# Fiber optic near-infrared Raman spectroscopy for clinical noninvasive determination of water content in diseased skin and assessment of cutaneous edema

**Natalja Skrebova Eikje**
Tokushima University
Department of Dermatology
School of Medicine
Tokushima 770-8503, Japan
and
Tartu University
Clinic of Dermatology
Tartu 57305, Estonia
and
Erasmus University Medical Center
River Diagnostics B.V.
Ee1979
Dr. Molewaterplein 50
3015 GE Rotterdam
The Netherlands

**Yukihiro Ozaki**
Kwansei-Gakuin University
Department of Chemistry
School of Science and Technology
Sanda 669-1337, Japan

**Katsuo Aizawa**
Tokyo Medical University
Department of Physiology
Tokyo 160-8402, Japan

**Seiji Arase**
Tokushima University
Department of Dermatology
School of Medicine
Tokushima 770-8503, Japan

**Abstract.** Currently, measuring Raman spectra of tissues of living patients online and in real time, collecting the spectra in a very short measurement time, and allowing diagnosis immediately after the spectrum is recorded from any body region, are specific advantages that fiber optic near-infrared Raman spectroscopy (NIR RS) might represent for *in vivo* clinical applications in dermatology. We discuss various methodological aspects and state of the art of fiber optic NIR RS in clinical and experimental dermatology to outline its present advantages and disadvantages for measuring skin *in vivo*, particularly its water content. Fiber optic NIR Fourier transform (FT) RS has been introduced to dermatological diagnostics to obtain information regarding the molecular composition of the skin up to several hundred micrometers below the skin surface in a relatively fast nondestructive manner. This has been especially important for probing for *in vivo* assessment of cutaneous (intradermal) edema in patients patch test reactions. Fiber optic NIR FT Raman spectrometers still require further technological developments and optimization, extremely accurate water concentration determination and its intensity calculation in skin tissue, and for clinical applications, a reduction of measurement time and their size. Another promising option could be the possibility of applying mobile and compact fiber optic charge-coupled device (CCD)-based equipment in clinical dermatology.  [DOI: 10.1117/1.1854682]

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## 1 Introduction

In the last quarter of the 20th century, progress in technology promoted applications of Raman spectroscopy (RS) in many areas of science and technology, including medicine.[1] Currently it is employed in the fields of angiology, anesthesiology, cell biology, dentistry, gynecology, laboratory medicine, neurology, oncology, ophthalmology, orthopedics, pathology, physiology, urology, virology, and also in dermatology.[1–5] Reports in the literature have mainly described near-infrared (NIR) Fourier transform (FT) RS for clinical applications in dermatology, although mostly for *in vitro* and *ex vivo* analysis of the molecular and conformational nature of skin and its appendages associated with different dermatological conditions and diseases.[6–13] It has recently become possible to apply *in vivo* fiber optic NIR FT Raman spectrometry within the field of contact dermatitis to spectroscopically evaluate patch test reactions directly in patients.[14] It was demonstrated as an alternative method for *in vivo* noninvasive quantification of cutaneous edema in various degrees of positive patch test reactions in comparison to visual grading of reaction intensity in readings at 48 h and 72 h on the patients.[15] Although the approach of measuring Raman spectra directly from the skin showing contact hypersensitivity reactions is still in its introductory and developmental stage, Raman spectra obtained from negative and positive patch test sites display distinct spectral features mainly attributed to variations in the concentrations of water and proteins in the skin.[14] Moreover, since the intensity of the 3250 $cm^{-1}$ band was shown to be proportional to the concentration of water in the skin by fiber optic NIR FT RS,[16] the mean values of relative water content were obtained in Raman spectra *in vivo*, and that showed a possibility to noninvasively quantify cutaneous edema at positive patch test sites with continuous data grading of reaction intensity suitable for clinical studies *in vivo*.[15]

Address all correspondence to Natalja Skrebova Eikje, Urnesveien 11, N-5565 Tysvaervaag, Norway. Fax: 4752777777; E-mail: eikjen@yahoo.co.uk

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Examination of skin lesions by visual examination and palpation is essential, but subjective.[17] Objective methods are required to detect and quantify any changes in different layers of the skin. Sensitive bioengineering techniques are required to quantify and precisely monitor the dynamics of skin lesions *in vivo* to increase our understanding of skin physiology and biophysics.[14,15,18] This review describes the advantages of coupling fiber optics to RS for facilitating clinical *in vivo* applications, and compares that method with other noninvasive methods for measuring dermal edema. Future trends concerning the use of charge-coupled device (CCD)-equipped Raman spectrometers for spectrographic evaluation of cutaneous edema are also described.

## 2 Development of Fiber Optic Raman Instrumentation for *In Vivo* Skin Applications

Since the discovery of the Raman effect in 1928,[19] and the wide introduction of RS as a laboratory method to widespread areas of science and technology, it has not been known to various fields in medicine, including dermatology, until numerous innovations (FT-RS, confocal Raman microscope) and technical developments (new lasers, photomultipliers, CCD detectors, holographic filters, fiber optic probes) have allowed the recording of Raman spectra of living tissues not only *ex vivo* and *in vitro*, but also *in vivo*, thus demonstrating potential of RS as a new clinical diagnostic tool.[20–29]

All commercially available Raman spectrometers use lasers as a radiation source. The selection of an exciting line is normally confined to the ultraviolet-visible-near-infrared regions of the electromagnetic spectrum, being dictated by instrumental constraints and the relative intensity of the scattered radiation. Excitation from an ultraviolet (UV) source at 300 nm ($33.3\times10^3\ cm^{-1}$) produces Raman scattering 160 times more intense than that from the NIR source at 1064 nm ($9.4\times10^3\ cm^{-1}$) for the same laser irradiances. However, when visible excitation is used, tissue exhibits strong broadband fluorescence that can obscure the tissue Raman spectrum. Compared to the Raman signal, the fluorescence signal is intense, and even a weak fluorescence signal may be strong enough to mask the Raman signals completely. One way to diminish the fluorescence is by increasing the wavelength of the exciting radiation, since the fluorescence is wavelength dependent, whereas the Raman signal follows the excitation wavelength. Formerly, this way of reducing fluorescence was not a possibility because of the lack of detectors, which were sensitive in the NIR region. Today, with the development of interferometers and CCD detectors, it is no longer a problem to record Raman spectra of tissues with NIR excitation. According to the fourth power dependence of the scattering intensity, the excitation at 785 nm ($=12739\ cm^{-1}$) should yield 3.4 times stronger Raman bands than the excitation at 1064 nm ($=9398\ cm^{-1}$). In this point, dispersive RS with excitation in the 700- to 800-nm range is more advantageous than FT-RS.

Noninvasive Raman technology, based mainly on NIR RS,[4] was introduced to skin research by two types of spectrometers: dispersive Raman spectrometers and FT-Raman spectrometers.[6,27] Both consist of four basic instrument components: 1) a radiation source such as a tunable titanium:sapphire laser for a CCD-Raman system and the Nd:YAG (neodynium-doped yttrium aluminum garnet) laser emitting the 1064 nm line for an FT-Raman system; 2) optics to focus the laser beam on a sample and to collect the Raman scattered light from the sample; 3) a monochromator (dispersive) or an interferometer (FT); and 4) a detector with peripherals (fiber optics) for the acquisition of data.[6,27,30–33]

FT-Raman spectrometers are based mainly on a Michelson interferometer.[6,20] The primary information is coded in the interferogram, which is Fourier-transformed to obtain the spectrum.[6] NIR FT Raman spectrometers offer several advantages suitable for the investigations of the skin, because excitation at 1064 nm manifests a global optimum: the reduction of fluorescence is quite efficient; the sensitivity of the liquid-nitrogen cooled gallium arsenide (GaAs) and germanium (Ge) detectors employed in the NIR region is very high; transmittance of quartz fibers is large enough; and low-intensity light in the NIR region is noninvasive and allows studies of the molecular conformation of the compounds in their intact microenvironment as far as several hundred micrometers below the skin surface. All these advantages have been discussed at length elsewhere.[4,6–9,21,30] However, current fiber optic NIR FT Raman systems have certain limitations that prevent them from being easily accepted in a clinical setting: they are not yet compact and mobile,[27,31] they have technical limitations in performing *in vivo* measurements on the skin below 250 $cm^{-1}$ and in distinctively resolving the 3400 $cm^{-1}$ band,[16] and in order to obtain *in vivo* spectra with acceptable signal-to-noise ratios, they require a lengthy measuring time (5 to 10 min).[14,15,34]

Efficient and quick detection of the Raman signal is important for *in vivo* clinical applications, when long signal collection times are generally not opportune by NIR FT Raman spectrometers.[27] The only way to attain an acceptable recording time is to optimize every component of FT-Raman spectrometer carefully: multireflection arrangements and cross-section transformers, combined with optimized spectrometers.[5,9] Further, the optical conductance has to be as large as possible. Large optical conductance can be realized using an interferometer (FT), equipped with a liquid nitrogen-cooled GaAs detector in reliable long-wavelength laser excitation (1064 nm).[6,24] Improving the light conductance of the spectrometer by decreasing the spectral resolution from 8 to 16 $cm^{-1}$ might lead to a reduction of measuring time from 7 to 3.5 min.[9,15]

Another important prerequisite is the reproducible measurement of tissue spectra with a high (adequate) signal-to-noise ratio (SNR). The SNR of a Raman spectrum is related directly to the applied laser power and signal collection (integration) time. An increase in laser power will improve the SNR in a given time interval, but may require prohibitive excitation powers and/or result in sample photodegradation.[5,34] Therefore, it is necessary to perform detailed measurements to ensure safe excitation powers for *in vivo* applications and/or investigate the stability of the sample in relation to the intensity of the laser source.[32]

CCD array detectors are now being successfully used for dispersive Raman spectroscopy with NIR excitation, due to compact solid state semiconductor lasers, offering superior sensitivity compared to FT-Raman spectroscopy with 1064 nm excitation, low noise level, and rapid spectra collection.[1,6,27,32,35] On the other hand, the sensitivity of CCD

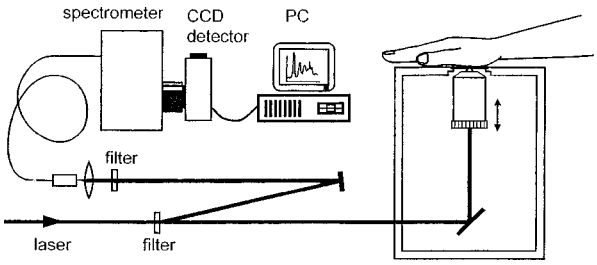


**Fig. 1** Confocal Raman setup for *in vivo* experiments. Laser light (with 100 mW laser power on the skin) from a titanium-sapphire laser (730 nm) is transmitted by a short-pass filter and focused on the skin by an inverted microscope objective. Raman scattered light is collected by the same objective, reflected by the short-pass filter, filtered by a laser rejection filter (either a notch filter or a color glass filter), and focused onto the core of an optical fiber. The fiber guides the light into a multichannel spectrometer equipped with a CCD camera. Signal collection time for each spectrum is 3 s.

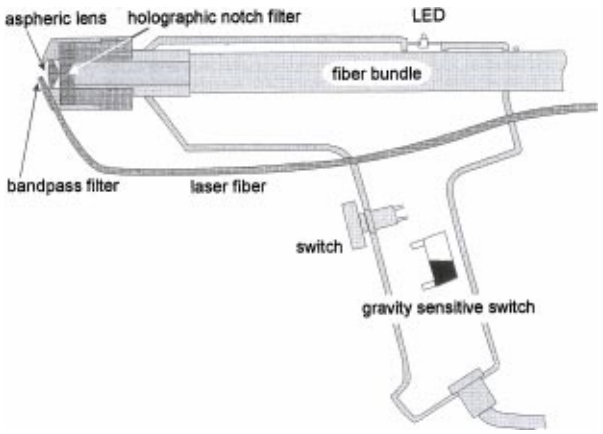


**Fig. 2** Fiber optic probe for *in vivo* NIT FT Raman spectroscopy (schematic). A circular fiber bundle (Fiberware GmbH, Berlin) with a diameter of 10 mm is the main part of the fiber optic probe. A bandpass filter (1064 nm, Bruker Analytic GmbH, Karlsruhe) covering the excitation laser fiber separates laser radiation from the interfering fiber Raman signal. A holographic notch filter (1064 nm, Kaiser Optical Systems Incorporated, Ann Arbor, Michigan) in front of the fiber bundle prevents Rayleigh scattering and back-reflected laser radiation from entering.

detectors decays sharply above 1000 nm (CCDs are transparent to light with wavelengths greater than 1000 nm), so it is rather difficult to use excitation wavelengths above 900 nm since it puts an upper limit to the NIR-excitation wavelengths that can be employed.[6] Therefore, a solution is the usage of different laser excitation wavelengths for measuring skin *in vivo*.[26]

In a CCD-based spectrometer, the different wavelengths of the scattered light are dispersed by an optical grating and projected on a CCD detector. This is essentially a large array of detectors that enables simultaneous recording of a complete Raman spectrum in a single exposure.[6,35] The resulting detection efficiency is greatly enhanced as compared to FT-Raman systems. By combining CCD detectors with electronically addressable notch filters, high fidelity 2-D Raman images can be produced, which allow a direct visualization of tissue morphology and the spatial distributions of various molecular constituents in the selected tissue areas.[1,20]

Sufficient spatial resolution in the depth direction is a prerequisite for discriminating between the different skin layers.[35] Puppels et al.[25] applied the principle of confocal Raman microspectroscopy in the design of a sample stage for *in vivo* investigation of the skin. His group described *in vivo* confocal Raman spectroscopy as a method to measure molecular concentration profiles in the skin (Fig. 1).[26] They demonstrated that with an axial resolution of 5 $\mu$m (full width at half maximum), the method can be used to determine the water concentration in the stratum corneum as a function of distance to the skin surface, and *in vivo* water concentration profiles have been determined for normal skin of the arm and the thenar. The same setup was adapted to allow the rapid automated determination of molecular concentration profiles.[36] Their most recent instrumental refinement, a combination of *in vivo* confocal scanning laser microscopy with Raman spectroscopy, allows the skin morphology to be visualized and (subsurface) structures in the skin to be targeted for Raman measurements simultaneously.[33]

The throughput advantage of interferometer-based FT and CCD-based RS is that the light input and collection optics can also be built into probes connected to a main unit via fiber optic cables. These allow flexible delivery and collection of light in any anatomical location, even in hard to reach anatomical sites, which is especially important for *in vivo* measurements in clinical dermatology.

The design of fiber optic probes plays an important role in the studies, since it can affect the light delivery and propagation into the tissue, the collection efficiency, and the origin of collected light.[37] In general, an optical fiber consists of a core, a doped cladding, and a protective jacket.[29] It is characterized by its overall design configuration (single fiber, bifurcated), the number, core size, and numerical aperture (NA) of the illumination (source) and collection (detector) fibers, and the (center-to-center) source-to-detector separation (SDS).[38]

In a recent review, Utzinger and Richards-Kortum presented a large variety of designs for fiber optic probes for RS.[29] In it, all of the described Raman probes included imaging optics to enhance the collection efficiency, due to the fact that background signal originating from the laser source, the fibers, and all optical components can fill the dynamic range of the detector and overwhelm the Raman signal. These signals must be reduced with filters to accomplish sensitive *in vivo* measurements.[29]

Here, we focus on the designs of fiber optic probes for *in vivo* skin characterization. Previous studies[8,9,24,39–41] describe optimization of a 10-mm active measurement area fiber optic probe (Fig. 2) for an NIR FT spectrometer with excitation at 1064 nm designed for *in vivo* Raman scattering and testing of different types of optodes (Fig. 3), i.e., the coupling element between the fiber bundle and the sample. This group demonstrated the optical conductance of an optical fiber 6.25 times larger with a diameter of 10 mm than of a fiber bundle with a diameter of 4 mm. Several possibilities for reducing the fiber background signal by the use of optical fibers have been described: a bandpass filter to separate laser radiation from the interfering filter and Raman signal; a holographic notch filter to cut off Rayleigh scattering; and back-reflected laser radiation from entering. The same group recognized that aspherical lenses with a large numerical aperture or transparent parabo-

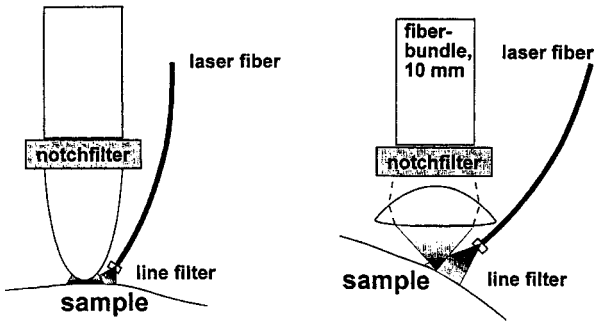


**Fig. 3** Probe for the investigation of the Raman spectra of tissues *in vivo* on surfaces with a paraboloid concentrator from calcium fluoride and with an aspheric lens combined with a fiber bundle. A notch filter reduces the Raman scattering of the fibers, and the illumination is performed via a line filter to eliminate the Raman spectrum of the laser fiber.

loids of calcium fluoride or silica are the most suitable optodes for Raman measurements of skin, because such a compound parabolic concentrator improves the effectiveness of light collection. Although with such a fiber optic probe attached to NIR FT RS *in vivo* spectra can be obtained in 7 min from 600 to 3500 $cm^{-1}$,[13–15] the main technical drawback in these fiber probes is the presence of filters that suppress the Rayleigh line and spectral bands below 250 $cm^{-1}$, thus limiting studies on skin water structure in the 60- to 350-$cm^{-1}$ region.[16]

Shim et al.[32,34] described silica and sapphire fiber optic probes for a CCD-based Raman system that could obtain spectra *in vivo* in less than 30 s, tested in a turbid solution to simulate *in vivo* use. They demonstrated a method that may be used to substract the effects of fiber contamination and studied the effects of tissue geometry. This includes a study of the probe length that must be further optimized for skin, and the probe design that must be continued for minimizing the generation and collection of silica Raman while maximizing the collection of the Raman signal from the tissue.

Recently, Kaminaka, Ito, and Hamaguchi[28] introduced another option for obtaining spectra directly from human normal skin within 64 s with a fiber optic dispersive NIR Raman spectrometer, equipped with a new multichannel detector, image intensifier, and CCD linear sensor. Their "6+1" fiber optic probe, consisting of a central fiber for Raman excitation and six Raman collecting fibers surrounding it, was 2 m long and made of low-OH silica fibers of 400 $\mu$m diameter each. Such a fiber optic probe was set 3 mm away from the sample surface and the 1064 nm laser beam irradiated a circular area of 2 mm diameter in the fiber configuration. Visionex Incorporated developed a biomedical Raman probe based on fibers with beveled and flat tips,[29] that was tried in connection to a built in-house Raman spectroscopic system for *in vivo* measurements, including skin.[42] Excellent collection efficiency was reported from this small diameter probe, consisting of a central flat delivery fiber (400 $\mu$m diameter) surrounded 7 collection fibers (300 $\mu$m diameter); incorporated onto the core of the fibers dielectric a bandpass and a longpass filter, but unfortunately without demonstration of obtained spectra.

In spite of shown advantages of a variety of optical fiber devices to CCD-based or multichannel NIR Raman spectrometers for facilitating *in vivo* applications, those fiber optic NIR Raman systems are still new in clinical dermatology.

In the sample stage for skin measurements by *in vivo* confocal Raman microspectroscopy, the laser light is focused via a microscope objective in a small region in the skin. Scattered light that emerges from this irradiated volume is collected by the microscope objective and projected onto the core of an optical fiber. The core of the optical fiber acts as a pinhole and permits only light emerging from the region where the laser light is focused to enter the fiber, whereas light from out-of-focus regions is blocked. The focus from which scattered light is detected and the focus irradiated by the laser coincide, hence the name "confocal".[33,35,36,43,44,58–60] By using a depth increment of 2 $\mu$m, *in vivo* spectra by a confocal Raman microspectrometer from normal stratum corneum of the arm can be recorded in the 400 to 1850 $cm^{-1}$ region within 30 s, and in the 2500 to 3800 $cm^{-1}$ region within 3 s.[26]

## 3 Evolving Technology Increases Applications of NIR Raman Spectroscopy in Clinical and Experimental Dermatology

Dermatology has become one of the prime targets of RS during the last decade.[45] A couple of pioneering works appeared earlier using NIR FT RS for the investigations of the skin, hair, and nail *in vitro*. Williams et al.,[46,47] and Barry, et al.[48] were the first who reported and assigned Raman bands due to specific vibrational modes of chemical bonds in proteins and lipids in the stratum corneum. Importantly, they showed differences in Raman spectra between normal and diseased skin, and thus opened the possibility of using NIR FT RS for noninvasive dermatological diagnosis. Since then, NIR RS has been increasingly used as a tool for characterizing the molecular structure of components in normal and diseased skin, and also in its appendages not only *in vitro* and *ex vivo*, but also *in vivo*.[6,9–16,30,40,41,49–56] Papers on the prospects of *in vivo* confocal Raman microspectroscopy as a method to measure molecular concentration profiles in the stratum corneum for fundamental skin research, pharmacology (percutaneous transport), and dermatology, have lately begun to appear in the literature.[26,33,35,36,57–59]

### 3.1 *In Vivo Spectra from the Skin with Emphasis to Estimate Water Content*

The skin is a very complex and highly nonhomogeneous tissue consisting of numerous different molecules. To aid in the interpretation of the NIR Raman spectra from the whole skin, various reference spectra have been recorded *in vitro*, *ex vivo*, and *in vivo*.[30] It has been found that there is a great similarity between the spectrum of the whole skin and the spectrum of dermis;[9,30] sample handling does not influence Raman spectra;[12] NIR FT Raman spectra *in vitro* and *in vivo* from the skin via optic fibers are virtually identical in the region of 600 to 3500 $cm^{-1}$.[12]

Figure 4 depicts *in vivo* Raman spectrum of normal human skin by fiber optic NIR FT RS. Major protein-specific bands, amide I and III (amide II is very weak), identified in NIR FT-Raman spectra of the whole skin are located in the 1620 to 1680 $cm^{-1}$ (approximately at 1661 $cm^{-1}$) and 1230 to 1310 $cm^{-1}$ (approximately at 1271 $cm^{-1}$) regions, respectively.[49] Combinations of their frequencies indicate that the majority of

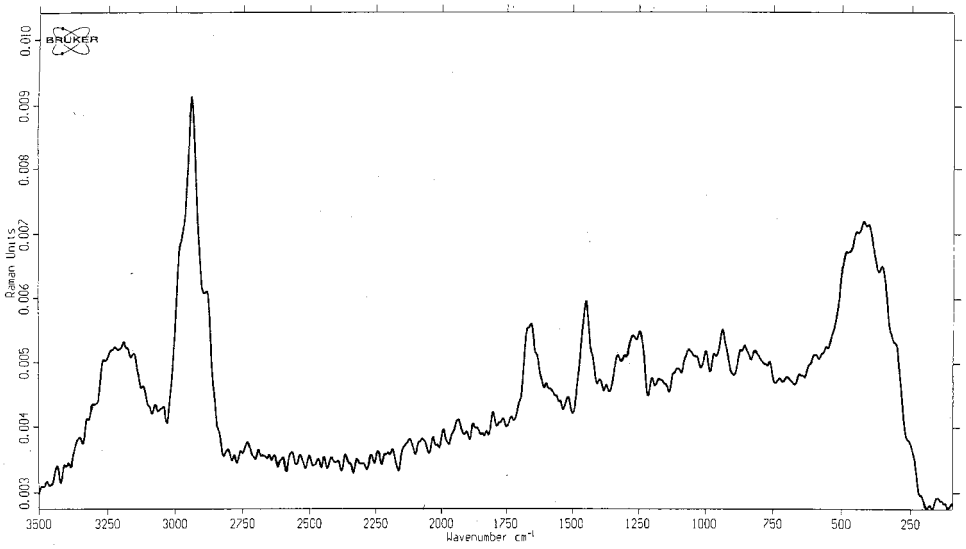


**Fig. 4** An example of an *in vivo* NIR-FT Raman spectrum in the range of 250 to 3500 $cm^{-1}$ obtained on the back from normal human skin by a fiber optic probe.

the proteins in the whole skin are in the $\alpha$-helix conformation (a secondary protein structure type).[5,49] Moreover, a strong C-C stretch band at ~935 $cm^{-1}$, which is typical for $\alpha$-helix, is invariably presented in the *in vivo* NIR FT Raman spectra. Collagen is a major protein of the skin and dominantly contributes to Raman spectra of the whole skin.[9,30] Elastin contributes to the Raman spectrum of the skin to a smaller degree than collagen.[16]

In Raman spectra the strong band centered at 1100 $cm^{-1}$ in the whole skin has been assigned as a $CH_2$ twisting deformation of methylene groups of intracellular lipid acyl chains.[49] In the region between 2700 and 3100 $cm^{-1}$, the bands due to $CH_2$ symmetric ($\upsilon_s$) stretching vibrations at 2900 $cm^{-1}$, $CH_3$ asymmetric ($\upsilon_{as}$) and symmetric ($\upsilon_s$) stretching vibrations, at 2980 and at 2940 $cm^{-1}$ respectively, are known as protein specific; whereas the 2890 and 2850 $cm^{-1}$ bands are said to originate from the lipids in the skin.[12,60] A Raman signal with medium strength that occurs between 1440 and 1453 $cm^{-1}$ (at ~1451 $cm^{-1}$ in the whole skin), is assigned to $CH_2$ scissoring modes of proteins and lipids.[16] Focusing on some representative examples associated with skin diagnostics by NIR FT RS, various major and minor spectral changes indicating alterations in the protein conformation and in the molecular structure of the lipids have been already marked as certain bands shifting and specific combinations of band shapes changing *in vitro* Raman spectra obtained from biopsies of chronically aged and photoaged skin;[12] some benign, premalignant, and malignant skin lesions;[10] *in vitro* and *in vivo* Raman spectra from nail samples before and after soaking in water;[13] *in vivo* Raman spectra obtained from positive patch test reactions at 48 h and 72 h;[14] and *in vitro* and *in vivo* Raman spectra from cutaneous tophi and skin calcifications in three patients with gout.[50]

Water in Raman spectra of the whole skin has been described in two regions: 1) 60 to 350 $cm^{-1}$, and 2) 3200 to 3700 $cm^{-1}$.[16,49] For the purpose of water structure evaluation *in vitro*, the most interesting region is between 60 and 350 $cm^{-1}$, because it contains a band at 180 $cm^{-1}$ specific to water molecules organized in tetrahedrons, i.e., so-called free water to describe water molecules hydrogen bonded to other water molecules.[16,60–63] Bound water is the term given to describe water molecules that form heterogeneous structures with other molecules, such as proteins, via hydrogen bonds.[12,49] To study those water-specific interactions and to estimate water content in the skin *in vivo*, the second region is the most important, since it contains two broad peaks at approximately 3250 and 3400 $cm^{-1}$ originating from symmetric [$\upsilon_s$(OH)] and asymmetric [$\upsilon_{as}$(OH)] O-H stretching modes, respectively.[12,13,16,26,49,60] (Note that the latter band is not distinctively resolved in the *in vivo* spectra by fiber optic NIR FT RS because of a drop of the detector intensity in the 2500 to 3500 $cm^{-1}$ region.)[12,14,15,49] The intramolecular vibrations of hydrogen-bound water also include the bending mode near 1645 $cm^{-1}$, which is weaker in intensity than the mentioned stretching bands and strongly overlaps with the amide I vibrations around 1650 $cm^{-1}$ in Raman spectra of the skin.[60]

To compare intensity of bands in the region of 0 to 1800 $cm^{-1}$ and 2500 to 3500 $cm^{-1}$ by fiber optic NIR FT RS, *in vivo* spectra of the skin is needed to be equalized for the 1450 and the 2940 $cm^{-1}$ band intensity, respectively, as a reference. The C-H bands (at 1450 and 2940 $cm^{-1}$) protrude outside the protein chain and do not take part in strong intermolecular interactions, therefore the C-H band is not modified by alterations in the secondary protein structure and is used for equalization of the spectra. It is necessary to equalize the spectra in two different ranges using both 1450 and 2940 $cm^{-1}$ bands because of the fact that self-absorption of water in investigated tissue influences spectral regions differently.[12,64]

The literature describes two ratios, $I_{2940}/I_{3250}$ and $I_{3250}/I_{2940}$, to estimate water content in the skin and its appendages by NIR FT Raman spectroscopy. The ratio $I_{2940}/I_{3250}$ [intensity (peak height) of the 2940 $cm^{-1}$ band/ intensity (peak height) of the 3250 $cm^{-1}$ band] has been used by Gniadecka et al. to determine water content (skin hydration) *in vitro* in normal, photoaged, and chronically aged skin

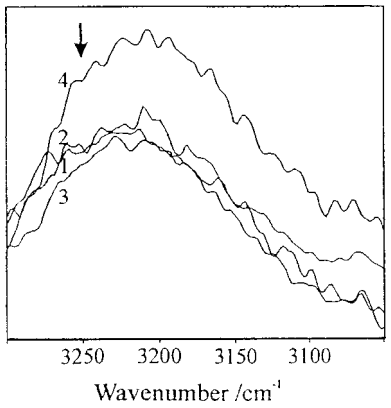


**Fig. 5** *In vitro* NIR FT Raman spectra of young (curves 1 and 2) and aged (curves 3 and 4) individuals. Curves 1 and 3, sun-protected buttock skin; and curves 2 and 4, sun-exposed dorsal forearm skin. An increase in intensity of O-H stretching band in water in photoaged skin is marked with the arrow.

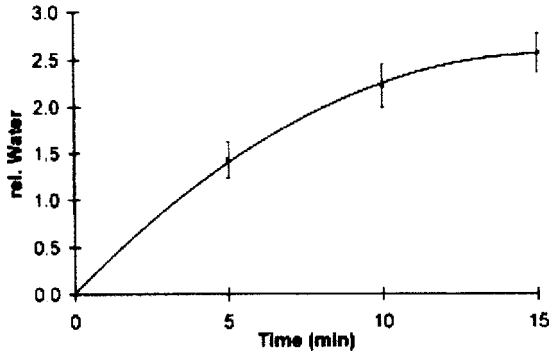


**Fig. 7** Time-dependent water uptake of toenail samples (distal part). The values are calculated from the *in vitro* Raman spectra of toenails using Eq. (1) (relative water$=I_{OH,water}/I_{CH,protein}$) and Eq. (2) [water uptake$=$(relative water$_{wet}-$relative water$_{dry}$)100]. Nails absorbed water and showed a saturating effect after 10 min soaking in water.

(Fig. 5).[12,49] The same group monitored *in vitro* and *in vivo* water uptake in normal nail samples (distal part of the nail) by calculating the intensity ratio of the $\upsilon(OH)/\upsilon(CH_2)$ bands [$\upsilon$(OH) mode at 3250 cm$^{-1}$, and the $\upsilon$(CH) mode at 2930 cm$^{-1}$] (Figs. 6, 7, and 8).[13] It must be noted that this is the inverse of the previously described ratio $I_{2940}/I_{3250}$.[5,12,16,49] For assessment of water content *in vivo* skin at patch test sites with various degrees of inflammatory reaction intensity at 48 h and 72 h, the intensities of the peaks at 3250 and 2940 cm$^{-1}$ ($I_{3250}/I_{2940}$) were calculated using baseline corrections from edge to edge between 3350 and 3100 cm$^{-1}$ for the $\upsilon$(OH) mode, and between 3030 and 2830 cm$^{-1}$ for the $\upsilon$(CH) mode (Fig. 9).[14,15]

The ratio of the intensities of the Raman bands at 3390 and 2935 cm$^{-1}$ can also be used to calculate the water-to-protein ratio in tissue.[26,65–67]

Confocal Raman microspectroscopy has been demonstrated as an *in vivo* method to understand the actual skin layer from which the Raman signal is collected and which can be applied to noninvasively determine water concentration profiles in the normal thin stratum corneum of hand (on the volar aspect of the forearm) and in the normal thick stratum corneum of the palm (on the thenar) (schematically presented in Fig. 1).[26,36] The spectral ranges from 3350 to 3550 cm$^{-1}$ and from 2910 to 2965 cm$^{-1}$ ($I_{3350-3550}/I_{2910-2965}$) were chosen to calculate water-to-protein ratios at 3390 and 2935 cm$^{-1}$, respectively, in the normal stratum corneum (Fig. 10).[26,36]

## 4 Comparison of NIR Raman Spectroscopy with Other Spectroscopic Techniques to Study Water Molecular Properties, Water Interaction, and Estimation of Water Content in the Skin

Spectroscopic techniques are sophisticated tools for the direct and specific determination of water, allowing estimation of water content and studies on water molecular properties and interactions.[16]

We can divide spectroscopic techniques according to the speed at which the data are collected. The crucial value is $10^{-11}$ s, which is the average duration of a hydrogen bond responsible for intra- and intermolecular structure of

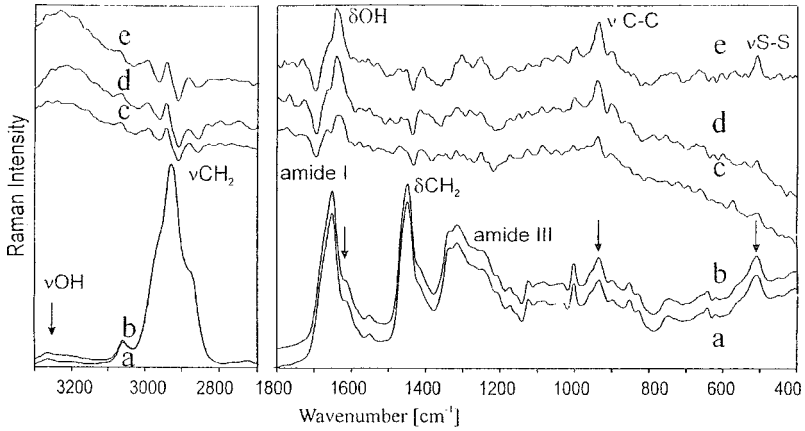


**Fig. 6** *In vitro* normal NIR FT Raman spectra (distal part) before and after soaking in water for 15 min: (a) untreated nail; (b) moistened nail; (c), (d), and (e) are the difference spectra of a wet minus untreated nail after 5, 10, and 15 min water treatment. Water is identified by the $\nu$(OH) band at 3250 cm$^{-1}$ and the $\delta$(OH) band at 1630 cm$^{-1}$.

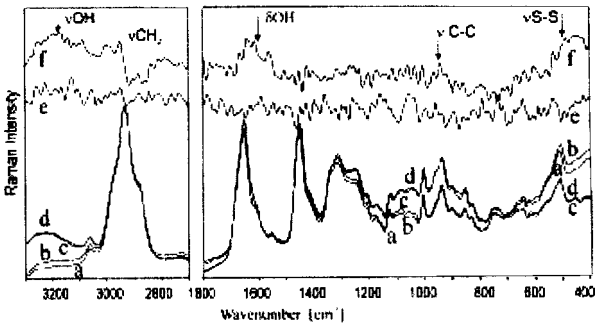


**Fig. 8** *In vivo* NIR FT Raman spectra for the proximal and distal part of the fingernails (*a*,*b*) proximal, (*c*,*d*) distal part of the nail. Spectra *a* and *c* were before and spectra *b* and *d* after soaking in water for 15 min. *e* is the difference of spectrum *b* and *a*, *f* is the difference of spectrum *d* and *c*. *In vivo* NIR FT Raman spectra of the distal end of the nail did not show major differences in comparison with *in vivo* Raman spectra. Water was identified at the band at 3250 $cm^{-1}$. The water uptake of the nails was increasing with increasing treatment time, although *in vivo* it was smaller than *in vitro*. The value for the wet fingernails *in vivo* was about 27±6% in comparison with 45±6% *in vitro* for the toenails.

water.[16,68,69] The most rapid techniques, infrared (IR) spectroscopy and RS, measure at a time scale of $10^{-13}$ to $10^{-16}$ s and will thus yield instantaneous information about water structure.[16] The techniques that require longer measurement time will give information about the average state of water molecules in the sample. An example of such a technique is nuclear magnetic resonance (NMR) (time scale $10^{-3}$ to $10^{-6}$ s), which has been used to determine the proportion of water molecules bound to the macromolecules (bound or immobile water in contrast to free or freely exchangeable water) in various normal and diseased tissues.[70–72]

The advantage of NMR spectroscopy and imaging in dermatological research is the spatial resolution with the possibility to quantitate water content and average molecular kinetics *in vivo* in epidermis, dermis, and subcutis.[73–76] The limitations of NMR for clinical applications are, however, its inability to yield information about instantaneous water structure and an ability to provide information only about the average state of water molecules in the sample, long measurement time; and expensive, large-sized instrumentation that needs a special environment.[16] IR and RS are complementary techniques.[77]

Absorption of infrared light at approximately 3200 $cm^{-1}$ depends on the stretching of the covalent O-H bond in water and therefore reflects water content.[78] IR-spectroscopy is rapid and allows the obtaining of spectra noninvasively from the skin surface,[77] but only the most superficial layer; in practice, stratum corneum can be studied for *in vivo* quantitative measurement of water.[77–79] The use of attenuated total reflectance (ATR) FT-IR spectroscopy is restricted to the upper few microns of the stratum corneum because of the limited penetration depth of mid- and far-IR light in skin.[77,79]

RS has certain advantages: it can be applied to obtain information regarding the molecular composition of the skin down to several hundred micrometers below the skin surface; it has relative insensitivity to water (in Raman spectra, water shows only weak bands and backgrounds and, consequently, the tissue signal is not swamped by the water spectrum); and the Raman spectrum is highly molecule specific.[26,80,81] In NIR Raman spectrum, the intensity of spectral bands at 3250 and 3400 $cm^{-1}$ are proportional to the concentration of water in the skin, originating from symmetrical and asymmetrical OH stretching, respectively, and thus enabling the recording of *in vivo* changes in the water content in the skin.[14–16,60] The spectral band at 180 $cm^{-1}$ represents water molecules organized in tetrahedrons, which yield a possibility to collect information about chemical water structure in the skin.[16,49,68] However, due to the presence of filters in the fiber optic probe used for measurements *in vivo*, studies on water structures in the 60 to 350 $cm^{-1}$ region are not yet possible.[16] Moreover, because the 3400 $cm^{-1}$ band is not distinctively resolved in the spectra by NIR FT RS due to the drop in the detector intensity in the 2500 to 3500 $cm^{-1}$ region, the intensity of that spectral line also cannot be calculated.[12,14,15,49]

## 5 Noninvasive Methods to Assess and Quantify Cutaneous Edema in Patch Test Reactions

Cutaneous infiltration and edema are important signs in reading patch test reactions.[82,83] Edema formation is one of the essential features of inflammation of the skin. It becomes clinically apparent and palpable because of the increase in skin thickening from extravasation of plasma, as a result of the type IV allergic reaction (delayed type), which is the basic process in positive patch test reactions.[84]

Patch testing is a well-established method of diagnosing delayed type hypersensitivity in allergic contact dermatitis, with the aim of provoking the disease in miniature, resembling the real one.[83,85] In spite of developments in clinical medicine, the patch test, which is a relatively crude and primitive procedure, still remains the classical method for the diagnosis of contact allergies.[83]

A small number of *in vivo* methods have been developed to evaluate cutaneous edema. The literature describes several methods capable of measuring edema in patch test reactions: the Harpenden skin-fold caliper, xeroradiography, high-frequency ultrasound, and recently, fiber optic NIR FT RS.[14,15,17,84,86–90] However, some have severe drawbacks. For example, pressure from the clamps of the caliper inevitably compress areas of edema, creating uncertainty about the layer of the skin being included in the fold and measured.[88] Xeroradiography cannot be carried out on the back, and moreover, the patch test area is too small for radiological projections.[82,89]

High-frequency ultrasound for assessment of allergic and irritant reactions directly on the patients was initiated in the last two decades.[82,84,86,87,91–94] Primarily, 20-MHz high-frequency ultrasound in the A mode (i.e., 1-D scanning to measure skin thickness, which corresponds to the interval between the epidermis echo and that of the interface between the dermis and subcutis) has been used for differentiation of positive patch test reactions on the basis of skin thickness, which increased proportionally to the intensity of the reaction.[82,84] The next step was the introduction of 2-D scanning, called B mode, which allows the measurement of the thickness and acoustic properties of a particular region of the skin, offering the advantage of supplying data for the evaluation of patch test reactions of every degree of intensity.[16,87,91]

These studies revealed that affected areas of the inflamed skin are characterized by low echogenicity, particular in the

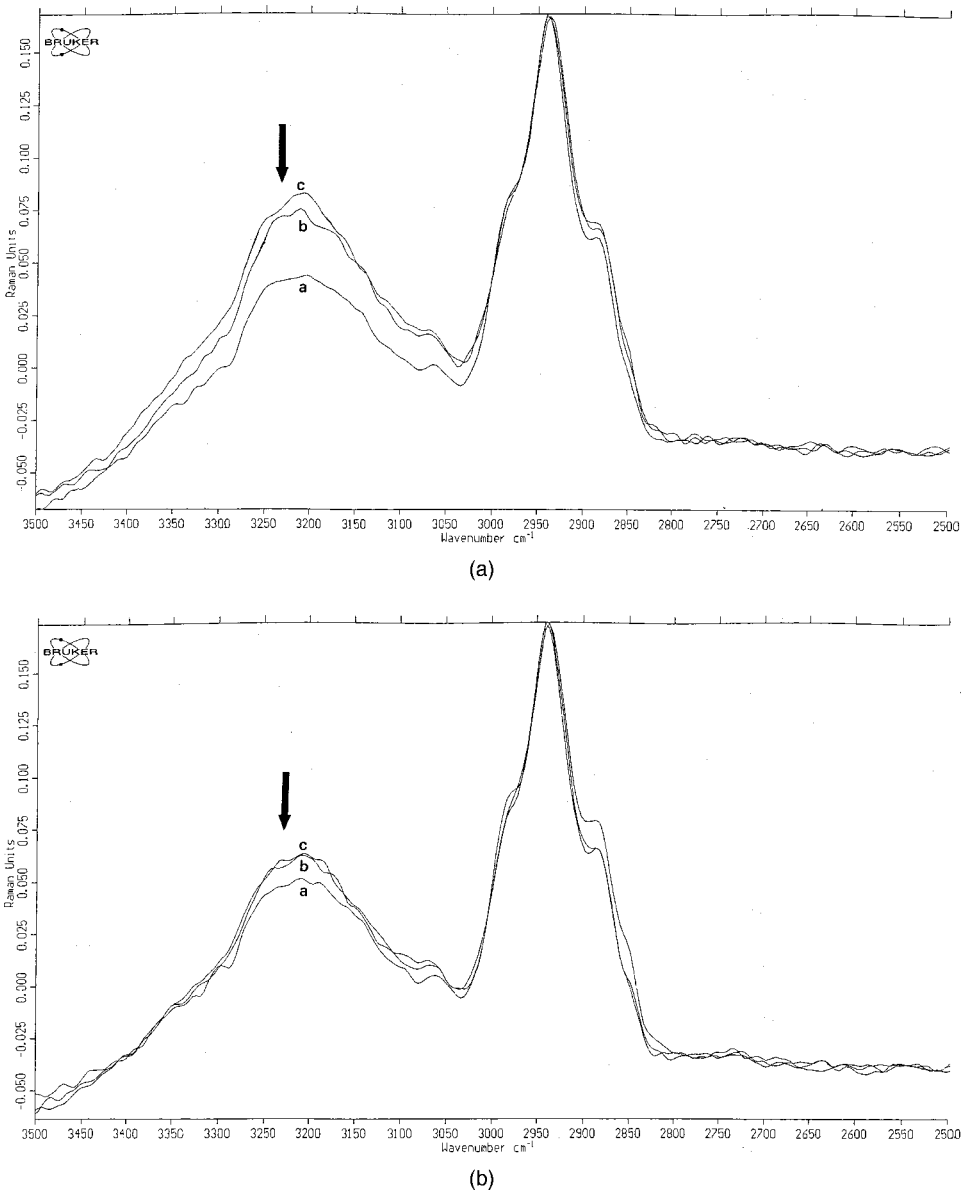


**Fig. 9** *In vivo* NIR FT Raman spectra (mean) in the range of 2500 to 3500 $cm^{-1}$ of normal skin (line *a*), (a) Positively (++,++/+++) scored reactions and (b) positively (?+,?+/+,+) scored reactions at 48 h (line *b*) and 72 h (line *c*) to different allergens in a group of patients with contact dermatitis. The band at 3250 $cm^{-1}$ is significantly increased, showing higher water content in strongly positive patch test reactions, when compared to weak positive reactions at 48 and 72 h.

upper part of the dermis. This subepidermal low echogenic area was thought to represent inflammatory edema. However, the resolution of 20-MHz scanners is still too low to enable measuring the thickness of the epidermis and the stratum corneum. Therefore, a 100-MHz scanner has been developed.[31] Nevertheless, direct evidence that echogenicity of the dermis correlates with water content is lacking. In spite of those technical limitations, ultrasonography has been shown to be a suitable method for quantification of cutaneous edema in patch test reactions, because by use of portable, relatively cheap equipment, one can evaluate cutaneous edema from any region of the body in a relatively short time.[84] Also, ultrasound measurements are less dependent on high technical expertise, and therefore this method can be easily adopted in the clinical routine.[16,31]

NIR FT Raman spectroscopy does not have the same drawbacks as high-frequency ultrasonography. The feasibility of NIR FT Raman spectroscopy in conjunction with sensitive fiber optic probes to directly estimate relative water content in various degrees of patch test reactions in comparison to visual grading of reaction intensity in readings at 48 h and 72 h have been demonstrated.[14,15] Figure 11 provides examples of *in vivo* NIR FT Raman spectra over the wavenumber range 2500 to 3500 $cm^{-1}$ obtained with fiber optics on backs of patients at patch tests sites, showing various degrees of reaction intensity to the same and different allergens, at 48 h and at 72 h.[15] The main changes in NIR FT Raman spectra in the 2500 to 3500 $cm^{-1}$ region from normal and reacted to applied allergens tissue were found to agree with each other in band positions and their shapes, but with various intensities (peak

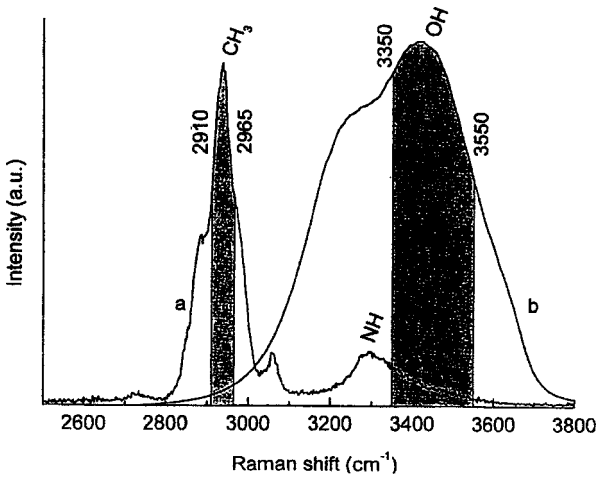


**Fig. 10** Raman spectra of water and dry stratum corneum. Raman spectrum of freeze-dried stratum corneum (line *a*). Raman spectrum of water (line *b*). The gray areas highlight the spectral intervals that are used in the calculation of water content in the skin. Water to protein ratios in the stratum corneum were calculated as the ratio between the Raman signal intensity of water (due to OH-stretching vibrations) integrated from 3350 to 3550 $cm^{-1}$ and that of protein (due to $CH_3$-stretching vibrations) integrated from 2910 to 2965 $cm^{-1}$.

height) of the 3250 $cm^{-1}$ peak related to the water content in the skin, when compared visually to reaction intensities (negative, ?+; +; ++; +++) as recommended by the International Contact Dermatitis Research Group.[83] The band at 3250 $cm^{-1}$ is significantly increased, showing higher water content in strongly positive patch test reactions, when compared to weak positive reactions, at 48 h and 72 h. Since the intensity of the 3250 $cm^{-1}$ band was shown to correlate with water content in the skin according to the visual results of patch test evaluation, by using the ratio $I_{3250}/I_{2940}$ described earlier, the mean values of relative water content were obtained (Fig. 12), and showed the possibility of noninvasively quantifying cutaneous edema at positive patch test sites with continuous data grading of reaction intensity suitable for clinical studies, which is important for monitoring the dynamics of these reactions at 48 and 72 h *in vivo*.[14,15] Nevertheless, application of fiber optic NIR FT RS for precise water concentration determination in the skin cannot be considered as a very accurate technique at present due to some technical limitations described. Additionally, self-absorption of scattered light by water should prove not to be an influencing factor when the intensity of water is calculated in various skin models with high water content. Certainly advantageous would be the development of multivariate statistical methods on the basis of measured NIR-Raman spectra in the 2600 to 3800 $cm^{-1}$ region to predict the water fraction of skin tissue with better accuracy.[42,67]

## 6 Advantages and Disadvantages of Fiber Optic NIR Raman Spectroscopy in Clinical Dermatology

Fiber optic NIR RS represents a potentially viable approach for widespread clinical use, since *in vivo* measurements may be performed nondestructively under physiological conditions on nonhomogeneous samples, and the acquired data can be rapidly analyzed with ''trained'' software.[42] Fiber optic NIR RS offers a high degree of information qualitatively and quantitatively at the molecular level. Spectra collected with fiber optics may effectively localize the volume of tissue that is studied, from a very small measurement volume by confocal Raman microspectroscopy, to a 10-mm active measurement area by fiber optic NIR FT RS. Raman spectra obtained by fiber optic NIR RS can reflect the changes in molecular structure and composition underlying transformation from normal to diseased states within tissues, that may be used for elucidation of the etiology and progress of diseased states, thus improving clinical diagnosis and treatment. It can also characterize the interactions of certain chemical agents with tissues, which can be of particular use in studies of skin barrier properties, penetration enhancers, and other topical applications. A recent example is the monitoring of dynamic processes and intermolecular interactions in various degrees of visually assessed positive patch test rections at 48 h and 72 h after cutaneous application and penetration of various standardized allergens, underlying *in vivo* cutaneous transformation from normal to inflamed states.

NIR spectroscopy has been particularly important for recent dermatological applications of RS, since it not only avoids interference from fluorescence and photodegradation, but also provides an opportunity to probe larger tissue depths up to several hundred micrometers below the skin surface with assessment of high-quality spectral data due to the fact that in NIR Raman spectra, water shows only weak bands and backgrounds, and so the tissue signal is not swamped by the water spectrum. So, the NIR FT RS method, in conjunction with sensitive fiber optical probes, may be a suitable, powerful tool for the diagnosis of the whole human skin tissue *in vivo*, allowing extensive data collection at different anatomical locations that is essential in the development of *in vivo* clinical applications. An introductory interpretation of the changes in the spectral range 2500 to 3500 $cm^{-1}$ obtained from skin reactions to patches in patients with suspected allergic contact dermatitis is suggested to further demonstrate the applicability of remote NIR RS as an alternative method for assessing and quantifying cutaneous edema by directly measuring water concentration in the whole skin tissue *in vivo*,[15] although this area still has some unanswered questions. Though the described method certainly does not enable a very accurate water concentration determination presently in the skin, our introductory approach is supported by a recent study by Wolthius et al.,[67] aimed at quantifying water content in the brain tissue as a first step toward the development of an *in vivo* tool for monitoring brain edema in patients with intracranial neoplasms and cerebrovascular pathology. An experimental fiber optic CCD-based NIR in-house-built Raman system has shown the possibility of measuring, in the 2600 to 3800 $cm^{-1}$ region, the concentration of water in porcine brain tissue postmortem with an accuracy better than 0.01 in the range 0.75 to 0.95. Thus, similar assessment of the applicability of Raman spectroscopy as an alternative method for assessing brain edema, measuring the water concentration in the tissue directly, has also been introduced to neurology.

The situation is complicated for measuring skin *in vivo*, because different portions of normal skin have different water content. With sufficient spatial resolution in the depth direction, discrimination between the different skin layers and

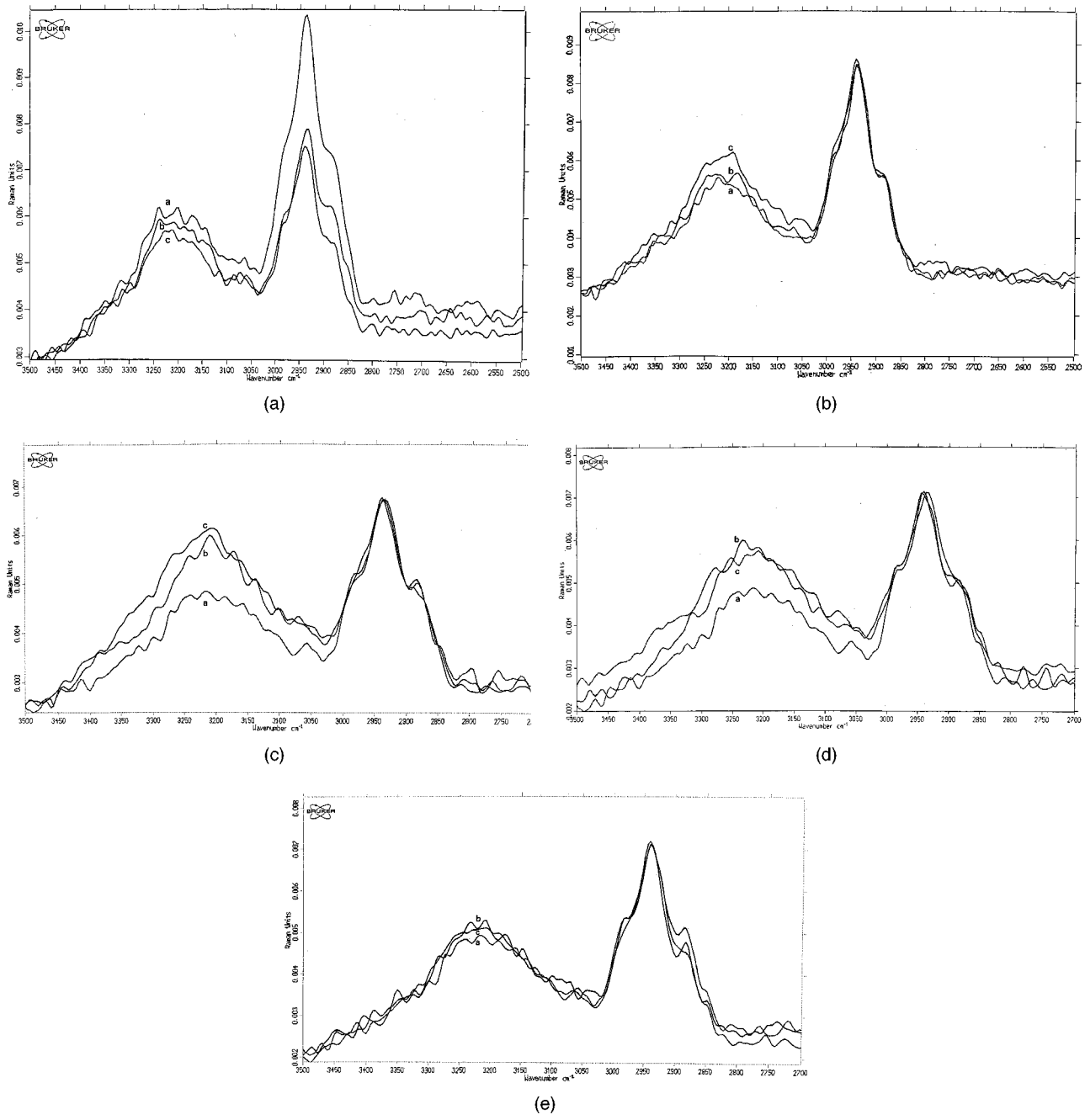


**Fig. 11** An example of *in vivo* NIR FT Raman spectra in the range of 2500–3500 $cm^{-1}$ of: (a) normal skin (line *a*), positively (?+) scored reaction (line *b*), and (++) scored reaction (line *c*) of the same patient to different allergens after 48 h. (b) Normal skin (line *a*), positively (?+) scored reaction (line *b*), and (+) reaction (line *c*) of the same patient to different allergens after 72 h. (c) Normal skin (line *a*), positively (++) scored reaction at 48 h (line *b*), and (++,++/+++) reaction at 72 h (line *c*) to neomycin sulphate in the same patient. (d) Normal skin (line *a*), positively (++) scored reaction at 48 h (line *b*), and (++) scored reaction at 72 h (line *c*) to potassium dichromate in the same patient. (e) Normal skin (line *a*), positively (?+) scored reaction at 48 h (line *b*), and 72 h (line *c*) to nickel sulphate-hexahydrate in the same patient.

studies on molecular variations within the whole skin are possible. At present, we have detailed information only on the water concentration in the outermost layer of the skin, in the stratum corneum, as a function of distance by confocal Raman spectroscopy. In combination with confocal scanning laser microscopy, for the first time a detailed *in vivo* concentration profile was shown for the stratum corneum in relation to skin architecture.[33]

Higher spatial resolution may increase the accuracy of diagnosis of contact dermatitis based on patch testing and provide guidance for subsequent measurements from inflamed portions of the whole skin *in vivo*. Here we only have the model of patch test reactions to demonstrate fiber optic NIR FT RS *in vivo* Raman spectra from patches visually showing various degrees of cutaneous inflammation on the backs of the patients.

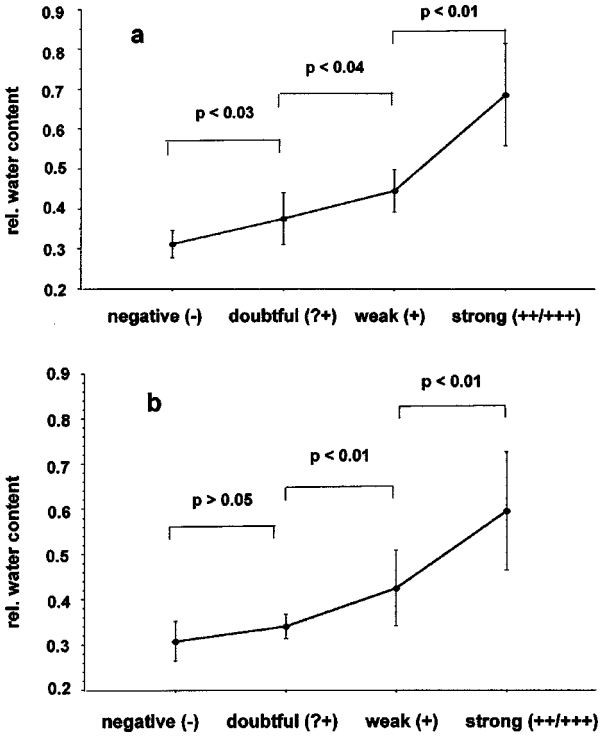


**Fig. 12** Mean relative water content values and SD in contrast to visual gradings of patch test reactions intensity (–;?+;+;++;+++) at (a) 48 h and (b) 72 h. By using the ratio $I_{3250}/I_{2940}$, the mean values of relative water content showed the possibility to noninvasively quantify cutaneous edema at positive patch test sites (?+;+;++;+++) with continuous data grading of reaction intensity suitable for clinical studies, which is important for monitoring the dynamics of these reactions at 48 h and 72 h *in vivo*.

Water content is higher in the normal dermis. The interaction between water molecules and connective tissue components of the dermis are the main determinants of water homeostasis in the skin, and edema obviously manifests a disturbance in skin water balance. Although an occlusive probe has been used to noninvasively quantify cutaneous edema in patch test reactions that could influence the water content in the upper layers of the skin during acquisition time of 7 min, we assume that such a lengthy measurement time cannot influence water content in dermis and an established inflammatory condition in the skin. Skin hydration experiments have been already described by Caspers et al.,[36] showing higher water content in the stratum corneum after skin hydration for 45 min. However, in the viable cells of epidermis (about 15 to 20 $\mu$m below the surface), the water content was shown to reach a constant value, as in nonhydrated skin. To eliminate this as a factor, it would be optional to use a fiber optic probe set away from the skin surface, as described by Kaminaka, Yamazaki, and Hamaguchi[28] and Shim,[32,34] and/or use CCD-based equipment for quick spectra acquisition in high wavenumber region, as was recently demonstrated by Caspers et al.[26,33]

One more important issue is that current fiber optic NIR FT Raman systems are not yet portable, although they do not require a special environment apart from wearing protective goggles against laser light. Recent CCD-based equipment offers miniaturization and integration of optical and optoelectronics instruments to achieve portable, personal, and handheld instrumentation with minimal power consumption and high reliability that can be used in a clinical setting. That trend certainly suits decentralization in the hospital, with the associated demand for small-sized instrumentation close to the patient.

## 7 Conclusions

The development of objective methods has been delayed in dermatology compared to other fields of medicine and biophysics. For dermatologists, bioengineering is not absolutely necessary to make a diagnosis, since it is much easier, rapid, and inexpensive to see and touch skin lesions directly. However, the application of bioengineering techniques to the quantification and monitoring of skin lesions could help see the dynamics and characteristics of living skin lesions, which have never been described in textbooks of dermatology before. For this purpose, appropriate measurement systems are needed to deliver objective, accurate, reliable, and reproducible results at any time to increase our understanding of *in vivo* skin physiology and biophysics. A newcomer to the medical instrumental market is Raman spectroscopy, which has already been probed in a variety of medical applications, and appears to be a particularly promising *in vivo* diagnostic tool for feasible, nondestructive measurements in dermatology.

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