Enhancement appears to be more likely if the two eyes share a line of threshold acuity and perhaps especially if the first eye assessed has the better acuity. In addition, routinely asking subjects to read from the top line increases the frequency of exposure to the letters and the level of passive memory that can be developed.

Consequently, if the best of multiple measures is enhanced by passive memory, any apparent deterioration at a subsequent examination may be partly due to the loss of letter subset memory. However, a retroactive interference method for the inhibition of passive chart memory has been shown to be effective.[5] The successful technique involved a sham acuity assessment with a near-point contrachart that had been constructed from the 16-letter subset that was not involved in the construction of the chart used for distance acuity assessment.[5] Reading just 4 lines of this contrachart resulted in memory scores that were not significantly different from the scores of control subjects (who guessed which letters would be involved, not having had recent exposure to any letter chart).[5] This result suggests that memory of the distance chart letter subset had been inhibited or neutralized during this 30-second procedure.[5] Use of such a contrachart may increase the reliability of acuity scores in clinical and research settings that involve multiple measures within a single examination session and/or multiple examination sessions over a short period. Control of passive letter subset memory might be achieved if the contrachart is presented at distance; however, near presentation of a handheld version can be more convenient.

Significant variations in individual letter readability and legibility[6,7] mean that using charts constructed from different letter subsets can introduce variations in chart and line difficulty. For example, for many chart designs involving 5 letters per line, these variations have been found to result in lines of the same nominal acuity level having different levels of difficulty, depending on the chance combinations of easy and hard letters.[6,7] Interval scaling and the potential to create alternative equivalent charts cannot be achieved using a 5-letters-per-line design, but can be achieved when a full subset of (10?) letters is used in each line.[6,7] Nevertheless, irrespective of chart design, retroactive inhibition of passive letter subset memory may eliminate memory-associated enhancement of best acuity performance and improve the reliability of the assessment of changes in visual acuity.

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## On the Presence and Role of Polar Lipids in Meibum

I read with interest the paper by Chen et al.[1] published online on July 29, 2010. The paper is concerned with shotgun (or direct-infusion) lipidomic analysis of normal human meibum by means of electrospray ionization mass spectrometry. I noticed with pleasure that Chen et al., along with providing new and important information on the meibomian lipidome, largely confirmed our results reported in articles published from 2007 to 2010 and presented at ARVO Annual Meetings from 2006 to 2010 with regard to the lipid composition of normal human meibum, especially its wax esters (WEs),[2–4] cholesteryl esters (CEs),[5,6] (*O*-acyl)-omega-hydroxy fatty acids [OAHFAs]),[3] and phospholipids.[2,3,7,8] I was also pleased that the authors arrived at the same idea that had been proposed, explained, and illustrated in a review paper published in 2009 about the putative role of OAHFAs as amphiphilic anionogenic compounds that may facilitate the spread of meibum on the ocular surface and thus be responsible for the tear film (TF) and TF lipid layer stabilization.[8] This function had been routinely ascribed to phospholipids, which, according to new data, apparently are not present in meibum in quantities sufficient to play such a role.

However, I could not help but notice a few items that I want to comment on, as they are important for future progress in this area of ocular science, and, if left uncorrected, could lead to confusion and erroneous physiological conclusions.

First, Chen et al.[1] claimed that they observed a large presence of free fatty acids (FFAs) in normal human meibum, specifically those with C20 to C28 chain lengths. Of importance, the mass spectrometry (MS) signals of these FAs were observed in direct-infusion experiments, which was the basis for the claim. There is no doubt that these signals were correctly identified as those of FAs. However, the origin of these FA signals in normal human meibomian lipid samples seems to have been misjudged. Contrary to the statement by Chen et al., I am confident that these signals of FAs originated from a range of more complex lipids (such as CEs, triacylglycerols [TAGs], and di- and triesters), due to the spontaneous in-source losses of FFA residues during the MS analysis (Fig. 1). This effect is well known in MS of TAGs (which are known to spontaneously produce strong signals of their deacylated derivatives) and was recently discussed and used to identify and quantitate various CEs in human meibum.[6] The in-source behavior of these lipids is such that under the conditions of positive and negative ion mode atmospheric pressure ionization MS, part of those complex lipid esters lose some of their FA residues, which then become visible as ions of FFAs. This loss may be further facilitated by ammonium hydroxide, which was a reagent of choice in the experiments of Chen et al. However, in no way can these data be interpreted as a confirmation of the presence of the stated amounts of FFAs in the meibomian samples (up to 3% of the meibum weight, per Chen et al.) and thus should be treated as an experimental artifact. Notably, the FA signals reported by Chen et al. closely match those of the CEs reported in our recent paper[6] and the data on CEs presented by Chen et al. themselves. The other major FA signals, those of C16- and C18-FAs in particular, most likely originated from TAGs and even more complex lipids such as di- and triesters. Corroborating this interpretation of the data of Chen et al. are the results of our HPLC-MS experiments, in which only minor signals of true FFAs were observed, with their total amount being typically $<0.1\%$ of

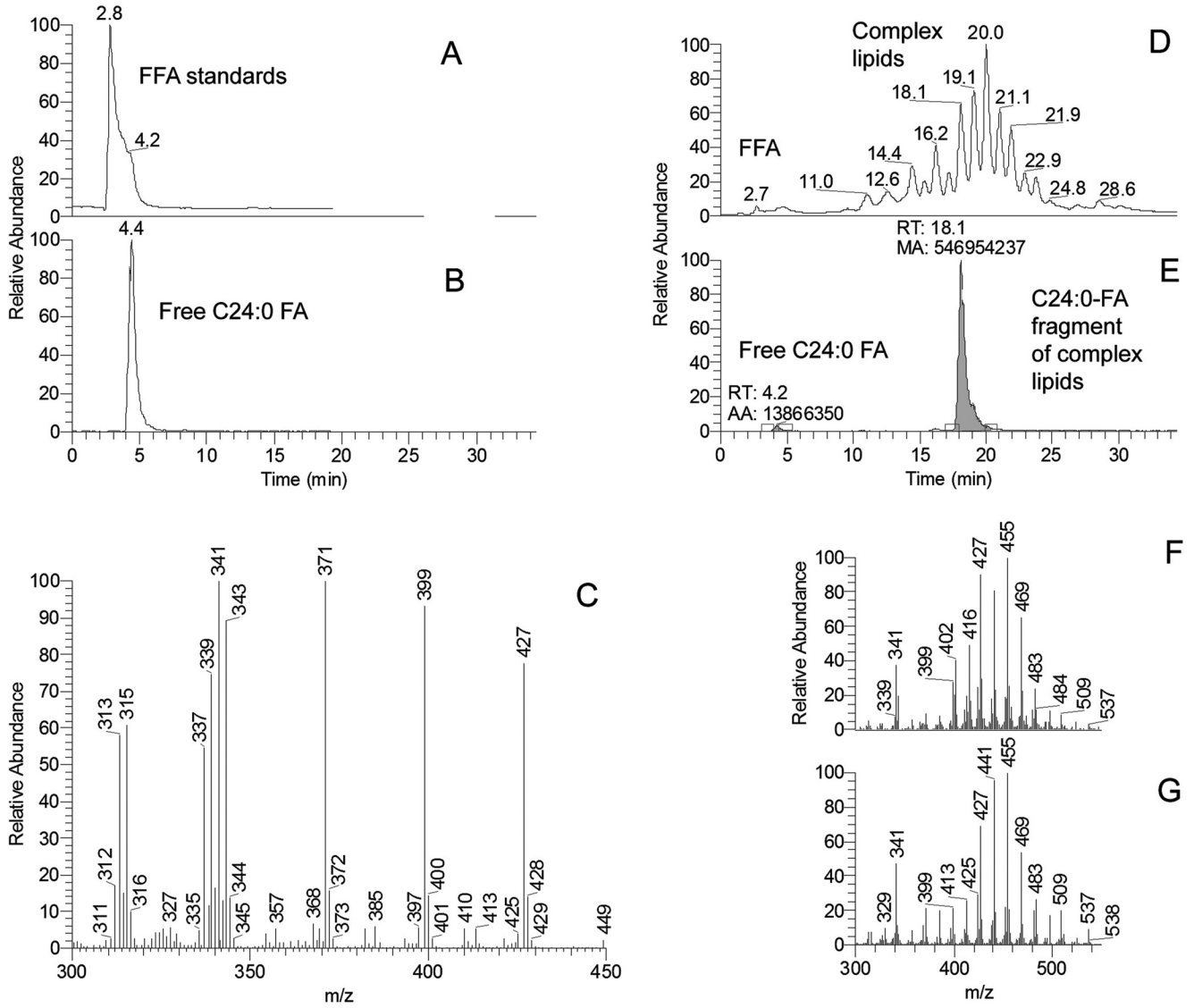


**Figure 1.** LC-MS analyses of human meibum and free fatty acids standards conducted in negative ion mode. Reverse phase HPLC separation of analytes on a $C_{18}$ column (Hypersil Gold, 2.1 × 150 mm, 5 μm) in an acetic acid–containing eluent with atmospheric pressure chemical ionization MS detection was conducted as described earlier.[6] (**A**) Total ion chromatogram of FFA standards recorded in the *m/z* range of 200 to 550. All FFA were detected as adducts with acetic acid ($M + CH_3COO^-$). Nine saturated and unsaturated FFA were tested as an equimass mixture: palmitoleic acid ($C_{16:1}$, *m/z* 313), palmitic acid ($C_{16:0}$, *m/z* 315), linolenic acid ($C_{18:3}$, *m/z* 337), linolenic acid ($C_{18:2}$, *m/z* 339), oleic acid ($C_{18:1}$, *m/z* 341), stearic acid ($C_{18:0}$, *m/z* 343), arachidic acid ($C_{20:0}$, *m/z* 371), docosanoic acid ($C_{22:0}$, *m/z* 399), and tetracosanoic acid ($C_{24:0}$, *m/z* 427). (**B**) Extracted ion chromatogram of tetracosanoic acid ($C_{24:0}$, *m/z* 427, retention time RT = 4.4 min). (**C**) Averaged mass spectrum of the chromatographic peak of FFA standards eluted between 2 and 6 minutes. (**D**) Total ion chromatogram of normal human meibum recorded for the *m/z* range of 200 to 550. Note extremely small HPLC peaks with RT of 2.7 and 4.5 minutes in the elution region of FFA, and very intense HPLC peaks in the elution region of complex lipids.[6] (**E**) Extracted ion chromatogram of ion *m/z* 427 (tetracosanoic acid, $C_{24:0}$) detected in meibum. Note that the ion eluted as two separate HPLC peaks: the first peak had RT of 4.2 minutes and coeluted with authentic tetracosanoic acid standard, while the second peak had an RT of 18.1 minutes, similar to those of cholesteryl esters.[6] Also note that the HPLC peak area of free tetracosanoic acid in meibum (peak area 13866350 arbitrary units) is only 2.6% of the one of tetracosanoic acid formed in-source from more complex lipids due to their spontaneous fragmentation (546954237 arbitrary units). The absolute amounts of true FFA in meibum were quantified using calibration curves obtained with authentic FFA standards. (**F**) Averaged mass spectrum of the chromatographic peak of meibomian FFA eluted between 2 and 6 minutes. (**G**) Averaged mass spectrum of the chromatographic peak of complex meibomian lipids eluted between 10 and 25 minutes. Note that the spectra presented in panels (**F**) and (**G**) are quite similar.

meibum (weight/weight), if present at all. We anticipated these problems with the inadvertent in-source fragmentation of complex lipids at the very beginning of our project. Thus, our HPLC-MS protocols differ from the shotgun experiments of Chen et al. in that the physical separation of meibomian lipids during the HPLC step occurred before the MS analysis. This approach allowed us to reliably distinguish between the signals of true FFAs present in meibum and those of other lipids, including those that are prone to spontaneous in-source fragmentation. In our experiments, the reverse-phase HPLC retention times of CEs, TAGs, and OAHFA (between 10 and 25 minutes) were dramatically different from those of the FFAs (retention times, ~3–5 minutes), which made their correct structural assignments an easy task. Clearly, without the HPLC separation step, Chen et al. could not unequivocally determine the origin of these FFA signals and described them as true FFAs present in meibum, rather than the in situ–

generated fragmentation products of more complex lipids. In addition, the HPLC-MS methods adopted in our laboratory are designed to provide quantitative results, as we use standard curves to quantify the analytes where possible (see, e.g., our recent paper on CEs[6]). Our HPLC-MS approach used for FFA quantitation showed that their total content in normal human meibum, though varying from sample to sample, was typically <0.1% (weight/weight).

Thus, the FA signals reported by Chen et al. most likely were in situ–generated artifacts—namely, products of the spontaneous in-source fragmentation of more complex meibomian lipids such as CEs, TAGs, and di- and tri-esters. This observation is critical for our understanding of the chemical composition of normal human meibum and the role of FFAs in tear film physiology and pathology.

Another related problem seems to be the presence of a mixture of isobaric C16:1- and C18:1-based WEs reported by Chen et al. According to their report (see Fig. 3 of their paper online), C16:1-WE could be present in quantities approaching those of isobaric C18:1-WE. This was not the case in our HPLC-MS experiments, in which the palmitoleic acid–based WEs were indeed detected, but in quantities typically below 10% of their C18:1-based counterparts. In fact, the typical presence of C16:1-WE was so small that an analysis was deemed to be unnecessary at the time. Again, the HPLC step greatly facilitated identification of the WEs. Thus, the claimed observation of the intense product peaks of C16:1-WE could be partly a result of the presence of unknown isobaric compounds (not necessarily of the WE nature) with palmitoleic acid in their structures that had not been chromatographically separated from WE before the MS analysis.

A few minor comments concern mostly the diligence in referring to the recent work done in the area. (1) The FA composition of meibomian CEs described by Chen et al.[1] closely matched the one that had already been described in an earlier paper on the topic,[6] where exactly the same major saturated FAs (C24:0, C25:0, and C26:0) were reported and quantified. (2) The possible role of OAHFAs, exactly as described by Chen et al., had already been described in a recent review.[8] Unfortunately, these two papers were not referenced and discussed by Chen et al.

In conclusion, it seems that the experimental approach described by Chen et al., though very informative, still has limitations that can lead to inadvertent but still erroneous interpretation of the data: In this case, a 10- to a 100-fold overestimation of the presence of endogenous FFAs and a large overestimation of the presence of C16:1-WE in normal human meibum.

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## Author Response: On the Presence and Role of Polar Lipids in Meibum

The truth often comes from debating. We are glad to discuss the issues Dr. Butovich et al. has raised about our paper.[1] We recognize Dr. Butovich's contribution to the identification and quantitation of wax esters and cholesteryl esters. However, it is important to gather information on all the major species in meibomian lipids.

The first comment was regarding the origin of the free fatty acids (FFAs) that we observed in normal human meibum. Dr. Butovich suggested that the FFAs we reported were from in-source decay of complex lipids, such as cholesteryl esters, triacylglycerols, and di- and triesters. We also thought about this when reading the paper of Butovich,[2] published recently (made available on the publisher's website on May 12, 2010, 1 month after the submission of our manuscript). We agree that the relative intensities of the FFAs of C20 to C28 chain length appear to match those of the cholesteryl esters with the same fatty acid moiety compositions (Fig. 3 of our paper[1]) and are somewhat similar to the fatty acid compositions of the cholesteryl esters reported by Butovich.[2] It is probably true that these fatty acids mainly originate from cholesteryl esters. It is also possible that considerable amounts of C18:0, C18:1, and C16:1 FFAs are from the dissociation of diesters or triacylglycerols. Currently, we are working on an alternative way for determining the amount of FFAs.

The second comment is about the amount of C16:1-based wax esters. We agree that for some wax esters (e.g., wax esters with chain length longer than C43), C18:1 is the dominant fatty acid moiety and that the C16:1-based wax esters are less than 10% of their C18:1-based counterparts. However, for meibum samples with wax esters of more than 40 different isobaric *m/z* values, the relative intensities of C16:1- and C18:1-based wax esters vary significantly. Depending on the *m/z* values of the wax esters, the quantities of some C16:1-based wax esters can be even greater than the isobaric C18:1-based wax esters (e.g., C40:2, C40:1, and C41:1), and their analysis should not be deemed to be "unnecessary." Overall, as we indicated in our paper, the relative amounts of C18:1- and C16:1-based wax esters were consistent with a previous report in which they accounted for 57.39% and 11.66% of the total wax esters, respectively.[3] In addition, the fragmentation patterns show that the structures of these isobaric species are those of wax esters and are very likely difficult to separate from their C18:1 counterparts with the resolution of HPLC separation that Dr. Butovich used. In fact, the extracted ion chromatograms of two wax esters in meibum as shown in a paper by Butovich et al.[4] (Figs. 3D, 3F; the latter corresponds to the same wax ester species as indicated in Fig. 3 of our paper) display peaks