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Development and comprehensive greenness assessment of HPLC method for quality control of β-sitosterol in pharmaceutical ointments with trio-color coded evaluation

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Abstract

A simple, rapid, and reproducible high-performance liquid chromatography (HPLC) method has been developed and validated for the determination of β-sitosterol in the pharmaceutical dosage form of moist exposed burn ointment (MEBO). This method involved an effective sample procedure for extraction of β-sitosterol from MEBO using an alkali saponification agent composed of 0.8 N ethanolic NaOH and diethyl ether. The chromatographic separation was achieved on a C18 column (50×3.0 mm, 2.5 µm), using a mobile phase composed of methanol and acetonitrile (70:30 v/v) pumped in an isocratic mode at a flow rate of 0.7 mL/min. The column temperature was maintained at 40 °C, the injection volume was 10 µL, and the detection wavelength was 203 nm. Employing these conditions, the retention time was found to be 2.10 min. The developed method was validated for its specificity, linearity, accuracy, precision, the limit of detection, the limit of quantification, robustness, and solution stability based on International Council for Harmonisation (ICH) guidelines Q2 (R1). Our proposed method demonstrated superior performance compared to other reported methods. It exhibited a linearity range of 30 to 500 µg/mL and improved detectability with a limit of detection (LOD) of 4.65 µg/mL, highlighting its high sensitivity. Additionally, the separation was achieved in a remarkably short analysis time of just 2.1 min, which not only enhanced throughput but also significantly minimized waste and solvent consumption, thereby making it a more sustainable and effective alternative for β-sitosterol extraction. Moreover, in the light of green and white analytical chemistry, a comprehensive ecological and sustainable tri-color coded assessment protocol was established. The proposed method has been successfully applied to quantify β-sitosterol in commercial products (MEBO®, Avomeb® and BISTROI®) demonstrating its suitability for routine quality control analysis of β-sitosterol in pharmaceutical ointment dosage forms.

Highlights

- Quality control for evaluating MEBO anti-burn ointment is crucial
- The first study for the determination of β-sitosterol in MEBO

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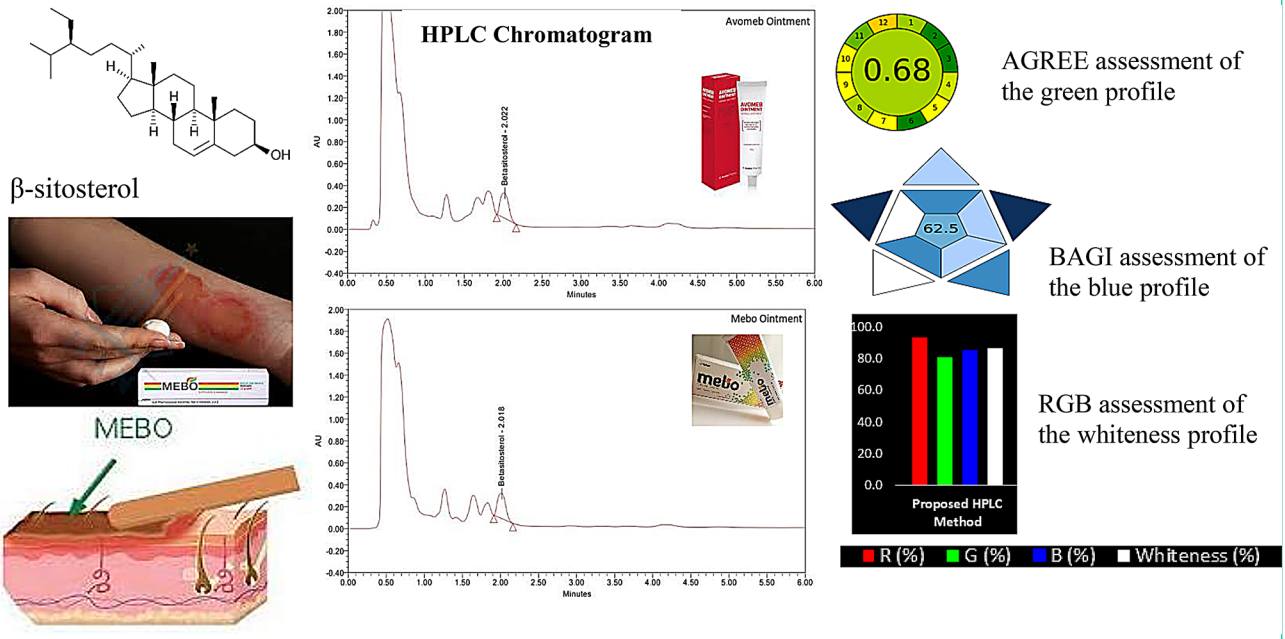
- *A challenge in its extraction from the complex oily matrix of MEBO ointment*
- Analytical Greenness, Blueness, and whiteness evaluation

Graphical Abstract



Keywords β-sitosterol, HPLC, Sample preparation, Green, blue and white assessment

Introduction

Burns are generally perceived as skin injuries resulting from excessive exposure to heat. The severity and location of these injuries can significantly impact burn patients, affecting various aspects such as human life, pain, disability, and financial consequences. Given the variability of burn injuries, managing less severe cases may involve employing basic first-aid treatments at home. These measures might entail the application of cold water and the use of topical remedies like burn ointments [1]. One widely used burn ointment for managing certain types of burns is MEBO (Moist Exposed Burn Ointment). MEBO, a traditional Chinese medicine, that can significantly enhance burn wound healing and pressure ulcers [2]. It aids in wound soothing, maintains moisture at the burn site, and relieves pain and discomfort [3]. MEBO is an herbal product comprising ingredients such as beeswax, sesame oil, and β-sitosterol, with the latter serving as the primary active component within the ointment [3]. β-sitosterol (Fig. 1), chemically known as [17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1 H-cyclopenta[a]phenanthren-3-ol], belongs to the phytosterol class, and shares structural similarities with cholesterol [4]. This compound exhibits

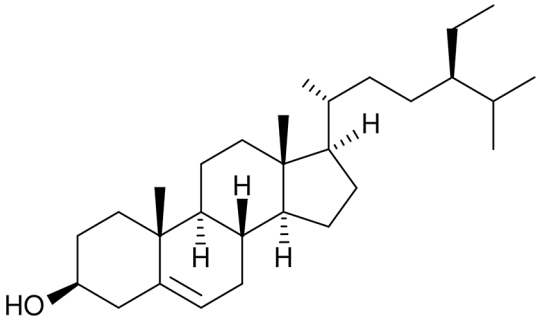


Fig. 1 Chemical structure of β-sitosterol

notable pharmacological properties, such as anti-inflammatory, antioxidant, and immunomodulatory effects [5, 6]. Research comparing the topical application of MEBO and silver sulfadiazine for treating second-degree thermal burns, showed that MEBO produces more favourable outcomes. This is attributed to its ability to promote rapid re-epithelialization at the burn site [7].

The importance for MEBO led to the pharmaceutical product being produced from numerous pharmaceutical companies. With the product's continuous presence in the market, pharmaceutical companies and regulatory authorities must uphold rigorous

quality control measures for all product formulations. This ensures that all produced pharmaceutical products, are safe and therapeutically active, with consistent and predictable results in terms of their performance. Thus, the development of precise and accurate analytical procedures for evaluating pharmaceutical products is necessary to meet this demand.

Only a few HPLC techniques have been reported for the analysis of β-sitosterol in herbal medicines, vegetable oils [8], supplements [9]and ointment [10]. β-sitosterol was also estimated after extraction from plants parts using HPLC [11–13] and HPTLC [14]. However, most of these methods are qualitative, lack crucial sample preparation procedures, are not extremely sensitive, or tend to detect β-sitosterol at very prolonged retention time. Consequently, these methods require to consume a considerable amount of organic solvents, resulting in increase of the cost and time required for each sample analysis. Moreover, the non-polar nature of β-sitosterol presents a challenge in its extraction from the formulation, highlighting the need for better and improved methods for recovering the compound from ***the complex oily matrix of MEBO ointment***. To address this gap, this study was devoted to improving β-sitosterol recovery and develop a new sensitive, simple and rapid HPLC method for the detection of β-sitosterol in the various pharmaceutical formulations of MEBO products. This method was validated in compliance with the recommendations of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [15]. Analytical Greenness metric (AGREE) [16], Chloroform-oriented Toxicity Estimation Scale (Chlortox) [17], Blue Applicability Grade Index (BAGI) [18], Red green and blue (RGB12) [19] and RGBfast [20] were used in this investigation to evaluate the greenness, blueness, and whiteness of the entire process, from extraction to quantitation.

Methodology

Chemicals and reagents

A pure reference standard of β -sitosterol (assigned purity 98.0%) was obtained from USP. The working standard grade of β -sitosterol (assigned purity 78.3%) was provided by Aja Pharma Pharmaceuticals Co., Ltd. Moist Exposed Burn Ointment 0.25% w/w was purchased from the local market. Commercial batches of moist exposed burn ointment (MEBO Julphar, Ras Al Khaimah, United Arab Emirates), (Avomeb Avalon pharma, Riyadh, KSA) and (BISTROl : AJA Pharma, Hail, KSA) were purchased from a local pharmacy. HPLC-grade methanol and diethyl ether were purchased from Fisher Chemical. HPLC-grade acetonitrile and absolute ethanol were purchased from Honeywell and Chem-Lab NV, respectively. Analytical grade sodium hydroxide pellets were purchased from Panreac Applichem. Ultrapure water was obtained from a Milli-Q Element purification system with 0.22 µm filters (Millipore, Molsheim, France).

HPLC instrumentation and software

Method development and validation were conducted using an HPLC system (Waters-Aquity Arc, Waters Alliance XC e2695). The system was equipped with a quaternary solvent manager-R as a solvent delivery unit, a sample manager FTN-R autosampler, a 2998 PDA detector, and 2489 UV/VIS detectors for acquiring chromatograms and UV spectra. Empower® 3 Build 3471 Software was used for data acquisition and monitoring. Analytical separation was performed on an Avantor ACE C18 column (50 mm × 3.0 mm, particle size 2.5 µm). The UV/VIS detector was set at 203 nm.

Chromatographic conditions

The mobile phase used for separation was a mixture of methanol and acetonitrile (70:30 v/v). The mobile phase was filtered through 0.45 µm membrane filters and sonicated for 10 min to remove air bubbles. The analysis was performed on a Waters-Aquity Arc and Waters Alliance XC e2695 HPLC system. A C18 column (2.5 µm, 50 × 3.0 mm) was used for separation, with a detection wavelength of 203 nm. The column temperature was set at 40 °C. The flow rate was maintained at 0.7 mL/min, and the injection volume was 10 µL. The total runtime of the analysis was 6 min.

Preparation of standard solution

A standard solution of β-sitosterol was prepared by dissolving an accurately weighed amount of β-sitosterol working standard (32 mg, equivalent to 25 mg β-sitosterol) in a 100 mL volumetric flask containing approximately 75 mL of absolute ethanol. The mixture was shaken and placed in a sonicator bath for 15 min. The volume was completed with ethanol, and the mixture was shaken and filtered using a 0.22 µm PVDF filter, resulting in a standard solution of β-sitosterol with a concentration of 250 µg/mL.

Sodium hydroxide solution 50% (w/v)

Sodium hydroxide (50.0 g) was accurately weighed and transferred into a 100 mL volumetric flask. Approximately 70 mL of water was added to the flask, and the solution was shaken to dissolve the sodium hydroxide, aided by sonication for 10 min. Water was added to reach a final volume of 100 mL.

Preparation of 0.8 N ethanolic NaOH solution

For the preparation of a 0.8 N ethanolic NaOH solution, 250 mL of ethanol (96% or absolute) was mixed with 16 mL of 50% (w/v) NaOH.

Preparation of sample solution

The sample solution was prepared by accurately weighing and transferring 2.5 g of MEBO containing 0.25% (w/w) sample (equivalent to 6.25 mg of β-sitosterol) into a 500 mL round-bottom flask. Approximately 50 mL of 0.8 N ethanolic NaOH was added to the flask. The round-bottom flask was placed in a heated rotary evaporator water bath at approximately 70 °C until the sample completely dissolved. After the complete evaporation of ethanol, the flask was removed from the rotary evaporator and allowed to cool. The residue was dissolved in approximately 150 mL of diethyl ether by vigorous manual shaking until the large solid particles reduced in size. All the contents from the flask were moved to a 250 mL separating funnel, and the flask was rinsed with 50 mL of water, with the rinse added to the same separating funnel. The contents of the separating funnel were shaken until the solution transformed into an oily or homogeneous mixture, and all solid particles nearly dissolved. The mixture was allowed to stand for a few minutes to separate the aqueous layer and the ether layer. After separation, the aqueous layer (lower layer) was discarded, and the ether layer was collected using a Whatman® filter paper directly into the round-bottom flask. The diethyl ether was then evaporated using a rotary evaporator set at 30 °C. The residue was dissolved in 5 mL of absolute ethanol, and the entire content was transferred into a 25 mL volumetric flask. The volume was completed with ethanol. The sample was then centrifuged for 10 min at 6000 RPM, filtered with a 0.22 µm PVDF filter, and an aliquot was injected for analysis.

Method validation

The method was validated according to the ICH guideline [15], with specificity, linearity, range, accuracy, precision, sensitivity (LOQ and LOD), robustness, and solution stability as validation parameters.

Specificity

Specificity, an essential characteristic of HPLC, refers to the method's ability to distinguish between the analyte and other components in a complex mixture. Specificity was assessed by injecting 10 µL of each solution, including the standard, sample, blank, and placebo.

Linearity and range

To test the linearity and range of the method, a series of standard solutions with varying concentrations of β-sitosterol (30, 62, 125, 250, 375, and 500 µg/mL, covering 12%, 25%, 50%, 100%, 150%, and 200% of the target concentration, respectively) were prepared by diluting the standard stock solution with the diluent. Three injections from each concentration were analyzed, and the linearity of the calibration curve was determined using the least-squares linear regression approach.

Accuracy

Recovery studies were performed at three concentration levels (50%, 100%, and 150% of the target concentration: 125, 250, and 375 µg/mL), with three samples injected at each concentration to determine the accuracy of the assay method. The percentage recovery of added β -sitosterol and RSD were calculated for each replicate sample.

Sensitivity

The limit of detection (LOD) and limit of quantitation (LOQ) of β -sitosterol were determined by analyzing different solutions and measuring the signal-to-noise ratio. The LOQ is the concentration resulting in a signal-to-noise ratio of approximately 10:1 with a percent RSD ($n = 5$) of less than 10%. The LOD is the concentration resulting in a signal-to-noise ratio of around 3:1.

Precision

The system precision and method/intermediate precision of the analytical methods were evaluated by measuring the standard solution and sample solution, respectively. System precision was assessed by performing six measurements of the standard solution at 100% concentration on the same day. Method precision was determined by conducting six assay determinations of the sample solution at 100% concentration on the same day, and another six assay determinations on a different day were used to determine intermediate precision. The RSD of the acquired results was calculated to assess repeatability and reproducibility.

Robustness

The robustness of the method was determined by applying minor and deliberate changes in experimental parameters, such as column temperature (± 5 °C), flow rate (± 10%), wavelength (± 3 nm), and mobile phase composition (± 2% absolute of the minor component). The changes were made to evaluate the method's ability to remain unaffected by minor variations. RSD and recovery percentages were calculated to evaluate the data for each case.

Stability of analytical solutions

The standard and sample preparations were analyzed at 0 h and after one day (up to 24 h) under different storage conditions: ambient room temperature exposed to light, ambient room temperature protected from light, and a refrigerator (2–8 °C). The stability of the analytical solutions was evaluated by calculating the % RSD and percent recovery for each case.

Trio color-coded sustainable assessment (green, blue and white assessment)

AGREE [16] and BAGI [18] were performed using a freely available software (https://mostwiedzy.pl/AGREE) and web application BAGI making the assessment procedure easy and straightforward. In addition, in case of whiteness assessment using RGB [19] and RGBfast [20], freely available Excel spreadsheets were used in order to simultaneously evaluate and compare different methods.

Results and discussion

Method development and optimization

Several physicochemical properties and chromatographic condition factors, such as detection wavelength, were collected from literature research to aid in the development of a liquid chromatographic method for the identification of β-sitosterol [11]. The HPLC method was developed by evaluating various chromatographic conditions, including the stationary phase, mobile phase, flow rate, injection volume, column temperature, UV detector wavelength, and sample preparation procedures.

Development and optimization of chromatographic condition

Initial trials were conducted using different experimental parameters, with the aim to achieve the best chromatographic resolution and symmetric peak shape for the analyte. Two different columns were evaluated: an Inertsil ODS-4 HP (2.1 × 150) mm, with 3 µm HP particle size column, and an Avantor® ACE Ultra Core 2.5 super C18 (50 × 3.0 mm) with 2.5 µm particle size column. To develop the ideal chromatographic conditions, various HPLC flow rates and sample injection volumes varying between 5 and 20 µL were assessed. HPLC mobile phase consisting of mixtures of methanol and acetonitrile were commonly reported for HPLC analysis of β-sitosterol [9, 13]. For the chromatographic method development here, various mobile phases mixtures consisting of methanol and acetonitrile ranging from 80:20 (v/v) to 70:30 (v/v) were also evaluated, with higher methanol ratios showing poor peak resolution. A UV wavelength of 203 nm was selected for detecting β-sitosterol, based on experimental findings and scientific literature, which commonly identified it as the optimal absorbance wavelength [12]. The optimized chromatographic conditions for analyzing β-sitosterol were established using an Avantor® ACE Ultra Core 2.5 Super C18 (50 mm × 3.0 mm) column with a methanol: acetonitrile (70:30, v/v) mobile phase in isocratic mode, at a flow rate of 0.7 mL/min. The injection volume was 10 µL, with the sampler maintained at ambient temperature (25 °C). Detection was carried out at a wavelength of 203 nm, with the column temperature set at 40 °C, and the total run time was less than 6 min. The optimization of these chromatographic conditions is summarized in Fig. 2.

Development of sample preparation

After a comprehensive review for the related literature, sample preparation trials were conducted by testing various phytosterol extraction procedures mentioned in publications involving vegetable oils and herbal medicines [8, 21]. It was observed that several variables, including extraction instrumentation techniques, alkali saponification agent, and extracting agent, influenced the experimental processes for the yield and purity of phytosterols, such as β-sitosterol extracted from vegetable oil and herbal medicines [8, 21]. These variables were assessed to determine the optimal response consistent with our sample.

Extraction instrumentation techniques Previous researchers have utilized both Soxhlet Extraction (reflux condenser) and rotary evaporator' extraction method to extract phytosterol from vegetable oils and herbal medicines [8, 21]. In our experiments comparing both extraction methods, we found that the rotary evaporator' extraction method proved to be more ideal method compared to Soxhlet Extraction. This was attributed to its simpler setup process and higher recovery rates. However, the installation of the reflux condenser parts during Soxhlet Extraction was time-consuming, and no satisfactory sample recoveries were obtained.

Selection of alkali saponification agent

Saponification can use any of the following alkali hydroxide solutions: lithium hydroxide, sodium hydroxide, potassium hydroxide, or rubidium hydroxide [21]. During the development of sample preparation KOH and NaOH were initially chosen as saponification agents. This was due to their availability and cost-effectiveness. Following several trials, it was determined that NaOH would be the alkali saponification agent used for the sample preparation. This was based on the higher yield and better purity of β -sitosterol achieved with NaOH compared to KOH.

Extracting agent Different solvents have varying effects on the yield of unsaponifiable analytes. Past studies have reported the use of aromatic hydrocarbon solvents (such as benzene and toluene) and aliphatic hydrocarbon solvents (such as n-hexane, cyclohexane, and diethyl ether) to extract phytosterols, including β -sitosterol, from saponification residues [21]. For the method development stage of sample preparation in this study, two aliphatic hydrocarbon solvents (cyclohexane and diethyl ether), were initially chosen as extracting agents. However, through multiple trials, it was discovered that the

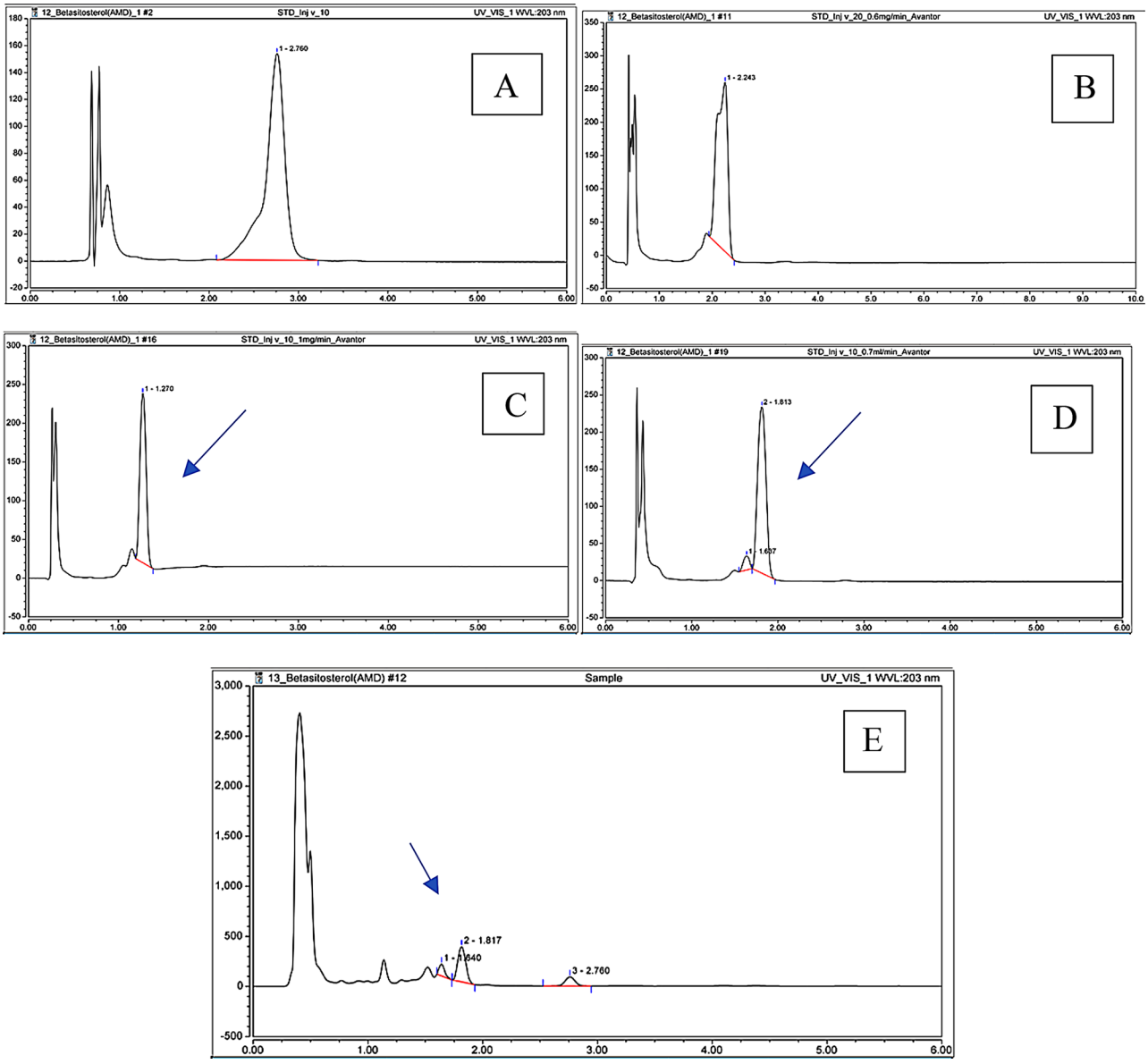


Fig. 2 Analytical method development steps using working standard grade of β-sitosterol **A**, use Inertsil ODS-4 HP (2.1 × 150 mm), 3 µm column with methanol: acetonitrile (80:20, v/v), injection volume 10 µL by 0.6 mL/min flow rate. **B**, use Avantor ACE Ultra Core 2.5 super C18 (50 × 3.0 mm) column with methanol: acetonitrile (80:20, v/v), 20 µL injection volume by 0.6 mL/min flow rate **C**, use Avantor ACE Ultra Core 2.5 super C18 (50 × 3.0 mm) column with methanol: acetonitrile (80:20, v/v) 10 µL injection volume by 1.0 mL/min flow rate. **D**, use Avantor ACE Ultra Core 2.5 super C18 (50 × 3.0 mm) column, with. methanol: acetonitrile (70:30, v/v) 10 µL injection volume by 0.7 mL/min flow rate, while **E** the developed method has been applied on sample solution, by used optimize condition Avantor ACE Ultra Core 2.5 super C18 (50 × 3.0 mm) column, with methanol: acetonitrile (70:30, v/v) 10 µL injection volume, by 0.7 mL/min flow rate

mixing of cyclohexane and water in the separatory funnel, resulted in the formation of an emulsion layer, making separation difficult. This emulsion formation could potentially lead to some amount of the analyte being suspended in the emulsion layer, resulting in a decrease in the β -sitosterol assay result. However, when diethyl ether was used as the extracting agent, two distinct and separate layers of diethyl ether and water were formed without emulsification. Moreover, diethyl ether exhibited a quicker evaporation rate compared to cyclohexane; this is likely attributable to diethyl ether lower boiling point of 34.60 °C compared to cyclohexane boiling point of 80.75 °C. Consequently, diethyl ether was chosen as the extracting agent over cyclohexane.

Optimized sample preparation

For this study, the extraction method of rotary evaporators with the aid of a vacuum pump was employed as the extraction instrumentation technique. A saponification agent was prepared using ethanolic sodium hydroxide

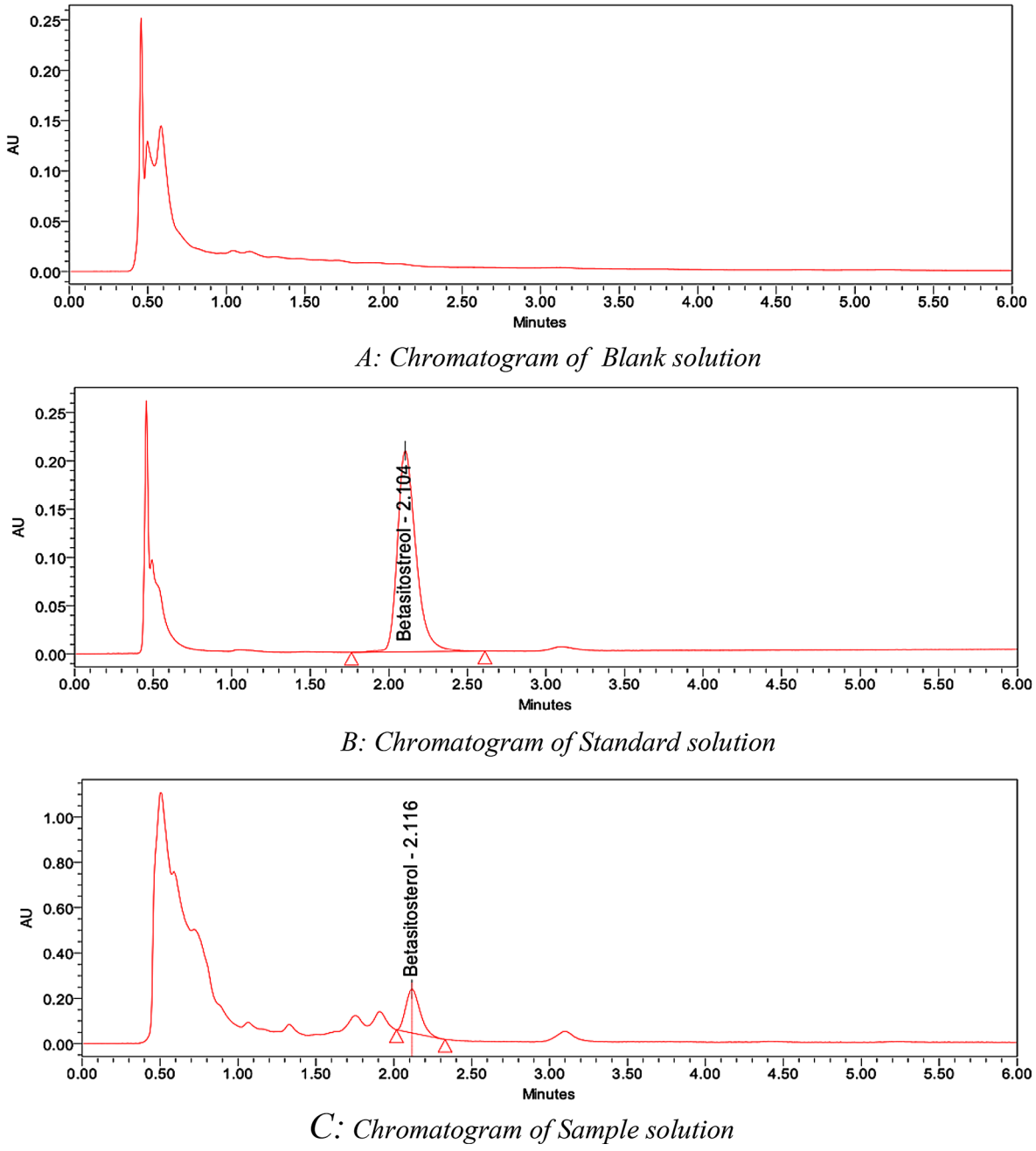


Fig. 3 Specificity Chromatogram of (***A***) *blank*, (***B***) A pure reference standard *solution* (250 µg/mL)and (***C***) sample solution

(NaOH 0.8 N) solution. Diethyl ether was selected as the extracting agent based on its favourable separation characteristics and faster evaporation rate compared to cyclohexane.

Validation of methods

Method Validation is the process of determining whether the performance characteristics of an analytical procedure meet the requirements for its intended applications through laboratory testing [15]. In accordance with the ICH guideline Q2 (R1), assay procedures need to be validated as they involve the quantitative measurement of major components in the sample [15].

Specificity

The specificity of the method was assessed by comparing the chromatograms of the blank (placebo solution), standard solution, and sample solution (250 µg/mL β -sitosterol). Each solution was separately injected into the HPLC system, and the resulting chromatograms are shown in Fig. 3, No coeluting peaks were observed at the retention time of β -sitosterol, indicating the purity

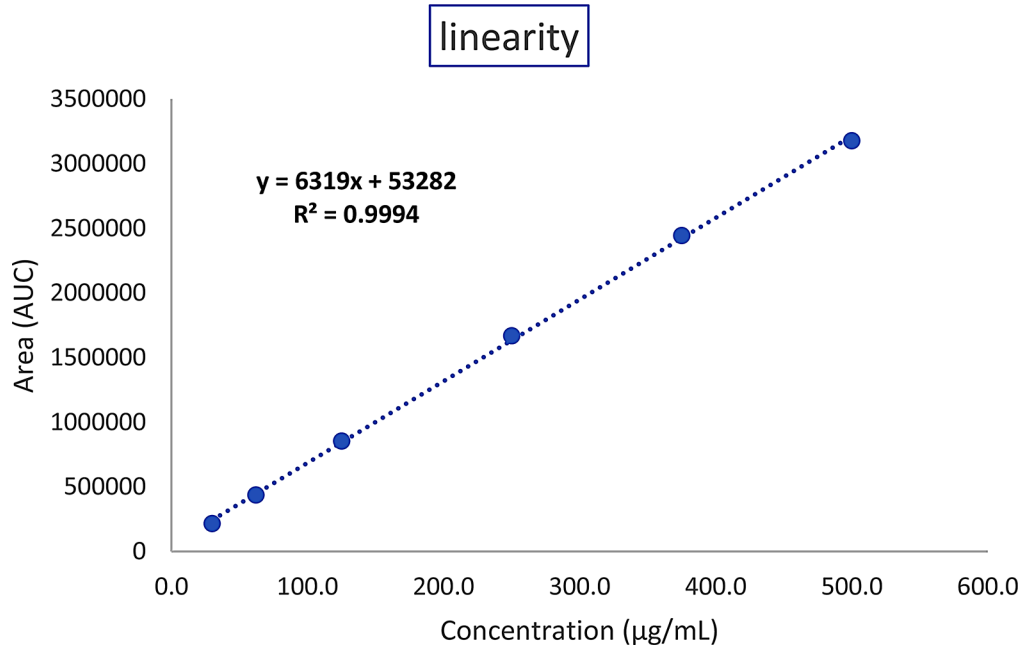


Fig. 4 The standard curve of β-sitosterol

Table 1 Validation parameter for the determination of standard solution of β-sitosterol using the proposed method

Parameter	β-sitosterol
Concentration range (µg/mL)	30 – 500
Intercept (a)	53,282
Slope (b)	6319
Correlation coefficient (R^2)	0.9994
LOD (µg/mL)	4.65
LOQ (µg/mL)	15.50

Y is the dependent variable

a is the Y-intercept

b is the slope of the regression line

X is the independent variable

Table 2 Accuracy and precision data for β-sitosterol in ointment formulations

	Conc. Added (µg/mL)	Conc. Found (µg/mL) ± SD ($n = 6$)	Error %	RSD%
Intra-day	125.00	125.36	0.29	0.29
	250.00	250.33	0.13	0.28
	375.00	375.73	0.19	0.18
Inter-day	125.00	125.54	0.43	0.35
	250.00	250.56	0.23	0.20
	375.00	375.08	0.02	0.10

Error % = ((Estimated Number – Actual Number)/ Actual number) x 100

Relative standard deviation (RSD%) = 100SD/ $\bar{x}$

of the analyte peak and confirming the method's sensitivity. (Acceptance criteria: Peak purity angle < Purity threshold).

Linearity and range

The linearity study (Fig. 4) for β -sitosterol over the concentration range of 30–500 µg/mL exhibited a linear relationship. The high regression coefficient ($R^2 = 0.9994$) confirmed the linear correlation between analyte concentrations and peak areas. (Table 1).

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

The LOD and LOQ for β -sitosterol were determined using signal-to-noise ratio (S/N) values of 3 and 10, respectively. The calculated LOD and LOQ values for β -sitosterol were 4.65 µg/mL and 15.50 µg/mL, respectively (Table 1). The %RSD for 5 replicate injections of the LOQ solution was 0.49.

Precision and accuracy

A summary of the accuracy and precision results is given in Table 2. The acceptance criteria (within-run and between-run % RSD of < 2% and an accuracy between 98 and 102% with percentage error less than 5%) were met in all cases. The Precision and Accuracy of the method were determined by using spiked standard solutions at three levels (Table 2). The intra-day precision and accuracy ($n = 6$) as expressed by relative standard deviation (%RSD) and percentage error were 0.180–0.288% and 0.131–0.292%, respectively. The inter-day precision and accuracy ($n = 6$) as expressed by relative standard deviation (%RSD) and percentage error were 0.103–0.349% and 0.021–0.432%, respectively. The percentage recovery and %RSD values were within acceptable limits of 98.0–102.0% and not more than 2.0%, respectively, indicating the suitability of the method for routine drug analysis.

Table 3 Robustness data of the developed HPLC method

Parameter	Conditions	%RSD of standard solution peak area	% Recovery
Column temperature ± 5° C	35 °C	0.48	102.2
	40 °C (Control)	0.33	98.6
	45 °C	0.60	99.0
Wavelength ± 3 nm	200 nm	0.82	100.0
	203 nm (Control)	0.33	98.6
	206 nm	0.27	102.8
Mobile phase composition ± 2%	Methanol: Acetonitrile (68:32)	1.16	99.5
	Methanol: Acetonitrile (70:30) (Control)	0.33	98.6
	Methanol: Acetonitrile (72:28)	0.52	100.9
Flow rate ± 10%	0.63 ml/min	0.36	101.5
	0.70 ml/min (Control)	0.33	98.6
	0.77 ml/min	0.74	100.8
Average	*NMT 2.0%	0.59	100.6
%RSD		0.21	1.43

*NMT: not more than

Robustness

The robustness of the analytical method was evaluated by assessing the effect of slight modifications in HPLC conditions on the system suitability parameters. As described in Sect. Robustness, variations in method conditions such as mobile phase composition, temperature, flow rate, and wavelength were tested. The results of the robustness testing (Table 3) demonstrated that slight changes in method conditions did not significantly affect the separation of β -sitosterol. The percentage recovery remained within acceptable limits, and the %RSD was less than 2% in all cases.

Stability of analytical solutions

Stability studies were conducted on the standard solution and sample solution under the conditions described in Sect. Stability of Analytical Solutions. The recovery percentage was within the range of 98.0–102.0%, and the %RSD was not more than 2.0%, indicating good stability of the sample and standard solutions for 24 h under all conditions (Table 4).

Application of the method: analysis of commercial samples

The newly validated method was used to determine the β -sitosterol content in three commercial batches of burn ointment, containing 0.25% (w/w) obtained from a local pharmacy. The ointments analyzed were MEBO® (Julphar Pharma), Avomeb® (Avalon Pharma) and BISTROl® (AJA Pharma). The percent recoveries for β -sitosterol were 98.3%, 99.1% and 99.9%for MEBO, Avomeb and BISTOL, respectively. The chromatograms, concentration, and % assay results of β -sitosterol in MEBO, AVOMEB and BISTOL are summarized in Fig. 5; Table 5.

Comparison with reported HPLC methods

Je-Chiuan Ye et al. [8] published a method for extraction and quantitation of β- sitosterol in herbal medicines with a linearity range of 0.25-2.0 mg/mL. The method was tedious and time consuming as the duration of extraction took more than 10 days. Moreover, the HPLC method used in the previous study [8] separated the β- sitosterol with a retention time of 36.8 min which led to more waste and solvent consumption. Additionally, Khonsa, K. et al. [9] reported an HPLC method for the separation of β- sitosterol from simple matrix (supplement tablets). This method had a linearity range of 15–90 µg/mL with LOD 3.26 µg/mL. Besides, the HPLC separated the β-sitosterol with a retention time of 13 min. Furthermore,

Table 4 Solutions stability data of the developed HPLC method

Parameter	Condition	RT (min.)	Peak area	Recovered %	Tailing factor	Number of theoretical plates
Standard solution	**Control standard solution at 0 h.**	2.06	1,335,002	-	1.2	1373
	Standard solution after 24 h. in the autosampler	2.06	1,328,295	99.5	1.3	1355
	Standard solution after 24 h. exposed to light	2.06	1,337,535	100.2	1.2	1387
	Standard solution after 24 h. protected from light	2.06	1,327,404	99.4	1.2	1347
	Standard solution after 24 h. at refrigerator	2.06	1,345,591	100.8	1.2	1388
Average		**2.06**	**1,334,765**	**99.7**	**1.2**	**1370**
%RSD		**0.00**	**0.56**	**0.66**	**0.00**	**1.35**
Sample solution	**Control sample solution at 0 h.**	2.09	1,222,159	-	1.3	1850
	Sample solution after 24 h. in the autosampler	2.07	1,216,241	99.5	1.3	1829
	Sample solution after 24 h. exposed to light	2.08	1,211,997	99.2	1.2	1842
	Sample solution after 24 h. protected from light	2.07	1,222,027	100.0	1.3	1823
	Sample solution after 24 h. at refrigerator	2.08	1,210,712	99.1	1.2	1782
Average		**2.08**	**1,216,627**	**99.45**	**1.3**	**1825**
%RSD		**0.40**	**0.44**	**0.41**	**0.06**	**1.45**

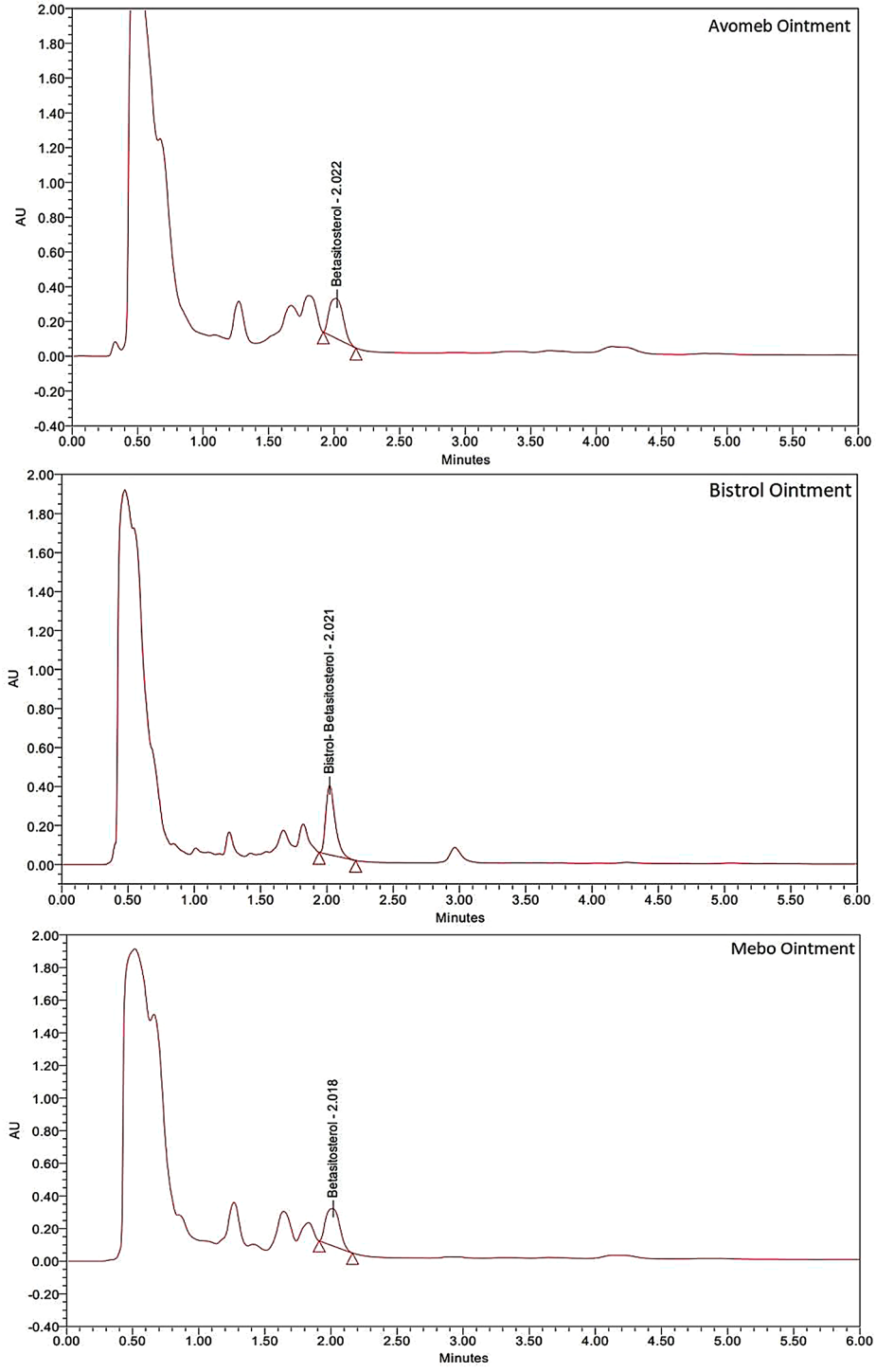


Fig. 5 Commercial batches (Avomeb, Bistrol and MEBO) chromatograms, by used optimize conditions Avantor® ACE Ultra Core 2.5 super C18 (50 × 3.0 mm) column, with Methanol: Acetonitrile (70:30, v/v) 10 µL inj.vol, by 0.7 mL/min flow rate

Alkelani, A. et. al. [10] developed an HPLC method for the determination of β-sitosterol in a pharmaceutical product (MEBO).This method had a high linearity range of 0.0125–0.0375 mg/mL. Besides, β-sitosterol was separated with retention time 35 min consuming more solvent and energy. Finally, our proposed method succeeded in the extraction of β- sitosterol from ***the complex oily matrix*** of MEBO ointment. The method had a linearity range of 30 to 500 μg/mL with LOD of 4.65 µg/mL highlighting its high sensitivity, also the separation

Table 5 Recovery study of β-sitosterol from commercial batches of the pharmaceutical dosage forms (MEBO®, Avomeb®, and Bistrol®)

MEBO (Julphar pharma) sample results obtained				
Replicate number	**Peak area**	**mg/g**	**% w/w**	**% Assay**
1	1,679,027	2.47	0.25	**99.0**
2	1,668,103	2.46	0.25	**98.3**
3	1,655,835	2.44	0.24	**97.6**
Average %RSD	**1,667,655**	**2.46**	**0.25**	**98.3**
	0.70	**0.70**	**0.70**	**0.70**
Avomeb (Avalon pharma) sample results obtained				
Replicate number	**Peak area**	**mg/g**	**% w/w**	**% Assay**
1	1,683,951	2.47	0.25	**98.8**
2	1,688,088	2.48	0.25	**99.1**
3	1,692,933	2.48	0.25	**99.4**
Average %RSD	**1,688,324**	**2.48**	**0.25**	**99.1**
	0.27	**0.27**	**0.27**	**0.27**
Bistrol (Aja pharma) sample results obtained				
Replicate number	**Peak area**	**mg/g**	**% w/w**	**% Assay**
1	1,649,409	2.47	0.25	**98.9**
2	1,658,078	2.49	0.25	**99.9**
3	1,675,324	2.52	0.25	**100.6**
Average %RSD	**1,660,148**	**2.49**	**0.25**	**99.9**
	0.70	**0.86**	**0.86**	**0.86**

was performed in a short analysis time (2.1 min) which minimizes the amount of waste and solvent consumed during the HPLC analysis. Accordingly, the proposed method offered improved detectability with the least solvent consumption compared with the reported methods [8–10].

Trio color-coded sustainable assessment (green, blue and white Assessment)

Environmental and green approaches have gained greater attention recently in different analytical techniques, including spectroscopy [22, 23], chromatography [24–28], and capillary electrophoresis [29, 30]. Moreover, the trio color-coded protocol [31, 32] offers all-inclusive assessment of each analytical stage. It is imperative that the analytical method address the issues of environmental safety and sustainable development goals. As a result, the suggested method's greenness (ecological), blueness (practicality), and whiteness (sustainability) were thoroughly compared with other reported HPLC procedures for the analysis of β-sitosterol [8–10].

A variety of evaluation tools were employed, such as Analytical Greenness metric (AGREE) [16] and Chloroform-oriented Toxicity Estimation Scale (Chlortox scale) [17] for the Greenness assessment; blue applicability grade index (BAGI) [18] was used to evaluate the economic and practical aspects. Additionally, RGB 12 model [19] and RGBfast [20] were put into practice to address every aspect of the previously discussed paradigms. These comprehensive evaluation tools assessed the levels of Whiteness or Greenness at various stages, making the method's environmental impact and sustainability quotient simple to comprehend. It is crucial to ensure that analytical methodologies are environmentally friendly before conducting practical laboratory trials to lessen the amount of chemical hazards released into the environment. Furthermore, it is highly advised to include different assessment tools for the evaluation of greenness and whiteness of the analytical methods as part of method validation procedures [33, 34].

The AGREE calculator has the merits of being automated, informative and the ability to identify the weakest points in the analytical methods that require further greenness enhancements. It is preferred to analytical eco scale in terms of consideration of method throughput, sample size and toxic hazards and bio-based solvent usage. Additionally, AGREE is better than GAPI in terms of automation and simplicity, allowing for the acquisition of comparable findings regarding the greenness of the approach with less effort [33]. The result of AGREE is summarized in Fig. 6 as a clock-like graph that illustrates the twelve Green Analytical Chemistry (GAC) principles in an insightful way. The reported HPLC method [9] had a slightly higher AGREE score this is due to the simple extraction from tablet and not from a challenging highly viscous matrix, MEBO. Moreover, being a green method does not mean very functional in the red or blue area, so it may will not represent the desired compromise.

The ChlorTox Scale is a global benchmark method for assessing greenness by calculating the relative hazard of several reagents. The ChlorTox value is calculated from the following equation.

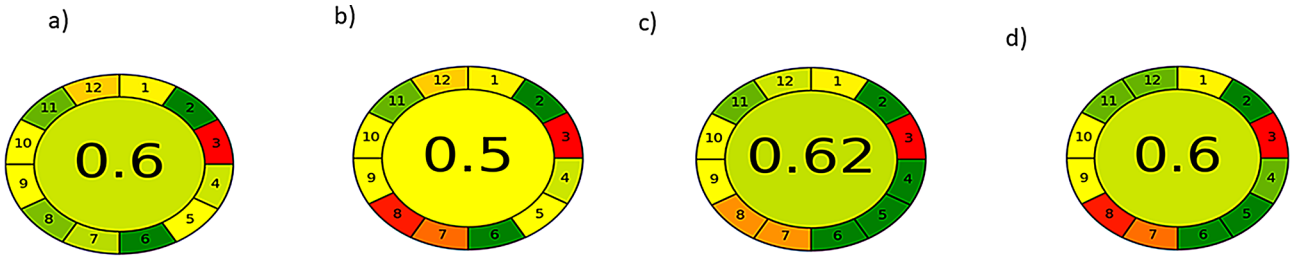


Fig. 6 AGREE assessment of the green profile of the evaluated procedures for determination of β-sitosterol by (**a**) the proposed HPLC method, with (**b**) reported HPLC [8], (**c**) reported HPLC [9] and (**d**)reported HPLC [10] methods

Table 6 Results of ChlorTox scores for the proposed method in comparison with reported methods in terms of the relative hazards with respect to chloroform (CHsub/$CHCHCl_3$) derived using the WHN model

Method	Stage	Compound	Relative hazard (CHsub/$CHCHCl_3$) (WHN)	msub (mg)	ChlorTox (g)	Total ChlorTox (g) (WHN)	Reference
Proposed HPLC analysis	Sample Extraction	Ethanol	0.26	19,750	5.13	38.96	Proposed method
		Diethyl ether	0.30	106,500	31.95		
		Sodium Hydroxide	0.61	1500	0.92		
	HPLC analysis	Methanol	0.57	1120	0.64		
		Acetonitrile	0.39	817	0.32		
Reported HPLC analysis	Sample Extraction	Potassium hydroxide	0.56	2800	1.57	12.41	[8]
	HPLC analysis	Ethanol	0.26	4526	1.18		
		Acetonitrile	0.39	24,780	9.66		
Reported HPLC analysis	Sample Extraction	Chloroform	1	1485	1.49	29.57	[9]
		Methanol	0.57	20,000	11.40		
		Acetonitrile	0.39	19,650	7.66		
	HPLC analysis	Methanol	0.57	14,480	8.25		
		Acetonitrile	0.39	1984	0.77		
Reported HPLC analysis	Sample Extraction	Ethanol	0.26	79,000	20.54	27.78	[10]
	HPLC analysis	Ethanol	0.26	5735	1.49		
		Acetonitrile	0.39	14,740	5.75		

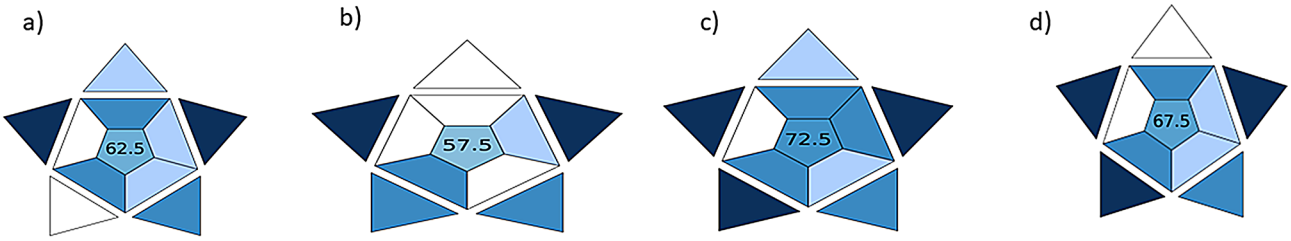


Fig. 7 BAGI assessment of the blue profile of the performance of the proposed HPLC method for determination of β-sitosterol by (**a**) the proposed HPLC method, with (**b**) reported HPLC [8], (**c**) reported HPLC [9] and (**d**) reported HPLC [10] methods

$$ChlorTox = \frac{CHsub}{CH_{CHCl3}} \cdot msub$$

Where, CH_{sub} is the substance-of-interest's overall chemical hazard, CH_{CHCl3} is the standard chloroform's chemical hazard and $msub$ is the mass of the material of interest.

The Sigma Aldrich safety data sheet was utilized to calculate the values of CH_{sub} and CH_{CHCl3} using the weighted hazards number (WHN) approach [17]. It is important to determine the correct amount of reagents required for each step of the process for example method calibration, instrument preparation, and instrument rinsing to avoid underestimating the overall procedure's risk. The results of the ChlorTox were illustrated in Table 6, the reported HPLC method [8] had the lowest total ChlorTox value of 12.41 g, this is attributed to the use of potassium hydroxide only in the sample preparation procedure but at the same time the extraction of β-sitosterol took more than 10 days which is considered as time consuming method and makes the method unapplicable. Moreover, the ChlorTox score lacks information about the total analysis time, energy consumption and validation criteria which are considered of the main principals of Green and white analytical chemistry.

The results showed that our method had a lower score in the greenness assessment compared to the other chromatographic method [8–10]. This is attributed to the fact that our sample preparation procedure necessitates the use of organic solvents related to the the non-polar nature of β-sitosterol and the complex matrix of MEBO (ointment). Conversely, the other reported methods extract β-sitosterol from supplements tablets, took more than 10 days to extract β-sitosterol from herbal medicines or the β-sitosterol was separated at a high retention time, causing higher waste production and greater solvent consumption. Moreover, the greenness assessment tools do not put into consideration the total duration of the sample extraction, applicability and excludes the validation criteria of the analytical procedure (reproducibility, accuracy, and precision).

For blueness assessment, the innovative BAGI software makes it simple to assess the effectiveness of the analytical technique and to pinpoint a method's strong and weak aspects in terms of application and practicality. Our method is deemed practicable with a score of 62.5 in the middle of the asteroid pictogram (> 60 points) (Fig. 7).

Table 7 RGB12 profiles of the proposed HPLC method and other reported methods

Method name	R (%)	G (%)	B (%)	Whiteness (%)
Proposed HPLC Method	93.8	80.8	85.0	86.5
Reported HPLC method [8]	20.0	80.8	72.5	57.8
Reported HPLC method [9]	95.0	84.6	78.3	86.0
Reported HPLC method [10]	70.0	82.1	72.9	75.0

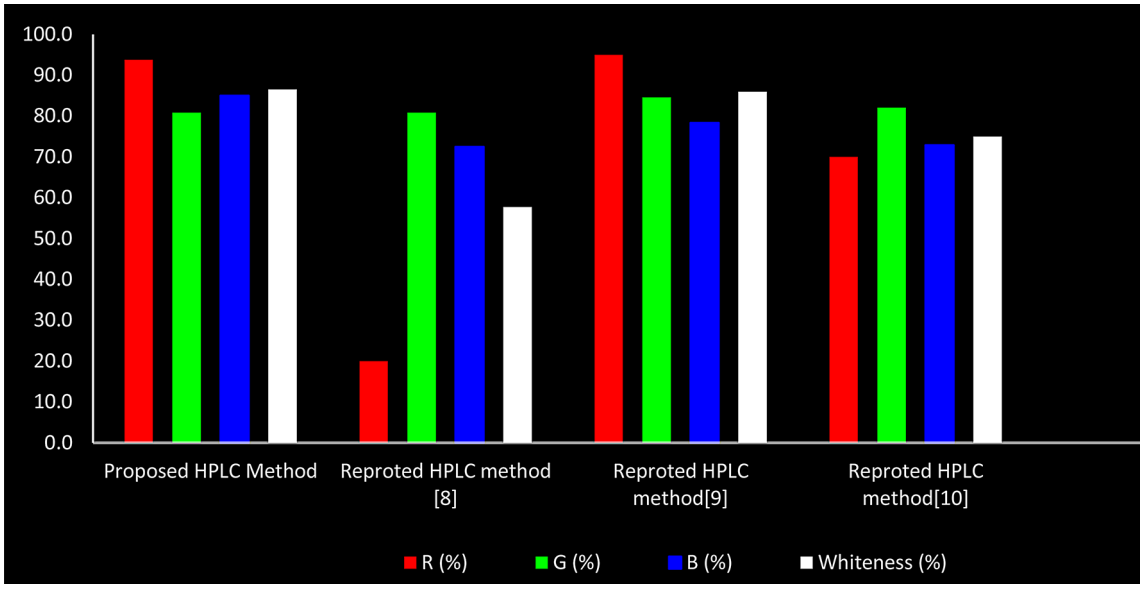


Fig. 8 Comparison of the main evaluation outcomes obtained from RGB12 analysis for the proposed HPLC method for determination of β-sitosterol with the reported methods. The white bar (whiteness %) indicates the arithmetic mean of the three other bars (red, green and blue)

Since each strategy has its drawbacks, using many models simultaneously seems like a reasonable solution because their unique characteristics might enhance one another. With the aim of creating a complete image of the environmental safety and sustainability of the method, the two versions of the RGB model will be evaluated as a sustainable assessment tool.

The RGB-12 multicriteria model was employed to obtain an exhaustive evaluation of the entire analytical procedure with respect to the validation criteria, GAC's principles and economic aspects. As shown in Table 7; Fig. 8, our suggested approach had the greatest blueness score of 85.0 and an acceptable greenness and redness score (80.8) and (93.8), respectively, and the highest arithmetic mean (86.5) for the whiteness score.

RGBfast is an improved version of RGB designed to make determining whiteness easier to use and more intuitive. It has six evaluation criteria and uses the Chlor-Tox Scale as its primary greenness indicator. As shown in Table 8, the recommended approach had the greatest whiteness score (58), compared with the reported methods [8–10].

Table 8 RGBfast profiles of the proposed HPLC method and other reported methods

Method	Redness	Greenness	Blueness	Whiteness
Proposed HPLC Method	83.6	47.0	50.4	58
Reported HPLC method [8]	28.7	58.6	10.6	26
Reported HPLC method [9]	73.4	45.4	55.3	57
Reported HPLC method [10]	57.9	52.6	60.6	57

The explanation of the high whiteness score obtained in both RGB and RGBfast is that the reported chromatographic HPLC methods [8–10] had long analysis time ranging from (13 min to 37 min), so high amount of reagent and waste consumption. On the contrary, our proposed HPLC method had the shorted analysis time (2.1 min) with minimum volume of waste and energy consumption. Moreover, it had a high red score due to the full validation with acceptable accuracy, precision and high sensitivity.

Finally, through the tri-color coded comprehensive strategy which considers a number of factors, such as the validation standards, practical and economic issues and fulfils the main objectives of the analytical chemistry. Our proposed HPLC method outperformed other alternatives techniques for determination of β-sitosterol in complex ointment matrix, in term of whiteness and applicability with acceptable green score.

Conclusion

This study developed a simple, accurate, precise, robust, and sensitive HPLC method for the detection of β -sitosterol in MEBO containing 0.25% β -sitosterol. The developed method offers several benefits, including a short run time, outstanding sensitivity, and the capacity to handle variations in chromatographic conditions without compromising the precision and accuracy of the results. These attributes render it a valuable instrument for routine pharmaceutical laboratory analysis. In addition to the application of the AGREE metrics, Chlortox, BAGI, RGB 12 and RGBfast for the greenness, blueness and whiteness appraisal demonstrated a synergy between analytical, environmental, and practical aspects.

Abbreviations

AGREE	Analytical Greenness metric
BAGI	Blue Applicability Grade Index
Chlortox	Chloroform-oriented Toxicity Estimation Scale
HPLC	High-performance liquid chromatography
ICH	International Council for Harmonisation
LOD	Limit of detection
LOQ	Limit of quantitation
MEBO	Moist Exposed Burn Ointment
R^2	Regression coefficient
RGB12	Red, green and blue
RSD	Relative standard deviation
UV	Ultraviolet

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Author contributions

H.I.A.: devised the project, the main conceptual ideas and proof outline, Visualization, Formal analysis, Validation, Writing—review & editing.A.M.A.H.: Visualization, Formal analysis, Validation, Writing—review & editing.S. A. A., S.A.: Investigation, Visualization, Formal analysis, original draft, Writing—review & editing. H.M.M.: Formal analysis, Validation, Writing—review & editing.A.R.A.: Formal analysis, Validation, Writing—review & editing.All authors have read and agreed to the published version of the manuscript.All authors reviewed the manuscript.

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Data availability

The datasets used during the study are available from the corresponding author on request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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