REVIEW ARTICLE

Novel aspects of Raman spectroscopy in skin research

Dominique Lunter[1] | Victoria Klang[2] | Dorottya Kocsis[3] |
Zsófia Varga-Medveczky[3] | Szilvia Berkó[4] | Franciska Erdő[3,5]

[1]Department of Pharmaceutical
Technology, Institute of Pharmacy and
Biochemistry, Eberhard Karls University
of Tübingen, University of Tübingen,
Tübingen, Germany

[2]Division of Pharmaceutical Technology
and Biopharmaceutics, Department of
Pharmaceutical Sciences, Faculty of Life
Sciences, University of Vienna, Vienna,
Austria

[3]Faculty of Information Technology
and Bionics, Pázmány Péter Catholic
University, Budapest, Hungary

[4]Institute of Pharmaceutical Technology
and Regulatory Affairs, Faculty of
Pharmacy, University of Szeged, Szeged,
Hungary

[5]EA 6295 Nanomédicaments et
Nanosondes, University of Tours, Tours,
France

Correspondence
Franciska Erdő, Faculty of Information
Technology and Bionics, Pázmány Péter
Catholic University, Práter u. 50A,
Budapest 1083, Hungary.
Email: erdo.franciska@itk.ppke.hu

Funding information
National Research, Development and
Innovation Office of Hungary; Studium
Loire Valley-Institute for Advanced
Studies

Abstract

The analytical technology of Raman spectroscopy has an almost 100-year history. During this period, many modifications and developments happened in the method like discovery of laser, improvements in optical elements and sensitivity of spectrometer and also more advanced light detection systems. Many types of the innovative techniques appeared (e.g. Transmittance Raman spectroscopy, Coherent Raman Scattering microscopy, Surface-Enhanced Raman scattering and Confocal Raman spectroscopy/microscopy). This review article gives a short description about these different Raman techniques and their possible applications. Then, a short statistical part is coming about the appearance of Raman spectroscopy in the scientific literature from the beginnings to these days. The third part of the paper shows the main application options of the technique (especially confocal Raman spectroscopy) in skin research, including skin composition analysis, drug penetration monitoring and analysis, diagnostic utilizations in dermatology and cosmeto-scientific applications. At the end, the possible role of artificial intelligence in Raman data analysis and the regulatory aspect of these techniques in dermatology are briefly summarized. For the future of Raman Spectroscopy, increasing clinical relevance and in vivo applications can be predicted with spreading of non-destructive methods and appearance with the most advanced instruments with rapid analysis time.

KEYWORDS
artificial intelligence, cosmetoscience, Raman spectroscopy, skin composition, skin diagnostics, skin research, topical drug penetration

1 | INTRODUCTION

Imaging modalities help in the development of topical drugs and cosmetics and also contribute to the early diagnosis, monitoring and grading of various pathological or physiological conditions in the skin. Different techniques are available in the toolbox of dermatologists and cosmeto-scientists including dermoscopy, confocal microscopy, optical coherence tomography, high-frequency ultrasound, Raman spectroscopy, fluorescence imaging and multispectral opto-acoustic tomography. All these methods have advantages and limitation which are summarized in details by Schneider et al, 2019a and 2019b.[1,2] This review article aimed to focus on Raman spectroscopy in skin research. This technology uses near-infrared lasers which generate photons from the test preparation (e.g. skin tissue) that scatter at the same and at different energies at each chemical bond within the structure. The changes are detected by the device and

translated into highly specific and characteristic graphs (spectra). It can be applied both on in vivo and ex vivo/in vitro samples. The main strength of Raman spectroscopy is that it is a non-invasive, non-destructive method, which makes possible the in vivo sample analysis and further investigation of ex vivo samples after Raman analysis. Also, the coupling of Raman spectroscopy with confocal microscope enables the possibility of making axial screening. These screening steps can be combined with automatic focus which enables the measurements to be made automatically. No sample preparation is required which simplifies the method and shortens the time collecting the experimental results.[3]

In the current paper after some introductory words about the history and principle of Raman techniques, the main types of the Raman spectroscopy in dermatological research, the most important application possibilities in skin analysis, and the use of machine learning in data analysis are summarized. Finally, a short chapter about the regulatory aspects is presented.

2 | HISTORY OF RAMAN SPECTROSCOPY

The principle of Raman spectroscopy is based on the Raman effect which has been discovered more than 90 years ago. The phenomenon of inelastic scattering of light (Raman scattering) was discovered by Dr. C.V. Raman (1888–1970) in 1928. Sir Chandrasekhara Venkata Raman was an Indian physicist known about his work in the field of light scattering.[4] He developed a spectrograph, and together with his student, K. S. Krishnan discovered that when light traverses a transparent material, a shift is happening in the deflected light in its wavelength and frequency.[5] This phenomenon is called "modified scattering" which was subsequently termed as Raman effect or Raman scattering. Raman received the Nobel Prize in 1930 in Physics for this discovery, and he was the first Asian to receive a Nobel Prize in any branch of sciences.

Originally, extraordinary measures were required to obtain Raman spectra due to the low sensitivity of the technique. Typically, the sample was held in a long tube and illuminated along its length with a beam of filtered monochromatic light generated by a gas discharge lamp. The photons scattered by the sample were collected through an optical flat at the end of the tube. To maximize the sensitivity, the sample was highly concentrated and relatively large volumes (5 ml or more) were used. The use of Raman spectroscopy diminished when commercial infrared (IR) spectrophotometers became available in the 1940s. However, the discovery of the laser in the 1960s resulted in simplified Raman spectroscopy instruments and also boosted the sensitivity of the technique. This has renewed the use of Raman spectroscopy as a common analytical technique.[6]

In the late 1970s, Raman spectroscopy combined with an optical microscope was introduced and was used for microanalysis in several fields. Micro-Raman spectroscopy became an important tool in biology, especially for single-cell studies. Now, many researchers in various fields use micro-Raman spectroscopy. The application of this technology is widespread in analytical fields for industrial, biological, medical and environmental samples[7] and also for art and archeology.[8] Non-destructive Raman analysis has been used for cultural heritage diagnostics[9] and to evaluate the ageing and degradation of plenty of precious artefacts, paints and statues.[10] During the last almost one century, the market for Raman spectroscopy has had plenty of time to mature, and users are continuously working on to find new application areas.

The continuous development of the technology resulted in (1) increased light transmission due to the more advanced optical components, such as the lens, mirror and, especially, optical filters for the removal of scattered light; (2) improved stability due to developments in the spectrometer; and (3) enhanced light detection sensitivity with the use of detectors such as charge-coupled devices (CCDs).

Developments in coherent Raman scattering microscopy applications such as stimulated Raman scattering and coherent anti-Stokes Raman scattering microscopy offer exciting potential in biological imaging applications (see later in the article). Moreover, they offer huge potential for sub-diffraction limited microscopic approaches such as tip-enhanced Raman scattering (TERS), which are now crossing boundaries of spatial resolution that have previously not been accessible with conventional Raman microscopy. Another advancement is surface-enhanced Raman spectroscopy (SERS). It uses nanostructured metal surfaces to generate Raman spectra that are several orders of magnitude higher than regular ones.

3 | THE PRINCIPLE OF RAMAN SPECTROSCOPY

Among the methods of material science, non-destructive vibration spectroscopy (including Raman spectrometry) is known, which is a set of structural analysis methods based on the excitation of vibrational energy transitions and suitable for the production of chemical maps also. Raman scattering is an indirect way to excite vibrational transitions. Photons emitted by irradiated (monochromatic) high-intensity laser light undergo a change in frequency when they collide inelastically with a molecule. The magnitude of the frequency shift is the same as the vibration frequencies characteristic of the molecules of the test substance, so that the vibrations of the test substance can be identified from the spectra. The condition is a molecular structure, or a system of atoms connected by covalent bonds, as these result in a spectrum of sufficiently narrow bands to facilitate the identification of a given substance. Raman spectrometry usually detects molecular vibrations in the range of 3200–200 cm^{-1}.

Raman spectrometry complements infrared spectroscopy well, as both analyse the molecular vibrations of matter. The relative sensitivity of the two methods is different, so it is possible to study different molecular bonds and bond groups. There are several vibrational transitions that are inactive in infrared spectroscopy but active in Raman spectrometry, and vice versa. Raman spectrometry is mainly concerned with the most common chromophore groups, that is C-O, C-N, C-S, S-S, C-Cl bonds and C=C,

C=O, C=N, N=O, C=S and C≡C bonds and bond groups, while IR spectroscopy is more sensitive to polar bonds and functional groups. Water with an intense IR absorption spectrum shows only a weak Raman scattering, so its presence during the measurement is not disturbing.

Chemical mapping is currently one of the most dynamically evolving areas of spectroscopy. The term "chemical mapping" refers primarily to methods based on vibrational (infrared and Raman) spectrometric techniques.[11] Chemical mapping techniques combine the advantageous properties of a spectrometer and a microscope. The information carried by the vibration spectra, which is very characteristic of each molecule, can be combined with the possibility of spatial mapping, so the concentration and location of the components can be determined in heterogeneous systems. This method is becoming increasingly important in many fields of science and industry, from the semiconductor industry,[12] medicine,[13] the paper industry,[14] the food industry[15] and the plastics industry[16] to pharmaceutical technology.[11,17,18,19]

A practical advantage of Raman spectrometry is that in most cases no sample preparation is required. Another major advantage is that due to the high power of the irradiated light, it is also possible to measure the samples through glass and plastic. The disadvantage of this method is that it can cause degradation in more sensitive samples and some substances show fluorescence, which significantly impairs the signal-to-noise ratio.

4 | RAMAN SPECTROSCOPIC TECHNIQUES

Raman spectroscopic investigation of the skin, changes in skin composition due to age or diseases, or analysis of drug delivery to the skin are mostly done by either acquiring spectra along a line perpendicular to the skin surface or by two-dimensional imaging. The former gives penetration depth profiles similar to those obtained by conventional methods such as tape stripping. The latter enables the visualization of drug (or other substances) distribution in the skin.

4.1 | Types of data acquisition

In general, spectra may be acquired in a point-by-point mapping (using a spot focus), line scanning (using a line focus) or global imaging approach (using wide-field illumination). Point-by-point mapping is the most frequently used approach. Here, spectra are acquired in each point in an x-y-coordinate system. In each point, only a small dimension (typically below 1 μm) is illuminated with the laser and a full spectrum from each point is recorded. Subsequently, a data cube is generated comprising the spectra and x-y-coordinates. From this datacube, parts of the information are extracted to form, for example a 2D colour-coded image of a drug substance inside a skin sample. This part of information

may, in the easiest case, contain the intensities of the signal in one single wavenumber at each point in the x-y-coordinate system. In line scanning, the sample is illuminated along a line and the detector simultaneously detects the signals along this line. This is achieved either by conversion of the circular beam to a line focus using a cylindrical lens or by using a point focus to scan along a line.[20] In global imaging, a two-dimensional area of the sample is illuminated. To this end, the laser is defocused which in turn leads to lower intensity of the incident light at the sample. The light is guided towards a detector comprising many thousand elements that acquires all signals simultaneously. Here, each wavenumber is detected one after the other. For imaging purposes, an area of interest may thus be mapped in a point-by-point approach or imaged by global imaging (depending on the type of instrument used). In point-by-point mapping, a full spectrum is acquired in each point. While the acquisition of one spectrum is very fast, the need for acquiring many spectra slows down the process. In global imaging, all points are illuminated at the same time but only one wavenumber is detected. In general, less light is guided to the detector than in point-by-point mapping, thus requiring longer acquisition times. This means that in the case that only one signal at one wavenumber is of interest, this approach is faster. But, if a full spectrum needs to be acquired, the process is slowed down substantially and may then be even slower than point-by-point mapping.

4.2 | Transmittance Raman spectroscopy

In transmittance Raman spectroscopy, the whole sample or a large part of it (typically several mm) is illuminated and the transmitted light is detected. The detected spectrum contains an average spectrum of the sample that it diffused through. It is thus not useful for generating spatially resolved information like it is aimed, for example in skin penetration research. If alterations in the overall spectrum are high enough, it may still be used, for example for quick detection of cancerous tissue. The main area of application remains non-destructive quantitative analysis of dosage forms like ointments, tablets or capsules.[21]

4.3 | Coherent Raman scattering microscopies

Coherent Raman spectroscopy comprises two techniques: coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS). Both are stimulated and thus need two lasers: "pump" and "Stokes." The frequency difference between the two lasers is tuned to match the molecular vibration of interest, that is a specific vibration of a drug substance or excipient. By creating a coherent, stimulated condition, signals many orders higher than in conventional Raman spectroscopy are created.[22,23] In CARS, the molecular vibration, the lasers have been tuned to, creates a photon in the anti-Stokes part of the spectrum which is then detected.[22-24] In SRS, the energy transfer from the Stokes

to the pump laser is detected via a modulation transfer scheme. The CARS spectrum may be contaminated by non-resonant contribution which makes quantification challenging. Further, signal-concentration dependence is not linear but quadratic which needs to be compensated for using appropriate algorithms.[22,23,25,26] The SRS spectrum does not contain non-resonant contamination and the signal-concentration dependence is linear.[27] Both methods are most powerful when a specific molecular vibration of the substance under investigation either falls within the range of 2000–2700 cm^{-1}, where there are no vibrations of the skin itself, or if they are deuterium labelled which makes vibrations fall into this range, too.[24,25,26,28,29,30] In this range, the methods show limits of detection around 100 mM but this may increase drastically when signals overlapping with those of the skin are to be used.[23] Tuning the lasers to multiple frequencies, that is acquiring hyperspectral data would improve this situation as more than one wavenumber could be used for evaluation but this drastically slows down acquisition speed. Further, many setups have limited tuning range.[31]

4.4 | Surface-enhanced Raman scattering (SERS)

Raman spectroscopy of the skin is often hampered by fluorescent background in the Raman spectrum and superposition of the signals of interest with the spectrum of the skin itself, making it difficult to generate high signal-to-noise ratios. Surface-enhanced Raman spectroscopy (SERS) uses nanostructured metal surfaces to generate Raman spectra that are several orders of magnitude higher than regular ones. To this end, a very thin sample specimen is adsorbed to a nanostructured metal surface like gold or silver.[32,33] When illuminating the sample with an excitation wavelength that matches the plasmon resonance of the atoms at the surface of the specimen, a strong electromagnetic field is induced. The generated electromagnetic wave propagates along the surface of the specimen. As the Raman signal intensity relates to the amplitude of the electromagnetic field, the Raman modes of the molecules close to the metal surface are strongly enhanced. As a result, the incident and the scattered lights intensities are increased.[33,34] Raman signal intensity enhancement can be explained by the high intensity of the electromagnetic field near the plasmonic substrate and thus by classical electromagnetic theory. On the contrary, there are also chemical enhancement effects. They are derived from the interactions of the samples' molecules with the SERS substrate and can be explained by quantum mechanical electronic structure theory.[33] SERS requires the sample to be prepared as a very thin sheet, carefully fixed to the metal surface which makes sample preparation rather elaborate. SERS substrates themselves require multiple factors to be carefully balanced which makes them elaborate to prepare as well. Furthermore, depth profiling is not feasible unless a large number of such very thin samples are prepared. The technique is therefore only rarely used in skin penetration analysis.[32,35,36] Technical progress such as tip-enhanced SERS or the fabrication of microneedle
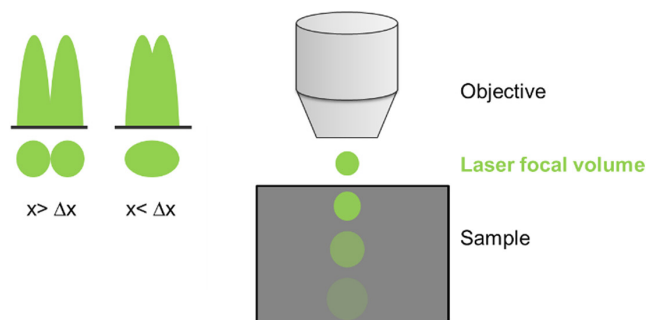
FIGURE 1 Left: schematic of the Rayleigh criterion, right: illustration of depth-dependent attenuation and laser focal volume broadening inside a sample

SERS probes will probably lead to more versatile instrumentation in the future making the method more convenient and increase its use in skin research.[33,35,37]

4.5 | Confocal Raman spectroscopy/microscopy

CRS is by far the most frequently used Raman technique to investigate penetration of exogenous substances into the skin or to analyse the skin in healthy or diseased state. Combined with a movable scan stage that enable precise positioning of the sample in three dimensions, imaging in two or three dimensions can be performed, also referred to as confocal Raman microscopy. A confocal Raman microscope consists of a laser light source, objective(s), beam splitter, pinhole, motorized scan stage, detector and optical fibres to guide the light from the laser source to the objective and from the objective to the detector. Furthermore, the fibres may also be used as pinhole to achieve confocality. The principle of confocality and the factors affecting it as wells as resolution will be explained briefly hereafter.

As in every optical method, the resolution is defined by the Rayleigh criterion (Equation 1).[38]

$$\Delta x = 0.61 * \lambda / NA \qquad (1)$$

where Δx is the distance or resolution or laser focal volume [nm], λ is the wavelength of the light [nm] and NA is the numerical aperture of the objective.

Figure 1 shows an illustration of the Rayleigh criterion. On the left, the distance x between two point-like light sources is larger Δx so that the two points can be differentiated. The situation in the middle shows two point-like structures at a distance x which is smaller than Δx. The two points cannot be distinguished and appear as one spot. The distance Δx beyond which two point-like light sources cannot be detected as two points depend on the wavelength of the light and the numerical aperture of the objective. The longer the wavelength of the light and the smaller the numerical aperture of the objective the larger Δx will become. As a result, resolution decreases. The used wavelength is determined by the laser used and can only be altered if different lasers are at hand. The numerical

aperture is defined by the objective used and can more easily be altered by simply attaching different objectives to the microscope. In general, water or oil immersion objectives show higher numerical apertures compared to metallurgical objectives.[39-42] Typically, green (532 nm) or read (785 nm) lasers are used in confocal Raman spectroscopy. With these lasers and an oil immersion objective with the numerical aperture of 1.25, the maximum theoretical resolution which can be achieved is 260 nm or 383 nm, respectively. In practice, the resolution also depends on the quality of the objective as well as on the sample and the interaction of the light with the sample. In depth profiling, a laser is focused to different depths inside the skin and spectra are acquired from these depths. Inside the sample, the light is scattered and absorbed which leads to depth-dependent signal attenuation. Furthermore, the laser focal volume broadens and the resolution therefore decreases with increasing depth inside the sample (Figure 1, right). As a result, spectra can only be obtained from up to 50 μm inside the skin. To counteract the effect of depth attenuation, the spectra acquired from deeper depths inside the skin need to be normalized to the signal at the skin surface. Otherwise, the decrease in drug concentration with increasing depth in the skin will be overestimated.[43,44]

When light is focused into a sample of different refractive index, the incident light suffers from refraction at the sample surface. As shown in Figure 2 left, the laser light will be focused into deeper depth of the sample if the laser is directed from a medium with the lower refractive index into a medium with higher refractive index (the sample). Although the light is theoretically focused into the depth Δ, it will be refracted and focused into the depth z. To avoid this effect, an immersion medium with the same refractive index as the sample should be used as shown in Figure 2 middle.[39-44] In this case, no refraction takes place at the interface between immersion medium and sample and the depth z the laser is actually focused to equals the theoretical depth Δ. In the case of skin, oil immersion objectives are helpful as the refractive index of the immersion oil (1.51) is close to that of the stratum corneum (SC) (1.47).[45]

The light which is focused onto or into the sample by the objective of the microscope interacts with the sample and is scattered. The scattered light is then collected by the same objective and guided towards the detector. To achieve confocality, an optical pinhole is installed between the objective and the detector. The principal setup is shown in Figure 2-right. Only light from the focal plane will be allowed to pass through the pinhole and will be guided on towards the detector. Light from different depths, for example the reference plane will not be able to pass the pinhole. The size of the pinhole determines the height of the focal plane and thus determines the degree of confocality and depth resolution.[39-42] A small pinhole and high degree of confocality are of pivotal importance in skin depth profiling in order to reject all rays from out of focus regions. Otherwise, spectra of substances, for example the drug on the skin surface may be erroneously detected inside a certain skin depth and lead to false penetration profiles.[43,44]

In principle to types of microscope setups exist, upright and inverted microscopes. For skin Raman analysis, upright microscopes are used for in vitro or ex vivo studies whereas inverted microscopes are used for in vitro cell culture and in vivo measurements. In the upright setup, the skin sample is placed onto a movable scan stage and beneath the objective.[43,44,46,47,48,49] The distance between objective and sample can be adjusted by the scan stage. Thereby, the laser will be focused into different depths of the skin. In most cases, the skin is incubated beforehand in Franz diffusion cells or transwell plates. The samples are then withdrawn from the cells or plates and mounted onto special sample holders which guarantee skin hydration throughout the Raman analysis.[43,44,50,51] A more recent development is a modified diffusion cell which can be mounted directly beneath the objectives of the microscope. The setup (Figure 3B) enables the incubation of the skin with the respective solution of formulation directly underneath the Raman microscopes objective. Thereby, Raman spectra can be acquired at any timepoint, enabling in situ analysis of penetration kinetics.[46,52]

For in vivo analysis, an inverted microscope setup is used. It comprises an oil immersion objective which is located beneath a measuring window onto which the respective body part may be placed for analysis. The depth into which the laser is focused is adjusted through the distance between objective and measuring window.[53-55] The use of the inverted setup shows the advantage that the skin is always in full contact with the measuring window and will thus not be moved out of focus due to involuntary movements of subjects. A disadvantage is that (at least to date) this system does not allow for measurements in
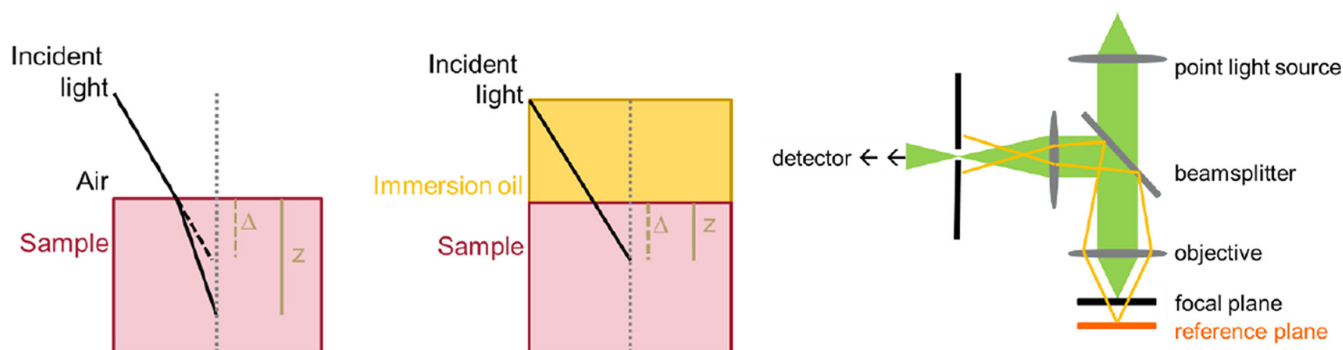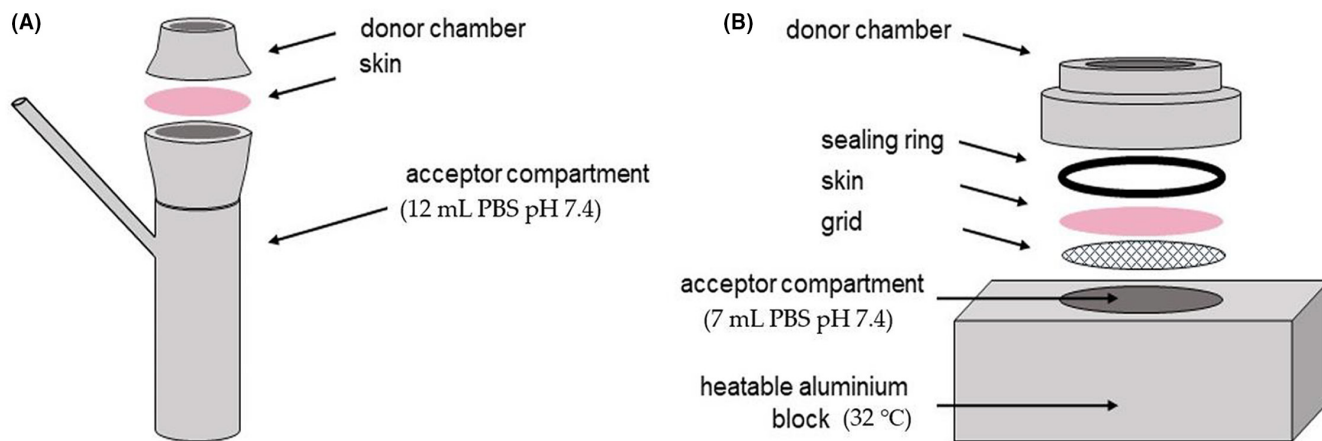

FIGURE 2 Illustration of reflection—sample surface, and microscope setup of confocal microscopy. Left: metallurgical objective, middle: oil immersion objective and right: the propagation of light through a confocal microscope

FIGURE 3 The schematic profiles of the off-line device (A) of Franz diffusion cell for skin incubation and in-line device (B) of skin incubation cells used (reproduced from Pharmaceutics 2021, 13, 67. https://doi.org/10.3390/pharmaceutics13010067; Creative Commons Attribution (CC BY) licence (https://creativecommons.org/licenses/by/4.0/))[46]
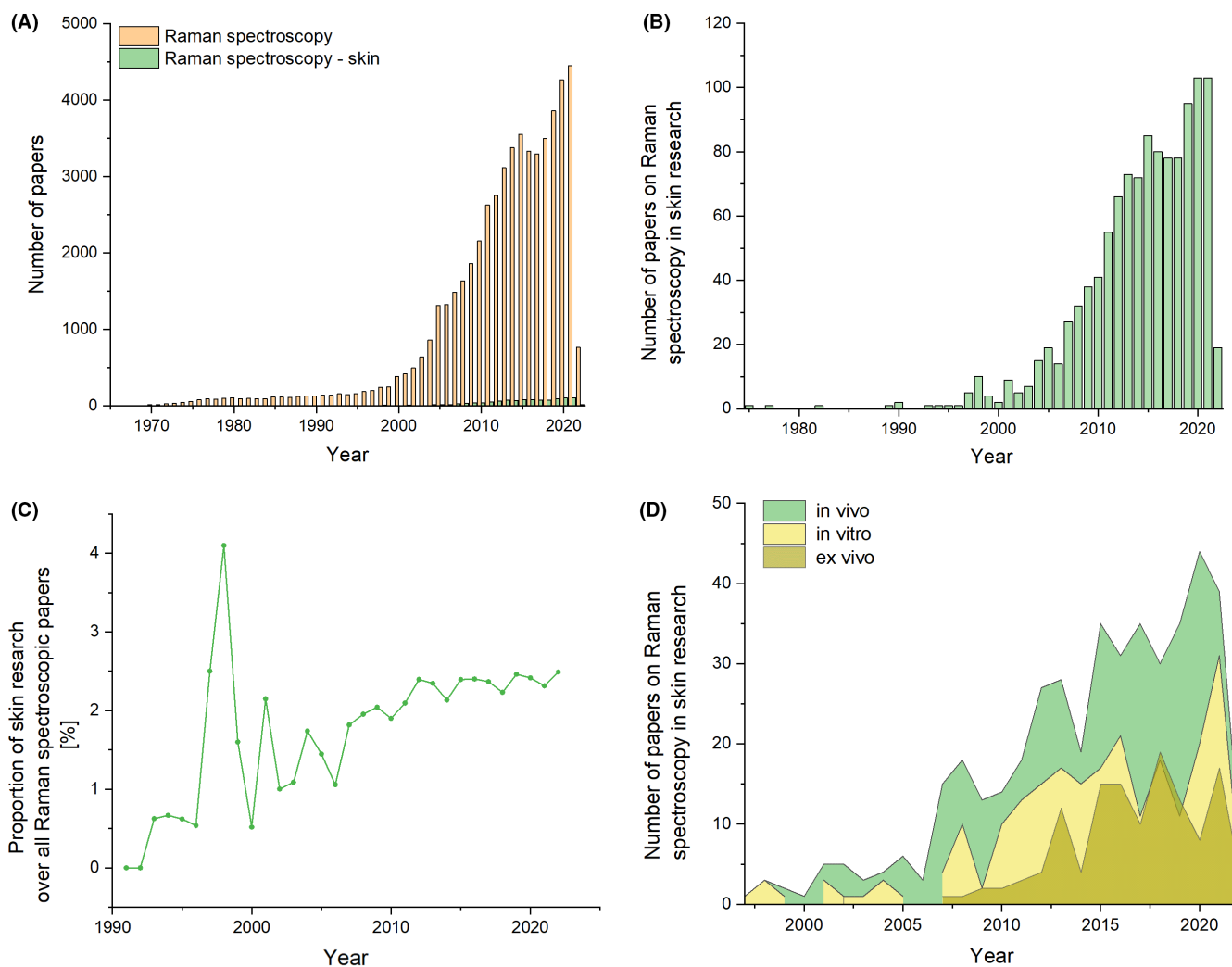
FIGURE 4 Number of publications in PubMed database with the search term (A) "Raman spectroscopy" and (B) "Raman spectroscopy skin." Changes over time (C) in the proportion of skin research in all Raman spectroscopy articles and (D) in the number of publications on Raman spectroscopy in skin research regarding in vivo/ in vitro/ ex vivo applications (The PubMed search was finalized by 25 February 2022)

two or three dimensions thereby prohibiting imaging/mapping. Solving this issue should only be a matter of software capabilities, and it can be expected that this drawback will be resolved in the future.

5 | RAMAN SPECTROSCOPY IN SKIN RESEARCH FROM THE BEGINNINGS BASED ON PUBMED DATABASE

Though the Raman effect underlying the Raman spectroscopy was described in 1928, it could become widespread only after the invention of lasers in the 1960s. The PubMed database contains 15 publications between 1947 and 1960 with the keywords "Raman spectroscopy." In the next decade, an average of less than 5 publications per year was published, followed by a few dozen articles per year in the 1970s, when the microscopic Raman with an optical microscope equipped with a Raman spectrometer has also been invented. Since the introduction of the near-infrared region Fourier transform Raman spectroscopy enabled the application of the technique in biomedical studies, the number of relevant papers has steadily increased, reaching more than 1000 articles in 2005 and over 4400 in 2021, as it is shown in Figure 4A,B.

There are a few articles between 1975 and 1990 which are related to skin studies, but in these cases, they are mostly about the study of different molecules isolated from the skin (e.g. peptides isolated from the skin of Xenopus laevis,[56] or proteodermatan sulphate from pig skin[57]). The first studies on the skin composition were published in the late 1990s (e.g. comparing the structure of water, proteins and lipids in intact human skin,[58] or analysing the molecular structure of 5200-year-old skin[59]). The annual number of papers on the use of Raman spectroscopy in skin research has already reached 100 in recent years. Which means that, although this is only a few per cent (2%–3%) of all Raman spectroscopy papers, its proportion is slowly increasing, presented in Figure 4C,D.

Nearly one third of hits on Raman spectroscopy in skin research are not labelled either in vitro, in vivo or ex vivo, including, for example, structural studies of molecules isolated from skin. A further one third of the articles deals with in vivo applications, such as diagnosis of skin cancer,[60,61] or monitoring the changes due to ageing.[62] The remaining part of the relevant papers used ex vivo analysis for instance for cancer discrimination,[63] or for classification of burn severities,[64] and in vitro applications, such as using in vitro skin substituents.

6 | UTILIZATION OF RAMAN SPECTROSCOPY IN SKIN RESEARCH

6.1 | Skin composition studies

Confocal Raman Spectroscopy (CRS) is being extensively used to analyse skin composition. The basic analysis of skin compounds under physiological conditions comprises a large body of data. Among other vibrational spectroscopic techniques available for this task such as Attenuated Total Reflection Fourier Transform Infrared Spectroscopy, CRS has been shown to provide significantly richer spectroscopic detail and to differentiate between SC and underlying epithelial layers in skin tissue sections of human skin and artificial skin models.[65] Data on skin biochemistry by CRS has stepwise been established for intact and diseased skin in the last 20 years. The steps along the way are discussed in the following section and recent examples are given.

CRS has been used to study the molecular composition of human skin in vivo,[66,67] tissue sections ex vivo[65,68,69] and skin substitutes in vitro.[70-72] The Raman spectrum of human SC is dominated by the vibrational bands of its structural proteins, amino acids and lipids. Both human skin and animal skin can be analysed by CRS (Figure 5).

Spectral bands were first assigned by Barry et al.[73] and are summarized in recent literature.[35,74] The most prominent bands are given in Table 1.

Changes in barrier function of a reconstructed human epidermis model caused by frozen storage have been studied.[75] Both storage time and temperature were relevant factors. Molecular properties of human skin in vivo have been analysed for different age groups; differences in skin biochemistry have been shown for elderly[76,77] and infant skin.[78]

Apart from human skin, animal skin models have been assessed. Porcine skin is perhaps most frequently used due to its relative similarity to human skin in morphology and barrier function.[79-81] Differences of skin models to their natural counterpart based on CRS analysis of their molecular composition provide insights into the underlying cause of lower barrier function as known for reconstructed skin models and porcine skin ex vivo. Choe et al.[79] conducted an in-depth analysis of SC molecular profiles of human skin in vivo and porcine ear skin. The SC depth profiles of porcine skin exhibited lower natural moisturizing facture (NMF) content throughout the entire SC and depth-dependent differences in hydrogen bonding states of water. A higher content of keratin in β-sheet form and higher hexagonal lateral packing order of intracellular lipids was observed at 10%–50% SC depth, which permits penetration of both hydrophilic and lipophilic entities more easily than in human skin.

Of particular interest when investigating the effect of dermal preparations is skin lipid composition.[53,82] The effect of surfactants on the SC lipid matrix can be observed by CRS through changes in lipid content via lipid/protein ratio, changes in conformation and in lateral packing order.[83,84] Non-ionic polysorbate emulsifiers and ethoxylated ethers with a small number of oxyethylene groups were found to cause smaller lipid perturbations than, for example PEG-20 ethers.[84] Further, the NMF as a collection of hygroscopic molecules derived from the protein filaggrin in the SC can be analysed by CRS; the contribution of free amino acids such as serine, glycine, pyrrolidone-5-carboxylic acid, arginine, ornithine, citrulline, alanine, histidine and urocanic acid is usually taken into account to obtain information on the state of skin hydration and barrier function.[82] The analysis of NMF in atopic skin has been validated against tape stripping/HPLC analysis; comparable results were obtained with both methods.[85]
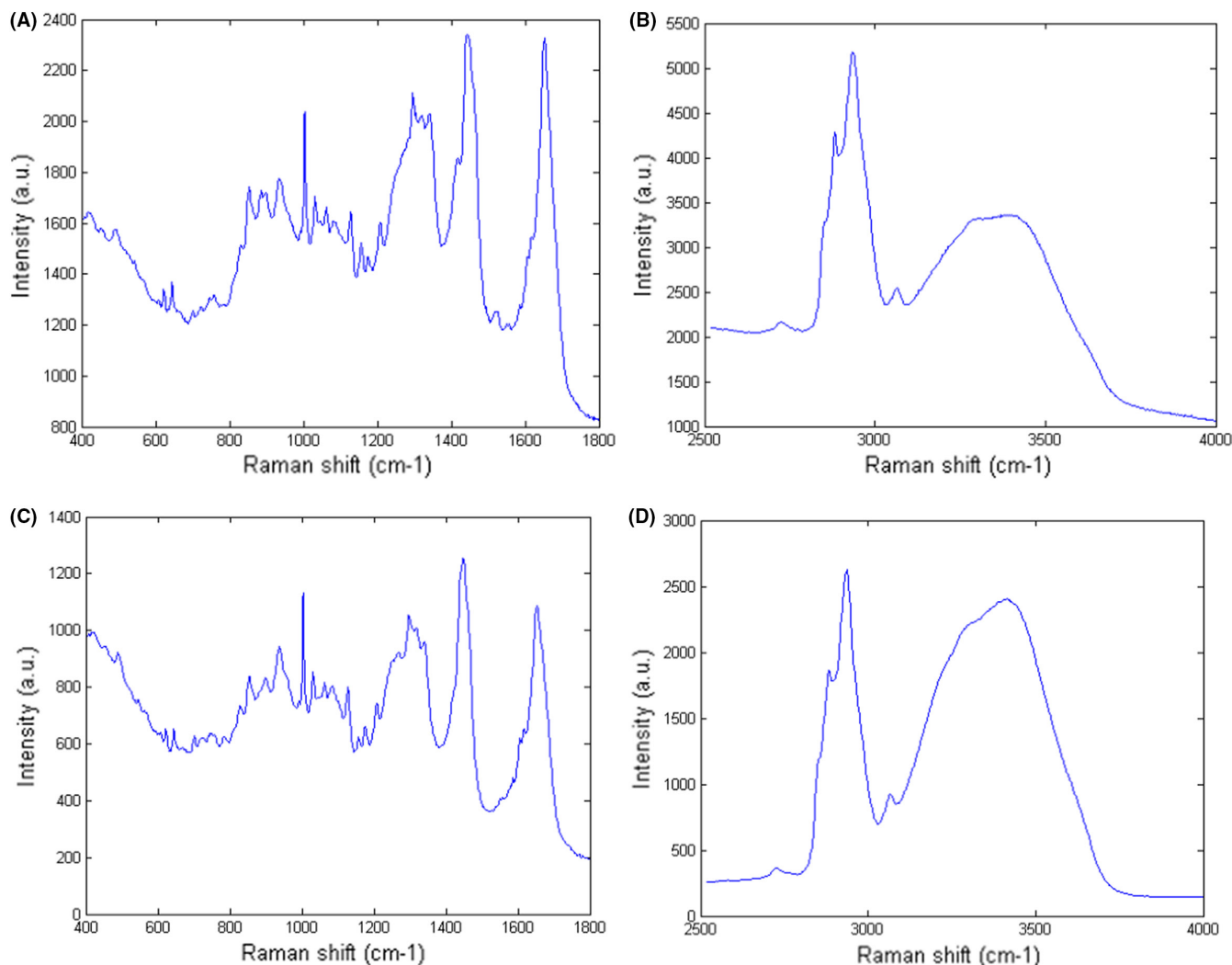
FIGURE 5 Model Raman spectra of fingerprint region (left) and high wavenumber region (right) of healthy human skin in vivo (A, B) and porcine ear skin ex vivo (C, D). Images were taken with a gen2-SCA Raman spectroscope (River D., the Netherlands)

The water content within the skin, in particular the SC, is important to keep the skin barrier functional and is therefore routinely investigated in dermatological studies. CRS can be used to calculate the water mass concentration profile as a function of depth based on the ratio between the intensity of the OH vibration and keratin vibration.[82] Skin hydration, that is the water molecules present in the SC that are involved in maintaining elasticity and physiological molecular structures, has been intensively studied by CRS.[77,86,87] For the mentioned aspects, the bound water content is of particular importance since it is associated with intracellular lipids and proteins via hydrogen bonds.[86] In this context, the hydrogen bonding state of water as determined by CRS was found to reach a maximum at a SC depth of 30% of its entire thickness, correlating well with the maximum lateral packing order of the intercellular lipid matrix and the NMF content.[86] Atmospheric relative humidity was reported to affect the partially bound states of water within the SC.[88] At intermediate relative humidity around 60% both lipid organization and protein deployment were optimal, representing the maximal SC water binding capacity. An increased content of unbound water

within the SC, however, was associated with disordered lipid and protein states. Recently, the application of heavy water was investigated by CRS[89]; different ethoxylated emulsifiers were dissolved in D_2O to probe their effect on skin biochemistry while avoiding additional effects on the OH bond of the skin spectrum through externally applied water. The D_2O distribution within the skin was well discernible due to its OD stretching band and was clearly affected by the co-applied selected ethoxylated emulsifiers in dependence of their chemical structure and the positive control sodium laureth sulphate (SLES).

The effect of dermally applied preparations on hydration status and NMF levels has been explored by CRS for washing procedures with different cleaning products[90,91] and cosmetic emollients,[92] but also for drug delivery systems in vivo.[93,94] Normal skin, but also the lip region have been investigated.[95] Titanium dioxide nanoparticles, as frequently used in cosmetics for sun protection, were found to promote structural rearrangement of the SC lipid bilayers under controlled indoor illumination,[96] which underlines the advantages of CRS in toxicological investigations.

TABLE 1 Assignment of spectral bands in healthy human skin after[35]

Raman shift [cm^{-1}]	Assignment	Raman shift [cm^{-1}]	Assignment
526	ν (SS)	1274	ν (CN), δ (NH) amide 3 α-helix
600	ρ (H)	1296	δ (CH$_2$)
623	ν (CS)	1385	δ (CH$_3$) symmetric
644	ν (CS) amide 4	1421	δ (CH$_3$)
746	ρ (CH$_2$) in phase	1438	δ (CH$_2$) scissoring
827	δ (CCH) aliphatic	1552	δ (NH), ν (CN) amide 2
850	δ (CCH) aromatic	1585	ν (C=C) alkenic
883	δ (CH$_2$), ν (CC), ν (CN)	1652	ν (C=O) amide 1 α-helix
931	ρ (CH$_3$) terminal, ν (CC) α-helix	1743	ν (C=O) amide 1 lipid
956	ρ (CH$_3$), δ (CCH) alkenic	1768	ν (COO)
1002	ν (CC) aromatic ring	2723	ν (CH) aliphatic
1031	ν (CC) skeletal cis	2852	ν (CH$_2$) symmetric
1062	ν (CC) skeletal trans	2883	ν (CH$_2$) asymmetric
1082	ν (CC) skeletal random	2931	ν (CH$_3$) symmetric
1126	ν (CC) skeletal trans	2958	ν (CH$_3$) asymmetric
1155	ν (CC), δ (COH)	3050	ν (CH) alkenic
1172	ν (CC)	3280	ν (OH) of H$_2$O
1244	δ (CH$_2$) wagging, ν (CN)amide 3 disordered		

Note: ν, stretch; ρ, rock; δ, deformation.

Apart from the effect of chemical stressors, the impact of physical procedures such as tape stripping, microneedling or hair removal, is possible, but less promising since not all physical stressors evoke significant changes in the chemical composition of the skin. In addition, potential changes in SC thickness due to physical removal of corneocytes have to be taken into account in data evaluation.[97,98] In a recent study, the effect of iontophoresis and sonophoresis on barrier function alone and in combination with a chemical enhancer was shown for a model permeant using mouse skin and surface-enhanced Raman spectroscopy[99]; the best option for oxaprozin delivery was found to be sonophoresis combined with azone as enhancer.

Chemical and physical influences held aside; skin is also affected by photo-stress, such as solar radiation. Studies with artificial skin sections subjected to simulated solar radiation cell culture studies have shown that induced biochemical changes, for example in SC lipid profile can be detected significantly earlier by CRS than by conventional cytotoxicological analyses.[100] Despite biochemical differences of the probed model to actual human skin, this approach using multivariate data analyses is an interesting prospect for diagnosis of subclinical skin damage due to the high sensitivity of CRS. Recent studies confirmed similar effects caused by infrared irradiation in human skin in vivo,[101] including increased SC lipid content and decreased basal deoxyribonucleic acid (DNA) peaks. The observed spectral shift of the amide I peak of collagen in the dermis indicates structural changes and functional loss. In a recent study, advanced glycation end products as sign of photo-damage and skin ageing were successfully analysed in human biopsies by CRS.[102]

6.2 | Skin penetration studies

In analogy to its role in skin composition analysis, CRS has found a solid place in skin penetration studies. In comparison with other vibrational spectroscopic techniques used to analyse drug penetration, for example the ATR-FTIR-spectroscopy/tape stripping method, it is non-invasive, faster and requires less sample processing.[54] Thus, CRS has become an important asset in cosmetic and pharmaceutical formulation development. Likewise, it is used to evaluate potentially hazardous permeants in toxicological studies. For these tasks, different membranes are used: human skin in vivo,[103] human skin ex vivo,[104,105] in vitro skin substitutes[75] and porcine skin.[52,54,106,107,108,109] Rodent skin[110] is rarely due to its inherent differences to human skin.

Caffeine,[52,106,109,111,112,113] lidocaine,[104] procaine,[107,114] tetracaine,[115] salicylic acid,[113] ibuprofen,[103] flufenamic acid,[105] imiquimod,[116] trans-retinol,[117,118] resorcinol,[75] niacinamide,[119] sulfathiazole sodium,[54] triamcinolone acetonide[120] and oxaprozin are among the most investigated permeants in the CRS penetration studies.[99] Next to these drugs, the penetration of enhancers such as DMSO,[54,121] propylene glycol,[103,106,107,118] polyoxyethylene-23-lauryl ether[107] and other ethoxylated emulsifier systems[114,122] has been evaluated.

The skin penetration of classic anionic surfactants such as sodium dodecyl sulphate (SDS) or sSLES has likewise been investigated in numerous studies, often as positive control.[54,123,124] Regarding skin penetration of additives used in cosmetic or pharmaceutical preparations, data on the penetration behaviour of oils or waxes,[95] preservatives[120] or sunscreen agents[125] have been presented.

In dependence of the experimental setup used, both two- and three-dimensional information on the location of applied permeants is obtained. To facilitate visualization of drugs next to solvents or enhancers such as octanol or propylene glycol and to obtain lateral spatial distribution of the applied materials, the use of deutered compounds is a valid approach.[105] When using complex vehicles, overlapping of formulation compounds with numerous skin bands has to be avoided.[104] However, monitoring the spatial distribution of chemically similar species in CRS penetration studies, for example prodrug and drug, is possible without labelling for compounds with a suitable spectroscopic profile.[125]

The experimental conditions for CRS penetration studies should be kept constant during the analysis. Artefacts can arise from changes in skin biochemistry during analysis caused by prolonged measurement time (occlusion, changes in temperature, etc.). Skin temperature and hydration of porcine skin samples were found to affect results,[109] potentially due to crystallization of the applied drug. This should be kept in mind when comparing results obtained with different experimental setups.

An important question in CRS penetration studies is how to obtain quantitative drug penetration profiles. So far, most studies have delivered information on permeant distribution within the skin on a relative scale. Approaches for quantitative analysis of permeants have been proposed.[126] In recent years, Caspers et al.[119] have moved forward in this respect; the first fully quantitative approach analysing the skin penetration of niacinamid from various formulations into human skin was studied both in vitro using classic Franz-type diffusion cells and in vivo by quantitative CRS. The correlation after linear regression between cumulative drug amounts in vitro and penetrated drug at 2 μm SC depth in vivo was reported with $R^2 = 0.98$. Further research will provide more insight into quantitative CRS analysis for different applications to determine its role in future bioequivalence studies.

Among the most important conclusions derived from the summarized penetration data is the importance of selecting suitable experimental parameters for CRS instrumentation such as choice of objective and pinholes,[43,44] and skin samples. Recently, penetration studies performed in parallel with a common multi-purpose confocal Raman spectroscope by WiTec (alpha 500) and the River Diagnostics device (gen2 SCA) were found to deliver comparable outcomes.[127] Binder et al. obtained similar enhancement ratios for procaine HCl when using polyoxyethylene23-lauryl ether to promote skin penetration. This is promising considering that instruments of two different suppliers were used in different laboratories, working with different objectives, pinholes and laser wavelengths. Aspects to be kept in mind are the specific mode of data evaluation and substance-specific aspects such as resonant effects.[127]

6.3 | Skin diseases

As mentioned before, articles on the applicability and utility of Raman spectroscopy for dermatological purposes first appeared in the 1990s.[1,2] Shortly afterwards, increasing number of results were published on Raman spectroscopic investigation of cancerous skin tissues.

Next to healthy skin, analysis of pathological conditions has been the target of numerous CRS investigations, for example on atopic dermatitis[128,129] and psoriatic lesions.[130] A very specific application in context with unphysiological skin conditions is the identification of multicoloured pigments in tattooed human skin preceding laser removal.[131] The effect of applied substances on skin biochemistry, in particular on the SC lipid matrix and barrier function, has been investigated for numerous common additives in dermal preparations: oils,[95,132] emulsifiers,[54,83,84,133,134] solvents or other penetration enhancers.[52] Strategies have been proposed on how to optimize biochemical analysis of the skin by CRS considering spectral variability,[69] impact of applied chemicals[87] or keratin distribution throughout the SC.[135]

Skin cancer is one of the common cancers. Several types of melanomal and non-melanomatous (basal cell carcinoma (BCC) and squamous cell carcinoma (SCC)) cancers[136] can be distinguished. The first step in the medical diagnosis of the disease is a visual examination,[137] followed by skin biopsy and histopathology.[136,137] Thanks to increasingly sophisticated data processing methods, it became possible to fully differentiate between cancerous and non-tumorous tissues[3-5] and between different types of skin cancers, and to determine a demarcation line of the diseased tissue.[6-9] The technical advances have made it possible to apply the technique clinically for examination and diagnosis of various, primary skin cancers.[10-15] On application of Raman spectroscopy for diagnostic purposes, some sample papers are shown in Table 2.

6.4 | Applications in cosmetoscience

In addition to the dermatological use of Raman spectroscopy, its use for cosmetology purposes is gaining ground, as it is becoming important to know the mechanism, exact extent and depth of penetration of different active ingredients and drugs furthermore provides information on the distribution of these molecules in different layers of the skin. In vivo Raman spectroscopy is a non-invasive, sensitive method that offers an effective, easy-to-use solution for all these requirements.

The penetration of several popular molecules in the cosmetic industry has been investigated by Raman spectroscopy. The tested active ingredients provide solutions to various skin problems (e.g. acne, skin ageing, hydration, anti-inflammatory problems and sun protection). Essendoubi et al. studied three different hyaluronic acid derivatives (Cristalhyal (1000–1400 kDa), Bashyal (100–300 kDa) and Renovhyal (20–50 kDa)) on plastic abdominal skin samples removed by plastic surgery.[138] Tfaili et al.[139] performed penetration

TABLE 2 Examples for diagnostic application of Raman spectroscopy in dermatology (modified from Franzen et al. and Zhao et al.[35,160])

Type of investigation	Type of the skin	Result, conclusion	Ref.
In vitro	Human SC samples	Comparison of Raman spectroscopy and infrared spectroscopy based on spectra taken from SC, supporting the dermatological applicability and utility of Raman spectroscopy	73,161
In vitro	Biopsies BCC	Differentiation of basal cell carcinoma (BCC) from non-tumorous tissue based on Raman spectra	162-164
In vitro	Biopsies healthy and cancerous skins	Discrimination of diseased and non-cancerous tissues, differentiation of skin cancer types	165
In vitro	Biopsies and normal skin samples	Investigation the differences between the Raman spectra of malignant melanoma (MM), basal cell carcinoma (BCC), pigmented naevi (PN), seborrheic keratoses (SK) and normal skin for diagnostic purposes	166-169
In vitro	Healthy SC samples, atopic dermatitis, psoriasis	Analysis of skin samples from patients with atopic dermatitis and psoriasis by Raman spectroscopy	130
Ex vivo	SC isolated from human abdominal skin	Investigation and understanding of the hydration process	88
In vivo	Psoriatic skin	Examination of psoriatic skin by Raman spectroscopy	170
In vivo	Allergic skin	Application of Raman spectroscopy in patients suffering from nickel allergy	171
In vivo	Atopic dermatitis	Use of Raman spectroscopy in patients suffering from atopic dermatitis	172
In vivo	Skin cancer	Application of Raman spectroscopy for in vivo cancer diagnosis	137

studies with caffeine, also on human abdominal skin samples. The penetration of cholesterol- and linoleic-containing creams has been studied in the skin of healthy volunteers. It was determined that the active ingredients could penetrate into the stratum corneum and have a repairing and regenerating effect during their release from the topical cream formulation.[140] Dancik[75] examined the effect of freezing temperature and duration on the resorcinol penetration on reconstructed human epidermis (RHE) samples. To evaluate the safety and the synergistic effects of tea tree, lavender, eucalyptus and tangerine essential oils in combination on the skin[141] Infante and co-workers (2022) used ex vivo confocal Raman spectroscopy and in in vitro and in vivo conditions. Skin penetration was evaluated after direct application of essential oils to pig ears. The authors concluded that the essential oils in combination were safe and effective in the improvement of the hydrolipidic balance and morphological properties of the skin. Micek and co-workers (2021)[142] investigated the effects of two creams: 3% S. balsamita extract and 3% Taxifolin (TXF) on the function of adult skin. The percutaneous penetration of creams was also examined with the use of electrospray ionization mass spectrometry (ESI-MS) and confocal Raman spectroscopy. The 3% S. balsamita extract cream reduced hyperpigmentation, erythema and elevated pH. A higher penetration rate was revealed for the 3% TXF cream than for the 3% S. balsamita extract cream by Raman spectroscopy. Based on the results, the authors concluded that both extracts can be considered as ingredients of skincare products for adults. Pany and co-workers (2021)[143] tested the effect of hydroxypropyl-β-cyclodextrin (HP-β-CD) in cosmetic submicron emulsions and submicron emulsion gels on physiological skin parameters during regular application in a clinical setup. Confocal Raman spectroscopy was employed to monitor urea and NMF levels. The

authors reported that no statistically significant effects on any of the observed parameters were obtained, indicating good skin tolerability of all tested formulations.

In addition to penetration studies, Raman spectroscopy is also used in formulation studies (e.g. for glycolic acid, *Punica granatum* seed oil hydroxyphenethyl ester [PHE] and raspberry seed oil).[144-147] Effects of glycolic acid on the skin were assessed by measurement of biophysical skin parameters in in vivo studies (5-h, 4-h and 7-day).

Organic pigments are favoured for tattooing because of their high tinting strength, light fastness, enzymatic resistance, dispersion and relatively inexpensive production costs. A methodology was established by Poon and co-workers (2008)[148] using micro-Raman spectroscopy on an animal model to correctly identify the constituents of a selection of modern, organic tattoo inks in situ or postprocedure, within the skin. The results reported by Persechino et al (2019) and Carlson et al (2016) confirm the high sensitivity and great expendability of Raman spectroscopy for identification of pigments present in tattoo inks and the relative quality control (as composition control).[149,150] The aim of this research was to assess the feasibility of Raman spectroscopy as a screening technique for chemical characterization of tattoo pigments in pathologic reacting tattoos and tattoo ink products to depict unsafe pigments and metabolites of pigments.

7 | ARTIFICIAL INTELLIGENCE IN ANALYSIS OF RAMAN SPECTRA

The large amount of data from Raman spectroscopy allows, and at the same time may require the use of artificial intelligence in data analysis

to extract meaningful information and make predictions based on the data collected. In the literature, a myriad of different application fields is available, where the measured Raman spectra were the input data of machine learning, mostly by classification algorithms. A large portion of the studies focuses on non-medical applications, with an emphasis on adulteration detection in the food industry. Other part of the articles describe Raman spectroscopic techniques combined with machine learning algorithms to improve diagnostic measures for identifying diseases such as infections, cancer, neurodegenerative disorder or different skin conditions. A summary on application of artificial intelligence in Raman spectroscopic data analysis can be found in Table 3.

8 | REGULATORY ASPECTS OF RAMAN SPECTROSCOPY IN SKIN TESTING

Modelling drug permeation through the skin is a complex challenge. Although there are many quantitative and qualitative methods for following-up skin penetration, the different techniques are not fully equivalent but complement each other. Therefore, the regulation of dermal and transdermal formulations has received increasing attention nowadays. There are more and more guidelines to provide harmonization for dermal and transdermal testing. The Organization for Economic Cooperation and Development (OECD) published several documents about this topic, including: Guidance Notes on Dermal

TABLE 3 Summary of recent studies combining Raman spectroscopy with machine learning algorithms

	Sample	Details	Machine Learning methods	Ref.
Non-medical applications	Butter	Detection of adulteration of butter with margarine	PCA, PCR, PLS, ANN	173
	Caviar	Discrimination between three different caviar types	PCA, LDA, ANN	174
	Edible oils	Edible oil authentication (sesame, hemp, walnut, linseed, pumpkin, sea buckthorn)	Ensemble classifier-subspace KNN when the PCA was disabled	175
	Fruit distillates	Trademark fingerprint differentiation, geographical discrimination	DT, DA, SVM, KNN other ensemble classifiers	176
	Honey	Detection of low-concentration adulterated Suichang native honey	CNN, PNN, SVM	177
	Milk	Differentiation between milk from different species (cow, buffalo, goat and human)	PCA, RF	169
	Minerals	Recognition of minerals and estimation their elemental composition	CNN, KNN, SVM, extremely randomized trees, weighted-neighbours	178
	Poplar	Prediction of the lignin content in poplar wood samples	DT, SVM ensemble classifiers (LightGBM, CatBoost, XGBoost)	179
Medical applications	Alzheimer's disease	Alzheimer's disease diagnosis based on the analysis of cerebrospinal fluid	ANN, SVM-DA	180
	Alzheimer's disease	Alzheimer's disease diagnosis based on the analysis of saliva	ANN, GA	181
	COVID-19 infection	Diagnosis of COVID-19 infection based on saliva samples	MIL	182
	Tuberculosis infection	Distinction between tuberculosis positive (diseased), negative (cured) and control (healthy) serum samples	PCA, HCA	183
	Breast cancer	Classification of breast cancer subtypes	PCA-DFA, PCA-SVM	184
	Colorectal cancer	Prediction the effect of immunotherapy	SVM, RF	185
	Lung cancer	Cytopathological diagnosis of lung cancer	KNN, SVM	186
	Skin cancer (basal/squamous cell carcinoma)	Distinction between basal cell carcinoma, squamous cell carcinoma and healthy skin tissues and cells	CNN, LR, SVM	187
	Skin cancer (melanoma)	Distinction between benign versus malignant melanoma tissues	LightGBM, KNN, XGBoost	188
	Atopic dermatitis	Stratification of severity in atopic dermatitis	SVM	189
	Burn injury	Classification of burn injury	LR, SVM, RF	190

Absorption (No. 156),[151] Test Guideline 427 (in vivo methods)[152] Test Guideline 428 (in vitro methods),[153] and Guidance Document for the Conduct of Skin Absorption Studies.[154] There are some other documents: World Health Organization International Programme on Chemical Safety (WHO/IPCS) Environmental Health Criteria 235,[155] European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) Monograph 20,[156] United States Environmental Protection Agency (USEPA) report on dermal exposure assessment,[157] European Food Safety Agency (EFSA) Guidance on dermal absorption for plant protection products,[158] and the European Medicines Agency (EMA) document, Draft Guideline on Quality and Equivalence of Topical Products.[159] There are two methods proposed in these guides: the widely used diffusion cell and tape stripping methods. The Raman spectroscopy is mentioned only in the latest EMA recommendation, so it is very important to investigate the method's applicability, and in the future, if there will be a sufficiently favourable investigation, it might be recommended by all authorities.

9 | SUMMARY

Simplicity in using and collecting data, as well as conducting analyses without the need for prior labelling and complicated sample preparation, has resulted in increased interest and a significant increase in the use of Raman spectroscopy in the field of life sciences including skin research and dermatologic diagnosis. Raman spectroscopy techniques used for research on biological material, that is skin tissues, provide insight into the structure and organization of the dermal barrier. Raman microscopy is a precise tool for the study of the structural polymers, metabolites, lipids, proteins and water content in the tissues. Owing to its exceptional sensitivity, this method can distinguish even subtle differences among regions with different chemical composition and structure. It allows the researcher to study the processes taking place in the skin and the effectivity of different topical formulations. The introduction of new and improved Raman techniques by researchers, as well as continued technological development of the apparatus, has resulted in an increased significance of Raman spectroscopy in the discovery and definition of tissues and the processes taking place inside them. The Raman technique is a promising direction for science resulting in the discovery of skin and artificial skin tissues as well as their identification and characterization.

This method offers extensive potential for use in further scientific work on dermatological disease diagnosis, drug and cosmetic penetration studies and evaluation of enhancer technologies (both chemical and physical methods). In the future, special attention should be paid to the use of Raman techniques for carrying out the research on the impact of various internal and external factors, such as ageing, disease conditions, environmental changes and environmental stress on biological processes in the skin barrier.

AUTHOR CONTRIBUTIONS

DL: Raman spectroscopic techniques, VK: Utilization of Raman spectroscopy in skin research, DK: Analysis of PubMed database for Raman spectroscopy; artificial intelligence for evaluation of Raman results, FE, ZV-M: Applications of Raman spectroscopy in skin diseases and cosmeto-science; ZV-M: list of references SB: Principle of Raman spectroscopy and regulatory aspects, FE: History of Raman spectroscopy, introduction and summary.

CONFLICT OF INTEREST

No conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

ORCID

Victoria Klang https://orcid.org/0000-0003-2561-4378
Dorottya Kocsis https://orcid.org/0000-0001-7908-3248
Franciska Erdő https://orcid.org/0000-0001-6265-3777

REFERENCES

1. Schneider SL, Kohli I, Hamzavi IH, Council ML, Rossi AM, Ozog DM. Emerging imaging technologies in dermatology: part i: basic principles. *J Am Acad Dermatol*. 2019;80:1114-1120. doi:10.1016/j.jaad.2018.11.042
2. Schneider SL, Kohli I, Hamzavi IH, Council ML, Rossi AM, Ozog DM. Emerging imaging technologies in dermatology: part II: applications and limitations. *J Am Acad Dermatol*. 2019;80:1121-1131. doi:10.1016/j.jaad.2018.11.043
3. Förster M, Bolzinger M-A, Montagnac G, Briançon S. Confocal Raman microspectroscopy of the skin. *Eur J Dermatol*. 2011;21:851-863. doi:10.1684/ejd.2011.1494
4. Smith R, Wright KL, Ashton L. Raman spectroscopy: an evolving technique for live cell studies. *Analyst*. 2016;141:3590-3600. doi:10.1039/c6an00152a
5. Raman CV, Krishnan KS. A new type of secondary radiation. *Nature*. 1928;121:501-502. doi:10.1038/121501c0
6. Editorial, B.R. A history of raman spectroscopy. Accessed April 9, 2022. https://blog.bccresearch.com/a-history-of-raman-spectroscopy
7. Kudelski A. Analytical applications of Raman spectroscopy. *Talanta*. 2008;76:1. doi:10.1016/j.talanta.2008.02.042
8. Vandenabeele P, Edwards HGM, Moens L. A decade of Raman spectroscopy in art and archaeology. *Chem Rev*. 2007;107:675-686. doi:10.1021/cr068036i
9. Analytical Methods Committee Amctb No. Null Raman spectroscopy in cultural heritage: background paper. *Anal Methods*. 2015;7:4844-4847. doi:10.1039/c5ay90036k

10. Casadio F, Daher C, Bellot-Gurlet L. Raman spectroscopy of cultural heritage materials: overview of applications and new Frontiers in instrumentation, sampling modalities, and data processing. *Top Curr Chem (Cham)*. 2016;374:62. doi:10.1007/s41061-016-0061-z

11. Gowen AA, O'Donnell CP, Cullen PJ, Bell SEJ. Recent applications of chemical imaging to pharmaceutical process monitoring and quality control. *Eur J Pharm Biopharm*. 2008;69:10-22. doi:10.1016/j.ejpb.2007.10.013

12. Yang XM, Tryk DA, Hasimoto K, Fujishima A. Surface enhanced Raman imaging of a patterned self-assembled monolayer formed by microcontact printing on a silver film. *Appl Phys Lett*. 1996;69:4020-4022. doi:10.1063/1.117857

13. Ferris DG, Lawhead RA, Dickman ED, et al. Multimodal hyperspectral imaging for the noninvasive diagnosis of cervical neoplasia. *J Low Genit Tract Dis*. 2001;5:65-72. doi:10.1046/j.1526-0976.2001.005002065.x

14. Tatzer P, Wolf M, Panner T. Industrial application for inline material sorting using hyperspectral imaging in the NIR range. *Real-Time Imaging*. 2005;11:99-107. doi:10.1016/j.rti.2005.04.003

15. Robert P, Bertrand D, Devaux MF, Sire A. Identification of chemical constituents by multivariate near-infrared spectral imaging. *Anal Chem*. 1992;64:664-667. doi:10.1021/ac00030a017

16. Markwort L, Kip B, Silva ED, Roussel B. Raman imaging of heterogeneous polymers: a comparison of global versus point illumination. *Appl Spectrosc*. 1995;49:1411-1430. doi:10.1366/0003702953965452

17. Gendrin C, Roggo Y, Collet C. Pharmaceutical applications of vibrational chemical imaging and chemometrics: a review. *J Pharm Biomed Anal*. 2008;48:533-553. doi:10.1016/j.jpba.2008.08.014

18. Amigo JM. Practical issues of hyperspectral imaging analysis of solid dosage forms. *Anal Bioanal Chem*. 2010;398:93-109. doi:10.1007/s00216-010-3828-z

19. Gordon KC, McGoverin CM. Raman mapping of pharmaceuticals. *Int J Pharm*. 2011;417:151-162. doi:10.1016/j.ijpharm.2010.12.030

20. Sil S, Gautam R, Umapathy S. Chapter 6 - applications of Raman and infrared microscopy to materials and biology. In: Gupta VP, ed. *Molecular and Laser Spectroscopy*. Elsevier; 2018:117-146 ISBN 978–0–12-849883-5.

21. Ohashi R, Fujii A, Fukui K, Koide T, Fukami T. Non-destructive quantitative analysis of pharmaceutical ointment by transmission Raman spectroscopy. *Eur J Pharm Sci*. 2022;169:106095. doi:10.1016/j.ejps.2021.106095

22. Evans CL, Potma EO, Puoris'haag M, Côté D, Lin CP, Xie XS. Chemical imaging of tissue in vivo with video-rate coherent anti-stokes Raman scattering microscopy. *Proc Natl Acad Sci USA*. 2005;102:16807-16812. doi:10.1073/pnas.0508282102

23. Pena A-M, Chen X, Pence IJ, et al. Imaging and quantifying drug delivery in skin - part 2: fluorescence Andvibrational spectroscopic imaging methods. *Adv Drug Deliv Rev*. 2020;153:147-168. doi:10.1016/j.addr.2020.03.003

24. Evans CL, Xie XS. Coherent anti-stokes Raman scattering microscopy: chemical imaging for biology and medicine. *Annu Rev Anal Chem (Palo Alto Calif)*. 2008;1:883-909. doi:10.1146/annurev.anchem.1.031207.112754

25. Cheng J-X, Volkmer A, Xie XS. Theoretical and experimental characterization of coherent anti-stokes Raman scattering microscopy. *J Opt Soc Am B*. 2002;19:1363-1375. doi:10.1364/JOSAB.19.001363

26. Ganikhanov F, Evans CL, Saar BG, Xie XS. High-sensitivity vibrational imaging with frequency modulation coherent anti-stokes Raman scattering (FM CARS) microscopy. *Opt Lett*. 2006;31:1872-1874. doi:10.1364/ol.31.001872

27. Freudiger CW, Min W, Saar BG, et al. Label-free biomedical imaging with high sensitivity by stimulated Raman scattering microscopy. *Science*. 2008;322:1857-1861. doi:10.1126/science.1165758

28. Xie XS, Yu J, Yang WY. Living cells as test tubes. *Science*. 2006;312:228-230. doi:10.1126/science.1127566

29. Saar BG, Contreras-Rojas LR, Xie XS, Guy RH. Imaging drug delivery to skin with stimulated Raman scattering microscopy. *Mol Pharm*. 2011;8:969-975. doi:10.1021/mp200122w

30. Belsey NA, Garrett NL, Contreras-Rojas LR, et al. Evaluation of drug delivery to intact and Porated skin by coherent Raman scattering and fluorescence microscopies. *J Control Release*. 2014;174:37-42. doi:10.1016/j.jconrel.2013.11.002

31. Brinkmann M, Fast A, Hellwig T, Pence I, Evans CL, Fallnich C. Portable all-fiber dual-output widely tunable light source for coherent Raman imaging. *Biomed Opt Express*. 2019;10:4437-4449. doi:10.1364/BOE.10.004437

32. Zhu Y, Choe C-S, Ahlberg S, et al. Penetration of silver nanoparticles into porcine skin ex vivo using fluorescence lifetime imaging microscopy, Raman microscopy, and surface-enhanced Raman scattering microscopy. *J Biomed Opt*. 2015;20:51006. doi:10.1117/1.JBO.20.5.051006

33. Cialla D, März A, Böhme R, et al. Surface-enhanced Raman spectroscopy (SERS): Progress and trends. *Anal Bioanal Chem*. 2012;403:27-54. doi:10.1007/s00216-011-5631-x

34. Le Ru EC, Blackie E, Meyer M, Etchegoin PG. Surface enhanced Raman scattering enhancement factors: a comprehensive study. *J Phys Chem C*. 2007;111:13794-13803. doi:10.1021/jp0687908

35. Franzen L, Windbergs M. Applications of Raman spectroscopy in skin research--from skin physiology and diagnosis up to risk assessment and dermal drug delivery. *Adv Drug Deliv Rev*. 2015;89:91-104. doi:10.1016/j.addr.2015.04.002

36. Falamas A, Dehelean CA, Cinta Pinzaru S. Monitoring of Betulin Nanoemulsion treatment and molecular changes in mouse skin cancer using surface enhanced Raman spectroscopy. *Vib Spectrosc*. 2018;95:44-50. doi:10.1016/j.vibspec.2018.01.004

37. Yuen C, Liu Q. Towards in vivo intradermal surface enhanced Raman scattering (SERS) measurements: silver coated microneedle based SERS probe. *J Biophotonics*. 2014;7:683-689. doi:10.1002/jbio.201300006

38. Rayleigh XXXI. Investigations in optics, with special reference to the spectroscope. *Lond Edinb Dublin Philos Mag J Sci*. 1879;8:261-274. doi:10.1080/14786447908639684

39. Everall NJ. Confocal Raman microscopy: why the depth resolution and spatial accuracy can be much worse than you think. *Appl Spectrosc*. 2000;54:1515-1520. doi:10.1366/0003702001948439

40. Everall, N. Depth profiling with confocal raman microscopy, Part I. *Spectroscopy*. 2004:19.

41. Everall, N. Depth profiling with confocal raman microscopy, Part II. *Spectroscopy*. 2004:19.

42. Everall NJ. Modeling and measuring the effect of refraction on the depth resolution of confocal Raman microscopy. *Appl Spectrosc*. 2000;54:773-782.

43. Lunter DJ. Determination of skin penetration profiles by confocal Raman microspectroscopy: statistical evaluation of optimal microscope configuration. *J Raman Spectrosc*. 2017;48:152-160. doi:10.1002/jrs.5001

44. Lunter DJ. How confocal is confocal Raman microspectroscopy on the skin? Impact of microscope configuration and sample preparation on penetration depth profiles. *Skin Pharmacol Physiol*. 2016;29:92-101. doi:10.1159/000444806

45. Knüttel A, Boehlau-Godau M. Spatially confined and temporally resolved refractive index and scattering evaluation in human skin performed with optical coherence tomography. *J Biomed Opt*. 2000;5:83-92. doi:10.1117/1.429972

46. Krombholz R, Liu Y, Lunter DJ. In-line and off-line monitoring of skin penetration profiles using confocal Raman spectroscopy. *Pharmaceutics*. 2021;13:67. doi:10.3390/pharmaceutics13010067

47. Miloudi L, Bonnier F, Tfayli A, et al. Confocal Raman spectroscopic imaging for in vitro monitoring of active ingredient penetration and distribution in reconstructed human epidermis model. *J Biophotonics*. 2018;11:e201700221. doi:10.1002/jbio.201700221

48. Stella A, Bonnier F, Tfayli A, et al. Raman mapping coupled to self-modelling MCR-ALS analysis to estimate active cosmetic ingredient penetration profile in skin. *J Biophotonics*. 2020;13:e202000136. doi:10.1002/jbio.202000136

49. Tfaili S, Josse G, Angiboust J-F, Manfait M, Piot O. Monitoring caffeine and resveratrol cutaneous permeation by confocal Raman microspectroscopy. *J Biophotonics*. 2014;7:676-681. doi:10.1002/jbio.201300011

50. Ashtikar M, Matthäus C, Schmitt M, Krafft C, Fahr A, Popp J. Non-invasive depth profile imaging of the stratum corneum using confocal Raman microscopy: first insights into the method. *Eur J Pharm Sci*. 2013;50:601-608. doi:10.1016/j.ejps.2013.05.030

51. Bonnist EYM, Gorce J-P, Mackay C, Pendlington RU, Pudney PDA. Measuring the penetration of a skin sensitizer and its delivery vehicles simultaneously with confocal Raman spectroscopy. *Skin Pharmacol Physiol*. 2011;24:274-283. doi:10.1159/000328729

52. Krombholz R, Lunter D. A new method for in-situ skin penetration analysis by confocal Raman microscopy. *Molecules*. 2020;25:E4222. doi:10.3390/molecules25184222

53. Caspers PJ, Lucassen GW, Bruining HA, Puppels GJ. Automated depth-scanning confocal Raman microspectrometer for rapid in vivo determination of water concentration profiles in human skin. *J Raman Spectrosc*. 2000;31:813-818. doi:10.1002/1097-4555(200008/09)31:8/9<813::AID-JRS573>3.0.CO;2-7

54. Binder L, Kulovits EM, Petz R, et al. Penetration monitoring of drugs and additives by ATR-FTIR spectroscopy/tape stripping and confocal Raman spectroscopy - a comparative study. *Eur J Pharm Biopharm*. 2018;130:214-223. doi:10.1016/j.ejpb.2018.07.007

55. Mateus R, Moore DJ, Hadgraft J, Lane ME. Percutaneous absorption of salicylic acid – in vitro and in vivo studies. *Int J Pharm*. 2014;475:471-474. doi:10.1016/j.ijpharm.2014.08.061

56. Williams RW, Starman R, Taylor KM, et al. Raman spectroscopy of synthetic antimicrobial frog peptides magainin 2a and PGLa. *Biochemistry*. 1990;29:4490-4496. doi:10.1021/bi00470a031

57. Renugopalakrishnan V, Zheng S, Tu AT, Damle SP. Secondary structure of pig skin Proteodermatan sulfate: a perspective from Raman spectroscopic studies in aqueous solution. *Biopolymers*. 1989;28:1935-1938. doi:10.1002/bip.360281110

58. Gniadecka M, Faurskov Nielsen O, Christensen DH, Wulf HC. Structure of water, proteins, and lipids in intact human skin, hair, and nail. *J Invest Dermatol*. 1998;110:393-398. doi:10.1046/j.1523-1747.1998.00146.x

59. Williams AC, Edwards HG, Barry BW. The "iceman": molecular structure of 5200-year-old skin characterised by Raman spectroscopy and electron microscopy. *Biochim Biophys Acta*. 1995;1246:98-105. doi:10.1016/0167-4838(94)00189-n

60. Bratchenko IA, Bratchenko LA, Moryatov AA, et al. In vivo diagnosis of skin cancer with a portable Raman spectroscopic device. *Exp Dermatol*. 2021;30:652-663. doi:10.1111/exd.14301

61. Kourkoumelis N, Balatsoukas I, Moulia V, Elka A, Gaitanis G, Bassukas ID. Advances in the in vivo Raman spectroscopy of malignant skin tumors using portable instrumentation. *Int J Mol Sci*. 2015;16:14554-14570. doi:10.3390/ijms160714554

62. Ali SM. In vivo confocal Raman spectroscopic imaging of the human skin extracellular matrix degradation due to accumulated intrinsic and extrinsic aging. *Photodermatol Photoimmunol Photomed*. 2021;37:140-152. doi:10.1111/phpp.12623

63. Schmälzlin E, Moralejo B, Gersonde I, et al. Nonscanning large-area Raman imaging for ex vivo/in vivo skin cancer discrimination. *J Biomed Opt*. 2018;23:105001. 1, 11. doi:10.1117/1.JBO.23.10.105001

64. Ye H, Rahul, Kruger U, et al. Raman spectroscopy accurately classifies burn severity in an ex vivo model. *Burns*. 2021;47:812-820. doi:10.1016/j.burns.2020.08.006

65. Ali SM, Bonnier F, Lambkin H, et al. A comparison of Raman, FTIR and ATR-FTIR micro spectroscopy for imaging human skin tissue sections. *Anal Methods*. 2013;5:2281-2291. doi:10.1039/C3AY40185E

66. Choe C, Lademann J, Darvin ME. A depth-dependent profile of the lipid conformation and lateral packing order of the stratum corneum in vivo measured using Raman microscopy. *Analyst*. 2016;141:1981-1987. doi:10.1039/c5an02373d

67. Quatela A, Miloudi L, Tfayli A, Baillet-Guffroy A. In vivo Raman microspectroscopy: intra- and Intersubject variability of stratum corneum spectral markers. *Skin Pharmacol Physiol*. 2016;29:102-109. doi:10.1159/000445079

68. Ali SM, Bonnier F, Tfayli A, et al. Raman spectroscopic analysis of human skin tissue sections ex-vivo: evaluation of the effects of tissue processing and dewaxing. *J Biomed Opt*. 2012;18:061202. doi:10.1117/1.JBO.18.6.061202

69. Franzen L, Windbergs M. Accessing Raman spectral variability in human stratum corneum for quantitative in vitro depth profiling. *J Raman Spectrosc*. 2014;45:82-88. doi:10.1002/jrs.4428

70. Leroy M, Labbé J-F, Ouellet M, et al. A comparative study between human skin substitutes and Normal human skin using Raman microspectroscopy. *Acta Biomater*. 2014;10:2703-2711. doi:10.1016/j.actbio.2014.02.007

71. Tfayli A, Piot O, Draux F, Pitre F, Manfait M. Molecular characterization of reconstructed skin model by Raman microspectroscopy: comparison with excised human skin. *Biopolymers*. 2007;87:261-274. doi:10.1002/bip.20832

72. Bakar J, Michael-Jubeli R, El Khoury R, et al. Assessment of the skin barrier function in the reconstructed human epidermis using a multimodal approach at molecular tissue and functional levels. *Analyst*. 2021;146:4649-4658. doi:10.1039/d1an00465d

73. Barry BW, Edwards HGM, Williams AC. Fourier transform Raman and infrared vibrational study of human skin: assignment of spectral bands. *J Raman Spectrosc*. 1992;23:641-645. doi:10.1002/jrs.1250231113

74. Pezzotti G, Boffelli M, Miyamori D, et al. Raman spectroscopy of human skin: looking for a quantitative algorithm to reliably estimate human age. *J Biomed Opt*. 2015;20:065008. doi:10.1117/1.JBO.20.6.065008

75. Dancik Y, Kichou H, Eklouh-Molinier C, et al. Freezing weakens the barrier function of reconstructed human epidermis as evidenced by Raman spectroscopy and percutaneous permeation. *Pharmaceutics*. 2020;12:E1041. doi:10.3390/pharmaceutics12111041

76. Choe C, Schleusener J, Lademann J, Darvin ME. Age related depth profiles of human stratum corneum barrier-related molecular parameters by confocal Raman microscopy in vivo. *Mech Ageing Dev*. 2018;172:6-12. doi:10.1016/j.mad.2017.08.011

77. Egawa M, Tagami H. Comparison of the depth profiles of water and water-binding substances in the stratum corneum determined in vivo by Raman spectroscopy between the cheek and volar forearm skin: effects of age, seasonal changes and artificial forced hydration. *Br J Dermatol*. 2008;158:251-260. doi:10.1111/j.1365-2133.2007.08311.x

78. Nikolovski J, Stamatas GN, Kollias N, Wiegand BC. Barrier function and water-holding and transport properties of infant stratum corneum are different from adult and continue to develop through the first year of life. *J Invest Dermatol*. 2008;128:1728-1736. doi:10.1038/sj.jid.5701239

79. Choe C, Schleusener J, Lademann J, Darvin ME. Human skin in vivo has a higher skin barrier function than porcine skin ex vivo-comprehensive Raman microscopic study of the stratum

corneum. *J Biophotonics*. 2018;11:e201700355. doi:10.1002/jbio.201700355

80. Tfaili S, Gobinet C, Josse G, Angiboust J-F, Manfait M, Piot O. Confocal Raman microspectroscopy for skin characterization: a comparative study between human skin and pig skin. *Analyst*. 2012;137:3673-3682. doi:10.1039/c2an16292j

81. Mahrhauser D-S, Nagelreiter C, Gehrig S, et al. Assessment of Raman spectroscopy as a Fast and non-invasive method for Total stratum corneum thickness determination of pig skin. *Int J Pharm*. 2015;495:482-484. doi:10.1016/j.ijpharm.2015.09.018

82. Caspers PJ, Lucassen GW, Carter EA, Bruining HA, Puppels GJ. In vivo confocal Raman microspectroscopy of the skin: noninvasive determination of molecular concentration profiles. *J Invest Dermatol*. 2001;116:434-442. doi:10.1046/j.1523-1747.2001.01258.x

83. Purohit P, Chandar P, Vilinska A, Ananthapadmanabhan KP, Somasundaran P. Effect of mixed surfactants on stratum corneum: a drying stress and Raman spectroscopy study. *Int J Cosmet Sci*. 2014;36:379-385. doi:10.1111/ics.12139

84. Liu Y, Lunter DJ. Systematic investigation of the effect of non-ionic emulsifiers on skin by confocal Raman spectroscopy-a comprehensive lipid analysis. *Pharmaceutics*. 2020;12:E223. doi:10.3390/pharmaceutics12030223

85. Koppes SA, Kemperman P, Van Tilburg I, et al. Determination of natural moisturizing factors in the skin: Raman microspectroscopy versus HPLC. *Biomarkers*. 2017;22:502-507. doi:10.1080/1354750X.2016.1256428

86. Choe C, Lademann J, Darvin ME. Depth profiles of hydrogen bound water molecule types and their relation to lipid and protein interaction in the human stratum corneum in vivo. *Analyst*. 2016;141:6329-6337. doi:10.1039/c6an01717g

87. Choe C, Schleusener J, Choe S, Lademann J, Darvin ME. A modification for the calculation of water depth profiles in oil-treated skin by in vivo confocal Raman microscopy. *J Biophotonics*. 2020;13:e201960106. doi:10.1002/jbio.201960106

88. Vyumvuhore R, Tfayli A, Duplan H, Delalleau A, Manfait M, Baillet-Guffroy A. Effects of atmospheric relative humidity on stratum corneum structure at the molecular level: ex vivo Raman spectroscopy analysis. *Analyst*. 2013;138:4103-4111. doi:10.1039/c3an00716b

89. Liu Y, Lunter DJ. Tracking heavy-water-incorporated confocal Raman spectroscopy for evaluating the effects of PEGylated emulsifiers on skin barrier. *J Biophotonics*. 2020;13:e202000286. doi:10.1002/jbio.202000286

90. Perticaroli S, Meyers JL, Wireko FC, et al. Cleansers' mildness: stratum corneum lipid organization and water uptake after a single wash. *J Raman Spectrosc*. 2020;51:795-806. doi:10.1002/jrs.5841

91. Wu J, Polefka TG. Confocal Raman microspectroscopy of stratum corneum: a pre-clinical validation study. *Int J Cosmet Sci*. 2008;30:47-56. doi:10.1111/j.1468-2494.2008.00428.x

92. Stettler H, Kurka P, Wagner C, et al. A new topical panthenol-containing emollient: skin-moisturizing effect following single and prolonged usage in healthy adults, and tolerability in healthy infants. *J Dermatolog Treat*. 2017;28:251-257. doi:10.1080/09546634.2016.1218417

93. Wolf M, Klang V, Stojcic T, Fuchs C, Wolzt M, Valenta C. NLC versus Nanoemulsions: effect on physiological skin parameters during regular in vivo application and impact on drug penetration. *Int J Pharm*. 2018;549:343-351. doi:10.1016/j.ijpharm.2018.08.007

94. Binder L, Klang V, Sheikh Rezaei S, et al. Topical application of highly concentrated water-in-oil emulsions: physiological skin parameters and skin penetration in vivo - a pilot study. *Int J Pharm*. 2019;571:118694. doi:10.1016/j.ijpharm.2019.118694

95. Bielfeldt S, Blaak J, Laing S, et al. Deposition of plant lipids after single application of a lip care product determined by confocal Raman spectroscopy, Corneometry and Transepidermal water-loss. *Int J Cosmet Sci*. 2019;41:281-291. doi:10.1111/ics.12533

96. Turci F, Peira E, Corazzari I, Fenoglio I, Trotta M, Fubini B. Crystalline phase modulates the potency of nanometric TiO_2 to adhere to and perturb the stratum corneum of porcine skin under indoor light. *Chem Res Toxicol*. 2013;26:1579-1590. doi:10.1021/tx400285j

97. Pany A, Klang V, Brunner M, Ruthofer J, Schwarz E, Valenta C. Effect of physical and chemical hair removal methods on skin barrier function in vitro: consequences for a hydrophilic model permeant. *Skin Pharmacol Physiol*. 2019;32:8-21. doi:10.1159/000493168

98. Pany A, Klang V, Peinhopf C, Zecevic A, Ruthofer J, Valenta C. Hair removal and bioavailability of chemicals: effect of physicochemical properties of drugs and surfactants on skin permeation ex vivo. *Int J Pharm*. 2019;567:118477. doi:10.1016/j.ijpharm.2019.118477

99. Liu S, Bao X, Zhang S, et al. The study of ultrasound and iontophoresis on Oxaprozin transdermal penetration using surface-enhanced Raman spectroscopy. *Drug Deliv Transl Res*. 2020;10:83-92. doi:10.1007/s13346-019-00664-9

100. Ali SM, Bonnier F, Ptasinski K, et al. Raman spectroscopic mapping for the analysis of solar radiation induced skin damage. *Analyst*. 2013;138:3946-3956. doi:10.1039/c3an36617k

101. Ali SM, Khalid SG. To study the effect of acute infrared radiation-induced alterations in human skin at cellular and molecular level using in vivo confocal Raman spectroscopy. *Photodermatol Photoimmunol Photomed*. 2022;38:44-52. doi:10.1111/phpp.12714

102. Pereira AFM, Rodrigues BVM, Neto LPM, et al. Confocal Raman spectroscopy as a tool to assess advanced glycation end products on solar-exposed human skin. *Vib Spectrosc*. 2021;114:103234. doi:10.1016/j.vibspec.2021.103234

103. Mateus R, Abdalghafor H, Oliveira G, Hadgraft J, Lane ME. A new paradigm in Dermatopharmacokinetics - confocal Raman spectroscopy. *Int J Pharm*. 2013;444:106-108. doi:10.1016/j.ijpharm.2013.01.036

104. Bakonyi M, Gácsi A, Kovács A, Szűcs M-B, Berkó S, Csányi E. Following-up skin penetration of lidocaine from different vehicles by Raman spectroscopic mapping. *J Pharm Biomed Anal*. 2018;154:1-6. doi:10.1016/j.jpba.2018.02.056

105. Pyatski Y, Zhang Q, Mendelsohn R, Flach CR. Effects of permeation enhancers on Flufenamic acid delivery in ex vivo human skin by confocal Raman microscopy. *Int J Pharm*. 2016;505:319-328. doi:10.1016/j.ijpharm.2016.04.011

106. Mujica Ascencio S, Choe C, Meinke MC, et al. Confocal Raman microscopy and multivariate statistical analysis for determination of different penetration abilities of caffeine and propylene glycol applied simultaneously in a mixture on porcine skin ex vivo. *Eur J Pharm Biopharm*. 2016;104:51-58. doi:10.1016/j.ejpb.2016.04.018

107. Lunter D, Daniels R. Confocal Raman microscopic investigation of the effectiveness of penetration enhancers for procaine delivery to the skin. *J Biomed Opt*. 2014;19:126015. doi:10.1117/1.JBO.19.12.126015

108. Meyer S, Heinsohn G, Wolber R, Pörtner R, Nierle J. Confocal Raman investigation of diffusion processes in monolithic type transdermal drug delivery systems. *Drug Deliv*. 2015;22:1103-1110. doi:10.3109/10717544.2014.889778

109. Liu Y, Krombholz R, Lunter DJ. Critical parameters for accurate monitoring of caffeine penetration in porcine skin using confocal Raman spectroscopy. *Int J Pharm*. 2021;607:121055. doi:10.1016/j.ijpharm.2021.121055

110. Suzuki T, Aoki T, Saito M, et al. Enhancement of skin permeation of a hydrophilic drug from acryl-based pressure-sensitive adhesive tape. *Pharm Res*. 2021;38:289-299. doi:10.1007/s11095-021-02996-z

111. Alonso C, Carrer V, Barba C, Coderch L. Caffeine delivery in porcine skin: a confocal Raman study. *Arch Dermatol Res*. 2018;310:657-664. doi:10.1007/s00403-018-1854-4

112. Franzen L, Anderski J, Windbergs M. Quantitative detection of caffeine in human skin by confocal Raman spectroscopy--a systematic in vitro validation study. *Eur J Pharm Biopharm*. 2015;95:110-116. doi:10.1016/j.ejpb.2015.03.026

113. Muhammad F, Riviere JE. Differential effects of some natural compounds on the transdermal absorption and penetration of caffeine and salicylic acid. *Int J Pharm*. 2015;483:151-157. doi:10.1016/j.ijpharm.2015.02.029

114. Liu Y, Lunter DJ. Confocal Raman spectroscopy at different laser wavelengths in analyzing stratum corneum and skin penetration properties of mixed PEGylated emulsifier systems. *Int J Pharm*. 2022;616:121561. doi:10.1016/j.ijpharm.2022.121561

115. Dennis AC, McGarvey JJ, Woolfson AD, McCafferty DF, Moss GP. A Raman spectroscopic investigation of bioadhesive tetracaine local Anaesthetic formulations. *Int J Pharm*. 2004;279:43-50. doi:10.1016/j.ijpharm.2004.04.012

116. Alvarez-Figueroa MJ, Narváez-Araya D, Armijo-Escalona N, Carrasco-Flores EA, González-Aramundiz JV. Design of Chitosan Nanocapsules with Compritol 888 ATO® for imiquimod transdermal administration. Evaluation of their skin absorption by Raman microscopy. *Pharm Res*. 2020;37:195. doi:10.1007/s11095-020-02925-6

117. Pudney PDA, Mélot M, Caspers PJ, Van Der Pol A, Puppels GJ. An in vivo confocal Raman study of the delivery of trans retinol to the skin. *Appl Spectrosc*. 2007;61:804-811. doi:10.1366/000370207781540042

118. Mélot M, Pudney PDA, Williamson A-M, Caspers PJ, Van Der Pol A, Puppels GJ. Studying the effectiveness of penetration enhancers to deliver retinol through the stratum Cornum by in vivo confocal Raman spectroscopy. *J Control Release*. 2009;138:32-39. doi:10.1016/j.jconrel.2009.04.023

119. Iliopoulos F, Caspers PJ, Puppels GJ, Lane ME. Franz cell diffusion testing and quantitative confocal Raman spectroscopy: in vitro-in vivo correlation. *Pharmaceutics*. 2020;12:E887. doi:10.3390/pharmaceutics12090887

120. Sigg M, Daniels R. Impact of Alkanediols on stratum corneum lipids and triamcinolone acetonide skin penetration. *Pharmaceutics*. 2021;13:1451. doi:10.3390/pharmaceutics13091451

121. Caspers PJ, Williams AC, Carter EA, et al. Monitoring the penetration enhancer dimethyl sulfoxide in human stratum corneum in vivo by confocal Raman spectroscopy. *Pharm Res*. 2002;19:1577-1580. doi:10.1023/a:1020481305420

122. Liu Y, Lunter DJ. Profiling skin penetration using PEGylated emulsifiers as penetration enhancers via confocal Raman spectroscopy and fluorescence spectroscopy. *Eur J Pharm Biopharm*. 2021;166:1-9. doi:10.1016/j.ejpb.2021.04.027

123. Mao G, Flach CR, Mendelsohn R, Walters RM. Imaging the distribution of sodium dodecyl sulfate in skin by confocal Raman and infrared microspectroscopy. *Pharm Res*. 2012;29:2189-2201. doi:10.1007/s11095-012-0748-y

124. Hoppel M, Kwizda K, Baurecht D, Caneri M, Valenta C. The effect of a damaged skin barrier on percutaneous absorption of SDS and skin hydration investigated by confocal Raman spectroscopy. *Exp Dermatol*. 2016;25:390-392. doi:10.1111/exd.12950

125. Tippavajhala VK, de Oliveira Mendes T, Martin AA. In vivo human skin penetration study of sunscreens by confocal Raman spectroscopy. *AAPS PharmSciTech*. 2018;19:753-760. doi:10.1208/s12249-017-0852-8

126. Franzen L, Selzer D, Fluhr JW, Schaefer UF, Windbergs M. Towards drug quantification in human skin with confocal Raman microscopy. *Eur J Pharm Biopharm*. 2013;84:437-444. doi:10.1016/j.ejpb.2012.11.017

127. Binder L, Valenta C, Lunter D. Determination of skin penetration profiles by confocal Raman microspectroscopy: evaluation of interindividual variability and Interlab comparability. *J Raman Spectrosc*. 2020;51:1037-1043. doi:10.1002/jrs.5871

128. Verzeaux L, Vyumvuhore R, Boudier D, et al. Atopic skin: in vivo Raman identification of global molecular signature, a comparative study with healthy skin. *Exp Dermatol*. 2018;27:403-408. doi:10.1111/exd.13388

129. Mlitz V, Latreille J, Gardinier S, et al. Impact of filaggrin mutations on Raman spectra and biophysical properties of the stratum corneum in mild to moderate atopic dermatitis. *J Eur Acad Dermatol Venereol*. 2012;26:983-990. doi:10.1111/j.1468-3083.2011.04198.x

130. Wohlrab J, Vollmann A, Wartewig S, Marsch WC, Neubert R. Noninvasive characterization of human stratum corneum of Undiseased skin of patients with atopic dermatitis and psoriasis as studied by Fourier transform Raman spectroscopy. *Biopolymers*. 2001;62:141-146. doi:10.1002/bip.1006

131. Darvin ME, Schleusener J, Parenz F, et al. Confocal Raman microscopy combined with optical clearing for identification of inks in multicolored tattooed skin in vivo. *Analyst*. 2018;143:4990-4999. doi:10.1039/c8an01213j

132. Choe C, Lademann J, Darvin ME. Confocal Raman microscopy for investigating the penetration of various oils into the human skin in vivo. *J Dermatol Sci*. 2015;79:176-178. doi:10.1016/j.jdermsci.2015.05.004

133. Zhang Z, Lunter DJ. Confocal Raman microspectroscopy as an alternative method to investigate the extraction of lipids from stratum corneum by emulsifiers and formulations. *Eur J Pharm Biopharm*. 2018;127:61-71. doi:10.1016/j.ejpb.2018.02.006

134. Zhang Z, Lunter DJ. Confocal Raman microspectroscopy as an alternative to differential scanning calorimetry to detect the impact of emulsifiers and formulations on stratum corneum lipid conformation. *Eur J Pharm Sci*. 2018;121:1-8. doi:10.1016/j.ejps.2018.05.013

135. Choe C, Choe S, Schleusener J, Lademann J, Darvin ME. Modified normalization method in in vivo stratum corneum analysis using confocal Raman microscopy to compensate nonhomogeneous distribution of keratin. *J Raman Spectrosc*. 2019;50:945-957. doi:10.1002/jrs.5596

136. Linares MA, Zakaria A, Nizran P. Skin cancer. *Prim Care*. 2015;42:645-659. doi:10.1016/j.pop.2015.07.006

137. Lui H, Zhao J, McLean D, Zeng H. Real-time Raman spectroscopy for in vivo skin cancer diagnosis. *Cancer Res*. 2012;72:2491-2500. doi:10.1158/0008-5472.CAN-11-4061

138. Essendoubi M, Gobinet C, Reynaud R, Angiboust JF, Manfait M, Piot O. Human skin penetration of hyaluronic acid of different molecular weights as probed by Raman spectroscopy. *Skin Res Technol*. 2016;22:55-62. doi:10.1111/srt.12228

139. Tfaili S, Gobinet C, Josse G, et al. Vibrational spectroscopies for the analysis of cutaneous permeation: experimental limiting factors identified in the case of caffeine penetration. *Anal Bioanal Chem*. 2013;405:1325-1332. doi:10.1007/s00216-012-6512-7

140. Zhang Z, Lukic M, Savic S, Lunter DJ. Reinforcement of barrier function - skin repair formulations to deliver physiological lipids into skin. *Int J Cosmet Sci*. 2018;40:494-501. doi:10.1111/ics.12491

141. Infante VHP, Maia Campos PMBG, Gaspar LR, et al. Safety and efficacy of combined essential oils for the skin barrier properties: in vitro, ex vivo and clinical studies. *Int J Cosmet Sci*. 2022;44:118-130. doi:10.1111/ics.12761

142. Micek I, Nawrot J, Seraszek-Jaros A, et al. Taxifolin as a promising ingredient of cosmetics for adult skin. *Antioxidants (Basel)*. 2021;10:1625. doi:10.3390/antiox10101625

143. Pany A, Wohlgenannt M, Klopprogge S, et al. Effect of hydroxypropyl-β-cyclodextrin in fluid and semi-solid submicron emulsions on physiological skin parameters during regular in vivo application. *Int J Cosmet Sci*. 2021;43:263-268. doi:10.1111/ics.12674

144. Lukic M, Filipovic M, Pajic N, Lunter D, Bozic D, Savic S. Formulation of topical acidic products and acidification of the skin - contribution of glycolic acid. *Int J Cosmet Sci*. 2021;43:419-431. doi:10.1111/ics.12707

145. Munnier E, Al Assaad A, David S, et al. Homogeneous distribution of fatty Ester-based active cosmetic ingredients in hydrophilic thin films by means of Nanodispersion. *Int J Cosmet Sci*. 2020;42:512-519. doi:10.1111/ics.12652

146. Gledovic A, Janosevic Lezaic A, Krstonosic V, et al. Low-energy Nanoemulsions as carriers for red raspberry seed oil: formulation approach based on Raman spectroscopy and textural analysis, physicochemical properties, stability and in vitro antioxidant/ biological activity. *PLoS One*. 2020;15:e0230993. doi:10.1371/journal.pone.0230993

147. Van Gheluwe L, Munnier E, Kichou H, et al. Confocal Raman spectroscopic imaging for evaluation of distribution of Nano-formulated hydrophobic active cosmetic ingredients in hydrophilic films. *Molecules*. 2021;26:7440. doi:10.3390/molecules26247440

148. Poon KWC, Dadour IR, McKinley AJ. In situ chemical analysis of modern organic tattooing inks and pigments by micro-Raman spectrosc. *J Raman Spectrosc*. 2008;39:1227-1237. doi:10.1002/jrs.1973

149. Persechino S, Toniolo C, Ciccola A, et al. A new high-throughput method to make a quality control on tattoo inks. *Spectrochim Acta A Mol Biomol Spectrosc*. 2019;206:547-551. doi:10.1016/j.saa.2018.08.037

150. Hutton Carlsen K, Køcks M, Sepehri M, Serup J. Allergic reactions in red tattoos: Raman spectroscopy for "fingerprint" detection of chemical risk spectra in tattooed skin and culprit tattoo inks. *Skin Res Technol*. 2016;22:460-469. doi:10.1111/srt.12287

151. OECD. Guidance notes on dermal absorption No. 156. Draft Second Edition 2019, Paris.

152. OECD. *Test No 427: Skin Absorption In Vivo Method*. OECD Publishing; 2004. doi:10.1787/9789264071063-en

153. OECD. *Test No 428: Skin Absorption In Vitro Method*. OECD Publishing; 2004. doi:10.1787/9789264071087-en

154. OECD. *Guidance Document for the Conduct of Skin Absorption Studies; OECD Series on Testing and Assessment*. OECD; 2004. ISBN 978-92-64-07879-6.

155. Kielhorn J, Mangelsdorf I, Organization, W.H. *Dermal Absorption*. World Health Organization; 2006. ISBN 978-92-4-157235-4.

156. European Centre for Ecotoxicology and Toxicology of Chemicals. Percutaneous Absorption, Monograph No20. European Centre for Ecotoxicology and Toxicology of Chemicals - ECETOC; 1993. ISSN-0773-6347-20.

157. U.S. Environmental Protection Agency (EPA). *Dermal exposure assessment: a summary of EPA approaches*. U.S. Environmental Protection Agency. Accessed April 1, 2019. http://www.epa.gov/ncea

158. European Food Safety Authority (EFSA); Buist, H.; Craig, P., et al. Guidance on dermal absorption. *EFSA J* 2017, 15, 4873. doi:10.2903/j.efsa.2017.4873

159. FDA. *Draft Guideline on Quality and Equivalence of Topical Products*. European Medicines Agency; 2018. EMA/CHMP/QWP/708282/2018. https://www.ema.europa.eu/en/quality-equivalence-topical-products

160. Zhao J, Zeng H, Kalia S, Lui H. Using Raman spectroscopy to detect and diagnose skin cancer in vivo. *Dermatol Clin*. 2017;35:495-504. doi:10.1016/j.det.2017.06.010

161. Williams AC, Edwards HGM, Barry BW. Fourier transform Raman spectroscopy a novel application for examining human stratum corneum. *Int J Pharm*. 1992;81:R11-R14. doi:10.1016/0378-5173(92)90022-T

162. Nijssen A, Bakker Schut TC, Heule F, et al. Discriminating basal cell carcinoma from its surrounding tissue by Raman spectroscopy. *J Invest Dermatol*. 2002;119:64-69. doi:10.1046/j.1523-1747.2002.01807.x

163. Nijssen A, Maquelin K, Santos LF, et al. Discriminating basal cell carcinoma from perilesional skin using high wave-number Raman spectroscopy. *J Biomed Opt*. 2007;12:34004. doi:10.1117/1.2750287

164. Short MA, Lui H, McLean D, Zeng H, Alajlan A, Chen XK. Changes in nuclei and peritumoral collagen within nodular basal cell carcinomas via confocal micro-Raman spectroscopy. *J Biomed Opt*. 2006;11:34004. doi:10.1117/1.2209549

165. Lieber CA, Majumder SK, Billheimer D, Ellis DL, Mahadevan-Jansen A. Raman microspectroscopy for skin cancer detection in vitro. *J Biomed Opt*. 2008;13:24013. doi:10.1117/1.2899155

166. Gniadecka M, Nielsen OF, Wulf HC. Water content and structure in malignant and benign skin Tumours. *J Mol Struct*. 2003;661–662:405-410. doi:10.1016/j.molstruc.2003.08.030

167. Gniadecka M, Philipsen PA, Wessel S, et al. Melanoma diagnosis by Raman spectroscopy and neural networks: structure alterations in proteins and lipids in intact cancer tissue. *J Invest Dermatol*. 2004;122:443-449. doi:10.1046/j.0022-202X.2004.22208.x

168. Sigurdsson S, Philipsen PA, Hansen LK, Larsen J, Gniadecka M, Wulf HC. Detection of skin cancer by classification of Raman spectra. *IEEE Trans Biomed Eng*. 2004;51:1784-1793. doi:10.1109/TBME.2004.831538

169. Amjad A, Ullah R, Khan S, Bilal M, Khan A. Raman spectroscopy based analysis of Milk using random Forest classification. *Vib Spectrosc*. 2018;99:124-129. doi:10.1016/j.vibspec.2018.09.003

170. Egawa M, Kunizawa N, Hirao T, et al. In vivo characterization of the structure and components of Lesional psoriatic skin from the observation with Raman spectroscopy and optical coherence tomography: a pilot study. *J Dermatol Sci*. 2010;57:66-69. doi:10.1016/j.jdermsci.2009.10.006

171. Alda J, Castillo-Martinez C, Valdes-Rodriguez R, Hernández-Blanco D, Moncada B, González FJ. Use of Raman spectroscopy in the analysis of nickel allergy. *J Biomed Opt*. 2013;18:61206. doi:10.1117/1.JBO.18.6.061206

172. Baclig AC, Bakker Schut TC, O'Regan GM, et al. Possibilities for human skin characterization based on strongly reduced Raman spectroscopic information. *J Raman Spectrosc*. 2013;44:340-345. doi:10.1002/jrs.4198

173. Uysal RS, Boyaci IH, Genis HE, Tamer U. Determination of butter adulteration with margarine using Raman spectroscopy. *Food Chem*. 2013;141:4397-4403. doi:10.1016/j.foodchem.2013.06.061

174. Mohamadi Monavar H, Afseth NK, Lozano J, Alimardani R, Omid M, Wold JP. Determining quality of caviar from Caspian Sea based on Raman spectroscopy and using artificial neural networks. *Talanta*. 2013;111:98-104. doi:10.1016/j.talanta.2013.02.046

175. Berghian-Grosan C, Magdas DA. Raman spectroscopy and machine-learning for edible oils evaluation. *Talanta*. 2020;218:121176. doi:10.1016/j.talanta.2020.121176

176. Berghian-Grosan C, Magdas DA. Application of Raman spectroscopy and machine learning algorithms for fruit distillates discrimination. *Sci Rep*. 2020;10:21152. doi:10.1038/s41598-020-78159-8

177. Hu S, Li H, Chen C, et al. Raman spectroscopy combined with machine learning algorithms to detect adulterated Suichang native honey. *Sci Rep*. 2022;12:3456. doi:10.1038/s41598-022-07222-3

178. Jahoda P, Drozdovskiy I, Payler SJ, Turchi L, Bessone L, Sauro F. Machine learning for recognizing minerals from multispectral data. *Analyst*. 2021;146:184-195. doi:10.1039/D0AN01483D

179. Gao W, Zhou L, Liu S, Guan Y, Gao H, Hui B. Machine learning prediction of lignin content in poplar with Raman spectroscopy. *Bioresour Technol*. 2022;348:126812. doi:10.1016/j.biortech.2022.126812

180. Ryzhikova E, Ralbovsky NM, Sikirzhytski V, et al. Raman spectroscopy and machine learning for biomedical applications: Alzheimer's disease diagnosis based on the analysis of cerebrospinal fluid. *Spectrochim Acta A Mol Biomol Spectrosc*. 2021;248:119188. doi:10.1016/j.saa.2020.119188

181. Ralbovsky NM, Halámková L, Wall K, Anderson-Hanley C, Lednev IK. Screening for Alzheimer's disease using saliva: a new approach based on machine learning and Raman Hyperspectroscopy. *J Alzheimers Dis*. 2019;71:1351-1359. doi:10.3233/JAD-190675

182. Ember KJ, Daoust F, Mahfoud M, et al. Saliva-based detection of COVID-19 infection in a real-world setting using reagent-free Raman spectroscopy and machine learning. *J Biomed Opt*. 2022;27:25002. doi:10.1117/1.JBO.27.2.025002

183. Ullah R, Khan S, Chaudhary II, Shahzad S, Ali H, Bilal M. Cost effective and efficient screening of tuberculosis disease with Raman spectroscopy and machine learning algorithms. *Photodiagnosis Photodyn Ther*. 2020;32:101963. doi:10.1016/j.pdpdt.2020.101963

184. Zhang L, Li C, Peng D, et al. Raman spectroscopy and machine learning for the classification of breast cancers. *Spectrochim Acta A Mol Biomol Spectrosc*. 2022;264:120300. doi:10.1016/j.saa.2021.120300

185. Paidi SK, Rodriguez Troncoso J, Raj P, et al. Raman spectroscopy and machine learning reveals early tumor microenvironmental changes induced by immunotherapy. *Cancer Res*. 2021;81:5745-5755. doi:10.1158/0008-5472.CAN-21-1438

186. Zheng Q, Li J, Yang L, et al. Raman spectroscopy as a potential diagnostic tool to analyse biochemical alterations in lung cancer. *Analyst*. 2020;145:385-392. doi:10.1039/C9AN02175B

187. Wu M, Wang S, Pan S, Terentis AC, Strasswimmer J, Zhu X. Deep learning data augmentation for Raman spectroscopy cancer tissue classification. *Sci Rep*. 2021;11:23842. doi:10.1038/s41598-021-02687-0

188. Araújo DC, Veloso AA, de Oliveira Filho RS, et al. Finding reduced Raman spectroscopy fingerprint of skin samples for melanoma diagnosis through machine learning. *Artif Intell Med*. 2021;120:102161. doi:10.1016/j.artmed.2021.102161

189. Ho CJH, Yew YW, Dinish US, et al. Handheld confocal Raman spectroscopy (CRS) for objective assessment of skin barrier function and stratification of severity in atopic dermatitis (AD) patients. *J Dermatol Sci*. 2020;98:20-25. doi:10.1016/j.jdermsci.2020.02.001

190. Rangaraju LP, Kunapuli G, Every D, Ayala OD, Ganapathy P, Mahadevan-Jansen A. Classification of burn injury using Raman spectroscopy and optical coherence tomography: an ex-vivo study on porcine skin. *Burns*. 2019;45:659-670. doi:10.1016/j.burns.2018.10.007