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1 Effects of high-pressure treatment on the muscle structure of salmon (*Salmo*  
2 *salar*)

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5

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11

12 **Abstract**

13 High pressure (HP) is a non-thermal treatment that is generally used to reduce the  
14 microbiological contamination of food products, such as Atlantic salmon (*Salmo salar*).

15 However, HP is known to alter the stability of proteins and can therefore affect the quality  
16 of salmon flesh. In this study, the effects of HP treatment for 5 min at 200, 400 and 600

17 MPa on the structure of Atlantic salmon were investigated. Transversal histological

18 sections revealed a decrease in the fibre size from 200 MPa associated with an expansion

19 of the extracellular spaces. Connective tissue was found to be modified from 400 MPa,

20 resulting in an increase in its surface area. Fourier transform infrared (FT-IR)

21 microspectroscopy revealed a reduction in the  $\alpha$ -helix content and an increase in the

22 aggregated  $\beta$ -sheet structure content with increasing pressure, reflecting a change in the

23 secondary structure of proteins from 200 MPa.

24

25 **Keywords**

26 High pressure, Salmon, Histology, Infrared microspectroscopy, Protein secondary  
27 structure, Muscle structure

28

## 29 **1. Introduction**

30 Atlantic salmon (*Salmo salar*) is the third most consumed fish in the European Union, with  
31 an apparent consumption per capita in 2018 of 2.24 kg live weight equivalent (Directorate-  
32 General for Maritime Affairs and Fisheries (European Commission), 2020). However, due  
33 to microbial, chemical and enzymatic activity, salmon is a highly perishable product with a  
34 shelf life of approximately ten days on ice (Fogarty et al., 2019). Among the numerous  
35 technologies that are being investigated to increase this shelf life, de Alba et al. (2019) and  
36 Park et al. (2015) have shown a significant effect of high pressure (HP) on microbial  
37 reduction. High-pressure treatments have been used since 1990 in Japan to process jams,  
38 jellies and sauces and are currently used for processing vegetables, meat and fish  
39 products, as well as juices and beverages (Oliveira et al., 2017). This technology  
40 represents a particularly interesting preservation technique because it tackles numerous  
41 issues of the food industry related to consumer expectations: it does not require the use of  
42 food additives to improve the product shelf life, it is more energy efficient than traditional  
43 technologies such as cooking (Truong et al., 2015) and it preserves fish nutrients, such as  
44 unsaturated fatty acids (Yagiz et al., 2009).

45 Nonetheless, this technology leads to modifications of food biopolymers, especially  
46 proteins, as a result of the thermodynamic effects of pressure. Indeed, according to the Le  
47 Chatelier principle, the application of HP favours changes causing a decrease in volume  
48 (Mozhaev et al., 1994). Consequently, phenomena such as disruption of hydrophobic and  
49 electrostatic interactions are promoted by HP, leading to changes in quaternary, tertiary  
50 and secondary structures. Covalent bonds, hydrogen bonds and disulphide bridges, on the  
51 other hand, have low compressibility and are expected to be resistant to pressure

52 (Aubourg, 2018; Knorr et al., 2006; Mozhaev et al., 1994; Oliveira et al., 2017).

53 Fish muscles are composed of approximately 20% proteins in total and are therefore  
54 particularly sensitive to HP. Fish meat consists mainly of myofibrillar proteins, which are  
55 responsible for muscle contraction; sarcoplasmic proteins, which are water soluble; and  
56 proteins from connective tissue such as collagen, which are insoluble extracellular  
57 proteins.

58 HP has been proven interesting to improve the quality of fish by reducing its susceptibility  
59 to oxidation (Yagiz et al., 2009) and by enhancing its sensory attributes such as taste,  
60 odour and texture (Truong et al., 2015). Moreover, HP has been used to ameliorate the  
61 freezing and thawing of different fish species, as it increases the kinetics of ice-water  
62 transitions, thus avoiding the formation of large ice crystals (Aubourg, 2018). The protein  
63 modifications caused by the pressure have also been used to improve the quality of fish  
64 gels (Truong et al., 2017; Uresti et al., 2004). However, several studies have shown  
65 unwanted effects of HP on the quality of fish fillets, such as an increased hardness and a  
66 change in colour with increased lightness after treatment at 150 MPa (Arnaud et al., 2018;  
67 Aubourg et al., 2013), giving the product a cooked appearance.-Even though the effects of  
68 HP on the sensory attributes of salmon have been widely described, the molecular  
69 mechanisms causing these modifications are still unclear. *In situ* approaches are therefore  
70 a powerful tool, as they allow precise analysis of the microscopic and biochemical  
71 alterations induced by treatment of the product while taking into account the complexity  
72 and high level of organization of the biological structures. The knowledge of the effects of  
73 HP on a microscopic and molecular scale should therefore help understanding the  
74 changes of fish quality and determine the ideal parameters of treatment to minimize the  
75 undesirable impacts of HP.

76 The purpose of this study was to investigate *in situ* the effects of a high-pressure treatment  
77 on both the microstructure and secondary structure of salmon muscle proteins.

78

## 79 **2. Materials & Methods**

80

### 81 **2.1. Biological material**

82 Twelve whole salmon (*Salmo salar*, farmed in Scotland) were purchased from a local  
83 supplier (Jean LEBEAUPIN, Nantes, France) one day after the harvesting date. The fish  
84 weighed 2.5 to 3 kg and were of “Label Rouge” quality, which is an official French label  
85 guaranteeing strict farming conditions and final product quality such as a lipid rate between  
86 10 and 16%.

87 The collagen content in the muscle of the salmon, which was evaluated by an external  
88 laboratory (Inovalys, Nantes, France) and calculated as [(L)-hydroxyproline content x 8],  
89 was equal to 0.3 %. From each fish, a slice approximately 5 cm wide was excised and cut  
90 into four 40 g parts (see the supplementary material), which were each vacuum packed  
91 (98% vacuum) in individual polyamide/polyethylene bags (La Bovida, Paris, France) and  
92 kept on ice before processing. The remaining parts of the fish were stored at -20°C until  
93 further biochemical analysis.

94

### 95 **2.2. Reagents and chemicals**

96 For the HES staining, the following chemicals and reagents were used: Harris  
97 Haematoxylin (Harris Hematoxylin Surgipath, Leica biosystem, Buffalo Grove, IL, USA),  
98 Eosin 0.5% (eosin Y 0.5% w/v aqueous solution, Sigma Aldrich, St Louis, MO, USA),  
99 Powdered saffron (Technical, VWR International, Radnor, PA, USA), Methylcyclohexane  
100 (for synthesis, Merck, Darmstadt, Germany), Eukitt (Eukitt® Quick-hardening mounting  
101 medium, Sigma Aldrich, St Louis, MO, USA) and Ethanol (99%, Carlo Erba Reagents, Val  
102 de Reuil, France).

103 For the Picrosirius red staining, the following chemicals and reagents were used: Acetone

104 (99%, Sigma Aldrich, St Louis, MO, USA), Picro-Sirius red (Sirius red F3B, Gurr BDH  
105 laboratory Supplies, Poole, England), Hydrochloric acid 0.01M (37%, VWR International,  
106 Radnor, PA, USA), Ethanol (99%, Carlo Erba Reagents, Val de Reuil, France) and  
107 Methycyclohexane (for synthesis, Merck, Darmstadt, Germany). A Picro-formalin solution  
108 was prepared with ethanol 95% and formaldehyde (37 %, Sigma Aldrich, St Louis, MO,  
109 USA) (180/25 v/v) and picric acid to saturation (Powdered, Sigma Aldrich, St Louis, MO,  
110 USA).

111

## 112 **2.3 High-pressure processing**

113 The packed samples (approximately 40 g each) were inserted in the stainless steel  
114 chamber of a custom-made high-pressure device (NOVA SWISS, Cesson, France). The  
115 chamber, equipped with a water jacket and a temperature regulator device, was filled with  
116 water at 20°C and pressure was generated by addition of water in the chamber using a  
117 hydrostatic pump. For each fish, the four pieces were processed with a balanced  
118 experimental design for 5 min at 200 MPa, 400 MPa, 600 MPa or unpressurized (control),  
119 meaning that for each level of pressure, different locations of the salmon were used. These  
120 pressure rates were chosen in order to investigate the process effect up to 600 MPa which  
121 is generally the maximum HP used in industry and was the maximum limit of the  
122 equipment. The compression rate was 3 MPa/s, and the depression rates were 4 MPa/s, 8  
123 MPa/s and 10 MPa/s respectively. During the pressurization process, the maximal  
124 temperatures reached were 24.7°C, 29.4°C and 33.6°C for the samples pressurized at  
125 200, 400 and 600 MPa, respectively. The samples were kept on ice as soon as the  
126 treatment ended and until histological sampling.

127

## 128 **2.4. Microstructure**

### 129 **2.4.1. Histochemical analysis**

130 A 10 mm wide cube was cut from each sample to identify the muscle fibre direction. The  
131 cubes were plunged and stirred for 30 seconds in 2-methyl-butane (-160°C) cooled with  
132 liquid nitrogen (-196°C). The cryofixed samples were then kept at -80°C until analysis.  
133 A cryomicrotome (Leica CM 1950, Leica Biosystems, Nussloch, Germany) was used to cut  
134 10 µm slices from each cube, transversally to the muscle fibre direction. The sections were  
135 mounted on SuperFrost microscope slides, air-dried (20°C) and stained with haematoxylin-  
136 eosin-saffron (HES) to contrast the tissue and visualize the general structure and with  
137 picosirius red to reveal connective tissue. The stained sections were mounted with  
138 synthetic resin (Eukitt, Kindler GmbH & Co, Freiburg, Germany) and protected by covering  
139 with a glass coverslip. Images were acquired using an Olympus BX 61 microscope  
140 coupled with a high-resolution digital camera (Olympus DP 71) and Olympus Cell Sens  
141 software (Olympus France SAS, Rungis, France).  
142 For each sample, at least ten images of x100-magnified HES staining and picosirius  
143 staining were captured.

144

#### 145 **2.4.2. Image analysis**

146 For each of the four conditions, ten images of HES staining and picosirius staining were  
147 analysed using open-source ImageJ software (<https://imagej.nih.gov/ij/>). Approximately 50  
148 muscle fibres were counted per optical field, which means that approximately 500 fibres  
149 per sample were analysed. For each picture, the software was used to calculate both the  
150 average size of the fibres and the percentage of surface occupied by the fibres, the  
151 connective tissue and the extracellular spaces (ECSs), per optical field.

152

#### 153 **2.5. Infrared spectroscopy data acquisition**

154 Histological cross-sections (6 µm thick) were obtained from the cryofixed muscle sample,  
155 collected on a BaF<sub>2</sub> window with IR spectroscopy compatibility (CRYSTRAN, Poole,

156 United Kingdom) and air-dried at room temperature. IR spectra were collected using an  
157 FT-IR microscope (Thermo Scientific, Nicolet iN10) scanning from 4000 to 675  $\text{cm}^{-1}$  with a  
158 spectral resolution of 4  $\text{cm}^{-1}$  and an aperture size set at 30 X 30  $\mu\text{m}$ . For each section, 10  
159 spectra were acquired in 10 different muscle fibres. Each spectrum resulted from 64  
160 accumulated scans. The accumulated spectra were averaged and subtracted from a  
161 background spectrum obtained at the start of the scan by accumulating 128 scans.

162

163

## 164 **2.6. Infrared spectra pre-treatment**

165 The spectra acquired were analysed using TQAnalyst software (V 9.5.0, Thermo Fisher  
166 Scientific Inc., Waltham, MA, USA). All spectra were first submitted to multiplicative signal  
167 correction (MSC) so that analytical artefacts would not be taken into account. The second  
168 derivative of the spectra was calculated, and a Savistky-Golay filter with 9 points and a  
169 third-degree polynomial was applied to improve the data resolution.

170

## 171 **2.7. Statistical analysis**

172 The analysis on the infrared spectra was focused on the 1600-1700  $\text{cm}^{-1}$  region mostly  
173 assigned to the amide I band of proteins (Barth & Zscherp, 2002). Principal component  
174 analysis (PCA) was performed on all spectra to explore similarities between the samples  
175 without prior assumptions, using the absorbance at each measured wavenumber as a  
176 variable. Principal component representation was used to determine which variables had  
177 the most weight to describe the variability between the samples and the pressure  
178 conditions. Measurement of the peak heights at 1655  $\text{cm}^{-1}$  and 1628  $\text{cm}^{-1}$  was performed,  
179 and the resulting data were shifted to improve the graphical representation.

180

181 For histological data, statistical analysis was carried out using Statgraphics Centurion 18



182 software (Statgraphics Technologies, Inc., The Plains, VA, USA). Multivariate analysis of  
183 variance (ANOVA) was performed on the data to determine significant differences between  
184 the levels of pressure applied while taking intraspecies variability into account. Multiple  
185 range tests were used to identify which means were significantly different according to  
186 Fisher's least significant difference (LSD) test ( $p < 0.05$ ).

187

188 The data in all the figures are expressed as the means  $\pm$  standard error.

189

### 190 **3. Results**

191

#### 192 **3.1. Effect of HP on the microstructure of salmon muscle**

193 Figure 1 shows representative histological images of the HES- and Picrosirius red stained  
194 cross-sections.

195 HES staining (Figure 1A) allowed us to assess the effects of HP on salmon microstructure.

196 The HES-stained control fibres appeared tight, with narrow spaces between cells. At 200

197 MPa, the ECS increased compared to that of the control. Similar results were found on

198 cold-smoked Atlantic salmon treated with HP above 400 MPa for 30 and 60 s

199 (Gudbjornsdottir et al., 2010) and on raw salmon muscle processed at 300 MPa

200 (Gudmundsson & Hafsteinsson, 2001). Conversely, Chéret et al. (2005) observed the

201 opposite effect on sea bass (*Dicentrarchus labrax L.*) fillet, with a decrease in the ECS with

202 increasing pressure from 200 to 500 MPa, which they attributed to gelation of the

203 myofibrillar proteins.

204 After pressurization at 400 and 600 MPa, the appearance of the fibres changes and

205 becomes smoother with brighter red staining, which could be a consequence of an

206 increase in protein density and/or protein coagulation.

207 Image analysis with ImageJ software was used to precisely quantify the changes in

208 microstructure. Collagen, which appears in purple in Figure 1A, was calculated as part of  
209 the ECS.

210 The high-pressure treatment resulted in shrinkage of the fibres (Figure 2A) and an  
211 increase in the ECS (Figure 2B), suggesting that this increase was at least partly a  
212 consequence of lateral shrinkage of the muscle fibres. Although significant differences in  
213 fibre size were demonstrated between the 200 MPa and 400 MPa samples, overall, the  
214 treated samples showed little cross-sectional area (CSA) variation among them. However,  
215 the treatment at 600 MPa resulted in a large increase in ECS, even though the average  
216 fibre size was not modified compared to that at 400 MPa. Similarly, Gudbjornsdottir et al.  
217 (2010) observed a significant increase in the ECS for samples pressurized to 600 MPa or  
218 more. However, the increase in ECS was related to the shrinkage of muscle fibres,  
219 contrary to our results. Our results could be explained by a disruption of the connective  
220 tissue, which no longer holds the fibres together, as suggested by Figure 1B.

221

### 222 **3.1.2 Picrosirius red staining**

223 The images of the picrosirius red-stained sections are shown in Figure 1B: the fibres are  
224 coloured yellow, and the connective tissue is coloured red. For the HES-stained sections,  
225 the control fibres appeared close to each other, with a thin layer of connective tissue  
226 between the cells. The fibre colour also changes with increasing pressure, becoming more  
227 orange. From 400 MPa, connective tissue appears thicker and brighter red.

228 Compared to HES staining, which was used to precisely evaluate the fibre characteristics,  
229 picrosirius red staining allowed estimating the amount of space occupied distinctively by  
230 the fibres, connective tissue and ECSs, as presented in Figure 3A, 3B and 3C,  
231 respectively.

232

233 For the unpressurized samples, the fibres represent 57.7% ( $\pm$  0.6%) of the total area, 3.1%

234 for the connective tissue and 3.0% for the ECS. Only the fibres that were fully inside the  
235 optical area were taken into account for the calculation, which explains why the sum was  
236 not equal to 100%.

237 The pressurization of the salmons at 200 MPa resulted in a marked increase in the area  
238 occupied by the ECS and a slight decrease in the fibre area, whereas no significant effect  
239 was observed for the connective tissue. At 400 MPa, no additional effect was observed on  
240 the fibres and ECS, but the percentage of area occupied by the connective tissue was  
241 doubled compared to that at 200 MPa, which we assumed could be explained by a  
242 loosening of the collagen structure.

243 At 600 MPa, significant decreases in the fibres and the connective tissue area were  
244 measured, correlated with a large increase in the ECS.

245

### 246 **3.2. Effects of high-pressure treatment on the secondary structure of salmon** 247 **proteins**

248 Mid-infrared spectroscopy is a powerful tool to characterize protein secondary structures  
249 based on the principle that the vibrational frequencies of a molecule are affected by the  
250 strength of its bonds and the mass of its vibrating atoms (Barth & Zscherp, 2002). In the  
251 present study, vibrational spectra were acquired from approximately 120 myofibres for  
252 each pressure condition.

253 PCA was performed on all the whole spectra (675-4000  $\text{cm}^{-1}$ ) and allowed identifying the  
254 1700-1600  $\text{cm}^{-1}$  band as the most discriminant for HP treatments.

255 This band is assigned to the amide I band of proteins and is more specifically related to  
256 the vibration of the stretching of C=O bonds. It is strongly related to the secondary  
257 structure of the proteins (Barth & Zscherp, 2002; Jackson & Mantsch, 1995).

258 Consequently, the data analysis focused on the 1700-1600  $\text{cm}^{-1}$  part of the spectra  
259 (Figures 4 - 5).

260

261 With increasing pressure, the second-derivative spectra (Figure 4) clearly show a  
262 decrease in absorbance at  $1655\text{ cm}^{-1}$  assigned to  $\alpha$ -helices (Barth, 2007; Jackson &  
263 Mantsch, 1995) and an increase in absorbance in the  $1640\text{-}1620\text{ cm}^{-1}$  range. The band  
264 occurring at  $1628\text{ cm}^{-1}$  is assigned to denatured aggregated  $\beta$ -sheet components  
265 (intermolecular), while the band between  $1630$  and  $1640\text{ cm}^{-1}$  is assigned to antiparallel  $\beta$ -  
266 sheet structures (intramolecular) (Jackson and Mantsch, 1995; Bocker et al., 2006, Wu et  
267 al. 2007).

268 PCA (Figure 5) confirms that the dissimilarities between the pressure groups were strongly  
269 correlated to the first principal component, which was mainly explained by bands at  $1655$   
270  $\text{cm}^{-1}$  and  $1628\text{ cm}^{-1}$  reflecting myofibre protein denaturation (Astruc et al. 2012).

271

272 Quantification of the peak heights at  $1655\text{ cm}^{-1}$  and  $1628\text{ cm}^{-1}$  was performed to study the  
273 evolution of the secondary structure with pressure (figure 6). Previous studies conducted  
274 on fish treated with HP (Larrea-Wachtendorff et al., 2015; Martínez et al., 2017) also  
275 focused on the amide I band of the FT-IR spectra and found a similar evolution of the  
276 secondary structure, i.e., a decrease in the  $\alpha$ -helix content and an increase in the content  
277 of aggregated  $\beta$ -sheet structures. Villamonte et al. (2015) focused on the effects of HP on  
278 sarcoplasmic proteins extracted from hake and observed the same evolution of secondary  
279 structure correlated to a decrease of their emulsifying properties. The changes of the  
280 sarcoplasmic proteins structure also caused their aggregation, which can result in an  
281 increase of the hardness of the fish meat. The decrease of  $\alpha$ -helix and increase of  
282 aggregated  $\beta$ -sheet structures was also measured by Cando et al. (2014) on myofibrillar  
283 proteins isolated from hake and treated by HP. The pressure treatment induced the  
284 formation of gels which were less rigid and softer than gels formed by thermal denaturation  
285 of the proteins. The authors suggested that the increase of aggregated  $\beta$ -sheet structures

286 could be the consequence of a gelling process.  
287 Pressure denaturation of muscle proteins resulted in similar modifications of their  
288 secondary structure than after thermal treatment (Astruc et al., 2012; Ovissipour et al.,  
289 2017). However, in the present study, the denaturation cannot be caused by temperature  
290 as it did not exceed 33.6°C. Protein denaturation occurs during HP due to destabilisation  
291 of non-covalent interactions in the tertiary and secondary structures, particularly  
292 hydrophobic and ionic interactions because their disruption is associated with a negative  
293 change of volume and thus a compaction of the molecule (Chapleau et al., 2004;  
294 Heremans, 1982; Mozhaev et al., 1994). High pressure acts by altering the balance of  
295 intermolecular and solvent–protein interactions. Contrary to thermal denaturation, HP  
296 treatments do not lead to the formation of intermolecular hydrogen bonds (Chapleau et al.  
297 2004).

298

#### 299 **4. Discussion**

300

301 The results of the histological analysis show that HP causes a decrease in the fibre size  
302 regardless of the level of pressure applied, which is likely to be responsible for the ECS  
303 increase. The shrinkage of the fibres could be related to water flows between the muscle  
304 cells and the ECS. Several authors have shown a decrease in the water holding capacity  
305 of fish submitted to HP, which supports this hypothesis (Chéret et al., 2005; Jiranuntakul et  
306 al., 2018; Lakshmanan et al., 2007; Ramirez-Suarez & Morrissey, 2006). Chéret et al.  
307 (2005) additionally studied the exudation of sea bass fillets after pressurization but did not  
308 observe any modification caused by HP.

309 Furthermore, we have shown that pressure above 400 MPa resulted in a change in  
310 connective tissue appearance and size. This alteration could be due to a change in the  
311 collagen structure. Collagen is formed of a triple helix mainly stabilized by hydrogen and

312 covalent bonds connecting hydroxyproline from one strand to amide carboxyl from another  
313 strand (Benjakul et al., 2012) and should therefore be relatively resistant to HP (Truong et  
314 al., 2015). However, Kaur et al. (2016) studied the effects of HP on the structure of bovine  
315 meat and suggested that pressure, by disrupting the non-covalent bonds, could disturb the  
316 molecule stability. Fish collagen contains smaller amounts of proline and hydroxyproline  
317 and is therefore less stable than mammalian collagen which supports our hypothesis  
318 (Benjakul et al., 2012).

319 Kaur et al. (2016) also observed a decrease in connective tissue on raw pork treated by  
320 HP (600 MPa, 10 min), which they attributed to a possible rise in temperature during high-  
321 pressure processing associated with a decrease in denaturation temperature of the  
322 collagen caused by the destabilization of non-covalent bonds. During the processing of our  
323 samples, we measured a rise in temperature from 20°C to 29.4°C and 33.6°C for the 400  
324 MPa and 600 MPa treatments, respectively. The denaturation of fish collagen was reported  
325 to occur at between 30°C and 40°C (Benjakul et al., 2012; Hastings et al., 1985), and the  
326 slight increase in temperature might have participated in the loosening of the connective  
327 tissue at 400 MPa and the partial denaturation at 600 MPa caused by the high-pressure  
328 treatment.

329 It is worth noting that after pressurization at 600 MPa, the ECS size is substantially  
330 increased even though the fibre cross-sectional area is not significantly modified. It is  
331 unlikely that the overall volume of the samples was expanded by the treatment because  
332 pressure rather favours molecular states which are related with a decreased volume  
333 (Mozhaev et al., 1994). However, this phenomenon was correlated with the decrease of  
334 the connective tissue area at 600 MPa compared to 400 MPa, and it is therefore likely that  
335 the pressure induced denaturation of collagen caused a partial disruption of the connective  
336 tissue which could no longer hold the fibres together, explaining the increase of the ECS.  
337 This could have possibly happened after depressurization, during the preparation of the

338 samples.

339

340 All levels of HP resulted in a modification of the salmon muscle secondary structure. After  
341 pressurization at 200 MPa, myofibres contained a smaller amount of  $\alpha$ -helices and more  
342 aggregated  $\beta$ -sheet structures, associated with the compaction of the fibres and larger  
343 ECS than that of the control. This modification of the secondary structure was enhanced at  
344 higher levels of pressure. After pressurization at 400 MPa, connective tissue integrity was  
345 altered and therefore occupied an increased surface area compared to that of the control  
346 and 200 MPa samples. When pressurization at 600 MPa was applied, connective tissue  
347 was found to be partially degraded, leading to an expansion of the ECS.

348 Myofibrillar proteins are the most abundant proteins in muscle cells, with 50-60% myosin  
349 (Venugopal & Shahidi, 1996) and are mainly organized in  $\alpha$ -helices (Wang et al., 2021; Xu  
350 et al., 2020). Several authors (Angsupanich & Ledward, 1998; Arnaud et al., 2018; Larrea-  
351 Wachtendorff et al., 2015) have studied the thermal denaturation of proteins from fishes  
352 previously treated by HP, and all found that myofibrillar proteins were denatured by  
353 pressures above 200 MPa, with denaturation enthalpies decreasing all the more that  
354 pressure was increased. The protein secondary structure is stabilized by hydrogen bonds,  
355 which are known to be resistant or even strengthened by HP (Mozhaev et al., 1994;  
356 Oliveira et al., 2017) because of the negative volume changes that are associated with the  
357 formation of these bonds. Boonyaratanakornkit et al. (2002) reported that these bonds  
358 were, however, modified by HP, with a reduced length and a possible promotion of  
359 intermolecular hydrogen bonds to the detriment of intramolecular hydrogen bonds. HP, by  
360 unfolding myofibrillar proteins and changing the properties of the hydrogen bonds, could  
361 therefore result in the destabilization of part of the  $\alpha$ -helices and an increase in the content  
362 of  $\beta$ -sheets. The consequent compression of myofibrillar proteins might explain the  
363 observed decrease in myofibre size.

364 These alterations in muscle structure are linked to pressure-induced modifications of fish  
365 sensory properties. Authors have reported significant changes in salmon colour after  
366 pressurization, especially increased lightness (Gudbjornsdottir et al., 2010; Yagiz et al.,  
367 2009). Although the mechanisms responsible for this discoloration are still unclear,  
368 evidence shows that it could be due to the denaturation or coagulation of myofibrillar  
369 proteins. The unfolding of these proteins could indeed modify their surface properties and  
370 affect the way they absorb and reflect light (Kruk et al., 2011; Suemitsu & Cristianini,  
371 2019). The decrease in  $\alpha$ -helical structures that we observed as well as the histological  
372 analysis, which show that the myofibre cross-sections become smoother with pressure,  
373 support the hypothesis that the change in colour caused by HP is linked to the  
374 denaturation of muscle proteins rather than the alteration of carotenoid pigments. These  
375 molecules are responsible for the distinctive orange colour of salmon and are likely  
376 unaltered by mechanical treatments such as HP (Martínez-Delgado et al., 2017).

377 In most cases, high-pressure treatments increase the hardness of fish muscles (Oliveira  
378 et al., 2017), and this change in texture has been associated with a higher compaction of  
379 the fibres after treatment and a preservation of collagen by pressure (Truong et al., 2015).  
380 In contrast, we observed an increase in the intercellular space and an alteration in  
381 connective tissue integrity in this study. Arnaud et al. (2018) suggested that the increased  
382 hardness observed on salmon after pressurization at 300 MPa could be a result of protein  
383 aggregation, which is consistent with the increase in the  $\beta$ -sheet structure that we  
384 measured.

385 In the present study, FT-IR spectral analysis could not be performed on the connective  
386 tissue because the spatial resolution of a laboratory FT-IR microspectrometer is too low  
387 (best acquisition area of 10  $\mu\text{m}$  x 10  $\mu\text{m}$ ) to precisely target the endomysium. Further  
388 analyses using a synchrotron infrared beamline (Astruc et al., 2012) or Raman  
389 microspectroscopy technique will allow us to achieve a sufficient spatial resolution (3x3  $\mu\text{m}$



390 for synchrotron-FTIR microspectroscopy; 1 x 1  $\mu\text{m}$  for Raman microspectroscopy) to  
391 investigate the intramuscular connective tissue in order to better understand the  
392 modifications of the connective tissue affected by high-pressure treatment.

393

## 394 **5. Conclusion**

395

396 Most interpretations of the pressure-induced modifications of fish muscles are based on  
397 physical rules such as Le Chatelier's principle (Mozhaev et al., 1994) or on experimental  
398 data obtained from extracted proteins. However, these interpretations fail to take into  
399 consideration the high level of organization and complexity of the muscular structure. The  
400 combination of histological and FT-IR microspectroscopy analysis allowed us to  
401 investigate the microstructure and myofibre protein macromolecular structure *in situ* and  
402 concomitantly. Supplementary analysis of quality parameters of Atlantic salmon treated by  
403 HP, such as colour, texture and water holding capacity, would provide helpful data to  
404 explain the relations between the microstructure and the technological properties of  
405 salmon meat.

406

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410 Ribette-Lancelot for the help to analyse the spectral data.

411

## 412 **Conflicts of interest**

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

415

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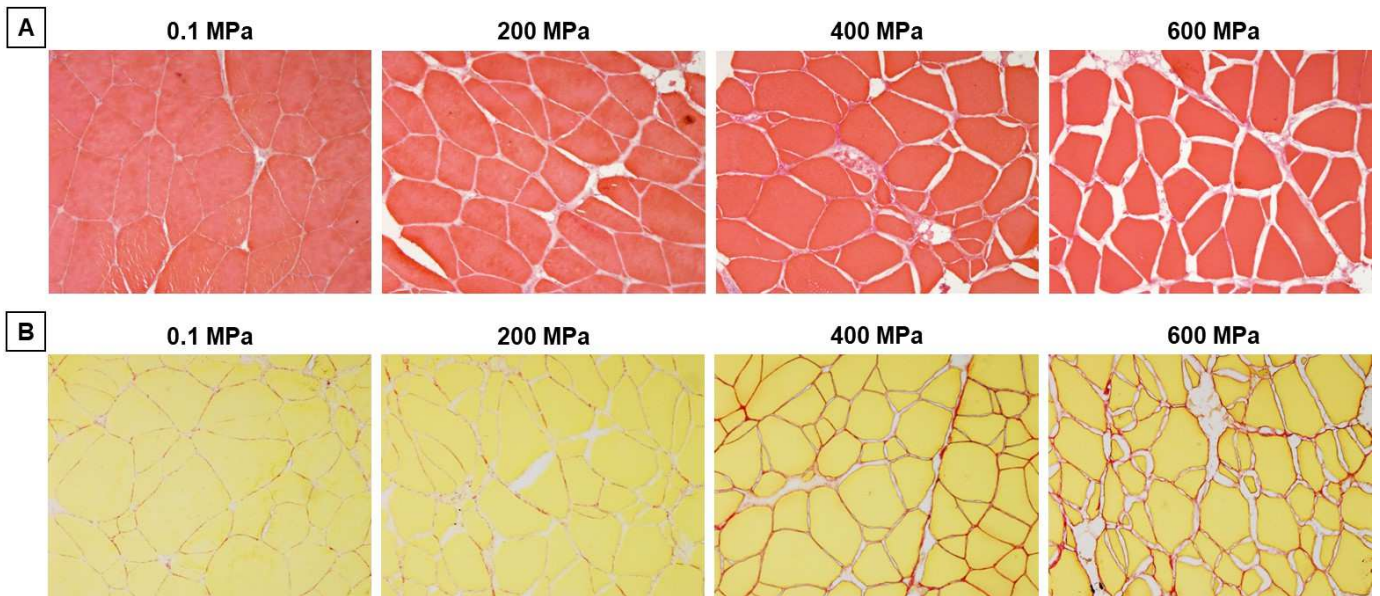
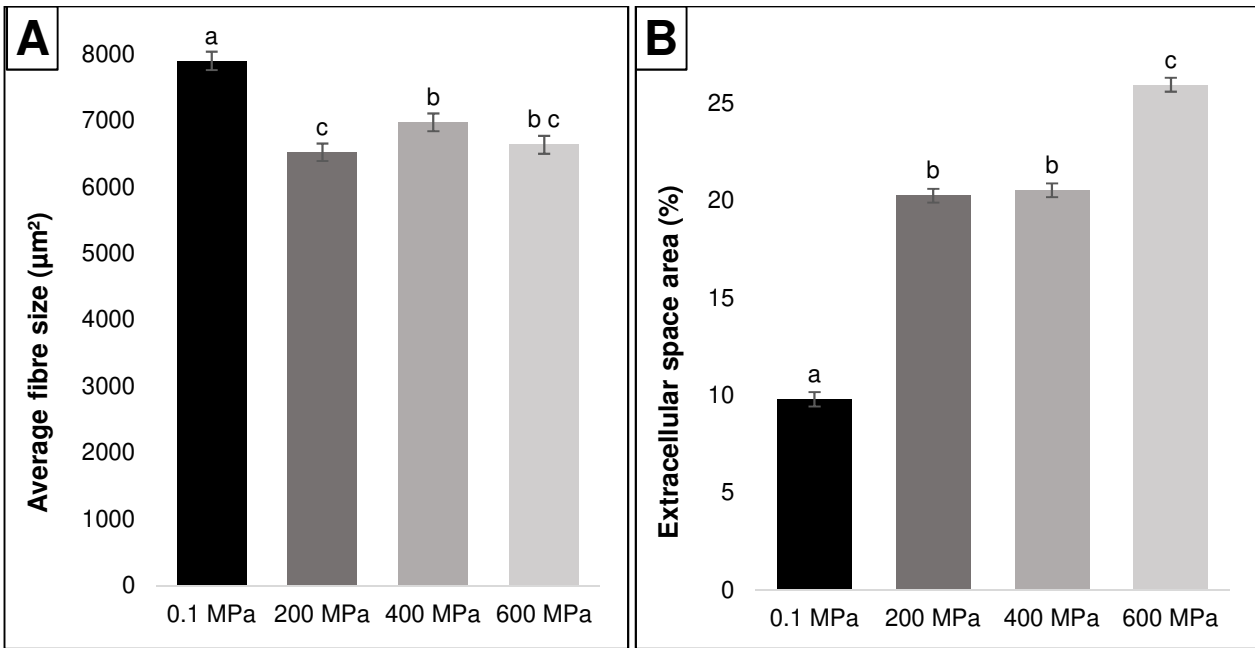
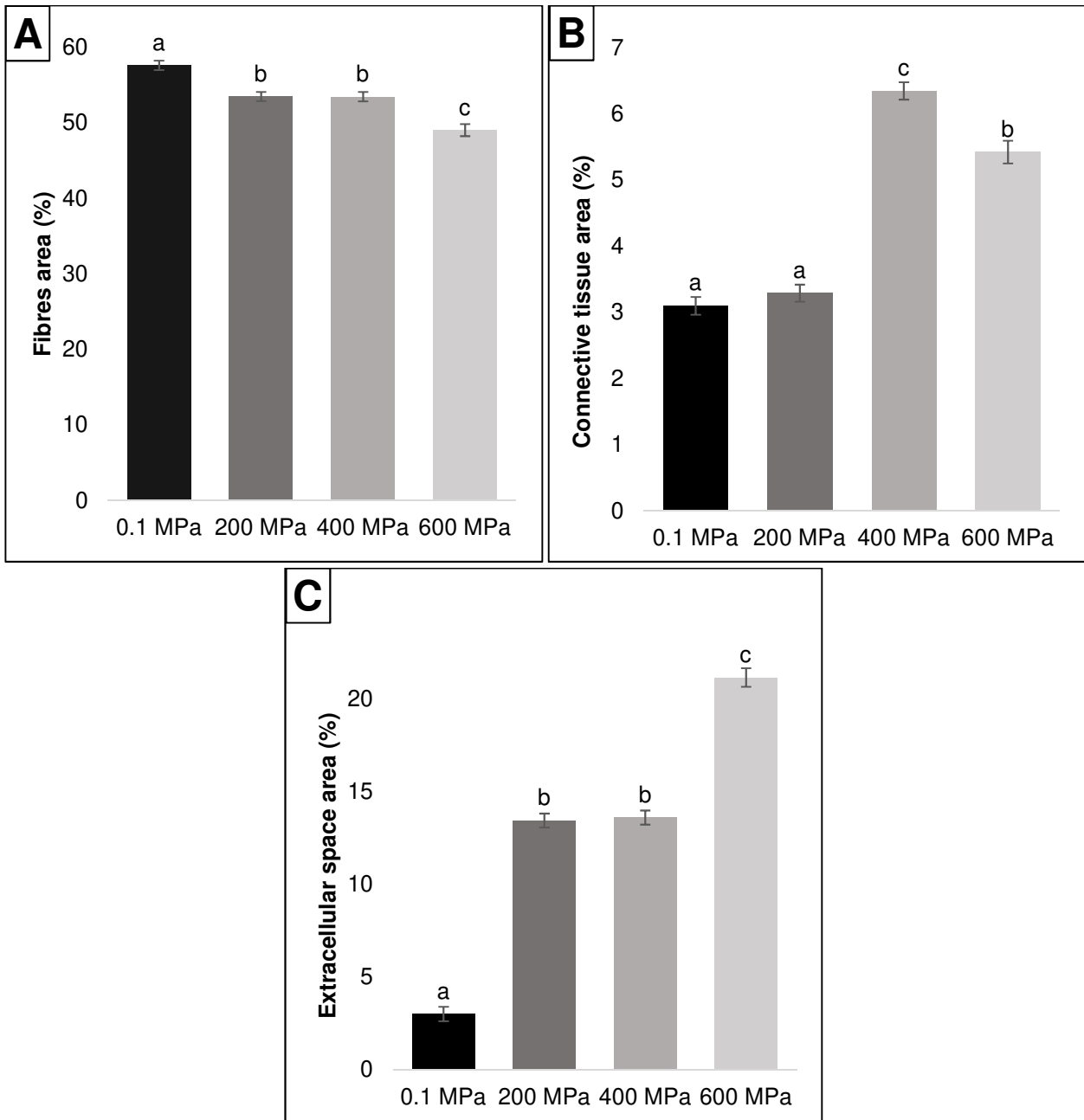


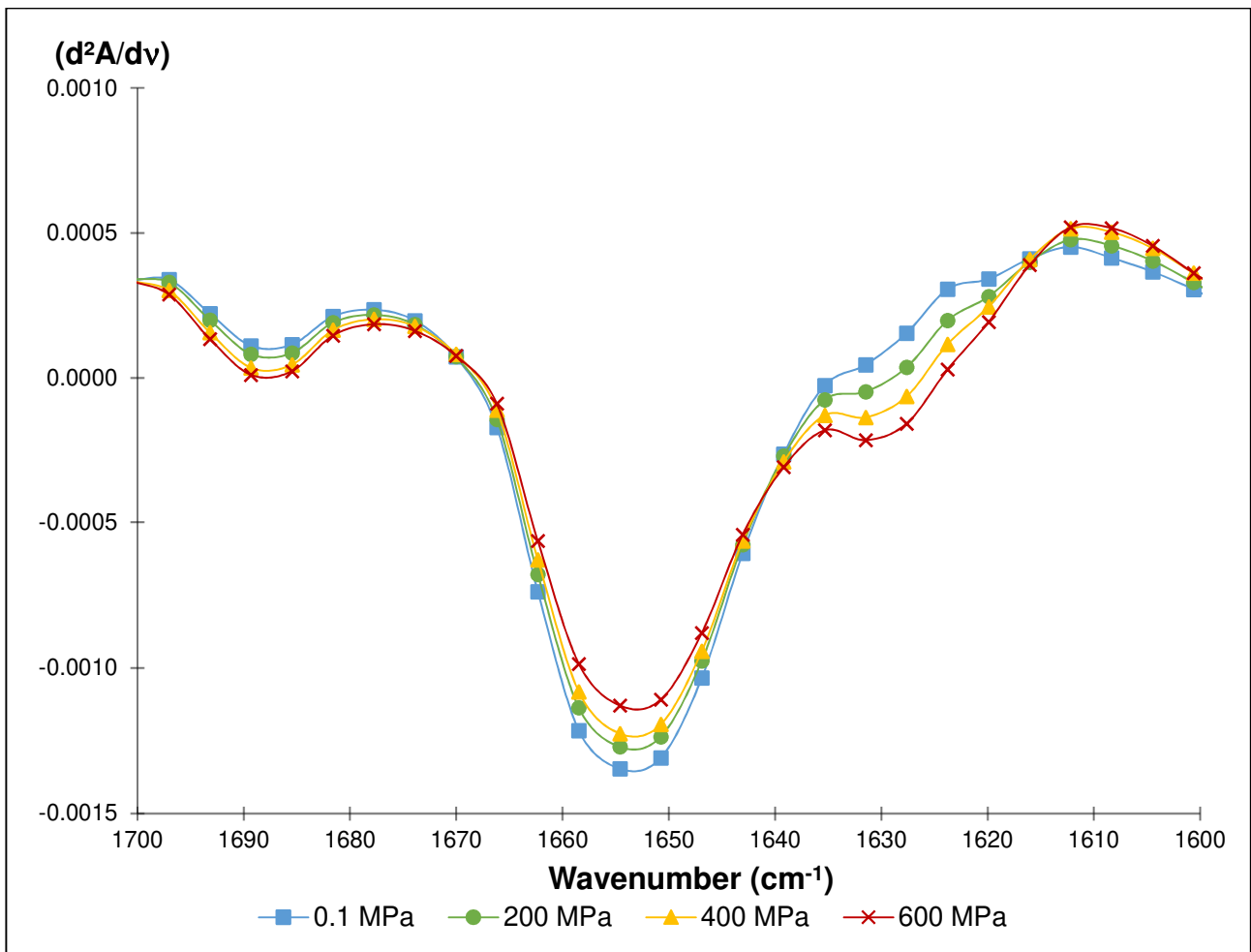
Figure 1: Histological sections of non-pressurized (0.1 MPa) and pressurized (200, 400 and 600 MPa, 5 min) salmon muscle. HES staining (A) colours the fibres red. Picrosirius red staining (B) colours the fibres yellow and the connective tissue red. Scale: 50 mm = 100  $\mu$ m



**Figure 2:** Average fibre size in  $\mu\text{m}^2$  (A) and area from extracellular space in % (B) for non-pressurized (0.1 MPa) and pressurized (200, 400 and 600 MPa, 5 min) salmon (n=12) measured on HES-stained sections. The results are expressed as the mean  $\pm$  standard error. Means with different letters are significantly different ( $p < 0.05$ ).



**Figure 3:** Percentage of the total section area from fibres (A), connective tissue (B) and extracellular space (C) for non-pressurized (0.1 MPa) and pressurized (200, 400 and 600 MPa, 5 min) salmon (n=12) measured on Picrosirius red-stained sections. The results are expressed as the mean  $\pm$  standard error. Means with different letters are significantly different ( $p < 0.05$ ).



**Figure 4:** Second-derivative mean infrared spectra (1700-1600 cm<sup>-1</sup>) obtained in muscle fibres of non-pressurized (0.1 MPa) and pressurized (200, 400 and 600 MPa, 5 min) salmons (n=12), each from approximately 120 spectra.



The principal component graph (B) describes what wavenumbers contribute the most in describing each principal component. PC1 is mainly described by two bands with maximums at  $1628\text{ cm}^{-1}$  and  $1655\text{ cm}^{-1}$  and describes 56.77% of the variability between all samples.

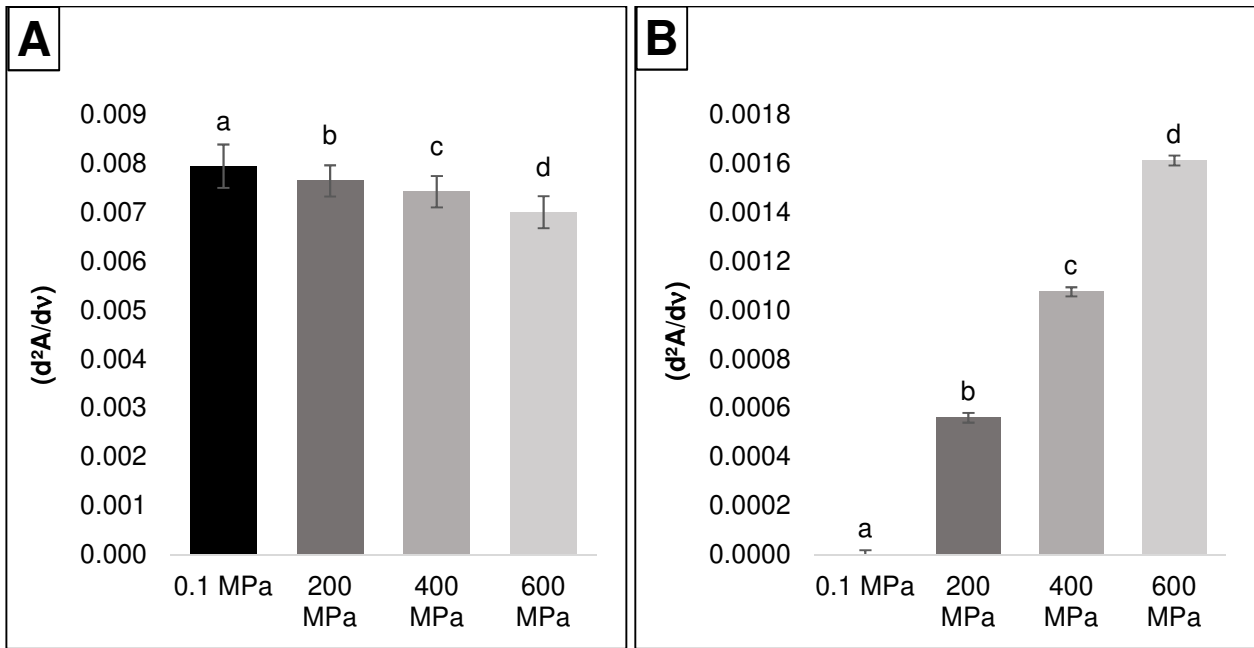
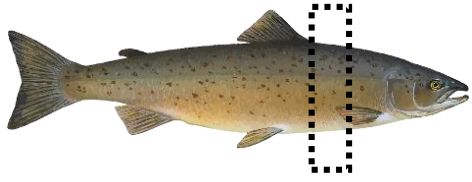


Figure 6: Evolution of  $\alpha$ -helices (A) and aggregated  $\beta$ -sheets (B) in salmon ( $n=12$ ) muscle fibres measured by the height of the second-derivative spectra peaks at  $1655\text{ cm}^{-1}$  and  $1628\text{ cm}^{-1}$ , respectively. The results are expressed as the mean  $\pm$  standard error. Means with different letters are significantly different ( $p < 0.05$ ).

# Effects of high-pressure treatment on the muscle structure of salmon (*Salmo salar*)



n = 12 salmons

## High-pressure processing

Control, 200 MPa,  
400 MPa, 600 MPa  
5 min, 20°C

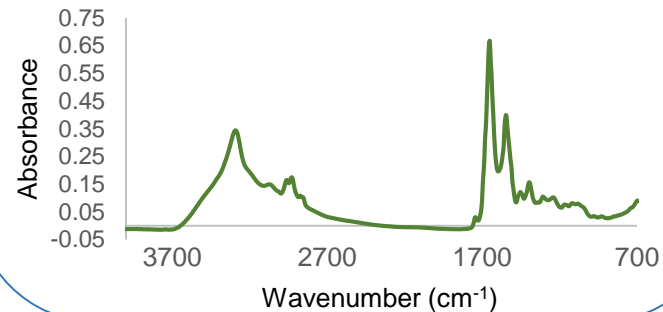


## Histochemical analysis

- average fibre size
- amount of space occupied by :
  - the extracellular spaces (ECS)
  - the connective tissue

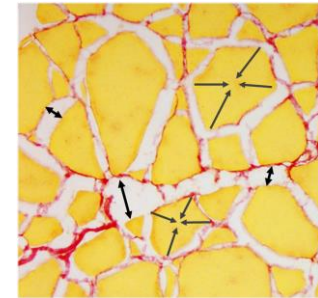
## Infrared spectromicroscopy

- Infrared spectra acquired in the muscle fibres
- Evaluation of  $\alpha$ -helix and aggregated  $\beta$ -sheet content



## Results

- Shrinkage of the fibres from 200 MPa
- Increase of the ECS from 200 MPa
- Modification of the connective tissue at 400 MPa



- Decrease of the  $\alpha$ -helix and increase of the aggregated  $\beta$ -sheet with pressure

