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# In vitro human oral bioaccessibility assessment of hazardous chemicals, including N, N′-substituted-*p*-phenylenediamines, coming from recycled tire crumb rubber

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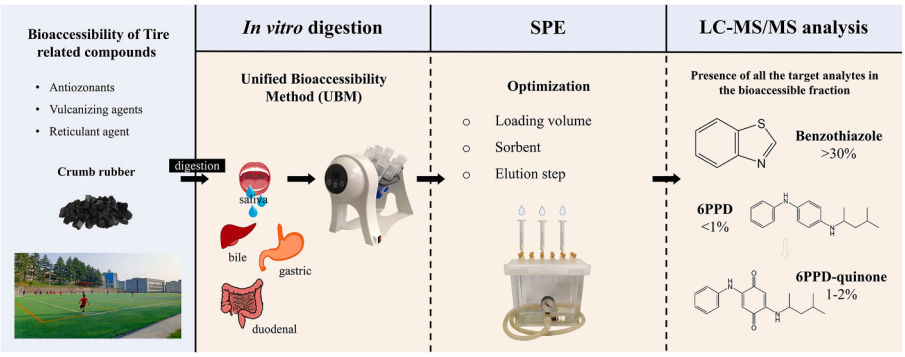
## HIGHLIGHTS

- In vitro oral bioaccessibility of antiozonants and other tire-related compounds is studied.
- Unified Bioaccessibility Method is used for the simulation of the ingestion.
- SPE method is optimized for the biological fluid followed by LC-MS/MS analysis.
- Human bioaccessibility of all target compounds is proven, including 6PPD and 6PPD-quinone.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Tires, apart from being formed by rubber and filling materials, contain organic compounds added to make them resistant and durable. The widely use of recycled tire crumb rubber (RTCR), main product of the shredding process of end-of-life tires, can cause human exposure to these chemicals due to its use in synthetic football fields and kid's playgrounds. In 2023, the European Commission banned the use of recycled tire crumb rubber in synthetic fields, giving eight years to replace the used material. This study intends to assess the oral bioaccessibility of antiozonants, crosslinking and vulcanizer agents present in RTCR. With this purpose, the Unified Bioaccessibility Method (BARGE) was used to simulate the material ingestion. RTCR is put into contact with the four simulated biological fluids including saliva, gastric and duodenal juice and bile, attempting to simulate human digestion. Afterwards, the organic compounds present in the fluid need to be extracted and Solid-phase extraction (SPE) was the technique selected after being optimized to obtain the best extraction conditions. Ultrasound assisted extraction was performed to evaluate the total concentration of the target compounds in the crumb rubber matrixes. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was employed to identify and quantify the target compounds. The results showed the bioaccessibility of all the studied analytes, with values ranging from 0.1 % up to 70 %. Benzothiazole was the compound with the highest

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bioaccessibility with a mean value of 40 % and concentrations reaching 32000 ng $g^{-1}$ in the bioaccessible fraction. N-(1,3-dimethylbutyl)-N′-phenyl-*p*-phenylenediamine and its transformation product ((4-Methylpentan-2-yl)amino)-5-(phenylamino)cyclohexa-2,5-diene-1,4-dione) showed an average bioaccessibility of 0.1 % and 1.8 %, respectively, the latter being present in all the analysed samples.

## 1. Introduction

End-of-life tires (ELTs) are a waste that need to be treated. Tires are composed by carbon black, steel wire, natural and synthetic rubber, and other compounds such as fabric, fillers, antioxidants and antiozonants (Rogachuk and Okolie, 2023). The most common way to recycle this material consists of a shredding process that gives rise to crumb rubber. The problematic of recycled tire crumb rubber (RTCR) has been gaining prominence in the last years due to its environmental hazard and toxicity (Perkins et al., 2019). This material is used on daily surfaces such as kids' playgrounds and other urban pavements being its main use as infill in synthetic football fields. Due to its size, below 5 mm, it is a microplastic (Frias and Nash, 2019) and it can be intentionally, or unintentionality ingested by the users of these surfaces, mainly children, which are highly exposed to RTCR (Llompart et al., 2013). A recent study performed in saliva reported that the exposure to this material caused the presence of environmental persistent free radicals (EPFRs), that may produce oxidative stress or inflammation (Huang et al., 2023). Other rubber microplastics coming from pneumatics are tire and road wear particles (TRWP), which are the sum of tire wear particles (TWPs), road wear particles (RWPs) and minerals deposited on the road (Kang and Kim, 2023). Other authors reported that 93 % of $PM_{10\text{-}80}$ recollected near roadways are traffic-related particles, including TWPs, RWPs and brake particles (Sommer et al., 2018).

RTCR is an ECHA (European Chemical Agency) (ECHA hot topic) hot topic. In September 2023, the European Commission decided to ban the use of RTCR on synthetic football fields alleging the largest source of intentional microplastics in the environment and giving a maximum period of eight years in order to ensure that a great number of synthetic sport surfaces that use this type of product can reach their natural end-of-life (Commission Regulation, 2023).

The concern about RTCR not only resides on the size of the particle, but also on its composition. Since these particles are the result of the tire shredding process, they have similar composition as pneumatics. Numerous studies have found polycyclic aromatic hydrocarbons (PAHs) and plasticizers in this matrix (Llompart et al., 2013) and toxic metals, including As, Cu or Pb (Graça et al., 2022). Tire related compounds, including N, N′-substituted-*p*-phenylenediamines (PPDs), used as antiozonants, vulcanizers and one crosslinking agent have been recently found in RTCR reaching concentrations up to 2000 μg $g^{-1}$ (Duque-Villaverde et al., 2024).

In addition, the ubiquity of RTCR and TRWP produces the migration of these compounds to environmental matrices, such as, air (Armada et al., 2021), soils or water leachates (Fořt et al., 2022; Celeiro et al., 2021).

The presence of antiozonant N-(1,3-dimethylbutyl)-N′-phenyl-*p*-phenylenediamine (6PPD) and its transformation product 6PPD-quinone (2-((4-Methylpentan-2-yl)amino)-5-(phenylamino)cyclohexa-2,5-diene-1,4-dione, 6PPDq) in water, issued from RTCR and TRWP, has been linked to the death of *Oncorhynchus kisutch* (Tian et al., 2021) and other salmonids at low concentrations. In zebrafish larvae, the toxicity study of both analytes did not reveal significant toxicity at environmental concentrations but, at higher concentrations, 6PPD and 6PPDq have been shown to enhance cardiotoxicity (Fang et al., 2023; Varshney et al., 2022). Regarding humans, these two chemicals have been found in children and adult urine, as well as in that of pregnant woman, in which the highest concentrations have been reported (Du et al., 2022). Currently, there is no specific regulation regarding 6PPD, but the Department of Toxic Substances Control of Canada considers it as a priority matter (Department of Toxic Substances Control, 2023). In the USA, some actions are being taken aiming at substituting this antiozonant by other safer chemicals (U.S. Tire Manufacturers Association) but this task remains difficult. Other PPDs present in RTCR are N, N′-Diphenyl-1,4-phenylenediamine (DPPD) and 4-Isopropylaminodiphenylamine (IPPD). The subproduct of 6PPD, 1,3-Dimethylbutylamine (DMBA), is also present.

Oral bioaccessibility is the fraction of an analyte that is released from the matrix onto the gastrointestinal tract (Intawongse and Dean, 2006). The bioaccessibility from crumb rubber was previously studied for PAHs (Armada et al., 2023), showing the oral bioaccessibility of 17 PAHs.

The number of works in biological fluids that includes the target compound of the present study is low. One of them investigated the migration of tire related compounds into artificial body fluids including sweat, saliva, and gastric juice, detecting vulcanizers Benzothiazole (BTZ) and 2-Mercaptobenzothiazole (MBTZ) in all samples studied. 6PPD was only found in sweat and vulcanizer 1,3-diphenyl guanidine (DPG) in saliva and sweat (Schneider et al., 2020). Simulated gastric fluid was used with crumb rubber samples as well. A study performed for the National Toxicological Program assessed the bioaccessible concentration of 32 compounds, including amines, plasticizers, PAHs and other compounds likely to be present in the sample, proving the bioaccessibility of some of these agents, including 6PPD, BTZ and MBTZ (National Toxicology Program, 2019).

Regarding other tire compounds, it is worth mentioning that vulcanizer agents, including DPG and 1,3 Di-o-tolylguanidine (DTG), were found in urines of children and adults from New York, in 73 % and 20 % of the samples, respectively (Li and Kannan, 2023).

Masset et al., (2022) studied the bioaccessibility of chemicals present in TWP using an in-vitro digestion model on *Oncorhynchus mykiss* demonstrating the bioaccessibility in fish.

Solid phase extraction (SPE) has demonstrated to be one of the best techniques to extract analytes from liquid matrices as an alternative to the conventional liquid-liquid extraction accomplishing in one-step the separation, preconcentration of the compounds and matrix clean-up. This technique was previously used within our research group (Armada et al., 2023) demonstrating suitability and reporting favourable recoveries for the extraction of analytes coming from biological fluids.

The main objective of the present work is to evaluate the bioaccessibility of eleven tire chemicals, including antiozonants, vulcanizers and one crosslinking agent (Hexamethoxymethyl melamine, HMMM) which are present in RTCR samples. To achieve this goal, the Unified Bioaccessibility Method (UBM) (BARGE-INERIS, 2010) is implemented to simulate the digestion of the material using gastric fluids. Afterwards, the analytes are extracted by SPE and analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). It is worth noting that previous bioaccessibility studies are very scarce and there are not focused on humans. In addition, these studies employ lab-made samples of rubber derivate, whereas the aim of the present work is the assessment of human bioaccessibility (in-vitro) using real RTCR samples recollected *in situ* on sport fields and playgrounds conceived with this kind of material.

## 2. Materials and methods

### 2.1. Reagents and materials

The eleven target compounds, their name and abbreviation are

included on Table S1. Additional information, including CAS numbers, molecular weights, structures, retention times and MS/MS transitions are also included. The purity and source of the solvents, materials, and standards utilized are detailed in Table S2 and Supplementary material.

### 2.2. Crumb rubber samples

The ten real samples (crumb rubber play surfaces) are described in Table S3, indicating the origin, data collection and type of sample. They include 7 football fields (FF), 1 park (kid's playgrounds) (P) and 2 commercial samples (Com) from local providers.

### 2.3. UBM method

The UBM method, established by the Bioaccessibility Research Group of Europe (BARGE), simulates the in vitro ingestion of the material. For this, it is necessary to prepare four different biological fluids in the laboratory: saliva, duodenal juice, gastric juice and bile. Each fluid is composed of two fractions, the inorganic fraction with inorganic salts and the organic fraction, which consists of an aqueous solution of organic compounds. First, both fractions are prepared according to the amounts indicated in Table S4. In the next step, the corresponding enzymes for each of the fluids are weighed into 100 mL vials and the previously prepared inorganic (25 mL) and organic (25 mL) fractions are added and shaken manually.

To simulate the digestion, 0.2 g of rubber granules are weighed into a 50 mL Falcon tube. The digestion process takes place in two phases: gastric and gastrointestinal digestion. On the solid sample, 3 mL of saliva and 4.5 mL of gastric juice are added, the pH is adjusted to 1.2 ± 0.1 and placed in the end-over-end shaker to simulate the movements of human digestion for 1h at 37 °C ± 2 °C. Once gastric digestion is completed, the pH is checked to be between 1.2 and 1.5 and gastrointestinal digestion is started. For this purpose, 9 mL of duodenal juice and 3 mL of bile are added to the Falcon tube and adjusted to a pH value of 6.3 ± 0.5. Then, end-over-end agitation is left for 4 h at 37 ± 2 °C and once finished, the pH of the sample is measured, which should be between 6.3 ± 0.5. Finally, it is centrifuged for 15 min at a frequency of 1479 rcf (relative centrifugal force), and the supernatant is collected and stored in a 20 mL vial. In addition, a small amount of methanol (MeOH) (0.5 mL) is added to prevent the wall effect (prevent compounds from being retained on the vial walls).

### 2.4. Solid-phase extraction (SPE)

Solid-phase extraction is performed with the aid of a vacuum system (SPE Vacuum Manifold) and three stages are carried out during the extraction of the analytes: conditioning, sample load and elution. In this stage, two sorbents are studied. The commercial cartridges provided by Waters (Milford, MA, USA) OASIS HLB (Hydrophilic-Lipophilic-Balanced) 30 mg, 1 cc, based on Hydrophilic-Lipophilic-Balanced technology consists of a water-attracting phase created from two monomers: the lipophilic divinylbenzene and the hydrophilic N-vinylpyrrolidone. This combined material can eliminate interfering substances like proteins, phospholipids, and salt from the surrounding matrix.

In addition, particulate cork by-product from the cork industry was also tested as sorbent, working on reversed phase. These cartridges are lab-made prepared in 2 mL polypropylene syringes, employing glass wool at the end of the syringe, a cellulose filter, 50 mg of cork and finally another cellulose filter. The cork is a heterogeneous material, coming from cork oak, whose biopolymers (lignin and suberin) and hydrophobic polysaccharides (cellulose) make cork a good material for the retention of organic compounds. Cork needs a thermic pretreatment before its use to improve the retention capacity (Celeiro et al., 2020).

For the cartridges conditioning, 2 mL of Acetonitrile:MeOH (90/10 v/v) and 2 mL of ultrapure water are used. Then, 2–5 mL of gastrointestinal fluid is then passed through the cartridge and when the entire amount of sample has been passed through, the cartridge is left to air dry (with the negative pressure active). Finally, the elution is accomplished by gravity with the different solvents studied and collecting 2x1 mL aliquots, both in volumetric flasks of 1 mL. They are transferred to vials of 1.8 mL for subsequent LC-MS/MS analysis.

Bioaccessibility: to calculate the oral bioaccessibility of the target compounds, it is necessary to quantify the analytes in the raw material. The % bioaccessibility is the concentration in the bioaccessible fraction divided by the concentration of analytes in the crumb rubber following Eq. (1) (Armada et al., 2023; Tokalıoğlu, 2023).

$$\%\ Bioaccessibility = \frac{Concentration\ of\ bioaccesible\ analytes\ (ng\ g^{-1})}{Total\ concentration\ of\ analytes\ in\ crumb\ rubber\ (ng\ g^{-1})}\ x\ 100 \quad (1)$$

To get the concentration of the bioaccessible analytes, referred to amount of material exposed to the fluid, the concentration in fluid (μg $L^{-1}$) is multiplied by the total amount of fluid (20 mL) and then divided by the amount of RTCR used for the simulation experiment (200 mg).

### 2.5. Ultrasound Assisted Extraction (UAE)

Ultrasound Assisted Extraction procedure was carried out to determine the concentration of the analytes in the crumb rubber samples (Duque-Villaverde et al., 2024). In brief, 0.2 g of sample are transferred to a 4 mL glass vial with 2 mL of ethyl acetate and then the extraction takes place in 15 min, with an ultrasound frequency of 50 KHz at 65 °C. Subsequently, the extract is filtered using PTFE filter and the extract is evaporated under nitrogen, reconstituted with MeOH, and diluted employing AP (Aqueous Phase, see section 2.6) to become ready to inject in the LC-MS/MS system.

### 2.6. LC-MS/MS analysis

The analyses are carried out with a Thermo Scientific (San Jose, CA, USA) liquid chromatograph coupled to a TSQ Quantum Ultra Triple Quadrupole TSQ mass spectrometer, working in multiple reaction monitoring (MRM) mode, with a HESI-II (Heated Electrospray ionization) ionization source working on positive mode. The selected MRM transitions and monitoring ions are given in Table S1 for each target compound.

Calibration curves based on twelve calibration points, ranging from 0.2 to 1000 μg $L^{-1}$, were injected weekly in order to check the linearity of the method. As regards the precision, one concentration level of the calibration curve was injected in triplicate in one day (intra-day) and at three different days (inter-day). Periodically, spiked sample experiments were performed to evaluate the accuracy and recoveries of the whole method. In addition, blank procedures were made for each sample batch; and regarding the chromatographic system, solvent blanks , including methanol and pure water, were injected within the sample sequences to avoid any possible memory effect.

The injection system was an automatic Accela Open injector with a

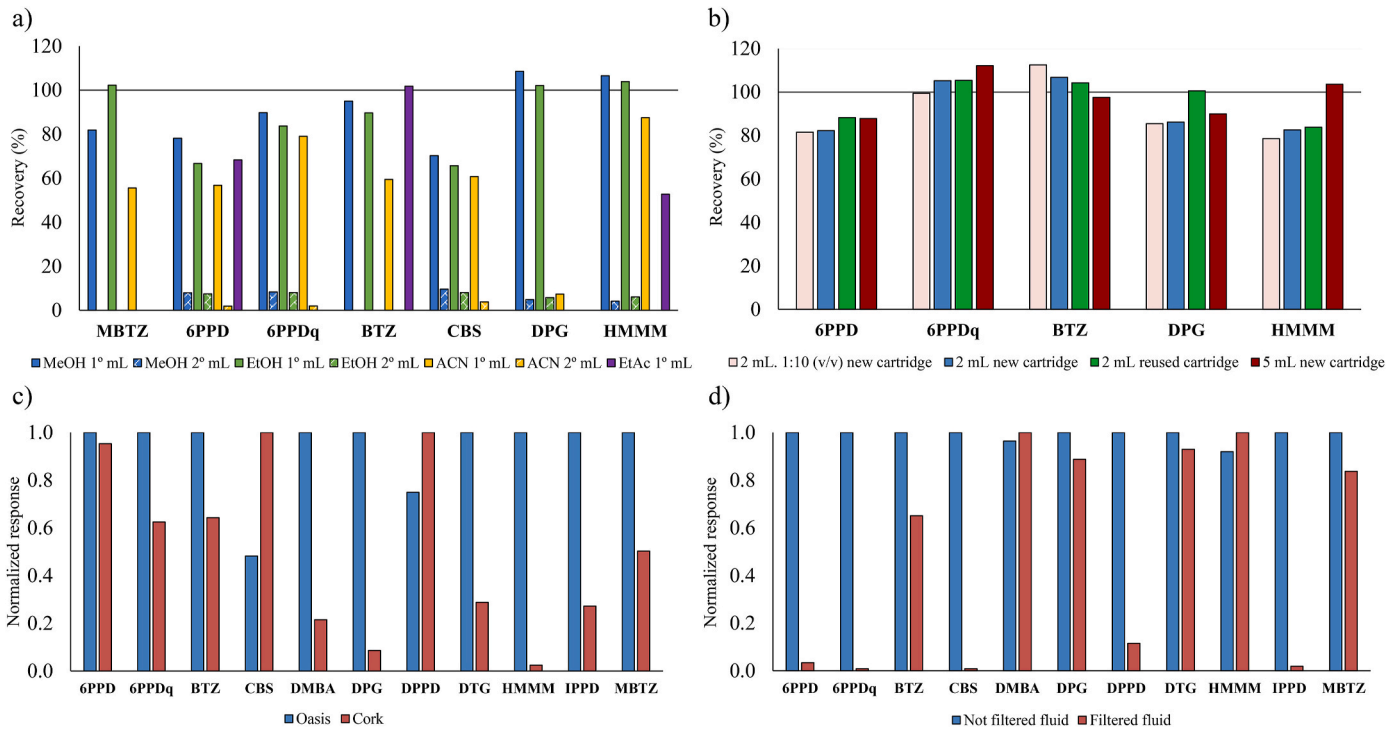


**Fig. 1.** a) Study of the different solvents and elution fractions. b) Evaluation of influence of the loading volume. c) Result comparison between commercial and cork sorbents. d) Result comparison between filtered and not filtered fluids.

20 μL loop and 10 μL were injected. Regarding chromatographic separation, a Kinetex $C_{18}$ column (2.6 μm, 100 × 2.1 mm), supplied by Phenomenex (Torrance, CA, USA) was used and the column temperature was set at 40 °C. The mobile phases were MeOH (Organic Phase, OP) and ultrapure water (Aqueous phase, AP) both with containing buffer (3 mM of Ammonium Formiate) and acidified with formic acid (0.1 % v/v). The elution programme started with 50 % of B for 1 min and then it was increased up to 90 % of B in 9 min and held for 1 min. Subsequently, the initial conditions (50 % B) were restored within 7 min. Lastly, during the last 2 min, the gradient remained isocratic at 50 % of B. The system was controlled using XCalibur 2.2 and TraceFinderTM 3.2 software.

## 3. Results and discussion

### 3.1. SPE optimization

The SPE process was optimized studying several factors with the aim of obtaining the best extraction results.

#### 3.1.1. Elution solvent and volume

The initial SPE conditions were adapted from a previous study (Armada et al., 2023). For the elution step 4 solvents were compared: MeOH, ethyl acetate (EtAc), ethanol (EtOH) and acetonitrile (ACN); collecting a first and a second aliquot, each one of 1 mL. 5 mL of ultrapure water spiked at 25 μg $L^{-1}$ with the target compounds were loaded onto the cartridge and eluted with the different solvents. Fig. 1a shows the obtained results.

In all cases, the second aliquot showed very low response or non-response (MBTZ or BTZ). Regarding the solvents, EtAc gave the worst results, being unable to elute several compounds. In contrast, all analytes were recovered with ACN, MeOH and EtOH. In addition, both alcohols enabled better recoveries than ACN, with MeOH giving better chromatographic peak shapes than EtOH. Therefore, the selected elution conditions comprised the use of 1 mL of MeOH.

#### 3.1.2. Loading volume

The applied volume of fluid on the SPE column was also studied. These experiments were carried out with OASIS HLB Prime 30 mg. To find out whether this material could be reused, seeking at generating less waste and lowering the cost of the analysis, new and reused cartridges were compared.

The studied volumes were 2 mL and 5 mL of gastrointestinal fluid. In addition, 2 mL of biological fluid diluted 1:10 (v/v) in ultrapure water were also processed since the dilution might facilitate sample loading and improve the performance. The obtained results depicted for some compounds in Fig. 1b show that the recoveries are quantitative in all cases and that there are no significant differences among results obtained with new and reused cartridges.

The dilution of the sample did not improve sample application. On the contrary, in the case of reused cartridge no data was obtained due to the difficulty and slowness of the elution step.

According to the data, 5 mL of gastric fluid was selected (although 2 mL is also appropriated) since it allowed a higher concentration factor, and no evidence of breakthrough volume was observed using 5 mL of sample.

#### 3.1.3. SPE sorbent

Looking for a greener analytical chemistry sustainable with the environment, a cork by-product material was tested as SPE sorbent as an alternative to commercial cartridges. The use of this material requires the preparation of lab-made cartridges since they are not commercially available. The device preparation is included in section 2. Fig. 1c shows the result comparison between cork and commercial cartridges (normalized response).

As can be seen, the commercial devices provided higher responses excluding N-cyclohexylbenzothiazole-2-sulfenamide (CBS) and DPPD. Cork cartridges appeared efficient for some of the analytes but failed for the extraction of other compounds such as DPG, DTG or HMMM. Therefore, commercial OASIS HLB Prime cartridges were selected, despite the cork "greenness" nature.

**Table 1**
LC-MS/MS performance. Linearity, precision, IDL and IQL. SPE-LC-MS/MS recoveries (%). UBM-SPE-LC-MS/MS precision (RSD, %).

| | Linearity | | Precision | | IDL (μg $L^{-1}$) | IQL (μg $L^{-1}$) | SPE-LC-MS/MS Recovery | | | SPE-LC-MS/MS Precision |
|---|---|---|---|---|---|---|---|---|---|---|
| | $R^2$ | Linear range (μg $L^{-1}$) | Intra-day precision (n = 3) | Inter-day precision (n = 6) | | | 10 μg $L^{-1}$ | 50 μg $L^{-1}$ | Mean recoveries | |
| 6PPD | 0.995 | 0.2–1000 | 5.2 | 12 | 0.043 | 0.14 | 90.7 | 74.5 | 82.6 | 8.9 |
| 6PPDq | 0.994 | 0.2–1000 | 8.4 | 11 | 0.016 | 0.054 | 92.1 | 107 | 99.6 | 4.7 |
| BTZ | 0.993 | 5–1000 | 8.3 | 11 | 0.79 | 2.6 | 110 | 130 | 120 | 12 |
| CBS | 0.998 | 0.2–100 | 2.6 | 16 | 0.050 | 0.17 | 106 | 63.4 | 84.7 | 5.9 |
| DMBA | 0.992 | 0.2–1000 | 8.1 | 13 | 0.025 | 0.08 | 89.5 | 82 | 85.8 | 7.0 |
| DPG | 0.997 | 0.2–1000 | 6.2 | 13 | 0.071 | 0.236 | 91.5 | 107 | 99.3 | 6.8 |
| DPPD | 0.987 | 0.2–200 | 5.4 | 11 | 0.043 | 0.14 | 90.4 | 87.8 | 89.1 | 3.5 |
| DTG | 0.997 | 0.2–1000 | 5.5 | 16 | 0.017 | 0.057 | 112 | 111 | 111 | 12 |
| HMMM | 0.991 | 0.2–1000 | 6.3 | 11 | 0.029 | 0.10 | 117 | 105 | 111 | 5.8 |
| IPPD | 0.997 | 0.2–1000 | 9.1 | 16 | 0.009 | 0.031 | 78.5 | 85.8 | 82.2 | 2.6 |
| MBTZ | 0.994 | 5–1000 | 2.7 | 15 | 0.42 | 1.41 | 60.6 | 79.7 | 70.1 | 8.8 |

**Table 2**
Concentration of the target analytes in the crumb rubber samples (mg $kg^{-1}$) and bioaccessible fraction (ng $g^{-1}$).

| Samples (n = 10) | Concentration in crumb rubber (mg $kg^{-1}$) | | Bioaccessible fraction (ng $g^{-1}$) | |
|---|---|---|---|---|
| Compounds | Range | Average | Range | Average |
| 6PPD | 1.1–2908 | 528 | 4.1–4209 | 867 |
| 6PPDq | 0.26–32.9 | 11.4 | 6.7–796 | 211 |
| BTZ | 1.3–80 | 28.8 | 286–32131 | 11847 |
| CBS | 0.03–0.62 | 0.20 | 3.5–4.25 | 4.0 |
| DMBA | 0.79–73.1 | 16.1 | 111–11567 | 3581 |
| DPG | 1.4–63.6 | 19.4 | 716–8226 | 3085 |
| DPPD | 0.23–82.9 | 18.6 | 9.4–98 | 30.8 |
| DTG | 0.04–0.56 | 0.18 | 2.3–15.5 | 10.6 |
| HMMM | 0.03–51.2 | 14.1 | 20.1–4626 | 1426 |
| IPPD | 0.03–10.2 | 2.3 | 2.7–544 | 105 |
| MBTZ | 1.9–90 | 28.5 | 603–10046 | 2474 |

### 3.2. Filtration of the SPE extracts

Some of the extracts obtained at the end of the elution process were muddy, making them inappropriate for direct injection into the chromatographic system. To solve this problem, a filtration step was considered. To evaluate possible losses during filtration, two aliquots of spiked gastric fluid were analysed, before and after filtration employing cellulose acetate filters. As can be seen in Fig. 1d (normalized responses), quite surprising low responses were obtained for the paraphenylenediamine family (IPPD, 6PPD, DPPD, 6PPDq) family and for CBS with values between 0.5 and 10 % indicating that these compounds were almost completely retained onto the filter. BTZ was also affected by filtration although in a minor grade. As regards the other analytes (DMBA, DPG, DTG and MBTZ), no retention problems occurred during filtration step.

Other type of filters, such as PTFE ones, were tested in aqueous solutions and led to better results. Even so, they still showed minor recovery loses for PPDs and CBS. Considering these filtration troubles, if required, a centrifugation step was performed instead of filtration.

### 3.3. SPE-LC-MS/MS method performance

Method validation included the assessment of the linearity, sensitivity, precision, and accuracy. The results are shown in Table 1.

The linearity was evaluated using the least square method. Twelve calibration levels were studied and injected in duplicate or triplicate (concentration ranges between 0.2 and 1000 μg $L^{-1}$). The concentrations of analytes were proportional to the chromatographic response, obtaining coefficients of determination ($R^2$) above 0.991 for all compounds.

Instrument precision was assessed over a single day (n = 3) and across multiple days (n = 6), with suitable relative standard deviation values (RSD) between 2 % and 18 % for method for intra-day and inter-day precision, respectively. In addition, instrumental detection, and quantification limits (IDLs and IQLs) were determined as the compound concentration giving signal-to-noise ratios (S/N) of 3 and 10, respectively. These values ranged from the low ng $L^{-1}$ (e.g., IPPD) to the low μg $L^{-1}$ (BTZ). The method limits of detection (LOD) and quantification (LOQ) are included in Table S5.

Since not any reference material containing all the target compounds is available, accuracy was evaluated by recovery studies. The gastric fluid was spiked at two concentration levels: 10 μg $L^{-1}$ and 50 μg $L^{-1}$, and the SPE-LC-MS/MS method was applied. The extracts were quantified by means of calibration curves prepared with standard solutions and the results were compared with the added concentrations. The recoveries were quantitative in all cases with mean values between 83 and 112 % (MBTZ 70 %). In addition, these recoveries demonstrated the absence of significant matrix effects, which allows using external calibration with standard solutions prepared in solvent (or in mobile phase) improving both method quality and throughput.

To evaluate the repeatability and reproducibility of the whole method including the digestion step (UBM-SPE-LC-MS/MS), six real

**Table 3**
Target compound concentrations in fluid (μg $L^{-1}$).

| | 6PPD | 6PPDq | BTZ | CBS | DMBA | DPG | DPPD | DTG | HMMM | IPPD | MBTZ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **FF-1** | 0.06 | 0.08 | 16.8 | _ | 1.6 | 0.93 | _ | 0.16 | 0.29 | _ | _ |
| **FF-2** | 41 | 2.9 | 270 | _ | 115 | 82.1 | 0.97 | 0.15 | 31.4 | 5.4 | 100 |
| **FF-3** | 1.7 | 7.8 | 126 | _ | 38.7 | 44.2 | 0.29 | 0.08 | 20.4 | 0.11 | 33.2 |
| **FF-4** | 15 | 2.1 | 195 | _ | 114 | 21.5 | 0.61 | 0.16 | 6.2 | 0.61 | 23.1 |
| **FF-5** | 0.04 | 1.3 | 12.5 | _ | 2.7 | 7.2 | _ | _ | 0.2 | _ | _ |
| **FF-6** | _ | 0.06 | 3.4 | 0.04 | 1.1 | _ | 0.10 | 0.07 | _ | _ | 5.6 |
| **FF-7** | _ | 0.07 | 3.9 | _ | 1.1 | _ | 0.10 | 0.02 | _ | _ | 7.9 |
| **P-1** | 0.08 | 0.91 | 5.4 | 0.04 | 13.2 | _ | 0.11 | _ | 0.71 | 0.03 | 26.4 |
| **Com-1** | 2.5 | 3.4 | 237 | <LOQ | 21.8 | _ | 0.27 | _ | 47.4 | 0.20 | 16.1 |
| **Com-2** | 7.8 | 2.1 | 329 | 0.04 | 50.7 | _ | 0.34 | _ | 8.9 | 0.99 | 11.5 |

_: Not identified.

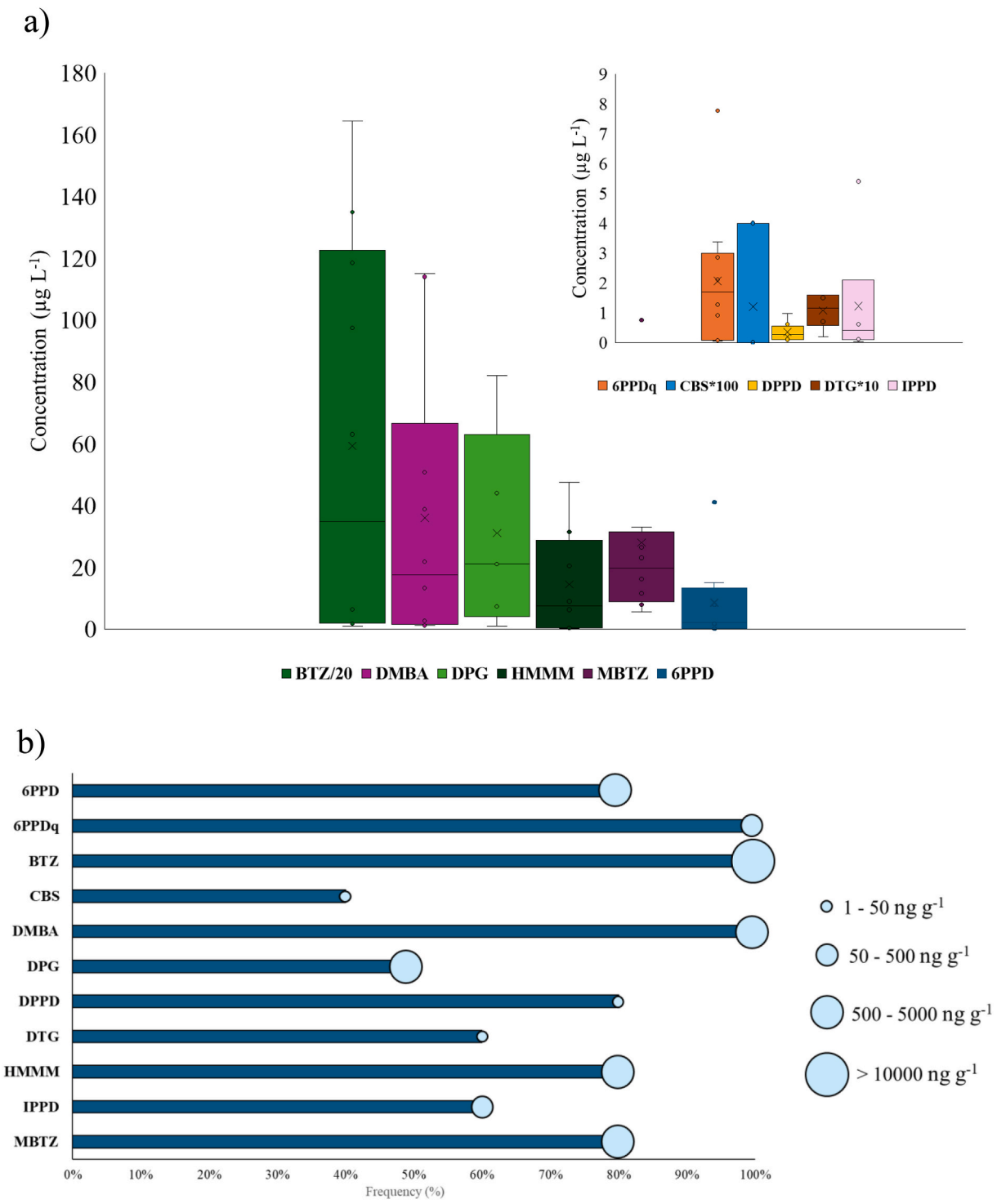


**Fig. 2.** a) Box-and-Whisker charts for the target analytes in the synthetic biological fluids (µg $L^{-1}$). b) Occurrence frequency (%) and mean concentration (ng $g^{-1}$) of the target analytes in the bioaccessible fraction. Note that the ocurrence frequency is in general equivalent to the crumb rubber samples (see supplementary material).

non-spiked crumb rubber samples were subjected to the complete procedure. The obtained % RSD values (Table 1) were satisfactory and below 15 % in all cases.

### 3.4. Real samples analysis. Bioaccessibility evaluation

Real crumb rubber samples were subjected to the UBM digestion process to assess the bioaccessibility of the target compounds. The concentration ranges and average values of the target analytes in the crumb rubber samples and the bioaccessible fraction are summarized in Table 2.

The individual concentrations in crumb rubber samples are given in Table S6 and a brief discussion of the results are included in the Supplementary Material. Table 3 includes the individual concentrations of the target analytes in the gastrointestinal fluids obtained after digestion of the ten crumb rubber samples. Fig. 2a depicts these data by means of box and whiskers charts. Table 4 includes the bioaccessibility fraction values of the target analytes.

**Table 4**
Bioaccessible fraction (ng $g^{-1}$).

| | 6PPD | 6PPDq | BTZ | CBS | DMBA | DPG | DPPD | DTG | HMMM | IPPD | MBTZ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **FF-1** | 5.9 | 8.2 | 1677 | _ | 161 | 92.4 | _ | 16.4 | 29.2 | _ | _ |
| **FF-2** | 4209 | 292 | 27073 | _ | 11567 | 8226 | 98 | 14.5 | 3137 | 544 | 10046 |
| **FF-3** | 171 | 796 | 12640 | _ | 3868 | 4290 | 29.4 | 7.5 | 2040 | 11.0 | 3293 |
| **FF-4** | 1538 | 217 | 19475 | _ | 11375 | 2101 | 60.6 | 15.5 | 617 | 60.8 | 2303 |
| **FF-5** | 4.07 | 127 | 1250 | _ | 266 | 716 | _ | _ | 20.1 | _ | _ |
| **FF-6** | _ | 6.7 | 288 | 4.3 | 114 | _ | 10.0 | 7.5 | _ | _ | 603 |
| **FF-7** | _ | 6.9 | 286 | _ | 111 | _ | 9.4 | 2.3 | _ | _ | 763 |
| **P-1** | 7.4 | 88 | 522 | 4.2 | 1283 | _ | 10.1 | _ | 70.2 | 2.7 | 2576 |
| **Com-1** | 245 | 359 | 23135 | <LOQ | 2129 | _ | 26.5 | _ | 4626 | 19.5 | 1564 |
| **Com-2** | 762 | 206 | 32131 | 3.6 | 4940 | _ | 33.6 | _ | 871 | 96.4 | 1122 |

_: Not identified.

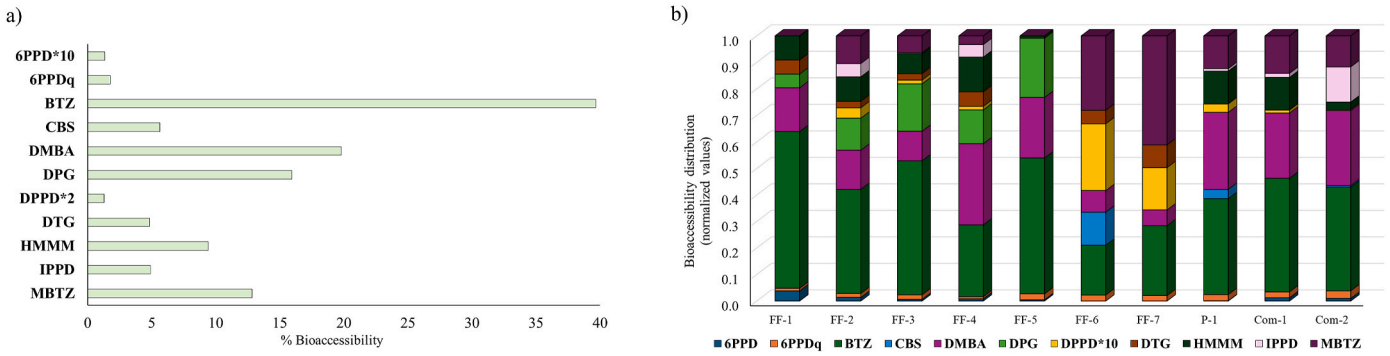


**Fig. 3.** a) Mean bioaccessibility values (%). b) Bioaccessibility distribution (normalized values) of the target compounds.

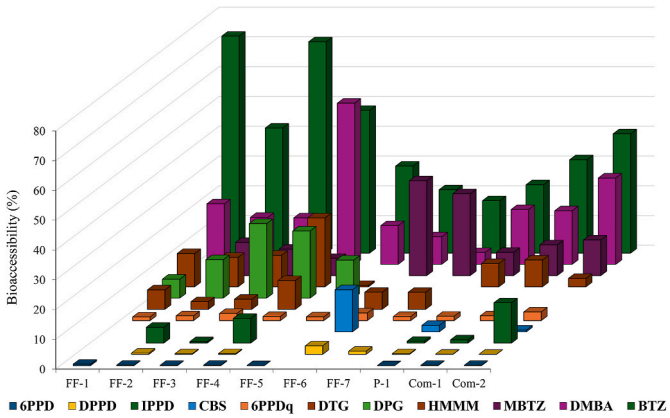


**Fig. 4.** Bioaccessibility values (%) for the target analytes obtained in the 10 analysed samples.

All the analytes were detected in the fluids, proving the oral bioaccessibility of the target compounds. The frequency of appearance of the analytes and the mean values of bioaccessible concentration are displayed in Fig. 2b. In general, all the compounds detected in the crumb rubber were detected in the bioaccesible fraction of the corresponding sample. In this sense, bioaccessibility can be considered 100 % excluding CBS (6 samples) and some other isolated cases where the analytes were detected in the crumb rubber but not in the corresponding biological fluid probably due to the low concentrations (see Table S6 and Table S7). DMBA, 6PPDq and BTZ were present in all samples and BTZ showed the highest concentration, up to 330 μg $L^{-1}$, while 6PPD and other paraphenylenediamines, such as, IPPD and DPPD were found in the biological samples fluid with concentrations between 0.1 μg $L^{-1}$ and 50 μg $L^{-1}$. 6PPD showed higher values, above 15 μg $L^{-1}$, including samples FF-4 and FF-2, this last showing the highest 6PPD concentration. In contrast, CBS was the analyte with the lowest concentrations in fluid, with values about 0.04 μg $L^{-1}$.Table S6

The values obtained for the bioaccessible fraction are included in Table 4. Mean bioaccessibility values and its distribution are indicated on Fig. 3a and b, respectively. The individual values of bioaccessibility are described in Fig. 4 and Table S7. BTZ was the analyte with the highest concentration in fluid, as previously commented, showing also the highest % bioaccessibility, with values between 20 and 50 % excluding 2 samples from 2 football fields (FF-1 and FF-3) with very high values up to 73 % (see Table S7 and Fig. 4). DMBA also showed very high bioaccessibility with mean values of 20 %, followed by MBTZ with 13 %. The *p*-phenylenediamine family showed lower bioaccessibility values than other compounds below, including IPPD and DPPD, with a medium value of 4.9 % and 0.6 %, respectively. 6PPD was the analyte with the lowest % bioaccessibility, less than 0.2 % (see Fig. 3a). This behaviour could be explained by the likely 6PPD transformation, especially that giving rise to 6PPDq or DMBA even though 6PPD was present in 80 % of the samples (see Fig. 2b). 6PPDq presented a medium value of bioaccessibility of 1.8 %, with a low range of bioaccessibility within the ten samples. Despite being present in only 4 samples, CBS showed a medium value of 5.6 %. Diphenyl compounds, including DTG and DPG had similar results, obtaining 5 % and 16 %, respectively. This last value is similar to HMMM (9.4 %). Fig. 3b describes the bioaccessibility distribution (accumulative figure) of the target compounds in the samples. As can be seen, BTZ is the most abundant analyte in all the samples, as its contribution on the fraction is above 40 % in most cases, reaching 31 % in FF-1. Owing to its low bioaccessibility, 6PPD, has the lowest contribution in the samples. DMBA contribution reaches 10 % in most samples, excluding FF-7.

Regarding literature, there is hardly any study devoted to the bioaccessibility of the target compounds from rubber, which makes very difficult the comparison of our results. The bioaccessibility of three of the target compounds, 6PPD, BTZ and MBTZ, was studied by the NTP (National Toxicology Program, from the United States Department of Health and Human Services) (NTP, 2019). They used one homogenised composite sample, provided by different manufacturers. To simulate the

ingestion, authors followed the Pavilonis method (Pavilonis et al., 2014) alleging its simple implementation. The main difference between our method and this method is the absence of bile and the lesser amount of reagents for fluid preparation. MBTZ was detected 0.62 μg $g^{-1}$ (μg in extract per g of crumb rubber extracted). BTZ was also present at a concentration of 27.8 μg $g^{-1}$, which is in consonance with our values for commercial crumb rubber samples (23.1 and 32.1 μg $g^{-1}$). 6PPD was also determined at 1.8 μg $g^{-1}$, but the blank response was too high to confirm this value.

Another study that tried to assess the presence of 6PPD, DTG, BTZ and MBTZ on simulated fluids after digestion is included in the European Risk Assessment Study on Synthetic Turf Rubber Infill (ERASSTRI) (Schneider et al., 2020). In this case, BTZ and MBTZ were found in the ten analysed samples of simulated gastric fluid after a simple digestion with hydrochloric acid. However, 6PPD could not be detected due to matrix interferences.

Finally, and more recently, Masset et al. (2022) studied fish (*Oncorhynchus mykiss*) bioaccessibility of tire related compounds including some of the target compounds included in our study. Although human and fish digestion are hardly comparable, we decided to include this study due to the lack of other studies conducted in humans. As rubber particulate material, they used a lab-made tire derived particle. BTZ showed the highest bioaccessibility, in consonance with our study with an average value of 40 %. MBTZ was reported with lower values but still quite high, 8.7 % although we obtained a higher value for MBTZ in the present study, 12.8 % (see Fig. 3a). Regarding the paraphenylenediamines family, the bioaccessibility was lower and quite similar to our values. 6PPD and 6PPDq showed values of 1 % and 2 %, respectively, in good agreement with our study. The last analyte covered in both studies is DPG, which presented higher human bioaccessibility, with values of 15.9 % (3.4 % for fish). Therefore, the bioaccessibility results obtained in both studies are quite similar although the comparison is made with the available fraction of two different species. In any case, both species are clearly affected by the tire related compounds, as demonstrated by the levels in the biological gastric fluids and the bioaccessibility values.

These results suggest the potential risk associated to the accidental ingestion of the target analytes from tire crumb rubber, especially for children, the mainly users of crumb rubber fields and playgrounds.

## 4. Conclusions

An analytical method was successfully developed and validated to evaluate the bioaccessibility of chemicals of emerging concern present in tire rubber. For this purpose, the Unified Bioaccessibility Method (UBM) was selected to simulate the ingestion of the material and then SPE-LC-MS/MS was carried out for the extraction and analysis.

Ten real samples taken from football fields, one park and commercial samples were analysed. All the target analytes were detected in the gastrointestinal fluids, demonstrating the bioaccessibility of these chemical agents.

Benzothiazole (BTZ) was the analyte with the highest concentration in fluids, up to 329 μg $L^{-1}$, giving the highest bioaccessibility values (40 % as average value). 6PPD, and its transformation products, 6PPDq showed an average bioaccessibility value of 0.1 % and 1.8 % respectively. 6PPDq, DMBA and MBTZ were present in all the analysed samples. The occurrence frequency of the remaining target compounds was above 50 % and in general the analytes were found in the fluid of those crumb rubber samples containing them.

This study assesses the human bioaccessibility of 6PPDq, DMBA, CBS, DPG, DTG, HMMM, IPPD and DPPD for the first time using real recycled tire crumb rubber samples.

## CRediT authorship contribution statement

**Sergio Sóñora:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Andres Duque-Villaverde:** Writing – review & editing, Visualization, Validation, Investigation. **Daniel Armada:** Investigation, Formal analysis. **Thierry Dagnac:** Writing – review & editing, Investigation, Funding acquisition, Data curation. **Maria Llompart:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors state that they do not have any identified conflicting financial interests or personal connections that could be perceived to impact the findings presented in this paper.

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## Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143534.

## Data availability

Data will be made available on request.

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