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Species identification of smoked and gravad fish products by sodium dodecylsulphate polyacrylamide gel electrophoresis, urea isoelectric focusing and native isoelectric focusing: a collaborative study

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Abstract

A collaborative study on the use of sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), urea-isoelectric focusing (urea-IEF) and native isoelectric focusing for the identification of species of smoked salmonids, gravad salmonids and smoked eels was carried out by eight laboratories. With SDS-PAGE, minor changes took place in the profiles of the processed salmonid species making it impossible or very difficult to identify closely related species. With urea-IEF, there were fewer changes in the profiles due to processing and the system generally had greater species-discriminating power for the processed salmonids than SDS-PAGE. The profiles of the eel species as obtained on SDS-PAGE or urea-IEF were not affected by smoking. Urea-IEF had greater species-discriminating power than SDS-PAGE for the eel species. Native IEF was useful in providing supplementary identification on species difficult to identify by SDS-PAGE or by urea-IEF in the case of cold smoked products.

1. Introduction

The identification of species of raw fish by protein electrophoresis, usually isoelectric focusing, is a well-established procedure for monitoring and controlling the authenticity of seafood [Association of Official Analytical Chemists (AOAC), (1995); Mackie, 1997; Rehbein, 1990].

As the method requires the proteins — the water-soluble sarcoplasmic proteins of muscle — to be in their native states, it is not suitable for identifying the species of cooked fish. Heat-denatured muscle proteins can, however, be extracted in denaturing solvents containing sodium dodecylsulphate (SDS) or urea and the extracted proteins can then be analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis (Scobbie & Mackie, 1988) or by urea-isoelectric focusing (An, Marshall, Otwell & Wei, 1988; Mackie, 1979). As for raw species, the identity is established by comparing the protein profiles obtained with those of reference species extracted and analysed under the same conditions. Although these techniques have been described (Seki, 1976; Mackie, 1979, Seki, Takayasu & Kokuryo, 1980; An et al., 1988; Scobbie & Mackie, 1988), they have not, until recently, been optimised or evaluated for routine use in Food Control Laboratories.

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Table 1
Smoked and reference samples supplied for the study

Species	Raw	Smoked	Supplied by[a]
Sea trout (*Salmo trutta*)	+ (1)	+ (1)	IFREMER
Salmon (*Salmo salar*)	–	+ (1)	NIFA
Rainbow trout (*Oncorhynchus mykiss*)	–	+ (1)	NIFA
Arctic char (*Salvelinus alpinus*)	–	+ (1)	NIFA
European eel (*Anguilla anguilla*)	+ (1)	+ (1)	FRCF
American eel (*Anguilla rostrata*)	+ (2)	+ (2)	FRCF
Short-finned eel (Australia/New Zealand) (*Anguilla australis*)	+ (2)	+ (2)	FRCF

[a] Raw reference samples of *Salmo salar*, *Oncorhynchus mykiss* and *Salvelinus alpinus* were also supplied by IFREMER.

Both the SDS-PAGE and the urea-IEF procedures for the identification of species of cooked fish have recently been optimised (Etienne, Jérôme, Fleurence, Rehbein et al., 1999; Piñeiro et al., 1999) and subsequent collaborative studies on identifying unknown samples (Rehbein, Kündiger, Malmheden-Yman, 1999; Etienne et al., 1999) have demonstrated the suitability of the techniques for routine use in Food Control Laboratories.

As part of an additional study into the effects of different forms of processing on the protein profiles, smoked and gravad products of salmonids and smoked eels were analysed by these two techniques and by native isoelectric focusing of the water-soluble proteins. As the proteins in the products are denatured to different extents, depending upon the conditions used for the smoking or gravad processes, it was important to establish to what extent (if any) the protein profiles were altered and whether any confusion in the identification of species might result. It has already been demonstrated that, if aqueous extracts of smoked fish are dialysed prior to isoelectric focusing, it is possible to identify the species by reference to the profiles of raw reference species (Sotelo, Piñeiro, Gallardo & Pérez-Martín, 1992).

2. Materials and methods

2.1. Fish samples

Smoked and raw reference samples of fish were supplied by IFREMER, the Norwegian Institute for Fisheries and Aquaculture, (NIFA) and the Bundesforschungsanstalt für Fisherei (FRCF) as indicated in Table 1.

Salmo salar, *S. trutta* and *Oncorhynchus mykiss* were cold-smoked while Arctic char (*Salvelinus alpinus*) was hot-smoked; the smoked samples of the *S. salar, O. mykiss* and *S alpinus* were prepared by NIFA while the smoked *S. trutta* was produced by a smoke house in Nantes. The three eel species were hot smoked by a smoke house in Hamburg.

Commercial gravad samples of *S. salar* and *O. mykiss* were supplied by the National Food Administration (NFA), Sweden (Table 2).

In all cases, the samples were frozen prior to distribution, sealed in plastic bags and packed in insulated containers with dry ice. They were then transported by air freight and arrived in good condition at the participating institutes within 36 h of being despatched.

2.2. Analytical methods

Standard operation procedures which had been drawn up from earlier collaborative studies on SDS-PAGE (Piñeiro et al., 1999) and on urea-IEF (Etienne et al., 1999) were used. As the standard operation procedure for identifying the species of raw fish has not been published previously, it is described in detail below.

All three procedures required the same basic equipment, comprising a flat bed electrophoresis system (Multiphor II from Pharmacia Biotech or equivalent, an electrophoresis power supply to be run at least at 2000 V, a thermostatically controlled circulator, a homogeniser (Polytron or Ultra-Turrax) a centrifuge to be used at $20{,}000 \times g$, a spectrophotometer capable of measurement at 280 nm with quartz cuvettes, a rocking platform, and an image analysis system.

2.2.1. Protein determination

For all three analytical methods the protein contents of the extracts were determined by the OD_{280} procedure. The principle of this determination is based on the

Table 2
Gravad samples supplied by NFA

Species	Type of sample
Salmo salar	Reference
Salmo salar	Retail sample 1
Salmo salar	Retail sample 2
Oncorhynchus mykiss	Reference
Oncorhynchus mykiss	Retail sample 1
Oncorhynchus mykiss	Retail sample 2

assumption that, if a solution gives A at 280 nm of 1, this means that the protein concentration is 1.0 mg/ml. This method of protein determination requires that aqueous extracts are not frozen before analysis. Urea and SDS extracts can, however, be frozen prior to protein determination.

Fish muscle extracts, bovine serum albumin (BSA), standard solution (10 mg/ml in 0.2% (w/v) SDS solution) and reagent control without protein (extraction solvent) were diluted 20-fold with 0.2% (w/v) SDS solution. The absorbance was measured at 280 nm in a spectrophotometer and the protein content (P) of the extracts (mg protein/ml) was calculated using the equation

$$P_{\text{sample}} = (A_{\text{sample}} - A_{\text{blank}}) \times 20$$

As a control, the difference (A_{BSA}-A_{SDS}) should be close to 0.33.

2.2.2. Standard operation procedure for analysis of fish by SDS-PAGE

The procedures used for extraction of proteins in SDS solution and for electrophoretic analysis by SDS-PAGE were as previously described (Piñeiro et al., 1999).

2.2.2.1. Loading order. Solutions of samples and of molecular weight marker proteins were applied to the gels according to the protocol provided by the Rowett Research Institute.

2.2.3. Standard operation procedure for analysis of fish by urea-IEF

The procedures used for extraction of proteins in urea solution and for isoelectric focusing in urea-IEF were as previously described (Etienne, Jérôme, Fleurence, Rehbein, Kündiger, Malmheden-Yman et al., 1999).

2.2.3.1. Loading order. Solutions of samples and of pI marker proteins were applied to the gels according to the protocol provided by the Rowett Research Institute.

2.2.4. Standard operation procedure for the analysis of raw fish flesh by isoelectric focusing of water-soluble proteins using ampholine PAG plates

2.2.4.1. Extraction of proteins. The sample of fish muscle to be analysed was cut into small pieces and 500 mg were homogenised in 1.0 ml pre-cooled distilled water for 30 s in an ice-bath. The mixture was centrifuged at 4°C for 20 min at 20,000×g. The supernatant solution was removed and dialysed (dialysis membrane pore size 2.4 nm)-12–14 kDa cut-off, (BDH, 275/1270/01) at +4°C overnight against a large volume of pre-cooled distilled water. The concentration of protein was adjusted to 8 mg/ml with distilled water and the solution then transferred to Eppendorf tubes and stored at +4°C until analysed (24 h maximum).

2.2.4.2. Preparation of pI calibration solution. The lyophilised broad pI calibration kit (Broad pI kit, pH 3.5–9.3, — Amersham Pharmacia Biotech 17-0471-01) was reconstituted in 100 μl distilled water.

Conditions for isoelectric focusing. Instrument and gel preparation. The electrophoresis unit was connected to the thermostatic circulator and the temperature set to 10°C. After a small volume of kerosene was spread from the centre to cover the whole surface of the cooling plate of the electrophoresis apparatus, the gel (Ampholine PAG plates 3.5–9.5, Amersham Pharmacia Biotech 80-112480 or Servalyt Precote (Rehbein et al., 1995) was positioned on the plate, making sure that no air bubbles were trapped beneath it. Excess of kerosene was removed by means of paper towels.

The electrode strips were soaked in the respective solutions for anode (1 M H_3PO_4) and cathode (1 M NaOH) and applied with the correct polarity to the long edges of the gel.

The solutions of samples and pI marker proteins (10 μl) were applied to the gel at positions approximately 10 mm from the cathode using sample application pieces (Pharmacia 80-1129-46) in the order specified in a protocol prepared by RRI. Isoelectric focusing was then carried out for 1.5 h at 1500 V, 50 mA, 30 W. After 45 min the application pieces were removed to avoid any smearing of the gel.

Fixing and staining the gel. Immediately after isoelectric focusing, the gel was placed in a fixing solution of 11.6% trichloroacetic acid and 3.4% sulphosalicylic acid for 30–60 min. The gel was washed once with destaining solution (ethanol:acetic acid:water — 50:16:134) for 5 min. It was then stained for 10 min in staining solution: 1 tablet Phastgel Blue R (Amersham Pharmacia Biotech 17-0518-01) in 400 ml destaining solution, pre-heated to 60°C. The gel was destained by changing the destaining solution several times until the background was clear. The gel was finally soaked in the preserving solution (25 ml glycerol made up to 250 ml in destaining solution) for 1 h and finally covered with a cellophane preserving sheet (Amersham Pharmacia Biotech 80-1129-38) and allowed to dry at room temperature or in a gel air-drying system (Gel air-dryer-Bio-Rad, 65-1772).

3. Results

3.1. Smoked and gravad salmonids

3.1.1. SDS-PAGE

It can be seen (Fig. 1) that with the exception of *S. alpinus*, the profiles of the smoked and gravad samples

changed to varying extents from those of the corresponding raw reference profiles as a result of the processing. For *S. trutta* and *O. mykiss*, some band shifts in the molecular weight range, 36 kDa→55 kDa were observed when compared with the raw reference profiles. That of *O. mykiss* showed similar band shifts resulting from both smoking and gravad processing. Observations varied from one laboratory to another, however, with some reporting no differences from the raw reference profiles.

All laboratories reported that differentiation of smoked *S. trutta* and *S. salar* was not possible as the profiles were very similar. Some minor differences were observed in the profiles of the *Salmo* species and of *O. mykiss* but they were not sufficient to enable differentiation to be made with any confidence. Smoked *S. alpinus* could, however, be readily differentiated from the other species when processed.

It is evident that SDS-PAGE on its own is not sufficiently discriminating to enable the processed salmonids examined to be differentiated. Variations in the profile of *O. mykiss*, possibly due to polymorphism, added to the problem. Nonetheless, it is important to note that all the raw reference species could be differentiated. The discriminating power of SDS-PAGE is reduced for the processed samples because of essentially minor changes in the band profiles.

3.1.2. Urea-IEF

All laboratories found that the profiles of the processed samples were closely similar to those of the corresponding raw reference species (Fig. 2). That for *O. mykiss* could readily be differentiated from those of the *Salmo* species but differentiation of the profiles of the two *Salmo* species was very difficult. Indeed, some laboratories found no difference between the profiles of these two species either as raw or smoked samples. It was of interest that the *O. mykiss* retail sample 1 was clearly shown not to be *O. mykiss* but likely to be a *Salmo* species. Urea-IEF showed greater power of differentiating smoked/gravad *O. mykiss* and the *Salmo*

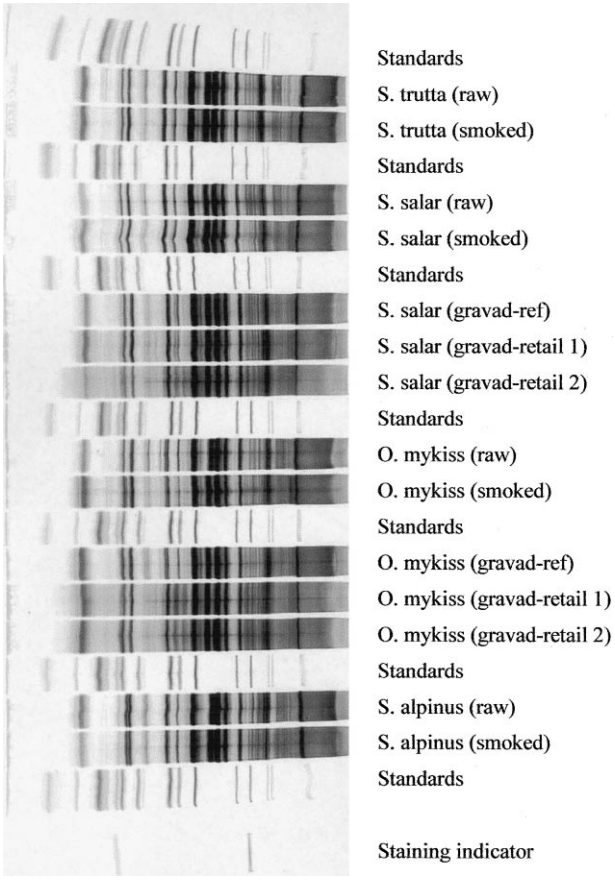

Fig. 1. SDS-PAGE: excel gel of raw, smoked and gravad salmonids.

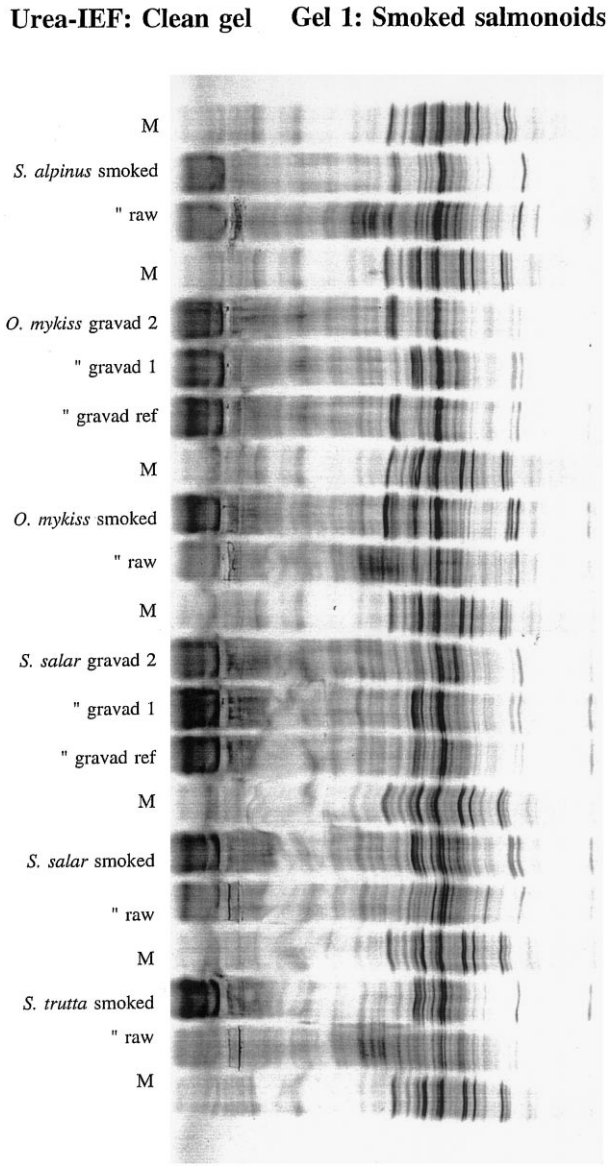

Fig. 2. Urea-IEF: clean gel of raw, smoked and gravad salmonids.

species than SDS-PAGE. However, *S. alpinus* and *O. mykiss* were more easily differentiated by SDS-PAGE.

3.1.3. Native isoelectric focusing

For all species, the treatments of either smoking or gravad production led to the disappearance of bands from the profiles (Fig. 3).

For the cold-smoked products, there was a selective loss of the heat-sensitive proteins, some of which are also species-specific. As a result, almost identical profiles were obtained for smoked *S. trutta*, smoked *S. salar* and gravad *S. salar*, making the species indistinguishable. The profiles of *O. mykiss* both smoked and gravad were closely similar but could be differentiated from those of the processed *Salmo* species. The profile of *O. mykiss*, gravad, retail sample 1, indicated that it was of a *Salmo* species, confirming the findings of urea-IEF analysis.

The effect of hot-smoking on *S. alpinus* was to denature most of the water-soluble proteins making the profile too faint to be of any value for species identification.

The profiles of all the raw reference samples could, however, be clearly differentiated.

3.2. Smoked eels

3.2.1. SDS-PAGE

In contrast to the profiles of the smoked salmonids which showed some changes resulting from smoking, those of the eels were indistinguishable from the profiles of the raw reference species (Fig. 4).

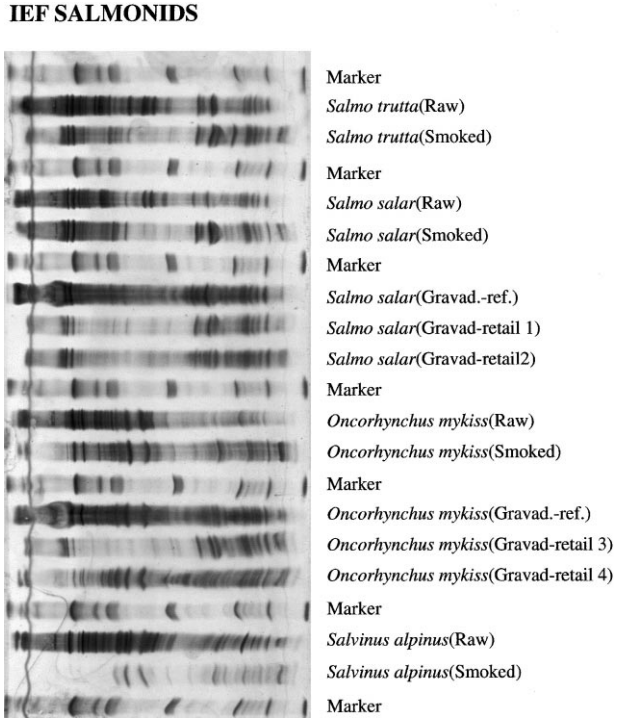

Fig. 3. Ampholine PAG plate, pH 3.5–9.5 IEF, of aqueous extracts of raw smoked and gravad salmonids.

All laboratories found that those of *A. anguilla* and *A. rostrata* were indistinguishable and that the profile for *A. australis* differed from the others significantly in having two characteristic narrower bands at two different positions.

3.2.2. Urea-IEF

Again, the profiles of the smoked products were found to be indistinguishable from those of the raw reference species (Fig. 5). Although the profiles of *A. anguilla* or *A. rostrata* were similar, most laboratories were able to differentiate them, while that of *A. australis* could readily be differentiated from the other two species.

3.2.3. IEF of water-soluble proteins

Although, as expected, the number of protein zones in the profiles of the smoked eels was reduced, it was found that an acidic band, characteristic of *A. australis* (Fig. 6) was not denatured on smoking and that its presence could be used to differentiate *A. australia* from *A. anguilla* and *A. rostrata*. The profiles of smoked *A. anguilla* and *A. rostrata* could not, however, be differentiated.

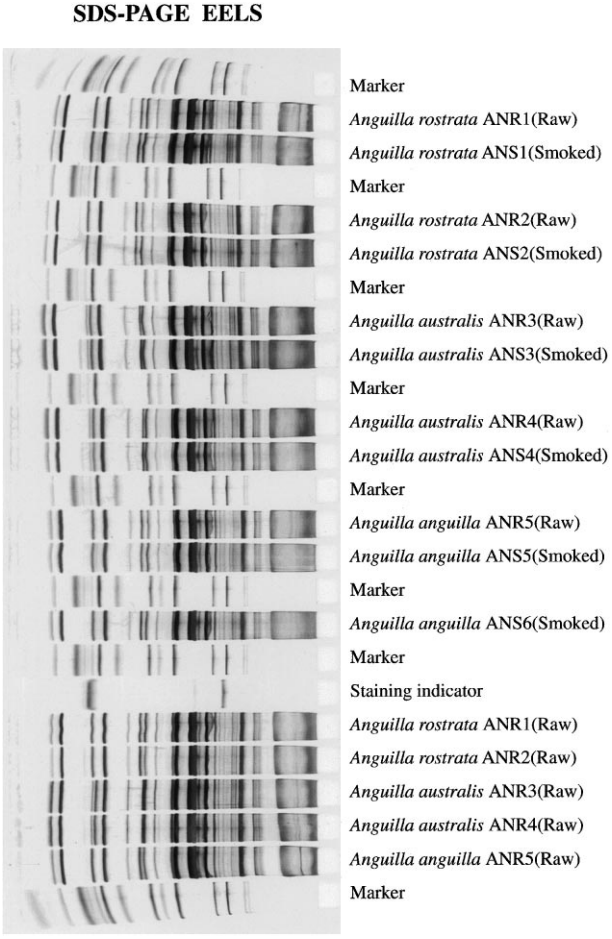

Fig. 4. SDS-PAGE: excel gel of raw and smoked eels.

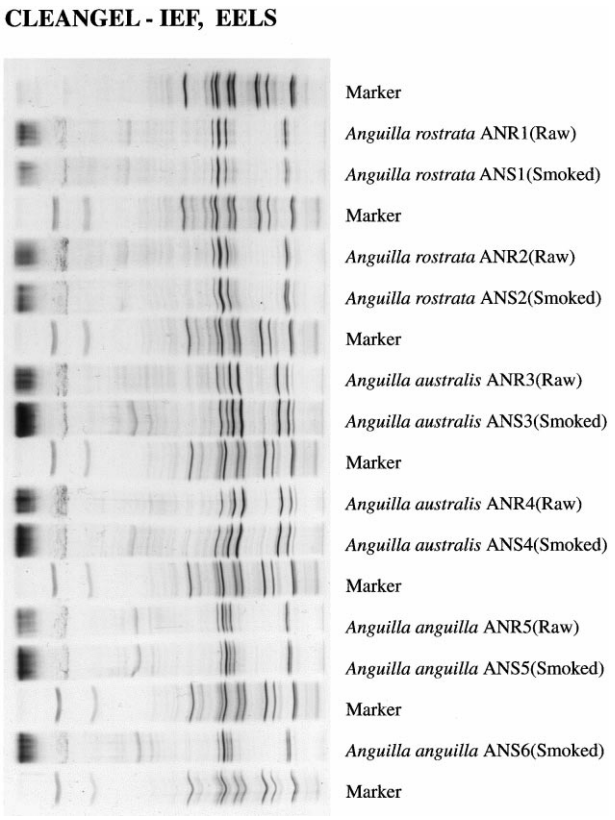

Fig. 5. Urea-IEF: Clean gel of raw and smoked eels.

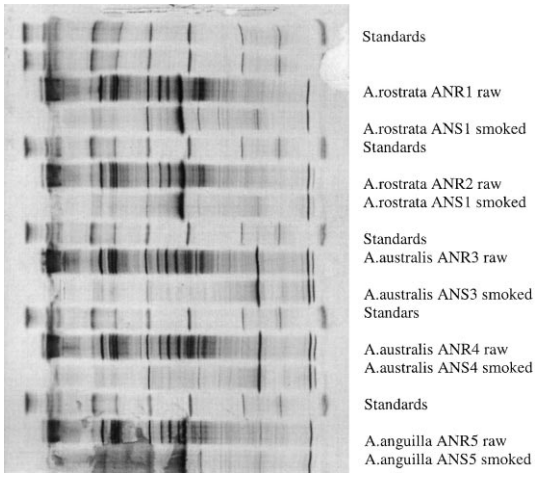

Fig. 6. Servalyte Precote pH 3–10 IEF of aqueous extracts of raw and smoked eels.

4. Conclusions

It appears that the effects of smoking or gravad processing are to introduce some relatively minor changes in the protein profiles obtained on SDS-PAGE and urea-IEF, making differentiation of closely related species more difficult. These changes apply particularly to the SDS-PAGE analysis.

Because of these problems, it is recommended that all three analytical systems be used to differentiate the *Salmo* and *Oncorhynchus* species.

Although more protein zones are obtained with SDS-PAGE than with urea-IEF, there are generally fewer that are species-discriminating. Urea-IEF is thus the preferred system for differentiating the *Salmo* and *Oncorhynchus* species. For some species-*S. alpinus* and *O. mykiss,* SDS-PAGE has greater differentiating power.

Native IEF cannot be used to differentiate smoked *S. trutta,* smoked *S. salar* or gravad *S. salar*. It is of limited value in differentiating *O. mykiss* and the salmon species when smoked or gravad processed but can be useful as a complimentary analysis.

For the eel species, it is recommended that both SDS-PAGE and urea-IEF are used. Urea-IEF has greater power than SDS-PAGE in differentiating the two Atlantic species — *A. anguilla* and *A. rostrata* and would be the preferred system. Native IEF can be used to differentiate *A. australis* from the other two *Anguilla* species as a species-identifying band remains after processing. However, as commercial conditions of smoking will vary, it is only recommended to provide supplementary information.

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References

An, H., Marshall, M. R., Otwell, W. S., & Wei, C. I. (1988). Electrophoretic identification of raw and cooked shrimp using various protein extraction systems. *Journal of Food Science, 53*, 313–318.

Association of Official Analytical Chemists (1995). *Official Methods of Analysis* (16th ed.). Vol II, 35.1.41. AOAC Official Method 980.16. Identification of fish species.

Etienne, M., Jérôme, M., Fleurence, J., Rehbein, H., Kündiger, R., Malmheden-Yman, I., Ferm, M., Craig, A., Mackie, I., Jessen, F., Smelt, A., & Luten, J. (1999). A standardised method for identification of raw and heat-processed fish by urea-isoelectric focusing: a collaborative study. *Electrophoresis, 20*, 1923–1933.

Etienne, M., Jérôme, M., Fleurence, J., Rehbein, H., Kündiger, R., Mendes, R., Pérez-Martín, R., Piñeiro-Gonzales, C., Craig, A., Mackie, I., Malmheden-Yman, I., Martinez, I., Jessen, F., Smelt, A. & Luten, J. (1999b). Fish species identification of cooked flesh by sodium dodecylsulphate, polyacrylamide gel electrophoresis and urea isoelectric focusing. (Manuscript submitted for publication.)

Mackie, I. M. (1979). A review of some recent applications of electrophoresis and isoelectric focusing in the identification of species of fish in fish and fish products. In J. J. Connell, *Advances in Fish Science and Technology* (pp. 444–450). Farnham, UK: Fishing News Books.

Mackie, I. M. (1997). Authenticity of fish. In P. R. Ashurst, & M. J. Dennis, *Food Authentication* (pp. 140–170). London: Blackie Academic and Professional.

Piñeiro, C., Barros-Velázquez, R., Pérez-Martín, R., Martinez, I.,

Jacobsen, T., Rehbein, H., Kündiger, R., Mendes, R., Etienne, M., Jérôme, M., Craig, A., Mackie, I., & Jessen, F. (1999). Development of a sodium dodecylsulphate polyacrylamide gel electrophoresis reference method for the analysis and identification of fish species in raw and heat-processed samples. A collaborative study. *Electrophoresis, 20*, 1425–1432.

Rehbein, H. (1990). Electrophoretic techniques for species identification of fishery products. *Zeitschrift für Liebensmittel-Untersuchung und Forschung, 191*, 1–10.

Rehbein, H., Kündiger, R., Malmheden-Yman, I., Ferm, M., Etienne, M., Jérôme, M., Craig, A., Mackie, I., Jessen, F., Martinez, I., Mendes, R., Smelt, A., Luten, J., Piñeiro, C., & Pérez-Martín, R. (1999). Species identification of cooked fish by urea isoelectric focusing and sodium dodecylsulphate polyacrylamide gel electrophoresis: a collaborative study. *Food Chemistry, 67*, 333–339.

Scobbie, A. E., & Mackie, I. M. (1988). The use of sodium dodecylsulphate polyacrylamide gel electrophoresis in fish species identification — a procedure suitable for cooked and raw fish. *Journal of the Science of Food and Agriculture, 44*, 343–351.

Seki, N. (1976). Identification of fish species by SDS polyacrylamide gel electrophoresis of the myofibrillar proteins. *Bulletin of the Japanese Society of Scientific Fisheries, 42*, 1169–1176.

Seki, N., Takayasu, M., & Kokuryo, H. (1980). Preparation of myosin light chains as an index for identification of fish species from raw muscle and cooked products. *Bulletin of the Japanese Society of Scientific Fisheries, 46*, 1001–1006.

Sotelo, C. G., Piñeiro, C., Gallardo, I. M., & Pérez-Martín, R. (1992). Identification of fish species in smoked fish products by electrophoresis and isoelectric focusing. *Zeitschrift für Liebensmittel-Untersuchung und Forschung, 195*, 224–227.