Comparison of protocols measuring diffusion and partition coefficients in the stratum corneum

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ABSTRACT: Partition (K) and diffusion (D) coefficients are important to measure for the modelling of skin penetration of chemicals through the stratum corneum (SC). We compared the feasibility of three protocols for the testing of 50 chemicals in our main studies, using three cosmetics-relevant model chemicals with a wide range of logP values. Protocol 1: SC concentration-depth profile using tape-stripping (measures KSC/v and DSC/HSC where HSC is the SC thickness); Protocol 2A: incubation of isolated SC with chemical (direct measurement of KSC/v only) and Protocol 2B: diffusion through isolated SC mounted on a Franz cell (measures KSC/v and DSC/HSC and is based on Fick’s laws). KSC/v values for caffeine and resorcinol using Protocol 1 and 2B were within 30% of each other, values using Protocol 2A were two-fold higher, and all values were within 10-fold of each other. Only indirect determination of KSC/v by Protocol 2B was different from the direct measurement of KSC/v by Protocol 2A and Protocol 1 for 7-EC. The variability of KSC/v for all three chemicals using Protocol 2B was higher compared to Protocol 1 and 2A. DSC/HSC values were comparable using different methods. Pig skin might be a good surrogate for human skin for the three chemicals tested. Copyright © 2017 The Authors Journal of Applied Toxicology published by John Wiley & Sons Ltd.

Keywords: diffusion; partition; coefficients; stratum corneum; protocols; comparison

Introduction

Determination of the bioavailability of chemicals via skin is a key part of the safety assessment of most cosmetic products. Skin absorption can be measured according to validated in vitro methods and guidelines (OECD, 2004b; SCCS, 2015). However, these methods are expensive and time-consuming; therefore, predictions of skin absorption using in silico models would help to address this.

We have initially focused on measuring penetration through the stratum corneum (SC); however, information about other skin layers is also important for the interpretation of the penetration of topically applied chemicals. Two main pathways for penetration through the SC have been established: the lipophilic pathway and the hydrophilic pathway, with the lipophilic pathway being the main route for penetration through the SC. In silico models that are based on the lipid pathway of penetration incorporate the logP and have been used to predict the percutaneous flux of many chemicals solely on the basis of their physicochemical properties (e.g. Potts and Guy, 1992). If another pathway is involved in penetration, such as the polar pathway, logP is not an appropriate parameter to predict the penetration. Penetration through the main skin barrier, the SC, depends mainly on the partitioning of the chemicals between the formulation and SC, as well as on the diffusion in the SC. The partition (K) and diffusion (D) coefficients are both key parameters for modelling skin penetration through this barrier (Anissimov et al., 2013) when used in combination with other physicochemical properties. Although different approaches have been described in the literature, these parameters are usually measured under infinite dose conditions, which are required for the measurement of a steady state flux through the SC and other...
isolated skin layers. Therefore, the diffusion coefficient is measured by using a kinetic diffusion assay based on steady flux values (Hansen et al., 2008) and lag times (Modamio et al., 2000). These parameters can also be measured using Fick’s second law based on diffusion profiles through the skin (Todo et al., 2013) or distribution profiles in the SC (Herkenne et al., 2006).

Partition coefficients (K) are measured experimentally either with isolated SC sheets (Raykar et al., 1988; Hansen et al., 2008; Wang et al., 2010) or on powdered human plantar SC (Wester et al., 1987; Hui et al., 2005). While K values are relatively well published, there are only a few diffusion coefficient values available in the literature; therefore, skin penetration in silico models have often been built using solely partition coefficients (Vecchia and Bunge, 2002; Hansen et al., 2011), which can contribute to a lack of performance of these models. For example, Vecchia and Bunge (2002) evaluated 18 different equations to predict skin permeability using K values. The lower predictivity of these equations could have been due the use of K values only, as well as the different sources of data and protocols used (animal vs, human skin, different solvents, finite vs. infinite does etc). Therefore, improvement of the predictivity of an in silico model for K and D coefficients requires more and better quality data using standardized methods.

As part of a larger project on dermal bioavailability measuring the K and D coefficients for 50 cosmetics-relevant chemicals, we first determined which protocol(s) would be used. Three protocols have been established and validated in two laboratories, and all are routinely used to measure K and D coefficients of cosmetics relevant chemicals (data not shown). The selection was then based on a number of the assay attributes, including relevance, reproducibility, practicality, ability to measure kinetics, and the relevance to in vivo skin. Protocol 1 was based on tape stripping study on ex vivo full thickness skin. The profile in the SC was then fitted to the determined partition coefficient for the SC in the vehicle (KSCV) and the diffusion coefficient in the SC (DSC) (Herkenne et al., 2006). Protocol 2A was used to measure KSCV only and Protocol 2B was used to measure KSCV and DSC. In method 2A, isolated SC was immersed in a solution of test chemical and the KSCV was directly measured as the ratio of the compound concentration in the tissue (isolated SC) versus the compound concentration in the buffer (i.e. the vehicle, DPBS) at equilibrium (after 24h). As the SC is very hygroscopic, dry SC was used in Protocol 2A to minimize the weight interference by water. The measured partition coefficient, defined as the mass of chemical per unit mass of dry SC relative to the mass of chemical in buffer per volume of buffer, needs to be corrected for tissue hydration and tissue density. This conversion is afforded by Nitsche et al. (2006), giving a partially hydrated value to reflect the in vivo skin hydration status. The second assay (2B) involved the use of isolated SC in an in vitro skin penetration cell. Determining DSC along with associated parameters, was based primarily on a non-linear regression of the accumulated penetration data of the solute migrating through the SC into the receptor fluid, relative to Fick’s 2nd law. It also involved the direct measurement of the SC thickness, HSC.

The chemicals tested in these assays were caffeine, resorcinol and 7-ethoxycoumarin (7-EC), the physicochemical properties of which are listed in Table 1. These chemicals were selected to ensure a large logP range was covered (~0.07 to 2.3), which, in our studies, partially correlates with K values (data not shown). All three chemicals were stable in the frozen human skin (Jacques-Jamien et al., 2016). They were also tested at the same time in skin penetration studies using human and pig skin in two laboratories (Gerstel et al., 2016). In addition, resorcinol was selected because it is a cosmetics ingredient and a known skin sensitizer (Basketter et al., 2007); and caffeine is a cosmetics ingredient and is a standard model chemical used for skin absorption assays and in silico modeling (Van de Sandt et al., 2004; Danck et al., 2013). Although only three chemicals were tested in this comparison, we considered this number sufficient to make a conclusion on which assay to use for further testing, since in addition to data comparisons, multiple practical aspects were evaluated, as mentioned above.

Pig skin has been used as a surrogate for human skin due to their structural, physiological and biochemical similarities (Simon and Maibach, 2000; Herkenne et al., 2006; Barbero and Frasch, 2009; Jung and Maibach, 2015) and is accepted by the Scientific Committee on Consumer Safety (SCCS) for use in skin penetration studies (SCCS, 2010). Therefore, we investigated whether pig skin (obtained as waste from the food industry) could be used as an alternative source of SC for KSCV and DSC measurements if the human skin was not available in sufficient quantities.

### Methods

### Chemicals

The same lot numbers of cold chemicals were used by both laboratories. The cold chemicals, 7-ethoxycoumarin (CAS 31005-02-4), caffeine (CAS 58-08-2) and resorcinol (CAS 108-46-3), were from Sigma-Aldrich (St Louis, MO, US). Radiolabelled chemicals were from ARC, Saint Louis, MO, USA, and were the same as those used in the skin penetration studies (Gerstel et al., 2016): 14C-7-ethoxycoumarin (7-ethoxycoumarin [phenyl ring-14C(U)]; specific activity: 77 mCi/mmol);14C-caf-feine (caffeine [8-14C]; specific activity: 17.5 mCi/mmol); and 14C-resorcinol (resorcinol [14C(I)]; specific activity: 194 mCi/mmol). All chemicals were tested in this comparison, we considered this number sufficient to make a conclusion on which assay to use for further testing, since in addition to data comparisons, multiple practical aspects were evaluated, as mentioned above.

The solvent for caffeine and resorcinol was ethanol/proplylene glycol/water (5/5/90) and the solvent for 7-EC was 1% ethanol in Dulbecco’s Phosphate-Buffered Saline [DBPS+ (with calcium and magnesium)] supplemented with 0.5 mg/ml sodium azide. For Protocol 2A and B, radiolabelled chemicals were mixed with cold

### Table 1. Physicochemical properties of caffeine, resorcinol and 7-EC. Predicted logP values using the SRC PhysProp Database, EpiWeb - WSKOW v1.41 (“a”) or BioByte v5.2 – ClogP (“b”)

| Chemical          | CAS number | MW       | logP    | Water solubility (mg/ml) | Melting Point (°C) |
|-------------------|------------|----------|---------|--------------------------|--------------------|
| Caffeine          | 58-08-2    | 194.2    | -0.07 (exp⁸) | 17.5                     | 194 (exp⁹)        |
| Resorcinol        | 108-46-3   | 110.1    | 0.8 (exp⁸) | 504                      | 111 (exp⁹)        |
| 7-Ethoxycoumarin  | 31005-02-4 | 190.2    | 2.3 (calc²) | 0.778                     | 92 (exp⁹)         |

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chemicals to achieve the final concentrations. The final dosing concentrations of cold and radiolabelled 7-EC, caffeine and resorcinol were 0.02%, 1% and 1% (w/v), respectively.

**Skin tissue**

For Protocol 1, the full-thickness human skin was obtained with consent from four donors undergoing abdominal plastic surgery (2 samples per donor, 3 females and 1 male, donor age ranged between 32 and 57). Flank pig skin was obtained from a local slaughterhouse (France). Pig and human skin were frozen at −20°C after sampling and stored at this temperature until use. Before use, hair was shaved from the pig skin using an electric razor and the thickness was adjusted to between 2 and 3 mm. After partial thawing of the skin, the fatty layer was removed using a surgical blade. For protocol 2, human cadaver skin (consented for research) from three donors (4 samples from the back or thigh per donor, female, Caucasian, donor age 40–65 years) was obtained from Allosource® (Centennial, CO, USA) and stored at −80°C for less than 6 months in a cryoprotective medium (containing glycerin, buffer and DMSO) to protect against freeze/thaw damage. The integrity of the SC was tested according to the method of Davies et al. (2004) using electrical resistance measurements. The electrical resistance of SC during dosing of these compounds was always greater than 3.94 kΩ/cm², the cut-off value reported by Davies et al. (2004) for whole skin. Although the cut-off value suggested by Davies et al. (2004) was specific to whole skin and not SC, historical data from our lab has shown that the electrical resistance for SC and dermated skin are similar and thus, the SC electrical resistance greater than 3.94 kΩ/cm² indicates that storage at −80°C did not compromise the skin integrity – or the SC integrity (which was measured during the course of the assay). The number of donors used in these studies is in keeping with OECD (at least three replicates to obtain an indication of variability) and SCCS (four donors) guidelines for skin penetration studies (OECD, 2004a and b; SCCS, 2010).

**Protocol 1**

**Overview of the method.** Protocol 1 was conducted on full-thickness skin with the topical application of the test chemical and measurement of the concentration-depth profile of a chemical in the SC by tape stripping after a specific exposure period (Herkenne et al., 2006). The penetration profile of a topically applied chemical can then be determined by a combination of tape-stripping and accurate measurement of the amount of SC, together with analysis of the amount of chemical present in each strip (Kalia et al., 1996; Bunge et al., 2006). In this protocol, the SC thickness, HSC, is a key parameter, which was measured by tape stripping in combination with transepithelial water loss (TEWL). As the corneocyte layers are removed from the SC, its barrier function is decreased, and this can be monitored by measuring TEWL before and after each tape strip until the rate of water loss reaches 4 times its initial value. The amount of water that evaporates from the skin surface increases as the SC barrier is damaged (Fluhr et al., 2006). When the entire SC barrier is lost, this is reflected in the TEWL measurement. The total SC thickness can be determined according to the change in TEWL as a function of the thickness of SC stripped, which is fitted to the equation according to Fick’s first law.

**Dermal penetration and SC sampling.** Skin discs (32 mm diameter) were mounted onto Franz diffusion cells, filled with 9 g l⁻¹ NaCl and allowed to equilibrate for 1 h at 32°C with stirring. An infinite dose of chemical was applied (350 μl cm⁻²) to the surface of the skin. After 30 min, the excess dosing solution was removed and the skins were dried with tissue. An area (18 mm diameter) of the SC was removed by tape stripping using pre-weighted standard D-Square discs (22 mm diameter). To reduce uncertainty on DSC the tape stripping was carried out as quickly as possible. Criteria defined by Reddy et al. (2002) for tape stripping time were adhered to. A total of 15 strips were removed and weighed before extraction of the chemical using methanol.

**Analytical method.** The extract solution was directly injected onto an LC/MS-MS system (Shimadzu Nexera LC system with a CTC PAL Autosampler coupled with a mass spectrometer API 3200 (ABSciex, Concord, Ontario, Canada). The analytical system was managed by the software Analyst version 1.6. The analytical column used was a Kinetex C18 from Phenomenex (Torrance, CA, USA) (50 x 3.0 mm, dp. 2.6 μm) and analysis are carried out with gradient elutions with mobile phases of 20 mM of ammonium acetate (A) and acetonitrile (B). The column temperature was fixed at 50°C, the volume of the injection was 10 μl and the flow rate at 0.8 ml/min. Ionization mode used was electrospray positive for caffeine and 7-EC and negative for resorcinol. Multiple Reaction Monitoring (MRM) was used for detection of the following transition 138 → 95.1, 109.1 → 65.0 and 191.1 → 163.1 for caffeine, resorcinol and 7-EC, respectively.

Each analytical method was evaluated according to criteria used in the bioanalytical method of Bansal and DeStefano (2007). The specificity of the analytical method was controlled with blank strip extract. The limits of quantitation (LOQ) were 8.5, 9.6 and 5.5 ng ml⁻¹ for caffeine, resorcinol and 7-EC, respectively. Linearity was determined between the LOQ and 10000 ng ml⁻¹, with an accuracy below ±15%, except at the LOQ, which was below ±20%. Accuracy and precision was determined at least at three QC theoretical concentrations: low (at 20 ng ml⁻¹), middle (at 200 ng ml⁻¹) and high (at 3500 ng/ml for caffeine and 7-EC and 8000 ng ml⁻¹ for resorcinol) with six replicates. All QCs remained within the acceptance criteria (CV % < 15%, accuracy% < ± 15%).

Matrix effects and extraction recovery were evaluated at three concentrations (10000, 1000 and 100 ng ml⁻¹) in triplicate by spiking tape strips of untreated SC with known amounts of chemicals and then extracting with methanol. The total recovery including matrix effect of caffeine, resorcinol and 7-EC were 110.0 ± 6.5%, 63.2 ± 10.9% and 92.8 ± 6.7%, respectively. A matrix effect was observed for resorcinol (which accounts for its lower total recovery); therefore, all calibrations for this chemical were carried out in the matrix.

**Determination of chemical concentration profiles.** The passive diffusion of a chemical through the SC is governed by Fick’s 2nd law, which can be solved by Eqn (1) when an infinite dose is used.

$$C_n = K_{SC} C_0 \left[ 1 - \frac{x}{H_{SC}} - \frac{2}{\pi} \sum_{m=1}^{\infty} \frac{1}{m^2} \sin \left( \frac{nx}{H_{SC}} \right) \exp \left( - \frac{D_{SC}}{H_{SC}^2} \pi^2 m^2 t \right) \right]$$  \hspace{1cm} (1)

where C is the concentration of the chemical, D is the diffusion coefficient, x is the position relative to the SC surface; n is a natural number, HSC is the total SC thickness and t is the exposure time. The curve of the penetration profile can be fitted to Eqn (1) r to estimate the KSC and DSC/HSC.

The depth in the SC (”x”) in which the amount of chemical was present in the nth tape of a total of 15 is expressed according to Eqn (2).
Diffusion and partition coefficients in the stratum corneum protocols

\[
x = \sum_{i=1}^{n} e_i
\]  

(2)

The thickness of SC removed after each strip was calculated using Eqn (2).

\[
e_i = \frac{m_i}{\rho_{SC} \times 5ST}
\]  

(3)

where \( m_i \) is the SC mass of the \( n \)th tape strip, \( \rho_{SC} \) is the SC density (1 g/cm³) (Anderson et al., 1976) and \( 5ST \) is the surface of the stripping area (2.54 cm²). The density of the SC is an estimate used by others (Russell et al., 2008). It may vary as a function of depth depending on the level of hydration (Egawa et al., 2007) which could lead to some uncertainties of the estimation of the SC thickness. The concentration of a chemical in each strip is then plotted as a function of the relative depth in the stratum corneum (i.e., \( x/H_{SC} \) with \( H_{SC} \) the SC thickness).

**Total SC thickness – TEWL measurements.** TEWL measurement combined with tape stripping was used to measure the SC thickness (i.e. \( H_{SC} \) ) (Kalia et al., 2001). The TEWL was measured using a Biox Aquaflux AF200 closed-chamber evaporimeter before and after each tape strip was removed until the rate of water loss reached 4 times its initial value. The change in TEWL as a function of the SC thickness was fitted to Eqn (4) (according to Fick’s 1st law).

\[
\text{TEWL} = \frac{1}{H_{SC}} \times K_{SC,w} \times D_{SC,w} \times \Delta C_w
\]  

(4)

where \( x \) is the thickness of the SC removed, \( H_{SC} \) is the total SC thickness, \( K_{SC,w} \) is the SC-viable tissue partition coefficient of water, \( D_{SC,w} \) is the diffusion coefficient of water in the SC and \( \Delta C_w \) is the water concentration gradient between superior and inferior surfaces of the SC. In line with the updated protocol proposed by Russell et al. (2008), a simple non-linear model was used which also fits the data directly to Fick’s first law equation (Eqn (4)). When the TEWL tends to infinity, \( x \) tends to \( H_{SC} \).

**Protocol 2**

**Overview of the method.** Protocols 2A and 2B were used to measure \( K_{SC,v} \) and \( D_{SC} \). In method 2A, isolated SC was immersed in a solution of test chemical and the \( K_{SC,v} \) was directly measured as the ratio of the compound concentration in the tissue (isolated SC) versus the compound concentration in the buffer (i.e. the vehicle, DPBS) at equilibrium (after 24 h). As the SC is a very hygroscopic material, dry SC was used in Protocol 2A to minimize the weight interference by water. The measured partition coefficient, as defined as the mass of chemical per unit mass of dry SC relative to the mass of chemical in buffer per volume of buffer, needs to be corrected for tissue hydration and tissue density. This conversion is afforded by Nitsche et al. (2006), giving a partition coefficient (Eqn (5a)). A partition coefficient was calculated for each skin replicate. This directly measured partition coefficient value (PC) using dry SC weight was converted to a partially hydrated \( K_{SC,v} \) value according to Eqn (5a) described by Nitsche et al. (2006). A similar conversion by Nitsche et al. to a fully hydrated \( K_{SC,v} \) value was afforded using Eqn (5b).

\[
K_{SC,v} \text{(partially hydrated)} = \text{PC}/1.198
\]  

(5a)

\[
K_{SC,v} \text{(fully hydrated)} = \text{PC}/3.518
\]  

(5b)

The partially hydrated \( K_{SC,v} \) would be more indicative of in vivo skin, while the fully hydrated value would probably be more comparable to the state that exists when a subject is immersed in water (e.g. in a bath) or during the diffusion coefficient determination procedures lasting several hours (wet environment on both sides of the skin sample).

**Protocol 2B - Dermal penetration and determination of diffusion coefficient, \( D_{SC,w} \).** SC pieces were mounted on SuPor® membranes (0.22 µm pore size, Pall Corporation, Port Washington, New York, USA), and the SC thickness (\( H_{SC} \)) was measured between two microscope slides, using a digital micrometer (Conrad Electronic GmbH, Hirschau, Germany). The SuPor® membranes were used to provide an inert support, with nearly negligible resistivity, for the thin SC layer, thus keeping the SC level and uniform. The SC membrane was fitted onto flow-through diffusion cells (0.64 cm² exposed surface area), according to Hansen et al. (2008). The permeation experiments were conducted based on the OECD guidelines (SCCS, 2015; OECD, 2004a, b) with slight modifications. The
mounted cells were placed as a group inside a heated incubator (32°C) and equilibrated with receptor fluid (DPBS+) for at least 30 min before dosing with compound (receptor fluid flow rate of 25 μl/min, with a magnetic stirrer).

The integrity of the SC layers was confirmed according to the method of Davies et al. (2004) using electrical resistance measurements. Radiolabeled chemical (~800 μl) was then added to the donor chamber, which was occluded with Parafilm. Receptor fluid fractions from each cell were collected every 2 h up to a total of 22 h. Samples of the dosing solution and of the solution in the donor chamber were removed after the 22-h incubation to determine pre- and post-dose concentrations. The receptor fluid fractions were mixed with 15-mM sodium hydroxide and the amount of radioactivity was measured as all wash solutions were collected. The SC was dissolved in 1 M sodium hydroxide and the amount of radioactivity was measured as described above. The receptor fluid fractions were mixed with 15-mL Ultima Gold scintillation cocktail. All washes, rinses, swabs, Parafilm, dosing solutions, fractions, and dissolved stratum corneum and compound standards were mixed with a cocktail and counted in a Packard 2550 TR/LL liquid scintillation counter.

Successful samples had an overall mass balance of 100%.

The receptor fluid was mixed with a cocktail of Ultima Gold scintillation cocktail. All washes, rinses, swabs, Parafilm, dosing solutions, fractions, and dissolved stratum corneum and compound standards were mixed with a cocktail and counted in a Packard 2550 TR/LL liquid scintillation counter. Successful samples had an overall mass balance of 100 ± 10%.

Protocol 2B: Graphical manipulation to determine partition, KSCV and diffusion, DSC coefficients. SC thicknesses were measured manually with a digital micrometer. Steady state flux (JSS ng/cm²/h) was determined from the slope of the earliest linear portion of the plot (Hansen et al., 2008). Eqn (6) was used to calculate the flux of the SC alone, based on the relationship between resistance and flux (Zhang et al., 2009; Miller and Kasting, 2012):

\[ J_{SC} = (J_{SC + M} - J_M)/(J_M - J_{SC + M}) \]  

where M is the membrane and SC + M is the SC and the SuPor membrane together. The membrane was determined to have only negligible resistance. The permeability coefficient, kp, was calculated from the steady state flux (using the linear portion of the curve), and the donor fluid concentration, C, according to Eqn (7) (Zhang et al., 2009).

\[ k_p = J_{SC}/C \]  

The diffusion coefficient, DSC, can be calculated using three different approaches. First, using the lag time, tlag, which would be calculated from the x-intercept [Time (h)] of the linear portion of the plot. HSC, the thickness of the SC, would be directly measured by hand using a micrometer. The DSC could then be calculated using Eqn (8), which is from a term in the solution of Fick’s 2nd law:

\[ D_{SC} = H_{SC}^2/6\text{tlag} \]  

A second approach used Eqn (9) (Hansen et al., 2008) and the measured SC thickness, HSC, the KSCV measured in protocol 2a and the permeability coefficient, kp, measured at steady state.

\[ D_{SC} = k_p^*H_{SC}^2/K_{SCV} \]  

For the third approach, which was used for the reported values from Protocol 2B in Table 2, Fick’s 2nd law was used to determine KSCV and DSC. Specifically, KSCV = HSC and DSC/HSC² were calculated using data from Protocol 2B from the permeation of the chemical through the isolated SC by non-linear regression of the cumulative amounts absorbed per time (Q) (using software JMP Pro 10 (SAS Institute)), according to Eqn (10). These values are reported in Table 2. (Protocol 2B).

\[ Q(t) = K_{SC}H_{SC}C_v \left[ \frac{D_{SC}}{H_{SC}^2} t - \frac{1}{6} - \frac{2}{\pi^2} \sum_{n=1}^{\infty} \left( -1 \right)^n \frac{1}{n^2} e^{-\frac{H_{SC}^2}{4D_{SC}t}} \right] \]  

The non-linear regression was performed against the cumulative penetration profile throughout the portion of the plot which showed good linearity and there was good mass balance (90–110%). Using the manually measured values of the SC thicknesses, individual values of KSCV and DSC were also calculated.

### Results

#### Comparison of protocols for the determination of the partition coefficient

KSCV values for caffeine and resorcinol from the tape stripping method (Protocol 1) and direct measurement (Protocol 2B) were within 30% of each other. Values obtained with protocol 2A were approximately two-fold higher (see Table 2). Whereas Protocol 1 and 2B measurements were carried out in the same conditions.

### Table 2. Diffusion and partition values in human and pig SC for caffeine, resorcinol and 7-EC measured using different protocols. Values are mean with the %CV in parentheses

| Chemical          | Caffeine | Resorcinol | 7-Ethoxycoumarin |
|-------------------|----------|------------|------------------|
|                   | KSCV     | DSC/HSC² (h⁻¹) | KSCV   | DSC/HSC² (h⁻¹) | KSCV       | DSC/HSC² (h⁻¹) |
| Protocol 1 Pig skin | 1.27 (31%) | 0.23 (49%)   | 5.18 (14%) | 0.19 (49%)   | 89.5 (4.6%) | 0.10 (44%)   |
| Protocol 1 Human skin | 2.68 (20%) | 0.21 (26%)   | 5.35 (28%) | 0.19 (37%)   | 39.5 (19%)  | 0.030 (55%)  |
| Protocol 2A Human skin | 5.88 (18%) | N.A.         | 8.41 (12%) | N.A.         | N.D.       | N.D.         |
| Protocol 2B Human skin | 2.63 (70%) | 0.056 (23%)  | 4.07 (47%) | 0.047 (9%)   | 0.019 (34%) | 0.078 (26%)  |
| Literature values | 5.62², 9.62¹ | N.D.         | 1.8², 3.6¹  | N.D.         | N.D.       | N.A.         |

¹Corrected with Eqn (5a); ²Equation (10) was used to calculate both Dsc/Hsc² and for KSCV; ³Hansen et al., 2008; ⁴Surber et al., 1990; ⁵Anderson et al., 1976; ⁶Wolfram and Maibach, 2005 N.A.: not applicable, N.D.: no data available from the literature.
manner (i.e. topical application of a solution), protocol 2A was quite different from the SC sheet was incubated in a buffer. Nevertheless, all values were of the same order of magnitude i.e. within a factor of 10. Protocol 2A is equivalent to the “flask shaking” method used to measure logP. The guideline (OECD, 1995) claims that the typical uncertainty for the water/octanol partition coefficient, measurement by flask shaking, is approximately a factor 2, and any difference between different protocols below this uncertainty means that data are equivalent.

An unexpected difference of three orders of magnitude was observed between Protocol 1 and 2B for the measurement of $K_{SC/V}$ for 7-EC. Whereas the $K_{SC/V}$ for caffeine and resorcinol was higher with protocol 2A compared to Protocol 1, the opposite was observed for 7-EC, with a higher value with Protocol 1. The variability (expressed as the %CV) for determination of $K_{SC/V}$ for all three chemicals by Protocol 2B was much higher compared to the tape stripping method (Protocol 1) and direct measurement (Protocol 2A).

**Comparison of protocols for the determination of diffusion coefficient**

The $D_{SC}/H_{SC}$ values for the three model chemicals were of the same order of magnitude using all three protocols. The $D_{SC}/H_{SC}$ values for caffeine, resorcinol and 7-EC in human and pig SC ranged between 0.06-0.23 h$^{-1}$, 0.05-0.19 h$^{-1}$ and 0.03-0.1 h$^{-1}$, respectively (Table 2).

In Protocol 2B, the rate of the accumulative absorption of caffeine and resorcinol through the SC into the receptor fluid was relatively constant over the incubation time (Fig. 1A and B). In contrast, the flux of 7-EC deviated slightly from linearity after 10 h (especially in skin discs with lower absorption); therefore, the initial flux rate was taken from the first part of the curves. As previously described, two sets of equations can be used to measure $K_{SC/V}$ and $D_{SC}$. Using Fick’s 2nd law, $D_{SC}$ can be calculated using either the lag time (Eqn (8)) or by combining $k_p$ and known $K_{SC/V}$ (Eqn (9)). Using the Fick’s 2nd law, $K_{SC/V}$ and $D_{SC}$ can be calculated using a non-linear regression with Eqn (10). Parameters determined from the graphs using protocol 2B are shown in Table 3 and were used to calculate $K_{SC/V}$ and $D_{SC}$ values, which are shown in Table 2 and are compared with the corresponding values measured using Protocol 1. When Eqn (8) was used to calculate $D_{SC}$, there was relatively high variability, because there were not enough data points to achieve an accurately measured value of the tlag (which contained some negative values for 7-EC, which were, in reality, not physically possible) (Table 3). A second approach using Eqn (9) (Hansen et al., 2008), calculated $D_{SC}$ from directly measured $H_{SC}$ and $K_{SC/V}$ (Protocol 2A) and $k_p$ from Protocol 2B, using non-linear regression of the cumulative amounts over time. This non-linear regression resulted in much lower variability in $K_{SC/V}$, $H_{SC}$ and $D_{SC}/H_{SC}$ values and was therefore used in comparing protocols from the two laboratories.

**Comparison of human and pig skin**

A comparison of $K_{SC/V}$ and $D_{SC}$ in human and pig skin was made using Protocol 1. The preliminary assay compared the SC thickness between pig and human using TELW measurement combined with tape stripping (Fig. 2). Using this method, the thickness of abdominal human and flank pig SC was determined to be 11 ± 1.1 μm ($n = 3$) and 10.8 ± 2.3 μm, respectively ($n = 4$). Pig and human skin were compared using Protocol 1 only. The difference between these types of skin was not more than 2-fold. Considering the small number of skin samples, no significant difference was observed between SC thickness on back pig skin and abdominal human skin.

Figure 3 shows the concentration-depth profiles for each of the model chemicals in SC from human skin (data not shown for pig SC). The profiles for caffeine and resorcinol were consistent with the theoretical Eqn (1), such that the concentration decreased to...
zero as the depth neared the final layers of the SC. By contrast, the concentration of 7-EC in the lower SC layers did not decrease to zero and remained constant in the lower SC layers. The curve fitting was therefore adjusted manually to fit the first tape strips, which accounted for the majority of the curve. The resulting values for $K_{SC}/v$ and $D_{SC}/H_{SC}^2$ for each chemical in human and pig SC are shown in Table 2. The concentration-depth profiles in pig SC were not significantly different from those in human SC; however, the $K_{SC}/v$ and $D_{SC}/H_{SC}^2$ for 7-EC were two- and three-fold higher in pig than human SC, respectively.

Protocol 1: Effect of exposure time

As seen in Fig. 3C, at 30 min, the curvature for the 7-EC curve was stronger than caffeine and resorcinol. Moreover, the concentration of 7-EC in the lower SC layers did not tend to zero and such behaviour is not consistent with the theoretical profile. One hypothesis was that the 30-min exposure was too short. Therefore, the protocol was repeated using skin from a single human donor and a 90-min exposure period (Fig. 4). The curvature was less pronounced, in keeping with the theory. Despite the longer time, the concentration of 7-EC in the lower SC layers remained constant. Moreover, the deviation between experimental and theoretical fitting became more pronounced in deeper SC layers. Thus, fitting to Eqn (1) was done using the first strips to estimate $K_{SC}/v$ and $D_{SC}/H_{SC}^2$. Despite the uncertainties of the fitting, the coefficients were unchanged ($K_{SC}/v$ was $34.5 \pm 6.4$ and $46.0 \pm 1.4$ with a 30 and 90 min exposure, respectively and $D_{SC}/H_{SC}^2$ was $0.03 \pm 0.01$ and $0.08 \pm 0.04$ with a 30 and 90 min exposure, respectively). No clear explanation was found for the deviation to the theory.

Discussion

These studies were designed to determine whether the partition and diffusion coefficients in SC could be measured (a) using protocols with different incubation and sample collection methods and (b) using pig skin should the supply of human skin become limited. The methods used employed both label-free (Protocol 1) and radiolabelled (Protocols 2A and B) chemicals, which is unlikely to have an impact on the comparisons made here as the limit of quantitation was not a limiting factor for either analytical method. Indeed, the protocols could theoretically be used with either label-free or radiolabelled chemical, providing the analytical method was shown to exhibit sufficient sensitivity. Moreover, the cutaneous absorption profiles of these three chemicals are not affected by the detection method, as shown by Gerstel et al. (2016). In the same way, cutaneous distribution obtained on pig skin is very similar to those obtained on human skin.

The $K_{SC}/v$ values for resorcinol measured with both pig and human SC was within two-fold across the assays (between 4.07 and 6.78).
8.41 using all three protocols), which we considered to be within the uncertainty of the protocols. Similarly, an uncertainty of two-fold is also applied for the measurement of octanol/water partition coefficients (OECD, 1995). The difference in KSC/v values is supported by the data available in the literature (Table 2). Measured esorcinol Ksc/v values using Protocols 1, 2A and 2B with human skin were in the range 1.1 to 4.7-fold of the values reported by Anderson et al. (1976) and Wolfram and Maibach (2005) (Table 2). The measured KSC/v values for caffeine using Protocol 1 with pig and human skin (1.27 and 2.68, respectively) are equivalent, and all results obtained on human skin, whatever the protocol used, are also equivalent (between 2.63 and 5.88). The range of KSC/v values for caffeine using Protocols 1, 2A and 2B with human skin were within the range 1.1- to 3.7-fold of the value reported by Hansen et al. (2008) and Surber et al. (1990). The KSC/v value for 7-EC was similar in pig and human SC (89.5 and 39.5, respectively) using Protocol 1 and in human skin using Protocol 2A with direct measurement of KSC/v but not using Protocol 2B. Such a finding was unexpected, especially since protocol 2B employed non-linear regression and Protocol 1 used similar equations. Flux out of the SC is calculated by derivating Eqn (1) as a function of time for x = HSC. This flux is then integrated as a function of time to obtain Eqn (10). These equations can only be used for the infinite condition of use (which were adhered to in these studies) and sink conditions. Sink conditions mean that chemical diffusion is not limiting by its solubility in the receptor fluid or any binding with any components of the set-up or the skin. Solubility in the receptor fluid could be excluded as a possible explanation for the lack of correlation of the value of KSC/v for 7-EC using Protocol 2B vs. Protocols 1 and 2A, which was demonstrated in the previous study (Gerstel et al., 2016). Some degree of unexpected binding could explain the deviation of the concentration-depth profile observed with Protocol 1 from the theoretical profile (i.e. the concentration of 7-EC in the lower SC layers did not decrease to zero and remained constant in the lower SC layers). Increasing the exposure time did not modify this behaviour. This effect could be related to lipophilicity of 7-EC (the predicted logP is 2.3 – compared to the relatively hydrophilic resorcinol and caffeine, which had a logP of 0.8 and -0.07, respectively), which prevented it from entering the relatively

Figure 3. Stratum corneum (SC) concentration versus relative SC depth profiles for caffeine (A), resorcinol (B) and 7-EC (C) after 30 min exposure time with human SC in protocol 1. Experimental data for each donor are described by ○, ●, △, ▲, respectively. The corresponding curves fitted to Eqn (1) are described by ---, ---, ---, ---, ---. The relative depth is defined as the fraction of the total thickness of the SC, where 1 is equivalent to the distance between the first tape strip and the last strip taken before reaching the epidermis.

Figure 4. Stratum corneum (SC) concentration versus relative SC depth profiles for 7-EC after 30 and 90 min exposure times with human SC in Protocol 1. The comparison was made using one donor in duplicate. Experimental data at 30 and 90 min are described by ○, ●, △, ▲, respectively. Mean experimental fitting and theoretical fitting defined by Eqn (1) are described by --- and --- at 30 and 90 min, respectively. The relative depth is defined as the fraction of the total thickness of the SC, where 1 is equivalent to the distance between the first tape strip and the last strip taken before reaching the epidermis.
hydrophilic environment of the epidermis. As no another lipophilic compound was evaluated, it’s difficult to make a firm conclusion on this assumption. Nevertheless, this chemical exhibited other unexpected outcomes, such as a lower mass balance observed with Protocol 2B. Thus, the value obtained with Protocol 2B is not consistent with classical Potts & Guy relationship; however, no clear explanation for this was confirmed. Possible reasons are the protocol, the skin sample preparation, or a specific behaviour of 7-EC itself.

An advantage of using protocol 2A is that the $K_{SCV}$ determination is a direct measurement that is robust with a small variation. Nevertheless, the value obtained includes any potential adverse binding. The ratio of the $K_{SCV}$ referred to dried SC weight; however, the SC in vivo is not dried; therefore, the value is then corrected considering volume variation between partial or full hydration and dried SC using the equation of Nitsche et al. (2006). Different correction factors reflecting dry (factor of 1), partially dry (factor of 1.198) and fully hydrated (factor of 3.52) result in lower $K_{SCV}$ values with increasing hydration (for example, the $K_{SCV}$ values for resorcinol are 10.08, 8.41 and 2.86 for dry, partially hydrated and fully hydrated skin, respectively). The values of $K_{SCV}$ for the partially hydrated SC would be best suited for direct in vivo comparisons, while those for the fully hydrated SC would be best suited for comparisons and modelling using a skin which is fully hydrated, such as found in infinite dose skin penetration studies.

The $D_{SC}/H_{SC}^2$ values for the three model chemicals were similar using Protocols 1 and 2B. In Protocol 2B, the calculation of $D_{SC}$ can be made using either $t_{lag}$ and $H_{SC}$ or $k_p, H_{SC}$ and the directly determined $K_{SCV}$ value. Although there is variability due to the determination of the $k_p$ and $H_{SC}$ values, the $K_{SCV}$ value is directly determined in Protocol 2A, and no assumptions or calculations are necessary. By contrast, calculations based on the penetration kinetics in Protocol 1 and 2B assumes that sink conditions are respected, and no covalent or non-covalent binding occurs with the SC. The chemical should not significantly modify the barrier function properties of the SC as a function of time. If such adverse effects do take place, Fick’s law cannot strictly be used. Otherwise, it could lead to undesired variation or inaccurate parameters. In addition, Protocol 1 is conducted under conditions that are nearer to the in vivo application and does not involve separation of the skin layers. When using the non-linear regression analysis method in protocol 2B, the values for $D_{SC}/H_{SC}^2$ had relatively low variability for each of the tested compounds versus those in Protocol 1, with %CVs ranging from only 9.2% to 26% vs. 44% to 49%. Using the direct measurement of $K_{SCV}$ from protocol 2A provided a more precise determination of $D_{SC}/H_{SC}^2$ in Protocol 2B, compared to the values determined in Protocol 1.

One critical aspect of the Protocol 1 that was addressed was the exposure time. This is very important because it influences the curvature of the concentration-depth profile. For example, if the exposure is too long, the exponential factors in Eqn (1) become negligible, and the profile becomes linear, and $D_{SC}/H_{SC}^2$ no longer applies to the equation and therefore cannot be determined. Conversely, if the exposure time is too short, the curvature of the profile is too pronounced, and the concentration of the chemical in

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**Table 4. Comparison of protocols**

- **Application method:** In Protocol 1 and 2B, the dosing is topical and therefore relevant to the exposure to the skin. Different formulations can be tested using Protocol 1 and 2B but not using Protocol 2A (since the incubation is in DPBS). Moreover, penetration enhancers should be avoided for Protocol 2B.

- **Establishment in labs:** Easy to implement. Protocol 1 has been described in the literature (Herkenne et al., 2006). Its transferability and reproducibility has been evaluated (data not shown). Protocol 2A is simple and Protocol 2B is based on a standard skin penetration study for which test guidelines exist.

- **Throughput:** For $K_{SCV}$ measurement, the highest throughput is obtained with a simple partition protocol (i.e. Protocol 2A). Unlike protocol 2B, Protocol 1 would require a pre-test to identify the correct exposure time as well as LC-MS analysis to quantify the unlabelled chemicals, thus limiting the throughput.

- **Skin preparation for test chemical application:** In Protocol 1, the skin remains in its native state; whereas, in Protocol 2A and B, the SC (and any additional layers) must be separated from each other. The use of native skin reduces preparation time for the experiment and may better represent the in vivo architecture of the skin than skin layers since the presence of the epidermis and dermis may impact the diffusion of the chemical through the skin (based on the more hydrophilic nature of the environment compared to the SC). The measurement using skin layers allows for a more empirical measurement in each layer without the impact of other layers. Both intact native skin and SC layers can be used in Protocol 1 and 2B for the topical application of chemicals in different formulations.

- **Application to different skin layers:** Individual layers of the skin can be tested for both $K_{SCV}$ and $D_{SC}$ in Protocol 2A and B but only values for the SC can be measured in Protocol 1 (not the epidermis or dermis).

- **Measurement considerations:** For Protocol 1 only, the exposure times may need adjusting according to the chemical. There is some uncertainty of the SC thickness determined by weighing individual tape strips in Protocol 1. In Protocol 2A and B, the thickness of the SC and other layers is afforded by a direct measurement. Unlike Protocol 2B, in Protocol 1, the skin layers require no mounting or structural support. In Protocol 2A for $K_{SCV}$ the SC is accurately weighed by removing any added water by drying. This gives a more accurate measurement of the tissue layer mass.

- **Data handling:** For Protocol 1, manual adjustment of curve fitting is sometimes needed. For Protocol 2A, a correction to partially hydrated SC is afforded by equation of Nitsche et al. (2006). This correction for hydration is not necessary for other tissue layers. The determination of $K_{SCV}$ in Protocol 2B is based on an indirect determination, using non-linear regression analysis method and results in more variable values. By contrast, in Protocol 2A, $K_{SCV}$ determination is a direct measurement and exhibits low variability. In Protocol 2B, using the graphical analysis method, D determination was slightly more variable and less well correlated with the spread of the data.
the SC layers is too low to be able to quantify. The exposure time should also be optimized according to the chemical tested. In line with this, the optimal exposure time for 7-EC was addressed in these studies. The curve using 30 min incubation time was considered to be too short, suggesting the exposure time could be increased to improve the accuracy of the measurement; however, an additional hour of exposure did not change the outcome of the assay.

There are a number of advantages of each protocol used in these studies, and these are summarized in Table 4. One of the main aspects of Protocol 1 is that it uses the whole native skin, which allows the measurements to be made using skin with the same architecture as that in vivo. Once the initial test concentration is optimized in pre-test(s), the incubation and sample preparation involved in Protocol 1 may be less time-consuming (main procedure is complete in <3 h), although the analysis of the samples taken longer (as the chemicals are unlabelled) than those in Protocol 2A and B (which employ radiolabelled chemicals). In contrast, Protocol 2A and 2B involve much longer incubation times (up to 24 h) and use isolated SC layers that take some time to prepare and may not directly reflect the in vivo situation. The use of isolated skin layers, though, comes with notable advantages, such as low variability in the data generated for $K_{SCV}$ and the possibility to apply the same procedures to the other isolated skin layers (unlike Protocol 1). Moreover, two different values are obtained for $K_{SCV}$ based on different models and assumptions.

A comparison of measured parameters from pig and human skin using Protocol 1 support the findings of Gerstel et al. (2016) such that the distribution of chemicals was similar in pig and human skin. Indeed, there were only small differences observed in the values generated for the two species, especially for caffeine and resorcinol. A greater difference was observed for 7-EC, which showed some deviation from the theoretical profile. Interestingly, the SC thickness measured on full thickness pig skin using the TEWL protocol was not significantly different from the thickness of human SC. This result is consistent with other observations on SC thickness between pig and human skin (Herkenne et al., 2006), in contrast, a marked difference was observed between human skin from plastic surgery used in Protocol 1 (i.e. 10.8 ± 2.3 μm) and human cadaver skin measured in protocol 2B (i.e. 54 ± 10 μm). These differences could be due to a number of factors. First, in Protocol 1, the skin from plastic surgery was from the abdomen; whereas, cadaver skin was taken from the back or the thigh for Protocol 2A and 2B. This may be a major contributory factor since skin penetration is known to be dependent on the anatomical site (Rougier et al., 1968; Wester et al., 2005). In both cases, the skin was frozen before use (at -20°C and -80°C, respectively).

Second, two different kinds of sample preparation were used to measure SC thickness: either on full thickness abdominal skin from plastic surgery or on isolated SC sheets from cadaver skin. Thirdly, two different protocols were used to measure SC thickness: an indirect measurement with tape stripping combined with TEWL for abdominal plastic surgery; and a direct measurement with digital micrometer for cadaver skin. Recent results (Grégoire et al., 2014) have shown that SC thickness measured with the indirect approach (tape stripping combined with TEWL) is correlated with a direct optical measurement (using Optical Coherence Tomography). Unfortunately, the methods used here cannot be directly compared; digital micrometers cannot be used on full thickness skin to measure SC thickness only, and tape stripping combined with TEWL cannot be used on isolated SC sheet. To identify the origin of the differences, an additional study could be carried out. Firstly, the SC thickness is measured on full thickness skin with tape stripping combined with TEWL. After this, the SC can be isolated from the same skin samples and the SC thickness measured with a micrometer. If no difference is observed, differences previously observed are likely to be related to the skin source (i.e. cadaver vs. plastic surgery). If a difference is observed, differences previously observed are likely to be related to either preparation or protocol measurement. No additional studies can be performed to distinguish between these two explanations for the reasons previously described. Fifteen human skins and three pig skins were measured. The percentage of the difference between the two methods was between -20% and 39%. The greatest differences were observed for the thinner stratum corneum. Thus, despite the indirect approach, the method by tape stripping combined with TEWL provides accurate values for SC thickness.

In conclusion, the protocols described here have advantages and disadvantages; however, they all produced similar values for $K_{SCV}$ and $D_{SC}$ for the three model chemicals, caffeine, resorcinol and 7-EC. These initial studies suggest that pig skin can be used as an alternative to human skin if sourcing of human tissue is limited.

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Conflict of interest

The authors state no conflict of interest.

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