Comprehensive determination of phthalate, terephthalate and di-*iso*-nonyl cyclohexane-1,2-dicarboxylate metabolites in wastewater by solid-phase extraction and ultra(high)-performance liquid chromatography-tandem mass spectrometry

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1 **Abstract**

2   Plasticizers are chemical compounds used in the production of flexible plastics for a large

3   variety of applications. They are present in most of the environments and, hence, we are

4   highly exposed to them via several routes (ingestion, inhalation, etc). Due to the endocrine

5   disruption potential of some of these chemicals and the unknown toxicological effects of

6   their alternatives, assessing human exposure to these contaminants is an issue of emerging

7   concern. Herein we propose an analytical methodology for the determination of several

8   plasticizer metabolites in wastewater as a non-invasive, cheap, and fast exposure

9   monitoring tool complementary to the analysis of urine. A solid-phase extraction

10   procedure followed by an ultra(high)-performance liquid chromatography-tandem mass

11   spectrometry method was optimized and validated for 21 analytes among phthalate,

12   terephthalate, and di-*iso*-nonyl cyclohexane-1,2-dicarboxylate metabolites. Method

13   quantification limits ranged from 0.079 to 4.4 ng L^{-1}. The method was applied to the

14   analysis of seven daily composite wastewater samples collected in the NW of Spain.

15   Metabolites of low molecular weight phthalates and of di-2-ethylhexyl phthalate were

16   quantified in all samples, despite the existing regulations limiting the use of phthalates.

17   Metabolites of terephthalates, introduced at the end of the 20th century as phthalate

18   substituents, were also quantified in all samples, being the first time that they were

19   detected in this matrix. Exposure back-calculation highlighted di-2-ethylhexyl

20   terephthalate as the second most common plastic additive after diethyl phthalate in the

21   population considered, reflecting the increasing substitution of di-2-ethylhexyl phthalate

22   by its analogous terephthalate.

23

1. INTRODUCTION

Plasticizers are large-scale production chemicals used as additives in plastic polymers in order to improve their properties, representing in some cases up to 80% of the plastic weight [1]. Esters of the 1,2-benzenedicarboxylic acid (phthalic acid), also known as phthalates, are the most widely used plasticizers due to their low volatility, water resistance, inexpensive prize, and excellent compatibility with a great variety of plastics [1]. They can be divided in two groups: low molecular weight (LMW) phthalates, with six or less carbon atoms in the ester chain, and high molecular weight (HMW) phthalates, with more than six carbon atoms in the ester chain [2]. Since these compounds are mixed with the polymeric material but not chemically bonded to it, they can be easily released into the surrounding environment. Hence, people are frequently exposed to them [3]. The most common exposure route is direct ingestion, derived from the use of these additives in toys and from the consumption of contaminated foods and water [4]. Inhalation and dermal contact are other exposure routes affected by several factors, like temperature and packaging, cosmetics, or clothes composition [5].

From a toxicological approach, phthalates are endocrine disruptors causing a large variety of harmful effects. The most dangerous ones are the LMW phthalates with linear ester chains of 4-6 carbon atoms. Phthalates are related to respiratory problems, but they mainly interfere with the production of sex hormones causing the *phthalate syndrome,* responsible for disorders in the development of the male reproductive system, infertility, and even fetus malformations [6,7]. Allergy, asthma, and obesity found in babies and children between 2 and 8 years old have been attributed to both prenatal and postnatal exposure to phthalates [8,9]. Due to all these negative effects, a European legislation set in 2007 is being regularly updated to control the concentration of these plasticizers in toys, childcare articles [10], and food contact materials [11]. Also, the European Food

53 Safety Authority (EFSA) and the Environmental Protection Agency of the United States

54 (US-EPA) have set Tolerable Daily Intakes (TDI) [12–16] and Oral Reference Doses

55 (RfD)[17–23] for several phthalate derivatives. The European Commission and,

56 subsequently, the US-EPA have developed an action plan to substitute phthalates by other

57 less harmful plasticizers such as terephthalates, citrates, adipates, or trimellitates [2,24].

58 This study is focused on two groups of phthalates (LMW and HMW derivatives), and on

59 two alternative plasticizer families: terephthalates and di-*iso*-nonyl cyclohexane-1,2-

60 dicarboxylate (DINCH). Some of the HMW phthalates entered the market in the 1980s

61 as a new and less harmful alternative to LMW phthalates and to di-2-ethylhexyl phthalate

62 (DEHP). Examples of these derivatives are di-*iso*-nonyl phthalate (D*i*NP) and di-*iso*-

63 decyl phthalate (D*i*DP), which are actually sold as technical mixtures that contain several

64 positional isomers [25,26]. Terephthalates are esters of the 1,4-benzenedicarboxylic acid

65 (terephthalic acid) that were introduced at the end of the 20th century. Despite their similar

66 structure to LMW phthalates, their toxicological profiles differ considerably, not showing

67 a remarkable reproductive toxicity [27]. DINCH was introduced in 2002 by the chemical

68 company BASF as a plasticizer for medical devices, since HMW phthalates did not have

69 the required flexibility and viscosity at low temperature. Plasticizer properties of DINCH

70 are similar to those of DEHP, but its migration rate is lower and it has no demonstrated

71 negative effects on fertility at high concentrations [28]. Phthalates, terephthalates, and

72 DINCH metabolites are excreted primarily as the monoester following enzymatic hydrolysis,

73 though further oxidation may also occur prior to excretion. Both the monoesters and the

74 oxidized forms are used to estimate human exposure to the parent plasticizers by

75 measuring their concentrations in urine [29–32]. This human biomonitoring (HBM)

76 approach can give a reliable and comprehensive picture since, e.g., population data can

77 be stratified by sex, age, etc. Conversely, the analysis is expensive, laborious, and

restricted to a limited number of people. The use of wastewater as an integrated and diluted sample of urine of an entire location is an interesting tool that allows to obtain comprehensive results by analyzing fewer samples in a faster and less expensive way. This methodology, known as wastewater-based epidemiology (WBE), was initially applied to estimate the use of illicit drugs in different cities [33–35]. In 2010, the European Monitoring Centre for Drugs and Drugs Addiction (EMCDDA) encouraged the creation of SCORE (Sewage Analysis Core group Europe), which leads the performance of an annual wastewater monitoring campaign to estimate the consumption of illicit drugs in an ever growing number of cities worldwide (www.score-network.eu) (Sewage Analysis CORe group Europe (SCORE)). Recently, WBE has been extended to the measurement of the metabolites of environmental contaminants, such as pesticides, plasticizers, flame retardants or bisphenols [37–42], to estimate not human consumption, but human exposure to the parent chemicals.

In this context, the aim of this study was to develop an analytical method to determine 21 plasticizer metabolites in wastewater by solid-phase extraction (SPE) and ultra(high)-performance liquid chromatography (U(H)PLC) coupled to tandem mass spectrometry (MS/MS). The target analytes are the metabolites of 12 different plasticizers belonging to four different groups: LMW phthalates, HMW phthalates, terephthalates, and DINCH. Extraction, separation, and detection conditions were optimized, and the method was validated and applied to the analysis of seven daily composite raw wastewater samples collected in the NW of Spain. To the best of authors' knowledge, there are four WBE-derived studies dealing with the determination of phthalate metabolites in wastewater [39,43–45], and the current one is the first including the assessment of alternative plasticizers such as terephthalates and DINCH.

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

The target analytes and their corresponding parent chemicals are displayed in the Supplementary Material, Table S1. Analytical standards of monomethyl phthalate (MMP), monoethyl phthalate (MEP), mono-*n*-butyl phthalate (M*n*BP), monobenzyl phthalate (MBzP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), and the deuterated analogues MMP-d4, M*n*BP-d4, and MEHHP-d4 were supplied by AccuStandard (New Haven, CT, USA). Mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), and mono-*iso*-butyl phthalate (M*i*BP) were supplied by Toronto Research Chemicals (Toronto, ON, Canada). Monomethyl terephthalate (MMTP), mono-(2-ethyl-5-hydroxhexyl) terephthalate (MEHHTP), mono-(2-ethyl-5-oxohexyl) terephthalate (MEOHTP), mono-(2-ethyl-5-carboxypentyl) terephthalate (MECPTP), and their deuterated analogues MMTP-d_4, MEHHTP-d_4, MEOHTP-d_4, and MECPTP-d_4 were supplied by CanSyn (Toronto, ON, Canada). Monocyclohexyl phthalate (MCHP), mono-hydroxy-*iso*-nonyl phthalate (MHINP), mono-(4-methyl-7-oxooctyl) phthalate (MMOOP), mono-(4-methyl-7-carboxyheptyl) phthalate (MMCHP), mono-carboxy-*iso*-nonyl phthalate (MCINP), mono-(4-methyl-octyl) cyclohexane-1,2-dicarboxylate (MINCH), mono-(7-carboxy-4-methyl-heptyl) cyclohexane-1,2-dicarboxylate (cx-MINCH), mono-(4-methyl-hydroxy-octyl) cyclohexane-1,2-dicarboxylate (OH-MINCH), and mono-(4-methyl-7-oxo-octyl) cyclohexane-1,2-dicarboxylate (oxo-MINCH) were supplied by MuseChem (Fairfield, USA). Analytical standards of the parent compounds dimethyl terephthalate (DMTP), DEHTP, dicyclohexyl phthalate (DCHP), D*i*NP, and D*i*DP were supplied by Sigma-Aldrich (Steinheim, Germany). DINCH was supplied by Toronto Research Chemicals. Individual stock standard solutions were prepared in methanol (MeOH) at a concentration

128 of 1,000 μg mL^{-1}. Mixed stock solutions containing 10 μg mL^{-1} of all the analytes or all

129 the deuterated analogues (used as surrogate or internal standards, IS), were prepared in

130 MeOH and stored in the dark at -20 °C until use.

131 Ultrapure water was obtained with a Milli-Q Gradient A-10 system (Merck Millipore,

132 Bedford, USA). HPLC-grade MeOH, acetic acid (100%), hydrochloric acid (37%) and

133 ammonia solution in water (25%) were supplied by Merck (Darmstadt, Germany). Formic

134 acid (95-97%) and ammonia in MeOH (7 N) were supplied by Sigma-Aldrich.

135

2.2. Samples

141

142 Composite 24-hour raw wastewater samples were collected after the fine screen

143 pretreatment of an urban wastewater treatment plant (WWTP) located in Santiago de

144 Compostela (Spain). The WWTP serves a population of ca. 136,500 inhabitants. Samples

145 were collected during seven consecutive days (May 21st-28th, 2019) with an automatic

146 sampler (Sigma SD900, Hach, USA) working in time proportional mode (120 mL

147 collected every 10 min, from 9.00 a.m. to 9.00 a.m. of the following day). Composite-

148 sample aliquots were solid-phase extracted daily following the protocol showed in section

149 2.3.1.

150

2.3. Method development

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2.3.1. Solid-phase extraction

152

153 Aliquots of 100 mL of wastewater were vacuum-filtered through 0.7 μm glass microfiber

154 filters GF/A (47 mm diameter, Whatman, Kent, UK) and 0.45 μm cellulose filters (47

155 mm diameter, Merck Millipore).Samples were spiked with 20 ng of every IS after

156 filtration. SPE was performed using a standard vacuum manifold Visiprep™ (12-port

157 model, Supelco, Steinheim, Germany) maintaining a pressure of approx. 5 inches Hg.

Samples were loaded onto mixed-mode reversed-phase strong anion-exchange cartridges (Oasis MAX-60 mg, Waters, Milford, MA, USA) previously rinsed with 3 mL of MeOH followed by 3 mL of ultrapure water. Sorbents were dried under nitrogen during 30 min and analytes eluted with 5 mL of 2% formic acid in MeOH. Eluates were evaporated to dryness under nitrogen using a Turbo-Vap II (Zymark, Hopkinton MA, USA) and a Mini-Vap (Supelco, Steinheim, Germany) concentrator. Extracts were reconstituted in 100 µL of MeOH, filtered through 0.22 µm PVDF syringe-driven filters (Millex, Merck Millipore) and injected into the UPLC® system.

2.3.2. Ultra(high)-performance liquid chromatography-tandem mass spectrometry

Instrumental analyses were performed with a Waters Acquity UPLC® H class system (Milford, MA, USA) equipped with a quaternary solvent pump, a thermostatted LC column compartment, and a sample manager. The UPLC® system was interfaced to a triple quadrupole mass spectrometer Xevo TQD from Waters.

The chromatographic separation was performed at 45 °C on a Raptor Biphenyl column (150×2.1 mm I.D., 1.8 µm particle size) from Restek (Bellefonte, PA, USA). A Kinetex® Phenyl-Hexyl column (150×2.1 mm I.D., 1.7 µm particle size) from Phenomenex (Torrance, CA, USA) was also tested at the initial stage of the chromatographic optimization. Under final conditions, a dual eluent system consisting of (A) 0.1% acetic acid in ultrapure water and (B) 0.1% acetic acid in MeOH was used at a flow rate of 0.3 mL min^{-1}, reaching a pressure of 11,000 psi under initial gradient conditions. The gradient lasted 27 min and consisted of the following stages: 0 min (50% B), ramped to 100% in 17 min, which was maintained for 5 min, then, rapidly returned to initial conditions (50% B) in 0.05 min, which was maintained for 5 min for column back-conditioning (total time 27 min, including back-conditioning). Injection volume was set at 2 µL.

183 The interface between the UPLC® system and the Xevo TQD mass spectrometer was an

184 electrospray ionization (ESI) source operating in negative mode at a fixed capillary

185 voltage of 3 kV and a temperature of 150 °C. Nitrogen, provided by a nitrogen generator

186 from Peak Scientific Spain (Barcelona, Spain), was used as desolvation gas at 600 L h^{-1}

187 and 450 °C (desolvation temperature), and as cone gas at 10 L h^{-1}. Analyses were

188 performed by MS/MS in Selected Reaction Monitoring (SRM) mode acquiring three

189 precursor/product ion transitions per analyte (except for MMP, MEP, MiBP, MnBP,

190 MBzP, MEHHP, MEOHP, and MECPP, for which two transitions were acquired) and

191 one transition per IS. Argon was used as collision gas. Table 1 compiles chemical

192 formulae, retention times (RT), SRM transitions (Q_n), optimal collision energies (CE),

193 and cone voltages (CV) for every analyte.

194

195 **2.4. Quantification and method validation**

196 MMP, MnBP, MEHHP, MMTP, MEHHTP, MEOHTP, and MECPTP were quantified

197 using their deuterated analogues as IS. For the remaining analytes, for which no

198 deuterated analogues were available, different surrogate standards were tested (Table 1).

199 The one providing the best results in terms of matrix effects and accuracy was selected.

200 Instrumental detection and quantification limits (IDLs and IQLs) were estimated from the

201 lowest calibration standards as the concentrations providing a signal-to-noise ratio (S/N)

202 of 3 and 10, respectively. Noise height was measured on both sides of the peak in a ca. 1

203 min window. Calibration curves were prepared in MeOH at seven concentration levels

204 ranging from the IQL (0.03-2.3 ng mL^{-1}, see Section 3.3) to 5,000 ng mL^{-1} for MEP, and

205 from the IQL to 1,000 ng mL^{-1} for the remaining compounds (IS level: 200 ng mL^{-1}).

206 Instrumental precision was assessed by the relative standard deviation (%RSD) of five

207 injections of two calibration standards, containing 10 ng mL^{-1} and 100 ng mL^{-1} of all

208   analytes and 200 ng mL^{-1} of IS. Injections were performed within the same day for the

209   intra-day precision studies and in five different days within a month for the inter-day

210   precision studies.

211   The validation of the SPE-UPLC-MS/MS method was performed in terms of trueness,

212   precision, method detection limits (MDL), and method quantification limits (MQL).

213   Trueness and precision were assessed by recovery studies performed in ultrapure water

214   and wastewater spiked with 50 ng L^{-1} and 500 ng L^{-1}, respectively, of all analytes and 200

215   ng L^{-1} of IS (n=3). Wastewater aliquots spiked only with IS were processed

216   simultaneously to account for analyte background levels in this matrix (n=3). Matrix

217   effects (ME) were calculated as the signal (analyte peak area) percentage in a 500 ng mL^{-1}

218   ^{1} spiked (pooled mix) wastewater extract, after non-spiked signal subtraction and referred

219   to the signal of a 500 ng mL^{-1} standard. Thus, a value below 100% implies signal

220   suppression due to matrix effects in the ESI source. MDLs and MQLs were calculated

221   from IDLs and IQLs, respectively, considering MEs and the concentration factor

222   achieved with the SPE (1,000):

$$\text{MDL}(^{ng}/_{L}) = \frac{\text{IDL}(^{ng}/_{mL})}{^{ME}/_{100}} \times 1000 \qquad \text{MQL}(^{ng}/_{L}) = \frac{\text{IQL}(^{ng}/_{mL})}{^{ME}/_{100}} \times 1000$$

223   Instrumental blanks (standard solutions containing only IS) were run at the beginning of

224   every sequence. Procedural blanks were assessed together with every set of samples by

225   solid-phase extracting ultrapure water aliquots (100 mL) spiked with 200 ng L^{-1} of every

226   IS and analyzing them normally. When concentrations were >MQL in procedural blanks,

227   these values were subtracted from levels found in samples.

228

229   ## 2.5. Stability and filtration loss tests

230 Stability tests were carried out for metabolites and their precursor plasticizers in order to

231 determine if (a) metabolites are stable in wastewater against potential adsorption and/or

232 degradation processes occurring during sampling (24 hours); and (b) precursor

233 plasticizers can be degraded or hydrolyzed in wastewater causing the formation of the

234 metabolites selected as human exposure biomarkers. Since the stability of DMP, DEP,

235 D*n*BP, BzBP, DEHP and their metabolites has been verified by González-Mariño et al.

236 [39], tests were conducted only for the remaining plasticizers and their corresponding

237 biomarkers. Potential analyte adsorption to filters during wastewater filtration was also

238 assessed.

239 To address scenarios (a) and (b), three replicates of 10 mL of unfiltered wastewater (n=3)

240 were spiked with 500 ng mL^{-1} of all metabolites (scenario a) or all precursor plasticizers

241 (scenario b) and stored in amber glass vials for 48 hours. Storage was performed at room

242 temperature (22 $\pm$ 2 $^{\circ}$C) in both scenarios, and also at 4 $^{\circ}$C in scenario (a). Aliquots of 1

243 mL were collected at the beginning of every experiment (time 0) and after 1.5, 3, 5, 8, 24

244 and 48 hours. They were filtered through 0.22 μm hydrophilic PTFE syringe-driven

245 filters, spiked with 200 ng mL^{-1} of IS, and analyzed by direct injection into the UPLC$^{\circledR}$-

246 MS system following the method described in section 2.3.2.

247 To assess potential losses due to analyte sorption onto sample filters, six aliquots of 100

248 mL of ultrapure water were spiked with 500 ng L^{-1} of all the analytes. Three replicates

249 were then filtered through glass microfiber filters and cellulose filters, spiked afterwards

250 with 200 ng L^{-1} of IS and submitted to SPE. Another three replicates were spiked with IS

251 and processed without filtering. A Student's t-test (α= 0.05) was applied to compare

252 responses in filtered and unfiltered samples. No significant differences were observed in

253 any case.

254

2.6. Estimation of plasticizer exposure through WBE

Metabolite concentrations found in 24-hour composite influent samples were used to estimate daily exposure levels to the parent plasticizers (in µg day^{-1} inhabitant^{-1}), To this end, metabolite concentrations (in µg L^{-1}) were multiplied by the wastewater daily flow rates (L day^{-1}) measured by the WWTP operators and by a correction factor (CF) that takes into account the fraction of plasticizer excreted as that specific metabolite. Exposure levels were further normalized to the population served by the WWTP (see equations below).

$$\text{Human daily exposure in µg day}^{-1}\text{inh}^{-1} = \frac{\text{Concentration (µg L}^{-1}) \times \text{Flow rate (L day}^{-1})}{\text{number of inhabitants}} \times \text{CF} \quad (1)$$

$$\text{CF} = \frac{\text{Molecular weight}_{\text{Plasticizer}} / \text{Molecular weight}_{\text{Metabolite}}}{\text{Molar excretion fraction}} \quad (2)$$

CF values are recorded in Table S1. For DMP, DEP, DiBP, DnBP, BzBP and DEHP, they were obtained from [39], where existing metabolism studied were already compiled. For the remaining compounds, they were calculated following the same approach. First, average molar excretion factors were calculated by weighting the excretion factors published up to date in human metabolism studies by the number of participants involved in every study (Table S2). CFs, as depicted in Eq. (2), were then calculated by dividing the molecular weight of the plasticizer between the molecular weight of the metabolite, and this value between the average molar excretion fraction obtained (Table S1). For those plasticizers with more than one metabolite, individual exposure levels were calculated from every single metabolite and the average exposure was obtained.

3. RESULTS AND DISCUSSION

3.1. UHPLC-MS/MS optimization

278 MS/MS parameters (precursor/product ion transitions, CV and CE) were optimized by

279 direct injection analysis of individual standard solutions (5 µg mL^{-1}) in ultrapure

280 water:MeOH (1:1) (Table 1). The structural similarity between most phthalates and

281 terephthalates led them to share some common products, such as *m/z* 121, corresponding

282 to the benzoate anion, and *m/z* 77, corresponding to the benzene anion [31,46]. DINCH

283 metabolites shared the product *m/z* 153, corresponding to cyclohexane-1,2-dicarboxilic

284 acid anhydride (Figure S1) [46].

285 Separation was initially attempted on a Kinetex® Phenyl-Hexyl column (150×2.1 mm I.D,

286 1.7 µm particle size) using a dual eluent system of (A) 0.1% acetic acid in ultrapure water,

287 (B) 0.1% acetic acid in MeOH, and a flow rate of 0.35 mL min^{-1}. These conditions result

288 from extrapolating to UPLC® our previously published HPLC method for the separation

289 of eight phthalate metabolites on a Luna® Phenyl-Hexyl column (150×2.1 mm I.D., 3 µm

290 particle size) [39]. Several gradients and flow rates were tried (Figure S2) until 18 out of

291 the 21 analytes eluted at times > 3 min. Only MMP, MEP and MMTP eluted earlier.

292 These conditions (gradient (c), flow rate 0.3 mL min^{-1}) were tested for comparison on a

293 Raptor Biphenyl column (150×2.1 mm I.D., 1.8 µm particle size), which led to higher

294 retention (Figure S2 (d)) and, in most cases, slightly lower IDLs (0.14-3.3 ng mL^{-1} versus

295 0.10-5.0 ng mL^{-1} obtained with the Phenyl-Hexyl column using the same gradient and

296 flow conditions, data not shown). However, MECPTP and MHINP, isobaric compounds

297 sharing two SRM transitions (307>121 and 307>77, actually the two most intense

298 transitions for MHINP) overlapped. Different gradients were tested again on the Raptor

299 Biphenyl column (Figure S3) until they were baseline fully resolved (Figure S3 (d)). As

300 an example, a chromatogram of a 500 ng mL^{-1} standard analysed with this gradient is

301 displayed in Figure S4.

302

3.2. Solid-phase extraction sorbent selection

Given the acidic character of target analytes, two different sorbents were assessed for their extraction in wastewater (pH ca. 7-8): the hydrophilic-lipophilic balance reversed-phase polymeric sorbent Oasis HLB, and the mixed-mode reversed-phase anion exchange sorbent Oasis MAX (strong anion exchange). Both were tested as 60 mg sorbents in commercial SPE syringe cartridges and following the generic method conditions (regarding conditioning, equilibration and elution solvent selection) recommended by the manufacturer (Waters). Aliquots of 100 mL of ultrapure water (n=3 for each sorbent) were spiked with 500 ng L^{-1} of all analytes and processed following the SPE protocols recommended by the manufacturer. Briefly, Oasis HLB sorbents were conditioned with 3 mL of MeOH followed by 3 mL of pH 2.0 ultrapure water; samples were adjusted to pH 2.0 with diluted hydrochloric acid to neutralize analyte acidic groups and increase reversed-phase interactions, and elution was performed with 5 mL of MeOH. Oasis MAX sorbents were conditioned with 3 mL of MeOH followed by 3 mL of ultrapure water; samples were extracted at their natural pH and elution was performed with 5 mL of 2% formic acid in MeOH. With the Oasis MAX, a washing step with 5 mL of MeOH was included before the elution to wash off neutral interferences. All washes and extracts were concentrated to dryness and reconstituted in 100 µL of MeOH for instrumental analysis. The comparison of the analyte areas in the SPE extracts/washes to the areas in a 500 ng mL^{-1} standard provided the absolute recoveries of the analysis. As it is displayed in Figure S5, DINCH metabolites were partially or totally recovered in the washing fraction of the Oasis MAX and, hence, this step had to be skipped (i.e. all analytes were directly recovered with 5 mL of 2% formic acid in MeOH). Matrix effects and absolute recoveries achieved with this sorbent when skipping the washing step were compared to the values obtained with the Oasis HLB (n=3 in every case). To this end, six aliquots of 100 mL of

328   wastewater were extracted with each sorbent; in each case, three SPE extracts were

329   directly injected into the UPLC®-MS system, and another three were spiked with 50 ng

330   of all analytes prior to the instrumental analysis. MEP was excluded from this test due to

331   the high concentrations found in wastewater (section 3.5), notably higher than the spiking

332   level selected (500 ng L^{-1} referred to sample). Oasis MAX provided statistically

333   significant higher absolute recoveries (p-value < 0.05) for all analytes but MECPP,

334   MMCHP, MCINP, MECPTP, and cx-MINCH, for which non-significant differences

335   were observed between both sorbents (p-value between 0.2 and 0.8, Figure 1(a)).

336   Statistically significant lower matrix effects (i.e. higher ME recoveries, p-value < 0.05)

337   were also obtained with the Oasis MAX for all analytes but MMP, M*i*BP, M*n*BP, the

338   three DEHP metabolites, and MMTP (p-values > 0.05, Figure 1(b)). Thus, the Oasis

339   MAX sorbent was selected to extract the 21 plasticizer metabolites in wastewater

340   following a single elution with 5 mL of 2% formic acid in MeOH (section 2.3.1.).

341

3.3. Method validation

343   UPLC®-MS/MS method performance parameters (linearity, IDLs, IQLs, and intra- and

344   inter-day precision) are displayed in Table 2. The representation of the analyte response

345   (analyte area/IS area) versus analyte concentration (IQL-5,000 ng mL^{-1} range for MEP

346   and IQL-1,000 ng mL^{-1} range for the remaining compounds) fitted a linear model at a

347   95% level of confidence (p-values for a Lack of fit test performed with 6 calibration

348   curves varied between 0.057 and 0.997). IDL and IQL values varied between 0.010 ng

349   mL^{-1} and 0.70 ng mL^{-1}, and between 0.032 ng mL^{-1} and 2.3 ng mL^{-1}, respectively. %RSD

350   values were below 17% for the intra-day precision studies and below 18% for the inter-

351   day precision studies. Concentrations in instrumental blanks were below the IDL in all

352   cases.

353 Trueness and precision of the whole SPE-UPLC®-MS/MS method were assessed through

354 recovery studies performed in ultrapure water and raw wastewater. IS-corrected

355 percentages of recovery (%R) for triplicate analyses of ultrapure water samples spiked

356 with 50 ng L^{-1} of all analytes and 200 ng L^{-1} of IS varied between 81% and 136%, with

357 %RSD comprised between 4% and 23%. In raw wastewater samples spiked with 500 ng

358 L^{-1} of all analytes and 200 ng L^{-1} of IS, %R varied between 74% and 130%, and %RSD

359 between 1% and 21%. MDLs ranged from 0.024 ng L^{-1} to 1.3 ng L^{-1}, and MQLs from

360 0.079 ng L^{-1} to 4.4 ng L^{-1} (Table 2). Table S3 compares the performance of the proposed

361 method with that of other available SPE-LC-MS/MS methods for the determination of

362 plasticizer metabolites in (i) wastewater (whole method performance comparison); and

363 (ii) urine (instrumental performance comparison only). The IQLs achieved with our

364 UPLC®-MS/MS method are in the same order of magnitude than the ones reported in

365 other studies [39,47,48]. MQLs are also comparable to the ones achieved by other SPE-

366 LC-MS/MS procedures developed for the extraction and determination of phthalate

367 metabolites in wastewater [39,43,44]. Trueness, assessed from relative recovery

368 experiments, was also similar: %R varied between 74-136% (this study) versus 76-100%

369 [39], 100-105% [43], and 64-98% [44].

370 Procedural blanks (section 2.4) showed the variable occurrence of MMP and M*i*BP at

371 levels up to 2.8 ng L^{-1} and 3.8 ng L^{-1}, respectively. These values stand for the 0.8% and

372 1.4% of the average concentrations found in wastewater, pointing to the absence of strong

373 contamination issues. Nevertheless, blank concentrations were subtracted from levels

374 measured in wastewater samples (section 3.5). M*n*BP was also detected in some

375 procedural blanks but at concentrations varying between the MDL and MQL; thus, no

376 action was performed in this case.

377

3.4. Plasticizer and plasticizer metabolites stability in wastewater

Stability tests were conducted for the target plasticizers and their metabolites in wastewater, excluding those compounds whose stability has been verified by González-Mariño et al. [39]. All metabolites (scenario (a), section 2.5) were stable at room temperature but terephthalate metabolites, which were stable for 24 hours but underwent a remarkable drop after 48 hours (Figure 2(a)). When performing the same experiments at 4 °C, no signal drop was observed (Figure 2(b)), proving that the biodegradation occurring at room temperature is slowed down by cooling the sample. Since the maximum time that wastewater spends in the autosampler is 24 hours (first 120 mL aliquot taken by the sampler), and samples were extracted within 2 hours after collection, no significant degradation is expected to occur. Only MINCH showed, both at room temperature and 4 °C, a high variability between the responses of different time aliquots (Figure 2). This phenomenon was attributed to adsorption processes occurring onto suspended particle matter, since MINCH is the most apolar analyte in this study. Hence, MINCH is not rendered a suitable biomarker for the assessment of human exposure to DINCH through the analysis of wastewater, and the three oxidative metabolites OH-MINCH, oxo-MINCH, and cx-MINCH must be used instead.

The potential degradation/hydrolysis of the precursor plasticizers in wastewater (scenario (b), section 2.5) showed that DMTP was completely hydrolyzed into MMTP after 48 hours (Figure S6, in molar percentage). This prevented it from being considered, as in the case of MINCH, a suitable biomarker of exposure to DMTP.

3.5. Application to real samples

The validated method was applied to the analysis of composite wastewater samples of 24 hours collected over seven consecutive days (n=3). Figure 3 shows the chromatogram

403   (quantifier transition) of the analytes found in the sample of May 28th. To guarantee the

404   appropriate identification of all analytes, the deviation of the Q_2/Q_1 ratio in a sample,

405   relative to the average Q_2/Q_1 ratio in the calibration standards, was ensured to be less than

406   30% [49].

407   Twelve compounds were positively quantified in all samples (Table 3): the LMW

408   phthalate metabolites MMP, MEP, MiBP, MnBP, and MBzP; the three DEHP

409   metabolites MEHHP, MEOHP, and MECPP; and the terephthalate metabolites MMTP,

410   MEHHTP, MEOHTP, and MECPTP. To the best of our knowledge, this is the first time

411   that any terephthalate metabolite is detected in wastewater. None of the other nine

412   analytes was found in any case. The highest concentrations were measured for the LMW

413   phthalate metabolites, particularly for MEP (2,656-3,690 ng L^{-1}), followed by MMP (226-

414   714 ng L^{-1}), and the butylated derivatives MiBP (223-334 ng L^{-1}) and MnBP (219-795 ng

415   L^{-1}). However, considerable variations were observed in some cases, where the level on

416   one of these analytes increased or decreased in one single day in comparison with the

417   previous and further samples. Further and larger monitoring campaigns will help to

418   confirm whether this is a usual observation or not. BzBP and DEHP metabolites

419   (MEHHP, MEOHP, and MECPP) were quantified at lower levels. Phthalate metabolite

420   concentrations were compared to the few existing studies quantifying the occurrence of

421   phthalate metabolites in influent wastewater. González-Mariño et al. measured high

422   concentrations of MEP (300-1,599 ng L^{-1}), MMP (48-1,885 ng L^{-1}), and the isomers

423   MiBP and MnBP (67-277 ng L^{-1} and 55-274 ng L^{-1}, respectively) in samples collected in

424   several WWTPs of the same region of the one sampled here (Santiago de Compostela

425   [39]. In a recent study involving thirteen cities across Spain, even higher levels of MEP

426   (up to 12,700 ng L^{-1}), MMP (up to 3,828 ng L^{-1}), and the butylated derivatives (up to

427   1,974 ng L^{-1} for MiBP and 867 ng L^{-1} for MnBP) were found [45], likely due to the

428 inclusion of samples collected at larger cities. On a national scale, and considering

429 medium-size locations only, levels measured in this study are in line with the levels

430 reported previously. In several cities across China, Du et al. found MnBP at the highest

431 concentrations (93-6,921 ng L^{-1}), followed by MMP (23-2,670 ng L^{-1}), MiBP (<LOD-

432 2,600 ng L^{-1}) and MEP (6-1,581 ng L^{-1}) [43]. Finally, Tang et al. quantified several

433 phthalate metabolites in wastewater samples from South East Queensland (Australia).

434 Among the substances shared with our study, MMP was the one found at the highest

435 levels (up to 11,000 ng L^{-1}), followed by MEP (over 2,000 ng L^{-1}), and then MiBP (1,900

436 ng L^{-1}) and MnBP (1,500 ng L^{-1}) [44].

437 To the best of author's knowledge, this is the first time that a terephthalate metabolite is

438 detected in wastewater. Among them, MMTP concentrations ranged from 47 ng L^{-1} to

439 230 ng L^{-1}, MEHHTP from 16 ng L^{-1} to 26 ng L^{-1}, MEOHTP from 13 ng L^{-1} to 17 ng L^{-}

440 ^{1}, and MECPTP from 125 to 169 ng L^{-1}. According to the excretion factors listed in Table

441 S1, the three DEHP metabolites are excreted in a similar percentage (just slightly higher

442 for MEHHP) following DEHP exposure. Thus, their concentrations in sewage are

443 expected to be similar. Conversely, the higher excretion factor of MECPTP (0.130)

444 compared to that of MEHHTP (0.018) and MEOHTP (0.010), points to the first one

445 occurring in wastewater at concentrations between 7 and 13 times higher than the

446 remaining two metabolites. Levels listed in Table 3 match these observations for both

447 DEHP and DEHTP metabolites.

448

449 **3.6. Estimation of human exposure to phthalate plasticizers.**

450 Metabolite concentrations in wastewater were used to estimate daily exposure levels to

451 the parent plasticizers. As explained in section 3.4, all the selected metabolites are, in

452 principle, suitable biomarkers of exposure with the exception of MMTP and MINCH.

453 Thus, DMTP and DINCH were excluded from exposure calculations (DINCH

454 metabolites were not detected in any case, Table S4). In addition, since both Tang et al.

455 [44] and González-Mariño et al. [45] have pointed to the likely existence of sources other

456 than human excretion contributing to MMP occurrence in wastewater, DMP was also

457 excluded from these calculations. As it is observed in Figure 4, the highest exposure was

458 attributed to DEP (above 1,800 μg day^{-1} inhabitant^{-1}), which is more than three times the

459 average exposure observed for this compound in two previous sampling campaigns

460 performed in the same city in 2016 (559 μg day^{-1} inhabitant^{-1}) [39] and 2018 (717 μg day^{-1}

461 inhabitant^{-1}) [45]. The following highest exposure was reported for DEHTP (average of

462 524 μg day^{-1} inhabitant^{-1}), whose metabolites were found in quantifiable concentrations

463 despite their low excretion factors (Table S1). To the best of author's knowledge, this is

464 the first time that DEHTP metabolites are found in wastewater, and also the first time that

465 DEHTP exposure is estimated at population level by applying WBE principles. While

466 there is only one study assessing the excretion kinetics of this plasticizer [50], exposure

467 calculations derived from single MEHHTP, MEOHTP, and MECPTP concentrations are

468 relatively similar, providing an average value of 524 μg day^{-1} inhabitant^{-1}. The exposure

469 to its positional isomer DEHP is lower (average of 68 μg day^{-1} inhabitant^{-1}) and in the

470 same order of magnitude that the levels reported in the same city by González-Mariño et

471 al. in the sampling campaign of 2016 [39] and in 2018 [45]. For DiBP, DnBP, and BzBP,

472 average exposure values were 147 μg day^{-1} inhabitant^{-1}, 205 μg day^{-1} inhabitant^{-1}, and 11

473 μg day^{-1} inhabitant^{-1}, respectively.

474

4. CONCLUSIONS

476 A new SPE-UPLC®-MS/MS method has been developed for the determination of 21

477 plasticizer metabolites (among phthalate, terephthalate and DINCH metabolites) in

478 wastewater. This is the first time that terephthalate and DINCH metabolites are included

479 in a method for their determination in sewage. Analytes have been successfully extracted

480 from raw wastewater using the SPE sorbent Oasis MAX, which has provided a better

481 performance than Oasis HLB in the retention of polar and acidic compounds. The use of

482 a Raptor Biphenyl reversed-phase column with an appropriate mobile phase gradient has

483 been very effective in the separation of isomers and isobaric compounds sharing

484 transitions. The analysis of composite samples of 24 hours collected along one week in

485 Santiago de Compostela (Spain) has shown the ubiquity of LMW phthalate and DEHP

486 metabolites in wastewater, as well as provided the first quantification of terephthalate

487 metabolites in this matrix. The estimation of human exposure to the parent plasticizers

488 highlighted the high use of DEHTP when compared to its positional isomer DEHP, and

489 the high exposure to DEP. This is the first time that a terephthalate metabolite is detected

490 in wastewater, and also the first time that human exposure to the parent terephthalate is

491 estimated following the WBE principles. The performance of additional monitoring

492 campaigns along the year is needed to compare exposure values on different seasons and

493 properly assess human exposure. Moreover, the extension of the campaigns to different

494 locations will help to compare potential spatial variations. Finally, further toxicokinetic

495 studies are also recommended as data for some of the newest plasticisers is still very

496 limited.

497

498 **Supplementary Material**

499

500 **Declarations of interest: Authors declare they have no conflicts of**

501 **interest**

502

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Table 1. Chemical formula, retention time (RT), transitions (Q$_n$) used for quantification (Q$_1$) and confirmation (Q$_2$ and Q$_3$), ratio between these transitions, optimal collision energy (CE) and cone voltage (CV) values, and deuterated compounds used as surrogate or internal standards (IS).

	Compound	Chemical formulae	RT (min)	[M-H]⁻	CV (V)	Q$_1$ (m/z)	CE (eV)	Q$_2$ (m/z)	CE (eV)	Q$_2$/Q$_1$	Q$_3$ (m/z)	CE (eV)	Q$_3$/Q$_1$	IS
LOW MOLECULAR WEIGHT PHTHALATES	MMP	C$_9$H$_8$O$_4$	2.29	179	27	179>107	8	179>77	17	0.65	-	-	-	MMP-d$_4$
	MEP	C$_{10}$H$_{10}$O$_4$	2.95	193	22	193>77	15	193>121	10	0.6	-	-	-	MnBP-d$_4$
	MiBP	C$_{12}$H$_{14}$O$_4$	5.08	221	27	221>77	16	221>134	12	0.81	-	-	-	MnBP-d$_4$
	MnBP	C$_{12}$H$_{14}$O$_4$	5.35	221	23	221>77	17	221>177	9	0.4	-	-	-	MnBP-d$_4$
	MBzP	C$_{14}$H$_{10}$O$_4$	6.52	255	27	255>77	20	255>183	10	0.94	-	-	-	MnBP-d$_4$
	MCHP	C$_{14}$H$_{16}$O$_4$	6.96	247	40	247>97	14	247>77	18	0.9	247>147	15	0.27	MEOHTP-d$_4$
HIGH MOLECULAR WEIGHT PHTHALATES	MEHHP	C$_{16}$H$_{22}$O$_5$	5.64	293	32	293>145	13	293>121	20	0.9	-	-	-	MEHHP-d$_4$
	MEOHP	C$_{16}$H$_{20}$O$_5$	6.28	291	27	291>121	16	291>143	12	0.91	-	-	-	MEHHP-d$_4$
	MECPP	C$_{16}$H$_{20}$O$_6$	5.75	307	23	307>159	10	307>113	27	0.17	-	-	-	MEHHP-d$_4$
	MHINP	C$_{17}$H$_{24}$O$_5$	6.80	307	44	307>121	14	307>77	29	0.67	307>157	28	0.05	MEHHP-d$_4$
	MMOOP	C$_{17}$H$_{22}$O$_5$	7.54	305	43	305>121	25	305>157	13	0.48	305>77	32	0.45	MEHHP-d$_4$
	MMCHP	C$_{17}$H$_{22}$O$_6$	6.97	321	38	321>173	16	321>121	24	0.17	321>77	48	0.06	MEHHP-d$_4$
	MCINP	C$_{18}$H$_{24}$O$_6$	8.40	335	37	335>187	14	335>121	25	0.16	335>77	35	0.08	MEHHP-d$_4$
TERE-PHTHALATES	MMTP	C$_9$H$_8$O$_4$	3.12	179	35	179>135	11	179>120	20	0.13	179>76	27	0.10	MMTP-d$_4$
	MEHHTP	C$_{16}$H$_{22}$O$_5$	6.48	293	47	293>121	17	293>77	21	0.19	-	-	-	MEHHTP-d$_4$
	MEOHTP	C$_{16}$H$_{20}$O$_5$	7.46	291	47	291>121	17	291>77	25	0.23	291>165	14	0.04	MEOHTP-d$_4$
	MECPTP	C$_{16}$H$_{20}$O$_6$	6.97	307	41	307>165	14	307>121	25	0.34	307>77	35	0.11	MECPTP-d$_4$
DINCH	MINCH	C$_{17}$H$_{30}$O$_4$	12.2	297	35	297>153	14	297>109	26	0.33	297>81	29	0.03	MEHHP-d$_4$
	cx-MINCH	C$_{17}$H$_{28}$O$_6$	8.38	327	39	327>173	16	327>153	27	0.24	327>109	28	0.21	MEHHP-d$_4$
	OH-MINCH	C$_{17}$H$_{30}$O$_5$	8.19	313	42	313>153	17	313>109	30	0.32	313>125	31	0.02	MEHHP-d$_4$
	oxo-MINCH	C$_{17}$H$_{28}$O$_5$	8.95	311	40	311>153	15	311>109	32	0.26	311>81	22	0.01	MEHHP-d$_4$
DEUTERATED SPECIES	MMP-d$_4$	C$_9$H$_4$O$_4$D$_4$	2.29	183	27	183>111	8	-	-	-	-	-	-	-
	MnBP-d$_4$	C$_{12}$H$_{10}$O$_4$D$_4$	5.35	225	23	225>81	17	-	-	-	-	-	-	-
	MEHHP-d$_4$	C$_{16}$H$_{18}$O$_5$D$_4$	5.64	297	32	297>149	13	-	-	-	-	-	-	-
	MMTP-d$_4$	C$_9$H$_4$O$_4$D$_4$	3.12	183	35	183>139	11	-	-	-	-	-	-	-
	MEHHTP-d$_4$	C$_{16}$H$_{18}$O$_5$D$_4$	6.48	297	47	297>125	17	-	-	-	-	-	-	-
	MEOHTP-d$_4$	C$_{16}$H$_{16}$O$_5$D$_4$	7.46	295	47	295>125	17	-	-	-	-	-	-	-
	MECPTP-d$_4$	C$_{16}$H$_{16}$O$_6$D$_4$	6.97	311	41	311>169	14	-	-	-	-	-	-	-

Table 2. Method performance parameters: linearity, intra- and inter-day instrumental precision, instrumental quantification and detection limits (IQL and IDL), trueness, method precision, and method detection and quantification limits (MDL and MQL).

Compound	R^2 [a]	Instrumental precision [b]				IDL (ng mL⁻¹)	IQL (ng mL⁻¹)	Trueness and Precision (%R and %RSD) [c]		MDL (ng L⁻¹)	MQL (ng L⁻¹)
		Intra-day (%RSD)		Inter-day (%RSD)				Ultrapure water (50 ng L⁻¹)	Wastewater (500 ng L⁻¹)		
		10 ng mL⁻¹	100 ng mL⁻¹	10 ng mL⁻¹	100 ng mL⁻¹						
MMP	0.9985	10	8.3	8.3	15	0.12	0.39	110 (15)	104 (21)	0.17	0.56
MEP	0.9972	16	16	15	17	0.19	0.62	101 (21)	92 (15)	0.20	0.56
MiBP	0.9996	4.4	5.2	11	12	0.45	1.5	112 (6)	124 (2)	0.77	2.6
MnBP	0.9987	14	5.1	9.3	12	0.59	2.0	119 (8)	99 (4)	1.3	4.4
MBzP	0.9992	11	5.4	6.7	11	0.14	0.47	122 (18)	112 (4)	0.28	0.92
MCHP	0.9998	16	3.8	17	17	0.22	0.44	103 (8)	117 (6)	0.44	1.5
MEHHP	0.9993	16	5.2	9.0	16	0.13	0.44	108 (8)	107 (8)	0.40	1.1
MEOHP	0.9995	17	5.7	12	18	0.18	0.61	136 (11)	130 (14)	0.33	1.3
MECPP	0.9973	16	3.1	16	16	0.12	0.39	106 (23)	114 (8)	0.24	0.81
MHINP	0.9996	8.8	3.1	16	6.6	0.13	0.42	111 (11)	80 (13)	0.35	1.2
MMOOP	0.9923	17	4.1	16	14	0.15	0.49	109 (11)	97 (11)	0.37	1.2
MMCHP	0.9991	17	3.8	14	7.0	0.20	0.66	81 (10)	118 (10)	0.37	1.2
MCINP	0.9998	13	3.4	16	14	0.089	0.29	103 (9)	118 (7)	0.17	0.56
MMTP	0.9989	16	3.2	8.5	16	0.70	2.3	117 (9)	110 (12)	1.3	4.2
MEHHTP	0.9998	9.3	4.5	10	8.9	0.17	0.56	103 (9)	95 (3)	0.42	1.4
MEOHTP	0.9965	12	17	9.4	14	0.13	0.42	96 (4)	97 (6)	0.30	1.0
MECPTP	0.9994	13	6.4	17	15	0.086	0.29	101 (11)	95 (1)	0.20	0.66
MINCH	0.9995	14	15	11	17	0.041	0.14	85 (15)	74 (11)	0.12	0.41
cx-MINCH	0.9999	17	9.7	14	15	0.14	0.48	102 (21)	112 (8)	0.28	0.93
OH-MINCH	0.9993	5.3	4.1	11	13	0.010	0.032	111 (12)	85 (11)	0.024	0.079
oxo-MINCH	0.9999	12	6.7	11	15	0.10	0.32	109 (11)	84 (12)	0.25	0.83

[a] Determination coefficient for a 10-point calibration curve. Linear range: IQL–5,000 ng mL⁻¹ for MEP, IQL–1,000 ng mL⁻¹ for the remaining analytes

[b] Relative Standard Deviation (%RSD) for five injections of a standard over 24 hours (intra-day precision or repeatability) or over one month (inter-day precision)

[c] IS-corrected recovery (%R) from the nominal spiking value and %RSD from the average measured concentration (i.e. repeatability). Experiments performed in triplicate. Spiking level: 50 ng L⁻¹ of analytes + 200 ng L⁻¹ of IS (ultrapure water), 500 ng L⁻¹ of analytes + 200 ng L⁻¹ of IS (wastewater)

Table 3. Concentrations measured in composite raw wastewater samples collected over one week in May 2019

Compound	Average concentration ± SD (ng L^{-1})								Q$_2$/Q$_1$		Relative deviation (%)
	21-May	22-May	24-May	25-May	26-May	27-May	28-May	Average	Standard	Real Samples	
MMP	243 ± 65	297 ± 63	262 ± 89	253 ± 86	226 ± 31	435 ± 69	714 ± 18	347 ± 26	0.65	0.65	0
MEP	3345 ± 54	3690 ± 185	3427 ± 85	3255 ± 81	2656 ± 99	3576 ± 292	3430 ± 66	3340 ± 86	0.60	0.61	1.7
MiBP	268 ± 5	334 ± 33	267 ± 19	230 ± 9	223 ± 30	272 ± 3	256 ± 19	264 ± 12	0.81	0.83	2.5
MnBP	219 ± 10	260 ± 5	249 ± 17	295 ± 12	795 ± 62	241 ± 10	247 ± 21	329 ± 19	0.40	0.43	7.5
MBzP	19 ± 2	19.3 ± 0.6	23 ± 4	14.8 ± 0.9	19 ± 8	23 ± 3	22 ± 5	20 ± 2	0.94	0.92	2.1
MEHHP	19 ± 2	27 ± 2	25.2 ± 0.8	20 ± 1	18 ± 2	26 ± 1	24 ± 3	22.5 ± 0.8	0.90	0.78	13
MEOHP	17.0 ± 0.7	24 ± 1	19 ± 2	16 ± 2	14 ± 3	17 ± 2	16 ± 5	18 ± 1	0.91	0.86	5.5
MECPP	20 ± 3	23.8 ± 0.3	26 ± 2	20.1 ± 0.7	19 ± 2	24 ± 3	24 ± 3	22 ± 1	0.17	0.17	0
MMTP	230 ± 19	107 ± 5	65 ± 8	54 ± 8	47 ± 8	55 ± 10	47 ± 4	86 ± 5	0.13	0.13	0
MEHHTP	18 ± 3	21 ± 3	26 ± 9	19 ± 2	16 ± 2	16 ± 2	17 ± 1	19 ± 3	0.19	0.15	21
MEOHTP	13 ± 2	13.2 ± 0.9	13.7 ± 0.2	13 ± 2	13 ± 3	16 ± 2	17 ± 2	14 ± 1	0.23	0.20	13
MECPTP	125 ± 14	139 ± 7	169 ± 13	135 ± 8	137 ± 5	151 ± 7	152 ± 12	144 ± 4	0.34	0.31	8.8

Figure captions

Figure 1. Absolute recoveries (a) and matrix effects (expressed in percentage of recovery, (b)) observed when performing the SPE with Oasis HLB versus with Oasis MAX cartridges. For Oasis MAX, a single elution with 5 mL of 2% formic acid in MeOH was considered. Error bars represent the standard deviation. The star (*) above the bars means that the p-value of a Student´s t-test is lower than 0.05 (*), 0.01 (**), or 0.001 (***), implying the existence of statistically significant differences between both sorbents.

Figure 2. Stability of terephthalate metabolites and one or two metabolites representative of each family (MMP, MEHHP, MECPP, MCINP, MINCH and OH-MINCH) in wastewater (a) at room temperature, and (b) at 4 °C. Error bars represent the standard deviation.

Figure 3. Chromatogram with the quantifier (Q1) transition of all the analytes positively quantified in the wastewater sample of May 28th.

Figure 4. Individual levels of exposure to the target plasticizers estimated from the concentration of their metabolites in daily raw wastewater samples. Blue dots: average values. Red dots: values outside the 1.5 interquartile range from the box (expected outliers).

Figure 1

(a)

(b)
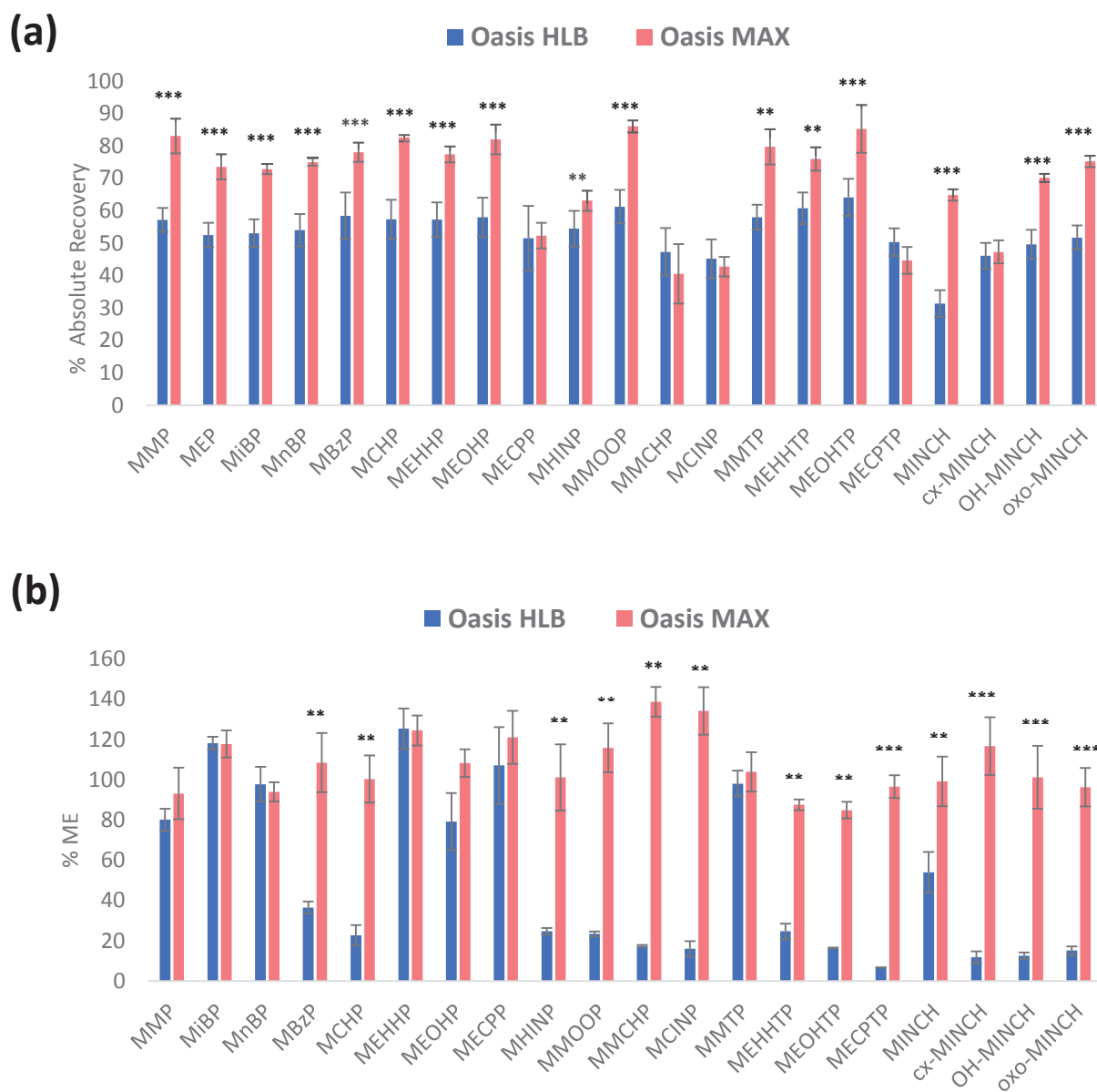
Figure 2

(a)
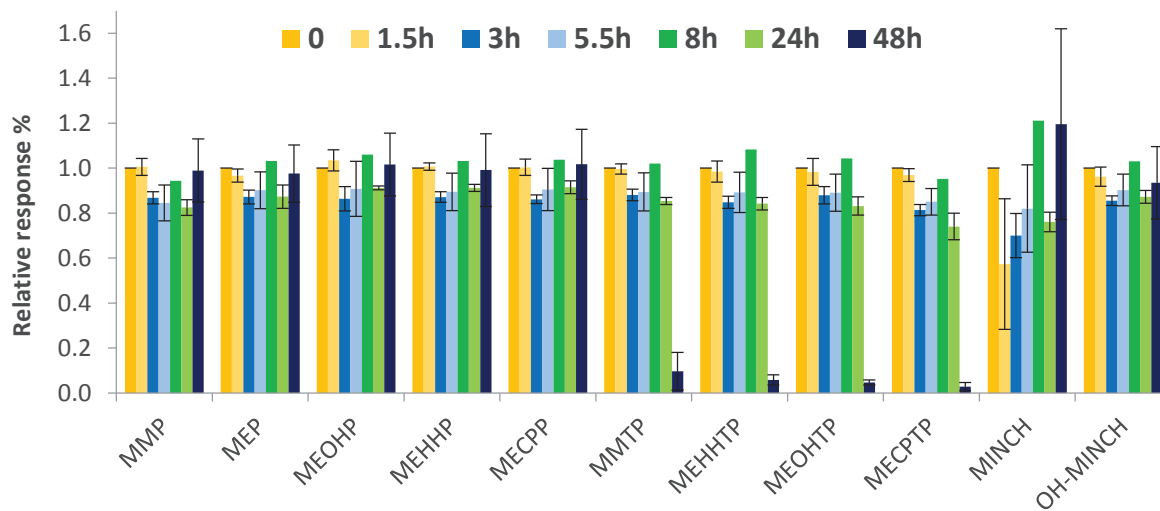
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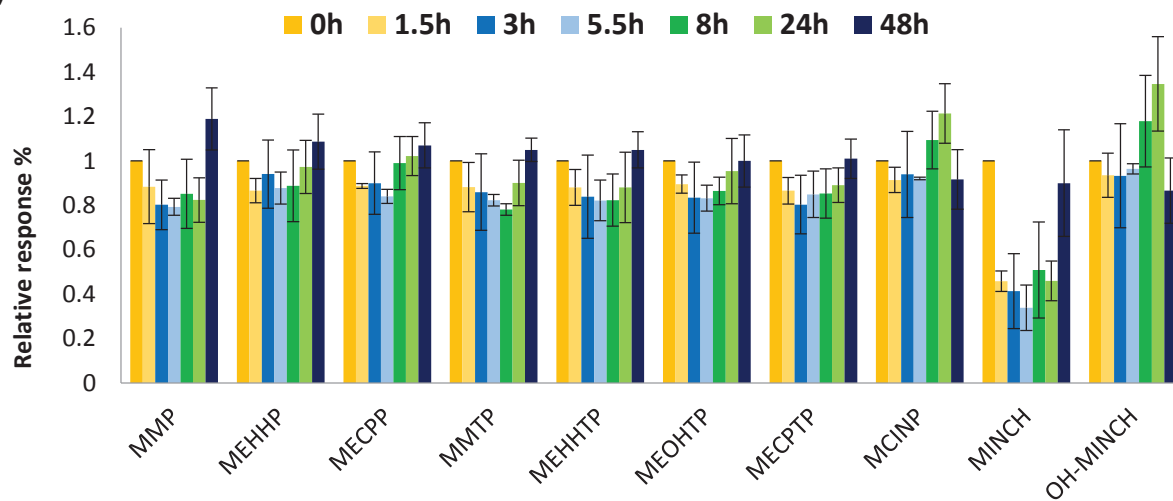
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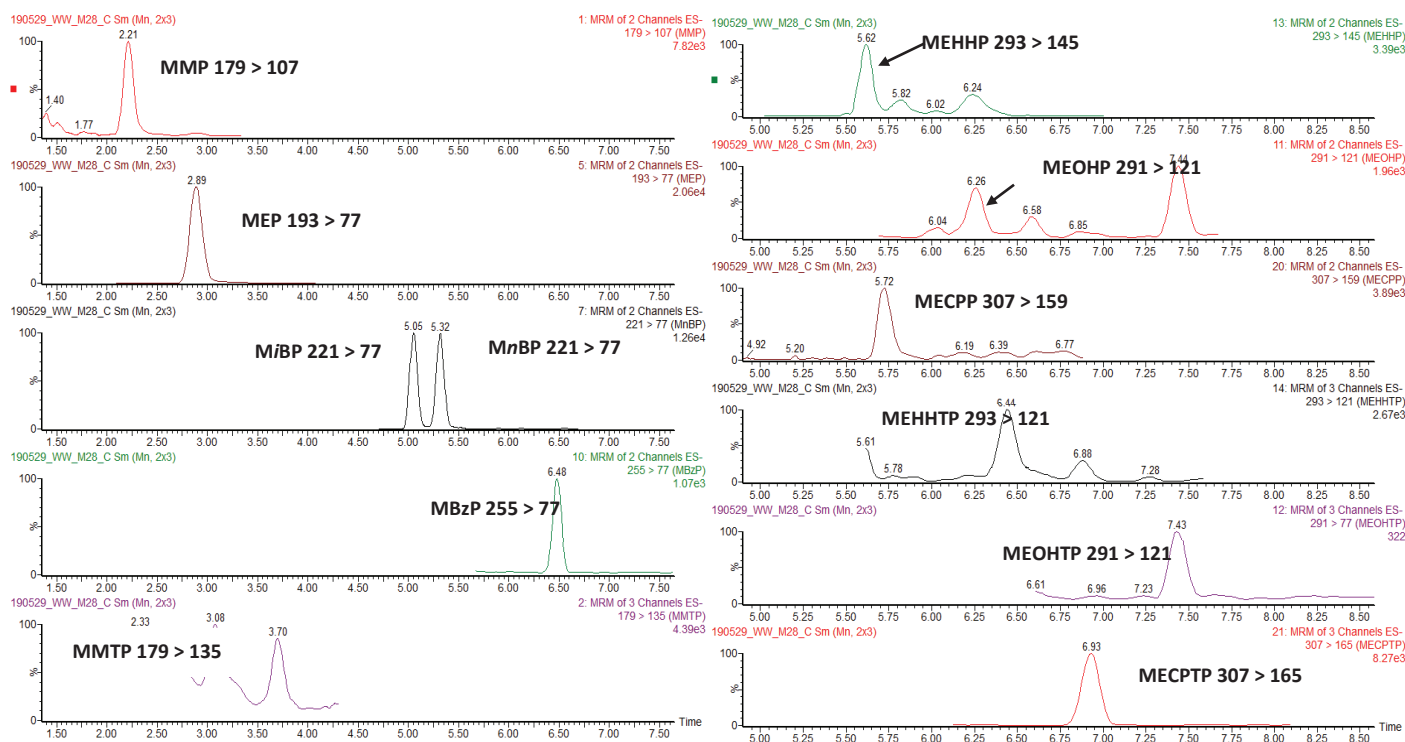
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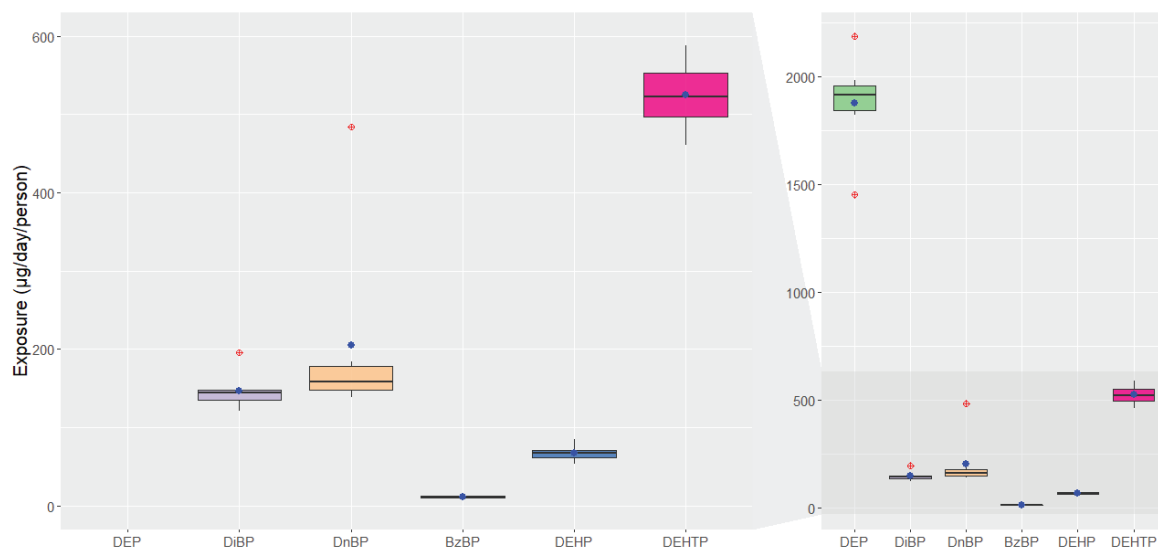
Supplementary Material

Comprehensive determination of phthalate, terephthalate and di-iso-nonyl cyclohexane-1,2-dicarboxylate metabolites in wastewater by solid-phase extraction and ultra(high)-performance liquid chromatography-tandem mass spectrometry

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Materials and methods

Table S1. Chemical structure of target phthalate and phthalate-substituent plasticizers, metabolites used as human exposure biomarkers, average percentage of excretion, and correction factors (CF) applied to convert metabolite concentrations in wastewater into levels of exposure to the parent plasticizers.

Table S2. Studies considered calculating participant-weight average excretion factors.

Table S3. Comparison of the performance of the proposed method with that of other multi-residue analytical methods for the determination of plasticizer metabolites in wastewater (whole method performance comparison) and urine (instrumental method performance comparison only).

Table S4. Daily exposure levels to phthalate and terephthalate plasticizers estimated from their metabolite concentrations in wastewater.

Figure S1. Product ion spectra at 20 eV of one representative analyte of the three families considered: phthalate, terephthalate, and DINCH metabolites.

Figure S2. Retention behaviour of all analytes on a Kinetex® Phenyl-Hexyl column using different gradients (a, b, c) and on a Raptor Biphenyl column (d).
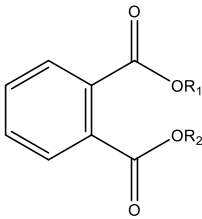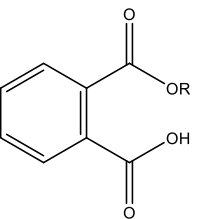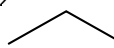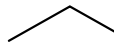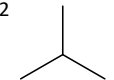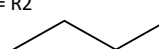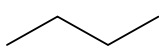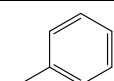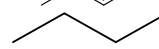
Figure S3. Separation between MHINP (left peak) and MECPTP (right peak) on the Raptor Biphenyl column using different gradients.

Figure S4. Chromatogram of a 500 ng mL^{-1} standard under final chromatographic conditions.
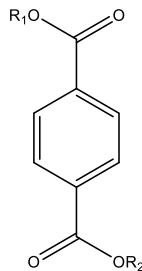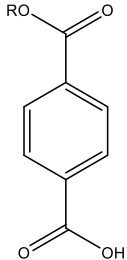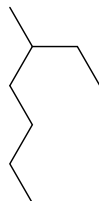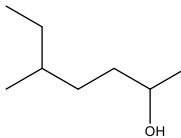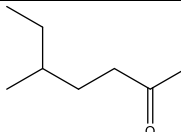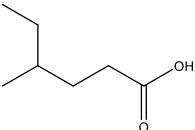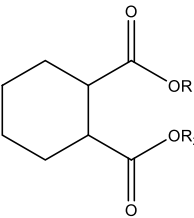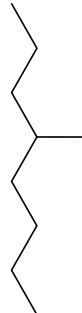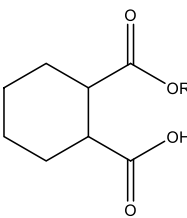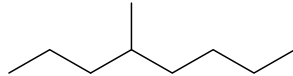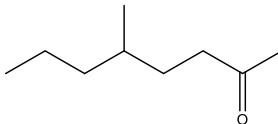
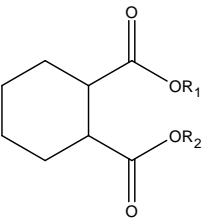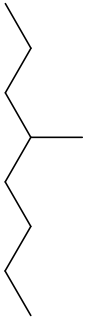**Figure S5**. SPE recovery with Oasis MAX cartridges in ultrapure water
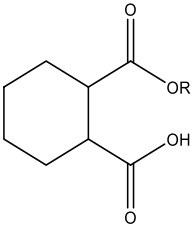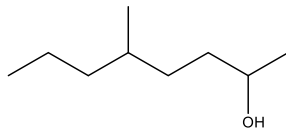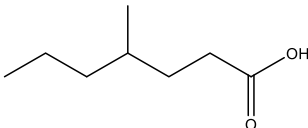
Figure S6. Percentage of molar formation of MMTP from its precursor plasticizer DMTP due to hydrolysis/degradation in wastewater at room temperature

Table S1. Chemical structure of target phthalate and phthalate-substituent plasticizers, metabolites used as human exposure biomarkers, average percentage of excretion, and correction factors (CF) applied to convert metabolite concentrations in wastewater into levels of exposure to the parent plasticizers.

Parent plasticizer	Chemical structure	R1 = R2 in all cases but BzBP	Metabolite	Chemical Structure	R	Average percentage of excretion (24 h)	CF
Dimethyl phthalate (DMP)		R1 = R2	Monomethyl phthalate (MMP)			69 [a]	1.55
Diethyl phthalate (DEP)		R1 = R2	Monoethyl phthalate (MEP)			69 [a]	1.65
Di-*iso*-butyl phthalate (D*i*BP)		R1 = R2	Mono-*iso*-butyl phthalate (MiBP)			71 [a]	1.76
Di-*n*-butyl phthalate (D*n*BP)		R1 = R2	Mono-*n*-butyl phthalate (MnBP)			63 [a]	1.8
Benzyl butyl phthalate (BzBP)		R1 / R2	Monobenzyl phthalate (MBzP)			73 [a]	1.68
Dicyclohexyl phthalate (DCHP)		R1 = R2	Monocyclohexyl phthalate (MCHP)			-	-

Parent plasticizer	Chemical structure	R1 = R2 in all cases but BzBP	Metabolite	Chemical Structure	R	Average percentage of excretion (24 h)	CF
Di-2-ethylhexyl phthalate (DEHP)		R1 = R2	Mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP)			16 [a]	8.40
			Mono-(2-ethyl-oxohexyl) phthalate (MEOHP)			11 [a]	11.8
			Mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP)			14 [a]	9.01
Di-iso-nonyl phthalate (DiNP)		R1 = R2 (mixture of positional isomers)	Mono-hydroxy-iso-nonyl phthalate (MHINP)			20.2 [b]	11.3
			Mono-(4-methyl-7-oxooctyl) phthalate (MMOOP)			10.6 [b]	20.1
			Mono-(4-methyl-7-carboxyheptyl) phthalate (MMCHP)			10.7 [b]	11.8
Di-iso-decyl phthalate (DiDP)		R1 = R2 (mixture of positional isomers)	Mono-carboxy-iso-nonyl phthalate (MCINP)			10.7 [b]	12.1

Parent plasticizer	Chemical structure	R1 = R2 in all cases but BzBP	Metabolite	Chemical Structure	R	Average percentage of excretion (24 h)	CF
Dimethyl terephthalate (DMTP)		R1 = R2	Monomethyl terephthalate (MMTP)			-	
Di-2-ethylhexyl terephthalate (DEHTP)		R1 = R2	Mono-(2-ethyl-5-hydroxyhexyl) terephthalate (MEHHTP)			1.8 [c]	73.7
			Mono-(2-ethyl-5-oxohexyl) terephthalate (MEOHTP)			1.0 [c]	134
			Mono-(2-ethyl-5-carboxypentyl) terephthalate (MECPTP)			13 [c]	9.74
Di-iso-nonyl cyclohexane-1,2-dicarboxylate (DINCH)		R1 = R2	Mono-(4-methyl-octyl) cyclohexane-1,2-dicarboxylate (MINCH)			0.65 [d]	206
			Mono-(7-carboxy-4-methyl-octyl) cyclohexane-1,2-dicarboxylate (cx-MINCH)			1.67 [d]	68.1

Parent plasticizer	Chemical structure	R1 = R2 in all cases but BzBP	Metabolite	Chemical Structure	R	Average percentage of excretion (24 h)	CF
			Mono-(4-methyl-7-hydroxy-octyl) cyclohexane-1,2-dicarboxylate (OH-MINCH)			9.55 [d]	13.8
			Mono-(4-methyl-7-oxo-octyl) cyclohexane-1,2-dicarboxylate (oxo-MINCH)			1.85 [d]	59.1

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[b] S.M. Hays, L.L. Aylward, C.R. Kirman, K. Krishnan, A. Nong, Biomonitoring Equivalents for di-isononyl phthalate (DINP), Regul. Toxicol. Pharmacol. 60 (2011) 181–188.

[c] F. Lessmann, A. Schütze, T. Weiss, A. Langsch, R. Otter, T. Brüning, H.M. Koch, Metabolism and urinary excretion kinetics of di(2-ethylhexyl) terephthalate (DEHTP) in three male volunteers after oral dosage, Arch. Toxicol. 90 (2016) 1659–1667.

[d] H.M. Koch, A. Schütze, C. Pälmke, J. Angerer, T. Brüning, Metabolism of the plasticizer and phthalate substitute diisononyl- cyclohexane-1,2-dicarboxylate (DINCH®) in humans after single oral doses, Arch. Toxicol. 87 (2013) 799–806.

Table S2. Studies considered to calculate participant-weight average excretion factors.

Parent plasticizer	Metabolite	Number of participants	Molar excretion percentage (in 24 h)	Reference
DMP	MMP	see reference	69	
DEP	MEP	see reference	69	
D*i*BP	M*i*BP	see reference	71	
D*n*BP	M*n*BP	see reference	63	González-Mariño et al., 2017 [a]
BzBP	MBzP	see reference	73	
	MEHHP	see reference	16	
DEHP	MEOHP	see reference	11	
	MECPP	see reference	14	
	MHINP	1	18	
D*i*NP	MMOOP	1	10	Koch et al., 2007 [b]
	MMCHP	1	9.1	
	MHINP	20	12	
D*i*NP	MMOOP	20	6.6	Anderson et al., 2011 [c]
	MMCHP	20	11	
D*i*DP	MCINP	21	11	Kransler et al., 2013 [d]
	MEHHTP	3	1.8	
DEHTP	MEOHTP	3	1.0	Lessmann et al., 2016 [e]
	MECPTP	3	13	
	MINCH	3	0.65	
DINCH	cx-MINCH	3	1.7	Koch et al., 2013 [f]
	OH-MINCH	3	9.6	
	oxo-MINCH	3	1.9	
	MINCH	3	0.72	
DINCH	cx-MINCH	3	2.0	Schütze et al., 2017 [g]
	OH-MINCH	3	10	
	oxo-MINCH	3	2.6	

[a] I. González-Mariño, R. Rodil, I. Barrio, R. Cela, J.B. Quintana, Wastewater-Based Epidemiology as a New Tool for Estimating Population Exposure to Phthalate Plasticizers, Environ. Sci. Technol. 51 (2017) 3902–3910.

[b] H.M. Koch, J. Angerer, Di-iso-nonylphthalate (DINP) metabolites in human urine after a single oral dose of deuterium-labelled DINP, Int. J. Hyg. Environ. Health. 210 (2007) 9–19.

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[d] K.M. Kransler, A.N. Bachman, R.H. McKee, Estimates of daily di-isodecyl phthalate (DIDP) intake calculated from urinary biomonitoring data, Regul. Toxicol. Pharmacol. 65 (2013) 29–33.

[e] F. Lessmann, A. Schütze, T. Weiss, A. Langsch, R. Otter, T. Brüning, H.M. Koch, Metabolism and urinary excretion kinetics of di(2-ethylhexyl) terephthalate (DEHTP) in three male volunteers after oral dosage, Arch. Toxicol. 90 (2016) 1659–1667.

[f] H.M. Koch, A. Schütze, C. Pälmke, J. Angerer, T. Brüning, Metabolism of the plasticizer and phthalate substitute diisononyl- cyclohexane-1,2-dicarboxylate (DINCH®) in humans after single oral doses, Arch. Toxicol. 87 (2013) 799–806.

[g] A. Schütze, R. Otter, H. Modick, A. Langsch, T. Brüning, H.M. Koch, Additional oxidized and alkyl chain breakdown metabolites of the plasticizer DINCH in urine after oral dosage to human volunteers, Arch. Toxicol. 91 (2017) 179–188.

Table S3. Comparison of the performance of the proposed method with that of other multi-residue analytical methods for the determination of plasticizer metabolites in wastewater (whole method performance comparison) and urine (instrumental method performance comparison only).

Reference	Target plasticizer metabolites	Sample preparation		Separation and detection	%R	IQL [a] (ng mL⁻¹)	MQL [a] (ng L⁻¹)
		Pretreatment	Extraction	LC-MS			
This study	(1) LMW phthalate metabolites: MMP, MEP, MBzP, M*n*BP, M*i*BP, MCHP (2) HMW phthalate metabolites: MEHHP, MEOHP, MECPP, MHINP, MMOOP, MMCHP, MCINP (3) Terephthalate metabolites: MMTP, MEHHTP, MEOHTP, MECPTP (4) DINCH metabolites: MINCH, OH-MINCH, oxo-MINCH, cx-MINCH	100 mL of wastewater Filtration through GF/A 0.7 µm filters + 0.45 µm cellulose filters Addition of IS	SPE on Oasis MAX 60 mg Filtration of the extract through 0.22 µm PVDF filters	UPLC-(ESI-)-MS/MS on QqQ (MRM) Raptor Biphenyl column (150 × 2.1 mm, 1.8 µm) Mobile phase: 0.1% acetic acid in water - 0.1% acetic acid in MeOH	(IS corrected %R) 74-136%	(1) 0.39-2.0 (2) 0.29-0.66 (3) 0.043-0.78 (4) 0.032-0.48	(1) 0.56-4.4 (2) 0.56-1.3 (3) 0.10-1.4 (4) 0.079-0.93
González-Mariño et al. 2017 [d]	(1) LMW phthalate metabolites: MMP, MEP, MBzP, M*n*BP, M*i*BP (2) HMW phthalate metabolites: MEHHP, MEOHP, MECPP	100 mL of wastewater Filtration through GF/A 1.6 µm filters + 0.45 µm cellulose filters Acidified to pH 2.0 with 37% HCl Addition of IS	SPE on Oasis HLB 60 mg	HPLC-(ESI-)-MS/MS on QqQ (MRM) Luna Pheny-Hexyl column (150 × 2 mm, 3 µm) Mobile phase: 0.1% acetic acid in water - 0.1% acetic acid in MeOH	(IS corrected %R) 76-100% (> 80% except MEP)	(1) 0.01-0.31 (2) 0.07-0.11	(1) 0.5-8.1 (2) 1.7-3.2
Du et al. 2018 [e]	(1) LMW phthalate metabolites: MMP, MEP, MBzP, M*n*BP, M*i*BP (2) HMW phthalate metabolites: MEHHP	50 mL of wastewater Filtration through GF/A 1.6 µm filters Acidified to pH 2.0 with 37% HCl Addition of IS	SPE on Oasis HLB 60 mg Filtration of the extract through 0.2 µm centrifugal filters	UFLC[b]-(ESI-)-MS/MS on QqQ (MRM) Phenomenex Gemini C18 column (100 × 2 mm, 3 µm) Mobile phase: 0.1% acetic acid in water - MeOH	(IS corrected %R) 100-105%	-	(1) 5.0-10 (2) 1.0

Tang et al. 2020 [f]	(1) LMW phthalates metabolites: MMP, MEP, MBzP, MnBP, MiBP (2) HMW phthalates metabolites: MEHHP, MEOHP, MECPP	100 mL of wastewater Filtration through GF/A 47 mm filters + cellulose filters Acidified to pH 2.0 with 37% HCl Addition of IS	SPE on Oasis HLB 60 mg	UPLC-(ESI-)-MS/MS on QTRAP[c] (MRM) Kinetex F5 column Mobile phase: 0.1% acetic acid in 99-1 water-MeOH - 0.1% acetic acid in 5-95 water-MeOH	(IS corrected %R) 64-98%	-	(1) 3.2-240 (2) 4.4-1,900
Servaes et al. 2013 [g]	(1) LMW phthalate metabolites: MEP, MBzP, MnBP, MiBP (2) HMW phthalate metabolites: MEHHP, MEOHP	1 mL of urine Addition of amonium acetate buffer (pH 6.5) Addition of IS Incubation	SPE on Oasis HLB 200 mg	UPLC-(ESI-)-MS/MS on QqQ (MRM) Acquity UPLC BEH phenyl column (100 × 2.1 mm, 1.7 µm) Mobile phase: water - acetonitrile	-	(1) 0.20-0.50 (2) 0.10	-
Been et al. 2019 [h]	(3) Terephthalate metabolites: MEOHTP (4) DINCH metabolites: MINCH, OH-MINCH, cx-MINCH	1 mL of urine Addition of phosphate buffer (pH 6) Addition of IS Incubation	SPE on Oasis HLB 60 mg	HPLC-(ESI-)-MS/MS on QqQ (MRM) Phenomenex Kinetex Biphenyl column (100 × 2.1 mm, 2.6 µm) Mobile phase: 0.05% formic acid in water - 0.05% formic acid in acetonitrile	-	(3) 0.10 (4) 0.05-0.30	-

[a] IQL and MQL ranges for: (1) LMW phthalate metabolites; (2) HMW phthalate metabolites; (3) Terephthalate metabolites; (4) DINCH metabolites

[b] Ultra-fast liquid chromatography

[c] Hybrid quadrupole / ion trap

[d] I. González-Mariño, R. Rodil, I. Barrio, R. Cela, J.B. Quintana, Wastewater-Based Epidemiology as a New Tool for Estimating Population Exposure to Phthalate Plasticizers, Environ. Sci. Technol. 51 (2017) 3902–3910.

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[f] S. Tang, C. He, P. Thai, S. Vijayasarathy, R. Mackie, L.M.L. Toms, K. Thompson, P. Hobson, B. Tscharke, J.W. O'Brien, J.F. Mueller, Concentrations of phthalate metabolites in Australian urine samples and their contribution to the per capita loads in wastewater, Environ. Int. 137 (2020).
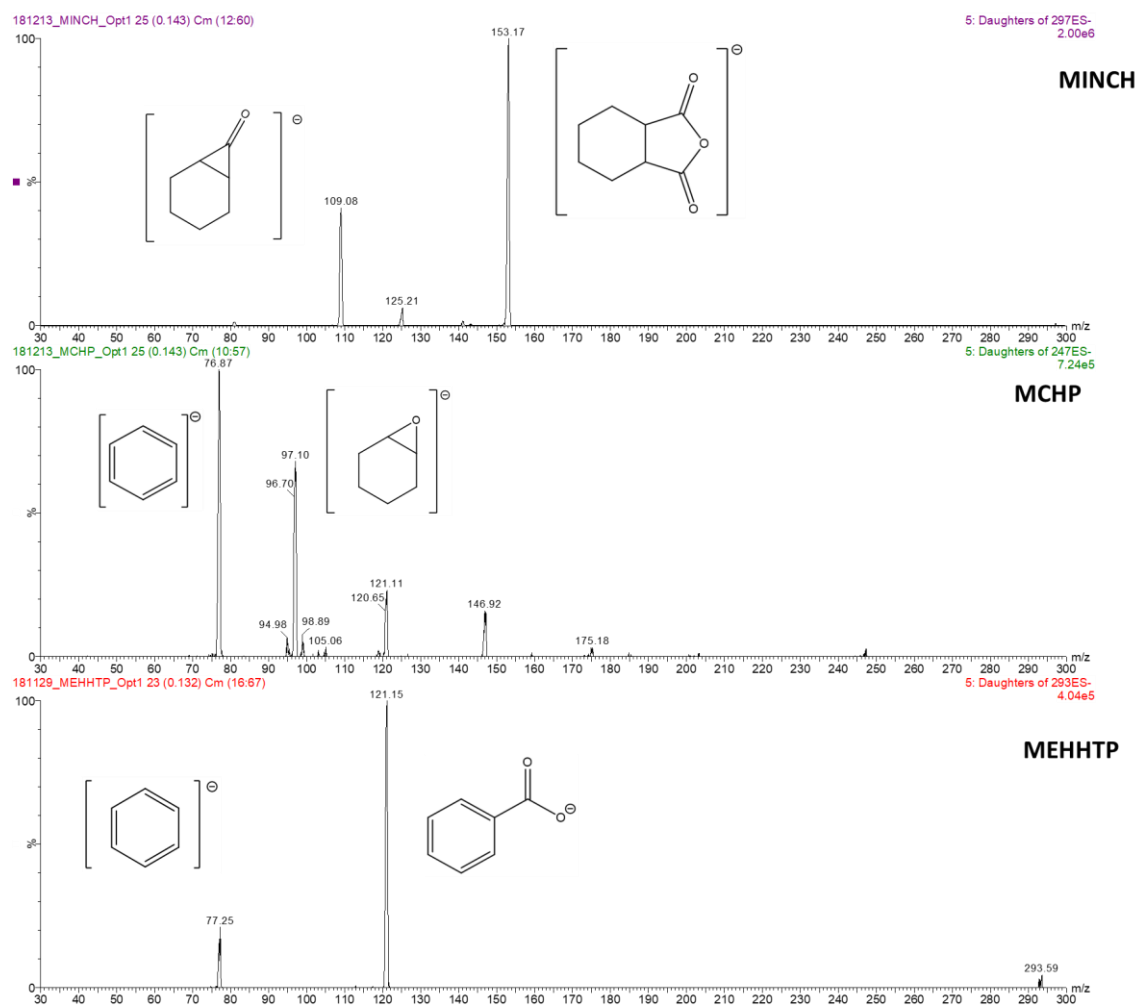
[g] K. Servaes, S. Voorspoels, J. Lievens, B. Noten, K. Allaerts, H. Van De Weghe, G. Vanermen, Direct analysis of phthalate ester biomarkers in urine without preconcentration: Method validation and monitoring, J. Chromatogr. A. 1294 (2013) 25–32.

[h] F. Been, G. Malarvannan, M. Bastiaensen, S. Yin, A.L.N. van Nuijs, A. Covaci, Development and validation of a bioanalytical assay based on liquid chromatography-tandem mass spectrometry for measuring biomarkers of exposure of alternative plasticizers in human urine and serum, Talanta. 198 (2019) 230–236. https://doi.org/10.1016/j.talanta.2019.02.024.
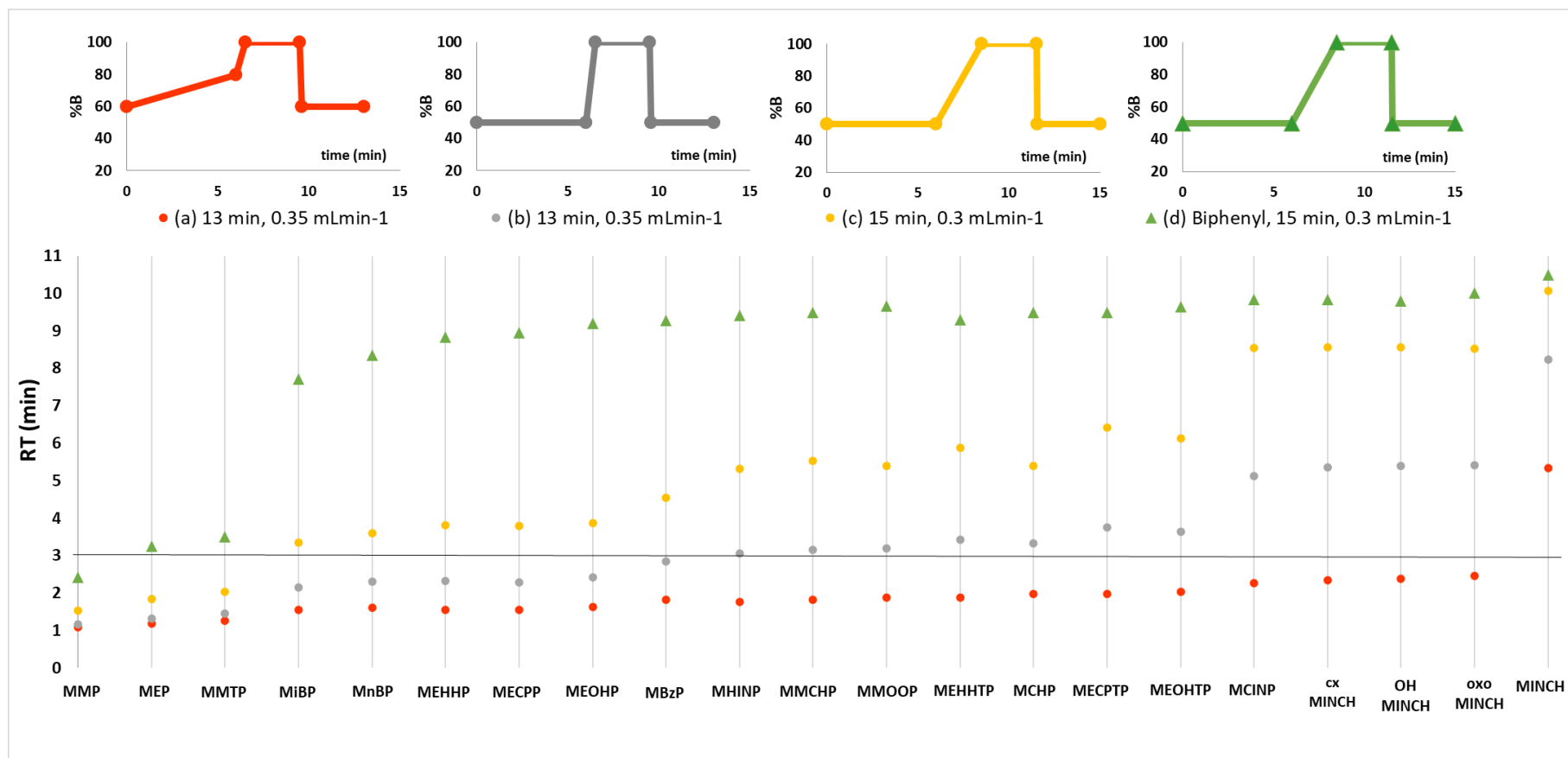
Table S4. Daily exposure levels to phthalate and terephthalate plasticizers estimated from their metabolite concentrations in wastewater.

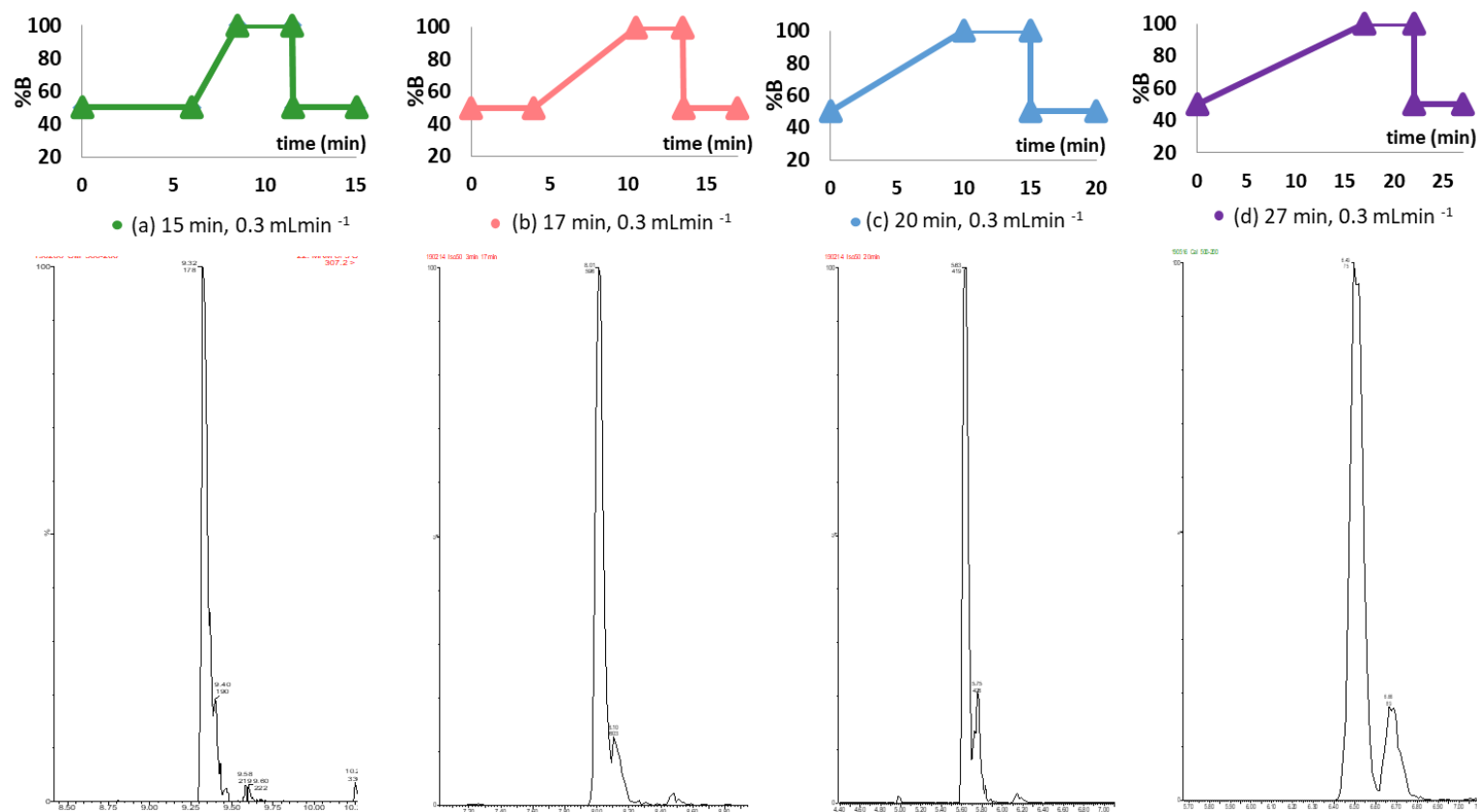| Parent plasticizer	Metabolite	Average exposure load ± SD (ug day^{-1}inh^{-1})							
		21-May	22-May	24-May	25-May	26-May	27-May	28-May	Average
DMP	MMP	131 ± 35	166 ± 35	134 ± 46	133 ± 45	117 ± 16	222 ± 35	389 ± 10	185 ± 14
DEP	MEP	1917 ± 31	2190 ± 112	1864 ± 46	1822 ± 45	1453 ± 54	1933 ± 158	1984 ± 38	1880 ± 47
DiBP	MiBP	151 ± 3	196 ± 19	143 ± 10	127 ± 5	121 ± 16	145 ± 2	146 ± 11	147 ± 7
DnBP	MnBP	139 ± 6	172 ± 3	151 ± 10	184 ± 8	484 ± 38	145 ± 6	159 ± 13	205 ± 12
BzBP	MBzP	11 ± 1	11.5 ± 0.4	13 ± 2	8.3 ± 0.5	11 ± 4	13 ± 2	13 ± 3	11 ± 1
DEHP	MEHHP	54 ± 5	80 ± 7	70 ± 2	55 ± 4	51 ± 7	70 ± 4	69 ± 9	64 ± 2
	MEOHP	69 ± 3	100 ± 5	74 ± 8	65 ± 8	56 ± 12	65 ± 9	65 ± 21	71 ± 6
	MECPP	63 ± 11	76.8 ± 0.9	76 ± 7	61 ± 2	55 ± 7	69 ± 8	75 ± 9	68 ± 3
	Average	62 ± 4	85 ± 3	73 ± 3	61 ± 3	54 ± 3	68 ± 3	70 ± 7	68 ± 2
DEHTP	MEHHTP	453 ± 77	551 ± 67	505 ± 81	473 ± 59	381 ± 46	395 ± 50	445 ± 33	458 ± 17
	MEOHTP	603 ± 71	633 ± 41	600 ± 8	583 ± 107	561 ± 142	692 ± 93	802 ± 101	639 ± 45
	MECPTP	422 ± 47	485 ± 23	541 ± 42	444 ± 27	440 ± 16	480 ± 23	518 ± 42	476 ± 12
	Average	493 ± 16	556 ± 22	549 ± 36	500 ± 40	461 ± 66	523 ± 35	588 ± 37	524 ± 16

Figure S1. Product ion spectra at 20 eV of one representative analyte of the three families considered: phthalate, terephthalate, and DINCH metabolites.
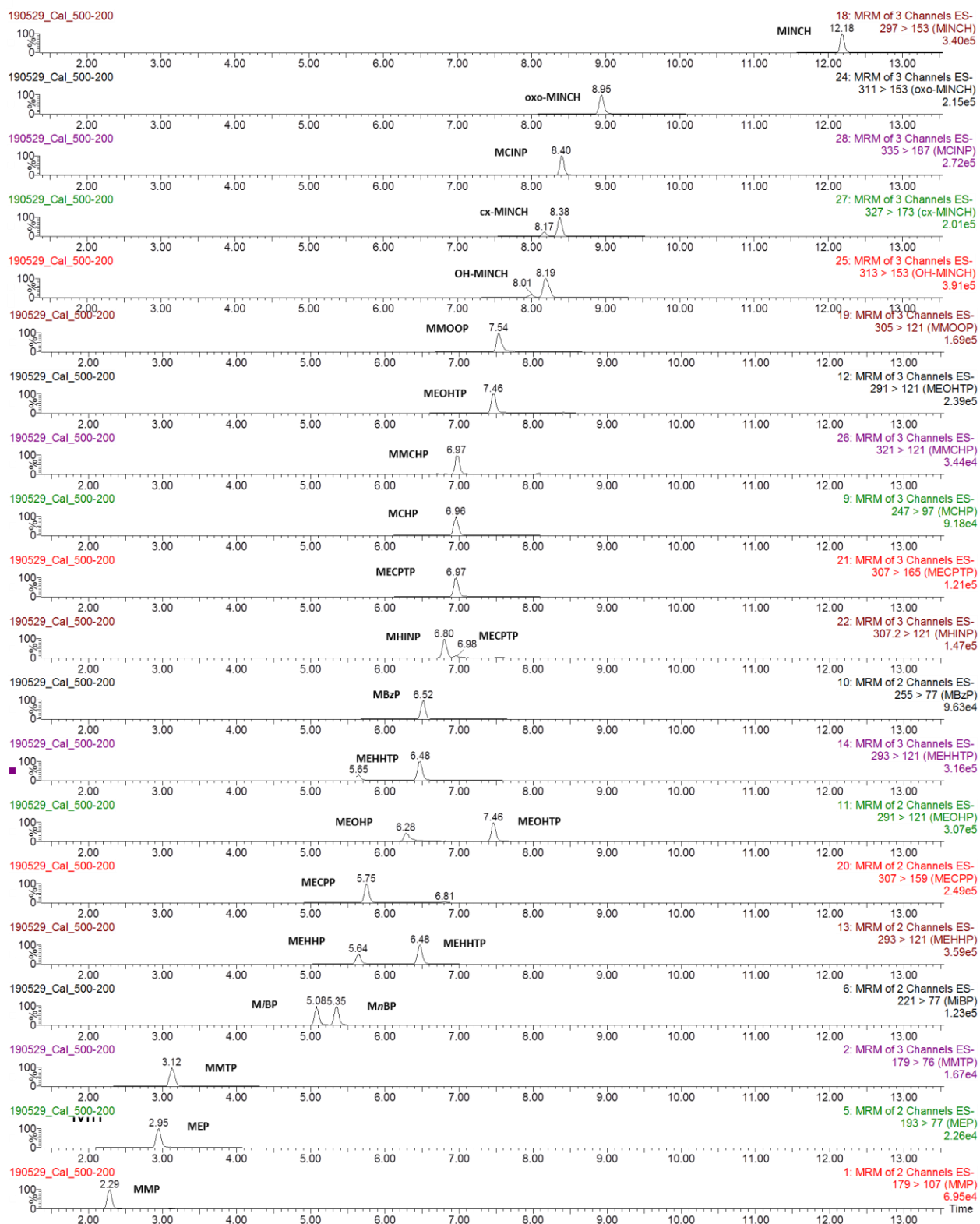
Figure S2. Retention behaviour of all analytes on a Kinetex® Phenyl-Hexyl column using different gradients (a, b, c) and on a Raptor Biphenyl column (d).
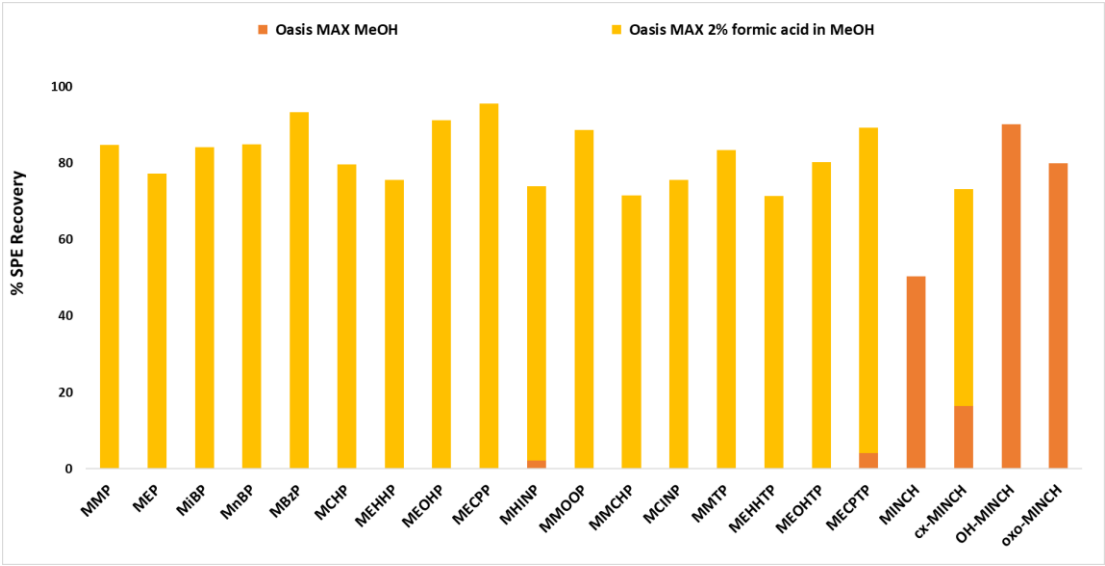
Figure S3. Separation between MHINP (left peak) and MECPTP (right peak) on the Raptor Biphenyl column using different gradients.

(a) 15 min, 0.3 mLmin^{-1} (b) 17 min, 0.3 mLmin^{-1} (c) 20 min, 0.3 mLmin^{-1} (d) 27 min, 0.3 mLmin^{-1}

Figure S4. Chromatogram of a 500 ng mL^{-1} standard under final chromatographic conditions.

Figure S5. SPE recovery with Oasis MAX cartridges in ultrapure water

Figure S6. Percentage of molar formation of MMTP from its precursor plasticizer DMTP due to hydrolysis/degradation in wastewater at room temperature.