# Composition of the neutral lipids of bovine meibomian secretions

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**Abstract** Lipids secreted from the bovine meibomian glands were assigned to the following classes: cholesteryl esters (A) (fatty acyl chain lengths from 15 to 27 carbon atoms), 41%; wax esters, 29%; triacylglycerols, 10%; and cholesteryl esters (B) (fatty acyl chain lengths from 14 to 18 carbons), 15%. The remaining 5% consisted of cholesterol, fatty acids, and highly polar material. Analysis of the lipids showed that only the cholesteryl esters (A) contained fatty acyl chains $\geq C_{20:0}$. One-third of the fatty acyl chains of the wax esters and the cholesteryl esters (B) was $iC_{15:0}$. The fatty alcohol moieties of the wax esters were found to be branched chain, $C_{23:0}$–$C_{27:0}$. A computer-based programmable gas–liquid chromatographic procedure using 10% apolar 10C on Gas Chrom Q could be used to distinguish both *iso* and *anteiso* fatty alcohols and esters of fatty acids.

**Supplementary key words** wax esters · cholesteryl esters · branched chain fatty acids · branched chain alcohols · precorneal tear film lipids · meibomian lipids

The oily secretions of the meibomian glands are a major constituent of the precorneal tear film. These glands, located at the upper and lower margins of the eyelid, secrete materials that are thought to be responsible for the lubrication between the lid margin and the cornea and that act as a hydrophobic barrier preventing evaporation of water from the tear film (1).

The oils from the human meibomian glands are composed mainly of nonpolar lipids, principally cholesteryl esters and wax esters (2, 3), but they also appear to contain a surface active component (4). Analysis of the composition of the principal ester fraction from human meibomian secretions showed the major fatty acyl moieties to be $nC_{18:1}$, $aC_{25:0}$, $iC_{26:0}$ and $nC_{18:2}$, while the major alcohols are cholesterol, $iC_{26:0}$, $nC_{26:0}$, $iC_{24:0}$, and $nC_{24:0}$ (2).

In this paper, we shall describe the characterization of bovine meibomian secretions. The principal nonpolar lipids, in particular the wax esters and cholesteryl esters, were separated by TLC and then the resulting fatty acyl and alcohol moieties were analyzed by GLC.

## MATERIALS AND METHODS

Silica gel G was obtained from Brinkmann (A. G. Merck, Darmstadt). MgO (adsorptive, catalytic grade, 200 mesh) was from Matheson, Coleman, and Bell. All solvents used for TLC and GLC were redistilled. Branched chain fatty acid methyl esters (from $iC_{14:0}$ to $aC_{31:0}$) and mixtures of saturated, monounsaturated and polyunsaturated fatty acid methyl esters were from Applied Science Lab., Inc. State College, Pa. Saturated and monounsaturated fatty alcohols were obtained commercially (Nu Chek Prep, Elysian, Minn.); branched chain alcohols were prepared from the branched chain fatty acid methyl esters according to Nystrom and Brown (5).

### Preparation of bovine meibomian lipids

Approximately 100 eyelids and surrounding tissue were removed from cattle within 5 min after slaughter. The lids were placed on ice. They were then thoroughly rinsed with 0.9% NaCl buffered with 10 mM imidazole–HCl (pH 7.0) and warmed to 37°C in a humidified incubator. The meibomian glands were expressed by applying pressure to both the inner and outer portions of the lids. The secretions were collected on a spatula washed with $CHCl_3$–$CH_3OH$ 2:1 (v/v) and immediately placed in freshly distilled benzene containing 0.001% (w/v) butylated hydroxytoluene as an anti-oxidant.

### Separation and analysis of meibomian lipids

The neutral lipids (200–400 $\mu$g) were separated by TLC on 20 × 36 cm plates of silica gel G (thickness 0.5 mm) using a double development system (6). The lipid-containing spots were visualized by exposure to $I_2$ vapor, scraped off the plate and quantified by charring (7). Lipids for analysis by GLC (about 5 mg) were separated on the same plates as

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; *i*, iso; *a*, anteiso; *n*, normal; tr, trace.

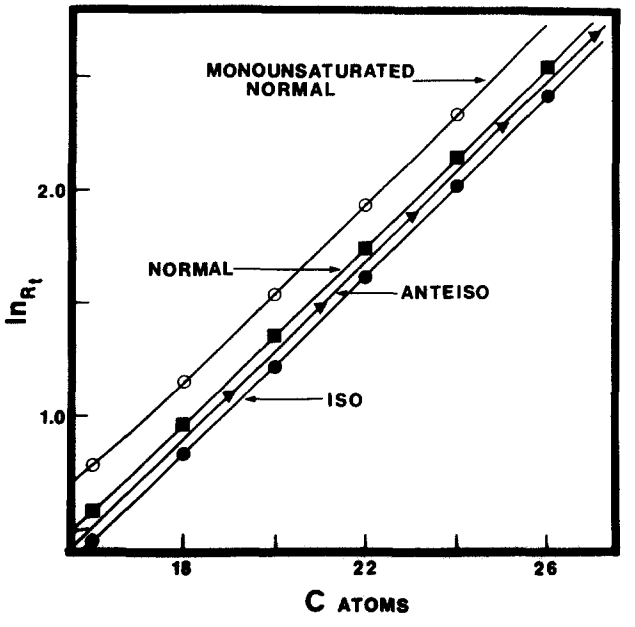


**Fig. 1.** Analysis of fatty alcohol standards by GLC. The alcohols were analyzed on the 10% Apolar 10C on Gas Chrom Q column, as described in Materials and Methods. $R_t$, retention time in min.

described above under a $N_2$ atmosphere. The lipids were visualized with UV light after spraying an edge of the plate with 0.001% rhodamine 6G in water. Lipids were eluted from the plate using the development solvents. Wax esters and cholesteryl esters, which run together on Silica gel G, were then separated by TLC on 20 × 20 cm plates containing MgO (8) (thickness, 0.3 mm) by development with 1% acetone in hexane. The wax esters and cholesteryl esters were visualized as described above and then eluted.

The isolated lipids were saponified in 1N KOH in 95% ethanol; the fatty acids and alcohols were separated and the fatty acids were methylated (9).

### Gas–liquid chromatography of fatty acid methyl esters and alcohols

Analysis by GLC was done in a computerized Hewlett-Packard 5830A gas chromatograph equipped with dual flame ionization detectors (Hewlett-Packard, Avondale, Pa.). The computerized gas chromatograph reported the retention times and the areas under each peak and calculated the percentage area for each peak.

Fatty acid methyl esters were analyzed using the following conditions: I, glass columns, 6 ft × 2 mm; 15% HIEFF-1BP (diethylene glycol succinate) on CWAWDMCS (Chromosorb W—acid washed and dimethyldichlorosilane treated), 60/80 mesh (Applied Science Lab., Inc.); 20 ml $N_2$ per min; 150°C for 0.5 min, 2°C rise in temperature per min, 190°C for 40 min; and II, glass column, 6 ft × 2 mm; 10% Apolar 10C (silicone) on Gas Chrom Q, 100/120 mesh (Applied Science Lab., Inc.); 20 ml $N_2$ per min; 150°C for 0.5 min, 1°C rise in temperature per min, 200°C for 20 min. The detector response when using these columns was checked with GLC Reference Mixtures 17A and 20A (Nu Chek Prep) that contained the following *normal* fatty acid methyl esters: 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 22:0, 22:1, and 24:0. The response of each fatty acid methyl ester was between 96 and 106% on the 15% HIEFF-1BP on CWAWDMCS column and between 95 and 107% on the 10% Apolar 10C on Gas Chrom Q column. The assignment as to *iso-* or *anteiso-* were determined on the 10% Apolar 10C on Gas Chrom Q column. Unsaturated fatty acid methyl esters were confirmed by their disappearance after hydrogenation with $PtO_2$ in $CH_3OH$ and by the appearance of increased amounts of the corresponding saturated species.

Fatty alcohols and cholesterol were analyzed as follows: III, glass columns, 6 ft × 4 mm; 3% OV-1 (methyl silicone) on Gas Chrom Q, 80/100 mesh (Applied Science Lab., Inc.); 40 ml $N_2$ per min; 240°C (isothermal); and IV the same as II but at 200°C (isothermal). The detector response of the 3% OV-1 on Gas Chrom Q column was checked with GLC Reference Standards 32A and 34A (Nu Chek Prep) that contained even number alcohols, saturated and monounsaturated, from 16–24 carbon atoms and with a known cholesterol standard mixed with GLC Reference Standard 32A. The response of each fatty alcohol or cholesterol was between 91 and 107%. The 10% Apolar 10C on Gas Chrom Q column was used to determine the *iso-* and *anteiso-*assignments (**Fig. 1**).

No correction factors were used in the reporting of the composition of the fatty acyl and alcohol moieties of the meibomian lipids; thus the numbers in Tables 2 and 3 are only reliable to within a deviation of 6–8%, e.g., $aC_{25:0} = 25.5 \pm 1.8$.

## RESULTS

### Neutral lipids of bovine meibomian secretions

The meibomian secretions were composed mainly of neutral lipids which were quantified by TLC (**Table 1**). Of the total secretions, 95% of the lipids can be accounted for by wax and cholesteryl esters and triacylglycerols. Cholesteryl esters were found not only in the spot with the highest $R_f$ but also in the spot directly below it. The cholesteryl esters with the highest $R_f$ (identical with that of the wax esters) are referred to as cholesteryl esters

(A) and the spot below (which contained no wax esters) are referred to as cholesteryl esters (B). The remaining 5% was free cholesterol, free fatty acids, and material remaining at the origin consisting primarily of glycolipids and protein.[1]

### Fatty acid analysis of neutral meibomian lipids

After isolation of the wax esters, cholesteryl esters (A), cholesteryl esters (B), and triacylglycerols by TLC, the fatty acyl moieties were analyzed by GLC. Striking differences were noted among the different classes of neutral lipids (**Table 2**). Acyl chains with 20 carbon atoms or more were found only in cholesteryl esters (A) with the major species, $aC_{25:0}$. The major components in the wax esters and cholesteryl esters (B) were $aC_{15:0}$, $nC_{16:1}$, and $nC_{18:1}$. The triacylglycerols contained mostly $nC_{16:0}$, $nC_{16:1}$, and $nC_{18:1}$. The presence of glycerol following the saponification of the triacylglycerol fraction was demonstrated by the assay with chromotropic acid (10, 11), and by formation of the trimethylsilyl derivative of glycerol with Tri-Sil 'Z' (Pierce Chemical Co. Rockford, Ill.) and GLC analysis on a glass column of 3% SE-30 (methyl silicone) on Gas Chrom Q, 100/120 mesh.

### Fatty alcohol analysis of neutral meibomian lipids

The alcohol moieties of the wax esters and cholesteryl esters (A) and (B) were determined by GLC (**Table 3**). The main sterol nucleus of the cholesteryl esters (A) and (B)[2] was cholesterol. Although another unknown peak was detected, which co-chromatographed with a species of oxidized cholesterol, it did not correspond to any of the following sterols: coprostanol, cholestanol, desmosterol, or lanosterol.

The wax ester fraction contained only five fatty alcohols. These were identified as $aC_{23:0}$, $iC_{24:0}$, $aC_{25:0}$, $iC_{26:0}$, and $aC_{27:0}$. The relative amounts of the fatty alcohols were determined using the 3% OV-1 on Gas Chrom Q column and the assignment as to *iso*- or *anteiso*- was determined on the 10% Apolar 10C on Gas Chrom Q column.

## DISCUSSION

The preponderance of branched chain fatty acids and alcohols found in the meibomian secretions is not unexpected since the tear film is exposed directly to

[1] Baron, C., and H. A. Blough, unpublished results.

[2] In some of the preparations of the meibomian secretions, the cholesteryl ester (B) component contained 50–70% of six distinct GLC species whose assignments could not be made. Recent preparations did not contain these species, so further analysis will have to wait until we obtain a preparation which contains these components. The variations may be due to the diets of the animals from which we obtained the meibomian secretions.

TABLE 1. Composition of meibomian lipids

| Lipid | $R_f$ | Percent Composition | Range |
|---|---|---|---|
| Cholesteryl esters (A) + Wax esters | 0.67 | 69.7 (± 5.5) | 60.4–75.2 |
| Cholesteryl esters (B) | 0.53 | 15.4 (± 2.7) | 12.1–20.2 |
| Triacylglycerols | 0.43 | 9.8 (± 1.6) | 7.0–11.8 |
| Cholesterol | 0.29 | 1.5 (± 1.4) | 0.0–3.9 |
| Fatty Acids | 0.21 | 1.0 (± 0.8) | 0.2–2.5 |
| Origin | 0.0 | 1.7 (± 1.0) | 0.5–4.1 |

The following standards were used in the charring assay: cholesterol, cholesteryl stearate, cholesteryl palmitate, cetyl palmitate, stearic acid and tristearin.

TABLE 2. Fatty acyl moieties of bovine meibomian neutral lipids

| Fatty Acid | Cholesteryl Esters (A) | Wax Esters | Cholesteryl Esters (B) | Triacylglycerol |
|---|---|---|---|---|
| $nC_{14:0}$ | tr | tr | 1.9 | 2.4 |
| $aC_{15:0}$ | 3.9 | 33.4 | 17.0 | 4.2 |
| $iC_{16:0}$ | tr | 0.8 | 1.5 | tr |
| $nC_{16:0}$ | 1.9 | 2.4 | 6.7 | 25.0 |
| $nC_{16:1}$ | 2.8 | 14.9 | 23.1 | 10.0 |
| $aC_{17:0}$ | 1.6 | 6.0 | 4.1 | 1.3 |
| $iC_{18:0}$ | 1.6 | 3.2 | 2.7 | 1.5 |
| $nC_{18:0}$ | 0.5 | tr | 0.7 | 3.7 |
| $nC_{18:1}$ | 9.5 | 32.9 | 31.8 | 47.6 |
| $aC_{19:0}$ | 2.4 | | | |
| $nC_{18:2}$ | 1.6 | 5.3 | 9.9 | 3.3 |
| $nC_{20:1}$ | 1.9 | | | |
| $aC_{21:0}$ | 5.6 | | | |
| $iC_{22:0}$ | 1.0 | | | |
| $nC_{22:1}$ | 2.3 | | | |
| $iC_{23:0}$ | 6.1 | | | |
| $iC_{24:0}$ | 4.7 | | | |
| $nC_{24:0}$ | 7.3 | | | |
| $aC_{25:0}$ | 25.5 | | | |
| $iC_{26:0}$ | 5.3 | | | |
| $nC_{26:0}$ | 2.8 | | | |
| $aC_{27:0}$ | 10.0 | | | |

Fatty acid methyl esters were analyzed on the GLC columns as described in Materials and Methods.

TABLE 3. Fatty alcohol moieties of bovine meibomian neutral lipids

| Fatty Alcohol | Cholesteryl Esters (A) and Wax Esters[a] | Cholesteryl Esters (A) | Wax Esters | Cholesteryl Esters (B) |
|---|---|---|---|---|
| $aC_{23:0}$ | 1.8 | | 6.1 | |
| $iC_{24:0}$ | 2.4 | | 6.0 | |
| $aC_{25:0}$ | 18.3 | | 48.6 | |
| $iC_{26:0}$ | 7.2 | | 14.3 | |
| $aC_{27:0}$ | 10.7 | | 24.0 | |
| Cholesterol | 54.6 | 86.3 | | 68.4 |
| Unknown sterol | 5.0 | 13.7 | | 31.6 |

Fatty alcohols were analyzed on the GLC columns described in Materials and Methods.

[a] Before separation by TLC on MgO plates.

the air and if a large amount of polyunsaturated species were present, they would probably undergo extensive oxidation. In other organs, where lipids are exposed to air, the fatty acyl chains consist primarily of saturated and branched species: skin (12) and preen glands of birds, viz. wax secretions (13).

Analysis of the lipids of bovine meibomian secretions raises certain questions concerning the synthesis of these lipids. It is probable that the enzyme system(s) responsible for the synthesis of cholesteryl esters is regulated in such a way as to acylate with long chain branched or *normal* fatty acids as well as shorter chain ones. A considerable amount (approximately 48%) of the fatty acids esterified to cholesterol were $\geq C_{20:0}$. The absence of these from the other neutral lipids suggests a possible limiting chain length for the condensation of fatty acid moieties with fatty alcohols and/or glycerol in the meibomian gland.

The meibomian secretions are major components of the precorneal tear film. The structure(s) that these lipids assume in the film is unknown. A structure for the tear film has been proposed by Holly (14) in which the film may have three or four distinct layers: a bottom layer of absorbed mucin, an intermediate aqueous layer which may contain two phases, and an outermost layer which is a superficial bilayer. Much of this model is based on studies of meibomian oils and artificial tear solutions on water, artificial surfaces, and corneal surfaces (4, 15). We would like to add to this the possibility that structures similar to plasma lipoproteins may exist, that is, the apolar lipids exist in an inner phase or core while protein and polar lipids occupy all or most of the surface (16). The determination of whether or not this is the actual structure of the film will have to await direct examination of the intact tear film.

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