



Quantitative Determination of thymoquinone in *Nigella Sativa* and its nano formulation using validated stability indicating HPTLC densitometric method

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ABSTRACT

An accurate and stability indicating high-performance thin layer chromatographic method (HPTLC) was developed and validated for the quantification of thymoquinone (TQ) as per the ICH guidelines. The analysis was carried out on the aluminum plate using n-hexane: ethyl acetate: methanol (7:2:1 v/v/v) as the mobile phase and the densitometric determination was carried out by TLC scanner (CAMAG) at 254 nm. The developed method was validated for different parameters like linearity, precision, recovery, robustness, and stressed stability study. The developed analytical method was found to be linear in the concentration range of 75-500 ng band⁻¹ with regression value closer to unity ($r^2 = 0.997$). The developed system was found to give compact spots for thymoquinone ($R_f 0.77$) with the limit of detection and limit of quantification (18 and 54 ng band⁻¹) respectively. Further, the study showed accuracy, precision and repeatability were all within the required limits. The stress degradation study of TQ showed well separated degraded peak from the pure thymoquinone. The mean recoveries measured at three concentrations were more than 95% with $RSD \leq 3\%$. The method has been successfully applied in the analysis and routine quality control of herbal material and formulations containing TQ.

Key Words: *Nigella Sativa*, thymoquinone, HPTLC, validation, stress degradation, Nano formulation.

INTRODUCTION

Nigella sativa Linn. (Ranunculaceae) very often known as black seed or black cumin, is an herbaceous plant, mostly found in the middle east, Central Europe, and Western Asia. It is broadly used in indigenous system of medicine for treatment of numerous disorders for over 2000 years (Alam *et al.*, 2013). Thymoquinone (TQ, **Figure 1**) is the precious constituent of the volatile oil of *Nigella Sativa* seeds, has shown vital medicinal properties, and has long been used in traditional and folk medicines (Ragheb *et al.*, 2009). The main bioactive constituent of *Nigella Sativa* is TQ and various biological activities have been reported in different research (Akhtar *et al.*, 2014; Sayeed *et al.*, 2017). TQ belongs to the family of monocyclic monoterpenes, having the molecular formula (C₁₀H₁₂O₂) and IUPAC name 2-methyl-5-propane-2-yl-cyclohexane-2, 5-diene-1,4-dione phenol. Most properties of the whole seeds or their extracts are mainly attributed to the presence of quinone constituents in the volatile oil and the most abundant component found is thymoquinone (about 27-57%) (Ali *et al.*, 2003). Other pharmacologically active constituents, present were dithymoquinone, thymohydroquinone and thymol in low content (Ghosheh *et al.*, 1999).

The most preferred analytical tool for fingerprints and quantification of marker compounds in herbal drugs used is HPTLC. This technique has shown accuracy, sensitivity, suitability for high-throughput screening, reliability in the quantification of analytes from nanogram to microgram levels (Tayade *et al.*, 2007; Kaur *et al.*, 2008). It has several advantages in comparison to high-

performance liquid chromatography (HPLC), it can analyze several samples, use the small quantity of mobile phase, as well as it facilitates repeated detection (scanning) of the chromatogram, analyze different kinds of samples, which reduces the time, and cost of analysis (Kaur *et al.*, 2008) (Dixit *et al.*, 2008). Although several methods such as thin-layer chromatography (AbouBasha *et al.*, 1995) gas chromatography (Houghton *et al.*, 1995). HPLC (Ghosheh *et al.*, 1999). HPTLC (Michelitsch *et al.*, 2003). and differential pulse polarography (Reelma *et al.*, 2011) have been used to quantify the TQ in black seed oil. But till now not a single stress induced HPTLC method was developed to quantify TQ.

The International Council for Harmonisation (ICH) guideline entitled Stability Testing of New Drug Substances and products requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance (Stolarczyk *et al.*, 2010). There are different stability parameters (oxidation, hydrolytic and the photolytic) used for the stress stability studies. An ideal stability- indicating method is one that quantifies the standard drug alone and also resolves its degradation products (Zammataro *et al.*, 2010). The basic acceptance criteria for evaluation of validation experiments based on practical experience for planar chromatographical procedures, which may be used at different levels either in qualitative identity testing, assays, semi-quantitative limit tests or quantitative determination of impurities (Bakshi *et al.*, 2011). Form this approach, the aim of the present study to develop and optimize a simple and selective HPTLC method for the quantification of TQ for purity assessment in bulk as well as in in-house developed nanoformulation. The proposed method was validated as per ICH guidelines (Ranganathan *et al.*, 2002; Sethi *et al.*, 1996) and its updated international convention (ICH, Q2A, 1994).

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MATERIALS AND METHODS

Materials

Pure Thymoquinone and Mature seed powder of *Nigella Sativa* were purchased from Sigma Aldrich, Mumbai and from Sunpure extract private Ltd. (Delhi) respectively. Pre-coated silica gel 60 F254 HPTLC plates were purchased from E. Merck, Germany. All the solvents used were of chromatography grade and other chemicals used were of analytical reagent (AR) grade. Water was purified by a Milli-Q system (Millipore Corporation, Bedford, MA, USA).

Methods

HPTLC instrumentation and condition.

The samples were spotted in the form of bands of width 6 mm with a Camagmicrolitre syringe on pre-coated silica gel aluminum plate 60 F-254, (20 cm × 10 cm with 250 μm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai). Methanol was used to wash the plates and activated at 60°C for 5 min prior to the study. A constant sample application with the rate of 140 nL s⁻¹ was employed and space between two bands was 6 mm maintained. The slit dimension was kept at 5 mm × 0.45 mm and 20 mm/s scanning speed was employed. The monochromatic bandwidth was set at 20 nm, each track was scanned thrice and a baseline correction was used. The optimized mobile phase composition (20mL) consisted n-hexane: ethyl acetate: methanol (7:2:1 v/v/v) was used for the analysis. Linear ascending development was carried out in 20 cm × 10 cm twin trough pre-saturated glass chamber (Camag, Muttenz, Switzerland). The HPTLC plate development was carried out for twice with the same mobile phase to get high resolution. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25 ± 2°C) at the relative humidity of 60 ± 5%. Densitometric scanning was performed on Camag TLC scanner III in the reflectance-absorbance mode at 254 nm and operated by WinCATS® software (Version 1.2.0).

Preparation of standard and quality control (QC) samples

A standard stock solution of TQ (10 mg/mL) was prepared in methanol and further dilution of the standard was done in the concentration range of 0.1 to 1.0 mg/mL. The calibration of TQ standard solution (1-10 μL) was applied to an HPTLC plate to furnish amounts in the range 75-500 ng band⁻¹. The data of peak area vs. drug concentration were treated by linear least square regression analysis. QC samples as low, medium and high at the concentration level of 250, 350 and 400 ng band⁻¹ were taken to carry out validation of the method. Three replications of each calibration standard were performed (n = 3).

Extraction process

The weighed quantity of NS seed powder was packed in a muslin cloth and kept in the beaker containing sufficient quantity of methanol for 72 hrs. Thereafter the methanolic extracts were filtered through Whatman paper no. 42 and the resultant filtrates were concentrated under reduced pressure using the rotary evaporator to yield a concentrated mass which was labeled as meth-NS and preserved in airtight amber colored glass container at 4°C until use. The fingerprinting of methanolic TQ extract was enacted by spotting 10 μL of suitably diluted sample solution on an HPTLC plate.

Method validation

The developed method was validated as per ICH guidelines for linearity range, precision, accuracy as recovery,

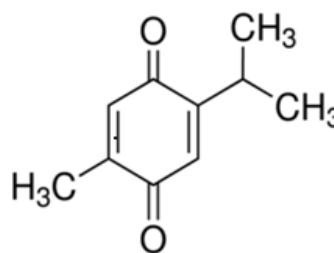


Figure 1: Chemical structure of Thymoquinone.

robustness, limits of detection (LOD), limits of quantification (LOQ) and stability study (Ali *et al.*, 2016; Mittal *et al.*, 2015).

Precision

The inter-day precision study was done by repeating the study on same day whereas in intraday assay the same study was repeated for three different days. In both, the study, precision of the developed method was evaluated by performing replicate analyses (n = 6) of QC samples at low (250 ng band⁻¹), medium (350 ng band⁻¹) and high levels (400 ng band⁻¹). The result of precision was expressed as the percent recovery with the coefficient of variation of measured concentrations for each level.

Accuracy

The accuracy was determined by standard additions method at three different levels, *i.e.*, by multiple level recovery studies. The recovery studies were performed with the addition of extra percentage level of 50, 100 and 150% from the initial level (Ali *et al.*, 2016). It was performed by application of test sample (n=6) of known concentrations of TQ that had been prepared from stock solutions. The percent recovery was calculated using regression equation at different levels in the sample.

Limit of detection (LOD) and quantification (LOQ)

The limit of detection and limit of quantification are applied for the determination of low levels of the compound in sample matrices and is used particularly for the determination of impurities and degradation product in the quantitative assay. To estimate the limits of detection (LOD) and quantification (LOQ), blank methanol was applied (n=6) and the standard deviation (σ) of the analytical response was determined. The LOD and LOQ values were calculated from the calibration curves as $k\sigma/b$ where $k = 3$ for LOD and 10 for LOQ, σ is the standard deviation of the intercept and b is the slope of the calibration curve.

Robustness

Robustness was determined by introducing small changes in the different parameters such as mobile phase composition, mobile phase saturation time, and mobile phase volume and their effects on the results were examined. The study was performed in triplicate at 350 ng band⁻¹ and the effects of changes were examined on the results of peak areas and R_f value. The mobile phase composition n-hexane: ethyl acetate: methanol in different proportions (7.5:1.8:0.7 and 6.8:2.1:1.1 v/v/v) and mobile phase volume (13, 17 and 15 ml) and duration of saturation (10, 20 and 30 min) were investigated. Further, the plates were prewashed with methanol and activated at 60 ± 5°C for 2, 5 and 7 minutes.

Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spot for TQ in the sample was confirmed by comparing the R_F and spectra of the spot with that of the standard. The peak purity of TQ was assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M) and peak end (E) positions of the spot.

Analysis of TQ from Developed nano Formulation

The validated developed method was applied to the quantification of TQ in-house developed TQ loaded nanoformulation. The estimation of the contents of TQ in nanoformulation, 5 mL of nanoformulation was extracted with 10 mL methanol. To ensure complete extraction of the drug, it was sonicated for 30 min and centrifuged at 3000 rpm for 10 min. The supernatant of each sample was suitably diluted to give desired concentration (400 ng spot⁻¹). After that, it was applied on TLC plate followed by the development. The result of the peak area obtained corresponding to TQ was used for quantification in samples using regression equation. The results of the triplicate analysis were expressed as an average amount of TQ in % w/w. The possibilities of excipient interference in the analysis were studied.

Stability studies

The stress testing of the drug substance can help identify the likely degradation products, the stability of the molecule and also validate the stability and specificity of the analytical procedures. The degradation studies were carried out as per by subjecting the standard thymoquinone sample to oxidation, wet heat, dry heat and photo-degradation to evaluate the stability indicating properties of the developed HPTLC method.

Hydrogen peroxide-induced degradation

To 25 ml of a methanolic stock solution of thymoquinone, 10 ml of 50 % w/v hydrogen peroxide were added. The solution was heated in boiling water bath for 15 min to remove completely the excess of hydrogen peroxide and then refluxed for 2h at 70°C. The resultant solution (200 ng band⁻¹) was applied to TLC plate and the chromatograms were run for the analysis as described above.

Dry heat and wet heat degradation

The standard drug was placed in the oven at 100°C for 7 days to study dry heat degradation, and the stock solution was refluxed for 12h on boiling water bath for wet heat degradation. The resultant solution (200 ng band⁻¹) was applied on TLC plate. Further, the HPTLC study was performed and the chromatograms were run for the analysis.

Photochemical and UV degradation

The photochemical stability of the thymoquinone was also studied by exposing the stock solution (250 g ml⁻¹) to direct sunlight for 3 days on a wooden plank and kept on the terrace. The thymoquinone solution was also exposed to UV radiation for 15 days in UV stability chamber. One micro-litre (200 ng band⁻¹) from each sample was applied on TLC plate and chromatograms were run as described above.

RESULTS AND DISCUSSION

Mobile phase optimization

There are different solvent systems were tried for the separation of thymoquinone on the TLC plates. Initially, the different solvent system used as the solvent system to get a high-resolution peak, and get better peak resolution. The

Table 1: Linear regression analysis data for the developed HPTLC method of Thymoquinone.

Parameter	Values
R _F	0.77
Linearity range (ng band ⁻¹)	75-500
Regression equation	16.58x - 224.2
Correlation coefficient (r ²)	0.997
Slope ± SD	0.0068±0.0067
LOD	18ng band ⁻¹
LOQ	54 ng band ⁻¹
Recovery (%)	99.15 ± 1.44
Robustness	Robust
Specificity	Specific

optimized mobile phase system was found n-Hexane: ethyl acetate: methanol in the ratio of (7:2:1 v/v/v). The R_F value found with this mobile phase composition was 0.77 for all the samples shown in **Figure (2 & 3)**. The resolution between spots of standard and deprecates appeared better, when the TLC plates were pretreated with methanol, activated at 60°C for 30 min, and were saturated with conc. ammonia vapors for 30 min in TLC chamber prior to application. It was required to eliminate the edge effect and to avoid unequal solvent evaporation losses from the developing plate that can lead to various types of random behavior usually resulting in lack of reproducibility in R_F values. So the attempt has been taken to develop and validate a cost-effective simple and robust HPTLC technique to quantify thymoquinone in the methanolic extract of *Nigella Sativa*. The methanolic extract peak of thymoquinone was well resolved at R_F value 0.77 depicted in **Figure 3**. The developed method was found to be quite selective with good baseline resolution of each compound.

Method Validation

Linearity

The drug concentration versus peak area was plotted between 75 - 500 ng band⁻¹ (n = 3) to construct a standard curve of TQ (**Figure 4**). The graph was found to be linear, i.e., adherence to the system to Beer's law. The polynomial regression for the calibration plots showed the good linear relationship with the coefficient of correlation r² = 0.997 and regression equation 16.58x - 224.2 over the concentration range studied depicted in in **Table 1**. There is no significant difference (ANOVA; P < 0.05) observed in the slopes of the standard curves in this range. The LOD and LOQ of the developed method were (18 and 54 ng band⁻¹) which showed that the method was sensitive to detect and quantitative the thymoquinone.

Precision

The intra-day and inter-day precision of TQ were expressed as % recovery and percentage relative standard deviations (% RSD) at three different levels were shown in **Table 2**. The percentage recovery for both was found to be in the range of (98.5-99.7 %) indicates the method was accurate by satisfying the acceptance criteria. The results for intra-day and inter-day variation of TQ recovery were studied at three different concentration levels 250, 350 and 400 ng spot⁻¹. The % RSD value was found to be <2% in all the cases. The low values of % RSD are indicated the high precision of the method.

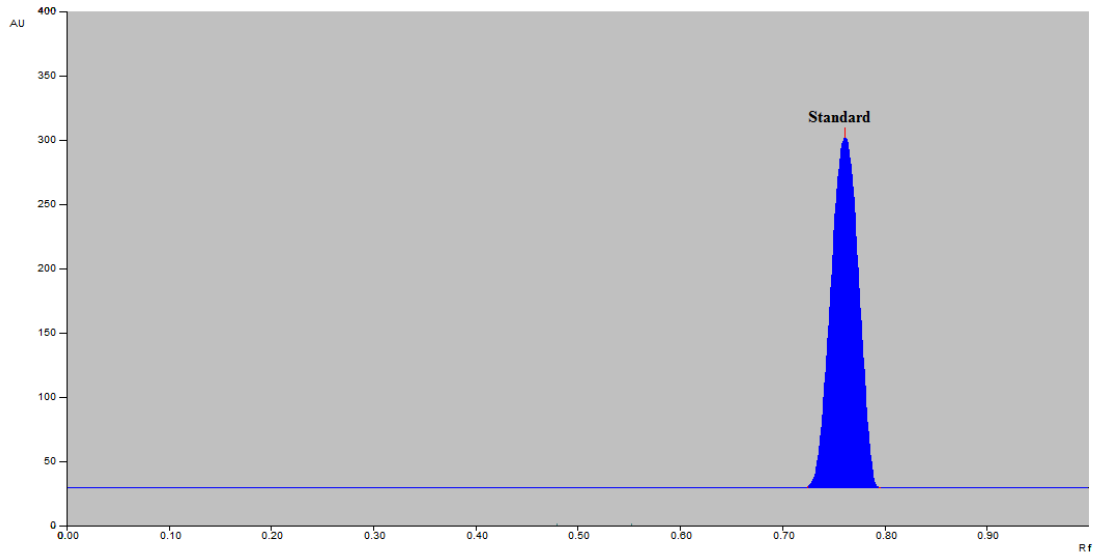


Figure 2: HPTLC chromatogram of standard thymoquinone (R_f 0.77).

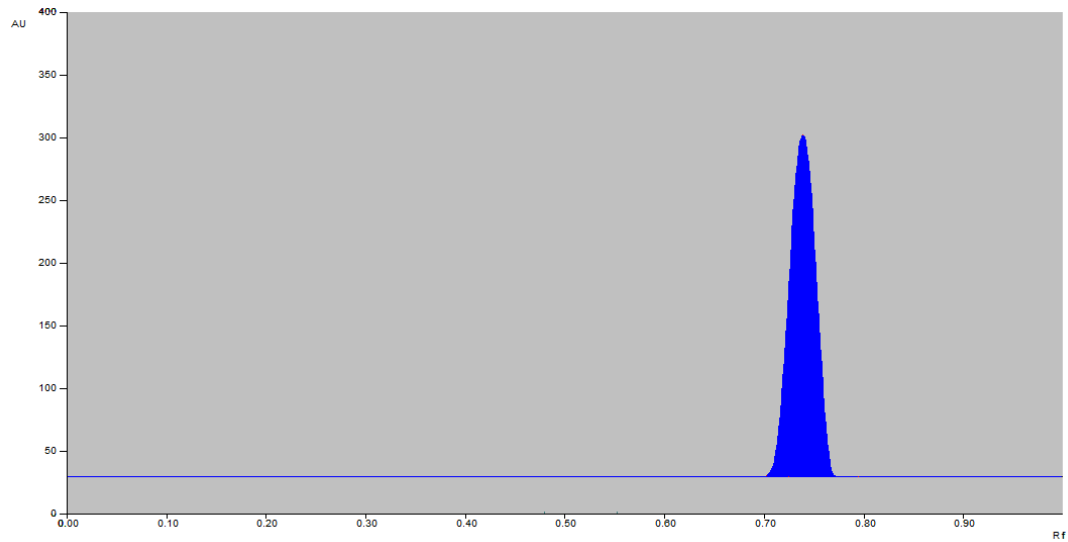


Figure 3: HPTLC chromatogram of thymoquinone extract (R_f 0.77).

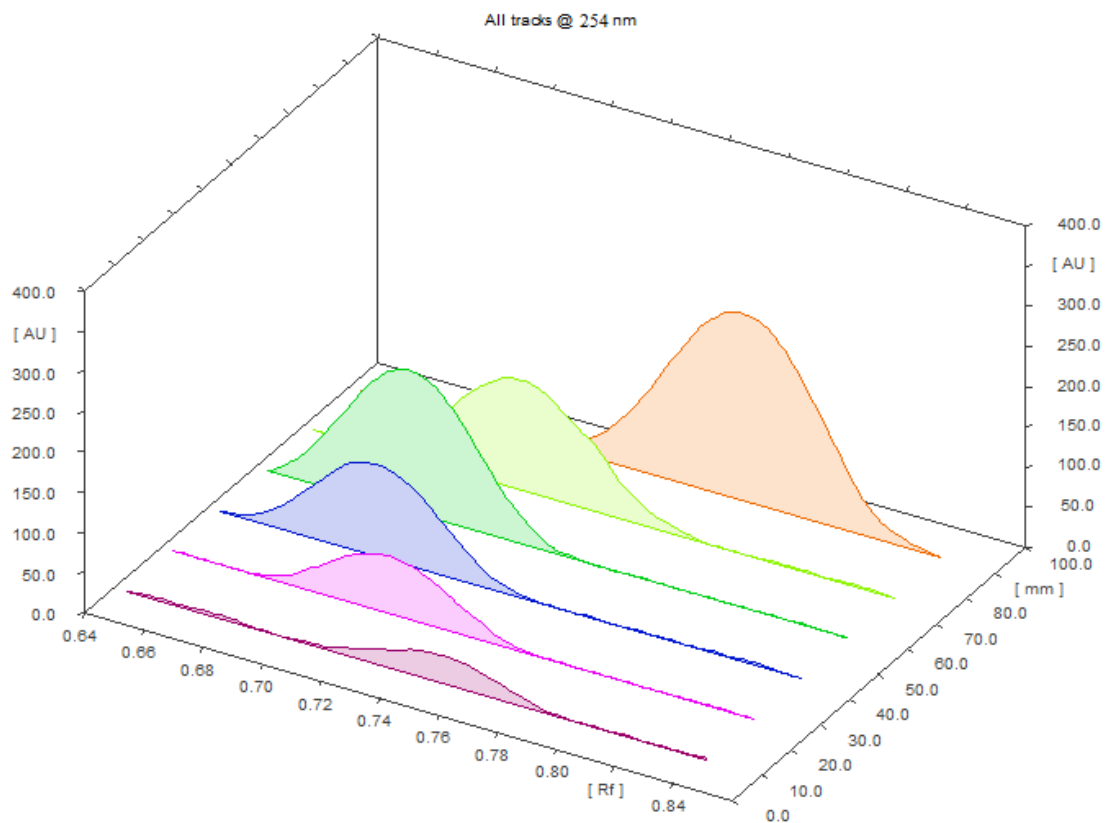


Figure 4: Comparative 3D scan of thymoquinone extract.

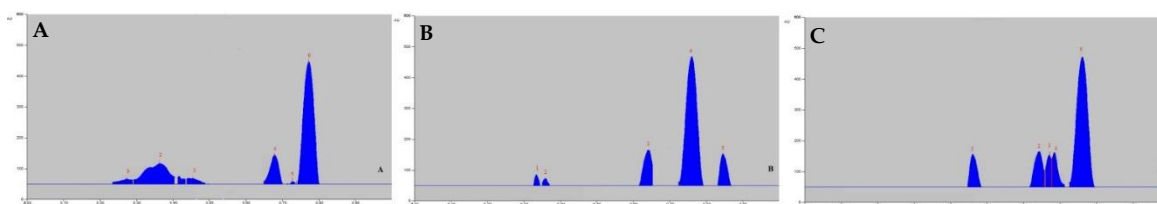


Figure 5: Degraded Chromatogram (A). H₂O₂ treated thymoquinone peak with degraded peak 1: degraded, R_f : 0.29, peak 2, R_f : 0.36, peak 3: degraded, R_f : 0.46, peak 4: degraded, R_f : 0.68, peak 5: degraded, R_f : 0.73); (B). dry heat and wet heat treated thymoquinone with degraded peak (peak 1: degraded, R_f : 0.33, peak 2, R_f : 0.36, peak 3: degraded, R_f : 0.64, peak 4: degraded, R_f : 0.85); (C). photochemical and uv rays treated thymoquinone with degraded peak (peak 1: R_f : 0.48, peak 2: R_f : 0.64, peak 3: R_f : 0.67, peak 3: R_f : 0.69).

Table 2: Intra-day and Inter-day precision data of the Thymoquinone (N=6).

Nominal concentration	Intra-day batch					Inter-day batch				
	Final conc.	Precision	% Recovery	SD	%CV	Final conc.	Precision	% Recovery	SD	%CV
250	247.25	0.90	98.9	0.81	0.28	247.75	0.71	99.1	0.58	0.23
350	348.25	0.37	99.5	0.67	0.19	344.75	0.20	98.5	0.29	0.08
400	398.80	0.48	99.7	0.81	0.20	397.80	0.31	99.4	0.41	0.10

SD- Standard Deviation, cv- Coefficient Variation

Table 3: Accuracy data of the Thymoquinone (n=6).

Conc. added to analyte (%)	Theoretical (ng)	Added (ng)	Detected (ng)	Recovery (%)	SD	%CV
50	250	125	374.01	99.73	0.055	0.014
100		250	498.86	99.77	0.364	0.073
150		375	623.95	99.83	0.756	0.121

Accuracy (%) = [concentration found]/(nominal concentration) × 100

Table 4: Robustness of the developed HPTLC method of Thymoquinone (n=6).

Mobile Phase						
Conc. ng/ml	n-Hexane: ethyl acetate : methanol		Results			
	Original	Used	Area	SD	%RSD	R _f
350	(7:2:1)	7.5:1.8:0.7	154.83	1.24	0.80	0.75
		7:2:1	156.86	1.40	0.90	0.77
		6.8:2.1:1.1	155.75	1.32	0.85	0.76
Mobile Phase Volume (15 ± 2 ml)						
Conc. ng/ml	Volume (ml)	Result				
		Area	SD	%RSD	R _f	
350	17	156.83	1.39	0.89	0.78	
	15	155.86	1.49	0.96	0.77	
	13	157.75	1.73	1.1	0.76	
Duration of Saturation						
Conc. ng/ml	Time (min)	Result				
		Area	SD	%RSD	R _f	
350	10	157.83	1.89	1.2	0.77	
	20	158.86	1.41	0.89	0.79	
	30	155.75	1.49	0.96	0.76	
Activated at 60 ± 5°C						
Conc. ng/ml	Time (min)	Result				
		Area	SD	%RSD	R _f	
350	2	155.83	1.21	0.78	0.78	
	5	156.86	1.28	0.82	0.77	
	7	154.75	1.47	0.95	0.76	

Specificity

The specificity of the method was ascertained by analyzing the pure TQ and methanolic extract. The spot for TQ in the sample was confirmed by comparing the R_f values and spectra of the spot with that of the standard. The peak purity of TQ was assessed by comparing the spectra at three different levels, *i.e.*, peak start (S), peak apex (M) and peak end (E) positions of the spot, *i.e.*, r^2 (S, M) = 0.989 and r^2 (M, E) = 0.991. A good correlation ($r^2 = 0.997$) was also obtained between standard and sample spectra of TQ, therefore, the method was considered specific.

Accuracy

The recoveries of the drugs were determined by standard addition method. The proposed method was used for extraction and subsequent estimation of TQ after spiking with 50, 100 and 150% of the additional drug in the extract, and the afforded recovery was obtained 99.49 to 100.37% (Table 3). The result suggests that the method can be considered accurate, as the %RSD of all the determinants were to be <2% which indicated that the method was accurate and also there was no interference of the excipients present in tablets.

Robustness

Table 4 describes the robustness of the proposed method and their SD and % RSD was calculated for the change in mobile phase composition, mobile phase volume, and duration of saturation and activation of prewashed-TLC plates at concentration levels of 350 ng band⁻¹ (in triplicate). The result of the study showed that the small deliberate change in the chromatographic condition gives no significant effect on the area and R_f value. The low values of % RSD (less than 3) indicated the robustness of the developed method.

Estimation of TQ in nanoformulation

A single spot at $R_f = 0.77 \pm 0.03$ was observed in the chromatogram of the TQ isolated from the extract along with other constituents. The presence of single spot confirms that there was no interference from the excipients found in the developed nanoformulation. The drug content was found to be 99.15 ± 1.44 with % RSD of 0.0877 for nanoformulation for three replicate. The low value of % RSD indicated the suitability of this method for routine analysis of TQ in pharmaceutical dosage forms.

Stability studies

The results of the forced degradation study of thymoquinone using are summarized in Table 5. Hydrogen peroxide-induced degradation. The sample degraded with 50 % w/v hydrogen peroxide showed additional peaks at R_f value of 0.29, 0.36, 0.46, 0.68 and 0.73 (Figure 5A). The spots of degraded products were well resolved from the parent compound spot.

Dry heat and wet heat degradation

The samples degraded under dry heat and wet heat conditions showed additional peaks at R_f values of 0.33, 0.36, 0.64 and 0.85, respectively (Figure 5B). The spots of degraded products were well resolved from the parent compound spot.

Photochemical and UV degradation

The photodegraded sample showed one additional peak at R_f value of 0.48, 0.64, and 0.67 (Figure 5C) when thymoquinone solution was left in daylight for 3 days. The

Table 5: Stress induced stability studies data for the developed method.

Degradation condition	No. of degradation products	R_f value
Peroxide-induced degradation	3	0.38, 0.40, 0.45
Dry heat-wet heat (100°C)	4	0.50, 0.55, 0.64, 0.69
Photochemical degradation	1	0.74
UV degradation	1	0.79

thymoquinone was degraded when exposed to UV irradiation for 15 days and showed additional peaks at R_f value of 0.69. The spot of UV degraded product was well resolved from the standard.

CONCLUSION

HPTLC is a simple, rapid and accurate method for analyzing plant material. This method can be used for phytochemical profiling of plants and quantification of compounds present in plants, with increasing demand for herbal products as medicines and cosmetics there is an urgent need for standardization of plant products. The presented study clearly gave evidence of the bioactive thymoquinone in methanolic extracts of *Nigella Sativa*. The developed method is simple, precise, specific, sensitive, and accurate. Further, this method can be effectively used for routine quality control of herbal materials as well as formulations containing this compound.

CONFLICT OF INTEREST

None.

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REFERENCES

- AbouBasha, L., Rashed, M.S., Aboul-Enein, H.Y. (1995). TLC assay of thymoquinone in black seed oil (*Nigella sativa* Linn) and identification of dithymoquinone and thymol. *J Liq Chromatogr Relat Technol* 18: 105–115. [\[DOI\]](#)
- Akhtar, M., Imam, S.S., Ahmad, M.A., Najmi, A.K., Mujeeb, M., and Aqil, M., (2014). Neuroprotective study of *Nigella sativa*-loaded oral provesicular lipid formulation: in vitro and ex vivo study. *Drug Deliv*, 21(6): 487–494. [\[DOI\]](#)
- Alam, P., Yusufoglu, H., Alam, A., (2013). HPTLC densitometric method for analysis of thymoquinone in *Nigella sativa* extracts and marketed formulations *Asian Pac. J. Trop Dis.*; 3(6): 467–471. [\[DOI\]](#)
- Ali, B.H., Blunden, G., (2003). Pharmacological and toxicological properties of *Nigella sativa*. *Phytother Res* 17: 299–305. [\[DOI\]](#)
- Ali, S., Taleuzzaman, M., Gilani, S.J., Ahmed, M.L., and Hafeez, A., (2016). Quantitative estimation of Donepenzil hydrochloride tablet by HPLC. *IJPC* 06 (02).
- Bakshi, M., Singh, S., (2011). Development of validated stability-indicating assay methods--critical review. *J. Pharm. Biomed. Anal.* 28 1011–1040. [\[DOI\]](#)
- Dixit, R.P., Barhate, C.R., Nagarsenker, M.S. (2008). Stability-indicating HPTLC method for simultaneous determination of ezetimibe and simvastatin. *Chromatographia* 67: 101–107. [\[DOI\]](#)
- Ghosheh, O.A., Houdi, A.A., Crooks, P.A., (1999). High performance liquid chromatographic analysis of the pharmacologically active quinones and related compounds in the oil of the black seed (*Nigella sativa* L.). *J Pharm Biomed Anal* 19: 757–762. [\[DOI\]](#)

- Ghosheh, O.A., Houdi, A.A., Crooks, P.A., (1999). High performance liquid chromatographic analysis of the pharmacologically active quinones and related compounds in the oil of the black seed (*Nigella sativa* L.). *J Pharm Biomed Anal* 19: 757–762. [\[DOI\]](#)
- Houghton, P.J., Zarka, R., DelasHeras, B., Hoult, J.R. (1995). Fixed oil of *Nigella sativa* and derived thymoquinone inhibit eicosanoid generation in leukocytes and membrane lipid peroxidation. *Planta Med* 61: 33–36. [\[DOI\]](#)
- ICH, Q1A (October 1993). Stability Testing of New Drug Substances and Products, International Council for Harmonisation, Geneva.
- ICH, Q2A, (October 1994). Validation of Analytical Procedure: Methodology, International Council for Harmonisation, Geneva.
- Kaur, A.D., Ravichandran, V., Jain, P.K., Agrawal, R.K., (2008). High-performance thin layer chromatography method for estimation of conessine in herbal extract and pharmaceutical dosage formulations. *J Pharm Biomed Anal* 46: 391–394. [\[DOI\]](#)
- Michelitsch, A., Rittmannsberger, A. (2003). A simple differential pulse polarographic method for the determination of thymoquinone in black seed oil. *Phytochem Anal* 14: 224–227. [\[DOI\]](#)
- Mittal, A., Imam, S.S., Parmar, S., Gilani, S.J. and Taleuzzaman, M., (2015). Design of Experiment based Optimized RP-HPLC Method for Simultaneous Estimation of Amlodipine and Valsartan in Bulk and Tablet Formulations. *Austin J Anal Pharm Chem*, 2(6): 1-6.
- Ranganathan, T.V., Kulkarni, R.P., (2002). Food Chemist. A simple method for the analysis of trehalose using HPTLC. 77 263–265.
- Reelma, M., Pereira, V., Chandrashekar, R., Barhate, R., Kulkarni, G., Jagtapa, A., (2011). Validated High-performance Thin-layer Chromatographic Method for the Quantification of Thymoquinone in *Nigella Sativa* Extracts and Formulations. *Phytochem. Anal.*, 22, 367–373. [\[DOI\]](#)
- Sayeed, S., Imam, S. S., Najmi, A.K., Aqil, M., and Akhtar, M., (2017). Nonionic surfactant based thymoquinone loaded nanoprinosomal formulation: invitro physicochemical evaluation and in vivo hepatoprotective efficacy. *Drug Development and Industrial Pharmacy*, 43, 9, 1413–1420. [\[DOI\]](#)
- Sethi, P.D., (1996) High Performance Thin Layer Chromatography, Quantitative Analysis of Pharmaceutical Formulations, CBS Publishers, New Delhi, India.
- Stolarczyk, M., Maalanka, A., Apola, A., Krzek, J., (2010). Analysis of hypotensive compounds occurring in complex agents *Acta Pol. Pharm. Drug Res.* 67 441–454.
- Tayade, N.G., Nagarsenker, M.S. (2007). Validated HPTLC method of analysis for artemether and its formulations. *J Pharm Biomed Anal* 43: 839–844. [\[DOI\]](#)
- Zammataro, A., Saletti, R., Civiale, C., Muccilli, V., Cunsolo, V., Foti, S., (2010) Simultaneous quantification of carteolol and dorzolamide in rabbit aqueous humor and ciliary body by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.*; 878 (9-10):807-14. [\[DOI\]](#)