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Bestuur voor Landbouwkundig Onderzoek
**Rijkscentrum voor Landbouwkundig Onderzoek - Gent**
**RIJKSSTATION VOOR ZEEVISSERIJ - OOSTENDE**
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# Identification of cod fillets by HPLC: a screening method.

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Mededelingen van het Rijksstation voor Zeevisserij (C.L.O.-Gent)
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Summary.

Water soluble sarcoplasmic proteins of nine Gadoid species were chromatographed using a $C_4$ wide pore reversed phase column and gradient elution with trifluoracetic acid, water and acetonitrile. In most cases, nine peaks could be identified in cod (Gadus morhua), with four major peaks always present. Direct visual comparison of unknown chromatograms with a cod chromatogram kept in memory by the data processor allowed the elimination of most non-cod Gadoids. Appearance of peaks not found in cod confirmed this elimination. In case of doubt, however, confirmation by electrophoretic technics is recommended. HPLC appeared to be a quick screening technique for identifying cod by eliminating non-cod species.

Samenvatting

Wateroplosbare sarcoplasma-eiwitten van negen kabeljauwachtigen werden gechromatografeerd gebruik makend van een $C_4$ "wide pore reversed-phase" - kolom en gradiëntelutie met trifluorazijnzuur, water en acentonitril. In de meeste gevallen konden negen pieken in kabeljauw (Gadus morhua) worden geïdentificeerd. Hiervan waren vier hoofdpieken altijd aanwezig.
Rechtstreekse visuele vergelijking van onbekende chromatogrammen met een kabeljauwchromatogram die in het geheugen van de data processor werd opgeslagen liet de uitschakeling van de meeste niet-kabeljauw Gadiformes toe. Het verschijnen van pieken die niet in kabeljauw werden aangetroffen bevestigden deze eliminatie. In geval van twijfel echter is bevestiging met electroforetische technieken aangeraden. HPLC bleek een snelle screening techniek te zijn voor de identificatie van kabeljauw door het elimineren van niet-kabeljauw soorten.

## Introduction

Increasing amounts of fish fillets from different parts of the world are now being marketed in countries where they were unknown only a few years ago. Several cheaper "new" fish species however are often substituted for more expensive traditional ones creating a need for reliable identification methods of fish species.

This identification is usually carried out with electrophoretic techniques (Mackie 1980, Williams 1984). It necessitates a large collection of authentic reference electropherograms when an unknown species has to be identified. When on the other hand the question to be answered is "Is this (e.g.) cod ?", the problem is somewhat simplified, the identity of the possible non-cod fish species present being of less importance.

Due to diminishing catches and high prices of cod (Gadus morhua) other similar species are now frequently sold under the same name. In Belgium, this is especially the case with ling (Molva spp.), saithe (Pollachius virens), hake (Merluccius spp.), Alaska pollack (Theragra chalcogramma) and hoki (Macruronus novaezelandiae).

When a whole fillet or a large part of it is available, shape of the fish, flesh colour (e.g. dark for saithe) and the pattern of the myotomes may very often differentiate cod from other fish (Kietzmann et al. 1969). With small pieces however, or minced fish, this is not feasible. Also the fat content may give useful information. In cod it seldom exceeds 1 % whilst e.g. some hake species contain 3 % and more fat. It should further be taken into consideration that cod will not be replaced by more expensive fish species. This is also a limiting factor when screening fish species.

In most cases however, determination of the water soluble sarcoplasmic proteins is necessary. They show a species dependent pattern. Besides electrophoresis and isoelectro-focusing, HPLC has been proposed as an identification method for fish species (Ashoor and Knox 1985 ; Osman et al. 1987 ; Rehbein 1990a, b). The purpose of the present investigation was to evaluate the usefulness of HPLC for eliminating non-cod species as a screening test for identifying cod.

## Materials and methods.

- Fish : Nine Gadoid species were used :
  Cod (Gadus morhua), haddock (Melanogrammus aeglefinus), whiting (Merlangius merlangus), ling (Molva molva), saithe (Pollachius virens) and pout (Trisopterus luscus) were purchased fresh. Hake (Merluccius spp.), Alaska pollack (Theragra chalcogramma) and hoki (Macruronus novaezelandiae) were obtained in the frozen state.

- Extraction of sarcoplasmic proteins :
  Twenty grams of fish muscle were blended with 40 ml water for 30 sec (Waring blender) and filtered through a folded filter (Whatman No 4).

A portion of the filtrate was passed through a 0.45 µm filter membrane before injection into HPLC.

- Apparatus :
  HPLC was performed with a Varian Vista 5500 chromatograph (Sunnyvale, CA, U.S.A.) equipped with a PE Nelson data processor model 1020 (Norwalk, CT, U.S.A.).
  The HPLC-column was 250 x 4,6 mm I.D. $C_4$ Hi-Pore reversed-phase column RP-304 with guard column (Bio-Rad Labs, Richmond, CA, U.S.A.). High column efficiency should be maintained by frequent solvent cleaning and guard column changes.

- Reagents :
  HPLC mobile phase : solvent A was 0,1 % trifluoracetic acid (TFA) in water and solvent B as 0,1 % TFA in acetonitrile.
  Ribonuclease-solution (internal standard) (Rehbein 1990a,b): 4 mg/ml in water.

- Chromatographic conditions : according to Ashoor and Knox (1985) with modifications.
  A volume of 20 µl containing internal standard (0,5 ml per ml filtrate) was injected.
  A linear gradient of 5-90 % solvent B in 30 min with a flow rate of 1,7 ml/min was used. Column temperature was maintained at 30 °C.
  Detection was performed at 280 nm.

Results and discussion.

Thirty samples of cod were taken at random over a period of one year. They were caught in the southern and central parts of the North Sea.

In most cases, nine peaks could be identified (table 1, fig. 1-3. Four major peaks (No. 2, 3, 6, 9) were always present and could be considered as key peaks. The others were sometimes missing. The concentration of proteins (peak areas) varied greatly, with no clear pattern. Retention times (Rf-values) on the other hand were very reproducible giving narrow confidence intervals (table 1). Some overlapping was noted only between peaks 4 and 5 as well as between peaks 6 and 7. In some cases peak 3 was split into two only partially separated peaks (fig.3).

The internal standard ribonuclease showed a surprisingly lower reproducibility than the fish proteins. With the exception of No 8 and 9, the standard deviations of the Rf-values of the cod peaks were significantly lower than that of ribonuclease (F-test, 95 % probability). The reason for this is not clear. Therefore, relative retention times as applied by Ashoor and Knox (1985) and Rehbein (1990a) were not calculated. This internal standard however could be useful in case of changing performance of the HPLC column.

It was found that direct visual observation of the chromatograms gave the best results. Unknown samples were compared on the screen of the data processor with a cod sample chromatographed immediately beforehand and kept in memory.

The presence of peaks 2, 3, 6, and 9 was first ascertained. This allowed the elimination of most Gadoids under investigation in the present study. Appearance of peaks not found in cod confirmed this elimination (table 2, fig. 4 to 11).

It should be remarked that peak 3 was present in all Gadoids investigated.

Haddock and Alaska pollack showed the four key cod peaks. In this case however, visual quantitative evaluation gave additional information. In haddock (fig. 10) peak 3 was small (and split) whereas in cod it was always large. In Alaska pollack (fig. 11) peak 6 was distinctly smaller and it showed a large peak 7, which was often lacking in cod. Nevertheless, in such cases a confirmation by electrophoretic techniques is recommended.

As a result of the rather large variation found in the concentration of cod proteins, some additional parameters were investigated. Fig. 1 to 3 show chromatograms of cod of increasing size. No clear differences were noted.

The influence of the degree of freshness was also tested. A cod fillet was kept for 3 days at room temperature. From fig. 12 it appears that spoilage did not significantly change the chromatographic pattern.

The possible influence of frozen storage was not studied. However, according to Osman et al. (1987), qualitative differences are limited. Quantitative changes however, especially the loss of high molecular weight proteins appear to occur (Leblanc and Leblanc 1989).

Although further work on a larger number of samples, including fish from different fishing grounds and seasons, should be carried out, HPLC as a screening method for identifying cod by eliminating non-cod species appears to be useful. The HPLC procedure has several advantages, i.e. quick equipment preparation, speed of component separation and ability to rapidly assess the soluble protein profile.

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Table 1. Retention times (min) of cod protein peaks (a)

| Peak (b) | Average | s | CI |
|---|---|---|---|
| Ribonuclease (c) | 12,61 | 0,223 | 12,16-13,06 |
| 1 | 15,19 | 0,115 | 14,96-15,42 |
| 2 | 16,05 | 0,086 | 15,87-16,22 |
| 3 | 16,64 | 0,093 | 16,46-16,83 |
| 4 | 17,08 | 0,086 | 16,91-17,25 |
| 5 | 17,28 | 0,092 | 17,07-17,44 |
| 6 | 18,38 | 0,079 | 18,22-18,54 |
| 7 | 18,66 | 0,107 | 18,45-18,88 |
| 8 | 19,52 | 0,181 | 19,15-19,88 |
| 9 | 20,42 | 0,180 | 20,06-20,78 |

(a) s = standard deviation.
CI = Confidence interval of Rf-value (95) %.
(b) Main peaks underlined.
(c) Internal standard.

Table 2. Absence (-) of main cod peaks (No 2,3,6,9) and presence (+) of additional non-cod peaks in some Gadoids (a).

| | Peak No | | | | | | |
|---|---|---|---|---|---|---|---|
| | 2 | 2b | 3 | 5b | 6 | 7b | 9 |
| Hake | | + | | | | + | - |
| Saithe | - | + | | | | | |
| Whiting | | | | + | - | | |
| Ling | | | | + | - | | |
| Hoki | | | | + | | | - |
| Pout | | + | | + | - | + | |

(a) Rf peak 2b : 16,32-16,39
Rf peak 5b : 17,47-18,20
Rf peak 7b = 18,90-18,95

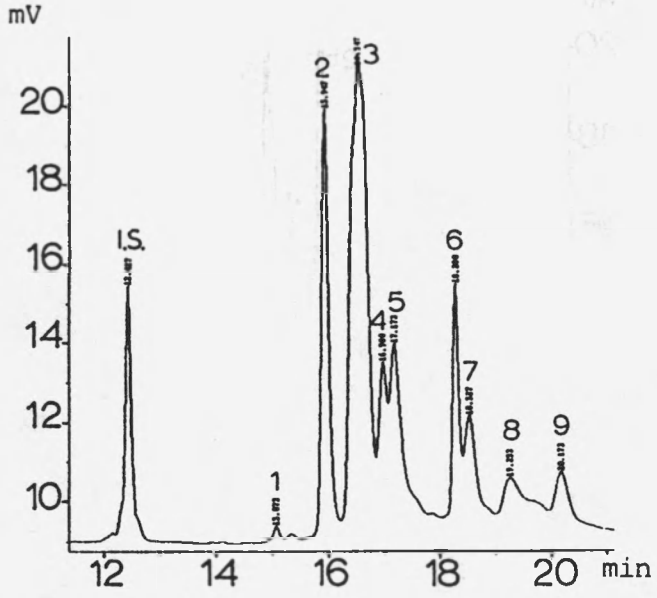


Fig. 1. Cod - 48 cm - 1,1 kg
2 years

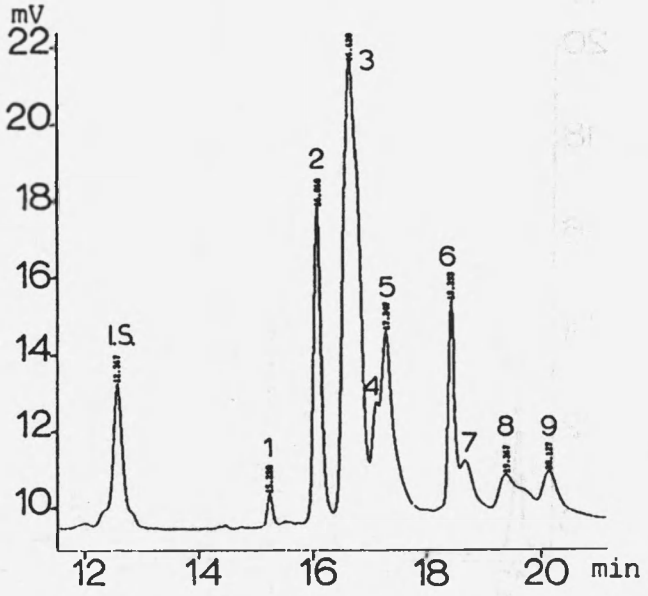


Fig. 2. Cod - 70 cm - 3,1 kg
3 years

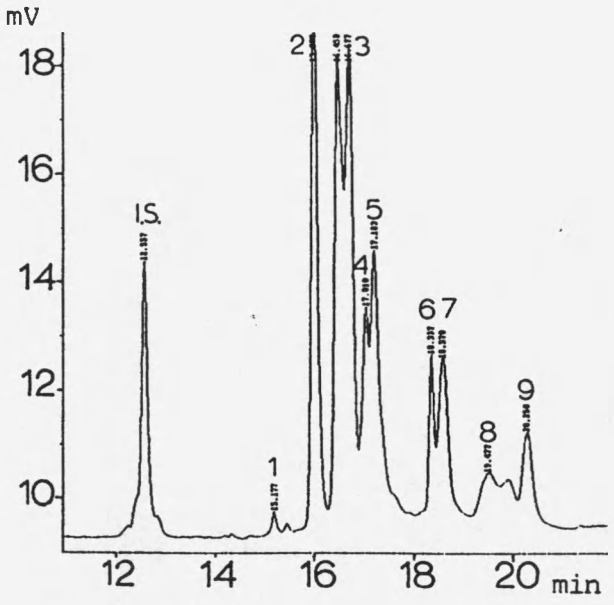


Fig. 3. Cod - 83 cm - 5,1 kg
4 years

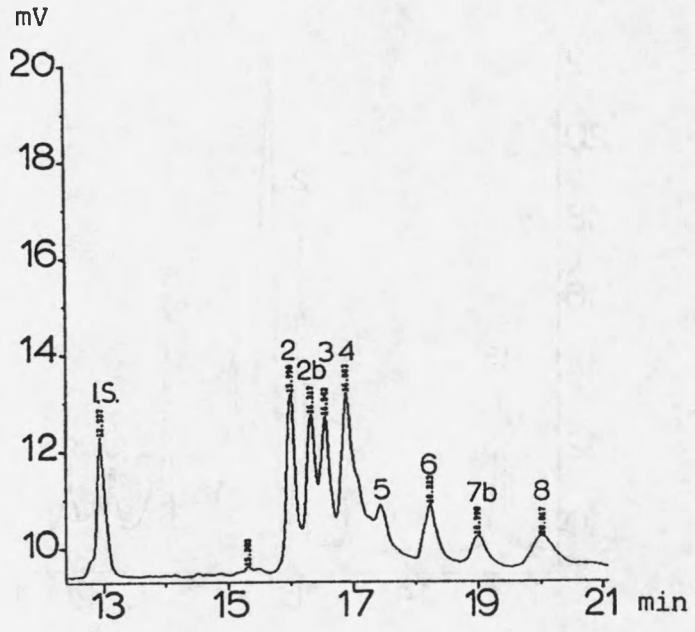


Fig. 4 Hake

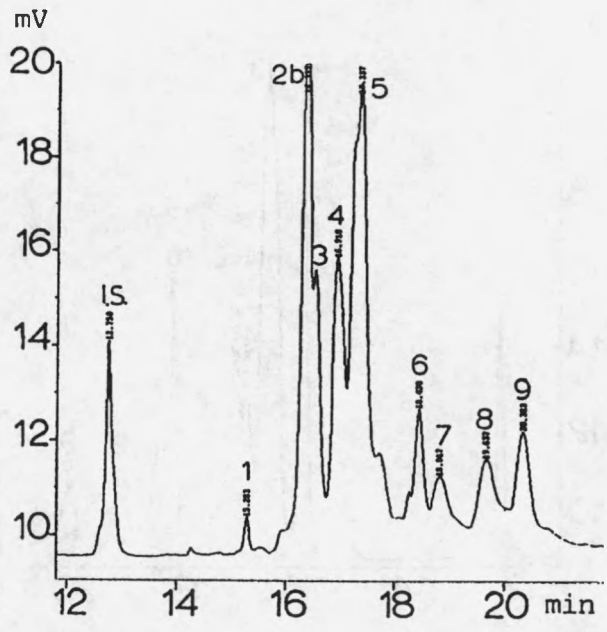


Fig. 5 Saithe

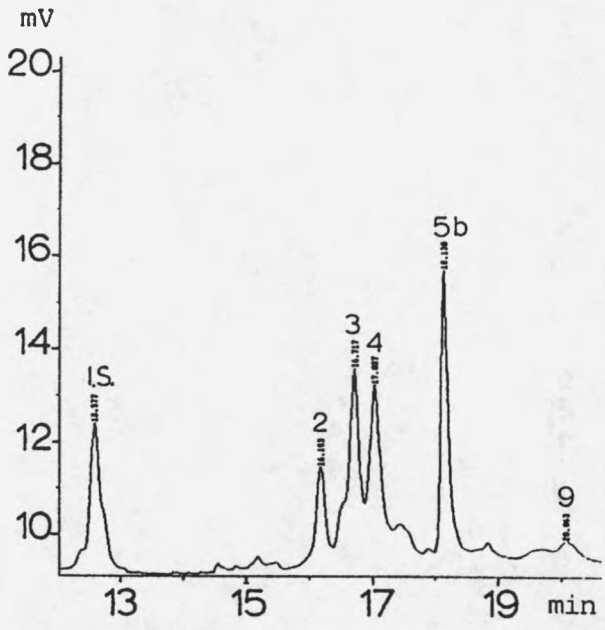


Fig. 6 Whiting

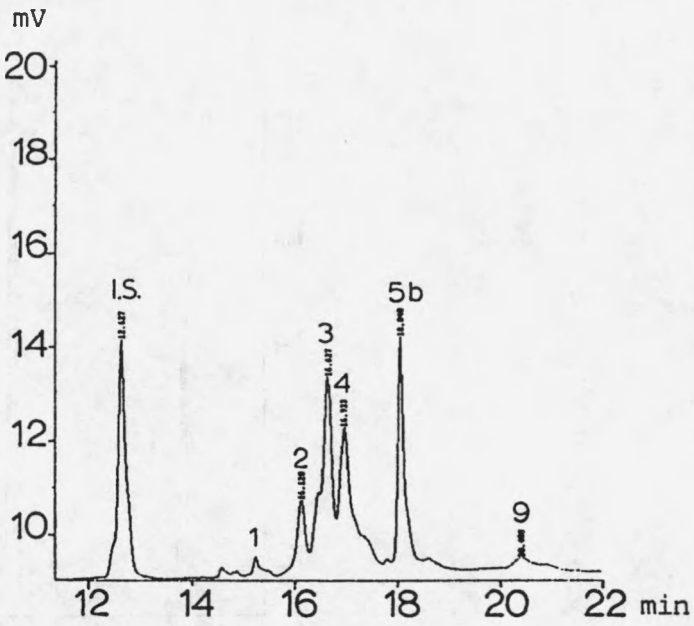


Fig. 7 Ling

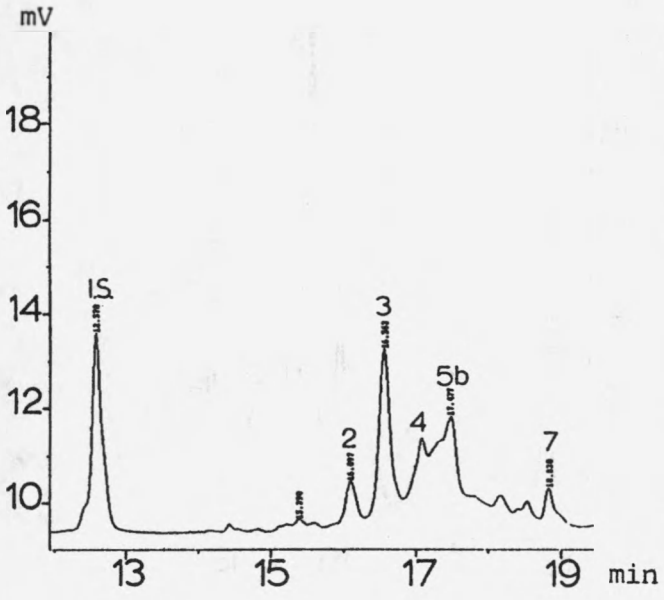


Fig. 8 Hoki

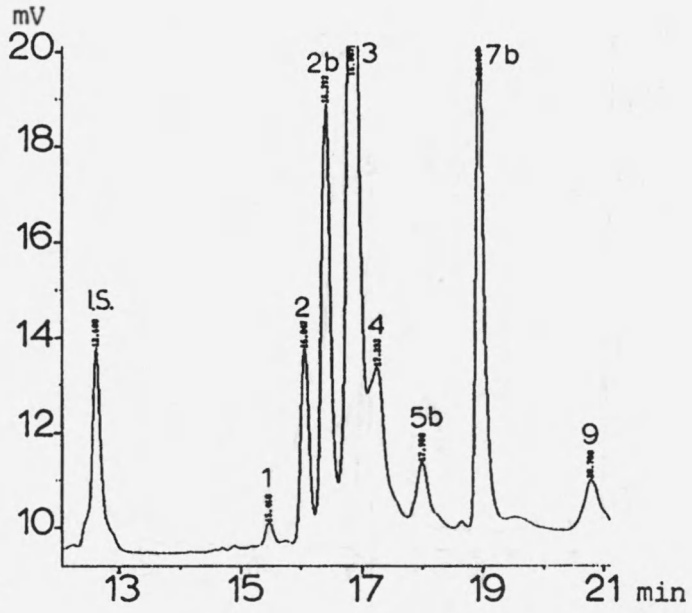


Fig. 9 Pout

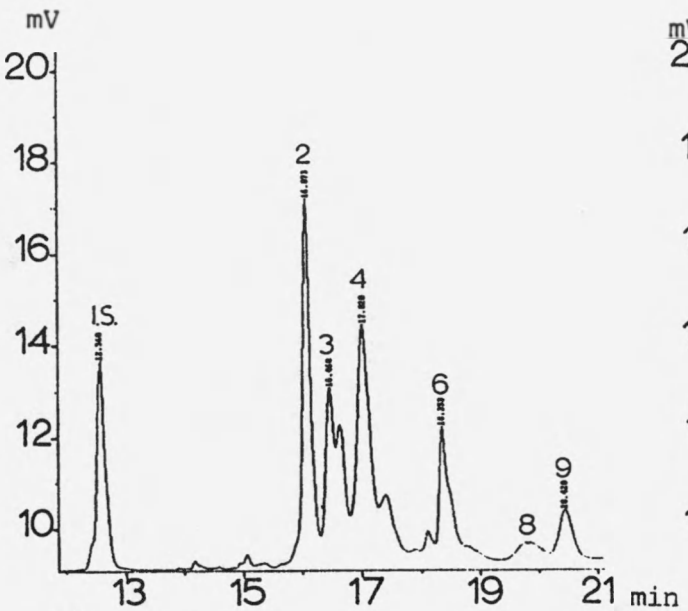


Fig. 10 Haddock

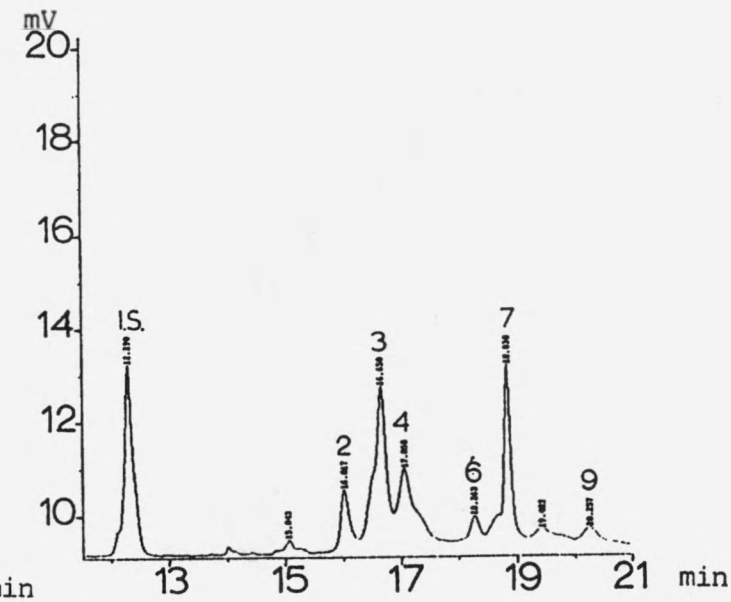


Fig. 11 Alaska pollack

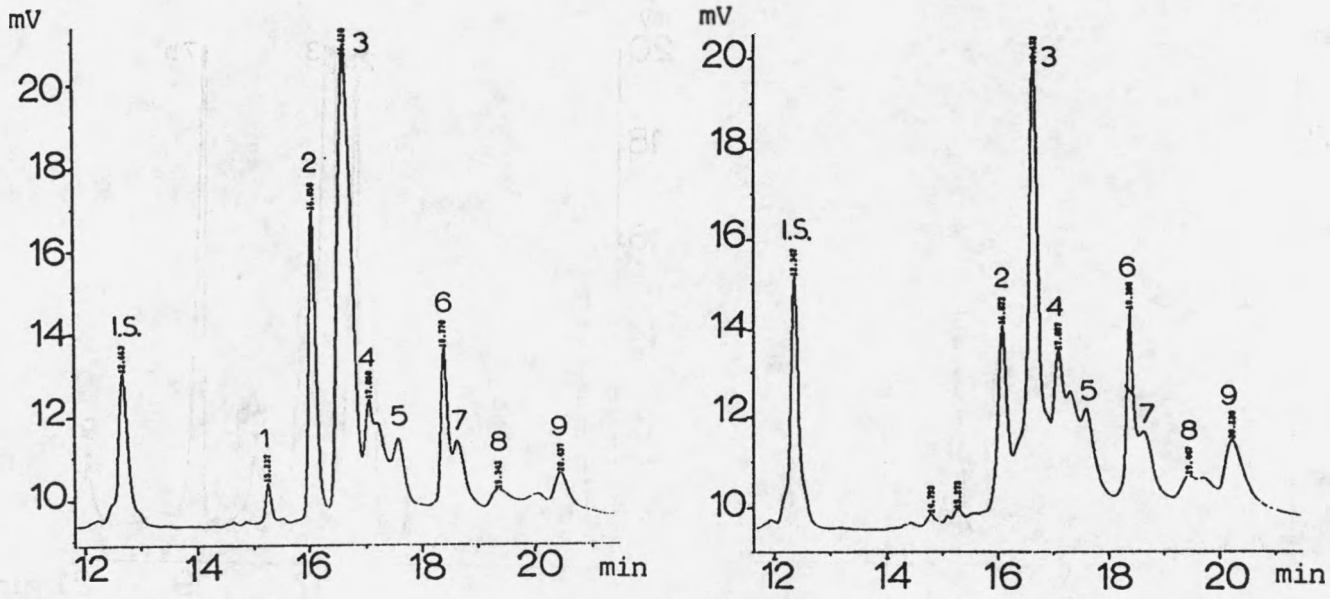


Fig. 12 Cod 0 day (left) and after 3 days At room temperature (right)