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Presence of parabens in children's faeces. Optimization and validation of a new analytical method based on the use of ultrasound-assisted extraction and liquid chromatography-tandem mass spectrometry

--Manuscript Draft--

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Abstract:	Due to their antimicrobial properties, parabens are a family of synthetic chemical compounds widely used as preservative additives in food and cosmetics. For this reason, humans are highly exposed to them. These substances are capable of altering the proper functioning of the endocrine system and are classified as endocrine disrupting chemicals (EDCs). Traditionally, urine has been the typical matrix studied as an excretion route. However, faeces contain valuable information. In the present study, the presence of methyl-, ethyl-, isopropyl-, propyl-, isobutyl-, butyl- and phenylparaben in stool samples from children has been evaluated. A new analytical method has been optimised and validated. The method is based on the use of ultrasound-assisted extraction followed by clean-up of the extracts by dispersive solid phase extraction (d-SPE). Parabens were analysed by ultrahigh performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The matrix effect was evaluated and a significant effect was observed for all analytes. Therefore, calibration and validation were performed by addition of different concentrations of analytes to faecal blanks. The coefficient of determination (%R2) for calibration curves was higher than 98.9% in all cases. The limits of detection and quantification were between 0.2-0.4 and 0.6-1.0 ng g-1 respectively. The recovery for accuracy assessment had values between 89.0 and 112.7% with an RSD of less than 15% in all cases. The method was successfully applied to 14 samples from children volunteers, 100% of which showed contamination by at least one of the analysed compounds.
Suggested Reviewers:	Julia Martín-Bueno, PhD Professor, University of Seville jbueno@us.es Dr. Martin-Bueno has published in the subject of this research. She has work with biological samples similar to the ones proposed in the present research.
	Hilde Kristin Vindenes, PhD Researcher, Haukeland University Hospital hilde.kristin.vindenes@helse-bergen.no Dr. Vindenes has published in the subject of this research. The profile of the researcher is the prototype to which this work would be destined.
Response to Reviewers:	

December 20th, 2022

Dear Editor,

Please find attached the revised version of the manuscript entitled ***"Presence of parabens in children's faeces. Optimization and validation of a new analytical method based on the use of ultrasound-assisted extraction and liquid chromatography-tandem mass spectrometry"*** by *Inmaculada Moscoso-Ruiz, Alberto Navalón, Ana Rivas, Alberto Zafra-Gómez*, which we are submitting for publication in the journal ***Journal of Pharmaceutical and Biomedical Analysis.***

According to the reviewer comment, the minor change suggested has been included in the new version of the text.

I look forward to hearing from you.

Dr. Alberto Zafra-Gómez

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Reviewer #1:

Suggestion: Please add the reply for comment 3 (explanation for the double peaks in Figure 1) to the manuscript.

Thank you for your suggestion. The explanation has been included in the final version of the manuscript, Page 6, Line 200: *"The chromatograms showing double peaks belong to the separate isomers iPPB and PPB at retention times 9.71 and 10.02 min, respectively; and iBPB and BPB at 11.27 and 11.39 min, respectively. The peaks appear together because their most sensitive transitions coincide with each other; however, they can be differentiated by the retention time and the ratio between transitions. In the figure, the peak corresponding to each compound is marked with the retention time and the corresponding compound is shown on the right."*

Reviewer #2:

The revised manuscript is now suitable for publication in this esteemed journal. Therefore, I can recommend this manuscript for publication.

Thank you for your revision for improving the quality of our research.

Reviewer #3:

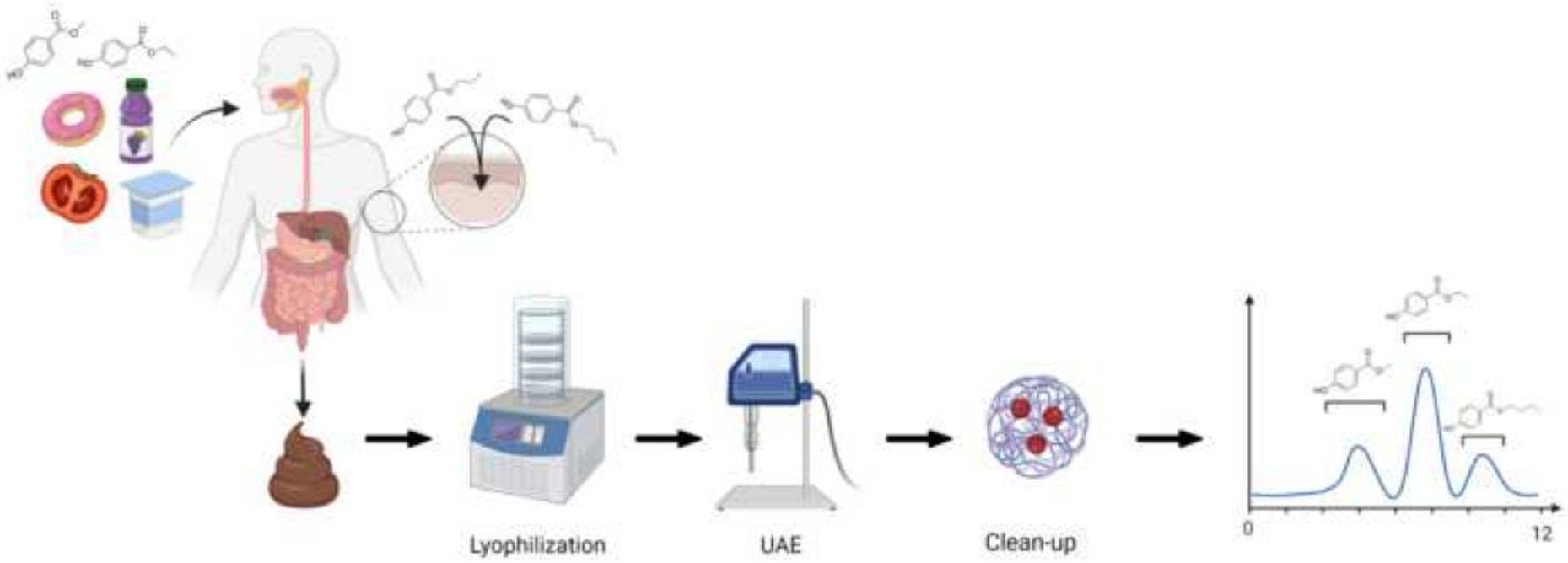
The authors were answered all of my concerns.

Thank you for your revision for improving the quality of our research.

Highlights

- An ultrahigh performance liquid chromatography coupled to tandem mass spectrometry method for the determination of parabens in human faeces is proposed.
- For analyte isolation ultrasound assisted extraction is proposed.
- The highest amounts found were of methylparaben, ethylparaben and propylparaben in all samples.
- The method has been validated and the analytical quality parameters described.
- Faeces may be considered as a good biomarker for the human exposure to parabens.

Graphical Abstract



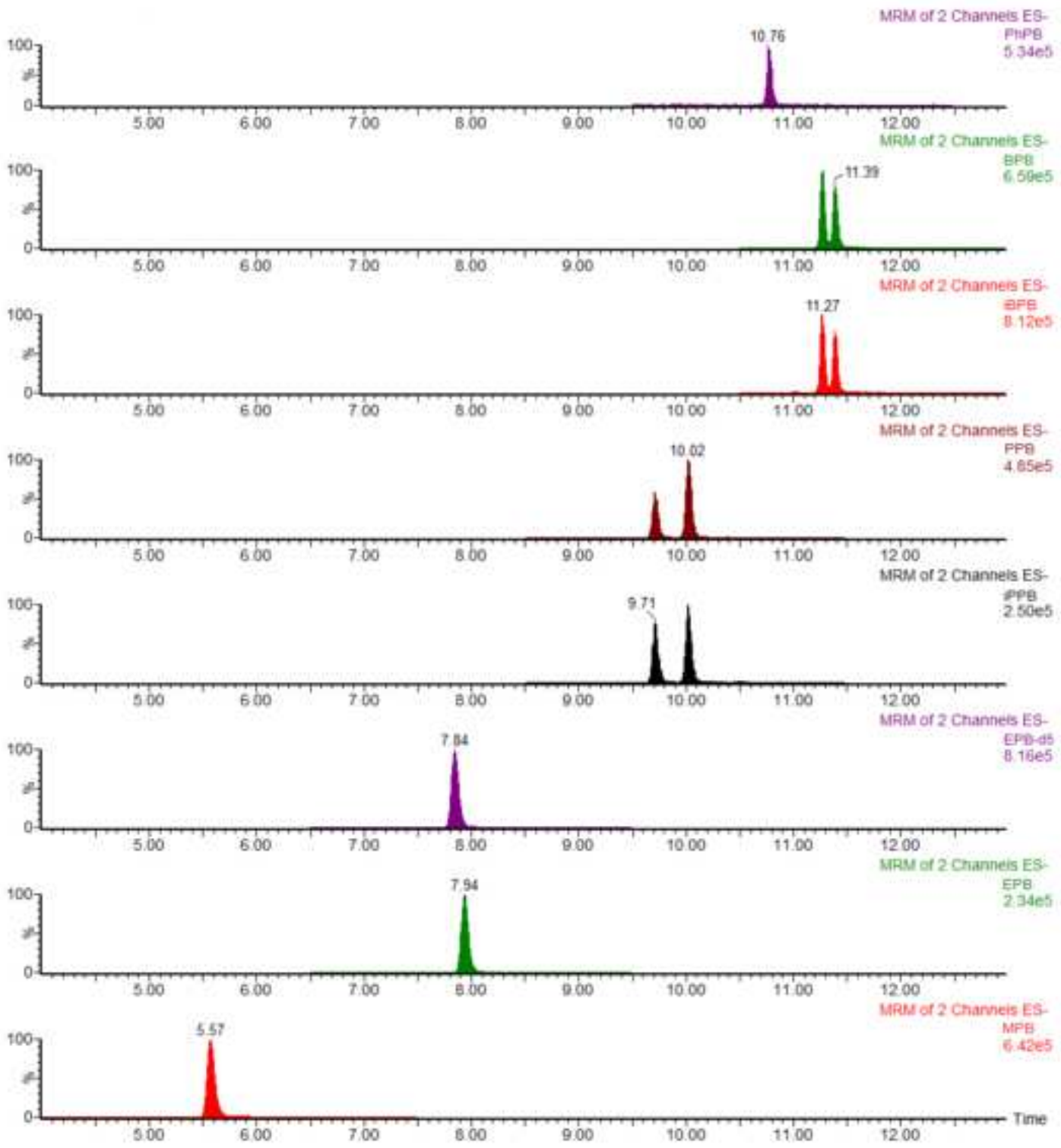
Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Figure

Click here to access/download;Figure;JPBA - Figure 1.tif



Figure

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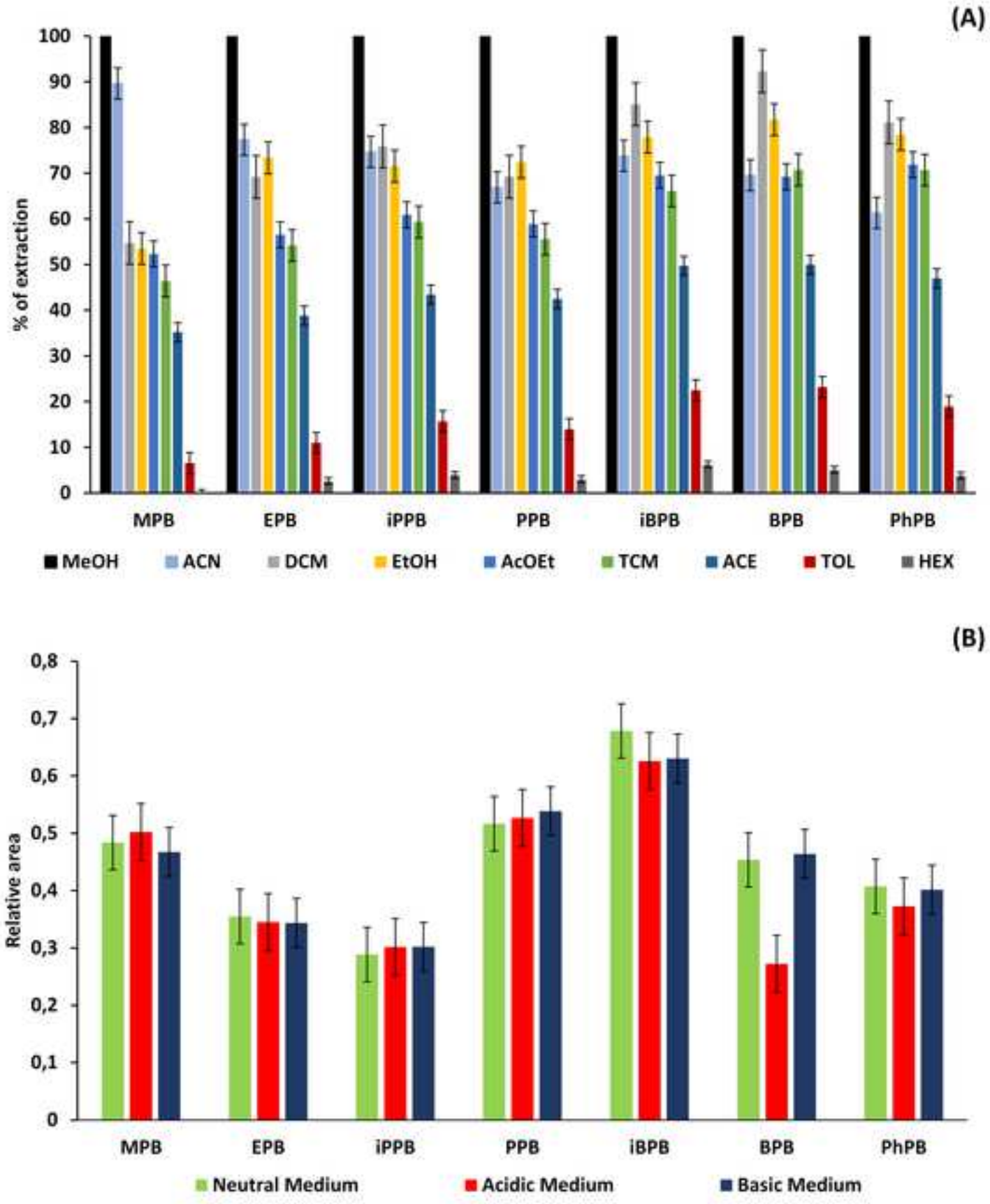

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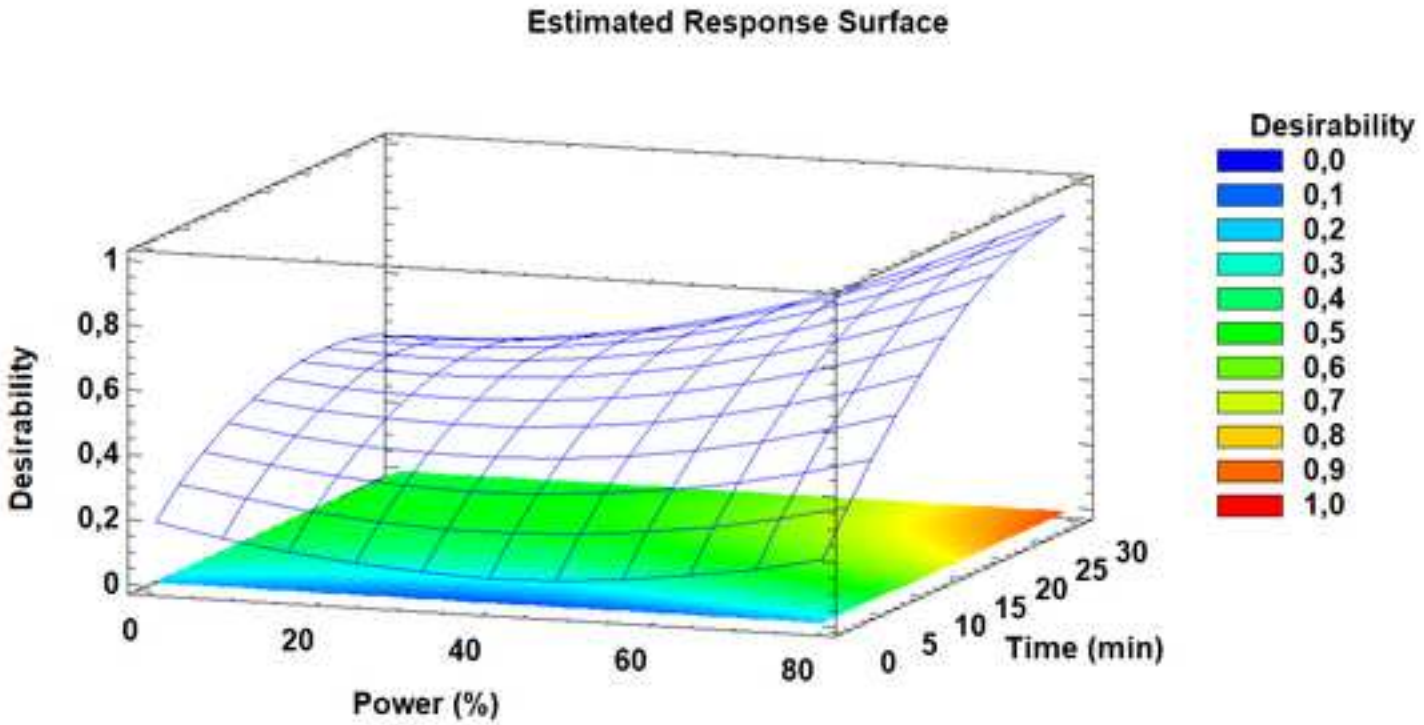

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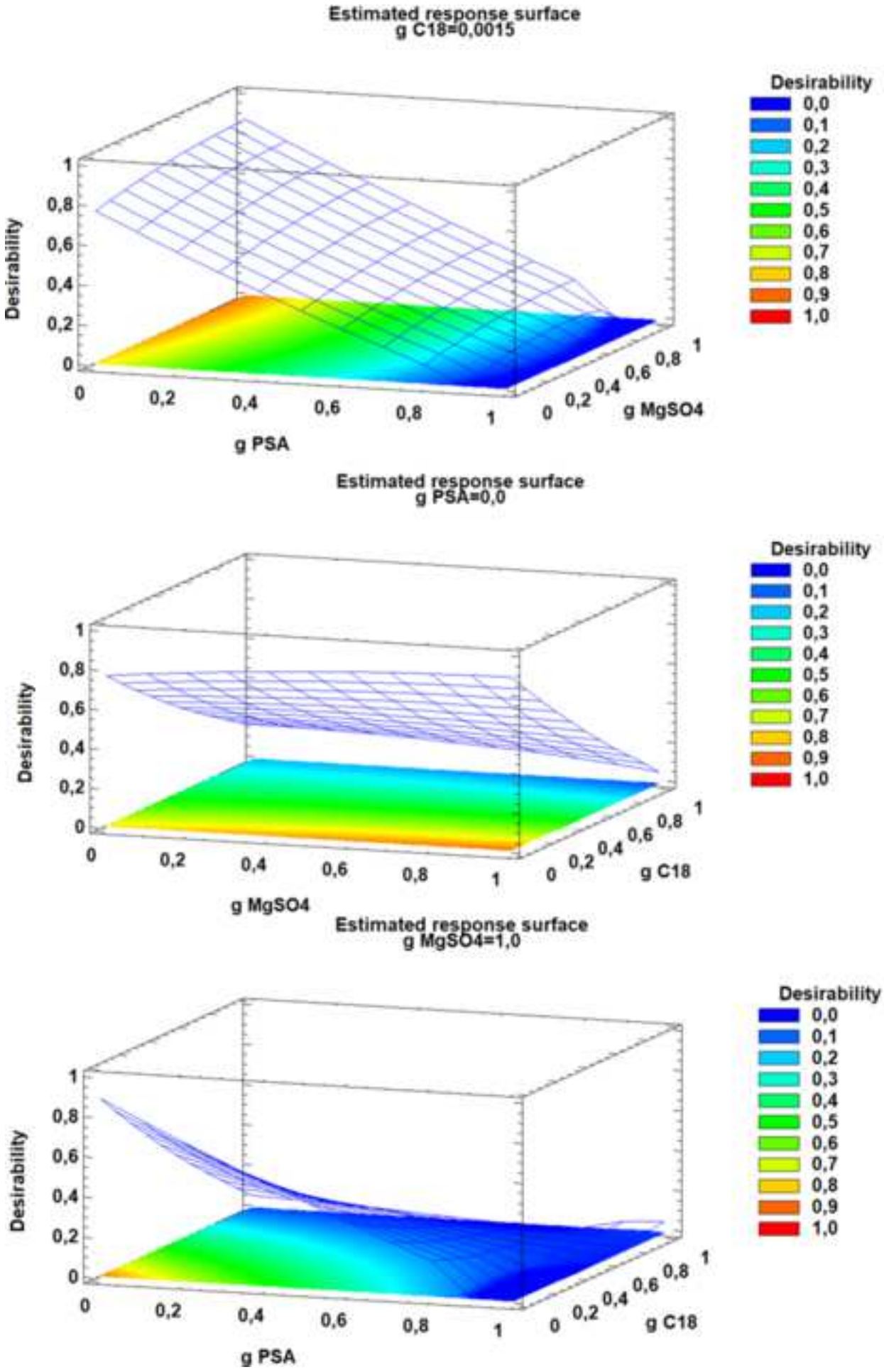

Figure

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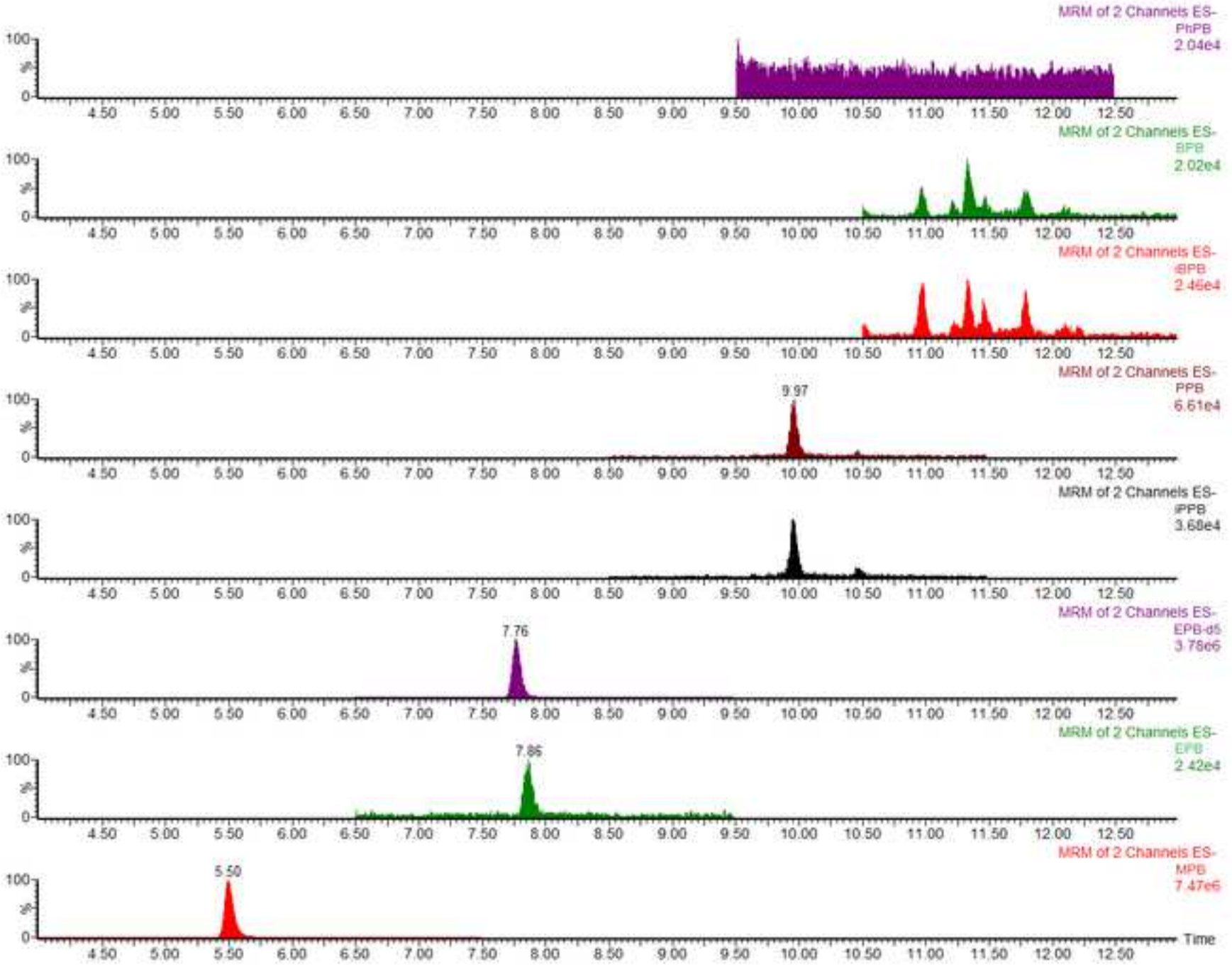

Table 1. UHPLC-MS/MS parameters for parabens.

	t_R (min)	MRM Transitions (m/z)	r_{ab}	CV (V)	CE (eV)	Precursor ions (m/z)	Fragment ions
MPB	5.6	151.0 → 92.0[a] 151.0 → 135.9[b]	2.3	-6 -6	-18 -14	$[M-H]^-$ 151.0	$[M-H-COOCH_3]^-$ 92.0 $[M-H-CH_3]^-$ 135.6
EPB-d_5	7.8	170.2 →92.0[a] 170.2 → 138.0[b]	1.9	-6 -16	-22 -14	$[M-H]^-$ 170.2	$[M-H-COOCD_2CD_3]^-$ 92.0 $[M-H-CD_2CD_3]^-$ 138.0
EPB	7.9	165.1 → 92.2[a] 165.1 → 136.7[b]	1.8	-16 -16	-20 -14	$[M-H]^-$ 165	$[M-H-COOCH_2CH_3]^-$ 92.2 $[M-H-CH_2CH_3]^-$ 136.7
iPPB	9.7	179.05 →92.6[a] 179.05 →136.1[b]	3.0	-12 -12	-18 -18	$[M-H]^-$ 179.05	$[M-H-COOCH(CH_3)_2]^-$ 92.6 $[M-H-CH(CH_3)_2]^-$ 136.7
PPB	10.0	179.1 → 92.1[a] 179.1 →136.4[b]	3.8	-8 -8	-20 -14	$[M-H]^-$ 179.1	$[M-H-COO(CH_2)_2CH_3]^-$ 92.1 $[M-H-(CH_2)_2CH_3]^-$ 136.4
PhPB	10.8	213.1 → 92.8[a] 213.1 → 64.9[b]	191.6	-12 -12	-38 -14	$[M-H]^-$ 213.1	$[M-H-COOC_6H_5]^-$ 92.0 $[C_5H_5-]^-$ 65.0
iBPB	11.3	193.6 → 92.0[a] 193.6 → 136.1[b]	2.5	-14 -14	-22 -16	$[M-H]^-$ 193.6	$[M-H-COOCH_2CH(CH_3)_2]^-$ 92.6 $[M-H-CH_2CH(CH_3)_2]^-$ 136.1
BPB	11.4	193.1 → 92.2[a] 193.1 → 136.5[b]	4.5	-6 -6	-22 -14	$[M-H]^-$ 193.1	$[M-H-COO(CH_2)_3CH_3]^-$ 92.2 $[M-H-(CH_2)_3CH_3]^-$ 136.5

Capillary Voltage: 3 kV; Source temperature: 150 ºC; Desolvation temperature: 600 ºC; Cone gas flow: 150 L h^{-1}; Desolvation gas flow: 500 L h^{-1}; Collision gas flow: 0.15 mL min^{-1}; Nebulizer gas pressure: 7 bar; Cone/desolvation gas: N_2 (≥99.995 %); Collision gas: Ar (≥99.995 %); Dwell time: 25 ms; Inter-scan delay: 3 ms.

[a]Transition used for quantification; [b]Transition used for confirmation; t_R retention time; r_{ab} ratio between transitions (a/b); CV cone voltage; CE collision energy.

Table 2. Study of matrix effect. Matrix calibration parameters. Accuracy, recovery and precision

	% ME	b g ng^{-1}	R^2 %	LOD ng g^{-1}	LOQ ng g^{-1}	Recovery study (RSD, %, n=21) 1 ng g^{-1}	 10 ng g^{-1}	 100 ng g^{-1}	 250 ng g^{-1}
MPB	89.6	$3.01 \cdot 10^{-3}$	99.4	0.3	0.9	101.3 (12.1)	110.8 (12.2)	105.7 (10.5)	99.6 (2.4)
EPB	90.0	$2.69 \cdot 10^{-3}$	99.9	0.3	0.9	94.4 (9.0)	95.2 (6.9)	102.9 (1.0)	98.4 (1.3)
PPB	77.4	$4.19 \cdot 10^{-3}$	98.5	0.2	0.7	100.4 (14.8)	89.0 (11.7)	101.9 (13.2)	97.5 (4.5)
iPPB	84.6	$1.77 \cdot 10^{-3}$	99.4	0.4	1.0	108.9 (15.0)	91.8 (10.4)	100.1 (11.7)	98.6 (4.2)
BPB	81.6	$5.37 \cdot 10^{-3}$	99.4	0.2	0.6	95.7 (14.5)	109.1 (9.0)	92.9 (3.6)	100.1 (1.2)
iBPB	82.6	$7.41 \cdot 10^{-3}$	98.9	0.2	0.6	107.8 (14.5)	106.8 (8.1)	96.4 (6.2)	104.1 (3.8)
PhPB	62.6	$5.95 \cdot 10^{-3}$	99.5	0.2	0.6	112.7 (4.2)	109.5 (7.3)	91.4 (2.1)	101.7 (4.3)

ME: Matrix effect; Linear dynamic range: LOQ-250 ng mL^{-1}; n: calibration levels; b: slope of matrix calibration; % R^2 determination coefficient; LOQ: Limit of quantification; LOD: Limit of detection; RSD: Relative Standard Deviation.

Table 3
Detection of parabens in children's faeces.

	MPB	EPB	iPPB	PPB	iBPB	BPB	PhPB
1	D (>R)	2.7 (7.6)	ND	5.3 (15.8)	ND	ND	ND
2	35.7 (2.3)	1.9 (8.6)	ND	96.2 (2.9)	ND	ND	ND
3	94.0 (13.9)	2.3 (12.4)	ND	4.4. (7.9)	ND	ND	ND
4	83.8 (8.3)	4.1 (10.8)	ND	7.2 (7.8)	ND	ND	ND
5	149.4 (3.8)	6.2 (9.1)	ND	8.5 (11.1)	ND	ND	ND
6	137.0 (10.5)	2.3 (9.0)	ND	7.0 (11.7)	ND	ND	ND
7	182.0 (9.2)	2.2 (9.9)	D	6.8 (9.4)	ND	ND	ND
8	190.1 (1.9)	3.1 (3.7)	ND	5.2 (15.5)	ND	ND	ND
9	43.6 (6.1)	2.7 (15.5)	ND	6.9 (0.8)	ND	ND	ND
10	48.7 (4.2)	2.5 (4.4)	ND	4.9 (11.7)	ND	ND	ND
11	59.6 (2.6)	1.5 (9.2)	ND	3.8 (8.3)	ND	ND	ND
12	92.2 (6.0)	1.7(13.1)	ND	3.8 (8.8)	ND	ND	ND
13	ND	1.0 (9.0)	3.9 (1.1)	16.3 (8.0)	0.6 (12.4)	0.6 (14.5)	ND
14	D (>R)	2.2 (7.1)	ND	5.2 (13.2)	ND	ND	ND

D (>R): detected but at concentrations above the upper limit of the calibration; D: Detected (LOD < x < LOQ); ND: Not detected (<LOD)

Inmaculada Moscoso-Ruiz: Formal analysis, Investigation, Methodology, Data curation, Writing – original draft. **Alberto Navalón**: Conceptualization, Formal analysis, Investigation, Writing–original draft. **Ana Rivas**: Formal Analysis, Funding acquisition, Writing-review and editing. **Alberto Zafra-Gómez**: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Data curation, Writing–original draft, Writing-review, editing and supervision.

Presence of parabens in children's faeces. Optimization and validation of a new analytical method based on the use of ultrasound-assisted extraction and liquid chromatography-tandem mass spectrometry

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ABSTRACT Due to their antimicrobial properties, parabens are a family of synthetic chemical compounds widely used as preservative additives in food and cosmetics. For this reason, humans are highly exposed to them. These substances are capable of altering the proper functioning of the endocrine system and are classified as endocrine disrupting chemicals (EDCs). Traditionally, urine has been the typical matrix studied as an excretion route. However, faeces contain valuable information. In the present study, the presence of methyl-, ethyl-, isopropyl-, propyl-, isobutyl-, butyl- and phenylparaben in stool samples from children has been evaluated. A new analytical method has been optimised and validated. The method is based on the use of ultrasound-assisted extraction followed by clean-up of the extracts by dispersive solid phase extraction (d-SPE). Parabens were analysed by ultrahigh performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The matrix effect was evaluated and a significant effect was observed for all analytes. Therefore, calibration and validation were performed by addition of different concentrations of analytes to faecal blanks. The coefficient of determination (%R^2) for calibration curves was higher than 98.9% in all cases. The limits of detection and quantification were between 0.2-0.4 and 0.6-1.0 ng g^{-1} respectively. The recovery for accuracy assessment had values between 89.0 and 112.7% with an RSD of less than 15% in all cases. The method was successfully applied to 14 samples from children volunteers, 100% of which showed contamination by at least one of the analysed compounds.

Keywords Endocrine disruption; Excreta Stool; Parabens; Ultra-high performance liquid chromatography-tandem mass spectrometry

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1. Introduction

Endocrine disrupting chemicals (EDCs) are synthetic substances present in the environment mainly due to industrial activity. They are widely used in all kinds of applications such as the manufacture of plastics and resins, as additives in personal care products (PCPs) and even as preservatives in food and cosmetics. These substances can be found in the environment (soil, water and air) as well as in food and PCPs, reaching living organisms mainly through the diet, although there are other routes, such as dermal or inhalation [1]. EDCs are increasingly studied for their ability to modulate hormonal response, disrupt the endocrine system and produce different types of toxicity [1,2]. Children are a particularly vulnerable population to EDCs because they are at a critical stage of development.

Parabens are alkyl or aryl homologues of the *p*-hydroxybenzoic acid. Before year 2000, based on various scientific studies, scientific community determined they were non-toxic to human health [3]. However, in the last years new studies have shown that this family of compounds is capable of altering the endocrine system of individuals, which has led some parabens to be considered as EDCs [4]. Nowadays, it has been proved that parabens provoke, among others, alterations in reproductive system and in adipose tissue accumulation [5]. Parabens most used are methylparaben (MPB), ethylparaben (EPB), propylparaben (PPB) and butylparaben (BPB). These substances are used as antimicrobial preservatives due to its properties, especially in personal care products (PCPs) and foods. In 2013, it was regulated by the European Union the maximum amount of parabens in PCPs, being 0.14 % for PPB and BPB, 0.4 % for MPB and EPB and 0.8 % for mixtures [6]. In food, MPB and EPB and their sodium salts are allowed as E numbers (E218 and E219 for MPB and Na-MPB; E214 and E215 for EPB and Na-EPB, respectively), meanwhile PPB and BPB were forbidden [7].

Parabens are well-studied compounds in different kinds of matrixes. There are lots of works which determine these substances both in the environment and in biological matrixes [8-11]. Isopropyl paraben (iPPB) and isobutyl paraben (iBPB) are isomers of PPB and BPB respectively, and they have been found in outdoor environments and in PCPs named as "green" [12, 13]. As one of the main routes of exposure is the diet, there are multiple scientific papers studying parabens in foods and also in invasive and non-invasive biological human matrixes, such as breast milk, plasma, urine, hair, saliva or nails [14-19]. Urine is the most used matrix as a biomarker. In the first 24 h, 80% of parabens are excreted, but in 48 h

some studies show more recovery of MPB in urine than BPB, suggesting the larger is the carbon chain radical, the larger stays in the body [20]. However, parabens in faeces have been scarcely studied, probably due to the complexity of this matrix. Only two recent papers have studied parabens in stool [21, 22]. One of them analyzes MPB and PPB in poultry manure using gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) [21]. The second one analyses MPB, EPB, PPB, BPB and benzylparaben (BzPB) in penguin excreta from the Antarctic region, using high performance liquid chromatography with ultraviolet detection (HPLC-UV) [22]. To our knowledge, there are still no studies on the determination of parabens in human faeces samples. Because people are surrounded by EDCs, in particular parabens, faeces can provide valuable data on daily intake and dermal absorption of these substances as does urine, as well as complementary information to that provided by urine. Further, the objectives of the present work are first to optimize an analytical method to determine 7 parabens (MPB, EPB, iPPB, PPB, iBPB, BPB and phenylparaben (PhPB)) with endocrine disrupting activity in children's faeces using liquid chromatography-tandem mass spectrometry, then to validate the proposed method following appropriate international guidelines, and finally to apply the method to samples of children's faeces to determine the content in parabens and to assure the applicability of the method to natural samples.

2. Materials and methods

2.1. Chemical reagents

For the optimization it was used standard reagents and all were analytical grade: MPB, EPB, PPB and BPB (≥99% purity) and iPPB and iBPB (≥98% purity) were supplied by Alfa Aesar (Thermo Fisher Scientific, Kandel, Germany). Deuterium labelled EPB (EPB-d_5) (≥98% purity) was from Toronto Research Chemicals (NY, Canada). Table S1 shows the structure and CAS number of those compounds. For the usage of ultra-pure water (18.2 M□) it was used an in-house Milli-Q Plus® system (Merck Millipore). It was prepared working standard solutions in methanol (MeOH) for each target analyte at 4.0 µg mL^{-1}, and EPB-d_5 and was prepared at a concentration of 0.5 µg mL^{-1}. Methanol (MeOH, 99.9%), dichloromethane (DCM, 99.9%), acetonitrile (ACN, 99.9%) and n-hexane (HEX, 97.0%) were purchased from Honeywell (Madrid, Spain); trichloromethane (TCM, 99.9%), acetone

(ACE, 99.9%) and sodium hydroxide (NaOH) in pellets from Panreac (Barcelona, Spain); ethanol (EtOH, 99.9%) and acetic acid (glacial 99-100%) from JT Baker (Madrid, Spain); and toluene (TOL, 99.9%) and ethyl acetate (AcOEt, 99.9%) from Sigma-Aldrich. For the clean-up of extracts procedure, it was used Primary Secondary Amine (PSA) bonded silica (Supelco), C18 (Supelco) and magnesium sulfate ($MgSO_4$, 96%, Panreac). It was used ammonium acetate (≥ 98% purity, Honey-well) for the mobile phase.

2.2. Instrumentation and software

The determination of the target analytes was carried out using an ultrahigh performance liquid chromatography system UPLC™ H-Class, coupled with a triple quadrupole mass spectrometer Xevo TQ-XS equipped with a StepWave ion guide and an orthogonal Z-spray™ electrospray ionization (ESI) source from Waters, Manchester, UK (UHPLC-MS/MS). The separation was performed on an Acquity UPLC® BEH C18 column (2.1 mm x 100 mm, 1.7 µm particle size).

For the optimization of the extraction, an ETHOS SEL microwave Labstation (Sheldon, CT, USA) at 2455 MHz with the Easy CONTROL-280 software was used for microwave assisted extraction (MAE), and for ultrasound assisted extraction (UAE) it was used a 400 W digital sonifier with a 12.7 mm (0.5-inch) probe and 20 kHz operating frequency (from Branson Ultrasonic Corporation, Danbury, CT, USA). Other laboratory equipment was: a ScanVac Coolsafe™ freeze dryer (Lynge, Denmark), a Mettler-Toledo GX400 balance (Columbus, OH, USA), a Labnet Spectrafuge™ 24D centrifuge (New Jersey, USA), an IKA vortex-mixer (Staufen, Germany) and a Stuart sample concentrator (OSA, UK). For calibration purposes, Hamilton® syringes of 100, 50 and 10 µL were used (Supelco).

Regarding software, for UHPLC-MS/MS data treatment MassLynx v.4.1 (Waters, Manchester, UK) was used; for experimental design it was used Statgraphics plus v.5.0 (Statpoint Technologies, VA, USA); and for statistic treatment it was used Microsoft Excel. Graphical abstract and image of procedure have been done with BioRender.

2.3. Experimental

2.3.1. Collection of faeces, initial sample treatment and blank selection

The samples were taken directly at the time of defecation. To facilitate the collection, it was carried out by placing a piece of aluminum foil in the toilet and then it was transferred to

a 100 mL polypropylene cup, the typical for urine analysis. Faeces were immediately stored at -20 ºC until analysis. Samples were accurately handled in order to avoid external contamination.

On the other hand, as faeces is a complex matrix, a blank was sought by analyzing some of the samples. To select experimental blanks, it was used a mixed procedure of the one proposed by Sturm *et al.* (2020) and García-Córcoles *et al.* (2018) [23,24]. Briefly, initially the sample was lyophilized, shredded into powder and 0.5 g were weight into a 15 mL glass tube. Then, it was added 8 mL of ACN, vortexed for 2 min and ultrasonicated for 13 min. After sonication, it was vortexed 1 min more and centrifugated during 5 min at 4000 rpm (2594 × *g*). Once the supernatant was separated, the solid was again extracted using 2 mL of ACN following the same procedure. Both supernatants were mixed and evaporated under N_2 stream at 40 ºC. For the clean-up step, it was added 6 mL of ACN, 0.6 g $MgSO_4$ and 0.15 g PSA to the dry residue, vortexed for 1 min, centrifugated and the supernatant evaporated. The resulting solid residue was dissolved, vortexing for 1 min, with 200 µL of a H_2O:MeOH (70:30, v:v) mixture. Finally, the extract was centrifuged at 13000 rpm (16300 × *g*), filtered through a 0.22 µm nylon filter and injected into the UHPLC system.

2.3.2. Preparation of the fortified samples

As there are not certified reference materials (CRMs), blank samples selected were spiked with different amounts of analytes. Since 0.5 g is an excessive amount of sample and it provided high matrix effect, 0.1 g of dry faeces (dry weight, dw) was set as the amount of sample for future experiments. The samples were spiked, at a final concentration of 4 µg g^{-1}, by spreading on the sample 100 µL of a methanolic solution (4.0 µg mL^{-1}) of the target analytes. The tube was then well-mixed for 1 min to guarantee maximum interaction. Tubes were left into darkness for 24 h at room temperature to allow complete evaporation of solvent and to simulate a real strong interaction between the analytes and the matrix.

2.3.3. Comparison of extraction techniques

Given that the research group has extensive experience in the extraction of chemical compounds from very dirty and complex matrices, based on the procedure followed by Dorival *et al.*, the comparison of 2 extraction techniques was proposed. MAE and UAE efficiencies were compared [25]. For MAE, 0.1 g of sample was placed into the microwave vessel and it was added 5 mL of solvent, being extracted at 90 ºC during 10 min and 1000 W

of power. For UAE, 0.1 of sample was ultrasonicated in glass tubes for 20 min at 70%. After that, the procedure was the same as for the blank selection.

2.3.4. Design of experiments-response surface

Two different types of experimental design (DoE) have been used during the method optimization. A 3^2 model was used for the optimization of the UAE parameter, where it is optimized 2 factors at same time (ultrasound power and extraction time) at 3 levels (low, medium and high), with 3 central points (number of experimental runs: 11). Power ranged between 10% and 70% (the maximum allowed for the equipment) and time ranged from 1 to 30 min. This model can provide the maximum desirability in the extraction in terms of UAE parameters by response surface for each paraben and the whole, and their graphic representation as well as the matrix of experimental runs obtained is shown in Supplementary Material (Table S2 and Figure S2). For the clean-up step, a Box-Behnken model was selected. In this type of DoE three factors are optimized involving 3 blocks, in which 2 of the factors vary across the 4 possible combinations of high and low. Number of experimental runs were 15 with three central points. Factors under study were $MgSO_4$, PSA and C_{18} amount, ranged between 0 and 1 g. Matrix of DoE and graphic representation are also shown as Supplementary Material (Table S3 and Figure S3).

2.3.5. Ultrahigh performance liquid chromatography–tandem mass spectrometry

The chromatographic method was based on the previously published by Moscoso-Ruiz *et al.* [18]. Briefly, the column operated at 40 ºC and solvent A and B were 2 mM solutions of ammonium acetate in water and a MeOH, respectively. Injection volume was 4 µL and flow rate 0.3 mL min^{-1}. Chromatographic gradient was as follows: 0.0 to 2.0 (isocratic), 75% solvent A; 3.0 min, 70% A; 7.5 min, 52% A; 9.5 min, 40% A and 14.0 min, 10% A. After that, 7 min are included for column cleaning and condition. The procedure is as follows: 14.5 min 0% solvent A; 14.5-16.5 min, 0% A; 17.0 min, 75% A and finally 17.0-21.0, 75% solvent A. A chromatogram of a spiked sample is shown in Figure 1.

Figure 1

The chromatograms showing double peaks belong to the separate isomers iPPB and PPB at retention times 9.71 and 10.02 min, respectively; and iBPB and BPB at 11.27 and 11.39 min, respectively. The peaks appear together because their most sensitive transitions coincide

with each other; however, they can be differentiated by the retention time and the ratio between transitions. In the figure, the peak corresponding to each compound is marked with the retention time and the corresponding compound is shown on the right.

Regarding detection, it was carried out in multiple reactions monitoring mode (MRM). For each compound two transitions were selected: one for quantification (the most abundant) and the other one for confirmation. Transition ratios from a pure pattern were also used to confirm the identity of the contaminant. For developing of the spectrometric method, individual pure standards of each paraben, at a concentration of 1 μg mL^{-1} in MeOH, were directly infused into the MS/MS to characterize each paraben in separate. The MS/MS system used, described in *section 2.2*, is able to determine optimal transitions and voltages automatically. With this optimization, it can be scanned multiple and narrow mass windows by MRM, "Multiple reaction monitoring", whose main objective is to detect and quantify specific molecules in complex mixtures by choosing selective transitions. Furthermore, since each paraben has a specific ratio between transitions (quantification / confirmation) and in order to guarantee the nature of the peak, this parameter was also established. Fragments of each transition match in all cases with a m/z of 92 and almost all cases with a m/z of 136, and it has been attributed by the loss of -COO-R, and the loss of the radical itself respectively (R= CH_3, CH_2CH_3, $CH(CH_3)_2$, $CH_2)_2CH_3$, $COOC_6H_5$, $CH_2CH(CH_3)_2$, $-(CH_2)_3CH_3$ for MPB, EPB, iPPB, PPB, iBPB and BPB). PhPB had additionally a fragment ion with m/z of 65, attributed to C_5H_5- group (MassBank of North America (ucdavis.edu)). MRM transitions, retention times, transition ratios, proposed fragments and UHPLC–MS/MS parameters are shown in Table 1.

Table 1

2.3.6. Method validation

To ensure that the method works correctly, an accurate validation was carried out. The following Quality Assurance/Quality Control (QA/QC) procedures were taken into account. Blanks were tested to assure there were not target parabens or the amounts were below the detection limits and they were used for optimization and calibration.

Calibration was made by spiking blanks of faeces in different concentration in a range expected for the natural samples. A calibration in solvent was also made in order to study the matrix effect. Initially, 10 concentration levels were established: 1, 2.5, 5, 10, 25, 50, 100, 125, 200 and 250 ng g^{-1}. At each level, three experimental and three instrumental replicates

were made (number of replicas in each point of calibration: 9). Standard solutions of target parabens were made in MeOH, at 10 ng mL^{-1}, 100 ng mL^{-1} and 500 ng mL^{-1}. After weighting 0,1 g of blank stool, different volumes of the standard solutions were taken to spik the faeces. With the solution of 10 ng mL^{-1}, it was made the points 1, 2.5, 5 and 10 ng g^{-1} by adding 10, 25, 50 and 100 µL respectively. With the solution of 100 ng mL^{-1}, the points of 25, 50 and 100 ng g^{-1} by adding 25, 50 and 100 µL; and finally, with the more concentrated standard solution (500 ng mL^{-1}) it was made calibration points of 125, 200 and 250 ng g^{-1} by adding 25, 40 and 50 µL respectively. Deuterated EPB was added to all the samples at the same concentration (250 ng g^{-1}). All the calibration process was carried out with Hamilton® syringes. To evaluate the matrix effect (ME), expressed as a percentage, the ratio between the slopes of the calibration curves obtained in matrix and solvent for each compound, was calculated. A ME close to 100% means no matrix effect, meanwhile analytes who show higher or lower than 100% show remarkable ME.

Parameters of validation were obtained using the calibration in blank matrix. Linearity was determined with the correlation coefficients (% R^2) of calibration curves and the P-value of the *lack-of-fit* test was also calculated to ensure the randomness of the residuals. Analytical sensitivity was obtained by the slope of the calibration curve, as well as with the limits of detection (LODs) and quantification (LOQs). Selectivity was evaluated by comparing a chromatogram of the blank with the corresponding to a calibration level (100 ng g^{-1}). Finally, a recovery assay using spiking blank samples at low, medium and high concentrations, was developed to determine trueness (recovery) and precision (% RSD) for the method accuracy.

2.3.7. Basic Procedure

The final procedure for the determination of parabens in children's faeces samples is as follow. In a 15 mL capacity glass tube, it was weighted 0.1 g (dw) of the sample. It was added 50 µL of a 0.5 ng L^{-1} methanolic solution of the internal standard (surrogate, EPB-d_5) to obtain a final concentration of 250 ng g^{-1} in sample. For extraction, 1 mL of MeOH was added and the parabens were extracted using UAE for 30 min at maximum power (70%). After centrifugation at 4000 rpm (2594 × *g*) for 5 min, the supernatant was transferred to a clean glass tube and the extraction process was repeated. The supernatants were pooled and evaporated to dryness at 35 ºC under a N_2 stream. For cleaning step, 6 mL of ACN, 0.1 g of PSA and 0.5 g of $MgSO_4$ were added. After vigorously mixed for one minute in vortex, the extract was centrifuged at 4000 rpm (2594 × *g*) for 5 min and the supernatant transferred to a clean glass tube and evaporated to dryness at 35 ºC under N_2. Then, 200 µL of a mixture of

H_2O:MeOH, 70:30 (v/v) were added. The sample was sonicated for 5 minutes in an ultrasonic bath to guarantee maximum dissolution and transferred to an Eppendorf tube to centrifuge at 13,000 rpm (16,300 × *g*) for 30 min. The supernatant was filtered through a 0.22 µm Nylon filter into a chromatographic vial prior to the injection in the UHPLC-MS/MS system. A schedule of the experimental procedure is included as supplementary information (Figure S1).

3. Results and discussion

3.1. Optimization of the extraction procedure

The first experiment carried out was the determination of the amount of sample to analyze. Sometimes the increase in the amount of sample causes an increase in the extraction of substances from the matrix and a decrease in the analytical signal due to the appearance of ion suppression. Because in previous experiments it was observed that 0.5 g of sample provided very dirty extracts and a high ionic suppression, 0.10, 0.25 and 0.50 g of sample were analyzed and the results compared. It was observed that 0.1 g of sample was enough, since clear and clean extracts were obtained without notable loss of analytical signal (lower than 10% in all cases).

Once the amount of sample was fixed, the efficiency of two extraction techniques, MAE and UAE, was compared. However, in the first experiment for solvent selection, it was observed that MAE offered low recoveries for parabens as well as very unclear extracts with a significant loss of analytical signal. Furthermore, it was very difficult to interpret the chromatograms since some important interferences appeared. This can be explained due to MAE has greater extraction power than UAE, since it combines the traditional solvent extraction with the microwave energy. In addition to parabens, this technique is capable of extracting large concentrations of substances that accompany the analyte in the matrix. According to the scientific literature, one of the parameters that most influences MAE is matrix type [26]. With this information, MAE was discharged as extraction technique and UAE was selected for further experiments. The experimental parameters optimized were type of solvent, volume, alkalinity/acidity for the extraction, parameters affecting UAE (power and time), clean-up procedure and reconstitution of extracts prior to.

304 *3.1.1. Extraction solvent optimization*

305 Nine different solvents were tested: ACE, ACN, AcOEt, MeOH, EtOH, DCM, TCM,
306 HEX and TOL. Figure 2A shows the results obtained. MeOH offered the highest extraction
307 yield for all analytes, and it was selected as solvent for extraction.

308

309 **Figure 2**

310

311 Considering MeOH as 100% of extraction efficiency, ACN provided 60 to 90% of the
312 extraction yield and the values for DCM, EtOH, AcOEt, TCM and ACE were 35 to 85%, in
313 the case of TOL and HEX the extraction percentages were minimal. Once the solvent was
314 selected, it was checked the number of extraction cycles (between 1 and 3). It was observed
315 that when 2 extraction cycles were performed, the signal reaches high values in relation to the
316 use of a single cycle. However, the increase achieved when three cycles are applied is not
317 significant (lower than 5%), notably increasing the sample handling time. Two cycles of
318 extraction were set for later experiments.

319 Then, it was studied the influence of alkalinity or acidity of the extraction media. For the
320 basic medium it was added 100 µL of NaOH (aq) 0.1 M, and for the acidic medium 100 µL of
321 acetic acid 0.1 M. The results obtained are shown in Figure 2B. There were no significant
322 differences in the extraction of compounds, and extracts were dirtier when adding acid or
323 base. It was decided to work with neutral medium.

324

325 *3.1.2. UAE and clean-up optimization. Design of experiments*

326 Ultrasound power and time of irradiation are the two variables that must be taken into
327 account when optimizing the UAE procedure. For simultaneous optimization of both
328 variables, an experimental design 3^2 to optimize 2 factors at 3 different concentrations was
329 selected (Figure S2). The values for power were varied from 10 to 70 % (minimum and
330 maximum allowed by the ultrasound probe) and times from 1 to 30 min were checked. For
331 longer ultrasound times, an excessive overheating of the sample was observed with the
332 consequent drawbacks of loss of solvent and difficulty in sample handling. The experimental
333 design used is included as supplementary material (Table S2). Figure 3 shows the three-
334 dimensional estimated response surface obtained.

335

336 **Figure 3**

337

The results demonstrated that the greater the time and the power, the greater the desirability. The maximum desirability for the model was 82% with a power of 70 % and 30 min. With a 95 % of confidence level, ANOVA analysis showed % R^2 between 65 and 80 and P values were greater than 0.05 in all cases. Pareto diagrams are also included as supplementary information (Figure S4). Considering these results, it was decided that UAE offers good extraction recoveries at 70 % power during 30 min.

The possibility of carrying out a cleaning process of the extract prior to the chromatographic analysis was studied. A dispersive solid phase extraction (dSPE) procedure based on QuEChERS technique was optimized. A Box-Behnken model experimental design was proposed. The addition of $MgSO_4$, PSA and C18 with 6 mL of ACN strongly mixed in vortex for 1 min, was assayed. $MgSO_4$ eliminate traces of water, PSA helps to eliminate organic acids, sugars and fatty acids, and C18 eliminate lipids. Box-Behnken matrix generated was at three levels (0, 0.5 and 1 g) with three central points to estimate that experimental error does not depend of fitted model. The design is included as supplementary material (Table S3 and Figure S3). The three-dimensional estimated response surface helps to understand the behavior of two variables when it is setting constant the third one. Once made the experiments, it is setting each variable at maximum desirability given by the program. Desirability helps us to find the optimum values of all variables simultaneously during the optimization of analytical methods. This value can vary between 0 and 1, being 1 the maximum desirability. Figure 4 shows each response surface graphic when maintaining constant each reagent at maximum desirability.

Figure 4

Optimal values were 0.0015 g of C18, 0 g for PSA and 1 g for $MgSO_4$, with a desirability of 88%. ANOVA analysis show values between 70 and 85%. Pareto diagrams show that PSA negatively affects the signal in the case of MPB, EPB, PPB, iBPB and PhPB, and that C18 and $MgSO_4$ are not dependents (Figure S5). C18 is close to be significant and $MgSO_4$ is the less notable variable in all cases. Therefore, the optimal values are 0 g for C18 and PSA, and 1 g for $MgSO_4$. However, with the objective of protect the UHPLC–MS/MS equipment, it was decided to add 0.1 g of PSA, amount for which the loss of signal is not significant and eliminates interference that could affect the chromatographic column and the equipment in general. The contours of the estimated response surface when setting 0.1 g PSA are also included as supplementary material (Figure S6). The desirability ranged between 70

and 80%. Lastly, as $MgSO_4$ helps to eliminate water and this procedure uses lyophilized stool, an experiment with 1 g and 0.5 g $MgSO_4$ was done, and results were practically the same in terms of recuperation, and it was decided to use 0.5 g of $MgSO_4$.

3.1.3. Final step. Dissolution of the extract

The final extract was initially dissolved using 40 µL of MeOH and 160 µL of H_2O, subjecting the sample to an ultrasonic bath for 5 min, into an Eppendorf tube. The extract was then centrifuged at 13,000 rpm (16,300 × *g*) for 30 minutes. However, it was observed that when adding H_2O to the final residue after clean-up, a dirty and cloudy solution was formed. Figure S7 in Supplementary Material shows the aspect of the final extracts. Therefore, it was tested the possibility of only centrifuging or centrifuging and filtering through a 0.22 µm Nylon filter to improve the extracts and thereby protect the equipment. The results are included as supplementary material (Figures S8 and S9). The figures show Eppendorf tubes after centrifugation at 16,300 × *g* for 30 minutes and the vials after filtration ready for the analysis in the UHPLC–MS/MS. The results are also shown as supplementary material (Figure S10). Filtration offered improvements in the analytical signal for MPB, EPB, PPB and iPPB; for the other three parabens there were no significant differences. Moreover, with filtration more particles stay into the filter and the equipment remains more protected; therefore, it was set 30 min of centrifugation + filtration.

3.2. Validation

An analytical calibration was made in a range from 1 to 250 ng g^{-1} for each paraben by spiking blanks of faeces. EPB-d_5, used as surrogate, was added at a final concentration in sample of 250 ng g^{-1}. To determine the matrix effect, it was also compared a calibration in solvent (initial mobile phase) with a calibration made in the matrix. Table 2 shows the calibration parameters obtained. %ME values and LODs/LOQs for each analyte are also included.

Table 2

The %MEs are in all cases lower than 100%. It ranges from 62.6% for PhPB to 90.0% for EthPB. These data show that there are matrix effects in all cases and it is necessary to validate

405 the method using matrix calibration in order to obtain reliable results in the quantification of
406 parabens in faeces children's samples.

407 The selectivity was studied by comparing the chromatogram corresponding to a blank
408 with the one of a standard of the calibration curve (100 ng g^{-1}). The chromatogram of the
409 blank is included as supplementary information (Figure S11). In all cases, it is observed the
410 absence of signal at the retention time of the target analytes. Therefore, the method is highly
411 selective.

412 The linearity was evaluated using $\%R^2$ and the P-value of the *lack-of-fit* test. $\%R^2$ was
413 ranged between 98.9 (iBPB) and 99.9 (EPB), and P_{lof} values were higher than 5% for all
414 analytes. Therefore, there is an excellent linearity also fulfilling the homoscedasticity
415 condition.

416 The analytical sensitivity was established in terms of the slope of the calibration curve.
417 Furthermore, LODs and LOQs were also calculated. LODs and LOQs were calculated by
418 interpolating, in the calibration curve, the signal corresponding to three times the signal-to-
419 noise ratio given by the UPHPLC–MS/MS system for the LODs, and 10 times for the LOQs.
420 Table 2 shows the slope (b) and both parameters, ranging LODs between 0.2 and 0.4 ng g^{-1}
421 and LOQs between 0.6 and 1.0 ng g^{-1}. Therefore, the proposed method is highly sensitive for
422 the determination of parabens in stool samples from children.

423 Finally, and since it does not exist a certificated reference material, the accuracy of the
424 method was tested using a recovery assay with spiked blank sample (ISO 5725-1). Four
425 concentration levels (1, 10, 100 and 250 ng g^{-1}) were studied. It was checked the intra-day
426 deviation by analyzing three samples in the same day and inter-day deviation by analyzing
427 those concentrations in 7 different days, a total of 21 analyses were carried out. Trueness was
428 studied in terms of recovery data, interpolating each result from the spiked sample in the
429 calibration curve, dividing with the real concentration added and expressed in percentage.
430 Precision was evaluated by calculating % RSD. The results are also shown in Table 2. The
431 recoveries obtained were between 89.0 and 112.7% with RSD under 15% in all cases.
432 Moreover, with these data which includes several analyses in different days and different
433 conditions, it can be conclude the robustness and ruggedness of the method is also
434 appropriate. Linear graphs of calibration curves can be seen in Supplementary Material,
435 Figure S12. With those data it can be concluded that the method for the determination of
436 parabens in a complex matrix such as human feces is accurate and truthful, and therefore
437 meets the criteria established in the validation guide to conclude that it is an accurate method.
438

4. Method application

Fourteen samples of human faeces, collected from children volunteers, were evaluated to determine the content of parabens. The age of these children was between 2 and 13 years, with 6 boys and 8 girls among them. Results are shown in Table 3.

Table 3

While EPB and PPB were detected in 100% of the samples, MPB appeared in 93% of them. However, MPB is the contaminant with the highest average concentration, with a mean value of 102.2 ng g^{-1} and a maximum above the upper limit of the linear range studied. The mean value of EPB concentration found was 2.95 ng g^{-1}, with a minimum and maximum of 1.0 and 6.2 ng g^{-1}, respectively. The mean value of the concentration in the case of PPB is 6.8 ng g^{-1} with values between 3.8 and 16.3 ng g^{-1}, except for sample number 2 where the concentration found was 96.2 ng g^{-1}. One of the samples did not contain MPB, but the rest of the parabens were detected at low concentrations, except for PhPB, which was not detected in any of the stool samples. Figure 5 shows the chromatogram obtained for a real sample (number 1).

Figure 5

Some important matches have been observed. Children who are relatives present similar concentrations in some of the PBs. Samples 1 and 14 are brothers and both of them had a concentration of MPB superior to the established linear range. Samples 7, 8 and 9 are also siblings and their MPB concentration is similar and higher than the found in other samples. Volunteers 2, 10, 11 and 12 are cousins and their MPB concentration is lower than the rest, besides having an EPB concentration very similar between them. It has been studied other possible similarities such as age and sex, however, correlations could not be established.

MPB is the paraben whose concentrations are higher than the rest. This fact has a clear explanation since this compound is allowed as additive in foods and as antimicrobial in PCPs. Numerous studies on this substance in food and in the body have already been published in the scientific literature [14-19]. On the other hand, all the analyzed samples contained EPB,

and this is also consistent because legislation also allows the presence of EPB in food and in PCPs. Next, PPB was also presented in all studied samples. This compound is limited in PCPs and forbidden in food due to its toxic potential discovered in recent years by researchers [27]. Although the concentrations are not as high as those found for MPB, they are higher than the amounts of EPB determined. This could be due to the fact that parents often apply high amounts of body creams to their children, especially in summer, the season of the year in which the samples were taken. Furthermore, the highest concentration corresponds to a 13-year-old girl who, in addition to this type of body cream, uses shampoo, conditioner and make-up regularly.

It is difficult to compare the results obtained in the present study with previous published studies since, to the best of our knowledge, this is the first systematic study carried out for the optimization of an analytical method capable of detecting PBs in fecal samples, and specifically in human feces. Aznar *et al.* in 2017 analyzed MPB in poultry manure from a farm in Spain, among other contaminants [21]. They used ultrasound-assisted matrix solid-phase dispersion as extraction technique and gas chromatography-tandem mass spectrometry as analytical technique. They obtained a LOD and a LOQ of 1.2 and 3.3 ng g^{-1} respectively, and recoveries between 81 and 103 %. Out of 23 samples, 48% were found to be contaminated with MPB. Subsequently, Tekin *et al.* (2022) detected parabens in penguin scat samples from two Antarctic islands [22]. The method they proposed is based on a dispersive solid-phase extraction with reduced graphene oxide modified iron nanoparticles and the use of liquid chromatography coupled to UV as a separation and detection technique. In this scientific work, LODs between 0.03 and 0.16 ng mL^{-1} and LOQs between 0.06 and 0.24 ng mL^{-1} with recoveries between 85.6 and 121% were achieved. They analyzed 14 fecal samples and found no parabens in them. When these results are compared with those obtained in the work we propose in the present article, it can be affirmed that this new method, based on the use of UHPLC-MS/MS combined with ultrasound-assisted extraction and cleaning of the extracts by dispersive solid phase extraction (dSPE), provides better LODs and LOQs. In addition, while the first author cited observed MPB in some of the samples, Tekin *et al.* did not obtain positive results for these substances, unlike the present study, where positive samples were found for more than two contaminants in all the cases analyzed.

5. Conclusions

To our knowledge, this is first analytical method developed and validated for determining parabens in human faeces. The optimized method is based on an ultrasound extraction step followed by cleaning of the extracts by d-SPE and analysis by UHPLC-MS/MS. The method offers excellent characteristics in terms of sensitivity, selectivity, linearity and accuracy (trueness and precision). The method is easy to apply and provides important knowledge on EDC exposure, particularly of parabens. To date, the scientific community has mainly considered only urine as a route of elimination of EDCs. Faeces can provide very important information on the excretion of compounds that can enter our body via dermal or dietary routes.

It is noteworthy that this method was applied to 14 volunteer samples, all of which tested positive for three or more parabens. The matrix studied is currently very little studied and could be a good biomarker to complement data obtained in urine or saliva samples, matrices widely studied in the scientific literature. Due to the limited number of studies available, more research is needed in this field to have a complete picture of EDCs in the body and their exposure/removal/accumulation pathways. Therefore, all this demonstrates the need for further work to develop rapid, sensitive, reliable methods at the lowest possible economic cost for those matrices that have been little studied or even not studied at all. The goal should be to accurately and reliably know the (bio)exposure to EDCs, especially in the early stages of human development.

Credit authorship contribution statement

Inmaculada Moscoso-Ruiz: Formal analysis, Investigation, Methodology, Data curation, Writing – original draft. **Alberto Navalón**: Conceptualization, Formal analysis, Investigation, Writing–original draft. **Ana Rivas**: Formal Analysis, Funding acquisition, Writing-review and editing. **Alberto Zafra-Gómez**: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Data curation, Writing–original draft, Writing-review, editing and supervision.

Declaration of interests

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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Declarations

The present study has been approved by the ethics committees of the University of Granada and of the Provincial Biomedical Research of Granada (CEI), Spain (reference 1939-M1-22, Andalusian Biomedical Research Ethics Portal); and the study have been performed in accordance with the ethical standards. Also, all subjects gave written informed consent and had parental permission to participate.

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Figure captions

Figure 1. Chromatogram of a spiked sample (100 ng g^{-1}).

Figure 2. Solvent optimization (A) and study of alkalinity, acidity or neutral medium (B).

Figure 3. Estimated response surface of 3^2 for UAE parameters.

Figure 4. Box-Behnken DoE for clean-up step.

Figure 5. Chromatogram of a volunteer sample (Nr. 1)

Presence of parabens in children's faeces. Optimization and validation of a new analytical method based on the use of ultrasound-assisted extraction and liquid chromatography-tandem mass spectrometry

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ABSTRACT Due to their antimicrobial properties, parabens are a family of synthetic chemical compounds widely used as preservative additives in food and cosmetics. For this reason, humans are highly exposed to them. These substances are capable of altering the proper functioning of the endocrine system and are classified as endocrine disrupting chemicals (EDCs). Traditionally, urine has been the typical matrix studied as an excretion route. However, faeces contain valuable information. In the present study, the presence of methyl-, ethyl-, isopropyl-, propyl-, isobutyl-, butyl- and phenylparaben in stool samples from children has been evaluated. A new analytical method has been optimised and validated. The method is based on the use of ultrasound-assisted extraction followed by clean-up of the extracts by dispersive solid phase extraction (d-SPE). Parabens were analysed by ultrahigh performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The matrix effect was evaluated and a significant effect was observed for all analytes. Therefore, calibration and validation were performed by addition of different concentrations of analytes to faecal blanks. The coefficient of determination (%R^2) for calibration curves was higher than 98.9% in all cases. The limits of detection and quantification were between 0.2-0.4 and 0.6-1.0 ng g^{-1} respectively. The recovery for accuracy assessment had values between 89.0 and 112.7% with an RSD of less than 15% in all cases. The method was successfully applied to 14 samples from children volunteers, 100% of which showed contamination by at least one of the analysed compounds.

Keywords Endocrine disruption; Excreta Stool; Parabens; Ultra-high performance liquid chromatography-tandem mass spectrometry

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1. Introduction

Endocrine disrupting chemicals (EDCs) are synthetic substances present in the environment mainly due to industrial activity. They are widely used in all kinds of applications such as the manufacture of plastics and resins, as additives in personal care products (PCPs) and even as preservatives in food and cosmetics. These substances can be found in the environment (soil, water and air) as well as in food and PCPs, reaching living organisms mainly through the diet, although there are other routes, such as dermal or inhalation [1]. EDCs are increasingly studied for their ability to modulate hormonal response, disrupt the endocrine system and produce different types of toxicity [1,2]. Children are a particularly vulnerable population to EDCs because they are at a critical stage of development.

Parabens are alkyl or aryl homologues of the *p*-hydroxybenzoic acid. Before year 2000, based on various scientific studies, scientific community determined they were non-toxic to human health [3]. However, in the last years new studies have shown that this family of compounds is capable of altering the endocrine system of individuals, which has led some parabens to be considered as EDCs [4]. Nowadays, it has been proved that parabens provoke, among others, alterations in reproductive system and in adipose tissue accumulation [5]. Parabens most used are methylparaben (MPB), ethylparaben (EPB), propylparaben (PPB) and butylparaben (BPB). These substances are used as antimicrobial preservatives due to its properties, especially in personal care products (PCPs) and foods. In 2013, it was regulated by the European Union the maximum amount of parabens in PCPs, being 0.14 % for PPB and BPB, 0.4 % for MPB and EPB and 0.8 % for mixtures [6]. In food, MPB and EPB and their sodium salts are allowed as E numbers (E218 and E219 for MPB and Na-MPB; E214 and E215 for EPB and Na-EPB, respectively), meanwhile PPB and BPB were forbidden [7].

Parabens are well-studied compounds in different kinds of matrixes. There are lots of works which determine these substances both in the environment and in biological matrixes [8-11]. Isopropyl paraben (iPPB) and isobutyl paraben (iBPB) are isomers of PPB and BPB respectively, and they have been found in outdoor environments and in PCPs named as "green" [12, 13]. As one of the main routes of exposure is the diet, there are multiple scientific papers studying parabens in foods and also in invasive and non-invasive biological human matrixes, such as breast milk, plasma, urine, hair, saliva or nails [14-19]. Urine is the most used matrix as a biomarker. In the first 24 h, 80% of parabens are excreted, but in 48 h

some studies show more recovery of MPB in urine than BPB, suggesting the larger is the carbon chain radical, the larger stays in the body [20]. However, parabens in faeces have been scarcely studied, probably due to the complexity of this matrix. Only two recent papers have studied parabens in stool [21, 22]. One of them analyzes MPB and PPB in poultry manure using gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) [21]. The second one analyses MPB, EPB, PPB, BPB and benzylparaben (BzPB) in penguin excreta from the Antarctic region, using high performance liquid chromatography with ultraviolet detection (HPLC-UV) [22]. To our knowledge, there are still no studies on the determination of parabens in human faeces samples. Because people are surrounded by EDCs, in particular parabens, faeces can provide valuable data on daily intake and dermal absorption of these substances as does urine, as well as complementary information to that provided by urine. Further, the objectives of the present work are first to optimize an analytical method to determine 7 parabens (MPB, EPB, iPPB, PPB, iBPB, BPB and phenylparaben (PhPB)) with endocrine disrupting activity in children's faeces using liquid chromatography-tandem mass spectrometry, then to validate the proposed method following appropriate international guidelines, and finally to apply the method to samples of children's faeces to determine the content in parabens and to assure the applicability of the method to natural samples.

2. Materials and methods

2.1. Chemical reagents

For the optimization it was used standard reagents and all were analytical grade: MPB, EPB, PPB and BPB (≥99% purity) and iPPB and iBPB (≥98% purity) were supplied by Alfa Aesar (Thermo Fisher Scientific, Kandel, Germany). Deuterium labelled EPB (EPB-d_5) (≥98% purity) was from Toronto Research Chemicals (NY, Canada). Table S1 shows the structure and CAS number of those compounds. For the usage of ultra-pure water (18.2 M□) it was used an in-house Milli-Q Plus® system (Merck Millipore). It was prepared working standard solutions in methanol (MeOH) for each target analyte at 4.0 µg mL^{-1}, and EPB-d_5 and was prepared at a concentration of 0.5 µg mL^{-1}. Methanol (MeOH, 99.9%), dichloromethane (DCM, 99.9%), acetonitrile (ACN, 99.9%) and n-hexane (HEX, 97.0%) were purchased from Honeywell (Madrid, Spain); trichloromethane (TCM, 99.9%), acetone

(ACE, 99.9%) and sodium hydroxide (NaOH) in pellets from Panreac (Barcelona, Spain); ethanol (EtOH, 99.9%) and acetic acid (glacial 99-100%) from JT Baker (Madrid, Spain); and toluene (TOL, 99.9%) and ethyl acetate (AcOEt, 99.9%) from Sigma-Aldrich. For the clean-up of extracts procedure, it was used Primary Secondary Amine (PSA) bonded silica (Supelco), C18 (Supelco) and magnesium sulfate ($MgSO_4$, 96%, Panreac). It was used ammonium acetate (≥ 98% purity, Honey-well) for the mobile phase.

2.2. Instrumentation and software

The determination of the target analytes was carried out using an ultrahigh performance liquid chromatography system UPLC™ H-Class, coupled with a triple quadrupole mass spectrometer Xevo TQ-XS equipped with a StepWave ion guide and an orthogonal Z-spray™ electrospray ionization (ESI) source from Waters, Manchester, UK (UHPLC-MS/MS). The separation was performed on an Acquity UPLC® BEH C18 column (2.1 mm x 100 mm, 1.7 µm particle size).

For the optimization of the extraction, an ETHOS SEL microwave Labstation (Sheldon, CT, USA) at 2455 MHz with the Easy CONTROL-280 software was used for microwave assisted extraction (MAE), and for ultrasound assisted extraction (UAE) it was used a 400 W digital sonifier with a 12.7 mm (0.5-inch) probe and 20 kHz operating frequency (from Branson Ultrasonic Corporation, Danbury, CT, USA). Other laboratory equipment was: a ScanVac Coolsafe™ freeze dryer (Lynge, Denmark), a Mettler-Toledo GX400 balance (Columbus, OH, USA), a Labnet Spectrafuge™ 24D centrifuge (New Jersey, USA), an IKA vortex-mixer (Staufen, Germany) and a Stuart sample concentrator (OSA, UK). For calibration purposes, Hamilton® syringes of 100, 50 and 10 µL were used (Supelco).

Regarding software, for UHPLC-MS/MS data treatment MassLynx v.4.1 (Waters, Manchester, UK) was used; for experimental design it was used Statgraphics plus v.5.0 (Statpoint Technologies, VA, USA); and for statistic treatment it was used Microsoft Excel. Graphical abstract and image of procedure have been done with BioRender.

2.3. Experimental

2.3.1. Collection of faeces, initial sample treatment and blank selection

The samples were taken directly at the time of defecation. To facilitate the collection, it was carried out by placing a piece of aluminum foil in the toilet and then it was transferred to

a 100 mL polypropylene cup, the typical for urine analysis. Faeces were immediately stored at -20 ºC until analysis. Samples were accurately handled in order to avoid external contamination.

On the other hand, as faeces is a complex matrix, a blank was sought by analyzing some of the samples. To select experimental blanks, it was used a mixed procedure of the one proposed by Sturm *et al.* (2020) and García-Córcoles *et al.* (2018) [23,24]. Briefly, initially the sample was lyophilized, shredded into powder and 0.5 g were weight into a 15 mL glass tube. Then, it was added 8 mL of ACN, vortexed for 2 min and ultrasonicated for 13 min. After sonication, it was vortexed 1 min more and centrifugated during 5 min at 4000 rpm (2594 × *g*). Once the supernatant was separated, the solid was again extracted using 2 mL of ACN following the same procedure. Both supernatants were mixed and evaporated under N_2 stream at 40 ºC. For the clean-up step, it was added 6 mL of ACN, 0.6 g $MgSO_4$ and 0.15 g PSA to the dry residue, vortexed for 1 min, centrifugated and the supernatant evaporated. The resulting solid residue was dissolved, vortexing for 1 min, with 200 µL of a H_2O:MeOH (70:30, v:v) mixture. Finally, the extract was centrifuged at 13000 rpm (16300 × *g*), filtered through a 0.22 µm nylon filter and injected into the UHPLC system.

2.3.2. Preparation of the fortified samples

As there are not certified reference materials (CRMs), blank samples selected were spiked with different amounts of analytes. Since 0.5 g is an excessive amount of sample and it provided high matrix effect, 0.1 g of dry faeces (dry weight, dw) was set as the amount of sample for future experiments. The samples were spiked, at a final concentration of 4 µg g^{-1}, by spreading on the sample 100 µL of a methanolic solution (4.0 µg mL^{-1}) of the target analytes. The tube was then well-mixed for 1 min to guarantee maximum interaction. Tubes were left into darkness for 24 h at room temperature to allow complete evaporation of solvent and to simulate a real strong interaction between the analytes and the matrix.

2.3.3. Comparison of extraction techniques

Given that the research group has extensive experience in the extraction of chemical compounds from very dirty and complex matrices, based on the procedure followed by Dorival *et al.*, the comparison of 2 extraction techniques was proposed. MAE and UAE efficiencies were compared [25]. For MAE, 0.1 g of sample was placed into the microwave vessel and it was added 5 mL of solvent, being extracted at 90 ºC during 10 min and 1000 W

of power. For UAE, 0.1 of sample was ultrasonicated in glass tubes for 20 min at 70%. After that, the procedure was the same as for the blank selection.

2.3.4. Design of experiments-response surface

Two different types of experimental design (DoE) have been used during the method optimization. A 3^2 model was used for the optimization of the UAE parameter, where it is optimized 2 factors at same time (ultrasound power and extraction time) at 3 levels (low, medium and high), with 3 central points (number of experimental runs: 11). Power ranged between 10% and 70% (the maximum allowed for the equipment) and time ranged from 1 to 30 min. This model can provide the maximum desirability in the extraction in terms of UAE parameters by response surface for each paraben and the whole, and their graphic representation as well as the matrix of experimental runs obtained is shown in Supplementary Material (Table S2 and Figure S2). For the clean-up step, a Box-Behnken model was selected. In this type of DoE three factors are optimized involving 3 blocks, in which 2 of the factors vary across the 4 possible combinations of high and low. Number of experimental runs were 15 with three central points. Factors under study were $MgSO_4$, PSA and C_{18} amount, ranged between 0 and 1 g. Matrix of DoE and graphic representation are also shown as Supplementary Material (Table S3 and Figure S3).

2.3.5. Ultrahigh performance liquid chromatography–tandem mass spectrometry

The chromatographic method was based on the previously published by Moscoso-Ruiz *et al.* [18]. Briefly, the column operated at 40 ºC and solvent A and B were 2 mM solutions of ammonium acetate in water and a MeOH, respectively. Injection volume was 4 µL and flow rate 0.3 mL min^{-1}. Chromatographic gradient was as follows: 0.0 to 2.0 (isocratic), 75% solvent A; 3.0 min, 70% A; 7.5 min, 52% A; 9.5 min, 40% A and 14.0 min, 10% A. After that, 7 min are included for column cleaning and condition. The procedure is as follows: 14.5 min 0% solvent A; 14.5-16.5 min, 0% A; 17.0 min, 75% A and finally 17.0-21.0, 75% solvent A. A chromatogram of a spiked sample is shown in Figure 1.

Figure 1

The chromatograms showing double peaks belong to the separate isomers iPPB and PPB at retention times 9.71 and 10.02 min, respectively; and iBPB and BPB at 11.27 and 11.39 min, respectively. The peaks appear together because their most sensitive transitions coincide

with each other; however, they can be differentiated by the retention time and the ratio between transitions. In the figure, the peak corresponding to each compound is marked with the retention time and the corresponding compound is shown on the right.

Regarding detection, it was carried out in multiple reactions monitoring mode (MRM). For each compound two transitions were selected: one for quantification (the most abundant) and the other one for confirmation. Transition ratios from a pure pattern were also used to confirm the identity of the contaminant. For developing of the spectrometric method, individual pure standards of each paraben, at a concentration of 1 μg mL^{-1} in MeOH, were directly infused into the MS/MS to characterize each paraben in separate. The MS/MS system used, described in *section 2.2*, is able to determine optimal transitions and voltages automatically. With this optimization, it can be scanned multiple and narrow mass windows by MRM, "Multiple reaction monitoring", whose main objective is to detect and quantify specific molecules in complex mixtures by choosing selective transitions. Furthermore, since each paraben has a specific ratio between transitions (quantification / confirmation) and in order to guarantee the nature of the peak, this parameter was also established. Fragments of each transition match in all cases with a m/z of 92 and almost all cases with a m/z of 136, and it has been attributed by the loss of -COO-R, and the loss of the radical itself respectively (R= CH_3, CH_2CH_3, $CH(CH_3)_2$, $CH_2)_2CH_3$, $COOC_6H_5$, $CH_2CH(CH_3)_2$, $-(CH_2)_3CH_3$ for MPB, EPB, iPPB, PPB, iBPB and BPB). PhPB had additionally a fragment ion with m/z of 65, attributed to C_5H_5- group (MassBank of North America (ucdavis.edu)). MRM transitions, retention times, transition ratios, proposed fragments and UHPLC–MS/MS parameters are shown in Table 1.

Table 1

2.3.6. Method validation

To ensure that the method works correctly, an accurate validation was carried out. The following Quality Assurance/Quality Control (QA/QC) procedures were taken into account. Blanks were tested to assure there were not target parabens or the amounts were below the detection limits and they were used for optimization and calibration.

Calibration was made by spiking blanks of faeces in different concentration in a range expected for the natural samples. A calibration in solvent was also made in order to study the matrix effect. Initially, 10 concentration levels were established: 1, 2.5, 5, 10, 25, 50, 100, 125, 200 and 250 ng g^{-1}. At each level, three experimental and three instrumental replicates

were made (number of replicas in each point of calibration: 9). Standard solutions of target parabens were made in MeOH, at 10 ng mL^{-1}, 100 ng mL^{-1} and 500 ng mL^{-1}. After weighting 0,1 g of blank stool, different volumes of the standard solutions were taken to spik the faeces. With the solution of 10 ng mL^{-1}, it was made the points 1, 2.5, 5 and 10 ng g^{-1} by adding 10, 25, 50 and 100 µL respectively. With the solution of 100 ng mL^{-1}, the points of 25, 50 and 100 ng g^{-1} by adding 25, 50 and 100 µL; and finally, with the more concentrated standard solution (500 ng mL^{-1}) it was made calibration points of 125, 200 and 250 ng g^{-1} by adding 25, 40 and 50 µL respectively. Deuterated EPB was added to all the samples at the same concentration (250 ng g^{-1}). All the calibration process was carried out with Hamilton® syringes. To evaluate the matrix effect (ME), expressed as a percentage, the ratio between the slopes of the calibration curves obtained in matrix and solvent for each compound, was calculated. A ME close to 100% means no matrix effect, meanwhile analytes who show higher or lower than 100% show remarkable ME.

Parameters of validation were obtained using the calibration in blank matrix. Linearity was determined with the correlation coefficients (% R^2) of calibration curves and the P-value of the *lack-of-fit* test was also calculated to ensure the randomness of the residuals. Analytical sensitivity was obtained by the slope of the calibration curve, as well as with the limits of detection (LODs) and quantification (LOQs). Selectivity was evaluated by comparing a chromatogram of the blank with the corresponding to a calibration level (100 ng g^{-1}). Finally, a recovery assay using spiking blank samples at low, medium and high concentrations, was developed to determine trueness (recovery) and precision (% RSD) for the method accuracy.

2.3.7. Basic Procedure

The final procedure for the determination of parabens in children's faeces samples is as follow. In a 15 mL capacity glass tube, it was weighted 0.1 g (dw) of the sample. It was added 50 µL of a 0.5 ng L^{-1} methanolic solution of the internal standard (surrogate, EPB-d_5) to obtain a final concentration of 250 ng g^{-1} in sample. For extraction, 1 mL of MeOH was added and the parabens were extracted using UAE for 30 min at maximum power (70%). After centrifugation at 4000 rpm (2594 × *g*) for 5 min, the supernatant was transferred to a clean glass tube and the extraction process was repeated. The supernatants were pooled and evaporated to dryness at 35 ºC under a N_2 stream. For cleaning step, 6 mL of ACN, 0.1 g of PSA and 0.5 g of $MgSO_4$ were added. After vigorously mixed for one minute in vortex, the extract was centrifuged at 4000 rpm (2594 × *g*) for 5 min and the supernatant transferred to a clean glass tube and evaporated to dryness at 35 ºC under N_2. Then, 200 µL of a mixture of

H_2O:MeOH, 70:30 (v/v) were added. The sample was sonicated for 5 minutes in an ultrasonic bath to guarantee maximum dissolution and transferred to an Eppendorf tube to centrifuge at 13,000 rpm (16,300 × *g*) for 30 min. The supernatant was filtered through a 0.22 µm Nylon filter into a chromatographic vial prior to the injection in the UHPLC-MS/MS system. A schedule of the experimental procedure is included as supplementary information (Figure S1).

3. Results and discussion

3.1. Optimization of the extraction procedure

The first experiment carried out was the determination of the amount of sample to analyze. Sometimes the increase in the amount of sample causes an increase in the extraction of substances from the matrix and a decrease in the analytical signal due to the appearance of ion suppression. Because in previous experiments it was observed that 0.5 g of sample provided very dirty extracts and a high ionic suppression, 0.10, 0.25 and 0.50 g of sample were analyzed and the results compared. It was observed that 0.1 g of sample was enough, since clear and clean extracts were obtained without notable loss of analytical signal (lower than 10% in all cases).

Once the amount of sample was fixed, the efficiency of two extraction techniques, MAE and UAE, was compared. However, in the first experiment for solvent selection, it was observed that MAE offered low recoveries for parabens as well as very unclear extracts with a significant loss of analytical signal. Furthermore, it was very difficult to interpret the chromatograms since some important interferences appeared. This can be explained due to MAE has greater extraction power than UAE, since it combines the traditional solvent extraction with the microwave energy. In addition to parabens, this technique is capable of extracting large concentrations of substances that accompany the analyte in the matrix. According to the scientific literature, one of the parameters that most influences MAE is matrix type [26]. With this information, MAE was discharged as extraction technique and UAE was selected for further experiments. The experimental parameters optimized were type of solvent, volume, alkalinity/acidity for the extraction, parameters affecting UAE (power and time), clean-up procedure and reconstitution of extracts prior to.

304 *3.1.1. Extraction solvent optimization*

305 Nine different solvents were tested: ACE, ACN, AcOEt, MeOH, EtOH, DCM, TCM,
306 HEX and TOL. Figure 2A shows the results obtained. MeOH offered the highest extraction
307 yield for all analytes, and it was selected as solvent for extraction.

308

309 **Figure 2**

310

311 Considering MeOH as 100% of extraction efficiency, ACN provided 60 to 90% of the
312 extraction yield and the values for DCM, EtOH, AcOEt, TCM and ACE were 35 to 85%, in
313 the case of TOL and HEX the extraction percentages were minimal. Once the solvent was
314 selected, it was checked the number of extraction cycles (between 1 and 3). It was observed
315 that when 2 extraction cycles were performed, the signal reaches high values in relation to the
316 use of a single cycle. However, the increase achieved when three cycles are applied is not
317 significant (lower than 5%), notably increasing the sample handling time. Two cycles of
318 extraction were set for later experiments.

319 Then, it was studied the influence of alkalinity or acidity of the extraction media. For the
320 basic medium it was added 100 µL of NaOH (aq) 0.1 M, and for the acidic medium 100 µL of
321 acetic acid 0.1 M. The results obtained are shown in Figure 2B. There were no significant
322 differences in the extraction of compounds, and extracts were dirtier when adding acid or
323 base. It was decided to work with neutral medium.

324

325 *3.1.2. UAE and clean-up optimization. Design of experiments*

326 Ultrasound power and time of irradiation are the two variables that must be taken into
327 account when optimizing the UAE procedure. For simultaneous optimization of both
328 variables, an experimental design 3^2 to optimize 2 factors at 3 different concentrations was
329 selected (Figure S2). The values for power were varied from 10 to 70 % (minimum and
330 maximum allowed by the ultrasound probe) and times from 1 to 30 min were checked. For
331 longer ultrasound times, an excessive overheating of the sample was observed with the
332 consequent drawbacks of loss of solvent and difficulty in sample handling. The experimental
333 design used is included as supplementary material (Table S2). Figure 3 shows the three-
334 dimensional estimated response surface obtained.

335

336 **Figure 3**

337

The results demonstrated that the greater the time and the power, the greater the desirability. The maximum desirability for the model was 82% with a power of 70 % and 30 min. With a 95 % of confidence level, ANOVA analysis showed % R^2 between 65 and 80 and P values were greater than 0.05 in all cases. Pareto diagrams are also included as supplementary information (Figure S4). Considering these results, it was decided that UAE offers good extraction recoveries at 70 % power during 30 min.

The possibility of carrying out a cleaning process of the extract prior to the chromatographic analysis was studied. A dispersive solid phase extraction (dSPE) procedure based on QuEChERS technique was optimized. A Box-Behnken model experimental design was proposed. The addition of $MgSO_4$, PSA and C18 with 6 mL of ACN strongly mixed in vortex for 1 min, was assayed. $MgSO_4$ eliminate traces of water, PSA helps to eliminate organic acids, sugars and fatty acids, and C18 eliminate lipids. Box-Behnken matrix generated was at three levels (0, 0.5 and 1 g) with three central points to estimate that experimental error does not depend of fitted model. The design is included as supplementary material (Table S3 and Figure S3). The three-dimensional estimated response surface helps to understand the behavior of two variables when it is setting constant the third one. Once made the experiments, it is setting each variable at maximum desirability given by the program. Desirability helps us to find the optimum values of all variables simultaneously during the optimization of analytical methods. This value can vary between 0 and 1, being 1 the maximum desirability. Figure 4 shows each response surface graphic when maintaining constant each reagent at maximum desirability.

Figure 4

Optimal values were 0.0015 g of C18, 0 g for PSA and 1 g for $MgSO_4$, with a desirability of 88%. ANOVA analysis show values between 70 and 85%. Pareto diagrams show that PSA negatively affects the signal in the case of MPB, EPB, PPB, iBPB and PhPB, and that C18 and $MgSO_4$ are not dependents (Figure S5). C18 is close to be significant and $MgSO_4$ is the less notable variable in all cases. Therefore, the optimal values are 0 g for C18 and PSA, and 1 g for $MgSO_4$. However, with the objective of protect the UHPLC–MS/MS equipment, it was decided to add 0.1 g of PSA, amount for which the loss of signal is not significant and eliminates interference that could affect the chromatographic column and the equipment in general. The contours of the estimated response surface when setting 0.1 g PSA are also included as supplementary material (Figure S6). The desirability ranged between 70

and 80%. Lastly, as $MgSO_4$ helps to eliminate water and this procedure uses lyophilized stool, an experiment with 1 g and 0.5 g $MgSO_4$ was done, and results were practically the same in terms of recuperation, and it was decided to use 0.5 g of $MgSO_4$.

3.1.3. Final step. Dissolution of the extract

The final extract was initially dissolved using 40 µL of MeOH and 160 µL of H_2O, subjecting the sample to an ultrasonic bath for 5 min, into an Eppendorf tube. The extract was then centrifuged at 13,000 rpm (16,300 × *g*) for 30 minutes. However, it was observed that when adding H_2O to the final residue after clean-up, a dirty and cloudy solution was formed. Figure S7 in Supplementary Material shows the aspect of the final extracts. Therefore, it was tested the possibility of only centrifuging or centrifuging and filtering through a 0.22 µm Nylon filter to improve the extracts and thereby protect the equipment. The results are included as supplementary material (Figures S8 and S9). The figures show Eppendorf tubes after centrifugation at 16,300 × *g* for 30 minutes and the vials after filtration ready for the analysis in the UHPLC–MS/MS. The results are also shown as supplementary material (Figure S10). Filtration offered improvements in the analytical signal for MPB, EPB, PPB and iPPB; for the other three parabens there were no significant differences. Moreover, with filtration more particles stay into the filter and the equipment remains more protected; therefore, it was set 30 min of centrifugation + filtration.

3.2. Validation

An analytical calibration was made in a range from 1 to 250 ng g^{-1} for each paraben by spiking blanks of faeces. EPB-d_5, used as surrogate, was added at a final concentration in sample of 250 ng g^{-1}. To determine the matrix effect, it was also compared a calibration in solvent (initial mobile phase) with a calibration made in the matrix. Table 2 shows the calibration parameters obtained. %ME values and LODs/LOQs for each analyte are also included.

Table 2

The %MEs are in all cases lower than 100%. It ranges from 62.6% for PhPB to 90.0% for EthPB. These data show that there are matrix effects in all cases and it is necessary to validate

405 the method using matrix calibration in order to obtain reliable results in the quantification of
406 parabens in faeces children's samples.

407 The selectivity was studied by comparing the chromatogram corresponding to a blank
408 with the one of a standard of the calibration curve (100 ng g^{-1}). The chromatogram of the
409 blank is included as supplementary information (Figure S11). In all cases, it is observed the
410 absence of signal at the retention time of the target analytes. Therefore, the method is highly
411 selective.

412 The linearity was evaluated using $\%R^2$ and the P-value of the *lack-of-fit* test. $\%R^2$ was
413 ranged between 98.9 (iBPB) and 99.9 (EPB), and P_{lof} values were higher than 5% for all
414 analytes. Therefore, there is an excellent linearity also fulfilling the homoscedasticity
415 condition.

416 The analytical sensitivity was established in terms of the slope of the calibration curve.
417 Furthermore, LODs and LOQs were also calculated. LODs and LOQs were calculated by
418 interpolating, in the calibration curve, the signal corresponding to three times the signal-to-
419 noise ratio given by the UPHPLC–MS/MS system for the LODs, and 10 times for the LOQs.
420 Table 2 shows the slope (b) and both parameters, ranging LODs between 0.2 and 0.4 ng g^{-1}
421 and LOQs between 0.6 and 1.0 ng g^{-1}. Therefore, the proposed method is highly sensitive for
422 the determination of parabens in stool samples from children.

423 Finally, and since it does not exist a certificated reference material, the accuracy of the
424 method was tested using a recovery assay with spiked blank sample (ISO 5725-1). Four
425 concentration levels (1, 10, 100 and 250 ng g^{-1}) were studied. It was checked the intra-day
426 deviation by analyzing three samples in the same day and inter-day deviation by analyzing
427 those concentrations in 7 different days, a total of 21 analyses were carried out. Trueness was
428 studied in terms of recovery data, interpolating each result from the spiked sample in the
429 calibration curve, dividing with the real concentration added and expressed in percentage.
430 Precision was evaluated by calculating % RSD. The results are also shown in Table 2. The
431 recoveries obtained were between 89.0 and 112.7% with RSD under 15% in all cases.
432 Moreover, with these data which includes several analyses in different days and different
433 conditions, it can be conclude the robustness and ruggedness of the method is also
434 appropriate. Linear graphs of calibration curves can be seen in Supplementary Material,
435 Figure S12. With those data it can be concluded that the method for the determination of
436 parabens in a complex matrix such as human feces is accurate and truthful, and therefore
437 meets the criteria established in the validation guide to conclude that it is an accurate method.
438

4. Method application

Fourteen samples of human faeces, collected from children volunteers, were evaluated to determine the content of parabens. The age of these children was between 2 and 13 years, with 6 boys and 8 girls among them. Results are shown in Table 3.

Table 3

While EPB and PPB were detected in 100% of the samples, MPB appeared in 93% of them. However, MPB is the contaminant with the highest average concentration, with a mean value of 102.2 ng g^{-1} and a maximum above the upper limit of the linear range studied. The mean value of EPB concentration found was 2.95 ng g^{-1}, with a minimum and maximum of 1.0 and 6.2 ng g^{-1}, respectively. The mean value of the concentration in the case of PPB is 6.8 ng g^{-1} with values between 3.8 and 16.3 ng g^{-1}, except for sample number 2 where the concentration found was 96.2 ng g^{-1}. One of the samples did not contain MPB, but the rest of the parabens were detected at low concentrations, except for PhPB, which was not detected in any of the stool samples. Figure 5 shows the chromatogram obtained for a real sample (number 1).

Figure 5

Some important matches have been observed. Children who are relatives present similar concentrations in some of the PBs. Samples 1 and 14 are brothers and both of them had a concentration of MPB superior to the established linear range. Samples 7, 8 and 9 are also siblings and their MPB concentration is similar and higher than the found in other samples. Volunteers 2, 10, 11 and 12 are cousins and their MPB concentration is lower than the rest, besides having an EPB concentration very similar between them. It has been studied other possible similarities such as age and sex, however, correlations could not be established.

MPB is the paraben whose concentrations are higher than the rest. This fact has a clear explanation since this compound is allowed as additive in foods and as antimicrobial in PCPs. Numerous studies on this substance in food and in the body have already been published in the scientific literature [14-19]. On the other hand, all the analyzed samples contained EPB,

and this is also consistent because legislation also allows the presence of EPB in food and in PCPs. Next, PPB was also presented in all studied samples. This compound is limited in PCPs and forbidden in food due to its toxic potential discovered in recent years by researchers [27]. Although the concentrations are not as high as those found for MPB, they are higher than the amounts of EPB determined. This could be due to the fact that parents often apply high amounts of body creams to their children, especially in summer, the season of the year in which the samples were taken. Furthermore, the highest concentration corresponds to a 13-year-old girl who, in addition to this type of body cream, uses shampoo, conditioner and make-up regularly.

It is difficult to compare the results obtained in the present study with previous published studies since, to the best of our knowledge, this is the first systematic study carried out for the optimization of an analytical method capable of detecting PBs in fecal samples, and specifically in human feces. Aznar *et al.* in 2017 analyzed MPB in poultry manure from a farm in Spain, among other contaminants [21]. They used ultrasound-assisted matrix solid-phase dispersion as extraction technique and gas chromatography-tandem mass spectrometry as analytical technique. They obtained a LOD and a LOQ of 1.2 and 3.3 ng g^{-1} respectively, and recoveries between 81 and 103 %. Out of 23 samples, 48% were found to be contaminated with MPB. Subsequently, Tekin *et al.* (2022) detected parabens in penguin scat samples from two Antarctic islands [22]. The method they proposed is based on a dispersive solid-phase extraction with reduced graphene oxide modified iron nanoparticles and the use of liquid chromatography coupled to UV as a separation and detection technique. In this scientific work, LODs between 0.03 and 0.16 ng mL^{-1} and LOQs between 0.06 and 0.24 ng mL^{-1} with recoveries between 85.6 and 121% were achieved. They analyzed 14 fecal samples and found no parabens in them. When these results are compared with those obtained in the work we propose in the present article, it can be affirmed that this new method, based on the use of UHPLC-MS/MS combined with ultrasound-assisted extraction and cleaning of the extracts by dispersive solid phase extraction (dSPE), provides better LODs and LOQs. In addition, while the first author cited observed MPB in some of the samples, Tekin *et al.* did not obtain positive results for these substances, unlike the present study, where positive samples were found for more than two contaminants in all the cases analyzed.

5. Conclusions

To our knowledge, this is first analytical method developed and validated for determining parabens in human faeces. The optimized method is based on an ultrasound extraction step followed by cleaning of the extracts by d-SPE and analysis by UHPLC-MS/MS. The method offers excellent characteristics in terms of sensitivity, selectivity, linearity and accuracy (trueness and precision). The method is easy to apply and provides important knowledge on EDC exposure, particularly of parabens. To date, the scientific community has mainly considered only urine as a route of elimination of EDCs. Faeces can provide very important information on the excretion of compounds that can enter our body via dermal or dietary routes.

It is noteworthy that this method was applied to 14 volunteer samples, all of which tested positive for three or more parabens. The matrix studied is currently very little studied and could be a good biomarker to complement data obtained in urine or saliva samples, matrices widely studied in the scientific literature. Due to the limited number of studies available, more research is needed in this field to have a complete picture of EDCs in the body and their exposure/removal/accumulation pathways. Therefore, all this demonstrates the need for further work to develop rapid, sensitive, reliable methods at the lowest possible economic cost for those matrices that have been little studied or even not studied at all. The goal should be to accurately and reliably know the (bio)exposure to EDCs, especially in the early stages of human development.

Credit authorship contribution statement

Inmaculada Moscoso-Ruiz: Formal analysis, Investigation, Methodology, Data curation, Writing – original draft. **Alberto Navalón**: Conceptualization, Formal analysis, Investigation, Writing–original draft. **Ana Rivas**: Formal Analysis, Funding acquisition, Writing-review and editing. **Alberto Zafra-Gómez**: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Data curation, Writing–original draft, Writing-review, editing and supervision.

Declaration of interests

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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Declarations

The present study has been approved by the ethics committees of the University of Granada and of the Provincial Biomedical Research of Granada (CEI), Spain (reference 1939-M1-22, Andalusian Biomedical Research Ethics Portal); and the study have been performed in accordance with the ethical standards. Also, all subjects gave written informed consent and had parental permission to participate.

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Figure captions

Figure 1. Chromatogram of a spiked sample (100 ng g^{-1}).

Figure 2. Solvent optimization (A) and study of alkalinity, acidity or neutral medium (B).

Figure 3. Estimated response surface of 3^2 for UAE parameters.

Figure 4. Box-Behnken DoE for clean-up step.

Figure 5. Chromatogram of a volunteer sample (Nr. 1)