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Note

Gas–liquid chromatographic method for the determination of marine wax esters according to the degree of unsaturation[a]

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Wax esters constitute an important lipid class in the marine environment and are found in many marine organisms[1], in particulate[2] and dissolved matter[3] and in sediments[4]. They are energy-storage molecules in the marine food chain, especially in calanoid copepods, which represent a large fraction of the zooplankton biomass in most parts of the world's oceans. Calanoid copepods from high latitudes generally contain high levels of wax esters with high degrees of unsaturation[5–9].

Marine wax esters are composed of long-chain saturated and monounsaturated fatty alcohols and fatty acids with up to six double bonds. Therefore, the wax esters may exhibit up to seven double bonds in the molecule. In previous work various phases for the separation of wax esters were described, such as OV-1[10,11], OV-101[12], Dexil 300[13] and SE-52[14]. With these phases separations could be achieved based on chain length but hardly on the degree of unsaturation.

In order to obtain clear separations also of the various unsaturated wax esters, high-temperature gas chromatography (GC) on a special triglyceride stationary phase using a capillary column was applied in this work.

EXPERIMENTAL

The method was developed by using a complex mixture of wax esters isolated from lipids of the calanoid copepod *Calanus hyperboreus* originating from the Greenland Sea (Arctic). The organisms had to be sorted immediately after catching and preserved in chloroform–methanol (2:1) to avoid deterioration and loss of substance. Samples were frozen and stored at −25°C until analysis[9].

Lipid was extracted by homogenizing the animals in the storage solution with a Potter homogenizer (Braun, Melsungen, F.R.G.) at 1000 U/min. Insoluble particles settled out within a few hours or after slight centrifugation. From an aliquot of the supernatant, wax esters were separated by thin-layer chromatography (TLC) on silica gel 60 (Merck, Darmstadt, F.R.G.) with hexane–diethyl ether–acetic acid (90:10:1)[15].

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Bands corresponding to wax esters were scraped off and eluted with chloroform, evaporated and dissolved in hexane. Additional TLC separations were made on silica gel 60 impregnated with 3% silver nitrate to differentiate between the polyunsaturated and the mono- and diunsaturated wax esters. Plates were developed with chloroform–methanol (95:5)[16].

GC of the wax esters was carried out under the following conditions: instrument, Carlo Erba Model 5370 Mega Series; carrier gas, hydrogen at 100 kPa; column, 25 m × 0.25 mm I.D. wall-coated open-tubular bonded fused-silica column coated with a 0.10-μm film of 50% methyl–50% phenylpolysiloxane [Triglyceride Analysis Phase (TAP); Chrompack, Mühlheim, F.R.G.]; injection, 0.2–0.5 μl of hexane solution

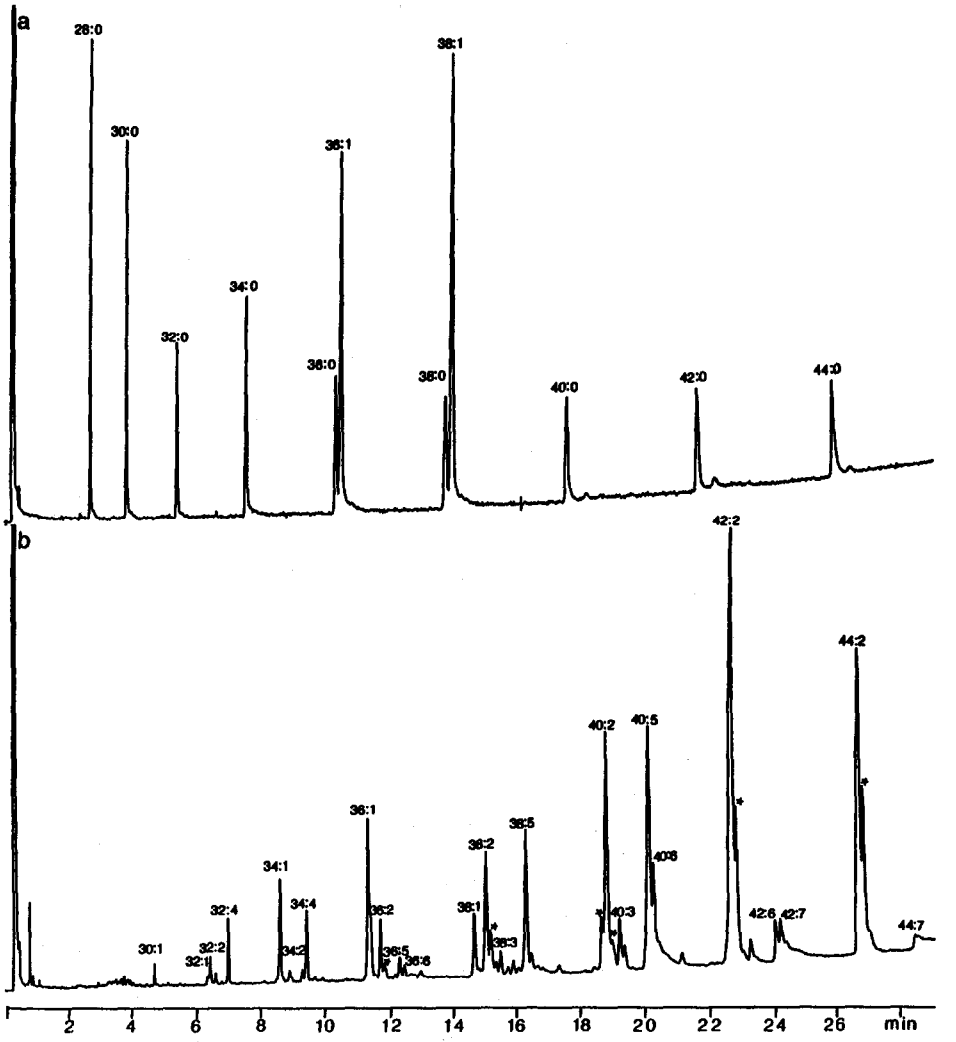

Fig. 1. Gas chromatograms of wax esters. (a) Commercial mixture; (b) natural wax esters isolated from lipids of *Calanus hyperboreus*. Asterisks indicate corresponding isomers.

on-column at 280°C oven temperature with 30-s auxiliary cooling; temperature programme, 2 min at 280°C, increased at 1°C/min to 320°C; detector, flame ionization (380°C).

To confirm the identification of peaks, commercial standards of saturated wax esters from C_{28} to C_{44} and monounsaturated 36:1 and 38:1 wax esters (Sigma, Deisenhofen, F.R.G.) were used. For the determination of possible wax ester combinations, an aliquot of the wax ester extract was transesterified. The resulting fatty acid methyl esters and fatty alcohols were analysed by GC according to the method described by Kattner and Fricke[17].

RESULTS

The samples of *Calanus hyperboreus* contained a mixture of wax esters in the range C_{30}–C_{44} with different degrees of unsaturation. In Fig. 1 the chromatogram of these wax esters is compared with that of a standard mixture of commercially available saturated and monounsaturated wax esters. Saturated wax esters were not detected in

TABLE I

ALL POSSIBLE COMBINATIONS OF THE WAX ESTERS OF *CALANUS HYPERBOREUS* CALCULATED BY ALCOHOL AND FATTY ACID ANALYSIS

C:X = number of carbon atoms:number of double bonds; wax ester combination = alcohol/fatty acid.

C:X	*Main compounds*	*Minor compounds*	*Traces*	*C:X*	*Main compounds*	*Minor compounds*	*Traces*
28:0	14:0/14:0			38:1	22:1/16:0	16:0/22:1	20:1/18:0
30:0	14:0/16:0			38:2	20:1/18:1		16:1/22:1
	16:0/14:0				22:1/16:1		
30:1	14:0/16:1	16:1/14:0		38:3	20:1/18:2		22:1/16:2
30:2	14:0/16:2			38:4	20:1/18:3		22:1/16:3
30:3	14:0/16:3			38:5	20:1/18:4		22:1/16:4
30:4	14:0/16:4			38:6	16:0/22:6		16:1/22:5
32:0	16:0/16:0		14:0/18:0	40:2	20:1/20:1	22:1/18:1	
32:1	16:0/16:1	14:0/18:1	16:1/16:0	40:3	22:1/18:2		
32:2	14:0/18:2	16:0/16:2	16:1/16:1	40:4	22:1/18:3		
32:3	14:0/18:3	16:0/16:3	16:1/16:2	40:5	22:1/18:4		
32:4	14:0/18:4	16:0/16:4	16:1/16:3	40:6	20:1/20:5		
32:5	16:1/16:4			42:2	20:1/22:1		
34:1	20:1/14:0	14:0/20:1	16:0/18:1		22:1/20:1		
34:2	16:0/18:2		16:1/18:1	42:6	22:1/20:5		20:1/22:5
34:3	16:0/18:3		16:1/18:2	42:7	20:1/22:6		
34:4	16:0/18:4		16:1/18:3	44:2	22:1/22:1		
34:5	14:0/20:5		16:1/18:4	44:6	22:1/22:5		
36:1	22:1/14:0	16:0/20:1		44:7	22:1/22:6		
	20:1/16:0	14:0/22:1					
36:2	20:1/16:1		16:1/20:1				
36:3	16:0/18:3		16:1/18:2				
36:4	20:1/16:3						
36:5	20:1/16:4	16:0/20:5	14:0/22:5				
36:6	14:0/22:6	16:1/20:5					

the copepod. The 36:1 and 38:1 wax esters were directly identified by comparison of their retention times. By using the standard mixture (Fig. 1a) the range of retention times for the unsaturated wax esters could be deduced, as it has already been established that TAP retards the unsaturated more than the saturated components.

Identification is further possible from the known linear relationship between the logarithm of the retention times and the number of carbon atoms for members of a homologous series. Most important for the identification was the analysis of the fatty acid and alcohol components of the wax esters. On the basis of these results all possible wax ester combinations can be calculated (Table I). The compositions were classified on the basis of the amounts of the major fatty acids and alcohols. Traces of fatty acids and alcohols may contribute very small amounts to some wax ester peaks. These combinations are also given in Table I. In Table II the wax ester composition of *Calanus hyperboreus* in combination with the resulting fatty acid and alcohol composition is presented. The fatty alcohols were less complex than the fatty acids. Only five alcohols were found, of which the principle components were the 20:1 and 22:1 alcohols. Nineteen fatty acids were identified with about seven major acids. Owing to their different proportions, the identities of the major wax esters could be established.

TABLE II

COMPOSITION OF WAX ESTERS, FATTY ACIDS AND ALCOHOLS OF *CALANUS HYPERBOREUS*

C:X ($n-y$) = number of carbon atoms:number of double bonds; y is the number of carbon atoms between the methyl end and the first double bond. Trace amounts are neglected.

Wax esters	*Content) (%,w/w)*	*Fatty acids*	*Content (%,w/w)*	*Alcohols*	*Content (%,w/w)*
30:1	0.42	14:0	3.84	14:0	2.22
32:1	0.51	16:0	2.12	16:0	3.23
32:2	0.21	16:1 ($n-7$)	5.37	20:1 ($n-9$)	27.97
32:4	1.31	16:2 ($n-6$)	0.51	22:1 ($n-11$)	65.61
34:1	3.04	16:3 ($n-3$)	0.43		
34:2	0.32	16:4 ($n-3$)	0.66		
34:4	2.12	18:0	0.32		
36:1	5.45	18:1 ($n-9$)	2.73		
36:2	2.09	18:1 ($n-7$)	0.45		
36:5	0.54	18:2 ($n-6$)	2.44		
36:6	0.24	18:3 ($n-3$)	1.28		
38:1	1.95	18:4 ($n-3$)	19.94		
38:2	5.64	20:1 ($n-9$)	20.81		
38:3	0.39	20:1 ($n-7$)	1.45		
38:5	6.17	20:5 ($n-3$)	4.79		
40:2	9.98	22:1 ($n-11$)	21.10		
40:3	1.99	22:1 ($n-9$)	4.69		
40:5	8.73	22:5 ($n-3$)	1.14		
40:6	5.05	22:6 ($n-3$)	5.83		
42:2	21.79				
42:6	1.28				
42:7	1.90				
44:2	17.38				
44:7	1.40				

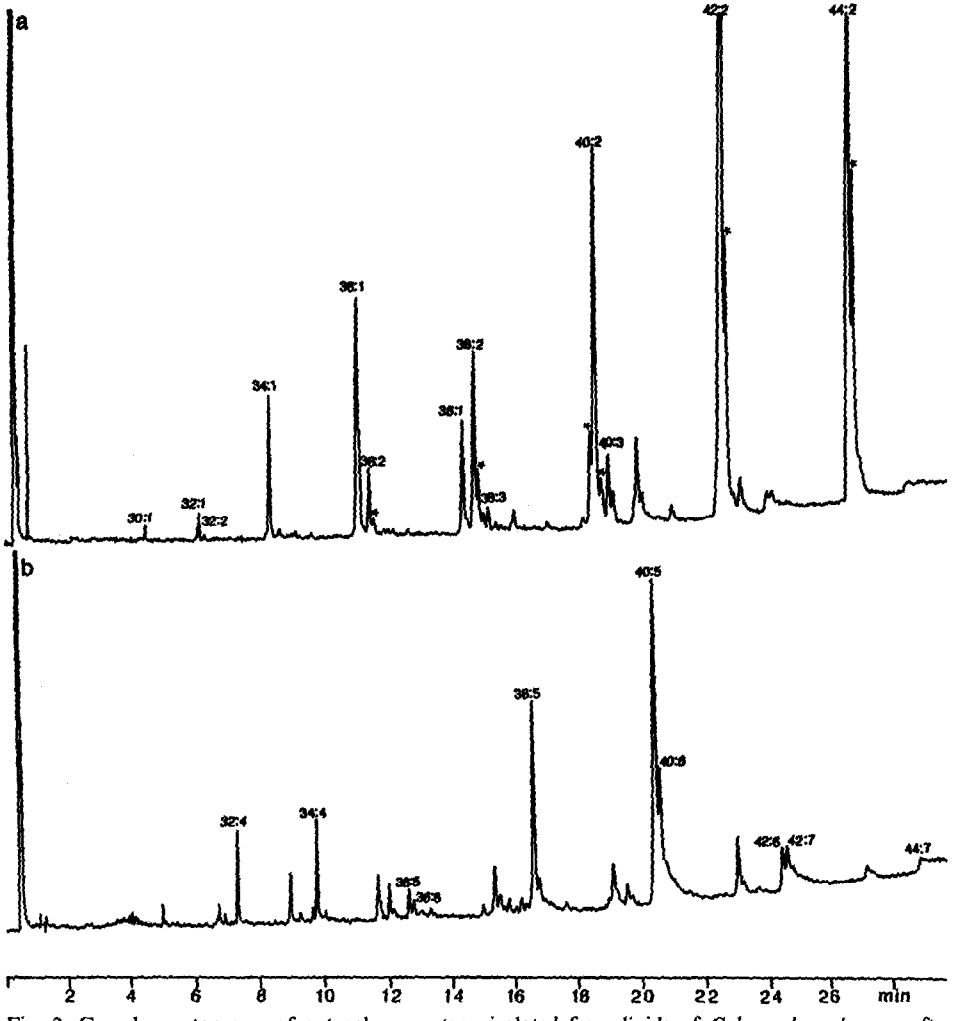

Fig. 2. Gas chromatograms of natural wax esters, isolated from lipids of *Calanus hyperboreus*, after separation by silver ion thin-layer chromatography. (a) Mono- and diunsaturated wax esters; (b) polyunsaturated wax esters. Asterisks indicate corresponding isomers.

Small amounts of the different isomers of the wax esters were found but are not listed in Table I. They appear as peak shoulders and as broadening of the bases of the corresponding peaks (Figs. 1 and 2). For the 42:2 wax ester, for example, theoretically at least eight isomers are possible owing to the different double bond positions in the fatty acids and alcohols.

From combined TLC and GC it could be concluded that the major mono- and diunsaturated wax esters are 36:1, 38:1, and 40:2, 42:2, 44:2, respectively (Fig. 2a). In the polyunsaturated fraction the 40:5 and 38:5 wax esters are most abundant as a combination of the 20:1 and 22:1 alcohol with the 18:4 fatty acid. The 40:6 and the 38:6 wax esters are not clearly separated from 40:5 and 38:5 (Fig. 2b). Owing to incomplete TLC separation in both chromatograms small amounts of the other fraction are detected.

DISCUSSION

In previous studies, high-temperature GC was performed to characterize especially commercial waxes according to chain length with and without derivatization[13]. Most data on marine wax esters are based on the GC identification of fatty acids and alcohols after hydrolysis. The calculation of possible wax ester combinations from those data has some limitations, of course. On the other hand, the few methods used so far were performed on columns with non-polar phases. They provided only total values for all wax esters having the same carbon number and gave no information about their degree of unsaturation[10–13] or at most they only differentiated between saturated and mono- and diunsaturated wax esters[14].

The Triglyceride Analysis Phase used in our method allows the separation of wax esters of the same chain length according to the number of double bonds without derivatization. The peaks appear in order from the saturated to the most unsaturated components, similarly to standard fatty acid methyl ester separation methods[17]. It is also possible to separate isomeric components. However, most of the peaks may be composed of a number of wax esters which differ in the alkyl and acyl moieties but having the same total carbon number and the same number of double bonds. Supported by the analysis of fatty acids and alcohols, most of the wax esters can be identified as a major single component (Table I). Hence the combination of the two methods allows a nearly complete elucidation of the wax ester composition of the studied material. Quantification is possible by adding internal standards, *e.g.*, a saturated wax ester such as 40:0 or 42:0.

This method was developed to study the wax esters of marine calanoid copepods from temperate and high latitudes, which are known to contain high amounts of wax esters often with large moieties of polyunsaturated fatty acids[7–9]. Now it will be possible to investigate, *e.g.*, species variabilities and distributions of copepods based on more detailed data for intact wax ester molecules.

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