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1 **Differentiation between fresh and frozen-thawed sea bass (*Dicentrarchus labrax*) fillets**  
2 **using two-dimensional gel electrophoresis**

3 Short title: Use of 2D-electrophoresis to differentiate fresh from thawed sea bass fillets

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18 **Abstract**

19 This study aimed to identify a protein marker that can differentiate between fresh skinless and  
20 frozen-thawed sea bass (*Dicentrarchus labrax*) fillets using the two-dimensional  
21 polyacrylamide gel electrophoresis (2-DE) technique. Distinct gel patterns, due to proteins  
22 with low molecular weight and low isoelectric points, distinguished fresh fillets from frozen-  
23 thawed ones. Frozen-thawed fillets showed two specific protein spots as early from the first  
24 day of the study. However, these spots were not observed in fresh fillets until at least 13 days  
25 of storage between 0 and 4°C, fillets were judged, beyond this period, unfit for human  
26 consumption as revealed by complementary studies on fish spoilage indicators: total volatile  
27 basic nitrogen and biogenic amines. Mass spectrometry identified the specific proteins as  
28 parvalbumin isoforms. Parvalbumins may thus be useful as markers of differentiation between  
29 fresh and frozen-thawed sea bass fillets.

30

31 **Keywords:** Sea bass (*Dicentrarchus labrax*), 2D-electrophoresis, frozen-thawed, fresh,  
32 biogenic amines

33

34 **Chemical compounds studied in this article:**

35 3-[(3-Cholamidopropyl)dimethylammonium]-1-propanesulfonate (PubChem CID 107670);  
36 Acetonitrile (PubChem CID 6342); Diaminopropane (PubChem CID 6567); Dithiothreitol  
37 (PubChem CID 19001); Ethanol (PubChem CID 702); Formic acid (PubChem CID 284);  
38 Iodoacetamide (PubChem CID 3727); Perchloric acid (PubChem CID 24247);  
39 Tris(hydroxymethyl)aminomethane hydrochloride (PubChem CID 93573); Urea (PubChem  
40 CID 1176)

## 41 **1. Introduction**

42 Over the past several years, wild-caught fish have become rare although consumer demand  
43 for them has increased. The development of frozen storage has allowed producers and  
44 suppliers to commercialize products worldwide, and thus meet consumer demand.  
45 Nevertheless, a common practice consists in thawing frozen fish and selling it as fresh fish;  
46 this practice is illegal under current regulations in the European Union, as mentioned in  
47 Directive 2000/13/EC, which lays down the rules for the labelling of foodstuffs including  
48 specifications as to their physical condition and the specific treatment that they have  
49 undergone (*e.g.* deep-frozen). There are several methods to differentiate between fresh and  
50 frozen-thawed fish (Duflos, Le Fur, Mulak, Becel, & Malle, 2002; Karoui, Thomas, &  
51 Dufour, 2006). Some methods are suitable for whole fish and skin-on fillets, but difficulties  
52 remain in distinguishing fresh from frozen-thawed skinless fillets. Currently, no accurate,  
53 sensitive, rapid and inexpensive method is available for producers, suppliers and official  
54 authorities to determine directly if a skinless fish fillet has been frozen. Freezing and thawing  
55 causes sensory changes and quality decay (Matsumoto, 1979; Tironi, De Lamballerie, & Le-  
56 Bail, 2010; Tironi, LeBail, & De Lamballerie, 2007), increases protein denaturation (Benjakul  
57 & Bauer, 2001), lipid oxidation (Baron, Kjaersgard, Jessen, & Jacobsen, 2007; Duun &  
58 Rustad, 2007) and drip loss (Zhu, Ramaswamy, & Simpson, 2004). For moderate subfreezing  
59 temperatures, extracellular water usually freezes and cells may suffer from severe osmotic  
60 contraction. During freezing, the formation of large ice crystals leads to high mechanical  
61 damage in cells (Martino, Otero, Sanz, & Zaritzky, 1998; Tironi, LeBail, & De Lamballerie,  
62 2007), affecting their membranes and other ultrastructural elements (Mazur, 2010; Pegg,  
63 2010) and causing them to release intracellular protein exudates. Furthermore, during  
64 thawing, the diluted external medium causes an increase in cell volume, and the plasma  
65 membrane cannot withstand such high hydrostatic pressure (Tchir & Acker, 2010).

66 To differentiate fresh from frozen-thawed fish, proteomics was used — a technique applied  
67 for analyzes such as species identification (Berrini, Tepedino, Borromeo, & Secchi, 2006;  
68 Etienne et al., 2001), tissue and development stage identification (Martinez & Friis, 2004),  
69 and more specifically, for documentation of changes in cod muscle during frozen storage  
70 (Kjaersgard, Norrelykke, & Jessen, 2006) or differentiation between wild and farmed cod  
71 (Martinez, Slizyte, & Dauksas, 2007). Regarding sea bass, studies have used 2D-  
72 electrophoresis to evaluate freshness (Verrez-Bagnis, Ladrat, Morzel, Noël, & Fleurence,  
73 2001) or to provide detailed characterization of its specific protein expression profile (Terova,  
74 Pisanu, Roggio, Preziosa, Saroglia, & Addis, 2014).

75 Here, farmed sea bass (*Dicentrarchus labrax*) was chosen as a model because it has several  
76 advantages: low genetic variation, identical feed, environmental conditions and temperature,  
77 same age pyramid, graded weight, and known date of death.

78 The protein composition of the sea bass fillet exudates was tested after freezing-thawing to  
79 assess whether changes are recorded. To avoid selecting potential, non-specific post-mortem  
80 markers that are also found in fresh fillet exudates, spoilage indicators were monitored over a  
81 period of 15 days. Biogenic amines (BAs) and total volatile basic nitrogen (TVB-N) in fish  
82 are quality indices used for food safety assessment. Among the most important BAs,  
83 putrescine, cadaverine and histamine are known for their significant negative impact on fish  
84 freshness and human health. The purpose of this study was to compare electrophoresis  
85 profiles of fresh *versus* frozen-thawed fillets, to detect spots of interest and to characterize  
86 them using mass spectrometry, so as to identify protein markers and determine the fresh or  
87 frozen status of stored fillets.

## 88 **2. Materials and methods**

### 89 *2.1. Fish material*

90 Sampled fish came from the Aquanord sea farm (Gravelines, France). Sea bass (*D. labrax*)  
91 were raised in tanks containing 25,000 sea bass at a maximum concentration of 40 kg per  
92 cubic meter. The tanks were connected to a seawater recirculation system with the following  
93 strictly controlled conditions: temperature  $18 \pm 5^\circ\text{C}$ , pH 8.2, total ammonia  $< 30 \mu\text{mol/L}$ , and  
94 dissolved oxygen level over 99% (v/v) saturation (7 ppm). On day 0, 8 to 10 fish (average  
95 body weight  $500 \pm 150 \text{ g}$ ) were removed from the water and slaughtered via  
96 asphyxia/hypothermia (immersion in ice-cold water at a 3:1 fish-to-ice ratio). The fish were  
97 then packed in ice and transported (1 h trip) from Gravelines to Boulogne-sur-Mer (France).  
98 The fish were immediately processed (CFPMT, Boulogne-sur-Mer) to obtain skinless fillets.  
99 Cling-film protected fillets were stored in polystyrene boxes with crushed ice and kept in cold  
100 storage, between 0 and  $+2^\circ\text{C}$ . All stages of the processing, storage and freezing treatments  
101 were performed under conditions typical of a fish-processing plant.  
102 Fillets were examined over 15 days, stored between 0 and  $+4^\circ\text{C}$ . Each sampling day (day 0, 3,  
103 6, 9, 13 and 15 after slaughter), fillets were divided into two groups. The first one is  
104 composed of a fresh fillets group for immediate analysis: 2-DE profiles and spoilage  
105 indicators. During storage of the fresh fillets, water was drained off through drainage holes in  
106 the boxes and fresh ice was provided every day. The second one is composed of a frozen-  
107 thawed fillets group: the fillets were frozen at  $-30^\circ\text{C}$  at the Plateforme Nouvelles Vagues  
108 processing plant (Boulogne-sur-Mer, France) and stored at  $-20^\circ\text{C}$  for less than 15 days.  
109 Afterward, the frozen fillets were removed from the freezer and stored for 24 h between 0 and  
110  $+4^\circ\text{C}$  to thaw completely and the same analyses as those for the fresh fillets groups were  
111 carried out.

## 112 2.2. *Fish exudate preparation and protein extraction*

113 Exudates are extracts from flesh juice (Morel, 1979) obtained after centrifugation (Ayala et  
114 al., 2005; Duflos, Le Fur, Mulak, Becel, & Malle, 2002; Tironi, De Lamballerie, & Le-Bail,  
115 2010). Briefly, sampling for both groups of fish involved taking a piece of white muscle  
116 tissue (1 cm<sup>3</sup>) from the lateral-dorsal (epiaxial) muscle quadrant of each fish. Only the dorsal  
117 white flesh of the fillets was used in this study. The preparation of fish exudates was based on  
118 soft solubilization of proteins (Duflos, Le Fur, Mulak, Becel, & Malle, 2002). Each stage of  
119 the protocol was performed at +4°C. Soluble proteins were obtained from 20 g of diced white  
120 flesh (cubes of approximately 1 cm on each side) of fresh or frozen-thawed fillets placed in  
121 one volume (w/v) of phosphate buffer (10 mM, pH 7.4) containing anti-proteases: 1%  
122 phenylmethylsulphonyl fluoride (PMSF) (10 mg/mL isopropanol), 0.2% pepstatin (0.35  
123 mg/mL ethanol) and 0.1% leupeptin-aptinin (combination of leupeptin 0.5 mg/mL and  
124 aptinin 1 mg/mL). After gentle stirring, suspensions were centrifuged at 34,000 ×g, for 30  
125 min at +4°C to obtain exudates. Lipids were discarded by pipetting and the supernatant was  
126 filtered twice on a 0.45 µm membrane (Millipore, Guyancourt, France). The amount of  
127 proteins was determined using the Bradford method (Bradford, 1976) with the Bio-Rad  
128 reagent (Bio-Rad, Marnes-la-Coquette, France) and bovine serum albumin (BSA) as  
129 standards. Proteins were concentrated on a 10 kDa filter in an Amicon cell 8003 (Millipore).  
130 Rehydration buffer containing 8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonium]-1-  
131 propanesulfonate (CHAPS), 20 mM dithiothreitol (DTT), 0.2% ampholytes (100X Biolyte 3-  
132 10 Ampholyte, Bio-Rad) was added to reach 6.66 mg/mL.

## 133 2.3. *Two dimensional gel electrophoresis*

134 Each solution was filtered on a 0.45 µm filter and prepared with ultrapure water (18 MΩ) and  
135 all products met the quality requirements for proteomics and genomics.

136 *2.3.1. First dimension isoelectric electrophoresis focusing (IEF)*

137 Isoelectric electrophoresis focusing (IEF) was performed according to [Hochstrasser,](#)  
138 [Patchornik, and Merrill \(1988\)](#) with ReadyStrip immobilized pH gradient (IPG) Strips (Bio-  
139 Rad), 17 cm in length (pH gradient = 3-10). According to the manufacturer's instructions, the  
140 IPG strips were rehydrated for 6 h (passive rehydration) in a mixture of 300  $\mu$ L of the protein  
141 sample and 200  $\mu$ L of rehydration buffer with 0.001% bromophenol blue used to track  
142 migration. Then, 2 mg of protein was loaded in a volume of 500  $\mu$ L on each strip. The  
143 rehydrated strips were then positioned in the Protean IEF Cell (Bio-Rad) on the focusing plate  
144 for 15 h at 50 V (active rehydration). Proteins were focused for 27 h for a total of 85,000 Vh  
145 without exceeding an intensity of 50  $\mu$ A per strip.

146 After focusing, all strips were incubated in two equilibration buffers for 10 min. The first  
147 equilibration buffer contained 6 M urea, 2% sodium dodecyl sulphate (SDS), 125 mM  
148 Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5) and 50 mM DTT. The  
149 second equilibration buffer had the same composition except 150 mM iodoacetamide was  
150 used instead of DTT.

151 *2.3.2. Second dimension electrophoresis*

152 Equilibrated strips were loaded on 12.5% SDS gels (19.5 x 19.5 x 0.05 cm) and underwent  
153 SDS-polyacrylamide gel electrophoresis. The second dimension was performed as described  
154 in [Hochstrasser, Harrington, Hochstrasser, Miller, and Merrill \(1988\)](#). Electrophoresis was  
155 carried out without exceeding 30 mA per gel and with a voltage increasing gradually to 350  
156 V.

157 *2.3.3. Staining*

158 After migration, gels were stained with a silver nitrate staining kit (Sigma-Aldrich, Saint  
159 Quentin Fallavier, France). The gels were scanned with GS-800™ Calibrated Densitometer

160 and PD Quest software (Bio-Rad) The spots of interest were excised and destained with the  
161 destaining solution (Sigma-Aldrich) containing 1% potassium hexacyanoferrate III  
162 ( $C_6FeK_3N_6,3 H_2O$ ) and 1.6% sodium thiosulphate. The gels were dried between two  
163 cellophane membranes and archived.

#### 164 2.4. Mass spectrometry analysis

##### 165 2.4.1. Sample preparation and mass spectrometry analysis

166 *In situ* digestion of the gel spots was performed with an automated protein digestion system,  
167 MassPREP Station (Waters, Milford MA, USA) as previously described (Buhr, Carapito,  
168 Schaeffer, Hovasse, Van Dorsselaer, & Viville, 2007). The gel plugs were washed three times  
169 with a mixture of 50%/50%  $NH_4HCO_3$  (25 mM)/acetonitrile. Cysteine residues were reduced  
170 with DTT at 57°C for 30 min and alkylated with iodoacetamide at room temperature for 20  
171 min. After dehydration with acetonitrile, proteins were digested in gel with 20  $\mu$ L of 12.5  
172 ng/ $\mu$ L modified porcine trypsin (Promega, Madison, WI, USA) in 25 mM  $NH_4HCO_3$   
173 overnight at room temperature. Then, a double extraction was performed, first with 60% (v/v)  
174 acetonitrile in 5% (v/v) formic acid and then with 100% acetonitrile. The resulting tryptic  
175 peptides were analyzed using nano liquid chromatography-tandem mass spectroscopy (nano  
176 LC-MS/MS). These analyses were performed on an HCT Ultra ion trap (Bruker Daltonics,  
177 Bremen, Germany) equipped with a 1100 high-performance liquid chromatography (HPLC)  
178 system and a chip cube (Agilent Technologies, Palo Alto, CA, USA) as previously described  
179 (Fauquenoy et al., 2008). After loading, the peptide mixture was first concentrated and  
180 washed at 4  $\mu$ L/min in a 40 nL enrichment column (Agilent Technologies chip), with 0.1%  
181 formic acid as an eluent. Chromatographic separation was then carried out on a C18 reverse-  
182 phase capillary column (75 $\mu$ m x 43 mm in the chip cube) at a flow rate of 300 nL/min. For  
183 tandem MS experiments, automatic switching between MS and MS/MS modes was set as

184 follows: on each MS spectrum the three most abundant peptides, preferably doubly and triply  
185 charged ions, were selected for further isolation and collision-induced dissociation.

186 Mass data acquisitions were monitored by ChemStation (Agilent Technologies),  
187 EsquireControl (Bruker Daltonics) and DataAnalysis (Bruker Daltonics) software.

#### 188 2.4.2. Data interpretation and protein identification

189 Mass data collected by nano LC-MS/MS were processed, converted into .mgf peaklist files  
190 using DataAnalysis 4.0 (Bruker Daltonics) and interpreted using the Mascot 2.4.3 algorithm  
191 (Matrix Science, London, UK) run on a local server. Searches were performed without any  
192 molecular weight or isoelectric point restrictions against an in-house generated protein  
193 database composed of all vertebrate protein sequences extracted from UniProtKB (Release-  
194 2014\_02, TaxonomyID=7742, 2187035 entries). The database was concatenated in-house  
195 using the Mass Spectrometry Data Analysis (MSDA) software suite (Carapito et al., 2014).  
196 Known contaminant proteins such as human keratins and porcine trypsin were included in this  
197 vertebrate database and therefore only reversed copies of all sequences were added to obtain a  
198 target-decoy database of 4,374,070 entries (Elias & Gygi, 2010). This large database was used  
199 because the *D. labrax* genome is represented by only 2456 protein entries in UniProtKB.  
200 Trypsin was selected as the cleavage enzyme and searches were carried out with a mass  
201 tolerance of 250 ppm in MS mode and of 0.5 Da in MS/MS mode. A maximum of a single  
202 missed cleavage was allowed and some modifications were taken into account:  
203 carbamidomethyl (C), acetyl N-terminus of proteins, oxidation (M). Identifications were  
204 validated for Mascot Ion scores higher than 45, no decoy hit was identified with this ion score  
205 filter; all validated MS/MS spectra were manually checked.

206 2.5. *Assessment of the degree of fillet spoilage*

207 Two indicators were studied: BAs (Duflos, Dervin, Malle, & Bouquelet, 1999) according to  
208 the European regulation No. 1441/2007 and TVB-N (Castro, Penedo Padron, Caballero  
209 Cansino, Sanjuan Velazquez, & Millan De Larriva, 2006; Malle & Poumeyrol, 1989). The  
210 samples were analyzed in triplicate at different times after fish slaughter: day 0, 3, 6, 9, 13,  
211 and 15.

212 The HPLC method was used for BAs determination in fresh or frozen-thawed fillets.

213 First, 5 g of flesh were weighed and ground with Ultraturax in 10 mL of 0.2 M perchloric acid  
214 and 100  $\mu$ L of 0.8 mg/mL 1,3 diaminopropane. The homogenized mixture was centrifuged at  
215 7000  $\times$ g for 5 min at 4°C. In a safe-locked tube, 100  $\mu$ L of supernatant was added to 300  $\mu$ L  
216 of saturated sodium carbonate solution and 400  $\mu$ L of 7.5 mg/mL dansyl chloride. After  
217 stirring and incubation at 60°C for 5 min in the dark, samples were cooled under tap water.  
218 Then, 100  $\mu$ L of a 100 ng/mL proline solution was added and, after stirring, the tube was kept  
219 15 min in the dark. Afterwards, 500  $\mu$ L of toluene was added and, after stirring, the mixture  
220 was stored at -20°C for 30 min. The organic phase was recovered and the remaining phase  
221 was evaporated under a stream of nitrogen. The pellet was finally dissolved with 200  $\mu$ L of  
222 acetonitrile.

223 Separation was performed using a C18 Kromasil column (5  $\mu$ m, 100 A, 25 cm x 4.6mm). The  
224 reversed-phase column was eluted with an acetonitrile gradient. Absorbance was measured at  
225 254 nm. The quantity of BAs was calculated by linear regression against a series of standard  
226 solutions (25, 50, 100, 250 ppm).

227 To portray temporal patterns of the BAs and TVB-N content in fresh and frozen-thawed  
228 fillets, the cumulated function method was used (Ibanez, Fromentin, & Castel, 1993). The  
229 calculation consists in subtracting a reference value (here, the mean of the series) from the  
230 date, and then successively adding the residuals, forming a cumulative function. Successive

231 negative residuals produce a decreasing slope indicating values lower than the overall mean,  
232 whereas successive positive residuals create an increasing slope indicating higher values than  
233 the overall mean. Values that are only slightly different from the mean show no slope.

### 234 **3. Results**

#### 235 *3.1. 2D-electrophoresis comparison of fresh and frozen-thawed fillets*

236 In a preliminary study to identify potential biochemical markers, 2D-electrophoresis (2-DE)  
237 was used to compare protein maps of fresh versus frozen-thawed European sea bass fillet  
238 exudates. Experiments were performed on 3-day-old fillets. Two representative 2-DE maps  
239 are shown in Fig. 1. Both show numerous spots, mainly distributed in the basic part of the pH  
240 gradient, but their patterns clearly differed. The 2-DE profiles of frozen-thawed exudates  
241 showed more spots than the fresh profiles. Focusing in particular on protein spots with low  
242 isoelectric values and low molecular weights (Fig. 1), the 2D-E profiles of the frozen-thawed  
243 fillets are distinctive with respect to two spots — hereafter called 1 and 2 —, which were  
244 absent in the fresh fillet gel profiles. To confirm that these differences appeared regardless of  
245 the degree of spoilage, time-course analyses were carried out.

#### 246 *3.2. 2-DE time-course profiles of exudates*

##### 247 *3.2.1. 2-DE time-course profiles of fresh fillet exudates*

248 From slaughtering day (day 0) to day 15, analyses were performed in triplicate. 2D-E on the  
249 fresh fillet exudates showed that from day 0 to day 9, there were no spots located in the area  
250 of interest of the protein profiles (Fig. 2). Only a faint spot (noted 2) appeared on day 15 in  
251 the fresh fillet profile (Fig. 2A).

##### 252 *3.2.2. 2-DE time-course profiles of frozen-thawed fillet exudates*

253 In contrast to the profiles of fresh fillet exudates, the frozen-thawed fillet 2-DE profiles  
254 clearly showed two spots (1 and 2) as of day 0. The intensity of the two spots gradually  
255 increased over time (Fig. 2B).

### 256 3.3. Time course of spoilage indicators

257 To distinguish spoiled fillets from thawed fillets and to check that the potential markers  
258 revealed by 2-DE could not also be considered as indicators of spoilage, freshness was  
259 evaluated using normalized methods. Therefore, BAs and TVB-N levels were determined on  
260 each day of the experiment (Fig. 3).

#### 261 3.3.1. Fresh fillet analysis

262 Changes in BAs rates and in TVB-N content of fresh fillets are given in Fig. 3A. All values  
263 increased slightly or remained similar, never exceeding 10 ppm for BAs or 25 mg/100 g for  
264 TVB-N until day 9. After this period, levels increased significantly. Histamine and putrescine  
265 never exceeded the upper legal limit, although TVB-N and cadaverine showed increases that  
266 exceeded American limits (respectively, 25 mg/100 g and 50 mg/kg) and European limits  
267 (100 mg/kg). Examination of the related cumulated sums (Fig. 3B) highlighted two distinct  
268 periods: from day 0 to day 9, and after day 9. In the second period (day 13 and day 15), two  
269 markers (cadaverine and TVB-N) indicated that the fish quality was not acceptable according  
270 to US and European regulations. As shown in Fig. 3A regarding fresh fillets, from day 9 after  
271 slaughter, there was significant increase in histamine and cadaverine. Between day 9 and day  
272 13, histamine, cadaverine and putrescine markedly increased, reaching very high levels on  
273 day 13. The same phenomenon was observed for TVB-N (data not shown).

#### 274 3.3.2. Frozen-thawed fillet analysis

275 Changes in BAs concentrations and TVB-N were different for the frozen-thawed fillets (Fig.  
276 3C). Only putrescine concentrations showed change, quite similar to the one observed for the

277 fresh fillets. The samples, that had been stored in ice for zero to six days before freezing, then  
278 thawed, showed very few changes in their BAs levels, close to zero, whereas the TVB-N  
279 levels were relatively stable at approximately 22 mg/100 g. Then, as of day 9, cadaverine and  
280 TVB-N levels exceeded the upper legal limit. Cadaverine and histamine levels were  
281 approximately 2.4 and 10 times higher at the end of the experiments, exceeding 200 mg/kg.  
282 The related cumulated sums revealed two different periods, depending on the indicator (Fig.  
283 3D). The periods during which histamine was observed in the frozen-thawed fillets were  
284 identical to the ones observed in fresh fillets. For the other indicators (cadaverine, putrescine  
285 and TVB-N), the first period (D0 to D6) was shorter than for the fresh samples. This means  
286 that by considering the indicators that exceeded the legal limits (cadaverine and TVB-N),  
287 thawed fillets became unfit for human consumption sooner (as of day 9) than fresh fillets.  
288 These results confirm that freezing and thawing facilitate development of microflora (data not  
289 shown) and promote spoilage of fish.

#### 290 3.4. Protein identification

291 Priority was given to characterizing proteins located in the area of interest, corresponding to  
292 an isoelectric point between 3 and 4 and molecular weights comprised between 10 and 12  
293 kDa. The 2-DE gels of frozen-thawed fish were repeated (n=10) to collect a sufficient amount  
294 of spots 1 and 2 to ensure the reproducibility of their content (Fig. 2).

295 Nano LC-MS/MS analyses provided unambiguous identification of parvalbumin isoforms in  
296 all samples of spots 1 and 2 as summarized in Table 1.

297 Although the genome of *D. labrax* has been sequenced  
298 (<http://genomesonline.org/project?id=8120>), its annotation is not complete and only 2456  
299 proteins are present in the UniProtKB database (2375 protein entries are recorded in the  
300 NCBI nr database). Therefore, a large database including all vertebrate protein entries was  
301 used to allow for cross-species protein identifications. Thus, two groups of parvalbumin

302 isoforms were identified in spots 1 and 2 based on three unique peptides. Peptides  
303 LFLQNFSAGAR and SGFIEEDELK unambiguously matched 16 parvalbumin proteins  
304 recorded for the following species in UniProtKB: *Cyprinus carpio*, *Boreogadus saida*,  
305 *Siniperca chuatsi*, *Hypomesus transpacificus*, *Thunnus albacares*, *Danio rerio*, *Carassius*  
306 *auratus*, *Macruronus magellanicus*, *Macruronus novaezelandiae*, *Fundulus heteroclitus*,  
307 *Fundulus grandis*, *Ictalurus punctatus*. Peptides AFAIIDQDK and SGFIEEDELK  
308 unambiguously matched 14 parvalbumin proteins recorded for the following taxa in  
309 UniProtKB: *Cyprinus carpio*, *Micropterus salmoides*, *Gillichthys mirabilis*, *Gillichthys seta*,  
310 *Siniperca chuatsi*, *Ictalurus furcatus*, *Hypophthalmichthys nobilis*, *Hypophthalmichthys*  
311 *molitrix*, *Kryptolebias marmoratus*, *Xiphias gladius*, *Ictalurus punctatus*.

312 A representative MS/MS spectrum of each identified peptide is provided in Fig. 4.

313 Here, 431 out of 886 parvalbumins present in the database belong to bony fish species and all  
314 species with matching peptides in this study belong to this category.

315 Two parvalbumins had been previously identified by proteomic profiling of sea bass muscle  
316 using 2-DE (Terova, Pisanu, Roggio, Preziosa, Saroglia, & Addis, 2014). In addition,  
317 parvalbumins are proteins with low molecular weight (10 to 12.5 kDa) and an acidic  
318 isoelectric point (4.1 to 5.2), correlating with the region in which the spots were found on the  
319 2-DE gels.

#### 320 **4. Discussion**

321 To differentiate a fresh sea bass fillet from a frozen-thawed one, exudates were studied, the  
322 supernatant present after fillets have been centrifuged. The 2-DE patterns from these exudates  
323 revealed areas of differentiation between fresh and frozen-thawed fish. This study focused  
324 only on the protein composition of the exudates, which contains soluble proteins that may be  
325 released after freezing and thawing (Martinez & Friis, 2004; Terova, Pisanu, Roggio,  
326 Preziosa, Saroglia, & Addis, 2014). Instead of studying the total proteome, this approach

327 concentrated on a limited number of proteins that can be used to characterize easily the  
328 differences between fresh and frozen-thawed fillets. Results indicated that proteins of low  
329 molecular weight and low acid isoelectric values showed the most significant differentiation.  
330 Repeated 2-DE gels of exudates from frozen-thawed fish fillet revealed the same two  
331 differentiating spots compared with 2-DE of exudates from fresh fish fillets. These two spots  
332 appear in fresh fish fillet 2-DE profiles only 13 days after slaughter and beyond. To check the  
333 degree of spoilage of sea bass fillets, the time course of spoilage indicators such as BAs and  
334 TVB-N were also studied to ensure that the observed proteins did not correspond to proteins  
335 normally produced during post-mortem storage as described in previous 2-DE studies ([Terova  
336 et al., 2011](#); [Terova, Pisanu, Roggio, Preziosa, Saroglia, & Addis, 2014](#); [Verrez-Bagnis,  
337 Ladrat, Morzel, Noël, & Fleurence, 2001](#)).

338 BAs and TVB-N content show spoilage for fresh fillets and for frozen-thawed fillets from day  
339 9 as revealed by related cumulated sums. After nine days, fresh or frozen-thawed fillets show  
340 spoilage beyond the legal limits of consumption. Nevertheless, the 2-DE analyses could  
341 differentiate between fresh and frozen-thawed fish fillets up until day 13. The 2-DE method is  
342 therefore valuable for testing fish fillets up until their use-by date.

343 The two spots of interest identified in the 2-DE from frozen-thawed fillet were characterized  
344 by MS/MS as parvalbumin isoforms. Parvalbumins, which are sarcoplasmic proteins, were  
345 found in the frozen-thawed fillet exudates because membranes rupture following the  
346 formation of ice crystals inside cells during freezing ([Morel, 1979](#); [Tironi, De Lamballerie, &  
347 Le-Bail, 2010](#)). These proteins are known as the main fish allergens ([Swoboda et al., 2002](#);  
348 [Van Do, Hordvik, Endresen, & Elsayed, 2005](#)). Parvalbumin shares similarities in numerous  
349 species, possibly indicating that this protein is ubiquitous ([Chen, Hefle, Taylor, Swoboda, &  
350 Goodman, 2006](#)).

351 Fish parvalbumins are already used as markers to detect fish derivatives in final food products  
352 (Faeste & Plassen, 2008), to differentiate commercial hake varieties (Carrera, García-López,  
353 Martín del Río, Martínez-Rodríguez, Solé, & Mancera, 2007) and to identify different fish  
354 species (Rehbein, Kundiger, Pineiro, & Perez-Martin, 2000).

## 355 **5. Conclusion**

356 The present study provides a 2-DE comparative analysis of the exudates to differentiate  
357 frozen-thawed sea bass fillets from fresh sea bass fillets. Membrane disruption after freezing  
358 leads to protein release and thus their presence in exudates. LC-MS/MS analysis identified  
359 two protein spots as parvalbumin isoforms, and these were specifically recovered in frozen-  
360 thawed fillets of sea bass at each day of analysis from the beginning of the study. These same  
361 spots were not observed in fresh fillets until at least 13 days of storage between 0 and 4°C.  
362 These two proteins could not be mistaken with spoilage indicators since the fillets were  
363 considered as unfit for the human consumption earlier in the time course analysis of biogenic  
364 amines and TVB-N. This study demonstrated that 2-DE is a relevant tool to differentiate  
365 skinless fresh fillets from frozen-thawed ones. It was therefore suggested that parvalbumin  
366 could be used as a marker to differentiate fresh from frozen-thawed sea bass fillets and should  
367 be investigated in other fish species of high commercial value. This work opens the way for  
368 future studies to develop a rapid and accurate test and to ultimately provide it to producers  
369 and official authorities.

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- 497  
498

499 **Figure captions**

500 **Fig. 1.** Overlay of 2D gel electrophoresis profiles. A: exudates of fresh sea bass fillets stored  
501 on ice for 3 days (D3). B: exudate of 3-day-old (D3) frozen-thawed sea bass fillet. 12.5%  
502 polyacrylamide gels were used with isoelectric points from 3 to 10 and stained with silver  
503 nitrate. The triangle is a mark and the box indicates the area of interest in which the two  
504 studied spots referred to as 1 and 2 appear. MW: Molecular Weight

505 **Fig. 2.** Changes in the area of interest (bottom left-hand part of the 2-DE gels). A: exudates of  
506 fresh sea bass fillets (D0) stored on ice for 3, 6, 9, 13 or 15 days before analysis. B: exudates  
507 of frozen-thawed sea bass fillets. Fillets were stored on ice for 0, 3 and 6 days, then frozen for  
508 less than 15 days and thawed on ice 24 h before analysis.

509 **Fig. 3.** Changes in biogenic amines and total volatile basic nitrogen levels in fresh (A) and  
510 frozen-thawed (C) fillets and cumulative sums in fresh (B) and frozen-thawed (D) sea bass  
511 fillets.

512 Analyses were performed in triplicate at different time periods after fish slaughter: day 0, 3, 6,  
513 9, 13, 15

514 A. Changes in biogenic amine levels and in the TVB-N content of fresh fillets

515 B. Plot of the related cumulated sums (Cum. sum.) in fresh fillets

516 C. Changes in biogenic amine levels and in the TVB-N content of frozen-thawed fillets

517 D. Plot of the related cumulated sums (Cum. sum.) in frozen-thawed fillets

518 Storage period indicated in days corresponds to:

519 - the analysis day (0, 3, 6, 9, 12 or 15) after fish slaughter for fresh fillets stored at 0 to  
520 +4°C (A, B)

521 - the analysis day after storage on ice for 0, 3, 6, 9, 12 or 15 days followed by a freezing  
522 step at -30°C and storage at -20°C for less than 15 days before thawing for 24 h at  
523 between 0 and +4°C for the frozen-thawed fillets (C, D)

524 **Fig. 4.** MS/MS spectra allowing the identification of parvalbumin isoforms

525 A. Representative MS/MS spectrum of the SGFIEEDELK peptide

526 B. Representative MS/MS spectrum of the LFLQNFSAGAR peptide

527 C. Representative MS/MS spectrum of the AFAIIDQDK peptide

528 **Table 1**

529 Identification of sea bass protein spots based on LC-MS/MS analysis and cross-species

530 matching with database sequences

Spot	Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Peptide sequences
Spot 1	Parvalbumin alpha ( <i>Cyprinus carpio</i> ) <sup>a</sup>	P09227	11 444	2	LFLQNFSA GAR - SGFIEEDELK
	Parvalbumin beta-2 ( <i>Cyprinus carpio</i> ) <sup>b</sup>	P02618	11 429	2	AFAIIDQDK - SGFIEEDELK
Spot 2	Parvalbumin alpha ( <i>Cyprinus carpio</i> ) <sup>a</sup>	P09227	11 444	2	LFLQNFSA GAR - SGFIEEDELK
	Parvalbumin beta-2 ( <i>Cyprinus carpio</i> ) <sup>b</sup>	P02618	11 429	2	AFAIIDQDK - SGFIEEDELK

<sup>a</sup> and <sup>b</sup>: Lists of proteins identified with identical set of peptides:

<sup>a</sup> Parvalbumin (*Boreogadus saida*) tr|C0LEL4; Parvalbumin beta-1 (*Cyprinus carpio*) tr|E0WD92; Parvalbumin 4 (*Siniperca chuatsi*) tr|D2KQG3; Parvalbumin (Fragment) (*Hypomesus transpacificus*) tr|C3UUG3; Parvalbumin (*Thunnus albacares*) tr|C6GKU3; Parvalbumin 3 (*Danio rerio*) tr|Q7ZT36; Parvalbumin 1 (*Carassius auratus*) tr|G8GWA2; Parvalbumin (*Cyprinus carpio*) tr|Q8UUS3; Parvalbumin-2 (*Danio rerio*) sp|Q9I8V0; Parvalbumin (*Siniperca chuatsi*) tr|B9VJM3; Parvalbumin beta 3 (Fragments) (*Macruronus magellanicus*) sp|P86740; Parvalbumin beta 3 (Fragments) (*Macruronus novaezelandiae*) sp|P86742; Parvalbumin (*Fundulus heteroclitus*) tr|C0LEL6; Parvalbumin (*Fundulus grandis*) tr|C0LEL8; Parvalbumin-2 (*Ictalurus punctatus*) tr|E3TGD0

<sup>b</sup> Parvalbumin (*Micropterus salmoides*) tr|C0LEL5; Parvalbumin beta-2 (*Cyprinus carpio*) tr|E0WD93; Parvalbumin 1 (Fragment) (*Gillichthys mirabilis*) tr|C1J0K6; Parvalbumin 1 (Fragment) (*Gillichthys seta*) tr|C1J0K7; Parvalbumin 2 (*Siniperca chuatsi*) tr|D2KQG1; Parvalbumin beta (*Ictalurus furcatus*) tr|E3TBW7; Parvalbumin (*Hypophthalmichthys nobilis*) tr|B5TTU7; Parvalbumin (*Hypophthalmichthys molitrix*) tr|B6UV98; Parvalbumin 1 (*Kryptolebias marmoratus*) tr|Q6B4H8; Parvalbumin (*Cyprinus carpio*) tr|Q8UUS2; Beta-parvalbumin (*Xiphias gladius*) tr|B9W4C2; Parvalbumin 4 (*Hypophthalmichthys molitrix*) tr|R9R015; Parvalbumin (Fragment) (*Ictalurus punctatus*) tr|Q804Z0







