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High performance liquid chromatographic analysis of the pharmacologically active quinones and related compounds in the oil of the black seed (*Nigella sativa L.*)

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Abstract

An HPLC method for quantifying the putative pharmacologically active constituents: thymoquinone (TQ), dithymoquinone (DTQ), thymohydroquinone (THQ), and thymol (THY), in the oil of *Nigella sativa* seed is described. Extraction of the constituents from the oil was carried out using C_{18} PrepSep mini columns followed by quantification of the recovered constituents by HPLC on a reversed-phase μBondapak C_{18} analytical column, using an isocratic mobile phase of water:methanol:2-propanol (50:45:5% v/v) at a flow rate of 2 ml min^{-1}. UV detection was at 254 nm for TQ, DTQ, and THY, and at 294 nm for THQ. The above four compounds were separated with good resolution, reproducibility, and sensitivity under these conditions. This analytical method was used to quantify the above four constituents in a commercial sample of *N. sativa* seed oil, and provides a good quality control methodology for the pharmacologically active components in this widely used natural remedy.

Keywords: *Nigella sativa*; Quinones; Thymoquinone; Dithymoquinone; Thymohydroquinone; Thymol; Reversed-phase chromatography

1. Introduction

The seeds of *Nigella sativa*, commonly known as black seed, black cumin, and kalunji have been extensively investigated in recent years [1]. This seed has a rich historical and religious background. Millions of people in the Mediterranean region and on the Indian subcontinent use the oil from the seed daily, as a natural protective and curative remedy. Notable pharmacological properties that have been reported are: antitumor [2], immunopotentiation [3], antihistaminic [4], antidiabetic [5], antihypertensive [6], antiinflammatory [7], and antimicrobial activity [8]. Many of these activites have been attributed to quinone constituents in the seed. Mahfouz and El-Dakhakhny [9] reported the isolation of the antihistaminic

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'nigellone' [4], a substance extracted from the oil of *N. sativa* seed, using Girard's reagent. El-Dakhakhny [10] also isolated thymoquinone (TQ) from the essential oil of the seed, and determined that the 'nigellone' isolated earlier was a dimer of thymoquinone, which they named dithymoquinone (DTQ). DTQ is formed during various stages of extraction and separation of the quinones from the seed. Studies in our own laboratories have shown that TQ and DTQ have pronounced in vitro antitumor activity [11]. TQ has also been shown to have effects on the circulatory system[6], and on the respiratory system [12]. Houghton et al. [7] have measured the content of TQ in *N. sativa* seed oil samples from different origins by gas chromatography analysis, and found it to be in the range of 0.13–0.17% w/v of the oil. El-Alfy et al. [8] reported the isolation of the antimicrobial quinone, thymohydroquinone (THQ) from *N. sativa* seed volatile oil. Abou-Basha et al. [13] have developed a thin layer chromatographic assay to quantify TQ and to identify DTQ and thymol (THY) in *N. sativa* seed. This same group [14] has recently developed a normal phase HPLC method for the determination of TQ in the seed oil, and found significant variations of TQ content between different commercial samples of *N. sativa* seed oil.

In this report, a simple and sensitive isocratic high performance liquid chromatographic method for determining TQ, THQ, DTQ, and THY in commercial *N. sativa* seed oil is described, using a reversed-phase column and a mobile phase of water, methanol, and 2-propanol. A UV detector operating at wavelengths of 254 and 294 nm was utilized for solute quantification, and diphenyl sulfone (DPS) was used as a calibration standard. This method represents the first comprehensive analytical methodology for quantifying DTQ, THQ, and THY in *N. sativa* seed oil using HPLC. Moreover, the method described here provides the most sensitive HPLC method available for quantifying TQ, and provides an analytical protocol that can be utilized to quantify the comparative levels of the pharmacologically active constituents in the seed oil and seed extracts of *N. sativa* under different manufacturing conditions.

2. Materials and methods

2.1. Chemicals and materials

HPLC grade methanol and 2-propanol (Fisher Scientific, Pittsburgh, PA) were used. Millipore filtered water was obtained by passing distilled water through a Milli-Q system (Millipore Corp., Milford, MA). Thymoquinone, thymol (Sigma, St. Louis, MO), and diphenyl sulfone (Aldrich, Milwaukee, WI) were purchased. Dithymoquinone was prepared from thymoquinone according to the method described by Smith and Tess [15]. Thymohydroquinone was prepared by reducing thymoquinone, as described by El-Dakhakhny [10]. The highly purified synthetic components were analyzed by NMR spectroscopy and melting point to confirm their identity and purity. The commercial *Nigella sativa* seed oil '*Albadawia*' was purshased from Egypt.

2.2. Apparatus

An HPLC system composed of a model 5700 solvent delivery system (ESA, Bedford, MA) and a model 7125 sample injector (Rheodyne, Cotati, CA) connected in series to a model 480 lambda variable wavelength UV detector (Waters, Milford, MA) was utilized. The output signals were monitored on a model 3392A reporting integrator (Hewlett–Packard, Avondale, PA). Extractions were conducted using C_{18} PrepSep solid phase extraction columns (Fisher Scientific, Pittsburgh, PA).

2.3. Column

A C_{18} reversed-phase μBondapak analytical column (300 × 3.9 mm, 10 μm particle size, Waters Chromatography Division, Milford, MA) connected to a C_{18} reversed-phase guard column (30 × 4.6 mm, 10 μm particle size, Bioanalytical Systems, W. Lafayette, IN) was used in all HPLC studies.

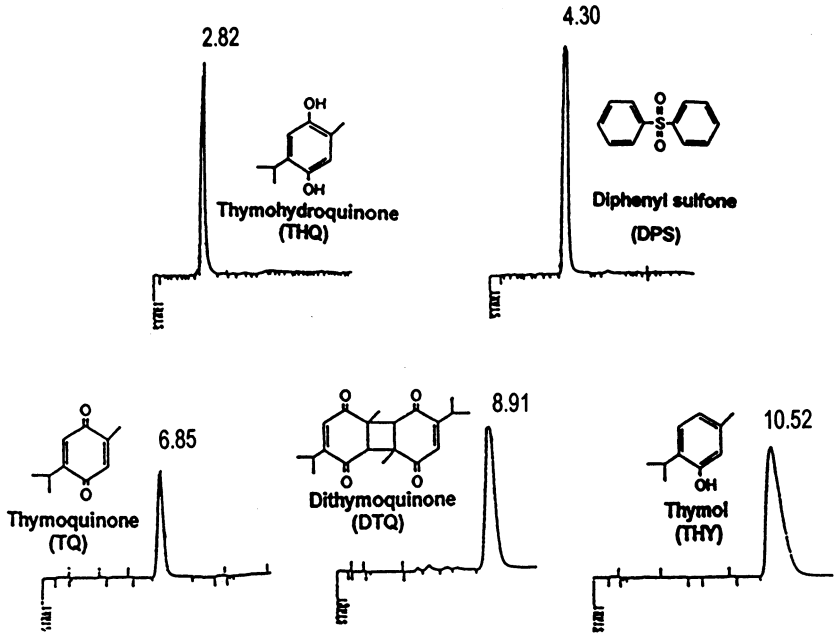

Fig. 1. HPLC chromatograms of separate runs of THQ, DPS, TQ, DTQ, and THY. The chromatographic conditions are mentioned in the Section 2, where detection was at λ_{254} for all compounds except THQ, which was detected at λ_{294}. The structure of each component is shown above its chromatogram.

2.4. Chromatographic conditions

The isocratic mobile phase utilized was composed of water:methanol:2-propanol (50:45:5% v/v), and was filtered through a 0.45 μm Millipore filter and deaerated before use. Analyses was performed at room temperature. UV monitoring of the eluted solutes was carried out at 254 nm for TQ, DTQ, and THY. THQ (which has a low absorptivity at 254 nm) was detected at 294 nm. A flow rate of 2.0 ml min^{-1} was used. Prior to analysis, precautions were taken to assure stability of the analysis samples, which are light and heat sensitive, since quinones of this type undergo facile formation of radicals when exposed to light. Thus, immediately after preparation, vials containing seed oil extract were refrigerated and covered by aluminum foil, to protect from light. Under these conditions the extracts were stable for at least 2 months.

2.5. Calibration curves

Calibration curves of peak area ratios were obtained by co-injecting different quantities of each analyte with a constant amount of diphenyl sulfone, the calibration standard (Fig. 2). At least three analyses were utilized for each point on the calibration curve, and each calibration curve had at least four points; all curves had $r^2 > 0.99$. The limit of detection for each of the analytes was according to our assay methodology: TQ (2.15×10^{-4}% w/w); DTQ (2.12×10^{-4}% w/w); THQ (6.46×10^{-4}% w/w); and THY (4.43×10^{-3}% w/

Table 1
Intra- and inter-day variation in the analysis of thymoquinone, dithymoquinone, thymohydroquinone, and thymol

Component	Intra-day analysis variation (CV%)	Inter-day analysis variation (CV%)
Thymoquinone	3.1	1.0
Dithymoquinone	10.3	8.7
Thymohydroquinone	5.4	2.2
Thymol	9.8	9.3

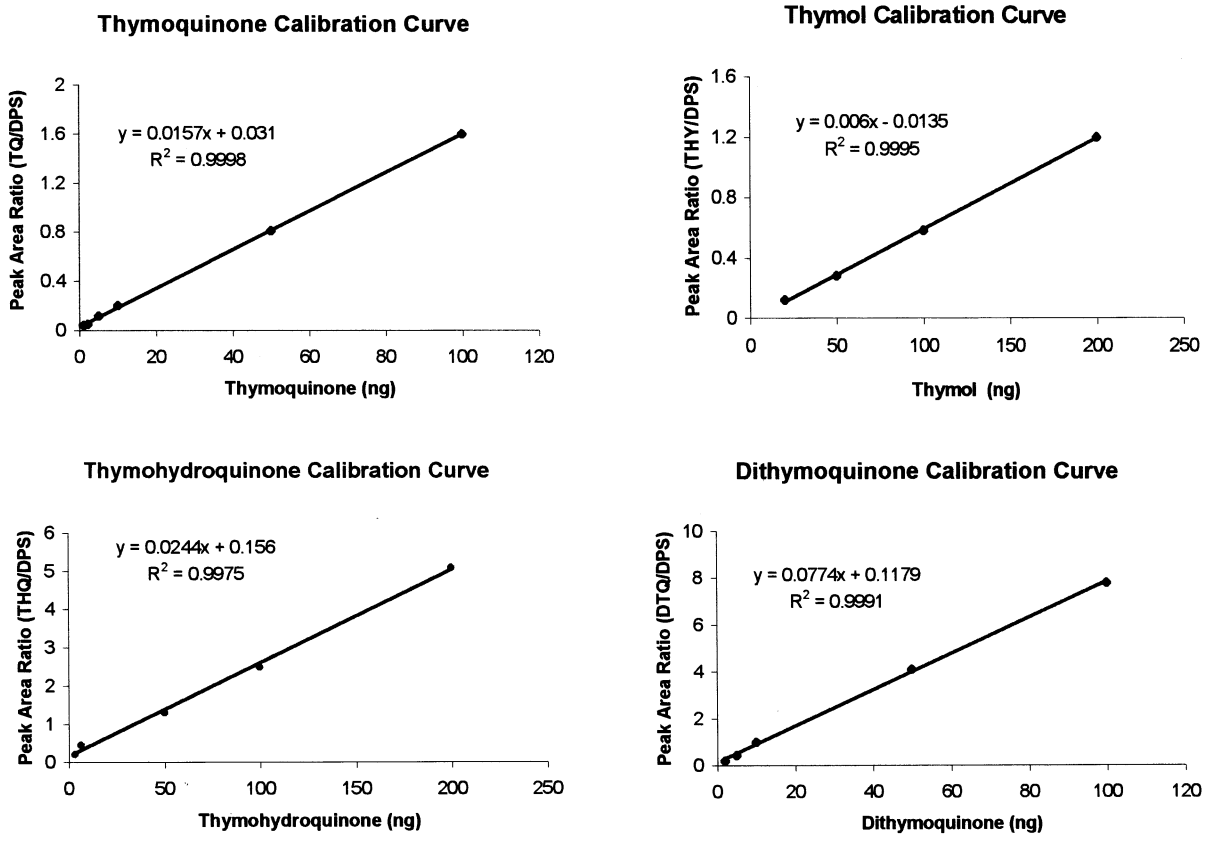

Fig. 2. Calibration curves for the thymoquinone, thymol, thymohydroquinone, and dithymoquinone. For the calibration curves of thymol, thymoquinone, and dithymoquinone the calibration standard used was 30 ng of diphenylsulfone (DPS), and UV detection was at 254 nm. For the calibration curve of thymohydroquinone 3.0 μg of DPS was used and UV detection was at 294 nm.

w). These values correspond to 1, 1, 3, and 20 ng, respectively in each 20 μl analyte solution (detection limits on column). Day-to-day intervariability and within-day intervariability were calculated for all constituents (Table 1). In the case of TQ, DTQ, and THY, UV detection was accomplished at a wavelength of 254 nm, whereas for THQ, UV detection were conducted at 294 nm, due to the low absorptivity of THQ at 254 nm compared to 294 nm.

2.6. Purification procedure

N. sativa seed oil was purified by passing the oil through a C_{18} PrepSep solid phase extraction column (preeluted with methanol) prior to HPLC analysis; 20 μl samples of seed oil followed by 800 μl (400 μl × 2) of methanol were passed through the column to afford an eluate free from greasy and fatty materials. The calibration standard, DPS, was added to the PrepSep eluate, and 20 μl was injected onto the HPLC column. The recovery from the extraction procedure was carried out for different amounts of each pure analyte ($20–2 \times 10^7$ ng). The recovery was determined by eluting 20 μl of a methanolic solution of each pure authentic analyte through the solid phase extraction column with 800 μl (400 μl × 2) of methanol eluent. To an accurate volume of the resulting eluate was added an accurate amount of DPS as the calibration standard, and 20 μl of this solution was injected onto the HPLC analytical system. The results were then compared to the values obtained using a similar procedure in which the solid phase extrac-

tion was omitted. Recovery was found to be $>95\%$ for all analytes over the mass range used ($20-2\times10^{7}$ ng).

3. Results and discussion

3.1. Chromatography

The HPLC chromatogram obtained after analysis of the seed oil, using the HPLC method described above showed well resolved peaks with no interference. The run time for total analysis of all four components required <15 min. The retention times of the components were: THQ 2.8 min; TQ 6.8 min; DTQ 8.9 min; THY 10.5 min; and the calibration standard (DPS) eluted at 4.3 min (Fig. 1).

3.2. Quantification

Using the above method, the quantification of quinone constituents in a sample of commercial *N. sativa* seed oil was achieved. Assays of samples taken from the seed oil are shown in Table 2 and Fig. 3. It was found that the amount of TQ present in this source of commercial oil was about one third that found in other commercial sources of oils [7]. In this commercial oil, DTQ was not detectable, however, the presence of DTQ is still an important constituent in seed oil, especially with regard to the nature of the extraction and manufacturing procedures utilized to generate the seed oil. In earlier studies, the major quinone constituent of the seed was thought to be DTQ (termed 'nigellone' by earlier workers), and was shown to be formed via photodimerization of TQ as a consequence of exposure to sunlight during separation and extraction procedures [10]. Various storage conditions are expected to make a difference in the amounts of the quinone constituents of the oil, especially if seed oil samples are exposed to heat and light. Thus the analytical methodology described here may find utility as a quality control method for the determination of pharmacologically active quinones in this *N. sativa* seed oil.

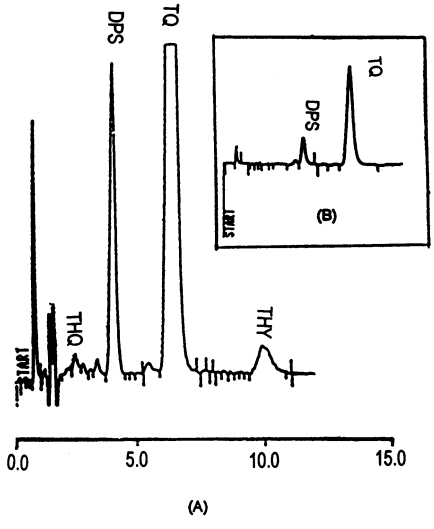

Fig. 3. (A) A chromatogram of purified *Nigella sativa* seed oil sample, detection was carried out at λ_{254}; other chromatographic conditioned are mentioned in Section 2. (B) Same sample as in (A) but run at higher attenuation.

Table 2
Quantification of quinones in '*Albadawia*', a commercial oil from *Nigella sativa* seed

Component	Content of oil % w/w
Thymoquinone	$5.26\times10^{-2}\pm2.59\times10^{-3}$
Thymohydroquinone	$7.67\times10^{-4}\pm5.49\times10^{-5}$
Dithymoquinone	Not quantifiable[a]
Thymol	$9.12\times10^{-3}\pm1.38\times10^{-3}$

[a] Below the limits of quantification (2.12×10^{-4}% w/w).

4. Conclusion

The analytical method described in this study provides an HPLC assay for quantifying the major quinone and related constituents in *N. sativa* seed oil and seed extracts. This method also provides a monitoring system for determining the relative amounts of the quinone constituents in the seed oil, and extracts of seeds from different origins, and may also afford a means for quality control, especially when seed extract or seed oil has been subjected to different storage, extraction and isolation conditions.

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