Short Communication

Simultaneous determination of β-sitosterol and gallic acid in *Nigella Sativa* seeds using reverse phase high performance liquid chromatography

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Received: 29 May 2020 / Accepted: 14 October 2020 / Published online: 23 October 2020
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Abstract
HPLC determination of β-Sitosterol and Gallic acid in seeds of *Nigella sativa* was carried out using reversed-phase Grace C18 column (250 mm × 4.6 mm id, 5 µm particle size), with UV detection at 210 nm. The mobile phase for column consisted of 60:40 v/v acetonitrile–water at a flow rate of 0.5 ml/min. The pH of the mobile phase was adjusted to 3.00, with 0.05% ortho-phosphoric acid. The linear response was examined in the concentration range of Gallic acid and β-Sitosterol between 0.5 and 2.5 µg/mL, and linear correlation coefficients of 0.9983 for Gallic acid, and 0.9993 for β-sitosterol, respectively, were observed. The detection limit was 4.8 µg/mL, and the recovery test in the determination of β-Sitosterol and Gallic acid revealed 98–101% for both compounds.

Keywords RP-HPLC · *B*-sitosterol · Gallic acid · ICH guidelines · *Nigella sativa*

1 Introduction

Spices extracted from the edible parts of a plant are widely used in food for flavoring, giving aroma, pungency, or coloring. These spices are normally extracted from fruits, seeds, bark or roots of plants [1]. Spices have huge contribution not only to the food industry, but also to medicine or herbal drug formulation due to their medicinally important properties [2], and being a rich source of essential oils.

Nigella sativa (Fam. Ranunculaceae), also called black cumin, is a herbaceous, dicotyledon annual flowering plant, and a miraculous herb with a rich historical and religious background. The fruit provides several seeds that are black or gray from outside and white inside. The seeds of a typical size of 2–3.5 × 1–2 mm are angular, stimulant, diuretic, and are used in puerperal fever. These seeds showed antispasmodic, analgesic, and CNS depressant activities [3, 4]. In one of the Prophetic hadith, black seeds were mentioned in connection with the cure of all diseases [5]. The seeds and their oil exhibited favorable medicinal activities as immunomodulatory [6], anticancer [7, 8], antimicrobial [9, 10], analgesic, anti-inflammatory [11], gastroprotective [12], hepatoprotective [13], antioxidant [14] and renal protective properties [15]. The seeds can also be used for the treatment of several diseases such as diarrhea, bronchitis, rheumatism, asthma, skin diseases, and liver problems. In addition, they can be used as appetite stimulant, agent to increase the milk of pregnant women, and support of the overall immune system [16]. Also, methanolic extract of *Nigella sativa* exhibited strong antifungal activity on different strains of *Candida albicans* [17].

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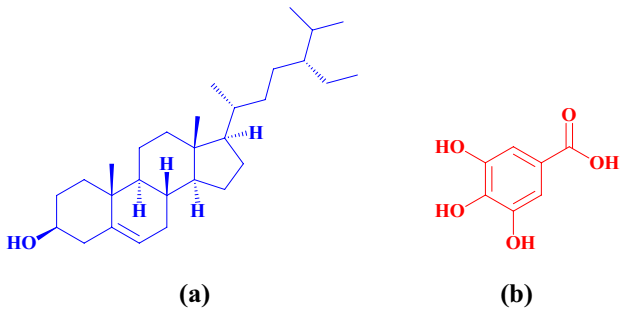

Fig. 1 **a** Structure of β-sitosterol; **b** structure of gallic acid

A number of active chemical compounds in *N. sativa* seeds have been isolated, identified and studied. Among these are thymoquinone (found with abundance of 30–48%), 4-terpineol, thymohydroquinone, p-cymene, dithymoquinone, carvacrol, sesquiterpene, thymol and α-pinene. In the *N. sativa* seeds studied by Ramadan and Morsel [18], β-Sitosterol was found to be the main component of the phytosterols.

The literature survey revealed that no method has been reported for simultaneous quantification of β-Sitosterol (Fig. 1a) and Gallic acid (Fig. 1b) from the seeds alcoholic extract of *N. sativa*. Therefore, the aim of the present work was to develop and validate a new simple, accurate and rapid analytical method for the simultaneous determination of Gallic acid and β-Sitosterol with short run time. The present work includes using a reversed-phase HPLC method with UV detection at 210 nm for the determination of Gallic acid and β-Sitosterol in *N. sativa* seeds, which can be reliably employed as a practical method for pharmaceutical study of any dosage form containing Gallic acid and β-Sitosterol. The validation parameters have been studied as per ICH guidelines [19–25].

2 Materials and methods

2.1 Standards and reagents

β-Sitosterol (Potency = 99.7%) and Gallic acid (Potency = 99.0%) were sourced from Sigma Aldrich (Germany). Acetonitrile (HPLC grade) and Ortho-phosphoric acid (AR grade) were supplied by Merck (Mumbai, India). Water was purified using Milli-Q Millipore system. All the solvents were filtered through a 0.45 µ membrane filter paper (MDI make).

2.2 Apparatus, analytical column and chromatographic conditions

A Younglin (S.K) Gradient HPLC System with UV Detector and manual injector with 20 µl was employed for the experiments. The HPLC separation was achieved on a reversed phase chromatographic column C18 (Make-Grace). The column dimensions were 4.6 mm × 250 mm id, with 5 µm particle size. The chromatographic system was operated with Autochro-3000 software for data acquisition and processing. A mixture of acetonitrile and water in a volume ratio 60:40 v/v was used as a mobile phase. The pH of a mobile phase was adjusted to 3.00 using 0.05% ortho-phosphoric acid. The flow rate of the mobile phase was 0.5 mL/min. The detector wavelength was set at 210 nm. The analysis was performed at ambient temperature (Table 1).

2.3 Plant material

Nigella sativa seeds were collected from Krishi Kendra, Nagpur, India. The seeds were botanically recognized, confirmed and authenticated by the Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, India; (Authentication number is 9990). The collected seeds were dried in air, and then ground to a fine powder in a mixer (Havells make), sieved, weighed and stored in airtight container at room temperature.

2.4 Preparation of plant extract

After the seeds were air-dried and ground to fine powder, 1.0 g of the *N. sativa* powder was accurately weighed in 20 mL vial followed by the addition of 10 mL of methanol. The methanol extract was filtered through Whatmann filter paper (No. 1), and then distilled under reduced pressure to remove the volatile solvent. The obtained residue was diluted to furnish a final concentration of 100 µg/ml. This solution was subjected to HPLC analysis for simultaneous quantification of Gallic acid and β-Sitosterol.

Table 1 Chromatographic conditions

HPLC system	Younglin (S.K) gradient system UV detector
Software	Autochro-3000
Detector	UV 730 D
Wavelength	210 nm
Pump	SF 930 D
Stationary phase	C18 (Grace)
Mobile phase	Acetonitrile: water 60:40 (v/v) (0.05% OPA)
Flow rate	0.5 ml/min
Injection volume	20 µl
Diluent	Methanol (HPLC grade)
Column length	4.6 × 250 mm
Particle size packing	5 µ
Column temperature	Ambient

2.5 Preparation of standard solutions

A stock solution of Gallic acid (1000 µg/ml) was prepared by dissolving 10 mg of Gallic acid in 10 ml methanol in a volumetric flask. Similarly, stock solution of β-sitosterol (1000 µg/mL) was prepared by the same method using 10 mg of β-Sitosterol. The series of working standard solutions were prepared by diluting appropriate aliquots of the stock solution with methanol, to yield the concentration of Gallic acid and β-sitosterol in the range of 0.5–2.5 µg/mL.

2.6 Method validation

The method validation parameters such as linearity, precision, accuracy, assay, robustness, repeatability, recovery limits of detection (LOD) and limits of quantification (LOQ) were evaluated using international conference on harmonization ICH Q2 (R1) procedures. The linearity was investigated over the range of 0.5–2.5 µg/L (in steps of 0.5) µg/ml for both β-Sitosterol and Gallic acid. The LOD and LOQ values were calculated based on the standard deviation (SD) of the response of the standard blank and the slope (s) of calibration curve at signal-to-noise ratio of 3:1 and 10:1, respectively, where LOD = 3.3 (SDs) and LOQ = 10 (SDs). The recovery studies were carried out using β-Sitosterol and Gallic acid at three fortification levels (80%, 100% and 120%) to pre-quantified sample solution of β-Sitosterol and Gallic acid. For each recovery level, three samples were prepared and analyzed, and the percentage of recovery was calculated. To evaluate the precision of the developed method, a sample with concentration level 120 µg/L was processed in replicates (n = 6) and subjected to HPLC analysis. The relative standard deviation (% RSD) is less than 2%. Repeatability, expressed as relative standard deviation (% RSD), was done by extracting six replicates at concentration levels of 0.5-2.5 µg/mL of Gallic acid and β-Sitosterol. The acceptance standard was ±2% for the percent relative standard deviation (% RSD) for the peak area and retention times for Gallic acid and β-Sitosterol.

3 Result and discussion

Ionizable compounds (acids and bases) separation depends on the pH of the mobile phase in terms of column efficiency, selectivity and retention. On a non-polar column, the retention is typically improved by adjusting the pH so that the analytes are isolated in their unionizable forms. Furthermore, the interaction between the analyte and the silica surface of the column packing, which leads to poor peak shape, could be reduced by choosing the correct mobile phase composition and adjust the pH.

3.1 Development of RP-HPLC method

In order to examine the best conditions for the RP-HPLC chromatographic separation of Gallic acid and β-Sitosterol from the *N. sativa* Seeds, the chromatograms of Gallic acid and β-Sitosterol on C18 column, in a mobile phase with varying the composition of mobile phase, flow rate, wavelength, and pH were tested to identify the best results for peak separation, retention time and peak shape. A satisfactory resolution of β-Sitosterol and Gallic acid was obtained with mobile phase 60:40 v/v acetonitrile-water, with pH adjusted to 3.0 using 0.05% Ortho-phosphoric acid, at a flow rate of 0.5 ml/min to obtain better reproducibility. Typical chromatograms of Gallic acid and β-Sitosterol are shown in (Figs. 2, 3).

3.1.1 System suitability

The system suitability was assessed by six replicate injection of Gallic acid and β-Sitosterol at a concentration of 0.5–2.5 µg/ml, giving %RSD values for retention time and peak area within 2%, indicating low variation of the measured values (Table 2). The tailing factors (T) for Gallic acid and β-Sitosterol were 1.51 and 1.17, respectively, demonstrating symmetry of all peaks ($T < 2$). The resolution (R) between Gallic acid and β-Sitosterol was 8.595, indicating a high degree of peak separation ($R > 2$). The efficiency of the column, as expressed by the number of theoretical plates, was more than 4900 for Gallic acid and 8500 for β-Sitosterol. These results indicate the suitability of the HPLC system and conditions, which were then used for further validation and sample analysis. Moreover, as seen from Figs. 2 and 3 the chromatographic peaks are well resolved, indicating the suitability of the proposed method for the retention study of Gallic acid and β-Sitosterol with good repeatability of retention times, thus confirming the suitability of the proposed method for the stability study.±

3.2 Linearity, limits of detection (LOD) and quantification (LOQ)

The developed method showed linear response in the concentration range from 0.5 to 2.5 µg/mL for both Gallic acid and β-Sitosterol (Figs. 4, 5). The calibration curves were determined from the best linear fit to the experimental data in the tested concentration range, and the fitting parameters obtained from the regression analysis are shown in Table 3. The regression equations were $y = 2120.879\ x - 27.866$ ($r^2 = 0.9983$) and $y = 1286.921\ x - 322.145$ ($r^2 = 0.9993$) for Gallic acid and β-Sitosterol, respectively. The correlation coefficients were all greater than 0.99, indicating high degrees of correlation and good linearity. The LOD was 0.0128 µg/mL for Gallic

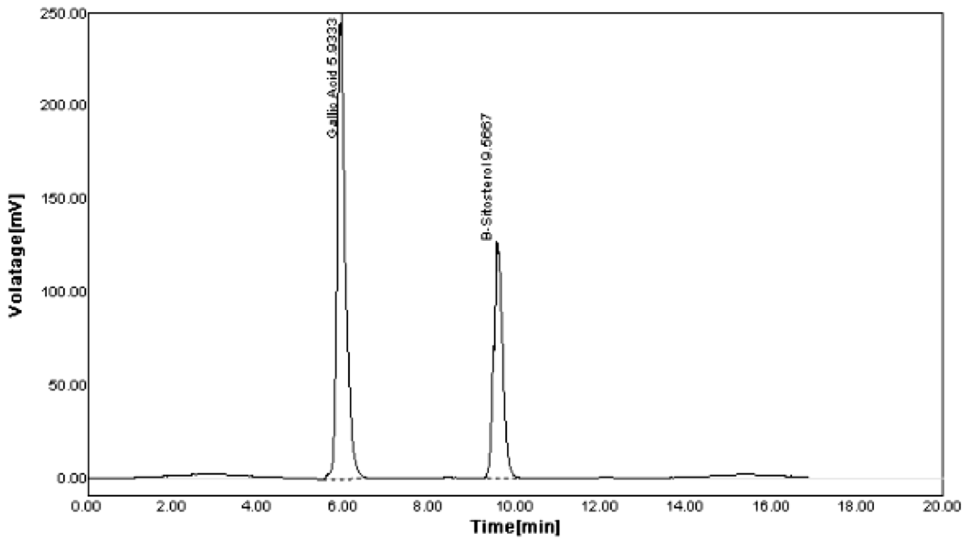

Fig. 2 Chromatogram of gallic acid and β-sitosterol in standard solution

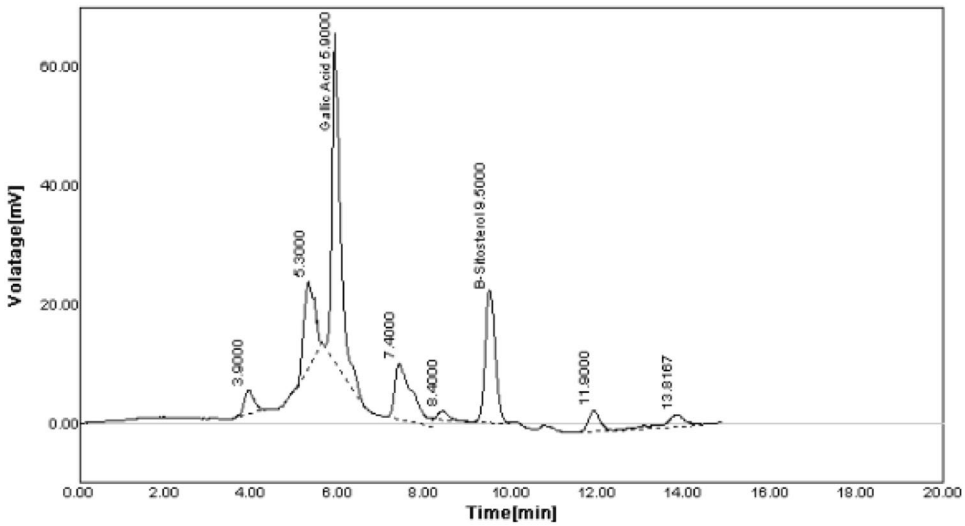

Fig. 3 Chromatogram of methanolic extract of *Nigella sativa* seeds

Table 2 System suitability parameters for gallic acid and β-sitosterol

Parameters	Gallic acid ± RSD (n = 6)	β-Sitosterol ±RSD (n = 6)
Retention time (min)	5.90 ± 0.463	9.61 ± 0.570
Tailing factor	1.51 ± 0.561	1.17 ± 0.768
Theoretical plates	4960.9 ± 0.942	8521.28 ± 1.0817
Resolution	8.595 ± 0.395	

RSD relative standard deviation

acid and 0.0227 µg/mL for β-Sitosterol, while the LOQ was 0.0389 µg/mL for Gallic acid and 0.0689 µg/mL for β-Sitosterol (Table 4). These results indicate that the method provided adequate sensitivity.

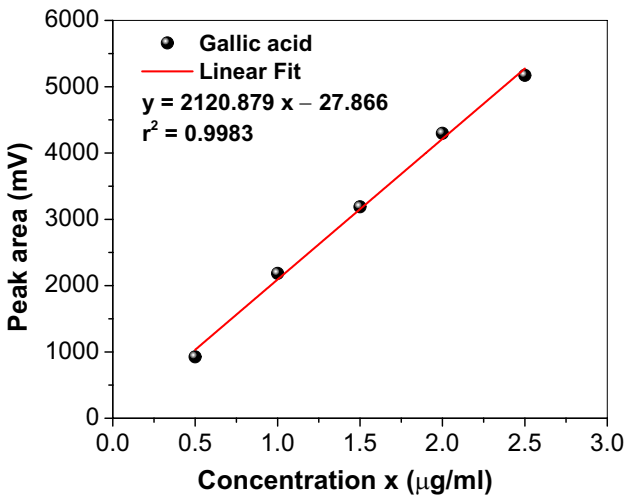

Fig. 4 Calibration curve for gallic acid

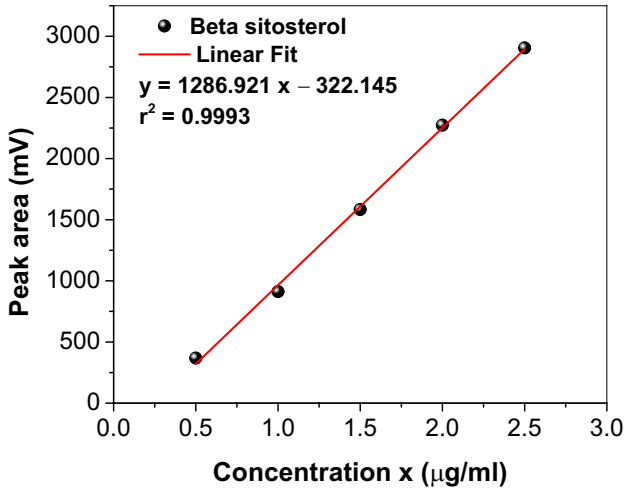

Fig.5 Calibration curve for β-sitosterol

Table 3 Regression analysis of calibration curves for gallic acid and β-sitosterol (n = 6)

Parameters	Gallic acid	β-Sitosterol
Linearity range (μg /ml)	0.5–2.5 μg/ml	0.5–2.5 μg/ml
Slope (m)[a]	2120.879	1286.921
Intercept (c)[a]	− 27.866	− 322.145
Correlation coefficient (r^2)	0.9983	0.9993

[a]Equation $y=mx + c$, where y is peak area, m is the slope, x is the concentration and c is the intercept

Table 4 LOD and LOQ value of gallic acid and β-sitosterol

Standard	LOD (μg/ml)	LOQ (μg/ml)
Gallic acid	0.0128	0.0389
β-sitosterol	0.0227	0.0689

3.3 Precision

The intra-day and inter-day precision are given in Table 5. The % RSD values for intra-day and inter-day precision were 0.35–1.45 and 0.41–1.32, respectively, for Gallic acid, while for the β-Sitosterol the intra-day and inter-day values were 0.64–1.23 and 0.89–1.21, respectively. The low values of %RSD (< 2%) indicate that the precision of this method is high.

3.4 Recovery (accuracy)

The percentage recoveries for intra-day and inter-day are given in Table 6. The % RSD values are less than 2% and the percentage recoveries were 99.08–101.84 and 98.75 –100.13 for Gallic acid and β-Sitosterol, respectively. All percentage recoveries were within 98–102%, indicating reliable accuracy of the method.

Table 5 Precision study of gallic acid and β-sitosterol

Standard	Level (n = 3)	Intraday amount found (%) ± RSD	Interday amount found (%) ± RSD
Gallic acid	1	101.60 ± 1.45	100.82 ± 1.32
	2	99.22 ± 0.35	99.74± 0.41
	3	98.87 ± 0.39	99.94 ± 0.96
β-sitosterol	1	98.70 ± 0.64	99.32 ± 1.21
	2	99.22 ± 1.23	99.93 ± 1.14
	3	100.10 ± 1.08	99.64 ± 0.89

Table 6 Recovery study of gallic acid and β-sitosterol

Standard	Level (n = 3) (%)	% Recovery ± % RSD	Mean recovery
Gallic acid	80	99.08 ± 0.64	100.26
	100	99.86 ± 1.08	
	120	101.84 ± 0.35	
β-sitosterol	80	98.75 ± 1.08	99.62
	100	99.98 ± 0.94	
	120	100.13 ± 0.96	

Table 7 Assay study of Gallic acid and β-Sitosterol in *Nigella sativa* seeds extracts

Sample	Content of marker compound (μg/ml)	
	Gallic acid	β–Sitosterol
Methanolic extract of *Nigella sativa* seeds	0.4736	0.4247

3.5 Assay study

The result of Assay study of *N. sativa* seeds extracts revealed marker compounds like Gallic acid and β-Sitosterol as shown in Table 7.

3.6 Robustness

The robustness of this method was examined for the chromatographic separation of Gallic acid and β-sitosterol from the *N. sativa* seeds, by deliberate changes in the method conditions like alteration in pH of the mobile phase, varying percentage of acetonitrile, or using different wavelength for the detector. The data involving these changes

Table 8 Robustness study of Gallic acid and β-Sitosterol

Robust conditions	Variation	RT (min)		System suitability parameters of gallic acid		System suitability parameters of β-sitosterol	
		Gallic acid	β-sitosterol	TP	TF	TP	TF
Flow rate (ml/min)	0.4	5.88	9.45	4088.60	1.55	10548.6	1.25
	0.6	5.91	9.95	4121.57	1.50	10380.2	1.26
Mobile phase	59:41	5.91	9.68	4135.13	1.50	9550.2	1.03
	61:39	5.93	9.57	4280.41	1.29	9636.8	1.33
Wavelength	209	5.90	9.60	3545.44	1.55	9386.6	1.29
	211	5.91	9.95	3548.93	1.53	9389.1	1.29

TP theoretical plate, *TF* tailing factor

and the effect of the changes on the Theoretical Plate (TP), and Tailing Factor (TF) are summarized in Table 8. It has been found that chromatograms did not change dramatically after these minor changes, indicating robustness of the method.

4 Conclusion

In this study, a simple RP-HPLC method for the simultaneous determination of individual and total β-Sitosterol and Gallic acid from seeds of *N. sativa* was successfully developed and validated with excellent precision, accuracy and linearity. The experimental conditions, including the diluting solvent, mobile phase composition with changing the pH and flow rate, were optimized to provide high-resolution and reproducible peaks. In this method, the mobile phase is uncomplicated and isocratic, and the sample preparation is simple and fast. Hence, we suggest that this method can be used for routine analysis of β-Sitosterol and Gallic acid from *N. sativa* seeds.

Acknowledgements The author (P. G. Bhishnurkar) gratefully acknowledges to the Director, Institute of Science, Nagpur for providing the necessary research facilities to carry out the work. Also thanks to Dr. Subhash Banerjee and Dr. R. G. Chaudhary for constant encouragement given.

Funding The corresponding author (TLL) acknowledges funding support from DST grant no (IF120418).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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