1 **LESSENING OF HIGH-PRESSURE-INDUCED CHANGES IN ATLANTIC SALMON MUSCLE**
2 **BY THE COMBINED USE OF A FISH GELATIN-LIGNIN FILM**
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12 ** This centre has implemented and maintains a Quality Management System which fulfils the
13 requirements of the following standard ISO 9001:2008.
14
15

16 **Abstract**

17 Salmon muscle is considerably affected by cooking with the resulting loss of its appealing red
18 colour. The combined use of high pressure with fish gelatin-lignin film is proposed as an
19 alternative to the more aggressive thermal processing procedures, with the aim of improving the
20 appearance and overall quality of salmon fillets in ready-to-eat or semi-prepared dishes. The
21 effects of high pressure processing (300 MPa, 10 min, 5ºC or 40ºC) and conventional heating
22 (90ºC, 10 min) were evaluated in terms of colour changes, protein denaturation, as well as
23 protein and lipid oxidation, by comparison with raw muscle. The stability of the processed
24 products was assessed by monitoring changes in microbial growth and total volatile basic
25 nitrogen and thiobarbituric acid reactive substances during 23 days of chilled storage. Fourier
26 transform infrared spectroscopy (FTIR), apparent viscosity and dynamic oscillatory studies
27 revealed notable differences in the overall degree and nature of protein aggregation between
28 high pressure and heating treatments, especially when performed at 5ºC instead of 40ºC. SDS-
29 PAGE of the protein fraction solubilized in 0.8M NaCl showed MHC and α-actinin to be the main
30 myofibrillar proteins denatured by high pressure processing at 40ºC, while actin was more
31 denatured when pressurized at 5ºC. The film attenuated colour changes associated with high
32 pressure treatment, especially at 5ºC, where redness was more preserved without jeopardizing
33 the appearance of a ready-to-eat product. High pressure processing at 5ºC in combination with
34 gelatin-lignin film was found to improve protein quality of salmon fillets. The film reduced the
35 levels of carbonyl groups formed immediately after processing, and prevented lipid oxidation
36 from taking place at advanced stages of chilled storage. However, the effect on microbial
37 growth was negligible, since total counts were similar for muscle with or without the film.

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40 Keywords: salmon, high pressure processing, colour, protein denaturation, FTIR, dynamic
41 oscillatory studies, oxidation, microbial growth.

42

43 1. **Introduction**

44 High hydrostatic pressure has been widely applied as a minimal processing technique to
45 prolong the shelf-life of additive-free foods, with its major advantage being the conservation of
46 micronutrients such as amino acids and vitamins, as well as flavour compounds (Knorr, 2000).
47 Although it is claimed that high pressure processing has less impact on colour compared to
48 thermal processing treatments (Ohshima, Ushio & Koizumi, 1993), it is well recognized that
49 pressurizing above 200 MPa leads to changes in colour and texture, giving fish muscle a
50 cooked-like appearance (Montero & Gómez-Guillén, 2005). With this idea in mind, high
51 pressure processing could be considered as an alternative to conventional heating treatments
52 for the preparation of ready-to-eat or semi-prepared dishes, with a minimum loss of
53 micronutrients and flavour compounds.

54 High pressure induces dramatic changes in the colour of Atlantic salmon muscle as this fish
55 species is probably one of the most sensitive in this respect (Amanatidou et al., 2000; Yagiz et
56 al., 2009). Besides haeme proteins, carotenoids (mainly astaxanthin and canthaxanthin) are
57 mainly responsible for the typical red colour of Atlantic salmon muscle (Andersen, Bertelsen,
58 Christophersen, Ohlen & Skibsted, 1990). In particular, the reduction in redness in pressurized
59 smoked salmon has recently been linked with astaxanthin oxidative degradation, coupled with
60 the formation of deoxy-myoglobin or deoxy-haemoglobin (Tintchev et al., 2009). Though early
61 studies reported that carotenoids were bound to actomyosin in salmon muscle (Henmi, Hata &
62 Hata, 1989), α-actinin was later identified as the target myofibrillar protein for binding with
63 astaxanthin (Matthews, Ross, Lall & Gill 2006). Thus, the degree of protein denaturation in
64 salmon muscle is relevant, not only affecting textural properties, but also influencing the strong
65 colour changes. High pressure has been shown to modify the palatability and functional
66 properties of meat and fish products by inducing denaturation and muscle protein gelation
67 (more specifically myosin and actin) (Cheftel & Culioli 1997). Yamamoto, Hayashi & Yasui
68 (1993) reported that pressure-induced aggregation was similar to that induced by thermal
69 treatment at the level of myosin head interaction. However, the nature of such interactions
70 differs somewhat, since pressure induces the formation of heat labile hydrogen-bonded
71 structures while the contribution of disulfide bonds and hydrophobic interactions plays a key role
72 in the stabilization of heat-induced structures (Angsupanich, Edde & Ledward, 1999). Fourier

73 transform infrared spectroscopy (FTIR) provides information about the chemical composition
74 and conformational structure of food components, and in particular, it is a useful tool for
75 monitoring changes in secondary protein structures (Willard, Merritt, Dean & Settle, 1981;
76 Jackson & Mantsch, 1995). Recently, FTIR has been successfully applied in the study of both
77 salted and salted smoked salmon muscle denaturation (Böcker, Kohler, Aursand & Ofstad,
78 2008; Carton, Bocker, Ofstad, Sørheim, & Kohler, 2009), however, to our knowledge, the effect
79 of high pressure treatment on fish muscle proteins has not yet been studied by FTIR.
80 Haeme proteins, which are key catalysts of lipid oxidation in fish muscle (Hultin, 1994), can also
81 be denatured by either heat or high pressure treatment, and become pro-oxidative. The
82 oxidation occurs mainly by the release of the haeme pigment from the haeme-proteins, since
83 free haeme pigment is known to be a very potent pro-oxidant (Everse & Hsia, 1997). Although it
84 is generally assumed that high pressure may induce some lipid oxidation, this effect would
85 largely depend on factors such as pressure level, fish species and subsequent storage. In
86 salmon muscle, the presence of asthaxantin, which has been described as a potent antioxidant
87 (Shimidzu, Goto, & Miki, 1996) may provide considerable protection from lipid oxidation. Yagiz
88 et al. (2009) reported that a high pressure treatment of 300 MPa and ~ 25ºC average
89 temperature reduced the oxidation susceptibility of Atlantic salmon muscle,, probably as a result
90 of pressure-induced changes in muscle structure, making astaxanthin more available to protect
91 lipids from oxidation. However, when pressurized at 300 MPa and ~ 7ºC, a significant increase
92 in TBARs was found for salmon carpaccio (Gómez-Estaca, López-Caballero, Gómez-Guillén,
93 López de Lacey, & Montero, 2009). Thus, the temperature at which high pressure processing is
94 carried out may also be a key factor affecting the oxidative stability of pressurized products.
95 The use of edible films helps to maintain product quality, enhance sensory properties, improve
96 product safety, and increase the shelf life of various ready-to-eat food products (Beverlya,
97 Janes, Prinyawiwatkula, & No, 2008). These films act as oxygen and water barriers thereby
98 slowing oxidation reactions and retaining moisture, thus enhancing quality and extending
99 storage life (Gennadios, Hanna, & Kurth, 1997).To control lipid oxidation in foodstuffs, there is a
100 growing interest in the formulation of edible and/or biodegradable active films, which are based
101 on the inclusion of an antioxidant or antimicrobial compound in the polymeric matrix. Although
102 there is increasing literature available regarding the development of active biodegradable films

103 (Guilbert, Gontard, & Gorris, 1996; Oussalah, Caillet, Salmiéri, Saucier, & Lacroix, 2004;
104 Gómez-Guillén, Pérez-Mateos, Gómez-Estaca, López-Caballero, Giménez, & Montero, 2009),
105 limited work has been done in relation to their application (in the form of films or coatings) to
106 extend the shelf-life of fish products (Jeon, Kamil, & Shahidi, 2002 , Ouattara, Sabato & Lacroix,
107 2001; López-Caballero, Gómez-Guillén, Pérez-Mateos, & Montero, 2005; Sathivel, Liu, Huang,
108 & Prinyawiwatkul, 2007; Ojagh, Rezaei, Razavi, & Hosseini, 2010). The application of active
109 plant extract-gelatin composite edible films in combination with high pressure processing in
110 cold-smoked sardine has already been shown to be a very promising technology for fish
111 preservation (Gómez-Estaca, Montero, Giménez, & Gómez-Guillén, 2007). However, more work
112 is necessary in this field to assess the composition and suitability of films, and thereby ensure
113 their resistance to high pressure processing while at the same time taking into account the wet
114 nature of the fish surface.
115 Lignin is found in plant cell walls in association with cellulose and hemi-cellulose, and is an
116 abundant waste product in the pulp and paper industry. In the present study, and based on
117 preliminary work carried out in our laboratory, a mixture of gelatin and lignin was used to
118 prepare the protective film, which was found to be flexible, transparent, with a relatively strong
119 brownish-yellow coloration and having reasonably high water resistance (unpublished data).
120 Lignin and derivatives have the right chemistry to be used in making coatings and composites
121 given their small particle size, hydrophobicity and their ability to form stable mixtures (Park,
122 Doherty, & Halley, 2008). Lignin has already been successfully used in the preparation of
123 gelatin-lignin films with high swelling capacity (Vengal & Srikumar, 2005). Moreover, lignins
124 have been shown to have efficient antibacterial and antioxidative properties (Ugartondo,
125 Mitjans, & Vinardell, 2009).
126 The objective of the present work was to evaluate the effects on Atlantic salmon fillets of two
127 high pressure treatments at different temperatures (300 MPa at 5ºC and 40ºC), in combination
128 with gelatin-lignin biodegradable film, compared with conventional heating treatment in an oven
129 at 90ºC. The degree of muscle alteration as a result of the different processing treatments was
130 assessed by comparing the extent of colour changes, protein denaturation, as well as protein
131 and lipid oxidation with raw muscle, The stability of the processed products was evaluated by

132 monitoring changes in microbial growth and total volatile basic nitrogen and thiobarbituric acid
133 reactive substances during 23 days of chilled storage.

134 **2. Materials and Methods**

135 **2.1. Preparation of film**

136 The gelatin-lignin film forming solution (FFS) was prepared by dissolving a commercial warm-
137 water fish gelatin (Rousselot S.A.S., Puteaux, France) in distilled water (3.4 % w/v) at 40ºC, with
138 sorbitol (15 g/100 g gelatin) and glycerol (15 g/100 g gelatin) as plasticizers. The commercial
139 lignin powder (Protobind 1000, Granit R&D SA, Lausanne, Switzerland) was added to a final
140 concentration of 0.6% w/v in the FFS. This concentration was selected according to previous
141 experiments. The mixture was stirred at 40 ºC for 15 min and was alkalinized to ~pH=11 to
142 obtain a good blend with total solubility. The films were made by casting an amount of 40 ml
143 over a plate of 12x12 cm^2 and drying at 45 ºC in a forced-air oven for 15 h to yield a uniform
144 thickness of 100 µm. Prior to covering the fish, films were conditioned over a saturated solution
145 of KBr in desiccators for 5 d. The film retained its structural integrity when applied on the surface
146 of the fillet and was not altered by the high pressure treatment, allowing to be easily separated
147 from the muscle for further analyses.

148 **2.2. Fish sample preparation and High Pressure Processing (HPP)**

149 Salmon fillets were acquired at a local market. The fillets were cut into 85–100 g portions (~ 5-6
150 cm thickness) and divided randomly to prepare six lots: (i) untreated raw muscle, R; (ii) cooked
151 muscle, C; (iii) muscle pressurized at 5ºC, HP5; (iv) muscle covered with a gelatin-lignin film
152 and subsequently pressurized at 5ºC, HPF5; (v) muscle pressurized at 40ºC, HP40 and (vi)
153 muscle covered with a gelatin-lignin film and subsequently pressurized at 40ºC, HPF40. In the
154 covered samples, each portion was covered individually with the gelatin-lignin film.

155 For HPP, all lots were vacuum-packaged into flexible plastic bags by using a Multivac
156 packaging machine (A-NG, 85021, GS, Germany). Vacuum packed samples were treated in an
157 pilot high-pressure unit (ACB 665, GEC Alsthom, Nantes, France), the temperature of the
158 immersion medium (distilled water) being regulated by a thermocouple connected to a
159 programmed temperature control equipment (model IA/2230AC, INMASA, Barcelona, Spain).
160 HPP was carried out for 10 min at 300MPa and at two temperature levels (5ºC and 40°C).

161 Pressure was increased by 2.5MPa/s and after high pressure treatment was completed, the
162 time for pressure dropping back to atmospheric was around 3 s.
163 The C lot was prepared by cooking the vacuum packed muscle portions at 90 °C for 10 min in a
164 Rational oven (mod. Combi-Master CM6, Suffolk, UK). All lots were stabilized at -80°C for
165 further analysis, except for microbiological, TVBN (total volatile basic nitrogen) and TBARs
166 (thiobarbithuric reactive substances) analyses, where a study of 23 days of chilled storage at
167 7ºC was performed.

168 **2.3. Colour measurements**

169 The colour parameters lightness (L*), redness (a*), and yellowness (b*) were measured using
170 a Konica Minolta CM-3500d colorimeter (Osaka, Japan). Measurements were taken at a
171 number of locations in different muscle portions and each point is the mean of at least ten
172 measurements.

173 **2.4. Protein solubility**

174 One gram of muscle (without connective tissue) was homogenized with 40 ml of 0.8M NaCl in
175 an Omni-Mixer homogenizer model 17106 (Omni International, Waterbury, CT) for 1 min at
176 setting 6. The resulting homogenates were stirred at ~4°C for 30 min and then centrifuged for
177 30 min at 4000xg in a Multifuge 3 L-R Heraeus (Hanau, Germany). The protein concentration in
178 the supernatant was determined by using the BCA kit (Meridian RD., Rockford, IL, 61101 USA).
179 Soluble protein was expressed as the percent protein solubilized with respect to total muscle
180 protein, which was determined according to AOAC (1984).

181 **2.5. Apparent Viscosity**

182 Apparent viscosity of muscle was determined according to Borderías, Jiménez-Colmenero &
183 Tejada (1985). Previously thawed samples (50 g) were homogenized with 150 mL of 5% NaCl
184 (w/v) in 0.2 M sodium phosphate buffer (pH 7.0). Apparent viscosity of the resulting
185 homogenate was measured by taking 3 readings at 1-min intervals at the speed of 12 rpm using
186 a Brookfield-LV viscometer (Brookfield Engineering NABS, Stoughton, Mass., U.S.A.).
187 Alternatively, the homogenate was filtered through gauze and centrifuged for 10 min at 345xg in
188 a Multifuge 3 L-R Heraeus (Hanau, Germany). All the procedures were carried out at ~4 °C. The
189 values obtained were expressed in centipoises (cP), and the results presented are averages of
190 at least 3 replicates.

191 **2.6. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)**

192 Salt-soluble protein was analyzed by SDS-PAGE in a Mini Protean II unit (Bio-Rad Laboratories,
193 Hercules, CA) at 20 mA/gel, using 12 % polyacrylamide gels. Samples were treated according
194 to Hames (1985) (2.5% SDS, 5% mercaptoethanol, 10mM Tris-HCl, 1mM EDTA and 0.002%
195 bromophenol blue) and then heated for 5 min in a boiling water bath. The protein concentration
196 was adjusted to 1 mg/ml, except in the cooked sample (due to lower protein concentration in the
197 supernatant), and the loading volume was 20 μl in all lanes, except for cooked sample, where
198 30μl were loaded. Protein bands were stained with Coomassie brilliant blue R-250. As reference
199 for molecular weights, two standards were used: SDS-PAGE Standard High Range (Cat. Num.
200 161-0309) (std 1) and SDS-PAGE Standard Low Range (Cat. Num. 161-0305) (std 2), (BioRad,
201 CA, USA).

202 **2.7. FTIR-Attenuated Total Reflectance (ATR) spectroscopy**

203 Infrared spectra between 4000 and 650 cm-1 were recorded using a Perkin Elmer Spectrum
204 400 Infrared Spectrometer (Perkin Elmer Inc, Waltham, MA, USA) equipped with an ATR prism
205 crystal accessory. For each spectrum 32 scans of interferograms were averaged and the
206 spectral resolution was 4 cm-1. Background was subtracted using the Spectrum software
207 version 6.3.2 (Perkin Elmer Inc.). Measurements were performed at room temperature using
208 extremely thin layers of semi-thawed muscle, which were placed on the surface of the ATR
209 crystal, and pressed with a flat-tip plunger until spectra with suitable and stable peaks were
210 obtained. All experiments were performed at least in duplicate.

211 **2.8. Dynamic oscillatory studies**

212 Salmon muscle batches of 100 g were homogenized with 2% NaCl in a domestic Braun
213 Minipimer homogenizer at ~4ºC for 3 min. Dynamic viscoelastic studies were performed on a
214 Bohlin CVO-100 rheometer (Bohlin Instruments Ltd., Gloucestershire, UK) using a cone-plate
215 geometry (cone angle 4º, gap=0.15 mm). A dynamic frequency sweep was done at 10ºC by
216 applying oscillation amplitude within the linear region (γ=0.005) over the frequency range 0.1-
217 10Hz. The Elastic modulus (G'; Pa), Viscous modulus (G''; Pa) and Complex modulus (G*, Pa)
218 were plotted as a function of frequency. The dynamic temperature sweep was done by heating
219 from 10 ºC to 90 ºC at a scan rate of 1 ºC/min, frequency 0.1 Hz, and target strain γ=0.005. G'

220 (Pa), G'' (Pa) and phase angle (δ; º) were plotted as a function of temperature. Results were
221 averages of at least 2 determinations.

222 **2.9. Protein carbonyl groups**

223 Protein carbonyls were assayed as hydrazone derivatives by reacting proteins with 2,4-
224 dinitrophenylhydrazine(DNPH) (Sigma Chemical Co., St. Louis, MO, USA) as described by
225 Levine et al. (1990). Results of carbonyl groups content were expressed in nm/mg protein, and
226 were the average values of at least three determinations.

227 **2.10. Microbiological assays**

228 The microbiological analyses were as follows: a total amount of 10 g of muscle, from at least 3
229 different packages, was collected and placed in a sterile plastic bag (Sterilin, Stone,
230 Staffordshire, UK) with 90 ml of buffered 0.1 % peptone water (Oxoid, Basingstoke, UK) in a
231 vertical laminar-flow cabinet (mod. AV 30/70 Telstar, Madrid, Spain). After 1 min in a Stomacher
232 blender (model Colworth 400, Seward, London, UK), appropriate dilutions were prepared for the
233 following microorganism determinations: (i) total bacterial counts (TBC) on spread plates of Iron
234 Agar (Scharlab, Barcelona, Spain), 1% NaCl incubated at 15 ºC for 3 days; (ii) H2S-producers
235 organisms, as black colonies, on pour plates of Iron Agar incubated at 15 ºC for 3 days; (iii)
236 luminescent bacteria on spread plates of Iron Agar 1% NaCl incubated at 15 ºC for 5 days; (iv)
237 Total aerobic mesophiles on pour plates of Plate Count Agar, PCA (Oxoid) incubated at 30 ºC
238 for 72 h; (v) Pseudomonas on spread plates of Pseudomonas Agar Base (Oxoid) with added
239 CFC (Cetrimide, Fucidine, Cephalosporine) supplement for Pseudomonas spp. (Oxoid)
240 incubated at 25 ºC for 48 h; (vi) Enterobacteriaceae on double-layered plates of Violet Red Bile
241 Glucose agar (VRBG, Oxoid) incubated at 30 °C for 48 h [after first adding 5 ml of Tryptone Soy
242 Agar (Merck, Darmstadt, Germany) and incubating at room temperature for 1 h]; (vii) lactic acid
243 bacteria on double-layered plates of MRS Agar (Oxoid) incubated at 30 °C for 72 h. All
244 microbiological counts are expressed as the log of the colony-forming units per gram (log cfu/g)
245 of sample. All analyses were performed in triplicate.

246 **2.11. Total volatile basic nitrogen (TVB-N)**

247 Total volatile basic nitrogen (TVB-N) determinations were carried out in triplicate over the
248 storage period using the method of Antonacopoulos & Vyncke (1989). Analyses were performed
249 at least in triplicate, and results were expressed as mg TVB-N/100g muscle.

250 **2.12. Thiobarbituric acid reactive substances (TBARS)**

251 Samples were analysed by the method of Vyncke (1970). A standard curve was prepared using
252 1,1,3,3-tetraethoxypropane. Analyses were performed at least in triplicate, and results were
253 expressed as µmol malondialdehyde (MDA) per kg of muscle..

254 **2.13. pH**

255 Approximately 5-10 grams of muscle were homogenized with distilled water (1:2, w/v). After five
256 minutes at ambient temperature, pH was determined with a pHm93 pH-meter and a combined
257 pH electrode (Radiometer, Copenhagen, Denmark). The experiments were repeated at least in
258 triplicate.

259 **2.14. Statistical analysis**

260 Statistical tests were performed using the SPSS® computer program (SPSS Statistical Software
261 v.18, Inc., Chicago, Ill.) One-way analysis of variance was carried out. The difference of means
262 between pairs was resolved by means of confidence intervals using a Tukey-b test at a level of
263 significance of $p \leq 0.05$.

264

265 **3. Results and Discussion**

266 **3.1. Colour analysis**

267 Colour measurement results expressed in terms of L* (lightness), a* (redness) and b*
268 (yellowness) parameters of both raw salmon muscle and differently processed muscles are
269 shown in Table 1. As expected, both pressurized and cooked samples showed significant
270 ($p \leq 0.05$) increases in L* values and decreases in a* values compared with the raw muscle, as
271 well as presenting a cooked-like appearance in all cases. The lightness of pressurized muscle
272 was not affected by the pressurization temperature, which was, significantly lower ($p \leq 0.05$) than
273 in the cooked muscle. When covered with film a noticeable decrease in lightness was observed,
274 which was more pronounced when high pressure treatment was carried out at 40ºC. High
275 pressure at both temperatures induced a smaller decrease in redness than by cooking.
276 Significant differences in a* values ($p \leq 0.05$) were found as a function of the pressurizing
277 temperature and film covering. When high pressure was applied at 5ºC, the presence of the film
278 led to higher redness whereas the opposite was observed at 40ºC. All processed samples
279 exhibited significant increases ($p \leq 0.05$) in b* values compared to the raw muscle, which also

280 contributed to their cooked-like appearance. In this case, pressurizing temperatures did not
281 produce any differences, however, yellowness increased slightly in muscle covered with film.
282 Consistent with our results, thermally treated salmon muscle had previously been reported as
283 presenting considerably higher lightness and lower redness when compared with muscle
284 pressurized at 300 MPa (Yagiz et al., 2009). On the other hand, increased L* values and
285 decreased a* values as a result of applying high hydrostatic pressure on fish muscle is well
286 documented (Matser, Stegeman, Kals, & Bartels, 2000; Chevalier, Le Bail & Ghoul, 2001), and
287 more specifically so in the case of salmon muscle (Amanatidou et al., 2000; Yagiz et al., 2009).
288 Covering samples with film significantly affected L* and a* values showing that the film had a
289 certain preservative effect on the colour of the pressurized muscle. However, the slightly higher
290 b* values for the covered samples could be the result of yellow being transferred from the film.

291 **3.2. Protein solubility**

292 The percentage of salt-soluble protein in raw salmon muscle was high (73.75%) as would be
293 expected for fresh fish (Table 2). However, all processed samples showed a pronounced
294 reduction in salt-soluble protein compared with the raw muscle, especially the cooked muscle
295 which had values of around 7%. High pressure treatment at both 5 and 40°C produced ~40%
296 decrease in salt-soluble protein, with no significant variations ($p>0.05$) as a result of pressure
297 temperature or film covering. Changes in muscle protein solubility are a measure of protein
298 denaturation, as solubility is decreased due to the formation of insoluble protein aggregates that
299 can no longer be extracted. Thus, the observed lower protein solubility suggested substantial
300 denaturation of proteins, induced by either high pressure processing or cooking. Our results are
301 in agreement with Carlez, Borderías, Dumay & Cheftel (1995) who found, by measuring the total
302 enthalpy of denaturation, that high pressure processing of bream surimi (300 MPa, 5ºC, 15 min)
303 caused much less protein denaturation (23%) than thermal treatment at 90ºC for 30 min (67%).

304 **3.3. Apparent viscosity**

305 Measurement of the apparent viscosity of a fish muscle homogenate has been proposed as an
306 indicator of protein quality, and has been reported to be more sensitive than protein solubility
307 (Barroso, Careche, & Borderias, 1998). Apparent viscosity values of muscle homogenates, with
308 and without a previous filtration/centrifugation step, are presented in Table 2, and were obtained
309 from both raw muscle and differently processed muscles. As expected, all processed samples

310 showed a dramatic reduction in viscosity compared to the raw fish, and this can be attributed to
311 extensive protein aggregation. No significant differences ($p>0.05$) were observed between
312 cooked muscle and muscle pressurized at 5ºC, with or without a film covering. However, when
313 high pressure treatment was carried out at 40ºC, a significant ($p\leq0.05$) decrease in viscosity
314 was observed, revealing additional heat-induced protein denaturation. By comparing the
315 viscosity values registered with and without filtration, no differences were found in the cooked
316 muscle. However, upon filtration, the muscles pressurized at 5ºC and 40ºC exhibited a ,~55%
317 and ~23% decrease respectively in viscosity. The results for apparent viscosity were not related
318 with those for salt-soluble protein, especially in the case of the cooked sample, which registered
319 extremely low protein solubility but viscosity comparable to HP5, and even slightly higher than
320 HP40. Such differences could be due to the distinct nature and size of protein aggregates
321 obtained under cooking and high pressure conditions. The nature of the heat-induced
322 aggregates did not interfere with the filtration process, most likely because they are strong small
323 aggregates. However, pressurizing at 5ºC led to large protein aggregates being retained in the
324 gauze, possibly as a result of higher protein unfolding favouring subsequent protein-protein
325 interactions. The viscosity values of the muscle pressurized at 40ºC suggested an intermediate
326 state between high pressure- and heat-denaturation as denoted by the strong aggregation and
327 presence of large aggregates. Covering the muscle with film did not affect apparent viscosity
328 when pressurizing at either 5ºC or 40ºC.

329 **3.4. SDS-PAGE**

330 The electrophoretic profiles of the salt-soluble protein fractions extracted from the raw muscle
331 and the muscle processed under different conditions are shown in Figure 1. In accordance with
332 the pronounced percentage decrease in soluble protein in the cooked muscle, the
333 electrophoretic profile was characterized by the nearly total disappearance of the majority of
334 bands. The band at ~200KDa, corresponding to the myosin heavy chain (MHC), was hardly
335 visible, whereas the bands at ~105 KDa and ~45 KDa, tentatively assigned to α-actinin and
336 actin respectively, as well as a pool of lower molecular weight proteins, in which troponins and
337 myosin light chains (MLC) are included, disappeared completely, denoting extensive heat-
338 induced protein aggregation of the main myofibrillar proteins. Specifically, α-actinin denaturation
339 could be largely involved in the strong muscle colour changes produced by heating, since it has

340 recently been shown to be the target myofibrillar protein where astaxanthin, which is mainly
341 responsible for the intense red colour of salmon, is bound (Tintchev et al., 2009).
342 The salt-soluble protein fractions from all pressurized muscles presented a great number of
343 bands, resembling to some extent the profile of the raw muscle, and revealing a considerably
344 lower degree of protein denaturation than that of the cooked muscle. Slight differences could be
345 observed with respect to the pressurization temperature, but none could be observed as far as
346 the film covering was concerned. The profile corresponding to the muscle pressurized at 40ºC
347 (with and without film) was characterized by the practical disappearance of protein bands in the
348 range between 200 and 63KDa, including the MHC and to some extent, the α-actinin. This
349 effect was less pronounced when the muscle was pressurized at 5ºC, indicating slightly lower
350 protein aggregation within this molecular weight range. The intensity of the actin band
351 decreased markedly with high pressure treatment, compared to the raw sample, although more
352 markedly when pressure was applied at 5ºC, suggesting a higher degree of actin denaturation
353 when pressure was applied at low temperature. The protein at ~63 KDa, tentatively assigned to
354 tropomyosin, and proteins below 45 KDa, were scarcely affected by high pressure as denoted
355 by the preserved intensity of the corresponding bands, suggesting strong resistance of
356 tropomyosin, troponins, MLC as well as sarcoplasmic proteins to high pressure treatment. The
357 importance of the role of MHC and actin polymerization, as opposed to troponins, tropomyosin
358 and MLC in pressure-induced protein aggregation has been previously reported (Carlez et al.,
359 1995; Angsupanich et al., 1999).

360 **3.5. FTIR spectroscopy**

361 The FTIR spectra between 4000 and 1000 cm-1 of raw salmon muscle as well as muscle
362 subjected to the different processing conditions are depicted in Figure 2. Generally, all spectra
363 exhibited similar features to spectra acquired from raw salmon myofibres as reported by Böcker
364 et al. (2008). The amide A band (~3100-3600cm-1), attributed fundamentally to OH and NH
365 stretching vibrations, exhibited a broadened absorbance in consonance with the high water
366 content in the muscle. In fact, the greatest contribution of this band in raw muscle, as opposed
367 to processed muscles, could be largely indicative of a higher water-holding capacity.
368 The amide I band, located in the region between 1600-1700 cm-1, arises predominantly from
369 C=O stretching vibrations, being weakly coupled with in-plane N-H bending and C-N stretching

370 vibrations. Studying changes in the amide I band was put forward as a helpful tool for
371 determining specific secondary structures within proteins (Jackson & Mantsch, 1995). Spectral
372 changes in the amide I region have been associated with myofibrillar protein conformational
373 changes as a result of either heating pork or salting salmon muscle (Bertram, Kohler, Böcker,
374 Ofstad, & Andersen, 2006; Böcker et al., 2008). In the present study, the amide I band showed
375 maximum IR absorbance at 1639 cm-1 in raw muscle, whereas it shifted to lower frequency
376 values in the pressurized muscles (~1631 cm-1), and more markedly in the cooked one (1623
377 cm-1). To enhance the spectral resolution, a second derivative spectrum (Figure 3) was used to
378 investigate the amide I region (1700-1600 cm-1). The bands located at 1695 cm-1, 1683 cm-1,
379 1668 cm-1, 1658 cm-1, 1652 cm-1, 1635 cm-1, 1625 cm-1, 1618 cm-1 and 1612 cm-1
380 coincided for the most part with those described previously for raw Atlantic salmon muscle
381 (Böcker et al., 2008). According to these authors, the major peak at 1652 cm-1 was indicative of
382 the contribution of the α-helical structure to protein conformation. As shown in Fig. 3, this peak
383 was diminished in the cooked sample compared with the raw muscle. In contrast, all
384 pressurized muscles showed increased intensity for this peak, especially when high pressure
385 took place at 5ºC without film covering (HP5). A similar trend was observed regarding IR
386 intensity at 1683 cm-1, although in this case no differences were found among high pressure
387 treatments. The peak at 1683 cm-1 was related to changes in intramolecular antiparallel β-sheet
388 structures in salted and smoked salmon muscle (Carton et al., 2009). The increase in α-helix
389 and β-sheet structures in all pressurized samples was attributed to the denaturing effect of high
390 pressure, leading to protein unfolding. Pressure levels above 200 MPa are known to cause
391 protein unfolding and reassociation of subunits from dissociated oligomers, inducing changes in
392 both secondary and tertiary structures (Lullien-Pellerin & Balny, 2002). An appreciable loss of
393 loop structures could also be found in the cooked muscle, as denoted by the diminished
394 intensity at 1658 cm-1. Pressure-induced changes in loop structures were much less evident;
395 nevertheless, these structures tended to decrease slightly in comparison with raw muscle when
396 high pressure was applied at 5ºC, whereas the opposite was found to be the case at 40ºC. On
397 the other hand, the cooked muscle exhibited a considerable increase in peak intensity at both
398 1695 cm-1 and ~1625 cm-1, the latter together with a noticeable shift towards lower frequency
399 values, denoting increased intramolecular aggregation of β-sheet structures. Moreover, the

400 higher intensity at 1618 cm-1 in the cooked sample would also indicate β-sheet aggregation at
401 the intermolecular level (Carton et al., 2009). The muscles subjected to high pressure showed
402 both intramolecular and intermolecular aggregation of β-sheet structures, as shown by the
403 increased intensity at 1695, 1625 and 1618 cm-1,when compared with the raw muscle, but less
404 pronounced than in the cooked sample. Differences among all pressurized samples were in
405 general scarce, although HP40 (muscle pressurized at 40ºC without film) showed a slight
406 tendency to exhibit higher intermolecular β-sheet aggregation. The band at 1667 cm-1, which
407 was tentatively assigned to non-hydrogenated C=O groups, reportedly increased in salmon
408 muscle after salting (Carton et al., 2009); however, this band was scarcely affected by either
409 cooking or high pressure treatments.

410 **3.6. Dynamic oscillatory study**

411 The degree of protein denaturation and/or aggregation was also assessed by dynamic
412 oscillatory studies. The raw muscle, as well as the muscles which had been processed under
413 different conditions, were subsequently solubilized with NaCl (salted ground muscle) and the
414 viscoelastic properties of the resulting doughs were determined. The extent of protein
415 solubilisation after homogenization with salt would depend on the degree and type of muscle
416 protein denaturation/aggregation resulting from the processing conditions, and would influence
417 the viscoelastic behaviour of the corresponding dough.

418 Fig. 4 represents the mechanical spectra, in terms of elastic (G'), viscous (G'') and complex (G*)
419 moduli as a function of the angular frequency at 10ºC, of the different salted ground muscles,
420 which had previously been subjected to a short setting period of 90 min at the same
421 temperature. In all cases, elasticity prevailed over viscosity (G'>G'') throughout the studied
422 frequency range. Thus, the resulting doughs could be considered as self-aggregated disordered
423 colloidal networks with the consistency of a viscoelastic gel (Badii & Howell, 2002). The G'
424 values in the samples pressurized at 5ºC were very close to the raw muscle G' value, especially
425 when covered with film (HPF5) (Fig. 4a). The elastic modulus of the salted ground muscle
426 increased noticeably when the samples had been pressurized at 40ºC, increasing more
427 markedly in the samples with film (HPF40), being highest in the cooked sample. A similar trend
428 was found for G* as well as for G'', the latter being clearly lowest in the raw sample (Fig. 4b,c).
429 This would confirm a higher degree of protein aggregation in the cooked muscle, followed by the

430 muscle which had been pressurized at 40ºC, as compared to the raw muscle and muscle
431 pressurized at 5ºC. Phase angle (δ) values were significantly ($p \leq 0.05$) lower in the raw sample
432 (Table 3), indicating better gel consistency, which could be attributed to higher self-aggregation
433 due to a more preserved protein conformation. On the other hand, values of δ were higher
434 ($p \leq 0.05$) in salted ground muscle from all pressurized samples, compared with the cooked one,
435 suggesting a more disordered protein network in the former.
436 The frequency dependence of G', G''and G* at 10ºC varied considerably among the different
437 samples. Table 3 shows other viscoelastic parameters, derived from G', G'' and G* mechanical
438 spectra, which had been calculated after fitting the power law, as previously described (Campo
439 & Tovar, 2008). According to Zhou & Mulvaney (1998) and Campo & Tovar (2008), G_0' and G_0''
440 would indicate the resistance of the material to elastic and viscous deformation, respectively, at
441 an angular frequency of 1rad/s. The difference G_0'-G_0'' and An are magnitudes related with the
442 viscoelastic gel strength, as well as with the strength of rheological unit interaction (Gabriele, De
443 Cindio, & D'Antona, 2001; Campo & Tovar, 2008). Both G_0'-G_0'' and An were highest($p \leq 0.05$) in
444 the cooked sample, followed by the HPF40 sample, while all the other pressurized samples
445 registered values closer to the raw muscle. The higher viscoelastic gel strength in the cooked
446 sample is attributed to the presence of more aggregated protein resulting from the heat
447 treatment, which in turn led to stronger protein interactions (mainly disulfide bonds), which were
448 not destroyed after solubilization with NaCl. In general, this effect was not observed in
449 pressurized samples, especially if the high pressure treatment was carried out at 5ºC,
450 suggesting the predominance of non-covalent interactions in pressure-induced protein
451 aggregation. In particular, hydrogen bonds have reportedly been largely involved in cod
452 myofibrillar protein denaturation at high pressure levels above 200 MPa, with additional
453 stabilization by disulphide bonds (Angsupanich et al., 1999).
454 The power law exponent n' is related to structural stability and protein network conformation in
455 the studied samples: the higher the n' values, the higher the instability of the matrix against
456 frequency changes (Campo &Tovar, 2008). As shown in Table 3, n' was significantly higher
457 ($p \leq 0.05$) in the pressurized samples when compared with both raw and cooked samples,
458 indicating that the pressurized protein network was more unstable, irrespective of pressurization
459 temperature. This suggests a considerably lower level of conformational order in the salted

460 ground muscle leading to higher matrix discontinuity as a result of high pressure treatment. This
461 confirmed once again, that the protein interactions in the pressurized muscle were weaker than
462 in the cooked one. Moreover, the structure is considerably more unstable than in the raw
463 muscle, again confirming noticeable pressure-induced changes in protein conformation. Among
464 pressurized samples, no significant differences were found in n' values ($p>0.05$) due to
465 pressurization temperature or film covering.
466 Fig. 5 shows the thermal aggregation ability of the different salted ground muscles in terms of
467 the elasticity modulus (G'), the viscosity modulus (G'') and phase angle (δ) as a function of
468 heating temperature (from 10ºC to 90ºC). As expected, the raw muscle presented the highest
469 thermal aggregation capacity, as deduced from the sharp increase in both G' and G'' values at
470 temperatures from 45ºC upwards. This would indicate extensive protein network stabilization
471 produced by the formation of heat stable bonds. Furthermore,, the pronounced phase angle
472 peak values between 30 and 45ºC revealed noticeable myofibrillar protein conformational
473 changes during this temperature interval, which could be attributed to moderate heat-induced
474 protein unfolding as well as to some muscle autolytic activity, which might be more active at
475 these temperatures. Similar results were previously reported for thermal aggregation properties
476 of squid mantle proteins (Gómez-Guillén, Martínez-Alvarez, & Montero, 2003). The viscoelastic
477 behaviour of the salted ground muscle from the cooked sample revealed that no protein
478 unfolding took place at moderate temperatures, which was consistent with most of the protein
479 aggregation having taken place during the previous cooking treatment. Moreover, the possible
480 contribution of muscle autolytic activity might also have been hindered by previous cooking. The
481 sharp increase in both G'' and phase angle at temperatures above 70ºC was attributed mainly
482 to extensive protein dehydration at such high temperatures, revealing the water-holding
483 inefficacy of the protein network. Regarding high pressure treatments, the thermal aggregation
484 capacity was considerably diminished in all pressurized muscles compared to the raw muscle,
485 as revealed by the considerably lower G' and G'' values reached at the end of the heating ramp.
486 Furthermore, conformational protein changes at ~30ºC were only observed when samples had
487 been pressurized at 5ºC, and to a much lesser extent than with the raw muscle. This effect can
488 once again be largely attributed to the protein denaturing effect of high pressure, being higher at
489 40º than at 5ºC. With regard to the effect of covering muscle with film, the highest G' values

490 were registered by the HPF5 sample. This finding confirmed that film could prevent protein
491 denaturation to some extent when high pressure treatment was applied at 5ºC (not a denaturing
492 temperature); however, this effect was not observed at 40ºC.

493 **3.7. Protein oxidation**

494 Carbonyl group content, as a measure of protein oxidation in the different muscles, is presented
495 in Table 2. In the fresh raw sample, the carbonyl group content was lowest (0.087 nmol/mg
496 protein), but protein oxidation increased significantly ($p \leq 0.05$) in all treated samples, reaching
497 maximum values in the muscle pressurized at 40ºC (HP40) and in the cooked muscle (0.728
498 and 0.654 nmol/mg protein, respectively). The muscle pressurized at 5ºC HP5 showed a
499 carbonyl content significantly lower ($p > 0.05$) than that recorded with the HP40 treatment. In the
500 latter, however, covering the muscle with film led to a significant reduction ($p \leq 0.05$) in carbonyl
501 content. The high pressure and cooking treatments used in the current study have been shown
502 to produce significant myofibrillar protein denaturation. Moreover, haeme proteins would most
503 likely have been denatured as well at the pressure levels used and become more pro-oxidative.
504 Therefore it is possible that the increase in protein carbonyl groups during pressurization and
505 cooking could have been due to catalysis by haeme proteins, which are known to induce protein
506 oxidation in fish muscle (Eymard, Baron, & Jacobsen, 2009). For film-covered samples, slightly
507 lower carbonyl content was detected compared to non-covered samples, showing that the film
508 offers some protection against protein oxidative changes. This protection was found to be
509 significant ($p \leq 0.05$) in the case of HPF40 treatment. Both the antioxidant and oxygen barrier
510 properties of gelatin-lignin composite film may have contributed to the reduction of protein
511 oxidation in the film-covered samples.

512 **3.8 Lipid oxidation**

513 The TBARS method was used to monitor changes in the level of secondary lipid oxidation
514 products in salmon muscle as a result of the different processing treatments, and during the
515 subsequent 23 days of chilled storage (Table 4). The TBARS value of the raw salmon was
516 3.22±0.19 µmol MDA/Kg muscle, which was in agreement with the low values reported by
517 Andersen et al. (1990) (2.8 µmol MDA/Kg muscle) and Yagiz et al. (2009) (3.78 µmol MDA/Kg
518 muscle). The cooking treatment induced a pronounced increase in TBARS, whereas the high
519 pressure treatment at 5ºC (with or without film) did not modify significantly the level of lipid

520 oxidation or even reduce it slightly when pressurizing at 40ºC. Unexpectedly, the gelatin-lignin
521 film increased TBARS levels in HPF40, however, this effect tended to disappear during chilled
522 storage. The level of TBARS remained practically unchanged in HP5 and HP40 samples for the
523 first 15 days of chilled storage, consistent with a presumably high level of carotenoids, which
524 has been said to act as a strong endogenous antioxidant system in salmon muscle (Andersen et
525 al., 1990). In contrast to the present work, Gómez-Estaca et al. (2009) found a significant
526 increase in TBARS after pressurizing salmon carpaccio at 300 MPa/ 7ºC/15 min. The different
527 results could be put down to the sample size (very thin slices *vs* thick fillet portions) as well as to
528 the duration of treatment (15 min *vs* 10 min). TBARS value increased drastically at day 23 of
529 chilled storage in uncovered pressurized muscle, especially in HP5. However this increase was
530 significantly ($p \leq 0.05$) minimized by film covering, suggesting a possible inhibitory effect against
531 lipid oxidation of lignin, as reported by Ugartondo et al. (2009).

532 **3.9. pH**

533 The salmon registered a pH of 6.5 on day 0 and then remained practically constant even after
534 cooking, applying the film or high pressure treatment. During storage all the lots evolved in the
535 same way, reaching around 6.6 on day 23.

536 **3.10. Total volatile basic nitrogen**

537 TVB-N in the raw salmon was 19.1 mg TVB-N/100 g muscle, whereas Aubourg et al. (2007) on
538 the other hand found 11.2 mg TVB-N/100 g muscle in Coho salmon on day 0. Some changes
539 were observed after applying the various treatments. Thus, total volatile compounds decreased
540 after cooking while the lots treated with high pressure, in combination or not with the film, barely
541 changed the TVB values (Fig. 6). This fact can be attributed to the release of exudates as a
542 consequence of protein aggregation during the cooking process. Throughout the storage period,
543 TVB production increased in all lots, but the limit of 30 mg TVB-N/100 g of muscle (the
544 established limit for fresh fish) was only exceeded by the HP5 lot ($p \leq 0.05$). The lignin film
545 helped to reduce basic compound production in the lot pressurized at 5 ºC (Fig. 6). However,
546 this behaviour was not observed in lots pressurized at lower temperatures ($p \leq 0.05$).

547 **3.11. Microbiological analysis**

548 The microbial counts are shown in Table 5. In spite of the high microbial load of raw salmon (≈ 7
549 cru/g on Iron Agar spread plates), the number of microorganisms was reduced after high

550 pressure treatment. Thus, the total viable bacteria in Iron Agar was reduced to about 4 or 5 log
551 cru/g when the high pressure treatment was at 40 ºC or 5 ºC, respectively. In the case of total
552 aerobic mesophiles there was also a reduction in the number of microorganisms by
553 approximately 3 logarithmic cycles. Pseudomonas spp. and Enterobacteriaiceae were below the
554 detection limit after high pressure treatment while lactic acid bacteria registered a lower
555 reduction (1.5 log cycle). This was to be expected if we take into account the higher resistence
556 of gram-positive flora to high pressure treatment. Black colonies (presumptive Shewanella
557 putrefaciens, López-Caballero et al., 2007) were not detected in the raw material before
558 treatment nor after treatment. S. putrefaciens sensitivity to high pressure was previously
559 described by López-Caballero et al. (2000). Yagiz et al. (2009) obtained total counts with
560 reductions of 3 log cycles and counts even below the detection limits for Atlantic salmon after
561 pressurization at 150 and 300 Mpa/15 min at room temperature. In this same species, reduction
562 in the total count for S. putrefaciens and lactic acid bacteria were reported by Amanatidou et al
563 (2000) after high pressure treatment (150 MPa, 10 min, 5 ºC). Luminiscent colonies, considered
564 as presumptive P. phosphoreum (López-Caballero et al. 2002), were also reduced after high
565 pressure treatment (table 5). Paarup et al. (2002) reported that the number of luminiscent
566 colonies decreased after pressurization of squid at 300 MPa for 15 min at room temperature,
567 though not so sharply as in our work. Pressure resistance, psychrofilic characteristics and
568 luminous properties of these marine vibrios have been described previously (Paarup et al.,
569 2002). The cooking process reduced the numbers of all the micro-organisms studied, which
570 remained below the detection limit throughout the period studied.
571 The number of micro-organisms increased during storage ($p \leq 0.05$). The HP treatment
572 temperature (5 or 40 ° C) affected these micro-organisms differently depending on the type of
573 organism involved. Thus, the total counts of viable, aerobic and lactic acid bacteria were lower
574 at 5 ºC on day 23, even though the counts for these microorganisms were less at 40 °C up to
575 the 15th day of storage. However, S. putrefaciens and P. phosphoreum concentrations
576 decreased more at 40 °C, which seems logical if we take into account the psychrofilic character
577 of these micro-organisms and P. phosphoreum sensitivity to non-chilling temperatures. This fact
578 explains the difference of almost 2 logarithmic cycles between the surface counts obtained for
579 PCA and those of Iron Agar (Table 5). Differences in total microorganism counts depending on

580 the medium and incubation temperature have already been described (López-Caballero et al
581 2000).
582 Although in recent years, the effect of high pressure on micro-organisms has been published
583 extensively, literature on high pressure treatment combined with films has been less abundantly
584 reported. Previous work in our laboratories has demonstrated the antimicrobial effect on fish
585 storage of combining high pressure treatment with films (Gómez-estaca et al., 2007). However,
586 to our knowledge there is no information about the effect of gelatin films incorporating lignin on
587 the spoilage flora of fish, when used in conjunction or not with high pressure. In this study the
588 effect of film on the growth of the micro-organisms studied has not been very noticeable since
589 the counts are similar for batches with or without film (Table 5).
590
591 **4. Conclusions**
592 HPP was found to produce a noticeably lower overall degree of protein aggregation compared
593 with conventional heating treatment, especially when performed at 5ºC instead of 40ºC. FTIR
594 analysis and dynamic oscillatory studies provided complementary information about the protein
595 unfolding effect of HPP. High pressure processing at 5ºC in combination with the gelatin-lignin
596 film was found to preserve redness coloration and minimally prevent protein denaturation and
597 oxidation, without jeopardizing the appearance of a ready-to-eat product. The film reduced the
598 level of lipid oxidation arising during advanced stages of chilled storage; however it was
599 ineffective at inhibiting microbial growth.
600
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604
605 **6. References**
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758

759 LEGEND TO FIGURES

760 **Figure 1.-** SDS-PAGE of proteins solubilized in 0.8M NaCl of raw salmon muscle and muscle
761 subjected to different processing treatments.

762 (a) C, muscle cooked at 90ºC for 10 min; (b) R, raw muscle; (c) HP5, muscle pressurized at 5ºC
763 for 10 min; (d) HPF5, muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min;
764 (e) HP40, muscle pressurized at 40ºC for 10 min; (f) HPF40, muscle covered with gelatin-lignin
765 film and pressurized at 5ºC for 10 min.
766 std 1: standard of high molecular weight; std2: standard of low molecular weight.
767

768 **Figure 2.-** FTIR spectra of raw salmon muscle and muscle subjected to different processing
769 treatments.

770 R, raw muscle; C, muscle cooked at 90ºC for 10 min; HP5, muscle pressurized at 5ºC for 10
771 min; HPF5, muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min; HP40,
772 muscle pressurized at 40ºC for 10 min; HPF40, muscle covered with gelatin-lignin film and
773 pressurized at 5ºC for 10 min.
774

775

776 **Figure 3.-** Second derivative of Amide I band (1700-1600 cm-1) from FTIR spectra of raw
777 salmon muscle and muscle subjected to different processing treatments.

778 (1) R, raw muscle; (2) C, muscle cooked at 90ºC for 10 min; (3) HP5, muscle pressurized at 5ºC
779 for 10 min; (4) HP40, muscle pressurized at 40ºC for 10 min; (5) HPF5, muscle covered with
780 gelatin-lignin film and pressurized at 5ºC for 10 min; (6) HPF40, muscle covered with gelatin-
781 lignin film and pressurized at 5ºC for 10 min.
782

783 **Figure 4.-** Elastic modulus (G', Pa), Viscous modulus (G'', Pa) and Complex modulus (G*, Pa)
784 as a function of the angular frequency of salt-ground muscles at 10ºC with a prior setting time of
785 90 min.

786 R, raw muscle; C, muscle cooked at 90ºC for 10 min; HP5, muscle pressurized at 5ºC for 10
787 min; HPF5, muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min; HP40,
788 muscle pressurized at 40ºC for 10 min; HPF40, muscle covered with gelatin-lignin film and
789 pressurized at 5ºC for 10 min.
790

791 **Figure 5.-** Elastic modulus (G', Pa), Viscous modulus (G'', Pa) and phase angle (º) as a function
792 of temperature of salt-ground muscles from 10ºC to 90ºC.

793 R, raw muscle; C, muscle cooked at 90ºC for 10 min; HP5, muscle pressurized at 5ºC for 10
794 min; HPF5, muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min; HP40,
795 muscle pressurized at 40ºC for 10 min; HPF40, muscle covered with gelatin-lignin film and
796 pressurized at 5ºC for 10 min.
797

798 **Figure 6.-** Total Basic Volatile Nitrogen Microbial (mg TBV-N/100g) in muscle subjected to
799 different processing treatments and stored at 7ºC for 23 days.

800 C, muscle cooked at 90ºC for 10 min; HP5, muscle pressurized at 5ºC for 10 min; HPF5,
801 muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min; HP40, muscle
802 pressurized at 40ºC for 10 min; HPF40, muscle covered with gelatin-lignin film and pressurized
803 at 5ºC for 10 min.
804 Different letters (a, b, c...) in the same column indicate significant differences ($p \leq 0.05$) as a
805 function of treatment; different letters (x, y, z...) in the same row indicate significant differences
806 ($p \leq 0.05$) as a function of storage time.

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Table 1.- Colour parameters (L*, a*, b*) of muscle subjected to different processing treatments

Treatment	L*	a*	b*
R	41.55±0.12[a]	16.82±0.03[a]	9.29±0.04[a]
C	74.71±0.58[b]	9.50±0.35[b]	14.91±0.45[b]
HP5	65.64±0.44[c]	11.04±0.02[c]	12.20±0.49[c]
HPF5	60.70±0.86[d]	13.71±0.62[d]	17.65±0.76[d]
HP40	66.39±0.25[c]	12.12±0.73[e]	12.35±0.42[c]
HPF40	56.31±0.31[e]	10.44±0.32[bc]	14.45±0.07[b]

R, raw muscle; C, muscle cooked at 90ºC for 10 min; HP5, muscle pressurized at 5ºC for 10 min; HPF5, muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min; HP40, muscle pressurized at 40ºC for 10 min; HPF40, muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min.

Different letters (a,b,c…) in the same column indicate significant differences ($p \leq 0.05$)

Table 2.- Protein functionality parameters of muscle subjected to different processing treatments

Treatment	Protein solubility (%)	Viscosity (cp) with filtering	Viscosity (cp) without filtering	Carbonyl group (nm/mg protein)
R	73.54±2.00[a]	*11140±135.27	*11140±135.27	0.087±0.009[a]
C	6.73±0.73[b]	47.92±1.07[a]	50±2.46[a]	0.654±0.044[bc]
HP5	30.29±0.94[c]	27.84±3.20[b]	48.61±2.50[a]	0.567±0.026[bd]
HPF5	31.36±0.74[c]	25.92±1.90[b]	47.5±3.27[a]	0.490±0.037[d]
HP40	30.20±0.90[c]	5.83±0.83[c]	26.66±2.84[b]	0.728±0.061[c]
HPF40	31.14±0.84[c]	6.16±1.36[c]	26.12±2.71[b]	0.610±0.057[b]

R, raw muscle; C, muscle cooked at 90ºC for 10 min; HP5, muscle pressurized at 5ºC for 10 min; HPF5, muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min; HP40, muscle pressurized at 40ºC for 10 min; HPF40, muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min.
Different letters (a,b,c…) in the same column indicate significant differences ($p \leq 0.05$)
*value not included in the analysis of variance

Table 3.- Rheological parameters derived from the frequency sweep test of the different salt-ground muscles at 10ºC with a prior setting time of 90 min.

	G_0'x10^3	G_0'' x10^3	(G_0'-G_0'')x10^3	A_n x10^3	n'	δ (º)
R	6.03±0.05[a]	1.04±0.04[a]	4.99±0.56[a]	6.13±0.51[a]	0.132±0.027[a]	9.44±0.25[a]
C	16.47±0.27[b]	4.38±0.00[b]	12.09±0.27[b]	17.05±0.27[b]	0.144±0.005[a]	13.75±0.37[b]
HP5	5.67±0.14[a]	1.58±0.00[c]	4.09±0.14[c]	5.89±0.13[ac]	0.185±0.007[b]	15.13±0.24[c]
HPF5	5.06±0.15[c]	1.29±0.03[d]	3.77±0.18[c]	5.23±0.14[c]	0.190±0.005[b]	14.93±0.04[c]
HP40	6.90±0.25[d]	2.07±0.02[e]	4.83±0.39[a]	7.21±0.35[d]	0.202±0.018[b]	15.41±0.49[c]
HPF40	11.04±0.22[e]	3.28±0.03[f]	7.72±0.26[d]	11.52±0.19[e]	0.181±0.009[b]	14.91±0.01[c]

R, raw muscle; C, muscle cooked at 90ºC for 10 min; HP5, muscle pressurized at 5ºC for 10 min; HPF5, muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min; HP40, muscle pressurized at 40ºC for 10 min; HPF40, muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min.

Different letters (a,b,c…) in the same column indicate significant differences ($p \leq 0.05$)

Table 4.- TBARS (µmol MDA/ Kg muscle) of muscle subjected to different processing treatments and stored at 7ºC for 23 days

Treatment	Days of chilled storage			
	1	7	15	23
C	11.20 $^{a/x}$	13.91 $^{a/y}$	7.06 $^{a/z}$	7.07 $^{a/z}$
HP5	3.34 $^{b/x}$	2.78 $^{b/x}$	1.85 $^{b/x}$	8.40 $^{b/y}$
HPF5	3.32 $^{b/x}$	2.14 $^{b/yz}$	1.49 $^{b/y}$	2.38 $^{c/z}$
HP40	1.32 $^{c/x}$	2.64 $^{b/y}$	1.35 $^{b/x}$	4.27 $^{d/z}$
HPF40	6.30 $^{d/x}$	8.58 $^{c/y}$	1.05 $^{b/z}$	2.19 $^{c/z}$

C, muscle cooked at 90ºC for 10 min; HP5, muscle pressurized at 5ºC for 10 min; HPF5, muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min; HP40, muscle pressurized at 40ºC for 10 min; HPF40, muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min.

Different letters (a,b,c…) in the same column indicate significant differences ($p \leq 0.05$) as a function of treatment; different letters (x,y,z…) in the same row indicate significant ($p \leq 0.05$) differences as a function of storage time.

Table 5.- Microbial counts (log UFC/g) in muscle subjected to different processing treatments and stored at 7ºC for 23 days

	Treatment	Days of chilled storage			
		1	7	15	23
Total viable Count (Iron Agar 1% NaCl)					
	C	$<2^{c}$	$<2^{e}$	$<2^{d}$	$<2^{d}$
	HP5	$2.00^{a/x}$	$5.70^{a/y}$	$7.88^{a/z}$	$7.74^{a/z}$
	HPF5	$2.00^{a/x}$	$5.10^{b/y}$	$7.20^{b/z}$	$7.37^{b/z}$
	HP40	$3.33^{b/w}$	$3.87^{c/x}$	$5.67^{c/y}$	$8.22^{c/z}$
	HPF40	$3.33^{b/x}$	3.04^{d7x}	$5.43^{c/y}$	$8.26^{c/z}$
H_2S-producers microorganisms					
	C	<2	$<2^{c}$	$<2^{d}$	$<2^{d}$
	HP5	<2	4.08^{a}	5.08^{a}	5.08^{a}
	HPF5	$<2^{x}$	$4.71^{b/y}$	$5.28^{a/z}$	$5.49^{a/z}$
	HP40	<2	$<2^{c}$	2.30^{b}	2.30^{b}
	HPF40	$<2^{x}$	$<2^{c/x}$	$3.39^{c/y}$	$3.39^{c/y}$
Luminous colonies					
	C	<2	$<2^{e}$	$<2^{e}$	<2
	HP5	$<2^{x}$	$5.70^{a/y}$	$7.60^{a/z}$	$<2^{x}$
	HPF5	$<2^{x}$	$4.77^{b/y}$	$6.47^{b/z}$	$<2^{x}$
	HP40	$<2^{x}$	$2.15^{c/y}$	$2.15^{c/y}$	$<2^{x}$
	HPF40	$<2^{x}$	$2.93^{d/y}$	$4.04^{d/z}$	$<2^{x}$
Pseudomonas spp					
	C	<2	$<2^{a}$	$<2^{d}$	<2
	HP5	$<2^{x}$	$<2^{a/x}$	$2.30^{a/y}$	2.00^{y}
	HPF5	$<2^{w}$	$3.82^{b/x}$	$4.98^{b/y}$	2.00^{z}
	HP40	$<2^{x}$	$2.42^{c/y}$	$2.00^{a/y}$	2.00^{y}
	HPF40	$<2^{x}$	$2.39^{c/y}$	$3.59^{c/y}$	3.00^{y}
Lactic acid bacteria					
	C	$<1^{c}$	$<1^{e}$	$<1^{c}$	$<1^{c}$
	HP5	$2.52^{a/w}$	$3.98^{a/x}$	$7.06^{a/y}$	$7.48^{a/z}$
	HPF5	$2.52^{a/x}$	$4.84^{b/y}$	$6.44^{ab/x}$	$7.15^{a/z}$
	HP40	$2.02^{b/x}$	$1.80^{c/x}$	$5.25^{b/y}$	$8.57^{b/z}$
	HPF40	$2.02^{b/w}$	$3.58^{a/x}$	$5.51^{b/y}$	$8.25^{b/z}$
Enterobacteriaceae					
	C	<1	$<1^{a}$	$<1^{a}$	$<1^{a}$
	HP5	<1	$<1^{a}$	$<1^{a}$	$<1^{a}$
	HPF5	$<1^{x}$	$2.04^{b/y}$	$3.03^{b/z}$	$2.74^{b/z}$
	HP40	<1	$<1^{a}$	$<1^{a}$	3.00^{b}
	HPF40	$<1^{x}$	$1.00^{c/x}$	$4.18^{c/y}$	$3.48^{b/y}$
Total mesophilic counts					
	C	$<1^{b}$	$<1^{e}$	$<1^{c}$	$<1^{c}$
	HP5	$2.74^{a/x}$	$4.13^{a/y}$	$7.15^{a/z}$	$7.64^{a/z}$
	HPF5	$2.74^{a/x}$	$4.83^{b/y}$	$7.19^{a/z}$	$7.32^{a/z}$
	HP40	$2.63^{a/x}$	$2.50^{c/x}$	$5.84^{b/y}$	$8.48^{b/z}$
	HPF40	$2.63^{a/w}$	$3.46^{d/x}$	$5.73^{b/y}$	$8.35^{b/z}$

R, raw muscle; C, muscle cooked at 90ºC for 10 min; HP5, muscle pressurized at 5ºC for 10 min; HPF5, muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min; HP40, muscle pressurized at 40ºC for 10 min; HPF40, muscle covered with gelatin-lignin film and pressurized at 5ºC for 10 min.

Different letters (a,b,c…) in the same column indicate significant differences ($p \leq 0.05$) as a function of treatment; different letters (x,y,z…) in the same row indicate significant ($p \leq 0.05$) differences as a function of storage time.

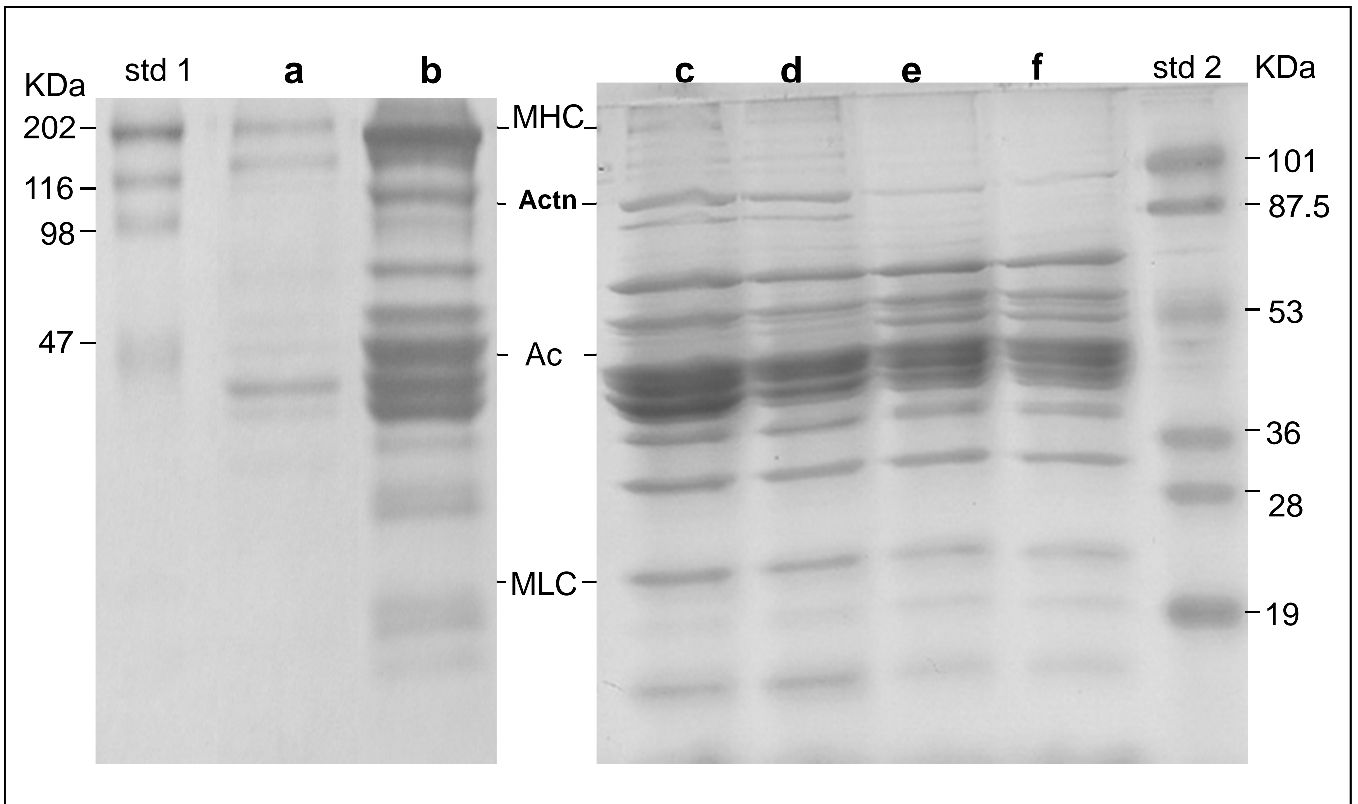


Fig. 1

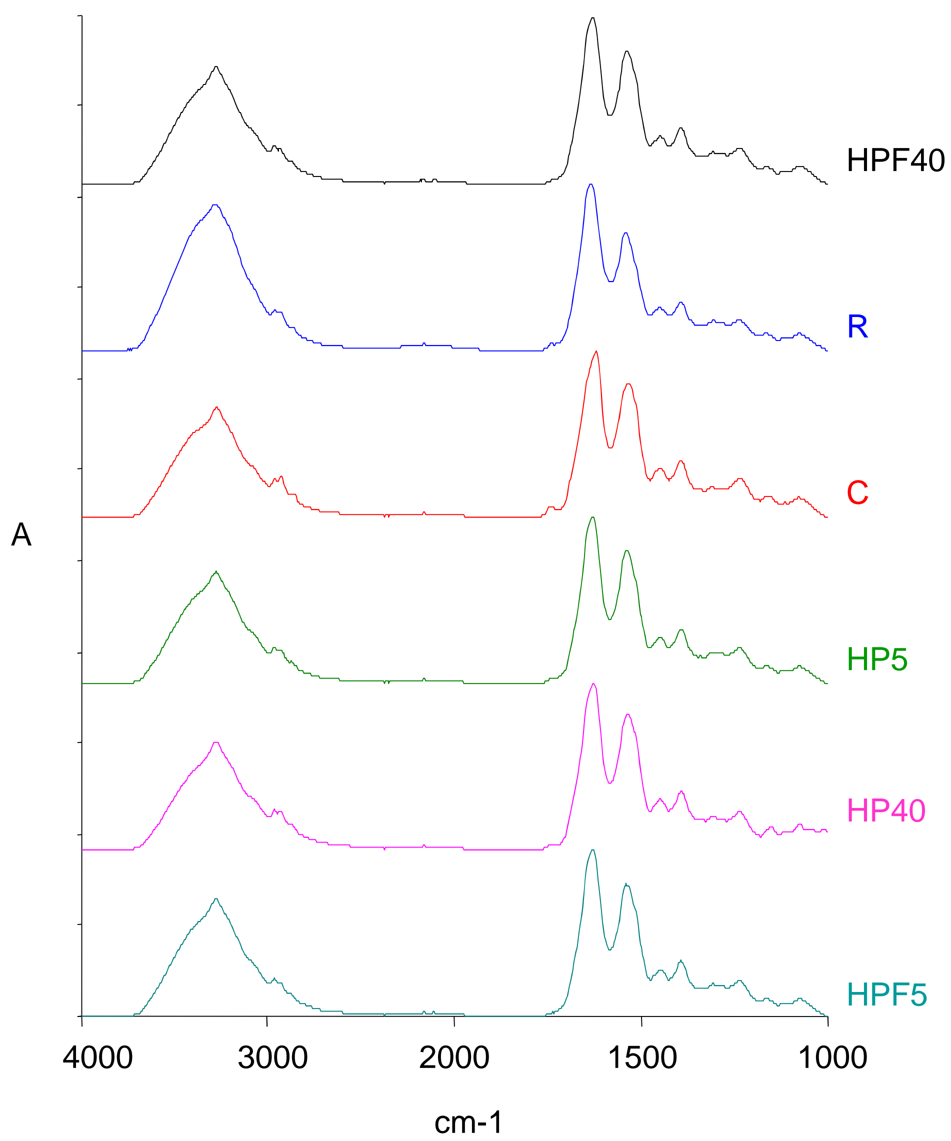


Fig. 2

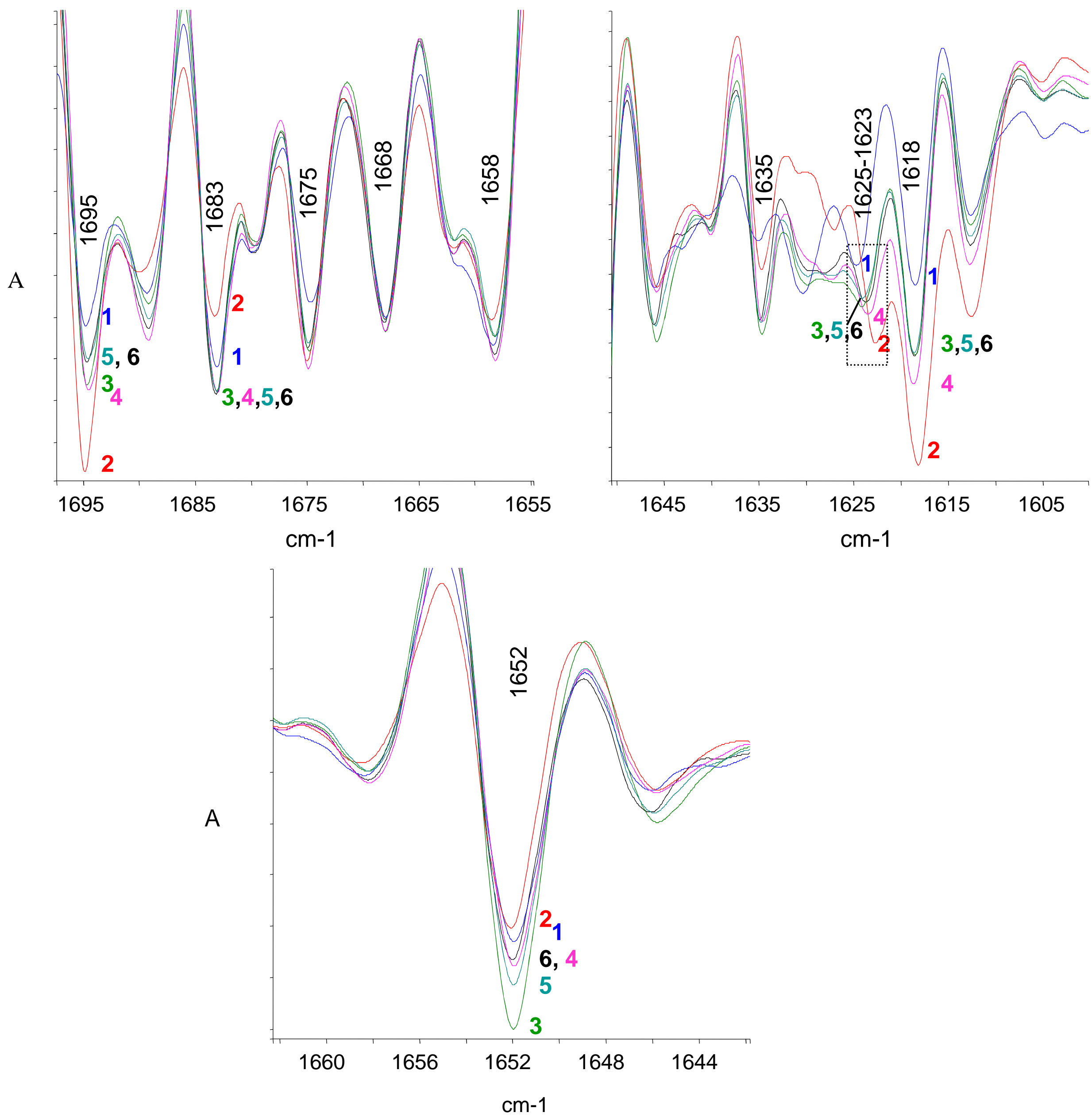


Fig. 3

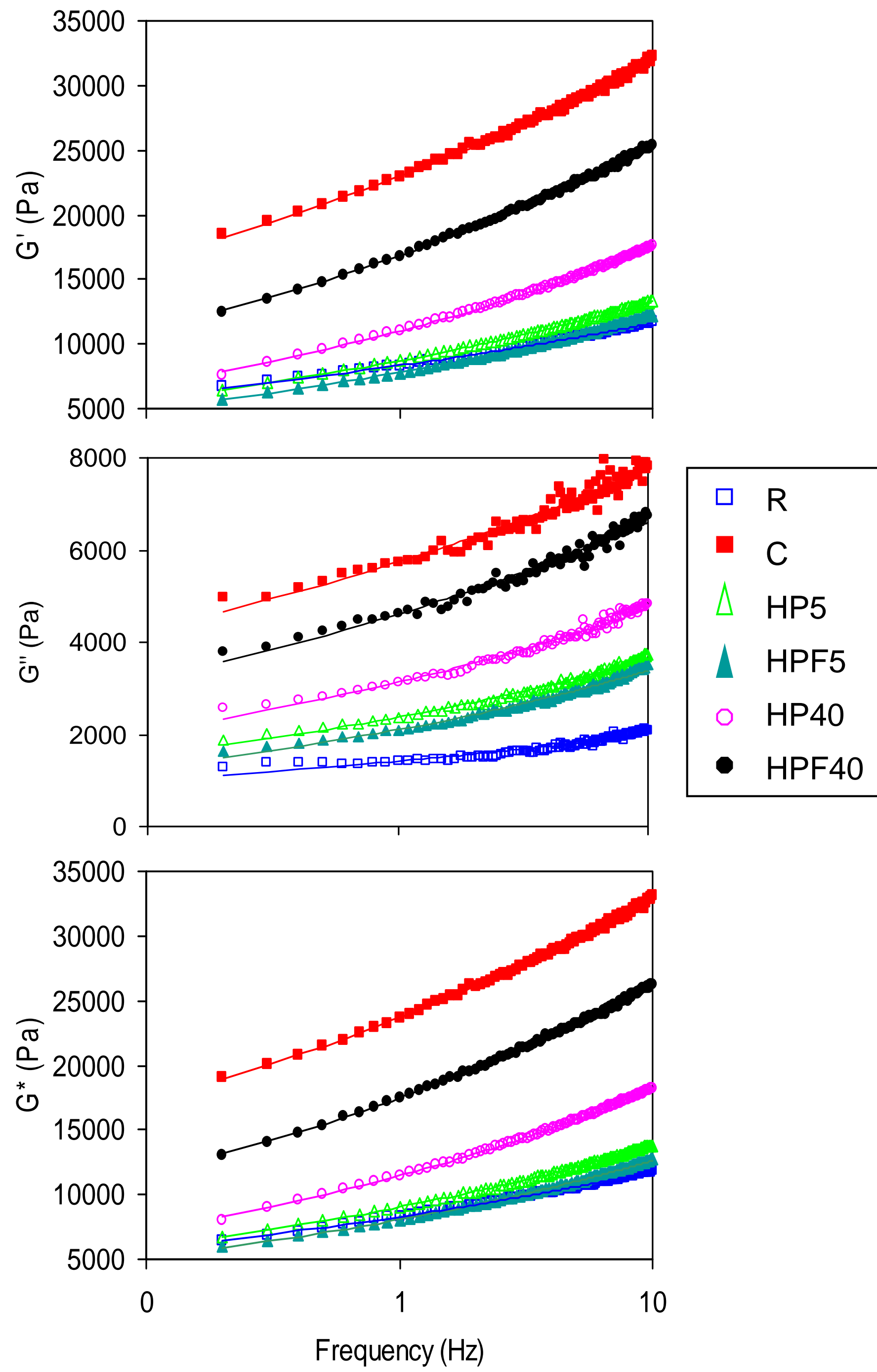


Fig. 4

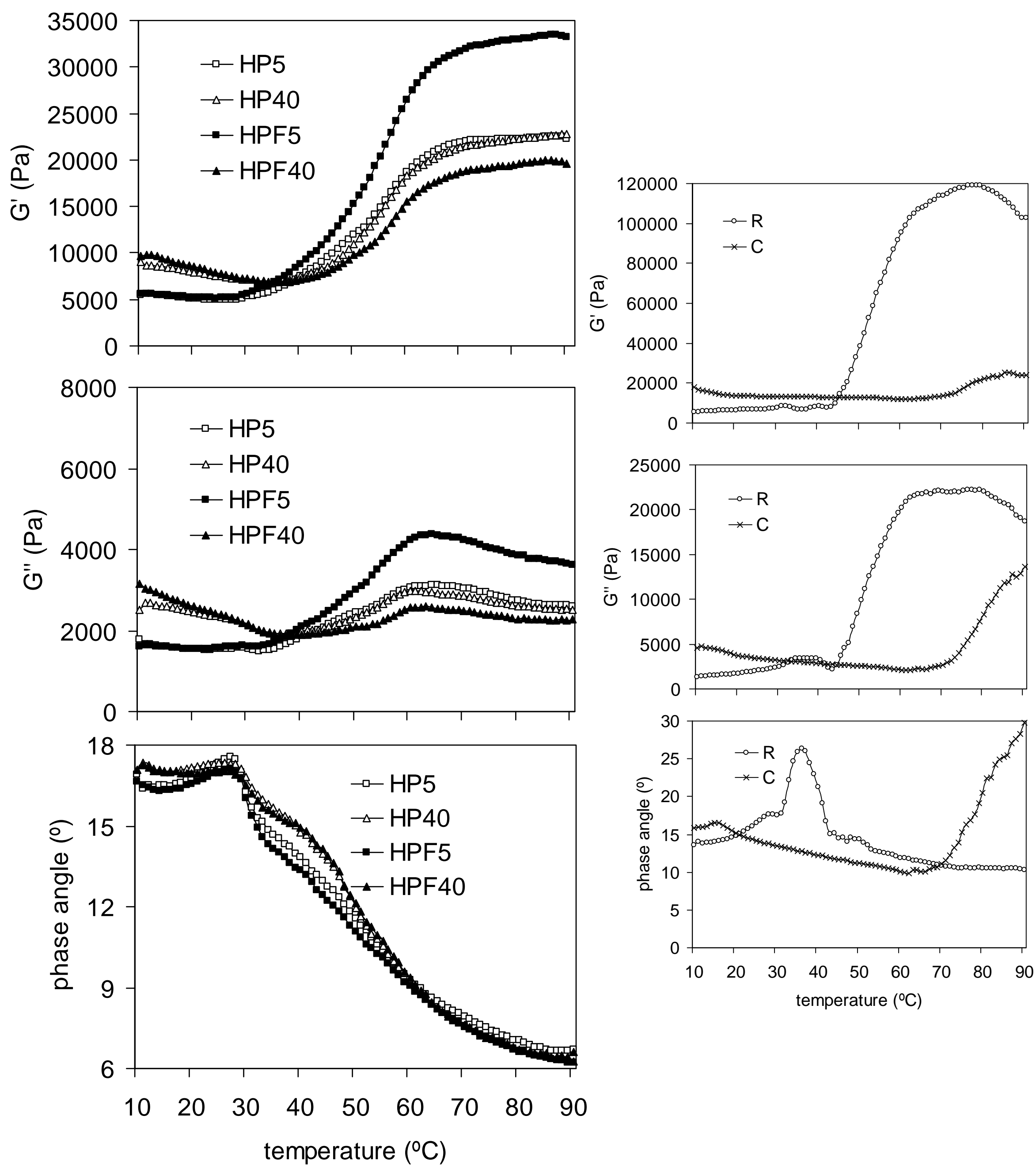
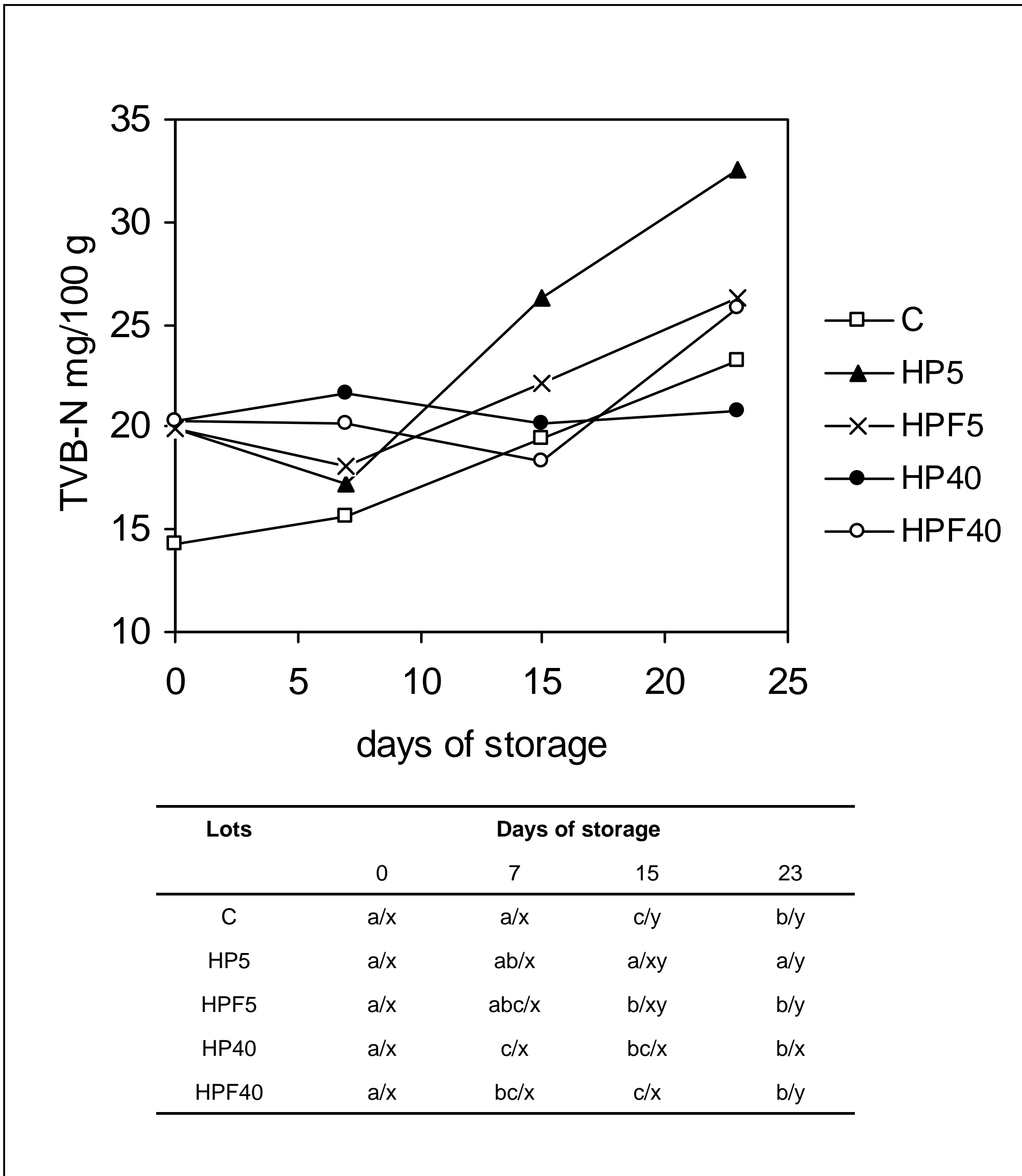


Fig. 5



Lots	Days of storage			
	0	7	15	23
C	a/x	a/x	c/y	b/y
HP5	a/x	ab/x	a/xy	a/y
HPF5	a/x	abc/x	b/xy	b/y
HP40	a/x	c/x	bc/x	b/x
HPF40	a/x	bc/x	c/x	b/y

Fig. 6