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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 $K_{SC/v}$ and D_{SC}/H_{SC}^2, where H_{SC} is the SC thickness); Protocol 2A: incubation of isolated SC with chemical (direct measurement of $K_{SC/v}$ only) and Protocol 2B: diffusion through isolated SC mounted on a Franz cell (measures $K_{SC/v}$ and D_{SC}/H_{SC}^2, and is based on Fick's laws). $K_{SC/v}$ 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 $K_{SC/v}$ by Protocol 2B was different from the direct measurement of $K_{SC/v}$ by Protocol 2A and Protocol 1 for 7-EC. The variability of $K_{SC/v}$ for all three chemicals using Protocol 2B was higher compared to Protocol 1 and 2A. D_{SC}/H_{SC}^2 values for the three chemicals were of the same order of magnitude using all three protocols. Additionally, using Protocol 1, there was very little difference between parameters measured in pig and human SC. In conclusion, $K_{SC/v}$, and D_{SC} 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

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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 ($K_{SC/v}$) and the diffusion coefficient in the SC (D_{SC}) (Herkenne *et al*., 2006). Protocol 2A was used to measure $K_{SC/v}$ only and Protocol 2B was 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 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 D_{SC}, 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, H_{SC}.

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-Jamin *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* modelling (Van de Sandt *et al*., 2004; Dancik *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 $K_{SC/v}$ and D_{SC} 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-ethoxy coumarin (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): ^{14}C-7-ethoxycoumarin (7-ethoxycoumarin [phenyl ring-^{14}C(U)]; specific activity: 77 mCi/mmol); ^{14}C-caffeine (caffeine [8-^{14}C]; specific activity: 55 mCi/mmol); and ^{14}C-resorcinol (resorcinol [^{14}C(U)], specific activity: 55 mCi/mmol). The purity of the radiolabelled chemicals in the respective solvent was tested at 0 h and 24 h at 32°C and was 100%, 100% and >97.5% for ^{14}C-7-ethoxycoumarin, ^{14}C-caffeine and ^{14}C-resorcinol, respectively. All other chemicals and solutions used were from Sigma-Aldrich.

The solvent for caffeine and resorcinol was ethanol/propylene 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 (exp[a])	17.5	194 (exp[a])
Resorcinol	108-46-3	110.1	0.8 (exp[a])	504	111 (exp[a])
7-Ethoxycoumarin	31005-02-4	190.2	2.3 (calc[b])	0.778	92 (exp[b])

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/cm^2, 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 dermatomed skin are similar and thus, the SC electrical resistance greater than 3.94 kΩ/cm^2 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, $H_{SC,}$ 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-weighed standard D-Squame discs (22 mm diameter). To reduce uncertainty on D_{SC}, 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 validated 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 % $< \pm$ 15%, accuracy% $< \pm$ 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.

$$C_x = K_{SC/v} C_v \left[1 - \frac{x}{H_{SC}} - \frac{2}{\pi} \sum_{n=1}^{\infty} \frac{1}{n} \sin\left(n\pi \frac{x}{H_{SC}}\right) \exp\left(-\frac{D_{SC}}{H_{SC}^2} n^2 \pi^2 t\right)\right] \quad (1)$$

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, H_{SC} 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 $K_{SC/v}$ and D_{SC}/H_{SC}^2.

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).

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$$x = \sum_{i=1}^{n} e_i \quad (2)$$

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

$$e_i = \frac{m_i}{\rho_{SC} \times S_{ST}} \quad (3)$$

where m_i is the SC mass of the n^{th} tape strip, ρ_{SC} is the SC density (1 g/cm^3) (Anderson *et al*., 1976) and S_{ST} is the surface of the stripping area (2.54 cm^2). 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).

$$TEWL = \frac{1}{H_{sc} - x} \times K_{SC,w} \times D_{SC,w} \times \Delta C_w \quad (4)$$

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 ΔC_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- thelinear 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 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 D_{SC}, 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, H_{SC}.

SC preparation. The skin pieces (1.5 × 1.5 cm) were dropped into 60°C water for 40–60 s and the dermis was peeled away and discarded. The epidermis + SC was laid epidermal-side-down on a pre-wetted SuPor membrane (wetting agent: DPBS (without calcium and magnesium) with sodium azide at 0.5 mg ml^{-1}), which were then placed on filter paper soaked in trypsin solution (0.02% in DPBS- with sodium azide) for approximately 24 h at room temperature. After trypsinization, the intact SC was peeled away from the viable epidermis, leaving the disrupted viable epidermis attached to the membrane. Any remaining epidermal cells on the epidermal surface of the SC were gently removed with a cotton swab. The intact SC was then washed in a trypsin inhibitor solution (0.02% in DPBS+ with sodium azide), followed by three washes in DPBS+ with sodium azide, to fully stop the trypsin action.

Protocol 2A – Direct measurement of partition coefficient, $K_{SC/v}$. The SC was blotted dry, weighed, and dried under a stream of nitrogen gas for 24 h. The dried SC pieces were re-weighed and placed in 2 ml of the appropriate dose solution, similar to the procedure of Corley *et al*. (2005). The test vials were capped and gently rocked in a 32°C incubator for 24 h. After incubation, the SCs were removed from the buffer, gently blotted to remove the excess external moisture, and dissolved in 2 ml 1 N sodium hydroxide at 80°C. After the SC had dissolved, the solution was neutralized with 2 ml 1 M HCl. A volume of 15 ml Ultima Gold XR scintillation cocktail was added to each tissue solution, vortexed and the amount of radioactivity in the entire solution was counted in a Packard 2550 TR/LL liquid scintillation counter. The test buffers were centrifuged at 4000 rpm (3635 *g*) for 30 min and three 25-µl aliquots were mixed with 10 ml Ultima Gold scintillation cocktail. The amount of radioactivity was counted in a Packard 2550 TR/LL liquid scintillation counter.

The partition coefficient (PC) is a ratio of the compound concentration in the tissue (per gram of dried tissue weight) versus the compound concentration in the buffer at equilibrium (24 h). The dried tissue weight is used because of the futility in accurately measuring the hydrated/partially-hydrated weight of the SC piece. A minute amount of extraneous water being present can change the SC 'weight' several fold. 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).5a

5b

$$K_{SC/v}\ (\text{partially hydrated}) = PC/1.198 \quad (5a)$$

$$K_{SC/v}\ (\text{fully hydrated}) = PC/3.518 \quad (5b)$$

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 cm^2 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. Radiolabelled 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 diffusion chambers were disassembled and the SC, Parafilm used for occlusion, and 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 ± 10%.

Protocol 2B: Graphical manipulation to determine partition, $K_{SC/v}$ and diffusion, D_{SC} coefficients. SC thicknesses were measured manually with a digital micrometer. Steady state flux (J_{ss}, ng/cm^2/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):

$$J_{SC} = (J_{SC+M} * J_M)/(J_M - J_{SC+M}) \quad (6)$$

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, k_p, 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).

$$k_p = J_{ss}/C \quad (7)$$

The diffusion coefficient, D_{SC}, 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. H_{SC}, the thickness of the SC, would be directly measured by hand using a micrometer. The D_{SC} could then be calculated using Eqn (8), which is from a term in the solution of Fick's 2nd law:

$$D_{SC} = H_{SC}^2/6tlag \quad (8)$$

A second approach used Eqn (9) (Hansen *et al.*, 2008) and the measured SC thickness, H_{SC}, the $K_{SC/v}$ measured in protocol 2a and the permeability coefficient, k_p, measured at steady state.

$$D_{SC} = k_p * H_{SC}/K_{SC/v} \quad (9)$$

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 $K_{SC/v}$ and D_{SC}. Specifically, $K_{SC/v} * H_{SC}$ and D_{SC}/H_{SC}^2 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).

$$Q(t) = K_{SC} H_{SC} C_v \left[\frac{D_{sc}}{H_{SC}^2} t - \frac{1}{6} - \frac{2}{\pi^2} \sum_{n=1}^{\infty} \frac{(-1)^n}{n^2} e^{-\frac{D_{SC}}{H_{SC}^2} n^2 \pi^2 t}\right] \quad (10)$$

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 $K_{SC/v}$ and D_{SC} were also calculated.

Results

Comparison of protocols for the determination of the partition coefficient

$K_{SC/v}$ 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

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	
	$K_{SC/v}$	$D_{SC}/H_{SC}^2(h^{-1})$	$K_{SC/v}$	$D_{SC}/H_{SC}^2(h^{-1})$	$K_{SC/v}$	$D_{SC}/H_{SC}^2(h^{-1})$
Protocol 1Pig skin	1.27 (31%)	0.23 (49%)	5.18 (14%)	0.19 (49%)	89.5 (4.6%)	0.10 (44%)
Protocol 1Human skin	2.68 (20%)	0.21 (26%)	5.35 (28%)	0.19 (37%)	39.5 (19%)	0.030 (55%)
Protocol 2AHuman skin[a]	5.88 (18%)	N.A.	8.41 (12%)	N.A.	13.3 (8.0%)	N.A.
Protocol 2BHuman skin[b]	2.63 (70%)	0.056 (23%)	4.07 (47%)	0.047 (9%)	0.019 (34%)	0.078 (26%)
Literature values	5.62[c], 9.62[d]	N.D.	1.8[e], 3.6[f]	N.D.	N.D.	N.A.

[a]Corrected with Eqn (5a);
[b]Equation (10) was used to calculate both Dsc/Hsc^2 and for $K_{SC/v}$;
[c]Hansen *et al.*, 2008;
[d]Surber *et al.*, 1990;
[e]Anderson *et al.*, 1976;
[f]Wolfram and Maibach, 2005 N.A.: not applicable, N.D.: no data available from the literature.

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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}^2 values for the three model chemicals were of the same order of magnitude using all three protocols. The D_{SC}/H_{SC}^2 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}^2 values and was therefore used in comparing protocols from the two laboratories.

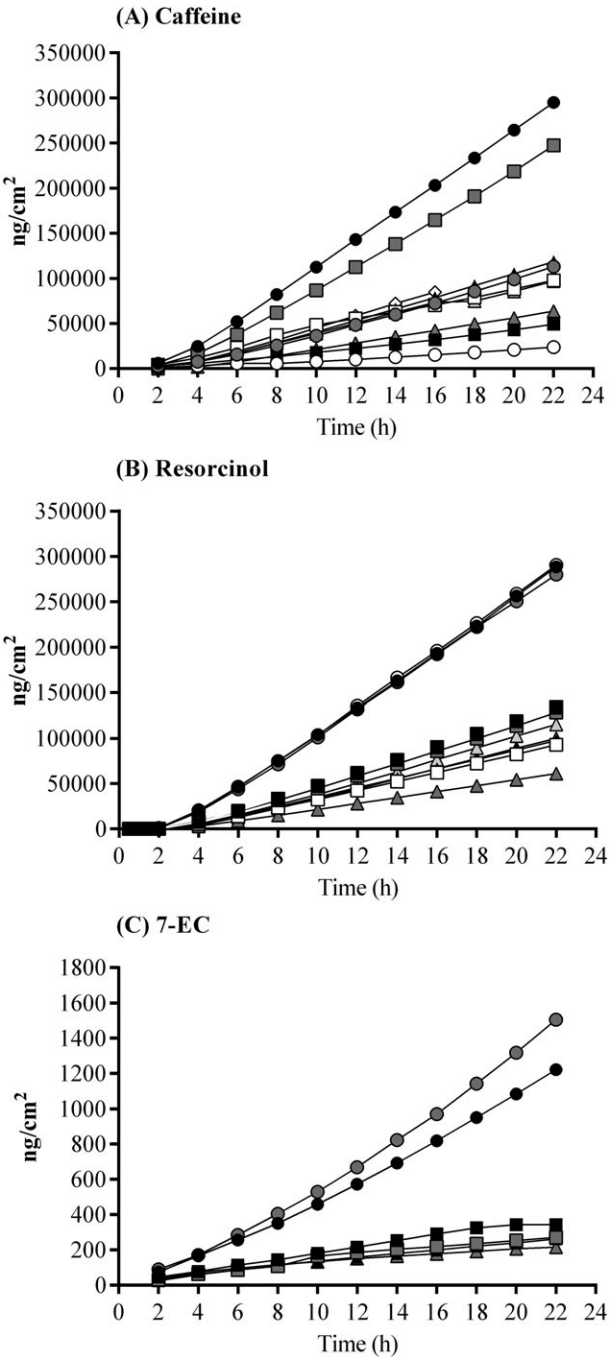

Figure 1. Measured cumulative penetration of resorcinol, caffeine and 7-ethoxycoumarin through human stratum corneum (SC) into receptor fluid. The different lines represent different cells with SC layers isolated from skin from 3 different donors, according to protocol 2B: Donor 1 replicate 1 (●), 2 (●) and 3 (○); Donor 2 replicate 1 (■), 2 (■) and 3 (□); Donor 3 replicate 1 (▲), 2 (▲), 3 (△) and 4 (△).

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 TEWL 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

Table 3. Parameter determinations for caffeine, resorcinol and 7-EC generated from the graphs in Fig. 1 and used to calculate D_{SC} values by protocol 2B

Parameter measured	Caffeine	Resorcinol	7-EC
t_{lag} (h)	1.82 ± 1.02	2.13 ± 0.16	0.001 ± 0.004
H_{SC} (μm)	53 ± 0.4	44 ± 0.9	56 ± 10
J_{ss} (ng/cm^2)	1261429	2699031	225
k_p (cm/h)	$5.56x10^{-4} \pm 4.00x10^{-4}$	$8.4x10^{-4} \pm 4.80x10^{-4}$	$3.42x10^{-4} \pm 6.78x10^{-4}$
Mass balance (%)	99.7 ± 1.3	99.2 ± 2.5	97.3 ± 1.4
SCs (n)	10	10	6

t_{lag} (h) is the lag time, as determined mathematically using the solution to Fick's 2nd law (non-linear regression of the cumulative amount penetrated vs. time data), J_{ss} is the steady state flux through the SC, k_p is the permeation coefficient, H_{SC} is the thickness of the SC and SCs (n) is the number of SCs with a recovery >95% used in the calculations.

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.

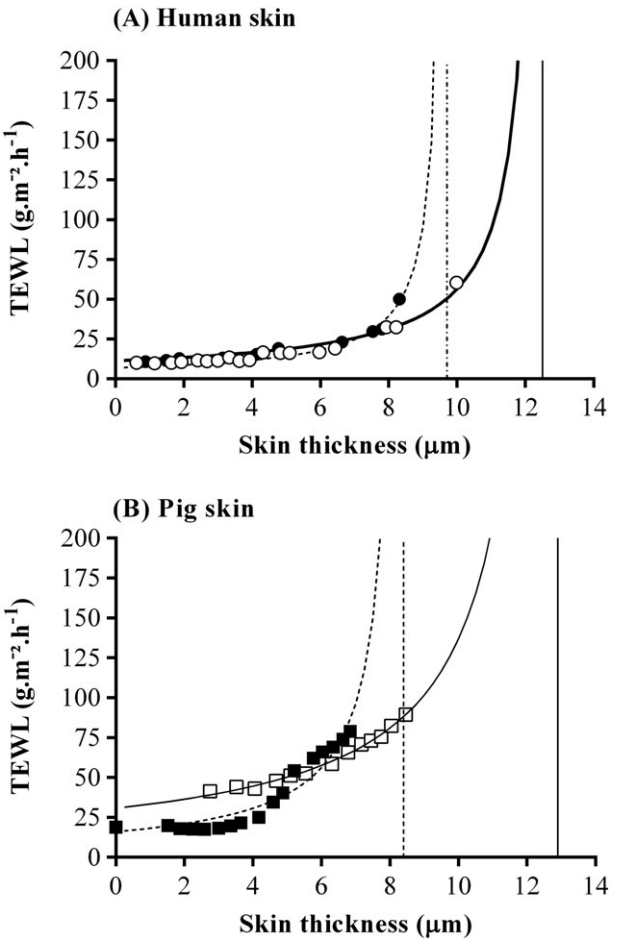

Figure 2. Total stratum corneum (SC) thickness of abdominal human (A) and back pig (B) skin measured by tape stripping and TEWL in protocol 1. For human skin, experimental data are described for two donors by ○ and ●, respectively. The corresponding curves fitted to Eqn (4) are described by ——, — · · ·. The vertical lines mark the estimated SC thickness values respectively at 12.5 and 9.7 μm. For pig skin, experimental data for two donors are described by □ and ■, respectively. The corresponding curves fitted to Eqn (4) are described by —, — · · ·. The vertical lines mark the estimated SC thickness values respectively at 8.4 and 12.9 μm.

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 ± 6.4 and 46.0 ± 1.4 with a 30 and 90 min exposure, respectively and D/H_{SC}^2 was 0.03 ± 0.01 and 0.08 ± 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

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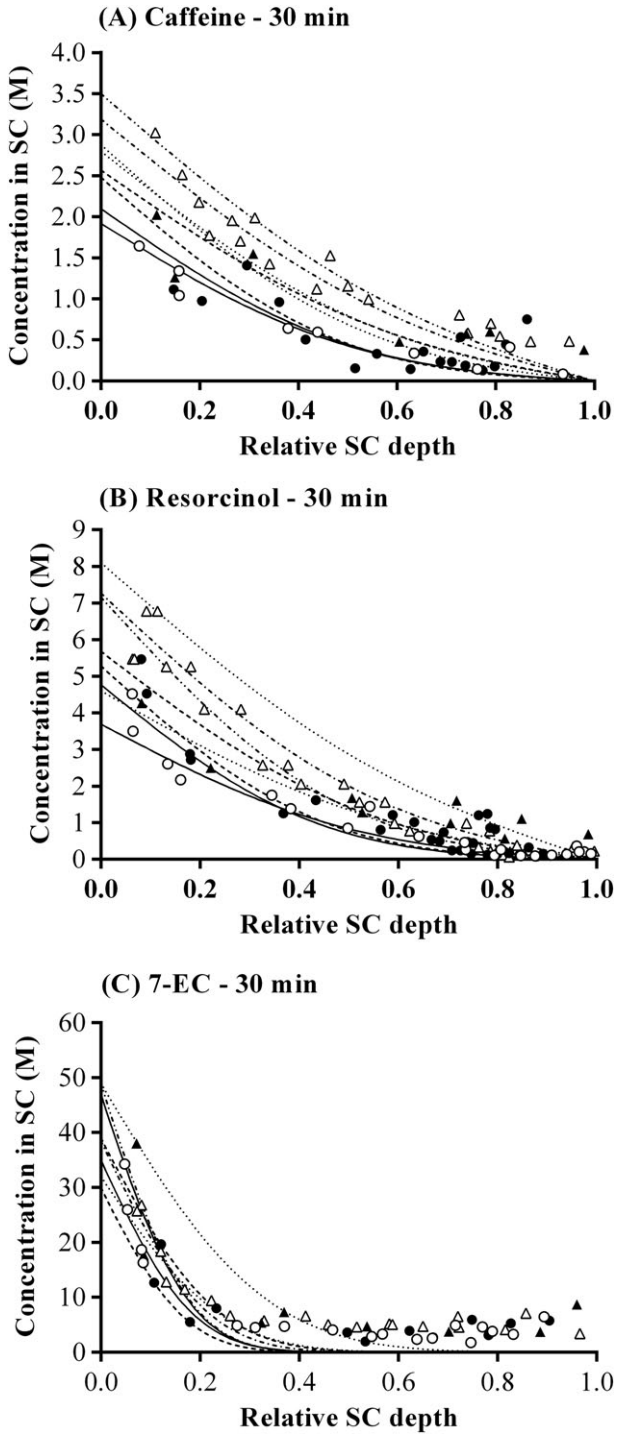

Figure 3. Stratum corneum (SC) concentration versus relative SC depth profiles for caffeine (A), resorcinol (B) and 7-EC (C) after 30 min exposure time with human SC in protocol 1. Experimental data for each donor are described by ○, ●, △. ▲, respectively. The corresponding curves fitted to Eqn (1) are 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.

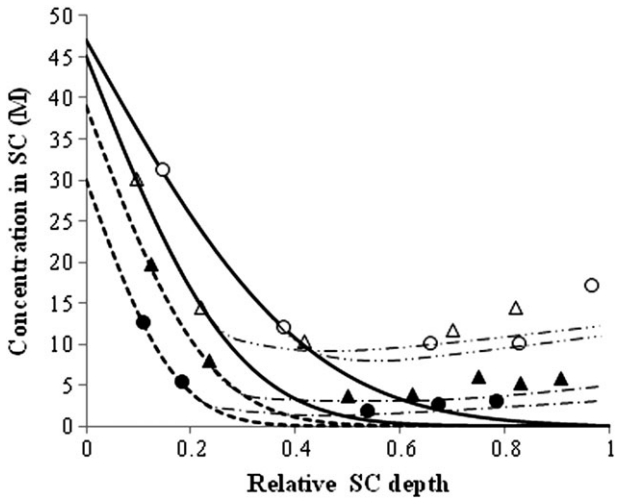

Figure 4. Stratum corneum (SC) concentration versus relative SC depth profiles for 7-EC after 30 and 90 min exposure times with human SC in Protocol 1. The comparison was made using one donor in duplicate. Experimental data at 30 and 90 min are described by ○, △ and ●, ▲, respectively. Mean experimental 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.

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 $K_{SC/v}$ 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 $K_{SC/v}$ 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 $K_{SC/v}$ 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 $K_{SC/v}$ 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 $K_{SC/v}$ 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 $x = H_{SC}$. 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 wereadhered 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 $K_{SC/v}$ 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

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 $K_{SC/v}$ 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 $K_{SC/v}$ 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 $K_{SC/v}$ values with increasing hydration (for example, the $K_{SC/v}$ values for resorcinol are 10.08, 8.41 and 2.86 for dry, partially hydrated and fully hydrated skin, respectively). The values of $K_{SC/v}$ 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 tlag and H_{SC}, or k_p, H_{SC} and the directly determined $K_{SC/v}$ value. Although there is variability due to the determination of the k_p and H_{SC} values, the $K_{SC/v}$ 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 $K_{SC/v}$ 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

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 $K_{SC/v}$ 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 $K_{SC/v}$ and D 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 $K_{SC/v}$, 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 $K_{SC/v}$ 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, $K_{SC/v}$ 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 takes 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 $K_{SC/v}$ and the possibility to apply the same procedures to the other isolated skin layers (unlike Protocol 1). Moreover, two different values are obtained for $K_{SC/v}$ 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. Firstl, 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. TTo 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 TWEL provides accurate values for SC thickness.

In conclusion, the protocols described here have advantages and disadvantages; however, they all produced similar values for $K_{SC/v}$ 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.

References

Anderson RA, Triggs EJ, Roberts MS. 1976. The percutaneous absorption of phenolic compounds 3. Evaluation of permeability through human stratum corneum using a desorption technique. *Aust. J. Pharm. Sci.* **NS5**: 107–1100.

Anissimov YG, Jepps OG, Dancik Y, Roberts MS. 2013. Mathematical and pharmacokinetic modelling of epidermal and dermal transport processes. *Adv. Drug Deliv. Rev.* **65**: 169–190. DOI:10.1016/j.addr.2012.04.009.

Bansal S, DeStefano A. 2007. Key elements of bioanalytical method validation for small molecules. *The AAPS Journal*. **9**: E109–E114. DOI:10.1208/aapsj0901011.

Barbero AM, Frasch HF. 2009. Pig and guinea pig skin as surrogates for human *in vitro* penetration studies: a quantitative review. *Toxicol. In Vitro*. **23**: 1–13. DOI:10.1016/j.tiv.2008.10.008.

Basketter DA, Sanders D, Jowsey IR. 2007. The skin sensitization potential of resorcinol: experience with the local lymph node assay. *Contact Dermatitis.* **56**: 196–200. DOI:10.1111/j.1600-0536.2006.01008.

Bunge AL, Touraille GD, Marty JP, Guy RH. 2006. Modeling dermal absorption from soils and powders using stratum corneum tape-stripping in vivo. In *Dermal Absorption Models in Toxicology and Pharmacology*, Riviere JE (ed). CRC Press; 191–212 .DOI: 10.1201/9780203020821.ch11

Corley RA, Bartels M, Carney E, Weitz KK, Soelberg JJ, Gies RA, Thrall KD. 2005. Development of a physiologically based pharmacokinetic model for ethylene glycol and its metabolite, glycolic acid, in rats and humans. *Toxicol. Sci.* **85**: 476–490. DOI:10.1093/toxsci/kfi119.

Dancik Y, Miller MA, Jaworska J, Kasting GB. 2013. Design and performance of a spreadsheet-based model for estimating bioavailability of chemicals from dermal exposure. *Adv. Drug Deliv. Rev.* **65**. DOI:10.1016/j.addr.2012.01.006.

Davies DJ, Ward RJ, Heylings JR. 2004. Multi-species assessment of electrical resistance as a skin integrity marker for in vitro percutaneous absorption studies. *Toxicol. In Vitro* **18**: 351–358. DOI:10.1016/j.tiv.2003.10.004.

Egawa M, Hirao T, Takahashi M. 2007. In vivo estimation of stratum corneum thickness from water concentration profiles obtained with Raman spectroscopy. *Acta Derm. Venereol.* **87**: 4–8. DOI:10.2340/00015555-0183.

Fluhr JW, Feingold KR, Elias PM. 2006. Transepidermal water loss reflects permeability barrier status: validation in human and rodent in vivo and ex vivo models. *Exp. Dermatol.* **15**: 483–492. DOI:10.1111/j.1600-0625.2006.00437.x.

Gerstel D, Jacques-Jamin C, Schepky A, Cubberley R, Eilstein J, Grégoire S, Hewitt N, Klaric M, Rothe H, Duplan H. 2016. Cosmetics Europe Skin Bioavailability and Metabolism project 1: Comparison of protocols measuring skin penetration in human and pig skin. *Toxicol. In Vitro* **34**. DOI:10.1016/j.tiv.2016.03.012.

Grégoire S, Jungman E, Formanek F, Cotovio J. 2014. 14th Perspectives in Percutaneous Penetration, La Grande Motte, France, 23-25 April.

Hansen S, Henning A, Naegel A. 2008. In-silico model of skin penetration based on experimentally determined input parameters. Part I: Experimental determination of partition and diffusion coefficients. *Eur. J. Pharm. Biopharm.* **68**: 352–367. DOI:10.1016/j.ejpb.2007.05.012.

Hansen S, Selzer D, Schaefer UF, Kasting GB. 2011. An extended database of keratin binding. *J. Pharm. Sci.* **100**: 1712–1726. DOI:10.1002/jps.22396.

Herkenne C, Naik A, Kalia YN, Hadgraft J, Guy RH. 2006. Pig ear skin ex vivo as a model for in vivo dermatopharmacokinetic studies in man. *Pharmaceut. Res.* **23**: 1850–1856. DOI:10.1007/s11095-006-9011-8.

Hui X, Wester RC, Zhai H, Maibach HI. 2005. Chemical partitioning into powdered stratum corneum: A useful in vitro model for studying interaction of chemicals and human skin. In *Percutaneous Absorption: Drugs-Cosmetics-Mechanisms-Methodology*, Bronaugh RL, Maibach HI (eds), Fourth edn. Marcel Dekker, Inc.: New York; 291–301.

Jacques-Jamin C, Duplan H, Rothe H, Viallant O, Eilstein J, Grégoire S, Cubberley R, Gerstel D, Klaric M, Hewitt N, Schepky A. 2016. Comparison of the dermal penetration of three metabolically stable chemicals using fresh and frozen human skin. *Skin Pharmacol. Physiol.* Submitted for publication.

Jung EC, Maibach HI. 2015. Animal models for percutaneous absorption. *J. Appl. Toxicol.* **35**: 1–10. DOI:10.1002/jat.3004.

Kalia YN, Pirot F, Guy RH. 1996. Homogeneous transport in a heterogeneous membrane: Water diffusion across human stratum corneum in vivo. *Biophys. J.* **71**: 2692–2700. DOI:10.1016/S0006-3495(96)79460-2.

Kalia YN, Alberti I, Naik A, Guy RH. 2001. Assessment of topical bioavailability in vivo: The importance of Stratum Corneum thickness. *Skin Pharmacol. Appl. Skin Physiol.* **14**: 82–86 .DOI: 56394

Miller MA, Kasting G. 2012. A measurement of the unstirred aqueous boundary layer in a Franz diffusion cell. *Pharm. Dev. Technol.* **17**: 705–711. DOI:10.3109/10837450.2011.572895.

Modamio P, Lastra CF, Mariño EL. 2000. A comparative in vitro study of percutaneous penetration of beta-blockers in human skin. *Int. J. Pharm.* **194**: 249–259.

Nitsche JM, Wang TF, Kasting GB. 2006. A two-phase analysis of solute partitioning into the stratum corneum. *J. Pharm. Sci.* **95**: 649–666. DOI:10.1002/jps.20549.

OECD Environmental Health and Safety Publications Series on testing and Assessment No. 28. Guidance Document for the conduct of skin absorption studies, 2004a.

OECD Guidelines for the Testing of Chemicals, Section 1: Physical-Chemical properties No. 107: Partition Coefficient (n-octanol/water): Shake Flask Method. OECD Editions. 1995.

OECD Guideline for the testing of chemicals. Skin Absorption: In vitro method, Test Guideline 428 2004b.

Potts RO, Guy RH. 1992. Predicting skin permeability. *Pharm. Res.* **9**: 663–669. DOI:10.1023/A:1015810312465.

Raykar PV, Fung MC, Anderson BD. 1988. The Role of Protein and Lipid Domains in the Uptake of Solutes by Human Stratum Corneum. *Pharm. Res.* **5**: 140–150.

Reddy MB, Stinchcomb AL, Guy RH, Bunge AL. 2002. Determining Dermal Absorption Parameters in Vivo From Tape Strip Data. *Pharm. Res.* **19**: 292–298.

Rougier A, Dupuis D, Lotte C, Roguet R, Wester RC, Maibach HI. 1968. Regional variation in percutaneous absorption in man: measurement by the stripping method. *Arch. Derm. Res.* **278**: 465–469. DOI:10.1007/BF00455165.

Russel LM, Wiedersberg S, Delgado-Charro MB. 2008. The determination of stratum corneum thickness An alternative approach. *Eur. J. Pharm. Biopharm.* **69**: 861–870.

SCCS, 2010 (SCCS/1358/10) SCCS (Scientific Committee on Consumer Safety), basic criteria for the in vitro assessment of dermal absorption of cosmetic ingredients, 22 June 2010.

SCCS, 2015. The SCCS's Notes of Guidance for the Testing of Cosmetic Substances and their Safety Evaluation 9th Revision. Adopted 29 September 2015.

Simon GA, Maibach HI. 2000. The Pig as an Experimental Animal Model of Percutaneous Permeation in Man: Qualitative and Quantitative Observations – An Overview. *Skin Pharmacol. Appl. Skin Physiol.* **13**: 229–234. DOI:10.1159/000029928.

Surber C, Wilhelm KP, Hori M, Maibach HI, Guy RH. 1990. Optimization of Topical Therapy: Partitioning of Drugs into Stratum Corneum. *Pharm. Res.* **7**: 1320–1324. DOI:10.1023/A:1015958526423.

Todo H, Oshizaka T, Kadhum WR, Sugibayashi K. 2013. Mathematical model to predict skin concentration after topical application of drugs. *Pharmaceutics* **5**: 634–651. DOI:10.3390/pharmaceutics5040634.

van de Sandt JJ, van Burgsteden JA, Cage S, Carmichael PL, Dick I, Kenyon S, Korinth G, Larese F, Limasset JC, Maas WJ, Montomoli L, Nielsen JB, Payan JP, Robinson E, Sartorelli P, Schaller KH, Wilkinson SC, Williams FM. 2004. *In vitro* predictions of skin absorption of caffeine, testosterone, and benzoic acid: a multi-centre comparison study. *Regul. Toxicol. Pharmacol.* **39**: 271–281. DOI:10.1016/j.yrtph.2004.02.004.

Vecchia BE, Bunge AL. 2002. Partitioning of Chemicals into Skin: Results and Predictions. In *Transdermal Drug Delivery, Second Edition, Revised and Expanded*, Guy RH, Hadgraft J (eds). Marcel Dekker: Inc. New York; 143–198.

Wang L, Chen L, Lian G, Han L. 2010. Determination of partition and binding properties of solutes to stratum corneum. *Int. J. Pharm.* **398**. DOI:10.1016/j.ijpharm.2010.07.035.

Wester RC, Maibach HI. 2005. Regional variation percutaneous absorption: Principles and application to human risk assessment. In *Percutaneous Absorption: Drugs-Cosmetics-Mechanisms-Methodology*, Bronaugh RL, Maibach HI (eds), 4th edn. Marcel Dekker: New York: Chap. **5**: 85–93.

Wester RC, Mobayen M, Maibach HI. 1987. In vivo and in vitro absorption and binding to powered stratum corneum as methods to evaluate skin absorption of environmental chemical contaminants from ground and surface water. *J. Toxicol. Environ. Health* **21**: 367–374. DOI:10.1080/15287398709531025.

Wolfram JL, Maibach HI. 2005. Hair Dye penetration in Monkey and Man. In *Percutaneous Absorption* drugs-Cosmetics-Mechanisms-Methodology, Bronaugh RL, Maibach HI (eds), Fourth edn. Taylor & Francis: Boca Raton; 623–634.

Zhang Q, Grice JE, Li P, Jepps OG, Wang GJ, Roberts MS. 2009. Skin solubility determines maximum transepidermal flux for similar size molecules. *Pharm. Res.* **26**: 1974–1985. DOI:10.1007/s11095-009-9912-4.