These observations suggest that MGS experiments with API MSn detection of the analytes (HPLC and authentic lipid standards were analyzed in direct infusion and high-pressure liquid chromatography (HPLC) experiments with API MSn detection of the analytes (HPLC API MSn)). The major precursor ions were isolated and subjected to further sequential fragmentation in MSn experiments, and their fragmentation patterns were compared with those of authentic lipid standards. Multiple precursor ions were observed in the positive-ion mode. Among those, previously identified cholesterol (Chl; m/z 369; [M − H2O + H]+) and oleic acid (OA; m/z 283; [M + H]+) were found. The other major compounds of the general molecular formula CnH2n−2O2 were consistent with wax esters (WEs), with OA as fatty acyl component. Accompanying them were two homologous series of compounds that fit the molecular formulas CnH2n+1O2 and CnH2nO2. Subset 2 was found to be a homolog series of linoleic acid-based WEs, whereas subset 3 was apparently, a mixture of stearic acid-based WEs. HPLC API MSn analysis revealed the presence of large quantities of cholesteryl esters (Chl-Es) in all of the tested samples. Less than 0.1% (w/w) of oleamide was detected in human MGS. In the negative-ion mode, three major compounds with m/z values of 729, 757, and 785 that were apparently related to anionogenic lipids of the diacylglycerol family were found in all of the samples. Common phospholipids and ceramides (Cers) were not present among the major MGS lipids. Phosphocholine-based lipids were found in MGS in quantities less than 0.01% (w/w), if at all. This ratio is two orders of magnitude lower than reported previously. These observations suggest that MGS are a major source of nonpolar lipids of the WE and Chl-E families for the tear film lipid layer, but not of its previously reported (phospho)lipid, Cer, and fatty acid amide components.—Butovich, I. A., E. Uchiyama, and J. P. McCulley. Lipids of human meibum: mass-spectrometric analysis and structural elucidation. J. Lipid Res. 2007. 48: 2220–2235.

Meibomian gland (MG), found in the eyelids of humans and other mammals (1), is a major source of various lipids that participate in formation of the tear film lipid layer (TFLL) (2). The latter is believed to play a critical role in protecting the ocular surface from dehydration by creating a physical barrier, where lipids, due to their poor miscibility with water and lower density, tend to locate at the air/aqueous phase interface (2, 3). The protective efficacy of the TFLL, therefore, should directly relate to the chemical composition of the lipid layer, and its thickness. There is evidence that irregularities in meibomian gland secretions (MGS) are one of the main causes of a pathological condition commonly known as dry eye syndrome (DES) [(4, 5) and references cited therein]. The chemical composition of MGS was evaluated by a wide range of analytical methods, but surprisingly limited information was obtained using the current de facto standard of lipidomic analysis, mass spectrometry (MS) with direct infusion of the analytes or in combination with high-pressure liquid chromatography (HPLC) (6). In our recent studies, we implemented MS and HPLC with atmospheric pressure ionization MS (API MS) detection to evaluate the major lipid classes of MGS (7–9). The HPLC API MS experiments with human MGS produced clear evidence of the presence of very hydrophobic compounds similar to the tear film lipid layer (TFLL) (2). The protective efficacy of the TFLL, therefore, should directly relate to the chemical composition of the lipid layer, and its thickness. There is evidence that irregularities in meibomian gland secretions (MGS) are one of the main causes of a pathological condition commonly known as dry eye syndrome (DES) [(4, 5) and references cited therein]. The chemical composition of MGS was evaluated by a wide range of analytical methods, but surprisingly limited information was obtained using the current de facto standard of lipidomic analysis, mass spectrometry (MS) with direct infusion of the analytes or in combination with high-pressure liquid chromatography (HPLC) (6). In our recent studies, we implemented MS and HPLC with atmospheric pressure ionization MS (API MS) detection to evaluate the major lipid classes of MGS (7–9). The HPLC API MS experiments with human MGS produced clear evidence of the presence of very hydrophobic compounds similar to...
wax esters (WEs), cholesteryl esters (Chl-Es), free cholesterol (Chl), and possibly free fatty acids (FAs) and triacylglycerols (TAGs). To our surprise, no detectable amounts of compounds that would coelute with authentic monoacylglycerols (MAGs), diacylglycerols (DAGs), ceramides (Cers), and phospholipids (PLs) were detected (9). Previously, DAG (10, 11), PL, and Cer (12) were reported to be present in human MGS, whereas in rabbits, increased levels of Cer and free Chl were indicative of MG dysfunction, apparently due to hyperkeratinization of their eyelids (13). No structural evaluations of the intact lipids were performed in any of those studies. A decade ago, \(^{31}\)P-nuclear magnetic resonance spectroscopy (\(^{31}\)P-NMR) was implemented to analyze the PL composition of homogenized tarsal plates of rabbits (14, 15). Several PL species were detected, among which phosphatidylincholine (PC) and phosphatidylethanolamine comprised almost 50% of the overall PL pool. However, considering the very low sensitivity of the \(^{31}\)P-NMR technique, the duration of the experiments, and the method of collecting the sample material (dissection of eyelids), that approach seemed to be unsuitable for human studies. Moreover, it was suggested that animals (rabbits, in particular) are poor models of human TFLL and DES (16, 17).

Therefore, to 1) further evaluate the structures of the compounds detected in normal human MGS collected from individual subjects, and 2) corroborate our observation of the lack of DAG, Cer, and PL in human MGS, we conducted ion trap API MS\(^n\) analyses with direct infusion of the lipid samples. DAG, Cer, and PL were products of Thermo Electron Corp. (San Jose, CA). We further evaluated the PL composition of the lipid samples by direct infusion of the lipid extracts. The ionization (APCI) ion source, operated under the XCalibur softwar, were products of Waters Corp. (Milford, MA). An LCQ Deca XP Max ion trap spectrometer equipped, depending on the application, with either an electrospray ionization (ESI) or an atmospheric pressure chemical ionization (APCI) ion source, operated under the XCalibur software, were products of Thermo Electron Corp. (San Jose, CA).

**Materials and Methods**

Reagents and equipment

HPLC-grade acetic acid, chloroform, n-hexane, methanol, propan-2-ol, and water were purchased from Aldrich (Milwaukee, WI) and/or from Burdick and Jackson (Muskegon, MI) through VWR (West Chester, PA). Lipid standards were products of Avanti Polar Lipids, Inc. (Alabaster, AL) and Sigma Chemical Co. (St. Louis, MO). 1,2-Di-O-acyl-sn-glycero-3-phosphatidylcholine (PC, 22:1/16:0) and phosphatidylethanolamine (PE) were also a product of Avanti. A Lichrosorb Si-60 silica gel column (3.2 × 125 mm, 5 μm) was a product of Supelco (Bellefonte, PA). A Lichrosphere Diol HPLC column (3.2 × 125 mm, 5 μm) was obtained from Phenomenex (Torrance, CA). An Alliance 2695 HPLC Separations Module was from Waters Corp. (Milford, MA). An LCQ Deca XP Max ion trap spectrometer equipped, depending on the application, with either an electrospray ionization (ESI) or an atmospheric pressure chemical ionization (APCI) ion source, operated under the XCalibur software, were products of Thermo Electron Corp. (San Jose, CA).

Collection of MGS samples

Volunteers (five male and five female, median age 34 ± 3 years), who underwent standard clinical tests for DES and showed no signs of ocular diseases, participated in the study. Samples collected from different volunteers were analyzed individually. The study was approved by the Institutional Review Board. The samples were collected as follows. A dry glass vial preweighed on an analytical microbalance was filled with 1 ml of MS-grade chloroform. The MGS were expressed from a subject’s four eyelids using a plastic conformer and a cotton swab. The lipid samples were harvested from the MG orifices using a platinum spatula. The samples were never in contact with the conformer or the swab. Samples collected from all four eyelids of an individual were pooled. The secretions that had been expressed from the glands immediately solidified at room temperature to assume a typical waxy texture. The sample was transferred into the vial with chloroform, dissolved, and the solvent was evaporated at room temperature under a stream of dry nitrogen. Then, the vial with the sample was reweighed to determine the weight of the dry lipid material. Depending on the donors, the dry samples were between 0.1 and 3 mg. The standard error of weighing was ±5% for each of the dry samples. The average weight of the nine samples collected from ten volunteers was 0.45 mg. The samples were stored under nitrogen at −80°C. The samples were stable for at least 3 months. The study was approved by the Institutional Review Board of The University of Texas Southwestern Medical Center at Dallas. The informed consents were obtained from the human subjects.

**Mass spectrometric analyses**

Mass spectra of the compounds were obtained in both positive- and negative-ion modes using both the ESI and the APCI ion sources. The m/z ratios were used to calculate molecular masses (M) of the parent compounds and their fragments. In the positive-ion mode experiments, the compounds were typically detected as either proton (M + H)\(^+\), or sodium (M + Na\(^+\) adducts. In the negative-ion mode, acidic compounds were visible as (M − H\(^−\)) or (M − Cl\(^−\)) species. An interesting exclusion was a group of compounds of PC and SM families. To comply with current views, for zwitterionic compounds of PC and SM families with quaternary amino groups [−N\(^+(CH\(_3\))\(_3\)]\(^−\), M was assumed to be the mass of the species with dissociated phosphoric acid residues [−O−P(O)(O\(^−\))−O−], which resulted in electroneutral lipid species. To be visible in MS experiments, those electroneutral species must be further ionized either by losing a proton (to acquire an overall negative charge, [M − H\(^−\)], or forming various adducts with already-ionized particles (see below). The samples were dissolved in methanol-chloroform (MC) 2:1 (v/v) solvent mixture for the ESI experiments, and in the n-hexane-propan-2-ol-acetic acid (HPA) 95:5:0.1 (v/v/v) solvent mixture for the APCI analyses. The HPA solvent mixture was also used for HPLC separation of nonpolar lipids. The solvent compositions were chosen to ensure complete solubility of all the analyzed lipid samples in the indicated concentration ranges, as well as their effective ionization under the tested conditions. To minimize the chances of sample degradation due to hydrolysis or solvolysis, no modifiers (acids, bases, or water) were typically added to the MC solvent in the direct-infusion ESI experiments, unless stated otherwise. For APCI experiments in n-hexane-based solvent mixtures, between 0.1% and 1% (v/v) of acetic acid had to be added to achieve good ionization of the analytes. High-purity nitrogen was utilized as sheath gas throughout the experiments. To achieve the highest possible precision, the m/z ratios of the precursor compounds were routinely recorded in the zoom scan modes, in accordance with Thermo Electron’s recommendations. To obtain more-detailed information on the structures, the major precursor ions were isolated and subjected to further sequential fragmentation in the MS\(^n\) mode. The analyses were performed using the settings that were individually optimized for each analyte. Addition of metal ions
(Na\(^+\) or Li\(^+\)), often used to promote the formation of charged adducts (18, 19), was avoided at this stage of initial characterization of MGS because, at the time, their effects on the resulting MS spectra could not be predicted. Often, such modifiers lead to more complex MS spectra, because they do not guarantee complete conversion of all the lipid species present in the mixture into one particular type of adduct (18, 19). In our preliminary experiments, tested lipids readily ionized in either of the solvents (MC or HPA), and the MS spectra of the major components obtained in the two solvents were quite similar.

**API MS\(^n\) analyses of lipids using the direct infusion method**

The great variability of lipids that were reported to be present in MGS and a need to separate lipid classes in HPLC experiments necessitated the use of two different API ionization methods, ESI and APCI. First, standard lipids and their mixtures were analyzed by ESI MS\(^n\) in the positive-ion mode, and the analytes were detected as proton and/or sodium adducts. A sample of a lipid (0.1–10 \(\mu\)g/ml, depending on the analyte) dissolved in the MC solvent mixture was infused at a flow rate of 3–5 \(\mu\)l/min. The MS parameters were as follows: source current 3 \(\mu\)A; source voltage 4–5 kV; sheath gas flow of 6 to 10 arbitrary units; auxiliary/sweep gas 0; capillary temperature 250 \(^\circ\)C for PLs and 325 \(^\circ\)C for nonpolar lipids; capillary voltage 10–45 V. For the negative-ion mode ESI experiments, the capillary voltage was maintained between –30 and –45 V. The rest of the parameters were optimized for each individual analyte or their mixtures using the automatic tuning procedure built into the XCalibur software (Thermo Electron). PLs were analyzed with and without 0.1% aqueous ammonium formate. The full MS spectra were collected typically between \(m/z\) values of 150 and 2,000 for a period of at least 1 min and then were averaged using the Excalibur Qual Browser built-in routine. Monoisotopic molecular masses of the analytes were determined in the zoom scan mode in the range of \(m/z\pm 10\).

Second, the lipids were analyzed using the APCI ion source. For the APCI type of experiments, the source voltage was 4.4 kV (positive-ion mode) and 1.5 kV (negative-ion mode), source current was 5 \(\mu\)A for the positive- and 6 \(\mu\)A for the negative-ion modes; vaporizer and capillary temperatures were set at 375 \(^\circ\)C and 300 \(^\circ\)C, and the capillary voltage was 12 V (for the positive-ion mode) and –15 V (for the negative one). The lipids were dissolved either in the MC (for ESI experiments) or in the HPA solvent mixture (for APCI experiments; see legends to the corresponding figures). Monoisotopic molecular masses of the analytes were determined in the zoom scan mode in the range of \(m/z\pm 10\).

Once the \(m/z\) ratios had been measured, the individual components of the sample were subjected to fragmentation in a collision-induced dissociation experiment at the collision energy of 25–100 V (see legends to the corresponding figures).

Then, nonpooled samples of MGS collected from 10 normal subjects were analyzed individually. The initial characterization of the lipid samples was performed as described above for lipid standards. A sample was dissolved in either MC solvent (for ESI experiments) or the HPA solvent mixture (for APCI experiments) to make, depending on the application, a solution of ~0.05 mg to 0.3 mg dry sample/ml, and then directly infused into the mass spectrometer at a flow rate between 2 \(\mu\)l/min and 10 \(\mu\)l/min. The samples were analyzed in both the negative- and the positive-ion modes as described above for standard lipids.

**Normal-phase HPLC MS analysis of lipids**

The standard nonpolar lipids and MGS samples were analyzed by normal-phase HPLC (NP-HPLC) on the Lichrosphere Diol column. Lipids were isocratically eluted from the column in Water

**TABLE 1.** Gradient HPLC analysis of phospholipids on a silica gel Lichrosorb Si-60 column

| Step | Time (min) | Flow (ml/min) | Propan-2-ol (%) | 5 mM NH\(_4\)\(^+\) COO\(^-\) in Water (%) | Hexane (%) |
|------|------------|---------------|-----------------|----------------------------------------|------------|
| 1    | 0          | 0.5           | 40              | 2                                      | 58         |
| 2    | 5          | 0.5           | 40              | 2                                      | 58         |
| 3    | 25         | 0.5           | 40              | 5                                      | 55         |
| 4    | 40         | 0.5           | 40              | 5                                      | 55         |
| 5    | 41         | 1             | 40              | 5                                      | 55         |
| 6    | 60         | 1             | 40              | 5                                      | 55         |
| 7    | 61         | 0.5           | 40              | 2                                      | 58         |
| 8    | 70         | 0.5           | 40              | 2                                      | 58         |

HPLC, high-pressure liquid chromatography.

**Fig. 1.** Normal-phase high-pressure liquid chromatography (NP-HPLC) analysis of model nonpolar lipids with the positive-ion mode atmospheric pressure chemical ionization (APCI) MS detection. Reconstructed single-ion monitoring chromatograms of cholesterol (Chl) (RT 7.4 min; \(m/z\) 369.4, [M + H – oleic acid\(^+\)])\(^+\)); cholesterol (Chl) (RT 7.4 min; \(m/z\) 369.4, [M + H – H\(_2\)O\(^+\)])\(^+\)); tripalmitin (RT 3.5 min; \(m/z\) 551.6, [M + H – palmitic acid\(^+\)])\(^+\)); behenyl oleate (RT 3.4 min; \(m/z\) 591.5, [M + H\(^+\)])\(^+\)); 1,2-dipalmitin (RT 5.6; \(m/z\) 551.7, [M + H – H\(_2\)O\(^+\)])\(^+\)); C\(_{16}\)ceramide (RT 11.7 min; \(m/z\) 548.5, [M + H – H\(_2\)O\(^+\)])\(^+\)); 1-monomyristoyl-glycerol (RT 21.8; \(m/z\) 285.5, [M + H – H\(_2\)O\(^+\)])\(^+\)); and oleamide (OAm) (RT 23.1; \(m/z\) 282.2, [M + H\(^+\)])\(^+\)). Hexane-propan-2-ol-acetic acid (HPA) solvent mixture was used as HPLC solvent.
with the HPA solvent mixture at 30°C and a flow rate of 0.3 ml/min. The entire flow of the effluent was directed to the APCI ion source and analyzed in either the positive- or the negative-ion modes. The lipid standards (1–20 μg of individual lipid/ml) and MGS samples (0.1–3 mg/ml) were dissolved in the mobile phase. Between 1 μl and 20 μl of the sample solutions were injected.

The samples of standard PLs were analyzed by NP-HPLC using the Lichrosorb Si-60 HPLC column. HPLC analyses of the lipids were performed using gradient elution by n-hexane-propan-2-ol 5 mM aqueous ammonium formate mixtures at 30°C according to the protocol presented in Table 1. The entire flow of the effluent was directed to the APCI ion source and analyzed in either the positive- or the negative-ion modes as described above. The sample (less than 0.1 mg dry sample/ml) was dissolved in a hexane-propan-2-ol 95:5 (v/v) solvent mixture. Between 0.5 and 10 μl of the sample solutions were injected. The same procedure was performed on the samples of MGS.

RESULTS

NP-HPLC MS analysis of lipid standards and MGS

A model mixture of nonpolar lipids composed of several standard lipids whose analogs had been reported to be present in human meibum (10–15, 20, 21) was analyzed using NP-HPLC with APCI detection of the analytes in the positive-ion mode (Fig. 1). The tested compounds included cholesteryl oleate (Chl-O), Chl, tripalmitin (TP), behenyl oleate (BO), 1,2-dipalmitoylglycerol (DP), C₁₈-ceramide (C₁₈-Cer), 1-miristoyl glycerol (MG), and oleamide (OAm). The lipid mixture was separated on a Lichrosphere Diol HPLC column (3.2 × 150 mm, 5 μm). The retention times (RTs) of the lipid standards were used to map the corresponding lipid classes that were expected to be observed in MGS. Surprisingly, MGS produced only one major elution peak whose RT was identical to those of standard WE, Chl-E, and TAG (Fig. 2) The peak showed a wide range of MS signals (Fig. 2, insert A), which were further analyzed using the direct infusion method (see below). A small peak of free Chl (m/z 369.4, [M – H₂O + H]⁺, visible only under the conditions of a single-ion monitoring experiment or in extracted chromatograms; Fig. 2, insert B), along with an equally small peak of OAm (m/z 282.1, [M + H]⁺; see below) were detected. No perceptible peaks that would coelute with MAG, DAG, and Cer were observed. At the same time, we cannot rule out the possible existence of very small pools of other lipids in meibum, whose presence was too small to be detected without optimizing the conditions of the analyses for those particular compounds.

To verify the recent findings of Nichols et al. (22) regarding OAm in meibum, we also conducted appropriate HPLC MS studies of the human meibum with selective ion monitoring at m/z 282 (Fig. 3). Compared with the other

Fig. 2. Total ion chromatogram of nonpolar lipids present in human meibomian gland (MG) secretions with the positive-ion mode APCI mass spectrometry (MS) detection. Insert A: Mass spectrum of the peak with RT 3.3 min. Insert B: Reconstructed ion chromatogram of ion m/z 369.3 (Chl – H₂O + H)⁺. HPA solvent mixture was used as HPLC solvent.

Lipids of human meibum 2223
lipids present in meibum, very little, if any, OAm was detected in all tested MGS samples. By using a standard curve for authentic OAm (not shown), its presence in MGS was found to be below 0.1% (w/w, dry weight).

In the negative-ion mode, three major compounds were detected, which possessed $m/z$ values of 729.8 ± 0.3, 757.8 ± 0.3, and 785.8 ± 0.2 ($n = 44$) (Fig. 4). The compounds eluted as one peak, and their RTs were between those of WE and Chl.

A model mixture of standard PLs was analyzed on a Lichrosorb Si-60 column using a solvent gradient (Table 1). RTs of several standard PLs are presented in

![Fig. 3](image1.png)

**Fig. 3.** Single-ion monitoring chromatogram of OAm present in human MG secretions with positive-ion mode APCI MS detection at $m/z$ 282. Insert: Mass spectrum of the peak with RT 23.3 min. HPA solvent mixture was used as HPLC solvent.

![Fig. 4](image2.png)

**Fig. 4.** NP-HPLC analyses of human MG secretions with negative-ion mode APCI MS detection. Insert: Mass spectrum of the peak with RT 4.6 min. HPA solvent mixture was used as HPLC solvent.
Table 2. When MGS were analyzed by HPLC MS using the conditions optimized for PL analysis, they showed no noticeable HPLC peaks that would be indicative of PL (data not shown). The compounds with \( m/z \) ratios of 729.8, 757.8, and 785.8 eluted very quickly (RT \(~\sim\) 1.7 min) and did not coelute with any of the PL standards. Such short RTs put the analytes in the category of rather non-polar compounds, and ruled out a possibility for them to be standard PLs.

**MS analyses of lipid standards and MGS using direct infusion of the samples**

A typical MS spectrum of MGS in the positive-ion mode is presented in Fig. 5. Note a striking resemblance of this ESI spectrum to the APCI spectrum presented in Fig. 2. This means that 1) most of the ions detected in the direct infusion ESI experiments eluted from the column very quickly as a major HPLC peak with an RT of \(~\sim\) 3.3 min (Fig. 2) and, therefore, were nonpolar lipids; and 2) the type of ionization procedure did not explicitly affect the major detected MS signals. Among the prominent MS signals in the peak with RT 3.3 min, there were groups of ions with \( m/z \) values of 283.1; 369.3; 535.4; 549.5; 561.5; 563.6, and 565.5; 575.6, 577.6, and 579.4; 589.6, 591.6, and 593.5; 603.6, 605.6, and 607.4; 617.5, 619.6, and 621.5; 631.5, 633.6, and 635.6; 645.5 and 647.5; 659.7, 661.6, and 663.5; 673.6 and 675.5; and 701.6.

The ion with \( m/z \) 283.2 was found to be similar to authentic OA (monoisotopic mass 283.2, \([M+H]^+\)). The ion was fragmented in an MS\(^n\) mode to give the following ions: 283 (MS1) \(\rightarrow\) 265 (MS2) \(\rightarrow\) 247 (MS3) (sequential loss of two \(\text{H}_2\text{O}\)). Subsequent fragmentation of ion \( m/z \) 247 (MS4) produced prominent product ions that were identical to the fragments of authentic OA (Fig. 6). Occasionally observed ions with \( m/z \) 282.3 and 304.4 (see Fig. 3) were similar to authentic OAm (monoisotopic mass 282.3, \([M+H]^+\)) and its sodiated derivative (monoisotopic mass 304.5, \([M+Na]^+\)), although the low abundance of the ions precluded their direct structural analysis at this time.

In both ESI and APCI experiments, ions with \( m/z \) values ranging from 535 to 675 (Figs. 2, 5; Table 3) showed the presence of three groups of compounds that differed within each group by 14 mass units. The latter was indicative of a methylene group. The first major series of compounds were nonpolar lipids of the possible general

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**Table 2.** NP-HPLC retention times of phospholipids

| Phospholipid                                | Retention Time |
|---------------------------------------------|----------------|
| 1-palmitoyl-2-oleoyl-phosphatidylglycerol   | 20–21          |
| 1,2-dipalmitoylphosphatidylethanolamine     | 25–26          |
| 1-palmitoyl-2-arachidonoyl-phosphatidylinositol | 29–30        |
| 1-palmitoyl-2-oleoyl-phosphatidic acid      | 29–34 (broad peak) |
| 1-stearoyl-2-oleoyl-phosphatidylerine       | 34–36          |
| 1-palmitoyl-2-oleoyl-phosphatidylcholine    | 37–38          |
| C\(_{16,0}\)_sphingomyelin                   | 39–40          |

NP-HPLC, normal-phase HPLC.

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**Fig. 5.** Positive-ion mode electrospray ionization MS (ESI MS) spectrum of the whole human MG secretions. Methanol-chloroform (MC) solvent mixture was used as solvent.
molecular formula \( \text{C}_n\text{H}_{2n-2}\text{O}_2 \) (subset 1; \( n = 36-48; m/z \)
535.4, 549.5, 563.6, 577.6, 591.6, 605.6, 619.6, 633.6, 647.6,
661.7, and 675.5; all \([M+H]^+\)); for more information on
the peaks see Table 3. Accompanying them were two homol-
gous series of compounds which fit the molecular formu-
las \( \text{C}_n\text{H}_{2n-4}\text{O}_2 \) (subset 2; \( n = 38-46; m/z \)
561.5, 589.6, 603.6, 617.6, 631.6, 645.5, 659.7, and 673.6; \([M+H]^+\)), and
\( \text{C}_n\text{H}_{2n}\text{O}_2 \) (subset 3; \( n = 38-46; m/z \)
565.5, 579.6, 593.6, 607.6, 621.6, 635.6, and 663.7; \([M+H]^+\)), with subsets
2 and 3 typically less prominent then subset 1. Their
mean \( m/z \) values and the corresponding standard errors
are presented in Table 3. The above molecular formulas
are consistent with WE's that have two oxygen atoms in
their structures. Importantly, authentic WE's [BO, stear-
stearate (SS), and SO] tested in preliminary experiments
produced exclusively proton adducts, and were never seen

### Table 3. Monoisotopic masses of representative compounds of human meibum detected in the positive-ion mode

| Experimental m/z | Empirical Formula | Theoretical Monoisotopic Mass | Da |
|------------------|-------------------|--------------------------------|-----|
| 563.58 ± 0.046   | \( \text{C}_{36}\text{H}_{75}\text{O}_2 \) | 563.57                         |     |
| 577.58 ± 0.053   | \( \text{C}_{38}\text{H}_{77}\text{O}_2 \) | 577.59                         |     |
| 591.61 ± 0.054   | \( \text{C}_{40}\text{H}_{79}\text{O}_2 \) | 591.61                         |     |
| 605.63 ± 0.061   | \( \text{C}_{42}\text{H}_{81}\text{O}_2 \) | 605.62                         |     |
| 619.61 ± 0.053   | \( \text{C}_{43}\text{H}_{83}\text{O}_2 \) | 619.64                         |     |
| 633.59 ± 0.053   | \( \text{C}_{45}\text{H}_{85}\text{O}_2 \) | 633.65                         |     |
| 647.55 ± 0.071   | \( \text{C}_{46}\text{H}_{87}\text{O}_2 \) | 647.67                         |     |
| 661.72 ± 0.141   | \( \text{C}_{48}\text{H}_{89}\text{O}_2 \) | 661.69                         |     |
| 673.58 ± 0.043   | \( \text{C}_{49}\text{H}_{92}\text{O}_2 \) | 673.68                         |     |
| 701.65 ± 0.048   | \( \text{C}_{51}\text{H}_{94}\text{O}_2 \) | 701.72                         |     |

*Average +/- standard error; \( n = 23 \) to 25 measurements.

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**Fig. 6.** Positive-ion mode APCI MS\(^4\) analysis of a peak with \( m/z \)
283.2 found in human meibomian gland secretions (MGS). Ions with
\( m/z \) 283, isolated during the MS\(^1\) phase of the experiment,
were fragmented as follows: 283 \( \rightarrow \) 265 (MS\(^2\), loss of water) \( \rightarrow \) 247
(MS\(^3\), loss of water) \( \rightarrow \) a series of fragments (MS\(^4\)). Collision energy
was 35 V at every fragmentation step. HPA solvent mixture was used
as solvent.

**Fig. 7.** Positive-ion mode APCI MS\(^4\) analysis of a compound from
human MGS with \( m/z \) 591.6. Ions with \( m/z \) 591.6, isolated during
MS\(^1\) phase of the experiment, were fragmented as follows: 591 \( \rightarrow \) 283 (MS\(^2\), loss of behenyl alcohol) \( \rightarrow \) 265 (MS\(^3\), loss of water) \( \rightarrow \) a
series of fragments (MS\(^4\)). Collision energy was 32 V to 35 V at every
fragmentation step. HPA solvent mixture was used as solvent.
as sodium adducts. For example, a compound with an m/z value of 591.6 was positively identified as BO (theoretical m/z value of its [M + H]^+ adduct is 591.6) by using MS^n sequential fragmentation of the precursor ion (Fig. 7). Authentic BO fragmented producing exactly the same fragment ions (not shown). Note that among the fragmentation products of both compounds (authentic BO and the MGS lipid with m/z 591.6), there was an ion with m/z 283, whose fragmentation pattern matched that of authentic OA (Figs. 6, 7). Similarly, another MGS compound with an m/z value of 535.4 was positively identified as SO (theoretical m/z value 535.5 ([M + H]^+)), by comparing their MS^n fragmentation spectra (not shown). Therefore, ion m/z 283 was unequivocally attributed to a proton adduct of OA. Similar experiments were conducted with all the major species listed in Table 3. Each of them fragmented similarly to BO and SO, producing ion m/z 283. Therefore, it was concluded that all of them had an OA residue in them. This suggests that they were homologous WEs of general formula C\textsubscript{n}H\textsubscript{2n-2}O\textsubscript{2} formed of OA and a series of fatty alcohols with varying lengths of alkyl chains (Scheme 1). This conclusion was corroborated by NP-HPLC experiments, in which all of the major components that belong to the C\textsubscript{n}H\textsubscript{2n}O\textsubscript{2}, C\textsubscript{n}H\textsubscript{2n-2}O\textsubscript{2}, and C\textsubscript{n}H\textsubscript{2n-4}O\textsubscript{2} families coeluted with standard WEs. Importantly, all of the major ions described above were proton adducts of the corresponding compounds. Although ESI experiments tend to produce sodiated adducts of some of the lipid classes as prominent ions, standard WEs were observed exclusively as protonated species. In addition, the ESI and APCI spectra of MGS were virtually identical, which further confirms our conclusion.

Fragmentation of authentic SS proceeded similarly to that of BO, with the major difference a product ion with m/z value of 285.1, which was attributed to a proton adduct of stearic acid (SA; theoretical m/z 285.3). Similar ions were observed among fragmentation products of the compounds of the C\textsubscript{n}H\textsubscript{2n}O\textsubscript{2} series. Therefore, the latter were tentatively identified as saturated SA-based WEs. Because of a) low abundance of these compounds, and b) the fact that their MS signals overlapped with isotopic peaks of the major C\textsubscript{n}H\textsubscript{2n-2}O\textsubscript{2} series of compounds, their complete structural analysis has not been performed at this time. The last series of homologs fit the general formula C\textsubscript{n}H\textsubscript{2n-4}O\textsubscript{2} and produced a characteristic ion, m/z 281.3.

Scheme 1. Proposed structures and the corresponding theoretical m/z values of the major C\textsubscript{18:1}-based wax esters present in human MGS.

Fig. 8. Positive-ion mode ESI analysis of trimyristin (TM) and dipalmitin. A: Observation MS spectrum of TM. B: Fragmentation pattern of TM in an MS^2 experiment at relative energy of 36 V. C: Observation MS spectrum of dipalmitin. D: Fragmentation pattern of dipalmitin in an MS^2 experiment at relative energy of 38 V.
Scheme 2. Fragmentation of trimyristin (upper scheme) and dipalmitin (lower scheme) in the positive-ion mode. Proton and sodium ions were assumed to form adducts with the most electronegative atoms in the structures (carbonyl oxygens).
[proton adduct of a C_{18:2} fatty acid, e.g., linoleic acid (LA)]. This was positively confirmed for at least two compounds with \( \text{m/z} \) 617.6 and 645.5.

On the other hand, standard TAGs ionized differently, and their fragmentation pathways led to a different set of product ions. A standard TAG, trimyristin (TM), produced singly charged sodiated monomeric (theoretical monoisotopic mass 745.5 \([\text{M} + \text{Na}]^+\)) and two different dimeric adducts under the conditions of direct infusion ESI experiments (Fig. 8A, B; Scheme 2). Fragmentation of ion \( \text{m/z} \) 745.5 resulted in two major fragments, \( \text{m/z} \) 495.3 and 517.2, which were consistent with the following structures (Scheme 2). In APCI experiments, TM was observed as an ion with \( \text{m/z} \) value of 495.6 \((\text{M} + \text{H} - 229)^+\) formed as a result of neutral loss of myristic acid. Subsequent fragmentation of the ion produced a characteristic product ion of a protonated aldehyde \([\text{C}_{14}\text{H}_{22}\text{O} + \text{H}]^+\) (Scheme 2).

No signals of protonated myristic acid (which should have been seen if the compound was a WE) were found during fragmentation of TM in either of the experiments. Therefore, although standard TAG did coelute with standard WE and the nonpolar fraction of MGS lipids in HPLC experiments, the obvious differences in the fragmentation patterns of TAG and WE allowed us to reach a conclusion that the (vast) majority of MGS species belong to the group of WEs. Intact DAGs that should have been visible as a separate HPLC peak(s) with RT 5–6 min, were not observed. In preliminary ESI experiments (Fig. 8C, D), DP produced signals with \( \text{m/z} \) 551 \((\text{[M} - \text{H}_2\text{O} + \text{H}]^+)\), 591.4 \((\text{[M} + \text{Na}]^+)\), and 1,159.2 \((\text{[2M} + \text{Na}]^+; \text{theoretical m/z} 1,160.1\)). Fragmentation of ion \( \text{m/z} \) 591.4 gave product ions \( \text{m/z} \) 313 and 335 \((\text{M} + \text{H} - 256)^+\) and \((\text{M} + \text{Na} - 256)^+\); neutral loss of palmitic acid). In APCI experiments, DP produced a major signal, \( \text{m/z} \) 551.4 \((\text{[M} - \text{H}_2\text{O} + \text{H}]^+)\), which, in an MS2 experiment, gave a product ion \( \text{m/z} \) 239 (a proton adduct of an aldehyde with molecular formula \( \text{C}_{16}\text{H}_{30}\text{O} \)). Similar to the experiments with TM, no signals of free protonated palmitic acid were detected in these experiments.

![Fig. 9](image-url)  
**Fig. 9.** Positive-ion mode APCI MS² analysis of a peak with \( \text{m/z} \) 369.4 found in human MGS. A: Zoom scan of the ion with \( \text{m/z} \) 369.4. B: Ions with \( \text{m/z} \) 369, produced by authentic Chl \((\text{[M} + \text{H} - \text{H}_2\text{O}]^+)\), fragmented at collision energy of 37 V. C: Ions with \( \text{m/z} \) 369, isolated during MS¹ phase of the experiment with meibum, fragmented at collision energy of 100 V. HPA solvent mixture was used solvent.
experiments. Therefore, we concluded that TAG and DAG were not among the major lipids of human MGS.

The ion with \( m/z \) 369.3 was positively identified as a product ion of Chl dehydration. In preliminary ESI and APCI MS experiments, we showed that authentic Chl (monoisotopic mass 387.4, \([M + H]^+\)) was never observed as the \([M + H]^+\) adduct, but always as a dehydro-Chl ion (monoisotopic mass 369.4, \([M - H_2O + H]^+\); loss of water). Additional evidence that confirmed our observation came from the MS\(^n\) experiments; the fragmentation pattern of the naturally occurring ion with \( m/z \) 369.4 matched that of synthetic Chl (Fig. 9). Because very little free Chl was detected in our HPLC MS experiments (Fig. 2), the vast majority of these signals apparently came from the pool of Chl-Es.

In the negative-ion mode, three major compounds were detected, which possessed the \( m/z \) values of 729.7 ± 0.3, 757.8 ± 0.3, and 785.8 ± 0.2 (n = 44) (Figs. 4, 10A). MS\(^n\) analyses revealed that the three compounds were apparently structurally related to each other. Collision-induced fragmentation of the compound with \( m/z \) 729 produced prominent product ions at \( m/z \) 253.6, 281.4, 465.6, and 493.5, depicted in Fig. 10B. The compound with the \( m/z \) value of 757 gave ions \( m/z \) 281.5 and 493.5 (Fig. 10C), and the ion with \( m/z \) 785 resulted in the formation of ions \( m/z \) 281.3, 309.4, and 521.0 (Fig. 10D). A negatively charged product ion, \( m/z \) 281, consistent with formula \( \text{C}_{18}\text{H}_{33}\text{O}_2 \), was identified as oleate that had been released from the precursor lipid molecules; the very same fragment was observed for authentic 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) in the negative-ion mode (Scheme 3). A detailed discussion of the possible fragmentation pathways of POPG was provided by Hsu and Turk (23). The fragment \( m/z \) 253, in all likelihood, was palmitoleate (monoisotopic mass 253.2, \([M - H]^+\)). The larger fragments were tentatively identified as the fragments carrying the second esterified fatty acid moiety, glycerol, and a negatively charged head group(s) (Scheme 4).

To further elucidate the question as to which, if any, PLs were present in human MGS, we conducted comparative ESI MS analyses of a model mixture of PL (Fig. 11) and of intact human meibum (Fig. 12) using the direct infusion method in both the negative- and the positive-ion modes. When a mixture of standard lipids composed of 1 \( \mu \)g/ml of each of OAm, 1,2-dipalmitoylphosphatidyl-ethanolamine (PPPE), 1-palmitoyl-2-oleoyl-phosphatidic acid (POPA), \( \text{C}_{18:0}-\text{SM} \), POPC, POPG, 1-stea-royl-2-oleoyl-phosphatidylserine (SOPS), and semi-purified bovine liver phosphatidylinositol [mainly, 1-stea-royl-2-arachidonoyl-phosphatidylinositol (SAPI)] was analyzed in the positive-ion mode, it produced a series of ions with \( m/z \) values of 282.2 (\([\text{OAm} + H]^+\)), 391.0 (solvent peak), 563.2 (\([2 \times \text{OAm} + H]^+\)), 585.1 (\([2 \times \text{OAm} + \text{Na}^+\)), 692.3 (\([\text{PPPE} + H]^+\)), 731.4 (\([\text{C}_{18:0}-\text{SM} + H]^+\), weak), 760.4 (\([\text{POPC} + H]^+\)), 782.5 (\([\text{POPC} + \text{Na}^+\]), and 813.5 (unknown) (Fig. 11A). In the negative-ion mode, the following ions were observed in the presence of 0.1% ammonium formate: 673.4 (\([\text{POPA} - H^-]\)), 690.4 (\([\text{PPPE} - H^-]\)), 747.5 (\([\text{POPG} - H^-]\)), 788.5 (\([\text{SOPS} - H^-]\)), 804.4 (\([\text{POPC} - \text{HCOO}^-]\)), and a group of very weak signals with \( m/z \) values of 885.4 ± 2 (\([\text{SAPI} - H^-]\) and its analogs of vary-
ing degrees of unsaturation) (Fig. 11B). The ion \( m/z \) 671 was probably a contamination from \( C_{34:2} \) phosphatidic acid. In a collision-induced dissociation experiment conducted in the positive-ion mode, the entire PL mixture was initially fragmented at the collision energy of 30–100 V to produce a product ion of phosphocholine (theoretical \( m/z \) 184.1). The presence of the phosphocholine ion with \( m/z \) 184.2 was confirmed and proven to be indicative of the occurrence of PC and/or SM in the samples. The same experiments were conducted with human meibum. Very little to no presence of such a fragment was routinely observed in human samples. To verify this observation, a sample of the lipids from MGS was mixed with SSPC-D9 (final concentrations, respectively, 300 \( \mu \)g/ml and 1 \( \mu \)g/ml in the MC solvent with 1% formic acid). Immediately, a strong signal with \( m/z \) 799.6 was observed in an MS1 experiment which matched that of SSPC-D9 (monoisotopic mass 799.7; Fig. 12A). Upon the collision-induced fragmentation in an MS2 experiment, SSPC-D9 that had been premixed with a human lipid sample should have released a deuterated phosphocholine ion \{phospho-\[(CD_3)\_3N^+\]-choline\} with a monoisotopic \( m/z \) value of 193.2. The latter is nine mass units heavier than the corresponding ion of the natural phosphocholine \((m/z \) 184.1). Then, by comparing the relative intensities of both the product ions, their relative ratio could have been estimated. Indeed, a fragment \( m/z \) 193.14 was detected in our experiments (Fig. 12B). Along with the deuterated phosphocholine ion, an extremely low intensity signal of the natural phosphocholine with \( m/z \) 184.2 was observed. Its intensity was less than 5% of the intensity of its deuterated counterpart. Considering that 1) the signal with \( m/z \) value of 184.1 was not detected in pure meibum, and 2) the isotopic purity of commercial SSPC-D9 was approximately 98%, the signal of natural phosphocholine \( m/z \) 184.1 was attributed mainly to an isotopic impurity in the preparation of SSPC-D9. Therefore, the combined presence of PC and SM in MGS was estimated to be negligibly small, at less than 0.015% (w/w), if any. This ratio is at least two orders of magnitude lower than was previously reported.

Scheme 3. Characteristic fragments of 1-palmitoyl-2-oleoyl-phosphatidylglycerol (negative-ion mode).
With the exception of the signals m/z 701.6 and 729.6 (weak), which belong to the family of WEs discussed above, there were no strong signals in the range of 700 to 850 of the mass spectra of MGS samples, where signals of typical PLs were expected to be (24).

DISCUSSION

The preliminary MS analysis of the model lipid mixtures showed that both nonpolar and polar lipids and their mixtures could be successfully detected using API MS at the concentrations of 1 μg of individual lipid/ml and below. The choice of the analytical standards used in the study was based on the previously reported compositions of MGS (10–15, 20–22, 25–31). Thus, BO, Chl-O, and TP are typical representatives of, correspondingly, WE, Chl-E, and TAG, similar to the species previously found in MGS. OA, POPC, and SM were also reported to be present in human meibum (15, 25, 28). Other lipids mentioned in the text were typical representatives of the corresponding lipid classes. It was noticed that high concentrations of the

Scheme 4. Characteristic fragments of the compounds with m/z 729 (Panel A), 757 (Panel B), and 785 (Panel C) detected in the negative-ion mode and their putative structures. Only one of the two possible positional isomers of the 1,2-diacylglycerol fragment is shown for each of the compounds. Shown are hypothetical dehydration product ions. R, unknown radical.
Lipids had detrimental effects on the measured m/z values; typically, they shifted up by 0.1–0.3 units with the increase in the concentration of the analytes. Therefore, it was critical to maintain dilution of the samples in the proper range, which was found to be about 0.1–0.3 mg total lipid/ml for MGS for direct infusion ESI experiments, and below 0.1 mg total lipid/ml for HPLC MS experiments with APCI detection. The working concentrations of standard lipids were typically kept at 1 \( \mu g/ml \) and below.

A prominent feature of the API mass spectra of MGS was a group of signals with m/z values of 550 to 700 that were visible in the positive-ion mode (Figs. 2, 5). The HPLC RTs and fragmentation patterns of those were indicative of WE (Figs. 1, 2). Indeed, the homologous compounds of the \( C_nH_{2n-2}O_2 \) series were identified as OA-based WEs (Table 3 and Scheme 1). Quite similar but often not identical signals were reported in earlier studies (29, 30) and were attributed to a complex mixture of DAG, TAG, and nonpolar lipids, full structural analyses of which were not performed at the time (27, 29–31). The variations in the m/z values between our findings and the earlier data could be caused by the differences in the implemented techniques, which resulted in a better signal-to-noise ratio in our experiments due to the advances in MS technology. Contrary to the previous findings (27, 29, 30), we did not find appreciable presence of MAG, DAG, or TAG in the analyzed MGS samples.

Occasional observation of two positive ions with m/z 282.1 and 304.1 in several (but not all) of our samples qualitatively corroborated an earlier report by Nichols et al. (22), who had proposed that OAm might be an intrinsic part of the human MGS and, in addition to being a lipid signaling factor, could serve a structural role in the formation and functioning of human TFLL. However, our experiments showed that the OAm presence was very minimal compared with other detected nonpolar lipids (Figs. 2, 3). If OAm is present in meibum in such low quantities, it is unclear whether the compound could have any structural role in maintaining the integrity of the TFLL, leaving its putative signaling role as the most viable option.

Free Chl was also identified among the components of MGS. A strong signal with m/z 369.3 was routinely observed in all of the samples during the direct infusion (Fig. 5) and HPLC (Fig. 2) experiments. It originated, at least in part, from free Chl and from other Chl-containing lipids.
species that coeluted with standard Chl-E. It remains to be seen whether those species are indeed Chl-E, Chl ethers, or a dehydroxylated derivative of Chl itself. Quantification of the lipid species observed and identified in the positive-ion mode is currently under way and will be reported separately.

The results of our MS\textsuperscript{n} experiments conducted in the negative-ion mode suggested that there was a group of apparently novel anionogenic lipid compounds present in MGS that have not been described in meibum before (Fig. 10 and Scheme 4). Considering that the experiments were performed over an extended period of time, the averaged m/z values were computed (see above). The compounds were detected in a variety of solvents, including methanol, MC mixtures, and HPA mixtures. Therefore, it is more likely that they are true anions rather than adducts of neutral molecules with anions, e.g., acetate or chloride. Effective ionization of these compounds in the negative-ion mode necessitates the presence in them of labile protons to those found in phosphoric or carboxylic acid residues. Fragmentation patterns of these acidic lipid species (Fig. 10) resembled fragmentation of phosphorylated DAGs and suggested that there were two fatty acid residues in their structures. We consider it plausible that the transformation 729\rightarrow (253 + 281 + 465 + 493) could be indicative of a compound with 1-palmitoyl-2-oleoyl-glyceryl fragment (Scheme 4A). Indeed, if the fragmentation pattern of the authentic sample of POPG (Scheme 3) is used as a template to explain the fragmentation of the compound m/z 729, the signals 253 and 281 could be attributed to, respectively, sn-1 palmitate and sn-2 olate, the most common fatty acids in various lipid species, whereas fragments 465 and 493 are the larger fragments of compound A (Scheme 4A). The corresponding assignments of the fatty acyl chains were based on an established opinion that common fatty acid residues derived from the sn-2 position produce a stronger signal than those released from the sn-1 position of PLs [(24) and references cited therein].

Similarly, transformation 757\rightarrow (281 + 493) shows that the precursor compound could be 1,2-dioleoyl-glyceryl-containing lipid B (Scheme 4B), whereas transformation 785\rightarrow (281 + 309 + 521 + 503) points toward 1-oleoyl-2-eicosenoyl-glyceryl-containing compound C (Scheme 4C). Note that the geometry and localization of the double bonds are assumed to be the common cis-\Delta\textsuperscript{2}. Neither of these compounds could be classified as a WE, CE, MAG, DAG, or TAG, inasmuch as a) none of those standard lipids were visible in the negative-ion mode, b) the fragmentation patterns of anionogenic compounds A–C did not resemble those of the standard nonpolar lipids, and c) the RT values of compounds A–C were different from those of WE, CE, or TAG. Although the RT values of compounds A–C were similar to those of DAGs, both the inability of standard DAGs to effectively ionize under the conditions of negative-ion mode MS analyses and the distinctive differences in their fragmentation patterns suggested that compounds A–C were not simple DAGs. Currently, the nature of the radical(s) of compounds A–C remains unknown. More-detailed structural analysis of compounds A–C is under way.

The physiological role of the negatively charged lipids in MGS remains to be investigated, but considering that the bulk of the TFLL is composed of nonpolar lipids, amphiphilic compounds similar to the anionogenic lipids described above could, if present in the proper ratio to nonpolar lipids, facilitate their spreading across the ocular surface and/or improve the stability of the TFLL (32–34). To our surprise, human subjects in MS experiments did not show a discernable presence of common PL species (Figs. 5, 10, 12) in their MGS. Although in our MS experiments, mixtures of the various lipid standards could be routinely and reliably analyzed at the 1 \mu g/ml level and below, the spectra of MGS taken in the positive-ion mode showed no major ions between m/z 700 to 850, which would be indicative of typical PLs. The virtual absence of the phosphocholine ion with m/z ratio of 184 also corroborated the above observation (Fig. 12). Therefore, neither HPLC APCI MS analysis (7–9) nor the direct-infusion APCI and ESI MS\textsuperscript{n} experiments described above produced solid evidence of the presence of typical PLs in the analyzed samples in the mass ratios reported earlier. This unexpected finding could be explained by experimental differences, primarily the unfortunate choice of ultraviolet detection of PLs at 220 nm during earlier HPLC analyses (12, 28) [instead of the originally sug-
gested 205 nm (35), and the much more sensitive and specific methods of API MS analyses implemented here. Currently, the more in-depth qualitative and quantitative lipidomic analyses of detected lipid species are underway, the results of which will be reported separately, especially with regard to the possible branching of the WEs (36).

In conclusion, ion trap API MS\textsuperscript{+} spectrometry has proven to be an excellent tool for evaluating the lipid composition of the human MGS. An apparently new group of lipid compounds that were tentatively identified as DAG-based anionogenic lipids were found in the secretions. Lipid compounds that were tentatively identified as DAG-based anionogenic lipids were detected in MGS by API MS\textsuperscript{+}. The dominant species of the nonpolar lipids were choline-containing lipids were detected in MGS by API MS\textsuperscript{+}. The dominant species of the nonpolar lipids were OA-, SA-, and LA-based WEs with fatty alcohol moieties ranging from C\textsubscript{18:0} to C\textsubscript{30:0}. The other major species were Chl-E, OA, and Chl. These observations suggest that MGs are a major source of nonpolar lipids for human TFLL, whereas more-polar PL, ceramide, and fatty acid amide components of the TFLL should be supplied through other secretory mechanisms.

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