

Evaluation of in vitro vs. in vivo methods for assessment of dermal absorption of organic flame retardants

Abdallah, Mohamed Abou-Elwafa; Pawar, Gopal; Harrad, Stuart

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1 **Evaluation of *in vitro* vs. *in vivo* methods for assessment of dermal**
2 **absorption of organic flame retardants: A review**

3
4 Mohamed Abou-Elwafa Abdallah^{1,2*}, Gopal Pawar¹ and Stuart Harrad¹

5
6 ¹Division of Environmental Health and Risk Management,

7 College of Life and Environmental Sciences,

8 University of Birmingham,

9 Birmingham, B15 2TT,

10 United Kingdom.

11
12 ²Department of Analytical Chemistry

13 Faculty of Pharmacy, Assiut University

14 71526 Assiut,

15 Egypt

16
17 * Corresponding author

18 Email mae_abdallah@yahoo.co.uk

19 Tel. +44121 414 7297

20 Fax. +44121 414 3078

21

22

23 **Abstract**

24 There is a growing interest to study human dermal exposure to a large number of chemicals,
25 whether in the indoor or outdoor environment. Such studies are essential to predict the
26 systemic exposure to xenobiotic chemicals for risk assessment purposes and to comply with
27 various regulatory guidelines. However, very little is currently known about human dermal
28 exposure to persistent organic pollutants. While recent pharmacokinetic studies have
29 highlighted the importance of dermal contact as a pathway of human exposure to brominated
30 flame retardants, risk assessment studies had to apply assumed values for percutaneous
31 penetration of various flame retardants (FRs) due to complete absence of specific
32 experimental data on their human dermal bioavailability. Therefore, this article discusses the
33 current state-of-knowledge on the significance of dermal contact as a pathway of human
34 exposure to FRs. The available literature on *in vivo* and *in vitro* methods for assessment of
35 dermal absorption of FRs in human and laboratory animals is critically reviewed. Finally, a
36 novel approach for studying human dermal absorption of FRs using *in vitro* three-
37 dimensional (3D) human skin equivalent models is presented and the challenges facing future
38 dermal absorption studies on FRs are highlighted.

39

40 **Keywords**

41 Flame retardants, dermal absorption, human exposure, human skin equivalents,
42 bioavailability.

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List of Acronyms

BFRs	brominated flame retardants
BPA	bisphenol A
BTBPE	1,2-bis(2,4,6 tribromophenoxy)ethane
DBDPE	Decabromodiphenylethane
EU	European Union
EVCAM	european centre for validation of alternative methods
FRs	flame retardants
FT	full-thickness skin
HBCD	hexabromocyclododecane
HSE	human skin equivalent
KC	keratinocytes
K _{ow}	octanol/water partition coefficient
LC	langerhans cells
NBFRs	novel brominated flame retardants
OATP	organic anion transporting polypeptides
OECD	organisation for economic co-operation and development
PA	percutaneous absorption
PBDEs	polybrominated diphenyl ethers
PBT	persistent, bioaccumulative and toxic
PCBs	polychlorinated biphenyls
PFRs	organophosphate flame retardants
PK	pharmacokinetic
POPs	persistent organic pollutants
RDP	resorcinol bis-diphenylphosphate
SC	stratum corneum
RHE	reconstructed human epidermis
TBB	2-ethylhexyl 2,3,4,5-tetrabromobenzoate
TBBPA	tetrabromobisphenol A
TBPH	Bis(2-ethylhexyl)tetrabromophthalate
TCEP	tris(2-chloroethyl) phosphate
TCIPP	tris(2-chloro-1-methylethyl) phosphate
TDCPP	tris(1,3-dichloro-2-propyl) phosphate
TRIS	tris (dibromopropyl) phosphate

USEPA

United States environment protection agency

48

49 **Introduction**

50 Organic flame retardants (FRs) are a diverse group of chemicals used to prevent or reduce
51 the flammability and combustibility of polymers and textiles. The major members of this
52 group are polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD),
53 tetrabromobisphenol A (TBBP-A), novel brominated flame retardants (NBFRs), as well as
54 organophosphate flame retardants (PFRs) (Ghosh, et al. 2011; van der Veen and de Boer
55 2012).

56 Although polychlorinated biphenyls (PCBs) were mainly applied as heat transfer fluids in
57 electric equipment, capacitors and transformers, one of their major advantages as heat
58 transfer fluids was flame-retardancy. Thus, PCBs were highly desirable for applications
59 where fire was a threat to life and property, such as in electrical equipment in commercial
60 buildings, hospitals, in hydraulic systems in foundries, and in heat transfer systems.
61 Furthermore, PCBs were also applied to flame-proof polyimide (nylon-type) and polyolefin
62 yarns. Due to their persistent, bioaccumulative and toxic (PBT) properties, the production and
63 usage of PCBs were banned throughout most of the industrialized world in the 1970s
64 (Erickson and Kaley 2011; Fiedler 2001).

65 PBDEs have found wide application as FRs for plastics, textiles, electronics casings and
66 circuitry. The fully brominated product (DecaBDE) dominated worldwide production with a
67 global demand of 56,100 t in 2001, compared to 7,500 and 3,790 t for the less brominated
68 PentaBDE and OctaBDE formulations, respectively (BSEF 2013). In 2001, the world market
69 demand for HBCD was 16,700 tons, 57% of which was in Europe (Covaci, et al. 2006). The
70 principal application of HBCD is in expanded and extruded polystyrene foams used for
71 building insulation , but it has also been used to flame retard textiles and housing for
72 electrical items (KEMI (National Chemicals Inspectorate) 2008). TBBP-A is the most widely
73 used BFR with a production volume of 170,000 tons in 2004, applied mainly for epoxy resins

74 used in printed circuit boards of electric and electronic equipments (Covaci, et al. 2009a). As
75 PBDEs, HBCD, and ~20% of the production of TBBP-A are blended physically within (and
76 referred to as “additive” FRs) rather than bound chemically (and known as “reactive” FRs) to
77 polymeric materials; they migrate from products, following which their persistence and
78 bioaccumulative character leads to contamination of the environment including humans
79 (Harrad, et al. 2010a). This is of concern owing to their potential environmental and
80 toxicological risks including: endocrine disruption, neurodevelopmental and behavioural
81 disorders, hepatotoxicity and possibly cancer (Darnerud 2008; Hakk 2010; Wikoff and
82 Birnbaum 2011). Moreover, the few data available from human epidemiological studies
83 imply effects on: male reproductive hormones (Johnson, et al. 2013; Meeker, et al. 2009),
84 semen quality (Akutsu, et al. 2008), thyroid hormone homeostasis (Turyk, et al. 2008),
85 cryptorchidism (Main, et al. 2007), hormone levels and fecundability in adult women
86 (Harley, et al. 2010), as well as lower birth weight and length (Chao, et al. 2007; Lignell, et
87 al. 2013). Such evidence has contributed to complete EU bans for the Penta- and Octa-BDE
88 formulations, and restrictions on the use of Deca-BDE (Roberts, et al. 2012). In addition,
89 PBDEs associated with Penta- and Octa-BDE are listed under the UNEP Stockholm
90 Convention on POPs, while Deca-BDE is currently under consideration for listing under
91 Annexes A, B and/or C of the convention (Stockholm convention on POPs 2009).
92 Furthermore, HBCD will be phased out following its recent listing under Annex A of the
93 Stockholm Convention (Stockholm convention on POPs 2013). Despite such restrictions on
94 their production and use, human exposure to PBDEs and HBCD is likely to continue for
95 some time, given the ubiquity of flame retarded products remaining in use and entering the
96 waste stream, coupled with the environmental persistence of these BFRs (Harrad and
97 Diamond 2006).

98 These restrictions on the use of PBDEs and HBCD have paved the way for the use of NBRs
99 as replacements with an estimated global production volume of 100,000 tonnes in 2009
100 (Harrad and Abdallah 2011). Major NBRs are: DBDPE (Decabromodiphenylethane),
101 BTBPE (1,2-bis(2,4,6 tribromophenoxy)ethane), TBB (2-ethylhexyl 2,3,4,5-
102 tetrabromobenzoate), and TBPH (Bis(2-ethylhexyl)tetrabromophthalate) (further details are
103 provided in Table SI-1). While information regarding the environmental occurrence of
104 several NBRs has become available recently (Covaci, et al. 2011), very little is known about
105 their toxicological properties and the pathways and magnitude of human exposure to these
106 chemicals. Nevertheless, several NBRs bear striking structural similarity to PBDEs (e.g.
107 DBDPE is a very close analogue of BDE-209) and are reported to have similarly low vapour
108 pressures and water solubilities, as well as high K_{OW} values, and PBT characteristics (Covaci,
109 et al. 2011; Harrad and Abdallah 2011).

110 In addition to BFRs, PFRs have been associated with a wide range of applications (Table SI-
111 1). Likely linked to the aforementioned restrictions on PBDEs, EU market demand for PFRs
112 increased from 83,700 tons in 2004 to 91,000 tons in 2006 (EFRA 2007). Tris(2-chloroethyl)
113 phosphate (TCEP), tris(2-chloro-1-methylethyl) phosphate (TCIPP) and tris(1,3-dichloro-2-
114 propyl) phosphate (TDCPP) were all subject to an EU risk assessment process under an
115 Existing Substances Regulation (EEC 793/93) (Regnery and Puttmann 2010). Despite less
116 stability and overall environmental persistence than PBDEs, they were classified as persistent
117 organic compounds in the aquatic environment and reported to fulfil PBT criteria. In
118 addition, several studies have reported them to display adverse effects including reproductive
119 toxicity and carcinogenic effects on lab animals (Regnery, et al. 2011). Hence TCEP is
120 classified by the EU as a “potential human carcinogen” (Regnery and Puttmann 2010), while
121 TDCPP is classified under regulation EC 1272/2008 as a category 2 carcinogen (ECHA
122 2010).

123

124 ***Human exposure to FRs.*** Several studies have reported on levels of different FRs in various
125 environmental and human matrices (Covaci, et al. 2011; Covaci, et al. 2009b; Harrad, et al.
126 2010b; Law, et al. 2014; van der Veen and de Boer 2012). Current understanding is that non-
127 occupational human exposure to BFRs occurs mainly via a combination of diet, ingestion of
128 indoor dust, dermal contact with dust/consumer products, and inhalation of indoor air (Figure
129 1) (Abdallah, et al. 2008a; Frederiksen, et al. 2009; Watkins, et al. 2011). The exact
130 contribution of these pathways varies substantially between chemicals, between individuals
131 according to lifestyle, and is further complicated by international variations in FR use
132 (Abdallah and Harrad 2009; Abdallah, et al. 2008a; Abdallah, et al. 2008b; Harrad, et al.
133 2008b). While it is established that the main exposure route to several POPs (e.g. PCBs and
134 DDT) is through diet, studies from North America report indoor dust (via ingestion or dermal
135 contact) as the major exposure pathway for all age groups to PBDEs contributing 70-80% to
136 the average overall daily exposure (Lorber 2008; Trudel, et al. 2011). Elsewhere, while dust
137 ingestion appears particularly important for toddlers and young children; other exposure
138 pathways make substantial contributions to the overall adult intake of BFRs (Abdallah, et al.
139 2008a; Harrad, et al. 2010b; Harrad, et al. 2008a; Roosens, et al. 2009). In contrast to PBDEs,
140 only a few studies are available that address human exposure to NBFRs and PFRs (Ali, et al.
141 2012; Covaci, et al. 2011; Stapleton, et al. 2011). Currently very little is known about dermal
142 exposure as a route of human exposure to FRs in indoor dust or FR-treated products. This
143 paucity of information was evident in the EU risk assessment reports on TBBPA (EU Risk
144 Assessment Report 2006) and BDE-209 (EU Risk Assessment Report 2002) where the lack
145 of experimental data has led to the assumption of dermal absorption efficiencies based on
146 consideration of compound-specific physicochemical properties and extrapolation from data
147 available for PCBs. Furthermore, several authors have discussed the absence of experimental

148 data on dermal absorption of various FRs and highlighted the potential inaccuracies of
149 current estimates of human exposure to these FRs owing to a general lack of knowledge on
150 the percutaneous route (Boyce, et al. 2009; Garner, et al. 2006; Trudel, et al. 2011; U.S. EPA
151 1992). Therefore, the lack of experimental information on human dermal uptake of FRs from
152 dust and source materials, represents an important research gap that hampers accurate
153 assessment of human exposure to FRs. However, efforts to fill this gap are hindered by
154 several difficulties including: ethical issues encountered with human studies, inter-species
155 variation in dermal structure and uptake that cast doubt on the accuracy of extrapolation or
156 allometric scaling of animal data to humans, and tighter regulations on *in vivo* tests involving
157 animals.

158 Against this backdrop, this paper: (a) provides a critical review of the current state-of-
159 knowledge on dermal absorption of FRs, (b) discusses the paradigm shift in toxicity testing
160 from *in vivo* to *in vitro* dermal bioavailability studies and (c) suggests effective novel
161 approaches to studying human dermal uptake of FRs, with special emphasis on *in vitro* 3D
162 human skin percutaneous assays, that are finding increasing application in the pharmaceutical
163 and cosmetics sectors (Gibbs, et al. 2013; Kandarova, et al. 2013; Tornier, et al. 2010).

164

165 **Skin as a barrier for systemic exposure to xenobiotic chemicals.**

166 Skin is the largest body organ, with a surface area of $\sim 2 \text{ m}^2$ and weighing about 5 kg in adult
167 humans (Godin and Touitou 2007). This multi-layered organ acts mainly to protect the body
168 from the surrounding environment, thus forming an efficient permeation barrier for
169 exogenous molecules. Human skin is formed of 3 main layers, namely: epidermis, dermis and
170 hypodermis (Figure 2). The epidermis (outermost) is a non-vascular layer, which has a
171 protective role as a barrier to penetration of chemicals to the underlying vascular dermis. The
172 healthy human epidermis comprises 4 layers (stratum corneum, stratum granulosum, stratum

173 spinosum and stratum basale) separated from the dermis by the basement membrane
174 (Breitkreutz, et al. 2013). The barrier properties of the skin lie mainly within the stratum
175 corneum (SC), which has about 16 layers and takes about two weeks to completely
176 desquamate (Hoath and Leahy 2003). This highly hydrophobic layer is composed of
177 differentiated non-nucleated cells, corneocytes, which are filled with keratins and embedded
178 in the lipid domain. Percutaneous penetration of molecules through the SC occurs mainly via
179 passive diffusion but may also occur via sweat glands and hair follicles directly to the dermis.
180 Although little is known about the expression and function of influx transport proteins in
181 human skin and their role in dermal uptake of xenobiotics, The role of organic anion
182 transporting polypeptides (OATP) in mediating the active transport process of large organic
183 cations via human keratinocytes was highlighted (Schiffer, et al. 2003). Chemical residues
184 limited to the epidermis will be eliminated from the exposed skin by desquamation and
185 willnot be available for systemic distribution (Aggarwal, et al. 2014).

186

187 **Significance of dermal absorption as a pathway of human exposure to FRs.**

188 Although several studies have highlighted the importance of indoor dust ingestion as a
189 pathway for human exposure to various FRs, few reports have discussed human dermal
190 exposure to such contaminants (Stapleton, et al. 2012; Stapleton, et al. 2008; Watkins, et al.
191 2011). Watkins *et al.* (Watkins, et al. 2011) reported a strong positive correlation between
192 PBDE levels on hand wipes (assumed to result from hand contact with contaminated dust or
193 flame-retarded products) and PBDE concentrations in serum from American adults. While
194 concentrations of PBDEs in indoor dust were strongly correlated with those in hand wipes,
195 and infrequent hand-washers had 3.3 times the levels of PBDEs in their handwipes than did
196 frequent hand-washers; correlation could not be established directly between PBDE
197 concentrations in indoor dust and their levels in serum (Watkins, et al. 2011). In a more

198 recent contribution, significant associations between concentrations of TCEP, TCIPP,
199 TDCPP, HBCD, TBB and TBPH in children handwipes and house dust were observed
200 (Stapleton, et al. 2014). Another recent study reported 2-3 times increase of median
201 concentrations of penta-BDE, TBB, and TBPH in paired handwipe samples of 11 gymnasts
202 after practice compared to before (Carignan, et al. 2013). This opens up the possibility that
203 FRs in dust may also be an indicator of another exposure pathway, such as direct dermal
204 uptake of FRs present in treated goods (e.g. games consoles, remote controls, and fabrics). A
205 pivotal issue for risk assessment studies is the influence of indoor contamination with FRs on
206 human body burdens. Understanding of this remains incomplete. One approach is that of
207 Lorber (Lorber 2008) who used a simple pharmacokinetic (PK) model to predict the body
208 burdens of PBDEs in American adults using intake data from different exposure pathways.
209 Predicted body burden were compared with measured data and the relationship between
210 external and internal exposure discussed. Since then, a few studies have applied similar PK
211 models with slight adjustments to further understanding of the relationship between
212 concentrations of PBDEs, HBCD and TBBP-A in the environment and human body burdens
213 (Abdallah and Harrad 2011; Johnson-Restrepo and Kannan 2009; Trudel, et al. 2011).
214 Further to identifying various research gaps including the bioavailability of FRs following
215 ingestion of indoor dust and the elimination half-lives of these compounds in human, One
216 major outcome of such PK studies is the highlighted potential importance of dermal contact
217 with indoor dust and/or FR-containing items as a pathway of exposure to BFRs. To illustrate,
218 dermal uptake was reported as the 2nd most important contributor(following dust ingestion)
219 to PBDE body burdens of Americans. This was despite a very conservative assumption –
220 *made in the absence of experimental data* - that only 3% of PBDEs with which dermal
221 contact occurred (via adherence of indoor dust to the skin) were absorbed (Lorber 2008).
222 Moreover, a recent PK model reported ingestion of diet and dust, as well as dermal exposure

223 to dust to constitute the major factors influencing human body burdens of PBDEs in both
224 Americans and Europeans. Once again, these conclusions were founded on low assumed
225 values of dermal absorption efficiency (2.5-4.8%) (Trudel, et al. 2011). Neither study
226 considered potential dermal absorption following contact with FR-treated items and assumed
227 percutaneous penetration fractions based on values reported for dermal absorption of dioxins
228 and PCBs from soil in laboratory animal models (Lorber 2008; Trudel, et al. 2011). Boyce et
229 al. (2009) applied a Monte Carlo-based mathematical approach for assessment of human
230 exposure to TBBPA, DBDPE and BDE-209 via indoor dust ingestion and dermal contact.
231 Based on physicochemical properties, analogy with data for PCBs and the absence of any
232 chemical-specific studies, dermal absorption values of 10%, 0.1% and 1% were used for
233 TBBPA, DBDPE and BDE-209, respectively. Results revealed dermal contact with indoor
234 dust made significant contributions (15 - 40%) to estimates of overall human exposure to
235 these BFRs in North America and Europe. The authors highlighted that at such significant
236 contribution levels; inaccuracies in the dermal absorption factors applied could have dramatic
237 effects on exposure assessments (Boyce, et al. 2009).

238

239 **Transdermal metabolism of xenobiotics.**

240 Besides the role of the stratum corneum as the major structure for epidermal barrier function,
241 there is increasing evidence that xenobiotic metabolizing enzymes and transport proteins
242 function as a second biochemical barrier of the skin (Esser and Goetz 2013; Gundert-Remy,
243 et al. 2014; Wiegand, et al. 2014). Currently, very little is known about the transdermal
244 metabolism of flame retardant chemicals. Garner and Matthews confirmed extrahepatic
245 dermal metabolism of mono- to hexa- PCBs in F-344 male rats. However, the exact chemical
246 structure of the formed metabolites was not confirmed (Garner, et al. 2006). Another *in vitro*
247 study reported the dermal metabolism of BDE-209 and TDCPP to be minimal in adult female

248 mice (Hughes, et al. 2001). However, an extensive literature exists on the capacity of human
249 skin to metabolise various chemical compounds. Recent findings indicate that human skin
250 possesses not only multiple cytochrome P450 isoenzymes, but also influx and efflux
251 transporter proteins. While the pattern of cytochrome P450 isoenzymes in the skin differs
252 from the pattern in the liver, It seems likely that the skin can participate in both Phase I (e.g.
253 oxidation, reduction and hydrolysis) and Phase II (e.g. glucuronidation and acetylation)
254 metabolic reactions (Gundert-Remy, et al. 2014; Merk 2009). Moreover, human skin
255 cells contained at least five different esterases reported to act on simple ester bonds in
256 organophosphate compounds (paraoxon and bis(4-nitrophenyl)phosphate). Therefore, dermal
257 biotransformation may play an important role in the ultimate fate and bioavailability of FRs in
258 the skin, especially for PFRs and NBFs which have labile functional groups.

259

260 ***In vivo* dermal bioavailability studies**

261 While the most reliable method for assessment of dermal absorption for human risk
262 assessment would involve study of human volunteers; technical and ethical constraints means
263 their use has been and will likely remain limited (Jakasa and Kezic 2008). Although the use
264 of *in vivo* animal models has been strongly discouraged (European Commission and
265 absorption 2004; Howes, et al. 1996), their application for dermal risk assessment is of value
266 because they represent an intact physiological and metabolic system when the use of human
267 volunteers is not possible. Furthermore, *in vivo* animal models (especially rats) have long
268 been used by different industrial and regulatory institutions to provide data on various
269 toxicokinetic and toxicodynamic parameters, as well as dermal absorption (Zendzian 2000).
270 While dermal uptake of environmental contaminants (e.g, polycyclic aromatic hydrocarbons,
271 phthalates and pesticides) from soil and sediment has been reviewed (Spalt, et al. 2009), very
272 little is known about the uptake of flame retardants via skin (Table 1). Schmid et al. studied

273 the dermal absorption of PCBs in one human volunteer (52 year old male, 65 kg body
 274 weight) (Schmid, et al. 1992). The volunteer was exposed to a mixture of 8 tetra- to hepta-
 275 ¹³C-PCBs for different time spans using cotton cloth and aluminium foil as carrier materials
 276 to mimic real life situations of skin contact with PCB-contaminated clothes or metal surfaces.
 277 After exposure the skin was washed subsequently with water and ethanol. Non-absorbed ¹³C-
 278 PCBs were determined in the washing solvents and in the carrier materials, while the
 279 bioavailable fraction was measured in plasma samples collected at 0.5-6 days post-exposure.
 280 Results revealed low percutaneous absorption (PA) of target PCBs equivalent to 6 % of the
 281 absorption after oral intake of the same amount. The absorption rate was largely dependent
 282 on the site of administration, on the carrier material (higher from the aluminium foil than the
 283 cotton cloth) and almost not on the amount administered where the percentage uptake
 284 remained constant at long (8 hours) and short (10 min) exposure times(Schmid, et al. 1992).
 285 Similar PA values (3.4-4.5 %) were reported in Rhesus monkeys exposed to PCB-
 286 contaminated soil for 24 h (Mayes, et al. 2002). The difference between the calculated PA
 287 values for soil PCBs in this study and the 14% dermal absorption factor used by the USEPA
 288 (U.S. EPA 1992) was attributed mainly to soil organic content in addition to particle size,
 289 skin residence time and contaminant “aging” in the soil. The percutaneous absorption of ¹⁴C-
 290 Aroclor 1260 in test monkeys was determined by measuring the radioactivity in excreta
 291 (equation 1) (Mayes, et al. 2002).

$$\% \text{ Dose Absorbed} = \left(\frac{\% \text{ Topical Dose Excreted } (^{14}\text{C-urine} + ^{14}\text{C feces})}{\% \text{ Intravenous Dose Excreted } (^{14}\text{C-urine} + ^{14}\text{C feces})} \right) \times 100 \dots(1)$$

292
 293 An important point is that the model used in equation 1 and in all *in vivo* studies in humans or
 294 surrogate species where the animal is not sacrificed, cannot account for any test compounds
 295 sequestered within the skin (Mayes, et al. 2002; Spalt, et al. 2009). This may lead to
 296 substantial underestimation of the actual dermal uptake of persistent lipophilic compounds
 297 which would eventually (within days) be systemically absorbed from the skin depot of the

298 exposed organism. For such compounds, for which the outcome of concern is typically not
299 acute toxicity, inclusion of skin burden is necessary (Spalt, et al. 2009). While adjustment for
300 excretion following intravenous administration may be employed, this has associated
301 uncertainty and presumes no difference in the excretory pattern associated with dermal and
302 intravenous administration used as a reference. The importance of this concept of
303 contaminant skin depot was confirmed by Garner and Mathews (Garner and Matthews 1998).
304 These authors applied 0.4 mg/kg body weight of a mixture of radiolabeled mono- to hexa-
305 PCBs in acetone to a 1 cm² hairless skin area at the back of adult male F-344 rats.
306 Distribution of radioactivity in the dose site and selected tissues was determined by serial
307 sacrifice at time points up to 2 weeks. Results revealed the dermal penetration of test
308 compounds to vary inversely with degree of chlorination and at 48 h ranged from ca. 100%
309 for mono-PCB to ca. 30% for hexa-PCB. Although the maximum internal exposure to Mono-
310 PCB was at 4 h (37% of the dose present in tissues), only 0.2% of the absorbed dose
311 remained in the tissues after 2 weeks. In contrast, tetra-PCB internal exposure was the
312 greatest with ca. 85% of the total absorbed dose present in tissues 72 h postadministration.
313 Furthermore, hexa-PCB equivalents in tissues continued to rise through 2 weeks postdose
314 (~15% of absorbed dose) since systemic absorption from epidermis depots was still
315 incomplete when the study was terminated. While rat skin favoured the rapid absorption of
316 lower chlorinated PCBs; their relatively rapid metabolism and elimination, suggests lower
317 body burdens of the less chlorinated congeners compared to higher molecular weight PCBs
318 which penetrate less rapidly, but persist at the site of exposure and slowly enter the systemic
319 circulation (Garner and Matthews 1998). In another contribution, Garner et al (Garner, et al.
320 2006) used the same animal model to study the disposition of mono- to hexa- PCBs following
321 dermal administration. Results confirmed higher chlorinated PCBs to be slowly absorbed and
322 accumulated in the adipose and skin. Interestingly, excretion and metabolic profiles following

323 dermal dosing tended to differ from profiles following equivalent intravenous doses. This
324 was attributed to first pass metabolism occurring at the dermal dose site. The study further
325 suggested that the rate of absorption, and consequently disposition of PCBs following dermal
326 exposure, may be mediated, either in part or fully, by transdermal metabolism (Garner, et al.
327 2006).

328 The dermal absorption of the flame retardant resorcinol bis-diphenylphosphate (RDP) was
329 investigated in rats and monkeys. Sprague-Dawley rats and cynomolgus monkeys were
330 dermally exposed to 100 mg of ¹⁴C-RDP spread over a shaved area representing about 20%
331 of the animal's surface area. Results revealed ~ 20% of the dermal dose was absorbed in rats,
332 whereas primates absorbed only 10% of the applied dermal dose (Freudenthal, et al.
333 2000). Very little is known about the dermal absorption of BFRs. In an early report, Ulsamer
334 et al. studied the dermal absorption of the banned flame retardant tris (dibromopropyl)
335 phosphate (TRIS) in rabbits. The test animals were exposed to radiolabelled ¹⁴C-TRIS via
336 sections of fabric (10 x 12 cm) placed in contact with skin for 96 h. Results revealed that up
337 to 17% of the applied dose was absorbed when the fabric was wetted with urine. Only 6% of
338 the dose was absorbed when the cloth was wetted with simulated sweat, which was slightly
339 higher than the absorption (4%) from a dry cloth (Ulsamer, et al. 1978). A more recent study
340 used a female C57BL/6 mice model to assess the dermal bioavailability of BDE-47. Test
341 animals were exposed to 1 mg/kg body weight of ¹⁴C-BDE 47 in acetone applied to a hairless
342 2 cm² skin patch. Results revealed ~62% absorption of the administered dose after 5 days
343 while 15% remained at the site of application where skin and adipose were reported as the
344 major depot tissues (Staskal, et al. 2005).

345

346 **Paradigm shift – *in vivo* to *in vitro* dermal bioavailability studies**

347 Due to the ethical and technical issues arising from the use of lab animals in toxicology
348 studies, the use of *in vivo* animal models is increasingly strongly discouraged (Jakasa and
349 Kezic 2008). Therefore, focus has shifted to developing and validating alternative *in vitro* test
350 methods, which also provide a better platform for development of predictive pharmacokinetic
351 models. Several guidance documents for conducting *in vitro* skin absorption studies (OECD
352 2004; U.S. EPA 2004; WHO 2006) are currently available rendering the application of *in*
353 *vitro* skin models increasingly acceptable for research and regulatory purposes.

354 Different types of skin may be used, for example, human excised skin from surgery or from
355 cadavers (*ex vivo* skin) or animal (e.g. pig) skin. Various types of diffusion cells have been
356 employed in *in vitro* studies to date, and the composition of receptor fluids may vary. All
357 these factors can influence the results of *in vitro* experiments (Jakasa and Kezic 2008). While
358 several papers have reported on *in vitro* dermal absorption of environmental contaminants
359 such as: polycyclic aromatic hydrocarbons, phthalates, as well as organochlorine and
360 organophosphate pesticides (Hopf, et al. 2014; Hughes and Edwards 2010; Spalt, et al. 2009);
361 very few *in vitro* studies of the dermal absorption of FRs exist. In one such study, Hughes et
362 al. (Hughes, et al. 2001) used skin from adult hairless female mice (SKH1) mounted in flow-
363 through diffusion cells to study the absorption of ¹⁴C-BDE-209 and ¹⁴C-TDCPP at 3
364 concentration levels. HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid))-
365 buffered Hanks' balanced salt solution (pH 7.4) with 10% fetal bovine serum was used as
366 receptor fluid. Following 24 h exposure, the skin patches were washed with solvent prior to
367 analysis of receptor fluid, skin wash and skin for chemical-derived radioactivity. BDE-209
368 showed low penetration (0.3%) into the receptor fluid while up to 20% of the dose remained
369 in skin after 24 h. TDCPP displayed higher penetration (39–57%) to the receptor fluid, while
370 28–35% of administered dose remained in the skin. This was mainly attributed to its lower
371 molecular weight and K_{OW} than BDE-209 (Hughes, et al. 2001). The dermal absorption of

372 BDE-47 was studied using *in vitro* split-thickness skin membranes (350–410 μm , stratum
373 corneum uppermost) of human and rat skin exposed to a single dose of ca. 10 mg/cm^2 of ^{14}C -
374 BDE-47 for 24 h. The skin patches were mounted in flow-through cells while receptor fluid
375 (NaCl, 0.9%, w/v in water) was pumped through the receptor chambers at ca. 1.5 ml/h
376 (Roper, et al. 2006). The dose recovered from the receptor fluid was 2% and 15% of
377 administered BDE-47 to human and rat skin, respectively. The difference between the results
378 of this *in vitro* study (Roper, et al. 2006) and the higher (62%) sorption observed in an *in vivo*
379 study of dermal absorption in mice (Staskal, et al. 2005) (Table 1) may be attributed mainly
380 to the use of 0.9% NaCl solution in water as a receptor fluid, as this may greatly reduce
381 diffusion of the lipophilic BDE-47 to the receptor fluid (Wilkinson and Williams 2002) and
382 does not accurately mimic actual biological conditions. Possible evidence of this is provided
383 by the high residual levels of BDE-47 detected in the cells (57% and 33% for human and rat
384 skin, respectively) that appeared not to diffuse to the receptor fluid (Roper, et al. 2006).
385 While no data exists on dermal absorption of TBBP-A, a recent *in vitro* study reported on the
386 percutaneous bioavailability of its precursor, bisphenol A (BPA) from human and pig skin
387 (Zalko, et al. 2011). Viable human and pig skin patches (500 μm thickness) were maintained
388 at the air/liquid interface using Transwell inserts while dermal/epidermal feeding was
389 achieved via diffusion of nutrients from a modified Dulbecco's Eagle culture medium which
390 kept the cells alive during 72 h exposure experiments. BPA was efficiently absorbed (65%
391 and 46% from pig and human skin, respectively) and metabolised by the cultured skin
392 indicating the trans-dermal route contributes substantially to human exposure to BPA (Zalko,
393 et al. 2011). However, it should be noted that TBBP-A has a much higher molecular weight
394 and consequently, different physico-chemical properties (e.g. water solubility, partition co-
395 efficient and vapour pressure) than BPA. Furthermore, the lack of halogen atoms in BPA is

396 likely to enhance the rate of its percutaneous absorption compared to its tetra-brominated
397 derivative (Garner and Matthews 1998).

398 Given the growing evidence that suggest dermal absorption to be a potentially significant
399 pathway of human exposure to FRs, the paucity of data on dermal bioavailability of such
400 ubiquitous contaminants may be attributed to a combination of ethical, technical and
401 economic issues. One alternative method with the potential to overcome such difficulties is
402 the use of 3D human skin equivalent (HSE) models which provide a relatively cheap,
403 commercially available, ethical, and reliable method for dermal absorption studies that is
404 capable of producing data of relevance to human exposure.

405 **Human Skin Equivalent models (HSE)**

406 ***Rationale.*** Although the Organisation for Economic Co-operation and Development (OECD)
407 and the European Centre for Validation of Alternative Methods (ECVAM) describe methods
408 for assessing dermal absorption using excised *in vitro* human and animal skin, the lack of
409 correlation in transdermal permeation of chemicals across species imparts a high degree of
410 uncertainty when extrapolating results from animal models to humans. This is mainly due to
411 variations in the stratum corneum thickness, intercellular subcutaneous lipids and/or between-
412 species differences in metabolic enzymes and their activity (Schafer-Korting, et al. 2008a).
413 Therefore, excised *in vitro* human skin is preferable to animal skin (e.g. rat or pig skin) for
414 dermal absorption testing, but is clearly less available. To overcome this shortage, HSE have
415 been developed to provide an alternative to human skin in testing of compounds for
416 transdermal permeability (Mertsching, et al. 2008). A protocol was developed and validated
417 according to the OECD guidelines for percutaneous absorption by using commercially
418 available HSE models (Table 2). The permeability of tested HSE models were compared to
419 that of excised human epidermis, pig skin and bovine udder skin, using 9 compounds widely
420 varying in physicochemical characteristics, including the OECD standards: testosterone,

421 caffeine and benzoic acid. Results revealed HSE models closely mimic the histological and
422 physiological character of viable human skin, allowing their use for *in vitro* skin penetration
423 studies, taking product-specific overpredictability into account (Hartung, et al. 2004; Schafer-
424 Korting, et al. 2008a). Consequently, several validated methods using HSE models have been
425 approved by OECD and ECVAM for testing skin absorption, phototoxicity, corrosion and
426 irritation by xenobiotic chemicals (Ackermann, et al. 2010; Buist, et al. 2010).

427 **Composition.** HSE models can be generally classified into 2 main types:

428 1- *Reconstructed Human Epidermis (RHE)*: RHE is a human skin tissue obtained
429 from human keratinocytes cultured on an inert polycarbonate medium. One key advantage is
430 that it permits growth of donor epidermal cells in a serum-free culture environment. After
431 rapidly proliferating preparative keratinocyte cultures have been obtained, the epidermal cells
432 yielded are seeded on inert filter substrates, which are then raised to the air-liquid interface in
433 a humidified-air incubator. A fully-defined nutrient medium feeds the basal cells through the
434 filter substratum. After 14 days, a stratified epidermis is formed that closely resembles human
435 epidermis *in vivo* (Figure 3) (Boelsma, et al. 2000).

436 Morphologically, these cultures exhibit a well-stratified epithelium and cornified epidermis
437 with significantly improved barrier function and metabolic activity (Boelsma, et al. 2000).
438 Differentiation markers such as suprabasal keratins, integrin b4, integrin a6, fibronectin,
439 involucrin, filaggrin, trichohyalin, type I, III, IV, V and VII collagen, laminin, heparan sulfate
440 and membrane-bound transglutaminase are expressed similar to those of the human epidermis
441 (Brinkmann, et al. 2013; Mehul, et al. 2004).

442 Several RHE models are now commercially available. The different models share the air-
443 exposed culture conditions, but differ in the support used as a dermal equivalent on which the
444 human keratinocytes are grown (Table 2). Numerous histological and biochemical features
445 are shared by these models, in particular epidermal stratification and differentiation, and all

446 produce a well-defined stratum corneum as a result of tightly regulated expression of
447 differentiation-related genes (Boelsma, et al. 2000; Zhang and Michniak-Kohn 2012).

448 2- *Full-Thickness skin (FT)*: Paracrine signaling between dermal fibroblasts (FB) and
449 epidermal keratinocytes (KC) is believed to modulate skin responses during contact irritant or
450 allergic reactions. Dermal FB also play an important role in photo-aging, photo-damage,
451 wound healing and cancer progression. To enable *in vitro* investigation of these and other
452 dermal phenomena in which FB-KC interactions are important, FT skin models composed of
453 a FB-containing dermis/KC-containing epidermis have been developed (Schafer-Korting, et
454 al. 2008b; Semlin, et al. 2011). In order to test possible immunological reactions on skin,
455 Langerhans cells (LCs) can be introduced into FT skin substitutes (Regnier, et al. 1997).
456 Percutaneous absorption of chemicals is due to two different routes of passive diffusion. The
457 first is trans-epidermal diffusion via inter- or trans- cellular pathway across the stratum
458 corneum, whereas the second is trans-appendageal diffusion via hair follicles and associated
459 sebaceous glands. The presence of appendages in the FT models may represent another
460 advantage added to their superiority over RHE models for biotransformation-linked toxic
461 endpoints (Ackermann, et al. 2010; Curren, et al. 2006). However, the scarce information
462 available to date, indicates a complex relationship between percutaneous absorption, skin
463 thickness and lipophilicity of test compounds (Wilkinson, et al. 2006). This is further
464 compounded by factors like: exposure vehicle, diffusion cell design and receptor fluid
465 (Schafer-Korting, et al. 2008b).

466 ***General Protocol for in vitro percutaneous absorption studies.*** Each HSE model is supplied
467 with its respective receptor/culture fluid and its percutaneous absorption protocol. Generally,
468 the protocol involves mounting the fully-developed skin patches at the air-liquid interface of
469 a permeation device (e.g. Franz-cell type diffusion cells, Mattek[®] permeation device, see SI
470 section for further details) while in contact with the receptor fluid. The test compound is then

471 applied to the surface of the stratum corneum and incubated for the required exposure time
472 (usually 24 h). The receptor fluid is sampled and replaced at fixed time intervals. At the end
473 of the exposure period, the skin surface is washed/wiped clean of any residual contaminant
474 remaining, prior to collection of the receptor fluid and cell culture for chemical analysis
475 (Figure 4).

476 **Future perspectives and challenges facing dermal absorption studies of FRs**

477 Although current commercially available HSE models may provide a useful alternative to
478 study the human dermal absorption of FRs, there remains several challenges and research
479 gaps that need to be addressed in the near future. These include:

- 480 • The lack of experimental data –either *in vivo* or *in vitro*– relevant to the dermal
481 bioavailability of a wide range of FRs in human. Such paucity of information
482 regarding the dermal pathway hinders the current efforts for accurate risk assessment
483 of various FRs. Furthermore, it complicates the pharmacokinetic modelling studies
484 aiming to understand the relationship between external exposure and human body
485 burdens of FRs.
- 486 • The diverse nature and wide range of physico-chemical parameters of organic FR
487 chemicals (Table SI-1). Contaminant properties like: Log K_{OW} , molecular weight, size
488 and water solubility were reported to affect the dermal absorption of PCBs (Garner
489 and Matthews 1998). Furthermore, the difference in protein binding affinities of
490 various FRs may also influence their permeation through the skin barrier. This will be
491 of particular interest if OATPs were involved in mediating the active transport process
492 of FRs across the human epidermis. Therefore, the chemical diversity and co-
493 existence of various BFRs and PFRs in different environmental samples are likely to
494 present a challenge to environmental scientists trying to mimic *in vivo* scenarios.

- 495 • FR chemicals with similar/comparable molecular weight, size and Kow can exist in
496 different isomeric forms (e.g. HBCD isomers), which might adopt various structural
497 characteristics (e.g. planarity) and exhibit different physico-chemical properties (e.g.
498 water solubility). This is also likely to constitute an important factor influencing the
499 dermal bioavailability of such iso-baric compounds.
- 500 • Despite the huge advances in production and validation of HSE models in the past
501 few years, further improvements are still required to closely mimic the *in vivo*
502 situation. The presence of hair follicles, sweat and sebaceous glands provides further
503 potential pathways for percutaneous penetration. The dermis *in vivo* is continuously
504 perfused by the subcutaneous vasculature, which can rapidly remove permeants
505 reaching the epidermal-dermis interface, allowing for further diffusion of the
506 permeant through the skin layers. This system can be mimicked *in vitro* via the use of
507 dynamic in-line flow through diffusion cells (Table SI-2). However, further validation
508 and standardisation of test protocols using this model is still required to gain the
509 approval of the regulatory bodies and research organisations.
- 510 • Transdermal metabolism has been reported as a major mediator for percutaneous
511 absorption of PCBs (Garner, et al. 2006). Currently, very little is known about the
512 dermal biotransformation of BFRs and PFRs (Hughes, et al. 2001). Enhanced
513 understanding of percutaneous metabolic pathways and identification of the
514 metabolites thus formed in humans thus appears important, if the reliability of risk
515 assessment of these contaminants is to be improved.
- 516 • The excretion of xenobiotic chemicals and their metabolites in sweat and hair follicles
517 has been well documented in literature (De Giovanni and Fucci 2013; Parle and Jadhav
518 2007). Therefore, biotransformation may not be the only dermal contaminant-removal
519 mechanism in human. Further research is required to understand the role of eccrine

520 sweat and hair follicles as excretion routes for FRs. Consequently, the *in vitro* human
521 skin models may consider the dermal bioavailability of FRs as an equilibrium process.

- 522 • While HSE models have been widely exploited in the pharmaceutical and cosmetic
523 fields; to the authors' knowledge, they are yet to be applied for studying dermal
524 absorption of FRs or any other organic contaminants. This is likely to create several
525 challenges for analytical method development, exposure protocols and modelling of
526 the results. Furthermore, *in vitro* dermal studies carried out for the purpose of risk
527 assessment should also include scenarios that mimic real life exposure to the test
528 compounds. This includes exposure to environmentally-relevant concentrations via
529 appropriate exposure media. Previous studies have shown that dermal absorption of
530 PCBs from contaminated soils was different from direct application of PCBs in
531 solution to the skin (Mayes, et al. 2002). In addition, dermal bioavailability has also
532 been shown as influenced by the age of the contaminant in soil and its organic content
533 (Spalt, et al. 2009). Similar factors are likely to affect percutaneous absorption of
534 BFRs and PFRs. Therefore, several exposure scenarios addressing dermal uptake
535 from a range of environmental media (e.g. indoor dust, soil, sweat and consumer
536 products) will be needed for full characterisation of the exposure arising from human
537 dermal exposure to FRs.

538

539

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546 **Supplementary data**

547 Specific details on physico-chemical parameters, uses, toxicokinetic profiles and main
548 exposure pathways of key brominated and phosphorous flame retardants in addition to
549 different *in vitro* dermal absorption protocols are available as supplementary data.

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865 Table 1: Summary of *in vivo* and *in vitro* methods applied for studying dermal absorption of FR chemicals.

Compound	Skin type	Study type	Dosing	Exposure time	Absorption (% of administered dose)	Ref.
PCBs # 52, 101, 108, 118, 138, 153, 170, 180	Human	<i>In vivo</i>	PCBs (5 mg) were dissolved in DCM and applied to the carrier (4cm ² cotton cloth or 28cm ² aluminium foil) prior to fixing to the skin	0.66-1 day	Up to 6% for PCB-153	(Schmid, et al. 1992)
PCBs (¹⁴C-Aroclor 1260 mixture)	Rhesus monkeys	<i>In vivo</i>	500 mg of 70 µg/g PCB-spiked soil applied to 12 cm ² of skin	12-24 h	3.43 ± 0.35% for 12 h and 4.26 ± 0.52% for 24 h	(Mayes, et al. 2002)
PCBs # 4, 15, 47, 155	Male F-344 rats	<i>In vivo</i>	0.4 mg/kg bw applied to 1 cm ² of skin	1, 4, 8, 12, 24, 48, 72, 96, and 336 h	From ca. 100% for PCB-4 to ca. 30% for PCB-155.	(Garner and Matthews 1998)
BDE-47	Female C57BL/6 mice	<i>In vivo</i>	1 mg/kg bw applied to 2 cm ² of skin	5 days	62%	(Staskal, et al. 2005)

BDE-209 and TDCPP	female mice (SKH1)	<i>In vitro</i>	6, 30 and 60 nmol in THF for BDE-209; 20, 100 and 200 pmol in acetone for TDCPP	24 hrs	2–20% in skin, 0.07-0.34% in receptor fluid for BDE-209. 39–57% in skin and 28–35% in receptor fluid for TDCPP	(Hughes, et al. 2001)
BDE-47	Human and rat skin (350–410 μm)	<i>In vitro</i>	10 mg/cm ² applied in acetone.	24 hrs	2-15% in 0.9% NaCl receptor fluid; 57% and 33% remained in cells for human and rat skin, respectively.	(Roper, et al. 2006)
BISPHENOL-A (Precursor to TBBP-A)	Pig Ear Skin and Human skin	<i>In vitro</i>	50, 100, 200, 400 and 800 nmol were applied in 60 μL ethanol/phosphate buffer (pH 7.4)	24, 48 and 72 h	Human skin (45.6 ± 6.2%), pig skin (65.3 ± 8.2%) BPA–glucuronide formed in human skin , corresponding to 7 ± 2, 16 ± 3 and 30.± 3 nmol at 24, 48 and 72 h, respectively.	(Zalko, et al. 2011)

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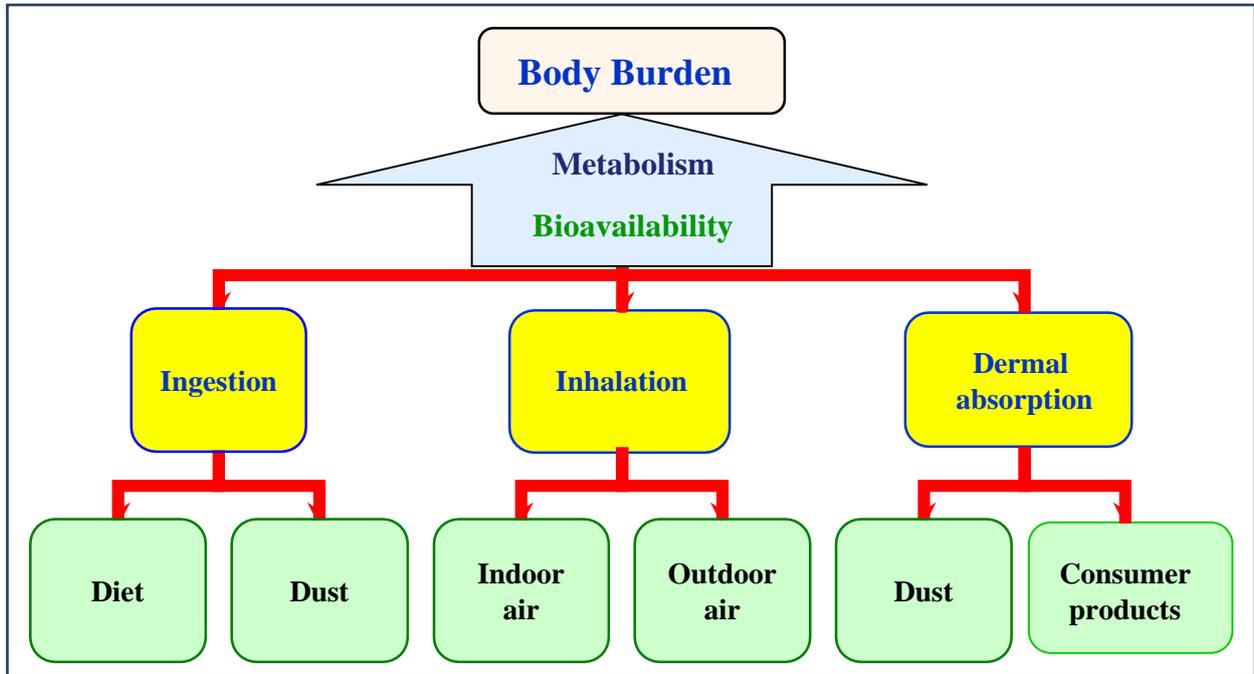
872 **Table 2: Characteristics of commercially available HSE models.**

Brand Name	Scaffold material	Source	Dermis	Manufacturer
Episkin™	Collagen (0.38 cm ²)	Keratinocytes(Mammary/Abdominal samples obtained from healthy consenting Donors during plastic surgery)	NO	L'Oreal, Nice,France
Skinethic™	Polycarbonate membrane (0.5 cm ²)	Keratinocytes (neonatal foreskin tissue or adult breast tissue)	No	L'Oreal, Nice,France
Epiderm™	Collagen coated Polycarbonate (9mm diameter)	Human keratinocytes (neonatal foreskin adult breast skin)	No	MatTek Corporation, MA, USA
EpidermFT™	Collagen	Human keratinocytes (neonatal foreskin adult breast skin) human fibroblasts (neonatal skin, adult skin)	Yes	MatTek Corporation, MA, USA
EST-1000	Polycarbonate membrane	Keratinocytes (neonatal foreskin)	No	CellSystems, Troisdorf Germany
AST-2000	Collagen	Human Keratinocytes	Yes	CellSystems, Troisdorf Germany
Phenion® FT Model	Bovine, cross linked,lyophilized collagen (1.3 cm dia)	Primary human keratinocytes (neonatal foreskin), human fibroblasts (neonatal foreskin)	Yes	Henkel, Duesseldorf, Germany
StrataTest®	Collagen I (0.6 cm ²)	immortalized, human NIKS® keratinocytes dermal fibroblasts	Yes	Stratatech Corporation Madison WI, USA
Epistem® LSE	Collagen	Primary human keratinocytes and dermal fibroblasts.	Yes	Epistem limited, Manchester, UK.
StratiCell® EPI/001	Polycarbonate membrane	Primary human keratinocytes	No	Straticell Corporation, Gembloux, Belgium.
StratiCell® Mel/001	Polycarbonate membrane	Primary human keratinocytes and melanocytes.	No	Straticell Corporation, Gembloux, Belgium.

874 **Figures**

875 **Figure 1: Major pathways of human exposure to FRs.**

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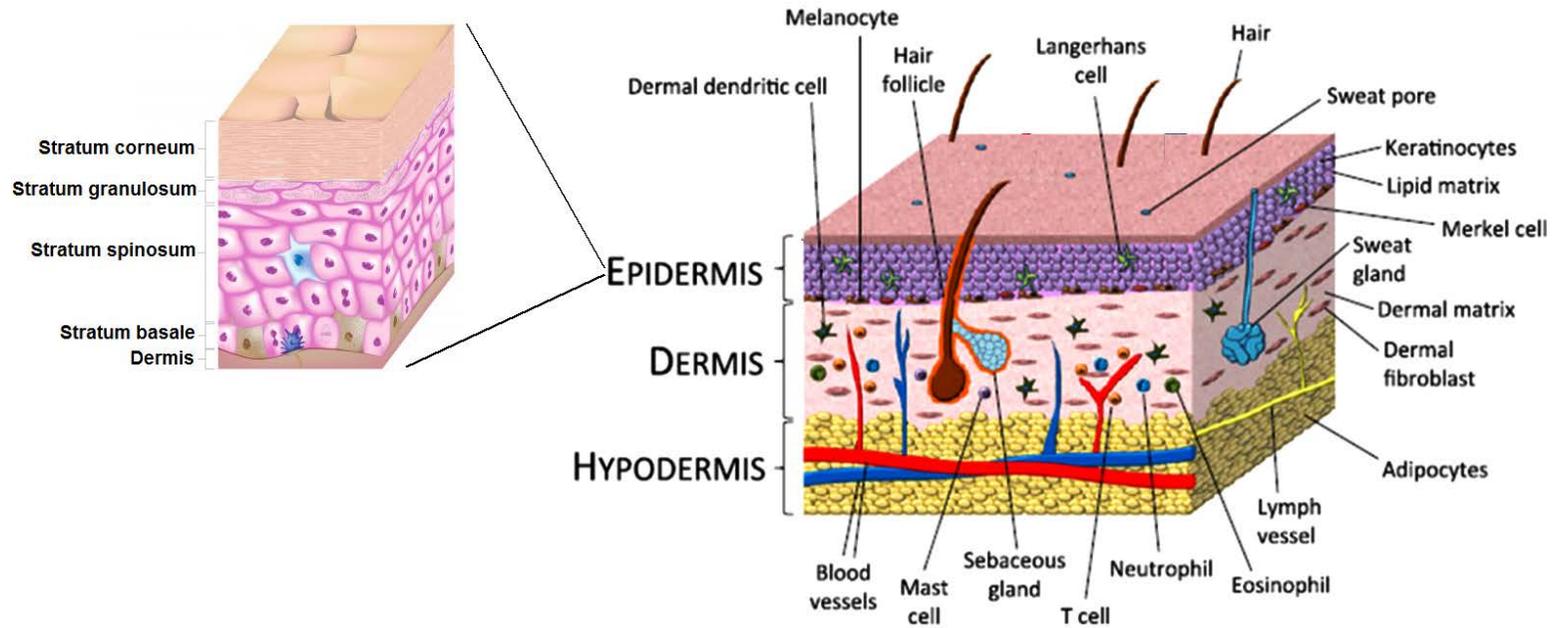
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891 Figure 2: Anatomy of the human skin.

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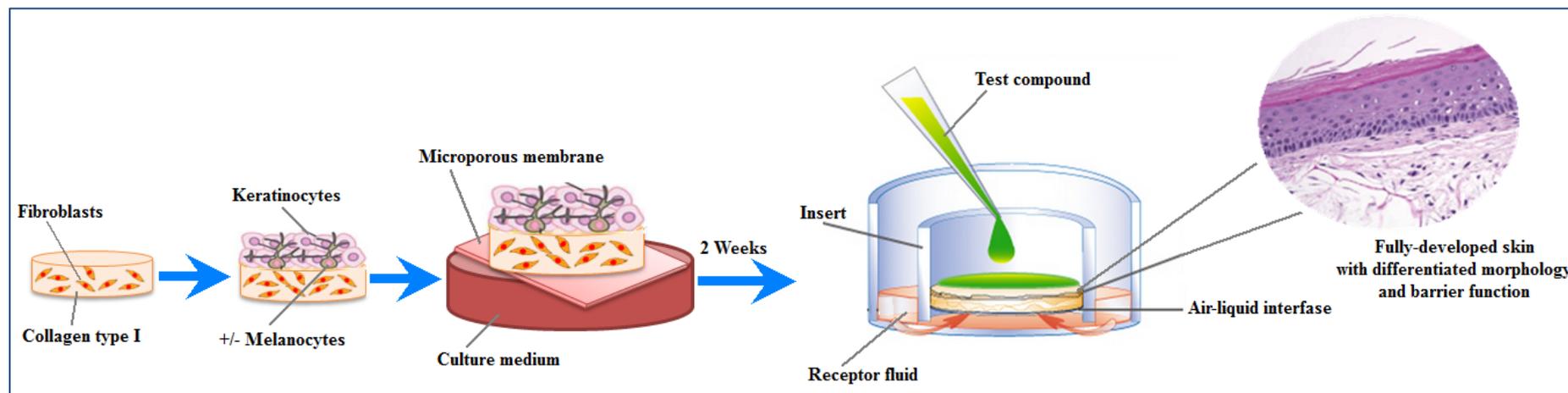
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899 **Figure 3: General stages of development of HSE model.**



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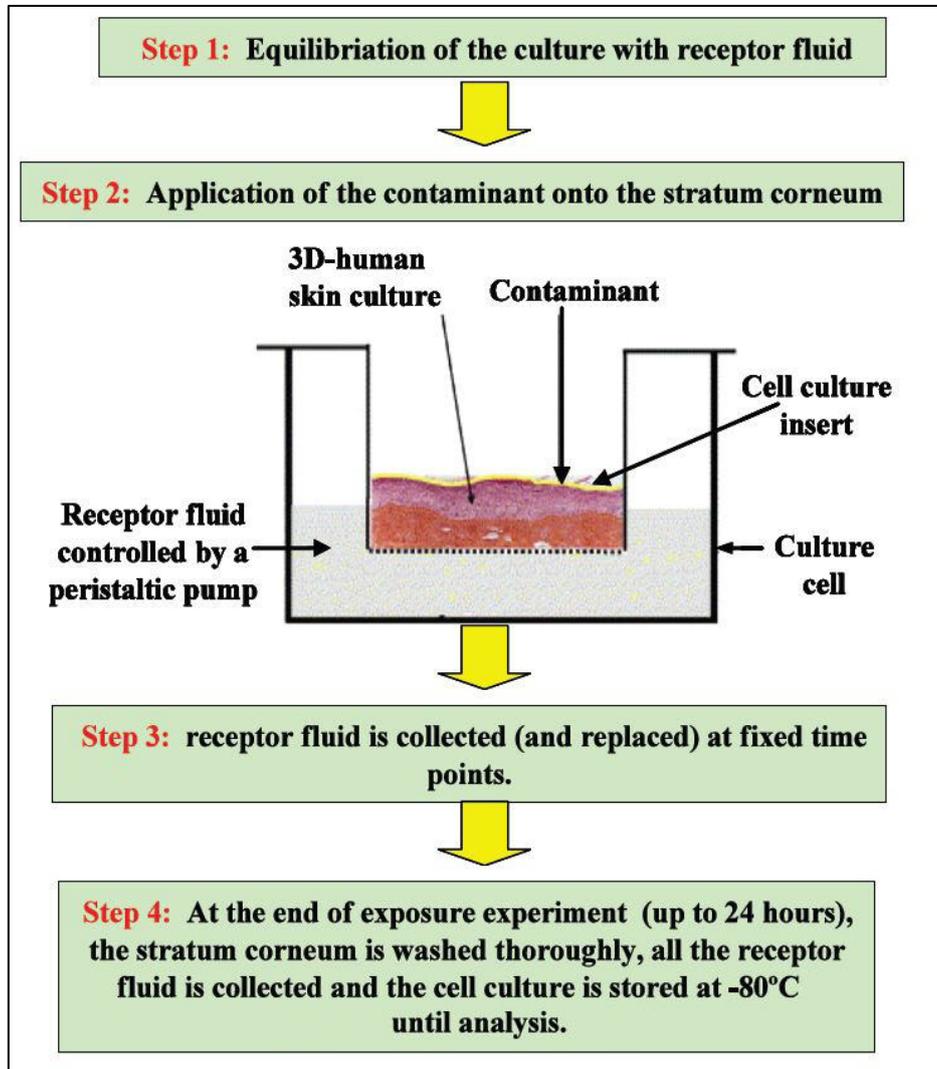
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910 **Figure 4: General protocol for percutaneous absorption studies using *in vitro* HSE**
911 **models.**

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