

# Dual Response Optimization of Ultrasound-Assisted Oil Extraction from Milkfish By-Products using D-Limonene as A Bio-Based Solvent

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## Abstract

Fish oil has been reported to have positive health effects because it is rich in n-3 essential fatty acids. This study aimed to determine the optimal conditions for achieving a high oil yield and omega-3 content from milkfish by-products using ultrasound-assisted extraction (UAE) combined with a d-limonene solvent. The Box-Behnken design (BBD) combined with response surface methodology was successfully used to determine the optimum extraction conditions for omega-3 fatty acids based on several factors: Temperature (30, 60 and 90 °C), solvent-to-sample ratio (2:1, 4:1 and 6:1 mL/g) and time (6, 38 and 70 min). The ANOVA results showed that sonication time ( $p < 0.05$ ) and temperature ( $p < 0.05$ ) had a significant influence on omega-3 fatty acids and the yield of extracted oil. The optimal extraction conditions for UAE were 68 min, 84 °C, and a solvent-to-sample ratio of 3:1 mL/g resulting in high oil recovery (21.95 %) containing omega-3 compounds (12.50 %). Additionally, the resulting oil was also further characterized by quality parameters (acid, peroxide, anisidine-total oxidation and iodine values) as well as the fatty acid composition. The developed milkfish oil met the IFOS™ (International Fish Oil Standards) criteria hence applicable for industrial oil production.

**Keywords:** Milkfish oil, Fatty acids, Omega-3 compounds, Response surface methodology, Ultrasound-assisted extraction

## Introduction

In recent years, the annual marine fish catching and aquaculture production has attained a figure of 100 million metric tons. Approximately 53 % of global fishery products are utilized for human consumption, whereas the remaining portion is designated for non-culinary applications, including fish feed [1]. Milkfish are commonly utilized in Indonesian cuisine and are prized for their delectable flavor and high fat content, which contribute to their appeal as a culinary ingredient. The fatty acid profile of milkfish comprises unsaturated fatty acids (50.74 %), including polyunsaturated fatty acids (34.47 %) and monounsaturated fatty acids (16.27 %) [2]. Furthermore, it provides a notable amount of protein, amino acids, vitamins and essential fatty acids. The growing interest in milkfish oil is attributable to its numerous nutritional benefits.

Fish oils are high in PUFA, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Fish oils are predominantly derived from mackerel, salmon, sardine, cod and anchovies [3]. The consumption of omega-3 fatty acids has been empirically demonstrated to have numerous positive effects on human health, such as their capacity to fight inflammatory and cardiovascular disorders [4] and promote antioxidant activity [5].

The utilization of milkfish as a raw material in the production of fillets yields by-products, the quantity of which may reach up to 40 % of the total output. In the course of processing fresh fish for human consumption in production settings, a substantial amount of both edible and non-edible by-products, such as heads, offal, bones and scales, is generated [6]. Thus, the potential for extracting fish oil from milkfish by-products as a source of omega-3-rich fish oil is significant.

Milkfish oil is commonly extracted using a combination of chloroform and hexane through the Soxhlet extraction method. Although this approach is widely used, it poses a risk to both human health and the environment due to the combustible nature of these organic solvents. Recently, agrochemical solvents have emerged as a feasible alternative to petroleum-based solvents for the extraction of specific components. Vegetable oils, limonene and turpentine have been proposed as alternatives to hazardous petroleum-based solvents for extracting natural raw materials. Limonene is widely recognized as a naturally occurring solvent that offers potential solutions to environmental challenges and promotes enhanced utilization capabilities.

D-limonene is a monoterpene hydrocarbon and the predominant constituent of orange peel essential oil [7]. It has been explored as a substitute for hazardous solvents in the Soxhlet extraction of fats and oils. Its water immiscibility, high extraction efficiency for target analytes and adherence to environmental criteria make it a promising alternative. Successful oil extraction from rice bran [8] and anchovy fillet by-product [9] utilizing d-limonene solvent has been accomplished without impairing oil quality. Hence, it is essential to devise reliable methodologies that employ environmentally friendly solvents for oil extraction.

The oil extraction from milkfish using environmentally friendly solvents presents numerous challenges, particularly in the development of reliable methods for extracting oil containing omega-3 fatty acids with strong bioactive capacities. The disadvantages of currently available extraction methods include extended processing periods, high energy consumption and high solvent usage. Therefore, it is essential to adopt green chemistry principles to develop methods that employ safer solvents and reduce solvent consumption while achieving shorter processing times [10]. UAE technology can address these challenges by accelerating mass transfer and improving extraction kinetics [11]. The application of UAE in the recovery of bioactive compounds from fish oils has been reported for omega-3 and nutritional components in *Sparus aurata* [12] and *Monopterus albus* [13]. The utilization of ultrasound-induced cavitation in UAE offers a unique advantage for extracting specific compounds from solid matrices. However, some factors influence the performance of UAE (extraction time, solvent composition and temperature). Therefore, the objective of this research was to optimize the UAE conditions for milkfish oil extraction to achieve a high recovery of bioactive components. Multivariate data analyses have effectively assessed and refined the variables most likely to impact the efficiency of UAE procedures [14].

## Materials and methods

### Chemicals and reagents

D-limonene was obtained from Sigma-Aldrich (St. Louis, MO, USA). The solvents and reagents utilized for chromatography and analysis were obtained from Merck (Darmstadt, Germany).

### Samples preparation

Milkfish by-product samples for the optimization process were collected from Pati (−6.633068, 111.121094), Central Java, Indonesia. In contrast, optimal extraction conditions were collected from several Indonesian fish farmers, namely Juwana (−6.674390, 111.153550), Rembang (−6.695100, 111.254048), Semarang (−6.947425, 110.392738), Tuban (−6.820642, 112.006450) for real applications.

The collected samples were cleaned and dried using a cabinet dryer (AM-TD6, PT. Khalifah Niaga Lantabura, Yogyakarta, Indonesia) at 55 °C until their water content reached 10 %. The dried samples were then mashed using Philips blender HR2116/40 glass green 350 W (Amsterdam, Netherlands) and stored in the freezer at −25 °C until they were used for extraction.

### Milkfish oil extraction using UAE

The UAE procedure was carried out in a Bransonic Ultrasonic (8510E MTH, Brookfield, Connecticut, USA) chamber at a frequency of 25 kHz and a power of 200 W. In each experiment, a combination of 25 g of milkfish by-product and d-limonene was placed in an Erlenmeyer flask and subjected to ultrasonic extraction for a specified extraction time according to the experimental design. Following extraction, the resulting mixture was centrifuged at 6,000 rpm for 5 min, and the d-limonene was removed using a rotary vacuum evaporator set to 40 °C and 40 mbar. The extracted oil was stored at 4 °C before use. The yield of the oil was determined using Eq. (1) [12,15].

$$\text{Yield (\%)} = \frac{\text{Total crude oil (g)}}{\text{Milkfish by-product powder (g)}} \times 100 \% \quad (1)$$

### Experimental design

The BBD was utilized to optimize the extraction of fish oil using d-limonene and the UAE system, to maximize total yields and omega-3 fatty acids. The variables examined in the UAE optimization included extraction time ( $X_1$ ), temperature ( $X_2$ ) and solvent-to-solid ratio ( $X_3$ ), each having 3 levels (−1, 0 and +1). The independent variables and their corresponding coding levels for oil extraction from milkfish by-products using UAE are presented in **Table 1**, while the full run of BBD is presented in **Table 2**. The dual responses of BBD were the extraction yield (%) and omega-3 fatty acid (%) levels in the resulting oil.

**Table 1** Independent variables coding level and real value for BBD.

Variable	Symbol	Code value		
		−1	0	1
Time (min)	$X_1$	6	38	70
Temperature (°C)	$X_2$	30	60	90
Solvent-to-sample ratio (mL/g)	$X_3$	2:1	4:1	6:1

**Table 2** BBD for oil extraction from milkfish by-products using UAE system.

Run	Extraction variables			Responses			
	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	Yield (%)		Omega-3 fatty acid (%)	
				Observed	Predicted	Observed	Predicted
1	-1	1	0	11.00	11.26	7.33	7.72
2	1	0	-1	21.05	21.02	11.42	11.26
3	1	1	0	21.98	21.98	12.20	12.17
4	1	0	1	20.95	21.23	10.98	11.57
5	0	-1	-1	14.21	14.49	6.01	6.85
6	0	0	0	14.78	14.88	8.93	8.95
7	0	0	0	14.86	14.88	9.28	8.95
8	0	0	0	15.01	14.88	9.22	8.95
9	-1	0	1	11.04	11.07	6.58	6.76
10	-1	0	-1	11.02	10.74	6.78	6.20
11	1	-1	0	20.92	20.67	11.20	10.66
12	-1	-1	0	10.95	10.95	5.36	5.24
13	0	1	-1	16.00	16.02	10.28	10.61
14	0	1	1	15.85	15.57	9.70	9.28
15	0	-1	1	15.50	15.48	8.96	9.05

A 2<sup>nd</sup>-order quadratic polynomial regression model was utilized to predict the Y responses, specifically the extraction yield and the content of omega-3 fatty acids. The model is described by Eq. (2), which provides an explanation for the behavior of Y.

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^k \beta_{ij} X_i X_j + \varepsilon \quad (2)$$

where Y is the predicted response function (extraction yield and omega-3 content),  $\beta_0$  is the constant term,  $\beta_i$  is the coefficient of the linear term,  $\beta_{ii}$  is the coefficient of the quadratic term,  $\beta_{ij}$  is the interaction coefficient, and  $X_i, X_j, \dots, X_k$  are the studied independent factors, and  $\varepsilon$  is a random error. The statistical significance of the factor and the evaluation of the fitting quality of the polynomial model were defined based on analysis of variance (ANOVA). Furthermore, the correlation impact of the independent variables and the responses was analyzed using the 3-dimensional curves of the response surface graphs.

### Chemical characterization and quality parameters of milkfish by-product oil

#### Determination of acid value

The milkfish by-product oil was precisely weighed (1 g) and transferred into an Erlenmeyer flask. Subsequently, 50 mL of 95 % ethanol solution was added, and the mixture was thoroughly shaken until the ethanol was fully dissolved. The titration process was performed using a 0.1 N potassium hydroxide (KOH)-ethanolic solution, and completion of the process was indicated by the phenolphthalein indicator. The acid value (AV) was calculated using Eq. (3), as described in reference [16].

$$\text{Acid value (mg KOH/g)} = \frac{\text{KOH volume (mL)} \times \text{N KOH} \times 56.1}{\text{Milkfish by-product oil (g)}} \quad (3)$$

#### ***Determination of peroxide value (PV)***

The oil (1.0 g) was placed in an Erlenmeyer flask. Subsequently, 30 mL of chloroform: Acetic acid (2:3) was added to the sample and the mixture was agitated until it became homogenous. Subsequently, a saturated potassium iodide solution (0.5 mL) was added to the mixture and left to stand for 1 min. The mixture was then titrated using a 0.1 N sodium sulfite ( $\text{Na}_2\text{SO}_3$ ) solution with 0.5 % starch solution (1 mL) serving as the indicator. The titration was terminated when the blue color of the mixture faded. The PV was calculated using Eq. (4) [1].

$$\text{Peroxide value (mEq O}_2\text{/kg)} = \frac{\text{Na}_2\text{SO}_3 \text{ volume (mL)} \times \text{Na}_2\text{SO}_3 \text{ N}}{\text{Milkfish by-product oil (g)}} \times 1000 \quad (4)$$

#### ***Anisidine value and total oxidation value (Totox)***

A UV-Vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) was employed to measure the absorbance ( $A_1$ ) at 350 nm of 1 g of the oil sample in 25 mL of n-hexane. Subsequently, the solution was transferred to a test tube and supplemented with 1 mL of *p*-anisidin in glacial acetic acid. The solution was then shaken for 10 min, followed by the measurement of absorbance ( $A_2$ ) at the same wavelength. The anisidine value was determined using Eq. (5) [16].

$$\text{Anisidine value (mEq O}_2\text{/kg)} = 25 \times \frac{(1.2 \times A_2 - A_1)}{\text{Milkfish by-product oil (g)}} \quad (5)$$

Oil degradation was also measured using Eq. (6) to calculate the total oxidation value (Totox value).

$$\text{Totox value (mEq } \frac{\text{O}_2}{\text{kg}}) = 2\text{PV} + \text{Anisidine value} \quad (6)$$

#### ***Determination of iodine value***

The milkfish by-product oil was weighed (0.3 g) and placed in an Erlenmeyer flask. The oil was dissolved in 10 mL of chloroform and combined with 25 mL of Wijs reagent, which is a solution containing 1 % iodine chloride in glacial acetic acid. The sample mixture was supplemented with 50 mL of  $\text{CO}_2$ -free aquadest and 15 % potassium iodide solution (10 mL) following a 30-min exposure to darkness. Subsequently, a titration procedure was conducted with a standardized 0.1 N sodium thiosulfate solution, forming a faint yellow hue. A starch solution (0.5 %) was used to indicate the final titration point when the blue color of the mixture disappeared. Titration against the blanks was also performed. The iodine value was determined using Eq. (7) [16].

$$\text{Iodine value (g I}_2\text{/100 g)} = \frac{(\text{Na}_2\text{SO}_3 \text{ volume of blank} - \text{Na}_2\text{SO}_3 \text{ volume of sample}) \times \text{Na}_2\text{SO}_3 \text{ N} \times 12.69}{\text{Milkfish by-product oil (g)}} \quad (7)$$

#### ***Fatty acid composition***

Fatty acid profiles were determined using gas chromatography flame ionization detector (GC-FID Agilent 7890B, Santa Clara, USA) following the AOAC method [17] with slight modifications. The initial process in determining the composition of fatty acids is the derivatization of methyl esters to form fatty acid methyl esters (FAME). A total of 0.5 mL of milkfish oil was collected, and 1.50 mL of methanolic

sodium solution was added. The solution was heated at 60 °C for 10 min and then cooled. The mixture was subjected to the addition of 2 mL BF<sub>3</sub>, followed by heating at 60 °C for 10 min and subsequent chilling. Next, introduce 1 mL of saturated sodium chloride (NaCl) and 1 mL of n-hexane into the mixture and agitate vigorously using a vortex. The top layer was transferred to a new vial. In addition, a sample solution volume of 1 µL was introduced into a GC-FID fitted with a DB-WAX column (Agilent HP-88, Santa Clara, USA) using a programmed oven temperature of 50 - 230 °C with a temperature increase rate of 3 °C/min.

### ***Statistical analysis***

The statistical BBD employed in this work was assessed using Design Expert 13 (Stat-Ease, Inc., USA). The study employed ANOVA to analyze the experimental design statistically and assess the interaction between variables and their influence on the observed outcomes. The parameters' statistical significance and the model's adequacy were evaluated using the F-test, *p*-values and the regression coefficient (*R*<sup>2</sup>).

### **Results and discussion**

The extraction of fish oil from milkfish by-products involves mechanical pressing or solvent extraction. The commonly used solvent extraction approach typically achieves higher recovery rates than mechanical techniques for the extraction process. D-limonene is a sustainable alternative to conventional solvents such as n-hexane because it offers lower toxicity, flammability and environmental effects [18-20]. Salmon oil was subjected to d-limonene and hexane solubility tests to assess its efficacy in dissolving certain oil components. The results revealed that d-limonene is a more effective solvent than hexane for diacylglycerols, triacylglycerols, free fatty acids and ergosterols. Additionally, a recent study suggested that d-limonene has the potential to serve as an alternative to conventional solvents sourced from petroleum and has been successfully applied to efficiently extract omega-3 fatty acids from fish [21].

Several fish oil extraction methods, including UAE, have been developed to increase oil production and shorten extraction time. Ultrasound waves generate high-frequency pressure waves in the extraction solvent. These waves cause tiny bubbles to form and collapse rapidly during cavitation. The cavitation effect and improved mass transfer resulted in higher extraction rates than those of the traditional methods. This study was conducted to extract milkfish oil rich in omega-3 using the UAE method, employing d-limonene as the extraction solvent from milkfish by-products. The processing industries can contribute to sustainability by reducing milkfish by-products by extracting fish oil from the by-products. Additionally, this practice can provide economic benefits by creating value-added products from what would otherwise be considered as by-product materials.

### **Evaluation of the effects of extraction variables**

A BBD was developed for the UAE of milkfish by-product oil. The extraction variables that may affect the efficiency of UAE were time (*X*<sub>1</sub>), temperature (*X*<sub>2</sub>) and solvent-to-sample ratio (*X*<sub>3</sub>). The levels of each variable (*X*<sub>1</sub>: 6, 38 and 70 min; *X*<sub>2</sub>: 30, 60 and 90 °C; and *X*<sub>3</sub>: 2:1, 4:1 and 6:1 mL solvent/g sample) were chosen based on the literature reporting fish oil extraction [12,13,15]. The extraction yields of oil from milkfish by-products using the specified experimental design ranged from 10.95 to 21.98 %, with omega-3 content from 5.36 to 12.2 % (**Table 2**). To determine the source of this variability, ANOVA (**Table 3**) was conducted to assess the impact of UAE factors on the extraction yield and omega-3 content of milkfish by-product oil.

**Table 3** Analysis of variance (ANOVA) of the quadratic polynomial model for extraction yield (%) and omega-3 content (%).

Variable	Yield (Y <sub>1</sub> )			Omega-3 (Y <sub>2</sub> )		
	Mean square (MS)	F-value	p-value	Mean square (MS)	F-value	p-value
Model	23.9	252.36	< 0.0001	10.08	33.79	< 0.0001
<b>Linear effect</b>						
X <sub>1</sub> (min)	209	2207.23	< 0.0001	48.76	163.51	< 0.0001
X <sub>2</sub> (°C)	1.32	13.94	0.01	7.96	26.69	0.0009
X <sub>3</sub> (mL/g)	0.14	1.48	0.28	0.37	1.25	0.29
<b>Interaction effect</b>						
X <sub>1</sub> X <sub>2</sub>	0.255	2.69	0.162	0.23	0.78	0.40
X <sub>1</sub> X <sub>3</sub>	0.04	0.04	0.863	0.01	0.05	0.83
X <sub>2</sub> X <sub>3</sub>	0.51	5.47	0.006	3.12	10.45	0.01
<b>Quadratic effect</b>						
X <sub>1</sub> <sup>2</sup>	3.53	37.23	0.002			
X <sub>2</sub> <sup>2</sup>	0.45	4.83	0.07			
X <sub>3</sub> <sup>2</sup>	0.09	0.93	0.38			
Lack of fit	0.15	10.91	0.08	0.39	11.02	0.08
R <sup>2</sup>		0.99			0.96	

The influencing variables provided significant main (sonication time and temperature), interaction (temperature×solvent-to-sample ratio), and quadratic (time×time) effects. Sonication time and temperature showed a positive influence of the extraction variables on the yield, which means a high extraction yield was achieved by increasing the respective variables. Ultrasonic waves can disintegrate cell membranes, increasing the contact between the solvent and solid and leading to a greater oil concentration on the surface. Ultrasonic pulses considerably affect the solvent penetration stage, which is characterized by a high mass transfer rate [22]. Extraction at higher temperatures can potentially enhance mass transfer and improve solubility and diffusivities [23]. A previous study on fish oil extraction from *Sparus aurata* by-products also confirmed that sonication time and higher temperature significantly positively affected the extraction yield [12]. Some studies have demonstrated that the solvent-to-sample ratio substantially impacts the UAE method for extracting oil from various sources [23,24].

Furthermore, the influence of the variables on omega-3 content showed that sonication time and temperature positively affected the response. In contrast, temperature×solvent-to-sample ratio had a negative effect on omega-3 content. Previous studies have reported that time and temperature variables significantly affect the EPA of omega-3 extracted from microalgae [25] and lemuru fish by-products [26]. Omega-3 fatty acids have double bonds and are susceptible to oxidative rancidity, which can be enhanced by high-temperature exposure. Furthermore, high temperatures can cause fatty acid hydrolysis, which implies changes in the fatty acid composition. Omega-3 fatty acid content of the extracted milkfish by-product oil was significantly higher than that of the fish oils extracted from tilapia and catfish [27].

### Optimization of the UAE method

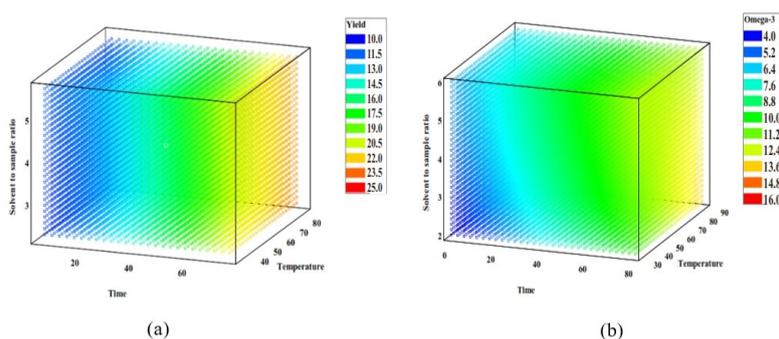
The optimization of milkfish by-product oil and omega-3 fatty acid production using UAE was accomplished by examining the significant effects of main, interaction and quadratic factors. This method entailed the development of 2<sup>nd</sup>-order polynomial equations to reduce the excessive variability. Eqs. (8) and (9) represent the 2<sup>nd</sup>-order polynomial models employed in response surface modeling for the yield and omega-3 content, respectively.

$$Y_{\text{yield}} = 14.88 + 5.11X_1 + 0.41X_2 - 0.36X_2X_3 + 0.98X_1^2 \quad (8)$$

$$Y_{\text{omega-3}} = 8.95 + 2.47X_1 + 0.99X_2 - 0.88X_2X_3 \quad (9)$$

The 2<sup>nd</sup>-order polynomial model was validated by the high values of the coefficient of determination for extraction yield ( $R^2$ , 0.99) and omega-3 ( $R^2$ , 0.93), representing the confidence that the regression model fitted the observed data [29]. Additionally, model validation was performed by a lack-of-fit test comparing the variability of the current model residuals to the variability between observations at replicate settings for the variables. A non-significant lack-of-fit ( $p > 0.05$ ) is a desirable statistical parameter to prove the fit of the model to the responses. The  $p$ -values for the lack-of-fit in the ANOVA table of yield (0.0856) and omega-3 (0.0851) were insignificant ( $p > 0.05$ ), which means that the models fit well with the observed data.

The response surface in **Figure 1** illustrates the relationship between the extraction yield and omega-3 fatty acids, based on the studied variables. The prediction surface was plotted using RSM to determine the best combination of variables for achieving the highest extraction yield and omega-3 content. The desirability function method was employed for numerical optimization to identify the optimal conditions for extracting the milkfish by-product oil. The desirability function measures the degree of desirability on a scale from 0 to 1, with 1 being the most desirable. The predicted extraction yield and omega-3 content under the optimal conditions of 84 °C, sonication time of 68 min, and solvent-to-sample ratio of 3:1 were 21.95 and 12.50 %, respectively.



**Figure 1** Response surface plot showing the effect of UAE variables on the responses of (a) extraction yield and (b) omega-3 content.

The predictions made by the model were validated through the conduct of 3 repetitions under identical conditions. The outcomes obtained revealed values of 19.85 % for extraction yield and 11.35 % for omega-3 content. According to the findings of the experiment, the mean absolute deviation between the actual and predicted values for yield and omega-3 content was 2.08 and 1.15 %, respectively.

### Fatty acids profile of milkfish by-product oils

Omega-3 fatty acids are crucial to the human body because they significantly promote human health and prevent diseases. Omega-3 fatty acids consist of 3 types: Long-chain alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). ALA is classified as an essential fatty acid owing to its inability to synthesize and its reliance on dietary intake or supplementation. The amount of omega-3 fatty acids in fish oil is an important parameter for determining the quality of fish oil. The fatty acid composition of milkfish by-product oil from various regions of Juwana, Semarang, Tuban and Rembang is shown in **Table 4**.

**Table 4** Fatty acids composition of milkfish by-product oil in several regions of Indonesia.

Fatty acid profile	Milkfish in several regions			
	Juwana	Semarang	Rembang	Tuban
laurostearic acid C12:0	0.99 ± 0.01	1.02 ± 0.07	0.97 ± 0.04	1.00 ± 0.56
Tridecanoic acid C13:0	1.08 ± 0.01	1.20 ± 0.05	1.02 ± 0.04	1.70 ± 0.01
Myristic acid C14:0	3.59 ± 0.01	3.51 ± 0.05	3.40 ± 0.01	3.40 ± 0.07
Pentadecanoic acid C15:0	3.92 ± 0.02	3.97 ± 0.12	3.68 ± 0.06	3.70 ± 0.14
Palmitic acid C16:0	20.17 ± 0.05	24.46 ± 0.38	24.41 ± 0.42	23.20 ± 0.99
Heptadecylic acid C17:0	1.79 ± 0.07	1.81 ± 0.06	1.78 ± 0.08	1.70 ± 0.05
Octadecanoic acid C18:0	5.48 ± 0.05	5.68 ± 0.16	5.66 ± 0.04	5.30 ± 0.08
Arachidic acid C20:0	1.06 ± 0.02	1.07 ± 0.04	1.08 ± 0.07	1.00 ± 0.01
Heneicosanoic acid C21:0	1.61 ± 0.02	1.70 ± 0.02	1.72 ± 0.07	1.60 ± 0.03
Docosanoic acid C22:0	0.75 ± 0.01	0.77 ± 0.01	0.68 ± 0.09	0.70 ± 0.05
Trichosanoic acid C23:0	0.17 ± 0.01	0.18 ± 0.02	0.17 ± 0.02	0.10 ± 0.01
Lignoceric acid C24:0	1.74 ± 0.04	1.76 ± 0.04	1.60 ± 0.11	1.00 ± 0.65
<b>Total SFA</b>	<b>42.34 ± 0.36</b>	<b>47.12 ± 1.40</b>	<b>46.17 ± 1.10</b>	<b>44.4 ± 2.20</b>
Myristoleic acid C14:1	0.53 ± 0.04	0.53 ± 0.03	0.52 ± 0.06	0.50 ± 0.03
Oleopalmitic acid C16:1	4.40 ± 0.05	5.25 ± 0.11	5.14 ± 0.36	5.10 ± 0.14
Cis-10-heptadecanoic acid C17:1	0.66 ± 0.01	1.20 ± 0.29	1.13 ± 0.04	1.70 ± 0.05
(E)-Oleic acid C18:1n-9t	1.74 ± 0.02	0.32 ± 0.09	1.03 ± 0.04	0.30 ± 0.04
Oleic acid C18:1n-9c	15.62 ± 0.07	17.82 ± 0.06	17.99 ± 0.02	16.80 ± 0.14
Cis-11-eicosanoic acid C20:1	0.51 ± 0.02	0.54 ± 0.03	0.48 ± 0.12	0.50 ± 0.08
Erucic acid C22:1	1.25 ± 0.02	1.18 ± 0.06	1.13 ± 0.02	1.10 ± 0.06
cis-15-Tetracosenoic acid C24:1	0.26 ± 0.01	0.29 ± 0.04	0.09 ± 0.02	0.10 ± 0.01
<b>Total MUFA</b>	<b>24.98 ± 0.27</b>	<b>27.13 ± 0.74</b>	<b>27.51 ± 0.72</b>	<b>26.10 ± 0.58</b>
Linolelaidatic acid C18:2n-9t	2.74 ± 0.02	2.80 ± 0.07	2.77 ± 0.07	2.50 ± 0.07
Linoleic acid C18:2n-6c	12.99 ± 0.07	5.06 ± 0.66	8.25 ± 0.19	10.20 ± 0.56
γ-linolenic acid C18:3n-6	0.26 ± 0.02	0.24 ± 0.02	0.24 ± 0.04	0.10 ± 0.03
Linolenic acid C18:3n-3	0.83 ± 0.05	0.86 ± 0.02	0.81 ± 0.09	0.80 ± 0.02
Cis-11,14-eikosadienoic acid C20:2	0.43 ± 0.03	0.33 ± 0.07	0.41 ± 0.05	0.40 ± 0.07
Cis-8,11,14-eikosatrienoic acid C20:3n-6	1.21 ± 0.28	1.27 ± 0.01	1.23 ± 0.02	1.00 ± 0.05

Fatty acid profile	Milkfish in several regions			
	Juwana	Semarang	Rembang	Tuban
Arachidonic acid C20:4n-6	0.15 ± 0.01	0.18 ± 0.02	0.18 ± 0.05	0.10 ± 0.04
Cis-13,16-doxosadinoic acid C22:2	0.68 ± 0.02	0.73 ± 0.02	0.71 ± 0.02	0.70 ± 0.06
Cis-5,8,11,14,17-eicosapentaenoic acid C20:5n-3	0.13 ± 0.02	0.14 ± 0.02	0.15 ± 0.04	1.00 ± 0.14
Cis-4,7,10,13,16,19-dokosaheptaenoic acid C22:6n-3	9.25 ± 0.11	3.09 ± 0.07	7.04 ± 0.58	6.02 ± 0.62
<b>Total PUFA</b>	<b>28.66 ± 0.41</b>	<b>14.71 ± 1.03</b>	<b>21.77 ± 1.19</b>	<b>22.90 ± 1.69</b>
Omega-3 fatty acids	11.42 ± 0.21	5.36 ± 0.14	9.78 ± 0.74	8.96 ± 0.84
Omega-6 fatty acids	14.50 ± 0.14	6.55 ± 0.79	9.22 ± 0.35	11.40 ± 0.72

The omega-3 fatty acids include linoleic acid (C18:3n-3), eicosatrienoic acid (C20:3n-6), eicosapentaenoic acid (C20:5n-3) and docosaheptaenoic acid (C22:6n-3). Omega-6 fatty acids consist of specific compounds, including linoleic acid (C18:2n-6), γ-linolenic acid (C18:3n-6), eicosadienoic acid (C20:2) and arachidonic acid (C20:4n-6). The total polyunsaturated fatty acids (PUFA) in milkfish by-product oil ranged from 14.71 (Semarang) to 28.66 % Juwana. The monounsaturated fatty acids (MUFA) in milkfish by-product oil ranged from 24.98 (Juwana) to 27.51 % (Rembang). The milkfish oil analyzed in this study contained significant levels of saturated fatty acids (SFA), with Juwana accounting for 42.34 %, Semarang for 47.12 %, Rembang for 46.17 % and Tuban for 44.40 % of the total SFA content. The major fatty acids present in the milkfish by-product oil were palmitic acid (C16:0), oleic acid (C18:1n-9) and linoleic acid (C18:2n-6) (**Table 4**).

The predominant fatty acids in milkfish oils are palmitic, oleic and linoleic, which together account for more than half of the total fatty acids [29]. The variations in the fat and lipids of milkfish may be attributed to several factors, including origin, harvesting seasons, fish feed and extraction procedures [30]. The composition of fatty acids in oil has a significant impact on its stability, flavor and shelf life.

#### Quality assessment of milkfish by-product oil

The extraction of high-quality milkfish by-product oil was achieved using a combination of UAE and the biosolvent d-limonene. Factors such as temperature, time and oxygen levels can affect the oxidation of oil, and these parameters are critical for determining the quality of milkfish oil [31]. **Table 5** compiles the extraction yield data and the chemical properties of milkfish oil from 4 different regions in Indonesia: Juwana, Semarang, Rembang and Tuban, which were obtained by applying the proposed UAE method.

**Table 5** Extraction yield and chemical properties of crude milkfish by-products oil.

Parameters	Origin of milkfish by-product oil			
	Juwana	Semarang	Rembang	Tuban
Yield (%)	20.35 ± 1.03 <sup>a</sup>	16.28 ± 1.90 <sup>b</sup>	16.60 ± 1.46 <sup>b</sup>	18.76 ± 1.15 <sup>c</sup>
AV (mg KOH/g)	1.63 ± 0.02 <sup>a</sup>	1.59 ± 0.02 <sup>a</sup>	1.61 ± 0.01 <sup>a</sup>	1.60 ± 0.06 <sup>a</sup>
PV (mEq O <sub>2</sub> /kg)	4.40 ± 0.23 <sup>a</sup>	3.87 ± 0.03 <sup>b</sup>	3.97 ± 0.03 <sup>ab</sup>	3.85 ± 0.01 <sup>b</sup>
Anisidin Value (mEq O <sub>2</sub> /kg)	14.18 ± 0.19 <sup>a</sup>	12.42 ± 0.76 <sup>a</sup>	13.95 ± 0.10 <sup>a</sup>	13.35 ± 0.47 <sup>a</sup>
IV (g I <sub>2</sub> /100 g)	100.56 ± 1.10 <sup>a</sup>	70.28 ± 0.56 <sup>d</sup>	95.22 ± 0.95 <sup>b</sup>	89.42 ± 0.89 <sup>c</sup>

Parameters	Origin of milkfish by-product oil			
	Juwana	Semarang	Rembang	Tuban
Totox Value (mEq O <sub>2</sub> /kg)	22.98 ± 0.64 <sup>a</sup>	20.16 ± 0.81 <sup>b</sup>	21.89 ± 0.04 <sup>ab</sup>	21.05 ± 0.49 <sup>ab</sup>
Total SFA (%)	42.34 ± 0.98 <sup>a</sup>	47.12 ± 0.20 <sup>c</sup>	46.17 ± 0.45 <sup>b</sup>	44.40 ± 0.8 <sup>bc</sup>
Total MUFA (%)	24.98 ± 1.40 <sup>a</sup>	27.13 ± 1.25 <sup>b</sup>	27.51 ± 0.80 <sup>b</sup>	26.10 ± 0.06 <sup>ab</sup>
Total PUFA (%)	28.66 ± 1.38 <sup>a</sup>	14.71 ± 1.70 <sup>b</sup>	21.77 ± 1.30 <sup>c</sup>	22.90 ± 1.49 <sup>c</sup>

The AV of milkfish by-product oil, which measures the quantity of free fatty acids produced as a result of hydrolysis damage, may have been affected during the extraction process. However, the AV of the milkfish by-product oils from the 4 regions were not significantly different from one another. The AV of milkfish by-product oil met the criteria set by IFOS™, the International Fish Oil Standards (IFOS), which is less than 3 mg KOH/g of free fatty acids. The peroxide value (PV) is widely used to assess the degree of oil damage. When unsaturated fatty acids react with oxygen, hydroperoxides are formed during lipid oxidation. The findings revealed that the peroxide value of Juwana milkfish by-product oil was the highest among the 4 oils and was significantly different from the others. This difference can be attributed to the higher unsaturated fat content of Juwana milkfish by-product oil, as per the IFOS criteria [32]. The peroxide value is defined as the maximum amount of fish oil that can be consumed and is equal to or less than 5 mEq O<sub>2</sub>/kg. Therefore, a purification process was necessary to reduce the peroxide value of the milkfish by-product oil obtained in this study, as it was still in a crude form.

The anisidine value is an indicator of the quality of fish oil during storage because it reflects the extent of secondary oxidation. The results of this study showed that the anisidine values of milkfish by-product oil from the 4 locations were not statistically significant, and they all met the IFOS criteria for fish oil quality (20 mEq O<sub>2</sub>/kg).

Additionally, the Totox value represents the relationship between the primary and secondary oxidation. The Totox value of oil from the 4 regions was the highest for Juwana milkfish by-product oil and significantly differed from that of the other milkfish by-product oils. The Totox value of the 4 milkfish by-product oils ranged from 20.16 to 22.90 mEq O<sub>2</sub>/kg, which is still within the maximum value set according to the IFOS™ criteria (< 20 mEq O<sub>2</sub>/kg). This is reasonable because the peroxide value of crude milkfish by-product oil remains high.

The iodine value measures the quantity of unsaturated fatty acids in the oil, either in their free form or as esters. The study showed that Juwana milkfish by-product oil had the highest iodine number, followed by Rembang, Semarang and Tuban. This was related to the PUFA and MUFA unsaturated fatty acid content of Juwana milkfish by-product oil, which was higher than that of the others. A higher iodine value indicates that the unsaturated fatty acids absorb more iodine to form saturated compounds. The IFOS™ criteria for iodine number for fish oil are 95 - 118 g I<sub>2</sub>/100 g.

## Conclusions

The use of d-limonene, a biodegradable and minimally toxic agrochemical solvent, has enabled the substitution of conventional petroleum-based solvents for the extraction of fish oils from milkfish by-products. In collaboration with response surface methodology and dual response optimization, UAE has been successfully used to extract oil containing omega-3 fatty acids from milkfish by-products. The influencing variables provided significant main (sonication time and temperature), interaction

(temperature×solvent-to-sample ratio), and quadratic (time×time) effects. The optimal conditions for these variables, which provided 21.59 % extraction yield and 12.50 % omega-3 content, were determined to be 68 min of extraction time at 84 °C using a solvent-to-sample ratio of 3:1 mL/g. The UAE method has proven effective in extracting omega-3 content from milkfish by-products across various growth regions. These findings demonstrate that the UAE method, which incorporates the bio-solvent d-limonene, is a reliable, cost-effective and rapid approach for obtaining higher quantities of omega-3 content from milkfish by-products. However, further research and an increased scale of research are required. In practical terms, it is imperative to study the industrial potential of milkfish oil by examining its physical, chemical and phytochemical properties.

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