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Effect of enzymatic degumming process on the physicochemical and nutritional properties of fish byproducts oil

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MUFAs, monosaturated fatty acids
SFAs, saturated fatty acids

ABSTRACT

The aim of this work focuses on the study for the degumming of the multi specie and ray liver oils obtained by fish industry trough phosphoric acid and enzymes process.

The enzymes used as degumming agents were Lecitase Ultra® phospholipase A1 and LysoMax® Oil acyltransferase. The effect on the phosphorous content, acidity, peroxide value, anisidine index, color and density were evaluated. Then, neutralization with NaOH was carried out to eliminate free fatty acids (FFA); followed by washing, drying and bleaching to remove soaps and other trace elements in order to evaluate the complete refining process. After that the parameter were evaluated again and the fatty acid profile was analyzed by gas chromatography. The contents of eicosapentaenoic acid (EPA) and docosahexaenoic acids (DHA) were outstanding in all samples, being around 8% and 23% for multi species oil and 11% and 45% for ray liver oil. All the refining processes improved the physical-chemical characteristics of the oils, highlighting the treatments using enzymes with respect to phosphorus content, color and appearance. This suggests that the enzyme refining process could be a valid alternative to improve the physicochemical characteristics of crude oil, without change the nutritional profile.

1. Introducción

Fish oil is the most important source of omega-3 polyunsaturated fatty acids (ω−3 PUFAs). The ω−3 PUFAs mainly eicosapentaenoic acid (EPA c20:5) and docosahexaenoic acid (DHA c22:6) have several benefits for human health, being essential for the development and functionality of vital organs and metabolic processes (Boran et al., 2006; Morais et al., 2015).

EPA is well known about this benefits in the treatment of some cardiovascular diseases and has a protective effect against thrombosis, atherosclerosis and some inflammatory diseases (Schmidt, Arnsen et al., 2005, b). On the other hand, DHA registered an effective role in preventing skin disorders and in brain development (Huang et al., 2018; Weiser et al., 2016).

Recently different studies have reported that the consumption of omega 3 might be associated with the improve on the treatment and recovery of severe SARS-COV-2 infected patients by reducing inflammatory response and excessive blood coagulation (Rogero et al., 2020), as well as to reduce the risk of complications, progressing to serious outcomes like acute respiratory distress syndrome, with the need for artificial ventilation in intensive care unit (Weill et al., 2020).

Fish oil is obtained by different extraction methods from whole capture fisheries or by-products of the fish industry. Once the crude oils are obtained, they need to be purified to meet the standards of production and generation of quality oils acceptable for human consumption (Hafidi et al., 2005). Also, the PUFAs are susceptible to oxidative processes, resulting in hydroperoxides. When hydroperoxides decompose, secondary oxidation products are created, such as aldehydes, ketones and acids, which have a fishy and rancid flavor (Oterhals & Vogt, 2013). Phospholipids, normally derived from the tissues of the fish, readily undergo hydrolysis to liberate free fatty acids (FFA) during storage of the oil and imparts dark color to the oil at exposure to oxygen or light (Charanyaa et al., 2017; Lamas et al., 2016). The refining process eliminates undesirable compounds such as mono and diglycerides, phosphatides, steroids, vitamins, hydrocarbons, pigments, carbohydrates, proteins, and their degradation products, guaranteeing the purity and stability of the oil. refining includes the following steps: degumming, which removes gums and phospholipids; neutralization with caustic soda for the elimination of FFA; washing, drying and bleaching to remove soap and trace elements; and deodorization by vacuum distillation for the removal of residual FFA, aldehydes, ketones, alcohols and other compounds (Berdeaux et al., 2007; Charanyaa et al., 2017; Crexi, Souza-Soares & Pinto, 2009; Hafidi et al., 2005; Lamas & Massa, 2019). In recent decades, technological alternatives have been developed that are not only more efficient than traditional methods but are also considered "green" and do not pollute the environment. In this sense, one of the technological alternatives studied is the use of enzymes, which are

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highly specific, biodegradable and easily removable biological catalysts. Specifically, during the oil refining degumming process, phospholipase-type enzymes are used. These enzymes hydrolyze phospholipids, without hydrolyzing triglycerides, releasing fatty acids that will remain in the oil, resulting in less oil loss (Lamas & Massa, 2017). This procedure has been shown to be carried out under mild conditions, does not have a negative environmental impact and has a higher yield compared to chemical degumming (Lamas et al., 2016; Lamas et al., 2014).

The objective of this work was to study and compare chemical and enzymatic degumming in the refining process of oils from fishery by-products. The physicochemical characteristics and the performance of the process were compared. In addition to that, the physicochemical and nutritional characteristics after refining were also studied.

2. Materials and methods

2.1. Raw material

The oil samples studied were obtained from by-products of different species: oil samples from multispecies by-products, consisting mainly of hake (*Merluccius hubbsi*) and to a lesser extent by residues of sole (*Paralychtis sp.*), batfish (*Percophis brasiliensis*), pollock (*Genypterus blacodes*), whiting (*Cynnoscion striatus*) and ray liver oil of different ray species captured in the varied coastal area of Buenos Aires. The oil samples were obtained by enzymatic extraction procedure and they were supplied by the company OmegaSur S.A, Mar del Plata, Argentina.

The storage was at −18 ˚C in a freezer, in the dark and free of oxygen until it uses.

All reagents used were of analytical grade.

2.2. Enzymes

The enzyme phospholipase A1, PLA1, Lecitase Ultra® from *Termomyces lanuginosus*, expressed in a strain of *Aspergillus oryzae* was provided by Novozymes, (Denmark). This microbial enzyme has an optimum reaction pH in the range of 5 to 6.5 and an optimum temperature of 50 ˚C according to the manufacturer's specifications. Sodium citrate buffer pH 5, enzyme reaction medium; was prepared from sodium citrate solution ($Na_3C_6H_5O_7$) and 0.5 M sodium hydroxide (NaOH).

The enzyme LysoMax Oil® is an acyltransferase LAT with PLA2 activity of bacterial origin produced by *Aeromonas spp*. The optimum reaction pH is 5 and the optimum temperature is in the range 50–60 ˚C. It was provided by the company Danisco (Arroyito, Córdoba, Argentina).

2.3. Oil refinement methodology

The degumming process: The oil samples were conditioned in a 100 ml jacketed batch reactor, connected to a thermometer and a water pump. The chemical degumming process was carried out with addition of 1.0% of phosphoric acid (85%v/v) in relation to the oil mass. The temperature was set at 85 ˚C and the magnetic stirring was constant during the 30 min of reaction. The biological degumming process was carried out using the enzymes phospholipase A 1 Lecitase Ultra® or acyltransferase LysoMax® Oil, as degumming agents. The reaction temperature was 50 ˚C for 30 min. The reaction was carried out by the addition of 2% buffer/substrate ratio and 200 U/kg of oil enzyme dosage solution, according to Lamas et al. (2014). The mixture was stirred with a mechanical mixer to provide a safe large surface area through emulsification. Then, the mixture was heated for 10 min at 100 ˚C to stop the enzymatic reaction. And finally, they were centrifuged (10 min at 2400 g) to recover oil and water phases.

To evaluate the efficiency of enzymatic degumming processes, the residual phosphorus content in crude and degummed oil optimized samples was used. The efficiency of each degumming process was estimated based on its ability to reduce the phosphorus content using the following equation:

$$\textbf{Efficiency} = (\textbf{Pi} - \textbf{Pr})/\textbf{Pi} \quad (1)$$

where Pi = initial phosphorus content of the crude oil (mg / kg) and Pr = actual content of phosphorus in degummed oils (mg / kg).

In order to calculate the yield of each degumming process, the percentage phosphorus content reduction was calculated using the following equation:

$$\textbf{Yield process}(\textbf{g}/\textbf{100g}) = \textbf{Efficiency} \times \textbf{100} \quad (2)$$

The oil yield of each degumming processes was experimentally obtained by measuring the recovery of degummed oil. The crude oil was weighed before the treatment into a container. After the degumming and centrifugation processes, the upper oil phase was weighed. The degummed oil recovery was calculated using the following equation:

$$\textbf{Oil yield}(\%) = (\textbf{m2}/\textbf{m1}) \times \textbf{100} \quad (3)$$

where m1 = mass of crude oil sample and m2 = mass of degummed oil.

The neutralization step: was performed for 20 min, at 40 ˚C and agitation of 500 rpm, with the addition of sodium hydroxide solution (20% w/w, using 4.0% excess in relation to the acidity value after degumming step). Then, the material was centrifuged for 20 min at 4000 rpm for oil separation. Washing consisted of adding 10% water in relation to the mass of oil, at 90 ˚C, for a contact time of 10 min, with constant stirring and maintaining the oil temperature at 50 ˚C.The drying procedure: was carried out for 20 min at a temperature of 90 ˚C with stirring. The bleaching stage was carried out at 50 ˚C and 40 rpm, with the addition of 5% adsorbents (diatomaceous earth, with a contact time of 20 min). Filtration was done with a Büchnner funnel with a pre-layer of diatomaceous earth.

Fig. 1 describes the general refinement process.

2.4. Physicochemical and nutritional characterization of oils

The fish oils obtained were characterized according to the American Oil Chemists' Society. Phosphorus content was analyzed by spectrophotometric determination of the blue phosphomolybdic complex at 650 nm (AOCS, Ca 12a-02, 2009). Moisture and volatile material content were evaluated by the vacuum oven method (AOCS, Ca 2d-25, 2009), and acidity index (AI) by titration (AOCS, Ca 5a-40, 2009). The peroxide value (PV) (AOCS, Cd 8–53, 2009) and p-anisidine value (AV) (AOCS, Ca 5a-40, 2009) were carried out in order to evaluate the oxidation, and total oxidation was determined by the TOTOX index (TOTOX = 2PV + AV). The relative density was determined using a pycnometer calibrated at 20 ˚C. The oil color was determined using the Gardner color scale (Gardner-Delta Color Comparator, Florida, USA, AOCS Td 1a-64, 2009).

To determine the fatty acid profile, the crude and refined oils were subject to methylation and subsequent gas chromatography (ISO 12966–2, 2011). Briefly, 60 mg of lipid sample was mixed with 2 mL hexane and 0.3 mL of KOH/MeOH reagent in a glass tube in order to convert fatty acids to methyl esters (FAMEs). The sample was mixed vigorously; then, 2 mL of NaCl and 2 mL of hexane were added and mixed again. The sample was allowed to stand for 5 min, and the upper hexane layer was separated and transferred to a clean tube. The FAMEs were determined with a Shimadzu GC2010 (Kyoto, Japan) equipped with a flame-ionization detector (260 ˚C) and capillary column (30 m x 0.32 mm; 0.25 μm film thickness; Omegawax 320, Darmstadt, Germany). A split injector (50:1) at 250 ˚C was used. The column temperature was programmed starting at a constant temperature of 120 ˚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. The separation was carried out with nitrogen as carrier gas. The oven temperature was increased to 240 ˚C at a rate of 5 ˚C/min and held for 5 min.

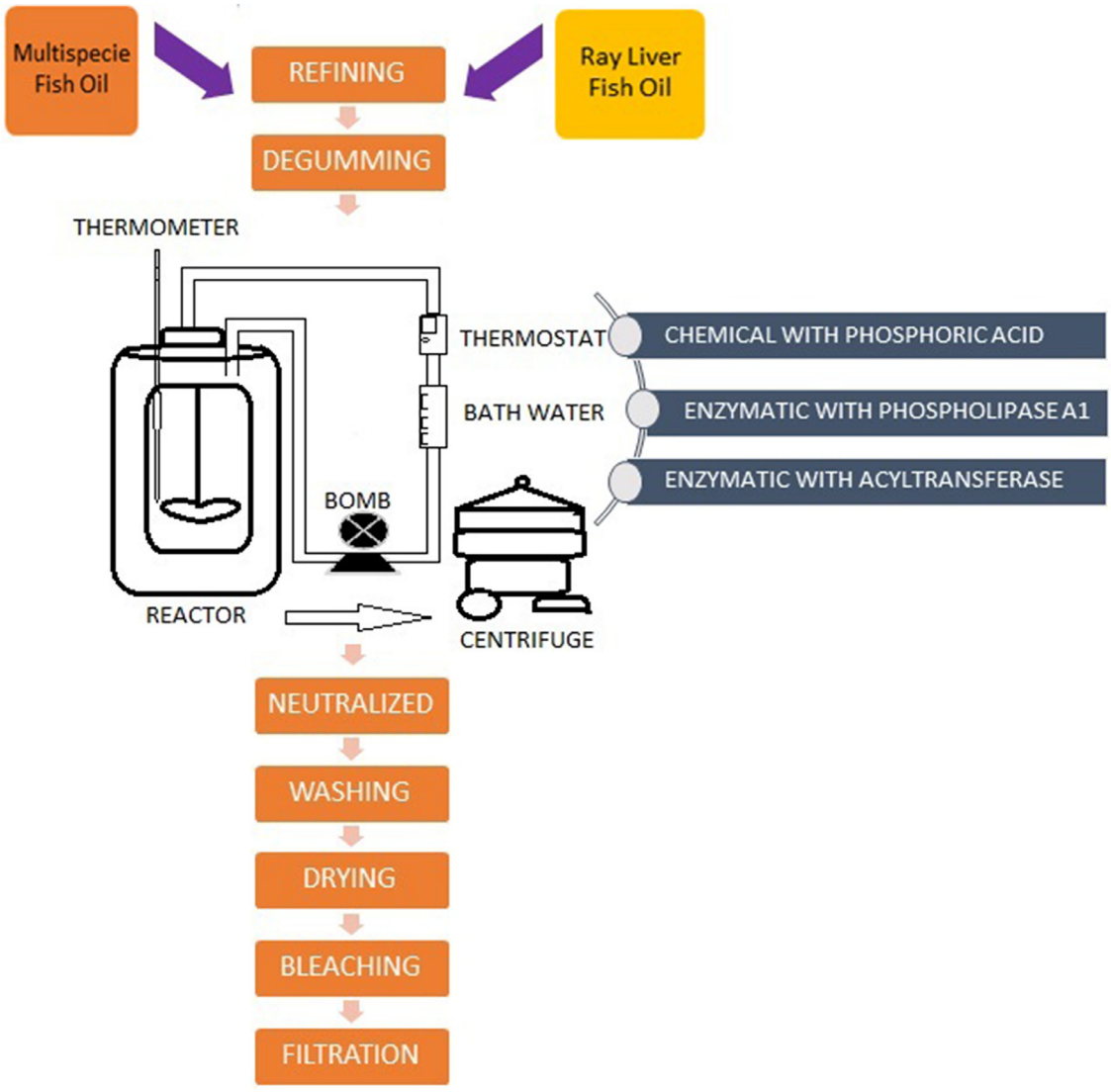

Fig. 1. Descriptive flowchart of oil refining method carried out.

A volume of 1 μL of sample was manually injected (in duplicate), and FA peaks were identified by comparison of their retention times with those of external reference standards (Supelco FAME Mix C4-C24 + PUFA N°1 Marine Source, Pennsylvania, USA). Retention times and peak areas were processed by Shimadzu GC Solution software.

2.5. Statistical analysis

All analyses were performed in triplicate and were expressed as mean value ± standard deviation. The difference between the mean values was evaluated by the Duncan (1955) test, and it was considered significant with a p-value of 5%. For the statistical analysis, Infostat software (2017) Córdoba, Argentine, of general application developed under the Windows platform was used.

3. Results and discussion

Fish oil is a valuable byproduct, and among its main applications focused on aquaculture, human consumption, industrial, and pharmaceutical uses and others (Gunstone et al., 2007; Soldo et al., 2019). The main objective of refining process is to remove the undesirable compounds that adversely affect the oil quality, its shelf life, and most importantly, consumer acceptance. Tables 1 and 2 summarized the values obtained of physicochemical parameters for crude, degummed and refined oil samples.

3.1. Phosphorous content

Degumming is the initial step in edible oil refining which removes phospholipids mucilaginous substances and trace metals (Charanyaa et al., 2017; Vaisali et al., 2015) .

Phospholipids consist of a glycerol-3-phosphate esterified at its sn-1 and sn-2 positions with fatty acids. Its phosphoryl group can be esterified with head groups such as choline, serine, ethanolamine, or inositol.

These compounds constituted impurities in fish oil that generate problems for its storage and processing. In addition, their presence damages the appearance of the oil, darkening the final product and reducing performance due to flocculation and emulsion (Autino, 2009). The removal of phospholipids, or a low initial level of phospholipids in the crude oil, is needed to minimize emulsion loss during the alkali-refining step and to optimize the performance of the bleaching clay (Oterhals & Vogth 2013). Phospholipids have a positive effect on oxidative stability in fish lipid systems (Saito & Ishihara, 1997); this effect is primarily attributed to the side-chain moieties that contain amine and hydroxyl groups or to decomposition products such as choline and ethanolamine. So, phosphatidic acid derivatives did not show any antioxidative effect.

Table 1
Effect of degumming and refining process on physicochemical parameters of crude multi species oil.

Oil samples								
Parameter	Crude	Phosphoric acid Degummed	L. Ultra® Degummed	L. Oil® Degummed	Phosphoric acid Refined	L. Ultra® Refined	L. Oil® Refined	CODEX STAN 329–2017Refined Fish Oil*
PC mg/Kg	782.86c±10.44	191.43b±7.87	77.30a±2.35	84.60a±3.65	189.66b±4.87	76.50a±3.22	81.43a±2.42	–
AI mg KOH/g	3.90c±0.22	4.64d±0.18	4.88d±0.13	4.76d±0.21	2.44b±0.12	1.31a±0.02	1.35a±0.01	≤ 3
PV meq/Kg	4.26cd±0.37	4.70d±0.28	3.73bc±0.16	4.02c±0.35	5.44e±0.10	3.04a±0.11	3.24ab±0.08	≤ 5
AV	10.23c±0.59	10.16c±0.29	9.94c±0.20	8.81b±0.18	8.56b±0.13	8.19ab±0.11	7.85a±0.12	≤ 20
totox	18.76	19.57	17.41	16.85	19.45	14.27	14.33	≤ 26
color	11–12	9–10	9–10	9–10	7–8	5–6	5–6	–
Density g/cm^3	0.983b±0.01	0.963b±0.01	0.959b±0.01	0.957b±0.01	0.922a±0.02	0.916a±0.01	0.914a±0.01	–

Different letters within the same line indicate significant differences ($P < 0.05$), a means the lowest value.
PC- phosphorous content; AI – acidity index; PV – peroxide value; AV –anisidine value.
Results are mean values of two determinations ± standard deviation.
* CODEX Standard for Fish Oil.

Table 2
Effect of degumming and refining process on physicochemical parameters of crude ray liver oil.

Oil samples								
Parameter	Crude	Phosphoric acid Degummed	L. Ultra® Degummed	L. Oil® Degummed	Phosphoric acid Refined	L. Ultra® Refined	L. Oil® Refined	CODEX STAN 329–2017 Refined Fish Oil*
PC mg/Kg	590.55d±13.36	89.70c±6.27	51.30b±3.77	31.04a±2.65	90.41c±3.67	49.70b±3.56	31.41a±2.40	–
AI% ac oleic	2.13d±0.13	2.48e±0.12	2.56e±0.12	2.04d±0.03	1.12c±0.06	0.83b±0.01	0.58a±0.01	≤ 3
PV meq/Kg	4.73c±0.15	5.72d±0.25	3.71b±0.05	3.86b±0.13	4.71c±0.27	3.69b±0.38	3.10a±0.10	≤ 5
AV	8.94e±0.09	10.24de±0.18	10.35cd±0.38	9.53bc±0.56	8.42b±0.27	8.25ab±0.10	7.65a±0.30	≤ 20
totox	18.185	22.04	17.85	17.435	17.72	16.175	13.715	≤ 26
color	9–10	7–8	5–6	5–6	7–8	5–6	5–6	–
Density g/cm^3	0.941c±0.01	0.933bc±0.01	0.930abc±0.01	0.935c±0.00	0.920abc±0.01	0.911ab±0.01	0.909a±0.0	–

Different letters within the same line indicate significant differences ($P < 0.05$). a means the lowest value.
PC- phosphorous content; AI – acidity index; PV – peroxide value; AV –anisidine value.
Results are mean values of two determinations ± standard deviation.
* CODEX Standard for Fish Oil.

Phospholipids have an amphiphilic nature (Oterhals & Vogt, 2013), that causes the oil losses due this type of molecules could to transport oil in the form of emulsions (Gahlardo et al., 2010). There are two types of phospholipids: hydratable (HPL) and non-hydratable (NHPL), most phospholipids in crude oil can be removed by water degumming, but NHPLs require complex higher temperature processes and the use of citric acid or other degumming substances (Noriega-Rodríguez et al., 2009; Suseno et al., 2016). Citric or phosphoric acid is used not only for the decomposition of metal salts with NHPL, but also as a chelating agent to keep the metals in a water-soluble complex (Suseno et al., 2016). In the enzymatic degumming, the phospholipase A1 removes the fatty acid from position 1 y with respect to glycerol (Dijkstra, 2010; Lamas et al., 2014), while the lipid acyltransferase catalyzes the transfer of an acyl moiety from the 2-position in a phosphatide to a sterol present in the oil in order to convert it into a sterol ester (Dijkstra, 2010). Both enzymes lead to lower oil retention by the gums, which contributes to an improved oil yield (Lamas et al., 2014).

The phosphorous content is usually used as indirect measure of phospholipids (Lamas & Massa, 2017; Lamas et al., 2016). According to Young, (1986) phosphorous level in crude fish oil is 5–100. As shown in Table 1, the phosphorus content of the multi-species crude oil was extremely high, this may be due to the fact that the raw material is made up of non-edible parts, rich in membranes made up of phospholipids or sources other than phospholipids such as meal residues, including inorganic phosphorous that could increase the value (Lamas et al., 2016). Using phosphoric acid, the initial phosphorus content was reduced by 75%, that are certainly higher than reported data for catfish oil refined with citric acid (Sathivel et al., 2003) and carp oil refined with phosphoric acid (Crexi et al., 2010) being 10 and 50%, respectively. Charanyaa et al. (2017) observed a significant reduction in the phospholipid content to 261.5 ppm from 612.66 ppm with 5% (w/w) of orthophosphoric acid. The degumming of the ray liver oil achieved high removal percentages using phosphoric acid as a degumming agent, yielding residual phosphorus values of 15% with respect to the initial content (Table 2).

The phosphorous content using the enzymes L. Ultra® and L. Oil® was reduced from 781 mg/Kg to 77 and 84 mg/Kg, respectively, in the case of multi-species oil. Slightly higher values for phosphorus reduction with the same enzymes can be observed in the ray liver oil. That is consistent with previous work on ray liver oil species captured in the same geographical area degummed with the phospholipase A1 (Lamas & Massa, 2017). Enzymes exhibit specificity for the bond they cleave and act interchangeably on hydratable and non-hydratable phospholipids. This constitutes an advantage for the following refining steps, and for the general performance of the process (Lamas & Massa, 2017; Lamas et al., 2014, 2016). Enzymatic degumming generates reaction by-products of a rigid consistency, facilitating the separation of the phases after centrifugation. In general, hydratable and nonhydratable phospholipids present in the oil become hydrophilic at low pH, and forms sludge, which is easily separated by centrifugation. The enzymatic modification produced in the phospholipid molecule eliminates the emulsifying properties of these compounds, preventing oil losses due to entrainment (Gahlardo et al., 2010).

3.2. *Degumming process characterization*

The yield of reaction and oil yield of degumming processes are listed in table 3. Compared to the acid degumming process, higher efficiency was observed by using enzymes treatments. The reaction yield was around 90% in multi species oil and higher than 90% in ray liver oil using both enzymes. The results of oil yield showed that there would be approximately 5.5% of oil loss during the acid degumming process

Table 3
Yield reaction and oil yield of acid phosphoric and enzymatic degummed samples under optimal conditions.

Oil Sample	Yield reaction (g/100 g)	Oil yield (g/100 g)
L.® Ultra Multispecies Degummed Oil	90.13b ± 0.18	95.88 *b* ± 0.16
L.® Oil Multispecies Degummed Oil	89.20b ± 0.61[b]	96.63 *b* ± 0.27
Phosphoric Acid Multispecies Degummed oil	75.55a ± 0.70	94.45 *a* ± 0.45
L.® Ultra Ray liver Degummed Oil	91.30b ± 0.84	96.44 *b* ± 1.08
L.® Oil Ray liver Degummed Oil	94.74b ± 0.57	97.08 *b* ± 1.11
Phosphoric Acid Ray liver Degummed oil	84.82a ± 0.72	94.11 *a* ± 0.51

Results are mean values ± standard deviation of 2 replicates analyzed by duplicate.
The means within a column followed by the same letter are not significantly different (test t, $\alpha = 0.05$).

and less oil loss was found in enzymatic degumming samples. This value is slightly lower than the loss obtained in sunflower oil using water as a degumming agent (Lamas et al., 2022). Others authors reported high yield for enzymatic oil degumming. Jiang et al., (2015) reported a high oil yield in soybean, camellia and rapeseed oil using a mixture of phospholipase A1/C respect to citric acid as degummed agent. The lower oil losses of the enzymatic treatments compared to others degumming process was also reported by Ambrosewicz-Walacik et al., (2015). The problem with acid degumming is that the acid decomposed the NHPL but it is not enough, so it is also necessary to avoid the re-forming of the NHPL when the pH is raised either by the addition of NaOH during the neutralization (Dijkstra, 2010). therefore, in this process, the HPL are eliminated, but the NHPL, probably remain in the oil phase after re-forming. The phospholipase A1, hydrolyzed the phospholipids to oil-soluble diacylglycerol and water-soluble phosphate esters. Since these diacylglycerols remain in the oil during refining, they contribute to the oil yield. Also, fatty esters formed as a consequence of the phosphatide hydrolysis catalyzed by the acyltransferase remain in the oil phase (Dijkstra, 2010). In addition, both enzymes cause less oil to be retained by the gums by decreasing the quantity of gums and/or their oil retention, which also contributes to an improved oil yield.

3.3. Acidity index

Oil acidity is an important quality parameter that is related to the presence of free fatty acids (FFA) and other non-lipid compounds. Generally, lower FFA content is associated with better fish oil quality (Fang et al., 2018) since FFAs are mainly the result of triacylglycerol hydrolysis reactions. In addition, during the degradation of the raw material, acetic acid is produced. In this way, the acidity depends on several factors related to the freshness of the raw material, the composition of the oil, the extraction procedure and the refining process (Rubio-Rodríguez et al., 2012).

The acidity increases with all degumming agents, in both samples. This is mainly due to the release of FFA. The temperature used during chemical degumming reaches 85 °C; such heating could accelerate the hydrolysis of triacylglycerols to FFA (Moghanjoghi et al., 2014). Also, the addition of phosphoric acid, which may not have completely reacted, could be contributed (Menegazzo et al., 2014). The acyltransferase enzyme was the one that showed the lowest degree of increase. This could be due to its transferase action, since it has the ability to transfer FFA to glycerol molecules (Lamas et al., 2014). During enzymatic degumming, FFAs bound to glycerol are released from phospholipids primarily and secondarily due to hydrolysis of triglycerides. Consequently, the acidity increases, which is then neutralized by the addition of NaOH. If the phospholipids content is high, the release of FFA is large due to enzymatic action, and the neutralization to be carried out must consider the initial acidity, plus the excess generated by this reaction.

However, throughout the refining, acidity decreased significantly for both oils compared to the crude oil ($P > 0.05$) using phosphoric acid. In the case of the oil refined using enzymatic degumming, the multispecies oil showed lower acidity values compared to traditional refining. In the case of the oil obtained from ray liver, the refined oil showed a remarkably decreased when the enzyme acyltransferase was used for degumming up to 0.58% of oleic acid. Similarly, degumming with the phospholipase A1 enzyme followed by the steps corresponding to refining, yielded significantly lower values, compared to crude oil.

It should be noted that all samples yielded acidity values that are below the limits established by the Codex for fish oils and fish liver oils suitable for human consumption (3 mg KOH /g oil). Furthermore, acceptable levels of FFA in refined fish oils were reported to range from 1.8% to 3.5% oleic acid (Sathivel et al., 2003) data that are above the values obtained in the present work for all refined samples.

3.4. Oil oxidation parameters

PUFA oxidation products are harmful to human health (Fang et al., 2018). The peroxide value is an important parameter for evaluating oxidation in fish oils. The values of peroxides obtained from the degumming process with phosphoric acid increased slightly with respect to the crude oil in both species. This may be because heating increases the rate of formation of hydroperoxides from PUFAs and accelerates decomposition (Crexi, Grunennvaldt, Souza-Soares & Pinto, 2009). Previous studies carried out using citric acid at 60 °C to degumming ray liver oil from the same geographical area, showed a significant reduction in the peroxide value, evaluating only the degumming step (Lamas & Massa, 2017). Although the degumming time was longer than the time used in the present work, the temperature applied in this chemical process was. So, the results suggest that the temperature used influences the formation of hydroperoxides. Contrary, with the Lecitase Ultra® enzyme, the peroxide value was significantly reduced ($P < 0.05$) in both oil samples and with the acyltransferase it did not undergo significant changes. This could be due, in part, to the ability of hydratable phospholipids to complex metal ions that are catalysts for oxidation reactions. During acid degumming, the phospholipid content is reduced, leaving a remainder that normally corresponds to non-hydratable phospholipids that form salts with metal ions. With enzymes, both types of phospholipids are lost by dragging metals into the gums during centrifugation. However, at the end of the degumming stage, both oils lose a large content of phospholipids, which on the one hand represents an advantage, since they complex metals and other substances, but on the other hand, they are known to have antioxidant properties (Lamas et al., 2014). Another factor to consider is that enzymatic degumming is carried out under mild reaction conditions while chemical degumming uses temperatures of 85 °C. Throughout the refining process, the results obtained showed values that are below the permitted limit (10 meq O_2/kg oil) for oils used in animal feed indicated by Masson (1994). Likewise, all oil samples are below the peroxide index range that establishes oils as oxidized, (7–26 meq O_2/kg oil) indicated by the same author. The anisidine value is a measure of high molecular weight, saturated and unsaturated end oxidizing products (Aidos et al., 2003). It is associated with the second stage of oxidation represented by a greater degrada-

tion of lipids. An acceptable AV for good quality crude fish oils is less than 20 (Masson et al., 1994). Both species yielded values lower than 20 for the crude oils, however, in all cases an increase in the AV could be observed after refining. Although the values are within the limits, the steps to which the oil is subjected during refining involve temperatures that produce the oxidation of the oils. This suggests that the use of antioxidants and the optimization of the process parameters are required, highlighting the reduction of the exposure time at temperatures above 40 °C and the adaptation to environments free of oxygen and light.

So, the raw material under study is suitable for being subjected to refining processes, under controlled conditions, and considering the addition of antioxidants. Finally, all the oils in this study are below the permitted limits for crude and refined fish oils indicated by Masson (1994). Usually, at the end of refining antioxidants are added under nitrogen to prevent oxidation of fatty acids and improve the shelf life of fish oil (Liu et al., 2020).

3.5. Oil physicochemical parameters

The crude oil from hake waste showed an orange, opaque, cloudy hue. The characteristic yellow-orange color of fish oil is the result of deposition of carotenoids in the diet (Luterotti et al., 1999), which are lipid-soluble antioxidants (Hidalgo et al., 2006).

Ray liver oil showed a yellow, translucent and bright hue, with values from 7 to 8 on the Gardner scale, falling within the quality standards for crude fish oils that indicate maximum values for the Gardner scale of 14 (Lamas & Massa, 2019).

After degumming process both samples improved the color. Among the degumming agents the acyltransferase reached clearer and transparency of multispecies oil, and phospholipase A1 achieved clearer ray liver oil. The phosphoric acid degumming agent improved the color of the oil respect to the crude; however, the really remarkable difference was glimpsed at the end of the process. Chakraborty and Joseph (2015) also reported an improvement in the color after phosphoric acid degumming process in sardine fish oil.

After refining the color change in oils is due to the capacity of the adsorbents used for bleaching, which can remove pigments and other impurities, such as trace metals, phospholipids and oxidation products (Rossi et al., 2003). While hake residue oil was most clear with all degumming agents, ray liver oil only cleared after refining with enzymes as degumming agent. However, in all cases, the transparency of the studied oils increased.

The density of crude oil from hake by-products was close to 1, probably due to the content of impurities dragged during the collect of the raw material. In the ray liver oil, the density was 0.941 mg/mL, according to the results obtained in other ray liver oil obtained by different species from the same geographical area (Lamas & Massa, 2019). This characteristic is consistent with the raw material of origin, because this oil comes only from livers, and not from other remains that may contain other impurities that increase density. No significant differences were found after degumming step. That is close to the results reported by Menegazzo et al. (2014) for fish oil of Nile Tilapia. The density of all oil samples was corrected after the refining process, in which impurities and unwanted substances are removed.

3.6. Fatty acids profile of oils

The fatty acid composition of crude and refined multispecies and cartilaginous fish oils are shown in Tables 4 and 5 respectively. Fatty acids that were less than 1% were omitted.

The levels of saturated fatty acids (SFAs) were found to be lower than the levels of both monounsaturated fatty acids (MUFAs) and PUFAs in multispecies by-products oils. Betwixt the saturated fatty acids (SFAs), palmitic acid (C16:0) achieved the highest value. That is in accordance with the results reported by Rubio-Rodríguez et al. (2008) for hake by-products oil obtained by hexane and supercritical fluid extraction. The most abundant MUFAs established were oleic acid (C18:1), eicosenoic and palmitoleic acid (C16:1). Karoud et al. (2020) reported similar values for oleic and palmitoleic acid, but lowest for eicosenoin in hake head fish oil. The percentages of PUFAs found were around 39% after all the refining process applied. Interestingly, the major ω−3 PUAFs were EPA (C20:5) and DHA (C22:6) with contents reached 8.7% and more than 20% respectively. The level of EPA is slight superior than that of salmon heads studied by Gbogouri et al. (2006). Besides, these results are highest to those mentioned by Karoud et al., (2020) for hake head oil. The individual fatty acids and the SFA, MUFA and PUFA fractions did not showed significant differences between the crude and refined byproducts oils using enzymes or phosphoric acid as degumming agents. In addition, the refining treatments maintained the percentage content of individual fatty acids with respect to the crude oil.

In the ray liver oil PUFAs constituted the major group followed by MUFAs and SFAs. Among the SFAs, palmitic acid (C16:0) showed predominance followed by stearic acid (C18:0) and myristic acid (C14:0). These results agree with others found for liver oils extracted from other cartilaginous species (Navarro-García et al., 2009; Ould El Ould El Kebir et al., 2007; Sellami et al., 2018, Lamas & Massa, 2019). Oleic and palmitoleic fatty acids were predominant in the MUFAs fraction and the values obtained are consistent with data previously reported for *Z. Flavirostris* and *A. Castelnaui* (Lamas & Massa, 2019). The percentages of PUFAs found in were important (more than 40%). Interestingly, as in multispecies oil, the major ω−3 PUFAs were EPA (C20:5) and DHA (C22:6) with contents around 10% and 30% respectively. Both fatty acids are highest than that of salmon heads studied by Gbogouri et al. (2006) and other species such as G. marmorata (Navarro-García et al., 2009) cuttlefish (Shen et al., 2007), and Dosyatis violacea (Sellami et al., 2018). Furthermore, the contents achieved appear to be at admissible levels when compared to oils from ray livers from *Z. Flavirostris* (Lamas & Massa, 2019).

It has been shown that ω−3 PUFAs have antiatherosclerotic efficacy and can reduce diseases of the circulatory system (De Caterina, 2011; Martínez-González & Badimon, 2006). Also, they decrease total serum triacylglycerols, changes the balance of eicosanoids in favor of antiaggregant fractions, reduces platelet aggregation, prolonging the time of bleeding and blood pressure. On the other hand, ω−3 PUFAs may also contribute to the prevention of Alzheimer's disease (Gu et al., 2010) and age-related degeneration (Hodge et al., 2006). Likewise, the consumption of these fatty acids by pregnant women contributes to the correct development and health of the fetus and the newborn (Noakes et al., 2012; Palmer et al., 2012).

In the case of ray liver oils, all fractions were modified after refining. The traditional refining showed significant losses in the PUFAs fraction, mainly DHA. This may be due to the increase in temperature used during degumming, which caused the oxidation and loss of unsaturated fatty acids, while in the saturated fraction, a minimum increase in palmitic acid was associated. Other studies conducting deodorizing on tuna and squid oil have shown losses in polyunsaturated content explained that the high temperatures used in this process cause the breaking of double bonds of fatty acids (Fang et al., 2018; Fournier et al., 2007). Applying enzymatic degumming, the decrease in palmitic acid was accompanied by a slightly increase in EPA, while DHA was maintained with both enzymes. In addition, the increase in polyunsaturated could be attributed to the lipase action of the phospholipase A1 enzymes, its site of attack being the sn-1 position, favoring the sn-2 position of EPA and DHA bound to triglycerides, causing a slight enrichment (Cui et al., 2017). In the case of acyltransferase, this enzyme specifically hydrolyzes the 2-position of phospholipids. However, there are no studies that indicate its ability to hydrolyze triglycerides, allowing removing only acyl from specific macromolecules (Soe & Brown, 2011).

As mentioned above, PUFAs ω−3 prevent diseases and are important in the development and maintenance of neuronal activities, requiring an adequate relationship with their n-6 antagonists (Cortés et al., 2013). Several investigations about the ω−3 /n-6 ratio and its influence

Table 4
Fatty acids composition (w/w%) of crude and refined multispecies oil using different degumming treatments.

	Oil samples			
Fatty acids	Crude	Phosphoric acid	L. Ultra®Degummed	L. Oil®Degummed
14:0	3.30±0.20	3.37±0.10	3.41±0.57	3.36±0.27
15:0	0.41±0.0	0.41±0.02	0.41±0.05	0.40±0.03
16:0	19.24±0.34	19.78±0.32	20.23±0.70	20.07±1.32
17:0	0.37±0.06	0.40±0.02	0.42±0.04	0.41±0.04
18:0	2.30±0.11	2.41±0.42	2.47±0.22	2.53±0.21
Σ SFA	25.63a±0.50	26.37a±0.64	26.94a±1.50	26.77a±1.87
16:1	6.47±0.46	5.92±0.45	6.25±0.60	5.97±0.25
17:1	0.28±0.0	0.31±0.01	0.35±0.04	0.30±0.02
18:1n9	21.02±1.44	21.43±0.64	21.68±0.54	22.00±1.22
20:1n11	6.72±0.50	6.73±0.41	6.33±0.16	6.57±0.20
Σ MUFA	34.50a±0.55	34.40a±0.66	34.61a±0.04	34.84a±1.56
18:2n6	3.07±0.10	3.23±0.14	3.40±0.04	3.21±0.57
18:3n3	1.32±0.05	1.37±0.10	1.46±0.25	1.40±0.03
20:4n6 ARA	0.97±0.0	0.95±0.23	0.98±0.26	0.95±0.03
20:5n3 EPA	8.66±0.0	8.84±0.24	8.62±0.56	8.75±0.13
22:1n9	2.02±0.03	2.00±0.31	1.95±0.38	2.10±0.01
22:6n3 DHA	23.85±0.06	22.84±0.34	22.10±0.94	22.03±1.15
Σ PUFA	39.88a±0.06	39.24a±0.02	38.51a±1.4	38.43a±1.21

Different letters within the same line indicate significant differences ($P < 0.05$).
Σ SFA – sum of saturated fatty acids; Σ MUFA – sum of monounsaturated fatty acids; Σ PUFA – sum of polyunsaturated fatty acids.
Results are mean values of two determinations ± standard deviation.

Table 5
Fatty acids composition (w/w%) of crude and refined ray liver oil using different degumming treatments.

	Oil samples			
Fatty acids	Crude	Phosphoric acid Degummed	L. Ultra®Degummed	L. Oil®Degummed
14:0	2.54±0.30	3.075±0.22	2.44±0.14	2.43±0.33
15:0	0.30±0.01	0.431±0.01	0.38±0.01	0.37±0.00
16:0	18.25±0.33	19.46±0.13	15.63±1.10	15.10±1.47
17:0	0.45±0.05	0.51±0.04	0.46±0.02	0.50±0.14
18:0	2.47±0.37	2.89±0.17	2.98±0.32	2.87±0.17
Σ SFA	24.01b±0.37	26.37b±0.32	21.89a±0.64	21.28a±1.37
16:1	6.76±0.21	7.11±0.15	6.44±0.74	6.39±0.35
17:1	0.15±0.02	0.08±0.01	0.33±0.0	0.45±0.04
18:1n9	19.85±0.10	21.087±0.29	20.43±0.40	19.76±0.54
20:1n11	3.63±0.27	3.77±0.32	3.87±0.18	3.70±0.25
Σ MUFA	30.38a±0.17	32.047b±0.78	31.07b±1.32	30.30b±1.10
18:2n6	1.54±0.16	2.045±0.12	2.06±0.13	2.07±0.13
18:3n3	0.84±0.12	0.785±0.13	0.922±0.11	0.98±0.13
20:4n6 ARA	1.05±0.10	1.27±0.10	2.045±0.16	2.12±0.18
20:5n3 EPA	10.46±0.50	10.3 ± 0.40	11.04±0.73	11.08±1.40
22:1n9	0.63±0.05	0.67±0.15	1.20±0.27	1.33±0.29
22:6n3 DHA	31.60±0.62	26.67±0.58	29.74±2.40	30.56±0.21
Σ PUFA	46.11b±1.54	41.74a±1.30	47.00ab±1.85	48.16ab±1.40

Different letters within the same line indicate significant differences ($P < 0.05$).
Σ SFA – sum of saturated fatty acids; Σ MUFA – sum of monounsaturated fatty acids; Σ PUFA – sum of polyunsaturated fatty acids.
Results are mean values of two determinations ± standard deviation.

in different disease. Simopoulos (2009) reported that for the secondary prevention of cardiovascular disease, a ratio of 1:4 has been associated with a 70% reduction in total mortality, while Griffin et al. 14 concluded that the ω−3/ω−6 ratio is practically of no use in predicting changes in cardiovascular risk. Other studies reported that an adequate intake of both FFA, ω 6 and ω3, is essential for good health and for reducing the percentage of cardiovascular diseases (García-Ríos et al., 2009; Willett, 2007).

According to Bell et al. (1991), the increase in the dietary ω3/ω6 fatty acid ratio in favor of ω3 fatty acids also seems to be effective in preventing shock syndrome and cardiomyopathy. In the case of cancer, there is agreement regarding the need to increase the ω−3/ω−6 ratio, and according to some authors the ideal ratio may be 1:1 or 1:2 (García-Ríos et al., 2009). It was well demonstrated that ω−3 suppressing the development of most cancer processes, as breast, colon, prostate, liver and pancreatic (Kelavkar et al., 2006; Shannon et al., 2007; Simopoulos, 2009: Binukumar & Mathew 2005). Regarding to rheumatoid arthritis, several authors suggested the potential benefits that could be obtained by combining drug treatment with an adequate ω−3/ω−6 ratio, reporting significant changes particularly in inflammatory markers. Taking into account, the increased in ω−3 fatty acid consumption help to prevent different diseases. Increased ω−3 fatty acid consumption can be achieved with an increase in fish consumption

According Geri et al., (1995) common carp reared in warm water showed a higher ω−3/ω−6 PUFA ratio with 1.52 than carp reared in water of natural temperature with 0.47. Fajmonova et al. (2003) found ω−3 /ω−6 ratio as 0.5 in the carp fillets, and Guler et al. (2008) reported that the average of the carp ω−3 /ω−6 ratio is near 1 in winter, spring

and summer and decreased until 0.5 in autumn. In our study hake waste oil yielded values close to 8 for this ratio and for ray liver oils, the values obtained are in the range 17–22.

These results suggest that the oils under study could be refined for human consumption, since they have important nutritional properties.

4. Conclusion

All refining processes improve the physicochemical characteristics of crude oil. The use of enzymes during the degumming process is more effective in reducing the phosphorus content and also has several advantages, such as the use of low temperatures that prevent oxidation and loss of free fatty acids, and the easy separation of the gums obtained, avoiding oil losses.

In the case of multispecies oil, enzymatic degumming reduced the phosphorus content more efficiently than the traditional process, although for ray liver oil, phosphoric acid was a good degumming agent, reducing 90% of phosphorus initial content.

The refined oils obtained by both processes did not differ significantly in the oxidative level parameters, presenting adequate values for refined fish oils.

Density was corrected for all samples, probably after filtration. color and appearance were noticeably changed after refinement probably due the bleaching earth treatment that increased transparency and gloss, and remove pigments that impart brown tones. In addition, in both samples an additional improvement of this parameter could be observed when enzymes were used during the degumming process.

EPA and DHA were the predominant PUFAs in all oil samples. After multispecies oil refining, there were not significant differences among the SFA, MUFA, and PUFA fractions. As for the crude ray liver oil, the saturated fraction was slightly lower and the polyunsaturated fraction increased in the refined oil that had been degummed with enzymes, although the opposite effect was observed with traditional refineries.

Based on the results obtained in this work, the refining process using enzymatic degumming as an initial step could be a valid alternative to improve the physicochemical characteristics of crude while maintaining its nutritional profile.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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