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Design of a Validated HPLC Methodology for the Measurement of Linoleic Acid and Beta Sitosterol in *Solanum nigrum*

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ABSTRACT: *Solanum nigrum*, a member of the Solanaceae family, has a long history of medicinal use. It has immunomodulatory, anti-ulcerogenic, hepatoprotective, and anti-hyperlipidemic effects. It has been used to treat measles, malaria, and cancer. The plant contains alkaloids, coumarins, flavonoids, tannins, saponins, proteins, carbohydrates, glycosides, and phytosterols. *S. nigrum* is notable for its linoleic acid content, and the plant itself has a higher lipid content compared to other plants. *S. nigrum* is an essential ingredient in various herbal and Ayurvedic preparations. The objective of the study was to develop a reliable HPLC method for simultaneous quantification of linoleic acid and beta-sitosterol in *S. nigrum* extract berries. One potential challenge of the study could be optimizing the separation and detection parameters for accurate quantification of linoleic acid and beta-sitosterol. A validated RP-HPLC method was developed in accordance with ICH guidelines. The separation was achieved using an isocratic approach on a C_{18} column with specific conditions. The developed method demonstrated satisfactory linearity, with correlation coefficients of 0.9982 and 0.9971 for LA and BS, respectively. The LOD and LOQ values indicated the sensitivity of the method for both compounds. Overall, the HPLC method proved to be efficient, precise, and reproducible, offering advantages such as speed, cost-effectiveness, and the ability to maintain isocratic conditions throughout the analysis. The study contributes to the field by providing a validated HPLC methodology for accurately quantifying linoleic acid and beta-sitosterol in *S. nigrum*, which can aid in quality control and therapeutic applications.

Keywords: *Solanum nigrum*, linoleic acid, beta-sitosterol, HPLC, validation.

INTRODUCTION

The interest in utilizing plant-based products has experienced a significant rise in developed nations, driven by the increasing demand for their application as medicinal products, nutraceuticals, and cosmetics (Patil *et al.*, 2011). The increasing global demand for herbal treatments has been accompanied by a decline in quality and a rise in demand, which can be attributed to insufficient drug regulations (Rasheed and Gupta 2010). Identification and quality evaluation are essential prerequisites for crude herbal drugs. Given the intricate and variable nature of these materials, analytical control of crude medicines must consider their complex composition. The analytical requirements for crude herbal drugs are less precise compared to those for a single chemical entity. To achieve satisfactory quality, it is necessary to employ chemical, physicochemical, and instrumental procedures. The World Health Organization (WHO) has underscored the importance of using advanced analytical techniques and establishing physicochemical criteria to ensure the quality of herbal medicines (Ali *et al.*, 2016).

Solanum nigrum, a member of the Solanaceae family commonly referred to as black nightshade, is a plant that flourishes in diverse soil conditions, such as moist environments and various soil types. These soil types include dry, shallow, rocky, deep soils, and others (Kiran *et al.*, 2009). It is utilized in healthcare to address a range of ailments. These conditions include tonsillitis, ringworms, pneumonia, and tumors (Noumedem *et al.*, 2013; Jain *et al.*, 2013). The medicinal plant is commonly incorporated as a crucial element in cancer therapies within traditional Chinese medicine (An *et al.*, 2006). The juice extracted from the berries of *S. nigrum* is utilized for treating various

conditions such as diarrhoea, eye problems, and hydrophobia. It is also employed in managing heart ailments and anasarca. The berries are believed to possess tonic, diuretic, and cathartic properties (Khattak *et al.,* 2012).

Linoleic acid (LA) is a type of polyunsaturated omega-6 fatty acid that exhibits several beneficial physiological characteristics. These include anti-atherosclerotic, anti-cancer, anti-menorrhagic, hepatoprotective, and immunomodulatory properties, etc. (Chakraborty *et al.,* 2016). β-sitosterol (BS) is a notable phytosterol known for its beneficial physiological effects, including anorectic, anti-inflammatory, antiandrogenic, anticancer, antigonadotropic, antibacterial and antilymphomic properties (Chakraborty *et al.,* 2016). *S. nigrum* has been identified as a source of both BS (Ramanpreet and Kaur 2019) and LA (Chakraborty *et al.,* 2016).

S. nigrum is an important component of many herbal remedies and is used to cure a variety of diseases. The essential components, BS and LA, are commonly present in numerous herbal remedies employed for the treatment of a wide range of conditions. Many herbal formulations are not standardized, which can lead to safety concerns. Standardization is important to ensure compliance with regulations and promote safe use. A literature review identified the utilization of HPLC (Sriraman *et al.,* 2015; Valerian *et al.,* 2022), HPTLC (Dhruv and Tatke 2013; Mallick and Dighe 2014), and GC (Sorenson and Sullivan 2006) for beta-sitosterol quantification. Linoleic acid quantification employed HPLC (Melis *et al*., 2001; Nishikiori *et al.,* 2014), HPTLC (Chakraborty *et al.,* 2016); GC (Gangopadhyay *et al.,* 2016). A comprehensive analysis revealed that GC (Pantano *et al*., 2016; Gangopadhyay *et al.,* 2016), GC-MS (Williams *et al*., 2021), and HPTLC (Chakraborty *et al.,* 2016) were the sole documented methods for concurrent quantification of both BS and LA. GC-MS is not a practical method for routine quality control testing because it is time-consuming and expensive. HPLC is a more practical method for quantifying LA and BS in *S. nigrum* berries. This study is the first to validate an HPLC method for simultaneously quantifying LA and BS in *S. nigrum*.

The objective of this study was to develop a validated HPLC method for simultaneously determining linoleic acid (LA) and beta-sitosterol (BS) in *Solanum nigrum* berry (SNB) extract. The research established a technique using a C_{18} column as the stationary phase and a mobile phase composed of a mixture of 0.05% phosphoric acid and acetonitrile in a ratio of 90:10 (v/v). The analytical procedure underwent validation according to the guidelines set by the International Council for Harmonization (ICH).

MATERIALS AND METHODS

Instrumentation, Chemicals and Reagents. The chromatographic analysis was performed using an HPLC system consisting of specific components. The system utilized was the DionexUltiMate 3000, which included a titanium quaternary pump (LPG-3400AB), an autosampler (WSSIN3000TBPL), and a photodiode array detector (DAD-3000) manufactured by Thermo Fisher Scientific in New York, NY. The data acquisition and control were managed by Chromeleon 7.0 software, developed by Dionex (Thermo Fisher Scientific, New York, NY). For the separation of compounds, an Acclaim® 120 A C_{18} column with dimensions of 250 mm × 4.6 mm inner diameter and 5 µm particle size (Thermo Fisher Scientific, New York, NY), was employed. This column type is designed for reversed-phase chromatography and facilitates the separation of analytes based on their hydrophobic characteristics. By utilizing this specific HPLC system configuration and the Acclaim® 120 A C_{18} column, the analysis aimed to achieve efficient separation and detection of the target compounds. The combination of the components and column is tailored to meet the requirements of the analytical method and ensure reliable and accurate results in the analysis of the samples.

The reference standards used for linoleic acid (LA) and β-sitosterol (BS) were provided by Innovative Chemical Exchange Pvt. Ltd (Carbino). The LA standard had an accuracy of 97%, while the BS standard had an accuracy of 98%. All the chemicals utilized in this experiment were of analytical grade, ensuring their high purity and suitability for analytical purposes. The solvents used were of spectroscopic quality, meeting the necessary standards for spectroscopic analysis. The chemicals included HPLC grade methanol, petroleum ether, phosphoric acid, acetonitrile, and methanol, all of which were sourced from Merck in Mumbai, India.

Plant Material. The *Solanum nigrum* barriers (SNB) were collected in January and verified by a taxonomist. They were air dried, filtered through 10 mesh, and finely pulverized to achieve a powdered form suitable for further analysis or processing.

Standard Preparation. LA and BS were measured and mixed with methanol to create concentrated standard solutions. To prepare the working standard solutions, the stock solutions were diluted using the mobile phase. The concentrations of LA in the standard solutions ranged from 5 to 25 µg/ml, while the concentrations of BS ranged from 3 to 15 µg/ml. Prior to chromatographic analysis, all standard solutions underwent filtration using a 0.45 µm nylon syringe filter membrane. Subsequently, all solutions were stored at a temperature of 4°C. The calibration curve was established in adherence to the guidelines specified by the ICH guideline (Ngamkhae *et al*., 2022).

Sample Preparation. The dried course powder of SNB was subjected to continuous heat extraction for six hours at 60-80°C for three days by a soxhlet apparatus using petroleum ether (PE). The extract was filtered and evaporated in a vacuum to remove the solvent, yielding a semisolid product. A desiccator was employed to preserve the dry substance during the studies. After concentrating the air-dried powdered crude material, the percentage yield of the extract was determined to be 1.8% (w/w). To prepare the SNB extract solution, 5 mg of the extract were dissolved in 1 ml of PE and adjusted to a total volume of 12 ml using the mobile phase. The

solution was then sonicated for 15 minutes and filtered through a 0.45 µm nylon syringe filter membrane. Finally, 20 µl samples of the filtered solution were injected into the HPLC system for analysis.

HPLC condition. The RP-HPLC system employed for separation utilized an Acclaim® 120 A C_{18} column (4.0 mm × 250mm, 5µm, Thermo Fisher Scientific in NY, USA). A sample injection volume of 20 µl was used, and the column temperature was maintained at ambient conditions. For detection, the wavelengths were set at 256 nm specifically for the analysis of LA and BS. The mobile phase consisted of a mixture of 0.05% phosphoric acid and acetonitrile in a ratio of 90:10 (v/v), and the flow rate was set at 1 ml/min using isocratic elution. The total run time for the analysis was 30 minutes.

Method validation. Following ICH criteria, the analytical procedure was validated (Ngamkhae *et al*., 2022). The validation of the analytical method encompassed several parameters, including specificity and sensitivity, linearity, range, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and robustness.

Specificity. The method's specificity was assessed by introducing 20 µl solutions of standard, sample, and blank into the chromatographic system.

Linearity. Linearity was confirmed by creating standard solutions at five different concentrations. Working standards were created for LA within a range of 5 to 25 µg/ml and for BS within a range of 3 to 15 µg/ml. In the HPLC system, 20 µl of each concentration were injected. Regression analysis was then performed on the peak area plotted against the concentration to determine the calibration equations and coefficient of determination.

Precision. The precision of the method was evaluated through two measures: repeatability (within-day) and intermediate precision (between-day). Within-day precision was determined by calculating the percent relative standard deviation (%RSD) for assays conducted on the same day. On the other hand, between-day precision was assessed by comparing the assays conducted on five different days, and the standard deviation (SD) and %RSD were calculated for this comparison.

Accuracy. The accuracy of the method was assessed by performing successive analyses (n = 3) of three different concentrations of LA (5, 10, and 20 µg/ml) and BS (3, 6, and 12 µg/ml) using the developed method. The accuracy was determined by calculating the percent recovery (% recovery) of the analytes. To be considered acceptable, the mean recovery should fall within the range of 97.0–103.0 %.

Limit of detection (LOD) and limit of quantification (LOQ). LOD is the smallest concentration of an analyte in a sample that can be reliably detected. In this study, the LOD was determined by achieving a signal-to-noise ratio of 1:3. On the other hand, LOQ refers to the lowest concentration of an analyte that can be determined with acceptable precision and accuracy. In this particular case, the LOQ was determined based on a signal-to-noise ratio of 1:10.

Robustness. The robustness of the method was assessed by conducting the analysis with varying flow rates, with a deviation of ±0.1 ml/min, while keeping all other chromatographic conditions constant. This evaluation aimed to determine the method's ability to remain reliable and consistent even under slight changes in the flow rate parameter.

RESULTS AND DISCUSSION

Selection of wavelength. The UV spectrum of a standard compound, LA, displayed its highest absorbance at 255.82 nm. UV spectrum of a reference material named BS, however, revealed that its absorbance occurred at two distinct wavelengths, respectively 258.72 nm and 293.09 nm (Fig. 1). The wavelength of 256 nm was chosen for the assay chromatogram of both LA and BS. This wavelength was selected because it provided a clear and stable baseline, and it was also close to the common wavelength response observed for both LA and BS.

Accuracy. The accuracy of the method was assessed by calculating the % recovery, and the results are summarized in Table 1. The mean percentage recoveries of LA and BS were observed to range from 99.77% to 100.27% and 100.53% to 100.87%, respectively. The percentage recovery results fell within the acceptable limits of 97.0–103.0 %.

Precision. The precision of the method was evaluated by determining the percent relative standard deviation (% RSD) for measurements made within the same day (within-day) and across multiple days (between-day). To evaluate within-day precision, standard solutions were injected on the same day, while between-day precision was assessed by repeating the measurements on five different days. The % RSD values were determined for three concentration levels in the precision studies. The obtained % RSD values for both within-day and between-day assays of all compounds demonstrated excellent precision, with values below 2%. The acceptable precision threshold was set at 2.0% for the % RSD. The detailed results can be found in Table 2.

Linearity. The linearity of the LA and BS methods was assessed by conducting linear regression analysis, and the findings are presented in Table 3. During the analysis, the concentration ranges investigated were 5-25 µg/ml for LA and 3-15 µg/ml for BS. All calibration curves exhibited excellent linearity, with coefficient of determination (R^2) values exceeding 0.997. The high R^2 values, close to 1.00, indicate a strong linear relationship between the concentration of each compound and its corresponding peak area. The calibration curves for LA and BS can be observed in Fig. 2.

Limit of Detection (LOD) and Limit of Quantification (LOQ). The results showed the LOD for LA and BS were 1.01, and 1.60 µg/ml, respectively and the LOQ of these compounds were 0.96 and 2.90 µg/ml, respectively (Table 3).

Robustness. The robustness of the method was assessed by introducing variations in the flow rate during the chromatographic analysis. To ensure

acceptable robustness, the criterion set was a relative standard deviation (RSD) value of no more than 2.0%. The results obtained from the robustness study, as shown in Table 4, revealed that altering the flow rate did not have any significant impacts on the chromatographic parameters. Therefore, the method demonstrated robustness, indicating its ability to provide consistent and reliable results even in the presence of slight variations in the flow rate.

Determination of LA and BS in SNB extract. Chromatograms of LA and BS detected under the developed HPLC conditions are shown in Figure 3. The figure shows that a good separation was accomplished in less than 30 minutes. The retention times for LA, and BS were 6.375, and 10.607 min, respectively.

The analytical HPLC method that was developed and validated was successfully applied to analyse LA and BS simultaneously in SNB petroleum ether extract. The peak areas of triplicate samples were determined using a regression equation derived from the calibration plot, allowing for the quantification of LA and BS contents in the samples. The amounts of LA and BS in the SNB petroleum ether extract ware determined to be 28.14 and 1.76 % w/w, respectively. To confirm the identification of LA and BS peaks in the sample, their retention times were compared with those of standard compounds. Furthermore, the confirmation was reinforced by comparing the UV spectra of the peaks in the sample with the respective UV spectra of the LA and BS standards. A visual representation of the peaks observed in Figure 4.

The HPLC method was validated according to the guidelines of the International Conference on Harmonisation (ICH) (Ngamkhae *et al.,* 2022). The % RSD values found in precision study depicted in Table 2 showed that the proposed method provides acceptable within-day and between-day variations for LA and BS in their simultaneous determination. Results are summarized in Table 2 where %RSD was within the limit ≤ 2 (Rahman *et al*., 2014). According to USP, the correlation coefficient (R^2) for a calibration curve must be ≥ 0.995 (Rahman *et al.,* 2014). The correlation coefficient was found to be greater than 0.995 for both LA and BS, indicating good linearity of the calibration curve. The linearity curves are shown in Figure 2.For LA and BS, the percent recovery is shown in Table 1. All experimental results are in the range of acceptability for accuracy (97.0–103.0%) (Rahman *et al*., 2014). Additional validation parameters, such as the limit of quantification (LOQ), limit of detection (LOD), and robustness, were also determined and are presented. The results from all these validation parameters indicated that the analytical method employed was reliable and suitable for analyzing the two compounds. The presence of LA in *S. nigrum* has been reported in a study by Chakraborty *et al.* (2016) ;Chakraborty *et al*. (2016). In addition to the aforementioned study, another research conducted by Ramanpreet and Kaur (2019); Ramanpreet and Kaur (2019) also identified the presence of BS in *S. nigrum*. These findings were successfully confirmed through the HPLC research methodology. Subsequently, this HPLC method was utilized to quantify the concentrations of the two active compounds in the petroleum ether extract of SNB. These chemical markers are crucial for quality control purposes, enabling standardization of the herbal extract and the formulation. Therefore, the development of an appropriate and efficient method to determine the quality of the chemical markers was of significant importance. The two major compounds identified in the SNB extract were precisely quantified using the developed HPLC method. Consequently, this method holds broader applicability and facilitates time-savings in the analytical process.

Table 1: Percentage recovery of LA and BS.

Compounds	Concentration (µg/ml)	Concentration founded (µg/ml) (mean ± SD)	% Recovery (n=3)
LA	5	5.007 ± 0.045	100.13 ± 0.90
	10	9.976 ± 0.047	99.76 ± 0.47
	20	20.053 ± 0.04	100.26 ± 0.21
BS	3	3.027 ± 0.07	100.53± 1.51
	6	6.073 ± 0.16	100.76 ± 1.61
	12	12.053 ± 0.04	100.86 ± 1.02

Table 2: Within-day and between-day precision data of LA and BS.

Compounds	Concentration (µg/ml)	Within-day precision (n=3)		Between-day precision (n=3)	
		Area of peak (mAU)	%RSD	Area of peak (mAU)	%RSD
LA	5	6719.33 ± 30.74	0.46	6763.10 ± 50.26	0.74
	10	13509.67 ± 38.27	0.28	13533.10 ± 39.40	0.29
	20	26385.33 ± 37.22	0.14	26402.01 ± 58.92	0.22
BS	3	9281.33 ± 43.65	0.47	9284.66 ± 43.18	0.46
	6	18946.67 ± 37.52	0.20	18965.02 ± 55.04	0.29
	12	37280.66 ± 45.72	0.12	37300.66 ± 34.35	0.09

Table 3: Regression formula, correlation value, linearity ranges, correlation coefficient and LOD and LOQ of calibration curves for LA and BS.

Compounds	Regression formula	Coefficient of determination (R^2)	Linearity range (µg/ml)	LOD (µg/ml) (n=3)	LOQ (µg/ml) (n=3)
LA	y = 1278.x + 671.8	0.9988	5-25	1.05 ± 0.42	0.96 ± 0.33
BS	y = 3052.x + 672.6	0.9971	3-15	1.60 ± 0.31	2.90 ± 0.15

Table 4: Results of the robustness study from the variation of flow rate.

Compounds/Flow rate	0.9 ml/min		1.0 ml/min		1.1ml/min	
	value	%RSD	value	%RSD	value	%RSD
LA (10 µg/ml)						
Retention time	7.31	0.31	6.35	0.04	5.64	0.61
Peak area	13638	0.35	13565	0.25	13320	0.72
%Recovery	101.46	0.41	100.39	0.21	97.15	0.81
BS (10 µg/ml)						
Retention time	11.61	0.49	10.62	0.05	9.24	0.77
Peak area	19240	0.52	18984	0.36	18652	0.65
%Recovery	102.14	0.39	99.92	0.18	96.69	0.86

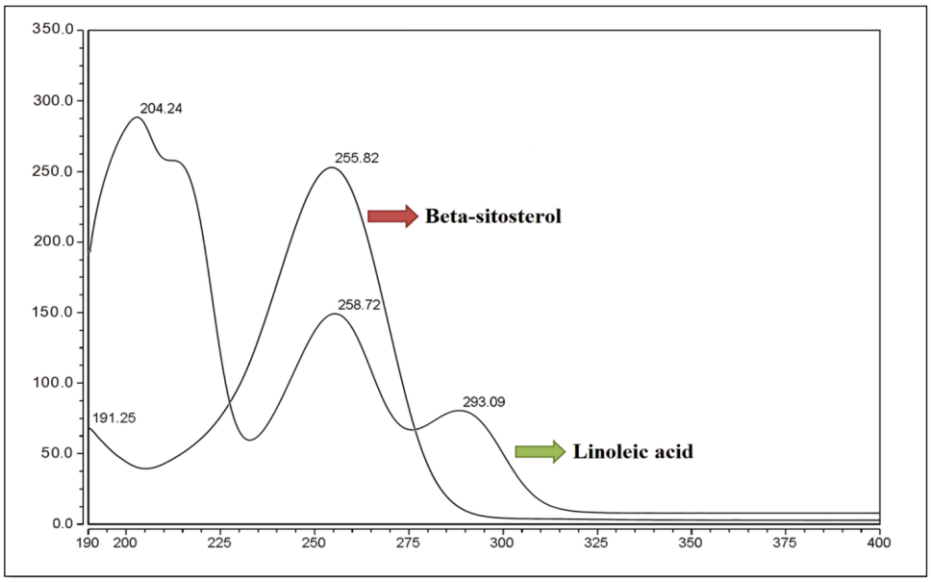

Fig. 1. Scanning of LA and BS from UV range 200 - 400 nm.

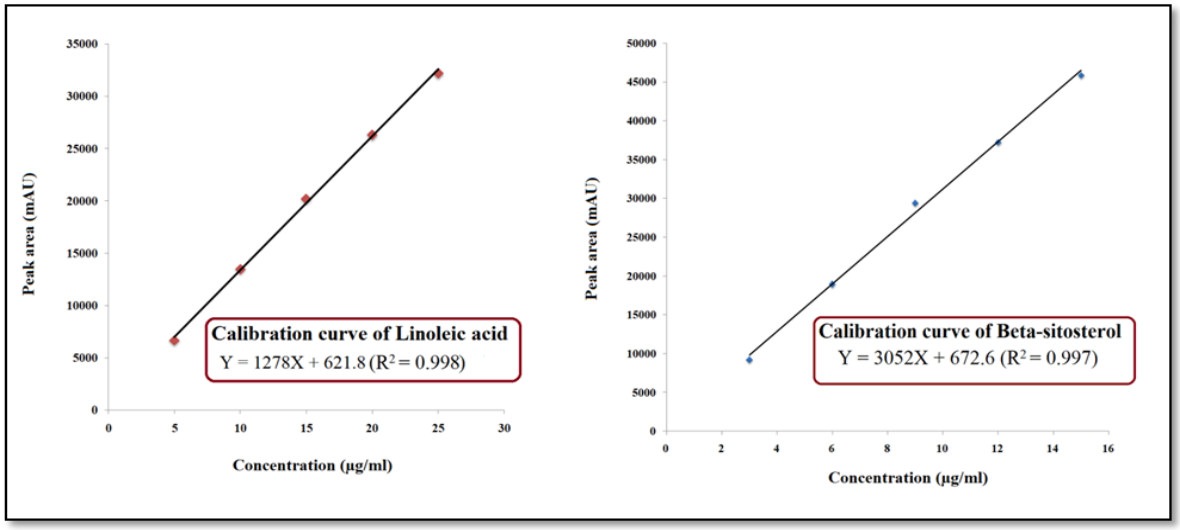

Fig. 2. Calibration curve of LA, and BS solution.

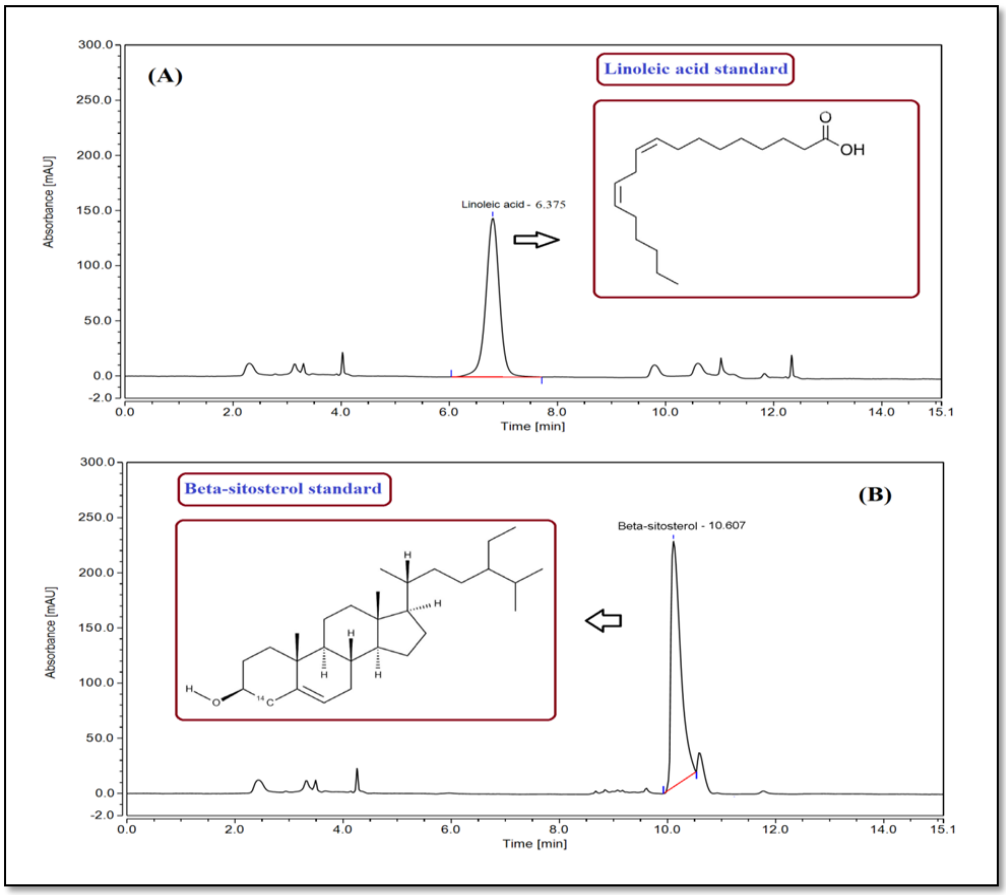

Fig. 3. HPLC chromatogram at wavelength 256 nm: (A) standard LA (B) standard BS.

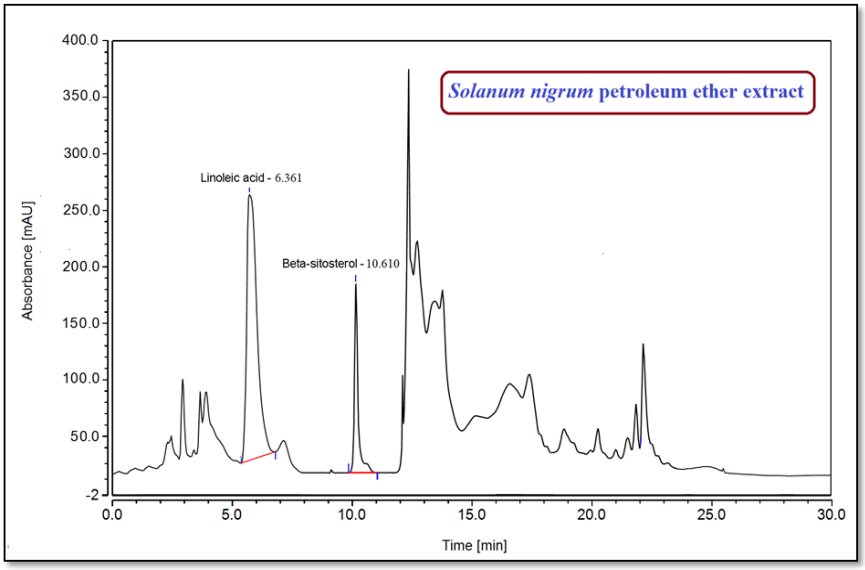

Fig. 4. HPLC chromatogram of SNB petroleum ether extract at wavelength 256 nm.

CONCLUSIONS

A precise and reliable HPLC method has been successfully developed and validated for the quantification of two active compounds, including LA and BS, in SNB extract. This method is deemed suitable for routine quality control analysis of SNB extract. The analytical conditions employed in the method offer excellent resolution for the separation of LA and BS, ensuring accurate quantification. The validation of the method was performed following the guidelines set by the ICH, and it successfully met all the crucial parameters, including robustness. The findings of this study are expected to serve as a valuable quality control reference for the standardization of these specific components in formulations or herbal raw materials.

FUTURE SCOPE

Future scope includes method optimization, robustness testing, application to different matrices, stability studies, method comparison, inter-laboratory studies,

and method standardization to enhance accuracy and expand application areas.

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Conflict of Interest. None.

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