# Confocal Raman Microscopy
# in
# Skin Drug Delivery Research

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# 1 Short Summary

In drug research, growing scientific and regulatory demands increase the need for novel analytical methods. Especially in skin drug delivery research, advanced analytical techniques are urgently needed. The ability of contactless, label-free and chemically selective detection and quantification of substances predestines confocal Raman microscopy to fill this scientific gap.

In this work, we implement confocal Raman microscopy in skin drug delivery research regarding reevaluation of existing *in vitro* approaches and acquisition of quantitative substance depth profiles in skin tissue. At first, we use Raman imaging to verify the suitability of porcine ears as *in vitro* model for follicular uptake. Furthermore, we investigate Raman signal attenuation as major drawback for quantitative depth profiling via the development of an artificial skin surrogate and evaluate the variability of Raman data acquired from different human skin donors. Moreover, we perform a systematic proof-of-concept study highlighting the possibility of quantitative depth profiling inside skin. Finally, we present freeze-drying as customized sample preparation technique to simplify and standardize quantitative depth profiling.

Overall, the key parameters necessary for a standardized implementation of confocal Raman microscopy are understood and we elucidate crucial interdependences. This newly gained knowledge will help to advance current analytics in skin drug delivery research beyond the state-of-the-art.

# 2 Kurzzusammenfassung

In der Arzneimittelforschung steigern wachsende wissenschaftliche und regulatorische Anforderungen den Bedarf nach neuen analytischen Methoden. Besonders in der dermalen Galenik sind die Ansprüche an die Analytik gestiegen. Durch die Fähigkeit zur kontaktfreien und chemisch selektiven Detektion und Quantifizierung von Substanzen hat konfokale Raman Mikroskopie das Potenzial diese Anforderungen zu erfüllen.

In dieser Arbeit implementieren wir Raman Mikroskopie für die Neubewertung existierender *in vitro* Methoden und die Aufnahme quantitativer Tiefenprofile in Haut. Wir nutzen bildgebende Raman Mikroskopie um die Eignung von Schweineohren als *in vitro* Modell für follikulare Aufnahme zu bestätigen. Zudem bestimmen wir Raman-Signaldämpfung, das Haupthindernis bei der Erfassung von quantitativen Tiefenprofilen und bewerten die Variabilität von Raman-Spektren verschiedener menschlicher Hautproben. Ebenso zeigen wir in einer systematischen Machbarkeitsstudie erste quantitative Raman-Messungen in Haut und stellen Gefriertrocknung als maßgeschneiderte Probenaufbereitung für die vereinfachte und standardisierte Erfassung quantitativer Tiefenprofile vor. Insgesamt werden die entscheidenden Parameter für eine standardisierte Anwendung von Raman Mikroskopie verstanden und wichtige Zusammenhänge aufgeklärt. Auf dieser Grundlage kann sich konfokale Raman Mikroskopie durch weiter vereinfachte Messroutinen und spezialisierte Instrumente zu einer Standardmethode in der dermalen Arzneimittelforschung weiterentwickeln.

# 3 Introduction

## 3.1 State-of-the-art in skin drug delivery research

The human skin comprises one of the largest organs in the human body. With an estimated area of 2 $m^2$, skin represents the main barrier between an organism and its environment. Today, the human skin faces the challenge of constant exposure to chemical, physical and biological hazards. Besides forming the first line of defense of the immune system, skin also regulates the water content of the human body and functions as heat insulation. This results in a sophisticated need to balance permeability and impermeability. To fulfill this complex task, skin exhibits a layered structure with individual composition. The skin's outside is covered with a hydrolipid film consisting of fatty acids and amino acids providing a low pH environment as antimicrobial feature. The main barrier function is constituted by the stratum corneum as the outermost skin layer. This layered structure is 10 µm – 40 µm thick in the areas relevant for percutaneous absorption like abdomen, upper arm and neck (1). The stratum corneum consists of cornified cells embedded in a coherent lipid matrix in a 'brick and mortar' like structure (2), which impedes substance penetration. The dead corneocytes are surrounded by a lipid layer consisting of ceramides, fatty acids and cholesterol derivatives (3). Most skin absorption studies focus on the stratum corneum as the main limiter for substance penetration and permeation. The cornified cells derive from the living epidermis located beneath and are bordered by the basal membrane. Appendices like sweat glands and hair follicles are located in the adjoining dermis. The complex assembly and versatile function of the skin is reflected in a diverse field of skin related research ranging from cosmetics to drug delivery. With the advantage of avoiding the first pass effect, the painless and non-invasive administration of drugs via the skin is a convenient alternative to parenteral or oral drug application. Furthermore, the easy access to most skin areas facilitates local application of drugs for fast and targeted treatment of the skin and the tissues below. However, overcoming the skin barrier is a challenging task for skin drug delivery research. Therefore, effective skin research demands extended knowledge of substance penetration and permeation behavior. Especially for development and optimization of skin therapeutics, a suitable analytical setup is an essential prerequisite (4).

Since *in vivo* testing is expensive and subject to strict obligations, *in vitro* studies are a mandatory requisite in formulation development. Accessing rate and extent of substance penetration into the skin *in vitro* is commonly performed by tape stripping and extraction. After topical substance application to the skin sample, adhesive tape is used to remove the stratum corneum layer by layer.

Subsequently, the substance of interest is extracted from each tape and quantified. Further tissue segmentation by micro-cryotome gives additional information on substance penetration behavior into the deeper skin layers including viable epidermis and dermis (5). Despite recent approaches an exact determination of sample depth remains a challenge during the tape stripping procedure, since the removed layer thickness is mostly indistinct (6). *In vitro* permeation through the skin is accessed by the Franz diffusion cell as the most common setup. The cell comprises a skin layer separating a donor and an acceptor compartment. By analyzing the substance concentration in the acceptor compartment, the permeated amount can be quantified (7). These techniques demand a great deal of manual work by a skilled and trained experimenter. A reliable automated measurement system with defined spatial resolution would speed up and standardize analysis and improve the transferability of acquired results. Furthermore, state-of-the-art penetration and permeation testing is destructive and requires large amounts of sample material. In the light of limited availability of excised human skin and ethical restrains on the use of animal skin, a reduction of sample size and number is advantageous. Thus, there is a strong demand for novel non-destructive techniques reducing sample size and an instrumental implementation for automated measurements.

In this context, optical methods such as fluorescence microscopy, infrared and Raman spectroscopy provide the advantages to fill these scientific gaps. The contact-free measurement mode and immediate analytical readout led to first implementations of optical techniques in skin research. Starting in the late 90s, several optical techniques have been applied to study skin pathophysiology as well as for permeation and penetration studies (8, 9). Recently, microscopy based optical methods like confocal laser scanning microscopy (CLSM) and multiphoton microscopy (MPM) have been used to track substances upon penetration into skin *in vivo* and *in vitro*. CLSM and MPM provide spatially resolved information on substance penetration depth and molecular interaction between substance and skin (10-12). Both techniques require fluorescent signals from the substances of interest, which is mostly achieved by labelling. However, the used dye or fluorescent label can interfere with a strong autofluorescence emitted by the skin itself, mostly caused by melanin and collagen. In addition, the use of a label might introduce alterations to substance penetration behavior and transferability of the results is impaired. In contrast, vibrational spectroscopy provides direct molecular information without labelling. Infrared spectroscopy (IR), as a well-established technique in vibrational spectroscopy, is based on the absorption of light. In skin drug delivery research, IR has successfully been applied *in vivo* and *in vitro* for physiological investigations and follow-up of penetrating substances (13-16). However, the accuracy of IR measurements is limited to 5-10 µm and the asymmetric OH vibration of water interferes with signals from many chemical substances. This impedes spatially high resolved measurements in hydrated biological tissue. Raman spectroscopy as

complementary vibrational spectroscopic technique provides chemically selective information about the sample composition similar to IR. In contrast to IR, Raman spectroscopy provides a spatial resolution below 1 µm in a confocal setup and is not interfered by strong water signals, bearing the potential for data acquisition inside skin.

## 3.2 From the Raman effect to a new analytical technique

The effect of Raman scattering was first reported in 1928 by the Indian physicist Sir C.V. Raman, who later was awarded the Nobel prize in physics for this discovery (17). When light interacts with matter, the effect of light scattering is the most infrequent among other phenomena like transmission and absorption. Scattering only occurs when photons and matter collide on atomic scale. In case of an elastic collision, the energy amounts of incoming and outgoing photons remain unchanged. This type of scattering is called Rayleigh scattering. A different effect reported by C.V. Raman occurs in case of inelastic collisions between incoming photons and dense electron systems like molecular functional groups. If the scattered photons loose energy compared to the incident photons, it is called Stokes scattering. In the rare case of higher energy levels in the outgoing photons, the effect is described as anti-Stokes scattering. Both, Stokes and anti-Stokes effects can be detected by monitoring the frequency and therefore the energy content of the scattered photons. Stokes scattering is detected at lower frequency (lower energy) and anti-Stokes scattering at higher frequency (higher energy) compared to the incoming photons. A schematic drawing of the energy levels of incoming and outgoing photons for the different scattering effects is depicted in figure 1 A. Because of the rare occurrence of anti-Stokes scattering, conventional Raman spectroscopy utilizes only Stokes scattering. In Raman spectroscopy, the energy shift of the scattered photons is measured against their quantity of occurrence. Therefore, a Raman spectrum is depicted as energy shift in wavenumbers on the x-axis and scattering intensity as dependent value, having the advantage that the correlation of wavenumber, frequency and energy content is linear. The energy amount onto which the photons are shifted after light matter interaction is characteristic for molecular vibrations representing specific molecular functional groups. The Raman scattering pattern is specific for a chemical structure and resembles a ‘molecular fingerprint’. Furthermore, the count of scattered photons at a defined Raman shift and therefore the signal intensity correlates linear with the quantity of the represented molecule. This enables a linear correlation between Raman signal intensity and substance concentration. Thus, Raman spectroscopy allows chemically selective identification and quantification without any labelling. The described scattering process is provoked by large delocalized electron systems, resulting in strong Raman bands from non-polar functional groups and aromatic structures. Most small

molecule drugs involve aromatic structures and are strong Raman scatterer and therefore easy to monitor. Biological tissues such as skin have a high variability in chemical composition (18). The resulting large number of different functional groups leads to a highly complex Raman pattern with weak characteristics and strong background signals. As only 1 in $10^7$ scattered photons is relevant for the Raman effect, high detector efficiency and sophisticated optical engineering were necessary to make Raman spectroscopy interesting for skin research.

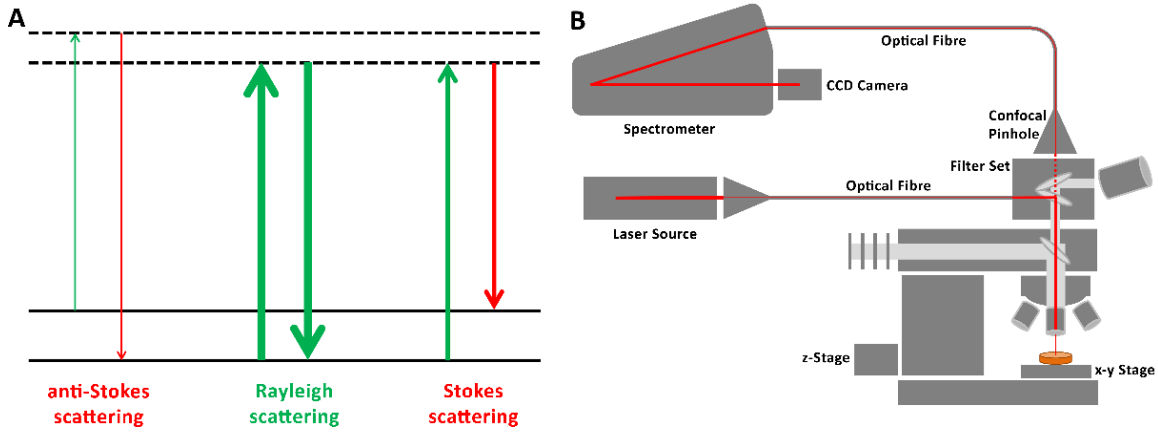


**Figure 1**: **A** Energy level diagram illustrating different types of scattering, line thickness corresponds with the probability of appearance. **B** Schematic of a confocal Raman microscopic setup, red lines indicate the way of excitation laser light and gathered Raman scattering. Reproduced with permission from (19).

In the course of the necessary advancement, Raman spectroscopy was implemented in other optical instruments like fiber probes and microscopes (20, 21). The combination of Raman spectroscopy and confocal microscopy allows the compilation of spatially resolved Raman information. Confocal Raman microscopy (CRM) usually includes a laser source and a spectrometer as detection unit built in a confocal microscope. The laser beam is focused through the microscope objective, allowing the light to interact with the sample on the microscope stage. The scattered photons are collected through the objective and directed towards the detection unit by optical fibers. Cameras equipped with charge coupled devices detect the scattered photons and visualize the energy pattern as Raman spectrum. A confocal pinhole rejects photons from out of focus planes, limiting the gain of spectral information to one voxel. This volumetric pixel, where the Raman information is acquired can freely be moved in all three dimensions by an automated sample positioner. A schematic of a typical confocal Raman microscope is illustrated in figure 1 B. The localized and reproducible acquisition of Raman spectra enables a broad panel of measurement modes. A single Raman spectrum provides chemical information on the sample composition in one exact spot. A combination of subsequently acquired

Raman spectra allows insight in changes in chemical composition as a function of spatial position. In a one directional assembly, Raman spectra can be collected along a line. With this line going into the sample, a series of Raman spectra can be collected in different depths beneath the sample surface. These depth profiles display relative or absolute changes in specified peak intensities over depth. Two dimensional series of data acquisition can be transformed into spectral maps. Single spectra are collected step by step in a defined plane (x-y or y-z) and the spectral information from each pixel is converted into false color images. These false color images are an important visualization tool for sample composition and component distribution. Three dimensional measurements include the assembly of multiple maps to a picture stack. This measurement mode is time consuming and requires advanced data processing. A summary of the different measurement modes and data evaluation methods is presented in figure 2.

Virtual three dimensional measurements can be achieved by combining CRM with optical profilometry. In optical profilometry, a white light source and an assembly of lenses with high chromatic aberration are used to acquire a surface map of an opaque sample. The measurement principle is based on white light reflectance and the wavelength of the detected light can be correlated to the specific distance between light source and sample surface. Based on this information, the software focusses the laser on the exact surface of the sample without the necessity of manual focusing (22).

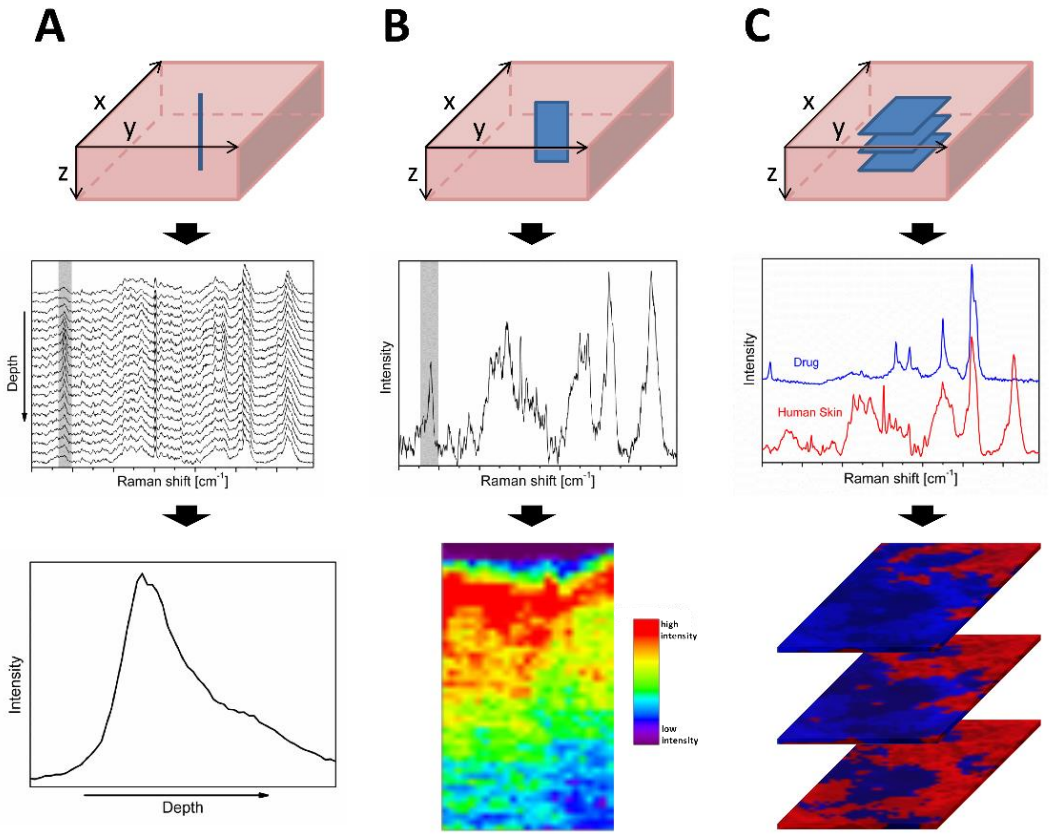


**Figure 2**: **A** Depth profiling: Raman spectra are recorded along a z-directed line, peak intensities are depicted as intensity profiles against depth. **B** y-z intensity mapping: in y-z direction acquired Raman spectra are presented by color coded peak intensities in a two dimensional picture creating a virtual cross section. **C** z-stack false color imaging: series of x-y maps are stacked to a three dimensional data set, different components are assigned to different colors. Reproduced with permission from (19).

In confocal Raman microscopic imaging, the laser spot size defines the spatial resolution. Derived from the point spread function, lateral resolution (LR) can be estimated as LR = 0.82λ/NA (NA = numerical aperture of the objective). The axial resolution (AR) is impacted by the refractive index of the immersion medium (n): AR = $2.2n\lambda/\pi(NA)^2$ (23). In state-of-the-art Raman microscopes, resolution can be down to about 200 nm in lateral and about 500 nm in axial direction. For depth profiling of excised human skin, an ideal penetration depth has been reported for an excitation wavelength of 785 nm and the usage of air interface objectives is necessary to avoid interactions of the sample with the immersion medium (24). After recording of Raman spectra, further data processing becomes necessary. However, most measurement software automates the removal of cosmic ray related peaks. For quantitative evaluation, adjusting the spectral baseline is required. For biological samples, a polynomic fitting functions describes the baseline and facilitates further data processing (25, 26).

## 3.3 Confocal Raman microscopy in skin research – potential and challenges

As a first application of CRM in dermal research, initial studies focused on the physiological investigation of the skin. As pioneers, the group of Puppels et al. established CRM to access the skin hydration status in the late 90s (27, 28). For implementing CRM in skin drug delivery research, analysis and evaluation of *in vitro* skin models is a primary objective. Besides previously investigated animal skin models like shed snake skin (29, 30), porcine skin represents the most commonly applied skin model for *in vitro* testing. Some studies already focused on the physiological evaluation of porcine skin by CRM (31, 32). Tfaili et al. concluded a general comparability of human and porcine skin regarding its spectral features (33). Although human skin is still generally considered as gold standard for penetration and permeation experiments, porcine ear skin is considered superior for testing of follicular uptake (34, 35). The transfollicular route for administration of drugs recently came in the center of attention, especially since it has been shown that nanoparticle based drug delivery systems facilitate uptake into hair follicles and act as drug reservoir (36, 37). In addition, the presence of immune cells bears the potential for trans-follicular vaccination (38). In contrast to excised human skin, porcine ear cartilage prevents the closure of hair follicles after excision allowing particle uptake close to the *in vivo* situation. However, it is important to know to which extent porcine and human hair follicles are comparable. Anatomically both comprise an invagination of epidermis, an inner and outer root sheath and sebaceous glands connected to the follicle (39). To compare the composition of human and porcine hair follicles on a chemically selective level, CRM provides the possibility of label-free localization and characterization of the main components (40). Special focus is required on the sebum's composition, since it might influence release behavior and depot sustainability of applied drug delivery systems (41).

Although porcine skin has proven beneficial for testing of follicular penetration, excised human skin is the most suitable model for *in vitro* penetration experiments (42). While previous studies utilized skin obtained from cadavers, human skin removed during plastic surgery is considered the gold-standard skin sample (42). To assure transferability of results obtained from excised human skin, most laboratories limit the usage to abdominal skin samples from healthy female volunteers. Nevertheless, skin remains a highly complex tissue with variable composition (43). Results of penetration studies obtained from different skin donors by tape stripping are known to exhibit high variability (44). The use of a standardized and automated analytical technique like CRM might prove beneficial regarding reduced result variability and increased transferability. However, it has been reported that recorded Raman spectra reflect the high variability of skin composition *in vivo* (45). To implement CRM in the field of penetration studies, the variability of the obtained spectra has to be evaluated towards

reliability and transferability of obtained results. Furthermore, it is necessary to differentiate between the disagreement of multiple measurements on one sample and the disagreement in spectral information obtained from different donors to comprehensively evaluate spectral variability. The proof of transferability of the obtained results is an essential prerequisite for drug detection and quantification inside the human skin samples *in vitro*.

The use of CRM to analyze substance penetration behavior is the most worthwhile and most demanding application for CRM in skin research. It has been shown that CRM is capable of following a substance in relation to other substances by relative spectral depth profiling. While Forster et al. focused on the tracking of cosmetic relevant compounds (46), and Broding et al. followed chemicals for risk assessment (47), others adapted the method for pharmaceutical research (47-50). The lab of Mendelsohn succeeded in monitoring the ratio between drug and prodrug during skin penetration (51, 52). However, to compete with state-of-the-art analytical techniques, quantification of the penetrating substance is vital. The fact, that the acquired Raman signal intensity is linear to the substance concentration, enables quantitative CRM measurements as previously shown (53). However, transferring this potential to the complex situation of measurements inside biological tissue is a challenging task. Drawbacks and pitfalls in depth profiling with confocal Raman spectroscopy have previously been summarized (54-56). Everall et al. highlight the uncertainty of depth determination during depth profiling. Mendelssohn et al. used layered polymer films to determine the depth uncertainty between 11-16% and Piot et al. evaluated various mathematical correction algorithms (57, 58). The remaining major drawback to implement quantitative depth profiling is Raman signal attenuation. With ongoing depth the Raman signal loses intensity due to absorption, reflection and scattering effects inside the sample. This loss of intensity inside a sample causes the loss of the linear correlation between signal intensity and substance concentration. The effect of signal attenuation on spectral measurements is illustrated in figure 3. Knowledge about rate and extent of the signal attenuation under certain conditions is essential to perform quantitative Raman measurements beneath the sample surface. So far, Raman signal attenuation has been circumvented by relating the peak of the substance of interest to a skin representing Raman peak. This method premises that both peaks are affected by the Raman signal attenuation to similar extend. However, this requires the substance from which the peak is derived, to be constant throughout all layers and depths of the skin sample. An ultimate proof-of-concept that valid quantitative measurements by CRM in skin are possible by peak relation is still missing. This includes a systematic study that comprises a validation of quantitative data acquired by CRM against a standard quantification method and thorough evaluation and selection of peaks suitable as internal standards.

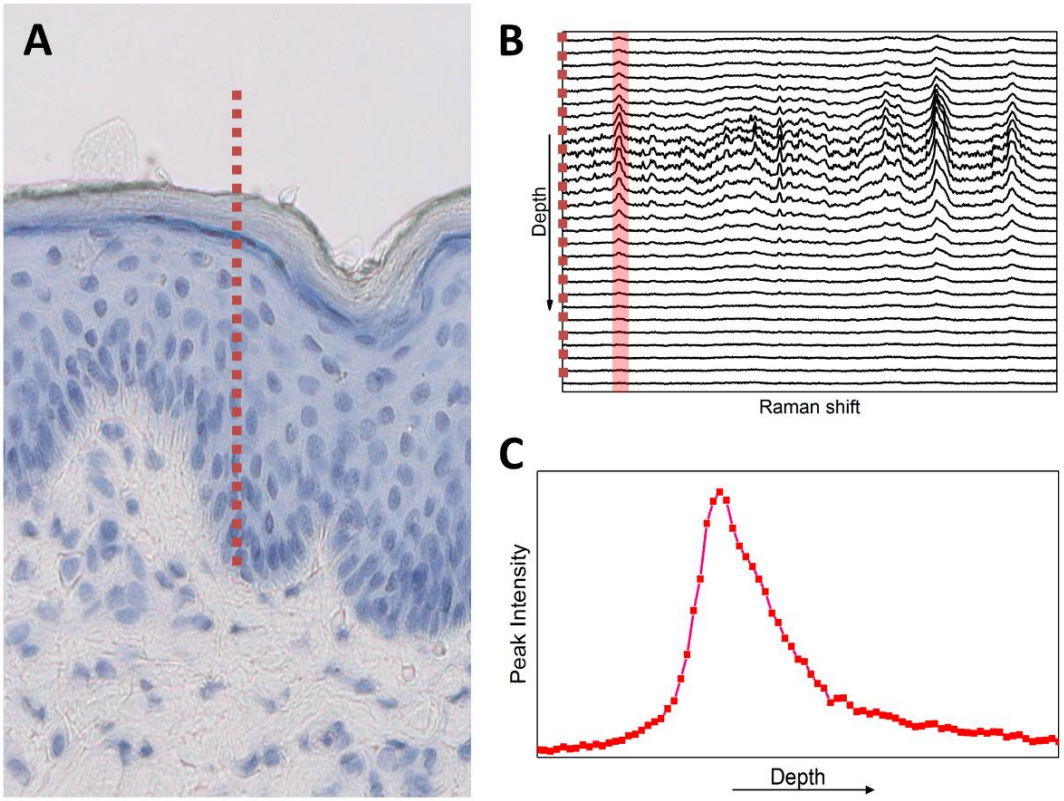


**Figure 3**: Schematic explanation of Raman signal attenuation. **A** Basic principle of depth profiling in skin. **B** Raman spectra recorded along the red line in A. **C** Intensity depth profile of a selected Raman peak highlighted in B. With ongoing depth, the Raman signal decays due to a reduced yield of collected photons. Reproduced with permission from (19).

Besides these major challenges the implementation of confocal Raman microscopic depth profiles is impeded by other drawbacks. Although automated, CRM measurements are time consuming and the sample is constantly irradiated by laser light. This gives way to further diffusion of the penetrated substance, microbial growth and sample movement caused by thermal changes and drying effects. These new challenges follow the recent developments of CRM in skin depth profiling. In order to establish CRM as novel analytical tool, new ways of sample preparation and preservation are necessary specifically addressing the aforementioned issues.

# 4 Aims

From the current state-of-the-art in skin research it becomes eminent that novel analytical strategies are needed to cope with the increasing requirements. Confocal Raman microscopy (CRM) already indicated its potential for versatile analytical implementation especially in skin drug delivery research. One of the most demanding and challenging applications in skin drug delivery research is the determination of substance concentration depth profiles. Obtaining information about rate and extent of substance penetration into skin is of major importance to evaluate and optimize drug delivery as well as to assess health risks of chemicals. Determining the concentration of the respective substance in a defined depth is currently performed by a labor-intensive sequence of removal and extraction of different skin layers. The ability of CRM to acquire chemical information from inside a sample bares the potential to localize and quantify a substance in skin. The advantages of utilizing CRM for substance depth profiling include a label-free, non-destructive and automated mode of operation. Different groups around the world already leaped forward and employed CRM in conventional analytical setups. However, to master the existing challenges rethinking of conventional approaches and redesigning of standard procedures is necessary. The overall goal of this thesis is to fully exploit the benefits of CRM in skin drug delivery research and establish CRM as standardized analytical method for quantitative drug depth profiling *in vitro*. To achieve this goal, we state four major aims:

I. To solve existing problems and eliminate uncertainties with the novel insights offered by CRM
II. To understand and overcome current limitations for substance depth profiling in skin
III. To establish CRM as tool for quantitative depth profiling
IV. To simplify and standardize CRM measurements

The first aim includes the reevaluation of existing approaches for skin penetration analysis. CRM can provide new insights in current methods and eliminate existing doubts. Especially sophisticated *in vitro* models still lack a proof of validity and CRM can vindicate their application.

The second aim focusses on CRM for substance depth profiling. Gathering detailed spectral information from inside a skin sample is impeded by numerous factors. Previous studies already identified Raman signal attenuation as major drawback. When light enters a non-transparent medium like skin, the signal intensity is reduced. In Raman spectroscopy, this reduced signal causes a loss of the linear correlation between signal intensity and substance concentration. Furthermore, the high

variability of *in vitro* skin samples in composition and penetration behavior is well known. A significant impact on Raman spectroscopic investigations is expected. To understand and rate the impact of such factors on CRM measurements is a mandatory prerequisite for further developments.

The third aim comprises the quantification of substances as exploitation of the full potential of CRM for substance depth profiling. The linear correlation between Raman signal intensity and substance concentration theoretically enables the acquisition of quantitative concentration depth profiles in skin. The understanding of aforementioned limitations should enable the implementation of quantitative measurement routines. The possibility to acquire concentration depth profiles contact-less and label-free in *in vitro* skin samples would be an important advancement of current analytics.

Finally, the fourth aim pursues the implementation of quantitative depth profiling in a novel simplified and standardizable analytical setup. It is expected that the usage of conventional preparation of human skin samples for CRM *in vitro* depth profiling introduces a number of unwanted drawbacks in sample behavior and measurement inaccuracies. To facilitate the implementation and to increase the transferability of results, customized approaches from sample preparation to data evaluation are needed.

# 5 Scientific Outcome

## 5.1 Advanced chemical imaging and comparison of human and porcine hair follicles for drug delivery by confocal Raman microscopy

This chapter refers to:

***L. Franzen****, *C. Mathes**, *S. Hansen*, *M. Windbergs*, 'Advanced chemical imaging and comparison of human and porcine hair follicles for drug delivery by confocal Raman microscopy', *J Biomed Opt*, 18 (2013) 61210
*both authors contributed equally to this work

As novel approach for drug administration, the transfollicular route has recently gained a lot of attention. The reported increase in penetration rate of nano-sized drug delivery systems and a potential reservoir formation led to numerous novel applications (36, 59, 60). As an invagination of the epidermis, a hair follicle provides high surface area with numerous blood vessels and antigen presenting cells in the adjoining tissue. This facilitates drug uptake und bares the potential for a triggered immune response (38). To develop new drug delivery systems tailored for the transfollicular route, a suitable *in vitro* model is necessary. Unfortunately, excised human skin, as gold-standard model for *in vitro* penetration and absorption, was found to be unfavorable for this task. A contraction of tensile fibers subsequent to the excision closes the follicle and impedes the uptake of an applied formulation. In contrast to human skin, the skin of a porcine ear is fixed on the cartilage, which prevents the follicle from closing. In combination with comparable results in penetration and absorption studies and a broad availability from abattoir refuse, without ethical concerns, porcine ear skin comprises the preferred *in vitro* model for follicular absorption studies (34). To quantify the extent of follicular penetration, cyanoacrylate skin surface stripping is performed. After application of superglue to a skin area, corneocytes, the stratum corneum and the follicular cast are stripped off (61).

With the introduction of this new analytical setup, the question of comparability of human and porcine follicles arises. Furthermore, a complete removal of the hair follicle by cyanoacrylate biopsies has to

be assured for reliable quantification. In this scientific gap, confocal Raman microscopy can prove its versatile values for skin drug delivery research due to its previously discussed features.

At first, we used Raman microscopy to analyze the chemical composition of a sample in a defined location. Despite an apparent anatomical similarity depicted in histological cross sections in figure 4 A and B, a chemical equivalence is important to mimic the simulated body side in humans. Especially the sebum as potential release medium for an applied drug delivery system needs to be considered. The targeted acquisition of Raman spectra from the four major components of a hair follicle allowed the comparison of the chemical composition of respective components. The spectra are presented in figure 4 C and D. A detailed analysis, including spectral subtraction revealed little to no difference in the Raman spectra of human and porcine, epidermis, dermis and hair. In sebum a slight discrepancy in the wavenumber range of 1050 $cm^{-1}$ and 1090 $cm^{-1}$ indicated conformational differences between the lipids in human and porcine sebum (62). Overall, these results prove a general comparability of human and porcine hair follicles and indicate the suitability of porcine ears as *in vitro* model.

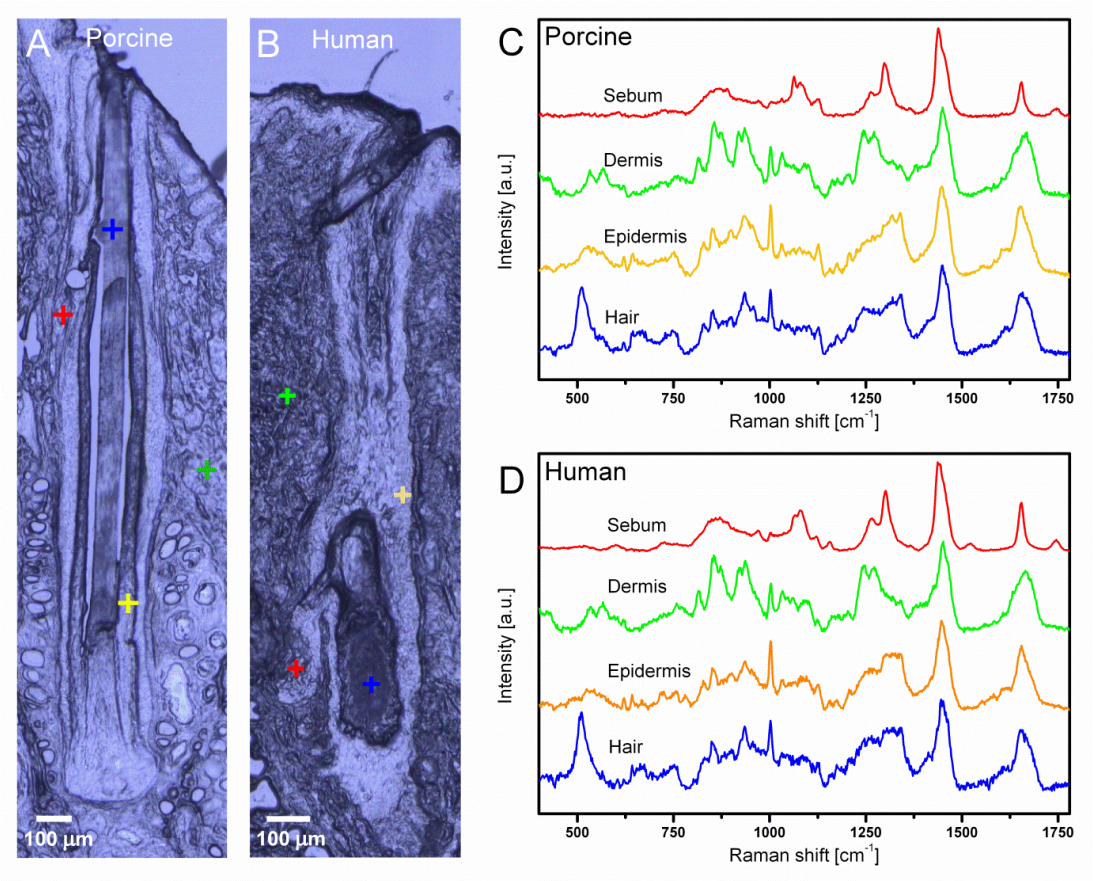


**Figure 4:** Microscopic pictures of porcine **A** and human **B** hair follicle cross sections. Raman spectra obtained from the areas marked by the colored crosses displayed in corresponding colors from porcine **C** and human **D** follicles. Reproduced with permission from (63).

Furthermore, we used Raman imaging to identify and localize the main components of a hair follicle in a histological cross section. Usually visualization of the different components is performed by

complex steps of different dyeing procedures. In contrast Raman microscopy classifies spectra by chemical similarity and delivers a false-color image depicting the different components in different colors. By combining CRM with an optical profilometer, we employed Raman imaging to visualize a follicular cast after removal by cyanoacrylate biopsy. Figure 5 D reveals an intact epidermis shell around the removed follicle. Therefore, a complete extraction of the formulation can be assumed and the suitability of differential stripping to quantify penetrated drug and delivery system is confirmed.

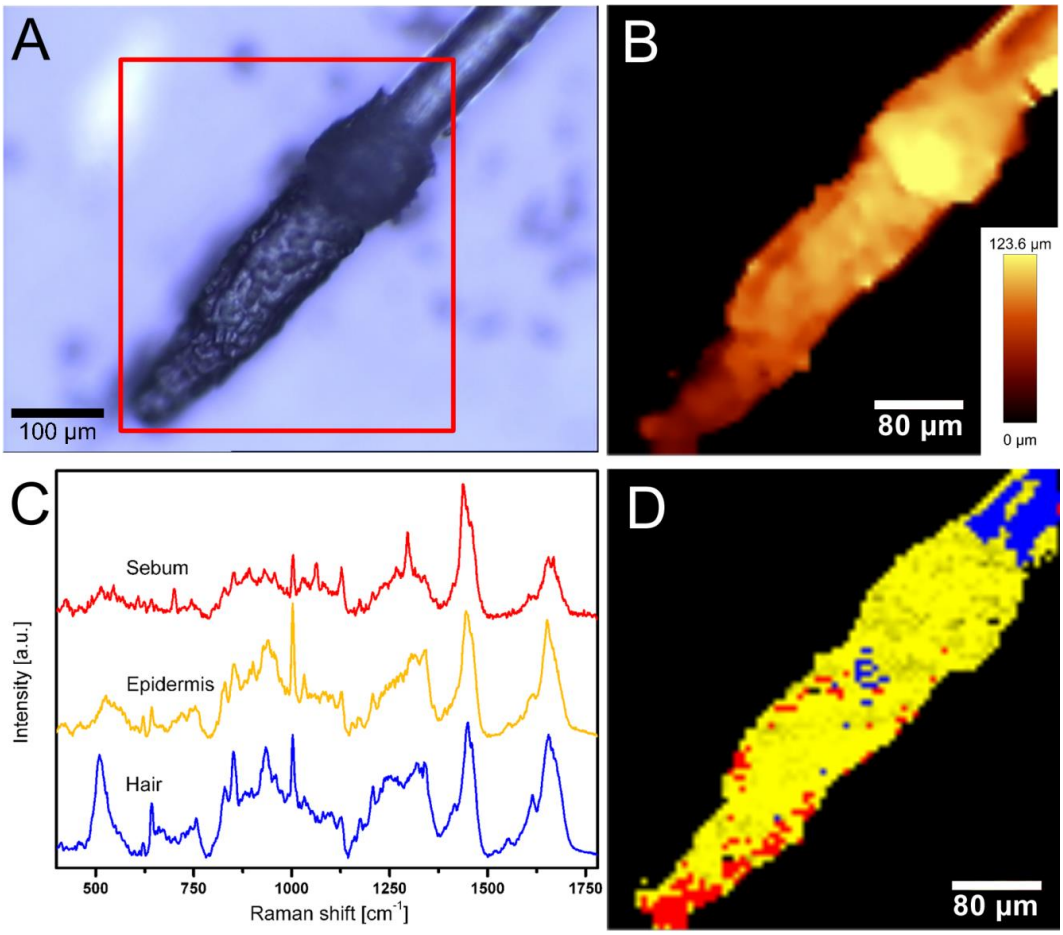


**Figure 5**: **A** Light microscopy picture of an excised hair follicle. **B** Surface topography map of the area indicated by the red rectangle in **A**. **C** Raman spectra of the individual chemical components in the hair follicle. **D** Raman map displaying the component distribution on the excised hair follicle. Reproduced with permission from (63).

In conclusion, CRM was able to provide valuable insights in an existing *in vitro* model. By utilizing CRM remaining doubts were eliminated and the analytical course of action was approved. This versatile and complex application demonstrates the potential of CRM in skin drug delivery research.

## 5.2 Towards drug quantification in human skin with confocal Raman microscopy

This chapter refers to:

***L. Franzen,*** *D. Selzer*, *J.W. Fluhr*, *U.F. Schaefer*, *M. Windbergs*, 'Towards drug quantification in human skin with confocal Raman microscopy', *Eur J Pharm Biopharm*, 84 (2013) 437-444

Not only in skin drug delivery research but also for risk assessment of chemicals, the knowledge of rate and extent of substance penetration is highly important. However, the acquisition of substance depth profiles is one of the most challenging analytical procedures in skin research. In this context CRM is considered a promising option to advance analytics beyond the labor-intensive and destructive state-of-the-art. CRM has already been applied for first studies tackling penetration analysis in skin (28, 48, 49, 52). However, all previous studies limit themselves to relative depth profiling by comparing the penetration depth of two components. The lack of quantitative studies originates from Raman signal attenuation as main drawback. Upon penetration into the skin the exciting light is weakened by reflectance, absorbance, diffraction and other optical effects. This causes a decrease in scattered photons necessary for detection of the Raman effect. This imponderable attenuation of the signal leads to a loss of the linear correlation between signal intensity and substance concentration. In this chapter we present a first investigation focusing on Raman signal attenuation. Aim of this study was to gain insight knowledge and derive methods to circumvent Raman signal attenuation.

To deeply understand Raman signal attenuation, we developed a skin surrogate comprising the main components of human skin, similar in optical properties but homogenous in composition. In this homogeneous skin surrogate, we included keratin as main protein and a mixture of fatty acids, triglycerides and cholesterol. After assuring similarity regarding spectral properties and refractive index, we acquired Raman depth profiles inside the surrogate. By absence of concentration gradients, the homogeneous mixture assures, that the decay in Raman signal is solely caused by signal attenuation. The measured Raman signal attenuation was fitted mathematically and exposed to be approximated by an exponential function. Figure 6 exhibits the mean Raman intensity depth profile from the skin surrogate and its mathematical fit as described in equation 1.

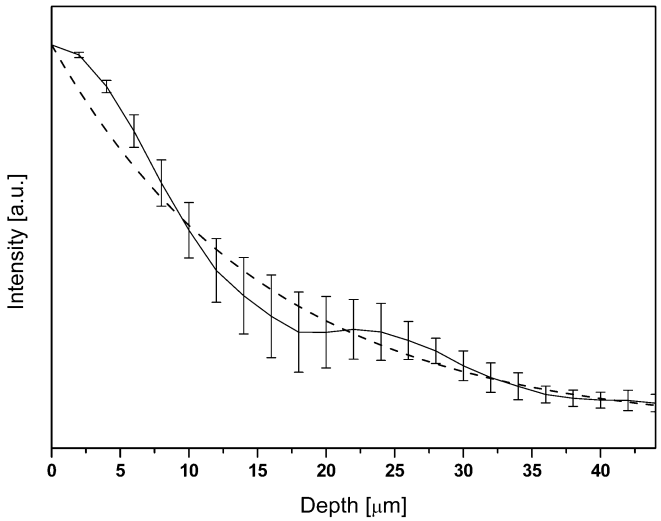


**Figure 6**: Raman mean drug intensity depth profile based on three skin surrogates (solid line mean ± SD, n = 3) and fitting function (dashed line). Reproduced with permission from (64).

$$D(x) = 0.9997 * \exp\left(-\frac{\mathrm{x}}{15.7581}\right) + (1 - 0.9997)$$

Equation 1

This first mathematical description of Raman signal attenuation in our surrogate was confirmed by reevaluation of measurements made in excised human skin. The mathematical description of the signal attenuation was translated into a re-attenuation algorithm, which succeeded in eliminating the Raman signal attenuation from Raman depth profiles acquired in human skin. The original intensity depth profiles and the corrected profiles of a model drug in two human skin samples are depicted in figure 7 A and B.

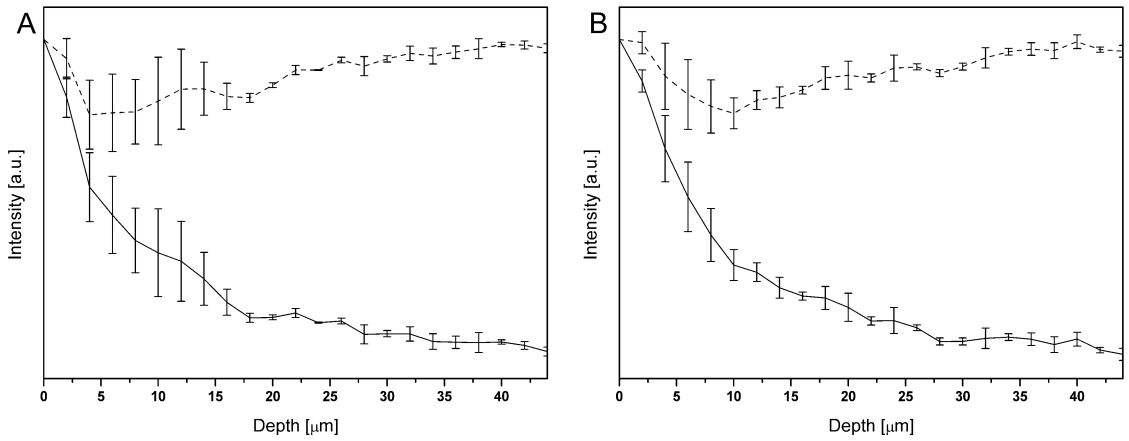


**Figure 7: A** and **B** Raman mean intensity depth profile for caffeine in two skin donors (solid line, mean ± SD, n = 3) and corrected profile (dashed line, mean ± SD, n = 3). Reproduced with permission from (64).

To conclude, this study offers deeper understanding of the effect of Raman signal attenuation. In addition, we offer a mathematical way to cope with signal attenuation, serving as a first step to overcome this major drawback.

## 5.3 Accessing Raman spectral variability in human skin for quantitative depth profiling

This chapter refers to:

***L. Franzen,*** *M. Windbergs*, 'Accessing Raman spectral variability in human skin for quantitative depth profiling', *J Raman Spectrosc*, 45 (2014) 82-88

Besides the optical issue of Raman signal attenuation, the skin as target tissue exhibits some unique challenges. Due to ethical concerns and economic considerations *in vivo* screening of substance penetration is not suitable for many tasks. Hence, elaborate *in vitro* models are necessary for rational development of skin drug delivery systems. Excised human skin from plastic surgery presents the favored gold-standard model for *in vitro* penetration testing (42). However, excised human skin as primary biological tissue is known to exhibit a high variability in composition and structure (43) reflected in a complex Raman spectrum (45). The implementation of CRM as analytical tool for *in vitro* depth profiling in excised human skin might be affected by these variabilities and the obtained results could reflect the deviation. This can ultimately lead to a lack of comparability and transferability of stated findings obtained from different skin sources. Furthermore, one of the most common approaches to overcome Raman signal attenuation is to relate the Raman peak of the substance under investigation to a Raman peak derived from a skin component. This requires a certain Raman signal to be constant throughout all skin layers with a minimum of variation. Therefore, we discuss in this chapter the results of a systematic study targeting the variability of Raman spectra obtained from multiple skin donors.

By detailed investigation of an averaged Raman spectrum of human stratum corneum, we identified four major spectral bands representing four major skin components as displayed in figure 8. First, marked in green, the C-C stretch vibrations represent the ring breathing of the aromatic amino acids phenylalanine and tyrosine, detectable in the wavenumber range 995-1018 $cm^{-1}$. Second, the $CH_2$ deformation, mainly originating from alkyl chains in triglycerides and free fatty acids incorporates skin lipids. The wavenumber region for peak investigation was selected as 1288-1314 $cm^{-1}$ and is highlighted in blue. As third peak, assigned in orange, the unspecific and therefore most intense C-H deformation in the wavenumber range 1388-1497 $cm^{-1}$ was evaluated. C-H deformations can be found in lipids as well as in proteins throughout the skin. Fourth is the magenta labeled N-C=O stretch

vibration embodying amid I as found in proteins. This protein representing peak was defined as the wavenumber range of 1559-1721 $cm^{-1}$. The above mentioned peaks are representative for the major skin components, the selected wavenumber areas were used for detailed investigation of Raman spectral variability of human skin. For potential quantification the relation of peak intensity and substance amount is of uttermost importance, therefore we focused our investigations on the integrated intensity of the selected wavenumber ranges.

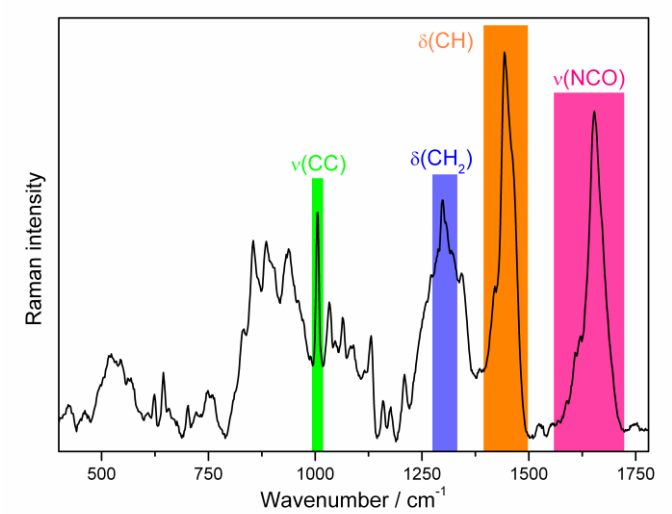


**Figure 8:** Raman spectrum of untreated human stratum corneum. Average of 27 spectra from three donors. The marked regions represent the four major peaks selected for further analysis. green: aromatic amino acids, blue: lipids, orange: lipids and proteins, magenta: proteins. Reproduced with permission from (65).

One of the biggest concerns regarding the variability of Raman spectra of human skin includes the transferability of results obtained using skin from different donors. Due to limited amounts from one individual, often skin from multiple donors is utilized for a single study. By acquiring Raman spectra from different skin samples from different skin donors, we assessed the intra- and interindividual variability of the selected Raman peaks. The average Raman spectrum of untreated human stratum corneum is plotted in figure 9 A (black line). The symbols visualize the calculated mean peak intensities +/- standard deviation (SD) of each donor. We observed high variability for each individual donor, but no statistically significant difference between the three donors was detected. Furthermore, the intraindividual variability was assessed by evaluation of differences between individual samples obtained from the same donor. In figure 9 B, the symbols visualize the calculated mean peak intensities +/- SD of three samples from donor 1. We observed similar variability, but no significant differences between the samples could be noted. Our observations confirm the expected high

variability in Raman spectra, but a similar variability between different donors. Thus transferability of findings stated by utilizing skin from different individuals is assured.

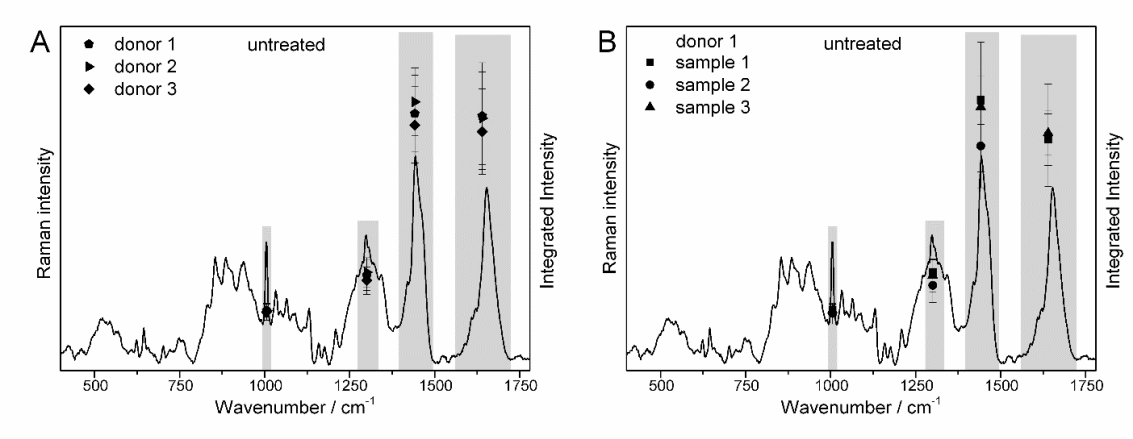


**Figure 9: A** Inter- and **B** intraindividual variability of untreated human SC. The symbols represent the marked peak area integral +/- SD. **A** for three different donors (n=9) **B** for three different samples of donor 1 (n=3). No significant differences between samples or donors could be detected. Reproduced with permission from (65).

Furthermore, we monitored the variability of the selected peaks upon depth profiling. All peaks revealed an increase in relative standard deviation in deeper skin layers. However, the lipid derived peak in the spectral range from 1288 $cm^{-1}$ to 1314 $cm^{-1}$ exhibited abnormal behavior in a plotted intensity depth profile. A steeper decay of the signal intensity compared to peaks of other components indicated a concentration gradient of skin lipids. This excluded this specific peak from being suitable as internal standard, since a constant signal throughout the depth profile is not given.

Overall we were able to gain deep understanding of spectral variability of CRM measurements during depth profiling in human skin. We confirmed excised human skin as suitable *in vitro* model and assured transferability of results obtained from different donors. Furthermore, our extended knowledge in skin depth profiling allowed us to eliminate a candidate peak as potential reference for quantitative depth profiling. This proceeds the understanding and overcoming of pitfalls, impeding quantitative depth profiling in human skin by CRM.

## 5.4 Quantitative detection of caffeine in human skin by confocal Raman spectroscopy – a systematic *in vitro* validation study

This chapter refers to:

***L. Franzen,*** *J. Anderski*, *M. Windbergs*, 'Quantitative detection of caffeine in human skin by confocal Raman spectroscopy – a systematic *in vitro* validation study ', *Eur J Pharm Biopharm*, 95, Part A (2015) 110-116

After elucidating the current limitations for quantitative Raman depth profiling in human skin, the newly found knowledge had to be translated into solutions to overcome them. As mentioned earlier, the most common way to cope with Raman signal attenuation is the relation of two peaks, resulting in relative depth profiles. The intensity of a Raman peak representing the penetrating substance is related to a peak representing a skin component. Assuming that both peaks are equally affected by signal attenuation, the result is a profile of how deep the substance penetrated into the skin. This peak correlation method requires a constant and reliable skin component representing peak as internal standard. The objective of this chapter was to validate the approach of peak correlation regarding its transferability to quantitative measurements.

At first, the capability of our instrument to perform quantitative measurements was assured by acquisition of spectra in drug solutions of different concentration. A prominent drug representing peak was integrated and Raman intensity proved to be linearly related to drug concentration. Furthermore, we developed a sample incubation procedure that provided a homogeneous drug concentration throughout the whole skin sample. Hence, we expect a constant peak ratio independent of depth for each drug concentration. This assures a valid reference to be correlated with our internal skin peak.

Since the lipid representing peak was already ruled out as internal standard in the previous chapter (65), the three aforementioned skin representing peaks were evaluated in terms of suitability as internal reference. The C-C stretch vibration represents the ring breathing of aromatic amino acids (AAA), detectable in the wavenumber range of 995-1018 $cm^{-1}$. The unspecific and therefore most intense C-H deformation is represented in the wavenumber range 1388-1497 $cm^{-1}$ and the N-C=O stretch vibration embodying amid I, as found in proteins, is detectable in a wavenumber range of 1559-1721 $cm^{-1}$ (18).

We calculated the ratios of the integrated peak intensities of drug and the respective internal skin peaks in different sample depths. However, the peak ratios derived from AAA exhibit high divergence

even in the upper layers as depicted in figure 10 B. The peak ratios derived from amid I bindings show a similar divergence after a depth of 7.5 µm is reached, as illustrated in figure 10 C. Due to high error margins and the high variability in deeper tissue layers, both skin peaks cannot provide the expected results. These findings impede the utilization of AAA and the amid I vibration as endogenous internal standard.

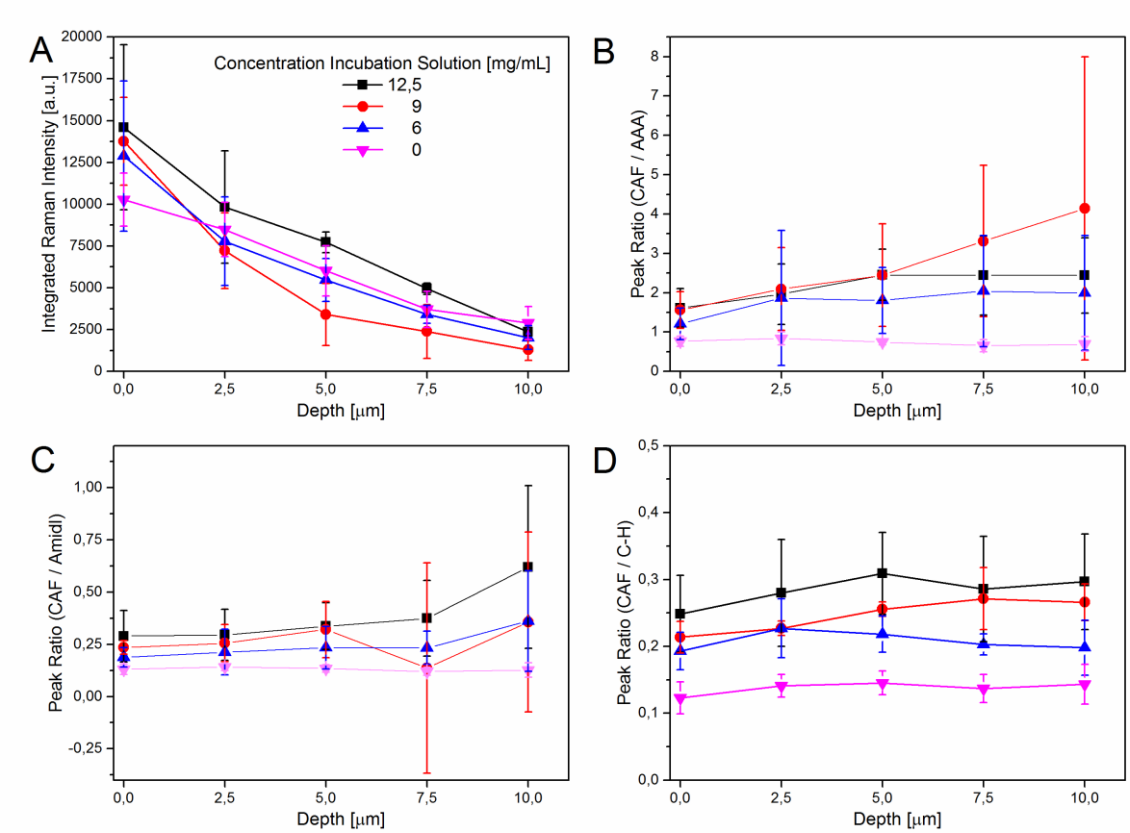


**Figure 10: A** Raman signal intensity of the caffeine representing peak as a function of depth for four different caffeine concentrations. **B, C, D** Ratios of the skin derived peak intensities of **B** aromatic amino acids (AAA), **C** amid I, **D** the unspecific C-H vibration and the caffeine representing peak intensity as a function of depth for four different caffeine concentrations (mean ± SD, n = 9). Reproduced with permission from (66).

Fortunately, a correlation of the drug derived peak with the Raman peak representing the unspecific C-H deformation features constant values as a function of depth. A horizontal curve progression visualizes the constant peak ratio independent from depth for all drug concentrations in figure 10 D. To further exploit the C-H / CAF relation we averaged the obtained peak ratios over depth for each drug concentration. After correlating the results with the drug concentration in skin, we achieved an excellent accordance of the applied linear fit, as depicted in figure 11. The derived correlation allows for converting Raman intensities obtained from skin depth profiling into absolute drug concentration.

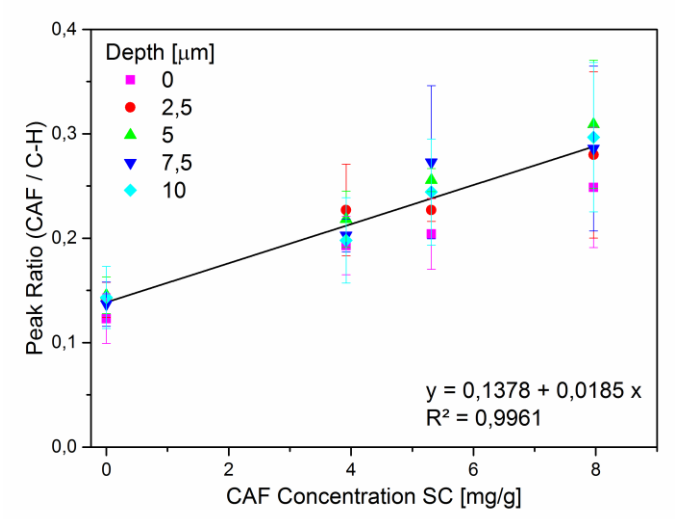


**Figure 11:** Ratio of the C-H and caffeine representing peak intensity determined by CRM as a function of caffeine concentration per mass stratum corneum determined by HPLC for five different depths with applied linear fit (mean ± SD, n = 9). Reproduced with permission from (66).

To conclude this chapter, we finally present a proof-of-concept study that translates the peak correlation method to a quantitative measurement setup. We were able to directly relate Raman data with actual drug concentrations in human skin *in vitro*. Based on our previous work this concludes a series of investigations focusing on understanding and overcoming the main drawbacks of Raman depth profiling. Furthermore, this study serves as another important step towards reliable and reproducible quantitative measurements by CRM in human skin *in vitro*.

## 5.5 Freeze-drying as tailored preparation technique for human skin depth profiling by confocal Raman microscopy

This chapter refers to:

***L. Franzen,*** *L. Vidlarova*, *K.H. Kostka*, *U.F. Schaefer*, *M. Windbergs*, 'Freeze-drying as a preserving preparation technique for in vitro testing of human skin', *Exp Dermatol*, 22 (2013) 54-56

***L. Franzen***, *J. Anderski*, *V. Planz*, *K.H. Kostka*, *M. Windbergs*, 'Combining confocal Raman microscopy and freeze-drying for quantification of substance penetration into human skin', *Exp Dermatol*, 23 (2014) 942-944

To ultimately implement CRM as analytical tool in skin drug delivery research, a reliable and standardized setup is necessary. In the previous chapters, we already discussed major pitfalls impeding quantitative depth profiling by CRM in human skin *in vitro*. However, the day-to-day measurement routine bares some challenges itself. The combination of conventional sample preparation and the advanced analysis by CRM gives way to avoidable uncertainties. During the time-consuming acquisition of depth profiles, the diffusion of the penetrated substance continues, introducing inaccuracies in localization and quantification. Furthermore, the constant exposure to laser radiation and the need to operate at air interface, cause drying effects in the excised skin samples. This slow drying process can provoke drug crystal growth on the sample surface and leads to a shrinking of the sample, which impedes the determination of measurement depth. To conclude, a standardized and simplified application of CRM demands a tailored preparation of the *in vitro* skin samples. In this chapter, we present freeze-drying as preserving preparation technique for human skin and its application in quantitative CRM depth profiling.

By freeze-drying an *in vitro* skin sample after incubation, we transform the skin into a solid matrix. This 'mummification' stops ongoing diffusion, and prevents further drying effects. Based on a comprehensive comparison by thermal and spectroscopic analysis, we excluded significant changes in structure and composition after freeze-drying. The results of comparing fresh and freeze-dried stratum corneum by differential scanning calorimetry (DSC), IR and Raman spectroscopy are depicted in figure 12. Moreover, we ruled out the occurrence of drug crystals on the sample surface. This comparison confirms the suitability of freeze-drying as preserving preparation technique.

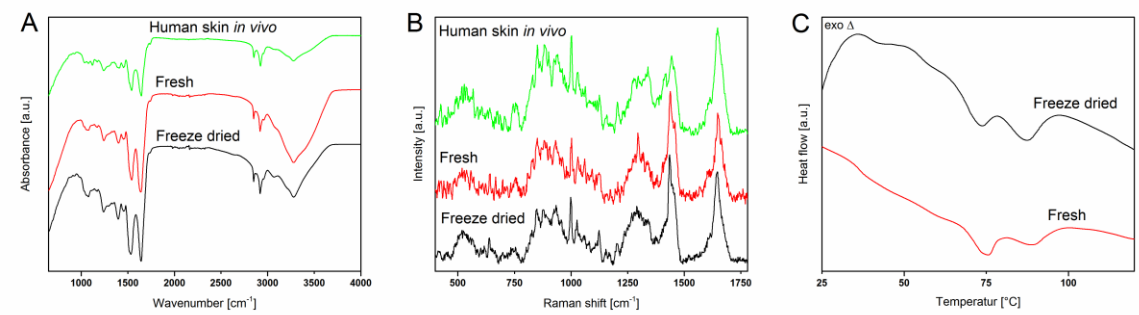


**Figure 12: A** DSC thermograms, **B** Raman spectra and **C** IR spectra of stratum corneum before and after freeze-drying compared to human skin *in vivo*. Modified with permission from (67).

In addition, we evaluated freeze-dried skin samples towards their capability to facilitate quantitative depth profiling by CRM. After loading skin samples with a defined amount of drug, the drug concentration in fresh and freeze-dried samples was determined by HPLC. By a simple correction for the weight loss, we were able to determine the original drug concentration in the skin samples prior to freeze-drying. The drug concentration in the skin samples as a function of the applied drug amount is presented in figure 13 A before and after correction for the weight loss. After acquisition of CRM depth profiles, we applied the peak correlation method presented in the previous chapter. Again, we were able to establish a linear correlation between Raman signal intensity and drug concentration independent from measurement depth, as exhibited in figure 13 B.

To sum up, freeze-drying of the skin samples after incubation allowed for stopping ongoing diffusion, preventing crystal growth and unwanted drying effects. Moreover, we maintain the capability of quantitative detection of substances by CRM in freeze-dried samples. This combination of tailored sample preparation and advanced data evaluation introduces the necessary simplification and standardization for CRM depth profiling.

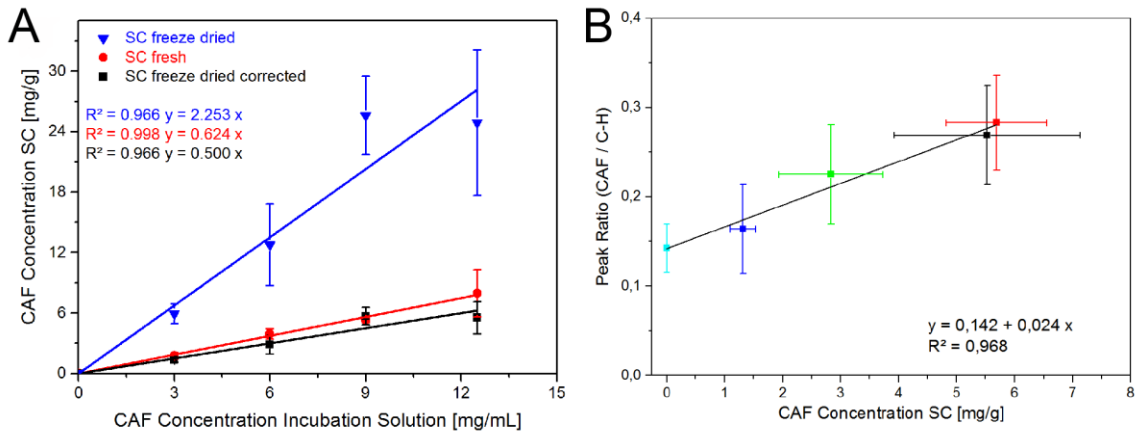


**Figure 13: A** Concentration of drug in skin determined by HPLC as a function of drug concentration in the incubation solution. The drug amount in the freeze- dried skin (blue) was corrected by the weight loss (black) to represent the drug amount in fresh skin (red) with applied linear fit (mean ± SD, n = 9). **B** Ratio of the unspecific C-H and drug representing Raman peak intensity as a function of drug concentration per mass stratum corneum with applied linear fit (mean ± SD, n = 9). Modified with permission from (68).

# 6 Conclusion

Confocal Raman microscopy is an upcoming analytical method in many fields of research. The ability for contactless detection and visualization of the chemical sample composition without addition of dyes or labels makes CRM a valuable analytical tool. In the course of this thesis, we investigated and evaluated different applications of CRM in skin drug delivery research ranging from physiological investigations to quantitative depth profiling. We highlighted benefits, exposed limitations and overcame drawbacks of confocal Raman microscopy for *in vitro* analytical tasks related to skin drug delivery.

In the first featured paper, we used confocal Raman imaging to reevaluate the suitability of porcine ear skin as *in vitro* model for analysis of follicular uptake. This study utilizes CRM to gain new valuable insights of an established *in vitro* model and to remove existing doubts. It serves as an example for the versatile benefits of an application of CRM in skin drug delivery research. This matches with our first initially stated aim.

Not only for risk assessment of chemicals, but especially for skin drug delivery research, the knowledge about rate and extent, with which a substance overcomes the skin barrier is essential. First studies already showed the potential of CRM to become a valuable tool for substance follow-up *in vitro*. However, to acquire quantitative information, a linear dependency between signal intensity and substance quantity has to be assured during the entire depth profiling process. Raman signal attenuation as main drawback reduces the signal intensity inside the skin, causing a loss of the linear correlation between Raman signal and substance amount. This issue was addressed in the second paper presented in this work. We created an artificial skin surrogate with homogeneous composition of the main skin components and were able to assign a mathematical function to the extent of signal attenuation. This sophisticated study serves as a first approach to understand and characterize Raman signal attenuation. The newly gained knowledge helps to better understand the effect of Raman signal attenuation as major drawback in depth profiling and facilitates methods to circumvent it.

In the third featured paper we addressed arising uncertainties in Raman spectroscopic measurements in skin and reviewed the usage of excised female human abdominal skin as standard *in vitro* model. We exposed the expected high but tolerable variability but results gathered from different donors varied to a similar extend. This demonstrates that results obtained from different donors are directly comparable and a general transferability of the acquired data is given. By investigating the variability of excised skin samples, we resolve another major uncertainty in CRM depth profiling, as intended in our second aim.

The possibility to quantify substance concentration in skin over depth was a missing key feature of CRM. Although the potential of CRM for this purpose has been broadly acknowledged, a systematic validation study as ultimate proof remained undone. Referencing our extended knowledge from the previous works, the fourth presented paper envisaged a proof-of-concept for quantitative depth profiling in human skin *in vitro*, as predetermined in our third aim.

Numerous previous studies circumvented Raman signal attenuation by relating the drug representing peak to a peak representing a skin component. This requires a skin component, which gives a strong Raman signal and is equally represented throughout the skin. In the fourth study we approached quantitative depth profiling directly by systematically validating multiple skin representing Raman peaks towards their suitability as internal standard. This study finally provides a suitable and reliable reference peak to broadly establish depth profiling. Furthermore, our systematic proof-of-concept study pioneers quantitative measurements in skin by CRM and ultimately overcomes the drawback of Raman signal attenuation.

The novel application of CRM for *in vitro* depth profiling in human skin also comes with novel demands on sample preparation and handling. The time consuming CRM measurements with steady laser irradiation afflict the excised human skin and new particular drawbacks arise. During *in vitro* testing these drawbacks like ongoing drug diffusion and changes in the hydration state impede the analysis of human skin samples. Meeting our fourth aim, we engaged these drawbacks by establishing a tailor-made sample preparation method adapted for CRM depth profiling in the featured papers five and six. By freeze-drying the skin samples immediately after the penetration experiment and prior to the analysis, we conserve the substance localization inside the skin. We ultimately combined freeze-drying as tailored sample preparation technique for CRM and quantitative depth profiling. This study finally allows the acquisition of quantitative depths profiles without being pressed by time. In combination with the previous studies, this work promotes a standardized analytical procedure for substance depth profiling.

In conclusion, we present a comprehensive work addressing the versatile applications of confocal Raman microscopy in skin drug delivery research. We present new applications like chemically selective 3D imaging of hair follicles and overcome existing limitations like signal attenuation. We introduce quantitative depth profiling and offer the combination with freeze-dried skin as further development, towards a standardized analytical technique. Overall, this thesis facilitates the basic understanding of numerous influencing factors and serves as a first step to establish CRM as analytical technique in skin drug delivery research.

# 7 Outlook

For the near future the possibility of label-free and non-destructive acquisition of concentration depth profiles is the most tempting feature of CRM. The progress we made and the hurdles we overcame regarding *in vitro* depth profiling paved the way for future applications. In particular, we present the first proof-of-concept for acquisition of quantitative penetration depth profiles and provide an analytical model tailor-made for CRM. With this tools CRM can be utilized for quantitative follow-up of substances into the skin *in vitro*, an essential analytical procedure in risk assessment and drug delivery research. In a next step the simultaneous tracking of drug and excipient of drug delivery systems will become possible. Thus, CRM can not only support researchers in evaluating and optimizing drug delivery systems, but also satisfy the increasing demands in pharmacovigilance of regulatory agencies like Food and Drug Administration (FDA) and European Medicines Agency (EMA). However, further improvements are needed to establish confocal Raman microscopy as standard analytical technique in drug delivery laboratories. The simplification of quantitative measurement routines and further development of instrumental designs specialized for the desired application are necessary. With dedicated software and hardware solutions a variety of affordable and easy-to-handle Raman systems is conceivable. Specialized instruments for analysis of skin composition (RiverD®, Rotterdam, NL) and skin disease diagnosis (Verisante Aura®, Richmond, BC, CA) are already arriving on the market. A Raman apparatus devoted to quantitative *in vitro* substance follow-up is highly anticipated and the presented results might provide key solutions for instrument developers.

# 8 Featured Papers

8.1 **L. Franzen***, C. Mathes*, S. Hansen, M. Windbergs, 'Advanced chemical imaging and comparison of human and porcine hair follicles for drug delivery by confocal Raman microscopy', *J Biomed Opt*, 18 (2013) 61210; *both authors contributed equally to this work

8.2 **L. Franzen**, D. Selzer, J.W. Fluhr, U.F. Schaefer, M. Windbergs, 'Towards drug quantification in human skin with confocal Raman microscopy', *Eur J Pharm Biopharm*, 84 (2013) 437-444

8.3 **L. Franzen**, M. Windbergs, 'Accessing Raman spectral variability in human skin for quantitative depth profiling', *J Raman Spectrosc*, 45 (2014) 82-88

8.4 **L. Franzen**, J. Anderski, M. Windbergs, 'Quantitative detection of caffeine in human skin by confocal Raman spectroscopy – a systematic in vitro validation study ', *Eur J Pharm Biopharm*, 95, Part A (2015) 110-116

8.5 **L. Franzen**, L. Vidlarova, K.H. Kostka, U.F. Schaefer, M. Windbergs, 'Freeze-drying as a preserving preparation technique for *in vitro* testing of human skin', *Exp Dermatol*, 22 (2013) 54-56

8.6 **L. Franzen**, J. Anderski, V. Planz, K.H. Kostka, M. Windbergs, 'Combining confocal Raman microscopy and freeze-drying for quantification of substance penetration into human skin', *Exp Dermatol*, 23 (2014) 942-944

## 8.1 Advanced chemical imaging and comparison of human and porcine hair follicles for drug delivery by confocal Raman microscopy

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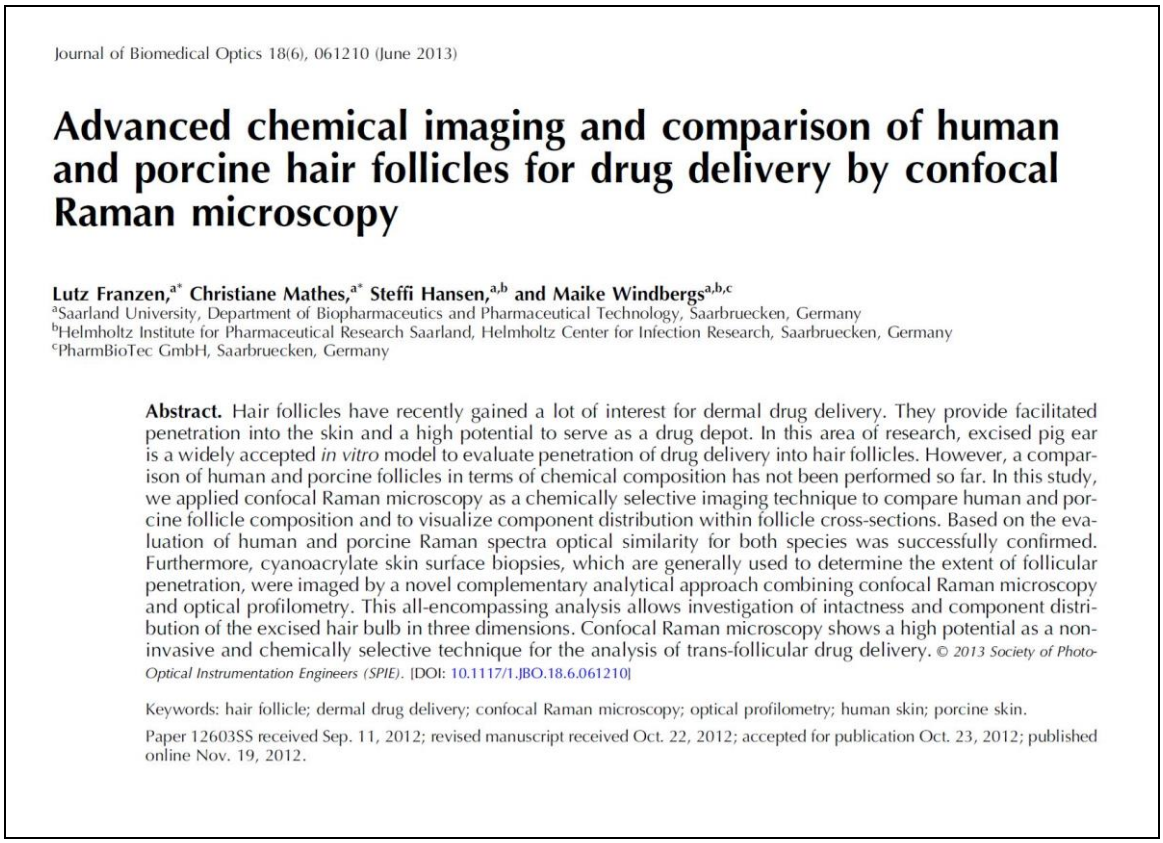

Journal of Biomedical Optics 18(6), 061210 (June 2013)

# Advanced chemical imaging and comparison of human and porcine hair follicles for drug delivery by confocal Raman microscopy

**Lutz Franzen,[a*] Christiane Mathes,[a*] Steffi Hansen,[a,b] and Maike Windbergs[a,b,c]**
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**Abstract.** Hair follicles have recently gained a lot of interest for dermal drug delivery. They provide facilitated penetration into the skin and a high potential to serve as a drug depot. In this area of research, excised pig ear is a widely accepted *in vitro* model to evaluate penetration of drug delivery into hair follicles. However, a comparison of human and porcine follicles in terms of chemical composition has not been performed so far. In this study, we applied confocal Raman microscopy as a chemically selective imaging technique to compare human and porcine follicle composition and to visualize component distribution within follicle cross-sections. Based on the evaluation of human and porcine Raman spectra optical similarity for both species was successfully confirmed. Furthermore, cyanoacrylate skin surface biopsies, which are generally used to determine the extent of follicular penetration, were imaged by a novel complementary analytical approach combining confocal Raman microscopy and optical profilometry. This all-encompassing analysis allows investigation of intactness and component distribution of the excised hair bulb in three dimensions. Confocal Raman microscopy shows a high potential as a non-invasive and chemically selective technique for the analysis of trans-follicular drug delivery.  [DOI: 10.1117/1.JBO.18.6.061210]

Keywords: hair follicle; dermal drug delivery; confocal Raman microscopy; optical profilometry; human skin; porcine skin.

Paper 12603SS received Sep. 11, 2012; revised manuscript received Oct. 22, 2012; accepted for publication Oct. 23, 2012; published online Nov. 19, 2012.

For full text please refer to the following link:

http://biomedicaloptics.spiedigitallibrary.org/article.aspx?articleid=1392641

## 8.2 Towards drug quantification in human skin with confocal Raman microscopy

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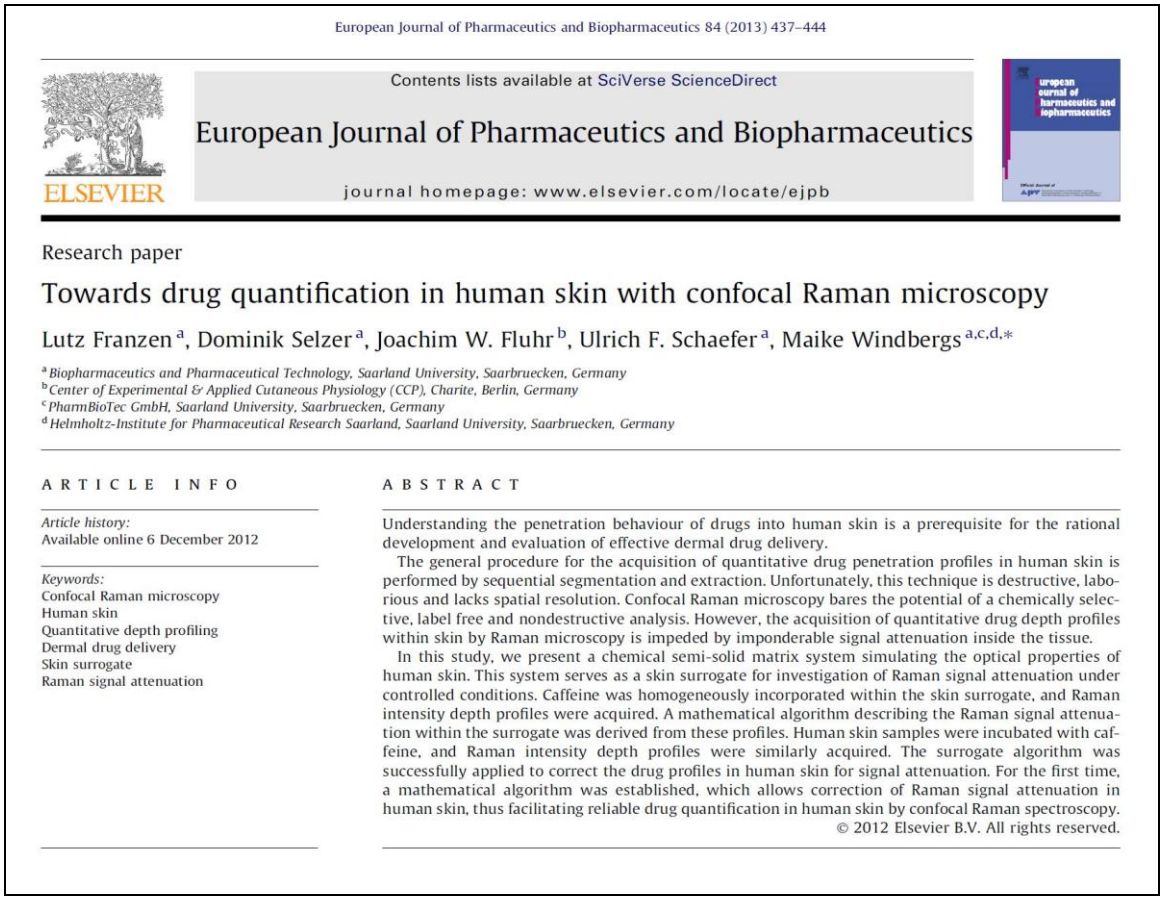

European Journal of Pharmaceutics and Biopharmaceutics 84 (2013) 437–444

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Research paper

Towards drug quantification in human skin with confocal Raman microscopy

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ABSTRACT

Understanding the penetration behaviour of drugs into human skin is a prerequisite for the rational development and evaluation of effective dermal drug delivery.

The general procedure for the acquisition of quantitative drug penetration profiles in human skin is performed by sequential segmentation and extraction. Unfortunately, this technique is destructive, laborious and lacks spatial resolution. Confocal Raman microscopy bares the potential of a chemically selective, label free and nondestructive analysis. However, the acquisition of quantitative drug depth profiles within skin by Raman microscopy is impeded by imponderable signal attenuation inside the tissue.

In this study, we present a chemical semi-solid matrix system simulating the optical properties of human skin. This system serves as a skin surrogate for investigation of Raman signal attenuation under controlled conditions. Caffeine was homogeneously incorporated within the skin surrogate, and Raman intensity depth profiles were acquired. A mathematical algorithm describing the Raman signal attenuation within the surrogate was derived from these profiles. Human skin samples were incubated with caffeine, and Raman intensity depth profiles were similarly acquired. The surrogate algorithm was successfully applied to correct the drug profiles in human skin for signal attenuation. For the first time, a mathematical algorithm was established, which allows correction of Raman signal attenuation in human skin, thus facilitating reliable drug quantification in human skin by confocal Raman spectroscopy.



For full text please refer to the following link:

http://www.sciencedirect.com/science/article/pii/S0939641112003839

## 8.3 Accessing Raman spectral variability in human stratum corneum for quantitative *in vitro* depth profiling

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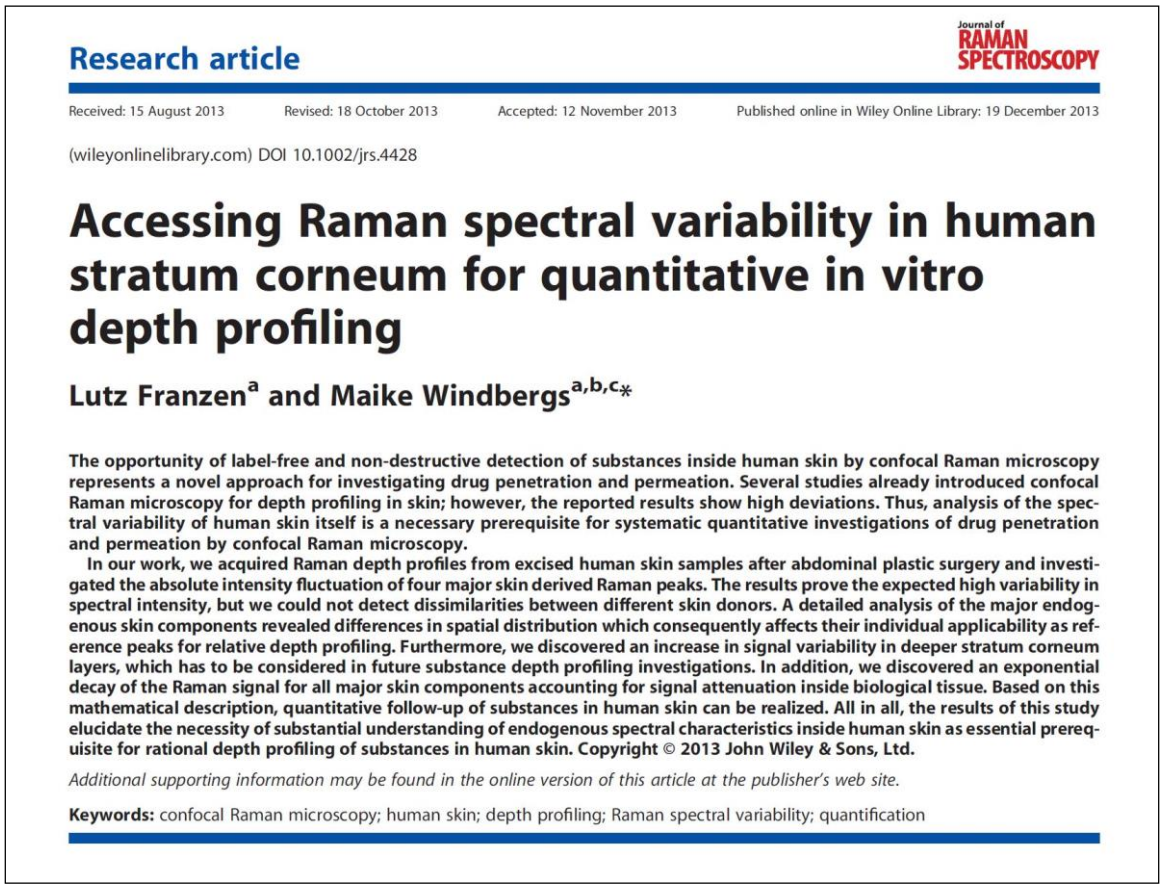
[2] Helmholtz Centre for Infection Research, Helmholtz Institute for Pharmaceutical Research Saarland, Department of Drug Delivery, Saarbruecken, Germany
[3] PharmBioTec GmbH, Saarbruecken, Germany



Research article

Journal of RAMAN SPECTROSCOPY

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(wileyonlinelibrary.com) DOI 10.1002/jrs.4428

# Accessing Raman spectral variability in human stratum corneum for quantitative in vitro depth profiling

**Lutz Franzen[a] and Maike Windbergs[a,b,c]***

**The opportunity of label-free and non-destructive detection of substances inside human skin by confocal Raman microscopy represents a novel approach for investigating drug penetration and permeation. Several studies already introduced confocal Raman microscopy for depth profiling in skin; however, the reported results show high deviations. Thus, analysis of the spectral variability of human skin itself is a necessary prerequisite for systematic quantitative investigations of drug penetration and permeation by confocal Raman microscopy.**

**In our work, we acquired Raman depth profiles from excised human skin samples after abdominal plastic surgery and investigated the absolute intensity fluctuation of four major skin derived Raman peaks. The results prove the expected high variability in spectral intensity, but we could not detect dissimilarities between different skin donors. A detailed analysis of the major endogenous skin components revealed differences in spatial distribution which consequently affects their individual applicability as reference peaks for relative depth profiling. Furthermore, we discovered an increase in signal variability in deeper stratum corneum layers, which has to be considered in future substance depth profiling investigations. In addition, we discovered an exponential decay of the Raman signal for all major skin components accounting for signal attenuation inside biological tissue. Based on this mathematical description, quantitative follow-up of substances in human skin can be realized. All in all, the results of this study elucidate the necessity of substantial understanding of endogenous spectral characteristics inside human skin as essential prerequisite for rational depth profiling of substances in human skin. Copyright © 2013 John Wiley & Sons, Ltd.**

*Additional supporting information may be found in the online version of this article at the publisher's web site.*

**Keywords:** confocal Raman microscopy; human skin; depth profiling; Raman spectral variability; quantification

For full text please refer to the following link:

http://onlinelibrary.wiley.com/doi/10.1002/jrs.4428/abstract

## 8.4 Quantitative detection of caffeine in human skin by confocal Raman spectroscopy – a systematic *in vitro* validation study

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Research Paper

# Quantitative detection of caffeine in human skin by confocal Raman spectroscopy – A systematic *in vitro* validation study

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ABSTRACT

For rational development and evaluation of dermal drug delivery, the knowledge of rate and extent of substance penetration into the human skin is essential. However, current analytical procedures are destructive, labor intense and lack a defined spatial resolution. In this context, confocal Raman microscopy bares the potential to overcome current limitations in drug depth profiling. Confocal Raman microscopy already proved its suitability for the acquisition of qualitative penetration profiles, but a comprehensive investigation regarding its suitability for quantitative measurements inside the human skin is still missing.

In this work, we present a systematic validation study to deploy confocal Raman microscopy for quantitative drug depth profiling in human skin. After we validated our Raman microscopic setup, we successfully established an experimental procedure that allows correlating the Raman signal of a model drug with its controlled concentration in human skin. To overcome current drawbacks in drug depth profiling, we evaluated different modes of peak correlation for quantitative Raman measurements and offer a suitable operating procedure for quantitative drug depth profiling in human skin.

In conclusion, we successfully demonstrate the potential of confocal Raman microscopy for quantitative drug depth profiling in human skin as valuable alternative to destructive state-of-the-art techniques.



For full text please refer to the following link:

http://www.sciencedirect.com/science/article/pii/S0939641115001599

## 8.5 Freeze-drying as a preserving preparation technique for *in vitro* testing of human skin

Lutz Franzen[1], Lucie Vidlarova[1,2], Karl-Heinz Kostka[3], Ulrich F. Schaefer[1] and Maike Windbergs[1,4,5]

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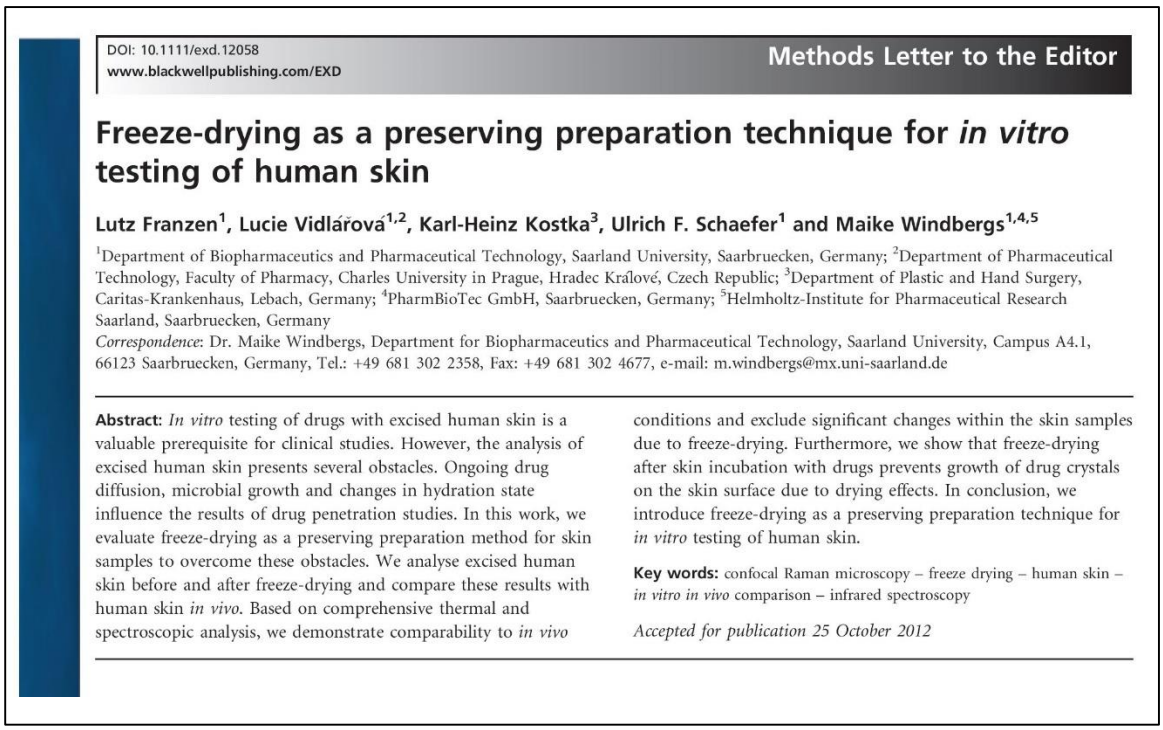

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Methods Letter to the Editor

**Freeze-drying as a preserving preparation technique for *in vitro* testing of human skin**

**Lutz Franzen[1], Lucie Vidlářová[1,2], Karl-Heinz Kostka[3], Ulrich F. Schaefer[1] and Maike Windbergs[1,4,5]**

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**Abstract**: *In vitro* testing of drugs with excised human skin is a valuable prerequisite for clinical studies. However, the analysis of excised human skin presents several obstacles. Ongoing drug diffusion, microbial growth and changes in hydration state influence the results of drug penetration studies. In this work, we evaluate freeze-drying as a preserving preparation method for skin samples to overcome these obstacles. We analyse excised human skin before and after freeze-drying and compare these results with human skin *in vivo*. Based on comprehensive thermal and spectroscopic analysis, we demonstrate comparability to *in vivo* conditions and exclude significant changes within the skin samples due to freeze-drying. Furthermore, we show that freeze-drying after skin incubation with drugs prevents growth of drug crystals on the skin surface due to drying effects. In conclusion, we introduce freeze-drying as a preserving preparation technique for *in vitro* testing of human skin.

**Key words:** confocal Raman microscopy – freeze drying – human skin – *in vitro in vivo* comparison – infrared spectroscopy

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## 8.6 Combining confocal Raman microscopy and freeze drying for quantification of substance penetration into human skin

*Lutz Franzen*[1], *Juliane Anderski*[1], *Viktoria Planz*[1,2], *Karl-Heinz Kostka*[3], *Maike Windbergs*[1,2,4]

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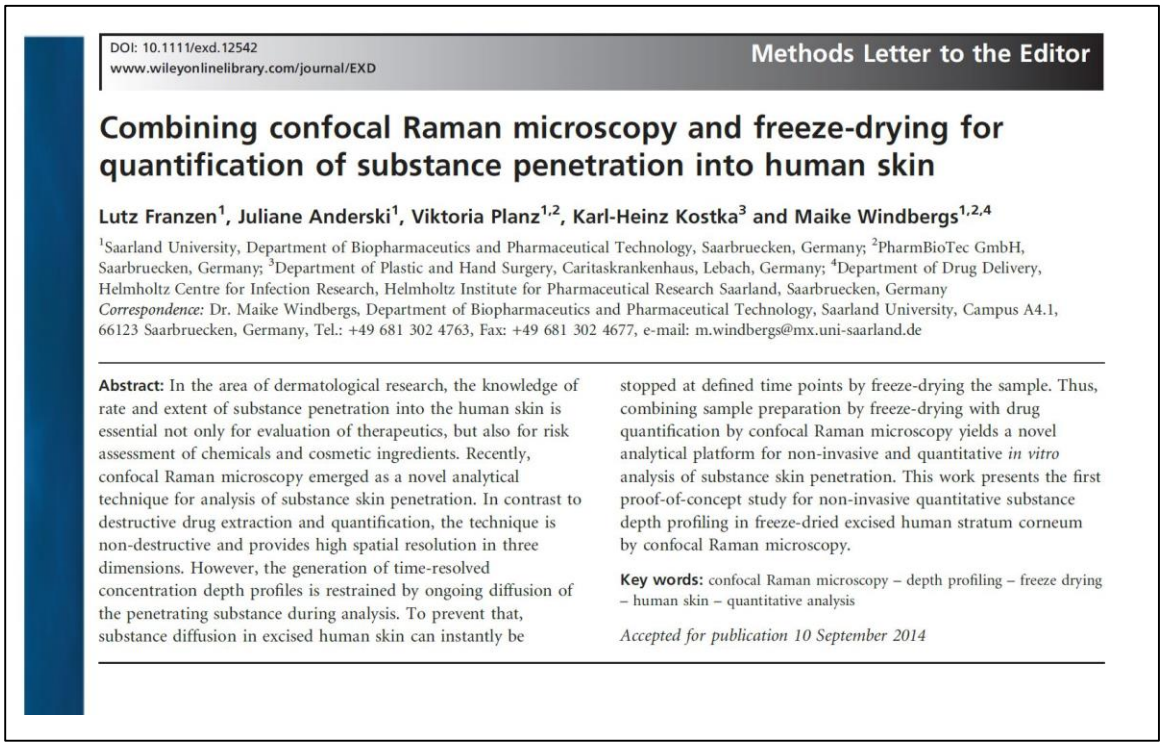

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Methods Letter to the Editor

### Combining confocal Raman microscopy and freeze-drying for quantification of substance penetration into human skin

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**Abstract:** In the area of dermatological research, the knowledge of rate and extent of substance penetration into the human skin is essential not only for evaluation of therapeutics, but also for risk assessment of chemicals and cosmetic ingredients. Recently, confocal Raman microscopy emerged as a novel analytical technique for analysis of substance skin penetration. In contrast to destructive drug extraction and quantification, the technique is non-destructive and provides high spatial resolution in three dimensions. However, the generation of time-resolved concentration depth profiles is restrained by ongoing diffusion of the penetrating substance during analysis. To prevent that, substance diffusion in excised human skin can instantly be stopped at defined time points by freeze-drying the sample. Thus, combining sample preparation by freeze-drying with drug quantification by confocal Raman microscopy yields a novel analytical platform for non-invasive and quantitative *in vitro* analysis of substance skin penetration. This work presents the first proof-of-concept study for non-invasive quantitative substance depth profiling in freeze-dried excised human stratum corneum by confocal Raman microscopy.

**Key words:** confocal Raman microscopy – depth profiling – freeze drying – human skin – quantitative analysis

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*Exp Dermatol*, 23 (2014) 942-944

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# 10 Curriculum Vitae

## *Lutz Franzen*

**15.09.1985 in Düren*

| | |
|---|---|
| Aug. 2014 – Jun. 2015 | DAAD Research Fellow, **School of Engineering and Applied Sciences** (Prof. D. Weitz), **Harvard University**, Cambridge, MA, USA |
| Since Jan. 2011 | PhD in Pharmaceutical Technology, **Saarland University**, Saarbrücken |
| May – Oct. 2010 | Pharmacist in Training, **"Apotheke im Hauptbahnhof"** and **"Dom Apotheke**", Cologne |
| Nov. 2009 – Apr. 2010 | Pharmacist in Training, **Department of Pharmaceutical Development, Bayer Healthcare AG**, Berlin |
| Dec. 2010 | Approbation as Pharmacist, **Apothekerkammer Nordrhein**, Düsseldorf |
| Oct. 2009 | Graduation in Pharmacy, **Rheinische Friedrich-Wilhelms University**, Bonn |
| Jun. – Jul. 2008 | Research-Student Fellow of "Graduiertenkolleg 677", **Pharmaceutical Chemistry** (Prof. G. Bendas), **Rheinische Friedrich-Wilhelms University**, Bonn |
| Jun. 2005 | Abitur, **Gymnasium am Wirteltor**, Düren |

# 11 List of Scientific Publications

## Articles in international peer-reviewed journals

**L. Franzen,** J. Anderski, M. Windbergs, 'Quantitative detection of caffeine in human skin by confocal Raman spectroscopy – a systematic *in vitro* validation study ', Eur J Pharm Biopharm, 95, Part A (2015) 110-116

**L. Franzen,** M. Windbergs, 'Applications of Raman spectroscopy in skin research – from skin physiology and diagnosis up to risk assessment and dermal drug delivery ', Adv Drug Deliv Rev, 89 (2015) 91-104

**L. Franzen**, J. Anderski, V. Planz, K.H. Kostka, M. Windbergs, 'Combining confocal Raman microscopy and freeze-drying for quantification of substance penetration into human skin', *Exp Dermatol*, 23 (2014) 942-944

V. Planz, **L. Franzen**, M. Windbergs, 'Novel *in vitro* Approaches for the Simulation and Analysis of Human Skin Wounds', *Skin Pharmacol Physiol*, 28 (2014) 91-96

S. Seif, **L. Franzen**, M. Windbergs, 'Overcoming drug crystallization in electrospun fibers - Elucidating key parameters and developing strategies for drug delivery', *Int J Pharm*, 478 (2014) 390-397

**L. Franzen,** M. Windbergs, 'Accessing Raman spectral variability in human skin for quantitative depth profiling', *J Raman Spectrosc*, 45 (2014) 82-88

**L. Franzen*,** C. Mathes*, S. Hansen, M. Windbergs, 'Advanced chemical imaging and comparison of human and porcine hair follicles for drug delivery by confocal Raman microscopy', *J Biomed Opt*, 18 (2013) 61210

**L. Franzen,** L. Vidlarova, K.H. Kostka, U.F. Schaefer, M. Windbergs, 'Freeze-drying as a preserving preparation technique for *in vitro* testing of human skin', *Exp Dermatol*, 22 (2013) 54-56

**L. Franzen,** D. Selzer, J.W. Fluhr, U.F. Schaefer, M. Windbergs, 'Towards drug quantification in human skin with confocal Raman microscopy', *Eur J Pharm Biopharm*, 84 (2013) 437-444

**L. Franzen,** M. Windbergs, S. Hansen, 'Assessment of Near-Infrared Densitometry for in situ Determination of the Total Stratum Corneum Thickness on Pig Skin: Influence of Storage Time', *Skin Pharmacol Physiol*, 25 (2012) 249-256

## Contributions to international conferences

*Oral presentations*

**L. Franzen**, 'Evaluating uptake mechanisms of liposomal drug carrier systems in mammalian cells', 62nd New England Complex Fluids Meeting, Yale University, New Haven, CT, USA, 2015

**L. Franzen**, 'Drug tracking in human skin – a challenge for confocal Raman microscopy', Deutsche Pharmazeutische Gesellschaft (DPhG) Annual Conference, Freiburg, Germany, 2013

**L. Franzen**, 'Confocal Raman microscopy as a quantitative analytical technique for dermal drug delivery', 7th Annual Pharmaceutical Solid State Research Cluster (PSSRC) Symposium, Lille, France, 2013

**L. Franzen**, 'Non-invasive drug depth profiling in human skin by confocal Raman microscopy - understanding and overcoming pitfalls', 6th Annual Pharmaceutical Solid State Research Cluster (PSSRC) Symposium, Lisbon, Portugal, 2012

**L. Franzen**, 'The use of artificial skin surrogate to overcome uncertainties in human skin depth profiling by confocal Raman microscopy', 5th Annual Pharmaceutical Solid State Research Cluster (PSSRC) Symposium, Helsinki, Finland, 2011

*Poster presentations*

**L. Franzen,** M. Windbergs, 'Non-invasive substance quantification in biological tissue – Evaluating the potential of confocal Raman microscopy', American Association of Pharmaceutical Scientists (AAPS) Annual Meeting, San Diego, CA, United States, 2014

**L. Franzen,** J. Anderski, M. Windbergs, 'Non-invasive drug quantification in human skin by confocal Raman microscopy' 9th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Lisbon, Portugal, 2014

**L. Franzen,** M. Windbergs, 'The application of confocal Raman microscopy in dermal drug delivery research– From anatomy to depth profiling', American Association of Pharmaceutical Scientists (AAPS) Annual Meeting, San Antonio, TX, United States, 2013

**L. Franzen,** M. Windbergs, 'Direct quantification of drugs in human skin by confocal Raman microscopy', Gordon Research Conference: Barrier Function of Mammalian Skin, Waterville Valley, NH, United States, 2013

**L. Franzen,** M. Windbergs, 'Accessing spectral variability of Raman depth profiles in excised human skin', Gordon Research Conference: Barrier Function of Mammalian Skin, Waterville Valley, NH, United States, 2013

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