Mimicking human ingestion of microplastics: Oral bioaccessibility tests of bisphenol A and phthalate esters under fed and fasted states

Javier López-Vázquez[1], Rosario Rodil[1*], María J. Trujillo-Rodríguez[2], José Benito Quintana[1], Rafael Cela[1], Manuel Miró[2*]

[1] *Department of Analytical Chemistry, Institute of Research on Chemical and Biological Analysis (IAQBUS), University of Santiago de Compostela, E-15782 Santiago de Compostela, Galicia, Spain*

[2] *FI-TRACE Group, Department of Chemistry, Faculty of Science, University of the Balearic Islands, Carretera de Valldemossa km 7.5, E-07122 Palma de Mallorca, Illes Balears, Spain*

[*] Corresponding authors: rosario.rodil@usc.es (R. Rodil) & manuel.miro@uib.es (M. Miró)

Abstract

Notwithstanding the fact that microplastic fragments were encountered in the human stool, little effort has been geared towards elucidating the impact of chemical additives upon the human health. In this work, standardized bioaccessibility tests under both fasting and fed conditions are herein applied to the investigation of human oral bioaccessibility of plastic additives and monomers (i.e. eight phthalate esters (PAEs) and BPA) in low-density polyethylene (LDPE) and polyvinyl chloride (PVC) microplastics. The generation of phthalate monoesters was evaluated in the time course of the bioaccessibility tests. Maximum gastric and gastrointestinal bioaccessibility fractions are obtained for dimethyl phthalate, diethyl phthalate and BPA, within the range of 55-83%, 40-68% and 37-67%, respectively, increasing to 56-92% and 41-70% for dimethyl phthalate and diethyl phthalate, respectively, whenever their hydrolysis products are considered. Bioaccessibility fractions of polar PAEs are dependent upon the physicochemical characteristics of the microplastics, with greater bioaccessibility for the rubbery polymer (LDPE). With the method herein proposed, oral bioaccessible pools of moderately to non-polar PAEs could be also accurately assessed for risk-assessment explorations, with values ranging from 1.8% to 32.2%, with again significantly larger desorption percentages for LDPE. Our results suggested that the highest gastric/gastrointestinal bioaccessibility of the eight PAEs and BPA was reached under fed-state gastrointestinal extraction conditions because of the larger amounts of surface-active biomolecules. Even including the bioaccessibility factor within human risk assessment/exposure studies to microplastics, concentrations of dimethyl phthalate, di-n-butyl phthalate and BPA exceeding 0.3% (w/w) may pose severe risks after oral uptake in contrast to the more hydrophobic congeners for which concentrations above 3% (w/w), except for diethylhexyl phthalate, would be tolerated.

Keywords: Bioaccessibility, bisphenol A, chromatography, hydrolysis products, mass spectrometry, microplastics, phthalates.

1. Introduction

Ingestion, inhalation or dermal uptake of (micro/nano)plastic particles are significant pathways of human exposure and uptake of plastic additives, such as plasticizers, flame retardants, light and thermal stabilizers, antioxidants, pigments, surfactants, lubricants, and residual monomers amongst other (ad)sorbed compounds from the surrounding medium (Cox et al., 2019; Ivleva et al., 2017; Jiménez-Skrzypek et al., 2021; Rodrigues et al., 2019). Plasticizers are widely used across the manufacturing process of a wide variety of plastic products to increase their flexibility and softness (González-Mariño et al., 2019; Hauser and Calafat, 2005; Lim, 2020; Oteef and Elhassan, 2020; Xu et al., 2020). There are several plasticizer classes, among which phthalate esters (PAEs) are the most frequent organic substances (Lowell, 2011; Ventrice et al., 2013). PAEs are considered endocrine disruptors and primarily target the male reproduction system (Diamanti-Kandarakis et al., 2009). The European Parliament Directive 2005/84/EC banned diethylhexyl phthalate (DEHP), di-n-butyl phthalate (DnBP), and benzylbutyl phthalate (BzBP) at concentration levels above 0.1% by mass in toys and child-care articles (EC, 2014b). For higher-molecular mass PAEs, namely, diisononyl phthalate (DiNP), diisodecyl phthalate (DiDP), and di-n-octyl phthalate (DnOP), the Directive ban only applies to toys that can be put into children mouths (EC, 2014a). Bisphenol A (BPA) is another yet common organic species in polymer manufacturing and is used in polycarbonate plastics and epoxy resins (Staples et al., 1998). BPA is known as oestrogen agonist and androgen antagonist with a broad range of effects on the human reproductive system (Park et al., 2020; Wu et al., 2020). The European Union regulation limited BPA to 0.02% (w/w) in thermal paper in 2020, and had previously banned BPA in polycarbonate drinking containers for infants and toddlers (EC, 2016).

It should be however noted that the total amount of an ingested contaminant (intake) does not always reflect the amount that is available to the body because it is influenced by at least three factors: (i) the release of the contaminant from the carrier matrix in the gastrointestinal tract (GIT), (ii) the absorption rate and (iii) the metabolism of the contaminant in the intestine and liver (Brandon et al., 2006). Thus, the hazardous effects of potentially contaminated environmental solid substrates should be linked to oral bioaccessible and bioavailable contaminant fractions (Brandon et al., 2006; Fedotov and Miró, 2008; Quintana et al., 2017; Trujillo-Rodríguez et al., 2020). Bioaccessibility is the percentage of a total contaminant that is extractable in the GIT and thus becomes potentially available for absorption following ingestion (Heaney, 2001; Holmes et al., 2020; Trujillo-Rodríguez et al., 2020). To evaluate the bioaccessibility of chemicals from solid materials *in-vitro* physiologically based extraction tests (PBETs) that mimic a number GIT compartments using body fluid surrogates have been reported in the literature (Collins et al., 2015; Holmes et al., 2020; Liu et al., 2020; Lu et al., 2021; Minekus et al., 2014; Rodríguez-Navas et al., 2017; Trujillo-Rodríguez et al., 2020) in line with the specifications of ISO/TS 17924:2018 (ISO, 2018). Among them, Versantvoort *et al.* (Versantvoort et al., 2005) proposed a seminal *in-vitro* digestion model to estimate the oral bioaccessibility of contaminants from food in the

human GIT that is simulated through three different compartments (mouth, stomach and upper intestine), with the secretion of saliva, gastric acid, bile and pancreatic fluids. Furthermore, the Bioaccessibility Research Group in Europe (BARGE) has proposed more recently the so-called Unified Bioaccessibility Method (UBM) (BARGE, 2011), in which physiological conditions are simulated during human digestion using the same three compartments from Versantvoort but under fasted conditions. Human PBETs have been usually resorted to risk exposure/assessment of legacy contaminants in environmental matrices or food-borne targets (Collins et al., 2015; Dean and Ma, 2007; Hur et al., 2011; Koch and Reimer, 2012; Lucas-González et al., 2018). In the case of exposure to microplastics (MPs), efforts have been geared towards mimicking the GIT of marine organisms or using avian body fluids which do not resemble those of the human GIT (Bridson et al., 2021). Very few recent reports focused on simulating human physiological conditions, yet either employed overly simplistic gut fluids without addition of inorganic and organic GIT constituents (Liu et al., 2020) or do not evaluate both fasted and fed conditions for a variety of plastic materials (Sixto et al., 2021). In addition, the detectability of chromatographic methods coupled to optical detection systems might not suffice for accurate determination of the human bioaccessible pools of the most hydrophobic, high molecular-mass PAEs in MP pellets (Sixto et al., 2021). Also, to the best of our knowledge, none of the previous articles investigated the potential degradation/hydrolysis of leachable compounds from MPs under biorelevant PBETs notwithstanding the fact that hydrolysed compounds must be ascertained for accurate determination of the overall bioaccessible and potentially bioavailable pools of plasticizers from plastic particles.

The aim of this work is to evaluate the human bioaccessibility of BPA and PAEs from MPs and the potential generation of hydrolysis/transformation products under *in vitro* physiologically relevant digestion conditions for the gastric and small intestine compartments in a risk assessment framework using two scenarios: (i) the fed state exploiting the Versantvoort model, and (ii) the UBM fasted-state model. For that purpose, two certified reference materials (CRM) containing PAEs and BPA with a broad range of polarities were selected: (i) low-density polyethylene (LDPE) and (ii) polyvinyl chloride (PVC) that differ each other on structural rigidity, surface properties and particle size. Critical variables and interactions thereof that drive the extent of release of target compounds physically sorbed onto MPs were assessed by multifactor ANOVA tests.

2. Material and methods

2.1. Reagents and materials

Ethyl acetate (AcOEt) GC-MS grade was purchased from Panreac (Castellar del Vallès, Spain) and methanol (MeOH) HPLC-MS grade from Fisher scientific (Portsmouth, NH, USA). Dichloromethane (DCM) Pestinorm grade was obtained from VWR (Radnor, PA, USA). Acetic acid and formic acid

HPLC-MS grade were purchased from Scharlau (Sentmenat, Spain). Alumina (Al_2O_3), hydrochloric acid 37% and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Merck KGaA (Darmstadt, Germany).

Analytical standards of BPA, dimethyl phthalate (DMP), diethyl phthalate (DEP), DnBP, BzBP, DEHP, DnOP, DiNP, DiDP and deuterated standards used as internal standard (IS) (i.e., DMP-d4, DnBP-d4, BPA-d16 and DEHP-d4) were purchased from Merck KGaA. Analytical standards of phthalate monoesters, namely, monomethyl phthalate (MMP), monoethyl phthalate (MEP), monobutyl phthalate (MBP), monobenzyl phthalate (MBzP), mono-(2-ethylhexyl) phthalate (MEHHP), mono-(2-ethyl-5-carboxylpentyl) phthalate (MECPP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono-(hydroxyisononyl) phthalate (MHINP) were purchased from AccuStandard (New Haven, CT, USA) and potassium hydrogen phthalate was purchased from Merck. MMP-d4, MBP-d4 and MEHHP-d4, used as IS, were purchased from Toronto Research Chemicals (Toronto, ON, Canada). All standards were of a purity ≥ 97%. Individual stock standard solutions of ca. 1000 mg/L were prepared in AcOEt and MeOH for further separation and detection by gas chromatography-mass spectrometry (GC-MS) and ultra-high performance liquid chromatography- tandem mass spectrometry (UHPLC-MS/MS), respectively. All standard solutions were stored at -20ºC pending use.

Four distinct GIT fluids mimicking saliva, gastric, duodenal and bile phases were prepared according to Versantvoort (Versantvoort et al., 2005) and UBM (BARGE, 2011) *in-vitro* digestion models. Those complex human body fluid surrogates were composed of inorganic salts, organic compounds and a variety of enzymes, all of analytical grade purchased from Merck with a purity ≥ 97%. Each individual extractant (saliva, gastric, duodenal and bile fluids) was a composite reagent of 100 mL (50 mL for bile) obtained by mixing the so-called 'inorganic solution' and 'organic solution' (see chemical composition in the Supplementary Material, Table S1), to which a given number of solid enzymes (see Table S1) were added prior to orbital mixing using amber glass bottles. The mock-digestive fluids were prepared the day before performing the tests to ensure the dissolution and activation of all the enzyme components. Prior to undertaking the *in-vitro* bioaccessibility testing, the pH of each surrogate body fluid was adjusted by dropwise addition of NaOH (1 M) or HCl (37%) to ensure the pH in the tolerance range specified by Versantvoort and UBM (Table S1). The fluids were kept overnight at room temperature and heated to 37 ± 2 ºC one hour prior to carrying out the bioaccessibility tests.

Two certified reference materials (CRM) of LDPE (CRM-PE002) and PVC (CRM-PVC001) MPs (Spex CertiPrep, Stanmore, UK), with average particle sizes of 110 µm and 140 µm (see SEM images in the Supplementary Material Fig. S1 and S2), respectively, with certified concentrations of DiDP and DiNP at ca. 30,000 µg/g level, and DMP, DEP, DnBP, BzBP, BPA (only in LDPE), DEHP and DnOP at ca. 3,000 µg/g level were used in this study (see actual certified concentrations in Table S2).

To minimize contamination, all glassware were baked at 300 °C for 12 hours before use, and alumina (3% (w/w)) was added to ethyl acetate (González-Mariño et al., 2019).

2.2. *In-vitro* fed and fasted human bioaccessibility models

The digestion process in the GIT of humans is herein simulated by applying physiologically relevant extraction conditions, i.e. the complex chemical composition of the digestive fluids, pH, and residence periods expected in every GIT compartment. Fed (Versantvoort) (Versantvoort et al., 2005) and fasted (UBM) (BARGE, 2011) models encompass a three-step additive procedure mimicking the GIT transit of the chyme, and the sequential extraction processes of ingested material in mouth, stomach, and small intestine, as these compartments are accounting for the largest percentage of bioaccessible pools, which can ultimately reach the systemic circulation.

A diagram of the workflow of both fed and fasted state tests is illustrated in Fig. 1. In brief, the oral bioaccessibility tests were performed by accurately weighing 0.1 g of LDPE or PVC MPs into glass test tubes by triplicate. Then, 1.2 mL or 1.5 mL (fed/fasted) of saliva fluid was added and mixed manually for 10 seconds. Thereafter, 2.3 mL of gastric fluid was added, and the pH adjusted by the addition of 1 M NaOH or 37% HCl within the pH interval between 2-3 for the fed state and pH =1.20 ± 0.05 for the fasted state. Then, the samples were incubated at 37 ± 2 ºC for 2 hours (fed state) or 1 hour (fasted state) under agitation using an end-over shaker at 37 rpm. For estimation of the gastric bioaccessible fraction, the gastric extracts were retrieved by sample centrifugation at 1500 rcf for 30 minutes, whereupon an aliquot of supernatant was collected in a glass vial.

For assessment of the gastrointestinal bioaccessible fractions, 2.4 mL or 4.6 mL (fed/fasted) of duodenal fluid and 1.2 mL bile and, only under fed conditions 0.4 mL of 1 M $NaHCO_3$, were added to the gastric phase. The pH was adjusted to the interval of 6.5-7 in the fed state or to 6.3 ± 0.5 in the fasted state. The gastrointestinal extraction lasted 2 hours (fed state) or 4 hours (fasted state) under physiological temperature and identical shaking conditions as those of the gastric phase. Finally, the MP suspension was centrifuged at 1500 rcf for 30 min and an aliquot of supernatant was collected in a glass vial.

SEM images of LDPE and PVC after gastric and gastrointestinal extractions for both PBETs (Figure S1 and S2) revealed that there are no appreciable changes on neither the average particle size nor the characteristic spherical-shaped and brain-shaped particles for LDPE and PVC MPs, respectively.

2.3. Determination of the bioaccessible fraction of PAEs and BPA in microplastics

The determination of the bioaccessible fraction of PAEs was performed by dilute and shoot with a 1:100 (v/v) dilution of the gastric extracts and 1:40 (v/v) of the gastrointestinal extracts taking into account the larger volume of gastrointestinal phase, with the subsequent potential dilution of the extracted

species. In both cases ultrapure water/methanol (80:20, v/v) was used as diluent. The percentage of methanol was selected to minimize the sorption of PAEs onto the surface of the borosilicate glass and tubing of the analytical detection instruments. ISs were added to the final extract at a concentration level of 700 µg/L. 1 mL-aliquots of the extracts were filtered through hydrophilic polytetrafluoroethylene (PTFE) filters (Ø 13 mm, 0.22 µm) from Phenomenex (Torrance, CA, USA) followed by percolating 250 µL methanol through the filters to prevent losses of the target species. The extracts of the PBETs (including the filtered methanol) were further analysed by UHPLC-MS/MS.

For determination of the oral bioaccessible BPA, a prior liquid-liquid extraction (LLE) was performed. To this end, 100 µL of the gastric or gastrointestinal extracts containing 700 µg/L IS was extracted with 2 mL of AcOEt. A volume of 20 µL of the extracts was derivatized with 30 µL of MSTFA at 60 °C for 1 hour and further analysed by GC-MS.

Detection and quantification of potential degradation/hydrolysed products (viz., phthalate monoesters) of the bioaccessible PAEs were performed by dilute and shoot with a dilution 1:7.5 of the gastric extract or 1:3 of the gastrointestinal extract using ultrapure water/methanol (80:20) with a final concentration of 200 µg/L of IS-metabolites. Aliquots of 500 µL of the extracts were filtered through hydrophilic PTFE filters (Ø 13 mm, 0.22 µm) and after that 125 µL methanol was percolated through the filter. The IS containing extracts and washing methanol were analysed by UHPLC-MS/MS.

2.4. Determination of the non-bioaccessible fraction of PAEs and BPA in microplastics

The residual MPs after the PBETs were transferred to a 20 µm-steel mesh (3 × 3 cm) (Filtra Vibración, Badalona, Spain), washed with 4 mL of ultrapure water and dried at 40ºC overnight. Then, the MPs were transferred to a glass vial and extracted with 2 mL of DCM by ultrasonic solvent extraction (USE) during 30 min at room temperature. The supernatant (1 mL) was filtered through hydrophobic PTFE filters (Ø 13 mm, 0.22 µm).

An aliquot of 10 µL of the DCM extract was diluted with ethyl acetate (1:200, v/v) and ISs were added at a final concentration of 700 µg/L prior to determination of PAEs by GC-MS. The determination of non-bioaccessible BPA in LDPE was undertaken following a derivatization reaction at 60 °C for 1 hour with the addition of 30 µL MSTFA to 20 µL extract.

Another 10 µL aliquot of the DCM extract was diluted 1:2000 (v/v) with methanol and ISs were added at a final concentration of 700 µg/L for further determination of non-bioaccessible DiNP and DiDP by UHPLC-MS/MS.

2.5. GC-MS analysis

GC-MS determination of oral bioaccessible BPA and non-bioaccessible BPA and PAEs, excepting DiNP and DiDP, was carried out by a 7890A gas chromatograph interfaced with a triple-axis Detector mass spectrometer (MSD 5975C, Agilent Technologies, Santa Clara, CA, USA). Separation was performed onto a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) supplied by Agilent. The GC oven temperature was programmed as follows: 60 °C for 1 min, then ramped to 250 °C at 15 °C/min and held for 10 min, and finally increased to 280 °C at 5 °C/min and held for 10 min. Two microliters of the extract were injected in splitless mode using an Agilent 7693 series autosampler. Injection port, transfer line, quadrupole and source temperatures were set at 280°C, 280ºC, 150ºC and 230ºC, respectively. Helium 99.9999% (Nippon Gases) at a flow rate of 1 mL/min was used as a carrier gas with a solvent delay set at 7.5 min.

Acquisition was performed with an electron impact ionization (EI) source at 70 eV and operated under selected-ion monitoring mode (SIM) (see Table S3). The instrument was controlled by Agilent Chemstation E.02, and MassHunter Quantitative Analysis MS software v.10.1 (Agilent) was used for MS data treatment.

2.6. UHPLC-MS/MS analysis

UHPLC-MS/MS analyses were performed in a Waters Acquity UPLC H class system (Milford, MA, USA), equipped with a sample manager, a quaternary solvent pump, and a column oven thermostated at 40ºC, coupled to a triple quadrupole mass spectrometer Xevo-TQD (Waters) with an electrospray ionization (ESI) source. Nitrogen, used as desolvation and cone gas, was provided by a nitrogen generator (Peak Scientific, Barcelona, Spain), and argon used for the collision induced dissociation, was purchased from Nippon Gases (Tokyo, Japan). Ionization was performed in positive mode using the following parameters: 4 kV (capillary voltage), 150 °C (source temperature), 500 °C (desolvation temperature), 1000 L/h (desolvation gas flow, N_2) and 50 L/h (cone gas flow, N_2). Collision energy (CE) and cone voltage (CV) values were adjusted individually for every compound. Analyses were done in selected reaction monitoring (SRM) mode recording one (IS) or two (analytes) precursor/product ion transitions per compound. Selected transitions, together with their corresponding CE and CV values, retention times (RT) and labelled compounds used as IS are listed in the Supplementary Material, Tables S4 and S5.

Separation of PAEs and BPA (in preliminary tests) was carried out on a Synergi 4u Fusion-RP 80 Å C18 column (100 mm × 2.0 mm × 4.0 μm) from Phenomenex with a dual eluent system consisting of (A) ultrapure water containing 0.1% of formic acid and (B) MeOH containing 0.1% of formic acid at a flow rate of 0.4 mL/min. The gradient elution started with 5% B, increased linearly to 100% B in 10 min,

and held at 100% B for 4 min. Returning to initial conditions (5% B) was performed in 0.1 min and held for 6 min for column reconditioning. Injection volume was set to 1 μL.

Separation of phthalate monoesters was carried out on a Raptor Biphenyl 90 Å C18 column (150 × 2.1 mm × 1.8 μm) from Restek (Bellefonte, PA, USA) as described elsewhere (Estévez-Danta et al., 2021). Briefly, a dual eluent system consisting of (A) ultrapure water containing 0.1% of acetic acid and (B) MeOH containing 0.1% of acetic acid at a flow rate of 0.3 mL/min was used. The linear gradient elution started with 50% B, increased to 100% B in 17 min, held at 100% B for 5 min, and finally returned to initial conditions (50% B) in 0.05 min and held for 5 min for column reconditioning. Injection volume was set to 2 μL.

The software MassLynx v4.1 and TargetLynx v4.1 (Waters) were used for control and data treatment, respectively.

2.7. Statistical analysis

Statistical data treatment was performed using the Statgraphics Centurion XVIII software (Statpoint Technologies, Warrenton, VA, USA). Analysis of variance (ANOVA) was conducted to evaluate those factors that could potentially influence the oral bioaccessibility of PAEs and BPA, i.e. body fluids (gastric vs gastrointestinal compartments), MP type (LDPE vs PVC) and *in-vitro* (fed vs fasted) test model. The statistical significance boundary was set to $\alpha = 0.05$ in all cases.

3. RESULTS AND DISCUSSION

3.1. Evaluation of the analytical performances of the chromatographic and extraction methods

The liquid and gas chromatographic methods using internal calibration as indicated in Tables S3, S4 and S5 were evaluated in terms of linearity, precision and limits of quantification (LOQs) for the target compounds.

For GC-MS, the dynamic linear range of all compounds in H_2O/MeOH (80/20, v/v) spanned between 1 μg/L and 10 mg/L, except BPA up to 5 mg/L, BzBP and DnOP from 5 μg/L and DiNP and DiDP from 0.5 to 40 mg/L, obtaining determination coefficients in all instances higher than 0.9990. Repeatability, expressed as relative standard deviation of 5 replicates at a concentration of 50 μg/L (1 mg/L for DiNP and DiDP), ranged between 5 and 19%, and LOQs, calculated for a signal-to-noise ratio of 10, ranged from 0.01 to 1.35 μg/L, except for DiNP and DiDP with LOQs of 500 and 300 μg/L, respectively (Table S3).

For UHPLC-MS/MS, the dynamic linear range spanned between 1 μg/L and 5 mg/L, except for long-chain PAEs (DiNP and DiDP) up to 10 mg/L, BPA from 0.5-10 mg/L and DnOP from 0.1-10 mg/L,

obtaining determination coefficients in all instances higher than 0.9990. Repeatability at 100 µg/L (1 mg/L for BPA) with 5 replicates, was below 19%. LOQs, calculated for a signal-to-noise ratio of 10, ranged from 0.10 and 0.70 µg/L, except for DnOP (67 µg/L) and BPA (500 µg/L) (Table S4).

Based on the above results, GC-MS was used for the determination of BPA and the non-bioaccessible fraction of PAEs except for DiNP and DiDP, and UHPLC-MS/MS for the determination of the bioaccessible fraction of PAEs and the non-bioaccessible fraction of DiNP and DiDP.

For the extraction of the residual PAEs and BPA from MPs to estimate the non-bioaccessible fraction, various solvents (AcOEt and DCM) were tested by USE. The results of the analysis of the CRM MPs, expressed as absolute recoveries, are summarized in Table S6. The extraction recoveries with DCM were improved for BzBP, DnOP and DiDP. Therefore, DCM was selected for the further extraction of non-bioaccessible fractions with recoveries from total certified concentrations on LDPE and PVC ranging from 57 to 90% and 77 to 117%, respectively. Repeatability, expressed as RSD, was below 20%. LOQ values, calculated for a signal to noise ratio of 10, ranged from 0.05 to 7.45 µg/g (see Table S6).

Matrix effects for the determination of oral bioaccessible PAEs by UHPLC-MS/MS were evaluated by comparing the analytical responses of spiked GIT fluids against those of standards prepared in H_2O/MeOH (80/20, v/v) at a concentration level of 400 µg/L. The experimental results revealed that the responses of the long-chain phthalates (DEHP, DnOP, DiNP and DiDP) were those most affected and ranged from 61 to 87% for the gastric fraction, and 73 to 93% for the gastrointestinal fraction as compared to the responses of the standards. Signal suppression was below 40% for all the compounds but compensated with the isotopologues as indicated in Tables S3 and S4.

The LLE method for the extraction of BPA from both gastric and gastrointestinal extracts to estimate the bioaccessible fraction was performed with different solvents (AcOEt and DCM). To this end, an aliquot of 100 µL of body fluids spiked with BPA (700 µg/L) was extracted with 2 mL of AcOEt or DCM. Recoveries were similar for DCM (111-113%) and AcOEt (108-120%). However, AcOEt was selected for LLE extraction because of its suitability for further analyte derivatization. Repeatability, calculated at 700 µg/L by triplicate and expressed as RSD, was below 5%. LOQ values, calculated for a signal to noise ratio of 10, were 0.25 and 0.35 µg/L BPA for gastric and gastrointestinal fluids, respectively.

The UHPLC method for the separation and determination of phthalate monoesters has been validated previously by Estévez-Danta *et al.* (Estévez-Danta et al., 2021) (Table S5). Briefly, the dynamic liner range spanned from LOQ-1000 µg/L, LOQs ranged between 0.01 µg/L and 6 µg/L and RSDs at 10 µg/L were below 19%. Matrix effects for metabolites were between 85 to 98% and 72 to 88% for gastric

and gastrointestinal fractions, respectively, yet were offset using the deuterated IS as indicated in Table S5.

3.2. Stability of the target PAEs and BPA in GIT fluids

Preliminary tests were performed to investigate the stability of the PAEs and BPA under gastric and gastrointestinal conditions for fed and fasted oral bioaccessibility tests. For that purpose, gastric and gastrointestinal fluids were spiked by triplicate with 7.5 mg/L and 3 mg/L, respectively, of the target PAEs and BPA, to obtain a final concentration of 0.075 mg/L after dilution, and incubated at physiological conditions as described in Section 2.2 and determined as section 2.3. Absolute recoveries after gastrointestinal incubation ranged between 82 and 113% (Figure S3a). Phthalate monoesters were also determined to elucidate their potential generation from the parent phthalate diesters in both gastric and gastrointestinal compartments. Experimental findings demonstrated that MMP, MEP and phthalic acid were the only compounds formed in the incubated samples. Assuming that MMP and MEP are only formed by the hydrolysis of DMP and DEP, respectively, and phthalic acid is equally obtained from both DMP and DEP, the molar conversion percentages are reported in Figure S3b. Experimental results indicated that up to a 10% of hydrolysis occurs for DMP and DEP under gastrointestinal extraction with significantly higher percentages under fasted conditions than those of fed conditions (down to 0.5%). This fact could be attributed to the more acidic gastric phase in the UBM test (pH 1.2 ± 0.5) (Fig.1) since pH affects the hydrolysis rates of PAEs (Harris and Sumpter, 2001). In order to evaluate if the transformation of DMP and DEP is due to the enzymatic activity or the chemical hydrolysis, *in-vitro* digestion was performed under fasted conditions without the addition of enzymes. No statistically significant differences were observed in the extent of generation of MMP, MEP and phthalic acid. This confirms that degradation of DMP and DEP is mainly occasioned by chemical hydrolysis and triggered under fasted conditions.

3.3. Fed and fasted human oral bioaccessibility tests

The bioaccessible fractions of PAEs and BPA were calculated related to the certified concentrations provided by the CRMs. The extent of release of the compounds from MPs during human digestion was elucidated by the measurement of the leachable compounds in the respective biorelevant gut fluid (gastric and gastrointestinal phases). Note that the bioaccessible fraction represents the maximum amount of compound amenable to be bioavailable and reach the systemic circulation. The percent of bioaccessibility of PAEs and BPA in LDPE and PVC using fed and fasted PBET conditions is presented in Table 1, and exemplarily summarized in Figure 2 for DMP and DiDP. Bioaccessibility values ranged between 2% and 83% with the highest bioaccessibility corresponding to DMP, DEP and BPA compared

to the other PAEs (Table 1). Hydrolysis of PAEs during the bioaccessibility tests was also evaluated. MMP, MEP and phthalic acid were the only degradation products identified across the varied GIT fluids as discussed in the previous section, with concentrations of hydrolytic products ranging from 0.7 and 7% (w/w) of the total DMP and from 0.3 and 3% (w/w) of the total DEP (Table 1 & Figure 2). Total bioaccessibility (sum of bioaccessible and hydrolysis fractions) ranged between 1.8% for DiDP from PVC in the gastric fraction under fasted conditions to 90% for DMP from LDPE in the gastrointestinal fraction under fed conditions. These results are similar to those previously reported using a dynamic *in-vitro* PBET for PAEs and BPA (Sixto et al., 2021), to those of inhalation (lung) bioaccessibility (Kademoglou et al., 2018) and also to those of GIT bioaccessibility of PAEs in indoor dust (He et al., 2016). Regardless of the polymer type, the % bioaccessibility is inversely correlated with the hydrophobicity of the compounds (log K_{ow}), with Spearman correlation *p-values* <0.0004. The mathematical model that better fits the experimental data is %bioaccessibility = a + b/log K_{ow} (Figure S4) with correlation coefficients spanning between 0.9087 and 0.9668 for the two types of MPs and PBET methods.

The residual fraction of phthalates and BPA in MPs (non-bioaccessible fraction) was evaluated by the analysis of the MPs after the PBET as explained in Experimental. Total non-bioaccessible fractions ranged from 13 to 108 % (Table 1 & Figure 2).

Finally, a mass balance study was performed by considering the three fractions: bioaccessible fraction, non-bioaccessible fraction and hydrolysed fraction (see Figure 2 and Table 1). The percentages for LPDE under gastric extraction ranged from 78 to 112% and 82 to 117% for the fasted and fed scenarios, respectively. The percentages for LPDE under gastrointestinal extraction spanned from 84-118% and 84-126% for the fasted and fed conditions, respectively. As to PVC, the percentages under gastric and gastrointestinal extraction ranged from 68-112% and 69-94%, respectively, for the fasted state and 62-114% and 70-102%, respectively, for the fed state. It should be noted that absolute recoveries down to 70% are encountered, in some instances, for DEP, BzBP, and DINP for all of which congener isotopologues were used.

3.4. Evaluation of critical parameters influencing oral bioaccessible fractions

The effect of the polymer type, the *in-vitro* PBET method and the GIT compartment on the magnitude of the bioaccessible fraction was investigated using multifactor ANOVA. For BPA, the effect of MP composition could not be evaluated since BPA is only certified in LDPE MPs. As seen in Table 2, the ANOVA test revealed that all the factors are statistically significant (*p-values* <0.05) for all of the studied compounds. For example, the experimental findings indicated that the lowest bioaccessibility in both gastric and gastrointestinal compartments and both PBETs is encountered for the glassy PVC microplastics (Table 1, Figure S5 and Fig S6), which is in good agreement with previous observations

for other xenobiotics (Liu et al., 2020). In case of the most polar PAEs, because of the small differences in average particle size of LDPE against PVC MP the lower bioaccessibility from PVC could be attributed to the large heteroatom/C ratio in PVC because of the chloride content of the material as compared to LDPE (only contains alkyl chains) that facilitates strong polar interactions with the less hydrophobic species as previously observed by (Liu et al., 2020) . Regarding the PBET method, bioaccessibility using fed conditions is significantly higher than that of fasted conditions and, this is likely due to the elevated concentration of enzymes and bile salts acting as surfactants in the gastrointestinal fluids thereby increasing analyte solubility in the gut fluid and triggering displacement from the MP surface. Bioaccessibility also increases whenever the two compartments (gastric + intestinal) are considered as compared to the gastric phase alone (Figure S5), which is in good agreement with previous literature results (Raffy et al., 2018).

Two-factor interactions were also studied in this work (Table 2). Interaction between the MPs composition and the PBET method is significant for DMP, DEP and BzBP (*p-value* <0.05) and shows greater differences between the two PBET methods for LDPE MPs against PVC MPs (see Fig. 3a and S6a for DEP and BzBP, respectively). Interaction between the MPs composition and the GIT fluid is significant for all the compounds but DMP and DEHP. In the case of DEP, the increase of bioaccessibility during the intestinal step is more acute in PVC than that in LDPE (Figure 3b). On the contrary, for the other compounds, intestinal bioaccessibility increases more sharply in LDPE (Figure S6b). However, the interaction between the PBET method and the GIT fluid is not significant for any of the compounds.

3.5. Human health risk assessment

To assess the potential human health risks from PAEs and BPA via MPs ingestion, the average daily intake (ADI) of PAEs and BPA per person could be estimated from the average mass of MPs ingested per day (MPM), the total concentration of the PAEs or BPA in the MPs (C) and the oral bioaccessible fraction of each compound (BF) according to the following equation:

$$ADI = MPM \cdot C \cdot BF$$

Previous papers in the literature have estimated the number of MPs ingested by humans per time unit. For example, Cox *et al.* estimated that North Americans ingest averagely between 39,000 and 52,000 MPs per year (Cox et al., 2019), Zhang *et al.* estimated an ingestion rate up to 77,700 MPs per year from salt and water (Zhang et al., 2020) and Senathirajah *et al.* between 11,845 and 193,200 MPs/year from shellfish, salt, water and beer (Senathirajah et al., 2021). Drinking water (tap and bottled) was deemed the greatest contributor to the number of plastic particles ingested by humans. However, the number of MPs ingested by an individual will depend on a combination of highly variable parameters,

e.g., age, demographics, cultural heritage, geographic location, nature of the development of the surrounding environment and lifestyle options (Rahman et al., 2021). Moreover, Senanthirajah *et al.* provided a preliminary calculation of the potential mass of MPs that may be ingested by humans (Senathirajah et al., 2021). After the estimation of the average number of MPs ingested, they calculated the mass of an individual MP particle using a volume density approach. Considering three scenarios, the global average rate of MP mass ingestion ranged between 7.7 g and 287 g per person per year (0.021 – 0.786 g per person per day) (Senathirajah et al., 2021).

The concentration of PAEs and BPA in MPs can differ significantly by the origin and ageing of the MPs ranging from the low ng/g in MPs sampled from sea water to mg/g in raw plastic materials (Table S7). In fact, it is known that plastic materials usually contain 0.1–5% of phthalates as the certified MPs considered in this work (Paluselli et al., 2019). Therefore, three scenarios were considered to calculate the ADI of PAEs and BPA, namely, low (1 ng/g), medium (10 µg/g) and high (3 mg/g) content of PAEs and BPA in MPs.

The BFs used for ADI calculations were the gastrointestinal bioaccessibility data reported in Table 1, and included the sum of bioaccessible and hydrolysis fractions for the two types of MPs. Both UBM and Versantvoort tests were considered. The human ADI of PAEs and BPA via MPs per person considering a high exposition level (0.786 g MP/(person·day)) ranged from 0.04 - 0.7 ng/(person·day) under the first scenario, 0.4 - 7 µg/(person·day) under the second one and 124 - 2128 µg/(person·day) under the third one. Results were compared against the human safe reference values based on either the oral reference doses (RfDs) provided by the United States Environmental Protection Agency (U.S. EPA) (EPA) or the tolerable daily intakes (TDI) provided by European Food Safety Authority (EFSA) (EFSA, 2015, 2019) and considering an average adult body weight of 70.8 kg (Walpole et al., 2012). As shown in Table 3, the levels of exposure to PAEs and BPA were far below the safe reference values even under the third scenario (high level content of additives, *viz.* 3000 µg/g), except for DMP, DnBP and BPA. For DMP, the daily intake at the high level content of plasticizer is always higher than the safe reference value for adults considering the US EPA RfD regardless of the type of MP and the fed/fasted gastrointestinal digestion conditions. By considering the distinct scenarios for BF calculation, the ADI of DnBP at the high level content of plasticizer is close to or slightly higher than the safe reference value based on the EFSA TDI but lower if the US EPA RfD is considered. Moreover, the estimated ADI of BPA at the high level content was between 4 and 6 times higher than the safe reference value based on the EFSA TDI but did not exceed the limit posed by US EPA RfD. Very recently, there is a public consultation about EFSA draft opinion proposing lowering the TDI of BPA to 0.04 ng/(kg·day) (EFSA, 2021), leading to a human safe reference value for an adult of $2.8 \cdot 10^{-3}$ µg/(adult·day), which is far below the ADI under the second scenario. In summary, the human uptake of primary MP might pose severe health risks to humans because of the leachability of the most polar

additives, namely, DMP, DnBP and BPA, at expectable concentrations in plastic materials under gastrointestinal digestion conditions.

4. Conclusions

This article is aimed at shedding light on the human oral bioaccessibility of PAEs and BPA with different range of polarities (log K_{ow} 1.98-9.65) from LDPE and PVC MPs by *in-vitro* PBETs tests using fed and fasted conditions. The oral bioaccessibility of PAEs and BPA in the gastric compartment usually accounts for more than 65% of overall bioaccessibility and increases significantly for those compounds with log K_{ow} < 4.0, with the highest leachability values for DMP, DEP and BPA. It should be however noted that DMP and DEP were partially hydrolysed under gastrointestinal conditions with the subsequently formation of MMP, MEP and phthalic acid. In addition, PAEs and BPA were released to a larger extent from LDPE than from PVC, which is most likely attributed to the differential chemical sorptive properties of PVC against LDPE, including the structural rigidity of the glassy PVC that might lead to significant desorption irreversibly and low diffusion kinetics of the most hydrophobic compounds from the rigid pores, and the increased surface polarity of PVC against the rubbery LPDE that fosters adherence of the most polar additives. The superior surface area in contact with the body fluids of LDPE vs PVC on account of the significantly higher density of the latter and the lower average particle size of LDPE MPs (110 μm for LDPE vs 140 μm for PVC) might also contribute to the greater oral bioaccessibility of the plastic additives from LDPE MPs. In addition, our results signalled that the larger amounts of enzymes in suspension and bile salts that lead to the formation of micelles under fed state conditions may account for the observed enhancement of the bioaccessibility of plastic-borne organic compounds compared to fasted state conditions.

The estimated human ADI, taking into account the overall oral bioaccessibility data calculated in this work, indicated that the accidental ingestion of MPs exceeding 3000 μg/g (i.e. 0.3% (w/w)) of DMP, and DnBP or BPA might generate a real risk to human health on account of the US E.P.A RfD and/or EFSA TDI values.

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