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Substitution of fish species detected by thin-layer isoelectric focusing and a computer-assisted method for the evaluation of gels

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Abstract

Fourteen fish species susceptible to substitution were analysed by the thin-layer isoelectric focusing technique on polyacrylamide gels of pH 3.5–9.5. Four fish per species were run on the same gel to verify the possibility to differentiate them according to their protein banding patterns. The occurrence of intraspecific differences due either to electrophoretic variations or to protein polymorphism was also observed. In fact, some of them showed few dissimilarities among their protein profiles. However, the differentiation was possible for all species, even for those belonging to the same order, family and genus. Computer-based tools combined with statistical analysis were implemented and usefully applied to avoid a subjective evaluation of the isoelectric focusing gels, and to verify the reliability of the preliminary visual comparison of protein patterns.

Keywords: Fish; Food analysis; Cluster analysis; Chemometrics; Isoelectric focusing

1. Introduction

In recent years, fish and seafood consumption in Italy has been constantly increasing, as reported by the national statistical reports [1]. The great demand for important fish species may often determine substitution by lower value ones and mislabeling also occurs quite frequently [2]. Thus, an accurate fish identification plays a fundamental role in the veterinary inspection praxis. Isoelectric focusing (IEF) methods are widely used for the separation of sarcoplasmic proteins, peptides and enzymes, which are applied to a polyacrylamide gel or agarose gel and separated into distinctive banding patterns with a variety of experimental conditions and materials [3]. During the last 20 years a large number of studies have been reported in the field of fish species identification using IEF [4–17].

In these studies the isoelectric focusing techniques proved to be fast and reliable. IEF can be usefully applied not only to raw fish, but also to cooked fish [18,19], smoked fish products [20], canned fish [21], crabs [22,23] and sturgeon's eggs, or caviar [24,25].

The aim of the present work was both to verify the possibility to differentiate those fish species susceptible to the fraud of substitution and to show that, in spite of their protein profile variability due to protein polymorphism or electrophoretic variations (i.e. gel distortions), it was possible to differentiate them

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comparing the banding patterns of the fish species along the gel's pH gradient. The protein patterns of 14 different fish species were compared by the thin-layer isoelectric focusing technique on pH 3.5–9.5 polyacrylamide gels (PAGs). Different categories of fish were chosen both to test the reliability of the method and to give a panorama of some fraudulent fish substitutions that often occur in Italy. A specific statistical method for the objective evaluation of protein bands was studied and implemented. This procedure was applied to avoid the simple visual comparison of the protein patterns and to check the correct grouping of fishes, according to the species they belong to, despite their evident intraspecific variability.

2. Experimental

2.1. Materials

The fish used in the present work were collected from the Wholesale Fish Market of Milan and identified by their external characters. Each species is listed in Table 1. The nomenclature of fish follows the specifications given by the Italian Ministry of Mercantile Marine [26]. The commercial denominations of fish were translated into English according to the Multilingual Dictionary of Fish and Fish Products [27].

Two fish species, four fish each, were analysed in a single gel along with the p*I* standards; the more important species was placed on the left half of the gel and it was compared with the lower value one. Five grams of light muscle were minced with a knife and homogenised with 5 ml of distilled water by means of an Ultra-Turrax (Jankle & Kunkle). The total mixing time was 50 s at 9500 rev./min. The homogenate was then transferred into 1.5-ml plastic centrifuge tubes and centrifuged for 3 min at 14 737×*g* (13 000 rev./min) in a table centrifuge (Microcentrifugette 4214-ALC, Milan, Italy) at room temperature. The supernatant was kept in the refrigerator until used for IEF (no longer than 24 h).

2.2. Isoelectric focusing

Isoelectric focusing was carried out on thin layer gels according to the AOAC method [28], although minor modifications were applied, using Multiphor-II, Multi-Temp II, Multi-Drive XL electrophoretic system (Amersham Pharmacia Biotech, Uppsala, Sweden). Precast gels (Ampholine PAG plates pH 3.5–9.5) along with broad p*I* Markers calibration kit (Amersham Pharmacia Biotech) were used [29]. Fifteen μl of p*I* standard and 5 μl of sample extract were applied either on plastic or filter paper ap-

Table 1
List of fish species[a]

No.	Species	Order	Family	Wholesale price (È/kg)
1	European seabass (*Dicentrarchus labrax*)	Perciformes	Serranidae	9.30[b]–23.24
2	Spotted seabass (*Dicentrarchus punctatus*)	Perciformes	Serranidae	6.71
3	Common pandora (*Pagellus erythrinus*)	Perciformes	Sparidae	9.30
4	Red pandora (*Pagellus bellottii bellottii*)	Perciformes	Sparidae	6.20
5	Striped Red mullet (*Mullus surmuletus*)	Perciformes	Mullidae	9.30
6	Red mullet (*Mullus barbatus*)	Perciformes	Mullidae	5.16
7	European anchovis (*Engraulis encrasicolus*)	Clupeiformes	Engraulidae	4.65
8	European pilchard (*Sardina pilchardus*)	Clupeiformes	Clupeidae	1.55
9	Chinook (King salmon) (*Oncorhynchus tschawitscha*)	Clupeiformes	Salmonidae	7.75[c]
10	Atlantic salmon (*Salmo salar*)	Clupeiformes	Salmonidae	6.20[b]
11	Common sole (*Solea vulgaris*)	Pleuronectiformes	Soleidae	9.30
12	European flounder (*Platychtis flesus flesus*)	Pleuronectiformes	Pleuronectidae	4.65
13	Smoothhound (*Mustelus mustelus*)	Squaliformes	Triakidae	4.13
14	Picked dogfish (*Squalus acanthias*)	Squaliformes	Squalidae	3.10

[a] Source: Wholesale Fish Market of Milan.

[b] Reared.

[c] Frozen.

Table 2
Isoelectric focusing experimental conditions[a]

Run	V	mA	W	Time (min)
Prefocusing	200	25	25	15
Phase II	2000	25	10	15
Phase III	2000	25	15	75

[a] Precast Ampholine PAG gels pH 3.5–9.5. Broad p*I* markers 3.5–9.5. Fish tissue dilution: 1:1 (5 g/5 ml H_2O). Cooling temperature: 16°C. Anolyte solution: 0.04 *M* D,L-aspartic acid. Catholyte solution: 0.1 *M* NaOH. Sample application: 5 μl. p*I* standard application: 15 μl.

plicators. The electrical parameters of the IEF run are reported in Table 2.

After the run the gel was transferred to the fixing solution for 45 min, then washed for 5 min in a destaining solution. The fixed and washed gel was put into a freshly made Coomassie blue R350 staining solution for 15 min, then destained in destaining solution until the background was sufficiently clear (approx. three washes in ca. 20 min) [30]. Each gel was then covered with polyester film and scanned at 600 ppi with a flatbed scanner (SNAP*SCAN 600*, Agfa-Gevaert, Brussels, Belgium). After 10–12 h the polyester film layer was removed and replaced with a clean one. This procedure was repeated 2–3 times until the background was almost transparent [31]. Finally, the gel was scanned again and the image was stored in the computer for future use.

2.3. Gel analysis

The measurement of the isoelectric points of each sample was performed by means of the SIGMA-GEL Software (SPSS Science, Chicago, IL, USA).

The first step of the gel analysis was to create a standard calibration curve according to the p*I* standard (Broad p*I* Standard, Amersham Pharmacia Biotech).

The sample protein bands, whose optical densities were lower than a pre-defined threshold, were not considered for the statistical analysis.

The p*I* values were then imported in the SPSS software (SPSS Science) and elaborated by making a contingency table to show the presence and the number of bands at their corresponding isoelectric points in the gel pH gradient.

A cluster analysis (SPSS proximities procedure), carried out according to the average linkage between groups method based on squared euclidean distance, was applied to the data in order to produce a graphic output (dendrogram) which could group together the lanes belonging to a certain species.

3. Results and discussion

Fig. 1 shows the seven gels where the 14 species were analysed. Table 1 shows the wholesale market quotation of the examined fish species. Some species have a remarkably different wholesale price, although for sample 9 (King Salmon, *Oncorhynchus tschawitscha*) and sample 10 (Atlantic Salmon, *Salmo salar*), the price comparison is questionable because they arrive at the wholesale fish market of Milan either wild or reared, and in a different commercial form: frozen and fresh, respectively.

In the graphical elaboration of the contingency table, the light-grey colored boxes show the bands disposition along the pH gradient, giving a representation of each sample protein profile (see Fig. 2). Little differences among samples belonging to the same species, most likely due to the above mentioned electrophoretic variations or protein polymorphism, are visible (e.g. sample 1, lanes 3 and 4; sample 8, lane 5; sample 9, lane 4 and sample 12, lane 7).

Even if some species showed an evident intraspecific polymorphism (i.e. sample 4, lane 7 and sample 11, lane 3) or some discrepancies most likely due to unpredictable band distortions, it was therefore possible the differentiation between the compared species, as proved by the SPSS proximities procedure. The graphic output of the cluster analysis (dendrogram) shows the grouping of the species and the dissimilarities among fish as well (see Fig. 3). The vertical lines define the groupings, and the horizontal lines define the distances both among clusters and elements (lane or fish): the greater the difference among them (in this case fish species or single lanes), the longer the line is and vice versa. The difference in length among elements shows the above mentioned intraspecific variability, while the difference in length among clusters shows the interspecific differences. However, the most relevant fact

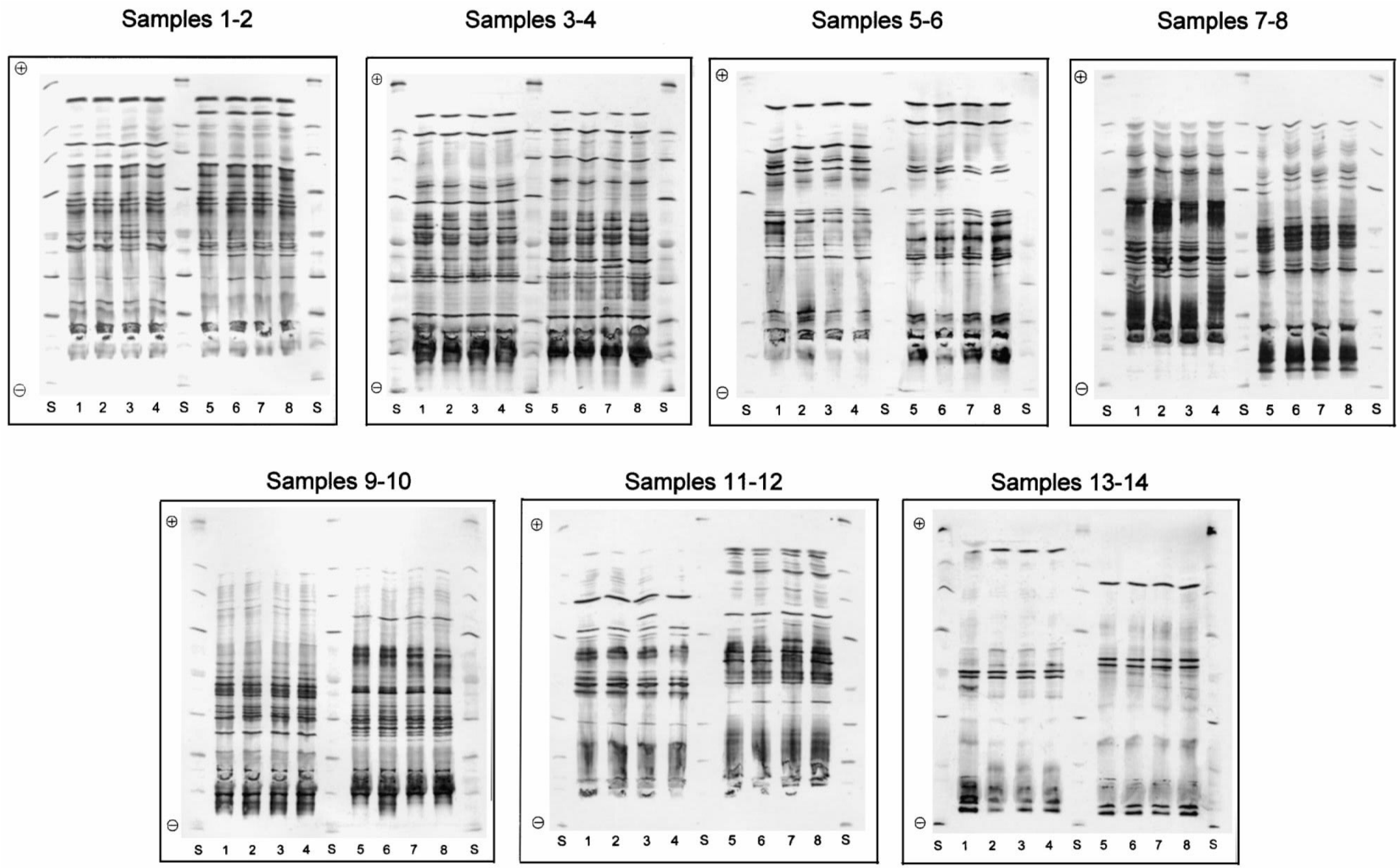

Fig. 1. Protein patterns of fish samples (S, p*I* Standard; 1–4, important fish species; 5–8, lower value fish species).

Samples 1-2

pI	1	2	3	4		5	6	7	8
3,9	1	1	1	1		1	1	1	
4,0									1
4,2						1	1	1	
4,3									1
4,9	1	1							
5,0			1	1					
5,4	1	1	1	1		1	1	1	1
5,5	1	1							
5,6			1	1		1	1	1	1
5,7								1	
5,8	1	1				1	1	1	1
5,9		1	1	1		1	1		1
6,0	2	1	1	1		1	1	2	1
6,1	1	1	1	1		1	1		1
6,2			1	1					
6,4			1						
6,5	1	1	1						
6,6			1	1		1			
6,7		1					1	2	1
6,8	1	1	1	2		2	1		1
6,9	1		1				1	1	1
7,0						1	1		
7,3								1	1
7,4	1	1	1	1					
7,5							1		
7,9	1	1	1	1					

Samples 3-4

pI	1	2	3	4		5	6	7	8
4,1						1		1	1
4,2	1	1	1	1			1		
4,5						1			
4,6	1	1	1	1			1	1	1
5,1						1			
5,2							1	1	1
5,5						1			
5,6							1	1	1
5,7	1	1	1	1					
5,9	1		1	1					
6,0		1							
6,1	1	1	1	1					
6,2	1	1	1	1		1	1	1	1
6,3	1	1	1	1		1	1	1	1
6,4	1	1				1		1	
6,5	1		2	2		1	2	2	3
6,6		1							
6,9	1	1	1	1		1	1	1	1
7,0								1	
7,1	1	1	1	1					
7,2	2	1	1	2		1		1	1
7,3	1	1	2	1			1		
7,4		1							
8,0	1							1	
8,1		1	1	1		1	1		1

Samples 5-6

pI	1	2	3	4		5	6	7	8
4,1			1			1	1	1	1
4,2	1	1		1					
4,5						1	1	1	1
5,1		1	1	1					
5,2	1								
5,3			1						
5,4	1	1		1		1	1	1	1
5,5	1	1	1	1		1	1	1	1
6,1	1	2	2	2		2	2	2	1
6,2	1	1	1	1		1			1
6,3	1						1	1	1
6,5						1	1	1	1
6,6	1	1	1	1					
6,7						1	1	1	1
6,8	1	1	1	1		1	1	1	1
6,9						1	1	1	
7,0									1
7,9	1	1	1	1		1			
8,0							1	1	1

Samples 7-8

pI	1	2	3	4		5	6	7	8
4,5	1	1	1			1	1	1	1
4,6				1					
4,7						1			
4,8							1	1	1
5,3						1	1	1	1
5,5	1					2	1	1	1
5,6		1	1	1			1		
5,7	1	1	1	1					
5,8	1	1	1						
5,9				1					
6,0						1	1	1	1
6,1						1	1		
6,3	1	1	1	1					
6,4	1	1	1	1					
6,5	1	1	1	1					
6,6	1		1						
6,7	2	2	1	1		1	1	1	1
6,8		1	1	2		1	2	1	1
6,9									1
7,0	1	1	1			1	1	1	
7,1	2	1	1	2		1	1	1	1
7,2	1	1	1	1		1	1	1	1
7,3						1	1	1	1
7,6						1			1
7,7						1			1
7,9						1			
8,0						1			
8,9	1	1	1	1					
9,0		1							
9,1	1		1	1					

Samples 9-10

pI	1	2	3	4		5	6	7	8
5,6						1		1	
5,7							1		1
6,2						1	1	1	1
6,3						2	1	1	1
6,4							1	1	1
6,6	1	1	1						
6,7				1					
6,8	1	1	1	1					
6,9						1	1	1	1
7,0	1	1	1	1					
7,2	1	1	1	1		1	1	1	1
7,3	1	1	1			2	2	2	2
7,4	1	1	1	2		1	1	1	1
7,5						2	2	2	2
7,6						1	1	1	1
7,7	1	1	1			1	1	1	1
7,8				1		1	1	1	1
8,7	1		1	1		1	1	1	1

Samples 11-12

pI	1	2	3	4		5	6	7	8
4,1						1	1	1	1
4,2						1	1	1	
4,3						1	1	1	2
4,4						1	1	1	
4,5						1	1	1	1
4,6						1	1	1	1
4,7									1
5,2	1	1	2	1					
5,5			1			1	1	1	1
5,7			1						
5,8	1	1		1					
5,9	1	2	1	1				1	
6,0	1		1						
6,1						1	1	2	1
6,2	1	1	1	1		1	1	1	1
6,3	1	1	1	1		1	1	1	
6,4						1	1	1	2
6,5	1	1	1			1	1	1	1
6,6	1			1					
6,7		1	1	1					
6,8	1	1	1	1		1	1		1
6,9	1	1	1					1	
7,0				1					
7,4	1								
7,5		1	1	1					

Samples 13-14

pI	1	2	3	4		5	6	7	8
4,1		1							
4,2	1		1	1					
5,0						1	1	1	1
6,3						1	1	1	1
6,4						1	1	1	1
6,5			1						
6,6	2	1		2					
6,7		1	1						
6,8	1								
7,0	1	1	1	1					
8,9								1	
9,0						1	1		1
9,1	1	1	1	1		1	1	1	1

Fig. 2. Electrophoretic patterns of fish samples. Lanes 1–4, important species; lanes 5–8, lower value species (fish species are indicated according to Table 1). Boxes containing a number larger than 1 indicate more than a single band in its pH zone (ΔpH = 0.1).

is that the clear distinction between fish species could be achieved. The method we developed can do this reliably, giving the veterinary inspector a useful tool to reveal the fraud of fish substitution.

From our experience, another advantage of this statistical method is the possibility of comparing the data with those obtained after new IEF analysis of fish species, provided that the same analytical procedures are applied.

The differentiation among all species has been possible in all analysed groups, even for closely related species considering their taxonomic classification. In fact, species ranging from 1 to 6 belong to the same order, family and genus (see Table 1). It

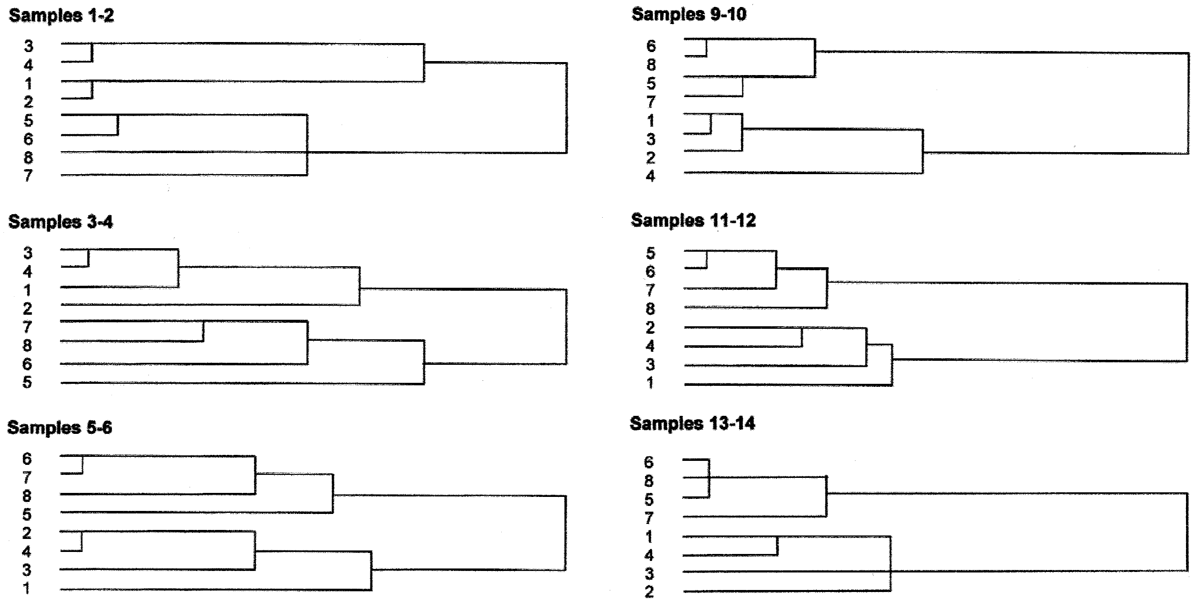

Fig. 3. Dendrograms obtained after cluster analysis (SPSS proximities procedure).

seems that the objective evaluation of IEF protein patterns has not been held in due consideration so far in the field of fish identification; in fact, the visual comparison of protein bands is commonly accepted. We think that even the most experienced visual approach to IEF banding patterns may lead to some incorrect results. Furthermore, some major companies have been developing PC-based tools and software especially designed for the image evaluation of electrophoretic gels; if appropriately set up, they can give consistent and reproducible results. The US Food and Drug Administration (FDA), for example, does use these tools to evaluate the IEF and Phast gels (Amersham Pharmacia Biotech) and furthermore includes the polymerase chain reaction (PCR)–restriction fragment length polymorphism analysis (RFLP) to confirm the results obtained with IEF and Phast [32]. This kind of procedure indirectly proves the weakness of the sheer visual observation of IEF banding patterns.

In conclusion, we believe that such an objective approach could go beyond any uncertain estimation of the electrophoretic patterns. We look forward to defining a standard method for the evaluation of electrophoretic gels to improve the reliability and the consistency of the results.

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