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High resolution two-dimensional electrophoresis as a tool to differentiate wild from farmed cod (*Gadus morhua*) and to assess the protein composition of klipfish

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Abstract

Tris and CHAPS–urea extracts from wild and farmed cod muscle and from rehydrated cod klipfish fillets were analyzed by one (1DE) and two-dimensional electrophoresis (2DE). 2DE maps of tris extracts from farmed cod differed from the wild in a series of spots of Mw 35 and 45 kDa. The CHAPS–urea extracts from farmed cod had a several spots of Mw between 100 and 45 kDa, which were hardly detectable in wild cod and very prominent in klipfish. Klipfish was clearly different from the other samples: the myosin heavy chain was hardly detectable in these samples, and the tris extracts contained fewer, and the CHAPS–urea more spots than the corresponding extracts from the raw muscles. Further identification of these potentially diagnostic spots will make it easier the differentiation of farmed from wild cod and the evaluation of klipfish processing on the protein content of the product.

Keywords: Wild cod; Farmed cod; Klipfish; Fish muscle; Myofibrillar proteins; Two-dimensional electrophoresis

1. Introduction

European citizens are entitled by law (CR-EC Nos. 2065/2001 and 104/2000) to information on the scientific name, method of production (farmed or wild), and the area in which wild fish was caught or farmed fish underwent the final developmental stage. Additional legal requirements for the implementation of traceability systems in the food and feed supply chains in Europe, are laid down in the General Food Law, Regulation 178/2002/EC, whose article number 18 referring to traceability has become effective since 1st January 2005. The EU Food Law defines traceability as "the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution".

Unfortunately, globalization and the consequent availability of unknown, novel species in certain markets, together with the consumer's willingness to pay higher prices for certain production methods (i.e. organically produced or environmentally friendly) and the lack of analytical methods to verify some product claims, allows the intervention of opportunistic elements and the fraudulent falsification of product information. The consequences can be very serious, including allergies and death, in the case of spoiled or contaminated products.

There are several methods suitable for species identification in seafood, but not for the unequivocal determination of wild and farmed cod, or for the life-history of the product, which are data necessary to verify the traceability documentation of a product and to detect fraud (Martinez et al., 2003).

We are interested in developing analytical techniques to verify product composition and claims that may be of relevance for the customers. Our previous works have dealt with the analyses of proteins and DNA for species and

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breeding stock identification (Martinez et al., 2003; Martinez & Friis, 2004) and application of nuclear magnetic resonance techniques for the authentication of fish products (Aursand, Rainuzzo, & Grasdalen, 1994; Martinez et al., 2003) and of small water soluble bioactive molecules (Martinez et al., 2005). Complementing these works, we decided to examine the suitability of muscle protein analysis to differentiate farmed from wild cod due to the fact that proteins are the most abundant component in cod muscle and also because it has been shown that the feed composition, and in particular the content of vegetable protein components in the feed, induce alterations in the protein expression in rainbow trout liver (Martin et al., 2003). However, we are not aware of works examining the protein expression in muscle, which is the most common edible part and therefore available for analysis all along the production chain, from the farm to the dish.

The protein profile of klipfish was also examined in order to map changes due to the processing and preserving conditions because that information may be of relevance regarding the protein composition of these products. In our recent work we have documented that klipfish is poorer in bioactive components than fresh fish (Martinez et al., 2005). Proteins from marine organisms have been shown to exert positive effects on human health, for example on the blood lipid profiles (Wergedahl et al., 2004) and skeletal muscle insulin responsiveness (Tremblay, Lavigne, Jacques, & Marette, 2003). However, these works used isolated and heat-treated salmon protein hydrolysates and purified cod protein respectively, while human diets are made up, usually, of processed fish containing whole proteins and therefore the protein profile resulting from certain processing conditions deserves closer examination. The suitability of a proteomics approach to map the composition of seafoods and changes in fish muscle due to loss of freshness has already been examined by several authors (Kjærsgård & Jessen, 2003; Martinez & Friis, 2004; Piñeiro, Barros-Velázquez, Vázquez, Figueras, & Gallardo, 2003). However, we are aware of only one work analyzing the effect of the processing conditions on the protein profile of surimi (Martinez, Solberg, Lauritzsen, & Ofstad, 1992), and of none aiming at identifying the protein profile of klipfish, a popular product consumed by millions of persons in many European and American countries.

This work had two aims: the first was to examine the suitability of two-dimensional electrophoresis of muscle extracts to differentiate farmed from wild cod and the second to assess the effect of klipfish processing on the protein composition of cod muscle.

2. Materials and methods

2.1. Fish samples

The cod (*Gadus morhua*) used were wild specimens captured in the Trondheimsfjord in April ($n = 10$), farmed cod from the Trondelag region slaughtered in February ($n = 5$) and rehydrated klipfish ($n = 5$). The wild and farmed fish arrived iced at our laboratory within a few hours (under 5 h) of having been captured or slaugthered. The cod were eviscerated and washed, and chops of about 3 cm in width were cut and immediately frozen and stored at −80 °C. For protein extraction, the chops were placed in a freezer at −20 °C and about 100 mg of white muscle were scraped while the muscle was still frozen. The klipfish had been purchased in different local shops and upon arrival to our laboratory they were soaked in a ratio fish:cold water of 1:8 for 2 days with one change of water after 24 h. Cubes of approximately $4 \times 4 \times 2$ cm were frozen stored at −80 °C and treated as the rest of the cod samples for protein extraction.

2.2. Protein extraction

The procedure described by Molloy et al. (1998) was followed using the ReadyPrep Sequential Extraction Kit of Bio-Rad. The muscle (75 mg) were scraped and placed into a 2 ml Eppendorf tube in ice, where 1 ml of Solution 1 (40 mM Tris, measured pH 10.5) was added. The samples were homogenized with the tubes in ice and centrifuged at 15,000 rpm in an Eppendorf bench centrifuge for 5 min. The supernatants (Tris-extracts) were immediately collected and frozen stored at −80 °C, except for an aliquot left to measure protein content. To the remaining pellet, 500 μl of Solution 2 (8 M urea, 4% 3-((3-cholamidopropyl) dimethylamino)-1-propanesulfonate (CHAPS), 2 mM tributylphosphine (TBP), 40 mM Tris and 0.2% Pharmalyte 3–10 ampholines) was added. The samples were homogenized for 30 s, centrifuged at 15,000 rpm for 5 min, and the supernatants (CHAPS–urea extracts) were immediately frozen stored at −80 °C, except for an aliquot left to measure protein content. Protein concentration was measured with the Bio-Rad RC DC Protein Assay.

2.3. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of the extracts

The sequential extracts were analyzed by SDS–PAGE according to Laemmli (1970) in 14 cm × 16 cm, 0.75 mm thick slab gels. The stacking gel contained 5% acrylamide and 0.13% piperazine diacrylamide and the separating gels 15% acrylamide and 0.087% piperazine diacrylamide (Anderson, Baum, & Gesteland, 1973; Hochstrasser, Harrington, Hochstrasser, Miller, & Merril, 1988; Hochstrasser, Patchornik, & Merril, 1988). The extracts were diluted to 0.4 mg/ml in Laemmli buffer (4.8% SDS, 1 mM ethylenediaminetetraacetic acid (EDTA), 125 mM Tris–HCl, pH 6.8, 5% β-mercaptoethanol, 20% glycerol and some bromophenol blue). The samples were boiled for 5 min, cooled down and frozen stored at −20 °C. They were centrifuged prior to loading on the gels to remove undissolved matter. One microgram of protein per sample was loaded and electrophoresis was carried out at 20 mA/gel until the front reach about 0.5 cm from the bottom of

the gel. The gels were silver stained (Ansorge, 1983) and dried between two sheets of cellophane. The Low Molecular Weight Calibration Kit for SDS electrophoresis (Amersham Biosciences) was used as marker. It contains a mixture of markers of molecular mass: 97, 66, 45, 30, 20.1 and 14.4 kDa.

2.4. Two-dimensional electrophoresis

2.4.1. First dimension: Isoelectric focusing (IEF)

IEF was performed on 13 cm Immobiline DryStrips pH 3–10 (Amersham Biosciences). The strips were individually rehydrated overnight at 4 °C in 250 μl of 8 M urea, 2 M thiourea, 50 mM dithiothreitol (DTT), 1.5% CHAPS, 2% Pharmalyte 3–10, 10 mM Tris–HCl, pH 8.3 and some Orange G containing 100 μg of protein. IEF was performed in the Protean IEF System according to the instructions of the manufacturer (Bio-Rad, 2001) at 15 °C as follows: 30 min at 250 V, followed by 1 h at 4000 V; then the voltage was increased to 9000 until 70,000 Vh were reached and afterwards the voltage was decreased to 500 V until the run was manually stopped. The strips were stored frozen at −80 °C until ready to run the second dimension (SDS–PAGE). Prior to SDS–PAGE, the strips were equilibrated in 6 M urea, 2% SDS, 50 mM Tris, pH 8.8, 30% glycerol and 1% DTT for 10 min followed by another 10 min using the same buffer but with 4.5% iodoacetamide instead of DTT. All samples were analyzed individually and then as pools of 5 fish.

IEF was also performed in rods following O'Farrell's (1975) procedure as described by Martinez and Christiansen (1994) in 6 cm × 1 mm rod gels containing 3% acrylamide 0.08% piperazine diacrylamide (Hochstrasser et al., 1988), 9 M urea, 2% Nonidet P-40, and 2% 3–10 IsoDalt Servalyt ampholines (spread in the 5–7 range). Forty microgram of protein diluted in 9.5 M urea, 2% Nonidet P-40, 2% 3–10 IsoDalt Servalyt ampholines and 5% β-mercaptoethanol were loaded onto the rods. IEF was performed for about 2700 Vh in a Mini Protean III unit (Bio-Rad) according to the instructions of the manufacturers. After IEF, the rods were extruded, shortly equilibrated in 62.5 mM Tris–HCl (pH 6.8), 2.3% SDS, 5% β-mercaptoethanol and 20% glycerol, containing bromophenol blue and either immediately loaded onto the second dimension slab gels or stored frozen at −20 °C for later analysis. All fish were analyzed individually.

2.4.2. Second dimension: SDS–PAGE

SDS–PAGE was performed according to Laemmli (1970) in 14 cm × 16 cm, 1 mm thick slab gels. The stacking gel contained 5% acrylamide and 0.13% piperazine diacrylamide and the separating gels 12.5% acrylamide and 0.1% piperazine diacrylamide (Hochstrasser, & Patchornik, et al.). Electrophoresis was carried out at 20 mA/slab until the front reached about 0.5 cm from the bottom of the gel. The stacking gel was omitted in the analyses of the Immobiline DryStrips. The gels were silver stained according to Ansorge (1983) and dried between 2 sheets of cellophane.

2.5. Identification of myosin subunits, actin and tropomyosin

No spots have been sequenced as part of this work. However, the bands and spots corresponding to the myosin heavy chains, fast (white) and slow (red) myosin light chains types 1f, 2f, 3f, 1s and 2s, actin and tropomyosin, from several muscle types (skeletal (white and red) and cardiac) and fish species, including cod skeletal white muscle, had been previously identified by one and two-dimensional electrophoresis of electrophoretically isolated native myosin and actomyosin (Martinez, Christiansen, Ofstad, & Olsen, 1991; Martinez, Ofstad, & Olsen, 1990; Martinez et al., 1992; Martinez, Bang, Hatlent, & Blix, 1993).

3. Results and discussion

3.1. Analyses of wild and farmed cod

Fig. 1 shows the 1D SDS–PAGE analyses of the Tris (Fig. 1a) and CHAPS–urea (Fig. 1b) extracts from farmed and wild cod. The bands corresponding to the myosin heavy chain, actin, tropomyosin and fast myosin light chain types 1, 2 and 3, which have been identified by electrophoretic analyses of purified native myosin and actomyosin in previous works (Martinez et al., 1990, 1991, 1992, 1993) are indicated in the figure.

Some of the differences between samples of unprocessed wild and farmed cod may be attributed to individual polymorphisms or to differences in the relative amount of protein loaded. Consistent differences between groups, on the other hand, may be attributed to differences in the production or processing method. We could not detect any band in the Tris extracts with potential diagnostic value for the production method. In the CHAPS–urea extracts, there was one band with such potential (labelled with an asterisk in Fig. 1), but the fact that it was present in detectable amounts in only 4 of the 5 farmed samples, limits is value.

2DE analyses of Tris– and CHAPS–urea extracts are shown in Fig. 2. Actin and tropomyosin were present in both types of extracts. Solubilization of fish myofibrillar proteins at very low (pH 2–3) and very high (pH 10.5–11) pH values due to electrostatic repulsion has been described by Kristinsson and Hultin (2003, and references therein) and this explains the presence of these myofibrillar proteins in the Tris extracts, which had a pH of 10.5. As expected (Martinez et al., 1992), the CHAPS–urea extract were heavily dominated by actin, tropomyosin and the fast myosin light chain types 1, 2 and 3 (Martinez et al., 1990, 1992). The myosin heavy chain could also be identified in the CHAPS–urea extracts but this protein does not enter easily the first dimension gels, the shape of the spot usually shows horizontal streaking and its size under-represents the true amount of myosin heavy chain in the sample (the author's unpublished results).

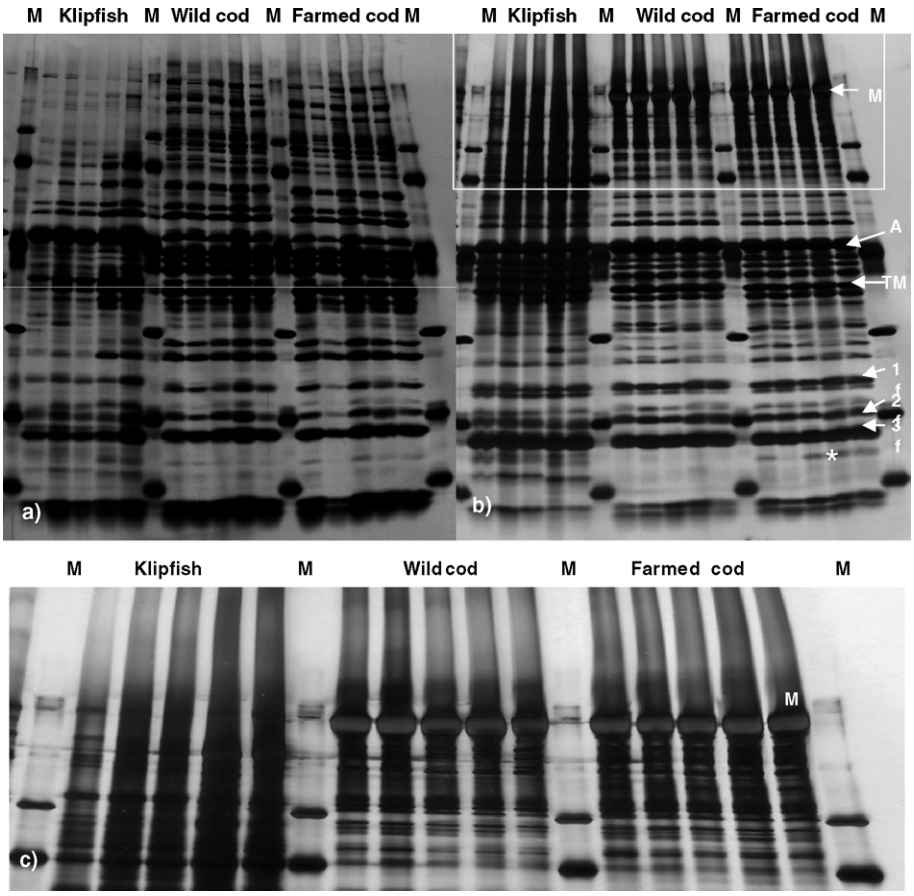

Fig. 1. 15% SDS–PAGE analyses of klipfish and wild and farmed cod: (a) Tris extracts and (b) CHAPS–urea extracts. The area enclosed in the square in (b) is shown enlarged in (c). Each lane is one individual fish. Lanes M contain mixture of markers of molecular mass: 97, 66, 45, 30, 20.1 and 14.4 kDa (Low Molecular Weight Calibration Kit for SDS Electrophoresis, Amersham Biosciences). Identified protein bands are A, actin, T, tropomyosin, M, myosin heavy chain; 1f, 2f and 3f are the fast myosin light chains type 1, 2 and 3, respectively. The square in the CHAPS–urea extract of klipfish indicates a smear where the myosin heavy chain would be in an intact sample.

The main difference between the 2DE patterns of wild and farmed fish was the presence of additional spots in the farmed fish of Mw between 35 and 45 kDa and from 50 to about 100 kDa, in the Tris and in the CHAPS–urea extracts, respectively (labelled with squares in Fig. 2). The latter spots were much more clearly visible when IEF was performed using rods (Fig. 3) than when using strips (Fig. 2).

Current work is in progress to identify the origin of these spots. Some of these discriminatory spots may correspond to proteins induced by the farming conditions as shown by Martin et al. (2003) in liver, while others may be the result of proteolytic degradation of higher molecular mass muscle proteins, since faster post mortem deterioration suffered by farmed than by wild fish has already been reported by Olsson, Olsen, and Ofstad (2003). In any case, our results are in contrast to those of Carpené, Martin, and Dalla Libera (1998) who did not find any significant differences in the protein composition of wild muscle from farmed and wild sea bream. This may be an indication of more resilience and/or better farming conditions for sea bream, a species that has been the subject of cultivation for longer time than cod: farmed Atlantic cod presents some characteristics that deviate from those considered normal in the wild fish (reviewed by Martinez, 2006) such as higher condition factor, larger liver and smaller head as well as backbone malformations in farmed specimens. Farmed cod flesh is less firm and has higher water content, lower functional properties and different muscle fibre structure than wild maturing cod (Ofstad, Kidman, Mykelbust, Olsen, & Hermansson, 1996). Similarly, farmed halibut muscle had more small fibres, larger intra- and inter-myofibrillar spaces and more abundant extracellular matrix, post mortem degradation proceeded faster and it had lower functional properties than wild halibut (Olsson et al., 2003). Differences in the living conditions between wild and farmed specimens have shown to have unexpected effects on the growth, development and protein expression of several tissues (Gornati et al., 2005; Martin et al., 2003). Protein expression and turnover in fish has been shown to be affected by the water temperature (Hall, Cole, & Johnston, 2003; Martinez, Dreyer, Agersborg, Leroux, & Boeuf, 1995; Martinez & Pettersen, 1992) and morphological studies have shown that certain farming conditions may induce lesions in the muscle (Christiansen, Martinez, Jobling, & Amin, 1992). Thus, we believe that our results may indicate both altered expression of some

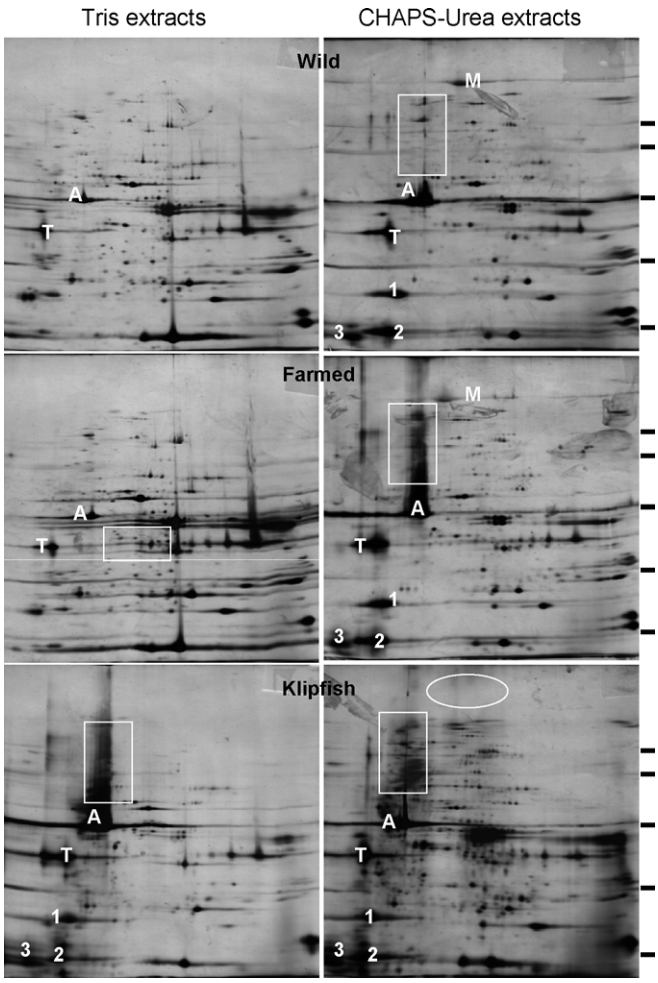

Fig. 2. 2DE analyses (Immobiline DryStrips, pH 3–10; 12.5% SDS–PAGE) of wild and farmed cod and klipfish samples, as indicated in the figure. To the left, Tris- and to the right CHAPS–urea extracts. The samples shown are pools of 5 individuals. A, actin, T, tropomyosin, M, myosin heavy chain; 1, 2 and 3 are the fast myosin light chains type 1, 2 and 3, respectively. The circle in the CHAPS–urea extract of klipfish indicates the area where the myosin heavy chain would be in an intact sample. Spots mentioned in the text, are enclosed in the squares. The black lines to the right of the upper gel indicate the position of the markers of molecular mass: 97, 66, 45, 30 and 20.1 kDa.

proteins and higher muscle protein degradation and turnover in farmed than in wild cod.

3.2. Analyses of klipfish

Fig. 1 shows the 1D SDS–PAGE analyses of the Tris (Fig. 1a) and CHAPS–urea (Fig. 1b) extracts from klipfish. The Tris extract from klipfish showed a loss of most of the proteins present in the Tris extracts from the unprocessed muscles, with the exception of the band corresponding to actin that was more prominent. The CHAPS–urea extract of these products were characterized by large smears and prominent bands in particular in the region above 50 kDa (absent in the CHAPS–urea extracts of unprocessed cod) and absence of the band corresponding to myosin heavy chain, which was notorious in the unprocessed

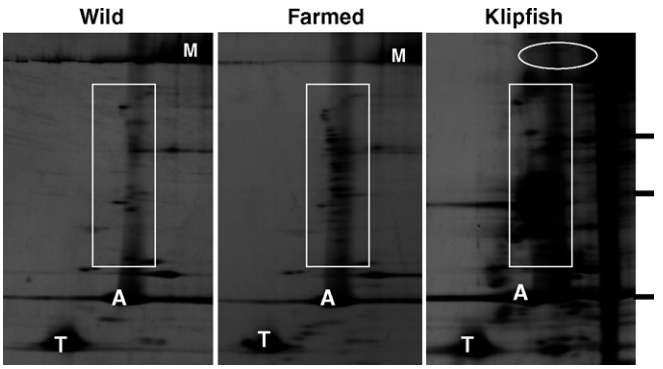

Fig. 3. 2DE analyses (Mini IEF-rods pH 3–10; 12.5% SDS–PAGE) of CHAPS–urea extracts of wild and farmed cod and klipfish samples, as indicated in the figure. Each gel corresponds to one fish. A, actin, T, tropomyosin, M, myosin heavy chain. The circle shows the area where the myosin heavy chain should have been. Spots mentioned in the text are enclosed in the squares. Only the relevant part of the gels is shown. The black lines to the right of the upper gel indicate the position of the markers of molecular mass: 97, 66, and 45 kDa.

samples. Also in the 2DE gels of klipfish most of the spots characteristic of the Tris extracts from unprocessed muscle were absent. Both the Tris and the CHAPS–urea extracts of klipfish were dominated by actin, tropomyosin and the myosin light chains (Fig. 2). Interestingly, the CHAPS–urea extracts had many more spots than their unprocessed counterparts, including those corresponding to proteins extractable only with tris in the muscle samples.

High resolution ^{1}H NMR analysis of these samples revealed the presence of dimethylamine (DMA) in all of them (Martinez et al., 2005). Formaldehyde is formed concomitantly with DMA, and formaldehyde is known to induce a toughening of the fillet in gadoids because it reacts with the myofibrillar proteins and accelerates their denaturation and aggregation rates (Ang & Hultin, 1989; Del Mazo, Huidobro, Torrejón, Tejada, & Careche, 1994; Tejada, Torrejón, Del Mazo, & Careche, 1997). In addition, drying and salting increase protein oxidation and further decrease protein extractability (Lauritzsen, 2004). Thus, proteins that in their native state were low- or high-ionic strength soluble, and that had suffered denaturation, aggregation and proteolysis due to drying, salting and storage, may have been brought back into solution by the harsher CHAPS–urea buffer, in addition to many other spots that may originate from the proteolytic degradation of higher molecular mass proteins, including myosin heavy chain, whose band at 200 kDa was absent in these samples. Some of the spots resembled those found in farmed cod (labelled with squares in Fig. 3, klipfish), but they were more abundant and presented higher intensity in klipfish than in farmed fish. Although loss of solubility and protein aggregation may have contributed to decreasing the amount of myosin extractable by CHAPS–urea, we believe that proteolysis may have a no less significant role in the origin of the mentioned the spots of molecular mass between 100 and 50 kDa (Martinez, Friis, & Careche, 2001). Current work is under way to identify the origin of these spots.

4. Conclusions

The protein patterns of wild and farmed cod seemed to indicate that farmed cod muscle had a different protein expression and/or different post mortem degradation pattern than wild cod. This may be due to stress during cultivation, to differences in post mortem muscle conditions (for example pH, that is altered by feed intake at around the time of death), and/or to qualitative and quantitative differences in the expression or regulation of proteases with a role in post mortem muscle tenderdization. In any case, it is important to identify the origin of these discriminatory spots to improve the farming conditions and obtain an optimal muscle protein composition.

As could be expected due to the heavy processing conditions, the protein pattern of klipfish was very different from that of the unprocessed muscles with the most notorious features being the loss of the myosin heavy chain band from the CHAPS–urea extracts and the loss of many protein bands from the Tris extracts that appeared in the CHAPS–urea extracts. These are the first results we have in the characterization of the effect of salting and drying on the protein composition of the product. Practical application of this work requires the inclusion of more samples manufactured under known standard and commercial conditions in order to find a relationship between the protein map, the manufacturing procedure and the quality of the final product.

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