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DEVELOPMENT AND VALIDATION OF LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRIC METHOD FOR SIMULTANEOUS QUANTITATION OF B-SITOSTEROL, CAMPESTEROL AND STIGMASTEROL FROM DRIED LEAF POWDER EXTRACT OF *PROSOPIS CINERARIA* (LINN.) DRUCE

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ABSTRACT

A rapid, sensitive, and accurate liquid chromatography-tandem mass spectrometric (LC-MS/MS) method is developed for simultaneous quantitation of β-sitosterol, campesterol and stigmasterol from dried leaf powder extract of *Prosopis cineraria* (Linn.) Druce. The chromatographic separation was carried out using a Inertsil C8 (50 mm L x 4.6 mm ID; 3 µ particle size) column. The mobile phase used was methanol at a flow rate of 0.8 mL/min in an isocratic elution mode. Nexera UHPLC system coupled with LCMS-8040 triple quadrupole mass spectrometer with Atmospheric Pressure Chemical Ionization (APCI) source was used as a detector in Multiple Reaction Monitoring (MRM) mode. The method was found to be simple, precise, accurate, fast, specific, and sensitive that can be used for routine quality control check of dried leaf powder extract of *Prosopis cineraria* (Linn.) Druce.

KEYWORDS: LC-MS/MS, phytosterols, MRM, *Prosopis cineraria* (Linn.) Druce.

INTRODUCTION

Plant derived products are increasingly being sought out as medicinal products, nutraceuticals and cosmetics and are available in health food shops and pharmacies over the counter as or also as drugs prescribed in the non-allopathic systems. Herbal drug technology is used for

converting botanical materials into medicines, where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge is important. Every single herb needs to be quality checked to ascertain that it confirms to quality requirement and delivers the properties consistently. Standardization assures that products are reliable in terms of quality, efficacy, performance, and safety.[1]

Chromatographic techniques such as high-performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC), and gas chromatography (GC), liquid chromatography-mass spectrometry (LC-MS) are used to efficiently determine the quality of the herbs by developing fingerprints and estimation of biomarkers. In the present research work, liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed and validated for simultaneous quantitation of the β-sitosterol, campesterol and stigmasterol from dried leaf powder extract of *Prosopis cineraria* (Linn.) Druce.

Prosopis cineraria (Linn.) Druce is known as a boon tree of the Thar desert by its multiple uses and its medicinal values. *Prosopis cineraria* (Linn.) Druce belongs to family Leguminosae, grows in dry and arid regions of Arabia and in India mainly Rajasthan, Haryana, Punjab, Gujarat, Western Uttar Pradesh and drier parts of Deccan and extends as far as South India. It is also known as Khejri, Jand, Janti and Sangri in Rajasthan, Jand in Punjab, Kandi in Sindh, Banni in Karnataka, Vanni or Jambu in Tamil Nadu, Sami and Sumri in Gujarat. The tree holds an important place in the rural economy in the northwest region of Indian subcontinent. Since all parts of the tree are useful, it is called ‘Kalptaru’. It is also known as “Golden tree” or “Wonder tree” of the desert. It is used as antihyperlipidemic, antioxidative, anthelmintic, antibacterial, antifungal, antiviral, anticancer, in treatment of dysentery, bronchitis, asthma, leukoderma, piles, leprosy, muscular tremors and wandering of the mind. It has analgesic and antipyretic activities. It is also used as a remedy for rheumatism. Applied on boils and blisters, mouth ulcers in livestock and on open sores on the skin, good for eye, prevent miscarriage, anti-diabetic agent, help in preventing protein calorie malnutrition and iron calcium deficiency in blood. Various phytoconstituents like tannins (gallic acid), Flavone derivatives (Prosogerin A, B, C, D and E), alkaloids (spicigerine, prosophylline), steroids (stigmasterol, campesterol, sitosterol) etc. has been isolated from the plant.[2]

Number of GC, GC-MS, HPLC-PDA, HPLC-ELSD methods have been reported for quantification of phytosterols from various medicinal plants and other matrices. Also, LC-MS/MS methods have been reported for phytosterols analysis from human serum, edible oils, sediments etc.[3-5] After a systematic review of literature, it was found out that no validated LC-MS/MS method was reported for simultaneous quantitation of the β-sitosterol, campesterol and stigmasterol from dried leaf powder extract of *Prosopis cineraria* (Linn.) Druce to the best of our knowledge. Hence, the aim of the present research work was to develop and validate LC-MS/MS for simultaneous quantitation of the β-sitosterol, campesterol and stigmasterol from dried leaf powder extract of *Prosopis cineraria* (Linn.) Druce.

MATERIALS AND METHODS

Experimental reagents

LCMS grade methanol was purchased from J.T. Baker, Mumbai, India. HPLC grade ethyl acetate, methanol and chloroform were obtained from LiChrosolv Merck, India.

Reference standards

Reference standards of β-sitosterol, campesterol and stigmasterol were procured from Sigma-Aldrich Chemie GmbH (Aldrich Division, Steinbeim, Germany).

Plant material

The plant material of *Prosopis cineraria* (Linn.) Druce was collected from Mumbai, Maharashtra, India. The plant material was authenticated from Botanical Survey of India, Pune, India (voucher specimen no. DB-1). Leaves of *Prosopis cineraria* (Linn.) Druce were dried at room temperature, under shade and then ground in a mixer to a fine powder. Powder was then passed through an ASTM BSS mesh (size 85) and stored in an airtight container at room temperature.

Preparation of stock solutions

About 20 mg of β-sitosterol was weighed in 1.5 mL microcentrifuge tube and 1000 µL of ethyl acetate was added to it. Solution was vortexed vigorously for 2 minutes for complete dissolution of β-sitosterol. This gives a stock solution of β-sitosterol with concentration of 20000 µg/mL. About 1 mg of campesterol was weighed in 1.5 mL microcentrifuge tube and 1000 µL of ethyl acetate was added to it. Solution was vortexed vigorously for 2 minutes for complete dissolution of campesterol. A stock solution of campesterol with concentration of

1000 µg/mL was, thus, prepared. About 20 mg of stigmasterol was weighed in 1.5 mL microcentrifuge tube and 1000 µL of chloroform: methanol (1:1 v/v) was added to it. Solution was vortexed vigorously for 2 minutes for complete dissolution of stigmasterol. Hence, a stock solution of campesterol with concentration of 20000 µg/mL was prepared.

These stock solutions were further diluted using methanol to prepare mix standard solutions of β-sitosterol, campesterol and stigmasterol.

Preparation of sample solution

About 1 g of dried leaf powder of *Prosopis cineraria* (Linn.) Druce was weighed and transferred to a 50 mL centrifuge tube. Fifteen mL of methanol was added to it and the flask was sonicated in an ultrasonic bath for 15 minutes. The contents of the tube were then centrifuged at 5000 rpm and supernatant was collected in a separate tube. Same procedure was repeated with the remaining residue and filtrates were combined. Hence, 1g of sample was extracted in overall 30 mL of methanol. Sample solution was then filtered using nylon syringe filter (0.22 µ). Filtered sample was then diluted in ratio of 1:10 using methanol and analyzed using developed LC-MS/MS method.

Instrumentation and analytical conditions

LC-MS/MS analysis was performed using Shimadzu Nexera UHPLC chromatograph coupled with LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Corporation, Japan). Nexera system was equipped with binary gradient pump (LC-30AD), auto sampler (SIL-30 AC) and oven (CTO-20 AC). A reversed phase, Inertsil C8 (50 mm L x 4.6 mm ID; 3 µ particle size) was used for the chromatographic separation and column temperature was maintained at 40° C. Mobile phase used for analysis was methanol in isocratic elution mode and flow rate used was 0.8 mL/min. Injection volume used was 20 µL. LCMS-8040 was equipped with APCI source and analysis was carried out in positive mode. Nitrogen was used as nebulizing as well as drying gas and flow rate used was 2.5 L/min and 5 L/min respectively. Interface temperature, desolvation line temperature and heat block temperature were maintained at 300 ° C, 200 ° C and 300 ° C respectively. Argon was used as CID gas at a pressure of 230 kPa. Capillary voltage used was 4 kV. Quantification was performed using MRM mode with transition of 397.40 >161.00 for β-sitosterol, 383.40 >147.00 for campesterol and 395.40 >83.20 for stigmasterol. The peak widths of precursor and product ions were maintained at 0.7 Da at FWHM in the MRM mode.

OPTIMIZATION OF MS PARAMETERS

Full scan MS analysis was carried out using APCI source in positive mode to determine precursor ion m/z of selected phytosterols. As shown in Figures 1, 2 and 3, the most intense m/z of 397.40, 383.45, and 395.30 in the full scan MS spectrum corresponded to the dehydrated protonated species $[(M-H_2O+H^+)^+]$ of β-sitosterol, campesterol and stigmasterol respectively.

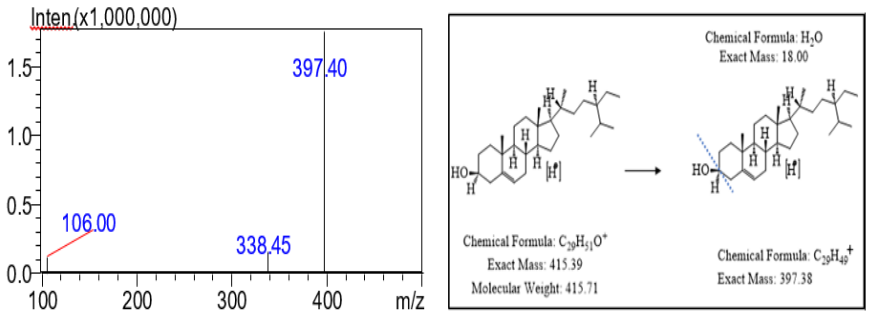

Figure 1: Full scan MS spectrum of β-sitosterol in APCI positive mode.

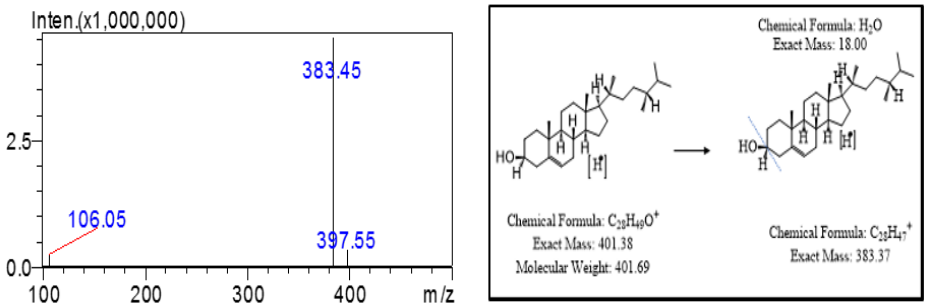

Figure 2: Full scan MS spectrum of campesterol in APCI positive mode.

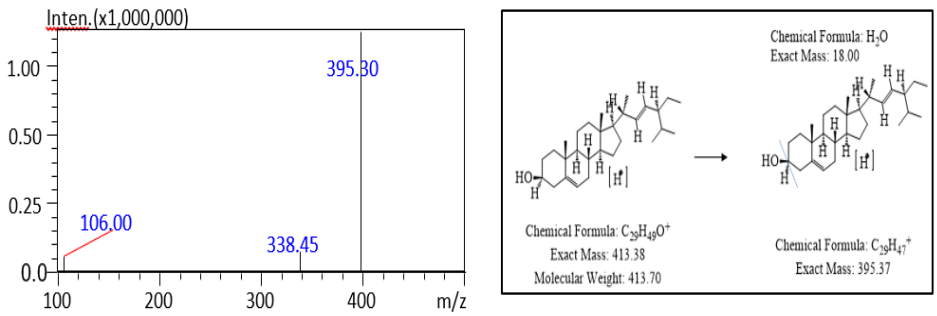

Figure 3: Full scan MS spectrum of stigmasterol in APCI positive mode.

Selection of most abundant and consistent product ions was carried out using 'Automatic MRM Optimization' tool of LabSolutions software. Representative product ion scan spectra

of β-sitosterol, campesterol and stigmasterol are shown in Figures 4, 5 and 6 respectively. For further quantitative analysis, MRM transitions of 397.40 >161.00 for β-sitosterol, 383.40 > 147.00 for campesterol and 395.40 >83.20 for stigmasterol were used. Selected MRM transitions are in accordance with the MRM transitions reported in the literature for the given phytosterols.[6,7] Furthermore, MS/MS parameters including fine adjustments of precursor and product ions, collision energies and, Q1 & Q3 pre-bias voltages were carried out to improve the overall sensitivity.

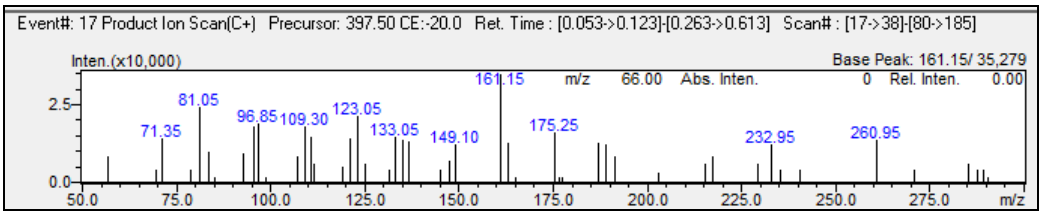

Figure 4: Representative product ion scan spectrum of β-sitosterol.

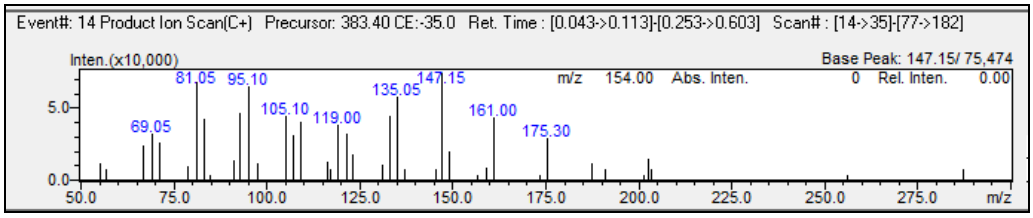

Figure 5: Representative product ion scan spectrum of campesterol.

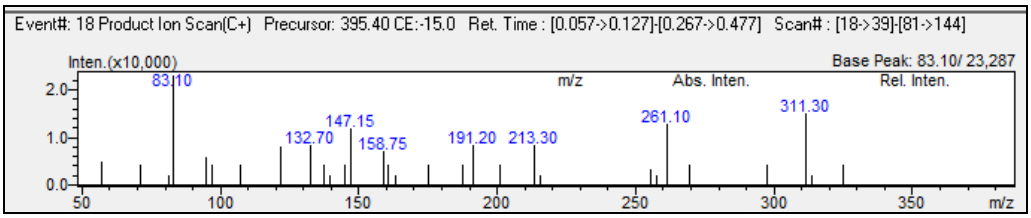

Figure 6: Representative product ion scan spectrum of stigmasterol.

METHOD VALIDATION PARAMETERS

Linearity

Linearity of method for simultaneous quantitation of β-sitosterol, campesterol and stigmasterol was determined by analyzing mix standard solutions of concentration levels of 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL, 500 ng/mL, 1000 ng/mL, and 2000 ng/mL on LC-MS/MS system in triplicates, under the optimized analytical conditions. The peak areas obtained for each analyzed concentration were noted. The calibration curves of all the analytes were obtained by plotting graphs of mean peak areas of each standard versus corresponding concentration (Figures 7, 8 and 9). The values of correlation coefficient,

intercept and slope were determined from the corresponding graphs of mean peak area of β-sitosterol, campesterol and stigmasterol (Y axis) against injected concentrations of β-sitosterol, campesterol and stigmasterol (X axis) by applying weighted ($1/A^2$) least-squares linear regression analysis. The results, listed in Table 1, show that within the concentration range indicated, there was a good correlation between mean peak area and concentration of standards.

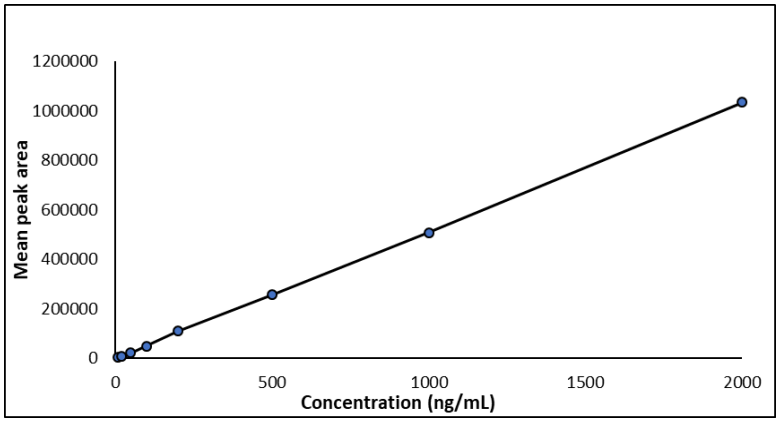

Figure 7: Graph of mean peak area v/s concentration for β-sitosterol.

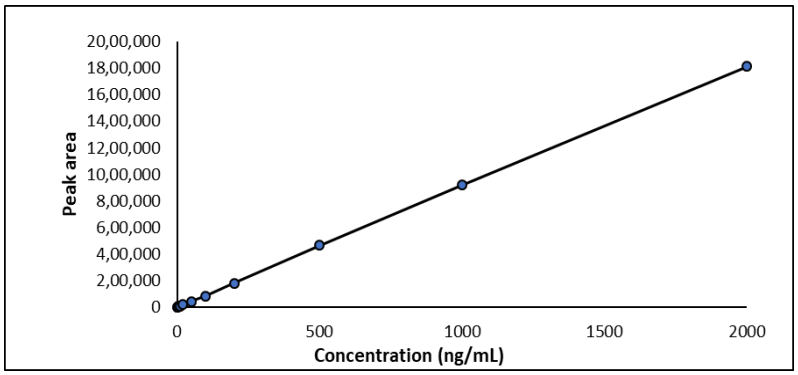

Figure 8: Graph of mean peak area v/s concentration for campesterol.

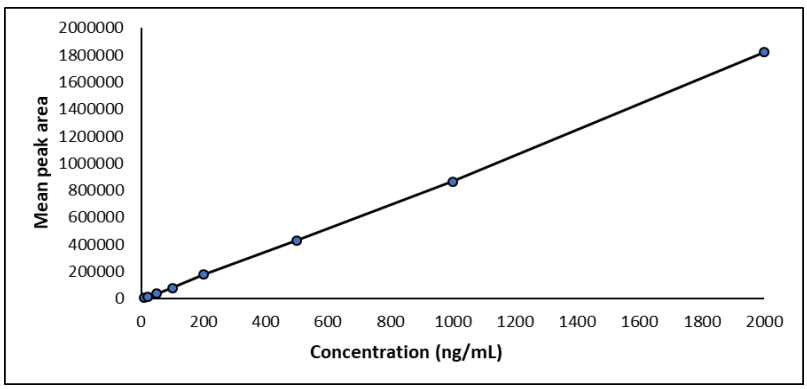

Figure 9: Graph of mean peak area v/s concentration for stigmasterol.

Limit of detection (LOD) and limit of quantitation (LOQ)

The Limit of Detection (LOD) is defined as a concentration where signal-to-noise (S/N) ratio obtained is 3:1. LOD was found to be 1 ng/mL for β-sitosterol, campesterol and stigmasterol. The Limit of Quantification (LOQ) is defined as a lowest concentration point of calibration curve where signal-to-noise (S/N) ratio obtained is 10:1 with accuracy within 80-120 % and precision below 20 %. LOQ was found to be 10 ng/mL for β-sitosterol, campesterol and stigmasterol. Results are tabulate in Table 1.

System suitability

System suitability was carried out to verify that reproducibility of the system was acceptable for the analysis. System suitability was determined by injecting, mix standard solution of β-sitosterol, campesterol and stigmasterol at concentration level of 200 ng/mL into the LC-MS/MS system, under the optimized analytical conditions in six replicates. The system suitability parameters like retention time reproducibility, peak area reproducibility, peak tailing factor, and column efficiency were evaluated. Results of the system suitability are presented in Table 1.

Specificity

The specificity of the proposed LC-MS/MS method was ascertained by injecting diluent (methanol) to observe for interference at the retention times of peaks of interest. Figures 10, 11 and 12 show overlay of MRM chromatograms of methanol and standard solution of β-sitosterol, campesterol and stigmasterol at LOQ level (10 ng/mL) respectively, to check that the interference, if any, is below LOQ level for both the analytes. No interference from the diluent was observed for all the analytes.

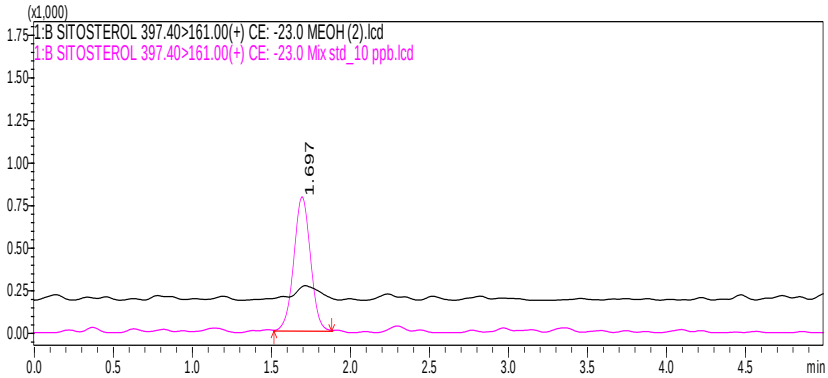

Figure 10: Overlay of MRM chromatograms of methanol and standard solution of β-sitosterol at LOQ level.

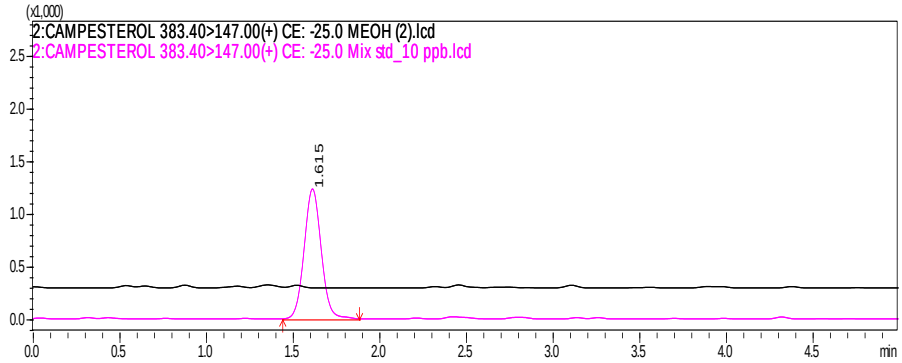

Figure 11: Overlay of MRM chromatograms of methanol and standard solution of campesterol at LOQ level.

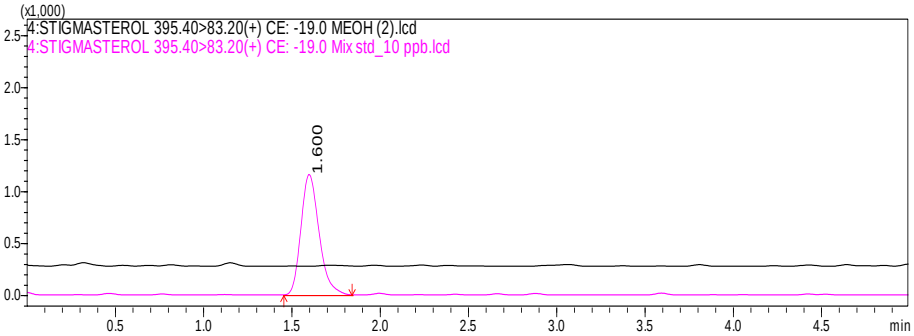

Figure 12: Overlay of MRM chromatograms of methanol and standard solution of stigmasterol at LOQ level.

Precision

The precision of the method was studied by determining repeatability and intermediate precision. Repeatability was evaluated by analyzing sample solution of dried leaf powder of *Prosopis cineraria* (Linn.) Druce in six replicates, on the same day. The intermediate precision of the method was evaluated by analyzing the sample solution in six replicates on three different days, under the specified chromatographic conditions. The precision results were expressed as percentage relative standard deviations of peak areas for β-sitosterol, campesterol and stigmasterol. The results, listed in Table 1, indicate that the proposed method is precise and reproducible.

Standard stability

The stabilities of β-sitosterol, campesterol and stigmasterol standards were determined by comparing the peak areas of mix standard solution of β-sitosterol, campesterol and stigmasterol of concentration level of 100 ng/mL at different time intervals, for a period of minimum 48 hours, at room temperature. Values of percentage relative standard deviation for

area counts (less than 5 %) indicates no significant degradation for β-sitosterol, campesterol and stigmasterol for 48 hours.

Assay

The developed and validated LC-MS/MS method was used for quantitation of β-sitosterol, campesterol and stigmasterol from dried leaf powder extract of *Prosopis cineraria* (Linn.) Druce.

The identities of peaks of β-sitosterol, campesterol and stigmasterol in the sample solution were confirmed by comparing the retention times of peaks found in sample solution of with that of the standard solution of β-sitosterol, campesterol and stigmasterol. Figure 13 shows MRM chromatogram of mix standard solution of β-sitosterol, campesterol and stigmasterol at concentration level of 50 ng/mL. Figure 14 shows MRM chromatogram of sample solution. Amount of β-sitosterol, campesterol and stigmasterol present in the sample solution was determined from their respective calibration curves, by using the peak area of β-sitosterol, campesterol and stigmasterol present in the sample solution. To ascertain the repeatability of the method, the assay experiment was repeated six times. The results of assay experiment are shown in Table 1.

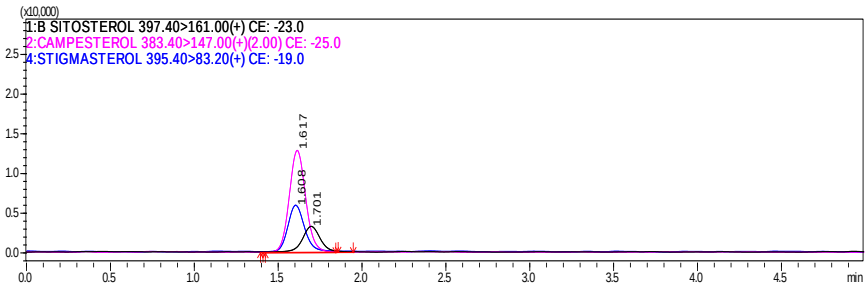

Figure 13: MRM chromatogram of β-sitosterol, campesterol and stigmasterol mix standard at concentration level of 50 ng/mL.

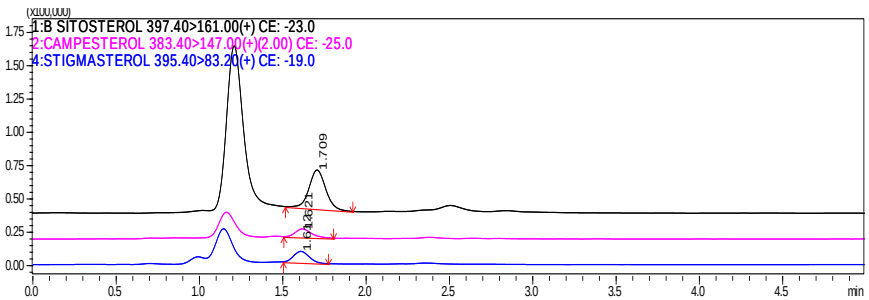

Figure 14: MRM chromatogram of β-sitosterol, campesterol and stigmasterol present in dried leaf powder extract of *Prosopis cineraria* (Linn.) Druce.

Recovery

Recovery of the method was ascertained by spiking the pre-analyzed samples with known concentration of standards and then analyzing samples under optimized analytical conditions. The spiking was done at three different concentration levels at 80 %, 100 % and 120 % of the assay concentration level of sample. The average percentage recovery at each concentration level was evaluated and tabulated in Table 1.

Table 1: Method validation results for simultaneous quantification of β-sitosterol, campesterol and stigmasterol from dried leaf powder extract of *Prosopis cineraria* (Linn.) Druce.

Method validation parameters	β-sitosterol	Campesterol	Stigmasterol
Linear range (ng/mL)	10-2000	10-2000	10-2000
Correlation coefficient (r^2)	0.9901	0.9954	0.9922
Slope (m)	493.712	860.636	832.625
Intercept (c)	391.313	-18.206	-248.703
LOD (ng/ mL)	1	1	1
LOQ (ng/ mL)	10	10	10
Stability of standard solution	48 hours	48 hours	48 hours
System suitability (% RSD area counts)	1.94	2.94	1.32
System suitability (% RSD retention time)	0.26	0.37	0.33
Column efficiency	34591	28162	24515
Peak tailing	1.052	1.154	1.143
Repeatability (% RSD area counts)	8.82	6.86	7.87
Intermediate precision (% RSD area counts)	9.30	7.96	12.71
Assay (µg/g)	123.55	13.31	20.74
% Recovery	Level 1-105.42 Level 2-105.95 Level 3-105.33	Level 1-88.59 Level 2-91.45 Level 3-101.20	Level 1-110.17 Level 2-113.17 Level 3-113.64

DISCUSSION

In the present research work, a LC-MS/MS method was developed and validated for simultaneous quantitation of the β-sitosterol, campesterol and stigmasterol from dried leaf powder extract of *Prosopis cineraria* (Linn.) Druce. This developed and validated LC-MS/MS method for simultaneous quantification of β-sitosterol, campesterol and stigmasterol over concentration range 10-2000 ng/mL has significant advantages in terms of sensitivity

(LOQ- 10 ng/mL) and selectivity (use of MRM mode), shorter run time (5 mins) without need of any derivatization.

An introduction of tandem mass spectrometric detection (MS/MS) has led to a significant improvement in detection ability and selectivity by employing MRM data acquisition in recent years. Due to the high selectivity of MRM mode, sample pre-treatment and optimization of chromatographic separation are not essential or crucial any more. This could be considered a major advantage over conventional HPLC methods, which usually require additional time for chromatographic method development and results in substantial losses of analytes due to multiple steps involved in sample pre-treatment.

CONCLUSION

A precise, accurate and reproducible LC-MS/MS method has been developed for simultaneous quantitation of the β-sitosterol, campesterol and stigmasterol from dried leaf powder extract of *Prosopis cineraria* (Linn.) Druce. The developed method has been validated according to the US FDA guidelines. This method can be used as an analytical tool for quality evaluation of plants and formulations containing β-sitosterol, campesterol and stigmasterol as chemical markers. It is an efficient method to assess quality of dried leaf powder extract of *Prosopis cineraria* (Linn.) Druce.

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