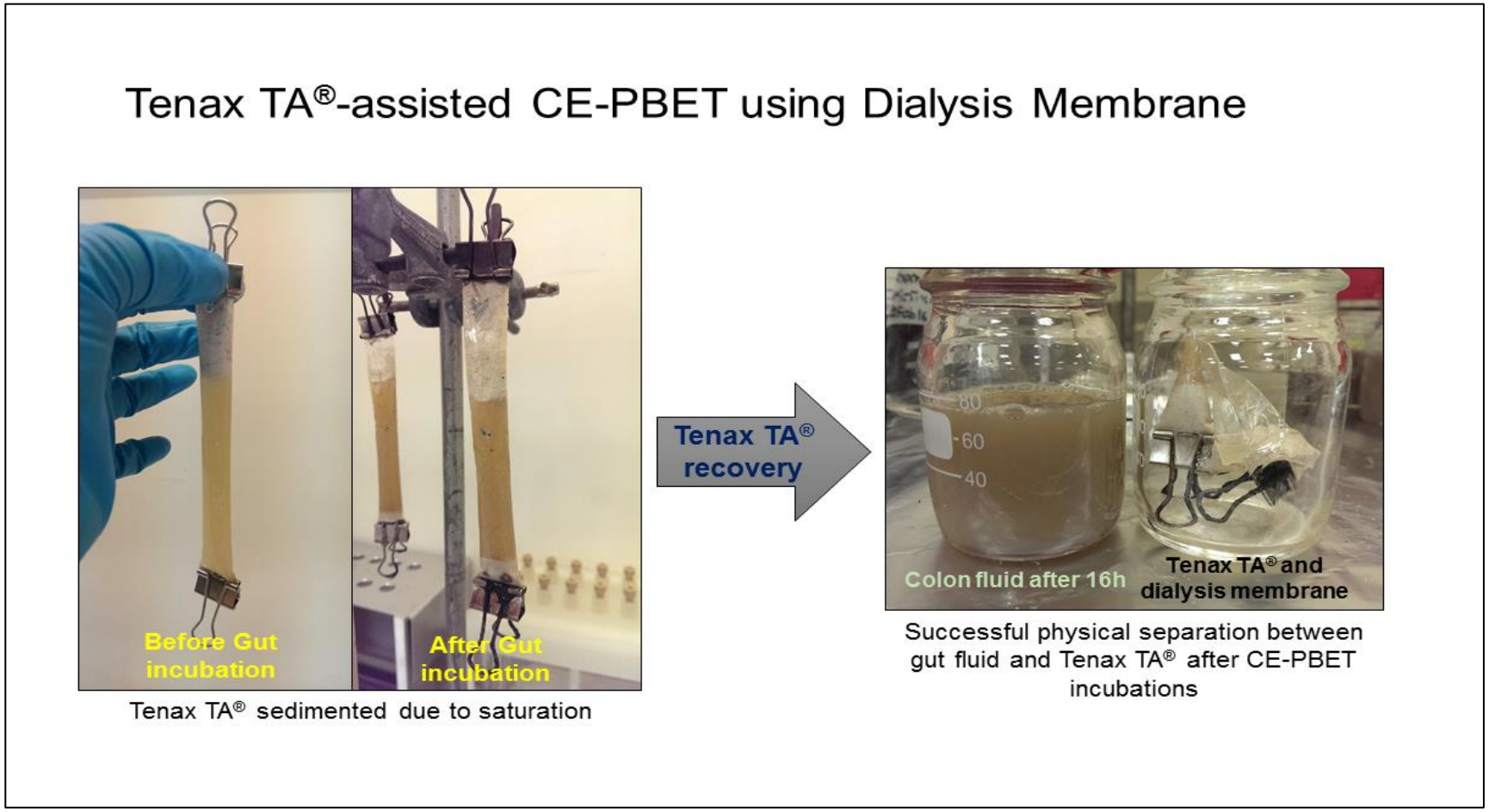
Bioaccessibility of PBDEs present in indoor dust: a novel dialysis membrane method with a Tenax TA® absorption sink

Graphical abstract



Highlights

- First method employing dialysis membrane for physical separation between Tenax TA® and dust
- Tenax TA® used as an absorption sink trapped in dialysis membrane mimics the situation *in vivo*
- CE-PBET performance was tested under different Tenax TA® loadings (0.25, 0.5 & 0.75 g)
- Two to three-fold bioaccessibility increase with Tenax TA® inclusion for all PBDEs
- Colon sorption to Tenax TA® was similar to small intestine for BDE28, but was higher than small intestine sorption for other PBDEs

16 Bioaccessibility of PBDEs present in indoor dust: A novel dialysis membrane method with a
17 Tenax TA® absorption sink

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27

Abstract

Human uptake of flame retardants (FRs) such as polybrominated diphenyl ethers (PBDEs) via indoor dust ingestion is commonly considered as 100% bioaccessible, leading to potential risk overestimation. Here, we present a novel *in vitro* colon-extended physiologically-based extraction test (CE-PBET) with Tenax TA® as an absorptive "sink" capable to enhance PBDE gut bioaccessibility. A cellulose-based dialysis membrane (MW cut-off 3.5kDa) with high pH and temperature tolerance was used to encapsulate Tenax TA®, facilitating efficient physical separation between the absorbent and the dust, while minimizing re-absorption of the ingested PBDEs to the dust particles. As a proof of concept, PBDE-spiked indoor dust samples (n=3) were tested under four different conditions; without any Tenax TA® addition (control) and with three different Tenax TA® loadings (*i.e.* 0.25, 0.5 or 0.75 g). Our results show that in order to maintain a constant sorptive gradient for the low MW PBDEs 0.5 g of Tenax TA® are required in CE-PBET.. Tenax TA® inclusion (0.5 g) resulted in 40% gut bioaccessibility c.for BDE153 and BDE183, whereas greater bioaccessibility values were seen for less hydrophobic PBDEs such as BDE28 and BDE47 (~60%). When tested using SRM 2585, our new Tenax TA® method did not present any statistically significant effect ($p>0.05$) between non- spiked and PBDE- spiked SRM 2585 treatments. Our study describes an efficient method where due to the sophisticated design, Tenax TA® recovery and subsequent bioaccessibility determination can be simply and reliably achieved.

Keywords: bioaccessibility, Tenax TA®, dialysis membrane, PBDEs, indoor dust

1. Introduction

Despite the strict legislative measures on the use of Penta-BDE and Octa-BDE formulations in both the EU and USA in consumer products (*e.g.* carpets, electronic appliances and furniture polyurethane foam) (Dodson et al., 2012; European Commission, 2003) and their listing as persistent organic pollutants (POPs) under the Stockholm Convention (Stockholm Convention, 2009a, 2009b), polybrominated diphenyl ethers (PBDEs) are legacy flame retardants (FRs) being detected in considerable levels in indoor dust from China (Cao et al., 2014; Sun et al., 2016), France (Raffy et al., 2017), the UK (Kademoglou et al., 2017; Tao et al., 2016), the Czech Republic, USA and Canada (Venier et al., 2016). Under this regime, human health concerns remain a critical issue, given the well-known PBDE potential to induce endocrine and thyroid disruption (Legler, 2008) and neurodevelopmental disorders in children (Bellinger, 2013; Costa and Giordano, 2007).

Total pollutant concentration of a contaminated solid matrix is perceived as the bioavailable fraction after ingestion and it is frequently used in human risk and exposure assessment (Semple et al., 2004). However, the assumption that 100% of the ingested toxicant within a matrix being available is unrealistic (Collins et al., 2015). Animal bioavailability studies (*e.g.* rodents or swine) are representative of the *in vivo* situation, but are often hindered due to financial and ethical restrictions (Oomen et al., 2003; Ruby et al., 2002). To avoid risk overestimation, bioaccessibility, *i.e.* the maximal fraction of an organic pollutant released from an ingested matrix (*e.g.* dust) into the gastro-intestinal tract (GIT) fluids of the organism has been proposed as a more realistic but conservative approach in human exposure assessment of persistent organic pollutants (POPs), serving as a surrogate to bioavailability (Brandon et al., 2006; Dean and Ma, 2007; Oomen et al., 2000). Several physiologically-based extraction tests (PBET) have been proposed to assess organic pollutant release and uptake from an ingested matrix via the GIT fluids *in vitro* (Brandon et al., 2006; Cave et al., 2010; Gouliarmou and Mayer, 2012; Tilston et al., 2011; Van de Wiele et al., 2004), as a substitute to *in vivo* studies (James et al., 2011) or for high-throughput estimates of bioaccessibility when animal studies are not feasible (Rodríguez-Navas et al., 2017; Ruby et al., 1996). Due to the non-polar and hydrophobic nature of hydrophobic organic compounds (HOCs) such as PBDEs, sorption to indoor dust is likely to occur via volatilisation, abrasion or fragmentation (Cao et al., 2014; García-Alcega et al., 2016), marking dust ingestion as a potential major route of exposure to FRs for humans (Alves et al., 2014; Jones-Otazo et al., 2005). Hence, *in vitro* bioaccessibility studies have been deployed, assessing human exposure

to contaminated indoor dust on a wide spectrum of HOCs including brominated flame retardants (BFRs) (Abdallah et al., 2012), organophosphate FR (OPFRs) (He et al., 2016; Quintana et al., 2017), pesticides and polychlorinated biphenyls (PCBs) (Ertl and Butte, 2012) and polybrominated diphenyl ethers (PBDEs) (Yu et al., 2012). However, the lack of an adsorption sink in the various test formats may lead to risk underestimation due to the absence of constant concentration gradient (Collins et al., 2015). Sink conditions better mimic the sorption/desorption processes in the human GIT *in vivo* and, coupled with the lipid-rich environment of the GI lumen and a long matrix:fluid contact time, may improve the bioaccessibility estimates of HOCs, such as PBDEs (Collins et al., 2015; Zhang et al., 2015, 2016).

A colon-extended PBET system (CE-PBET) with a carbohydrate-rich colon compartment as a "sink", favouring polycyclic aromatic hydrocarbons (PAHs) desorption from soil has been described (Tilston et al., 2011). Strong adsorbents such as silicone-activated contaminant traps, cyclodextrins and silicone rods have also been proposed as "absorption sink" materials in PBET systems, to improve bioaccessibility estimates for PAH-contaminated soil and biochar (Gouliarmou et al., 2013; Mayer et al., 2016; Zhang et al., 2015). As part of the International Organization for Standardization (ISO) guideline on bioavailability, an extended (20h) Tenax-based extraction method achieved increased mobilisation (*i.e.* bioaccessibility) of HOCs from soils and sediments onto this infinite sink and has been proposed for standarisation (ISO, 2015; Ortega-Calvo et al., 2015). Tenax TA® is a versatile absorption sink with large surface area and high sorption capacity for HOCs and was thus used as an "infinite" sink in PBET systems, studying the uptake of FRs and PAHs via indoor dust (Fang and Stapleton, 2014) and soil (Li et al., 2015), respectively. Cornelissen et al (1997) employed Tenax TA® studying sorption/desorption kinetics of PAHs, alkylbenzenes and PCBs from dredged sediments; the sink captured the organic pollutants from the solid matrix but the Tenax TA® beads adhered to the glassware with consequent problems for physical separation and recovery of Tenax TA® from the matrix (Cornelissen et al., 1997). The variability in Tenax TA® mass recovery, its separation from the matrix and the design of an appropriate vessel for Tenax TA® inclusion (*e.g.* stainless steel net) during PBET incubation has discouraged further applications of Tenax TA® in environmental exposure studies (Li et al., 2016; Mayer et al., 2016). In the work presented here, we describe a novel *in vitro* method capable to overcome the aforementioned challenges concerning physical separation and recovery of Tenax TA® from the matrix, while facilitating its successful inclusion and

performance as an adsorption sink in a previously established bioaccessibility test, namely CE-PBET, for the assessment of oral bioaccessibility of PBDEs from indoor dust.

To separate aqueous and solid matrices, a regenerated cellulose (RC) dialysis tubing method was employed, studying the sorption and dissolution of perchloroethane and PAHs from clay-rich materials and sewage sludges, respectively (Allen-King et al., 1995; Woolgar and Jones, 1999). RC membranes present high pH and temperature tolerances, carry no fixed charge and are highly resistant to halogenated hydrocarbons, such as PBDEs (Pollard, 1987). Tubing characteristics including length, width, membrane sealing method and molecular weight cut off (MWCO) have been evaluated. For example, 2.5 g of contaminated sewage sludge were introduced into 10 cm of dialysis tubing with a 3.5 kDa MWCO (Woolgar and Jones, 1999). Alternatively, 20 cm of dialysis tubing (29 mm width; 12-14 kDa MWCO) was used to ensure that at least 30% of the analyte mass would remain in the solid phase after equilibration (Allen-King et al., 1995). The solid material in the tubing was then introduced inside glass bottles with synthetic groundwater spiked with the HOCs of interest. During equilibration, all non-settling particles were retained inside the dialysis membrane, while dissolved organic pollutants could permeate through the membrane and equilibrate across the dialysis tubing by passive diffusion (Allen-King et al., 1995).

Our study aims are to systematically (a) develop an efficient method to separate Tenax TA® and indoor dust as a matrix whilst enabling desorption of PBDEs to the Tenax TA® and (b) optimise Tenax TA® as an absorption sink for PBDEs in a colon-extended gastro-intestinal bioaccessibility *in vitro* system (CE-PBET).

2. Materials and methods

2.1 Target analytes and indoor dust

An indoor dust sample was collected in 2013 from a pre-existing vacuum cleaner bag in an office at Reading (UK) and was used during method development tests and the results are presented in sections 3.1 and 3.2. The dust sample was sieved to <250 μm, a particle cut off likely to be ingested by humans (Yu et al., 2012), using a hexane-washed, metallic sieve and stored in hexane-washed, amber glass bottles at +4 ^{o}C. Concentrations of all target analytes in all dust samples were determined using methods described elsewhere (Kademoglou et al., 2017). Briefly, 30 mg of dust was extracted with 2.5 mL hexane:acetone (3:1) using ultra-sonication extraction for 10 min and vortexing for 1 min three times. The combined extract

was concentrated to 1 mL and loaded on aminopropyl (NH_2) silica cartridges (500 mg, 3 mL, Agilent, USA) and further eluted with 10 mL hexane. The eluate was then further concentrated, following a clean-up on an acidified silica cartridge (5%, 1 g, 6 mL) and elution with 12 mL dichloromethane. The dust extracts were then evaporated, reconstituted with 100 µL of iso-octane and filtered using a micro centrifuge filter lined with 0.45 µm pore size nylon filter (1.5 mL volume capacity) . Finally, the extracts were transferred to injection vials and analyzed on GC-ECNI-MS. Standard reference material for indoor dust SRM 2585 (organic contaminants in house dust), purchased from the US National Institute of Standards and Technology (NIST, USA), was used to assess method performance and the results are presented in section 3.3. . Both SRM 2585 (used for method performance assessment; 0.5 g) and dust samples (0.5 g) (used for method development) were spiked at environmentally relevant concentrations (200 ng; 200µL of PBDEs native standard mix 1 ng/µL prepared in iso-octane) and the validity of the spiking was confirmed analytically for both the SRM 2585 and the dust (Table SI 2). After spiking, samples were shaken for 2h on an orbital shaker and allowed to stand inside a fumehood for 6h before the gastro-intestinal extraction for the solvent to evaporate, thus facilitating compound interactions with the matrix (Ballesteros-Gómez et al., 2016).

2.2 Dialysis membrane

Approximately 16 cm of standard grade, flexible and transparent regenerated cellulose (RC) dialysis membrane with 3.5 kDa MWCO and 18 mm flat width (1.1mL/cm) (Spectra/Por™ 3, SpectrumLabs Inc., USA) was used to encapsulate the Tenax TA® beads. The membrane length and flat width were selected for the sample volume to be added in the membrane using an online tool provided by SpectrumLabs Inc. (http://www.spectrumlabs.com/dialysis/dtCalc.html), allowing for tube sealing with 19 mm metallic clips. MWCO selection for the RC membrane is primarily governed by the molecular weight (MW) of the biological molecules of the GI compartments and the target analytes of our study. To maximize the rate of dialysis, the membrane with the largest MWCO which will not cause excess loss of the desired analytes was used. Hence, the MWCO was selected to be over three-fold higher of the MW of the heaviest target analyte studied here (*i.e.* BDE183; MW= 722) (SpectrumLabs Inc., personal communication). The diffusion of PBDEs across the membrane was aided by the addition of 10 mL of GIT fluid (*i.e.* stomach, small intestine, colon) inside the RC membrane/Tenax TA® system.

2.3 Gastro-intestinal Extraction

The gastro-intestinal extraction test involved three compartments, namely stomach (1h; pH=2.5), small intestine (SI) (4h; pH=7) and colon (16h; pH=6.5) tested in sequential mode (Fig. 1). Fed CE-PBET conditions were achieved by the addition of dietary components such as mucin, lipid-rich carbohydrates and bile salts into stomach, SI and colon incubations as described in Table SI-3 according to (Tilston et al., 2011) and all media were prepared in deionised H_2O (dH_2O). All experiments were conducted in triplicate. Gut media aliquots (80 mL) were added into clean, amber 100 mL Duran® glass bottles, sealed with PTFE-lined screw caps and stored at -20°C prior use if necessary. Tenax TA® beads were cleaned prior use to remove fine particles by ultrasonication with 40 mL acetone (x2), 40 mL acetone:hexane 1:1 (x2) and 40 mL hexane (x 2) for 10 min in each sonication step. Tenax® TA was then allowed to air-dry at 105 °C overnight and was stored in a hexane-washed, Duran® bottle inside a desiccator. A short video demonstration of the Tenax TA® inclusion in the RC dialysis membrane is available online https://figshare.com/s/e7312fa7d177b35bc7d0 (video used for demonstration purposes only; the RC membrane is sealed using 19 mm metallic clips; see below). Before employment, the RC dialysis membrane was soaked in ultra-pure H_2O at room temperature for 45 min under continuous stirring to remove any preservatives such as glycerine and sodium azide. The RC membrane was then thoroughly rinsed with dH_2O and one side sealed with a 19 mm hexane-washed, metallic clip. Using a small glass funnel, Tenax TA® (0.5 g) was added inside the RC membrane, followed by 10 mL of stomach medium. The tubing was then sealed using another metallic clip. Then, 0.5 g of indoor dust were added in the remaining 70 mL of stomach fluid and the RC membrane/Tenax TA® system was introduced to the bottle (Fig 1A). A solid-to-liquid (S/L) ratio 1:140 was achieved, thus preventing any bioaccessibility underestimation due to poor dissolution of contaminants from dust (Abdallah et al., 2012; Dean and Ma, 2007). The bottles were placed at 45° angle inside a temperature-controlled waterbath at 37 °C and rotated at 130 rpm for 1h, mimicking the GIT peristaltic movement. After 1 h, the samples were removed from the waterbath and, due to the continuous character of CE-PBET, stomach fluid was converted to small intestine media (SI) by addition of bile salts (0.5 g/L) and pancreatine (1.78 g/L) with pH adjusted to 7 using saturated $NaHCO_3$. The small intestine incubation continued as above for 4h (Fig 1 B). The stomach medium was converted to small intestine only outside the membrane, given the assumption that bile salts and pancreatine would permeate to the inner barrier of the RC membrane during the 4-h small intestine incubation step in order to reach a pH equilibrium between inside and outside

of the RC membrane/Tenax TA® system to sustain sorption/desorption by passive diffusion. According to Spectrum Labs Inc. (USA) instructions to users, a first-order permeability rate is observed provided that the RC tubing system is well stirred and the solvent (in our case gut fluid) is changed several times during the dialysis procedure (Spectrum Labs Inc., personal communication). The RC membrane/Tenax TA® system was then removed from the bottle and was allowed to sediment for 15 min. Due to its hydrophobic character, Tenax TA® floats on top of the small intestine fluid inside the membrane (Fig. SI 1). Tenax TA® was trapped on the one side of the membrane, while the other side was carefully unsealed. The small intestine fluid inside the membrane was carefully collected (≈8 mL), was subsequently combined with the remaining 70 mL from the incubation and stored at +4 °C prior to liquid-liquid extraction (LLE).

The transition between the small intestine and colon compartments was achieved by physical transfer: the dust was recovered from the 70 mL of small intestine media by centrifugation (3500 rpm, 15 min), then added to 70 mL of colon medium. Using the same RC membrane and Tenax TA® as in the small intestine compartment, approximately 8 mL of pre-warmed colon medium were added and sealed with the metallic clips as described for the stomach compartment, re-introduced into the bottle where the indoor dust was re-suspended using the colon medium and incubated for 16 h (Fig 1C). At the end of the colon incubation, the dust pellet was recovered by centrifugation as before and stored at -20 °C for extraction. Finally, Tenax TA® was recovered using clean cotton wool filtration, the colon fluid was passed through cotton wool, combined with the remaining 70 mL of colon fluid and stored at +4 °C for LLE (Fig SI-2). The cotton wool pieces from filtration together with the Tenax TA®, the RC membrane and the metallic clippers were collected in one bottle for ultra-sonication assisted extraction. More details on the RC membrane/ Tenax TA® system, Tenax TA® filtration and recovery are available at Fig. SI 1 and Fig.SI 2, respectively.

2.4 Tenax TA® sorption capacity

An assessment of PBDE release via the gut and Tenax TA® sorption capacity with respect to the three CE-PBET compartments was conducted per batch (*i.e.* a single Tenax TA® sorption experiment was conducted separately relative to the CE-PBET compartment and its incubation duration), not in sequential mode (*i.e.* continuous Tenax TA® sorption; total incubation duration 21 h). Briefly, a fresh Tenax TA® sample (0.5 g) was incubated using a new RC dialysis membrane before the initiation of each CE-PBET compartment. Each Tenax TA® sample was finally harvested and subjected to extraction and clean up, along with the gut fluids and the residual dust as described in section 2.5.2. 5 Extraction and clean up

Before extraction, all samples were spiked with 200 ng of internal standard (ISTD) mix (100 μL of 2 ng/μL) prepared in toluene (BDE77 for BDE28, 47 and 100 and BDE128 for BDE153, 154 and 184 quantifications, respectively) and shaken on an orbital shaker for 1h. Gut fluids were subjected to a LLE using 30 mL hexane/ethyl acetate 3:1 v/v twice (Fig. SI2 – step 1). Two mL of acetone were added to enhance separation, when necessary. A gel-like emulsion bilayer (mainly lipid and carbohydrates) was developed, especially in the colon compartment. Oven-baked Na_2SO_4 (400 ^oC; powder) was added in the combined LLE extracts to absorb all remaining water residues and dissolve the gel-like emulsion. All samples were then allowed to settle for 1h at room temperature and the extracts were collected by centrifugation (3500 rpm, 15 min). The residual dust and the recovered Tenax TA® beads (together with the glass wool and the metallic clips) were subjected to ultra-sonication assisted extraction for 15 min using 30 mL acetone/hexane 1:3 v/v twice (Fig. SI-2 – step 2 & 3). After each step, the extracts were collected by centrifugation (3500 rpm, 15 min). All extracts collected from each step were combined, evaporated to 1mL hexane using Syncore ® Analyst evaporator (Buchi, Switzerland) and then loaded onto Florisil® cartridges (2 g, 6 mL), using a slightly modified method published elsewhere (Van den Eede et al., 2012) (Fig. SI 2 – step 4). Briefly, Florisil® cartridges were pre-cleaned with 10 mL ethyl acetate and 6 mL of hexane; our target analytes were eluted using 20 mL hexane. This eluate was further concentrated to 1mL (in hexane) and then subjected to SPE clean-up on 5% acidified silica (5% AS) (2 g, 6 mL). The 5% AS cartridges were pre-cleaned with 6 mL hexane and 3 mL dichloromethane and then all extracts from the Florisil® step were loaded onto the SPE silica column. Our target analytes were eluted using 16 mL hexane and 8 mL dichloromethane and after collection, all eluates were concentrated near dryness under a gentle stream of N_2, reconstituted in 100 μL of toluene and then filtered using a micro

centrifuge filter lined with 0.45 μm pore size nylon filter (1.5 mL volume capacity) . Finally, the samples were transferred to injection vials, biphenyl (40 ng) was added as an injection recovery standard and analysed by GC-EI/MS. Further details about instrumental analysis are available at SI.

2.6 Data analysis

Bioaccessibility can be expressed as a mass (*e.g.* ng of a contaminant solubilised in the GI tract), a concentration (ng/g of a contaminant in dust) or as a fraction expressed in percentage (BAF%) (Guney and Zagury, 2016). In our study, bioaccessibility was determined according to (García-Alcega et al., 2016) using Eq. 1, where mass FR (SI+colon+Tenax TA®) is set as the sum of FR mass (ng) determined in small intestine (SI), colon and Tenax TA® compartments of CE-PBET system and mass FR (dust residual) is the FR mass (ng) determined in the dust residual collected after 16h-incubation of CE-PBET colon compartment which is considered as the non-bioaccessible fraction.

$$Bioaccessibility\ \%\ (BAF\%) = \frac{mass\ FR\ (SI + Colon + Tenax\ \text{TA®})}{mass\ FR\ (SI + Colon + Tenax®) + mass\ FR\ (dust\ residual)}\ x\ 100 \quad \text{(Eq.1)}$$

GraphPad Prism® version 7.04 for Windows (GraphPad Software, La Jolla CA, USA) was used for statistical analysis. Prior to statistical analysis, all BAF% were converted into fractions and arc-sine transformed. This mathematical transformation is necessary for statistical analysis of results set in percentages in order to equalise variances among treatments (Sokal and Rohlf, 1995). Multiple t-tests (unpaired; $p<0.05$) were performed to assess statistically significant differences among the different Tenax TA® amounts added (sections 3.1 and 3.2), whereas ordinary two-way ANOVA (Uncorrected Fisher's test, $p<0.05$) was performed to assess statistical differences for bioaccessibility with and without the addition of Tenax TA® in SRM 2585 method validation (section 3.3).

2.7 Quality assurance and quality control

All samples were analysed in triplicate together with oven-baked, laboratory-grade sand (procedural blank) and SRM 2585 (n=3, NIST, USA) was used for method validation and QC testing. Concentrations of our target analytes in method blanks were all below method limit of detection (mLOD) (0.05 ng/μL). RC membrane and Tenax TA® blanks were extracted for

FR background contamination prior use and all values were found below mLOD. No weight correction on bioaccessibility values with respect to potential Tenax TA® mass losses was employed in our study. According to the ISO 16751 method on organic pollutant bioavailability (2015), correction for such losses is recommended by air drying and weighing the dry amount of Tenax TA® after extraction (ISO, 2015). In our study separating Tenax TA® beads from the glass wool and the RC membrane post extraction was not feasible due to the character of Tenax TA® to adhere in any surface it comes in contact with during filtration (e.g. glass wool).Extraction efficiency (%) was assessed for SI, colon, Tenax TA® and residual dust compartments by spiking experiments (see SI Table 2). Briefly, 100 ng of native PBDEs (100 μL of 1 ng/μL) in iso-octane were spiked to SI and colon media, Tenax TA® (0.5 g) and dust (0.5 g). All samples were shaken on an orbital shaker for 1h. Finally, 30 mL of the corresponding extraction medium was added in each compartment, following the same sample preparation processes as before. Finally, biphenyl (40ng) was added as an injection recovery standard and the samples were analysed by GC-EI/MS. Extraction efficiency values for all target analytes were >60% in all CE-PBET compartments, except BDE100 efficiency which was 52% and 54% in Tenax TA® and residual dust, respectively. Such phenomena may be attributed to potential mass loses of Tenax TA® during glass wool filtration steps. Despite the moderately lower extraction efficiency for BDE100 in comparison to the other target analytes, the relative standard deviation (RSD%) of the method for BDE100 was 6%. Given the low deviation and variability, no correction was performed for BDE100 (Table SI 3). Glass test tubes were cleaned by soaking for at least 12 h in an alkali solution. After washing, the tubes were rinsed with water and dried at 100 ºC for at least 12 h and burnt at 400°C to remove all traces of contamination.

3. Results and discussion

3.1 Tenax TA® optimisation

The addition of Tenax TA® in CE-PBET considerably increased the bioaccessible fraction (%BAF) of all target analytes, illustrating the value of Tenax TA® as an adsorbent matrix for HOCs. Different masses of Tenax TA® were added to the CE-PBET system to optimise the adsorbent sink to ensure exhaustive FR desorption from indoor dust. PBDE-spiked indoor dust samples (n=3) were tested under four different conditions; (A) without any Tenax TA® addition (control) and with three different amounts of Tenax TA®, namely 0.25 g (B), 0.5 g (C) and 0.75 g (D). The same length of RC dialysis membrane (16cm) and mass of dust (0.5

g) was used in all treatments. Our results show that Tenax TA® enhanced gut bioaccessibility for PBDEs by approximately two-fold (Fig. 2) and the bioaccessible fraction was significantly different ($p<0.001$) between the controls (no Tenax) and with Tenax TA® addition, for all target analytes (Fig. 2). For example, with no Tenax TA® (control), the bioaccessible fraction of the low brominated PBDEs, BDE28 and BDE47, was 37.7% and 32.8%, respectively, whereas their BAF% increased with 0.25 g Tenax TA® inclusion to 55.1% and 54.9%, respectively. A trend to decreasing BAF% with increasing degrees of bromination for PBDEs can be seen for the control treatments and the different amounts of Tenax (Fig 2). Such findings are in agreement with Fang and Stapleton (2014), where a negative relationship between gut bioaccessibility and PBDE physicochemical properties such as degrees of bromination, MW and log Kow was described (Fang and Stapleton, 2014). Few studies describe the influence of Tenax TA® inclusion on gut bioaccessibility of organic pollutants from solid matrices such as indoor dust or soil. CE-PBET and Tenax TA® were employed to assess FR gut bioaccessibility and for a wide range of low and high MW FRs present in indoor dust including BDE47, BDE100 and BDE183; in their experimental design, Fang and Stapleton (2014) used 0.5 g of Tenax as an absorptive sink but the effects of varying Tenax TA® content were not reported (Fang and Stapleton, 2014). In a study assessing PAHs bioaccessibility in soils from China, 0.25 g of Tenax TA® were added into a PBET *in vitro* system (Li et al., 2015). According to Li et al (2015), this mass was five-fold higher than the small intestine organic matter (OC), thus allowing sufficient sorption capacity for the PAHs mobilized during their study (Li et al., 2015). Zhang et al (2017) reported fast and efficient sorption only for high MW PAHs (*i.e.* 3 -5 benzene ring) using 0.1 g of Tenax TA® studying PAH soil bioaccessibility; poor extraction efficiencies were noted for volatile PAHs such as naphthalene, acenaphthylene and acenaphthene, possibly as a result of an air drying step during Tenax TA® collection and separation from the gut fluid (Zhang et al., 2017). Varying the content of Tenax TA® (0.25, 0.5 and 0.75 g) in the CE-PBET system studied here, showed few statistically significant differences for our analyte recoveries. Here, statistically significant differences among the three Tenax TA® amounts tested were found only for BDE28 bioaccessibility as an exception; some increase in BDE28 BAF% with Tenax TA® content, rising from 55.1% with 0.25 g Tenax TA® to 66.7% with 0.5 g (0.25 g vs 0.5 g; $p=0.017$) and 69.9% with 0.75 g Tenax TA® added (0.25 g vs 0.5 g; $p=0.006$) was observed. These results reflect the physicochemical properties of this FR as a low MW tri-BDE congener; Tenax TA® is a hydrophobic sink and the calculated log K_{ow} (EpiWeb) shows that BDE28 (log Kow 5.88) is less hydrophobic than BDE47 (log Kow 6.77) and hence greater

amounts of the adsorbent may be needed to capture all of the released BDE28. For all other analytes, there were no statistically significant differences in BAF% among the varying Tenax TA® amounts tested. In other words, for BDE28 being the least hydrophobic of our target analyte list, we propose that Tenax TA mass loading greater than 0.25 g is required in order maximise the sortion potentialof low MW PBDEs such as BDE28 to Tenax TA®. Given the a) high sorption capacity of Tenax TA®, b) the broad range of physical properties (MW, water solubility and log K_{ow}) of our FRs mobilised from the ingested matrix and c) the relatively high Tenax TA® mass recovery (SI Fig 3), 0.5 g of Tenax TA® were selected and subsequently used below. Our results show that in order to maintain a constant sorptive gradient for the low MW PBDEs, a larger mass of Tenax TA® is required, since 0.25 g of Tenax TA® was not enough to sustain an exhaustive *in vitro* gut extraction for all target analytes.

3.2 Tenax TA® sorption capacity to PBDEs in CEPBET components

Here the influence of the gut media on Tenax sorption was being tested. Each compartment (i.e. stomach, small intestine colon) was tested independently so Eq. 1 was not suitable for calculating PBDE sorption capacity on the different Tenax TA® batches. Hence, Eq.1 was to determine the Tenax® loadings as fractions of the total concentration in each CE-PBET compartment PBDE sorption capacity (%) was determined using equation 2 (Eq. 2), where mass FR in Tenax TA® is the FR mass (ng) determined in each Tenax TA® sample for each CE-PBET compartment and mass FR in compartment is FR mass (ng) determined in CE-PBET gut fluids separately.

$$Tenax\ Sorption\ (\%) = \frac{\text{mass FR in Tenax TA®}}{mass\ FR\ \text{in Tenax TA®} + mass\ \text{FR in compartment}}\ x\ 100 \quad \text{(Eq. 2)}$$

Shown in figure 3 are the results from PBDEs sorption to Tenax TA® in the three different CE-PBET compartments with respect to their incubation step. PBDE sorption to Tenax TA® results should not be considered as total PBDE bioaccessibility, but as the component attributable to Tenax TA® as an absorptive sink. Within the stomach compartment, BDE28 and BDE47 presented higher sorption on Tenax TA® (43.7 % and 25.6%, respectively) compared to PBDEs with higher bromine content such as BDE154 and BDE183 where Tenax TA® sorption ranged from 7.0 % to 8.8 %, respectively. Comparing stomach and colon absorption, statistically significant relationships ($p<0.01$) were noted for all target analytes, apart from BDE28 and BDE47 ($p>0.05$). Fundamental differences between stomach and

colon media formulae, ingredient concentrations (Table SI 3) and incubation times can be considered as the driving factors for the interpretation of such results. Small intestine absorption to Tenax TA® was similar to the colon for BDE28 (66.2 % and 60.0 %, respectively, whereas it was found repeatedly lower than the colon for all the other target analytes (Fig. 3) without any considerable differences. Tenax TA® sorption in the colon was higher than SI overall, but was not statistically significant for individual compounds exceptBDE183 sorption on Tenax TA® which was nearly two-fold higher in the colon in comparison to small intestine (52.6 % and 36.1 %, respectively, p=0.045). We believe that such findings are influenced by the addition of food components and bile salts as surfactants to the small intestine and colon compartments (Table SI 3). Such biological phenomena are able to enhance FR solubility and desorption potential from the dust to the gut (*i.e.* more released and freely available FR in the gut fluids), promoting thus higher FR mobilisation and sorption onto the Tenax TA sink (Oomen et al., 2004; Zhang et al., 2015). Compared to the small intestine, incubation times and the concentration of compounds enhancing desorption (e.g. mucin) are higher in the colon. All these factors combine to increase the release from the dust that a higher concentration of PBDE is in solution and hence available for subsequent sorption onto Tenax TA® .Hence, both the "solvent" capacity of the medium and the "sink" capacity of the Tenax TA® are required to achieve optimum extraction of FRs from dust as a matrix. Besides Tenax TA®, our results further support the idea of dietary components addition in CE-PBET acting as additional mechanism enhancing FR mobilisation, especially in the lipid-rich colon compartment as reported by (Tilston et al., 2011).

3.3 Method performance using SRM 2585

The selected CE-PBET parameters as well as the overall performance of our new method were assessed using SRM 2585 serving as a well-characterised and homogenous dust sample. PBDE bioaccessibility was studied using a) CE-PBET without the Tenax TA® adsorption sink, b) CE-PBET with the addition of 0.5 g of Tenax TA® and c) PBDE-spiked SRM 2585 (100 ng spike) to evaluate greater FR contamination levels under environmentally realistic conditions using SRM 2585 as the same homogenous dust sample. As observed for BAF% using a dust sample from Reading (section 3.1), statistically significant differences (p=0.03) were found in %BAF% for all target analytes when comparing CE-PBET without Tenax TA® addition (Fig. 4 A) and with Tenax TA® addition (Fig 4 B & C). The BAF% when Tenax TA® was used as an adsorption sink rerose between approximately two-fold (BDE153 and BDE183) with greater increases seen for the low-brominated and less hydrophobic FRs such

as BDE28 and BDE47 (nearly 3-fold bioaccessibility increases, respectively) (Fig. 4 B & C). No statistically significant effect ($p>0.05$) was found between the two SRM 2585 treatments (spiked and non-spiked) which both included 0.5 g of Tenax TA® and different FR contamination levels did not present any considerably different bioaccessibility values from the same dust matrix (Fig. 4 B & C). Finally, compared to the control treatments (*i.e.* no Tenax TA inclusion), the performance of the novel CE-PBET method described here using SRM 2585 offers two to three-fold gut bioaccessibility increase for a wide range of PBDEs with diverse physicochemical profiles, following a similar pattern to the indoor dust tested in section 3.1.

3.4 Proposing a unified test approach

This study describes an efficient method to physically separate Tenax TA® as an absorbent sink and indoor dust for *in vitro* bioaccessibility testing, and our model allows assessment of FRs (and potentially other HOCs) bioaccessibility from a solid matrix using artificial gastro-intestinal fluids. Previous methods used a self-designed stainless steel sieve to separate and recover Tenax TA® beads (Fang and Stapleton, 2014; ISO, 2015; Li et al., 2015, 2016). Our approach, using RC dialysis tubing provides some important benefits. Dialysis tubing is readily available, reproducible (quality controlled) and can be sourced with a wide range of molecular weight cut offs. This allows investigators to select a membrane with a MW cut off sufficient to permit free diffusion of the analytes of interest, whilst restricting passage of larger macromolecules such as enzymes or proteins that may be added to simulated GI fluids. By restricting the passage of these unwanted materials, the sorption capacity of the Tenax TA® is predominantly used for the organic pollutants rather than media components and clean up and desorption is thus simplified. The tubing functions effectively to physically separate the Tenax TA® from the solid matrix (dust) and has high pH and temperature tolerance. Our study also shows the benefits of using an adsorption sink in the CE-PBET system. Compared to controls with no Tenax TA®, inclusion of the resin increased gut bioaccessibility for PBDEs with diverse physicochemical profiles. For the low brominated BDE28, 0.25 g of Tenax TA® were insufficient for exhaustive *in vitro* gut absorption, illustrating that the amount of Tenax TA® added to the modified CE-PBET system should be optimized with respect to the physicochemical properties (e.g. $LogK_{ow}$, water solubility) of the target analytes tested. Other than BDE28, for the (hydrophobic) FR's studied here, 0.5 g of Tenax TA® was shown to be an appropriate amount to add in order to ensure released pollutants were readily adsorbed. A proposed rule can be a Tenax TA® mass loading of 0.5 g for

organic compounds with $LogK_{ow} < 6$ (e.g. BDE28, low MW PAHs etc.), while 0.25 g of Tenax TA® mass loading can be employed for very lipophilic compounds such as penta- and octa- BDEs ($LogK_{ow} > 6$).

3.5. Future work

Given the assumption that first-order permeability rates can be obtained provided that the RC tubing system is well stirred and the gut fluid is changed several times during the dialysis procedure, no kinetic characterisation was conducted in the present study. However, we believe that further kinetic characterisation of the diffusion rates of the RC membrane system should be encouraged and explored in the future. Additionally, the proposed unified gut bioaccessibility method should be further examined against different matrices (e.g. soil) and groups of emerging organic pollutants with diverse physicochemical properties. Fang and Stapleton (2014) proposed their method to be employed for flame retardants with log Kow>5 using 0.5 g of Tenax TA® mass loading in the test settings. Additionally, the ISO method on bioavailability was designed for non-polar organic compounds with log Kow>3 (ISO, 2015). Potentially, our unified method on *in vitro* gut bioaccessibility could be proposed to a wide range of organic pollutants and a rule of Tenax TA® mass loading could be established with respect to a pollutant's log Kow and water solubility values, e.g. 0.25 g of Tenax TA® mass loading should be used when testing for compounds with log Kow greater than 6 (*i.e.* more hydrophobic), whereas 0.5 g of Tenax TA® mass should be employed for organic compounds with log Kow lower than 6. Given the infinite sink inclusion, bioaccessibility parameters including a reduction of the S/L ratio could be potentially explored on the basis of mass transfer from the outside to inside of the membrane being quicker since the concentration outside would reach earlier the solubility limit.

3.6 Conclusion

Under the influence of the ISO 16751 method on the environmental availability of non-polar compounds being currently approved for registration, we propose a novel test format for assessing *in vitro* bioaccessibility of PBDEs with Tenax TA® addition as an adsorptive sink. Our data also show that the existing default assessment of risk (*i.e.* all the ingested pollutant in a solid matrix being bioavailable) is an overestimate and that the BAF% varies between ~60% (BDE47) and ~50% (BDE153). This study reveals that colon sorption to Tenax TA® for low MW BDEs was similar compared to small intestine sorption for BDE28, unlike other more hydrophobic PBDEs where colon sorption was higher than small intestine sorption.

Well designed *in vitro* bioaccessibility tests thus provide a simple approach for initial human risk assessments from ingested solid matrices giving a conservative, yet realistic indication of risk.

Supporting Information

Further details on chemicals and reagents, sample preparation and instrumental analysis are provided at supporting information.

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Artwork and Tables with Captions

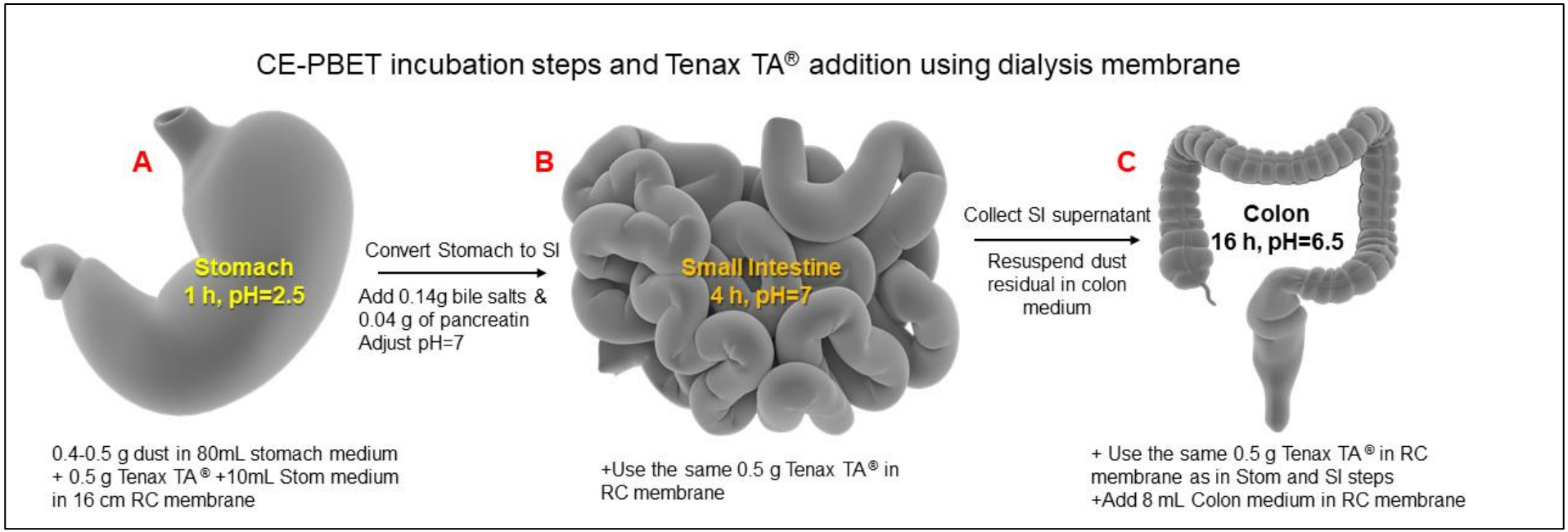

Figure 1 – Schematic representation of CE-PBET gut compartments and parameters (*i.e.* stomach (1 h, pH = 2.5), small intestine (SI) (4 h, pH = 7) and colon (16 h, pH = 6.5)) using 0.5 g Tenax TA® added in 16 cm of RC dialysis membrane.

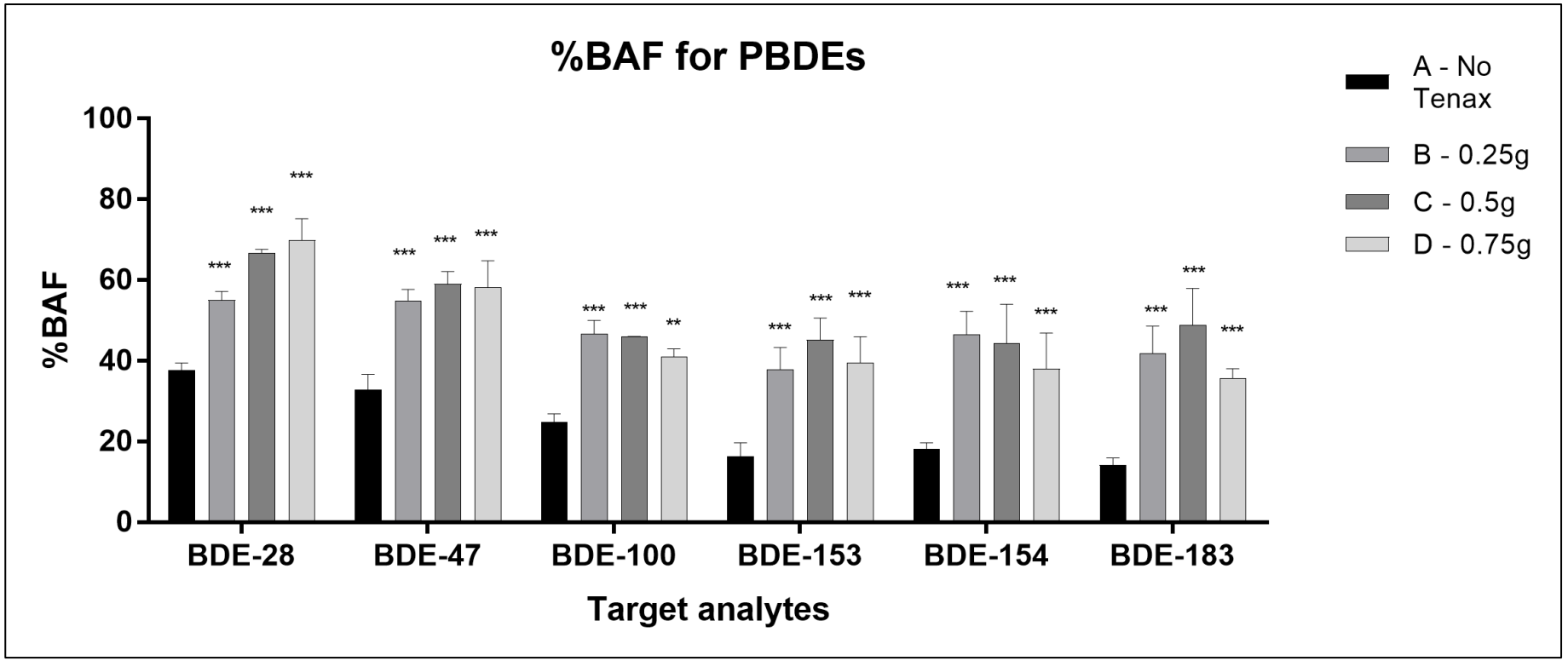

Figure 2 – CE-PBET bioaccessibility fraction (%BAF) of PBDEs without any Tenax TA® addition (control, A) and CE-PBET with Tenax TA® addition in three different amounts; i.e. 0.25 g (B), 0.5 g (C) and 0.75 g (D). Statistically significant differences shown here (**; $p<0.01$ and ***; $p<0.001$) were established between the control (A) and all Tenax TA® treatments (B, C, D). Bar charts represent average values of triplicates. Error bars represent one standard deviation.

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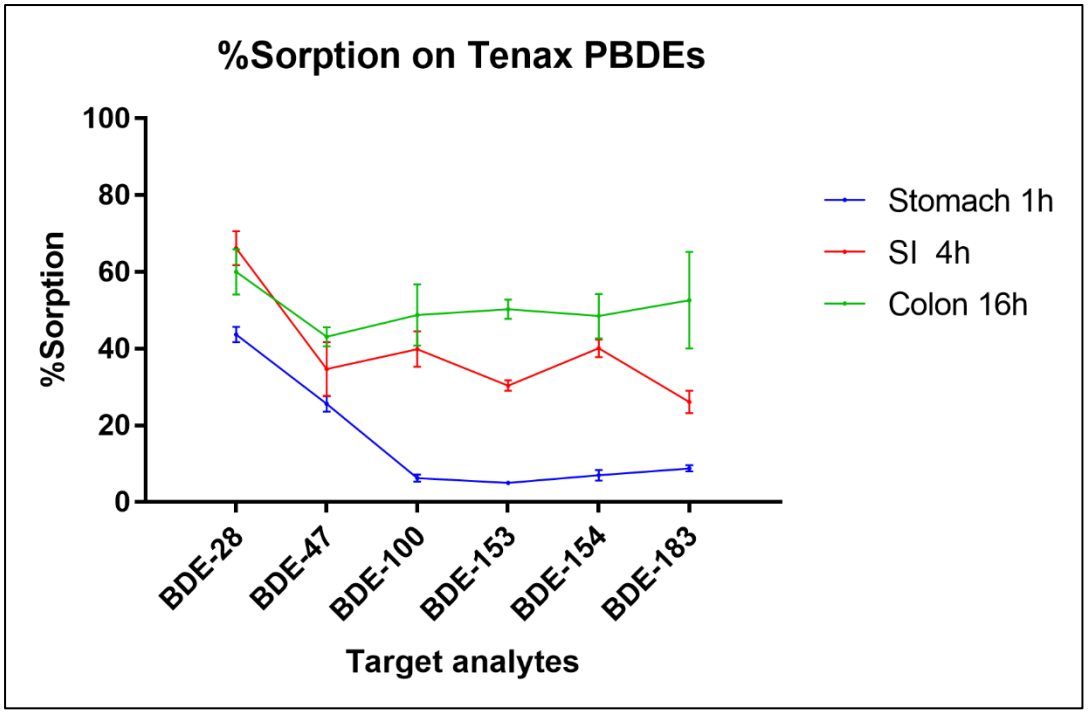

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713 **Figure 3 –** Line plots presenting FR sorption on Tenax TA® separately in stomach (1h), small intestine (SI; 4h) and colon (16h) compartments. Line plots
714 represent average values of triplicates. Error bars represent one standard deviation.

715

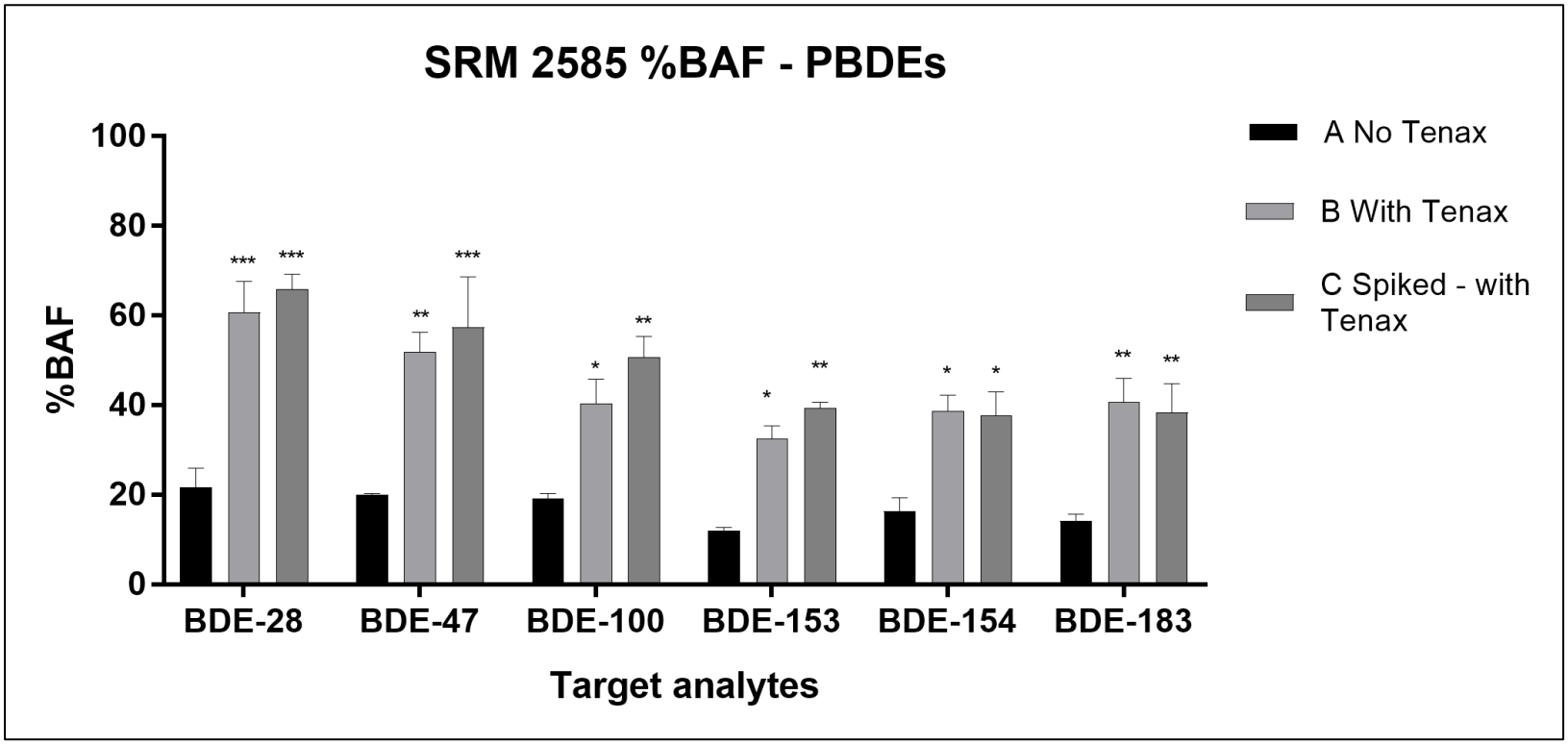

Figure 4 – Method performance of CE-PBET and bioaccessibility fraction (%BAF) using SRM 2585 without Tenax TA® inclusion (control; A), with Tenax TA® inclusion (B) and artificially spiked SRM 2585 and Tenax TA® inclusion (C). Statistically significant differences shown here (*; $p<0.05$, **; $p<0.01$ and ***; $p<0.001$) were established between control treatments of SRM 2585 without Tenax TA® inclusion (A) and treatments B and C with Tenax TA® inclusion . Bar charts represent average values of triplicates. Error bars represent one standard deviation.

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