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Supercritical fluid extraction of fish oil from fish by-products: a comparison with other extraction methods

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Abstract

Fish and fish by-products are the main natural source of omega-3 polyunsaturated fatty acids, especially EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), both of them with a great importance in the food and pharmaceutical industries. Comparing to conventional fish oil extraction processes such as cold extraction, wet reduction or enzymatic extraction, supercritical fluid extraction with carbon dioxide under moderate conditions (25 MPa and 313 K) may be useful for reducing fish oil oxidation, especially when fish oil is rich in omega-3 such as salmon oil, and the amount of certain impurities, such as some species of arsenic. Furthermore, taking profit of the advantages of supercritical carbon dioxide as extractive solvent, a coupled extraction - fractionation process is proposed as a way to remove free fatty acids and improve fish oil quality, alternatively to physical and chemical refining procedures.

Keywords

Fish oil. Omega-3 fatty acids. Supercritical fluid extraction. Carbon dioxide.

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1. Introduction

The fish industry is a wide sector that includes several production processes such as filleting, curing, salting, smoking, canning, etc. Nowadays, it is estimated that more than 70 % of the total fish captures are processed, generating a large amount of solid wastes and by-products, which often represent more than 50 % of the total fish weight (Shahidi, 2007) (*see* Figure 1). On the other hand, production of high quality fish oil has acquired a great importance since it is considered one of the main natural sources of omega-3 PolyUnsaturated Fatty Acids (PUFA), which benefits in human health have been extensively reported in the literature (Chow, 2000). Production of omega-3 rich fish oils has become a good opportunity for valorising fish by-products and increasing the competitiveness of the fish industry. In the last years, by-products from different types of fishes, such as tuna (Chantachum et al., 2000), herring (Aidos et al., 2002), salmon (Wu & Bechtel, 2008), or walleye pollock (Wu & Bechtel, 2009), have been proposed as raw materials for fish oil production. However, the production of high quality fish oil as source of omega-3 involves, not only searching for an omega-3 rich raw material, but also developing a suitable extraction procedure.

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, 1986). 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. Other processes, such as enzymatic reaction with proteases, have been studied for obtaining crude oil from fish by-products (Linder et al., 2005). In the last years, supercritical fluid extraction (SFE) has become an attractive technology for obtaining high quality fish oil from some by-products (Letisse et al., 2006, Rubio-Rodríguez et al., 2008), not only because it uses moderate temperatures and provides an oxygen free media, which aim to reduce the omega-3 oxidation during the extraction process, but also because it allows extracting selectively low polar lipid compounds, avoiding the co-extraction of polar impurities such as some inorganic derivatives with heavy metals. Furthermore, the tunability of the supercritical carbon dioxide (SC-CO_2) regarding density, and therefore solvation power, by changing temperature and/or pressure, makes fish oil de-acidification possible, alternatively to conventional physical

and chemical fish oil refining (Catchpole et al., 2000, Jakobsson et al., 1991, Jakobsson et al., 1994, Kawashima et al., 2006, Yuqian & Huashi, 2001).

The main limitation of the SFE process is the high cost at production scale, not only due to the use of high pressure equipment, but also because the raw material should be freeze-dried in order to reduce its moisture to values below 20 % and keep unaltered the omega-3 PUFA and the fish structure (Rubio-Rodríguez et al., 2008). Taking this into account, a study of the quality of the oil obtained by SFE and non-SFE methods would illustrate on the competitiveness of SFE from a commercial point of view.

The aim of this work is to compare different extraction processes (i.e.: cold extraction, wet reduction, enzymatic extraction and supercritical fluid extraction) to obtain oil from different fish by-products, at a laboratory scale, taking into account, not only the extraction yield, but also the oil quality.

2. Material and methods

2.1. Raw material and pretreatment

The raw materials studied were four different fish by-products supplied by Pescanova, a Spanish fish company located in Pontevedra (Spain), specifically, offcuts from hake (*Merluccius capensis – Merluccius paradoxus*) (H), orange roughy (*Hoplostethus atlanticus*) (OR) and salmon (*Salmo salar*) (S), and livers from jumbo squid (*Dosidicus gigas*) (JS). The offcuts consisted mainly of skin with some stuck muscle, obtained by peeling fishes with a TRIO™ peeler in open seas, whereas the livers were obtained during the evisceration process. For each species, the by-products used as raw material came from a unique batch (related to a certain place, season of fish capture and processing batch), which was delivered frozen at 253 K. In order to minimize the variability due to the different fish individuals and to improve the extraction rate, each batch received in the laboratory was cut in small pieces (1-10 mm equivalent diameter) with a cutter, packed in individual plastic bags under vacuum and kept frozen until the experiments were performed.

2.2. Oil extraction methods

Oil from each fish by-product was obtained in parallel by four different methods: Cold Extraction (CE) or centrifuging, Wet Reduction (WR), Enzymatic Extraction (EE) and Supercritical Fluid Extraction (SFE). The amount of raw material used in each extraction method was approximately 100 g. The experimental conditions used in each case are reported in Figure 2.

In cold extraction, wet reduction and enzymatic extraction, fish offcuts were previously thawed at room temperature during 12 hours, and the water co-extracted together with the oil was removed by centrifuging (Centrikon T-124, Kontron Instruments).

Enzymatic extraction was carried out following the method proposed by Gbogouri et al. (2006). The enzyme used was a food-grade protease, Alcalase 2.4 L (bacterial protease from *Bacillus licheniformis*), provided by Novozyme (Bagsvaerd, Denmark). The enzyme / substrate ratio was 0.05 w / w protein.

SFE was carried out in a semi-pilot plant under the optimal extraction conditions (p = 25 MPa, T = 313 K) found in a previous study (Rubio-Rodríguez et al., 2008). The raw material used for this extraction method was previously freeze-dried (FreeZone 12 L Console Freeze Dry System with drying chamber, Labconco). Some of the SFE experiments included fractionation of the extract by depressurization in two consecutive separators: the first separator (S1), which was kept at a pressure of 9 ± 0.5 MPa and a temperature of 308 ± 1 K, and the second separator (S2), which was kept at a pressure of 5 ± 0.5 MPa and a temperature 283 ± 1 K. Thus, the SC-CO_2 density in S1 was in the range 650 ± 50 kg / m^3, well below its density in the extractor (880 ± 5 kg / m^3) and above that in S2 (below critical density, $\rho_c = 468$ kg / m^3). This way, most of the triglycerides were recovered in S1 and most of the free fatty acids were recovered in S2. In all cases, the oil fractions were stored in closed vessels in darkness at -18 °C in order to minimize spoiling before characterization.

2.3. Analytical methods

2.3.1. Characterization of fish by-products

Fish by-products were characterized by determining their water, protein and fat content in order to establish their profitability as raw materials for oil extraction (see Table 1). Water and protein content were determined by the AOAC Official Methods 934.01 and 981.10 respectively (2000). Total fat content was determined by Soxhlet extraction using petroleum ether as solvent in a Büchi extraction system (model B-811). Soxhlet extraction was performed over freeze-dried samples in 140 minutes distributed in three stages: extraction (120 min), rinsing (10 min) and drying (10 min).

The amount of trace metals (Fe, Cu, Zn, As, Cd, Hg and Pb) was also determined. A wet digestion was firstly carried out over the samples in order to destroy the organic matter. A sample of about 20 mg was treated with 10 mL of HNO_3 65 % suprapur® (Merck, Germany) in a microwave oven (Ethos Sel, Milestone) provided with ten Teflon vessels (HPR-1000/10 S). The temperature program selected involved three heating steps (from room temperature to 80 °C in 4 min, from 80 °C to 130 °C in 7 min and from 130 °C to 170 °C in 5 min) followed by a constant heating at 170 °C for 10 min and a final ventilation step. After digestion, the samples were diluted to 25 mL with Milli-Q water, and analysed by ICP-MS (Agilent 7500 i).

2.3.2. Oil characterization

The quality of the oil obtained by the different extraction methods was evaluated by determining several parameters, i.e.: moisture and volatile matter content, neutral lipid composition, fatty acid profile, acidity value, peroxide value, anisidine value, volatile compounds profile and trace metals.

Moisture and volatile matter content was determined according to the IUPAC Standard Method (1964) by weight loss after heating in an oven at 105 °C during 30 min.

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 × 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 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.

The fatty acids profile was determined following the AOAC method (1995) as previously described by Rubio-Rodríguez et al. (2008).

The acidity value and the peroxide value (PV), were determined according to the AOCS Official Methods Ca 5a-40 and Cd 8-53 respectively (1990), whereas the anisidine value (AV) was evaluated according to the British Standard method BS 684-2.24 (2008). The TOTal OXidation value (TOTOX) was determined according to the expression (2 PV + AV) (Perrin, 1996).

Volatile compounds were analyzed by GC-MS after Solid Phase Dynamic Extraction (SPDE) sampling. The SPDE device (Chromtech, Idstein, Germany) was equipped with a needle coated with a non-polar 50 μm film of polydimethylsiloxane with 10 % embedded activated carbon phase (PDMS/AC). Samples were incubated for 1 min at 70 °C; and after equilibration, extraction was performed (50 aspiration cycles, extraction speed 40 μL/s). Gas chromatography analyses were carried out with a 6890N Series GC System coupled to a 5973i mass spectrometer (Agilent Technologies, Palo Alto. CA.USA). The SPDE needle was injected and thermally desorbed at 250 °C. Compounds were separated on a HP5 capillary column (50 m length x 0.32 mm I.D, fused silica capillary column coated with a 1.05 μm film thickness. Quadrex Corporation. New Haven. USA). The temperature of the column was increased at a rate of 3 °C / min from 40 to 240 °C.

The amount of trace metals (Fe, Cu, Zn, As, Cd, Hg and Pb) in fish oil was determined following the same procedure described above for fish by-products.

2.3.3. Oil sensory analysis

An off-odour comparison among the oils extracted by different methods was carried out both by electronic nose and by sensory analysis. The overall smell print was determined by an electronic nose αFOX 4000 (AlfaMOS, Toulouse, France) with a sensor array of 18 metal oxide sensors. Vials with the samples were incubated 5 minutes under stirring (500 rpm, cycles 5 s on and 2 s off) in an oven at 50 °C for generating the equilibrated headspace. The injection temperature was 60 °C; the carrier gas was synthetic air with a flow of 150 mL/min. Sensory characterization of oil was carried out by 10 panellists trained in descriptive analysis of fish off-flavours. A total of six sensory descriptors were used (fishy, rancid, boiled, acid, sweet and other off-flavours), which were measured on a structured intensity scale with a range from a minimum of zero to a maximum of five. Samples (0.5 g of oil) were presented randomized at room temperature in blind vials numbered with a code of three digits.

3. Results and discussion

3.1. General features on the extraction procedures used in this work

Extraction with SC-CO_2 has been proposed as a good method for obtaining fish oil with a high amount of omega-3 fatty acids, since it involves the use of a non-oxidant atmosphere and mild temperatures, which prevent the oxidation of the polyunsaturated fatty acids. Previous studies (Rubio-Rodríguez et al., 2008) have concluded that it is possible to totally extract the oil contained in hake offcuts by using SC-CO_2 at a pressure of 25 MPa and a temperature of 313 K. The highest yield and extraction rate were reached when the by-products were previously cut and freeze-dried to a moisture content below 20 % in order to improve the oil - SC-CO_2 contact and minimize oil – water interaction in the supercritical phase. The extraction curves obtained were well fitted to the empirical model proposed by Kandiah and Spiro (1990), which assumes that the process is controlled by two diffusion stages depending on the amount of oil accessible to the SC-CO_2. At the beginning, the amount of the most accessible oil is high, thus the internal mass transfer resistance is low and the extraction rate is large.

However, after the most accessible oil has been extracted, the remaining oil, less accessible to the solvent, is removed more slowly due to the higher internal mass transfer resistance (Rubio-Rodríguez et al., 2008).

SFE from other freeze-dried fish by-products i.e.: orange roughy offcuts, salmon offcuts and jumbo squid liver, has been also shown to be feasible under the same experimental conditions. Figure 3 shows the extraction curves where the amount of oil extracted along time can be observed for each species. In all cases, it was observed that, at the beginning of the process, the oil extracted depended linearly on the amount of SC-CO_2 that flows through the extractor, which may indicate either that the internal mass transfer is negligible and the process is controlled by the oil solubility in SC-CO_2 or that the internal mass transfer is constant and the extraction rate depends on the internal structure of the solid matrix. The values of the slopes of the extraction curves estimated at zero time are reported in Table 2. It is observed that these slopes differ significantly among the fish by-products studied, which may be attributed not only to the different internal structure, which affects the internal mass resistance in the extraction process, but also to the different solubility of the fish oil in SC-CO_2 due to the different neutral lipid composition and fatty acid profile, as can be seen in Tables 3 and 4 respectively.

The internal mass transfer resistance can be considered negligible for fish by-products with mostly extracellular oil as is the case of orange roughy oil (Phleger, 1998), or oil weakly bound to the protein matrix, as observed for salmon oil; therefore, the initial slope of their SFE curves in Figure 3 can be assumed to be close to the oil solubility in SC-CO_2, which, moreover, depends on the oil composition. Thus, for a fish oil rich in triacylglycerides (i.e.: salmon oil, see Table 3) this slope would be lower than for fish oil rich in wax esters (i.e.: orange roughy oil, see Table 3), which solubility in SC-CO_2 is usually higher than that of triacylglycerides (Gupta & Shim, 2007).

In the case of fish by-products, in which the oil is strongly bounded to the protein matrix (intracellular oil), the internal mass transfer resistance is important, reason for which, the slopes of the extraction curves are lower than the oil solubility values. This hypothesis may explain why in the case of hake offcuts or jumbo squid livers, both with an oil rich in triacylglycerides, the values of the initial slopes of the extraction curves (see Table 2) were not as high as the value obtained for salmon oil.

When using other methods, different than SFE (non-SFE methods), to obtain fish oil, the by-products were used just after being defrost, without freeze-drying.

Cold extraction was the easiest way for obtaining fish oil since it only involves a mechanical phase separation (solid, water and oil) by centrifuging. However, by using this procedure, in our laboratory, only oil from orange roughy and salmon offcuts could be obtained. This could be expected, since the oil of those fish by-products is the most weakly bound to the protein matrix, out of the four species considered in this work.

Other non-SFE methods, such as wet reduction, in which the protein matrix is previously denaturised by the action of heat in the cooking step, or enzymatic extraction, in which the protein matrix is hydrolysed by the action of the protease in the enzymatic reaction step, are expected to provide higher yields than cold extraction. The experiments performed in our laboratory showed that these two methods were suitable for obtaining oil from fatty fish by-products such as orange roughy or salmon offcuts, but not from lean fish by-products such as hake offcuts or jumbo squid liver. In these last cases, most of the oil appeared emulsified in either a cream or a skim fraction, stable even after a centrifugation step, probably due to the emulsifying effect of some fish proteins. A similar effect was observed after the aqueous extraction of oils from seeds, such as soybean, coconut or peanut (Rosenthal et al., 1996). In this case, the authors proposed a demulsification step (freezing and thawing, clear oil addition, high shear stress…) to break down the stable oil-in-water emulsion. Thus, among the by-products explored in this work, only those from fatty species, with a high amount of oil weakly bound to the solid matrix (orange roughy and salmon offcuts), were suitable for obtaining fish oil by any of the four methods proposed.

Figure 4 shows an estimation of the mass balance that results when obtaining oil from salmon offcuts by the four different extraction methods carried out in our laboratory. This estimation was observed to be also valid for oil extraction from orange roughy offcuts. It can be observed that SFE coupled with freeze-drying generates oil and a dry solid phase, rich in proteins (fish meal), whereas when using cold extraction or wet reduction, a high amount of oil still remains in the wet solid (press cake) obtained after centrifuging. This solid would require a subsequent treatment in order to obtain a dry and defatted fish meal. When using the enzymatic method, almost the total amount of

oil can be separated from the aqueous phase containing the protein hydrolysed by the action of the protease. The aqueous phase may be subjected to a subsequent drying step in order to obtain a dry fish protein hydrolysate.

3.2. Oil quality parameters

A comparison of the quality parameters of the oils obtained from orange roughy and salmon offcuts, by the four different methods considered in this work, was carried out. In the case of hake offcuts and jumbo squid livers, the only successful method for obtaining oil was SFE, the rest of the methods did not provide enough oil for the comparison to be made.

Some oil properties, such as colour, neutral lipid composition and fatty acid profile, were observed to be similar, regardless the extraction method used. Thus, the advantages and disadvantages of the different methods have been established by comparing other properties such as oil acidity, total oxidation value (TOTOX), volatile compounds, sensory properties and heavy metal content for orange roughy and salmon oils.

3.2.1. Oil acidity

Oil acidity is an important quality parameter related to the presence of Free Fatty Acids (FFA) and other non-lipid acid compounds. FFA are mainly generated by a hydrolysis reaction of triacylglycerides, whereas non-lipid acid compounds, such as acetic acid, may be generated during spoilage of the raw material. Thus, oil acidity depends on several factors related to oil composition, the extraction procedure and the raw material freshness.

Figure 5 shows the acidity values found for the oils obtained from orange roughy and salmon offcuts by the four methods used in our laboratory. Focusing on oil composition (*see* Tables 3 and 4), we observed that, on the average, oil obtained from salmon offcuts, with a high amount of triacylglycerides and PUFA, presented a higher acidity than oil obtained from orange roughy, which was mainly composed by wax esters and a low amount of PUFA. Focusing on the oil extraction method, it can be observed that, when salmon offcuts were used as raw material, the oil obtained by SFE presents lower

acidity than the oils obtained by non-SFE procedures, which may indicate that, in this case, the hydrolysis of triacylglycerides, and therefore the release of FFA, was less extended. However, in the case of orange roughy offcuts, it was observed that, in spite of having a negligible FFA content, as detected by the neutral lipid analysis (see Table 3), the oil obtained by SFE shows a higher acidity value than the oils obtained by non-SFE methods. These experimental results may be explained taking into account that some acidic compounds, such as acetic acid, were co-extracted together with the oil when SC-CO_2 was used as solvent in a closed system, while they were not obtained by the non-SFE methods carried out in open vessels (see section 3.2.3).

3.2.2. Total oxidation value (TOTOX)

The total oxidation value is a quality parameter related to the presence of different compounds such as hydroperoxides, aldehydes, ketones.., which are mainly generated by PUFA degradation under pro-oxidant conditions, especially high temperatures, oxygen, metal compounds and light. The TOTOX value is therefore intrinsically related to the PUFA amount in the oil and to the extraction procedure. Figure 6 shows that, regardless the extraction method, salmon oil, with a higher PUFA content than orange roughy oil, presents also a higher TOTOX value. On the other hand, the SFE method, which was carried out under lower oxidising conditions (mild temperatures, non-oxidant atmosphere, darkness) than the non-SFE procedures, made possible to reduce significantly the TOTOX value in both salmon and orange roughy oil.

3.2.3. Volatile compounds and sensory properties

Sensory properties related to odour and flavour in fish oil, are strongly dependent on the presence of volatile compounds such as organic acids, amines or aldehydes, which are mostly responsible for the main fishy off-flavours. Some of these volatile compounds, such as hexanal or nonanal, are mostly generated as a consequence of a lipid auto-oxidation process, and, therefore, their presence in fish oil is intrinsically affected by the extraction parameters, especially temperature, oxygen in the media, light or metal content. On the contrary, other volatile compounds are produced during fish storage or spoilage by bacterial and / or enzymatic action over protein, aminoacids and

carbohydrates, and thus, their presence in the oil may be attributed to the raw material freshness (*see* Table 5). That is the case of trimethylamine which is mainly produced by the action of specific spoilage bacteria, such as *Shewanella putrefaciens*, of dimethylamine, mainly produced by endogenous enzymes during fish storage, or of the acetic acid which can be produced by anaerobic degradation of aminoacids (Huss, 1995).

As can be observed in Table 6, where the volatile compounds found in the oil obtained from orange roughy offcuts by the four different methods essayed in this work are presented, hexanal and nonanal, mainly generated by lipid oxidation, were only detected in the oil obtained by non-SFE methods, which explain the highest level of rancid odour detected by sensory analysis of these oils, especially in that obtained by enzymatic extraction (*see* Figure 7). These results show that, due to the use of mild temperatures and a non-oxidizing atmosphere, SFE made possible to reduce the lipid oxidation more than the non-SFE processes. On the contrary, dimethylamine, responsible for the highest level of fishy odour detected in this oil by sensory analysis (*see* Figure 7), and acetic acid, responsible for the unexpected high acidity value (see section 3.2.1.), were only detected in the oil obtained by SFE (*see* Table 6). These experimental results could be explained taking into account that these volatile compounds, soaked in the raw material, can be easily extracted by SC-CO_2 due to their high vapour pressure, and, since the process takes place in a solvent recirculation system, they may partially remain absorbed by the oil. In any case, the amount of these volatile compounds may be significantly reduced by coupling SFE with other separation process such as countercurrent fractionation or adsorption, as has been previously reported in the literature (Rubio-Rodríguez et al., 2010).

The different volatile compounds profile found in the oil obtained from orange roughy offcuts by SFE, compared with that profile for the oils obtained by the non-SFE methods, is also observed in the results obtained from the electronic nose analysis, as illustrated by the Principal Component Analysis (PCA) of the data shown in Figure 8, as well as was found by the trained panel (Figure 7).

These results show that the freshness of the raw material is crucial for obtaining good quality sensory properties in the oil, especially if oil production is carried out in a closed system and with SC-CO_2 as solvent.

3.2.4. Toxic heavy metals

Heavy metals, such as As, Cd, Hg and Pb, are toxic compounds that, in some cases, may be accumulated in some fish parts, such as fish offcuts or livers, due to water pollution (*see* Table 7). In this work, focus has been brought into As since it was detected at a level higher than 1 mg / kg in oil fraction in all the by-products explored in this work.

Due to the high selectivity of SC-CO_2 for non-polar compounds, the amount of heavy metals extracted together with the oil by SFE was negligible, as was the case of Cd, Hg and Pb, or significantly reduced, as in the case of As (*see* Table 7).

Total As content includes inorganic and organic derivatives, which are present in sea water due to natural processes (Smedley & Kinniburg, 2002) and pollution. These As derivatives may bio-accumulate in marine organisms, being the water soluble form, arsenobetaine, the main species found in fish (Ackley et al., 1999). However, recent studies have also found considerable amounts (4.3 – 10.5 ppm) of non polar lipid bound As compounds or arsenolipids in ten different crude fish oils (Schemeisser et al., 2005). Figure 9 shows the As concentration found in orange roughy and salmon oils obtained by the four different methods proposed in this work. It can be observed that, in the case of orange roughy oil, SFE made possible to reduce significantly the amount of As, whereas in the case of salmon oil, this reduction was not significant compared with the oil extracted by the non-SFE methods. These results suggest that the success of SFE for reducing the amount of As in oil is strongly dependent on the type of As species present in the raw material. Reduction down to the recommended values could be achieved by countercurrent SFE.

3.3. Enhancement of oil quality through SFE-fractionation

SFE followed by fractionation in two separators was studied as a way to refine fish oil and reduce the amount of impurities, especially free fatty acids, which, in general, are more soluble in SC-CO_2 than the relative triacylglycerides.

Fractionation was only applied to the species that provided the best oil regarding triacylglycerides and omega-3 fatty acids content, that is, hake and salmon offcuts and jumbo squid liver SFE.

Table 8 shows the average amount of oil recovered in each separator for different experiments together with the calculated mass percentage. It can be observed that, in all cases, a higher amount of oil is recovered in S1, although this amount varies from 63 % in hake oil to 86 % and 83 % in salmon and jumbo squid liver oil respectively, which may be attributed to the different fish oil composition, as it is reported in Tables 3 and 4, and to the experimental CO_2 density fluctuations in Separator 1 (S1).

The mass percentage distribution of triacylglycerides (TAG) and FFA between the two separators is also presented in Figure 10. It can be observed that, in all cases, most of TAG are collected in S1, which can be explained by considering their higher molecular weight and lower vapour pressure, and therefore their lower solubility regarding FFA. However, the distribution of fatty acids varies significantly among different fish oils, which may be attributed to the different fatty acid profile (see Table 4). Thus, in the case of hake oil and salmon oil, in which palmitic and oleic are the main fatty acids, the majority of FFA reach Separator 2 (S2), whereas in the case of jumbo squid oil, in which palmitic acid and EPA are the most common fatty acids, a large among of FFA remain in S1. These experimental results indicate again that fractionation is highly affected by fish oil composition.

Finally, it is observed that, in all cases, the mass ratio FFA / TAG increases noticeably in the fraction recovered in S2, and decreases in the fraction recovered in S1 (*see* Figure 11), although in the case of jumbo squid liver oil, this fraction still remains fairly high in S1.

A comparison of the fatty acid profile of the different oil fractions and the oil without fractionation is presented in Figure 12. It can be observed that, in general, the concentration of fatty acids is higher in the fraction recovered in S1, which is related to the fact that most neutral lipids remain in that fraction. The long chain (LCFA) to short chain fatty acids (SCFA) ratio and the saturated (SFA) to unsaturated (MUFA and

PUFA) fatty acids ratio do not show a significant variation between the two lipid fractions (*see* Figure 13).

Finally, as can be observed in Figure 14, fractionation in two separators may offer the possibility of obtaining a fraction in the first separator with a lower acidity value and total oxidation value (TOTOX) than in the fish oil without fractionating. In any case, a countercurrent oil fractionation would be much more effective that the simple fractionation after extraction carried out in this work, as it has been observed in previous studies (Rubio-Rodríguez et al., 2010).

3.4. Economical considerations

During the last decades, different supercritical fluid technologies have been established as interesting for safely processing natural products in the food and pharmaceutical industries. Nowadays, several processes such as coffee decaffeination, hops extraction, essential oils extraction, cork cleaning, pesticides removal from rice, etc., are carried out at commercial scale in different parts of Europe, US and Asia (Brunner, 2010, Perrut, 2000). Some studies have shown that, in spite of requiring a high investment cost, supercritical fluid extraction of essential oils requires lower processing costs and downstream processing making this process competitive regarding steam distillation (Pereira & Meireles, 2007). Concerning the processing of fats and oils, SFE may also compete with traditional processes in the case of specialty oils such as nut oils (almond, peanut...), seed oils (apricot, borage, grape, sesame...), cereal oils (wheat germs, rice bran...) or fruit oils (cloudberry, tomato...), which contain bioactive lipid compounds interesting in the food and pharmaceutical industries (Temelli, 2009). In the case of fish oil extraction, although SFE leads to high quality oil, the drying step, required before extraction, increases noticeable the production cost and minimizes competitiveness against alternative extraction processes. Thus, the industrial application of supercritical fluid technology in omega-3 processing should be focused not only on an isolated SFE procedure but on a whole process involving the use of SC-CO_2 in fish oil extraction, fractionation, omega-3 concentration and / or formulation (Rubio-Rodríguez et al., 2010) in order to obtain small volumes of high value omega-3 concentrates used as ingredients in functional foods or as active principles in pharmacology.

4. Conclusions

The valorisation of fish by-products by recovering their oil has a great interest in the fish industry, especially when the oil is rich in triglycerides and has a high content of omega-3 polyunsaturated fatty acids. The extraction process used to obtain omega-3 rich oils has been also shown to be important to obtain the best oil quality regarding lipid oxidation, pollutants content and sensory properties. In addition, the extraction method may not only affect the oil extraction yield and quality, but also the quality of the fish protein or fish meal obtained, which has also a great interest as add value ingredient.

A comparison of the oils obtained by SFE over freeze-dried fish by-products and by other methods carried out in the laboratory (cold extraction, wet reduction and enzymatic extraction), shows that SFE may be a useful method to prevent lipid oxidation, especially in fish oils rich in omega-3 such as salmon oil, and, to reduce significantly the amount of certain pollutants such as some arsenic species (mainly polar derivatives). Nonetheless, it has been observed that SFE may involve the co-extraction of some endogenous volatile compounds soaked in the raw material, such as amines or short chain organic acids, when performed in a closed system, which reduce oil quality by increasing the fishy odour and the acidity. That suggests that the success of a SFE method is highly dependent on the quality and freshness of the raw material and, in some cases, coupling a subsequently deodorization step would be required. On the other hand, SFE over freeze dried fish made possible to extract oil from by-products with a low fat content such as hake offcuts or jumbo squid liver, avoiding production of water wastes rich in proteins or fat, which have an important interest both from an economical and an environmental point of view. Therefore, in spite of involving higher inversion costs, SFE presents some advantages over other extraction processes such as cold extraction, wet reduction or enzymatic extraction. Furthermore, fractionation of the extract in two separators after SFE is an easy way to enhance fish oil quality by reducing the amount of free fatty acids as well as some oxidation products.

5. Acknowledgements

The authors gratefully acknowledge the Ministry of Education and Science (CTQ2005-07301), Junta de Castilla y León (GR 167) and Pescanova S.A. (Spain) for financial support.

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539 (*Theragra chalcogramma*) Byproducts. *JAOCS, Journal of American Oil Chemists' Society*, 86 903-908.

540 Wu, T.H., & Bechtel, P.J. (2008). Salmon by-product storage and oil extraction. *Food Chemistry*, 111
541 868–871.

542 Yuqian, H. & Huashi, G. (2001) Preparation of high-grade fish smell-eliminating fish oil. Patent
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544

545 Table 1. Composition of the different by-products studied (H: Hake offcuts, S: Salmon
546 offcuts, OR: Orange Roughy offcuts, JS: Jumbo Squid livers) as potential sources of
547 marine oil.

Marine by-products	Water (%)	Fat (%)	Protein (%)	Oil / water ratio
H	79 ± 1	4.0 ± 0.1	16 ± 1	0.1
OR	55 ± 2	32 ± 1	13 ± 1	0.6
S	57 ± 3	27 ± 5	17 ± 4	0.5
JS	70 ± 1	8 ± 3	22 ± 4	0.12

548

549 Table 2. Initial slope, w_p, of the fish oil extraction curves presented in Figure 3.

	H	OR	S	JS
w_p (g oil / kg CO_2)	3.0	10.0	5.2	3.0

550

551 Table 3. Neutral lipids profile in marine oils obtained by SFE from different fish by-
552 products: H: Hake offcuts, S: Salmon offcuts, OR: Orange Roughy offcuts, JS: Jumbo
553 Squid livers

Neutral lipids	% wt. in oil			
	H	OR	S	JS
Wax esters (WE)	n.d.	> 99	n.d.	0.6
Triacylglycerides (TAG)	67	n.d.	97.1	30.4
Free Fatty Acids (FFA)	3.8	n.d.	1.3	7.1
Cholesterol (CHOL)	1.8	n.d.	0.7	4.9

Concentration expressed using palmityl palmitate, tripalmitine and palmitic acid as standards for obtaining the calibration curves of wax esters, triacylglycerides and fatty acids respectively. n.d. not detected.

554

555 Table 4. Fatty acid profile in marine oils obtained by SFE from different marine by-
556 products: H: Hake offcuts, S: Salmon offcuts, OR: Orange Roughy offcuts, JS: Jumbo
557 Squid livers.

Fatty acids	mg of fatty acids / g fish oil			
	H	OR	S	JS
C14:0 (myristic acid)	19 ± 2	4.0 ± 0.3	40.4 ± 0.1	39 ± 3
C16:0 (palmitic acid)	129 ± 12	6.4 ± 0.4	143 ± 0.4	141 ± 12
C16:1 (palmitoleic acid)	34 ± 6	44 ± 3	59 ± 1	43 ± 4
C18:0 (stearic acid)	21 ± 2	2.5 ± 0.2	46.4 ± 0.1	43 ± 4
C18:1n-9 (oleic acid)	142 ± 13	213 ± 13	146 ± 2	42 ± 4
C18:1n-7 (vacenic acid)	22 ± 2	24 ± 2	28.9 ± 0.1	23 ± 2
C18:2n-6 (LA)	7.0 ± 0.7	4.7 ± 0.3	93 ± 1	5.8 ± 0.5
C18:3n-6 (GLA)	1.9 ± 0.2	1.4 ± 0.1	5.5 ± 0.1	3.1 ± 0.2
C18:3n-3 (ALA)	2.6 ± 0.3	n.d.	14.0 ± 0.2	2.9 ± 0.3
C18:4n-3 (stearidonic acid)	3.2 ± 0.4	n.d.	5.9 ± 0.1	2.0 ± 0.1
C20:1n-9 (gadoleic acid)	37 ± 3	50 ± 3	13.1 ± 0.4	24 ± 2
C20:3n-6 (DGLA)	0.82 ± 0.03	1.5 ± 0.1	3.2 ± 0.2	1.6 ± 0.2
C20:4n-6 (araquidonic acid)	5.5 ± 0.6	n.d.	6.7 ± 0.1	127 ± 10
C20:5n-3 (EPA)	36 ± 4	3.2 ± 0.2	79 ± 1	127 ± 10
C22:1n-11	28 ± 2	19 ± 1	n.d.	5.6 ± 0.1
C22:1n-9	4.2 ± 0.3	6.5 ± 0.4	n.d.	1.5 ± 0.1
C22:4n-6 (adrenic acid)	4 ± 2	n.d.	n.d.	5.4 ± 0.4
C22:5n-3 (DPA)	8.0 ± 0.7	n.d.	38.4 ± 0.7	22 ± 1
C22:6n-3 (DHA)	82 ± 8	5.2 ± 0.3	63 ± 1	130 ± 9
C24:1 (nervonic acid)	7.8 ± 0.6	2.9 ± 0.1	2.5 ± 0.1	2.8 ± 0.3
Total fatty acids	595 ± 53	388 ± 24	789 ± 7	691 ± 74
Total Saturated Fatty Acids (SFA)	168 ± 15	13 ± 1	230 ± 1	223 ± 19
Total MonoUnsaturated Fatty Acids (MUFA)	275 ± 24	359 ± 22	250 ± 3	156 ± 32
Total PolyUnsaturated Fatty Acids (PUFA)	151 ± 14	16 ± 1	309 ± 5	312 ± 23
Total ω3 fatty acids	132 ± 14	8 ± 1	100 ± 3	284 ± 20
Total ω6 fatty acids	19 ± 1	11 ± 1	108 ± 2	29 ± 2

558

559 Table 5. Volatile compounds produced by different fish degradation processes (adapted
560 from Huss (Huss, 1995).

Process	Substrate	Compounds produced
Bacterial degradation	Trimethylamine oxide	Trimethylamine
	Cysteine	H_2S
	Methionine	CH_3SH, $(CH_3)_2S$
	Carbohydrates and lactate	Acetate, CO_2, H_2O
	Inosine	Hypoxanthine
	Glycine, serine, leucine	Esters, ketones, aldehydes
	Urea	NH_3
Enzymatic action	Trimethylamine oxide	Dimethylamine
Autooxidation process	Lipids	Aldehydes
		Ketones
		Alcohols
		Short-chain organic acids
		Alkanes
Anaerobic (spoilers)	Aminoacids	NH_3, acetic acid, butyric acid, propionic acid

561

562 Table 6. Volatile compounds found in the oil obtained from orange roughy offcuts by
563 different methods: Cold Extraction (CE), Wet Reduction (WR), Enzymatic Extraction
564 (EE) and Supercritical Fluid Extraction (SFE)

Compound		Odor characteristics	Extraction method			
			CE	WR	EE	SFE
Alkanes	Decane	---	✓	✓	✓	✓
	2-methyl-Decane	---	×	×	×	✓
	3-methyl-Decane	---	×	×	×	✓
	Undecane	---	✓	✓	✓	✓
	Dodecane	---	✓	✓	✓	✓
	Tridecane	---	✓	✓	✓	✓
	Pentadecane	---	✓	✓	✓	✓
	Cyclohexadeccane	---	×	×	×	✓
	2,6,10,14-tetramethyl-Pentadecane	---	✓	✓	✓	✓
Aldehydes	Heptanal	Waxy, green	×	✓	×	×
	Hexanal	Green	✓	✓	✓	×
	Nonanal	Fatty, floral	×	×	✓	×
Acids	Acetic acid	Vinegar-like	×	×	×	✓
Amines	Dimethylamine	Fishy	×	×	×	✓

✓ found × not found

565

566 Table 7. Heavy metals in marine oils obtained by SFE from different marine by-
567 products: Hake (H), Salmon (S) and Orange Roughy (OR) offcuts and Jumbo Squid
568 (JS) livers.

Raw material		mg / kg (in oil fraction)						
		Fe	Cu	Zn	As	Cd	Hg	Pb
H	A	83.4 ± 0.9	11.6 ± 0.4	114.7 ± 0.3	33.8 ± 0.1	n.d.	3 ± 1	n.d.
	B	n.d.	0.07 ± 0.04	1 ± 1	0.05 ± 0.04	n.d.	n.d.	n.d.
OR	A	5.3 ± 0.1	0.8 ± 0.3	31.3 ±0. 3	2.6 ± 0.1	n.d.	n.d.	n.d.
	B	n.d.	n.d.	1.5 ± 0.6	0.26 ± 0.03	n.d.	n.d.	n.d.
S	A	22.3 ± 0.4	1.9 ± 0.2	27.2 ± 0.1	1.5 ± 0.2	n.d.	0.5 ± 0.2	n.d.
	B	2 ± 1	0.10 ± 0.01	n.d.	0.89 ± 0.05	n.d.	n.d.	n.d.
JS	A	$> 10^3$	$> 10^3$	726 ± 1	207 ± 1	$> 10^3$	12 ± 1	5 ± 1
	B	10.3 ± 0.2	0.48 ± 0.01	1.1 ± 0.1	6.7± 0.3	n.d.	n.d.	0.07 ± 0.01

A: in fish by-products; B: in fish oil obtained by SFE

n.d.: not detected

569

570 Table 8. Lipid fraction recovered in each separator in SFE-fractionation of different fish
571 oils obtained from different by-products: Hake (H) and Salmon (S) offcuts and Jumbo
572 Squid (JS) livers. Extraction conditions: 25 ± 0.5 MPa / 313 ± 1 K. S1: 9 ± 0.5 MPa /
573 308 ± 1 K. S2: 5 ± 0.5 MPa / 283 ± 1 K.

	g oil /100 g dry material			%	
	S1	S2	total	S_1	S_2
H	11 ± 1	7 ± 2	18 ± 1	63	37
S	44 ± 2	7 ± 1	51 ± 1	86	14
JS	14 ± 1	3 ± 1	17 ± 1	83	17

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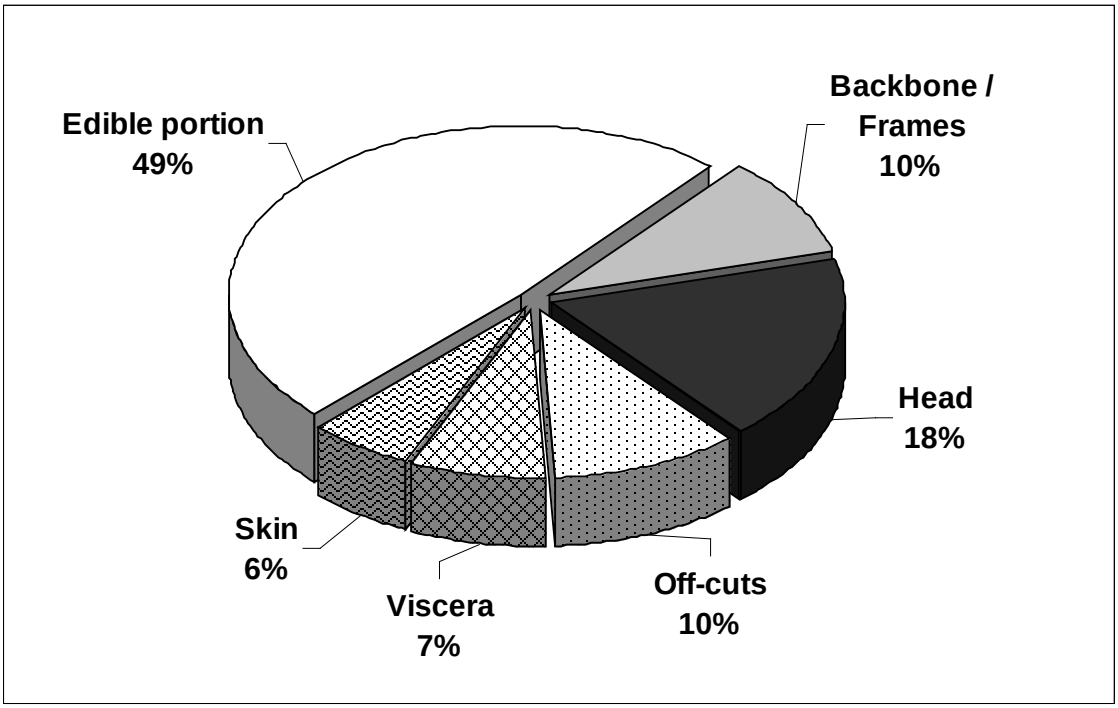
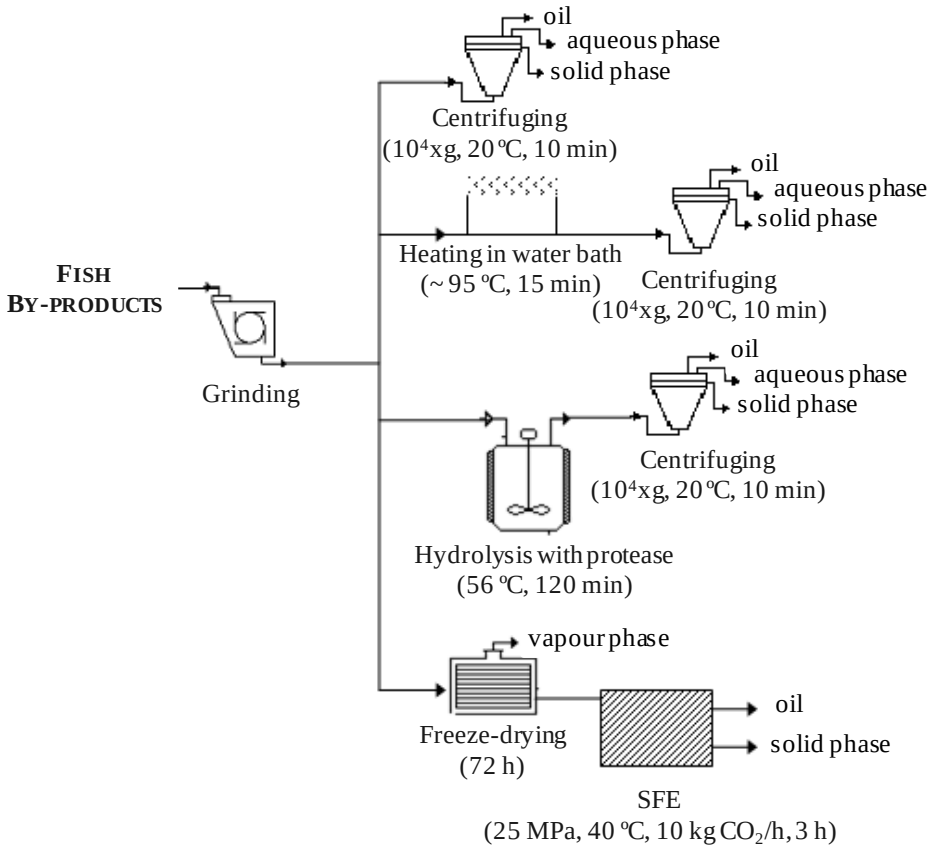


575

576 Figure 1. Average distribution of edible portion and by-products in fish (Data taken
577 from Rustad (2007)

578



579

580 Figure 2. Scheme of the different fish oil extraction procedures studied in this work.

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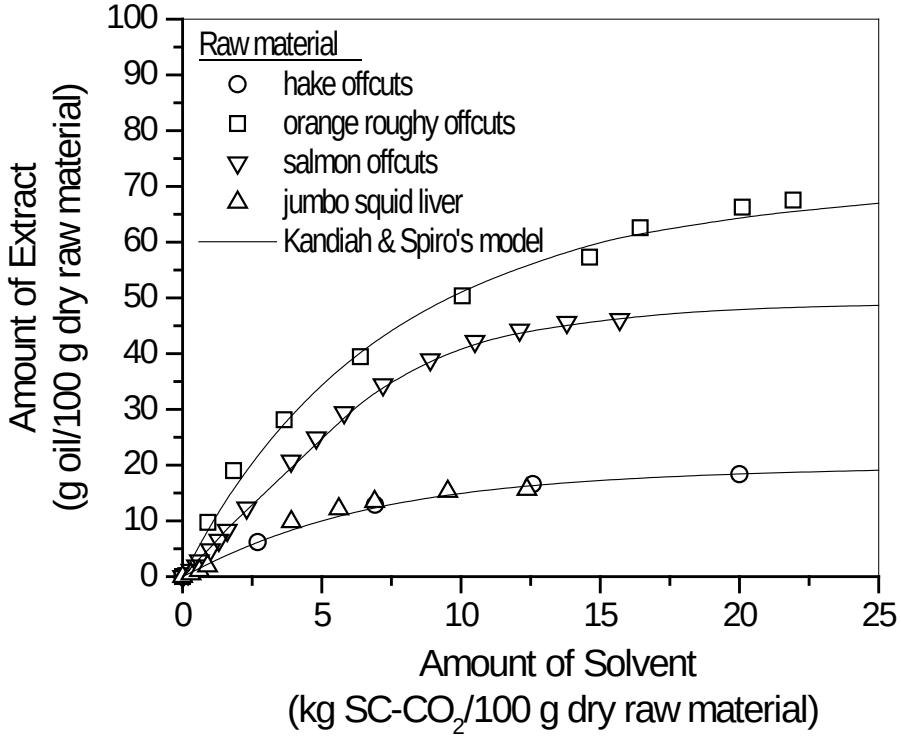

582

583 Figure 3. Extraction curves obtained for SFE of oil from different fish by-products. The
584 continuous lines represent the correlation of the experimental data through the model
585 proposed by Kandiah & Spiro (1990). Extraction conditions: 25 ± 0.5 MPa / 313 ± 1 K.

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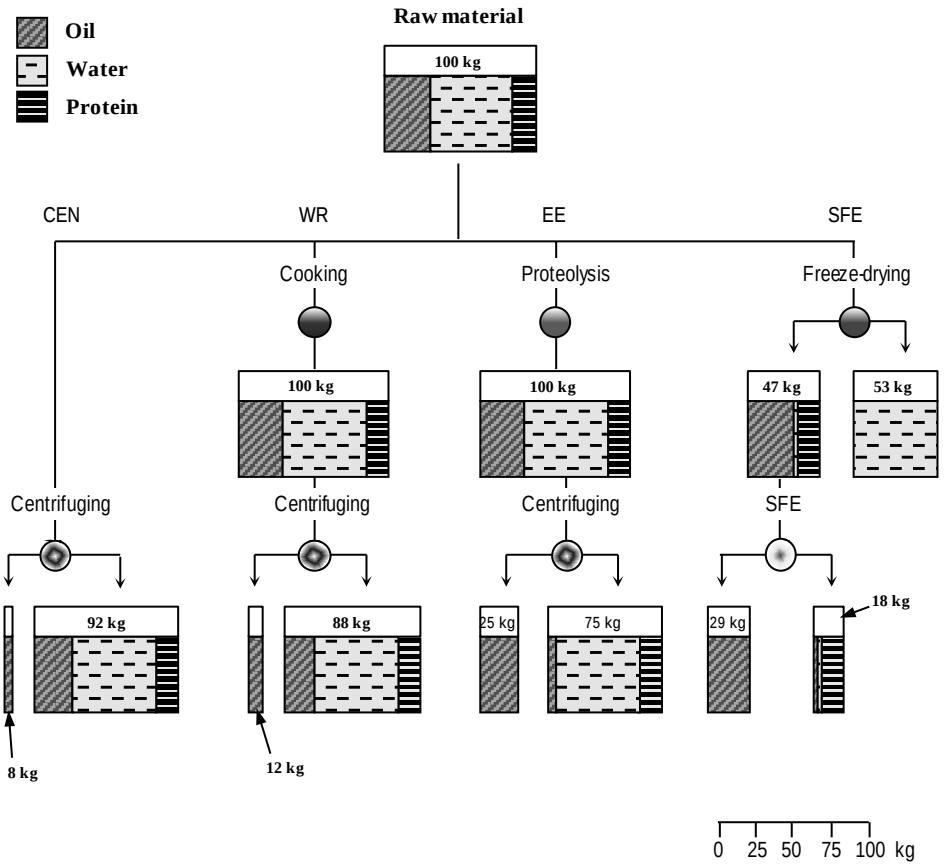
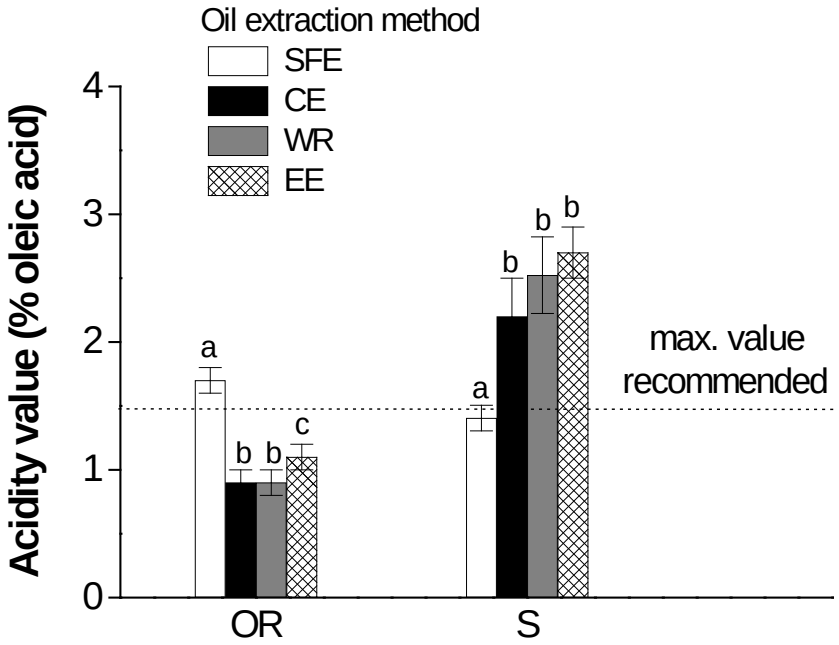


588

589

590 Figure 4. Estimation of the mass balance that results when obtaining oil from salmon
591 offcuts by the four different extraction methods carried out at laboratory scale: Cold
592 Extraction (CE), Wet Reduction (WR), Enzymatic Extraction (EE) and Supercritical
593 Fluid Extraction (SFE)

594



595

596 Figure 5. Acidity value of the oil extracted from orange roughy (OR) and salmon (S)
597 offcuts by different methods: Cold Extraction (CE), Wet Reduction (WR), Enzymatic
598 Extraction (EE) and Supercritical Fluid Extraction (SFE). Determinations were carried
599 out in triplicate and the results are the average values ± standard deviation. Means with
600 the same letter within the same species are not significantly different ($p > 0.05$).

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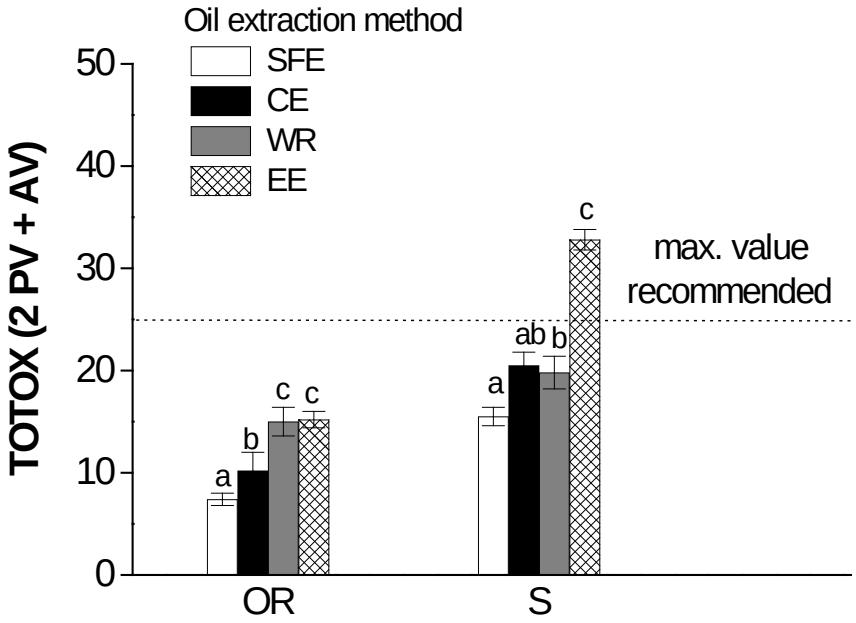
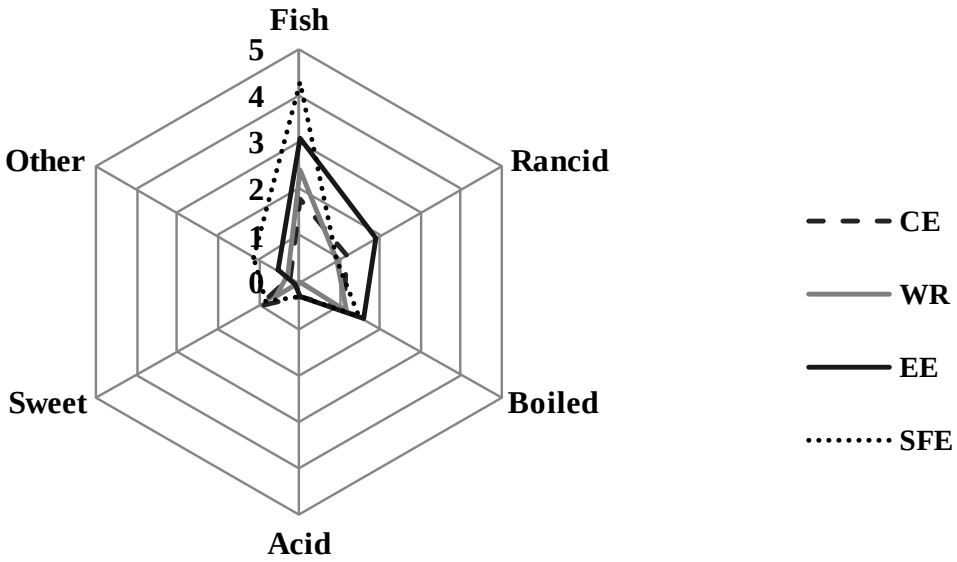


602

603 Figure 6. Total oxidation value (TOTOX) of the oil extracted from orange roughy (OR)
604 and salmon (S) offcuts by different methods: Cold Extraction (CE), Wet Reduction
605 (WR), Enzymatic Extraction (EE) and Supercritical Fluid Extraction (SFE).
606 Determinations were carried out in triplicate and the results are the average values ±
607 standard deviation. Means with the same letter within the same species are not
608 significantly different ($p > 0.05$).

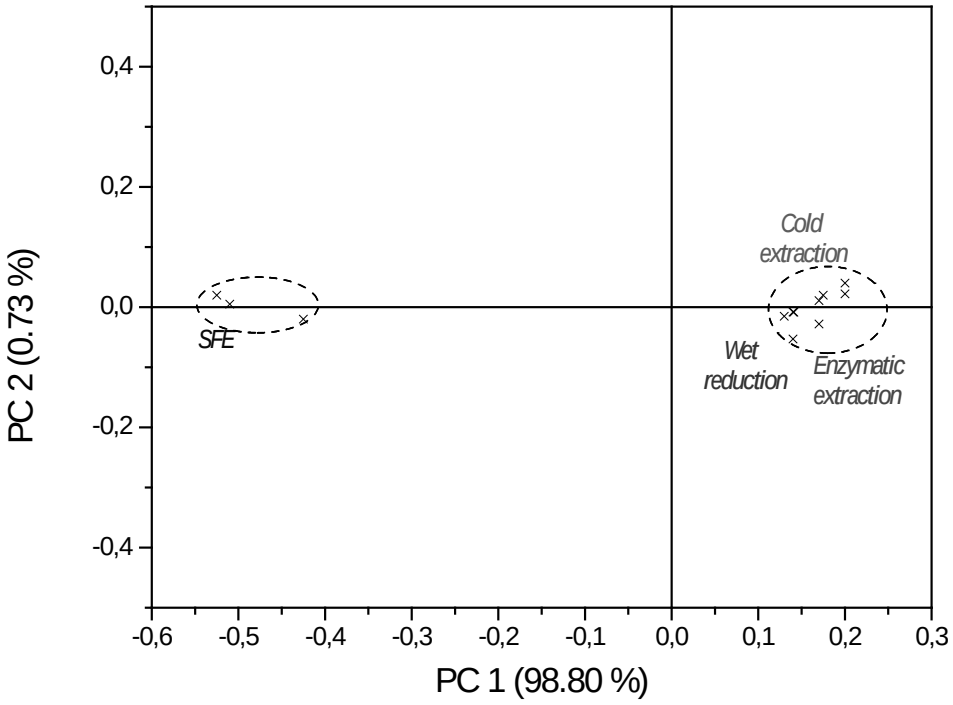
609



610

611 Figure 7. Sensory analysis of the oil extracted from orange roughly offcuts by different
612 methods: Cold Extraction (CE), Wet Reduction (WR), Enzymatic Extraction (EE) and
613 Supercritical Fluid Extraction (SFE)

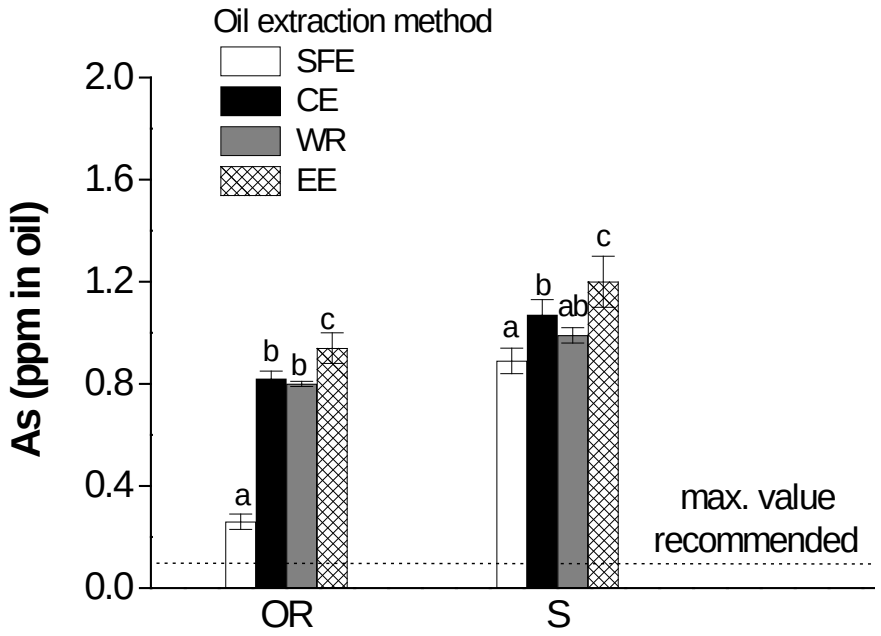
614



615

616 Figure 8. Principal Component Analysis (PCA) of the data obtained with the electronic
617 nose for the oil extracted from orange roughly offcuts by different methods.

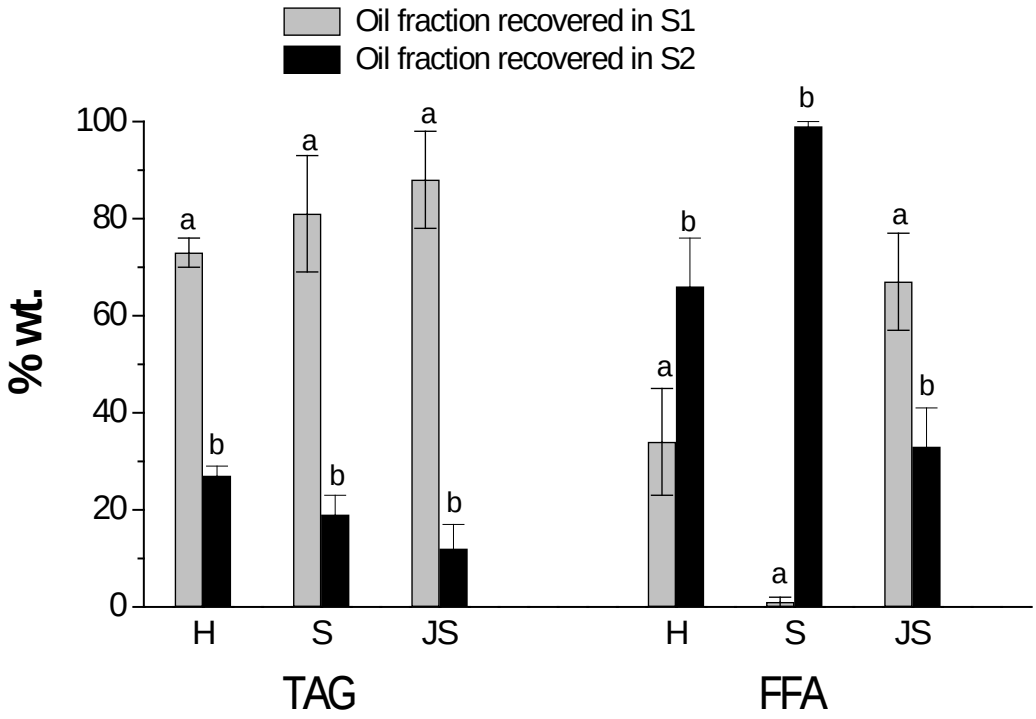
618



619

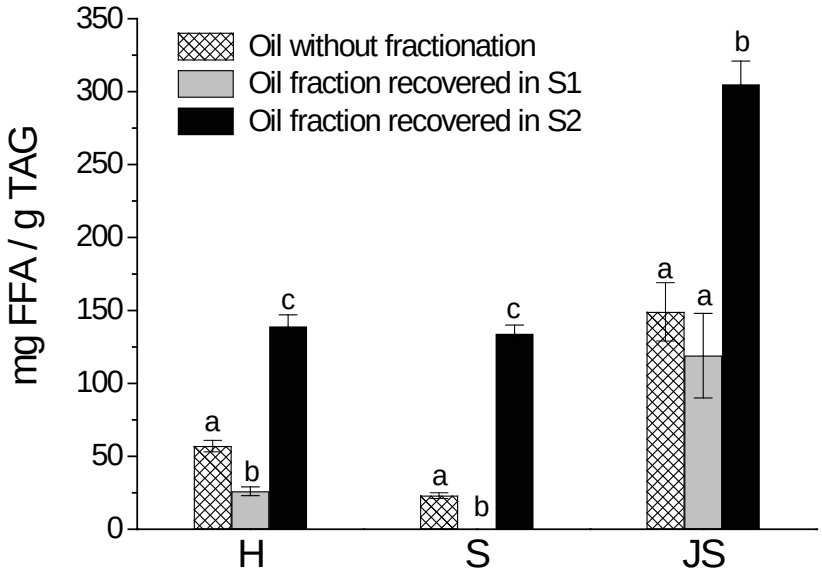
620 Figure 9. Total arsenic content found in the oil extracted from orange roughy (OR) and
621 salmon (S) offcuts by different methods: Cold Extraction (CE), Wet Reduction (WR),
622 Enzymatic Extraction (EE) and Supercritical Fluid Extraction (SFE). Determinations
623 were carried out in triplicate and the results are the average values ± standard deviation.
624 Means with the same letter within the same species are not significantly different ($p > 0.05$).

626



627

628 Figure 10. Mass percentage distribution of triacylglycerides (TAG) (left) and free fatty
629 acids, (FFA) (right) between both separators in fish oil fractionation. H: Hake oil. S:
630 Salmon oil. JS: Jumbo squid oil. Determinations were carried out in triplicate and the
631 results are the average values ± standard deviation. Means with the same letter within
632 the same species are not significantly different ($p > 0.05$).



633

634 Figure 11. FFA (free fatty acids) to TAG (triacylglycerides) mass ratio in fish oils
635 before and after fractionation. H: Hake offcuts oil. S: Salmon offcuts oil. JS: Jumbo
636 squid livers oil. Determinations were carried out in triplicate and the results are the
637 average values ± standard deviation. Means with the same letter within the same species
638 are not significantly different ($p > 0.05$).

639

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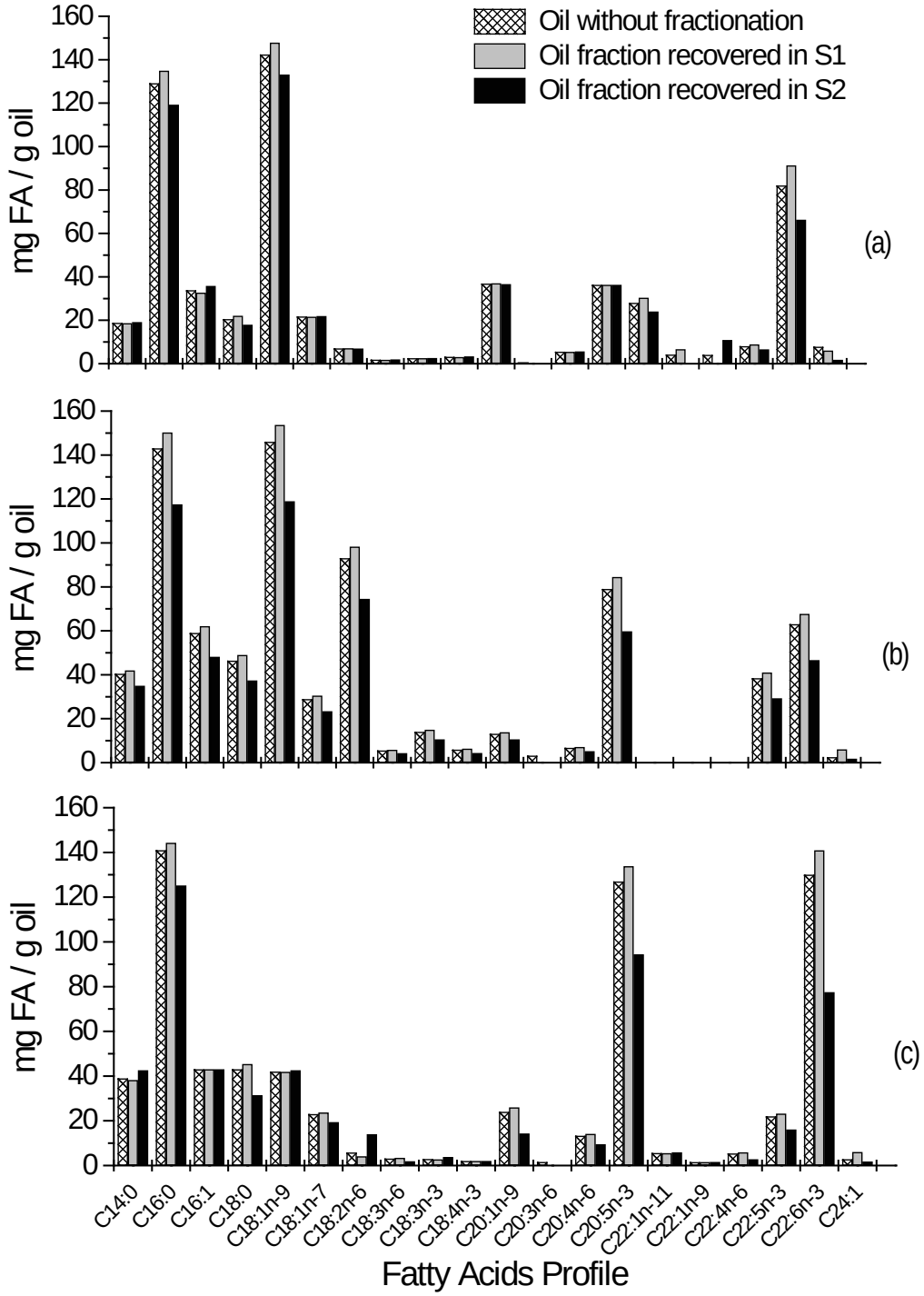


641

642

643 Figure 12. Comparison among the fatty acid profiles of the lipid fractions obtained after

644 fractionation of (a) hake offcuts oil, (b) salmon offcuts oil and (c) jumbo squid liver oil.

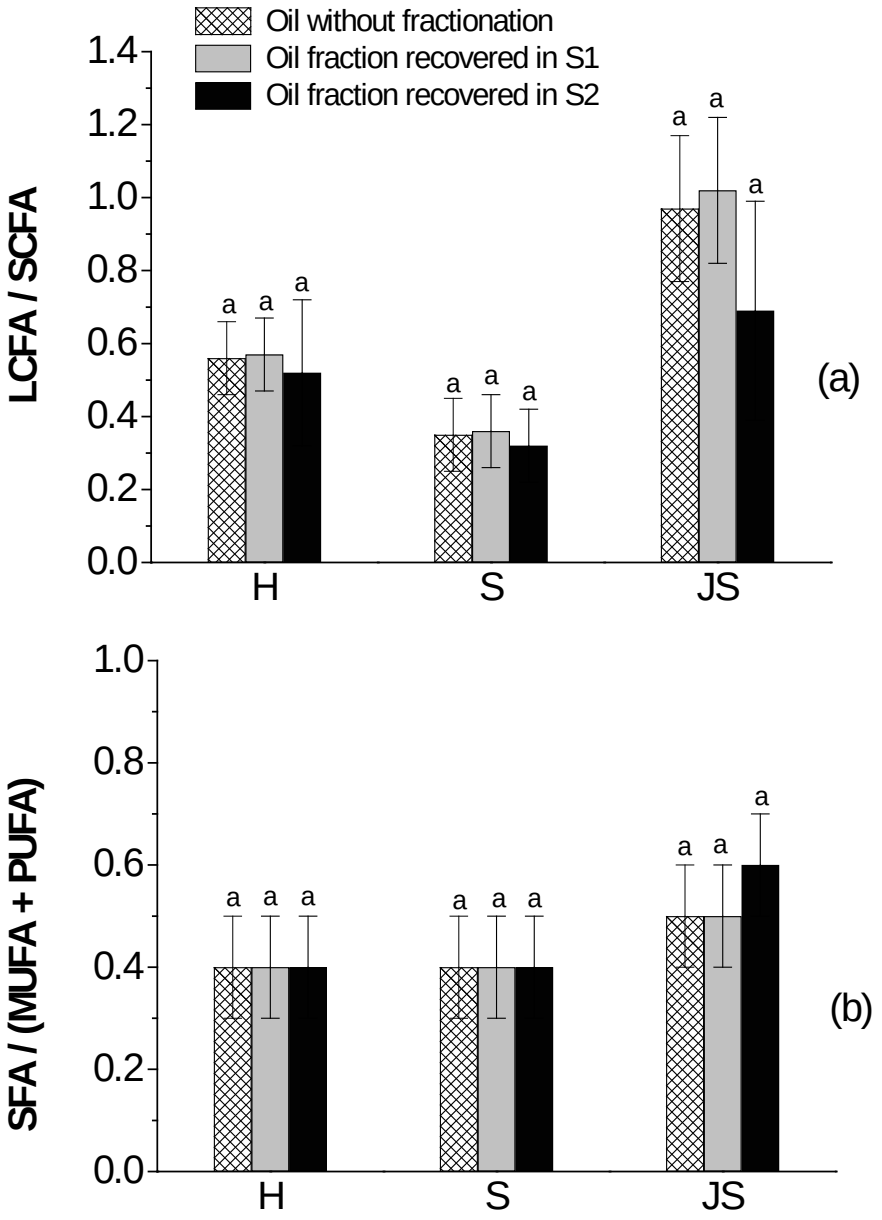
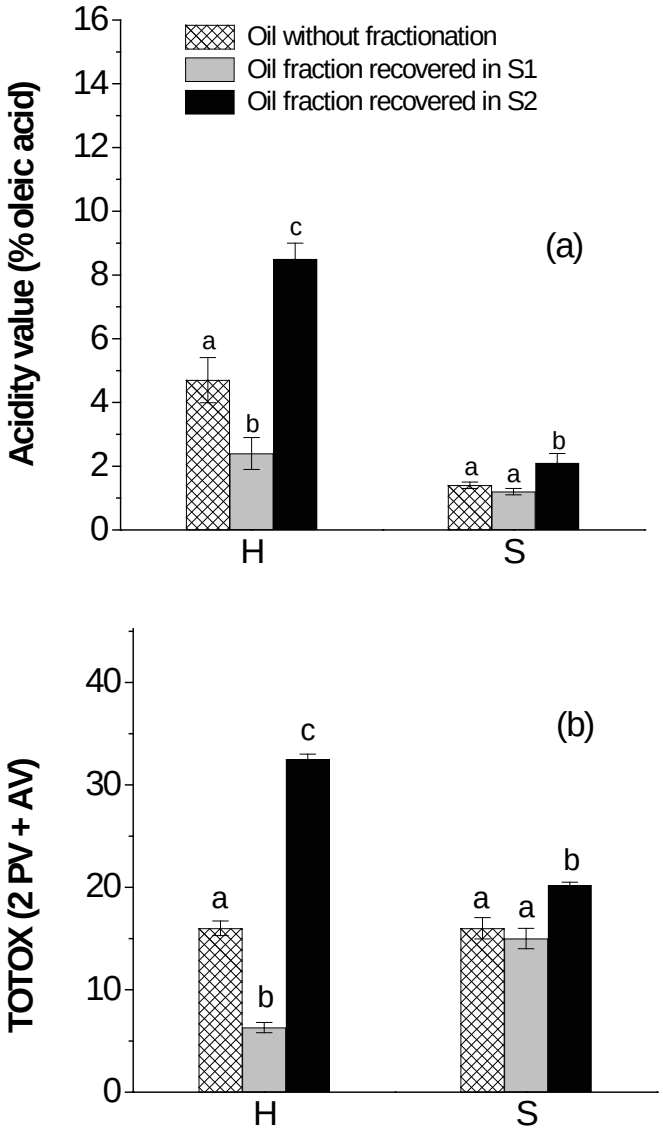


Figure 13. (a) LCFA/SCFA ratio in fish oil with and without fractionation. (b) SFA / (MUFA + PUFA) ratio in fish oil with and without fractionation. LCFA are considered fatty acids with a carbon number, C > 18, whereas SCFA are considered those with a carbon number, C ≤ 18. H: Hake oil. S: Salmon oil. JS: Jumbo squid oil. Determinations were carried out in triplicate and the results are the average values ± standard deviation. Means with the same letter within the same species are not significantly different ($p > 0.05$).



654

655

656 Figure 14. (a) Comparison between the acidity value and (b) total oxidation value
657 (TOTOX), determined in oil fractions recovered in separator 1, S1, and in separator 2,
658 S2; and oil without fractionation obtained from hake (H) and salmon (S) offcuts
659 respectively. Determinations were carried out in triplicate and the results are the average
660 values ± standard deviation. Means with the same letter within the same species are not
661 significantly different ($p > 0.05$).