Tolylfluanid permeates human skin slowly and as dimethylamino sulfotoluidid (DMST)

Aurélie Berthet[a]*, Julie Hechon[b], Nancy B. Hopf[a]

[a] Center for Primary Care and Public Health (Unisanté), University of Lausanne, Switzerland, University of Lausanne, Switzerland. Aurelie.Berthet@unisante.ch; Nancy.Hopf@unisante.ch;

[b] Unit of Toxicology, University Centre of Legal Medicine (CURML), Universities of Lausanne and Geneva, Lausanne, Switzerland. Julie.Hechon@chuv.ch

*corresponding author: Aurélie Berthet
Center for Primary Care and Public Health (Unisanté)
Route de la Corniche, 2
1066 Epalinges
aurelie.berthet@hospvd.ch
Tel.: +41 (0)21 314 74 21

Date: December 13, 2019

Abstract

Tolylfluanid (TF) is a sensitizing biocide used in antifouling products and wood preservatives. Paint application is associated with skin exposure; however, the importance of this exposure route is uncertain as TF skin permeation rates are lacking in the peer-reviewed scientific literature. TF is a lipophilic powder that hydrolyses rapidly in contact with water to dimethylamino sulfotoluidid (DMST). DMST is also a TF metabolite. We characterized TF and DMST skin permeation using an *ex vivo* flow-through diffusion system with viable and frozen human skin. TF permeated as DMST with a low permeation rate (0.18±0.05 µg/cm^2/h) and a moderate time lag (7.1±1.4 h) in viable human skin. Applying DMST gave a 3.5-fold lower permeation rate (0.05±0.01 µg/cm^2/h) compared to TF under a similar experimental setting. We simulated paint activities in an exposure chamber to understand a possible skin exposure from airborne TF concentrations. Although, paint can deposit onto the skin during work activities, TF permeation when paint was applied to human skin *ex vivo* was very low (as TF: 0.004±0.005 µg/cm^2/h, and as DMST: 0.02±0.001 µg/cm^2/h). Our results show that TF can permeate skin, and consequently, can contribute to sensitization, which support previous reports on sensitization in TF exposed workers.

Keywords: Tolylfluanid, Dimethylamino sulfotoluidid (DMST), human skin, dermal permeation, biocide.

Introduction

Health effects reported for tolylfluanid (TF) (CAS no. 731-27-1) includes promoting insulin resistance (Sargis et al., 2012) and metabolic disruption (Neel et al., 2013; Sargis et al., 2012) as well as endocrine disruption in mice (Regnier et al., 2014). TF was marketed in the early 1970s as a fungicide but was later repealed by the EU in 2010 (European Food Safety Authority, 2013). It is now used as a biocide. In Europe, biocides are regulated under EU 528/2012 (European Parliament, 2012) and are classified into 22 biocidal product types (PT). TF is used in antifouling products (PT 21) and wood preservatives (PT 8).

From animal studies, it is known that TF is easily absorbed from the gastro-intestinal route, and is widely distributed, especially in liver, kidney, and thyroid (European Food Safety Authority, 2005). Urine elimination (60-90%) is rapid, and mainly as its metabolites. One of the major metabolites is N,N-dimethyl-N′-tolylsulfonyldiamide (DMST, CAS no. 66840-71-9) (FAO/WHO, 2002). The main physical and chemical properties of TF and DMST are summarized in Table 1.

Although exposure through contaminated food and water (dietary) has diminished (Cesnik et al., 2006; Stensvand and Christiansen, 2000), occupational skin exposure is still expected for workers applying marine paint containing TF in the boating industry (Links et al., 2007; Tielemans et al., 1999).TF skin acute systemic toxicity after dermal contact is low (EU Regulation 528/2012; European Parliament, 2012), while allergic skin disease cases have been reported among workers at a TF manufacturing plant (Faul, 1982, 1989). When the workers stopped handling TF, the allergic skin symptoms disappeared. This temporality is an indication that these symptoms were work-related. Skin exposure is from airborne paint or by touching contaminated surfaces. Painting is associated with skin exposures (Anderson and

Meade, 2014). Workers can be sensitized to TF, if their skin is exposed to the paint containing TF during their work activities, *and* if TF migrates from the paint, cross the *stratum corneum*, and presents itself to the Langerhans cells that alert the immune system (Reby et al., 2015).

TF readily hydrolyzes at room temperature (Wilmes, 1982) producing hydrophilic substances such as DMST. No data regarding hydrolysis rates have been published in the peer-reviewed scientific literature. Skin exposure during painting activities might therefore be to both TF and DMST. Furthermore, TF is highly lipophilic (octanol-water partition coefficient, pK_{ow}, 3.9), and will likely cross the skin barrier (*stratum corneum*), but not easily pass through the hydrophilic epidermis layer. Skin can metabolize xenobiotic substances, and consequently, TF can metabolize to DMST and be absorbed. Thus, DMST is not only a hydrolysis product, but also a metabolite from TF exposures (Assessment report to Substances in Annex I or IA to Directive 98/8/EC (ECHA, 2014)). Few studies have characterized TF absorption accounting for DMST in exposure as a hydrolysis product and in the epidermis as a metabolite.

A useful method in characterizing skin absorption is the dynamic flow-through diffusion cell system mounted with *ex vivo* human skin (Fasano and McDougal, 2008). This method is designed to measure penetration of TF into the skin (crossing the *stratum corneum*) and its subsequent permeation across the skin (epidermis and dermis) into a receptor liquid (OECD Technical Guidance Document 428; (OECD, 2004c)). Fresh human ex vivo skin is necessary to test metabolism in this system. This requires a physiologically conducive receptor liquid, which is often cell culture media or water with polyethylene glycol (PEG). Using water in the receptor liquid may hamper the diffusion process for lipophilic substances and result in an

underestimate of the permeation rate. If this is the case, using frozen skin is an option. Our study objectives were therefore to:

(1) Characterize TF and DMST human skin permeation including rates (J), lag times (T_{lag}), and permeation coefficients (Kp) obtained from *in vitro* human skin experiments with TF alone at different concentrations and in formulation.

(2) Determine if TF and DMST were detected in air samples obtained while simulating spraying and rolling antifouling paint work activities in our exposure chamber.

Methods

Chemicals

Tolylfluanid (N-Dichlorofluoromethylthio-N',N'-dimethyl-N-p-tolylsulfamide; CAS no. 731-27-1; purity of 98.5 %) was bought from Dr. Ehrenstorfer (Augsburg, Germany). DMST (DMST in acetonitrile, 100 ng/µL, purity ≥ 98%) was obtained from Sigma-Aldrich (Pestanal®, Buchs, Switzerland). Sodium chloride (NaCl) (>99% purity) was purchased from Fluka (Sigma-Aldrich, Buchs, Switzerland), polyethylene glycol (PEG) (Brij® O10, CAS no. 9004-98-2, Sigma-Aldrich, Buchs, Switzerland), Roswell Park Memorial Institute medium (RPMI) 1640 media (HEPES modification, with 25 mM HEPES, without l-glutamine), and acetonitrile (ACN) from Sigma-Aldrich (analytical grade, Buchs, Switzerland). These chemicals were used for the receptor liquid solutions. For the extraction procedure, the solvents dichloromethane and n-heptane were analytical grade and purchased from Sigma-Aldrich (Buchs, Switzerland). All stock and working solutions were prepared in purified water (MilliQ

water treatment system, Millipore, Merck, Darmstadt, Germany). To measure skin permeation of a commercially available product, an antifouling paint (paint product 1: A4 T.Speed, Nautix, Guidel, France) with 5% TF (according to the material safety data sheet or MSDS) were bought. An antifouling paint product (paint product 2: Alpina, Growe Holzschutz AG, Belp, Suisse) containing TF (4 g/L) locally used by professionals preparing boats was used in the paint simulation study. Other ingredients listed on the safety data sheets (SDS) were pigments, extenders, copper oxide, colored pigments, xylene, and naphtha.

Flow-through diffusion cell experiments

Human skin

Skin permeation experiments were performed using human abdominal skin obtained from abdominoplasty ("tummy-tuck") surgeries from the Lausanne university hospital (CHUV) and the Department of Musculoskeletal Medicine (DAL) biobank (ethical protocol 264/12). The surgeon obtained the written consents from five patients, and skin was collected anonymized immediately following surgery. The viable skin was rinsed with physiological water (saline water; 0.9% NaCl) and dermatomed to a thickness of 800 µm using an electrical dermatome (Acculan®II, B. Braun/Aesculap, Sempach, Switzerland). The skin flap was cut into circular discs (skin contact area of 1.7 cm^2) before mounted on the flow-through diffusion cells (PermGear®, SES Analytical System, Bechenheim, Germany). No more than two hours elapsed following the surgery-end to skin mounting, unless it was frozen at -20°C directly after dermatoming it.

Solubility

TF is highly soluble in organic solvents (54 g/L in n-heptane and >250 g/L in ACN) and has a very low solubility in water (0.9 mg/L at 20ºC). The choice of receptor liquid is determined by the solubility of the test substance as specified in the Organisation for Economic Co-operation and Development (OECD) guidelines (OECD, 2004a, b). TF's solubility was assessed for the following receptor liquids: water, water with 6% polyethylene glycol (PEG; Brij® O10), and ACN:H_2O 50:50 at 20ºC (environmental test conditions) and 32ºC (skin experiment temperature). The TF powder was added to an Erlenmeyer flask (2 L) filled with water or water mixed with 6% PEG until the reported water solubility concentration was reached (0.9 mg/L). After stirring (5-10 minutes), the solution was heated in a water bath to 32°C for 5-10 minutes. If TF did not solubilize, we added liquid (200 mL), heated, and continued stirring. This dilution procedure was repeated until TF was dissolved or up to 2 L. ACN:H_2O solution was prepared by mixing ACN (5 mL) with water (5 mL). TF powder was added to 1 mL ACN:H_2O solution. After stirring (few seconds), the ACN:H_2O solution was added in 1 ml increments and stirred and repeated until dissolved.

We used ACN:H_2O solution as the receptor liquid for frozen human skin experiments. To keep the skin viable, we could not use an organic solvent (ACN) in the receptor liquid (Barba et al., 2016) therefore we added 6% PEG to water as the receptor liquid (12 mL) (Krohn, 1988a). All experimental parameters are summarized in Table 2.

Hydrolysis

Hydrolyses rates for TF to DMST in ACN:H_2O 50:50 (our receptor fluid) have not

been published in scientific peer reviewed literature. We needed to assess the amount of TF and DMST in the donor chamber and receptor fluid during our experiments. We, therefore, quantified TF and DMST in TF solution (2.04 mg TF added to ACN:H_2O 50:50 (15 mL)) at ambient temperature up to 24 hours. The TF solution was divided into 8 vials (≈1.8 mL) and kept at 20°C until chemical analyses. We analyzed TF and DMST with a liquid chromatography (LC) equipped with a UV detector (Ultimate 3000, Thermo fischer, Reinach). TF and DMST were separated using a C18 column (3 x 50 mm, 1.8 µm) (Zorbax Eclipse Plus, Agilent, Morges, Switzerland) heated to 30°C. The mobile phase was ACN:H_2O 50:50 at a flow rate of 0.3 mL/min. Both compounds were detected at 193 nm. The retention time for DMST was 1.9 min and 12.1 min for TF. The 8 vials were used in injecting the following time points (vial no) to assess hydrolysis rate: 0 (vial 1), 0.5 (vial 1), 1 (vial 2), 2 (vial 3), 3 (vial 4), 4 (vial 5), 8 (vial 6), 12 (vial 7), and 24(vial 8) h (vial 1 was injected twice as the LC run was 15 minutes).

Skin permeation experiments

The flow-through diffusion cell system consisted of a rack of six jacketed cells (12 ml receptor chamber) operated by a peristaltic pump (8 channels, Ismatec IPC-N, IDEX Health & Science GmbH, Wertheim-Mondfeld, Germany) at a rate of 3.6 mL/h (complete liquid turnover after 3.3 hours) and kept at 32°C with a circulating water bath.

Cell culture media is known to protect the skin integrity; however, it was too difficult to chemically analyze these samples therefore we switched to water with 6% PEG. TF was applied in ACN:H_2O (50:50, v/v) and DMST in ACN as this was the solvent in which the standard was bought.

Six mounted skin discs for each donor were stabilized for approximately 30 minutes prior to skin barrier integrity assessment by measuring the transepidermal water loss (TEWL; mean measured value was 7.5 ± 2.7 g/m^2/h) using a VapoMeter (Delfin Technologies Ltd., Kuopio, Finland). Skin discs with values above 11 g/m^2/h were considered damaged (Pinnagoda et al., 1990), and replaced before topical applications of standard solutions or commercial products. Experiments were performed using infinite doses (i.e., the added volume of the tested compounds were sufficient for the duration of the experiment) for TF (1 mL) and DMST (0.2 mL) standard solutions. Undiluted commercial product (1 mL, product 1) and TF in ACN:H_2O (1mL) were tested under the same experimental conditions. For frozen skin, the receptor liquid was ACN:H_2O as this would ensure the sink effect for the lipophilic TF and no metabolism was expected. Exposure times were either 8 hours to simulate a working day and 16 hours to represent TF residues not washed off after work. These were thus convenience-sampling times. We were limited by the number of programmable collection times (eight time points) as well as the maximum volume collected per time point. Table 2 summarizes the main experimental parameters. We repeated the TEWL measurements at the end of the experiment to assess skin integrity after exposure to TF and ACN. We did not observe obvious differences.

Tolylfluanid and DMST quantification

The receptor samples (4 mL) were liquid-liquid extracted with dichloromethane (2 mL). The samples were agitated (vortex, 20 min), and then centrifuged (400 rpm, 10 min). The organic phase was transferred to a second vial. This procedure was repeated twice; and the organic phases combined. The organic

phase was then gently evaporated to dryness under nitrogen. The sample was reconstituted in n-heptane (200 µl) before analysis. TF and DMST liquid extraction recovery rates were greater than 90%.

TF and DMST were analyzed by a capillary gas chromatograph with mass spectroscopy (GC-MS) detection according to Rasmussen et al. (2003). Briefly, the sample (1 µL) was injected in splitless mode (injector set at 250°C), and separation was achieved with a low polarity column (30 m, ID 0.32 mm, film 0.25 µm, a DB1701 from Agilent, Morges, Switzerland). The following GC program was used: starting temperature 160°C (1 min), 15°C/min until 260°C (8 min). The MS (Scion 456, Bruker Daltonics, Brement, Germany) operated in electron ionization (E.I.) mode at 70 eV, quantifying the following mass to ratio (m/z) fragments: TF (m/z 347/137) and DMST (m/z 215/106). The total analysis time was 10 min/sample with retention times of 7.4 min for TF and 5.5 min for DMST. Limit of detection for TF was 0.1 µg/mL and 0.05 µg/mL for DMST.

Exposure Simulation Experiments

Paint tasks were simulated in an exposure chamber (10 m^3) with a general ventilation (180 m^3/h) corresponding to a well ventilated industrial setting indoors. Airborne TF concentrations were measured during paint rolling (polyamide fiber, 10 cm wide) and paint spraying using a pressurized gun (generic brand from the local hardware store). Product 2 was applied on two pressed wooden boards (150 x 50 cm) mounted side-by-side. There are no validated air sampling methods for TF. We therefore sampled TF in air actively (MSA Escort Elf pump, MSA Auer, Wangen, Switzerland, 1 mL/min) using two glass impingers in series filled with ACN (15 mL) and following the published method of Bohn et al. (2000). The impingers were placed

next to the laboratory technician that simulated the paint tasks. Samples were collected during rolling (5 min) and spray-painting (3 min) and the following 1 h. A sample (1 mL) was collected from each impinger, directly injected into the GC-MS, and analyzed as described earlier.

Data analysis

Fick's first law of diffusion is a quantitative description of human skin permeation and simplified as followed from Pirot et al. (1997):

$$J = K_p \, C_V \qquad \text{(Eq. 1)}$$

where J is the permeation coefficient (ng/cm^2/h), C_V is the concentration of the applied chemical (ng/cm^3), and Kp is the skin permeability coefficient (cm/h).

Human skin permeation rates or permeation coefficient (J), time lag (T_{lag}), and permeation coefficients (Kp) were calculated based on the skin permeation curves generated from TF and DMST concentrations in the reservoir liquid. Means and standard deviations for the replicates are given in Table 2. Although, steady-state conditions were not reached in the experiments J and T_{lag} can still be calculated from cumulative amount of TF or DMST absorbed per unit skin area per time course for each permeation cell. Specifically, the slope (J) (µg/cm^2/h) was determined from the steepest linear part of the permeation curve; and where this part of the curve intercepted the time axis was determined as T_{lag} (h). Dividing J by the initial TF or DMST concentration gave Kp (cm/h). For TF in water, which was a supersaturated solution, we calculated Kp assuming TF saturation concentration (0.9 µg/mL). TF and DMST were not quantified in the skin or in the donor chamber at the end of the experiment.

Results

TF solubility

We did not manage to solubilize TF according to its reported solubility in water (0.9 mg/L). We therefore had to use a solvent in the receptor liquid. We measured TF's solubility in water with 6% PEG as 0.45 mg/L and in ACN:H_2O (50:50) as 100 mg/L.

Hydrolysis

We detected DMST immediately in the prepared TF solution (T0) reducing the TF concentration by about 10%, and after 24 hours about 15%. The DMST concentration doubled after 30 minutes (T0.5), and slightly increased until 24 hours (Figure 1). Consequently, around 10% of applied TF penetrated the stratum corneum as DMST during 8-16 hour experiments at 20°C.

Ex-vivo human skin permeation

Skin permeation results are presented in Table 2 and permeation curves in Figure 2. Applied TF was only detected and quantified as DMST in viable human skin with a low permeation rate (0.18 ± 0.05 µg/cm^2/h) and a moderate apparent T_{lag} (7.1 ± 1.4 h). The permeation rate increased 10-fold using frozen skin, and T_{lag} was reduced to half. Contrary to viable skin, we quantified TF in receptor liquid with frozen skin, although at very low concentration.

Applying DMST to the viable skin gave a very low permeation rate (0.05 ± 0.01 µg/cm^2/h), which is about a quarter of the TF rate. Although, the amount applied differed by a factor of 5, both DMST and TF was in infinite dose for the skin, and the *J*s should be comparable. The apparent DMST T_{lag} (7.38 ± 0.25 h) was about the

same as when TF was applied. Permeation rates increased more than 100-fold in frozen skin and T_{lag} was less than 2 hours. Steady state was achieved after 12 hours.

The permeation rate across frozen skin was 100 times lower for the paint product compared to TF measured as DMST. The apparent T_{lag} however, was immediate compared to the observed 4 hours for TF applied in solution. We also quantified TF in the receptor liquid when paint was applied. T_{lag}s were similar for paint and TF in solution while the permeation rate was 20 times lower when paint was applied.

Kp could not be calculated for TF in viable skin because only DMST was quantified in the receptor liquid. Kp was calculated for TF in frozen skin experiments by reducing the applied TF dose 10% to adjust for the TF hydrolyzes process when diluted in ACN:H_2O (see Figure 1). TF applied dose was not adjusted for the paint product that did not contain any water.

TEWL skin measurements before and after the experiments did not differ including TF applied in ACN:H_2O (data not shown). Consequently, we conclude that the skin integrity was intact.

Air concentrations during simulated paint jobs

Amount of airborne DMST quantified in the impinger liquid during roller painting was 7.6 µg for 122 g of paint used. No TF was detected. When the plywood boards were spray-painted, 887 g of paint was used and both DMST (17.1 µg) and TF (0.13 µg) were quantified in the impinger liquid. The expected hydrolysis ratio of 1:9 DMST:TF ratio was not observed. Although we can only speculate, this might be related to the presence of humidity (~36%) in air that have large contact surfaces

with the paint. These experiments showed that small amounts of aerosolized DMST and TF are airborne during manipulation of TF containing paint, and consequently, skin exposures from aerosol deposition are possible. Minute DMST amounts (0.5 µg) were quantified in the 1 h sample collected after completion of the paint tasks.

Discussion

TF permeated as DMST through viable human skin at a low permeation rate (0.18 µg/cm^2/h), and a moderate T_{lag} (7 h). The permeation rates and T_{lag} were not comparable between frozen and viable skin experiments. We adjusted for TF hydrolysis as this influenced the skin absorption results.

To permeate through the skin, the first step is to cross the stagnant layer of delivery vehicle adjoining the surface of the skin, and then through the *stratum corneum* followed by the epidermis. The final step is to diffuse through the aqueous phase of the dermis and into the capillaries (Brown et al., 2016). In our experiments, skin was dermatomed to 800 µm and included the *stratum corneum* (about 20 µm) and parts of the dermis underneath. The expected permeation mechanism for TF is that TF readily permeates through the *stratum corneum*, and metabolizes in the epidermis to the more hydrophilic DMST. This in turn diffuses into the receptor liquid. In contrast, DMST diffuses slowly into the *stratum corneum* from the applied vehicle due to its hydrophilicity. This is supported by the similar T_{lag}s for TF and DMST as well as the much faster TF permeation rate compared to DMST (Table 2). TF was detected in receptor liquid only in frozen skin and not in viable skin. From this, we conclude that TF is metabolized completely to DMST in the skin. Furthermore, the driving force for viable skin permeation was probably TF metabolism.

In addition to the effects of freezing the skin, our assays show that other possible effects such as solvents in the receptor liquid contributes to permeation differences observed. We wanted to keep the sink effect and maximize the diffusion across the skin, and to do so, we used solvents in the receptor liquid. This would render viable skin not metabolically active, therefore we chose to use frozen skin, which is not very metabolically active, to assess this effect. The receptor liquid for these assays were 50:50 ACN:H_2O. TF permeation rate was greater and T_{lag} was shorter than expected if metabolism was the only driving force across skin. TF's lipophilicity could contribute to the diffusion from the hydrophilic epidermis into the solvent containing receptor liquid. We conclude that for frozen skin, the solvents in the receptor liquid affected TF's skin permeation.

We computed an apparent absorbed dose based on our skin permeation experiments. The amount of DMST (mole) determined in the receptor liquid at 16 hours was divided with the amount of TF (mole) applied, and gave an absorbed TF dose of 4%. Our results gave a smaller absorbed TF dose than ECHA (2014) reported in their 2014 report. They calculated an absorbed TF dose of 71% after 24 hours as described "*In vitro* dermal absorption study in human epidermis using a mineral-oil based formulation of tolylfluanid (0.7% by weight) was applied for 6 h.". Performing the same calculations (amount of DMST in the receptor liquid divided by amount of TF applied) for our frozen skin experiments, gave an apparent absorbed TF dose of 37%. Unfortunately, we are unable to comment further on this difference as description of the ECHA study is missing.

The TF permeation rate was lower for the commercial paint product compared to TF in solution while the T_{lag} was immediate. Commercial products and formulations contain ingredients that may reduce or facilitate permeation. We have

previously observed this with pesticide and their formulations such as isoproturon (Berthet et al., 2014). This could potentially be described by the thermodynamic activity of TF in the different formulations. This activity is defined as the tendency of the molecule to leave the delivery vehicle environment and enter the stratum corneum. The SDSs for the commercial products do not give specific ingredients nor concentration information to assess how these would influence TF skin permeation.

We wanted to know if two common skin permeation models could predict permeation rates for TF. One simple model by Fiserova-Bergerova et al. (1990) was developed based on theoretical diffusivity of chemicals through a hypothetical human reference skin (Korinth et al., 2012) and require the following parameters: molecular weight, Log P and the water solubility of TF (Eq.2).

$$J = \frac{C_{sat}}{15}\,(0.038 + 0.153\,P)\,e^{-0.016\,MW} \qquad \text{(Eq. 2)}$$

$$J\,(mg/cm^2/h) = \frac{0.9 \times 10^{-3}}{15}\,(0.038 + 0.153\,\times 7943)\,e^{-0.016\,\times 347.3}$$

$$J\,(\mu g/cm^2/h) = 0.28$$

A second model by Guy and Potts (1993) used Kp values from experimental databases (Flynn, 1990) (Eq.3 and 4).

$$J\, = K_p\,\times\,C_{sat} \qquad \text{(Eq. 3)}$$

$$J\,(mg/cm^2/h) = \,[0.0018\,\times\,P^{0.71}\,\times\,e^{-0.014\,MW}]\,\times\,C^{sat} \qquad \text{(Eq. 4)}$$

$$J\,(mg/cm^2/h) = \,[0.018\,\times 7943^{0.71}\,\times\,e^{-0.014\,\times 347.3}]\,\times (0.9\,\times\,10^{-3})$$

$$J\,(\mu g/cm^2/h) = 0.07$$

Where, K_p is the permeability coefficient, C_{sat} is the concentration of saturated aqueous solution of the chemical (mg/ml; 0.9 mg/L for TF (Krohn, 1985)), P is the octanol-water partition coefficient (log P_{ow} = 3.9 for TF (Krohn, 1988b), and MW is the molecular weight (g/mol; 347.3 g/mol for TF).

The Fiserova-Bergerova modeled rate (0.28 µg/cm^2/h) overestimated the TF permeation rate by 55% while the Guy and Potts model (0.07 µg/cm^2/h) underestimated by 39% compared to our experimentally determined TF permeation rate (0.18 µg/cm^2/h). The variability in the predictive values could be due to metabolism and the heterogeneous database used to derive the model (Korinth et al., 2012).

The skin permeation rates obtained can be used for risk assessment purposes where internal doses are calculated and compared to toxicity. For a typical paint job, we assume an 8-hour workday with constant exposures (not showering or washing of hands and face during work hours) where both hands and forearms and face are exposed. This gives a skin surface area of about 4,000 cm^2. This would give an estimated internal dose (4000 cm^2 x 8 h work x 0.18 µg/cm^2/h) from TF skin exposure of 5.8 mg. This is an unrealistic estimate for several reasons. Workers would probably be exposed to paint and not pure TF and not paint 8 hours per day but perform other activities in between (e.g. sanding the boat before applying the antifouling agent). Skin exposure to deposited aerosols would not be an infinite dose thus the skin permeation rate would probably be lower than the one we used which is for steady state. It does show, however, that internal exposure will likely occur. Risk assessments for TF have been performed within the EU biocide regulation context (Article 89 of Regulation (EU) No 528/2012) by European Chemicals Agency (ECHA) (ECHA, 2014). Skin absorption was estimated to be during spray painting 60

mg/person/day and 13.2 mg/person/day during brushing or rolling paint, given a bodyweight value of 60 kg for an adult, a skin delivery rate of 3.3 % determined by an example product containing 2.76 % TF w/w. Although, these comprehensive risk assessment estimates are far greater than our simplistic assessment, they support the conclusion that TF may enter the body.

We acknowledge that there are several limitations to our study:

- We used a simplified occupational hygiene exposure measurement to determine possible TF exposure during spray or rolling paint in the exposure chamber. We did not characterize the liquid aerosol phase separately from the vapor phase, which would have required a filter and absorbent tube, respectively. Currently, no TF air sampling method has been published, and developing such a method was outside the scope of this study. This was the reason why we sampled using an impinger. Although aerosols containing TF were not characterized, we give a total amount of TF generated in air during these short work activities.
- We dissolved TF, a solid, in ACN as TF can only be partially dissolved in water. ACN, as all organic solvents, is probably damaging to the skin, but we could not assess this, as our TEWL readings did not show a difference before and after exposure. In the case of TF, antifouling paint usually contain solvents (in the products tested it was naphtha), consequently, by adding ACN this would mimic antifouling paint exposure.
- We did not wash off TF and DMST skin residues at the end of the experiment (no mass-balance was performed). Although no rinse off was used, we stopped the experiment after 8 hours assuming a full work day and after a 16 hours assuming a full work day and no handwashing until 8 hours after work.

We are in accordance with the OECD TGD 428 with respect to human skin, experimental flow-through diffusion cell set-up, skin integrity, TF preparation, temperature, duration of exposure, receptor liquid, but not for assessing overall recovery. However, mass-balance studies are not required for infinite dose experiments (art.21).

TF has shown sensitizing properties in several animal studies (ECHA, 2014). At a TF manufacturing plant, 2/60 workers developed TF skin allergies but only after a number of years (unspecified) (Faul, 1989). Both cases resolved when the workers were not packing TF goods. Others have reported no sensitization among workers dipping or brushing wood preservatives with TF concentrations of 0.8–0.9% (Roos, 1993), nor in customers using the product at home or in individuals in contact with the treated wood (Imsgard, 1993; Olloz, 1993; Schneeberger, 1993). The discrepancies in sensitization rates among TF exposed populations could be due to amount and frequency of TF containing products as well as their formulations. We encourage risk assessors to use measured *in vitro* permeation rates for untested dilutions or products, and consider permeation of possible metabolites, especially for experiments conducted with metabolically active skin.

Conclusion

TF permeated human viable skin as DMST and the skin permeation rates were fairly low (0.18 µg/cm^2/h). Predictive skin permeation models were close to the measured *in vitro* rates obtained in our experiments. Further research should investigate the DMST toxicity as this is lacking from the scientific literature.

Acknowledgements

The authors thank Laure-Elise Forel and Nicole Charrière for their assistance in skin experiments and in obtaining the commercial products as well as Simon Deslarzes for his contribution to the chemical analyses.

Funding

This work was supported by the Swiss Federal Office of Public Health (FOPH).

Disclosure statement

The authors report no conflicts of interest.

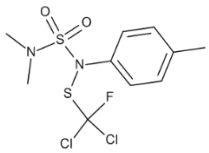
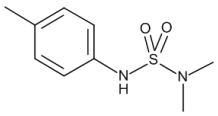
Ethical standards

The manuscript does not contain clinical studies or patient data.
Human abdominal skin was obtained as surgical waste of abdominoplasty from DAL biobank (Lausanne University Hospital (CHUV), Lausanne, Switzerland), under anonymous donation, in accordance with its regulation and the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The protocol 264/12 was accepted by the State Ethics Commission. The surgeon obtained the general consent of patients for the use of the removed skin and the skin samples were deidentified before collection.

Research involving human participants and/or animals

This article does not contain any studies with human participants or animals performed by any of the authors.

Table 1 Physical and chemical properties of tolylfluanid (reported from ECHA (2014)) and of DMST (reported from PubChem; (National Center for Biotechnology Information (NCBI)))

		
	Tolylfluanid	DMST
IUPAC name	N-(Dichlorofluoromethylthio)-N',N'-dimethyl-N- p-tolylsulfamide	1-(dimethylsulfamoylamino)-4-methylbenzene
Molecular formula	$C_{10}H_{13}Cl_2FN_2O_2S_2$	$C_9H_{14}N_2O_2S$
CAS number	731-27-1	66840-71-9
Molecular weight (g/mol)	347.3	214.3
Log K_{OW}	3.9 at 20°C (independent of the pH)	NA.
Water solubility	0.9 mg/L at 20°C and pH 4-9	NA
Solubility in organic solvents[a]	> 250 g/L	NA
Solubility in n-heptane	54 g/L (20°C)	NA

NA: not available.

[a] In acetone, acetonitrile, dichloromethane, dimethylsulfoxide, and ethylacetate

Table 2 Summary of ex vivo skin permeation protocols including duration (D), number of donor skins (N), and number of replicates (n) as well as skin permeation coefficient (J), Tlag, and permeability coefficient (Kp) means and standard deviations (SD).

Applied			Compound analyzed	Experiment				J (µg/cm²/h)		T_{lag} (h)		Kp (cm/h)	
substance	Concentration (µg/µL)	Amount (µg)		Receptor liquid	D (h)	N	n	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Viable skin													
TF (ACN:H_2O)	0.10	100	DMST	H_2O+6% PEG	16	3	13	0.176	0.05	7.10	1.41	NA[a]	NA[a]
DMST (ACN)	0.10	20	DMST	H_2O+6% PEG	16	2	4	0.050	0.01	7.38	0.25	5.44E-04	1.23E-04
Frozen skin													
TF (ACN:H_2O)	0.10	100	TF	ACN:H_2O	16	1	2	0.088	0.08	11.2	0.15	9.96E-04[b]	9.38E-04[b]
			DMST					2.12	0.23	4.16	0.92	NA[a]	NA[a]
DMST (ACN)	0.10	20	DMST	ACN:H_2O	16	1	2	7.18	4.85	<2	NA[c]	7.18E-02	4.84E-02
Paint product	50.0	50,000	TF	ACN:H2O	16	1	2	0.004	0.005	10.0	1.88	8.58E-08	9.45E-08
			DMST					0.02	0.001	NA[d]	NA[d]	NA[a]	NA[a]

ACN: Acetonitrile;
ACN:H2O: 50:50 v/v solution,
DMST : 1-(dimethylsulfamoylamino)-4-methylbenzene;
NA: not available
PEG: polyethylene glycol (Brij® O10)
TF : tolylfluanid;

[a] Cannot compute since DMST was quantified while TF was applied
[b] The TF dose was adjusted down 10% because TF hydrolyzes immediately to DMST when diluted in ACN:H_2O
[c] Time point at which we quantify DMST in the receptor liquid
[d] Permeates immediately

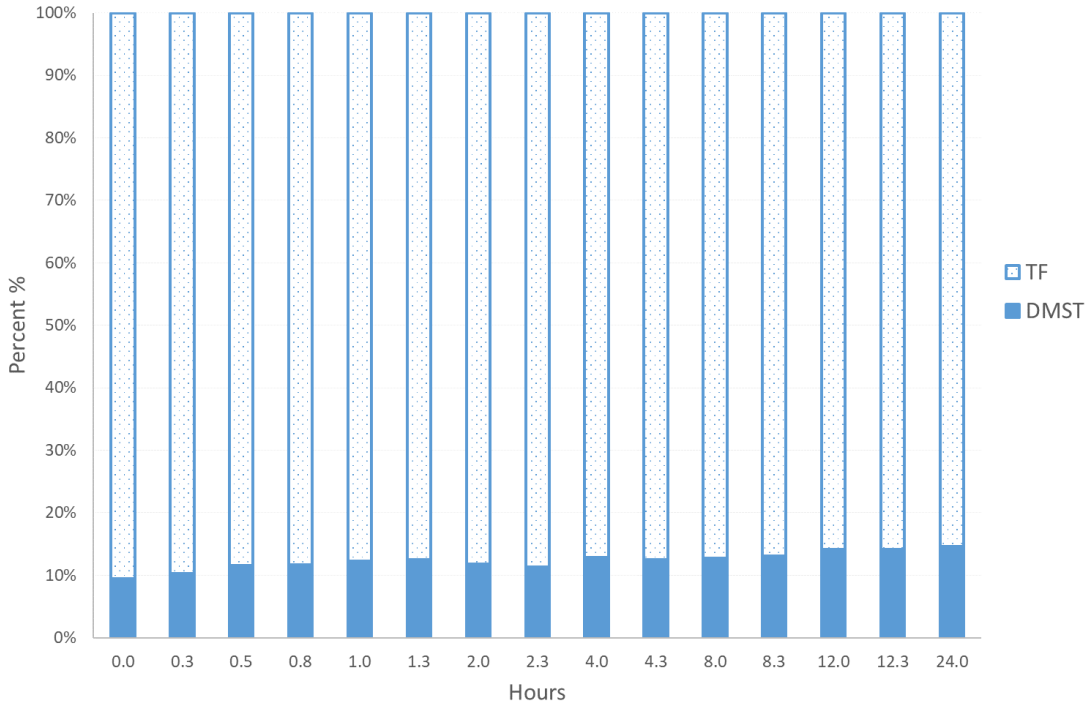
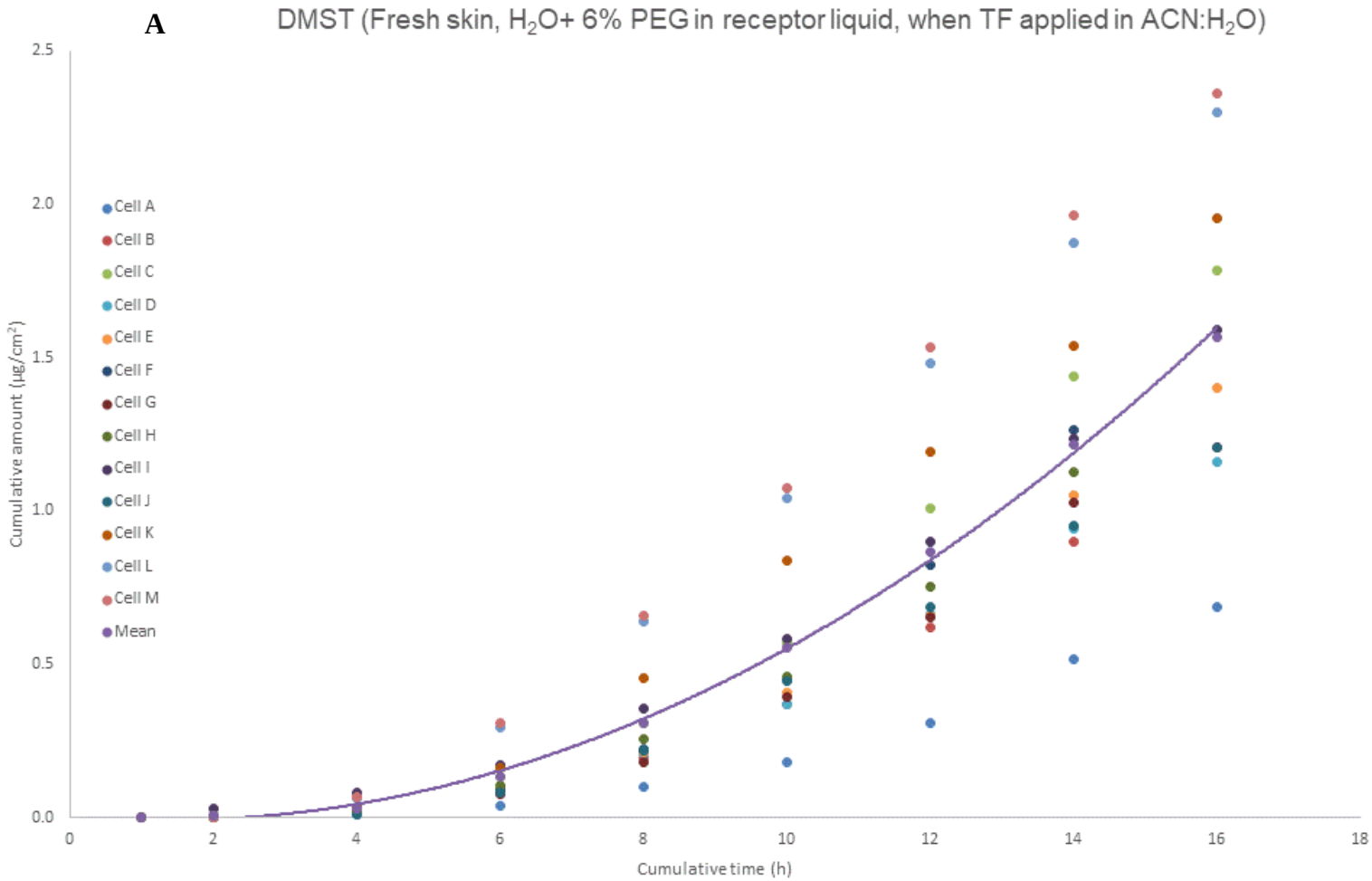


Figure 1 Percent of TF (y-axis) hydrolyzed to DMST in H2O over time (x-axis) at 20 °C.

A


DMST (Fresh skin, H2O+ 6% PEG in receptor liquid, when TF applied in ACN:H2O)
Cumulative amount (µg/cm²)
Cumulative time (h)
Cell A
Cell B
Cell C
Cell D
Cell E
Cell F
Cell G
Cell H
Cell I
Cell J
Cell K
Cell L
Cell M
Mean
0.0
0.5
1.0
1.5
2.0
2.5
0
2
4
6
8
10
12
14
16
18

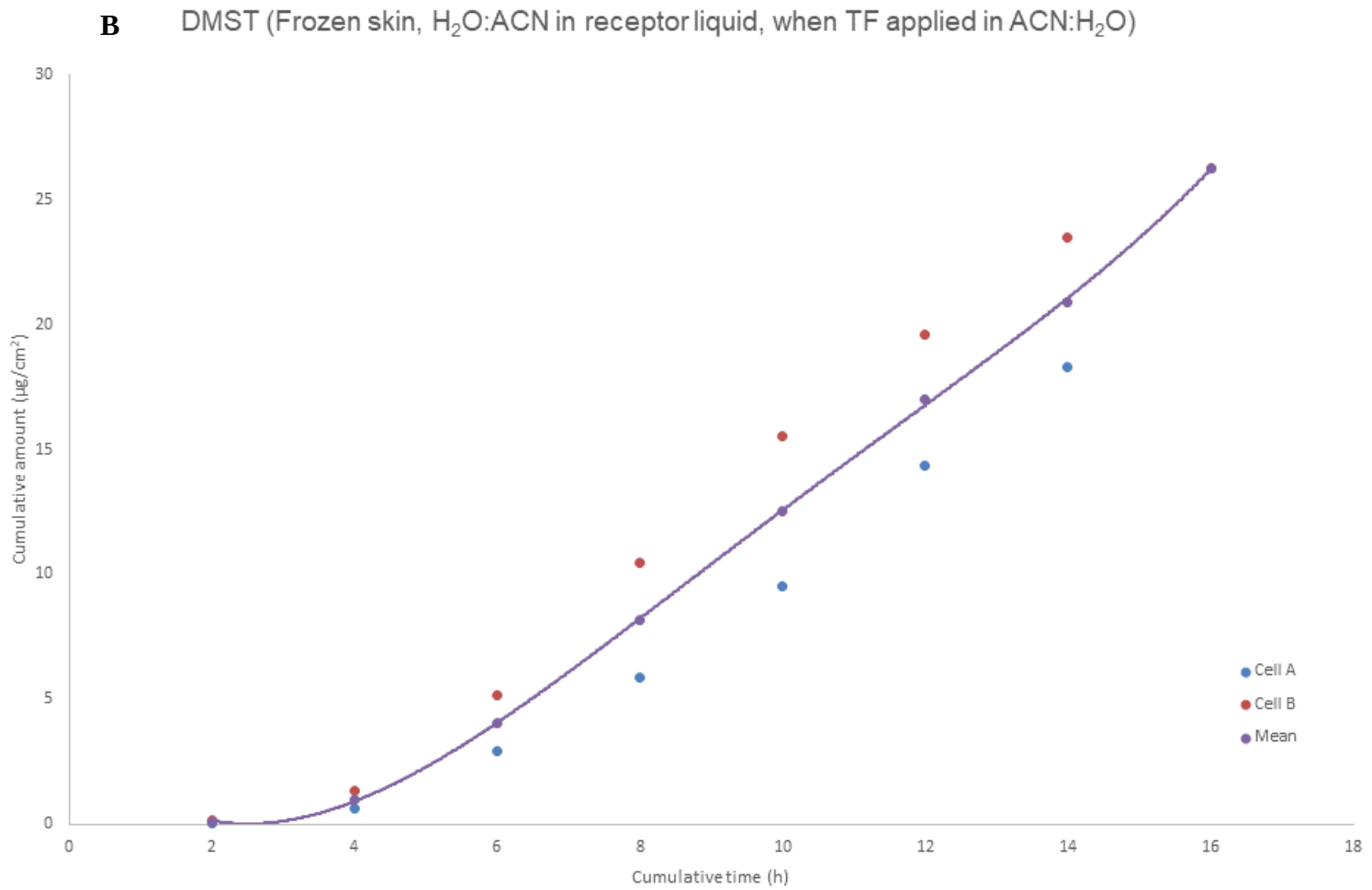


Figure 2 Skin permeation curves for cumulative amount of DMST (y-axis) over time (x-axis) for viable skin (A) and frozen skin (B) flow-through diffusion cell experiments when TF was applied to the skin. Individual dots are flow-through diffusion cell results and the orange line is the mean.

References

Anderson, S.E., Meade, B.J., 2014. Potential health effects associated with dermal exposure to occupational chemicals. Environmental health insights 8, 51-62.

Barba, C., Alonso, C., Martí, M., Manich, A., Coderch, L., 2016. Skin barrier modification with organic solvents. Biochimica et Biophysica Acta (BBA) - Biomembranes 1858, 1935-1943.

Berthet, A., Hopf, N.B., Miles, A., Spring, P., Charriere, N., Garrigou, A., Baldi, I., Vernez, D., 2014. Human skin in vitro permeation of bentazon and isoproturon formulations with or without protective clothing suit. Arch Toxicol 88, 77-88.

Bohn, S., Niederer, M., Brehm, K., Bircher, A.J., 2000. Airborne contact dermatitis from methylchloroisothiazolinone in wall paint. Abolition of symptoms by chemical allergen inactivation. Contact Dermatitis 42, 196-201.

Brown, T.N., Armitage, J.M., Egeghy, P., Kircanski, I., Arnot, J.A., 2016. Dermal permeation data and models for the prioritization and screening-level exposure assessment of organic chemicals. Environ Int 94, 424-435.

Cesnik, H.B., Gregorcic, A., Bolta, S.V., Kmecl, V., 2006. Monitoring of pesticide residues in apples, lettuce and potato of the Slovene origin, 2001-04. Food Addit Contam 23, 164-173.

ECHA, 2014. Assessment Report - Tolylfluanid Product-type 21 (Antifouling products). In: ECHA (Ed.) Regulation (EU) No 528/2012 concerning the making available on the market and use of biocidal products.

European Food Safety Authority, E., 2005. Conclusion regarding the peer review of the pesticide risk assessment of the active substance tolylfluanid. EFSA Journal 3, 29r-n/a.

European Food Safety Authority, E., 2013. Reasoned opinion on the review of the existing maximum residue levels (MRLs) for tolylfluanid according to Article 12 of Regulation (EC) No 396/2005. EFSA Journal 11, 3300-n/a.

European Parliament, C.o.t.E.U., 2012. Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. In: EUR-Lex (Ed.), vol. 528/2012. Official Journal of the European Union, p. 123 pages.

FAO/WHO, 2002. Pesticide residues in food 2002 : Evaluations Part 1 - Residues, Volume 2. vol. 2. Food and Agriculture Organization of the United Nations, Rome, Italy, pp. 1301-1388.

Fasano, W.J., McDougal, J.N., 2008. In vitro dermal absorption rate testing of certain chemicals of interest to the Occupational Safety and Health Administration: summary and evaluation of USEPA's mandated testing. Regulatory toxicology and pharmacology : RTP 51, 181-194.

Faul, J., 1982. Statement to Pkt IV/1.2.2 of the BBA application form 'Details of effects on man, internal company experience'. Unpublished report 28 April 1982 from Bayer AG, Submitted to WHO by Bayer AG, Bayerwerk, Germany.

Faul, J., 1989. Euparen and Euparen M—In-company occupational medical experience. Submitted to WHO by Bayer AG, Bayerwerk, Germany.

Fiserova-Bergerova, V., Pierce, J.T., Droz, P.O., 1990. Dermal absorption potential of industrial chemicals: criteria for skin notation. Am J Ind Med 17, 617-635.

Flynn, G.L., 1990. Physicochemical determinants of skin absorption. In: Gerrity TR, H.C. (Ed.) Principles of route-to-route extrapolation for risk assessment. Elsevier, New York, pp. 93–127.

Guy, R.H., Potts, R.O., 1993. Penetration of industrial chemicals across the skin: A predictive model. American Journal of Industrial Medicine 23, 711-719.

Imsgard, F., 1993. Overview concerning the use of Preventol A4 (tolylfluanid) by GORI. GORI, Kolding, Denmark, Submitted to WHO by Bayer AG, Bayerwerk, Germany.

Korinth, G., Schaller, K., Bader, M., Bartsch, R., Göen, T., Rossbach, B., Drexler, H., 2012. Comparison of experimentally determined and mathematically predicted percutaneous penetration rates of chemicals. Archives of Toxicology 86, 423-430.

Krohn, J., 1985. Water solubility of DMST at 20°C. Bayer AG.

Krohn, J., 1988a. Dissociation constant of tolylfluanid. Bayer AG.

Krohn, J., 1988b. Partition coefficient of tolylfluanid (Methyl-Euparen) at 21°C. Bayer AG.

Links, I., Van Der Jagt, K.E., Christopher, Y., Lurvink, M., Schinkel, J., Tielemans, E., Van Hemmen, J.J., 2007. Occupational exposure during application and removal of antifouling paints. Ann Occup Hyg 51, 207-218.

NCBI, Tolylfluanid. In: Information, N.C.f.B. (Ed.). PubChem Compound Database.

Neel, B.A., Brady, M.J., Sargis, R.M., 2013. The Endocrine Disrupting Chemical Tolylfluanid Alters Adipocyte Metabolism via Glucocorticoid Receptor Activation. Molecular Endocrinology 27, 394-406.

OECD, 2004a. Guidance Document for the Conduct of Skin Absorption Studies. OECD Series on Testing and Assessment Number 28, Paris, Organisation for Economic Co-operation and Development.

OECD, 2004b. OECD guideline 428 for the testing of chemicals. Skin absorption: in vitro method. OECD Series on Testing and Assessment Number 428, Paris, Organisation for Economic Co-operation and Development.

OECD, 2004c. Test No. 428: Skin Absorption: In Vitro Method.

Olloz, F., 1993. Preventol A5/tolylfluanide. Pentol AG, Switzerland, Submitted to WHO by Bayer AG, Bayerwerk, Germany.

Pinnagoda, J., Tupker, R.A., Agner, T., Serup, J., 1990. Guidelines for transepidermal water loss (TEWL) measurement. A report from the Standardization Group of the European Society of Contact Dermatitis. Contact Dermatitis 22, 164-178.

Pirot, F., Kalia, Y.N., Stinchcomb, A.L., Keating, G., Bunge, A., Guy, R.H., 1997. Characterization of the permeability barrier of human skin in vivo. Proc Natl Acad Sci U S A 94, 1562-1567.

Rasmussen, R.R., Poulsen, M.E., Hansen, H.C.B., 2003. Distribution of multiple pesticide residues in apple segments after home processing. Food Additives and Contaminants. 20 (11) (pp 1044-1063), 2003. Date of Publication: November 2003.

Reby, D., Patatian, A., Bader, T., Durand, C., Gasser, P., Scalvino, S., Percoco, G., Peno-mazzarino, L., Benech, P., Lati, E., 2015. Skin sensitization measured in an ex vivo skin model. IFSCC Conference, Zurich, Switzerland.

Regnier, S.M., Zhang, X., El-Hashani, E., Kirkley, A., Williams, A., Kamau, W., Thomas, C., Sargis, R.M., 2014. Effects of the endocrine disruptor tolylfluanid on global energy metabolism. Endocrine Reviews. Conference: 96th Annual Meeting and Expo of the Endocrine Society, ENDO 2014 Chicago, IL United States. Conference Start: 20140621 Conference End: 20140624. Conference Publication: (var.pagings). 35 (no pagination), 2014. Date of Publication: 2014.

Roos, B., 1993. Tolylfluanid VP OC 3049. Geveko Oy, Finland, Submitted to WHO by Bayer AG, Bayerwerk, Germany.

Sargis, R.M., Neel, B.A., Brock, C.O., Lin, Y., Hickey, A.T., Carlton, D.A., Brady, M.J., 2012. The novel endocrine disruptor tolylfluanid impairs insulin signaling in primary rodent and human adipocytes through a reduction in insulin receptor substrate-1 levels. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1822, 952-960.

Schneeberger, R., 1993. Unpublished report. Blaser & Co. AG, Switzerland, Submitted to WHO by Bayer AG, Bayerwerk, Germany.

Stensvand, A., Christiansen, A., 2000. Investigation on fungicide residues in greenhouse-grown strawberries. J Agric Food Chem 48, 917-920.

Tielemans, E., Louwerse, E., de Cock, J., Brouwer, D., Zielhuis, G., Heederik, D., 1999. Exposure to Fungicides in Fruit Growing: Re-Entry Time as a Predictor for Dermal Exposure. Am Ind Hyg Assoc J 60, 789-793.

Wilmes, R., 1982. Fate/behaviour of crop protection products in water. Bayer AG.

Figure captions

Figure 1. Percent of TF (y-axis) hydrolyzed to DMST in H2O over time (x-axis) at 20 °C.

Figure 2. Skin permeation curves for cumulative amount of DMST (y-axis) over time (x-axis) for viable skin (A) and frozen skin (B) flow-through diffusion cell experiments when TF was applied to the skin. Individual dots are flow-through diffusion cell results and the orange line is the mean.