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Fatty acid composition of cholesteryl esters of human meibomian gland secretions

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Abstract

Very long chain cholesteryl esters (CE) are a major group of lipids found in meibomian gland secretions (MGS, also called meibum). MGS are produced by the meibomian glands of human and animal eyelids. They are a critical part of the tear film which covers the exposed ocular surface and serves various physiological roles. The composition of CE of MGS is complex, and still remains poorly understood. Here, a liquid chromatography-ion trap mass spectrometry (LC-MS) procedure developed to analyze CE is described, and a detailed composition of human meibomian CE is reported.

MGS were collected from donors, analyzed without any modifications by LC-MS in positive and negative ion modes (PIM and NIM), and quantified using lipid standards where available.

CE comprised about 30% of human meibum by mass. More than 40 individual CE species were found and characterized. In PIM, CE were observed as spontaneously in-source generated product ions m/z 369. The signals of the proton adducts of intact CE $(M+H)^+$ were of very low intensity. In NIM, all tested CE spontaneously fragmented in-source producing signals of their respective FA. By combining the LC and MS information, the most abundant CE were found to be based on FA ranging from C16 to at least C32 in the following order C26:0>C25:0>C24:0>C27:0>C24:1=C18:1=C20:0>other CE.

We conclude that the FA composition of CE can be successfully established in LC-MS experiments conducted in NIM. Meibomian CE have a large presence of both saturated and unsaturated FA with an average molar ratio of 4 to 1, respectively.

Keywords

Fatty acids; Very long chain cholesteryl esters; Human meibum; Meibomian secretions; High performance liquid chromatography; Mass spectrometry

1. Introduction

Meibomian gland secretions (MGS, also known as *meibum* since 1981 [1]) are produced by a variety of sebaceous glands called Meibomian glands [2] that are located in the eyelids of humans and animals. Once excreted from the glands and delivered to the lid margin, MGS

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are taken up onto the surface of the aqueous tear film (TF) in the upstroke of each blink. TF itself originates from lachrymal glands. MGS form an outermost part of TF, usually called tear film lipid layer (TFLL) which retards evaporative water loss from the eye [3]. The chemical composition of MGS is extremely complex and the overall number of individual lipid species is easily in the hundreds, if not thousands [4]. The role of these lipids in TFLL is poorly understood, and their exact qualitative and quantitative compositions are yet to be determined. However, chemical changes in MGS have been found to be associated with the onset of *dry eye disease* in humans, and have been linked to the diminished stability of TF and TFLL [5–8].

To elucidate the correlation between the changes in meibum and the instability of TF, one needs to characterize the major lipid species present in meibum and TF, both qualitatively and quantitatively. With the exception of one report [9], there is a general consensus that CE are always present in meibum [5–8], and comprise, on average, one-third of the overall meibomian lipid pool [5]. However, in earlier reports human WE and CE were analyzed together by gas chromatography after they had been hydrolysed and transesterified [9]. These procedures are known to cause inadvertent isomerization, cyclization, and decomposition of long chain FA, especially those of polyunsaturated nature [10–12], which is probably why the higher molecular weight unsaturated FA were not reported even in recent publications [13,14].

We, on the other hand, were able to detect CE species with FA components ranging from C16 to C32 in high pressure liquid chromatography-ion trap mass spectrometry experiments (HPLC-MSⁿ) conducted in the positive ion mode (PIM) [15]. The peculiarity of the proposed method was a low intensity of CE signals caused by the spontaneous fragmentation of CE in the ion source of the mass spectrometric detector through the neutral loss of their FA moieties. Ironically, but fortunately, the very same spontaneous fragmentation of the CE in a PIM MS experiment produced a very convenient and universal analytical ion for detection of all CE as a lipid class – ion m/z 369 – which is an ion of dehydrated cholesterol ($M - H_2O + H$)⁺. As the FA generated in this reaction were not visible under the conditions of our PIM experiments [15], we speculated that they might be detected as *anions* in a negative ion mode (NIM) experiment. No conclusions about the effects of gender, age, hormonal status etc were to be made: this study is concerned with the development of a HPLC-MS procedure suitable for evaluation and quantitation of CE in complex extracts of biological origin, and presents for the first time data on the FA composition of normal, non-dry eye human meibomian CE.

2. Experimental

2.1. Materials and equipment

Lipid standards were purchased from Nu-Chek Prep, Inc. (Elysian, MN) and from Sigma Chemical Co. (St. Louis, MO). HPLC grade solvents from Honeywell Burdick & Jackson and Mallinckrodt Baker, Inc. were used throughout. A Waters Alliance 2695 HPLC Separations Module (Waters Corp., Milford, MA) was interfaced to an LCQ Deca XP Max ion trap mass spectrometer manufactured by ThermoElectron (San Jose, CA) equipped with an atmospheric pressure chemical ionization (APCI) ion source. The mass spectrometer was operated under an Xcalibur software package v. 1.4 from ThermoElectron. A reversed-phase Hypersil Gold HPLC column (2.1 × 150mm, 5μm) was purchased from ThermoElectron.

2.2. Sample collection and preparation

Samples of MGS were collected from five healthy, non-dry eye human donors without signs of any ocular surface or eyelid pathology. A previously described protocol of meibum collection using a gentle soft-squeezing technique and a platinum spatula was used [16,17].

The procedures were approved by the Institutional Review Board of the University of Texas Southwestern Medical Center in Dallas and performed in accordance with the principles of the Declaration of Helsinki. Four males and one female (39 ± 5 years old) donated 0.47 ± 0.05 mg of meibum each. Briefly, eyelids were cleansed with a cotton swab and then human meibum was expressed from Meibomian glands of the lower eyelids of a donor. Being expressed as transparent oil, meibum immediately assumed a waxy texture upon contact with the metal spatula at room temperature. The combined samples from the left and the right eyelids were dissolved in about 1 mL of hexane:propan-2-ol (1:1, v/v) solvent mixture (HP) placed in a HPLC vial pre-weighed on an analytical balance. The solvent then was evaporated to dryness and the vial was re-weighed to determine the weight of the collected sample. The average dry sample weight was about 0.5 mg. The samples were kept in a dry state under nitrogen at -70°C and were reconstituted in a HPLC mobile phase (see below) before the experiments. No sample degradation was observed during at least six months of storage.

2.3. HPLC-MSⁿ experiments

Both authentic CE standards and biogenic CE of MGS were chromatographically separated using a hexane:propan-2-ol:aqueous ammonium formate gradient solvent system as described in our earlier paper [15], and in a similar solvent system where the ammonium formate solution was replaced with 0.1% aqueous acetic acid. A dry sample of MGS was reconstituted in the HP solvent mixture to make a sample stock solution of known concentration (depending on the application, between 0.1 and 1 mg/mL), and between 0.5 and 7 μL of the sample stock solution were injected onto the HPLC column. The effluent was continuously monitored mass spectrometrically in either PIM or NIM. Three types of experiments were conducted.

First, the mass spectrometer was set to scan the ions in the range of m/z 150 to 2,000 in PIM and to store the collected spectra. This produced a total ion chromatogram (TIC) which was used post-experimentum to evaluate the overall lipid composition of the sample. The stored spectra were then used to extract signals of the ions of interest and plot them as extracted ion chromatograms (EIC).

Second, the mass spectrometer was tuned to monitor a specific fragment ion (in the case of CE, ion m/z 369 [15,16]) in PIM to produce single ion monitoring (SIM)-style chromatograms of all CE present in the sample. This effectively increased the sensitivity of the analysis and minimized background noise thus producing better defined HPLC peaks.

Third, the samples were analyzed in NIM in the range of m/z 200 to 550. This produced TIC chromatograms, from which signals of particular ions could be extracted and plotted as EIC. An equimolar mixture of six authentic CE, with FA components ranging from C_{11} to C_{24} , plus free cholesterol (Chl) was used to create calibration curves suitable for quantitation of CE in MGS. This mixture was analyzed as described above for MGS.

2.4. HPLC-MSⁿ data analysis

Theoretical m/z values for FA ranging from $\text{C}_{12:n}$ to $\text{C}_{36:n}$ ($n = 0-6$) and their possible adducts with various anions (such as formate, acetate, chloride, etc) were computed using the ChemBioDraw Ultra v. 12.0 software package (CambridgeSoft, Cambridge, MA). Then, TIC chromatograms of authentic CE and MGS were analyzed for the presence of FA anions and FA adducts and detected signals were plotted as EIC. The resulting HPLC peaks of detected ions were integrated using the Avalon built-in routine of the Xcalibur software. Chemical noise was subtracted from the mass spectra of the analytes as described earlier

[16]. The theoretical mass spectra of CE and their isotopic ratios were computed using the Xcaliber's isotope simulation routine.

3. Results

When analyzed by RP HPLC-MSⁿ using the acetic-acid based gradient solvent system, an equimolar mixture of standard CE was effectively separated according to the overall hydrophobicity of CE species. Corroborating our earlier report, Chl eluted first, followed by CE in the order of their increasing hydrophobicity: C_{11:0}-CE, C_{16:1}-CE, C_{18:1}-CE, C_{18:0}-CE, C_{22:1}-CE, and, finally, C_{24:1}-CE (Fig. 1A). Note the almost identical peak areas of EIC of ion *m/z* 369 for every CE tested. The peak area *ratios* remained constant and close to 1:1:1:1:1 regardless of the amount of the injected sample. When the same mixture was analyzed in NIM, a different TIC pattern was observed (Fig. 1B). The TIC peaks did have the same retention times (RT) as those detected in PIM (with a notable exception of Chl, which was not visible in NIM), but their intensities steadily rose from peak RT 8.1 min to peak RT 16.4 min. The MS spectra of all the peaks are shown in Fig. 2A. One can easily see that they were the spectra of FA adducts with acetic acid. When plotted as EIC, they produced a pattern of HPLC peaks whose RT differed by 1.5 to 2 min (Fig. 2B). Obviously, the FA adducts were generated *in situ* from CE in the ion source of the mass spectrometer due to spontaneous fragmentation of CE molecules. By comparing the RT of ion *m/z* 369 in PIM with the RT of FA adducts in NIM, one can easily deduce the nature of the intact CE molecules (Scheme 1). The same HPLC and MS patterns were observed in the eluent with ammonium formate, though the RT of the HPLC peaks were slightly different, and the observed mass spectra were those of FA adducts with formate instead of acetate (see below). Importantly, the relative abundance of these adducts also increased with the increase in the molecular weight of CE (Fig. 3). Thus, we computed a correction factor for our calibration curves which took into account this tendency of FA adducts to produce more and more intense MS signals with the increasing length of their FA moieties. For our testing conditions and compounds, the dependence of the intensity of the MS signal (*I*_{MS}) on the FA length (*C*_N) is given by the following Equation 1:

$$I_{MS} = 0.68 \times C_N - 6.75 \quad (R^2 = 0.910) \quad \text{Equation 1}$$

As the impact of (*m/z* + 2) isotopic peaks on the main *m/z* signal of a FA adduct was found to be about 2%, inclusion of such a correction in Equation 1 was deemed unnecessary. Importantly, the degree of unsaturation of homologous pairs of FA of general formulas C_nH_{2n}O₂ and C_nH_{2n-2}O₂ had very little effect on the relative intensity of their MS signals (Figs. 3 and 4), and the observed small differences were related to the random weighing errors while making the lipid solutions.

Then, human MGS were tested. In PIM IEC experiments, human meibum samples produced a familiar pattern (Fig. 5, upper trace) indistinguishable from one reported earlier [15]. More than 20 HPLC peaks that corresponded to different CE species were detected. Note that some individual species of CE could co-elute as they might have identical overall hydrophobicity [15]. A representative chromatogram of the same MGS sample recorded in NIM produced a complex pattern (Fig. 5, lower trace) from which one can draw two conclusions: 1) a large number of CE species present in meibum were of higher molecular weight than the longest tested standard compound, C₂₄-CE (Figs. 1 and 4), and 2) the observed HPLC peaks had reassuringly similar patterns in both PIM and NIM experiments. Indeed, the RT of C_{24:1}-CE and C_{24:0}-CE standards were about 17.3 and 19.5 min, respectively (Figs. 2 and 4), while the major HPLC peaks of meibomian CE had RT of 19.3 and 21.2 min with clearly seen species with even longer RT of 22–25 min (Fig. 5).

The integrated NIM mass spectra of two samples of meibomian CE recorded as acetic acid (Panel A) and formic acid (Panel B) adducts are shown in Fig. 6. Signals of more than 20 major FA species derived from human CE were detected, with another 20 or so minor ones. The FA species ranged from C₁₈ to C₃₂. The observed CE FA produced the following MS pattern: C_{26:0}>C_{25:0}>C_{24:0}>C_{27:0}>C_{24:1}≈C_{18:1}>rest of CE. As the degree of unsaturation of homologous FA had negligible effects on the relative intensities of the MS signals of authentic CE (Figs. 3 and 4), and the relative intensities of their MS signals rose almost linearly with the increase in lengths of their FA chains (Fig. 3), their *molar* ratio in human MGS were estimated by comparing the intensities of the signals of the corresponding homologs as shown in Fig. 7. The upper graph (Panel A) shows the IEC peak areas of the corresponding compounds as measured by HPLC-MS (as shown in Fig. 4). The *lower graph* represents the same data after the peak areas had been corrected using Equation 1 and normalized against endogenous C_{18:1}-CE present in every meibomian sample tested. For compounds C_{24:1}-CE (fragment ion *m/z* 411, RT 16.9 min) and C_{24:0}-CE (fragment ion *m/z* 413, RT 19 min), for example, the molar ratio was calculated to be about 1:3. Thus, in this particular sample of normal human meibum, there were three molecules of C_{24:0}-CE and one molecule of C_{24:1}-CE per one molecule of C_{18:1}-CE (fragment ion *m/z* 327, RT 11.8 min) detected. No C_{18:0}-CE (fragment ion *m/z* 329, projected RT 13.8 min) was observed above the noise level. After correction for the chain length effect, four very long chain FA still clearly dominated the CE pool of normal human meibum: C_{24:0}, C_{25:0}, C_{26:0}, and C_{27:0}, with the most abundant unsaturated FA being C_{18:1} and C_{24:1} (Fig. 7, Panel B).

Five tested human MGS samples produced qualitatively similar HPLC-MSⁿ patterns (Fig. 8), corroborating our earlier observations on the quite conservative nature of normal human meibum, at least with regard to its WE and (O-acyl)-omega-hydroxy-fatty acids [17]. However, we noticed some variations in the molar ratios of some of the CE. Specifically, C_{18:1}-CE and C_{20:1}-CE changed from sample to sample more than the other compounds. Studies are in progress to answer questions with regard to the nature of this variability.

Finally, we tested meibomian samples for the presence of CE with double unsaturated FA, C_{n:2}-CE. Corroborating our preceding paper, only minor amounts of those were detected (not shown). Typically, they represented less than one molecule of C_{n:2}-CE per 30 to 200 molecules of the corresponding saturated C_{n:0}-CE, depending on the compound and the sample. Even more unsaturated compounds of the C_{n:3}-CE to C_{n:6}-CE family were not found by this method. Future studies will show whether any of these highly unsaturated CE are present in human meibum or not.

4. Discussion

For decades, the only viable way of analyzing the FA composition of CE was GC in combination with either a flame ionization detector (FID) or a MS detector (MSD). Both methods required the prior hydrolysis and transesterification of CE to convert them into volatile derivatives suitable for GC. Both methods have some limitations. The GC-FID approach provides information on the RT of the analytes, but tells nothing about their molecular weights or structures. The only parameter that can be used for identification purposes in such experiments is the RT of the analyte. Thus, this technique requires the use of authentic lipid standards to provide the reference points for the identification of unknowns in the biological sample. In case of a compound that does not have an authentic standard, its identification relied heavily on the procedures of extrapolating and interpolating the RTs using a parameter called equivalent chain length (ECL) introduced by Miwa et al. [18,19]. In many cases, this approach worked quite well. However, in other cases its limitations started to show when the analytes became more complex than simple saturated fatty acids of moderate chain lengths [20]. Indeed, addition of *iso* or *anteiso* branched-chain

compounds to the mix greatly complicates the interpretation of chromatograms of the FA mixtures as the RTs of various branched FA follow their own patterns which may overlap with the pattern for straight chain FA. Adding double bonds to the structures of FA adds another level of complexity as not only does it introduce another unknown – the effect of the double bond on the RT – but it also dramatically diminishes the stability of FA during a GC experiment. Long exposure of the analytes to high temperatures during a GC experiment causes them to decompose, and the longer and the more unsaturated the FA chain, the more severe the FA decomposition [10,12]. This is a very likely explanation of why only five species of meibomian CE were detected by LC-FID with their ECL numbers ranging from 19.1 to only 23.2[20], leaving higher molecular weight, higher boiling, and less stable C₂₄- to C₃₂-CE undetected.

To partly overcome these problems with traditional GC-FID, one can use a MSD. This approach is far superior to FID as in many cases it enables a researcher to determine the molecular masses of the analytes, and to study their fragmentation patterns. The latter are vital for the correct structure elucidation of unknowns and were used in human meibomian lipids studies [13,14]. However, GC-MS has the same limitations as GC-FID in terms of the analyte's stability during the analysis. This, apparently, was a factor in earlier studies [13,14] on human MGS, where no human meibomian very long chain unsaturated FA were reported. Considering the high temperature (up to 225°C in the column and 250°C in the injector) and the overall duration of the analysis (more than an hour) sample decomposition and/or isomerization could have been a factor.

The last, but not least, major limitation of GC methods is that they are not capable of analyzing native, underivatized lipid molecules because most of them are not volatile, cannot be eluted from the GC column under any conditions, and quickly decompose in the column at high temperatures. Thus, lipids are typically hydrolyzed prior the GC analysis to their building components such as FA, fatty alcohols, sterols, glycerol etc, and transesterified to make these fragments volatile. By doing so, the structures of the original complex lipid molecules become scrambled, and the information on the original structures is irreversibly lost. To partly overcome this limitation, one can separate the lipid into classes before the GC analysis, but in many cases it was deemed to be impracticable, especially with samples as small as human MGS. Notoriously difficult to separate from each other are WE and CE. Therefore, even in the latest studies using GC-MS only the combined figures for their FA compositions were reported [13,14].

Unlike GC-FID and GC-MSD, separation of analytes in HPLC-MSⁿ experiments is conducted at much lower temperatures, typically between 20 and 50°C. We routinely use the column temperatures not higher than the bodily 35°C [15,16]. This temperature is safe for the lipids and does not cause their isomerization or decomposition. Also, HPLC-MSⁿ analysis does not require prior manipulations with the samples. The nature of human meibum, which typically is an almost pure lipid with very little protein material in it allows the samples, dissolved in a proper organic solvent (chloroform:methanol or hexane:propan-2-ol mixtures, for example), to be analyzed without any preparations, i.e. “as is”. This also minimizes the chances of losing minor components as the collected sample is immediately transferred directly into the organic solvent and stored in a freezer.

Our previous PIM HPLC-MSⁿ results, obtained by monitoring analytical ion m/z 369, clearly demonstrated that CE of human meibum are very heterogeneous and range from C₁₈ to C₃₂ [15]. Yet, in several cases we could not determine the ratios of saturated to unsaturated CE because certain saturated and unsaturated CE co-eluted producing one combined HPLC peak of two species (e.g. pairs C_{24:0}-CE and C_{26:1}-CE, C_{26:0}-CE and C_{28:1}-CE, etc [15]), and the intensities of their molecular ions were too low for their

accurate quantitation. By performing the NIM experiments described in the current manuscript this limitation has been overcome and those previously unresolved pairs of CE became clearly visible as acetate or formate adducts of their FA fragments (Figs. 2–4, and 6). The spontaneous loss of its FA residue by a CE made it detectable in a NIM experiment, which in combination with a separate PIM experiment made it possible to reconstruct the starting CE. Some MS instruments are capable of operating in alternating polarity mode with a sub-second frequency sufficient to gather the same information in one experiment instead of two. Such more capable instruments are destined to provide even more accurate information in shorter periods of time with less sample material consumed.

After calculating the relative molar contributions of the 20 or so major CE described in this paper, the overall molar ratio of very long chain saturated to unsaturated CE was found to be 4 to 1 (Fig. 8), i.e. there were 4 molecules of $C_{n:0}$ -CE per 1 molecule of $C_{n:1}$ -CE detected. This ratio slightly changed on a sample-to-sample basis, and may also slightly change if one takes into account the even smaller fractions of $C_{n:2}$ - and $C_{n:3}$ -CE found in human meibum [15]. However, considering the low abundance of the latter compounds (around 1 molecule of $C_{n:2}$ -CE per 30 molecules of $C_{n:0}$ -CE, or less), the differences ought to be minor. For this first study on the FA composition of human very long chain CE, a limited number of donors (four males and one female) were recruited. Thus, no conclusions about the possible effects of gender, age, hormonal status etc. were to be drawn and were left for upcoming studies. However, our main goal – to develop an HPLC-MS procedure suitable for evaluation and quantitation of CE in complex extracts of biological origin such as meibum – has been achieved. The exact physiological role of CE in meibum is not known. However, a proper balance between the lower-melting unsaturated CE and their higher-melting saturated analogues might be important for keeping the melting point (or range) of meibum within physiologically acceptable limits.

In conclusion, reverse phase HPLC in combination with NIM APCI MS has proven to be an effective tool for structural characterization and quantification of CE in general, and meibomian CE in particular. Meibomian CE analyzed in this study were demonstrated to be based mostly on very long chain saturated FA with the total saturated to total unsaturated CE ratio being about 4:1. The major CE ranged in the following order of their molar ratios in normal meibum samples $C_{26:0} > C_{25:0} > C_{24:0} > C_{27:0} > C_{24:1} \approx C_{18:1} \approx C_{20:0} > \text{rest of CE}$.

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Figure 1. Extracted ion chromatograms of a mixture of cholesterol and of six authentic cholesteryl ester standards

Panel A. The HPLC trace of ion m/z 369 recorded in the positive ion mode. Standard lipids eluted in the following order: cholesterol (RT 4.3 min), $C_{11:0}$ -CE (8.0 min), $C_{16:1}$ -CE (9.8 min), $C_{18:1}$ -CE (11.3 min), $C_{18:0}$ -CE (13.2 min), $C_{22:1}$ -CE (14.7 min), and $C_{24:1}$ -CE (16.5 min). Note the almost identical peak areas for all tested CE. Injected: 5 μ L of a stock solution of 100 μ M (each CE). Acetic acid-based HPLC solvent mixture was used.

Panel B. The HPLC trace of ions m/z 200–450 recorded in the negative ion mode. The same lipid mixture as in *Panel A*.

Figure 2. Mass spectra and chromatograms of authentic cholesteryl esters

Panel A. Mass spectra of the HPLC peaks depicted in Fig. 1, Panel B. The major MS peaks m/z 245, 313, 341, 343, 397, and 425 were identified as acetic acid adducts of $C_{11:0}$, $C_{16:1}$, $C_{18:1}$, $C_{18:0}$, $C_{22:1}$, and $C_{24:1}$ fatty acids derived from the corresponding CE.

Panel B. Total ion chromatogram and the extracted ion chromatograms of individual major ions m/z 245 to 425

Figure 3. The integrated mass spectrum of ions m/z 245 to 425

Note that: 1) the equimolar mixture of six CE produced the signals with rising intensities; 2) the signals of saturated and unsaturated CE ($C_{18:0}$ -CE and $C_{18:1}$ -CE) were very close.

Figure 4. Extracted ion chromatograms of characteristic signals of four saturated and unsaturated CE and their corresponding mass spectra

Upper panel. Extracted ion chromatograms of an equimolar mixture of C₁₈:1-CE, C₁₈:0-CE, C₂₄:1-CE, and C₂₄:0-CE. The retention times (RT) of the peaks, and their areas (AA) are shown next to each peak. Note that the detector response for the pair of C₂₄-based CE is much higher than that for the C₁₈-based CE.

Lower panel. Mass spectra of the corresponding C₁₈- and C₂₄-CE pairs. Note that the intensities of the signals of saturated and unsaturated CE were almost identical.

Figure 5. Extracted ion chromatograms of a sample of human meibum

Upper trace. Ion m/z 369 was extracted from a positive ion mode chromatogram.

Lower trace. Ions m/z 250–550 were extracted from a negative ion mode chromatogram of the same sample.

Figure 6. Combined mass spectra of human meibum recorded in the negative ion mode HPLC-MS experiment

Panel A. Acetic acid-based HPLC solvent produced acetic acid adducts of meibomianlipids

Panel B. Formic acid-based HPLC solvent produced formic acid adducts of meibomian lipids

Figure 7. The fatty acid distribution within the class of cholesteryl esters detected in human meibum of a 32-years old healthy female

Panel A. Uncorrected signals of fatty acids as recorded by mass spectrometer in the HPLC-MS experiment in the negative ion mode.

Panel B. The same data as in Panel A after the correction of the effect of the fatty acid chain length using Equation 1. The results are plotted as molar ratios.

Figure 8. The average fatty acid composition of cholesteryl esters detected in human meibum
Five samples were analyzed three times each. The sum of all depicted C₁₈ to C₃₂ saturated and unsaturated meibomian compounds was assumed to be one. The results are presented as mean $\pm$ standard deviation.

Panel A. Unsaturated fatty acids.

Panel B. Saturated fatty acids.

Scheme 1. Fragmentation patterns of cholesteryl esters in negative and positive ion modes