A Critical Comparison of Some Raman Spectroscopic Techniques for Studies of Human Stratum Corneum

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Received October 20, 1992; accepted April 6, 1993

This study evaluates a variety of techniques and sampling conditions for Raman spectroscopic investigations of human stratum corneum. Using a Fourier-transform Raman system and samples of stratum corneum *in vitro*, we demonstrated minimal inter- and intracadaver variations in molecular vibrations. We have also shown Raman spectroscopy to be relatively insensitive to the hydration state of human stratum corneum, indicating that the technique should be valuable for monitoring the transdermal delivery of drugs from aqueous solutions. The stability of human stratum corneum to near-infrared laser excitation was verified by spectral collection for approximately 1 hr. We have also compared FT-Raman spectra from human stratum corneum *in vitro* and *in vivo*. Of the different types of Raman instruments used in this study (visible-light excitation microprobe, visible-light excitation macroscopic sampling, and Fourier-transform Raman), the FT-Raman system provided good-quality spectra with high sample throughput, but systems using visible-light excitation should provide unique information for use in specialist applications.

KEY WORDS: Raman; stratum corneum; techniques; skin; spectroscopy; Fourier transform.

INTRODUCTION

The outermost layer of the skin, the stratum corneum, is the major barrier to transdermal delivery of most drugs. A variety of spectroscopic techniques has recently been employed to study the structure of this horny layer and its modification by drug or chemical enhancer interactions. Electron spin resonance (ESR) investigations have probed stratum corneum lipid domains (1) and penetration enhancer effects on human stratum corneum (2), but these methods can be used only *in vitro*, whereas nuclear magnetic resonance has been employed for skin *in vivo* (3). Fourier transform infrared (FTIR) is currently the most popular spectroscopic technique for investigating stratum corneum structure, and this method allows *in vitro* or, using attenuated total reflectance, *in vivo* protocols (4,5). We recently reported the first use of FT-Raman spectroscopy for assessing the molecular nature of human stratum corneum *in vitro* (6). Additionally, we have described the Raman vibrational modes of human horny layer, compared these with infrared vibrations, and illustrated some of the advantages and disadvantages of these two complementary spectroscopic techniques for probing the structure of human stratum corneum (7).

Fourier transform techniques offer rapid spectral collection, but "conventional" spectroscopic techniques can still provide valuable information regarding molecular structures. Here we compare various Raman spectroscopic techniques for studying the molecular nature of human stratum corneum, including sample excitation using visible and infrared lasers and microscopic and macroscopic sampling, and for the first time we present a comparison of Raman spectra gathered from human stratum corneum *in vitro* and *in vivo*.

MATERIALS AND METHODS

Preparation of Human Stratum Corneum Membranes

Caucasian abdominal skin (male and female, 27–73 years) obtained postmortem was stored at −20°C (8). Epidermal membranes were prepared by the method of Kligman and Christophers (9); skin samples were immersed in water at 60°C for 45 sec, after which the epidermal membranes were teased off the underlying dermis. Stratum corneum samples were prepared from epidermal membranes (9); these were floated overnight on an aqueous solution of trypsin (0.0001%, w/v) and sodium hydrogen carbonate (0.5%, w/v) at 37°C. The enzyme digests the nucleate epidermal tissue, allowing the remnants to be removed by swabbing. The stratum corneum membranes were rinsed in water before being stored dry in a dessicator.

Raman Spectra

Raman spectra of human stratum corneum were collected using three spectrometers.

Visible-Light Excitation, Microprobe (MOLE)

Spectra were collected using a Jobin-Yvon Instruments Raman microprobe MOLE (Molecular Optical Laser Examiner), with sample excitation using a 2 W Spectra-Physics 164 argon-ion laser operating at 488.0 nm (visible light, blue/green region). The laser power was nominally 100 mW, representing some 20 mW at the sample, and an RCA type C 13034 photomultiplier detector was used. Samples were viewed with a Leitz microscope and a Hitachi color TV camera with color monitor; with a 100× microscope objective, a magnification of 3000× was achieved at the monitor. Spectra were collected from a 10 μm diameter spot at the stratum corneum surface, and typically 20 scans were collected per sample.

Visible-Light Excitation, Macroscopic Sampling (SPEX)

Stratum corneum samples were excited with a Spectra-Physics Model 2020/5 argon ion laser operating at 488.0 nm with a power of 100 mW at the sample. The scattered radiation was analyzed using a SPEX Industries Model 1401 Czerny-Turner 0.85 m spectrometer with a spectral slit width

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0724-8741/93/1100-1642$07.00/0

of 4 cm^{-1}. Photon counting detection was employed with an EMI 9789 QA photomultiplier tube. Spectra were collected from a 50 μm diameter spot of the stratum corneum sample, and typically, 20 scans per sample were recorded.

Near-Infrared Excitation, Macroscopic Sampling (FT-Raman)

Spectra were collected using a Bruker FRA 106 FT-Raman module on a Bruker IFS 66 infrared system. Samples were excited using an Nd:YAG laser operating at 1064 nm (near-infrared radiation). Spectra were acquired over the wavenumber range 3500–200 cm^{-1} using a liquid nitrogen-cooled germanium detector with an extended spectral bandwidth. Spectra were obtained from a 100 μm diameter spot of the stratum corneum sample packed into a stainless-steel sampling cup. A laser power of 350 mW was used, with, typically, 200 scans being collected at 4 cm^{-1} resolution. *In vivo* FT-Raman spectra were acquired from the dorsal surface of the forefinger, 500 scans being collected at 8 cm^{-1} using a focused 300 mW laser beam. Surface layers of stratum corneum and possible environmental contaminants were removed by the application of adhesive tape to the skin surface; 4, 8, 16, and 24 strippings of the skin surface were made.

Because data were generated from three different instruments, the spectra were transferred to a PC and were analyzed using Lab Calc. (Galactic Industries Software Corporation).

RESULTS

We have previously described the vibrational modes obtained from Raman spectroscopic studies of human stratum corneum (7). To facilitate interpretation of the results from the present study, the major FT-Raman vibrational modes for human stratum corneum are given in Table I (abstracted from Ref. 7).

Before the different Raman spectroscopic methods for human stratum corneum *in vitro* were compared, the inherent variability of the technique was assessed. Intrasample variability of FT-Raman spectra was determined by recording spectra of four samples of stratum corneum from the same donor. This assessment was repeated using three different donors, and example spectra are shown in Fig. 1.

Spectra obtained from a single donor were essentially the same. Intersample variability was assessed by comparisons of FT-Raman spectra from different donors (Fig. 2).

This study illustrates that intersample variations in Raman spectra were minimal; some minor differences in band intensities were noted but peak wavenumber positions were consistent.

The effects of tissue hydration on FT-Raman spectra were also assessed for three donors; water bands are major features in the FT-infrared spectrum of hydrated stratum corneum. FT-Raman spectra of dry stratum corneum (<10% water) samples were recorded. The tissues were then hydrated (>150% water) and the spectra rerecorded. Figure 3 illustrates, for two typical donors, that water is a weak feature in the Raman spectrum and causes little change in molecular vibration intensities or wavenumber positions of the spectral bands.

Table I. FT-Raman Spectroscopic Wavenumbers (cm^{-1}) and Approximate Descriptions of Vibrational Modes for Human Stratum Corneum, Abstracted from Ref. 7[a]

Wavenumber (cm^{-1})	Assignment
424 w, br	δ (CCC) skeletal backbone
526 mw, br	ν (SS)
600 w, br	ρ (CH) wagging
623 w	ν (CS)
644 mw	ν (CS); amide IV
746 w, br	ρ (CH_2) in phase
827 w	δ (CCH) aliphatic
850 mw	δ (CCH) aromatic
883 mw	ρ (CH_2)
931 w, br	ρ (CH_3) terminal; ν (C-C) α-helix
956 w	ρ (CH_3); δ (CCH) olefinic
1002 m	ν (CC) aromatic ring
1031 mw	ν (CC) skeletal cis conformation
1062 mw	ν (CC) skeletal, trans conformation
1082 mw	ν (CC) skeletal, random conformation
1126 mw	ν (CC) skeletal, trans conformation
1155 w	ν (CC); δ (COH)
1172 w	ν (CC)
1207 mw	—
1244 w, sh	δ (CH_2) wagging; ν (CN) amide III disordered
1274 mw	δ (NH) and ν (CN) amide III α-helix
1296 ms	δ (CH_2)
1336 m	—
1385 vw	δ (CH_3) symmetric
1421 w, sh	δ (CH_3)
1438 s	δ (CH_2) scissoring
1552 w	δ (NH) and ν (CN) amide II
1585 w	ν (C=C) olefinic
1602 w	—
1652 s	ν (C=O) amide I α-helix
1743 vw	ν (C=O) lipid
1768 vw	ν (COO)
2723 w	ν (CH) aliphatic
2852 m	ν (CH_2) symmetric
2883 ms	ν (CH_2) symmetric; ν (CH_2) asymmetric
2931 s	ν (CH_2) asymmetric; ν (CH_3) symmetric
2958 m, sh	ν (CH_3) asymmetric
3000 vw, sh	—
3060 w	ν (CH) olefinic

[a] v, very; s, strong; m, medium; w, weak; sh, shoulder; br, broad; δ, deformation; ν, stretch; ρ, rock.

Additionally, the stability of human stratum corneum samples on exposure to the laser and the effects of the number of scans on the FT-Raman spectra were assessed. A spectrum from a sample of stratum corneum was collected from 200 scans with a spectral slit width of 4 cm^{-1}. The same sample was analyzed after 4000 scans at 4 cm^{-1}. While the signal:noise ratio of the second spectrum was improved, no sample degradation was observed, tissue fluorescence was not evident and band positions and intensities were invariant with the different collection parameters (Fig. 4).

We compared FT-Raman spectra of human stratum corneum *in vitro* and *in vivo* (Fig. 5). *In vivo* spectra were obtained from the forefinger dorsal surface, 500 scans at 8 cm^{-1} being collected in 7 min using a focused laser beam at 300 mW. Figure 5 shows some interesting differences be-

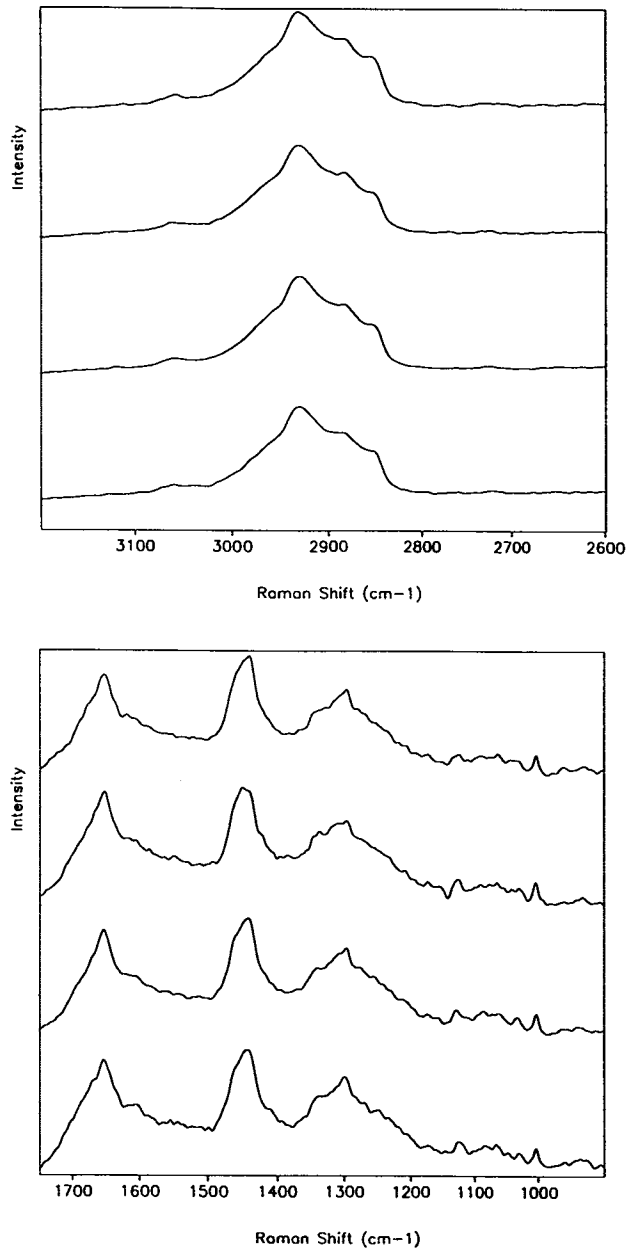

Fig. 1. FT-Raman spectra of four samples of human stratum corneum from the same donor. Top: Spectra obtained over the wavenumber range 2600–3200 cm^{-1}. Bottom: Spectra derived over the wavenumber range 900–1750 cm^{-1}.

tween *in vitro* and *in vivo* spectra, notably the peak around 3230 cm^{-1} evident from *in vivo* tissue.

A comparison of Raman spectra from human stratum corneum sampled microscopically and macroscopically *in vitro* using visible-light excitation, with spectra excited using a near-infrared laser (FT-Raman), is shown in Fig. 6. Spectral differences are again evident using the different techniques but are not attributable to the type of excitation laser used.

DISCUSSION

Our results show minimal intra- and intersample variability in peak position of FT-Raman spectra obtained from human stratum corneum samples *in vitro* (Figs. 1 and 2). The differences in band intensities between different donors is expected because lipid content of human stratum corneum alters between cadavers (10). Variations in C–H stretching frequencies of human stratum corneum lipids *in vivo* over the wavenumber range 2850–2920 cm^{-1} have recently been reported using attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (11). The authors report a 10 cm^{-1} range for C–H stretching features and attribute this to natural variability in the degree of stratum corneum lipid ordering between subjects. While the intensities of lipid

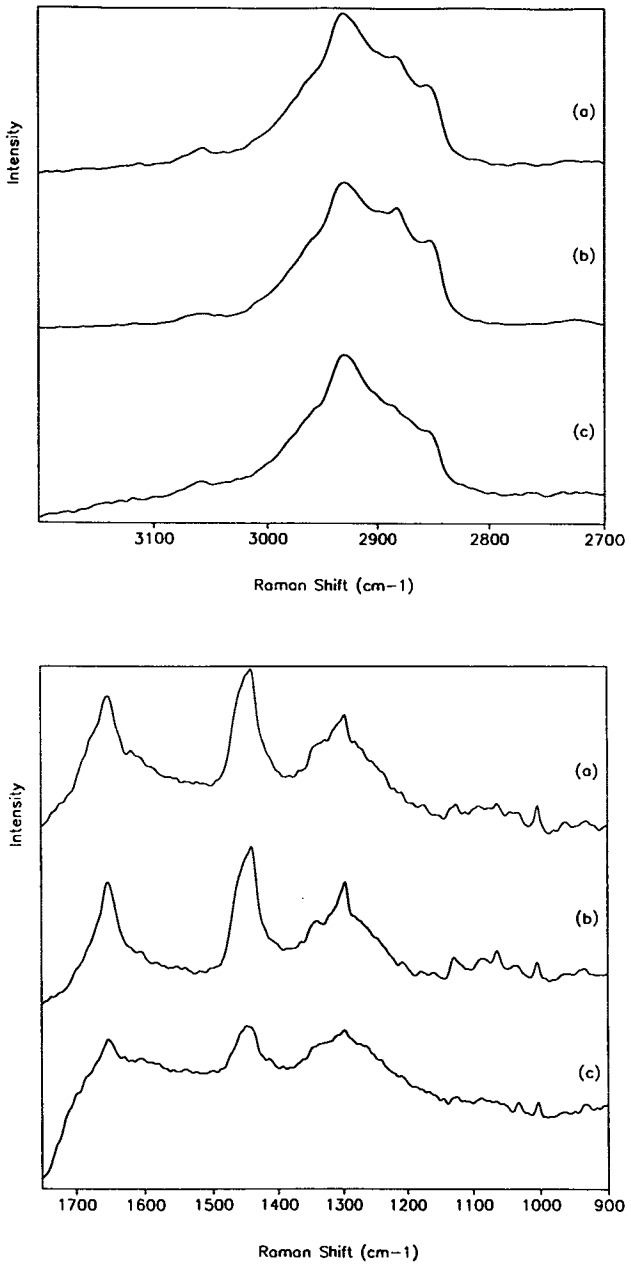

Fig. 2. FT-Raman spectra of human stratum corneum from three different donors. Top: Spectra derived over the wavenumber range 2700–3200 cm^{-1}. Bottom: Spectra gathered over the wavenumber range 900–1750 cm^{-1}.

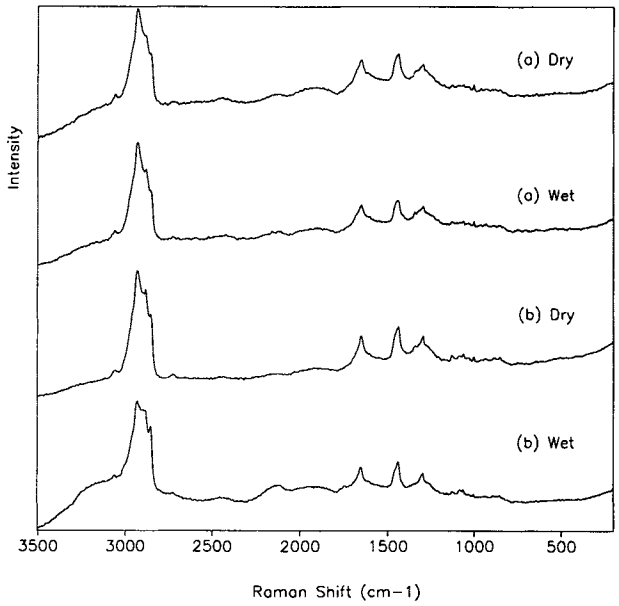

Fig. 3. FT-Raman spectra of dry and wet samples of human stratum corneum collected from two different donors.

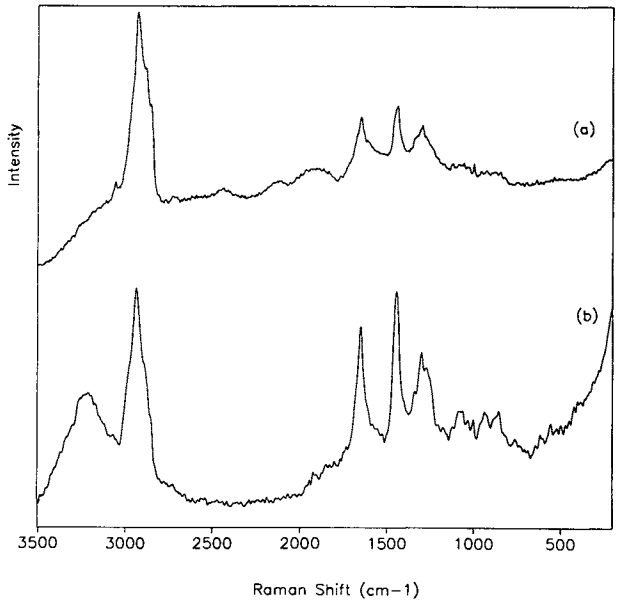

Fig. 5. FT-Raman spectra of human stratum corneum collected *in vitro* (a) and *in vivo* (b).

C–H vibrations at approximately 2880 and 2920 cm^{-1} vary between donors in our FT-Raman study, the band positions do not vary between the different cadavers. These contrasting sets of results may indicate some of the differences between these two spectroscopic techniques; Raman spectra are essentially obtained from the surface of a sample, whereas infrared spectra are collected from within a sample. Additionally, FT-IR spectra are susceptible to deviations in molar absorption coefficients between different donors, for example, where skin is reddened or tanned, which can cause alterations in band intensities. Artificial variations in peak positions can be introduced in both spectroscopic techniques by inappropriate software manipulations of the data, such as overdeconvolution, and by the use of some smoothing routines and some center of gravity algorithms for selecting peak maxima of asymmetrical bands.

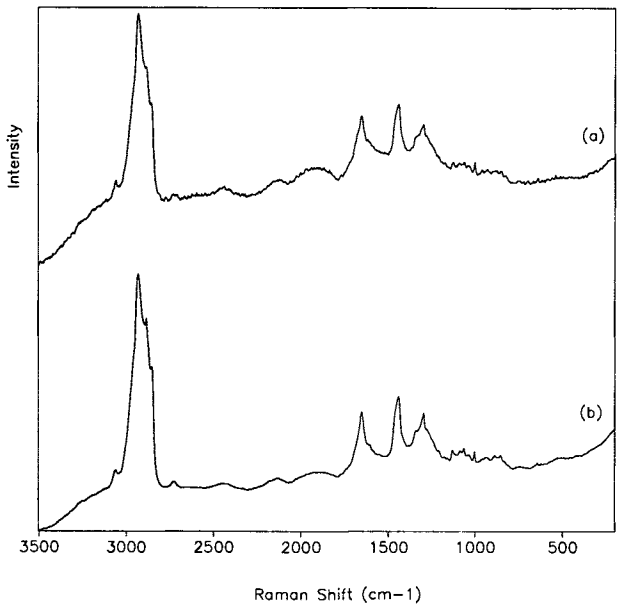

Fig. 4. FT-Raman spectra of the same sample of human stratum corneum obtained under different collection parameters: (a) 200 scans at 4 cm^{-1}; (b) 4000 scans at 4 cm^{-1}.

The strong absorption of infrared radiation by water presents problems in FTIR spectroscopy of hydrated human stratum corneum, especially where band intensities and positions are measured from spectral subtraction of water absorption; such problems are minimized in these studies because of the weak Raman spectrum of water (12). However, water itself is a penetration enhancer; increased hydration of the stratum corneum usually leads to an increased flux of most drugs. Consequently, on increasing the water content of the tissue, some alterations to the molecular nature of the corneum might be expected. Figure 3 shows that this was not the situation for Raman spectroscopy and that no change in lipid CH stretching or protein CONH bending and stretching vibrations were seen. Recent small-angle X-ray scattering studies have shown that the spacing between lipid bilayers in the stratum corneum is invariant to hydration (13), but wide-angle studies on lipid chain order with water content variations are still to be reported. We are currently performing further FT-Raman studies on the molecular interactions of stratum corneum components with respect to varying degrees of sample hydration.

The spectra in Fig. 4 illustrate the improvement in signal:noise ratio gained by collecting 4000 scans of a sample compared with our usual gathering of 200 scans. While the signal:noise ratio improves as a function of the square root of the number of scans, a compromise is necessary between spectral quality gain and sample throughput. Spectral features evident after 200 scans are clarified after 4000, but no differences are evident in band positions or relative intensities. The collection of 4000 scans is useful to discern very weak Raman features that are, nevertheless, real and that can provide valuable structural information on the nature of the stratum corneum (7). However, once these weak features

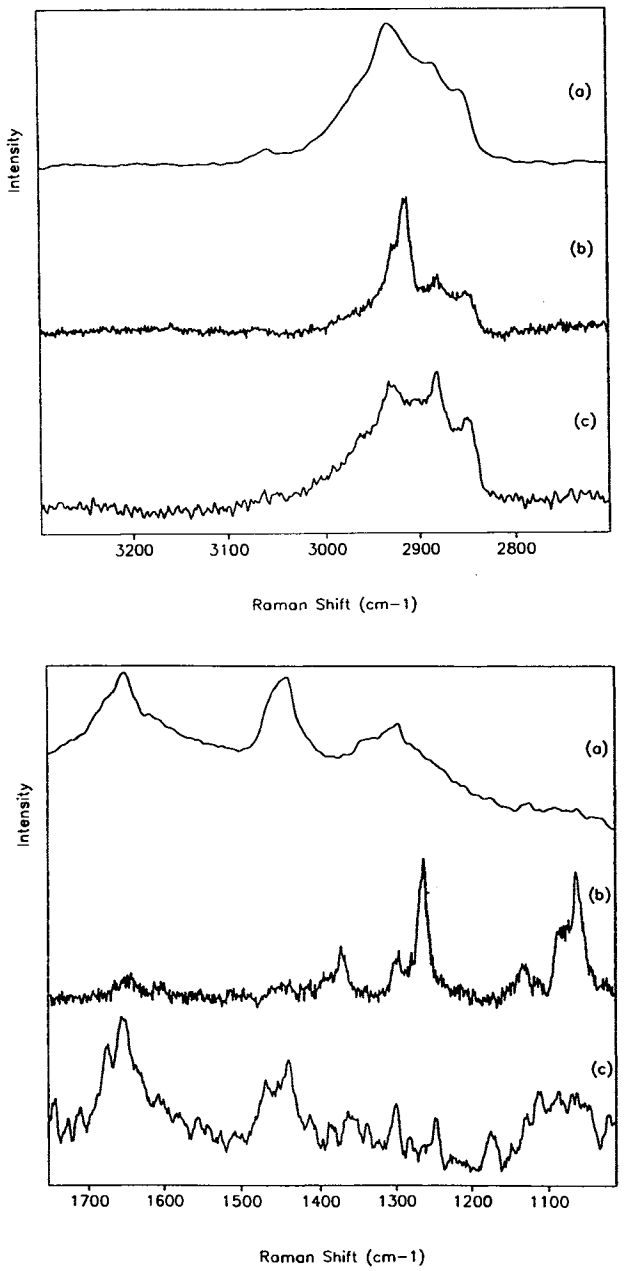

Fig. 6. Raman spectra of human stratum corneum determined using three spectroscopic techniques: (a) FT-Raman; (b) visible excitation, macroscopic sampling; (c) visible excitation, microscopic sampling. Top: Spectra collected over the wavenumber range 2700–3300 cm^{-1}. Bottom: Spectra gathered over the wavenumber range 1000–1750 cm^{-1}.

are accurately defined, then all the information obtained from the extensively scanned sample is already evident after 200 scans of the tissue. The main advantage from collecting 200 scans compared with 4000 is sample throughput; 200 scans at 4 cm^{-1} resolution require approximately 3 min, whereas 4000 scans at 4 cm^{-1} require almost 1 hr using FT-Raman. However, throughput cannot compensate for poor quality spectra and hence sampling parameters should be selected to ensure that all necessary features are evident and accurate. The invariance between spectra in Fig. 4 also demonstrates that the sample is stable to prolonged excitation by the 1064 nm laser.

The FT-Raman spectra collected from human stratum corneum *in vivo* and *in vitro* show significant differences (Fig. 5). A major difference between the spectra is the relatively more intense C–C stretching vibrations (between 1030 and 1130 cm^{-1}) associated with lipid skeletal backbones evident in the *in vivo* spectrum. However, the main difference between the spectra in Fig. 5 is the presence, *in vivo,* of a broad feature centered around 3230 cm^{-1} that is not seen in spectra from stratum corneum *in vitro*. This vibration is unlikely to be due to water; hydrated tissue *in vitro* shows no such band. Sequential tape stripping of the stratum corneum did not remove or diminish the intensity of this peak (relative to other spectral features), indicating that this band is not due to surface contamination of the skin *in vivo,* and although the spectra in Fig. 5 are from different body sites (*in vitro* from the abdomen, *in vivo* from the dorsal forefinger surface), we have also noted a broad Raman band around 3230 cm^{-1} in spectra from other body sites *in vivo* (ongoing studies). The likely assignment for the feature around 3230 cm^{-1} is an N–H stretching vibration. A fundamental vibration from N–H stretching is expected around 3236 cm^{-1}, but also an overtone of the amide II at 1552 cm^{-1} (7) may be seen at 3104 cm^{-1} (2 × 1552 cm^{-1}). However, the peak is a real spectral feature that is not seen in human stratum corneum *in vitro*. Studies aimed at clarifying the origins of this vibration are ongoing are considering factors such as viable skin proteins (enzymes), site to site variations, sweat, and the presence of bacteria.

The spectra of human stratum corneum *in vitro* excited using visible or near-infrared radiation provide an interesting comparison (Fig. 6). For the three techniques used, no shift in the frequencies of spectral features was noted. This again provides good evidence that the samples tested did not degrade, as the same molecular vibrations are seen when using lasers with different power outputs. The major variations in the spectra are seen in the relative band intensities, notably around 2800–3000 cm^{-1} and over the range 1000–1300 cm^{-1}. We ensured that the intensity differences were not due to variations in the lipid content of samples by obtaining Raman spectra from the same sample of stratum corneum using both visible microscopic (MOLE) and visible macroscopic (SPEX) techniques. Thus intensity differences are likely to arise from sample orientation effects. The FT-Raman and MOLE techniques present the sample at 180° to the incident laser and the back-scattered radiation is collected along the axis of the laser. Figure 6 shows that the spectra from these two techniques are essentially the same with only very minor differences in band intensities. The visible macroscopic technique held the stratum corneum at an angle of 63° to the laser (the Brewster angle), at which the scattered radiation and incident laser beam do not coincide. The polarization of, for example, a C–H bond by the laser beam will depend on the orientation of the bond in the radiation path. If the bond is aligned with the laser, then maximum bond polarization occurs, resulting in strong Raman scattering. Figure 6 shows marked intensity differences when the sample is held at the Brewster angle compared with a 180° alignment of sample to laser. Thus, by using a macroscopic visible excitation technique and altering the angle

between incident laser and sample, we are able to obtain information concerning the average orientation of functional groups, C–H backbones, and C–C skeletal groups in stratum corneum relative to the skin surface. We are currently investigating this aspect of Raman spectroscopy of human stratum corneum using macroscopic visible excitation; the angle of sample presentation cannot be simply altered using the FT-Raman and MOLE techniques.

The three techniques allow spectra to be collected from various sample areas; the FT-Raman system from a 100-μm-diameter spot, the SPEX system from a 50-μm-diameter spot, and the MOLE system from a 10-μm-diameter spot (although the manufacturer claims spectra can be collected from a 3-μm diameter). In comparison to typical infrared sampling, the above areas may all be considered to be minimal, and a diameter of 3 μm may be required only for highly specialized studies on human skin.

Another consideration relevant to the three techniques is sample throughput. As previously described, the Fourier transform system allows very rapid spectral collection. Indeed, with specialized software, up to 50 scans/sec can be collected using FT-Raman but typical sampling times are between 2 and 3 min (200 scans at 4 cm^{-1}). In contrast, the visible Raman studies typically last 2 hr (20 scans at 4 cm^{-1} over a 1000-cm^{-1} range).

For most applications, the FT-Raman system provides good-quality spectra in a relatively short time, and unless investigation of very small sample areas is required, there is little merit in using the MOLE system instead of the FT instrumentation. Spectra obtained from the SPEX system differ from those derived from the FT-Raman in relative band intensities. By alteration of sample alignment, molecular vibrations of particular interest may be enhanced using the visible excitation, a facility that is not available on the FT system. However, for most applications and for *in vivo* studies the speed of spectral collection of the FT-Raman is valuable.

In conclusion, we have shown the benefits of Raman spectroscopy for studies of human stratum corneum. We have considered some of the aspects of spectral collection techniques and shown that inter- and intracadaver variations in FT-Raman spectra are minimal. Water in the tissue has been shown to yield only a weak Raman effect, indicating that tissue hydration need not be as strictly controlled during Raman studies as may be necessary in infrared investigations. The stratum corneum samples have also been proved to be stable to the laser excitation. We have presented, for the first time, FT-Raman spectra from human stratum corneum *in vivo* and compared it to tissue *in vitro*. Of the different types of instruments used in this study, the FT-Raman system provided good quality spectra with a high-sample throughput, but the instruments using visible light excitation provide unique information for specialist applications.

ACKNOWLEDGMENTS

The authors thank Dr. P. Turner and Dr. A. Wigley for assistance in obtaining the FT-Raman spectra and I. R. Lewis for assistance with the spectral analysis.

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