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Influence of variation in methodology on the reliability of the isoelectric focusing method of fish species identification

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The reliability of isoelectric focusing (IEF) of sarcoplasmic proteins for fish species identification was evaluated by a collaborative study among eight European laboratories. Each laboratory used its own method of IEF to identify 10 unknown samples of raw muscle by means of reference material. In 93% of cases the assignment between sample and reference was correct.

In a second study, the influence of extractant (water, low ionic strength buffer, or detergent) and the position of sample application on the protein pattern was examined. Working with light muscle of rainbow trout (*Oncorhynchus mykiss*), it was found that the type of extractant did not influence the protein pattern. Comparison of the patterns of samples, which had been applied near the anode, in the middle, or near the cathode, revealed differences in the number and position of the protein bands under the experimental conditions applied by most laboratories. This effect was not observed with the Phast System.

INTRODUCTION

Species identification of fishery products is nowadays mostly performed by isoelectric focusing (IEF) of sarcoplasmic proteins (Rehbein, 1990). In comparison with other electrophoretic methods, IEF has several advantages.

(1) During electrophoresis the proteins focus into sharp zones. This effect improves resolution and sensitivity.
(2) At the end of the electrophoretic run the system is in equilibrium; the proteins have reached fixed positions within the gel, according to the pH-gradient and their isoelectric points (pIs). Variations in experimental parameters (sample application technique, separation time, applied voltage or current) should, at least in theory, have only minor influence on the protein pattern (Lundstrom, 1979).
(3) IEF can be modified in many respects to meet special analytical requirements (Righetti, 1983).

Either agarose or polyacrylamide may be used as stabilising media (Laird *et al.*, 1982) and pH-gradients can be established by means of a great variety of commercially available ampholytes. These can be either wide range (pH 3–10) or narrow range (e.g. pH 3–6, useful for the analysis of gadoid fish). Addition of urea or non-ionic detergents is possible, and may be necessary for the analysis of denatured proteins, e.g. those extracted from cooked fish (Mackie, 1980) or crab (Krynowek & Wiggin, 1979).

Table 1. Procedures for extraction of water-soluble proteins from fish muscle. Cooled, demineralized or distilled water was used as extractive

Laboratory	Ratio of muscle to extract	Type of mixer	Conditions of centrifugation
1	10 g/20ml	Ultra-Turrax	10000 rpm, 30 min, 0°C
2	50 g/50 ml	Turmix	10000 rpm, 15–20 min, 8°C
3	1 part/3 parts	—	25000 *g*, 40 min
4	1 g/2 ml	Ultra-Turrax	20000 *g*, 20 min, 4°C
5	2·5 g/5 ml	—	(1) 3500 rpm, 15 min (2) 13000 rpm, 15 min
6	Instead of an extract, press juice was prepared by centrifugation of muscle		22000 *g*, 15 min, 3°C
7	1 part/2 parts	—	—

The suitability of IEF for fish species identification has been demonstrated by two collaborative studies, where unknown samples were identified by comparison with photographs of protein patterns from authentic species (Lundstrom, 1980, 1983). In these studies each laboratory had to use exactly the same method.

This procedure has the disadvantage that the same type of gel, which has been used for establishing the protein patterns of the references, has also to be used in the analysis of unknown samples. It is not possible to make use of technical innovations in IEF, e.g. newly developed apparatus or types of ready-to-use gels.

The present study was undertaken to examine which parameters of IEF had to be standardised for fish species identification, and which steps in the analytical procedure were not so critical for the reliability of the results. Reference material and unknown samples of raw fish muscle were sent to seven other laboratories by the Institute of Biochemistry and Technology, Hamburg, and each participant had to apply the variant of IEF normally used in the respective laboratory for species identification. Furthermore, the influence of extractant (water, buffer or detergent) and the position of sample application on the protein patterns were studied in detail.

MATERIALS AND METHODS

Fish samples

The fish used in the present study were either collected from the North Atlantic and the North Sea during research cruises of the German research vessel or obtained from the local fish market and identified by their external biological characters.

Specimens of 12 fish species, viz. (1) cod (*Gadus morhua*), (2) haddock (*Melanogrammus aeglefinus*), (3) saithe (*Pollachius virens*), (4) redfish (*Sebastes marinus*), (5) North Atlantic hake (*Merluccius merluccius*), (6) halibut (*Hippoglossus hippoglossus*), (7) herring (*Clupea harengus*), (8) spotted catfish (*Anarchichas minor*), (9) blue ling (*Molva dipterygia*), (10) ling (*Molva molva*), (11) Alaska pollack (*Theragra chalcogramma*) and (12) rainbow trout (*Oncorhynchus mykiss*), were analysed.

Light muscle was separated from fillet, frozen and distributed as frozen material to the collaborators.

Procedure of the first collaborative study

Each laboratory received 10 references (species Nos 1–10) and 10 unknown samples. Samples and references were from different specimens. The participants were instructed that different samples could belong to the same fish species and that one of the samples was possibly not represented by a reference.

Each laboratory used its own analytical procedures (preparation of extracts, procedure for electrophoresis) for fish species identification. The methods are summarised in Table 1 (extraction) and Table 2 (electrophoresis). As an example, the method used by the distributing laboratory (Hamburg) to check the samples (see Figs 1 and 2) is described in detail.

Preparation of extracts

Five grams of light muscle were cut into small pieces and homogenised with 15 ml of precooled distilled water by means of an Ultra-Turrax. The total mixing time, including two interruptions, was 2 min; the speed of rotation increased gradually, and warming of the mixture was avoided. The homogenate was centrifuged (e.g. using the Eppendorf 5412 Table Centrifuge for 4 min at room temperature; 12000 rpm = 8000 *g*), and the supernatant was kept in the refrigerator, for not longer than 2 days, until used for IEF.

Table 2. Electrophoretic methods used for fish species identification. Laboratories 1 and 2 used the Phast System (Pharmacia). In all laboratories proteins were stained with Coomassie dye, but following different protocols. In three laboratories (Nos 1, 2 and 5) the protein patterns were evaluated by densitometry or image analysis

Laboratory	PAGIF	AGIF	Thickness of the gel (mm)	pH-gradient
1	+		0·45	3–9
2	+		0·45 and 1·00	3–9 and 3·5–9·5
3	+		0·30 and 1·00	5–7 and 3·5–9·5
4	+		2·0	3–10
5	+		0·5	4–7
6		+	0·4	4–6·5
7	+		—	4–6 and 3·5–10

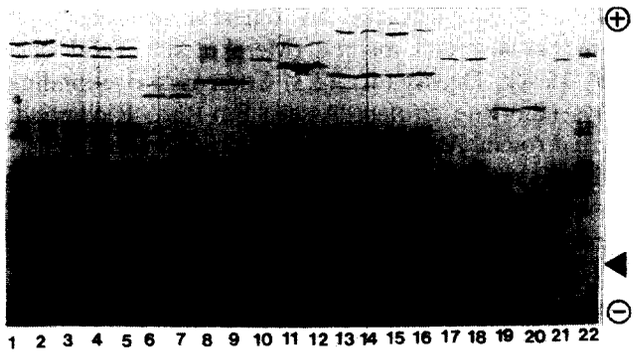

Fig. 1. Patterns obtained by IEF of sarcoplasmic proteins from fishes used in the first collaborative study. References (R) and samples (S; to be identified) from the following species were compared using a Servalyte® Precote® 3–10, 150 μm: cod, R, S (lanes 1, 2); haddock, R, S, S (3–5); saithe, R, S (6, 7); redish, R, S (8, 9); halibut, R, R, S (10, 17, 18); North Atlantic hake, R, S (11, 12); ling, R, S, S (13, 14, 16); blue ling, R (15); herring, R, S (19, 20); spotted catfish, R (21); Alaska pollack, S (22). The extracts contained 7–11 mg/ml of protein, determined by means of the Coomassie dye-binding assay (Bio-Rad). Extract (7·5 μl) was applied to the gel at the position marked by the arrow. Examples of polymorphic proteins are marked by a star (✳). The volt hour product was 7350 Vh.

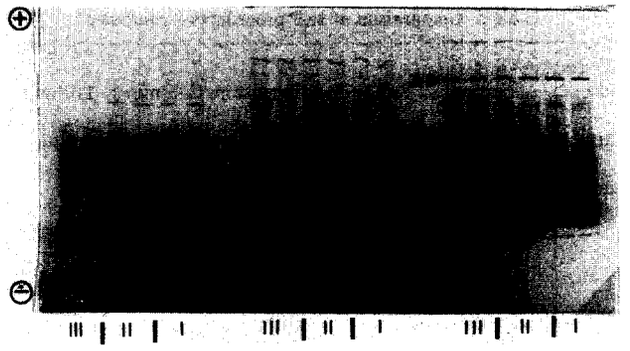

Fig. 2. Influence of the position of sample application on the protein pattern. Different extracts of light muscle of rainbow trout were applied to the gel near the anode (A), in the middle (M), or near the cathode (C). By means of applicator strips, 7·5 μl (containing about 130 μg of protein) of different extracts (with distilled water (I), 20 mM Na-phosphate pH 7·0 (II), or 0·2% Triton X-100 (III) as extractant) was placed on the gel. Conditions of IEF: Servalyte® Precote® 3–10, 300 μm; at the end of the run voltage was 1270 V, volt hour product was 4850 Vh.

Isoelectric focusing

IEF was performed with Servalyte® Precotes® 3–10, dimensions 245 mm × 125 mm × 0·15 mm, generally following the instructions given by SERVA, Heidelberg. The anode fluid was made 10 mM with $CaCl_2$ for sharpening the bands of acidic proteins; after prefocusing (30 min, setting: 250 V, 15 mA, 4 W), 7·5 μ of extracts was pipetted into the slots of the applicator strip 7 mm × 1 mm, and electrophoresis was continued (setting: 2000 V, 15 mA, 4 W). Focusing was completed when the product of voltage and time had reached about 6000 Vh. SERVA Violet 49 (100 mg/100 ml universal solvent) was used for staining; the universal solvent, also used for destaining, contained methanol/acetic acid/water (25/10/65, v/v/v) (Radola, 1980).

Procedure of the second collaborative study

In this study the influence of extractant and position of sample application on the protein patterns were evaluated. Each laboratory received frozen fillets from rainbow trout and instructions for the preparation of extracts and procedures of IEF.

Extraction of sarcoplasmic proteins from trout muscle

Extracts were made as described above using three different extractants: precooled distilled water (I) or 20 mM Na-phosphate pH 7·0 (II), or 0·2% (w/v) Triton X-100 (III).

Isoelectric focusing

Within the scope of the following guidelines each laboratory used its own method (Table 3).

Table 3. Comparison of IEF procedures

Laboratory No. 1	
Gel:	Phast Gel IEF 3–9, dimensions 43 mm × 50 mm × 0·35 mm; Pharmalyte
Run:	Maximal voltage 2000 V, power × time 245 AVh, with prefocusing
Sample:	Volume 1 μl, protein content adjusted to 10 mg/ml
Staining:	The proteins were fixed by trichloroacetic acid (TCA) and stained by 'Phast blue R'; the gels were destained by methanol/acetic acid and water. The protein bands were evaluated densitometrically by the 'Phast Image System'
Laboratory No. 2	
Gel:	(I) Servalyte® Precote® 3–10, dimensions 125 mm × 125 mm × 0·3 mm (II) PAG plate (Pharmacia-LKB), Ampholine 3·5–9·5, thickness 1 mm
Run:	(I) Maximal voltage 2000 V, voltage × time 3000 Vh, no prefocusing (II) Maximal voltage 1300 V, no prefocusing
Sample:	(I) Volume 7·5 μl (II) Volume 10 μl, protein content adjusted to 10 g/ml, the samples were applied with a SMI micropettor B syringe
Staining:	(I) Fixing solution 200 g TCA in 1000 ml ethanol 95% Staining solution: 100 mg Coomassie R-250 in 250 ml destaining solution Destaining solution: ethanol 95%/acetic acid/water, 4/5/1, v/v/v (II) Fixing and staining were carried out simultaneously in the following solution: Coomassie R-250 0·25 g methanol 75 ml water 155 ml sulphosalicylic acid (SSA) 8 g TCA 25 g Destaining solution: ethanol 95%/acetic acid/water, 375/120/1000, v/v/v
Laboratory No. 3	
Gel:	PAG plate (Pharmacia-LKB), Ampholine 3·5–9·5, dimensions 245 mm × 110 mm × 1 mm
Run:	Settings: 1500 V, 50 mA, 30 W, 1·5 h, no prefocusing
Sample:	Volume 7·5 μl, application by means of pieces of filter paper
Staining:	Fixing solution: 57·5 g of TCA + 17·25 g of SSA + 500 ml water Staining solution: 0·46 g Coomassie Blue R in 400 ml destaining solution Destaining solution: 500 ml ethanol + 160 ml acetic acid to 2 litres of water

continued

Table 3. Comparison of IEF procedures—*continued*

Laboratory No. 4	
Gel:	Pharmalyte 3–10 (7%), dimensions 245 mm × 110 mm × 0·3 mm
Run:	Settings: 2000 V, 15 mA, 8 W; no prefocusing, voltage × time 3000 Vh
Sample:	Application by means of pieces of filter paper
Staining:	Fixing solution: 10% TCA, 5% SSA Staining solution: 0·04% Coomassie Blue R-250 in destaining solution Destaining solution: ethanol/acetic acid/water, 4/1/6, v/v/v
Laboratory No. 5	
Gel:	Ampholine 3·5–9·5, thickness 0·5 mm
Run:	Settings: 1500 V, 50 mA, 25 W, 1 h; no prefocusing
Sample:	Volume 7·5 µl, application by means of pieces of filter paper
Staining:	Fixing solution: 57·5 g of TCA + 17·25 g of SSA + 500 ml water Staining solution: 0·46 g Coomassie Blue R-250 in 400 ml destaining solution Destaining solution: 500 ml ethanol + 160 ml acetic acid + water add 2 litres of water
Laboratory No. 6	
Gel:	PAG plate (Pharmacia-LKB), Ampholine 3·5–9·5, dimensions 245 mm × 100 mm × 1 mm
Run:	Settings: 15 W for the first 30 min and 20 W for the rest of the run, no prefocusing
Sample:	Volume 7·5 µl, application by means of pieces of filter paper
Staining:	Fixing solution: 11·5% TCA, 3·5% SSA Staining solution: 0·115% Coomassie Blue R-250 in destaining solution Destaining solution: 25% ethanol, 8% acetic acid
Laboratory No. 7	
Gel:	Ampholine 3·5–10, thickness of the gel 0·5 mm
Run:	Prefocusing (if used): 500 V, 20 W, 50 mA; after application of samples: 500 V, 20 W, 50 mA; after removing of strips: 1200 V, 20 W, 50 mA, 4 h
Sample:	Volumes 7·5, 10 or 18 µl; application by means of pieces of filter paper
Staining:	Fixing solutions: (I) ethanol/acetic acid, 5/1, v/v (II) 20% TCA Staining solution: 290 mg of Coomassie Blue R-250 or G-250 (Serva Blau R or G) in 250 ml of destaining solution Destaining solution: ethanol/acetic acid, 25/8, v/v (?)
Laboratory No. 8	
Gel:	Servalyte® Precote® 3–10, dimensions 245 mm × 125 mm × 0·3 mm
Run:	Prefocusing with settings: 250 V, 30 mA, 8 W, 30 min; focusing, after application of samples, with settings: 2000 V, 30 mA, 8 W, voltage × time: 5000 or 7000 Vh
Sample:	Volume 7·5 µl, applicator strip (slots 7 mm × 1 mm; silicone rubber)
Staining:	Fixing solution: 20% TCA Staining solution: 0·1% of SERVA Violett 49 in destaining solution Destaining solution: methanol/acetic acid/water, 25/10/65, v/v/v

Polyacrylamide gels (thickness of the gel: 0·3, 0·5 or 1 mm) with a pH-gradient 3–10 had to be used. Sample application was by means of an applicator strip, pieces of filter paper, or a syringe. 7·5 µl of extracts I, II and III had to be applied to the gel at the following positions: in front of the cathode, in the middle of the gel, and in front of the anode. Protein bands were visualised by staining with Coomassie dye and documented by photography or densitometry.

Protein determination

Each laboratory was free to apply its own method of measuring the concentration of sarcoplasmic proteins in the different extracts. Six different methods were used: (1) the biuret method (Merckotest® 'Total Protein', Merck, Darmstadt), (2,3) Coomassie dye-binding assays (Bio-Rad Protein Assay, Bio-Rad. Richmond; Pierce Protein Assay, Pierce Europe, BA Oud Beijerland), (4) measurement with the folin–phenol reagent (Lowry *et al.*, 1951), (5) the Kjeldahl method, (6) measurement of the difference in absorbance at 235 and 280 nm (UV method) (Whitaker & Granum, 1980).

In each case, bovine serum albumin served as the protein standard.

RESULTS AND DISCUSSION

Identification of unknown samples: First collaborative study

Fish species identification by isoelectric focusing comprised two steps: extraction of proteins and separation by electrophoresis. The procedures used in the different laboratories are compiled in Tables 1 and 2. Cooled water was the extractant in all laboratories, with the exception of laboratory No. 6, working with press juice (centrifuged tissue fluid). The type of gels used for IEF varied considerably between the laboratories. Relatively thick (1–2 mm) as well as ultra thin (0·15 mm, Fig. 1) gels were in use with narrow (slightly acidic) or wide range pH-gradients. Laboratory No. 6 used agarose gel isoelectric focusing (AGIF).

Figure 1 shows the protein patterns of all fish, i.e. references as well as samples. Each species had a unique pattern, but in some cases (e.g. hake) the patterns of reference and sample were not completely identical. The intensity, or even the number of bands. of corresponding proteins varied. The last occurrence. known as protein polymorphism, had also been observed in a former collaborative study (Lundstrom, 1980), where monkfish (*Lophius americanus*) were not identified correctly. The results of the present study are summarised in Table 4. The assignment between sample and reference (including Alaska pollack, where it should have been stated

Table 4. Summary of the results of the collaborative study on fish species identification using reference material and different IEF methods

Laboratory	Code of samples[a]									
	A	B	C	D	F	G	H	I	J	K
1	+	+	+	+	✳	+	+	+	+	+
2	+	+	+	+	✳	+	+	+	+	+
3	+	+	+	+	✳	+	+	+	+	=
4	+	+	–	+	✳	+	=	+	–	+
5	+	+	+	+	✳	+	+	+	+	+
6	+	+	+	+	✳	+	+	+	+	+
7	=	+	+	+	✳	+	+	+	+	+

+, Fish species was correctly identified; ✳, fish species was designated as not included in the references; –, fish species was not identified, although it was included in the references; =, fish species was not correctly identified.

[a]A, halibut; B, North Atlantic hake; C, cod; D, herring; F, Alaska pollack; G and H, haddock; I, redfish; J, saithe; K, ling.

that the pattern of the sample could not be found within the references) was correct in 93% of cases. Only five samples were incorrectly assigned, either because the fish species could not be identified or it was incorrectly identified. All samples of hake were correctly assigned and none of the collaborators complained about difficulties due to protein polymorphism.

Influence of extracting conditions and sample application on protein patterns: Second collaborative study

Critical inspection of the gels of the first study disclosed some variation in the quality of the protein patterns. Therefore, two steps of the procedure, extraction and sample application were examined in respect of their importance for the protein patterns. Some other points, e.g. the staining methods listed in Table 3, may be more relevant for quantitative work.

It was found that extraction of light muscle of rainbow trout with water, or 20 mM Na-phosphate pH 7·0. or 0·2% Triton X-100, resulted in nearly identical protein patterns. The pattern was characterised by many strong bands located in the basic and neutral part of the gel, whereas only a few bands appeared in the anodic region (Fig. 2).

The position of sample application had a great influence on the protein pattern, especially on the bands in the anodic and basic regions (Fig. 2), under the conditions of IEF used in most of the laboratories (Table 3). However, working with the Phast System gave the result that different positions of sample application did not alter the protein pattern. Inspection of the gels from the various laboratories revealed that the protein pattern depended also on several other factors: (i) type of ampholyte, (ii) protein content of the sample, (iii) volt hour product, (iv) staining procedure.

Some of these factors have been studied recently by Toom *et al.* (1982), by working with gels in tubes. These authors reported that the method of extracting protein was critical for subsequent species identification, a result in sharp contrast to our findings. They recommended extraction of muscle protein with a buffer containing 0·6 M NaCl. An extract of such a high ionic strength has two disadvantages: (i) besides the water-soluble proteins, most of the myofibrillar proteins are also extracted, but these proteins will not enter the gel unless gels containing 6–8 M urea are used; (ii) the high NaCl concentration of the extractant will disturb the uniformity of the electrical field within the slab gel with the consequence of wavy and distorted protein bands (Allen *et al.*, 1984).

In the present study the protein concentration of the extracts was measured with six different methods in the eight participating laboratories. The values reported varied to a very large extent, e.g. by the factor of 18 for detergent as extractant (Table 5). The values from laboratory No. 4 were considered to be far too low, whereas the protein content determined with the biuret method seemed to be too high. Protein determination in extracts requires to be improved and standardised. It is desirable for the following reasons to know the 'true' protein content: (i) explanations for distortion of bands; (ii) conclusions about the sensitivity of staining methods; (iii) interpretation of quantitative analysis, performed by IEF in combination with densitometry; and (iv) assessment of the suitability of extraction procedures.

Table 5. Protein content (mg/ml) of extracts from trout muscle

Laboratory	Extractant			Method of protein determination
	Water	Buffer	Detergent	
1	11·8	12·4	10·9	Coomassie, Pierce
2	31·7	23·3	33·3	Biuret
3	11·9	11·7	14·1	Coomassie, Bio-Rad
4	5·2	7·1	1·9	Coomassie, Pierce
5	16·1	21·3	22·2	Folin–phenol
6	11·9	13·9	12·0	Coomassie, Bio-Rad
7	17·0	19·0	18·0	Kjeldahl
	22·0	29·0	nd	UV (E_{235}–E_{280})
8	15·4	18·6	18·0	Coomassie, Bio-Rad

nd, Not determined.

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