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SIMPLE GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF FATTY ACIDS AND ALCOHOLS IN WAX ESTERS OF MARINE ORGANISMS

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SUMMARY

A rapid and simple gas–liquid chromatographic analysis of fatty acids and alcohols of lipid extracts is described. Calanoid copepods were chosen because they contain large amounts of wax esters. Lipid extracts were analysed directly after transesterification and subsequent hexane extraction. In a single chromatogram fatty acid methyl esters and fatty alcohols were analysed simultaneously on capillary columns coated with SILAR 10 C and CP SIL 5 CB. The major and most of the minor compounds can be determined. Time-consuming separation and purification steps, *e.g.* by thin-layer chromatography, which can cause loss of polyunsaturated fatty acids and alcohols, are thus avoided.

INTRODUCTION

Detailed analyses of lipids in marine organisms, particulate and dissolved organic matter have been often described. Wax esters are found in many marine animals (reviewed by Sargent *et al.*[1]), in particulate matter[2] and in surface waters that originate from zooplankton lipids[3,4].

Wax esters are the dominant class of lipids in calanoid copepods[5–8]. Especially high concentrations are found in copepods from high latitudes, when the lipids may consist of more than 90% of wax esters, which is about half of the dry weight[9–11].

Extensive analyses of the fatty acid and alcohol content of wax esters have been published (reviewed by Sargent *et al.*[1]). Fatty alcohols were generally separated after saponification and converted into their trifluoroacetate derivatives[12] or acetate esters[13]. These methods need a lot of derivatization steps, followed by separation and purification and separate gas chromatographic (GC) analysis of the fatty acid methyl ester and the fatty alcohol derivatives.

The method described in this report allows identification and quantification of fatty acid methyl esters and fatty alcohols as separate peaks on a single gas chromatogram, after transmethylation of wax esters or whole lipid extracts without the use of additional chromatographic methods.

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EXPERIMENTAL

A mixture of lipid extracts of Arctic copepods was used to test the method. The extracts were mainly from the calanoid copepods *Calanus hyperboreus* and *C. finmarchicus*, as adults and developmental stages V and IV. The organisms had to be sorted immediately after catching and preserved in chloroform–methanol (2:1), because preservation (*e.g.* by formaldehyde) and later sorting will cause deterioration and unreproducible loss of substance. Samples were frozen and stored at −25°C until analysis.

The copepods were crushed and extracted in a mortar in the chloroform–methanol mixture. Before crushing an internal standard should be added. All the following steps were performed under nitrogen. Reagents were at least of analytical grade quality. Insoluble particles settled out within some hours or after slight centrifugation. Aliquots of the supernatant were transferred into 10-ml tubes (Sovirell) and evaporated to dryness. For transesterification 0.2 ml of benzene or hexane and 1 ml of 3% concentrated sulphuric acid in methanol were added to the dried extracts. The tubes were closed and heated at 80°C for at least 4 h. After cooling, 4 ml of double-distilled water were added and the fatty acid methyl esters and fatty alcohols were extracted three times with 3 ml of hexane. The combined extracts were transferred into a flask with a tapering neck. The solvent was evaporated, the residue diluted with hexane, and 1 μl of sample taken for the GC analysis.

Fatty acid methyl esters and fatty alcohols were analysed in a single run by gas–liquid chromatography (GLC). The analysis was performed with a Hewlett Packard 5711 gas chromatograph on a 50 m × 0.325 mm I.D. wall-coated open tubular (WCOT) glass capillary column coated with SILAR 10 C (Macherey and Nagel, Düren, F.R.G.). Temperature programming from 180°C for 8 min and then at 4°C/min to 220°C and at 220°C with final hold for 8 min was employed. For comparison the analysis was repeated using a Packard 428 gas chromatograph equipped with a fused-silica column of 50 m × 0.22 mm I.D. (WCOT) (Chrompack, Müllheim, F.R.G.), coated with CP SIL 5 CB and temperature programmed from 140°C to 280°C at 3°C/min. Helium was used as the carrier gas. The temperatures of the injector and the flame-ionization detector were maintained at 250°C and 300°C, respectively.

To check the analysis, the fatty acid methyl esters and fatty alcohols were separated by thin-layer chromatography (TLC) on Kieselgel 60 (Merck, Darmstadt, F.R.G.) with hexane–diethyl ether–acetic acid (80:20:1)[14]. Bands corresponding to fatty acid methyl esters and fatty alcohols were scraped off, eluted with hexane, evaporated and submitted to GC analysis.

Peaks were identified by means of reference standards, by comparison of relative retention times and by comparison with extracts of well-known fatty acid composition (*e.g.* fatty acid composition of krill[15] and turbot larvae[16]). For fatty acids and alcohols equal response factors from the flame-ionization detector were assumed.

RESULTS

Fig. 1a shows the gas–liquid chromatogram of fatty acid methyl esters and alcohols of a total extract of mainly *Calanus hyperboreus* and *C. finmarchicus*. Fig.

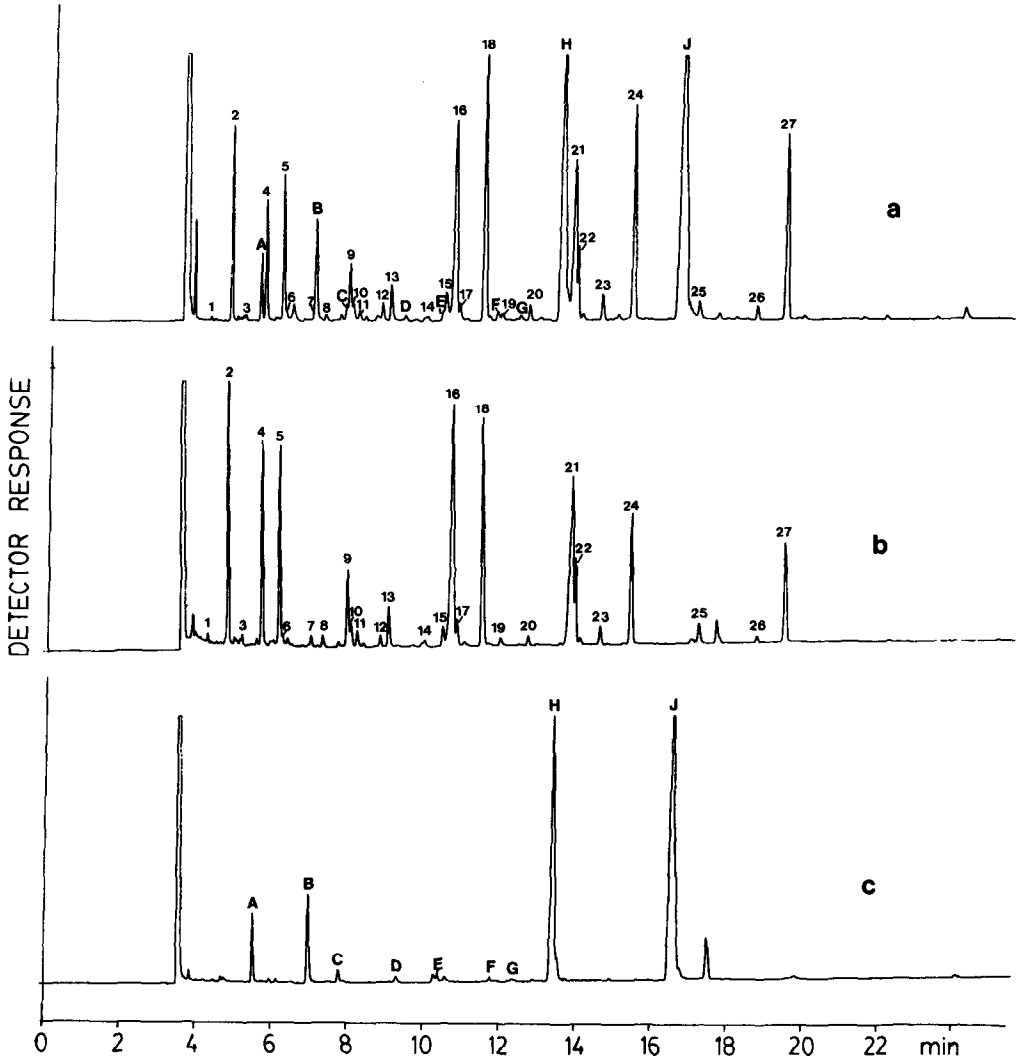

Fig. 1. Gas–liquid chromatograms: (a) fatty acid methyl esters and fatty alcohols; (b) fatty acid methyl esters after TLC separation; (c) fatty alcohols after TLC separation. Glass capillary column (50 m) coated with SILAR 10 C (WCOT), temperature-programmed at 180°C for 8 min, then at 4°C/min to 220°C and 8 min hold at 220°C. Peak numbers and letters refer to Table I. Plots are from Hewlett Packard Integrator 3390 A.

1b shows the chromatogram of fatty acid methyl esters and Fig. 1c that of fatty alcohols, which had been separated by TLC. All major and nearly all minor compounds are clearly separated. The 16:1 ($n-7$) fatty alcohol is only separated as a shoulder from the 18:1 ($n-9$) fatty acid, but both components can be identified clearly. The pairs of isomers of the monounsaturated fatty acids are not baseline-separated but can be well quantified. The peaks containing the main fatty alcohols 20:1 ($n-9$) and 22:1 ($n-11$) seem to be accompanied by the same isomers as their

TABLE I

FATTY ACID AND FATTY ALCOHOL ANALYSES OF LIPIDS ON DIFFERENT GAS–LIQUID CHROMATOGRAPHS

Capillary columns coated with (a) SILAR 10 C and (b) CP SIL 5 CB. Data are expressed as wt. %. + = Trace amount.

No.	*Fatty acids*	*a*	*b*
1	12:0	+	+
2	14:0	5.80	6.42
3	15:0	0.23	+
4	16:0	4.47	5.37
5	16:1 (*n*−7)	5.94	7.30
6	16:1 (*n*−5)	+	0.00
7	16:2 (*n*−6)	+	+
8	18:0	0.36	0.30
9	18:1 (*n*−9)	3.21	3.23
10	18:1 (*n*−7)	1.05	1.06
11	16:3 (*n*−?)	0.70	0.00
12	16:4 (*n*−3)	0.96	0.90
13	18:2 (*n*−6)	1.93	2.18
14	20:0	+	0.00
15	18:3 (*n*−3)	0.41	0.00
16	20:1 (*n*−9)	13.90	13.68
17	20:1 (*n*−7)	1.10	0.91
18	18:4 (*n*−3)	18.05	18.75
19	20:2 (*n*−?)	+	+
20	18:5 (*n*−3)	0.88	0.84
21	22:1 (*n*−11)	12.04	11.93
22	22:1 (*n*−9)	3.17	3.18
23	20:4 (*n*−3)	1.46	1.77
24	20:5 (*n*−3)	11.58	11.38
25	24:1 (*n*−?)?	1.22	0.88
26	22:5 (*n*−3)	0.76	0.00
27	22:6 (*n*−3)	10.80	9.94
	Fatty alcohols		
A	14:0	3.03	3.30
B	16:0	6.17	6.47
C	16:1 (*n*−7)	1.03	1.12
D	18:0	0.50	1.70
E	18:1 (*n*−9)	+	+
F	18:2 (*n*−6)	0.68	0.00
G	20:0	0.43	0.00
H	20:1 (*n*−9)	33.01	31.50
J	22:1 (*n*−11)	55.15	55.91

corresponding fatty acids but to a much lesser extent. Therefore they were not extra-calculated.

The comparison of the analysis on the different gas chromatographs equipped with capillary columns coated with the highly polar SILAR 10 C phase and with the non-polar CP SIL 5 phase, respectively, shows very similar quantitative results (Table I). The only differences were not significant, like a slight increase in shorter-chain fatty acids on the CP SIL 5 phase. The accuracies, calculated as the S.D. of both

runs, are averaged and compared with double determinations on the same system given in parenthesis. For major components (more than 10%) the S.D. was of the order of 2.4% (2.2%) and for moderate-sized components (1–10%) of the order of 7.4% (2.6%); minor components (less than 1%) may have larger relative errors. The accuracy is just as good for the double determinations, especially for the major components.

Free alcohols are retained longer on polar phases than fatty acid methyl esters with the same chain length, because of hydrogen-bonding interactions with the stationary phase. On non-polar phases the free alcohols are eluted before the corresponding fatty acid methyl esters.

The specific loss of polyunsaturated fatty acids during TLC and extraction from the silica can be seen in the different fatty acid compositions, as shown in Fig. 1a and b. The reason might be poor extraction from the silica or oxidation of double bonds, because our separations were not performed under a nitrogen atmosphere, which is possible with special constructions. No significant change occurred for fatty alcohols because no significant amounts of polyunsaturated alcohols were detected.

DISCUSSION

The results show that it is not necessary to separate fatty acid methyl esters and fatty alcohols and to derivatize the alcohols prior to GLC analysis. Fatty alcohols are usually separated and converted into their trifluoroacetate derivatives, *e.g.* Lee *et al.*[6] and Sargent and Lee[17], and Sargent and Falk-Petersen[18], trimethylsilyl-ether derivatives[4] or acetate esters[5,18–20]. The method for trifluoroacetate derivatives was published by Wood and Snyder[12] and for acetate ester derivatives by Farquhar[13]. He had already compared the GC behaviour of free fatty alcohols and their acetates. The free alcohols have a much greater retention time on a polar phase, which can be explained by the tendency to associate by hydrogen-bonding, resulting in decreased volatility and in tailing. But the free alcohols were successfully chromatographed on a non-polar stationary phase; columns with polar phase needed a thoroughly conditioning. The use of the glass capillary system together with temperature programming prevents loss of substance and tailing of free alcohol peaks. Morris and Sargent[21] also carried out analytical and preparative GC on major methyl esters and free fatty alcohols, but efforts to find more rapid methods for the derivatization of alcohols have continued[22].

The main advantages of the method described here are the short time of analysis and the fewer steps of preparation of the material for GC analysis. This reduces the undefinable loss of components, as shown for polyunsaturated fatty acids. The method can also be used to analyse the fatty acid composition of any particulate matter and of dissolved material (*e.g.* after extraction with solvents). In the case of polluted water, hydrocarbons and phthalic esters may interfere with the identification and quantitation because of additional peaks in the chromatogram.

The short time of analysis makes it possible to investigate large numbers of natural samples, which are very variable in time and region[11]. The method described here for the simultaneous GC analysis of fatty acid methyl esters and free fatty alcohols is now being applied to the analysis of copepods from the Arctic and North Sea: the results will appear at a later date.

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REFERENCES

1 J. R. Sargent, R. F. Lee and J. C. Nevenzel, in P. E. Kolattukudy (Editor), *Chemistry and Biochemistry of Natural Waxes*, Elsevier, Amsterdam, 1976, p. 50.
2 S. G. Wakeham, *Mar. Chem.*, 17 (1985) 213.
3 R. F. Lee and J. C. Nevenzel, *J. Fish. Res. Board Can.*, 36 (1979) 1519.
4 J. K. Volkman, R. R. Gatten and J. R. Sargent, *J. Mar. Biol. Assoc. U.K.*, 60 (1980) 759.
5 R. G. Ackman, B. A. Linke and J. Hingley, *J. Fish. Res. Board Can.*, 31 (1974) 1812.
6 R. F. Lee, J. C. Nevenzel and G. A. Paffenhöfer, *Mar. Biol.*, 9 (1971) 99.
7 R. F. Lee, J. C. Nevenzel and G. A. Paffenhöfer, *Naturwissenschaften*, 59 (1972) 406.
8 R. F. Lee and J. Hirota, *Limnol. Oceanogr.*, 18 (1973) 227.
9 R. F. Lee, *Mar. Biol.*, 26 (1974) 313.
10 J. R. Sargent, R. R. Gatten and R. J. Henderson, *Oceanis*, 7 (1981) 623.
11 G. Kattner, M. Krause, H. J. Hirche and S. L. Smith, *Mar. Chem.*, in prep.
12 R. Wood and F. Snyder, *Lipids*, 1 (1966) 62.
13 J. W. Farquhar, *J. Lipid Res.*, 3 (1962) 21.
14 V. P. Skipski, A. F. Smolowe, R. C. Sullivan and M. Barclay, *Biochem. Biophys. Acta*, 106 (1965) 386.
15 H. Fricke, G. Gercken, W. Schreiber and J. Oehlenschläger, *Lipids*, 19 (1984) 821.
16 U. Witt, G. Quantz, D. Kuhlmann and G. Kattner, *Aquacult. Eng.*, 3 (1984) 177.
17 J. R. Sargent and R. F. Lee, *Mar. Biol.*, 31 (1975) 15.
18 J. R. Sargent and S. Falk-Petersen, *Mar. Biol.*, 62 (1981) 131.
19 J. R. Sargent, R. McIntosh, A. Bauermeister and J. H. S. Blaxter, *Mar. Biol.*, 51 (1979) 203.
20 R. J. Henderson, S. Falk-Peterson and J. R. Sargent, *Mar. Biol.*, 70 (1982) 7.
21 R. J. Morris and J. R. Sargent, *Mar. Biol.*, 22 (1973) 77.
22 I. C. Barta and T. Kömives, *J. Chromatogr.*, 287 (1984) 438.