# Two-Dimensional Electrophoretic Study of the Water-Soluble Protein Fraction in White Muscle of Gadoid Fish Species

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Eight gadoid fishes—five hakes (*Merluccius* spp.), cod (*Gadus morhua*), pollack (*Pollachius pollachius*), and blue whiting (*Micromesistius poutassou*)—were characterized in their water-soluble protein profiles by two-dimensional (2D) electrophoresis both by nondenaturing isoelectric focusing (IEF) in the 4−6.5 pH range and gradient SDS−PAGE in the 12−14% range. Species-specific parvalbumin patterns (MW < 13 kDa, p*I* < 4.6) were observed in most of the species. 2D analysis of mixed extracts revealed that *M. merluccius* had one parvalbumin (10.4 kDa, p*I* 3.9) in common with *M. australis* and *M. hubbsi*, and two parvalbumins (10.3 kDa, p*I* 4.1; 10.4 kDa, p*I* 3.9) in common with *M. capensis*, respectively. 2D electrophoresis allowed detailed characterization of both the isoelectric points and molecular weights of the major water-soluble proteins and proved to be a valuable tool for the differential characterization among closely related *Merluccius* spp.

**Keywords:** *Identification; species; 2D electrophoresis; isoelectric focusing; fish; Merluccius spp.; gadoids*

## INTRODUCTION

The identification of fish species is becoming increasingly important in the seafood industry because of the labeling regulations imposed by many countries throughout the world (Ashoor and Knox, 1985; Mermelstein, 1993). These regulations are designed to prevent the substitution of fish species of higher commercial value by others of less value (Summer and Mealy, 1983). The handling of fillets or minced fish—instead of whole specimens—as raw material is becoming more important in the manufacturing industry. This complicates the identification of the fish species, which has been traditionally based on the analysis of external anatomical and morphological features.

In Spain and in other countries gadoid fish represent an important percentage of the overall amounts of fish consumed (*FAO 1994 yearbook*, 1996; Piñeiro et al., 1997). From the commercial point of view, some members of the genus *Merluccius* are more appreciated than others as fresh fish because of their excellent organoleptic features. Among them, *Merluccius merluccius*, commonly known as European hake, is the most appreciated species and has the highest commercial value in Spain, being preferred to other species of this genus and to other gadoids. Additionally, species such as *M. capensis* have high commercial value as frozen fish with respect to other *Merluccius* spp. Nevertheless, and although the European Comission allows the commercialization of several *Merluccius* spp. under the label of "hake", the possible substitution of *M. merluccius* by species of less value must be controlled from both the organoleptic and economic points of view.

Currently, with a view to developing new strategies for the identification and characterization of fish species, powerful techniques based on biochemical methods are being put into practice (Sotelo et al., 1993). Thus, high-performance liquid chromatography (HPLC) (Ashoor and Knox, 1985; Armstrong and Leach, 1992; Osman et al., 1987; Piñeiro et al., 1997), isoelectric focusing (IEF) (Mackie, 1980; An et al., 1989; Rehbein, 1990; Sotelo et al., 1992; Plowman and Herbert, 1992), and, more recently, capillary zone electrophoresis (CZE) (Gallardo et al., 1995), and the amplification of selected DNA sequences by the polymerase chain reaction (PCR) (Rehbein et al., 1995; Quinteiro et al., 1998) have been applied to the identification of certain groups of fish species.

Until now, two-dimensional (2D) electrophoresis has not been considered for the characterization and identification of gadoid fish species, although some 2D electrophoretic methods have been applied to analyze proteins in other fish species (Martínez et al., 1992; Martínez and Christiansen, 1994; Huang et al., 1995; Gangar et al., 1996). Moreover, the 2D electrophoretic analyses reported to date have always been carried out in the presence of urea, a compound that enhances protein separation and resolution but prevents determination of the native p*I* of the proteins, thus limiting the information afforded by the technique. In this work, a 2D electrophoresis method was applied in the absence of urea, to the characterization of five *Merluccius* spp., *Gadus morhua*, *Pollachius pollachius*, and *Micromesistius poutassou*. This strategy allowed us to determine both the p*I* and MW of the major water-soluble proteins, and to perform protein characterization in the gadoid fish species studied. Additionally, we carried out a comparative analysis of the 2D electrophoretic profiles with a view to distinguishing among closely related species.

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## MATERIALS AND METHODS

**Fish Material.** Eight different gadoid fish species were employed in this study, these were: five hake species (*M. merluccius*, *M. australis*, *M. hubbsi*, *M. gayi*, and *M. capensis*), cod (*G. morhua*), pollack (*P. pollachius*), and blue whiting (*M. poutassou*). *M. merluccius*, *P. pollachius*, and *M. poutassou* were caught near the Galician coast (Northwestern Spain) in May 1997. *G. morhua* was caught in Gran Sol fishing bank in May 1997. Specimens belonging to these four species were purchased at a local market as fresh fish, frozen at our lab, and kept at −30 °C until processing. *M. australis* and *M. gayi* were caught near the coast of Chile in Autumn 1996. *M. hubbsi* and *M. capensis* were caught near the coasts of Argentina and South Africa, respectively, in Autumn 1996. The latter four *Merluccius* spp. were shipped on ice by overnight delivery, frozen at our lab and kept at −30 °C until processing. The weight of the specimens studied were in the range of 3−6 kg. The whole fish specimens were classified before freezing, according to their anatomical and morphological features. Four specimens of each fish species were used in this study. The analyses were carried out by triplicate (IEF) or by duplicate (SDS−PAGE).

**Preparation of Water-Soluble Protein Extracts.** White muscle was separated from each fish specimen and portions of 5 g were minced and homogenized with 10 mL of bidistilled water for 3 min at 9000 rpm—with interruptions of 30 s to avoid warming the samples—by means of an Ultra-Turrax homogenizer. The mixture was then spun at 4 °C for 15 min at 12500*g*. Supernatants were recovered, filtered, and maintained at −80 °C until the electrophoretic analyses were carried out. When required, mixed extracts of two species were prepared by combining equal volumes of each individual protein extract. Protein concentrations in the extracts were determined by the bicinchoninic acid (BCA) method (Pierce Laboratories, Rockford, IL).

**Isoelectric Focusing.** IEF was carried out at 15 °C in a Multiphor II electrophoresis unit (Pharmacia Biotech Europe, Uppsala, Sweden) provided with a MultiTemp III refrigerated bath circulator (Pharmacia Biotech). Precast polyacrylamide 245 × 110 × 1 mm gels (Ampholine PAGplate pH 4.0−6.5, Pharmacia Biotech) for analytical IEF were employed. The anode solution was 0.1 M glutamic acid in 0.5 M phosphoric acid, and the cathode solution was 0.1 M $\beta$-alanine. Ten to fifteen micrograms of total protein was loaded on each sample applicator paper. Run conditions were as follows: 2000 V/25 mA/25 W until at least 4000 V h were reached. First dimension IEF gels were stained with 0.1% Coomassie Blue (Merck) according to the Pharmacia-Biotech staining protocol. A protein standard in the 2.5−6.5 pH range (low p*I* standard from Pharmacia-Biotech) was included in the IEF gels. This p*I* standard comprised the following proteins (p*I* values are indicated in brackets): human carbonic anhydrase B (6.55), bovine carbonic anhydrase B (5.85), $\beta$-lactoglobulin A (5.20), soybean trypsin inhibitor (4.55), glucose oxidase (4.15), amyloglucosidase (3.50), and pepsinogen (2.80). When required, IEF strips corresponding to individual lanes were cut immediately after the runs had been completed and stored frozen at −80 °C for later analysis by SDS−PAGE.

**Two-Dimensional Analysis by Gradient SDS−PAGE.** Equilibration of IEF gel strips was carried out at room temperature as follows. The strips were placed for 10 min in sample buffer (Laemmli, 1970) containing 0.75% dithiothreitol and then for another 10 min in sample buffer containing 4.5% iodoacetamide. Once equilibrated, the strips were subjected to 2D analysis by gradient SDS−PAGE at 15 °C in the Multiphor II electrophoresis system (Pharmacia Biotech). Precast polyacrylamide 245 × 180 × 0.5 mm gradient gels (ExcelGel XL SDS 12−14) for horizontal electrophoresis were employed. Anode and cathode buffer strips (ExcelGel SDS Buffer Strips, Pharmacia Biotech) were employed. Run conditions were as follows: 1000 V/40 mA/40 W, for 165 min. Once the bromophenol blue had reached the anode, the gels were fixed and stained by a standard silver staining protocol (Pharmacia Biotech). A low molecular weight protein standard (14−67 kDa) from Pharmacia Biotech was employed as reference. This comprised the following proteins (MW values are indicated in brackets): phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

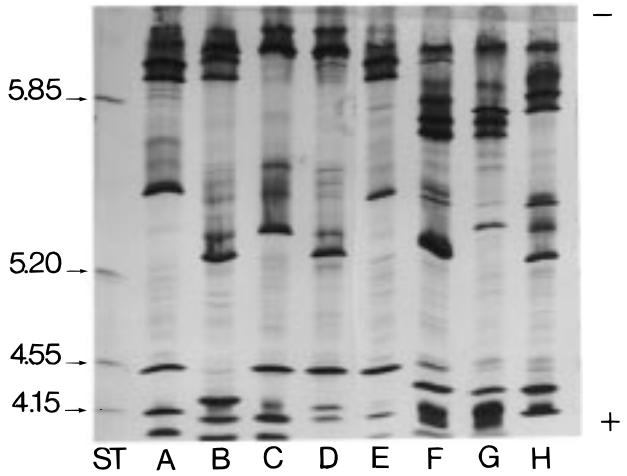


**Figure 1.** Isoelectric focusing (4−6.5 pH range) of the water-soluble proteins in the eight gadoid fish species studied: (A) *M. merluccius*, (B) *M. australis*, (C) *M. hubbsi*, (D) *M. gayi*, (E) *M. capensis*, (F) *P. pollachius*, (G) *M. poutassou*, (H) *G. morhua*; (ST) isoelectric focusing standard. The cathodic (−) and anodic (+) sides are indicated.

**Data Processing.** The mobility of the bands obtained with both IEF and SDS−PAGE was determined using the Whole Band Analyzer Software (BioImage Systems Corp., MI) in a Sun SPARCstation 5 (Sun Microsystems Inc.) equipped with a Scanmaster 3+ device (Howtek Inc., NH), and the corresponding p*I* and MW were calculated by comparison with the IEF and SDS−PAGE protein standards.

## RESULTS AND DISCUSSION

**IEF Characterization of the Protein Profiles.** The first goal of this work was to characterize the major water-soluble proteins of the selected gadoid fish species by IEF. Preliminary work aimed at elucidating the optimal pH range for IEF analysis was carried out at our laboratory, and comprised the analysis of the water-soluble proteins from the eight gadoid fish species in both the 3.5−9.5 (broad) and 4−6.5 (narrow) pH ranges. The results obtained clearly indicated that the most significant differences among species were observed in the 4−6.5 pH interval (data not shown). These results agree with those of other authors, who found that an acidic pH range resolved more characteristic protein patterns than other pH ranges in several gadoid fish species (Bhushana Rao et al., 1969; Rehbein and Kündiger, 1984; Girija and Rehbein, 1988). Accordingly, the 3.5−9.5 pH range was not further considered in this study.

As seen in Figure 1, all the fish species analyzed by IEF showed characteristic protein patterns. The most significant differences among species were observed on the anodic side of the gel, where the acidic proteins with p*I* < 4.6 yielded species-specific profiles in most species tested (Figure 1). These proteins proved to be heat-resistant (80 °C for 20 min), a well-known feature of parvalbumins (Capony and Pechère, 1973; Closset, 1976; Closset and Gerday, 1976; Keenan and Shaklee, 1985), while the rest of the proteins aggregated and precipitated after heat treatment (data not shown). The number of major parvalbumins observed in the species tested varied significantly, from two in *G. morhua* to

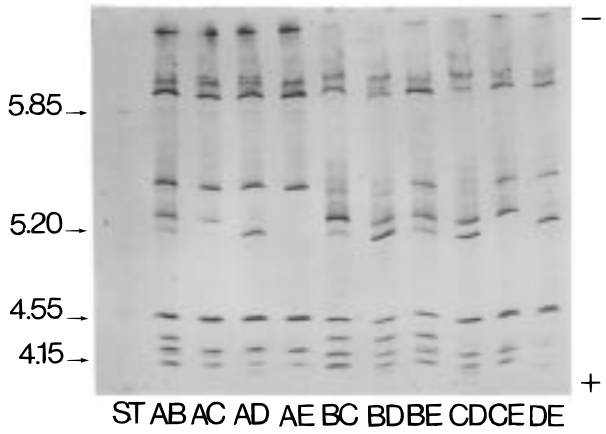


**Figure 2.** Isoelectric focusing (4−6.5 pH range) of the water-soluble proteins in combined extracts of pairs of *Merluccius* spp.: (AB) *M. merluccius* + *M. australis*, (AC) *M. merluccius* + *M. hubbsi*, (AD) *M. merluccius* + *M. gayi*, (AE) *M. merluccius* + *M. capensis*, (BC) *M. australis* + *M. hubbsi*, (BD) *M. australis* + *M. gayi*, (BE) *M. australis* + *M. capensis*, (CD) *M. hubbsi* + *M. gayi*, (CE) *M. hubbsi* + *M. capensis*, (DE) *M. gayi* + *M. capensis*, (ST) isoelectric focusing standard. The cathodic (−) and anodic (+) sides are indicated.

five in *M. poutassou* and *P. pollachius* (Figure 1). A major parvalbumin with a p*I* of 4.52 was detected in *M. merluccius*, *M. hubbsi*, *M. gayi*, and *M. capensis* but not in *M. australis*, *G. morhua*, *P. pollachius*, or *M. poutassou*. Nevertheless, IEF alone did not reveal whether these parvalbumins with the same p*I* were identical or not, especially in the case of closely related species such as *M. merluccius* and *M. capensis*.

The cathodic region of the IEF gels—that is the 6.0−6.5 pH range—revealed the presence of several major protein bands in all species tested, especially in those belonging to the genus *Merluccius*. The number of protein bands in this pH interval was lower in *G. morhua*, *P. pollachius*, and *M. poutassou* than in the *Merluccius* spp. tested.

Valuable information was obtained in the 5.2−5.7 pH range, where at least one specific major protein was observed in all the species. A major protein with a p*I* of approximately 5.5 was detected in the European hake (*M. merluccius*) and a protein with apparently identical p*I* was also observed in *M. capensis*. Thus, the IEF protein profile in this pH region was apparently identical in *M. merluccius* and *M. capensis*. By contrast, the major proteins of the other species in this pH range had p*I* values below or above 5.5 (Figure 1).

The level of similarity among some of the proteins from the *Merluccius* spp. studied was further explored by IEF separation of binary mixtures of water-soluble protein extracts. Analysis of these mixtures would allow than to be distinguished among proteins from different species with similar but not identical p*I* values. Figure 2 shows the IEF profiles obtained for the mixed protein extracts of pairs of species. The results obtained confirmed that some of the parvalbumins with close p*I* values were species-specific. By contrast, the parvalbumin with a p*I* value of 4.52 seemed to be identical in *M. merluccius*, *M. hubbsi*, or *M. gayi*, since the binary mixtures prepared from these species did not yield two protein bands at this pH value. Moreover, the mixture of *M. merluccius* and *M. capensis* yielded only three parvalbumins (lane AE), this suggesting that these three parvalbumins were apparently identical in both species. On the other hand, at least two different major proteins within the 5.2−5.7 pH interval were observed in the mixed extracts from pairs of species, except for the mixture of *M. merluccius* and *M. capensis*, in which a single protein band with a p*I* value of 5.5 was observed.

As stated above, the main goal of this work was to accomplish the identification of several gadoid fish species of commercial interest by electrophoretic techniques in the absence of urea. As described above, and as can be observed in Figures 1 and 2, IEF analysis of combined protein extracts from pairs of species significantly increased the discriminatory power of this technique and showed that certain proteins from different species were not identical. Nevertheless, although this strategy proved to be a valuable approach, certain parvalbumins of different species were seen to have equal p*I* values and migrated as single protein bands even in the mixed protein extracts analyzed by IEF, indicating that both proteins were apparently identical. This was especially significant in the cases of *M. merluccius* and *M. capensis*, a pair of closely related species that could not be distinguished by IEF alone. This prompted us to develop a 2D strategy by gradient SDS−PAGE, in which the information about the p*I* values of the major water-soluble proteins was not altered and in which the resolution of apparently equivalent protein bands was improved.

**Two-Dimensional Electrophoretic Analysis of Water-Soluble Proteins.** As mentioned above, the fact that the one dimensional analysis by IEF and further equilibration steps were carried out in the absence of urea implied that the two-dimensional analysis of the IEF strips by gradient SDS−PAGE would afford both the p*I* and MW of each individual protein. Besides, IEF allowed the identification of most of the fish species studied, but seemed to fail in the differentiation of two closely related species as in the case of *M. merluccius* and *M. capensis*, a pair of species which displayed apparently identical IEF profiles, either in the individual (Figure 1) and combined protein extracts (Figure 2). Additionally, apparently identical protein bands from different species, as determined by IEF, would be further investigated by 2D electrophoresis, with the aim of confirming whether these bands were identical or not.

To achieve characteristic 2D protein profiles, two types of two-dimensional analyses were considered: (i) individual analysis of each species (Figure 3); and (ii) analysis of selected pairs of species (Figure 4). This double strategy was carried out not only to obtain the characteristic 2D protein profiles of each species, but also to elucidate whether apparently equivalent proteins, as determined by IEF, were identical or not among closely related species. In this part of the study, the European hake was considered as the reference species for comparison with all the other *Merluccius* spp. studied.

As seen in Figure 3A, *M. merluccius* showed a characteristic 2D electrophoretic pattern that allowed its identification with respect to other *Merluccius* spp. and the other gadoid fish species studied. The cathodic (left) side of the 2D pattern of *M. merluccius*, corresponding to p*I* values above 6.0, was characterized by the presence of two proteins with a p*I* value of 6.5 and with MW of 63 and 50 kDa, respectively (Figure 3A, proteins **a** and **b**). Both proteins were also observed in *M. capensis* (Figure 3E, proteins **a** and **b**), but not in

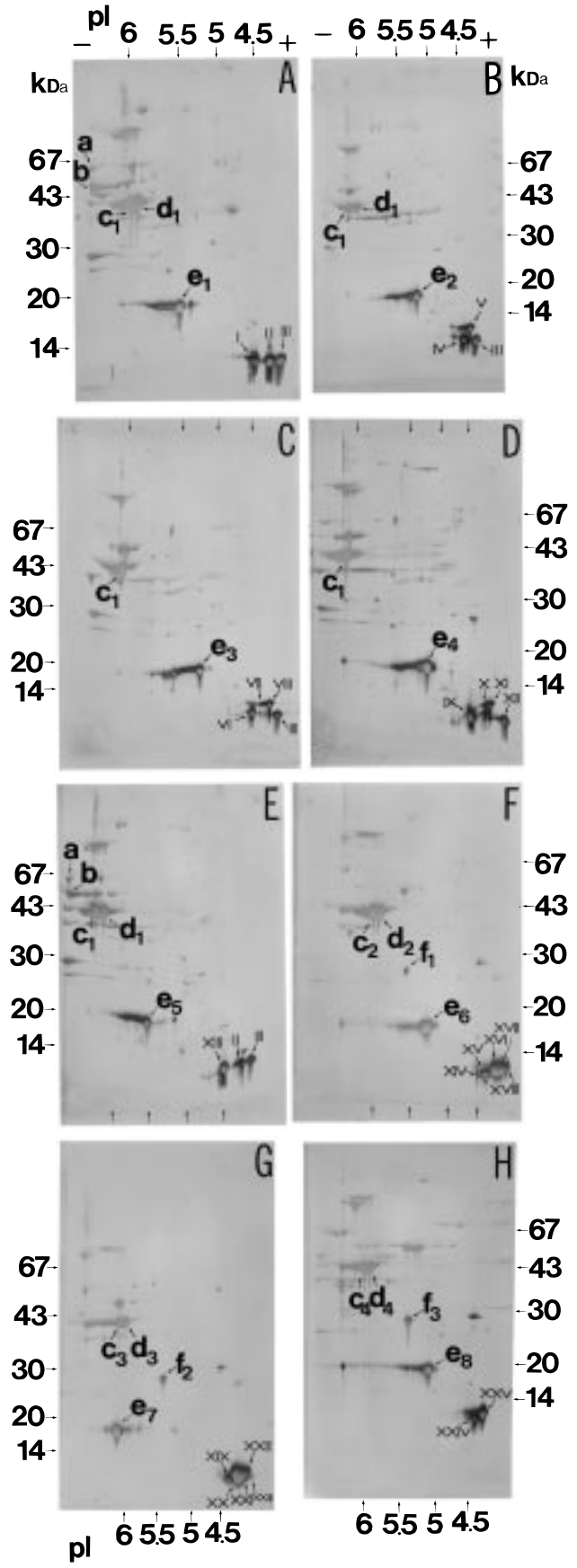


**Figure 3.** Two-dimensional electrophoretic analysis of the water-soluble protein fraction in the eight gadoid fish species tested, labeled as in Figure 1. Characteristic major proteins are indicated with black arrows. The cathodic (−) and anodic (+) sides are indicated.

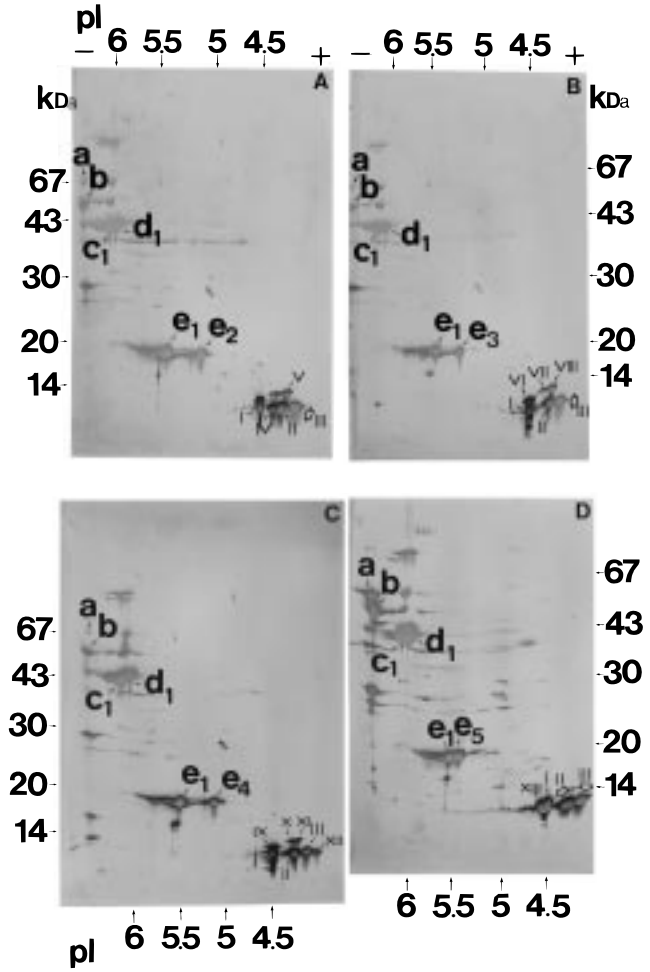


**Figure 4.** Two-dimensional electrophoretic analysis of the water-soluble protein fraction in combined extracts of European hake and other *Merluccius* spp.: (A) *M. merluccius* + *M. australis*, (B) *M. merluccius* + *M. hubbsi*, (C) *M. merluccius* + *M. gayi*, (D) *M. merluccius* + *M. capensis*. White arrows indicate apparently identical parvalbumins in each mixture of hakes studied. The cathodic (−) and anodic (+) sides are indicated.

any of the other species studied. Additionally, two major proteins of moderately high p*I* values (6.10 and 5.99, respectively) and an average MW of 42.5 kDa were present in *M. merluccius* (Figure 3A, proteins $\mathbf{c_1}$ and $\mathbf{d_1}$), *M. australis* (Figure 3B, proteins $\mathbf{c_1}$ and $\mathbf{d_1}$), and *M. capensis* (Figure 3C, proteins $\mathbf{c_1}$ and $\mathbf{d_1}$), while this pattern was not observed in any of the other *Merluccius* spp. tested (Figure 3, part C and D). Thus, *M. hubbsi* (Figure 3C) and *M. gayi* (Figure 3D) displayed a single major protein with a p*I* value of 6.10 and a MW of 42.5 kDa although this protein (represented as protein $\mathbf{c_1}$) appeared to be identical to its counterpart (p*I* 6.10, 42.5 kDa) present in European hake, as deduced from the 2D analysis of the mixed extracts obtained by combining *M. merluccius* with either *M. hubbsi* or *M. gayi*, respectively (Figure 4, parts A and B). By contrast, *P. pollachius* (Figure 3F), *M. poutassou* (Figure 3G), and *G. morhua* (Figure 3H) displayed a pair of major proteins of 42.5 kDa, represented as proteins $\mathbf{c_{2-4}}$ and $\mathbf{d_{2-4}}$, but their p*I* values were in the 5.73−5.92 pH interval, this being significantly below the p*I* values determined for $\mathbf{c_1}$ and $\mathbf{d_1}$.

The central region of the 2D electrophoretic patterns displayed a major protein slightly below 20 kDa, with species-specific pI values lying in the 5.2−5.7 pH range

**Table 1. Parvalbumins Present in the Five *Merluccius* spp. Studied and Their p*I* Values[a]**

| | p*I* value | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| fish species | I | II | III | IV | V | VI | VII | VIII | IX | X | XI | XII | XIII |
| *M. merluccius* | 4.5 | **4.1** | **3.9** | | | | | | | | | | |
| *M. australis* | | | **3.9** | 4.2 | 4.1 | | | | | | | | |
| *M. hubbsi* | | | **3.9** | | | 4.5 | 4.2 | 4.1 | | | | | |
| *M. gayi* | | | | | | | | | 4.5 | 4.2 | 4.1 | 3.6 | |
| *M. capensis* | | **4.1** | **3.9** | | | | | | | | | | 4.5 |

[a] The parvalbumins that are present in more than one species appear in bold characters.

**Table 2. Parvalbumins Present in *P. pollachius, M. poutassou,* and *G. morhua* and Their p*I* Values with Respect to *M. merluccius***

| | p*I* value | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| fish species | I | II | III | XIV | XV | XVI | XVII | XVIII | XIX | XX | XXI | XXII | XXIII | XXIV | XXV |
| *M. merluccius* | 4.5 | 4.1 | 3.9 | | | | | | | | | | | | |
| *P. pollachius* | | | | 4.6 | 4.4 | 4.2 | 4.0 | 3.9 | | | | | | | |
| *Micr. poutassou* | | | | | | | | | 4.6 | 4.3 | 4.2 | 4.0 | 3.9 | | |
| *G. morhua* | | | | | | | | | | | | | | 4.3 | 4.2 |

(Figure 3, protein $\mathbf{e_{1-8}}$). Thus, both *M. merluccius* and its closely related species *M. capensis* displayed a major protein with an isoelectric point of 5.5, and average molecular weights of 17.5 kDa (Figure 3, parts A and E). Nevertheless, analysis by 2D electrophoresis of a combined extract of both species revealed that these two proteins did not migrate as a single protein spot, but rather as two partially overlapping proteins, indicating that they were not identical (Figure 4D). Thus, the analysis of the mixed extract of *M. merluccius* and *M. capensis* by 2D electrophoresis allowed us to elucidate that proteins $\mathbf{e_1}$ and $\mathbf{e_5}$ were not identical. In consequence, such a small difference between proteins $\mathbf{e_1}$ and $\mathbf{e_5}$ was successfully resolved by 2D electrophoresis (Figure 4D).

Likewise, individually *M. australis, M. hubbsi,* and *M. gayi* displayed major proteins of about 18 kDa in the 5.2−5.7 pH range, but their p*I* values were below or above 5.5 (Figure 3, parts B−D; proteins $\mathbf{e_{2-4}}$). Analysis of mixed protein extracts allowed us to confirm that in all cases the characteristic protein band of 17.5 kDa and the isoelectric point of approximately 5.5 of *M. merluccius* was unique and that this protein was not identical to any of those present in the other species tested (Figure 4). This characteristic allowed unambiguous identification of European hake with respect the other gadoid fish species studied, even in the case of closely related *Merluccius* spp. The other gadoid fish species tested—*G. morhua, P. pollachius,* and *M. poutassou*—displayed a major protein band of about 18 kDa in the 5.2−5.7 pH range (Figure 3, parts F−H; proteins $\mathbf{e_{6-8}}$) but the relative position of this band was also quite different with respect to that characterized for the European hake (Figure 3A). Additionally, a second major protein of about 30 kDa in the 5.39−5.52 pH range was observed in these three gadoids (Figure 3, parts F−H; proteins $\mathbf{f_{1-3}}$) but not in the five *Merluccius* spp. tested, this proving to be a remarkable characteristic feature in the 2D protein profiles of the above three gadoid fish species studied with respect to the five *Merluccius* spp. studied.

The relevance of the parvalbumin patterns in the identification of frozen fish species has been previously reported by other authors (Bhushana Rao et al., 1969; Girija and Rehbein, 1988). Indeed, parvalbumins from frozen cod and other frozen gadoid fish species had been studied by these authors by IEF (Girija and Rehbein, 1988) and other biochemical techniques. Besides, Owusu-Ansah and Hultin (1992) found that the sarcoplasmic proteins of red hake muscle were less susceptible to insolubilization during frozen storage than contractile proteins. Moreover, these authors reported that the water-soluble proteins with MW < 28 kDa suffered insolubilization to a lesser extent. These results agree with Sotelo et al. (1994), who did not find any significant loss of solubility of the sarcoplasmic proteins of European hake with storage time at −5, −12, and −20 °C. Other works carried out at our lab confirmed that the parvalbumins of several gadoid fish species did not suffer significant changes during 12 months of frozen storage (unpublished data).

As a consequence of this, special interest was focused on the 2D electrophoretic analysis of the parvalbumin fraction. This technique afforded species-specific protein patterns in all the frozen fish species tested, even in the cases of *M. merluccius* and *M. capensis*. The small size (below 13 kDa) of this heat-resistant acidic protein fraction was confirmed at this point by 2D analysis, which confirmed the parvalbumin nature of this group of proteins (Figure 3, proteins **I** to **XXV**). Although some differences had been observed in the parvalbumin profiles with IEF, analysis of both the individual (Figure 3) and mixed (Figure 4) extracts by 2D electrophoresis allowed us to accomplish a differential characterization of all the species tested based on their parvalbumin patterns. The three major parvalbumins of European hake muscle were in the 10−11 kDa range (Figure 3A: proteins **I** to **III**), *M. capensis* showing a similar parvalbumin pattern to that of European hake (Figure 3E, proteins **XIII**, **II**, and **III**). Despite this, four different proteins were observed in the mixed extract of *M. merluccius* and *M. capensis*, revealing that the parvalbumins with isoelectric points of 4.5 were not identical in both species (Figure 4D). Analysis of mixed extracts also allowed us to conclude that *M. australis* and *M. hubbsi* had one parvalbumin in common with *M. merluccius*, respectively; this was the parvalbumin with the lowest isoelectric point (p*I* 3.9), which migrated as a single protein spot in the 2D electrophoresis gel corresponding to mixed extracts obtained by combination of pairs of these species (Figure 4, parts A and B).

On the other hand, *M. gayi* displayed a characteristic parvalbumin profile, with a central protein of higher molecular weight (12−13 kDa). Up to six different protein spots, corresponding to six different

parvalbumins, were observed upon 2D analysis of the mixed extracts of *M. merluccius* with *M. gayi*, indicating that neither species had any parvalbumin in common (Figure 4C). The parvalbumin profiles of *G. morhua*, *P. pollachius*, and *M. poutassou* were significantly different from those observed in the six species belonging to the genus *Merluccius*. Table 1 (*Merluccius* spp.) and Table 2 (other gadoids) summarize all the different parvalbumins described in this work and show their corresponding p*I* values.

Briefly, the results obtained in the 2D analysis allowed the classification of the species studied in three groups: (i) *M. merluccius* and *M. capensis*; (ii) *M. australis*, *M. hubbsi* and *M. gayi*; and (iii) *G. morhua*, *P. pollachius*, and *M. poutassou*.

## CONCLUSIONS

The results obtained by IEF and 2D electrophoresis demonstrated that the gadoids tested displayed species-specific protein patterns. The species of the genus *Merluccius* studied were clearly distinguished from one another and from other gadoid species not belonging to this genus. This was achieved by comparing, respectively, both the 2D electrophoretic parvalbumin profiles and the presence in *G. morhua*, *P. pollachius*, and *M. poutassou* of a major protein of about 30 kDa, this protein being absent in the *Merluccius* spp. tested.

A characteristic 2D parvalbumin profile with three major proteins was observed in *M. merluccius* although this species had one parvalbumin in common with *M. australis* and *M. hubbsi* and two parvalbumins in common with *M. capensis*, respectively. Additionally, a specific major protein of p*I* 5.5 and MW 17.5 kDa was observed in *M. merluccius* (protein $\mathbf{e_1}$), this being distinguished from that of *M. capensis* (protein $\mathbf{e_5}$, with similar p*I* an MW) by 2D analysis of a mixed extract of both species. Thus, 2D electrophoresis allowed us the identification of closely related species—as in the case of *M. merluccius* and *M. capensis*—showing apparently identical IEF protein patterns.

As stated above, the analysis of the 2D electrophoretic protein profiles of gadoid fish species proved to be a valuable tool in the discrimination between closely related frozen species in which IEF alone was not determinant. The fact that the parvalbumin fraction was species-specific in all cases may be of interest for the identification of fish species in heat treated fish. Additionally, 2D electrophoresis in the absence of urea allowed us to characterize both the p*I* and MW of the water-soluble proteins in the frozen gadoids studied.

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