Species identification of cooked fish by urea isoelectric focusing and sodium dodecylsulfate polyacrylamide gel electrophoresis: a collaborative study

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Abstract

The suitability and reliability of urea IEF and SDS-PAGE for the identification of cooked fish flesh was tested by a collaborative study among nine laboratories. Urea IEF was performed with CleanGels as well as with ImmobilineGels, and ExcelGels were used for SDS-PAGE, enabling all three types of gels to be run in the same flat bed electrophoresis chamber. By strictly following optimised standard operation procedures (SOPs), five unknown cooked samples had to be identified with each technique using a set of 10 raw reference samples. With urea IEF, only one out of 35 identifications was incorrect, and with SDS-PAGE a similar result was obtained. It was concluded that methods, as now developed, are suitable for checking the species declaration of fishery products.

1. Introduction

The identification of the species of raw fish by protein electrophoresis, mainly isoelectric focusing of the sarcoplasmic proteins, is a well established technique, which is used in the control of seafood authenticity (Mackie, 1996). For analysis of heat-processed fish, however, the method is applicable only to those species which possess characteristic patterns of the heat-stable parvalbumins (Plowman & Herbert, 1992; Rehbein, 1992).

Heat-denatured fish muscle proteins may be solubilise by urea or sodium dodecylsulfate (SDS) and separated b urea isoelectric focusing (IEF) (An, Wei, Zhao, Marshal & Lee, 1989) and sodium dodecylsulfate gel electrophoresis (SDS-PAGE) (Scobbie & Mackie, 1988) respectively.

Both techniques are not widely used in routine analysis today, mainly because they are demanding of time and technical skills. Until now, the methods have not been standardised and evaluated by collaborative studies. As a first step to this objective, protocols for urea IEF and SDS-PAGE for the identification of cooked fish using commercially available precast gels, CleanGels, Immobiline Dry Plates and ExcelGels were optimised (Etienne et al., 1999; Pineiro et al., 1999), resulting in standard operation procedures being set down. The suitability of these optimised procedures was then evaluated by a collaborative study, the results for which are reported here.

2. Materials and methods

2.1. Fish samples

Samples of the fish species listed in Table 1 had been collected either on research cruises of the Institute of Biochemistry and Technology, Hamburg or bought as wet fish at the local fish market in Hamburg-Altona.

2.1.1. Cooking of samples

Pieces of fillet were vacuum-sealed in plastic bags and heated in a water bath of 85ºC. When this temperature was reached in the core of the sample, heating was continued for a further 2 min. The samples were then removed from the bath, allowed to cool to

ambient temperature and stored deep-frozen at about -20ºC. The heating regime was followed by continuously measuring the temperature inside the muscle pieces by means of thermocouples.

2.1.2. Distribution of samples

Frozen samples, provided with dry ice, were delivered by air freight and arrived in good condition within 36 h at the participating institutions.

2.2. Analytical methods

2.2.1. Standard operation procedure for analysis of raw or heated fish fillet by means of urea CleanGel IEF

2.2.1.1. Extraction of proteins. A fish fillet was cut into small pieces and 500 mg of light muscle were homogenized for 1 min with 4 ml of extraction solution (8 M urea, 0.1 M 1,4-dithiothreitol (DTT), 20 mM sodium phosphate, pH 6.5) by using an Ultra-Turrax homogenizer fitted with a small rod. The mixture was kept at room temperature for at least 30 min, then undissolved material was removed by centrifugation (e.g. Sorvall RC 28S, rotor F28/13, 20ºC, 15 min, 20 000xg_{max}). The extract could be stored for analysis by IEF and protein assay up to three days at ambient temperature.

2.2.1.2. Conditions of urea IEF

2.2.1.2.1. Materials and reagents. Flat-bed electrophoresis, power supply, thermostatic circulator. Clean-Gels for IEF, GelPool (both from Amersham Pharmacia Biotech); electrode wicks (extra long), applicator strips 7x1 mm, silicon rubber, ampholytes: Servalyte 2-4 (40% w/v), Servalyte 4-6 (40%), Servalyte 4-9 T (40%), SERVA Violet 17 (all from Boehringer Ingelheim Bioproducts).

Anode fluid pH 3: 0.025 M aspartic acid, 0.025 M glutamic acid, 10 mM $CaCl_2$; cathode fluid pH 10: 2 M ethylene diamine, 0.025 M arginine, 0.025 M lysine.

2.2.1.2.2. Rehydration of CleanGels. A full size Clean-Gel was rehydrated in 21 ml of 8 M urea, 1% (w/v) Servalyte 2-4, 1.5% Servalyte 4-6 and 0.5% Servalyte 4-9T according to the following procedure.

The rehydration solution was degassed and poured into the large chamber of the GelPool. Starting at the edge, the gel film, with the surface downwards, was set into the rehydration solution and slowly lowered. The gel was moved to and fro several times during the first 15 min. Then the pool was covered with a glass plate (and a wet towel, if the humidity of the atmosphere was low), and allowed to stand overnight on a horizontal table to complete re- swelling of the gel.

Directly before use, the rehydrated gel was taken out of the pool. Droplets were wiped off the gel surface with a sheet of filter paper.

2.2.1.2.3. IEF run. The gel support plate was cooled to 15ºC by means of a thermostatic circulator. To avoid crystallisation of urea during the run within the gel, a small volume of water was poured onto the bottom of the electrophoresis chamber. Electrode wicks were cut to the length suitable for the gel and soaked with an appropriate volume of anode or cathode fluid. After evenly dispensing a small volume of kerosene in the centre of the cooling plate of a flat-bed electrophoresis apparatus, the gel was placed on the plate, excess kerosene was removed by means of paper towels, and the electrode wicks were applied to the gel.

The settings for IEF were: prefocusing: 500 V, 8 mA, 8 W, 30 min; sample entrance: 500 V, 8 mA, 8 W, 20 min; focusing: 2000 V, 14 mA, 14 W, 5000 Vh.

After prefocusing, the applicator strip was placed about 2 cm in front of the cathodic wick, and 7.5 ml of sample solution (containing 5-10 mg of protein) were placed into the slots of the strip. After about 1000 Vh, when the proteins had entered the gel, the applicator strip was removed to avoid bleeding or smearing of the proteins.

2.2.1.2.4. Protein staining. When the IEF run was finished at about 5000 Vh, electrode wicks were removed, and the proteins were visualised by staining with a Coomassie dye. The gel was successively shaken in 200 ml of the following solutions.

Fixation: 20% (w/v) trichloroacetic acid, 30 min; washing: destaining solution, 30 min; staining: % (w/v) SERVA Violet 17, dissolved in destaining solution; destaining: methanol/acetic acid/distilled water (25/10/65) (v/v/v), changing the destaining solution several times until the background was clear and colourless; impregnation: 1% (w/v) glycerol (87%), 10 min; drying: overnight at room temperature.

2.2.2. Standard operation procedure for analysis of raw or heated fish fillet by means of urea immobiline dry plate IEF

2.2.2.1. Extraction of proteins. Extraction of proteins was performed as described in Section 2.2.1.1.

2.2.2.2. Conditions of urea immobiline dry plate (IDP) IEF

2.2.2.2.1. Material and reagents. Flat-bed electrophoresis, power supply, thermostatic circulator. Immobiline Dry Plate 4-7, Reswelling Cassette for IDP, GelPool, electrode wicks, sample application pieces (all from Pharmacia), applicator strips 7x1 mm, silicon rubber (Boehringer Ingelheim Bioproducts), Ampholine 3.5-9.5, Ampholine 3.5-5.0, PhastGel Blue R (all from Pharmacia), Coomassie Brilliant Blue R-250 (Sigma).

2.2.2.2.2. Rehydration of IDPs. The IDP was rehydrated overnight either in the reswelling cassette or in the GelPool of Pharmacia. A full size IDP 4-7 was rehydrated with 20 ml of a solution prepared by dissolving 9.6 g urea, 0.1 g CHAPS [3-((3-Cholamidopropyl)dimethylammonio)-1-propane-sulfonate], and 60 mg DTT in 12 ml distilled water, adding 0.5 ml Ampholine 3.5-9.5 and 0.1 ml Ampholine 3.5-5.0 and filling up with water to the final volume of 20 ml.

The cassette or the pool was covered with a glass plate (and a wet towel, if the humidity of the atmosphere was low), and stood overnight on a horizontal table for reswelling of the gel. Directly before use the rehydrated gel was taken out of the cassette. Droplets were wiped off the gel surface with a sheet of filter paper.

2.2.2.2.3. IEF run. A small volume of kerosene was dispensed in the centre of the cooling plate, and the temperature of the thermostatic circulator was set to 15ºC. The electrode strips were cut to a suitable length for the gel, soaked with distilled water, and afterwards freed from excess water until they appeared to be “dry". The gel was placed on the plate (the support film had a pre-cut corner indicating the anodic side of the gel), excess kerosene was removed, and the electrode wicks were applied to the gel

The settings for IEF were: 3500 or 3000 V, 5 mA, 15 W during 2.5 h or 3, respectively. The samples (10 ml) were applied about 1 cm in front of the cathodic wick; after half of the total focusing time, the application pieces or strips were removed from the gel.

2.2.2.4. Protein staining. After removal of the electrode wicks the gel was successively shaken in 200 ml of the following solutions.

Fixation: 11.5% (w/v) trichloroacetic acid, 3.5% (w/v) sulphosalicylic acid, 30-60 min; washing: destaining solution: 5 min; staining: preheated (60ºC) staining solution, 10 min; the staining solution was prepared by dissolving 0.29 g Coomassie Blue R 250 in 250 ml of destaining solution, or alternatively one tablet PhastGel Blue R in 400 ml of destaining solution under stirring and heating to 60ºC; the solutions were filtered before use; destaining: ethanol/acetic acid/water (25/8/67) (v/v/v), changing the destaining solution several times; impregnation: 10% (v/v) glycerol in destaining solution, 1h; drying: the gel was covered with a cellophane preserving sheet and allowed to dry at room temperature or in a gel air-drying system (BioRad).

2.2.3. Standard operation procedure for analysis of raw or heated fish fillet by means of SDS-PAGE

2.2.3.1. Extraction of proteins. A fish fillet was cut into small pieces and 300 mg of the flesh were homogenised in 4 ml of 2% (w/v) SDS, 0.1 M DTT, 60 mM Tris-HCl, pH 7.5 using a Polytron or Ultra Turrax homogeniser equipped with a small rod for 30-60 s at low speed to avoid foaming. Then the samples were boiled in a water bath (100ºC) for 2 min and after-wards homogenised while being hot for 30 s. Finally the samples were centrifuged (20 000xg_{max}, 20ºC, 15 min).

2.2.3.2. Preparation of samples for SDS-PAGE. The protein contents of the extracts were determined using the OD280-procedure (see Section 2.2.4.1). The protein concentration of extracts was adjusted to 0.3 mg/ml with Laemmli buffer [4.8% (w/v) SDS, 1 mM EDTA, 0.1 M DTT, 20% (v/v) glycerol, 125 mM Tris-HCl, pH 6.8, 0.05% (w/v) bromphenol blue]. The molecular weight marker, NOVEX Mark 12, was diluted 10 times with Laemmli buffer, and the staining indicator, containing 1.6 mg bovine plasma albumine and 1.6 mg egg white lysozyme in 1 ml of 2% SDS, 0.1 M DTT, 60 mM Tris-HCl, pH 7.5, was diluted 215 times with Laemmli buffer.

2.2.3.3. Conditions of SDS-PAGE

2.2.3.3.1. Materials and reagents. Flat-bed electrophoresis, power supply, thermostatic circulator. Excel- Gel SDS Homogeneous 15%, ExcelGel SDS buffer strips (Pharmacia).

2.2.3.3.2. SDS-PAGE. The gel support plate was cooled to 15ºC, about 1 ml of kerosene was poured onto the plate, and the gel was positioned on the plate with the wells at the cathodic side. The white cathodic buffer strip was positioned above the wells with the narrow side against the gel. Similarly, the yellow anodic buffer strip was positioned at the other side of the gel.

Ten µl of samples, molecular weight markers and staining indicator were applied in the wells of the gel. The running conditions were: 600 V, 30 mA, 30 W; when the bromphenol blue front started to enter the yellow electrode strip the electrophoresis was continued for another 20 min and then stopped. The electrode strips were removed and the reverse side of the gel was cleaned using filter paper moistened with ethanol to remove kerosene.

2.2.3.3.3. Protein staining. The proteins were fixed and stained using the Silver Staining Kit Protein of Pharmacia essentially according to the procedure described in the instruction manual with one modification: the stop solution was made 5% (v/v) acetic acid to reduce browning of the background. The developing step was stopped, when the bands of the staining indicator became visible.

2.2.4. Protein determination

Two methods were used for measuring the protein concentration of extracts, reading of OD_{280} and a Coo-massie dye binding method (Bio-Rad Protein Assay).

2.2.4.1. OD280 test. Fish muscle extracts, bovine serum albumin (BSA) standard solution (10 mg/ml), and reagent control without protein (i.e. extraction buffer, 8 M urea, 0.1 M DTT, 20 mM sodium phosphate, pH 6.5, respectively 2% SDS, 0.1 M DTT, 60 mM Tris-HCl, pH 7.5) were diluted 20-fold with 0.2% (w/v) SDS.

OD_{280} was read using quartz cuvettes, and the protein content of extracts was calculated using the equation:

$P_{Sample} = (OD_{Sample} - OD_{Control}) \times 20$, assuming that 1 mg protein per ml gives the OD_{280} of 1.

As a control, OD_{280} was read also for the bovine serum albumin standard solution and the 0.2% SDS solution, and the difference $OD_{BSA} - OD_{SDS}$ should be close to 0.33.

2.2.4.2. Coomassie dye-binding method. The standard assay procedure as outlined in the instruction manual of the Bio-Rad Protein Assay was used, diluting extracts or BSA standard (1 mg/ml) with 6 M urea, 20 mM Tris-HCl, pH 6.5.

3. Results and discussion

Nine European laboratories involved in control and quality determination of fishery products participated in the collaborative study, the samples being prepared and distributed by one of these institutes and analysed by eight laboratories.

Three sets of samples had been distributed (Table 1), one set with 10 references (set “R") and two sets with five unknown samples each, to be identified either by urea IEF (set “I"), or by SDS- PAGE (set “S"). The participants were informed that the sets “I" and “S" might contain samples not included in the references, as well as two samples of the same species.

3.1. Protein content of extracts

The results for the protein determinations performed in the institute, which had prepared the samples, are compiled in Table 2. Cooking resulted in about 50% reduced solubilisation of proteins on extraction with urea containing buffer. On the other hand, with the SDS solution the same amount of protein was extracted from raw or cooked fish.

3.2. Species identification by urea IEF

Two different type of gels were used for urea IEF, CleanGels and ImmobilineGels, giving protein patterns characterised by strong bands in the acidic part of the gel, as shown in Figs. 1 and 2. These bands may represent parvalbumins (H. Rehbein, 1998, unpublished results), as well as myosin light chains (Ochiai, Watabe & Kashimoto, 1988) and troponin C (Hirabayashi, 1981).

The protein pattern of raw fish muscle was not significantly changed by cooking the fish, thus allowing raw muscle to be used as reference material for identification of cooked fish.

The inter-species differences of pI-values were sufficient to allow discrimination between all fish species tested within this exercise. Both types of gels were suited for fish species differentiation by urea IEF, but Clean-Gels may be preferentially used in routine analysis, as they are cheaper than ImmobilineGels.

Urea IEF turned out to be a very reliable technique for the identification of cooked fish, as demonstrated by Table 3. Only in one out of the 35 identifications was the species incorrectly assigned.

3.3. Species identification by SDS-PAGE

For practical reasons, i.e. to perform IEF and SDS-PAGE with the same electrophoresis equipment, ready-to-use ExcelGels were run in flatbed electrophoresis systems. To

avoid precipitation of myofibrillar proteins, e.g. of the myosin heavy chain, in the entrance zone of the gel, only low amounts of protein (about 3 mg per lane) were applied, requiring silver staining. For standardisation of silver staining, low amounts of two proteins (74 ng of BSA and lysozyme per lane) were also run on the gels, and the developing step was stopped when these proteins became visible.

As can be seen in Fig. 3, the protein patterns can be divided into two parts with respect to their usefulness for species discrimination.

The high molecular weight region, reaching from actin (42 kDa) to the myosin heavy chain (200 kDa), was characterised by a few strong bands, being at the same position for all species, and a number of faint bands showing some variability between species. This variability was considered to be too small for species identification.

On the other hand, species-specific protein patterns were produced by the low molecular weight proteins (Suzuki, 1981) comprising the three myosin light chains (14-23 kDa), troponins (19-30 kDa) and parvalbumins (about 12 kDa). It has been previously reported (Civera & Parisi, 1991; Scobbie & Mackie, 1988; Seki, 1976; Seki, Takayasu & Kokuryo, 1980; Sotelo, Pineiro, Gallardo & Perez-Martin, 1992) that differentiation of fish and shellfish species by SDS-PAGE was possible, considering proteins of molecular weight lower than 30 kDa.

In the present study, differentiation between all of the species examined was achieved, despite the fact that some of them are genetically closely related, e.g. cod, whiting, haddock, and saithe, which all belong to the same family (Gadidae); hake and haddock also differed only in the position of one protein band.

Table 3 shows that in one case only (out of 40), the unknown sample had been misidentified.

3.4. Concluding remarks: comparison of urea IEF and SDS-PAGE

Both methods, using precast gels, proved to be reliable and well suited for species identification of heated fishery products. It may depend on the type of product to be

analysed which of these techniques gives better results. Extensively washed products, like kamaboko and other surimi-based products, may be preferentially analysed by SDS-PAGE, because most of the parvalbumins have been washed out leaving mainly myosins.

On the other hand, urea IEF may be preferred for differentiation of closely related species being rich in parvalbumin isoforms.

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Table 1

Fish species used for the collaborative study

Sample set “R" (reference material, raw muscle)	
Code	Fish species
R01	Gadus morhua, cod
R02	Pollachius virens, saithe
R03	Merluccius merluccius, North Atlantic hake
R04	Molva molva, ling
R05	Merlangius merlangus, whiting
R06	Melanogrammus aeglefinus, haddock
R07	Pleuronectes platessa, plaice
R08	Solea solea, sole
R09	Platichthys flesus, flounder
R10	Sebastes marinus, red fish
Sample set “I" (samples for urea IEF, cooked muscle)	
Code	Fish species
I01	Plaice
I02	Cod
I03	Haddock
I04	Red fish
I05	Ling
Sample set “S" (Sample for SDS-PAGE, cooked muscle)	
Code	Fish species
S01	Flounder
S02	Haddock
S03	Cod
S04	Whiting
S05	North Atlantic hake

Table 2

Protein extractability of raw or cooked fish muscle

A. Extracting solution: 8 M urea, 0.1 M DTT, 20 mM sodium phosphate, pH 6.5

		Protein content[a]	
Sample code	Fish species	(mg/ml of extract)	(g/100 g wet weight)
References (raw)			
R01	Cod	7.2	6.5
R02	Saithe	10.6	9.5
R03	NA hake	11.7	10.5
R04	Ling	12.6	11.3
R05	Whiting	10.0	9.0
R06	Haddock	9.6	8.6
R07	Plaice	9.2	8.3
R08	Sole	8.6	7. 7
R09	Flounder	6.6	5.9
R10	Red fish	15.3	13.8
Set “I" (cooked)			
I01 Plaice 5.6 5.0			
I02 Cod 5.6 5.0			
I03 Haddock 5.4 4.9			
I04 Red fish 5.1 4.6			
I05 Ling 5.5 5.0			

B. Extracting solution: 2% SDS, 0.1 M DTT, 60 mM Tris-HCl, pH 7.5

		Protein content[b]	
Sample code	Fish species	(mg/ml of extract)	(g/100 g wet weight)
References (raw)			
R01	Cod	15.2	21.7
R02	Saithe	14.3	20.5
R03	NA hake	12.9	18.5
R04	Ling	12.0	17.2
R05	Whiting	13.7	19.6
R06	Haddock	11.4	16.3

R07	Plaice	14.8	21.2
R08	Sole	13.3	19.1
R09	Flounder	12.1	17.3
R10	Red fish	15.6	22.4
Set “S" (cooked)			
S01	Flounder	12.7	18.2
S02	Haddock	14.8	21.2
S03	Cod	15.2	21.7
S04	Whiting	13.9	19.9
S05	NA hake	18.1	25.9

a Determined by the Coomassie dye binding method.

b Determined by the OD280 test.

Table 3

Results of the collaborative study for identification of cooked fish by urea IEF and SDS-PAGE

Samples	Laboratory							
	1	2	3	4	5	6	7	8
Urea IEF[a]								
I01 Plaice	+	+	+	+	nd[c]	+	+	+
I02 Cod	+	+	+	+	nd	+	+	+
I03 Haddock	+	+	+	-	nd	+	+	+
I04 Red fish	+	+	+	+	nd	+	+	+
I05 Ling	+	+	+	+	nd	+	+	+
SDS-PAGE								
S01 Flounder	+	+	+	+	+	+	+	+
S02 Haddock/Hake[b]	+[b]	+	+[b]	+	+[b]	+[b]	+[b]	+
S03Cod	+	+	+	+	+	+	+	+
S04 Whiting	+	+	+	+	+	+	+	+
S05 NA hake	+	+	-	+	+	+	+	+

a For each laboratory results from urea CleanGel IEF and urea Immobiline IEF were identical, and therefore combined.

b Five of the participants had obtained hake, and three received haddock.

c nd, not determined.

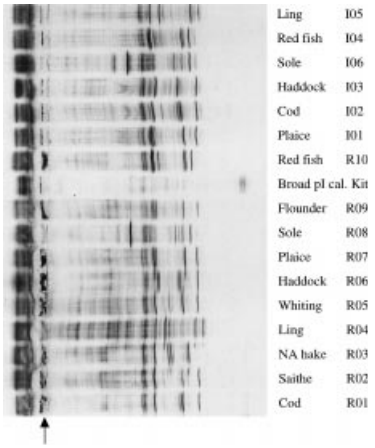

Fig. 1. Urea IEF with CleanGel. Extracts of raw (refs R01-R10) or cooked (samples I01-I06) fish muscle were run on CleanGel rehydrated with 8M urea, 1% (w/v) Servalyte 2-4, 1.5% Servalyte 4-6, 0.5% Servalyte 4-9. Broad pI calibration kit was from Pharmacia. The anode is on the right side in all of the figures; the position of sample application is indicated by the arrow.

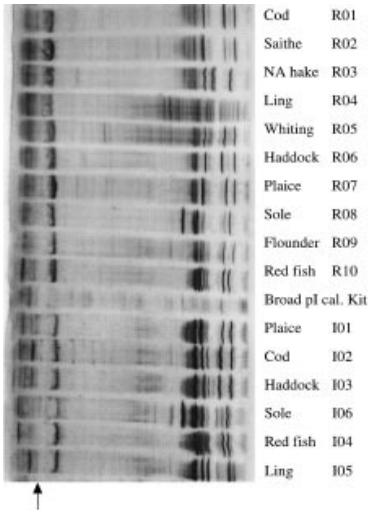

Fig. 2. Urea IEF with ImmobilineGel. Extracts of raw (refs R01-R10) or cooked (samples I01-I06) fish muscle were run on re-swollen Immobiline Dry Plate 4-7. Other details are as in Fig. 1. Here the reference sample of ling showed an unusual pattern

(due to protein degradation during thawing of the raw reference material), which was not observed with other extracts.

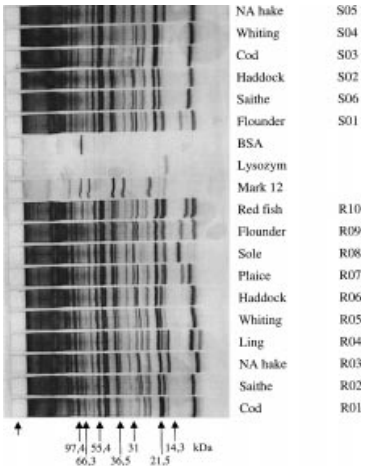

Fig. 3. SDS-PAGE. Extracts of raw (refs R01-R10) or cooked (samples S01-S06) fish muscle were run on ExcelGel Homogeneous 15%. Mark 12 was used as calibration kit, the molecular weights are indicated at the bottom of the figure. BSA (bovine serum albumin) and lysozyme were used as staining indicators.