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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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Abstract

A sensitive and reliable analytical method was developed for the simultaneous determination of five phthalate diesters and corresponding monoesters in human milk samples and infant formulas. The method involved a liquid-liquid extraction with a $CH_2Cl_2/CH_3OH/NaCl$ 30% 2/1/0.5 (*v/v/v*) mixture, the clean-up of the extract by size-exclusion chromatography (swelling and elution solvent: cyclohexane/ethyl acetate 9/1 *v/v*), the derivatization of monoesters by trimethylsilyl-diazomethane and instrumental analysis by gas chromatography coupled with mass spectrometry. Recovery was in the range of 83-115% and precision was found between 9% and 21%. For phthalate diesters, method detection limits (MDLs) ranged from hundreds of ng/kg to 4.2 µg/kg on a fresh weight milk (f.w.) basis, depending on blank contribution evaluated in matrix. Lower MDLs (0.03-0.8 µg/kg f.w.) were achieved for corresponding monoesters. The proposed method was applied to the determination of target compounds in nine human milk samples and four infant formulas, confirming their presence in all samples. However, a generally higher contamination was assessed in artificial milk than in breast milk samples.

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

1 Introduction

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

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

The endocrine disrupting properties of phthalates, together with their ubiquitous presence in the environment, make the determination of both parent compounds and metabolites of paramount importance in human milk and infant formula, as they represent the unique nourishment for newborns in a crucial developmental period of their life. The determination of phthalate diesters and corresponding metabolites in food intended for infants should comply with toxicological evaluations that assess, for instance, tolerable daily intakes in adults of 10 µg/kg b.w. for di-n-butyl phthalate [12]. In this regard, the omnipresence of phthalate diesters may give rise to blank contributions that strongly affect the actual sensitivity of the analytical method, making difficult the quantification of these analytes in human milk at ppb and especially sub-ppb levels.

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

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

Human milk is a very complex matrix, due to the high content of lipids, mostly represented by esters of fatty acids, which exhibit physicochemical properties similar to target analytes. Accordingly, the analysis of phthalates in milk represents a great analytical challenge. A number of different analytical approaches have been adopted for the analysis of phthalates diesters and/or monoesters in milk samples. In this regard, it is worth mentioning that, when both phthalate diesters and monoesters were analysed, two completely different analytical approaches have been proposed.

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

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

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

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

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

Data concerning the levels of phthalate diesters and their metabolites in milk are necessary for assessing their potential impact on nursing mothers and their children. Accordingly, the main purpose of this study was to develop and validate an extraction and clean-up protocol for the simultaneous determination of both phthalate diesters and monoesters in human milk. The proposed method involved LLE of total fats and phthalates and their separation by SEC. In this regard, it should be remarked that phthalate diesters are hydrophobic compounds strongly partitioned in the lipid phase of milk [24] and therefore the extraction of total fats from milk samples is a recommendable procedure for their reliable analysis. Using this method, we performed for the first time the monitoring of target analytes in various milk samples collected from Tuscan donors of the Human Milk Bank of the Florence Children's Hospital. Furthermore, some infant formula widely commercialized in Italy were analysed, in order to compare the exposure to phthalates due to artificial milk consumption with that associated to breastfeeding.

2 Experimental

2.1 Standards, solvents and materials

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

Labelled phthalate diesters and monoesters were obtained as following specified. Diethyl phthalate (ring-1,2,3,4-d_4) (DEP-d4), di-n-butyl phthalate (ring-1,2,3,4-d_4) (DBP-d4), benzyl butyl phthalate (ring-1,2,3,4-d_4) (BzBP-d4) and di-2-ethyl-hexyl phthalate (ring-1,2,3,4-d_4) (DEHP-d4) were purchased from Cambridge Isotope Laboratories, Inc., whereas di-isobutyl phthalate (ring-1,2,3,4-d_4) (DiBP-d4) and di-isononyl phthalate (ring-1,2,3,4-d_4) (DiNP-d4) were obtained from Toronto Research Chemicals (Toronto, Canada). Mono-ethyl phthalate (ring-1,2-$^{13}C_2$, dicarboxyl-$^{13}C_2$) (MEP-C4), mono-n-butyl phthalate (ring-1,2-$^{13}C_2$, dicarboxyl-$^{13}C_2$) (MBP-C4), mono-benzyl phthalate (ring-1,2-$^{13}C_2$, dicarboxyl-$^{13}C_2$) (MBzP-C4), mono-2-ethylhexyl phthalate (ring-1,2-$^{13}C_2$, dicarboxyl-$^{13}C_2$) (MEHP-C4) and mono-isononyl phthalate (ring-1,2-$^{13}C_2$, dicarboxyl-$^{13}C_2$) (MiNP-C4) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, U.S.A.). Mono-isobutyl phthalate (ring-1,2,3,4-d_4) (MiBP-d4) was supplied by Toronto Research Chemicals. Note that all methyl esters derivatives of phthalate monoesters are indicated throughout the manuscript with the aforementioned compound abbreviation followed by "Me".

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

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

Phosphoric acid 85% and Florisil® were supplied by Merck (Darmstadt, Germany).

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

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

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

Glass fibre filters with nominal porosity of 0.45µm were obtained from Whatman (Springfield-Mill, Kent, UK).

Activated acid aluminium oxide was supplied by Sigma-Aldrich.

2.2 Precautions for minimizing phthalate background contaminations

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

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

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

Purified water was obtained from HPLC grade water by replicated extraction with 3x30 mL of n-hexane. Phosphoric acid 1 M used for esterase inhibition was obtained via dilution of H_3PO_4 85% with purified water.

The commercially available cyclohexane was treated with purified aluminium oxide (30 g of Al_2O_3 per one litre of solvent) by manually shaking for 30 seconds and finally through purified glass fibre filters.

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

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

2.3 Instrumentation

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

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

The GC-MS analysis was carried out with a Shimadzu GCMS-QP2010 Plus mass spectrometer. The gas chromatographic system was provided with AOC-20i auto injectors, equipped by a Shimadzu AOC-20s auto sampler, and a split-splitless (SSL) injector.

2.4 Milk samples and infant formula

A pooled milk sample (hereafter denominated Pool) obtained by mixing the breast milk from four donors recruited by the human milk bank of the “Meyer” Children’s Hospital (Florence, Italy) was used for the development of the analytical method. The milk samples used for the preparation of the Pool were collected by mothers in glass containers containing 5.0 mL of phosphoric acid 1M and kept in the fridge (+4°C) until transported to the laboratory, where the samples were mixed and further stored at -20°C until analysis.

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

2.5 Sample pre-treatment

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

2.6 Extraction of phthalate diesters and monoesters

The extraction protocol is derived from a previously published procedure for the analysis of polycyclic aromatic hydrocarbons (PAHs) in human milk [31], modified by replacing chloroform with dichloromethane and aqueous 0.7% NaCl with 30% NaCl, as part of the extraction mixture. Furthermore, no back-extraction step is required. Briefly: 5 grams of milk, previously acidified with 0.65 mL phosphoric acid 1M, were spiked with a labelled phthalate standard solution (25 µL, approximately 1 µg/mL in methanol), corresponding to about 5 µg/kg fresh weight (f.w.), and vortex mixed for 5 min. The mixture was extracted once with 21 mL of CH_2Cl_2/CH_3OH/NaCl 30% 2/1/0.5 (*v/v/v*) and twice with 6 mL of CH_2Cl_2. The extracts were pooled, dried over anhydrous sodium sulphate and evaporated to a constant weight thus obtaining a fatty residue.

2.7 Clean-up of the extract

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

2.8 Derivatization of phthalate monoesters

The derivatization of phthalate monoesters consisted in the addition of 4 µL of TMSDM and 12 µL of CH_3OH to 50 µL of the F2 fraction of the cleaned-up extract; the obtained mixture was kept at room temperature for 60 min, purified according to the protocol reported by Herrero et al. [32] and finally analysed by GC-MS.

2.9 GC-MS analysis

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

Different oven temperature programs were adopted for the analysis of phthalate diesters and monoesters. As regards phthalate diesters the following temperature program was used: starting 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 at 10°C/min, from 280°C to 320°C at 40°C/min, and finally an isotherm for 2 min. For the analysis of phthalate monoester derivatives, the temperature program was the following: starting 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 at 3°C/min, from 190°C to 320°C at 60°C/min. and finally an isotherm for 5 min. Under these instrumental conditions the elution of phthalate diesters and monoesters was achieved with analysis time of about 10 min and 14 min, respectively (see **Table 1**).

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

2.10 Blank procedure

In order to evaluate the blank contribution to the analysis of phthalate diesters and monoesters, two blanks were analysed along with each batch of samples (usually consisting of 5-8 milk aliquots) and their mean results were subtracted from those of the corresponding samples. In this regard, it should be noted that the analytical response of a certain analyte present in solvents and/or released from materials used for the analysis, might be influenced by the presence of matrix. Accordingly, in this study we calculated the matrix effect of samples (ME%) and if, for a certain analyte within a batch, a blank chromatographic area (A_{blank}) with signal-to-noise ratio (s/n) > 3 was found, this area was corrected for ME% by applying the equation (1), thus obtaining a chromatographic area accounting for the effect of blank contribution in the matrix (A^{ME}_{blank}).

$$A^{ME}_{blank} = A_{blank} + \left(A_{blank} \cdot ME\%\right) \quad (1)$$

Accordingly, only chromatographic areas which were at least double, compared to A^{ME}_{blank} were taken into account for compound detection and quantification.

Among the analytes investigated in human milk samples and infant formula, DiBP (0.79-1.08 µg/Kg), DBP (0.27-0.36 µg/Kg) and DEHP (0.88-1.14 µg/Kg) were detected in the blanks.

2.11 Quantification

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

2.12 Data analysis

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

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

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

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

3 Results and Discussion

3.1 Optimisation of the liquid/liquid extraction

The method herein adopted for fat extraction from human milk samples was a modification of a protocol previously applied to the analysis of PAHs in breast milk [31]. More in detail, the three-component extraction mixture, based on $CHCl_3$/CH_3OH/0.7% NaCl aqueous solution 2/1/0.5 (*v/v/v*), was modified by replacing $CHCl_3$ with CH_2Cl_2, the latter being less toxic, and the concentration of the aqueous NaCl solution was increased up to 30% in order to improve the phase separation, thus making unnecessary the final back-extraction step.

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

3.2 Optimisation of the clean-up procedure

SEC was adopted as clean-up technique for the separation of phthalate diesters and monoesters from the fat residue, in turn derived from the liquid/liquid extraction of milk samples. Bio-Beads® S-X3 and S-X8 stationary phases, which are neutral, porous styrene divinylbenzene polymers with 3% and 8% average degree of crosslinking, were tested to this aim. A wide range of swelling volumes were obtained by using different solvents, highlighting that very different size exclusion properties can be provided by these resins, depending on the swelling solvent used. As a general consideration, S-X8 swelled less than S-X3; for instance, using benzene as swelling solvent, bed volumes of about 3.0 and 4.5 mL/g were obtained with the former and the latter resins, respectively. Among the solvents tested, benzene, toluene and dichloromethane provided the highest swelling volumes (about 4.5 mL/g for Bio-Beads® S-X3), whereas cyclohexane and ethyl acetate gave rise to a lower swelling (about 3.0 mL/g for Bio-Beads® S-X3). Furthermore, it is possible to achieve a more accurate modulation of the resin swelling by using binary solvent mixtures, which may in turn also allow for obtaining separation performance better matching the chromatographic problem under examination. In this regard, it should also be noted that the solvent adopted for resin swelling and packing, as well as for isocratic elution, should be the same, in order to avoid shrinking or expansion of the resin during the analysis.

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

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

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

Fig. 2 illustrates the chromatograms obtained with the S-X3 resin, under the experimental conditions described in paragraph 2.6, for: (A) a phthalate diester standard solution (containing DEP, BzBP, DBP, DiBP, DEHP and DiNP dissolved in cyclohexane/ethyl acetate 9/1 (*v/v*); total injected amount = 50 μg), (B) a phthalate monoester standard solution (containing MEP, MBzP, MBP, MiBP, MEHP and MiNP dissolved in cyclohexane/ethyl acetate 9/1 (*v/v*); total injected amount = 5 μg), and (C) 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*).

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

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

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

3.3 Optimisation of the derivatization of phthalate monoesters

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

Much homogeneous signals were observed for all target analytes dissolved in the aforementioned solvent mixture, by using TMSDM and methanol, as derivatization reagents. In this regard, it should be remarked that with this derivatization reagent the reaction proceeds spontaneously at room temperature. Accordingly, methanolic TMDSM was chosen for proceeding with the optimization of the derivatization procedure. Derivatization tests were performed on 50 µL of the 2nd fraction of the cleaned-up extract of the Pool, spiked with the labelled phthalate monoesters (see paragraph 2.1) at 5 µg/kg, by using different TMSDM-to-methanol ratios and a fixed derivatization time of 90 min. Under these conditions, the best results were obtained with 4 µL of TMSDM and 12 µL of CH_3OH.

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

3.4 Figures of merit of the proposed method

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

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

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 and monoesters, respectively. More in detail, the highest limits were achieved for DBP (1.4 µg/kg), DiBP (3.1 µg/kg) and DEHP (4.2 µg/kg), as the result of the contributions determined for these analytes in procedural blanks and above all owing to their correction for signal enhancement in matrix (see paragraph 2.10).

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

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

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

3.5 Phthalate diesters and monoesters in human milk and infant formula

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

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

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

Our data evidenced that among the phthalate diesters, DEHP and DiBP were the predominant compounds in four samples each, whereas in one case the highest concentration was exhibited by DiNP. In agreement with our results, the prevalence of DEHP in breast milk has been evidenced by other authors [13, 20, 21]. Conversely, for the branched butyl phthalate a lesser presence has been reported in the only study investigating its occurrence in human milk [21]. To the best of our knowledge, DiNP has never been investigated before in human milk; however, data elsewhere reported regarding the occurrence of MiNP in Danish and Finnish women [18], suggest a high abundance in breast milk of the corresponding diester, as well.

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

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

3.6 Phthalate intake estimation and risk evaluation for newborns

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

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

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

4 Conclusions

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

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

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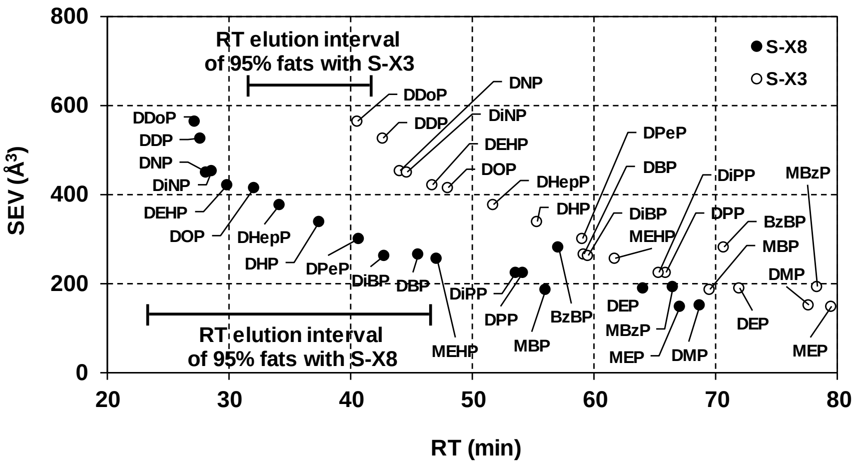

Figure 1 – Retention time (RT, min) of phthalate diesters and monoesters on Bio-Beads® S-X8 and S-X3 size exclusion stationary phases swelled and eluted with cyclohexane/ethyl acetate 9/1 (*v/v*), as a function of the Connolly solvent excluded volume (SEV, Å^3). Horizontal bars indicated the RT interval 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".

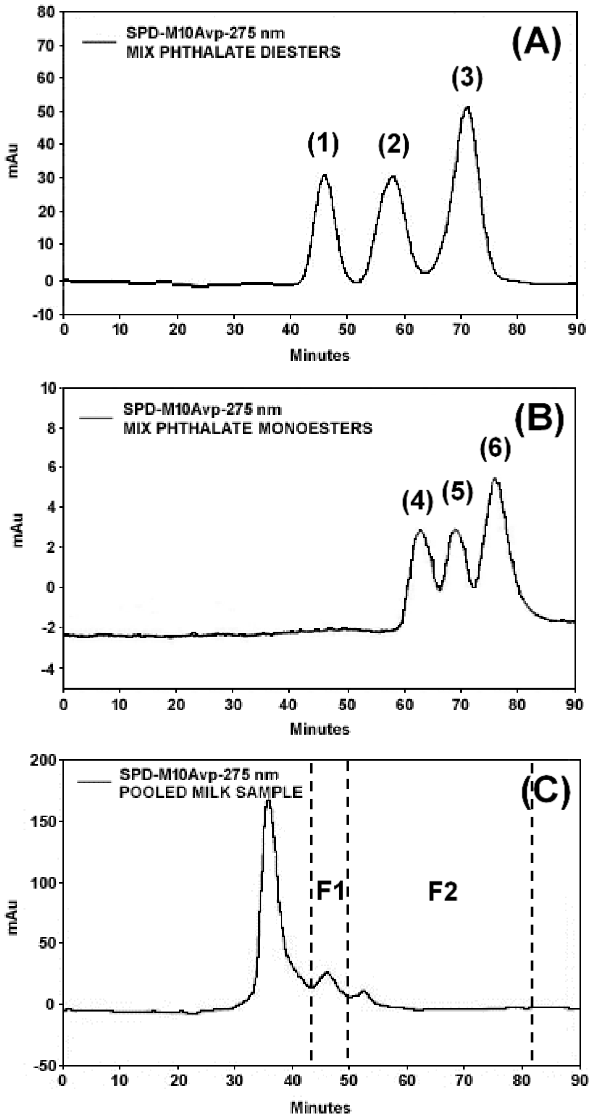

Figure 2 – Size exclusion chromatograms obtained on Bio-Beads® S-X3 with cyclohexane/ethyl acetate 9/1 (*v/v*) as swelling and elution solvent. (A) Standard solution of phthalate diesters (peak 1: DiNP and DEHP; peak 2: DiBP and DBP; peak 3: BzBP and DEP; 1 mL injected; total phthalate diester concentration = 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.

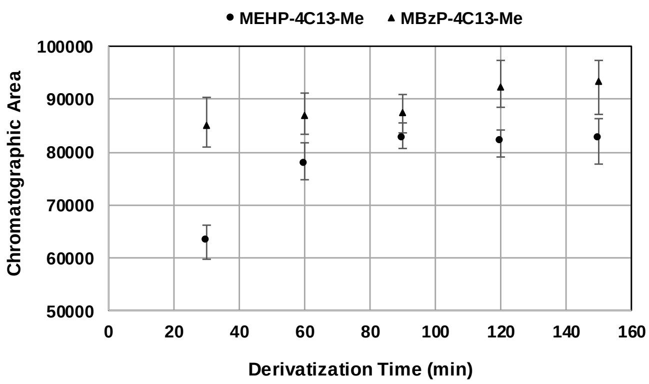

Figure 3 – Chromatographic areas of quantifier ions of MEHP-4C13-Me (m/z 167) and MBz-4C13-Me (m/z 93) (see section 2.1 "Standards, solvents and materials" for the meaning of compound abbreviations) as a function of derivatization time. Derivatization conditions: 50 µL of the second fraction of the cleaned-up extract of the Pool, spiked with labelled phthalate monoesters at 5 µg/kg, derivatized with 4 µL of TMSDM and 12 µL of CH_3OH.

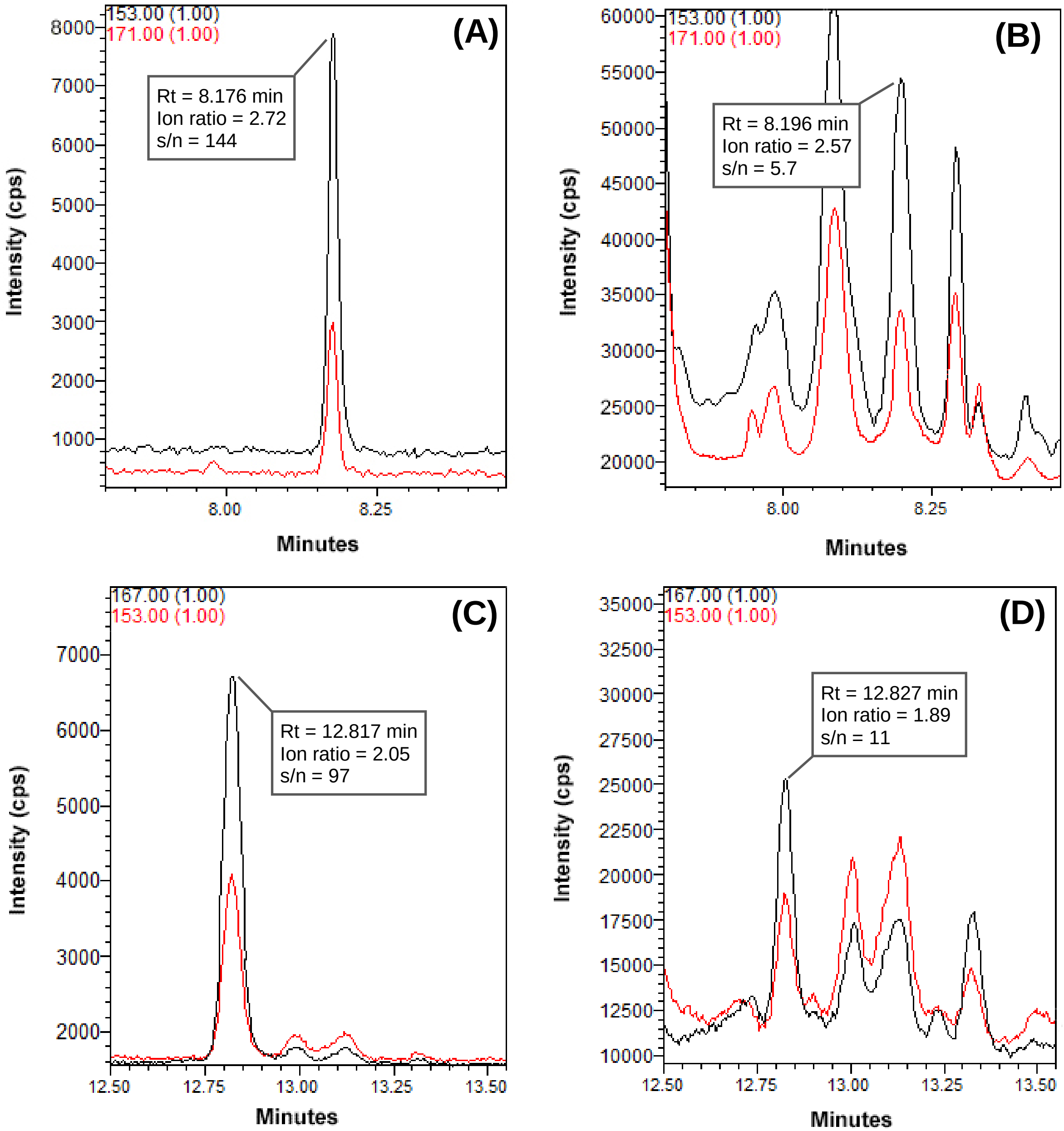

Figure 4 – Overlapped SIM quantifier (*black line*) and qualifier (*red line*) ions, retention time (Rt), quantifier-to-qualifier ion ratio and signal-to-noise ratio of: (A) DEHP-d4 standard (1.0 µg/L); (B) DEHP-d4 in the Pool of human milk samples spiked with MEHP-4C13 at 1.0 µg/kg level; (C) methyl 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.

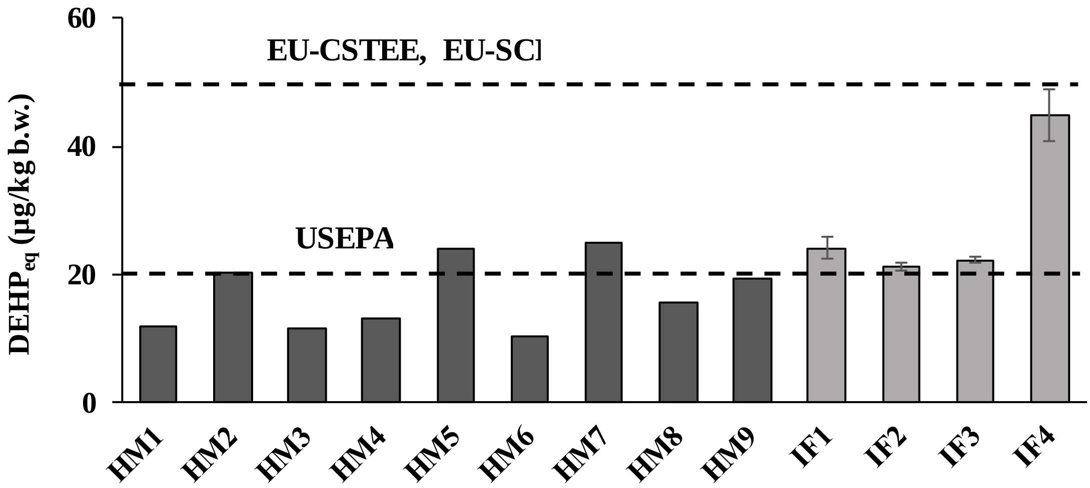

Figure 5 – Total daily intakes expressed as µg of di-2-ethylhexyl phthalate equivalent (DEHPeq) per kg of body weight (b.w.), compared to the maximum acceptable daily intakes (dotted lines) proposed by the European Scientific Committee on Toxicity, Ecotoxicity and the Environment (EU-CSTEE), European 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 (μg/kg milk)	Linear range (μg/kg milk)[a]	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

[a] 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 addiction 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 [a]	3.1[b]	1.4[b]	0.7	4.2[b]	0.5	0.7	0.8	0.03	0.3	0.5	This study
HM and IF	LLE + SPE	GC-MS	0.033[c]	0.033[c]	1.6[c]	0.16[c]	n.r.[d]	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[n]	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]

[a] Phthalate monoesters were analysed after derivatizion.
[b] These limits take into account of blank contribution, corrected for the signal enhancement due to the matrix effect.
[c] These limits were extrapolated by dividing the quantification limits reported in the manuscript for three.
[d] DiNP was investigated but its quantification and detection limits were not reported in the manuscript.

Table 4 – Mean phthalate concentrations (μg/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
Phthalate diesters			
DiBP	37	11 - 77	9/9
DBP	7.1	< 3.5[a] - 19	9/9
BzBP	1.7	< 2.0[a] - 3.2	9/9
DEHP	34	< 13[a] - 94	9/9
DiNP	20	6.3 - 51	9/9
Phthalate monoesters			
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

[a] 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 products1996, 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