Surface Lipids of *Ligia oceanica*; Part II: The Ester Fractions

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ABSTRACT

The surface lipids of *Ligia oceanica* have been isolated and fractionated into long chain wax esters, secondary alkylacetates, sterol esters, triglycerides, and alkenyl acyl diol esters. The fatty acid composition of these classes have been determined by gas liquid chromatography on Apiezon L, diethyleneglycol succinate and show that there are different biosynthetic pools of fatty acid for wax esters and triglycerides. The surface lipids have unusual constituents which may be important in water proofing the crustacean.

INTRODUCTION

The crustacean *Ligia oceanica* is a member of the order Isopoda, subclass Malacostraca in the phylum Arthropoda. Previous studies of the lipids of crustaceans have been restricted to total lipids (1,2). Little is known of the lipids of the arthropod cuticle, and it was felt that it might be useful to consider this animal since it occupies the middle ground between the terrestrial and the marine environments.

In the first paper (3) of this series we showed that the hydrocarbons of *L. oceanica* consisted of a number of unusual components including heptadeca-8, 13-diene, and cholestadiene. The present paper concerns the remaining fractions which consist of esterified fatty acids.

EXPERIMENTAL PROCEDURES

The collection of specimens of *Ligia oceanica* and the extraction of surface wax were as reported earlier (3).

Column Chromatography

The surface lipid (1.14 g) was placed on a column of silica gel (50 g 0.2-0.5 mesh) in light petroleum (bp 40-60 C) (10 cm^3). Elution with solvents of increasing polarity gave the following components: hydrocarbons (80.0 mg 7.0%), aromatic hydrocarbons (50 mg 4.3%), long chain esters (127.5 mg 11.4%), secondary acetates (17.5 mg 1%), alkenyl acyl diol esters (36.0 mg 3%), triglycerides (230.0 mg 20%), free acids and alcohols (644.0 mg 56%).

Thin Layer Chromatography (TLC)

Silica gel plates (5) loaded with the surface lipid (100 mg) were developed in petroleum ether (bp 40-60 C): diethyl ether (19:1 v/v). Components were located by spraying with 2,7-dichlorofluorescein. The bands were scraped from the plates into sintered filters and extracted with either cold or warm chloroform (100 cm^3). Rf values were: hydrocarbons (0.79), cholesteryl esters (0.64), aromatic hydrocarbons (0.55), wax esters (0.61), secondary alkyl acetates (0.54), alkenyl acyl diol esters (0.37), triglycerides (0.19), free acids, and alcohols (0.04).

Gas Liquid Chromatography (GLC)

All analyses were performed on a Pye 104 dual flame ionization detector instrument. Sterol analyses were conducted by either 1% OV-1 at 250 C, 1% OF-1 at 230 C, or 1% cyclohexanedimethanol succinate at 250 C on 5 ft x ¼ in. columns. Short chain constitutents were analyzed on a Porapak Q column (18 in. x ¼ in.). Fatty acid analyses were carried out by 3% Apiezon L or 13% diethylene glycol succinate. Intact wax esters and triglycerides and alkenyl-ester lipids were analyzed by 5 ft x ¼ in. 1% OV-1 on Gas Chrom Q.

Saponification of Ester Lipids

The lipids (100 mg) were refluxed with 0.5% methanolic potassium hydroxide (10 cm^3) for 2½ hr after which time methanol (7 cm^3) was removed by distillation. Water (10 cm^3) was added to the solution which was then extracted with diethyl ether (4 x 10 cm^3). The ether extracts were combined, back washed with water, and finally dried over anhydrous sodium sulphate. After filtration the ether was removed by distillation to yield the unsaponifiable material. The fatty acids were liberated by the addition of mineral acid to the aqueous phase above and extracted with diethyl ether.

Acetylation of Alcohols

The alcohols (10 mg) in pyridine (3 cm^3) and acetic anhydride (1 cm^3) were stirred in a 10 cm^3 conical flask for 18 hr at room temperature. Water (7 cm^3) was added to the reaction mixture which was then extracted with ether (3 x 10 cm^3). The ether extracts were washed with water and dried over anhydrous sodium sulphate and the solvent removed by distillation to yield the acetates.

Preparation of Trimethylsilyl Ethers

Fatty alcohols (1 mg) were placed in a "Reactivial" containing bistrimethylsilylacetamide (100 µl) and carbon tetrachloride (500 µl) for 1 hr. An aliquot (10 µl) could then be injected directly onto the GLC unit.

Qualitative Analysis of Steroids

Steroids give a characteristic color with different locating reagents whereas long chain esters show no such coloration, e.g., one locating solution is prepared by dissolving $FeCl_3 6H_2O$ (50 mg) in water (90 cm^3), acetic acid (5 cm^3), and conc. H_2SO_4 (5 cm^3). After spraying this solution onto a steroid on a TLC plate, the plate was heated to 100 C for 2-3 min when steroids and triterpenes gave a red violet color. The sample of *Ligia* also showed the same color.

RESULTS AND DISCUSSION

Wax Ester Fraction

The hydrocarbons from the surface lipids were removed by column chromatography and their analyses have been reported previously (3). The wax ester fraction from column chromatography appeared as three overlapping bands on TLC. One of these bands (Rf 0.64) exhibited a strong red-violet color when it was sprayed with 2.5% sulphuric acid and heated to 100 C for 2 to 3 min, a characteristic of sterol esters. The esters of fatty acids and alcohols do not show any color. Another of these bands (Rf 0.61) corresponded to long chain wax esters while the third (Rf 0.54) was similar in retention to a secondary alkyl acetates.

The total ester band showed infrared absorption bands at 2940 cm^{-1} (CH_2 stretch), 2860 cm^{-1} (CH_3 stretch),

TABLE I

Wax Esters of *Ligia*

Retention index	Area %
2030	0.9
2240	2.4
2350	2.3
2430	2.3
2550	1.1
2640	5.5
2720	1.7
2820	7.6
2900	0.6
2980	4.3
3560	6.7
3700[a]	13.5
3900	5.2
4140	4.1
4320	8.1
4500	8.1
4680	10.8
4920	13.5
5100	1.3

[a]Corresponds to C_{34} ester.

1740 cm^{-1}, 1730 cm^{-1} (C=O stretch), 1460 cm^{-1} (CH_2 bend), 1380 cm^{-1} (CH_3 bend) 1240 cm^{-1} (C–O stretch of acetate). By temperature-programmed GLC, it was found that the esters ranged from retention index (RI) 2030 to retention index (RI) 5100 with the major constituents having RI 3700 and 4920 (Table I) where C_{34} ester corresponds to RI 3700.

TLC normally proves a very valuable technique for the separation of the major wax classes (4). However, in the wax esters of *Ligia oceanica*, we encountered a situation which may be more common than is frequently appreciated. The esters contain not only the long chain esters ($C_{20} + C_{20}$) but also the short chain esters where the acid or alcohol component may be C_2 to C_9. In addition there are sterol esters and secondary alkyl acetates. In such a complex mixture the difference in polarity between two of these ester classes may be counterbalanced by the band spreading caused by differences in molecular weight. The result is that the three bands intermingle on the TLC plate, and we found it necessary to saponify the total ester fraction (12% of total lipid extract). After saponification, the long chain acid and alcohol components could be isolated as usual by TLC and subsequent GLC of their derivatives. The short chain constituents both in the saponifiable fraction and in the unsaponifiable fraction were analysed by injection of aliquots directly onto a Porapak Q column (5). The long chain acids (36.0% of the ester saponification mixture) and the long chain alcohols (34.8%) constitute the largest proportion of the esters with the short chain acids (8.0%), short chain alcohols (14.0%), and sterols (7.2%) making up the remainder.

The long chain acids were isolated, converted to their methyl esters and separated on $AgNO_3$ TLC into saturated (41.1%), monounsaturated (42.5), and diunsaturated esters (16.4%). The distribution of the fatty acids in each of these fractions (Table II) showed that C_{16} and C_{18} acids were the major components with palmitoleic, oleic, and linoleic acid present in substantial amounts. Such components are very unusual in surface waxes where more saturated components usually predominate.

However, in the crustaceans *Calanus finmarchicus, Calanus crustatus, Homarus americanus, Euphasia superba,* and *Euphasia pacifica* the major components of the total lipids are $C_{16:0}$ and $C_{18:1}$ and $C_{18:2}$. Another species, *Ommastergles solani*, contains $C_{14:0}$ and $C_{20:0}$ (6).

The total long chain alcohol fraction obtained by saponification showed a wide range of constituents from C_{10} to C_{30} with cholesterol (13.8%) of total alcohols

TABLE II

Analysis of Fatty Acids of Wax Esters

Chain length	Area %
14:0	4.4
14:1	0.6
15:0 br	0.4
15:0 n	0.4
15:1	tr[a]
15:2	0.2
16:0 br	tr
16:0 n	14.7
16:1	6.2
16:2	0.3
17:0 br	0.4
17:0 n	tr
17:1	0.6
17:2	0.3
18:0 br	1.1
18:0 n	6.4
18:1	26.2
18:2	13.6
19:0 br	tr
19:1	tr
19:2	2.1
20:0 n	4.5
20:1	4.9
21:0 n	1.0
22:0 n	7.9
22:1	4.0

[a]tr = trace.

TABLE III

Primary Alcohols and Sterols Acetates on 3% Apiezon

Chain length	Area %
10:0	tr[a]
10:1	6.9
11:0	tr
11:1	tr
12:0	14.2
14:1	0.7
15:1	0.7
16:0	5.7
16:1	0.8
16:2	0.3
17:0	tr
18:1	24.5
19:1	3.0
20:1	3.4
21:0	0.5
22:0	1.7
22:1	0.3
24:1	1.6
26:0	2.2
28:1	5.5
30:0	13.8
Sterols	13.8

[a]tr = trace.

(Table III).

It is interesting to note that the fatty alcohol chain length distribution bears no resemblance to the acid chain length distribution (7). This may indicate that where the lipid is for specialist uses, the direct interconversion of fatty alcohols and acids sometimes reported (8) does not occur.

The sterols were isolated from the unsaponifiable portion of the wax ester fraction by TLC on silica gel using petroleum ether:diethyl ether (1:1 v/v). The sterols were then analysed as the free alcohol (9), as their acetates (10), and trimethylsilyl ethers (11). By comparison of their GLC retention times with standards, (Table IV) the sterols were identified as cholesterol (94.6%), campesterol (0.6%), and fucosterol (4.8%). It is not surprising to find cholesterol as the major sterol in *Ligia oceanica* since cholesterol is the major sterol in the marine environment (12). *L. oceanica*, being a detritus eater, feeds on members of the *Rhodo-*

TABLE IV

Sterol Analyses[a], Retention Time

Standards	(a) 1% OV-1[b] Acet	Free	TMSi	(b) 1% QF-1[b] Acet	Free	TMSi	(a) 1% CHDMS[b] Acet	Free	TMSi
Cholestane	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol	2.55	1.83	2.17	4.36	2.80	2.10	7.10	8.10	2.00
Brassicasterol	2.83			4.90			7.30		
Ergosterol	3.00	2.17		5.45	3.33		10.20	12.3	2.90
Campesterol	3.28	2.29	2.84	5.90	3.39	4.10	8.60		
Stigmasterol	3.52	2.60	3.02	7.09	3.83	2.80	8.80	10.3	2.50
Fucosterol	4.07	2.86	3.48	6.72	4.20	3.20	11.80	13.6	3.20
B-Sitosterol	4.09			7.09			9.70		
Ligia I	2.52	1.80	2.17	4.45	2.80	2.10	7.20	8.10	2.10
II	3.21	2.30	2.88		3.46	4.10			
III	4.05	2.86	3.48	6.82	4.40	3.20	11.40		3.25

[a]GC characteristics: column temperature (a) 250 C, (b) 230 C; detector temperature 300 C; carrier gas, nitrogen at a flow rate of 80 cm^3/min; column support Gas Chrom Z (80-100 mesh).

[b]Author identify OV-1, QF-1, CHDMS.

TABLE V

Fatty Acids of Triglycerides from *Ligia*

Chain length	Area %
12:0	0.2
14:0	3.1
14:1	0.2
14:2	0.1
15:0	0.3
15:1	tr[a]
15:2	0.3
16:0	28.4
16:1	8.8
16:2	1.5
17:0	tr
17:1	0.4
18:0	2.0
18:1	30.9
18:2	23.7

[a]tr = trace.

TABLE VI

Distribution of the Fatty Moieties in the Alkenyl Acyl Diol Esters Fraction, Area %

Chain length	Methyl esters	Ether linked moiety
11:0	-	tr[a]
11:1	-	5.7
12:0	tr	17.5
12:1	-	3.2
13:0	4.1	tr
13:1	-	1.9
14:0	2.1	2.6
15:0	2.3	tr
15:1	4.7	-
16:0	15.7	14.1
16:1	13.7	11.9
17:0	0.6	tr
17:1	-	4.2
18:0	6.0	tr
18:1	14.0	36.9
18:2	36.8	-

[a]tr = trace.

phyta, the red algae, which have cholesterol as the major component. Equally the presence of fucosterol may be related to one of the other foods of *L. oceanica*, the brown algae *Fucus vesciculosus*. It seems likely that both of these sterols are derived from the food of the crustacean. The presence of campesterol in the crustacean is unexpected but since the biotransformation of fucosterol to cholesterol is unknown it may be that campesterol is involved.

The short chain acids analysed by GLC on Porapak Q consisted primarily of C_5 (90%), isovaleric acid, and C_2 (7%) acetic acid with traces of C_7, C_8, and C_9. Such short chain components have not been found previously in waxes although butyl butanoate has been reported in coreoid bugs (13). In addition, long chain alkyl acetates have been found in a number of plant species (14) and as pheromones in insects (15). It seems likely that many earlier analyses of waxes would not determine alkyl acetates because a saponification step was included in the analyses. Extraction of acetic acid from the saponification mixture is very difficult to achieve, and subsequent handling of the acids probably allowed the loss of the volatile short chain acids.

The presence of isovaleric acid has been reported in the porpoise (16) and other marine animals, but in such examples the short chain acid is present in the triglyceride fraction, though there is also a report of a hexadecyl isovalerate (17). Such triglycerides have been extracted from the "melon," the special fat body in the head which is associated with echo location. It may be that the surface lipids of *L. oceanica* also require an unusual building block to provide a wax with better waterproofing qualities such as would be required by an isopod inhabiting the seashore.

The short chain alcohols consisted primarily of heptanol (49%) with hexanol (20%) and pentanol (20%) as minor components. While free hexanol has been found in weaver ants (18), heptanol has not been reported in waxes. The related terrestrial wood louse *Porcellio scaber* contains unsaturated alcohols dec-3-en-1-ol (slaterol) and non-3-en-lol (19). No evidence was obtained for the presence of slaterol in the esters of *L. oceanica*.

The remaining class found in the wax esters was the secondary alkyl esters. After saponification of the esters, the alkyl acetates (Rf 0.44) and the sterol acetates (Rf 0.37) were readily separated from the secondary alkyl acetates (Rf 0.54) on silica gel TLC. By GLC, the composition of the secondary alkyl acetates ranged from C_{20}-C_{29} with C_{26} (25%) and C_{29} (14%) as the major components. The position of the alcohol group on the hydrocarbon chain was not determined. Such components have been found in the cuticular lipids of insects *Melanoplus packardii* and *Malanoplus sanguinipes* (20).

Triglycerides

The triglycerides (present as 20.2% of the total extract) range from C_{42} to C_{54}. The fatty acids, produced by saponification, were methylated and separated by $AgNO_3$ TLC into saturated (34.1%), monounsaturated (40.3%), and

diunsaturated acids (25.6%). By GLC on 1% Apiezon L and 13% Diethylene glycol succinate columns, the fatty acids were identified as ranging from C_{12} to C_{18} (Table V).

Comparison of the fatty acids of the triglycerides and the wax esters suggests that there are two different biosynthetic pools from which the esters and the triglycerides take their fatty acids. Thus although the diunsaturated acids of both triglycerides and esters are similar, there is a difference in the presence of $C_{19:2}$ acid in the wax esters and its absence in the triglycerides. For both monounsaturated and saturated acids, there is an obvious difference in the absence of branched chain fatty acids and longer chain acids C_{20}-C_{22} in the triglycerides.

Alkenyl Acyl Diol Esters

This fraction, though slightly higher on the TLC plate (Rf 0.39) than a standard alkyldiglyceride (0.29), showed a typical yellow coloration when the TLC spot was sprayed with 2,4-dinitrophenylhydrazine, which suggests the presence of an alk-1-enyl ether.

On GLC the intact diol plasmalogen ranged from 3540-4870 with the major components at RI 4500 (47%), RI (4670 (28%), and RI 4300 (15.5%).

After saponification, the alkenyl ethers were converted into the trimethylsilyl derivatives and analysed by GLC and the liberated fatty acids also analysed as their methyl esters. The composition of these two fractions (Table VI) showed that the fatty acids were of the usual type (e.g., as found in mouse) (21) C_{12}-C_{18} though not as widespread as is usual in the marine environment. However the fatty chain on the ether part showed a high proportion of a $C_{12:0}$ constituent. There are many examples of the presence of plasmalogens in the marine environment (22) but this is the first report of their presence on the surface of a crustacean.

Our results show that the surface covering of *Ligia oceanica* is comprised of a mixture of lipids each class of which is somewhat different from the composition expected by comparison either with insects or with marine organisms. It would appear that in the middle ground between sea and land this crustacean has developed lipid mixtures modified by its unusual environment.

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