Graphical Abstract (for review)

1 A comparative study of the *in vitro* permeation of 2-
2 phenoxyethanol in the skin PAMPA model and mammalian skin

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Abstract

For permeation studies that use excised skin, experimental data may show variability associated with the use of biological tissues. As a consequence, achieving reproducible results and data interpretation may be challenging. The skin parallel artificial membrane permeability assay (skin PAMPA) model has been proposed as a high-throughput tool for predicting skin permeation of chemicals. A number of skin cleansing wipe formulations for the diaper area of infants contain 2-phenoxyethanol (PE) as a preservative and cetylpyridinium chloride (CPC) as a surfactant with antimicrobial activity. However, information regarding cutaneous absorption of PE and CPC in the scientific literatures is remarkably limited. The main aim of the present study was to assess the suitability of the skin PAMPA model for prediction of skin permeation of PE. A secondary aim was to investigate the influence of CPC on the dermal absorption of PE. PE (1% w/w) was prepared in two vehicles, namely propylene glycol (PG) and water-PG (WP). Permeability of PE was investigated *in vitro* using the skin PAMPA membrane, porcine skin and human skin under finite dose conditions. The highest permeation of PE was observed for the water-PG preparation with 0.2% w/w of CPC. This finding was consistently observed in the skin PAMPA model and in Franz cell studies using porcine skin and human skin. Permeation of CPC was not detected in the three permeation models. However, permeation of PE increased significantly ($p < 0.05$) in the presence of CPC compared with formulations without CPC. When comparing the skin PAMPA data and the mammalian skin data for the cumulative amount of PE permeated, the r^2 values for PAMPA-porcine skin and PAMPA-human skin were 0.84 and 0.89, respectively. The findings in this study demonstrate the capability of the skin PAMPA model to differentiate between various doses and formulations and are encouraging for further applications of this model as a high throughput screening tool in topical formulation development.

Keyword: dermal absorption, PAMPA, porcine skin, human skin, preservative, surfactant.

1. Introduction

Dermal absorption of ingredients in topical formulations intended for infants has not been widely studied. The importance of assessing skin permeation of such ingredients has recently been reviewed (Rahma and Lane, 2022; Stamatas et al., 2021). Baby wipes are used regularly in the infant population, and mainly comprise a high percentage of water (>90%), preservative(s), and surfactant(s). Phenoxyethanol (PE) is the most commonly used preservative ingredient in baby wipe formulations and is typically included at a concentration of 0.5-1% w/v. PE has been shown to demonstrate significantly lower adsorption ($p < 0.05$) to the fabric component of baby wipes in comparison with methylparaben (Endo et al., 2020). In

addition, PE (log P 1.2) has a comparatively higher aqueous solubility compared with other preservatives commonly used in topical preparations such as parabens and benzoic acid (Sheskey et al., 2020). These are important considerations for formulations with high water content such as baby wipes. Although PE is extensively used in baby wipe formulations (Salama et al., 2021), it has also been reported to act as a penetration enhancer (Ibrahim and Li, 2009; Ibrahim and Li, 2010). The haemolytic effects of PE have long been acknowledged (European Union Scientific Committee on Consumer Safety, 2016). Following oral administration, PE undergoes rapid first pass metabolism and is then converted into 2-phenoxyacetic acid (European Union Scientific Committee on Consumer Safety, 2016). Therefore, the systemic bioavailability of PE is very low (European Union Scientific Committee on Consumer Safety, 2016). By contrast, percutaneous absorption resulted in higher concentrations of PE in blood (Kim et al., 2015). In 2012, the French National Agency for the Safety of Medicines and Health Products (ANSM) submitted a risk assessment concerning the use of PE as a preservative ingredient in cosmetic formulations (French National Agency for the Safety of Medicines and Health Products, 2019). The ANSM proposed that PE should be excluded from topical formulations intended for the diaper area of infants and children aged under 3 years. The main reasons were the haematotoxicity and hepatotoxicity potential and insufficient margins of safety (MoS) for children under 3 years old (Dreno et al., 2019; European Union Scientific Committee on Consumer Safety, 2016).

Some baby wipe formulations also contain cetylpyridinium chloride (CPC), a quaternary surfactant with antimicrobial activity. For example, three commercial baby wipes listed in Table 1 contain PE and CPC. To date there is no information in the published scientific literature regarding the skin permeation of PE in the presence of surfactant(s). Although several authors have reported skin permeation of PE these studies used rodent skin. Therefore, the experimental data cannot be extrapolated to humans (Lane, 2013).

Table 1. Commercial baby wipes containing PE and CPC

Ingredient list	Cussons baby wipes pure & gentle	Paseo baby wipes	Pigeon baby wipes
Water	*	*	*
PE	*	*	*

Ingredient			
PG	*	*	
Polyhexamethylene biguanide		*	
Benzalkonium chloride			*
CPC	*	*	*
Croduret		*	
PEG-40 hydrogenated castor oil	*		
PPG-5-ceteth-20	*		
Sodium lactate	*		
Sodium citrate			*
Citric acid		*	*
Sodium benzoate			*
Disodium EDTA	*		
PEG-45 palm kernel glycerides	*		
Sodium lauryl sarcosinate	*	*	
Olea europaea fruit oil	*		
Cyclodextrin	*		
Methylparaben	*		
l-menthol	*		
Ethylparaben	*		
Butylparaben	*		
Propylparaben	*		
Polydimethylsiloxane	*		
Chamomile extract		*	

| Perfume | * |
| *Prunus   serrulata* flower extract | * - |

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Excised human skin is the most preferred tissue for *in vitro* skin permeation studies that are intended to estimate results in human (Dick and Scott, 1992; Schmook et al., 2001). The major challenges in using excised human skin are (1) difficulty in sourcing the skin samples and (2) intra- and inter-specimen variability (Dick and Scott, 1992; Schmook et al., 2001). The latter may also be problematic when interpreting the results from permeation studies (Oliveira et al., 2010; Zhang et al., 2019). Porcine skin has been considered as an appropriate surrogate for human skin, with reference to the close representation of the permeability properties of porcine tissue to human skin compared to other animal models (Dick and Scott, 1992; Schmook et al., 2001). However, porcine skin reportedly has a lower barrier function than human skin (Barbero and Frasch, 2009; Schmook et al., 2001; Yoshimatsu et al., 2017). In addition, ethical concerns over the use of animals in experiments is rising (Hopf et al., 2020; Pistollato et al., 2021). In light of these challenges, there has been a growing interest in exploring high throughput permeation models that are capable of generating reproducible data that may be extrapolated to man (Zhang et al., 2019).

The skin Parallel Artificial Membrane Permeation Assay (PAMPA) model has been developed for prediction of percutaneous absorption. The development of the skin PAMPA membrane and its use as percutaneous absorption model was first described by Ottaviani et al. (Ottaviani et al., 2006). The membrane composition was then refined by Sinkó et al. to achieve better representation of the intercellular stratum corneum (SC) region (Sinkó et al., 2012). The skin PAMPA membrane is essentially a filter substrate coated with synthetic lipids (certramides, free fatty acids, and cholesterol) arranged in a 96-well plate. Several studies have reported positive correlations for the permeability parameters of compounds using the skin PAMPA model and conventional *ex vivo* permeation studies (Balázs et al., 2016; Kovács et al., 2021; Sinkó et al., 2021; Zhang et al., 2019). However, these studies only investigated permeation of actives under infinite dose conditions.

It is important to assess skin permeation using clinically relevant doses, where the applied dose represents the actual exposure "in use". Clinically relevant doses demonstrated a finite dose profile or "plateau" for *in vitro* permeation studies (Franz, 1975, 1983; Lehman, 2014). This reflects the depletion of the applied dose of active over time (Franz, 1983; Lehman, 2014). Most recently Zhang and co-workers reported that permeation data for niacinamide in the skin

PAMPA model correlated well with porcine and human skin *in vitro* studies under finite dose conditions but not under infinite dose conditions (Zhang et al., 2019). Thus, the primary objective of the present work was to investigate the suitability of the skin PAMPA model for determination of dermal absorption of PE in the presence and absence of CPC in model formulations. To this end, *in vitro* studies were conducted in the skin PAMPA model and in porcine skin or human skin using Franz diffusion cells under finite dose conditions. A secondary objective was to elucidate the influence of CPC on the dermal absorption of PE.

2. Materials and Methods

2.1. *Materials*

PE, CPC, and propylene glycol (PG) were purchased from Sigma-Aldrich Co., USA. HPLC grade solvents (acetonitrile, methanol, water, and trifluoroacetic acid) were purchased from Fischer Scientific, UK. Phosphate-buffered saline (PBS) tablets were purchased from Oxoid Ltd., UK. Porcine ear skin was obtained from a local abattoir. Excised abdominal human skin from a single donor was obtained from a tissue bank with informed consent and institutional ethical approval (Research Ethics Committee reference 07/H1306/98). Skin specimens were stored at -20 °C prior to use. The skin PAMPA sandwich plate, skin PAMPA collector plates, standard hydration solution, stirring disks and Gut-Box™ were supplied by Pion Inc. Billerica, USA.

2.2. Methods

2.2.1. *Preparation of test solutions*

PE solutions were prepared in the presence or absence of CPC, as shown in Table 12. PG was chosen as a single solvent system, whereas water-PG 97:3 (WP) was chosen as a vehicle that better represents the cleansing liquid in baby wipes. In addition, selected formulations contained CPC either at 0.2% (w/w) or 1% (w/w). These concentrations represent the typical concentration of surfactants used in baby wipes (Cunningham et al., 2008; Cunningham et al., 2016; Sheehan, 2016). The full details of the formulations used in this study are listed in Table 12.

Table 12. PE solutions used in permeation studies.

Formulation	PE (% w/w)	CPC (% w/w)	Vehicle
PG1	1	-	PG
PG2	1	0.2	PG
PG3	1	1	PG
WP1	1	-	WP
WP2	1	0.2	WP
WP3	1	1	WP

138

139   The solutions were freshly prepared before the experiments. For PG2, PG3, WP2, and

140   WP3, mixing of the ingredients was performed with mild stirring to avoid foam formation by

141   CPC.

142

143   *2.2.2. Skin PAMPA studies*

144   *Influence of CPC on PAMPA membrane integrity*

145   In order to assess the compatibility of the skin PAMPA membrane with CPC, permeation

146   of CPC in the PAMPA model was monitored over 6 h. For these tests, CPC was dissolved in

147   PG at a final concentration of 0.2% w/w and 1% w/w. The permeation studies were performed

148   using the same setup as described in the previous section. Eight different doses were used,

149   covering finite and infinite conditions: 6, 10, 18, 30, 103, 515, 181, and 909 $\mu g/cm^2$. To achieve

150   these doses, CPC was applied at application volumes of 1, 3, 17, and 30 μL.
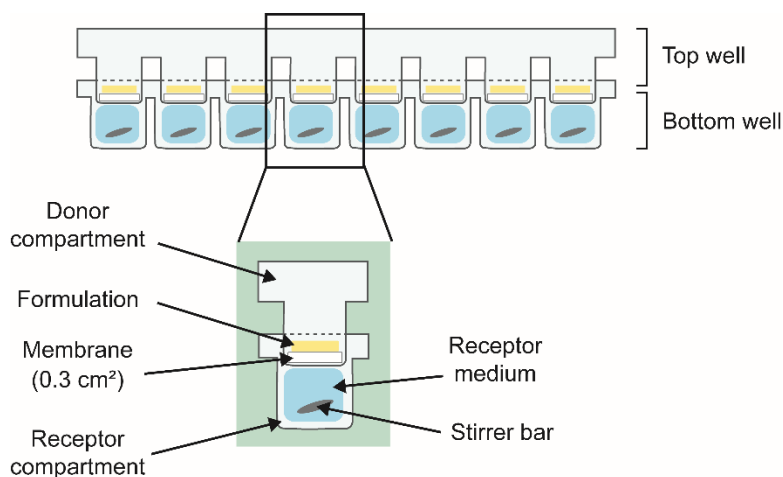
151

152   *Permeation of PE in the skin PAMPA membrane*

153   Permeability of PE was investigated in the skin PAMPA membrane using a modified

154   procedure reported by Luo et al. and Zhang et al. (Luo et al., 2016; Zhang et al., 2019). The

155   setup for this assay is illustrated in Fig. 1. The top side of the skin PAMPA sandwich plate was

156   set as the donor compartment, where the test solution was applied. For pilot studies, the dose

157   of PE applied was varied from 3 to 6 9 $\mu L/cm^2$ to assess the suitability of the experimental

158   design. Based on the results, an applied dose of 6 9 $\mu L/cm^2$ was selected for the remaining

159   experiments.

160   The collector plates were added with stirring disks and filled with 180 µl of degassed

161   PBS (pH 7.30 ± 0.10). Following application of the test solution, the two plates were assembled

162   and incubated in the Gut-Box™ stirring unit at 32 ± 1 °C for 1 h. The collector plate was

163   replaced with a new plate prefilled with receptor solution at 1, 2, 5, 10, 15, 20, 30, 45, and 60

164   min.

165

166

167   **Fig. 1.** Experimental setup for skin PAMPA studies.

168

169   *2.2.3. Franz cell studies*

170 *Skin permeation studies*

171   Permeation studies were conducted using full-thickness porcine skin and heat-

172   separated human epidermis in Franz-type diffusion cells. Porcine skin was prepared as

173   described by Cristofoli et al. (Cristofoli et al., 2022). The epidermis of human skin was isolated

174   by the heat separation technique as described by Oliveira et al. (Oliveira et al., 2012). The

175   receptor medium was similar to that of the skin PAMPA assay. The permeation study was

176   conducted for 24 h at 32 ± 1 °C. Temperature was controlled by using a water bath (Sub Aqua

177   26 Plus, Grant™, Fischer Scientific, UK). Prior to the experiments, the skin impedance was

178   measured to assess the skin barrier integrity, as described previously (Oliveira et al., 2012).

179   The skin was then allowed to equilibrate to reach 32 ± 1 °C. The temperature of the skin was

180   measured using a digital thermometer (TM-22 Digitron, RS Components, UK). 10 µL of the

181   test solution, representing a finite dose of 10 µL/cm^2 (corresponding to 100 µg PE per cm^2),

182   was applied evenly on the donor compartment. 100 µL of sample was withdrawn at 0, 0.5, 1,

183   2, 4, 6, 8, 10, 12, and 24 h. After sample collection, volume replacement was carried out by

184   adding 100 µL of receptor solution (maintained at 32 ± 1 °C) to the receptor compartment. All

185   samples were analyzed for PE and CPC content using a validated HPLC method (section

186   2.2.4).

187

188   *Mass balance studies*

189   At the end of the permeation study, the receptor medium was immediately removed. Each

190   donor compartment was washed with 1 mL of methanol-water (85:15) mixture and the washing

191   solution was transferred to a 2 mL microcentrifuge tube. The cap of the tube was sealed with

192   Parafilm® to prevent evaporation. The washing procedure was performed 3 times. The Franz

193   diffusion cells were then disassembled, and each skin membrane was transferred to a 2 mL

194   microcentrifuge tube. 1 mL of methanol-water (85:15) was added to the microcentrifuge tube

195   and the cap of the tube was sealed with Parafilm® to prevent evaporation. Extraction of PE

196   and CPC was carried out by placing the tubes in a bench shaker (VWR International, USA) at

197   800 rpm for 12 h. All samples were centrifuged using an Eppendorf® microcentrifuge 5415R

198   (Eppendorf, UK) at 13,200 rpm for 20 min. Subsequently, the samples were analyzed for PE

199   and CPC content using a validated HPLC method. The percentage of the two ingredients

200   recovered from the washing solution, extraction solution, and the receptor solution was

201   determined.

202

203   *2.2.4. Quantification of PE and CPC*

204   The determination of PE and CPC content was performed using HPLC with a diode-array

205   detector (Agilent Technologies 1200 Series). Data acquisition was performed using

206   Chemstation software (Agilent Technologies, USA). A Kinetex® XB-C_{18} column (250 x 4.60

207   mm, Phenomenex, UK) was used for the analysis. All the samples and standard solutions

208   were stored in amber HPLC vials during the assay. Optimized HPLC conditions were achieved

209   using a gradient elution system. The elution conditions are shown in Table 23. The flowrate,

210   injection volume, and column temperature were set as 1 ml/min, 20 µL, and 40 °C,

211   respectively. The detection wavelengths were 258 nm (for CPC) and 270 nm (For PE).

212

213

214   **Table 23.** Elution conditions for PE and CPC.

Time	Volume ratio of mobile phase (%)	
	acetonitrile	0.1% TFA
0 – 4	28	72
5 – ~~9~~12	80	20
~~10 – 12~~	~~28~~	~~30~~
13 – 16	28	72

Validation of the analytical method was carried out in accordance with the guidelines outlined by the International Conference on Harmonization (ICH) regarding quantitative assays for determination of the content of active compounds (ICH Harmonised Tripartite, 2005). The following parameters were assessed: linearity, range, accuracy, intra-day and inter-day precision, limit of detection (LOD), limit of quantification (LOQ), and robustness. In addition, a system suitability test was performed to ensure that consistent chromatographic behavior was achieved for each analytical procedure (US Food and Drug Administration, 1994).

2.2.5. Statistical analysis

Statistical analysis was performed using Origin 8.0 (OriginLab Corp., Northampton, MA). Data were assessed for normal distribution and homogeneity using the Shapiro-Wilk Test and Levene's Test, respectively. Sets of data that met the assumptions of normality and homogeneity were analyzed using one-way ANOVA followed by Tukey's post hoc test. The Kruskal–Wallis H Test was used for datasets that did not follow the assumptions of normality and/or homogeneity of variance. The level of significance was set at a p-value lower than 0.05 ($p < 0.05$). The correlation assessment was performed using the Pearson correlation coefficient (r^2).

3. Results

3.1. HPLC method validation

The results of method validation are shown in Table ~~3~~4. The retention times of PE and CPC using the validated method were 6.01 and 9.40 min, respectively. The linearity for PE and CPC was obtained for a concentration range of 1 to 100 µg/mL. The calibration curve from this range of concentrations exhibited a linear regression line. The LOD and LOQ for PE

were calculated as 0.49 µg/mL and 1.49 µg/mL, respectively. The LOD and LOQ for CPC were 0.51 µg/mL and 1.55 µg/mL, respectively.

Table 3̶4. Summary of validation parameters for PE and CPC analysis using HPLC

Parameters	PE	CPC
Linearity range (µg/mL)	1 – 100	1 – 100
r^2	>0.99	>0.99
LOD (µg/mL)	0.49	0.51
LOQ (µg/mL)	1.49	1.55
Accuracy		
Recovery (%)	100.07 ± 0.34	100.42 ± 0.59
RSD (%)	0.03 – 0.65	0.04 – 0.89
Precision		
Intra-day (%RSD)	0.17	0.42
Inter-day (%RSD)	0.25	1.14
Robustness (r^2)	>0.99	>0.99
System suitability		
peak area (%RSD)	0.16	0.59
RT (%RSD)	0.10	0.10
Peak symmetry (%RSD)	0.99	0.18

RSD = relative standard deviation

The accuracy, which represents the closeness between the experimentally measured values and the true values, was determined by measuring the response of blank samples spiked with PE and CPC at 5, 50, and 100 µg/mL, representing the low, medium, and high concentrations. The average recovery value for PE was 100.07 ± 0.34 %, with a relative standard deviation (RSD) value <1%. As for CPC, the recovery value was 100.42 ± 0.59 %, with an RSD value <1.00%. The precision of the method was assessed based on intra-day and inter-day precision assessments. The RSD of the measured concentrations was used to express the inter-assay variability. Acceptable precision was achieved with corresponding RSD values of <1.00% and <2.00% for intra-day and inter-day assessment, respectively.

The robustness of an analytical method is investigated to ensure that the capability of the HPLC system remains unaffected with minor variations in chromatographic conditions.
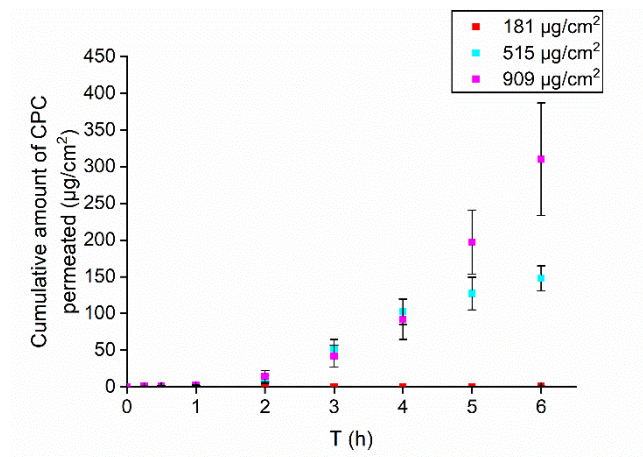
258 The robustness test was performed on standard solutions containing PE and CPC within a
259 concentration range of 1-100 µg/mL. Despite the minor variations (2% change) in injection
260 volume, flow rate, column temperature, mobile phase composition, and detection wavelength,
261 r^2 values remained ≥0.99. A system suitability test was performed by determining the RSD of
262 the injection repeatability, as described in the Reviewer Guidance on Validation of
263 Chromatographic Methods (US Food and Drug Administration, 1994). Six injections of
264 solutions with PE and CPC at a concentration of 50 µg/mL were determined for the peak area,
265 peak symmetry, and retention time. The RSD values for these parameters were ≤ 1.00% for
266 PE and CPC.

267

268 *3.2.   Influence of CPC on PAMPA membrane integrity*

269   Fig. 2 compares the amount of CPC permeated from different applied doses. When the
270 applied dose was ≤103 µg/cm², permeation of CPC was not detected over 6 h. For the 181
271 µg/cm² dose, permeation of CPC was evident from 5 h. The amount of CPC permeated for
272 this dose was 0.06 ± 0.07 µg/cm² at 5 h. At the higher dose of CPC (≥ 515 µg/cm²), the
273 permeation of CPC was observed at earlier timepoints.

274

275

276   **Fig. 2.** The amount of CPC permeated through the skin PAMPA membrane under various
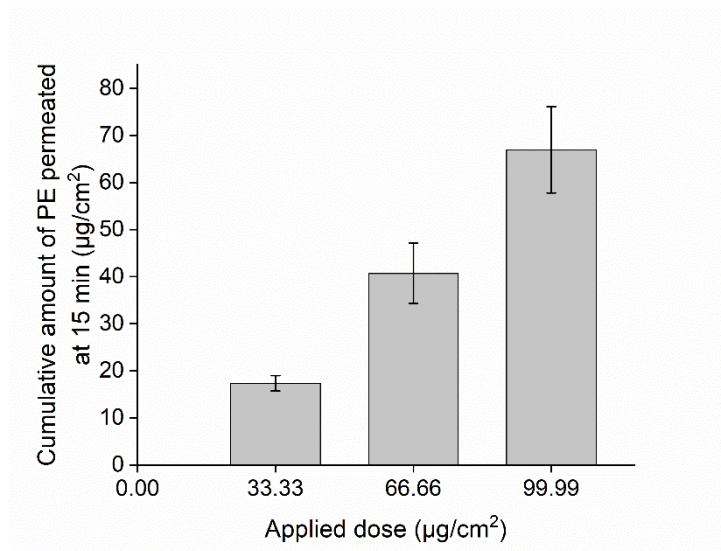277   applied doses (mean ± SD, n = 6).

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279

280   *3.3.   Permeation of PE in the skin PAMPA membrane*

Cumulative amounts of PE permeated through the skin PAMPA membrane from different applied doses are shown in Fig. 3. Three PE doses were used in this set of experiments – 1, 2, and 3 µL. These doses corresponded to 3, 6, and 9 µL/cm².
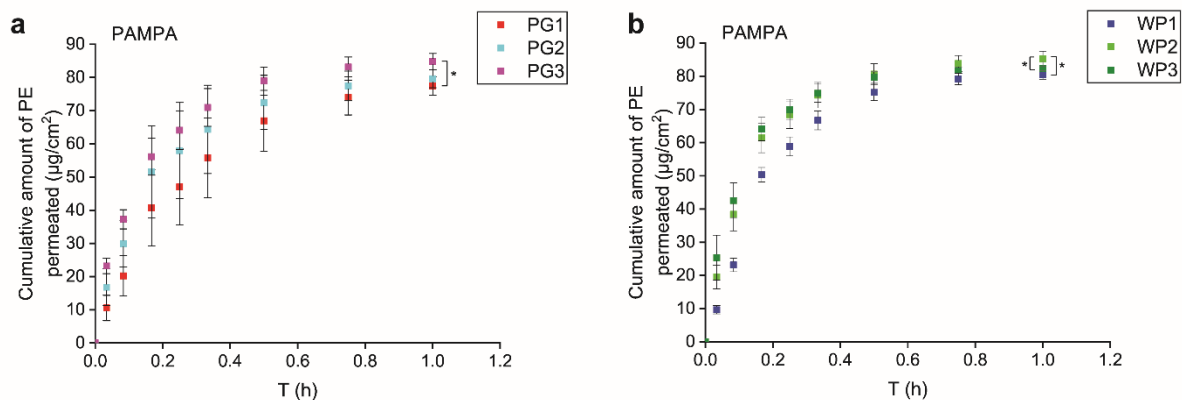

Fig. 3. Cumulative amounts of PE permeated through the skin PAMPA membrane from the different applied doses (mean ± SD, n = 6).

The permeation profiles of PE in the skin PAMPA model are shown in Fig. 4. Permeation of PE was evident from 1 min. The highest permeation of PE was observed for the solution containing CPC 0.2% with water-PG as the vehicle. The cumulative amount of PE that permeated from this preparation was 85.30 ± 2.19 µg/cm². For preparations with neat PG as the solvent, a significantly higher amount ($p<0.05$) of PE permeated from the vehicle with CPC 1%, compared with the PE solution with no CPC.

297   **Fig. 4.** Permeation profiles of PE from (a) PG and (b) Water-PG in the skin PAMPA

298   membrane (mean ± SD, n = 6). * ~~indicates~~Indicates statistical significance (p<0.05)

299   compared to the ~~rest of~~other preparations.

300

301   Interestingly, for water-PG preparations, the cumulative amount of PE permeated was

302   significantly higher (p<0.05) in the presence of CPC 0.2% compared with all other PE

303   solutions. These findings suggested that the increase in PE permeation from water-PG was

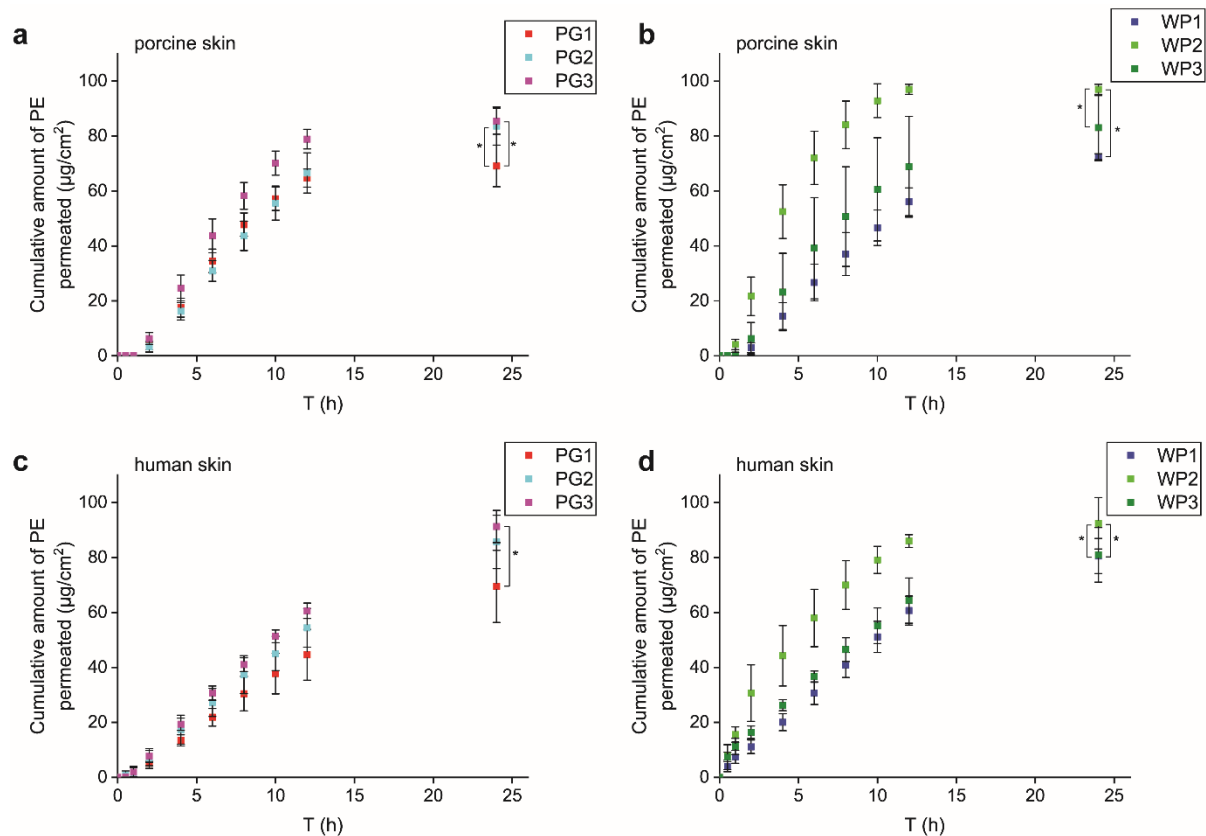304   not necessarily proportional to the concentration of CPC.

305

306   *3.4.   Franz cell studies*

307   *3.4.1.   Permeation of PE in porcine skin and human skin*

308   The permeation profiles of PE in porcine skin and human skin are shown in Fig. 5. We

309   observed a trend which was consistent with the skin PAMPA data, where the highest

310   permeation of PE was observed for water-PG with <u>0.</u>2% of CPC. The cumulative amounts of

311   PE that permeated in porcine skin and human skin from this preparation at 24 h were 96.98 ±

312   1.83 µg/cm^2 and 92.46 ± 9.34 µg/cm^2, respectively. These values corresponded to more than

313   97% of the applied dose. The lowest permeation of PE was observed for PG without CPC,

314   both in porcine skin and human skin (69.09 ± 7.54 µg/cm^2 and 73.99 ± 9.70 µg/cm^2,

315   respectively). These results are consistent with the skin PAMPA data.

316

Fig. 5. Permeation profiles of PE from various preparations in porcine skin and human skin. (a) PG vehicle applied to porcine skin; (b) Water-PG vehicle applied to porcine skin; (c) PG vehicle applied to human skin; (d) Water-PG vehicle applied to human skin. Mean ± SD, n = 5-6. * ~~indicates~~Indicates statistical significance (p<0.05) compared to the ~~rest of~~other preparations.

Regarding the effect of CPC on the permeation of PE, the results from porcine skin and human skin studies were consistent with the trends observed in the skin PAMPA studies. For PG, a significantly higher cumulative amount of PE permeation was evident in the presence of CPC 1% compared to preparations with no CPC (p<0.05). This was observed both in porcine skin and human skin. For water-PG, the cumulative amount of PE permeated was significantly higher (p<0.05) in the presence of CPC 0.2% compared with all other PE vehicles.

3.4.2. Mass balance studies for PE and CPC in porcine skin and human skin

The results of the mass balance studies for PE are shown in Table ~~4~~5. The amount of PE that remained on the surface of porcine skin was ~~1-2%~~a maximum of 2.01% of the applied dose for the PG vehicle. In contrast, PE was not detected in the washing solution for all water-

335 PG preparations. For human skin data, the amount of PE that remained on the skin surface

336 for the PG vehicle was ~~up to~~ a maximum of 27.08% of the applied dose. Similar to the findings

337 from porcine skin, PE was not detected in the washing solution for all water-PG preparations.

338 Regarding the skin retention of PE, the percentages of the active recovered from the porcine

339 skin for PG vehicle were ~~around~~ 8.19-9.37% of the applied dose. For the water-PG

340 preparations, ~~around~~ 8.61-13.25% of PE was retained in porcine skin. The percentages of PE

341 retained in human skin were lower than in porcine skin. At the end of the permeation studies,

342 3.08–3.57% of PE from the PG preparations were recovered in human skin. For the water-PG

343 preparations, the percentage of PE extracted from human skin was 1.10–1.27%.

344

345 **Table 45.** Mass balance results for PE in porcine skin and human skin (mean ± SD, n = 5).

Preparation	PE recovered (% of applied dose)			
	Receptor fluid	Skin surface	Skin	Total recovery
		Porcine skin		
PG1	81.32 ± 6.03	1.47 ± 1.09	9.20 ± 2.44	92.00 ± 4.97
PG2	85.23 ± 5.22	2.01 ± 0.57	9.37 ± 0.98	96.61 ± 4.96
PG3	92.51 ± 3.39	ND	8.19 ± 0.82	100.70 ± 2.83
WP1	79.00 ± 4.04	ND	13.25 ± 2.99	92.25 ± 1.77
WP2	95.81 ± 9.45	ND	8.61 ± 0.94	104.49 ± 8.56
WP3	88.29 ± 10.28	ND	10.06 ± 2.86	98.36 ± 13.14
		Human skin		
PG1	71. 40 ± 12.62	27.08 ± 8.17	3.57 ± 0.12	102.06 ± 4.65
PG2	83.63 ± 2.48	11.17 ± 3.09	3.41 ± 0.28	98.23 ± 0.64
PG3	97.34 ± 3.59	4.24 ± 3.82	3.08 ± 0.55	104.67 ± 0.73
WP1	89.07 ± 2.75	ND	1.11 ± 0.17	90.19 ± 2.92
WP2	97.79 ± 6.66	ND	1.10 ± 0.16	98.89 ± 6.68
WP3	87.90 ± 7.05	ND	1.27 ± 0.12	89.18 ± 7.02

346 ND = not detected

347

348 In contrast to PE, permeation of CPC was not detected in porcine skin and human skin

349 studies for all preparations, as was observed in the skin PAMPA model. Mass balance results

350 using porcine skin (Table 56) showed that the amount of CPC that remained on the skin

351 surface varied between 61.31 to 95.28% of the applied dose, whereas the amount of CPC

352 retained in the skin was 10.28-38.72% of the applied dose. In contrast, mass balance studies

353 using human skin confirmed that more than 80% of the applied dose of CPC was recovered
354 from the skin surface for all vehicles (Table 56).

355

356 **Table 56.** Mass balance results for CPC in porcine skin and human skin (mean ± SD, n = 5).

Preparation	Receptor fluid	CPC recovered (% of applied dose)		
		Skin surface	Skin	Total recovery
Porcine skin				
PG2	ND	83.21 ± 8.44	21.12 ± 7.68	104.33 ± 1.19
PG3	ND	95.28 ± 4.29	10.28 ± 4.45	105.57 ± 0.16
WP2	ND	61.31 ± 0.87	38.72 ± 1.99	100.04 ± 2.16
WP3	ND	82.71 ± 3.25	13.00 ± 2.52	95.71 ± 2.28
Human skin				
PG2	ND	85.06 ± 8.07	8.81 ± 1.13	93.88 ± 7.01
PG3	ND	91.40 ± 14.03	3.33 ± 3.00	94.74 ± 11.48
WP2	ND	90.04 ± 9.87	7.72 ± 2.33	97.77 ± 7.84
WP3	ND	89. 92 ± 7.25	1.71 ± 91.64	91.64 ± 8.13

357 ND = not detected

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359 *3.5.   Comparative evaluation of the permeation data*

360   Cumulative amounts of PE that permeated the skin PAMPA membranes, porcine skin, and
361 human skin are shown in Fig. 6. Statistical differences in the amount of PE that permeated
362 from various preparations were assessed within the same vehicles rather than all vehicles.
363 This approach should provide a clearer insight into possible active-vehicle-membrane
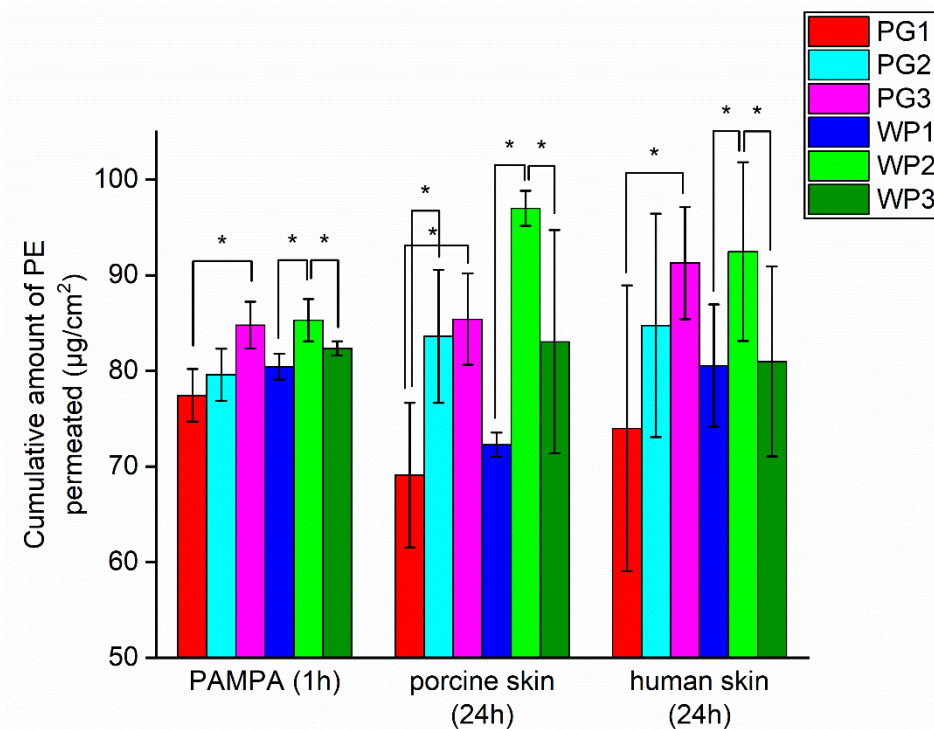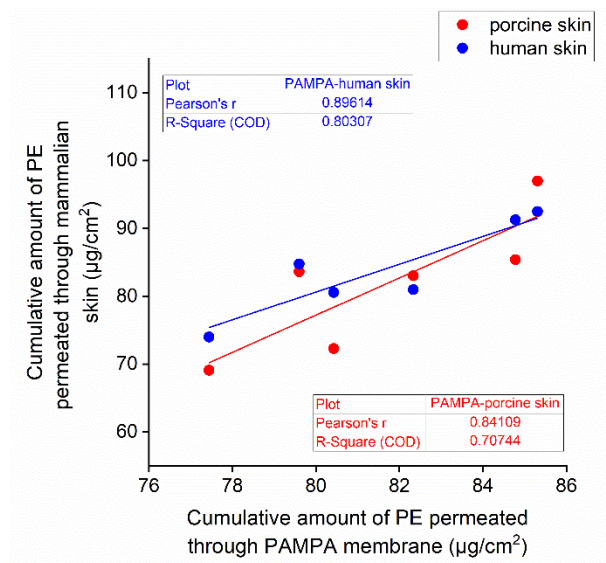364 interactions.

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Fig. 6. Cumulative amounts of PE that permeated the membranes (mean ± SD, n = 5-6). * indicates statistical significance (p<0.05) compared to the ~~rest of~~other preparations within the same vehicle and within the same membrane model.

Correlations for the cumulative amount of PE permeated between the skin PAMPA data and the mammalian skin data are shown in Fig. 7. When comparing the skin PAMPA data and the porcine skin data for cumulative amount of PE permeated, the r^2 value was 0.84. This value is consistent with previously reported data by Zhang et al. (Zhang et al., 2019). For the correlation between the skin PAMPA and human skin data for cumulative amount of PE permeated the corresponding r^2 value was 0.89.

Fig. 7. Correlation between cumulative amounts of PE that permeated through skin PAMPA and mammalian skin. Each point represents the mean value (n = 5-6).

4. Discussion

Synthetic membranes mimicking the stratum corneum (SC) have been investigated extensively as surrogate models for *in vitro* skin permeation studies (Čuříková et al., 2017; de Jager et al., 2006b; Groen et al., 2011; Sinkó et al., 2012; Uchida et al., 2015). The membranes are composed of a porous substrate coated with synthetic SC lipids, which serve as the rate-determining barrier for skin permeation. In the present work, preliminary assessment of skin PAMPA compatibility with CPC was conducted prior to permeation studies for CPC-containing preparations. It is important to evaluate the compatibility of synthetic membranes with formulations, particularly when the formulation contains penetration enhancers (Köllmer et al., 2019; Kovács et al., 2021). Surfactants have been reported to have the potential to disrupt the organization of the SC lipids, although the mechanism underlying this is not fully understood (Gloor et al., 2004; Jiang et al., 2003). The interaction between surfactants and SC lipids is complex, depending on the duration of exposure, the nature, and the concentration of the surfactants (De la Maza et al., 1997; Imokawa, 2004; Rhein et al., 1986). Köllmer et al. recently investigated the compatibility of the skin PAMPA membrane with different surfactants (Köllmer et al., 2019). In their study, the skin PAMPA membrane was incubated with blank formulations which contained different surfactants (1% Brij™ S2 + 0.15% Brij™ S721 and 1% Brij™ S2 + 1% Brij™ S721). After 4 h of pre-incubation, a permeation test was performed for methylparaben, ethylparaben, and propylparaben for 4 h. The results indicated that pre-incubation with these surfactants did not increase the permeation of the parabens. In addition,

405 the rank order for permeation remained unchanged (methylparaben > ethylparaben >
406 propylparaben). The authors suggested that the surfactants used in the study did not alter the
407 permeability of the skin PAMPA membrane towards the parabens (Köllmer et al., 2019).

408   In the present study, permeation of CPC was not detected over 6 h when the application
409 dose was ≤103 μg/cm^2. The concentration of CPC used in the permeation studies for PE-
410 containing vehicles using the skin PAMPA model was not more than 30 μg/cm^2. In addition,
411 the duration of the experiment was only 1 h. Permeation of CPC from all vehicles was not
412 detected in the receptor solution over 1 h. More importantly, a comparison with the Franz cell
413 studies confirmed that the permeation data from the skin PAMPA model showed a good
414 correlation with the porcine skin and human skin data (Fig. 7). Therefore, no evident
415 incompatibility was observed between the skin PAMPA membrane with the vehicles used in
416 this study under previously mentioned conditions.

417   Possible membrane-excipient interactions such as membrane blockage by the
418 formulation, disorganization or solubilization of the membrane lipids have also been
419 recognized (Köllmer et al., 2019). Two recent studies reported the effects of several excipients
420 on the permeation of actives in the skin PAMPA membrane (Kovács et al., 2021; Zhang et al.,
421 2019). Zhang et al. reported that niacinamide permeated the skin PAMPA membrane from
422 PEG 400 and PEG 600, whereas it did not permeate in porcine and human skin studies (Zhang
423 et al., 2019). These authors suggested that this might reflect the interaction between those
424 solvents and the skin PAMPA membrane.

425   Finite dose experiments using the skin PAMPA model reported by Luo et al. and Zhang et
426 al. included application doses as low as 3 μL/cm^2 (Luo et al., 2016; Zhang et al., 2019). These
427 studies reported comparable results for permeation profiles ~~The latter study reported a good~~
428 ~~correlation for cumulative amount~~ of the actives (ibuprofen, niacinamide) between the skin
429 PAMPA model and porcine skin as well as human skin. In the current study, permeation of PE
430 at the 3 μL/cm^2 dose in the skin PAMPA membrane had reached a plateau at 45 min. As
431 frequent sampling within such a short period was impractical and permeation data showed
432 high variability, a dose of 9 μL/cm^2 was selected as the final dose for PE. As shown in Fig. 3,
433 it is evident that by using the skin PAMPA model, a 1 h study time was sufficient for PE to
434 demonstrate comparable finite dose kinetics to those observed in porcine skin and human skin
435 in 24 h. Even with relatively high permeation of PE over a short period, the skin PAMPA model
436 was also capable of discriminating between different preparations. This was reflected by the
437 statistical differences between the formulations for the cumulative amounts of PE permeated,
438 as was observed in the porcine skin and human skin studies (Fig. 6). Such high-throughput
439 prediction in a time efficient manner is expected to be particularly useful for screening

numerous topical formulations (Luo et al., 2016; Neupane et al., 2020; Sinkó et al., 2012; Zhang et al., 2019). Consistent with previously reported studies, the time required for the active to permeate the artificial membrane was shorter compared with porcine skin or human skin (Čuříková et al., 2017; Kovács et al., 2021; Luo et al., 2016; Zhang et al., 2019). In addition, the variability in the skin PAMPA data, represented by the standard deviation, was lower than in the Franz cell studies with mammalian tissue.

As shown in Fig. 4, the highest permeation of PE in the skin PAMPA model was observed for water-PG with 0.2% of CPC. This finding was consistent with observations in the Franz cell studies using porcine skin and human skin (Fig. 5). This concentration of CPC is typically used in commercial baby wipe formulations, which comprise more than 90% of water and a low concentration of surfactant (< 0.5%). On the other hand, the three permeation models confirmed that the lowest permeation of PE was observed for neat PG without CPC. These results also correspond with the findings from a study by Luo et al., where the highest amount of active (ibuprofen) that permeated the skin PAMPA model was observed for the formulation which delivered the highest amount of active in human skin under finite dose conditions (Luo et al., 2016).

While permeation of CPC was not detected in the skin PAMPA model and Franz cell studies, the presence of CPC enhanced the permeation of PE significantly ($p < 0.05$) compared with preparations without CPC. Interestingly, better enhancement for permeation of PE was observed when the CPC concentration was 1% for PG and 0.2% for water-PG. The results of permeation studies using human skin showed that the cumulative amounts of PE permeated from PG in the presence of CPC 0.2% and 1% were 14% and 23% higher, respectively, compared with PE alone. For water-PG, the presence of CPC at 0.2% and 1% resulted in enhanced PE permeation by 14% and 0.5%, respectively, compared to PE alone. This might indicate that CPC influences the permeation of PE from PG and water-PG by different mechanisms. In general, it has been thought that the alkyl chains of surfactants may intercalate with the hydrophobic regions of the SC lipids (Gloor et al., 2004; Jiang et al., 2003). The ability of surfactants to increase the permeation of various actives is well documented (Merwe and Riviere, 2005; Riviere et al., 2010; Shokri et al., 2001). Clearly, it should be noted that surfactants are able to form micelles, enhance the solubility of permeant and thus reduce its thermodynamic activity (Lane, 2013). The critical micellar concentration of CPC was reportedly 1 mM (approximately 0.34 mg/mL) in water at 25 °C (Wang et al., 1999). In the present work, the concentrations of CPC used were 2 mg/mL and 10 mg/mL. For WP, the vehicle with 97% water, CPC was present above the CMC which may explain the results observed.

Regarding the correlation between the skin PAMPA data and mammalian skin data, the r^2 values for skin PAMPA-porcine skin and skin PAMPA-human skin were 0.84 and 0.89, respectively (Fig. 7). Previously, Zhang et al. reported that the correlation for the permeation of niacinamide between the skin PAMPA studies and porcine skin studies was 0.88 under finite dose conditions. However, the corresponding r^2 value for human skin was lower compared to porcine skin, namely 0.71.

It is important to note that artificial membranes are not intended to provide an estimation of the amount of actives that permeate the human skin since these membranes do not fully represent the biological complexity of human skin (Čuříková et al., 2017; Neupane et al., 2020). Such models can be used as an initial screening tool before performing permeation studies using human skin (Neupane et al., 2020; Zhang et al., 2019). Recently, a comprehensive overview of the findings from permeation studies using synthetic membranes was reported by Neupane et al. (Neupane et al., 2020). The main advantages of artificial membranes are less variability in the thickness and composition compared with biological tissues, and ease in storage (Neupane et al., 2020). A number of studies have demonstrated the ability of artificial membranes to correlate with *ex vivo* permeation studies using porcine skin or human skin (Balázs et al., 2016; Čuříková et al., 2017; de Jager et al., 2006a; Groen et al., 2011; Sinkó et al., 2021; Sinkó et al., 2012; Uchida et al., 2015). However, most of the studies were conducted under infinite dose conditions. On the other hand, finite dose experiments are more relevant for prediction of dermal absorption with reference to the amount of formulation applied in the real clinical setting. In finite dose experiments, the implications of evaporation and residence time of the solvents, as well as the solubility of the active in the remaining formulation should be taken into consideration (Lane, 2013). Correlations observed in comparative studies using infinite dose conditions should not be directly assumed to hold true for finite dose situations. As mentioned earlier, Zhang and co-workers reported that the skin PAMPA data showed correlations with the porcine skin and human skin data, for the amount of active permeated, under finite dose conditions but not under infinite dose conditions (Zhang et al., 2019).

The importance of lipid composition for permeability of artificial membrane models has been studied extensively. Groen et al. described the importance of lipid compositions (cholesterol free fatty acid, and ceramide) for the permeability of skin lipid models (Groen et al., 2011). In this study, the authors investigated the permeability of a stratum corneum substitute (SCS) with various ratios of SC lipids. This was done by assessing the permeation of benzoic acid under infinite dose conditions. It was found that the permeability of SCS to benzoic acid was higher when the SCS membrane contained a high content of free fatty acids. In contrast, permeation of benzoic acid was lower for SCS with high cholesterol or ceramide

content compared to SCS with equimolar composition of cholesterol free fatty acid, and ceramide.

A synthetic ceramide named certramide is used in the skin PAMPA membrane (Sinkó et al., 2012). Certramides are long-chain tartaric acid diamide derivatives. In the skin PAMPA membrane, the length of the alkyl chains in the certramides are 8 and 18 (C8-C18). The ceramides was are used as ceramide analogs with long alkyl chainscombined with stearic acid and cholesterol (Sinkó et al., 2012). The ratio of the membrane components in the skin PAMPA model (certramides, stearic acid and cholesterol) was chosen to represent the intercellular lipid matrix of the SC (Sinkó et al., 2012). The membrane , which demonstrated positive correlation with human SC for permeation of various compounds (Sinkó et al., 2012). The effects of molecular structure of ceramides on the permeability of a SC lipid model membrane has have previously been reported (Čuříková et al., 2017; Školová et al., 2013). Školová and co-workers reported a decrease in permeability of the studied synthetic membrane towards for two drugs models (theophylline and indomethacin) when natural long-chain ceramides (24-acyl chains/, Cer24) was were incorporated into the membrane compared to a control model without Cer24. Increased permeation for both model compounds was observed when short-chain ceramides (4-6 acyl chains) were used rather than the long-chain ceramides. Čuříková et al. investigated the potential of simplified SC membranes to predict the effects of permeation enhancers on the permeation of actives (Čuříková et al., 2017). This membrane model is composed of stearic acid, cholesterol, cholesterol sulfate, and ceramides. The ceramides used in the study was were simplified, consisting of N-2-hydroxystearoyl phytosphingosine (CER[AP]) and/or N-stearoyl phytosphingosine (CER[NP]), rather than more complex ceramides mixtures as developed earlier by de Jager et al. (de Jager et al., 2006b). In the study, theThe optimized membrane, containing an equimolar mixture of CER[AP] and CER[NP], was used to examine the permeation of two active models, indomethacin and theophylline from formulations with different permeation enhancers. For both actives, the enhancement of permeation by N-dodecyl azepan-2-one (Azone) and (S)-N-acetylproline dodecyl ester (L-Pro2) observed in the membrane model was consistent with the porcine skin studies conducted under infinite dose conditions.

5. Conclusions

This work confirms the potential of the PAMPA model as an important tool to discriminate between different vehicles for permeation of actives. In addition, a positive correlation between the skin PAMPA model and the Franz cell studies using porcine skin and human skin was observed for the cumulative amount of PE permeated. Both the skin PAMPA model and Franz

546   cell studies using mammalian skin showed that the highest permeation of PE was observed

547   for CPC 0.2% in water-PG, which is the typical amount of CPC used in commercial baby wipe

548   formulations. Considering that skin cleansing using baby wipes is carried out regularly, the

549   findings of the current study may have implications for the safety evaluation of such products.

550   The three permeation models showed that the presence of CPC enhanced the permeation of

551   PE significantly ($p<0.05$) compared with preparations without CPC. A greater enhancement

552   for permeation of PE was observed when the CPC concentration was 1% for PG and 0.2% for

553   water-PG. No evident incompatibilities between the skin PAMPA membrane and the examined

554   preparations were observed. Based on the present study and previous PAMPA publications it

555   is worth noting that the optimum experimental conditions for this model may vary for different

556   compounds. Future work will expand the range of actives to be studied in the model and further

557   investigate the application of the PAMPA model in high-throughput screening studies for

558   development of dermal formulations.

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Conflict of Interest

The authors declare no conflict of interest.

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1 # A comparative study of the *in vitro* permeation of 2-
2 # phenoxyethanol in the skin PAMPA model and mammalian skin

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Abstract

For permeation studies that use excised skin, experimental data may show variability associated with the use of biological tissues. As a consequence, achieving reproducible results and data interpretation may be challenging. The skin parallel artificial membrane permeability assay (skin PAMPA) model has been proposed as a high-throughput tool for predicting skin permeation of chemicals. A number of skin cleansing wipe formulations for the diaper area of infants contain 2-phenoxyethanol (PE) as a preservative and cetylpyridinium chloride (CPC) as a surfactant with antimicrobial activity. However, information regarding cutaneous absorption of PE and CPC in the scientific literatures is remarkably limited. The main aim of the present study was to assess the suitability of the skin PAMPA model for prediction of skin permeation of PE. A secondary aim was to investigate the influence of CPC on the dermal absorption of PE. PE (1% w/w) was prepared in two vehicles, namely propylene glycol (PG) and water-PG (WP). Permeability of PE was investigated *in vitro* using the skin PAMPA membrane, porcine skin and human skin under finite dose conditions. The highest permeation of PE was observed for the water-PG preparation with 0.2% w/w of CPC. This finding was consistently observed in the skin PAMPA model and in Franz cell studies using porcine skin and human skin. Permeation of CPC was not detected in the three permeation models. However, permeation of PE increased significantly ($p<0.05$) in the presence of CPC compared with formulations without CPC. When comparing the skin PAMPA data and the mammalian skin data for the cumulative amount of PE permeated, the r^2 values for PAMPA-porcine skin and PAMPA-human skin were 0.84 and 0.89, respectively. The findings in this study demonstrate the capability of the skin PAMPA model to differentiate between various doses and formulations and are encouraging for further applications of this model as a high throughput screening tool in topical formulation development.

Keyword: dermal absorption, PAMPA, porcine skin, human skin, preservative, surfactant.

1. Introduction

Dermal absorption of ingredients in topical formulations intended for infants has not been widely studied. The importance of assessing skin permeation of such ingredients has recently been reviewed (Rahma and Lane, 2022; Stamatas et al., 2021). Baby wipes are used regularly in the infant population, and mainly comprise a high percentage of water (>90%), preservative(s), and surfactant(s). Phenoxyethanol (PE) is the most commonly used preservative ingredient in baby wipe formulations and is typically included at a concentration of 0.5-1% w/v. PE has been shown to demonstrate significantly lower adsorption ($p<0.05$) to the fabric component of baby wipes in comparison with methylparaben (Endo et al., 2020). In

48 addition, PE (log P 1.2) has a comparatively higher aqueous solubility compared with other
49 preservatives commonly used in topical preparations such as parabens and benzoic acid
50 (Sheskey et al., 2020). These are important considerations for formulations with high water
51 content such as baby wipes. Although PE is extensively used in baby wipe formulations
52 (Salama et al., 2021), it has also been reported to act as a penetration enhancer (Ibrahim and
53 Li, 2009; Ibrahim and Li, 2010). The haemolytic effects of PE have long been acknowledged
54 (European Union Scientific Committee on Consumer Safety, 2016). Following oral
55 administration, PE undergoes rapid first pass metabolism and is then converted into 2-
56 phenoxyacetic acid (European Union Scientific Committee on Consumer Safety, 2016).
57 Therefore, the systemic bioavailability of PE is very low (European Union Scientific Committee
58 on Consumer Safety, 2016). By contrast, percutaneous absorption resulted in higher
59 concentrations of PE in blood (Kim et al., 2015). In 2012, the French National Agency for the
60 Safety of Medicines and Health Products (ANSM) submitted a risk assessment concerning
61 the use of PE as a preservative ingredient in cosmetic formulations (French National Agency
62 for the Safety of Medicines and Health Products, 2019). The ANSM proposed that PE should
63 be excluded from topical formulations intended for the diaper area of infants and children aged
64 under 3 years. The main reasons were the haematotoxicity and hepatotoxicity potential and
65 insufficient margins of safety (MoS) for children under 3 years old (Dreno et al., 2019;
66 European Union Scientific Committee on Consumer Safety, 2016).

67 Some baby wipe formulations also contain cetylpyridinium chloride (CPC), a quaternary
68 surfactant with antimicrobial activity. For example, three commercial baby wipes listed in Table
69 1 contain PE and CPC. To date there is no information in the published scientific literature
70 regarding the skin permeation of PE in the presence of surfactant(s). Although several authors
71 have reported skin permeation of PE these studies used rodent skin. Therefore, the
72 experimental data cannot be extrapolated to humans (Lane, 2013).

73

74 Table 1. Commercial baby wipes containing PE and CPC

75

Ingredient list	Cussons baby wipes pure & gentle	Paseo baby wipes	Pigeon baby wipes
Water	*	*	*
PE	*	*	*

Ingredient			
PG	*	*	
Polyhexamethylene biguanide		*	
Benzalkonium chloride			*
CPC	*	*	*
Croduret		*	
PEG-40 hydrogenated castor oil	*		
PPG-5-ceteth-20	*		
Sodium lactate	*		
Sodium citrate			*
Citric acid		*	*
Sodium benzoate			*
Disodium EDTA	*		
PEG-45 palm kernel glycerides	*		
Sodium lauryl sarcosinate	*	*	
Olea europaea fruit oil	*		
Cyclodextrin	*		
Methylparaben	*		
l-menthol	*		
Ethylparaben	*		
Butylparaben	*		
Propylparaben	*		
Polydimethylsiloxane	*		
Chamomile extract		*	

Perfume	*
Prunus serrulata flower extract	*

76

Excised human skin is the most preferred tissue for *in vitro* skin permeation studies that are intended to estimate results in human (Dick and Scott, 1992; Schmook et al., 2001). The major challenges in using excised human skin are (1) difficulty in sourcing the skin samples and (2) intra- and inter-specimen variability (Dick and Scott, 1992; Schmook et al., 2001). The latter may also be problematic when interpreting the results from permeation studies (Oliveira et al., 2010; Zhang et al., 2019). Porcine skin has been considered as an appropriate surrogate for human skin, with reference to the close representation of the permeability properties of porcine tissue to human skin compared to other animal models (Dick and Scott, 1992; Schmook et al., 2001). However, porcine skin reportedly has a lower barrier function than human skin (Barbero and Frasch, 2009; Schmook et al., 2001; Yoshimatsu et al., 2017). In addition, ethical concerns over the use of animals in experiments is rising (Hopf et al., 2020; Pistollato et al., 2021). In light of these challenges, there has been a growing interest in exploring high throughput permeation models that are capable of generating reproducible data that may be extrapolated to man (Zhang et al., 2019).

The skin Parallel Artificial Membrane Permeation Assay (PAMPA) model has been developed for prediction of percutaneous absorption. The development of the skin PAMPA membrane and its use as percutaneous absorption model was first described by Ottaviani et al. (Ottaviani et al., 2006). The membrane composition was then refined by Sinkó et al. to achieve better representation of the intercellular stratum corneum (SC) region (Sinkó et al., 2012). The skin PAMPA membrane is essentially a filter substrate coated with synthetic lipids (certramides, free fatty acids, and cholesterol) arranged in a 96-well plate. Several studies have reported positive correlations for the permeability parameters of compounds using the skin PAMPA model and conventional *ex vivo* permeation studies (Balázs et al., 2016; Kovács et al., 2021; Sinkó et al., 2021; Zhang et al., 2019). However, these studies only investigated permeation of actives under infinite dose conditions.

It is important to assess skin permeation using clinically relevant doses, where the applied dose represents the actual exposure "in use". Clinically relevant doses demonstrated a finite dose profile or "plateau" for *in vitro* permeation studies (Franz, 1975, 1983; Lehman, 2014). This reflects the depletion of the applied dose of active over time (Franz, 1983; Lehman, 2014). Most recently Zhang and co-workers reported that permeation data for niacinamide in the skin

PAMPA model correlated well with porcine and human skin *in vitro* studies under finite dose conditions but not under infinite dose conditions (Zhang et al., 2019). Thus, the primary objective of the present work was to investigate the suitability of the skin PAMPA model for determination of dermal absorption of PE in the presence and absence of CPC in model formulations. To this end, *in vitro* studies were conducted in the skin PAMPA model and in porcine skin or human skin using Franz diffusion cells under finite dose conditions. A secondary objective was to elucidate the influence of CPC on the dermal absorption of PE.

2. Materials and Methods

2.1.   *Materials*

PE, CPC, and propylene glycol (PG) were purchased from Sigma-Aldrich Co., USA. HPLC grade solvents (acetonitrile, methanol, water, and trifluoroacetic acid) were purchased from Fischer Scientific, UK. Phosphate-buffered saline (PBS) tablets were purchased from Oxoid Ltd., UK. Porcine ear skin was obtained from a local abattoir. Excised abdominal human skin from a single donor was obtained from a tissue bank with informed consent and institutional ethical approval (Research Ethics Committee reference 07/H1306/98). Skin specimens were stored at -20 °C prior to use. The skin PAMPA sandwich plate, skin PAMPA collector plates, standard hydration solution, stirring disks and Gut-Box™ were supplied by Pion Inc. Billerica, USA.

2.2.   Methods

2.2.1. *Preparation of test solutions*

PE solutions were prepared in the presence or absence of CPC, as shown in Table 2. PG was chosen as a single solvent system, whereas water-PG 97:3 (WP) was chosen as a vehicle that better represents the cleansing liquid in baby wipes. In addition, selected formulations contained CPC either at 0.2% (w/w) or 1% (w/w). These concentrations represent the typical concentration of surfactants used in baby wipes (Cunningham et al., 2008; Cunningham et al., 2016; Sheehan, 2016). The full details of the formulations used in this study are listed in Table 2.

139 **Table 2.** PE solutions used in permeation studies.

Formulation	PE (% w/w)	CPC (% w/w)	Vehicle
PG1	1	-	PG
PG2	1	0.2	PG
PG3	1	1	PG
WP1	1	-	WP
WP2	1	0.2	WP
WP3	1	1	WP

140

141   The solutions were freshly prepared before the experiments. For PG2, PG3, WP2, and
142 WP3, mixing of the ingredients was performed with mild stirring to avoid foam formation by
143 CPC.

144

145   *2.2.2. Skin PAMPA studies*

146 *Influence of CPC on PAMPA membrane integrity*

147   In order to assess the compatibility of the skin PAMPA membrane with CPC, permeation
148 of CPC in the PAMPA model was monitored over 6 h. For these tests, CPC was dissolved in
149 PG at a final concentration of 0.2% w/w and 1% w/w. The permeation studies were performed
150 using the same setup as described in the previous section. Eight different doses were used,
151 covering finite and infinite conditions: 6, 10, 18, 30, 103, 515, 181, and 909 µg/cm^2. To achieve
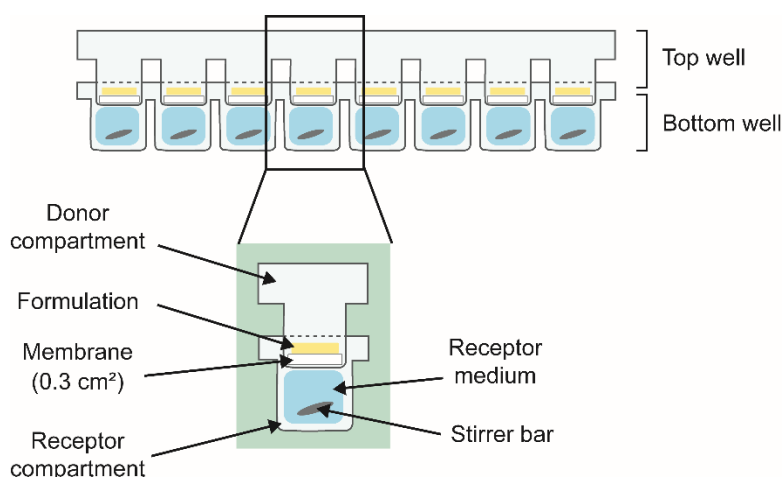152 these doses, CPC was applied at application volumes of 1, 3, 17, and 30 µL.

153

154 *Permeation of PE in the skin PAMPA membrane*

155   Permeability of PE was investigated in the skin PAMPA membrane using a modified
156 procedure reported by Luo et al. and Zhang et al. (Luo et al., 2016; Zhang et al., 2019). The
157 setup for this assay is illustrated in Fig. 1. The top side of the skin PAMPA sandwich plate was
158 set as the donor compartment, where the test solution was applied. For pilot studies, the dose
159 of PE applied was varied from 3 to 9 µL/cm^2 to assess the suitability of the experimental

160   design. Based on the results, an applied dose of 9 µL/cm^2 was selected for the remaining

161   experiments.

162   The collector plates were added with stirring disks and filled with 180 µl of degassed

163   PBS (pH 7.30 ± 0.10). Following application of the test solution, the two plates were assembled

164   and incubated in the Gut-Box™ stirring unit at 32 ± 1 °C for 1 h. The collector plate was

165   replaced with a new plate prefilled with receptor solution at 1, 2, 5, 10, 15, 20, 30, 45, and 60

166   min.

167

168

169   **Fig. 1.** Experimental setup for skin PAMPA studies.

170

171   *2.2.3. Franz cell studies*

172   *Skin permeation studies*

173   Permeation studies were conducted using full-thickness porcine skin and heat-

174   separated human epidermis in Franz-type diffusion cells. Porcine skin was prepared as

175   described by Cristofoli et al. (Cristofoli et al., 2022). The epidermis of human skin was isolated

176   by the heat separation technique as described by Oliveira et al. (Oliveira et al., 2012). The

177   receptor medium was similar to that of the skin PAMPA assay. The permeation study was

178   conducted for 24 h at 32 ± 1 °C. Temperature was controlled by using a water bath (Sub Aqua

179   26 Plus, Grant™, Fischer Scientific, UK). Prior to the experiments, the skin impedance was

180   measured to assess the skin barrier integrity, as described previously (Oliveira et al., 2012).

181   The skin was then allowed to equilibrate to reach 32 ± 1 °C. The temperature of the skin was

182   measured using a digital thermometer (TM-22 Digitron, RS Components, UK). 10 µL of the

183   test solution, representing a finite dose of 10 µL/cm^2 (corresponding to 100 µg PE per cm^2),

was applied evenly on the donor compartment. 100 µL of sample was withdrawn at 0, 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 h. After sample collection, volume replacement was carried out by adding 100 µL of receptor solution (maintained at 32 ± 1 °C) to the receptor compartment. All samples were analyzed for PE and CPC content using a validated HPLC method (section 2.2.4).

Mass balance studies

At the end of the permeation study, the receptor medium was immediately removed. Each donor compartment was washed with 1 mL of methanol-water (85:15) mixture and the washing solution was transferred to a 2 mL microcentrifuge tube. The cap of the tube was sealed with Parafilm® to prevent evaporation. The washing procedure was performed 3 times. The Franz diffusion cells were then disassembled, and each skin membrane was transferred to a 2 mL microcentrifuge tube. 1 mL of methanol-water (85:15) was added to the microcentrifuge tube and the cap of the tube was sealed with Parafilm® to prevent evaporation. Extraction of PE and CPC was carried out by placing the tubes in a bench shaker (VWR International, USA) at 800 rpm for 12 h. All samples were centrifuged using an Eppendorf® microcentrifuge 5415R (Eppendorf, UK) at 13,200 rpm for 20 min. Subsequently, the samples were analyzed for PE and CPC content using a validated HPLC method. The percentage of the two ingredients recovered from the washing solution, extraction solution, and the receptor solution was determined.

2.2.4. Quantification of PE and CPC

The determination of PE and CPC content was performed using HPLC with a diode-array detector (Agilent Technologies 1200 Series). Data acquisition was performed using Chemstation software (Agilent Technologies, USA). A Kinetex® XB-C$_{18}$ column (250 x 4.60 mm, Phenomenex, UK) was used for the analysis. All the samples and standard solutions were stored in amber HPLC vials during the assay. Optimized HPLC conditions were achieved using a gradient elution system. The elution conditions are shown in Table 3. The flowrate, injection volume, and column temperature were set as 1 ml/min, 20 µL, and 40 °C, respectively. The detection wavelengths were 258 nm (for CPC) and 270 nm (For PE).

217 **Table 3.** Elution conditions for PE and CPC.

Time	Volume ratio of mobile phase (%)	
	acetonitrile	0.1% TFA
0 – 4	28	72
5 – 12	80	20
13 – 16	28	72

218

219   Validation of the analytical method was carried out in accordance with the guidelines
220 outlined by the International Conference on Harmonization (ICH) regarding quantitative
221 assays for determination of the content of active compounds (ICH Harmonised Tripartite,
222 2005). The following parameters were assessed: linearity, range, accuracy, intra-day and
223 inter-day precision, limit of detection (LOD), limit of quantification (LOQ), and robustness. In
224 addition, a system suitability test was performed to ensure that consistent chromatographic
225 behavior was achieved for each analytical procedure (US Food and Drug Administration,
226 1994).

227

228   *2.2.5. Statistical analysis*

229   Statistical analysis was performed using Origin 8.0 (OriginLab Corp., Northampton, MA).
230 Data were assessed for normal distribution and homogeneity using the Shapiro-Wilk Test and
231 Levene's Test, respectively. Sets of data that met the assumptions of normality and
232 homogeneity were analyzed using one-way ANOVA followed by Tukey's post hoc test. The
233 Kruskal–Wallis H Test was used for datasets that did not follow the assumptions of normality
234 and/or homogeneity of variance. The level of significance was set at a p-value lower than 0.05
235 ($p<0.05$). The correlation assessment was performed using the Pearson correlation coefficient
236 (r^2).

237

238 **3. Results**

239   *3.1.   HPLC method validation*

240   The results of method validation are shown in Table 4. The retention times of PE and CPC
241 using the validated method were 6.01 and 9.40 min, respectively. The linearity for PE and
242 CPC was obtained for a concentration range of 1 to 100 µg/mL. The calibration curve from

243 this range of concentrations exhibited a linear regression line. The LOD and LOQ for PE were
244 calculated as 0.49 µg/mL and 1.49 µg/mL, respectively. The LOD and LOQ for CPC were 0.51
245 µg/mL and 1.55 µg/mL, respectively.

246

247 **Table 4.** Summary of validation parameters for PE and CPC analysis using HPLC

Parameters	PE	CPC
Linearity range (µg/mL)	1 – 100	1 – 100
r^2	>0.99	>0.99
LOD (µg/mL)	0.49	0.51
LOQ (µg/mL)	1.49	1.55
Accuracy		
Recovery (%)	100.07 ± 0.34	100.42 ± 0.59
RSD (%)	0.03 – 0.65	0.04 – 0.89
Precision		
Intra-day (%RSD)	0.17	0.42
Inter-day (%RSD)	0.25	1.14
Robustness (r^2)	>0.99	>0.99
System suitability		
peak area (%RSD)	0.16	0.59
RT (%RSD)	0.10	0.10
Peak symmetry (%RSD)	0.99	0.18

248 RSD = relative standard deviation

249

250   The accuracy, which represents the closeness between the experimentally measured
251 values and the true values, was determined by measuring the response of blank samples
252 spiked with PE and CPC at 5, 50, and 100 µg/mL, representing the low, medium, and high
253 concentrations. The average recovery value for PE was 100.07 ± 0.34 %, with a relative
254 standard deviation (RSD) value <1%. As for CPC, the recovery value was 100.42 ± 0.59 %,
255 with an RSD value <1.00%. The precision of the method was assessed based on intra-day
256 and inter-day precision assessments. The RSD of the measured concentrations was used to
257 express the inter-assay variability. Acceptable precision was achieved with corresponding
258 RSD values of <1.00% and <2.00% for intra-day and inter-day assessment, respectively.
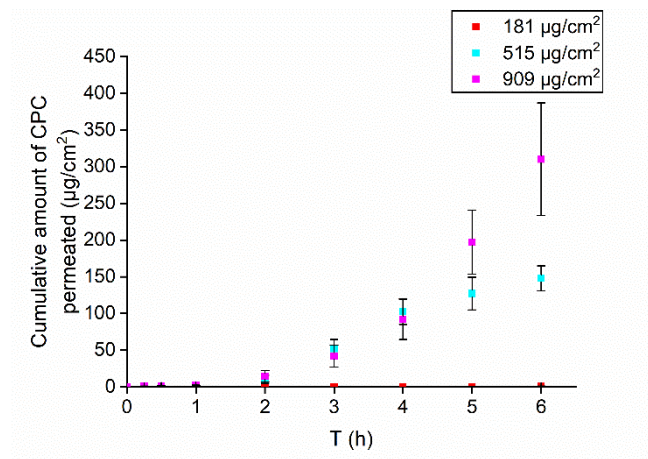
259   The robustness of an analytical method is investigated to ensure that the capability of
260   the HPLC system remains unaffected with minor variations in chromatographic conditions.
261   The robustness test was performed on standard solutions containing PE and CPC within a
262   concentration range of 1-100 µg/mL. Despite the minor variations (2% change) in injection
263   volume, flow rate, column temperature, mobile phase composition, and detection wavelength,
264   r^2 values remained ≥0.99. A system suitability test was performed by determining the RSD of
265   the injection repeatability, as described in the Reviewer Guidance on Validation of
266   Chromatographic Methods (US Food and Drug Administration, 1994). Six injections of
267   solutions with PE and CPC at a concentration of 50 µg/mL were determined for the peak area,
268   peak symmetry, and retention time. The RSD values for these parameters were ≤ 1.00% for
269   PE and CPC.

270

271   *3.2.   Influence of CPC on PAMPA membrane integrity*

272   Fig. 2 compares the amount of CPC permeated from different applied doses. When the
273   applied dose was ≤103 µg/cm^2, permeation of CPC was not detected over 6 h. For the 181
274   µg/cm^2 dose, permeation of CPC was evident from 5 h. The amount of CPC permeated for
275   this dose was 0.06 ± 0.07 µg/cm^2 at 5 h. At the higher dose of CPC (≥ 515 µg/cm^2), the
276   permeation of CPC was observed at earlier timepoints.

277

279   **Fig. 2.** The amount of CPC permeated through the skin PAMPA membrane under various
280   applied doses (mean ± SD, n = 6).
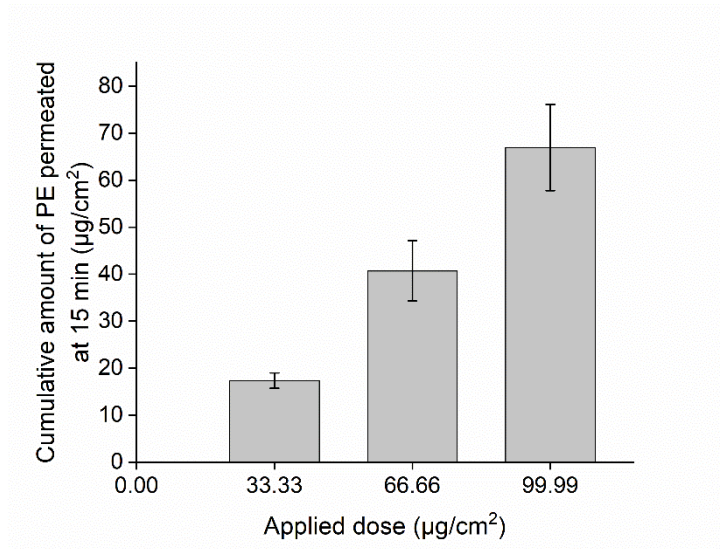
281

282

283   *3.3.   Permeation of PE in the skin PAMPA membrane*

284   Cumulative amounts of PE permeated through the skin PAMPA membrane from different

285   applied doses are shown in Fig. 3. Three PE doses were used in this set of experiments – 1,

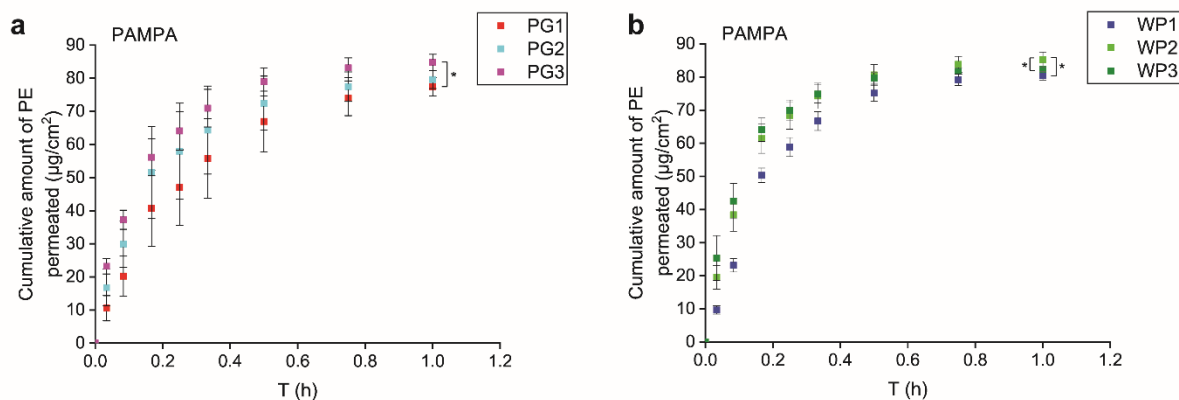286   2, and 3 µL. These doses corresponded to 3, 6, and 9 µL/cm^2.

287

288

289   **Fig. 3.** Cumulative amounts of PE permeated through the skin PAMPA membrane from the

290   different applied doses (mean ± SD, n = 6).

291

292   The permeation profiles of PE in the skin PAMPA model are shown in Fig. 4.

293   Permeation of PE was evident from 1 min. The highest permeation of PE was observed for

294   the solution containing CPC 0.2% with water-PG as the vehicle. The cumulative amount of PE

295   that permeated from this preparation was 85.30 ± 2.19 µg/cm^2. For preparations with neat PG

296   as the solvent, a significantly higher amount ($p<0.05$) of PE permeated from the vehicle with

297   CPC 1%, compared with the PE solution with no CPC.

298

299

Fig. 4. Permeation profiles of PE from (a) PG and (b) Water-PG in the skin PAMPA membrane (mean ± SD, n = 6). * Indicates statistical significance (p<0.05) compared to the other preparations.

Interestingly, for water-PG preparations, the cumulative amount of PE permeated was significantly higher (p<0.05) in the presence of CPC 0.2% compared with all other PE solutions. These findings suggested that the increase in PE permeation from water-PG was not necessarily proportional to the concentration of CPC.

3.4.   Franz cell studies

3.4.1. Permeation of PE in porcine skin and human skin

The permeation profiles of PE in porcine skin and human skin are shown in Fig. 5. We observed a trend which was consistent with the skin PAMPA data, where the highest permeation of PE was observed for water-PG with 0.2% of CPC. The cumulative amounts of PE that permeated in porcine skin and human skin from this preparation at 24 h were 96.98 ± 1.83 $\mu g/cm^2$ and 92.46 ± 9.34 $\mu g/cm^2$, respectively. These values corresponded to more than 97% of the applied dose. The lowest permeation of PE was observed for PG without CPC, both in porcine skin and human skin (69.09 ± 7.54 $\mu g/cm^2$ and 73.99 ± 9.70 $\mu g/cm^2$, respectively). These results are consistent with the skin PAMPA data.

Fig. 5. Permeation profiles of PE from various preparations in porcine skin and human skin. (a) PG vehicle applied to porcine skin; (b) Water-PG vehicle applied to porcine skin; (c) PG vehicle applied to human skin; (d) Water-PG vehicle applied to human skin. Mean ± SD, n = 5-6. * Indicates statistical significance (p<0.05) compared to the other preparations.

Regarding the effect of CPC on the permeation of PE, the results from porcine skin and human skin studies were consistent with the trends observed in the skin PAMPA studies. For PG, a significantly higher cumulative amount of PE permeation was evident in the presence of CPC 1% compared to preparations with no CPC (p<0.05). This was observed both in porcine skin and human skin. For water-PG, the cumulative amount of PE permeated was significantly higher (p<0.05) in the presence of CPC 0.2% compared with all other PE vehicles.

3.4.2. Mass balance studies for PE and CPC in porcine skin and human skin

The results of the mass balance studies for PE are shown in Table 5. The amount of PE that remained on the surface of porcine skin was a maximum of 2.01% of the applied dose for the PG vehicle. In contrast, PE was not detected in the washing solution for all water-PG preparations. For human skin data, the amount of PE that remained on the skin surface for

338 the PG vehicle was a maximum of 27.08% of the applied dose. Similar to the findings from
339 porcine skin, PE was not detected in the washing solution for all water-PG preparations.
340 Regarding the skin retention of PE, the percentages of the active recovered from the porcine
341 skin for PG vehicle were 8.19-9.37% of the applied dose. For the water-PG preparations, 8.61-
342 13.25% of PE was retained in porcine skin. The percentages of PE retained in human skin
343 were lower than in porcine skin. At the end of the permeation studies, 3.08–3.57% of PE from
344 the PG preparations were recovered in human skin. For the water-PG preparations, the
345 percentage of PE extracted from human skin was 1.10–1.27%.

346

347 **Table 5.** Mass balance results for PE in porcine skin and human skin (mean ± SD, n = 5).

Preparation	PE recovered (% of applied dose)			
	Receptor fluid	Skin surface	Skin	Total recovery
Porcine skin				
PG1	81.32 ± 6.03	1.47 ± 1.09	9.20 ± 2.44	92.00 ± 4.97
PG2	85.23 ± 5.22	2.01 ± 0.57	9.37 ± 0.98	96.61 ± 4.96
PG3	92.51 ± 3.39	ND	8.19 ± 0.82	100.70 ± 2.83
WP1	79.00 ± 4.04	ND	13.25 ± 2.99	92.25 ± 1.77
WP2	95.81 ± 9.45	ND	8.61 ± 0.94	104.49 ± 8.56
WP3	88.29 ± 10.28	ND	10.06 ± 2.86	98.36 ± 13.14
Human skin				
PG1	71. 40 ± 12.62	27.08 ± 8.17	3.57 ± 0.12	102.06 ± 4.65
PG2	83.63 ± 2.48	11.17 ± 3.09	3.41 ± 0.28	98.23 ± 0.64
PG3	97.34 ± 3.59	4.24 ± 3.82	3.08 ± 0.55	104.67 ± 0.73
WP1	89.07 ± 2.75	ND	1.11 ± 0.17	90.19 ± 2.92
WP2	97.79 ± 6.66	ND	1.10 ± 0.16	98.89 ± 6.68
WP3	87.90 ± 7.05	ND	1.27 ± 0.12	89.18 ± 7.02

348 ND = not detected

349

350 In contrast to PE, permeation of CPC was not detected in porcine skin and human skin
351 studies for all preparations, as was observed in the skin PAMPA model. Mass balance results
352 using porcine skin (Table 6) showed that the amount of CPC that remained on the skin surface
353 varied between 61.31 to 95.28% of the applied dose, whereas the amount of CPC retained in
354 the skin was 10.28-38.72% of the applied dose. In contrast, mass balance studies using

human skin confirmed that more than 80% of the applied dose of CPC was recovered from the skin surface for all vehicles (Table 6).

Table 6. Mass balance results for CPC in porcine skin and human skin (mean ± SD, n = 5).

Preparation	CPC recovered (% of applied dose)			
	Receptor fluid	Skin surface	Skin	Total recovery
Porcine skin				
PG2	ND	83.21 ± 8.44	21.12 ± 7.68	104.33 ± 1.19
PG3	ND	95.28 ± 4.29	10.28 ± 4.45	105.57 ± 0.16
WP2	ND	61.31 ± 0.87	38.72 ± 1.99	100.04 ± 2.16
WP3	ND	82.71 ± 3.25	13.00 ± 2.52	95.71 ± 2.28
Human skin				
PG2	ND	85.06 ± 8.07	8.81 ± 1.13	93.88 ± 7.01
PG3	ND	91.40 ± 14.03	3.33 ± 3.00	94.74 ± 11.48
WP2	ND	90.04 ± 9.87	7.72 ± 2.33	97.77 ± 7.84
WP3	ND	89. 92 ± 7.25	1.71 ± 91.64	91.64 ± 8.13

ND = not detected

3.5.   Comparative evaluation of the permeation data

Cumulative amounts of PE that permeated the skin PAMPA membranes, porcine skin, and human skin are shown in Fig. 6. Statistical differences in the amount of PE that permeated from various preparations were assessed within the same vehicles rather than all vehicles. This approach should provide a clearer insight into possible active-vehicle-membrane interactions.

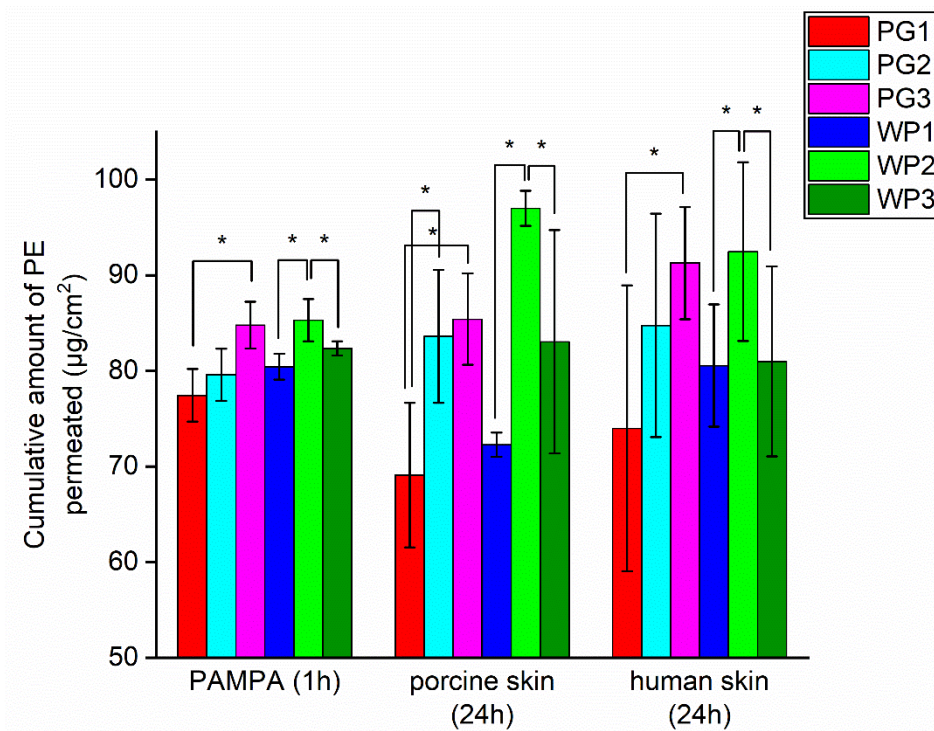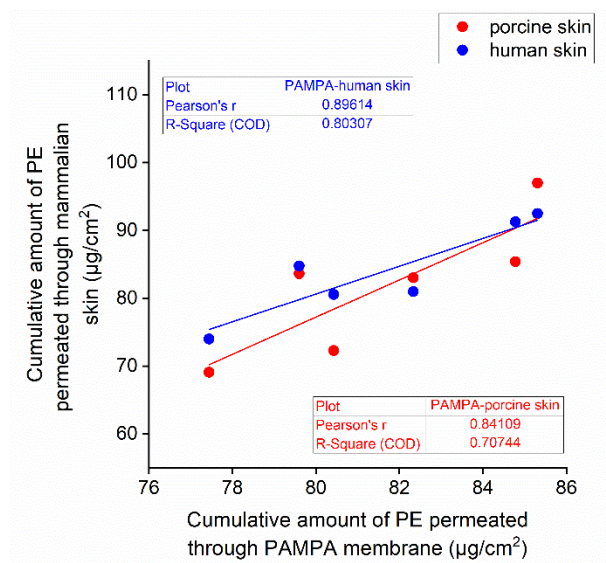**Fig. 6.** Cumulative amounts of PE that permeated the membranes (mean ± SD, n = 5-6). * Indicates statistical significance ($p < 0.05$) compared to the other preparations within the same vehicle and within the same membrane model.

Correlations for the cumulative amount of PE permeated between the skin PAMPA data and the mammalian skin data are shown in Fig. 7. When comparing the skin PAMPA data and the porcine skin data for cumulative amount of PE permeated, the r^2 value was 0.84. This value is consistent with previously reported data by Zhang et al. (Zhang et al., 2019). For the correlation between the skin PAMPA and human skin data for cumulative amount of PE permeated the corresponding r^2 value was 0.89.

Fig. 7. Correlation between cumulative amounts of PE that permeated through skin PAMPA and mammalian skin. Each point represents the mean value (n = 5-6).

4. Discussion

Synthetic membranes mimicking the stratum corneum (SC) have been investigated extensively as surrogate models for *in vitro* skin permeation studies (Čuříková et al., 2017; de Jager et al., 2006b; Groen et al., 2011; Sinkó et al., 2012; Uchida et al., 2015). The membranes are composed of a porous substrate coated with synthetic SC lipids, which serve as the rate-determining barrier for skin permeation. In the present work, preliminary assessment of skin PAMPA compatibility with CPC was conducted prior to permeation studies for CPC-containing preparations. It is important to evaluate the compatibility of synthetic membranes with formulations, particularly when the formulation contains penetration enhancers (Köllmer et al., 2019; Kovács et al., 2021). Surfactants have been reported to have the potential to disrupt the organization of the SC lipids, although the mechanism underlying this is not fully understood (Gloor et al., 2004; Jiang et al., 2003). The interaction between surfactants and SC lipids is complex, depending on the duration of exposure, the nature, and the concentration of the surfactants (De la Maza et al., 1997; Imokawa, 2004; Rhein et al., 1986). Köllmer et al. recently investigated the compatibility of the skin PAMPA membrane with different surfactants (Köllmer et al., 2019). In their study, the skin PAMPA membrane was incubated with blank formulations which contained different surfactants (1% Brij™ S2 + 0.15% Brij™ S721 and 1% Brij™ S2 + 1% Brij™ S721). After 4 h of pre-incubation, a permeation test was performed for methylparaben, ethylparaben, and propylparaben for 4 h. The results indicated that pre-incubation with these surfactants did not increase the permeation of the parabens. In addition,

407   the rank order for permeation remained unchanged (methylparaben > ethylparaben >
408   propylparaben). The authors suggested that the surfactants used in the study did not alter the
409   permeability of the skin PAMPA membrane towards the parabens (Köllmer et al., 2019).

410   In the present study, permeation of CPC was not detected over 6 h when the application
411   dose was ≤103 µg/cm^2. The concentration of CPC used in the permeation studies for PE-
412   containing vehicles using the skin PAMPA model was not more than 30 µg/cm^2. In addition,
413   the duration of the experiment was only 1 h. Permeation of CPC from all vehicles was not
414   detected in the receptor solution over 1 h. More importantly, a comparison with the Franz cell
415   studies confirmed that the permeation data from the skin PAMPA model showed a good
416   correlation with the porcine skin and human skin data (Fig. 7). Therefore, no evident
417   incompatibility was observed between the skin PAMPA membrane with the vehicles used in
418   this study under previously mentioned conditions.

419   Possible membrane-excipient interactions such as membrane blockage by the
420   formulation, disorganization or solubilization of the membrane lipids have also been
421   recognized (Köllmer et al., 2019). Two recent studies reported the effects of several excipients
422   on the permeation of actives in the skin PAMPA membrane (Kovács et al., 2021; Zhang et al.,
423   2019). Zhang et al. reported that niacinamide permeated the skin PAMPA membrane from
424   PEG 400 and PEG 600, whereas it did not permeate in porcine and human skin studies (Zhang
425   et al., 2019). These authors suggested that this might reflect the interaction between those
426   solvents and the skin PAMPA membrane.

427   Finite dose experiments using the skin PAMPA model reported by Luo et al. and Zhang et
428   al. included application doses as low as 3 µL/cm^2 (Luo et al., 2016; Zhang et al., 2019). These
429   studies reported comparable results for permeation profiles of the actives (ibuprofen,
430   niacinamide) between the skin PAMPA model and porcine skin as well as human skin. In the
431   current study, permeation of PE at the 3 µL/cm^2 dose in the skin PAMPA membrane had
432   reached a plateau at 45 min. As frequent sampling within such a short period was impractical
433   and permeation data showed high variability, a dose of 9 µL/cm^2 was selected as the final
434   dose for PE. As shown in Fig. 3, it is evident that by using the skin PAMPA model, a 1 h study
435   time was sufficient for PE to demonstrate comparable finite dose kinetics to those observed
436   in porcine skin and human skin in 24 h. Even with relatively high permeation of PE over a short
437   period, the skin PAMPA model was also capable of discriminating between different
438   preparations. This was reflected by the statistical differences between the formulations for the
439   cumulative amounts of PE permeated, as was observed in the porcine skin and human skin
440   studies (Fig. 6). Such high-throughput prediction in a time efficient manner is expected to be
441   particularly useful for screening numerous topical formulations (Luo et al., 2016; Neupane et

442 al., 2020; Sinkó et al., 2012; Zhang et al., 2019). Consistent with previously reported studies,

443 the time required for the active to permeate the artificial membrane was shorter compared with

444 porcine skin or human skin (Čuříková et al., 2017; Kovács et al., 2021; Luo et al., 2016; Zhang

445 et al., 2019). In addition, the variability in the skin PAMPA data, represented by the standard

446 deviation, was lower than in the Franz cell studies with mammalian tissue.

447 As shown in Fig. 4, the highest permeation of PE in the skin PAMPA model was observed

448 for water-PG with 0.2% of CPC. This finding was consistent with observations in the Franz cell

449 studies using porcine skin and human skin (Fig. 5). This concentration of CPC is typically used

450 in commercial baby wipe formulations, which comprise more than 90% of water and a low

451 concentration of surfactant (< 0.5%). On the other hand, the three permeation models

452 confirmed that the lowest permeation of PE was observed for neat PG without CPC. These

453 results also correspond with the findings from a study by Luo et al., where the highest amount

454 of active (ibuprofen) that permeated the skin PAMPA model was observed for the formulation

455 which delivered the highest amount of active in human skin under finite dose conditions (Luo

456 et al., 2016).

457 While permeation of CPC was not detected in the skin PAMPA model and Franz cell

458 studies, the presence of CPC enhanced the permeation of PE significantly (p <0.05) compared

459 with preparations without CPC. Interestingly, better enhancement for permeation of PE was

460 observed when the CPC concentration was 1% for PG and 0.2% for water-PG. The results of

461 permeation studies using human skin showed that the cumulative amounts of PE permeated

462 from PG in the presence of CPC 0.2% and 1% were 14% and 23% higher, respectively,

463 compared with PE alone. For water-PG, the presence of CPC at 0.2% and 1% resulted in

464 enhanced PE permeation by 14% and 0.5%, respectively, compared to PE alone. This might

465 indicate that CPC influences the permeation of PE from PG and water-PG by different

466 mechanisms. In general, it has been thought that the alkyl chains of surfactants may

467 intercalate with the hydrophobic regions of the SC lipids (Gloor et al., 2004; Jiang et al., 2003).

468 The ability of surfactants to increase the permeation of various actives is well documented

469 (Merwe and Riviere, 2005; Riviere et al., 2010; Shokri et al., 2001). Clearly, it should be noted

470 that surfactants are able to form micelles, enhance the solubility of permeant and thus reduce

471 its thermodynamic activity (Lane, 2013). The critical micellar concentration of CPC was

472 reportedly 1 mM (approximately 0.34 mg/mL) in water at 25 °C (Wang et al., 1999). In the

473 present work, the concentrations of CPC used were 2 mg/mL and 10 mg/mL. For WP, the

474 vehicle with 97% water, CPC was present above the CMC which may explain the results

475 observed.

476   Regarding the correlation between the skin PAMPA data and mammalian skin data, the r^2
477   values for skin PAMPA-porcine skin and skin PAMPA-human skin were 0.84 and 0.89,
478   respectively (Fig. 7). Previously, Zhang et al. reported that the correlation for the permeation
479   of niacinamide between the skin PAMPA studies and porcine skin studies was 0.88 under
480   finite dose conditions. However, the corresponding r^2 value for human skin was lower
481   compared to porcine skin, namely 0.71.

482   It is important to note that artificial membranes are not intended to provide an estimation
483   of the amount of actives that permeate the human skin since these membranes do not fully
484   represent the biological complexity of human skin (Čuříková et al., 2017; Neupane et al.,
485   2020). Such models can be used as an initial screening tool before performing permeation
486   studies using human skin (Neupane et al., 2020; Zhang et al., 2019). Recently, a
487   comprehensive overview of the findings from permeation studies using synthetic membranes
488   was reported by Neupane et al. (Neupane et al., 2020). The main advantages of artificial
489   membranes are less variability in the thickness and composition compared with biological
490   tissues, and ease in storage (Neupane et al., 2020). A number of studies have demonstrated
491   the ability of artificial membranes to correlate with *ex vivo* permeation studies using porcine
492   skin or human skin (Balázs et al., 2016; Čuříková et al., 2017; de Jager et al., 2006a; Groen
493   et al., 2011; Sinkó et al., 2021; Sinkó et al., 2012; Uchida et al., 2015). However, most of the
494   studies were conducted under infinite dose conditions. On the other hand, finite dose
495   experiments are more relevant for prediction of dermal absorption with reference to the
496   amount of formulation applied in the real clinical setting. In finite dose experiments, the
497   implications of evaporation and residence time of the solvents, as well as the solubility of the
498   active in the remaining formulation should be taken into consideration (Lane, 2013).
499   Correlations observed in comparative studies using infinite dose conditions should not be
500   directly assumed to hold true for finite dose situations. As mentioned earlier, Zhang and co-
501   workers reported that the skin PAMPA data showed correlations with the porcine skin and
502   human skin data, for the amount of active permeated, under finite dose conditions but not
503   under infinite dose conditions (Zhang et al., 2019).

504   The importance of lipid composition for permeability of artificial membrane models has
505   been studied extensively. Groen et al. described the importance of lipid compositions
506   (cholesterol free fatty acid, and ceramide) for the permeability of skin lipid models (Groen et
507   al., 2011). In this study, the authors investigated the permeability of a stratum corneum
508   substitute (SCS) with various ratios of SC lipids. This was done by assessing the permeation
509   of benzoic acid under infinite dose conditions. It was found that the permeability of SCS to
510   benzoic acid was higher when the SCS membrane contained a high content of free fatty acids.
511   In contrast, permeation of benzoic acid was lower for SCS with high cholesterol or ceramide

content compared to SCS with equimolar composition of cholesterol free fatty acid, and ceramide.

A synthetic ceramide named certramide is used in the skin PAMPA membrane (Sinkó et al., 2012). Certramides are long-chain tartaric acid diamide derivatives. In the skin PAMPA membrane, the length of the alkyl chains in the certramides are 8 and 18 (C8-C18). The ceramides are combined with stearic acid and cholesterol (Sinkó et al., 2012). The ratio of the membrane components in the skin PAMPA model was chosen to represent the intercellular lipid matrix of the SC (Sinkó et al., 2012). The membrane demonstrated positive correlation with human SC for permeation of various compounds (Sinkó et al., 2012). The effects of molecular structure of ceramides on the permeability of a SC lipid model membrane have previously been reported (Čuříková et al., 2017; Školová et al., 2013). Školová and co-workers reported a decrease in permeability of the studied synthetic membrane for two drugs (theophylline and indomethacin) when natural long-chain ceramides (24-acyl chains, Cer24) were incorporated into the membrane compared to a control model without Cer24. Increased permeation for both model compounds was observed when short-chain ceramides (4-6 acyl chains) were used rather than the long-chain ceramides. Čuříková et al. investigated the potential of simplified SC membranes to predict the effects of permeation enhancers on the permeation of actives (Čuříková et al., 2017). This membrane model is composed of stearic acid, cholesterol, cholesterol sulfate, and ceramides. The ceramides used in the study were simplified, consisting of N-2-hydroxystearoyl phytosphingosine (CER[AP]) and/or N-stearoyl phytosphingosine (CER[NP]), rather than more complex ceramides mixtures as developed earlier by de Jager et al. (de Jager et al., 2006b). The optimized membrane, containing an equimolar mixture of CER[AP] and CER[NP], was used to examine the permeation of two active models, indomethacin and theophylline from formulations with different permeation enhancers. For both actives, the enhancement of permeation by N-dodecyl azepan-2-one (Azone) and (S)-N-acetylproline dodecyl ester (L-Pro2) observed in the membrane model was consistent with porcine skin studies conducted under infinite dose conditions.

5. Conclusions

This work confirms the potential of the PAMPA model as a tool to discriminate between different vehicles for permeation of actives. In addition, a positive correlation between the skin PAMPA model and the Franz cell studies using porcine skin and human skin was observed for the cumulative amount of PE permeated. Both the skin PAMPA model and Franz cell studies using mammalian skin showed that the highest permeation of PE was observed for CPC 0.2% in water-PG, which is the typical amount of CPC used in commercial baby wipe

547 formulations. Considering that skin cleansing using baby wipes is carried out regularly, the
548 findings of the current study may have implications for the safety evaluation of such products.
549 The three permeation models showed that the presence of CPC enhanced the permeation of
550 PE significantly ($p<0.05$) compared with preparations without CPC. A greater enhancement
551 for permeation of PE was observed when the CPC concentration was 1% for PG and 0.2% for
552 water-PG. No evident incompatibilities between the skin PAMPA membrane and the examined
553 preparations were observed. Based on the present study and previous PAMPA publications it
554 is worth noting that the optimum experimental conditions for this model may vary for different
555 compounds. Future work will expand the range of actives to be studied in the model and further
556 investigate the application of the PAMPA model in high-throughput screening studies for
557 development of dermal formulations.

558

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Conflict of Interest

The authors declare no conflict of interest.

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699

Table 1. Commercial baby wipes containing PE and CPC

Ingredient list	Cussons baby wipes pure & gentle	Paseo baby wipes	Pigeon baby wipes
Water	*	*	*
PE	*	*	*
PG	*	*	
Polyhexamethylene biguanide		*	
Benzalkonium chloride			*
CPC	*	*	*
Croduret		*	
PEG-40 hydrogenated castor oil	*		
PPG-5-ceteth-20	*		
Sodium lactate	*		
Sodium citrate			*
Citric acid		*	*
Sodium benzoate			*
Disodium EDTA	*		
PEG-45 palm kernel glycerides	*		
Sodium lauryl sarcosinate	*	*	
Olea europaea fruit oil	*		
Cyclodextrin	*		
Methylparaben	*		

l-menthol	*
Ethylparaben	*
Butylparaben	*
Propylparaben	*
Polydimethylsiloxane	*
Chamomile extract	*
Perfume	*
Prunus serrulata flower extract	*

Table ~~1~~2. PE solutions used in permeation studies.

Formulation	PE (% w/w)	CPC (% w/w)	Vehicle
PG1	1	-	PG
PG2	1	0.2	PG
PG3	1	1	PG
WP1	1	-	WP
WP2	1	0.2	WP
WP3	1	1	WP

Table 23. Elution conditions for PE and CPC.

Time	Volume ratio of mobile phase (%)	
	acetonitrile	0.1% TFA
0 – 4	28	72
5 – 9	80	20
10 – 12	28	30
13 – 16	28	72

Table 34. Summary of validation parameters for PE and CPC analysis using HPLC

Parameters	PE	CPC
Linearity range (µg/mL)	1 – 100	1 – 100
r^2	>0.99	>0.99
LOD (µg/mL)	0.49	0.51
LOQ (µg/mL)	1.49	1.55
Accuracy		
Recovery (%)	100.07 ± 0.34	100.42 ± 0.59
RSD (%)	0.03 – 0.65	0.04 – 0.89
Precision		
Intra-day (%RSD)	0.17	0.42
Inter-day (%RSD)	0.25	1.14
Robustness (r^2)	>0.99	>0.99
System suitability		
peak area (%RSD)	0.16	0.59
RT (%RSD)	0.10	0.10
Peak symmetry (%RSD)	0.99	0.18

RSD = relative standard deviation

Table 45. Mass balance results for PE in porcine skin and human skin (mean ± SD, n = 5).

Preparation	PE recovered (% of applied dose)			
	Receptor fluid	Skin surface	Skin	Total recovery
Porcine skin				
PG1	81.32 ± 6.03	1.47 ± 1.09	9.20 ± 2.44	92.00 ± 4.97
PG2	85.23 ± 5.22	2.01 ± 0.57	9.37 ± 0.98	96.61 ± 4.96
PG3	92.51 ± 3.39	ND	8.19 ± 0.82	100.70 ± 2.83
WP1	79.00 ± 4.04	ND	13.25 ± 2.99	92.25 ± 1.77
WP2	95.81 ± 9.45	ND	8.61 ± 0.94	104.49 ± 8.56
WP3	88.29 ± 10.28	ND	10.06 ± 2.86	98.36 ± 13.14
Human skin				
PG1	71. 40 ± 12.62	27.08 ± 8.17	3.57 ± 0.12	102.06 ± 4.65
PG2	83.63 ± 2.48	11.17 ± 3.09	3.41 ± 0.28	98.23 ± 0.64
PG3	97.34 ± 3.59	4.24 ± 3.82	3.08 ± 0.55	104.67 ± 0.73
WP1	89.07 ± 2.75	ND	1.11 ± 0.17	90.19 ± 2.92
WP2	97.79 ± 6.66	ND	1.10 ± 0.16	98.89 ± 6.68
WP3	87.90 ± 7.05	ND	1.27 ± 0.12	89.18 ± 7.02

ND = not detected

Table 5̶6. Mass balance results for CPC in porcine skin and human skin (mean ± SD, n = 5).

Preparation	Receptor fluid	CPC recovered (% of applied dose)		
		Skin surface	Skin	Total recovery
Porcine skin				
PG2	ND	83.21 ± 8.44	21.12 ± 7.68	104.33 ± 1.19
PG3	ND	95.28 ± 4.29	10.28 ± 4.45	105.57 ± 0.16
WP2	ND	61.31 ± 0.87	38.72 ± 1.99	100.04 ± 2.16
WP3	ND	82.71 ± 3.25	13.00 ± 2.52	95.71 ± 2.28
Human skin				
PG2	ND	85.06 ± 8.07	8.81 ± 1.13	93.88 ± 7.01
PG3	ND	91.40 ± 14.03	3.33 ± 3.00	94.74 ± 11.48
WP2	ND	90.04 ± 9.87	7.72 ± 2.33	97.77 ± 7.84
WP3	ND	89. 92 ± 7.25	1.71 ± 91.64	91.64 ± 8.13

ND = not detected

Fig. 1

Top well

Bottom well

Donor compartment

Formulation

Membrane (0.3 cm²)

Receptor compartment

Receptor medium

Stirrer bar

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Plot	PAMPA-human skin
Pearson's r	0.89614
R-Square (COD)	0.80307

Plot	PAMPA-porcine skin
Pearson's r	0.84109
R-Square (COD)	0.70744

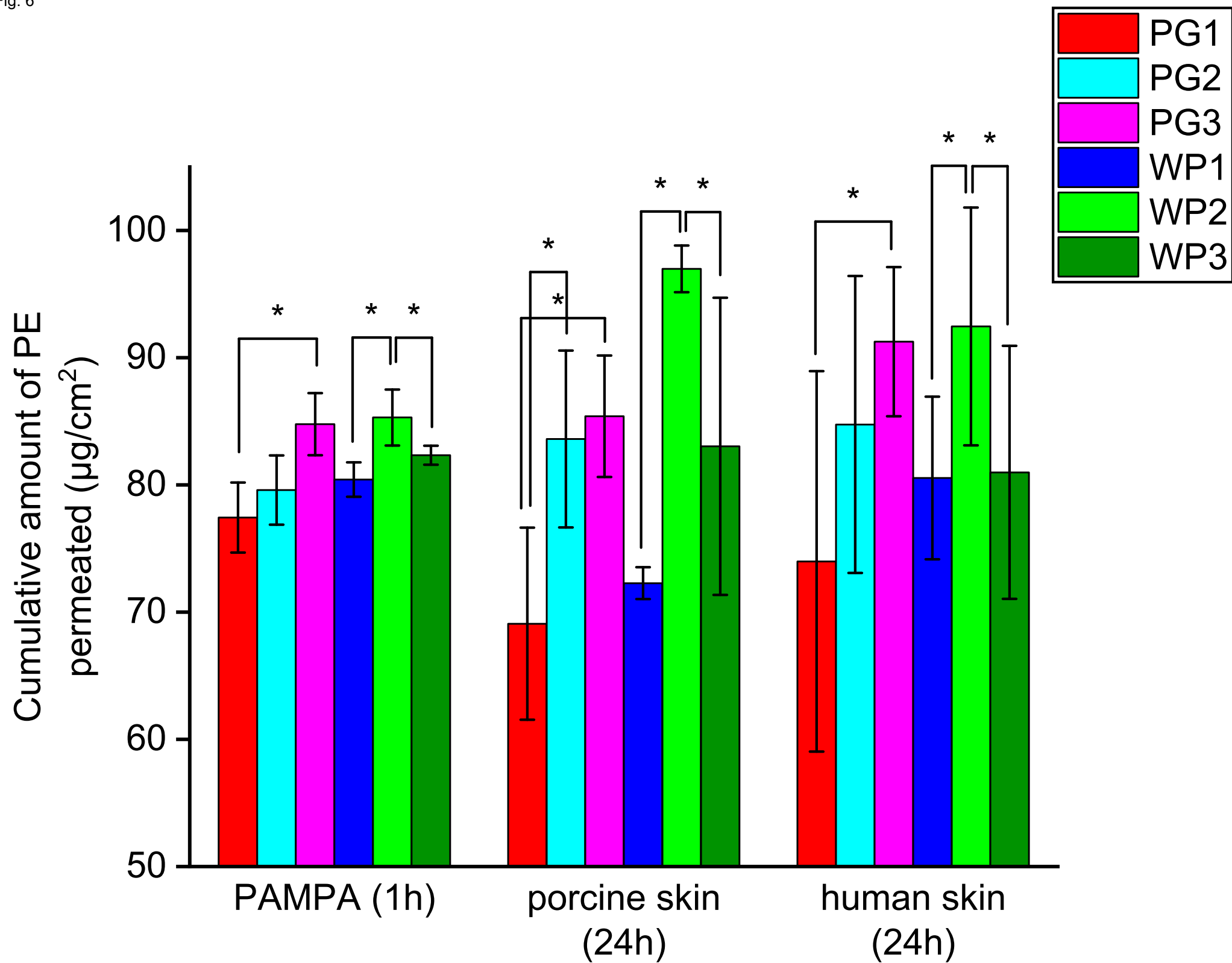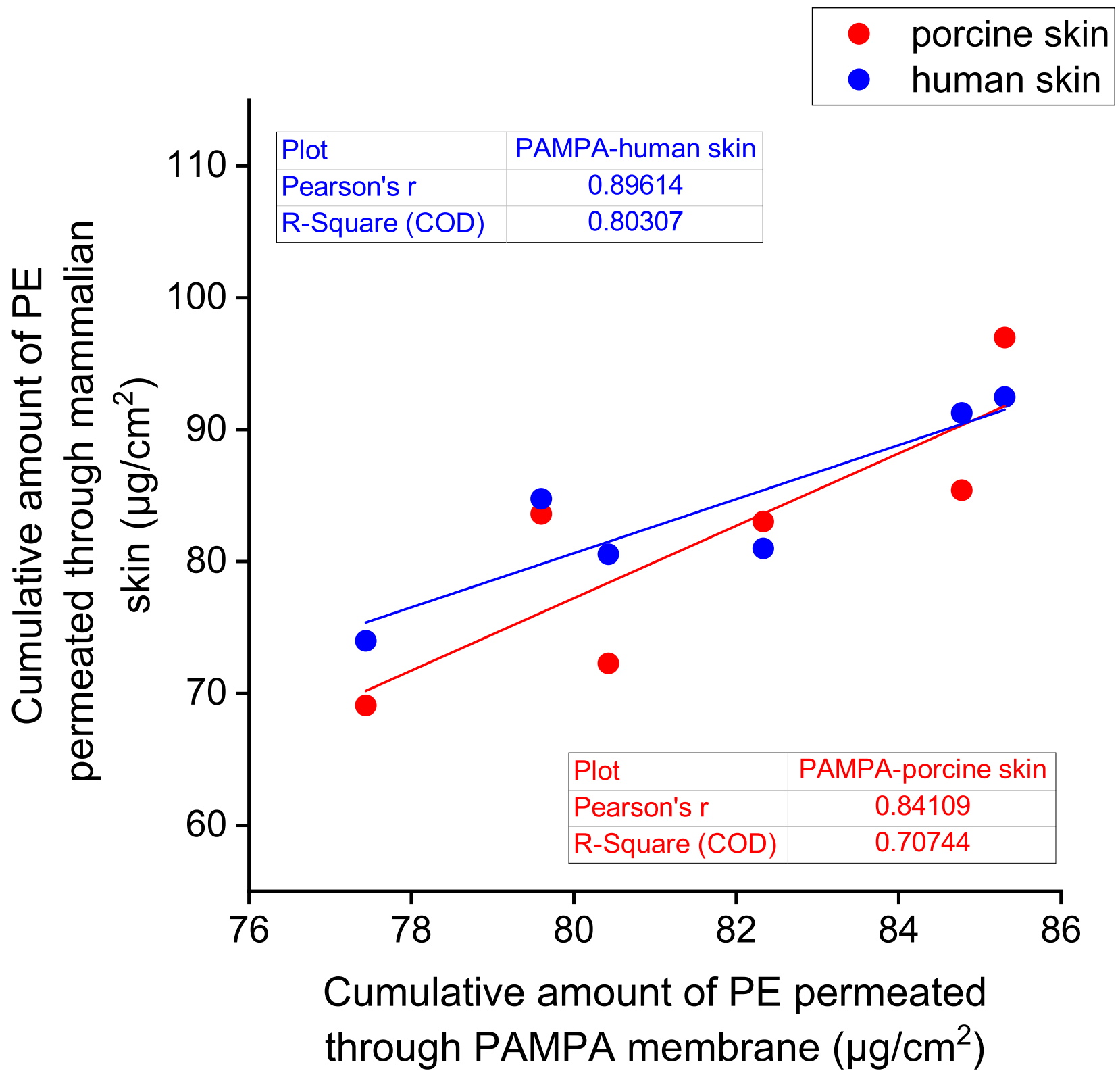Cumulative amount of PE permeated through PAMPA membrane ($\mu g/cm^2$)

CRediT authorship contribution statement

Annisa Rahma: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Writing – original draft. **Majella E. Lane:** Conceptualization, Resources, Supervision, Writing – review & editing. **Bálint Sinkó:** Conceptualization, Methodology, Formal analysis, Resources.