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Application of Chemometrics for the simultaneous estimation of stigmasterol and β-sitosterol in Manasamitra Vatakam-an ayurvedic herbomineral formulation 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 C_{18} column (250 x 4.6 mm; 5 µm); mobile phase was surged at isocratic mode with a flow 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. Amount of Stigmasterol and β-Sitosterol was quantified and found to be 51.0 and 56.3 µg/mg respectively. The r^2 value of 0.9971 and 0.9960 was found in the linear range of 80-130 µg/mL for Stigmasterol and β-Sitosterol respectively. LOD and LOQ were 6.951, 21.063 µg/mL and 6.048, 18.328 µg/mL for Stigmasterol and β-Sitosterol respectively. The relative standard deviation for the system 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 were performed and found in the range of 95-105% indicates the accuracy of the developed method. The developed chromatographic method is the first report for the concurrent estimation of Stigmasterol and β-Sitosterol in Manasamitra Vatakam.

INTRODUCTION

ICH Q_8 (R) defines QbD approach as a "systematic commence for the development that begins with predefined objectives which conspicuous on the product, process understanding, and process control based on scientific attributes and quality risk management" (Pande *et al.*, 2017).

Analytical methods are widely used in pharmaceutical development for divergent formulations of all categories which involves elution of active analytes and its separation with minimal resolution criteria (Sivakumar *et al.*, 2007). Optimization of a single response with varying all the factors at a single approach, the chemometric analysis makes the best choice of separation (Mannemala *et al.*, 2016), which helps in hasting the method development and extensively explains the chromatographic nature of the eluent. The different approaches to chemometric analysis include the pareto-optimality, path of steepest ascent, Derringer's desirability function and coerce acceleration of the procedure. The alley of gradient ascent can be exerted when the obtained responses are linear (Sivakumar *et al.*, 2007).

The potent therapeutic agents are prepared from the traditional plants where herbal drugs and the chemical constituents from the plants form the major traditional claim in Ayurvedic, homeopathic, naturopathic and other medicine systems (Benzie *et al.*, 2011). Due to the extensive and unknown side effects of allopathic systems, the herbal drug manufacturers have become relatively more as they are obtained from the natural origin

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which is less toxic reactions. Polyherbal formulations (PHF) have 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, upon 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. In the global market of all the available medical formulation, 25% of the potent drugs are been synthesized and prescribed are from plants of higher therapeutic use (Sahoo *et al.*, 2010).

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. Manasamitra Vatakam (MMV), texted in Sahasrayogam (Krishna Kumar, 2008) a classical Ayurvedic polyherbal formulation available in the market as tablet dosage form is 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 (Goldberg *et al.*, 2013). 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 antioxidant properties (Thirunavukkarasu *et al.*, 2013).

Phytosterols comprise several bioactive properties like deprivation of cholesterol levels, regulating the LDL levels, inhibiting the tumor growth (Naiyer *et al.*, 2017). Literature review explores that the inclusion of phytosterols in the diet can prevent and reduce the cancerous growth. The commonly occurring phytosterols in the diet are stigmasterol, β-sitosterol, and campesterol (Scholz *et al.*, 2015). In the extent to the analytical applications, few chromatographic methods were available for the simultaneous estimation of phytosterols in various herbal formulations (Sandhiya *et al.*, 2015; Nair *et al.*, 2006; Careri *et al.*, 2001), HPLTC method validation of stigmasterol in the leaf and stem of *Bryophyllum pinntum* (Anjoo *et al.*, 2015). 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.

MATERIALS AND METHODS

Experimental conditions

HPLC Instrumentation

The samples were analyzed using HPLC Shimadzu Prominence model comprising of 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 C_{18} column at the wavelength maximum of 208 nm.

Design Expert was extensively used for the Chemometric measures, factorial analysis, Perturbation plots and desirability function calculations using 11.0 version and the MS Excel 2010 was used for the data analysis.

Materials

Stigmasterol (99% w/w) and β-sitosterol, depicted in Fig. 1 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 stock solution

The standard with the concentration of 200 µg/mL using methanol as a diluent was prepared for the construction of calibration curve for stigmasterol and β-sitosterol, the working standard solution was prepared in the linear range of 80–130 µg/mL. Both the stock and 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

Accurately weighed about twenty tablets and evenly powdered, from which weighed and transferred 1 g of powder into a 10 mL standard flask. 5 ml of methanol as a diluent was added and complete extraction was exerted by sonication for about 30 min and made to the mark with the diluent. The sample matrix prepared was then subjected to prior filtration with Whatman filter paper and further filtration was done with 0.2 µm membrane filter and the filtered solution was injected with a volume of 20 µl for LC analysis.

Chromatographic parameters

The optimized chromatographic measures performed indulges the eluent composition of MeOH and ACN in the quotient of 95:5% v/v with a flow rate of 1.0 mL min^{-1} and degassed the mobile phase for 15 min using ultrasonicator. Phenomenex C_{18} column was used as stationary phase. 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

Liquid chromatographic method optimization

Central composite design (CCD) was initiated for the better optimization of the method and understanding the interactions for the factors selected to identify the chromatographic behavior of the elutes (Bezerra *et al.*, 2008). The selection of key factors for optimization was based on preliminary experiments (Wang *et al.*, 2006; Sivakumar *et al.*, 2007). The chromatographic

optimization includes the factors like wavelength (A), % MeOH concentration (B) and flow rate (C). The selected variables range was diversified in the array 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}. For the proper optimization of the method under varied conditions, "the responses which highly alter the chromatographic nature were selected and identified are a resolution between the stigmasterol and β-sitosterol (R_1), retention time of stigmasterol and β-sitosterol (R_2 & R_3) and peak ratio of stigmasterol (R_4)".

Table 1: Central composite rotatable 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	1.0	2.957	18.632	16.595	1.10
7	Factorial	206	98	1.2	2.812	14.566	12.989	1.00
3	Factorial	206	98	0.8	3.119	21.207	18.930	1.42
1	Factorial	206	92	0.8	3.282	22.802	20.291	1.53
12	Centre	208	95	1.0	2.899	18.453	16.435	1.00
4	Factorial	210	98	0.8	2.830	21.200	18.930	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	1.0	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	1.0	2.891	18.245	16.255	0.99
14	Axial	210	95	1.0	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	1.0	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	1.0	2.938	18.617	16.591	1.18
19	Centre	208	95	1.0	2.868	18.202	16.220	1.00
20	Centre	208	95	1.0	2.855	18.180	16.206	0.99
16	Axial	208	98	1.0	2.978	17.258	15.400	1.01

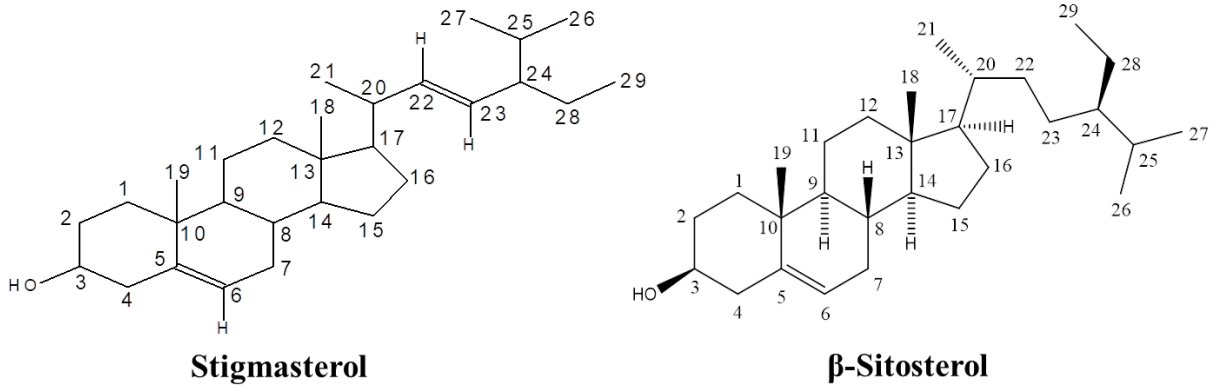


Fig. 1: Chemical structures of stigmasterol and β-sitosterol.

All the analytical factors were done in randomized order to reduce the upshots of unbridled bias in all the variables which causes bias in the measurement of the responses. (Giriraj *et al.*, 2014). The standard experimental error was estimated by selecting the replicates of central axis points. The experimental design for factorial analysis includes linear, quadratic and cross terms can be expressed as

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_{21} + \beta_{22}X_{22} + \beta_{33}X_{23},$$

where Y is the response to be modeled, β is the regression coefficient and X1, X2, and X3 represents factors A, B, and C respectively.

The factors and the selected responses were analyzed using a "standard least squares" model. Calculated coefficients for the response model and the P (< 0.05) were tabulated in Table 2 and for the coefficients of P > 0.05 were effused from the model using

"backward elimination process" to gain a sensible and rational model (Kalariya *et al.*, 2017). The adjusted $R^2 \geq 0.80$ reveals the experimental data exhibited a good fit with the second-order polynomial equations (Janardhanan *et al.*, 2016). The reduced models after backward elimination orienting the factorial analysis with a p-value < 0.05, were implied as significant. The adequate precision values were in the assortment of 11.28-108.93, indicates the adequacy of the model as eloquent. The % CV for the factorial models was found to be < 5 % indicates the fidelity of the method (Kuhnt *et al.*, 2013; Xiao *et al.*, 2015).

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.

The affirmative interaction between B and C was "eloquent and statistically significant" with the P < 0.004 for response R_3 indicates a change in MeOH concentration from nadir to high results shows a marginal decline in the elution time of β-sitosterol either at the increasing or by decreasing the flow rate. On the later stage at a higher level of factor C, rapid dwindling of the retention time was observed inferred that the foster interaction with largest absolute coefficient B and C among the fitted model was 0.205. The utilization of all the variable interactions emphasizes the necessity to carry out active multifactor experiments for optimization of the chromatographic behavior of the analytes.

The predicted models presented in Fig. 2 as the perturbation plot (Decaestecker *et al.*, 2004) explains better about the optimization of the chromatographic nature by depicting the "change in response to the particular factor which gets mobilized from the selected reference point, where the other factors were kept constant at the reference value". Higher the sensitivity of the response towards the factor, steeper is the slope. Based on the interpretations from the perturbation plots it was evident that factor C mostly affected the elution time (R_3), followed by factor B and A. Hence of all responses, R_3 was highly sensitized by the factorial design analysis and the perturbation plot was examined for the better understanding of the independent factors on a peculiar response (Hatambeygi *et al.*, 2011).

Derringer's desirability function

Derringer's desirability function was used for the further optimization of responses using different factors. The Derringer's desirability function D is defined as the geometric mean, weighed or otherwise, of the individual desirability functions (Derringer *et al.*, 1980). Desirability function scales the response of 0–1. On the scale of 0 indicates an undesirable and 1 the most desirable response. The optimum conditions for the method were chosen based on the desirability value nearer to 1. At the wavelength of 207.386 nm, MeOH -93.314 (% v/v) and flow rate of 0.948 mL min^{-1}, the maximum desirability of 0.967 was obtained within a difference of <4% (Costa *et al.*, 2011), stipulates a good correlation between the predicted and the experimental responses. The desirability graph was depicted in Fig. 3.

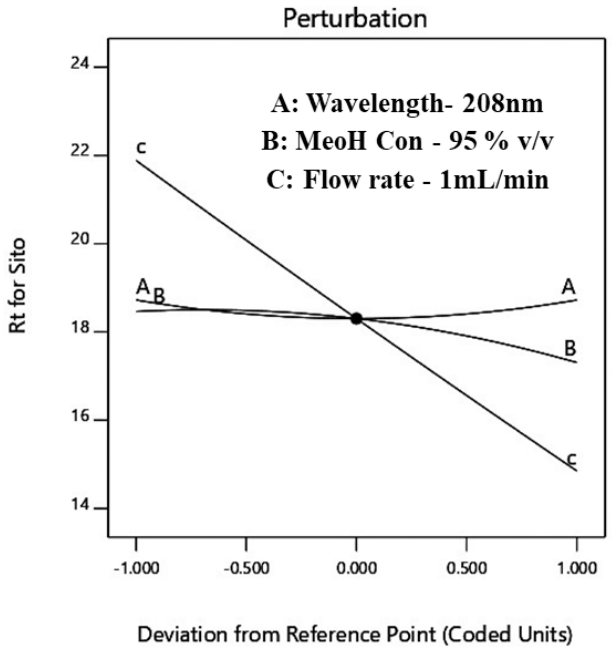


Fig. 2: Perturbation plot showing the effect of the independent variables on response R_3 by keeping other variables constant.

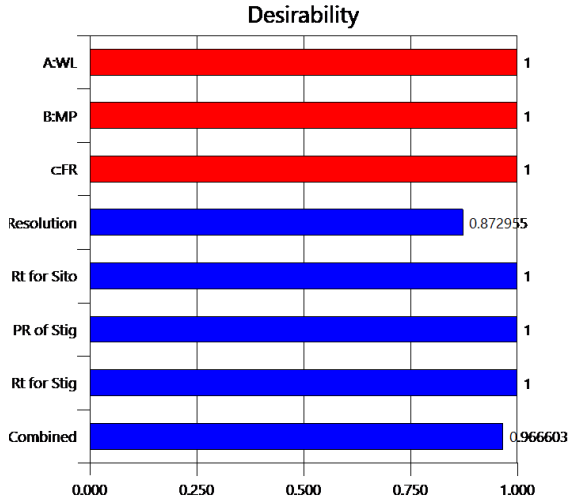
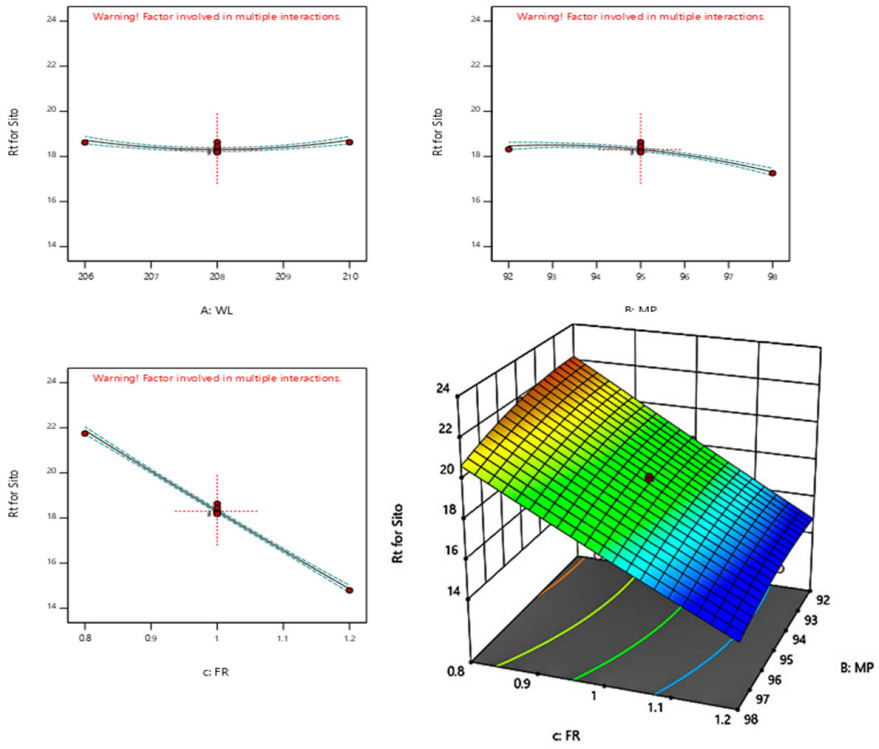


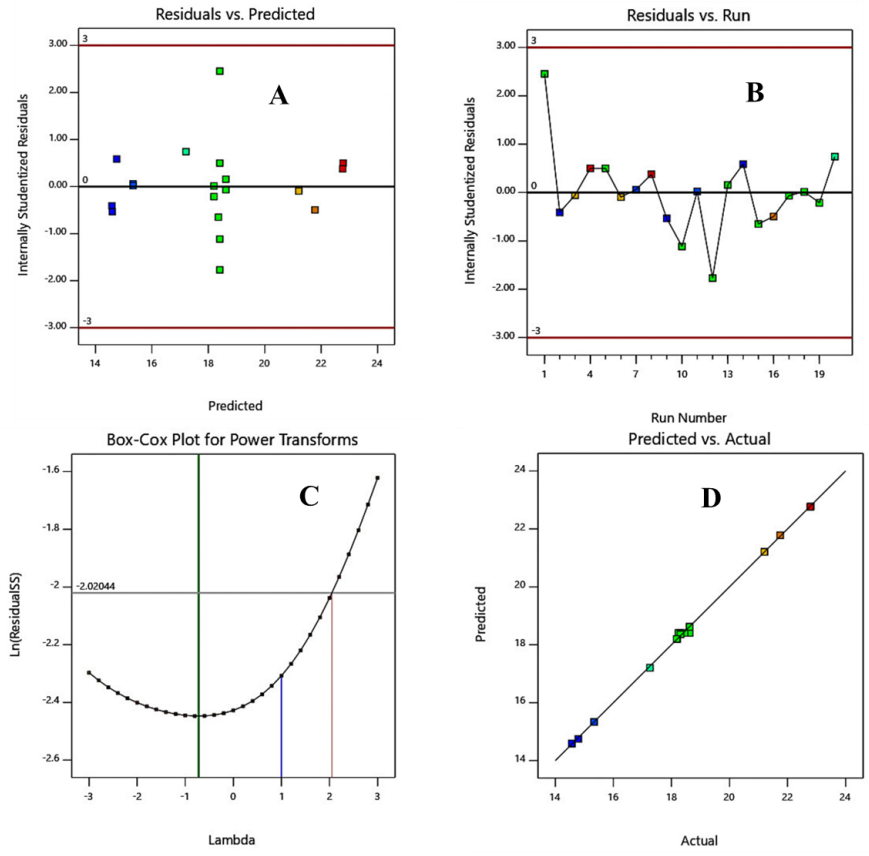
Fig. 3: Derringer's Desirability functions for the factors and responses.

Response surface plots

The optimization conditions for the factors selected and the responses were obtained by the regression equation (Tian *et al.*, 2017). The three-dimensional response surface figures were attained 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 Fig. 4a. In the response surface figures, response R_3 was by amased using the 2 continuous variables where the other variable was kept 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 Fig. 4a.



(a)



(b)

Fig. 4: a. Diagnostic plots and response surface plots for model adequacy. b. Residual plots and box-cox plot for the model.

Model adequacy diagnostics

The applicability of the experimental model was diagnosed using Model adequacy test (Tahmouzi, 2014). The diagnostic figures for model adequacy were depicted in Fig. 4a. The Figs. 4b (A-D), shows all the diagnostic measures of the model which explains residual and predicted values. The internally studentized residuals versus predicted values were displayed in Fig. 4b (A). The consistency of all the values was interpreted from internally studentized residuals (Thangam *et al.*, 2014). The experimental run numbers versus internally studentized residuals were shown in Fig. 4a (B). 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 Fig. 4b (C).

Method validation

As per ICH Q2b validation guidelines, the developed RP HPLC method was validated for the parameters like linearity, specificity, LOD, LOQ, accuracy, precision, and robustness were performed (ICH, 2005).

Linearity

The stock solution with the concentration range of 80-130 µg/mL for stigmasterol and β-sitosterol respectively was selected for the calibration curve in the range of 100% with a deviation of ±20% in the standard concentration used for accuracy and was analyzed in triplicate. Concentration and peak area were plotted to obtain least square regression analysis and to calculate regression equation. The regression coefficient (r^2) was found to be 0.9971 and 0.9966 eliciting the linear response over the range and was represented in Fig. 5 and depicted in Table 3.

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	0.9900	0.0001	2.23	53.0604

LOD and LOQ

A calibration curve was constituted in the range of 80-130 µg/mL from the nominal analyte concentration. The LOD and LOQ were calculated from the regression equation of the calibration with the formula of LOD as 3.3 σ/s and LOQ as 10 σ/s. Based on the given formula, the LOD and LOQ were calculated and were found to be 6.951, 21.063 µg/mL and 6.048, 18.328 µg/mL of stigmasterol and β-sitosterol respectively.

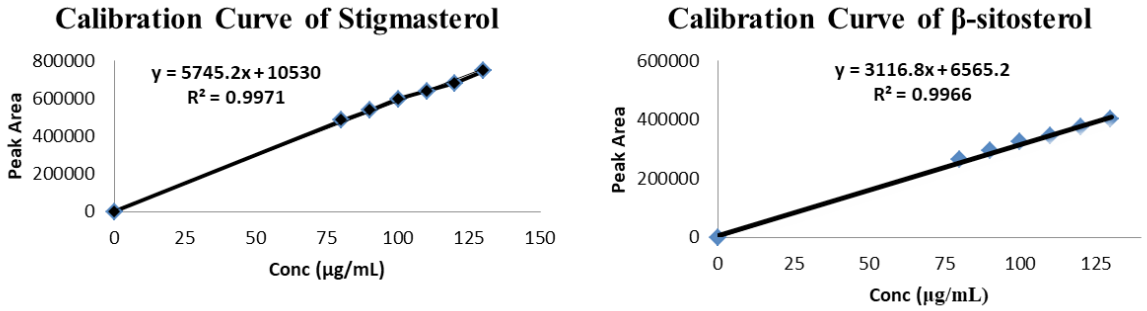


Fig. 5: Calibration curve of stigmasterol and β-sitosterol.

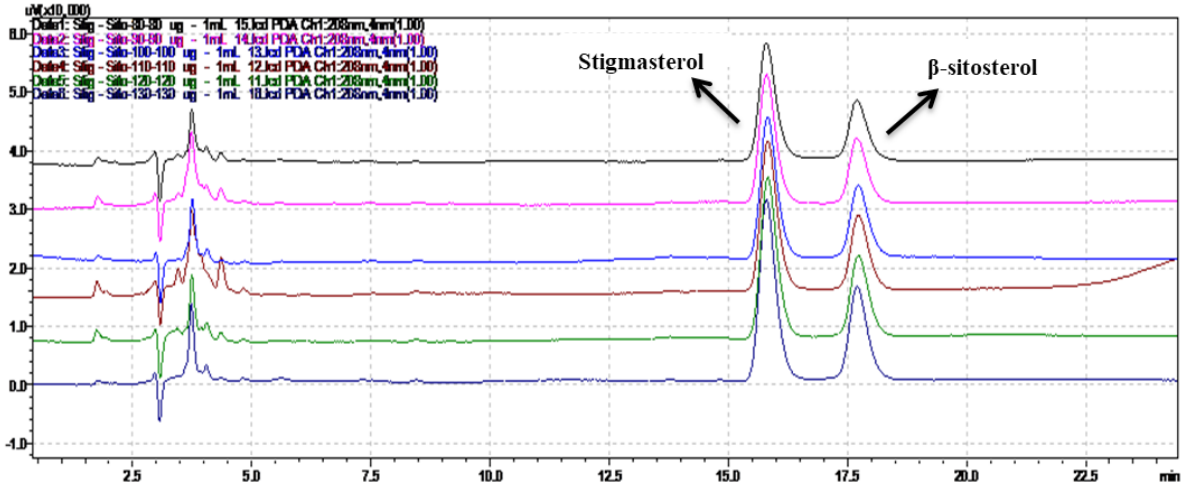


Fig. 6: Overlay of System Precision chromatograms.

Precision

The method repeatability was calculated in terms of % RSD using six replicates at 100% concentration of the standards for the determination of both intraday and interday precision. The data was reported in Tables 4 & 5 shown in Fig. 6. % RSD NMT 2 specifies the repeatability and reproducibility of the developed method.

Table 4: Linearity, precision, accuracy and assay data of stigmasterol and β-sitosterol.

Validation data of stigmasterol and β-sitosterol			
	Parameters	**Stigmasterol**	**β-sitosterol**
Linearity (n = 3) 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
Accuracy (n = 3)	% Level of addition	Mean % Recovery (RSD)	Mean % Recovery (RSD)
	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)	0.40	1.09
Assay in mg (n = 3)	Mean	0.051	0.056

Table 5: 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
16.220	650053	16.522	640324	18.202	352299	18.361	356292
16.209	638781	16.121	648378	18.180	349914	18.438	349419
0.92	0.94	1.22	0.98	0.96	1.51	1.00	1.83

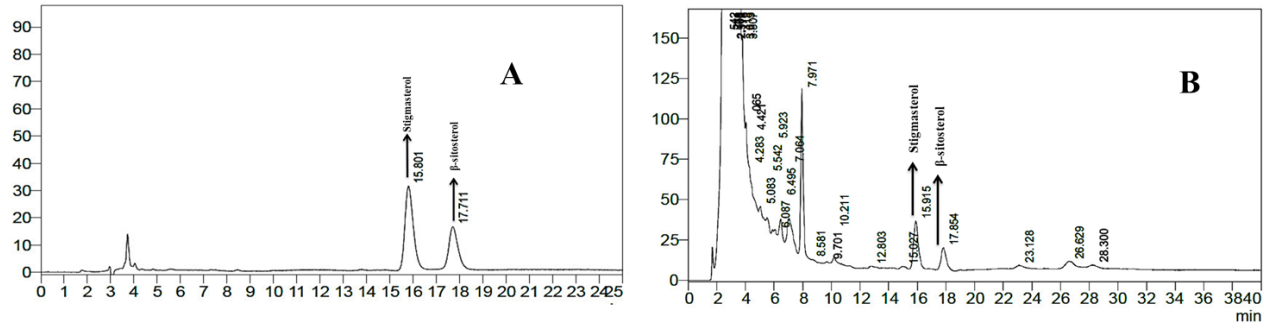


Fig. 7: Chromatogram of stigmasterol and β-sitosterol in standard and MMV.

Accuracy

To ensure the consistency and accuracy of the method, the standard addition method was followed to perform the recovery studies by spiking the unknown concentration of the standards spiked at 100 ± 20% level of the nominal concentration of the test solution of 51.0 and 56.3 µg/mg of stigmasterol and β-sitosterol respectively. Triplicate analysis of the study at each level was studied; the standard and the sample chromatograms were represented in Fig. 7 and tabulated in Table 4.

Robustness

To perform the robustness of the study, all the independent variables like mobile phase, wavelength, and flow were premeditatedly studied to identify the influence of the independent factors on the proposed method. With the change in flow rate, mobile phase there is a significant change in the elution time and no alteration of the qualitative parameter with the change in wavelength.

Appendix A: Robustness data of Stigmasterol and β-sitosterol.

Chemical Marker	Parametres	Change	Rt (min)	K'	ASF	R_s	Plate number
Nominal Concentration							
Stigmasterol	--	--	16.220	4.561	1.178	NA	9589
β-sitosterol	--	--	18.202	2.786	1.128	2.868	10247
Stigmasterol	Mobile Phase A	Low (92)	16.314	4.027	1.150	NA	11385
		High (98)	15.400	2.516	1.179	NA	10546
β-sitosterol		Low (92)	18.315	4.219	1.164	3.126	12016
		High (98)	17.258	2.373	1.137	2.978	11355
Stigmasterol	Flow rate (ml min^{-1})	Low (0.8)	19.426	4.322	1.146	NA	11640
		High (1.2)	13.197	2.638	1.192	NA	9560
β-sitosterol		Low (0.8)	21.746	4.481	1.104	3.121	12878
		High (1.2)	14.793	2.814	1.183	2.833	10155
Stigmasterol	Wavelength	Low (206)	16.591	4.751	1.160	NA	9941
		High (210)	16.593	2.552	1.158	NA	9912
β-sitosterol		Low (206)	18.617	4.490	1.203	2.938	10878
		High (210)	18.631	2.625	1.153	2.954	10934

Where Rt: Retention time, K': Capacity factor, ASF: Tailing factor, R_s: Resolution, Plate number: Theoretical Plate number.

The symmetrical parameters like theoretical plate number, capacity factor were significant and tabulated within the limits and were depicted in Table 6. From the results obtained, it implies the method was robust with the change in chromatographic parameters.

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

DISCUSSION

For the simultaneous estimation of stigmasterol and β-sitosterol, application of chemometrics evince to be a significant approach in optimizing the selectivity of the independent variables. The significant factors were optimized by response surface methodology, central composite design and model adequacy diagnosis explains about the extent of application for the experimental model. The elution and the runtime for the elutes were diagnosed both qualitatively and quantitatively using the derringers D value and the perturbation plots. The CCD provides the better insight into the prior and proper understanding of the chromatographic behavior, the sensitivity of the independent variables in the process of chromatographic separation. Of all the factors used in the design, "factor C was shown highly affecting the experimental method as per the CCD results, in which six replicates were taken as central and axial points, 14 factorial points" as a datum for the prediction of the results.

CONCLUSION

The developed method is the first report for the simultaneous estimation of stigmasterol and β-sitosterol in Manasamitra Vatakam. In the present study, a simple, efficient, precise and accurate RP-HPLC method was optimized and validated for the simultaneous estimation of the Stigmasterol and β-sitosterol respectively. Highly sensible method, reducing the chromatographic run time, economic usage of chromatographic solvents, the adequate resolution between the two elutes emphasize that the developed method was qualitatively and quantitatively determined and demonstrates the necessity of the method to further contemplate for the future analytical applications. The developed HPLC method was found to be sensitive, simple, linear, precise, selective and accurate. Hence, the method can be successfully adopted for "conventional qualitative and quantitative analysis of stigmasterol and β-sitosterol in ayurvedic formulations".

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CONFLICTS OF INTEREST

None of the authors declare a conflict of interest.

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