EXPOSURE TO DI-2-ETHYLHEXYL PHTHALATE, DI-N-BUTYL PHTHALATE AND BISPHENOL A THROUGH INFANT FORMULAE

Teresa Cirillo[1], Giuseppe Latini[2], Maria Antonietta Castaldi[3], Lucia Dipaola[2], Evelina Fasano[1], Francesco Esposito[1], Gelsomina Scognamiglio[1], Fabio Di Francesco [4,2], Luigi Cobellis[3]

[1]*Department of Agriculture, "Federico II" University, Via Università, 100–80055 Portici, Naples, Italy.*

[2]*Clinical Physiology Institute, National Research Council of Italy (IFC-CNR), Italy.*

[3]*Department of the Woman, the Child, and General and Specialized Surgery, Second University of Naples.*

[4]*Department of Chemistry and Industrial Chemistry, University of Pisa*

Running title: DEHP, DnBP and BPA in Infant Formulae

Corresponding Author: Teresa Cirillo Department of Agriculture - via Università, 100-80055 Portici - Naples, ITALY; e-mail: tcirillo@unina.it, Phone:+390812539450 Fax:+390812539407.

Abstract

Background: Phthalates and Bisphenol A (BPA) are ubiquitous contaminants identified as endocrine disruptors. Phthalates are worldwide used as plasticizers, in particular to improve the mechanical properties of polymers such as polyvinyl chloride. Since they are not chemically bound to the polymer, they tend to leach out with time and use. Di-2-ethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DnBP) are two most common phthalates. BPA is an estrogenic compound used to manufacture polycarbonate containers for food and drink, including baby bottles. It can migrate from container into foods, especially at elevated temperatures. Diet is a predominant source of exposure for phthalates and BPA, especially for infants.

Objective: to test the presence of DEHP, DnBP and BPA in infant formulae.

Methods: DEHP, DnBP and BPA concentrations were measured in 22 liquid and 28 powder milks by gas chromatography with flame ionization detection and high performance liquid chromatography with fluorimetric detection respectively.

Results: DEHP concentrations in our samples were between 0.005 and 5.088 µg/g (median 0.906 µg/g), DnBP concentrations were between 0.008 and 1.297 µg/g (median 0.053 µg/g), and BPA concentrations were between 0.003 and 0.375 µg/g (median 0.015 µg/g). Concentrations of the investigated contaminants in liquid and powder milks were not significantly different, even though samples were packed in different types of containers.

Conclusions: These data point out potential hazards for infants fed with baby formulae. Contamination seems more related to the production of formulae than to a release from containers.

Key Words

Di-2-ethylhexyl phthalate, di-n-butyl phthalate, bisphenol A, infant formula.

Introduction

Endocrine Disruptors (EDs) are chemicals known to mimic steroid hormones' action and to interfere with the synthesis, secretion, transport, activity or elimination of natural hormones [Cobellis et al. 2003, Jenkins et al. 2009, Habert et al. 2009, Latini et al. 2006, Latini et al. 2010]. In particular, they modify the programming of the normal endocrine-signaling pathways during pre- and early post-natal life, thus determining adverse health effects such as neurological and immune effects, reproductive disorders, cancers, lowered fertility and increased incidence of endometriosis [Cobellis et al. 2003, Jenkins et al. 2009, Habert et al. 2009, Latini et al. 2006, Latini et al. 2010]. Recent papers show that EDs pose the greatest risk during prenatal and early postnatal development, when organ and neural systems are forming [Jenkins et al. 2009, Habert et al. 2009]. The possible relationships between combined exposures to environmental contaminants and diseases are now attracting attention, especially if they occur early in life [Sathyanarayana et al. 2013, Wang et al. 2014]. Recently, some studies correlated the combined exposure to phthalates and BPA with human health risks [Sathyanarayana et al. 2013, Wang et al. 2014].

Phthalates are widely used in many products to impart softness, flexibility, transparency and longevity to an otherwise rigid polyvinyl chloride (PVC). Since there is not a chemical bond with the polymer, they leach out with time and use, thus becoming ubiquitous environmental contaminants [Latini 2005]. Di-2-ethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DnBP) are two of the most common phthalates [Latini 2005]. Human exposure occurs through ingestion, inhalation, and dermal contact during the whole lifetime, including intrauterine life, but exposure in children exceeds that in adults. Phthalates determine toxic effects in laboratory animals, especially on the developmental and reproductive systems [Sun et al. 2006]. Human studies correlated phthalate exposure with adverse health effects such as liver, kidney and lung damage as well as sexual developmental abnormalities [Cobellis et al. 2003, Latini et al. 2006a, Latini et al. 2006b, Yavaşoğlu et al. 2012, Lovekamp-Swan and Davis 2003 1, 5, 10-12]. Moreover, phtalates may alter the methylation status of DNA and consequently the DNA sequence itself, thus transmitting these

effects to future generations [Singh and Li 2012]. Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl) propane, is at the same time an estrogenic compound and a main monomer for the synthesis of polycarbonate and epoxy resins. Polycarbonate is used for many products like water and baby bottles, children's toys, sport equipment, medical and dental devices etc., whereas coatings of many food and beverage containers consist of epoxy resins [Jenkins et al. 2009, Latini et al. 2005]. BPA tends to migrate from cans containers into foods, especially at elevated temperatures [Jenkins et al. 2009, Oldring et al. 2014]. As a consequence, potential risks of exposure to BPA raised concern over the years due to suspicion to affect reproduction, development, and metabolism. There is a consensus that infants are at the greatest risk of harm, even with a low level exposure to BPA [Jenkins et al. 2009]. Recent studies of National Toxicology Program (NTP) and US Food and Drug Administration (FDA) pointed out the potential BPA effects on brain, behavior, and prostate gland in fetuses, infants, and young children [FDA 2010]. Indeed, BPA can affect the hormone-mediated neurologic and behavioral development in early life [Bashore et al. 2001, Chapin et al. 2008, FDA 2010, Hengstler et al. 2011, Vom saal and Hughes 2005]. In addition, high BPA exposure has been associated with heart disease, diabetes, abnormally high levels of liver enzymes, and alterations of the thyroid function [Belcher et al. 2012, Rubin 2011, Sriphrapradang et al. 2011]. For these reasons, BPA containing baby bottles have been banned in Europe since March 2011 [Commission Directive 2011/08/EU].

Diet remains the predominant source of exposure for both phthalates and BPA especially for infants, since these compounds have been found in breast milk and in baby formulae [Cirillo et al. 2011, Cirillo et al. 2013, Latini et al. 2004, Latini et al. 2009]. The present paper analyzed the presence of DEHP, DnBP and BPA in infant formulae to assess possible neonatal exposure and reduce the gap of knowledge in this field.

MATERIALS AND METHODS

Sampling

Fifty infant formula samples were collected at different neonatal nurseries in Naples hospital during three months (May-July 2013). Liquid ready to use (n=22) and powder (n=28) milk samples were collected. Among them, there were 7 special milk samples, i.e. milks for infant with gastrointestinal problems (n=3), rice milk formulae (n=3) and a premature formula. Liquid samples were packed in polyethylene terephthalate (PET) and Tetrapak™, whereas milk powders were contained in aluminum (Al) containers.

The infant formula samples were collected in glass vials and rapidly transferred to the laboratory of the Department of Agriculture, where analytical samples were obtained for the different procedures.

All samples were labelled. For DEHP and DnBP analysis, aliquots (15 mL) of liquid milk were lyophilized and stored a -18°C until analyses, whereas powder sample aliquots (1 g) were just stored in the dark. For BPA determination, aliquots (5 mL) of liquid milk were stored at -18°C until analyses, whereas powder samples aliquots (2 g) were reconstituted with HPLC water (15 mL), split into 5 mL aliquots and stored at -18°C until analyses. For each reconstituted vial, an additional 5 mL vial with HPLC water was stored at -18°C as negative control to avoid possible bias due to a contamination of HPLC water.

DnBP and DEHP

Chemical reagents

Acetonitrile, n-hexane, acetone for organic trace analysis and anhydrous Na_2SO_4 were supplied by Merck (Darmstadt, Germany). Florisil (60/100 mesh) was furnished by Supelco (Bellefonte, PA, USA), and Bondesil (PSA 40UM) by Varian (Palo Alto, CA, USA). Standard solutions of DnBP and DEHP were purchased from Sigma Aldrich (St. Louis, MO, USA).

Instrumental parameters

The analyses of phthalates (PAEs, Phthalic Acid Esters) were carried out by a Shimadzu GC-17 (Shimadzu, Kyoto, Japan) capillary gas chromatography with a Flame Ionization Detector (GC-FID) and an HP-5 (Crosslinked 5% PHME Siloxane, 30 m length, 0.32 i.d., 0.25 μm film thickness) glass-capillary column. Helium was used as carrier and a hydrogen/air mixture was used to sustain the flame. The volume of injection was 1 μl in splitless mode, the injector and detector temperatures were 260°C and 310°C respectively. The temperature program was 100°C for 1 min, increase of 15°C/min up to 280°C, retention of this temperature for 10 min.

DnBP and DEHP measurement

Because of PAE ubiquity, any contact with plastic was avoided. All the glassware was thoroughly washed, rinsed twice with acetone and n-hexane, heated at 250°C for 2 h and finally stored away from any environmental contamination.

In accordance with the method by Cirillo et al. 2013, the lyophilized samples were: 1) extracted three times with 15 mL of acetonitrile in an ultrasound bath for 15 min, 2) centrifuged at 2000 rpm for 10 min and the acetonitrile layer transferred to a separatory funnel, 3) added with 10 mL of n-hexane saturated with acetonitrile and the funnel was vigorously shaken for 5 min. The acetonitrile phase was transferred into a flask and dried under vacuum at 55°C. The extracts were reconstituted by 5 mL of n-hexane and purified through a column containing 2 g of Florisil activated for 2 hours at 200°C, 0.5 g of Bondesil and 1 g of anhydrous Na_2SO_4. The column was eluted three times with 10 mL of n-hexane/acetone mixture (100:5 v/v). The eluates were collected in a flask, evaporated under vacuum at 40°C and reconstituted with 1 mL of n-hexane for GC analysis.

The calibration curves were obtained using standard solutions at 0.625, 1.250 and 2.500, 5.00 and 10.00 μg/mL for DEHP, and at 0.312, 0.625, 1.250, 2.500 and 5.00 μg/mL for DnBP. The regression coefficients (R) were >0.99 for both contaminants. The PAE concentrations in the

samples were obtained by comparing the relevant peak areas with calibration curve.

Limits of Detection (LODs) and Quantification (LOQs) were evaluated as the mean blank value plus three blank standard deviations and three times the LOD. LODs and LOQs were 5.0 ng/g and 15.0 ng/g for DEHP, and 7.5 ng/g and 22.5 ng/g for DnBP respectively.

A run without sample was carried out every six determinations to reduce the instrumental background due to contamination. Moreover, solvents used to wash the syringe were frequently replaced.

The intra- and inter-day repeatability of the method were evaluated by injecting standard solutions at three different concentration levels (2.50, 5.00 and 10.00 µg/mL for DEHP and 1.25, 2.50 and 5.00 µg/mL for DnBP) five times during a day (intra-day) and during five consecutive days (inter-day). The intra-day repeatability ranged from 7.0 to 9.5% for DEHP and from 5.5 to 8.5% for DnBP, whereas inter-day repeatability varied from 6.0 to 8.5% for DEHP and from 4.5 to 6.5% for DnBP.

Samples with DEHP and DnBP concentrations lower than LOD were used for recovery tests. Three liquid and three powder milk samples (each in triplicate) were spiked with standard solutions at concentration 2.0, 4.0 and 8.0 µg/mL for DEHP and 1.0, 2.0 and 4.0 µg/mL for DnBP, and then processed as milk samples. Recoveries were for 98 ± 10 % for DEHP and 98 ± 9 % for DnBP.

Because of the ubiquity of these compounds, a blank sample (only solvents) for each batch was analysed and the average concentration value was subtracted from PAE detected values.

Bisphenol A

Chemical reagents

Acetonitrile, methanol and water (HPLC grade) were supplied by Merck (Darmstadt, Germany). Solid phase extraction cartridges (Bond Elut C18 SPE, 1g/6mL) were purchased from Agilent Technologies (Palo Alto, CA, USA). A BPA standard (purity ≥ 99%) was purchased from Sigma

Aldrich (St. Louis, MO, USA).

Instrumental parameters

BPA detection was performed through an HPLC (LC-10AT VP Shimadzu, Kyoto, Japan) equipped with a fluorescence detector (Shimadzu RF-10A XL) and a reversed-phase column (Ascentis C18. L × I.D.: 15 cm × 4.6 mm; particle size: 5 μm, Supelco, Bellefonte, PA). The column was kept at a constant temperature of 40°C. The mobile phase consisted of 60% of acidified water (1% of acetic acid), 35% of acetonitrile and 5% of methanol. The flow rate of mobile phase was set at 0.950 mL/min (isocratic run). The fluorimetric detection was carried out at an excitation wavelength of 275 nm and an emission wavelength of 305 nm.

BPA measurement

BPA measurement was performed by adapting the procedure by Sun et al. 2006. An aliquot of each sample (5 mL) was inserted into a 250 mL glass round-bottom flask and added with acetonitrile (20 mL). Flasks were placed onto a Heidolph Promax 2020 shaker for 25 min. The content of each flask was then filtered through a filter paper and transferred into a separatory funnel. The flask was rinsed with 5 mL of acetonitrile, which were added to the funnel. Afterwards, 35mL of n-hexane were also added to the separatory funnel, and the resulting mixture was shaken for 25 min. The acetonitrile layer was removed from the funnel and stored in a round-bottom flask, whereas the hexane layer was washed twice with acetonitrile (firstly with 15 mL, then with 10 mL) which was collected and added in the same round-bottom flask. The solvent was removed from the extract through a rotavapor, then the flask was washed with 3 mL of a methanol: water (5:95 v/v) solution to be processed by solid phase extraction. SPE cartridges were firstly conditioned with 5 mL of methanol and then with 5 mL of water. Later the sample was loaded, and the elution was carried out at a flow rate of 3-4 mL/min using a Supelco Visiprep SPE vacuum manifold. The cartridges were then

washed with 2 mL of a methanol:water solution (30:70 v/v) and dried under vacuum pump for 1 min. Finally, the BPA retained in the cartridge was eluted with 3 mL of a methanol:water (80:20 v/v) solution. The eluate was dried by a rotavapor, dissolved with 1 mL of methanol and finally collected in an amber vial before the HPLC analysis.

A calibration curve with a correlation coefficient of 0.998 was obtained by injecting standard solutions of BPA at concentrations 10.0, 20.0, 30.0, 40.0 and 50.0 µg/L. An instrumental LOD equal to 0,003 µg/g dry weight (dw) was calculated using the standard deviation of the response (σ) and the slope of the calibration curve (S) according to the formula: 3.3 σ/S. Similarly, a LOQ equal to 0,009 µg/g dw was calculated as: 10 σ/S.

Recovery percentages at three concentration levels were assessed on six samples (3 liquid and 3 powder milk samples with BPA level below the LOD) by spiking each sample with BPA solutions in methanol at concentrations 50.0, 100.0 and 1000.0 µg/L. The recoveries were 87 ± 3%. BPA quantification was performed comparing the peak areas obtained in the samples with the BPA standard calibration curve.

For each batch of samples, a blank sample was processed according to the procedures mentioned before. A total of 16 blanks were analyzed and all of them showed BPA concentrations well below the LOD value.

BPA confirmation by LC MS/MS

Since BPA measurements could be affected by matrix related interferences, a confirmation by LC MS/MS was carried out according to the Shao et al. 2005 method.

Instrumental parameters

Identification was carried out using an alliance 2695 (Waters, USA) liquid chromatography equipped with a Quattro Ultima Pt (Micromass, UK) tandem mass spectrometer and a symmetry C-18 column (150mm×2.1mm i.d., 3.5m). The temperature of the column oven was set at 40 °C, the

flow rate was 0.2 mL/min and the injection volume was 10 µL. Mobile phases consisted of methanol and water with 0.1% ammonia. The methanol was linearly increased from 10 to 55% in 10 min, then increased to 85% in 10 min and held for 7.5 min, finally brought back to 10% and held for 15 min before the following injection. The mass spectrometer was operated in negative mode electrospray ionization in multiple-reaction monitoring (MRM) mode. The capillary voltage was 3.5 kV, the cone voltage was 70V and the multiplier voltage was 650V. Nitrogen was used as nebulizing, desolvation and cone gas. In particular, the nebulizing gas was adjusted to the maximum, whereas the flow of the desolvation gas and cone gas were set to 550 L/h and 80 L/h respectively. The source temperature and the desolvation gas temperature were held at 100 and 300 ◦C respectively. The RF lens 1 and 2 were set at 50 and 0.5, the ion energy 1 and ion energy 2 were both 0.5, the entrance and exit were zero, the collision gradient was 3.2 eV. UHP argon was used as the collision gas for the tandem mass spectrometric analysis, and the pressure in the collision chamber was kept at 2.8×10−3 mbar.

A calibration curve in the concentration range 1 to 100 ng/g was obtained by linear regression of the normalized (to the internal standard area) standard solution areas against BPA concentrations. The correlation coefficient was ≥ 0.999.

The intra- and inter-day repeatability of the method were evaluated by injecting standard solutions at three different concentration levels (10, 50 and 100 ng/g) five times during a day (intra-day) and during five consecutive days (inter-day). The intra-day reproducibility ranged from 4.0 to 6.5%, while inter-day reproducibility varied from 4.5 to 6.2%.

Statistical analysis

A power calculation was undertaken to determine an appropriate sample size for this study. Based on literature data [MAFF 1998], considering DEHP as the most abundant phthalate in infant formula, a two-sided test power calculation was performed. Two double of the range value was used as the sigma (0,780 µg/g dry weight). This power calculation indicated that 11 samples in each

group would be necessary to detect a 15% difference in the DEHP concentration with a power of 80% at a 5% level of significance.

Data distribution was assessed with the Shapiro Wilk's test. One-way ANOVA was performed with SPSS 20.0 software (IBM) to assess the differences between DEHP, DnBP and BPA concentrations in liquid and powder milks. Significance was set at $p < 0.05$. The concentrations below LOD were assumed to be equal to LOD.

Dietary intake assessment for Italian infants (age 0–4 months)

Daily intake was estimated as:

Intake = (C concentration × V volume of milk per day) /BW body weight (1)

to evaluate DEHP, DnBP and BPA exposure of young children through artificial milk.

Dietary exposure was calculated using the blueprint to the budget method (BM) model [WHO 2009] in accordance with FAO/WHO, and with the help of weight growth charts by WHO 2006 and pediatric nutrition suggestions for our range of age. We considered two possible scenarios: 1) median concentrations of contaminants, infants with average weight to development at the 50th percentile or at the 95-97th percentile (according to the growth curve by WHO (2006)) who introduce daily a medium quantity of milk (medium case); 2) maximum concentrations of contaminants, children who have grown at the 50th percentile or at the 95-97th percentile and introduce daily a higher quantity of milk (worst case).

RESULTS

Most milk samples showed detectable levels of DEHP (92%, 86% of liquid and 96% of powder milks), DnBP (90%, 82% of liquid milks and 96% of powder milks) and BPA (58%, 52% of liquids milks and 67% of powder milks) (**Table 1**).

The average concentration of DEHP in all milk samples was 1.327 ± 0.724 μg/g dw, and in particular it was 1.112 ± 0.716 μg/g dw in liquid milks and 1.496 ± 0.729 μg/g dw in powder milks.

For DnBP, the average concentration in all milk samples was 0.354 ± 0.305 μg/g dw, namely 0.384 ± 0.385 μg/g dw in liquid milks and 0.330 ± 0.229 μg/g dw in powder milks. The average concentration of BPA in all milk samples was 0.021 ± 0.022 μg/g dw, it was 0.019 ± 0.037 μg/g dw in liquid milks and 0.023 ± 0.028 μg/g dw in powder milks (**Table 1**).

DEHP concentrations varied from 0.092 to 3.552 μg/g (median=1.136 μg/g), DnBP concentrations from 0.008 to 1.624 μg/g (median=0.244 μg/g) and BPA concentrations from 0.003 to 0.169 μg/g (median=0.008 μg/g) (**Table 1**).

Similar concentrations of the three analytes were found in liquid and powder milks, even though containers were of different types (**Figure 1**). DEHP, DnBP and BPA concentrations in the HLPC water samples stored as negative controls for reconstituted powder milk were below the LODs.

The concentration of DEHP, DnBP and BPA in liquid and powder milks together with the type of packaging are reported in **Table 2** and **3** respectively**.**

Estimates of dietary exposure to DEHP, DnBP and BPA in the medium and worst case are shown in **Table 4** and **5**. The daily intake of DEHP in the medium case ranged from 19.84 to 24.85 μg/kg bw day at 50th percentile and from 17.63 to 19.14 μg/kg bw day at 97th percentile. In the worst case, DEHP intake varied between 42.57 at 54.68 μg/kg bw day at the 50th percentile and between 38.80 at 46.52 μg/kg bw day at the 97th percentile (**Table 4-5**). Estimates of dietary exposure to DnBP in the medium case ranged from 4.15 μg/kg bw day to 5.34 μg/kg bw day at the 50th percentile and from 3.79 μg/kg bw day to 4.54 μg/kg bw day at the 97th percentile. In the worst case, the DnBP intake varied between 13.62 and 17.50μg/kg bw day at the 50th percentile and between 12.41 and 14.89 μg/kg bw day at the 97th percentile (**Table 4** and **5**). BPA intake in the medium and worst case are shown in **Table 4** and **5**. In the medium case, values ranged from 0.14 to 0.17 μg/kg bw day at the 50th percentile and from 0.12 to 0.15 μg/kg bw day at the 97th percentile. In the worst case, the BPA intake varied between 0.99 and 1.27 μg/kg bw day at the 50th percentile and between 0.90 and 1.08 μg/kg bw day at the 97th percentile. Both in the medium and worst case the highest intake occurred at the 30th day of life, because the amount of consumed milk starts increasing while

baby's weight is still pretty low. As for BPA, for both DnBP and DEHP the higher values of intake occurred in children at 30 days of age (**Tables 4-5**).

DISCUSSION

Our data indicate the presence of DEPH, DnBP and BPA in infant formulae. Data relevant to all contaminants showed a wide variability but we found no significant concentration differences for the investigated contaminants between liquid and powder milks, even though samples were packed in different types of containers. This finding would suggest the DEHP, DnBP and BPA contamination to arise from raw materials or manufacturing processes rather than from packaging. Phthalates, in particular, may contaminate milk during the production or preparation of formulae. A main source of contamination results from migration of phthalates from products in contact with food during processing. A number of studies concerned the migration of DEHP from the PVC tubing of the milking machine used in dairy farms [Castle et al. 1990, Feng et al. 2005, Ruuska 1987]. PVC tubing contains up to 40% DEHP by weight. A Norwegian study showed a clear difference in DEHP levels between raw milk collected by hand milking (about 5 μg/kg) and machine milking involving PVC tubing (30 μg/kg in milking chamber and 50 μg/kg in collection tank) [Feng et al. 2005].

Dietary intake assessment for Italian infants (age 0–4 months)

In order to assess post-natal exposure to phthalates and BPA, the estimation of daily dietary intake of these contaminants was carried out in 0-4 month old children, as milk is the only food introduced in this age group.

Four possible nutrition scenarios were possible, namely nutrition with infant powder, liquid formula, breast milk or a combination of these, but we only considered artificially fed babies assuming liquid or powder formulas (or both). The European Food Safety Authority (EFSA) established a Tolerable Daily Intake (TDI) of 50 μg/kg bw for DEHP and 10 μg/kg bw for DnBP [EFSA, 2005a; 2005b]. As expected, the highest intakes of DEHP and DnBP were estimated among infants with growth at the 50th percentile, who have a lower body weight than those at the 97th percentile.

Daily intake of DEHP in the medium case varied between 20-25% and 18-21% of TDI at 50th and 97th percentile respectively. In the worst case, intake was also lower than TDI, except for the 50th percentile infants aged 30 and 45 days (**Table 5**).

Daily intake of DnBP in the medium case varied between 42-53% and 38-45% of TDI at 50th and 97th percentile respectively. In the worst case, instead, intake always exceeded TDI, up to 175%.

Muller et al. 2003 estimated for 0-6 month old Danish infants a daily intake via infant formulae of 9.8 μg/kg bw/day for DEHP and 16.4 μg/kg bw/day for DnBP [Muller et al. 2003]. Our values for DEHP intake were higher than Muller's both in the medium and worst case, whereas DnBP intake levels were lower in the medium case and similar in the worst case. Our estimates of DnBP and DEHP daily intake were higher than those reported by MAFF (1998) for infants 0-3 months old, i.e. 13 μg/kg bw/day for DEHP and 2.4 μg/kg bw/day for DnBP.

Our estimated BPA daily intakes were well below the temporary Tolerable Daily Intake (t-TDI) established by EFSA in 2014 (5.0 μg/kg bw) [40]. In the medium case, our intakes ranged 2.8-3.4% and 2.4-3.0% of t-TDI for the 50th and 97th percentile respectively, which increased in the worst case to 20-25% and 18-22% of t-TDI for the 50th and 97th percentile respectively.

EFSA t-TDI for BPA refers to the adult population, and there isn't a specific TDI for children or infants. Diet is the main source of exposure to BPA in infants aged 0-4 months [EFSA 2014]. Minor pathways of introduction could be the inhalation or ingestion of dust, the dermal contact and the mouthing of toys. Until a few years ago, babies could introduce BPA from polycarbonate baby bottles, especially when bottles were heated and reused multiple times [Jenkins et al. 2009, Wang et al. 2014]. The EU Regulation No. 321/2011 imposed not to use BPA in the manufacture of baby bottles, thus reducing exposure.

In 2008, a report of the U.S. National Toxicology Program (NTP) provided daily exposure estimates for infants, children and adults based on realistic scenarios [NTP 2008]. The highest daily exposure to BPA was estimated to occur in infants and children. Formula-fed infants (0-6 months of age) had estimated daily intakes of 1-11 μg/kg bw.

In 2010, the FAO and WHO jointly held an Expert Meeting on BPA, whose final report was published in 2011 [FAO/WHO 2011]. The report identified 0-6 month infants fed with liquid formulae in polycarbonate bottles as the sub-population with the highest dietary exposure to BPA, namely 2.4 μg/kg bw per day (average) and 4.5 μg BPA/kg bw per day (95th percentile).

In 2012, a probabilistic exposure assessment using data from recent Canadian surveys suggested that daily exposure to BPA in children ranged from 0.083 μg/kg bw (0-1 month old) to 0.164 μg/kg bw (children 4-7 months of age) [Health Canada 2012].

Our data resemble those of Health Canada but are lower than those of NTP and FAO/WHO, probably because the problem of BPA migration from baby bottles in Europe has been solved.

The different packages (Tetrapack™, PET and aluminum) represent a possible bias of the present study. However, the studied contaminants can be found not only in Tetrapack™ and PET but also in aluminum packages, as these are often internally coated with plastic derivatives.

CONCLUSIONS

Our data show a widespread contamination of infant formulae from the three investigated contaminants, either of environmental or process origin. Our findings demonstrate that infant formulae may represent a main source for the simultaneous exposure to DEHP, DnBP and BPA in babies. This risk is particularly relevant for DEHP and DnBP because intake from formulated milk could exceed in the worst case the TDI from EFSA. In conclusion, potential hazards exist for infants fed with baby formula, as these endocrine disruptors show the highest toxicity in infant population. EFSA established TDIs for the three investigated contaminants only referring to an adult population. We believe that specific TDIs for children would help the protection of the most vulnerable part of the population from a severe public health hazard.

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Table 1. DEHP, DnBP and BPA concentrations in μg/g dry weight (mean ± sd, median and range).

Sample	DEHP				DnBP				BPA			
	POS (%)	Mean ± sd	Median	Min-max	POS (%)	Mean ± sd	Median	Min-max	POS (%)	Mean ± sd	Median	Min-max
Liquid Milk (n = 22)	86	1.112 ± 0.716	0.926	0.092 – 2.919	82	0.384 ± 0.385	0.280	0.008 – 1.624	43	0.019 ± 0.037	0.003	0.003 – 0.169
Powder Milk (n = 28)	96	1.496 ± 0.729	1.159	0.702 – 3.552	96	0.330 ± 0.229	0.212	0.101 – 0.812	67	0.023 ± 0.028	0.011	0.003 - 0.108
Total (n = 50)	80	1.327 ± 0.724	1.136	0.092 – 3.552	90	0.354 ± 0.305	0.244	0.008 – 1.624	60	0.021 ± 0.022	0.008	0.003 - 0.169

Table 2. Concentrations of Bisphenol A (BPA), di-n-butylphthalate (DnBP) and di(2-ethylhexyl)phthalate (DEHP) in liquid milk samples and type of packaging.

Product	Type	Packaging	DEHP (µg/g dry weight)	DnBP (µg/g dry weight)	BPA (µg/g dry weight)
C1	Infant formula	Tetrapak™	0.696	0.075	0.003
C2	Infant formula	PET	0.092	0.082	0.030
C3	Infant formula	Tetrapak™	1.831	0.067	0.003
C4	Infant formula	Tetrapak™	0.219	0.084	0.020
C6	Infant formula	Tetrapak™	0.633	0.142	0.009
C7	Infant formula	Tetrapak™	2.067	0.0075	0.003
C9	Infant formula	PET	1.456	0.287	0.003
C10	Infant formula	PET	0.301	0.067	0.003
C11	Infant formula	PET	2.099	0.624	0.003
C12	Infant formula	PET	0.784	0.482	0.058
C13	Infant formula	PET	0.606	0.216	0.014
C17	Infant formula	PET	1.877	0.787	0.003
C18	Infant formula	PET	1.202	0.351	0.003
C19	Infant formula	PET	0.923	0.899	0.017
C20	Infant formula	PET	0.256	0.14	0.018
C21	Infant formula	PET	0.929	0.088	0.003
C22	Infant formula	PET	2.919	1.624	0.169
C23	Infant formula	PET	0.852	0.423	0.030
C24	Infant formula	PET	1.428	0.384	0.003
C25	Infant formula	PET	0.796	0.807	0.003
C26	Infant formula	PET	1.137	0.272	0.003
C27	Infant formula	PET	1.371	0.548	0.003

Table 3. Concentrations of Bisphenol A (BPA), di-n-butylphthalate (DnBP) and di(2-ethylhexyl)phthalate (DEHP) in powder milk samples and type of packaging.

Product	Type	Packaging	DEHP (µg/g dry weight)	DnBP (µg/g dry weight)	BPA (µg/g dry weight)
C5	Infant formula	Alluminium	1.408	0.321	0.003
C8	Infant formula	Alluminium	1.134	0.199	0.003
C14	Premature formula	Alluminium	0.997	0.201	0.003
C15	Infant formula	Alluminium	0.702	0.155	0.003
C16	Infant formula	Alluminium	0.871	0.212	0.028
C28	Infant formula	Alluminium	1.274	0.161	0.008
C29	Infant formula	Alluminium	0.883	0.137	0.003
C30	Infant formula	Alluminium	3.552	0.809	0.011
C31	Infant formula	Alluminium	2.909	0.765	0.100
C32	Infant formula	Alluminium	1.023	0.101	0.009
C33	Infant formula	Alluminium	1.142	0.356	0.022
C34	Infant formula	Alluminium	0.981	0.392	0.003
C35	Infant formula	Alluminium	1.024	0.161	0.003
C36	Infant formula	Alluminium	0.922	0.337	0.043
C37	Infant formula	Alluminium	1.052	0.709	0.054
C38	Infant formula	Alluminium	1.018	0.575	0.026
C39	Infant formula	Alluminium	2.341	0.187	0.012
C40	Infant formula	Alluminium	0.982	0.123	0.003
C41	Infant formula	Alluminium	1.723	0.118	0.016
C42	Infant formula	Alluminium	1.899	0.704	0.003
C43	Infant formula	Alluminium	1.175	0.148	0.035
C44	Infant formula for gastrointestinal problems	Alluminium	1.213	0.301	0.003
C45	Infant formula for gastrointestinal problems	Alluminium	1.897	0.812	0.108
C46	Rice milk formula	Alluminium	1.723	0.211	0.003
C47	Rice milk formula	Alluminium	2.871	0.321	0.003
C48	Rice milk formula	Alluminium	0.951	0.184	0.046
C49	Infant formula for gastrointestinal problems	Alluminium	1.821	0.201	0.018
C50	Infant formula	Alluminium	2.409	0.349	0.041

Table 4. Medium case, estimated daily dietary intake of Bisphenol A (BPA), di-n-butylphthalate (DnBP) and di(2-ethylhexyl)phthalate (DEHP) in newborns fed with liquid or powder formulae according to the 50th and 97th of infant weight growth curve by WHO (2006).

Age (days)	Infant's average weight (kg)		Milk assumption (g dry weight / day)		DEHP intake (µg/kg bw day)		DnBP intake (µg/kg bw day)		BPA intake (µg/kg bw^2 day)	
	50th pctl[1]	97th pctl	50th pctl	97th pctl	50th pctl	97th pctl	50th pctl	97th pctl	50th pctl	97th pctl
15	3.70	4.75	67.61	76.06	20.78	18.21	4.46	3.91	0.15	0.13
30	4.25	5.45	92.96	101.41	24.85	21.14	5.34	4.54	0.17	0.15
45	4.76	6.20	101.41	109.86	24.23	20.15	5.20	4.33	0.17	0.14
60	5.41	6.84	98.59	105.63	20.72	17.54	4.45	3.77	0.15	0.12
75	5.76	7.26	105.63	112.68	20.83	17.64	4.47	3.79	0.15	0.12
90	6.10	7.65	112.68	126.76	20.98	18.82	4.51	4.04	0.15	0.13
120	6.70	8.35	114.08	129.58	19.34	17.63	4.15	3.79	0.14	0.12

[1]pctl = percentile

[2]kg bw = kg body weight

Table 5. Worst case, estimated daily dietary intake of Bisphenol A (BPA), di-n-butylphthalate (DnBP) and di(2-ethylhexyl)phthalate (DEHP) in newborns fed with liquid or powder formulae, according to the 50th and 97th of infant weight growth curve by WHO (2006).

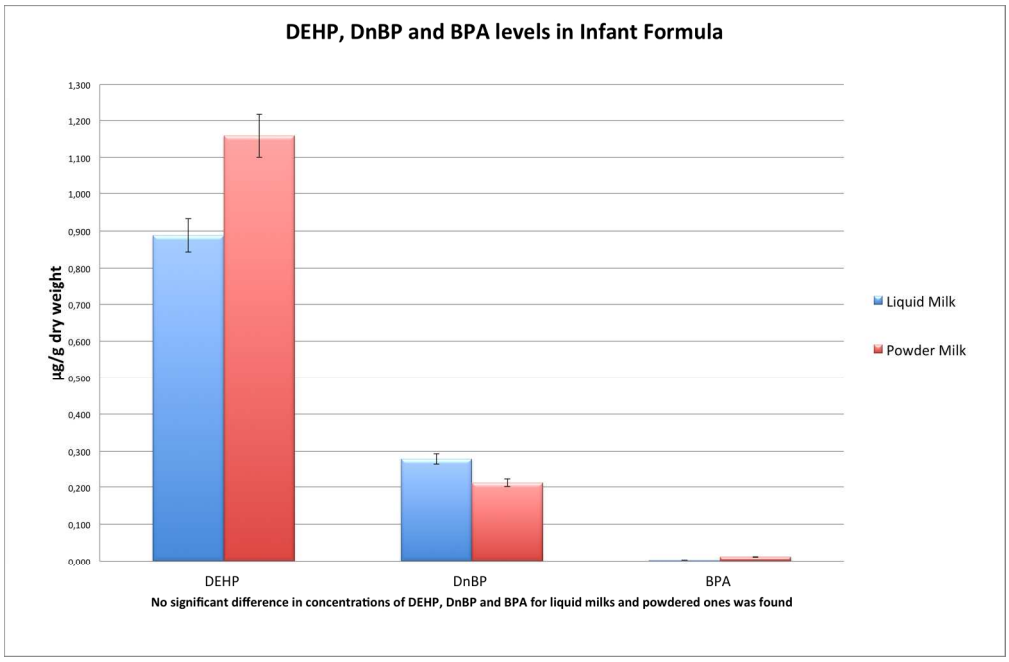
Age (days)	Infant's average weight (kg)		Milk assumption (g dry weight / day)		DEHP intake (µg/kg bw day)		DnBP intake (µg/kg bw day)		BPA intake (µg/kg bw[2] day)	
	50th pctl[1]	97th pctl	50th pctl	97th pctl	50th pctl	97th pctl	50th pctl	97th pctl	50th pctl	97th pctl
15	3.70	4.75	67.61	76.06	45.74	40.07	14.64	12.82	1.06	0.93
30	4.25	5.45	92.96	101.41	54.68	46.52	17.50	14.89	1.27	1.08
45	4.76	6.20	101.41	109.86	53.32	44.33	17.06	14.19	1.24	1.03
60	5.41	6.84	98.59	105.63	45.60	38.61	14.59	12.35	1.06	0.90
75	5.76	7.26	105.63	112.68	45.85	38.83	14.67	12.42	1.06	0.90
90	6.10	7.65	112.68	126.76	46.18	41.43	14.78	13.26	1.07	0.96
120	6.70	8.35	114.08	129.58	42.57	38.80	13.62	12.41	0.99	0.90

[1]pctl = percentile

[2]kg bw = kg body weight

Figure legends

Figure 1. Concentrations of DEHP, DnBP and BPA in liquid and powder milk samples. Data are expressed as median and percentage of SE.



Concentrations of DEHP, DnBP and BPA in liquid and powder milk samples. Data are expressed as median and percentage of SE.