Sniffing out the plastic: inhalation bioaccessibility of phthalate esters and alternative plasticisers present in indoor dust using simulated lung fluids

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Introduction:

Phthalate esters (PEs) are used as plasticisers in consumer products. 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 and used as colouring or fragrance additives [1,2]. 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 [3,4]. Their low migration stability has resulted into the classification of PEs as major indoor contaminants [5]. Human exposure to PEs in the indoor environment is of growing concern due to the potentially adverse health effects of PEs such as di-(2-ethylhexyl) phthalate (DEHP), di-n-butyl phthalate (DnBP) and di-iso-butyl phthalate (DiBP) in adults. These include disrupted endocrine and thyroid homeostasis, reduced fertility and reproduction [6,7]. Due to PE's ubiquitous character and adverse health effects to humans and especially children, non-phthalate alternative plasticisers have been introduced into the market since 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 [8]. Physiologically-based extraction tests (PBET) have been employed to assess the oral bioaccessibility (*i.e.* uptake) of PEs via dust ingestion. However, no studies exist regarding the inhalation bioaccessibility of organic pollutants. Artificial pulmonary fluids have been previously employed in inhalation bioaccessibility studies of water-soluble metals and nanoparticles. Artificial lysosomal fluid (ALF, pH=4.5) represents the acidic (*i.e.* inflammatory) intracellular lung environment inhaled particles come into contact with after phagocytosis by alveolar and interstitial macrophages. Gamble's solution (GMB, pH=7.4) is a surrogate fluid for deep lung deposition of particles within the interstitial (*i.e.* extracellular) environment under healthy conditions [9]. The objectives of this 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.

Materials and methods:

Pre-existing vacuum cleaner dust samples (N=10) were collected in Oslo, Norway during winter 2013 – spring 2014 [10]. All dust samples 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) [11]. All lung fluid extractions were conducted in duplicate. Both pulmonary fluids were freshly prepared 24 h before the initiation of each test in ultra-pure H_2O (18.2 Ω) as described elsewhere [9], pH-

adjusted using HCl 1 M and NaOH 1 M, stored at 4°C and checked for background phthalate contamination prior to use. 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)[12]. To avoid particle agglomeration due to dust overloading, 0.2 g of indoor dust (< 63 µm) were combined in glass test tubes with 20 mL of each artificial lung fluid separately, maintaining 1:100 solid-to-liquid (S/L) ratio between the incubated matrix and the pulmonary fluid [13]. All sample test tubes were covered 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 [14,15]. 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 (Figure 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 formed during LLE, a 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, charcoal-filtered N_2 stream at room temperature. The residual dust extracts were cleaned-up using ENVI-Florisil SPE cartridges (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 charcoal-filtered N_2 flow at room temperature. 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.

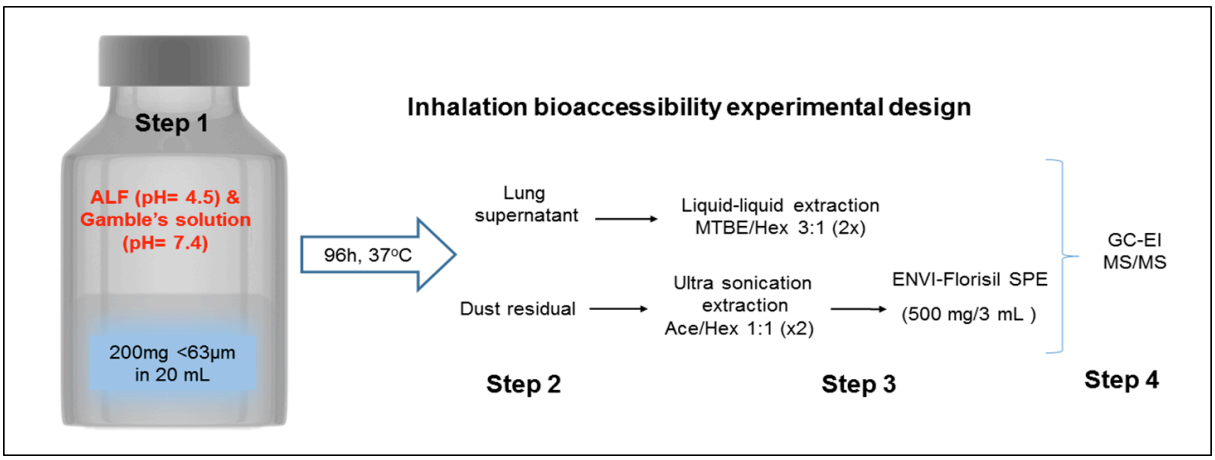

Figure 1 - Schematic representation of inhalation bioaccessibility test using two separate artificial lung fluids, namely a) Gamble's solution (pH=7.4) and b) artificial lysosomal fluid (pH=4.5). Shown in the figure are the different steps of the experimental procedure; lung fluid incubation for 96h at 37oC (step 1), sample collection using centrifugation for 3 min at 1500 rpm (step 2), sample preparation and clean-up (step 3) and GC-EI MS/MS instrumental analysis (step 4)

Results and discussion:

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 fluids (Figure 2). Statistical comparison of IBAF between the two pulmonary fluids 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 ($p>0.05$) in Gamble's solution and ALF, respectively. We found that inhalation bioaccessibility of LMW PEs was 2 to 3-fold higher compared to previously reported PE uptake via the gut. Therefore, inhalation can be an important route of exposure for LMW PEs. Gamble's solution mimics

the extracellular fluid deep within the lung and ALF represents the acidic intracellular lung environment following phagocytosis by alveolar and interstitial macrophages [9]. Hence, with the exception of DMP, considerable pulmonary uptake of plasticisers occurs via the extracellular lung matrix and no phagocytosis of inhaled dust particles seems necessary for PEs to reach blood circulation. Compared to lung uptake, HMW PEs were more bioaccessible via the gut [16], strongly influenced by their hydrophobic character and low water solubility, alongside the lipid-rich gut environment which facilitates higher desorption rates. However, no consensus exists regarding pulmonary fluid composition for inhalation bioaccessibility studies of organics. Employing modified lung fluid formulations with the addition of biologically relevant pulmonary surfactants such as albumin, mucin and dipalmitoylphosphatidylcholine (DPCC) have been proposed [17].

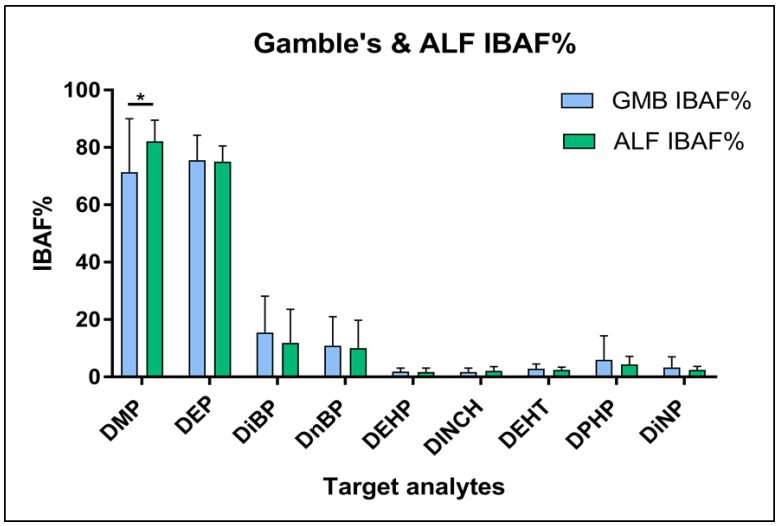

Figure 2 – In vitro inhalation bioaccessibility (IBAF%) of phthalate esters and alternative plasticisers present in indoor dust samples (N=10), using two different simulated lung fluids, namely Gamble's solution (GMB) and artificial lysosomal fluid (ALF). Statistically significant differences shown here (*; $p<0.05$). Bar charts represent average values in duplicates. Error bars represent 1 standard deviation (STDEV).

In this study we proposed an *in vitro* method to assess the inhalation bioaccessibility of PEs and alternatives plasticisers via indoor dust. Inhalation may be a considerable route of exposure for LMW and less hydrophobic PEs. Our results show that inhalation bioaccessibility of organic pollutants is primarily governed by hydrophobicity and water solubility. Future research should therefore aim towards unified and biologically relevant in vitro inhalation bioaccessibility tests for organics related to lung dust deposition and diffusion mechanisms, human lung function and inflammation alongside animal studies, necessary for the *in vitro* method validation.

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