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Original research paper

Stability study of thymoquinone, carvacrol and thymol using HPLC-UV and LC-ESI-MS

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The aim of this study was to investigate the stability of three major antioxidants of *Nigella sativa*: thymoquinone (TQ), carvacrol (CR) and thymol (THY), under different stress conditions using HPLC and LC-MS/MS. Forced degradation for each compound was performed under different conditions, including oxidation, hydrolysis, photolysis and thermal decomposition. The results showed that both CR and THY were stable under the studied conditions, whereas TQ was not affected by acidic, basic and oxidative forced conditions but the effect of light and heat was significant. The degradation products of TQ were further investigated and characterized by LC-MS/MS. HPLC-UV method has been fully validated in terms of linearity and range, the limit of detection and quantitation, precision, selectivity, accuracy and robustness. The method was successfully applied to quantitative analysis of the principal antioxidants of *Nigella sativa* TQ, CR and THY in different phytopharmaceuticals.

Keywords: thymoquinone, carvacrol, thymol, HPLC-UV, LC-MS/MS, stability

Black seed (*Nigella sativa*, family Ranunculaceae) (NS) is a valuable medicinal plant that exhibits several biological activities, including anti-cancer, anti-microbial, anti-inflammatory, anti-atherogenic and cardioprotective (1–3), as well as antioxidant properties (4–6). Oxidative stress is generated due to a disturbance in the balance between the production of free radicals and the endogenous antioxidants. It plays a crucial role in the pathology of various conditions, including aging, cancer and neurodegenerative disorders (7–9). Natural antioxidants with protective activity against oxidative stress and cellular damage have gained much interest.

NS seeds contain a variety of constituents, mainly oils. Thymoquinone (TQ) (2-isopropyl-5-methyl-1,4-benzoquinone) is the major active constituent in the volatile oil (2). The

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therapeutic value of TQ could be attributed to its antioxidant effects. In comparison with TQ, carvacrol (CR) (5-isopropyl-2-methylphenol) and its isomer, thymol (THY) (2-isopropyl-5-methylphenol), are found in lower amounts in the volatile oil as phenolic antioxidants. They are both considered promising compounds (10, 11).

The quality of phytopharmaceuticals is essential for consumer safety. Improper storage of phytopharmaceuticals may lead to the degradation of products. The presence of these degraded products may have a significant negative effect on phytopharmaceuticals quality, efficacy and integrity. This occurs through changing the chemical, toxicological and pharmacological properties of the active constituents. Thus, maintaining the stability of phytoconstituents confirms the delivery of the proper therapeutic doses to consumers.

Through the literature, several analytical methods have been described for the analysis of TQ alone or in combination with THY including HPLC (12–15), UPLC (16), TLC (17) and HPTLC (18). CR and THY were analyzed using differential pulse voltammetry (19). Few GC methods were used for the estimation of active constituents in NS (4, 20).

Although there are various analytical reports for qualitative and quantitative analysis of NS active constituents, only two research papers were reported on the stability-indicating method for TQ (16, 21), using UPLC for the determination of TQ in formulation or in biomatrices such as plasma/serum. The two published methods (16, 21) stated that TQ degrades under different forced conditions. However, these methods (16, 21) did not identify any degradation products of TQ and did not study the stability of other components (CR, THY).

The goal of the current research was to investigate the stability of the principal antioxidants of NS (TQ, CR and THY) by using the HPLC technique, and to investigate and characterize the degradation products using LC-MS/MS.

EXPERIMENTAL

Materials and reagents

Authentic standards of TQ, CR and THY were obtained from Merck KgaA (Germany) and purity of ≥ 99.0, ≥ 98.0 and ≥ 99.0 %, resp., was certified for each standard. Methanol (HPLC grade) was purchased from Carlo Erba Reactifs-SDS SAS (France). Formic acid (99 %) was provided by Carlo Erba (Italy). Sodium hydroxide, 30 % hydrogen peroxide and hydrochloric acid of analytical grade were all purchased from Merck.

Baraka® soft gelatin capsules, three different batches (Batch no 412, 418, 422) used, were manufactured by TechnoPharma Egypt, Egypt, and packed by Pharco Pharmaceuticals, Egypt. Each capsule was labeled to contain 450 mg of NS seed oil. Black seed® oil used was manufactured by El Hawag, Egypt (company for row oils), and Black cumin® oil used was manufactured by Zamzam, Egypt (company for extracting natural oils, herbs and cosmetics). Each bottle contains 25 mL of *Nigella sativa* oil.

Analytical methods

The amounts of TQ, CR and THY were quantified using the HPLC-UV apparatus (Hitachi LaChrom Elite, Japan) consisting of EZChrom Elite software package, two L-2130

pumps, autosampler L-2200, column oven L-2300 and UV–Visible detector, model L-2420. The separation was done on a C_{18} column (Inertsil ODS-3v, 250 mm × 4.6 mm i.d., 5-μm particle diameter, GL Sciences, Japan). The mobile phase contained 0.1 % aqueous formic acid and methanol, 40:60 (*V*/*V*). Twenty microliters of each sample were injected at a flow rate of 1.5 mL min^{-1}. The temperature was adjusted to 25 °C and the detection wavelength was fixed to 254 nm.

In order to analyze the degraded samples of TQ, CR and THY LC-ESI-MS system incorporating an HP 1100 Series HPLC instrument (Agilent Technologies, Germany) and a Finnigan LCQ ADVANTAGE MAX mass spectrometer coupled with ESI source (Thermo Fisher Scientific, USA), controlled by Xcalibur 1.4 software was used. Chromatographic separation was made on a YMC^{TM} ODS-AQ 2 × 150 mm C_{18} column (Waters, USA). Mass spectra were acquired by electrospray ionization in positive mode mass spectrometry (ESI/MS) in the mass/charge ratio (*m*/*z*) range of 50–1000 during the complete chromatographic run. An injection of 20 μL was used and the source voltage was 4.5 kV, source current 100 μA, capillary voltage 3 V and capillary temperature 200 °C, were adjusted for optimal detection.

Using LC-ESI-MS, the degradation products were identified by comparing sample chromatograms at different degradation times to those of the standard prior to degradation. Detection of new peaks in the sample chromatograms obtained after degradation times is an indication of degradation products.

Standard solutions preparation

Stock standard solutions were prepared by dissolving 10.0 mg TQ, CR and THY, separately, in 10 mL of methanol. The standard solutions of 100 μg mL^{-1} were obtained by serial dilutions of the stock with methanol.

Forced degradation conditions

To develop the forced degradation assays, the following conditions were applied to the standard solutions.

Hydrolytic conditions: acid/base-induced degradation. – The degradation was carried out by putting 2 mL of the solution containing 100 μg mL^{-1} of each compound separately in a 10-mL volumetric flask covered with aluminum foil to exclude the effect of light. The solution was treated with 2 mL 2 mol L^{-1} HCl or 2 mL 1 mol L^{-1} NaOH, resp. The flasks were sealed and placed at room temperature for a month. Later, the solutions were neutralized as needed (with 2 mol L^{-1} NaOH or 1 mol L^{-1} HCl). The final volume was made up with mobile phase. In the end, the solutions were filtered by using a 0.45-μm syringe filter and injected into the HPLC system. The study was carried out in triplicate.

Oxidative conditions: hydrogen peroxide-induced degradation. – The degradation was performed by pipetting 2 mL of a 100 μg mL^{-1} solution of each compound, separately, in 10-mL volumetric flask covered with aluminum foil. The solution was treated with 0.5 mL of hydrogen peroxide solution (30 %, *V*/*V*). The flasks were closed and stored at room temperature for a month. Before injecting the solution into the chromatographic system, the volume was completed with the mobile phase and filtered using a 0.45-μm syringe filter.

Photodegradation studies. – The degradation was carried out by pipetting 2 mL of each compound (100 μg mL^{-1}) into a 10-mL transparent volumetric flask and completing the volume with methanol. The flasks were exposed to direct sunlight for 6 h. Then, the solutions were filtered through a 0.45-μm syringe filter and further injected into the chromatographic system. The experiment was carried out in triplicate.

*Thermal degradatio*n. – The thermal decomposition was performed by pipetting 2 mL of each compound (100 μg mL^{-1}) in a 10-mL volumetric flask coated with aluminum foil and completed to volume with methanol. The flasks were heated to 85 °C for 2 h and filtered using a 0.45-μm syringe filter, before injecting the solution into the chromatographic system. The degradation studies were also carried out in triplicate.

Method validation

Validation of the proposed HPLC-UV method was performed with respect to linearity concentration range, limits of detection and quantitation, selectivity, precision, accuracy and robustness, as per the guidelines of ICH (22).

Linearity and range. – The linearity of the developed HPLC-UV method was judged by analyzing seven concentrations in the range 0.4–500 μg mL^{-1} for TQ and 0.1–100 μg mL^{-1} for each CR and THY. Each concentration was prepared three times. The analysis was done according to the previous conditions. The linearity was evaluated by plotting peak area measured at 254 nm against corresponding concentrations of each drug.

Selectivity. – The selectivity of the HPLC-UV method was achieved with analyses of different laboratory prepared mixtures of TQ, CR, and THY within the linearity range, spiked with different levels of light and heat degradation products of TQ.

Precision. – For 1.0, 50.0 and 100 μg mL^{-1} of each compound, the analysis procedure previously described under experimental conditions, was repeated three times within a day to determine the repeatability (intra-day precision) and three times on different days to determine the intermediate precision (inter-day precision) of the method. The relative standard deviation (RSD) values for TQ, CR and THY for intra-day and inter-day precision were calculated.

Detection and quantitation limit. – Limit of detection (*LOD*) and limit of quantification (*LOQ*) were calculated according to the current ICH guidelines (22) as the ratio of 3.3 and 10 standard deviations of the blank ($n = 7$), resp., and the slope of the calibration line (14).

Accuracy. – The accuracy of the HPLC-UV method was tested by adding known amounts of the studied compounds to a known concentration of the commercial products that were previously analyzed by the proposed method (standard addition method). The resulting mixtures were analyzed and the mean percentage recoveries and their standard deviations for three replicates were calculated.

Robustness. – The robustness of the method was tested by variation of the organic strength of the mobile phase by ± 2 % and the mobile phase flow rate by ± 0.2 %. The effect

of these factors on percent recovery of 10 μg mL^{-1}, retention time and resolution of the studied compounds was measured. All measurements were done in triplicates.

Sample preparation

The content of twenty capsules from each capsule formulation was weighed and mixed. A portion equivalent to the mass of one capsule was accurately weighed, dissolved and diluted to 5 mL with methanol. For oil preparations, 0.2 mL oil was transferred into a 10-mL volumetric flask separately and the volume was completed with methanol. Finally, sample solutions were filtered and diluted with the mobile phase to reach the calibration range. The calibration procedure described above was followed and the concentration of each compound was calculated.

RESULTS AND DISCUSSION

HPLC-UV optimization

The chromatographic conditions of the HPLC-UV method were studied and optimized as a function of methanol concentration in the mobile phase. The chosen chromatographic conditions gave good separation of the studied compounds. The optimum conditions for the separation were obtained using a C_{18} column at 25 °C and mobile phase composed of 0.1 % formic acid in water and methanol, in the ratio 40:60 (*V*/*V*). Using mobile phase containing methanol in a ratio higher than 60 % caused the overlap between CR and THY peaks. When trying mobile phase containing methanol, less than 40 % separation was achieved but with increased retention time and peak tailing for TQ, CR and THY.

The optimized method can be utilized for the analysis of TQ, CR, THY and TQ degradation products. Chromatographic parameters of the optimized HPLC-UV method are given in Table I.

Table I. Chromatographic parameters of the optimized HPLC-UV method

Compound	Retention time (min)	Capacity factor (k')	Resolution R_s	Plate count (N)[a]	Tailing factor
TQD1	3.41	2.92	1.87	90,456	0.98
TQD2	3.83	3.40	2.54	97,213	1.06
TQD3	6.40	6.36	3.10	100,567	1.12
TQD4	9.66	10.10	2.97	105,608	1.03
TQ	13.14	14.10	8.53	122,315	1.14
CR	20.20	22.21	1.51	131,334	1.07
THY	21.10	23.25	–	150,446	1.13

[a] Per meter.

HPLC-UV method validation

Linearity and range. – The linearity of the calibration curves was confirmed by the high value of correlation coefficient of the regression line of 0.9999 for TQ, CR and THY (Table II).

Selectivity. – using the HPLC-UV method. These data show that the results were unaffected by the presence of the TQ-degradation products (Table III). The obtained chromatogram from the laboratory prepared mixtures (Fig. 1) did not show any interference of TQ

Table II. Characteristic parameters for the regression equations of the proposed HPLC method for determination of TQ, CR and THY

Parameter	TQ	CR	THY
Linearity range (μg mL^{-1})	0.4–500	0.1–100	0.1–100
LOD (μg mL^{-1})	0.006	0.006	0.027
LOQ (μg mL^{-1})	0.018	0.018	0.081
Regression equation[a]			
Slope (b)			
Slope (b)	3.15×10^5	7.10×10^3	6.26×10^3
Slope (b)			
Standard deviation of the slope (S_b)	1.89×10^2	5.21×10^{-1}	6.13×10^{-1}
Intercept (a)	-3.11×10^3	2.78×10^3	1.51×10^4
Correlation coefficient (*R*)	0.9999	0.9999	0.9999

[a] $Y = a + b\gamma$, where γ is the concentration in μg mL^{-1} and Y is the peak area, $n = 7$.

Table III. Model recovery of TQ, CR and THY in laboratory prepared mixtures using the proposed HPLC-UV method

Mixture No.	Concentration (μg mL^{-1})			Recovery (%)		
	TQ	CR	THY	TQ	CR	THY
1	50.0	50.0	50.0	99.0	99.3	99.1
2	0.4	0.1	0.1	102.3	102.5	101.8
3	500.0	100.0	100.0	99.8	100.2	100.7
4	30.0	20.0	40.0	99.7	99.5	99.2
5	70.0	80.0	0.5	100.1	100.4	101.9
6	180.0	70.0	90.0	99.6	99.5	100.6
7	80.0	70.0	50.0	99.8	99.3	100.6
Mean				100.0	100.1	100.6
SD[a]				1.1	1.1	1.1

[a] $n = 7$

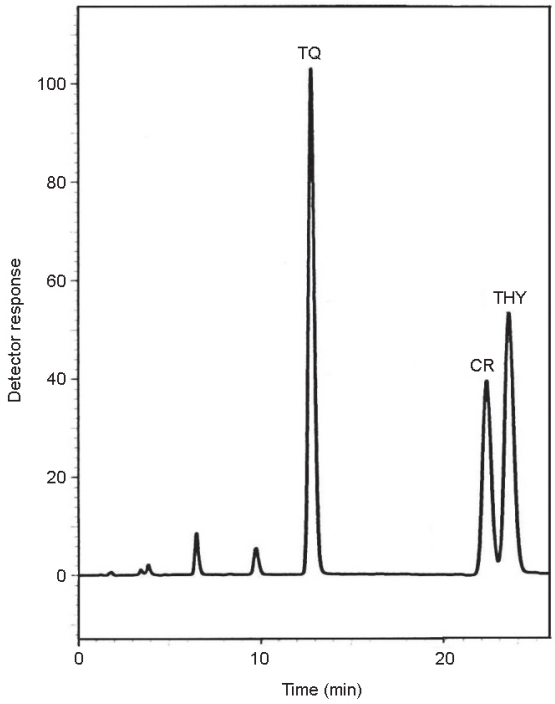

Fig. 1. HPLC chromatogram of 20-μL injection of laboratory prepared mixture of TQ, CR, THY and TQ degradants.

Table IV. Intra- and inter-day model validation of the proposed HPLC-UV method through the recovery of TQ, CR and THY

Compound	Concentration (μg mL^{-1})	Intra-day precision		Inter-day precision	
		Recovery (%)[a]	RSD (%)	Recovery (%)[a]	RSD (%)
TQ	1.0	100.2 ± 0.6	0.6	99.7 ± 1.1	1.1
	50.0	99.8 ± 0.5	0.5	100.1 ± 0.8	0.8
	100.0	100.0 ± 0.4	0.4	100.1 ± 0.7	0.7
CR	1.0	99.9 ± 0.7	0.7	99.7 ± 1.0	1.0
	50.0	97.6 ± 0.1	0.1	100.1 ± 0.8	0.8
	100.0	100.0 ± 0.4	0.4	100.1 ± 0.7	0.7
THY	1.0	103.2 ± 0.3	0.3	99.9 ± 0.1	0.1
	50.0	99.9 ± 0.6	0.6	99.9 ± 0.9	0. 9
	100.0	100.0 ± 0.4	0.4	100.1 ± 0.8	0.8

[a] Mean ± SD for five determinations.

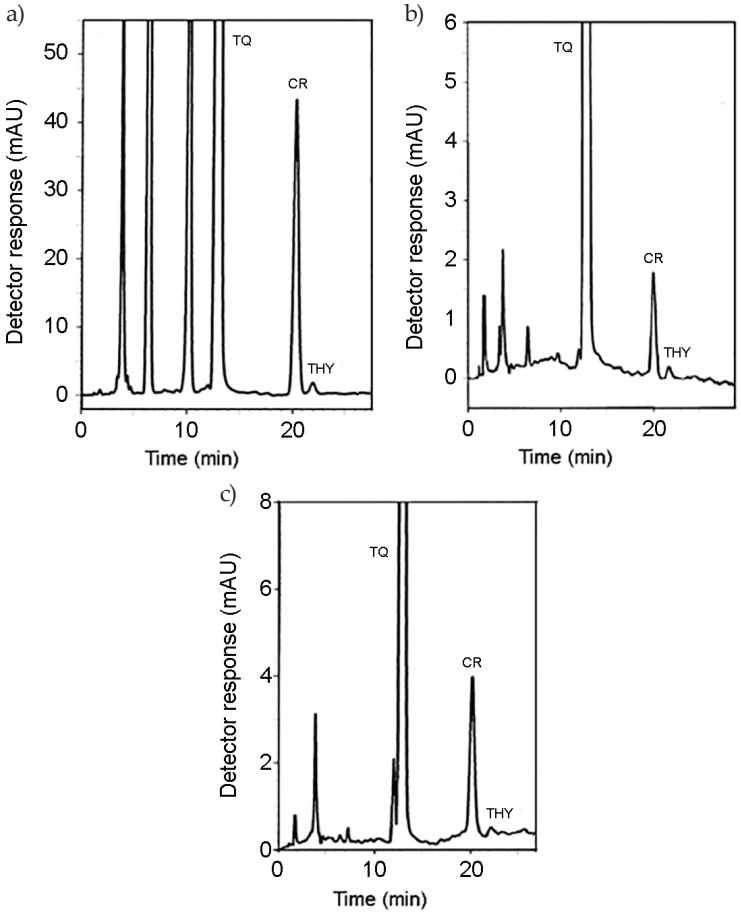

Fig. 2. Typical HPLC chromatograms of 20 μL injection of: a) Baraka® capsules, b) Black seed® oil, c) Black cumin® oil phytopharmaceuticals.

degradation products and the three studied compounds. In addition, selectivity was also verified by observing the obtained chromatograms from real samples (Fig. 2) and they show good resolution between TQ, CR, and THY peaks and the other components or excipients present in the herbal matrix.

Precision. – RSD values as shown in Table IV for TQ, CR and THY, were 0.3–0.7 % for repeatability and 0.1–1.1 % for intermediate precision, resp., indicating reasonable precision of the HPLC-UV method.

Detection and quantitation limits. – The limits of detection and quantification were found to be 0.006 and 0.018 μg mL^{-1} for both TQ and CR, as well as 0.027 and 0.081 μg mL^{-1} for THY (Table II).

Table V. Application of the standard addition technique to the analysis of TQ, CR and THY on: a) Baraka® capsules, b) Black seed® oil, c) Black cumin® oil by the proposed LC-UV method

a)	Analyzed conc. (μg mL^{-1})			Added conc. (μg mL^{-1})			Recovery of added analyte (%)		
Exp. No.	TQ	CR	THY	TQ	CR	THY	TQ	CR	THY
1	45.0	4.0	1.0	40.0	15.0	20.0	97.8	98.9	99.2
2	20.0	9.0	6.0	10.0	1.0	2.0	98.3	99.6	100.1
3	25.0	6.0	3.0	15.0	10.0	25.0	100.0	100.5	99.3
4	2.0	25.0	5.0	1.0	1.0	20.0	98.0	99.0	100.7
5	60.0	10.0	4.0	3.0	3.0	6.0	97.9	99.9	100.8
6	40.0	15.0	3.0	20.0	2.0	4.0	98.9	100.0	99.5
					Mean		98.5	99.7	99.9
					SD		0.8	0.6	0.7

b)	Analyzed conc. (μg mL^{-1})			Added conc. (μg mL^{-1})			Recovery of added analyte (%)		
Exp. No.	TQ	CR	THY	TQ	CR	THY	TQ	CR	THY
1	120.0	77.0	4.4	2.0	4.0	20.0	99.8	99.6	100.6
2	60.0	3.0	15.0	9.0	10.0	6.0	99.5	99.4	100.2
3	35.0	6.5	4.0	6.0	10.0	30.0	99.1	99.7	99.7
4	55.0	4.0	10.0	25.0	1.0	15.0	100.3	99.6	99.5
5	40.0	3.0	3.0	10.0	3.0	40.0	100.6	100.2	99.6
6	30.0	5.0	4.0	15.0	2.0	3.0	100.1	100.3	99.3
					Mean		99.9	99.8	99.8
					SD		0.6	0.4	0.5

c)	Analyzed conc. (μg mL^{-1})			Added conc. (μg mL^{-1})			Recovery of added analyte (%)		
Exp. No.	TQ	CR	THY	TQ	CR	THY	TQ	CR	THY
1	100.0	15.0	30.0	4.0	40.0	30.0	100.3	100.3	99.8
2	10.0	1.0	10.0	10.0	10.0	15.0	99.7	99.4	99.7
3	20.0	10.0	25.0	20.0	20.0	20.0	98.9	99.8	100.9
4	30.0	10.0	45.0	30.0	30.0	5.0	100.2	100.8	101.2
5	50.0	20.0	5.0	40.0	8.0	7.0	100	101.2	100.5
6	5.0	20.0	1.0	55.0	6.0	10.0	100.5	99.6	101.1
					Mean		99.9	100.2	100.5
					SD		0.6	0.7	0.7

$n = 6$.

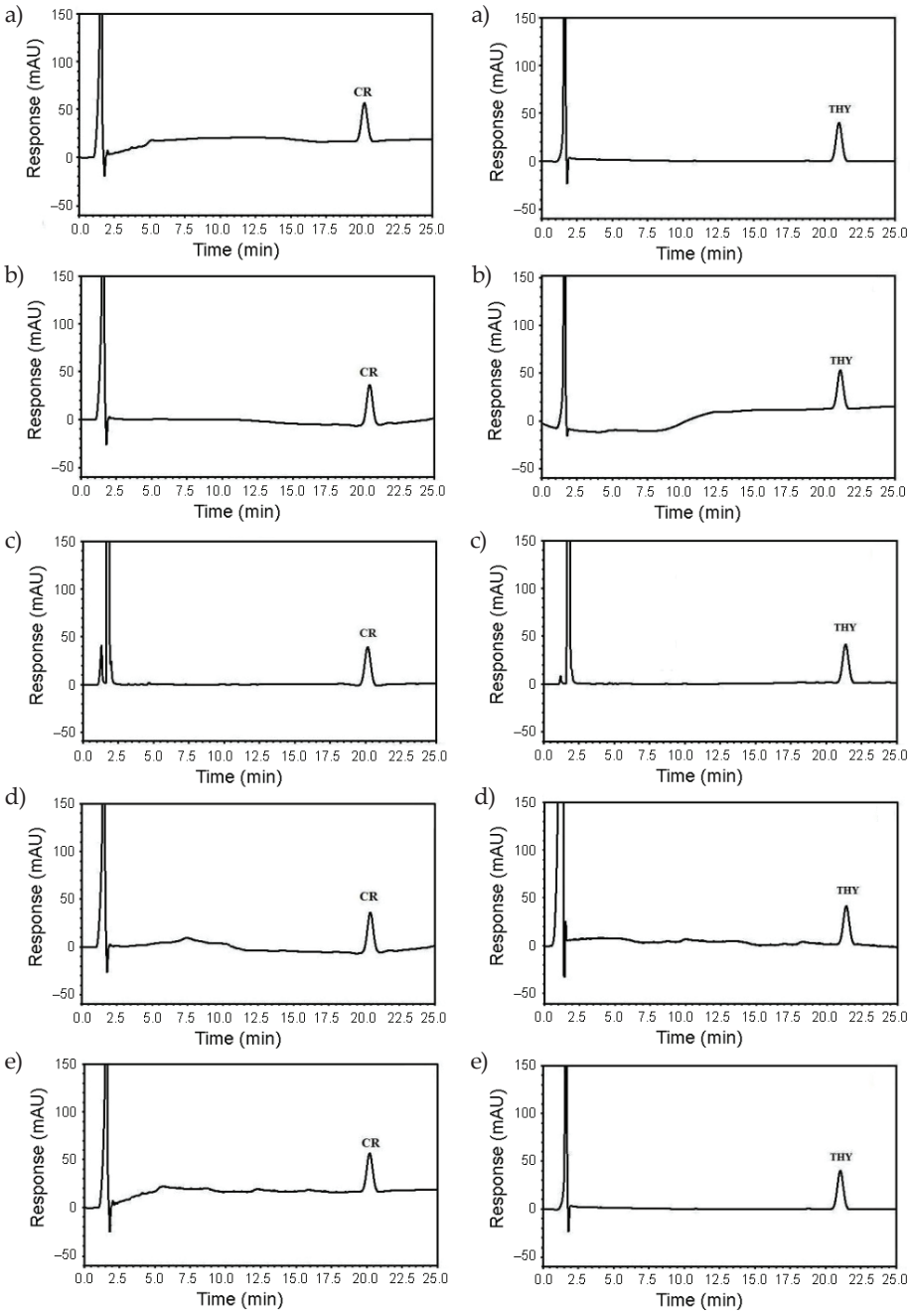

Fig. 3. HPLC chromatograms of 20-μL injection of: a) acid, b) base, c) oxidative, d) light and e) thermally induced forced degradation of CR and THY.

Table VI. HPLC-UV method robustness for TQ, CR and THY

Factor	Level of changes	TQ			CR			THY		
		Recovery (%)	Capacity factor	Resolution	Recovery (%)	Capacity factor	Resolution	Recovery (%)	Capacity factor	Resolution
Mobile phase composition (*V*/*V*) 0.1 % aqueous formic acid: methanol	42:58	100.5	14.15	8.61	99.6	22.27	1.52	100.3	23.31	–
	38:62	99.2	14.04	8.49	99.9	22.15	1.50	100.6	23.20	–
Flow rate (mL min^{-1})	1.3	101.1	14.17	8.44	99.2	22.29	1.51	100.1	23.29	–
	1.7	100.8	14.02	8.46	99.4	22.17	1.51	101.4	23.19	–

Table VII. Determination of TQ, CR and THY in commercial phytopharmaceuticals using the proposed HPLC-UV method

Formulation	Mean ± SD[a]					
	New HPLC-UV method			Reported HPLC-UV method (15)		
	TQ	CR	THY	TQ	CR	THY
Baraka® capsules	20.95 ± 0.90	0.76 ± 0.07	0.04 ± 0.01			
t	1.79	2.09	1.97	20.18 ± 0.70	0.85 ± 0.09	0.03 ± 0.009
F	1.65	1.65	1.23			
Black seed® oil	2.10 ± 0.09	0.73 ± 0.05	0.09 ± 0.01			
t	2.05	2.03	1.18	1.99 ± 0.11	0.79± 0.06	0.10 ± 0.02
F	1.49	1.44	4.0			
Black cumin® oil	1.81 ± 0.06	0.98 ± 0.03	0.07 ± 0.009			
t	1.72	2.12	1.96	1.75 ± 0.07	1.02 ± 0.04	0.08 ± 0.01
F	1.36	1.78	1.23			

[a] Concentration found in mg per capsule or in 25 mL oil; $n = 7$.
Theoretical values for t and F at $p = 0.05$: 2.18 and 4.28, resp.

Accuracy. – According to the obtained results (Tables IV and V) a good accuracy was observed for this method. Satisfactory, mean percentage recoveries were obtained for application of the standard addition technique to the analysis of TQ, CR and THY, respectively, and the results obtained were as follow, 98.5, 99.7, 99.9 % for standard addition on Baraka® capsules, 99.9, 99.8, 99.8 % for standard addition on Black seed® oil and 99.9, 100.2, 100.5 % for standard addition on Black cumin® oil.

Robustness. – Robustness of the method was judged from the obtained results, it was concluded that the studied factors did not affect percent recovery of 10 μg mL^{-1}, retention time and resolution of the studied compounds (Table VI).

Sample analysis

The proposed HPLC-UV method was applied to the quantification of principal antioxidants in three NS commercial phytopharmaceuticals without the interference of other active components present in NS. One batch of Baraka® capsules and one batch of Black seed® oil and Black cumin® oil were selected. Every batch was analyzed in triplicate (Table VII). The results of the analysed samples were obtained by the developed HPLC-UV method and were statistically compared to the published HPLC method (15), using Student's *t*-test and the *F*-ratio test at 95 % confidence level (Table VII). It is obvious that there is no significant difference between the performances of the developed and the reported method (15).

Identification of the degradation products by LC-ESI-MS

CR and THY were stable under the studied stress conditions (previously described in experimental section) since no new peaks developed (Fig. 3).

TQ was not affected by acidic, basic and oxidative forced conditions, but the effects of light and heat were significant (Fig. 4). Under light and thermal stress conditions, TQ (retention time 13.14 min) was degraded to give TQD1, TQD2, TQD3 and TQD4 peaks at 3.41, 3.83, 6.40 and 9.66 min, resp. These results are in contradiction to the data reported by Pathan *et al.* (16) who stated that TQ degraded in acidic, basic, oxidation and under UV light stress conditions.

TQ is sensitive to heat and light; Smith and Tess (23) stated that after exposure to light for five days, TQ converted gradually to dithymoquinone (70–80 %), which undergoes redox cycling reaction to give other degradation products.

TQ degraded sample was analyzed by LC-MS/MS. The standard solution of TQ was directly injected in the positive ion scan mode in the ESI source. TQ gave protonated molecular ion at *m*/*z* 165.46 $(M+H)^+$ and also fragmental ions at *m*/*z* 150.33, 148.37, 136.92 and 122.04, attributed to the loss of a methyl group (*m*/*z* 15), hydroxyl group (*m*/*z* 17), carbon monoxide (*m*/*z* 28) and isopropyl (*m*/*z* 43), resp. Also, TQ gave a peak at *m*/*z* 107.13 which is due to the loss of $(CH_3)_2CO$ plus the radical hydrogen (Fig. 5).

The fragmentation patterns obtained in the mass spectra were used for the characterization of the parent compound and its degradation products. The degradation product TQD1 gave molecular ion at *m*/*z* 109.09 $(M+H)^+$, pointing out that it has a molecular mass of 108.09. TQD1 had a fragment ion at *m*/*z* 81.97 attributed to the liberation of carbon monoxide. This finding suggested that TQD1 was benzoquinone (Fig. 6). The degradation product

TQD2 exhibited molecular ion at *m*/*z* 111.11 $(M+H)^+$ corresponding to the molar mass of 110.11. TQD2 had a fragment ion at *m*/*z* 94.02 attributed to the liberation of hydroxyl group. This finding suggested that TQD2 was hydroquinone (Fig. 7). The degradation product TQD3 exhibited an $(M+H)^+$ ion at *m*/*z* 329.96, suggesting that two TQ molecules are linked together generating a dimer of TQ. This degradation product was found to be the major compound among the analyzed degradation products. This finding suggested that the

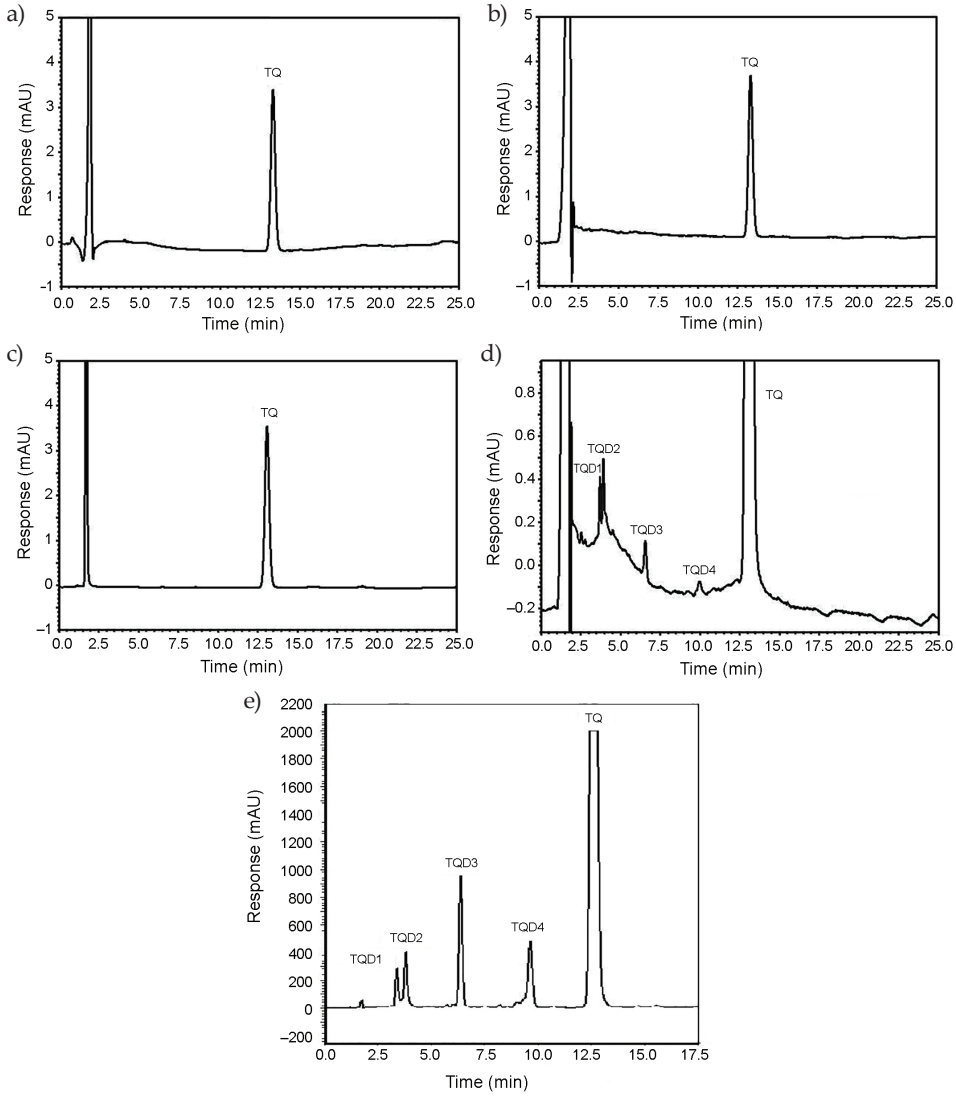

Fig. 4. HPLC chromatograms of 20-μL injection of: a) acid, b) base, c) oxidative, d) thermal and e) light-induced forced degradation of TQ.

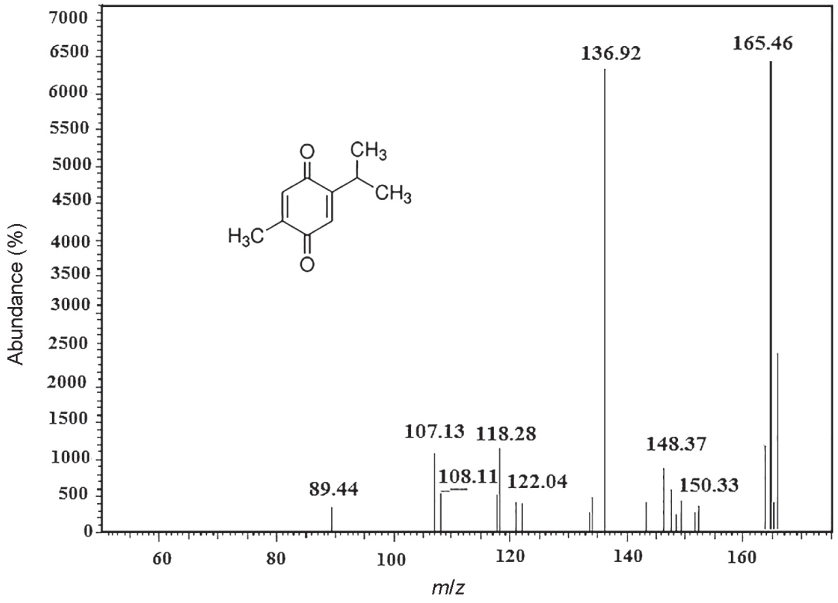

Fig. 5. MS/MS product ion spectrum of TQ standard after direct injection in the (+) ESI source.

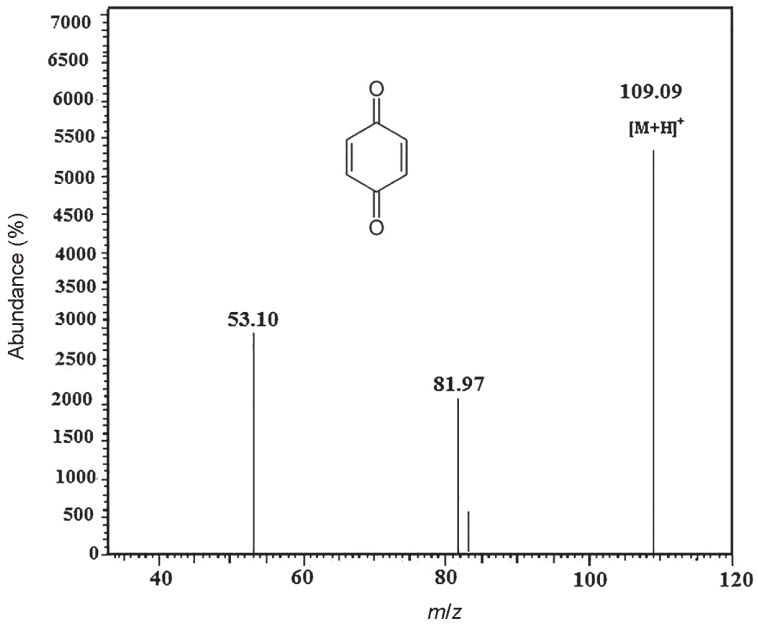

Fig. 6. Product ion mass spectrum of $(M+H)^+$ ions of TQD1.

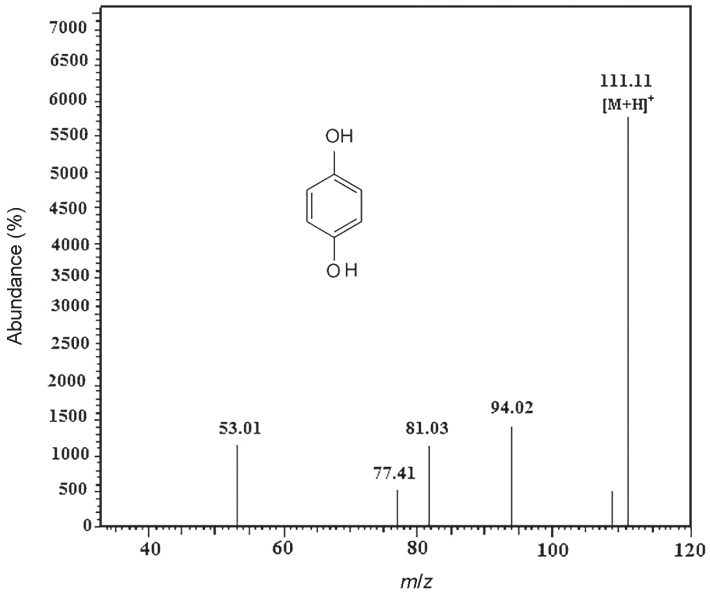

Fig. 7. Product ion mass spectrum of $(M+H)^+$ ions of TQD2.

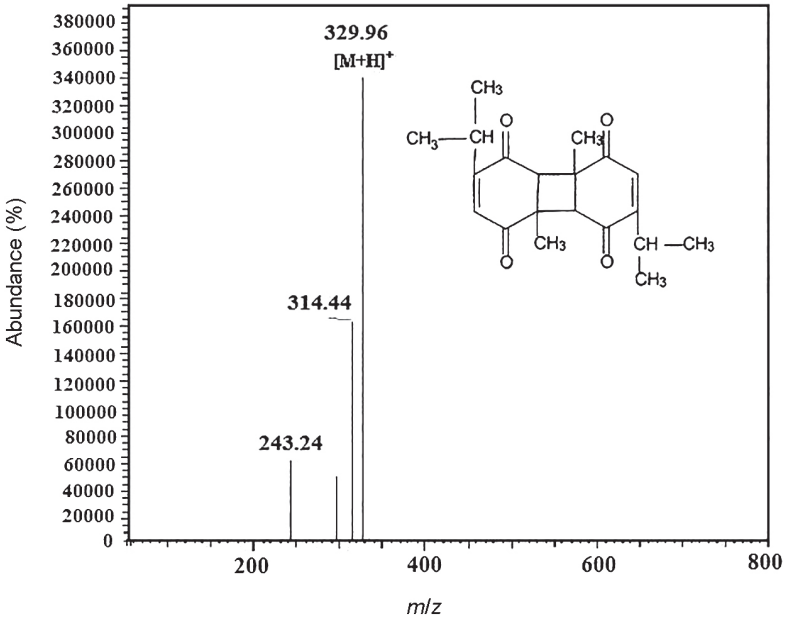

Fig. 8. Product ion mass spectrum of $(M+H)^+$ ions of TQD3.

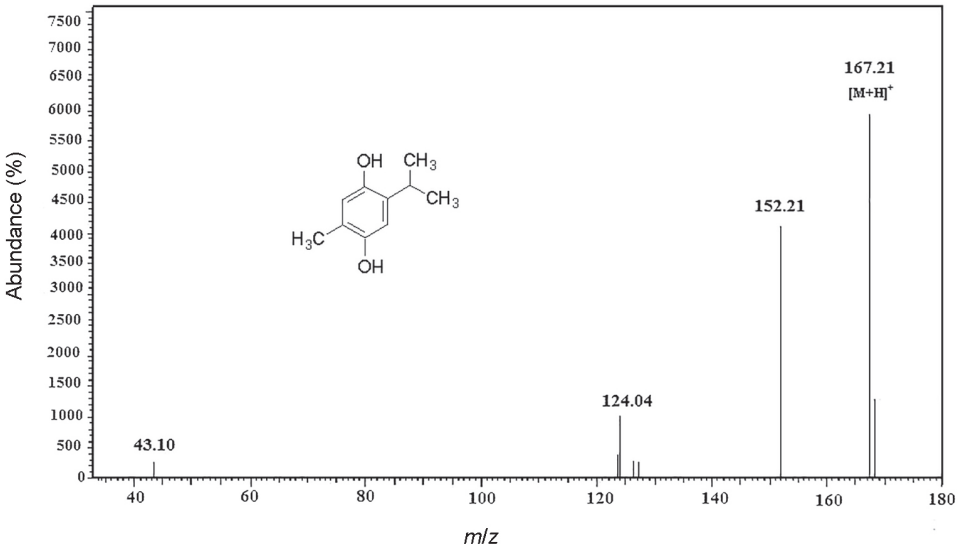

Fig. 9. Product ion mass spectrum of $(M+H)^+$ ions of TQD4.

Table VIII. Comparison of chromatographic methods for thymoquinone, carvacrol and thymol assay

Method used	Analyzed compound	Stability studies	Reference
HPLC-UV/ LC-ESI-TOF	TQ and THY	No stability studies	13
HPLC-UV	TQ and THY	No stability studies	14
HPLC-UV	TQ , CR and THY	No stability studies	15
HPLC-UV	TQ	Study stability of TQ only; no identification degradation products	16
TLC	TQ	No stability studies	17
HPTLC	TQ	No stability studies	18
GC_MS	TQ and CR	No stability studies	20
UPLC	TQ	Study stability of TQ only; no identification degradation products	21
HPLC-UV/ LC-ESI-MS	TQ , CR and THY	Study stability of TQ, CR and THY; identification of degradation products	This paper

degradation product was dithymoquinone (Fig. 8). The degradation product TQD4 involved an $(M+H)^+$ ion at *m/z* 167.21, so, it has a molecular mass of 166.21. TQD4 had a fragment ion at *m/z* 152.21 referring to the loss of methyl group. In addition, low-intensity peaks appeared at *m/z* 124.04 and 43.10, indicating the loss of the isopropyl group as well

as the formation of isopropyl cation, resp. As seen from Fig. 9 the TQD4 was elucidated to be thymohydroquinone.

A detailed comparison between the developed methods and the published ones is given in Table VIII. It is clear that chromatographic methods in refs. 13–15 and 17–20 did not study the stability of TQ, CR or THY, whereas only two methods in refs. 16 and 21 studied the stability of TQ only but did not identify its degradations products. To the best of our knowledge, the proposed method is the only method that investigates the stability of three principal antioxidants of NS (TQ, CR and THY) by using HPLC technique and characterize the degradation products of TQ using LC-MS/MS.

CONCLUSIONS

A study of the behavior of TQ, CR and THY under different stress conditions was established. The developed HPLC-UV method is simple, accurate, precise and selective for determination of TQ, CR and THY in pure form or in commercial preparations without interference from other constituents/excipients/degradants present. The LC-ESI-MS was used to confirm the structure and mass of the degradants of TQ. The method proved to be simple and selective for giving a distinct separation and identification of TQ and its degradants when exposed to forced degradation conditions.

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