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Comprehensive investigation of penetration ehnancers with complementary analytical techniques.

Komplexní analytická studie akcelerantů transdermální penetrace.

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DECLARATION

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ABSTRAKT

Transdermální aplikace léčiv je v posledních letech stále centrem zájmu. Pro účinnou transdermální aplikaci léčivo musí překonat vrchní bariéru kůže, stratum corneum. Pro usnadnění trasdermální aplikace mohou být vlastnosti stratum corneum pozměněny například aplikací akcelerantů transdermální penetrace.

Cílem této práce bylo charakterizovat vybrané akceleranty transdermální penetrace a zjistit jejich mechanismus účinku. Pro tyto účely byly využity různé techniky jako diferenciální skenovací kalorimetrie (DSC), infračervená spektroskopie (IR) a konfokální Ramanova spektroskopie (CRS) jako nová metoda ve výzkumu trasdermální aplikace.

Pro experimenty s akceleranty transdermální penetrace byly použity vzorky lidské kůže. Novým přístupem bylo využití metody lyofilizačního procesu poté, co bylo stratum corneum inkubováno akceleranty. Tímto postupem bylo zamezeno vzniku rozdílů, které mohou nastat při různém stupni hydratace vzorků stratum corneum.

V této studii byly vzorky kůže inkubovány dimethylsulfoxidem, propylenglykolem, ethylenglykolem, ethylenglykolem-d4 a olejovými kyselinami. Bylo zpozorováno, že některé akceleranty např. olejová kyselina, působí na lipidovou část stratum corneum, kdežto například dimethylsulfoxid způsobil změny také v konformaci bílkovin. Na závěr byl také prozkoumán možný synergický efekt akcelerantů s odlišným mechanismem účinku.

Souhrnně řečeno, byly úspěšně kombinovány různé analytické metody, které umožnily detekci akcelerantů s různým mechanismem účinku. Diferenciální skenovací kalorimetrie poskytla monitorování změn lipidové části stratum corneum. Infračervená spektroskopie umožnila detekci lipidové i proteinové části. Ramanova spektroskopie byla vyhodnocena jako vhodný nástroj pro prozkoumání bílkovinné konformace, ale detekce změn lipidů vyžaduje další šetření.

ABSTRACT

Transdermal drug delivery system is in the centre of attention in recent years. For efficient dermal drug delivery the drug has to overcome the barrier of the outermost layer of the skin, the stratum corneum. For facilitating dermal drug transport, the barrier properties of the stratum corneum can be varied by applying chemical penetration enhancers.

The aim of this work was to characterize various penetration enhancers and investigate their mechanism of action. We combined well established techniques like differential scanning calorimetry (DSC) and infrared spectroscopy (IR) with confocal Raman microscopy (CRM) as an upcoming technique in skin research. CRM offers the possibility of label-free and non-destructive, chemically selective analysis of stratum corneum lipids and proteins.

We used isolated human stratum corneum for incubation with the penetration enhancers. As a novel approach, the samples of treated stratum corneum were freeze dried to avoid any discrepancies which might come up with differences in the hydratation state of stratum corneum (SC). Furthermore, the structure of lipids and proteins in the stratum corneum was analyzed.

In our study, stratum corneum was treated with dimethyle sulphoxide, propylene glycol, ethylene glycol, ethylene glycol-d4 and oleic acid. We observed that enhancers like oleic acid mainly acted on lipid constitution, whereas others, for example DMSO caused changes in protein conformation. Finally the potential synergistic effect was investigated by a mixture of penetration enhancers with different mechanism of action.

To sum up, different analytical techniques were successfully combined and offered a comprehensive insight into various penetration enhancers' mechanism of action. Differential scanning calorimetry offered the possibility of monitoring changes in lipid structure and barrier properties of stratum corneum. With infrared spectroscopy the disruption of lipid and protein part was detectable but the spectra of protein peaks were similar for most of penetration enhancers. Raman spectroscopy was a suitable tool for investigation changes in protein conformation of stratum corneum while the detection of lipid changes needs further investigation.

1. INTRODUCTION

Drug delivery via the skin, either for systemic or for local therapy, bears a lot of potential for different therapeutically applications. The first transdermal system for systemic delivery was launched in the USA in 1976 as a three-day patch which delivered scopolamine for therapy of motion sickness. After several years also nicotine patches for smoking cessation were presented. Nowadays, several drugs for example estradiol, testosterone, lidocain, fentanyl are used in transdermal delivery systems not only in transdermal patches but also in ointments, creams, lotions and gels.[1,2]

Dermal delivery exhibits several advantages as avoiding the first-pass effect in the liver or reduced side effects. The application is in most cases non-invasive, painless and convenient for the patient and all these attributes may improve patient compliance.

However, drug delivery via the skin is constraint by some limitations. Only a small number of drugs can be administrated by this route, high lipophilicity, low molecular mass (<500 Da) and small dose (up to milligrams) are required. Another challenge is enzymatic degradation of the drug molecules within the skin, which can inactivate the drug. Furthermore, not every drug is convenient for skin application, as many chemicals cause irritation or sensitization of the skin. One of the major challenges in this field is still the application of large hydrophilic drugs.

The main barrier for dermal drug delivery is the outermost layer of the skin, the stratum corneum, which exacerbates skin absorption as well as penetration to the deeper skin layers. There was a strong need to develop strategies to overcome the skin barrier and facilitate absorption.

One of the possibilities to promote skin absorption is represented by penetration enhancers. Penetration enhancers are molecules which reversibly change the organisation of stratum corneum, which is mainly composed of proteins and lipids. Various analytical techniques were applied to analyse these changes and to elucidate the mechanism of action. [3,4]

Since the late 60s there are several reports which describe the investigation of the physical properties of the stratum corneum for example by differential scanning calorimetry (DSC). The first study aimed on distinguishing the bound and unbound water in stratum corneum.[5]

A comprehensive thermal analysis of the complete stratum corneum was conducted by

Barry and Van Duzze in 1975.[6] State of the art techniques for the analysis are DSC and attenuated total reflectance-Fourier transformed infrared spectroscopy (ATR-FTIR) which helps to understand the changes in stratum corneum structure.[7]

An upcoming technique for the analysis of human skin is confocal Raman spectroscopy which gained a lot of interest in recent years. In comparison to infrared spectroscopy water is not interfering with Raman experiments. Therefore, Raman spectroscopy is a suitable analytical tool for the investigation of biological samples and offers promising opportunities, like non destructive and chemically selective investigation of penetration and permeation studies for drugs. A study based on Fourier transformed Raman and infrared spectroscopy study on human skin was described in 1992 by Williams, Edwards and Barry.[8] Wartewig and Neubert published a report in 2005 which deals with the pharmaceutical applications of IR and Raman spectroscopy.[9]

The objective of the present study was to combine analytical techniques for a comprehensive investigation of the effect of different chemical penetration enhancers on human skin. The suitability of the individual techniques was evaluated. Furthermore, mixtures of different penetration enhancers were investigated to analyse potential synergistic effects.

2. THEORETICAL PART

2.1. Structure of the skin

The skin is the largest organ of the human body. The total size of the human skin is 1.8 m² in average. The skin represents the main physical and mechanical barrier protecting the body and it is also involved in thermoregulation and metabolism.

Within the skin several different anatomical structures coexist. The skin tissue can be subdivided into these tissue layers: epidermis, dermis and fatty tissue.

Epidermis

Epidermis can generally be divided in stratum corneum and viable epidermis.

The stratum corneum is 10-20 μm thick but can swell in hydrated state. It consists of cornified cells which consist of keratin accounting for about 5% of stratum corneum weight. These corneocytes are surrounded by various lipids as ceramides, cholesterol, cholesterol esters, free fatty acids or triglycerides in the intercellular regions. In total amount SC mass comprises 75-80 % proteins, 5-15 % lipids and 5-10% other material. 70% of protein fraction is represented by α-keratin, about 10% is β-keratin and 5% are cell wall envelopes. A well known model for the stratum corneum describes this unique structure as "brick and mortar" (figure 1).
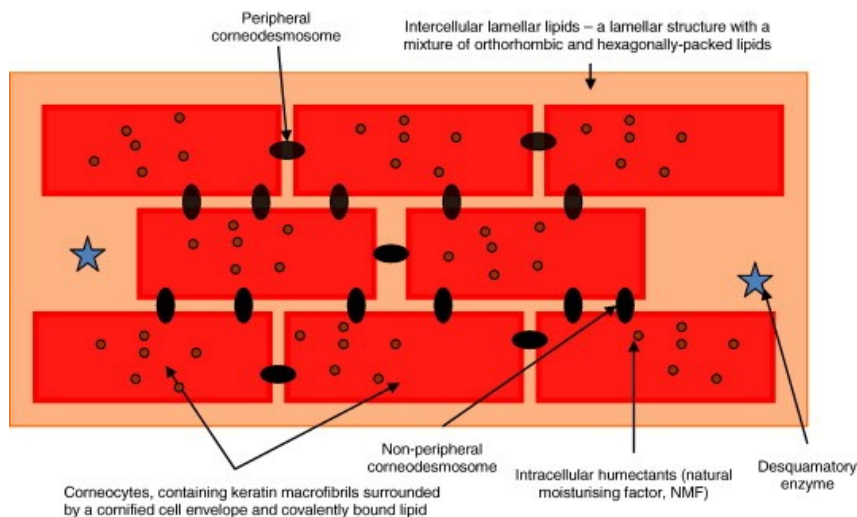

Figure 1: Structure of stratum corneum – brick and mortar model.[10]

The viable epidermis is formed by several layers; the stratum germinativum, the stratum spinosum, the stratum granulosum and the stratum lucidum, which can only be found at the palm of the hand and the sole of the foot. In the viable epidermis, melatonin is produced by melanocytes and Langerhans cells, of the immune system are localized.

Dermis

The thickness of the dermis is about 3-5 mm. This connective tissue is compound of collagen, elastin and reticulin which occurs in skin appendages. Furthermore, nerves, lymphatic and blood vessels are located there.

Subcutaneous fatty tissue

The subcutaneous fatty tissue is mainly composed of connective tissue with lipocytes. The thickness of this layer differs in each human individuum. The main functions of the fatty tissue are thermal isolation, mechanical cushion and the storage of energy.[11,12,13,14,15]

2.2. Analytical techniques for testing skin absorption

As in vivo skin absorption experiments are widely limited due to ethical, economical and analytical aspects, in vitro studies on human skin samples are considered to be gold standard for testing skin absorption. Different systems as excised human skin, animal skin or artificial model membranes were investigated to simulate in vivo conditions. Howe at al. showed a physiological hierarchy of in vitro methods according to their resemblance of the in vivo situation (figure 2).
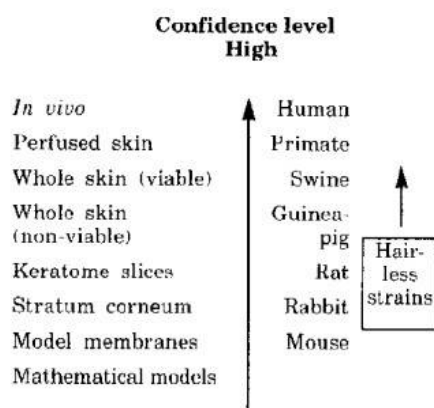

Figure 2: Hierarchy of different setup for measuring percutaneous absorption. [16]

In vitro analysis bears several advantages in comparison to in vivo studies. For example, the test conditions can be very well controlled, multiple experiments can be run at the same time and even toxic or harmful chemicals can be investigated. However, in vitro studies can not fully simulate in vivo conditions.

Animal skin was favoured for skin irritation and toxicity testing as the access to human skin samples is limited. Several animal species have already been used for the analysis of dermal drug absorption. Rat skin is mostly used the most, however, porcine skin is considered as the best substitute for human skin. Even though animal skin is easily available it differs from human skin in several aspects as stratum corneum thickness, number of hair follicles, water content and number of corneocyte layers. As these characteristics largely determine skin absorption, the correlation of animal skin data and the situation in human skin is difficult.

Human skin is generally considered to be gold standard for in vitro testing. Human skin samples usually stem from plastic surgery, amputations or cadavers. For in vitro testing abdominal, back, leg and breast skin is used.

After excision, fatty tissue is carefully removed and the skin is stored frozen at -20 to -30 °C. Skin samples may be stored for several months without changing their barrier function. However, thawing and freezing cycles should be avoided.

For in vitro testing full-thickness skin, dermatomed skin, separated epidermis or isolated stratum corneum are used (figure 3).

The main advantage of using full-thickness skin experiments is avoidance of additional modifications which may occur due to different way of storage, excision and experimental manipulation.

By cutting full-thickness skin using a dermatome, samples with different thickness can be obtained usually in the range of 200-600 μm. The sample contains stratum corneum, epidermis and small part of dermis.

Epidermis samples are obtained by complete removal of the dermis, which can be achieved by several mechanical, thermal and chemical techniques. The most frequently used method of heat separation placing the skin sample in water of 60°C for about one minute and then peeling of the epidermis layer.

For further separation, digestion of the connective epidermal tissue for 24 hours using trypsin in buffer solutions of ph 7.4 results in isolated stratum corneum. [17]
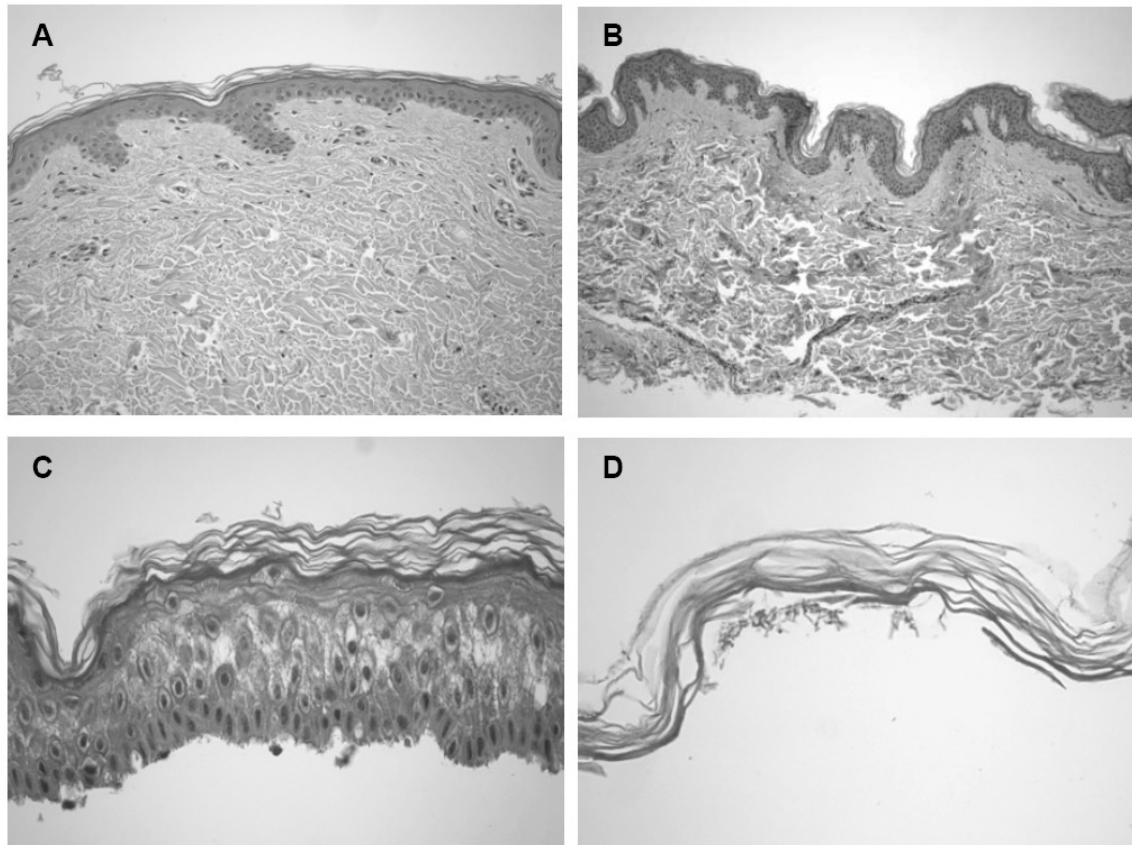
Figure 3: Cross-sections of (A) full thickness skin, x100; (B) dermatomed skin, x100; (C) heat-separated epidermis, x400 and (D) trypsin isolated stratum corneum, x400 (Images courtesy of Leon Muijs, Biopharmaceutics and Pharmaceutical Technology, Saarland University, Germany).

2.3. Penetration through the skin

Stratum corneum as a horny layer represents the major biological barrier and the passage is considered to be the rate-limiting step in the penetration process. For penetration through the skin there are two general options: the transepidermal route and the route via pores.

Transepidermal route

The transepidermal route is subdivided into transcellular and intercellular route. Transcellular transport requires penetration of the drug through the lipid barrier of the SC as well as the cytoplasm of dead corneocytes. Thus, the drug has to penetrate lipophilic as well as hydrophilic structures. In contrast, for intercellular transport, which occurs more often, the drug has to penetrate through the intercellular room between the corneocytes. Polar

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drugs can penetrate by the transcellular route. However, as most drugs are non-polar, the main pathway for transport is the intercellular route. However, for most penetrators of intermediate polarity a variety of routes depending on skin condition and diffussional time-scale is suggested.

Intercellular and transcellular ways of absorption are shown in figure 4.
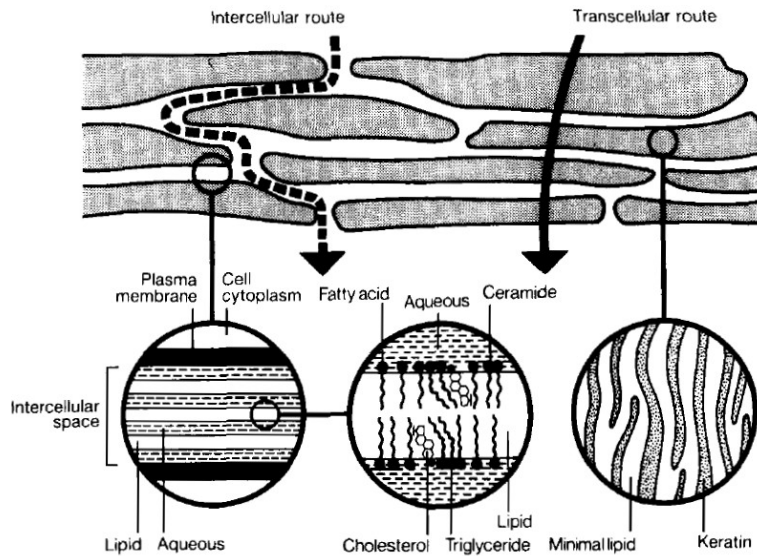
Figure 4: Ways of skin absorption.[18]

Penetration through appendages

Even though appendages cover just 0.1% of the skin surface, recent publications emphasised the penetration via appendages especially, for the absorption of liposomes and nanoparticles. Many factors affect this pathway; skin metabolism, density of follicles and sweat glands, thickness of horny layer, physiochemical properties of the drug and the time-scale on penetration.

2.4. Chemical penetration enhancers

To promote skin penetration and reduce the barrier properties of the stratum corneum, chemical penetration enhancers were identified or even developed. These enhancers are molecules which reversibly decrease the barrier function of the stratum corneum and facilitate drug penetration into the skin tissue.

Ideal penetration enhancer should exhibit the following properties:

1) No toxic, allergenic or irritating potential.

2) Reversible alteration of the skin barrier properties.

3) The barrier function should be reduced in one direction only.

4) The duration of its effect should be predictable and reproducible.

5) The substance is supposed to be pharmacologically inert.

6) The substance should be inexpensive, odourless, tasteless and colourless.

7) The enhancer should be compatible with many drugs and pharmaceutical adjuvants.

8) Incorporation into formulations like lotions, suspensions, creams, gels, skin adhesives should be possible.

So far, no penetration enhancer has yet been discovered exhibiting all these properties, although some chemicals demonstrate several of the above mentioned qualities. [16,19]

The stratum corneum consists of a complex composition of different components. The major fraction is formed by neutral lipids (78%) and sphingolipids (18%) with a small amount of polar lipids. Non-polar material as squalene and n-alkanes are represented with 11% fraction. Many lipids in the horny layer form bilayer structure as figure 5 depicts.
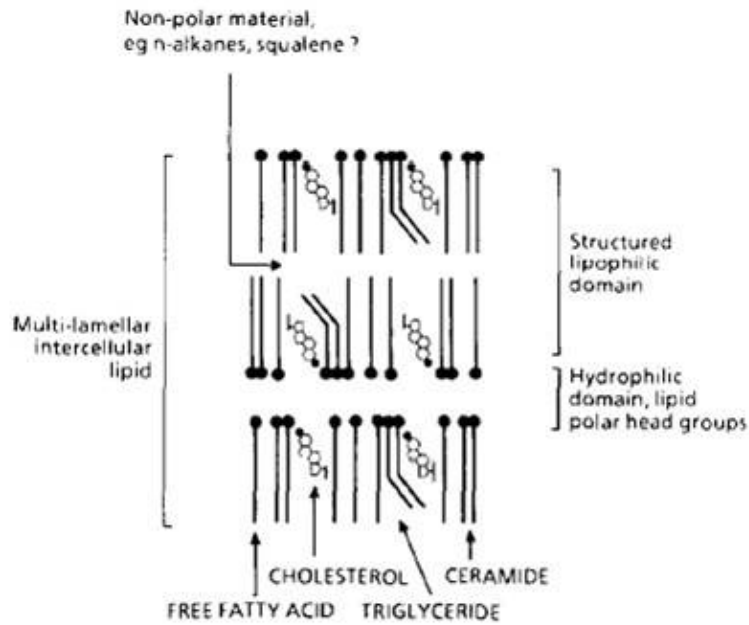
Figure 5: Suggested model representing the structure of lipid bilayers within the intercellular spaces of human stratum corneum. [3]

In previous studies it was postulated that the mechanism of action of penetration enhancers is based on their molecular locations as shown in figure 6 below. Accelerants may interact with the polar head groups of the lipid (site A), within the aqueous region between lipid leaflets (site B), and between the hydrophobic tails of the bilayer (site C).
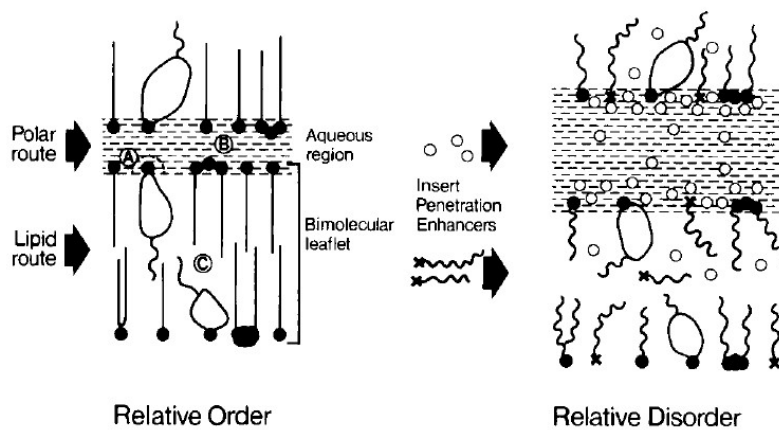

Figure 6: Suggested sites for penetration enhancers to act in the intercellular space of the stratum corneum. There is a change from relative order to disorder on insertion of accelerants. Small circles represent polar solvents such as DMSO, 2-pyrrolidone and

propylene glycol; linear chains represent azone and bent chains correspond to cis-unsaturated oleic acid.[4]

In this study dimethylsulphoxide, propylene glycol, ethylene glycol, deuterated ethylene glycol, oleic acid and a mixture of dimethylsulphoxide with ethylene glycol, oleic acid respectively were investigated with respect to their penetration enhancing properties. To detect changes in stratum corneum lipid structure, techniques of differential scanning calorimetry, infrared spectroscopy and Raman spectroscopy were combined.

3. EXPERIMENTAL PART

3.1. Materials

Dimethylsulphoxid, propylene glycol, ethylene glycol, ethylene glycol-d4, oleic acids were ordered from Sigma-Aldrich, Stenheim, Germany. Human skin was obtained from plastic surgery of female Caucasians from the Department of Plastic and Hand Surgery, Caritaskrankenhaus, Lebach, Germany.

3.2. Methods

Skin punches of about 25 mm diameter were gained from frozen skin and then placed on filter paper soaked with distilled water. Thereafter, the skin samples were heat separated. Full-thickness skin was incubated water of 6O°C for 90 seconds. Then the skin was removed from water, placed on filter paper and the dermis was peeled of with forceps.

For isolation of stratum corneum, the epidermis was incubated in a 0.15 % trypsin solution in phosphate-buffered saline (composed of 0.2mg potassium chloride, 8.0g sodium chloride, 1.44 g disodium hydrogen phosphate dihydrate, 0.2 mg potassium dihydrogen phosphate in a litre of purified water).

For complete separation incubation for 24 hours in this solution at 32°C is needed. Then, the stratum corneum was washed 3 times with purified water and dried with filter paper. After this step the stratum corneum was placed in a Petri dish filled with the penetration enhancer for at least 14 hours. After that, the sheet of stratum corneum was placed on a Teflon sheet and freeze dried for 48 hours.

3.3. Instruments

Differential Scanning Calorimetry (DSC)

For the thermal characterisation of the skin samples, differential scanning calorimetry was performed (DSC Q100, TA Instruments, USA). For each measurement, we used 6-10mg freeze dried stratum corneum or heat separated epidermis and 10-15 mg for non freeze dried samples. The samples were placed into hermetically sealed aluminium T-zero pans (Hermetic Pans, TA Instruments, USA) and analyzed in the temperature range of 20 – 150°C with a heating rate of 10 °C/min under nitrogen flow.

Fourier transformed infrared spectroscopy

Infrared spectra were acquired using an attenuated total reflectance unit (Spectrometer 400 ATR-IR, Perkin Elmer, USA). The skin samples were placed on the ATR-FTIR with the SC facing the ATR crystal. Spectra were recorded in the range of 500-4,000 cm^{-1} with 4 accumulations for each measurement.

Confocal Raman spectroscopy

For Raman analysis, the sheets of skin were fixed on glass slides. Raman analysis was performed with a confocal Raman microscope (alpha300R+, WITec GmbH, Ulm, Germany). Raman spectra were collected in the range of 400-1780 cm^{-1} with 10 seconds acquisition time with 10 accumulations. The data were processed with WITec Project Plus software (WITec GmbH, Ulm, Germany).

4. RESULTS AND DISCUSSION

4.1. Differential Scanning Calorimetry (DSC)

DSC is a well established technique to investigate phase transitions of skin components. In previous studies, the measurements were performed within the temperature range of 20-110 °C. Untreated skin exhibits four main thermic events (T_1-T_4) at 40 °C, 75 °C, 85 °C, 107 °C.[5, 6, 20, 21, 22, 23]

The first peak which occurs around 40 °C is attributed to melting of sebaceous lipids or to lipid lamella phase transition from a crystalline to a gel like phase.[3,7] With increasing temperature, the lipid gel-like phase changes to a more liquid state.[7] Two peaks are reported corresponding to a disorganization of the lipid chains in bilayer structure (T_2 around 75 °C) and as a complete disruption of any associations within the lipid polar head region (T_3 around 85 °C).[3] Furthermore, keratin denaturation can be detected as a thermic event in the temperature range of 105 – 120 °C.

4.1.1. Stratum corneum

Before every analysis samples of untreated and treated SC were freeze dried for 48 hours. This procedure should avoid any discrepancies which might come up with different hydratation states of SC. Comparison of fresh heat separated epidermis, SC stored in desiccator and freeze dried SC is shown in figure 7.

The intensity of the heat flow which is detected was influenced by the weight of the sample (figure 8).
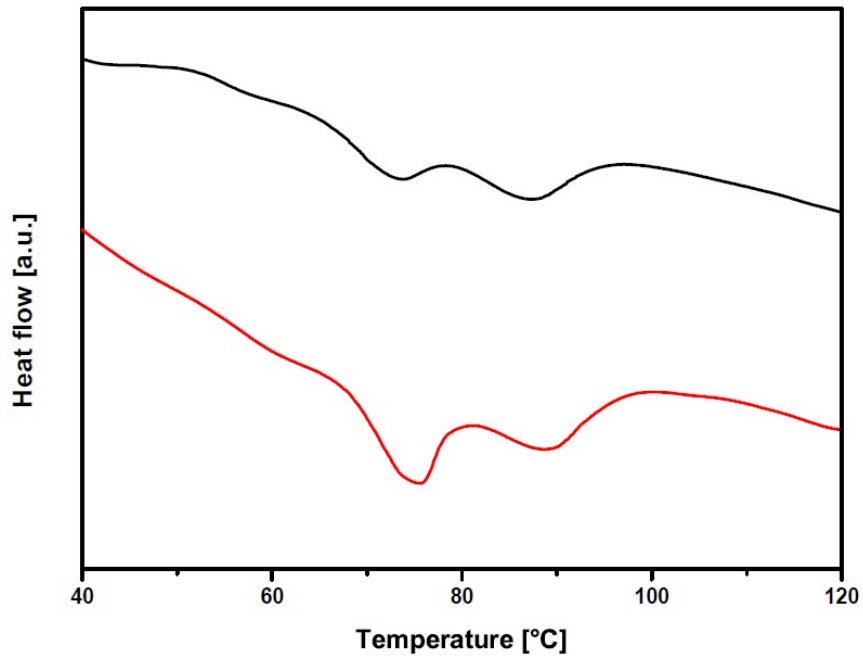
Figure 7: Comparison of freeze dried stratum corneum (black line) and stratum corneum stored in desiccators for 48 hours (red line) analysed by DSC.
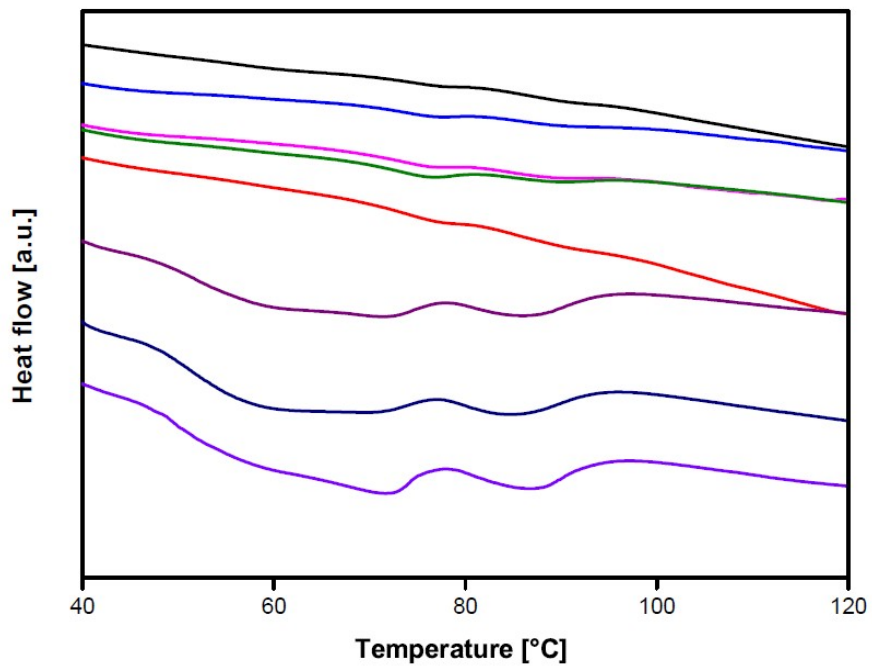

Figure 8: Signal intensity of freeze dried stratum corneum influenced by weight of sample: the upper five curves (1-2mg) in comparison with three bottom curves (5-7mg).

In our results of freeze dried stratum corneum two endothermic peaks were detected. According to literature these peaks corresponds to T_2 and T_3 endothermic transition. Previous studies reports that observation of the peak corresponding to T_1 required 15% moisture content in the skin sample. In our results this peak was not detectable because freeze drying lead to evaporation of water.[23, 24] The peak related to denaturation of keratin T_4 is also water dependent and never occurred in our results.

4.1.2. Stratum corneum treated with dimethylsulphoxide (DMSO)

Penetration enhancers are able to change protein or lipid structure of stratum corneum. As mentioned in literature, shift of T_2 and T_3 to lower temperature shows the disruption of the lipid bilayer and removal of T_4 is probably caused by keratin denaturation.

DMSO belongs to the widely studied penetration enhancers. As previously postulated DMSO changes the stratum corneum structure when administered in concentrations above 60%. In recent studies, the lipid endotherm T_2 increased linearly up to 90% concentration of DMSO. Furthermore, the peak abolished after treatment of pure DMSO. With T_3 endotherm a splitting of the peak was observed at 70% and with higher concentration broadening of the peak was evident.[14, 25]

In this project, 100% concentration of DMSO was used for experiments. Stratum corneum was treated with DMSO and then freeze dried. As we can see in figure 9 in our experiment with DMSO only T_3 was observed. This endotherm was broaden and moved to higher temperature.

The effect of DMSO on stratum corneum was compared with freeze dried skin and skin stored in desiccator for 48 hours as shown in figure 10. As we can see in DSC thermograms the freeze dried stratum corneum exhibits more pronounced peak than the stratum corneum stored in desiccator.
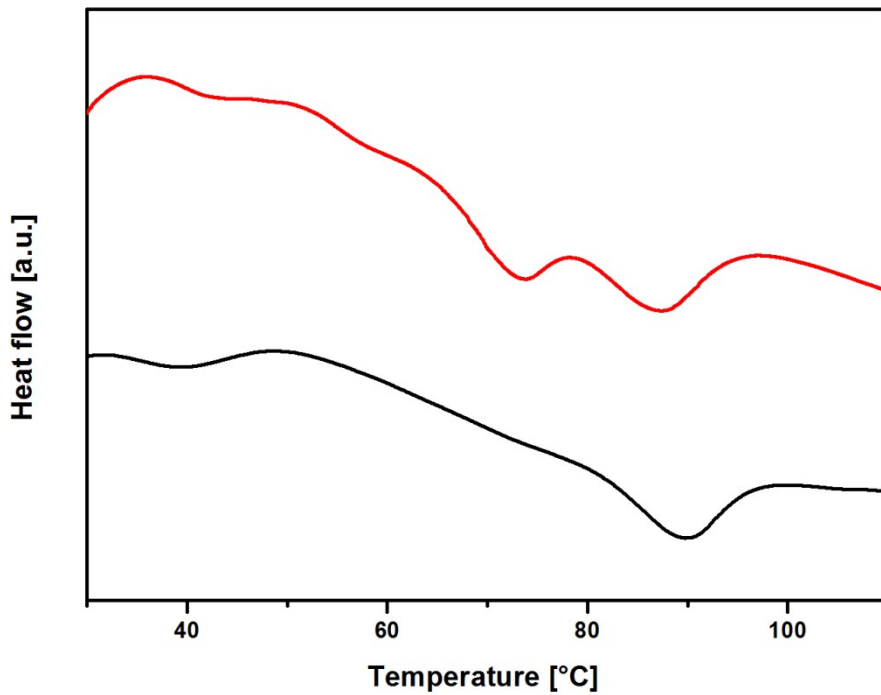
Figure 9: Comparison of freeze dried stratum corneum (red line) and stratum corneum treated with 100% DMSO then freeze dried (black line) and measured by DSC.
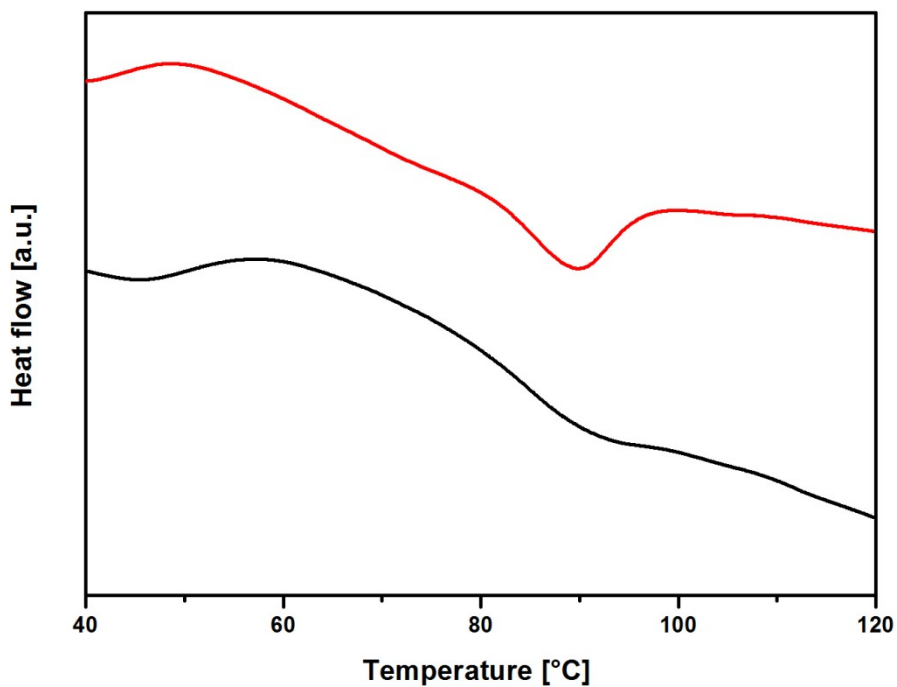

Figure 10: DSC thermograms of stratum corneum treated with 100% DMSO, stored for 48 hours in desiccators (black line) and stratum corneum treated with 100% DMSO and then freeze dried (red line).

4.1.3. Stratum corneum treated with propylene glycol (PG)

Previous reports which studied the effect of PG on stratum corneum detected only small changes in thermic events T_2 and T_3 to lower temperature which could appear probably due to disturbance of lipid-protein complex.

In our experiments 100% PG was used for stratum corneum treatment. The effect of PG was evaluated on freeze dried stratum corneum and stratum corneum stored in desiccator. DSC thermograms of these samples were compared with freeze dried stratum corneum as shown in figure 11.

Because the peaks of stratum corneum samples are better detectable and pronounced while the samples are freeze dried, we excluded the storing of stratum corneum in desiccator in next experiments and worked only with freeze dried samples.
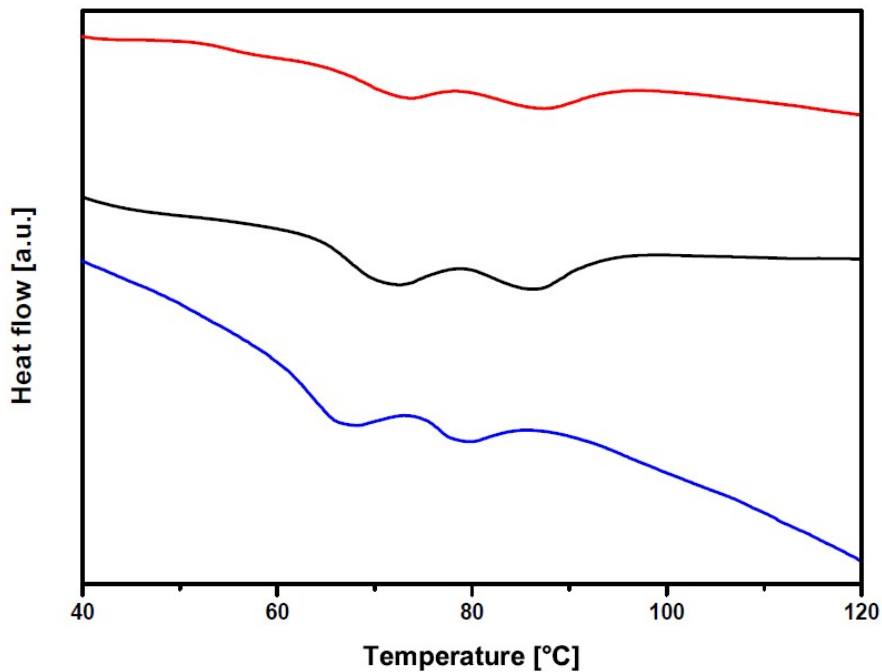

Figure 11: DSC thermograms of freeze dried stratum corneum (red line), stratum corneum treated with PG and then freeze dried (black line) and stratum corneum treated with PG then stored in desiccator (blue line).

4.1.4. Stratum corneum treated with ethylene glycol (EG)

No data for stratum corneum treated with ethylene glycol and measured by technique of DSC were found in literature.

In our experiments with stratum corneum treated with 100% of EG edothermic events T_2 and T_3 were only slightly shifted to lower temperature, on the other hand the intensity of the detected heat flow increased as shown in figure 12.

Some studies reports that reduction of the intensity of the detected heat flow is related to fluidization of lipid bilayers while increasing of the intensity of the detected heat flow may be due to strengthening of the lipid organization. An increased the intensity of the detected heat flow is attributed to the mechanism of action of retardants, substances which act in opposite way than penetration enhancers. [27]

However, as mentioned above signal intensity might also be influenced by the amount of sample. While the weight of untreated freeze dried stratum corneum was about 4mg, the weight of stratum corneum treated with EG was about 7 to 9 mg.
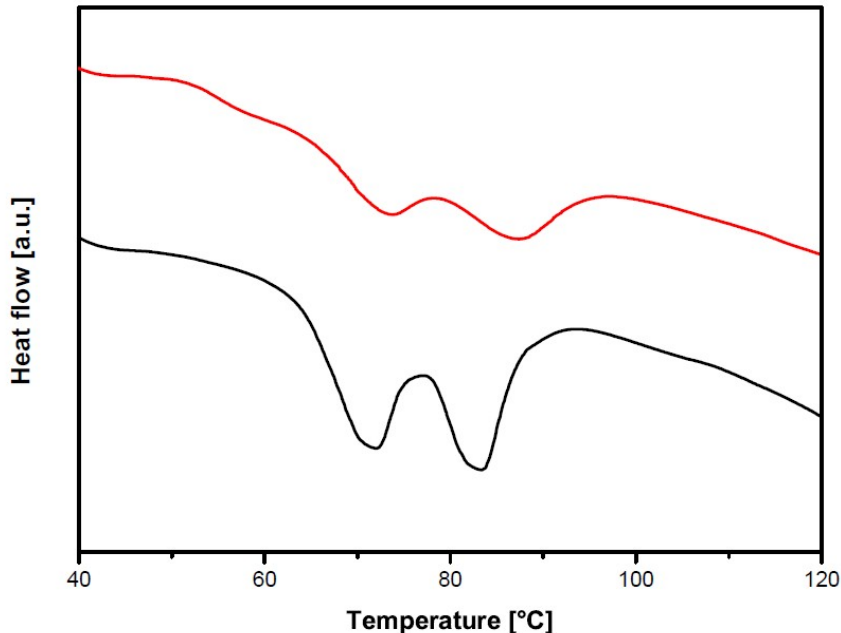

Figure 12: DSC results of freeze dried stratum corneum (red line) compared with freeze dried stratum corneum treated with ethylenglycol (black line).

4.1.5. Stratum corneum treated with mixture of DMSO and EG

The mixture of 50% DMSO and 50% ethylene glycol was used for penetration studies considering a potential synergistic effect. [28, 29, 30] DSC thermograms of this mixture compared with freeze dried stratum corneum are shown in figure 13. Thermic events T_2 and T_3 of the mixture are slightly shifted to lower temperature and the intensity of the detected heat flow is increased. These attributes show probably the effect of EG and no effect of DMSO as the DMSO itself acts only in concentration above 60%.
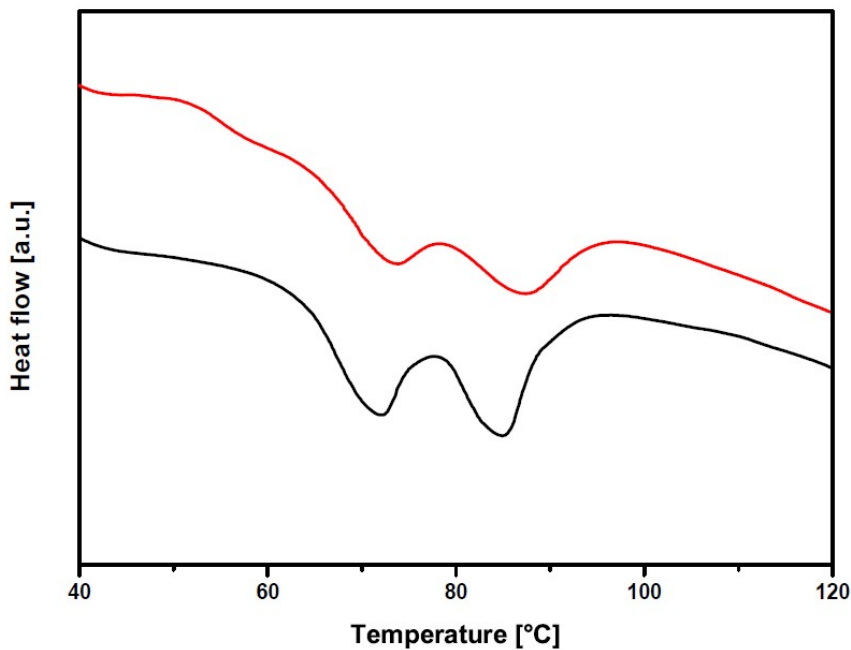

Figure 13: DSC thermograms of freeze dried stratum corneum (red line) and stratum corneum treated with amixture of DMSO and EG (black line).

4.1.6. Stratum corneum treated with oleic acid (OA)

By other workers was previously reported that 5% OA in propylenglycol caused changes in lipid stucture of stratum corneum detactable in DSC thermograms as shifts of T_2 and T_3 thermic events to lower temperature and reducing of endotherm size. [3,31]

In the present study stratum corneum was treated with pure OA, then freeze dried and compared with freeze dried stratum corneum as shown in figure 14. Endothermic transitions T_2 and T_3 of oleic acid treated stratum corneum were remarkably shifted to lower temperature and intensity of the detected heat flow was reduced. These results are in agreement with literature data.
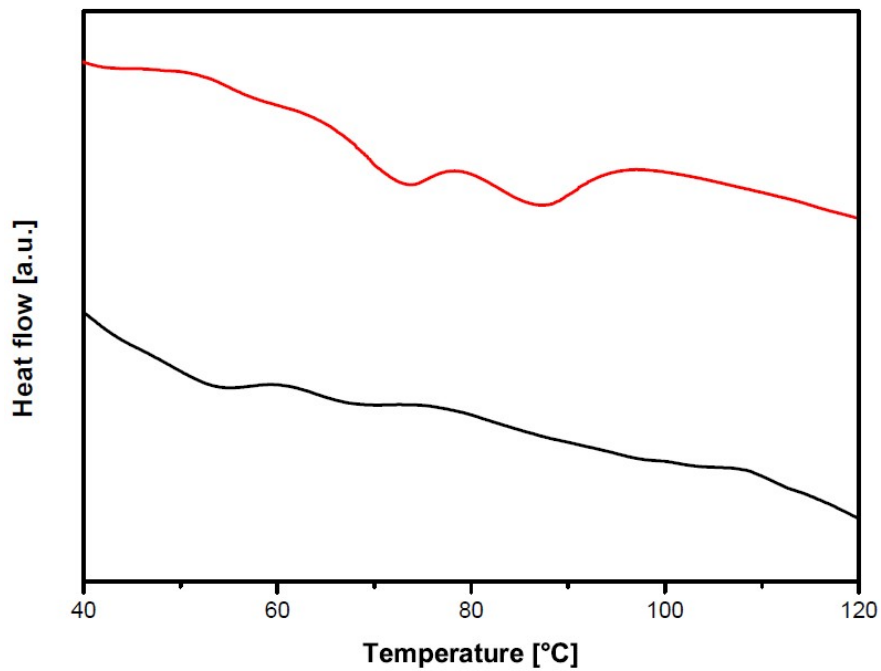
Figure 14: Freeze dried stratum corneum (red line) and stratum corneum treated with 100% oleic acid then freeze dried (black line) and measured by DSC technique.

4.1.7. Stratum corneum treated with mixture of DMSO and OA

The mixture of 25% oleic acid in DMSO was used to treat stratum corneum to investigate possible synergistic effect. As we can see in figure 15, this mixture caused changes in the lipid structure of stratum corneum detectable as lowering and reducing of the endothermic event T_2 and abolishing of T_3. Shift to lower temperature of T_2 is probably due to OA effect and abolishing of T_3 might be the effect of DMSO.
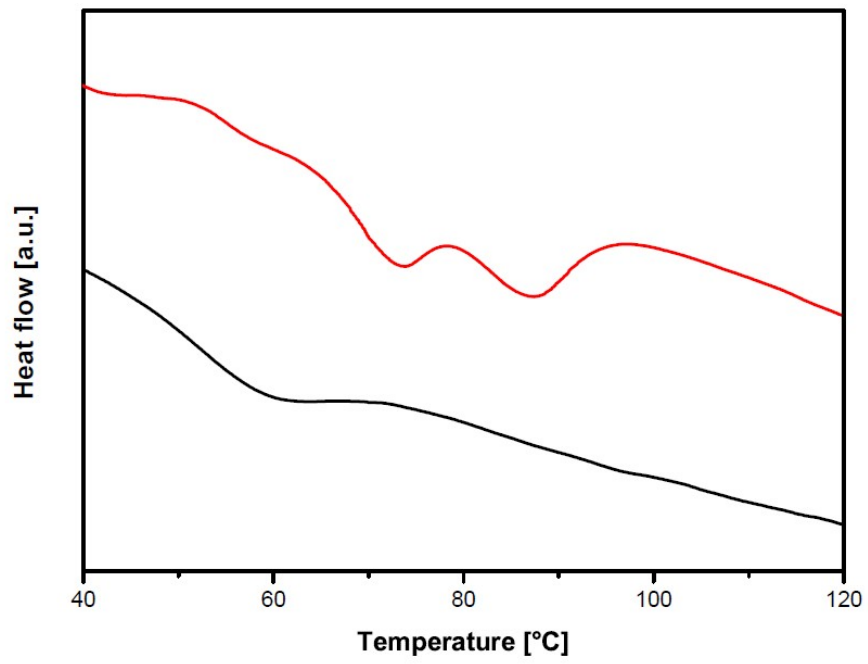
Figure 15: DSC thermograms of untreated freeze dried stratum corneum (red line) and stratum corneum treated with mixture of 25% OA in DMSO (black line).

4.2. Infrared spectroscopy

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy was used to detect changes in the lipids and proteins structure of the horny layer of the skin. Stratum corneum with different hydratation level as well as stratum corneum treated with various penetration enhancers was investigated.

4.2.1. Stratum corneum (SC)

Literature reports several stratum corneum peaks of particular interest.[8,32,33] Vibrations in the region 3100 – 2700 cm^{-1} : asymmetric C-H vibrations (2920 cm^{-1}) and symmetric C-H vibrations (2850 cm^{-1}) provides the information about intercellular lipid bilayer. The major contribution to these peaks is the absorbance of hydrocarbon chains of the lipids.

After treatment of stratum corneum with penetration enhancers, disorder of lipid structure can be found in this area as shift of the C-H stretching absorbance to higher wavenumber (blue shift).

Another indicies of lipid acyl chain disorder is the ratio of the intensity of the C-H stretching absorbance or the bandwidth at 70% height of the C-H stretch absorbance.

Characteristic peaks related to protein conformation can be found in the region 1650 cm^{-1} which corresponds to C=O stretching frequency of amide I (carbonyl group of proteins) and in the region 1550 cm^{-1} which corresponds to C-N stretching of amide II. Amide I band is sensitive to protein conformation and the change in wavenumber might significate protein denaturation.

In the FTIR spectrum of stratum corneum a peak in the area of 1740 cm^{-1} can be also found. This peak occurs due to C=O stretch of lipid polar head groups and it is assigned to endogenous lipids. However, observation of this peak is variable and usually depends on the preparation technique of the skin.

We investigated the fresh stratum corneum, freeze dried stratum corneum and fresh heat separated epidermis as shown in figure 16. No changes in lipid or protein peak could be observed. Peak broadenings are related to the different water content within the samples.
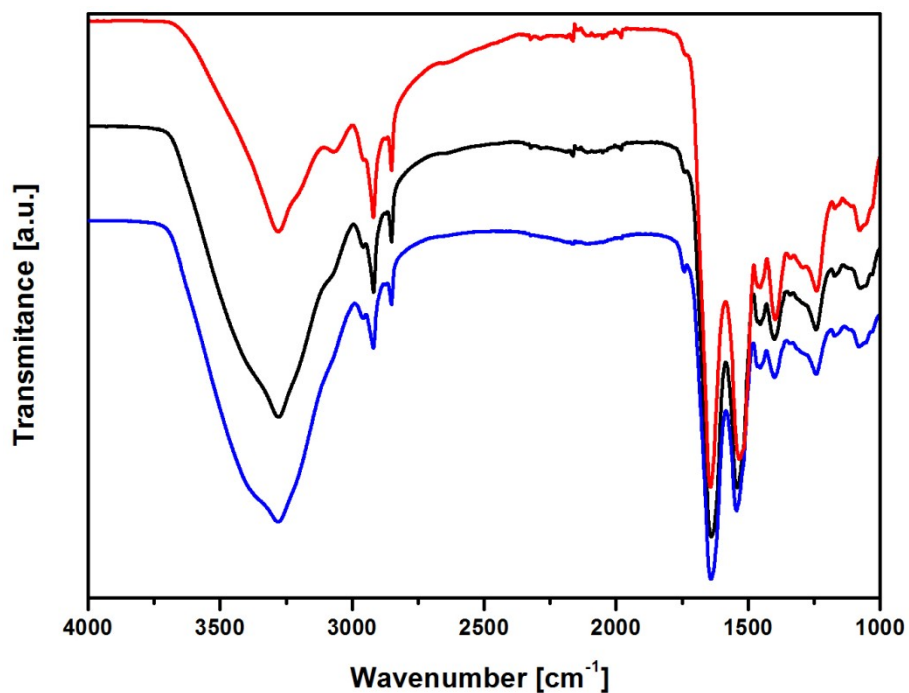
Figure 16: FTIR spectra of fresh stratum corneum (black line), heat separated epidermis (blue line) and freeze dried stratum corneum (red line).

4.2.2. Stratum corneum treated with dimethyle sulphoxide (DMSO)

Previous studies suggested that DMSO may cause extensive lipid extraction and also protein denaturation.[32]

In the present study, DMSO was used to characterise changes in barrier properties of stratum corneum. As shown in figure 17, amide I peak of DMSO treated stratum corneum splitted in two peaks and shift to higher wavenumber was detectable. These attributes corresponds to altering of stratum corneum proteins. Furthermore, C-H stretching absorbance was reduced probably due to lipid extraction as shown in figure 18.
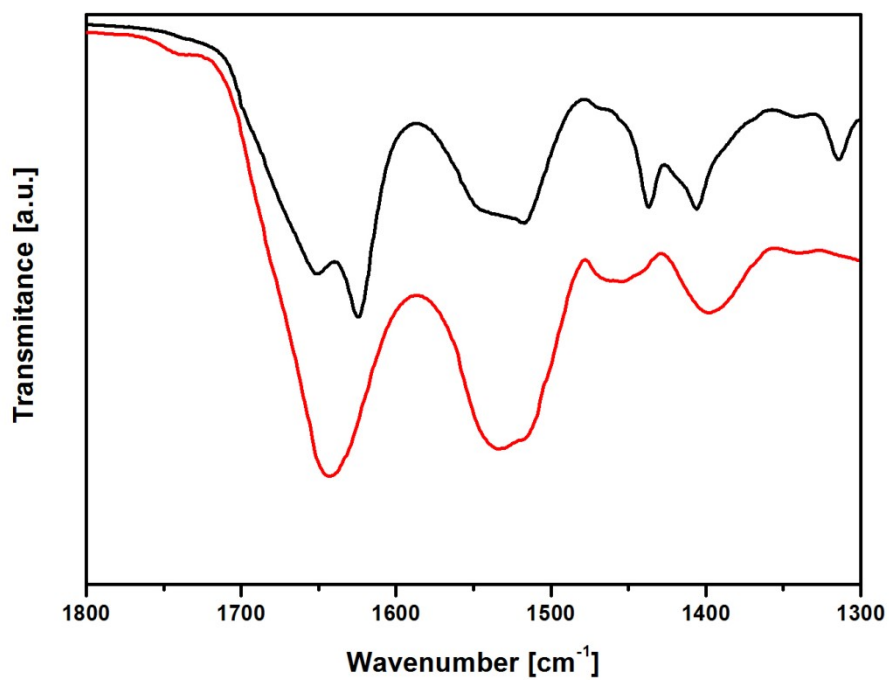
Figure 17: FTIR spectra of amide I peak of freeze dried stratum corneum (red line) and freeze dried stratum corneum treated with DMSO (black line).
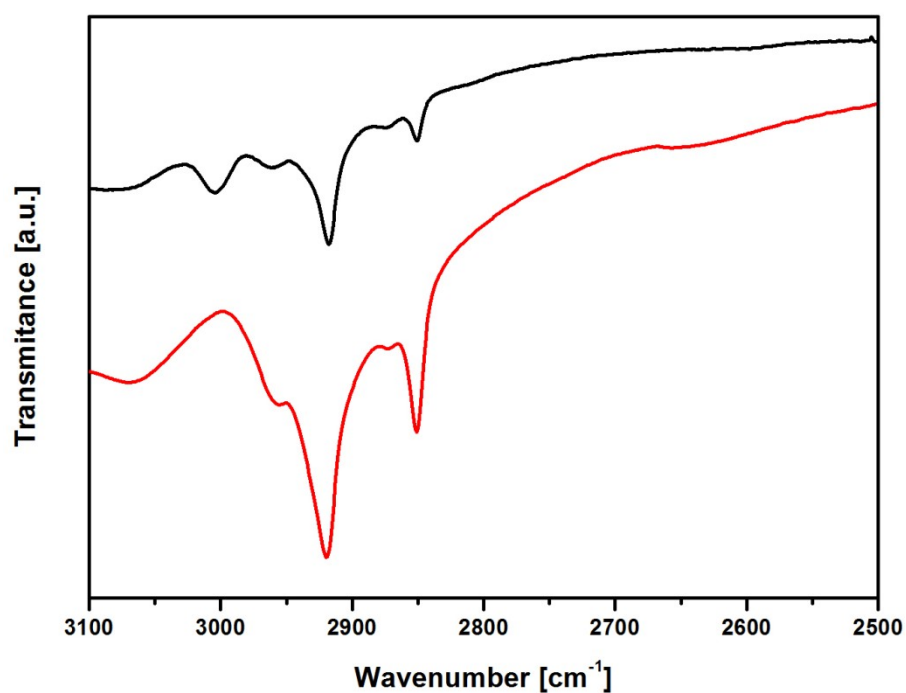

Figure 18: FTIR spectra of freeze dried stratum corneum (red line) and freeze dried stratum corneum treated with DMSO (black line) detail of C-H stretching absorbance.

4.2.3. Stratum corneum treated with propylene glycol (PG)

Previous studies reports whether PG by itself increases skin permeability or not. The fact that PG acts as cossolvent for other accelerants is well known. Research work of B.W.Barry indicated that PG may act as penetration enhancer especially in cases where the stratum corneum is not fully hydrated.[10] Ramesh Panchagula et al.[35] treated rat skin with PG and in their FTIR results there was no significant shift or broadening of lipid peaks. They explained that effect of PG is water dependent and while in a solvent system is 100% of PG it leads to extraction of water from the lipid bilayer and corneocytes and leads to increasing in the barrier property of SC. Research study of D.Kuashik and B.M.Michniak-Kohn[18] demonstrated that treating of stratum corneum with PG caused blue shift in amide I peak and no changes in lipid peaks. Figure 19 depicts our result of stratum corneum treated with 100% of PG. As we can see PG treatment leads to splitting of amide I peak, however, no significant changes in C-H stretching absorbance peak were detectable as shown in figure 20.
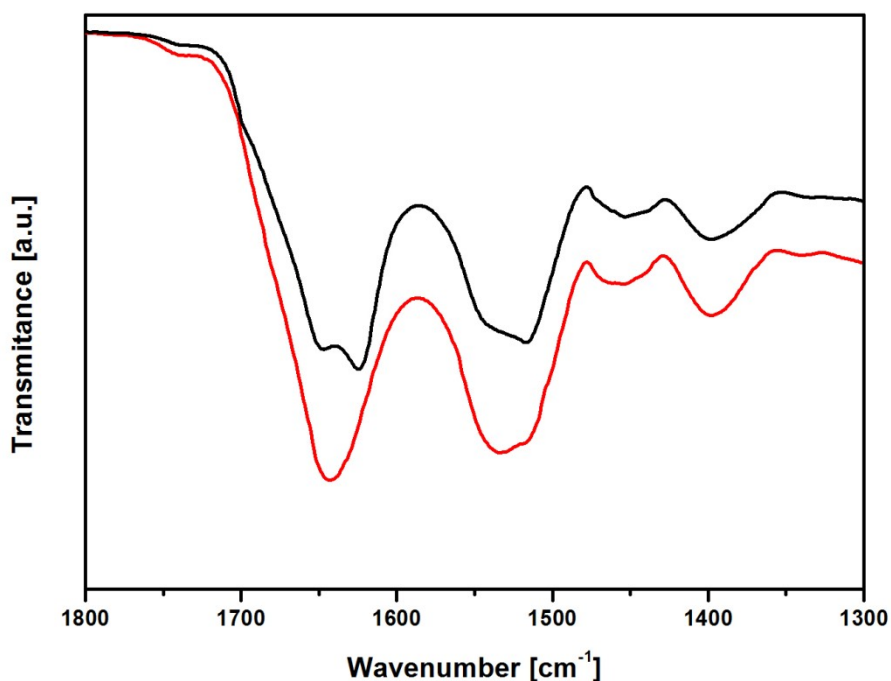

Figure 19: FTIR spectra of freeze dried stratum corneum (red line) and freeze dried stratum corneum after treatment with PG (black line) detail of amide I peak.
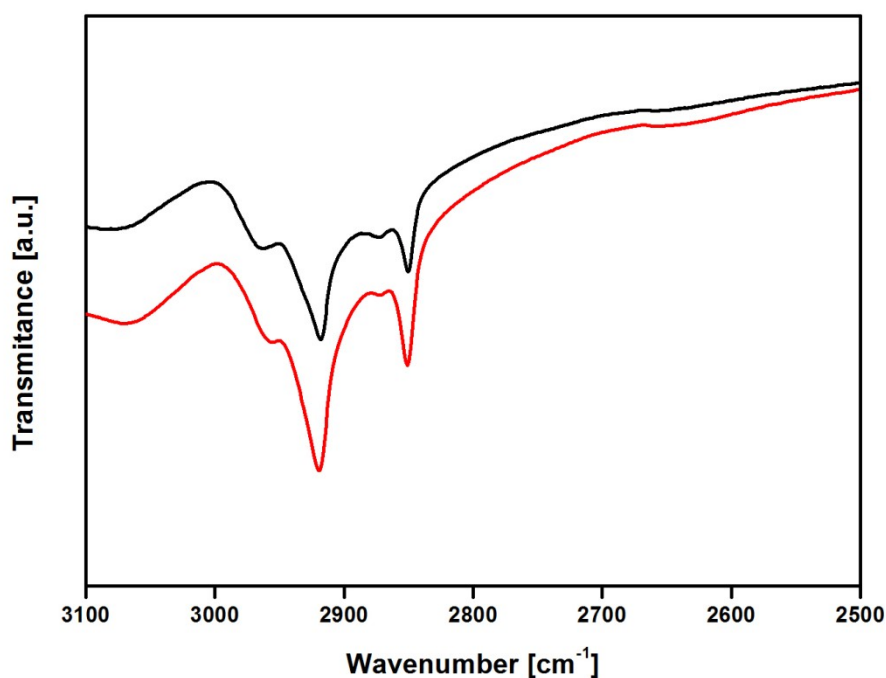
Figure 20: FTIR spectra of lipid peaks of freeze dried stratum corneum (red line) and freeze dried stratum corneum treated with PG (black line).

4.2.4. Stratum corneum treated with ethylene glycol and deuterated ethylene glycol (EG and EG-D4)

No literature data were found about treating the stratum corneum with ethylene glycol. For our experiments we used EG and fully deuterated EG as well, because we considered the fact that C-H vibrational frequencies of stratum corneum can interfere with C-H frequency of EG. [34]

As demonstrated in figure 21, no effect on stratum corneum lipids was detectable with both, EG or EG – d4. However, treatment with these penetration enhancers leads to splitting of amide I peak as shown in figure 22.
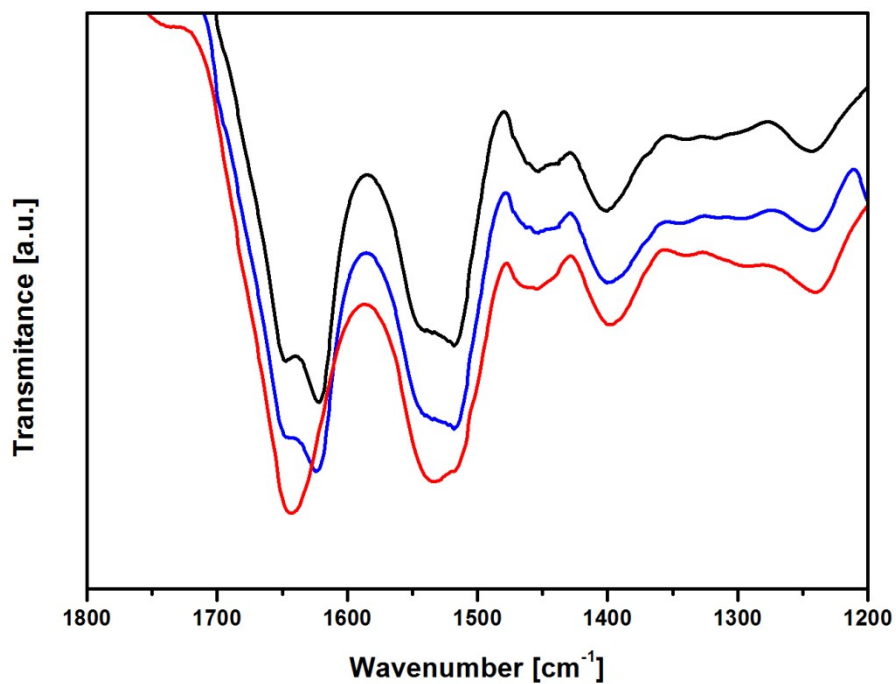
Figure 21: FTIR spectra of freeze dried SC (red line) compared with freeze dried stratum croneum treated with EG (blue line) and EG d-4 (black line) detail of amid I peak.
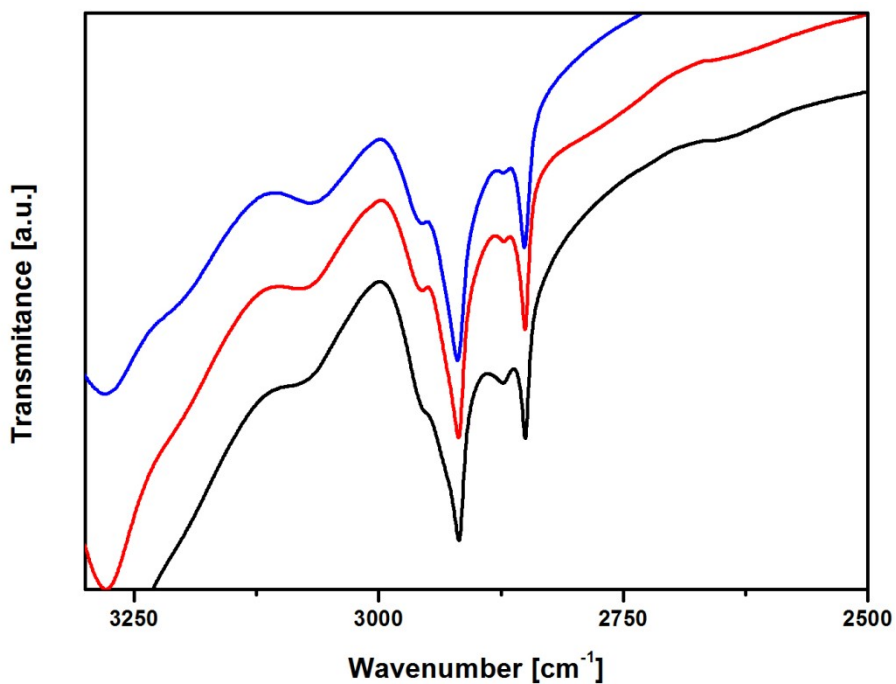

Figure 22: FTIR spectra of freeze dried stratum corneum (red line) compared with stratum corneum treated with EG (black line) and EG d-4 (blue line) detail of lipid peaks.

4.2.5. Stratum corneum treated with mixture of dimethyle sulphoxide and ethylene glycol (DMSO/EG)

The potential synergistic effect of a mixture DMSO/EG was investigated in our work. Stratum corneum was incubated with the solution of 50% DMSO and 50% EG. The same mixture was used with EG–d4. As demonstrated in figure 23 there are no changes in lipid peaks but as figure 24 depicts amide I peak is splitted after stratum corneum was treated with this mixture.
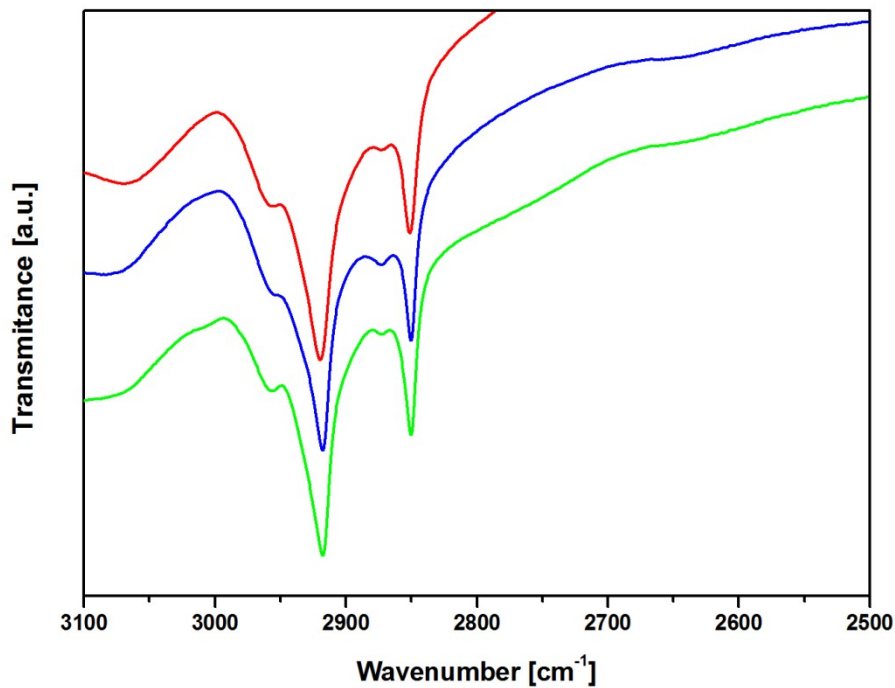

Figure 23: FTIR spectra focused on lipid peak: freeze dried stratum corneum (red line), freeze dried stratum corneum treated with a mixture of EG and DMSO (blue line) and freeze dried stratum corneum treated with a mixture of EG-d4 and DMSO (green line).

Figure 24: FTIR spectra focused on protein amide I peak: freeze dried stratum corneum (red line), freeze dried stratum corneum treated with a mixture of EG/DMSO (blue line) and treated with a mixture of EG-d4/DMSO (green line).

4.2.6. Stratum corneum treated with oleic acid (OA)

In previous studies it is postulated that OA only interacts with the lipid structure of stratum corneum and in FTIR investigation it causes a blue shift in lipid peaks but no changes in protein peaks.[14, 36]

As figure 25 depicts no splitting in protein peak was detectable and figure 26 demonstrates a slight shift to higher wavenumber.

Figure 25: FTIR spectra of protein peak: freeze dried stratum corneum (red line) and freeze dried stratum corneum treated with OA (black line).
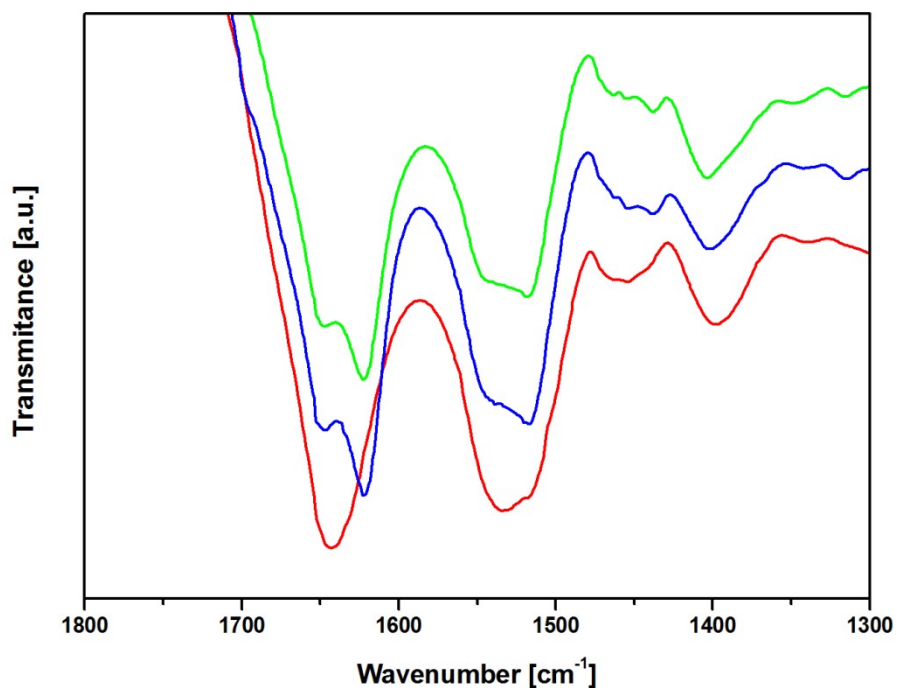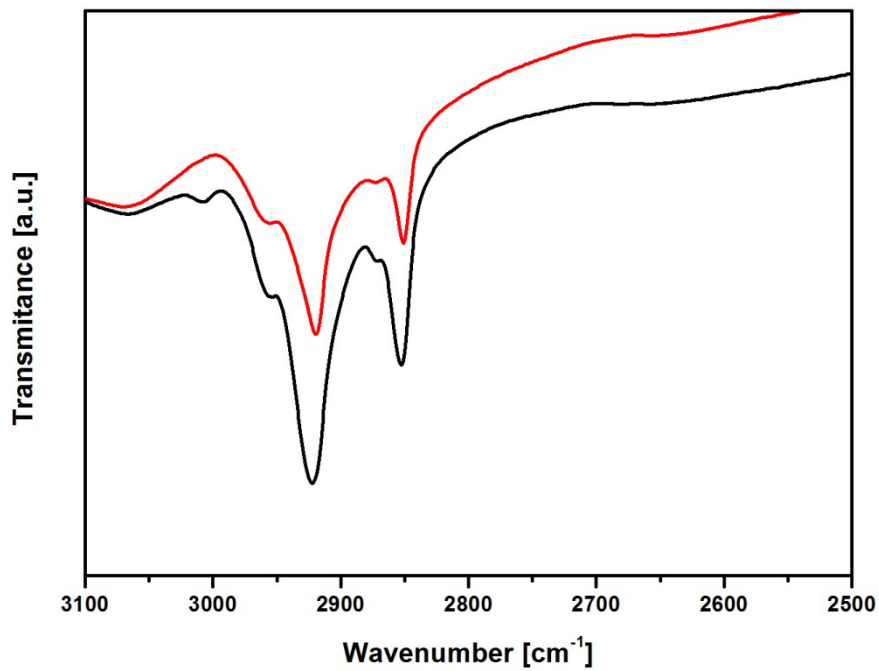

Figure 26: FITR spectra of lipid peak: freeze dried stratum corneum (red line) and freeze dried stratum corneum treated with OA (black line).

4.2.7. Stratum corneum treated with mixture of OA and DMSO

For the next experiment stratum corneum was incubated in 25% of OA and 75% of DMSO. Figure 27 and figure 28 shown the effect of this mixture on stratum corneum. The lipid peak was shifted to higher wavenumber and protein peak splitted which may be associated with protein denaturation.

Figure 27: FITR spectra of lipid peak: freeze dried stratum corneum (red line) and freeze dried stratum corneum treated with a mixture of DMSO/OA (black line).
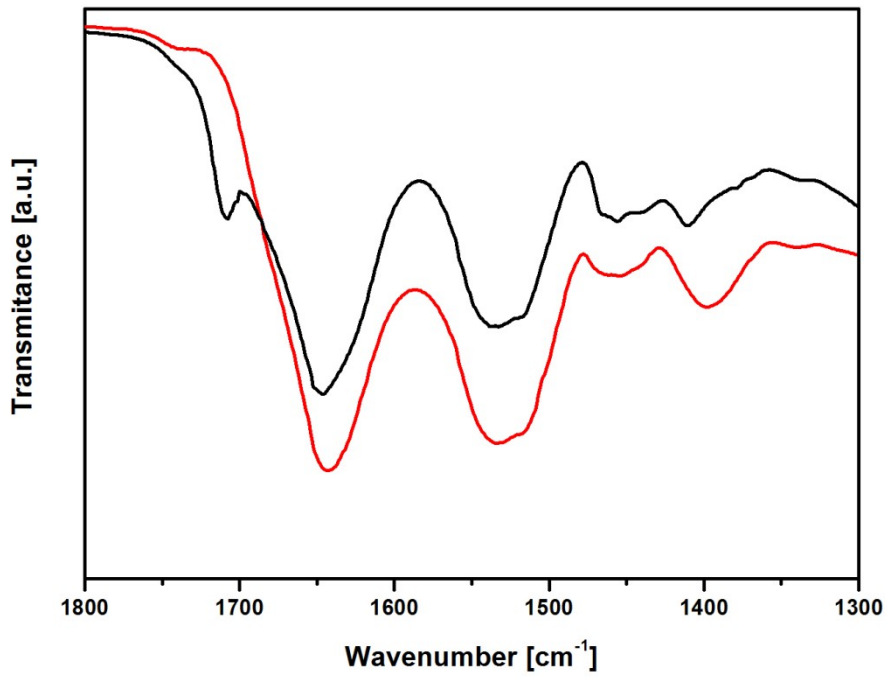
Figure 28: FTIR spectra of protein peak: freeze dried stratum corneum (red line) and freeze dried stratum corneum treated with a mixture DMSO/OA (black line).
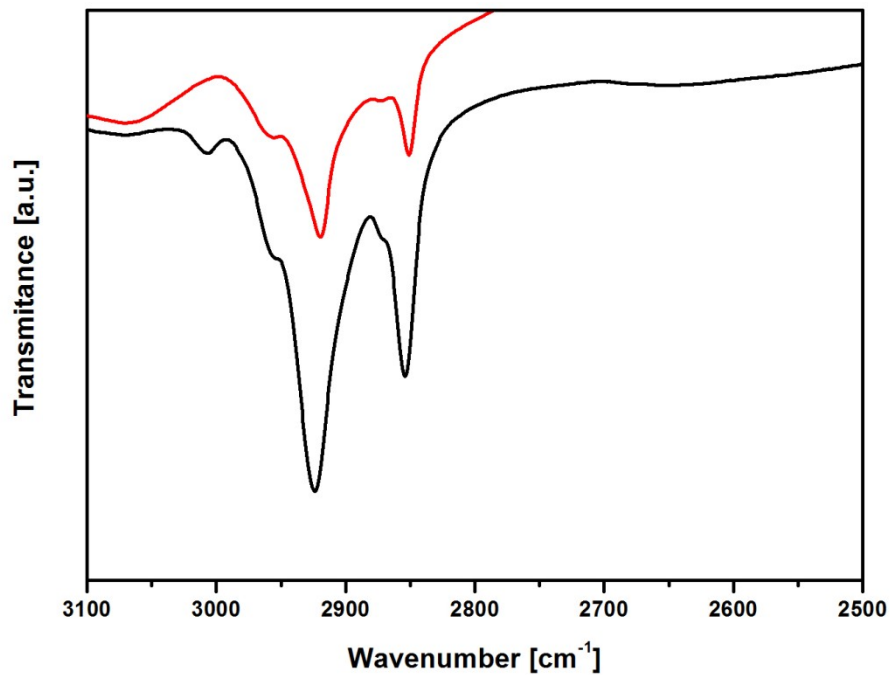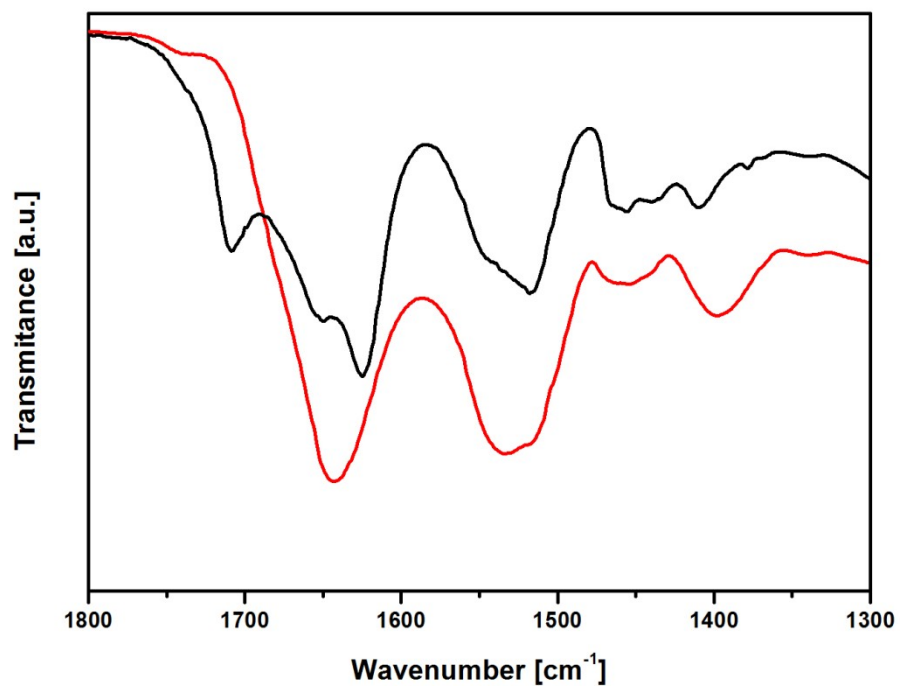
4.3.   Confocal Raman microscopy (CRM)

Raman spectra of human stratum corneum and their changes induced by penetration enhancers were recorded. Stratum corneum was investigated untreated (fresh or freeze dried) or incubated with penetration enhancers.

4.3.1. Stratum corneum (SC)

The study of Williams, Edwards and Barry [37, 38] confirmed that the techniques IR and CRS are complementary. Both spectra of human skin show C-H stretching in the range 2700-3100 cm^{-1} which is associated with lipid alkyl chains of stratum corneum. The principal features of IR are amide I and amide II bands. Raman spectrum exhibits three amide bands. The amide I and amide II which can be found in region about 1655 cm^{-1} and 1550 cm^{-1} are reduced in intensity and amide III is present in Raman spectrum in region about 1300 cm^{-1} but it is absent in IR spectrum.

In present study we used for our experiments freeze dried stratum corneum. At the first we compared spectra of fresh stratum corneum and freeze dried stratum corneum as demonstrated in figure 29.
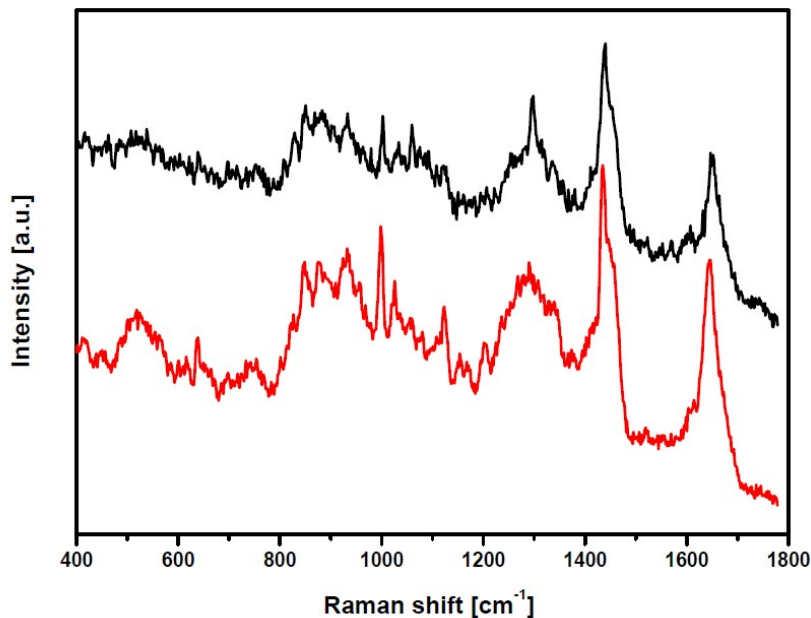

Figure 29: Raman spectra of freeze dried stratum corneum (red line) and fresh stratum corneum (black line).

4.3.2. Stratum corneum treated with dimethyle sulphoxide (DMSO)

In previous studies effect of DMSO on stratum corneum was investigated. Results of Raman spectroscopy investigations demonstrated that DMSO show minor changes in C-H stretching vibrations. All bands in the region about 3200-2700cm^{-1} were broadened after stratum corneum was treated with DMSO. The amide I peak significantly shifted to higher wavenumbers and split in two peaks. [34, 39]

The present work shows the effect of DMSO in freeze dried stratum corneum samples. Fresh and freeze dried stratum corneum treated with DMSO were compared as figure 30 depicts. Because no significant changes were detected between these two graphs in further experiments only freeze dried stratum corneum was investigated.
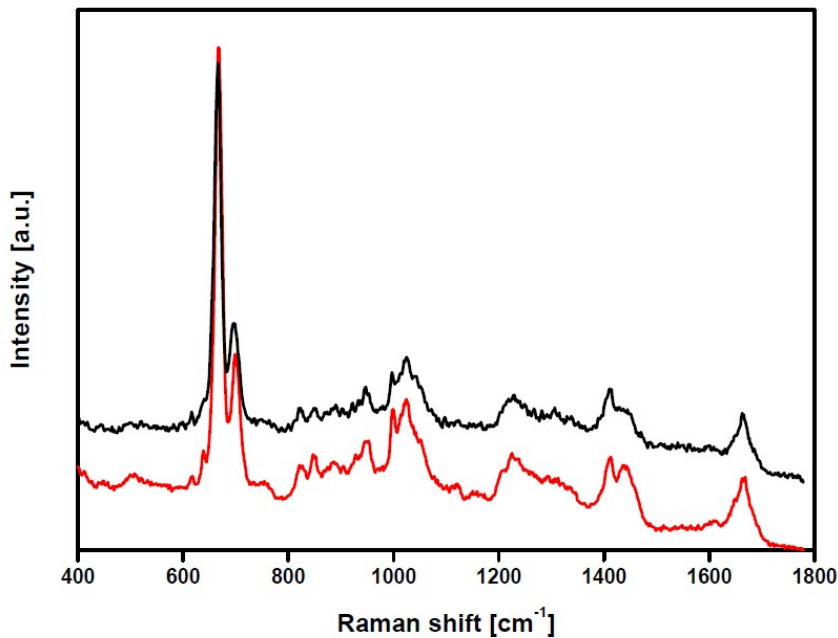
Figure 30: Raman spectra of DMSO treated stratum corneum fresh (black line) and freeze dried (red line).

The amide I band of untreated stratum corneum and stratum corneum treated with DMSO is shown in figure 31. Treatment of DMSO led to obvious and reproducible shift to higher wavenumbers and splitting of the amide I peak.

Figure 31: Raman spectra of amide I peak: freeze dried stratum corneum (black line) and freeze dried stratum corneum treated with DMSO (red line).

4.3.3. Stratum corneum treated with propylene glycol (PG)

No data were found in literature dealing with PG investigations by Raman spectroscopy. In our experiments stratum corneum was treated with PG and a slight shift in amide I peak was detected as shown in figure 32.
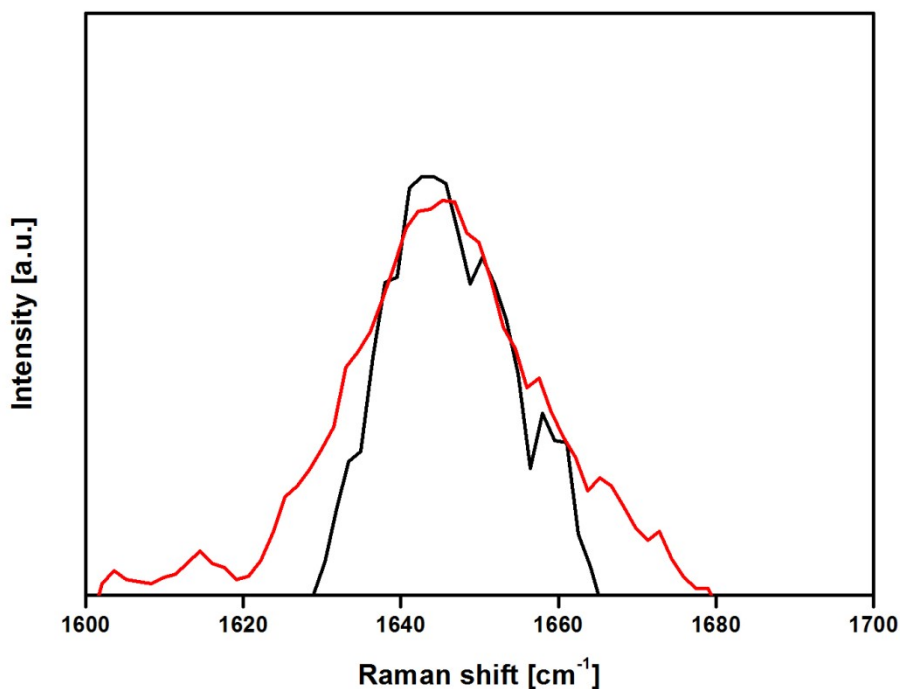
Figure 32: Raman spectra of stratum corneum (red line) and stratum corneum treated with PG (black line).

4.3.4. Stratum corneum treated with ethylene glycol (EG) and ethylene glycol – d4 (EG-d4)

EG was used for treatment of stratum corneum and as figure 33 indicates a slight shift in amide I peak is visible. For our experiments stratum corneum was incubated also with fully deuterated EG, due to fact that heavy atoms occur in lower frequency and allow interference-free assessments of vibrational modes. However, no difference between the effects of EG and deuterated EG were detected.
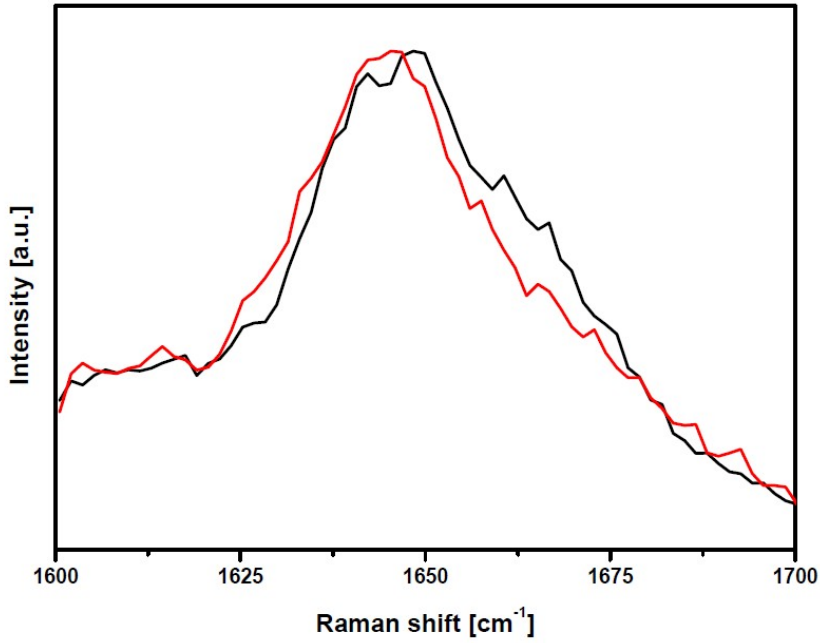
Figure 33: Raman spectra of amide I peak: freeze dried stratum corneum (red line) and freeze dried stratum corneum treated with EG (black line).

4.3.5. Stratum corneum treated with a mixture of DMSO and EG

To evaluate possible synergistic effects, stratum corneum was incubated with 50% of DMSO and 50% of EG. The effect of amide I peak is shown in figure 34.
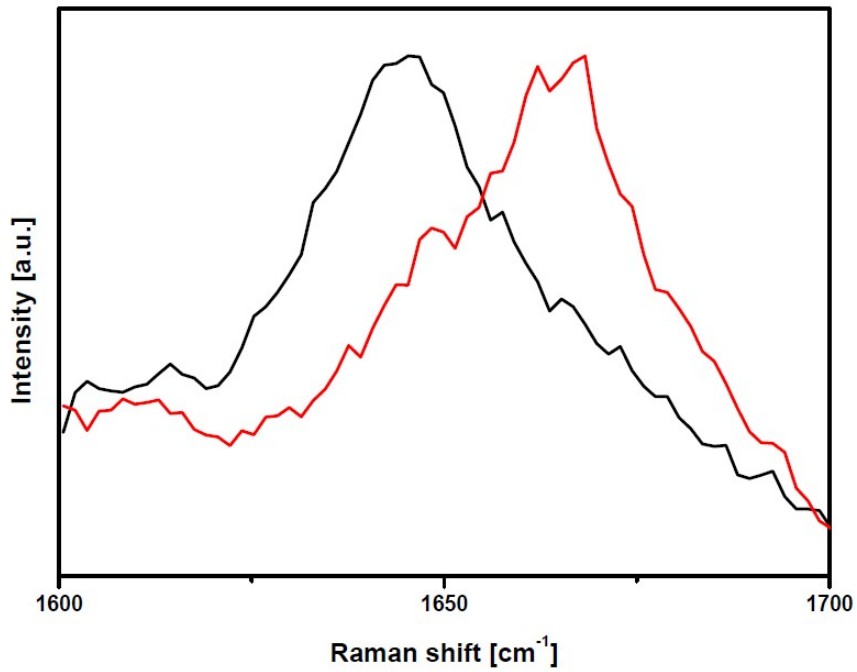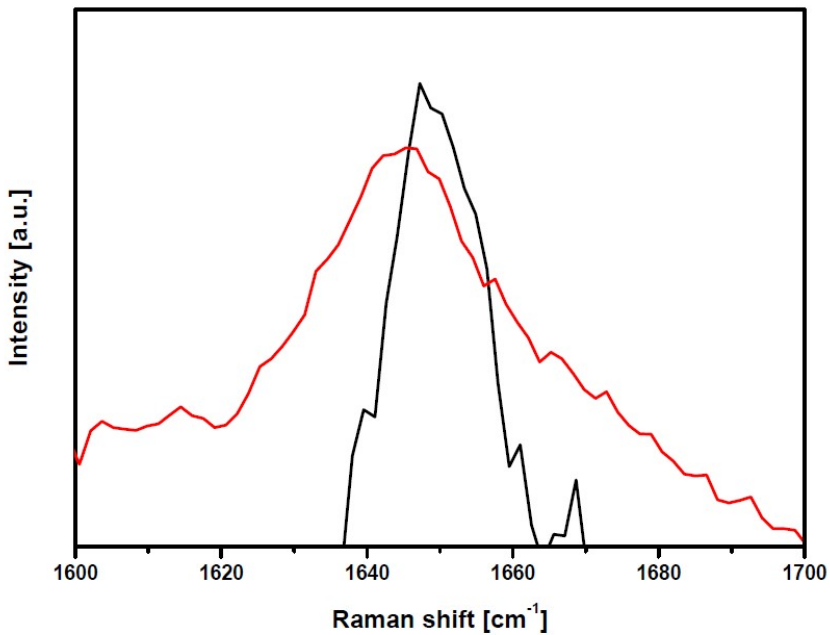

Figure 34: Raman spectra of amide I peak: freeze dreid stratum corneum (red line) and freeze dried stratum corneum treated with a mixture of DMSO and EG.

4.3.6. Stratum corneum treated with oleic acid (OA)

As postulated in previous studies OA acts in the lipid part of stratum corneum. In our experiments 100% of OA and a mixture of OA and DMSO were used. However, in both cases no Raman signals of stratum corneum were detected since OA could not be eliminated from the system and buried all other bands.

5. CONCLUSION

The aim of this work was to investigate various penetration enhancers and characterise their effect on human stratum corneum structure. We combined well established techniques like differential scanning calorimetry (DSC) and infrared spectroscopy (IR) with confocal Raman microscopy (CRM) as an upcoming technique in skin research. Moreover, we tested freeze drying as a novel technique for sample preparation in analysis of human skin structure.

We determined that freeze drying of stratum corneum samples was a suitable method for penetration experiments. We wanted to avoid any discrepancies which might occur with different levels of hydration of SC because this structure might extend and swallow with an increasing amount of water. With IR and Raman investigation we confirmed that freeze drying did not changed the chemical structure of untreated SC and we avoided the influence of water. In DSC thermograms we observed better pronounced, detectable and reproducible peaks.

With respect to the penetration enhancers, we proved that DMSO and PG acted as postulated in literature before. DMSO caused lipid disruption which was confirmed by DSC and IR. Furthermore, DMSO caused protein denaturation confirmed by IR and Raman studies.

With PG, small changes were detected in lipid structure by DSC and protein changes were observed in IR studies while Raman experiments did not show significant influence on protein structure. This attribute shows that PG might cause some changes in protein structure (IR study) but does not cause protein denaturation (Raman study).

As we considered a similar structure to PG, EG was also investigated. In our comprehensive study EG showed similar results to PG. The effect on protein conformation was detected in IR spectra, but Raman spectra did not show the significant shift related with protein denaturation. DSC detected small changes in lipid structure.

The effect of fully deuterated EG observed by IR and Raman did not differ from the effect of EG. Therefore, it can be concluded that deuteration has no influence on the EG mechanism of action. However, the unrestricted detectability of non-deuterated EG, makes deuteration for our particular unnecessary.

Our study is in agreement with theoretical claims that oleic acid acts only on lipids. We observed changes with DSC and IR on lipids but no changes on proteins were confirmed with IR.

We considered a potential synergistic effect of a mixture of DMSO with EG. In DSC thermograms, the heat flow of lipid thermic events was increased. SC treated with this mixture exhibited a shift in IR spectra but no significant shift in Raman data. This reflects the results of pure EG. The concentration of DMSO used in these experiments might have influenced the results. We used only 50% but DMSO acts only above 60%.

The second mixture used in this project was a mixture of DMSO and OA. This mixture showed the synergistic effect. DMSO was used in a concentration of 75 % and therefore acted as explained earlier. DSC and IR proved lipid changes caused by OA and also protein changes caused by DMSO were confirmed by IR.

In our study the combination of all techniques was successfully applied for investigation of stratum corneum structure. The lipid changes were well detectable with DSC technique. However, in DSC thermograms protein changes cannot be observed. With IR both lipid and protein changes were detectable. Raman spectra were more sensitive to protein conformation and shifts to higher wavenumber were observed. Lipid changes detected with Raman spectroscopy need further investigation.

To sum up, we established a comprehensive study of three complementary techniques to gain deeper insight in various penetration enhancers' mechanism of action. The confirmed literature results proved the suitability of our setup and additional findings indicated the potential of the introduced techniques for the future.

6. LIST OF ABBREVIATIONS AND SYMBOLS

SC	stratum corneum
HSE	heat separated epidermis
T_{1-4}	thermic events of differential scanning calorimetry thermogram
ΔH	enthalpy
DSC	Differential Scanning Calorimetry
IR	Infrared spectroscopy
CRM	Confocal Raman microscopy
DMSO	dimethyle sulphoxide
PG	propylene glycol
EG	ethylene glycol
EG-d4	deuterated ethylene glycol
OA	oleic acids

7. REFERENCES

1) M.R. Prausnitz, R. Langer, Transdermal drug delivery, *Nat Biotechnol*, 26 (2008) 1261-1268

2) H.A.E. Benson, Transdermal Drug Delivery: Penetration Enhancement Technique, *Current Drug Delivery*, 2 (2005) 23-33

3) B.W. Barry, Mode of action of penetration enhancers in human skin, *J Control Rel,* 6 (1987) 85-97

4) B.W. Barry, Action of skin penetration enhancers – the Lipid Protein Partitioning theory, *Int J Cosmetics*, 10 (1988) 281-283

5) K. Walkley, Bound water in stratum corneum measured by differential scanning calorimetry, *J Invest Dermatol*, 59 (1972) 225-227

6) B.F. Van Duzee, Thermal analysis of human stratum corneum, *J Invest Dermatol,* 65 (1975) 404-8

7) G.M. Golden, D. Guzek, R. Harris, J.E. McKie, R.O. Potts, Lipid thermotropic transitions in human stratum corneum, *J Invest Dermatol*, 86 (3) (1986) 255-9

8) A.C. Williams, H.G.M. Edwards, B.W. Barry, Fourier transform Raman spectroscopy: A novel application for examining human stratum corneum, *Int J Pharm*, 81 (1992) 11-14

9) S. Wartewig, R.H.H. Neubert, Pharmaceutical applications of Mid-IR and Raman spectroscopy, *Adv Drug Deliv Rev* , 57 (2005) 1144-1170

10) G.P. Moss, S.C. Wilkinson, Y. Sun, Mathematical modelling of percutaneous absorption, *Current Opinion in Colloid & Interface Science*, 14 (2012)

11) U. Schaefer, S. Hansen, M. Schneider, J.L. Contreras, C.M. Lehr, Drug absorptin studies: In situ, in vitro and in silico models, chapter 1, pg.3-33 (2008)

12) L. Koláčková, A comparison in vitro study of permeation of selected drugs from lipophilic solutions through human skin, Master thesis, Saarbrücken 2007

13) M. Mádlová, Investigation of water evaporation from creams and permeation of flufenamic acid through human epidermis, Master thesis, Saarbrücken 2003

14) J. Štecová, Study of human equivalents permeation properties with flufenamic acid, Saarbrücken 2002

15) E. Proksch, J.M. Brandner, J.M. Jensen, The skin: an indispensable barrier, *Exp Dermatol,* 17 (2008) 1063-1072

16) R.L. Bronaugh, H.I. Maibach, In vitro percutaneous absorption, CRS press, 1991

17) F. Netzlaff, Haut und Hautmodelle, Ph.D. thesis, Saarbrücken 2006

18) M.A. Lampe, M.L. Williams and P.M. Elias, Human epidermal lipids: characterization and modulations during differentiation, *J Lpid Res*, 24 (1983) 131

19) A.C. Williams, B.W. Barry, Penetration enhancers, *Adv Drug Del Rev*, 56 (2004) 603 – 618

20) J.D.Middleton, The mechanism of water binding in stratum corneum, *Br J Dermatol,* 80 (1968) 437-450

21) C.L. Gay, R.H. Guy, G.M. Golden, V.H.W. Mak, M.L. Francoeur, Characterization of Low-Temperature (i.e., <65°C) Lipid Transitions in Human Stratum Corneum, *Inv Dermatol*, 103 (1994) 233-239

22) S.M. Al-Saidan, B.W. Barry, A.C. Williams, Differential scanning calorimetry of human and animal stratum corneum membranes, *Int J Pharm,* 168 (1998) 17-22

23) T. Inoue, M.S. Kaoru Tsujii, K. Okamoto, K. Toda, Differential Scanning Calorimetric Studies on the Melting Behaviour of Water in Stratum Corneum, *J Invest Dermatol,* 86 (1986) 689-693

24) D. Kaushiki, B. Michniak-Kohn, Percutaneous Penetration Mofifiers and Formulation Effects: Thermal and Spectral Analyses*, Pharm Sci Tech,* 11 (2010)

25) C.S. Leopold, B.C. Lippold, An attempt to clarify the mechanism of the penetration enhancing effects of lipophilic vehicles with differential scanning calorimetry (DSC), *J Pharm Pharmacol,* 47 (1995) 276-81

26) Z.U. Khan, I.W. Kellaway, Diferential scanning calorimetry of dimethylsulphoxide-treated human stratum corneum, *Int J Pharm*, 55 (1989) 129 – 134

27) D. Kaushik, P. Batheja, B. Kilfoyle, V. Rai, B. Michniak-Kohn, Percutaneous permeation modifiers: ehnancement versus retardations, *Expert Opin Drug Deliv,* 5 (2008) 517-29

28) P. Karande, A. Jain, S. Mitragotri, Insights into synergistic interactions in binary mixtures of chemical permeation enhancers for transdermal drug delivery, *Journal Control Rel,* 115 (2006) 85-93

29) P. Karande, S. Mitragotri, Enhancement of transdermal drug delivery via synergistic action of chemicals, *Biochimica et Biophysica Acta*, 1788 (2009) 2362-2373

30) Y.C. Kim, J.H. Park, P.J. Ludovice, M.R .Prausnitz, Synergistic enhancement of skin permeability by N-lauroylsarcosine and ethanol, *Int J Pharm*, 352 (2008) 129-138

31) M.A. Yamane, A.C. Williams, B.W. Barry, Effects of terpenes and oleic acids as skin penetration enhancers towards 5-fluorouracil as assesed with time, permeation, partitioning and differential scanning calorimetry, *Int J Pharm,* 116 (1995) 237-251

32) M.J. Clancy, J. Corish, O.I. Corrigan, A comparison of the effects of electrical current and penetration enhancers on the properties of human skin using spectroscopic (FTIR) and calorimetric (DSC) methods, *Int J Pharm*, 105 (1994) 47-56

33) L. Brancaleon, M.P. Bamberg, T. Sakamaki, N. Kollias, Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy as a Possible Method to Investigate Biophysical Parameters of Stratum Corneum In Vivo, *J Invest Derm*, 116 (2001) 380-386

34) H. Trommer, R.H.H. Neubert, Overcoming the Stratum Corneum: The Modulation of Skin Penetration, *Skin Pharm Pharmacol*, 19 (2006) 106-121

35) R. Panchagnula, P.S. Salve, N.S. Thomas, A.K. Jain, P. Ramarao, Transdermal delivery of naloxone: effect of water, propylene glycol, theanol and their binary combinations on permeation through rat skin, *Int J Pharm*, 219 (2001) 95-105

36) A.N.C. Anigbougu, A.C. Williams, B.W. Barry, H.G.M. Edwards, Fourier transform Raman spectroscopy of interactions between the penetration enhancer dimethyl sulfoxide and human stratum corneum, *Int J Pharm*, 125 (1995) 265 – 282

37) B.W. Barry, H.G.M. Edwards, Fourier Transform Raman and Infrared Vibrational Study of Human Skin: Assignment of Spectral bands, *J Raman Spectrosc*, 23 (1992) 641-645

38) P.J. Caspers, A. Williams, E.A. Carter, H.G.M. Edwards, B.W. Barry, H.A. Bruining, G.J. Puppels, Monitoring the Penetration Enhancer Dimethyl Sulfoxide in Human Stratum Corneum in Vivo by Confocal Raman Spectroscopy, *Pharm Res*, 19 (2002)