In vitro inhalation bioaccessibility of phthalate esters and alternative plasticisers present in indoor dust using artificial lung fluids

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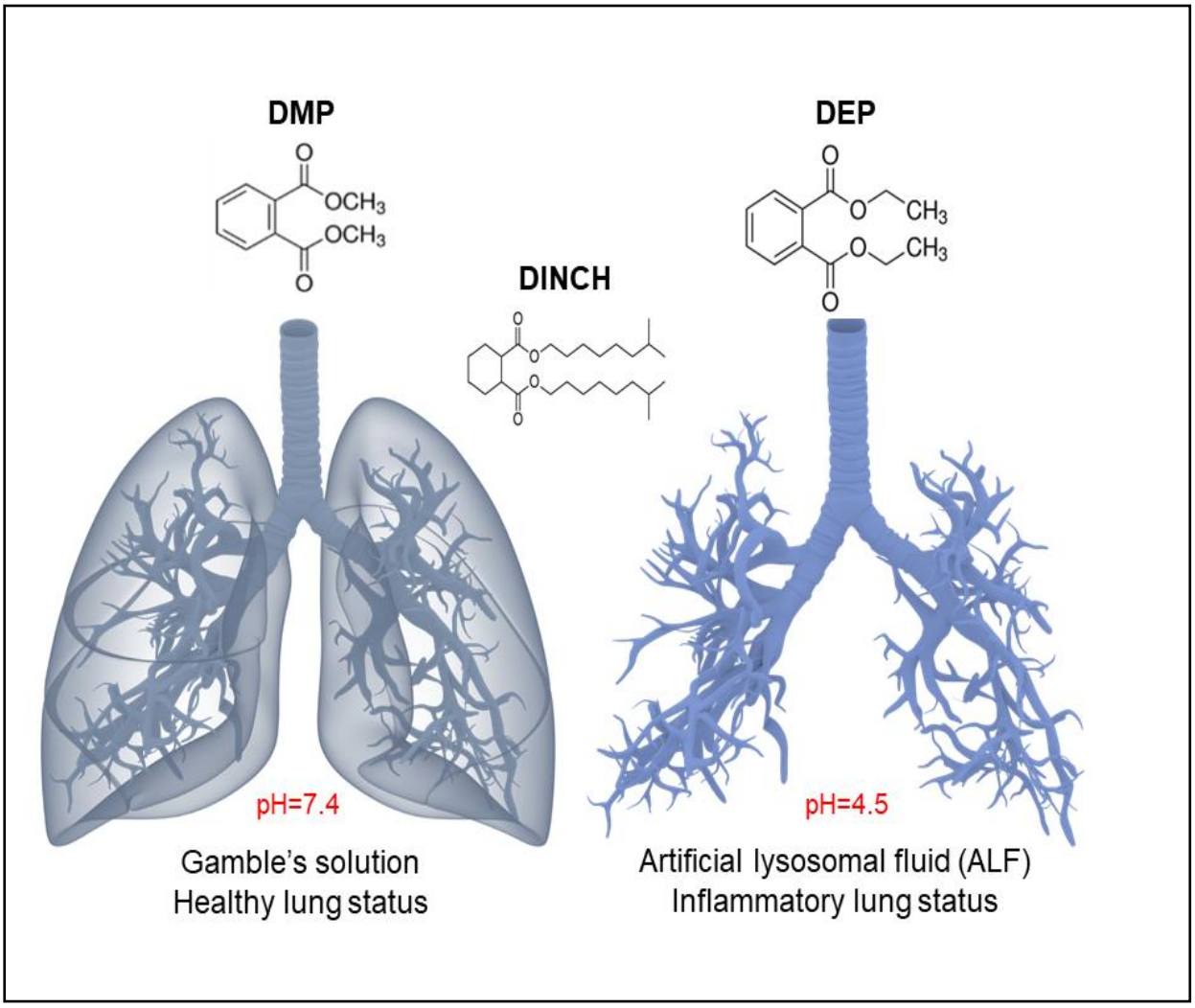
Graphical abstract



Highlights

- First study on *in vitro* inhalation bioaccessibility of organics from house dust
- Gamble's solution and artificial lung fluid were used as pulmonary surrogate media
- DMP and DEP were > 75 % bioaccessible in both lung media
- Alterative plasticisers DINCH and DEHT were < 5% bioaccessible
- Inhalation bioaccessibility was highly influenced by hydrophobicity

In vitro inhalation bioaccessibility of phthalate esters and alternative plasticisers present in indoor dust using artificial lung fluids

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Abstract

Phthalate esters (PEs) are plasticiser additives imparting durability, elasticity and flexibility to consumer products. The low migration stability of PEs along with their ubiquitous character and adverse health effects to humans and especially children has resulted in their classification as major indoor contaminants. This study assesses inhalation exposure to PEs via indoor dust using an *in vitro* inhalation bioaccessibility test (*i.e*. uptake) for of dimethyl phthalate (DMP), diethyl phthalate (DEP) and di-(2-ethylhexyl) phthalate (DEHP) and the alternative non phthalate plasticisers bis(2-ethylhexyl) terephthalate (DEHT) and cyclohexane-1,2-dicarboxylic acid diisononyl ester (DINCH), exposure. Using artificial lung fluids, which mimicktwo distinctively different pulmonary environments, namely artificial lysosomal fluid (ALF, pH = 4.5) representing the fluid that inhaled particles would contact after phagocytosis by alveolar and interstitial macrophages within the lung and Gamble's solution (pH = 7.4), the fluid for deep dust deposition within the pulmonary environment. Low molecular weight (MW) PEs such as DMP and DEP were highly bioaccessible (> 75 %) in both artificial pulmonary media, whereas highly hydrophobic compounds such as DEHP, DINCH and DEHT were < 5 % bioaccessible via the lung. Our findings show that the *in vitro* pulmonary uptake of PEs is primarily governed by their hydrophobicity and water solubility, highlighting thus the need for the establishment of a unified and biologically relevant inhalation bioaccessibility test format, employed within the risk assessment framework for volatile and semi-volatile organic pollutants.

Keywords: bioaccessibility, inhalation, phthalate esters, indoor dust, artificial lysosomal fluid, DINCH

Introduction

Phthalate esters (PEs) are plasticiser additives enhancing durability, elasticity and flexibility in consumer and polymeric products [1]. Low molecular weight (LMW) PEs such as dimethyl phthalate (DMP) and diethyl phthalate (DEP) are added as synthetic stabilisers to industrial solvents and personal care products they are also used as colouring or fragrance additives [2,3]. High MW (HMW) PEs such as di-(2-ethylhexyl) phthalate (DEHP) and di-iso-nonyl phthalate (DiNP) are primarily used in polyvinyl chloride (PVC) products including floor polishing, wall coatings, children's toys, medical products and food packaging [4–6]. Their low migration stability and vapour pressure influence PE release to the indoor environment, resulting in their classification as major indoor organic contaminants [7,8]. Consequently, considerably high levels of PEs have been found in indoor dust worldwide [5,9–13].

Human exposure to PEs in the indoor environment is a phenomenon of growing concern due to the potentially adverse health effects of PEs such as DEHP, di-n-butyl phthalate (DnBP) and di-iso-butyl phthalate (DiBP) in adults, such as disrupted endocrine and thyroid homeostasis, reduced fertility and reproduction [3,14,15]. Hence, the US and the EU have partly restricted the use of DiBP, DnBP, and DEHP in toys and childcare products [16,17]. Such actions paved the way for the introduction of less toxic, non-phthalate substitutes (*i.e.* alternative plasticisers) in consumer products in the early 2000s, such as di-isononyl-cyclohexane-1,2-dicarboxylate (DINCH; DEHP and DiNP replacement) and bis(2-ethylhexyl) terephthalate (DEHT), a structural isomer of DEHP [18–21]. However, due to their dominant use and rapid substitution, considerable levels of DINCH and DEHT have been reported in the indoor environment, raising concerns about their potential effects on humans [22–25].

Due to their critical and vulnerable developmental status, pre and postnatal children's exposure to PEs via indoor dust and PVC materials has been linked with chronic respiratory problems such as allergies, asthma, bronchial hyperactivity and inflammation, as well as neurodevelopmental disorders manifesting in adulthood [26–31]. Franken et al. (2017) reported the high occurrence of asthma in Belgian teenagers (especially girls) associated with high DEHP and DnBP exposure [32]. DEHT and DINCH administration to rodents revealed no signs of DEHP-like toxicity [33–35]. However, DINCH *in utero* exposure has been associated with signs of impaired liver metabolism and premature testicular aging such as decreased testosterone secretion, physical changes in seminal glands and testicular atrophy in rats and

their young offspring [36]. Thus, the debate regarding the safety of alternative plasticisers is ongoing especially during early-life exposure.

Physiologically-based extraction tests (PBET) have been employed to assess the oral bioaccessibility (*i.e.* uptake) of PEs via dust ingestion [37–39]. PE gut bioaccessibility decreased as $\log K_{ow}$ increased; LMW PEs such as DMP and DEP were found to be 32 % and 26 % bioaccessible, respectively, while DEHP was only 10 % bioaccessible via the gut [38]. In a comparative study between different dust size fractions and oral bioaccessibility, Wang et al. (2013) reported the highest gut uptake for LMW PEs in < 63 μm size fraction, compared to particles > 63 μm[39]. Dermal absorption of DEP and DnBP directly from air has been proposed by Weschler et al[40]. Since no studies exist regarding the inhalation bioaccessibility of organic pollutants, this calls for their development [41].

This is the first study we are aware of quantifying the inhalation bioaccessibility of PEs and alternative plasticisers employing two artificial lung fluids, mimicking two distinctively different interstitial lung conditions. Artificial pulmonary fluids have been previously employed in inhalation bioaccessibility studies of water-soluble metals and nanoparticles [42–46]. Artificial lysosomal fluid (ALF, pH=4.5) represents the fluid which inhaled particles come into contact with after phagocytosis by alveolar and interstitial macrophages within the lung. Gamble's solution (GMB, pH=7.4) is a surrogate fluid for deep dust deposition within the interstitial fluid of the lung [43,46]. The objectives of the present study are to evaluate the *in vitro* inhalation bioaccessibility of PEs, DINCH and DEHT present in indoor dust by employing two different artificial pulmonary fluids, *i.e.* Gamble's solution and ALF representing the healthy and inflammatory status of the tracheobronchial environment, respectively and to assess possible factors influencing inhalation bioaccessibility of PEs, DINCH and DEHT.

Material and methods

Sampling and dust particle properties

Details on the A-TEAM sampling protocols are given elsewhere[47]. Pre-existing vacuum cleaner dust samples (N=10) were passed through a methanol-washed, metallic sieve (< 63 μm) with respect to the inhalable aerodynamic particle cut off convention according to the International Organization for Standardization (ISO)[48]. Specific surface area and dust particle size were determined by laser diffraction spectroscopy (Mastersizer 3000, Malvern Ltd.,

UK), while total carbon (TC %) and nitrogen (TN %) contents were determined by Thermo Flash 2000 and organic matter content (OMC %) was determined by loss-on-ignition (LOI) as described elsewhere [49].

Dust extraction and clean-up

Details of the indoor dust extraction have been published previously [24,50]. Briefly, 100 mg of dust (< 63 μm) were extracted with 10 mL acetone: n-hexane (1:1 v/v) using microwave-assisted extraction (MAE) under controlled pressure and temperature. Prior to extraction, 400 ng ISTD mix prepared in n-hexane (DMP-d_4, DnBP-d_4 and DEHP-d_4) were spiked into all samples. The dust extracts were concentrated to 0.5 ml under a gentle nitrogen (N_2) stream which was filtrated through a glass Pasteur pipette tip containing charcoal in order to eliminate any traces of external contamination and the solvent was exchanged to n-hexane. This solution was loaded onto an ENVI-Florisil cartridge (500 mg / 3 mL, Biotage Isolute, Uppsala, Sweden) and 9 mL of n-hexane were added as a cleaning elution step. During the second elution, all target analytes were eluted using the 9 mL acetone: n-hexane (1:1) and the resulting eluate was concentrated to 1 ml with a gentle N_2 flow at room temperature, filtered as described above. Finally, all extracts were transferred to GC vials and biphenyl (300 ng) was added as an injection recovery standard prior to GC-MS/MS analysis (Fig SI 1). Further details about instrumental analysis are available in SI.

Lung fluid extraction

All lung fluid extractions were conducted in duplicate. Both media were freshly prepared 24 h before the initiation of each test in ultra-pure H_2O (18.2 Ω) as described elsewhere[43] (Table SI 3), pH-adjusted using HCl 1 M and NaOH 1 M, stored at 4°C and were checked for background phthalate contamination prior use. According to Boisa et al (2014), the experimental volume for simulated lung fluid extraction tests should be equal to 20 mL, given the pulmonary fluid volume capacity of healthy non-smoking adults (0.3 mL / kg; 70 kg body mass)[42]. In order to maintain 1:100 solid-to-liquid (S/L) ratio between the incubated matrix and the pulmonary fluid, 0.2 g of indoor dust (< 63 μm) were combined with 20 mL of each artificial lung fluid separately, as suggested by Schaider et al[51]. All samples were covered on top with oven-baked aluminium foil to avoid background phthalate contamination, followed by continuous incubation inside a thermostatic chamber (60 rpm; 37 °C) for 96 h , a time point relevant to the human alveolar clearance capacity [45,52]. After 96 h, the samples were separated by centrifugation (1500 rpm; 3 min) and the lung supernatants

were subjected to liquid-liquid extraction (LLE) using 7 mL Hexane: MTBE 3:1 twice, while ultrasonication-assisted extraction was employed for the residual dusts twice for 10 min using 7 mL of Acetone: Hexane 1:1. Prior to all extractions, all samples were spiked with 400 ng ISTD mix prepared in n-hexane (DMP-d_4, DnBP-d_4 and DEHP-d_4). To avoid any water residue and remove any gel-like emulsion formulated during LLE, sufficient amount of oven-baked Na_2SO_4 (powder) was added to all extracts, followed by 1 min vortexing and organic phase collection after centrifugation (1500 rpm; 3 min). All extracts were combined, solvent was exchanged to n-hexane and concentrated to 1 ml under a gentle N_2 stream at room temperature, filtered as described above. The residual dust extracts were subjected for clean-up through ENVI-Florisil SPE cartridge (500 mg / 3 mL, Biotage Isolute, Uppsala, Sweden), similarly to the dust extraction procedure described above. Briefly, the residual dust extracts were loaded onto the Florisil® columns, the first hexane eluate was discarded, while the second eluate was collected using 9 mL of MTBE. The resulting eluate was concentrated to 1 ml under a gentle N_2 flow at room temperature, filtered as described above. Finally, all extracts were transferred to oven-baked GC vials and biphenyl (300 ng) was added as an injection recovery standard prior to GC-MS/MS analysis (Fig SI 2).

Data analysis

Inhalation bioaccessibility (IBAF) was determined using Eq. 1, where mass phthalate (lung supernatant) is set as the phthalate mass (ng) determined in the lung supernatant of the *in vitro* pulmonary system and mass phthalate (dust residual) is the mass (ng) determined in the dust residual collected after the 96 h-incubation of the *in vitro* pulmonary system which is considered as the non-bioaccessible fraction.

$$\text{IBAF\%} = \frac{mass\ phthalate\left(\begin{matrix} lung \\ supernatant \end{matrix}\right)}{mass\ phthalate\ (\text{lung supernatant}) + mass\ phthalate\ (dust\ residual)}\ x\ 100 \quad \text{(Eq. 1)}$$

GraphPad Prism® version 7.00 for Windows, (GraphPad Software, La Jolla CA, USA) was used for statistical analysis. Prior to statistical analysis, all data were checked for normality using the Shapiro–Wilk test and not all data passed the normality test. All data were arc-sine transformed, as this mathematical transformation is necessary for statistical analysis of results set in percentages in order to equalise variances among treatments [53]. Ordinary two-way ANOVA (Uncorrected Fisher's test, $p<0.05$) was performed to assess statistically significant

differences of target analytes between both pulmonary fluids. Spearman's correlation ($p<0.05$) was employed to assess statistical dependence and correlation between artificial lung fluids and the physicochemical properties of all target analytes.

Quality assurance and quality control

The methods were evaluated using SRM 2585 as QC sample during dust (n=5) and lung fluid (n=4) extractions, respectively. Oven-baked, uncontaminated sand was used as a procedural blank during dust extractions; four blank lung fluid samples with no added matrix (two for each lung fluid) were sequentially incubated and analysed as procedural blanks. The results were blank-corrected for all target analytes by subtraction of the mean blank values from the raw target analytes values (expressed in ng g^{-1}) according to Abdhalah and Covaci[54]. Extraction efficiency for all target analytes ranged from 70 – 120% for both lung fluids respectively (Table SI 6). Method limits of detection (mLOD) were calculated as three times the standard deviation of the lung fluid blanks (Table SI 7).

Results and discussion

PEs and alternative plasticisers in indoor dust

Apart from DEHT, levels of PEs and DINCH from floor (N=61) and vacuum cleaner dust (N=58) from the ATEAM cohort have been previously reported [23] and were of the same order of magnitude as reported here (N=10; SI Table xxx). Besides the smaller dust particle size used in this study compared to Giovanoulis et al.[23] (< 63 µm and < 500 µm, respectively), the median values for all target analytes were marginally different apart from DINCH (this study: 17.06 µg g^{-1}, Giovanoulis et al.: 32.82 µg g^{-1}; $p<0.05$). Substantial differences between the maximum values of two studies were also found, e.g. DEP (this study: 54.2 µg g^{-1}, Giovanoulis et al: 240 µg g^{-1}) or DiNP (this study: 2470 µg g^{-1}, Giovanoulis et al: 1490 µg g^{-1}). These findings can be attributed to a) differences in sample size assessed and b) differences in particle size cut off and specific surface area which are likely to influence a pollutant's concentration in dust [39,55]. However, the aim of the present study is primarily to assess the inhalation bioacceessibility of PEs and their alternatives plasticisers, rather than report on their levels in dust.

Inhalation bioaccessibility

This is the first study on the *in vitro* inhalation bioaccessibility of PEs and alternative plasticisers via indoor dust. Inhalation bioaccessibility for DMP and DEP exceeded 70 % in both pulmonary media (Fig. 1). Statistical comparison of IBAF between the two pulmonary media did not reveal any statistically significant differences for any target analyte regarding the fluids' pH (pH Gamble's = 7.4; pH ALF = 4.5) and composition, apart from DMP ($p=0.017$) with 71 % and 82 % IBAF for Gamble's solution and ALF, respectively. DEP was also readily absorbed with 76 % and 75 % IBAF in Gamble's solution and ALF, respectively ($p>0.05$), showing thus that inhalation is an important route of exposure for LMW PEs. Gamble's solution is representative of the interstitial fluid of the deep lung area and ALF is representative of the more acidic environment following phagocytosis by alveolar and interstitial macrophages within the lung [42,43]. Hence, the inhaled dust particles would not have to be phagocytised before a considerable uptake of plasticisers occurs, with the exception of DMP.

Similarly to gut bioaccessibility which is partly governed by a pollutant's physico-chemical properties including MW and log K_{ow} [56,57], inhalation bioaccessibility of PEs decreased against the increasing trend in MW and log Kow (> 4). DiBP pulmonary uptake was 15.5 % and 12 %, in Gamble's solution and ALF, respectively, whereas DnBP and HMW PEs were 10 % and < 5 % bioaccessible in both media, including DEHP and its alternatives, DEHT and DINCH (Fig 1). Such findings endorse ingestion (food or dust) and dermal uptake as the predominant exposure routes for medium and HMW PEs, strongly influenced by their hydrophobic character and low water solubility [6,23,38]. However, no consensus exists regarding pulmonary media composition for inhalation bioaccessibility studies of organics. Employing modified media formulations with the addition of biologically relevant pulmonary surfactants such as albumin, mucin and dipalmitoylphosphatidylcholine (DPCC) have been proposed [41,42,58]; the case of DPCC makes biological sense and it should be thus systematically investigated along with other test parameters including S/L, incubation duration and particle size cut off [41,59], aiming towards a unified approach similarly to gut bioaccessibility[56].

Method performance using SRM 2585

Method performance was assessed using SRM 2585, since the pulmonary media used here were initially designed for nanoparticle and trace element inhalation bioaccessibility

studies[43,45,60]. IBAF > 75 % was found for LMW PEs, while DEHP and DiNP were the least bioaccessible (IBAF < 5 %) as highly hydrophobic compounds (Table 1), following a comparable pattern to the Norwegian house dust IBAF results. The SRM 2585 batch purchased in our study was prepared using a pool of dust samples collected during mid to late 1990s. Thus, DINCH and DPHP were not detected, since they were introduced in the market after 2000[18,61].

In this study we propose an *in vitro* method regarding the inhalation bioaccessibility of PEs and their alternatives via indoor dust. Low MW PEs such as DMP and DEP were highly bioaccessible in both artificial pulmonary media (> 75 %), regardless of the medium's pH and composition. Unlike DEP which presented similar pulmonary uptake in both media, DMP was more readily absorbed through ALF than Gamble's solution. HMW PEs along with DEHP alternatives, DEHT and DINCH did not exceed 5 % pulmonary uptake. Therefore, inhalation is a considerable route of exposure for LMW and less hydrophobic PEs. The lung uptake potential for compounds with comparable physico-chemical properties, *e.g.* LMW polycyclic aromatic hydrocarbons (PAHs) or organophosphates (PFRs) should be further assessed. Our results show that inhalation bioaccessibility of organic pollutants is primarily governed by hydrophobicity and water solubility. Future research should be targeted towards a unified and biologically relevant *in vitro* pulmonary uptake test for organics relevant to dust deposition in the lung, human lung function and inflammation *in vivo*. Finally, animal studies are more representative of the *in vivo* situation, marking them as necessary for the validation of *in vitro* inhalation bioaccessibility tests.

Conflict of interest

The authors declare no conflict of interest.

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Artwork and tables

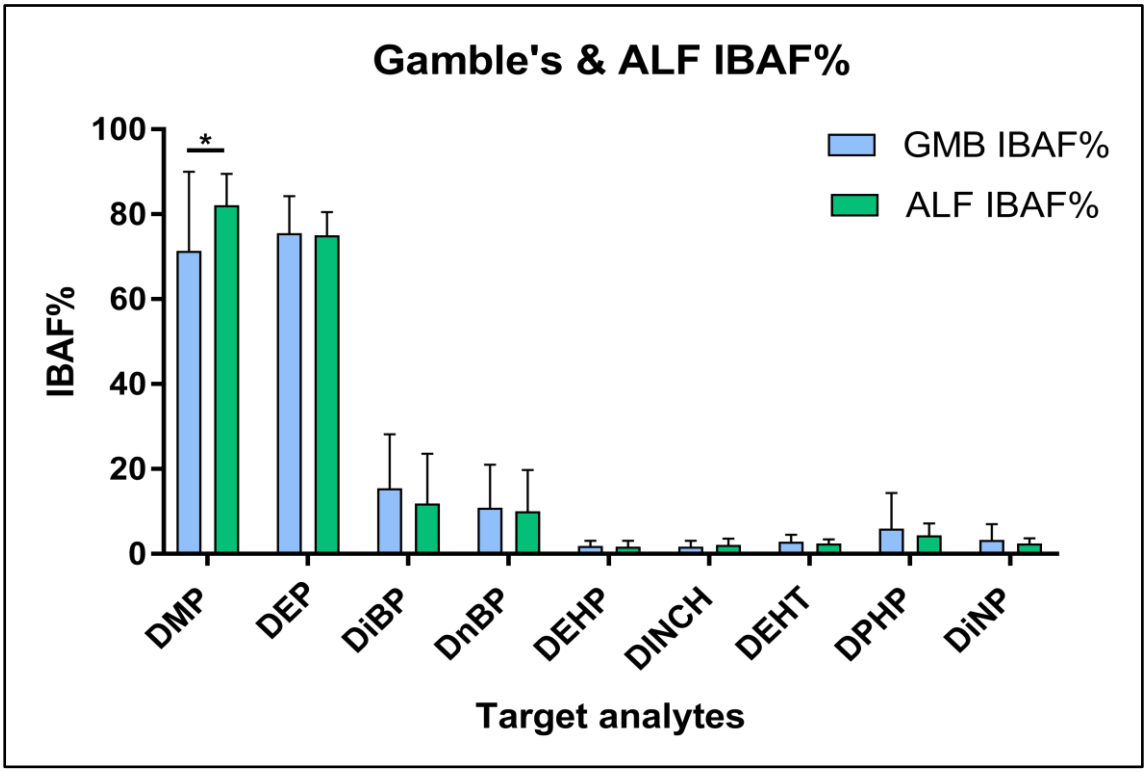

Figure 1 – *In vitro* inhalation bioaccessibility (IBAF%) of phthalate esters and alternative plasticisers present in indoor dust samples (N=10), using two different simulated lung fluids, namely Gamble's solution (GMB) and artificial lysosomal fluid (ALF). Statistically significant differences shown here (*; $p<0.05$). Bar charts represent average values in duplicates. Error bars represent 1 STDEV.

Table 1 - Lung fluid method performance using SRM 2585 (n=4) for Gamble's solution and artificial lysosomal fluid (ALF)

Target analytes†	Gamble's IBAF% (n=2)	STDEV	ALF IBAF% (n=2)	STDEV

DMP	89.9	1.8	89.5	0.3
DEP	80.7	1.2	73.7	1.0
DiBP	17.6	2.7	8.0	0.6
DnBP	9.8	1.3	6.2	0.5
BzBP	18.5	3.6	13.2	0.6
DEHP	3.1	1.6	2.0	0.2
DEHT	4.9	1.6	4.6	0.6
DiNP	3.9	1.0	3.5	0.3

282 †DINCH and DPHP not present in SRM 2585

283 Table 2 –Spearman's correlation between inhalation bioaccessibility (IBAF) in Gamble's
284 solution (GMB) and artificial lysosomal fluid (ALF) and the physicochemical properties of
285 plasticisers studied here

	GMB IBAF		ALF IBAF	
Physico-chemical properties†	Spearman's ρ	p value	Spearman's ρ	p value
MW	-0.561	0.096	-0.561	0.096
Log Kow	-0.705	0.027*	-0.705	0.027*
Log Koa	-0.588	0.081	-0.624	0.060
Vapour pressure	-0.535	0.115	-0.559	0.098
Water solubility	0.661	0.044*	0.636	0.054

286 *levels of statistical significance: $p<0.05$

287 † Physicochemical properties of plasticisers studied here can be found at Table SI xxx

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