Spectral Differences between Stratum Corneum and Sebaceous Molecular Components in the Mid-IR

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Despite a number of studies on the composition of the lipids of stratum corneum (SC) and sebum, questions remain about the detailed molecular arrangement of the two superficial components of human skin. The investigation of the molecular components of SC *in vivo* is important to understand the function of what was once thought to be a "dead" epithelium. We have investigated the molecular composition of SC and sebum *in vivo,* in the mid-infrared, with fiber-based attenuated total reflection Fourier transform infrared spectroscopy (ATR/FT-IR). This technique combines the sensitivity of infrared spectroscopy in detecting molecular composition and conformational order with the capability of probing surfaces to a depth of less than 1 μm. ATR/FT-IR is therefore particularly useful for the investigation of interfaces such as SC and the sebaceous layers. We found that with the use of ATR/FT-IR one can distinguish between the contribution of the molecular components of sebum and SC. The presence of spectral "signatures" of the lipids of sebum allowed us to improve the interpretation of some infrared bands of sebaceous origin as well as of SC *in vivo*. We also found that ATR/FT-IR can be used to separate the spectral contributions of sebum and SC, and as a method to study the early recovery of superficial lipids after the removal of sebum. Following calibration, a method can be developed to quantify the relative amount of fatty acid in sebum with the use of ATR/FT-IR. We observed that the sebaceous fatty acids that reach the surface of the skin recover at a slower rate than other sebaceous lipids. Our investigation shows that fiber-based ATR/FT-IR is a promising spectroscopic approach to the study of epithelial surfaces and surface contaminants *in vivo*.

Index Headings: **Attenuated total reflection Fourier transform infrared; ATR/FT-IR; Stratum corneum; Sebum.**

INTRODUCTION

The characterization of the permeability barrier of human skin represents a challenge.[1–5] The molecular composition and the conformational order of the molecules participating in the permeability barrier have been investigated in animal models and humans.[6–11] Stratum corneum (SC) has emerged as a complex tissue whose biology includes post-transcriptional[12,13] and enzymatic activity.[14,15] The characterization of the composition, the structure, and the conformational order of the molecules that form the SC is the key to understanding the functions of this tissue. In some areas of the body the investigation of the molecular characteristic and the biological activity of the SC is complicated by the presence of a layer of sebum on its surface. The role of sebum, its physiology, and the methods to measure its composition and secretion rates have been investigated without closure on a model for its function *in vivo*.[16–22] The lipids of the SC are characterized by the exclusive presence of sphingolipids and a large amount of cholesterol and triglycerides.[23,24] On the other hand, sebum is rich in waxes, squalene, and free fatty acids.[16,21] We investigated SC and sebum with attenuated total reflection Fourier transform infrared spectroscopy (ATR/FT-IR).[25,26] ATR/FT-IR in the spectral region 4000–1000 cm^{-1} provides information on the composition and the conformational order of proteins, lipids, and water.[27–31] This technique, which was previously used with internal reflectance elements (IREs)[32] of large areas and large numbers (>9) of reflections,[33] can now be applied *in vivo* with the use of a fiber launch terminating with a small conical IRE (3 mm in diameter). This extension reduces the area investigated and increases the potential for the detection of infrared signal *in situ*. In ATR experiments, the shallow penetration of the evanescent field (less than 1.5 μm in skin[33]) is ideal for investigating the spectra of the surface of skin and the sebaceous material, which is collected on the surface of the IRE when this touches the skin. The amount of sebum transferred from the surface of the skin to the IRE during the acquisition of an FT-IR spectrum is sufficient to record a second IR spectrum of the material collected. With this method we separated the contribution of SC and sebum to the infrared signal. A method was developed to separate the contribution of sebaceous lipids from that of SC lipids. Thus sebum and SC could be characterized *in vivo,* and the rate of recovery of sebaceous lipids could be measured. The fiber-based ATR/FT-IR is a versatile method for the study of biological interfaces.

MATERIALS AND METHODS

ATR/FT-IR. The experimental setup included an FT-IR spectrometer (Vector 22, Bruker Analytic GmbH, Hamburg, Germany) with a sample compartment for transmission experiments and a bifurcated fiber bundle (Remspec Corp., Sturbridge, MA) for reflectance and ATR experiments. For transmission experiments we used a deuterated triglycine sulfate (DTGS) detector. *In vivo* measurements were carried out with the bifurcated fiber attached to the FT-IR spectrometer, terminating with a ZnSe crystal (diameter = 3 mm) that represents the IRE.[32] The signal collected by the fiber was directed to a liquid nitrogen-cooled HgCdTe (MCT) detector (EG&G, Montgomeryville, PA). In this configuration the incident light undergoes two total reflections of 45° at the crystal/skin interface. The distance into the second medium at which the evanescent field has decayed to 1/e of the initial value is given by the equation[32]

$$d_p = [2\pi\nu n_1(\sin^2\theta - n_{21}^2)^{1/2}]^{-1} \quad (1)$$

where ν is the frequency of the incident radiation (4000–

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1000 cm^{-1} in this case), n_1 is the refractive index of the IRE crystal (2.42 for ZnSe),[33] θ is the angle of incidence (45°), and n_{21} is the ratio between the refractive index of the sample (1.55 for skin)[33] and the one of the crystal. Under these conditions the penetration of the evanescent field in the skin is between 0.25 and 1.2 μm.[33] In order to correct the data for the wavenumber-dependent penetration of the investigating beam, we divided the IR spectra with Eq. 1.

In transmission mode each spectrum was the average of 25 scans, while in ATR mode each spectrum was the average of 80 scans. Resolution was set at 2 cm^{-1}. Under these conditions each spectrum *in vivo* was collected in approximately 1 min with a signal-to-noise ratio of the averaged spectra better than 10^3.

Spectra were collected, analyzed, and fitted with the software OPUS 3.0 (Galactic, Salem, NH). Fitting procedures were applied to the spectra over regions of 300–500 cm^{-1} at a time. When available, the starting parameters for each absorption band (e.g., position of the absorption peak) were obtained from the literature.[8,11,29,34–38] A Gaussian shape was assumed for each component. The starting number of free parameters for the fitting (i.e., the starting number of Gaussians curves) was overestimated. After each fitting cycle, the components that contributed less than 1% to the overall area under the IR spectrum were cancelled and a new fitting cycle with the remaining free parameters was started. The best fit was chosen as the last curve that fitted the spectra without a significant change (more than 5%) of the mean square of the residuals. With this approach we obtained the best fitting of the spectra employing the minimum number of independent parameters. Since each Gaussian carries three independent parameters (intensity, spectral position, and width) the fitting was performed with the constraint that the number of free parameters in the final fitting cycle was much smaller then the number of experimental points (typically 10 times smaller).

Human Subjects and Spectra Recording. After written informed consent was obtained, 18 healthy volunteers [10 men (age 22–60) and 8 women (age 21–55) (chosen among the employees of the laboratory)] were enrolled in the study. The subjects did not have history of skin disorders and were not under any medication at the time of the experiment. The volunteers were also asked to refrain from the use of cosmetic products for 48 h prior to the experiment. For this study we selected five sites: forehead, nose, neck, finger, and forearm. The sites were cleaned one hour prior to the experiment, and subjects were instructed not to touch those areas before the experiment. The measurements were carried out after the subject had been inside the room, without performing any heavy physical activity for at least half an hour. This precaution was taken to avoid effects on sebaceous and sweat glands due to steep temperature change[39] and/or excessive transpiration. Before each experiment a background scan was taken with the ATR crystal separated from the skin surface; the sequence of measurements for each site included the following acquisitions: (1) With the IRE in contact with skin (this step provides the spectrum of the uncleansed skin surface). (2) After the IRE was withdrawn from the surface (this step provides the spectrum of the components adsorbed onto the IRE). After this procedure the IRE crystal was gently cleaned with chloroform to remove the adsorbed lipids. After the total evaporation of chloroform we proceeded to the acquisition of the remaining spectra as follows: (3) With the IRE in contact with the skin after the SC had been gently wiped with organic solvents (this step provides the spectrum of the skin surface after the removal of most of the sebaceous lipid). (4) After the IRE was withdrawn from the surface of the skin (this step provides the spectrum of the residual components adsorbed onto the IRE after the removal of sebum). No residual components were left on the IRE after such procedure as verified by the acquisition of a spectrum of the cleaned crystal.

In each anatomic area the superficial material was removed by either gentle wiping of the skin with a 70% isopropyl alcohol gauze (Prep Pad, Professional Disposables Inc., Missisagua, ON) or by gentle tape stripping 2–4 times with the D-Squame tape (CuDerm, Dallas, TX). The tapes were weighed before and after stripping to obtain an estimate of the amount of material removed. The number of tape strippings varied to achieve the same signal as that obtained by isopropanol cleansing near 2850 cm^{-1}, where the lipids have one of their absorption maxima. For comparison, in a few cases sebum was removed with acetone (with application of an acetone soaked gauze for 5 s) or with Sebutape (CuDerm, Dallas, TX) applied for 30 min and then gently removed from the surface of the skin. Sebutape removes the sebaceous material without removing the stratum corneum.

Each site was marked for reproducibility in positioning the crystal after it was removed. The ATR crystal was held at the center of a Plexiglass® holder (2 in. in diameter) in order to ensure that the pressure of the crystal on the skin was evenly distributed every time.

Methods. In agreement with previous studies,[22,40] superficial material is transferred to the crystal when the IRE is in contact with the skin. Our method records an IR spectrum of sebum after an IRE–skin contact of only one minute. We calibrated this method to understand the limitations in the collection of sebum. We investigated the experimental conditions that might affect the transfer of sebum onto the crystal—that is, whether the collection of sebum is limited by the amount of sebum on the surface of the skin, the sensitivity of the instrument, the time of contact, or the shape of the IRE. These points were addressed by transferring sebum to the IRE under different conditions (time of sebum collection, site of collection, consecutive contacts with the surface of the skin, and shape of the collecting object). The calibration was carried out by plotting the intensity of the maxima near 2920 cm^{-1} and 2850 cm^{-1} (common to all lipids)[11] as a function of the time of collection and number of consecutive contacts. The intensity of these bands as a function of the same parameters was also investigated by collecting sebum on a flat ZnSe windows. This comparison achieves two goals: it compares the signal in transmission (flat window) with that in ATR (IRE) and it yields the comparison between the adsorption on two object of very different shape (cone vs. flat). This calibration was used to determine the conditions under which the intensity of the lipid bands deviated from linearity. This information is necessary when the signal due to the total amount of lipids saturates. This calibration indicated that a single

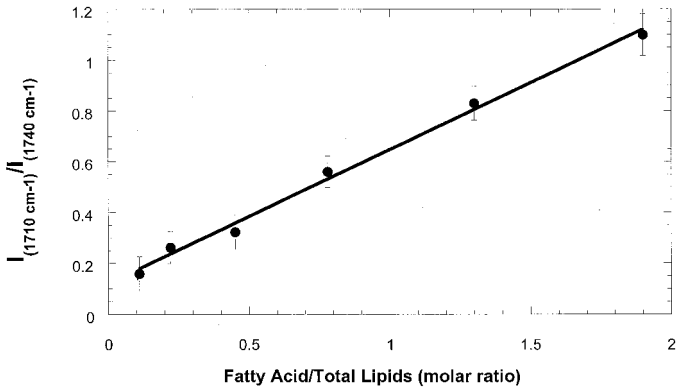

FIG. 1. Ratio of the IR intensity near 1710 cm^{-1} and near 1740 cm^{-1} as a function of the molar ratio of free fatty acids to total lipids. The spectrum was recorded in ATR mode. This plot provides a calibration of the amount of free fatty acids in a lipid mixture.

IRE–skin contact of one minute would ensure both a sufficient adsorption of sebum by the crystal and the linearity of the measurement.

Sebum Recovery. To follow the recovery of sebum, we cleaned two areas of the forehead—one with an isopropanol gauze, the other with gentle tape stripping. Immediately after the removal of surface lipids, the skin was touched with the IRE crystal for one minute, and the spectrum of the lipids adsorbed onto the crystal was taken. Successive readings were taken at 7, 15, 30, 60, and 120 min after the initial removal of the superficial lipids by collecting sebum on the IRE for 1 min. After each reading, the IRE was carefully cleaned with chloroform to completely remove the lipids of the previous reading.

Model Systems. The interpretation of the IR signals of SC and sebum was based on published data and on *in vitro* experiments on several lipids and lipid mixtures. The lipid mixtures included the most common SC and sebaceous lipids such as triglicerides, squalene, waxes, free fatty acids, methylated free fatty acids, ceramides, cholesterol, and cholesteryl ester. Each mixture contained lipids of different chain length. All lipids were purchased from Sigma (Sigma Chemical Company, St. Louis, MO) and used without further purification. Known amounts of lipids and lipid mixtures were dissolved in chloroform or in a 1:1 mixture of chloroform and methanol. For the experiments in the sample compartment, a layer of lipids was deposited on ZnSe windows by evaporation of the organic solvent. For ATR experiments, the IRE was immersed into the solution for 30 s, and the spectra were taken after the solvent evaporated.

The lipid mixtures were also used in the calibration necessary for the quantification of free fatty acids. Since free fatty acids are the only class of lipids that show an absorption maximum near 1710 cm^{-1},[41] we used a calibration method similar to one previously developed.[41] ATR/FT-IR spectra of lipid mixtures at known molar ratios of fatty acids were fitted with two components in the range 1800–1600 cm^{-1}. The variation of the ratio I_{1710}/I_{1740} provides the calibration to quantify the molar ratio of fatty acids with respect to the lipid pool (Fig. 1).

RESULTS AND DISCUSSION

Sebum vs. Stratum Corneum Signal. The superficial material is transferred abundantly from areas rich in sebaceous glands (i.e., forehead, nose, and neck), whereas little material (often below the detection threshold) remains attached to the probe after touching areas with few sebaceous glands (forearm and finger). The comparison of the spectra obtained with the probe in contact with the skin and after the contact is broken allowed us to isolate the contribution of sebum to the overall IR spectrum (Fig. 2). Acquisition of spectra of the superficial material transferred to the IRE is possible because a thin layer (<1 μm) of material gives a sufficiently strong signal (Eq. 1). The broad band near 3300 cm^{-1} due mostly to water (O–H stretch)[28] disappears in the spectrum of the superficial material transferred to the IRE (Fig. 2A). In areas rich in sebaceous glands, few changes are found in the intensity of the lipid bands between SC and the superficial material. The apparent decrease in intensity is mostly due to the absence of overlap with the broad band due to the OH stretch of water as confirmed by spectral fitting. The position of these peaks shifts towards higher frequencies in the spectrum of the surface lipids that are transferred to the IRE. The spectral fitting of the 3000–2800 cm^{-1} region shows that the shift is on average 2.2 ± 0.5 cm^{-1} for the asymmetric stretch (near 2920 cm^{-1}) and 1.9 ± 0.6 cm^{-1} for the symmetric stretch (near 2850

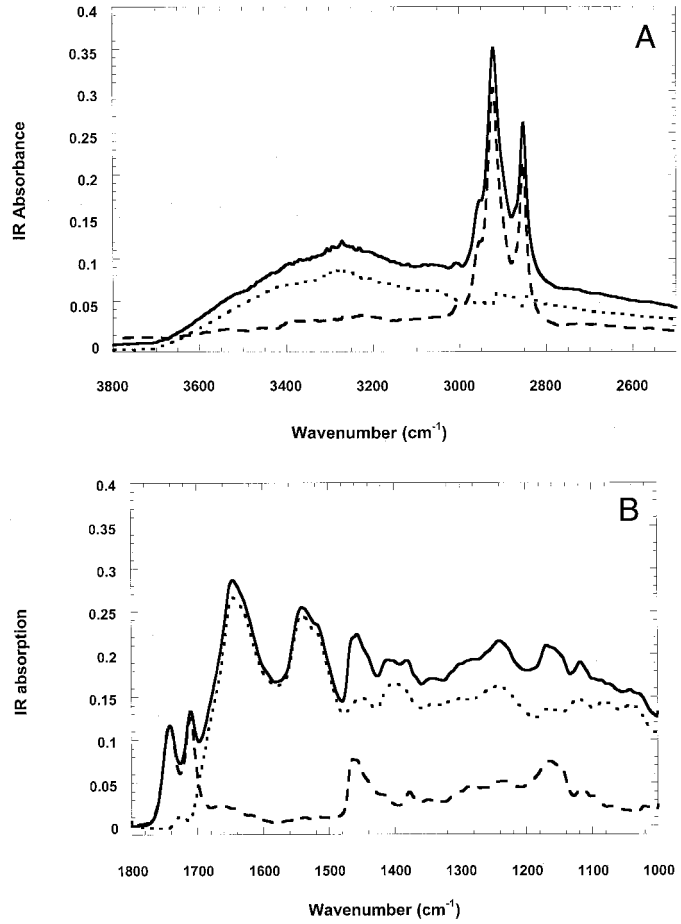

FIG. 2. *In vivo* ATR/FT-IR spectra of stratum corneum (———), sebum (– – –), and the difference spectrum of the two (·····). The spectrum of SC was taken with the probe touching the skin. The spectrum of sebum was acquired after the IRE was withdrawn from the surface of the skin. Each spectrum is the average of 10 subjects. The measurement site was the forehead. (**A**) Spectral range 3800–2500 cm^{-1}; (**B**) spectral range 1800–1000 cm^{-1}.

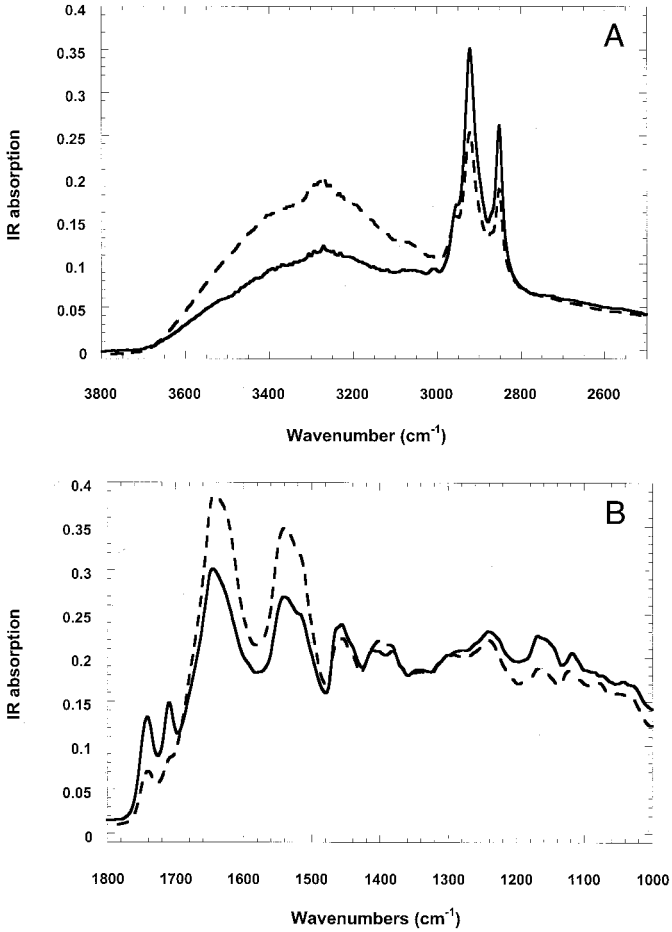

FIG. 3. *In vivo* ATR/FT-IR spectra of stratum corneum before (——) and after (– – –) cleansing the surface of the skin with isopropanol. Each spectrum is the average of 10 subjects. The measurement site is the forehead. (**A**) Spectral range 3800–2500 cm^{-1}; (**B**) spectral range 1800–1000 cm^{-1}.

cm^{-1}). In the region between 1800 and 1500 cm^{-1} (Fig. 2B) there are also differences between the spectra. For the peaks of the carbonyl of the ester linkages of lipids (near 1740 and 1710 cm^{-1})[25,40] the argument is similar to the one produced for the methylene peaks near 2920 and 2850 cm^{-1}. In the spectrum of SC the tail of the absorption band of the water OH stretch[28] overlaps to the absorption of the two peaks near 1720 and 1740 cm^{-1} and causes an apparent increase in the intensity of these bands in the SC. In the superficial material a broad band near 1465 cm^{-1} replaces a composite band with maximum near 1455 cm^{-1} (Fig. 2B). The spectral fitting of this region reveals that the superficial material presents two major peaks near 1378 cm^{-1} and 1465 cm^{-1}. In the spectrum of the SC in addition to these two peaks and the water peaks,[33] two more bands of comparable amplitude near 1457 cm^{-1} and 1406 cm^{-1} are needed for the fitting.

Clean Skin. Gentle delipidization of the skin surface with isopropanol 70% as well as with gentle tape stripping results in an essentially complete removal of superficial material from the skin (Fig. 3).[14,42,43] The intensity of the peak near 3300 cm^{-1} increases after the superficial material has been removed. A parallel increase occurs in the amide region of the spectrum. The magnitude of the increase is comparable for the two methods used to remove the superficial material. The increase also characterizes areas rich in sebum after its removal with Sebutape. The application of Sebutape does not change the intensity of this band in areas with a low content of sebum, such as the arm. In the SC, removal of sebum produces also a shift towards lower wavenumbers of the peaks near 2920 and 2850 cm^{-1} (Fig. 4). The spectral fitting shows that the extent of the shift depends on the site investigated; it was between 1 and 3 cm^{-1} and was maximal immediately after cleansing with isopropanol. Removal of the superficial lipids produces a dramatic reduction of the amount of material collected on the IRE, as shown by the decrease of the peaks in the 3000–2800 cm^{-1} region (Fig. 5A). In SC, cleaning of the surface of the skin produced a decrease in the intensity of the peaks near 1740 and 1710 cm^{-1} (at some locations it disappeared below the detection limit). The decrease is also evident in the material collected on the IRE (Fig. 5B). Removal of the superficial lipids modifies the ratio between the 1710 and 1740 cm^{-1} peaks. The I_{1710}/I_{1740} ratio in the sebum transferred to the IRE is consistently smaller after cleaning of the surface of the skin (Table I). In the spectrum of SC, the position of the peaks in the region of the amide bands (1700–1500 cm^{-1}) is not affected by the removal of the superficial material. In the spectrum of SC, the position of the peaks near 1456 cm^{-1} and 1406 cm^{-1} remained unchanged (Fig. 3B).

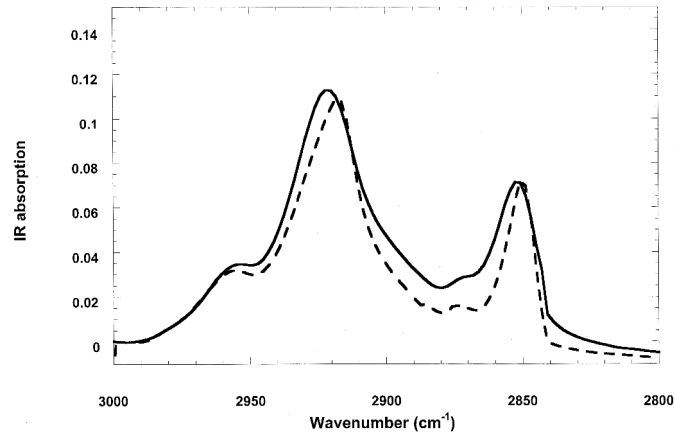

FIG. 4. Difference in the position of the methylene (CH_2) symmetric and asymmetric peaks. (——) SC before cleansing; (– – –) SC after cleansing. The position of the peaks after cleansing is shifted towards lower frequencies. The spectra are the average of 10 subjects. The measurement site was the nose.

Gender Differences. Clear differences between men and women were observed in the spectrum of sebum in the 1800–1700 cm^{-1} region. The amount of sebum transferred to the IRE in men was greater than in women at all sites investigated (Table I). The relative intensity of the peak near 1710 cm^{-1} is consistently smaller in women (Fig. 6 and Table I). The molar ratio of fatty acids, calculated from the calibration of Fig. 1, is on average 1.17 ± 0.13 in men and 0.55 ± 0.08 in women. Regional differences in the amount of sebum are under investigation. Gender differences occur also in the spectrum of SC. In men the peak near 1740 cm^{-1} is detectable at all sites investigated, whereas it disappears in the SC of women in the forearm and the finger.

Sebum Recovery. The superficial material on the fore-

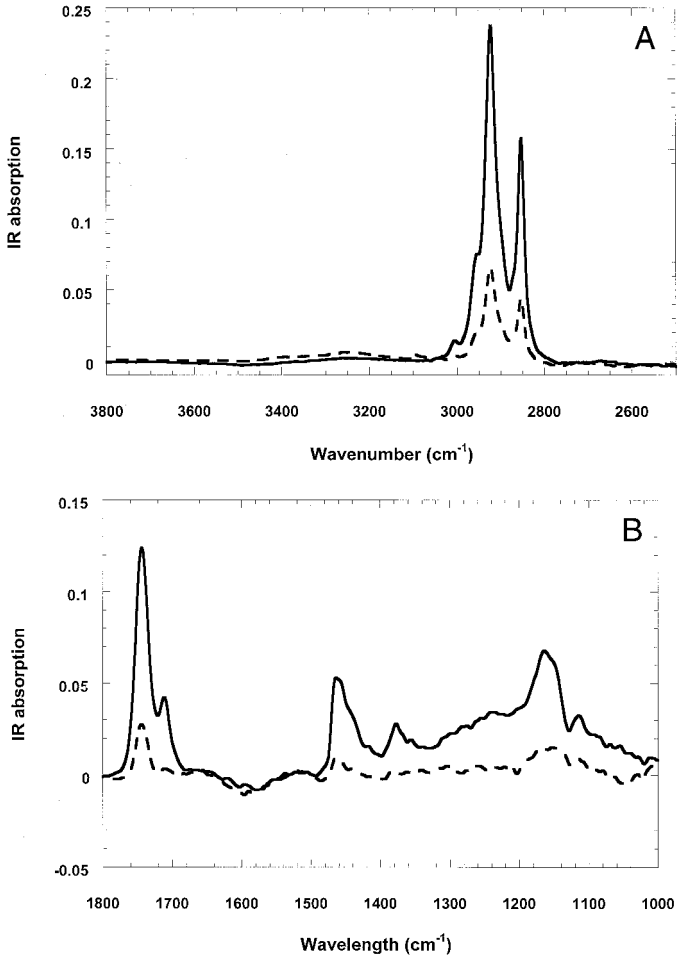

FIG. 5. Removal of sebum from the surface of the skin. The signal of the superficial lipids collected by the IRE after cleansing the skin with isopropanol (– – –) is much smaller than before cleansing the surface (———). (**A**) Spectral range 3800–2500 cm^{-1}; (**B**) spectral range 1800–1000 cm^{-1}.

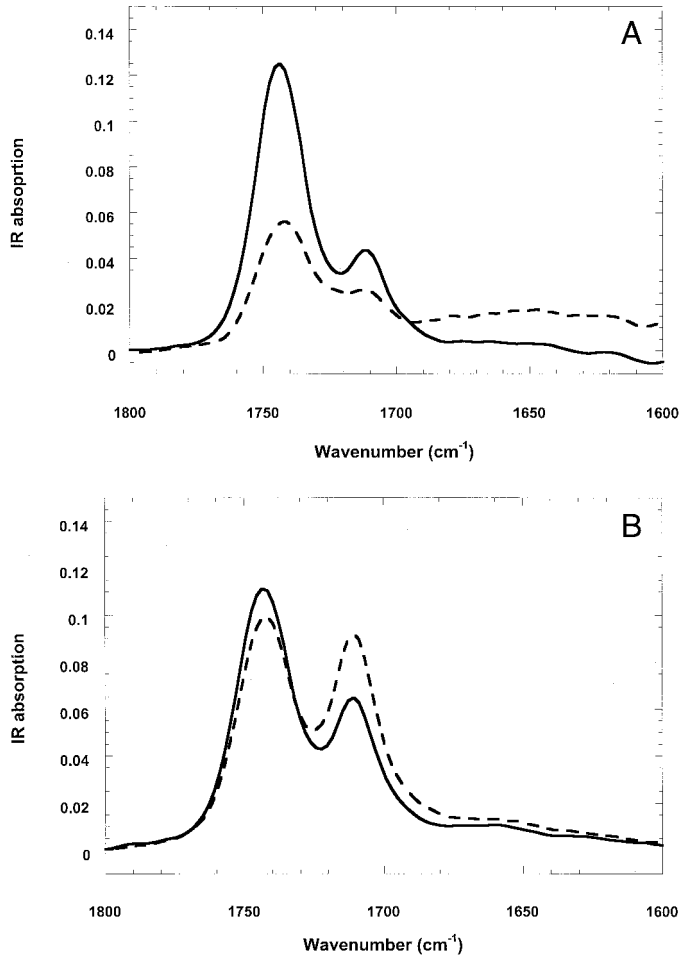

FIG. 6. Gender differences in the amount and composition of sebaceous excretion. The amount of sebum collected and the ratio between free fatty acids (peak near 1710 cm^{-1}) and the other esterified lipids (peak near 1740 cm^{-1}) is different in women (**A**) and men (**B**). (———) Forehead; (– – –) nose. The relative amount of fatty acids on the nose is on average bigger than in the forehead. Each spectrum is the average of 10 subjects. The spectra were recorded from the sebum collected by the IRE in 1 min of contact with the skin.

head was first removed with gentle tape stripping (2 to 4 times) or isopropanol; the recovery of the superficial material was then followed by measuring the spectrum of the material transferred in 1 min to the IRE after 7, 15, 30, 60, and 120 min. The intensity of the lipid absorption bands was followed over time (Fig. 7). The lipid absorption shows an initial increase and a plateau after 40 to 60 min. The results obtained by using ATR were parallel to those obtained in transmission experiments (where the superficial material is collected by using a flat ZnSe window). On the basis of the calibration used both in ATR and transmission mode, we believe that the intensity of the signal in the ATR spectra plateaus because the layer of sebum becomes thicker than the depth investigated by the evanescent field. At the same time, the amount of sebum collected reaches a maximum. Our calibrations, however, lead to the conclusion that the amount of sebum collected on the IRE crystal at the early stages ($t < 40$ min) of recovery is proportional to the amount of sebum actually present on the surface of the skin. It was determined (Fig. 7) that the rate of recovery of the carbonyl band of fatty acids (1710 cm^{-1}) is slower than for all other bands (Fig. 7 and Table II). The CH_2 stretching and bending modes (near 2920 and 1465 cm^{-1}, respectively) recover at the same rate as the absorption near 1740 cm^{-1}. Moreover the CH_2 bands and the peak near 1740 cm^{-1} recover to values close to the original one (0.95 ± 0.16),

TABLE I. Intensity and ratio of the carbonyl peaks at 1710 and 1740 cm^{-1} in areas rich in sebum.

		Before cleansing			After cleansing with isopropanol			After tape stripping		
Location	Gender	1740	1710	Ratio	1740	1710	Ratio	1740	1710	Ratio
Forehead	Men	0.124	0.067	0.54 ± 0.06	0.037	0.018	0.48 ± 0.05	0.038	0.011	0.3 ± 0.04
	Women	0.116	0.038	0.33 ± 0.02	0.028	0.005	0.19 ± 0.06	0.015	0.003	0.2 ± 0.03
Nose	Men	0.123	0.111	0.9 ± 0.01	0.049	0.033	0.67 ± 0.05	0.037	0.026	0.71 ± 0.03
	Women	0.073	0.062	0.85 ± 0.02	0.028	0.013	0.49 ± 0.02	0.016	0.09	0.55 ± 0.05

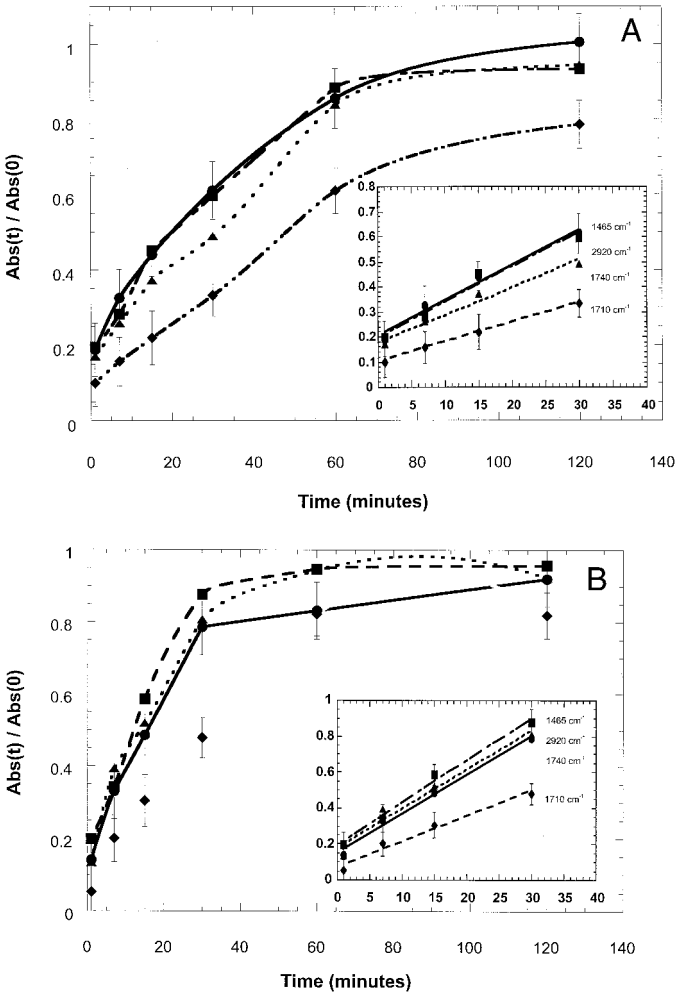

FIG. 7. Sebum recovery. The recovery of sebum on the surface of the skin as measured by the IRE is reported after its removal with isopropanol (**A**) and gentle (2–4 applications) tape stripping (**B**). (———) CH_2 near 2920 cm^{-1}; (– – –) CH_2 near 1465 cm^{-1}; (—·—) C=O near 1740 cm^{-1}, (·····) C=O near 1710 cm^{-1}. Then inserts in **A** and **B** represent the linear regression fit of the first four data points of each recovery curve.

while the intensity of the peak near 1710 cm^{-1} remains well below the initial value (0.8 ± 0.12). We also found that the method used to clean the skin affects the rate at which sebum recovers. In Table II it is shown that after removal with isopropanol the rate of recovery of all components of the IR spectrum is slower (about half) than after tape stripping.

Sebum Contribution and Spectral Assignment. The material transferred to the IRE is of sebaceous origin because (1) the signal intensity of superficial material collected correlates to the areas rich in sebaceous glands; (2) the signal from water and proteins (near 3300 cm^{-1}, 1645 cm^{-1}, and 1545 cm^{-1})[28,38] is absent from the spectrum of the superficial material transferred to the IRE (Fig. 2); and (3) gentle wiping of the skin surface with organic solvents, gentle tape stripping, or the application of Sebutape reduces or eliminates the amount of material that is transferred to the IRE (Fig. 5).

These results rule out the possibility that a significant amount of corneocytes is transferred to the IRE during the acquisition of the spectrum of the SC. In fact, a single

TABLE II. Rate of recovery of sebaceous molecules after removal of superficial material from the forehead.

Position of peak (cm^{-1})	Vibrational mode	Rate of recovery ($\times 10^{-4}$ s^{-1})	
		Cleansing with Isopropanol	Tape stripping 4 times
≅2920	CH_2 steretch	1.5 ± 0.04	3.1 ± 0.08
≅1740	C=O stretch	1.6 ± 0.06	3.2 ± 0.05
≅1710	C=O stretch	1.1 ± 0.03	2.3 ± 0.07
≅1465	CH_2 scissoring	1.5 ± 0.07	3.6 ± 0.06

layer of corneocytes, which contain most of the SC proteins,[3] has an average thickness of 0.5 to 0.8 μm[44] that, according to Eq. 1, is comparable to the penetration of the evanescent field into the SC (0.2–1.2 μm).[32,33] If corneocytes were transferred in large amounts to the IRE crystal, the signal of SC and that of the material transferred to the IRE would be similar in intensity. Since most of the SC lipids are either forming the cornified envelope or connecting the corneocytes,[1,10,45,46] we also assume that SC lipids do not transfer easily to the IRE. Moreover, since ceramides are the most abundant SC lipids we would expect to detect the bands of the lipid amide in the 1700–1500 cm^{-1} region (Fig. 8), which are instead absent from the spectra of the material adsorbed onto the IRE.

The absence of the water bands in sebum is consistent with a high content of lipids whose hydrophobicity excludes water from the environment. The almost exclusive presence of lipids in the material transferred to the probe is confirmed by the small decrease in intensity of the CH_2 bands between SC and sebum. The shift of the position of the CH_2 bands towards higher frequencies indicates a higher disorder of the aliphatic chains once sebum is on the IRE.[11] This apparent loss of conformational order suggests that sebaceous lipids may participate in an organized structure when they are on the surface of the skin and lose this structure when they are adsorbed by other materials. The difference in temperature between SC (about 32 °C) and the IRE (around room temperature, 22 °C) cannot account for the spectral shift, which in fact

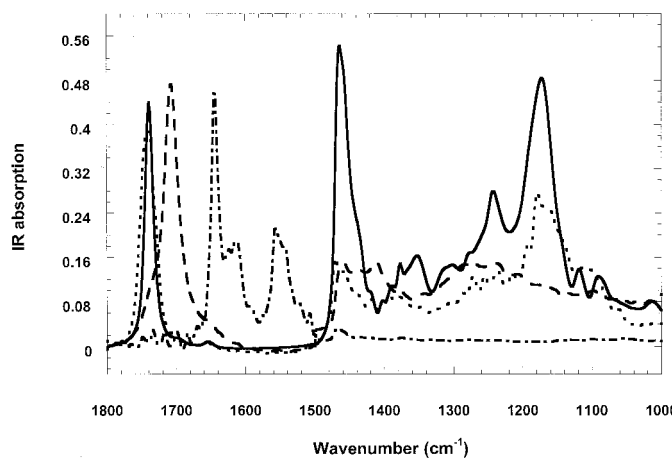

FIG. 8. Examples of IR absorption of lipid mixtures which are commonly attributed to sebum and SC are shown. (———) Triglycerides; (·····) waxes; (– – –) free fatty acids; (—·—) ceramides. Ceramides do not have a peak in the 1800–1700 cm^{-1} region, and free fatty acids are the only lipid mixture that shows an absorption near 1710 cm^{-1}.

would occur in the opposite direction.[11] The peaks near 1710 and 1740 cm^{-1} are the most characteristic absorption bands of sebum. The removal of the superficial lipids causes the decrease or the disappearance of these two bands (Figs. 3B and 5B). In the past there has been some uncertainty in their assignment.[40,47] The results we obtained with the lipid mixtures (Fig. 8) lead us to the conclusion that the peak around 1710 cm^{-1} comes from free fatty acids, because this mixture is the only one that gives a peak in the vicinity of that frequency. The data in Table I show that fatty acids are preferentially removed by the removal of the lipids from the surface of the skin, suggesting that they might be principally contributed from the sebaceous material.

The absence of peaks in sebum in the 1700–1500 cm^{-1} is consistent with the above-mentioned absence of water (OH bending mode absorbs near 1640 cm^{-1})[28] and proteins.[38] The presence of the peak near 1465 cm^{-1} and 1378 cm^{-1} in SC and sebum is consistent with the presence of CH_2 and CH_3 bending modes of lipids.[11,27,33,34] The absence of a doublet near 1470 cm^{-1} [36] is also consistent with a lower conformational order of the aliphatic chains. The absence of the peaks near 1455 cm^{-1} and 1400 cm^{-1} in sebum might also indicate that these peaks are due to proteins. These peaks were previously assigned to CH_3 scissoring modes.[33] However, their exclusive presence in the SC suggests instead that proteins might be responsible.

Effect of Removal of Surface Lipids. The increase of the water band near 3300 cm^{-1} (Fig. 3A) is due to an increase in the permeability of the SC when superficial lipids are removed or when the SC is even slightly disrupted.[2,13,14] We correlated the increase of the OH band to the increase in superficial water by using the Tagami hygrometer (manuscript in preparation). The increase in OH absorption occurs regardless of the method used to remove the lipids, which rules out artifacts due to the absorption of the organic solvent or to residues of the glue from the D-Squame and Sebutape. The removal of sebaceous lipids produces an effect on the spectrum of the SC: the maximum of the CH_2 absorption shifts towards lower frequencies. This result suggests that, compared to sebaceous lipids, SC lipids retain a higher level of conformational order. This observation is strong evidence for an interaction between sebaceous lipids and SC lipids in the superficial layers which changes their conformational order.

We have discussed in the previous paragraph the effect that the removal of sebum has on the intensity of the bands of the ester C=O of lipids. The disappearance of these bands could seem surprising at first, as most of the lipids in the SC and sebum contain at least one ester linkage between the head group and the aliphatic chains. However, the spectra of Fig. 8 show that the ceramides do not absorb in this region. The occasional disappearance of the C=O bands in the spectra of SC can then be explained with a much larger concentration of ceramides in the tissue.

This observation is also consistent with the fact that ceramides are abundant in SC.[24,44] The increased content of superficial water after skin cleansing produces an increase in the intensity of the amide bands between 1700 and 1500 cm^{-1} (Fig. 3B). This result is due to the increase of the band of the OH bending mode near 1640 cm^{-1}.[28] The spectral fitting of this region shows that there is no effect on the position of the bands, which in turn means that neither the application of isopropanol nor the increase in water content produces any appreciable effect on the secondary structure of the keratin filaments *in vivo*. This observation is in contrast with that from previous experiments that were conducted *ex vivo*,[35] where it was demonstrated that isopropanol (and other alcohols) produce a change in the secondary structure of proteins in the SC. We believe that the difference is due to the fast evaporation of the alcohol on the surface of the skin, which prevents isopropanol from changing the environment surrounding keratins. The fitting of the amide I region shows peaks near 1675 cm^{-1} and 1625 cm^{-1}, characteristic of antiparallel ß-sheets,[29,38] and a peak near 1652 cm^{-1} of the α-helix secondary structure.[37,38] This hypothesis is confirmed by the fitting of the amide II absorption band that shows peaks near 1516 cm^{-1} and 1545 cm^{-1} for the α-helix together with a peak of antiparallel ß-sheet near 1584 cm^{-1}.[48] However we cannot rule out that this peak is the result of the higher concentration of cysteine in the cornified layer.[49,50]

Gender Variations. The difference in the amount of fatty acids between men and women (Table I and Fig. 5) might be explained in terms of differences in hormonal activity or dietary habits, which are known to influence the composition and the excretion rate of sebum.[39] This difference is, however, important for future *in vivo* ATR/FT-IR investigations of biological differences between superficial lipids of women and men. The smaller signal of sebum collected from the surface of the skin in women could also be related to the use of cosmetics. Even though we asked the subjects to refrain from the use of cosmetics for 24 h, their daily application may permanently affect the production of sebum.

Sebum Recovery. The kinetics of recovery of sebum provide important clues not only about the rates of sebum production but also about other mechanisms involving sebaceous material. The consistency and the similarity of the value of the recovery rate of CH_2 bands with the recovery of the C=O band near 1740 cm^{-1} confirms the validity of our assumption of proportionality between the amount of sebum collected by the IRE and the amount of sebum present on the surface of the skin at early stages. We believe this method follows the recovery of sebum and does not record the recovery of SC lipids (in the eventuality that the superficial layer of SC is removed by our gentle cleansing of the surface of the skin). Since ceramides are the major components of SC, we expected to observe an increase in the amide peaks (see Fig. 8) in the 1700–1500 cm^{-1} region due to the recovery of the ceramides. This increase, however, did not occur. The slower rate of recovery of fatty acids (intensity near 1710 cm^{-1}) compared to that of the rest of the lipids confirms that the production of fatty acids in sebum follows a more elaborate path (Table II). It might indicate, therefore, that indeed the enzymatic cleavage of triglycerides into fatty acids occurs after secretion either within the SC or on the surface of the skin, resulting in the delay of the accumulation of fatty acids on the surface of the skin. This evidence would be consistent with a post-excretional step

in the production of fatty acids in sebum, which may depend on bacterial breakdown.

The results shown in Fig. 7 suggest that ATR/FT-IR *in vivo* can be used to investigate modification in non-steady-state sebum production. The recovery rate is affected by isopropanol. The recovery rate after tape stripping is about twice as fast as the recovery after cleansing with isopropanol. Isopropanol could penetrate the follicle and modify the lipids closer to the gland. Alternatively, the change in the conformational order of SC lipids[35] may delay the mechanism that triggers the excretion of sebum because the change in the order of the aliphatic chain may partly ''mask'' the removal of superficial lipids.

CONCLUSION

ATR/FT-IR is a potentially powerful technique to investigate skin functions *in vivo*. Our findings include: (1) The combination of superficial cleansing and sebum detection led to a different interpretation of the bands near 1400 cm^{-1} and 1455^{-1}. These were previously assigned to lipids, but according to our results they may be assigned to proteins. (2) The total amount of sebum on the surface of the skin is larger in men than in women, and the relative amount of free fatty acids in sebum is also larger in men. (3) After surface lipid removal, fatty acids recover at a slower rate than the rest of the lipids, suggesting an extra step in their synthesis such as cleavage of other sebaceous lipids (e.g., triglycerides). We have shown that fiber-based ATR/FT-IR may be a useful technique for the investigation of skin disorders that may affect the molecular arrangement of the SC.

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