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RESEARCH ARTICLE

A Validated HPTLC Method for Quantification of Linoleic Acid and Beta-Sitosterol in *Solanum nigrum* Extract

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Abstract

Solanum nigrum, a member of the Solanaceae family, has long been used as both food and medicine. This plant has been recognized for its immunomodulatory, anti-ulcerogenic, hepatoprotective, and anti-hyperlipidemic effects. It contains various chemical components such as alkaloids, coumarins, flavonoids, tannins, saponins, proteins, carbohydrates, glycosides, and phytosterols. The objective of the current work was to create a repeatable and consistent HPTLC-validated technique for concurrently detecting Beta-Sitosterol (BTS) and Linoleic Acid (LIA) in *S. nigrum* in accordance with ICH recommendations. The HPTLC process involved using silica gel 60 F_{254} TLC plates as the stationary phase and a mobile phase consisting of n-hexane, ethyl acetate, and formic acid in a ratio of 6:4:0.2 (v/v/v). The resulting bands were uniformly visualized after derivatization with anisaldehyde sulfuric acid. The calibration curves of standard LIA and BTS exhibited satisfactory linearity within concentration ranges of 400-1150 ng/spot and 350-1200 ng/spot, respectively, with correlation coefficients (r^2) of 0.99153 and 0.99389. LOD and LOQ were determined as 91.96 and 118.14 ng/spot for LIA, and 278.66 and 314.62 ng/spot for BTS, respectively. In conclusion, this HPTLC method proved to be efficient, simple, precise, and reproducible for measuring LIA and BTS in *S. nigrum*.

Keywords: β-sitosterol, HPTLC, Linoleic Acid, *Solanum nigrum*, Validation

1. Introduction

Insufficient regulations pertaining to pharmaceuticals have contributed to a global increase in the demand for herbal remedies, leading to a decline in product quality and an upsurge in demand[1]. Identification and quality assessment are essential for crude herbal drugs, considering their complex and variable composition. Analytical control for these medicines requires less precise methods due to their diverse nature. To ensure adequate quality, a combination of chemical, physicochemical, and instrumental techniques should be employed. The World Health Organization (WHO) emphasizes the importance of utilizing advanced analytical methods and establishing analytical criteria to maintain quality standards[2].

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Solanum nigrum, a member of the Solanaceae family, also called black nightshade, grows well in diverse soil conditions and moist environment[3]. Within the field of healthcare, *S. nigrum* is utilized for the treatment of various ailments, including tonsillitis, ringworms, pneumonia, and tumors[4,5]. This medicinal plant is commonly incorporated as a crucial element in cancer therapies within the realm of traditional Chinese medicine[6]. The juice extracted from the berries of this plant is believed to have curative properties for conditions like diarrhea, eye ailments, hydrophobia, heart disease, and anasarca. The berries are reputed to possess tonic, diuretic, and cathartic effects[7].

Linoleic acid (LIA) is an omega-6 polyunsaturated fatty acid that exhibits beneficial physiological properties such as anti-atherosclerotic, anti-cancer, anti-menorrhagic, hepatoprotective, and immunomodulatory effects, among others, as supported by scientific studies[8]. β-sitosterol (BTS), a phytosterol, is associated with numerous beneficial physiological effects, such as androgenic, antiadenomic, anti-inflammatory, antiandrogenic, anticancer, antibacterial, and antilymphomic properties, among others[9]. BTS[10] and LIA[9] were found in *S. nigrum*.

By employing various analytical techniques, including HPTLC[9] and GC-MS[11], the simultaneous detection and quantification of LIA and BTS were achieved. A previous HPTLC study quantified these compounds in a herbal hair gel formulation but skipped technique validation.

S. nigrum is an important ingredient in numerous herbal remedies used to treat various ailments. BTS and LIA, the key components, are found in many remedies that target various health problems. However, due to inadequate analysis techniques, most commercially available formulations lack standardization, impeding compliance with safety regulations. After conducting a literature search, it was found that the previously mentioned GC-MS[11] and HPTLC[9] methods were the only reported techniques for simultaneously analyzing BTS and LIA. However, GC-MS has drawbacks due to its expensive and time-consuming sample preparation process, making it unsuitable for routine quality control testing in laboratories. In contrast, HPTLC offers a simple and effective method for standardizing and estimating LIA and BTS in *S. nigrum*. This HPTLC approach can also be applied to evaluate these phytoconstituents in other plant products with similar components. Notably, there have been no published studies on the simultaneous estimation of LIA and BTS in *S. nigrum* using HPTLC, making this study the first of its kind.

An HPTLC approach for the simultaneous assessment of LIA and BTS in SNB is being developed and validated in this work. The procedure makes use of silica gel 60 F_{254} TLC plates with a mobile phase containing the proportions 6:4:0.2 (v/v/v) of n-hexane, ethyl acetate, and formic acid. Derivatization is followed by quantitative analysis employing densitometric scanning at a wavelength of 540 nm.

2. Materials and Methods

2.1 Instrumentation

The HPTLC instrument was employed to concurrently measure LIA and BTS in SNB. The specific details of the instrumentation are as follows:

- Sample application: Camag Linomat V sample applicator from Muttenz, Switzerland.
- Plate saturation: Under a twin-trough chamber.

2.1.1 Experimental Settings of HPTLC Instrument

- Slit dimension: 6 mm × 0.45 mm.
- Spraying rate: 10 s/L.
- Scanning speed: 20 mm/s.
- Monochromator bandwidth: 20 nm.
- Data resolution: 100 mm/step.
- Densitometer: Camag TLC Scanner III with WinCATS software.
- Light source: Deuterium lamp.
- Filter wavelength: 540 nm.
- Chromatographic plates: Silica gel 60 F_{254} TLC plates used are from E. Merck, measuring 20 cm × 20 cm with a thickness of 0.25 mm.

2.2 Reagents and Chemicals

The reference standards for LIA and BTS were obtained from Innovative Chemical Exchange Pvt. Ltd. (Carbino), with accuracy levels of 97% and 98%, respectively. All chemicals used in the study were of analytical grade, and the solvents were of spectroscopic quality, including methanol, petroleum ether, anisaldehyde, sulfuric acid, ethyl acetate, formic acid, and n-hexane, sourced from Merck in Mumbai, India.

2.3 Preparation of Plant Sample

Solanum nigrum Berries (SNB) were collected from Kolkata in January. A taxonomist verified and authenticated the

plant material. The collected berries were air-dried and then finely powdered after passing through a number 10 filter.

2.4 Preparation of Standard

To serve as quantification reference markers, approximately 1 mg of each reference standard, LIA and BTS, were separately dissolved in 2 mL of methanol (0.5 mg/mL). The calibration curve was established following the guidelines set by the International Conference on Harmonization (ICH)[12].

2.5 Preparation of Sample

Petroleum ether extract was obtained from 100 g of SNB's dried coarse powder using a soxhlet apparatus. The powder was tightly packed, subjected to continuous heat extraction at 60-80°C for two days, filtered, and the resulting filtrate was evaporated under reduced pressure. The end result was a semisolid substance. 50 mg of the extract was diluted with petroleum ether and added to a 10 mL volumetric flask for analysis.

2.6 Calibration Curve Preparation

The standard curve followed ICH guidelines. Each concentration of standards was spotted in triplicate on a 20 x 10 cm TLC plate, with 6 mm wide bands separated by 11.2 mm. The plate had bottom and side edges positioned 12 mm and 8 mm away, respectively. A mobile phase of n-hexane, ethyl acetate, and formic acid (6:4:0.2, v/v/v) was used for band development after 20 minutes of saturation. Anisaldehyde sulfuric acid was applied for derivatization, followed by air drying. The plate was heated at 110°C for 5 minutes and analyzed using a densitometric scanner at 540 nm. Linear regression analysis evaluated the peak area.

2.7 Sample Assay Preparation

Following the procedure mentioned earlier, the sample and standard solutions were prepared. They were then spotted on a TLC plate and developed under the same circumstances as those specified for the standard. The plates underwent development, derivatization, and drying in a hot air oven, ensuring complete separation of the analyte from other components. As a result, distinct peak regions corresponding to LIA and BTS were observed when scanning the linear and compact zones at a wavelength of 540 nm.

3. Results and Discussion

The resolution of different ratios of n-hexane, ethyl acetate, and formic acid in the ratio of (6:4:0.2 v/v/v) on TLC plates was satisfactory. After derivatization, a well-defined and symmetrical band for LIA and BTS was observed in the SNB extract on the TLC plate (Figure 1). Derivatization with anisaldehyde sulphuric acid reagent resulted in blue and pink color spots for BTS and LIA, respectively. The HPTLC chromatograms of the standard LIA (R_f = 0.56) and BTS (R_f = 0.78) are shown in Figures 2 and 3, respectively. Scanning at a wavelength of 540 nm provided the best scanning result within the range of 200 to 700 nm for BTS and LIA provided in Figure 4. Densitogram scanning of the LIA and BTS standards at 540 nm was depicted in Figure 5. The optimized HPTLC chromatographic parameters for the analysis of LIA and BTS are provided in Table 1. The stability of the solvent suitability for the mobile phase was determined to be consistent and unchanged over a period of 24 hrs.

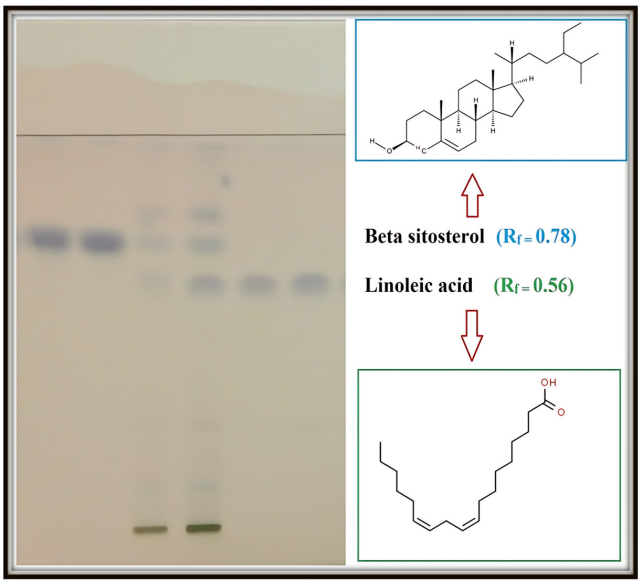

Figure 1. After derivatization standard linoleic acid, petroleum ether extract of *Solanum nigrum* berries, beta-sitosterol.

Table 1. Chromatographic optimization of LIA and BTS in HPTLC

Chromatographic factor		Conditions
Mobile phase		n-hexane, ethyl acetate, and formic acid at the ratio of (6:4:0.2 v/v/v)
Stationary phase		Silica gel 60 F_{254} (20 cm × 20 cm)
Temperature		27 ± 0.5 °C
Band width and separation		6 mm and 11.2 mm
Distance travel (mm) by mobile phase		80
Duration of chamber saturation (min)		20
Derivatization reagent		Anisaldehyde sulfuric acid
Derivatization method		Heat in hot air oven at 110°C for 5 minutes
Speed of scanning (mm/s)		20
Measuring wavelength (nm)		540
Retention factor (R_f)	LIA	0.56
	BTS	0.78
Diluent		Methanol

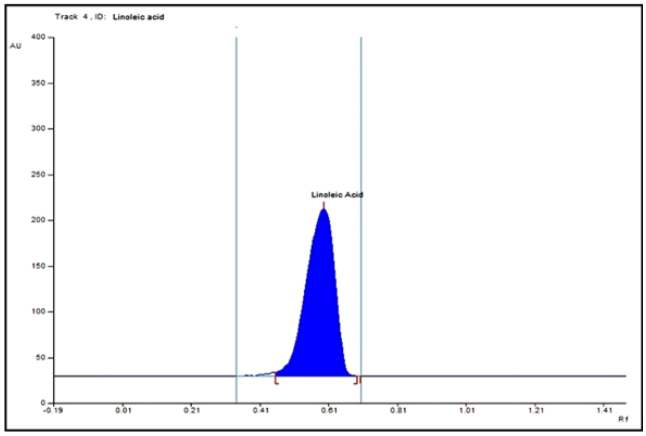

Figure 2. Standard linoleic acid HPTLC chromatogram.

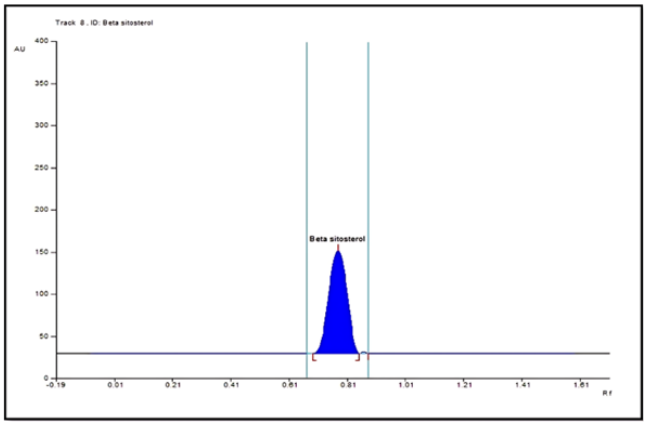

Figure 3. Standard β-sitosterol HPTLC chromatogram.

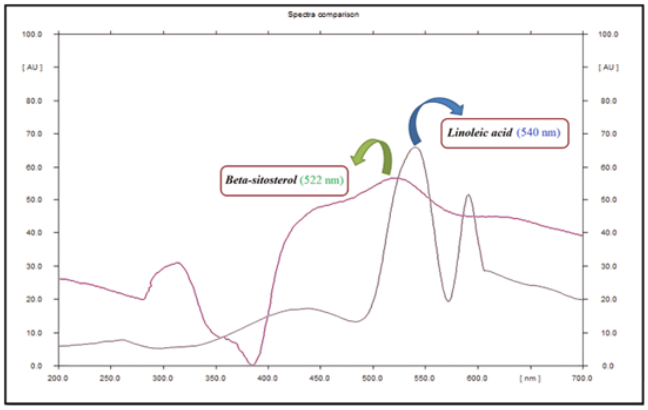

Figure 4. Scanning range of LIA and BTS from 200 - 700 nm.

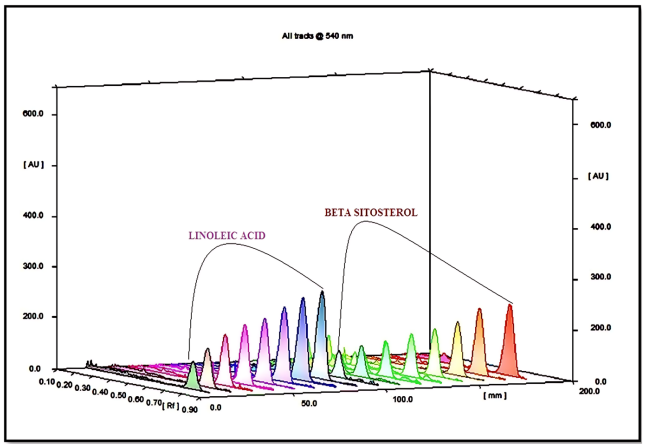

Figure 5. Densitogram scanning of LIA and BTS standard.

3.1 Linearity

By examining a number of varied concentrations for both LIA and BTS, each in triplicate, the linearity of the HPTLC technique was evaluated. The results showed that the standard curve for LIA exhibited a satisfactory linear relationship with the spot area over the concentration range of 400-1150 ng/spot, as indicated by the linear regression equation: y = 58.7305x - 3599.35, with a correlation coefficient (r^2) of 0.99153 (Figure 6). Similarly, BTS also demonstrated a good linear relationship with the spot area over the concentration range of 350-1200 ng/spot, with a linear regression equation of y = 28.5925x + 47.71622 and an r^2 value of 0.99389 (Figure 7).

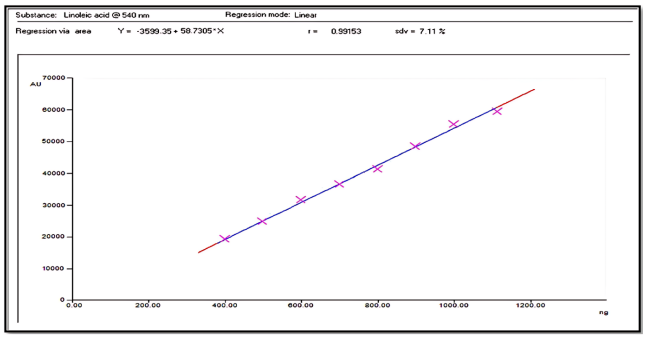

Figure 6. Linoleic acid standard calibration plot.

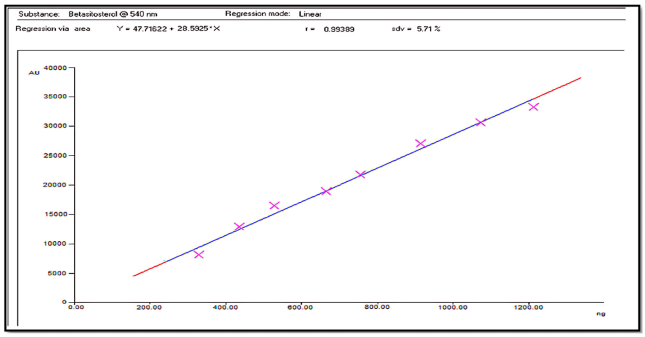

Figure 7. β-sitosterol standard calibration plot.

3.2 Method Precision (Repeatability)

Evaluation of method precision involved injecting reference standard solutions of LIA and BTS six times. The method's repeatability was tested by utilizing an automated spotter to apply the same sample solution on a plate six times, followed by repeated scans of the examined spot for LIA and BTS without moving the plate. The results were analyzed in terms of the Coefficient of Variation (CV), which is a measure of the variability. The precision and repeatability data are presented in Tables 2 and 3, respectively. The low CV values, as presented in Tables 2 and 3, indicate that the method exhibits high precision.

Table 2. Data from linear regression for LIA

Amount (ng/spot)	Area, mean ± SD* (n=6)	CV#
400	19480.66 ± 106.66	0.005
500	26247 ± 66.693	0.002
600	31798.16 ± 199.89	0.006
800	41266.33 ± 51.24	0.001
900	50465.83 ± 75.65	0.001
1000	57265.50 ± 60.09	0.001
1150	62490 ± 88.76	0.001

* SD: Standard deviation where n is number of samples (n=6); CV#: Coefficient of variation

Table 3. Data from linear regression for BTS

Amount (ng/spot)	Area, mean ± SD* (n=6)	CV#
350	8915.16 ± 207.37	0.023
450	13651.5 ± 62.73	0.004
600	17979.33 ± 93.07	0.005
750	21837 ± 99.82	0.004
950	27209 ± 38.08	0.001
1100	31505.50 ± 81.37	0.002
1200	33934.83 ± 84.45	0.002

* SD: Standard deviation where n is number of samples (n = 6); CV#: Coefficient of variation

3.3 Intermediate Precision (Reproducibility)

Intraday and interday precision evaluation of the proposed methods involved preparing standard solution mixtures of LIA and BTS at two different concentrations (450 and 750 ng/spot for LIA, and 500 and 800 ng/spot for BTS) three times on the same day and the next day. The relative standard deviation (RSD), which is a measure of the variability, was calculated for the obtained results. The precision data for intraday and interday variations are presented in Table 4. The RSD values, as indicated in Table 2, for both intra-day and inter-day measurements of LIA and BTS, do not exceed 2%. This indicates that the method demonstrates a high level of reproducibility and intermediate precision.

3.4 Accuracy (Percentage Recovery)

Method accuracy was evaluated by calculating the recovery of LIA and BTS using the standard addition approach. Three separate recovery trials were conducted at different levels (50%, 100%, and 125% inclusion of LIA and BTS) to assess the accuracy of the methods. The per cent recoveries and the total mean recoveries were calculated by determining the peak area values and applying them to the regression equations of the calibration curves. The levels of LIA and BTS were then calculated based on these results. The accuracy data, including percent recoveries and total mean recoveries, are presented in Table 5. The mean percentage recovery of LIA and BTS was 100.41 and 100.006.

Table 4. Intermediate precision of LIA and BTS

Marker	Concentration (ng / spot)	Intra-day (n = 3)		Inter-day (n = 3)	
		Concentration ± SD*	RSD (%)	Concentration ± SD	RSD (%)
LIA	450	451 ± 5.56	1.235	450 ± 3.46	0.770
	750	750 ± 7.21	0.961	752 ± 6	0.798
BTS	500	502.33 ± 5.68	1.132	502.33 ± 5.50	1.096
	800	805.66 ± 3.78	0.470	804.66 ± 3.05	0.380

* SD : standard deviation where n is number of times (n = 3)

Table 5. Recovery data of LIA and BTSBTS

Marker	Marker concentration level (ng)	Added marker concentration level (ng)	Detected marker concentration level (ng)	Recovery (%)	Mean recovery (%)
LIA	400	200	603.66 ± 4.50	100.50	100.41
	400	400	803.33 ± 4.72	100.41	
	400	425	827.66 ± 4.50	100.32	
BTS	450	225	677 ± 5	100.29	100.006
	450	450	898 ± 10.58	99.77	
	450	562	1011.66 ± 5.85	99.96	

* SD: standard deviation where n is the number of samples (n=3)

3.5 Limit of Detection and Limit of Quantification

The Limit Of Detection (LOD) and Limit Of Quantification (LOQ) were calculated for both substances in accordance with ICH recommendations. LOD was determined with a signal-to-noise ratio (S/N) of 3:1, while LOQ was computed with S/N of 10:1 using the specified equations:

$$\mathbf{LOQ = 3.3 \times \sigma/S}$$

$$\mathbf{LOQ = 10 \times \sigma/S}$$

Where the response of standard deviation is σ and S stands for the standard deviation of y-intercept of the regression line. LIA's LOD and LOQ were 91.96 and 118.14 ng/spot, respectively. For BTS, the LOD and LOQ values were 278.66 and 314.62 ng/spot, respectively.

3.6 Concentration Percentages of LIA and BTS in SNB Extract

Figure 8 displays the chromatogram obtained under the optimized HPTLC conditions, revealing the presence of LIA and BTS. The concentration of LIA and BTS in the SNB petroleum ether extract was determined to be 31.16% and 1.84% w/w, respectively. The peak areas of the sample were quantified using a regression equation derived from the calibration plot, enabling accurate determination of LIA and BTS contents. The R_f values of 0.56 and 0.78 confirmed the successful separation of LIA and BTS. Additionally, a comparison of R_f values between the standard and sample verified the specificity of the method.

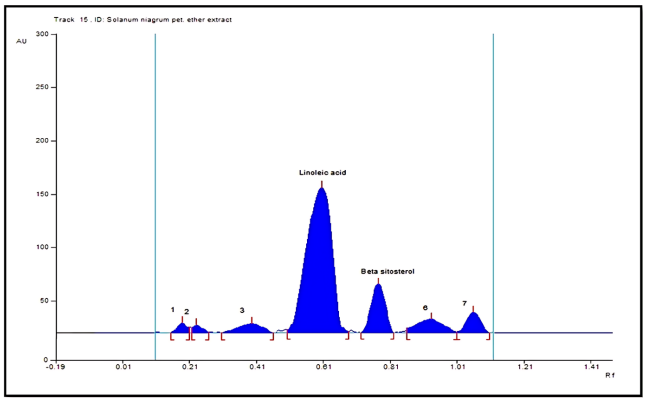

Figure 8. Chromatographic evaluation of *S. nigrum* berry extract in petroleum ether by HPTLC.

Validation of the HPTLC method was conducted in accordance with the International Conference on Harmonisation (ICH) guidelines[13]. Precision studies, showed % RSD values within acceptable limits (≤2), indicating good intra-day and inter-day variations for the simultaneous determination of LIA and BTS in SNB extract. Calibration curves demonstrated strong linear relationships with correlation coefficients (r^2) ≥ 0.99. Figures 6 and 7 depict the linearity curves. For LIA and BTS, the percent recovery is shown in Table 5. The experimental results obtained fall within the acceptable range for accuracy, which is between 97.0% and 103.0%[14]. Additionally, the validation process included the determination of important parameters such as the LOQ and LOD. The results obtained from these validation parameters provided strong evidence that the analytical method employed was reliable and suitable for the analysis of the two compounds. Consequently, the HPTLC method was used to quantify the concentrations of these active compounds in the petroleum ether extract of SNB. The same HPTLC method can be utilized to assess these phytoconstituents in comparable plant products with analogous components.

4. Conclusion

The HPTLC method for quantitative analysis of LIA and BTS in *S. nigrum* plant berries has an RSD of less than 2%, which is a reassuringly sufficient indication of its accuracy. The high recovery rates of LIA and BTS (99.77-100.29% and 100.32-100.50%, respectively) indicate that the technique is effective and reliable. The fingerprint profiling of chromatograms derived from extracts of *S. nigrum* is a quick, easy, and sensitive method that can be used to compare and assess commercial samples of plant berries. The HPTLC method is a sensitive method that can be used to detect even small amounts of LIA and BTS in *S. nigrum* berries. Validation of the method followed ICH guidelines, providing a quick, easy, and sensitive HPTLC approach to assess the quality of *S. nigrum* berries.

5. Acknowledgements

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6. References

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