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Comparison Between Gelatines Extracted From Mackerel and Blue Whiting Bones after Different Pre-treatments

Catherine Barry-Ryan

Technological University Dublin, Catherine.Barryryan@dit.ie

Zied Khiari

Dublin Institute of Technology

Daniel Rico

Public University Navarra, Pamplona, Spain

Ana Belen Martin-Diana

Dublin Institute of Technology, anabelen.martindiana@dit.ie

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1 **Comparison between gelatines extracted from mackerel and blue whiting bones**
2 **after different pre-treatments**

3

4 Zied Khiari*¹, Daniel Rico², Ana Belen Martin-Diana³ and Catherine Barry-Ryan¹.

5

6 ¹ School of Food Science and Environmental Health, Dublin Institute of Technology
7 (DIT), Cathal Brugha Street, Dublin 1, Ireland.

8

9 ² Food Technology Department, Public University Navarra, Pamplona, Spain.

10

11 ³ Agricultural Technological Institute of Castilla and Leon, Government of Castilla
12 and Leon, Finca Zamadueñas, Valladolid, Spain.

13

14 * Corresponding author/Present address: Zied Khiari, Department of Agricultural
15 Food & Nutritional Science (AFNS). 4-10, Agriculture/Forestry Centre. University of
16 Alberta. Edmonton, AB, Canada T6G 2P5.

17 Tel: +1 780 492 4614.

18 Email: khiari@ualberta.ca

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22 **Abstract**

23 Gelatines were extracted from mackerel and blue whiting bones after chemical
24 or enzymatic pre-treatments and their functional properties (solubility, foaming and
25 emulsifying properties) were analysed. The pre-treatment significantly ($p<0.05$)
26 affected the composition and the functional properties of the extracted gelatines. The
27 amino acid analyses showed that chemically pre-treated bone gelatines had higher
28 imino acids (proline and hydroxyproline) contents compared to those extracted after
29 the enzymatic pre-treatment, regardless of the fish species. It was observed that all
30 gelatines had higher solubility at low pH with a maximum value observed at pH 2. A
31 significant effect of ionic strength was observed. Increasing the NaCl concentration to
32 more than 1% resulted in a significant decrease of the solubility. Mackerel bone
33 gelatines showed lower foaming capacity (FC) and higher foaming stability (FS) than
34 blue whiting bone gelatines. Increasing the concentration of gelatine decreased the
35 emulsifying activity (EAI) but increased the stability (ESI) indices. The use of
36 enzymes in the pre-treatment process generated gelatines with significantly ($p<0.05$)
37 higher EAI and ESI.

38

39 **Key words:** mackerel, blue whiting, bones, gelatines.

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47 **1. Introduction**

48 Every year, significant amounts of waste are generated by the fish processing
49 industries. These wastes are regarded as low quality products and are discarded or in
50 the best case scenario processed into fishmeal and pet food (Kim & Mendis, 2006).
51 Fish waste is costly to dispose of and is typically discarded overboard in case of
52 onboard processing or buried to landfill for the on-shore processing.

53 Environmental legislation has contributed to the introduction of sustainable
54 waste management practices in the European Union. The European Directive
55 1999/31/EC on the landfill of waste (Council Directive, 1999) and the Regulation
56 (EC) No 1774/2002 restrict the disposal of untreated organic waste not intended for
57 human consumption. Therefore more sustainable alternatives are needed. Recycling
58 fish waste is of interest from an environmental point of view by reducing the organic
59 contaminant charge. Recent advances in fish waste management have resulted in their
60 examination as a source of ingredients with a potential application to the food
61 industry. Underutilised fish species along with fish processing discards may be
62 potential sources of bioactive and functional ingredients such as gelatine (Shahidi,
63 1994).

64 Gelatine is a biopolymer produced by extraction and hydrolysis of fibrous,
65 insoluble collagen. Sources for fish collagen can be fish skin, bones, scales or
66 connective tissue (Kim & Mendis, 2006). The industrial process of gelatine
67 manufacture involves either an acid or alkaline pre-treatment followed by extraction
68 with warm water. The heat denaturation converts collagen into gelatine. Further
69 clarification steps include filtration, concentration, drying and milling (Schrieber &
70 Gareis, 2007). The quality of gelatine preparation depends on its physicochemical

71 properties, which are influenced not only by the species or tissue from which it is
72 extracted, but also by the severity of pre-treatment and extraction process.

73 Atlantic mackerel (*Scomber scombrus*) is a pelagics species and is abundant in
74 cold and temperate shelf areas such as the North Atlantic Ocean (Collette & Nauen,
75 1983). The world catch of mackerel was estimated around 566 thousand tonnes in
76 2007 (FAO, 2009). Blue whiting (*Micromesistius poutassou*) is a typical lean species
77 and belongs to the *Gadidae* family along with cod and haddock. Blue whiting is an
78 underutilised fish with a global catch estimated to be about 1.7 thousand tonnes in
79 2007 (FAO, 2009).

80 In this study, mackerel and blue whiting, models for oily and white fish,
81 respectively, were investigated for gelatines extraction. The effect of the pre-treatment
82 of fish bones on the composition and functional properties of gelatines was evaluated.

83

84 **2. Materials and methods**

85 **2.1 Materials**

86 Atlantic mackerel (*Scomber scombrus*) caught in early March 2007, were
87 kindly provided by Bord Iascaigh Mhara (BIM, Ireland). Blue whiting
88 (*Micromesistius poutassou*) caught in January 2008, were provided by Donegal
89 Seafood. Both fish were caught in the area FAO 27 (Atlantic, Northeast). The average
90 weights for mackerel and blue whiting were 277 g and 116.8 g, respectively. Fillets
91 were manually removed after beheading and evisceration of fish, the remaining meat
92 was separated from the frame using a knife. Bones and scales were cut manually into
93 small pieces (1 to 2 cm length) using scissors. The bones were divided into batches
94 and kept in the freezer at - 20 °C for less than one month before use. All chemicals
95 used were analytical grade.

96

97 **2.2 Enzymes**

98 Flavourzyme is a fungal protease/peptidase complex obtained from
99 *Aspergillus oryzae*. Flavourzyme had a declared activity of 500 leucine
100 aminopeptidase units (LAPU)/g, where one LAPU is defined as the amount of
101 enzyme which hydrolyzes 1 μ mol of L-Ieucine-p-nitroanilide per minute. Alcalase is
102 an alkaline enzyme produced by *Bacillus licheniformis*. Alcalase had an activity of
103 2.4 Anson units (AU)/g, where one Anson unit is defined by Aspino *et al.* (2005) as
104 the amount of enzyme that releases 1.0 mEq of tyrosine from urea-denatured
105 hemoglobin per minute. Flavourzyme was produced by DSM Nutritional Products,
106 Inc. (Kaiseraugst, Switzerland) and Alcalase was produced by Novozyme Co.
107 (Copenhagen, Denmark). Both enzymes were supplied by Sigma-Aldrich, Inc.
108 (Dublin, Ireland).

109

110 **2.3 Proximate analysis of fish bones**

111 The proximate analysis was carried out according to the procedures of the
112 Association of Official Analytical Chemists (AOAC, 2000). Protein content was
113 determined by the Kjeldahl method using an automatic Kjeldahl system (Gerhardt,
114 Bonn, Germany). The protein content was calculated by using a conversion factor of
115 6.25. Moisture was determined by drying the sample using an oven (Qualivac,
116 Greenfield Oldham, UK) until reaching a constant weight (at 100 °C for 18 hours) and
117 ash by incineration in a muffle furnace (Carbolite, Bamford Sheffield, England) at
118 550 °C for 4 hours. Lipid content was determined according to the Bligh and Dyer
119 method (Bligh and Dyer, 1959). All analyses were performed in triplicate.

120

121

122 **2.4 Extraction of gelatine**

123 Different pre-treatment methods were used to extract gelatines from mackerel
124 and blue whiting bones.

125

126 **2.4.1 Pre-treatment**

127 **2.4.1.1 Chemical pre-treatment:**

128 Bones (250 g) used for gelatine extraction were treated with 0.1 N NaOH at a
129 ratio of 1/3 (w/v) for 30 min and this step was repeated 3 additional times to eliminate
130 non-collagenous proteins and fat.

131

132 **2.4.1.2 Enzymatic pre-treatment:**

133 Fish bones (250 g) were mixed with 0.1 M potassium phosphate buffer (pH 8)
134 at a ratio of 1/3 (w/v) then heat treated in a microwave oven model R-244 (Sharp
135 Electronics Ltd, Uxbridge, UK) for 5 min to inactivate the endogenous enzymes.
136 After air cooling, Flavourzyme or Alcalase were added at an enzyme/substrate ratio of
137 0.1% (v/w). The bones were hydrolysed for 4 h at 50 °C with continuous shaking at
138 150 rpm using a Gallenkamp orbital incubator (AGB, Dublin, Ireland). After
139 hydrolysis the samples were heat treated in the microwave oven for 5 min to
140 inactivate the enzymes. The mixture was allowed to cool down and filtered through a
141 1 mm pore size sieve to separate the bones from the protein hydrolysates. The clean
142 bones were collected and demineralised.

143

144 **2.4.2 Demineralisation and gelatine extraction**

145 Fish bones were demineralised at room temperature for 18 hours using 0.25 N
146 HCl (1/3, w/v). The demineralised bones were washed under running water from a tap
147 to remove the acid.

148

149 **2.4.3 Gelatine extraction**

150 The pre-treated bones were mixed with distilled water (at a ratio of 1/3, w/v)
151 and gelatine was extracted in a water bath at 45 °C for 18 hours. All extraction steps
152 were done with continuous stirring at 150 rpm. Extracted gelatine was filtered using a
153 Whatman No.4 filter paper (Whatman, Maidenstone, England). Gelatine was then
154 evaporated under vacuum at 45 °C using a Büchi Rotavapor model R-210 fitted with a
155 Büchi temperature-controlled water bath model B-491 and Büchi vacuum system
156 model V-700 (Büchi UK Ltd., Oldham, UK), freeze dried (Labconco corporation,
157 Kansas City, MO, USA) and ground. Gelatine extraction was done in triplicate for
158 each fish species and repeated for 3 batches. Gelatine extraction yield was calculated
159 as g of dry gelatine per 100 g of bones.

160

161 **2.5 Protein pattern of fish gelatines**

162 The electrophoresis procedure was carried out according to the method
163 described by Khiari *et al.* (2011). Gelatine solutions (5 mg/mL) were prepared in
164 distilled water at 60 °C and then diluted to a final concentration of 2 mg/mL with
165 sample buffer containing β -mercaptoethanol (Sigma, Dublin, Ireland). Gelatine
166 samples were heated to 85 °C for 10 min to denature the proteins. Samples and
167 molecular weight marker (10 μ L each) were loaded onto SDS-PAGE having a 4%
168 stacking gel and 10% resolving gel according to Laemmli (1970), the analysis was run
169 in an Atto Dual Mini-slab Size Electrophoresis System AE-6450 (Atto Corporation,
170 Tokyo, Japan) at a constant current of 25 mA/gel. Protein bands were stained with
171 Coomassie Brilliant Blue R250. The gel was de-stained using a mixture of
172 isopropanol, acetic acid and distilled water (12:10:78, v/v/v).

173 The molecular weight marker (Sigma, Dublin, Ireland) contained a lyophilised
174 mixture of six proteins: bovine carbonic anhydrase (29 kDa), egg albumin (45 kDa),
175 bovine albumin (66 kDa), phosphorylase B from rabbit (97.4 kDa), β -galactosidase
176 from *E. coli* (116 kDa) and myosin from rabbit muscle (200 kDa).

177

178 **2.6 Amino acids analysis**

179 The amino acid profile of gelatines was determined according to the method
180 described by Khiari *et al.* (2011). Briefly, 10 μ g of gelatine were subjected to
181 hydrolysis for 24 h at 110 °C, with 6 M HCl containing 0.1 % phenol in vacuum-
182 sealed hydrolysis vials. Norleucine (Sigma, Madrid, Spain) was added as an internal
183 standard. The amino acid composition was analysed using a cation exchange
184 Biochrom 20 amino acid analyzer (Pharmacia Biotech, Ltd., Cambridge, England)
185 with postcolumn derivatisation with ninhydrin. All amino acids were detected at an
186 absorbance of 570 nm, except for proline and hydroxyproline which were measured at
187 440 nm. Tryptophan and cysteine are completely lost by acid hydrolysis whereas
188 methionine can be destroyed to varying degrees (Lourenço *et al.*, 2002). Cysteine was
189 determined as cysteic acid by oxidation of the protein with formic acid prior to
190 hydrolysis, according to Hirs (1967). Results were averaged and presented as grams
191 (g) per 100 g amino acids. The amino acid analysis was done in the Service of Protein
192 Chemistry at the Centro de Investigaciones Biologicas (CSIC, Madrid).

193

194

195 **2.7 Protein solubility**

196 The effect of pH and ionic strength on the solubility of gelatines was
197 determined according to the method of Montero *et al.* (1991), with some
198 modifications.

199 **2.7.1 Effect of pH on gelatine solubility**

200 Gelatine samples were first dissolved in distilled water to a final concentration
201 of 0.3% (w/v, protein content). Eight mL of the gelatine solutions were added to a
202 glass test tube and the pH was adjusted ranging from 2.0 to 12.0 with either 1 M HCl
203 or 1 M NaOH using an Orion pH meter Model 420A (Orion Research Inc, Beverly,
204 MA. USA). The pH meter was calibrated at pH 4.0, 7.0 and 10.0 before the analysis.
205 The final volume was then adjusted to 10 mL by distilled water having the same pH
206 as the gelatine solution. Samples were centrifuged at 9,000×g for 15 min at 5 °C.
207 Protein content of the clear supernatant was determined according to the Biuret assay
208 as described by Gornall *et al.* (1949) and using bovine serum albumin (BSA, Sigma-
209 Aldrich, Inc., Dublin, Ireland) as a reference protein on a weight basis. Relative
210 solubility was calculated in comparison with that obtained at the pH giving the highest
211 solubility.

212
213

214 **2.7.2 Effect of NaCl on gelatine solubility**

215 Gelatines were dissolved in 50 mM potassium phosphate buffer at pH 7.5 to a
216 final concentration of 0.6% (w/v, protein content). Five mL of gelatine solution were
217 mixed with 5 mL of NaCl in 50 mM phosphate buffer at pH 7.5 at various
218 concentrations (0, 2, 4, 6, 8, 10 and 12% (w/v)). The mixture was stirred continuously
219 using a magnetic-stirrer device for 30 min at 5 °C, followed by centrifugation at
220 9,000×g for 15 min at 5 °C. Protein content of the clear supernatant was determined as
221 described above. Relative solubility was calculated in comparison with that obtained
222 at the NaCl concentration giving the highest solubility.

223
224

225

226 **2.8 Foaming properties**

227 Foaming properties including foaming capacity (FC) and foam stability (FS)
228 were determined by the method of Fernandez & Macarulla (1997) with minor
229 modifications. Gelatine solutions were prepared in 50 mM phosphate buffer at pH 7.5
230 to a final concentration of 0.3% (w/v, in protein content). Five mL of each sample
231 were homogenized with an Ultra-Turrax homogenizer, model T 25 (IKA Works, Inc.
232 Staufen, Germany) at 23,000 rpm for 1 min. FC was calculated as the percent increase
233 in volume of the protein dispersion upon mixing, while FS was estimated as the
234 percentage of foam remaining after 15 min.

235 **2.9 Emulsifying properties**

237 The emulsifying properties of gelatine samples were determined by the
238 method of Pearce & Kinsella (1978) with some modifications. Different
239 concentrations of gelatine solution were used. Gelatines were first dissolved in 50 mM
240 potassium phosphate buffer containing 0.3 M NaCl at pH 7.5 to the final
241 concentration of 0.05, 0.1 or 0.2% (w/v, in protein content). Then 2.0 mL of
242 commercial sunflower oil was mixed with 8.0 mL of each gelatine solution. The
243 mixture was vortexed in a plastic tube at 25°C and homogenized at 23,000 rpm for 1
244 minute. An aliquot (50 µL) of emulsion was diluted in 5 mL sodium dodecyl sulfate
245 (SDS) solution (0.1%, w/v) and the absorbance was measured at 500 nm (Milton Roy
246 Spectronic 1201, Rochester, NY, USA). To estimate the emulsion stability, the
247 emulsions were left for 15 min at 25 °C and then 50 µL of the emulsion were diluted
248 in 5 mL SDS solution (0.1%, w/v) and the absorbance was measured at 500 nm. The
249 emulsifying activity and emulsion stability were expressed as indexes.

250

251

252 EAI (m²/g) was defined as:

253

254

$$EAI(m^2/g) = \frac{2 \times 2.303}{C \times \phi \times 10^4} \times A_{500} \times Dilution$$

255

256 Where; A₅₀₀ represents the absorbance at 500 nm, C the protein concentration (g/mL)

257 before emulsification and Φ the oil volume fraction (v/v) of the emulsion (i.e. the

258 volume of emulsion droplets divided by the total volume of the emulsion, $\Phi=0.2$).

259 ESI (%) was calculated as the ratio of the turbidity measured at 500 nm of the

260 emulsion at time zero (A₀) and after 15 min (A₁₅) (Agyare *et al.*, 2009).

261

262

263

$$ESI(\%) = 100 \times \frac{A_{15}}{A_0}$$

264 **2.10 Statistical analyses**

265 ANOVA (Multifactor and one-way) was used to find differences between

266 treatments. Means were compared by significant difference (LSD) test, at a

267 significance level of p<0.05 using the Statgraphics Centurion XV software (version

268 15.1.02; StatPoint, Inc., Warrenton, VA, USA). Three independent trials were carried

269 out.

270

271 **3. Results and discussion**

272 **3.1 Characterisation of fish bones and gelatine extraction yield**

273

274 Mackerel and blue whiting bones had similar protein (19.8 and 19.5%,

275 respectively) and moisture (64.9 and 64.2%, respectively) contents. The ash content

276 was considerably high for both fish bones (8.9 and 16.0% for mackerel and blue

277 whiting, respectively) mainly due to the high content of minerals. The fat content of

278 mackerel bones was significantly (p<0.05) higher than blue whiting bones (5.5 and

279 0.8%, respectively), which could be due to the variation among the species (fatty and
280 lean fish).

281 The gelatine extraction yields varied depending on the pre-treatment used for
282 mackerel and blue whiting bones (Table 1). Regardless of fish, gelatine extracted
283 from chemically pre-treated bone, showed the lowest yield. Yields of 2.5 and 1.0%
284 were observed for mackerel and blue whiting respectively. Bones pre-treated
285 enzymatically generated significantly ($p < 0.05$) highest yields (~3.9 and 1.8%, for
286 mackerel and blue whiting, respectively).

287

288 **3.2 Protein pattern of fish gelatines**

289 The electrophoretic (SDS-PAGE) profiles of the various gelatine preparations
290 are shown in Figure 1.

291 The gel electrophoresis of chemically pre-treated fish bone gelatines (Figure 1
292 A & B, lane 4) showed the presence of the three bands (one β chain and two α chains).
293 These three chains are characteristics of type I gelatine. The β chain seems to be lower
294 or entirely absent in bone gelatines extracted after the enzymatic pre-treatment (Figure
295 1 A & B, lane 2 & 3), which could be due to the combined effect of enzymatic and
296 chemical hydrolysis of the gelatines as a consequence of the demineralisation step.

297 The presence of low molecular weight proteins was observed in gelatines
298 extracted after the enzymatic pre-treatment. This may indicate a partial hydrolysis of
299 gelatine during extraction (Giménez *et al.*, 2005).

300

301

302

303

304 3.3 Amino acid profile

305 The amino acid composition of gelatine from mackerel and blue whiting
306 bones, expressed as percentage of total amino acid, is shown in Table 2. Different
307 profiles were observed for fish bone gelatines depending on the pre-treatment.
308 Mackerel bone gelatine, pre-treated with sodium hydroxide, had high glycine content
309 (21.3%). Gelatines extracted after the enzymatic pre-treatment of mackerel bones,
310 showed significantly different amino acid profile. The glycine content was half of that
311 observed for chemically pre-treated bone gelatines (~11.5%). Blue whiting bone
312 gelatines, pre-treated with sodium hydroxide and Alcalase, had similar glycine
313 content (~20%). However, lower glycine content was observed with Flavourzyme
314 pre-treated blue whiting bone gelatines (17%).

315 For both fish, significant ($p < 0.05$) differences among the content of
316 hydrophobic amino acids (Ala, Val, Ile, Leu, Met, Phe, Tyr and Cys) were observed
317 for enzymatically pre-treated bone gelatines compared to chemically pre-treated bone
318 gelatines. Low content of imino acids (proline and hydroxyproline) were observed for
319 both fish bone gelatines, pre-treated enzymatically, compared to chemically pre-
320 treated fish bone gelatines. The imino acid content of chemically pre-treated bone
321 gelatines was similar to that observed for Japanese sea bass caudal fin (Nagai, 2004),
322 black drum (*Pogonia cromis*) and sheepshead seabream (*Archosargus*
323 *probatocephalus*) bone collagens (Ogawa *et al.*, 2003). The difference among proline
324 and hydroxyproline contents of mackerel bone gelatines may affect the rheological
325 properties of the gelatines. Gelatines with low proline and hydroxyproline level
326 usually show lower melting point and weaker gel network (Gilsenan & Ross-Murphy,
327 2000; Johnston-Banks, 1990).

328 Regardless of the pre-treatment, tryptophan was not detected in any gelatines
329 but cysteine was present in low levels. This may indicate some contaminations by
330 non-collagenous protein (Morimura *et al.*, 2002).

331

332 **3.4 Protein solubility**

333 The effect of pH and NaCl on the relative solubility of gelatines from
334 mackerel and blue whiting bones are shown in Figure 2.

335 All the gelatines, regardless of the pre-treatment used, showed similar pH
336 behaviour. The solubility was higher at low pH, with a maximum at pH 2. The lowest
337 solubility was observed close to neutral pH (Figure 2 A & B). Similar results were
338 also reported by Aewsiri *et al.* (2008) for gelatines from precooked tuna fin.

339 The effect of NaCl on the relative solubility is depicted in Figure 2 (C & D). In
340 general, the solubility of gelatines decreased gradually with increasing concentration.
341 The decrease in solubility with the increase of NaCl concentration is probably due to
342 the increase of the hydrophobic interactions and to the competition of ionic salts for
343 water (Vojdani, 1996).

344 The higher solubility of gelatines, from both fish bones pre-treated
345 enzymatically, compared to those pre-treated chemically could be due to the presence
346 of lower molecular weight peptides formed during the extraction process (enzymatic
347 hydrolysis and demineralisation step). The hydrolysis generally cleaves peptides with
348 more polar residues that may interact with water molecule through hydrogen bonds
349 and results in an increase in solubility (Gbogouri *et al.*, 2004).

350

351 **3.5 Foaming capacity and stability**

352 Foams are complex two-phase colloidal systems which contain at least a
353 continuous liquid phase and a gas phase dispersed as bubbles or air cells. The

354 properties of foams determine their industrial applications. In the food industry, the
355 determination of foaming properties has a significant impact on the processing and the
356 quality of some products (Exerowa & Kruglyakov, 1998).

357 The foaming capacity (FC) of gelatines, extracted from mackerel and blue
358 whiting bones, varied significantly ($p < 0.05$) depending on the source and the pre-
359 treatment. Regardless of the pre-treatment, gelatines from blue whiting bones showed
360 higher FC than mackerel bone gelatines. Mackerel bone gelatines showed similar
361 values for FC (~38 %, Figure 3 A) with no significant ($p > 0.05$) differences between
362 pre-treatments. Gelatines extracted from blue whiting bones, after enzymatic pre-
363 treatment, had significantly ($p < 0.05$) higher FC than chemically pre-treated bones
364 (Figure 3 B). All mackerel bone gelatines, regardless of the pre-treatment used had
365 very high foaming stability (Figure 3 C). Blue whiting bone gelatines had lower FS
366 than mackerel bone gelatines. The chemical pre-treatment of blue whiting bones
367 resulted in gelatines with significantly ($p < 0.05$) lower FS than enzymatic pre-
368 treatment.

369 Foaming properties (foaming capacity and stability) of a protein, including
370 gelatine, might be influenced by the source, intrinsic properties, the compositions and
371 conformations of the protein in solution (Wilde & Clark, 1996; Zayas, 1997). The
372 process of foam formation depends largely on the protein adsorption kinetics at the
373 air-water interface (Phillips *et al.*, 1994). The higher FC observed with enzymatically
374 pre-treated bone gelatines may be due to the higher amount of hydrophobic amino
375 acid residues (Ala, Val, Ile, Leu, Met, Phe, Tyr and Cys) compared to chemically pre-
376 treated bone gelatines (Table 2). The foaming agent, having an amphiphilic property,
377 adsorbs at the air-water interface and orients itself in such a way that the lipophilic
378 group orients towards the non-polar phase and the hydrophilic group towards the

379 aqueous phase. This phenomenon reduces the surface tension allowing the formation
380 of the foam (Liceaga-Gesualdo & Li-Chan, 1999). The lower FS observed with
381 chemically pre-treated mackerel and blue whiting bone gelatines compared to those
382 extracted after the enzymatic pre-treatment of bones, could be due to the lower
383 percentage of negatively charged amino acids (Asp and Glu). On average, the
384 enzymatic pre-treated bone gelatines had 21% negatively charged amino acids
385 compared to 16% from chemically pre-treated bone gelatines. Higher content of
386 negatively charged amino acids, observed with enzymatically pre-treated bone
387 gelatines, may have prevented the neutralisation of charge in gelatine molecules and
388 enhanced the FS.

389

390 **3.6 Emulsifying capacity and stability**

391 Emulsions are a major component of many foods and their properties
392 (emulsifying capacity and stability) play an important role in the formulation of food
393 products (Spyropoulos *et al.*, 2011). Emulsion activity index (EAI) and emulsion
394 stability index (ESI) for gelatines from mackerel and blue whiting bones at different
395 protein concentrations (0.05, 0.1 and 0.2%) are shown in Table 3.

396

397 **3.6.1 Emulsifying capacity**

398 The emulsifying activity index (EAI), a measurement of the area of interface
399 stabilized per unit weight of protein (m^2/g) relates to the ability of a protein to coat an
400 interface (Pearce & Kinsella, 1978). The results showed that the increase of the
401 concentration of gelatine solution decreased the emulsifying activity (EAI). Similar
402 results were reported by Binsi *et al.* (2009) for gelatine from skin of bigeye snapper.
403 The protein concentration is an important parameter that affects the emulsifying
404 activity. Low protein concentration favours higher EAI, due to the ability of the

405 protein to diffuse and adsorb at the oil-water interface (Cheftel *et al.*, 1985). While at
406 high protein concentration, the diffusion is limited as a result of the activation energy
407 barrier (Phillips, 1981).

408 For all the concentrations studied (0.05, 0.1 and 0.2%), gelatines extracted
409 from mackerel and blue whiting bones after pre-treatment with Alcalase and
410 Flavourzyme showed significantly ($p < 0.05$) highest EAI, while the chemical pre-
411 treatment gave gelatines with the lowest EAI (Table 3). This possibly resulted from
412 the difference in the intrinsic properties, composition and conformation among the
413 different gelatines (Cheftel *et al.*, 1985). Mechanisms of the emulsification process of
414 gelatines are correlated to the adsorption ability at the surface of freshly formed oil
415 droplets during homogenization and formation of a protective membrane that prevents
416 droplets coalescence. According to Rahali *et al.* (2000), the degree of insertion of
417 peptides in the interfacial layer mostly depends on the alternative distribution of
418 hydrophobic and charged amino acids. The flexibility of protein (or peptide) structure
419 may be an important structural factor governing the emulsification (Kato *et al.*, 1985).
420 It is also known that protein solubility plays an important role in emulsification
421 because rapid migration and adsorption at the interface are critical (Chobert *et al.*,
422 1988).

423

424 **3.6.2 Emulsifying stability**

425 For all gelatines, regardless of the source and the pre-treatment, a positive
426 correlation between the protein concentration and the ESI was found (increasing the
427 concentration of gelatine solutions increased the ESI). Similar results were previously
428 observed for whey proteins (Hung & Zayas, 1991). High protein concentrations result

429 in higher viscosity of the dispersion. This usually leads to a better emulsion stability
430 probably by reducing the coalescence rate (Sajjadi, 2007).

431 For both fish, gelatines extracted after the enzymatic pre-treatment of bones
432 showed significantly ($p < 0.05$) higher ESI compared to those extracted after the
433 chemical pre-treatment (Table 3). As discussed before, gelatines from mackerel and
434 blue whiting bones, pre-treated with Flavourzyme and Alacalse, had significantly
435 higher hydrophobic amino acid residues. According to Giménez *et al.* (2009), a higher
436 content in hydrophobic amino acid residues results in an effective distribution of
437 hydrophilic/hydrophobic amino acids which leads to an improvement of the
438 emulsifying properties of gelatines. Hence, the higher content of hydrophobic amino
439 acid residues, observed with enzymatically pre-treated bone gelatines, may have
440 resulted in better EAI and ESI in comparison to chemically pre-treated bone gelatines.

441 **4. Conclusion**

442 The composition and the functional properties of mackerel and blue whiting
443 bone gelatines were affected by the pre-treatment used in the extraction process. The
444 enzymatic pre-treatment of fish bones produced gelatines with higher content of
445 hydrophobic amino acid residues. The presence of these amino acids had a positive
446 effect on the functional properties of the gelatines. In general, higher foaming and
447 emulsifying properties were observed with enzymatically pre-treated bone gelatines.

448

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453

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624 **Figure Captions**

625

626 **Figure 1** SDS-PAGE patterns of mackerel (A) and blue whiting (B) bone gelatines.

627 Lane 1: Molecular weight marker (MW. 30,000 - 200,000); lane 2: gelatine from bone

628 pre-treated with Flavourzyme; lane 2: gelatine from bone pre-treated with Alcalase;

629 lane 4: gelatine from bone pre-treated with NaOH.

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631

632 **Figure 2** Foaming capacity (FC) and stability (FS) of gelatines from mackerel (A &

633 C) and blue whiting (B & D) bones extracted using different pre-treatments.

634

635 **Figure 3** Relative solubility of gelatines from mackerel (A & C) and blue whiting (B

636 & D) bones extracted using different pre-treatments. Solubility in the pH range 2 – 12

637 (A & B) and solubility as function of NaCl concentration (C & D).

638

639 **Table Captions**

640

641 **Table 1** Yield of gelatine extraction.

642

643 **Table 2** Average amino acid composition (g/100 g amino acids) of gelatines extracted

644 from mackerel and blue whiting bones using different pre-treatments.

645

646 **Table 3** Emulsifying activity (EAI) and stability (ESI) indexes of gelatines from

647 mackerel and blue whiting bones extracted using different pre-treatments at different

648 concentrations (0.05, 0.1 and 0.2%).

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650 **Khiari et al**

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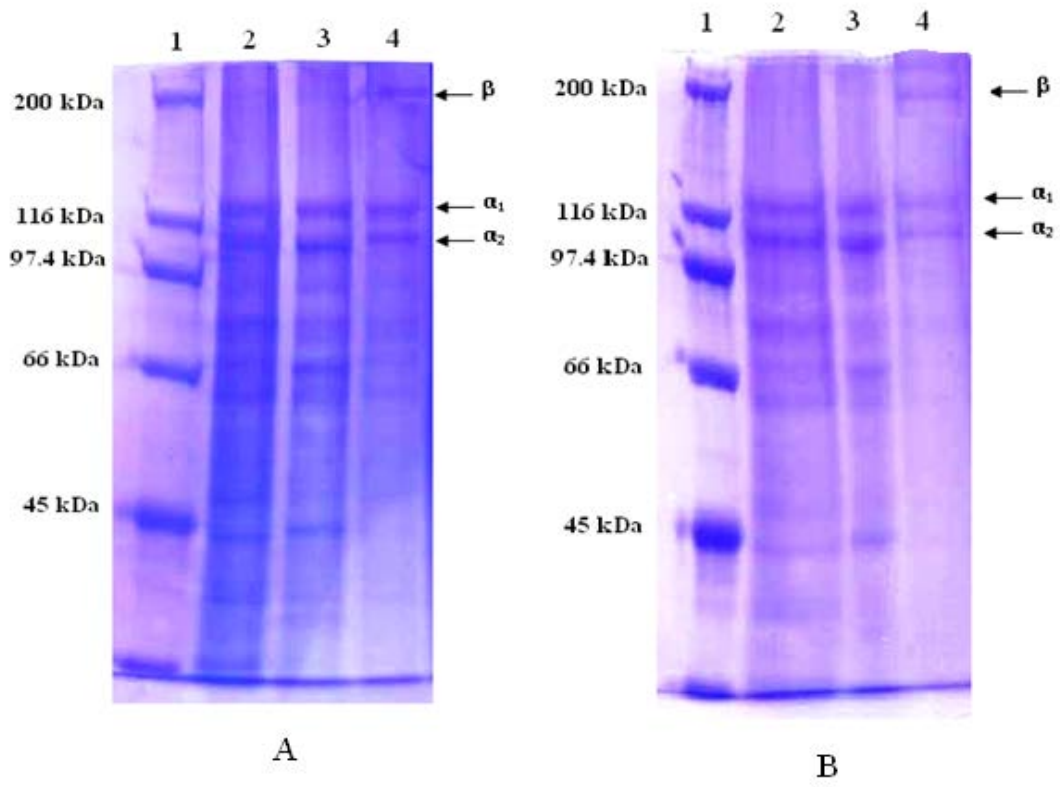
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653 Figure 1

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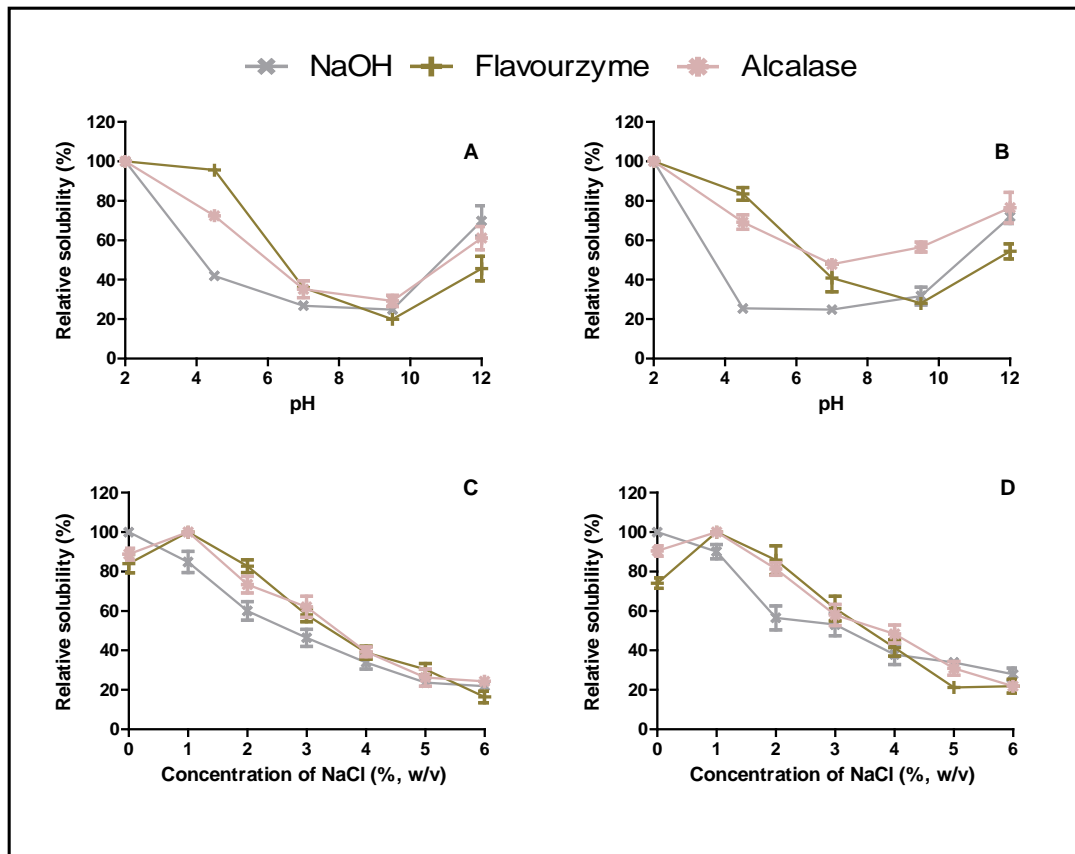


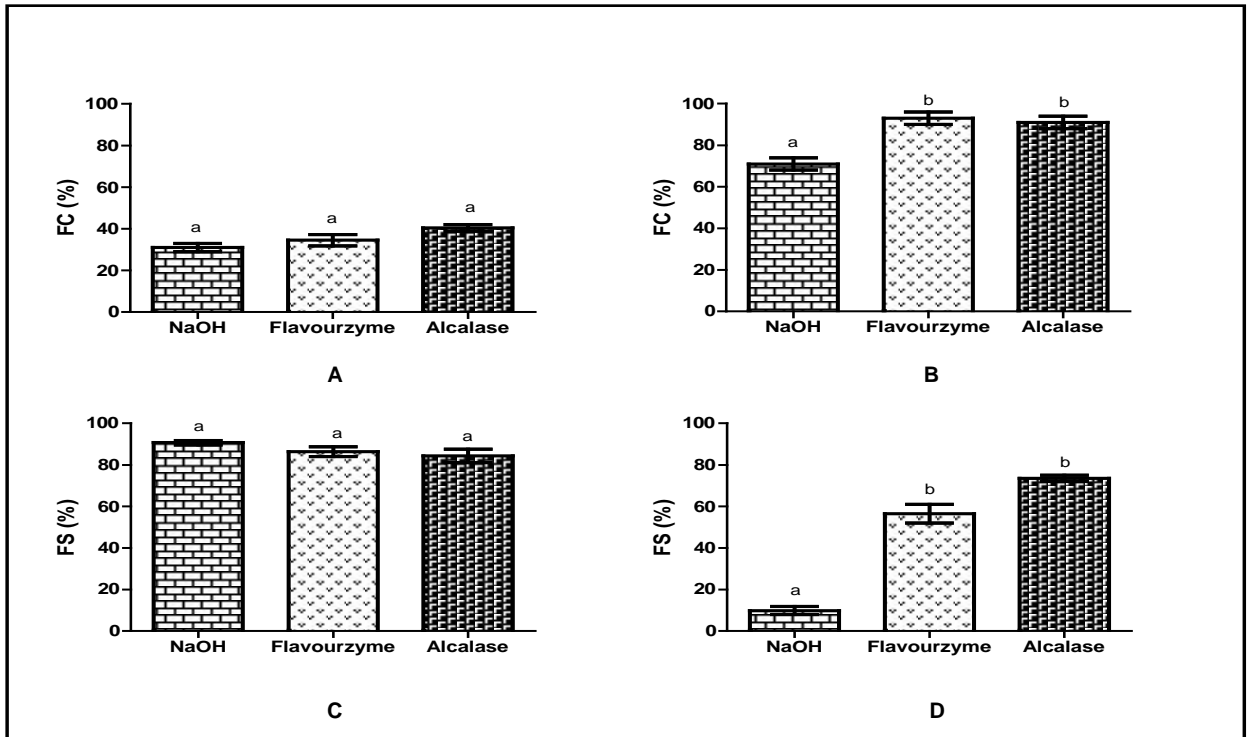
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662 Figure 2





742 **Khiari et al**

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744 Table 1

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757 Pre-treatment: NaOH: alkaline; Alcalase and Flavourzyme. Values are given as

758 mean \pm standard deviation. Different lower case letters in the same column indicate

759 significant ($p < 0.05$) differences between pre-treatments. Different upper case letters in

760 the same row, within the same pre-treatment (i.e. chemical or enzymatic), indicate

761 significant ($p < 0.05$) differences between fish species.

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Pre-treatment	Mackerel	Blue whiting
NaOH	2.5 \pm 0.1 ^{aB}	1.0 \pm 0.1 ^{aA}
Alcalase	3.7 \pm 0.2 ^{bB}	1.8 \pm 0.2 ^{bA}
Flavourzyme	4.0 \pm 0.1 ^{bB}	1.9 \pm 0.2 ^{bA}

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Table 2

Amino acids	Content (g/100 g amino acids)					
	Mackerel			Blue whiting		
	NaOH	Alcalase	Flavourzyme	NaOH	Alcalase	Flavourzyme
Asp	6.1	8.1	7.8	6.5	8.7	8.4
Thr	2.8	3.8	3.7	3.0	2.9	3.3
Ser	4.8	4.6	4.4	5.6	5.3	5.4
Glu	9.8	13.5	13.3	10.5	10.9	11.7
Gly	21.3	11.4	11.6	20.7	20	17.3
Ala	9.0	7.0	7.3	9.1	8.8	8.3
Cys	0.8	1.2	1.2	0.1	0.2	0.2
Val	2.3	4.0	3.7	2.3	2.5	2.8
Met	2.1	2.6	2.6	2.4	2.5	2.7
Ile	1.3	3.0	2.5	1.4	1.6	2.0
Leu	3.7	6.2	6.0	2.9	3.4	4.1
Tyr	1.1	2.7	2.3	0.7	1.1	1.5
Phe	2.6	3.3	3.1	2.4	2.4	2.7
His	1.2	2.3	2.3	1.6	1.6	1.8
Lys	4.1	6.7	7.0	3.7	4.1	4.8
Arg	8.4	7.3	7.5	8.6	8.8	8.4
Pro	10.3	6.9	7.7	9.7	9.4	8.4
Hyp	8.3	5.4	6.0	8.5	5.7	6.1

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Pre-treatment: NaOH: alkaline; Alcalase and Flavourzyme. The amino acid composition was performed in triplicate and data correspond to mean values.

806 **Khiari et al**

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808 Table 3

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		EAI			ESI		
		0.2%	0.1%	0.05%	0.2%	0.1%	0.05%
Mackerel	NaOH	8.3±1.5 ^{aA}	10.7±1.9 ^{aB}	15.3±2.4 ^{aB}	35.2±3.6 ^{bB}	26.2±3.7 ^{bB}	13.1±2.4 ^{aA}
	Alcalase	22.6±3.4 ^{cA}	35.7±3.1 ^{cB}	36.5±2.5 ^{bB}	54.4±4.5 ^{cB}	53.7±2.3 ^{cB}	24.9±0.9 ^{bA}
	Flavourzyme	23.1±2.7 ^{cA}	30.0±0.8 ^{cB}	35.0±5.5 ^{bB}	52.8±2.8 ^{cB}	48.1±1.6 ^{cB}	24.7±1.4 ^{bA}
Blue whiting	NaOH	16.4±1.2 ^{bA}	24.6±2.9 ^{bB}	39.3±4.8 ^{bC}	17.9±2.8 ^{aB}	11.4±2.2 ^{aA}	9.3±2.8 ^{aA}
	Alcalase	19.8±0.7 ^{cA}	33.5±2.7 ^{cB}	62.1±3.5 ^{cC}	37.5±3.4 ^{bB}	32.6±3.4 ^{bB}	10.4±2.7 ^{aA}
	Flavourzyme	20.8±2.5 ^{cA}	35.6±2.4 ^{cB}	54.6±2.4 ^{cC}	40.8±5.0 ^{bC}	27.7±3.1 ^{bB}	13.6±1.5 ^{aA}

822 Pre-treatment: NaOH: alkaline; Alcalase and Flavourzyme. Values are given as mean ± standard deviation. Different lower case letters in the

823 same column indicate significant (p<0.05) differences between pre-treatments. Different upper case letters in the same row, within the same

824 parameter (i.e. EAI or ESI), indicate significant (p<0.05) differences between concentrations.

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