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Original Article

COMPARATIVE EVALUATION OF THE STRUCTURE OF *VERNIX CASEOSA* AND HUMAN *STRATUM CORNEUM* BY TRANSMISSION-FTIR SPECTROSCOPY

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Abstract

In utero, during the last trimester of gestation, the fetus is covered by a protective biofilm called Vernix caseosa (VC). One of the major problems in preterm delivery is the immature epidermal barrier and the absence of VC. When examining the structure of VC with electron microscopic studies, it appeared that the structure is very similar to that of the upper protecting layer of the skin, the Stratum corneum (SC). From this point of view it is important to evaluate the structural similarities and differences of VC and SC, in order to examine the concept that VC functions as a "mobile phase" SC.
This study aimed to determine the water content of VC and to examine the lipid organization of VC and human SC by Transmission FTIR Spectroscopy. The initial water content of VC was 79.33 ±1.10%. The experimental Transmission-FTIR data obtained in this study indicated that the orthorhombic lipid chain packing in VC is absent which is different from the lipid phase behavior in SC.

Key words: *Vernix caseosa, Stratum Corneum, FTIR Spectroscopy*

Vernix Caseosa ve İnsan Derisi Boynuzsu Tabakası Yapısının Transmisyon FTIR Spektroskopisi ile Karşılaştırmalı İncelenmesi

Anne rahminde, hamileliğin son döneminde, fetusun derisi Vernix caseosa (VC) olarak adlandırılan koruyucu özellikte biyolojik bir film ile kaplanmaktadır. Erken doğumlarda karşılaşılan önemli bir problem, epidermal bariyerin gelişmemiş olması ve VC'nın eksikliğidir. Elektron mikroskobu çalışmaları VC yapısının, derinin en üstteki koruyucu tabakası Stratum corneum (SC) yapısı ile çok benzer olduğuna işaret etmektedir. Yapısal benzerlik ve farklılıkların değerlendirilmesi, VC nın "hareketli faz" SC olarak işlev gördüğü yaklaşımından dolayı önem taşımaktadır.
Bu çalışma, VC'nın su içeriğinin saptanması ve Transmisyon FTIR Spektroskopisi ile VC ve SC lipit düzeninin araştırılmasını amaçlamaktadır. VC su içeriği %79.33 ±1.10 olarak saptanmıştır. Deneysel Transmisyon FTIR Spektroskopisi verileri, SC lipit faz davranışından farklı olarak, VC da ortorombik zincir düzeninin varolmadığını göstermiştir.

Anahtar kelimeler: *Vernix caseosa, Boynuzsu tabaka, FTIR Spektroskopisi*

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INTRODUCTION

In *utero*, during the last trimester of gestation, the fetus is covered by a protective biofilm called *Vernix caseosa* (VC). VC is mainly composed of water, proteins and lipids (1-4) and it is a uniquely human material (2). One of the major problems in preterm delivery is the immature epidermal barrier and the absence of VC. This results in a much higher risk for infectious diseases and a poor temperature control of the newborn. For this reason preterm infants are often covered with occlusive barrier creams, which differ in some important aspects from that of the VC (5). There is a strong need to mimic VC more closely, especially with respect to its high water content. When examining the structure of VC with electron microscopic studies, it appeared that the structure is very similar to that of the upper protecting layer of the skin, the *Stratum corneum* (SC) (5).

The human SC consists of keratin filled dead cells, the corneocytes, which are entirely surrounded by crystalline lamellar lipid regions. The lipids of SC are unusual in their composition, structure and localization; they contain only ceramides, free fatty acids and cholesterol, and they form broad, multilamellar sheets (6,7). There is a correlation between the phase behavior of SC lipids and the properties of the skin barrier (8,9). In SC corneocytes are linked one to another via specific molecular structures called corneodesmosomes. VC is a simpler system composed of hydrated fetal corneocytes embedded in a hydrophobic lipid matrix. The lipids of VC have been reported to contain ceramides and cholesterol as well as squalene. Ceramides are epidermal barrier lipids, whereas squalene is a product of sebaceous origin (2).

Measurements of the water content of VC have revealed major differences between VC and standart wound ointments used in newborn care. The long-term goal is to design novel barrier creams, to improve the protection of preterm infants after birth and to facilitate the treatment of diseased skin in adults, which often parallels an impaired barrier function. From this point of view it is important to evaluate the similarities and differences of the structure of VC and SC, together with the results of the studies about the interaction of VC with human skin.

FTIR spectroscopy has been extensively used to study the phase behavior of lipid membranes (8,10,11). It is of great potential for studying the disorder of the SC lipids since it was shown that the degree of the alkyl chain disorder results in a shift to higher wave numbers of the asymmetric stretching vibration (ASSV) and symmetric stretching vibration (SSV) bands located around 2920 cm^{-1} and 2850 cm^{-1}, respectively (12). This might be related to the introduction of gauche conformers in the alkyl chain (12-14). The ASSV and SSV bands are affected differently by the degree of lipid packing and they require different packing arrangements to achieve free vibrational movement (15).

In this study, the water content of VC was investigated and the lipid organization of human SC and VC at various hydration levels was examined by Transmission FTIR (Fourier Transformed Infrared) Spectroscopy in order to examine the concept that VC functions as a "mobile phase" SC.

EXPERIMENTAL

Vernix caseosa

VC was harvested from 6 full-term infants in and stored at 4°C±0.5 until use. All samples were stored in tightly closed small vials to prevent the contact with air and the evaporation of intrinsic water as much as possible. Organoleptic controls of VC included evaluation of color change, drying, phase separation (water droplets on the surface), contamination with blood or meconium and visible bacteriological contamination. The collection of VC was conducted after written consent of the mother and was approved by the local ethical committee.

Human Stratum corneum

Female human abdominal skin was obtained immediately after plastic surgery with the written consent of the patients and dermatomed (Padgett Electro Dermatome, Kansas City, MO). The dermatomed skin was placed on filter paper which was previously soaked in 0.1% w/v trypsine in phosphate buffered saline (PBS) solution (pH 7.4) and stored overnight at 4°C. Then, skin was incubated at 37°C for 1 h. SC was carefully removed from the viable epidermis and washed with 0.1% anti-trypsin solution and then rinsed twice with distilled water. After the isolation procedure, SC samples were brought over a grid and stored in a desiccator over silicagel under nitrogen to remove traces of solvent. The experiments were performed on abdominal SC obtained from 3 donors. All samples were taken with adherence to the Declaration of Helsinki Principles.

Water content of Vernix Caseosa

To measure the natural water content of VC, the specimens were weighed and transferred into a vacuum chamber and desiccated over silicagel under nitrogen until a constant weight was obtained. During this period, the specimens were weighed at regular intervals (after 24, 48, 72, 96, 120, 144, 168 and 192 hour) using a Microbalance (Mettler TG 50 Thermobalance).

Transmission Fourier Transform Infrared (FTIR) Spectroscopy Studies on Human Stratum Corneum and Vernix Caseosa

The effects of temperature and different hydration levels on human SC and VC were investigated by Transmission FTIR spectroscopy in Leiden/Amsterdam Center for Drug Research (LACDR), Leiden University, Leiden, The Netherlands. The aim of these analyses was to investigate the phase behavior of VC lipids at various hydration levels and to compare it with that of the human SC lipids.

SC samples (1cm x1 cm) were cut from a single sheet of SC for each experiment. VC samples were spread on the middle of the IR windows on a 1cm x 1cm area. Because of the viscous creamy structure of VC specimens, samples were placed directly in the middle of the windows and attention was paid so that almost all sample remains on the way of the IR beam.

FTIR transmission spectra were recorded at 2 °C intervals from 20 °C to 100 °C as a function of increasing temperature in the frequency 4000-400 cm^{-1} with a Bio-Rad FTIR spectrophotometer. The samples were sandwiched between two CaF_2 windows, mounted on a specially designed heating/cooling cell. Temperature control between 20 °C and 100 °C was achieved by a thermocouple, which was in direct contact with the sample. Each spectrum resulted from the co-addition of 128 scans with a nominal resolution of 2 cm^{-1}. To obtain the information about the degree of conformational order characterizing the alkyl chains of SC and VC, the thermally induced variations in the wave numbers associated with the ASSV (2920 cm^{-1}), SSV (2850 cm^{-1}) and C-H_2 rocking (720 cm^{-1}) bands, were studied. Individual bands were monitored by sequentially subtracting each spectrum from the subsequent one (which was obtained at a higher temperature) and frequency peak positions were determined using the Bio-Rad software, which allowed positions to be determined within ±0.1 cm^{-1} deviation. All experiments were repeated at least 3 times.

RESULTS AND DISCUSSION

Vernix caseosa

Only one VC sample was contaminated with blood. This specimen was excluded.

Human Stratum corneum

When human SC specimens were physically controlled, no damage was observed on the surface of the specimens.

Water content of Vernix Caseosa

The loss of VC weight during 5 days is shown in Table 1. The data after a dehydration period of 144, 168 and 192 hours is not included because no further reduction in weight was observed.

Table 1. VC weight loss during the dehydration procedure

	Sample weight (mg)					
VC SAMPLE	**DAY 0**	**DAY 1**	**DAY 2**	**DAY 3**	**DAY 4**	**DAY 5**
VC 1	26,207 ±2.02	9,366 ±3.06	6,243±4.89	5,710±3.14	5,650±2.13	5,644±3.01
VC 2	30,328 ±3.56	11,880 ±2.45	7,171±5.45	6,448±3.67	6,393±2.15	6,390±2.08
VC 3	28,408 ±2.75	8,352 ±5.67	6,071±5.90	6,006±3.78	5,996±2.17	5,991±2.53
VC 4	30,552 ±3.04	8,735 ±3.90	6,600±2.34	6,511±2.56	6,510±2.04	6,509±2.09
VC 5	27,107 ±2.82	8,977 ±3.12	5,926±1.34	5,653±3.26	5,629±3.03	5,629±3.08
VC 6	25,775 ±4.07	7,467 ±2.44	4,766±2.35	4,727±1.90	4,713±3.01	4,713±2.10

Comparison between hydrated and dry VC revealed that the initial water content of VC was 79.33 ±1.10%. It was observed that 67.51±3.93% of water was rapidly released within the first 24 hours. This result is in accordance with the hypothesis that VC contains a large quantity of releasable water (5). Given this high water content of VC, its moisturizer capabilities were investigated. Application of VC to adult human volar skin resulted in increases in baseline surface hydration and this effect was confirmed with panel tests performed with Corneometer and ATR-FTIR Spectroscopy on normal and acetone induced dry skin (2, 16).

The complete dehydration of VC at room temperature took 5 days and the slow rate of dehydration is consistent with the finding that VC lipids are resistant to aqueous diffusion.

Transmission Fourier Transform Infrared (FTIR) Spectroscopy Studies on Human Stratum Corneum and Vernix Caseosa

Changes in the ASSV and SSV bands of the SC lipid alkyl chains as a function of temperature were compared for isolated human SC at 2 hydration levels, namely, dry and hydrated (Figure 1 and 2). In all cases, the lipids underwent a phase transition from ordered all-trans chains to conformational disordered chains above 60 °C, characterized by an increase up to 7 cm^{-1} in the ASSV and 5 cm^{-1} in the SSV bands. The sigmoidal shapes showed the change in fluidity associated with the phase transition of gel to liquid crystalline.

For dry SC, shifts in ASSV bands from 2916.8 to 2925.3 cm^{-1} and in SSV bands from 2849 to 2854.2 cm^{-1} were observed in the temperature range of 20 °C -100 °C. Hydrated SC samples showed shifts from 2916.7 to 2926 cm^{-1} in ASSV and from 2848.9 to 2854.7 cm^{-1} in SSV bands.

In general, SSV bands between 2848 cm^{-1} and 2850 cm^{-1} are representatives of a hexagonal type gel bilayer structure, whereas frequencies of 2852 cm^{-1} to 2854 cm^{-1} exist for liquid crystalline phases (17). As an illustration for the differences in SSV bands, the temperatures are provided at which this band reach 2850 cm^{-1}. This is at 46 °C and 36 °C for dry and hydrated SC, respectively. For dry SC, the shift to 2852 cm^{-1} was observed at 80 °C, while the same shift was detected at 72 °C for hydrated samples. For dry SC, bands are shifted to 2853.2 cm^{-1} between 60 °C and 90 °C. Upon heating, a conformational disorder is also introduced in the hydrated SC samples.

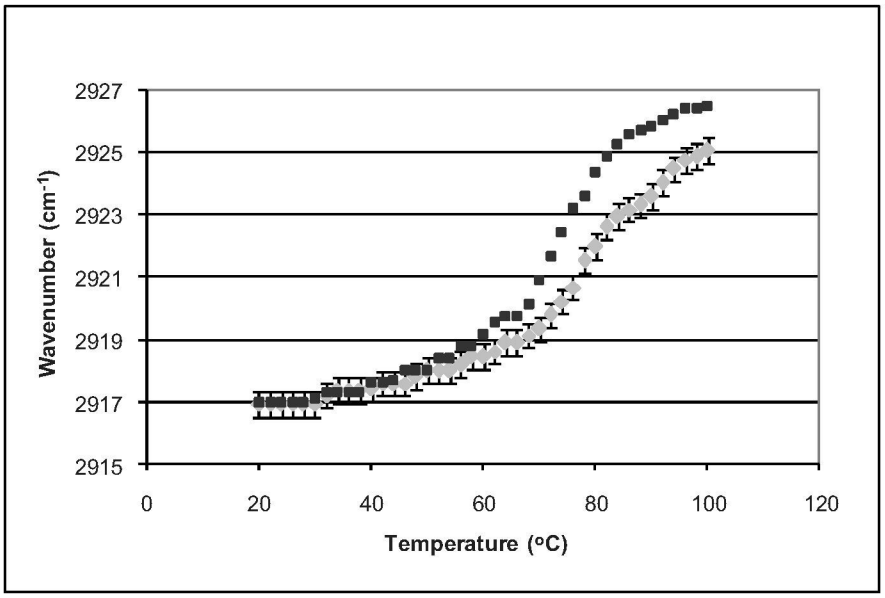

Figure 1. Changes in asymmetric stretching frequencies of SC lipid alkyl chains as a function of temperature for isolated human SC at 2 hydration levels, dry (black square) and hydrated (grey square)

The values observed after the last transition were approximately 2854.5 cm^{-1}, a value typical of a fluid phase. The shift to a wave number of 2854 cm^{-1} is observed at 86 °C and 98 °C for dry and hydrated SC, respectively. ASSV bands reached a value of 2920 cm^{-1} at 72 °C and 66 °C for dry and hydrated SC, respectively. The significant increase in ASSV and SSV band values above 60 °C (up to 7 cm^{-1} for ASSV and 5 cm^{-1} for SSV) may be related to the increase in the trans-gauche isomerization along the alkyl chains at high temperatures indicating a fluid phase.

Figure 3 and Figure 4 illustrate the thermally induced (20 °C - 100 °C) changes in ASSV and SSV bands of fresh (hydrated state) and dry VC. In fresh VC, the first increase in the frequency of ASSV band was at 26 °C from 2923.4 cm^{-1} to 2924 cm^{-1} and at 42 °C a second increase of 1 cm^{-1} was observed. Between 60 °C and 62 °C there is a small but definite inflection point from 2925 cm^{-1} to 2926 cm^{-1}. At 76 °C and 84 °C, two small shifts by 0,4 cm^{-1} and 0,5 cm^{-1} has been found, while the last shift was seen at 88 °C – 90 °C from 2926,8 cm^{-1} to 2927 cm^{-1}.

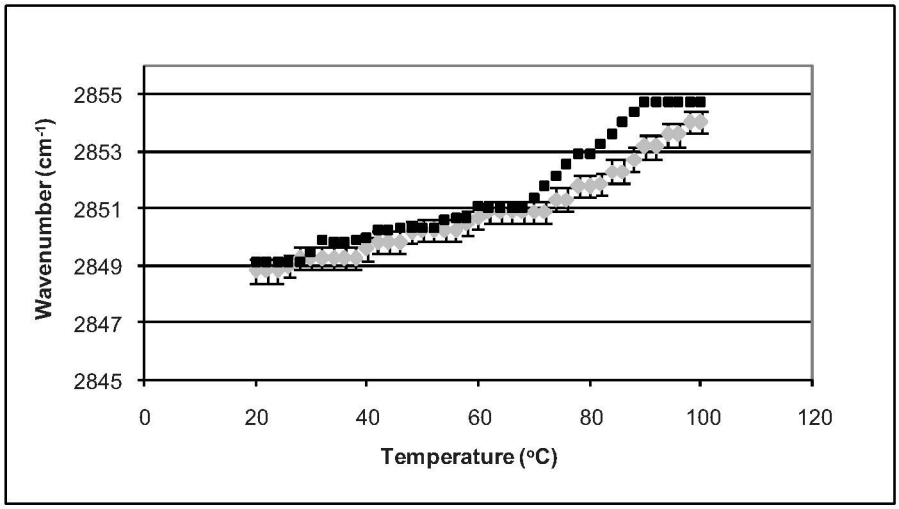

Figure 2. Changes in symmetric stretching frequencies of SC lipid alkyl chains as a function of temperature for isolated human SC at 2 hydration levels, dry (black square) and hydrated (grey square)

SSV bands obtained from fresh VC varied between 2851.8 cm^{-1} and 2853.5 cm^{-1} over the temperature range of 20 °C and 100 °C. The shift in frequencies is generally representative of liquid crystalline phase transitions (17). At 28 °C, SSV bands in fresh VC approached a frequency of 2851.8 cm^{-1} and 2852.2 cm^{-1} which indicates chains with a substantial fraction of disorder (6). Upon heating, two transitions were observed at 56 °C and 76 °C and the band position remained constant up to 100 °C at 2853.3 cm^{-1}. Spectral results obtained from the C-H_2 stretching region indicated that VC is mostly in a state which is intermediate between the hexagonal and liquid crystalline phase.

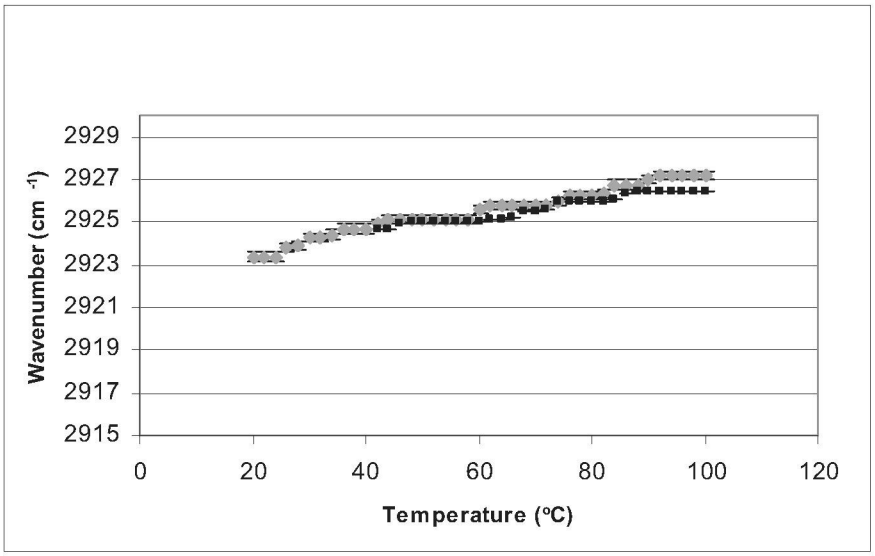

Figure 3. Changes in asymmetric stretching frequencies of VC lipid alkyl chains as a function of temperature at 2 hydration levels, dry (black square) and hydrated (grey square)

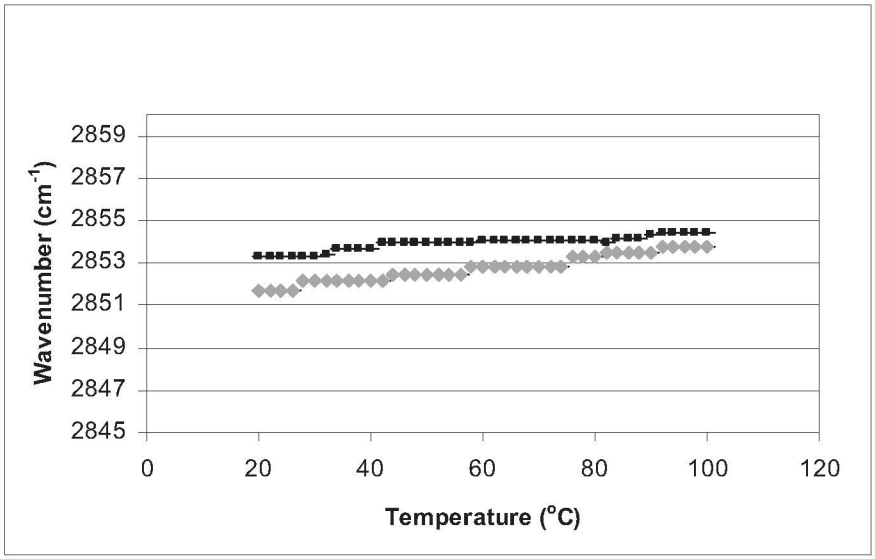

Figure 4. Changes in symmetric stretching frequencies of VC lipid alkyl chains as a function of temperature at 2 hydration levels, dry (black square) and hydrated (grey square)

At low temperatures, the wave numbers of ASSV and SSV bands are at a higher value in VC samples compared to that of SC. However, the shift toward higher frequencies upon heating was not very high in VC; ASSV bands showed a shift up to 5 cm^{-1} between 20 °C and 100 °C, while SSV bands shifted toward higher wave numbers by only 1-3 cm^{-1} in the same temperature range. At high temperatures, the wave numbers of SSV bands in VC were smaller than that of SC.

ASSV bands could not be detected in completely dried VC until 42 °C, while the SSV peaks were very broad and therefore very difficult to evaluate. ASSV band shifted from 2924.6 cm^{-1} to 2927 cm^{-1} between 42 °C and 100 °C. Depending on the degree of hydration, changes in the structure of sebaceous originated lipids which are mixed with the epidermal lipids in VC seem to cause a collapse of ASSV and SSV bands (Figure 5).

VC does not have the mechanical properties of the skin but it contains multiple cellular elements which allow VC to function as a semi-fluid viscous paste with temperature- dependent characteristics (18). In VC, structural alterations depending on the degree of dehydration affected the inter-chain interactions of epidermal lipids and a collapse of ASSV and SSV bands was detected in completely dehydrated VC samples. Increasing temperature caused immobilization of the lipids and the lipid structure was ordered probably with increased inter-chain interactions due to the higher mobility.

In previous studies it was reported that the lipid matrix material within VC is nonlamellar (2). However, in recent studies lamellar lipid regions was observed close to the surface of VC corneocytes (4). One approach is that, these lamellar lipid regions in VC regulate the water transport from the corneocytes to the surrounding lipid regions. Limitation of water transport across the developing epidermis facilitates skin barrier formation of fetus in utero. Spectral results obtained from the C-H_2 stretching region in this study indicates the existence of some lamellar region in the lipid part of VC, which is sensitive to the amount of the water content of the material.

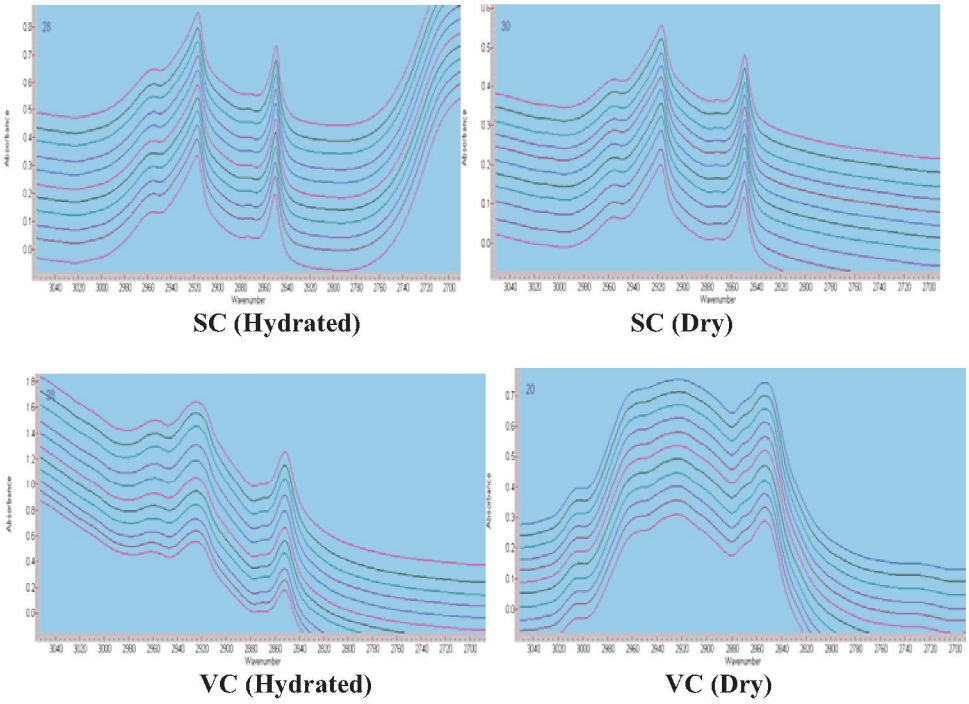

Figure 5. Asymmetric and symmetric stretching frequencies in human SC and VC between 20 °C and 40 °C

The lateral packing within the lamellae can be described by the methylene rocking vibrations located at approximately 720 cm^{-1} due to the short range coupling in the densely packed structure (12,19). At the physiological temperature, the C-H_2 rocking mode of the methylenes in the SC split into two components (group splitting). This indicates an ordered phase with orthorhombic perpendicular chain packing arrangement. Temperature-dependent collapse of the group splitting to a single peak identifies a transition from an orthorhombic to hexagonal packing of the chains (19). The investigations in this study revealed that the C-H_2 rocking vibration values observed for dry human SC constitute of two components at about 719.3 cm^{-1} and 729.2 cm^{-1} between 20 °C and 42 °C, following their collapse above 42 °C to a single component near 719.4 cm^{-1}. Temperature-dependent collapse of the group splitting to a single component is observed close to 720.3 cm^{-1} above 32 °C for fully hydrated SC (Figure 6).

In VC only a single component was observed in the entire temperature range of 20 °C -100 °C between 720.5 cm^{-1} and 722 cm^{-1} at different hydration levels. This indicates that VC acyl chains adopt a hexagonal packing at all the temperatures studied.

Barrier lipids, especially ceramides with long fatty acid chains play a key role in the characteristic organization of SC and the majority of lipids have straight saturated fatty-acid chains. In VC the majority of the fatty acid chains are either unsaturated or branched (Rissman). Transmission FTIR data obtained in this study supports the concept that VC functions as a "mobile-phase" SC and the functional significance of the structural differences remain to be further explored.

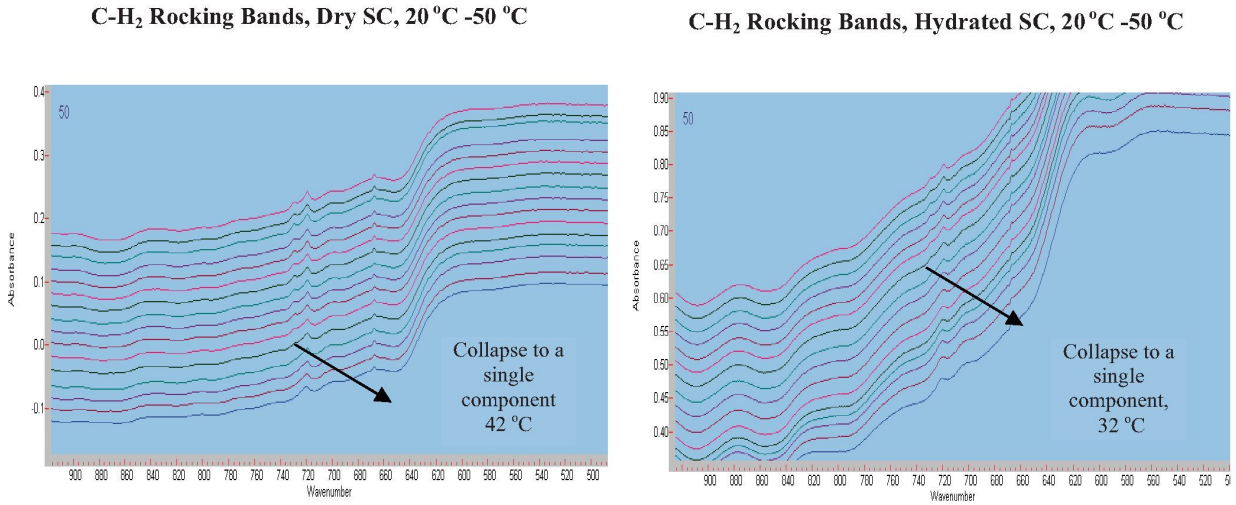

Figure 6. Temperature-dependent collapse of the group-splitting of C-H_2 rocking vibrations in dry and in hydrated human SC

CONCLUSION

Although VC is a simpler system than human SC, it has a complex biological role in the human fetus and newborn. The high water content distinguish VC from convantionel skin barrier creams. The experimental Transmission-FTIR data obtained in this study indicated that VC was mostly in a state which is intermediate between the hexagonal and liquid crystalline phase and no clear transition from gel to fluid phase was observed. The results from the C-H_2 rocking region appear to provide evidence that the orthorhombic lipid chain packing in VC is absent which is different from the lipid phase behavior in SC. In combination with the data gained from the studies about the hydration characteristics of human SC following topical application of VC, these results provide a framework for discussing the formulation parameters of novel barrier creams, which might be useful for improving and enhancing epidermal barrier function.

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