Automatic flow-through system for exploration of the human bioaccessibility of endocrine disrupting compounds from microplastics

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Abstract

This article reports on the first attempt towards investigating the leaching rates in the human gastrointestinal (GI) tract of plastic-borne contaminants that can be ingested accidentally using physiologically relevant body fluids. Oral bioaccessibility under fasted and fed state was undertaken under dynamic mode exploiting an automatic flow setup. The flow system is able to mimic the fast uptake of released species from the polymeric matrix by absorption in the human digestive system by in-line removal of leached species. Complex GI extractants based on the Unified Bioaccessibility Method (UBM, fasted-state) and Versantvoort test (fed-state) were brought through a microplastic-loaded metal microcolumn for semi-continuous leaching of plasticizers (phthalic acid ester congeners) and monomer/antioxidant species (bisphenol A, BPA) followed by in-line solid-phase extraction and clean-up of GI extracts prior to liquid chromatographic analysis. The temporal extraction profiles were fitted to a first-order kinetic model for estimation of maximum bioaccessibility pools and apparent leaching rates.

Among all studied contaminants, only BPA, dimethylphthalate and diethylphtalate were appreciably released under dynamic GI conditions from high-density polyethylene pellets (average size of 110 µm), with average bioaccessibility values spanning from 51 to 84%, and 48 to 87% for UBM and Versantvoort's methods, respectively. No statistically significant differences in oral bioaccessibility pools were found under fed and fasted-state dynamic extraction. The apparent kinetic constants under fed-state were greater by ≥ 30% as a consequence of the effect of the larger amounts of bile salts and digestive enzymes in the Versantvoort test on the leaching rates. The estimated average daily intakes, in which bioaccessibility data are

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contemplated, signaled that plastic materials exceeding 0.3 % (w/w) BPA might pose real risks to human health.

Introduction

Plastics are synthetic organic polymers whose properties such as versatility, strength and durability have boosted their use as a global consumer's product. In fact, the production of plastic materials exceeded 300 Mt/year in recent years.[1] A recent report by Borelle *et al.* in Science[2] foresees that between 20 and 53 Mt/year of plastic emissions could enter the world's aquatic ecosystems by 2030. Although the plastic waste generated is partially recycled, pellets from these materials still reach marine settings and fresh waters[3,4] because of inappropriate waste reduction, waste management, and environmental recovery[2] and enter into the food chain.

Covalent binding endows plastic materials with a high microbial and chemical degradation resistance, which in turn makes them behave as a new class of persistent organic pollutants. The main degradation path of plastic materials is through mechanical abrasion as a result of which smaller particles, such as mesoplastics, microplastics and nanoplastics are continuously generated, thus becoming ubiquitous contaminants of emerging concern.[5] As a consequence of their size (less than 5 mm, yet the definition has been recently revisited[6,7]), microplastics are considered potentially bioavailable to organisms along the food chain as demonstrated by the translocation of plastic particles from the external medium into tissues of aquatic invertebrates and fish.[8,9] Ingestion of microplastics can, therefore, foster introducing plastic additives and plasticizers into the base of the food chain, from where there is potential for bioaccumulation.[10,11] To tackle this situation, in 2016, the European Food Safety Authority (EFSA)[12] recommended to evaluate the occurrence of microplastics and nanoplastics in foods for which efforts were directed toward developing novel analytical procedures for isolation, identification and quantification of polymeric materials.[13,14]

By the end of the year 2018, an alarming global study that was echoed by all social media revealed for the first time the presence of a variety of microplastics (mostly, polypropylene and polyterephtalate) in human stool around the world.[15,16] Further studies also suggested that plastics from food processing and storage are making their way into the human gastrointestinal tract.[17] However, many questions arise regarding the actual sources and pathways of the plastic pellets in the human gut along with their toxicity and toxicokinetics.[18] Though microplastics have been hypothesised to act as vectors for the introduction of xenobiotics into the food chain, recent research seems to indicate that this role is negligible[19] compared to other routes of exposure, such as water and food commodities. Hence, (micro/nano) plastic toxicity derives from physical harm and the chemical effects of plastic additives and monomers.[20] To improve certain properties after extrusion (e.g., flexibility) plastic materials are usually modified during manufacturing with

several chemicals which are not chemically bonded and thus are potentially released in the environment and the human gastrointestinal tract. Among plastic additives, plasticizers (viz., esters of phthalic acid) might pose serious environmental and health risks because they are employed in high quantities[21] and are proven to behave as endocrine disruptors at concentration levels as low as the ng L^{-1} level.[22] To this end, several legislation and recommendations on exposure thresholds have been established. For example, the Water Framework Directive of the European Commission includes bis(2-ethylhexyl)phthalate (BEHP) in its priority substances list[23] and the United States Environmental Protection Agency (US-EPA) incorporated BEHP and five other phthalates as priority substances under the Clean Water Act.[24] Bisphenol A (BPA), used as antioxidant and monomer in food contact materials, is another yet plastic associated-chemical that needs to be monitored on account of its estrogen agonist and androgen antagonists effects on the human reproductive system.[25,26] In fact, the European Union banned BPA in baby bottles since June 2011 and has limited the BPA concentration to 0.02% (w/w) in thermal paper since January 2020.[27]

Because of the increasing evidence that an elevated total concentration of a potentially hazardous compound in an environmental solid may not be indicative of actual deleterious effects towards biota and human health there is a current paradigm shift towards evaluation of bioaccessible and bioavailable concentrations of contaminants[28-30] Bioaccessibility/availability tests are invaluable tools in human health risk assessment and exposure within the broad concept of exposomics.[31] Oral bioaccessibility refers to a measure of the physiological solubility of a given species in a solid matrix at the portal of entry into the body. This term should not be confused with bioavailability, which stands for the fraction of an ingested dose that crosses the gastrointestinal epithelium and thus becomes available for distribution to internal target tissues and organs.[32] To assess the bioavailability of compounds that are released into the gastrointestinal (GI) tract, *in vivo* methods have been developed and standardized over the past decades. *In vivo* testing is the most accurate method for estimation of bioavailability but must be carried out over an extended period to ensure that the compound is absorbed, retained, and excreted. Furthermore, methods using animal models are deemed ethically controversial, may not be sensitive enough to test environmentally relevant concentrations of contaminants, and required specialized personnel.[33,34] To this end, regulatory settings such as the registration, evaluation, authorization and restriction of chemicals (REACH) program of the European Union[35] suggested the replacement of *in vivo* assays by *in vitro* counterparts as cost-effective tools for oral bioaccesibility evaluation without the need of animal models. *In vitro* assays are simpler, faster, more reproducible, do not raise ethical concerns, and allow an experimental estimate of maximum bioavailable pools under 'worst case-scenarios'.[28]

There is a plethora of physiologically-based extraction tests developed for mimicking the human uptake of contaminant-borne food and environmental solids.[36-39] However, human

bioaccessibility of contaminants associated to microplastic pellets has not been to the best of our knowledge investigated as of yet. In fact, leaching tests for inorganic and organic species associated to plastic debris involved overly simplistic extraction media, including a gut surfactant mimic at varying pH and temperatures,[40,41] acidified pepsin solution,[42] or the combination of a limited number of enzymes or proteins and salts in buffer solutions,[19,43-45] which cannot reliably simulate the complexity of the human gastrointestinal tract. In this work, we are aimed at filling the gap by evaluating two *in vitro* extraction tests that are deemed most representative of the physicochemical conditions of the human gastrointestinal tract. The first method is so-called Unified Bioaccessibility Method (UBM)[46,47] and was launched by the Bioaccessibility Research Group of Europe (BARGE) as an operational procedure for harmonization of oral bioaccessibility tests under fasted conditions. The second *in vitro* digestion model, so-called Versantvoort´s test[48] modifies the UBM chemical composition, the volume of phases and the amount of enzymes and bile salts to simulate body fluid release in the GI tract under fed conditions.

In vitro tests are generally performed using batchwise protocols. However, oral bioaccessibility tests executed under end-point conditions suffer from two main shortcomings[49,50]: (i) the impossibility of mimicking the dynamic extraction processes in physiological compartments that entails the removal of desorbed compounds (e.g. by intestinal absorption); and (ii) the absence of pertinent information of the leaching kinetics at real-time, which might otherwise serve as an invaluable parameter for accurate risk assessment of accidental ingestion of plastic-borne contaminants. These shortcomings can be ameliorated by performing *in vitro* flow-through bioaccessibility assays with the aid of flow setups that are able to bring fresh portions of body fluids through the solid sample contained in a flow through chamber.[51-57]

In this paper, for the first time, in-line dynamic physiologically relevant extraction methods are developed for plasticizers and plastic additives in primary microplastic pellets, as demonstrated by the determination of oral bioaccessibility pools of phthalates and bisphenol A in low density polyethylene pellets as a proof-of-concept study. The automatic flow setup allows investigation of the leachability of plastic additives in fed and fasted state along with the in-line processing of the gastrointestinal extracts by solid-phase extraction for removal of matrix constituents prior to liquid chromatographic separations.

EXPERIMENTAL

Reagents, standards and samples

All solutions were prepared from analytical grade reagents. Ultrapure water (Millipore, Bedford, USA) with resistivity ≥18.2 MΩ·cm was employed to prepare the standard solutions and used as one of the components of the HPLC mobile phase. HPLC grade methanol and acetonitrile were

purchased from Fisher Scientific (Madrid, Spain). Analytical standards of bisphenol A (BPA), dimethylphthalate (DMP), diethylphthalate (DEP), benzylbutylphthalate (BBP), di-n-butylphthalate (DNBP), di-n-octyl phthalate (DNOP) were purchased from Sigma Aldrich/Merck KGaA (Darmstadt, Germany) and diluted or dissolved with acetonitrile to a stock concentration level of 10000 mg L^{-1} except for BBP which was diluted to a stock concentration of 2000 mg L^{-1}. An intermediate stock solution containing all the analytes in acetonitrile was prepared at the 300 mg L^{-1} concentration level. The intermediate stock solution was stored at 4 °C. 2,2-Bis(4-hydroxy-3-methylphenyl)propane (Bisphenol C, Merck KGaA) was selected as internal standard (IS). Working solutions were prepared daily by appropriate dilution of the intermediate stock with acetonitrile. The log K_{ow} values of the analytes are 3.32; 1.60; 2.47; 4.73; 4.50 and 8.10 for BPA, DMP, DEP, BBP, DNBP and DNOP respectively.[58]

Two physiologically relevant GI extractants based on the UBM[46] and Versanvoort's[48] tests were evaluated in this work. The chemical composition of the individual body fluids used for the preparation of the GI extractants is summarized in Tables S1 and S2. Each body fluid (namely, saliva, gastric and duodenal fluids and bile) is prepared the day prior to its use by mixing (i) an inorganic salt solution, (ii) an organic solution and (iii) several GI enzymes. These GI mixtures are subjected to magnetic agitation at 100 rpm for a minimum of 3 hours. The pH value of every individual fluid is then regulated with 1 mol L^{-1} NaOH or concentrated HCl, if necessary, following UBM or Versantvoort's recommendations. On the day of the dynamic GI extraction, the individual fluids are placed at 37°C for at least one hour for activation of the enzymes. The fasted-state UBM-like biofluid is obtained by mixing 9 mL of saliva, 13.5 mL of gastric fluid, 27 mL of duodenal fluid and 9 mL of bile in order to simulate the composition of the chyme in the gastrointestinal tract. If necessary, the final pH was adjusted to 6.3 ± 0.5. In the case of the fed-state GI extractant, the composite biofluid was prepared by mixing 10 mL of saliva, 20 mL of gastric fluid, 20 mL of duodenal fluid, 10 mL of bile and 3.3 mL of 1.0 mol L^{-1} $NaHCO_3$, the last one to adjust the final pH to 6.5.

The water-wettable co-polymeric N-vinylpyrrolidone-divinylbenzene Oasis HLB sorbent (average particle size of 30 μm, 80 Å pore size, Waters Corporation, Milford, Massachusetts, USA) with lipophilic/hydrophilic balance, and the Oasis Prime HLB reversed-phase sequel (Waters Corporation) composed of mixed polymeric and reversed-phase silica-type beads with average particle sizes of 30 μm and 22 μm, respectively, as identified by scanning electron microscopy equipped with energy-dispersive X-ray spectroscopy (see micrographs in Fig S1), were assayed as in-line sorptive materials for the matrix clean-up of the GI extracts.

Two certified reference materials (CRM) of low-density polyethylene (PE) pellets (CRM-PEBLK and CRM-PE002, SPEX-CertiPrep, Metuchen, New Jersey, USA) were used for analytical method development and validation. Polyethylene CRM-PE002 (with average particle size of 110

μm) containing, among others, DMP, DEP, DNBP, DNOP, BBP and BPA at a concentration level of ca. 3000 μg g^{-1}. In fact, the concentration of plasticizers in different types of plastics usually exceeds 10 % (w/w).[59] Polyethylene CRM-PEBLK did not contain the analytes (blank CRM) but was used for QC/QA purposes and evaluation of contamination sources.

Flow-based system

A schematic illustration of the flow manifold for automatic dynamic UBM and Versantvoort GI extraction of phthalates and BPA from PE microplastics is presented in Fig. 1. The flow analyser (MicroSIA, FIALab Inc., Seattle, USA) is composed of an automatic bi-directional syringe pump (SP, Cavro, Tecan, Sunnyvale, USA) equipped with a 5 mL gas-tight glass syringe (Hamilton, Bonaduz, Switzerland) and a low-pressure 26-position selection valve (VICI AG International, Schenkon, Switzerland). The SP was connected via a 700 cm long fluoropolymer tubing (FEP) of 1/16'' I.D, serving as a holding coil, to the central port of the selection valve, which by computer control allowed the aspiration of the GI fluid, the GI extractant, and the reagents for the in-line micro-solid phase extraction (μSPE) procedure.

The dynamic extraction of the chemical additives from microplastics using body fluids was facilitated by using a stainless-steel column (50 mm long, 4.6 mm ID, internal volume of ca. 0.85 mL), which was filled with 50 mg of PE microplastics and then connected in upright position to one of the ports of the selection valve (see Fig. 1). The PE pellets were weighed directly in the extraction column that was equipped with a bottom plug of melamine foam (approximately 5 mg) acting as a stopper (also of the insoluble enzymes) because of its three-dimensional porous structure. The outlet of the extraction column was connected to a 5-mL glass container (Fischer Scientific, Spain) nested to one of the ports of the selection valve (see Fig. 1) employing 30-cm long FEP tubing. The container serves to collect the GI extract fractions to which the internal standard (bisphenol C) and 9% (v/v) MeOH are automatically added prior to in-line SPE. The addition of MeOH avoided losses of the bioaccessible plastic additives by sorption onto the walls of the flow network tubing. A water bath with a digital thermoregulator (VELP Scientifica, Usmate Velate Monza e della Brianza, Italia) was used for adjusting the temperature of the holding coil, GI extractant and PE-containing column to 37.0 ± 2.0 °C.

In-line SPE was carried out using 120 mg of Oasis Prime HLB (or alternatively Oasis HLB) manually packed in 1-mL syringe body. The purified (eluate) fractions were automatically collected by a sample collector (AIM 3200, Aim Lab Automation Technologies, Virginia, Australia) equipped with a sample rack for 12 mL vials prior to at-line HPLC analysis.

The Cocosoft 5.5 user-friendly freeware [60] written in Python was used for automatic selection of the ports of the multiposition valve and the positions of the AIM 3200 autosampler and control of the flow-rate, volume and direction of the SP.

Automatic method for dynamic leaching of additives from PE microplastics under GI tract conditions in combination with in-line µSPE.

The automatic dynamic bioaccessibility method started by filling the tubing line connecting the artificial GI fluid reservoir with the selection valve (port #4), and the holding coil with the GI fluid and ultrapure water, respectively. Afterwards, 200 µL of air (port #2) were introduced into the holding coil to prevent mixing of the digestive fluid with the carrier solution. Thereafter, a metered volume of GI fluid (viz., 300 µL at 37ºC) was aspirated at 2 mL min^{-1} toward the holding coil whereupon 250 µL were brought by flow reversal to the microplastic-laden stainless steel column at 1 mL min^{-1} so as to fill the column and the tubing connecting with the 5-mL glass syringe (container attached to port #11) with GI fluid while keeping 50 µL of digestive fluid surplus and the air plug in the holding coil that were then directed to waste (port #1). This step is repeated with a GI fluid volume of 1.5 mL (fraction no. 1). The analyte containing GI extract (1.5 mL) was collected in the glass syringe and mixed with 150 µL of a methanolic solution of IS at the 160 mg/kg level (from port #9) employing a plug of air (1000 µL from port #2) for homogenization of the extract with the IS in methanol. A portion of 825 µL of the mixture containing 9% (w/w) methanol was transferred to the Oasis Prime HLB cartridge (at port #5) at a flow rate of 2 mL min^{-1}. The SPE sorbent was previously conditioned with 2 mL of 90:10 (v/v) acetonitrile:methanol from port #7, and 2 mL of ultrapure water from SP at a flow rate of 2 mL min^{-1}. After loading of the GI extract volume, the SPE sorbent was washed with 2 mL of 95:5 (v/v) water:methanol (from port # 6) and eluted with 3 mL of 90:10 (v/v) acetonitrile:methanol at 3 mL min^{-1}. The SPE eluate was collected in a given vial of the sample collector. Finally, the sorbent was rinsed with 4 mL of 98:2 (v/v) methanol:acetic acid from port #10 at 3 mL min^{-1} to eliminate potentially retained matrix components from the body fluid, and the glass container was washed with 4 mL of 100% methanol from port #8. The overall procedure was repeated 15 times for investigation of the leaching kinetics of the analytes, and the 15 GI extracts were subsequently subjected to at-line high-performance liquid chromatography-diode array detection (HPLC-DAD) analysis, as described in SM. Quantification of oral bioaccessible fractions was effected by external calibration with internal standardization using standards at the 0.5, 1, 2, 4, 7 and 10 mg kg^{-1} level containing 4 mg kg^{-1} IS in 90:10 (v/v) acetonitrile:methanol.

HPLC-DAD analysis

Determination of the oral bioaccesible pools, residual fraction and total amount of plastic additives from PE microplastics was performed by an LC-4000 HPLC system (Jasco, Tokyo, Japan) that was composed of a quaternary high-pressure pump (PU-4180), photodiode array detector (MD-4017), and Gecko 2000 column oven set at 25°C. The analytical column consisted of Pursuit PAH (4.6 × 250 mm, 5 µm; Agilent Technologies, Santa Clara, California, USA). The mobile phase consisted of water (A) and acetonitrile (B) at a flow rate of 1 mL min^{-1}. For determination of total concentrations and residual fractions of organic compounds in the pellets, the elution gradient ranged from 40 % to 100 % B in 25 minutes with further 10 minutes at 100 % B. Afterwards, the gradient returned to 40 % B in 1 min and the column was equilibrated for 5 min. For determination of bioaccessible concentrations of BPA, DMP, DEP, BBP and DNBP, including BPC as IS, the separation was optimized using the DryLab 4.2 software (Molnár Institute, Berlin). The elution gradient varied from 15% to 60% B in 20 min, raised to 88% B in 1 min and then to 100% B in 4 min with further 10 min at 100% B. Initial gradient conditions were reached in 1 min and column equilibration was effected for 5 more minutes prior to the ensuing analysis. The detection wavelength was set to 210 or 230 nm (depending on the sensitivity required) and the injection (eluate) volume was 10 µL. The retention times of the bioaccessible species, namely, DMP, BPA, DEP, and IS were 16.3 min, 18.6 min, 21.4 min and 22.5 min, respectively.

Microwave assisted extraction

A MARS 5 Digestion Microwave system (CEM Corporation, Matthews, North Carolina, USA) was employed to determine the residual (non-bioaccesible fraction) and the total amount of the analytes in CRM for mass balance validation. The microwave oven was equipped with a rotor for twelve high-pressure 100 mL-closed vessels made of modified polytetrafluoroethylene. The residual fraction or 50 mg of CRM-PE002 (or CRM-PEBLK) was extracted with 10 mL 30:70 (v/v) cyclohexane: acetone for determination of total concentrations of plastic additives. The heating programme of the microwave oven involved a ramp at ca. 12 °C min^{-1} until 140 °C in 10 min followed by extraction for 10 more minutes at 140 °C. [61] Magnetic stirring at medium speed was performed during the extraction. Prior to HPLC analysis, 2 mL of the organic solvent was evaporated at room temperature under N_2 atmosphere for exactly 10 min and was reconstituted with 2 mL of acetonitrile. Under these conditions only the more volatile analyte (viz., DMP) was influenced by the evaporation step but recoveries were in all instances > 91±2%.

RESULTS AND DISCUSSION

Configuration of the flow setup

Preliminary tests were undertaken so as to setup a flow system arrangement suitable for reliable in-line leaching of microplastic pellets unattended under physiologically relevant extraction conditions. Two flow-through devices were assayed for such purpose:

i) A large-bore column (22 mm of height, 47 mm of diameter and inner volume of ca. 3 mL as described elsewhere).[57] In brief, the microplastic-loaded container is composed of two threaded filter holders ended with fitting for standard ¼-28 female to male luer connection, a membrane filter, and two gaskets. Both filter holders contained 18 rectangular apertures (5 mm long, 1 mm width) to allow gut fluids flow freely while retaining the microplastic pellets and insoluble fluid components onto a polyvinylidiene difluoride membrane (5 µm pore size, Merck Millipore, Darmstadt, Germany).

ii) The small-bore stainless steel cylindrical column (ca. 0.85 mL inner volume) described in Experimental. A metered portion of 50 mg of CRM PE blank microplastics was employed in both devices to evaluate blank signals from the column and components of the flow system. Except for BEHP, none of the target phthalates were detected across the leachates in any configuration. The occurrence of BEHP in the leachates is a consequence of the polymeric nature of the nuts and tubing of the flow setup, and thus, the evaluation of BEHP bioaccessibility was proven unfeasible by the automatic flow-through method. Notwithstanding the fact that the polymeric large-bore column did not release phthalates at appreciable levels and none of the configurations lead to pressure drop effects, the stainless-steel holder was selected for further studies on account of its low internal volume which afforded high resolution data for near real-time monitoring of the leaching kinetics.

The coupling of the dynamic flow-through bioaccessibility test with further in-line SPE was carried out in an automatic at-line format. To this end, the GI extracts were collected in a reservoir attached to the multiposition valve with the purpose of adding a metered amount of methanol. This solvent enables compound stability in the GI extracts and the handling of a given volume of GI extract for SPE regardless of the exact volume of GI extract collected. Two reservoir formats were assayed: (i) a 5 mL-pipette-tip and a (ii) 5 mL-glass syringe barrel. Significant analyte carryover within consecutive fractions was detected with the pipette tip because residual liquid remained in the interface and because of potential adsorption of leached compounds. Therefore, the glass barrel reservoir was selected for the remainder of the studies.

With regard to the containers for packing SPE materials, 1 mL-glass and 1 mL-plastic syringes were compared in terms of potential contamination effects. Akin previous tests with components of the flow network, the release of the target compounds was not detected by HPLC-DAD for any syringe material type at levels above the ng mL^{-1} ng/mL level except for BEHP. In the final configuration, plastic syringes were attached to the multi-position selection valve because of the ease of tailoring the syringe dimensions and inner volume to the sorbent amount needed for in-line SPE of the GI extracts.

Evaluation of in-line SPE parameters

Selection of the sorbent material

Preliminary experiments were performed in a batch mode in order to assess the sorptive capacity and the clean-up effectiveness of Oasis HLB against Oasis Prime HLB as reversed-phase copolymeric materials. For this purpose, the UBM GI fluid was spiked with the target analytes (DMP, DEP, BPA, DNBP, BBP and DNOP) at the 6 µg mL^{-1} level and 2.0 mL of the GI fluid were loaded onto 60 mg of sorbent.

The retention capacity from the target compounds from the GI fluid was on a par for all of the analytes in both sorbents with recoveries ranging from 73 to 107 % and 71 to 119 % for Oasis Oasis HLB and Oasis Prime HLB, respectively. However, the matrix clean-up effectiveness was significantly superior for the Oasis Prime HLB, which afforded cleaner eluates with less HPLC interfering species from the complex GI fluid. Particularly, Oasis HLB did not serve to eliminate an interfering species from the gut fluids co-eluting with DNOP.

Sorption flow rate and analyte stabilization

The flow rate for analyte uptake by the sorptive material was studied from 0.5 to 2.0 mL min^{-1} using a sample volume of 2.0 mL of GI fluid spiked at the 6 µg mL^{-1} level, 60 mg of sorbent and elution with 1.0 mL of eluent at 2 mL min^{-1}. For BPA, DMP and DEP, no significant differences across extraction efficiencies were found with increasing flow rates, ranging from 93.7 to 96.4 % regardless of the loading flow rate. However, the extraction efficiency of DNOP increased about 28 % and those of DNBP and BBP decreased by 9 and 11%, respectively, with the decrease of flow rates from 2.0 mL min^{-1} down to 0.5 mL min^{-1}. This can be explained by the increase of the contact time of the UBM GI fluid with the sorbent, which favours the retention of DNOP, yet the unwanted sorption on the inner surfaces of the fluorinated tubing of the flow system is also boosted, as is the case for DNBP and BBP. This is most likely due to the fact that the latter compounds as opposed to DNOP seem less associated to proteins and organic constituents of the GI matrix, and, thus, DNBP and BBP are more prone to interact as free species with the PTFE tubing. A compromised flow rate of 1.0 mL min^{-1} was thus selected for further investigation of experimental parameters, but once it was shown that the leaching of DNOP from the microplastic pellets was negligible (see below), the flow rate was increased to 2.0 mL min^{-1} for amelioration of the extraction throughput.

To minimize the potential sorption of the target compounds onto the inner surfaces of the fluorinated tubing, the addition of methanol within the GI fluid was assayed. In order to prevent protein precipitation, methanol was added to the physiological fluid in quantities ≤ 20% (v/v). As expected, the effect of methanol onto the SPE efficiency was remarkable for DNBP and BBP. For

instance, the extraction efficiency of BBP increased from 66 ± 6 % (without the addition of methanol) to 92 ± 1 % when adding 9% (v/v) methanol and up to 94 ± 4% with 18% (v/v) methanol. The extraction efficiency of DNOP was not significantly different with the addition of methanol and this was the case for the rest of the compounds for which recoveries were quantitative using 0-18% (v/v) MeOH. Thus, the automatic addition of 9% (v/v) methanol to the external glass reservoir (see Fig. 1) that serves as a collector for the GI extracts was deemed necessary for stabilization of BBP and DNBP.

Breakthrough effects and sorbent amount

Analyte breakthrough was studied by loading increasing volumes from 0.5 to 6.0 mL of a spiked UBM GI solution with analytes at the 6 μg mL^{-1} level using both 60 and 120 mg of packed sorbent. The sampling flow rate was affixed to 1.0 mL min^{-1} throughout. The elution was performed with 4.5 mL of solvent at a flow rate of 2 mL min^{-1}. Surplus of solvent was employed to ensure quantitative elution of the extracted analytes. The HPLC peak areas vs sample volume plots showed a positive linear correlation for all of the analytes, thereby analyte breakthrough for the assayed sample volumes and analyte amounts was not observed for any of the compounds except for DNOP (in particular for 60 mg) with extraction efficiencies < 60% due to losses in the sampling stage. Quantitative uptake of DNOP by Oasis Prime HLB was not accomplished under flow-through conditions because of the hydrophilic-hydrophobic balance of the sorbent. Anyway, reliable measurements were still possible because of the highly repeatable extraction efficiencies obtained by the automatic flow-based method. On the other hand, the more polar compounds (BPA, DEP, DNBP, and especially the DMP) were near-quantitatively retained by the hydrophilic moieties of the Oasis Prime HLB and also rapidly eluted from 120 mg sorbent.

Elution volume and flow rate

As suggested by the sorbent's manufacturer, a mixture of 90:10 (v/v) acetonitrile:methanol was used as eluent of Oasis Prime HLB to prevent the concomitant elution of the analytes with fatty bile species from the GI matrix. In this test, 2.0 mL of 12 μg mL^{-1} spiked UBM GI fluid were brought to a 120 mg containing Oasis Prime HLB column at 1 mL min^{-1}. The elution volume was assayed between 0.5 and 3.0 mL at a flow rate of 2 mL min^{-1}, as Fig. S2 shows. Our experimental results revealed that extraction efficiencies above 95% were obtained for DMP, DEP, BPA, DNBP and BBP with a volume of eluent of 3.0 mL (see Fig. S2) that was adopted for further experiments. Larger eluent volumes are not recommended because of the excessive dilution of the eluates prior to HPLC analysis.

Further studies that involved the modification of the elution flow rate from 1.0 to 3.0 mL min^{-1} revealed the inexistence of differences across the recovery percentages for any of the target

compounds. The maximum relative percentage difference for all the conditions assayed and all the compounds was <11%. Therefore, an elution flow rate of 3 mL min^{-1} was then selected for the remainder of the work.

Temporal extraction profiles of bioaccessible pools of contaminant-borne plastic pellets using fasted and fed-state physiologically relevant tests

The main asset of the dynamic flow-through method herein proposed, as opposed to conventional bioaccessibility end-points of batch extraction, is its strict adherence to the bioaccessibility concept under conservative conditions.[62] This concept entails that the rate limiting step for human bioavailability is the release of compounds from the plastic surfaces, whereupon they are quickly uptaken by the intestinal epithelium. To this end, the automatic method was programmed to record the temporal extraction profiles until baseline level (i.e., concentrations < limit of detection (LOD)) or steady-state conditions. The latter was realized by plotting the accumulated bioaccessibility concentrations *versus* time or number of fractions (see Fig. 2a and b). Practically, the release of compounds from MP was deemed completed whenever the mass increase of each target species over the last five fractions amounted to less than 10% of the cumulative bioaccessibility pools.[50] The LODs of the phthalate congeners and BPA estimated from signal to noise ratios equal to 3 were 0.05 $mg \cdot kg^{-1}$, 0.06 $mg \cdot kg^{-1}$, 0.01 $mg \cdot kg^{-1}$, 0.02 $mg \cdot kg^{-1}$, 0.02 $mg \cdot kg^{-1}$ and 0.02 $mg \cdot kg^{-1}$ for DMP, DEP, BBP, DNBP and DNOP, respectively. Noise was calculated from peak-to-peak values adjacent to the chromatographic peaks of the target compounds. Preliminary assays were aimed at elucidating the compounds that were bioaccessible by UBM and Versantvoort assays. Out of the various MP-associated contaminants, only DMP, DEP and BPA were highly bioccessible in GI fluids. DNOP and BBP concentrations in all UBM and Versantvoort GI fractions were <LOQ and DNBP was only quantifiable in Versantvoort's test. However, the GI bioaccessibility of DNBP in several replicates was in all instances <13% and with relative standard deviation (RSD) of the non-bioaccessible fraction >20%. For this reason, it was excluded for further analytical validation and risk assessment/exposure studies. These results are in agreement with previous findings by Heinrich and Braunbeck[63] who stated that compounds with log k_{ow}<<5 might be readily leachable from MP because of hysteresis indexes tending to zero, thus indicating desorption reversibility.

The bioaccessible pools of DMP, DEP and BPA were calculated from the cumulative temporal extraction profiles illustrated in Fig. 2 and given in Tables 1 and 2. Fifteen fractions (1.5 mL GI fluid each) suffice for exhaustive extraction of bioaccessible amounts of overall plasticizes and BPA for both UBM and Versantvoort's methods. A schematic illustration of selected chromatographic runs of the UBM extraction pattern (fractions 1,5, 10 and 15) is shown in Fig.

S3. Analysis of pristine PE CRM material for QA/QC studies revealed that none of the target species nor potential matrix components after in-line SPE were detected in the chromatogram.

The relative oral bioaccessibility pools of DMP, DEP and BPA from CRM-PE MP in UBM and Versantvoort ranged from 51 to 81% and from 48 to 87%, respectively (see Tables 1 and 2). Notwithstanding the larger amounts of bile salts and increased concentrations of pancreatin and lipase under fed-state conditions, no statistically significant differences ($p>0.05$) were encountered for any of the compounds under fed or fasted dynamic extraction methods, even for BPA for which leachability percentages only ranged from 45-57%. Assessment of the trueness of the automatic flow method for oral bioaccessibility studies was ascertained by mass balance evaluation. Hereto, the sum of oral bioaccessible concentrations in either UBM or Versantvoort's method and those of the corresponding residual fractions as determined by microwave extraction were statistically evaluated against total concentrations for every compound in the MP pellets. Experimental results compiled in Tables 1 and 2 revealed the inexistence of statistically significant differences for any of the target compounds ($t_{observed} < t_{critical;}$ $p>0.05$). Therefore, we might conclude that the automatic flow-through dynamic method herein presented is free from bias and that additive or multiplicative interferences that would in turn jeopardise the quality of the reported bioaccessibility data are not observed.

Oral bioaccessibility values of the three leachable endocrine disruptors from PE MPs (Tables 1 and 2) are comparable to previously reported leaching data using seawater as extractant.[64] This result is in good agreement with earlier observations[63] and signaled that human risk might be posed by MP ingestion from bottled water, non-marine food commodities and airborne particulates but from secondary microplastics in seafood or those found in marine settings.

The temporal extraction profiles were fitted to a first order mathematical model with a single compartment that has been previously recommended for fractionation assays of trace metals in solid matrices.[65,66] The cumulative leachable concentrations C(t) (given in mg kg^{-1}) can be estimated by equation (1):

$$C_{(t)} = A\left(1 - e^{-kt}\right) \qquad (Eq.\,1)$$

in which A is the maximum concentration of leachable endocrine disrupting compounds (mg kg^{-1}); k is the associated apparent rate constant of A (min^{-1}); and t is the time coordinate (min). Coefficients of Eq. 1, estimated for the investigated compounds in both fed and fasted state digestive systems, are compiled in Table 3. The coefficients of determination (R^2) for DMP, BPA and DEP were above 0.98 in all instances. The *lack of fit* test of every individual compound and extraction method suggested that almost all variance was accounted for by the two variables specified in the model with $p > 0.05$ (see Table 3). The maximum theoretical bioaccessibility of the model, that is the term A in Eq. 1, was statistically compared for every individual compound and physiologically based extraction test against the experimental data as obtained by the dynamic

flow-through method (see Tables 1 and 2). Again, no statistically significant differences (p>0.05) were observed for any of the tested compounds using a t-test of comparison of means for heteroscedastic data. Notwithstanding the fact that both dynamic UBM and Versantvoort methods rendered virtually identical bioaccessible concentrations of fast leachable compounds, the apparent kinetic constants under fed state were 1.3-2.4 fold enhanced compared to fasted conditions, thus revealing a faster release (and potential uptake) of polar contaminants for food-borne ingested MPs against consumption of MPs by inhalation under fasted conditions.

Human risk assessment and exposure to intentionally added compounds in MPs (namely, BPA and DEP) was evaluated by comparing the average daily intakes (ADI) against maximum tolerable daily intakes (TDI). Both ingestion and inhalation are herein considered as human exposure pathways of MPs. In fact, Vianello *et al.*[67] reported a median value of airborne fragment-like MP of 68 μm, which is well aligned with the size of the primary MP studied in this work. Cox *et al.*[68] indicated a maximum annual consumption of MP in American adults of 52,000 particles and a maximum annual inhaled of 62,000, thus amounting a total amount of 312 MP·day^{-1}. As a worst case scenario, the MP intake ratio (IR) was estimated by assuming spherical shape particles with average size of 110 μm (as those of the CRM-PE-002) and density equal to medium-density PE, that is, 0.93 g·mL^{-1}, thereby obtaining a value of IR of 0.2 mg MP·day^{-1}. ADI is estimated from Eq. 2:

$$ADI = \frac{IR \cdot C \cdot EF \cdot f}{BW} \qquad (Eq. 2)$$

In which C is the total concentration of target analyte, EF is the exposure factor that is here equal 1 because of daily exposure to MPs, that is 365 days·year^{-1}, f is the bioaccessible fraction (see Tables 1 and 2), and BW is the body weight (taken as 70 kg for adults). Whilst ADI values calculated for DEP (see Table 4) are far below the TDI (viz., 5 mg kg^{-1} of body weight·day^{-1})[69]. ADIs for BPA, even including bioaccessibility data from UBM and Versantvoort tests, namely, 4.4 and 4.1 μg kg^{-1} of body weight·day^{-1}, respectively, for were slightly above the TDI, which is endorsed as 4.0 μg kg^{-1} of body weight·day^{-1}.[70,71] These findings indicate that plastics exceeding 0.3% (w/w) BPA pose threat to human health and that further ecotoxicological and epidemiological tests are needed for estimating TDI of recently introduced bisphenol congeners replacing BPA.

Conclusions

This manuscript reports the first flow-based dynamic physiologically-based extraction test for bioaccessibility studies of migrants from microplastic pellets. Extraction under fasted and fed conditions were assessed using complex, yet biorelevant gastrointestinal fluids. The proof-of-concept applicability of the proposed flow system was demonstrated by oral bioaccessibility assays of a low-density polyethylene certified reference material with average particle dimensions

of 110 µm. Validation (trueness) was effected by mass balance with recoveries ranging from 87 to 112%. The main asset of the automatic extraction system as a front-end to liquid chromatography is the fingerprinting of the temporal extraction profiles of the target compounds at near real time along with the estimation of the leaching rates of the accessible phthalates (DMP and DEP) and BPA under fed and fasted state. Further work is underway in our research group for expanding the applicability of the automatic flow setup to oral bioaccessibility studies of contaminants/migrants associated to weathered microplastics in aquatic settlings.

CRediT statements

A. Sixto: Investigation, Methodology, Formal Analysis, Validation, Visualization, Writing-original draft

B. El Morabit: Investigation, Formal Analysis, Validation

E.J. Carrasco-Correa: Formal Analysis, Methodology, Visualization, Supervision, Writing-review&editing

M. Trujillo-Rodríguez: Methodology, Supervision, Writing-review&editing

M. Miró: Conceptualization, Funding acquisition, Project Administration, Resources, Supervision, Writing-review&editing

Conflict of interest

The authors declare that they do not have any commercial or associative interest that represents a conflict of interest in connection with the manuscript submitted.

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Table 1. UBM bioaccessible concentrations of DMP, BPA and DEP from PE MPs and mass balance validation

Analyte	CRM (mg kg^{-1})	Bioaccessible fraction (mg kg^{-1})	Bioaccessibility (%)	Residual fraction (non-bioaccessible) (mg kg^{-1})	Bioaccessible + Residual fractions (mg kg^{-1})	Microwave assisted extraction (mg kg^{-1})	Recovery (mass balance) (%)	$t_{observed}$ *
DMP	3002 ± 178	2440 ± 360	81± 12	39 ± 26	2480 ± 350	2480 ± 120	100± 14	0.011
BPA	2995 ± 180	1520 ± 170	51 ± 6	1180 ± 230	2740 ± 320	2800 ± 280	98 ± 11	0.210
DEP	3002 ± 180	2520 ± 330	84 ± 11	190 ± 66	2720 ± 360	2550 ± 120	107± 14	0.620

* Comparing the results of the microwave assisted extraction against the sum of bioaccessible and residual concentrations. $t_{critical}$= 2.776

Table 2. Versantvoort bioaccessible concentrations of DMP, BPA and DEP from PE MPs and mass balance validation

Analyte	CRM (mg kg^{-1})	Bioaccessible fraction (mg kg^{-1})	Bioaccessibility (%)	Residual fraction (non-bioaccessible) (mg kg^{-1})	Bioaccessible + Residual fractions (mg kg^{-1})	Microwave (MW) extraction (mg kg^{-1})	Recovery (mass balance) (%)	$t_{observed}$*
DMP	3002 ± 178	2610 ± 290	87 ± 10	49 ± 29	2660 ± 260	2480 ± 120	107 ± 11	0.877
BPA	2995 ± 180	1450 ± 100	48 ± 3	975 ± 290	2420 ± 190	2800 ± 280	87 ± 7	1.549
DEP	3002 ± 180	2620 ± 290	87 ± 10	235 ± 6	2860 ± 290	2550 ± 120	112 ± 11	1.413

* Comparing the results of the microwave assisted extraction against the sum of bioaccessible and residual concentrations. $t_{critical}$= 2.776

Table 3. First-order mathematical model and kinetic parameters thereof for investigation of leaching rates of phthalate congeners and BPA from microplastic pellets

Analyte	**UBM method**					**Versantvoort's method**				
	***k* (min^{-1})**	**A_o (mg/kg)**	**A_{exp} (mg/kg)**	**R^2**	***p***	***k* (min^{-1})**	**A_o (mg/kg)**	**A_{exp} (mg/kg)**	**R^2**	***p***
DMP	0.0107 ± 0.0003	2530 ± 330	2560 ± 340	0.995	0.962	0.014 ± 0.002	2980 ± 430	3030 ± 520	0.997	0.998
DEP	0.006 ± 0.001	2780 ± 320	2590 ± 370	0.999	0.612	0.0093 ± 0.0012	2980 ± 480	2950 ± 530	0.980	0.999
BPA	0.0113 ± 0.002	1460 ± 190	1510 ± 210	0.980	1.000	0.027 ± 0.005	1480 ± 240	1500 ± 240	0.980	0.975

Table 4. Average daily intake (ADI) of BPA, DEP and DMP from ingested and inhaled PE MPs calculated without ($ADI_{non\text{-}corrected}$) and with ($ADI_{corrected}$) the bioaccessibility factor for UBM and Versantvoort tests

Analyte	**UBM method**	**Versantvoort's method**	**Without bioaccessibility data**	**Tolerable daily intake (µg analyte·kg^{-1} BW·day^{-1})**
	ADI corrected	**ADI corrected**	**ADI non-corrected**	
DMP	6.95 ± 0.06	7.46 ± 0.05	8.6 ± 0.5	
BPA	4.37 ± 0.03	4.11 ± 0.02	8.6 ± 0.5	4.0 [70]
DEP	7.21 ± 0.06	7.46 ± 0.05	8.6 ± 0.5	5000 [69,71]

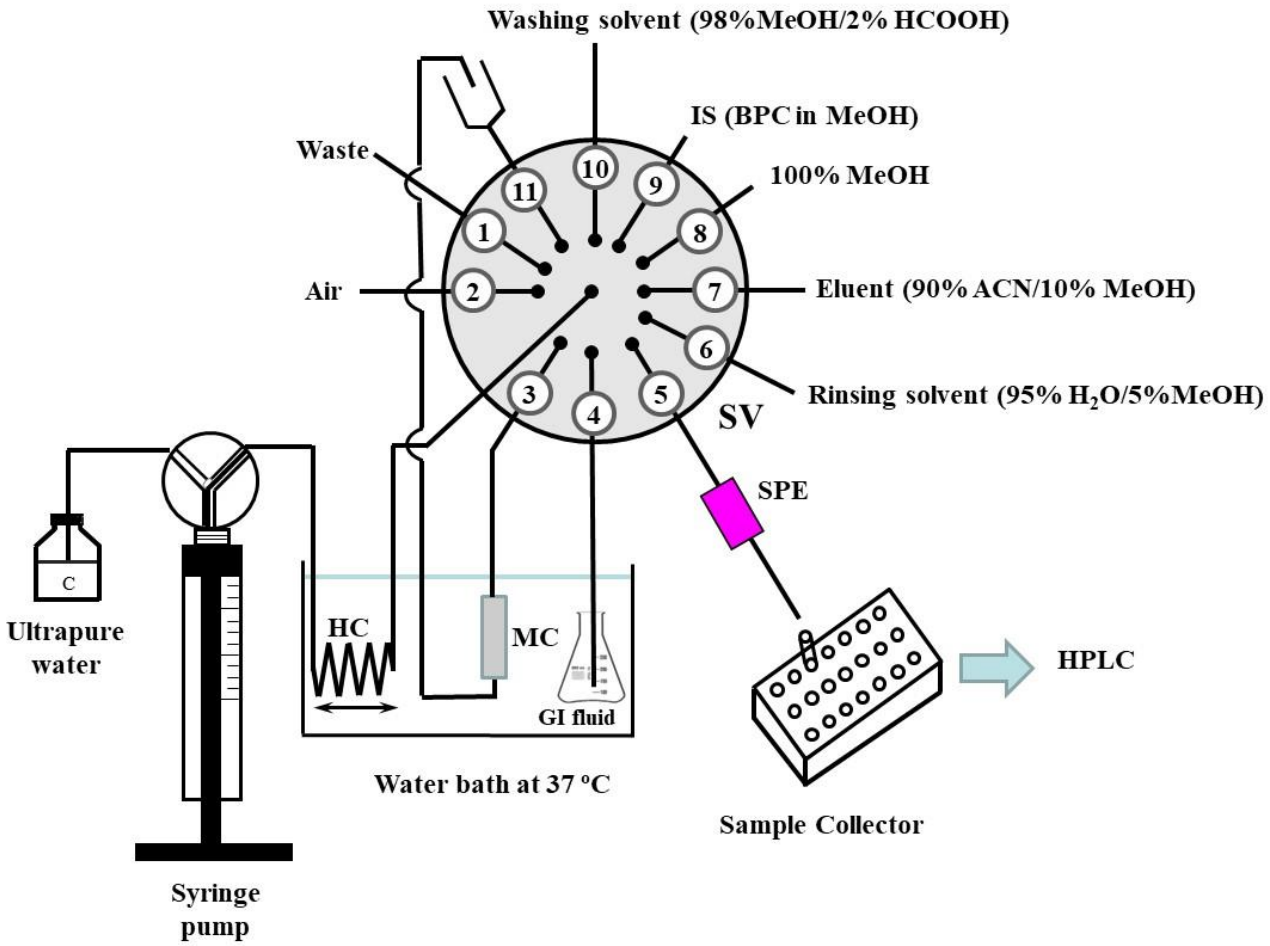
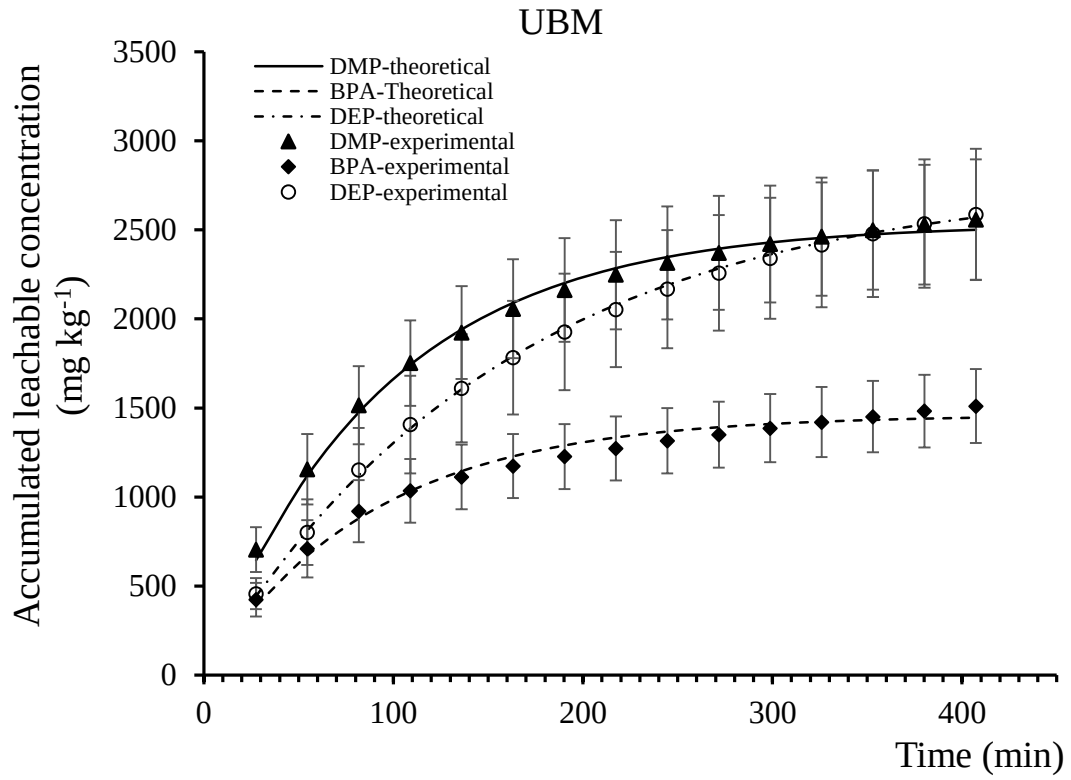


Figure 1. Diagrammatic description of the flow-based manifold for automatic physiologically-based extraction tests of endocrine disrupting compounds associated to microplastics. SV: Selection Valve, MC: Microcolumn, C: Carrier, HC: Holding Coil, GI: Gastrointestinal, IS: Internal Standard, HPLC: High-performance Liquid Chromatography

a)



b)

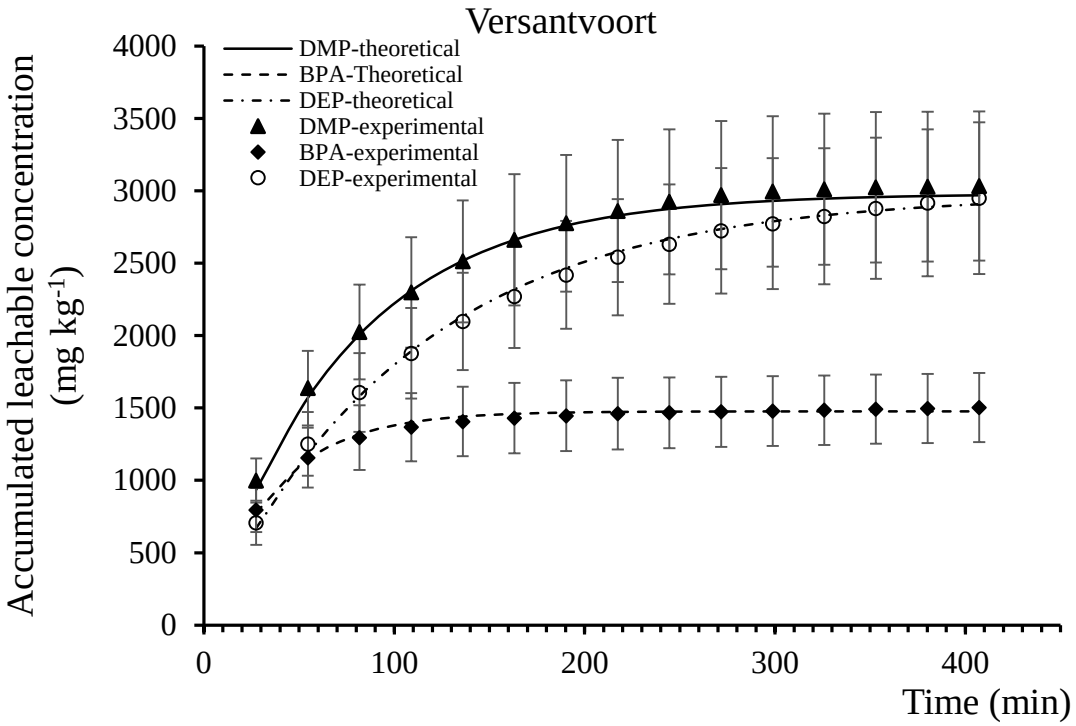


Figure 2. Experimental and theoretical extractrograms obtained for plastic-borne xenobiotics under fasted-state (a) and fed-state (b) using automatic flow-based methodology

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