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Assessment of the bioaccessibility of PAHs and other hazardous compounds present in recycled tire rubber employed in synthetic football fields

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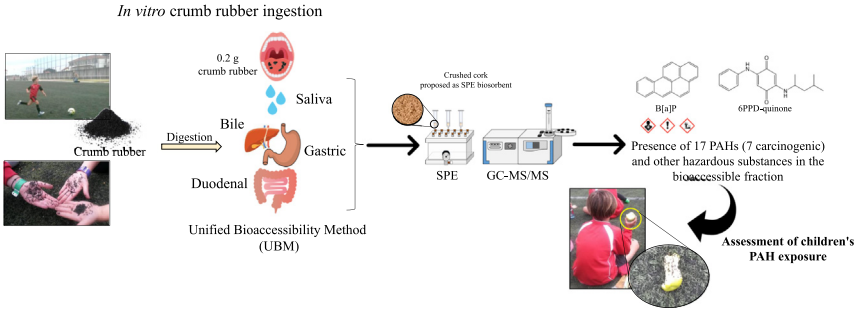
HIGHLIGHTS

- *In-vitro* oral bioaccessibility of PAHs from tire crumb rubber used in sports fields
- Unified Bioaccessibility Method employing human synthetic body fluids was applied.
- SPE-GC–MS/MS was optimized showing the feasibility of a new green cork biosorbent.
- Bioaccessibility of 17 PAHs and other hazardous chemicals (6PPD-quinone) is proven.
- Exposure assessment was carried out to estimate the potential risk to children.

GRAPHICAL ABSTRACT



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ABSTRACT

Recycled tire crumb rubber (RTCR) surfaces contain harmful and carcinogenic substances, which can be ingested by the users of these facilities, mainly athletes and children. In this work, the potential *in-vitro* oral bioaccessibility of eighteen polycyclic aromatic hydrocarbons (PAHs) from RTCR employed as infill in synthetic football fields was studied in human synthetic body fluids (saliva, gastric, duodenal and bile), prepared according the Unified Bioaccessibility Method. Solid-phase extraction (SPE) using commercial sorbents and a new green material based on cork (cork industry by-product) were used to isolate the bioaccessible PAHs before gas chromatography-tandem mass spectrometry analysis. The method was optimized and validated attending the analytical figures of merit. The feasibility of cork biosorbent for the extraction of the compounds was demonstrated, as well as the suitability of the UBM method to perform the digestion with good precision. The application to real samples collected from football fields demonstrated the presence of 17 of the 18 target PAHs in the biofluids. Most volatile PAHs such as NAP, ACY, ACE, FLU, PHN and ANC, achieved the highest bioaccessibility percentage levels. The carcinogenic B[*a*]P was detected in 75 % of the samples at concentrations up to 2.5 ng g^{-1} (bioaccessible fraction). Children exposure assessment was carried out to identify potential risk. Other hazardous and environmentally problematic compounds such as N-(1,3-Dimethylbutyl)-*N′*-phenyl-*p*-phenylenediamine-quinone (6PPD-quinone), recently related with the dead of coho salmon, and hexamethoxymethylmelamine (HMMM), among others, were also detected. This is the first study in which the bioaccesibility from real crumb rubber samples of 15 out of the 16 PAHs considered as priority pollutants by the United States Environmental Protection Agency (EPA) and the presence of 6PPD-quinone and HMMM in the bioaccessible fractions is reported.

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1. Introduction

Concern about the use of recycled crumb rubber as infill in synthetic turf football pitches is increasing due to the presence of multiple hazardous substances (ECHA granules and mulches, 2022), including polycyclic aromatic hydrocarbons (PAHs), plasticizers, antioxidants, vulcanisation additives, benzothiazoles, chlorinated paraffins, polychlorinated biphenyls (PCBs) and heavy metals (Bocca et al., 2009; Celeiro et al., 2018; Marsili et al., 2014; Massey et al., 2020; Skoczyńska et al., 2021). Their direct release into the environment and through the air and water leachates (Armada et al., 2021, 2022a; Celeiro et al., 2021b; Gomes et al., 2021; Rhodes et al., 2012) could cause an environmental problem and poses a risk to human health thought different exposure routes: dermal contact, inhalation and ingestion (Perkins et al., 2019). This recycled material is considered a microplastic itself due to its small particle size (1 nm to <5 mm), being the largest source of intentionally added microplastic pollution (ECHA microplastics, 2022). In addition, tire and road wear particles (TRWP), which have a similar chemical composition to recycled rubber materials, are one of the largest sources of microplastics to the urban environment (Klöckner et al., 2021; Rauert et al., 2022). The harmful organic compounds present in crumb and tire rubber can be found on a variety of daily use surfaces, such as synthetic football pitches and playground's flooring, being accessible to the general users and specially to children which, due to their hand-to-mouth nature, may accidentally or consciously ingest some of these materials facilitating oral intake. It was estimated that approximately 200 mg of soil and dust per day are ingested by 95 % of young children (Calabrese et al., 1997; EPA, 2017).

Oral bioaccessibility is the fraction of a chemical compound or element solubilized from the matrix sample employing *in-vitro* test methods that mimic the human gastrointestinal conditions (Dean and Ma, 2007). The assessment of *in-vitro* studies to evaluate the potential risk to humans caused by organic compounds or metal and metaloids present in recycled rubber is necessary. Recent studies indicate the risk of cancer associated with children who play over recycled tire rubber surfaces is 10 times higher than in uncovered surfaces (Tarafdar et al., 2020). Despite this, only a few investigations have assessed their bioaccessibility and the risk to human health (Oomen and de Groot, 2017; Pavilonis et al., 2014; Schneider et al., 2020; Zhang et al., 2008). Crumb rubber cytotoxicity was demonstrated in skin, lung and small intestine employing human cell lines representing the three main entry routes (dermal contact, inhalation and ingestion) (NTP, 2019a). These findings have prompted research into exposures in female mice fed chow adulterated with recycled crumb rubber, the first research of its kind studying the hazards and risks associated with this material (NTP, 2019b). On the other hand, further research on the bioaccessibility of metals present in tire particles in different fish species is currently underway (Masset et al., 2021).

Among the high number of harmful compounds present in rubber crumb, PAHs have been highlighted (Armada et al., 2022b; Celeiro et al., 2018, 2021a; Llompart et al., 2013). The European Chemicals Agency (ECHA) lists 8 of them (benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene and dibenzo[*a*, *h*]anthracene) as carcinogenic, among other harmful properties (ECHA PAHs, 2019). The United States Environmental Protection Agency (US-EPA), which classified as priority pollutants 16 PAHs, proposed, as an oral reference dose (RfD), 300 ng kg^{-1} day^{-1} for benzo[*a*]pyrene (EPA B[a]P, 2017). Several studies of players on synthetic turf football pitches revealed uptake of PAHs from crumb rubber used as infill (van Rooij and Jongeneelen, 2010). Furthermore, recent studies have demonstrated the bioaccessibility of PAHs from different matrices such as microplastics in the ocean or particulate matter in the air (Jiang et al., 2021; Sánchez-Piñero et al., 2022). On the other hand, other components of rubber and its transformation products, such as 6PPD-quinone and HMMM, have been shown to be harmful or toxic and have already been linked to the death of various aquatic species (Brinkmann et al., 2022; Tian et al., 2021; Varshney et al., 2022).

In recent years, different *in-vitro* procedures have been developed to simulate the human gastrointestinal tract digestion such as the Fed ORganic Estimation human Simulation Test (FOREhST) and the Unified BARGE (Bioaccessibility Research Group of Europe) Method (UBM), among others (BARGE-INERIS, 2010; Cave et al., 2010; Chen et al., 2022; Wragg et al., 2011). BARGE-UBM methods assess the bioaccessibility for accidental ingestion in a fasting individual employing four types of synthetic biofluids (saliva, gastric, duodenal and bile), mimicking the human digestion with high efficiency (Chen et al., 2020). The UBM method was previously used to study the *in-vitro* bioaccessibility of PAHs and other compounds from soils (Lorenzi et al., 2012; Lu et al., 2021; Różański et al., 2021).

Solid phase extraction (SPE) is a widely used sample preparation technique. It has been employed to extract PAHs from biological matrices using common sorbents as octadecyl silica (C18) and Oasis HLB, obtaining quantitative recoveries (Adetunde et al., 2018; Cave et al., 2010; Lorenzi et al., 2012; Riding et al., 2013). Nevertheless, its application to gastrointestinal fluids is rare, and the use of green materials as sorbents has not been reported.

In the present study, a UBM-SPE-GC–MS/MS methodology was developed to assess the *in-vitro* bioaccessibility of 18 PAHs present in recycled crumb rubber into human digestive fluids by ingestion. Additionally, a suspect screening was carried out to evaluate the presence of other harmful chemicals (6PPD-quinone and HMMM, among others) in these fluids. The method was extensively applied to real crumb rubber samples used as infill in synthetic turf football fields, demonstrating the bioaccessibility of PAHs and other hazardous substances present in tire rubber.

2. Materials and methods

2.1. Reagents and materials

The purity and commercial origin of the solvents, standards and materials used are specified in the Supporting Information. The target PAHs studied are listed in Table S1, in Supporting Information.

2.2. Crumb rubber samples

The crumb rubber samples employed as infill in synthetic turf football pitches were directly collected from football pitches (samples S1-S6) following previous sampling procedures (Celeiro et al., 2021a). Two other crumb rubber samples (CS1, CS2) were purchased from local suppliers. The specific characteristics of each crumb rubber samples are described in Table S2, in Supporting Information.

2.3. In-vitro simulation of digestion. UBM method

The protocol employed for the *in-vitro* simulation of human digestion, requires the preparation of four biological fluids: saliva, gastric juice, duodenal juice and bile according to UBM method (BARGE-INERIS, 2010). These phases consisted of an aqueous solution of inorganic salts (inorganic fraction), and an aqueous solution of organic components (organic fraction), both containing specific enzymes. The preparation of the four fluids mentioned above are described elsewhere (BARGE-INERIS, 2010), see supplementary material. The digestion was carried out in two steps. Firstly, the gastric stage in which 200 mg of rubber granulate sample were placed in a 50 mL polypropylene centrifuge tube. Over this material 3 mL of saliva and 4.6 mL of gastric juice were added, the pH was adjusted to 1.2 ± 0.1 and the sample was subjected to *end-over-end* agitation for 1 h at 37 ± 2 °C to simulate the peristaltic movements of human digestion. Afterwards, 9.2 mL of duodenal fluid and 3 mL of bile were added to the previous mixture and the pH was adjusted to 6.3 ± 0.5; next, the mixture was again shaken for 4 h at 37 ± 2 °C in the *end-over-end* system to ensure a good mixing. Afterwards, if the pH of the mixture was 6.3 ± 0.5 or greater, the mixture was centrifugated for 30 min at 1479 rcf. Finally, the supernatant was collected. MeOH (5 %, *v/v*) was added to prevent the adsorption of

PAHs and/or other organic compounds in the glass vial (wall effect), a phenomenon that would compromise the extraction efficiency of the compounds from the gastric fluid (Qian et al., 2011).

2.4. Extraction of the bioaccessible fraction. Solid-phase extraction (SPE)

After digestion, SPE was applied to isolate compounds from the gastrointestinal fluids. Firstly, 50 mg of Oasis HLB were packed in a 1 mL polypropylene cartridge containing glass wool (Sigma-Aldrich Chemie) and cellulose filters (Thermo Scientific, Waltham, MA, USA) at the bottom. Then, the cartridge was linked at the outlet tip with the SPE manifold (Visiprep™, Supelco). Before the sample load, the cartridge was conditioned with 2 mL of a mixture of ACN and MeOH (90:10, *v*/v) followed by 2 mL of ultrapure water. Then, the gastrointestinal fluid (1–5 mL) was loaded under vacuum at a nominal flow of 0.5 mL min^{-1}. Next, the cartridge was air dried under vacuum and eluted with 1 mL (or 2 × 1 mL) of EtAc by gravity flow. Finally, the obtained extract was analyzed by GC–MS/MS.

For method optimization and performance studies, the synthetic fluid was spiked with the target PAHs to give a final concentration of 0.2, 1 and 10 μg L^{-1}. To prevent contamination and interferences that could overestimate the obtained results, solvent blanks and whole procedure blanks employing gastrointestinal fluid were carried out.

To calculate the percentage of PAHs present in the bioaccessible fraction, it was necessary to determine the concentration of PAHs in the raw material (see Section 2.5). This percentage was expressed as the amount of PAH in the synthetic biological fluids per g of crumb rubber, divided by the initial concentration in the crumb rubber following Eq. (1) (BARGE-INERIS, 2010).

$$\%Bioaccessible = \frac{Concentration\ of\ bioaccessible\ PAH\ (ng\ g^{-1})}{Concentration\ of\ total\ PAH\ in\ crumb\ rubber\ (ng\ g^{-1})} x100 \quad (1)$$

The assessment of oral exposure to contaminants is usually calculated using the chemical concentration in a matrix (in this case recycled rubber from synthetic football fields), the mass of the matrix ingested per day, and the absorption factor of the chemical in the human body (% bioaccessibility in this study). Since the main users of synthetic football fields are children, a child between 3 and 6 years old exposure was estimated. The estimated daily uptake (EDU) which is employed to assess the daily exposure considering the bioaccessibility fraction, was calculated employing Eq. (2) (Arfaeinia et al., 2022; Lu et al., 2021) as follows:

$$EDU\left(\frac{ng}{day \times kg}\right) = \frac{C\left(\frac{ng}{g}\right) \times IR\ (g/day) \times Bioa}{BW\ (kg)} \quad (2)$$

Being BW the body weight of a child between 3 and 6 years (15.7 kg); C represents the concentration of the compound in the crumb rubber infill; IR means the daily ingestion rate of the granulate crumb rubber (0.2 g/day) and Bioa represents the bioaccessibility fraction (Oomen and de Groot, 2017).

The B[*a*]P equivalent carcinogenicity was calculated (Sánchez-Piñero et al., 2022) for the target PAHs using Eq. (3):

$$[B[a]P]_{equiv.} = \Sigma(C_{PAH} \times TEF_{PAH}) \quad (3)$$

where C_{PAH} is the concentration of the oral bioaccessibility (ng g^{-1}) and TEF_{PAH} is the toxic equivalence factor of target PAHs (relative to B[*a*]P).

2.5. PAH content in the crumb rubber samples. Ultrasound-assisted extraction

PAHs were extracted from the crumb rubber samples employed as infill in synthetic turf football fields by ultrasound-assisted extraction (UAE). The methodology was previously optimized by the authors (Celeiro et al., 2018). Briefly, 200 mg of crumb rubber were placed in a vial and 2 mL of EtAc were added. Then, UAE was carried out (ultrasonic bath at 50 kHz and 25 °C) for 20 min. The supernatant was filtered through 0.22 μm PTFE filters and diluted (1:10, *v*/v) before GC–MS/MS analysis.

2.6. GC–MS/MS analysis

Analysis was carried out by GC–MS/MS employing a gas chromatograph (Thermo Scientific Trace 1310) with an autosampler IL 1310 and coupled to a triple quadrupole mass spectrometer (TSQ 8000) from Themo Scientific. Compounds detection was performed by Selected Reaction Monitoring (SRM) acquisition mode, monitoring 2 or 3 transitions per compound (see Table S1, Supporting Information). For a more specific description of the chromatographic and spectrometry conditions see the supporting information files.

3. Results and discussion

The first step was the optimization of the SPE procedure to determine the target PAHs content in the synthetic biological fluids.

3.1. Optimization of SPE

The optimization of the SPE is a crucial step to ensure extraction with high efficiency and yield. Biological fluids are complex matrices; therefore, the selection of the adequate sorbent, the sample volume loaded, as well as the elution solvent and volume, are critical parameters to obtain proper results.

The selection of a suitable sorbent capable to retain the target compounds is crucial for the effectiveness of the method. Four sorbents were considered: C18, Oasis HLB, Oasis PRIME HLB and regranulated cork particles. The first three are commercially available. The technology Hydrophilic-Lipophilic-Balanced (HLB) involves a water-wettable phase made from two monomers, the hydrophilic N-vinylpyrrolidone and the lipophilic divinylbenzene. These sorbents can remove interferences such as proteins, phospholipids and salt from the matrix. Cork is a new biosorbent, providing a dual balance, favouring interaction with the analytes through hydrogen bonds (hydrophilic) and π- π stacking (hydrophobic). It has been recently proposed as a green material for SPE of fungicides from water (Celeiro et al., 2020). The comparative results obtained with the four sorbents are displayed in Fig. 1a (see experimental conditions in Section 2.4). Oasis HLB, Oasis PRIME HLB and cork produced generally favourable recoveries for all compounds. In contrast, C18 presented an insufficient retention with recoveries below 40 % in most cases. This might be attributed to the fact that the other sorbents provide a dual balance, promoting interactions with the PAH aromatic rings (Olivella et al., 2011; Pintor et al., 2012). Therefore, C18 was discarded for further experiments.

A quantitative, high-throughput elution that offers the highest possible sensitivity of the compounds of interest is very important. For this purpose, the results by eluting the target compounds with an ACN/MeOH mixture (90:10, *v*/v) and EtAc were compared. The latter solvent was selected based on previous studies in which these PAHs were assessed by GC–MS (Celeiro et al., 2018). The elution with EtAc offered higher response for some compounds for both cork and Oasis HLB sorbents (Fig. 1b).

In order to develop a sustainable methodology with the lowest consumption of organic solvent, the elution volume was assessed collecting two consecutive and separate fractions of 1 mL of extract. Results for Oasis HLB and cork are depicted in Fig. 1c. For both sorbents, all compounds were completely eluted with only 1 mL of solvent excluding B[*b*]F, B[*j*]F, which were detected in the second elution aliquot, at percentage lower than 3 %. Therefore, both sorbents were quantitatively eluted with only 1 mL of EtAc, contributing to miniaturize the sample preparation process.

Since the studied compounds are present at trace levels in the crumb rubber (general individual concentrations below 5 mg kg^{-1} (Armada et al., 2022b) the expected concentrations in the biological fluid (bioaccessible fraction) might be very low. The possibility of using a higher amount of biological fluid up to 5 mL instead of 1 mL could be an interesting option

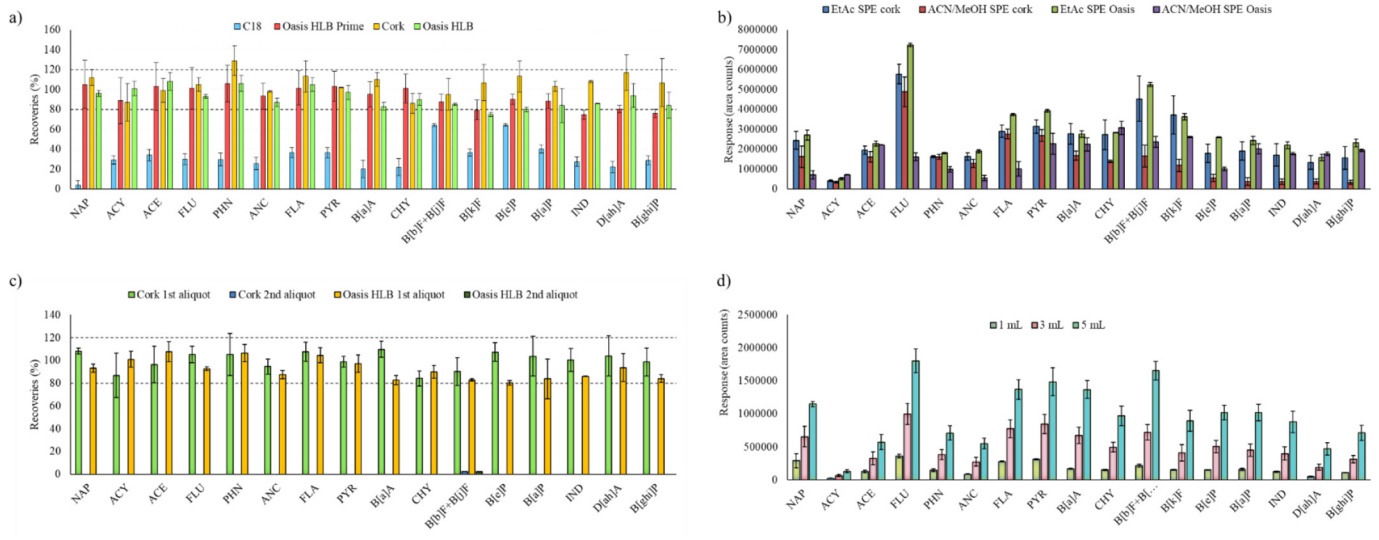

Fig. 1. a) Study of the different type of sorbent efficiency for the retention of the target PAHs, b) comparison of eluting using ethyl acetate (EtAc) or the mixture ACN/MeOH (90:10, *v*/v), c) evaluation of the elution volume on SPE efficiency for the target PAHs, and d) assessment of the gastrointestinal fluids volume on SPE efficiency for the studied PAHs.

to achieve higher sensitivity. Three different volumes, 1, 3 and 5 mL of gastrointestinal fluid were tested. As can be seen in Fig. 1d, the instrumental response increased proportionally to the sample volume. Therefore, and taking into account the volume of sample available and the sensitivity required, up to 5 mL of sample can be loaded in the SPE column obtaining equivalent performance. Higher volumes were not considered due to the limited amount of biological fluid and the possible cartridge clog.

The addition of a small amount of methanol (5 % *v*/v) to the sample (gastrointestinal fluid) can help to prevent the "wall effect" as has been previously described. Nevertheless, the presence of the organic solvent could affect extraction efficiency. This was studied employing a real crumb rubber sample subjected to the complete UBM (see Section 2.3 and 2.4). The comparison of the SPE extraction of the target compounds in the bioaccessible fraction with or without MeOH is depicted in Fig. S1, Supporting Information. Results were similar in both cases; thus, the addition of methanol can be used without disturbing extraction efficiency.

3.2. SPE-GC–MS/MS method performance

The proposed method was validated in terms of linearity, sensitivity, precision and accuracy. The results are shown in Table 1.

Linearity was evaluated employing calibration standards containing the 18 target PAHs prepared in EtAc (concentration range between 0.02 and 100 μg L^{-1}). The chromatographic response was proportional to the concentration of the PAHs, obtaining coefficients of determination (R^2) higher than 0.9974. Instrumental precision was evaluated within a day ($n = 3$) and among days ($n = 6$) for all calibration concentration levels, obtaining relative standard deviation (RSD) values lower than 11 % and 12 %, for intra-day and inter-day precision, respectively. Instrumental detection and quantification limits (IDLs and IQLs) were calculated as the compound concentration giving a signal-to-noise ratio (S/N) = 3 and 10, respectively, giving values at the low ng L^{-1}.

To assess the accuracy of the methodology, recovery studies were carried out at three concentration levels, 0.2, 1 and 10 μg L^{-1} using spiked

Table 1
GC–MS/MS performance. Linearity, precision, IDL and IQL. SPE-GC–MS/MS recoveries (%). UBM-SPE-GC–MS/MS precision (RSD, %) for cork and Oasis HLB.

Compounds	GC–MS/MS					SPE-GC–MS/MS Recovery						UBM-SPE-GC–MS/MS Precision		
	Linearity (R^2)	IDL μg L^{-1}	IQL μg L^{-1}	Intra-day precision ($n = 3$)	Inter-day precision ($n = 6$)	10 μg L^{-1}		1 μg L^{-1}		0.2 μg L^{-1}				
						Cork	Oasis	Cork	Oasis	Cork	Oasis	Cork	Oasis	Mean
NAP	0.9986	0.0028	0.0091	1.1	1.3	112	105	–	114	–	–	–	16	16
ACY	0.9984	0.0029	0.010	5.7	12	86.9	80.5	72.0	104	76.8	96.0	8.5	24	16
ACE	0.9988	0.0056	0.018	2.0	3.1	99.5	88.2	90.3	86.3	105	79.5	21	22	22
FLU	0.9986	0.0063	0.021	1.3	1.3	98.2	84.8	123	101	88.4	93.6	26	14	20
PHN	0.9995	0.0023	0.0076	0.26	0.90	103	97.2	106	94.1	156	125	3.8	12	7.9
ANC	0.9997	0.0059	0.019	1.2	1.8	91.3	85.3	87.4	96.1	84.1	111	17	10	14
FLA	0.9994	0.0019	0.0062	1.7	2.6	103	104.5	107.6	103	96.7	96.9	15	8.6	12
PYR	0.9993	0.0021	0.0070	1.8	2.3	101	95.2	118	102	105	97.6	18	14	16
B[*a*]A	0.9997	0.0035	0.012	3.0	7.1	110	90.0	125	112	109	91.7	7.7	8	7.8
CHY	0.9997	0.0021	0.0068	3.3	6.3	86.4	99.7	103	98.1	94.5	94.0	17	16	17
B[*b*]F + B[*j*]F	0.9976	0.0070	0.020	2.5	3.0	95.0	108	96.1	94.2	85.6	92.1	19	18	18
B[*k*]F	0.9989	0.0075	0.020	0.52	8.3	107	79.4	86.0	96.2	71.7	92.7	–	–	–
B[*e*]P	0.9986	0.0041	0.014	10	9.7	87.2	84.1	86.3	86.2	96.4	101	7.7	18	13
B[*a*]P	0.9978	0.0042	0.014	1.9	5.7	65.4	115.6	99.0	86.1	88.3	96.9	20	21	20
IND	0.9976	0.0024	0.0080	3.1	3.3	99.1	86.0	59.1	75.5	72.4	95.1	13	17	15
D[*ah*]A	0.9980	0.0025	0.0082	11	11	102	94.2	84.1	80.1	73.5	73.1	–	–	–
B[*ghi*]P	0.9974	0.0072	0.020	8.8	8.1	100	84.1	71.4	85.1	85.9	107	8.6	15	12

biological fluid. As can be seen in Table 1, mean recovery around 93 %, were obtained for most compounds, demonstrating method suitability for the two considered SPE sorbents (cork and Oasis HLB).

Limits of detection (LOD) and quantification (LOQ) of the SPE-GC–MS/MS method are depicted in Table S4, in Supporting Information. In general, they were calculated as the compound concentration giving a S/N ratio of 3 and 10, respectively. For the compounds detected in the procedure blanks (NAP, FLU, PHN and PYR) they were calculated as the average concentration corresponding to the signal of the blank plus 3 or 10 times the standard deviation.

To evaluate the feasibility and suitability of the *in-vitro* digestion, as well as the repeatability and reproducibility of the whole UBM-SPE GC–MS/MS method, eight real non-spiked crumb rubber samples from football fields were subjected to the complete procedure. Results for repeatability are included in Table 1. Mean RSD values were in general below 15 %. These results are satisfactory considering the complexity of the process and all the steps involved, as well as the low concentration of the analytes (ultra-trace level) in the biological samples. Some of the samples were extracted using cork and Oasis HLB as SPE sorbents obtaining similar results (Fig. S2), demonstrating the suitability of both sorbents, and showing the green cork material (by-products from the cork industry) as a sustainable alternative that favours the circular economy.

3.3. Analysis of real samples. Bioaccessibility

Table S5 includes the PAH concentrations in the biological fluids for the eight digested crumb rubber samples (see sample description in Table S2 in Supporting Information). To simplify visualization box and whiskers plots showing data distribution for each compound and statistical analysis are included in Fig. 2.

As can be seen, 17 out of 18 target PAHs (excluding D[*ah*]A) were detected in the biological fluid samples. In addition, all 17 compounds were found in 3 of the 8 samples. Seven compounds, including the carcinogen CHY, were found in all samples. The ECHA carcinogenic compounds B[*a*]A, B[*b*]F + B[*j*]F, B[*k*]F and B[*e*]P were present in 7 of the 8 analyzed samples; NAP, ANC, and B[*a*]P in 6 of them, whereas ACY in 5 samples. NAP was the compound that reached the highest concentrations in all samples (1 μg L^{-1}). Hence, the bioaccessibility of the compounds from the crumb rubber is demonstrated.

3.4. Human bioaccessibility of PAHs in crumb rubber from synthetic football fields

The bioaccessible fraction was calculated employing the Eq. (1) described in Section 2.4. The bioaccessible fraction of PAHs referred to the crumb rubber sample (ng g^{-1}) and the oral bioaccessible percentage (%) are included in Table S6. Table 2 shows the concentration ranges for the bioaccessible fraction for each PAH detected in the samples studied and the mean concentration value.

In addition, Fig. 3 shows the concentrations and frequency of PAHs in the bioaccessible fractions of the samples.

In general, the most volatile PAHs such as ACY, ACE, FLU, PHN and ANC presented the highest bioaccessibility rate between 0.1 and 6.6 % (excluding NAP, with much higher values) which was in line with a recent study about the bioavailability of PAHs in particulate matter (Sánchez-Piñero et al., 2022). A recent study that evaluated the dermal bioaccessibility of PAHs present in indoor dust showed similar bioaccessible percentages for NAP (Luo et al., 2020). In this study, ACY, ACE and FLU bioaccessibility percentage exceeded 1 % in 7 of the 8 analyzed samples, while for the other detected PAHs, it was lower than 1 %. These results were also consistent with those reported for the bioaccessibility of PAHs (5–13 %) of plastic pellets in coastal environments (Jiang et al., 2021). Fig. S3 (Supporting Information) represents the accumulative sum of PAHs found in the bioaccessible fraction (BF) for all samples.

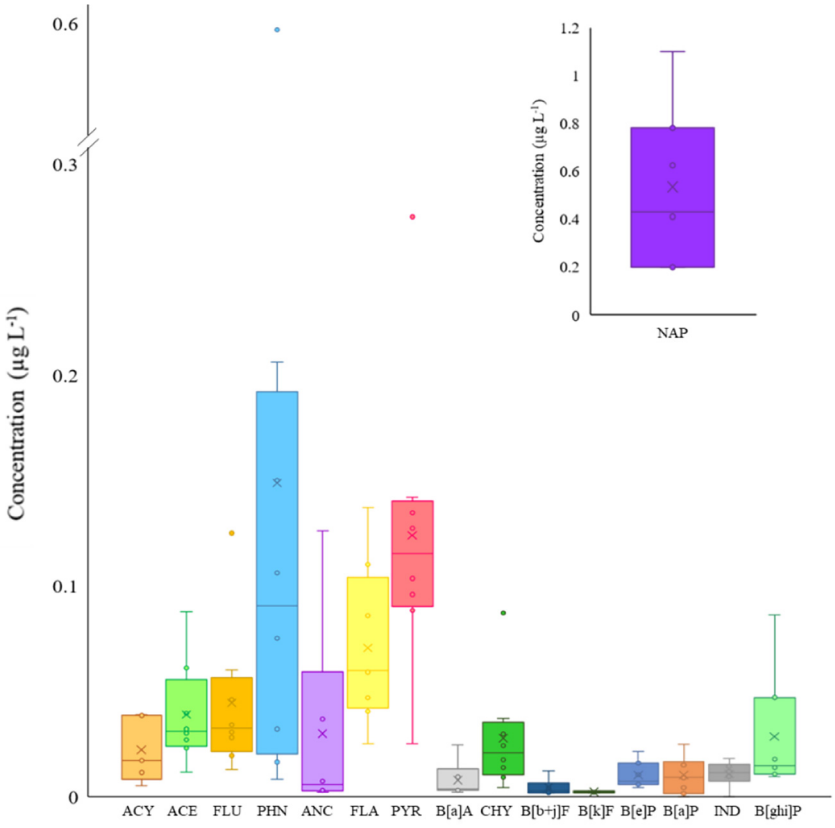

Fig. 2. Box-and-Whisker charts for the target PAHs detected in the synthetic biological fluids (μg L^{-1}).

Table 2
Concentration ranges and mean values of target PAHs in the crumb rubber infill (CR) (μg g^{-1}), in the bioaccessible fraction (BF) (ng g^{-1}) and the bioaccessible percentage (Bioac. %).

Samples (n = 8)	CR		BF		Bioac. %	
Compounds	Range	Average	Range	Average	Range	Average
NAP	0.061–0.71	0.18	20–51	34	19–49	33
ACY	0.042–0-54	0.20	0.52–3.9	2.2	0.31–2.0	0.92
ACE	0.023–2.2	0.44	1.2–8.8	3.9	0.39–6.6	3.2
FLU	0.070–2.4	0.86	1.3–13	4.7	0.25–2.1	1.3
PHN	0.81–24	7.3	1.6–60	17	0.035–0.84	0.29
ANC	0.11–9.7	2.0	0.43–13	4.4	0.090–0.46	0.22
FLA	4.0–20	8.1	2.5–14	7.0	0.053–0.15	0.099
PYR	11–23	15	4.0–28	13	0.035–0.12	0.084
B[*a*]A	0.30–4.6	1.4	0.22–2.5	0.79	0.016–0.16	0.074
CHY	1.7–7.8	4.2	0.41–8.7	2.8	0.011–0.11	0.065
B[*b*]F + B[*j*]F	0.37–1.9	0.9	0.39–1.6	0.9	0.039–0.10	0.072
B[*k*]F	0.074–0.73	0.27	–	–	–	–
B[*e*]P	1.1–4.5	2.6	0.42–2.1	1.0	0.015–0.10	0.044
B[*a*]P	0.72–4.2	1.6	0.43–2.5	1.4	0.017–0.19	0.081
IND	0.24–1.4	0.67	0.96–1.4	1.2	0.10–0.13	0.11
D[*ah*]A	0.083–0.71	0.36	–	–	–	–
B(*ghi*)P	1.2–6.1	3.3	0.94–8.6	2.8	0.029–0.19	0.083
ΣPAH	24–100	49	38–212	80	0.074–0.33	0.19

The observed behaviour might be related to compound hydrophobicity, which increases with the number of condensed rings in the molecule. NAP was the compound with the highest bioaccessibility percentage (between 19 and 49 %). The information about the possible bioaccessibility of these compounds in rubber granules is currently very limited and makes difficult to compare the present study with other investigations. For example, Zhang et al. (2008) evaluated the possible bioaccessibility of 2 rubber granulate samples in biological fluids, but the rubber samples contained too low concentrations of the PAHs which made the determination of the compounds in the gastrointestinal fluids impossible. For this reason, the percentage of bioaccessibility is only given for 3 compounds, offering similar results for NAP that the ones given in this research. Additionally, in other recent study the high LOQ and the rubber matrix interferences made it impossible to quantify several compounds present in the biological fluids (Schneider et al., 2020).

PHN, which is considered as a substance of very high concern (SVHC) and dangerous if ingested, was found in the bioaccessible fraction of all analyzed samples reaching concentrations up to 60 ng g^{-1} (bioaccesibility 0.25 %). B[*a*]P, considered as carcinogenic, mutagenic and toxic for reproduction(ECHA B[a]P, 2022), was detected in 75 % of the bioaccessible fractions at concentrations up to 2.5 ng g^{-1}. The comparison between the sum of target PAH concentrations found in the crumb rubber infill samples (μg g^{-1}) and the bioaccessible fraction (ng g^{-1}) is depicted in Fig. 4 for the eight analyzed samples. As can be seen, the distribution of the compounds is similar in the crumb rubber and in the bioaccessible portions. To provide more information about the distribution of the target compounds in the bioaccessible fractions, Fig. 5a shows the profiles for the bioaccessible fractions of samples S1, S2, S3, S4, S5 and S6. NAP, FLU, PHN, ANC, FLA and PYR were the compounds with the highest concentrations detected in crumb rubber bioaccessible fractions followed by the carcinogenic compound CHY.

It is important to mention that in the rubber granulate and in the bioaccessible fraction, the compounds with the highest concentrations were those with 4–6 rings (the heaviest and least volatile). In Fig. 5b the profiles for the concentration in the crumb rubber material (μg g^{-1}) and in the bioaccessible fraction (ng g^{-1}) for two samples from football fields (S1 and S6) are depicted. It is clear that the profile of the rubber granulates and the bioaccessible fractions are similar. As mentioned above, the compounds with 4–6 rings achieved the highest concentrations and many of these substances have toxic and harmful properties. Therefore, it is important be cautious and not only take into account the percentage of bioaccessibility, but also the detected bioaccessible concentrations for hazardous compounds.

Regarding the two commercial samples studied (CS1 and CS2), up to 16 out of the 18 PAHs studied were detected in the bioaccessible fractions. In the CS1 sample BF, 7 (B[*a*]A, CHY, B[*b*]F + B[*j*]F, B[*k*]F, B[*e*]P and B[*a*]P) of the 8 PAHs considered carcinogenic by ECHA were detected, reaching concentration up to 2.4 ng g^{-1} (CHY). It is also important to highlight that B[*a*]P has been detected in the bioaccessible fraction of these samples at a concentration of 0.70 ng g^{-1} (ECHA B[a]P, 2022). The total PAH bioaccessibility values ranged between 49 and 83 ng g^{-1} in the bioaccessible fractions for CS1 and CS2, respectively. Fig. 5c shows the relationship between the concentration profiles of PAHs in the commercial rubber granulates samples and their bioaccessible fractions, with a higher bioaccessibility for the CS1 sample than for CS2 in spite of the higher crumb rubber concentration of CS2. This is probably due to the lower particle size (1.8 mm) of CS1. CS2 showed a very high particle size (5.6 mm), much higher than CS1 and the samples collected in the football fields in this

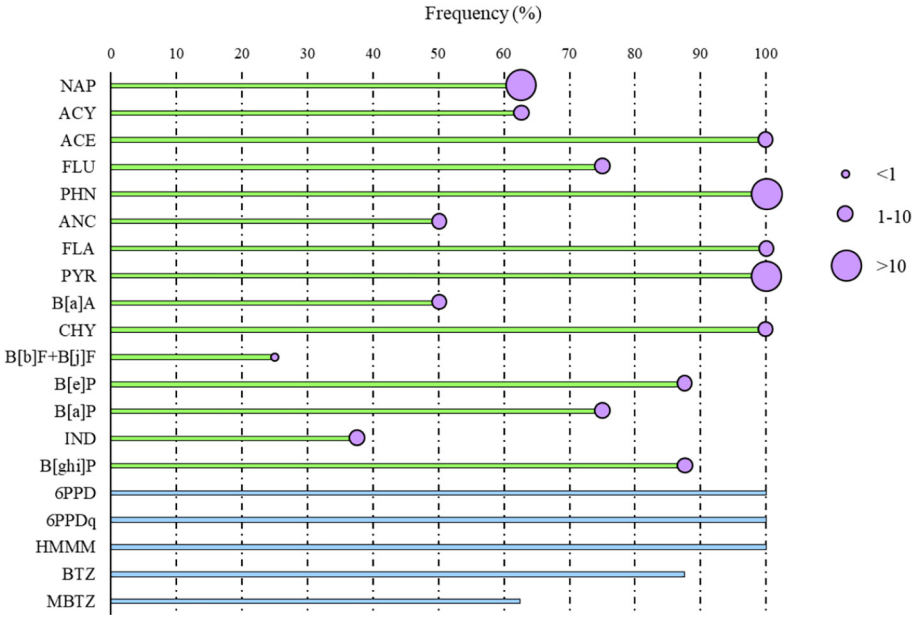

Fig. 3. Frequency (%) and mean concentration (ng g^{-1}) for PAHs in the bioaccessible fraction of the rubber granulate samples. The frequency (%, last 5 blue bars at the bottom of the figure) for other compounds detected in the bioaccessible fractions is also included.

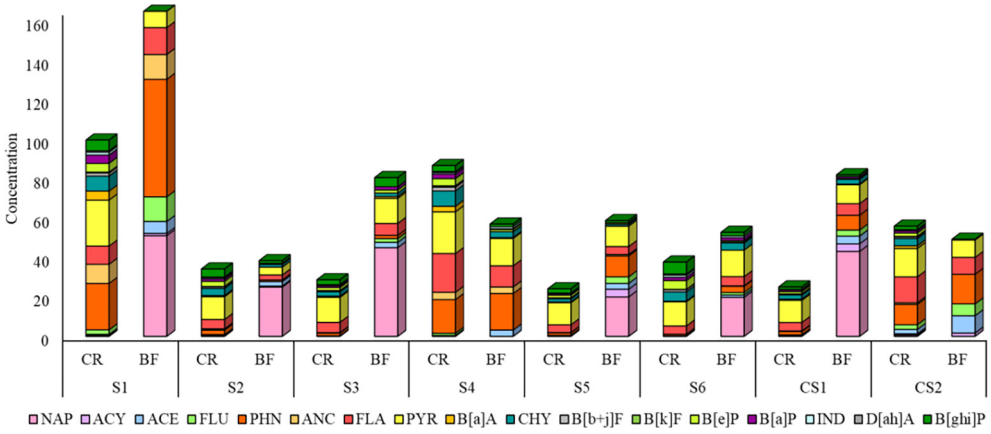

Fig. 4. Comparison of target PAHs concentration per sample in the crumb rubber (CR, μg g^{-1}) and in the bioaccessible fraction (BF, ng g^{-1}).

and in other previous studies. In samples directly collected on synthetic football pitches included in this study it is not possible to relate particle size to the bioaccessibility of the compounds, since all of them presented equivalent particle sizes (approx. 2 mm). In addition, other factors (age of the field, climatic conditions, *etc.*) might have modified the recycled crumb rubber properties. It was observed that the new pitches with ages

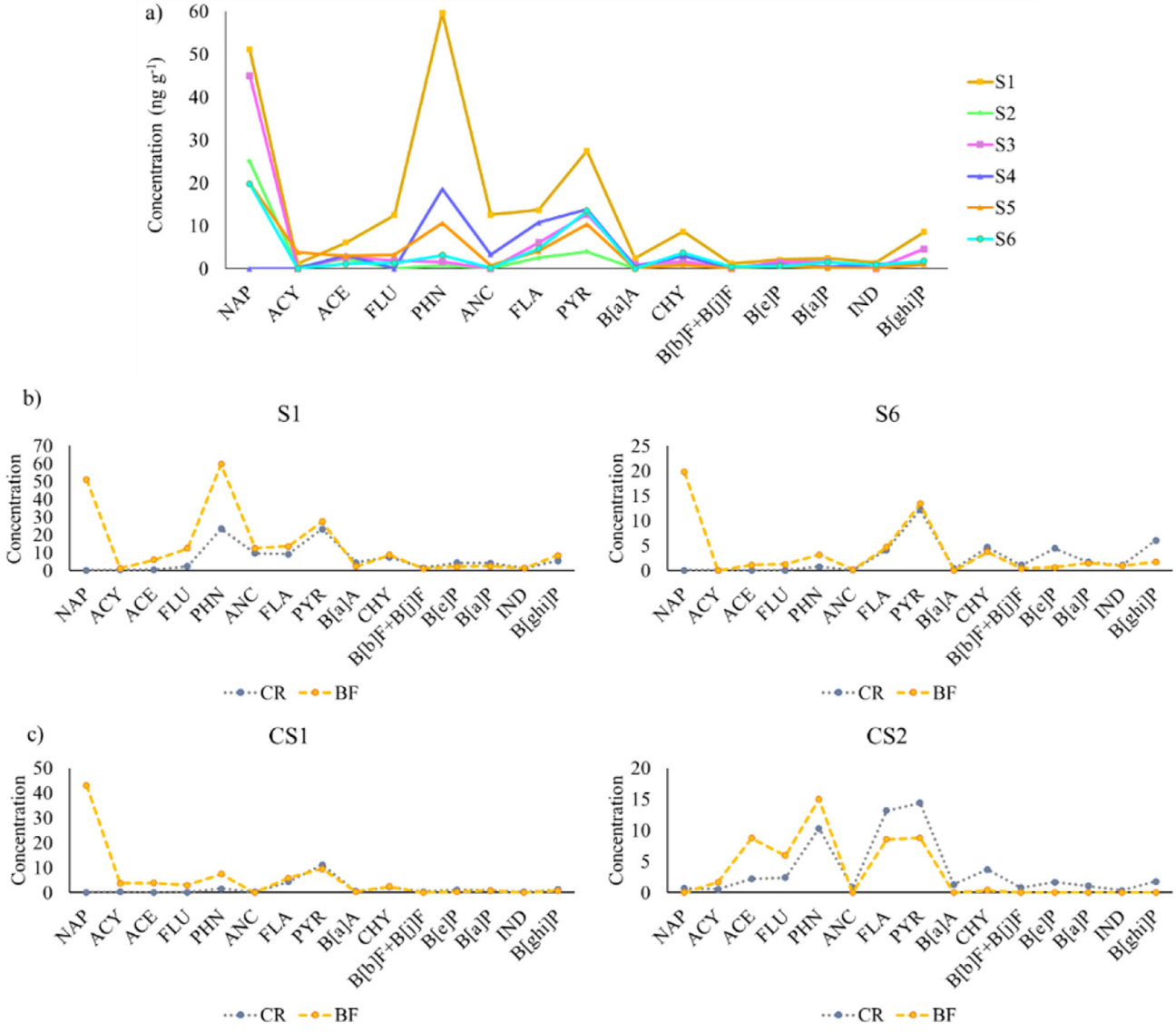

Fig. 5. a) Concentration profiles (ng g^{-1}) for individual PAHs in six crumb rubber bioaccessible fractions, b) PAH concentration profiles of the bioaccessible fraction (BF, ng g^{-1}) and in the crumb rubber employed as infill (CR, μg g^{-1}) for samples S1 and S6, and c) PAH concentration profiles of the bioaccessible fraction (BF, ng g^{-1}) and in the crumb rubber employed as infill (CR, μg g^{-1}) found in commercial samples CS1 and CS2.

ranging between 0.25 and 2 years showed higher bioaccessibility percentages than the older one. This was a slight approximation as only recycled rubber from one old pitch was assessed and the other pitches were quite new (<2 years old).

3.5. Detection of other bioaccessible substances from crumb rubber

Crumb rubber employed as infill in synthetic turf football pitches contains others hazardous chemicals such as plasticizers, antioxidants and vulcanisation additives (Armada et al., 2022b; Celeiro et al., 2018). Therefore, in addition to analyzing the 18 target PAHs, some other compounds related to the tire industry were identified in screening studies of the gastrointestinal fluids and their presence was confirmed by comparing the mass spectra found in the samples with those of the injection solutions prepared from the commercial standards (see Fig. S4 in Supporting Information). These compounds include 2 vulcanisers, BTZ and MBTZ, one antioxidant, 6PPD, and 1 accelerator and crosslinking agent HMMM. Additionally, 6PPD-quinone, a product of the reaction of the antioxidant 6PPD with ozone, was also found. Recent studies linked the presence of this compound in river water to the regular acute mortality of coho salmon and two types of trouts, as well as to early fish development (Brinkmann et al., 2022; McIntyre et al., 2021; Tian et al., 2021, 2022; Varshney et al., 2022). 6PPD, HMMM and 6PPD-quinone were detected in all the analyzed biological fluids. Furthermore, BTZ and MBTZ were detected in 7 and 5 samples, respectively (see Fig. 3). The presence of BTZ, MBTZ and 6PPD in synthetic body fluids (saliva and gastric juice) has been very recently reported (Schneider et al., 2020). The occurrence of 6PPD-quinone in the synthetic biological fluids is of great concern due to its lethal effect on the salmon species mentioned above. To the best of our knowledge, this study identifies for the first time HMMM and 6PPD-quinone in human gastrointestinal fluids obtained after the digestion of recycled crumb rubber materials.

3.6. Exposure assessment of children to PAHs present in crumb rubber in synthetic football fields

The estimated daily uptake (EDU) which is employed to assess the daily exposure considering the bioaccessibility percentage, was calculated employing Eq. (2) (Arfaeinia et al., 2022; Lu et al., 2021). Since the main users of synthetic football fields are children, a child between 3 and 6 years old exposure was estimated.

Table 3 shows the EDU values obtained for the target PAHs in the bioaccessible fraction of the 8 crumb rubber samples.

The US-EPA proposed a value of 300 ng kg^{-1} day^{-1} as the oral reference dose (RfD) for B[*a*]P (EPA B[a]P, 2017), but there is no RfD for other PAHs. The results for B[*a*]P were much lower than the RfD ($\leq$ 0.011 % respect to the RfD value or lower) but the presence of the other PAHs should be considered. For this reason, the sum of the uptake of all target PAHs was compared. It represented between 0.16 and 0.90 % of the RfD. These results must be taking as an estimation because not all target PAHs have the same harmful and damaging properties such as B[*a*]P, for example, PHN has cardiac impacts ((Brette et al., 2017). Nevertheless, it should be noted that many other PAHs including heterocyclic, sulfur-PAHs and alkylated PAHs (for example methyl-PAHs), have been found in this material, and the full latent risk due to PAH content should not be underestimated (Eriksson et al., 2022; US-EPA, 1993). Additionally, the B[*a*]P equivalent carcinogenicity was calculated (Sánchez-Piñero et al., 2022) for an evaluation of the potential carcinogenic properties considering the target PAHs using Eq. (3).

Results for B[*a*]P equivalents are shown in Table S7, in Supporting Information. In addition to B[*a*]P, other target PAHs have a significant influence on the carcinogenic potential such as B[*a*]A, B[*b*]F, B[*j*]F and IND with TEF value of 0.1, and B[*e*]P with a value of 1. Several studies reported the contribution to the relative equivalence of B[*a*]P of more PAHs than the 18 considered in this study, and as discussed above, different PAHs are usually found in these materials.

Therefore, there are probably more compounds harmful to health that were not considered. Additionally, a synergy effect between PAHs in complex mixtures, enhancing their toxic properties has been demonstrated (Samburova et al., 2017).

B[*a*]P equivalents of each EDU target PAH and their relation to the daily RfD are shown in Table S7. For this calculation, the EDU for each compound was multiplied by its corresponding TEF respect to B[*a*]P. The obtained values ranged between 0.0027 and 0.024 %. The samples with the highest percentage were S1, S3, S4 and S6, the first three corresponding to crumb rubber from new football fields and S6 to a sample collected in an indoor pitch.

In conclusion, these results suggest the potential risk of accidental ingestion for these substances (18 PAHs) present in recycled rubber, but it should be borne in mind that there are numerous hazardous compounds that can have detrimental effects on human or animal health.

4. Conclusions

This study demonstrates for the first time the bioaccessibility of most 16 EPA PAHs and 8 ECHA PAHs in real crumb rubber samples employed as

Table 3
Estimated daily uptake (EDU) of target PAHs *via* synthetic turf football field crumb rubber infill ingestion by children (3-6 years).

EDU (ng kg^{-1} day^{-1}) × 10^2	S1	S2	S3	S4	S5	S6	CS1	CS2	Statistical summary		
									N[a]	Range	Mean
NAP	66	32	57	–	25	25	56	–	6	25–66	44
ACY	1.5	0.65	–	–	4.9	–	4.99	2.1	5	0.66–4.99	2.8
ACE	7.4	2.9	3.4	3.9	3.9	1.5	5.3	11	8	1.5–11.0	4.9
FLU	16	–	2.5	–	4.2	1.6	3.7	7.7	6	1.6–16.0	5.9
PHN	75	1.0	2.1	24	13	4.1	9.6	20	8	1.0–75.0	19
ANC	16	–	–	4.2	0.93	0.27	–	–	4	0.27–16.00	5.4
FLA	18	3.2	8.1	14	5.1	5.8	7.7	11	8	3.2–17.8	9.0
PYR	36	5.1	16	18	13	17	12	11	8	5.1–35.6	16.1
B[*a*]A	3.1	–	1.2	0.57	–	–	0.49	–	4	0.49–3.14	1.3
CHY	11	1.8	2.2	3.8	1.2	4.7	3.0	0.47	8	0.47–11.00	3.5
B[*b*]F + B[*j*]F	1.6	–	–	–	–	0.55	–	–	2	0.55–2.03	1.29
B[*e*]P	2.7	0.73	2.0	1.4	0.94	0.85	0.55	–	7	0.55–2.72	1.31
B[*a*]P	3.2	–	2.1	0.50	0.18	1.9	1.2	–	6	0.18–3.18	1.5
IND	1.8	–	–	1.3	–	1.4	–	–	2	1.3–1.8	1.5
B(*ghi*)P	11	1.7	6.0	1.8	1.4	2.2	1.2	–	7	1.2–11.2	3.6
ΣPAH	270	48	104	82	76	68	105	63	–	48–270	102
ΣPAH/RfD (%)	90	16	35	27	25	23	35	21	–	16–90	34

[a] Number of samples on which each target compounds were detected (total number of samples 8).

infill in synthetic turf football fields. To digest the samples and analyze the synthetic gastrointestinal fluids a method based on UBM-SPE-GC–MS/MS was optimized and validated. The accuracy of the method was evaluated with recovery tests at three concentration levels (0.2, 1 and 10 μg L^{-1}) obtaining values around 93 % with an RSD of 13 %. The feasibility of a new green SPE biosorbent based on cork by-products for PAH extraction was demonstrated, as well as the suitability of the UBM method to perform the digestion of these type of matrices. In addition, the use of the biosorbent allows a new function for a waste product promoting circular economy.

The application of the UBM-SPE-GC–MS/MS protocol to RTCR samples taken from football fields demonstrated PAH bioaccessibility. All compounds were detected in the gastric fluids (excluding one) including the carcinogenic PAHs. The bioaccessible fraction achieved values of 2.5 ng g^{-1} for B[*a*]P and even higher levels for other PAHs (up to 60 ng g^{-1}). The risk assessment of accidental ingestion by a child between 3 and 6 years was also carried out. The presence in the bioaccessible fraction of emerging contaminants such as 6PPD, 6PPD-quinone, HMMM, BTZ, MBTZ is reported for the first time. Although this research has provided a better understanding of the bioaccessibility of PAHs present in recycled rubber granulate, further studies are needed to assess the possible bioaccessibility of other compounds present in the chemical composition of tires, such as various endocrine disruptors, reprotoxic compounds, and heavy metals, among others, which may be harmful to human health, to avoid risks underestimation. Besides, the study of the particulate matter (PM) fraction that could be inhaled, precipitated or ingested must be addressed in future works.

CRediT authorship contribution statement

Daniel Armada: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, review & editing, Visualization. **Antia Martinez-Fernandez:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft. **Maria Celeiro:** Methodology, Formal analysis, Investigation, Review & editing, Visualization. **Thierry Dagnac:** Review & editing. **Maria Llompart:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159485.

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