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NOVEL METHOD DEVELOPMENT AND VALIDATION OF RP-HPTLC METHOD FOR THYMOQUINONE AND CURCUMIN

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ABSTRACT
A method for the rapid, linear, precise, and robust quantitative analysis (RP-HPTLC) has been developed and validated. This method was used to separate the components of thymoquinone and curcumin, on a precoated, silica gel, aluminium plate with a 60F-254 stationary phase, consisting of a mobile phase composed of a 16:4 ratio of toluene: acetic acid. The detection wavelength of the mobile phase was chosen to be 260 nm, and the detection wavelength of the detection phase was 417 nm, with the Rf value for curcumin being found to be 0.41 and for thymoquinone to be 0.55. The method was able to accurately and linearly measure the concentration range from 100-350 nd/band. The method was validated according to the ICH guideline for specificity, linearity and accuracy, as well as for reproducibility and robustness.
Keywords: Curcumin, thymoquinone, HPTLC

INTRODUCTION
Curcumin is a bright yellow chemical that is produced by the plants of the *Curcuma longa* species. Curcumin is the main curcuminoid of turmeric, which belongs to the family of Zingiberaceae, which is known for its anti-oxidants, anti-inflammatory properties, anticarcinogens, anti-mutagens, antifungals, anti-viruses and anti-cancer.[1]

Nigella sativa Linn., also known as Black Seed or Black Cumin, is a plant species of the Ranunculaceae family. It is commonly used in traditional systems of medicine to treat a variety of ailments, including arthritis, lung disease, hypercholesterolaemia, and other conditions. Reported pharmacological effects of N. Sativa include a decrease in blood pressure, analgesia, uric acid reduction, bile production aid, pregnancy prevention, diabetes management, allergy relief, antioxidant activity, inflammation reduction, antimicrobial activity, anticancer activity, immunomodulation, and more.[2]

Kazia M., *et al.* (2018)[5] has developed a method for the simultaneous detection of Curcumin and THQ in lipid based self-nanoemulsifying systems and its application to the commercial product

using a UHPLC- UV-VIS spectrophotometer. The chromatographic separation of Curcumin was performed on the reversed-phase of Acquity®BEH C-18 in a methanol: 0.25% formic acid solution in water (60:40, v/v) as the mobile phase. **Taleuzzaman M., *et al.*** (2017)[6] developed and validated as a stability-indicated HPTLC density measurement method for the quantitative quantification of TQ according to the ICH guideline. The method was performed on an aluminum plate and was based on the following formula: n-hexane: ethyl acetate: methanol (7:2:1 v/v/v) as mobile phase Densitometric determination TLC scanner CAMAG 254 nm. **Srivastava S., *et al.*** (2009)[7] developed high-performance liquid chromatography (HPLC) method for the chemotaxonomic examination of certain species of Curcuma, as well as their commercial samples. The chromatographic analysis was carried out on silica gel 60F254 plates with chloroform:ethanol:acetic acid and 95:4:5v/v as the mobile phase. The detection and quantification was performed densitometrically at λ_{max} = 260 nm with curcumin as external standard. **Pathania V., *et al.*** (2007)[8] have developed an improved HPTLC method for curcuminoid detection from Curcuma longa. The method is based on the use of a mobile phase (Chloroform-Methanol) consisting of 98:2v/v chloroform-methanol on HPTLC (LiChrospheres) precoated aluminium plates (Si 60F254). The volume of the mobile phase is 20 ml with a chamber saturation time of 30 min. The densitometry is performed at 366 nm in adsorption reflection mode.

The HPTLC method has been widely adopted for the analysis of drugs and pharmaceuticals because of its simplicity, wide application, low cost, and relatively quick speed.[3,4] A literature survey also showed that there are no published analytical methods for the simultaneous quantification of both thymoquinones and curcumin, apart from a few HPLC methodologies. Therefore, we developed and validated a high-performance liquid chromatography (HPLC) method for the concomitant determination of the two compounds in the pharmaceutical dosage form.

MATERIAL AND METHODS

Materials

Pure Thymoquinone and Curcuminoids standards were purchased from Yucca Enterprises, Mumbai. Precoated silica gel 60 F 254 HPTLC plates were purchased from Merck, Germany. All the solvents used were of chromatography grade.

EXPERIMENTAL METHOD

HPTLC instrumentation and chromatographic conditions

The samples were spotted in bands of 8 mm width using a micro litre syringe, on a precoated silicon gel aluminium plate (60 F-254) with a constant sample application rate of 150 nl/s. The slit dimension was maintained at 6 mm×0.45 mm with a scanning speed of 20 mm/s. Optimized mobile phase (20 ml) composition (toluene: acetic acid) with 16:4 v/v was used for analysis. Linear ascending development was performed in 20 cm x 10 cm pre-saturated glass chamber (20 cm x 10 cm) with a pre-saturated chamber saturation time of 20 min at 25 ± 2°C with a relative humidity

60 ± 5%. Densitometric Scanning (Camag TLC Scanner 4) at 260 nm absorbance mode (WinCATS®) Acetic Acid Scanning (Toluene) at 417 nm (Curcumin) WinCATS® Software.

Assay method development

Preparation of standard solution

Measure 10 mg THYMOOQUINON and 10 mg CURCUMIN standard and transfer to 100 ml ambered color volumetric flask, dilute with 30 ml of solvent and sonicate for standard dissolving. Make up volume to mark with solvent and mix well for final concentration 100 µg/ ml THQ & CUR.

Preparation of sample solution

The net content of 20 capsules was accurately weighed and 761 mg was transferred into 100 ml volume flask. About 40 ml diluent was added and sonicated for 20 min (shaking after every 5 min). The flask was allowed to cool to room temperature, then the volume of the flask was made up by diluent until it reached 100 ml. The flask was centrifuged for 15 min at 3000 rpm and supernatant was collected. 10 ml of the above solution was diluted to 25 ml (dilution of 50 ml) using the diluent of the sample curcumin solution preparation. The total concentration of the solutions was 250 µg/ ml (50 µg/ ml). The chromatogram was recorded and the percentage assayed was calculated.

The amount of analytes present in the formulation were calculated by using the formula as given below,

Formula:

"% Assay = $\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{AW}{LC} \times 100$"

Where,

"*AT: Peak area response of test sample*"

"*AS: Peak area response of standard sample*"

"*WS: Weight of standard sample*"

"*DS: Dilution factor of standard sample*"

"*DT: Dilution factor of test sample*"

"*WT: Weight of test sample*"

"*AW: Average Weight*"

"*LC: Label Claim*"

"*P: Potency*"

Selection of mobile phase

Different experiments have been done to improve mobile phase composition, which will lead to sharper, more defined peaks.

Following Mobile phase compositions were tried:

Table No 1 Mobile phase composition trails for HPTLC method development

Sr. No.	Mobile phase composition
1.	n- Hexane: Ethyl Acetate: Methanol (7:2:1 %v/v/v)

2.	n-Hexane: Ethyl acetate: Methanol: Formic Acid (8:2:1: 2-3 drops %v/v/v)
3.	Chloroform: Methanol (8:2 %v/v)
4.	Chloroform: Ethanol: Glacial Acetic Acid (9.4:0.5 0.1 %v/v/v)
5.	Toluene: Acetic Acid (8:2 %v/v)

The solvent system for separation was selected on the basis of good separation, peak purity index, peak symmetry, Rf value etc. So, number of trails was performed to select a mobile phase.

Method validation[9]

The method proposed was rigorously tested according to ICH standards for linearity, repeatability, precision, and reliability.

Specificity

In order to confirm the specificity of the method, standard drugs, the sample, the diluent and the solvents were tested. The spot for the presence of thymoquinone and curcumin within the sample was compared with that of standards by comparing the residue fraction (Rf) and spectra. The effects of interference on the analysis were examined.

Linearity

Linearity is determined by applying 1.0 -3.5 µl/band (1.0, 1.5, 2.0, 2.5, 3.0, 3.5) of standard solution of thymoquinone and curcumin on the TLC plate from 100 µg/ml stock solution with the help of microliter syringe using an automatic sample applicator. Plate development, drying and densitometry at 260 nm and 417 nm are performed. Drug peak-area is calculated per concentration level and drug concentration graph plotted against peak area.

Reproducibility

The reproducibility of the system was evaluated by detecting 0.5 µl/band thymoquinone and 1.0 µl/band curcumin standard solutions on the TLC plate six times. Subsequently, the plate was developed and the maximum area for 6 bands was recorded. The method was further refined by analysing the standard solution in the concentrations of 50 µl/band and 100 µl/band for 3 consecutive days on the same day, and the inter-day accuracy was determined by comparing the corresponding standards for the following day.

Recovery

In accordance with ICH guidelines, a recovery study was conducted at three concentration levels (80%, 100%, and 120%) to confirm the accuracy of the method developed. Multiple level recovery studies were conducted using a replicate analysis (n = 3) to calculate the percent recovery.

Table No 2 Recovery Study

	Curcumin	**Thymoquinone**

Level (%)	Amount of std added (µl/band)	Amount of sample Added (µl/band)	Final concentration (ng/band)	Amount of std added (µl/band)	Amount of sample added (µl/band)	Final concentration (ng/band)
80	1.0	0.8	180	0.5	0.4	90
100	1.0	1.0	200	0.5	0.5	100
120	1.0	1.0	220	0.5	0.6	110

Robustness

The robustness of the results was determined by introducing minor alterations in the various parameters, including mobile phase composition and mobile phase saturation time, and the effects of these alterations on the results. The study was conducted at 50 nd/band and 100 nd/band for the thymoquinone and for the curcumin respectively. The effects of the alterations on the results of the peak areas and the Rf value were assessed. The duration of the saturation period (10, 20 or 30 min) was also studied.

RESULTS AND DISCUSSION

Mobile phase optimization

Different solvent systems have been tested for thymoquinone and curcumin separation on TLC plates and the optimal mobile phase system has been identified as Toluene:Acetic acid in the ratio (8:2 v/v) found to have the good separation. For curcumin, the Rf value is 0.41, and for thymoquinone, it is 0.55. For all the samples, the resolution between the spot of the standard and the spot of the sample appeared to be better. Therefore, an attempt has been made to create and validate a low-cost, simple, and reliable HPTLC method for quantifying thymoquinone, and curcumin. The method has been developed and is quite selective.

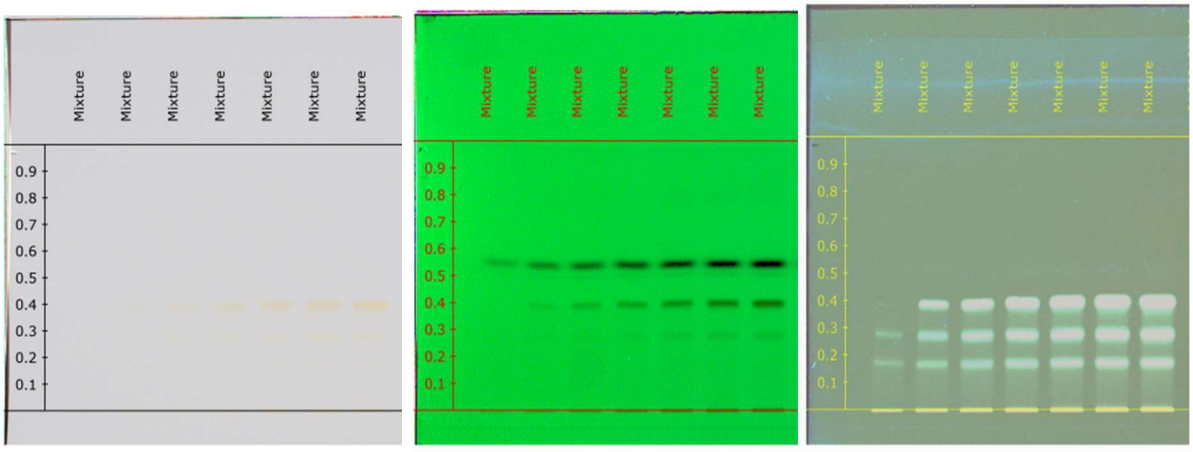


Figure no. 1 a) Developed TLC Plate images captured in TLC Visualizer at White light, b) at 254 nm & c) at 366 nm.

Assay Method

Table No 3 Assay method

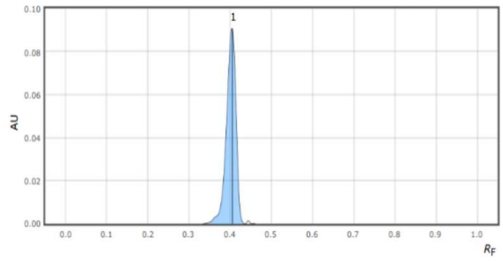
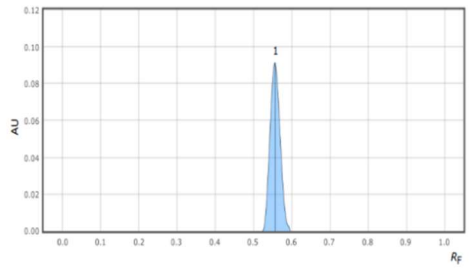
Chromatogram of Curcumin	Chromatogram of Thymoquinone
	

Figure No. 2 Chromatogram of Sample for Assay

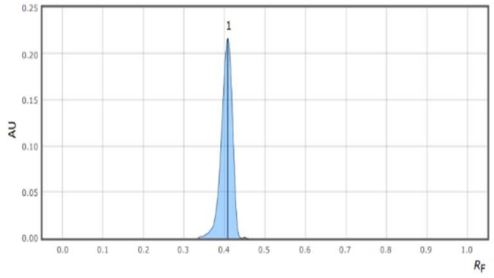
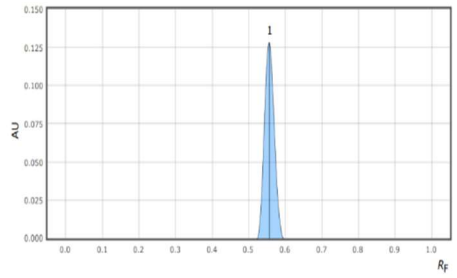
Chromatogram of Curcumin	Chromatogram of Thymoquinone
	

Figure No. 3 Chromatogram of Standard for Assay

The curcumin and thymoquinone content in the analyzed dosage form was 98.11% and 98.28%, respectively.

Method Validation

Specificity

In order to determine the specificity of the method, the pure TQ and CUR, the sample, the diluents, the mobile phase solvent, etc. were analyzed. The spot for TQ and CUR in the sample was verified by comparison of the Rf values and spectra with the standard. Peak purity of TQ and CUR was determined by comparison of the spectra. The method was therefore considered to be specific.

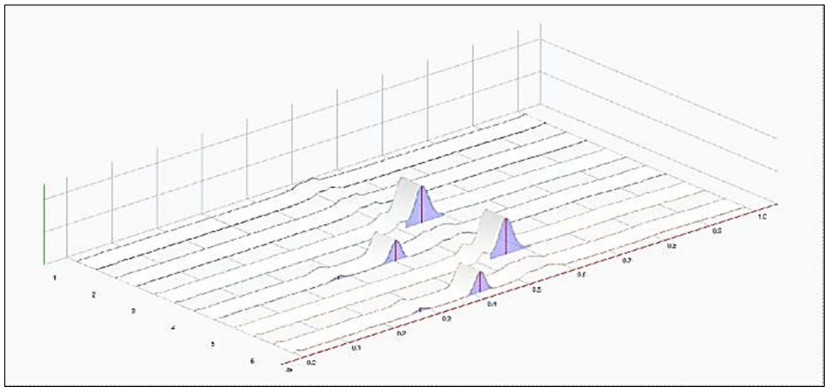


Figure No 4 Specificity

Linearity

The correlation coefficient of Thymoquinone (0.999) and Curcumin (0.9995) was determined, indicating a linear distribution of concentrations between 100-350 ng/band.

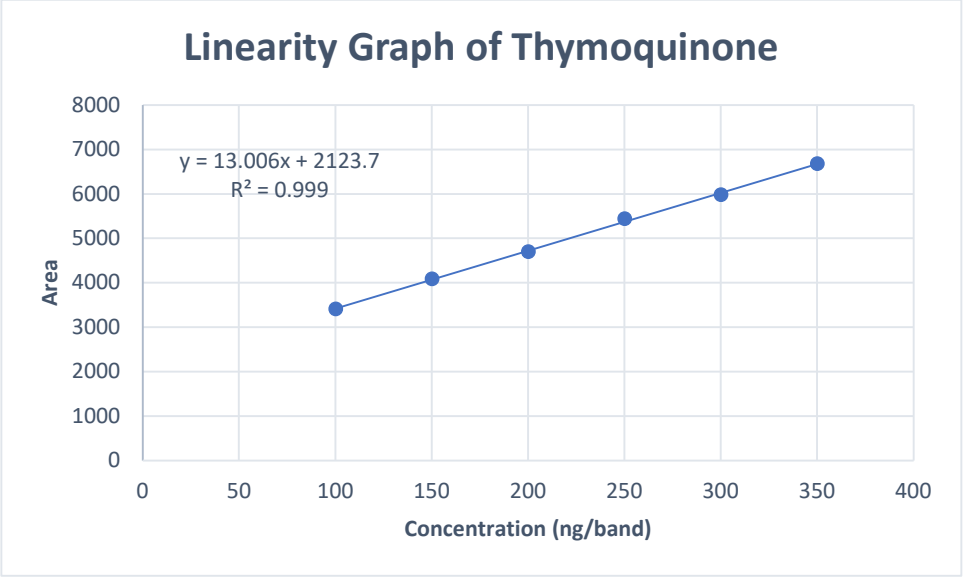


Figure No 5 Linearity graph of thymoquinone

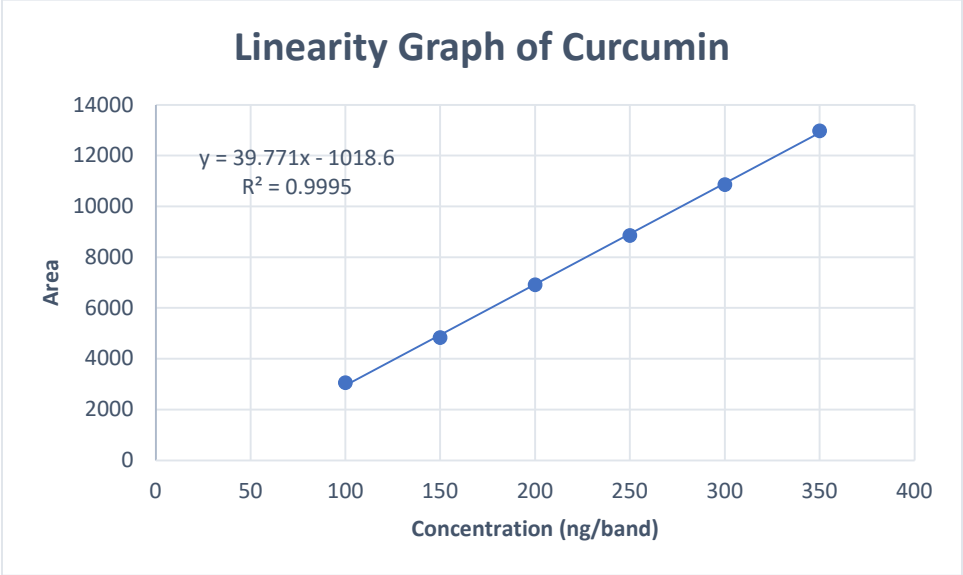


Figure No 6 Linearity graph of curcumin

LOD and LOQ

LOD and LOQ values were 0.28 ng/band & 0.84 ng/band respectively for curcumin. LOD and LOQ were 0.31 ng/band & 0.94 ng/band respectively. This indicates that the method is sensitive for the detection and quantification of the two compounds.

Reproducibility

The percent relative standard deviations (RSDs) for TQ and the percent RSD for CUR were found to be within the range, indicating that the method was correct by meeting the accept criteria. The reproducibility results for TQ and for CUR were analyzed at 50 nd/band and at 100 nd/band, respectively. For Curcumin, the percentage RSD was 1.52 %, and for CUR, it was 1.76 %.

Recovery

The drug recoveries were determined using the standard addition approach. The proposed approach was employed to estimate the TQ and the CUR after the addition of 80, 100, and 120% of the additional drug. The recovery was obtained for Curcumin and Thymoquinone to be 99.29 % (80%), 99.55 % (100%), 98.84 % (120%) and 101.34 % (80%), 98.13 % (100%), 98.99 % (120%) respectively.

Robustness

TQ and CUR robustness were assessed at 50 nd/band and 100 nd/band respectively for standard and 200 nd/band for sample.

Table No 4 Robustness Data

PARAMETERS	CUR (% RSD)	THQ (% RSD)
Saturation time (10 min)	1.87 %	1.95 %
Saturation time (30 min)	1.79 %	1.77 %
Saturation with filter paper	1.5 %	1.66 %
Saturation without filter paper	1.59 %	1.52 %

CONCLUSION

Thymoquinone and curcumin in dosage form can be determined using the developed HPTLC method, which is a simple, precise and accurate analytical method. On precoated silica-gel 60 F254 plates, a toluene:acetic acid (8:2%v/v) mobile phase was used to successfully separate the analytes. The technique was approved in accordance with ICH guidelines. Curcumin and thymoquinone recovered at 99.29% (80%) and 99.55% (100%), respectively. They also recovered at 98.84% (120%), and at 98.13% (100%), and 98.99% (120%), respectively. This means the method is good for measuring the analytes in dosage form. The recommended HPTLC is a robust, cost-effective, method of analysis that is appropriate for routine co-assessment of thymoquinone and curcumin in dosage forms.

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