A method for determining free fatty acids (FFA) in marine plankton and sediments by means of high performance liquid chromatographic analysis of their p-nitrobenzyl esters*

OCEANOLOGIA, No. 34
pp. 57–68, 1993.
PL ISSN 0078–3234

Free fatty acids (FFA)
p-nitrobenzyl esters
High performance liquid
chromatography (HPLC)
Marine plankton
Sediments

LEONARD FALKOWSKI, KSENIA PAZDRO,
JOLANTA LEWANDOWSKA
Institute of Oceanology,
Polish Academy of Sciences,
Sopot

CHRISTOPHER OSTERROHT
Institut für Meereskunde
an der Universität Kiel,
Germany

MAREK ŚLEBIODA
Institute of Organic Chemistry,
Technical University,
Gdańsk

Manuscript received June 23, 1993, in final form November 1, 1993.

Abstract

A method for determining free fatty acid content in marine plankton and sediments by HPCL analysis of their p-nitrobenzyl esters has been elaborated. The acids were extracted from lyophilised samples of plankton or sediment with a 2:1 chloroform-methanol mixture. They were isolated from the remaining lipids by extraction with alkaline water and reextraction, after acidification, with chloroform. The

* The investigations were part of a basic research programme of the Institute of Oceanology PAS, partially supported by the Polish Committee of Scientific Research, grant no. 6076899101.

solvent was evaporated, the remaining substances dissolved in dichloromethane (DCM) and estrified with N,N′-dicyclohexyl-O-p-nitrobenzyl isourea (DcH-pNB-iU). The reaction mixture was separated on a silicagel pad using a cyclohexane-ethyl acetate mixture, the fractions containing the esters were evaporated, dissolved in an appropriate solvent and analysed by means of HPLC.

1. Introduction

This paper is a part of a research project involving the identification and determination of biologically active substances in the marine environment. Particular attention has been given to fatty acids, common in every part of this environment (Ackman, 1989; Dawson and Liebezeit, 1981; Joseph, 1989; Morris and Culkin, 1989; Sargent, 1976). A number of methods for their identification and quantitative determination have been developed, in particular gas chromatography (GC) and high performance liquid chromatography (HPLC) (Christie, 1982, 1987; Hulshoff and Lingeman, 1985). Because the fatty acids in most natural samples are structurally similar and present in low concentrations, their separation from such samples and their subsequent analysis are difficult. Progress in analytical chemistry as well as in sampling and clean up procedures makes the search for new, simpler, more accurate methods necessary.

The paper describes a method of determining the free fatty acids in marine plankton and sediments by the HPLC analysis of their p-nitrobenzyl esters.

2. Experimental

2.1. Sampling

The plankton was taken from the Gulf of Gdańsk on three different occasions in June 1992 using a standard WP–2 net. Three sediment replicates were collected from the Pomeranian Bay (N 54° 04', E 14° 16') in June 1992 with a Van Veen grab. The samples were deep-frozen on board ship and lyophilised in the laboratory.

2.2. Instrumentation and materials

Lyophilisation was performed in a Chris Beta A apparatus. The UV spectra were measured on a Beckman DU Series 60 spectrophotometer. The HPLC instrumentation was by Hewlett Packard and consisted of a 1050 pump, a UV–VIS detector, an HP 3396 Series II integration recorder and a rheodyne injector with a 20 μl loop.

All the chemicals were analytical grade; the solvents were distilled prior to application and checked for UV-absorbing impurities. The N,N′-dicyclohexyl-carbodiimide and p-nitrobenzylalcohol were purchased from

Merck, the fatty acids from Aldrich. The N,N′-dicyclohexyl-O-p-nitrobenzyl isourea was prepared according to Hoffmann (1988).

2.3. Extraction procedure

A 1 g sample of plankton or sediment was disintegrated in 5 ml of 2:1 chloroform-methanol at room temperature for 5 min., centrifuged, the supernatant decanted and the deposit extracted once more in the same way. The organic solutions were combined and then extracted three times with 3 ml of water buffered to pH 12. Following this, these aqueous solutions were combined and acidified to pH 3 and extracted 3 times with 5 ml of chloroform. Finally, the combined organic phases were washed with water, dried over 4 Å molecular sieves, evaporated in a stream of argon and the remainder dispersed in 1 ml of dichloromethane (DCM).

2.4. Estrification procedure

The reagent solution was prepared by dissolving 358.5 mg (1 mM) of DcH-pNBt-iU in 10 ml of DCM and stored in a refrigerator at 4° C. Stock solutions of fatty acids were prepared in DCM at concentrations of 100 μM ml^{-1}. 0.5 ml of DCM, 100 μl of appropriately diluted FA solution or the plankton or sediment extract and 1 ml of reagent solution were poured into a 5 ml dark, teflon screw-cap bottle. The reaction was observed using TLC on Kieselgel GF_{254} plates. The spots of reagent and esters were located on illumination with UV light, the nonsubstituted acids on spraying the plate with a solution of cerium sulphate and sodium phosphomolybdate in 10% sulphuric acid and heating it to 110° C. The mixture was stored overnight at room temperature, after which the solvent was evaporated in a stream of argon. The substance remaining was dissolved in 5 ml of hexane-ethyl acetate mixture and passed through a silicagel pad. The fraction containing fatty acid esters was dried in a stream of argon and dissolved in an appropriate solvent – acetonitrile for reversed phase chromatography or DCM for normal phase system – and analysed by HPLC.

2.5. Chromatography

Reversed-phase chromatography was performed by applying a Lichrospher 100 RP–18,5 μ 250 × 4 mm column and an acetonitrile-water solvent system with isocratic or gradient flow. For normal-phase chromatography a Lichrospher Si 60 6 μm 250×4 mm column and an 80:10:2 hexane-chloroform-isopropanol solvent system were used. Both types of columns were provided with appropriate pre– and guard columns. The unknown esters in the samples were identified by means of internal standards and

quantified by the establishment of a correlation between the peak area of the ester in question and the amount of it introduced into the column.

3. Results and discussion

Free fatty acids occur commonly in the marine environment (Ackman, 1989; Dawson and Liebezeit, 1981; Joseph, 1989; Morris and Culkin, 1989; Sargent, 1976). Their presence in nature in low concentrations and the fact that this group of compounds possesses very similar properties makes their identification and quantitative determination very difficult. Until quite recently, their distribution was established mainly by GC analysis of suitable derivatives, usually their methyl esters (Christie, 1982). Progress in instrumentation and methods of obtaining derivatives have made the broader use of HPLC possible (Christie, 1987; Hulshoff and Lingeman, 1985). Introducting to the acid molecules a radical displaying high UV–VIS light absorbance or fluorescence emission makes it possible to determine specifically and accurately a single compound present at a very low concentration. Estrification is most frequently used to obtain derivatives of the acids (Hulshoff and Lingeman, 1985). The procedure should be simple, the yields high, the quantity of by-products low, the derivatives stable and exhibiting good chromatographic properties. Such requirements are fulfilled by the reaction of carboxylic acids with O-substituted N,N-dialkyl-isoureas with a chromophoric group (Mathias, 1979; Mikołajczyk and Kiełbasiński, 1991; Vowinkel, 1967). In the present work N,N′-dicyclohexyl-O-p-nitrobenzyl isourea was applied (Kayama *et al.*, 1989). Although its N,N′-diisopropyl analogue reacts faster, the yield of fatty acids esters is lower and the quantity of by-products higher. The p-nitrobenzyl esters of lauric (12:0), myristic (14:0), palmitic (16:0), stearic (16:0), arachidic (20:0), myristoleic (14:1), palmitoleic (16:1), oleic (18:1), linoleic (18:2, n–6), α–linolenic (18:3) acids were synthesised and their properties determined. The reactions of these acids with DcH-p-NB-IU were practically complete after a few hours. The yields were high (> 90%), the purification procedure simple and efficient.

The UV absorption spectrum of p-nitrobenzyl palmitate (Fig. 1) shows a broad absorption peak in the 240–300 nm range. The calculated molar extinction coefficient in methanol at a maximum absorption at 272 nm is 1260.

Esters are advantageous in that they are stable and give a good linear detector response over a broad range of concentrations. The minimum detection limit of p-nitrobenzyl palmitate determined on the RP–18 column with a 0.6 ml min^{-1} flow of 98:2 acetonitrile-water at 270 nm was established at 5 pM (2 ng) applied to the injector.

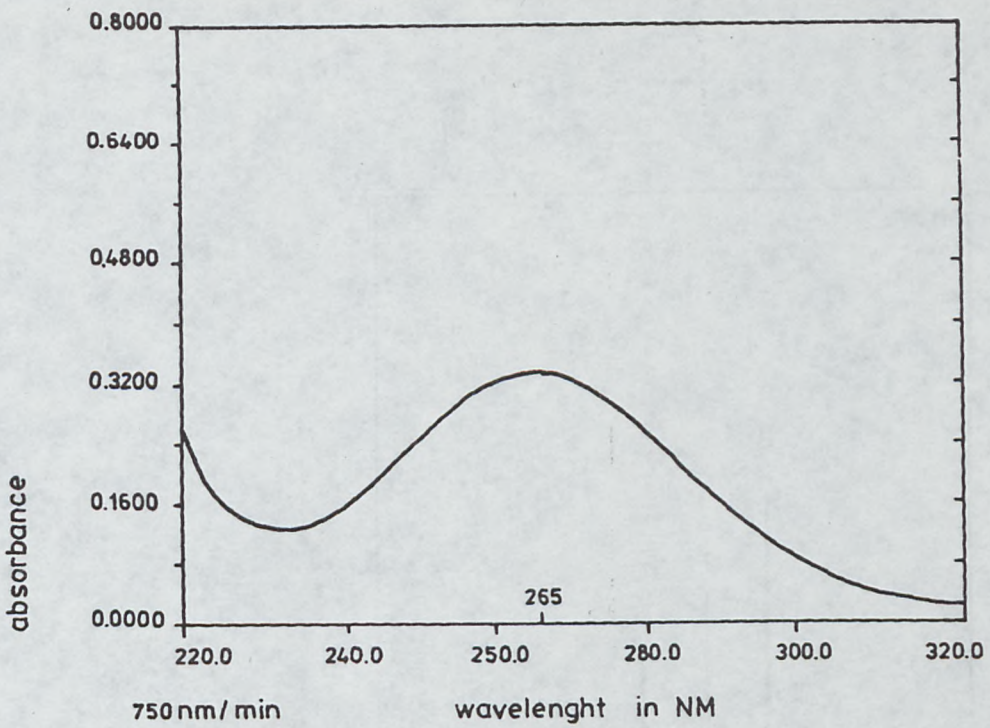
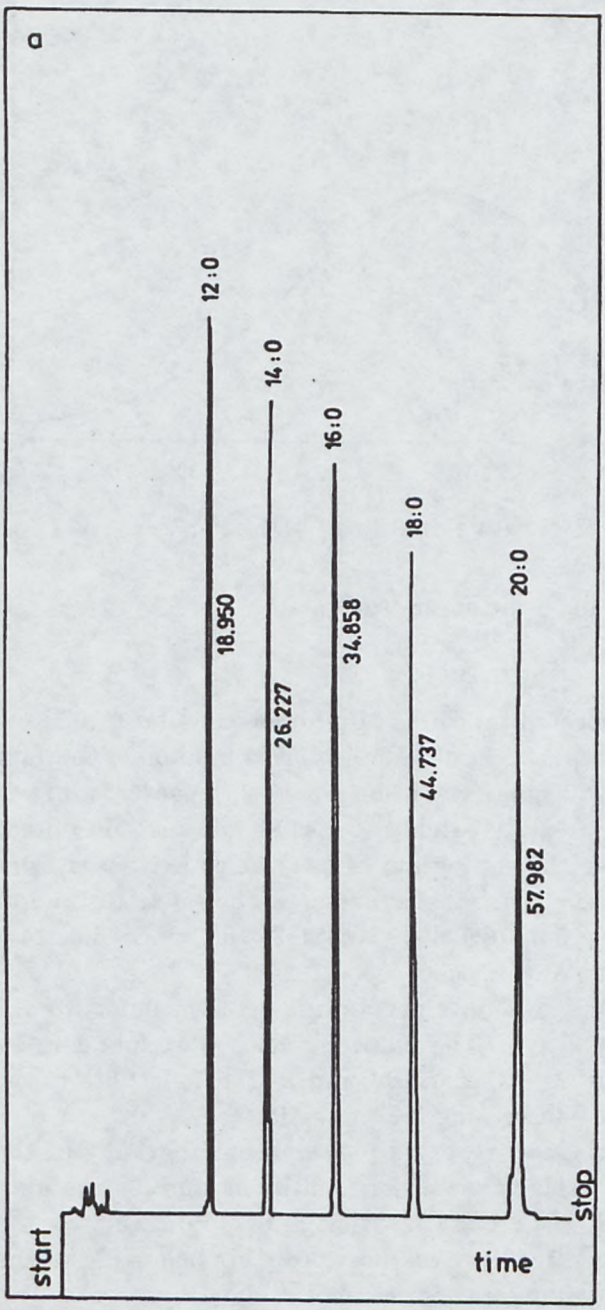


Fig. 1. UV absorption spectrum of p-nitrobenzyl palmitate

The chromatograms of selected mixtures of p-nitrobenzyl fatty acid esters (Fig. 2a–c) exhibit the separation of homologous compounds; the fact that acids 14:0 and 18:2 have similar retention times is, however, a drawback. The presence of low intensity satellite peaks should also be noted. Fig. 3 shows a reversed-phase chromatogram of fatty acids extracted from plankton and converted to their p-nitrobenzyl esters. The esters of the following acids were identified: 16:0, 18:1 n–9, 16:1 n–7, 18:3 n–3, 18:0, 14:0 plus 18:2 n–6, 12:0 plus 22:6 n–3, 14:1 n–5.

A chromatogram of FFA esters obtained in analogous fashion from a sediment sample is shown in Fig. 4. The following acids were found to be present: 18:1 n–3, 14:0 plus 18:2 n–6, 16:0, 18:0 and 16:1 n–7. In both cases, the acids are given in order of decreasing concentration.

The above results indicate that reversed-phase chromatography of the p-nitrobenzyl esters of fatty acids is useful for identifying and determining single compounds. However, their quantification is made difficult by the presence of satellite and unidentified peaks, most probably belonging to the light-absorbing derivatives of p-nitrobenzyl alcohol.


a
12:0
14:0
16:0
18:0
20:0
18.950
26.227
34.858
44.737
57.982
start
stop
time

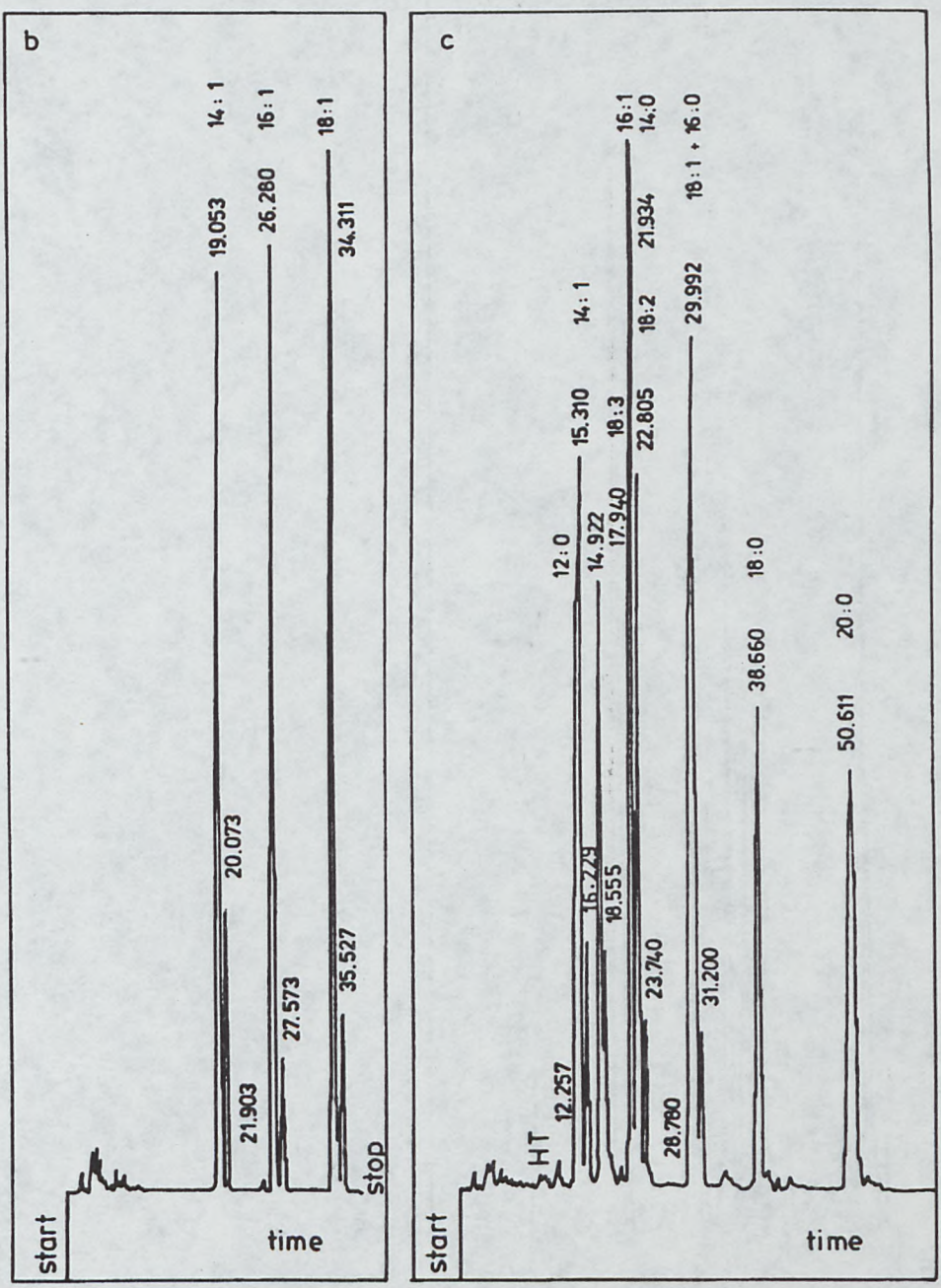


Fig. 2. Chromatograms of the p-nitrobenzyl esters of standard solutions of fatty acids

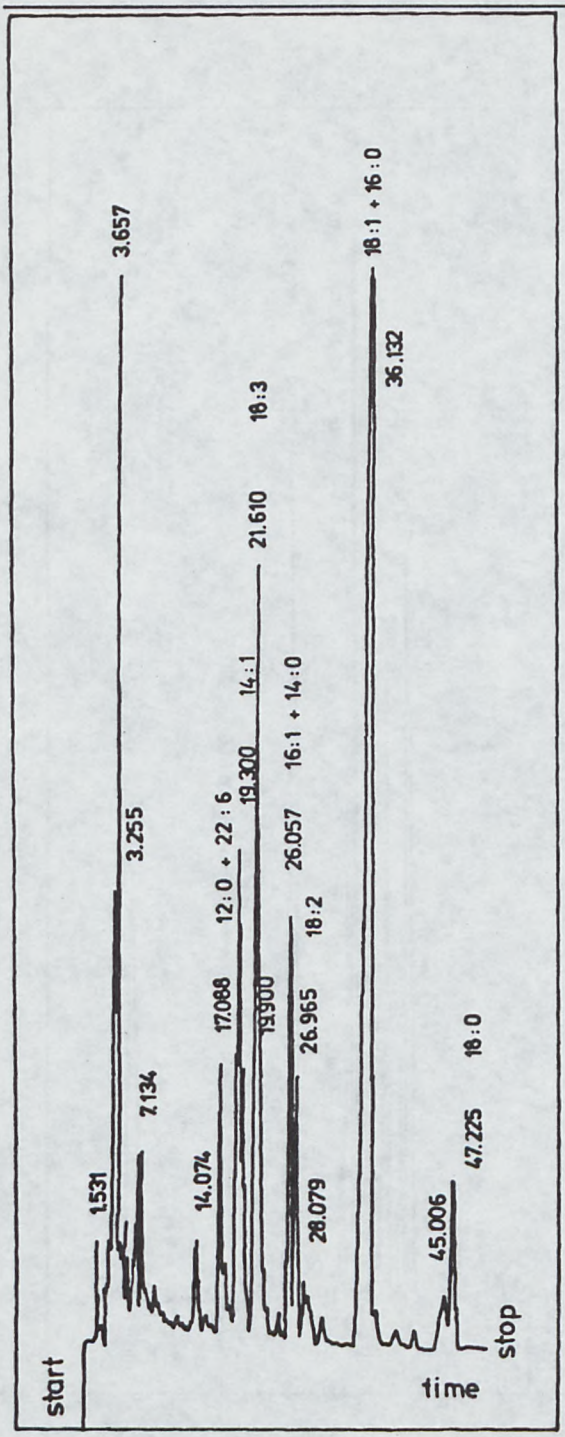


Fig. 3. Chromatogram of the p-nitrobenzyl esters of FFA separated from plankton samples from the Gulf of Gdańsk, May 1991

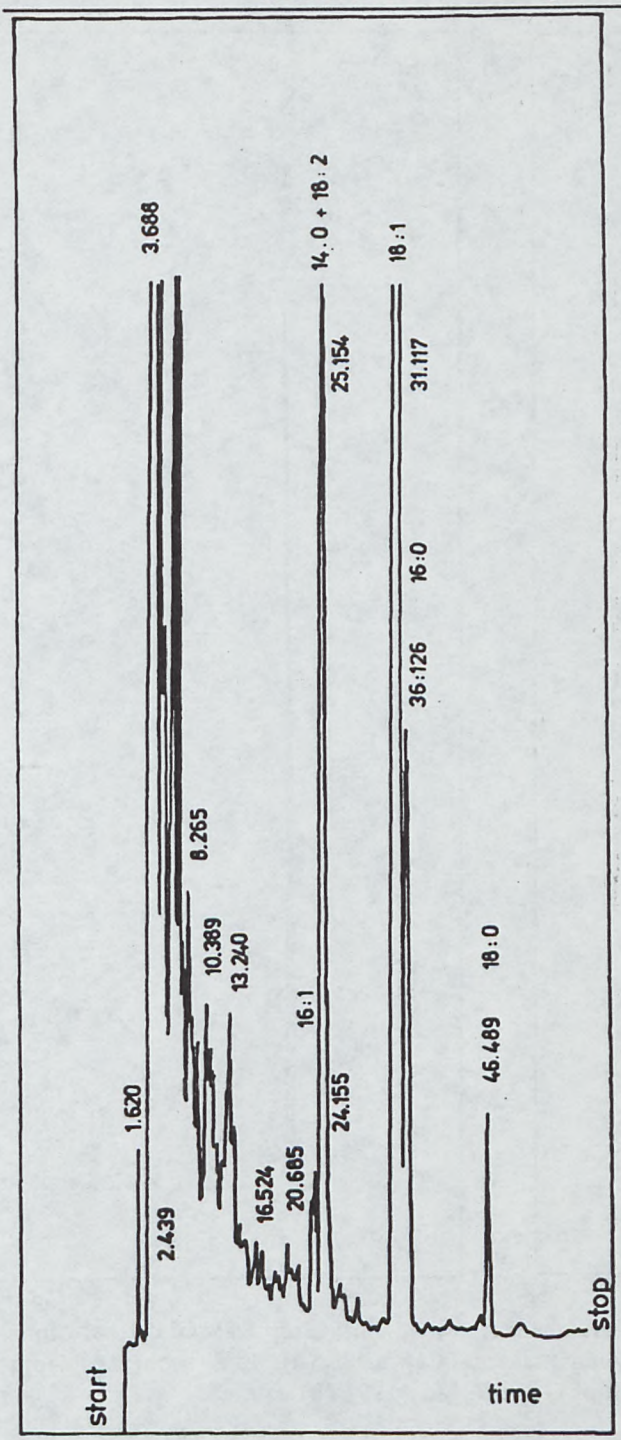


Fig. 4. Chromatogram of the p-nitrobenzyl esters of FFA separated from a sediment sample from the Pomeranian Bay, June 1991

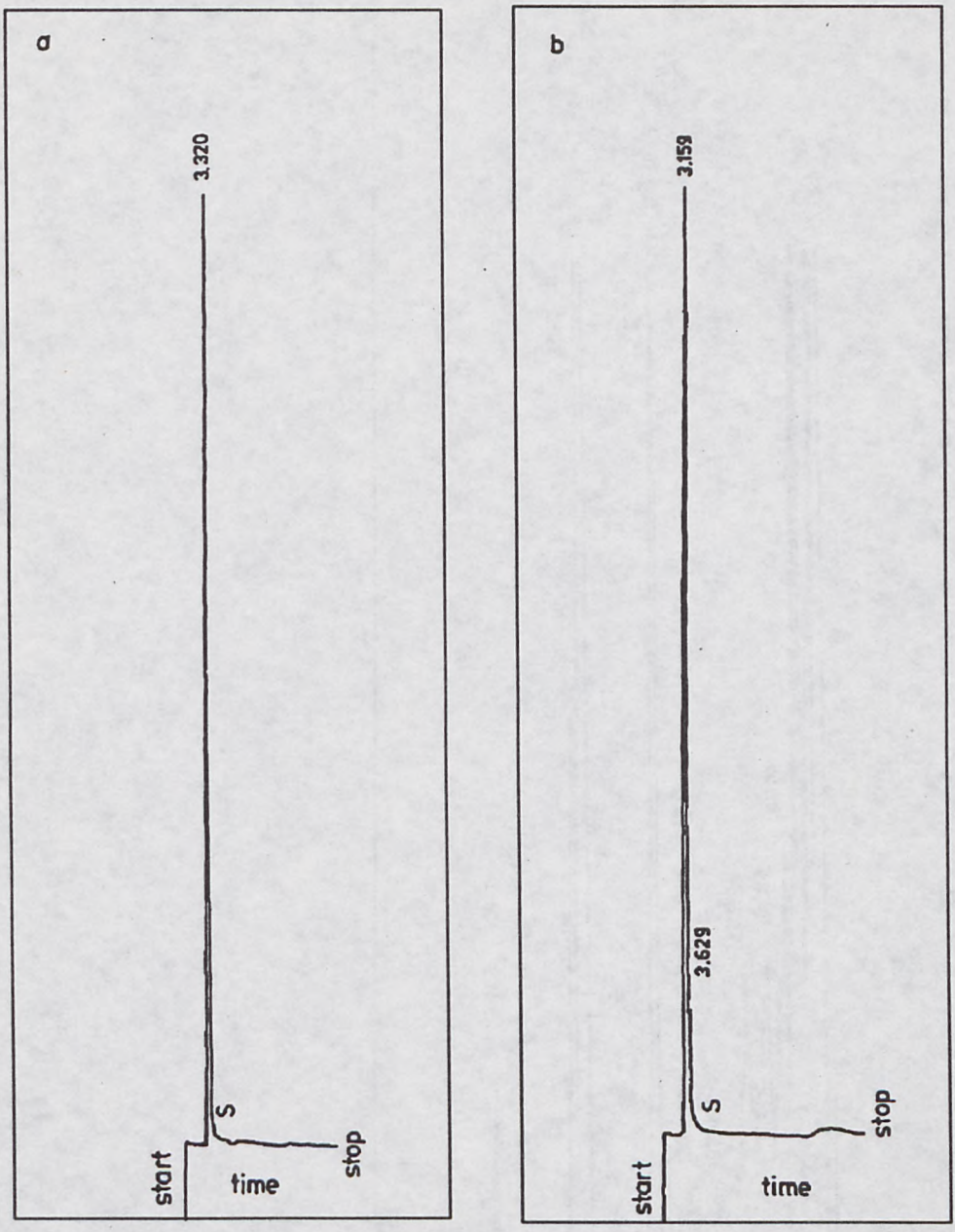


Fig. 5. Chromatograms of p-nitrobenzyl esters: mixture of standard solutions of saturated, mono– and polyunsaturated fatty acids (a), FFA separated from plankton samples from the Gulf of Gdańsk, May 1991 (b)

The application of HPLC in the identification and quantitative determination of p-phenylphenacyl esters of fatty acids separated from marine

particulate matter and seawater has been described by Osterroht (1987, 1993). The excellent resolution of this complex mixture of esters using reversed-phase chromatography on micro-bore columns should be pointed out.

Normal-phase chromatograms of p-nitrobenzyl esters of fatty acids done for standard mixtures as well as for natural samples display one peak only (Fig. 5). Using this technique we established the quantity of FFA in the analysed samples of plankton and sediments at $456 \pm 33\,\mu g\,g^{-1}$ (s) and $40 \pm 5\,\mu g\,g^{-1}$ of dry matter. These results were calculated as the average of three independent analyses.

4. Conclusions

1. The p-nitrobenzyl esters of fatty acids isolated from samples of marine plankton and sediments can be obtained and purified in a very simple procedure. The yields are high and the obtained compounds exhibit good chromatographic properties.
2. Reserved-phase HPLC is more useful for identifying and determining single compounds present in natural samples, whereas normal-phase chromatography allows the total concentration of FFA in the analysed samples to be established with precision.

Acknowledgments

We wish to express our especial gratitude to the Directors and employees of the Institute für Meereskunde, Kiel, for their help in starting this programme. One of us (Leonard Falkowski) greatly appreciates the grant from the Ministerium für Bildung, Wissenschaft, Jugend und Kultur des Landes Schleswig–Holstein which stimulated the realisation of our programme.

References

Ackman R. G., 1989, *Fatty acids*, [in:] *Marine biogenic lipids, fats and oils*, I. R. G. Ackman (ed.), CRC Press Inc., Boca Raton, Florida, 103–139.

Christie W. W., 1982, *The analysis of fatty acids*, [in:] *Lipids analysis*, W. W. Christie (ed.), Pergamon Press, Oxford, 73–90.

Christie W. W., 1987 *The analysis of fatty acids*, [in:] *HPLC and lipids*, W. W. Christie (ed.), Pergamon Press, Oxford, 133–166.

Dawson R., Liebezeit G., 1981, *The analytical methods for the characterisation of organics in seawater*, [in:] *Marine organic chemistry*, R. Dawson and E. K. Duursma (eds.), Elsevier, Amsterdam – Oxford – New York, 455–487.

Hoffmann M., 1988, *A simple efficient synthesis of dibenzyl and Di-p-nitrobenzyl-1-hydroxyalkane phosphates*, Synthesis, 62–63.

Hulshoff A., Lingeman H., 1985, *Fluorescence detection in chromatography*, [in:] *Molecular luminescence spectroscopy, methods and applications*, S. G. Schulmann (ed.), John Wiley and Sons Inc., 622–696.

Joseph J. D., 1989, *Distribution and composition of lipids in marine invertebrates*, [in:] *Marine biogenic and lipids, fats and oils*, R. G. Ackman (ed.), CRC Press Inc., Boca Raton, Florida, II, 49–140.

Kayama M., Araki S., Sato S., 1989, *Lipids of marine plants*, [in:] *Marine biogenic lipids, fats and oils*, R. G. Ackman (ed.), CRC Press Inc., Boca Raton, Florida, II, 3–48.

Mathias J., 1979, *Estrification and alkylation reactions employing isoureas*, Synthesis, 561–576.

Mikołajczyk M., Kiełbasiński P., 1991, *Recent developments in carbodiimide chemistry*, Tetrahedron, 37, 233–284.

Morris R. J., Culkin F., 1989, *Fish*, [in:] *Marine biogenic lipids, fats and oils*, R. G. Ackman (ed.), CRC Press Inc., Boca Raton, Florida, II, 145–178.

Osterroht C., 1987, *Determination of particulate fatty acids as p-bromophenacyl or phenyl phenacene esters using HPLC*, Chromatographia, 25, 417–422.

Osterroht C., 1993, *Extraction of dissolved fatty acids from sea water*, Fresenius J. Anal. Chem., (in press).

Sargent J. R., 1976, *The structure, metabolism and function of lipids in marine organisms*, [in:] *Biochemical and biophysical perspectives in marine biology*, D. C. Malins and J. R. Sargent (eds.), Academic Press, London – New York – San Francisco, 151–207.

Vowinkel S., 1967, *Darstellung von Carbonsäureestern mittels O-Alkyl-N,N-dicyclohexyl-izoharnstoffen*, Chem. Ber., 100, 16–22.