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Tear Film Lipids

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Abstract

Human meibomian gland secretions (MGS, or meibum) are formed from a complex mixture of lipids of different classes such as wax esters, cholesteryl esters, (O-acyl)- α -hydroxy fatty acids (OAHFA) and their esters, acylglycerols, diacylated diols, free fatty acids, cholesterol, and a smaller amount of other polar and nonpolar lipids, whose chemical nature and the very presence in MGS have been a matter of intense debates. The purpose of this review is to discuss recent results that were obtained using different experimental techniques, estimate limitations of their usability, and discuss their biochemical, biophysical, and physiological implications. To create a lipid map of MGS and tears, the results obtained in the author's laboratory were integrated with available information on chemical composition of MGS and tears. The most informative approaches that are available today to researchers, such as HPLC-MS, GC-MS, and proton NMR, are discussed in details. A map of the meibomian lipidome (as it is seen in reverse phase liquid chromatography/mass spectrometry experiments) is presented. Directions of future efforts in the area are outlined.

I. INTRODUCTION

Meibomian glands (or tarsal glands) are holocrine glands that populate the upper and the lower eyelids of humans and most animals. The glands were discovered and described in 1666 by a German physician/scientist Heinrich Meibom (Meibom, 1666). The glands are situated in the inner part of the tarsal plates of the eyelids. On average, there are about 31 meibomian glands found in the upper eyelids, and 26 – in the lower ones (Knop and Knop, 2009). The glands produce an oily, lipid -enriched secretion, often called *meibum* (Nicolaidis et al., 1981), which is excreted onto the ocular surface through orifices located at the eyelid's rim, next to the mucocutaneous junction. They are believed to excrete meibum onto the posterior lid margin either spontaneously, or upon blinking. An average amount of meibum stored in the meibomian glands is in the range of several hundred micrograms per eyelid (Bron et al., 2004). Once excreted, meibum mixes with aqueous tears that are produced by another type of ocular glands – lachrymal glands – to form the tear film. The relatively thin and dynamic tear film [whose depth was estimated to be 3.5 ± 0.8 μm (Kimball et al., 2010)] covers the entire ocular surface, though a large portion of aqueous tears is stored in the tear meniscus.

Aqueous tears, being a relatively hydrophilic environment, tend to separate from generally hydrophobic meibum. Due to their poor miscibility and because of the lower density of the latter, meibum tends to form the upper, outermost part of the tear film, called the tear film lipid layer (TFLL) (Figure 1), though some lipids may bind with proteins that reside either

in the bulk of the aqueous subphase, or are associated with the TFL (Glasgow et al., 2010; Miano et al., 2005; Saaren-Seppala et al., 2005). A mean thickness of the TFL was recently reported to be about 42 nm, with a range of 15 to 157 nm (King-Smith et al., 2010), though in an earlier report an average thickness of TFL in normal (i.e. non-dry eye) controls was measured to be 90–100 nm (Suzuki et al., 2006). Obviously, the thickness of this layer is orders of magnitude greater than the sizes of most typical lipid molecules, or even proteins, and hints at a complex, multilayered structure of the TFL. Because of uneven delivery of meibum over time, reflectory blinking, the drainage of the tears through the nasal ducts, and because of the overall thermodynamic instability of the oil/water mixtures, thickness of TFL may change over time.

The tear film is considered a vital structure whose main roles are to protect the ocular surface from desiccating caused by the tear film evaporation (King-Smith et al., 2009) and bacterial infections (Garreis et al., 2011), among others. The quality of the tear film was shown to be affecting the visual acuity (Kaido et al., 2012; Rolando et al., 1997), while artificial tears were reported to improve vision of dry eye patients (Ridder et al., 2005).

In certain ocular pathologies, such as dry eye syndrome (DES) and Sjogren syndrome, the tear film stability is severely diminished (Hong et al., 2013; Wakamatsu et al., 2013). Not a small part in it may be played by some unwelcome changes in the TFL (Hong et al., 2013; Rolando et al., 2008). Both thinning (Hosaka et al., 2011) and thickening (Hong et al., 2013) of the TFL were reported in conjunction with shortening the tear film breakup times (TFBUT). Experiments of Olson et al. (Olson et al., 2003) demonstrated that an increase in meibum delivery onto the ocular surface led to a concomitant increase in the TFL thickness, while Craig and Tomlinson (Craig and Tomlinson, 1997) reported that thicker TFL retarded evaporation from the tear film surface more effectively than thin or ruptured ones, and were more stable. Interestingly and controversially, Suzuki et al. (Suzuki et al., 2006) came to a conclusion that a thicker TFL in their cohort of allergic conjunctivitis patients led to a statistically significant decrease in the values of TFBUT. A plausible explanation of this controversy is that the *quality* of meibum and/or the tear film in those two different cohorts of subjects differed dramatically, which could be reflective of some major differences in their respective chemical compositions of meibum and the tear film. Therefore, a need for comprehensive knowledge of the chemical compositions of meibum and the tear film, and better understanding of what differentiates normal meibum from a pathological one are needed.

In this review, only recent developments in the area of human tear film and TFL studies will be discussed. Earlier reviews on the topic [(Bron et al., 2004; Bron and Tiffany, 1998; Butovich, 2009c, 2011a; Ohashi et al., 2006; Tiffany, 2008) and special issues of *Ocular Surface* and *IOVS* dedicated to the meibomian glands and the tear film in relation to the dry eye disease (2007; Green-Church et al., 2011) have extensively covered most of the preceding reports, and are strongly suggested to be considered alongside with this paper.

However, it is impossible not to mention the major contributions of scientists like Pes (Pes, 1897), Linton (Linton, 1961), Ehlers (Ehlers, 1965), Andrews (Andrews, 1973), Cory (Cory et al., 1973), Nicolaides (Nicolaides et al., 1981; Nicolaides and Santos, 1985), Tiffany (Tiffany, 1987) and many others that laid a solid foundation for future studies in the area, while discussing recent meibomian glands and tear film studies.

Despite those earlier efforts, major advances have been made in the area of qualitative and quantitative analyses of meibum, aqueous tears, and the tear film during the course of a last decade. Making this progress possible, and differentiating it from the past achievements, is, first and foremost, a new technological base that has provided researchers with analytical

capabilities unimaginable in the earlier years. New analytical methods, instrumentation, that boosts high sensitivity and selectivity, and a rapid progress in molecular biological techniques have made it possible to look into the details of the biochemistry, biophysics, and physiology of the meibomian and lachrymal glands, in an attempt to draw a comprehensive picture of the normal ocular surface, and visualize its changes upon onset of various ocular pathologies.

II. RECENT ADVANCES IN CHEMICAL ANALYSES OF MEIBUM AND TEAR FILM LIPIDS

1. GENERAL COMMENTS

1.1. METHODOLOGICAL ASPECTS—The chemical analyses of meibum performed over the period of a few decades provided a great deal of information on its chemical composition. It has been recognized that meibum is formed largely from nonpolar lipids, a smaller and, apparently, variable amount of amphiphilic (or polar) lipids, and undefined amounts of other types of molecules typically found in organisms, such as proteins, peptides, inorganic salts, etc.

By and large, technological limitations of the earlier analytical protocols precluded researchers from analyzing intact lipid molecules. Even the then-best analytical technique suitable for what we are now calling “lipidomics of meibomian gland secretions” – gas (liquid) chromatography in combination with mass spectrometry (GC/MS) – was not very effective in human meibum studies because of a mediocre sensitivity of the MS detectors, poor performance of GC columns at high temperatures, and, most importantly, insufficient volatility and stability of the most interesting and important analytes – intact lipids – in the conditions of a GC/experiment. Notably, meibum samples collected from individual donors could be analyzed by GC/MS (Shine and McCulley, 1996) or GC in combination with flame ionization detectors (GC/FID) (Dougherty and McCulley, 1986), no information is available to the author about attempts to characterize the lipid content of individual samples of human tears. Therefore, in the vast majority of studies the meibomian lipids were pooled and processed (i.e. hydrolyzed and/or transesterified) before the GC/MS or GC/FID analyses, which made the complete characterization of the material very cumbersome and all but impossible because of scrambling the initial structures (for a detailed discussion of this problem see our earlier review (Butovich, 2009c). A standout paper of McFadden et al. (McFadden et al., 1979), who, apparently, were the first to employ another, much more suitable for lipidomic analysis, approach – high performance lipid chromatography in combination with MS (HPLC/MS) – did not spark the interest of other researchers, and remained an isolated effort until early 2000-s when Sullivan et al. revitalized the efforts (Sullivan et al., 2000). Since then, HPLC/MS has become a popular technique with many distinctive advantages and a few weaknesses, some of which will be briefly discussed below. Note that other detection techniques (such as spectrophotometric and evaporative light scattering detectors) are available for HPLC studies, but are not recommended for experiments with intact lipids because of their generally poorer sensitivity and almost nonexistent selectivity (Butovich, 2009c, 2011a). Therefore, they will not be discussed in this review.

The number of contemporary analytical techniques available to researchers is staggering, and their discussion goes beyond the scope of this manuscript. However, it is important to note that one of the most informative, sensitive, and fast analytical techniques available today is atmospheric pressure ionization MS (API MS). Importantly, this technique is suitable for high throughput analyses of samples similar to meibum and tears. The general principle of the method is that a molecule of an analyte (MH) is converted in an ion by

either subtracting or adding another ion from M. For example, when a proton is subtracted from an electroneutral molecule M, the latter is converted into a charged particle usually presented as anion $(M - H)^-$. Alternatively, by adding a proton, M can be converted into a cation $(M + H)^+$. Instead of protons, other cations such as Li^+ , Na^+ , K^+ , and NH_4^+ can be added to the analytes to form $(M + Li)^+$, $(M + Na)^+$, $(M + K)^+$ or $(M + NH_4)^+$ charged complexes, while anions of MH can be made through a reaction of adducts formation by supplying an excess of Cl^- , Br^- , formate or acetate ions, to name a few. No matter the mechanism, the resulting ions are characterized by their *mass-to-charge ratio*, or *m/z*, which can be used to identify the analytes in complex mixtures. Other relevant techniques will be briefly explained and discussed later on in this review.

In this review we will mostly concentrate on papers that were published in the last few years, primarily because of a fast transition from earlier laborious (but absolutely necessary at that stage of meibum studies and wonderfully informative) approaches, to newer platforms such as HPLC/MS and alike.

The main strength of HPLC/MS (Figure 2), and its newer and much faster sibling ultra-high performance liquid chromatography-mass spectrometry, UPLC/MS), is their ability to spatially separate intact, underivatized analytes in mild conditions so that they reach the MS detector at different (and quite reproducible from experiment to experiment) times (called retention times, or RT). This 1) allows an experimenter to group the analytes according to their structures and/or physicochemical properties; 2) makes it possible to match the RT of an analyte with that of its synthetic standard or analog (if available and/or used); 3) separates analytes from chemical impurities present in the samples (if proper conditions of the run are chosen); 4) clearly identifies MS signals derived from the solvent, and the chemical and electronic noises produced by the MS detector itself (e.g. as a result of its inevitable contamination); 5) facilitates their accurate quantitation by minimizing the interference of analytes in the detector. The latter is a common cause of an undesirable effect called “ion suppression” (Rojo et al., 2012) which can inhibit signals of some compounds, thus making their accurate detection or quantitation all but impossible. Ion suppression of one group of analytes by the others is a common problem in mass spectrometry, especially in ESI (Jessome and Volmer, 2006) and direct infusion experiments (Yamada et al., 2013) in which all analytes are delivered to the MS detector at the same time (see below). Moreover, using customizable autoinjectors makes HPLC/MS and GC/MS suitable for unattended runs of multiple samples.

There is no surprise that HPLC, UPLC, GC, HPLC/MS and GC/MS in their multiple incarnations are the preferred *quantitative* bioanalytical approaches recommended by the Food and Drug Administration, Environmental Protection Agency, Center for Veterinary Medicine, and other US agencies, for pharmaceutical and biotechnological industries (2011; Biopharmaceutics et al., 2001). There is no reason to think that the same recommendations should not be followed in an academic laboratory. The main weaknesses of HPLC/MS is the length of the experiment, which typically takes between 10 min and an hour, or even longer. Fortunately, recent advances in the UPLC technology made it possible to achieve comparable resolution in a much shorter time, typically within a few minutes, with the added benefit of higher sensitivity because of the reduced molecular in-column diffusion and reduced zone-broadening.

However, other MS approaches do offer some distinctive advantages when quantitation is not the focus of the research, and when the mixtures are formed of a relatively small number of components. Two simpler and, sometimes, faster common approaches are called “direct injection” and “direct infusion” of the samples (Figure 3).

In a direct injection experiment, a constant flow of a solvent is maintained by a liquid pump and is pushed through an injector into an MS detector. This creates a flat baseline whose mass spectrum clearly shows the ions originating from the solvent. Then, an aliquot of a sample dissolved in a proper solvent is injected into the flow using the (auto)injector, and is carried by the flow into the ion source of the detector, where the solvent is vaporized, the analytes ionize and then are detected by the MS detector. This approach offers some advantages over HPLC/MS. First of all, direct injection experiments are considerably faster than most of the HPLC-based methods, as 1) there is no chromatographic step involved (the latter may take an hour or more to complete, though a newer approach – UPLC – reduces this runtime to a few minutes), and 2) autoinjectors allow for an unattended use of the instruments. Also, the direct injection approach makes isolating the solvent signals and the chemical and electronic noises an easy task as all the analytes in the injected sample are eluted as just one narrow peak separated by the baseline signals, which can be easily subtracted from the signals of the sample (Figure 4). However, the ion suppression remains a major problem as all of the analytes enter the detector concurrently. Moreover, no information on RT is gained during the experiment, thus rendering preliminary classification of the analytes impossible, which complicates distinguishing between similar compounds such as positional and structural isomers with the same elemental (C, H, N, O, P, etc.) composition, but geometrically different structures [so called *isobaric compounds*, Figure 5; see also Figure 7 in (Butovich et al., 2012a)]. Thus, exact quantitation of analytes in complex mixtures could be very challenging, and qualitative evaluation of the samples may produce ambiguous results because of the possibility of ion suppression. Structural analysis of individual lipids will be hampered, too, as there will be no simple way of differentiating between two or more different compounds with the same elemental composition.

Direct infusion is an approach when a sample is dissolved in a proper solvent and is infused into the ion source of a mass spectrometer with a help of a syringe pump (Figure 3). This creates a constant flow of the sample solution with a constant concentration of all the analytes from the beginning of the experiment to its end.

The main advantage of this approach is its simplicity and relative economy: typically, mass spectrometric detectors come equipped with a built-in syringe pump which is used to calibrate and tune the instrument. Thus, one may skip the costs of acquiring an HPLC or UPLC system. Another distinctive advantage of this style of analyses is that one can accumulate (i.e. amplify) signals of minor compounds for their structural elucidation in MS/MS or MSⁿ fragmentation experiments: the fast transient peaks of minor compounds in HPLC/UPLC experiments, sometimes, are not broad enough to be effectively analyzed by older and slower mass spectrometers. Another benefit of the direct infusion method is that it is relatively frugal with solvents as very small amounts of them are needed to run the experiments. Direct infusion MS is also a relatively fast procedure, though not as fast as the direct injection method, as reloading the syringe pump with a new sample and a thorough cleaning the system from the remnants of the previous sample takes considerably more time in the former than injecting a new sample in the constant flow of a pure solvent in the latter.

Some of the major deficiencies of the direct infusion approach are: 1) its inability to separate the solvent noise from the signals of the analytes (a separate run is required when the same solvent is being infused and analyzed by the MS detector); 2) a quite possible sample carry-over, making a thorough cleaning the instrument between the runs a necessity; 3) inability to separate isobaric compounds, especially structural isomers of the same compound, from one another, without resorting to yet uncommon and rare types of mass analyzers (such as ion mobility analyzers); 4) inability to identify and classify groups of analytes (such as various classes of lipids) based on their RT; 5) impossibility to separate analytes from impurities

during the experiment; 6) a likely problem with ion suppression; and 7) difficulties with quantitation of the analytes (see below).

An often publicized advantage of the direct infusion technique – its frugality with the samples (Chen et al., 2010) – loses its appeal upon close examination as one needs to take into account that though the *concentration* of the sample might be quite low, the *volume* of the sample solution that is needed for any kind of detailed analyses of complex mixtures is not, which negates the initially proclaimed advantage of the direct infusion procedure. In a typical direct infusion experiment, a seemingly low working concentration of a sample – below 10 μM (Han and Gross, 2005) – starts to look less impressive once one realizes how much sample is needed to fill up the syringe and capillaries of the instrument, which then needs to be infused into the MS detector at a constant speed of 10 to 100 $\mu\text{L}/\text{min}$ for the amount of time sufficient to generate reliable data. The overall amount of consumed a sample in this case is not much different from an HPLC/MS or UPLC/MS experiment, where injection volumes are few microliters, or less.

Also, now and then one can read or hear claims that the length of a direct infusion experiment (i.e. amount of time required to get a reasonable mass spectrum of a sample from the outset) is one minute or less, which seems to be faster than an average HPLC/MS experiment. However, this estimate does not take into account the time needed to prepare the instrument for the experiment: direct infusion methods are labor-intensive as the constant changes of samples in a syringe pump of a mass spectrometer and mandatory washing steps between the samples and standards make the speed advantage rather small, or non-existent. For example, in a recent paper of Dean and Glasgow (Dean and Glasgow, 2012) each analysis performed using the infusion technique took about 30 min, on top of the time needed to wash and equilibrate the system. Also, this perceived advantage became much less of a factor with the advances in the ultra-fast UPLC/MS technology. Moreover, chromatographic autoinjectors allow for unattended use of the HPLC/MS systems, while typical direct infusion experiments require the presence of an operator.

Last but not least important deficiency inherent to the direct infusion approach is its inability to determine the actual origin of the analytes that could either be present in the samples as its native components, or be produced in situ due to spontaneous inadvertent fragmentation of more complex, and less stable, compounds (e.g. triacylglycerols) in the ion source of the MS detector (Figure 6) (Butovich, 2010b, 2011b; Chen et al., 2010, 2011). This effect has been documented and discussed before, but still is often overlooked or ignored. A few examples of such complex lipids that undergo unintentional in-source fragmentation are: 1) regular cholesteryl esters (Chl-E) and cholesteryl esters of (O-acyl)- ω -hydroxy fatty acids (Chl-OAHFA), which produce ions m/z 369 ($\text{Chl} - \text{H}_2\text{O} + \text{H}$)⁺ or m/z 391 ($\text{Chl} - \text{H}_2\text{O} + \text{Na}$)⁺ which are indistinguishable from ions of Chl; 2) wax esters (WE), Chl-E, triacylglycerols (TAG), OAHFA and Chl-OAHFA, all of which produce a range of signals indistinguishable from those of free fatty acids (FFA); 3) Chl-OAHFA, which produce fragmentation ions identical to those of free OAHFA; 4) TAG that lose one of their fatty acid radicals and produce ions identical to those of diacylglycerols; 5) complex ceramides that can fragment releasing simple ceramides and sphingosine. These transformations are summarized in Table 1. Interestingly, Chen et al. (Chen et al., 2010), have not reported any numbers for Chl in their samples, and have not shown the parts of the mass spectra where those signals were supposed to be. As FFA and Chl were linked to the onset and/or progression of dry eye disease (Shine et al., 2003), the inability of the direct infusion method to evaluate these compounds is a serious handicap.

The list of compounds that can produce false results in the direct infusion experiments (Table 1) can be expanded. Nevertheless, even these examples are enough to understand that

the direct infusion approach is best suited for analyzing relatively simple mixtures with a limited number of stable, non-isobaric analytes, and for evaluating samples whose chemical components have already been identified in prior experiments. In any case, the deficiencies of the approach noted above make it a questionable choice for meibum and tear film studies, where the complexity of the samples is overwhelming, the analytes are very diverse and change unpredictably. HPLC or UPLC, on the other hand, resolves this problem by separating complex lipids mixtures *before* the MS step, and allows for easy discrimination between analytes that are present in the samples as free compounds, and those that are produced *in situ*.

Some transient techniques, such as HPLC with either spectrophotometric or evaporative light scattering detection of lipids, have been exhaustively discussed in earlier reviews on the topic (Butovich, 2009c, 2011a). Their usability for analyzing intact lipids, especially complex lipid mixtures, is limited by poor selectivity and inadequate sensitivity of the techniques. Generally, they should not be used in meibum studies because of a very high probability of misidentification of the analytes. Still, the spectrophotometric detection can provide some useful information on oxidized lipids, tocopherols, carotenoids (see below) or any other lipid that produce a distinctive UV/Vis absorption spectrum because of their unique chemical structures (the vast majority of lipids do not).

A number of other, less traditional, approaches (such as infrared and Raman spectroscopy, nuclear magnetic resonance spectrometry, and others) have recently been used to characterize meibum. These approaches will be discussed later in this review.

1.2. QUALITATIVE STUDIES vs. QUANTITATION—Quantitation of lipids has always been a difficult task with the main problems being lipid diversity, and complexity, which leads to a surprisingly high chemical stability of some types of lipids, and instability of the others. Currently, there is no simple and universal method of lipid quantitation that would work equally well with all (or even most) of the known classes of lipids. However, there are techniques that could be adapted for subsets of lipids, such as particular classes of meibomian and tear film lipids, to make their quantitation possible. But firstly, let's discuss two separate topics – (1) quantitation of a total lipid content of a sample, and (2) quantitation of a specific lipid (or a group of lipids) that is present in the sample.

Obviously, the first task requires an analytical method that would allow the researcher to visualize and quantify the whole lipidome, i.e. the *least* specific procedure, while the second task needs exactly the opposite – a method that will effectively isolate the signals of one particular lipid species from the others.

The least specific, and thus the most universal, procedure is gravimetric analysis. Unfortunately, using gravimetry in lipid analyses of meibum and the tear film samples requires a substantial amount of a sample, and an assumption that the sample is pure and dry lipid with no non-lipid contaminants, such as inorganic salts, peptides, proteins etc. These requirements are difficult, or even impossible, to meet in experiments with samples of biological origin, which are often contaminated with other materials. Thus, gravimetry is better suited for estimating the weight of the entire sample, as it is done when meibum samples are collected. In our hands, a median amount of dry meibum sample collected from two lower eyelids of a human donor is 0.6 ± 0.3 mg, with a range of values of 0.1 to 2.5 mg. Interestingly, Nicolaides et al. (Nicolaides et al., 1981) reported an average value of a meibum sample to be 5 mg per subject, though the samples were collected from all four eyelids of cadavers. Presumably, those weights were of the dry lipid material after extraction of the sample with the Folch solvent, and its subsequent evaporation. The median amount of 5 mg should be considered an average estimate of the *total* meibum content in four eyelids.

Unfortunately, no information on the distribution of that amount between the lower and the upper eyelids was reported.

With that information in hand, one needs to determine what percentage of total meibum sample is made of meibomian lipids, and how much of non-lipid material is present there. Answering this question is difficult as no universal protocol for the quantitative lipid analysis exists. However, a very old, but nevertheless poorly understood, sulfo-phospho-vanillin assay (SPVA) comes close to fulfilling this task. The assay was developed in 1937 by Chabrol and Charonnat (Chabrol and Charonnat, 1937) for total serum lipids determination. The complex chemical reactions that are responsible for the formation of chromophores in the reaction were later investigated by Knight et al. (Knight et al., 1972) and Johnson et al. (Johnson et al., 1977). The method has been demonstrated to work with unsaturated lipids, sterols, and free hydroxyl-containing compounds such as fatty alcohols. However, it was not clear whether it would be suitable for other lipids that do not have a free hydroxyl group ($R-CH_2-OH$), or an olefinic double bond ($-CH=CH-$). Nevertheless, SPVA was used in a number of studies, and was recently adopted for a microplate reader platform for high throughput analyses (Cheng et al., 2011; Inouye and Lotufo, 2006).

Considering a complex nature of the chemical reactions in the assay (Johnson et al., 1977; Knight et al., 1972; McMahon et al., 2013), it was obvious that a proper calibration standard was needed for the assay to be successful. A variety of lipids and lipid mixtures were tested in this capacity, many of which were natural animal and plant oils, while the others were artificial mixtures of various lipid standards (Vatassery et al., 1981) or individual chemically pure compounds such as oleamide (Pucker and Nichols, 2012). Meibum and tear film samples are complex mixtures, with hundreds of different lipids being present in various, and, possibly, changing proportions. Thus, it is intuitively clear that the closer the calibrants are to the mixture of analytes, the more reliable the estimation will be. Therefore, when we decided to explore the usability of SPVA for meibum analysis, we initially tested various lipids either as individual standards, or as complex mixtures that mimicked human meibum (McMahon et al., 2013), choosing the components that were as close to the meibomian lipids as possible. Our initial tests indicated that the reactivity of lipids varied wildly depending on their chemical structures. Interestingly, some lipids, such as saturated WE, were found to be SPVA-positive while, theoretically, they should not have been reactive as they had neither free hydroxyl groups, nor olefinic double bonds. However, saturated WE were found to be highly reactive in SPVA and showed molecular responses that were close to the reactivity of unsaturated WE and fatty alcohols. This observation prompted us to investigate the SPVA reaction in more details, and allowed us to hypothesize a generalized mechanism that explained the reactivity of all SPVA-positive compounds, and the lack of thereof with SPVA-negative ones, such as proteins (including mucins), ceramides, and saturated hydrocarbons and saturated TAG (McMahon et al., 2013).

Fortunately for those who work in the field of meibomian lipid studies, *normal* human meibum typically does not contain SPVA-negative lipids in appreciable quantities (see below), while an abnormal meibum may have some of them, but not as major components. For example, hydrocarbons that had been initially found in meibum samples (Tiffany, 1978), later were classified as exogenous contaminants (Nicolaidis et al., 1981). Ceramides are present in meibum only in very small amounts (Butovich, 2008; Butovich et al., 2007b; Lam et al., 2011; Nicolaidis et al., 1988). Most of TAG are of unsaturated nature (Butovich et al., 2012b; Chen et al., 2010; Lam et al., 2011) and, thus, are SPVA-positive. Thus, by choosing a proper mixture of standard lipids, and employing proper calibration curves with sufficient number of calibration concentration levels, it is indeed possible to estimate the total lipid content of a meibomian lipid sample with adequate accuracy. We developed a standard lipid mixture (MMx) with the following composition: behenyl oleate (BO) : behenyl stearate

(BS) : cholesteryl stearate (Chl-S) : cholesteryl oleate (Chl-O) : free cholesterol (Chl) = 0.41 : 0.08 : 0.40 : 0.10 : 0.01 (by weight), which was close to a natural distribution of saturated and unsaturated WE, Chl-E and Chl in normal human meibum [Table 2; from (McMahon et al., 2013)].

The actual levels of peptides and proteins in normal meibum remain unknown, but are certainly nonzero, and are elevated in pathological meibum (Butovich and Lu, unpublished). It is very likely that the protein content will change depending on the condition of a donor, and the sample collection technique (higher pressure applied to the eyelids during meibum expression can contaminate meibum due to the tissue damage). However, it is reasonable to assume that the protein content is relatively small as the normal secretions are clear, i.e. free from turbidity which would be a likely consequence of presence of proteins due to their poor miscibility with lipids.

Our SPV and gravimetric experiments with MMx as standard calibrant showed that about 80% of meibum sample were SPVA-positive lipids, while the remaining 20% or so were classified as SPVA-negative compounds. An important question is whether this difference can be a result of the presence of proteins, salts and/or other nonreactive compounds in the sample, or be caused by an unknown factor. While salts can be easily extracted from the meibum samples by using a procedure similar to the Folch extraction, we ruled against using it for the risk of losing more polar and amphiphilic lipids, which would be very difficult to detect and avoid with samples as small as human meibomian samples, and which might introduce more problems than it intended to solve. Developing of a reliable method of protein determination in lipid-enriched samples would facilitate addressing this uncertainty.

Thus, the SPVA protocol seems to be a viable approach, provided a proper calibration mixture has been chosen, and calibration curves have been generated.

A totally opposite approach to lipid quantitation is based on using individual chemical standards whose structures either match, or are close to, individual species of meibomian lipids. If a series of standard solutions that mimic meibum is made to cover the possible concentration ranges of prospective analytes, then a series of calibration curves for individual analytes can be generated which, in turn, can be used to quantitate individual lipids (or their classes) in meibum and tears (Butovich, 2009a, 2010b; Butovich et al., 2012a). This approach is a *de facto* standard approach recommended for bioanalytical studies by various regulatory agencies (2011; Biopharmaceutics et al., 2001), and is adhered to by bioanalytical, biotechnological, and pharmaceutical industries across the world. The official recommendations include a minimal number of concentration levels per analyte (a minimum of six in addition to a blank sample), their ranges (which start at the low limit of quantitation and end at a level that is higher than the highest level of the analyte in the study sample), a number of calibration curves (one per analyte), and other parameters.

Not every recommendation can be followed in case of samples that are either too complex, or which no chemical standards exist for. However, best efforts should be made to follow those recommendations when conducting quantitative studies, and available standards and their test conditions should be selected scrupulously to minimize possible errors. This potential problem can be illustrated by a paper of Lam et al. (Lam et al., 2011), where just one lipid standard per lipid class (e.g. deuterated cholesteryl stearate-26,26,26,27,27,27(d6) for Chl-E, d5-TAG 48:0 for TAG, palmityl palmitate for WE, etc.) was chosen to quantitate each respective lipid class. This approach did not take into account the fact that the ionization efficiency of the analytes depended on the length of their fatty acid and fatty alcohol chains and the degrees of their unsaturation, and also did not address a (possible) non-linearity of the instrument's response. It is not clear from the paper whether just one

concentration per standard was used, or whether proper calibration curves were generated for each reported lipid class. It would be interesting to evaluate the levels of Chl and FFA in the study samples as they might be different in normals and dry eye patients, but no such data was generated and/or reported by Lam et al. Also, the overall ratio of WE to Chl-E was found to be 25:67 for both normal donors and dry eye patients (Lam et al., 2011). This was opposite to our findings and the earlier data on the topic (Nicolaidis et al., 1981). Nevertheless, even though the absolute quantitation of meibomian lipids in that study has not been achieved, and the molar ratio of different lipid classes (such as waxes and Chl-E) probably was not measured correctly, one can consider the inter-sample variations in individual lipids or lipid classes reported in that study to be estimated more accurately, as those numbers are not affected by differences in ionization efficacy of different lipid classes.

A serious problem in lipid quantitation is spontaneous in-source fragmentation of complex lipids mentioned earlier in this review. This common problem is caused by general instability of complex molecules which becomes a factor upon their ionization in the ion source of a mass spectrometer. It mostly affects complex esters, which are far less stable than compounds with amide or ether bonds. Spontaneous fragmentation of Chl-OAHFA led to an overestimation of the amounts of OAHFA in meibum (Chen et al., 2010), while spontaneous fragmentation of OAHFA themselves – to an overestimation of the presence of FFA in meibum by a wide margin (Butovich, 2010b, 2011b; Chen et al., 2010, 2011). These inadvertent transformations are difficult to detect in direct infusion and direct injection experiments, unless the researchers are aware of them beforehand, in which case experiments can be modified to alleviate spontaneous in-source fragmentation of a particular group of lipids (typically, at the expense of other analytes). However, if a chromatographic step precedes the MS step, the presence (or absence) of those shorter fragments in the sample can be easily monitored as they will have retention times very different from the retention times of intact complex lipids, enabling their correct identification. This approach allowed us to measure separately: 1) free Chl and Chl-E in meibum samples by monitoring their common analytical ion m/z 369 ($M - H_2O + H$)⁺ (Butovich, 2009a, 2010a; Butovich et al., 2007b); 2) FFA and OAHFA, though the latter produce a range of ions shared with FFA (Butovich, 2010b, 2011b); and 3) OAHFA and Chl-OAHFA (Butovich, 2011b; Butovich et al., 2011; Butovich et al., 2012b). All this would have been impossible to achieve in direct infusion and direct injection experiments because all these analytes would have entered the ion source of the mass spectrometer simultaneously, and would have been analyzed together.

A very special experimental approach was chosen by Dean and Glasgow (Dean and Glasgow, 2012). To quantitate phospholipids (PL) in tears, the authors appropriately generated calibration curves for each group of studied PL, stating that at least six concentration levels were tested (though only three and four concentrations of, respectively, L- -phosphatidylcholine and phosphatidylserine were shown in the charts). The method used in the study seemed to be similar to the direct infusion or direct injection methods, whose pluses and minuses were discussed above. The major difference between the approach of Chen et al. (Chen et al., 2010) and that of Dean and Glasgow is that the latter team reportedly used a HPLC-style autoinjector to “infuse” (*sic*) the study samples in the amounts of 20 μ L at a flow rate of 100 μ L/min. As this procedure seems to be technically unfeasible (considering other technical details reported in that paper), one cannot unequivocally reconstruct the actual protocol that was used in that study, which could have been either “direct injection” of a sample in the flow of an organic solvent, or “direct infusion” of the samples dissolved in a solvent. Interestingly, the total amount of phosphocholines in tears was reported to be about 5 μ M (or between 3 and 4 ppm), with the most abundant intact phosphatidylcholine m/z 758 being present in a 194 ng/ml (or 0.2 ppm) concentration. The physiological relevance of these numbers will be discussed later in the

paper. Dean and Glasgow also pointed out that "... Four lysophosphatidylcholines (m/z 490–540) accounted for about 80% of the total integrated ion count" and that "The most abundant phospholipid in tears reported ... is lysophosphatidylcholine".

Another standard approach to quantitation of individual analytes in complex mixtures can be illustrated by our recent publication on quantitation of intact WE in human meibum using gas-liquid chromatography/mass spectrometry (or GLC/MS) (Butovich et al., 2012a). A range of chemical standards of WE (from palmityl palmitate to behenyl behenate for saturated WE, and from lauryl oleate to behenyl oleate for unsaturated WE), were used to generate calibration curves. The complex shapes of the latter strongly suggested that a simple linear or quadratic equation would not adequately represent the instrument response, which necessitated a more elaborate approach. First, the data were plotted as 3D graphs (i.e. *amount of analyte* vs. *m/z* vs. *signal*) (Figure 7). Then, a non-linear fitting routine was used to fit the data and determine the parameters of the model. Programs that can be used for these computations include, but are not limited to, SigmaPlot®, TableCurve®, Origin® and others. As a result, two smooth 3D calibration plots were generated for saturated and unsaturated compounds, which then were used to quantitate corresponding meibomian WE using the software's built-in mathematical routines. Importantly, the number of used standards and the number of tested concentration levels for each standard were sufficient to ensure statistical validity of the selected mathematical model. This approach, though rather lengthy and laborious, was much quicker than earlier techniques, and, most importantly, made it possible to determine the concentrations of intact individual WE in the individual, non-pooled study samples. It would have been beneficial to test other standards of WE, especially those that are longer than behenyl oleate and behenyl lignocerate – the longest unsaturated and saturated WE tested – and those that are branched. However, to the best of the author's knowledge, no such standards are presently available. Nevertheless, the smoothness of the generated 3D calibration plots ensured reliable interpolation, and (judicious) extrapolation, of the experimental data.

Yet another method of lipid quantitation – quantitative ^1H -NMR – was recently tested by Borchman et al. (Borchman et al., 2012a, b; Shrestha et al., 2011). The method is based on the assumption that it is possible in a ^1H -NMR experiment to select one or a few proton resonances (, ppm) that would be specific for an individual class of lipids, and would be absent in the others, thus allowing to monitor, and, using proper calibration curves, quantitate the lipids. For example, WE produced a resonance at 3.98, which was a signal from the first methylene group in the alcohol chain of all WE $\text{R}_1\text{-C(O)-O-CH}_2\text{-R}_2$. Chl-E were characterized using a broad resonance at 4.6 (ppm) of a proton attached to C3 of ring A of Chl-E, $\text{R}_1\text{-C(O)-O-(CH}_{\text{Chl}})$. TAG were observed by monitoring their resonances between 4.1 and 4.3 ppm, where four hydrogens of the glycerol backbone $\text{R}_1\text{-C(O)-O-CH}_2\text{-[CH-O-(O)C-R}_2\text{]-CH}_2\text{-O-(O)C-R}_3$ are visible. Borchman et al. reported the molar ratio of WE to Chl-E to TAG to be 1:0.57:0.19, with a decreased amount of Chl-E in MGD patients.

The NMR approach offers an uncomplicated view on meibum composition, which, in many cases is all what a researcher needs. However, a few limitations of this approach are as follows. 1) If a degree of unsaturation of an entire lipid class changed from, for example, one vinyl group -CH=CH- per molecule (as in monounsaturated fatty acids and alcohols) to two, three, or zero, it would be impossible to assign this change to that particular class of lipids, as only the overall degree of unsaturation of the whole sample could be approximated from the ^1H -NMR experiments. 2) It would be impossible to differentiate between simple Chl-E and Chl-OAHFA on the basis of their resonances at 4.6, as they will produce identical spectra in that region, precluding their separate quantitation. 3) Three different groups of lipids – WE, OAHFA, and Chl-OAHFA – would produce a common resonance at 3.98; thus, their molar ratios could not be estimated. 4) It seems that proton resonances

with between 4.1 and 4.3, attributed by Borchman et al. to TAG, could also originate in part from esterified long chain ω -diols, described by Nicolaides et al. (Nicolaides and Santos, 1985). This possibility is also supported, with certain reservations, by recent observations by Chen et al. (Chen et al., 2010), who reported that true TAG comprised not more than 0.005% of meibum lipids, but not by the data of Lam et al. (Lam et al., 2011), who reported a much higher number of 5%. 5) Quantitative NMR is known for its low accuracy because of the integration errors intrinsic to technique (this problem, however, is less of a factor when the mixtures are simple, the physical size of the sample is large, the resonances are sharp and well separated from each other, and the baseline is flat). Finally, (6) no differences in the individual lipid species can be monitored and calculated as the method does not have enough resolution power to tell apart two homologs of any of the tested lipids, especially if they are present as a mixture. Thus, one should use the ^1H -NMR strictly as a preliminary approach which may provide a broad view on the samples, but lacks in-depth information on their chemical composition. Nevertheless, ^1H -NMR have been used to evaluate changes in the meibomian lipids with aging, the onset of meibomian gland dysfunction, and effects of administering azithromycin and doxycycline (Borchman et al., 2012a; Foulks et al., 2013; Shrestha et al., 2011).

Interestingly, the NMR technique has not revealed any PL in any of the studied samples. The authors (Shrestha et al., 2011) explained this by their choice of a solvent for NMR studies – deuterated cyclohexane – because PL were not soluble in this solvent. It is not clear why deuterated chloroform – one of the most often used solvent in NMR, and arguably the best solvent for meibomian lipids, including PL – was not chosen instead.

In summary, mass spectrometry with HPLC/UPLC and GC provides the best combination of speed, sensitivity, and selectivity, with the added benefit of physical separation of lipid analytes *before* they enter the ion source of a mass spectrometer, thus minimizing uncertainties in determination of their nature. A huge selection of available chromatographic columns enables researchers to classify the analytes according to their polarity (when using reverse-phase or neutral straight phase columns), charge (when using silica gel columns or special ion-exchange matrices), chirality (with the help of special chiral phases), or molecular weight (in size-exclusion, or gel filtration chromatography). The preliminary chromatographic step greatly minimizes the chances of misinterpreting the results, minimizes lipid-lipid interactions by reducing the number of different types of lipids simultaneously entering the mass spectrometer, and, therefore, helps in getting better quantitative data on the analytes. Other techniques have their roles in quantitative studies, but their limitations need to be taken into account.

2. MEIBOMIAN AND TEAR FILM LIPIDOMES

Human meibomian gland secretions are formed from a complex mixture of lipids of different classes including (as main components) WE, Chl-E, OAHFA and Chl-OAHFA, TAG, FFA, Chl, and a smaller amount of other polar and nonpolar lipids whose chemical nature and the very presence in meibum have been a matter of intense debates. Let's discuss these lipids in more detail.

2.1. WAX ESTERS AND STEROL ESTERS—Chemically, WE are esters formed from relatively long fatty acids and alcohols, while Chl-E are esters of fatty acids and sterols (Figure 8). WE are common constituents of microorganisms (Finnerty et al., 1979), plants (Li et al., 2008; Vrkoslav et al., 2009; Vrkoslav et al., 2010), insects (Vrkoslav et al., 2009; Vrkoslav et al., 2010), animals (Robosky et al., 2008), and humans (Aitzetmuller and Koch, 1978; Robosky et al., 2008; Stewart, 1992) where most of them are found on the outer surfaces of the organisms and are involved in creating a sealing barrier that protects their

bodies from the harmful environment and desiccating. In humans, for example, WE are a major part of sebum. Chl-E, on the other hand, are found in human serum (Hidaka et al., 2007), adrenal glands (Connelly and Williams, 2004), atherosclerotic plaques (Stegemann et al., 2011), malignant neoplasms (Tosi and Tugnoli, 2005), and other tissues.

All typical WE and Chl-E are very hydrophobic, have characteristically poor solubility in water, and tend to form a separate phase once introduced into aqueous solutions. Despite having a mildly hydrophilic ester bond in their structures (the esters can form hydrogen bonds with molecules of water that increase their solubility in aqueous phases), the overall hydrophobicity of their fatty acid, fatty alcohol, and cholesteryl residues make them extremely hydrophobic, with only a modest tendency to interact with water.

WE and Chl-E also are a very diverse and prominent group of lipids of human meibum. Combined, they represent 65 to 70% of human meibomian lipids (Butovich, 2009a; Butovich et al., 2012a; Nicolaides et al., 1981). Initial publications on human meibomian WE and Chl-E (Nicolaides, 1965; Nicolaides et al., 1981) lacked information on *intact* compounds because the implemented analytical techniques – GC/MS and GC/FID – required complex and lengthy processing of the samples to make them analyzable. Importantly, after multistep purification and derivatization of the samples, it became impossible to determine the exact molecular combinations of sterols, fatty acids, and fatty alcohols which Chl-E and WE were composed of. Nevertheless, a wealth of information on the chemical nature of WE and Chl-E was obtained in those studies. Importantly, the types of fatty acids identified in human meibum included saturated straight chain fatty acids varying from C_{12:0} to C_{26:0}, unsaturated straight chain acids C_{14:1} to C_{26:1}, saturated *iso*-branched fatty acids *i*-C_{13:0} to *i*-C_{28:0}, saturated *anteiso*-branched fatty acids *ai*-C_{13:0} to *ai*-C_{29:0}, with much smaller amounts of branched monounsaturated fatty acids *i*-C_{15:1} to *i*-C_{20:1} and *ai*-C_{17:1} to *ai*-C_{25:1}. Only straight chain C_{18:2} and C_{18:3} fatty acids were detected and/or reported (Nicolaides et al., 1981).

Fatty alcohols found in human meibum are also highly branched and often unsaturated (Nicolaides et al., 1981). Almost all fatty alcohols were found to be in the WE pool, among which about 47% were saturated *iso*-branched, 23% - saturated *anteiso*-branched, and the rest were identified as straight chain monoenes (23%) and saturated straight chain (7%) compounds.

Chl-E showed an almost identical mixture of geometrical isomers of saturated fatty acids: 3% straight chain saturated, 45% – *iso*-branched, 29% – *anteiso*-branched, with the rest being monounsaturated ones. Along with cholesterol, another sterol – 5-cholest-7-en-3-ol – was found in meibum of bovines, but not of humans (Kolattukudy et al., 1985). Also, Tiffany and Marsden reported lanosterol and its esters in meibum of rabbits (Tiffany and Marsden, 1982), later confirmed in HPLC/MS experiments (Butovich et al., 2012b).

The analytical work of Nicolaides and his coworkers and collaborators yielded extremely valuable information on the chemical composition of meibum and laid a solid foundation for future bioanalytical studies of human meibum. However, the methods used in those earlier studies were very convoluted, laborious, and the physical amounts of meibum required for those experiments were rather high. The latter necessitated the use of pooled samples. Therefore, the traditional approaches utilized by Nicolaides et al., and by many researchers before and after them, seemed to be ill-fitted for modern clinical type studies where sensitive, high throughput techniques are welcome or mandatory, and where samples from individual patients are often to be evaluated.

Recent efforts undertaken during the last few years radically changed this situation (Butovich, 2008, 2009a, 2010a, 2011b; Butovich et al., 2012a; Butovich et al., 2007a;

Butovich et al., 2007b; Campbell et al., 2011; Chen et al., 2010; Dean and Glasgow, 2012; Joffre et al., 2008; Lam et al., 2011; Rantamaki et al., 2011; Saville et al., 2011; Saville et al., 2010; Souchier et al., 2008). The use of various kinds of modern mass spectrometric instruments and methods made it possible to visualize, evaluate, and monitor composition of meibum and tears on the level of individual lipid species (or their small groups), and, in certain cases, quantitate them. A quantitative summary of those findings is presented in Figure 9.

Meibomian WE are mostly based on moderately long fatty acids with C₁₅ to C₁₉ carbon chains with the vast majority of them being of C₁₆, C₁₇, and C₁₈ types (Butovich et al., 2012a). The molar ratio of major *saturated* fatty acids C_{16:0}, C_{17:0}, and C_{18:0} in wax esters was calculated to be 22:65:13, while for monounsaturated C_{16:1} and C_{18:1} fatty acids the ratio was 1:9. The degree of branching also varied between the lipid groups: C_{16:0} was a 2:8 mixture of straight chain and *iso*-branched isomers, C_{17:0} – a 7:93 mixture of *iso*- and *anteiso*-form, C_{18:0} – 43:57 mixture of straight and *iso*-branched isomers. The isomerism of fatty alcohol residues of WE was not established in that study. However, per Nicolaides et al. saturated straight chain alcohols comprised about 7% of the overall pool of fatty alcohols, saturated *iso*-branched alcohols – 47%, and saturated *anteiso* – 23% (Nicolaides et al., 1981). Monounsaturated fatty alcohols (~27% of all fatty alcohols) were all of the straight chain type. Importantly, it appeared from that study that all fatty alcohols were accumulated in the WE pool of human meibum.

Combining the results of Nicolaides et al. (Nicolaides et al., 1981) and our observations on individual human meibomian WE (Butovich et al., 2012a; Butovich et al., 2007b; Butovich et al., 2009), one can assemble a list of most common WE and Chl-E (Table 3), which, combined, accounted for more than 50% (w/w) of all lipids in meibum. For calculations that were based on the data of Nicolaides et al. (Nicolaides et al., 1981), an assumption was made that all the major WE were based on oleic acid, with the exception of a WE with *m/z* 620, which was based of *ai*-C_{17:0} fatty acid. Similar simple but tedious calculations can be performed for other WE and Chl-E, provided their structures are known, and the data of Nicolaides et al. and our recent report (Butovich et al., 2012a) are used together. Note that small variations in numbers are normal for this kind of complex studies and, also, could be reflective of interdonor variability of the meibum samples. It is clear that *all* of these major lipids shown in Table 3 were either unsaturated, or branched, or both.

Accurate quantitation of Chl-E in tears has not been accomplished yet. Saatci et al. measured the Chl level in tears to be about 560 mg/l, or 1.45 mM (Saatci et al., 1990). Combining this information with our recent data on the molar ratio of Chl to Chl-E in tears (about 9:100, respectively), we estimate the total Chl-E levels in basic tears collected from normal, non-dry eye subjects to be about 16.5 mM, or between 11 and 14 mg/ml, assuming a typical molecular mass of a meibomian Chl-E to be between 700 and 850.

2.2. (O-ACYL)- ω -HYDROXY FATTY ACIDS—Meibomian OAHFA is a group of relatively unknown lipids that are similar to WE and FFA (Figure 8 and Figure 10, Panel A). The terminal nonesterified carboxylic group is, at least partially, ionized at physiological pH, which gives the molecule of OAHFA a negative charge. Further increasing their amphiphilic properties is a mildly hydrophilic ester bond in the middle of their molecules. All esters are capable of forming hydrogen bonds with water, and, though the strength of these bonds is not sufficient to make long chain esters water-soluble, hydrogen bonds may help the OAHFA's carboxyl groups in making these compounds amphiphilic, and may be helping in orienting the molecules at the lipid/water interface in a certain way (Figures 1 and Figure 10, Panel B). The partial atomic charges of relevant atoms in OAHFA (a measure of their ability to interact with molecules of water and form hydrogen or ionic bonds) were

determined in a computer-assisted MM2/MMP2 molecular modeling experiment (Butovich, 2011a). Thus, it is a given that the carboxyl groups of OAHFA penetrate the TFLL/aqueous sublayer interface and anchor the lipid molecules at the interface. The two lipophilic parts of the molecule, on the other hand, can interact with nonpolar lipids inside the nonpolar lipid sublayer, thus keeping the structure from collapsing. It appears that OAHFA have all the prerequisites to be the main amphiphilic lipid in the TFLL – two very hydrophobic domains that should make them insoluble in water, and two hydrophilic domains (one of which is ionized at physiological pH) which would make them populate the air/water or lipid/water interfaces.

The MS signals of OAHFA were first reported in 2007 (Butovich et al., 2007b), but it was not until 2009 that their first chemical standard – (O-oleoyl)- ω -hydroxypalmitic acid – was made to allow their structures to be established and described (Butovich et al., 2009). Independent confirmations of their structures came from Chen et al. (Chen et al., 2010) and Lam et al. (Lam et al., 2011). Recently, we demonstrated that OAHFA were present not only in human meibum, but also in meibum of canines, rabbits, and mice (Butovich et al., 2011; Butovich et al., 2012b). Therefore, they can be considered a universal component of meibomian gland secretions.

Human OAHFA is a diverse class of homologous lipids. Chemically, human OAHFA are products of acylation of monounsaturated extremely long-chain ω -hydroxy fatty acids with (mainly) one of the typical monounsaturated fatty acids. A convenient way to name them is to follow this nomenclature: (O-FA₁)-(ω -hydroxy-FA₂). Six groups of OAHFA dominate the pool, specifically compounds with m/z values of 729.7, 745.7, 755.7, 757.7, 783.7 and 785.7 [all (M – H)[–] ions], with a large number of minor homologues also being present (Butovich et al., 2009). As with WE, each group is comprised of two or more structural isomers that differ in the lengths of their corresponding acylating fatty acid FA₁ and ω -hydroxy fatty acid FA₂. Several combinations of those were found to produce each of the six main MS signals of OAHFA. For example, ion m/z 729.7 was reported to originate from two major isobaric forms of OAHFA – (O-C_{16:1})- C_{32:1} and (O-C_{18:1})- C_{30:1}, ion m/z 757.7 – from (O-C_{16:1})- C_{34:1} and (O-C_{18:1})- C_{32:1}, while ion m/z 757.7 – chiefly from (O-C_{18:1})- C_{34:1} (Butovich et al., 2009). An even larger number of possible structures have been reported later on by Chen et al. (Chen et al., 2010), Lam et al. (Lam et al., 2011), and Butovich et al. (Butovich et al., 2011; Butovich et al., 2012b). Interestingly, animal OAHFA slightly differed from human ones in their lengths and degrees of unsaturation. For example, canine OAHFA matched human ones in terms of their overall lengths and degree of unsaturation, while rabbit OAHFA were found to be more saturated. The mouse OAHFA were two to four carbons longer than the human ones, but their major OAHFA had at least one more double bond.

The only study known to the author where an attempt was made to quantitate OAHFA was published by Lam et al. (Lam et al., 2011). Though the authors conducted a thorough characterization of human OAHFA, and their structural assignments were in agreement with other reports on the topic, the quantitation of all lipid classes, including OAHFA, was done using just one lipid standard per class, which might not be sufficient to properly quantitate the lipids. In case of OAHFA the standard was (O-oleoyl)- ω -hydroxypalmitic acid. It is not known whether the ionization efficacy of different types of OAHFA (and, hence, the results of their quantitation) depended on their fatty acid composition (i.e. lengths and degrees of unsaturation). Nevertheless, the reported value – 3 to 4% of all meibomian lipids, or 30,000 ppm – may be considered a preliminary estimate that needs to be refined in future experiments [compare this number with 4 ppm for another group of amphiphilic lipids – phosphocholines – reported by Dean and Glasgow (Dean and Glasgow, 2012) for tears; below]. Our preliminary estimation of the overall presence of OAHFA in human meibum

based on comparison of the signals of free OAHFA and Chl-OAHFA [$\sim 1:1$, (Butovich, 2011b)], and comparison of the relative amounts of Chl-E and Chl-OAHFA (see next Chapter) yielded a very similar 3–5% of all lipids.

2.3. CHOLESTERYL ESTERS OF (O-ACYL)- ω -HYDROXY FATTY ACIDS—

Besides being a major group of amphiphilic lipids, OAHFA are also found in esterified form as (chole)steryl esters in humans and animals alike (Butovich, 2011b; Butovich et al., 2011; Butovich et al., 2012b; Chen et al., 2010; Nicolaides and Santos, 1985). Firstly described by Nicolaides et al. (Nicolaides and Santos, 1985), the compounds have been forgotten for a quarter of a century, probably because of the complexity of their structures, and tedious analytical procedures required for their identification and quantitation. However, mass spectrometry greatly facilitated their analysis and visualization. Again, as with the first chemical standard for OAHFA – (O-oleoyl)- ω -hydroxypalmitic acid – a definitive proof of Chl-OAHFA structures was obtained once their first chemical standard – namely, cholesteryl (O-oleoyl)- ω -hydroxypalmitate – was synthesized and analyzed in HPLC/MSⁿ experiments along with the compounds of biological origin (Butovich et al., 2011).

The fatty acid composition of these lipids, as with other classes of meibomian lipids is very diverse. Using the same classification scheme as in Chapter 2.2., the compounds can be described as (O-FA₁)-(ω -hydroxy-FA₂)-Chl. Fatty acids from the FA₁ pool are mostly of C_{16:1} and C_{18:1} variety (~ 23 and 35% of all fatty acids of this group, respectively), *ai*-branched (mostly, *ai*-C_{15:0} and *ai*-C_{15:0}, $\sim 18\%$ total), or straight chain C_{16:0} and C_{18:0} ($\sim 9\%$ total) (Nicolaides and Santos, 1985). The nature of the FA₂ residues in the compounds, apparently, was not established in that study, as no fatty acid longer than *ai*-C_{29:0} was reported for any lipid class, while the longest unsaturated fatty acid reported for Chl-OAHFA of lipids was C_{22:1}. In an earlier study (Nicolaides et al., 1981), the longest detected unsaturated fatty acids were C_{25:1} and C_{26:1}. Importantly, the major ω -hydroxy-FA₂ in OAHFA and Chl-OAHFA established in our studies (Butovich et al., 2011; Butovich et al., 2009) and in the works of Chen et al. (Chen et al., 2010) and Lam et al. (Lam et al., 2011) were much longer mono- and di-unsaturated fatty acids C₃₀ to C₃₄.

To the best of the author's knowledge, no attempt has been made to date to quantify these compounds since Nicolaides et al. calculated them to comprise about 5% of all meibomian lipids (Nicolaides and Santos, 1985). A rough estimation, however, is possible if we assume that the analytical ion m/z 369 is produced by Chl-OAHFA with the same efficacy as all tested Chl-E (for which no difference in the spontaneous fragment generation was found across the board; see above). Then, a preliminary estimate of the molar ratio of standard Chl-E to Chl-OAHFA in one particular sample shown in Figure 11 is 9:1, i.e. Chl-OAHFA account for 2 to 3% (mol/mol), or between 3 and 5% of all meibomian lipids.

2.4. DIACYLATED α,ω -DIOLS—

Diacylated α,ω -diols, or DiAD, whose structure can be depicted as FA-(ω -hydroxy-FA₁- ω -hydroxy)-FA (Figure 8), were first reported in meibum by Nicolaides et al. (Nicolaides and Santos, 1985), who estimated that these lipids added up to about 3.6% of all meibomian lipids. Chen et al. (Chen et al., 2010) observed a range of DiAD (or α,ω -type II diesters, using terminology of Nicolaides et al.). Making a first chemical standard of a DiAD – namely, distearoyl-1,10-decanediol (Butovich et al., 2012b) – allowed us to study its fragmentation pattern and verify structural assignments made in the earlier reports. Since the work of Nicolaides and Santos, no quantitation of these lipids has been attempted. It is worth noting that DiAD dominate the mass spectrum of rabbit meibum as it was observed in normal phase HPLC/APCI MS experiments (Butovich et al., 2012b). They were also prominent components of the canine meibum, but less so of the human and the mouse ones.

Per Nicolaides et al., the most abundant fatty acids in DiAD were C_{18:1} and C_{16:1} (about 34 and 19% each), *ai*-C_{15:0}, *ai*-C_{17:0}, and straight chain C_{16:0} fatty acids. The *ai*-branched acids comprised, on average, 23% of all fatty acids in diacylated , -diols, while straight chain ones produced about 10%. In a separate study, the most common fatty , -diols in human meibum were shown to be of C_{30:1} to C_{34:1} type and estimated to produce more than 80% of all meibomian , -diols, with a C_{32:1} diol being the most common (Nicolaides et al., 1984). The nature of the fatty diols was recently re-visited by Chen et al. (Chen et al., 2010), who re-confirmed that a single C_{32:1} diol accounted for more than a half of all , -diols in the human DiAD.

2.5. PHOSPHOLIPIDS AND SPHINGOMYELINS—Phospholipids (PL) and sphingomyelins (SM) belong to an important class of meibomian lipids, namely polar (or, more correctly, amphiphilic) lipids. As meibomian glands are holocrine glands, PL and SM are expected to be present in their secretions. Indeed, the presence of detectable (but vary variable) amounts of amphiphilic lipids was reported in multiple papers published in the 20th century, and in 2000–2004 (for a detailed discussion of those studies see earlier reviews on the topic (Butovich, 2009c, 2011a)). However, considering multiple shortcomings of the analytical techniques employed in those earlier studies, not every amphiphilic lipid reported there may be called a PL or SM. In fact, in the vast majority of previous studies neither the chemical nature of those amphiphilic lipids, nor their origins were established with sufficient certainty.

In our recent HPLC/MS studies of normal human meibum, we estimated the presence of phosphatidylcholines (PC) – one of the most ubiquitous class of PL – to be a very low 0.015% (or 150 µg/g, or 150 ppm) in one study (Butovich et al., 2007b), and less than 0.05% (or <500 ppm) in another (Butovich et al., 2007a). Saville et al. reported a combined total PC and SM concentration in human meibum to be about 18 µg/g, or ~18 ppm (Saville et al., 2011). Lam et al. found that normal meibum contained a much higher 0.2% (or 2,000 ppm) of PC and SM combined (Lam et al., 2011). Lam et al. also pointed out that the amount of PL in the meibum samples might depend on the force which meibum samples had been expressed with (the latter could explain the variability in the literature data). This possibility had been illustrated and discussed earlier (Butovich et al., 2008) using bovine meibum as an example. Pertinently, in our laboratory the force that is applied to the eyelids of human donors to express meibum is minimal, and the samples typically do not produce any appreciable amounts of PC, SM, and other PL. It appears that meibum that can be expressed with minimal force is residing closer to the meibomian gland orifices, and is ready to be excreted through physiological mechanisms anyway, while an attempt to express meibum that resides deeper in the meibomian ducts (for example, with a goal to collect as much lipid material as possible) will produce meibum that is either not mature, or contaminated with cell debris from crashed cells that will be enriched with PL.

The overall amount of meibomian phosphocholines (of all types) in tears estimated in the study of Dean and Glasgow was about 5 µM, or about 4 ppm assuming an average molecular weight of a typical C_{16:0},C_{18:1}-PC being 761 Da (a typical *range* of the molecular weights of most common PC is 700 to 800, which does not affect our calculations in any meaningful way). Essentially identical number for combined fraction of PC in tears (6 µM, or about 5 ppm) was calculated by Saville et al., who also observed SM in the amounts of about 5 µM or so. Combined, the last two numbers brought the total presence of PC and SM in tears to about 11 ppm (Saville et al., 2011; Saville et al., 2010).

On the other hand, Rantamaki et al. found that choline-containing lipids populate meibum at a concentration of about 48 µM, or 38 ppm (Rantamaki et al., 2011). Their experimental approach of choice – the enzymatic determination of lipids – was very different from mass

spectrometric methods developed by other laboratories. Nevertheless, the concentration of choline-containing lipids estimated in this study was close to that of Saville et al., but differed markedly from other reports. As a note of caution, one needs to realize that the samples of aqueous tears collected in the study of Rantamaki et al. were treated similarly to the samples used in an earlier study of Nichols et al. (Nichols et al., 2007). In particular, samples of tears were stored and processed in plastic Eppendorf tubes. Since the lipids were extracted with a chloroform-containing solvent mixture, a heavy contamination of the samples with plastic extractives was very likely (Butovich, 2008; Carrott and Davidson, 1998; Cooper and Tice, 1995; Jenke et al., 2007; McDonald et al., 2008; Watson et al., 2009). As PL were quantitated enzymatically, the contaminations might or might not be influencing the results of the analyses.

Unexpectedly, Rantamaki et al. found only four classes of lipids in the tear film samples, namely PL, SM, ceramides, and TAG. This could be an indication of a potential problem with the initial handling of the samples of aqueous tears. Per Rantamaki et al., the collected aqueous tear samples were centrifuged at 4 °C at 15,000 rpm..." to remove the possible cell contamination of cell debris..." (Rantamaki et al., 2011). Then, the supernatants were collected and stored at -20°C. Considering that the most of meibomian lipids are either hydrophobic or amphiphilic, much of those lipids could have adhered to the walls of the centrifuge tubes, or other plastic- and glassware. This loss is the most logical explanation of the absence of any Chl, Chl-E, WE, and a range of other compounds that have been demonstrated to be in the human tear film (Butovich, 2008), in the samples tested by Rantamaki et al. Our recent data (Arciniega et al., 2013) clearly demonstrated that Chl and Chl-E are typical components of normal human aqueous tears, with the only difference being the higher ratio of the lower molecular weight Chl-E (not surprisingly, as their solubility in water is inversely proportional to their molecular weight), and an order of magnitude larger amount of Chl (the most water soluble compound of the group), compared with meibum.

Finally, Campbell and co-workers have not found measurable amounts of PL in tested human tear samples, with the low limit of detection of 1 to 4 µg/ml (or less than 1 to 4 ppm) (Campbell et al., 2011).

A few interesting points were made in the paper of Dean and Glasgow with regard to the fate of PL that are delivered onto the ocular surface with aqueous tears and meibum. First of all, the authors assumed that the major source of PL in tears was meibum. Second, Dean and Glasgow suggested that most of PL are bound to lipocalin (or tear specific pre-albumin) – a protein that had been previously shown to bind a range of compounds of lipid and non-lipid nature (Gasymov et al., 2008; Gasymov et al., 2001; Glasgow et al., 2010), including PL (Gasymov et al., 2005). Third they suggested, rather contradictorily considering the previous point, that the entire PC pool found in tears may form a monolayer of PC, thus fulfilling the goal proposed in the earlier work of McCulley and Shine (McCulley and Shine, 1997). Let's evaluate these points in more detail.

Human tear lipocalin-1 (HLC-1) is a small globular protein with a molecular mass of 19,250 ((UniProtKB)). Its concentration in tears is about 87 µM (Fullard and Snyder, 1990). The stoichiometry of lipid binding with HLC is not known to the author, but for a bacterial lipocalin (BLC) it was estimated to be 2 molecules of fatty acids per one molecule of BLC, or 2:1, or 1:1 for PL (Campanacci et al., 2004). BLC has a sole binding cavity 18 Å deep × 12 × 7 Å, which appears to be long enough to accommodate a long chain fatty acid of the C₁₄ to C₁₈ type. This binding capacity compares well with human serum albumin, a protein three times the size of HLC, which binds up to 4 molecules of FFA (Krenzel et al., 2013). On the surface, it seems that HLC must be capable of binding all molecules of PL and SM

that are present in tears [if we assume that the data of Lam et al. (Lam et al., 2011) are inflated]. However, HLC can bind a much broader range of molecules, including those lipids that are present in tears in much higher amounts than PL and SM. For example, concentration of Chl in human tears was estimated to be about 1.5 mM (Saatci et al., 1990), while the expected concentrations of Chl-E and WE are an order of magnitude higher (see above). These numbers are high compared with anything from 5 to 200 μ M for the PC/SM family. As all Chl-E and WE have fatty acid residues in their structures, one can expect them to be able to penetrate the binding cavity of lipocalins the same way PL and SM do. However, without knowing 1) the binding constants for all lipids that are present in meibum and tears, and 2) their accurate concentrations, it is impossible to determine the actual preferences of HLC in terms of lipid binding and the ratio of free HLC to HLC-lipid complexes, at this time.

Another factor that has not been considered yet is a simple *partitioning* of the lipids between the TFLL and aqueous tears. Generally, almost any two (or more) types of lipids can mix to form a more or less homogeneous mixture, but only when one of the components is present in sufficiently small amounts compared to the other (PL are). Some lipids are quite miscible, while the others have limited mutual miscibility. When a threshold of miscibility has been reached, the lipid mixtures start to segregate forming separate phases enriched with one of the components. Phase diagrams of these binary, ternary etc. types of mixtures are used to illustrate what state the mixture is in at a particular ratio of its components (2007).

When meibum is being excreted onto the ocular surface and mixed with aqueous tears, only a fraction of an already very small pool of meibomian PL and SM is expected to concentrate at the meibum/aqueous tear film interface: it is reasonable to assume that PL- and SM-binding proteins found in tears may extract (or “scavenge”) some PL from the interface, but not from the bulk of the lipid phase. However, there are two main reasons why this might not be happening.

The first reason is the overall thickness of the TFLL (about 42 nm, mean), which equals the combined length of about 9 fully stretched molecules of behenyl oleate (whose length is about 4.6 nm per molecule, Figure 12) oriented normally to the plane of the TFLL, and more than 14 – when they are folded. This number can be even greater considering that the lipids in the TFLL may assume all kinds of conformations, and can be *tilted* (i.e. not normal to the plane of the TFLL). The depth of this multilayered TFLL structure seems to be sufficient to create a relatively thick lipid layer with properties approximating those of bulk lipid. Also, 42 nm is a *mean* thickness, which is not necessarily uniform across the TFLL: earlier experiments showed that the thickness of the TFLL could be anywhere between 15 and 157 nm (King-Smith et al., 2010). The latter number makes our comparison of the TFLL with the bulk lipid phase even more justified. Therefore, there is a high probability that at least a part of PL absorbed by the bulk lipid phase would be shielded from the lipid-scavenging proteins, and preserved deep inside the lipid layer.

The second reason is that SM and phosphocholines (which share a common, positively charged choline fragment and a negatively charged phosphoric acid residue) can possibly form homo- or heterodimers with themselves or with anionic lipids, thus effectively becoming either electroneutral ion-pair complexes, or complexes with a decreased overall charge, all resulting in an increased hydrophobicity and a decreased tendency to stay at the lipid/water interface. For example, two PC molecules could aggregate to form a homodimer (PC-PC), in which their positive and the negative charges will be essentially neutralized by forming ion pairs, while molecules of PC and phosphatidic acid (PA) or phosphatidylglycerol (PG) could form heterodimers PC-PA or PC-PG with their overall electrical charges reduced from two negative and one positive charge to a net charge of -1

(Figure 13). Moreover, the number of molecules in the aggregates is not limited to two, and can be much higher, as in lamellas, micelles, and reverse micelles, which would lead to clustering of lipids based on their affinities to each other. Self-association of PL and SM in a nonpolar environment can also lead to a formation of structures typically called “reverse micelles” (Ohshima et al., 1983) or “reverse lamellas”, which would further facilitate their partitioning from the lipid/water interface into the bulk of the lipid phase.

There is no reason why OAHFA could not form these types of complexes with PL and SM, either. However, OAHFA – the predominant group of amphiphilic lipids in meibum – is present in quantities much larger than those of PL and SM. Therefore, OAHFA have better chances of populating the TFLL/aqueous interface.

A rough estimation of the largest possible ratio of the combined fraction of PC and SM to the total meibomian lipid fraction in the TFLL can be done as follows. If we assume that: 1) the mean thickness of the tear film is about 4.5 μm , 2) the mean TFLL thickness is about 42 nm (or 0.042 μm), and 3) the entire pool of PC and SM found in tears is associated with the TFLL, then their concentration in the TFLL would be (4.5/0.042) multiplied by 4 ppm, totaling 430 ppm (using data of Dean and Glasgow), or about 1,200 ppm (using the data of Saville et al.), or less than 100 to 400 ppm (per Campbell et al.).

If we take into account the volume of tears stored in the tear meniscus [10 μL ; from (Dean and Glasgow, 2012)] and not more than 5–7 μL in our hands), the numbers for PL will still be low. Indeed, one cannot realistically expect that all PL molecules will be located exclusively at the TFLL/aqueous subphase interface with an average area of about 2 cm^2 , as regular and frequent blinking thoroughly remixes and redistributes aqueous tears and the lipids of the pre-existing TFLL and fresh meibum, across the entire ocular surface and within the TFLL itself. Per Dean and Glasgow, the lipid-binding proteins found in TF may further reduce the interfacial concentration of PL.

Therefore, the average PL content in the TFLL can be estimated to be between 0.1 and 1 molecules of PL per 1,000 molecules of other lipids. These numbers are comparable with our data for meibum (<500 ppm) and the levels estimated by Rantamaki et al. (4,300 ppm) and Saville et al. (3,200 ppm). However, none of these estimates for the TFLL accounts for the PL pool that might be bound with HLC and other proteins in the tear film, in which cases the actual numbers for the free PL in the TFLL should be still lower. Also, the differences in the collection techniques (especially, the pressure applied to the eyelids upon expressing meibum, and the possibility of collecting debris of the crushed cells from the eyelid epithelium and meibomian glands) are unknown, and cannot be factored in at this time. The excessive force may crush the epithelial cells, meibocytes, etc. and is likely to skew the lipid balance toward PL, while expressing meibum from deeper parts of acini increases the chances of releasing an immature secretion.

The final conclusion of this brief discussion of PC and SM in the tear film is as follows. Given the lack of any chemical standards of OAHFA [except for (O-oleoyl)- - hydroxypalmitic acid (Butovich et al., 2009; Lam et al., 2011)], the accurate quantitation of these lipids is yet to be performed. Nevertheless, the concentrations of SM, PC, and other PL in meibum and tears discussed above seem to be small compared with 30,000 to 40,000 ppm estimate for OAHFA, especially considering uncertainties with the quality of meibum samples expressed in different conditions by different experimenters.

2.6. FREE FATTY ACIDS, STEROLS, CERAMIDES, AND TRIACYLGLYCEROLS

—Quantitation of FFA in meibum, and even their qualitative evaluation, are not as straightforward a task as one might think. Recent attempts to analyze the FFA content of

meibum (Chen et al., 2010, 2011; Nichols et al., 2007) were not successful because of the multitude of experimental issues from sample contamination with plasticizers to spontaneous in-source fragmentation of complex lipids to the lack of proper calibration curves. The issue of plasticizers, stabilizers, and other extractives that contaminate samples of lipids if their solutions in organic solvents (especially, chloroform) are put in contact with any plastic ware, has been pointed out in our previous publications, for example in (Butovich, 2008, 2009b). Contamination of biological samples with plastic extractives (that include surprisingly large amounts of FFA, fatty acid amides, and aromatic compounds that have molecular weights similar to meibomian lipids) is a recognized problem in analytical chemistry (Butovich, 2008; Butovich et al., 2007c; Cooper and Tice, 1995; Jenke et al., 2007; McDonald et al., 2008; Watson et al., 2009). When we reproduced the lipid handling procedure of Nichols et al. (Nichols et al., 2007), without using any meibomian lipids, a very high amount of fatty acid amides (including oleamide as a major component) in the samples was registered (Butovich, 2011a). The molecular pattern of the amides in our meibum-free control experiments was strikingly similar to the spectrum of compounds shown by Nichols et al. At the same time, our meibum samples, if stored and processed in glass, consistently showed little to no signals of these compounds (Butovich, 2008, 2009b; Butovich et al., 2007b). Notably, neither Nichols et al. in their later publications (Chen et al., 2010, 2011), nor independent laboratories have ever reproduced the results published by Nichols et al. in 2007. Thus, fatty acid amides seem to have no structural, lubricating, or moisturizing role in the tear film whatsoever, and, if found in the amounts exceeding those typical of signaling molecules, should be considered only as an inadvertent contamination originated from organic solvents or labware.

Later, Chen et al. reported that meibum had no less than 3% of FFA in its composition (Chen et al., 2010). However, in response to this assessment we have demonstrated that the vast majority of the signals that had been initially attributed by Chen et al. to FFA, were, actually, produced by more complex fatty acid-containing lipids, such as OAHFA, Chl-OAHFA, and, possibly, other classes of lipids, that undergo spontaneous in-source fragmentation during the mass-spectrometric experiment. This could have been established on the earlier stages of the project had the HPLC/MS techniques been utilized, but it was not. Instead, the shotgun MS approach with no chromatographic step was used, which made it impossible to determine the origins of the FFA signals which are identical regardless of whether they are produced by true FFA, OAHFA, Chl-OAHFA, TAG, Chl-E or any other type of lipid that spontaneously releases a FFA residue in the ion source of a mass spectrometer. A prior HPLC step [as in (Butovich, 2010b, 2011b)], on the other hand, made it possible to separate the lipids *before* they were subjected to the mass spectrometric analysis, thus allowing their classification by their retention times, which also made it possible to accurately quantitate them in the study samples using chemical standards and calibration curves.

Moreover, the use of proper calibration curves [a mandatory requirement for quantitative bioanalytical projects, especially those that fall into the category of GLP (or Good Laboratory Practice) studies (2011; Biopharmaceutics et al., 2001) are often not implemented, which was the case with experiments conducted by Chen et al. and Nichols et al. Therefore, the more accurate estimate for the presence of FFA in normal meibum – a few tenths of a percent – was obtained in our HPLC/MS experiments (Butovich, 2010b).

The very same problem with the discussed inability of the shotgun approach (which is based either on direct infusion, or direct injection experiments) to identify the origin of ion m/z 369 has been encountered while analyzing the ratios of Chl, Chl-E, and Chl-OAHFA in meibum and tears. Indeed, when a sample that contains an unknown combination of these three classes of compounds is being infused, all three of them produce one major ion m/z

369, which is a $(M - H_2O + H)^+$ ion of Chl. Thus, in case of Chl, which is a relatively minor, but diagnostically and functionally important, component in meibum, its signals will be overwhelmed by identical signals derived from major Chl-containing meibomian lipids Chl-E and Chl-OAHFA, making quantitation of Chl impossible. HPLC/MS, on the other hand, overcomes this problem by providing spatial separation of Chl and its esters during the HPLC step, thus rendering their separate quantitation a simple task (Arciniega et al., 2013; Butovich, 2009a, 2010a; Butovich et al., 2012b). There is no doubt that rapid evolution of mass spectrometric methodology will bring about new approaches that will make the analyses of complex samples easier. Notable is the development of ion mobility mass spectrometry that promises to make it possible to estimate the physical sizes of the analytical ions. However, this technique is in its early stage of development, which necessitates the use of more robust techniques, such as G(L)C, HPLC, and UPLC. Also, it is hard to imagine at this time that ion mobility MS will be able to resolve, for example, chiral (*R,S*) isomers of the same compound, geometrical (*cis,trans*) isomers of unsaturated lipids, isobaric WE or other equally similar structures.

Over the period of six years since our first publication on the topic (Butovich et al., 2007b), we observed various amounts of Chl in samples of normal human meibum, but its levels typically have been between 0.02 and 0.5%. In aqueous tears, on the other hand, the levels of Chl were markedly (as much as by an order of magnitude) higher than those in meibum samples collected from the same donors (Arciniega et al., 2013), making Chl one of the major lipids in human tears. The amounts of Chl can possibly be even higher in the tear film of dry eye patients with active bacterial infection or inflammation.

Ceramides is another minor, but very diverse, group of lipids, whose combined presence in meibum was estimated to be less than 0.1% by Lam et al. (Lam et al., 2011). Rantamaki et al. identified five simple ceramides with a combined concentration in tears of less than 1 μ M (or less than 1 ppm). At this level, ceramides are not likely to play any structural role in normal meibum and the tear film. The virtual lack of ceramides in the norm can be helpful in differentiating normal secretions from abnormal ones [for example, higher levels of these compounds could be expected in patients with ocular lesions (Arciniega et al., 2013)], or for evaluating the quality of collected samples [e.g. for ruling out the skin cell contamination (Butovich, 2008; Butovich et al., 2007b)].

Major TAG in meibum were compared with other lipids and estimated to comprise about 4% of meibum (Lam et al., 2011). The compounds ranged from fully saturated to highly unsaturated species with at least 5 to 7 double bonds per molecule. Once again, considering that Lam et al. used just one chemical standard per lipid class in their quantitation experiments (in case of TAG, it was deuterated tripalmitin), the estimate provided by Lam should be verified in independent experiments, and is most likely inflated. Indeed, the $(M + H)^+$ signals of a equimolar series of homologous unsaturated TAG rise proportionally to their molecular masses (Figure 14). Therefore, using just one, relatively short TAG standard, as in experiments of Lam et al., will skew the estimates of meibomian TAG toward the higher numbers, as most of the TAG reported by Lam et al. are of higher molecular weight than deuterated tripalmitin. Intriguingly, Lam et al. did not observe triolein – the most common TAG in meibum – in their study samples, as no TAG longer than C_{52:1} were reported. Notably, Chen et al. (Chen et al., 2010) provided a very different number of 0.05% as an estimate of all TAG in meibum. Though we have not attempted to quantitate TAG in our experiments yet, their signals indicated that triolein [*m/z* 885, $(M + H)^+$] was always the major TAG in the study samples (Butovich, 2008), which was in agreement with observations of Chen et al., who estimated it to comprise up to 20% of all TAG.

2.7. RANDOM OBSERVATIONS: OLEAMIDE, EPOXIDES, SQUALENE, CAROTENE AND CAROTENOIDS—A very complex chemical composition of meibum, composed of many hundreds, if not thousands of individual components, creates a lot of opportunities for an analytical chemist to demonstrate the power of modern analytical techniques by either analyzing the ever smaller samples, or by finding analytes that are present in the samples in ever-diminishing amounts. However, these exciting capabilities need to be used judiciously to avoid rather common pitfalls, some of which are discussed below.

Oleamide, and other fatty acid amides, are common reagents that are used in food, chemical, and publishing industries. Importantly, oleamide is used as lubricant to produce polyethylene (which Eppendorf tubes and other common laboratory plastic ware are made of), paper, emulsifiers, anti-static agents etc. (<http://www.chemicaland21.com/specialtychem/perchem/oleamide.html>). At the same time, oleamide is a well-known sleep-inducing agent in humans and animals alike (Cravatt et al., 1995). This duality of oleamide may create, and have created, a lot of confusion among biomedical scientists and clinicians who are not aware of its chemical and industrial uses.

In 2007, a report was published (Nichols et al., 2007) in which the data were presented that clearly demonstrated that the positive ion mode MS spectra of meibum samples evaluated in the study showed almost no other lipids but fatty acid amides. The second group of lipids revealed in that publication was a group of FFA with stearic and palmitic acids dominating the pool.

These data, however, raised some concerns, which resulted in our subsequent Letter to the Editor (Butovich et al., 2007c). In that letter, and in a series of later reports (Butovich, 2008, 2009b, 2010b), we questioned the findings of Nichols et al. and demonstrated that, 1) meibum did not have naturally derived oleamide (or any other fatty acid amide) in meaningful quantities, and was observed only sporadically, and 2) the makeup of FFA described by Nichols et al. was not representative of human meibum.

A careful examination of the report of Nichols et al. revealed that the culprit that led to the extremely pronounced presence of fatty acid amides in the study samples was the use of Eppendorf tubes to store and process lipid samples, and the use of chloroform and methanol to extract the lipids from the plastic containers in which they were stored. It seemed that the chloroform-containing solvent mixture was able to dissolve enough plastic to contaminate and change the study samples beyond recognition. To test this hypothesis, we evaluated the effects of chloroform and methanol (Butovich, 2011a), and demonstrated that if a chloroform-containing solvent mixture had been kept in Eppendorf tubes even briefly, the spectrum of fatty acid amides extracted from the clean tube duplicated the meibum spectrum published by Nichols et al. It is worth mentioning that even aqueous buffers were shown to extract oleamide and other plasticizer from plasticware (McDonald et al., 2008). Notably, neither oleamide nor any other fatty acid amides have been shown to be a part of human or animal meibum in any other publication to date, including a recent report from the same laboratory (Chen et al., 2010).

Thus, the amides of C_{14:0}, C_{16:0}, C_{18:0}, C_{18:1}, C_{18:2}, and C_{22:1} family are *not* major natural components of meibum, and, if reported above the levels typically reserved for signaling molecules, should be considered as an indicator of sample contamination. The makeup of meibomian FFA reported by Nichols et al. may also be not representative of meibomian lipids, where very long chain fatty acids of C₂₂-C₂₆ type dominate the pool (Butovich, 2010b).

Another group of lipids that have been confirmed to be absent from meibum as noticeable components is epoxides of various meibomian lipids. Two epoxidized fatty acids – 9,10- and 11,12-epoxy-C_{20:0} fatty acids were reported by Shine and McCulley (Shine and McCulley, 1993), both found in the combined WE/Chl-E fraction as esterified compounds. The results of our later experiments, however, disagree with this finding, as we were unable to detect the epoxides even though their chemical analogues were tested alongside the study samples (Butovich et al., 2009).

Squalene is an aliphatic branched highly unsaturated lipid that is a biosynthetic precursor of cholesterol. Squalene is a common component of skin. Traces of squalene have been identified in both human tears and meibum by using HPLC/MS/MS (Butovich, 2008). It has been noted that the tear samples have a larger pool of squalene than meibum, but no attempts to quantitate the compound was undertaken at the time.

A lesser-known class of lipids that was studied in the relation to meibum is carotenoids (Faheem et al., 2012; Foulks et al., 2013; Oshima et al., 2009). The most common members of carotenoids that are found in human bodies are β -carotene, lycopene, lutein, and zeaxanthin. These compounds are hydrophobic, lipid-soluble molecules that have multiple conjugated double bonds. The latter property gives them a distinctive coloration – the compounds are deeply orange or red in color. Some carotenoids (specifically, β -carotene) are precursors of vitamin A. The others are strong antioxidants (Kelkel et al., 2011). General consensus is that carotenoids are *not* produced in human bodies. Instead, they must be consumed with food (Maiani et al., 2009). Therefore, their levels in human tissues, other conditions being equal, strongly depend on the diet. Generally, carotenoids are accumulated in lipid-rich tissues, such as the skin and the retina. Excessive consumption of carotenoids leads to yellowing of the skin, a condition that is known as carotenosis, hypercarotenaemia, or hypercarotenoidaemia (Chaparro et al., 2003). Carotenoids possess antioxidant activity which is one of their suspected physiological roles in retina (Fletcher, 2010).

Recently, Oshima et al. interpreted changes in two Raman bands (1153 cm⁻¹ and 1520 cm⁻¹) that were observed in human meibum samples in Raman spectroscopy experiments as an indication of the presence of “carotenoid-like” compounds in the samples (Oshima et al., 2009). Potentially important, this conclusion, however, cannot be considered as final at this time as no definitive chemical assignments can be made on the basis of a Raman spectrum, especially if the analyte was present as a minor component of a complex mixture among other similar compounds. It is worth noting that many conjugated aliphatic dienes, trienes, and other unsaturated compounds may produce bands in their Raman spectra that are close to the bands reported by Oshima et al.

In a later paper, Borchman et al. referred to these compounds as “terpenoids” and linked them to a ¹H-NMR resonance δ 5.2 (Borchman et al., 2012b). This assignment, however, needs an independent verification. It is plausible that the proton resonance δ 5.2 might be produced by other unsaturated lipids, phenols [Ph-OH], alcohols (R-OH), and terminal vinyls (R-CH=CH₂) (Joseph et al., 2010), and not necessarily terpenoids and carotenoids *per se*. Foulks, Borchman et al. hypothesized that these carotenoid-like compounds (e.g. β -carotene) might be responsible for protecting meibum from oxidation, and reported that their amounts in meibum decreased with age (Oshima et al., 2009). The estimated amount of the compound(s) in meibum was about 90 μ g/g meibum, or 90 ppm, which is within reach of a typical MS analysis. Considering the very high molar extinction coefficient of carotenoids in the yellow-to-red region of spectrum [ϵ $\sim$ 100,000 M⁻¹cm⁻¹ (Zechmeister and Polgar, 1943)], this amount should be high enough to detect carotenoids in meibum by means of UV-Vis spectrometry as well. Interestingly, Bjørn Bjerkeng’s rather unexpected data on the levels of carotenoids in the tissues of salmonid fish (Bjerkeng, 2000) (available as a free

download from http://www.aces.edu/dept/fisheries/education/pond_to_plate/documents/CarotenoidPigmentationinTroutNorway.pdf) clearly demonstrated that if the compounds were present in tissues in the amounts as low as 1 to 7 ppm, the tissue were salmon-red, which is not the case with meibum, which per Borchman, Foulks et al., has about 90 ppm of carotenoids.

Thus, we were unable to find an independent confirmation of the observation of carotenoids in meibum in the published literature.

Also, it would be important to verify whether carotenoids can indeed slow down peroxidation of meibomian lipids if present in the amounts of ~100 ppm, or less, as at least one clinical trial showed no photoprotective effect of β -carotene on the skin of human subjects exposed to UV radiation (McArdle et al., 2004), whilst it is known that the skin is enriched with carotenoids and can be oversaturated with them to an almost pathological degree (Chaparro et al., 2003). Furthermore, vitamin A – a biologically active metabolite of β -carotene – was unable to improve the skin condition by increasing its ability to retain water (Leonardi et al., 2002), which might rule out another possibility for the compound to have a stabilizing effect on the ocular surface.

Considering that carotenoids are supplied to our bodies with food, their levels in human bodies should correlate, first and foremost, with the amount of vegetables and fruits consumed by humans. The link between meibomian “carotenoids” and/or “terpenoids” and the diet is yet to be established. Therefore, to be able to reliably identify the nature of the compounds that produce the two Raman bands (1153 cm^{-1} and 1520 cm^{-1}), and the ^1H -NMR resonance $\sim 5.2\text{ ppm}$, one needs to either isolate those compounds and subject them to rigorous chemical testing, or to confirm their structures in HPLC/MS experiments with proper chemical standards being analyzed alongside the unknowns.

3. IMPLICATIONS FOR THE TEAR FILM STUDIES AND FUTURE DIRECTIONS

The overall impact of recent studies on the field of tear film studies has been strong, but mixed. A lot of new (for the field) experimental techniques have been explored, and new ideas have been proposed. However, independent verification of many of the ideas proposed in recent papers that have been discussed in this review would greatly enhance their validity and value.

Arguably, the most important development in recent years was implementation and validation of contemporary analytical techniques such as API mass spectrometry. Both of its most common forms – the atmospheric pressure chemical ionization MS and the electrospray ionization MS – were shown to provide a wealth of information on the structures of *individual* meibomian lipid species in samples collected from *individual* human subjects. A great progress has been made in developing and validating experimental approaches that would eventually lead to a complete characterization and accurate quantitation of all meibomian lipids in the norm and pathology. At least three independent laboratories at three different institutions (at the Ohio State University, Singapore Eye Research Institute, and the UT Southwestern Medical Center) are in a good agreement now on the structural aspects of the major meibomian lipids analyzed so far.

Two 3D lipid maps presented in Figure 15 (Butovich, unpublished) show meibomian lipids as they are seen in reverse-phase HPLC experiments with an ion trap mass spectrometric detector equipped with an atmospheric pressure chemical ionization ion source operated in positive and negative ion modes. The most prominent lipid classes are labeled in the graphs. Note that the relative intensities of the peaks are *not* necessarily representative of the relative molar ratios of the analytes, at least when different classes of lipids are compared. However,

chromatograms of ions m/z 369 (an analytical ion for all cholesteryl-containing lipids) (Figure 15, Panel A) can be used to estimate the relative amounts of those compounds, and so can be ions of OAHFA (Figure 15, Panel B), which are analytical ions for free OAHFA and Chl-OAHFA. These types of analytical ions manifest themselves as horizontally oriented groups of peaks, and are not to be confused with chemical noise that originates either from the solvent, or the mass spectrometric detector, and which produces horizontal, almost continuous streaks of more or less uniform intensity. Series of homologous compounds, on the other hand, can be observed as regularly spaced, diagonally oriented sequences of peaks. As an illustration, one can consider OAHFA and Chl-OAHFA, which produce both the patterns. This kind of analysis makes it easier to visualize complex relationship between different MS signals and analytes.

Alternative approaches – such as NMR, Raman, and infrared (IR) spectroscopies – have a potential to provide an independent way of analyzing meibum, but given their poor selectivity, low (compared to MS) sensitivity, and ambiguity in assigning particular resonances or bands to particular lipid species in complex mixtures, are best suited for broad characterization of samples rather than their detailed analyses. Also, the NMR instruments are much costlier than the mainstream HPLC/MS systems, and rarer. However, an uncomplicated view on lipid mixtures, and suitability for studying phase transitions of lipids, make NMR a viable choice in certain situations. The ambiguity in interpretation of the Raman and IR spectra of complex lipid mixtures limits their use in qualitative and quantitative lipidomic studies, but, as with NMR, they can be used to study phase transitions in lipids.

New types of MS that are constantly being introduced and adapted for new uses will provide even better, simpler and more sensitive platforms for future studies. However, the major obstacle in the tear film and TFLL studies at this time is not the insufficient sensitivity of instruments: it is the lack of proper chemical standards for (rather atypical, if compared with most of other tissues) meibomian lipids, which hampers their *quantitation*. Undoubtedly, the best approach to quantitation of meibomian lipids would have been through the use their deuterated analogs, but, at this time, even non-label standards are not available for a number of lipid classes. Until these standards have become available, the uncertainties in the correctness of lipid quantitation will persist.

So far, most of the efforts have been made in the field of studying meibum. The tear film lipids were evaluated in a few recent studies (Arciniega et al., 2013; Butovich, 2008; Dean and Glasgow, 2012; Saville et al., 2010), though Dean and Glasgow, and Saville et al. devoted their attention to mostly PL and SM. In our study (Butovich, 2008), we demonstrated that tear lipids are largely based on meibomian lipids, with a noticeably higher presence of lower molecular weight components, such as shorter chain Chl-E, for example. In 2013, we estimated the molar ratio of Chl to Chl-E in tears to be an order of magnitude higher than in meibum, approaching 9% of all Chl-containing compounds, or at least 3% (or 33,000 ppm) of all lipids (Arciniega et al., 2013). The levels of PL and SM in tears – between 5 and 10 ppm were reported by Saville et al. and Dean and Glasgow (Dean and Glasgow, 2012; Saville et al., 2010). The possible impact of the latter (or the lack thereof) on the structural properties of the tear film in general, and the TFLL in particular, has been discussed earlier in this review.

The major difficulty in studying the tear film lipids is their relatively low concentration in the study samples: a typical sample of tears has no more than a few percents of lipid material (w/v). Thus, not every lipid component of the tears can be evaluated as rigorously as its meibum counterpart. There is no doubt that by extrapolating the results of structural evaluation of meibum lipids onto the tear film, this problem can be successfully overcome.

As a final note, the rate with which the field of meibomian lipid studies is progressing now virtually guarantees that remaining “unknowns” in the meibomian lipidome (shown in Figure 9) will be identified and quantified in a not-so-distant future. This, in turn, will allow future efforts to be aimed at using that information in biomedical studies to determine structure-function and quantity-function relationships between the lipids and the tear film in the norm and pathology, much like deciphering the human genome provided researchers with better tools to study molecular mechanisms of diseases. Another area where this information is critical is translational biomedical research with three targets – 1) finding or creating a proper model of the human tear film; 2) finding reliable markers of ocular diseases; and 3) getting exact information on how the composition and dynamics of the TF and TFL are correlated with various ocular diseases. The complete characterization of the meibomian lipidome is to help those efforts by identifying proper targets for future biomedical studies, and by excluding those that are less relevant to the tear film.

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- Here, recent developments in meibum and tear film lipid studies are summarized.
- Most informative experimental procedures related to the field are discussed.
- New concepts recently published in the literature are evaluated,
- Future directions of the studies are discussed.

Figure 1. Schematic representation of the tear film

Individual sublayers of the tear film are shown in different colors and are labeled. A microphotograph of the meibomian lipid film (field of view 1.6x2 mm) taken with a Brewster angle microscope is shown. Note the apparently microgranular, uniform, and very condensed structure of the lipid film. Below, a diagram shows a hypothetical organization of the amphiphilic lipid sublayer that separates the aqueous sublayer(below) from the bulk of the tear film lipid layer (above). The amphiphilic lipid sublayer is predominantly composed of extremely long chain (O-acyl)-omega-hydroxy fatty acids (OAHFA). As an example, (O-oleoyl)-ω-hydroxy-tetratriacontenoic acid is shown. Nonpolar regions of its molecules are shown in grey. The more polar carboxylic groups and ester bonds are shown in red. These groups have a pronounced affinity toward water and are anchoring the molecules of OAHFA at the interface between the aqueous sublayer and the nonpolar lipid sublayer.

The figure was originally published in *Progress in Retinal and Eye Research* (Butovich, 2009c), but has been modified from the original, and re-published with permission from Pergamon © 2009.

Figure 2. Representative reverse-phase chromatograms of human meibum and a negative ion mode atmospheric pressure chemical ionization mass spectrum of a Chl-OAHFA with MW of 1127.1

Panel A. Four HPLC traces are shown. *Trace 1* (m/z 369.3, positive ion mode) demonstrates the presence of multiple Chl-containing compounds in the sample. A peak of Chl has a retention time of about 4.5 min. Peaks of Chl-E have retention times between 10 and 25 min. Cholesteryl esters of OAHFA elute between 25 and 32 min into the experiment. *Trace 2* (m/z 1128.1, positive ion mode) shows a trace of Chl-OAHFA detected as an $(M + H)^+$ ion. *Trace 3* (m/z 757.8, negative ion mode) demonstrates a major elution peak with the same retention time as peaks m/z 369.3 and 1128.1. Signal m/z 757.8 is produced by an OAHFA fragment that originated from a parent Chl-OAHFA because of spontaneous in-source fragmentation of the compound. *Trace 4* (m/z 1126.1, negative ion mode) is the trace of the same Chl-OAHFA detected as an $(M - H)^-$ ion. The signal reconfirms the molecular weight of the analyte, 1127.1 Da.

Panel B. A characteristic fragmentation mass spectrum of the analyte taken in negative ion mode. The presence of several isobaric compounds of the same nature is revealed by several elution peaks with m/z 1126.1, and additional fragments seen around the main MS peak m/z 757.8

HPLC/MS workflow **Direct injection MS workflow** **Direct infusion MS workflow**

Figure 3. Comparison of three main techniques of introducing lipid samples that are dissolved in organic solvents into an ion source of a mass spectrometer – HPLC, direct injection, and direct infusion workflows

The HPLC technique is fundamentally different from the other two techniques because it employs an HPLC column (shown in red) which separates analytes according to their physical properties (such as size, charge, polarity, dipole moment, hydrophobicity, chirality, etc) *before* they reach the mass spectrometric detector. Therefore, the analytes are introduced into the MS detector one after another, according to their retention times, which minimizes the chances of ion suppression, and separates the analytes from contaminations. The retention times can be used to attribute the analytes to particular lipid classes (see Figure 2, for example).

Figure 4. A typical result of a direct injection mass spectrometric experiment

Three lipids were tested. Note that all the analytes arrive to the detector at the same time as a mixture.

Panel A. A raw mass spectrum of a test lipid mixture. Three lipids have been analyzed as an equimolar mixture: a wax ester behenyl oleate [m/z 535.6, $(M + H)^+$], tristearin [m/z 607.6, $(M - FA + H)^+$ and m/z 908.6, $(M + NH_4)^+$], and cholesteryl oleate (only one ion is visible, m/z 369 $(M - FA + H)^+$). Note the presence of an ion m/z 391 which may or may not be a sodium adduct of the cholesteryl oleate's product ion m/z 369 $(M - FA + Na)^+$. Also present are signals 1122–1622, which are residual signals of a standard solution that was used to calibrate and tune the mass spectrometer.

Panel B. A raw mass spectrum of the solvent in which the three lipids were dissolved. Note the prominent presence of ion m/z 391, and the signals of the MS calibrants 1122–1622. These baseline signals were obtained between sequential injections of the lipid mixture.

Panel 3. A differential mass spectrum of the lipid mixture that was obtained by subtracting the baseline signals shown in *Panel B* from the signals shown in *Panel A*. Note the absence of signals with m/z values of 391, and 1122–1622.

Similar results can be obtained in a direct infusion experiment. However, two separate runs are required – one for the sample solution, and another for the solvent itself.

Figure 5. A sample chromatogram of ion with m/z value of 981 detected in canine meibum in positive ion mode

Note the presence of several HPLC peaks with different retention times (16.3, 18.0, 19.7, 21.7, 27.7, and 29.0 min) that produced identical mass spectra, which confirmed the presence of several isobaric compounds with different chromatographic properties.

Figure 6. Spontaneous in-source fragmentation of a complex lipid (an extremely long-chain diacylated ω -diol, m/z 1053.8) in a positive ion mode MS experiment
The compound elutes as one sharp HPLC peak. In the ion source of the mass spectrometer, the compound spontaneously breaks up producing a series of fragments $(M - FA + H)^+$ and $(M - 2FA + H)^+$ shown in the insert.

Figure 7.

A three-dimensional (m/z versus lipid sample amount versus signal intensity) calibration plot obtained for seven standard wax esters. The plots of this type were used to identify and quantitate meibomian wax esters [from (Butovich et al, 2012a), with permission from ARVO © 2012].

Figure 8. Representative meibomian lipids of different classes identified in human meibum

1. A typical wax ester (note that the compound exists in meibum as at least three different geometrical isomers. The differences in the fatty alcohol residues are shown in bold. From left to right: *anteiso*-, *iso*-, and straight chain isomers are shown). **2.** A cholesteryl ester (again, at least three geometrical isomers exist). **3.** A typical OAHFA (many isobaric isomers exist with different combinations of fatty acids). **4.** A typical cholesteryl ester of OAHFA (isobaric isomers exist). **5.** A typical diacylated α,ω -diol (isobaric isomers exist). **6.** The major triacylglycerol of meibum, triolein (various TAG of the same molecular weight may exist).

Figure 9. Major lipid classes quantitated and/or identified in whole human meibum (aggregate numbers from multiple studies discussed in the review were used for this chart)

Nonpolar lipids include WE, Chl-E, Chl-OAHFA, TAG, and at least a part of unknowns (such as DiAD, diacylated , -diols and other, more complex lipids, whose quantitation and detailed structural characterization have not been completed yet).

Amphiphilic lipids include OAHFA, Chl, FFA, PL, and Cer in various proportions. Part of the unknowns might be of amphiphilic nature, too. However, at least a part of unknowns could be of nonlipid nature, for example denatured proteins, salts, etc.

Figure 10. Molecular modeling of one of the major amphiphilic lipid in human meibum, (O-oleoyl)-7-hydroxy-dotriacontenoic acid

Panel A. A stereoscopic view of the compound (computed using the ChemBio3D Ultra software; MM2-minimized for *in vacuo* conditions; other conformations are also possible). Oxygen atoms are shown in red. The free carboxylic group of the OAHFA is shown on the left.

Panel B. One of the possible conformations of the compound at the air-liquid interface, optimized taking into account the presence of water molecules (red spheres) capable of forming hydrogen bonds with themselves and with two hydrophilic sites in the OAHFA molecule – the ionized terminal carboxyl group and the ester bond in the middle of the molecule (both sites are encircled with yellow broken lines). Note that the nonpolar loops in the OAHFA molecule (shown in grey) tend to minimize their contact with molecules of water. For clarity, hydrogen atoms were removed from the structures after they had been optimized.

Figure 11. Sample HPLC/MS data that illustrate the difference in the Chl and Chl-E contents in human meibum and tears

Panel A. The trace of basic tear lipids is shown in red; the trace of meibum lipids is shown in black. Note how similar the chromatograms are starting with peak 4, and how different their peaks of free cholesterol, and peaks 1–3 are. Apparently, the difference is proportional to the water solubility of the compounds.

Panel B. Interdonor variability of the human tear lipid samples. Seven normal, non-dry eye donors were evaluated. Note the consistently high presence of Chl and shorter-chain Chl-E in the study samples.

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Figure 12. A molecule of behenyl oleate in extended conformation

Note the relatively polar oxygen atoms of the ester bond in the middle of the molecule. Though they are capable of binding a few molecules of water, their polarity is not sufficient to make behenyl oleate appreciably amphiphilic, as these types of interactions are weak compared to the effects introduced by an ionized carboxylic group.

Figure 13. Hypothetical homo- and hetero-dimers of phospholipids

Dimerization may occur spontaneously and can increase their solubility in hydrophobic media. The dimers are structurally analogous to reverse micelles and lamellas. Dimerization reduces the net electrical charge of the complexes (as with the PA-PC complex) or shields the existing charges by being its own counterion (as in the PC-PC dimer). These effects may make the dimers (or more complex aggregates) more soluble in the bulk of the nonpolar sublayer of the TFL, thus effectively reducing their presence in the aqueous phase and in the amphiphilic lipid sublayer.

Figure 14.

The effect of the chain length on the relative intensity of the $(M + H)^+$ signals of triacylglycerols. An equimolar mixture of four unsaturated triacylglycerols was analyzed. Note more than a 40% increase in the relative intensity of the signals from TAG C_{48:3} to C_{60:3} and beyond.

Figure 15.

A 3D map of meibomian lipids as they are detected in reverse-phase HPLC/atmospheric pressure chemical ionization MS experiments in positive (Panel A) and negative (Panel B) modes. Note that the overall intensity of the peaks depends not only on the concentration of the analytes, but also on their ionization efficiency.

Table 1

Some of the complex lipids susceptible to spontaneous in-source fragmentation in MS experiments and their common fragments indistinguishable from those of simple lipids

Intact lipid	Typical major fragments	Major observed species	Typical m/z	Ion mode
Chl-E	Cholesterol	$(\text{Chl} - \text{H}_2\text{O} + \text{H})^+$	369	PIM
Chl-OAHFA	Cholesterol	$(\text{Chl} - \text{H}_2\text{O} + \text{H})^+$	369	PIM
	OAHFA	$(\text{OAHFA} - \text{H})^-$	729, 731, 755, 757, 783, 785	NIM
WE, TAG, OAHFA, Chl-E, Chl-OAHFA	Fatty acids	$(\text{FA} + \text{H})^+$	Various $\text{C}_{16}\text{-C}_{34}$ fatty acids	PIM
		$(\text{FA} - \text{H})^-$		NIM
TAG	DAG	$(\text{TAG} - \text{FA} + \text{H})^+$	Various	PIM
		$(\text{TAG} - \text{FA} - \text{H})^-$		NIM

Table 2

Major lipid classes of human meibum *

Lipid class	% of total meibum lipid (w/w)	Ratio of saturated to unsaturated lipids within each class	References
Wax esters	41±8	1:4.6	(Butovich et al., 2012a)
Cholesteryl esters	~31	4:1	(Butovich, 2009a, 2010a)
Cholesterol	< 0.5	n/a	

* From (McMahon et al., 2013)

Table 3

Major wax esters and cholesteryl esters of human meibum

Major wax esters of human meibum							
m/z	Fatty acid residues found in WE ¹	Fatty alcohol residues found in WE ^{1,2}	WE, % of their respective classes (unsaturated ¹), B	WE, % of the total WE pool ¹ , B. Formulas for estimations: (B=A×0.82) _{unsat} (B=A×0.18) _{sat}	WE, % of total lipids, C (C=B×0.41) ¹	WE, % of total meibum, D (D=C×0.78) ³	WE, % of the total WE pool (calculated using data of Nicolaides et al. ²)
618.6	C _{18:1}	i-C _{24:0}	19.7	16.1	6.6	5.2	14.9
620.6	ai-C _{17:0}	i/ai-C _{25:0}	28.6	5.1	2.1	1.6	30 (the ratio of these WE was not established by Nicolaides et al.)
632.7	C _{18:1}	i/ai-C _{25:0}	22.4	18.4	7.5	5.8	
646.7	C _{18:1}	i-C _{26:0}	35.3	28.9	11.8	8.6	21.5
660.7	C _{18:1}	ai-C _{27:0}	8.9	7.3	3.0	2.3	5.8
674.7	C _{18:1}	i-C _{28:0}	5	4.1	1.7	1.3	2.1
Sum of all shown WE, %				74.8	32.7	24.8	74.3

Major cholesteryl esters of human meibum						
m/z	Fatty acid residues found in Chl-E ^{2,4}	Chl-E, % of the total Chl-E pool, (our data ⁴), <i>E</i>	Individual fatty acid residues, % of all fatty acid residues in Chl-E ²	Chl-E, % of total lipids, <i>F</i> (<i>F</i> = <i>E</i> ×0.31) ⁴	% of total meibum, <i>G</i> (<i>G</i> = <i>F</i> ×0.78) ³	Chl-E, % of the total Chl-E pool (calculated using data of Nicolaides et al. ²)
707.7 + 681.6	C _{22:1} + <i>i</i> -C _{20:0}	7.7	3.5 + 6.6	2.4	1.9	3.5 + 5.6 (total 10.1)
735.7 + 709.7	C _{24:1} + <i>i</i> -C _{22:0}	11.1	7.3 + 5.3	3.4	2.7	7.3 + 5.3 (12.6)
763.7 + 737.7	C _{26:1} + <i>i</i> -C _{24:0}	13.6	3.6 + 11.7	4.2	3.3	3.6 + 11.7 (15.3)
751.7	<i>ai</i> -C _{25:0} + <i>i</i> -C _{25:0}	12.7	12.5 + 1.3	3.9	3.0	12.5 + 1.3 (13.8)
791.7 + 765.7	C _{28:1} + <i>i</i> -C _{26:0}	16.4	N/D + 13.7	5.1	4.0	N/D + 13.7 (13.7)
821.8 + 793.8	C _{30:1} + <i>i</i> -C _{28:0}	8.3	N/D + 2.2	2.6	2.0	N/D + 2.2 (2.2)
Sum of all shown Chl-E, %		69.8		21.6	16.9	67.7

¹From (Butovich et al., 2012a);

²from (Nicolaides et al., 1981);

³(McMahon et al., 2013);

⁴(Butovich, 2009a).

N/D – not detected; n/a – not available

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