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Analysis of the Entrapment Efficiency of Thymoquinone in Microcapsules of Black Cumin Seed Oil (*Nigella sativa L.*) Using High Performance Liquid Chromatography

Ofa Suzanti Betha[1]; Supandi Supandi[2*]; Addina Syahida[1]; Boy Reynaldi Noor[1]; Nelly Suryani[1]; Sabrina Dahlizar[1]; Yuni Anggraeni[1]; Barita J. Siregar[1]; Zilhadia[1]

[1]Pharmacy Department, Faculty of Health Sciences, UIN Syarif Hidayatullah Jakarta, Ciputat, Banten, Indonesia

[2]Pharmacy Department, Faculty of Pharmacy and Science, Universitas Muhammadiyah Prof. Dr. HAMKA, South Jakarta, Indonesia

Abstract

Thymoquinone is the main active compound found in black cumin seed oil (*Nigella sativa*). This study intends to analyze the efficiency of its absorption in microcapsules by the ionic gelation of cross-linked alginate and the CaCl2 method, and loading with black cumin seed oil in a concentration of 20%. Thymoquinone was used as a marker to establish quality standards for this microcapsule preparation. Analysis of thymoquinone in microcapsules was performed using a pre-optimized, high-performance liquid chromatography method. Method optimization utilized an Acclaim® Polar Advantage II (C18) column with a flow speed of 1.5 mL/min, a UV detector wavelength of 252 nm, and an injection volume of 20 µL with an isocratic system on a methanol eluent composition: water (70:30). This method revealed a good linearity value (r = 0.9997) in the range of 0.5 – 500 µg/ml. The detection and quantitation limits were 8.67 µg/mL and 28.9 µg/mL, (%diff) was about -1.864 to 1.562, precision (% RSD) was 0.052 to 0.113%, and the recovery was 98.135 to 101.563%. The results of this method validation were then applied to determine thymoquinone loading in black cumin seed oil microcapsules. The results of the absorption efficiency value of black cumin seed oil microcapsules by the ionic gelation alginate and CaCl2 method, which contained 20% oil concentration in the formula, were 81.769%.

Keywords: Thymoquinone; black cumin oil; microcapsule; ionic gelation; alginate

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INTRODUCTION

Black cumin (*Nigella sativa L.*) is an annual flowering herbal plant widely grown in the Mediterranean, Middle East, Eastern Europe, and West Asia.[1] The seeds have abundant chemical content, namely fixed oil in the form of unsaturated fatty acids and essential oil, which consists of several substances, such as 4-terpineol, thymohydroquinone, thymoquinone, carcavol, carvone, and thymol. Thymoquinone is the main compound in *Nigella sativa* essential oil with diverse and important pharmacological activities.[2]

Among the results of testing the pharmacological activity of thymoquinone is that its activity is similar to that of methotrexate in rats induced to suffer from arthritis. This compound also has a cytotoxic effect on pancreatic cancer and can reduce the viability of pancreatic cancer cells. Nevertheless, there are several challenges in formulating black cumin seed oil in medicinal preparations, including that thymoquinone, as an active component, has low stability in water, at alkaline pH, and under exposure to intense light.[3] Several efforts have been made to increase the stability of the chemical content in black cumin seed oil, including through formulation.

One other effort that can be made to increase the stability of thymoquinone in black cumin seed oil is through microencapsulation, i.e., the technology of coating or coating a core substance with a polymer wall layer, so that it becomes small, micro-sized particles. The presence of a thin layer around the core can provide protection from external influences.[4]

Microencapsulation has emerged as an effective strategy to enhance the stability and controlled release of thymoquinone (TQ), a bioactive compound from *Nigella sativa* known for its poor stability and high sensitivity to light and pH. Studies have demonstrated that encapsulating TQ within polymer-based microcapsules, such as those made from alginate, chitosan, or Eudragit®, significantly protects the compound from degradation and improves its shelf-life. These microcapsules not only maintain more than 80% of TQ content after 30 days under accelerated storage conditions but also provide a sustained release profile for over 12 hours, thereby improving its pharmacological performance. Such formulations address the major limitations of TQ, including rapid degradation in aqueous environments and burst release,

and represent a promising approach for developing stable oral or targeted delivery systems for anticancer and anti-inflammatory therapies.

The ability of a microencapsulation method to adsorb the core material in a microcapsule is called adsorption entrapment. The adsorption efficiency of black cumin seed oil microcapsules can be determined through an entrapment test of thymoquinone as the main compound in the essential oil component of black cumin seed oil.[5,6] This innovative approach offers the entrapment of black cumin seed oil by the ionic gelation method of cross-linked alginate and $CaCl_2$.

METHOD

Materials

Thymoquinone (purity >99%) and sodium alginate were obtained from Sigma Aldrich USA, Black Cumin oil and tragacanth from PT Galenika Indonesian local source, distilled water and methanol for high-performance liquid chromatography, included calcium chloride and ethanol from Merck, Darmstadt, Germany.

Validation of the Thymoquinone Analysis Method Using High-Performance Liquid Chromatography Instrumentation and Chromatographic Conditions

The method development and validation were performed on an HPLC (Dionex Ultimate® 3000) system, connected to a UV detector (Hitachi U–2910). The LC system consisted of a dry vacuum pump (Welch), and the column used for the study was C18 (3 µm; 4.6 x 150 mm) (Acclaim® Polar Advantage II).

Linearity

The calibration range of thymoquinone was prepared by serial dilution of the stock solution (500 µg/mL). From the stock solution, further dilution was done in the mobile phase to get a final concentration range between 0.5 – 100 µg/mL. Finally, the plot between concentration vs. area was drawn, and linear regression analysis was performed. The LOD and LOQ values were determined using the slope of the calibration curve, and the data were calculated per the reported procedure.[7]

Accuracy

The accuracy of the developed method was assessed by standard addition methods using three samples at three different concentrations. The sample thymoquinone (500 µg/mL) was injected with the addition of 80, 200, and 375 µg/mL of the standard thymoquinone. The recovery (%) of samples at different concentrations was assayed, and the

recovered thymoquinone at each concentration was calculated.[11]

Determination of the Mobile Phase Composition of Thymoquinone Analysis

To determine the optimal mobile phase, three methanol-water mixtures (60:40, 65:35, and 70:30 v/v) were evaluated using HPLC with a UV-Vis detector (252 nm), a flow rate of 1.5 mL/min, and an injection volume of 20 µL.[12]

Determination of Thymoquinone in Black Seed Oil by HPLC

Samples were prepared by weighing a certain number of black cumin seed oil samples (replicated 3 times), then dissolved in methanol to a final volume of 10 mL, shaken with a vortex for 2 minutes, and then the mixture was allowed to stand for 1 minute until phase separation was seen. The methanol layer was taken and filtered using a syringe equipped with a 0.45 µm membrane filter. The filtered sample was injected into the HPLC as much as 20 µL. From the analysis, the peak area was observed, which was then substituted into the regression equation on the calibration curve as a Y value, so that the sample concentration was obtained in ppm units. Then, the % b / b levels were calculated.[4]

Formulation and Preparation Procedure of Black Cumin Oil Microcapsules

Black cumin oil containing thymoquinone microcapsules was prepared by the ionic gelation method. The preparation of microcapsules began with mixing 0.5% of sodium alginate and 0.3% of tragacanth in water using a homogenizer at 1000 rpm for 3 minutes or until the mucilage became transparent. Gradually, 20% black cumin oil was poured into the mixture while stirring with a homogenizer until a homogeneous emulsion was formed. Beads of microcapsules were formed by the extrusion technique, where black cumin seed oil emulsion was injected into Syringe no. 30 F and dripped into 0.5 M $CaCl_2$ solution so that a microcapsule bead was formed.[13] Beads were left submerged in $CaCl_2$ solution for 20 minutes, and then filtered and weighed.

Solvent Selection in the Thymoquinone Extraction Process in Black Cumin Seed Oil Microcapsules

Thymoquinone extraction was performed using two different solvents, i.e. using methanol and ethanol.[4,5] Solvent selection was carried out by testing the ability of the solvent to attract thymoquinone compounds contained in black cumin seed oil. A total of 100 µl of black cumin seed oil

was dissolved in methanol and ethanol to a volume of 10 ml. The preparation was repeated three times (triplo). Each solution was then analyzed using an HPLC instrument to observe the peaks and area of the chromatogram formed at the thymoquinone retention time. The solvent chosen was the one producing the best peak shape and/or chromatogram area at the greatest thymoquinone retention time

Determination of Thymoquinone Extraction Techniques in Black Cumin Seed Oil Microcapsules

Extraction techniques were determined by comparing several techniques on one sample at the same extraction time. Approximately 100 mg of microcapsules were carefully weighed, then crushed and dissolved with the selected solvent to a volume of 10 ml. Then, the sample solution was vortexed for 5 and 10 minutes, respectively. Using the same procedure, the sample solution was repeated three times to be extracted employing the sonication technique for the same time, 5 and 10 minutes each. Preparations for each time in each method were replicated three times (triplo). The supernatant from the extraction was taken. Then, all the extracted analytes were analyzed using HPLC, observing the peaks and area of the chromatogram formed at the thymoquinone retention time (RT).

Optimization of Thymoquinone Extraction Time in Black Cumin Seed Oil Microcapsules

Extraction time was determined by varying the extraction time for samples prepared with the selected solvent and technique. Carefully weighed, approximately 100 mg of microcapsules were crushed and dissolved in the chosen solvent to a volume of 10 ml. This mixture was made into four series, each repeated three times (triplo). Following that, the sample solution was sonicated using ultrasonics at a further time interval of 10 minutes, with an interval of 5 minutes at 15 minutes, 20 minutes, 25 minutes, and 30 minutes. The preparation for each time was replicated three times (triplo). Then, the supernatant from the extraction was taken, and all the analytes from the extraction were analyzed using HPLC, observing the peaks and area of the chromatogram formed at the retention time (RT) of thymoquinone. The optimal extraction time has been obtained if the analysis of extraction results using HPLC with an extended time (longer than the optimal time) does not result in an increase in area (AUC). After obtaining the optimal extraction time, extraction was

carried out again two times after the optimal time with an interval of 5 minutes.

Determination of the Entrapment Efficiency of Thymoquinone in Black Cumin Oil Microcapsules

Entrapment efficiency was determined by calculating the percentage of the comparison value of the extracted thymoquinone content with the actual level of thymoquinone.

RESULTS AND DISCUSSION

Results of Mobile Phase Determination for Thymoquinone Analysis

Mobile phase selection was carried out with three combination models of methanol and water as in Table 1, where the mobile phase used was methanol: water (60: 40) v/v in the first experiment, a ratio of (65: 35) in the second experiment, and a ratio of (70:30) in the third experiment, with a UV-Vis detector, wavelength 252 nm, flow rate 1.5 mL/minute, and with an injection volume of 20 µL. Based on experiments, the mobile phase composition chosen was a combination of methanol: water (70:30). This composition was chosen because it produced asymmetrical values that met the requirements when compared to other mobile phase compositions and had the fastest retention time (Fig. 1 A, B, C; Table 1) compared to other mobile phase compositions; thus, the analysis time was faster and more efficient.[9,12]

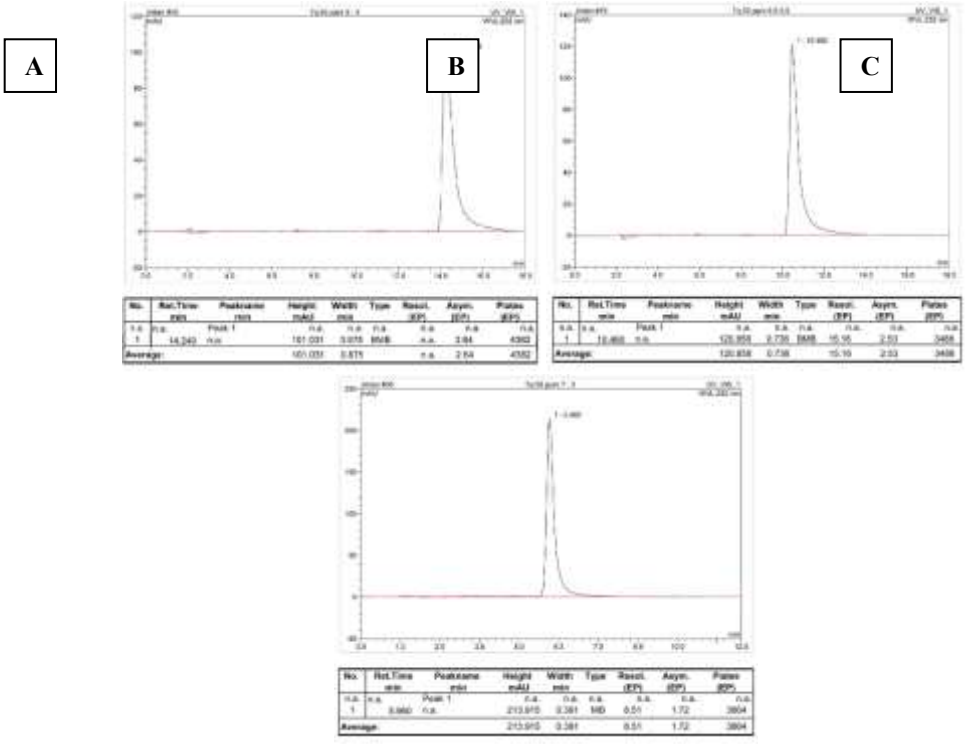


Figure 1. Chromatogram analysis results using mobile phase methanol-water (60:40) [A]; methanol-water (65:35) [B]; methanol-water (70:30) [C]

Table 1. Results of standard thymoquinone analysis by using three mobile phase combination models at a concentration of 50 µg/mL, flow rate of 1.5 mL/min, wavelength of 252 nm, and injection volume of 20 µL

Combination of mobile phase (v/v)	tR (minutes)	Peak area (mAU)	N	HETP	Asymmetric
60:40	14.240 ±0.029	63.4197 ±0.132	4382 ±0.098	0.0342 ±0.001	2.64 ±0.002
65:35	10.460±0.042	64.3771 ±0.112	3486 ±0.088	0.0430 ±0.001	2.53 ±0.002
70:30	5.960±55.750	61.9257 ±0.098	3864 ±0.089	0.0380 ±0.001	1.72 ±0.0029
Requirement	-	-	≥ 2500	-	≤ 2.5

Notes:
TR : *Time retention*
N : *Theoretical plate amount*
HETP : *Height Equivalent Theoretical Plate*

Validation of Thymoquinone Analysis Method

Linearity

Method validation began with constructing a calibration curve and assessing linearity. Thymoquinone (TQ) standard solutions were analyzed at concentrations of 0.5, 10, 10, 20, 30, 50, 100, and 500 µg/mL. Since the TQ content in the microcapsule formulation was unknown, the calibration curve was prepared over a wide concentration range. The analysis produced distinct chromatographic peak areas, which were used to generate the calibration curve shown in Figure 4.[7]

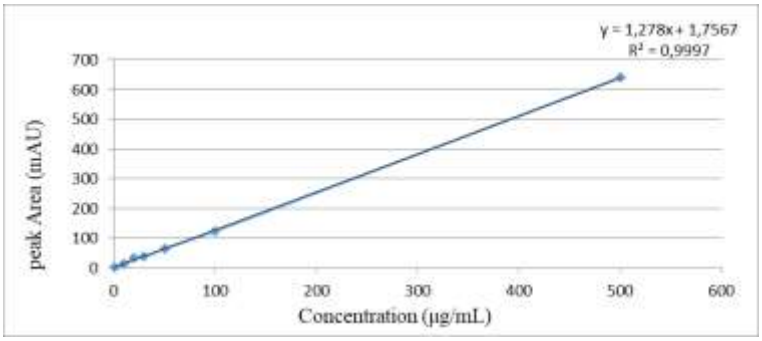


Figure 2. Calibration curve of thymoquinone analysis

From the calibration curve, the equation y = 1.278x + 1.7567 was obtained, producing an interceptive value symbolized by a = 1.7567. This indicates that the curve intersected the y-axis at the point + 1.7567 and the value b = 1.278, which represents the gradient slope value of the curve. The correlation coefficient value, r = 0.9997, was also obtained. This r value met the requirements for acceptance— the r value is > 0.9990.[7]

Accuracy

In testing the % diff at a concentration of 80 µg/mL, the average % diff was - 0.155%; at a concentration of 200 µg/mL, the average % diff was 1.562%; and at a concentration of 375 µg/mL, the average % diff was - 1.864 %.

Next, the recovery percentage value (% recovery) was obtained by comparing the measured value with the actual value. At a concentration of 80 µg/mL, the average % recovery was 99.844%; at a concentration of 200 µg/mL, the average % recovery was 101.563%; and at a concentration of 375 µg/mL, the average % recovery was 98.135%. The required value for (% diff) is no more than 2%, and the requirement for the recovery percentage is 97-103% [8, 17-18]. The results for the accuracy test have met the requirements for the analysis method, as presented in Table 2.

Table 2. Average accuracy test results (% diff and recovery)

Concentration of thymoquinone (µg/mL)	Real concentration of sample (µg/mL)	Concentration obtained (µg/mL)	Average of recovery value (%)	Average of diff (%)
80	81.5	79.875	99.844	-0.155
200		203.126	101.563	1.562
375		368.007	98.135	-1.864
Requirement	-		97-103 %	< 2%

Thymoquinone Levels in Black Seed Oil

After the specified method validation parameters met the requirements, this method was applied to determine thymoquinone levels in black cumin seed oil. Determination of thymoquinone levels was carried out 3 times by weighing a sample of 42.6 mg of black cumin seed oil. The sample was dissolved in methanol to a final volume of 10 mL, then homogenized with a vortex for 2 minutes, and left for 1 minute. The upper methanol layer was taken and filtered through a 0.45 µm filter [14-16, 18]. An amount of 20 µL of sample

(4.26 mg/mL) was then injected into HPLC, and then the concentration (% w/w) was calculated using 3 sample repetitions, as displayed in Table 3 and Figure 3.

Table 3. Thymoquinone concentration in black cumin seed oil

Sample replication	Black seed oil weighs (mg)	Peak area (mAU)	Thymoquinone concentration in sample (µg/mL)	Thymoquinone concentration in black cumin oil (%)
1	42.6	220.038	170.799	4.002
2		220.206	171.930	4.009
3		219.293	170.216	3.895
RSD (%)	-	-	-	0.22
Mean	-	-	-	3.968

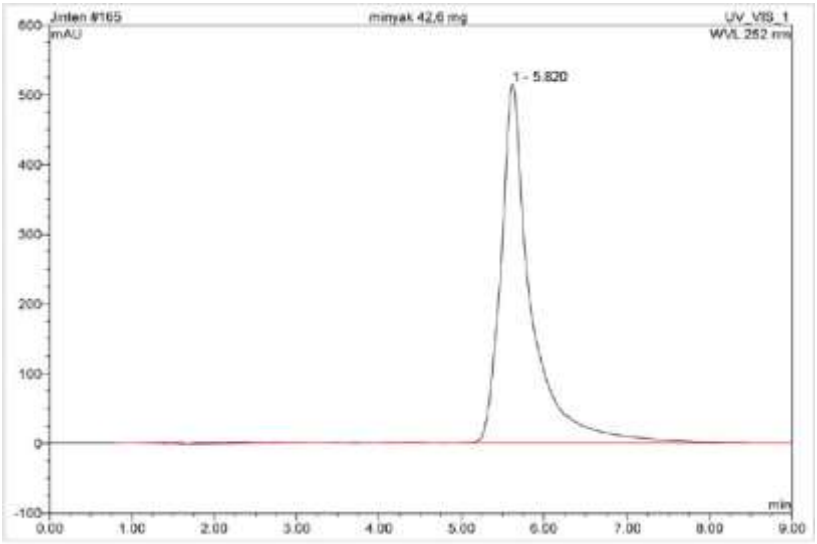


Figure 3. Chromatogram **of** thymoquinone in black cumin seed oil

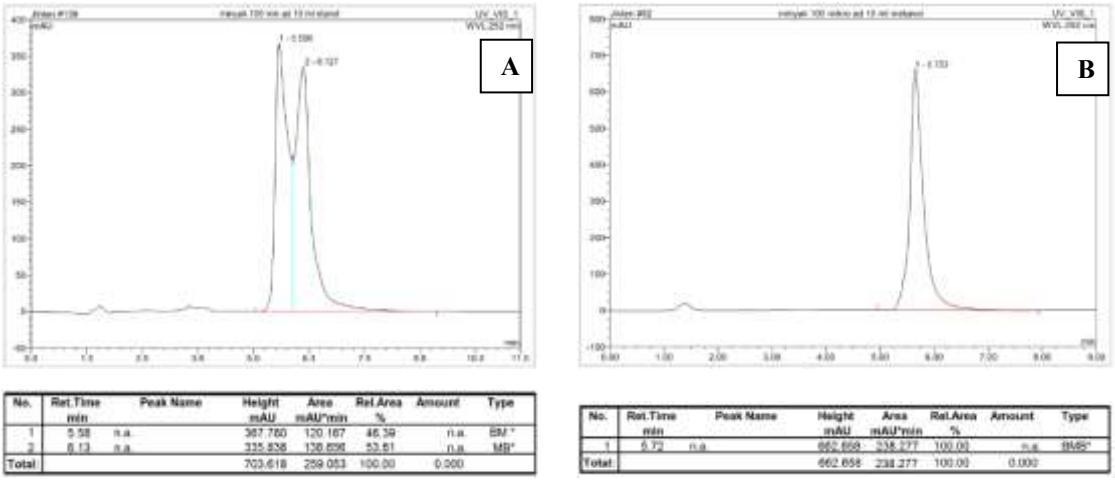
The average thymoquinone content in the black cumin oil sample was 3.968% (b/b) with an RSD value of 0.22%. This met the requirements—less than 2%, so the tested HPLC method provided accurate analysis results.[9]

Theoretical Thymoquinone Levels in Black Cumin Seed Oil Microcapsules

Based on the weight of black cumin seed oil used in the formulation (4.0016 g) and its thymoquinone (TQ) content of 3.98% (Table 3), the amount of TQ present in the oil was calculated to be 159.31 mg. By comparing this value with the total weight of the obtained microcapsules, the TQ content was determined to be 1.524%.[4,6] This TQ concentration requires further confirmation of its pharmacological activity in subsequent studies. Accordingly, 100 mg of microcapsules extracted with 10 mL of solvent would yield a TQ concentration of approximately 152.4 µg/mL.

Results of Solvent Selection in the Thymoquinone Extraction Process in Black Cumin Seed Oil Microcapsules

From the results of analysis using HPLC on a solution extracted from thymoquinone extraction from black cumin seed oil using methanol solvent, chromatogram results with a single peak were obtained. In the analysis with three injections, the retention time of each solution was obtained at 5.72, 5.68, and 5.73 minutes, and the area obtained was 238.277, 238.471, and 237.688 mAU. Meanwhile, from the analysis results using HPLC on the three analytes replicated from black cumin oil extraction preparations using ethanol solvent, chromatogram results were obtained with two peaks close together, each at a retention time of 5.5 minutes and 6.1 minutes, as seen in Figure 4.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	5.58	n.a.	367.760	120.167	46.39	n.a.	BM*
2	6.13	n.a.	335.836	138.896	53.61	n.a.	MB*
Total			703.618	259.053	100.00	0.000	

No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	6.72	n.a.	662.658	238.277	100.00	n.a.	BMB*
Total			662.658	238.277	100.00	0.000	

Figure 4. Chromatogram analysis of black cumin seed oil extraction with ethanol (A) and methanol (B)

In the extraction of thymoquinone in black cumin seed oil using methanol solvent, there was no complete solution when oil and methanol were mixed, or two layers were still formed—the methanol fraction was at the top. The oil layer was at the bottom. Meanwhile, in extraction using ethanol solvent, all oils could be dissolved by ethanol, so ethanol is thought to have the ability to dissolve compounds other than thymoquinone in black cumin seed oil, which has a retention time not much different from the retention time of thymoquinone.[5,9] Hence, the resulting chromatograms were squeezed together. The results of this clustered chromatogram indicate that the separation of compounds in the column did not occur properly.

During the extraction of thymoquinone (TQ) from black cumin seed oil using methanol, complete miscibility was not achieved; two distinct layers were observed, with the methanol phase on top

and the oil phase at the bottom. This occurred because highly polar methanol has a limited ability to dissolve nonpolar components of the oil, resulting in phase separation. In contrast, ethanol, which is less polar than methanol, was able to dissolve the entire oil phase. However, this higher solubilizing capacity means ethanol likely extracts not only TQ, but also other lipophilic compounds present in the oil. Some of these co-extracted compounds have retention times close to that of TQ (around 5.9 minutes), leading to overlapping peaks in the chromatogram. This peak clustering indicates poor resolution and incomplete separation of analytes on the column, compromising quantification accuracy.

Results of Thymoquinone Extraction Technique Determination in Black Cumin Seed Oil Microcapsules

From the results of analysis using HPLC on the solution extracted from microcapsules with the vortex technique for 5 minutes and 10 minutes, chromatogram results were obtained with increasing area with increasing extraction time, namely from an area of ± 116 mAU in 5 minutes of extraction to ± 136 mAU in 10 minutes of extraction. The area produced by the extraction by the vortex technique at 5 min and 10 min is shown in Table 4.

Table 4. Comparison of Average Area and Standard Deviation Value (SD) of Extracted Chromatogram by Vortex Technique and Ultrasonic Technique Using Methanol Solvent.

Extraction time	Replication to	Extraction Techniques			
		Vortex		Ultrasonic	
		Peak area (mAU)	SD (%)	Peak area (mAU)	SD (%)
5 minutes	1	116.706		126.082	
	2	116.325	0.195	125.443	0.793
	3	116.444		124.505	
10 minutes	1	136.623		145.300	
	2	136.318	0.339	145.424	0.321
	3	135.995		145.908	

Meanwhile, from the results of analysis using HPLC on the solution of microcapsule extraction with ultrasonic techniques for 5 minutes and 10 minutes, chromatogram results were also obtained with an increased area with increasing extraction time [20]. However, the area produced in this ultrasonic technique was larger than the vortex technique, from approximately 125 mAU at 5 min extraction to approximately 145 mAU at 10 min extraction. The area generated by

ultrasonic extraction at 5 min and 10 min is presented in Table 4.

Optimization of Thymoquinone Extraction Time in Black Cumin Seed Oil Microcapsules

Table 5. Mean Area and Standard Deviation (SD) Value of Optimization Results of Extraction Time with Methanol Solvent and Ultrasonic Technique at 15 Minutes to 30 Minutes

Extraction Time	Replication	Mean Area of Chromatogram (mAU)	SD (%)
15 minutes	1	151.492	0.242
	2	151.975	
	3	151.728	
20 minutes	1	160.212	0.123
	2	160.285	
	3	160.452	
25 minutes	1	159.764	0.551
	2	160.225	
	3	160.862	
30 minutes	1	160.293	0.271
	2	159.809	
	3	160.264	

From the results of the analysis using HPLC on the solution resulting from microcapsule extraction with ultrasonics for 15 minutes to 30 minutes with a 5-minute interval between times, the results showed that the area of the chromatogram increased from 151 mAU (15 minutes) to 160 mAU (20 minutes). Meanwhile, in the extraction with a time of 25 minutes and 30 minutes, the area produced did not increase, namely remaining at the chromatogram area of approximately 160 mAU. The area produced at each time is shown in Table 5.

From the results of the analysis of variance in each chromatogram area (AUC) at times 20, 25, and 30 minutes, it was found that there was no significant difference ($p>0.05$). With no significant difference, it can be concluded that at an extraction time of 20 minutes, methanol has been able to dissolve or extract all thymoquinone compounds contained in the sample. Therefore, these conditions are optimal extraction conditions and can be used to analyze thymoquinone in black cumin seed oil microcapsules quantitatively.

Results of Determination of Thymoquinone Entrapment Efficiency in Black Cumin Oil Microcapsules

Determination of microcapsule entrapment efficiency was carried out on the optimized extraction results, namely, in the extraction process with methanol solvent, ultrasonic agitation, and a 20-minute extraction time. The concentration of thymoquinone extracted was determined by substituting the AUC value of the chromatogram as the y value into the equation obtained from making the calibration curve, namely, y = 1.2792x + 0.9059. The concentration value of each extraction result, and its average, can be seen in Table 6.

From the calculation, the average concentration of thymoquinone in 100 mg of sample in 10 ml of methanol for each replication was 124.536 µg/ml, 124.592 µg/ml, and 124.723 µg/ml, with the average of the three replications being 124.617 µg/ml. The weight content of thymoquinone in microcapsules was 1.524%, so in 100 mg of microcapsules dissolved in 10 ml of methanol, there was a concentration of thymoquinone of 152.400 µg/ml.

By comparing the extracted thymoquinone concentration of 124.617 µg/ml to the theoretical thymoquinone concentration of 152.400 µg/ml, the efficiency value of black cumin seed oil microcapsules adsorption using the ionic gelation microencapsulation method and alginate and $CaCl_2$ crosslink coating with an oil concentration of 20% in the formula was 81.769%.

Table 6. Results of Black Cumin Seed Oil Microcapsule Adsorption Efficiency by Ionic Gelation Microencapsulation Method and Alginate and $CaCl_2$ Crosslink

Replication	Time Retention (min)	Area (mAU)	Mean Area (mAU)	Concentration (µg/ml)	Mean Concentration (µg/ml)	Entrapment Efficiency (%)
1	5.680	159.974	160.212±0.359	124.536	124.617±0.083	81.769%
	5.623	160.721				
	5.672	159.942				
2	5.687	159.697	160.285±0.434	124.593		
	5.553	160.735				
	5.621	160.423				
3	5.687	160.632	160.452±0.216	124.723		
	5.631	160.147				
	5.672	160.577				

CONCLUSION

The optimum conditions for the analysis of thymoquinone in black cumin seed oil using a chromatography system consisting of an Acclaim® Polar Advantage II (C18) column with a flow rate of 1.5 mL/min, a UV detector with a wavelength of 252 nm, an injection volume of 20 µL with an isocratic system at an eluent composition of methanol: water (70:30) were obtained.

Validation of the method performed gave linearity value results (r = 0.9997) in the 0.5 - 500 µg/ml range. The detection and quantitation limits were 8.67 µg/mL and 28.9 µg/mL, (% diff) was around -1.864 to 1.562, precision (% RSD) ranged from 0.052 to 0.113%, and recovery was 98.135 to 101.563%.

Thymoquinone analysis in black cumin seed oil samples had a content of 3.968% (w/w). The method of extracting thymoquinone from black cumin seed oil microcapsules (*Nigella sativa L.*) using the ionic gelation microencapsulation method and alginate and $CaCl_2$ crosslink coatings is optimal using methanol solvent, with a procedure of 100 mg of black cumin seed oil microcapsules being ground, methanol added to a volume of 10 ml, and with an ultrasonic stirring technique for 20 minutes.

Quantitative data on the results of thymoquinone extraction in black cumin seed oil microcapsules obtained can determine the value of thymoquinone adsorption efficiency in microcapsules, which was 81.769%.

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Integrated Laboratory of the Faculty of Health Sciences, UIN Syarif Hidayatullah, Jakarta.

Conflict of interest

Declare a conflict of interest.

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