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Note

Identification of fish species by high-performance liquid chromatography

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Identification of fish species is essential for conforming with legal regulations, meeting quality control requirements, and establishing fair pricing policies.

The identity of a fish species may be determined by the general appearance and the morphological characteristics of the whole fish. However, when fish is cooked or processed into fillet or other fish products, morphological identification becomes unreliable.

The present methods for the identification of fresh and cooked fish are based on the separation of water soluble sarcoplasmic proteins of fish by gel electrophoresis[1–5], and isoelectric focusing[6–8]. Different fish species yield different electrophoretograms which can be compared visually for qualitative identification. The electrophoretograms may also be scanned in a densitometer for quantitative identification[6,7]. Gel electrophoresis, however, is time consuming, and involves many preparation steps.

Considering the inherent advantages of high-performance liquid chromatography (HPLC), and the recent advances in HPLC columns used for protein analysis, HPLC seemed suitable for the task of fish identification. In this report, the HPLC conditions used for the identification of seven fish species are presented.

MATERIALS AND METHODS

Apparatus

HPLC was performed with a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph equipped with Model 6000 A pump, Model U6K injector and a data module. An Autochrom (Milford, MA, U.S.A.) OPG/S Model 111-2 one pump gradient controller was used for gradient elution. The detector used was a Gilson (Middleton, WI, U.S.A.) variable-wavelength Model 222 set at 280 nm and sensitivity of 0.05 a.u.f.s. The HPLC column was 250 × 4.6 mm I.D. Hi-Pore reversed-phase column RP-304 (Bio-Rad Labs., Richmond, CA, U.S.A.).

Reagents

HPLC mobile phase. The water used in the preparation of HPLC solvents was double distilled and passed through 0.45-μm filter membrane (HPLC-grade water). Solvent A was 0.1% trifluoracetic acid (TFA), and solvent B was acetonitrile–water–

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TFA (95:5:0.1). A linear gradient of 40–70% solvent B in 90 min with a flow-rate of 1.5 ml/min was used.

Bovine serum albumin (BSA) standard solutions. Five standard solutions with concentrations of 2–16 mg/ml were prepared from analytical-grade BSA (Sigma, St. Louis, MO, U.S.A.) and HPLC-grade water.

Sample preparation

Fish samples were purchased frozen from local stores and were kept frozen until analysis. The frozen fish was then thawed and 80 g were blended with 100 ml of distilled water for 5 min in a blender at a low speed. The fish blend was filtered through Whatman No. 4 filter paper and a volume of 1% sodium azide solution was added to the filtrate to give a final sodium azide concentration of 0.01% for preservation. A portion of the filtrate was passed through a 0.45-μm filter membrane before injection into HPLC.

HPLC analysis

A volume of 25 μl of the fish filtrate was co-injected with 10 μl of a BSA standard solution (2 mg/ml). BSA was used as an internal standard for qualitative and quantitative analysis of fish filtrates. The relative retention time (relative to BSA peak) of all peaks was determined and the equivalent amount of protein under each peak (equivalent to BSA) was estimated from the BSA calibration curve.

BSA calibration curve

A 10-μl volume of each of the five BSA standard solutions was injected three times into the HPLC system. The average area units were then plotted against the amount of BSA in μg injected and a BSA calibration curve was constructed.

RESULTS AND DISCUSSION

The use of BSA as an internal standard proved to be necessary for the qualitative identification of fish species. Retention times of the various peaks in each chromatogram were related to that of BSA peak, and the relative rather than absolute retention times were used for qualitative comparison of the various chromatogram. BSA was also used in estimating the total amount of proteins under each chromatogram for the quantitative data. Since the identity of each peak was not known, it was assumed that all peaks were of water soluble proteins with equal response factor to BSA. This assumption did not interfere with the main objective of the study. The BSA peak did not overlap with any of the indigenous peaks except in salmon. BSA was not, therefore, coinjected with salmon extract.

The results obtained from the HPLC analysis of the water soluble proteins of seven fish species are shown in Table I, and chromatograms of four fish species are shown in Fig. 1. These data indicate that there are major qualitative and quantitative differences among the seven fish species analyzed by the HPLC method. Five of the seven fish species had their major peak at a different relative retention time (1.07, 1.21, 1.88, 2.24, 2.40 for salmon, cod, monk, trout and rockfish, respectively). Catfish and shark had their major peak at the same relative retention time (1.48) but with an area ratio of approximately 2 (Table I). Salmon was the only species with a major

peak at a relative retention time of 1.07, and trout had the only major peak at a relative retention time of 2.24.

The linear gradient time was increased to 90 min to obtain high resolution required for accurate quantitative data (Fig. 1). The overall analysis time is, however, much shorter than that of any of the electrophoresis procedures. In addition, the method does not involve numerous preparation steps required in gel electrophoresis.

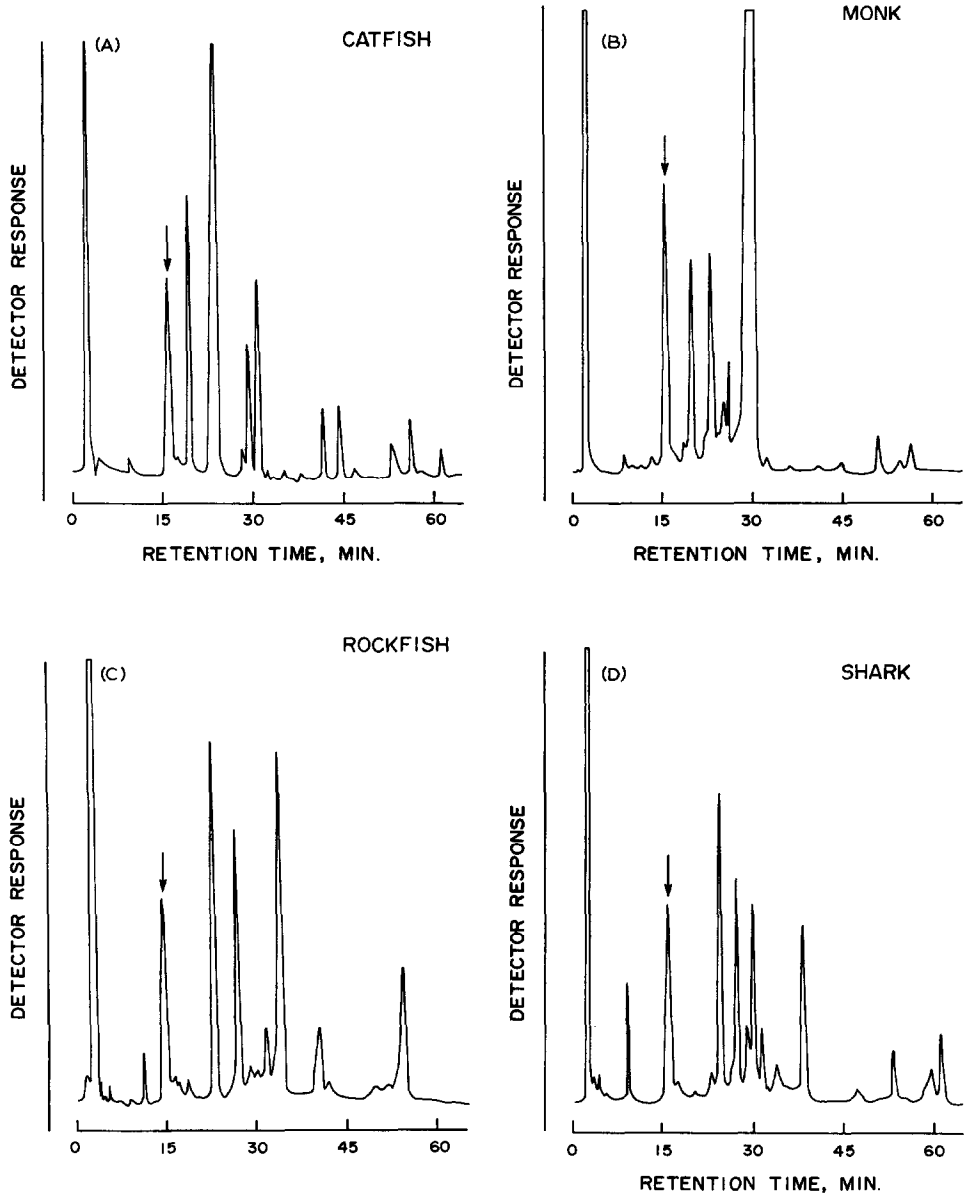

Fig. 1. HPLC chromatograms of selected fish species with BSA as the internal standard. The BSA is marked by an arrow.

TABLE I

ANALYSIS OF FISH WATER SOLUBLE PROTEINS WITH HPLC

*Relative retention time**	*Percent of total area*						
	Catfish	*Cod*	*Monk*	*Trout*	*Rockfish*	*Salmon*	*Shark*
0.57	**	—***	—	—	—	—	5.34
1.07	—	—	—	—	—	34.8	—
1.21	14.3	25.4	6.1	—	—	—	—
1.42	—	14.8	—	6.84	—	5.32	—
1.48	49.8	10.9	8.23	20.0	24.6	5.46	23.2
1.67	—	12.3	**	5.65	—	—	15.7
1.88	6.70	15.2	79.1	—	18.3	18.2	13.2
1.97	11.8	—	—	—	—	—	5.78
2.24	—	—	—	60.3	**	**	**
2.40	—	5.40	—	—	27.8	13.6	13.4
2.70	—	5.00	—	7.13	—	—	—
2.81	5.00	—	—	—	7.76	—	—
3.76	—	—	—	—	14.0	5.36	5.50
3.82	**	—	—	—	—	5.40	5.42

* Relative to retention time of BSA peak. Major peaks are underlined. Average of two determinations.

** Area is less than 5%.

*** Peak was not detected.

The most important advantage of the present method over the others is that it provides quantitative data needed for reliable identification of fish species. More fish species are being analyzed in our laboratory by the HPLC method, and it appears that the method is applicable to canned fish species and to all local fresh fish species. The method is also being used in our laboratory to determine the identity and the percentage of fish species in fish products and fabricated seafoods, *i.e.*, crab legs and crab flakes. Preliminary results are encouraging.

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