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**Determination of phthalate diesters and monoesters in human milk and infant formula by fat extraction, size-exclusion chromatography clean-up and gas chromatography-mass spectrometry detection**

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## UNIVERSITÀ DEGLI STUDI DI TORINO

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1       **Determination of phthalate diesters and monoesters in human milk and**  
2       **infant formula by fat extraction, size-exclusion chromatography clean-up**  
3       **and gas chromatography-mass spectrometry detection**

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25 **Abstract**

26 A sensitive and reliable analytical method was developed for the simultaneous determination of five phthalate  
27 diesters and corresponding monoesters in human milk samples and infant formulas. The method involved a liquid-  
28 liquid extraction with a CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH/NaCl 30% 2/1/0.5 (v/v/v) mixture, the clean-up of the extract by size-  
29 exclusion chromatography (swelling and elution solvent: cyclohexane/ethyl acetate 9/1 v/v), the derivatization of  
30 monoesters by trimethylsilyl-diazomethane and instrumental analysis by gas chromatography coupled with mass  
31 spectrometry. Recovery was in the range of 83-115% and precision was found between 9% and 21%. For phthalate  
32 diesters, method detection limits (MDLs) ranged from hundreds of ng/kg to 4.2 µg/kg on a fresh weight milk (f.w.)  
33 basis, depending on blank contribution evaluated in matrix. Lower MDLs (0.03-0.8 µg/kg f.w.) were achieved for  
34 corresponding monoesters. The proposed method was applied to the determination of target compounds in nine  
35 human milk samples and four infant formulas, confirming their presence in all samples. However, a generally higher  
36 contamination was assessed in artificial milk than in breast milk samples.

37

38 *Keywords:* Phthalate diesters; Phthalate monoesters; Size exclusion chromatography; Gas chromatography-mass  
39 spectrometry; Human milk; Infant formula

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## 52 **1 Introduction**

53 Diesters of 1,2-phthalic acid, commonly known as phthalates, are a group of industrial chemicals  
54 mainly employed as plasticizers in the production of polyvinylchloride (PVC) and, to a minor  
55 extent, in the synthesis of other polymers [1]. Phthalates are also employed in the manufacture  
56 of countless and various materials [2], including personal care products and medical devices [3].  
57 Over recent years, phthalates have been one of the most widely manufactured organic compound  
58 classes in the world, since their annual production is about 5 million tons [4]. As a consequence,  
59 such compounds have been found in atmospheric, terrestrial, and aquatic environments of  
60 anthropized regions [5], as well as repeatedly detected in various compartments of remote areas  
61 [6, 7]

62 Because of the diffused presence of phthalates in the environment and their frequent use in the  
63 above-mentioned products, humans are potentially exposed through inhalation, ingestion and  
64 dermal contact throughout their life. Phthalate contamination in humans has also been found as  
65 a consequence of drug administration [8]. When phthalates enter the organism they are  
66 hydrolysed into the corresponding monoesters and then further oxidized through complex  
67 pathways [9]. Even though it is not clear which molecules, among parent compounds and the  
68 various metabolites, are more toxic, several studies have highlighted endocrine disruption  
69 properties of phthalates in humans, pointing out an association between phthalate exposure and  
70 detrimental effects on sexual characters [10, 11].

71 The endocrine disrupting properties of phthalates, together with their ubiquitous presence in the  
72 environment, make the determination of both parent compounds and metabolites of paramount  
73 importance in human milk and infant formula, as they represent the unique nourishment for  
74 newborns in a crucial developmental period of their life. The determination of phthalate diesters  
75 and corresponding metabolites in food intended for infants should comply with toxicological

76 evaluations that assess, for instance, tolerable daily intakes in adults of 10 µg/kg b.w. for di-n-  
77 butyl phthalate [12]. In this regard, the omnipresence of phthalate diesters may give rise to blank  
78 contributions that strongly affect the actual sensitivity of the analytical method, making difficult  
79 the quantification of these analytes in human milk at ppb and especially sub-ppb levels.

80 In spite of their toxicological importance, phthalates and their metabolites have been  
81 investigated in human milk only to a limited extent. More in detail, two researches, which  
82 considered breast milk samples collected from Canadian [13] and German [14] women,  
83 monitored only phthalate diesters. Conversely, most of the published studies, performed in  
84 various regions of the planet (i.e. North-America, North and South Europe, East Asia), focused  
85 on the determination of phthalate monoesters [15-19], sometimes including also other polar  
86 metabolites [16, 17]. In this respect, it should be noted that metabolites originating from  
87 phthalate monoesters were sporadically detected in human milk samples and found in any case  
88 at concentrations much lower than their precursors [16, 17]. Surprisingly, the monitoring of a  
89 wide group of both phthalate diesters and monoesters was performed only in two studies,  
90 focusing on Swedish [20] and German [21] breast milk samples, whereas a recent research  
91 carried out in Italy was restricted to di-2-ethylhexyl phthalate and its corresponding monoester  
92 [22].

93 As far as infant formula is concerned, the monitoring of phthalate contamination focused mainly  
94 on the phthalate diesters [21, 23-26], even though monoesters have been sporadically analysed,  
95 as well [19, 21].

96 Human milk is a very complex matrix, due to the high content of lipids, mostly represented by  
97 esters of fatty acids, which exhibit physicochemical properties similar to target analytes.  
98 Accordingly, the analysis of phthalates in milk represents a great analytical challenge. A number  
99 of different analytical approaches have been adopted for the analysis of phthalates diesters

100 and/or monoesters in milk samples. In this regard, it is worth mentioning that, when both  
101 phthalate diesters and monoesters were analysed, two completely different analytical approaches  
102 have been proposed.

103 These protocols mainly include liquid-liquid extraction (LLE) [14, 18-26] and less frequently  
104 headspace solid-phase micro-extraction (HS-SPME) [27], QuEChERS extraction [28], solid-  
105 phase extraction (SPE) [15] and automated on-line SPE [16, 17].

106 LLE followed by purification of the organic extract by using various clean-up approaches has  
107 been the most widely adopted extraction technique and, even recently, often applied to the  
108 analysis of phthalates in human milk and infant formula. In some cases [22, 23, 26], several  
109 manual analytical steps were necessary for analyte extraction and extract purification (e.g.  
110 evaporation to dryness and successive reconstitution in a proper solvent or back-extraction  
111 processes). With these analytical procedures, blank values at ppb levels or higher were found  
112 for some phthalate diesters (e.g. di-n-butyl phthalate and di-2-ethylhexyl phthalate), thus  
113 limiting the method sensitivity. Easier LLE procedures were achieved by adopting more  
114 straightforward extraction protocols and using automated or automatable SPE clean-up  
115 strategies [14, 18, 19, 21, 24, 25]. However, background contaminations by phthalate diesters in  
116 procedural blanks were found in the ppb-level, as well [14, 25]. Size-exclusion chromatography  
117 (SEC) was also employed as clean-up strategy, after LLE of milk samples [20] and infant  
118 formula [29]. In this regard, it should be noted that SEC has been suggested as one of the elective  
119 purification strategies of fatty matrix, such as human milk, in a quite recent technical report  
120 published by the European Community [30]. With this analytical approach blank contributions  
121 between a few ppb and 110 ppb were reported, depending on the study and the compound  
122 investigated.

123 The applicability of the HS-SPME technique to the analysis of phthalate diesters in cow milk  
124 was investigated by Feng and co-workers [27], who reported the need of long extraction times  
125 (at least 60 min at 90°C) and detection limits varying from sub-ppb to ppb levels depending on  
126 the fat content of milk samples. In this regard, it should be underlined that lipids followed the  
127 same fate of phthalates during the enrichment process of the SPME fibre, thus giving rise to the  
128 presence of a great number of interfering peaks in the gas chromatogram.

129 The application of the QuEChERS extraction and clean-up method on the determination of  
130 phthalate diesters in bovine milk seemed to achieve lower background contaminations, since  
131 detection limits in the sub-ppb levels were reported [28].

132 Contrary to what generally reported for phthalate diesters, no significant background  
133 contamination was observed for the analysis of phthalate monoesters in both human milk and  
134 infant formula, irrespective of the overall analytical strategy employed for their determination  
135 [15-17, 19]. For the analysis of monoesters in human milk, detection limits in the sub-ppb range  
136 were achieved for most phthalate metabolites using SPE on a N-vinylpyrrolidone-  
137 divinylbenzene co-polymeric sorbent as extraction and clean-up strategy, and LC-MS/MS for  
138 the instrumental quantification [15]. On-line SPE-LC-MS/MS was also applied to the analysis  
139 of phthalate metabolites in human milk, obtaining sensitivities similar to those achieved by the  
140 off-line approach; however, it should be noted that the method adopted was developed for urine  
141 and no validation was performed on milk samples [16, 17].

142 Data concerning the levels of phthalate diesters and their metabolites in milk are necessary for  
143 assessing their potential impact on nursing mothers and their children. Accordingly, the main  
144 purpose of this study was to develop and validate an extraction and clean-up protocol for the  
145 simultaneous determination of both phthalate diesters and monoesters in human milk. The  
146 proposed method involved LLE of total fats and phthalates and their separation by SEC. In this



147 regard, it should be remarked that phthalate diesters are hydrophobic compounds strongly  
148 partitioned in the lipid phase of milk [24] and therefore the extraction of total fats from milk  
149 samples is a recommendable procedure for their reliable analysis. Using this method, we  
150 performed for the first time the monitoring of target analytes in various milk samples collected  
151 from Tuscan donors of the Human Milk Bank of the Florence Children's Hospital. Furthermore,  
152 some infant formula widely commercialized in Italy were analysed, in order to compare the  
153 exposure to phthalates due to artificial milk consumption with that associated to breastfeeding.

## 154 **2 Experimental**

### 155 *2.1 Standards, solvents and materials*

156 Analytical standards, dimethyl phthalate (DMP), diethyl phthalate (DEP), di-n-propyl phthalate  
157 (DPP), di-isopropyl phthalate (DiPP), di-n-butyl phthalate (DBP), di-isobutyl phthalate (DiBP),  
158 di-n-pentyl phthalate (DPeP), di-n-hexyl phthalate (DHP), di-n-heptyl phthalate (DHepP),  
159 benzyl-butyl phthalate (BzBP), di-n-octyl phthalate (DOP), di-2-ethyl-hexyl phthalate (DEHP),  
160 di-isononyl phthalate (DiNP), di-n-nonyl phthalate (DNP), di-n-decyl phthalate (DDP), di-n-  
161 undecyl phthalate (DUP) and di-n-dodecyl phthalate (DDoP) were supplied by Sigma Aldrich  
162 (Milwaukee, IW, U.S.A.). Mono-ethyl phthalate (MEP), mono-n-butyl phthalate (MBP), mono-  
163 iso-butyl phthalate (MiBP), mono-2-ethylhexyl phthalate (MEHP), mono-benzyl phthalate  
164 (MBzP) and mono-iso-nonyl phthalate (MiNP) were purchased from Cambridge Isotope  
165 Laboratories, Inc. (Andover, MA, U.S.A.).

166 Labelled phthalate diesters and monoesters were obtained as following specified. Diethyl  
167 phthalate (ring-1,2,3,4-d<sub>4</sub>) (DEP-d<sub>4</sub>), di-n-butyl phthalate (ring-1,2,3,4-d<sub>4</sub>) (DBP-d<sub>4</sub>), benzyl  
168 butyl phthalate (ring-1,2,3,4-d<sub>4</sub>) (BzBP-d<sub>4</sub>) and di-2-ethyl-hexyl phthalate (ring-1,2,3,4-d<sub>4</sub>)  
169 (DEHP-d<sub>4</sub>) were purchased from Cambridge Isotope Laboratories, Inc., whereas di-isobutyl

170 phthalate (ring-1,2,3,4-d<sub>4</sub>) (DiBP-d<sub>4</sub>) and di-isononyl phthalate (ring-1,2,3,4-d<sub>4</sub>) (DiNP-d<sub>4</sub>)  
171 were obtained from Toronto Research Chemicals (Toronto, Canada). Mono-ethyl phthalate  
172 (ring-1,2-<sup>13</sup>C<sub>2</sub>, dicarboxyl-<sup>13</sup>C<sub>2</sub>) (MEP-C<sub>4</sub>), mono-n-butyl phthalate (ring-1,2-<sup>13</sup>C<sub>2</sub>, dicarboxyl-  
173 <sup>13</sup>C<sub>2</sub>) (MBP-C<sub>4</sub>), mono-benzyl phthalate (ring-1,2-<sup>13</sup>C<sub>2</sub>, dicarboxyl-<sup>13</sup>C<sub>2</sub>) (MBzP-C<sub>4</sub>), mono-2-  
174 ethylhexyl phthalate (ring-1,2-<sup>13</sup>C<sub>2</sub>, dicarboxyl-<sup>13</sup>C<sub>2</sub>) (MEHP-C<sub>4</sub>) and mono-isononyl phthalate  
175 (ring-1,2-<sup>13</sup>C<sub>2</sub>, dicarboxyl-<sup>13</sup>C<sub>2</sub>) (MiNP-C<sub>4</sub>) were obtained from Cambridge Isotope  
176 Laboratories, Inc. (Andover, MA, U.S.A.). Mono-isobutyl phthalate (ring-1,2,3,4-d<sub>4</sub>) (MiBP-  
177 d<sub>4</sub>) was supplied by Toronto Research Chemicals. Note that all methyl esters derivatives of  
178 phthalate monoesters are indicated throughout the manuscript with the aforementioned  
179 compound abbreviation followed by “Me”.

180 Standard solutions were prepared in methanol for spiking experiments and in cyclohexane for  
181 external calibration curves.

182 Dichloromethane, ethyl acetate, methanol, n-hexane and 2-propanol (Ultra Resi-Analysed  
183 grade), cyclohexane and water (HPLC grade) were purchased from J.T. Baker (Avantor Ltd,  
184 Center Valley, PA, U.S.A.).

185 Phosphoric acid 85% and Florisil<sup>®</sup> were supplied by Merck (Darmstadt, Germany).

186 N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS), used  
187 for the conversion of phthalate monoesters into the corresponding trimethylsilyl (TMS)  
188 derivatives was purchased from Alltech (Deerfield, IL, U.S.A.). Trimethylsilyl-diazomethane  
189 (TMSDM), 2 M in diethyl ether, used for the conversion of phthalate monoesters into the  
190 corresponding methyl ester derivatives, was purchased from Sigma Aldrich.

191 The resins Bio-Beads<sup>®</sup> S-X3 and S-X8 (Bio-Rad, Hercules, CA, U.S.A.) used for SEC were  
192 styrene-divinylbenzene copolymers with 3% and 8% cross-linkages, respectively.

193 Sodium chloride (analysis grade) and anhydrous sodium sulphate (organic trace analysis) were  
194 purchased from Merck.

195 Glass fibre filters with nominal porosity of 0.45 $\mu$ m were obtained from Whatman (Springfield-  
196 Mill, Kent, UK).

197 Activated acid aluminium oxide was supplied by Sigma-Aldrich.

## 198 2.2 *Precautions for minimizing phthalate background contaminations*

199 Since phthalates are ubiquitous in the environment, they are present as contaminants in almost  
200 all laboratory equipment, solvents, and laboratory air. In this regard, a number of precautions  
201 were taken herein to avoid background contamination from phthalates.

202 All procedures of sample treatment were performed in a clean room (class 10,000) equipped  
203 with high efficiency air particulate filters (HEPA) and activated charcoal filters for vapour phase  
204 purchased from FAST (Padua, Italy). In addition, the room was kept over-pressurized so as to  
205 avoid air contamination from outside.

206 Sodium chloride was heated for 12 h at 450°C in a muffle furnace (Vittadini, Milan, Italy) and  
207 stored in a glass bottle until use. Anhydrous sodium sulphate and glass fibre filters underwent  
208 the same heating treatment and were then kept at 150°C until use. Activated acid aluminium  
209 oxide was purified at 450°C overnight and used immediately afterwards.

210 Purified water was obtained from HPLC grade water by replicated extraction with 3x30 mL of  
211 n-hexane. Phosphoric acid 1 M used for esterase inhibition was obtained via dilution of H<sub>3</sub>PO<sub>4</sub>  
212 85% with purified water.

213 The commercially available cyclohexane was treated with purified aluminium oxide (30 g of  
214 Al<sub>2</sub>O<sub>3</sub> per one litre of solvent) by manually shaking for 30 seconds and finally through purified  
215 glass fibre filters.

216 Glassware was cleaned before use by repeatedly washing with hot methanolic potassium  
217 hydroxide, chromic and hot concentrated sulphuric acid mixture and purified water, and finally  
218 dried at 300°C for 1 h.

219 All glassware used to collect and extract milk samples was deactivated by rinsing with 2-  
220 propanol before use.

### 221 2.3 *Instrumentation*

222 A Shimadzu (Duisburg, Germany) analytical balance, model AW120, with a precision of  $\pm 0.1$   
223 mg, and a BÜCHI (Flawil, Switzerland) Rotavapor R-200, equipped with a vacuum pump model  
224 Vac V-500 were used.

225 Low pressure SEC was performed with a system which includes a Shimadzu LC-10ADVP  
226 pump, a Rheodyne injector equipped with a 5 mL Teflon<sup>®</sup> loop, steel pre-column (1 x 10 cm)  
227 and column (2.5 x 50 cm) Alltech model Omnifit<sup>®</sup> and a Shimadzu diode array detector (DAD)  
228 SPD-M10AVP. Pre-column and column were packed with Bio-Beads resin, swollen overnight  
229 in the fractionation eluent. Chromatograms were acquired and processed by Shimadzu Class-VP  
230 5.032 software.

231 The GC-MS analysis was carried out with a Shimadzu GCMS-QP2010 Plus mass spectrometer.

232 The gas chromatographic system was provided with AOC-20i auto injectors, equipped by a  
233 Shimadzu AOC-20s auto sampler, and a split-splitless (SSL) injector.

### 234 2.4 *Milk samples and infant formula*

235 A pooled milk sample (hereafter denominated Pool) obtained by mixing the breast milk from  
236 four donors recruited by the human milk bank of the “Meyer” Children’s Hospital (Florence,  
237 Italy) was used for the development of the analytical method. The milk samples used for the  
238 preparation of the Pool were collected by mothers in glass containers containing 5.0 mL of

239 phosphoric acid 1M and kept in the fridge (+4°C) until transported to the laboratory, where the  
240 samples were mixed and further stored at -20°C until analysis.

241 After optimisation, the method was applied on human milk samples collected from nine healthy,  
242 non-smoking primiparae (age: 24-37 years; gestational age: 39-41 weeks) living in the city of  
243 Florence (Italy). The mothers were recruited after a full explanation of the project and consent  
244 was obtained. Milk samples were collected by manual expression, using glass breast pumps. The  
245 samples were acidified with phosphoric acid 1M (about 0.65 mL per 5 g of milk), stored at +4°C  
246 during transport from the donor's house to the laboratory and kept at -20°C until analysis. The  
247 method was also applied to four ready-to-use infant milk formulas, commercially available on  
248 the Italian market.

#### 249 *2.5 Sample pre-treatment*

250 Human milk contains enzymes which are involved in the conversion of phthalate diesters into  
251 the corresponding monoesters [15]. In order to prevent this conversion process that changes the  
252 ratio between phthalate diesters and monoesters, about 1.25 mL phosphoric acid 1M were added  
253 to 1 g of milk so as to adjust the pH of the samples to  $1.9 \pm 0.1$  [19]. It should also be noted that,  
254 at this pH value, the carboxylic group of phthalate monoesters is protonated and metabolites can  
255 be therefore extracted together with parent compounds with low polarity solvents.

#### 256 *2.6 Extraction of phthalate diesters and monoesters*

257 The extraction protocol is derived from a previously published procedure for the analysis of  
258 polycyclic aromatic hydrocarbons (PAHs) in human milk [31], modified by replacing  
259 chloroform with dichloromethane and aqueous 0.7% NaCl with 30% NaCl, as part of the  
260 extraction mixture. Furthermore, no back-extraction step is required. Briefly: 5 grams of milk,  
261 previously acidified with 0.65 mL phosphoric acid 1M, were spiked with a labelled phthalate

262 standard solution (25  $\mu\text{L}$ , approximately 1  $\mu\text{g}/\text{mL}$  in methanol), corresponding to about 5  $\mu\text{g}/\text{kg}$   
263 fresh weight (f.w.), and vortex mixed for 5 min. The mixture was extracted once with 21 mL of  
264  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}/\text{NaCl}$  30% 2/1/0.5 (v/v/v) and twice with 6 mL of  $\text{CH}_2\text{Cl}_2$ . The extracts were  
265 pooled, dried over anhydrous sodium sulphate and evaporated to a constant weight thus  
266 obtaining a fatty residue.

### 267 2.7 *Clean-up of the extract*

268 Fatty residue was dissolved in 5 mL of cyclohexane/ethyl acetate 9/1 (v/v), and a 1-mL aliquot  
269 is directly aspirated in the loop of the HPLC system and fractionated on Bio-Beads S-X3,  
270 previously swelled and packed in cyclohexane/ethyl acetate 9/1 (v/v), using the same solvent  
271 mixture as eluent. Elution was carried out under isocratic conditions at a flow rate of 4.5 mL/min,  
272 which corresponded to back pressure values close to, but below 300 psi, which is the maximum  
273 pressure allowed for this stationary phase. Two fractions (F1 and F2) containing analytes were  
274 collected separately (F1: 43-50 min and F2: 50-82 min), evaporated to 5 mL by Rotavapor,  
275 transferred to a glass conical vial and further reduced to 1 mL via cold-evaporation under a  
276 gentle nitrogen flow, using standardized conditions [31]. The resulting solutions were directly  
277 analysed by gas chromatography coupled with mass spectrometry (GC-MS) for phthalate  
278 diesters determination, whereas for monoesters the derivatization of an aliquot of F2 was  
279 performed before GC-MS analysis.

### 280 2.8 *Derivatization of phthalate monoesters*

281 The derivatization of phthalate monoesters consisted in the addition of 4  $\mu\text{L}$  of TMSDM and 12  
282  $\mu\text{L}$  of  $\text{CH}_3\text{OH}$  to 50  $\mu\text{L}$  of the F2 fraction of the cleaned-up extract; the obtained mixture was  
283 kept at room temperature for 60 min, purified according to the protocol reported by Herrero et  
284 al. [32] and finally analysed by GC-MS.

285 2.9 GC-MS analysis

286 GC-MS analysis was performed by injecting 1  $\mu\text{L}$  aliquots in splitless mode under the following  
287 instrumental conditions. High pressure injection mode (600 kPa); injector temperature: 280°C  
288 (sampling time of 1 min); linear velocity of carrier gas (He): 60  $\text{cm sec}^{-1}$ ; capillary column:  
289 Supelco (Bellefonte, PA, USA) SLB-5MS (length = 10 m; i.d. = 0.10 mm; film thickness = 0.10  
290  $\mu\text{m}$ ).

291 Different oven temperature programs were adopted for the analysis of phthalate diesters and  
292 monoesters. As regards phthalate diesters the following temperature program was used: starting  
293 period at 70°C for 1 min., linear increase from 70°C to 200°C at 40°C/min, from 200°C to 280°C  
294 at 10°C/min, from 280°C to 320°C at 40°C/min, and finally an isotherm for 2 min. For the  
295 analysis of phthalate monoester derivatives, the temperature program was the following: starting  
296 period at 80°C for 1 min., linear increase from 80°C to 140°C at 40°C/min, from 140°C to 190°C  
297 at 3°C/min, from 190°C to 320°C at 60°C/min. and finally an isotherm for 5 min. Under these  
298 instrumental conditions the elution of phthalate diesters and monoesters was achieved with  
299 analysis time of about 10 min and 14 min, respectively (see **Table 1**).

300 Electron impact mass spectra were obtained at 70 eV of ionization energy. The transfer line was  
301 set at 280°C and the ionization source at 230°C. Chromatograms were acquired and processed  
302 in single ion monitoring (SIM) mode with Shimadzu Lab Solution software, according to  
303 retention times and quantification and diagnostic ions reported in **Table 1**.

304 2.10 Blank procedure

305 In order to evaluate the blank contribution to the analysis of phthalate diesters and monoesters,  
306 two blanks were analysed along with each batch of samples (usually consisting of 5-8 milk  
307 aliquots) and their mean results were subtracted from those of the corresponding samples. In this

308 regard, it should be noted that the analytical response of a certain analyte present in solvents  
309 and/or released from materials used for the analysis, might be influenced by the presence of  
310 matrix. Accordingly, in this study we calculated the matrix effect of samples (ME%) and if, for  
311 a certain analyte within a batch, a blank chromatographic area ( $A_{\text{blank}}$ ) with signal-to-noise ratio  
312 ( $s/n$ ) > 3 was found, this area was corrected for ME% by applying the equation (1), thus obtaining  
313 a chromatographic area accounting for the effect of blank contribution in the matrix ( $A_{\text{blank}}^{\text{ME}}$ ).

$$314 \quad A_{\text{blank}}^{\text{ME}} = A_{\text{blank}} + (A_{\text{blank}} \cdot \text{ME}\%) \quad (1)$$

315 Accordingly, only chromatographic areas which were at least double, compared to  $A_{\text{blank}}^{\text{ME}}$  were  
316 taken into account for compound detection and quantification.

317 Among the analytes investigated in human milk samples and infant formula, DiBP (0.79-1.08  
318  $\mu\text{g}/\text{Kg}$ ), DBP (0.27-0.36  $\mu\text{g}/\text{Kg}$ ) and DEHP (0.88-1.14  $\mu\text{g}/\text{Kg}$ ) were detected in the blanks.

### 319 *2.11 Quantification*

320 The standard addition method was used for analyte quantification in real samples. Fractions F1  
321 and F2 from SEC clean-up were split into four aliquots, three of which were spiked with  
322 increasing concentration of standard solutions of target phthalates. Different spike levels were  
323 selected depending on target analyte, so that their final concentrations fell into the investigated  
324 linear range of the method. Furthermore, each sample was spiked with labelled phthalate  
325 diesters and monoesters at 5  $\mu\text{g}/\text{kg}$  milk f.w. for the recovery evaluation.

### 326 *2.12 Data analysis*

327 Data plots, histograms and linear regressions were performed using Microsoft Office® Excel  
328 2003 (Microsoft Corporation, Redmond, WA, USA).



329 Energy minimization of the structures of phthalate diesters and monoesters (MM2 force field  
330 method, RMS gradient = 0.01 kcal/mol) and successive calculation of the Connolly Solvent  
331 Excluded Volume (solvent radius selected for this calculation was that of cyclohexane, equal to  
332 2.9 Å) were performed by using the Chem3DPro software, version 12.0 (CambridgeSoft-Perkin  
333 Elmer, Waltham, MA, USA).

334 In order to assess the overall toxicity associated with phthalate content of human milk samples  
335 and infant formula, DEHP equivalent concentrations ( $DEHP_{eq}$ ) were calculated. The calculation  
336 of  $DEHP_{eq}$  for a given phthalate diester requires the use of its toxic equivalent factor (TEF),  
337 which represents the relative toxicity potency of the given phthalate, using DEHP as a reference  
338 compound to modify its original concentration. In this work TEFs reported by the international  
339 association Health Care Without Harm (HCWH) [33] were used; these values were selected by  
340 HCWH as the highest relative potencies determined by Gray and co-workers for reproductive  
341 and sexual developmental toxicity in male rats after administration of different doses of  
342 phthalate diesters [34]. The TEFs were the following: 0.3 for DiNP, 0.9 for DBP and DiBP, 1  
343 for BzBP and DEHP. Since the phthalate monoesters derived from hydrolysis of the  
344 corresponding diesters, their toxicity potency was estimated following a precautionary approach,  
345 by using the same TEFs of the diesters (i.e. 0.3 for MiNP, 0.9 for MBP and MiBP, 1 for MBzP  
346 and MEHP). For other phthalates TEFs were not available or were equal to zero (i.e. DEP and  
347 DMP).

348 In this work, only phthalate diesters characterized by TEFs different from zero, together with  
349 their corresponding monoesters, were considered for the analysis in milk samples and infant  
350 formula. Furthermore, based on TEFs, the toxicity potency of the total phthalates was assessed  
351 by calculating the sum of the  $DEHP_{eq}$  estimated for each phthalate.

## 352 **3 Results and Discussion**

### 353 *3.1 Optimisation of the liquid/liquid extraction*

354 The method herein adopted for fat extraction from human milk samples was a modification of  
355 a protocol previously applied to the analysis of PAHs in breast milk [31]. More in detail, the  
356 three-component extraction mixture, based on  $\text{CHCl}_3/\text{CH}_3\text{OH}/0.7\%$  NaCl aqueous solution  
357  $2/1/0.5$  (v/v/v), was modified by replacing  $\text{CHCl}_3$  with  $\text{CH}_2\text{Cl}_2$ , the latter being less toxic, and  
358 the concentration of the aqueous NaCl solution was increased up to 30% in order to improve  
359 the phase separation, thus making unnecessary the final back-extraction step.

360 The optimized method was compared with the AOAC Official Method 989.05 for the  
361 determination of fats in milk and the obtained results are shown in **Table S1** of the  
362 Supplementary Material section. The t-test performed on the two data sets clearly evidenced  
363 that the null hypothesis must be accepted and that therefore the two methods gave rise to  
364 identical results.

### 365 *3.2 Optimisation of the clean-up procedure*

366 SEC was adopted as clean-up technique for the separation of phthalate diesters and monoesters  
367 from the fat residue, in turn derived from the liquid/liquid extraction of milk samples. Bio-  
368 Beads<sup>®</sup> S-X3 and S-X8 stationary phases, which are neutral, porous styrene divinylbenzene  
369 polymers with 3% and 8% average degree of crosslinking, were tested to this aim. A wide range  
370 of swelling volumes were obtained by using different solvents, highlighting that very different  
371 size exclusion properties can be provided by these resins, depending on the swelling solvent  
372 used. As a general consideration, S-X8 swelled less than S-X3; for instance, using benzene as  
373 swelling solvent, bed volumes of about 3.0 and 4.5 mL/g were obtained with the former and the  
374 latter resins, respectively. Among the solvents tested, benzene, toluene and dichloromethane

375 provided the highest swelling volumes (about 4.5 mL/g for Bio-Beads<sup>®</sup> S-X3), whereas  
376 cyclohexane and ethyl acetate gave rise to a lower swelling (about 3.0 mL/g for Bio-Beads<sup>®</sup> S-  
377 X3). Furthermore, it is possible to achieve a more accurate modulation of the resin swelling by  
378 using binary solvent mixtures, which may in turn also allow for obtaining separation  
379 performance better matching the chromatographic problem under examination. In this regard, it  
380 should also be noted that the solvent adopted for resin swelling and packing, as well as for  
381 isocratic elution, should be the same, in order to avoid shrinking or expansion of the resin during  
382 the analysis.

383 Several tests were performed at different flow rates on phthalate diesters and monoesters, as well  
384 as human milk fats, employing various solvents and solvent mixtures, for swelling and elution.  
385 The first experimental conditions tested were those previously adopted for the analysis of PAHs  
386 in human milk, which consisted in the use of the S-X3 resin and dichloromethane as swelling  
387 and elution solvent [31]. However, this solvent provided the partial co-elution of phthalates and  
388 fats, due to its high swelling properties, moderate polarity and elution strength. Aromatic  
389 solvents, such as toluene, even when present at low percentages in mixtures with more polar  
390 solvents (e.g. ethyl acetate), provided the elution of phthalates in a quite narrow retention time  
391 window, due to the strong competition between eluent-phthalates and stationary phase-  
392 phthalates  $\pi$ - $\pi$  interactions. Under these experimental conditions, a significant overlap with the  
393 peaks of fats was observed. The use of cyclohexane, which is a solvent more compatible with  
394 the “green chemistry” approach, provided much lower swelling of the resin and elution strength.  
395 With this solvent, a general increase of retention times of both fats and phthalates, but also the  
396 improvement of the selectivity of the chromatographic method, was obtained. Accordingly, a  
397 significant gain in the resolution between fats and phthalates was achieved. Hence, further tests  
398 were performed by adding increasing percentages of ethyl acetate to cyclohexane, starting from

399 5% in volume. The choice of ethyl acetate was driven by its low toxicity, moderate polarity and  
400 the very low influence on the swelling of the resin, compared to the use of cyclohexane alone.  
401 The best resolution was achieved by using cyclohexane/ethyl acetate 9/1 (v/v), which was  
402 chosen as swelling and elution solvent mixture. With regard to the elution conditions, the effect  
403 of flow rate on the resolution between fats and target analytes was tested. Increasing flow rates,  
404 up to 4.5 mL/min, were adopted, without any detrimental effect on the resolution. Using this  
405 flow rate, a backpressure value little lower than 300 psi was obtained, which corresponded to  
406 the maximum pressure tolerated by the stationary phase.

407 **Fig. 1** illustrates the trend of the chromatographic retention of phthalate diesters and monoesters  
408 on Bio-Beads<sup>®</sup> S-X8 and S-X3 stationary phases following the experimental protocol described  
409 in the paragraph 2.6 (i.e. using cyclohexane/ethyl acetate 9/1 (v/v) as swelling and elution solvent  
410 mixture), as a function of the Connolly solvent excluded volumes (SEV). As expected, for both  
411 stationary phases, increasing retention times were generally observed with decreasing SEV,  
412 whereas MBzP and BzBP were more retained than expected on the basis of their SEV due to the  
413 extra  $\pi$ - $\pi$  interactions of the benzyl group. As clearly shown in the figure, the 8% cross-linked  
414 Bio-Beads<sup>®</sup> provided the earlier elution of both phthalates and fats, compared to the 3% cross-  
415 linked co-polymer. Moreover, S-X8 exhibited a wider elution window of fats that co-eluted with  
416 most phthalate diesters investigated. This behaviour is in accordance with the smaller pore size  
417 provided by the S-X8. Since the increase in pore size for this resin could be obtained only by  
418 using aromatic or chlorinated solvents, which are not in agreement with a “green” approach, the  
419 S-X8 phase was not further investigated.

420 **Fig. 2** illustrates the chromatograms obtained with the S-X3 resin, under the experimental  
421 conditions described in paragraph 2.6, for: (A) a phthalate diester standard solution (containing  
422 DEP, BzBP, DBP, DiBP, DEHP and DiNP dissolved in cyclohexane/ethyl acetate 9/1 (v/v); total

423 injected amount = 50  $\mu$ g), (B) a phthalate monoester standard solution (containing MEP, MBzP,  
424 MBP, MiBP, MEHP and MiNP dissolved in cyclohexane/ethyl acetate 9/1 (v/v); total injected  
425 amount = 5  $\mu$ g), and (C) 1 mL of a solution of the fatty residue from the extraction of 5 g of  
426 pooled milk dissolved in 5 mL of cyclohexane/ethyl acetate 9/1 (v/v).

427 All chromatograms were obtained at  $\lambda=275$  nm. Diesters gave rise to three baseline-resolved  
428 peaks, referring respectively to DiNP and DEHP (peak 1), DiBP and DBP (peak 2) and BzBP  
429 and DEP (peak 3). Similarly, the corresponding monoesters were eluted in three partially  
430 overlapping peaks, relative to MiNP and MEHP (peak 4), MiBP and MBP (peak 5) and MBzP  
431 and MEP (peak 6). The elution of the fatty milk extract showed one main tailed peak and two  
432 other peaks, characterized by much lower intensity. As **Fig. 2** clearly shows, under the  
433 chromatographic conditions adopted, monoesters were eluted at later retention times compared  
434 to the last peak of fats. On the other hand, peak 2 and above all peak 1 of phthalate diesters co-  
435 eluted with the less intense peaks of fats.

436 It should be noted that when human milk samples and infant formulas were analysed the  
437 sensitivity of UV detection was not sufficient for revealing the labelled phthalates used as  
438 reference standards at the concentrations spiked in the samples (5  $\mu$ g/kg fresh weight, f.w.). For  
439 this reason, based on the chromatographic profile of the phthalate standard mixture analysed  
440 before real samples, two fractions were collected at fixed time intervals: fraction 1 (F1) from 43  
441 to 50 min and fraction 2 (F2) from 50 to 82 min (see **Fig. 2C**). This procedure allowed for  
442 minimizing fat contamination of F2. In order to quantitatively determine the separation  
443 efficiency of phthalate diesters and monoesters from fats, five different fat aliquots of about 36-  
444 37 mg each, deriving from the extraction of about 5 g of the Pool, were eluted under the above-  
445 mentioned chromatographic conditions. Two fractions, corresponding to the most intense peak  
446 of fats (30-43 min) and to the phthalate diesters and monoesters elution window (F1 and F2, 43-

447 82 min, see **Fig. 2**) were separately collected and evaporated to about 0.5 mL by Rotavapor, and  
448 then to constant weight under a gentle nitrogen flow. The comparison of the weights of the  
449 collected fractions indicated that the fraction collected between 30 and 43 min contained about  
450 95% of total fats, and that the phthalate fractions were almost completely purified from fats.

### 451 3.3 *Optimisation of the derivatization of phthalate monoesters*

452 For derivatization of phthalate monoesters, BSTFA + 1% TMCS was initially tested, since it is  
453 widely used as a reagent for converting carboxylic acids into corresponding volatile  
454 trimethylsilyl derivatives. However, for BzBP, the derivatization reaction performed in  
455 cyclohexane/ethyl acetate 9/1 (v/v) produced very small analytical responses or no signal at all.  
456 This finding may be explained by the presence of steric hindrance, since in this kind of reactions  
457 (i.e. bimolecular nucleophilic substitution) an important amount of space is needed to form the  
458 transition state before the leaving group is ejected the opposite side and the final product is  
459 formed.

460 Much homogeneous signals were observed for all target analytes dissolved in the  
461 aforementioned solvent mixture, by using TMSDM and methanol, as derivatization reagents. In  
462 this regard, it should be remarked that with this derivatization reagent the reaction proceeds  
463 spontaneously at room temperature. Accordingly, methanolic TMSDM was chosen for  
464 proceeding with the optimization of the derivatization procedure. Derivatization tests were  
465 performed on 50  $\mu\text{L}$  of the 2<sup>nd</sup> fraction of the cleaned-up extract of the Pool, spiked with the  
466 labelled phthalate monoesters (see paragraph 2.1) at 5  $\mu\text{g}/\text{kg}$ , by using different TMSDM-to-  
467 methanol ratios and a fixed derivatization time of 90 min. Under these conditions, the best results  
468 were obtained with 4  $\mu\text{L}$  of TMSDM and 12  $\mu\text{L}$  of  $\text{CH}_3\text{OH}$ .

469 A further optimization step regarded the derivatization time. More in detail, reaction times of  
470 30, 60, 90, 120 and 150 min were investigated. The results obtained for MBzP-4C13-Me and  
471 MEHP-4C13-Me are illustrated in **Fig. 3**, as an example of the general trend observed for  
472 targeted phthalate monoesters. After 60 min of reaction time no significant increase in the  
473 chromatographic area of the quantifier ion was obtained and this time length was therefore  
474 selected.

#### 475 *3.4 Figures of merit of the proposed method*

476 As illustrated in **Fig. 4**, by the examples of DEHP-d4 (**Fig. 4 A-B**) and MEHP-4C13 (**Fig. 4 C-**  
477 **D**), the SIM GC-MS signal in matrix was characterized by a much higher noise than in solvent.  
478 Moreover, a strong signal enhancement, up to about 400% in the case of DEHP, was observed  
479 in milk samples due to the matrix effect. Accordingly, the evaluation of method performances –  
480 i.e. investigation of the linearity range, assessment of limits of detection (MDLs) and  
481 quantification (MQLs), as well as recovery – was performed in matrix. Since the presence of  
482 phthalates in procedural blanks is an issue widely recognized by the Scientific Community and  
483 highlighted also in this study (see paragraph 2.10), all the aforementioned figures of merit were  
484 evaluated by using labelled standards. To this aim, 5 g f.w. aliquots of the Pool were spiked with  
485 decreasing concentrations of methanol solutions of the following labelled standards of phthalate  
486 diesters and monoesters: DBP-d4, DiBP-d4, BzBP-d4, DEHP-d4, DiNP-d4, MBP-C4, MiBP-  
487 d4, MBzP-C4, MEHP-C4 and MiNP-C4. The lowest spiked concentrations were included  
488 between 0.10 and 1.0  $\mu\text{g}/\text{kg}$  and the highest between 10 and 100  $\mu\text{g}/\text{kg}$  (**Table 2**). The 5  $\mu\text{g}/\text{kg}$   
489 fortification level was included in this procedure and used as reference concentration for the  
490 recovery evaluation. All spiked samples underwent to the whole analytical protocol of  
491 extraction, clean-up, derivatization and GC-MS analysis mentioned in the experimental section.

492 In parallel, unfortified milk aliquots were extracted and fractionated by SEC, so as to obtain F1  
493 and F2 fractions, which were fortified at 5 µg/kg with each aforementioned target compound  
494 and finally analysed by GC-MS for phthalate diesters and monoesters, the latter after  
495 derivatization.

496 MDLs found in this study were included in the ranges 0.5-4.2 and 0.03-0.8 µg/kg f.w. for diesters  
497 and monoesters, respectively. More in detail, the highest limits were achieved for DBP (1.4  
498 µg/kg), DiBP (3.1 µg/kg) and DEHP (4.2 µg/kg), as the result of the contributions determined  
499 for these analytes in procedural blanks and above all owing to their correction for signal  
500 enhancement in matrix (see paragraph 2.10).

501 **Table 3** illustrates the MDLs found herein for phthalate diesters and monoesters in comparison  
502 with those determined elsewhere using various extraction, clean-up and detection techniques.  
503 The MDLs achieved in this study for phthalate diesters were in most cases lower or comparable  
504 with those previously reported in literature. The main exception was represented by the study of  
505 Fromme and co-workers [21] who reported sensitivities over one magnitude order higher for  
506 DiBP, DBP and DEHP. However, these limits seem to be unrealistic, since the same authors,  
507 using the same analytical protocol, reported in a successive study 0.34, 0.28 and 2.4 µg/kg as  
508 the lowest blank contributions for DiBP, DBP and DEHP, respectively [14].

509 Also for phthalate monoesters the limits provided by the proposed method were lower or  
510 comparable to the ones previously reported, except for MBP in the studies of Main and  
511 Mortensen [18, 19].

512 The mean recovery percentages varied from 83% (for MBP-4C13) to 115% (for DEHP-d4) and  
513 the inter-day precision ranged from 9 to 21% (see **Table 2**), which highlighted the reliability of  
514 the proposed analytical method.



515 3.5 *Phthalate diesters and monoesters in human milk and infant formula*

516 In order to make the best choice for feeding one's infant, data on both breast milk and formula  
517 need to be considered. Hence, the proposed method was applied to the determination of phthalate  
518 diesters and monoesters in nine breast milk samples and four infant formulas.

519 The mean concentrations of phthalate diesters and monoesters in breast milk, ranges and number  
520 of samples with concentrations higher than MDL (positive samples) are reported in **Table 4**. All  
521 samples were found to be positive for the presence of the investigated phthalate diesters and  
522 monoesters, highlighting the widespread human exposure to these pollutants. In accordance with  
523 our results a 100% detection rate was observed in Danish and Finnish women for all phthalate  
524 monoesters reported in **Table 4** [18]. High percentages of positive samples were also reported  
525 in Swedish, German and Italian breast milk [16, 20-22].

526 Minimum and maximum concentrations varied over about one magnitude order, indicating a  
527 quite low variability for both phthalate diesters and monoesters, compared to the results reported  
528 in literature [16, 18, 20]. This finding might be due to the low number of samples investigated  
529 in this study. Interestingly, diesters were generally determined at higher concentration, compared  
530 to corresponding monoesters. This result was also found by Hogberg and co-workers [20], who  
531 provided the same esterase inhibition protocol herein adopted, based on the addition of  
532 phosphoric acid immediately after milk sampling. Conversely, monoester concentrations higher  
533 than those of diesters were reported by Fromme et al. [21], who avoided this sample pre-  
534 treatment.

535 Our data evidenced that among the phthalate diesters, DEHP and DiBP were the predominant  
536 compounds in four samples each, whereas in one case the highest concentration was exhibited  
537 by DiNP. In agreement with our results, the prevalence of DEHP in breast milk has been  
538 evidenced by other authors [13, 20, 21]. Conversely, for the branched butyl phthalate a lesser

539 presence has been reported in the only study investigating its occurrence in human milk [21]. To  
540 the best of our knowledge, DiNP has never been investigated before in human milk; however,  
541 data elsewhere reported regarding the occurrence of MiNP in Danish and Finnish women [18],  
542 suggest a high abundance in breast milk of the corresponding diester, as well.

543 The mean concentrations of five replicated determinations of phthalate diesters and monoesters  
544 in four ready-to-use infant formulas are reported in **Table 5**. Analogously to breast milk, a 100%  
545 detection rate was also found for all target analytes in the investigated artificial milk samples.  
546 However, unlike what was determined in human milk, in infant formula a general prevalence of  
547 monoesters with respect diesters was highlighted. DEHP and its corresponding monoester  
548 MEHP were found to be the most abundant phthalates, being their concentrations respectively  
549 included in the ranges 18-75 and 35-72  $\mu\text{g}/\text{kg}$  f.w. High concentrations were also determined for  
550 DiBP (18-25  $\mu\text{g}/\text{kg}$  f.w.) and MiBP (5.7-43  $\mu\text{g}/\text{kg}$  f.w.), as well as for MiNP (10-23  $\mu\text{g}/\text{kg}$  f.w.),  
551 whereas the other investigated analytes were determined in most cases at ppb levels. Results  
552 found in this study highlighted an extent of phthalate diester contamination included in the very  
553 wide range of data reported in literature on powdered or ready-to-use infant formulas [23-26].

554 As far as we know, only two papers reported the analysis of phthalate monoesters in infant  
555 formulas [19, 21]. The results found in these researches highlighted a much lower contamination  
556 than that observed herein. More in detail, Mortensen and colleagues [19], in a study dedicated  
557 to the monitoring of phthalate monoesters only, detected MBP (0.6-3.9  $\mu\text{g}/\text{kg}$ ) and MEHP (5.6-  
558 9.1  $\mu\text{g}/\text{kg}$ ), whereas MBzP and MiNP were found always below the detection limit. Fromme  
559 and co-workers [21] did not detected any of the investigated monoesters (i.e. MiBP, MBP and  
560 MEHP), even though the corresponding diesters were determined at significant concentrations  
561 in all the artificial milk samples investigated (DEHP: 9.3-35.7  $\mu\text{g}/\text{kg}$ ; DBP: 1.7-5.5  $\mu\text{g}/\text{kg}$ ; DiBP:  
562 1.6-4.9  $\mu\text{g}/\text{kg}$ ).

563 3.6 *Phthalate intake estimation and risk evaluation for newborns*

564 Even though only a few samples were analysed in this work, the limited information regarding  
565 the contamination by phthalates of human milk from Italian women, and the lack of data  
566 concerning infant formulas commercialized in Italy, make the evaluation of their intake of great  
567 interest. It should also be noted that only two papers deal with the analysis of both phthalate  
568 diesters and monoesters in human milk [20, 21]; furthermore, no data are reported in literature  
569 on the analysis of both parent and degradation compounds in infant formula.

570 Based on DEHP<sub>eq</sub> found in milk samples and infant formula, the phthalate exposure of newborns  
571 due to food ingestion can be calculated. Assuming an infant body weight of 5 kg and daily milk  
572 feeding of 800 mL, the equivalent DEHP intake for human milk samples was approximately  
573 included between 10 and 25  $\mu\text{g kg}^{-1}$  body weight (b.w.), while, for infant formulas, the DEHP<sub>eq</sub>  
574 was in the range 21-45  $\mu\text{g kg}^{-1}$  b.w. (see **Fig. 5**).

575 Maximum acceptable daily intake (MADI) has been established for DEHP by the European  
576 Scientific Committee on Toxicity, Ecotoxicity and the Environment (EU-CSTEE), by the  
577 European Scientific Committee on Food (EU-SCF), and by the United States Environmental  
578 Protection Agency (USEPA). According to European organizations this limit is equal to 50  $\mu\text{g}$   
579  $\text{kg}^{-1}$  b.w., whereas a lower acceptable dose (22  $\mu\text{g kg}^{-1}$  b.w.) was proposed by USEPA. **Fig. 5**  
580 highlights how all the investigated breast milk samples correspond to equivalent ingested doses  
581 of DEHP much lower than the MADI proposed by European organizations. Furthermore, only  
582 two samples (i.e. HM5 and HM7) were associated to intakes slightly higher than the maximum  
583 dose established by the USEPA. With regard to infant formulas, most artificial milk samples  
584 (i.e. IF1-3) showed DEHP<sub>eq</sub> doses (21.2-24.1  $\mu\text{g kg}^{-1}$  b.w.) slightly higher than the USEPA  
585 MADI, whereas for the IF4 the equivalent DEHP dose (44.8  $\mu\text{g kg}^{-1}$  b.w.) was more than double  
586 the USEPA limit and very close to that fixed by the CSTEE and the SCF.

#### 587 4 Conclusions

588 This study provides a sensitive and reliable analytical method in which, for the first time, the  
589 same extraction and clean-up procedure are adopted for determining both phthalate diesters and  
590 monoesters in human milk and infant formula. The sensitivity (MDLs in the ranges 0.4-0.8 and  
591 0.03-0.8 µg/kg milk, for diesters and monoesters, respectively, without considering the phthalate  
592 contribution due to procedural blanks) and precision (CV = 9-21%) achieved with this method,  
593 make it suitable for monitoring these analytes at trace levels in epidemiological and toxicological  
594 studies. These limits are comparable or lower than those reported for the analysis of target  
595 compounds in milk and infant formula, and even in the presence of the highest procedural blank  
596 phthalate contaminations herein observed, concentrations included between 0.5 and 4.2 µg/kg  
597 can be detected. It should also be noted that the quantification limits achieved in this work are  
598 low enough to allow the evaluation of phthalate intake in infants by breast milk and infant  
599 formulas, well below the MADIs established by the European Food Safety Authority (see the  
600 various reports on <http://www.efsa.europa.eu/>).

601 Using this method, we determined phthalate diesters and monoesters in nine human milk  
602 samples from Italian women and four infant formulas, assessing the contamination extent due to  
603 both parent compounds and their primary metabolites. Our results indicate a significant presence  
604 of phthalates in both kinds of sample and demonstrate that an exposure to these endocrine  
605 disrupting molecules occur in the population. However, the calculated daily intake of phthalates  
606 by human milk for a newborn was in most cases lower than the most conservative MADI,  
607 established by the USEPA. It should also be remarked that the phthalate daily intake of an infant  
608 due to ingestion of infant formula was found to be generally higher than that due to breast milk.  
609 These findings highlight the importance of a wider investigation on infant formulas, as well as  
610 of a larger study on breast milk aimed at assessing the exposure to phthalates of nursing mothers

611 and their children, also attempting to understand the possible causes of the contamination, in  
612 order to improve the quality of this inimitable nourishment.

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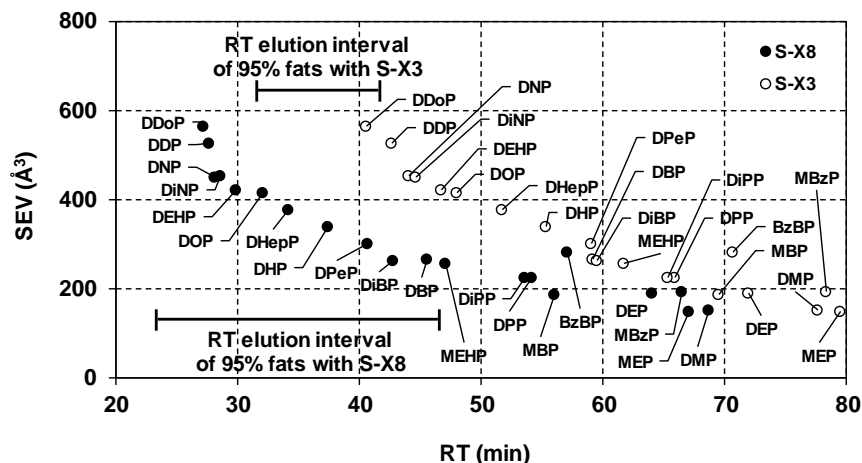
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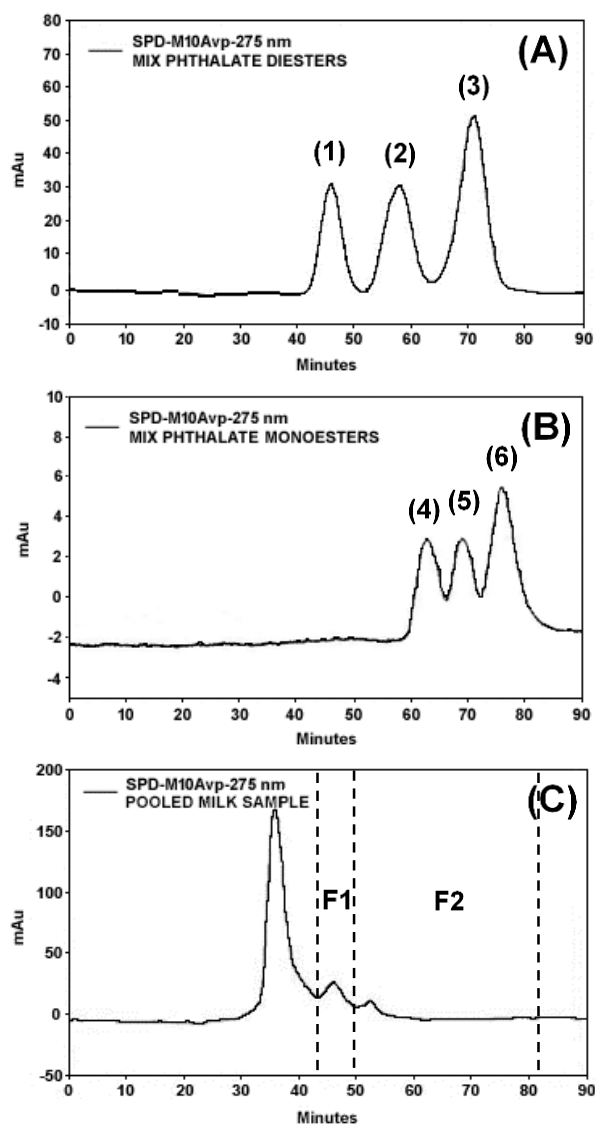
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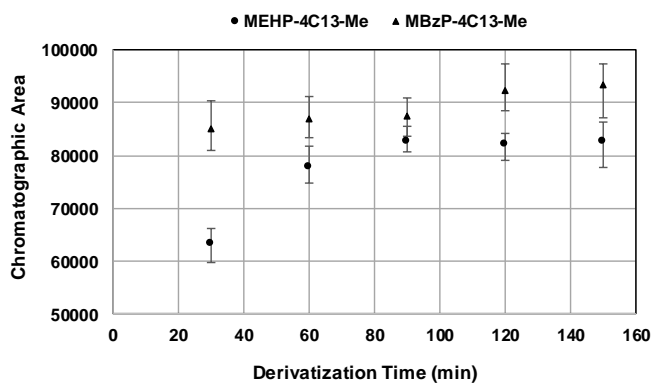


**Figure 1** – Retention time (RT, min) of phthalate diesters and monoesters on Bio-Beads® S-X8 and S-X3 size exclusion stationary phases swelled and eluted with cyclohexane/ethyl acetate 9/1 (v/v), as a function of the Connolly solvent excluded volume (SEV, Å<sup>3</sup>). Horizontal bars indicated the RT interval of elution of 95% of human milk fats with the two stationary phases. The meaning of compound abbreviations is reported in the section 2.1 “Standards, solvents and materials”.

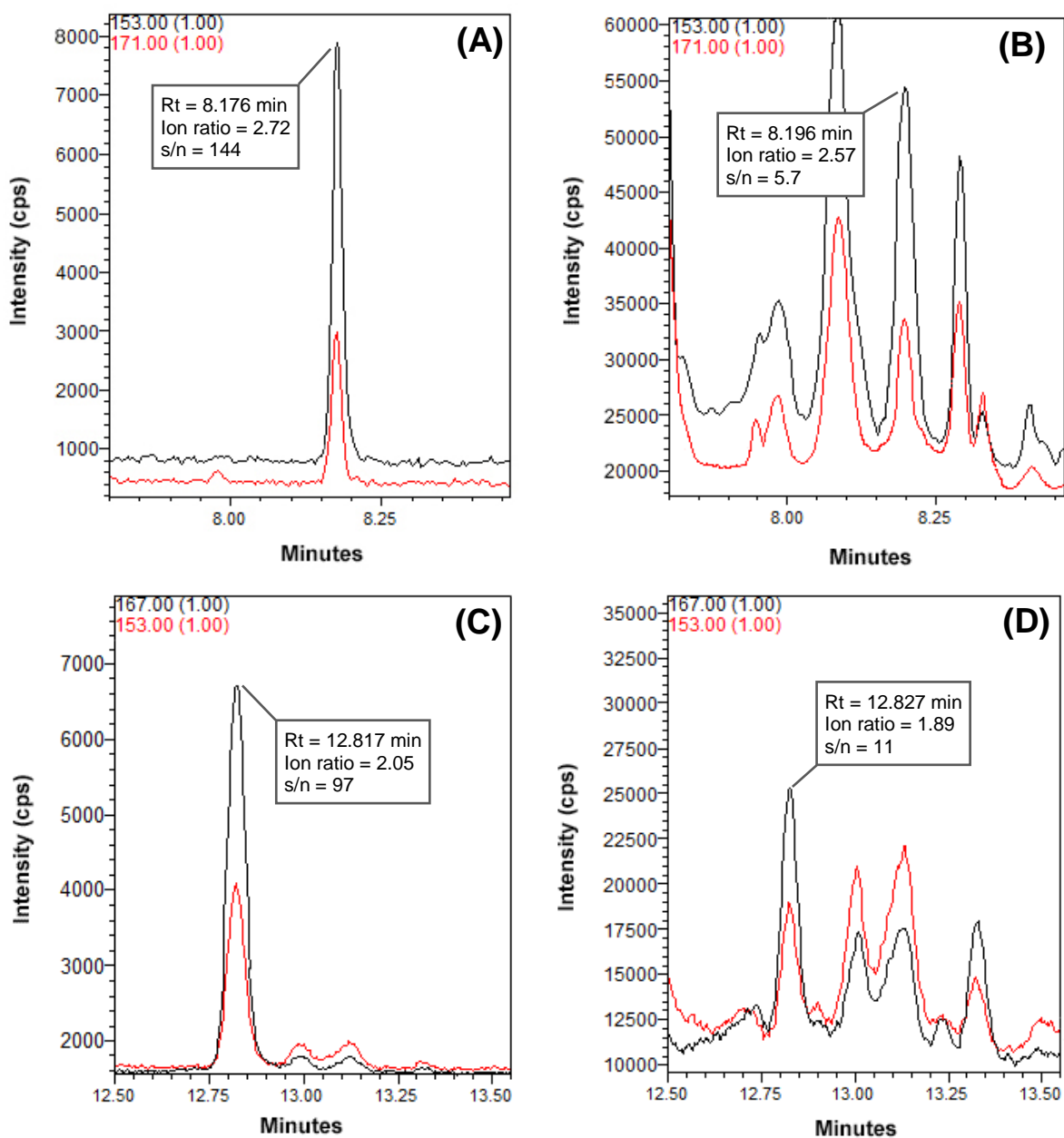




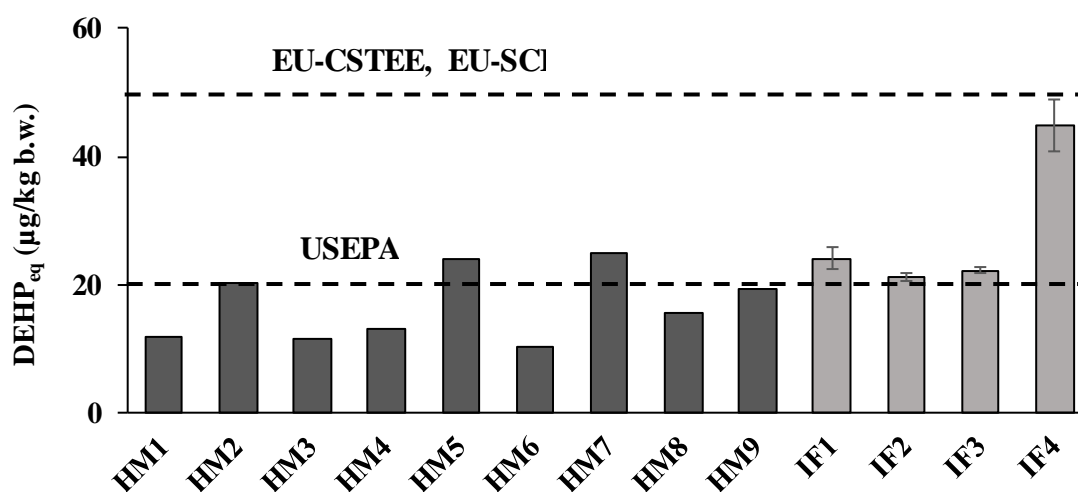
**Figure 2** – Size exclusion chromatograms obtained on Bio-Beads<sup>®</sup> S-X3 with cyclohexane/ethyl acetate 9/1 (v/v) as swelling and elution solvent. (A) Standard solution of phthalate diesters (peak 1: DiNP and DEHP; peak 2: DiBP and DBP; peak 3: BzBP and DEP; 1 mL injected; total phthalate diester concentration = 50 µg/mL); (B) standard solution of phthalate monoesters (peak 4: MiNP and MEHP; peak 5: MiBP and MBP; peak 6: MBzP and MEP; 1 mL injected; total phthalate monoester concentration = 5 µg/mL); (C) injection of 1 mL of a solution of the fatty residue from the extraction of 5 g of pooled milk dissolved in 5 mL of cyclohexane/ethyl acetate 9/1 (v/v). The dotted lines identify retention time intervals of the collected fractions F1 (43-50 min) and F2 (50-82 min). The meaning of compound abbreviations is reported in the section 2.1 “Standards, solvents and materials”. Note different scale on y-axes.



**Figure 3** – Chromatographic areas of quantifier ions of MEHP-4C13-Me (m/z 167) and MBz-4C13-Me (m/z 93) (see section 2.1 “Standards, solvents and materials” for the meaning of compound abbreviations) as a function of derivatization time. Derivatization conditions: 50  $\mu$ L of the second fraction of the cleaned-up extract of the Pool, spiked with labelled phthalate monoesters at 5  $\mu$ g/kg, derivatized with 4  $\mu$ L of TMSDM and 12  $\mu$ L of CH<sub>3</sub>OH.



**Figure 4** – Overlapped SIM quantifier (*black line*) and qualifier (*red line*) ions, retention time (Rt), quantifier-to-qualifier ion ratio and signal-to-noise ratio of: (A) DEHP-d4 standard (1.0 µg/L); (B) DEHP-d4 in the Pool of human milk samples spiked with MEHP-4C13 at 1.0 µg/kg level; (C) methyl ester derivative of MEHP-4C13 standard (0.8 µg/L) and (D) methyl ester derivative of MEHP-4C13 in the Pool of human milk samples spiked with MEHP-4C13 at 0.8 µg/kg level. The meaning of compound abbreviations is reported in the section 2.1 “Standards, solvents and materials”. Note different scale on y-axes.



**Figure 5** – Total daily intakes expressed as µg of di-2-ethylhexyl phthalate equivalent (DEHP<sub>eq</sub>) per kg of body weight (b.w.), compared to the maximum acceptable daily intakes (dotted lines) proposed by the European Scientific Committee on Toxicity, Ecotoxicity and the Environment (EU-CSTEE), European Scientific Committee on Food (EU-SCF), and United States Environmental Protection Agency (USEPA). Total daily intakes were calculated for a newborn weighing 5 kg, assuming 800 mL milk per day. HM1-9: human milk samples; IF1-4: infant formula (error bars represent the standard deviation associated with the mean of five replicates).

**Table 1** – GC/MS retention times, quantifier and qualifier ions of phthalate diesters and methyl ester derivatives (Me) of phthalate monoesters. The abundance percentage of the qualifier ion in respect to that of the quantifier is reported in bracket. The meaning of compound abbreviations is reported in the section 2.1 “Standards, solvents and materials”.

Compound	Retention times (min)	Quantifier ions (m/z)	Qualifier ions (m/z)
DEP	3.87	149	177 (23)
DiBP	4.67	149	223 (6)
DBP	5.03	149	223 (7)
BzBP	7.03	149	91 (65)
DEHP	8.19	149	167 (37)
DiNP	8.87	149	127 (30)
MEP-Me	4.26	163	149 (61)
MiBP-Me	6.49	163	149 (41)
MBP-Me	6.95	163	149 (57)
MBzP-Me	10.69	91	149 (33)
MEHP-Me	12.82	163	149 (51)
MiNP-Me	13.74	163	149 (50)
DEP-d4	3.87	153	181 (24)
DiBP-d4	4.67	153	227 (8)
DBP-d4	5.03	153	227 (5)
BzBP-d4	7.03	153	93 (70)
DEHP -d4	8.19	153	171 (34)
DiNP-d4	8.87	153	131 (28)
MEP-4C13-Me	4.26	167	153 (63)
MiBP-d4-Me	6.49	167	153 (46)
MBP-4C13-Me	6.95	167	153 (57)
MBzP-4C13-Me	10.69	93	153 (30)
MEHP-4C13-Me	12.82	167	153 (49)
MiNP-4C13-Me	13.74	167	153 (46)

**Table 2** – Method detection limits (MDLs, S/N = 3), linearity ranges, recoveries and inter-day precision of the proposed method. The meaning of compound abbreviations is reported in the section 2.1 “Standards, solvents and materials”. RSD% = relative standard deviation expressed as percentage; S/N = signal-to-noise ratio.

Compound	MDL ( $\mu\text{g}/\text{kg}$ milk)	Linear range ( $\mu\text{g}/\text{kg}$ milk) <sup>a</sup>	Recovery (%)	Inter-day precision (RSD%)
DiBP-d4	0.4	0.9-100	114	12
DBP-d4	0.4	1.0-100	112	11
BzBP-d4	0.7	2.0-100	107	19
DEHP -d4	0.8	1.8-100	115	15
DiNP-d4	0.5	1.3-100	108	14
MiBP-d4	0.7	1.9-100	85	16
MBP-4C13	0.8	2.3-100	83	21
MBzP-4C13	0.03	0.08-10	104	9
MEHP-4C13	0.3	0.8-10.0	103	14
MiNP-4C13	0.5	1.2-100	99	15

<sup>a</sup> The lower limit of the linearity range represents the Method Quantification Limit (S/N=10).

In the original version of Table 2 it is reported the inter-day precision expressed as coefficient of variation percentage. This value is the standard deviation expressed as percentage. In other words, for example for DiBP-d4, if one multiplies the recovery (114%) for 0.12, the value 13.7 is obtained, which is the standard deviation associated to 114. Hence, the addition of a standard deviation column seems to be redundant. However, in order to make clearer the table, the abbreviation CV% was replaced with RSD% and the explicit explanation was added in the caption. Furthermore, the measure unit of recovery was added.

**Table 3** – Main characteristics of the analytical method proposed herein, compared to the ones previously published and developed for the analysis of phthalate diesters and/or monoesters in human milk (HM) and/or infant formula (IF). The meaning of compound abbreviations is reported in the section 2.1 “Standards, solvents and materials”; LLE = liquid/liquid extraction; SEC = size exclusion chromatography; LLBE = liquid/liquid back extraction; SPE = solid phase extraction; n.i. = not investigated.

Matrix	Extraction + Clean-up	Instrumental Technique	MDL (µg/kg)											[Reference]
			DiBP	DBP	BzBP	DEHP	DiNP	MiBP	MBP	MBzP	MEHP	MiNP		
HM and IF	LLE + SEC	GC-MS <sup>a</sup>	3.1 <sup>b</sup>	1.4 <sup>b</sup>	0.7	4.2 <sup>b</sup>	0.5	0.7	0.8	0.03	0.3	0.5	This study	
HM and IF	LLE + SPE	GC-MS	0.033 <sup>c</sup>	0.033 <sup>c</sup>	1.6 <sup>c</sup>	0.16 <sup>c</sup>	n.r. <sup>d</sup>	n.i.	n.i.	n.i.	n.i.	n.i.	[21]	
HM and IF	LLE + SPE	LC-MS/MS	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	0.05	0.05	0.1	0.5	[18,19]	
HM and IF	LLE	LC-MS/MS	n.i.	n.i.	n.i.	n.i.	n.i.	0.3	0.3	n.i.	0.3	n.i.	[21]	
HM	LLE + SEC	GC-MS	n.i.	3	0.12	0.9	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	[20]	
HM	SPE	LC-MS/MS	n.i.	n.i.	n.i.	n.i.	n.i.	0.3	1.0	0.5	0.6	1.7	[15]	
HM	On-line SPE	LC-MS/MS	n.i.	n.i.	n.i.	n.i.	n.i.	1.0	1.0	0.3	0.3	n.i.	[16]	
HM	SPE	LC-MS/MS	n.i.	n.i.	n.i.	n.i.	n.i.	1.0	1.1	1.0	0.98	n.i.	[20]	
HM	LLE + LLBE	LC-MS <sup>a</sup>	n.i.	n.i.	n.i.	10	n.i.	n.i.	n.i.	n.i.	2	n.i.	[22]	
IF	LLE + LLBE + SPE	GC	n.i.	7.5	n.i.	5.0	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	[23]	
IF	LLE + SPE	GC-MS	5	5	5	8	10	n.i.	n.i.	n.i.	n.i.	n.i.	[24]	
IF	LLE + SEC	GC-MS	n.i.	100	4	70	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	[29]	
IF	LLE + SPE	GC-MS/MS	5	5	5	5	100	n.i.	n.i.	n.i.	n.i.	n.i.	[25]	
IF	LLE + LLBE + SPE	LC-MS/MS	n.i.	9	4	6	5	n.i.	n.i.	n.i.	n.i.	n.i.	[26]	

<sup>a</sup> Phthalate monoesters were analysed after derivatization.

<sup>b</sup> These limits take into account of blank contribution, corrected for the signal enhancement due to the matrix effect.

<sup>c</sup> These limits were extrapolated by dividing the quantification limits reported in the manuscript for three.

<sup>d</sup> DiNP was investigated but its quantification and detection limits were not reported in the manuscript.

**Table 4** – Mean phthalate concentrations ( $\mu\text{g}/\text{kg}$  milk, fresh weight), range and number of samples found to be positive (i.e. concentration higher than MDL) for the presence of each phthalate diester and monoester. The meaning of compound abbreviations is reported in the section 2.1 “Standards, solvents and materials”

	Mean	Range	No. of positive samples/analysed sample
<b>Phthalate diesters</b>			
DiBP	37	11 - 77	9/9
DBP	7.1	< 3.5 <sup>a</sup> - 19	9/9
BzBP	1.7	< 2.0 <sup>a</sup> - 3.2	9/9
DEHP	34	< 13 <sup>a</sup> - 94	9/9
DiNP	20	6.3 - 51	9/9
<b>Phthalate monoesters</b>			
MiBP	9.9	2.3 - 25	9/9
MBP	4.0	2.1 – 6.1	9/9
MBzP	0.80	0.15 - 1.2	9/9
MEHP	10	4.1 - 18	9/9
MiNP	7.6	1.5 - 29	9/9

<sup>a</sup> Method quantification limit



**Table S1** – Comparison between the proposed method and the AOAC Official Method 989.05 (AOAC International, Fat in Milk - Modified Mojonnier Ether Extraction Method, AOAC Official Methods of Analysis: Dairy products 1996, pp. 18-19) in the fat recovery from five aliquots (5 g each) of pooled human milk sample. Values are expressed as g fat/kg milk (fresh weight). *P*-value of the t-Test > 0.1.

	Proposed Method	AOAC Method 989.05
Aliquot 1	37.9	39.8
Aliquot 2	36.1	37.5
Aliquot 3	37.3	38.6
Aliquot 4	38.9	39.4
Aliquot 5	36.0	36.7
Mean ± Standard Deviation	37.6 ± 1.0	38.4 ± 1.3