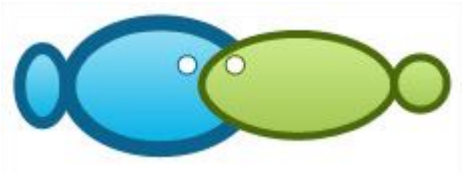

Investigation on fish oil extraction by enzyme extraction and wet reduction methods and quality analysis

Mohammad M. Taati, Bahareh Shabanpour, Mehdi Ojagh

Department of Fishery, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran. Corresponding author: B. Shabanpour, bshabanpour@yahoo.com

Abstract. Different methods of fish oil extraction could be effective on the yield and chemical composition of obtained oil. The purpose of the present study was the evaluation of the effect of 2 methods, namely wet pressing (WP) and enzymatic extraction (EE) on the yield of extracted oil from tuna fish by-products, as well as the amount of moisture and chemical parameters like neutral lipids (Wax esters: WE, Triacylglycerides: TAG, Free fatty acids: FFA, and Cholesterol: CHOL), fatty acid profiles and acid value. The yield rate showed that the amount of obtained oil by EE method was significantly higher than the WP method ($p < 0.05$). The moisture content of the oils did not show a significant difference in the two methods ($p > 0.05$). In neutral lipids, there was a significant difference between WE, TAG and FFA levels in EE and WP treatments ($p < 0.05$). Cholesterol levels in obtained oils from the two methods did not show significant differences ($p > 0.05$). The analyzed fatty acids in the two experimental groups, including the levels of Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), did not show significant differences between the treatments ($p > 0.05$). The acid value in EE treatment was significantly higher than other treatment ($p < 0.05$). Therefore, it can be stated in general that the results of this study indicate the appropriate potential of tuna by-products to extract fish oil for human consumption. Considering the features of the EE method, it can be considered a suitable method for fish oil extracting.

Key Words: fish oil, extraction methods, fatty acid profile, neutral lipids, enzyme extraction.

Introduction. Fish sources once appeared to be inexhaustible and by-products arising out of fish processing were looked as worthless garbage and discarded without an attempt of recovery, which created both disposal and pollution problems (Kristinsson & Rasco 2000). Fish by-products included viscera, head and skin, which had a lot of unexploited potential for value adding and some of them were being utilized at present (Bhaskar et al 2008; Kechaou et al 2009; Mbatia et al 2010). Tuna fishes (Scomberidae) are one of the most popular marine fishes in Iran due to their abundance, year-round availability, low cost, suitable size. By-products/wastes of fish processing industry were usually being discarded during processing and they had high oil content (Tsimidou et al 1995; Sarkardei & Howell 2007; Sahena et al 2010).

Fish oils are a rich natural source of omega-3 polyunsaturated fatty acids (PUFAs), mainly Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), which received great interest in the scientific community because of their positive roles on human health and nutrition (Saldanha et al 2009). Beneficial health effects of ω-3 PUFAs were well demonstrated and included the prevention of a number of diseases, such as coronary heart diseases, hypertension, arthritis, autoimmune disorders, cancer (Israel & Gorlin 1992; Russell & Tisdale 2005; Eslick et al 2009). Studies with newborns indicated that DHA was essential for the normal functional development of the retina, nerve and brain, particularly in premature infants (Ma et al 2010).

Fish oil could be produced by several methods which included hydraulic pressing, vacuum distillation, urea crystallization, supercritical fluid extraction, which all require high temperature or high pressure in processing or reduction of moisture content in sample prior to extraction (Mbatia et al 2010). The most common method used for fish oil production is wet reduction, which involves three basic steps: cooking at high

temperatures (85-95°C), pressing and centrifuging (FAO 2006). This process permits obtaining high volumes of crude fish oil, although subsequent refining steps are required in order to make the crude fish oil suitable for edible purposes. Enzymatic tissue disruption may be a valid alternative technique for releasing natural lipids from fish, which using commercial, low cost food grade neutral proteases provides an attractive alternative as reactions could be carried out under mild conditions for short periods of time. Previous studies (Ramakrishnan et al 2013; Deepika et al 2014; Qi-yuan et al 2016) had shown that, when compared to classical organic extraction, lipid extraction was enhanced by a pre-hydrolysis step using wide-spectrum neutral proteases and a part of the oil could be obtained after hydrolysis and centrifugation.

The aim of this work was to compare two extraction processes (wet pressing: WP and enzyme extraction: EE) to obtain oil from tuna by-products, at a laboratory scale, taking into account not only the extraction yield, but also some of chemical components of oil.

Material and Method

Fish sampling and processing. Approximately 30 kg of tuna by-products was obtained from Sahel Seyd Konarak (canned tuna factory), Chabahar, Iran, in January 2016. The by-products was transported on ice within 45 minutes of landing. The fish was then frozen at -30ºC to minimize the effects of biochemical changes during transportation from factory to the laboratory (located in Tehran). The fish was transported to the laboratory within 15 hours after sampling. The whole by-products were grinded and were stored under -20ºC until used.

Oil extraction methods

Wet reduction (wet pressing). In wet reduction method, grinded fish by-products were previously thawed at room temperature during 12 h, 1 L water was added to 200 g by-products and was cooked at 95°C for 15 minutes. After cooking the tissues were prresured, then, water co-extracted together with the oil was removed by centrifuging (Centrikon T-124, Kontron Instruments).

Enzyme extraction method. The amount of 1000 g of milled raw material and the enzyme protease (alkalase) with food grade (bacterial protease from *Bacillus licheniformis*, Novozyme, Denmark) and with a ratio of enzyme to substrate of 0.05 were added to the samples and at a temperature of 56°C were hydrolyzed for 120 minutes. Then centrifugation was performed at speed 10^4 g and temperature 20°C for 10 minutes, and the hydrophilic phase was separated (Gbogouri et al 2006) (Figure 1).

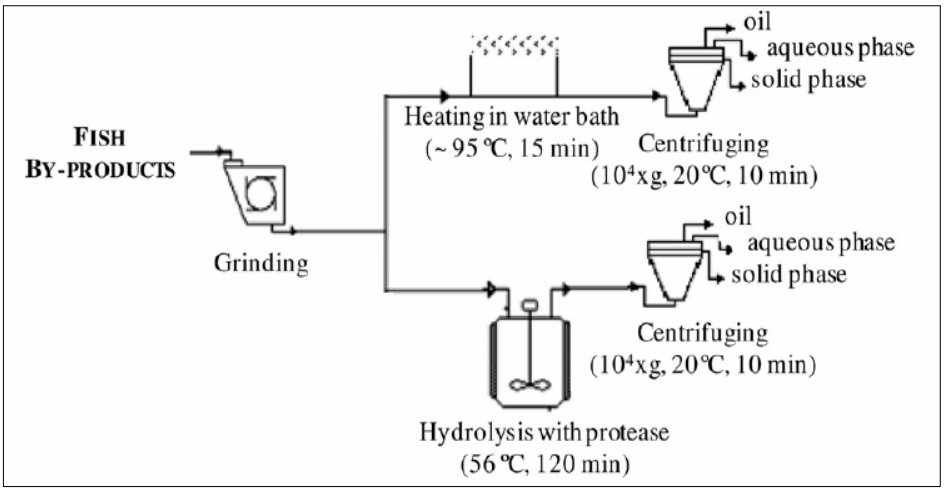

Figure 1. Scheme of the two procedures studied in this work (WP above and EE below).

Determination of yield. Yield was expressed as a percentage of oil separated from by-products tuna. Yield was calculated as follows:

$$\%Yield = \frac{\text{Wt: of crude oil} \times 100}{\text{Wt: of tuna by-products}}$$

Neutral lipids evaluation. The total amount of neutral lipids was determined by liquid chromatography in a HPLC system (Agilent 1200) formed by a quaternary pump and an auto-injector. The separations were carried out at room temperature in a column (Lichrospher Diol 5 mm, 4 x 250 mm) and the detection was performed in an evaporative light scattering detector (Agilent 1200 series) at 45°C and 3.5 bar. The mobile phase consisted of a mixture of solvents: (A) hexane/acetic acid (99.5/0.5 by volume) and (B) hexane/1-propanol/acetic acid/water (85/14.4/0.5/0.1 by volume). The solvent gradient used was as follow: first, solvent A was flowing for 1 min, after that, solvent B was added in three steps, up to 10% in 9 min, to 44% in 12 min and to 100% in 8 min. Finally, the stationary phase was rinsed with solvent A during 5 min. Total solvent flow rate was kept constant at 1 mL min^{-1} all along the analysis. Calibration was carried out using standards of palmitil palmitate (99%), tripalmitin (> 99%), dipalmitin (99%), monopalmitin (99%) and palmitic acid (99%) in hexane. The calibration curves showed a good correlation according to the exponential relationship described for an evaporative light scattering detector.

Fatty acid profile determination. The fatty acids profile was determined by the AOAC method (2005). The fatty acid methyl esters were first prepared and then analysed by gas chromatography (GC) in a Hewlett Packard gas chromatograph (6890N Network GC System) equipped with an auto-sampler (7683B series) and a flame ionization detector (FID). The separation was carried out with helium (1.8 mL min^{-1}) as carrier gas. A fused silica capillary column (OmegawaxTM-320, 30 m × 0.32 mm i.d.) was used. The column temperature was programmed starting at a constant temperature of 180°C during 20 min, heated to 200°C at 1°C/min, held at 200°C during 1 min, heated again to 220°C at 5°C/min and finally held at 220°C for 20 min. A split injector (50:1) at 250°C was used. The FID was also heated at 250°C. Most of the fatty acid methyl esters were identified by comparison of their retention times with those of chromatographic standards (Sigma Chemical Co.). Their quantification was made by relating the peak area to the area of an internal standard (methyl tricosanoate) as indicated by the AOAC method (2005). Calibration curves were made for each pair internal standard+chromatographic standards in order to find the corresponding response factors.

Acid value determination. The acidity value was determined according to the AOCS Official Methods Ca 5a-40 (AOCS 1990).

Statistical analysis. Data were presented as mean±standard deviation (SD) of three replicates and were subjected to analysis of variance (ANOVA). Significant means were compared by one-way procedure tests.

Results

Yield. The amount of obtained oil by WP and EE methods is showed in Figure 2. Yield in EE method was higher than WP process and this different was significant ($p < 0.05$).

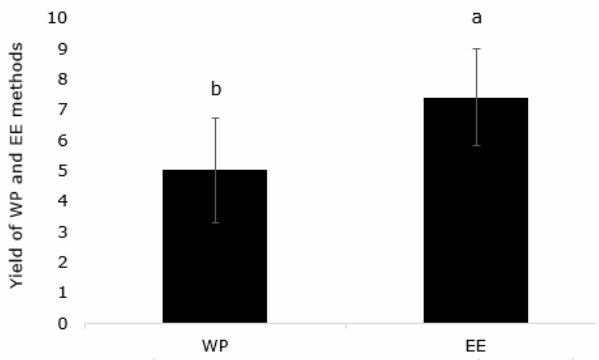

Figure 2. Yield (%) of obtained oils from tuna by-products by WP and EE methods.

Results of neutral lipids evaluation. As it is seen in Table 1, the levels of measured neutral lipids in this study showed a significant difference in most of the parameters ($p < 0.05$). Levels of wax esters (WE), triacylglycerides (TAG) and free fatty acids (FFA) in WP treatment were significantly lower than EE treatments ($p < 0.05$). By contrast, there was no significant difference in amount of cholesterol (CHOL) in the two treatments ($p > 0.05$).

Table 1

The levels of neutral lipids (%) in obtained oil from tuna by-products by WP and EE methods

Components	*WP*	*EE*
Wax esters (WE)	1.91±0.6	1.46±0.23
Triacylglycerides (TAG)	93.06±3.01	94.18±5.26
Free fatty acids (FFA)	3.43±0.9	4.68±2.37
Cholesterol (CHOL)	2.46±2.17	2.84±1.02

Results of fatty acids profile determination. The results of analysis of fatty acid content in fish oil extracted by WP and EE (Table 2) showed that there was not significant difference between the levels of all determined fatty acids in treatments. The amount of EPA and DHA in extracted oils were 6/05 and 21/25 for WP and 6/72 and 22/6 for EE respectively.

Table 2

The fatty acid profile in obtained oil from tuna by-products by WP and EE methods

Fatty acids	*WP*	*EE*
C12:0	0.07±0.02	0.05±0.03
C14:0	4.11±1.06	2.08±1.93
C15:0	0.73±0/2	0.76±0.36
C16:0	15.83±4/92	16.2±7.24
C16:1	6.28±2.8	6.53±4.62
C17:0	1.67±0.7	1.8±0.52
C18:0	4.95±1.7	3.41±2.06
C18:1c	16.56±3.89	16.35±6.02
C18:2c	1.35±0.6	2.14±1.03
C18:3 n6	1.03±0/05	1.58±0.69
C18:3 n3	1.96±0.8	2.18±0.96
C20:0	0.18±0.1	0.83±0.25
C20:1	0.42±0.29	0.76±0.48
C20:4 n3	1.74±0.6	1.42±0.53
C20:4 n6	1.9±0.76	1.78±0.4
C20:5 n3	6.05±2.19	6.72±2.94
C22:0	0.44±0.3	0.59±0.47
C22:5 n3	2.01±1.09	3.6±2.81
C22:6 n3	21.25±4/84	22.6±5.37
EPA+DHA	27.3	29.32

Results of acid value. There was a significant difference between the acidity value (Figure 3) in extracted fish oil by EE treatment with WP treatment ($p < 0.05$). The rate of acidity in EE treatment was significantly higher than the other treatment. On the other hand, the acidity value of obtained oil by WP treatment is significantly different with the maximum recommended acidity value by Codex (that it is shown in the first column of Figure 3) ($p < 0.05$) and was less than it. But, the acidity value of extracted fish oil by EE is not significantly different with the maximum recommended acidity value ($p > 0.05$).

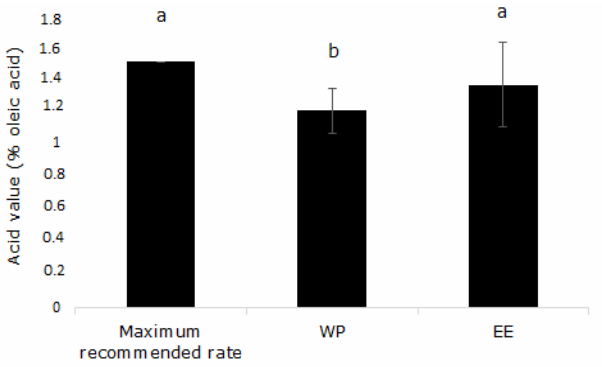

Figure 3. Acid value of obtained oils from tuna by-products by WP and EE methods.

Discussion. Fish oil production is important from two perspectives of human and animal consumption. The presence of this food in livestock feed, and in particular aquatic, is important for supplying the energy of the feed as well as essential fatty acids and is also considered in human consumption for providing essential fatty acids such as EPA and DHA. Therefore, the production of fish oil for two aims, yield and the quality of the product, was studied.

Yield. The results of oil yield coefficient in WP and EE showed that this value was higher in EE method and this difference was significant. The results of oil yield coefficient in WP and EE showed that the extracted oil content in the EE method was higher than WP method. In EE method, the protein matrix is hydrolyzed by the action of added protease to the mass of waste, then attached oils to the protein networks are purified after centrifugation (Liaset et al 2003). In the WP method, the mass is first baked at high temperature and then extracted by mechanical pressure and these two parameters result in the extraction of more oil from the bake mass. Heat contributes to the denaturation of the protein matrixes of the tissue that the oil strongly bonds to them. Following this process, solid particles and liquids can be extracted mechanically. Also, heat causes the opening of oil globules and fat cells, resulting in the release and fluidity of the oil, which can increase the yield (Chantachum et al 2000). The obtained results in this study showed that protease enzyme hydrolysis plays a more effective and efficient role in the separation of oil globules from tuna's protein network in comparison with high temperature and mechanical pressure. Therefore, its yield was significantly higher than WP method yield.

The results of oil extraction from fish by-products by 4 methods of WP, supercritical fluid extraction (SFE), EE and cold extraction (CE) showed that the efficiency of the SFE method was higher than the other methods and EE was higher than the two others. In the study of Rubio-Rodriguez et al (2012) there was a significant difference between the yield of EE and WP methods, which is in agreement with the results of the present study.

Neutral lipids. The results of analysis of neutral lipids in this study showed a significant difference in the levels of WE, TAG and FFA in extracted oils from tuna waste by WP and EE methods. Low levels of WE and high TAG levels can indicate low levels of intracellular fat and high levels of bonded extracellular fat to proteins in the raw material. Due to weak binding of the extracellular oils with protein matrices, the amount of oil extraction is increased and, consequently, the amount of triacylglycerol was increased and the wax esters were decreased (Gupta & Shim 2007). The appropriate rate of yield in these methods, as compared to the amount of oil in the raw material, can also show weak binding between oils and protein matrices in tuna waste. Also, high levels of TAG can indicate that PUFAs were high in oil (Liaset et al 2003). On the other hand, hydrolysis of TAG causes the formation of FFA. Therefore, the higher TAG and PUFA levels produce also more FFA (Linder et al 2005). In the study of the effect of time and temperature of cooking of tuna by-products in WP method on quantitative and qualitative parameters of extracted oil showed that with increasing TAG, levels of FFA also increased. In

Chantachum et al (2000) study, the levels of TAG and FFA in the treatment of cooking at 95°C for 30 minutes were significantly higher than other treatments.

In another study (Fiori et al 2012), the results of qualitative analysis of extracted oils from different rainbow trout parts by supercritical carbon dioxide showed that there was a significant difference between the levels of TAG and FFA in different parts. In this study, the amount of FFA of obtained oils from the head and spine was significantly higher than those in extractive oils from the intestines and viscera. Also, treatments that they had higher TAG had also higher FFA levels.

Fatty acids profile determination. In our study, there was no significant difference between the levels of fatty acids. Rubio-Rodriguez et al (2008) evaluated oil extraction from *Merluccius capensis*. by SFE and Soxhlet with hexane, and stated that there was no significant difference between levels of fatty acids. Therefore, the results of this study are in agreement with our results.

Acid value determination. Acidity value is one of the important parameters in the quality of oil, which is influenced by the amount of FFA and other non-lipid acids, such as acetic acid. This indicates that the acidity of the oil can be varied by factors such as oil compounds and extraction methods (Gbogouri et al 2006). In general, oils containing higher amounts of TAG and PUFA have higher levels of FFA, which can reduce acidity (Hajeb et al 2014). In the present study, the levels of TAG and FFA in EE treatment were slightly higher than WP. Therefore, due to the higher acidity value in EE treatments, it can be concluded that FFA probably have an effect on acidity, which has led to a higher acidity value in EE treatment than WP. On the other hand, the acidity value in WP treatment was significantly lower than the recommended acidity value for fish oil, and in contrast, acidity value of EE treatment had not significantly different with recommended acidity value. It was probably influenced by the higher levels of FFAs in this treatment. The obtained data and no higher acidity value than the recommended level in the two treatments can indicate the state of keeping the raw materials was good and the used methods for oil extraction in present study were suitable.

Conclusions. In the present study, the quantitative comparison and yield of extracted oil from tuna by-products by WP and EE methods revealed the suitability of each of the two methods for oil extraction in terms of quantity. In general, oil extraction methods, in addition to quantity, can be effective in the formation of compounds from oxidation of fats and various types of contaminants. In the present study, extracted oils by EE method, in terms of yield showed better conditions which can be considered as the special advantages of this method. Also, there was not significant difference between oil quality of WP and EE methods. Therefore, according to the results, it can be stated in the first that tuna by-product has a suitable potential for extracting oil with a human consumption approach. Also, considering the specifics of the EE process in oil extraction, it can be considered a suitable method for extracting fish oil.

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Authors:
Mohammad Mehdi Taati, Department of Fishery, Gorgan University of Agricultural Sciences and Natural Resources, Iran, Golestan, Gorgan, Shahid Beheshti Avenue, Postal code: 49138-15739, e-mail: taati.mehdi64@gmail.com.
Bahareh Shabanpour, Department of Fishery, Gorgan University of Agricultural Sciences and Natural Resources, Iran, Golestan, Gorgan, Shahid Beheshti Avenue, Postal code: 49138-15739, e-mail: bshabanpour@yahoo.com
Mehdi Ojagh, Department of Fishery, Gorgan University of Agricultural Sciences and Natural Resources, Iran, Golestan, Gorgan, Shahid Beheshti Avenue, Postal code: 49138-15739, e-mail: Ojagh@gau.ac.ir

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