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

Pierrette Ethuin, Sylvain Marlard, Mylène Delosière, Christine Carapito, Francois Delalande, Alain van Dorsselaer, Alexandre Dehaut, Valérie Lencel, Guillaume Duflos, Thierry Grard

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

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

Pierrette Ethuin[a*], Sylvain Marlard[a], Mylène Delosière[b], Christine Carapito[c], François Delalande[c], Alain Van Dorsselaer[c], Alexandre Dehaut[d], Valérie Lencel[a], Guillaume Duflos[d] and Thierry Grard[a]

[a] *Université du Littoral Côte d'Opale, équipe Biochimie des Produits Aquatiques (BPA), Boulevard du Bassin Napoléon, BP 120, 62327 Boulogne-sur-Mer, France*

[b] *INRA, UR 370 Qualité des Produits Animaux (QuaPA), Site de Theix, 63122 Saint-Genès-Champanelle, France*

[c] *Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO), IPHC, Université de Strasbourg, CNRS UMR7178, Strasbourg, France*

[d] *ANSES, Laboratoire de Sécurité des Aliments – Département des Produits de la Pêche et de l'Aquaculture, Boulevard du Bassin Napoléon, 62200 Boulogne-sur-Mer, France*

[*]Corresponding author: Tel: +33 3 21 99 45 20; Fax: +33 3 21 99 45 24.

E-mail address: pierrette.ethuin@univ-littoral.fr (P. Ethuin).

Published in *Food Chemistry* and available online at https://doi.org/10.1016/j.foodchem.2014.12.065

Abstract

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

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

Chemical compounds studied in this article:

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Fish material

Sampled fish came from the Aquanord sea farm (Gravelines, France). Sea bass (*D. labrax*) were raised in tanks containing 25,000 sea bass at a maximum concentration of 40 kg per cubic meter. The tanks were connected to a seawater recirculation system with the following strictly controlled conditions: temperature 18 ± 5°C, pH 8.2, total ammonia < 30 µmol/L, and dissolved oxygen level over 99% (v/v) saturation (7 ppm). On day 0, 8 to 10 fish (average body weight 500 ± 150 g) were removed from the water and slaughtered via asphyxia/hypothermia (immersion in ice-cold water at a 3:1 fish-to-ice ratio). The fish were then packed in ice and transported (1 h trip) from Gravelines to Boulogne-sur-Mer (France). The fish were immediately processed (CFPMT, Boulogne-sur-Mer) to obtain skinless fillets. Cling-film protected fillets were stored in polystyrene boxes with crushed ice and kept in cold storage, between 0 and +2°C. All stages of the processing, storage and freezing treatments were performed under conditions typical of a fish-processing plant.

Fillets were examined over 15 days, stored between 0 and +4°C. Each sampling day (day 0, 3, 6, 9, 13 and 15 after slaughter), fillets were divided into two groups. The first one is composed of a fresh fillets group for immediate analysis: 2-DE profiles and spoilage indicators. During storage of the fresh fillets, water was drained off through drainage holes in the boxes and fresh ice was provided every day. The second one is composed of a frozen-thawed fillets group: the fillets were frozen at -30°C at the Plateforme Nouvelles Vagues processing plant (Boulogne-sur-Mer, France) and stored at -20°C for less than 15 days. Afterward, the frozen fillets were removed from the freezer and stored for 24 h between 0 and +4°C to thaw completely and the same analyses as those for the fresh fillets groups were carried out.

2.2. Fish exudate preparation and protein extraction

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

2.3. Two dimensional gel electrophoresis

Each solution was filtered on a 0.45 µm filter and prepared with ultrapure water (18 MΩ) and 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 Merril (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 µL of the protein
141 sample and 200 µL 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 µL 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 µA 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 Merril (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

and PD Quest software (Bio-Rad) The spots of interest were excised and destained with the destaining solution (Sigma-Aldrich) containing 1% potassium hexacyanoferrate III ($C_6FeK_3N_6$,3 H_20) and 1.6% sodium thiosulphate. The gels were dried between two cellophane membranes and archived.

2.4. Mass spectrometry analysis

2.4.1. Sample preparation and mass spectrometry analysis

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

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

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

2.4.2. Data interpretation and protein identification

Mass data collected by nano LC-MS/MS were processed, converted into .mgf peaklist files using DataAnalysis 4.0 (Bruker Daltonics) and interpreted using the Mascot 2.4.3 algorithm (Matrix Science, London, UK) run on a local server. Searches were performed without any molecular weight or isoelectric point restrictions against an in-house generated protein database composed of all vertebrate protein sequences extracted from UniProtKB (Release-2014_02, TaxonomyID=7742, 2187035 entries). The database was concatenated in-house using the Mass Spectrometry Data Analysis (MSDA) software suite (Carapito et al., 2014). Known contaminant proteins such as human keratins and porcine trypsin were included in this vertebrate database and therefore only reversed copies of all sequences were added to obtain a target-decoy database of 4,374,070 entries (Elias & Gygi, 2010). This large database was used because the *D. labrax* genome is represented by only 2456 protein entries in UniProtKB. Trypsin was selected as the cleavage enzyme and searches were carried out with a mass tolerance of 250 ppm in MS mode and of 0.5 Da in MS/MS mode. A maximum of a single missed cleavage was allowed and some modifications were taken into account: carbamidomethyl (C), acetyl N-terminus of proteins, oxidation (M). Identifications were validated for Mascot Ion scores higher than 45, no decoy hit was identified with this ion score 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 µL of 0.8 mg/mL 1,3 diaminopropane. The homogenized mixture was centrifuged at
215 7000 ×g for 5 min at 4°C. In a safe-locked tube, 100 µL of supernatant was added to 300 µL
216 of saturated sodium carbonate solution and 400 µL 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 µL of a 100 ng/mL proline solution was added and, after stirring, the tube was kept
219 15 min in the dark. Afterwards, 500 µL 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 µL of
222 acetonitrile.

223 Separation was performed using a C18 Kromasil column (5 µm, 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*

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

3.3. Time course of spoilage indicators

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

3.3.1. Fresh fillet analysis

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

3.3.2. Frozen-thawed fillet analysis

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

fresh fillets. The samples, that had been stored in ice for zero to six days before freezing, then thawed, showed very few changes in their BAs levels, close to zero, whereas the TVB-N levels were relatively stable at approximately 22 mg/100 g. Then, as of day 9, cadaverine and TVB-N levels exceeded the upper legal limit. Cadaverine and histamine levels were approximately 2.4 and 10 times higher at the end of the experiments, exceeding 200 mg/kg. The related cumulated sums revealed two different periods, depending on the indicator (Fig. 3D). The periods during which histamine was observed in the frozen-thawed fillets were identical to the ones observed in fresh fillets. For the other indicators (cadaverine, putrescine and TVB-N), the first period (D0 to D6) was shorter than for the fresh samples. This means that by considering the indicators that exceeded the legal limits (cadaverine and TVB-N), thawed fillets became unfit for human consumption sooner (as of day 9) than fresh fillets.

These results confirm that freezing and thawing facilitate development of microflora (data not shown) and promote spoilage of fish.

3.4. Protein identification

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

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

5. Conclusion

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

Acknowledgements

This work was funded by the Nord-Pas de Calais regional council. The authors wish to thank Dorothée Vincent for her advice on statistical analyses and Emmanuelle Croquelois-Clément for her technical assistance. Sylvain Marlard is grateful to the Nord-Pas de Calais regional council for its financial support of his PhD studies.

References

Ayala, M. D., López Albors, O., Blanco, A., García Alcázar, A., Abellán, E., Ramírez Zarzosa, G., & Gil, F. (2005). Structural and ultrastructural changes on muscle tissue of sea bass, *Dicentrarchus labrax L.*, after cooking and freezing. *Aqualculture, 250*(1-2), 215-231.

Baron, C. P., Kjaersgard, I. V., Jessen, F., & Jacobsen, C. (2007). Protein and lipid oxidation during frozen storage of rainbow trout (*Oncorhynchus mykiss*). *J Agric Food Chem, 55*(20), 8118-8125.

Benjakul, S., & Bauer, F. (2001). Biochemical and physicochemical changes in catfish (*Silurus glanis* Linne) muscle as influenced by different freeze-thaw cycles. *Food Chemistry, 72*(2), 207-217.

Berrini, A., Tepedino, V., Borromeo, V., & Secchi, C. (2006). Identification of freshwater fish commercially labelled "perch" by isoelectric focusing and two-dimensional electrophoresis. *Food Chemistry, 96*(1), 163-168.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem., 72*, 248-254.

Buhr, N., Carapito, C., Schaeffer, C., Hovasse, A., Van Dorsselaer, A., & Viville, S. (2007). Proteome analysis of the culture environment supporting undifferentiated mouse embryonic stem and germ cell growth. *Electrophoresis, 28*(10), 1615-1623.

Carapito, C., Burel, A., Guterl, P., Walter, A., Varrier, F., Bertile, F., & Van Dorsselaer, A. (2014). MSDA, a proteomics software suite for in-depth Mass Spectrometry Data Analysis using grid computing. *PROTEOMICS, 14*(9), 1014-1019.

Carrera, E. P., García-López, A., Martín del Río, M. d. P., Martínez-Rodríguez, G., Solé, M., & Mancera, J. M. (2007). Effects of 17[beta]-estradiol and 4-nonylphenol on osmoregulation and hepatic enzymes in gilthead sea bream (*Sparus auratus*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 145*(2), 210-217.

Castro, P., Penedo Padron, J. C., Caballero Cansino, M. J., Sanjuan Velazquez, E., & Millan De Larriva, R. (2006). Total volatile base nitrogen and its use to assess freshness in European sea bass stored in ice. *Food Control, 17*(4), 245-248.

Chen, L., Hefle, S. L., Taylor, S. L., Swoboda, I., & Goodman, R. E. (2006). Detecting fish parvalbumin with commercial mouse monoclonal anti-frog parvalbumin IgG. *J Agric Food Chem, 54*(15), 5577-5582.

Duflos, G., Dervin, C., Malle, P., & Bouquelet, S. (1999). Use of biogenic amines to evaluate spoilage in plaice (*Pleuronectes platessa*) and whiting (*Merlangus merlangus*). *Journal of AOAC International, 82*(6), 1357-1363.

Duflos, G., Le Fur, B. L., Mulak, V., Becel, P., & Malle, P. (2002). Comparison of methods of differentiating between fresh and frozen-thawed fish or fillets. *Journal of the Science of Food and Agriculture, 82*(12), 1341-1345.

Duun, A. S., & Rustad, T. (2007). Quality changes during superchilled storage of cod (*Gadus morhua*) fillets. *Food Chemistry, 105*(3), 1067-1075.

Elias, J. E., & Gygi, S. P. (2010). Target-decoy search strategy for mass spectrometry-based proteomics. *Methods Mol Biol, 604*, 55-71.

Etienne, M., Jerôme, M., Fleurence, J., Rehbein, H., Kündiger, R., Mendes, R., Costa, H., & Martinez, I. (2001). Species identification of formed fishery products and high pressure-treated fish by electrophoresis: a collaborative study. *Food Chemistry, 72*(1), 105-112.

423 Faeste, C. K., & Plassen, C. (2008). Quantitative sandwich ELISA for the determination of
424 fish in foods. *J Immunol Methods, 329*(1-2), 45-55.
425 Fauquenoy, S., Morelle, W., Hovasse, A., Bednarczyk, A., Slomianny, C., Schaeffer, C., Van
426 Dorsselaer, A., & Tomavo, S. (2008). Proteomics and Glycomics Analyses of N-
427 Glycosylated Structures Involved in Toxoplasma gondii-Host Cell Interactions.
428 *Molecular & Cellular Proteomics, 7*(5), 891-910.
429 Hochstrasser, D. F., Harrington, M. G., Hochstrasser, A. C., Miller, M. J., & Merril, C. R.
430 (1988). Methods for increasing the resolution of two-dimensional protein
431 electrophoresis. *Analytical Biochemistry, 173*(2), 424-435.
432 Hochstrasser, D. F., Patchornik, A., & Merril, C. R. (1988). Development of polyacrylamide
433 gels that improve the separation of proteins and their detection by silver staining.
434 *Analytical Biochemistry, 173*(2), 412-423.
435 Ibanez, F., Fromentin, J.-M., & Castel, J. (1993). *Application de la méthode des sommes*
436 *cumulées à l'analyse des séries chronologiques en océanographie* (Vol. 316). Paris,
437 France: Elsevier.
438 Karoui, R., Thomas, E., & Dufour, E. (2006). Utilisation of a rapid technique based on front-
439 face fluorescence spectroscopy for differentiating between fresh and frozen-thawed
440 fish fillets. *Food Research International, 39*(3), 349-355.
441 Kjaersgard, I. V. H., Norrelykke, M. R., & Jessen, F. (2006). Changes in cod muscle proteins
442 during frozen storage revealed by proteome analysis and multivariate data analysis.
443 *PROTEOMICS, 6*(5), 1606-1618.
444 Malle, P., & Poumeyrol, M. (1989). A New Chemical Criterion for the Quality Control of
445 Fish: Trimethylamine/Total Volatile Basic Nitrogen (%). *Journal of Food Protection,*
446 *52*(6), 419-423.
447 Martinez, I., & Friis, T. J. (2004). Application of proteome analysis to seafood authentication.
448 *PROTEOMICS, 4*(2), 347-354.
449 Martinez, I., Slizyte, R., & Dauksas, E. (2007). High resolution two-dimensional
450 electrophoresis as a tool to differentiate wild from farmed cod (*Gadus morhua*) and to
451 assess the protein composition of klipfish. *Food Chemistry EFFoST 2005 Annual*
452 *Meeting: Innovations in traditional foods, 102*(2), 504-510.
453 Martino, M. N., Otero, L., Sanz, P. D., & Zaritzky, N. E. (1998). Size and location of ice
454 crystals in pork frozen by high-pressure-assisted freezing as compared to classical
455 methods. *Meat Science, 50*(3), 303-313.
456 Matsumoto, J. (1979). Denaturation of fish muscle proteins during frozen storage. In A. i. c.
457 series (Ed.), *Proteins at low temperatures* O. Fennema ed. ed., vol. 180): Washington,
458 D.C.
459 Mazur, P. (2010). A biologist's view of the relevance of thermodynamics and physical
460 chemistry to cryobiology. *Cryobiology, 60*(1), 4-10.
461 Morel, M. (1979). Une méthode pour déterminer si le poisson est à l'état frais ou décongelé.
462 *Science et Pêche, Bull. Inst. Pêches marit., n°288*, 13-17.
463 Pegg, D. E. (2010). The relevance of ice crystal formation for the cryopreservation of tissues
464 and organs. *Cryobiology, 60*(3 Suppl), S36-44.
465 Rehbein, H., Kundiger, R., Pineiro, C., & Perez-Martin, R. I. (2000). Fish muscle
466 parvalbumins as marker proteins for native and urea isoelectric focusing.
467 *Electrophoresis, 21*(8), 1458-1463.
468 Swoboda, I., Bugajska-Schretter, A., Verdino, P., Keller, W., Sperr, W. R., Valent, P.,
469 Valenta, R., & Spitzauer, S. (2002). Recombinant carp parvalbumin, the major cross-
470 reactive fish allergen: a tool for diagnosis and therapy of fish allergy. *J Immunol,*
471 *168*(9), 4576-4584.

Tchir, J., & Acker, J. P. (2010). Mitochondria and membrane cryoinjury in micropatterned cells: effects of cell-cell interactions. *Cryobiology, 61*(1), 100-107.

Terova, G., Addis, M. F., Preziosa, E., Pisanu, S., Pagnozzi, D., Biosa, G., Gornati, R., Bernardini, G., Roggio, T., & Saroglia, M. (2011). Effects of postmortem storage temperature on sea bass (*Dicentrarchus labrax*) muscle protein degradation: Analysis by 2-D DIGE and MS. *PROTEOMICS, 11*(14), 2901-2910.

Terova, G., Pisanu, S., Roggio, T., Preziosa, E., Saroglia, M., & Addis, M. F. (2014). Proteomic profiling of sea bass muscle by two-dimensional gel electrophoresis and tandem mass spectrometry. *Fish Physiology and Biochemistry, 40*(1), 311-322.

Tironi, V., De Lamballerie, M., & Le-Bail, A. (2010). Quality changes during the frozen storage of sea bass (*Dicentrarchus labrax*) muscle after pressure shift freezing and pressure assisted thawing. *Innovative Food Science and Emerging Technologies, 11*(4), 565-573.

Tironi, V., LeBail, A., & De Lamballerie, M. (2007). Effects of Pressure-Shift Freezing and Pressure-Assisted Thawing on Sea Bass (*Dicentrarchus labrax*) Quality. *Journal of Food Science, 72*(7), C381-C387.

Van Do, T., Hordvik, I., Endresen, C., & Elsayed, S. (2005). Characterization of parvalbumin, the major allergen in Alaska pollack, and comparison with codfish Allergen M. *Molecular Immunology, 42*(3), 345-353.

Verrez-Bagnis, V., Ladrat, C., Morzel, M., Noël, J., & Fleurence, J. (2001). Protein changes in post mortem sea bass (*Dicentrarchus labrax*) muscle monitored by one- and two-dimensional gel electrophoresis. *Electrophoresis, 22*(8), 1539-1544.

Zhu, S., Ramaswamy, H. S., & Simpson, B. K. (2004). Effect of high-pressure versus conventional thawing on color, drip loss and texture of Atlantic salmon frozen by different methods. *LWT - Food Science and Technology, 37*(3), 291-299.

Figure captions

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

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

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

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

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

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

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

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

Storage period indicated in days corresponds to:

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

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

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

A. Representative MS/MS spectrum of the SGFIEEDELK peptide

B. Representative MS/MS spectrum of the LFLQNFSAGAR peptide

C. Representative MS/MS spectrum of the AFAIIDQDK peptide

Table 1

Identification of sea bass protein spots based on LC-MS/MS analysis and cross-species matching with database sequences

Table(s)

Published in Food Chemistry and available online at https://doi.org/10.1016/j.foodchem.2014.12.065

Spot	Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Peptide sequences
Spot 1	Parvalbumin alpha (*Cyprinus carpio*)[a]	P09227	11 444	2	LFLQNFSAGAR - SGFIEEDELK
	Parvalbumin beta-2 (*Cyprinus carpio*)[b]	P02618	11 429	2	AFAIIDQDK - SGFIEEDELK
Spot 2	Parvalbumin alpha (*Cyprinus carpio*)[a]	P09227	11 444	2	LFLQNFSAGAR - SGFIEEDELK
	Parvalbumin beta-2 (*Cyprinus carpio*)[b]	P02618	11 429	2	AFAIIDQDK - SGFIEEDELK

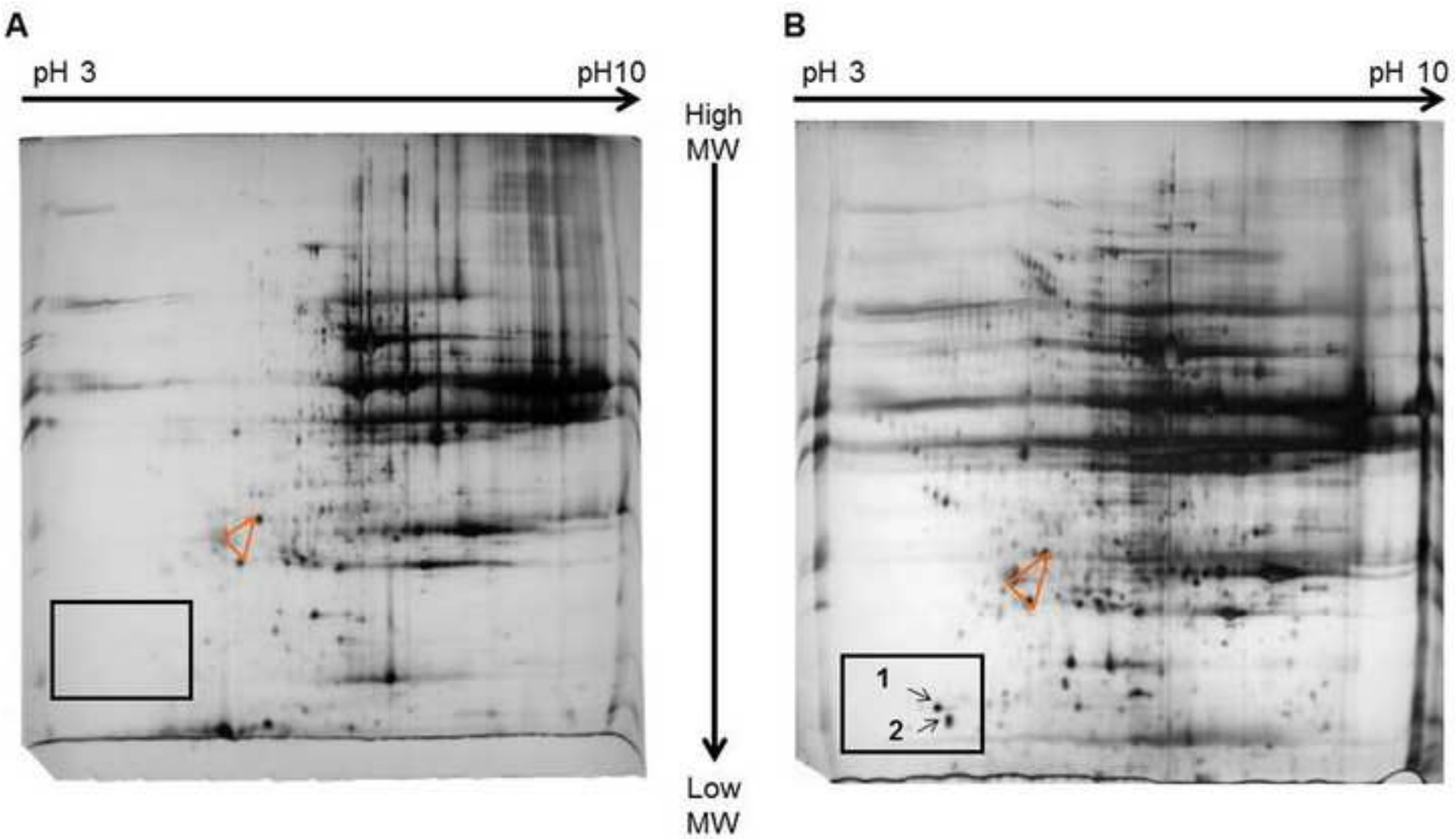
[a] and [b]: Lists of proteins identified with identical set of peptides:

[a] Parvalbumin (*Boreogadus saida*) tr|C0LEL4; Parvalbumin beta-1 (*Cyprinus carpio*) tr|E0WD92; Parvalbumin 4 (*Siniperca chuatsi*) tr|D2KQG3; Parvalbumin (Fragment) (*Hypomesus transpacificus*) tr|C3UVG3; 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

[b] 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

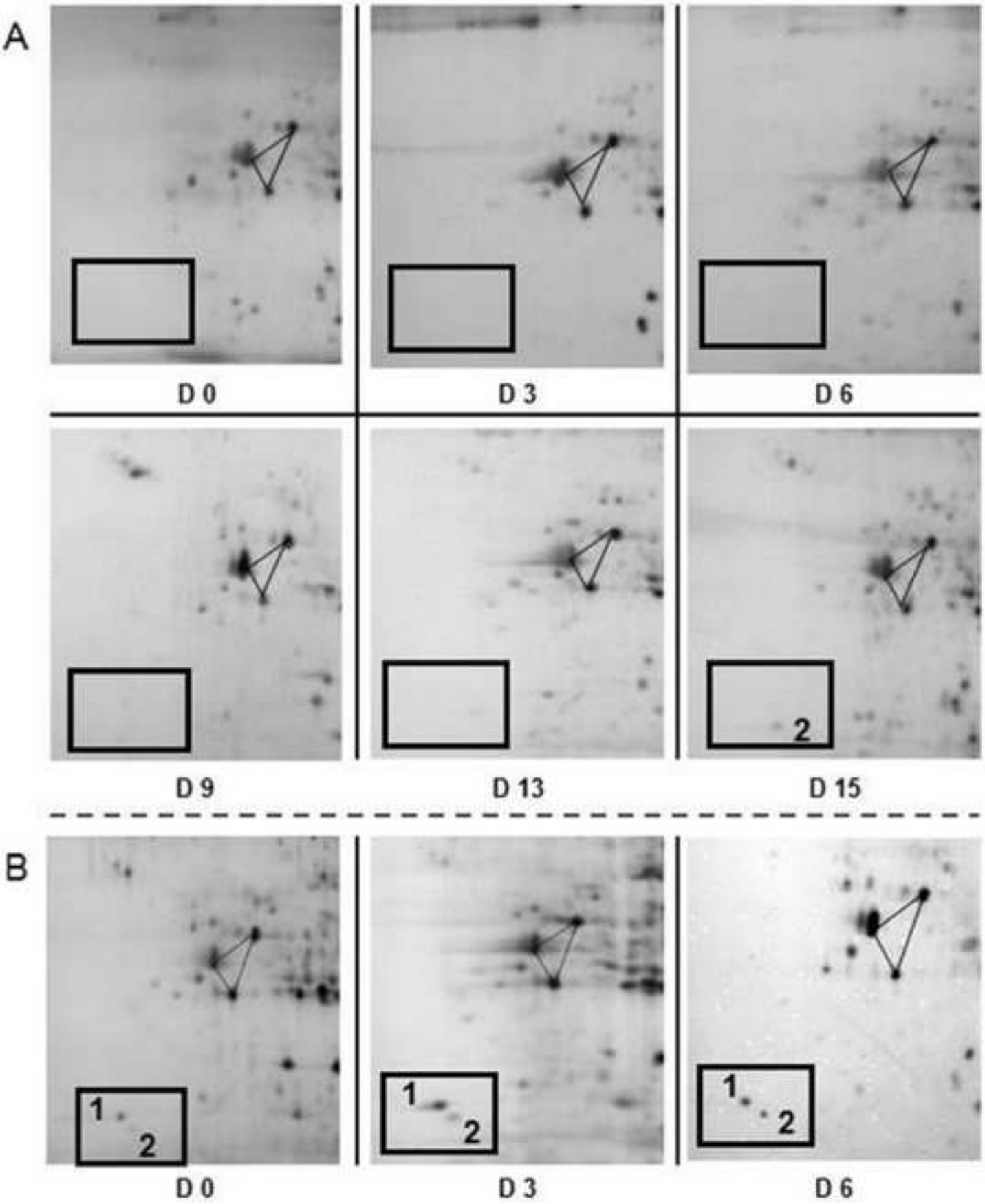
Figure(s)
Click here to download high resolution image

Published in Food Chemistry and available online at https://doi.org/10.1016/j.foodchem.2014.12.065



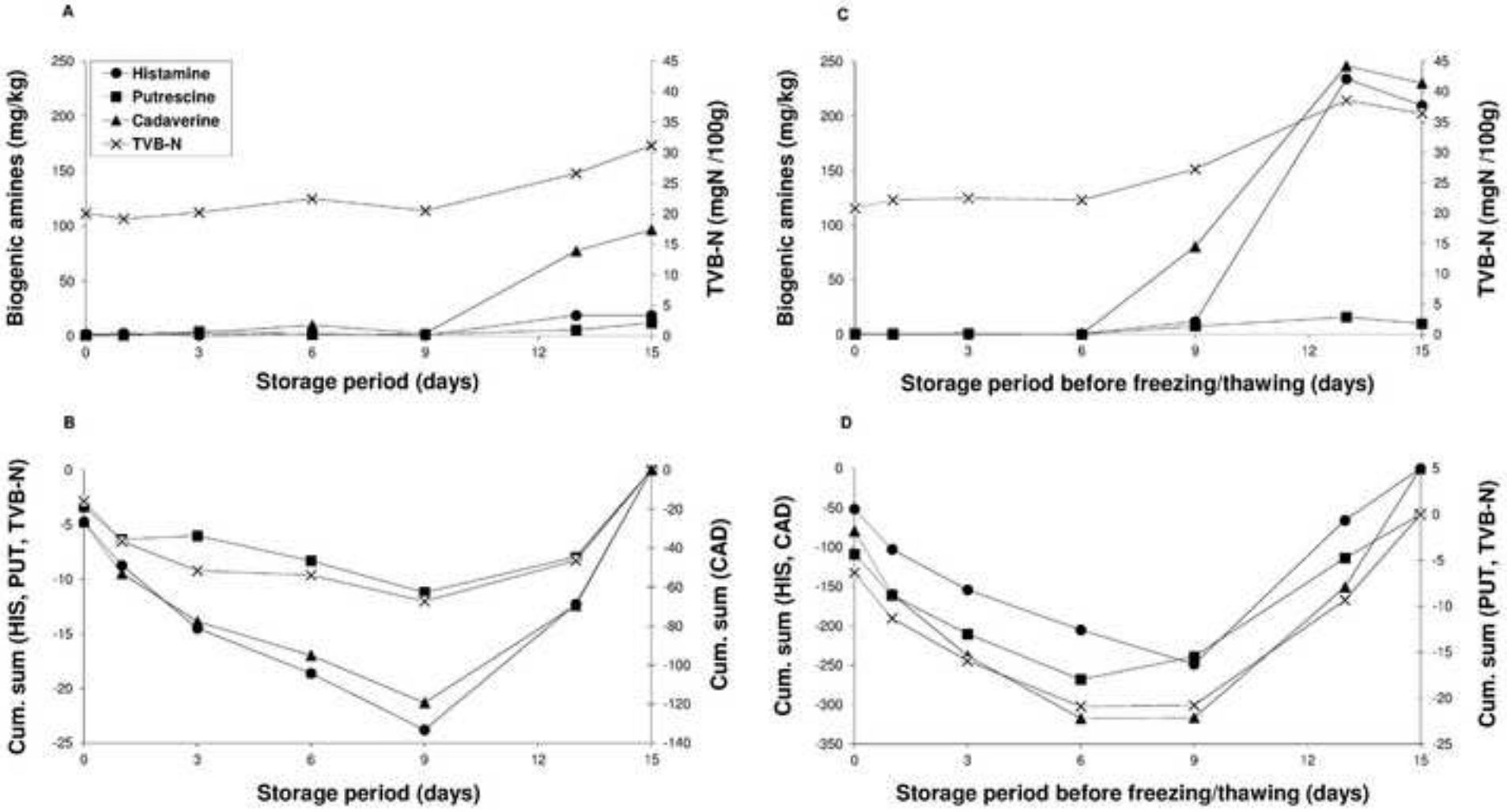
Figure(s)
Click here to download high resolution image

Published in Food Chemistry and available online at https://doi.org/10.1016/j.foodchem.2014.12.065



Figure(s)
Click here to download high resolution image

Published in Food Chemistry and available online at https://doi.org/10.1016/j.foodchem.2014.12.065



Figure(s)

Click here to download high resolution image

Published in Food Chemistry and available online at https://doi.org/10.1016/j.foodchem.2014.12.065