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Application of Chemometrics for the Simultaneous Estimation of Phytosterols in Manasamitra Vatakam Using HPLC-PDA Method

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ABSTRACT

A new research method has been developed to approach multiresponse optimization for simultaneously optimizing a large number of experimental factors. LC Chromatogram was optimized using Phenomenex RP C18 column (250 x 4.6 mm; 5 µm); mobile phase was pumped at isocratic mode with a flow rate of 1.0 mL/min using methanol and acetonitrile (95: 5% v/v) at the detection max of 208 nm with the retention time of 16.3 and 18.1 min for Stigmasterol and β-Sitosterol respectively. The amount of Stigmasterol and β-Sitosterol was quantified and found to be 51.0 and 56.3 µg/mg respectively. The method was found to be linear in the range of 80-130 µg/mL with r2 value of 0.9971 and 0.9960 for Stigmasterol and β-Sitosterol respectively. LOD and LOQ were 0.0507, 0.1537 µg/mL and 0.0594, 0.1800 µg/mL for Stigmasterol and β-Sitosterol respectively. The system precision and the method precision were found to be 0.94%, 0.40% and 1.51%, 1.1% (≤ 2%) for stigmasterol and β-Sitosterol. Recovery studies in the range of 80, 100 and 120% were performed and found in the range of 95-105% indicates the accuracy of the developed method. The developed method is the first report for the simultaneous estimation of Stigmasterol and β-Sitosterol in Manasamitra Vatakam.

Keywords: Fractional factorial design; Manasamitra vatakam; RP HPLC; Stigmasterol; β-sitosterol

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INTRODUCTION

ICH Q8 (R) defines quality by design (QbD) approach as a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management [1].

Analytical method development is widely used in pharmaceutical development for divergent formulations of all categories which involves elution of active analytes and its separation with minimal resolution criteria [2]. Optimization of a single response with varying all the factors at a single approach, the chemometric analysis makes the best choice of separation [3], which helps in hasting the method development and extensively explains the chromatographic nature of the eluent. The different approaches to chemometric analysis include the path of steepest ascent, constrained optimization procedure, pareto-optimality, utility function, Derringer's desirability function. The path of steepest ascent can be employed only when all the response models are linear [4].

Medicinal plants play an important role in the development of potent therapeutic agents. The herbal drug is a chief constituent in traditional medicine and a common constituent in ayurvedic, homeopathic, naturopathic and other medicine systems [5]. Herbs are usually considered as safe since they belong to natural sources. The use of herbal drugs due to toxicity and side effects of allopathic medicines has led to rapid increase in the number of herbal drug manufacturers. The use of poly herbal formulations (PHF) has become the test of time, whereas the vast utilization of herbs has driven the hostile approach. PHF has better acceptability and compatibility than allopathic formulations. On selection of high dose, the efficacy and safety increases and the adverse effects can be minimized. Traditional medicine provides an important health care service comparatively to allopathic medicine. On the whole 25% of the drugs synthesised and prescribed are from plants and are of higher therapeutic use [6].

India is rich in ethnic diversity and well-practiced knowledge in herbal medicines. Many classical ayurvedic formulations were texted in most of the contexts like Charakasamhita, Sahasrayogam, and Susrutasamhita. The article is mainly focused on the anti-epileptic activity of the plants in Manasamitra Vatakam, the classical ayurvedic formulation texted in Sahasrayogam [7]. MMV, a classical ayurvedic polyherbal formulation officially texted in sahasrayogam, Kerala Ayurvedic Pharmacopeia used for the treatment of convulsions, stress, anxiolytic and depression disorders. It helps in providing the treatment for Generalized Anxiety Disorder (GAD) and depression on prolonging usage of the drug. MMV is also a powerful memory enhancer showing its overall therapeutic effects on the central nervous system [8]. The major therapeutic indications include schizophrenia post-traumatic stress disorder, amnesia, Alzheimer's and cardiac arrhythmia due to anxiety. Literature survey reveals that MMV has the neuroprotective and anti-oxidant properties [9]. There is no scientific method available to quantify any of the chemical constituents present in MMV. Hence an attempt was made to quantify phytosterols present in the formulation and validation as per ICH Q2b guidelines (Figure 1).

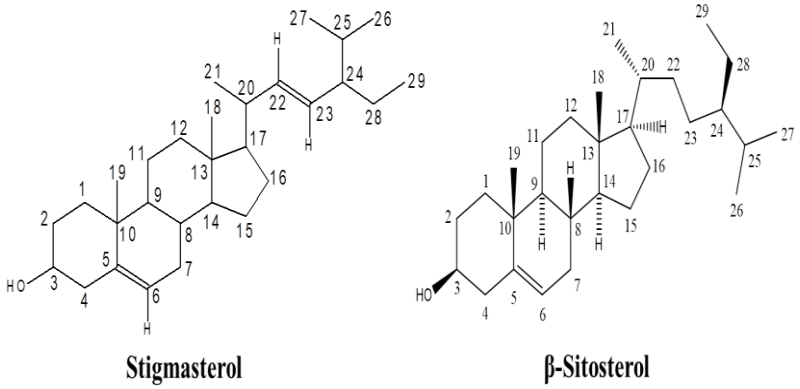

Figure 1: Chemical structures of stigmasterol and β-sitosterol

MATERIALS AND METHODS

Experimental Conditions

Instrumentation

The samples were analysed using HPLC Shimadzu (Tokyo, Japan) model which consisted of an LC20AD binary solvent delivery module, SPD M20A PDA detector, a Rheodyne injector (model 7125, USA) valve fitted with a 20 µl loop, CT0-20A Column oven. The system was controlled with the controller module equipped with CBM-20A Communications Bus Module and the data acquisition was set using the Lab solutions software (7.1 Version). Separation and quantification were done on Phenomenex C18 column (250 mm × 4.6 mm; 5 µm) as the stationary phase and at the wavelength maximum of 208 nm.

Chemometric measures, experimental design, data analysis and desirability function calculations and Perturbation plot were generated using Design expert®, 11.0 version (Trial Version). The rest of the calculations for the analysis were performed by the use of Microsoft Excel 2010 software.

Materials and reagents

Stigmasterol and β-sitosterol were purchased from M/S Natural Remedies, Bangalore, India. HPLC grade methanol and acetonitrile were used for the analyses. The mobile phase was vacuum filtered with a 0.45 µm membrane filter.

MMV was prescribed by the ayurvedic physician and was procured from the Ayurvedic pharmacy. The MMV used for the analysis further was manufactured by Kottakal.

Analytical Procedures

Preparation of standard solution

The standard stock solution was prepared with the concentration of 200 µg/mL using methanol as a diluent. To evaluate the linearity of stigmasterol and β-sitosterol, the working standard solution was prepared in the linear range of 80 – 130 µg/mL. The prepared stock and the working standard solutions were stored in the refrigerator and protected from sunlight. The working standards were freshly prepared on the day of validation. The calibration curve reported was taken against peak area vs. concentration (µg/mL) of the analyte.

Preparation of sample solution

Twenty tablets were accurately weighed and finely powdered. 1 g of triturated powder was accurately weighed and transferred to a 10 ml volumetric flask. Few ml of methanol was added and was subjected to sonication for 30 min for complete extraction and the solution was made up to the mark with methanol. The prepared sample matrix was then subjected to prior filtration with whatmann filter paper followed by the clear supernatant filtered through a 0.2 µm membrane filter and 20 µl of this solution was injected for HPLC analysis.

Chromatographic conditions

The LC chromatographic separations were performed using mobile phase conditions MeOH and ACN in the ratio of 95:5% v/v with a flow rate of 1.0 mL min-1 and mobile phase was degassed for 15 min using ultrasonicator. Phenomenex C18 column 250 mm × 4.6 mm ;(id) 5 µm (particle size) was used as RP C18 analytical column. All the determinations were done under ambient temperature conditions (25 ± 2°C) with an injection volume of 20 µL at the detection speck of 208 nm. The chromatographic conditions were maintained at an ambient temperature.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

Fractional factorial design by central composite design method was employed in this study for the optimization of chromatographic conditions and to understand the interaction of selected factors for the chromatographic behaviour of the compounds [10]. The selection of key factors for optimization was based on preliminary experiments [11,12]. The factors selected for optimization were wavelength (A),% MeOH concentration (B) and flow rate (C). The factor space of this design was expanded with in the following range of wavelength varied from 206 to 210 nm, MeOH concentration was varied from 92 to 98% v/v and flow rate from 0.8 to 1.2 ml min−1. In order to optimize the method under different conditions, the following responses of interest were selected and identified are a resolution between the stigmasterol and β-sitosterol (R1), the retention time of the stigmasterol and β-sitosterol (R2 and R3) and peak ratio of stigmasterol (R4).

All experiments were performed in randomized order to minimize the effects of uncontrolled bias of the variables that may introduce a bias on the measurements [13]. Replicates (n=6) of the central points were performed to estimate the experimental error. For an experimental design with three factors, the model including linear, quadratic and cross terms can be expressed as

$$Y=\beta0+\beta1X1+\beta2X2+\beta3X3+\beta12X1X2+\beta13X1X3+\beta23X2X3+\beta11X21+\beta22X22+\beta33X23$$

Where Y is the response to be modelled, β is the regression coefficient and X1, X2, and X3 represents factors A, B, and C respectively (Table 1).

Table 1: Central composite rotable design (CCD) coupled with fractional factorial design and responses

Standard	Space Type	Factors			Responses			
		A	B	C	R_1	R_2	R_3	R_4
11	Centre	208	95	1	2.957	18.632	16.595	1.1
7	Factorial	206	98	1.2	2.812	14.566	12.989	1
3	Factorial	206	98	0.8	3.119	21.207	18.93	1.42
1	Factorial	206	92	0.8	3.282	22.802	20.291	1.53
12	Centre	208	95	1	2.899	18.453	16.435	1
4	Factorial	210	98	0.8	2.83	21.2	18.93	0.85
5	Factorial	206	92	1.2	2.971	15.335	13.654	1.04
2	Factorial	210	92	0.8	3.257	22.784	20.293	0.97
8	Factorial	210	98	1.2	2.827	14.569	12.989	0.63
9	Centre	208	95	1	2.891	18.305	16.318	1.01
6	Factorial	210	92	1.2	2.986	15.335	13.654	0.66
10	Centre	208	95	1	2.891	18.245	16.255	0.99
14	Axial	210	95	1	2.954	18.631	16.593	0.76
18	Axial	208	95	1.2	2.833	14.793	13.197	0.93
15	Axial	208	92	1	3.126	18.315	16.314	1.04
17	Axial	208	95	0.8	3.121	21.746	19.428	1.31
13	Axial	206	95	1	2.938	18.617	16.591	1.18
19	Centre	208	95	1	2.868	18.202	16.22	1
20	Centre	208	95	1	2.855	18.18	16.206	0.99
16	Axial	208	98	1	2.978	17.258	15.4	1.01

The three factors wavelength, MeOH, and flow rate and the selected responses were then analysed using a "standard least squares" model. Calculated the coefficients for the response model and obtained P values are given in Table 2. The insignificant terms (P>0.05) were eliminated from the model through a "backward elimination process" to obtain a simple and realistic model [14]. The adjusted R2 was well within the acceptable limits of $R2 \geq 0.80$, which revealed that the experimental data show a good fit with the second-order polynomial equations [15]. For all the reduced models, P value<0.05 was obtained, implying that these models are significant. The adequate precision values were found to be in the range of 11.28-108.93, which indicates an adequate signal and therefore the model is significant for the separation process. The% CV for all the models was found to be<5% indicates the fidelity of the method [16,17].

Table 2: Reduced response model[a] (backward elimination process)

Response	Regression Model
R_1	$2.91-0.105B-0.118C-0.0996B^2$
R_2	$16.31-0.496B-3.138C+0.174BC+0.361A^2-0.373B^2$
R_3	$18.30-0.577B-3.514C+0.205BC+0.420A^2-0.417B^2$
R_4	$1.01-0.23A-0.033B-0.182C+0.047AC+0.02BC-0.0626A^2+0.087C^2$
[a]Only significant coefficients with P<0.05 are included. Factors are in coded levels	

Table 3: Statistical parameters obtained from ANOVA

Response	Adjusted R^2	Model P Value	% CV	Adequate Precision
R_1	0.8279	0.0008	2.01	11.2881
R_2	0.9984	0.0001	0.57	105.07
R_3	0.9986	0.0001	0.55	108.9307
R_4	0.99	0.0001	2.23	53.0604

The positive interaction between B and C is statistically significant (P<0.004) for the response R_3. The study reveals that changing the fraction of MeOH from low to high results in a marginal decline in the retention time of β-sitosterol either at the increasing or by decreasing the flow rate. Further at a higher level of factor C, rapid dwindles in the retention time was observed infers that the interaction term with largest absolute coefficient B and C among the fitted model was 0.205. The utilization of such interactions emphasizes the necessity to carry out active multifactor experiments for optimization of the chromatographic separation.

For a better understanding of the results, the predicted models were presented in Figure 2 as the perturbation plot [18]. For an optimization chromatographic design, this graph shows the change in response to the factor gets mobilized from the chosen reference point, was all other factors was held constant at the reference value. A steep slope or curvature of a factor indicates that the response is sensitive to that factor. Hence, the plot shows that factor A mostly affected the analysis time (R_3), followed by factor B and then factor C. Hence the perturbation plot was examined for response R_3 to understand the effect of independent factors on a specific response [19].

Derringer's Desirability Function

In the present study, to optimize the responses with different factors, Derringer's desirability function was used. The Derringer's desirability function D, is defined as the geometric mean, weighed or otherwise, of the individual desirability functions [20]. Desirability function carries the response variable to a 0 –1 scale. A response of 0 represents a completely undesirable response and 1 represents the most desirable response. The optimum conditions were chosen by total desirability as near as 1. The highest desirability value of 0.967 was achieved at wavelength of 207.386 nm, MeOH-93.314 (v/v) and flow rate of 0.948 mL min^{-1}, within a difference of<4% [21], indicating a good correlation between the experimental and the predicted responses. The desirability graph was depicted in Figure 3.

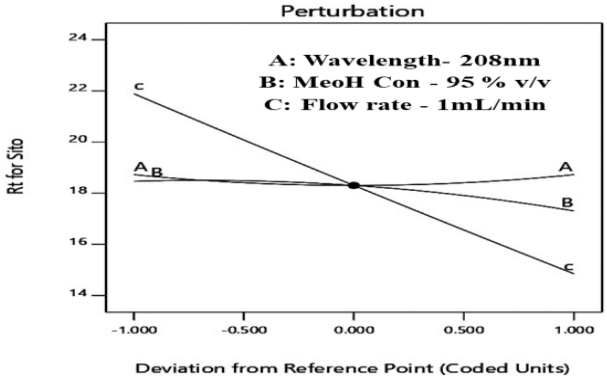

Figure 2: Perturbation plot showing the effect of the independent variables on response R_3 by keeping other variables constant

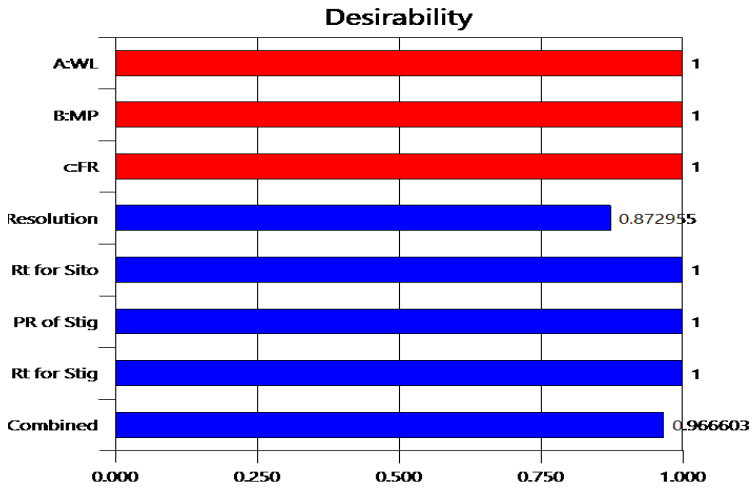

Figure 3: Derringer's Desirability functions for the factors and responses

Response Surface Plots

The optimization conditions for the factors selected and the responses were obtained by the regression equation [22]. The three-dimensional response surface figures were acquired using Design-Expert 11.0 version. The influence on the retention time of β-sitosterol by the variation of independent variables like wavelength, MeOH concentration and flow rate was displayed in Figure 4a. In the response surface figures, R_3 was by acquired using the two continuous variables whereas the other variable was kept at constant. All the factors that are responsible for the change in response R_3 were predicted at the confidence level of 95% confidence limit and represented in Figure 4a.

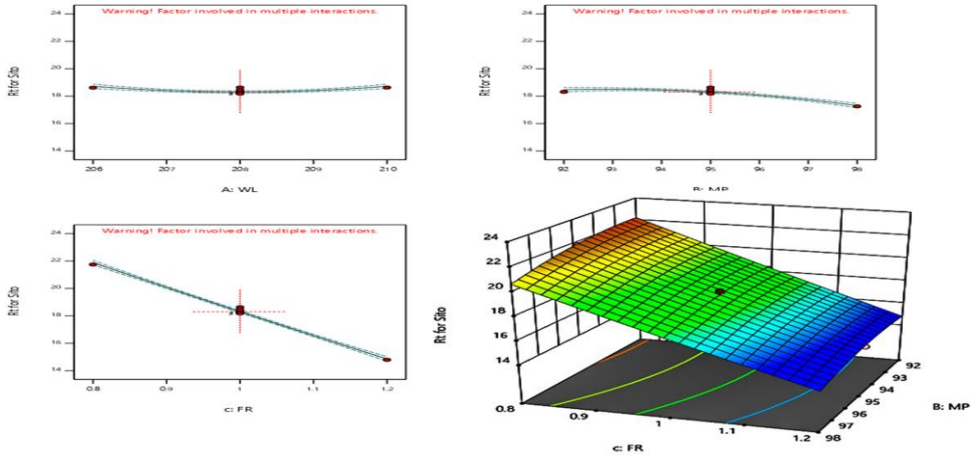

Figure 4a: Diagnostic plots and response surface plots for model adequacy

Model Adequacy Diagnostics

Model adequacy diagnostics [23] is necessary to check the applicability of the model to the existing model. Four diagnostics diagrams for model adequacy are shown in Figure 4a. The Figures 4b (A-D), shows all the diagnostic measures of the model which explains the predicted and the residual values. The spots of the predicted and actual values showed normal distribution and are close to the 45 line, attesting that the model has a good adaptation. The normal% probability plot of residuals for the normality assumption; the residual plot that approached a straight line proved that the normality assumption was appropriate. The internally studentized residuals versus predicted values were displayed in Figure 4b (A). The plots of the internally studentized residuals dispersed randomly showed that the original variance was constant for all values [24]. The internally studentized residuals versus experimental run numbers were shown in Figure 4a (B), and all the points are located within a limited range. All data indicated that the response surface model was applied to the ASP extraction and the model was significant and accurate. The

lambda value of Box-cox plot for power transforms shows 0.72, which implies the existing prediction model was fit and significant and no other Box-Cox transformation was recommended Figure 4b (C).

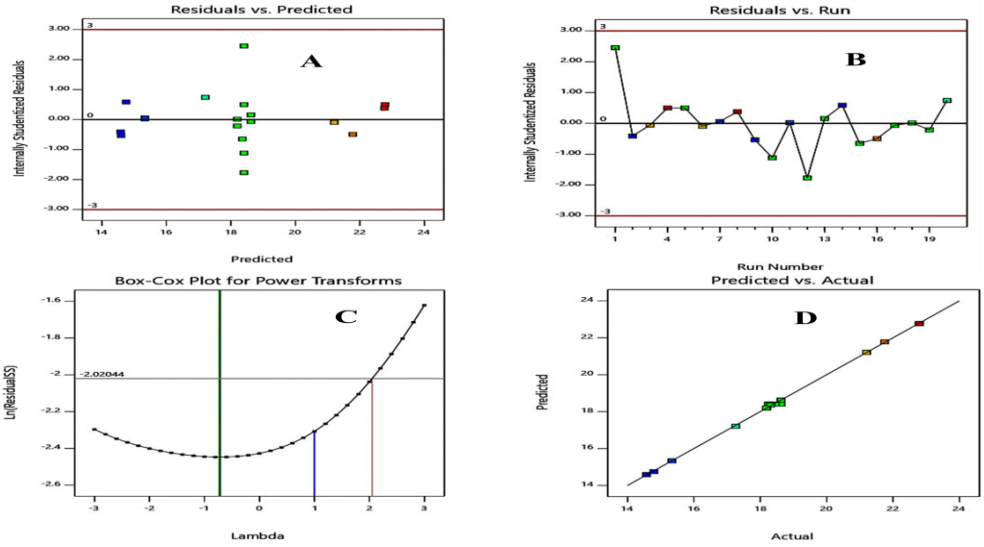

Figure 4b: Residual plots and box-cox plot for the model

Method Validation

The optimized method was validated according to ICH Q2b guidelines. The validation parameters like specificity, linearity, LOD, LOQ, accuracy, precision and robustness were performed [25].

Linearity

The range for the reliable quantification was set at the concentrations of 80-130 µg/mL for stigmasterol and β-sitosterol respectively. The range was selected based on 80-120% of the standard concentration used for accuracy and was analysed in triplicate. Peak area and concentrations were subjected to least square regression analysis to calculate regression equation. The regression coefficient (r^2) was found to be 0.9971 and 0.9966 indicating a linear response over the range used and represented in Figure 5 and depicted in Table 3.

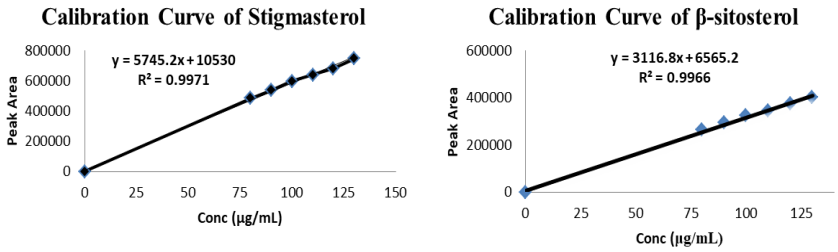

Figure 5: Calibration curve of stigmasterol and β-sitosterol

Limits of Detection And Quantitation

Calibration curves were plotted at six levels ranging from 80-130 µg/mL of the nominal analyte concentration. The residual standard deviation of the response (σ) and slope (s) of the calibration curve was used to calculate the LOD as 3.3 σ/s and LOQ as 10 σ/s. Using the above equations, the LOD and LOQ were 0.0507, 0.1537 µg/mL and 0.0594, 0.1800 µg/mL of stigmasterol and β-sitosterol respectively.

Precision

Precision was carried out in terms of repeatability. Repeatability of the standard application was assessed using six replicates at a concentration of 110 µg/mL of Stigmasterol and β-sitosterol respectively. The data was given in Tables 3-5 shown in Figure 6. The% RSD was found to be NMT 2, indicating the repeatability of the method.

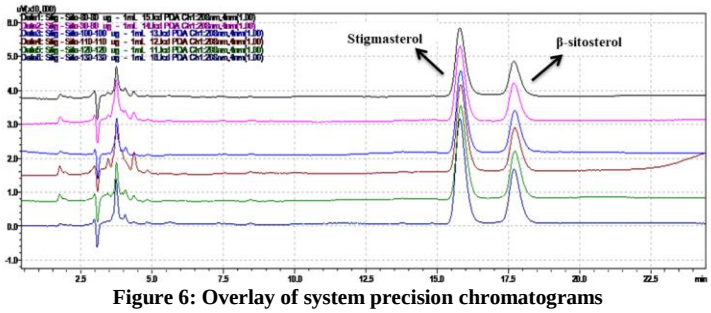

Figure 6: Overlay of system precision chromatograms

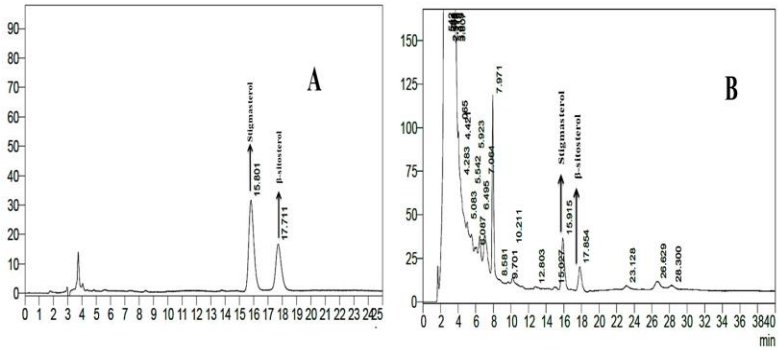

Figure 7: Chromatogram of stigmasterol and β-sitosterol in standard and MMV

Table 4: Intraday and interday precision data of stigmasterol and β-sitosterol

Stigmasterol (n=6)				β-sitosterol (n=6)			
Intraday		Interday		Intraday		Interday	
16.595	653052	16.692	635502	18.632	344085	18.457	358404
16.435	646928	16.509	649682	18.453	352550	18.759	355520
16.318	654240	16.324	642405	18.305	341937	18.264	343912
16.255	642429	16.547	652059	18.242	340983	18.658	343890
16.22	650053	16.522	640324	18.202	352299	18.361	356292
16.209	638781	16.121	648378	18.18	349914	18.438	349419
0.92	**0.94**	**1.22**	**0.98**	**0.96**	**1.51**	**1**	**1.83**

Accuracy

The accuracy of the proposed method was ascertained by performing recovery studies using external standard addition method by spiking the known quantities of the standard at 80%, 100% and 120% to the test solution of 51.0 and 56.3 µg/mg of stigmasterol and β-sitosterol respectively. These solutions were analysed in triplicate at each level of addition, the standard and the sample chromatograms were represented in Figure 7. The% RSD and the% recovery were within the acceptable limit in all the cases. It was evident from the results of accuracy study given in Table 4, that the proposed method enables very accurate quantitative estimation of stigmasterol and β-sitosterol respectively.

Table 5: Linearity, precision, accuracy and assay data of stigmasterol and β-sitosterol

Validation Data of Stigmasterol and β-sitosterol			
Linearity (n=3)	**Parameters**	**Stigmasterol**	**β-sitosterol**
80-130 µg/mL			
	Regression equation	Y=22826x-51498	Y=21669x-90599
	Regression coefficient (R^2)	0.996	0.998
	Standard Error of Slope	0.00017	0.00032
	Standard Error of Intercept	1.5652	1.7908
	Standard Error Estimate	2.5543	2.77005
	%Level of addition	Mean % Recovery (RSD)	Mean % Recovery (RSD)
Accuracy(n=3)	80	100.9 ± 0.68	100.1 ± 0.93
	100	98.3 ± 0.37	100.9 ± 0.97
	120	99.8 ± 0.64	102.4 ± 0.49
Precision(n=6)			
System Precision	Average Peak area of the standard sample (RSD)	0.94	1.51
Method Precision	Average peak area of the Assay sample (RSD)	0.4	1.09
Assay in mg (n=3)	Mean	0.051	0.056

Robustness

As part of the robustness, deliberate changes in the flow rate, mobile phase and wavelength were made to evaluate the impact on the method. Retention times were significantly changed with flow rate, mobile phase and there was no alteration of the retention time was observed with the change in wavelength. The symmetry parameters like capacity factor, theoretical plate number were significant and were within the limits and were depicted in Table 6. These results indicate that the method was robust in terms of change in flow rate, mobile phase, and wavelength.

Table 6: System suitability parameters of stigmasterol and β-sitosterol

SST Parameters	Stigmasterol (n=6)	β-sitosterol (n=6)	Limits
Resolution	NA	2.89	≥ 2
Asymmetric Factor	1.16	1.143	≤ 2
Capacity Factor	4.49	2.86	≥ 2
# Theoretical Plates	9679	10493	≥ 2000

CONCLUSION

The developed method is the first report for the simultaneous estimation of stigmasterol and β-sitosterol in Manasamitra Vatakam. In this paper, a simple, efficient, precise and accurate HPLC method was developed, optimized and validated for the simultaneous estimation of the Stigmasterol and β-sitosterol respectively. Higher sensitivity, shorter analysis time, use of organic solvents and adequate resolution of the developed method demonstrates that it can be extrapolated for the semi-preparative purpose. The proposed method was found to be linear, sensitive, selective, precise and accurate. Therefore, it could be successfully adopted for routine qualitative and quantitative analysis of divergent polyherbal formulations.

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CONFLICTS OF INTEREST

There is no conflict of interest.

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