



Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit



The impact of surface loading and dosing scheme on the skin uptake of fragrances

Q1 Fabienne Berthaud^a, Benjamin Smith^b, Mila Boncheva^{a,*}

^a Corporate R&D Division, Firmenich SA, Geneva, Switzerland

^b Toxicology and Scientific Services, Corporate Compliance, Firmenich SA, Geneva, Switzerland

ARTICLE INFO

Article history:

Received 3 June 2013

Accepted 5 September 2013

Available online xxxxx

Keywords:

Fragrance allergy

Fragrance uptake

In vitro skin penetration

Patch test

Repeated open application test

Stratum corneum lipids

ABSTRACT

This study compared the skin uptake of γ -undecalactone, decanol, and dodecyl acetate in an *in vitro*, un-occluded penetration assay in which they were applied to porcine skin at different finite loadings and application schemes. The pattern of fractional uptake differed between the chemicals and did not show the often assumed inverse correlation with surface loading. Furthermore, the mass uptake of identical cumulative amounts of the chemicals was not always additive. These results show that the uptake of fragrances in absence of occlusion and at finite loadings is chemical-specific and depends on the surface loading, the application scheme, and most probably, on the effects of the chemicals on the skin barrier efficiency. The observed lack of additivity might explain some of the differences in the responses observed in patch and repeated open application tests, and the boosting of the allergic state in sensitized individuals by sub-clinical exposures.

© 2013 Published by Elsevier Ltd.

1. Introduction

Both the assessment of the sensitization and systemic hazard potentials of chemicals following dermal exposure and the diagnostic tests for contact allergy rely on data on the skin uptake of the chemicals (Griem et al., 2003; Kimber et al., 2001). This *in vitro* study compared the skin uptake of three fragrance materials (FM) applied to the skin at different loadings and dosing schemes. The correlation between fractional skin uptake and surface loading and the influence of the dosing scheme on the mass uptake differed between the chemicals. These results suggest that the structure-specific, dose-dependent interactions between topical chemicals and the skin barrier lipids are an important factor to consider when interpreting the results of the practiced *in vivo* and *in vitro* assays.

Human skin uptake of FM *in vivo* is often estimated from the uptake measured in animal or *in vitro* studies performed at loadings and dosing schemes which differ from those found in

real-life exposure conditions (Kimber et al., 2001; Vecchia and Bunge, 2006). The two most commonly practiced diagnostic *in vivo* tests for contact allergy to FM, the patch test (PT) and the repeated open application test (ROAT) (Basketter, 2009; Hannuksela and Salo, 1986), often deliver different numbers of responses to identical cumulative doses of topically applied chemicals and different individual elicitation thresholds (Fischer et al., 2009; Villar-ama and Maibach, 2004). Several studies have suggested that the reasons behind these apparent discrepancies (Johansen et al., 1996a,b) and the causes for some effects observed in the tests [e.g., the boosting of the allergic state by sub-clinical exposures (Friedmann, 1994; Friedmann et al., 1990)] might be rooted in differences in the dermal uptake of the topically applied chemicals resulting from the different test designs (Fischer et al., 2009; Hostynek and Maibach, 2004). Clearly, detailed knowledge of all parameters that influence the dermal uptake is necessary to correctly evaluate the test results and relevance.

It is widely acknowledged that the surface loading of topically applied chemicals influences their dermal uptake (Buist et al., 2009; Kissel, 2011). The fractional uptake (i.e., the percentage of the applied dose that has been absorbed per unit time) of many chemicals applied to the skin neat or in rapidly evaporating solvents is inversely proportional to their surface loading (expressed as mass per unit area) (Brewster et al., 1989; Wester and Maibach, 1976). This correlation, however, holds only when the chemicals cover the skin surface completely, there is no

Abbreviations: C10, decanol; DA, dodecyl acetate; FM, fragrance material; GC/FID, gas chromatography with flame ionization detector; PBS, phosphate buffer saline; PBST, phosphate buffer saline containing 1% Tween-20; PT, patch test; ROAT, repeated open application test; SC, Stratum corneum; UL, γ -undecalactone.

* Corresponding author. Address: Corporate R&D Division, Firmenich SA, P.O. Box 239, Route des Jeunes 1, CH-1211 Geneva 8, Switzerland. Tel.: +41 22 780 3027; fax: +41 22 780 3334.

E-mail address: mila.boncheva@firmenich.com (M. Boncheva).

depletion of their source or skin saturation for the duration of the experiment, and they do not modify the skin permeability (Bunge, 2005; Kissel, 2011). These conditions are hardly ever fulfilled during the typical application of fragranced products: the volatile FM are applied to the skin surface at finite loadings (which decrease in the course of the application period because of evaporation of the chemicals) and are not uniformly distributed. Importantly, they might affect the permeability of the skin barrier layer, the Stratum corneum (SC). Several classes of FM (e.g., terpenes, alcohols, fatty acids, and fatty esters) are known to perturb the molecular organization of the SC lipids and/or proteins and thereby enhance the SC permeability to other co-formulated molecules and to themselves (Babita et al., 2006; Babu et al., 2006; Thakur et al., 2006; Williams and Barry, 2004). Since this effect is typically dose-dependent, one high dose of FM might cause a bigger perturbation of the SC permeability and thereby a higher uptake than the cumulative uptake obtained after several applications of smaller doses of FM. Thus, the fractional uptake of FM may depend not only on the surface loading but also on the dosing scheme of the chemical and the correlation between the three parameters is most probably chemical-specific. Indeed, a deviation from the inverse correlation between surface loading and fractional uptake has been reported for a number of known irritants and volatile chemicals (Buist et al., 2009).

In this work, we addressed the correlation between skin uptake, mass loading, and dosing scheme in the skin absorption of three FM— γ -undecalactone (UL), decanol (C10), and dodecyl acetate (DA)—in a 24-h, *in vitro* penetration study using porcine skin. We chose the FM to have different lipophilicities (as measured by their $\log P$ values) and chemical functionalities, and medium to low volatility. Recently, we have demonstrated that these FM perturb the molecular organization of the SC lipids in a dose-dependent manner (Groen et al., 2013). To mimic the typical in-use conditions for fragrances, we left the skin un-occluded and exposed to the ambient air. We applied the chemicals to the skin surface at six different surface loadings ranging between approximately 20 and 900 $\mu\text{g}/\text{cm}^2$. For the surface loadings of 78, 390, and 780 $\mu\text{g}/\text{cm}^2$ we used two different dosing schemes and applied the chemicals at once or in three consecutive single doses. At the end of the exposure period, we quantified the amounts of the three FM in the viable skin layers (dermis and epidermis) and in the receptor compartment. The sum of these amounts (denoted as systemic uptake) corresponds to the upper limit of the topically applied FM that can reach and interact with the viable skin tissues during the exposure period. To investigate if the correlation between fractional systemic uptake and surface loading was sensitive to the chemical structure of the three FM, we compared their fractional uptake at different surface loadings applied to the skin surface at once. To evaluate the influence of the dosing scheme on the systemic uptake, we next compared the uptake resulting from identical cumulative loadings of the FM applied following different dosing schemes.

2. Materials and methods

2.1. Fragrance materials (FM)

Table 1 shows relevant information on the three FM used in this study. We formulated them as 9:1 (v/v) ethanol/water solutions containing nominally 1%, 5%, and 10% (w/w) of FM. Before use, we quantified the exact concentration of FM in the solutions by GC/FID.

2.2. Skin samples

We collected the ears of domestic pigs at the local slaughterhouse (Gland, Switzerland) a couple of hours *post mortem*. After thoroughly washing the ears with cold water, we removed the skin from the outer sides of the ears using a scalpel, clipped the hairs using hair clippers, dermatomed the skin to a thickness of 350–400 μm using a 50-mm electric dermatome (Nouvag AG, Goldach, Switzerland), and wiped the SC side three times with cotton swabs wetted with cold (4 °C) heptane, to remove traces of sebum. The skin was stored at –20 °C wrapped in Parafilm and packed in Zip-Lock bags for not longer than 1 month prior to use. As recommended in the regulatory guidelines (OECD, 2009; SCCS, 2010), before use we assessed the integrity of the SC using measurement of transepidermal water loss, TEWL (Imhof, 2009). The criterion for barrier integrity that we used throughout this work was of TEWL lower than 12.2 $\text{g m}^{-2} \text{h}^{-1}$ (as measured by the closed-chamber AquaFlux instrument from Biox Systems, Ltd., UK). We have established that this criterion corresponds to a transcutaneous electrical resistance equal to 14 $\text{k}\Omega \text{cm}^2$, the most conservative cutoff value reported for porcine ear skin (Davies et al., 2004; data not shown).

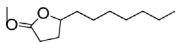
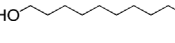
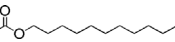
2.3. Diffusion cells

We used static jacketed, Franz-type, glass diffusion cells (PermeGear, Inc., Hellertown, USA) with a receptor volume of 8 mL, equipped with magnetic stirrers. Before use, the cells were cleaned thoroughly, plasma-activated, and gas-phase silanized with trichloro perfluorooctyl silane. As a receptor solution we used PBS buffer (150 mM, pH 7.4) containing 1% Tween-20 (denoted as PBST). All studied FM were soluble in this solution at concentrations exceeding their maximal possible concentrations (i.e., those corresponding to the total amount of applied FM dissolved in 8 mL, the volume of the receptor compartment).

2.4. Experimental design

We performed all experiments in an air-conditioned room with a temperature of 25 ± 2 °C, relative humidity of $50 \pm 4\%$, and average air velocity above the bench top of ≤ 0.1 m/s. At beginning of the experiments, we defrosted the skin samples for 30 min on

Table 1
Fragrance materials used in this study.

Fragrance material	CAS no.	Structure	Molecular weight	$\log P$	Vapor pressure
γ -Undecalactone (UL)	000104-67-6		184.28	3.06	0.55
Decanol (C10)	000112-30-1		158.28	4.57	1.45
Dodecyl acetate (DA)	000112-66-3		228.37	5.78	1.61

top of the receptor compartment of the diffusion cells filled with PBST maintained at 37 °C (Berthaud et al., 2011). On top of the skin surface we placed a PTFE spacer with a circular hole ($d = 1.1$ cm) in the middle, which delimited a test area of 0.95 cm^2 , and sealed its outer edge to the cell using high vacuum silicone grease (Dow Corning, Inc., USA). At the end of the equilibration period, we clamped the assembled cell tightly in place. To load the skin surface with one dose of FM, we applied 3 or 9 μL of its ethanol/water solution to the test area of 0.95 cm^2 using a 5- μL Hamilton syringe, and spread the liquid evenly. To load the skin surface with three consecutive doses of FM, we applied to the test area three aliquots of 3 μL each of the FM solutions at intervals of 2 h. The CV of the applied doses measured in ten separate applications using analytical balance was 1.7%.

After depositing the FM solution at the skin surface, we let the skin penetration proceed for 24 h (for the three doses applied at intervals of 2 h, we counted the 24-h period starting from the deposition of the first dose). At the end of this period, we dismantled the diffusion cells and placed the skin sample on a silicone support covered with protecting tapes. We removed the SC by stripping the skin surface 30 times using D-Squame tapes (Cu-Derm, Dallas, USA). We delimited the test area using adhesive tape, and after applying each stripping tape, we rolled a metal cylinder with a weight of 1 kg over the tape ten times to ensure its homogeneous adhesion to the skin, and removed it in one rapid movement. We performed all experiments using skin from five to seven different porcine ears in each set of experimental conditions.

2.5. FM distribution

At the end of the 24-h exposure period, we quantified the distribution of FM as follows:

2.5.1. Losses of the applied FM

To quantify the eventual losses of the applied FM due to its adsorption onto the PTFE spacer that delimited the application area, we extracted the spacer (together with the silicone grease remaining on it) with 1 mL iso-octane during 60 min under sonication and quantified the amount of FM in the extract by GC/FID (see below). To this amount we added also the amount of FM found on the skin surface under the PTFE spacer; to quantify this amount, we wiped this part of the surface with cotton swabs, extracted them for 60 min with 1 mL iso-octane/diethyl ether (1:1) under sonication, and quantified the amount of FM in the extract by GC/FID (see below). On the average, the FM losses varied between 2% and 11% of the applied doses.

2.5.2. FM retained in the viable skin layers

After tape-stripping, we extracted the skin samples (comprising the viable skin layers plus any remains of SC) by vortexing them in 2 mL iso-octane for 24 h and quantified the amount of FM in the extract by GC/FID (see below). To this amount we added also the FM retained on the protecting tapes that were in contact with the dermal side of the sample during tape-stripping.

2.5.3. FM penetrated through the skin

To quantify the amount of FM that had penetrated through the skin into the receptor fluid, we extracted two 3 mL-aliquots of the receptor fluid PBST with 10 mL of iso-octane/diethyl ether (9/1) by 30 min shaking in a Turbula multi-directional shaker (speed 32), centrifuged the mixtures (10 min at 3500g), and quantified the amount of FM in the supernatant by GC/FID (see below). To this amount we added also the amount of FM found on the rim of the glass cell that was in contact with the dermal side of the skin sample; to quantify it, we wiped this part of the surface with cotton swabs and extracted them as described above. We verified in

separate experiments that none of the studied FMs adsorbed on the magnetic stirrer or on the walls of the receptor compartment of the cells at the end of the exposure period.

We also quantified the amounts of FM that remained at the skin surface and that were retained in the SC at the end of the exposure period [data not shown; information on the overall mass balance and further methodological details can be found in (Berthaud et al., 2011)].

2.5.4. Quantification of FM in liquid extracts

We quantified the FM in all liquid extracts by GC/FID chromatography using a CP-3800 instrument (Varian, Inc.) with a He flow (2 mL/min) equipped with a Factor Four VF-1ms column (Varian, Inc.). We injected nominally 1 μL of the samples on-column, and normalized the injected amounts of FMs using ethyl decanoate and ethyl laurate as internal standards. The calibration curves for all FMs contained 12 points ($0.3\text{--}20\text{ }\mu\text{g/mL}$) and had $r^2 > 0.998$; thus, the method could reliably quantify the FMs in the extraction solvents in presence of contaminants resulting from the extraction procedures down to concentrations of $0.1\text{ }\mu\text{g/mL}$.

2.6. Systemic uptake of FM

We calculated the systemic uptake of FM as the sum of the amounts of FM that were retained in the viable skin layers and those found in the receptor fluid. Throughout this work, we presented the cumulative systemic uptake of FM after 24 h using the relevant for point-of-contact effects metric $\mu\text{g/cm}^2$ [mass uptake (Kimber et al., 2008)], or as a percentage of the applied dose [fractional uptake (Kissel, 2011)]. In calculating the fractional uptake from the surface loading, we took into account the losses of the FM due to their adsorption onto the PTFE spacer that delimited the application area.

3. Results

Fig. 1 compares the correlations between fractional uptake and surface loadings observed when the FM were applied to the skin surface in one dose. The nature of the correlation differed for the three FM. For UL (Fig. 1A), the fractional uptake increased with the surface loading up to approximately $250\text{ }\mu\text{g/cm}^2$ where it leveled off at around 19%. For C10 (Fig. 1B), the fractional uptake remained approximately constant and equal to 7% for surface loadings between 80 and $800\text{ }\mu\text{g/cm}^2$. For DA (Fig. 1C), however, the fractional uptake increased linearly with the loading throughout the whole studied range.

The fact that the observed correlations between fractional uptake and surface loading were far from the often assumed inverse correlation may have several causes. In our experimental design—aimed to reproduce the conditions encountered in typical use of fragranced products—the skin coverage with FM was most probably not homogeneous (especially at the low surface loadings) and the FM source at the skin surface was continuously depleted as a result of FM evaporation; thus, the flux of the FM into the skin was neither constant nor independent of the surface loading throughout the experiments (Kissel, 2011). In addition, we have a strong indication that the permeability of the SC was not constant throughout the experiments. In a recent study, we demonstrated that at high loadings the three FM cause dose-dependent fluidization of the hexagonally-packed SC lipids and swelling of the long periodicity phase in the lipid lamellar structure (Groen et al., 2013). These changes in the organization of the SC lipids typically correlate with increasing the SC permeability (Golden et al., 1987; Potts and Francoeur, 1990; Potts et al., 1991). Thus, even in the absence of evaporative losses of the load and homogeneous, complete

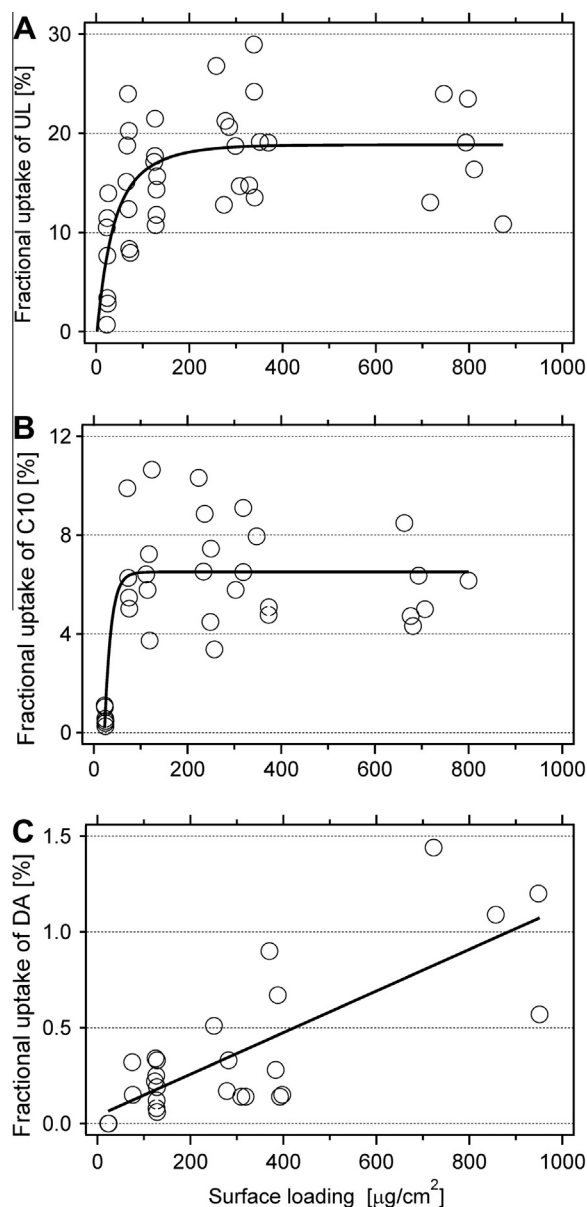


Fig. 1. Fractional systemic uptake of UL (A), C10 (B), and DA (C) as a function of their skin surface loading. The circles denote the experimental data obtained using skin from different porcine ears ($7 \geq n \geq 5$) in each set of experimental conditions. The solid trend lines [obtained by double-exponential (A and B) or linear (C) fits through the data points] are empirical fits to guide the eye.

coverage of the skin surface, the fractional uptake of these FM might be expected to depend heavily on the magnitude and the time scale of the perturbation that they cause on the SC permeability.

To investigate the influence of the dosing scheme on the uptake of FM, we next compared the mass uptake of the three FM when they were applied to the skin surface at cumulative loadings of 78, 390, and 780 µg/cm² in three doses (each of 26, 130, and 260 µg/cm², respectively) or at once. The results summarized in Fig. 2 show that the dosing scheme influenced the uptake to a different extent depending on the surface loading and the FM. For UL and C10, the mass uptake at a cumulative loading of 78 µg/cm² was lower when the FM were applied as three doses of 26 µg/cm² than when the same amount was applied at once (Fig. 2A and B, left panels); at higher loadings, the uptake resulting from the different loading schemes was identical (Fig. 2A and B, middle

and right panels). For DA, the dosing scheme had no influence on the mass uptake at all three loadings (Fig. 2C). Thus, while the mass uptake of all three FM at the highest studied loading was additive (i.e., the uptake of three single doses was approximately equal to the uptake of one triple dose), this was not always the case at the lowest surface loading.

We believe that the observed influence of the dosing scheme on the uptake is due to the effects of the FM on the molecular organization—and thereby, the permeability—of the SC lipid matrix. As mentioned above, the effect of UL on the conformational order and lamellar organization of the SC lipids is dose-dependent (Groen et al., 2013). It is also known that the larger the perturbation of the molecular organization of the SC lipids, the longer it takes for its reversal (Pensack et al., 2006). Thus, the application of UL in one dose of 78 µg/cm² is expected to perturb the native molecular organization (and, consequently, increase the permeability of the SC lipids) to a greater extent and for a longer period than the sequential application of the three 26 µg/cm²-doses. As a result, the amounts of UL that can penetrate through the SC into the viable skin layers will be higher following loading the skin surface with 78 µg/cm² than following sequential application of the same cumulative amount in small doses. The same arguments explain also the deviation from additivity in the uptake of C10 observed at the lowest surface loading. The additive uptake of UL and C10 observed at cumulative surface loadings of 390 and 780 µg/cm² indicates that the perturbations of the molecular organization in the SC lipid matrix caused by the application of 130–780 µg FM/cm² had similar magnitude and/or duration. The fact that the uptake of DA was additive at all studied loadings is in line with our earlier observation that, upon similar surface loadings, DA causes more extensive disordering of the SC lipids than UL, i.e., had a steeper dose-effect curve than UL (Groen et al., 2013). Thus, apparently at a loading of 26 µg/cm² the effect of DA on the molecular organization of the SC lipids was of similar magnitude as the effect caused by the higher loadings of 78–780 µg/cm², which in turn led to similar uptake of DA in the viable skin tissues. (Note that despite the considerable impact of DA on the molecular organization of the SC lipids, its penetration through the SC remained modest and significantly lower than the one of UL and C10 because of its considerable lipophilicity.)

4. Discussion

This study demonstrates that the skin uptake of topically applied FM in absence of occlusion is chemical-specific and varies with the skin surface loading and the dosing scheme. Taken together with our recent investigation of the interactions between the FM and SC lipids, our results indicate that the chemical specificity of the observed effects is—at least in part—due to reversible, structure-specific, and dose-dependent alterations of the molecular organization of the SC lipids.

These findings have important implications for the correct interpretation of *in vitro* and *in vivo* risk assessment and diagnostic assays based on the dermal uptake of topically applied FM. They demonstrate that inferring the typical human skin uptake from studies performed at different loadings and using different dosing schemes might easily lead to making unsafe or overly conservative risk assessment. Thus, one should carefully consider exposure-based estimates of individual elicitation thresholds based on tests of radically different design, such as PT and ROAT, or ROAT performed following a different dosing scheme. The assumption that the fractional uptake observed at high surface loadings—which are comparable to those used in PT—can automatically be transferred to the uptake at low surface loadings—which are comparable to those typically encountered in the everyday use of FM—

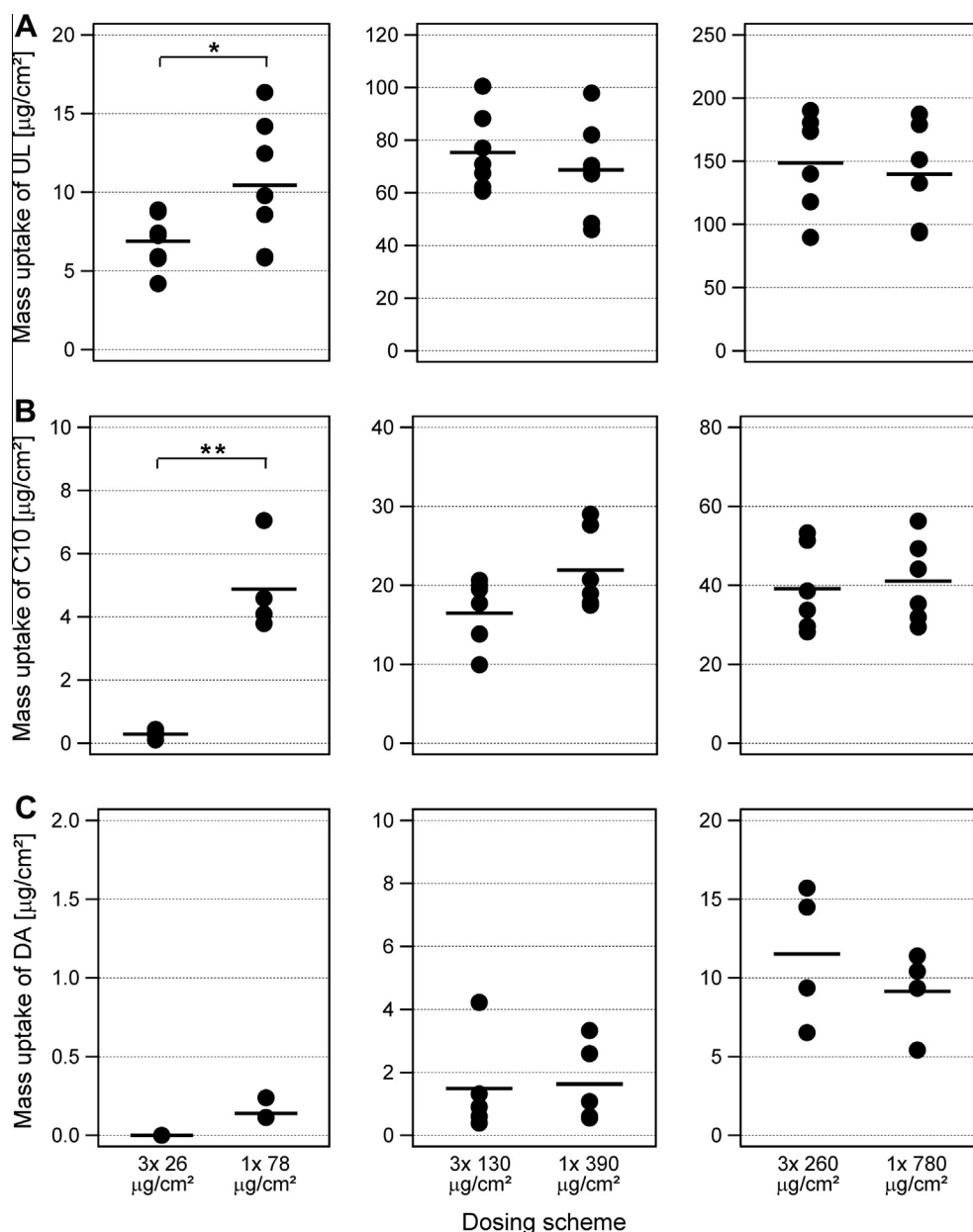


Fig. 2. Mass systemic uptake of UL (A), C10 (B), and DA (C) resulting from nominal cumulative surface loadings of 78 µg/cm² (left panels), 390 µg/cm² (middle panels), and 780 µg/cm² (right panels) applied in three consecutive doses or at once. The symbols denote the results obtained in experiments with skin from different porcine ears (7 ≥ n ≥ 5), and the horizontal bars—their average values. The symbols ** and * indicate differences significant at better than 1% and 5%, respectively.

can lead to many-fold overestimation of the systemic uptake of the chemicals. As an example, our results demonstrated that while such transfer of the fractional uptake is permissible for UL and C10 at loadings between 300 and 800 µg/cm², it is not correct at surface loadings of 26 µg/cm² for these two FM and at all loadings of DA.

Furthermore, our results lend further support to the hypothesis that the boosting effect in elicitation might be due to the accumulation of chemicals in the viable skin tissues following multiple applications (Friedmann, 1994; Friedmann et al., 1990). Since the nature of this accumulation—additive or non-additive—influences the magnitude of the observed effects, it is an important parameter to consider when attempting to correlate surface loading with clinical manifestations. Thus, for example, the accumulation of topically-applied paraphenylenediamine in the viable skin tissues was found to be additive: its levels in skin treated three times with

one dose were approximately three-fold higher than those found in skin treated once with one dose (White et al., 2007). Additive accumulation was inferred also for methylaldibromo glutaronitrile, based on the observed equal capability of the chemical to produce an allergic reaction in pre-sensitized individuals when applied at identical surface loading but following different dosing schemes (Jensen et al., 2005). The data presented here, however, show that for FM that can decrease the barrier efficiency of the SC lipids the accumulation might be even higher than the one expected if the dermal uptake were additive. For such chemicals, the accumulation will depend upon the interplay between the magnitude of SC barrier perturbation, its time course, and the duration of the contact between the FM and the SC lipids; this last parameter, in turn, depends upon the type of interactions between the FM and the lipids of the SC extracellular matrix, the presence of occlusion, and the volatility of FM. In the future it would be very interesting

to correlate our *in vitro* observations with *in vivo* studies on the influence of the dosing scheme and skin loading on the elicitation thresholds of FM with known effect on the SC permeability, and to investigate the absorption kinetics of these chemicals *in vitro*.

The results of this study contribute to the better understanding of the factors that govern the skin penetration of fragrances and, more generally, of volatile chemicals. Thus, our findings are relevant also for the hazard identification and exposure-based risk assessment—including systemic toxicology, sensitization, and irritancy—of several classes of chemicals besides fragranced cosmetic products, e.g., chemical warfare agent, toxic spills, industrial solvents, and pesticides, as well as for the design of performing and safe drugs for dermal and transdermal delivery.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgment

We thank Peter Cadby and William Troy for critically reading the manuscript.

References

- Babita, K., Kumar, V., Rana, V., Jain, S., Tiwary, A.K., 2006. Thermotropic and spectroscopic behavior of skin: relationship with percutaneous permeation enhancement. *Curr. Drug Deliv.* 3, 95–113.
- Babu, R.J., Singh, M., Kanikkannan, N., 2006. Fatty alcohols and fatty acids. In: Smith, E.W., Maibach, H.I. (Eds.), *Percutaneous Penetration Enhancers*. CRC Press, Boca Raton, FL.
- Basketter, D.A., 2009. The human repeated insult patch test in the 21st century: a commentary. *Cut. Occ. Toxicol.* 28, 49–53.
- Berthaud, F., Narancic, S., Boncheva, M., 2011. *In vitro* skin penetration of fragrances: trapping the evaporated material can enhance the dermal absorption of volatile chemicals. *Toxicol. in Vitro* 25, 1399–1405.
- Brewster, D.W., Banks, Y.B., Clark, A.M., Birnbaum, L.S., 1989. Comparative dermal absorption of 2,3,7,8-tetrachlorodibenzo-p-dioxine and three polychlorinated dibenzofurans. *Toxicol. Appl. Pharmacol.* 97, 156–166.
- Buist, H.E., Schaafsma, G., Van de Sandt, J.J.M., 2009. Relative absorption and dermal loading of chemical substances: consequences for risk assessment. *Regul. Toxicol. Pharmacol.* 54, 221–228.
- Bunge, A.L., 2005. Dermal absorption to solvent-deposited solids: is absorption proportional to the exposed dose? In: Poster Presented at the Second Conference on Occupational and Environmental Exposures of Skin to Chemicals, Stockholm, Sweden.
- Davies, D.J., Ward, R.J., Heylings, J.R., 2004. Multi-species assessment of electrical resistance as a skin integrity marker for *in vitro* skin percutaneous absorption studies. *Toxicol. in Vitro* 18, 351–358.
- Fischer, L.A., Menné, T., Avnstorp, C., Kasting, G.B., Johansen, J.D., 2009. Hydroxyisohexyl-3-cyclohexene carboxaldehyde allergy: relationship between patch test and repeated open application test thresholds. *Brit. J. Dermatol.* 161, 560–567.
- Friedmann, P.S., 1994. Clinical aspects of allergic contact dermatitis. In: Dean, J.H., Luster, M.I., Munson, A.E., Kimber, I. (Eds.), *Immunotoxicology and Immunopharmacology*. Raven Press, New York, N.Y., pp. 589–616.
- Friedmann, P.S., Rees, J., White, S.I., Mathews, J.N.S., 1990. Low-dose exposure to antigen induces sub-clinical sensitization. *Clin. Exp. Immunol.* 81, 507–509.
- Golden, G.M., McKie, J.E., Potts, R.O., 1987. Role of Stratum corneum lipid fluidity in transdermal drug flux. *J. Pharm. Sci.* 76, 25–28.
- Griem, P., Goebel, C., Scheffler, H., 2003. Proposal for a risk assessment methodology for skin sensitization based on sensitization potency data. *Regul. Toxicol. Pharmacol.* 38, 269–290.
- Groen, D., Berthaud, F., Bouwstra, J.A., Gooris, G.S., Chapuis, C., Boncheva, M., 2013. *In vitro* model systems for evaluating the impact of fragrance raw materials on the skin barrier lipids. Submitted for publication.
- Hannuksela, M., Salo, H., 1986. The repeated open application test (ROAT). *Contact Dermatitis* 14, 221–227.
- Hostynek, J.J., Maibach, H.I., 2004. Thresholds of elicitation depend on induction conditions. Could low level exposure induce sub-clinical allergic states that are only elicited under the severe conditions of clinical diagnosis? *Food Chem. Toxicol.* 42, 1859–1865.
- Imhof, R.E., 2009. Closed chamber transepidermal water loss measurement: microclimate, calibration and performance. *Int. J. Cosmet. Sci.* 31, 97–118.
- Jensen, C.D., Johansen, J.D., Menné, T., Andersen, K.E., 2005. Methylidibromoglutaronitrile contact allergy: effect of single versus repeated daily exposure. *Contact Dermatitis* 52, 88–92.
- Johansen, J.D., Andersen, K.E., Menné, T., 1996a. Quantitative aspects of isoeugenol contact allergy assessed by use and patch tests. *Contact Dermatitis* 34, 414–418.
- Johansen, J.D., Andersen, K.E., Rastogi, S.C., Menné, T., 1996b. Threshold responses in cinnamic-aldehyde-sensitive subjects: results and methodological aspects. *Contact Dermatitis* 34, 165–171.
- Kimber, I., Basketter, D.A., Berthold, K., Butler, M., Garrigue, J.-L., Lea, L., Newsome, R., Roggeband, R., Steiling, W., Stropp, G., Waterman, S., Wieman, C., 2001. Skin sensitization testing in potency and risk assessment. *Toxicol. Sci.* 59, 198–208.
- Kimber, I., Dearman, R.J., Basketter, D.A., Ryan, C.A., Gerberick, G.F., McNamee, P.M., Lalko, J., Api, A.M., 2008. Dose metrics in the acquisition of skin sensitization: thresholds and importance of dose per unit area. *Regul. Toxicol. Pharmacol.* 52, 39–45.
- Kissel, J.C., 2011. The mismeasure of dermal absorption. *J. Exp. Sci. Environ. Epidemiol.* 21, 302–309.
- Organization for Economic Cooperation and Development, OECD, 2009. Test No. 428: skin absorption: *in vitro* method. OECD Guidelines for the Testing of Chemicals 1, 1–8.
- Pensack, R.D., Michniak, B.B., Moore, D.J., Mendelsohn, R., 2006. Infrared kinetic/structural studies of barrier reformation in intact Stratum corneum following thermal perturbation. *Appl. Spectrosc.* 60, 1399–1404.
- Potts, R.O., Francoeur, M.L., 1990. Lipid biophysics of water loss through the skin. *Proc. Natl. Acad. Sci. USA* 87, 3871–3873.
- Potts, R.O., Mak, V.H.W., Guy, R.H., Francoeur, M.L., 1991. Strategies to enhance permeability via Stratum corneum lipid pathways. *Adv. Lipid Res.* 24, 173–210.
- Scientific Committee on Consumer Safety, SCCS, 2010. Basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients. Web 31 July, 2013. <http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_s_002.pdf>.
- Thakur, R.A., Wang, Y., Michniak, B.B., 2006. Essential oils and terpenes. In: Smith, E.W., Maibach, H.I. (Eds.), *Percutaneous Penetration Enhancers*. CRC Press, Boca Raton, FL, pp. 159–173.
- Vecchia, B.E., Bunge, A.L., 2006. Animal models: a comparison of permeability coefficients for excised skin from humans and animals. In: Riviere, J.E. (Ed.), *Dermal Absorption Models in Toxicology and Pharmacology*. Taylor and Francis Group, Boca Raton, FL, pp. 305–367.
- Villarama, C.D., Maibach, H.I., 2004. Correlations of patch test reactivity and the repeated open application test (ROAT)/provocative use test (PUT). *Food Chem. Toxicol.* 42, 1719–1725.
- Wester, R.C., Maibach, H.I., 1976. Relationship of topical dose and percutaneous absorption in rhesus monkey and man. *J. Invest. Dermatol.* 67, 518–520.
- White, J.M.L., Basketter, D.A., Pease, C.K., Sanders, D.A., McFadden, J.P., 2007. Intermittent exposure to low-concentration paraphenylenediamine can be equivalent to single, higher-dose exposure. *Contact Dermatitis* 56, 262–265.
- Williams, A.C., Barry, B.W., 2004. Penetration enhancers. *Adv. Drug Deliv. Rev.* 56, 603–618.