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Rubio-Rodríguez, N., De Diego, S.M., Beltrán, S., Jaime, I., Sanz, M.T., Rovira, J.

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

(2012) Journal of Food Engineering, 109 (2), pp. 238-248.

DOI: 10.1016/j.jfoodeng.2011.10.011

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

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8
9 **Abstract**

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

21 **Keywords**

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

23 | _____

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24 **1. Introduction**

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

40 The most common method used for fish oil production is wet reduction, which involves
41 three basic steps: cooking at high temperatures (85 – 95 °C), pressing and centrifuging
42 (FAO, 1986). This process permits obtaining high volumes of crude fish oil, although
43 subsequent refining steps are required in order to make the crude fish oil suitable for
44 edible purposes. Other processes, such as enzymatic reaction with proteases, have been
45 studied for obtaining crude oil from fish by-products (Linder et al., 2005). In the last
46 years, supercritical fluid extraction (SFE) has become an attractive technology for
47 obtaining high quality fish oil from some by-products (Letisse et al., 2006, Rubio-
48 Rodríguez et al., 2008), not only because it uses moderate temperatures and provides an
49 oxygen free media, which aim to reduce the omega-3 oxidation during the extraction
50 process, but also because it allows extracting selectively low polar lipid compounds,
51 avoiding the co-extraction of polar impurities such as some inorganic derivatives with
52 heavy metals. Furthermore, the tunability of the supercritical carbon dioxide (SC-CO₂)
53 regarding density, and therefore solvation power, by changing temperature and/or
54 pressure, makes fish oil de-acidification possible, alternatively to conventional physical

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

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

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

67 **2. Material and methods**

68 2.1. Raw material and pretreatment

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

82 2.2. Oil extraction methods

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

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

91 Enzymatic extraction was carried out following the method proposed by Gbogouri et al.
92 (2006). The enzyme used was a food-grade protease, Alcalase 2.4 L (bacterial protease
93 from *Bacillus licheniformis*), provided by Novozyme (Bagsvaerd, Denmark). The
94 enzyme / substrate ratio was 0.05 w / w protein.

95 SFE was carried out in a semi-pilot plant under the optimal extraction conditions
96 ($p = 25$ MPa, $T = 313$ K) found in a previous study (Rubio-Rodríguez et al., 2008). The
97 raw material used for this extraction method was previously freeze-dried (FreeZone
98 12 L Console Freeze Dry System with drying chamber, Labconco). Some of the SFE
99 experiments included fractionation of the extract by depressurization in two consecutive
100 separators: the first separator (S1), which was kept at a pressure of 9 ± 0.5 MPa and a
101 temperature of 308 ± 1 K, and the second separator (S2), which was kept at a pressure
102 of 5 ± 0.5 MPa and a temperature 283 ± 1 K. Thus, the SC-CO₂ density in S1 was in the
103 range 650 ± 50 kg / m³, well below its density in the extractor (880 ± 5 kg / m³) and
104 above that in S2 (below critical density, $\rho_c = 468$ kg / m³). This way, most of the
105 triglycerides were recovered in S1 and most of the free fatty acids were recovered in S2.
106 In all cases, the oil fractions were stored in closed vessels in darkness at -18 °C in order
107 to minimize spoiling before characterization.

108 2.3. Analytical methods

109 2.3.1. *Characterization of fish by-products*

110 Fish by-products were characterized by determining their water, protein and fat content
111 in order to establish their profitability as raw materials for oil extraction (see Table 1).
112 Water and protein content were determined by the AOAC Official Methods 934.01 and
113 981.10 respectively (2000). Total fat content was determined by Soxhlet extraction
114 using petroleum ether as solvent in a Büchi extraction system (model B-811). Soxhlet
115 extraction was performed over freeze-dried samples in 140 minutes distributed in three
116 stages: extraction (120 min), rinsing (10 min) and drying (10 min).

117 The amount of trace metals (Fe, Cu, Zn, As, Cd, Hg and Pb) was also determined. A
118 wet digestion was firstly carried out over the samples in order to destroy the organic
119 matter. A sample of about 20 mg was treated with 10 mL of HNO₃ 65 % suprapur®
120 (Merck, Germany) in a microwave oven (Ethos Sel, Milestone) provided with ten
121 Teflon vessels (HPR-1000/10 S). The temperature program selected involved three
122 heating steps (from room temperature to 80 °C in 4 min, from 80 °C to 130 °C in 7 min
123 and from 130 °C to 170 °C in 5 min) followed by a constant heating at 170 °C for
124 10 min and a final ventilation step. After digestion, the samples were diluted to 25 mL
125 with Milli-Q water, and analysed by ICP-MS (Agilent 7500 i).

126 2.3.2. *Oil characterization*

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

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

133 The total amount of neutral lipids was determined by liquid chromatography in a HPLC
134 system (Agilent 1200) formed by a quaternary pump and an auto-injector. The
135 separations were carried out at room temperature in a column (Lichrospher Diol

136 5 mm, 4 × 250 mm) and the detection was performed in an evaporative light scattering
137 detector (Agilent 1200 series) at 45 °C and 3.5 bar. The mobile phase consisted of a
138 mixture of solvents: (A) hexane/acetic acid (99.5/0.5 by volume) and (B) hexane/1-
139 propanol/acetic acid/water (85/14.4/0.5/0.1 by volume). The solvent gradient used was
140 as follow: first, solvent A was flowing for 1 min, after that, solvent B was added in three
141 steps, up to 10 % in 9 min, to 44 % in 12 min and to 100 % in 8 min. Finally, the
142 stationary phase was rinsed with solvent A during 5 min. Total solvent flow rate was
143 kept constant at 1 mL / min all along the analysis. Calibration was carried out using
144 standards of palmitil palmitate (99 %), tripalmitin (> 99 %), dipalmitin (99 %),
145 monopalmitin (99 %) and palmitic acid (99 %) in hexane. The calibration curves
146 showed a good correlation according to the exponential relationship described for an
147 evaporative light scattering detector.

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

150 The acidity value and the peroxide value (PV), were determined according to the AOCS
151 Official Methods Ca 5a-40 and Cd 8-53 respectively (1990), whereas the anisidine
152 value (AV) was evaluated according to the British Standard method BS 684-2.24
153 (2008). The TOTAl OXidation value (TOTOX) was determined according to the
154 expression (2 PV + AV) (Perrin, 1996).

155 Volatile compounds were analyzed by GC-MS after Solid Phase Dynamic Extraction
156 (SPDE) sampling. The SPDE device (Chromtech, Idstein, Germany) was equipped with
157 a needle coated with a non-polar 50 µm film of polydimethylsiloxane with 10 %
158 embedded activated carbon phase (PDMS/AC). Samples were incubated for 1 min at
159 70 °C; and after equilibration, extraction was performed (50 aspiration cycles,
160 extraction speed 40 µL/s). Gas chromatography analyses were carried out with a 6890N
161 Series GC System coupled to a 5973i mass spectrometer (Agilent Technologies, Palo
162 Alto, CA, USA). The SPDE needle was injected and thermally desorbed at 250 °C.
163 Compounds were separated on a HP5 capillary column (50 m length x 0.32 mm I.D,
164 fused silica capillary column coated with a 1.05 µm film thickness. Quadrex
165 Corporation, New Haven, USA). The temperature of the column was increased at a rate
166 of 3 °C / min from 40 to 240 °C.

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

169 2.3.3. *Oil sensory analysis*

170 An off-odour comparison among the oils extracted by different methods was carried out
171 both by electronic nose and by sensory analysis. The overall smell print was determined
172 by an electronic nose α FOX 4000 (AlfaMOS, Toulouse, France) with a sensor array of
173 18 metal oxide sensors. Vials with the samples were incubated 5 minutes under stirring
174 (500 rpm, cycles 5 s on and 2 s off) in an oven at 50 °C for generating the equilibrated
175 headspace. The injection temperature was 60 °C; the carrier gas was synthetic air with a
176 flow of 150 mL/min. Sensory characterization of oil was carried out by 10 panellists
177 trained in descriptive analysis of fish off-flavours. A total of six sensory descriptors
178 were used (fishy, rancid, boiled, acid, sweet and other off-flavours), which were
179 measured on a structured intensity scale with a range from a minimum of zero to a
180 maximum of five. Samples (0.5 g of oil) were presented randomized at room
181 temperature in blind vials numbered with a code of three digits.

182 **3. Results and discussion**

183 3.1. General features on the extraction procedures used in this work

184 Extraction with SC-CO₂ has been proposed as a good method for obtaining fish oil with
185 a high amount of omega-3 fatty acids, since it involves the use of a non-oxidant
186 atmosphere and mild temperatures, which prevent the oxidation of the polyunsaturated
187 fatty acids. Previous studies (Rubio-Rodríguez et al., 2008) have concluded that it is
188 possible to totally extract the oil contained in hake offcuts by using SC-CO₂ at a
189 pressure of 25 MPa and a temperature of 313 K. The highest yield and extraction rate
190 were reached when the by-products were previously cut and freeze-dried to a moisture
191 content below 20 % in order to improve the oil - SC-CO₂ contact and minimize oil –
192 water interaction in the supercritical phase. The extraction curves obtained were well
193 fitted to the empirical model proposed by Kandiah and Spiro (1990), which assumes
194 that the process is controlled by two diffusion stages depending on the amount of oil
195 accessible to the SC-CO₂. At the beginning, the amount of the most accessible oil is
196 high, thus the internal mass transfer resistance is low and the extraction rate is large.

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

200 SFE from other freeze-dried fish by-products i.e.: orange roughy offcuts, salmon offcuts
201 and jumbo squid liver, has been also shown to be feasible under the same experimental
202 conditions. Figure 3 shows the extraction curves where the amount of oil extracted
203 along time can be observed for each species. In all cases, it was observed that, at the
204 beginning of the process, the oil extracted depended linearly on the amount of SC-CO₂
205 that flows through the extractor, which may indicate either that the internal mass
206 transfer is negligible and the process is controlled by the oil solubility in SC-CO₂ or that
207 the internal mass transfer is constant and the extraction rate depends on the internal
208 structure of the solid matrix. The values of the slopes of the extraction curves estimated
209 at zero time are reported in Table 2. It is observed that these slopes differ significantly
210 among the fish by-products studied, which may be attributed not only to the different
211 internal structure, which affects the internal mass resistance in the extraction process,
212 but also to the different solubility of the fish oil in SC-CO₂ due to the different neutral
213 lipid composition and fatty acid profile, as can be seen in Tables 3 and 4 respectively.

214 The internal mass transfer resistance can be considered negligible for fish by-products
215 with mostly extracellular oil as is the case of orange roughy oil (Phleger, 1998), or oil
216 weakly bound to the protein matrix, as observed for salmon oil; therefore, the initial
217 slope of their SFE curves in Figure 3 can be assumed to be close to the oil solubility in
218 SC-CO₂, which, moreover, depends on the oil composition. Thus, for a fish oil rich in
219 triacylglycerides (i.e.: salmon oil, see Table 3) this slope would be lower than for fish
220 oil rich in wax esters (i.e.: orange roughy oil, see Table 3), which solubility in SC-CO₂
221 is usually higher than that of triacylglycerides (Gupta & Shim, 2007).

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

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

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

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

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

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

262 3.2. Oil quality parameters

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

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

274 3.2.1. Oil acidity

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

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

288 acidity than the oils obtained by non-SFE procedures, which may indicate that, in this
289 case, the hydrolysis of triacylglycerides, and therefore the release of FFA, was less
290 extended. However, in the case of orange roughly offcuts, it was observed that, in spite
291 of having a negligible FFA content, as detected by the neutral lipid analysis (see Table
292 3), the oil obtained by SFE shows a higher acidity value than the oils obtained by non-
293 SFE methods. These experimental results may be explained taking into account that
294 some acidic compounds, such as acetic acid, were co-extracted together with the oil
295 when SC-CO₂ was used as solvent in a closed system, while they were not obtained by
296 the non-SFE methods carried out in open vessels (see section 3.2.3).

297 3.2.2. *Total oxidation value (TOTOX)*

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

308 3.2.3. *Volatile compounds and sensory properties*

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

317 carbohydrates, and thus, their presence in the oil may be attributed to the raw material
318 freshness (*see* Table 5). That is the case of trimethylamine which is mainly produced by
319 the action of specific spoilage bacteria, such as *Shewanella putrefaciens*, of
320 dimethylamine, mainly produced by endogenous enzymes during fish storage, or of the
321 acetic acid which can be produced by anaerobic degradation of aminoacids (Huss,
322 1995).

323 As can be observed in Table 6, where the volatile compounds found in the oil obtained
324 from orange roughly offcuts by the four different methods essayed in this work are
325 presented, hexanal and nonanal, mainly generated by lipid oxidation, were only detected
326 in the oil obtained by non-SFE methods, which explain the highest level of rancid odour
327 detected by sensory analysis of these oils, especially in that obtained by enzymatic
328 extraction (*see* Figure 7). These results show that, due to the use of mild temperatures
329 and a non-oxidizing atmosphere, SFE made possible to reduce the lipid oxidation more
330 than the non-SFE processes. On the contrary, dimethylamine, responsible for the highest
331 level of fishy odour detected in this oil by sensory analysis (*see* Figure 7), and acetic
332 acid, responsible for the unexpected high acidity value (*see* section 3.2.1.), were only
333 detected in the oil obtained by SFE (*see* Table 6). These experimental results could be
334 explained taking into account that these volatile compounds, soaked in the raw material,
335 can be easily extracted by SC-CO₂ due to their high vapour pressure, and, since the
336 process takes place in a solvent recirculation system, they may partially remain
337 absorbed by the oil. In any case, the amount of these volatile compounds may be
338 significantly reduced by coupling SFE with other separation process such as
339 countercurrent fractionation or adsorption, as has been previously reported in the
340 literature (Rubio-Rodríguez et al., 2010).

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

346 These results show that the freshness of the raw material is crucial for obtaining good
347 quality sensory properties in the oil, especially if oil production is carried out in a closed
348 system and with SC-CO₂ as solvent.

349 3.2.4. Toxic heavy metals

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

354 Due to the high selectivity of SC-CO₂ for non-polar compounds, the amount of heavy
355 metals extracted together with the oil by SFE was negligible, as was the case of Cd, Hg
356 and Pb, or significantly reduced, as in the case of As (see Table 7).

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

371 3.3. Enhancement of oil quality through SFE-fractionation

372 SFE followed by fractionation in two separators was studied as a way to refine fish oil
373 and reduce the amount of impurities, especially free fatty acids, which, in general, are
374 more soluble in SC-CO₂ than the relative triacylglycerides.

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

378 Table 8 shows the average amount of oil recovered in each separator for different
379 experiments together with the calculated mass percentage. It can be observed that, in all
380 cases, a higher amount of oil is recovered in S1, although this amount varies from 63 %
381 in hake oil to 86 % and 83 % in salmon and jumbo squid liver oil respectively, which
382 may be attributed to the different fish oil composition, as it is reported in Tables 3 and
383 4, and to the experimental CO₂ density fluctuations in Separator 1 (S1).

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

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

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

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

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

412 3.4. Economical considerations

413 During the last decades, different supercritical fluid technologies have been established
414 as interesting for safely processing natural products in the food and pharmaceutical
415 industries. Nowadays, several processes such as coffee decaffeination, hops extraction,
416 essential oils extraction, cork cleaning, pesticides removal from rice, etc., are carried out
417 at commercial scale in different parts of Europe, US and Asia (Brunner, 2010, Perrut,
418 2000). Some studies have shown that, in spite of requiring a high investment cost,
419 supercritical fluid extraction of essential oils requires lower processing costs and
420 downstream processing making this process competitive regarding steam distillation
421 (Pereira & Meireles, 2007). Concerning the processing of fats and oils, SFE may also
422 compete with traditional processes in the case of specialty oils such as nut oils (almond,
423 peanut...), seed oils (apricot, borage, grape, sesame...), cereal oils (wheat germs, rice
424 bran...) or fruit oils (cloudberry, tomato...), which contain bioactive lipid compounds
425 interesting in the food and pharmaceutical industries (Temelli, 2009). In the case of fish
426 oil extraction, although SFE leads to high quality oil, the drying step, required before
427 extraction, increases noticeably the production cost and minimizes competitiveness
428 against alternative extraction processes. Thus, the industrial application of supercritical
429 fluid technology in omega-3 processing should be focused not only on an isolated SFE
430 procedure but on a whole process involving the use of SC-CO₂ in fish oil extraction,
431 fractionation, omega-3 concentration and /or formulation (Rubio-Rodríguez et al.,
432 2010) in order to obtain small volumes of high value omega-3 concentrates used as
433 ingredients in functional foods or as active principles in pharmacology.

434 **4. Conclusions**

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

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

462 **5. Acknowledgements**

463 The authors gratefully acknowledge the Ministry of Education and Science (CTQ2005-
464 07301), Junta de Castilla y León (GR 167) and Pescanova S.A. (Spain) for financial
465 support.

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541 868–871.
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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 ₂ S
	Methionine	CH ₃ SH, (CH ₃) ₂ S
	Carbohydrates and lactate	Acetate, CO ₂ , H ₂ O
	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 (spoilors)	Aminoacids	NH ₃ , acetic acid, butyric acid, propionic acid

561

562 Table 6. Volatile compounds found in the oil obtained from orange roughly 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	---	✓	✓	✓	✓
	Cyclohexadecane	---	×	×	×	✓
	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

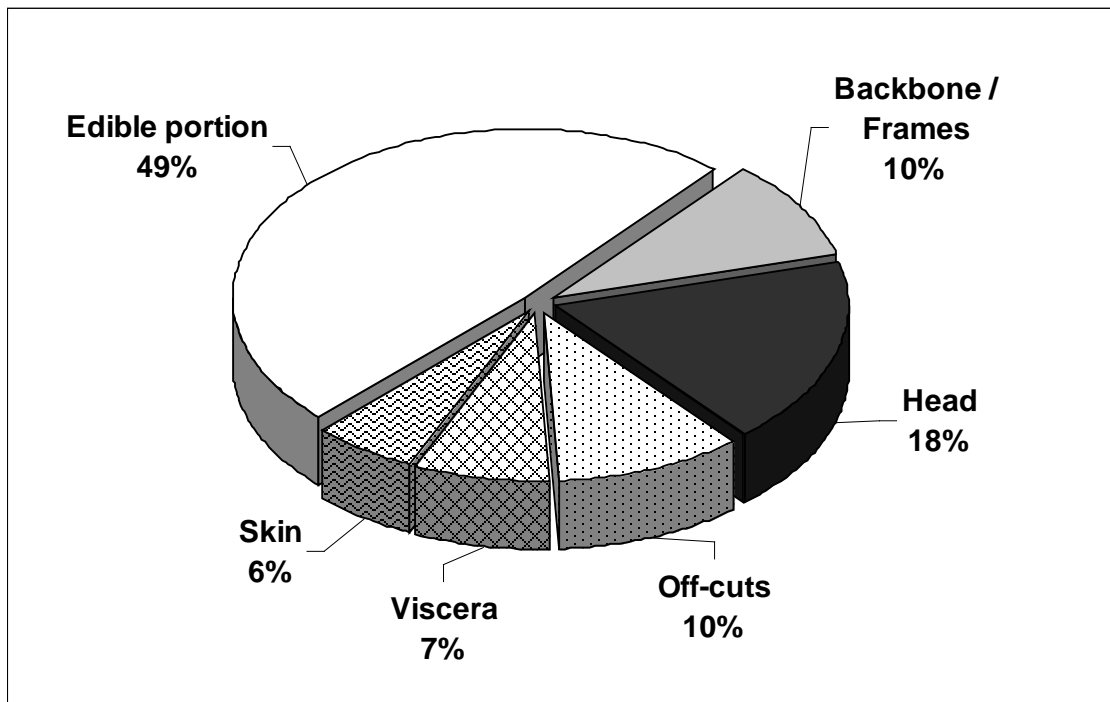
n.d.: not detected

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

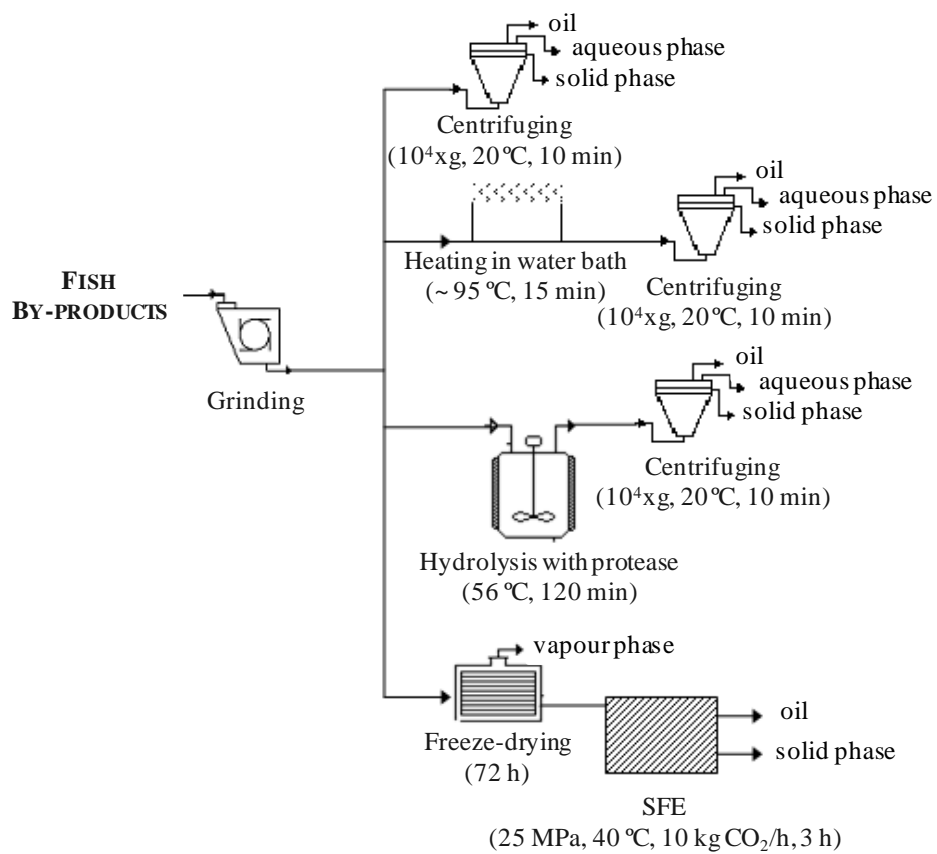
574



575

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

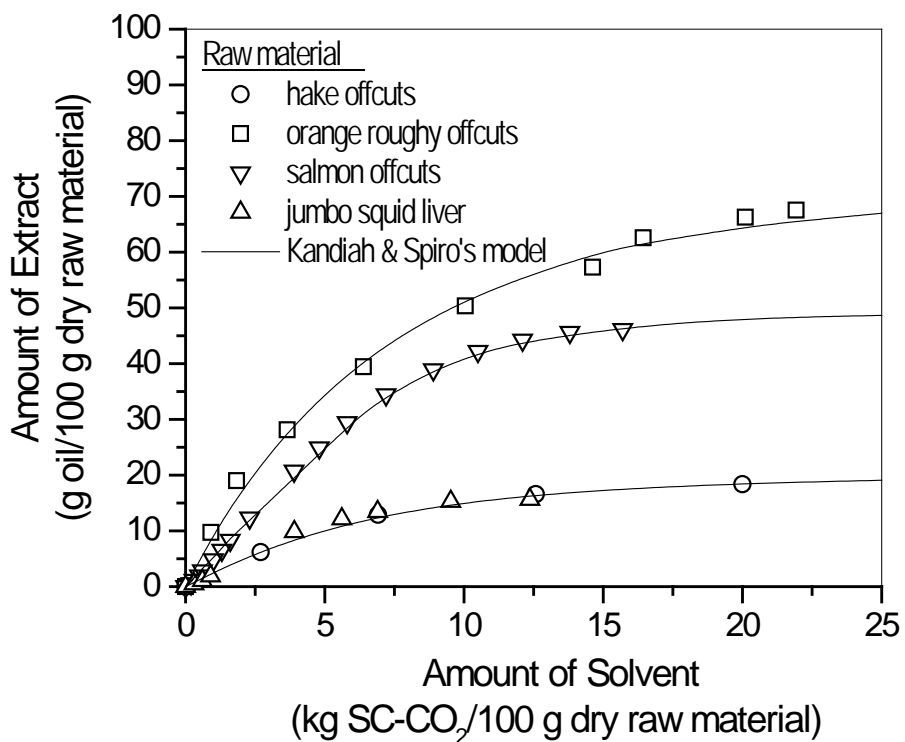
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579

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

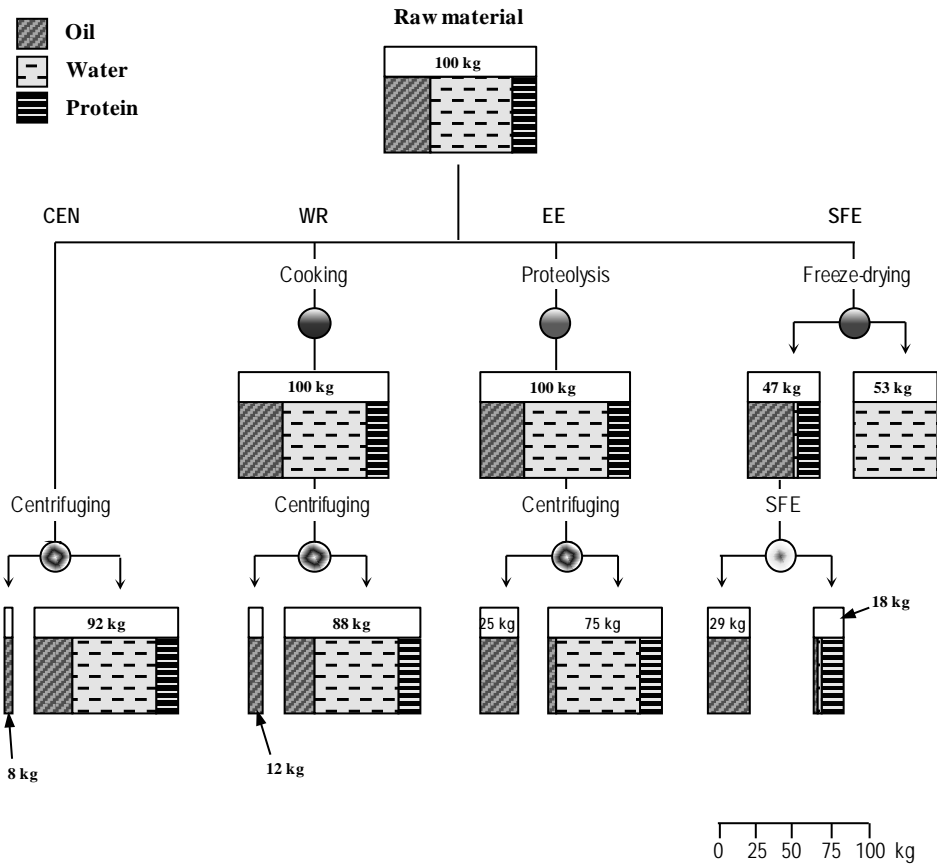
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582

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.

586

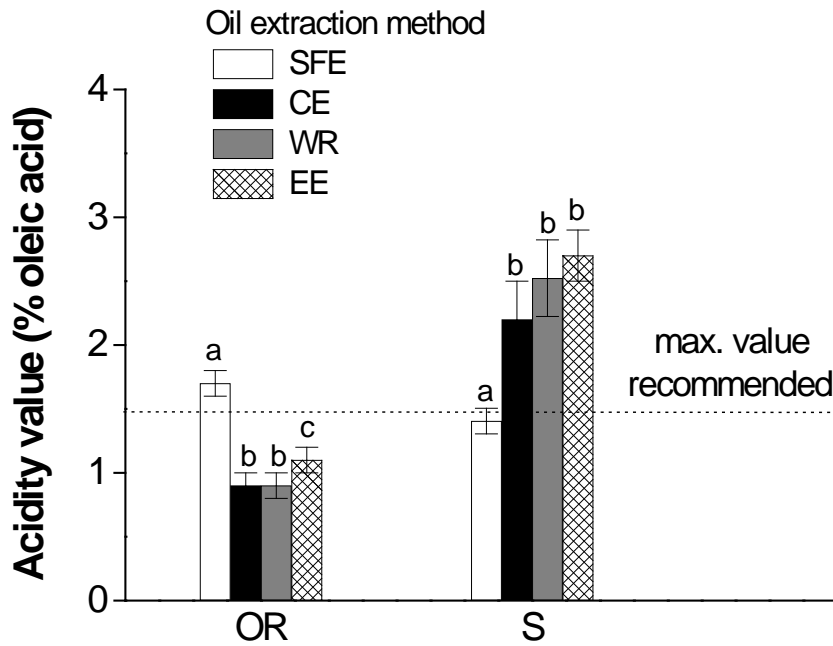


588

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)

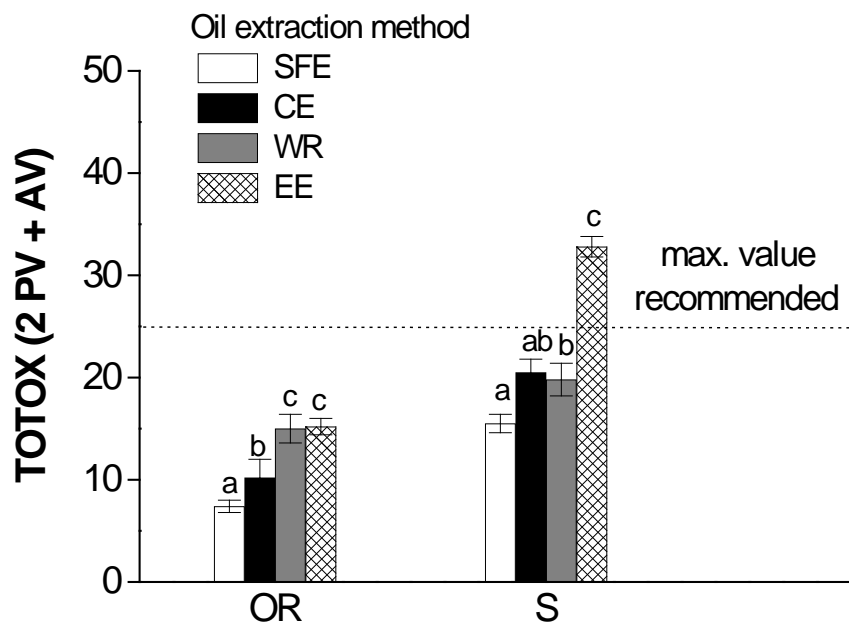
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595

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 \pm standard deviation. Means with
 600 the same letter within the same species are not significantly different ($p > 0.05$).

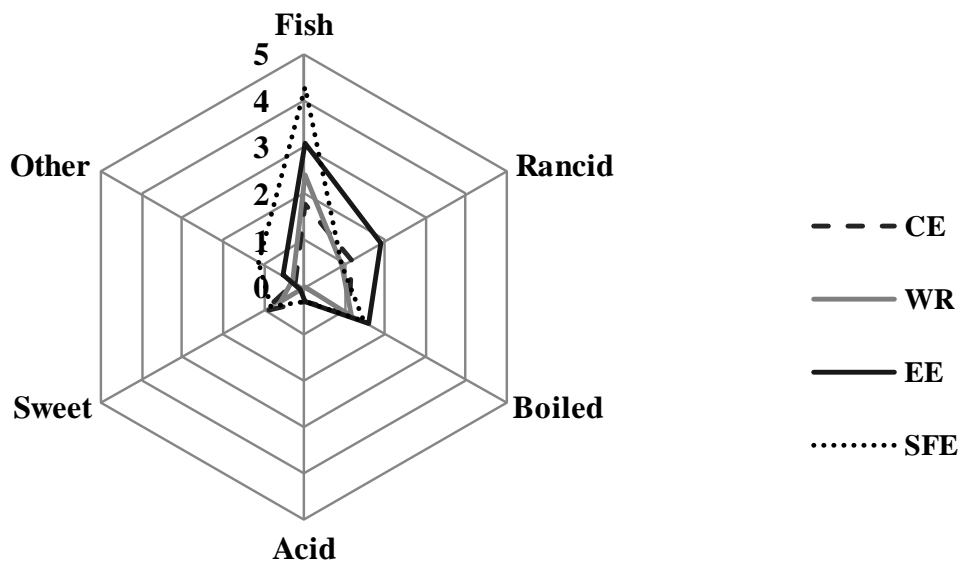
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602

603 Figure 6. Total oxidation value (TOTOX) of the oil extracted from orange roughly (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 \pm
 607 standard deviation. Means with the same letter within the same species are not
 608 significantly different ($p > 0.05$).

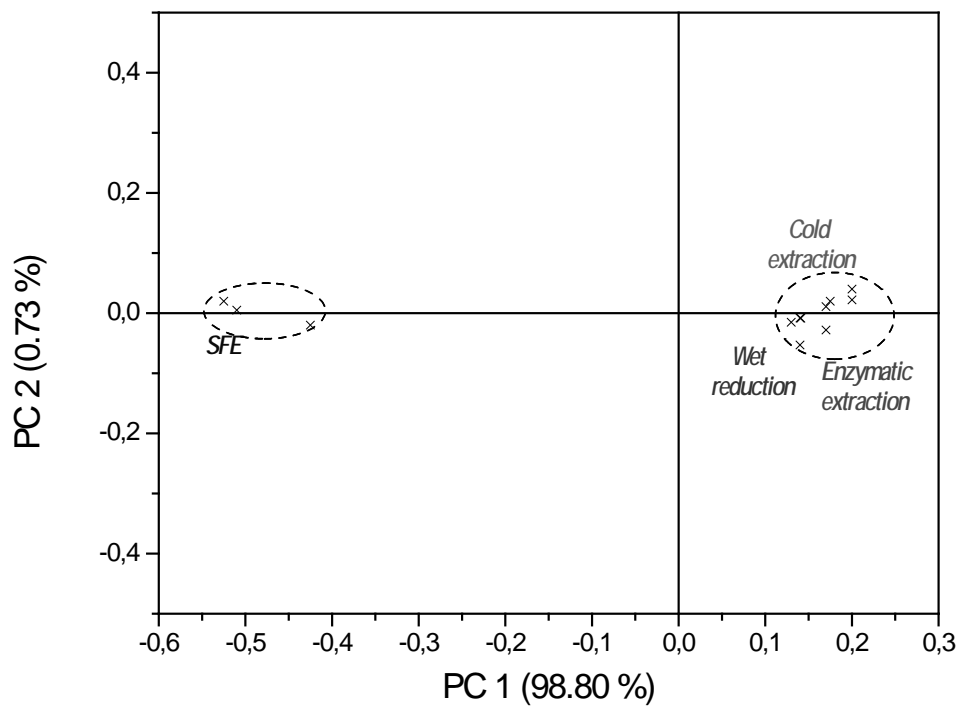
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610

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)

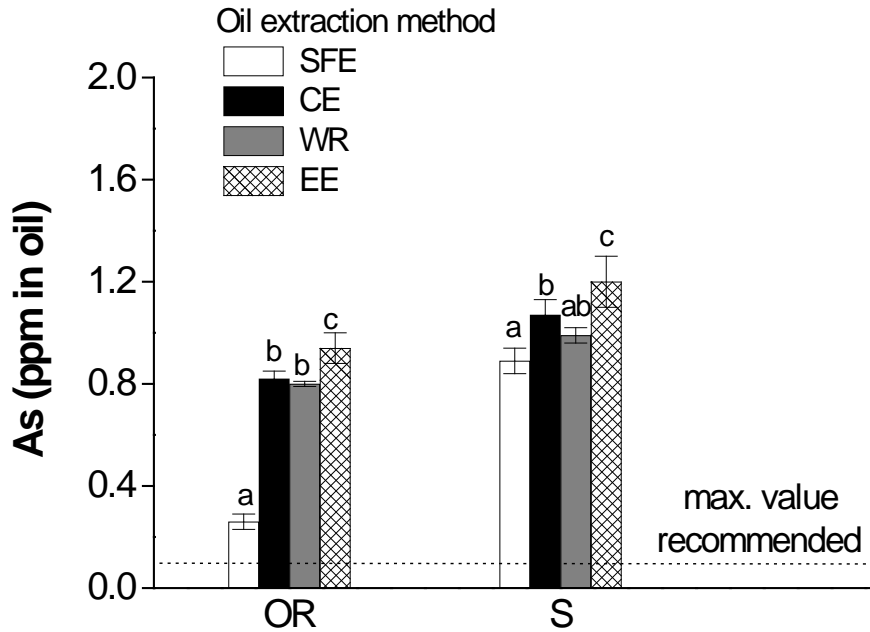
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615

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.

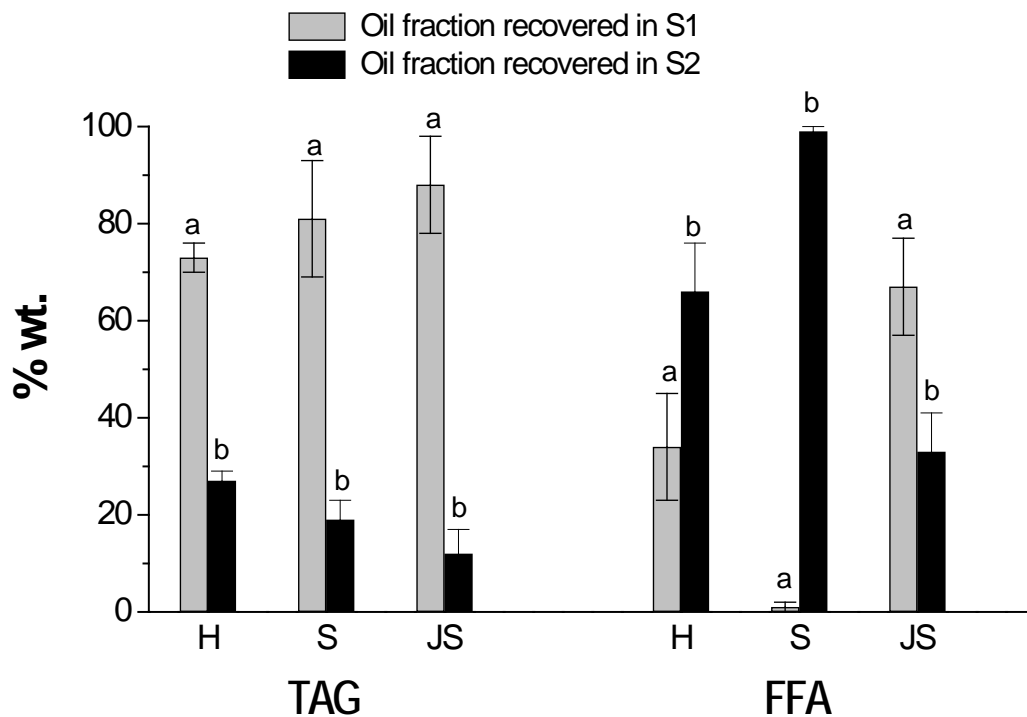
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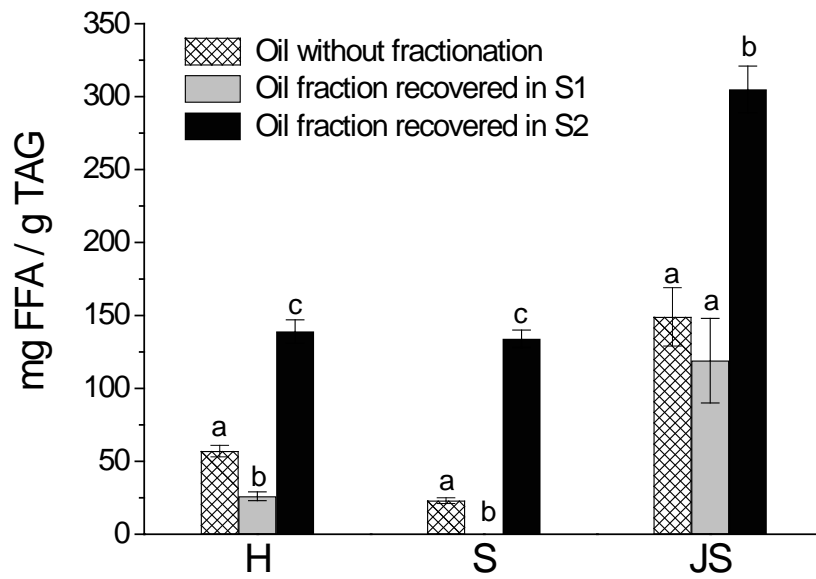
620 Figure 9. Total arsenic content found in the oil extracted from orange roughy (OR) and
 621 salmon (S) offcuts by different methods: Cold Extraction (CE), Wet Reduction (WR),
 622 Enzymatic Extraction (EE) and Supercritical Fluid Extraction (SFE). Determinations
 623 were carried out in triplicate and the results are the average values \pm standard deviation.
 624 Means with the same letter within the same species are not significantly different ($p >$
 625 0.05).

626



627

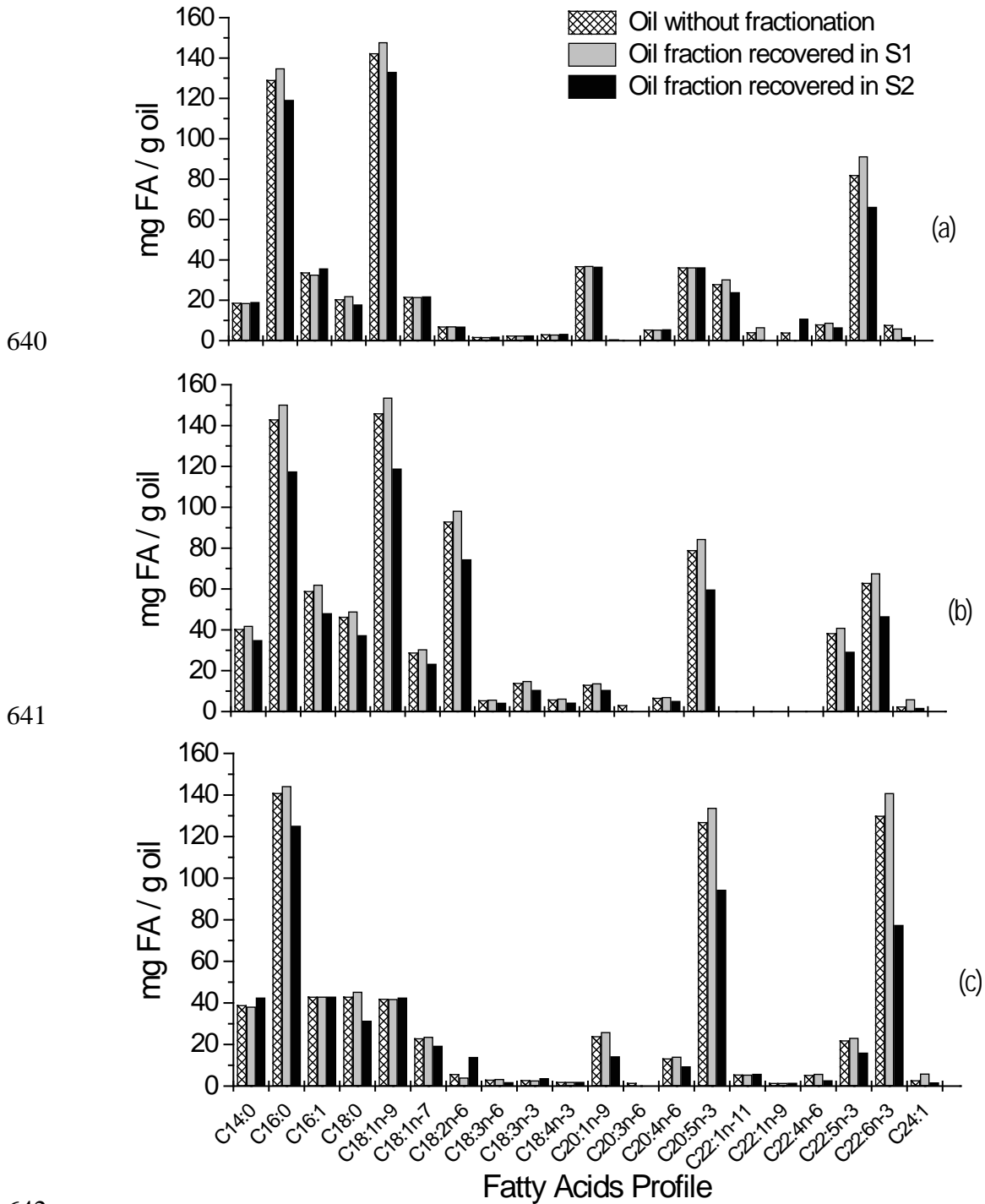
628 Figure 10. Mass percentage distribution of triacylglycerides (TAG) (left) and free fatty
 629 acids, (FFA) (right) between both separators in fish oil fractionation. H: Hake oil. S:
 630 Salmon oil. JS: Jumbo squid oil. Determinations were carried out in triplicate and the
 631 results are the average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



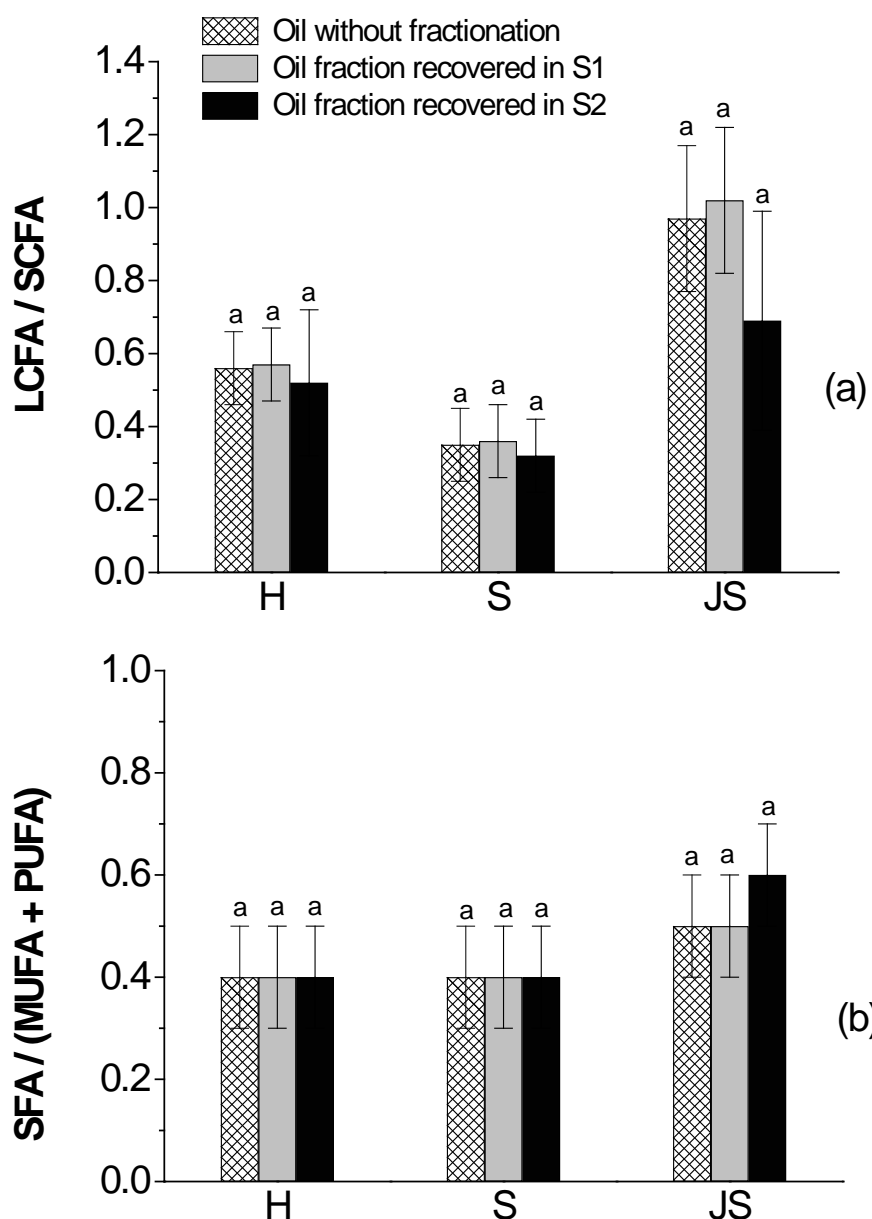
633

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 \pm standard deviation. Means with the same letter within the same species
 638 are not significantly different ($p > 0.05$).

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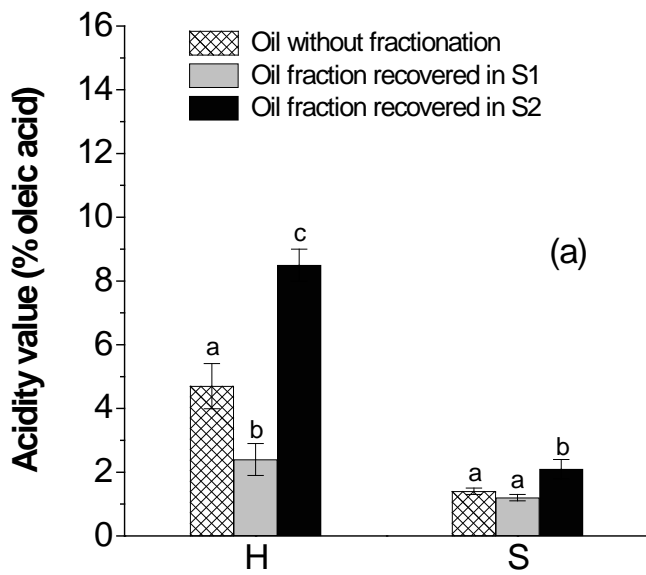
642
 643 Figure 12. Comparison among the fatty acid profiles of the lipid fractions obtained after
 644 fractionation of (a) hake offcuts oil, (b) salmon offcuts oil and (c) jumbo squid liver oil.



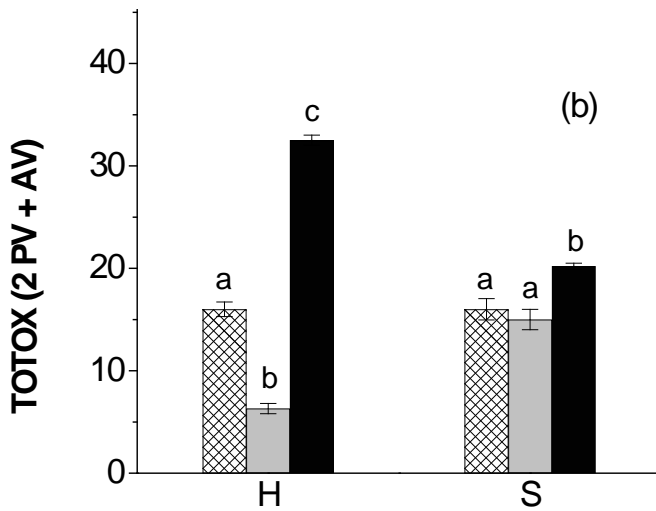
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647 Figure 13. (a) LCFA/SCFA ratio in fish oil with and without fractionation.
 648 (b) SFA / (MUFA + PUFA) ratio in fish oil with and without fractionation. LCFA are
 649 considered fatty acids with a carbon number, $C > 18$, whereas SCFA are considered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



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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 \pm standard deviation. Means with the same letter within the same species are not
 661 significantly different ($p > 0.05$).