# The effects of refining steps on Kilka (*Clupeonella delicatula)* fish oil quality

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**Abstract**

Kilka fish are known as the main industrial and pelagic species of Iran that are captured abundantly in the southern Caspian Sea. This study was conducted to survey the effectiveness of refinement steps on Kilka (*Clupeonella delicatula*) crude oil quality and fatty acid profiles. Neutralization, bleaching, winterization and deodorization were performed on crude oil with deodorization done under two different conditions of A: at 180˚C for 120 min and B: at 140˚C for 240 min. During refinement, peroxide value (PV) significantly decreased from 7.66 meq/kg in crude oil to 0.5 in deodorized oil A and to 0.21 in deodorized oil B ($p$<0.05). Significant reduction in free fatty acids (FFA) was recorded from 0.86% oleic acid in crude oil to 0.15% in deodorized oil A and to 0.33% in deodorized oil B ($p$<0.05). N-3 fatty acids content and fatty acids composition were determined by GC-FID and n-3 fatty acids content increased from 18.74% in crude oil to 21.58% in deodorized oil A and to 21.75% in deodorized oil B. The red color in oil significantly decreased from 5.4 to 0.4 and 0.5 in deodorized oil A and deodorized oil B, respectively ($p$<0.05). Results confirm that refinement steps improved oil quality and raised n-3 PUFA concentration and n-3/n-6, properly. Deodorization at 180˚C for 120 minutes (A) was more effective than deodorization at 140˚C for 240 minutes (B) in removing off flavors.

**Keywords:** *Clupeonella delicatula*, Fish oil, Refinement, Deodorization, N-3 fatty acids, Oxidation

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**Introduction**

Fish oil is known as the main dietary origin of long chain polyunsaturated fatty acids (PUFA) of omega-3. The major omega-3 PUFAs in fish oil include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Health benefits of EPA and DHA is the predominant cause of much attention to fish oil in scientific and industrial communities (Kolanowski and Weiβbrodt, 2007; Crexy *et al.*, 2010). DHA is a major fatty acid in the membrane of brain and retinal cells and plays an important role in infant growth during the development of the nervous system in early life. In addition, the beneficial effects of EPA intake is well demonstrated for prevention of cardiovascular diseases (Boran *et al*., 2006; Mazza *et al*., 2007; Murphy *et al.*, 2007; Wang *et al*, 2011).

The composition of fish oil varies not only with the type of fish involved, but also with factors including the season, geographic location and the diet of the fish (Allen, 1995). *Clupeonella* fish is named Kilka in Iran and is captured abundantly in the southern coast of the Caspian Sea. The catch volume of Kilka in Iran was 27110 tons in 2010, however the number of Kilka fish in the Caspian Sea has reduced in recent decades due to the invasion of *Mnemiopsis leidyi.* About 95% of captured Kilka fish is processed in fish meal plants. The fish oil obtained in these plants is a by-product of low commercial value. *C. delicatula* (with 5.5-11% oil) contains a higher percentage of oil than other species of Kilka like *C. grimmi* and *C. engrauliformis*. *C.delicatula* was the origin of the crude fish oil used in this study (Shojaei *et al*., 2001; Salmani, 2008; Iranian Fisheries Organization, 2011). Kilka fish oil can be considered as a suitable source of n-3 PUFA, especially DHA (Salmani, 2008). Crude oils require to be refined to meet the standards of quality for human consumption. Hence, refining steps including neutralization, bleaching and deodorization are indispensable (Ackman, 2005; Crexy *et al*., 2010; Noriega-Rodriguez *et al*., 2010). Neutralization is carried out with caustic soda to reduce FFA, and bleaching removes colour, oxidation products and trace metals. Deodorization by vacuum distillation removes aldehydes, ketones, alcohols, residual FFA and other compounds. Also, winterization can be used to increase PUFA concentration (Ackman, 2005; Kolanowski and Laufenberg, 2006; Crexy *et al.*, 2010).

PUFA and especially highly unsaturated EPA and DHA are very susceptible to oxidation. Consequently, this can cause rancidity and the fishy off flavor in fish oil. High temperature in the deodorization step may cause degradation of PUFA in fish oil, producing trans isomers, free radicals and polymers with an obvious loss in nutritional value. On the other hand, it is necessary to provide a required temperature to evaporate unacceptable volatile compounds (Vinter, 1995; Fiori *et al*., 2012).

Noriega-Rodriguez *et al*. (2010) showed that the refining process used on Sardine fish oil improved the quality of the oil by decreasing PV and FFA content and made a notable progress in color with no considerable difference in the fatty acid composition. According to the Shojaei *et al.* (2001), crude Kilka oil consists of 16.7% omega-3 fatty acids (DHA 10.76% , EPA

2.43% and Linolenic acid 3.49%) and the refining process had no effect on omega-3 fatty acids content of the oil. The present study was aimed to survey the quality and fatty acid profile changes of *Clupeonella delicatula* fish oil during refining under two different deodorizing situations of $D_A$; 180˚C for 120 min and $D_B$; 140˚C for 240 min.

## Materials and methods

### *Materials*

Crude fish oil extracted from *C.delicatula* was provided by Tadbir Co. (Bandar-Anzali, Iran). The fish oil was transported to Tehran in a 5 liter dark sealed container, stored at 4˚ C and kept away from sunlight until processed and analyzed. All of the chemical reagents were analytical grade (Merck, Germany).

### *Refinement*

This process consists of neutralization, washing, drying, bleaching, winterization and deodorization steps. In the neutralization step, 1 kg of crude fish oil was mixed with 24 g of NaOH solution (9.5%, 14 degrees baume) for 15 min, at room temperature and agitated at 300 rpm. Heating up to 65˚C under partial vacuum (9 mmHg) broke the emulsion and separated the soap easily. Oil separation was carried out by centrifuge for 20 min at 4000 g. The next stage, washing was accomplished in five steps by adding 10% water in relation to the oil mass, at 95˚C for a contact time of 10 min and agitation at 500 rpm, under vacuum. The washed oil was dried at 100˚C for 20 min under vacuum (9 mmHg) (Ghavami *et al*., 2003; Crexy *et al*., 2010; Noriega-Rodriguez *et al*., 2010). For bleaching, 3% of acid activated earth (tonsil) was added to oil and heated at 90˚C for 30 min with agitation at 50 rpm, under vacuum (9 mmHg). The mixture was then filtered 3 times in a büchner funnel using Whatman filter paper. Winterization was carried out in three stages in a refrigerated bath with water, alcohol and acetone mixture as refrigerants. At first, nucleation process occurred from 30˚C to 5˚C, with a rate of 0.62˚C/min and agitation at 500 rpm. Second and third stages, crystallization processes were carried out without agitation, from 5˚C to -4˚C at a rate of 2.7˚C/h, and from -4˚C to -5˚C at a rate of 0.25˚C/h, respectively. Later, centrifugation at 7000 g for 20 min was used to separate liquid olein and solid stearin fractions. Finally, deodorization was done in a round bottom balloon with three outlets under vacuum conditions. A continuous stream of water vapour (5% w/w oil) was directly injected to the oil. Deodorization A (known as $D_A$) and deodorization B (known as $D_B$) were performed at 180˚C for 120 min and at 140˚C for 240 min, respectively. Volatile compounds were condensed using a cold trap (0˚C) (Ghavami *et al*., 2003; Crexy *et al*., 2010; Noriega-Rodriguez *et al*., 2010; Pedro *et al*, 2013).

### *Fatty acid composition*

To identify fatty acids, gas chromatography was used for crude, bleached, winterized, and deodorized ($D_A$, $D_B$) oils. Fatty acid composition was determined by preparation of methyl esters according to AOAC method (1990). Gas chromatography (GC) analyses were conducted on a Shimadzu model 14A GC instrument fitted with a

flame ionization detector (FID) and a Restek fused silica capillary column (30 m, 0.25 mm i.d). Oven temperature was held at 180˚C then increased to 235˚C (for 15 min). Injector and detector temperatures were set at 250˚C and 270˚C, respectively. Flow rate of helium carrier gas was 50 ml/min. The standard fatty acid methyl esters (Applied Science and Sigma) were used for the identification of peaks. The areas of the peaks were measured and the relative amounts of the fatty acids were calculated by Waters 730 data module (AOAC, 1990).

*Proxide value (PV)*

The PV method was based on titration according to AOAC 965/33 with a sodium thiosulfate solution of the oil diluted with an acetic acid-chloroform mixture. Results are expressed as milli equivalents/kg oil (Firestone, 1990).

*Free Fatty Acid (FFA)*

FFA determination was performed based on titration according to AOAC 940/28. Samples were dissolved in a mixture of diethylether-ethanol (1:1 V/V). The mixture was titrated with a potassium hydroxide solution (phenolphetalein as an indicator). Results are expressed as percentage of oleic acid (Firestone, 1990).

*Colorimetry*

According to AOCS Cc13e-92, oil color was measured using the loviband tintometer, model F, with 1 inch cells. Results are reported based on red and yellow color units (Firestone, 1994).

*Statistical analysis*

PV, FFA and colorimetry analyses were run in triplicate. Results were statistically analyzed by using SPSS (version16) software. One way analysis of variance (ANOVA) and Duncan's multiple range test ($p<0.05$) were used to analyze the results obtained from all of the tests.

**Results**

PV was measured for six samples of crude, neutralized, bleached, winterized, deodorized ($D_A$ and $D_B$) oils (Fig. 1). The refining process reduced the PV of crude oil to 93.5% and 97.4% for $D_A$ and $D_B$, respectively. In the first step of the process of neutralization, PV decreased significantly from 7.66 to 6.83 (meq/kg) and after bleaching, a significant reduction of PV to 85% was observed ($p<0.05$). Significant reduction of PV occurred during deodorizations A and B ($p<0.05$). PV values of deodorized fish oils A and B were 0.50 and 0.21 meq/kg, respectively. Although there was no significant difference between deodorization A and B ($p<0.05$), the PV in both deodorized oils was below the demand level for human consumption of 5 meq/kg oil established by Codex (Codex Alimentarius Commission, 2013).

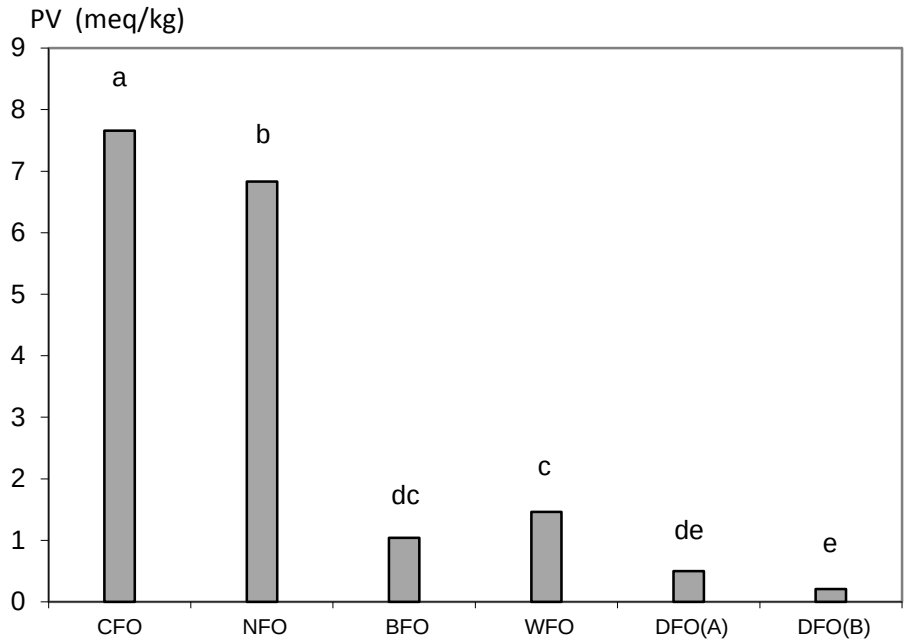


**Figure 1: Peroxide values (PV) of fish oil samples during refinement (meq/kg). CFO: crude fish oil, NFO: neutralized fish oil, BFO: bleached fish oil, $DFO_A$: deodorized fish oil A, $DFO_B$: deodorized fish oil B. Values with similar superscript letters represent no significant difference ($p$<0.05).**

FFA of six samples of crude, neutralized, bleached, winterized, deodorized ($D_A$ and $D_B$) oils is shown in Fig. 2. The most effective step was neutralization which showed 75.6% loss of FFA. Also, a significant reduction of FFA was observed in the bleaching step and there was a significant enhancement of FFA in deodorization B ($p$<0.05). The refining process reduced FFA from 0.86% (based on oleic acid%) in CFO to 0.15% and 0.33% in $DFO_A$ and $DFO_B$, respectively that was less than the maximum limit of oleic acid (1.5%) established by Codex in 2013 for human consumption (Codex Alimentarius Commission, 2013).

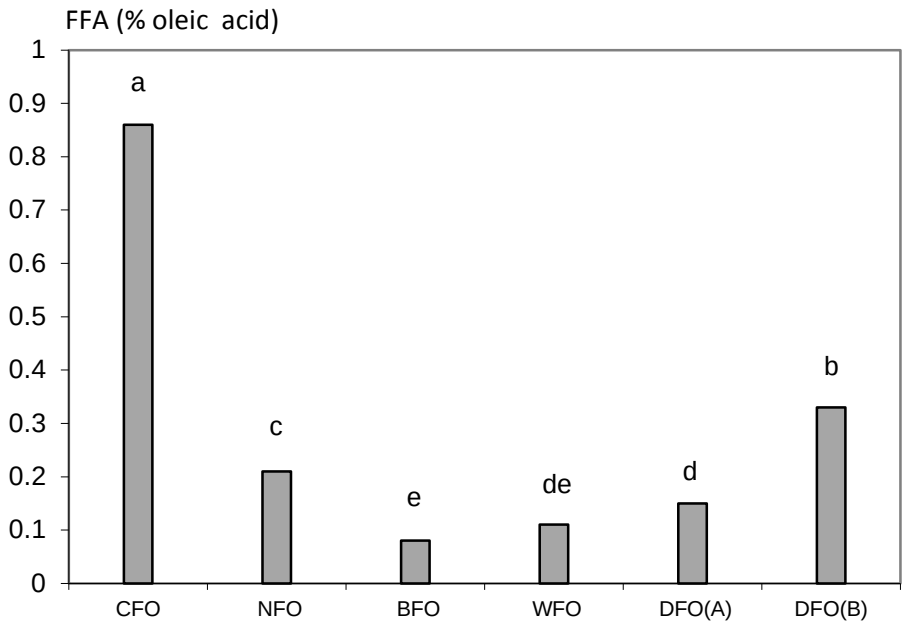


**Figure 2: Free fatty acids (FFA) of fish oil samples during refinement (%oleic acid). CFO: crude fish oil, NFO: neutralized fish oil, BFO: bleached fish oil, $DFO_A$: deodorized fish oil A, $DFO_B$: deodorized fish oil B. Values with similar superscript letters represent no significant difference ($p$<0.05).**

Fatty acids composition: Fatty acid profile of *C. delicatula* fish oil during refinement steps is shown in Table 1. The major fatty acid identified in this fish oil was oleic acid (C18:1) at 34% (g/100g fatty acids) in crude fish oil, followed by palmitic acid (C16:0). PUFA are important nutritionally which constitute around 22% and 24% in crude and refined fish oil, respectively. In this study EPA, DHA and total n-3 fatty acids of crude *C. delicatula* fish oil were found in amounts of 5.20, 10.80 and 19 %, respectively. EPA, DHA and total n-3 fatty acids decreased by 20%, 7%, and 9 %, respectively after bleaching, and increased by 37%, 42% and 36% after the winterization steps, respectively. Effect of deodorizing was compared under two conditions, $D_A$ and $D_B$. Under $D_A$ conditions (180˚C, 120 min), 17% of DHA and 9.4% of total n-3 fatty acids were reduced and no major difference was observed in EPA content. However under $D_B$ conditions (140˚C, 240 min), EPA, DHA and total n-3 were reduced by 9.5%, 9.8% and 8.7%, respectively. Therefore, refined oils produced by $DFO_A$ and $DFO_B$ showed a slight difference in total n-3 fatty acids content.

**Table 1: Fatty acid profiles (%) of *Clupeonella* fish oil samples during refinement.**

| % Fatty acids | Fish Oil Samples | | | | | |
|---|---|---|---|---|---|---|
| | **CFO** | **NFO** | **BFO** | **WFO** | **DFO$_A$** | **DFO$_B$** |
| C14:0 | 3.51 | 1.87 | 3.14 | 2.39 | 2.58 | 2.85 |
| C14:1 n-5 | 0.73 | 0.18 | 0.46 | 0.42 | 0.33 | 0.35 |
| C16:0 | 21.86 | 20.02 | 19.33 | 15.85 | 17.07 | 17.34 |
| C16:1 n-7 | 7.58 | 6.7 | 8.52 | 7.56 | 8.14 | 8.56 |
| C18:0 | 5.26 | 4.52 | 3.4 | 0.44 | 0.0 | 0.09 |
| C18:1 n-9 | 34.25 | 41.16 | 42.03 | 42.55 | 43.84 | 42.66 |
| C18:2 n-6 | 3.1 | 2.52 | 2.85 | 2.78 | 1.97 | 2.08 |
| C18:3 n-3 | 2.74 | 3.11 | 3.31 | 3.9 | 3.88 | 3.75 |
| C20:0 | 2.53 | 2.29 | 2.28 | 2.66 | 2.74 | 2.64 |
| C20:5 n-3(EPA) | 5.2 | 5.43 | 4.31 | 5.9 | 6.07 | 5.35 |
| C22:6 n-3(DHA) | 10.8 | 10.62 | 9.86 | 14.02 | 11.63 | 12.65 |
| ∑n UIFA | 2.17 | 1.58 | 0.87 | 1.53 | 1.73 | 1.68 |
| ∑n SFA | 33.16 | 28.7 | 27.79 | 21.34 | 22.39 | 22.92 |
| ∑n MUFA | 42.83 | 48.04 | 51.01 | 50.53 | 52.34 | 51.57 |
| ∑n PUFA | 21.84 | 21.68 | 20.33 | 26.6 | 23.55 | 23.83 |
| ∑n n-3 | 18.74 | 19.16 | 17.48 | 23.82 | 21.58 | 21.75 |
| ∑n n-6 | 3.1 | 2.52 | 2.85 | 2.78 | 1.97 | 2.08 |
| n-3/n-6 | 6.04 | 7.6 | 6.13 | 8.57 | 10.95 | 10.45 |

CFO: crude fish oil, NFO: neutralized fish oil, BFO: bleached fish oil, WFO: winterized fish oil, DFO$_A$: deodorized fish oil A, DFO$_B$: deodorized fish oil B, EPA: eicosapentaenoic acid, DHA, docosahexaenoic acid, UIFA: unidentified fatty acid, SFA: saturated fatty acid, MUFA: monounaturated fatty acid, PUFA: polyunsaturated fatty acid.

Results of Lovibond test for marked colors off red and yellow in crude, bleached and deodorized A and B oils are shown in Table 2. Both colors of red and yellow decreased significantly during bleaching.

**Table 2: Color characteristics of fish oil samples during refinement.**

| Color (Lovibond) | Fish Oil Samples | | | |
|---|---|---|---|---|
| | **CFO** | **BFO** | **DFO$_A$** | **DFO$_B$** |
| Red | $5.4 \pm 0.14^{a}$ | $0.04 \pm 0.0^{b}$ | $0.4 \pm 0.07^{b}$ | $0.5 \pm 0.14^{b}$ |
| Yellow | $6.55 \pm 0.07^{a}$ | $3.55 \pm 0.07^{c}$ | $3.3 \pm 0.14^{b}$ | $3.5 \pm 0.0^{bc}$ |

CFO: Crude fish oil, BFO: Bleached fish oil, DFO$_A$: Deodorized fish oil A, DFO$_B$: Deodorized fish oil B

Results are expressed as mean±standard deviation. Values with similar superscript letters represent no significant difference ($p$<0.05).

## Discussion

*PV*

At first, PV values decreased significantly during neutralization ($p$<0.05). Vacuum conditions and lack of degumming has caused to lower hydroperoxides to primary oxidation products (Ghavami *et al*, 2003; Ackman, 2005). Degumming is not required in fish oil refinement because of the low amounts of phospholipids (0.05-0.3%). Therefore, the phospholipid present in fish oil could help as an antioxidant to reduce oxidation with its synergistic

effects. (Ackman, 2005; Wang *et al*., 2011). The most effective step in decreasing PV was bleaching. Function of acid activated bleaching earth to absorb hydroperoxides and heating to 95˚C induced a huge loss of PV. Noriega-Rodriguez *et al*. (2010) found that PV of Sardine fish oil during refinement decreased during neutralization and increased during bleaching, while results of Crexy *et al*. (2010) showed PV of Carp fish oil decreased durung both neutralization and bleaching. It's supposed that in some studies different effects of bleaching on PV is related to type and amount of earth used during this process. Exposure of sensitive fish oil to air and light during the long time of winterization and agitation of nucleation stage may increase the PV, but in this study the PV did not increase significantly ($p$<0.05). Reduction of PV during deodorization was probably associated with decomposition of hydroperoxides at temperatures of 180˚C and 140˚C (Ghavami *et al*, 2003; Boran *et al*, 2006). PV of deodorized carp fishmeal oil was reported as 4.18 meq/kg oil by Crexy *et al*. (2010), which was much higher than results of this study (0.5 and 0.21 meq/kg). Similarly Noriega-Rodriguez *et al*. (2010) showed that PV of sardine oil reached 0.81 meq/kg after deodorization.

*FFA*

FFA in crude fish oil originate from enzyme activities of fish bodies. The main purpose of neutralization in refinement is to remove FFAs by adding NaOH and forming soap. Removing FFA and residual soap was continued in the bleaching step using bleaching earth. FFAs are related to the unpleasant flavor in oils. FFA value is known as an indicator of the efficiency of the deodorization, due to close vapor pressure of FFA with volatile compounds (aldehydes and ketones) (Dinamarca *et al*., 1990; Vinter, 1995; Ackman, 2005). In this study, FFA of winterized oil increased significantly in $D_B$ ($p$<0.05). It is supposed that the temperature in the $D_B$ step (140˚C for 240 min) was not efficient enough to vaporize FFAs and volatile compounds, where as heating could accelerate hydrolysis of triacylglycerols to FFAs. Refinement process of Sardine fish oil by Noriega-Rodriguez *et al*. (2010) decreased FFA content from 0.21 to 0.052% oleic acid through deodorization at 140˚C for 5 hr. In this study, FFA decreased from 0.87% to 0.33% during refinement, at the same temperature but in 4 hr.

*Fatty acids composition*

Monounsaturated fatty acids (MUFA) like oleic acid and palmitoleic acid are the most abundant oil fractions. Notable content of palmitoleic acid (16:1) in samples is one of the specifications of fish oils. Also, fish oils uniquely contain significant quantities of EPA (C20:5) and DHA (C22:6). EPA and DHA with 5 and 6 double bonds are very susceptible to oxidation by air, heat and metal ions (Allen, 1995). It is assumed that heating at 90˚C in the bleaching step deteriorated some of the EPA, DHA and total n-3 fatty acids. In the next step winterization, concentration of n-3 fatty acids was observed because of the elimination of saturated triacylglyceroles. Winterization is a thermo-mechanical process where the high and low-melting TAGs are separated by partial crystaliation, followed by centrifugation at -5˚C.

Dinamarca *et al*. (1990) reported that using temperatures over 140˚C probably causes a further reduction in EPA and DHA content in fish oil by the formation of trans fatty acids by thermal degradation. In this study, two conditions of deodorization step A (180˚C for 120 min) and step B (140˚C for 240 min), were compared. Results indicated that the refined oil obtained from $D_B$ contained higher amounts of EPA+DHA (0.3%) and total n-3 fatty acids (0.17%) than that from $D_A$. It seems that this slight difference may be related to the lower temperature used in $D_B$ compared to $D_A$. Since DHA contains 6 double bonds, it is therefore extremely susceptible to oxidation and higher temperatures than EPA. It is shown that PUFAs are more susceptible towards oxidation at temperatures above 180˚C. A critical temperature of about 185˚C for deodorizing of fish oils has been reported (Ackman, 2005; Noriega-Rodriguez *et al*, 2010). Temperatures above 200˚C causes cyclization of unsaturated fatty acids and triacylglyceroles polymers and dymers are formed in the polar fraction of oil at temperatures higher than 220˚C (Dinamarca *et al*., 1990; Ackman, 2005; Crexy *et al*., 2010; Fiori *et al*., 2012). In this study, during refinement of *C. delicatula* fish oil, n-3 fatty acids rose from 18.74% to 21.58% and 21.75% for $D_A$ and $D_B$ refined oils, respectively. This result shows the refinement process used in this study could improve refining process capability in comparison with a study by Shojaei *et al*. (2001) on *Clupeonella* fish oil refinement in which n-3 fatty acid content in the final refined oil was 17.89%.

*Colorimetry*

The red color in crude oil is due to the release of haem pigments, as the acid hydrolysis product of hemoglobin. Enzymatic and acid hydrolysis in crude fish oils enhance FFA which leads to protein-lipid complex formation and color increment. Use of acid activated earth in the bleaching stage could adsorb pigments and some impurities like trace metals, phospholipids and oxidation products (Crexy *et al.,* 2010; Noriega-Rodriguez *et al*., 2010; Pedro *et al*., 2013). It should be noted that the color changes in our study was somewhat different with the results reported by Crexy *et al*. (2010) and Noriega-Rodriguez *et al*. (2010).

Assessment of oil quality after each step of refining shows proper efficiency of methods. Deodorization A (at 180˚C for 120 min) seems more effective than deodorization B. It was concluded that deodorization B at 140 ˚C for 240 min was inefficient to reduce off flavors and FFAs properly. Enhancement of 16 percent in n-3 PUFA content confirms that vacuum condition and removing impurities during refinement steps specially winterization were useful. Final refined *C. delicatula* oil with n-3/n-6≅10-11 and n-3 fatty acid≅22% can be considered as a valuable source of omega-3 (especially DHA) nutritionally for public health. High production volume of crude *Clupeonella* (Kilka) oil as a by-product of fishmeal plants in Iran, provides a good opportunity to commercialize fish oil by refinement, microencapsulation, food enrichment and production of soft gelatin capsules.

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