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IDENTIFICATION OF MEIBOMIAN GLAND LIPIDS BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY: APPLICATION TO THE MEIBOMIAN LIPIDS OF THE MOUSE

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SUMMARY

Methods are described for the structural determination of the constituent alcohols, steroids and fatty acids from meibomian gland esters. The compounds, obtained from hydrolysed extracts, were separated and quantified as trimethylsilyl and methyl ester–trimethylsilyl derivatives by fused-silica capillary column gas chromatography. Structural information relating to the position of chain branching and the position of double bonds in the aliphatic chains of the acids and alcohols was obtained from the mass spectra of a number of other derivatives. Thus pyrrolidide–trimethylsilyl and picolinyl ester–trimethylsilyl derivatives provided information on both branching and unsaturation. The double-bond position in unsaturated fatty acids was also examined by use of trimethylsilyl derivatives of the derived glycols. The positions of chain branching of the alcohols were determined by the spectra of their acetate and nicotinate derivatives and by their gas–liquid chromatographic retention times. In meibomian gland lipids from the mouse, cholesterol was the major constituent; alcohols from the *n*-, *iso*-, *anteiso*- and unsaturated series were present with *iso*-C_{26} and *anteiso*-C_{27} being the most abundant. Mono-unsaturated fatty acids belonged mainly to the $\omega 9$ series and saturated acids belonged to the *iso*-, *anteiso*- and *n*-series. Several 1,2-diols were also identified. The most abundant of these had *iso*-C_{16} and *iso*-C_{20} chains. GC–MS studies on the intact wax esters showed them to be composed of the branched-chain alcohols and both branched-chain and unsaturated acids.

INTRODUCTION

The meibomian glands, which are found on the margins of mammalian eyelids, secrete an oily film over the surface of the eye to reduce evaporation of the tear film. Few studies have been made of the composition of this secretion but those that have been reported have revealed a complex mixture of sterol and wax esters con-

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sisting of normal, branched, unsaturated and hydroxy-fatty acids and alcohols. The relative proportion of these constituents appears to vary considerably with species. Baron and Blough[1], for example, have reported that the major acids of bovine meibomian secretions are *anteiso*-acids with chain lengths of 15, 25 and 27 carbon atoms and unsaturated acids with chain lengths of 16 and 18 carbon atoms. Human lipids, on the other hand, appear to contain more unsaturated acids, particularly 18:1ω9[2,3] although the pattern is subject to considerable interindividual variation[4]. ω-Hydroxy-fatty acids have recently been reported in both steer and human meibomian secretions[5]. Secretions from the rabbit eye have also been examined[6] and a major difference in the steroid profile has been reported in this species compared with the profile observed in most other animals[7].

We have recently studied the meibomian secretions from several mammalian species using gas–liquid chromatography (GLC) and combined gas chromatography–mass spectrometry (GC–MS) and have investigated the use of a number of derivatives for determining the structure of the alkyl chains from the constituent fatty acids and alcohols. The most common derivatives such as methyl esters and trimethylsilyl (TMS) ethers do not provide sufficient information on chain branching or on the positions of double bonds. A number of approaches to this problem have been investigated by other workers, particularly for acids[8] where the use of pyrrolidides[9–12] or ozonolysis followed by TMS ether formation[13] appear to be the most satisfactory methods for determining chain branching and double-bond position respectively. Picolinyl derivatives, which we are currently investigating[14], look particularly promising and applicable to both types of compound. Less work has been reported on derivatives for the structural determination of fatty alcohols[15] but information has been obtained from methyl ethers[16], acetates[15], and nicotinates[17]. In this paper we describe the use of several of these derivatives for investigating the structure of the constituent fatty acids and alcohols of the meibomian secretions of the mouse.

EXPERIMENTAL

Reference compounds

Branched-chain fatty acids (as methyl esters) were obtained from Applied Science Labs. (State College, PA, U.S.A.). Branched-chain alcohols were prepared from these compounds by reduction with lithium aluminium hydride. Normal and unsaturated fatty acids were obtained from Sigma (Poole, U.K.). Reduction of these compounds gave the *n*- and unsaturated alcohols respectively. 1,2-Diols were synthesised from the acids by the method described by Mangold[18]. Long-chain esters were prepared by condensation of the alcohols with the appropriate acid chloride in pyridine; the acid chlorides were prepared by heating the acid with an excess of thionyl chloride for 1 min at 60°C.

General

All extractions and hydrolysis reactions were performed in 4-ml screw-capped vials and Pasteur pipettes were used to transfer solvents. Derivatization reactions were performed in 0.3-ml screw-capped microvials.

Extraction of mouse meibomian lipids

The eyelids were removed from four mice (male, Charles River, CD-1, 25–30 g) and the meibomian glands were dissected out. These were washed with ethyl acetate to remove surface lipids by allowing them to stand in the solvent (1 ml) for 1 min. They were then either squeezed to express the lipids which were then dissolved in ethyl acetate (1 ml), or slit several times with a scalpel and stood in ethyl acetate (1 ml) for 5 min. The organic solution was removed from the residual tissue which was washed with ethyl acetate (1 ml). The combined ethyl acetate fraction was evaporated to dryness and reconstituted in 1 ml of ethyl acetate for storage in the dark at 4°C. Aliquots of this solution were examined by GC–MS after conversion to derivatives as described below.

Hydrolysis of lipids

An aliquot (0.5 ml) of the meibomian gland extract was blown to dryness (nitrogen stream), dissolved in methanol (0.2 ml) and heated at 80°C for 1 h with 1 *N* aqueous sodium hydroxide (0.05 ml). The cooled solution was then diluted with water (1.5 ml), acidified with 5 *N* sulphuric acid and the hydrolysed lipids were extracted with ethyl acetate (3 × 1 ml). The combined ethyl acetate fraction was washed with water (about 1 ml) and a saturated sodium chloride solution (about 1 ml), evaporated to dryness and dissolved in ethyl acetate (2 ml) for storage. Aliquots of this solution were derivatized as described below.

Preparation of derivatives

Pyrrolidides. An aliquot (0.25 ml) of the unhydrolysed meibomian gland extract was evaporated to dryness (nitrogen stream) and dissolved in pyrrolidine (0.1 ml)[9]. Acetic acid (0.02 ml) was added and the mixture was heated at 100°C for 2 h. The mixture was cooled, diluted with water (1.5 ml) and acidified with 5 *N* hydrochloric acid. The meibomian gland constituents were extracted with ethyl acetate (3 × 1 ml), and the combined extracts were washed with dilute hydrochloric acid, water and saturated sodium chloride solution (1 ml of each). The solvent was removed with a nitrogen stream and the residue was dissolved in ethyl acetate (1.0 ml). Aliquots of this extract were derivatized as described below.

TMS derivatives. Aliquots of the unhydrolysed extract (0.1 ml), the hydrolysed extract (0.25 ml) and the pyrrolidides (0.25 ml) were blown to dryness and converted into TMS derivatives by heating them at 60°C for 10 min with 10 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)–trimethylchlorosilane (TMCS)–acetonitrile (2:1:2, v/v/v). Samples of the resulting solution were examined by GC–MS directly.

[$^2H_9$]TMS derivatives[19]. The extracts were heated at 60°C for 10 min with [$^2H_{18}$]bis(trimethylsilyl)acetamide (5 μl) and acetonitrile (5 μl).

Methyl esters. An aliquot of the hydrolysed extract (0.25 ml) was blown to dryness with a nitrogen stream, mixed with an excess (0.3 ml) of ethereal diazomethane (prepared from Diazald, Aldrich) and allowed to stand at room temperature for 2 min. The solvents were then removed with a nitrogen stream. Alternatively the extract (0.25 ml) was heated at 60°C for 10 min with 25 μl of a 3% solution of methanol–HCl (prepared from methanol and acetyl chloride). Fractions derivatized in this way were either reacted further with the trimethylsilylating reagent as described above or were reacted with osmium tetroxide as described below.

Preparation of glycols. The methyl esters, prepared as above, were dissolved in 0.1 ml of a mixture of pyridine–dioxane (1:8) and allowed to react with 0.1 ml of a 2% solution of osmium tetroxide in dioxane at room temperature overnight. The glycols were extracted as described by Capella and Zorzut[13] and converted into TMS ethers as described above.

Acetates. An aliquot (0.25 ml) of the pyrrolidide sample was blown to dryness and heated for 1 h at 60°C with 0.1 ml of a 10% solution of acetic anhydride in pyridine. The cooled mixture was diluted with water (about 1.5 ml), extracted with ethyl acetate (3 × 1 ml) and washed with dilute sulphuric acid, water, and finally with saturated sodium chloride solution (1 ml of each). The dried extract was heated with BSTFA and TMCS as described above.

Nicotinates. Nicotinates of the alcohols present in 0.25-ml aliquots of the meibomian gland hydrolysate or pyrrolidide preparation were synthesised by their reaction with 0.1 ml of fresh nicotinoyl chloride[20] as described previously[15,17]. The products were reacted with BSTFA and TMCS as described above.

Picolinyl derivatives[14]. Aliquots (0.25 ml) of the hydrolysed sample were blown to dryness and allowed to stand for 1 min at room temperature with thionyl chloride (0.3 ml). The thionyl chloride was removed with a nitrogen stream and 20 μl of a 10% solution of 3-pyridylcarbinol in acetonitrile was added. This was allowed to stand at room temperature for 1 min, the acetonitrile was blown off and the alcohols and sterols in the residue were converted into their TMS ethers by heating them for 10 min at 60°C with 10 μl of BSTFA.

2-(Pyrid-2-yl)ethyl esters. These were prepared from the acids in the meibomian gland hydrolysates by the same method as that used for the preparation of the picolinyl esters except that 2-(pyrid-2-yl)ethanol was used in place of the 3-pyridylcarbinol.

Gas chromatography

GLC Separations of the TMS derivatives of the hydrolysed meibomian gland samples were performed with a Varian 2440 gas chromatograph fitted with a flame-ionization detector and a 50 m × 0.3 mm I.D. fused-silica capillary column coated with 0.52 μm OV-1 (Hewlett-Packard). Helium, at 2 ml/min, was used as the carrier gas and the column was programmed at 2°C/min from 100°C to 320°C. The sample was introduced by on-column injection and data were recorded with a Hewlett-Packard 3390A recording integrator. Peak areas were measured by the integrator and quantities of the individual compounds in Table II are expressed as percentages of the total integrated area.

GC–MS

GC–MS analyses were performed with a V.G. Micromass 12B mass spectrometer interfaced to a VG 2050 data system and via a glass jet separator to a Varian 2440 gas chromatograph. The GLC column was 2 m × 2 mm I.D. glass, packed with 3% SE-30 on 100–120 mesh Gas-Chrom Q (Applied Science Labs.). Operating conditions were: column oven temperature programmed from 150°C to 340°C at 4°C/min; injector, separator and ion source temperatures, 300°C, 300°C and 280°C respectively; carrier gas, helium, at 30 ml/min; accelerating voltage, 2.5 kV; electron energy, 25 eV; trap current, 100 μA; scan, 3 sec/decade, exponential, repetitive.

Capillary column GC–MS was performed with a V.G. Micromass 7070F mass spectrometer interfaced to a Varian 2440 gas chromatograph and to the above data system. The column was a 50 m × 0.3 mm I.D. fused-silica OV-1 (film thickness, 0.52 μm) capillary terminating inside the ion source. Operating conditions for the column were as above, those for the mass spectrometer were: electron energy, 70 eV; trap current 1 mA; accelerating voltage, 4 kV. Spectra were obtained at a scan speed of 1 sec/decade. Single-ion chromatograms were recorded with the mass spectrometer tuned to m/z 103 or m/z 74.

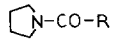
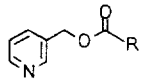
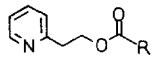
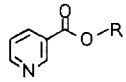
RESULTS AND DISCUSSIONS

Expression of the meibomian fluid from the glands of these small animals was difficult. However preparation of the extract by slitting the glands and extracting with ethyl acetate appeared to give a fairly pure sample with little contamination by lipids from surrounding tissues. This was verified by a corresponding preparation from the expressed lipids and from extracts of rabbit meibomian glands where a pure expressed sample could be obtained more easily. Thus this method is convenient for obtaining samples from small mammals where expression of the glands is difficult. Other workers have drawn similar conclusions from gland homogenates[3] which were shown to give similar profiles to the gland secretions.

Examination of the TMS (Table I, 1a) and methyl–TMS (Table I, 1b) deriv-

TABLE I

DERIVATIVES FOR THE IDENTIFICATION OF THE ACIDS AND ALCOHOLS FROM MEIBOMIAN GLAND LIPIDS

Group	*Derivative*	*No.*	*Structure*
COOH	Trimethylsilyl ester	1a	$(CH_3)_3Si\text{-}OCO\text{-}R$
	Methyl ester	1b	$CH_3\text{-}OCO\text{-}R$
	Pyrrolidide	1c	
	Picolinyl ester	1d	
	2-(Pyrid-2-yl)ethyl ester	1e	
OH	Trimethylsilyl ether	2a	$(CH_3)_3Si\text{-}O\text{-}R$
	Nicotinate	2b	
	Acetate	2c	$CH_3CO\text{-}OR$
Double bond	Glycol–TMS	3	R-CH-CH-R′ $(CH_3)_3SiO$ $OSi(CH_3)_3$

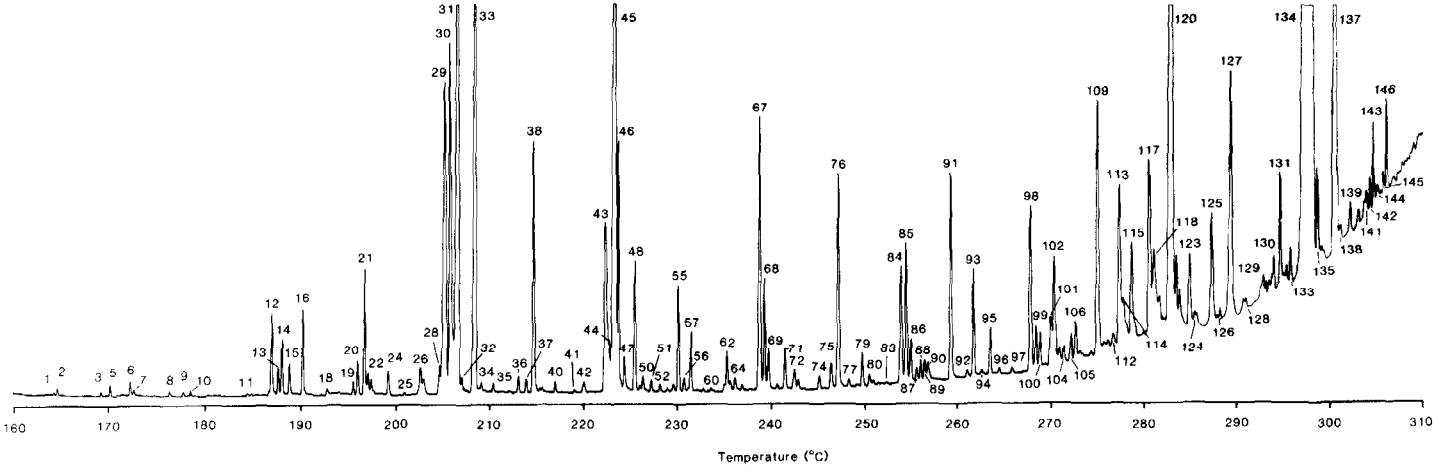


Fig. 1. Gas chromatogram of the methyl-TMS derivatives of the constituents of the hydrolysed extract of mouse meibomian glands. Separation was achieved on a 50 m × 0.3 mm fused-silica capillary column coated wtih OV-1. The separation conditions are listed in the Experimental section. Peaks are given in Table II. Peaks 1–11 are unknown compounds.

atives of the hydrolysed sample by capillary GLC revealed a complex profile of acids, alcohols, 1,2-diols and steroids (Fig. 1). These compounds are listed in Table II. The hydrolysis was complete under the conditions used as demonstrated by the absence of peaks produced by the intact esters. Retention increment shifts between the peaks produced by the two derivatives allowed those produced by the acids to be identified. Further confirmation of peak identity was obtained by plotting single-ion chromatograms of *m/z* 74 for acids and *m/z* 103 for alcohols. GC–MS confirmed these structural types but did not give full information on structural isomers.

The most rapid method for the structural determination of the constituent isomeric acids and alcohols from the extracts was by preparation of pyrrolidide-TMS (Table I, 1c) derivatives[9–12]. These could be prepared directly from the extracts and gave some detailed structural information on the esterified long-chain carboxylic acids and molecular weight information on both acids and alcohols. Free fatty acids were not converted into pyrrolidides under these conditions and eventually appeared as TMS esters in the chromatograms. Alcohols were converted directly into TMS ethers (Table I, 2a). Fig. 2 shows a reconstructed total-ion chromatogram of the pyrrolidide–TMS derivatives of the mouse meibomian gland extracts recorded with an SE-30 packed column. Single-ion chromatograms were plotted by the computer from the stored, repetitively scanned spectra to identify each lipid type. Thus *m/z* 103, $[CH_2=O\text{–}Si(CH_3)_3]^+$, (Fig. 3) was used to identify alcohols and diols, and the diols were differentiated by plots of *m/z* 147, $[(CH_3)_3Si\text{–}O=Si(CH_3)_2]^+$, and *m/z* 205, $[(CH_3)_3Si\text{–}O=CH\text{–}CH_2\text{–}O\text{–}Si(CH_3)_3]^+$. The ion at *m/z* 113, the base peak in the spectra of the pyrrolidides, was used to identify the esterified fatty acids (Fig. 4) and *m/z* 117 from the TMS derivatives of the fatty acids enabled the nonesterified acids to be determined (Fig. 5). The ion at *m/z* 129 localized cholesterol, but the ion was also present in the spectra of the TMS derivatives of the fatty acids. Figs. 6 and 7 show typical spectra of the pyrrolidide derivative of a fatty acid, and the TMS derivative of an alcohol respectively and Fig. 8 shows the spectrum of the TMS derivative of a 1,2-diol.

Double bond position was additionally located by preparation of TMS derivatives of glycols (Table I, 3) which were obtained by reaction of the fatty acid methyl esters with osmium tetroxide[13]. These derivatives generally gave superior results to those produced by the pyrrolidides and were particularly useful when mixtures of unsaturated isomers were present in a single GLC peak.

The spectra of the above derivatives of the major acids clearly showed that they belonged to the *n-*, *iso-*, *anteiso-* and unsaturated series; this was confirmed by comparison with authentic standards. Plots of carbon number against retention time were linear for each structural type. The identified compounds are listed in Table II. These derivatives did not, however, provide clear differentiation by packed column GC–MS, between the unsaturated acids and acids of the *iso*-series as these compounds were not completely separated and their spectra contained only very weak diagnostic ions. Other derivatives were thus investigated to provide more definitive information on the structure of these compounds. The most useful of these were the picolinyl esters (Table I, 1d)[14] prepared directly from the gland hydrolysates. These derivatives could be used to identify both branched and unsaturated acids and were usable for unsaturated acids of high molecular weight where glycol formation gave a product with too long a retention time to elute from the column. Although their

TABLE II

CONSTITUENT ACIDS, ALCOHOL AND STEROIDS OF MOUSE MEIBOMIAN GLANDS AS SEPARATED ON A 50-m OV-1 FUSED-SILICA CAPILLARY COLUMN

Peaks as numbered in Fig. 1. Alcohols and diols are indicated by -OH and diol, respectively. All other compounds except cholesterol are acids.

Peak number	*Compound*	*Retention time (min)*	*%*
12	*iso*-14:0	18.67	0.36
13	14:1,Δ^7	19.00	0.10
14	14:1,Δ^9	19.20	0.21
16	*n*-14:0	20.26	0.36
20	*iso*-15:0	23.10	0.21
21	*anteiso*-15:0	23.44	0.43
24	*n*-15:0	24.68	0.16
29	*iso*-16:0	27.58	2.15
30	16:1,Δ^7	27.98	1.66
31	16:1,Δ^9	28.22	10.02
33	*n*-16:0	29.16	3.67
37	*iso*-17:0	31.90	0.07
38	*anteiso*-17:0	32.29	1.31
40	*n*-17:0	33.25	0.06
43	*iso*-18:0	36.08	1.69
45	18:1,Δ^9	36.51	7.08
46	18:1,Δ^{11}	36.69	0.96
48	*n*-18:0	37.61	0.69
55	*iso*-16-diol	39.90	0.57
56	*iso*-19:0	40.20	0.15
57	*anteiso*-19.0	40.55	0.27
60	*n*-19:0	41.93	0.02
67	*iso*-20:0	44.19	1.54
68	20:1,Δ^{11}	44.45	0.36
69	20:1,Δ^{13}	44.65	0.21
71	*n*-20:0	45.54	0.22
75	*iso*-21:0	47.97	0.14
76	*anteiso*-21:0	48.35	1.25
78	*n*-21:0	49.32	0.02
84	*iso*-22:0	51.66	0.78
85	22:1,Δ^{13}	51.91	0.68
86	22:1,Δ^{15}	52.10	0.20
88	*iso*-22-OH	52.50	0.10
89	*n*-22:0	52.70	0.10
91	*iso*-20-diol	54.31	1.15
92	*iso*-23:0	55.19	0.07
93	*anteiso*-23:0	55.49	0.58
95	*anteiso*-23-OH	56.39	0.24
98	*iso*-24:0	58.54	1.25
99	24:1,Δ^{15}	58.82	0.14
100	24:1,Δ^{17}	59.10	0.13
101	*n*-24:0	59.62	0.12
102	*anteiso*-24-OH	59.82	0.37
106	*n*-24-OH	60.96	0.27
108	*iso*-25:0	61.79	0.04
109	*anteiso*-25:0	62.17	2.18
113	*anteiso*-25-OH	63.30	1.22

TABLE II (*continued*)

Peak number	*Compound*	*Retention time (min)*	*%*
117	*iso*-26:0	64.91	1.65
120	*iso*-26-OH	66.09	7.96
123	*n*-26-OH	67.03	0.62
125	*anteiso*-27:0	68.22	1.05
127	*anteiso*-27-OH	69.22	2.49
129	*iso*-28:0	70.83	0.12
130	*n*-28:0	71.39	0.27
131	*iso*-28-OH	71.74	0.68
134	*anteiso*-29:0	73.08	29.95
	Cholesterol	73.24	
136	*n*-29:0	73.86	0.23

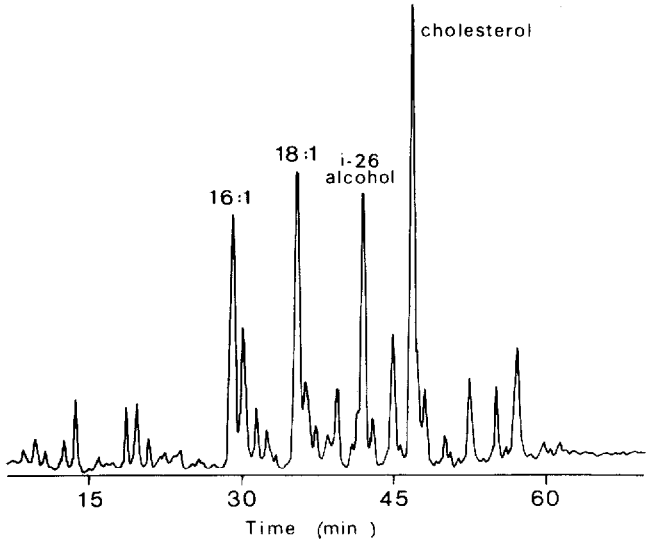


Fig. 2. Computer reprocessed total-ion chromatogram of the hydrolysed lipids from the meibomian glands of the mouse separated on a 2 m × 2 mm 3% SE-30 packed column as pyrrolidide–TMS derivatives. Conditions listed in the Experimental section; i = *iso*.

preparation involved a three-stage reaction, the resulting spectra were easier to interpret than those of the pyrrolidides and the diagnostic ions were of much higher relative abundance (Fig. 9). The spectra of all three derivatives showed that the major 16:1 unsaturated acid (Fig. 1, peak 31) had a Δ^9-double bond (ω7). The major monounsaturated octadecanoic acid (18:1, Fig. 1, peak 45) was also Δ^9 (ω9, oleic acid) and the other homologous unsaturated acids with even carbon numbers were mainly from the ω9 series. They were thus presumably synthesised by chain elongation of oleic acid (18:1,Δ9). The acid 16:1,Δ7 was also identified as a minor constituent (Fig. 1, peak 30) of the C_{16} unsaturated acids and chain elongation of this acid gave *cis*-vaccenic acid (*cis*-18:1,Δ^{11}, Fig. 1, peak 46).

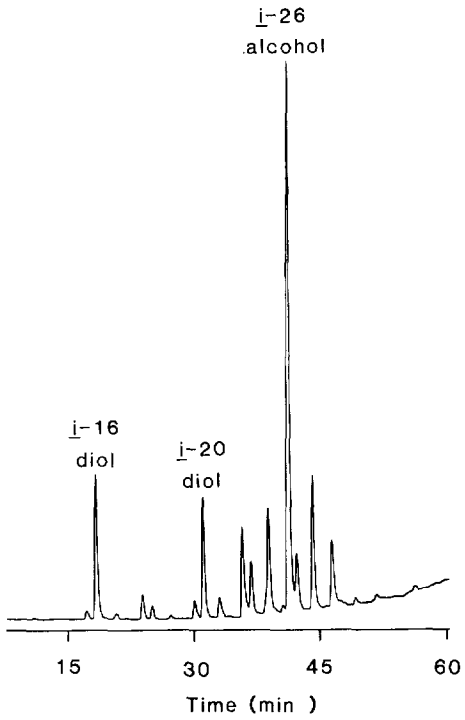


Fig. 3. Single-ion chromatogram of the ion at *m/z* 103, diagnostic for the TMS derivatives of the alcohols in the hydrolysed extract of mouse meibomian lipids. Conditions as for Fig. 2.

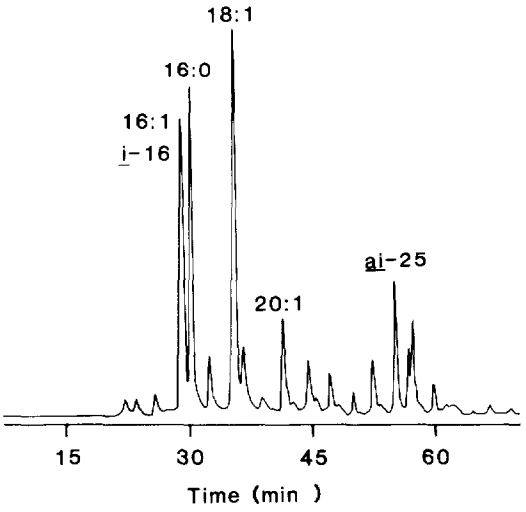


Fig. 4. Single-ion chromatogram of the ion at *m/z* 113, diagnostic for the pyrrolidide derivatives of the esterified fatty acids in the hydrolysed extract of mouse meibomian lipids. Conditions as for Fig. 2; ai = *anteiso*.

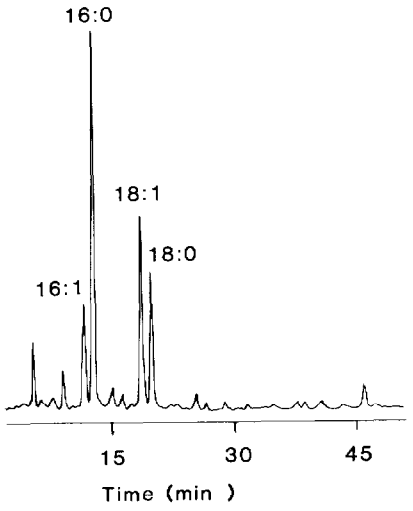


Fig. 5. Single-ion chromatogram of the ion at *m/z* 117 diagnostic for the TMS derivatives of the free fatty acids in the meibomian gland extracts from the mouse. Conditions as for Fig. 2.

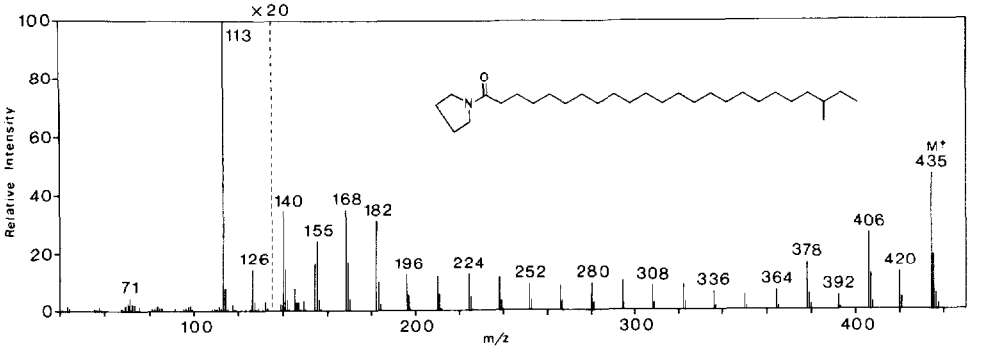


Fig. 6. Mass spectrum (25 eV) of the pyrrolidide derivative of *anteiso*-pentacosanoic acid.

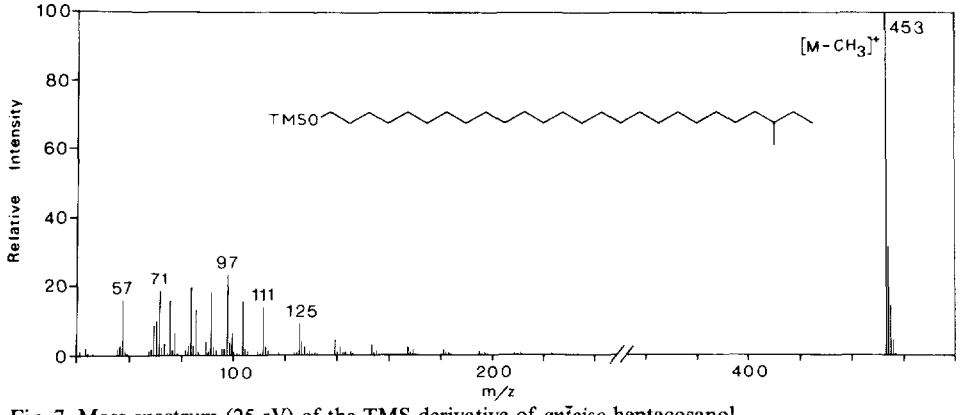


Fig. 7. Mass spectrum (25 eV) of the TMS derivative of *anteiso*-heptacosanol.

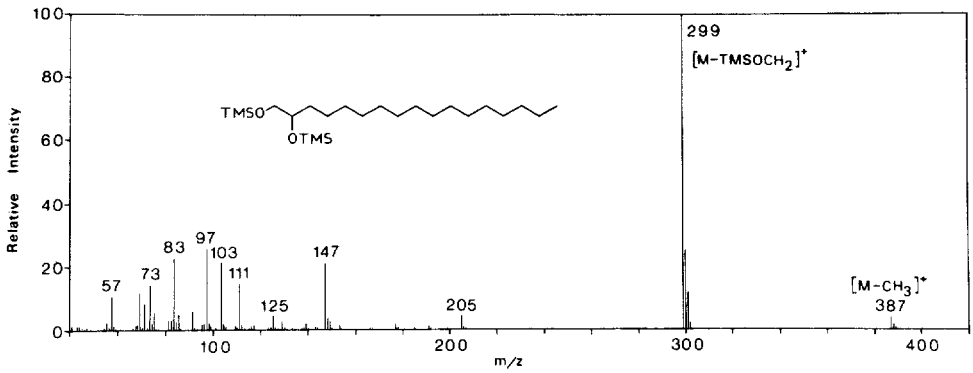


Fig. 8. Mass spectrum (25 eV) of the TMS derivative of 1,2-heptadecanol.

The spectra of the TMS derivatives (Table I, 2a) and [$^2H_9$]TMS derivatives of the alcohols showed that the major compound was cholesterol (Fig. 1, peak 134), identified by comparison with an authentic standard, and that the other compounds were mainly saturated alkanols and 1,2-diols. GLC retention times of the TMS derivatives indicated that the alkanols belonged to the *n*-, *iso*- and *anteiso*-series. Confirmation of this was obtained by preparation of the acetate (Table I, 2c)[15] or nicotinate (Table I, 2b) derivatives and by comparison with authentic standards. The compounds are listed in Table II. The major compound was *iso*-hexacosanol (Fig. 1, peak 120).

Two unsaturated alcohols with chain lengths of 30 and 32 carbon atoms were identified by GC–MS in the chromatograms of the pyrrolidide–TMS derivatives by comparison with standards prepared by reduction of mono-unsaturated acids with lithium aluminium hydride. However, the position of the double bond was not determined because of interference with ions from other compounds. These compounds were not located in the chromatogram obtained by capillary column GLC (Fig. 1).

The major 1,2-diols belonged to the *iso*-series. The compound producing peak 55 (Fig. 1) was identified as 1,2-dihydroxy-*iso*-hexadecane by comparison of its GC–MS properties with those of analogous compounds prepared by reduction of

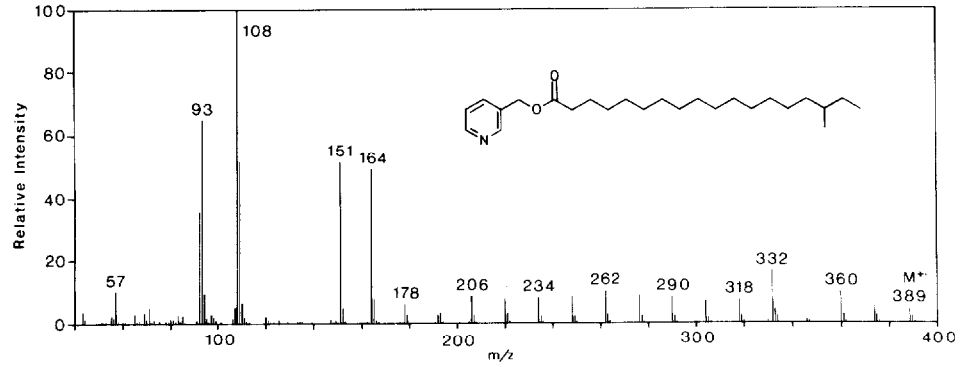


Fig. 9. Mass spectrum (25 eV) of the picolinyl derivative of *anteiso*-nonadecanoic acid.

2-hydroxypalmitic acid with lithium aluminium hydride or from the branched-chain acids by the method of Mangold[18]. The other abundant 1,2-diol (Fig. 1, peak 91) was the *iso*-C_{20}-diol. Traces of other diols were also present but were not identified. The most diagnostic ions for the location of these compounds were *m/z* 103, 147 and 205. Further evidence of the structure of the C_{16} and C_{20} diols was obtained from the spectra of their acetates which showed the absence of the $[M - 60 - 29]^+$ ion characteristic of compounds of the *anteiso*-series[15].

Experiments were conducted with the preparation of mixed picolinyl (Table I, 1d) and nicotinate (Table I, 2b) derivatives of acids and alcohols respectively in an attempt to obtain structural information on both types of compound from the same GC–MS run. However it was found that insufficient separation between the acids and alcohols was obtained on packed columns to enable structures to be determined; the derivatives were isomeric and thus produced ions of the same mass. Better GLC results were obtained by using the homologous 2(pyrid-2-yl)ethyl esters (Table I, 1e) of the acids but the spectra of these derivatives contained very weak diagnostic ions and were thus no better than those of the pyrrolidides.

For the examination of the structures of the intact esters, aliquots of the meibomian gland extracts were examined directly by packed column GC–MS. The cholesterol esters were not observed, but spectra were obtained from the wax esters with up to 48 carbon atoms. Identifications and mass spectral characteristics were determined by comparison with authentic samples synthesised by condensation of acids and alcohols of the different structural types. These spectra showed that molecular weight and hence carbon number information could be obtained from the rather weak molecular ion and that the constituent fatty acid could be identified by the mass of the acylium ion produced by saturated acids (Fig. 10)[21] or the alkenyl ion produced by the unsaturated acids. The chain length of the alcohol was obtained by difference. Verification of the type of chain branching was obtained from the high abundance of the ions at *m/z* 56 and 70 for the *iso*- and *anteiso*-compounds respectively.

Although GLC separation of the wax esters was essentially by carbon number, some separation of structural types was obtained within each peak obtained by packed column GLC. This was detected in the repetitively scanned spectra by plotting single ion traces of the acylium and alkenyl ions. The masses and relative retention times of these ions showed that the wax esters were composed mainly of branched and unsaturated acids and branched-chain alcohols. Esters of acids longer than C_{22} were not eluted from the column but the spectra of the other compounds showed a random distribution of lower-molecular-weight acids and alcohols among the esters with the abundance of the esters reflecting the acid and alcohol distribution observed above. Thus the major ester contained the *iso*-C_{16}-acid and the *iso*-C_{26}-alcohol. It was not possible to determine the chain-type of the constituent acids and alcohols from the spectrum of the intact ester alone as the diagnostic ions at *m/z* 56 and 70 could have come from either the acid or alcohol functions. Thus GC–MS analysis of the hydrolysed esters was essential in order to determine the structures of the constituents independently. The presence of *m/z* 56 or 70 in the mass spectrum of the ester, however, did provide confirmation of structure of these chains when their presence was suspected on the evidence of GLC retention times.

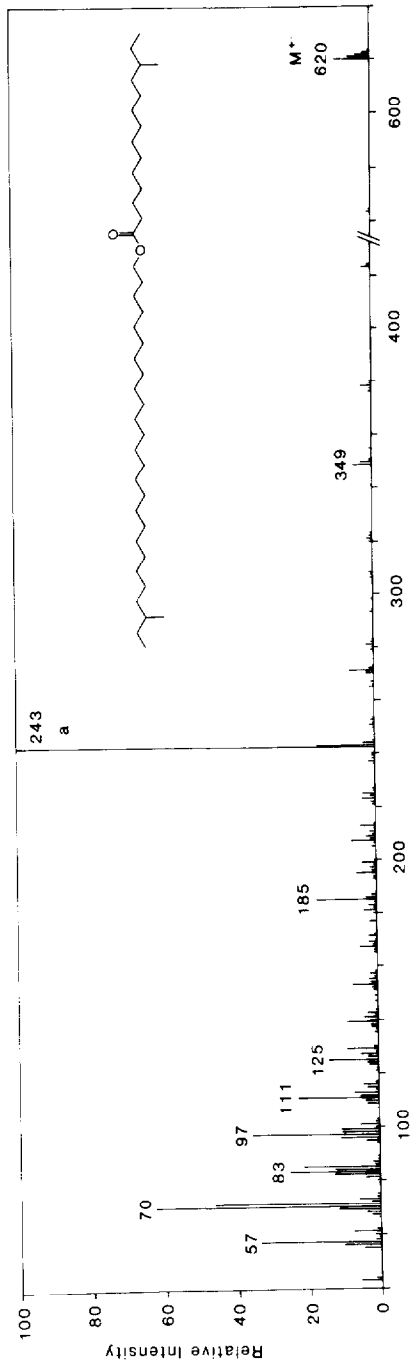


Fig. 10. Mass spectrum (25 eV) of *anteiso*-heptacosyl-*anteiso*-pentadecanoate. Ion a is the acylium ion.

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