Published in final edited form as:

Lipids. 2016 November ; 51(11): 1269–1287. doi:10.1007/s11745-016-4183-4.

Compositional Analysis of Wax Esters in Human Meibomian Gland Secretions by Direct Infusion Electrospray Ionization Mass Spectrometry

Jianzhong Chen¹, Kari B Green², and Kelly K Nichols¹

¹School of Optometry, University of Alabama-Birmingham, Birmingham, AL, 35294

²Department of Chemistry, University of Florida, Gainesville, FL, 32611

Abstract

Wax esters (WE) are one of the predominant lipid types in human meibomian gland secretions (meibum) and account for 40~50% of total meibum lipids. Recently, we managed to quantify 51 isomeric groups of intact WE in normal human meibum samples by direct infusion electrospray ionization mass spectrometry (ESI-MS), with each WE peak in the MS spectrum corresponding to one isomeric group (Chen et al., *Invest Ophthalmol Vis Sci*, 2013, 54(8):5730–53). However, the information of the isomeric composition in each group was not obtained. In this study, tandem mass spectrometry (MS/MS) was applied to quantify relative amounts of these isomers using the intensities of the corresponding diagnostic ions after appropriate correction and normalization. This data was combined with the previous obtained mole fraction of each isomeric group to total WE in human meibum to determine the corresponding percentage of each isomer. A total of 23 of the most abundant WE peaks of different molecular weights (corresponding to 85.3% of the total amount of WE) in human meibum were studied and resulted in quantification of 92 WE species. The quantitative information of composition of WE in human meibum will help better understand their role in the tear film.

INTRODUCTION

Human meibomian gland secretions (meibum) form the outmost layer of tear film and inhibit tear evaporation.[1] WE are one of the predominant lipid types in human meibum and account for 40~50% of total meibum lipids.[2–5] A detailed knowledge of the identity and amount of WE isomers in human meibum will elucidate their role in tear film maintenance.

Quantification of WE composition in human meibum has been attempted using gas chromatography (GC) and/or mass spectrometry (MS).[2, 3, 6–8] The most comprehensive study was performed by Nicolaides et al., who initially separated WE from other meibum lipids, followed by hydrolysis, derivatization, and then quantification by GC with flame ionization detection (FID) using a series of fatty acid (FA) or fatty alcohol (FAI) standards as a reference.[8] Information regarding the overall FA and FAI content of WE in meibum was

*Corresponding author: Jianzhong Chen, PhD, School of Optometry, The University of Alabama at Birmingham, Birmingham, AL 35294; jzchen@uab.edu; Phone: 205.934.8230.

obtained; however, information of how these FA and FAI components were combined to form intact WE was missing.[8]

Recently, the development of atmospheric pressure ionization MS has allowed analysis of individual intact WE (without hydrolysis) by direct infusion or coupled with front-end separation including liquid chromatography (LC) or GC.[2, 3, 6, 7, 9–12] Front-end separation enhances detection of individual components; however, the ionization efficiencies of components change during the separation due to continuous changes in experimental conditions (mobile phase composition in LC and temperature in GC). Additionally, GC is ineffective for analysis of many nonvolatile meibum species including polyunsaturated WE and very long chain WE.[2, 3] Therefore, it is more difficult to accurately quantify all meibum species with only a limited number of standards[3] using front-end separation. In contrast, direct infusion electrospray ionization (ESI)-MS analysis has several advantages including constant solution composition resulting in stable experimental conditions and the ease of performing tandem mass spectrometry (MS/MS) on all peaks of interest, which permit correction of ionization efficiencies and identification of unknown lipids more readily.[3] This direct infusion method can be further divided into two subgroups: targeted and nontargeted. Targeted MS analysis focuses on only a limited number of expected species by monitoring their transits (precursor/product ion pairs); therefore, many species are potentially missing.[6] We utilized nontargeted MS since it provides a more complete analysis of WE.[3]

More recently, we have successfully quantified intact WE of 51 different molecular weights (detected as sodium adducts), i.e., 51 isomeric groups of WE, via the nontargeted approach. [3] This was accomplished by adjusting the experimental conditions and instrument parameters so that ionization suppression was minimized, detection sensitivity was optimized and the ionization efficiency was only dependent on lipid class and saturation level while remaining independent of carbon chain length.[3] The quantities of these different molecular weight WE species sums up to account for $48 \pm 4\%$ of the total lipid mass.[3] However, for each molecular weight, multiple WE isomers exist, including different FA/FAI combinations, different double bond positions, and the presence of straight or branched (iso or anteiso) chains. In this work, we focused on determining the different FA/FAI combinations of WE isomers.

We recently reported a method for relative quantification of isomeric species in WE of the same molecular weight based on the peak intensity of characteristic product ions resulting from the dissociation of their WE parent ions.[13] For the study presented here, we improved the method by including corrections for peak overlapping and normalization based on product ion type, resulting in a more detailed and accurate profile of WE in human meibum.

EXPERIMENTAL

Chemicals

Chloroform (HPLC grade, >99.9%, with amylene as the stabilizer, either Burdick & Jackson or Sigma brand), methanol (HPLC grade, >99.9%, Riedel-de Haen) and ammonium acetate

(>98%, Sigma) were purchased from Sigma-Aldrich (St. Louis, MO). Ten WE standards, behenyl palmitate, behenyl stearate, palmityl behenate, behenyl palmitoleate, behenyl oleate, arachidyl oleate, stearyl oleate, oleyl stearate, palmitoleyl behenate and oleyl oleate were purchased from Nu-Chek Prep (Elysian, MN) and Sigma-Aldrich (St. Louis, MO). The shorthand notation for WE was the FA precedes the FAI connected with a slash (e.g., 16:1/18:0 for stearyl palmitoleate).[11, 14]

Meibum samples

The study protocol was approved by the Ohio State University IRB in accordance with the Declaration of Helsinki. Human non-dry eye meibum samples were collected from both eyes and sample preparation was carried out as described previously.[11] Briefly, meibum was collected in 0.5 μL $\times$ 32 mm glass microcapillary tubes and stored at -80°C prior to MS and MS/MS analysis.

Electrospray time-of-flight mass spectrometry analysis

A Quadrupole Time-of-Flight mass spectrometer (Waters Q-TOF II, Milford, MA) was used for all analyses. All samples were prepared in glass vials with glass syringes (Hamilton, Reno, NV) or micro-dispensers (Drummond, Broomall, PA). Contact of sample solutions with any plastics except PTFE (syringe plunger tip) was avoided. Stainless steel tubing was used to connect the syringe to the mass spectrometer to minimize adsorption of impurities.

Stock solutions of WE standards were prepared by dissolving 2.1–24 mg of the WE into 1 mL chloroform/methanol mixture (2:1, by vol). The working solutions were prepared by 100 or 1000 fold dilution of this stock solution with chloroform-methanol mixture (2:1, by vol), with the final concentrations of these WE standards being 25–53 μM containing 1% water and 1 mM ammonium acetate additive.

Meibum sample solutions were prepared similar to a previously described method.[3] At the time of analysis, the meibum stock solutions were prepared in chloroform-methanol solvent mixture (2:1, by vol) at an approximate concentration of 67 $\mu\text{g/mL}$. In this study, meibum from two individuals was analyzed. Eighteen tandem mass spectra were acquired with pooled samples of both eyes from one individual while the rest five spectra were acquired with meibum of one eye from the other individual. The final working solutions were prepared by diluting the stock solutions 5 or 10 fold with chloroform-methanol solvent mixture (2:1, by vol), containing approximately 6.7 or 13.4 $\mu\text{g/mL}$ total lipids and 1 mM ammonium acetate additive.

The working solutions of lipid standards and meibum samples were directly infused into the mass spectrometer at a flow rate of 10–20 $\mu\text{L/min}$ and detected in positive mode. The parameters of the mass spectrometer for acquisition of mass spectrum were as follows: cone voltage was set at 15 V; the source and desolvation temperatures were set at 100°C and 150°C , respectively; the capillary voltage was set at 3 kV. The instrument was calibrated with clustering ions of sodium iodide. Multiple cleaning cycles consisting of a series of three washes using methanol, chloroform/methanol (2:1, by vol) mixture, and chloroform, were performed to diminish carryover of the solutions. MS analysis was performed with a collision-energy of 5 eV to increase resolution without causing significant fragmentation.

MS/MS analysis was performed with collision-induced dissociation (CID) on the isolated peaks of interest; high mass and low mass resolutions were set at 15 which gave unit resolution at 20% peak height. In this work, a collision-energy of 20eV was found optimum to yield high signal to noise ratio of diagnostic product ions with insignificant interference from nonspecific product ions. The acquisition time was typically less than 1 min for each lipid standard and 1–5 minutes for each precursor ion of interest of meibum samples depending on the precursor ion intensity. The spectra were smoothed twice with the Savitsky Golay method and centered based on the peak height.

Determination of the relative amount of isomeric species of WE

Each single m/z peak of WE detected during MS analysis of human meibum was typically composed of ions from isomeric WE of the same elemental composition. Therefore, tandem mass spectrometry (MS/MS) analysis of ions corresponding to the peak often yielded several sets of diagnostic product ions derived from each isomer (Fig. 1).[13] The relative amount of the WE isomers were determined from the corresponding set of diagnostic product ions, assuming the summed intensities of these ions was proportional to the total product ions and in turn the amount of the corresponding WE. The diagnostic ions differed depending on the WE type: $[\text{RCOOH}_2]^+$ ion for saturated WE, three ions including $[\text{RCOOH}_2]^+$, $[\text{RCO}]^+$ and $[\text{RCO} - \text{H}_2\text{O}]^+$ for WE with unsaturated FA components only and for di-unsaturated WE with unsaturated FA and FAI components, and two ions including $[\text{RCOOH}_2]^+$ and $[\text{RCO}]^+$ for WE with unsaturated FAI components only.[13] To obtain more accurate quantitation of WE, two types of corrections were performed for these diagnostic product ions. These two corrections were: 1) monoisotopic diagnostic product ion peak of saturated WE ($P_{s, m}$) interfered with by the diagnostic product ions of the third isotopic peak of the corresponding monounsaturated WE ($P_{mu, m+2}$); and 2) different ratios of the summed intensities for these diagnostic product ions to the total product ions.

The relative amount of each isomer in each group of WE of a particular m/z to the total amount of WE in meibum was determined. It was calculated by multiplying the relative amount of each isomer at a particular m/z (as described above) by our previously determined percentage of that isomeric group of WE (corresponding to a particular m/z , composed of multiple isomers) to the total amount of WE.[3]

RESULTS

Corrections of intensities for peaks of diagnostic product ion from wax esters to determine relative amount of isomers

As shown in Fig. 2a, the monoisotopic diagnostic product ion peak of saturated WE ($P_{s, m}$) was often interfered with by the diagnostic product ions of the third isotopic peak of the corresponding monounsaturated WE ($P_{mu, m+2}$). This is because the difference between their precursor ions, $M_{s, m}$ and $M_{mu, m+2}$, was less than 10 mDa, where the labels m and $m+2$ indicate the monoisotopic peak (all carbon atoms in WE were composed of ^{12}C) or the third isotopic peak (all carbon atoms in WE were composed of ^{12}C except two were ^{13}C), respectively. For instance, the theoretical values of monoisotopic peak of FA 18:0/FAI 24:0 and the third isotopic peak of FA18:1/24:0 with ammonium ion adduct are m/z 666.7122 and

m/z 666.7033, respectively, and as a result the isolation of the former ion would also include the latter ion.

The correction was performed by subtracting the intensity of the third isotopic peak of the monounsaturated WE product ion ($P_{mu, m+2}$) from the intensity of the monoisotopic peak of the saturated WE product ion ($P_{s, m}$). P_{mu} and P_s are the diagnostic product ions resulting from dissociation of the monounsaturated WE precursor ions $M_{mu, m+2}$ and the corresponding saturated WE precursor ions $M_{s, m}$ (with the FA/FAI component composition differing only in saturation level), respectively. Fortunately, the theoretical isotopic peak pattern of P_{mu} (Fig. 2b) derived from precursor ion $M_{mu, m+2}$ is different from that derived from $M_{mu, m}$ (Fig. 2c), calculated using the combination of the isotopic peaks from the two complementary parts comprising the WE (Table 1) in a manner similar to the method previously described by Rockwood AL, et. al.[15] Therefore, the intensity of the overlapping peak $P_{mu, m+2}$ was determined using the intensity of one of its other isotopic peaks $P_{mu, m}$ or $P_{mu, m+1}$. Finally, the calculated intensity for $P_{mu, m+2}$ was subtracted from the apparent intensity of $P_{s, m}$ to determine the actual intensity of $P_{s, m}$. (Fig. 2)

The relative quantification of isomers for unsaturated WE based on diagnostic product ions is also influenced by the types of WE (presence or absence of a double bond in the FAI component). To account for the diagnostic product-ion intensity differences, the MS/MS spectra for a set of lipid standards with differing double bond locations were obtained and analyzed to determine the correction factor necessary. To minimize the interference of contaminants, only fragment peaks with a relative intensity of more than 0.5% of the most abundant peak were considered. The ratio of the sum of the peak intensities for the total product ions (including the ions of ammoniated and protonated molecules) to the diagnostic product ions, $R = I_{total\ ions} / I_{diag\ ions}$, was calculated for each standard. Similar ratio was found for the same type of WE, either unsaturated in the FA component only or in the FAI component only. For di-unsaturated WE with both the FA and FAI components unsaturated, the types of diagnostic product ions were the same as WE unsaturated in the FA component only; however, the ratio R was similar to those of WE with unsaturated FAI components only. The presence of a double bond in the FAI components may lead to a different fragmentation pathway which yields an increase in product ions other than the diagnostic product ions. Therefore, WE unsaturated in both FA and FAI components were grouped with WE unsaturated in FAI components only for R calculations but were quantified using the same diagnostic product ions as WE with unsaturated FA components (Table 2).

The relative amount of each isomer in unsaturated WE was calculated by using the following formula modified from a previous report[16] with inclusion of the normalization factor f (which is derived from R and described in detail below):

$$a(j)\% = \frac{Ia(j)}{\sum_1^n Ia(i) + f \sum_1^n Ib(i) + f \sum_1^n Ic(i)} \quad (1)$$

$$b(j)\% = \frac{fIb(j)}{\sum_1^n Ia(i) + f\sum_1^n Ib(i) + f\sum_1^n Ic(i)} \quad (2)$$

$$c(j)\% = \frac{fIc(j)}{\sum_1^n Ia(i) + f\sum_1^n Ib(i) + f\sum_1^n Ic(i)} \quad (3)$$

where a, b and c represent three types of unsaturated WE isomers with unsaturation in the FA component only, the FAI component only, or in both components (for di- and poly unsaturated WE), respectively; “i” represents any isomer of each type; j represents a specific isomer; Ia(i), Ib(i) and Ic(i) represent the sum of the intensities of diagnostic ions considered for each isomer of types a, b and c (the diagnostic ions considered for a and c were three ions including $[RCOOH_2]^+$, $[RCO]^+$ and $[RCO - H_2O]^+$; while for b were two ions $[RCOOH_2]^+$ and $[RCO]^+$); Σ represents the sum of all isomers for each type of wax esters; $f = R_p/R_{ab}$, R_{ab} and R_p were R values for unsaturated WE in the absence and in the presence of double bond(s) in the FAI component, respectively.

Isomeric composition of WE in human meibum

The relative amount of WE isomers contained at each of the 23 different m/z values that were present in MS spectra of human meibum were determined (Table 3). Characteristic diagnostic ions for WE isomers generated from MS/MS analysis were utilized to distinguish and quantify those species. Quantification based on the summed intensity of the diagnostic product ions, with and without the corrections for peak overlapping and different types of diagnostic product ions of the isomers, were compared.

Accurate quantitation of isomeric WE in the total amount of meibum WE was obtained by combining the relative amount of isomers present at each specific m/z with the relative quantitation information we previously obtained for WE at 23 different m/z values. In total, 92 WE species were identified and quantified (Tables 3, 4 and Fig. 3).

The FA (Fig. 4 and Table 5) and FAI (Fig. 5 and Table 6) components of WE in human meibum were compiled from the quantities of these 92 WE species (Table 3). The resulted composition was consistent with the report by Nicolaides et al.[8] using the gas chromatography method which is known to work robustly for quantifying these components after saponification and derivatization. The consistency supports the validity of our method to quantify intact WE in human meibum.

DISCUSSION

Performing relative quantification of isomeric WE based on their diagnostic product ions has been reported previously for electron ionization (EI) and chemical ionization (CI) MS analysis of WE.[16–19] In the present work, the method of relative quantification of isomers is extended to ESI-MS analysis of WE. One advantage of the direct infusion mass

spectrometry method over the LC-MS method is that the concentration of the ion of interest is constant instead of on-the-flight, which allows sufficient time to acquire high quality MS/MS spectra of the precursor ions[20] that can be utilized for reliable quantification. Two additional corrections were performed to increase the reliability of the quantification: one for peak overlapping from interfering peaks with diagnostic product ion peaks of saturated WE and another to normalize for the effect of double bond(s) location(s) on fragmentation behavior in unsaturated WE. There are also other interferences for relative quantification of WE isomers in human meibum; however, these interferences are negligible. See more detail below.

Note sodium iodide was used as the additive to convert different adduct forms to only one sodiated form and thus simplified the spectra in our earlier study;[3] however, sodiated wax esters are difficult to dissociate compared to ammoniated wax esters, and thus ammonium acetate was used as the additive in this work. Since in this work only isomeric composition was experimentally determined based on the corresponding product ions of the isomers, it was not necessary to use as low concentration of meibum lipids as possible to minimize the chain length effect. A relatively higher concentration of meibum lipids and ammonium acetate additive were used in this work which promoted the formation of one dominant ammonium adduct form.

Corrections for isomer composition determination

Since intensity of saturated WE (M_s) ions was typically much lower than that of the corresponding monounsaturated WE (M_{mu}) ions in human meibum, the overlapping for saturated WE apparently could not be neglected. With FA18:1 being the predominant moiety in monounsaturated WE, the most significant overlapping occurred to FA18:0-based saturated WE (Fig. 2). The relative change for the percentage of these FA18:0-based WE in each group of WE of the same elemental composition increased by 22.7% to 57.1% (an average of 37.1%) with the correction compared to without corrections (Table 3). However, as the FA18:0-based WE components in each group of saturated WE were typically less than 10%, the difference of the percentages of isomers in each group between with no correction and with the correction ranged only from -5.3% to 6.8% (Table 3). Moreover, since each group of saturated WE accounted for less than 2% of total WE, the difference of the percentages of each isomer to the total amount of WE between with no correction and with the correction was even less and in the range of -0.11% to 0.14% (Table 3).

The other correction is for the product ions resulted from different types of isomeric precursor WE ions. The assumption for the quantification of isomeric components based on product ions is that the ratio of the summed intensities for these product ions to the total product ions is constant, and therefore, the summed intensities of these diagnostic product ions is proportional to the amount of the precursor WE ions. The assumption of a constant ratio is reasonable when the dissociation pathways are the same; however, the ratio will change if the dissociation pathways are different and in fact, dissociation pathways differ depending on the types of WE.[13] As a result, normalization needs to be performed when different types of WE are present. Depending on whether the FA1 component did or did not contain a double bond in the FA1 component (for instance, WE44:2 mixture contains two

isomers WE18:2/26:0 and WE18:1/26:1), isomers of unsaturated WE in human meibum yielded different types of diagnostic product ions. When the double bond was located in the FAI component, the product ions in the low m/z region were of higher intensity,[13] and consequently the intensities of diagnostic product ions were lower.

Therefore, in contrast to significant overlapping for saturated WE, the correction for diagnostic product ions was only applicable to unsaturated WE. Since most of the monounsaturated WE in human meibum were unsaturated in the FA moiety (more than 90% and an average of 97% without the correction) and the correction factor was only 1.5 for the two types of WE (Table 2), the correction did not make significant changes for monounsaturated WE. The percentage of monounsaturated WE with unsaturation in FAI moiety in each group of isomeric WE increased by as high as around 33%, with the correction compared to without corrections (Table 3). However, as this type of monounsaturated WE in each group was typically less than 10%, the difference of the percentages of isomers in each group between with no correction and with the correction were only 0% to – 4.5% (Table 3). The difference of the percentages of each isomer to the total amount of WE between with no correction and with the correction was even less and in the range of –0.16% to 0.09% (Table 3). Di- and poly-unsaturated WE account for more changes; however, the difference of the percentages of each isomer to the total amount of WE between with no correction and with the correction ranged only from –0.47% to 0.53% (Table 3).

Quantification of WE with the additional corrections used in the present work offers a more accurate determination of the composition of WE. Relatively small absolute changes in the mole fractions were found for many individual WE as well as the FA and FAI components in these WE when comparing with and without corrections (Tables 5,6); however, relatively great changes in the mole fraction (more than $\pm 0.35\%$ and up to 1.38%) were observed for some species after the corrections (Tables 3, 7). To have a better understanding of the role of WE in the tear film, it is recommended to perform these corrections when quantifying meibum WE.

Other negligible interferences for relative quantification of WE isomers in human meibum

In addition to the interferences that have been accounted for with these two corrections discussed above, there were also three other interference sources including: 1) the diagnostic product ions of mono- and di-unsaturated WE are interfered with by the corresponding di- and tri-unsaturated WE of the same FA/FAI composition differing only in double bond number (e.g., $[C_{17}H_{33}COOH_2]^+$ from the di-unsaturated WE with the combination of FA18:1/FAI26:1 interfered with by $[C_{17}H_{31}COOH_2]^+$ from the tri-unsaturated WE with the combination of FA18:2/FAI26:1); 2) the diagnostic product ions of one isomer interfered with by another isomer differing only in the saturation level of the two components (e.g., $[C_{17}H_{33}COOH_2]^+$ from FA18:1/FAI26:1 interfered with by $[C_{17}H_{31}COOH_2]^+$ from FA18:2/FAI26:0); and 3) the diagnostic product ions of WE interfered with by product ions from related cholesteryl esters (CE). However, all these interferences were negligible in the scope of this work and a detailed discussion concerning why follows.

The first interference occurred between the monoisotopic peaks of diagnostic product ions from mono- and di-unsaturated WE ($P_{mu, m}$ and $P_{du, m}$) and the third isotopic peaks of the corresponding product ions of di- and tri-unsaturated WE ($P_{du, m+2}$ and $P_{tu, m+2}$) resulting from overlapping of their precursor ions (mono-isotopic peak of mono- or di-unsaturated WE, $M_{mu, m}$ and $M_{du, m}$, and the third isotopic peak of the corresponding di- or tri-unsaturated WE, $M_{du, m+2}$ and $M_{tu, m+2}$). This interference was negligible because ion intensity decreases with isotopic peak number while the intensities of WE containing the same total number of carbon atoms in human meibum decrease as unsaturation increases. For example, the intensity of a di-unsaturated WE peak ($M_{du, m}$) is typically less than 60% of the corresponding monounsaturated WE peak ($M_{mu, m}$). [3] Additionally, the theoretical intensity of the third isotopic peak $M_{du, m+2}$ is only ~8–15% of the monoisotopic peak $M_{du, m}$ and the intensity of the third isotopic peak for the major corresponding diagnostic product ion (e.g., $[C_{17}H_{33}COOH_2]^+357$) $P_{du, m+2}$ is only ~2% of the corresponding monoisotopic peak $P_{mu, m}$. As a result, overlapping from the product ion $P_{du, m+2}$ with $P_{mu, m}$ only accounted for approximately 1% of the peak intensity and was thus negligible in this work.

The second negligible interference occurred between the diagnostic product ions of one isomer with those of another isomer differing only in the saturation level of the FA and FAI components. The corresponding isomeric precursor ions for the overlapping peaks were typically di- or poly-unsaturated WE differing only in one or more double bond locations. For instance, WE18:2/26:0 (M_1) and WE18:1/26:1 (M_2) are both isomers of WE44:2 and were detected as one single peak in MS; dissociation of this peak resulted in overlapping protonated product ion FA18:2 (P_1) and FA18:1 (P_2) peaks. This interference was also negligible because ion intensity decreases with isotopic peak number while increases with degree of unsaturation for these WE in meibum. When M_1 and M_2 were of approximately the same intensity, the overlapping that occurred to $P_{2, m}$ from $P_{1, m+2}$ corresponded to approximately only 2% of the intensity of the $P_{2, m}$ peak. Moreover, this interference is even less because differences exist in relative abundance of WE, e.g., in human meibum, the abundance of FA18:2-based WE is much lower than the corresponding FA18:1-based isomers and therefore its influence on the intensity of protonated FA18:1 is typically negligible.

The third negligible interference occurred to the diagnostic product ions of WE from product ions of CE. Among the tandem mass spectra of 23 groups of isomeric ions, only two of them showed relatively high intensity product ions m/z 369.34 characteristic of CE. These two groups of WE correspond to WE 46:1 and WE 44:0 (Fig. 2), whose precursor ions overlapped with those of CE20:3 and CE18:2, respectively, with all these precursor ions being ammoniated adducts. However, dissociation of ammoniated CE generated only one dominant m/z 369.34 product ion and all the other product ions including those that might overlap with the diagnostic product ions of WE were negligible (see Fig. 2a for an example).

Additional potential interfere from other types of lipids such as phospholipids and triacylglycerols was negligible due to their much lower intensity and different m/z range. WE account for 40~50% of total meibum lipids.[2–5] In contrast, very tiny amount of phospholipids in meibum are reported in the literature, including 18 ± 5 ppm (18 ± 5 ng/mg of meibum), [21] $0.006 \pm 0.003\%$ [6] or 0.6%. [5] The amount of triacylglycerols in meibum

is higher than phospholipids, however, still less than 4% of total meibum lipids;[4, 6, 7, 11] in addition, the typical m/z range for triacylglycerols in meibum is 790–930, which is significantly different from the range of 590–750 discussed here for WE.

Distribution of WE molecular species in human meibum

Quantification of intact WE, including isomeric species, in a complex mixture such as meibum is challenging. There is no efficient chromatographic method capable of separating isomeric WE as their hydrophobicity differences are subtle.[3] Additionally, the ionization efficiency of each individual species often differs[22] and it is difficult to accurately correct for changes in ionization efficiency without using corresponding lipid standards. Published studies indicate that MS and MS/MS-based quantitative analysis of lipids is typically influenced by carbon chain length and saturation degree[23, 24], where differences in efficiencies of ionization and dissociation (for precursor ions scan, neutral loss scan, multiple reaction monitoring) may both have played roles in the quantification results obtained in those studies. Moreover, the constantly changing mobile phase composition over time and co-elution from the column during gradient LC-MS, can also affect ionization efficiency.[25] Isocratic LC avoids the mobile phase composition change; nonetheless, it is less frequently used for separation and the composition of the analytes eluted from the column still change with time. Therefore, one or more internal standards for each class of lipids are used to normalize the ionization and dissociation efficiencies; however, the limited number of standards available compared to the lipids of interest and the varying behavior between the standards and the sample lipids complicates efforts to obtain accurate quantification of meibum lipids.

Recently, we managed to quantify many more WE species (along with CE and diesters) using direct injection MS and only a limited number of standards.[3] Quantification in that work was based on peaks of accurate masses detected in single stage MS (not involving intended dissociation) with negligible in-source dissociation. Therefore, variation in quantification results due to dissociation efficiency (as mentioned above) was avoided. Additionally, ionization efficiency variation between the WE species due to solvent composition was minimized by directly infusing the samples into the MS without front-end HPLC separation. Moreover, the experimental conditions and instrument parameters were adjusted so that the ionization efficiencies of meibum lipids was only dependent on the saturation level and lipid class while remaining independent of carbon chain length, thus minimizing the number of standards required to correct for the different ionization efficiencies of meibum lipids.[3] It is interesting that the variation of abundance of each peak of WE (along with CE and diesters) between different samples is very small suggesting an almost constant composition of the lipids in normal meibum samples.[3]

However, one limitation of that work was that quantitative information for the different isomers of lipid species at each m/z was not obtained. In this work, we combined quantitative information from that study[3] and the newly acquired MS/MS spectra of individual WE peaks of two samples to obtain in-depth profiling of meibum WE.

One of the major functions of meibum is evaporation resistance. Very long chain saturated FA and FAI have been reported to demonstrate high evaporation resistance;[26, 27] however,

these very long chain FA and FAI also have low fluidity.[28] The composition of WE in human meibum (Table 4) appeared to combine these two apparent contradictory features that are important for the normal function of the TFLL:[29] high evaporation resistance due to very long to ultra-long saturated carbon chains in the FAI components[26, 27] and high fluidity (lower phase transition temperature) due to unsaturation[30, 31] and odd chain[32] in the FA components. A high percentage (78.9%) of FAI components in the total WE were saturated, thus conferring evaporation resistance to the TFLL (Tables 4, 6); while 79.2% of these FAI components (or 62.5% in total WE) were esterified to an unsaturated FA component (Table 4), which decreased the melting point and increased meibum fluidity.[30, 33] In addition, most of the rest 21.1% of FA components were either of odd chain or esterified to an odd chain FA component (Table 4), which also decreased the melting point. [32] The only outliers of WE containing even chain saturated both FA and FAI components (3.4% of total WE) were 14:0/26:0, 16:0/24:0, 18:0/22:0, 14:0/28:0, 16:0/26:0, 18:0/24:0, 16:0/28:0, 18:0/26:0, and 20:0/24:0 (Table 4). However, according to Nicolaides et al., most of these FA (57.4%, 78.4%, 63.1% and 87.0% of FA 14:0, 16:0, 18:0 and 20:0, respectively) or FAI (86.3%, 80.1%, 91.9% and 97.6% of FAI 22:0, 24:0, 26:0, and 28:0, respectively) components in meibum WE contained iso-methyl branched chains,[8] which also decreased the melting point of esters of FA.[34, 35] Furthermore, di- and poly-unsaturated WE account for greater than 24.7% of meibum WE (Table 4); these di- and poly-unsaturated WE have considerably lower melting points than saturated WE[30, 33], and contribute to the fluidity of meibum by further lowering its melting point.[36]

To the best of our knowledge, there is only one previous study reporting the quantitation of intact WE that included identification/quantification of the different isomeric species in meibum.[6] In that work, Brown et al. used the multiple-reaction monitoring (MRM) method with pre-defined transitions (a series of precursor and product ion pairs) to detect/quantify WE.[12, 22] However, only WE with one of 4 FA components (FA18:1, FA16:1, FA17:0 and FA16:0) and one of the 9 saturated FAI components (FAI 21:0 through FAI 29:0), corresponding to a total of only 36 species, were considered; therefore, that study did not provide a comprehensive picture of the composition of WE in human meibum. In contrast, 92 WE species were analyzed in the present work with each containing one out of the 15 FA components and one out of 20 FAI components.

No comprehensive study of WE composition of human meibum was available for direct comparison with our work. However, Nicolaides et al extensively separately studied FA and FAI compositions of WE in human meibum after saponification and derivation in their classic work. [8] The same sets of information can be compiled from the present study. The comparison shows close resembling between the two studies and supports the validity of our method to quantify isomeric composition and in-depth quantification of intact WE in human meibum. See the following discussion for more details.

FA composition of WE in human meibum

The percentage of each type of FA component (differing number of carbons and double bonds) was determined (Table 5) from the quantified 92 WE (Table 3). The top four most abundant FA components for these WE were either unsaturated (C18:1, C16:1, C18:2) or of

odd chain (C17:0), corresponding to approximately 90% of the total FA components (Fig. 4a). The percentages of saturated, mono-, di- and polyunsaturated FA components were 18.4%, 74.7%, 6.2%, and 0.8%, respectively; of the 18.4% of saturated FA components, 64.7% were of odd chain (Table 5). The high percentage of unsaturated[30, 33] or odd chain[32] FA components may contribute to the fluidity of meibum by lowering the melting point of the WE.

The profile of FA components in meibum we obtained is very similar to an early study by Nicolaides et al. (Fig. 4b), which was based on a series of time-consuming steps including thin layer chromatography pre-fractionation, hydrolysis, derivatization, and detection by gas chromatography-flame ionization detection.[8] The most abundant five FA components they identified (summed from those of different types of FA including with normal, iso, and anteiso branched chain and with different double bond positions) were C18:1, C16:1, C17:0, C16:0 and C18:2, corresponding to a total of 88.73% of meibum WE (Fig. 4).[8] These top five (with very similar percentages) were the same as our findings except that the order of the last two was switched. The percentage of C18:2 was lower in their work, likely due to the lower volatility of polyunsaturated FA derivatives and thus increased difficulty in detection by GC. Note the data in that work was mass%. However, in our present study, the mass% and mole% were quite close (Table 8). Compared to this early study, our method offers the advantage that information on intact WE, i.e., how FA and FAI components were combined, was obtained.

In another related study by Brown et al. as mentioned above, only WE with the combination of one of four FA components (FA18:1, FA16:1, FA17:0 and FA16:0) and one of the nine saturated FAI components were considered.[6] With only four FA components considered, a comprehensive picture of the composition of WE in human meibum is not possible. In addition, of the only four FA components considered, FA 16:1 was much more abundant in their study (20.7%) compared to the work of Nicolaides et al. (11.66%) and our work (11.0%). The significant difference observed in the percentage of FA 16:1 may be due to more interference to detection of FA 16:1-based WE from impurity peaks, background peaks and unusual peaks such as neutral molecule adducts with use of the MRM method in that work.[13]

In contrast to the above-discussed studies (Nicolaides et al.,[8] Brown et al.,[6] and the present work), Butovich et al. did not report the percentages of the FA components directly, rather they reported the percentages of unsaturated and saturated FA components separately. [2] C18:1 and C16:1 were the only two unsaturated FA components, corresponding to 90% and 10%, respectively; while C16:0, C17:0, C18:0 and C19:0, were the only four saturated FA components, corresponding to 24%, 62%, 11% and 3%, respectively (these percentages were the values in the main text; different values were reported in the abstract).[2] Several issues related to that work[2] which used GC-MS to quantify WE were discussed previously, including overfitting, inability to detect polyunsaturated WE, and potential decomposition/isomerization.[3] In comparison, our work identified/quantified seven unsaturated FA components including (in order of from high to low abundance) C18:1 (75.3%), C16:1 (13.5%), C18:2 (7.5%), C17:1 (2.0%), 18:3 (0.9%), 20:1 (0.8%) and 16:2 (0.1%); while eight saturated FA components were identified/quantified, including (in order of from high

to low abundance) C17:0 (58.6%), C16:0 (26.0%), C18:0 (7.6%), C15:0 (4.8%), C19:0 (1.3%), 14:0 (1.1%), 20:0 (0.3%), and 13:0 (0.2%). The numbers in the parentheses are the percentages of total amount of unsaturated and saturated FA, respectively.

FAI composition of WE in human meibum

The four most abundant FAI components in human meibum were saturated and included C26:0, C24:0, C25:0, and C27:0, corresponding to a total of 67.7%; followed by four monounsaturated FAI components including C26:1, C24:1, C30:1 and C28:1, corresponding to a total of 18.6% (Fig. 5a and Table 6). These top 8 FAI components corresponded to a total of 86.3%. The overall percentage of saturated, monounsaturated, and di-unsaturated FAI components in meibum WE were 78.9%, 20.7%, and 0.4%, respectively; of the saturated FAI components, 33.6% were of odd chain (Table 6). The abundance of odd chain FAI components decreased the melting point[32] while maintaining high evaporation resistance.[37]

Nicolaides et al. obtained similar results reporting the same eight most abundant FAI components and in the same order including C26:0, C24:0, C25:0, C27:0, C26:1, C24:1, 30:1 and 28:1, corresponding to a total of 84.6% (Fig. 5b).[8] Similar percentages of saturated (77.02%) and monounsaturated (22.00%) FAI components were also reported in that work.[8] 34.0% of the saturated FAI components determined in that work was of odd chain (ranging from C19 to C27, with the total amount corresponding to 26.17%),[8] which support our current finding that 33.6% of the saturated FAIs was of odd chain. Note the data in that work was mass%. However, in our present study, the mass% and mole% were quite close (Table 8).

In contrast to the studies by our group and Nicolaides et al., the percentages of only 9 FAI components in WE was determined by Brown et al.[6] Additionally, their study did not consider unsaturated FAI components, which prevented a comprehensive picture of the composition of WE in human meibum.

CONCLUSIONS

The work we present here increases our knowledge of the WE constituents of human meibum and their abundance. The structure of the TFLL, which is crucial for understanding the etiology of dry eye disease, is still unknown. However, the major WE molecular components of the human meibum WE quantified in this work suggests that a combination of an appropriate ratio of saturation and unsaturation in the FA/FAI components of WE are important factors for the fluidity and evaporation resistance functions of the TFLL. In-depth comparison of the WE profiles of meibum samples between normal and dry eye subjects will provide insight into the mechanisms of dry eye disease; this work is ongoing in our research group.

Acknowledgments

The authors thank NIH for funding (grant number: NEI R01EY015519, KKN) and Jeremy Keirseay at the Campus Chemical Instrument Center of the Ohio State University for critical comments and proofreading.

Abbreviations

CE	cholesteryl ester
CI	chemical ionization
CID	collision-induced dissociation
EI	electron ionization
ESI	electrospray ionization
FA	fatty acid
FAI	fatty alcohol
FID	flame ionization detector
GC	gas chromatography
LC	liquid chromatography
MS	mass spectrometry
MS/MS	tandem mass spectrometry
TFL	tear film lipid layer
WE	wax ester

REFERENCES

1. Knop E, Knop N, Millar T, Obata H, Sullivan DA. The international workshop on meibomian gland dysfunction: report of the subcommittee on anatomy, physiology, and pathophysiology of the meibomian gland. *Invest Ophthalmol Vis Sci.* 2011; 52:1938–1978. [PubMed: 21450915]
2. Butovich IA, Arciniega JC, Lu H, Molai M. Evaluation and quantitation of intact wax esters of human meibum by gas-liquid chromatography-ion trap mass spectrometry. *Invest Ophthalmol Vis Sci.* 2012; 53:3766–3781. [PubMed: 22531701]
3. Chen J, Green KB, Nichols KK. Quantitative profiling of major neutral lipid classes in human meibum by direct infusion electrospray ionization mass spectrometry. *Invest Ophthalmol Vis Sci.* 2013; 54:5730–5753. [PubMed: 23847307]
4. Mathers WD, Lane JA. Meibomian gland lipids, evaporation, and tear film stability. *Adv Exp Med Biol.* 1998; 438:349–360. [PubMed: 9634908]
5. Lam SM, Tong L, Duan X, Petznick A, Wenk MR, Shui G. Extensive characterization of human tear fluid collected using different techniques unravels the presence of novel lipid amphiphiles. *J Lipid Res.* 2014; 55:289–298. [PubMed: 24287120]
6. Brown SH, Kunnen CM, Duchoslav E, Dolla NK, Kelso MJ, Papas EB, Lazon de la Jara P, Willcox MD, Blanksby SJ, Mitchell TW. A comparison of patient matched meibum and tear lipidomes. *Invest Ophthalmol Vis Sci.* 2013; 54:7417–7424. [PubMed: 24135754]
7. Lam SM, Tong L, Yong SS, Li B, Chaurasia SS, Shui G, Wenk MR. Meibum lipid composition in Asians with dry eye disease. *Plos One.* 2011; 6:e24339. [PubMed: 22043274]
8. Nicolaides N, Kaitaranta JK, Rawdah TN, Macy JI, Boswell FM 3rd, Smith RE. Meibomian gland studies: comparison of steer and human lipids. *Invest Ophthalmol Vis Sci.* 1981; 20:522–536. [PubMed: 7194326]

9. Sullivan BD, Evans JE, Cermak JM, Krenzer KL, Dana MR, Sullivan DA. Complete androgen insensitivity syndrome: effect on human meibomian gland secretions. *Arch Ophthalmol*. 2002; 120:1689–1699. [PubMed: 12470144]
10. Butovich IA, Wojtowicz JC, Molai M. Human tear film and meibum. Very long chain wax esters and (O-acyl)-omega-hydroxy fatty acids of meibum. *J Lipid Res*. 2009; 50:2471–2485. [PubMed: 19535818]
11. Chen JZ, Green-Church KB, Nichols KK. Shotgun Lipidomic Analysis of Human Meibomian Gland Secretions with Electrospray Ionization Tandem Mass Spectrometry. *Invest Ophthalmol Vis Sci*. 2010; 51:6220–6231. [PubMed: 20671273]
12. Lam SM, Tong L, Reux B, Lear MJ, Wenk MR, Shui GH. Rapid and sensitive profiling of tear wax ester species using high performance liquid chromatography coupled with tandem mass spectrometry. *J Chromatogr A*. 2013; 1308:166–171. [PubMed: 23953715]
13. Chen J, Green KB, Nichols KK. Characterization of Wax Esters by Electrospray Ionization Tandem Mass Spectrometry: Double Bond Effect and Unusual Product Ions. *Lipids*. 2015; 50:821–836. [PubMed: 26178197]
14. Fitzgerald M, Murphy RC. Electrospray mass spectrometry of human hair wax esters. *J Lipid Res*. 2007; 48:1231–1246. [PubMed: 17277382]
15. Rockwood AL, Haimi P. Efficient calculation of accurate masses of isotopic peaks. *J Am Soc Mass Spectrom*. 2006; 17:415–419. [PubMed: 16458531]
16. Aasen AJ, Hofstett Hh, Iyengar BTR, Holman RT. Identification and Analysis of Wax Esters by Mass Spectrometry. *Lipids*. 1971; 6:502–507. [PubMed: 27519360]
17. Spencer GF. Alkoxy-Acyl Combinations in the Wax Esters from Winterized Sperm Whale Oil by Gas Chromatography-Mass Spectrometry. *J Am Oil Chem Soc*. 1979; 56:642–646.
18. Spencer GF, Plattner RD. Compositional Analysis of Natural Wax Ester Mixtures by Tandem Mass-Spectrometry. *J Am Oil Chem Soc*. 1984; 61:90–94.
19. Tava A, Cunico C, Cremona R, Piccinini E. Isomeric Composition of the Ester Fraction from Epicuticular Waxes of *Festuca arundinacea* Schreb. *J High Res Chromatogr: HRC*. 1996; 19:6.
20. Han X, Yang K, Gross RW. Multi-dimensional mass spectrometry-based shotgun lipidomics and novel strategies for lipidomic analyses. *Mass Spectrom Rev*. 2012; 31:134–178. [PubMed: 21755525]
21. Saville JT, Zhao Z, Willcox MD, Ariyavidana MA, Blanksby SJ, Mitchell TW. Identification of phospholipids in human meibum by nano-electrospray ionisation tandem mass spectrometry. *Exp Eye Res*. 2011; 92:238–240. [PubMed: 21195712]
22. Iven T, Herrfurth C, Hornung E, Heilmann M, Hofvander P, Stymne S, Zhu LH, Feussner I. Wax ester profiling of seed oil by nano-electrospray ionization tandem mass spectrometry. *Plant Methods*. 2013; 9:24. [PubMed: 23829499]
23. Han X, Gross RW. Quantitative analysis and molecular species fingerprinting of triacylglyceride molecular species directly from lipid extracts of biological samples by electrospray ionization tandem mass spectrometry. *Anal Biochem*. 2001; 295:88–100. [PubMed: 11476549]
24. Koivusalo M, Haimi P, Heikinheimo L, Kostianen R, Somerharju P. Quantitative determination of phospholipid compositions by ESI-MS: effects of acyl chain length, unsaturation, and lipid concentration on instrument response. *J Lipid Res*. 2001; 42:663–672. [PubMed: 11290839]
25. Zhou S, Hamburger M. Effects of solvent composition on molecular ion response in electrospray mass spectrometry: Investigation of the ionization processes. *Rapid Commun Mass Spectrom*. 1995; 9:1516–1521.
26. La Mer VK, Healy TW, Aylmore LAG. The transport of water through monolayers of long-chain n-paraffinic alcohols. *J Coll Sci*. 1964; 19:673–684.
27. Archer RJ, Mer VKLA. The rate of evaporation of water through fatty acid monolayers. *J Phys Chem*. 1955; 59:200–208.
28. Mer VK, Healy TW. Specification of Materials for the Retardation of Evaporation of Water-the Spreading of Hexadecanol Monolayers. *Proc Natl Acad Sci USA*. 1964; 52:679–683. [PubMed: 16591213]
29. King-Smith PE, Bailey MD, Braun RJ. Four characteristics and a model of an effective tear film lipid layer (TFLL). *Ocul Surf*. 2013; 11:236–245. [PubMed: 24112227]

30. Patel S, Nelson DR, Gibbs AG. Chemical and physical analyses of wax ester properties. *J Insect Sci.* 2001; 1:4. [PubMed: 15455064]
31. Borchman D, Foulks GN, Yappert MC, Bell J, Wells E, Neravetla S, Greenstone V. Human meibum lipid conformation and thermodynamic changes with meibomian-gland dysfunction. *Invest Ophthalmol Vis Sci.* 2011; 52:3805–3817. [PubMed: 21398284]
32. Boese R, Weiss HC, Blaser D. The Melting Point Alternation in the Short-Chain n-Alkanes: Single-Crystal X-Ray Analyses of Propane at 30 K and of n-Butane to n-Nonane at 90 K. *Angewandte Chemie.* 1999; 38:4.
33. Iyengar BT, Schlenk H. Melting points of synthetic wax esters. *Lipids.* 1969; 4:28–30. [PubMed: 5766845]
34. Dunn RO, Dunn RO. A Comprehensive Evaluation of the Melting Points of Fatty Acids and Esters Determined by Differential Scanning Calorimetry. *Journal of the American Oil Chemists' Society.* 2009; 86:843–856.
35. Yao L, Hammond EG. Isolation and melting properties of branched-chain esters from lanolin. *J Am Oil Chem Soc.* 2006; 83:547–552.
36. Kulkarni MG, Sawant SB. Physico-chemical properties of wax esters synthesised from corresponding alcohols using hydrobromic acid and hydrogen peroxide action. *Eur J Lipid Sci Technol.* 2002; 104:387–393.
37. Foulds EL Jr, Dressler RG. Performance of monolayer blends of odd and even carbon chain alcohols in water evaporation suppression. *Ind Eng Chem Prod RD.* 1968; 7:75–79.

Figure 1. Electrospray ionization (ESI) and collision-induced dissociation tandem mass (CID-MS/MS) spectra of ammonium ion adduct of wax esters of different elemental compositions: (a) WE41:0; (b) WE44:3; (c) WE44:2 and (d) WE44:1 at collision energy of 20 eV. The percentages of each isomer were listed in each panel.

Figure 2.

Correction of the monoisotopic diagnostic product ion peak $[C_{17}H_{33}COOH_2]^+$ for wax ester FA18:0/FAI26:0 in (a) MS/MS spectrum of ammonium ion adduct of WE44:0, based on theoretical isotopic pattern of product ion $[C_{17}H_{33}COOH_2]^+$ from dissociation of (b) M_{m+2} isotopic peak for ammoniated wax ester FA18:1/FAI26:0. As a comparison, theoretical isotopic pattern of the same product ion $[C_{17}H_{33}COOH_2]^+$ from dissociation (c) of M_m isotopic peak for ammoniated wax ester FA18:1/FAI26:0 is also shown. The percentages of

each isomer with and without the correction were listed in **(a)**. See the text for detailed discussion.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3.

The relative abundance distribution of the top 50 most abundant wax ester species in total wax esters from human meibum. Error bars indicate one standard deviation.

Figure 4.

The relative abundance of the major fatty acid components in human meibum wax esters as determined by: **(a)** non-targeted analysis of intact wax esters in this study; **(b)** analysis of fatty acids in wax esters after saponification by Nicolaides et al.[8] The data in **(b)** was summed from the percentages of fatty acids with the same elementary composition including normal, iso- and anteiso- as well as differing double bond positions. The composition in **(b)** was determined after saponification while in **(a)** the composition was determined from intact WE.

(a)

(b)

Figure 5.

The relative abundance of the major fatty alcohol components in human meibum wax esters as determined by: (a) non-targeted analysis of intact wax esters in this study; (b) analysis of fatty alcohols in wax esters after saponification by Nicolaides et al.[8] The “other” of the small pie chart in (b) includes 11 other components each less than 0.25% with 0.35% remaining unidentified. The data in (b) was summed from the percentages of WE with the same fatty alcohol elementary composition including normal, iso- and anteiso- as well as differing double bond positions. The composition shown in (b) was determined after saponification while data presented in (a) was determined from intact WE.

The matrix that was used to determine theoretical relative intensities of the isotopic product ion $[C_{17}H_{33}COOH_2]^+$ peaks resulting from dissociation of the third isotopic precursor ion peak (M_{m+2}) of ammoniated wax ester FA18:1/FAI26:0

Table 1

$[C_{17}H_{33}COOC_{26}H_{52}+NH_4]^+$		$C_{26}H_{52}+NH_3$ (N)		
		$N_m\%$	$N_{m+1}\%$	$N_{m+2}\%$
		74.4	22.4	3.2
$[C_{17}H_{33}COOH_2]^+$ (FA18:1, P)	$P_m\%$	81.3	18.2	2.6
	$P_{m+1}\%$	16.7	3.7	0.5
	$P_{m+2}\%$	2.0	0.4	0.1

The method is similar to the one previously described by Rockwood et al.[15] Since the third isotopic precursor ion (M_{m+2}) were composed of P_m , N_{m+2} , $P_m + 1/N_{m+1}$, and $P_m + 2/N_m$, the relative intensities of the monoisotopic, the second, and the third isotopic product ion $[C_{17}H_{33}COOH_2]^+$ were calculated as 2.6%, 3.7%, and 1.5% (highlighted), respectively. The corresponding relative intensities were 70.7%, 100% and 39.2% after normalization to the most abundant isotopic peak.

Table 2

The ratio of total ion intensity or total fragment ion intensity to the sum of diagnostic ion intensity, $R_1 = I_{\text{total ions}}/I_{\text{diag ions}}$ or $R_2 = I_{\text{total fragments}}/I_{\text{diag ions}}$, for wax esters of different types^{*}.

	Saturated WE	Unsaturated WE		
		Double bond in FAI		Correction factor f (ratio)
		Yes	No	
$R_1 = I_{\text{total ions}}/I_{\text{diag ions}}$	1.17±0.03	2.78±0.09	1.88±0.14	1.5 ± 0.1
$R_2 = I_{\text{total fragments}}/I_{\text{diag ions}}$	1.17±0.03	2.68±0.07	1.64±0.14	1.6 ± 0.1

^{*} : For WE unsaturated in both fatty acid (FA) and fatty alcohol (FAI) components, the diagnostic ions were the same as WE unsaturated in fatty acid components only, while the ratios (R_1 and R_2) were more similar to those determined for WE unsaturated in fatty alcohol components only. Therefore, they were quantified using the same diagnostic product ions as WE with unsaturated FA components only but grouped together with the WE with unsaturated FAI components only for R_1 and R_2 calculations.

Table 3

Quantification of wax ester (WE) isomers in human meibum*

WE	Mole % of total WEs[3]	FA	FAI	Isomer composition (%)						Mole % of total WEs		
				with correction for			Difference (none vs both)	Relative changes (% none vs both)	with correction for		Difference (none vs both)	
				None	Overlap.	Diag- ion type			both	None		both
39:0	0.79	13:0	26:0	3.4	3.5	3.4	3.5	-0.1	-2.9	0.03	0.03	0.00
		14:0	25:0	6.4	6.4	6.4	6.4	0	0.0	0.05	0.05	0.00
		15:0	24:0	31.3	31.7	31.3	31.7	-0.4	-1.3	0.25	0.25	0.00
		16:0	23:0	15.9	16.1	15.9	16.1	-0.2	-1.2	0.12	0.13	0.00
		17:0	22:0	37.6	38.0	37.6	38.0	-0.4	-1.1	0.29	0.30	0.00
		18:0	21:0	3.3	2.1	3.3	2.1	1.2	57.1	0.03	0.02	0.01
		19:0	20:0	2.1	2.2	2.1	2.2	-0.1	-4.5	0.02	0.02	0.00
40:0	1.47	14:0	26:0	5.4	5.5	5.4	5.5	-0.1	-1.8	0.08	0.08	0.00
		15:0	25:0	10.1	10.2	10.1	10.2	-0.1	-1.0	0.15	0.15	0.00
		16:0	24:0	62.4	63.1	62.4	63.1	-0.7	-1.1	0.92	0.93	-0.01
		17:0	23:0	18.9	19.1	18.9	19.1	-0.2	-1.0	0.28	0.28	0.00
		18:0	22:0	3.1	2.2	3.1	2.2	0.9	40.9	0.05	0.03	0.01
		14:0	27:0	0.8	0.8	0.8	0.8	0	0.0	0.02	0.02	0.00
		15:0	26:0	7.6	7.6	7.6	7.6	0	0.0	0.21	0.21	0.00
41:0	2.83	16:0	25:0	24.7	24.9	24.7	24.9	-0.2	-0.8	0.70	0.70	0.00
		17:0	24:0	65.0	65.3	65.0	65.3	-0.3	-0.5	1.84	1.85	-0.01
		18:0	23:0	1.9	1.4	1.9	1.4	0.5	35.7	0.05	0.04	0.01
		14:0	28:0	0.6	0.6	0.6	0.6	0	0.0	0.02	0.02	0.00
		15:0	27:0	2.9	3.0	2.9	3.0	-0.1	-3.3	0.10	0.10	0.00
		16:0	26:0	35.3	36.3	35.3	36.3	-1	-2.8	1.18	1.21	-0.03
		17:0	25:0	50.2	51.6	50.2	51.6	-1.4	-2.7	1.68	1.72	-0.05
42:0	3.34	18:0	24:0	10.5	8.1	10.5	8.1	2.4	29.6	0.35	0.27	0.08
		19:0	23:0	0.5	0.5	0.5	0.5	0	0.0	0.02	0.02	0.00

WE	Mole % of total WEs[3]	FA	FAI	Isomer composition (%)						Mole % of total WEs		
				with correction for				Difference (none vs both)	Relative changes (% none vs both)	with correction for		Difference (none vs both)
				None	Overlap	Diag- ion type	both			None	both	
43:0	3.49	15:0	28:0	1.0	1.0	1.0	1.0	0	0.0	0.03	0.04	0.00
		16:0	27:0	10.3	10.6	10.3	10.6	-0.3	-2.8	0.36	0.37	-0.01
		17:0	26:0	76.4	78.6	76.4	78.6	-2.2	-2.8	2.67	2.74	-0.07
		18:0	25:0	9.4	6.9	9.4	6.9	2.5	36.2	0.33	0.24	0.09
		19:0	24:0	2.8	2.9	2.8	2.9	-0.1	-3.4	0.10	0.10	0.00
		16:0	28:0	9.2	10.1	9.2	10.1	-0.9	-8.9	0.19	0.21	-0.02
44:0	2.07	17:0	27:0	49.2	54.5	49.2	54.5	-5.3	-9.7	1.02	1.13	-0.11
		18:0	26:0	36.7	29.9	36.7	29.9	6.8	22.7	0.76	0.62	0.14
		19:0	25:0	3.2	3.5	3.2	3.5	-0.3	-8.6	0.07	0.07	-0.01
		20:0	24:0	1.8	2.0	1.8	2.0	-0.2	-10.0	0.04	0.04	0.00
		16:1	22:0	12.2	12.2	12.2	12.2	0	0.0	0.15	0.15	0.00
		17:1	21:0	2.7	2.7	2.7	2.7	0	0.0	0.03	0.03	0.00
39:1	1.14	18:1	20:0	85.1	85.1	85.1	85.1	0	0.0	1.08	1.08	0.00
		16:1	23:0	22.3	22.3	21.9	21.9	0.4	1.8	0.26	0.25	0.00
		17:1	22:0	6.2	6.2	6.1	6.1	0.1	1.6	0.07	0.07	0.00
		17:0	22:1	4.0	4.0	5.9	5.9	-1.9	-32.2	0.05	0.07	-0.02
		18:1	21:0	67.5	67.5	66.2	66.2	1.3	2.0	0.77	0.76	0.02
		16:1	24:0	55.3	55.3	54.1	54.1	1.2	2.2	1.64	1.61	0.03
40:1	2.97	16:0	24:1	4.1	4.1	6.1	6.1	-2	-32.8	0.12	0.18	-0.06
		17:1	23:0	2.4	2.4	2.3	2.3	0.1	4.3	0.07	0.07	0.00
		18:1	22:0	37.2	37.2	36.5	36.5	0.7	1.9	1.10	1.08	0.02
		20:1	20:0	1.0	1.0	1.0	1.0	0	0.0	0.03	0.03	0.00
		16:1	25:0	40.4	40.4	38.5	38.5	1.9	4.9	1.48	1.41	0.07
		17:1	24:0	11.8	11.8	11.2	11.2	0.6	5.4	0.43	0.41	0.02
41:1	3.65	17:0	24:1	8.4	8.4	12.9	12.9	-4.5	-34.9	0.31	0.47	-0.16
		18:1	23:0	39.4	39.4	37.5	37.5	1.9	5.1	1.44	1.37	0.07

WE	Mole % of total WEs[3]	FA	FAI	Isomer composition (%)						Mole % of total WEs		
				with correction for			Difference (none vs both)	Relative changes (% none vs both)	with correction for	Difference (none vs both)		
				None	Overlap	Diag- ion type					None	both
42:1	11.18	16:1	26:0	19.9	19.9	19.8	19.8	0.1	0.5	2.22	2.21	0.01
		16:0	26:1	1.3	1.3	2.0	2.0	-0.7	-35.0	0.15	0.22	-0.07
		17:1	25:0	2.7	2.7	2.7	2.7	0	0.0	0.31	0.30	0.00
		18:1	24:0	76.0	76.0	75.5	75.5	0.5	0.7	8.50	8.44	0.06
43:1	9.67	16:1	27:0	7.7	7.7	7.6	7.6	0.1	1.3	0.75	0.74	0.01
		17:1	26:0	5.1	5.1	5.1	5.1	0	0.0	0.50	0.49	0.01
		17:0	26:1	2.3	2.3	3.4	3.4	-1.1	-32.4	0.22	0.33	-0.11
		18:1	25:0	84.9	84.9	83.9	83.9	1	1.2	8.21	8.12	0.09
44:1	13.03	16:1	28:0	2.5	2.5	2.5	2.5	0	0.0	0.32	0.32	0.00
		18:1	26:0	96.2	96.2	96.2	96.2	0	0.0	12.53	12.53	0.00
		20:1	24:0	1.4	1.4	1.4	1.4	0	0.0	0.18	0.18	0.00
		16:1	29:0	3.7	3.7	3.6	3.6	0.1	2.8	0.18	0.17	0.00
45:1	4.83	17:0	28:1	4.3	4.3	6.4	6.4	-2.1	-32.8	0.21	0.31	-0.10
		18:1	27:0	89.6	89.6	87.7	87.7	1.9	2.2	4.33	4.24	0.09
		20:1	25:0	2.4	2.4	2.4	2.4	0	0.0	0.12	0.11	0.00
		16:1	30:0	7.0	7.0	6.9	6.9	0.1	1.4	0.17	0.17	0.00
46:1	2.5	16:0	30:1	3.4	3.4	5.0	5.0	-1.6	-32.0	0.09	0.13	-0.04
		18:1	28:0	79.8	79.8	78.5	78.5	1.3	1.7	2.00	1.96	0.03
		20:1	26:0	9.8	9.8	9.6	9.6	0.2	2.1	0.24	0.24	0.00
		16:2	24:0	8.8	8.8	6.5	6.5	2.3	35.4	0.09	0.07	0.02
40:2	1.02	16:1	24:1	41.6	41.6	46.1	46.1	-4.5	-9.8	0.43	0.47	-0.05
		18:2	22:0	20.4	20.4	15.0	15.0	5.4	36.0	0.21	0.15	0.05
		18:1	22:1	29.3	29.3	32.4	32.4	-3.1	-9.6	0.30	0.33	-0.03
		16:1	26:1	10.8	10.8	12.3	12.3	-1.5	-12.2	0.53	0.61	-0.07
42:2	4.9	18:2	24:0	36.8	36.8	27.9	27.9	8.9	31.9	1.80	1.37	0.43
		18:1	24:1	52.4	52.4	59.7	59.7	-7.3	-12.2	2.57	2.93	-0.36

WE	Mole % of total WEs[3]	FA	FAI	Isomer composition (%)					Mole % of total WEs		
				with correction for			Difference (none vs both)	Relative changes (% none vs both)	with correction for		Difference (none vs both)
				None	Overlap	Diag- ion type	both		None	both	
44:2	5.75	16:1	28:1	7.3	7.3	8.5	8.5	-1.2	0.42	0.49	-0.07
		18:2	26:0	40.2	40.2	31.0	31.0	9.2	2.31	1.78	0.53
		18:1	26:1	52.4	52.4	60.6	60.6	-8.2	3.01	3.48	-0.47
46:2	3.01	16:1	30:1	16.7	16.7	17.7	17.7	-1	0.50	0.53	-0.03
		18:2	28:0	16.8	16.8	11.9	11.9	4.9	0.51	0.36	0.15
		18:1	28:1	66.4	66.4	70.4	70.4	-4	2.00	2.12	-0.12
48:2	2.91	16:1	32:1	8.1	8.1	8.5	8.5	-0.4	0.24	0.25	-0.01
		18:2	30:0	13.5	13.5	9.4	9.4	4.1	0.39	0.27	0.12
		18:1	30:1	78.4	78.4	82.1	82.1	-3.7	2.28	2.39	-0.11
50:2	1.32	18:2	32:0	11.3	11.3	7.9	7.9	3.4	0.15	0.10	0.05
		18:1	32:1	88.7	88.7	92.1	92.1	-3.4	1.17	1.22	-0.05
		18:3	24:0	38.5	38.5	29.4	29.4	9.1	0.37	0.29	0.09
42:3	0.97	18:2	24:1	48.4	48.4	55.5	55.5	-7.1	0.47	0.54	-0.07
		18:1	24:2	13.1	13.1	15.1	15.1	-2	0.13	0.15	-0.02
		18:3	26:0	39.5	39.5	30.3	30.3	9.2	0.46	0.35	0.11
44:3	1.16	18:2	26:1	47.3	47.3	54.5	54.5	-7.2	0.55	0.63	-0.08
		18:1	26:2	13.2	13.2	15.2	15.2	-2	0.15	0.18	-0.02
Total (%)	85.29								85.28	85.29	

* : The numbers in red indicate a change of isomer composition compared to the values with no correction.

Summary of fatty acid (FA) and fatty alcohol (FAI) compositions of wax esters (WE) with varying saturation level in human meibum

Table 4

Number of double bonds		Mole% with Corrections			
		WE	FA	FAI	both ¹
0	0	0	13.99	13.99	16.40
	0	0	49.10	48.55	57.58
1	0	1	1.14	1.70	1.34
	1	0	50.24	50.25	58.92
2	0	2	5.46	4.11	6.41
	1	1	13.46	14.81	15.78
3	0	3	18.92	18.92	22.19
	1	2	0.28	0.32	0.33
Total			85.28	85.29	100

¹ Determined directly from wax esters of 23 different molecular weights that comprised 85.29% of the total moles of wax esters in human meibum

² The data in ¹ was normalized by extending the pattern determined from 85.29% of total moles of wax esters to 100%.

Table 5

Fatty acid (FA) components (mole%) in wax esters from human meibum.

FA	No correction	With both correction	Difference	No correction ^{<i>I</i>}	With both correction ^{<i>I</i>}	Difference ^{<i>I</i>}
13:0	0.03	0.03	0.00	0.03	0.03	0.00
14:0	0.17	0.17	0.00	0.20	0.20	0.00
15:0	0.74	0.75	-0.01	0.87	0.88	-0.01
16:0	3.83	4.08	-0.25	4.49	4.78	-0.29
17:0	8.56	9.20	-0.63	10.04	10.78	-0.74
18:0	1.56	1.22	0.35	1.83	1.43	0.41
19:0	0.20	0.21	-0.01	0.23	0.24	-0.01
20:0	<u>0.04</u>	<u>0.04</u>	0.00	<u>0.04</u>	<u>0.05</u>	0.00
	15.13	15.69		17.74	18.40	
16:1	9.29	9.37	-0.09	10.89	10.99	-0.10
17:1	1.41	1.37	0.03	1.65	1.61	0.04
18:1	51.58	52.37	-0.79	60.48	61.41	-0.93
20:1	<u>0.57</u>	<u>0.56</u>	0.01	<u>0.67</u>	<u>0.66</u>	0.01
	62.84	63.68		73.69	74.67	
16:2	0.09	0.07	0.02	0.11	0.08	0.03
18:2	6.39	5.21	1.18	7.50	6.11	1.38
18:3	<u>0.83</u>	<u>0.64</u>	0.19	<u>0.97</u>	<u>0.75</u>	0.23
	7.31	5.91		8.58	6.94	
Total:	85.28	85.29		100	100	

^{*I*}The data for FA1 components were normalized by extending the pattern determined from 85.28% of total moles to 100%.

Table 6

Fatty alcohol (FAI) components (mole%) in wax esters from human meibum.

FAI	No correction	With both correction	Difference	No correction ^I	With both correction ^I	Difference ^I
20:0	1.13	1.13	0.00	1.32	1.32	0.00
21:0	0.83	0.81	0.02	0.98	0.95	0.03
22:0	1.88	1.79	0.09	2.20	2.10	0.10
23:0	2.24	2.15	0.08	2.62	2.53	0.10
24:0	16.51	15.80	0.71	19.35	18.52	0.83
25:0	13.08	12.88	0.20	15.34	15.11	0.23
26:0	23.19	22.49	0.70	27.19	26.37	0.82
27:0	6.57	6.60	-0.02	7.71	7.73	-0.03
28:0	3.07	2.91	0.16	3.60	3.41	0.19
29:0	0.18	0.17	0.00	0.21	0.20	0.00
30:0	0.57	0.45	0.12	0.67	0.52	0.14
32:0	0.15	0.10	0.05	0.18	0.12	0.05
	69.39	67.28	2.11	81.36	78.88	2.48
22:1	0.35	0.40	-0.05	0.40	0.47	-0.06
24:1	3.90	4.59	-0.69	4.57	5.38	-0.81
26:1	4.46	5.27	-0.80	5.23	6.17	-0.94
28:1	2.63	2.91	-0.28	3.08	3.42	-0.33
30:1	2.87	3.05	-0.18	3.37	3.58	-0.21
32:1	1.41	1.47	-0.06	1.65	1.72	-0.07
	15.62	17.69	-2.07	18.31	20.74	-2.43
24:2	0.13	0.15	-0.02	0.15	0.17	-0.02
26:2	0.15	0.18	-0.02	0.18	0.21	-0.03
	0.28	0.32	-0.04	0.33	0.38	-0.05
Total:	85.28	85.29		100.00	100.00	

^IThe data for FAI components were normalized by extending the pattern determined from 85.28% of total moles to 100%.

Table 7

Species whose determined percentage in wax esters from human meibum significantly changed after performing corrections to improve quantification reliability

Species		mole%		
		With corrections		Difference
		none	both	
WE (FA/FAI)	18:1/26:1	3.01	3.48	+0.47
	18:1/24:1	2.57	2.93	+0.36
	18:2/26:0	2.31	1.78	−0.53
	18:2/24:0	1.80	1.37	−0.43
FA ^I component	18:1	60.48	61.41	+0.93
	17:0	10.04	10.78	+0.74
	18:0	7.50	6.11	−1.38
	16:0	1.83	1.43	−0.41
FAI ^I component	26:1	5.23	6.17	+0.94
	24:1	4.57	5.38	+0.81
	24:0	19.35	18.52	−0.83
	26:0	27.19	26.37	−0.82

^IThe data for FA or FAI components were normalized by extending the pattern determined from 85.29% of total moles to 100%.

Table 8

Comparison of mass% and mole% of fatty acid (FA) and fatty alcohol (FAL) components in wax esters from human meibum.¹

FA	Mass%	Mole%	FAL	Mass%	Mole%
18:1	62.04	61.41	26:0	26.64	26.37
16:1	10.73	10.99	24:0	17.89	18.52
17:0	10.61	10.78	25:0	14.92	15.11
18:2	6.16	6.11	27:0	7.97	7.73
16:0	4.63	4.78	26:1	6.23	6.17
17:1	1.56	1.61	24:1	5.19	5.38
18:0	1.43	1.43	30:1	3.92	3.58
15:0	0.83	0.88	28:0	3.60	3.41 ²
18:3	0.74	0.75	28:1	3.59	3.42 ²
20:1	0.68	0.66	23:0	2.38	2.53
19:0	0.24	0.24	32:1	1.96	1.72 ³
14:0	0.19	0.20	22:0	1.94	2.10 ³
16:2	0.07	0.08	20:0	1.17	1.32
20:0	0.05	0.05	21:0	0.86	0.95
13:0	0.03	0.03	30:0	0.57	0.52
Total	100	100	22:1	0.43	0.47
			29:0	0.21	0.20 ⁴
			26:2	0.21	0.21 ⁴
			24:2	0.17	0.17
			32:0	0.14	0.12
			Total	100	100

¹The data for FA or FAL components have been normalized to 100% of total wax esters.
^{2,3,4}The order of the components from high to low were switched.