Exploratory *In Vivo* Biophysical Studies of Stratum Corneum Lipid Organization in Human Face and Arm Skin*

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*****We dedicate this paper to the memory of our colleague Dr Ken Walters who selflessly, and humorously, shared his insights and scientific expertise on skin delivery experiments with us on many occasions over the last 25 years.

Abstract

The purpose of this *in vivo* exploratory study was to investigate human stratum corneum (SC) lipid conformational order and chain packing in healthy face (cheek) skin as a function of stratum corneum depth using a combination of tape-stripping and horizontal attenuated total reflection Fourier transform infrared (HATR-FTIR) spectroscopy. Equivalent data were also collected from volar forearm skin as we, and others, have previously characterized forearm SC lipid order as a function of depth, therefore these data served as a comparison site and an experimental internal standard for the previously unmeasured *in vivo* face skin data. An SC depth profile was achieved by using tape strips to sequentially remove "layers" of SC. Trans epidermal water loss (TEWL) measurements were recorded following each sequential tape strip. *In vivo* HATR-FTIR spectra were collected after each tape strip, providing a depth profile of spectral data through the SC of both faces and arms. Spectral data were analyzed at five discrete SC depths corresponding to baseline and SC depths at which the increase in TEWL was 25, 50, 75, and 100 percent (%) from the baseline measurement. Analysis of the SC *in vivo* HATR-FTIR spectroscopic data utilized mean spectra generated by averaging the spectra from all panelists (n=10) at the five specific SC depths corresponding to when TEWL had increased by 25, 50, 75 and 100 % from baseline for each subject, respectively. While this is an exploratory study, and the data could be collated and processed in many ways, the average spectral data reveal clear trends in the face and arm SC. Increasing SC lipid order with depth is observed for face skin, as with arm skin, albeit with significant differences at all relative depths in the absolute lipid order between faces and arms. *In vivo* SC lipids are significantly more ordered at all depths in arms versus cheeks. The less ordered SC lipids of face skin can be in part attributed to the presence of a high amount of fluid sebaceous lipid species observed deep into the face SC. Interestingly, no evidence of orthorhombic packing is observed at any depth in face SC. This is consistent with high degree of disorder indicated from the chain fluidity measurements. These *in vivo* HATR-FTIR depth studies of face skin highlight how different SC barrier organization is in the face compared to the more widely studied body sites such as arms and legs. Further studies of the SC lipid barrier in faces are needed to understand the significant differences in lipid organization and its implication for the penetration of drug and cosmetic actives through face skin.

1. Introduction

Human stratum corneum (SC) provides the primary barrier function of the epidermis, preventing the permeation of external noxious agents and, as importantly, managing the loss of water from the body(Menon et al., 2012). The main lipid species of the SC are ceramides, fatty acids, and cholesterol (Moore and Rawlings, 2017)(Knox and O'Boyle, 2021). Most of the lipids in human SC are arranged into a mixture of orthorhombic and hexagonally packed lipid domains which coexist with more disordered (liquid crystalline) domains (Boncheva, 2014). It is well recognized, that human SC lipid organization and dynamics in the skin barrier are a function of chain conformational order, chain packing and head group hydrogen bonding interactions (Bouwstra and Gooris, 2014)(Bouwstra and Ponec, 2006) . A great deal of research continues to be focused on elucidating the complex molecular organization of SC lipids utilizing various biophysical methods, experimental models, and *in silico* simulations. In general, however, there are few published studies directed to measuring *in vivo* SC lipid organization and what previous research exists has focused primarily on changes in the SC lipid molecular organization on volar forearms (Bommannan et al., 1990)(Damien and Boncheva, 2010)(Yarovoy et al., 2019)(Boncheva et al., 2008). An early study suggesting the utility of attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy to study face skin and sebum was published by Nik Kollias and colleagues (Brancaleon et al., 2001). To our knowledge, there are no published studies directed to *in vivo* measurements of SC lipid organization as a function of SC depth in face skin with a comparison to forearm SC lipid organizational data from the same cohort of subjects.

In the current work *in vivo* HATR-FTIR spectra and trans epidermal water loss (TEWL) data were collected as a function of stratum corneum (SC) depth on face (cheek) and volar forearm sites. Sequential tape stripping was used to remove layers of SC and expose the deeper aspects of the SC. This work complements our previously published findings which focused on face barrier damage and recovery after 24 hours and 4 weeks after tape stripping and expands our recent work focused on barrier function and corneocyte morphology in face and arms (Gorcea et al., 2019, 2013b, 2013a).

Face skin, particularly cheek skin, is routinely exposed to cosmetic and pharmaceutical topical formulations containing actives and drugs intended to be delivered into, or across, the stratum corneum. In general, the vast majority of human skin *in vitro* permeation studies utilize torso or thigh skin. Therefore, it seems important to understand the differences that may exist in the molecular packing and chain fluidity of face SC lipids, as these are likely of importance to understanding and predicting the skin permeation of drugs and cosmetic actives in topical formulations applied to face skin. This communication

addresses the question of whether using an *in vivo* HATR-FTIR spectroscopy, tape stripping and TEWL methodology (previously utilized primarily for arm studies) could provide data of sufficient quality to provide insights into SC lipid dynamics at depth within face (cheek) SC. As the ATR-FTIR method samples the skin surface to a depth of 1-2 microns collecting spectra after each tape strip generates a spectroscopic depth profile into the SC. Previous studies by us and many others have used tape strip number, or the measured mass of protein removed with each tape, as the independent variable in SC depth studies. The current approach considers the % change in TEWL, which requires a different number of tape strips and does correspond to different absolute TEWL values for each subject, as the independent variable and asks whether useful trends are observed in the *in vivo* spectra. Specifically, this report explores whether there is utility in generating average (mean) spectra from all subjects (n=10) normalized to the % change in TEWL and using these mean data to analyze changes in SC lipid molecular dynamics as a function of depth.

2. Materials and methods

2.1. *Subject recruitment*

Ten healthy Caucasian subjects aged 29-56 years old (mean age 43 years, 4 females and 6 males) participated in this exploratory study, all signed consent forms prior to their participation. Subjects were instructed not to apply any cosmetic product to their faces and forearms for 24 hours prior to the study. The skin sites were not subjected to any special cleansing before commencing measurements and tape stripping. The study was conducted according to Camden and Islington Research Ethics committee approval (Reference 06/Q0511/26).

2.2. *Tape strips*

Adhesive tape strips (3M^{TM} Polyester Tape 8901, MN, US) were used to remove stratum corneum from arms and faces. This tape was used in previous studies as the infrared spectrum of its adhesive does not interfere with skin spectra. Spectra of tape strips were not collected or analyzed in the current work.

2.3. *Tape stripping*

Tape stripping was performed on the center of the face cheek (left side) and the center of volar forearms (left side). Sequential tape strips were collected until TEWL values had increased 100% from their respective baseline value (see below for the TEWL methodology). This required 5 - 6 tape strips for cheeks and 12 - 20 tape strips for volar forearms, depending on subject. For each anatomical skin site an

area of 7.5 cm^2 of skin was tape stripped to ensure that the *in vivo* cheek or forearm area placed on the ATR crystal has been completely tape stripped.

2.4. *TEWL measurements*

Tape stripping and TEWL measurements were performed at a room temperature of 21 ±1°C and 30 ±10% relative humidity. Subjects rested for 15-20 minutes before measurement collection to equilibrate with the room environment and minimize sweat gland activity. TEWL was measured with a closed chamber condenser instrument (AquaFlux AF -200, BioX, London, UK). TEWL measurements were performed at baseline and one minute after each tape strip was removed. TEWL data were processed with the AquaFlux software v.6.2 (Biox, London, U.K.). The % increase in TEWL (relative to baseline) was calculated for all subjects and skin sites following each tape strip. The subsequent data analysis which is presented in this report was limited to measurements collected at baseline and those corresponding to a 25, 50, 75 and 100% increase in TEWL. While "baseline" is a useful nomenclature for referencing the initial TEWL measurements, and the subsequent calculations of percent change in TEWL with tape stripping, it is noted that "baseline" corresponds to the very outer SC before the removal of any SC with tapes. For the *in vivo* HATR-FTIR spectroscopy measurements it is perhaps more useful to think of "baseline" as the outer most aspect of the SC (prior to any tape stripping).

2.5. *ATR-FTIR spectroscopy method*

To collect HATR-FTIR *in vivo* skin spectra subjects placed their upper left cheek or left volar forearm against the HATR crystal. Spectra were collected using an FTIR spectrometer (Nicolet™ 6700, Thermo, Madison, WI, US) equipped with a HATR Plus™ flat plate ZnSe crystal (Pike Technologies, Madison, WI, US) and a liquid nitrogen cooled mercury-cadmium-telluride (MCT) detector. Spectra were generated from the co-addition of 100 scans collected at 4 cm^{-1} resolution and processed with OMNIC™ 7.0 software (Thermo Electron, OH, US) and GRAMS 32/AI 7.0 software (Thermo Galactic, Salem, NH, US). Data plots were generated using Sigma Plot (Systat Software Inc., San Jose, CA, USA) and Excel (Microsoft Office, WA, USA). Spectra were collected following each tape strip thereby providing a depth profile of the SC in sequential IR spectra.

3. Results and discussion

Sequential tape stripping, followed by the collection of *in vivo* ATR-FTIR spectra, provides very high quality FTIR spectra at increasing depths into the SC. As noted above this measurement approach has been used to explore the molecular organization of SC in body skin. While tape stripping is a "destructive" technique it is very well tolerated by subjects. A host of additional biological and biophysical information can also be "extracted" from tape strips including compositional analysis of proteins, natural moisturizing factor (NMF) concentration, and corneocyte maturity. (Hughes et al., 2021)(Guneri et al., 2018) (Hulshof et al., 2019)

The focus of the current work was to apply the outlined *in vivo* experimental methods to face (cheek) skin and determine if the SC lipid organization in face skin was measurably different as a function of depth to the data reported for body skin (arms). As discussed above an average FTIR spectrum was generated by combining the spectra from all 10 subjects, this further increased the signal to noise ratio of the spectra. For the purposes of the current work the average spectrum was a useful approach for investigating *in vivo* depth changes in face skin. Given our experience with *in vivo* HATR-FTIR data from arms (and the published reports of others) we collected and analyzed *in vivo* arm spectra as a way to ensure our experimental methods were valid and provided the expected results.

The mean baseline (before tape stripping) face and arm spectra are shown in Figure 1. Several key peaks of interest are indicated including the two parameters that will be further interrogated in the current report, the $\nu_s CH_2$ and δCH_2 modes, sensitive primarily to chain fluidity and packing, respectively (Mendelsohn and Moore, 1998; Zhang et al., 2007). Several other differences between the spectra were noted although these are not explored further for this report; the major protein and lipid vibrational modes present in the spectra of skin have been tabulated in a 2013 review (Flach and Moore, 2013). The intensity ratio of the protein peaks (Amide I and II) to the lipid chain $\nu_{as} CH_2$ and $\nu_s CH_2$ region was very different, the much high lipid to protein ratio in the baseline (outer most SC layer) face spectrum was due to the presence of sebum (lipids) on face skin and not on arm skin. Further diagnostic of sebaceous lipids was the presence of the ester C=O and fatty acid C=O modes in the face spectrum. The sebaceous ester C=O peak was observed at all SC depths in face skin whereas it was only present at baseline in the arm spectra. This work did not focus on chemical composition or concentration profiles, but the sebum specific ester and acid peaks raise the possibility of generating relative concentration profiles of sebaceous lipids with SC depth by normalizing these peaks to protein. A more challenging possibility would be to determine the fraction of total lipid that is sebum within the SC as a function of depth and compare these data across body sites. Sheu et al., reported ultrastructural electron microscopy study of sebaceous lipids and the

mixing of sebaceous lipids with SC lamellae in the uppermost layers of SC and suggested that the surface sebaceous lipid film could be as thick as several microns in very sebum rich anatomical sites (Sheu et al., 1999).

As a first approach, the results obtained from processing individual spectra for the 10 subjects with those from the mean spectra were compared. Figure 2 shows the symmetric stretching methylene mode ($\nu_{sym}CH_2$) from both approaches at five SC depths corresponding to baseline, 25, 50, 75 and 100% increased TEWL. These data from arm spectra suggest that the approach of using an average spectrum reported the same information as processing the spectra individually. The $\nu_{sym}CH_2$ mode is sensitive to intra-molecular conformation (often referred to as chain fluidity); these data showed that arm SC lipids were highly conformationally ordered (< 2850 cm^{-1}) *in vivo* and this order initially increased with depth into the SC then remained constant until starting to sightly decrease again at a depth corresponding to 100% in TEWL (between 15-20 tape strips depending on the subject). This trend is consistent with previous reports using ATR-FTIR spectroscopy and indicated that at a certain SC depth lipid fluidity begins to increase near the boundary of the SC and the viable epidermis (Mendelsohn et al., 2006). A recent review discussed similar results for the profile of lipid conformational order across the SC obtained using confocal Raman spectroscopy (Darvin et al., 2022).

Superimposed average spectra showing the $\nu_{sym}CH_2$ peak are plotted in Figure 3 for cheeks and arms. It is evident that at all SC depths the face spectra had a higher value for $\nu_{sym}CH_2$ and the peaks were significantly broader, indicating more conformational chain disorder in the face SC. The values of $\nu_{sym}CH_2$ are plotted in Figure 3 and clearly show that at all SC depths the lipids in face (cheek) skin were significantly more fluid (less conformationally ordered) than arm skin, as indicated by the higher wavenumber values. Indeed, even at the lowest depths sampled in this work (100% increase in baseline TEWL) the SC lipids of the face were not conformationally ordered, i.e., remained fluid, and had already passed their maximum order of 2850.5 cm^{-1} at 75% TEWL. While there are reported differences in the amount and type of SC barrier lipids of face skin in sensitive skin the primary driver for this high lipid conformational disorder is the presence of sebaceous lipids in face SC which are observed at all SC depths of face skin (data not shown). It is interesting to consider the consequence of this much less ordered lipid matrix in face SC, compared to body (forearm) skin, in the context of skin barrier function and permeability. In combination with the smaller (less mature) corneocytes known to be present in face SC (Gorcea et al., 2019) a more fluid SC lipid matrix would lead to a less tortuous path for the permeation of materials through the SC. This is certainly relevant for skin permeation and the percutaneous penetration of drugs applied topically

to the face to treat local dermatological skin conditions. It is worth noting that there are conflicting reports in the literature on the importance of lipid packing and lamellar organization to barrier function. Bouwstra and colleagues reported that the permeation of benzoic acid through SC was not impacted by lipid packing i.e., permeability did not change with the transition from orthorhombic to hexagonal packing, however, Boncheva and colleagues did find a correlation to SC water permeability and lipid packing (Groen et al., 2011),(Damien and Boncheva, 2010).

Superimposed average spectra showing the δCH_2 spectral region are plotted in Figure 4 for cheeks and arms at baseline, 25, 50, 75 and 100% TEWL. The arm spectra clearly show peaks at 1472 and 1464 cm^{-1} sitting on the side of the main 1468 cm^{-1} peak for all spectra following baseline (i.e., the outer most SC). This suggests the presence of both orthorhombic and hexagonal lipid packing in the arm SC. In contrast, the average spectra from faces (cheek) displayed a single peak at ~1468 cm^{-1} without any suggestion of additional peaks with SC depth, suggesting very little or no orthorhombic lipid packing in face SC. To further elucidate the underlying spectral features, difference spectra were generated by subtracting the baseline (outer most SC) spectrum from that collected at an SC depth corresponding to a 25% TEWL increase. These differences spectra are also shown in Figure 4 for both arms and faces. In face skin the difference spectrum did not indicate any additional peaks indicative of crystalline orthorhombic packing. In contrast, and as previously reported, the difference spectra from arm skin showed a double of δCH_2 peaks at ~1472 and 1464 cm^{-1} corresponding to orthorhombic lipid packing within arm SC. Figure 5 presents the complete series of difference spectra for arm skin clearly showing the δCH_2 doublet resulting from the presence of orthorhombic chain packing was present at all SC depths in forearm skin, in marked contrast to face (cheek) skin. In addition, the plots of the peak frequencies for the both the hexagonal and orthorhombic packing in arm SC, and the single hexagonal/liquid peak frequency from arm SC, are plotted together in Figure 5.

Doucet at al., reported a gradient of hexagonal and orthorhombic packing across isolated abdominal SC using X-ray diffraction measurements, with the suggestion that orthorhombic packing was most abundant in the middle SC layers(Doucet et al., 2014). Our *in vivo* forearm data indicate that after the initial outer layers of SC, i.e., from the depth at which TEWL is increased 25% from baseline, orthorhombic and hexagonally packed lipids are both present at all SC depths.

4. Conclusions.

This exploratory study employed tape stripping, *in vivo* TEWL, and *in vivo* ATR-FTIR spectroscopy with spectral averaging from five depths within face SC, and the equivalent from arm SC for comparison, to probe molecular conformational order and chain packing within face (cheek) stratum corneum. No evidence of highly ordered chains, or crystalline chain packing, was observed at any depth within healthy face SC, indicating lipid organization in face SC is very different from SC at other body sites. Clearly there is still much to understand about the SC lipid barrier in face skin, however utilizing *in vivo* ATR-FTIR studies in combination with tape stripping and TEWL measurements, is a useful and productive approach. Future studies could employ the techniques used in this work to explore SC lipid molecular organization in face skin and how it changes as a function of disease and skin conditions, as well as the impact of topical therapies on the face SC barrier.

As this paper goes to press the authors note a new electron diffraction study is in press that explores stratum corneum structure as a function of body site and season. As with the vast majority of published studies this does not describe *in vivo* measurements nonetheless the authors report significant differences in lipid organization between forearm and face samples, consistent with the *in vivo* data in this work (Nakazawa et al., 2022).

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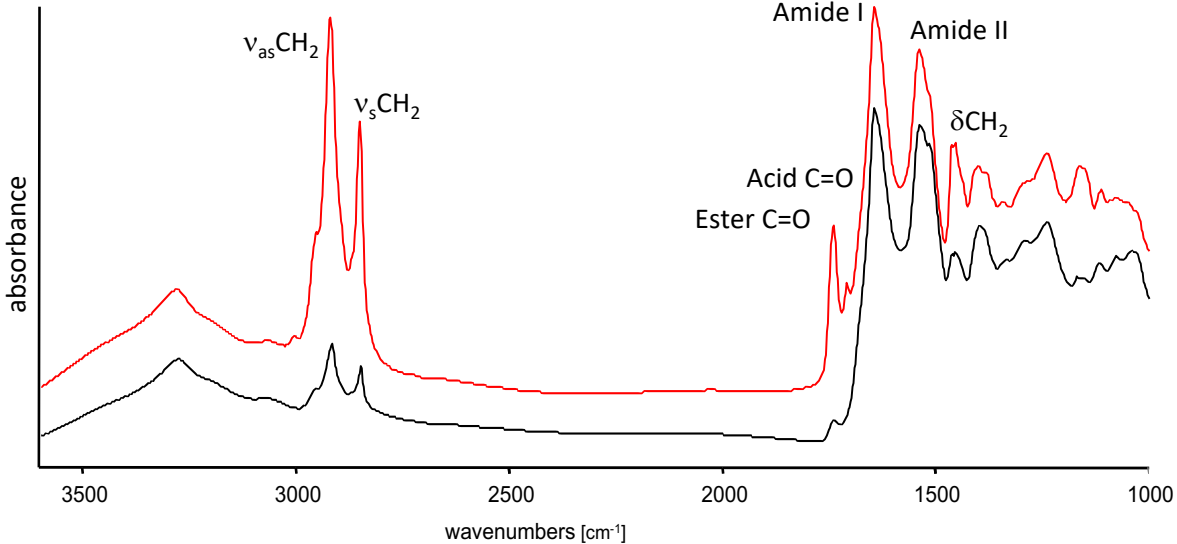
Figure 1. The mean ATR-FTIR *in vivo* spectra from face (red) and arm (black) collected at baseline (before any tape strips); some major spectral features are indicated. The clear spectral differences between these two skin sites at baseline are primarily due to the presence of sebaceous lipids on the face surface.

Figure 2. $\nu_{sym}CH_2$ value plotted as the mean +/- SD of 10 individual arm spectra collected at baseline compared to the $\nu_{sym}CH_2$ value from the single mean spectrum generated by averaging the same 10 individual spectra.

Figure 3. The left panel shows overlaid cheek (red) and arm (black) average spectra over the region 2880-2820 cm^{-1} at baseline and 25, 50, 75 and 100% TEWL. The right panels displays the ν_sCH_2 peak frequency plotted for cheeks and arms clearly showing the very significant difference in peak frequency between faces and arms.

Figure 4. The left panel displays the δCH_2 scissoring mode original spectral region (1474 -1462 cm^{-1}) for arms and faces SC depth corresponding to baseline, 25%, 50%, 75% and 100% increase in TEWL. The normalized average spectra have peaks at 1472 cm^{-1} and 1464 $cm^{-1}$of the CH_2 scissoring mode indicating orthorhombic and hexagonal packing coexist at all SC depths in the arm spectra (black). In contrast the face spectra (red) suggest only hexagonal/liquid packing is present in the SC of face SC. The right panels show difference spectra for arms and faces, resolving the presence of the 1472 cm^{-1} and 1464 cm^{-1} δCH_2 peaks in arm spectra and their absence in face spectra.

Figure 5. The left panel displays the δCH_2 scissoring mode difference spectra for arm skin following subtraction of the baseline spectrum at baseline, 25%, 50%, 75% and 100% increase in TEWL. The presence of the δCH_2 doublet indicates orthorhombic lipid phase packing is present. The right panel plots the frequency of the multiple δCH_2 modes for both skin sites as determined directly from *in vivo* spectroscopy shown in black for arm spectra and red for cheeks spectra.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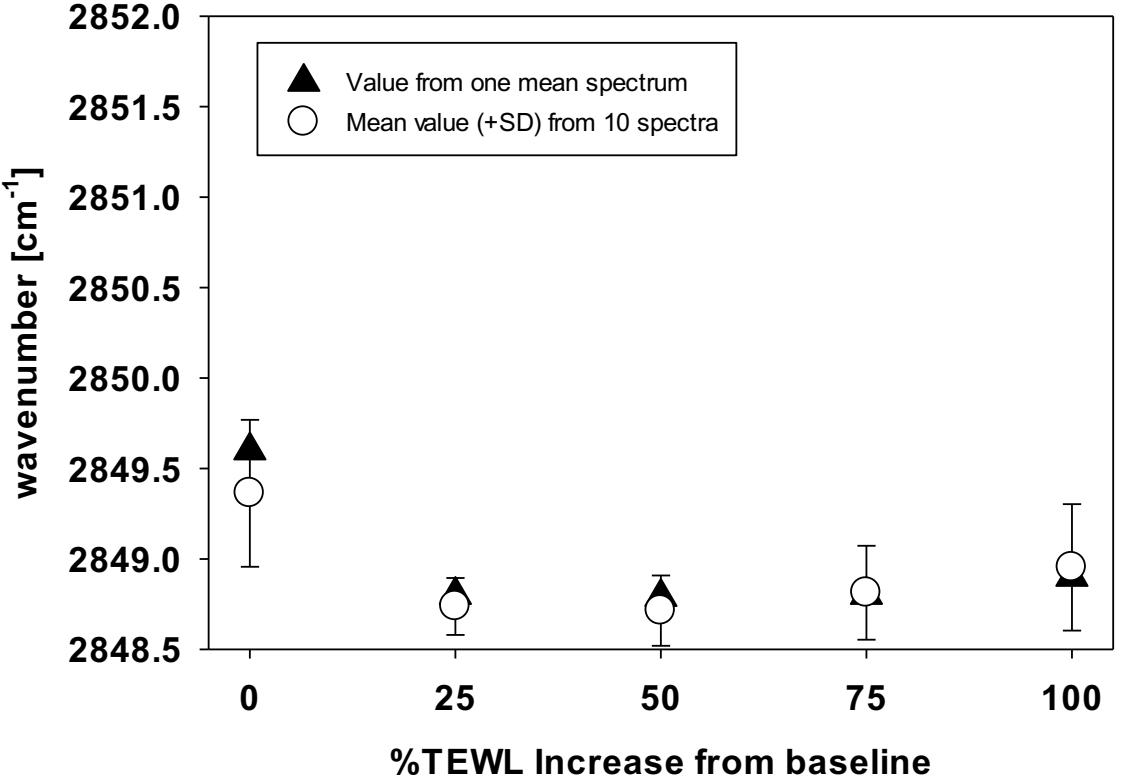
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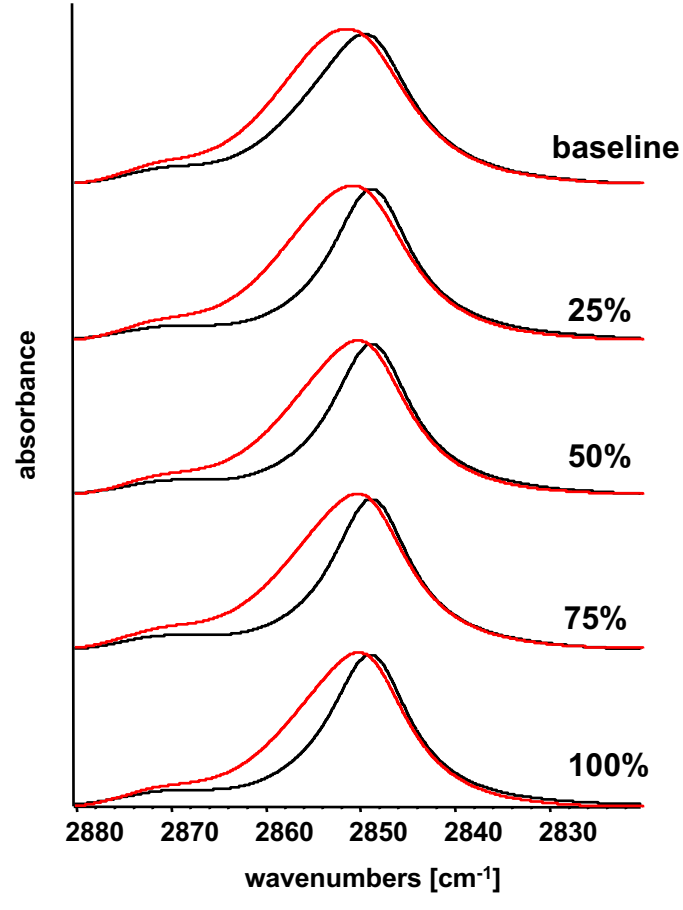
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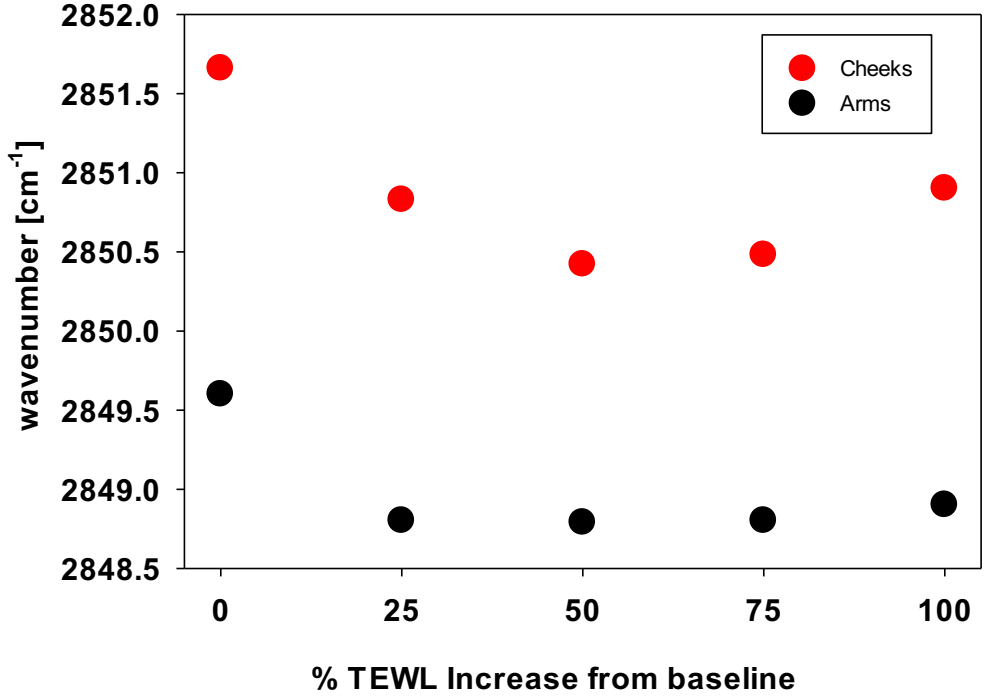
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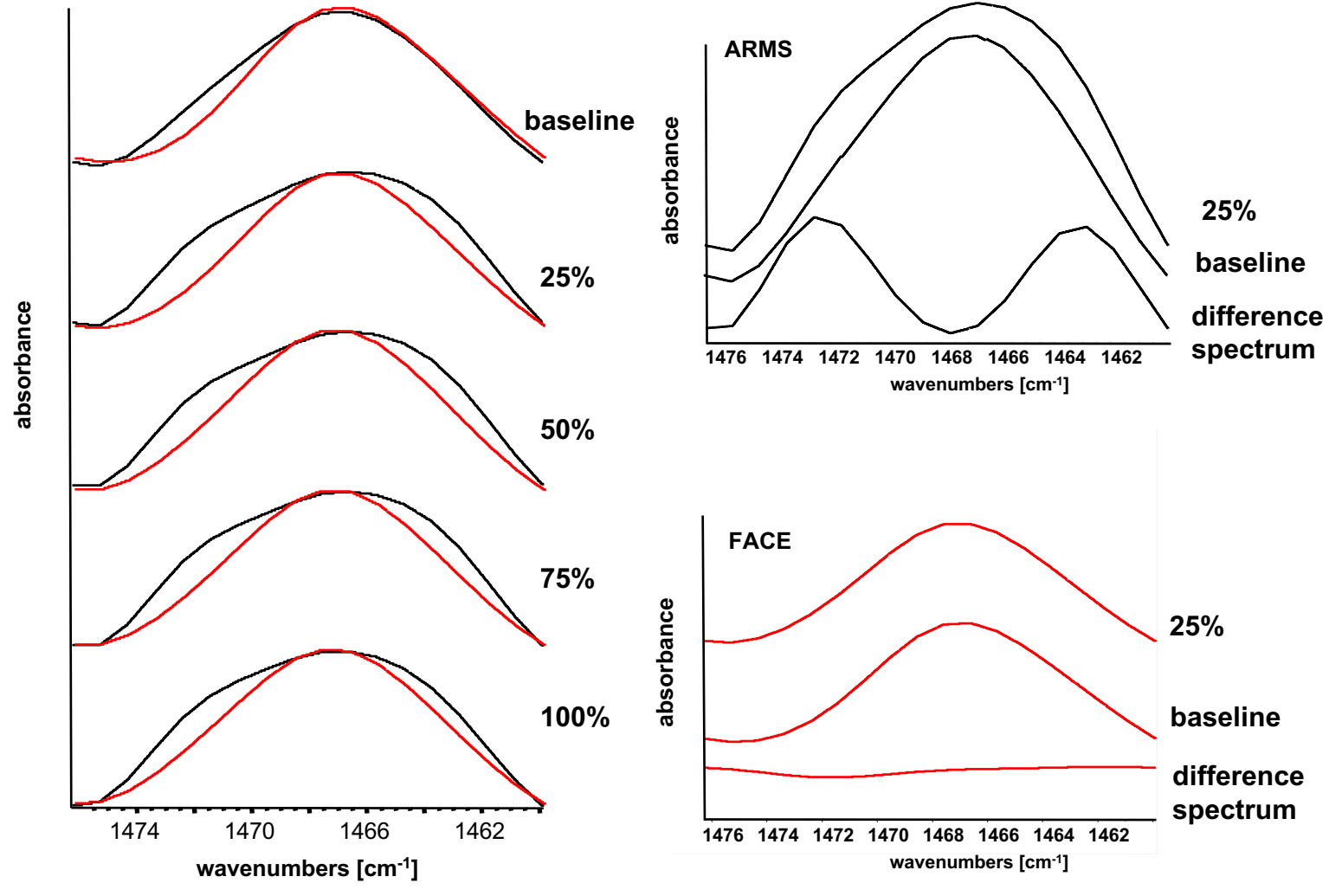
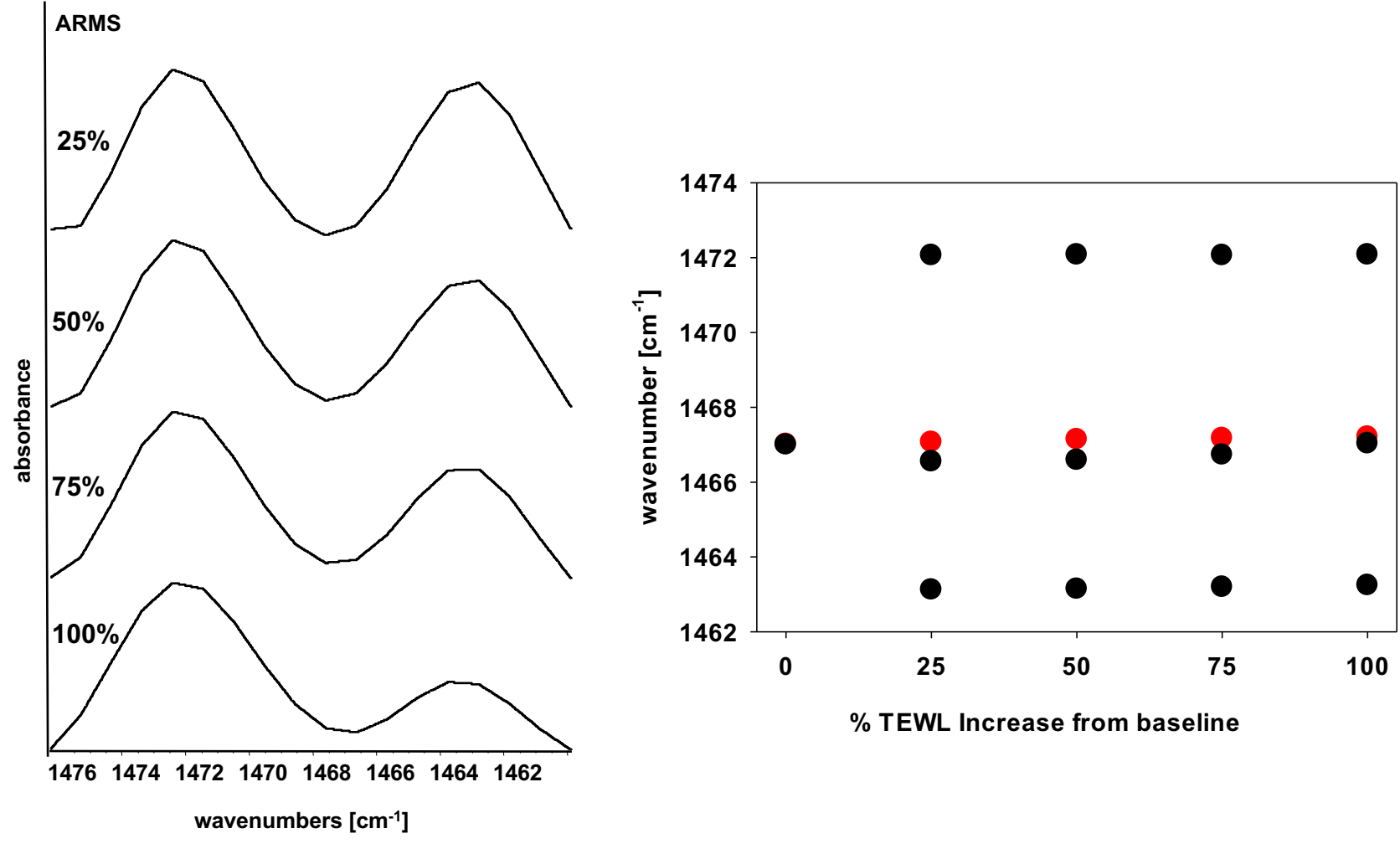
100
% TEWL Increase from baseline


absorbance
baseline
25%
50%
75%
100%
1474
1470
1466
1462
wavenumbers [cm-1]
ARMS
absorbance
25%
baseline
difference spectrum
1476 1474 1472 1470 1468 1466 1464 1462
wavenumbers [cm-1]
FACE
absorbance
25%
baseline
difference spectrum
1476 1474 1472 1470 1468 1466 1464 1462
wavenumbers [cm-1]


ARMS
25%
50%
75%
100%
absorbance
1476 1474 1472 1470 1468 1466 1464 1462
wavenumbers [cm-1]
1474
1472
1470
1468
1466
1464
1462
wavenumber [cm-1]
0
25
50
75
100
% TEWL Increase from baseline