RESEARCH ARTICLE

Validated HPTLC Analysis for Estimation of Quercetin in Seeds of *Anethum graveolens*

Sukeshini Lote[1*], Surendra Agrawal[1], Shirin Ghune[2], Pravina Gurjar[1,3]

[1]*Datta Meghe College of Pharmacy, Datta Meghe Institute of Higher Education & Research, Wardha, Maharashtra, India*
[2]*SPPSPTM, SVKM'S NMIMS, Mumbai, Maharashtra, India*
[3]*Sharadchandra Pawar College of Pharmacy, Pune, Maharashtra, India*

Received: 15th December, 2022; Revised: 18th January, 2023; Accepted: 26th March, 2023; Available Online: 25th June, 2023

ABSTRACT

A high-performance thin layer chromatography (HPTLC) method for the quantification of quercetin was developed on methanolic extract of dill (*Anethum graveolens)* seeds and subsequently validated. A suitable mobile phase was used to establish the HPTLC method, ethyl acetate, toluene, methanol, chloroform, and formic acid (2:3:3:2). A densitometric analysis was done at 366 nm of wavelength. Quercetin has an Rf value of 0.55. In the dilution range of 100 to 800 ng per band, quercetin revealed a linear connection with r^2= 0.9938 in the calibration's linear regression analysis. By conducting replication analysis on 2 separate days and one day, accuracy was verified. The standard addition method was used to conduct recovery studies to validate accuracy. The quercetin recovery rate was 98.60% on average. Five replicates of each of the three standards were used to detect the system suitability parameter. With regard to both the peak area and the Rf value, the %RSD was observed to be under 2%. The mobile phase concentration was altered from Toluene: Ethyl acetate: Chloroform: Methanol (2:3:3:2) to (3:2:2:3) with few drops of formic acid. It was found to have a %RSD of peak area below 10%, the robustness of the method was assessed. The developed HPTLC method was discovered to be easy to use, precise, accurate, suitable, and robust for estimating quercetin from dill seed extract.

Keywords: *Anethum graveolens*, HPTLC, Quercetin, Precision, Robust.

International Journal of Pharmaceutical Quality Assurance (2023); DOI: 10.25258/ijpqa.14.2.05

How to cite this article: Lote S, Agrawal S, Ghune S, Gurjar P. Validated HPTLC Analysis for Estimation of Quercetin in Seeds of *Anethum graveolens*. International Journal of Pharmaceutical Quality Assurance. 2023;14(2):274-278.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Dill or *Anethum graveolens* is a prominent herb frequently used as a spice and produces essential oil and flavonoids. It has been utilized in ayurvedic treatment from ancient times. Dill is a herb that is either annual or perennial. In the genus *Anethum* it is the only species.[1] Carvone is the main volatile component of dill seed, while terpenes like phellandrene, limonene, dill ether, and myristicin are the most significant components of dill herb. Coumarins, flavonoids, phenolic acids, and steroids are additional components extracted from seeds.[2] A flavonoid that is abundantly found in nature is quercetin. Quercetin functions as an antioxidant by scavenging destructive molecules in the body known as free radicals, which damage DNA, destroy cell membranes, and even lead to cell death.[3, 4] Additionally, quercetin helps prevent the oxidation of LDL (bad) cholesterol, which researchers believe may have a role in the development of heart disease.[5] Quercetin has anti-inflammatory and antihistamine properties and may help prevent cancer and heart disease.[6] Furthermore, quercetin can support in the stabilization of the histamine-releasing cells in the body, which has an anti-inflammatory activity.[7] Quercetin concentrations in various plant sources are determined using a variety of chromatographic techniques.[8] The HPTLC technique for determining and validating the method for identifying quercetin in the seeds of *A. graveolens* has not yet been reported in the literature. The HPTLC method developed here was validated using International Committee for Harmonization guidelines.[9]

Chemicals

The solvents used are of analytical grade were procured from Qualigens SD Fine Chemicals, Mumbai, India. Quercetin standard was procured from Sigma Aldrich (USA).

MATERIALS AND EQUIPMENT

The chromatographic Applicator was HPTLC- DESAGA Applicator AS 30, and the scanning system was HPTLC Densitometer CD60, 230V and Windows® software Pro Quant®.

**Author for Correspondence: sblote10@gmail.com*

Table 1: Percent extractive values of seeds of *A. graveolens* using different extraction methods

Method for extraction	*Extractive value (%)*
Maceration	3.02
Soxhlet	5.224

TLC was performed using Silica gel 60F 254 plates with dimensions of 20 x 20 cm and a thickness of 0.25 mm manufactured by Merck KGaA, Germany

Plant Material

A. graveolens seeds were obtained from local market from Mumbai, Maharashtra. Senior Scientist, Dr. M. S. Chandorkar from Zandu Foundation Gujarat, India did the authentication of seeds.

Method of Extraction

The seeds of *A. graveolens* were dried and powdered. The extraction was done by the soxhlet extraction apparatus. Approximately 500 gm of dried powder of dill seeds was used and extraction was performed with 2 liters of methanol solvent.[10,11] The collected extract was weighed, and the percent extractive value was noted in Table 1.

Preparation of Standard Solution

A standard quercetin sample of 1 mg was weighed and diluted in 100 mL methanol to produce (10 ppm), and the mixture was then sonicated for 10 minutes to create a stock solution of quercetin. Cool the solution to room temperature.

Preparation of Sample Solutions

An amount of 10 mg of dried methanolic extract were diluted in 1-mL of methanol, to make a concentration of 10000 ppm and passed through a 0.45 millipore membrane filter. The HPTLC assay was then carried out using this solution.

Chromatographic Method

The mobile phase finalized for separation of quercetin by TLC was Toluene: Ethyl acetate: Chloroform: Methanol in ratio of 2:3:3:2. Few drops of formic acid was added to mobile phase for better resolution of quercetin. The specified mobile phase was saturated in a glass twin-trough chamber (20 X10 X 4 cm) for 20 minutes. HPTLC plates of 100 ×100 mm aluminum-backed coated with 0.2 mm layers of silica gel 60F254 was used to carry out the study. A 100-L Hamilton syringe fitted in DESAGA Applicator AS30 was used to apply bands of samples that were 12.0 mm long, 6.0 mm wide, and 10.0 mm from the bottom line of the chromatographic plate. The DESAGA glass twin-trough chamber had been saturated with mobile phase vapors, and bands were ascending to a distance of 100 mm at room temperature. The HPTLC plates were dried, activated in an oven at 110°C for 15 minutes and examined at 366 nm.[9, 12]

Method Validation

Linearity and limits of Quantification

The prepared standard solution of quercetin (10, 20, 40, 60, and 80 µL/band) were applied and scanned to achieve linearity. A 10 µL of sample solution from 10000 ppm stock solution was applied as a band. The procedure was carried out thrice; the densitograms representing quercetin concentration were recorded. The corresponding concentrations (X axis) were displayed against the peak areas (Y axis). The Limit of detection (LoD) and Limit of Quantitation (LoQ) were calculated using standard formula.[12,13]

Precision

Any results of the analytical method can be impacted by either random or systematic error. Random errors are commonly linked to accuracy of the procedure. When the process is used frequently to take many samples of a homogenous sample, precision must be taken into account as the level of agreement among the individual test outcomes.

The precision can be determined by performing repeatability in which the accuracy is achieved over a brief period using the same process and the same condition is determined on the same test material. Repeatability is also termed intra-assay or inter-day precision. However, variations between laboratories are expressed with intermediate precision. Precision was achieved using the same process on the same test material but with change of laboratory, day, equipment, etc. It may also be referred to as intra-laboratory or inter-day precision. The intraday precision was assessed by analyzing six replicate applications of the standards quercetin at concentrations of 100 to 140 ng/band (100 to 140%) applied in three different volumes (10, 12, and 14 µL, respectively) on the same day. Inter-day accuracy was assessed by analysing six replicate standard solution applications at the identical concentrations described above on two distinct days. The single band was scanned ten times for instrument precision.[9,14,15]

Accuracy

Recovery studies using the standard addition method were conducted at three different levels to determine the accuracy of the developed HPTLC method. By spiking on the same plate in triplicate, known concentrations of quercetin (100, 200, and 400 ng/band) were added to 10 mL (10 mg/mL) of methanolic extract. The plate was created in Similar circumstances, and average quercetin recovery percentage values were computed.

System Suitability

System suitability parameters were evaluated to check for the overall performance of system developed. The test was run using 20 µL of each of the three standard quercetin solutions in five repetitions, giving a concentration 200 ng/band.[16]

Table 2: Validation data from the calibration curve of the quercetin Standard: Concentration (ng) on X-axis, peak area on Y-axis

Standard	*Equation derived from regression analysis*	*Coefficient of correlation (R2)*	*Conc Range (ng/band)*	*Detection Limit (ng/band)*	*Quantitation Limit (ng/band)*
Quercetin	y=70.709x-113.69	0.9938	100–800	87.34	234.68

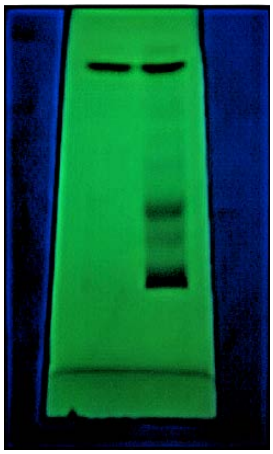

Figure 1A: Plate developed by TLC as observed under 280nm Band 1= Quercetin standard; Band 2= Methanolic extract of dill seeds

1B: Plate developed by TLC as observed under 366 nm fluorescence.

Robustness

Suppose one or more of the validated method parameters will significantly affect the analytical performance. In that case, the method must be modified until it is proven to be sufficiently robust, at which stage validation must begin all over again. The test for robustness comprised changing the mobile phase ratio of Toluene: Ethyl acetate: Chloroform: Methanol: Formic acid (2:3:2:2) to (3:4:4:3). In order to achieve a concentration of 200 ng/band, all three standard solutions were applied five times, showing the robustness of the developed method.[17-19]

Acceptance criterion

The intended use of the analytical method must serve as the basis for the acceptance criteria for the precision and accuracy of a procedure. Precision showing approximately 10% relative standard deviation (RSD) may be considered appropriate for analysing a phyto-consituent in a crude extract using a certain analytical method for assay. Any instrument intended to be used for analysis must have a precision value of less than 2%. Acceptance requirements typically vary from 97 to 101% in order to accurately identify the main phytoconstituents in the provided test crude extract.[20, 21]

RESULTS

Optimization of the Chromatography

Initially, flavonoids were separated employing known mobile phase systems in pilot research. The standards and phytoconstituents found in the methanolic extract of *A. graveolens* seeds had poor resolution when the mixture of ethyl acetate, chloroform, and methanol in the ratio of 5:4:1(v/v) was used.[22] Therefore, the composition of the mobile phase was modified using various combinations of toluene, ethyl acetate, chloroform and methanol. A ration of these solvents 3:3:2:2 (v/v) gave the best resolution of quercetin (Rf = 0.55). Formic acid improved resolution and hence few drops of formic acid included in mobile phase. A TLC scanner with high wavelength (366 nm) was used to observe the developed plates that showed quercetin's presence (Figure 1 A,B).

Method Validation

Linearity and Limits of Quantification and Detection

The AUC values obtained after scanning the linearity plates and analysis for regression analysis revealed a linear relationship in the range of 100 to 800 ng/band with a coefficient (r^2) of 0.9938 for quercetin. Quercetin has a LoD of 87.34 ng and a LoQ of 234.68 ng given in Table 2.The amount of quercetin found in the methanolic extract of *A. graveolens* seeds is shown in Table 3.

Precision

Relative standard deviation (RSD) was determined and expressed on the analyzed data from the repeated analysis of standard quercetin sample to determine the intra-day and inter-day precision. The relative standard deviation for intra-day was 1.7 to 2.1%, while Inter-day was between 1.5 to 2.7% given in Table 4.

Accuracy

The accuracy is expressed as the recovery studies deviation for standard of quercetin, by repeated analysis (*n*=3) at three different levels of 100, 200, 300 ng/spot. The average recovery was found to be 99.04% as given in Table 5.

Robustness

The change in mobile phase composition from Toluene: Ethyl Acetate: Chloroform: Methanol (2:3:3:2) to (3:2:2:3) and subsequent calculations of RSD within the limit of 10% as shown in Table 6 reveals the robustness.

Table 3: Contents of Quercetin in the methanolic extract of dill (n=3)

Standard	*Method of extraction*	*Peak area ± S. D.*	*Content mg /100gms of extract*
Quercetin	Soxhlet	647.76 ± 8.44	32.81
	Maceration	437.81 ± 11.78	49.68

Table 4: Intraday and interday Precision (n=6)

Parameter	*Conc (ng/spot)*	*Area under curve*		
		Mean	*SD*	*%RSD*
Intra day precision	100	765.34	16.122	2.105
	120	979.12	17.32	1.769
	140	1235.385	21.90	1.773
Inter day precision	100	778.08	21.22	2.728
	120	981.33	91.62	2.00
	140	1231.75	18.52	1.503

Table 5: Percent recovery of quercetin

% Level	*Amount added (ng)*	*Response*	*Amount recovered*	*% Recovery*	*Average% recovery*
100	100	763.94	98.79	98.79	99.04%
200	200	1650.61	297.34	98.67	
300	300	2779.68	298.72	99.68	

Table 6: System suitability test: %RSD of Quercetin (n=5)

Parameter		*200 ng/spot*	*Rf value*
System Suitability	AUC Mean	1673.28	0.54667
	AUC SD	13.881	0.01633
	AUC %RSD	1.871	1.987
Robustness	AUC Mean	1678.566	--
	AUC SD	19.078	--
	AUC %RSD	1.2978	--

System Suitability

The system suitability was assessed by considering the RSD of peak areas and RF value. The RSD for peak area and Rf value observed to be less than 2% as shown in Table 6. These reveals presence of minimum deviations between the process and instrument performance during validation.

DISCUSSION

HPTLC is used to identify and estimate the active constituents in the seeds of *A. graveolens* with the aid of a suitable mobile phase. It is very important to isolate other interfering phytoconstituents likely present in the sample extract from the active constituents. The acceptable values obtained for validation prove the effectiveness of the developed method. To increase band resolution and enable simple quantification of desired constituents, appropriate agents like formic acid/ acetic acid were applied.[23]

CONCLUSION

The study performed, proves that seeds of *A. graveolens* contain quercetin in abundance. This study provides a quick method for standardization of the plant, and assessing the effectiveness of *A. graveolens.* The validation parameters are found within acceptable limits and hence the HPTLC method developed is precise, accurate, robust and sensitive. Furthermore, the method can be used for standardization of the products containing quercetin. The study also provides an alternative source of quercetin extraction and isolation as large amount of quercetin is present in *A. graveolens.*

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