Use of Capillary Zone Electrophoresis for Fish Species Identification. Differentiation of Flatfish Species

José M. Gallardo,* Carmen G. Sotelo, Carmen Piñeiro, and Ricardo I. Pérez-Martin

Instituto de Investigaciones Marinas, CSIC, Eduardo Cabello 6, 36208 Vigo, Spain

A capillary zone electrophoresis (CZE) method is described for the analysis of fish sarcoplasmic proteins at low pH. Aqueous extracts of eight flatfish species, containing the sarcoplasmic proteins, were subjected to the action of an electric field in a capillary electrophoresis apparatus; the separated proteins were detected by means of an UV detector after less than 35 min. Acceptable resolution and reproducibility were obtained using low protein concentration (0.1 mg/mL) in the extracts analyzed. The CZE protein profiles that resulted were specific for each species and could be employed for differentiation and identification purposes.

Keywords: *Fish species identification; capillary electrophoresis; sarcoplasmic proteins*

INTRODUCTION

Identification of fish species in seafood products has become relevant because of the labeling regulations imposed by various countries (Mermelstein, 1993).

At the present time fish species identification in raw seafood products is usually carried out using different techniques of analysis of aqueous extracts (Rehbein, 1990; Mackie, 1979). Muscle aqueous extracts consist mainly of water soluble proteins, sarcoplasmic proteins, which account for 20–35% of total muscle proteins (Connell, 1968; Mackie, 1993). Most of these proteins are enzymes involved in metabolic processes; therefore, differences in amino acid composition of proteins should be expected in different species, and these differences are especially stressed in proteins performing specific functions which allow the particular exploitation of resources by different organisms. The techniques employed for detecting these differences are electrophoresis of proteins in denaturing conditions, which separates proteins on the basis of differences in molecular weights, isoelectrofocusing, which gives p*I* values of different protein bands, and reversed-phase chromatography, which relies on differences in protein hydrophobicity. As can be seen, all of the techniques employed for species determination take into account somehow the differences in amino acid composition of proteins.

Capillary electrophoresis (CE) is a relatively new technique which has a theroretical higher resolution than other instrumental analysis techniques, such as conventional HPLC. Analyses are quite rapid and reproducible. Quantitation, unlike other electrophoretic techniques, is performed on line, generally by means of UV detectors, and, in most cases, automation of the analysis is allowed. The high efficiency of CE is due to its characteristic flat flow profile, which allows for narrower peaks and therefore better resolution than conventional HPLC (Li, 1992). Another advantage is the low analysis cost; it does not employ large quantities of organic solvents. In fact, for most applications just milliliters of aqueous solvents are required. Also, in capillary zone electrophoresis (CZE), harmful substances, such as acrylamide, are not needed.

* Author to whom correspondence should be addressed (telephone 34-86-231930; fax 34-86-292762; e-mail gallardo@iim.csic.es).

Table 1. Common and Latin Names of the Flatfish Species Employed in This Study

common name	Latin name
megrim	*Lepidorhombus whiffiagonis*
sole	*Solea solea*
turbot	*Scophtalmus maximus*
brill	*Scophtalmus rombus*
four spotted megrim	*Lepidorhombus boscii*
witch	*Glytocephalus cynoglossus*
American plaice	*Hipoglossus platessoides*
European plaice	*Pleuronectes platessa*

Differences in electrophoretic mobility are expected due to differences in amino acid composition in different fish species. Although some methods for electrophoretic separation of proteins in capillaries have been developed (Grossman et al., 1989; Chen, 1991; Wu et al., 1992), the separation of sarcoplasmic proteins with the aim of species identification was not reported. The aim of the present work was to obtain reproducible water soluble protein profiles that could be useful for identification of fish species.

EXPERIMENTAL PROCEDURES

Apparatus. A capillary electrophoresis system P/ACE 2000 (Beckman, Palo Alto, CA) controlled with the System Gold (Beckman) was used.

Reagents. Cytochrome *c*, lysozyme, bovine serum albumin, and conalbumin were obtained from Sigma Biochemicals (St. Louis, MO), and *N,N*-dimethylformamide (DMF) was obtained from Merck (Darmstadt, Germany).

Procedure. *Fish Species.* Eight flatfish species were obtained from the local market (Table 1). Three specimens of each species were used in the study. Portions of the muscle from each individual were stored at −70 °C until required for analysis.

Sarcoplasmic Protein Extraction. Five grams of minced muscle was mixed with 10 mL of cold distilled and deionized (Milli-Q) water and homogenized with three bursts of 90 s in an UltraTurrax homogenizer. The mixture was then centrifuged (8000*g*, 15 min, 4 °C). Supernatant was taken and protein content determined by the Coomassie protein assay reagent (Pierce, Rockford, IL). Extracts were filtered through a 0.45 μm filter and diluted with 30 mM phosphate buffer, pH 2.44, to achieve a protein concentration of 1 mg/mL. Extracts were prepared fresh the same day of analysis.

0021-8561/95/1443-1238$09.00/0

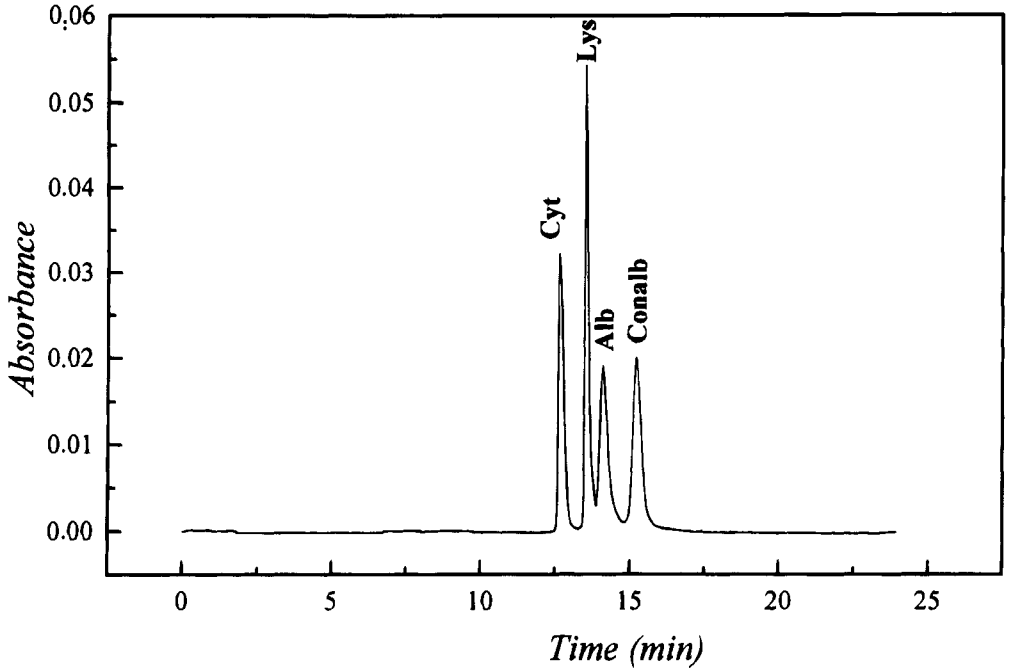

Figure 1. Separation of standard protein by CZE. Cyt, cytochrome; Lys, lysozyme; Alb, albumin; Conalb, conalbumin.

Table 2. Migration Time (MT) Mean, Standard Deviation (SD), and Variation Coefficient (VC%) and Percent Area, Standard Deviation (SD), and Variation Coefficient (VC%) of Each Standard Protein[a]

protein	MT mean	SD	VC%	% area	SD	VC%
cytochrome	12.8	0.08	0.63	21.35	1.58	7.38
lysozime	13.6	0.09	0.67	27.72	1.02	3.68
albumin	14.2	0.10	0.69	24.04	1.19	4.96
conalbumin	15.4	0.12	0.77	24.86	1.49	6.00

[a] Values shown are the result of eight replicate analyses.

Table 3. Turbot (*S. maximus*) Migration Time (MT) Mean, Standard Deviation (SD), and Variation Coefficient (VC%) of Each Peak

peak no.	MT mean	n[a]	SD	VC%
2	13.2	4	0.20	1.51
3	13.7	4	0.14	1.02
4	14.6	4	0.20	1.37
5	15.0	4	0.19	1.26
9	16.9	4	0.24	1.42
11	17.9	4	0.23	1.29
12	18.3	4	0.26	1.42
13	18.5	4	0.39	2.10
14	18.8	4	0.31	1.65
17	20.9	4	0.50	2.39

[a] n, number of individuals analyzed.

Capillary Electrophoresis. A 75 μm internal diameter and 57 cm total length fused silica capillary column from Beckman was used. The capillary was conditioned with a 1 M NaOH flush during 2 min, 0.1 M NaOH during 30 min, Milli-Q water during 5 min, 3 min flushes of phosphate buffer of decreasing molarity (100 and 75 mM phosphate buffer, pH 2.44), and finally a 35 min flush with 30 mM phosphate buffer, pH 2.44, which was also employed as electrolyte buffer.

Injection of the protein samples was carried out by a 3 s pressure injection. Capillary temperature was mantained constant at 25 °C. A three-step gradient voltage of 0–10 kV for 0.17 min, 10–15 kV for 22 min, and 15–25 kV for 1 min was employed. Proteins were monitored at 214 nm.

Between runs, the capillary was rinsed with 0.1 M NaOH for 3 min and with water for 1 min followed by a recondition step with the running buffer, 30 mM phosphate buffer, for 10 min.

Table 4. Megrim (*L. whiffiagonis*) Migration Time (MT) Mean, Standard Deviation (SD), and Variation Coefficient (VC%) of Each Peak

peak no.	MT mean	n[a]	SD	VC%
4	14.2	3	0.08	0.56
5	15.7	3	0.11	0.70
10	17.4	3	0.18	1.03
11	17.7	3	0.16	0.90
12	18.1	3	0.17	0.94
13	18.5	3	0.21	1.14
14	18.6	3	0.20	1.07
15	19.0	3	0.19	1.00
16	19.7	3	0.18	0.89
19	22.3	3	0.26	1.16

[a] n, number of individuals analyzed.

RESULTS AND DISCUSSION

The separation of sarcoplasmic proteins was carried out at low pH (pH 2.44). Most fish sarcoplasmic proteins have p*I* values higher than 2.44, and then, in theory, all sarcoplasmic proteins would be positively charged at 2.44 and would move toward the cathode edge, where the detector window is situated, when the electric field was established across the capillary (McCormick, 1988; Cobb et al., 1990). Another advantage of using low pH is reducing the adsorption of proteins to the capillary wall (Karger et al., 1989).

The injection of a neutral marker (0.01% DMF) showed a negligible electroosmotic flow; this marker passed the detector window after 52 min.

Reproducibility of separation was studied using a mixture of standard proteins (bovine serum albumin, lysozyme, cytochrome, and conalbumin). To improve the separation, standard proteins were solubilized in the electrode buffer (30 mM phosphate buffer, pH 2.44). Concentrations of 1 mg/mL of each protein were employed for these analyses. Figure 1 shows the separation obtained with the standard mixture; the peaks were identified by running each protein separately. Table 2 shows the reproducibility of the standards separation, migration time precisions (variation coefficient = stan-

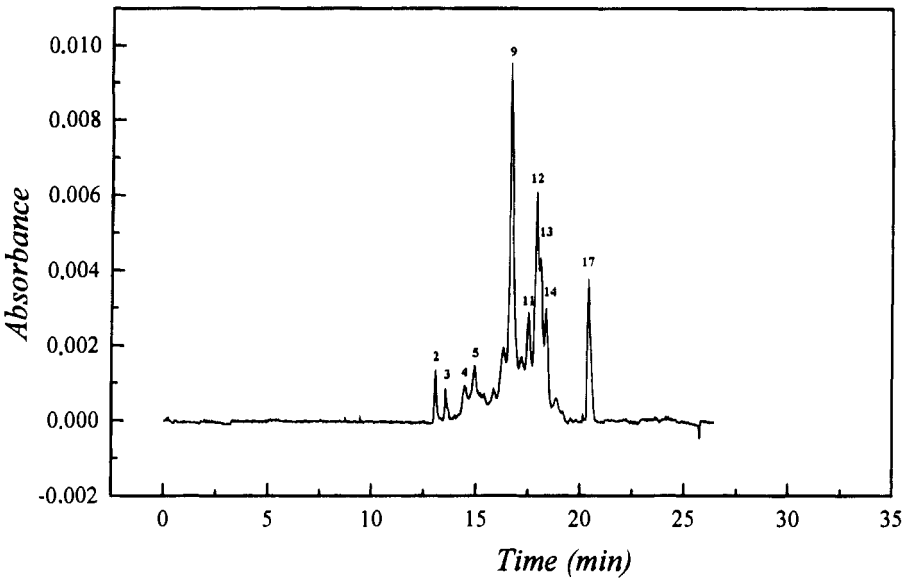

Figure 2. Separation of turbot (*S. maximus*) sarcoplasmic proteins by CZE. Peak numbers correspond to numbers in Table 3.

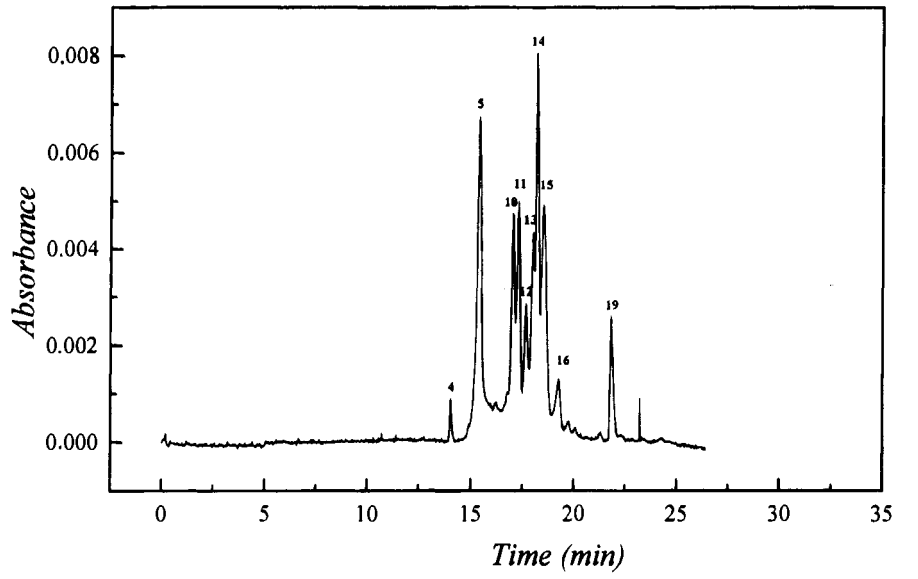

Figure 3. Separation of megrim (*L. whiffiagonis*) sarcoplasmic proteins by CZE. Peak numbers correspond to numbers in Table 4.

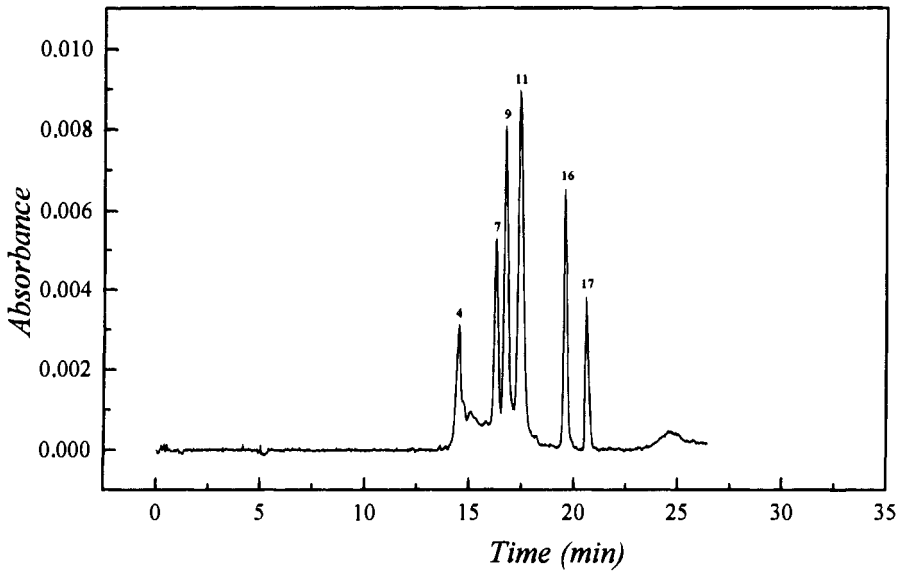

Figure 4. Separation of four spotted megrim (*L. boscii*) sarcoplasmic proteins by CZE. Peak numbers correspond to numbers in Table 5.

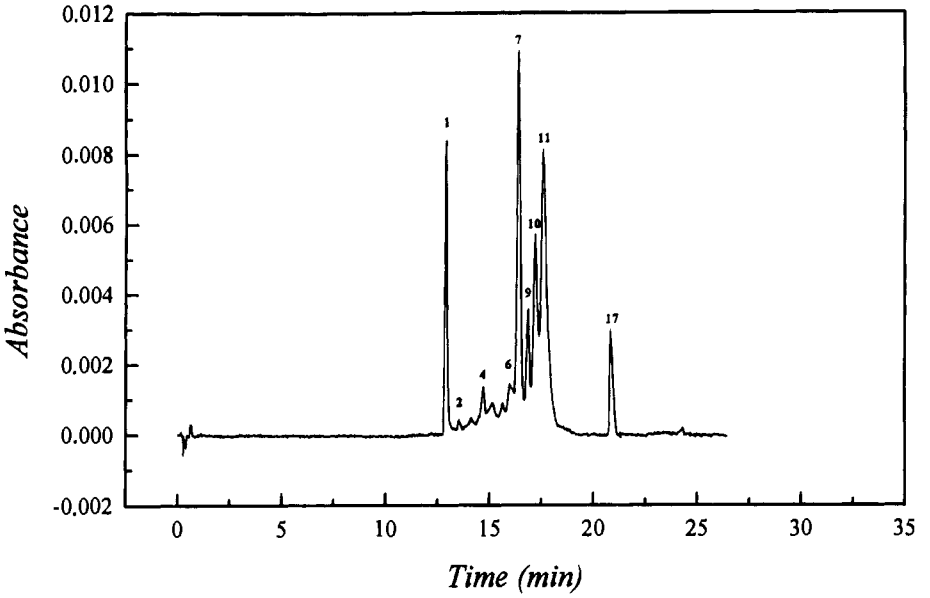

Figure 5. Separation of brill (*S. rombus*) sarcoplasmic proteins by CZE. Peak numbers correspond to numbers in Table 6.

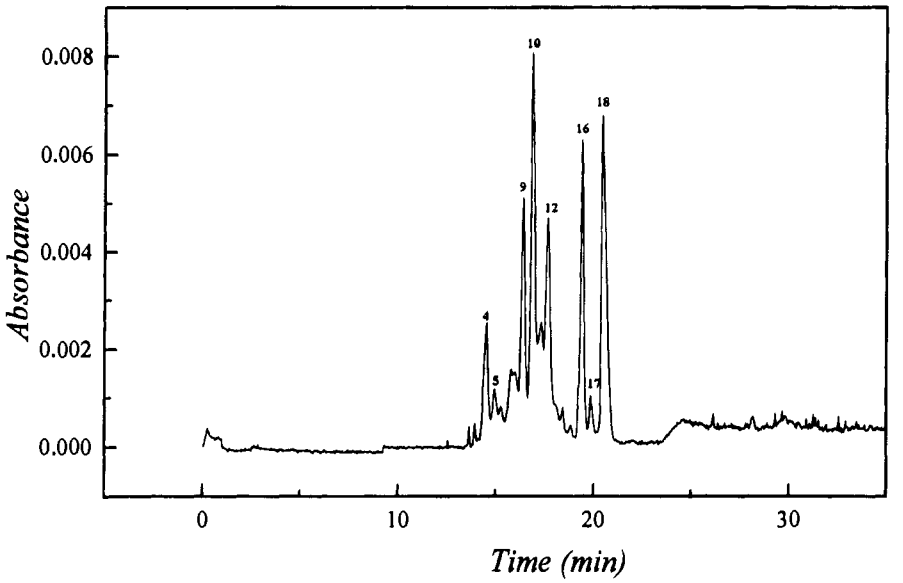

Figure 6. Separation of witch (*G. cynoglossus*) sarcoplasmic proteins by CZE. Peak numbers correspond to numbers in Table 7.

Table 5. Four Spotted Megrim (*L. boscii*) Migration Time Mean, Standard Deviation (SD), and Variation Coefficient (VC%) of Each Peak

peak no.	MT mean	n^a	SD	VC%
4	14.8	3	0.02	0.17
7	16.5	3	0.13	0.78
9	17.0	3	0.13	0.76
11	17.7	3	0.14	0.79
16	19.9	3	0.18	0.90
17	20.8	3	0.18	0.86

[a] *n*, number of individuals analyzed.

dard deviation/mean × 100) were all below 1%, increasing toward the end of the electropherogram. Quantitative reproducibility was also studied; variation was greater than migration time variation, and some proteins give percent area precisions above 5%.

The separation of these standard proteins was complete, and peaks are quite narrow, indicating that no significant protein–silica surface interactions have occurred. As can be seen in Figure 1, a complete separation of standard proteins was achieved in the first 20 min of the run.

Table 6. Brill (*S. rombus*) Migration Time Mean, Standard Deviation (SD), and Variation Coefficient (VC%) of Each Peak

peak no.	MT mean	n^a	SD	VC%
1	12.9	3	0.18	1.39
2	13.5	3	0.15	1.11
4	14.7	3	0.32	2.17
6	16.1	3	0.33	2.05
7	16.4	3	0.38	2.32
9	16.9	3	0.36	2.13
10	17.2	3	0.42	2.44
11	17.6	3	0.42	2.39
17	20.6	3	0.26	1.26

[a] *n*, number of individuals analyzed.

The separations of fish sarcoplasmic proteins of the eight flatfish species are shown in Figures 2–9. The employment of voltage ramps improves the separation of protein complex mixtures, as was shown already (McCormick, 1988); the gradual increase of voltage allows the attainment of equilibrium conditions slowly, thus improving the separation. It can be seen that

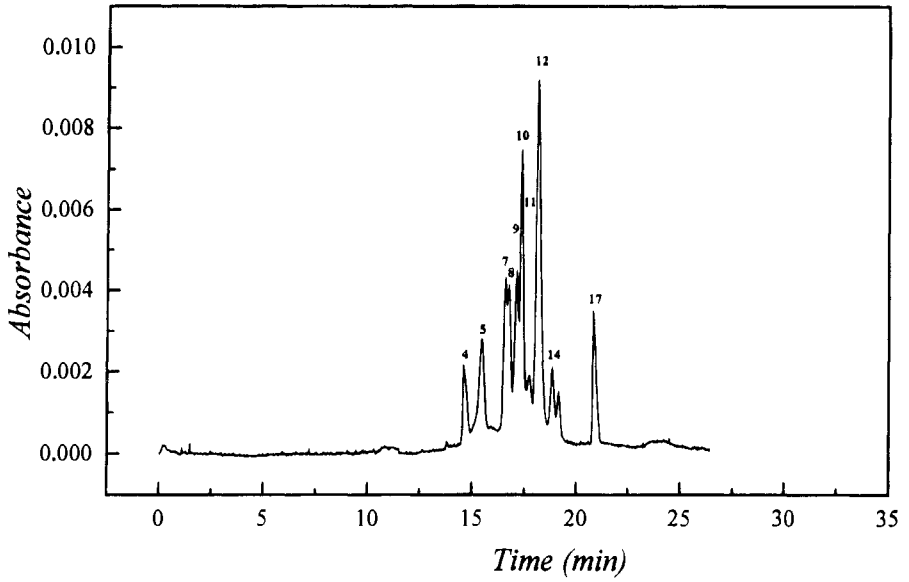

Figure 7. Separation of sole (*S. solea*) sarcoplasmic proteins by CZE. Peak numbers correspond to numbers in Table 8.

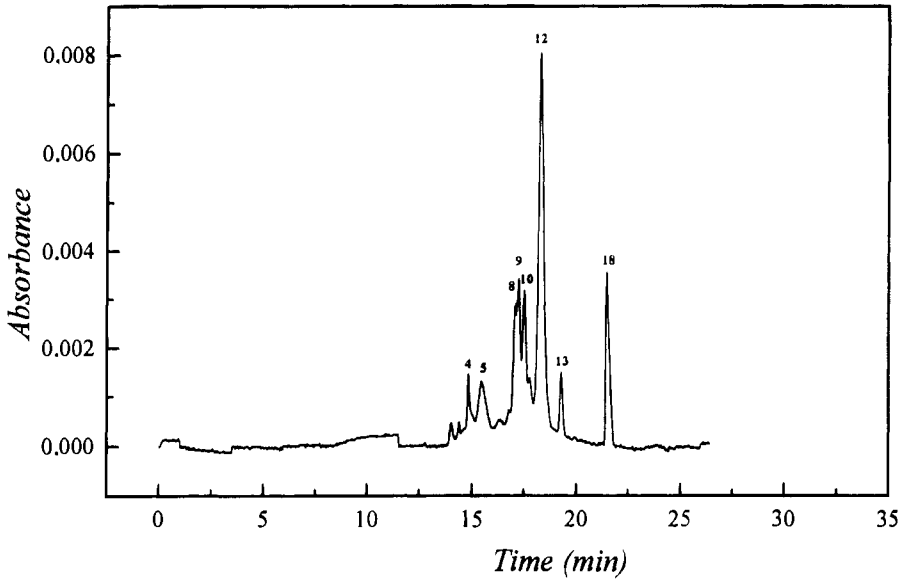

Figure 8. Separation of European plaice (*P. platessa*) sarcoplasmic proteins by CZE. Peak numbers correspond to numbers in Table 9.

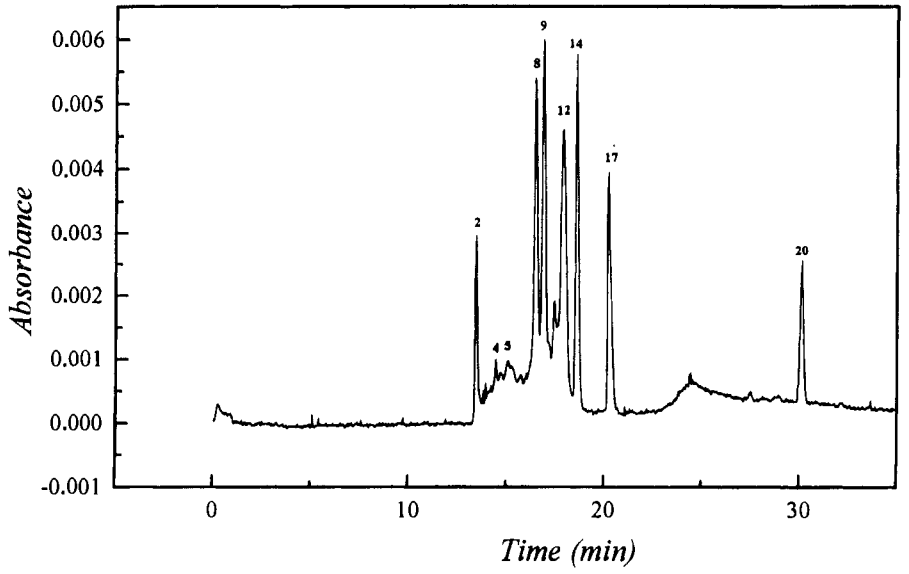

Figure 9. Separation of American plaice (*H. platessoides*) sarcoplasmic proteins by CZE. Peak numbers correspond to numbers in Table 10.

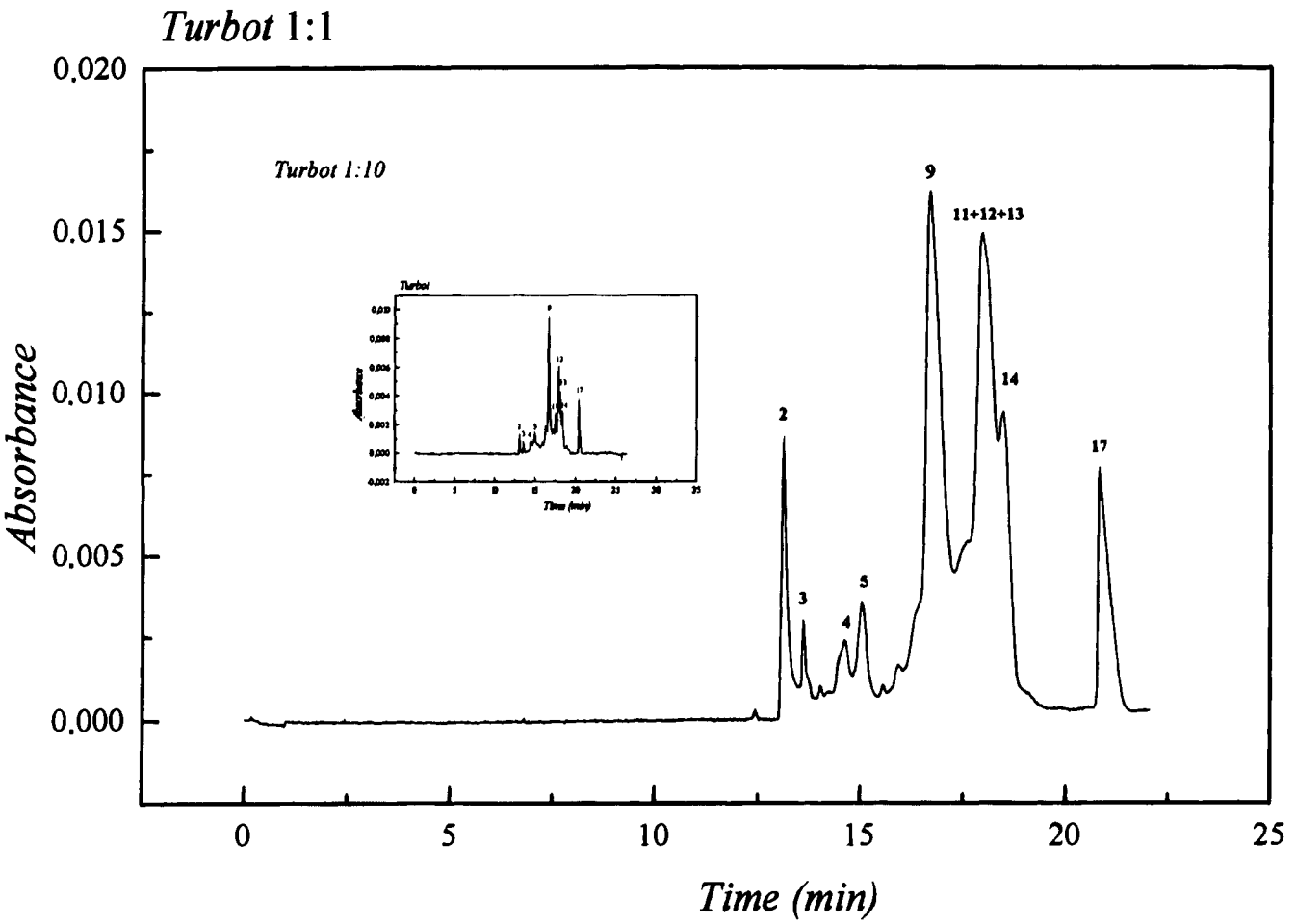

Figure 10. Comparison of turbot sarcoplasmic protein profiles at 1- (1:1) and 10-fold dilutions.

Table 7. Witch (*G. cynoglossus*) Migration Time Mean, Standard Deviation (SD), and Variation Coefficient (VC%) of Each Peak

peak no.	MT mean	n^a	SD	VC%
4	14.9	3	0.23	1.54
5	15.3	3	0.26	1.70
9	16.9	3	0.35	2.07
10	17.4	3	0.36	2.07
12	18.2	3	0.41	2.25
16	20.1	3	0.45	2.23
17	20.6	3	0.52	2.53
18	21.2	3	0.71	3.35

[a] n, number of individuals analyzed.

Table 8. Sole (*S. solea*) Migration Time Mean, Standard Deviation (SD), and Variation Coefficient (VC%) of Each Peak

peak no.	MT mean	n^a	SD	VC%
4	14.5	3	0.10	0.67
5	15.4	3	0.08	0.49
7	16.4	3	0.12	0.73
8	16.6	3	0.12	0.72
9	16.9	3	0.11	0.65
10	17.1	3	0.15	0.87
11	17.5	3	0.17	0.97
12	17.9	3	0.13	0.72
14	18.7	3	0.14	0.75
17	20.5	3	0.34	1.65

[a] n, number of individuals analyzed.

Table 9. European Plaice (*P. platessa*) Migration Time Mean, Standard Deviation (SD), and Variation Coefficient (VC%) of Each Peak

peak no.	MT mean	n^a	SD	VC%
4	14.7	3	0.35	2.38
5	15.0	3	0.54	3.59
8	16.7	3	0.58	3.46
9	16.9	3	0.52	3.07
10	17.2	3	0.51	2.95
12	17.9	3	0.59	3.29
13	18.5	3	0.14	0.75
18	21.0	3	0.92	4.36

[a] n, number of individuals analyzed.

Table 10. American Plaice (*H. platessoides*) Migration Time Mean, Standard Deviation (SD), and Variation Coefficient (VC%) of Each Peak

peak no.	MT mean	n^a	SD	VC%
2	13.5	3	0.36	2.66
4	14.6	3	0.32	2.18
5	15.2	3	0.52	3.41
8	16.6	3	0.62	3.72
9	17.0	3	0.70	4.10
12	18.1	3	0.91	5.00
14	18.7	3	0.91	4.85
17	20.3	3	1.03	5.08
20	30.8	3	1.98	6.43

[a] n, number of individuals analyzed.

when these conditions are used, the CZE protein patterns are specific. Also, each run could be completed in 40 min.

Sample dilution influences also the resolution of the different peaks. Figure 10 shows the comparison of the same extract injected 1- and 10-fold diluted; the separation is greatly improved using the 10-fold dilution of the raw extract. Some peaks (6–8) appear as only one peak in the 1-fold dilution. The effect of sample loading on

Table 11. CZE Migration Times for All of the Different Flatfish Species

peak no.	four spotted megrim	brill	turbot	megrim	witch	sole	E. plaice	A. plaice	peak no.	four spotted megrim	brill	turbot	megrim	witch	sole	E. plaice	A. plaice
1		12.9							11	17.7	17.6	17.9	17.7		17.5		
2		13.5	13.2					13.5	12			18.3	18.1	18.2	17.9	17.9	18.1
3			13.7						13			18.5	18.5			18.5	
4	14.8	14.7	14.6	14.2	14.9	14.5	14.7	14.6	14			18.8	18.6		18.7		18.7
5			15.0	15.7	15.3	15.4	15.0	15.2	15				19.0				
6		16.0							16	19.9			19.7	20.1			
7	16.5	16.4				16.4			17	20.8	20.6	20.9		20.6	20.5		20.3
8		16.9				16.6	16.7	16.6	18					21.2		21.0	
9	17.0		16.9		16.9	16.9	16.9	17.0	19				22.3				
10		17.2		17.4	17.4	17.1	17.2		20								30.8

capillary electrophoresis efficiency is explained by two mechanisms. One is related to the volume of sample injected and the other one to sample concentration. This second mechanism explains our results since the sample volume was the same in both cases. Sample concentration is related to the difference in electrical conductivity of the sample and the buffer. Perturbation of the potential field gradient can occur, and therefore the efficiency of the separation is affected with high sample concentrations (Li, 1992).

The reproducibility of the pattern was studied using three different individuals from each species. Tables 2–9 show the migration time mean, standard deviation, and precision of most of the peaks of the species studied. For most of the samples analyzed and peaks, variation was less than 2%, and all have less than 5%, which makes quite reproducible the patterns obtained.

The first remarkable difference between species is the number of peaks. Turbot, megrim, and sole present 10 peaks (Tables 3, 4, and 8, respectively); brill and american plaice, 9 peaks (Tabless 6 and 10); witch and European plaice, 8 peaks (Tables 7 and 9); and finally the four spotted megrim presented only 6 (Table 5).

Table 11 shows a comparison of the profiles in terms of migration times. It can be seen that although there are some peaks which could be considered common in all of the species, there are others that allow for the differentiation of the species.

In conclusion, the results presented here may be of utility for the differentiation and identification of fish species in refrigerated and frozen seafood products; the quantitation of individual proteins is also allowed.

ACKNOWLEDGMENT

We are indebted to Beckman Instruments España SA for technical support.

LITERATURE CITED

Asghar, A.; Pearson, A. M. Influence of ante- and postmortem treatments upon muscle composition and meat quality. *Adv. Food Res.* **1980**, *26*, 53–165.

Chen, F. A. Rapid protein analysis by capillary electrophoresis. *J. Chromatogr.* **1991**, *559*, 445–453.

Cobb, K. A.; Dolnik, V.; Novotny, M. Electrophoretic separations of proteins in capillaries with hydrolytically stable surface structures. *Anal. Chem.* **1990**, *62*, 2478–2483.

Connell, J. J. *Low temperature biology of foodstuffs*; Hawthorn, J., Rolfe, E. J., Eds.; Pergamon Press: Oxford, U.K., 1968; 333 pp.

Grossman, P. D.; Colburn, J. C.; Lauer, H. H.; Nielsen, R. G.; Riggin, R. M.; Sittampalam, G. S.; Rickard, E. C. Application of free-solution capillary electrophoresis to the analytical scale separation of proteins and peptides. *Anal. Chem.* **1989**, *61*, 1186–1194.

Karger, B. L.; Cohen, A. S.; Guttman, A. High-performance capillary electrophoresis in the biological sciences. *J. Chromatogr.* **1989**, *492*, 585–614.

Li, S. F. Y. *Capillary electrophoresis. Principles, practice and applications*; *Journal of Chromatography* Library; Elsevier: Amsterdam, 1992; Vol. 52.

Mackie, I. M. A review of some recent applications of electrophoresis and isoelectric focusing in the identification of species of fish in fish and fish products. In *Advances in fish science and technology*; Connell, J. J., Ed.; Fishing News Books: Farnham, Surrey, England, 1979.

Mackie, I. M. The effects of freezing on flesh proteins. *Food Rev. Int.* **1993**, *9*, 575–610.

McCormick, R. M. Capillary zone electrophoretic separation of peptides and proteins using low pH buffers in modified silica capillaries. *Anal. Chem.* **1988**, *60*, 2322–2328.

Mermelstein, M. H. A new era in food labelling. *Food Technol.* **1993**, *47*, 81–96.

Rehbein, H. Electrophoretic techniques for species identification of fishery products. *Z. Lebensm. Unters. Forsch.* **1990**, *191*, 1–10.

Wu, C.-T.; Lopes, T.; Patel, B.; Lee, C. S. Effect of direct control of electroosmosis on peptide and protein separations in capillary electrophoresis. *Anal. Chem.* **1992**, *64*, 886–891.

Received for review June 16, 1994. Revised manuscript received February 10, 1995. Accepted February 22, 1995.[⊗] We thank the Comisión Interministerial de Ciencia y Tecnología (CICYT) for the funding of the Project ALI 90-0773.

JF940325X

[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1995.