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Stable Isotope Dilution Gas Chromatography−Mass Spectrometry for Quantification of Thymoquinone in Black Cumin Seed Oil

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Supporting Information

ABSTRACT: Black cumin seed (*Nigella sativa* L.) is a widely used spice and herb, where thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) is the major bioactive compound. Here, a stable isotope dilution (SID) gas chromatography−mass spectrometry (GC−MS) technique was developed for the quantification of thymoquinone. A doubly deuterated thymoquinone ([$^2H_2$]-thymoquinone) was synthesized for the first time with more than 93% deuteration degree shown by mass spectrometry and proton nuclear magnetic resonance (^{1}H NMR). This compound was used as an internal standard for the quantification of thymoquinone using a SID GC−MS method. The validation experiment showed a recovery rate of 99.1 ± 1.1% relative standard deviation (RSD). Standard addition and external calibration methods have also been used to quantify thymoquinone, which cross-validated the developed stable isotope dilution assay (SIDA). In comparison to external calibration and standard addition methods, the SIDA method is robust and accurate. The concentration of thymoquinone in five marketed black cumin seed oils ranged between 3.34 and 10.8 mg/mL by use of SID GC−MS.

KEYWORDS: *Nigella sativa, thymoquinone, stable isotope dilution, GC−MS, quantification*

INTRODUCTION

Black cumin seed (*Nigella sativa* L.) is a widely used spice in Mediterranean countries, Pakistan, and India.[1,2] It is also used as a traditional medicine for the treatment of a range of diseases, such as diabetes, hypertension, fever, arthritis, inflammation, gastrointestinal disturbances, and cancer.[3] Studies into the biological activities of this plant have revealed that the volatile essential oil components, predominantly thymoquinone (**1**) (Figure 1),[4] possess antioxidant,[5] anti-inflammatory,[6] antidiabetic,[7] immunomodulatory,[8] and anti-tumor[9−11] activities.

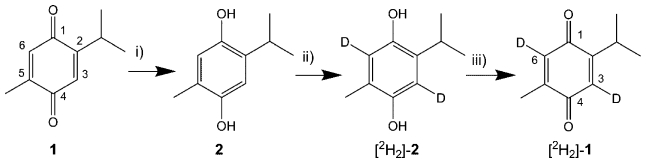

Figure 1. Scheme for synthesis of [$^2H_2$]-thymoquinone ([$^2H_2$]-**1**): (i) $Na_2S_2O_4$ in H_2O/MeOH at room temperature for 2 h, (ii) (a) 3.4 M D_2SO_4 in D_2O with reflux for 48 h and (b) cool to room temperature and workup with H_2O, and (iii) CrO_3 in 60% HOAc in H_2O at −4 °C for 2 h.

Thymoquinone was previously quantitated by means of high-performance liquid chromatography (HPLC),[12,13] high-performance thin-layer chromatography (HPTLC),[14] and a differential pulse polarographic method.[15] These methods could achieve high sensitivities and reproducibility, but they often need multi-steps of sample preparation and external calibrations; thus, the results could be influenced by complex matrices. Gas chromatography (GC) and/or gas chromatography−mass spectrometry (GC−MS) are preferred techniques for the analysis of volatile and semi-volatile compounds and have also been used for the quantification of thymoquinone[16] and identification of the composition of black cumin seed oil.[5,17] A stable isotope dilution assay/analysis (SIDA) should overcome the problem of the matrix effects, such as compound discrimination during extraction, chromatographic separation, and ultraviolet (UV)/mass spectrometry (MS)/electrochemical detection,[18,19] when a deuteriated thymoquinone is used as an internal standard. Stable isotope dilution coupled with GC−MS (SID GC−MS) has widely been used for determination of various compounds, e.g., methoxypyrazines in red wines,[19] dietary lignans in food,[20] natural estrogens in vegetables and fruits,[21,22] and alkypyrazines in coffee.[23] Here, we report synthesis of a stable deuterium (D or ^{2}H)-labeled thymoquinone (Figure 1) for the first time and its application as an internal standard for quantification of thymoquinone in black cumin seed oil by the means of SID GC−MS.

MATERIALS AND METHODS

Thymoquinone (99%), D_2-sulfuric acid (D_2SO_4, 99.5%), deuterated water [deuterium oxide (D_2O), 99.5%], sodium hydrosulfite ($Na_2S_2O_4$), and chromium trioxide (CrO_3) were purchased from Sigma-Aldrich. Hexane (analytical grade) was purchased from Fisher-Scientific, U.K. The following black cumin seed oils were purchased from Amazon: Manako black cumin oil, CEBRA *Nigella sativa* oil, Avena black cumin oil, Iman VERGIN black cumin oil, and This Health black seed oil.

Synthesis. The doubly deuteriated thymoquinone ([$^2H_2$]-**1**) (Figure 1) was prepared and purified according to a similar method

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for preparation of deuterated 1,4-benzoquinones,[24,25] with modification.

2-Isopropyl-5-methyl-1,4-benenediol (2). A solution of sodium hydrosulfite (348 mg, 2.0 mmol) in 2.0 mL of water was added to a solution of thymoquinone (164 mg, 1.0 mmol) in 2.0 mL of methanol. The mixture was stirred for 2 h at room temperature. The methanol was evaporated, and the resultant solution was extracted with ethyl acetate (3 × 10 mL). The combined organic phase was washed with water (3 × 10 mL) and then dried over sodium sulfate. After evaporation of ethyl acetate, 2-isopropyl-5-methyl-1,4-benenediol (thymohydroquinone, **2**, 70 mg, yield of 42%) was obtained. MS [electron ionization (EI)] *m/z* (relative intensity, %): 166.1 (43) $[M]^{\bullet+}$, 151.1 (100) $[M - CH_3]^{\bullet+}$, 137 (4), 123.1 (11), 95.1 (10), 77.1 (10), 53.0 (3). Proton nuclear magnetic resonance (^{1}H NMR) (300 MHz, $CDCl_3$) δ: 6.56 (s, 1H), 6.47 (s, 1H), 3.06 (m, 1H), 2.10 (s, 3H), 1.14 (d, 6H, J = 7.2 Hz, $-CH(CH_3)_2$).

2-Isopropyl-5-methyl-1,4-dideuterobenenediol ($[^2H_2]$-2). 2-Isopropyl-5-methyl-1,4-benenediol (**2**, 68 mg, 0.40 mmol) was refluxed for 48 h in 3.4 M D_2-sulfuric acid (D_2SO_4, 0.9 mL) in deuterated water (D_2O, 4.2 mL). After cooling to room temperature, water (H_2O, 10 mL) was added and the mixture was extracted with ethyl acetate (3 × 10 mL). The ethyl acetate extract was washed with water (H_2O, 3 × 10 mL) and then dried over sodium sulfate. After evaporation of the solvent, 2-isopropyl-5-methyl-1,4-dideuterobenenediol ($[^2H_2]$-**2**, 45 mg) was obtained. MS (EI) *m/z* (relative intensity, %): 168.1 (44) $[M]^{\bullet+}$, 153.1 (100) $[M - CH_3]^{\bullet+}$, 139.1 (3), 125.1 (11), 97.0 (10), 79.1 (8), 55.0 (3).

2-Isopropyl-5-methyl-1,4-dideuterobenzoquinone ($[^2H_2]$-thymoquinone ($[^2H_2]$-1). 2-Isopropyl-5-methyl-1,4-dideuterobenenediol ($[^2H_2]$-**2**, 42 mg, 0.25 mmol) was dissolved in 60% acetic acid (2 mL) at −4 °C. Chromium trioxide (150 mg, 1.5 mmol) in 0.5 mL of 30% acetic acid was added, and the mixture was stirred at −4 °C for 2 h. Water (10 mL) was then added to the mixture, followed by extraction with ethyl acetate (2 × 10 mL). After drying over sodium sulfate, the solvent was removed under vacuum to give crude doubly deuteriated thymoquinone, which was sublimed to give pure 2-isopropyl-5-methyl-1,4-dideuterobenzoquinone ($[^2H_2]$-thymoquinone, $[^2H_2]$-**1**, 28.0 mg, 43% overall yield based on thymohydroquinone (**2**)). The purity of compound $[^2H_2]$-**1** was determined to be 97.7% by GC−MS using full-scan mode (see Figure S1 of the Supporting Information). The extent of double deuteration according to ^{1}H NMR (Figure 2) and MS (see Figure S2 of the Supporting Information) was greater than 93%. MS [electron ionization (EI)] *m/z* (relative intensity, %): 166.1 (100) $[M]^{\bullet+}$, 151 (54) $[M - CH_3]^{\bullet+}$, 138.1 (62) $[M - CO]^{\bullet+}$, 123.1 (73) $[M - CH_3CHCH_3]^{\bullet+}$, 110.1 (19), 95.1 (63), 79.1 (19), 69.1 (22), 54.1 (27) (see Figures S1 and S3 of the Supporting Information). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.06 (m, 1H, $-CH(CH_3)_2$), 2.10 (s, 3H, CH_3), 1.10 (d, 6H, $-CH(CH_3)_2$) (Figure 2B).

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were recorded on a Bruker DPX 300 MHz spectrometer using $CDCl_3$ as the solvent. Chemical shifts are quoted on the δ scale in parts per million (ppm) using $CDCl_3$ as the internal standard. NMR data were analyzed using an ACD/Laboratories 12 software. Multiplicities of signals are described as singlet (s), doublet (d), triplet (t), quadruplet (q), and multiplet (m).

GC−MS. The GC−MS system consisted of an Agilent 7890A GC system with split injection (250 °C; 1:10), coupled to an Agilent MS model 5975C mass selective detector (MSD) with a triple axis detector (Agilent Technologies, Santa Clara, CA).

A 30 m HP5-MS column [(5% phenyl)-methylpolysiloxane, 0.25 mm inner diameter, film thickness (df) = 0.25 μm, Agilent Technologies, Santa Clara, CA] was used for the quantification of thymoquinone. The GC heating program was as follows: initially at 80 °C, raised to 160 °C at 20 °C/min, then raised to 300 °C at 50 °C/min, and finally held at 300 °C for 3.2 min under a constant helium pressure (10 psi). MS was fitted with an EI source operating at an electron energy of 70 eV held at a temperature of 230 °C. Mass spectra and selected ion monitoring (SIM) were acquired using MSD ChemStation (Agilent Technologies, Santa Clara, CA). Full-scan mode was used to identify thymoquinone in the black cumin oil and characterize the synthetic compounds **2**, $[^2H]_2$−**2** and $[^2H]_2$−**1**. After observation of the major ions for thymoquinone (**1**) and doubly deuteriated thymoquinone ($[^2H]_2$-**1**), SIM mode (*m/z* 164.1 for compound **1** and *m/z* 166.1 for deuterium-labeled compound $[^2H]_2$-**1**) was performed for quantification of thymoquinone using SID GC−MS analysis. SIM mode (three selected ions at *m/z* 164.1, 149.1, and 136.1 for compound **1**) was applied for the quantification of thymoquinone using SIM GC−MS with standard addition and external calibration methods.

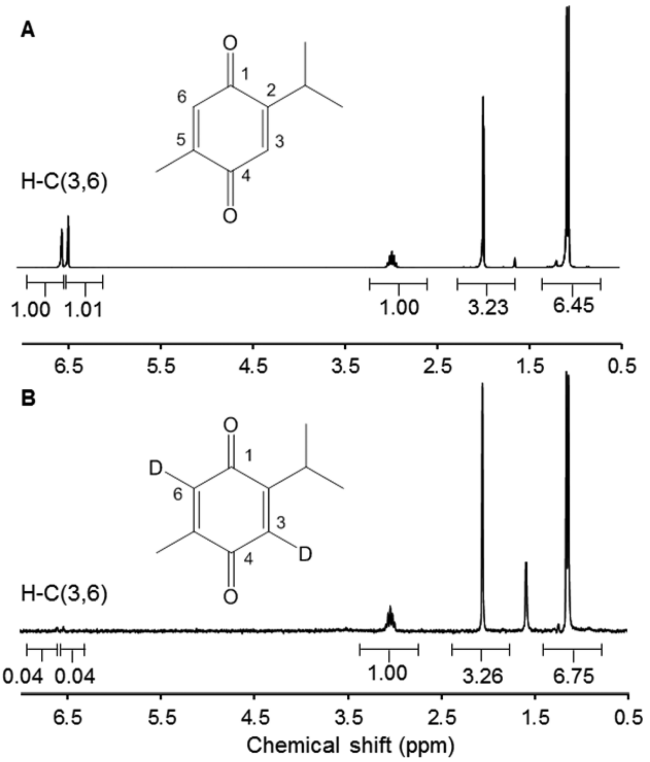

Figure 2. ^{1}H NMR spectra (300 MHz, $CDCl_3$) of (A) thymoquinone (**1**) and (B) $[^2H_2]$-thymoquinone ($[^2H_2]$-**1**). The disappearance of the signals at 6.52 and 6.45 ppm for H-3 and H-6 in the spectrum of compound $[^2H_2]$-**1** indicates >93% double substitution of deuterium for the two protiums of compound **1**.

Development of a SID GC−MS Method for Quantification of Thymoquinone. *Calibration.* The analyte thymoquinone (**1**) and the internal standard $[^2H_2]$-thymoquinone ($[^2H_2]$-**1**) were mixed in six ratios from 0.1 to 10 (5−500 μg/mL in hexane) and analyzed by means of GC−MS in triplicate. Calibration solutions were kept at 4 °C until analysis. The ratios of the area under the curve (AUC) of ion *m/z* 164.1 (AUC_{164}) for compound **1** to the AUC of ion *m/z* 166.1 (AUC_{166}) for compound $[^2H_2]$-**1** were plotted against the concentration ratio of compound **1** to compound $[^2H_2]$-**1** (see Figure S4 of the Supporting Information). The linearity of the calibration regression line was R^2 = 0.9952. The equation obtained was $y = 0.843x + 0.2297$ (**1**/$[^2H_2]$-**1**). The limit of detection (LOD) was 0.16 μg/mL with a signal-to-noise ratio of 3:1, and the limit of quantification (LOQ) (10:1)[26] was 0.50 μg/mL, respectively. When using splitless injection mode, LOD and LOQ for thymoquinone were 0.04 and 0.16 μg/mL, respectively.

Quantitation of Thymoquinone in the Black Cumin Seed Oils by SID GC−MS. A sample of black cumin seed oil (50 μL) was added to a 10 mL volumetric flask, and hexane was added to the mark. Aliquots of the diluted oil (200-fold) in hexane (980 μL) was spiked and mixed with a solution of compound $[^2H_2]$-**1** in hexane (20 μL, 1.0 mg/mL). A total of 1 μL of sample was injected into the GC−MS instrument. Each black cumin seed oil sample was prepared 3 times and analyzed with three replicates.

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Method Validation. Recovery experiments were performed with a sample of Manako black cumin seed oil. An aliquot of the diluted Manako oil (200-fold) in hexane (920 μL) was spiked with an increasing amount (20, 40, and 60 μL) of compound **1** dissolved in hexane (1.0 mg/mL) and mixed with 20 μL of compound [$^2H_2$]-**1** in hexane (1.0 mg/mL). Finally, hexane was added to give 1.0 mL of black cumin seed oil solution. Aliquots (1 μL) of the diluted oils were analyzed using the SID GC−MS method described above. Three replicates were performed.

To study the intra- and interday imprecisions of the SID GC−MS method, diluted Manako oil in hexane (200-fold) was analyzed. Relative standard deviations (RSDs) were determined for intra- and interday variations based on a set of 9 (in 1 day) and 15 (on 5 different days) measurements.

SIM GC−MS Analysis of Thymoquinone Using Standard Addition and External Calibration. *Standard Addition.* An aliquot of the diluted (200-fold) Manako and Avena oil (800 μL) was added with an increasing amount of compound **1** in hexane to make oil solutions (1.0 mL) that contained an additional 40, 60, 80, 100, 120, 140, and 160 μg/mL of compound **1**. Linear calibration curves (R^2 > 0.99) were obtained by plotting the AUC for the three selected ions (m/z 164.1, 149.1, and 136.1) versus the concentration. Each measurement was repeated in triplicate.

External Calibration. Quantification of thymoquinone using external calibration was carried out by comparing the AUC for the three selected ions (m/z = 164.1, 149.1, and 136.1) of compound **1** in the diluted oil samples to those defined standard solutions of compound **1** dissolved in hexane. A sample of oil (50 μL) was diluted with hexane (10 mL) in a 10 mL volumetric flask, and an aliquot (1 μL) of the diluted samples were analyzed via SIM GC−MS. Each measurement was performed in triplicate. The quantity of thymoquinone was determined using a five-point response curve based on the analysis of standard solutions. Those solutions contained the defined amount of thymoquinone in different concentrations ranging from 5 to 80 μg/mL. The calibration curve (see Figure S5 of the Supporting Information) obtained was $y = 2.2852x - 6.5186$ (R^2 = 0.9956), where y is the AUC/10^3 and x is the concentration of thymoquinone (μg/mL). The LOD was 0.10 μg/mL with a signal-to-noise ratio of 3:1, and the LOQ (10:1) was 0.31 μg/mL. When using splitless injection mode, LOD and LOQ were 0.02 and 0.08 μg/mL, respectively.

RESULTS AND DISCUSSION

Synthesis of a Stable Isotope-Labeled Thymoquinone. The scheme of synthesis of the doubly deuterated thymoquinone ([$^2H_2$]-**1**) is shown in Figure 1. Thymoquinone (**1**) was first reduced to thymohydroquinone (**2**) by sodium hydrosulfite. The two protiums (H-3 and H-6) of compound **2** were then exchanged by deuterium in 3.4 M D_2SO_4 in D_2O under refluxing for 48 h. After cooling to room temperature, the deuterated thymohydroquinone ([$^2H_2$]-**2**) was obtained after workup with H_2O rather than expensive D_2O. Finally, compound [$^2H_2$]-**2** was oxidized with CrO_3 in 60% HOAc to yield compound [$^2H_2$]-**1** (17% overall yield).

GC−MS shows a single peak with high purity (97.7%) based on GC−MS (see Figure S1 of the Supporting Information) and ^{1}H NMR (Figure 2) for compound [$^2H_2$]-**1**. A comparison of the EI−MS spectrum of thymoquinone (**1**) to that of compound [$^2H_2$]-**1** (see Figures S1 and S2 of the Supporting Information) showed that a mass increase of the molecule ion m/z 164.1 by two deuterium atoms to m/z 166.1, which confirmed [$^2H_2$]-**1** was the predominant isotopologue. The electron impact fragmentation pathways for compounds **1** and [$^2H_2$]-**1** are very similar because of the substitution of H-3 and 6 of compound **1** by two deuteriums (see Figure S3 of the Supporting Information). The isotopic distribution of compound [$^2H_2$]-**1** detected by GC−MS was found to be 2.0% natural [^{2}H] abundant compound **1**, 5.0% compound [$^2H_1$]-**1**, and 93.0% compound [$^2H_2$]-**1**. Further comparison of the ^{1}H NMR of compound **1** (Figure 2A) to that of compound [$^2H_2$]-**1** (Figure 2B) shows the disappearance of peaks for H-3 and H-6 because of the protium/deuterium exchange, again confirming the presence and position of two deuterium atoms in 2-isopropyl-5-methyl-1,4-dideuterobenzoquinone ([$^2H_2$]-**1**). It shows only minor protium abundance of 4.0% at position C-3 and 4.0% at position C-6 of compound [$^2H_2$]-**1** (Figure 2B), which is consistent with a high degree of double deuteration of compound [$^2H_2$]-**1** found by GC−MS (see Figure S2 of the Supporting Information).

Because only 2.0% natural [^{2}H] abundant compound **1** was present in the synthetic compound [$^2H_2$]-**1** and less than 2.0% of the third isotopic peak of compound **1** (m/z 166.1) was detected, the ratios of the AUC for ion m/z 164.1 for compound **1** to the AUC for ion m/z 166.1 for compound [$^2H_2$]-**1** were used for the calibration and quantification of thymoquinone (**1**) using the SID GC−MS method. Compound [$^2H_2$]-**1** was found to be stable in hexane for more than 6 months in the dark and, therefore, is suitable to be used as an internal standard for the SIDA.

Establishment of a SID GC−MS Method. The conditions for gas chromatographic separation were optimized to quantify thymoquinone (**1**) in black cumin seed oil within only 10 min (Figure 3). Figure 3A shows a typical total ion current (TIC) gas chromatogram of the Manako oil sample spiked with compound [$^2H_2$]-**1** using full-scan mode. A number of compounds can be detected. SIM (m/z 164.1 and 166.1 for compounds **1** and [$^2H_2$]-**1**, respectively) mode was used to quantify compound **1** in the oil using SID GC−MS (chromatogram not shown). Panels B and C of Figure 3

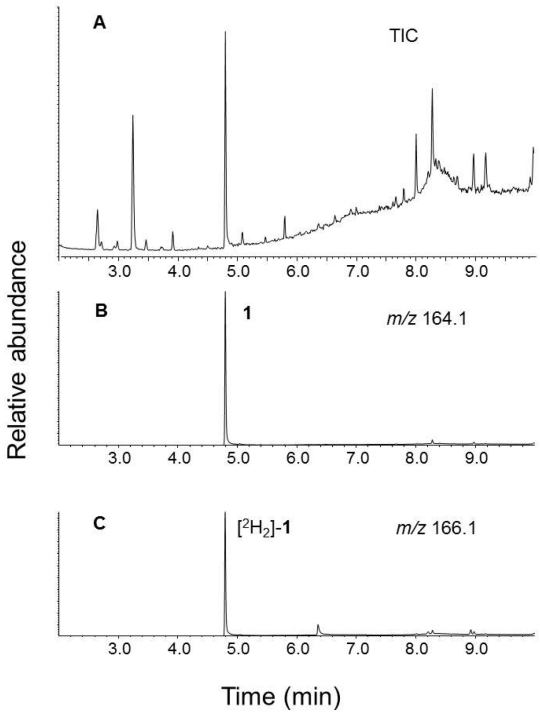

Figure 3. (A) Typical TIC chromatogram and reconstructed ion chromatograms of (B) thymoquinone (**1**) and (C) deuterium-labeled thymoquinone ([$^2H_2$]-**1**) in Manako black cumin seed oil spiked with compound [$^2H_2$]-**1**.

Table 1. Recovery Rates for the Quantification of Thymoquinone in the Diluted (1:200) Black Cumin Seed Oil (Manako) Determined by a SID GC−MS Method[a]

amount of compound **1** added (μg/mL)	concentration calculated (μg/mL)	concentration determined (μg/mL)	recovery (%)	average recovery (%)
		53.9 ± 0.01		
20.0	73.9 ± 0.01	73.3 ± 0.02	99.2 ± 0.02	
40.0	93.9 ± 0.01	94.0 ± 0.02	100.1 ± 0.02	99.1 ± 1.1
60.0	113.9 ± 0.01	111.5 ± 0.02	97.9 ± 0.02	

[a]Data are expressed as the mean ± RSD (n = 3).

show the reconstructed ion chromatograms of compounds **1** (m/z 164.1) and [$^2H_2$]-**1** (m/z 166.1) in the Manako black cumin seed oil. Compounds **1** and internal [$^2H_2$]-**1** were co-eluted at 4.81 min. A calibration graph (see Figure S4 of the Supporting Information) was plotted from a mixture of known mass ratios of compounds **1** and [$^2H_2$]-**1** and their corresponding peak area ratios in GC−MS by linear regression analysis. This calibration curve showing a linear response (R^2 = 0.9938) could enable the determination of the quantity of thymoquinone in the samples based on their measured ion intensities.

Recovery experiments were performed to check the trueness of the SIDA method. Specified quantities of thymoquinone (**1**) were added to the diluted Manako oil sample in three different concentrations before using SID GC−MS analysis. The quantity of compound **1** was measured and compared to those found in the blank diluted Manako oil sample as the control (Table 1). The mean recovery rate was determined to be 99.1 ± 1.1% RSD based on the amount of compound **1** added to the diluted oil sample. These results demonstrated that the developed SID GC−MS is a reliable, rapid, and accurate method for quantification of compound **1**. Thymoquinone in five marketed oil products was quantified, and their results are listed in Table 2. The concentration of thymoquinone ranged from 3.34 to 10.8 mg/mL. Manako oil has the highest content of thymoquinone, while CEBRA oil has the lowest content of thymoquinone.

Table 2. Quantification of Thymoquinone in Black Cumin Seed Oils via SID GC-MS, External Calibration, and Standard Addition Methods[a]

black cumin seed oil	concentration (mg/mL)		
	SID GC-MS	external calibration	standard addition
Manako	10.8 ± 0.01	8.95 ± 0.03	10.6 ± 0.13
Avena	7.27 ± 0.02	7.33 ± 0.08	7.17 ± 0.02
CEBRA	3.34 ± 0.03	2.96 ± 0.18	*b*
Iman	7.23 ± 0.02	6.02 ± 0.09	*b*
This Health	7.74 ± 0.04	7.03 ± 0.10	*b*

[a]Data are expressed as the mean ± RSD (n = 3). [b]Concentration not determined.

Cross-Validation of the SID GC−MS Method. The standard addition method can also overcome the matrix effects and is often used for the validation of the SIDA method.[18,27] To validate the SID GC−MS method developed above, the data obtained using SID GC−MS were compared to those found using standard addition and external calibration (Table 2). Three characteristic ions (m/z 164.1, 149.1, and 136.1) for compound **1** were selected for the quantification of thymoquinone using standard addition and external calibration. An external calibration curve with a linear response (R^2 = 0.9956) (see Figure S5 of the Supporting Information) was attained by linear regression analysis of the AUC versus the concentration.

Quantification based on the standard addition and external calibration showed 98.1 ± 1.2 and 82.9 ± 0.3% of compound **1** in Manako oil and 98.6 ± 0.4 and 100.8 ± 1.1% of compound **1** in Avena oil (calculated from data in Table 2) when compared to the results obtained by means of SID GC−MS, respectively. The standard addition and SID GC−MS results revealed very similar concentrations of compound **1** in Manako and Avena oils (differences are less than 2%), therefore validating the accuracy and advantage of the SIDA method. The concentrations of thymoquinone in all five oils determined by external calibration generally agreed with those determined from SIDA and/or standard addition methods (differences range between 1 and 17%). The slight discrepancies are probably due to matrix effects from the different preparations of these black cumin seed oils.[18]

The intraday precision of the SID GC−MS method for compound **1** showed a RSD of ±1.3% (n = 9) by analyzing diluted Manako oil in hexane in nine independent measurements in 1 day. Interday precision studies with diluted Manako oil revealed a RSD of ±5.9% (n = 15) in three independent measurements in each day for 5 days. These data again demonstrate that the SID GC−MS method is a reliable, rapid, and accurate analytic means for thymoquinone in black cumin seed oils.

In previous reports, thymoquinone was quantified by several other analytical techniques.[12−16] HPLC was used for quantification of thymoquinone in a commercial black cumin seed oil[12] and in different phytopharmaceuticals with a LOD of 0.006 μg/mL (10 μL of injection volume, ∼60 pg).[13] Quantification of thymoquinone in herbal extracts and oil was also achieved using HPTLC with LOD of 50 ng/spot.[14] A differential pulse polarographic method was developed for the determination of thymoquinone in black seed oil, where LOD was found to be 0.05 μg/mL (10 mL of volume, ∼500 ng).[15] The content of thymoquinone in black cumin seed oils was also found to be in the range of 0.13−0.17% (w/v) of the oil by GC.[16] In our GC−MS methods, where split injection mode (1:10) was used to avoid overloading samples, LODs of thymoquinone for SIM GC−MS and SID GC−MS were found to be 0.10 and 0.16 μg/mL (1 μL of injection volume, ∼100 and 160 pg), respectively. However, for trace analysis of thymoquinone, LODs can be further improved using splitless injection mode and range from 0.02 to 0.04 μg/mL (1 μL of injection volume, ∼20−40 pg). Therefore, the sensitivities of GC−MS methods are comparable to that of HPLC and are higher than those of HPTLC and differential pulse polarographic methods. Furthermore, GC−MS and/or SID GC−MS methods have advantages of identifying analytes and overcoming matrix effects over other analytical methods.[18,19]

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In summary, synthesis of a highly pure deuteriated thymoquinone has been achieved for the first time, which enables us to develop a SIDA of the biologically important thymoquinone in black cumin seed oil using GC−MS. Cross-validation using standard addition confirms the accuracy and reliability of the SIDA method, which can quantify a large number of black cumin seed oil products for their quality control in high-throughput means. Synthetic deuteriated thymoquinone and the SID GC-MS method should also be useful in the studies on the mechanism of binding thymoquinone to proteins,[24,28−30] *in vitro* and *in vivo* pharmacokinetics of thymoquinone, which are ongoing in our laboratory.[31]

■ ASSOCIATED CONTENT

Supporting Information

GC−MS chromatograms (Figure S1), mass spectra (Figure S2), possible electron impact fragmentation pathways of thymoquinone and doubly deuteriated thymoquinone (Figure S3), calibration curve for the SID GC−MS method (Figure S4), and external calibration curve for the SIM GC−MS method (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AUC, area under the curve; EI, electron ionization; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; GC, gas chromatography; GC−MS, gas chromatography−mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; SIM, selected ion monitoring; MSD, mass selective detector; ^{1}H NMR, proton nuclear magnetic resonance; RSD, relative standard deviation; SID, stable isotope dilution; SIDA, stable isotope dilution assay/analysis; TIC, total ion current

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