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RESEARCH ARTICLE

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ex vivo–in vivo comparison of drug penetration analysis by confocal Raman microspectroscopy and tape stripping

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Abstract

When it comes to skin penetration analysis of a topically applied formulation, the number of suitable methods is limited, and they often lack in spatial resolution. In vivo studies are pivotal, especially in the approval of a new product, but high costs and ethical difficulties are limiting factors. For that reason, good ex vivo models for testing skin penetration are crucial. In this study, caffeine was used as a hydrophilic model drug, applied as a 2% (w/w) hydrogel, to compare different techniques for skin penetration analysis. Confocal Raman microspectroscopy (CRM) and tape stripping with subsequent HPLC analysis were used to quantify caffeine. Experiments were performed ex vivo and in vivo. Furthermore, the effect of 5% (w/w) 1,2-pentanediol on caffeine skin penetration was tested, to compare those methods regarding their effectiveness in detecting differences between both formulations.

KEYWORDS
caffeine, confocal Raman spectroscopy, dermatology, drug delivery, skin barrier, skin penetration

1 | INTRODUCTION

Skin penetration analysis of topically applied drug delivery systems is still a challenging subject, as the number of suitable analytical methods is limited. As it is indispensable to know at which rate and to which extend active ingredients penetrate the skin, when it comes to formulation development the demand for new models for analysing skin penetration is high. Furthermore, there is a new approach in the approval of generic semisolid formulations, by establishing bioequivalence (BE).[1–3] As for most of the topically applied formulations, the target is in the skin, addressing BE through monitoring the drug concentration in blood or plasma, as it is the case for oral products, is not suitable.[4] In vivo studies of clinical efficacy are still the gold standard, even in approval of generic products, but due to their high costs, ethical burdens and inter-individual variability, especially in early stages of formulation development, they are not practicable. The ideal ex vivo model for skin penetration analysis, that gives reliable information about the drug penetration properties of a topically applied dosage form in vivo, is still to be developed. Franz diffusion cells, where exercised human skin or a suitable surrogate, most commonly postauricular pig skin,[5,6] is placed between an acceptor- and a donor compartment, are state of the art. After the incubation step in Franz diffusion cells, the skin can be analysed for drug content. Conventionally this is done, by segmenting the skin using for example tape stripping or a cryomicrotome.[7,8] Optical methods, above all confocal Raman microspectroscopy (CRM), show huge benefits as many active ingredients are Raman-active and hence can be detected, without any linkers or markers. Further, their non-invasive, non-destructive character makes the skin segmentation redundant, allowing continuous measurements and therefore save time and resources.[9]

As many different approaches are being made, to address the challenges of skin penetration studies, especially regarding BE of two formulations, the aim of this study was, to compare different techniques, confocal Raman microspecctroscpoy and tape stripping. Their effectiveness to monitor skin penetration was investigated by comparing caffeine skin penetration out of two different hydrogels. In one of them, 1,2-pentanediol was used as an penetration enhancer,[10] to see whether both methods are capable of detecting differences in caffeine skin penetration, when changing formulation composition.[11] Tape stripping with subsequent HPLC analysis, as a well-established but destructive method, and CRM, as a novel approach, were used to analyse caffeine skin penetration in vivo, as well as ex vivo. Furthermore, ex vivo CRM measurements were performed both on fresh and on frozen and thawed porcine ear skin, as for logistic reasons, porcine ear skin is usually stored at −28°C until usage. Topically applied caffeine as a cosmetic ingredient is well tolerated and no risk to health, it is used as a hydrophilic reference substance in dermal risk assessment studies[12] and was furthermore used in this study, as it is Raman-active and its penetration characteristics are well described.[13,14]

2 | MATERIALS AND METHODS

2.1 | Materials

Caffeine and Carbomer were obtained from Caesar & Loretz GmbH (Hilden, Germany), and 1,2-pentanediol was provided by BASF SE (Ludwigshafen, Germany). Sodium chloride, potassium chloride, disodium phosphate and monopotassium phosphate used for the preparation of phosphate-buffered saline (PBS) pH 7.4 and isotonic saline were all of European Pharmacopoeia Grade. Ethanol and Methanol were obtained from Sigma-Aldrich Corporation (HPLC gradient grade, St. Louis, USA). Parafilm was purchased from Bemis Company Inc. (Oshkosh, WI, USA), finn chambers from Epitest Ltd (Hyryla, Finland) and the tape strips (D-squame tapes Ø 2.2 cm) from CuDerm Corporation (Dallas, USA). All solutions used in this study were prepared with ultra-pure water (Elga Maxima, High Wycombe, UK). Porcine ear skin was provided by a local butcher.

2.2 | Preparation of caffeine gels

Table 1 shows the composition of the caffeine gels used in this study. Caffeine gels were prepared by a lab mixer (Unguator, Gako International GmbH, München, Germany), using the gel-program.[15]

2.3 | Preparation of porcine ear skin

For all ex vivo skin penetration studies, porcine ear skin was used, due to its well-described similarity in histology and morphology to human skin.[5,6,16]

TABLE 1 Composition of the caffeine gels used in this study (% w/w)

	Gel 1 [%]	Gel 2 [%]
Caffeine	2.0	2.0
1,2-pentanediol	-	5.0
Carbomer	0.5	0.5
NaOH-solution (5%)	3.0	3.0
Water	94.5	89.5

The porcine ears were obtained from a local butcher (Bio Metzgerei Griesshaber, Moessingen-Oeschingen, Germany) on the day of the animal's death and immediately prepared. The Department of Pharmaceutical Technology is registered for the use of animal products at the District Office of Tuebingen (registration number: DE 084161052 21). The full-thickness skin was cut off the cartilage, after cleaning the ears gently with isotonic saline and cotton swabs. After cutting the skin into about 4 cm wide strips, those were fixed with pins onto a Styrofoam block, which was wrapped in aluminium foil. The hair on the skin sheets was trimmed to approximately 0.5 mm, using a hair clipper (QC5115/15, Philips, The Netherlands), before the skin was cut to a thickness of 1 mm using a Dermatom (GA 630, Aesculap AG & Co. KG). Circles of 35 mm diameter were cut out for the in situ CRM incubation experiments, and circles of 25 mm were punched out for the Franz diffusion cell incubation experiments. Skin samples were used within 24 h after preparation for the fresh-skin measurements or stored at −28°C wrapped in aluminium foil until the day of usage.

2.4 | Incubation of porcine ear skin

Franz diffusion cells (Gauer Glas, Püttlingen, Germany) were used for incubating the porcine ear skin samples, which were segmented and quantified after the incubation step. For the in situ CRM measurements, porcine ear skin was incubated in a custom-built in-line device (mechanical workshop of the Institute for Pharmaceutical Sciences and the electronic workshop of the Institute of Chemistry, Tuebingen, Germany).[11]

For the incubation of skin samples in Franz diffusion cells, 12 ml of degassed and prewarmed (32°C) PBS was used as receptor medium, which was constantly stirred at 500 rpm. Skin samples were mounted and tightened with the donor compartment on top. A schematic presentation is given in Figure 1A. Before applying the gel to test, Franz diffusion cells were tempered to 32°C in a water bath for 30 min. Then, 173 mg of each caffeine gel was applied (98.8 µg/cm^2) using a finn chamber (Epitest Ltd Oy) without filter disk, and the cell was covered with a piece of parafilm to prevent water evaporation. After incubating the skin for 1, 2 and 3 h separately, the skin samples were removed and gently cleaned with a cotton swab, to remove excess formulation. The actual application area of 15 mm was punched out and equilibrated at ambient conditions for 20 min.[17]

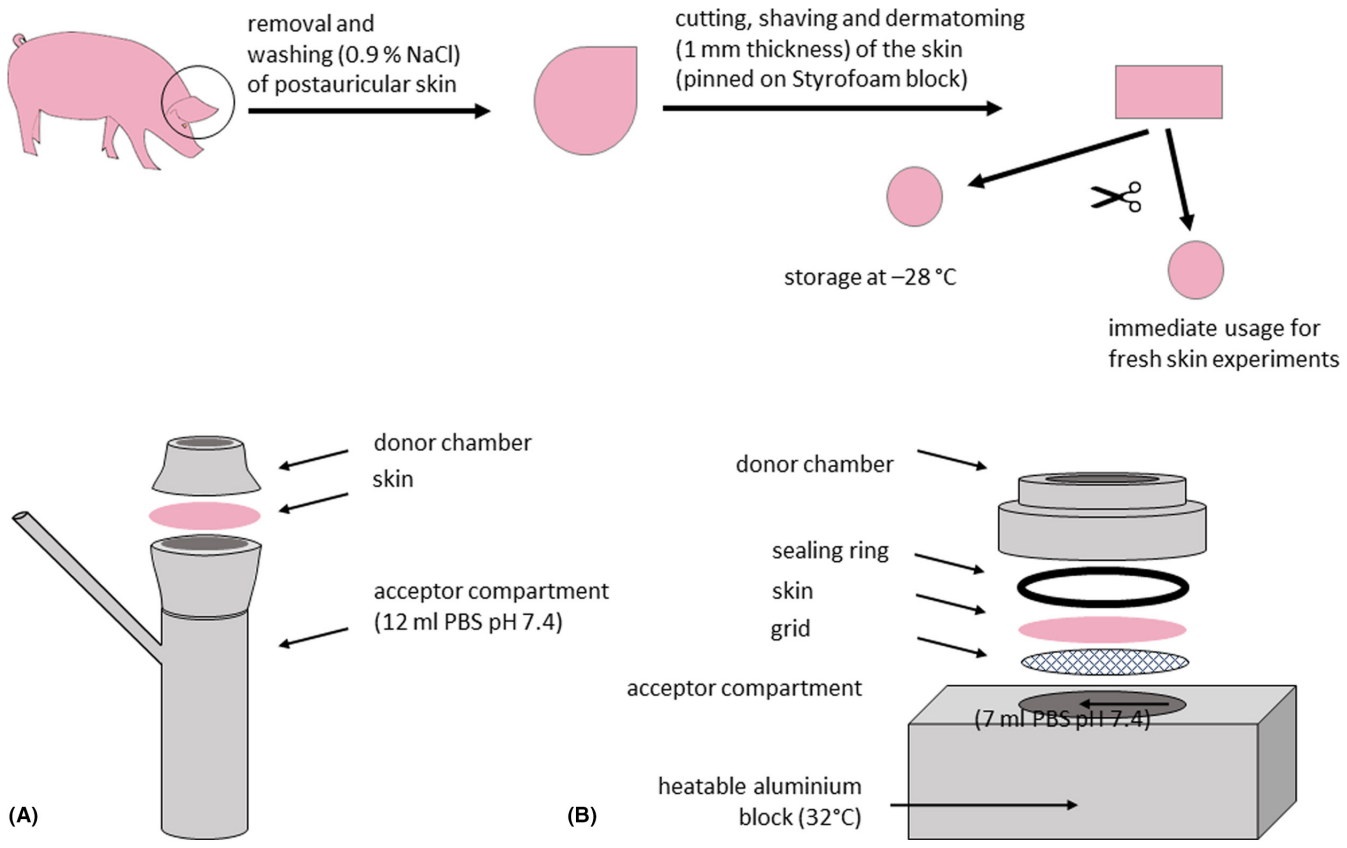

FIGURE 1 Preparation and incubation of porcine ear skin for ex vivo experiments, showing a Franz diffusion cell (A) and the custom-built CRM incubation cell (B).

For the in situ CRM measurements, a recently established method was used.[11,18] As shown in Figure 1B, 7 ml of PBS was used as a receptor medium, and a skin sample was placed on the grid above the acceptor compartment, ensuring the grid was properly soaked in PBS, to prevent any air inclusion between the skin and the acceptor medium. As with the Franz diffusion cell, the donor compartment was placed on top, holding the skin in place. Throughout the incubation time, the temperature of the incubation cell was held constantly at 32.0°C by two thermocouples with an external controlling device attached. After an equilibration time of 30 min, 694 mg of each caffeine gel to test was applied (98.8 μg/cm^2) and a piece of parafilm was tightened between the donor chamber and the objective of the Raman microscope to prevent water evaporation. The total incubation time was 3 h; measurements were performed after 1, 2 and 3 h. All experiments were performed in triplicate on separate days.

2.5 | Tape stripping ex vivo

Tape stripping was performed according the method described by T. Ilić et al.,[19] using 12 adhesive tapes per skin sample. Each tape was weighted before and immediately after stripping, to determine the mass of the skin removed using a high-precision analytical balance (Mettler Toledo XPE205 Delta Range, Mettler, Columbus, USA). As the stripped area (1.5 cm) and the density of the SC (1 g/cm^3) are known, the thickness of the removed skin per tape can be converted out of the difference in weight of each corresponding tape strip. Skin depth is displayed as the class-centre of the calculated thickness of the stratum corneum removed. For the actual stripping process, the tape is placed on top of the skin and pressed down constantly (140 g/cm^2) for 10 s. Each skin sample was stripped by 12 tapes, changing the orientation of each tape by 90° clockwise. The tapes were placed in separate centrifuge tubes (15 ml cellstar, Greiner Bio-One GmbH, Frickenhausen, Germany) afterwards, and caffeine was extracted using 2.0 ml of ethanol. The tubes were sonicated (Sonorex Super RK 510/H, Bandelin electronic GmbH & Co. KG) for 30 min and centrifuged at 4000 rpm for 10 min (Megafuge 1.0 R, Heraeus Holding GmbH, Hanau, Germany). Then, 1.0 ml of each obtained supernatant was analysed for caffeine content.

2.6 | Tape stripping in vivo

The in vivo study was performed in five healthy volunteers (female, BMI < 30, skin types I-III according to Fitzpatrick, no use of topical medicines within the last 14 days, no tattoos in the study site) after the written informed consent had been obtained from each volunteer. This non-medical study on healthy human subjects was executed according to the principal requirements of the declaration of Helsinki

and according to the main principles of Good Clinical Practice (GCP) and with approval of the local ethics committee. 250 µg of each caffeine gel was applied (98.8 µg/cm^2) by using an extra-large Finn chamber (Epitest Ltd Oy) without filter disk on the ventral surface of each forearm. After each time point, residual formulation was gently removed with a cotton swab. Tape stripping was performed according to the method described by T. Ilić et al.,[19] using 12 adhesive tapes per time point of each formulation. Each tape was weighted before and immediately after stripping, to determine the mass of the skin removed using an analytical balance (Sartorius BP210D, Sartorius AG, Goettingen, Germany). As the stripped area (1.5 cm) and the density of the SC (1 g/cm^3) are known, the thickness of the removed skin per tape can be converted out of the difference in weight of each corresponding tape strip. For the actual stripping process, the tape is placed on top of the skin and pressed down constantly (140 g/cm^2) for 10 s. Each skin sample was stripped by 12 tapes, changing the orientation of each tape by 90° clockwise. The tapes were placed in separate glass centrifuge tubes afterwards, and caffeine was extracted using 2.0 ml of ethanol. The tubes were sonicated for 30 min, and 1.0 ml of each obtained supernatant was analysed for caffeine content. Every incubation time was repeated five times for each formulation.

2.7 | Caffeine quantification by HPLC

Caffeine samples obtained by ex vivo tape stripping were quantified using the CBM-20A HPLC system (Shimadzu Europa GmbH, D Duisburg) and equipped with an UV detector. The HPLC column EC 125/4 Nucleosil 100–5 C18 (Macherey-Nagel GmbH & Co. KG) was used. The column-oven temperature was set to 35°C. The mobile phase consisted of 80% water and 20% acetonitrile with a flow rate of 0.8 ml/min. Aliquots of 10 µl were injected, the UV absorbance was measured at 273 nm.

Caffeine samples obtained by in vivo tape stripping were quantified using a Dionex Ultimate 3000 HPLC system (Thermo scientific, USA). The mobile phase was a mixture of 35% methanol and 65% water. The chromatographic column used was Zorbax Eclipse Plus C18, 4.6×150 mm, 5 µm (Agilent, Santa Clara, CA, USA); column temperature was set to 40°C and autosampler temperature to 10°C. The flow rate of the mobile phase was 1 ml/min. UV detection was performed on 275 nm.

2.8 | Confocal Raman microspectroscopy (CRM) ex vivo

For the in situ measurements, the incubation cell was placed on the scan table of an alpha 500 R confocal Raman microscope (WITec GmbH, Ulm, Germany). To ensure that the exact same position of the skin could be tracked over the whole incubation time, the incubation cell was held in place by four pins on the bottom of the device. The Raman microscope is equipped with a 532-nm excitation laser, a UHTS 300 spectrometer, a DV401-BV CCD detector and a 63× water immersion objective with numerical aperture of 1.0 (W "Plan-Apochromat" 63/1,0 M27, Carl Zeiss, Jena, Germany), which was placed into the donor compartment of the incubation cell, using the gel to test as immersion medium. The laser intensity was set to 25 mW, using an optical power meter (PM100D, Thorlabs GmbH, Dachau, Germany), which results in a strong signal without leading to thermal damage of the skin.

The DV401-BV CCD detector was cooled to −60°C, and a spectral range from 501 to 1635 cm^{-1}, obtained by an optical grating (1800 g/mm, spectral centre: 1100 cm^{-1}) was recorded. Two-dimensional image scans of 5 µm width and 25 µm depth, with an integration time of 1.5 s per spectra were performed, acquiring 10 spectra per line and 50 lines per vertical dimension. All recorded spectra were processed using the software Project Plus 5 (WiTec GmbH, Ulm, Germany), performing a cosmic ray removal and a background subtraction in the "shape" option (size: 400). Also, principal component analysis (PCA) was performed on all spectra for noise reduction.[20] For the determination of depth penetration profiles, the area under the fitted curve (AUC) of the caffeine band at 556 cm^{-1} (O=C-N deformation mode) was calculated, as this peak is selective for caffeine and not interfered with any signals of the skin. For the determination of the skin surface, as well as for the normalization of the caffeine signal, the aromatic amino acid peak at 1008 cm^{-1} (ring breathing mode) was used. As the measurements were started above the skin surface, all depth profiles were cropped to the skin surface, which was defined as the half maximum of the aromatic amino acid peak.[14] For normalizing the caffeine signal, the arithmetic mean of this peak at the skin surface was used, as it comes to signal attenuation in deeper skin regions and signal variations over the incubation time. Out of every two-dimensional scan, three depth profiles were extracted, which leads to a total of nine depth profiles for each formulation, which were used to calculate the mean penetration profile of the corresponding formulation. For the calculation of enhancement ratios, the areas under the curves of the mean depth profiles were determined using the trapezoidal method. The AUC corresponding to the formulation with penetration enhancer was divided by the AUC of the reference formulation, resulting in the enhancement ratio. Representative spectra of the stratum corneum incubated with caffeine are given in Figure 2.

To quantify the amount of caffeine in the skin, the method by Caspers et al. was adapted, as already shown for retinol in a previous publication.[21,22] The total amounts of caffeine were determined by calculating the area under the penetration profile.

2.9 | Confocal Raman microspectroscopy (CRM) in vivo

The in vivo study was performed in three healthy volunteers (male and female, BMI < 30, skin types I-III according to Fitzpatrick, no use of topical medicines within the last 14 days, no tattoos in the study site) after the written informed consent had been obtained from each volunteer. This non-medical study on healthy human subjects

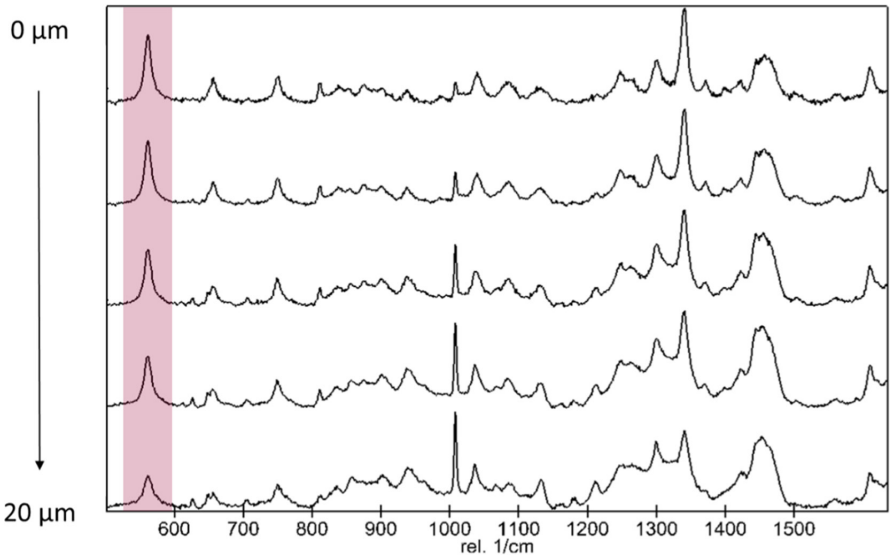

FIGURE 2 Raman spectra of the stratum corneum incubated with the 2% caffeine hydrogel, starting above the skin surface and gradually moving into the skin sample (532 nm excitation laser).

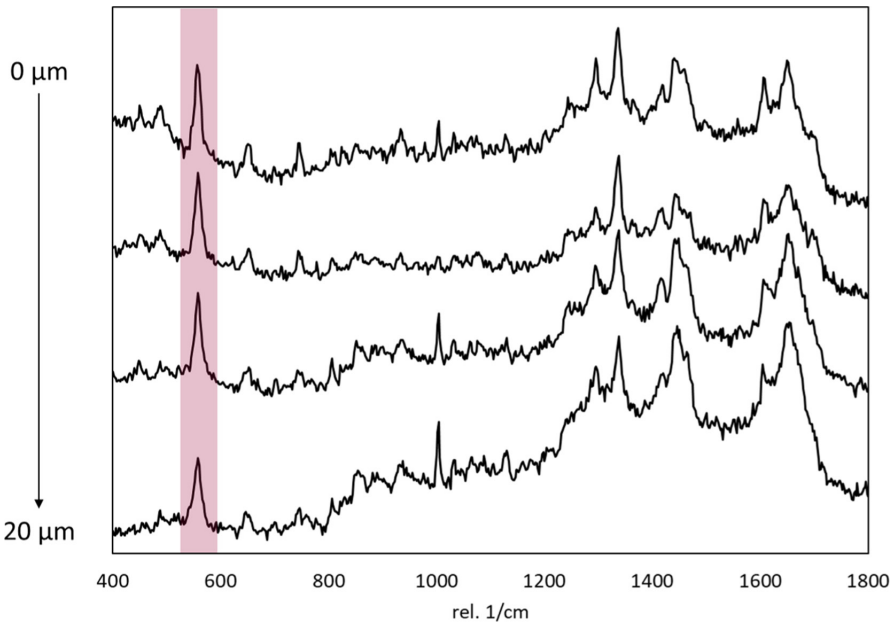

FIGURE 3 Raman spectra of the stratum corneum incubated with the 2% caffeine hydrogel, starting above the skin surface and gradually moving into the skin sample (785 nm excitation laser).

was executed according to the principal requirements of the declaration of Helsinki and according to the main principles of Good Clinical Practice (GCP).

Study was performed in controlled climate conditions (22 ± 2°C and at 50% ± 7.5% relative humidity). After 30 min of acclimatization, measurements were started in another air-conditioned examination room with the same temperature and relative humidity.

Three test areas for three different measurement time points (1.5, 3 and 4.5 h) were assigned to the left volar forearm of each subject. 250 µg of each caffeine gel was applied by using an extra-large Finn chamber (Epitest Ltd Oy) without filter disk. Three measurements were performed 1, 2 and 3 h after applying each caffeine gel. Before starting the measurements, the test area was cleaned with a water-moistened paper towel. For in vivo CRM measurements, the "gene2-SCA Ultimate" by RiverD International B. V., Rotterdam, Netherlands, was used. The device has two built-in wave class 3B lasers. The red light laser operates at a wavelength of 671 nm and is configured to measure at high wavenumbers (HWN) between 2500 and 3800 cm^{-1}. The second laser, a near-infrared laser, operates at a wavelength of 785 nm and measures in the "fingerprint region" with wavenumbers from 400 to 1800 cm^{-1}. A pinhole of 50 µm in diameter was used, and fingerprint profiles were recorded starting above the skin surface and going down to 28 +/− 5 µm deep into the SC with steps of 4 µm. Repeated measurements were taken from a skin region in the centre of each test area on a subarea of 500 × 500 µm. Eight to ten profiles were acquired with an integration time of 5 s per spectrum.[22] Representative spectra of the skin incubated with caffeine are given in Figure 3.

2.10 | Data evaluation and statistics

Data were found not to be distributed normally, and different numbers of samples/measurements were obtained from the four methods. To compensate for this, median +/− median absolute deviation

of the median were calculated and are displayed in all diagrams. No further statistical analysis was performed.

3 | RESULTS

3.1 | Ex vivo tape stripping

Figure 4 shows the results of caffeine skin penetration obtained by ex vivo tape stripping after a total incubation time of 3 h with gel 1 (A) and gel 2 (B).

As the skin is segmented by 12 tapes, this leads to 12 data points for each depth profile. The corresponding skin depth is displayed as the class-centre of the skin layers removed by each tape, as the amount of skin removed, and therefore, the calculated skin depth varies for every tape used. As the amount of caffeine within every tape is quantified (µg), displaying the amount of caffeine as a function of depth is common with tape stripping results; also, the amount of caffeine per skin area could be displayed as a function of depth (Figures S1 and S2). For better comparability to the results obtained by CRM, here the depth profiles show the amount of caffeine per skin volume (concentration; mg/cm^3) as a function of skin depth. In CRM measurements, the amount of drug is detected inside a volume (the laser focal volume) giving a concentration; therefore, the caffeine concentration in the skin was also calculated for all tape stripping results.

After incubating with gel 1 for 1 h, caffeine can be detected within the first 10 µm. As only little skin was removed by the tape strips, all 12 data points are located within the first 10 µm of skin depth. This is also the case after 2 h of incubation time, only the last data point shows a significant increase, indicating a concentration of over 60 mg/cm^3. That means, the last tape extracted showed only a small mass difference, which leads to this high concentration value, as the displayed caffeine concentration was calculated from the extracted amount of caffeine and the stratum corneum volume, determined by the mass difference of the tape before and after stripping. With other ways of data display (supplementary material), this effect is negligible. After 3 h of incubation time, the total penetration depth increases, caffeine can be detected even below 15 µm of skin depth. With the addition of 5% (w/w) 1,2-pentanediol, caffeine can be detected below 10 µm after 2 h of incubation time, while the course of the 1 h time point has similar penetration properties, as after incubating with gel 1. Despite the penetration enhancer, no caffeine can be detected below 15 µm after incubating for 3 h.

3.2 | In vivo tape stripping

Figure 5 shows the results of caffeine skin penetration obtained by in vivo tape stripping after a total incubation time of 3 h with gel 1 (A) and gel 2 (B).

For better comparability to the results obtained by CRM, the depth profiles again show the concentration of caffeine as a function of skin depth. As the amount of caffeine within every tape is quantified (µg), displaying the amount of caffeine as a function of depth is common with tape stripping results; also, the amount of caffeine per skin area could be displayed as a function of depth (Figures S3 and S4).

After incubation with gel 1 for 1 h, caffeine can be detected especially within the first 10 µm of skin depth, between 10 and 25 µm of skin depth, the caffeine concentrations are neglectable. After 2 h, the caffeine concentration increases on the skin surface, showing significantly higher amounts of caffeine within the

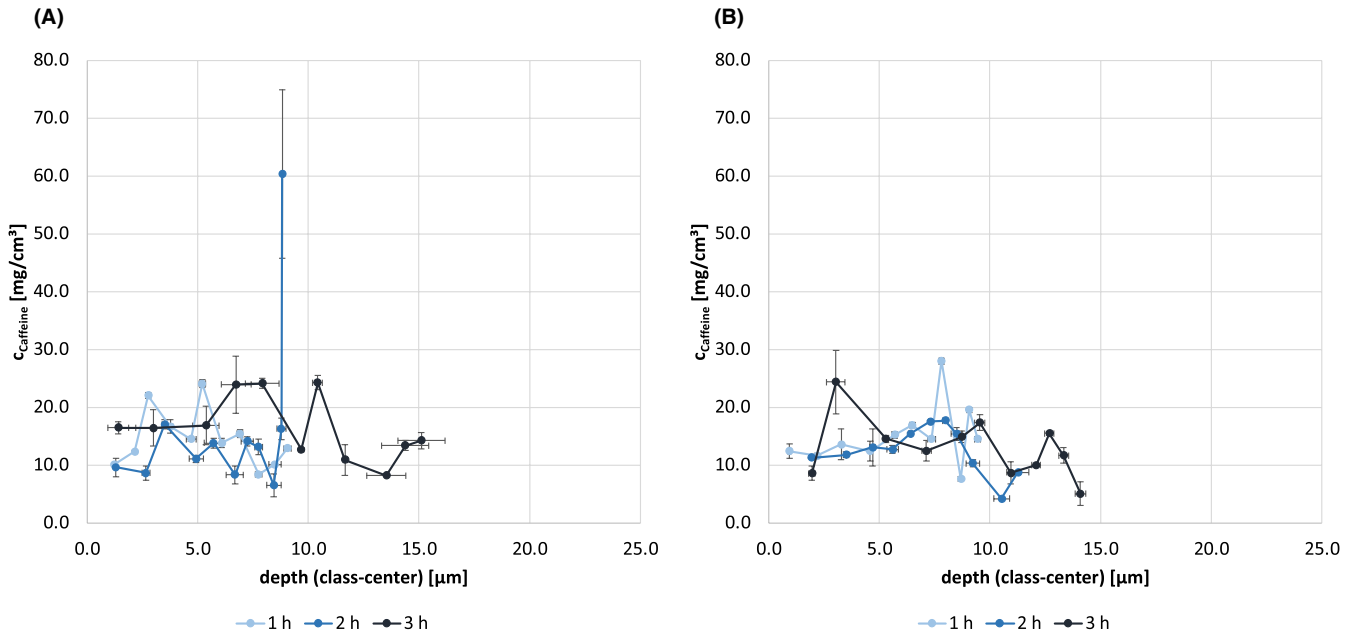

FIGURE 4 Skin penetration profiles of caffeine over 3 h incubation time with 2.0% caffeine hydrogel (A) and 2% caffeine hydrogel +5% 1,2-pentanediol (B) (% w/w); the error bars are showing the median absolute deviation. $n = 5$.

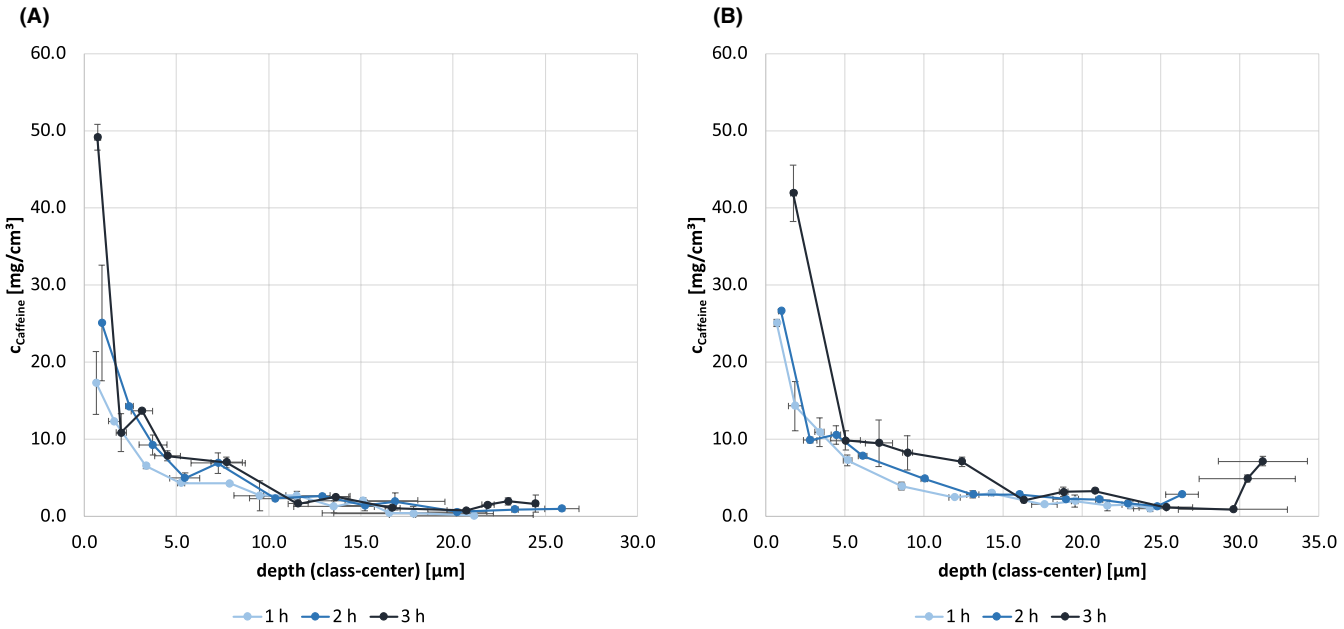

FIGURE 5 Skin penetration profiles of caffeine over 3 h incubation time with 2.0% caffeine hydrogel (A) and 2% caffeine hydrogel +5% 1,2-pentanediol (B) (% w/w); the error bars are showing the median absolute deviation. $n = 5$.

first 10 µm of skin depth, but no change in total skin penetration. Also, after 3 h of incubation time, only a slight increase of caffeine below 15 µm of skin depth is detected, while the concentration on the skin surface is remarkably higher, than compared to the previous time points. The addition of 5% (w/w) 1,2-pentanediol to the caffeine hydrogel leads to a clear difference in the skin penetration profile. As Figure 5B shows, caffeine skin penetration is generally increased for all three time points. After incubating for 1 h, caffeine is still found especially within the upper 10 µm of skin, the concentrations detected between 10 and 25 µm skin depth are higher, than after incubating with gel 1. Over the following 2 h of incubation time, a further shift of the penetration profiles towards deeper skin regions can be detected. While longer incubation times with gel 1 lead to differences in caffeine concentrations mostly between 0 and 10 µm of skin depth, the 2 h time point of gel 2 shows an increase in caffeine concentration at lower skin depths (below 10 µm). After 3 h, the shift towards deeper skin regions is even more pronounced.

3.3 | Ex vivo CRM

Figure 6 shows the results of ex vivo CRM measurements of caffeine skin penetration during 3 h of incubation time with the two caffeine gels described (A: gel 1; B: gel 2) on freshly prepared porcine ear skin. Starting at the skin surface, the amount of caffeine per skin volume is displayed as a function of depth.

The amount of caffeine per skin area could be displayed as a function of depth (Figure S5). Over the incubation time of 3 h with gel 1, a steady increase of the caffeine concentration in the skin over time is visible. After the first hour, most of the caffeine is located within the first 10 µm of skin depth, reaching a total penetration depth of approximately 15 µm. This total penetration depth also increases steadily over the following 2 h of incubation time. Especially between 15 and 20 µm of skin depth, there is a significant increase in caffeine concentration over time. The addition of 5% (w/w) 1,2-pentanediol as a penetration enhancer leads to an increased caffeine concentration within the skin for all three time points. After 1 h, the penetration profile shows similar properties than the penetration profile, after incubating with gel 1 for 3 h, where caffeine can be found even below 20 µm of skin depth. The increased caffeine concentrations in deeper SC depths are reached earlier compared to incubation with gel 1.

Figure 7 shows the results of ex vivo CRM measurements of caffeine skin penetration during 3 h of incubation time with the two caffeine gels described (A: gel 1; B: gel 2) on frozen and thawed porcine ear skin. Starting at the skin surface, the concentration of caffeine is displayed as a function of depth. The amount of caffeine per skin area could be displayed as a function of depth (Figure S6). After incubating the skin with gel 1, the penetration profiles of all three time points show the same characteristics as for the fresh porcine ear skin. While most of the caffeine is located within the first 10 µm of skin after 1 h, the depth profiles flatten after further incubation time, again a steady increase especially between 15 and 20 µm skin depth is clearly visible. The penetration-enhancing effect of 1,2-pentanediol on the contrary seems to be more distinct on frozen porcine ear skin. Already after 1 h of incubating with gel 2, the overall caffeine concentration in the skin is genuinely higher, than compared to gel 1, showing caffeine concentrations above 10 mg/cm^3 even below 20 µm. While there is not much change within the first 10 µm of skin depth over time, the increase in caffeine concentration is particularly visible between 15 and 20 µm.

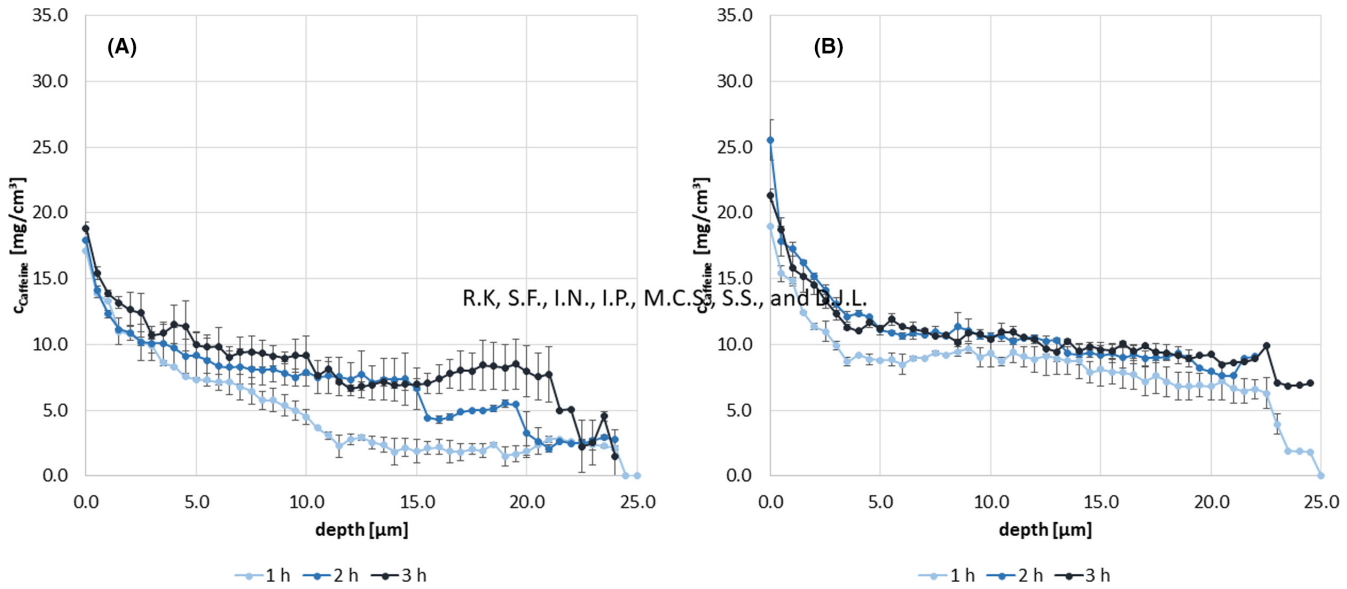

FIGURE 6 Skin penetration profiles of caffeine over 3 h incubation time with 2.0% caffeine hydrogel (A) and 2% caffeine hydrogel +5% 1,2-pentanediol (B) (% w/w); the error bars are showing the median absolute deviation. $n = 3$.

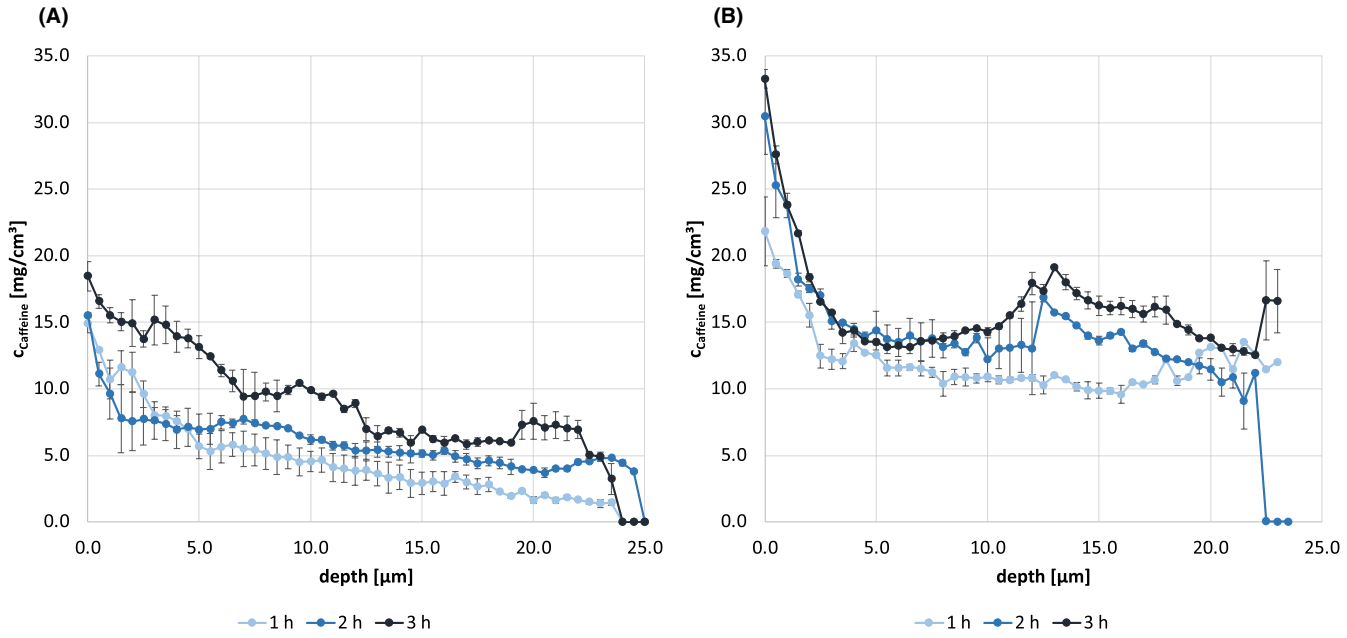

FIGURE 7 Skin penetration profiles of caffeine over 3 h incubation time with 2.0% caffeine hydrogel (A) and 2% caffeine hydrogel +5% 1,2-pentanediol (B) (% w/w); the error bars are showing the median absolute deviation. $n = 3$.

3.4 | In vivo CRM

Figure 8 shows the results of in vivo CRM measurements of caffeine skin penetration during 3 h of incubation time with the two caffeine gels described (A: gel 1; B: gel 2). Starting at the skin surface, the concentration of caffeine is displayed as a function of depth.

The amount of caffeine per skin area could be displayed as a function of depth (Figure S7). After 1 h of incubating with gel 1, caffeine can be detected within the first 12 µm of skin depth. The 2 h time point already shows higher caffeine concentrations within the skin, increasing only slightly further after 3 h. Total penetration depth is below 20 µm, despite constantly increasing, too. The effect of 1,2-pentanediol on caffeine skin penetration can be seen after 1 h of incubating with gel 2. Caffeine can be detected up to 16µm depth. This is 4 µm deeper than without penetration enhancer. After 2 h of incubation, caffeine concentrations within the skin increase a little, showing a bigger increase after 3 h of incubation time. Again, caffeine penetrates to a total depth of approximately 20 µm.

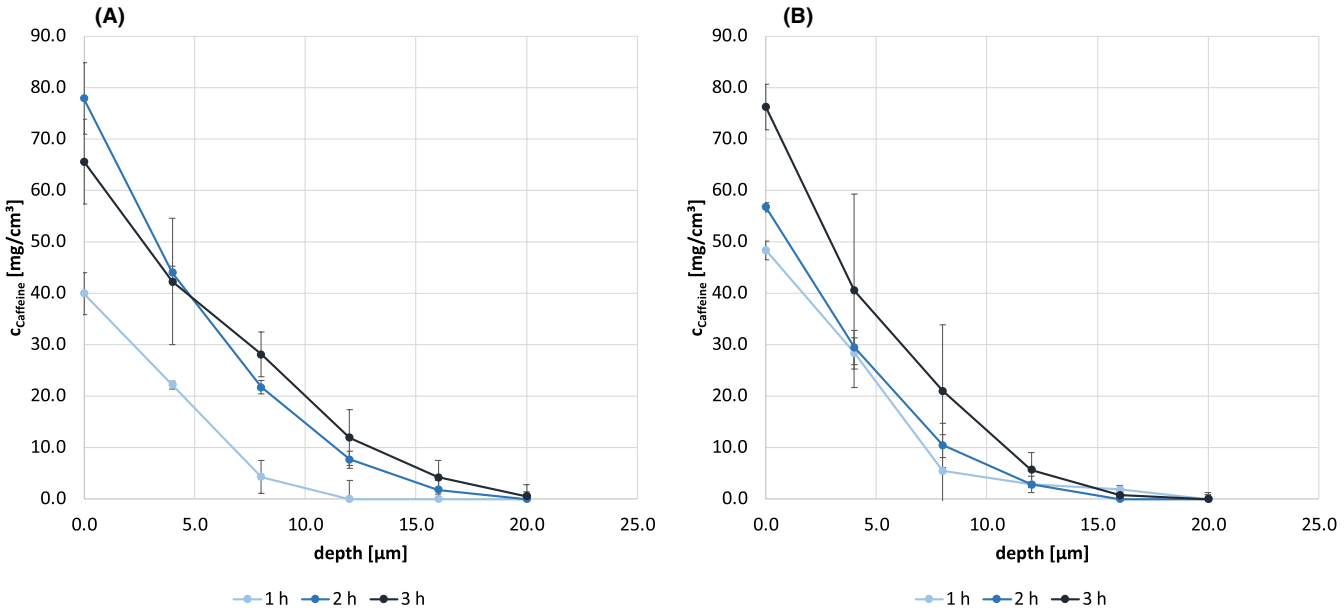

FIGURE 8 Skin penetration profiles of caffeine over 3 h incubation time with 2.0% caffeine hydrogel (A) and 2% caffeine hydrogel +5% 1,2-pentanediol (B) (% w/w); the error bars are showing the median absolute deviation. $n = 3$.

TABLE 2 Total amount of caffeine per skin area (median). (CRM: $n = 3$; tape stripping: $n = 5$)

		Gel 1	Gel 2	
		$c_{caffeine}$ [µg/cm²]	$c_{caffeine}$ [µg/cm²]	ER
In vivo CRM	1 h	18.58	25.09	1.35
	2 h	45.69	28.45	0.62
	3 h	47.98	42.45	0.88
Ex vivo CRM (fresh skin)	1 h	11.76	20.71	1.76
	2 h	16.92	24.21	1.43
	3 h	20.58	26.12	1.27
Ex vivo CRM (frozen skin)	1 h	11.28	27.89	2.47
	2 h	14.87	31.77	2.14
	3 h	21.98	37.00	1.68
In vivo tape stripping	1 h	7.17	10.89	1.52
	2 h	10.65	13.27	1.25
	3 h	12.35	20.92	1.69
Ex vivo tape stripping	1 h	13.20	13.86	1.05
	2 h	10.41	13.85	1.33
	3 h	24.93	18.60	0.75

3.5 | Total amounts of caffeine

Table 2 shows the total amounts of caffeine within the SC, normalized to the application area, as the area of skin incubated is different within all four methods.

Enhancement ratios are calculated by dividing the amount of caffeine within the SC after treatment with gel 2 by the amount of caffeine within the SC after treatment with gel 1 (reference).

The total amounts of caffeine within the SC range from 7.17 µg/cm², after incubating for 1 h with gel 1 (in vivo tape stripping) to 47.98 µg/cm² after incubating for 3 h with gel 1 (in vivo CRM). The enhancement ratios range from 0.62 (in vivo CRM, 2 h) to 2.47 (ex vivo CRM, frozen skin, 1 h). All methods show the highest enhancement ratio after 1 h, ranging from 1.35 (in vivo CRM) to 2.47 (ex vivo CRM, frozen skin), except for ex vivo tape stripping, where the effect of 1,2-pentanediol is most pronounced after 2 h and in vivo tape stripping, where the enhancement ratio is the highest after 3 h. For all methods, except for ex vivo tape stripping, the amount of caffeine within the SC increases over time. After 2 h of incubation time, for both gels, the ex vivo tape stripping results indicate a slight decrease.

4 | DISCUSSION

The skin penetration of caffeine from two 2% (w/w) hydrogels was analysed over 3 h by ex and in vivo tape stripping, ex vivo/in situ CRM on frozen and on fresh porcine ear skin, as well as by in vivo CRM. All methods lead to caffeine skin penetration profiles with similar characteristics and show total caffeine amounts within the SC in the same magnitude. Differences especially within the in vivo results can be attributed to inter-individual variability of the subjects.

Depth profiles obtained by ex vivo tape stripping differ from the other methods, as the total penetration depth determined is remarkably lower. This can be explained by the fact, that porcine ear skin is exposed to ambient conditions for 20 min after the incubation step in Franz diffusion cells, to prevent mass loss due to water evaporation while weighting the tapes. As the SC hydration state of the skin samples decreases to normal/equilibrium values, the stripped mass decreases, and as a result, the determined

total penetration depth is reduced. Furthermore, the amount of skin removed by adhesive tape is affected by the donor animal, as sometimes the applied pressure is not sufficient to remove the total SC. Caffeine may be present in deeper layers but will not be detected as it is not removed by the 12 tape strips. This leads to ex vivo tape stripping showing generally the lowest caffeine concentrations within the skin, ranging from 13.20 to 24.93 $\mu g/cm^2$ after incubating with gel 1. Regarding the penetration-enhancing effect of 1,2-pentanediole on caffeine skin penetration, ex vivo tape stripping and in vivo CRM only show a positive effect for one time point. As the ex vivo tape stripping results only show a penetration of caffeine to the upper 15 µm of skin depth, not all caffeine within the skin might be detected, leading to false enhancement ratios, as the other methods show differences in penetration profiles between the two gels used especially below 15 µm of skin depth. This supposedly contrary effect of 1,2-pentanediol on caffeine skin penetration also measured by in vivo CRM for the latter two time points can be explained by inter-individual variability, as only three subjects were participating in this study, while five subjects were part of the in vivo tape stripping experiments and all ex vivo experiments were carried out on the skin from one donor animal, respectively. Porcine ear skin is widely used as a skin surrogate in ex vivo studies, still there are differences in skin barrier function, that must be taken into account when comparing ex vivo porcine to in vivo human results.[23] Despite that, penetration curves show the same characteristics—especially when comparing the ex vivo to the in vivo CRM results, this becomes clear. After incubating with gel 1, the course of the 2 and 3 h time point is similar, showing a bigger increase in caffeine skin concentration between 1 and 2 h than compared to the 3 h value. After incubating with gel 2, the 1 and 2 h depth profiles show a bigger similarity, while the 3 h time point shows a stronger increase in caffeine skin concentration. This is shown not only by the in vivo results (tape stripping and CRM) but also by ex vivo CRM, on fresh, as well as on frozen porcine ear skin. Using frozen instead of fresh porcine ear skin leads to a more pronounced effect of 1,2-pentanediol on caffeine skin penetration, while the total amount of caffeine within the skin after incubating with gel 1 is not affected. For all three time points, 1,2-pentanediol leads to remarkably higher caffeine concentrations in beforehand frozen porcine ear skin. Previous studies already discussed the impact of freezing on skin permeability, showing increased permeability for drugs from aqueous vehicles.[24,25] In our case, freezing of the skin only had an impact on caffeine skin penetration in presence of 1,2-pentanediol.

5 | CONCLUSION

The aim of this study was to compare different methods of skin penetration analysis, two different ex vivo methods and two in vivo studies, by investigating the effect of 1,2-pentanediol on caffeine skin penetration, when applied as a 2% (w/w) hydrogel. In our previous work, we already found a positive effect of 1,2-pentanediol on caffeine skin penetration out of aqueous solutions and showed the potential and advantages of ex vivo/in situ CRM measurements for skin penetration analysis.[11,18,22] With the additional calibration step, according to a method described by Caspers et al.,[21] the obtained skin penetration profiles can be expressed as concentrations (as opposed to arbitrary units) and directly compared to other methods as for example tape stripping like we did in this study. Caffeine was used as a hydrophilic model drug, as it is used in cosmetics and well described as a Raman-active reference drug.[15,26,27] The depth profiles of caffeine skin penetration of both techniques used in this study, CRM, and tape stripping, both in- and ex vivo all showed similar courses and caffeine concentrations in the same range could be measured. All methods were suitable for analysing caffeine skin penetration, leading to comparable results, differing mostly in their time- and labour-consumption, spatial resolution, and regarding the in vivo studies in ethical aspects. As expected, a positive effect of 1,2-pentanediol on caffeine skin penetration could be shown. This positive effect seems to be more pronounced on beforehand frozen porcine ear skin, as the comparison of fresh and frozen skin of the same donor animal showed.

Especially in terms of formulation development, knowing rate and extend of drug penetration, as well as effects of penetration enhancers on penetration properties is crucial. As the results show, the amount of caffeine penetrating the SC can be substantially affected by the addition of 5% (w/w) 1,2-pentanediol to the formulation. In this study, we furthermore were able to show the advantages of ex vivo/in situ CRM measurements for analysing and understanding skin penetration of active ingredients. When it comes to show BE of a generic topically applied formulation, skin penetration studies by CRM have a huge potential, as it is a time-efficient and resource-saving technique, providing reliable real-time information.

AUTHOR CONTRIBUTIONS

Conceptualization: R.K. and D.L.; Data curation: R.K and S.F.; Funding acquisition: D.L. and S.S.; Investigation: R.K, S.F., I.N., I.P. and M.C.S.; Methodology: R.K, S.F., I.N., I.P., M.C.S., S.S., and D.L.; Project administration: D.L.; Supervision: D.J.L.; Writing—original draft: R.K.; Writing—review and editing: D.L. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1 Ex vivo tape stripping: skin penetration profiles of caffeine over 3 h incubation time with 2.0% caffeine hydrogel (A) and 2% caffeine hydrogel + 5% 1,2-pentanediol (B); the error bars are showing the median absolute deviation.

Figure S2. Ex vivo tape stripping: skin penetration profiles of caffeine over 3 h incubation time with 2.0% caffeine hydrogel (A) and 2% caffeine hydrogel + 5% 1,2-pentanediol (B); the error bars are showing the median absolute deviation.

Figure S3 In vivo tape stripping: skin penetration profiles of caffeine over 3 h incubation time with 2.0% caffeine hydrogel (A) and 2% caffeine hydrogel + 5% 1,2-pentanediol (B); the error bars are showing the median absolute deviation.

Figure S4. In vivo tape stripping: skin penetration profiles of caffeine over 3 h incubation time with 2.0% caffeine hydrogel (A) and 2% caffeine hydrogel + 5% 1,2-pentanediol (B); the error bars are showing the median absolute deviation.

Figure S5 Ex vivo CRM–fresh skin: skin penetration profiles of

caffeine over 3 h incubation time with 2.0% caffeine hydrogel (A) and 2% caffeine hydrogel + 5% 1,2-pentanediol (B); the error bars are showing the median absolute deviation.

Figure S6. Ex vivo CRM–frozen & thawed skin: skin penetration profiles of caffeine over 3 h incubation time with 2.0% caffeine hydrogel (A) and 2% caffeine hydrogel + 5% 1,2-pentanediol (B); the error bars are showing the median absolute deviation.

Figure S7 In vivo CRM: skin penetration profiles of caffeine over 3 h incubation time with 2.0% caffeine hydrogel (A) and 2% caffeine hydrogel + 5% 1,2-pentanediol (B); the error bars are showing the median absolute deviation.

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