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FELLOWSHIP FINAL REPORT

An investigation into the effect of freezing conditions on the barrier function of reconstructed human epidermis using Raman spectroscopy and percutaneous permeation

Yuri Dancik[1,2,*], Hichem Kichou[2], Christophe Eklouh-Molinier[2], Martin Soucé[2] , Emilie Munnier[2], Igor Chourpa[2] and Franck Bonnier[2,*]

[1] Le STUDIUM Institute of Advanced Studies, 1 rue Dupanloup, 45000 Orléans, France

[2] Faculté de Pharmacie, Université de Tours, 31 Avenue Monge, EA 6295 NanoMédicaments et NanoSondes, 37200 Tours, France

[*]Authors to whom correspondence should be addressed.

REPORT INFO

Fellow: **Yuri DANCIK**
From Singapore
Host laboratory in region Centre-Val de Loire: EA6295 NanoMédicaments et NanoSondes
Host scientist: **Franck BONNIER**
Period of residence in region Centre-Val de Loire: Oct. 2018 - June 2020

Keywords :
Reconstructed human epidermis, storage conditions, Raman spectroscopy, skin barrier, permeation, resorcinol

ABSTRACT

Reconstructed human epidermis (RHE) is an emerging skin model in pharmaceutical, toxicological and cosmetic sciences, yielding scientific and ethical advantages. RHEs remain costly, however, due to consumables and time required for their culture and a short shelf-life. Storing, i.e., freezing RHE could help reduce costs but little is known on the effects of freezing on the barrier function of RHE. We studied such effects using commercial EpiSkin™ RHE stored at −20, −80 and −150 °C for 1 and 10 weeks. We acquired intrinsic Raman spectra in the stratum corneum (SC) of the RHEs as well as spectra obtained following topical application of resorcinol in an aqueous solution. In parallel, we quantified the effects of freezing on the permeation kinetics of resorcinol from time-dependent permeation experiments. Principal component analyses discriminated the intrinsic SC spectra and the spectra of resorcinol-containing RHEs, in each case on the basis of the freezing conditions. Permeation of resorcinol through the frozen RHE increased 3- to 6-fold compared to fresh RHE, with the strongest effect obtained from freezing at −20 °C for 10 weeks. Due to the extensive optimization and standardization of EpiSkin™ RHE, the effects observed in our work may be expected to be more pronounced with other RHEs.

1- Introduction

Human skin equivalent (HSE) models are increasingly recognized as useful substitutes for human or animal tissue-based pharmacological and toxicological assays. HSEs have the potential to yield greater scientific and economic value, while at the same time avoiding ethical issues associated with use of animal tissues [**1**]. Reconstructed human epidermis (RHE) models are HSEs that recapitulate the epidermis specifically, that is, the *stratum corneum* (SC) and the viable epidermis of human skin. They are composed of normal human-derived keratinocytes seeded on a semi-permeable polycarbonate support membrane or a collagen or fibrin matrix. After an initial culture phase under liquid-covered conditions, a switch to culture at the air-liquid interface is performed, in which the apical (skin

Dancik, Y. ; Kichou, H. ; Eklouh-Molinier, C. ; Soucé , M. ; Munnier, E. ; Chourpa, I. ; Bonnier, F. An investigation into the effect of freezing conditions on the barrier function of reconstructed human epidermis using Raman spectroscopy and percutaneous permeation**,** *LE STUDIUM Multidisciplinary Journal*, **2020**, *4*, 79-88
https://doi.org/10.34846/le-studium.187.02.fr.06-2020

surface) side is exposed to air while the basal side of the culture remains in contact with the culture medium [2]. The exposure to air drives the corneocytes' differentiation and stratification, and hence the formation of a SC [3]. Common applications of RHEs mimicking normal human skin include *in vitro* toxicological assays, efficacy testing of topical actives and skin permeation studies [**2**]. Although recognized as possessing a significantly more permeable barrier than excised skin models [**4**,**5**,**6**], RHEs are useful for screening compounds and/or formulations. Ranking orders of penetration have been shown to be similar between RHEs and excised skin models [**7**,**8**]. High-quality RHE models are also useful for studies requiring lower biological variability than excised skin models [**6**]. A drawback of RHEs is the requirement that they be used soon after fabrication. The manufacturer of the commercially available EpiSkin™ RHE recommends use of the skin model within 48 h after reception. For in-house laboratory-grown RHEs with potentially greater intra- and inter-batch variability with respect to the formation of the skin barrier, internal recommendations may be even more stringent. Given the time frame in the order of 3 weeks to synthesize RHEs and, with regard to commercial RHEs, their significant costs, this requirement may severely limit the availability of RHE replicates for the testing or screening of topical compounds and/or formulations. This stands in stark contrast to excised skin, which is often stored at −20 °C for up to 3 months prior to use in permeability experiments. Despite the increasing prevalence of skin equivalents, the effects of storage conditions on their barrier function have not been extensively studied.

We sought to gain a deeper understanding of the effect of storage temperature and duration on the barrier function of RHEs using the commercially available EpiSkin™ RHE. We subjected the RHE to storage at −20, −80 and −150 °C for periods of 1 week and 10 weeks. Storage at −150 °C is meant to approach conditions of snap-freezing. The 10 week period was selected as a long-term storage condition similar to the 3 month period accepted for excised skin. In a first instance, we studied the intrinsic effects of these conditions on the SC of EpiSkin™ RHE samples by Raman spectroscopy (RS). RS constitutes a now widely accepted non-invasive biophotonic modality for the analysis of skin and percutaneous penetration. With respect to basic skin analysis, RS enables probing of several structural and compositional parameters which contribute to the barrier function of skin. These parameters include the lipid conformation and lateral packing order of SC lipids [**9**,**10**], skin hydration [**11**,**12**], differential water binding [**9**,**13**,**14**], SC keratin conformation, i.e., folding [**15**,**16**] and SC thickness [**17**]. These and other parameters have been investigated to probe differences in skin due to age, cutaneous diseases and the effects of topical formulation excipients. In addition to *in vivo* and *ex vivo* skin studies, RS is increasingly being applied to analyze reconstructed human skin equivalents and track chemicals therein [**18**-**23**].

We investigated the effects of the storage conditions on the permeation of resorcinol through the RHE using Raman spectroscopy as well as a time-dependent skin permeation protocol. Our results show that the SC barrier function is significantly altered as a function of freezing. Furthermore, different combinations of storage temperature and duration affect the barrier differently.

2- Experimental details

EpiSkin™ RHE (large model, 1.07 cm^2 diffusional surface area) replicates were purchased from EpiSkin (Lyon, France). EpiSkin™ RHE is a reconstructed epidermal membrane derived from human epidermal keratinocytes. The RHE are cultured and delivered in 12-well plates. In each well, a RHE replicate is supported by a matrix consisting of type I collagen which is coated with a thin layer of type IV collagen [**24**]. RHE were purchased at 13 days (J13) of maturity.

EpiSkin™ RHE Storage. Following instructions, upon reception in the laboratory,

the culture inserts containing the RHE replicates were removed from the 12-well plates and any remaining agarose was removed. The inserts were transferred under aseptic conditions and at room temperature to a new, sterile 12-well plate. Each well of the new plate was filled with 2 mL of fresh culture medium provided by the manufacturer. The RHE samples were then placed in an incubator (37 °C, 5% CO_2) overnight. Raman spectroscopy and the time-dependent resorcinol permeability experiment (see below) were conducted on the next day with the "fresh" RHE. The RHE replicates intended for freezing were equilibrated with 2 mL PBS placed in the receptor compartment of each culture well for 30 min, in order to remove any excess culture medium from the basal side. Following removal of the PBS, the RHE replicates were gently blotted dry and then stored at −20, −80, or −150 °C in their culture plates for either 1 or 10 weeks For spectroscopic characterization, n=2 EpiSkin™ RHE replicates were used per condition. For the resorcinol time-dependent permeation experiments, n=3 replicates were used per condition.

Non-Exposed EpiSkin™ RHE Preparation. After either 1 or 10 weeks of storage, the frozen RHE replicates were taken out of the freezers on the evening preceding the experiments and thawed overnight in a cold room (4 °C). On the next day, the RHE samples were removed from the cold room and equilibrated at room temperature for 30 min with 2 mL fresh PBS in contact with their basal side. Following equilibration, the RHE samples were visually inspected [**7**,**10**,**25**] for any defects. They were then removed from the culture inserts and carefully cut into 4 quarters with a scalpel. Each quarter was sectioned into 20 µm thin slices using a cryo-microtome (Leica CM 1850 UV, Nanterre, France). The slices were placed onto CaF_2 Raman-grade substrates (Crystran, Dorset, UK). These were stored at room temperature until spectroscopic analysis.

Permeation of resorcinol. On the day of the permeation experiment and following overnight thawing at 4 °C, the RHE samples were equilibrated at room temperature for 30 min with 2 mL fresh PBS in contact with the basal side of the RHE. Following equilibration, the integrity of the RHE was verified visually [**7**,**10**,**25**]. Permeation experiments were performed directly in the 12-well culture plates, without any handling of the RHE membranes, as in previously published studies with reconstructed skin equivalents [**22**,**26**]. RHE samples in their inserts were transferred to new sterile culture plates with each well containing 2 mL of fresh PBS as receptor solution. Resorcinol dissolved in PBS to a concentration of 5% *w*/*w* or 50 mg/mL served as the donor solution. The donor compartment consists of the insert into which the RHE was grown by the manufacturer. A volume of 200 µL of resorcinol donor solution was applied onto each RHE. The receptor compartment consists of the culture well in which the insert rests.

Permeation by Raman spectroscopy. The RHE samples remained exposed to the resorcinol solution for 12 h. At the end of the exposure phase, the donor solution was removed. The RHE and support membranes were carefully separated from the inserts using a punch. The donor (SC) side of the tissue and receptor side of the support membrane were gently swabbed with cotton tips to remove superficial resorcinol solution. The SC side of the tissue was then washed for 30 s with methanol to remove any resorcinol residue. The RHE tissue was then separated from the support membrane using tweezers. Each RHE sample was cut into 4 quarters with a scalpel. Each quarter was sectioned into 20 µm thin slices using a cryo-microtome (Leica CM 1850 UV, Nanterre, France) and the slices were placed onto CaF_2 Raman-grade substrates (Crystran, Dorset, UK). The substrates were stored at room temperature until spectroscopic analysis.

Analysis of Raman spectra. For each storage condition, spectra from each depth within each cryo-section were pooled and analyzed using the software Unscrambler® v. 11.0 (CAMO Software AS, Oslo, Norway). For each set of spectra from fresh and frozen RHE, following a linear baseline correction, unit vector

normalization was applied to remove multiplicative effects usually found in heterogeneous biological samples and arising from differences in the focus positions at which the spectra were acquired [**27**,**28**]. Principal components analysis (PCA) was performed on the mean-centered data using the Singular Value Decomposition (SVD) algorithm.

Time-dependent permeation in culture wells. At pre-determined times (every 0.5 h from 0.5 to 2.5 h and in 1 h intervals from 3 to 12 h) following application of the resorcinol solution, each RHE was transferred to a new well containing fresh receptor solution. After each transfer, the receptor solution in the used wells was collected for HPLC analysis. At the end of the permeation experiment, additional steps were performed for the determination of mass balances. The donor solutions were removed and set aside for analysis. RHE and support membranes were then carefully removed from the inserts using a punch and separated. The SC side of the RHE and receptor side of the support membrane were gently swabbed with cotton tips which were then immersed in 2 mL methanol for extraction and HPLC analysis. The SC side of the tissue was washed for 30 s with methanol to remove any resorcinol residue. Skin and support membranes were cut into small pieces and likewise immersed in 2 mL methanol for extraction and HPLC analysis.

Analysis of time-dependent permeation data. The solution to Fick's 2nd law of diffusion relating the permeant concentration in the RHE, C_{RHE}, to depth x in the RHE and time after application of the permeant was used. From this solution, the area-normalized cumulative amount Q_{RHE} at steady-state, written in terms of the steady-state flux J_{SS} and the lag time t_{lag} , is:

$$\frac{Q_{RHE}(t)}{A} = J_{ss}(t - t_{lag}) \quad \textbf{(1)}$$

The receptor solution concentrations obtained from the permeation experiments were converted to cumulative amounts using the formula

$$\frac{Q_{RHE}(t)}{A} = \frac{V_R}{A}(C_t + \sum_{i=0}^{t-1} C_i) \quad \textbf{(2)}$$

where V_R designates the receptor volume in the culture wells, C_t is the resorcinol concentration at each sampling time t, and C_i is the resorcinol concentration at prior samplings times i. Regression of Equation (8) against the linear part of the experimental cumulative amount time profiles obtained from Equation (9) yields J_{SS}, the lag time t_{lag} and the permeability coefficient $K_p = J_{ss}/C_d$.

Kinetic and mass balance results were analyzed with one-way analysis of variance (ANOVA) for multiple groups. Tukey's test was performed as a follow-up test to the multiple comparisons. The significance level was set at $p = 0.05$. Statistical analyses were conducted in GraphPad Prism v. 8 (GraphPad Software Inc., San Diego, CA, USA).

3- Results and discussion

The effects of freezing temperature and duration on the SC of EpiSkin™ RHE were investigated by Raman spectroscopy by comparing spectra from the interior of the SC of fresh, non-frozen RHE to those of RHE stored under the conditions described above. **Figure 1a** shows the well-defined SC and viable epidermis of a representative cryo-section of fresh EpiSkin™ RHE. A representative mean fingerprint region spectrum acquired at a depth of 2 µm inside the SC is shown in **Figure 1b**. The most intense peaks across the investigated depth (2 to 10 µm), occurring around 849, 933, 1002, 1126, 1297, 1440 and 1653 cm^{-1}, correspond to the major lipidic and protein components of human SC. The spectra of fresh EpiSkin™ RHE are also in good qualitative agreement with Tfayli et al.'s data of EpiSkin™ RHE [**22**].

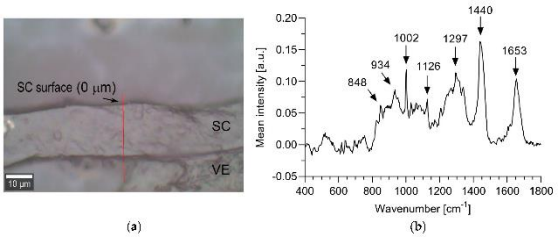

Figure 1 : (**a**) White light image of a representative section of EpiSkin™ RHE showing the stratum corneum (SC) and part of the viable epidermis (VE).

Spectra were acquired in increments of 2 µm along the red line. (**b**) Representative spectrum of SC showing major peaks assigned to SC lipids and proteins

The SC thickness at the locations at which the spectra were acquired was estimated to range from 16.6 ± 1.42 µm in the non-frozen EpiSkin™ RHE to 19.4 ± 2.08 µm in the EpiSkin™ RHE frozen at −80 ◦C for 10 weeks (**Figure 2**), with no statistical significance between the values. For reference, in vivo SC thicknesses measured by 2-photon microscopy in 20 healthy volunteers have been reported as 11.99 ± 2.13 µm in the abdomen, 12.55 ± 1.55 and 13.61 ± 1.43 µm in the volar and dorsal forearm, respectively, and 14.16 ± 1.9 µm in the sural region [**29**]. The SC thickness of excised human abdominal skin, frequently used in ex vivo permeation studies, has been determined to measure 13.2 ± 3.2 µm from histological sections [**30**].

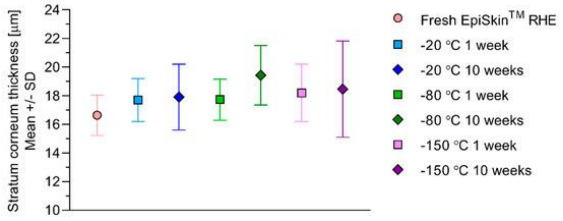

Figure 2: Stratum corneum thicknesses of fresh and frozen EpiSkin™ RHE estimated from the locations in the cryo-sections at which Raman spectra were acquired.

We employed PCA to explore whether the investigated storage conditions altered the composition or structure of the RHE's SC. **Figure 3** shows the scores and loading plots resulting from PCAs of fresh, non-frozen RHE vs. RHE stored for 1 week and 10 weeks at −20 (**Figure 3a,b**), −80 (**Figure 3c,d**) and −150 °C (**Figure 3e,f**). For each storage condition, these data encompass the totality of the spectra acquired at depths of 2 to 10 µm within the SC of the RHEs. The score plot of the fresh RHE vs. RHE stored at −20 °C show that discrimination based on the storage conditions occurs along the PC-2 axis. This discrimination accounts for 12% of the total explained variance. The loading corresponding to the PC-2 shows that the main peaks driving the discrimination between the fresh and −20 °C RHE spectra are the 1133 and 1435–1473 cm^{-1} peaks corresponding to proteins and lipids within the SC. Comparison of the fresh RHE spectra with that of RHEs stored at −80 °C yields inter-group discrimination along PC-2, accounting for 11% of the total variance (for visualization purposes, PC-2 is plotted against PC-3 in **Figure 3c**). The loading corresponding to PC-2 indicates that the discrimination is due to a preponderance of lipids and proteins in the fresh RHE compared to the ones frozen at −80 °C. The PCA of the fresh RHE vs. RHE stored at −150 °C resembles the PCA of fresh vs. −20 °C-stored RHE. Inter-group discrimination along PC-2 accounts for 13% of the total variance. The loading for PC-2 shows that this discrimination is driven by a preponderance of the non-specific protein and lipid peak around 1440 cm^{-1}.

From each of the PCAs, it is evident that the fresh RHEs are separate from the frozen ones on the basis of non-specific lipid and protein contents. Aside from the inter-group variance, there is significant intra-group biological variance, seen in the variances associated with PC-1. These variances range from 19% for the fresh RHE vs. the RHE stored at −80 °C to 28 °C and 29% in the comparison of fresh RHE vs. RHE stored at −20 and −150 °C, respectively.

To further probe the effect of freezing on the barrier function of EpiSkin™ RHE, we performed PCA on the spectra of EpiSkin™ RHE exposed to a 5% *w*/*w* aqueous PBS solution of resorcinol for 12 h. Resorcinol in aqueous solution displays particularly intense bands centered at 740 and 1000 cm^{-1}, which are associated with the molecule's aromatic ring vibrations (**Figure 4a**). A representative spectrum of EpiSkin™ RHE exposed to the same resorcinol solution for 12 h is shown in **Figure 4b**. The 740 cm^{-1} peak of resorcinol in powder form is consistently shifted to 748 cm^{-1} in the RHE tissue.

For each EpiSkin™ RHE, the Raman line scans yielded resorcinol intensities that were invariant

with depth (data not shown). This is attributable to the long exposure (12 h) of the RHEs to the resorcinol solution. As for the non-exposed RHE (**Figure 3**), PCA was used to explore the effect of storage on the absorption and penetration of resorcinol. For each RHE, the totality of the spectra acquired at depths of 2 to 10 µm within the SC were pooled to perform PCA. **Figure 5** shows the scores and loading plots resulting from PCAs of the resorcinol-exposed fresh RHE vs. RHE stored for 1 week and 10 weeks at −20 (**Figure 5a,b**), −80 (**Figure 5c,d**), and −150 °C (**Figure 5e,f**). On account of the overall high amounts of resorcinol contained in the tissues, discrimination between the fresh and frozen RHE along the PC-1 axes occurs due to the relative resorcinol intensities which dominate differences in the intrinsic tissue spectra. The corresponding PC-1 loadings show higher resorcinol intensities, demonstrated by the peaks at 748 and 1000 cm^{-1}, within the fresh RHE tissues compared to the frozen RHE tissues, driving the discrimination. While the 1000 cm^{-1} peak may partially be due to differences in the phenylalanine intensities in fresh vs. frozen RHE, it is likely mostly due to resorcinol, since phenylalanine was is not a major discriminant of fresh vs. frozen non-exposed RHE samples (**Figure 3b,d,f**). Differences in resorcinol intensities account for the greatest proportion of explained variance (44 to 52%) and are seen along the PC-1 axes. The less significant variance along PC-2 is due to intra-group differences in resorcinol amount and intrinsic tissue differences.

The time-dependent cumulative amount profiles of resorcinol permeated through the EpiSkin™ RHE tissues are summarized in **Figure 6**. All storage conditions yield a statistically greater cumulative amount of resorcinol permeated through the RHE into the receptor solution, compared to fresh RHE from 5 h after resorcinol application onward. After 12 h the cumulative amount of resorcinol in the RHE stored at −20 °C for 1 week, −80 °C for 1 and 10 weeks and −150 °C for 1 and 10 weeks ranges from 1.2 ± 0.15 to 1.6 ± 0.15 mg/cm^2. Within this group, the differences in the cumulative amount at 12 h are statistically non-significant except between the −20 °C 1 week and −80 °C 10 weeks conditions ($p = 0.0147$). On average, the cumulative amounts for the −20 °C 1 week, −80 °C (1 and 10 weeks) and −150 °C (1 and 10 weeks) conditions are three-fold greater than the cumulative amount in fresh RHE of 0.5 ± 0.11 mg/cm^2 (significantly different with $p \leq 0.0001$). The strongest effect on the permeation of resorcinol is obtained from the −20 °C 10 weeks condition. The cumulative amount for this storage conditions at 12 h is 2.9 ± 0.090 mg/cm^2, six-fold greater than the fresh RHE value (significantly different with $p \leq 0.0001$).

Tables 1a, b show the steady-state flux, permeability coefficient and lag time obtained from fitting **Equation (1)** to the cumulative amount data. Resorcinol fluxes through RHE stored at −20 °C for 1 week, −80 °C for 1 and 10 weeks and −150 °C for 1 and 10 weeks

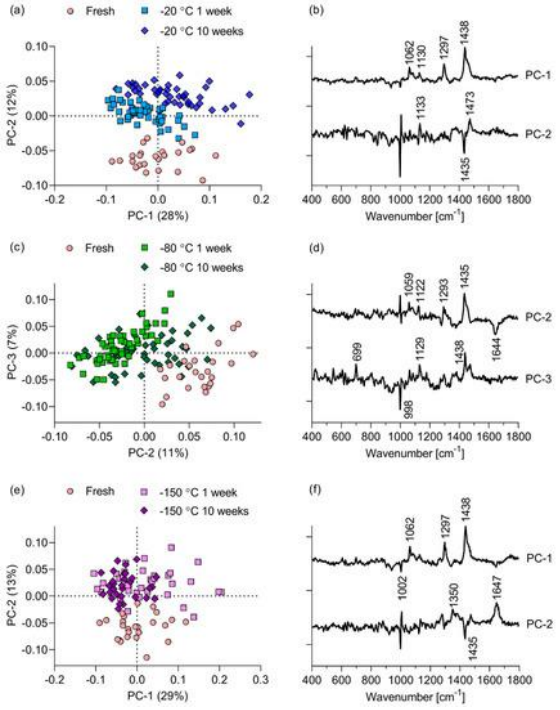

Figure 3 : Score and loading plots for the first 2 components obtained from the PCA of fresh EpiSkin™ RHE vs. the RHE stored for 1 and 10 weeks at (**a**,**b**) −20, (**c**,**d**) −80, and (**e**,**f**) −150 °C.

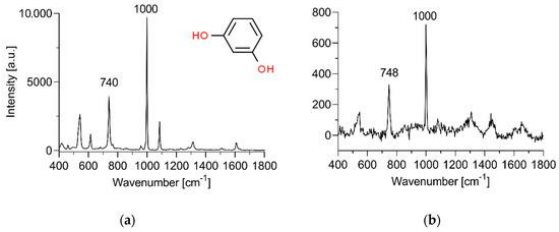

Figure 4 : Representative spectra of (**a**) resorcinol in PBS solution (5% w/w) and (**b**) the stratum corneum of EpiSkin™ RHE following a 12-h topical application of resorcinol in PBS solution (5% w/w).

average 0.17 mg/(cm^2 h), 2.3 times greater than the mean flux through fresh RHE (significantly different with $p \leq 0.0001$). The −20 °C 10 weeks storage conditions yields a mean flux of 0.29 mg/(cm^2 h), about four times greater than the flux through fresh RHE (significantly different with $p \leq 0.0001$). The lag times to steady-state are consistently smaller in the frozen RHEs compared to the fresh RHE. The mean lag times of the RHE stored at −20 °C for 1 week, −80 °C for 1 week and −150 °C for 1 and 10 weeks range from 2.6 ± 0.48 to 3.0 ± 0.54 h (not statistically different). They are 1.9 to 2.2 times shorter

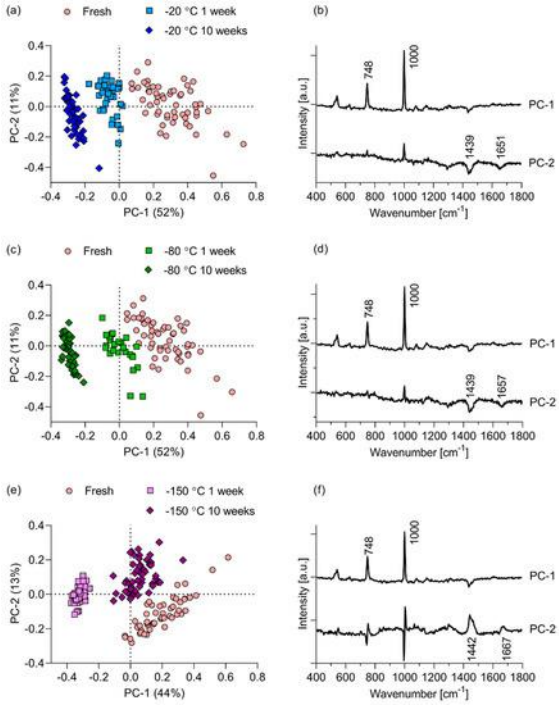

Figure 5 : Score and loading plots for the first 2 components obtained from the PCA of resorcinol-exposed fresh EpiSkin™ RHE vs. the RHE stored for 1 and 10 weeks at (**a**,**b**) −20, (**c**,**d**) −80, and (**e**,**f**) −150 °C.

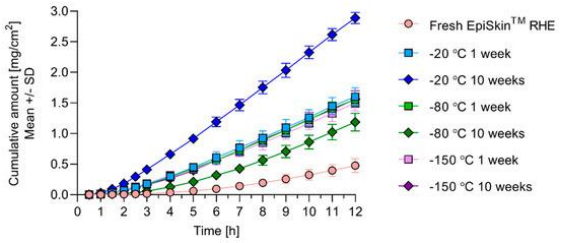

Figure 6 : Cumulative amounts of resorcinol permeated through fresh and frozen EpiSkin™ RHE.

than that in fresh RHE (significantly different with $p \leq 0.0001$). The mean lag time in the tissue stored at −80 °C for 10 weeks is about 20% smaller than in fresh RHE lag time (not statistically different). The −80 °C 10 weeks lag time is also significantly larger than the lag times of the −150 °C 1 week and 10 weeks conditions (p = 0.0095 and 0.0018, respectively) and the −20 °C and −80 °C 1 week conditions (p = 0.0017 and 0.0031, respectively). The lag time for the −20 °C 10 weeks storage condition is 1.8 ± 0.62 h, over three times shorter than in fresh RHE (significantly different with $p < 0.0001$). It is also statistically different from the lag time in the −80 °C 10 weeks condition ($p < 0.0001$).

Table 1a : Mean steady-state fluxes J_{ss} (mg/(cm^2 h)), permeability coefficients K_p (cm/h) and lag times t_{lag} (h) of resorcinol permeated through fresh and frozen EpiSkin™ RHE.

	Fresh	-20 °C		-80 °C	
Nbr. weeks ▶		1	10	1	10
J_{ss}	0.073	0.17	0.29	0.17	0.16
$K_p \cdot 10^3$	1.5	3.4	5.7	3.4	3.2
t_{lag}	5.6	2.6	1.8	2.7	4.6

Table 2b : Mean steady-state fluxes J_{ss} (mg/(cm^2 h)), permeability coefficients K_p (cm/h) and lag times t_{lag} (h) of resorcinol permeated through fresh and frozen EpiSkin™ RHE.

	-150 °C	
Nbr. weeks ▶	1	10
J_{ss}	0.17	0.17

$K_p \cdot 10^3$	3.3	3.3
t_{lag}	3.0	2.6

Donor solution depletion over the course of the permeation experiments was negligible, as verified during the analysis of the mass balance samples. The mass balances obtained at the end of the time-dependent permeation experiment. Overall resorcinol recovery ranges from 90 ± 0.4 to 99 ± 4.6% of the applied dose, in agreement with the OECD guideline for in vitro skin absorption experiments [**31**]. The percentage of applied resorcinol recovered in the fresh RHE tissue and support membrane samples is 1.4 to 3.5 times greater than the percentages recovered in the frozen tissues and membranes (in all cases statistically different with $p \leq 0.0007$).

4- Conclusion

The present study establishes that freezing alters the SC barrier function of the reconstructed human epidermis model EpiSkin™ RHE. Cumulative amounts of an infinite dose of resorcinol applied in aqueous solution are three- to six-fold lower in fresh EpiSkin™ RHE than in the same tissue stored at −20, −80 and −150 °C for 1 and 10 weeks. Among those conditions, storage at −20 °C for 10 weeks produces the most damage to the RHE's SC barrier function. As one of the most standardized and validated models available, it is reasonable to expect the EpiSkin™ RHE to be among the most resistant to external insults. We therefore expect the magnitude of barrier disruption measured herein to represent a lower limit, in particular when compared to most RHEs cultured in-house, for topical compounds exhibiting similar permeation kinetics to resorcinol applied in aqueous solution. Measured effects of freezing on the barrier function of reconstructed skin models depend on the composition of the SC, on the physico-chemistry of permeants of interest, and on the effect of the vehicles or formulations under consideration.

5- Perspectives of future collaborations with the host laboratory

Collaboration with the host laboratory has been ongoing, as evidenced by peer-reviewed articles published after 2020. In addition Y.D. is a recurring guest lecturer for the License Professionnelle: Formulation et Contrôle Qualité des Cosmétiques (FoQCos) led by Prof. Emilie Munnier at the University of Tours Faculty of Pharmacy.

6- Articles published in the framework of the fellowship

1. Bielfeldt S., Bonnier F., Byrne H.J., Chourpa I., Dancik Y., Lane M.E., Lunter D.J., Munnier E., Puppels G., Tfayli A., Ziemons E. (2022). Monitoring dermal penetration and permeation kinetics of topical products; the role of Raman microspectroscopy, TrAC Trends in Analytical Chemistry, Volume 156.
2. Kichou, H., Munnier, E., Dancik, Y., Kemel, K., Byrne, H. J., Tfayli, A., ... & Bonnier, F. (2022). Estimating the Analytical Performance of Raman Spectroscopy for Quantification of Active Ingredients in Human Stratum Corneum. *Molecules*, *27*(9), 2843.
3. Kichou, H., Dancik, Y., Eklouh-Molinier, C., Huang, N., Soucé, M., Gressin, L., ... & Bonnier, F. (2022). Highlighting the efficiency of ultrasound-based emulsifier-free emulsions to penetrate reconstructed human skin. *International Journal of Cosmetic Science*.
4. de Azevedo, J. R., Bolzinger, M. A., Briançon, S., Chevalier, Y., & Dancik, Y. (2021). Confocal Raman Microspectroscopy in the Assessment of Skin Barrier Function and Drug Penetration. In *Percutaneous Absorption* (pp. 799-836). CRC Press.
5. Dancik, Y., Kichou, H., Eklouh-Molinier, C., Soucé, M., Munnier, E., Chourpa, I., & Bonnier, F. (2020). Freezing weakens the barrier function of reconstructed human epidermis as evidenced by raman spectroscopy and percutaneous permeation. *Pharmaceutics*, *12*(11), 1041.

7- Acknowledgements

We gratefully acknowledge the financial support provided by the Cosmetosciences

programme and the Région Centre-Val de Loire (ARD 2020 programme).

The authors thank Françoise Debierre-Grockiego and the team “Immunologie Parasitaire et Vaccinologie, Biothérapies Anti-Infectieuses”, Université de Tours-INRAE, UMR 1282 Infectiologie et Santé Publique, for access to the cryo-microtome. Y.D. acknowledges the research fellowship awarded by Le STUDIUM Institute of Advanced Studies and Kamilia Kemel for assisting with experiments and for fruitful discussions.

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