Author Response: On the Presence of (*O*-Acyl)-Omega-Hydroxy Fatty Acids and Their Esters in Human Meibomian Gland Secretions

In the wake of our response[1] to his comments[2] on our recent paper,[3] Butovich followed with a second letter.[4] The title and contents of Butovich's letter relate to the presence of (*O*-acyl)-omega-hydroxy fatty acids (OAHFAs) and their esters in human meibum. He also discussed and criticized the shotgun mass spectrometry approach for meibum analysis. We would like to respond to these concerns.

Since the main topic of Butovich's comments was OAHFAs and their esters, we would like to discuss that issue first. We agree that there is no question of the existence of free OAHFAs. Our present study showed that complex lipids including cholesteryl esters were stable under our experimental conditions,[3] which suggests that the OAHFAs we detected were not from an in-source dissociation of ω-type I-St diesters or any other types of esterified OAHFAs (e-OAHFAs). We also found that the complex lipids of meibum were very stable in solutions with 1 mM ammonium acetate, and no significant hydrolysis was observed. It is known that esters can undergo hydrolysis in acidic or basic solutions. Although the stability of meibum lipids in ammonium hydroxide solution was not systematically studied, our analysis of meibum samples with ammonium hydroxide as the additive did show results similar to those with 1 mM ammonium acetate. For this analysis, we used a low concentration (10 ppm to 0.1%) of ammonium hydroxide as the additive and prepared the solution immediately before the analysis, to minimize possible hydrolysis. Butovich's new results shown in Figure 1A of his letter[4] confirmed our hypothesis[1] that OAHFAs can be formed from dissociation of e-OAHFAs under the same condition that fatty acids form from dissociation of cholesteryl esters.[5] Although Butovich showed that peaks of free OAHFAs were separated from those of OAHFAs dissociated from e-OAHFAs,[4] one question that remains from Butovich's results is whether there was a significant amount of free OAHFAs resulting from hydrolysis of e-OAHFAs during the injection. It seems unlikely, since the mobile phases were flowing through the column continuously, and the continuous hydrolysis would have left a long tail of chromatographic peaks. However, the hydrolysis may be affected by the solution composition and could be most significant at the beginning of the gradient.

Our recently reported estimate of free fatty acids (FFAs)[3] has been criticized by Butovich[2] in the previous letter as well as the current one and was used as evidence of the weakness of the shotgun mass spectrometry approach.[4] On the basis of his experience, Butovich suspected that the FFAs we detected were from in-source fragmentation of complex lipids. However, our experimental setup was different from his, including the ionization modes. He used atmospheric pressure chemical ionization (APCI), while we used electrospray ionization (ESI)—a softer ionization method.[6] Another important difference was the temperature used. We used 100°C to 150°C to help the ionization, whereas Butovich typically used 350°C. The dissociation of cholesteryl esters during chemical ionization has been reported previously, either in positive or negative mode.[7,8] Whether the APCI process has played a role in the dissociation is unknown; however, it is known that high temperature can cause significant fragmentation of intact lipids,[9,10] which may be one reason that Butovich observed significant dissociation in his experiments.[2] In contrast, in the experimental condition with much lower temperature and ESI, our recent study with standard esters including triolein, behenyl olate, cholesteryl stearate, and cholesteryl oleate showed that the dissociation was insignificant (<0.5%, if any). In addition, the estimated amount of FFAs reported by us (i.e., 3% of total lipids in meibum)[3] is in line with the 1% to 3% previously reported by three different groups.[11–13] The low concentration of FFAs claimed by Butovich (<0.1%)[2] may be due to the insensitive detection at the shorter retention time in their experiments (Fig. 1 of Ref . 5). Although the pattern of FFAs detected in negative mode seems to be similar to that of the fatty acid moieties in cholesteryl esters,[3] it does not necessarily suggest that these fatty acids must be from the dissociation. In fact, the pattern of the free OAHFAs that Butovich reported is also similar to that of the e-OAHFAs,[4] with the consideration of the high detection efficiency in negative mode at the later elution time (Fig. 1, Ref. 5). The similarity between the patterns may have occurred because these fatty acids were the precursors from which the esters were synthesized.

It is challenging to work with complex samples such as meibum. Both the shotgun method and LC-MS method have strengths and weaknesses. The LC-MS method has the advantage of adding one more parameter for identifying lipids; however, it takes a long time for separation and data analysis. In contrast, shotgun analysis is very fast and highly sensitive if performed appropriately. In the past, the species of complex samples could not be separated by mass spectrometry alone and had to be combined with separation approaches, such as GC and HPLC. The development of modern mass spectrometers makes it possible to detect different species directly. Compared to the ion-trap mass spectrometer used by Butovich, the Q-TOF mass spectrometer used by us has much higher resolution and mass accuracy, which makes the shotgun method appropriate. Therefore, investigators using mass spectrometers to analyze the lipids in meibum should take into consideration the resolution of the instrument used and experimental conditions when comparing results. In addition, modern mass spectrometers can add one more dimension, ion mobility, to separate ions based on their cross sections,[14] which may be of interest when analyzing complex samples such as meibum. Last, contrary to Butovich's claim, a direct-infusion system has fewer components than an LC-MS system; thus, we found it much easier to clean.

We are glad to see that Butovich[4] confirmed our observations that C16:1-based wax esters in some cases are of higher concentrations than C18:1-based wax esters with his LC-MS method. The trend of the higher ratio of C16:1-based wax esters for a low *m/z* wax ester may have some implications for the function of meibum, although the rationale is unknown at this time.

In summary, both free OAHFAs and FFAs, which could be important for maintaining the stability of tear film, exist in appreciable amount in meibum samples. High temperature may cause problems in the analysis of meibum lipids, and shotgun mass spectrometry has its own advantages for the analysis of meibum samples.

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