 and 54.95 ng/spot for BEB, PIP, SHO and ASA respectively. The developed HPTLC method was found to be simple, specific, accurate, precise and robust, thus can be used for routine analysis of SG for standardization with respect to selected active markers.

Keywords: Sarasvata ghrita; 6-Shogaol; Bebeerine; Piperine; β- Asarone

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INTRODUCTION

World Health Organization (WHO) stated that 70-95% of global population use traditional, alternative, complementary or non-conventional medicines for their health management [1]. Worldwide herbal medicines can enjoy a preference over the synthetic alternatives by customers on account of their benefits with minimal side effects. Herbal formulations are used as therapeutic agents for arthritis, diabetics, liver diseases, memory enhancers, cough remedies and adaptogens [2]. Herbal medicines have diverse medicinal properties however it is necessary to sustain its purity and quality in commercial market. These drugs are most often adulterated and fail to fulfil with standards prescribed for authentic drug [3]. But this field lacks the scientific exploration and most of the herbal medicines are used irrationally. Evidence-based verification of the efficacy of herbal medicinal products is still frequently lacking. There is lack of standardisation aspects of traditional medicines. Hence it is a necessary to develop suitable standardization method, which should be simple reproducible and reliable.

Standardisation of drug is nothing but approval of its identity and judgment of its purity and quality. In the beginning the crude drugs were analysed by comparing with the standard description available. Now a day's crude drugs are

analysed for its active constituents by various methods like chemical, botanical, spectroscopic and biological methods in addition to its physical constants [4].

Ayurveda, Indian medical science based on herbal remedies, is broadly admire for its global acceptance and uniqueness as it naturally treat diseases and promote health management [5]. Among the various formulations in Ayurveda, ghrita is one of the most potent Ayurveda formulation used in the treatment of chronic diseases like senile dementia [6], anxiety [7], antipsychotic and cardiac disorder [8], polycystic ovarian disease [9], learning and memory, anticonvulsant action, CNS depressant activity, anti-amnestic actions, antinociceptive action [10], anticancer activity [11]. For the ghrita preparation clarified butter (ghee) is boiled with prescribed keshaya (decoction), svarasa (fresh juice) or kalka (paste of crude plant powder in water) of drug as per Ayurveda formula [12]. Ghee is prominent ingredient which may help in extraction of active principles from plants. Sarasvata ghrita is one of the Ayurveda polyherbal preparations used as a memory enhancer. Though composition and method of preparation of sarasvata ghrita is available in ayurvedic text, there is lack of standardization aspects. Hence it is necessary to develop quality control method for standardisation of sarasvata ghrita. The formulation contains different herbs including *Zingiber officinale* Roscoe (ginger), *Terminalia chebula* Retz. (Hirda), *Piper nigrum* L.(Black pepper), *Acorus calamus* L. (Vekhand), *Cissampelos pareira* L. (Dhakti padaval), *Moringa pterygosperma* Gaertn (Drum stick) and *Piper longum* L. (Pipali) [13], and the principal constituents of which are 6-Shogaol [14], Chebulinic acid [15], Piperine [16], β-Asarone [17], Bebeerine [18], carotene [19] and Piperine [20] respectively.

The literature survey revealed that there is no analytical method available for the standardisation of SG and simultaneous analysis of active principles of SG. In recent days high performance thin layer chromatography (HPTLC) has attracted researchers due to its characteristics such as robustness and rapid analysis. Hence an attempt has been made to develop a new HPTLC method for the simultaneous determination of selected markers of plant ingredients of SG. The developed method was validated as per ICH guidelines [21,22] for the parameters accuracy, precision, specificity and robustness and successfully applied for standardisation of SG.

EXPERIMENTAL SECTION

Materials and Methods

Plant material and formulation:

All plants viz. *Zingiber officinale* Roscoe (rhizome), *Piper longum* L. (fruit), *Piper nigrum* L. (fruit), *Acorus calamus* L. (rhizome), *Moringa pterygosperma* Gaertn (root bark), *Terminalia chebula* Retz (whole plant), *Cissampelos pareira* L. (root) were collected from Mankarnika Aushadhalay, Pune, Maharashtra, India. All plants were authenticated by Botanical Survey of India (BSI), Pune, Maharashtra, India (specimen voucher no. MUS 01-07 respectively). The two marketed formulation of SG (Kottakkal Sarasvata Ghritam and Atharv's Sarasvata Ghritam) were purchased from local market (Pune, Maharashtra, India).

Chemicals and reagents:

Chemicals used were of analytical grade and purchased from Merck, USA. β-Asarone (Purity: 97.7% by HPLC), 6-shogaol (Purity: 97.8% by HPLC) and Piperine (Purity: 98.7% by HPLC) were purchased from Natural Remedies Pvt., Ltd., Banglore. Bebeerine (Purity: 98.3% by HPLC) was purchased from Baoji Herbest Bio-tech Co.,Ltd., China.

Preparation of Sarasvata ghrita (SG):

Laboratory formulation of sarasvata ghrita was prepared as per procedure mentioned in Ayurvedic Pharmacopoeia of India. All the plant parts were washed, dried, powdered and passed through sieve number 85. All the drug powders were taken in equal amount (2.4 g for 100 g) transferred to the wet grinder and 2.4 g/100 g of rock salt were added. The mixture was grinded with sufficient quantity of water to prepare a homogeneous blend (*Kalka*). Clarified butter from cow's milk (76.8 g for 100 g) was taken in stainless steel vessel, heated mildly and *kalka* was added to it. Goat milk (307 mL) and water (307 mL) were added with continuous stirring. The above mixture was heated for 3h with constant stirring; temperature was maintained between 50 - 90°C. The mixture was kept overnight. Heating of the mixture was started next day and observed the boiling mixture for subsidence of froth and constantly checked the *kalka* for formation of *vart* (*varti* ie. is prepared by rolling the *kalka* between fingers to get wick like shape). Heating was stopped when the froth subsides and *kalka* forms a *varti* which was confirmed by checking the absence of crackling sound. This was filtered while hot (about 80°C) through a muslin cloth and allowed to cool. This filtrate was analyzed by HPTLC [13].

Standardization of SG

HPTLC conditions:

TLC plates consisted of 20 × 10 cm; precoated with silica gel 60 F_{254} TLC plates (E. Merck) (0.2 mm thickness) with aluminium sheet support were used. The spotting was carried out with the help of CAMAG Linomat V Automatic Sample Spotter (Camag Muttenz, Switzerland); mounted with the 100 µL syringe (Hamilton, Switzerland). Application parameters were the length of the band was 6 mm, distance between bands was 10 mm, the application position along Y axis was 8 mm, and the start position along X axis was 15 mm, application rate of 150 nL/s. Linear ascending development was carried out in CAMAG glass twin trough chamber (20 × 10 cm) covered with stainless steel lid. The densitometer consisted of a CAMAG TLC Scanner 3 linked to winCATS Software was used. The slit dimensions were

5 × 0.45 mm and the scanning speed 20 mm/s. The optimized chamber saturation time for mobile phase was 25 min at room temperature and the plates were developed up to 80 mm using the solvent systems Toluene: Methanol: Triethylamine 9.2:0.5: 0.3 (v/v) as a mobile phase. The average development time was 20 min. After development the plate was air-dried for 15 min and optical densitometric scanning at λ_{max} = 282 nm was performed. After densitometric scanning, chromatograms were evaluated via peak area. Scanned peak areas were recorded for each sample at each concentration level.

Simultaneous Quantification of Markers

For the quantification 10 µL of sample solutions were spotted on a TLC plate. The plates were developed and scanned as mentioned above. The peak areas were recorded and the amount of all markers was calculated using the calibration curve. The analysis was carried out in triplicate.

Stock solution:

The stock solutions of pure compound ie. Bebeerine (BEB), Piperine (PIP), 6-Shogaol (SHO) and β-Asarone (ASA) (1000 µg/mL) were prepared by dissolving 10 mg of accurately weighed standards in small amount of methanol and diluted upto10 mL with methanol in standard volumetric flask.

For the calibration curve, standard solutions were prepared in 10 mL volumetric flask by appropriate dilutions of standard stock solution.

Sample preparation for SG:

Sample Preparation for in-house and two marketed formulations were optimized to extract the marker compounds efficiently and also to achieve good fingerprinting. The sample solutions were prepared as given below: accurately weighed 5 g formulations were extracted with 20 mL of methanol and 20 mL of hexane by means of separating funnel. The mixture was shaken vigorously and kept it for 5 min for separating the two layers. To get the sample free from fat methanolic layer was treated with 10 mL hexane. Hexane layers were leftover. The volume was made up to 25 mL with methanol by using volumetric flask and filtered through 0.45 µm membrane syringe filter. This solution was applied on TLC plate for HPTLC analysis.

Selection of Detection Wavelength

For UV spectra solutions of all marker compounds in the concentration of 10 ppm were spotted on HPTLC plate and allowed to scan over a range 200-400 nm. After the densitometric scanning spectra obtained were overlaid and appropriate common wavelength at 282 nm was used as detection wavelength for analysis (Figure 1).

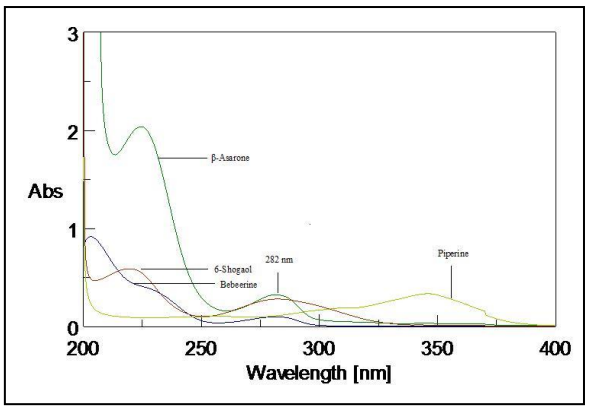

Figure 1: Overlay UV spectrum of bebeerine, piperine, 6- shogaol and β- asarone

Method Validation

The proposed method was validated according to the ICH guidelines. The method was validated for linearity, precision, accuracy, robustness, selectivity, limit of detection, limit of quantification.

Linearity:

Separate stock solutions of pure compound were used for the preparation of calibration curve. 10 µL of each standard solution was applied in triplicate on TLC plate to get final concentration 60-360 ng/spot for BEB, 40-240 ng/spot for PIP, 100-600 ng/spot for SHO and 60-360 ng/spot for ASA by using automatic sample spotter. The plate was developed, dried, and scanned as described above. After densitometric scanning the peak area was recorded for each concentration and a calibration plot was obtained by plotting average peak area against concentration (ng/spot). The slope and correlation coefficient were also determined. To confirm linearity, residual analysis was carried out.

Limit of detection and limit of quantification:

The limits of detection (LOD) and quantification (LOQ) were estimated using formula:

LOD = 3.3 X (Standard deviation of intercept/ Slope of the calibration plot)

LOQ =10X (Standard deviation of intercept/ Slope of the calibration plot)
The Standard deviation of the absorbance was calculated depends on the Standard deviation of y-intercepts of regression lines [23].

Precision:
Intraday precision was evaluated by analysis of six replicate applications of three concentrations 100, 120 and 140ng/band of freshly prepared solutions of the standards BEB, PIP, SHO and ASA (10µg/ml) and 10 µL, 20 µL and 40 µL of SG solution on the same day. Interday precision was evaluated by analysis of six replicate applications of standard solutions and SG solution of same concentrations as mentioned above on two different days. Instrumental precision was performed by scanning the single band ten times. The % RSD of peak areas was calculated.

Repeatability and reproducibility:
The repeatability of the method was done by analyzing 200 ng/spot of BEB, PIP, SHO and ASA individually on TLC plate (n = 6) and expressed as % R.S.D.

Accuracy by recovery:
Accuracy was determined by the standard addition method, where pre-analyzed SG formulation was spiked with extra 80, 100 and 120% of the standards and the mixtures were reanalyzed by the proposed method. The percentage recoveries were calculated. The experiment was conducted in triplicate.

Selectivity and specificity:
The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix for example impurities, degradation products, and matrix components. Specificity was ascertained by analyzing standard compounds and samples. The bands for BEB, PIP, SHO and ASA from sample solutions were confirmed by comparing the R_F and spectra of the bands to those of the standards. The peak purity of all the compounds (Data not shown) was analyzed by comparing the spectra at three different levels, i.e. start, middle, and end positions of the bands.

Robustness:
Robustness was studied by introducing small changes in the mobile phase composition, mobile phase volume and duration of chamber pre-saturation. Robustness study of the method was done in six replicates at a concentration level of 200ng/band for BEB, PIP, SHO and ASA. The % RSD of peak areas was calculated.

RESULTS AND DISCUSSION

Optimization of Chromatography
Different mobile phase containing various ration of ethylacetate, methanol, toluene, triethylamine, acetone, ethanol and n-hexane were tried for separation of the pure compounds. When toluene solvent was selected all peaks get eluted but there is slight difference in retention factor (R_F) hence to well resolved peaks ethyl acetate was used in different concentration. But Piperine and 6-Shogaol were not well resolved. The resolution of these peaks was obtained by addition of methanol to toluene. All marker compounds get well resolved with this solvent system but peak shape was not good hence to modify the peak shape triethylamine was added. Finally the mobile phase containing toluene: methanol: triethylamine (9.2:0.5:0.3 v/v) was selected which gave well resolved peaks. Optimum wavelength 282 nm was selected for detection and quantification. The retention factor for BEB, PIP, SHO and ASA were found to be 0.11 ± 0.02, 0.39 ± 0.02, 0.51 ± 0.02 and 0.81 ± 0.02 respectively (Figure 2).

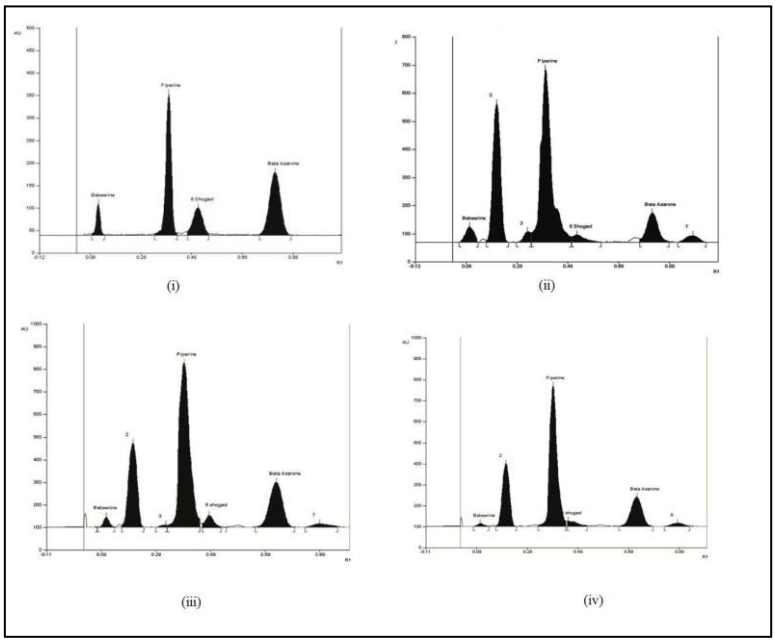

Figure 2: Densitogram obtained from (i) mixed standard solutions of bebeerine, piperine, 6- shogaol and β- asarone (ii) prepared sarasvata ghrita formulation (iii) kottakkal sarasvata ghritam (iv) atharv's sarasvata ghritam

The quantification results of the active phytoconstituents in in-house preparation and marketed formulation are given in Table 1. The assay results showed that concentration of BEB, PIP, SHO and ASA present in in-house preparation was found to be 0.48%, 0.20%, 0.73% and 0.21%; in Kottakkal Sarasvata Ghritam as 0.63%, 0.35%, 0.87% and 0.32% and in Atharv's Sarasvata Ghritam as 0.53%, 0.23%, 0.67% and 0.21% respectively.

Table 1: Content of Bebeerine, Piperine, 6- Shogaol and β- Asarone in in-house preparation and marketed formulations

Lipid	Drug content (%)			
	Bebeerine	**Piperine**	**6- Shogaol**	**β- Asarone**
Kottakkal Sarasvata Ghritam	0.63	0.35	0.87	0.32
Atharv's Sarasvata Ghritam	0.53	0.23	0.67	0.21
In-house preparation	0.48	0.2	0.73	0.21

HPTLC Method Validation

Linearity, limit of detection and limit of quantification:

The six-point calibration curves for BEB and ASA were found to be linear in the range of 60-360 ng/band and for SHO and PIP the range was found to be 100-600 ng/band and 40-240 ng/band respectively. These values revealed a good correlation coefficient for developed method (Table 2 and Figure 3). The residual analysis result of all markers revealed that Slope was significantly different from zero, and did not show trends thus confirm the linearity of the method. (Figure 4)

Table 2: Validation data from calibration curves of the standards- bebeerine, piperine, 6-shogaol and β-asarone

S No.	Parameter	Name of pure compound			
		Bebeerine	**Pipeerine**	**6-Shogaol**	**β-Asarone**
1	Linearity range (ng/band)	60-360	40-240	100-600	60-360
2	Correlation coefficient (r2)	0.999	0.999	0.999	0.998
3	Regression equation	y = 1.400x + 102.6	y =13.24x + 835.7	y = 2.75x + 120.1	y = 8.149x + 266.5
4	Limit of detection (ng/band)	10.89	8.124829	27.38375	18.134
5	Limit of quantification (ng/band)	33.04286	24.62069	82.98105	54.95153

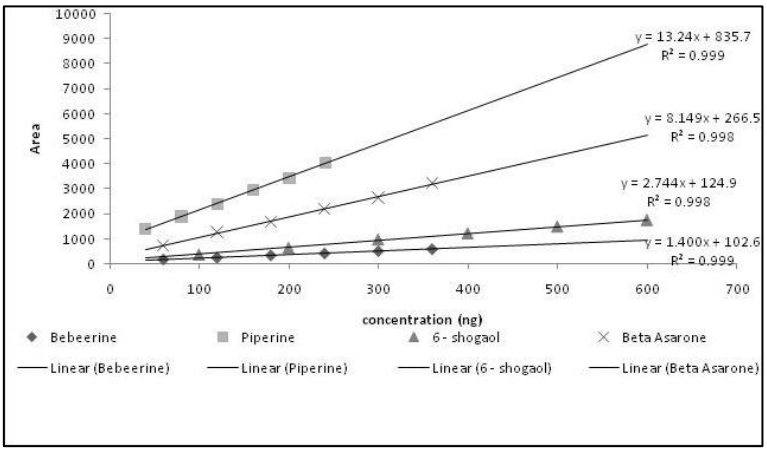

Figure 3: Linearity of bebeerine, piperine, 6- shogaol and β- asarone

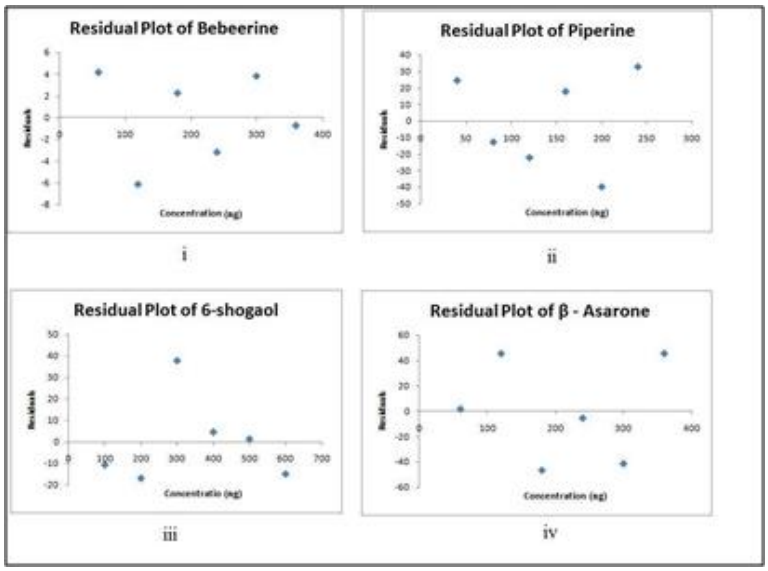

Figure 4: Concentrations versus residual plot of (i) bebeerine (ii) piperine (iii) 6- shogaol and (iv) β- asarone

Precision:

The intra-day and inter-day precision expressed as the % RSD for peak area were determined for standards BEB, PIP, SHO and ASA by repeated analysis (n = 6). Intra-day relative standard deviation of BEB, PIP, SHO and ASA were found between 1.53-1.76 %, 0.35-0.59%, 0.88-1.23% and 0.42-1.08% respectively for standard marker compound and 1.85-1.87% , 1.03-1.23%, 1.12-1.54%, 1.23-1.67% respectively for SG; while Inter-day relative standard deviation of BEB, PIP, SHO and ASA were found between 1.08-1.66%, 0.40-0.52%, 0.67-1.59% and 0.53-0.8% respectively for standard marker compound and 1.78-1.89%, 1.56-1.78%, 1.22-1.34% and 1.21-1.78% respectively for SG as shown in Table 3 and instrumental precision showed relative standard deviation of 1.07% for peak area.

Table 3: Intraday and interday precision (n=6)

Standards	Concentration (ng/band)	For standard solution		For SG solution	
		Intra-day RSD for peak area (%)	Inter-day RSD for peak area (%)	Intra-day RSD for peak area (%)	Inter-day RSD for peak area (%)
Bebeerine	100	1.76	1.08	1.87	1.78
	120	1.86	1.37	1.85	1.87
	140	1.53	1.66	1.67	1.89
Piperine	100	0.35	0.52	1.12	1.56
	120	0.59	0.4	1.23	1.78
	140	0.55	0.51	1.03	1.74
6- Shogaol	100	1.23	1.19	1.12	1.34
	120	0.88	1.59	1.54	1.32
	140	0.98	0.67	1.34	1.22
β- Asarone	100	1.08	0.6	1.67	1.34
	120	0.77	0.8	1.23	1.21
	140	0.42	0.53	1.53	1.78

Recovery:

The recovery experiments of the BEB, PIP, SHO and ASA were performed by spiking standards at known concentration in SG in triplicate. The recoveries of the standards were found to be 99.18% - 99.82%, 99.96% - 100.53%, 99.82% - 100.54% and 99.68% - 100.18% for BEB, PIP, SHO and ASA respectively as shown in Table 4.

Table 4: Percent recovery of bebeerine, piperine, 6-shogaol and β-asarone from sg formulation

Standards	Amount present in sample (ng)	Amount added (ng)	Average Recovery (%) ± S.D.*
Bebeerine	135	108	99.18 ± 0.71
	135	135	99.22 ± 1.49
	135	162	99.82 ± 1.80
Piperine	90	72	100.21 ± 0.45
	90	90	99.96 ± 1.06
	90	108	100.53 ± 1.24
6- Shogaol	145	116	99.85 ± 1.59
	145	145	100.54 ± 1.82
	145	174	99.82 ± 1.11
β- Asarone	160	128	100.18 ± 1.53
	160	160	99.68 ± 1.42
	160	192	99.90 ± 1.35

NOTE:* Values represented with average recovery ± standard deviation of the means of three independent experiments (n = 3).

Robustness:

% RSD for all the four standards viz. BEB, PIP, SHO and ASA, after changing the mobile phase composition, mobile phase volume, time from spotting to chromatography, time from chromatography to scanning, % RSD for peak area was calculated and found to be less than 2% as shown in Table 5.

Table 5: Robustness (n=6), Concentration- 200 ng/band

S No.	Parameter	% RSD			
		Bebeerine	Piperine	6- Shogaol	β- Asarone
1	Proportion of mobile phase (Toluene) ± 0.2 ml	0.8	1.08	0.62	1.25
2	Volume of mobile phase (± 2ml)	1.05	0.63	0.73	0.87
3	Time from spotting to chromatography	1.21	0.87	0.64	1.37
4	Time from chromatography to scanning	0.83	0.59	1.46	1.07

CONCLUSION

The identification and quantification of markers in SG evaluated by use of validated analytical methods. A new HPTLC method has been developed for the identification and quantification of BEB, PIP, SHO and ASA in in-house prepared and marketed formulations of SG. Fast, cost effective and satisfactory precise and accurate are the main features of this method. The method was validated as per ICH guidelines and the method is sensitive, specific, robust and repeatable. This method can be easily used for routine quality control analysis of all the four phytoconstituents for marketed formulations in Ayurvedic/Herbal industry.

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