



# The Comparison of Some Eco-Friendly and Conventional Methods of Extracting Common Kilka Oil in Terms of Quantity and Quality

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## ABSTRACT

**Aim:** The objective of the current study was to evaluate the effectiveness of various extraction techniques, namely supercritical carbon dioxide (SC-CO<sub>2</sub>), ultrasound, and enzymatic methods, in comparison to the conventional wet reduction method, in terms of oil yield, quality attributes, and fatty acid composition in common kilka oil.

**Materials & Methods:** Mentioned methods were used to extract fish oil. Then, their quality oxidation, including PV, TBA, FFA, and CD, and fatty acid composition were evaluated.

**Findings:** The study's findings revealed that the SC-CO<sub>2</sub> extraction method exhibited the most favorable outcome in terms of extraction yield, achieving a remarkable rate of 89.6%. The lowest oxidation indexes including PV (1.78 ± 0.19 mmol.kg<sup>-1</sup>), TBA (0.54 ± 0.03 mg MA.kg<sup>-1</sup>), FFA (35.49 ± 0.52 mg FFA.g TAG<sup>-1</sup>), and CD (7.61 ± 0.34 %) was found in oil extracted with SC-CO<sub>2</sub> method. The fatty acid profile of oil extracted by SC-CO<sub>2</sub> exhibited higher polyunsaturated fatty acid (PUFA) (29.81 ± 0.27) and lower saturated fatty acid (SFA) (27.64 ± 0.20) and monounsaturated fatty acid (MUFA) (34.78 ± 0.67) than those obtained by the other extraction methods (p > 0.05). The n-3/n-6, PUFA/SFA, and EPA + DHA/C16 ratios were higher for oil extracted by SC-CO<sub>2</sub> than the other three methods.

**Conclusion:** The common kilka oil, extracted through supercritical carbon dioxide (SC-CO<sub>2</sub>), can be regarded as an abundant source of crucial polyunsaturated fatty acids belonging to the n-3 series.

**Keywords:** Supercritical Fluid Extraction; Common Kilka; Omega-3 Fatty Acids; Fish Oil.

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## Introduction

Fish oil is widely acknowledged as a valuable reservoir of long-chain polyunsaturated fats from the n-3 series, notably encompassing eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). Polyunsaturated fatty acids (PUFAs) play a critical role in human nutrition and are, therefore, necessary to be acquired through dietary means<sup>[1,2]</sup>. Extensive research has demonstrated the numerous health advantages associated with omega-3 fatty acids, including enhancing cardiovascular and metabolic health and decreasing susceptibility to certain types of cancer, such as colon, breast, and prostate cancers<sup>[3,4]</sup>. DHA is a prominent fatty acid in the brain and retina, contributing significantly to neural functioning and visual activity<sup>[5]</sup>. On the other hand, EPA serves as a precursor to producing vital anti-inflammatory compounds, including prostaglandins, thromboxanes, and leukotrienes, which play crucial roles in vascular health and exhibit potent antithrombotic and anti-inflammatory properties<sup>[6,7]</sup>. Nutritional guidelines from the International Society for the Study of Fatty Acids and Lipids (ISSFAL) and the American Heart Association now provide recommendations for supplemental intake of EPA + DHA<sup>[6,7]</sup>.

Common kilka (*Clupeonella cultriventris caspia*) is an important fish species in the Caspian Sea (Iran), which contributes about 97% of the total catches<sup>[8]</sup>. Moreover, studies have indicated that common kilka oil is abundantly enriched with long-chain polyunsaturated fatty acids, particularly EPA and DHA, constituting approximately 5.67% and 9.93% of the oil composition, respectively<sup>[9]</sup>. This species is captured to produce fish oil and meal, utilized either in their raw form or as a direct source of human nourishment. Among the various techniques employed for fish oil extraction, the

prevalent approach involves a continuous wet reduction process comprising the stages of cooking, pressing, and centrifugation<sup>[10]</sup>. Other methods have been reported, such as solvent extraction, enzymatic reaction, and ultrasonic extraction<sup>[11]</sup>.

Fish oil extraction by solvent extraction method is widely used and efficient for obtaining essential fatty acids from fish tissues<sup>[12]</sup>. In this process, a solvent, usually hexane, dissolves the fish oil from the fish meal or waste. One advantage of fish oil extraction by solvent extraction method is that it allows for high extraction efficiency and a high yield of fish oil. Additionally, the solvent extraction method can be cost-effective and relatively quick compared to other extraction methods, making it suitable for large-scale production. However, one disadvantage of this method is that solvents may introduce impurities into the fish oil, affecting its quality and purity. Furthermore, the solvent extraction method can be potentially hazardous if proper safety measures are not in place, as some solvents used in the process can be toxic and flammable.

Also, fish oil extraction by an enzymatic process involves using specific enzymes to break down the fish tissues and release the oil from the cells<sup>[4]</sup>. This process is based on the principle of enzymatic hydrolysis, where enzymes such as proteases break down the fish's proteins into smaller peptides and amino acids<sup>[13]</sup>. These enzymes also help to break down the cellular structure and release the oil trapped in the cells. The enzymatic process offers several advantages over traditional methods, such as higher oil yields, reduced processing time, and minimal degradation of the oil's nutritional profile<sup>[14]</sup>. Additionally, this process is considered more environmentally friendly as it eliminates the need for organic solvents and reduces waste discharge<sup>[13]</sup>.

Ultrasonic extraction is a modern technique used to extract fish oil [15]. This method involves the application of high-frequency sound waves to break down the fish oil cells and release the lipid content [16]. The ultrasound waves create cavitation bubbles in the liquid, causing the cell walls to rupture and releasing the oil into the surrounding solvent. This process is highly efficient, as it reduces extraction time and increases the yield of fish oil [15]. Additionally, ultrasonic extraction offers numerous advantages, such as being a green and sustainable method, preserving the quality of the oil by reducing oxidative damage and allowing for the extraction of a wide range of bioactive compounds in the fish oil. Overall, ultrasonic extraction is a promising technique for fish oil extraction, offering high yields and superior quality of the extracted oil [16].

Over the past few years, supercritical fluid extraction techniques have been used for high-quality fish oil extraction [17,18]. Fish oil extraction by supercritical fluid extraction (SFE) techniques is highly efficient and environmentally friendly, with numerous advantages. In this process, supercritical fluids, usually carbon dioxide, are used as solvents to extract the oil from fish. The supercritical fluid behaves like a gas but has the density of a liquid, allowing it to penetrate the fish tissues and dissolve the oil with minimal damage or degradation. SFE techniques offer several benefits over traditional methods, such as shorter extraction times, higher selectivity, and the ability to extract a wide range of bioactive compounds [10,17,19]. Moreover, since supercritical fluids are non-toxic and non-flammable, they provide a safer alternative to conventional organic solvents, making this method efficient and eco-friendly [18,19]. While numerous studies have examined the nutritional advantages of fish oil consumption, limited research has focused

on the composition and quality of fish oil obtained through various extraction methods. Consequently, this investigation aimed to assess the impact of novel oil extraction techniques on the yield, quality, and fatty acid composition of common kilka fish oil.

## Materials & Methods

### Chemicals/Enzyme

Chloroform, ethanol, methanol, hexane, isooctane, pyridine, anhydrous sodium sulphate, and 2-Thiobarbituric acid were purchased from Merck (Darmstadt, Germany). Palmitic acid and Fatty acid methyl ester (FAME) standards were obtained by Sigma (St. Louis, MO, USA). Alcalase 2.4 L (serine protease) consisted of a food-grade enzyme from *Bacillus licheniformis* (Novozymes A/S, Bagsvaerd, Denmark). Carbon dioxide with a purity of 99.99% was obtained from ARAD GAS GOSTAR CO. (Tehran, Iran). All chemical reagents were of analytical grade.

### Sample preparation

Fresh Common kilka (*Clupeonella cultriventris caspia*) used in this research were collected from the special fishing region at Babolsar City (Mazandaran Province, Iran) in March 2021. The sample was kept between crushed ice in a Styrofoam box and transferred directly to the laboratory within 1 h. Once received in the laboratory, whole fish were rinsed with cold water, drained, and minced in an industrial mixer (1–3 mm diameter). Part of the sample was stored at -18 °C and freeze-dried (Operon, FDU-7012, Gyeonggi-do, South Korea) at 65 °C and 0.03 bar vacuum pressure. Another part of the minced fish sample was divided into plastic containers and frozen at -18 °C until analyzed. The dried samples were ground in a grinder (A11 Basic, IKA Instruments, Germany) for 5-10 seconds and then stored in a glass bottle at -18 °C until the

experiments were performed.

### Oil extraction methods

Oil was procured from the minced sample using four distinct methods: wet reduction, enzymatic extraction, ultrasound-assisted extraction (UAE), and supercritical fluid extraction (SFE). The experimental conditions employed for each method are outlined in Fig 1.

#### Wet reduction extraction method

A wet reduction extraction was conducted following the method of Hao et al. [20]. In this study, a batch of fish mince weighing 100 g was combined with 200 mL of water. Subsequently, the resulting mixtures were subjected to a water bath, specifically the WNB 14 model, by Memmert GmbH Co., Germany. The water bath was set at a temperature of 85 °C, and the mixtures were stirred every 10 min for 60 min. Following the completion of the heating process, the mixtures were centrifuged using a Universal 320R centrifuge from Tuttlingen, Germany. Centrifugation was carried out at a speed of 5000×g for 10 min, all conducted at a

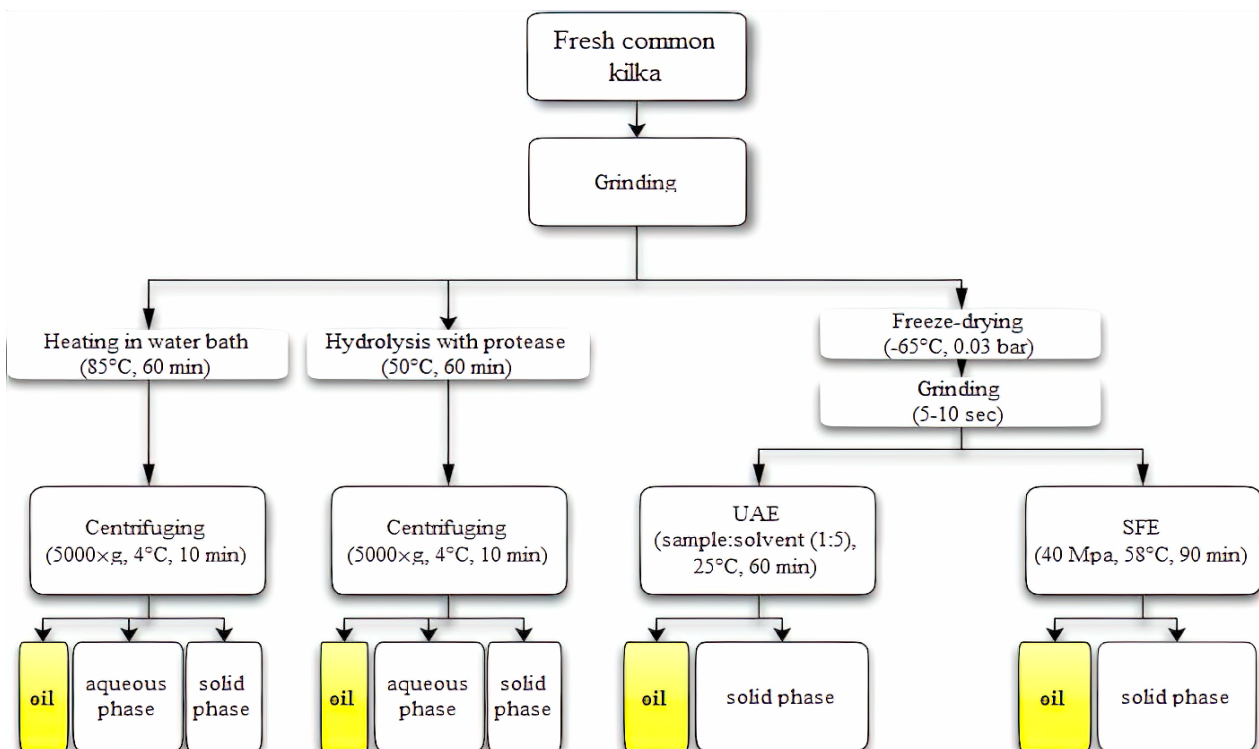
temperature of 4 °C. This procedure allowed for crude oil extraction, which accumulated as a top layer in the centrifuge tubes.

#### Enzymatic extraction method

An enzymatic extraction was conducted following the method of Ovissipour et al. [21] A 50 g of fish mince was homogenized in deionized water (1:2 w/v) using an Ultra-Turrax T25 homogenizer (IKA Instruments, Germany). The mixtures were heated in a shaking incubator (Comecta, SA, Barcelona, Spain) at a specific temperature of 50 °C for 60 min while continuously agitated at 150 rpm. Subsequently, enzymatic hydrolysis was initiated by adding 1% Alcalase 2.4L concerning the wet weight of the raw material. The resulting hydrolysates were cooled and centrifuged at 5000×g for 10 min at 4 °C. The supernatant was collected and preserved at -18 °C following centrifugation until further analysis.

#### Ultrasound-assisted extraction method

The experimental procedure for ultrasound-assisted extraction followed



**Figure 1)** The experimental conditions of fish oil extraction methods.

the methodology proposed by Bruno Siewe et al. [22] with some modifications. An ultrasonic water bath (Soner, 206H, Taiwan; 53 kHz; 180 W) with a usable capacity 6l (the internal dimensions: 30.0 × 15.0 × 15.0 cm) was employed for the extraction process. An 10 g of freeze-dried sample was placed in a 250 mL glass Erlenmeyer flask containing 50 mL ethanol. The mixture was immersed in an ultrasonic bath for 60 min at 25 °C. Subsequently, the mixture was extracted, followed by filtration and application of a rotary evaporator (RV 10 digital, IKA Instruments, Germany) to remove the solvent and obtain the oil.

### **Supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction method**

The supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction method was carried out in an SFE apparatus (Lab SFE 100 mL, Separex, Champigneulle, France) following the method reported by Sarker et al. [23]. A 40 g of freeze-dried sample was loaded into a 100 mL extraction vessel of SFE apparatus. The extraction was performed under the following condition: CO<sub>2</sub> and ethanol (as a co-solvent) at a flow rate of 2 mL.min<sup>-1</sup> (1.9 mL CO<sub>2</sub> and 0.1 mL ethanol.min<sup>-1</sup>, v.v<sup>-1</sup>), 40 MPa, and 58 °C for 90 min. The CO<sub>2</sub> with the dissolved fish oil is then passed into a separator, where the pressure is reduced. This reduction in pressure causes the CO<sub>2</sub> to revert to its gaseous state while the fish oil precipitates out of the CO<sub>2</sub> and separates from it. The fish oil is collected in a separate container, while the CO<sub>2</sub> is recycled for reuse in the extraction process. The collected fish oil may undergo further processing, such as filtration or purification, to remove any impurities or unwanted components.

### **Fatty acid determination**

Extracted crude oils by different extraction methods were transesterified into fatty acid methyl esters (FAME) as described by Cert et al. [24]. Fatty acid methyl esters (FAME)

were detected utilizing gas chromatography (Varian CP3800, Netherlands) equipped with a flame ionization detector and a fused silica capillary column (60 m × 0.32 mm SGE BPX70). The column temperature increased incrementally at 2°C per min, ranging from 180 to 250 °C. The detector and injection port were maintained at 280 and 240 °C, respectively. The carrier gas employed was helium, with a purity of 99.99%, while the make-up gas consisted of Nitrogen, with a purity of 99.9%. A split ratio of 1:100 was utilized. Identifying fatty acid components was accomplished by comparing their retention times to established standards. The Chromatography Workstation software was used to integrate peak areas, which were subsequently expressed as a percentage of total lipids.

### **Extraction yield determination**

The total yield was calculated as extractability (%) based on the method reported by Sahena et al. [25]. Extractability is based on the mass of extracted oil relative to the total mass of oil in the sample.

### **Determination of Peroxide value**

Peroxide value (PV) was measured according to the method described by Naghdi et al. [26]. PV was measured based on titration with 0.01 N sodium thiosulphate solution (1.0 % soluble starch as the indicator) of the oil, suitably diluted with an acetic acid-chloroform solution (3:2 v.v<sup>-1</sup>). The result was expressed as mmol.kg oil<sup>-1</sup>.

### **Determination of Thiobarbituric acid value**

Thiobarbituric acid value (TBA) was determined by the method of Naghdi et al. [26]. The measurement of malondialdehyde (MDA) was conducted through a spectrophotometric approach that relies on the formation of a pink complex after the reaction between MDA and 2-thiobarbituric acid (TBA). The quantification of MDA was expressed as milligrams of malondialdehyde per kilogram of oil.

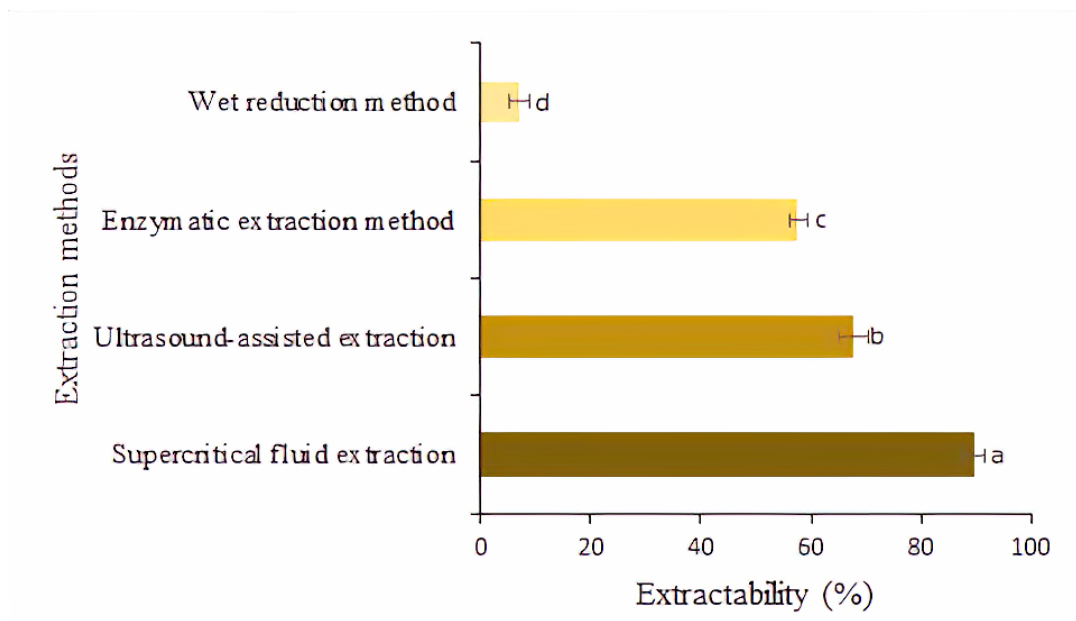


Figure 2) The extractability of different extraction methods.

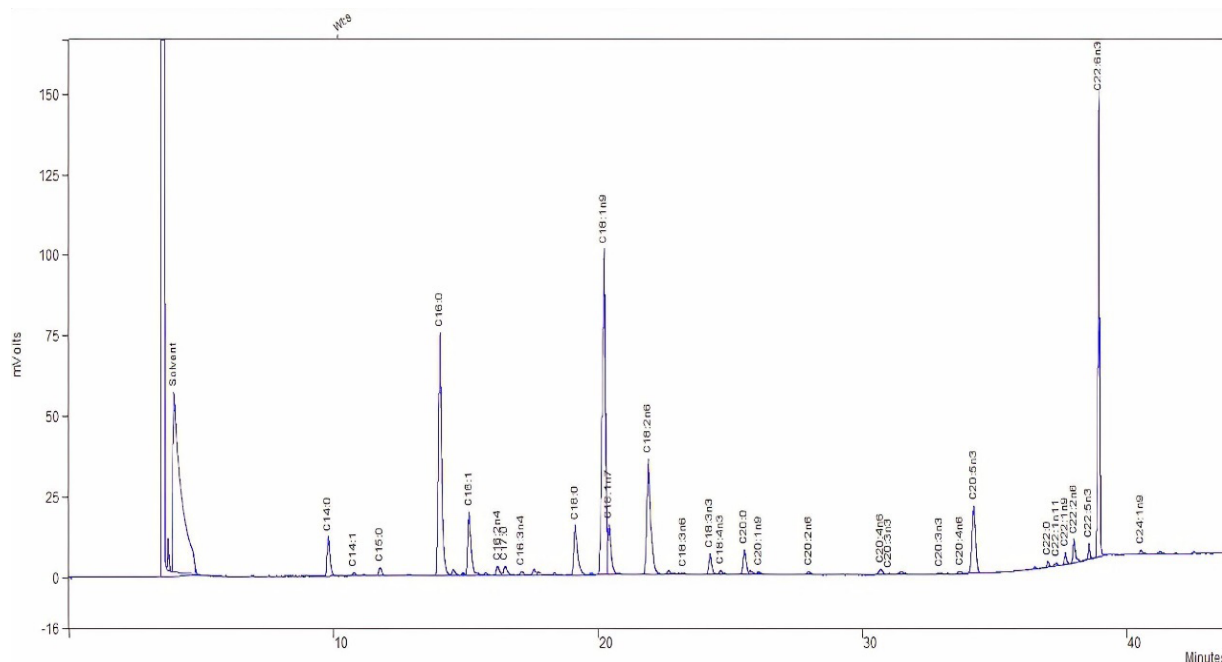


Figure 3) Chromatogram of the fatty acid composition of common kilka oil extracted by the SC-CO<sub>2</sub> extraction method.

**Determination of Conjugated diene value**

The conjugated diene (CD) value was measured spectrometrically according to the method proposed by Zuta et al. [27]. The sample (0.05 g) was diluted with isooctane, and the absorbance of the solution was determined against a blank made up of isooctane at 233 nm. The CD value was

calculated using the equation  $CD = A / (C \times L)$ , where A is the absorbance of the sample at 233 nm, C is the concentration of the sample (g/100 mL), and L is the length of the measuring cell (cm).

**Determination of Free fatty acid**

Free fatty acid (FFA) content of samples was determined according to the

method described by Senphan et al. [28]. A predetermined quantity of the extracted oil (0.1 g) was combined with 5 mL of isooctane and vigorously agitated to dissolve the sample. Subsequently, 1 mL of a 5% (w.v<sup>-1</sup>) cupric acetate-pyridine reagent was added to the mixture. The cupric acetate-pyridine reagent was prepared by dissolving 5 g of cupric acetate in 100 mL of distilled water, then filtration and adjusting the mixture's pH to 6.0-6.2 using pyridine. The resulting mixture was vigorously shaken for 90 seconds using a vortex (MS3 basic, IKA Instruments, Germany) and allowed to settle for 20 seconds. The absorbance of the upper layer was measured at 715 nm. A standard curve was generated using palmitic acid (C16:0) at 0 to 50 μmol concentrations of 5 mL<sup>-1</sup> in isooctane. The outcome was reported as milligrams of free fatty acids per gram of triacylglycerol (TAG).

#### Quantification of EPA and DHA

The quantification of long-chain omega-3 polyunsaturated fatty acids (EPA and DHA) was performed utilizing methyl tricosanoate (23:0) as an internal standard, following the method developed by Ozogul et al. [7]. The fatty acid contents were calculated in milligrams per gram of total lipids using the equation provided below:

$$\text{EPA or DHA (mg.g}^{-1}\text{)} = (A_X \times W_{IS} \times CF_X / A_{IS} \times W_S \times 1.04) \times 1000 \quad \text{Eq. (1)}$$

The peak area (EPA or DHA) is denoted as AX in this study, while AIS indicates the peak area of the internal standard (methyl tricosanoate). CFX is used as a theoretical correction factor for EPA or DHA. The weight of the internal standard added to the sample (mg) is referred to as WIS, and WS represents the weight of the sample (mg). Additionally, a conversion factor of 1.04 is applied in order to express the results as mg fatty acid/g oil rather than methyl esters.

#### Statistical analysis

The statistical software SPSS (version 16, Chicago, IL, USA) was employed for statistical analysis and data manipulation. The data were represented as mean values plus or minus the standard deviation and were subsequently subjected to analysis of variance (ANOVA). After conducting the one-way ANOVA test, a comparison of means was conducted using Duncan's test. The significance of differences amongst means was determined at a 95% confidence level.

#### Findings

##### Extraction yield and quality evaluation of extracted oil

The results obtained from the analysis of oil yields and quality attributes of different extraction techniques are presented in Fig 2. It was observed that the oil yield obtained through distinct extraction methods ranged from 7.04 to 89.60%. As can be seen from extraction results (Figure 2), extraction of oil with SC-CO<sub>2</sub> in this condition at 40 MPa and 58 °C by 89.60 % extractability presented the most efficient method for obtaining fish oil than other extraction methods, including ultrasound-assisted extraction, enzymatic extraction method, and wet reduction method were in the following ranks of extractability (p < 0.05). The results of FFA, PV, TBA, and CD values for the oils extracted by different methods are represented in Table 1.

##### Fatty acid composition

The fatty acid profiles of oil obtained through various extraction methods have been provided in Table 2. Statistical analysis did not reveal any substantial difference in the composition of fatty acids among the different extraction methods (p > 0.05). The primary identified fatty acids were C18:1n9 (oleic), C16:0 (palmitic), C22:6n3 (DHA), C20:5n3 (EPA), C16:1 (palmitoleic) and C18:0 (stearic) which constitute

**Table 1)** Characteristics of common kilka oil extracted by different methods.

Extraction methods	PV (mmol.kg <sup>-1</sup> )	TBA (mg MA.kg <sup>-1</sup> )	CD (%)	FFA (mg FFA.g TAG <sup>-1</sup> )
Supercritical fluid Extraction	1.78 ± 0.19 <sup>a</sup>	0.54 ± 0.03 <sup>a</sup>	7.61 ± 0.34 <sup>a</sup>	35.49 ± 0.52 <sup>a</sup>
Ultrasound-assisted Extraction	2.40 ± 0.20 <sup>b</sup>	1.80 ± 0.05 <sup>c</sup>	12.01 ± 0.51 <sup>c</sup>	166.48 ± 4.07 <sup>c</sup>
Enzymatic extraction method	6.10 ± 0.14 <sup>c</sup>	0.84 ± 0.06 <sup>b</sup>	12.75 ± 0.14 <sup>d</sup>	40.90 ± 0.60 <sup>b</sup>
Wet reduction method	2.25 ± 0.23 <sup>b</sup>	0.78 ± 0.04 <sup>b</sup>	8.48 ± 0.24 <sup>b</sup>	41.76 ± 1.45 <sup>b</sup>

Values are means ± standard deviations. Values in the same line labeled with a different letter are significantly different (p < 0.05).

around 74% of the total fatty acids. This is in parallel with the results of Pirestani et al. [29]. Palmitic acid was the most abundant saturated fatty acid (SFA), accounting for about 60% of total SFA (Table 2). Oleic and Palmitoleic acid were identified as the predominant monounsaturated fatty acids (MUFA) in the lipid extracted. In terms of polyunsaturated fatty acids (PUFA), it was found that DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid) exhibited the highest levels of abundance. Fig 3 shows the chromatogram of the fatty acid composition of common kilka oil extracted by the SC-CO<sub>2</sub> extraction method. Table 3 shows the n-3/n-6, PUFA/SFA, and EPA+DHA/C16 ratios in oils extracted with different methods. The ratios of n-3/n-6 and PUFA/SFA have been proposed as valuable indicators for assessing and comparing the nutritional qualities of fish oils. In this table, the PUFA/SFA and n-3/n-6 ratios in common kilka oil extracted with several methods were between 1.51 to 1.72 and 6.98 to 7.06, respectively.

## Discussion

Oxidative deterioration of polyunsaturated lipids (oxidative rancidity) is one of the most important spoilage mechanisms. Therefore, quality indicators such as PV, TBARS, CD,

and FFA may monitor oxidative changes in marine oils. Among these indicators, FFA is particularly important. FFA formation is usually associated with lipase activity and can be promoted by water and heat [28]. The findings demonstrated that the application of ultrasound-assisted extraction (UAE) yielded the highest free fatty acid (FFA) content. Additionally, the results indicated that the UAE had the highest FFA content (p < 0.05). This suggests that ultra-sonication can accelerate the deterioration of PUFA. Sarker et al. [23] reported that it is possible to recover up to 85% of the total oil contained in African Catfish viscera by using SC-CO<sub>2</sub> at a pressure of 40 MPa and a temperature of 57.5 °C. In a study conducted by Sahena et al. [25], different techniques of supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction were investigated to assess their impact on the extraction of oil from the ground skin of Indian mackerel (*Rastrelliger kanagurta*). They found that the oil yields with modifier (53.2%), soaking (52.8%), and pressure swing (52.3%) (dry basis) at 35 MPa and 75 °C were similar. In their study, Hao et al. [20] investigated the impact of various extraction techniques on sturgeon oil's composition and storage stability. The researchers found that the most efficient method for obtaining a high oil recovery



**Table 2)** Fatty acids profile (%) of common kilka oil extracted by different methods

Fatty acids	Wet reduction	Enzymatic extraction	Ultrasound-assisted extraction	Supercritical fluid extraction
C14:0	2.86 ± 0.01	2.94 ± 0.03	3.47 ± 0.09	2.82 ± 0.52
C15:0	0.63 ± 0.01	0.63 ± 0.01	0.67 ± 0.02	0.59 ± 0.01
C16:0	17.36 ± 0.10	17.38 ± 0.10	18.07 ± 0.42	17.30 ± 0.31
C17:0	0.75 ± 0.01	0.74 ± 0.01	0.79 ± 0.04	0.75 ± 0.01
C18:0	3.97 ± 0.01	3.79 ± 0.09	3.59 ± 0.05	3.63 ± 0.45
C20:0	2.25 ± 0.02	2.48 ± 0.02	2.40 ± 0.04	2.31 ± 0.24
C22:0	0.28 ± 0.01	0.26 ± 0.02	0.25 ± 0.02	0.22 ± 0.01
∑ SFA <sup>*</sup>	28.13 ± 0.11 <sup>a</sup>	28.23 ± 0.16 <sup>a</sup>	29.25 ± 0.63 <sup>b</sup>	27.64 ± 0.20 <sup>a</sup>
C14:1	0.31 ± 0.01	0.33 ± 0.01	0.35 ± 0.01	0.32 ± 0.02
C16:1	5.30 ± 0.12	5.54 ± 0.07	6.08 ± 0.16	5.57 ± 0.41
C18:1n9	26.24 ± 0.21	24.53 ± 0.35	25.01 ± 0.69	24.23 ± 0.46
C18:1n7	2.87 ± 0.06	3.90 ± 0.52	3.60 ± 0.39	3.01 ± 0.29
C20:1n9	0.12 ± 0.01	0.11 ± 0.01	0.06 ± 0.01	0.24 ± 0.03
C22:1n11	0.18 ± 0.01	0.16 ± 0.01	0.15 ± 0.02	0.19 ± 0.01
C22:1n9	0.59 ± 0.01	0.55 ± 0.01	0.50 ± 0.02	0.57 ± 0.01
C24:1n9	0.39 ± 0.01	0.61 ± 0.02	0.57 ± 0.10	0.62 ± 0.02
∑ MUFA <sup>**</sup>	36.01 ± 0.21 <sup>b</sup>	35.75 ± 0.19 <sup>b</sup>	36.33 ± 0.41 <sup>b</sup>	34.78 ± 0.67 <sup>a</sup>
C16:2n4	0.76 ± 0.01	0.74 ± 0.03	0.67 ± 0.01	0.62 ± 0.02
C16:3n4	0.29 ± 0.01	0.30 ± 0.01	0.34 ± 0.01	0.31 ± 0.01
C18:2n6	1.68 ± 0.02	1.70 ± 0.01	1.62 ± 0.04	1.57 ± 0.04
C18:3n6	0.16 ± 0.01	0.16 ± 0.01	0.18 ± 0.02	0.17 ± 0.01
C18:3n3	1.47 ± 0.01	1.51 ± 0.01	1.47 ± 0.01	1.47 ± 0.10
C18:4n3	0.24 ± 0.01	0.22 ± 0.01	0.20 ± 0.01	0.25 ± 0.01
C20:2n6	0.24 ± 0.01	0.21 ± 0.01	0.22 ± 0.01	0.24 ± 0.01
C20:4n6	0.43 ± 0.01	0.40 ± 0.01	0.51 ± 0.01	0.58 ± 0.01
C20:3n3	0.16 ± 0.01	0.15 ± 0.01	0.18 ± 0.01	0.21 ± 0.01
C20:4n3	0.23 ± 0.01	0.22 ± 0.01	0.15 ± 0.01	0.19 ± 0.01
C20:5n3	5.57 ± 0.18	5.42 ± 0.02	5.33 ± 0.04	5.50 ± 0.14
C22:2n6	0.96 ± 0.01	0.99 ± 0.01	0.74 ± 0.03	0.99 ± 0.02
C22:5n3	0.73 ± 0.01	0.76 ± 0.01	0.73 ± 0.03	0.83 ± 0.02
C22:6n3	16.18 ± 0.74	16.08 ± 0.16	14.96 ± 0.47	16.81 ± 0.22
PUFA <sup>***</sup> ∑	29.16 ± 0.86 <sup>b</sup>	28.91 ± 0.15 <sup>b</sup>	27.36 ± 0.59 <sup>a</sup>	29.81 ± 0.27 <sup>b</sup>

Values are means ± standard deviations. Values in the same line labeled with a different letter are significantly different ( $p < 0.05$ ).

\*∑ SFA: sum of saturated fatty acids.

\*\*∑ MUFA: sum of monounsaturated fatty acids.

\*\*\*∑ PUFA: sum of polyunsaturated fatty acids.

**Table 3)** Lipids classes (% of total fatty acids) of common kilka oil extracted by different methods.

Extraction methods	n3/n6	PUFA/SFA	EPA + DHA/C16
Supercritical fluid extraction	7.06 ± 0.07 <sup>a</sup>	1.72 ± 0.02 <sup>b</sup>	1.29 ± 0.01 <sup>b</sup>
Ultrasound-assisted extraction	7.01 ± 0.05 <sup>a</sup>	1.51 ± 0.06 <sup>a</sup>	1.12 ± 0.04 <sup>a</sup>
Enzymatic extraction method	6.98 ± 0.09 <sup>a</sup>	1.66 ± 0.01 <sup>b</sup>	1.23 ± 0.01 <sup>b</sup>
Wet reduction method	7.03 ± 0.13 <sup>a</sup>	1.67 ± 0.04 <sup>b</sup>	1.25 ± 0.04 <sup>b</sup>

Values are means ± standard deviations. Values in the same column labeled with a different letter are significantly different ( $p < 0.05$ ).

rate was through supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction. Ultrasound-assisted extraction has been considered an innovative and fast extraction method. UAE in a liquid medium uses acoustic cavitation to cause enhancement of contact between the solvent and the solid matrix [11]. Results showed that ultrasound-assisted extraction significantly increased the percentage of oil recovery compared with the other non-SFE methods ( $p < 0.05$ ). However, UAE gave poor extractability results compared to the SFE method ( $p < 0.05$ ). Xhaxhiu and Wenclawiak [24] studied the extraction efficiency of the orange peel essential oil using SC-CO<sub>2</sub> and ultrasonic. According to their report, a higher essential oil content was observed when utilizing Supercritical Fluid Extraction (SFE) compared to Ultrasound-Assisted Extraction (UAE). The low yield achieved with the wet reduction method can be attributed to the protein matrix likely denatured in the cooking process, leading to a tightly packed structure and prevention of oil release [25]. Alternatively, this lower percentage of wet reduction efficiency was obtained probably due to the emulsifying effect of fish proteins [14]. However, the wet reduction method can be observed to generate oil and a wet solid phase, in which a high amount of oil remains in the wet solid [14]. The solid in the statement can be used as

a potential raw material source for defatted fish meal production. In the enzymatic method, the proteins present in the solid, such as lipoproteins and protein-lipid complexes, are hydrolyzed using a protease enzyme during the enzymatic reaction. This process helps in separating the lipids from the proteins, allowing for the production of defatted fish meal.[23] As a result, enzymatic extraction can enhance extraction efficiency and facilitate oil separation. Generally, FFA formed was likely prone to oxidation. These results were coincidental with the increased lipid oxidation as indicated by the PV, TBA, and CD values. As shown in Table 1, the oil obtained by SFE had significantly lower FFA, PV, TBA, and CD values than those obtained by the other extraction methods ( $p < 0.05$ ). Hydroperoxide formation is influenced by heat, Oxygen, and other factors such as light, metal, and fatty acid composition [23]. Primary oxidation products (hydroperoxides) are unstable and decomposed to reactive carbonyl compounds determined by the TBA test [31]. Therefore, the increases in TBARS were coincidental with the decreased PV in oil obtained by UAE and the wet reduction method. TBARS of lipids extracted by different methods had the same trend as FFA.

Following the quality standards the Global Organization for EPA and DHA Omega-3 set,

fish oils intended for human consumption should adhere to a maximum peroxide value (PV) of 5 meq O<sub>2</sub>.kg<sup>-1</sup> lipid content.<sup>[32]</sup> It has been proposed that the maximum acceptability limit for FFA of crude fish oil is 4%<sup>[32]</sup>. The quality characteristics of oil extracted with SC-CO<sub>2</sub> were below these recommended amounts, indicating good quality.

Marine fish oils have been documented to exhibit significant quantities of EPA and DHA.<sup>[33,34]</sup> Nonetheless, slight disparities were noted in the fatty acid composition of oils extracted using varying techniques. In general, SC-CO<sub>2</sub> is likely to perform better on PUFA extraction. Fig 3 shows the fatty acid composition of common kilka oil extracted by the SC-CO<sub>2</sub> extraction method. When SC-CO<sub>2</sub> was used for extraction, the ratios of SFA and MUFA were lower, and that of PUFA was a little higher ( $p > 0.05$ ). These values are similar to the reported results by Rubio-Rodriguez et al.<sup>[14]</sup> and Hao et al.<sup>[20]</sup>. They documented that a high amount of PUFA could be obtained with the oil extracted by SC-CO<sub>2</sub>. In addition, Rubio-Rodriguez et al.<sup>[14]</sup> reviewed studies on producing omega-3 polyunsaturated fatty acid concentrates and concluded that SC-CO<sub>2</sub> is a suitable method for producing omega-3 oil. Similar values for the n-3/n-6, PUFA/SFA, and EPA+DHA/C16 ratios were obtained by Pirestani et al.<sup>[29]</sup>. The PUFA/SFA ratio was more than the minimum value (0.45) of the PUFA/SFA ratio recommended by Sahari et al.<sup>[34]</sup>. This is because lipids with higher ratios are considered biologically more critical. However, the World Health Organization recommends that the total daily diet n-3/n-6 ratio should not be less than 0.2 in the total human diet<sup>[29,35]</sup>. The augmentation of the n-3/n-6 ratio within the human diet assumes paramount importance as a protective factor against specific categories of neoplastic conditions and cardiovascular ailments, such as cancers and coronary heart diseases.<sup>[36]</sup>

There is a proposition that the EPA + DHA/C16 ratio is a highly effective indicator for evaluating the potential of lipid oxidation.<sup>[9]</sup> As shown in Table 3, for SFE, the ratio of EPA + DHA/C16 was a little higher ( $p > 0.05$ ), which is more susceptible to lipid oxidation.

## Conclusion

In conclusion, the study found that extracting common kilka oil using supercritical carbon dioxide (SC-CO<sub>2</sub>) was the most effective method in terms of both oil yield and quality characteristics. The SC-CO<sub>2</sub> method resulted in the highest extraction yield (89.60%) and the lowest oxidation indexes compared to the other extraction methods. Additionally, the oil extracted using SC-CO<sub>2</sub> contained a good content of polyunsaturated fatty acids (PUFA), saturated fatty acids (SFA), and monounsaturated fatty acids (MUFA). This suggests that common kilka oil extracted by SC-CO<sub>2</sub> is a rich source of essential polyunsaturated fatty acids, specifically of the n-3 series.

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## Ethical permissions

The authors of this study approve sending it to ECOPERSIA journal and declare that this study is not under revision in any other scholarly journals at the time of submission to the journal and will not be sent to any other scholarly journal during the revision at the journal until the definite answer about it. The authors chose Dr. Masoud Rezaei (Second author) as the corresponding author and delegated all the responsibility of the article to her regarding following the relation with ECOPERSIA.

## Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this study.

## Author Contribution

Behrouz Karim: Investigation, Methodology, Formal Analysis, Writing - Original Draft. Masoud Rezaei: Supervision, Project administration, Conceptualization, Resources, Writing - Review & Editing. Nader Bahramifar: Investigation, Review & Editing. Shahab Naghdi: Writing - Review & Editing.

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