Evaluation and Quantitation of Intact Wax Esters of Human Meibum by Gas-Liquid Chromatography-Ion Trap Mass Spectrometry

Igor A. Butovich,^{1,2} Juan C. Arciniega,¹ Hua Lu,¹ and Mike Molai¹

PURPOSE. Wax esters (WE) of human meibum are one of the largest group of meibomian lipids. Their complete characterization on the level of individual intact lipid species has not been completed yet. We obtained detailed structural information on previously uncharacterized meibomian WE.

METHODS. Intact WE were separated and analyzed by means of high-temperature capillary gas-liquid chromatography (GLC) in combination with low voltage (30 eV) electron ionization ion trap mass spectrometry (ITMS). 3D (mass-to-charge ratio [m/z] versus lipid sample weight versus signal intensity) calibration plots were used for quantitation of WE.

RESULTS. We demonstrated that GLC-ITMS was suitable for analyzing unpooled/underivatized WE collected from 14 individual donors. More than 100 of saturated and unsaturated WE (SWE and UWE, respectively) were detected. On average, UWE represented about 82% of the total WE pool. About 90% of UWE were based on oleic acid, while less than 10% were based on palmitoleic acid. The amounts of poly-UWE were <3% of their mono-UWA counterparts. SWE were based primarily on C₁₆-C₁₈ fatty acids (FA) in overall molar ratios of 22:65:13. A pool of C_{16:0}-FA was comprised of a 20:80 (mol/mol) mixture of straight chain and iso-branched isomers, while the corresponding ratio for C_{18:0}-FA was 43:57. Interestingly, C_{17:0}-FA was almost exclusively branched, with anteiso- and iso-isomers found in a ratio of 93:7.

CONCLUSIONS. GLC-ITMS can be used successfully to analyze more than 100 individual species of meibomian WE, which were shown to comprise 41 ± 8% (wt/wt) of meibum, which made them the largest group of lipids in meibum. (*Invest Ophthalmol Vis Sci.* 2012;53:3766-3781) DOI:10.1167/iov.11-9333

Wax esters (WE) have been demonstrated repeatedly to be an intrinsic and large group of meibomian gland lipids, which are known collectively as meibomian gland secretions, or meibum.¹ This secretion has a critical role in

protecting the surface of the human eye from desiccation by mixing with aqueous tears (produced by lacrimal glands) and forming the outermost part of the tear film layer, the tear film lipid layer. This lipid-rich layer “seals” the underlying aqueous layer of the tear film, keeping the ocular surface moist, which is critical for its health and good vision.^{2,3} Over a period of more than four decades, meibomian WE have been evaluated in a number of studies of animals and humans (for comprehensive reviews of earlier findings, see the most recent reviews on the topic⁴⁻⁷). However, for the purpose of our study, only information related directly to human meibum will be discussed. Earlier, human meibomian WE from normal (non-dry eye) and dry eye subjects were studied using gas (or gas-liquid) chromatography with flame ionization detection (GC-FID and GLC-FID), and GC- and GLC-mass spectrometry (GC- and GLC-MS).^{1,8-12} However, the molecular weights of meibomian waxes were not determined, and their exact molecular structures were not elucidated. Importantly, when G(L)C-MS was used, saponifiable meibomian lipids (such as WE and steryl esters) were transesterified, so that their structures (i.e., the exact combinations of their fatty acid [FA] and fatty alcohol [FAI] components) became scrambled. On the other hand, when G(L)C-FID was used with intact WE,¹⁰ the lipids were classified only on the basis of their retention times (RT), which then were compared to authentic lipid standards of straight chain saturated and unsaturated WE. This procedure, though useful, could not guarantee a positive identification of the analytes, as 1) not every standard was/is available; 2) the method requires extensive intra- and extrapolation of data, and 3) the likelihood was that some of the components of meibum might co-elute, making their detection and/or quantitation impossible by means of G(L)C-FID. A detailed discussion of those earlier findings is provided later in this study. Importantly, it was demonstrated previously that certain classes of meibomian WE could be analyzed by GLC-MS, and a good separation of saturated and unsaturated compounds could be achieved.⁷

Thus, the goals of our study were to evaluate unmanipulated human meibum collected from healthy volunteers for the presence of WE by using GLC-ion trap MS (GLC-ITMS), elucidate the structure of the intact lipids using fragmentation analysis, and develop (if possible) a WE quantitation procedure. No conclusions about the effect of sex, age, race, or diet on the inter-subject variability of meibomian lipid secretions were to be made.

MATERIALS AND METHODS

Samples of meibum were collected using a protocol described previously¹³ and the amounts of collected meibum were measured gravimetrically. All sample collection procedures that involved human subjects were approved by the UT Southwestern Institutional Review Board, and were conducted in accordance with the Declaration of

From the ¹Department of Ophthalmology and ²The Graduate School of Biomedical Sciences, University of Texas Southwestern Medical Center, Dallas, Texas.

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Corresponding author: Igor A. Butovich, Department of Ophthalmology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9057; Telephone 214-648-3523; Fax 214-648-9061; igor.butovich@utsouthwestern.edu.

Helsinki. We collected 14 samples of meibum from healthy, non-dry eye volunteers (nine males and five females), with an average age of 37 ± 6 years. The dry weights of individual samples ranged from 0.3 to 2.4 mg, with a median weight of 0.8 mg. Meibomian lipid samples were stored in dry state under nitrogen at -80°C in HPLC-MS certified glass vials with Teflon caps. Just before the analyses, each sample was dissolved in 1 mL chloroform. No deterioration of samples was observed during a 6-month storage of the samples. The meibomian lipids were quantitated using 3D (mass-to-charge ratio [m/z] versus lipid sample weight versus signal intensity) calibration plots.

Lipid standards were from Nu-Chek Prep, Inc. (Elysian, MN), Matreya, Inc. (Pleasant Gap, PA), and Sigma-Aldrich (St. Louis, MO).

A Trace GC Ultra gas chromatograph with a TG-5MS capillary column (length 30 m, internal diameter 0.25 mm, film coating 0.25 μm), and an ITQ 1100 ITMS detector (all from Thermo Scientific, Waltham, MA) were used for the WE analyses. The detector was operated in the electron ionization (EI) mode with electron energy (EE) set to -30 V and an ionization current of ~ 250 μA . The spectra were collected in the m/z range of 50 to 1000 with 3×25 ms microscans with a typical isolation width of 1.5 mass units. The temperature of the ion source was 250°C . High-purity helium was used as carrier gas. A Triple Trap (Thermo Scientific) was used to remove any possible traces of oxygen and water from the carrier gas. The injector temperature was maintained at 300°C . The MS-transfer line was kept at 325°C . Between 0.2 and 2 μL of samples dissolved in either *n*-hexane or chloroform were injected in a splitless mode using an AI3000 autoinjector (Thermo Scientific). The temperature gradient elution program was as follows: flow rate 1 mL/min (or 40 cm/min), starting temperature 250°C , and linear increase of temperature at a $2^{\circ}\text{C}/\text{min}$ rate for 50 minutes. The final temperature of 350°C was held for 10 minutes and then the column was cooled down to 250°C during the next 5 minutes. A two-minute hold time concluded the 67-minute run.

To make stock solutions with exact concentrations of standard lipids (typically between 1 and 3 mM), aliquots of the compounds were dissolved in chloroform and stored in glass vials in a -80°C freezer. The dry lipids and their solutions were handled using glass, stainless steel, and Teflon lab ware only.

The GLC-ITMS data were analyzed using the Xcalibur software version 2.1 (Thermo Scientific). Two different types of experiments were performed. In the first, total ion chromatograms (TIC) were recorded. Then, chromatograms of selected ions were extracted from TIC for each of the ions of interest and plotted as relative ion abundance versus RT. The resulting extracted ion chromatograms (EIC) were smoothed using the Xcalibur's built-in Avalon 5-point smoothing routine. Then, the detected chromatographic peaks were integrated with valley detection enabled. Only those peaks that exceeded 0.5% of the tallest peak in the chromatogram with a signal-to-noise ratio of 3 or above, were taken into consideration. Standard lipids were analyzed in the same fashion.

In the second type of experiments, selected ion monitoring of ions of interest was performed with or without their collision-induced dissociation. A range of MS/MS fragmentation experiments were conducted, mostly in selected reaction monitoring mode (e.g., m/z $620 \rightarrow 270/271$) with the Xcalibur's automatic optimization of fragmentation option selected. Finally, some lipids were analyzed in the data dependent scan mode. A preliminary study on this method has been reported recently.⁷

A SigmaPlot software package version 11 and a TableCurve 3D software version 4.0 (Systat Software, Inc., Chicago, IL) were used for statistical analysis of the data, curve-fitting, and quantitation of lipids.

RESULTS

Ionization, Fragmentation, and Chromatographic Behavior of Standard Lipids

Meibomian gland secretions were expected to be a complex mixture of WE, cholesterol, and cholesteryl esters, (O-acyl)-

omega-hydroxy FA, triacylglycerols, and other, even more complex compounds, such as "di- and tri-esters" proposed by Nicolaides et al.⁸ As the goal of our study was to develop a procedure for meibomian WE analysis, the first step was to optimize conditions of the analysis of intact (i.e., underivatized) WE by GLC-ITMS.

First, the effect of EE on the mass spectra of standard WE was tested. A standard EE of -70eV (Fig. 1A, upper panel) was demonstrated to cause a strong fragmentation of the analytes, and to lead to weak signals of molecular ions M^+ and/or pseudomolecular (protonated) ions $(\text{M}+\text{H})^+$. This situation potentially could harm the analyses of complex meibomian WE mixtures. Thus, in an attempt to minimize the fragmentation of the analytes, and increase the relative abundances of the M^+ and/or $(\text{M}+\text{H})^+$ ions of WE, lower EE of -30 and -50 eV were tested. At these lower EE, the relative abundances of the $(\text{M}+\text{H})^+$ ions increased noticeably, while the intensities of smaller product ions with m/z values below 200 decreased (Fig. 1A, lower panel). Also, we observed that at an EE of -30 eV, a 6- to 7-fold increase in the relative abundance of M^+ and/or $(\text{M}+\text{H})^+$ ions of unsaturated WE, and a 2- to 3-fold increase in the relative abundances of the corresponding ions of saturated waxes, were achieved (Figs. 1B, 1C). This approach was of particular help in studying unsaturated WE, which otherwise produced very weak M^+ ions (Fig. 1D) under all tested conditions, and fragmented much stronger than saturated WE. As these conditions are used in GLC-MS less frequently than a standard EE of -70 eV (see, for example, a study by Fitzgerald and Murphy¹⁴), we evaluated this low energy approach in more detail.

A 0.1 mM equimolar test mixture of lipids comprised of stearic acid; stearyl stearate; mono-, di-, and tri-stearins; free cholesterol; cholesteryl undecanoate; cholesteryl laurate, and cholesteryl oleate, was prepared and analyzed as described in the Materials and Methods section. A sample chromatogram of the mixture is shown in Figure 2. Under the tested conditions, only four of nine compounds eluted and/or were easily detectable on TIC (Fig. 2, panel A). These compounds eluted in the following order: cholesterol (m/z 386, M^+ , RT 14.2 minutes, panel B), stearyl stearate (m/z 536, M^+ , RT 28.3 minutes, panel C), cholesteryl undecanoate (m/z 368, $[\text{M} - \text{FA}]^+$, 40.2 minutes, RT 40.2 minutes, panel D), and cholesteryl laurate (m/z 368, $[\text{M} - \text{FA}]^+$, RT 42.7 minutes, panel D). The rest of the compounds either produced very weak signals, or did not elute at all. In the tested conditions, the RT of stearic acid (<4 minutes) was too short to be recorded as its peak overlapped with the injection peak. Note that it was possible to detect and quantitate stearic acid, along with other FFA, at lower GLC column temperatures (not shown). Two very weak GLC signals of di-stearin (m/z 606, $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$, RT 40.5 and 41.9 minutes, Fig. 2, panel E) clearly originated from its 1,2- and 1,3-isomers. Notably, the second GLC peak was that of 1,2-distearin, as in fresh preparations of the lipid this was the only isomer detected. With time, however, the lipid isomerized spontaneously in solution due to the known phenomenon of intramolecular migration of their acyl chains.¹⁵ Both isomers produced very weak $(\text{M} + \text{H})^+$ signals, but their much stronger $(\text{M} + \text{H} - \text{H}_2\text{O})^+$ ions made them detectable and identifiable. The last two detected GLC peaks were those of cholesteryl undecanoate and cholesteryl laurate. No $(\text{M} + \text{H})^+$ ions were observed for either of these compounds. However, the esters produced strong signals m/z 368 (dehydrated cholesterol) and m/z 353 (a demethylation product of the former), both of which could have been used as analytical ions. The absence of $(\text{M} + \text{H})^+$, $(\text{M} + \text{H} - \text{H}_2\text{O})^+$, and $(\text{M} - \text{cholesterol})^+$ ions, made the GLC retention times of cholesteryl esters the only parameter suitable for their identification. Mono-stearin produced very weak ions m/z 359 ($[\text{M} + \text{H}]^+$) and m/z 341 ($[\text{M} + \text{H} - \text{H}_2\text{O}]^+$),

FIGURE 1. Effects of electron energy on the mass spectra of standard wax esters. **(A)** Representative mass spectra of behenyl stearate (m/z 592.5) obtained with electron energy of -70 eV (upper spectrum) and -30 eV (lower spectrum). Note the much stronger fragmentation of the analyte, and a much weaker M^+ signal, at -70 eV. **(B)** Single reaction monitoring chromatogram of behenyl oleate (m/z 590.5) recorded with electron energy -70 eV (upper trace) and -30 eV (lower trace) using the same scale. Note the much smaller peak area and peak height at -70 eV compared to -30 eV. **(C)** Effects of electron energy on EIC of M^+ ions of selected saturated and unsaturated wax esters. Note the much stronger decline of the signals of unsaturated compounds with rising EE. **(D)** Representative mass spectra of behenyl oleate obtained with electron energy of -70 eV (upper spectrum) and -30 eV (lower spectrum). Note the much stronger fragmentation of the analyte, and the much weaker M^+ signal, at -70 eV.

but somewhat stronger protonated product ions of stearic acid (m/z 285) and those of the stearic acid dehydration product (m/z 267). When a much more concentrated 1.2 mM stock solution of mono-stearin was made, the compound became detectable on TIC and EIC as a distinctive peak with RT 7.8 with an m/z value of 341 (Fig. 2, panel F). Cholesteryl oleate and tri-stearin did not elute from the column as detectable peaks, most likely because of their lower volatility and/or stability under the conditions of GLC-MS analysis.

Notably, the intensities of the peaks of the analytes varied greatly, though the compounds were tested in equimolar quantities. The peaks with lowest intensity were from stearins,

while the most intense peak was produced by stearyl stearate. This was found to be true for all tested conditions, regardless of whether the peaks were detected in the TIC, EIC, or single reaction monitoring (SRM) modes (as M^+ , $[M + H]^+$, or $[M + H - H_2O]^+$ ions), the relative abundances of stearyl stearate ions were one to two orders of magnitude higher than those of acyl glycerols, and 3 to 4 times higher than those of cholesterol.

GLC-ITMS of Mixtures of Straight Chain Wax Esters

Next, a model mixture of ten standard WE was tested. The tested compounds were myristyl laurate, lauryl oleate,

FIGURE 2. Chromatographic and mass spectrometric analysis of a 0.1 mM equimolar mixture of eight standard lipids. Tested compounds included stearic acid; stearyl stearate; mono-, di-, and tri-stearins; free cholesterol; cholesteryl undecanoate; cholesteryl laurate; and cholesteryl oleate. (A) Total ion chromatogram of the mixture. Only four major peaks were detected. Their retention times (in the order of elution) were the same as for free cholesterol, stearyl stearate, cholesteryl undecanoate, and cholesteryl laurate. Other lipids either did not elute (cholesteryl oleate, tri-stearin), or produced very small TIC peaks (mono- and di-stearin). (B) EIC of ion m/z 386 (free cholesterol, M^+). (C) EIC of ion m/z 536 (stearyl stearate, M^+). (D) EIC of ion m/z 368 (dehydrocholesterol; fragmentation product of cholesteryl undecanoate and cholesteryl laurate). Note that cholesteryl oleate did not elute (E) EIC of ion m/z 606 (1,2- and 1,3-distearins). (F) EIC of ion m/z 341 [mono-stearin, $(M - H_2O + H)^+$].

palmityl oleate, stearyl oleate, stearyl stearate, arachidyl oleate, arachidyl stearate, behenyl oleate, behenyl stearate, and behenyl behenate, chosen on the basis of their similarities to meibomian WE.¹⁶ These saturated and unsaturated straight chain wax esters were mixed in equimolar quantities to produce a 0.1 mM solution of each of the analytes, and then analyzed as described above. The TIC and superimposed EIC of the $(M+H)^+$ ions of the esters are shown in Figure 3. Mono-unsaturated WE always eluted noticeably earlier than their saturated counterparts of the same carbon chain length (Fig. 3, panel A). Generally, regardless of the individual lengths of their FA and FAI parts, all WE eluted strictly in the order of their m/z values (Fig. 3, panel B, insert). The data plotted as retention time versus m/z were approximated by a linear equation 1:

$$\text{Retention Time (min)} = -58.69 + 0.16 \times m/z \quad (1)$$

with a correlation coefficient r^2 of 0.9997. This equation (within the limitations discussed below) was used to predict the RT values of WE with known m/z values and, conversely, to estimate their molecular masses using their RT.

Note that the TIC peak areas of pure lipid standards were quite similar for all tested pairs of saturated and unsaturated WE: $C_nH_{2n}O_2$ and $C_nH_{2n-2}O_2$ with the same number of carbons (n) produced TIC peaks in a ratio of roughly 1:1.2 (Fig. 3, panel A). At the same time, saturated WE produced much stronger signals of M^+ ions than their unsaturated counterparts: when EIC were plotted, the calculated peak areas of $C_nH_{2n}O_2$ and $C_nH_{2n-2}O_2$ pairs of lipids differed by more than one order of

magnitude (Fig. 3, panel B). The average ratio of the M^+ signals for the pairs of saturated and unsaturated WE of the same length was determined to be roughly 1:14.

In the tested conditions, the intensities of the MS signals of an equimolar mixture of standard WE increased proportionally, though non-linearly, to the m/z values of the analytes in the range of 450 to 676 arbitrary mass units (amu). The steepest increase in the signals was observed for saturated WE, which rose 5-fold with the increase in m/z from 450 to 676, while signals of unsaturated WE of similar masses increased only by a factor of ~ 2 . Another useful observation was made with regard to the relative abundances of the in-source generated FA residues: the intensities of the FA signals spontaneously generated from an equimolar mixture of five WE (behenyl heptadecanoate, behenyl stearate, behenyl arachidate, behenyl behenate, and behenyl lignocerate) increased proportionally to their molecular masses, but remained very close nevertheless. As most WE of meibomian gland secretions were expected to be in the range between m/z 500 and m/z 700 or so, with their FA fragments ranging mostly from C_{16} to C_{20} , these correlations seem to be a useful exploratory and analytical tool in evaluation of the relative abundances of these, or similar, compounds in human samples.

Fragmentation patterns of the standard saturated WE were further studied in a data dependent scan mode. Three WE were tested as an equimolar mixture: behenyl heptadecanoate (MW 578), behenyl arachidate (MW 620), and behenyl behenate (MW 648). These compounds produced M^+ ions m/z 578.4, 620.4, and 648.5, respectively. In the data dependent scan mode, these ions were fragmented to produce two types of clearly visible analytical fragments: M^+ ions of their FA residues (theoretical m/z 270.2, 312.3, and 340.3) and a common ion m/z 353.4, which was derived from their common behenyl alcohol moiety (Fig. 4). These simple and distinctive chromatographic and fragmentation patterns of tested standard WE, in conjunction with their clearly visible M^+ ions (under the condition of a lower than typical EE of -30 V), were used in the subsequent structural evaluation of meibomian WE.

The effects of double bonds on the mass spectra and fragmentation patterns of WE were studied with an equimolar mixture of six saturated and unsaturated WE. Three pairs of WE—stearyl oleate/stearyl stearate (m/z 534/536), arachidyl oleate/arachidyl stearate (m/z 562/564), and behenyl oleate/behenyl stearate (m/z 590/592)—were evaluated. The signals of M^+ and $(M + H)^+$ ions of saturated WE were an order of magnitude stronger than those of unsaturated WE, because of a much stronger spontaneous in-source fragmentation of the latter (Fig. 1, panels A, D).

Effects of Branching

Effects of branching on RT and fragmentation patterns were evaluated first with standard lipids. As very long chain branched WE are not available in pure form from any commercial provider, FFA and their methyl esters (FAME) were tested instead. These included straight chain methyl esters of $C_{12:0}$ to $C_{18:0}$ FA, such as methyl tetradecanoate ($sC_{14:0}$ -ME), methyl pentadecanoate ($sC_{15:0}$ -ME), methyl hexadecanoate ($sC_{16:0}$ -ME), methyl heptadecanoate ($sC_{17:0}$ -ME), methyl octadecanoate ($sC_{18:0}$ -ME), and methyl octadecenoate ($sC_{18:1}$ -ME), and a variety of branched FAME, namely methyl-11-methyl-dodecanoate ($iC_{13:0}$ -ME), methyl-12-methyl-tridecanoate ($iC_{14:0}$ -ME), methyl-13-methyl-tetradecanoate ($iC_{15:0}$ -ME), methyl-12-methyl-tetradecanoate ($aiC_{15:0}$ -ME), methyl-14-methyl-pentadecanoate ($iC_{16:0}$ -ME), methyl-14-methyl-hexadecanoate ($aiC_{17:0}$ -ME), and methyl-15-methyl-hexadecanoate ($iC_{17:0}$ -ME). Typical fragmentation patterns observed at EE -30 eV are shown in Figure 5. Saturated straight chain WE produced a bell-

FIGURE 3. Chromatographic and mass spectrometric analysis of a 0.1 mM equimolar mixture of ten standard wax esters. (A) TIC of the lipid mixture. Tested were myristyl laureate (peak 1a), lauryl oleate (2a), palmityl oleate (3a), stearyl oleate (4a), arachidyl oleate (6a), arachidyl stearate (7a), behenyl oleate (8a), behenyl stearate (9a), and behenyl behenate (10a). (B) EIC of M^+ ions of individual wax esters: myristyl laureate (peak 1b), lauryl oleate (2b), palmityl oleate (3b), stearyl oleate (4b), stearyl stearate (5b), arachidyl oleate (6b), arachidyl stearate (7b), behenyl oleate (8b), behenyl stearate (9b), and behenyl behenate (10b). *Insert:* Retention times of ten straight chain wax esters are correlated linearly with their m/z values (see equation 1 in the text). This approach also is known as the “ECL” approach.

shaped distribution of the daughter ions m/z 255, 241, 227, 213, 199, 185, 171, 157, 143, 129, and 115 derived from the carbon chain of the fatty acid fragment of $sC_{17:0}$ -ME. However, the *iso*-compound $iC_{13:0}$ -ME (m/z 229, $[M + H]^+$) produced a prominent product ion m/z 185 ($M - 44$, loss of a C_3H_8 fragment), while an *anteiso*-compound $aiC_{15:0}$ -ME (m/z 257, $[M + H]^+$) gave a prominent doublet of ions m/z 227 ($M - 30$, loss of a C_2H_6 fragment) and 199 ($M - 58$, loss of a C_4H_{10} fragment, Fig. 6). These distinctive fragmentation patterns were reproducible for all tested straight chain, *iso*-, and *anteiso*-compounds, and were used to identify homologous branched chain WE among meibomian lipids. Notably, branched chain FAME eluted earlier than the straight chain ones of the same molecular masses, with *anteiso*-isomers eluting faster than *iso*-isomers (not shown).

Calibration Curves

First, we determined the low limit of detection (LLoD) of intact WE. Two equimolar mixtures of ten standard WE (10 and 100 μ M each) were prepared. It was determined that the LLoD (defined here as the signal with an intensity of at least five

times the background noise) for most of the tested WE was in the low nanogram range (typically between 1 and 2 ng, or 2 and 3 pmol, for an individual WE per injection).

Next, dose-response (calibration) curves were generated using authentic WE standards. From those data, one could conclude that the calibration curves were nonlinear within much of the tested ranges of molecular masses and sample weights. The complex shape of the calibration curves called for a 3D-type of calibration graph (Fig. 7), which would depict the instrument's response to two changing factors (m/z values and physical amounts of injected lipids) simultaneously.

The calibration data were plotted in the TableCurve 3D software as a 3D graph in (m/z , sample mass, instrument response) system of coordinates to form a smooth “3-D calibration surface” (Fig. 7). Automatic non-linear curve-fitting of the data for saturated WE was performed using a standard transition equation 2 (#2132 from the TableCurves's equation database):

$$Z = a + EXVCUMX(b, c, d) + EXVCUMY(e, f, g) + EXVCUMX(h, c, d) \times EXVCUMY(1, f, g) \quad (2)$$

FIGURE 4. GLC-ITMS analysis of behenyl heptadecanoate. All major product ions (m/z 270/271, 353, and 579) coeluted. Note that the FA ion was detected as a pair of ions m/z 270/271, possibly due to their presence as a mixture of $(M + H)^+$ and M^+ ions.

which adequately approximated the GLC-ITMS experimental results with the following parameters: $a = 55,946$; $b = 6.1 \times 10^6$; $c = 161.9$; $d = 123.5$; $e = -447,352$; $f = 536.2$; $g = 68.3$; $h = 4.56 \times 10^7$ ($r^2 = 0.991$). The “Evaluate” subroutine of the software was used to evaluate and predict values of function Y (mass of

the analyte in nanograms) for known combinations of X (m/z) and Z (signal intensity). A low limit of quantitation (LLoQ) level of ~ 1 ng per injected sample was achieved for most WE, which was sufficient to analyze individual (non-pooled) samples of meibum collected from human volunteers.

Meibomian Lipids

A typical TIC recorded for meibum is shown in Figure 8. The vast majority of the meibomian lipid analytes had RT between 35 and 56 minutes, meaning that their molecular masses were

FIGURE 5. Fragmentation patterns of branched and straight chain methyl esters of fatty acids in single reaction monitoring experiments. (A) A straight chain methyl heptadecanoate. (B) Iso-branched methyl 11-methyl-dodecanoate. (C) Anteiso-branched methyl 12-methyl-tetradecanoate.

FIGURE 6. Straight chain, iso-, and anteiso-branched fatty acid methyl esters tested in GLC-ITMS experiments. Characteristic losses in single reaction monitoring experiments with branched lipids are shown.

FIGURE 7. 3D calibration plot for saturated wax esters. The plots were computed using TableCurve 3D fitting software. Extracted ion chromatograms were used for each individual wax ester. Calculated parameters are listed in the text. Plotted were the data for the following wax esters: palmityl palmitate (MW 480.4), arachidyl myristate (MW 508.5), stearyl stearate (536.5), arachidyl stearate (564.5), behenyl stearate (592.5), behenyl arachidate (620.5), and behenyl behenate (648.5). A similar plot was obtained for monounsaturated wax esters (not shown).

well above 500 (Fig. 3, Panel B). The complex nature of the TIC with multiple overlapping peaks implied a high level of complexity of meibomian lipids. As noted earlier in the text, neither triacylglycerols nor cholesteryl esters were expected to be eluted and/or detected under the experimental conditions used. Monoacyl glycerols, if present in meibum, would produce signals of a $C_nH_{2n}O_4$, $C_nH_{2n-2}O_4$, and $C_nH_{2n-4}O_4$ nature, and were expected to have very short RT (shorter than that of free cholesterol, Fig. 2, Panel B). However, no such compounds were detected upon examination of chromatograms of meibomian samples while evaluating their spectra for the presence of the M^+ , $(M + H)^+$, $(M - H_2O + H)^+$, and $(M - 2H_2O + H)^+$ ions of putative monoacyl glycerols. Their FA residues were not observed either. Free FA and hydrocarbons, which under the conditions of our experiments would have been eluted during the first 5 minutes of the run (data not

FIGURE 8. A representative positive ion mode total ion GLC-ITMS chromatogram of a meibomian sample. Conditions of the experiment were as follows. The scan range was 100 to 1000 amu. A TG-5MS column (0.25 mm $\times$ 30 m) was used. A sample (2 μ L of 1 mg of meibum/mL) was injected in splitless mode. The flow of helium was maintained at 1 mL/min (40 cm/min). The temperature of the injector was set at 300°C. The transfer line was kept at 325°C, while the analyzer was maintained at 250°C. Electron energy was set to be $-30V$. The temperature gradient is shown as broken line. The signals were collected in 3×25 ms microscans.

FIGURE 9. Retention times of wax ester standards and of meibomian wax esters. Shown are straight chain saturated and unsaturated WE standards (open circles, solid line), and meibomian $C_{16:0}$ -FA-based WE (gray circles, dash-dot-dash line) $C_{17:0}$ -FA-based WE (open squares, dashed line), $C_{18:0}$ -FA-based WE (open diamonds, dotted line), and $C_{18:1}$ -FA-based WE (open triangle, medium dash). The lines were non-linearly fitted to equation 3 (see text). For standard WE, the interception point was -58.69 , while the slope was 0.163 . For $C_{16:0}$ -FA-based WE these parameters were (-61.18) and 0.165 , for $C_{17:0}$ -FA-based WE these parameters were (-59.22) and 0.162 , and for $C_{18:1}$ -FA-based WE (-57.18) and 0.159 . Retention times of meibomian WE were about 1 minute longer than those of isobaric synthetic straight chain analogs.

shown), could not have contributed to the observed chromatographic patterns. Moreover, extremely large di- and triesters, being larger than typical cholesteryl esters and, judging from their proposed chemical structures, even more unstable, also were unlikely to be detected in the tested conditions because of their much longer RT and lower stability. Thus, it was reasonable to assume that many, if not all, of the peaks shown in Figure 8 were produced by WE. With this assumption in mind, the meibomian lipids were analyzed for the presence of WE. Based on the literature data,^{1,7-10} we expected to detect a wide range of meibomian WE with their FA ranging from C_{14} to at least C_{20} , and FAI ranging from C_{16} to C_{32} or so. We also expected to detect saturated and unsaturated WE, with straight and branched chains on both moieties—FA and FAI. These different possibilities could have resulted in hundreds, if not thousands, of combinations of various FA and FAI,⁸ some of which could have been present as major species, while the others only as minor ones. Thus, a systematic approach was implemented to characterize major WE that were expected to have the highest impact on the properties of meibum, and eventually on the human tear film.

First, a list of theoretical monoisotopic m/z values for saturated $C_nH_{2n}O_2$ and unsaturated $C_nH_{2n-2m}O_2$ WE was compiled ($n = 20-60$; number of double bonds $m = 1, 2, 3$; not shown). When co-plotted with a set of authentic straight chain WE, almost all analyzed meibomian WE had a RT shorter than those of isobaric synthetic straight chain WE standards (Fig. 9). This was clear evidence of the branched and/or unsaturated chain nature of most meibomian WE. Indeed, the

FIGURE 10. Representative chromatograms and product mass spectra of a meibomian wax ester m/z 620.5, and of its fatty acid product ions m/z 257, 271, 283, and 285. (A) Total ion chromatogram of ion m/z 620.5 (B) and (B') Single reaction monitoring MS² chromatogram of ion m/z 257.3 produced in the following automatic experiment: m/z 620.5@cid0.72@cid1.44@cid2.59 $\rightarrow$ m/z 256, and the mass spectrum of the GLC-ITMS peak with retention time of 41.12 min. Panels (C) and (C') Single reaction monitoring chromatogram of a product ion m/z 620.5 $\rightarrow$ m/z 271.3, and the mass spectrum of the GLC-ITMS peak with retention time of 41.12 minutes. (D) and (D') Single reaction monitoring chromatogram of a product ion m/z 620.5 $\rightarrow$ m/z 285.3, and the mass spectrum of the GLC-ITMS peak with retention time of 41.08 minutes. (E) and (E') For comparison purposes, an extracted ion chromatogram of ion m/z 283.3 (a product of spontaneous in-source fragmentation of oleic-acid based WE) is shown. The ion eluted as a GLC-ITMS peak with retention time of 41.7 minutes, and coeluted with another ion (m/z 618.5; not shown). The compound was identified as tertacosanyl oleate.

RT of straight chain saturated and unsaturated WE standards depended on their molecular masses in accordance with equation 1, while the RT values of all major meibomian WE were governed by the following equation 3 ($r^2 = 0.9960$):

$$\text{Retention Time (min)} = -60.19 + 0.16 \times m/z \quad (3)$$

Note that the slopes of both curves (0.163) were identical, and the only difference was their interception points (-58.69 vs. -60.19) with the Y-axis. Generally, the RT of WE were reproducible up to one to two seconds, if no changes in the condition of the GLC column, or other experimental conditions, had occurred. Thus, a 1 to 2-minute deviation from this equation could be caused only by meibomian WE branching, or by *cis,trans*-isomerism of the double bonds of unsaturated compounds. When analyzed individually, each of the individual subclasses of meibomian WE reproducibly showed identical slopes (0.16), but slightly different interception points ranging from -57 to 61 . These differences were large enough to conclude that meibomian WE were different structurally from their isobaric straight chain synthetic analogs.

Next, selected ion monitoring chromatograms (SIMC) of the detected WE species were plotted and examined. We observed 50 or so chromatographic peaks with intensities three times

(or more) the noise level. Considering that many of the meibomian lipids were expected to co-elute, the number of actual WE analytes in the sample was expected to be much higher than 50. The shortest detected compound had an m/z value of 508 ($C_{34:0}$), while the longest detected WE had an m/z value of 700 ($C_{50:2}$) with a RT of >55 minutes.

For most of the ions, SIMC produced complex patterns, clearly demonstrating that more than one isoform of the same molecular mass was present in the sample. Because of space constraints, only one ion m/z 620 ($C_{42:0}$) will be discussed below in detail. From its elution pattern, one can conclude that there were several isobaric isomers with the same m/z value of 620 present in the sample (Fig. 10, panel A). When this ion m/z 620 was evaluated in a GLC-MS/MS fragmentation experiment (Fig. 10, panels B-E), it became clear that it was produced by a mixture of isobaric WE based on several saturated FA (SFA). Among those, $C_{16:0}$ (m/z 256), $C_{17:0}$ (m/z 270), and $C_{18:0}$ (m/z 284) FA were identified as major constituents. A small amount of a $C_{20:0}$ -FA (m/z 312), and a large amount of co-eluting monounsaturated $C_{18:1}$ -FA and $C_{16:1}$ -FA-based WE (m/z 283/264 and 255/236, correspondingly) also were observed. The latter, however, were produced by a co-eluting WE with m/z 618 ($C_{42:1}$) with a RT of 41.5 to 42.5 minutes.

FIGURE 11. Fragmentation pattern of heptadecanoic acid (ion m/z 270/271) spontaneously formed in source from the corresponding meibomian WE in an SRM MS² experiment. The ion was fragmented in the following experiment: 270.3@cid0.57@cid1.13@cid2.04 experiment. Note that the ion produced characteristic fragments m/z 241 and 213, indicative of an anteiso-branched compound.

The fragment ion m/z 270.2 was clearly an M^+ ion of a $C_{17:0}$ -FA (heptadecanoic acid). However, its MS/MS fragmentation pattern (Fig. 11) did not match that of a straight chain behenyl heptadecanoate (Fig. 5A). The major difference between the two was that, in an MS² experiment, the M^+ ion of a meibomian compound m/z 270.2 (or, often, 271.2 $[C_{17:0} + H]^+$), showed a prominent characteristic fragment m/z 241.3 ($C_{17:0}$ -FA - 29)⁺, while its synthetic straight chain analog did not. Such a loss of a 28/29 amu fragment (CH_3CH_2 or $CH_2 = CH_2 + H^+$) is considered a characteristic feature of anteiso-FA ("b"-type of fragments).¹⁷ Interestingly, additional loss of a second 28 amu fragment produced yet another prominent ion with an m/z value of 213.2 ($[C_{17:0}$ -FA - 56 + $H]^+$, "a"-type of fragmentation¹⁷). This triplet of consecutive product ions (wax ester $\rightarrow m/z$ 270/271 $\rightarrow m/z$ [241 + 213]; m/z 241 $\rightarrow m/z$ 213) allowed us to identify anteiso- $C_{17:0}$ -FA-

containing WE among the meibomian lipids. Note that the shortest detected WE with anteiso- $C_{17:0}$ FA had a RT of 29.6 minutes and m/z value of 550, while the longest had an RT of more than 50 minutes, and an m/z of 704. The total number of chromatographic peaks of meibomian anteiso- $C_{17:0}$ WE was >30, of which 25 or so exceeded 0.1% of the area of the largest peak in the chromatogram with RT 42.4 minutes. Similarly, iso-compounds were identified as those whose FA fragments produced $(M - 44)^+$ ions in MS/MS experiments: in our hands, they typically produced "a"-type fragments, while anteiso-wax esters produced both "a" and "b"-types of fragments.¹⁷

An observation in chromatograms of ion m/z 283 ($[M+H]^+$, a $C_{18:1}$ -FA) for many WE, including ion m/z 618, prompted a more detailed analysis of this unsaturated species in GLC-MS/MS experiments. Authentic WE with oleic ($C_{18:1}$, n-9, m/z 283) and palmitoleic ($C_{16:1}$, n-7, m/z 255) FA moieties were fragmented in MS³ WE $\rightarrow$ FA $\rightarrow$ product ions experiments. Both FA produced a common product ion m/z 143, which was used as an analytical ion to determine the location of double bonds in their structures (Fig. 12). Then, meibomian WE were analyzed. It was confirmed that meibomian ions m/z 283 and 255 fragmented similarly to those formed from the authentic oleic- and palmitoleic-containing standard WE. In selected reaction monitoring (SRM) MS² experiments, spontaneously in-source generated FA ions of meibomian lipids were fragmented in a similar fashion and a corresponding analytical ion m/z 143 also was observed.

A combination of m/z values of intact WE (detected either as M^+ or $[M+H]^+$ ions), and their FA fragments allowed us to compute the length of the FAI fragment for each WE. However, to verify these calculations, we also analyzed mass spectra and chromatograms of standard and meibomian WE for the presence of ions related to their FAI moieties. Indeed, such analytical ions were detected (Fig. 13). This approach was used to corroborate our findings on the structures of meibomian WE.

Conveniently, $M^+/(M + H)^+$ FA and FAI ions and their fragments also could be observed in simple MS experiments due to a spontaneous in-source fragmentation of WE. This apparent instability of WE ions provided an interesting and convenient tool for studying meibum. An averaged MS spectrum of a portion of the chromatogram where WE elute

FIGURE 12. Fragmentation of the in-source generated fatty acid fragments of unsaturated wax esters under the conditions of GLC-ITMS. The common analytical fragment m/z 143 allows establishment of the location of double bonds in the FA.

FIGURE 13. Fatty alcohol fragment of a $C_{17:0}$: $C_{25:0}$ wax ester formed under the conditions of GLC-ITMS. The analytical fragment m/z 395 establishes the chain length of the alcohol moiety.

showed a range of signals for C_{15} to C_{18} FA residues generated spontaneously from meibomian WE (Fig. 14).

Then, SIMC, SRM, and EICs of major FA fragments of WE were evaluated. Detected were FA ions m/z 242/243 ($C_{15:0}$ ≤ 15 peaks), m/z 255 ($C_{16:1}$ ~ 6 peaks), m/z 256/257 ($C_{16:0}$ ~ 30 peaks), m/z 270/271 ($C_{17:0}$ ~ 20 peaks), m/z 282/283 and 264 ($C_{18:1}$ and $[C_{18:1} - H_2O]$ ~ 30 peaks), and m/z 284/285 ($C_{18:0}$ ~ 20 peaks, Fig. 15). Only small amounts of $C_{20:0}$ FA-based waxes were observed, with the largest one being a WE with an m/z value of 620.6 (not shown). Other minor FA, if present, were not observed and/or identified because of their unfavorable signal-to-noise ratios.

This approach was used to corroborate the results of our GLC-ITMS studies, and helped in estimating the ratio of meibomian WE based on different FA. The EIC or SIMC of the FA ions were plotted, and the corresponding GLC-ITMS peaks were integrated using the same Avalon algorithm as before. Preliminary experiments demonstrated that the $M^+/(M + H)^+$ ions of unsaturated and saturated FA produced by equimolar mixtures of unsaturated and saturated standard WE were detected, on average, in a 1:10 ratio. Importantly, the

intensities of the FA fragments of WE were very close ($\pm 10\%$) to each other for the equimolar mixtures of WE ranging from $C_{38:0}$ to $C_{46:0}$ (Fig. 16), which is an important range considering that the vast majority of meibomian WE fall in this category. Thus, by comparing the intensities of such signals in meibum samples, either as EIC or as SIMC, one could make conclusions about their mass ratios in meibum.

For 14 donors, TIC of meibomian lipids virtually overlapped. The overall amount of unsaturated WE (based predominantly on $C_{18:1}$ - and $C_{16:1}$ -FA) was found to be $82 \pm 2\%$ (mean $\pm$ SD of their total WE fractions (wt/wt), while the rest $18 \pm 2\%$ were saturated WE. Of all the unsaturated WE, the $C_{18:1}$ -FA-based ones gave more than 90% of the entire unsaturated WE pool.

Next, we evaluated the relative presence of branched and straight chain WE in meibum. First, three major groups of saturated WE were analyzed, namely those based on $C_{16:0}$, $C_{17:0}$, and $C_{18:0}$ -FA. Among all major $C_{16:0}$ -FA-based WE, on average $78 \pm 4\%$ of the species were based on *iso*- $C_{16:0}$ -FA, while the remaining $22 \pm 4\%$ had straight FA in their structure. For $C_{17:0}$ -FA-based WE, no straight chain FA were observed: $93 \pm 6\%$ of their FA were anteiso-branched, while the rest $7 \pm 2\%$ were confirmed to be iso-branched. Surprisingly, WE-derived $C_{18:0}$ -FA were observed as a complex mixture of $31 \pm 3\%$ iso-branched, $23 \pm 3\%$ straight chain, and $46 \pm 5\%$ as yet unidentified isomer of a $C_{18:0}$ FA.

A summary of our findings on the major WE detected in meibum is presented in Tables 1 and 2.

DISCUSSION

Generally, WE are believed to make up, on average, between 13 and 50% of meibum.^{18–22} Meibomian WE are very diverse chemically: Nicolaides et al. found that the overall number of different FA in meibum could reach 70 or so, while the number of corresponding FAI could be up to 40 or more, which could result in up to 2800 possible WE.⁸ Interestingly, Nicolaides et al. also reported that a large portion of the FA and FAI were comprised of branched and/or unsaturated species, and a large portion of the straight chain FA was unsaturated.⁸

In earlier experiments, two methods of choice in the lipid analysis of meibum were GC-FID and GLC-MS. However, in those experiments WE were not analyzed as intact molecules: the lipids were hydrolyzed and/or transesterified before the

FIGURE 14. Direct observation of fatty acid fragments of meibomian WE in a GLC-ITMS experiment. An average spectrum of a portion of a chromatogram from 35 to 55 min into the experiment is shown.

FIGURE 15. Total ion chromatogram of a human meibomian sample and extracted ion chromatograms of fatty acid ions generated due to spontaneous in-source fragmentation of WE. The nature of the ions is shown in the left upper corners of each panel. Peaks are labeled with the *m/z* values of predominant WE present in those peaks. Five dominant FA were palmitic (C_{16:0}), heptadecanoic (C_{17:0}), stearic (C_{18:0}), palmitoleic (C_{16:1}), and oleic (C_{18:1}) acids.

FIGURE 16. SRM MS² chromatograms of product ions originated from parent WE. The following reactions were followed. (A) Extracted ion chromatograms of fatty acid ions spontaneously generated from saturated WE: m/z 592.5 → 285 (peak a); m/z 620.5 → 313 (peak b); m/z 648.6 → 341 (peak c); m/z 676.7 → 369 (peak d). (B) Mass spectra of GLC-ITMS peaks a–d shown in (A).

analyses to make them more volatile under the conditions of the GC-FID and GC-MS experiments. Thus, information on the exact combinations of FA and FAI present in intact WE was lost.

Later, another group of researchers evaluated WE by GC-FID.¹⁰ Waxes and sterol esters were described in terms of their “equivalent chain lengths” (ECL), which, for WE, were a sum of the number of carbons present in the FA and FAI residues of a wax, while for sterol esters only carbons of the FA residues were counted. Osgood et al. found that the ECL values for WE ranged from 33.6 to 45.7, while for sterol esters the ECL values were between 19.1 and 23.2.¹⁰ Note that these fractional numbers differed from those of the saturated, straight chain WE standards used in the study (which were round), meaning that the meibomian lipids mostly were either unsaturated and/or branched. The exact structures of these WE, however, were not established in these earlier studies.

Another approach, that is electrospray ionization (ESI) MS in the presence of ammonium salts, has been tested previously by Fitzgerald and Murphy for human hair lipids,¹⁴ and showed promising results for tested lipid mixtures. However, they

skipped a chromatographic step in their experiments and used a direct infusion approach, which made it impossible to analyze separately isobaric compounds that were very likely to be present in complex biological mixtures, such as human hair or meibum, either as intact lipids or as products of their in-source fragmentation.²³ When a similar direct infusion approach with ammonium and sodium salts was tested by Chen et al. in direct infusion ESI experiments, ammonium adducts of meibomian WE were, indeed, observed.²⁴ However, Chen et al. did not attempt to separate the analytes chromatographically, which made analyzing and quantitating isobaric lipids difficult, if not impossible. Notably, compared to our current results, Chen et al. underestimated the overall presence of WE in meibum by more than 10% (wt/wt), most likely because of these difficulties and the use of just one standard, behenyl oleate, for quantitation of meibomian WE. In our preceding HPLC-ion trap atmospheric pressure chemical ionization (APCI) MS experiments,¹⁶ no ammonium adducts of meibomian wax esters had been detected, and the lipids were analyzed as proton adducts. However, neither our APCI MS

TABLE 1. Detected Major Meibomian WE

Observed M ⁺ Ions (MS Exp.)	Molecular Formula	RT, Mins.	Detected FA	Characteristic M ⁺ /(M+H) ⁺ Ions of FA (MS/MS Exp.)	Detected FAI	Characteristic (M + C ₂ H ₃) ⁺ Product Ions of FAI (MS/MS Exp.)	Relative Abundance, % of Total WE
536.6 (1) (2) (3) (4)	C ₃₆ H ₇₂ O ₂	26.9 27.3 27.5 27.8	<i>i</i> C _{16:0} <i>i</i> C _{15:0} , <i>ai</i> C _{17:0} <i>ai</i> C _{15:0} , <i>ai</i> C _{17:0} <i>s</i> C _{16:0}	256 242, 270 242, 270 256	C _{20:0}	325 <LLoD - -	0.04 (0.5% of sat. WE)
550.6 (1) (2) (3)	C ₃₇ H ₇₄ O ₂	29.3 29.6 30.5	<i>i</i> C _{15:0} , <i>i</i> C _{16:0} , <i>i</i> C _{17:0} <i>i</i> C _{15:0} , <i>i</i> C _{16:0} , <i>ai</i> C _{17:0} <i>s</i> C _{16:0} , <i>ai</i> C _{17:0}	243, 256, 270 242, 256, 270 256, 270	C _{20:0} , C _{21:0}	325, 339 <LLoD -	0.21 (2.2%)
562.6	C ₃₈ H ₇₄ O ₂	32.1	<i>s</i> C _{18:1}	283	C _{20:0}	407	4.33 (1.0% of unsat. WE)
564.6 (1) (2) (3) (4)	C ₃₈ H ₇₆ O ₂	31.7 31.9 32.3 32.5	<i>i</i> C _{16:0} , <i>i</i> C _{18:0} <i>i</i> C _{15:0} , <i>i</i> C _{17:0} , <i>ai</i> C _{17:0} <i>ai</i> C _{15:0} , <i>ai</i> C _{17:0} <i>s</i> C _{16:0} , <i>s</i> C _{18:0}	256, 284 242, 270 242, 270 284	C _{22:0} C _{21:0}	353 <LLoD 339 <LLoD	0.11 (1.3%)
576.6 (1) 1 (2) (3)	C ₃₉ H ₇₆ O ₂ C ₃₉ H ₇₈ O ₂	34.8 34.0 34.3	<i>s</i> C _{16:1} / <i>s</i> C _{18:1} (1:9) <i>i</i> C _{15:0} , <i>i</i> C _{16:0} , <i>i</i> C _{17:0} <i>ai</i> C _{15:0} , <i>i</i> C _{16:0} , <i>ai</i> C _{17:0} , <i>i</i> C _{18:0}	255, 283 242, 256, 270 242, 256, 270, 284	C _{21:0} C _{24:0} C _{22:0} , C _{23:0}	421 381 353, 367	0.85 (1.3%) 2.35 (0.2%)
(3)		35.2	<i>ai</i> C _{15:0} , <i>s</i> C _{16:0} , <i>ai</i> C _{17:0} , <i>s</i> C _{18:0}	242, 256, 270, 284		<LLoD	
590.6 (1) (2)	C ₄₀ H ₇₈ O ₂	36.8 37.9	<i>s</i> C _{16:1} / <i>s</i> C _{18:1} (1:5) <i>s</i> C _{16:1}	255, 283 255	C _{22:0} , C _{24:0} C _{24:0}	353, 381 381	1.62 (2.4%)
592.6 (1) (2) (3) (4)	C ₄₀ H ₈₀ O ₂	36.3 36.6 37.0 37.2	<i>i</i> C _{15:0} , <i>i</i> C _{16:0} , <i>i</i> C _{18:0} <i>i</i> C _{15:0} , <i>ai</i> C _{17:0} <i>i</i> C _{15:0} , <i>ai</i> C _{17:0} <i>s</i> C _{16:0} , <i>s</i> C _{18:0}	256 242, 270 242, 270 256, 284	<i>i</i> C _{24:0} <i>i</i> C _{23:0} <i>i</i> C _{23:0} <i>i</i> C _{24:0}	381 367 367 381	0.82 (6.9%)
604.6 (1) (2)	C ₄₁ H ₈₀ O ₂	39.4 39.6	<i>s</i> C _{16:1} / <i>s</i> C _{18:1} (1/4) <i>C</i> _{16:1} / <i>C</i> _{18:1} (5/4)	255, 283 255, 283	C _{23:0} , C _{25:0} C _{25:0}	367, 395 395	2.14 (2.7%)
606.6 (1) (2) (3) (4)	C ₄₁ H ₈₂ O ₂	38.6 39.0 39.5 39.8	<i>i</i> C _{15:0} , <i>i</i> C _{16:0} , <i>i</i> C _{17:0} <i>i</i> C _{16:0} , <i>ai</i> C _{17:0} <i>s</i> C _{15:0} , <i>C</i> _{16:0} , <i>s</i> C _{17:0} , <i>C</i> _{18:0} <i>ai</i> C _{15:0} , <i>s</i> C _{16:0} , <i>ai</i> C _{17:0} , <i>s</i> C _{18:0}	242, 256, 270 256, 270 242, 256, 270, 284 242, 256, 270, 284	<i>i</i> C _{24:0} , <i>i</i> C _{25:0} , <i>i</i> C _{26:0} <i>i</i> C ₂₄ C _{25:0:0} <i>i</i> C _{25:0} <i>i</i> C _{24:0} , <i>i</i> C _{25:0}	381, 395, 409 381, 395 395 381, 395	2.05 (19.9%)
9618.6 (1) (2)	C ₄₂ H ₈₂ O ₂	41.4 42.3	<i>C</i> _{16:1} / <i>C</i> _{18:1} (5/95) <i>s</i> C _{18:1}	255, 283 283	C _{24:0} C _{24:0}	381 381	16.1 (19.7%)
620.6 (1) (2) (3) (4)	C ₄₂ H ₈₄ O ₂	40.9 41.2 41.6 41.8	<i>i</i> C _{16:0} , <i>i</i> C _{17:0} , <i>i</i> C _{18:0} <i>i</i> C _{16:0} , <i>ai</i> C _{17:0} <i>ai</i> C _{17:0} <i>s</i> C _{16:0} , <i>s</i> C _{18:0}	256, 270, 284 256, 270 270 256, 284	<i>i</i> C _{24:0} , <i>i</i> C _{26:0} <i>i</i> C _{25:0} , <i>i</i> C _{26:0} <i>i</i> C _{25:0} <i>i</i> C _{24:0} , <i>i</i> C _{26:0}	381, 409 395, 409 395 381, 409	3.01 (28.6%)
632.6 634.7 (1) (2) (3) (4)	C ₄₃ H ₈₄ O ₂ C ₄₃ H ₈₆ O ₂	44.0 43.1 43.4 43.5 44.2	<i>s</i> C _{18:1} <i>i</i> C _{16:0} , <i>i</i> C _{17:0} , <i>i</i> C _{18:0} <i>ai</i> C _{15:0} , <i>i</i> C _{16:0} , <i>ai</i> C _{17:0} , <i>C</i> _{18:0} <i>s</i> C _{16:0} , <i>i</i> C _{18:0} , <i>ai</i> C _{17:0} <i>s</i> C _{16:0} , <i>ai</i> C _{17:0} , <i>s</i> C _{18:0} , <i>C</i> _{19:0}	283 256, 270, 284, 298 242, 256, 270, 284 256, 270, 284 256, 270, 284, 298	C _{25:0} <i>i</i> C _{26:0} , <i>i</i> C _{27:0} C _{25:0} , <i>i</i> C _{26:0} , C _{27:0} , <i>C</i> _{28:0} <i>i</i> C _{24:0} , <i>i</i> C _{25:0} , <i>i</i> C _{26:0} , <i>i</i> C _{27:0}	395 409, 423 395, 409, 423, 437 <LLoD 381, 395, 409, 423	18.9 (22.4%) 2.95 (26.5%)
646.7 648.7 (1) (2) (3)	C ₄₄ H ₈₆ O ₂ C ₄₄ H ₈₈ O ₂	46.0 45.2 45.6 46.0	<i>s</i> C _{18:1} <i>i</i> C _{16:0} , <i>i</i> C _{18:0} , C _{20:0} <i>all</i> C _{17:0} , <i>C</i> _{18:0} , <i>C</i> _{19:0} <i>s</i> C _{16:0} , <i>ai</i> C _{17:0} , <i>s</i> C _{18:0} , <i>C</i> _{19:0}	283 257, 285, 312 270, 284, 298 256, 270, 284, 298	C _{26:0} <i>i</i> C _{26:0} , <i>i</i> C _{28:0} <i>i</i> C _{27:0} <i>i</i> C _{26:0} , <i>i</i> C _{27:0} , <i>i</i> C _{28:0}	409 409, 437 423 409, 423, 437, 451	30.2 (35.3%) 1.2 (10.8%)
660.7 662.7 (1) (2) (3) (4)	C ₄₅ H ₈₈ O ₂ C ₄₅ H ₉₀ O ₂	48.3 47.3 47.6 48.0 48.5	<i>s</i> C _{18:1} <i>C</i> _{16:0} , <i>i</i> C _{17:0} , <i>C</i> _{18:0} , <i>C</i> _{19:0} , <i>C</i> _{20:0} <i>C</i> _{15:0} , <i>i</i> C _{16:0} , <i>ai</i> C _{17:0} , <i>C</i> _{18:0} , <i>C</i> _{20:0} <i>s</i> C _{16:0} , <i>ai</i> C _{17:0} , <i>s</i> C _{18:0} <i>s</i> C _{16:0} , <i>ai</i> C _{17:0} , <i>s</i> C _{18:0}	283 271, 298 243, 257, 271, 285, 299, 313 257, 271, 285 257, 271, 285	C _{27:0} C _{27:0} , C _{28:0}	423 <LLoD 423, 437 <LLoD -	7.86 (8.9%) 0.23 (3.1%)
674.7 (1) (2)	C ₄₆ H ₉₀ O ₂	50.0 50.2	<i>s</i> C _{18:1} <i>s</i> C _{16:1} / <i>s</i> C _{18:1} (1:2)	283 255, 283	C _{28:0} C _{28:0}	437 <LLoD	3.5 (5.0%)
688.7 (1) (2)	C ₄₇ H ₉₂ O ₂	52.5 52.7	<i>s</i> C _{18:1} <i>s</i> C _{18:1}	283 283	C _{29:0}	451 <LLoD	1.52 (1.7%)

TABLE 2. Wax Ester Composition of a Representative Sample of Human Meibum

Unsaturated WE of Human Meibum			Saturated WE of Human Meibum		
Mol. Mass	Structure	Mass, µg/mg Meibum	Mol. Mass	Structure	Mass, µg/mg Meibum
534	C ₃₆ H ₇₀ O ₂	<LLOQ	522	C ₃₅ H ₇₀ O ₂	<LLOQ
548	C ₃₇ H ₇₂ O ₂	-	536	C ₃₆ H ₇₂ O ₂	2.4
562	C ₃₈ H ₇₄ O ₂	-	550	C ₃₇ H ₇₄ O ₂	3.9
576	C ₃₉ H ₇₆ O ₂	-	564	C ₃₈ H ₇₆ O ₂	5.0
588*	C ₄₀ H ₇₈ O ₂	3.2	578	C ₃₉ H ₇₈ O ₂	7.8
590	C ₄₀ H ₇₈ O ₂	23.7	592	C ₄₀ H ₈₀ O ₂	14.3
602*	C ₄₁ H ₈₀ O ₂	8.7	606	C ₄₁ H ₈₂ O ₂	26.5
604	C ₄₁ H ₈₀ O ₂	28.0	620	C ₄₂ H ₈₄ O ₂	32.1
616*	C ₄₂ H ₈₀ O ₂	2.0	634	C ₄₃ H ₈₆ O ₂	30.0
618	C ₄₂ H ₈₂ O ₂	50.2	648	C ₄₄ H ₈₈ O ₂	20.3
630*	C ₄₃ H ₈₂ O ₂	3.6	662	C ₄₅ H ₉₀ O ₂	9.5
632	C ₄₃ H ₈₄ O ₂	55.9	676	C ₄₆ H ₉₂ O ₂	5.5
644*	C ₄₄ H ₈₄ O ₂	11.2	690	C ₄₇ H ₉₄ O ₂	<LLOQ
646	C ₄₄ H ₈₆ O ₂	61.1			
658*	C ₄₅ H ₈₆ O ₂	14.2			
660	C ₄₅ H ₈₈ O ₂	37.2			
674	C ₄₆ H ₉₀ O ₂	25.9			
688	C ₄₇ H ₉₂ O ₂	25.8			
702	C ₄₈ H ₉₄ O ₂	<LLOQ			
Total:		350.7			157.3

The sample was collected from a healthy (non-dry eye) 28-year old Caucasian male.

* The amounts of polyunsaturated WE were measured as described previously.¹⁶

approach nor the ESI MS used by other groups was compatible with GC experiments, which routinely offer a much better separation of (volatile and stable) analytes than HPLC needed to separate a large group of closely related, and often isobaric, compounds, such as WE. Thus, there was a need to develop an alternative robust GC-based approach that would yield qualitative and quantitative data on the meibomian WE.

Our current data demonstrated that GLC-ITMS can be used successfully for analyzing the structures and physical amounts of underivatized WE present in human meibum. A wealth of information was obtained by running various experiments simultaneously in TIC, SMC, SRM, MS/MS, and MSⁿ modes. The use of a lower than typical electron energy of -30 eV seems to help to detect better molecular ions of the intact WE, especially those of unsaturated nature. Direct quantitation of intact WE in tested samples demonstrated that unsaturated C_{18:1}-FA based WE comprised, on average, 70-80% of the entire WE pool, while about 20-30% were of a saturated nature. A study currently is underway to determine the variability of these numbers for a larger group of meibum donors.

When intact saturated WE were evaluated, a relatively narrow distribution of their FA was observed. The most common saturated FA was a C_{17:0} heptadecanoic acid. Per 100 molecules of all saturated WE, C_{17:0}-FA was found in about 62 molecules, C_{16:0}-FA in 24, C_{18:0} in 11, while C_{19:0}-FA was found in 3 molecules of WE. A C_{20:0} docosanoic acid formed the fifth largest group of the esters with less than 1 molecule per 100 molecules of all saturated WE.

The very high degree of FA branching detected for saturated WE was not surprising considering earlier reports,^{1,11,12} but the fact that, among all studied compounds the pool of C_{17:0}-FA-based WE was branched almost exclusively. We observed iso-C_{17:0}-FA and anteiso-C_{17:0}-FA in a molecular ratio of 7:93, with less than 1 mol percent of their straight chain analog present. Stearic acid was represented by a mixture of three geometrical isomers (iso-, straight, and an unidentified, but *not* straight chain) in the ratio of 32:24:44. Palmitic acid was found to be mostly in *iso*-form (80%), while the remaining 20% was identified as its straight chain isomer.

The obvious dominance of unsaturated WE in meibum (Tables 1 and 2) and the branched nature of most saturated WE might be responsible partly for making meibum melt at relatively low temperatures: both branching and an increase in the degree of unsaturation have been shown to lower the melting temperatures of long-chain lipids.^{25,26} Among unsaturated WE, two FA dominated the pool - oleic and palmitoleic ones. They were detected in an average molecular ratio of ~ 90:10, respectively. There is a possibility that a moderate change in the relative amounts of palmitoleic and oleic acids in meibomian WE can have an impact on its melting properties as the free palmitoleic acid melts at about 0.5°C, while oleic acid melts at 12-16°C.²⁷ However, if a change in the ratio is accompanied by a concomitant increase in the lengths of their FAI moieties, then the effect might be negated, and the melting point (or range) might not be affected, or at least would not change much. Interestingly, the shorter C_{16:1}-FA tended to be found in shorter WE than their C_{18:1} counterparts: the former maxed out at C₄₁H₈₀O₂ and was not found in WE longer than C₄₃H₈₄O₂, while the range of oleic acid-based waxes extended to at least C₄₇H₉₂O₂ and reached its maximum at C₄₄H₈₆O₂. Branching in WE chains similarly should lead to an analogous decrease in melting temperatures of meibum.

Previously, we reported the presence of small amounts of polyunsaturated (C_{18:2}, C_{18:3} and, possibly, C_{18:4}-FA-based) WE in human meibum, albeit in very small quantities (less than a few percent of their corresponding C_{18:1}-based homologs).¹⁶ These compounds were detected by using HPLC and atmospheric pressure chemical ionization MS (HPLC-MS). In our current GLC-ITMS experiments, these unsaturated compounds were observed only at low levels (certainly, below their LLOQ), most likely because of their much lower stability under the harsher conditions of GLC-ITMS. Indeed, the operating temperature of the column was changing between 250 and 350°C, while the length of each run was about 1 hour. These conditions caused a strong suppression of signals of even monounsaturated WE (the intensities of whose molecular ions were less than 10% of their fully saturated analogs). This trend is expected to become even more of a factor in case of

compounds with multiple double bonds. However, in Figure 14 one can observe these signals, albeit barely twice the noise level. Thus, it seems that HPLC-MS is better suited for detecting highly unsaturated WE due to its much milder experimental conditions.

A detailed comparison of various analytical techniques is beyond the size and scope of this paper. However, a few recent reports on the topic are worth noting. In a recent study, Shrestha et al. used ^1H -NMR spectroscopy to analyze test lipid mixtures and meibum.²⁸ They evaluated the NMR spectra of different lipid species, including WE, but did not provide the weight ratio, or the molar fraction, of WE in meibum. It is worth noting that, though the resolution of ^1H -NMR spectroscopy typically does not allow for a positive identification and/or quantitation of a compound, especially if present in complex mixtures, Shrestha et al. estimated the molar ratio of WE to cholesteryl esters to be 1:0.57,²⁸ which is in line with our current observations, if corrected for the differences in the molecular masses of meibomian WE and cholesteryl esters.

One of the contentious topics in meibum research is the alleged inter-donor and inter-sample variability of the lipid composition of meibum. This trend appears to originate from an earlier study of Tiffany,¹⁹ and was echoed in the later reports from various groups.^{10,11,29} Our current findings demonstrate convincingly that for a given group of meibum donors variations in their WE composition were minimal and did not exceed a few percentage points. Thus, it appears that at least the WE composition of normal (non-dry eye) meibum is very stable and does not change much from donor to donor. This conclusion corroborates our earlier reports on meibum in general^{30,31} and CE^{32,33} specifically, where no major differences in the lipid profiles were reported for groups of meibum donors of various types. Thus, the earlier reports on high inter-donor variability of meibum^{10,11,19,29} have not been confirmed in our studies.

A few comments must be made with regard to the study of Lam et al.²² First, the authors estimated WE to account for ~25% of meibum. However, this number is an underestimate because of the poor ability of HPLC-MS to detect saturated WE: note that no saturated WE were described in their study.²² Our current GLC-ITMS experiments, designed to overcome this deficiency of HPLC-MS, provided a much higher estimate of the total WE pool in meibum, about 40 to 45% (wt/wt) on average, of which ~70–80% were monounsaturated WE, <8% polyunsaturated WE, and the rest saturated ones. Thus, the number reported by Lam et al. must be corrected for this effect.²² Saturated and unsaturated cholesteryl esters (not evaluated in our current experiments, but quantified earlier^{32,33}) accounted for about 30% of meibum. It remains to be seen whether the possible differences (racial or other) in study groups could account for the reported differences in the chemical compositions of meibum presented in different reports.

Another potential problem in the study of Lam et al.²² arises from the fact that just one compound, a deuterated cholesteryl stearate, was used to quantify cholesteryl esters in their samples. This strategy invariably has led to an overestimation of the amount of cholesteryl esters as, in HPLC-MS experiments, the lower the molecular weight of the analyte, the lower the intensity of their $(\text{M} + \text{H})^+$ ions. In our hands, when an equimolar mixture of four cholesteryl esters with C_{16} , C_{18} , C_{22} , and C_{24} -FA-moieties was tested as described earlier,^{32,33} the response of the instrument rose in the following order: 1:1.3:2.3:3.0. Considering this trend, and the fact that the vast majority of meibomian cholesteryl esters are based on C_{24} - C_{28} FA, the choice of cholesteryl stearate as the only internal reference for this class of lipids invariably led to an overestimation of the presence of cholesteryl esters in human

meibum. More accurate approaches, the use of a range of authentic standards, or the use of a common cholesteryl ester fragment m/z 369 (which is formed with almost identical efficacy in HPLC-MS experiments from all tested compounds^{32,33}), are recommended instead. It is worth noting that other lipid groups were quantitated by Lam et al.²² in a similar fashion, that is using just one standard per a group of lipids. This could have led to uncontrollable over- or underestimation of their presence in meibum. It seems that, in quantitative experiments, there is no substitute for using multiple standards that cover the entire range of analytes, if possible. While finalizing our report, Lam et al. informed us in a personal communication that they were reconsidering their approach and started using multiple standards to determine the ratio of WE to CE. Their new numbers were much closer to those that had been/are reported in our studies.

Finally, we would like to acknowledge that, after the project had been completed, we came across a recent report by Urbanova et al., who used GC-EI MS/MS to analyze standard WE.³⁴ The readers should treat that study and our current work as complementary publications, comparing approaches and using them to the readers' benefit. However, several distinctive advantages of our approach over the methods described by Urbanova et al. must be mentioned. First, there was much milder fragmentation of molecular ions of WE under our conditions (EE of -30eV) compared to a more traditional, but much harsher, value of -70eV used by Urbanova et al. As a result, the molecular ions of intact WE were much more visible in our spectra, which is critical for qualitative analysis of complex lipid mixtures that are available in very small quantities, such as human meibum. Second, Urbanova et al. used a quadrupole mass spectrometer, which is incapable of MSⁿ analysis, and is limited to MS/MS studies only. The ability of ion trap instruments to perform multistage MS(1)/MS(2).../MS(n) fragmentation of parent (or precursor) ions is an invaluable tool in lipidomics, as shown in our study. Third, Urbanova et al. did not develop a quantitative approach to the WE analyses, while our method was designed and tested with this purpose in mind. However, a great number of mass spectra of WE standards reported in the study could be used as excellent references by those who use quadrupole or triple-quadrupole MS instruments.

Thus, as follows from our current and past experiments, the overall amount of WE in normal human meibum of the tested group of donors has been estimated to be (depending on the subject) about $40 \pm 10\%$ (wt/wt), of which about 80% is represented by unsaturated compounds. The cholesteryl ester pool is the second largest group of lipids in human meibum (about $30 \pm 10\%$), while the rest of the lipids remain insufficiently studied to be quantitated at this time. Overall, tested samples demonstrated a very low inter-donor variability, at least in terms of major types of WE. Future studies will demonstrate if race, sex, age, and so forth are important factors in the biochemistry and physiology of meibomian glands and the human tear film, and whether there are any major inter-donor differences in meibomian lipids on the level of individual lipid species.

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