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Abstract:	The aim of the study was to obtain data on the exposure of non-breast-fed infants to polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/F), polychlorinated biphenyls (PCB), organochlorine pesticides (OCP), and Bisphenol A (BPA) and its chlorinated derivatives through consumption of commercial infant

	<p>foods with largest shares of the market in 22 EU countries. The E-Screen bioassay was employed to assess the estrogenicity of the baby foods and the ethoxyresorufin-O-deethylase (EROD) induction was measured to determine the levels of PCDD/F and PCB. Consequently, the highest total effective xenoestrogen burden (TEXB) value of 73.60 pM Eeq/g was found in the soy-based formula and the EROD-bioassay was always below the LOQ (3.5 pg/g). Overall, the estimated dietary exposure to BPA via commercial baby foods was lower than the Tolerable Daily Intake (TDI) of 50 µg/kg body weight (bw). Furthermore, our findings indicated that the dietary exposure of 0-9-month-old infants through the products investigated here does not exceed the maximum Tolerable Daily Intake (TDI) of 4 pg WHO-TEQ (toxic equivalents)/kg bw. However, exposure to more than 2 pg WHO-TEQ/kg bw/day might occur for 0-4 month of age infants consuming "starting" hypoallergenic formula. Moreover, analysis of OCP indicated that the dietary exposure of non-breast-fed infants was not harmful. Considering the importance of early development and the vulnerability of infants and children, it is essential to determine their dietary exposure to contaminants in order to decide which efforts of risk reduction should receive highest priority.</p>

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Assessment of PCDD/F, PCB, OCP and BPA dietary exposure of non-breast-fed European infants

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Abstract

The aim of the study was to obtain data on the exposure of non-breast-fed infants to polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F), polychlorinated biphenyls (PCB), organochlorine pesticides (OCP), and Bisphenol A (BPA) and its chlorinated derivatives through consumption of commercial infant foods with largest shares of the market in 22 EU countries. The E-Screen bioassay was employed to assess the estrogenicity of the baby foods and the ethoxyresorufin-O-deethylase (EROD) induction was measured to determine the levels of PCDD/F and PCB. Consequently, the highest total effective xenoestrogen burden (TEXB) value of 73.60 pM Eeq/g was found in the soy-based formula and the EROD-bioassay was always below the LOQ (3.5 pg/g). Overall, the estimated dietary exposure to BPA via commercial baby foods was lower than the Tolerable Daily Intake (TDI) of 50 µg/kg body weight (bw). Furthermore, our findings indicated that the dietary exposure of 0-9-month-old infants through the products investigated here does not exceed the maximum Tolerable Daily Intake (TDI) of 4 pg WHO-TEQ (toxic equivalents)/kg bw. However, exposure to more than 2 pg WHO-TEQ/kg bw/day might occur for 0-4 month of age infants consuming “starting” hypoallergenic formula. Moreover, analysis of OCP indicated that the dietary exposure of non-breast-

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fed infants was not harmful. Considering the importance of early development and the vulnerability of infants and children, it is essential to determine their dietary exposure to contaminants in order to decide which efforts of risk reduction should receive highest priority.

Keywords: PCDD/F; PCB; OCP; BPA; food; infant

Introduction

Food is generally recognized as the main source of human exposure to persistent organic pollutants (POPs) such as polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F), polychlorinated biphenyls (PCB) and organochlorine pesticides (OCP) (Ahlborg et al., 1995; Weijs et al., 2006). Several health risk assessments were used by different authorities to propose safety limits for all human sub-population deriving from non-carcinogenic end-points and referring to different exposure periods. A WHO-expert consultation in May 1998 ended with the establishment of a Tolerable Daily Intake (TDI) of 1-4 pg WHO-TEQ (toxic equivalents)/kg body weight (bw) (WHO, 2000). In 2001, the Scientific Committee on Food of the European Commission established a Tolerable Weekly Intake (TWI) of 14 pg TEQ/kg bw (SCF, 2001) for the European population. More, recently the Joint FAO/WHO Expert Committee of Food Additives established a Provisional Tolerable Monthly Intake (PTMI) of 70 pg WHO-TEQ/kg bw/month (JECFA, 2002).

Children, particularly infants, can be biologically more sensitive to certain toxicant on the body weight basis than adults. Current understanding of the rates of maturation of metabolism and evidence from case studies indicated that infants up to the age of 6 months typically lack the capacity to detoxify and eliminate substances as readily as adults (Dourson et al., 2004). Moreover, the Scientific Committee on Food of the European Commission stated that the estimated dioxin dietary exposure of breast-fed infants is one to two orders of magnitude higher than the estimated adult dietary exposure (SCF, 2001). Although there is considerable evidence that the concentrations of PCDD/F and OCP in breast milk have fallen in recent years (Krauthacker et al., 2006; Mueller et al., 2008; Hsu et al., 2007). Since breast-feeding has a measurable positive influence on neurological and immunological development, a formula diet should not be recommended as a means of lowering dioxin intake. At the same time in some EU countries only about 50% of mothers initiate breast-feeding (Cattaneo, 2008). According to statistics from 2007 few European women breastfeed their infants exclusively at six months of age (EC, 2004) and only 33.1% of infants in the USA are exclusively breastfed up to 3 months of age (CDC). The increasing number of mothers who feed their babies industrially processed formula milk or solid foods as vegetables and meat or fish purée, has caused the baby market to grow significantly and with it, the assortment of products offered. According to the DONALD study, the sale of commercial infant food in Germany in 1996 was approximately 30% of the total sales of dietetic foods (Kersting et al., 1998).

Thus, commercial infant food plays an important role in the infant's diet, but few studies have examined the PCDD/F, PCB and OCP in formula milk consumed by infant (Hsu et al., 2007; Lorán et al., 2007; Chovancová et al., 2005; Ramos et al., 1989; Weijs et al., 2006; Mezcua et al., 2007). Additionally, it has been shown that exposure to bisphenol A (BPA) occur through the consumption of beverages or foods that have been in contact with polycarbonate plastic containers or epoxy resins in food packaging (Kubwabo et al., 2009; Maragou et al., 2006; Lopez-Espinosa et al., 2007). BPA is readily chlorinated by reaction with residual chlorine molecules, e.g. residual chlorine used as a disinfectant in drinking water (Zafra et al., 2003) or sodium hypochlorite, which is usually applied as a bleaching agent in paper factories (Fukazawa et al., 2002). Therefore, chlorinated BPA derivatives are potential contributors to inadvertent human exposure to bisphenols. In fact, tetrachlorobisphenol-A and lower chlorinated analogues (mono-, di- and tri-chloroBPA) were recently detected in the environment (Liu et al. 2009) and human tissues (Fernandez et al., 2007). In addition, chlorinated BPA derivatives have been shown to be estrogenic (Rivas et al., 2001), with affinities for estrogen receptors that are sometimes higher than those of the parent BPA compound (Takemura et al. 2005; Kitamura 2005). In its risk assessment on BPA adopted in 2006, the European Food Safety Authority (EFSA) set a Tolerable Daily Intake (TDI) of 50 µg/kg bw (EFSA, 2006) for this substance. EFSA re-confirmed the TDI in its 2010 opinion, while pointing out some uncertainties (EFSA, 2010). Successively, the European Commission has announced a ban on the use of BPA in plastic baby bottles from June 2011. The current study, as a part of the EU funded research project CASCADE (Chemicals as contaminants in the food chain; Network of Excellence, FP6 funded consortium, targeting health risks in food) is aimed at evaluating the current dietary exposure levels of non-breast-fed European infants. By the use of chemical target analysis, the scientific focus has in particular been on PCDD/F, PCB, OCP and BPA and some of its chlorinated derivatives. For this purpose, milk-, soy- and hypoallergenic-based infant formulae, as well as solid foods and beverages from the EU market were examined. Additionally, the E-Screen bioassay was used to assess the estrogenicity of the baby foods and the ethoxyresorufin-O-deethylase (EROD) induction was measured for determination of PCDD/F and PCB. Furthermore, the dietary exposure of non-breast-fed infants of age 0-9 months of age were estimated and compared with the above mentioned Tolerable Daily Intakes.

Materials and Methods

Baby food samples included in the study

Market baskets of baby foods designed for consumption during the first 9 months of life by an "average" EU baby fed with infant formulae and weaned (at the 5th month) with solid foods and beverages were put together (Table 1). The subsequent investigations concerned an infant whose diet consisted entirely of commercial baby foods, i.e. who was not breast fed at all. The 2007 market data made available by "Food for Thought" (www.fft.com) was used to identify the major brands of baby foods and their shares of the market in 22 EU countries considered to represent the entire EU.

1
2
3 109 A total of 6 pooled samples of “starting” infant formulae of milk-based (Mf), soy-based (Sf),
4 110 hypoallergenic-based (HAf) and “follow-on” infant formulae of milk-based (fMf), soy-based (fSf) and
5 111 hypoallergenic-based (fHAf) and 5 pooled samples of solid foods and beverages (SFB) designed for
6 112 5-9-month-old infants (SFB5, SFB6, SFB7, SFB8, and SFB9) were prepared separately for chemical
7 113 target analysis. The proportion of each product in the pooled sample was the same as its share of the
8 114 European market. Further details have been reported elsewhere (Piccinelli et al., 2010). In order to
9 115 assess the potential exposure to nuclear receptor through the whole diet, another 6 pooled samples of
10 116 fMf+SFB5, fMf+SFB9, fSf+SFB5, fSf+SFB9, fHAf+SFB5, and fHAf+SFB9 were further prepared.
11 117 (Table 1 here)
12 118
13 119 Lyophilisation was performed for baby jar and beverage products using freeze drying equipment
14 120 (Type BETA 1-16, Christ GmbH) with duration of about 36 h per sample. Consequently the fresh
15 121 weight (fw) of 20 g solid foods and beverages for SFB5, SFB6, SFB7, SFB8, and SFB9 resulted dry
16 122 weight (dw) of 9.44, 8.55, 8.01, 8.59, 7.87 g, respectively. The homogenisation of the pooled infant
17 123 formulae and solid food and beverages samples was done after the lyophilisation of the separate items.
18 124 The weighing procedures were performed in a purified glove box under nitrogen atmosphere and the
19 125 pooled samples were further homogenised in a roto shaker (Type Reax 20/8, Heidolph GmbH) for 8 h.
20 126 The samples were kept at room temperature and stored in brown glass jars protected from direct light.
21 127 In order to prevent any contamination the opening and closing of the sample collector was performed
22 128 under nitrogen atmosphere.
23 129
24 130 *Determination of PCDD/F and dioxin-like PCB amounts in baby food samples*
25 131 Extraction of 20 g of fw infant formulae and 15 g of freeze dried solid foods and beverages was
26 132 carried out on Accelerated Solvent Extractor (ASE 200, Dionex GmbH, Idstein, Germany) using a
27 133 mixture of n-hexane: acetone (75:25, v/v) at 120 °C and at a pressure of 12 MPa (Cok et al., 2009).
28 134 Two static cycles of 10 min were applied for a complete extraction. Cleanup encompassed sandwich,
29 135 carbon and alumina chromatographic columns. PCB and PCDD/F analyses were performed by
30 136 HRGC/HRMS. Detail information on clean-up procedure and instrumental parameters are given
31 137 elsewhere (Cok et al., 2009). The tetra to octa PCDD/F and tetra to hepta PCB were identified and
32 138 reported in pg WHO-TEQ/g fw. The older WHO 1998 Toxic Equivalency Factors (TEFs) values were
33 139 used because all regulatory limits were still based on them. The lower and upper bounds approach was
34 140 applied for quantification of the results as described elsewhere (WHO, 2003). For lower bound (LB)
35 141 values, the concentration was set equal to zero if it was below the limit of quantification (LOQ). For
36 142 upper bound (UB) values, the concentration was set equal to the LOQ if it was below the LOQ. So the
37 143 UB values present the highest possible amount of a contaminant based on the analytical results. The
38 144 analytical laboratory involved is quality assured according to DIN EN ISO/IEC 17025 and accredited
39 145 for the analysis of PCDD/F and PCB.

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Determination of organochlorine pesticides (OCP) in baby food samples

Extraction of 2 to 10 g of fw infant formulae and 15 g of freeze dried solid foods and beverages was performed in the same way as for the PCDD/F. Cleanup and instrumental analysis of OCP were executed according to a former described procedure (Wang et al., 2009). Only 6 of 26 OCP studied were below the detection limit and the concentration level was presented in pg/g fw.

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Micro-EROD bioassay

Samples of 20 g of fw infant formulae and 20 g of freeze dried solid foods and beverages were Soxhlet extracted with 800 ml toluene for 24 h. The extract was concentrated to 4 ml and then a clean-up step was carried out on a sandwich column. This column consisted of 10 g active silica gel, 20 g silica gel (44 % H₂SO₄), 40 g silica gel (4 % H₂O), and 10 g Na₂SO₄ from bottom to top. The sample was eluted with 900 ml n-hexane. The eluate was concentrated and transferred to 200 µl DMSO under N₂ stream at 45 °C. Subsequently, the concentrate was eluted to a mixture DMSO/Isopropanol 4:1 as an optimal carrier solvent for the bioassay.

EROD bioassay was performed using the rat liver cell line (HII4E) expressing cytochrome P4501A1 upon exposure to Aryl hydrocarbon receptor (AhR) agonists (Donato et al., 1993; Schwirzer et al., 1999). A H4IIE cell suspension was seeded into 96 well plates and incubated for 24 hours. Following this, the cells were exposed in parallel to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) standard concentrations and samples keeping the ratio standard/medium and sample/medium at 0.005. Blanks were also carried out. The EROD induction caused by sample extracts after 72 h of incubation (persistent compounds) was compared to the dose-response curve obtained with standards of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD). The results were given as pg 2,3,7,8-TCDD toxicity equivalent values (TE values) per gram sample.

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E-Screen bioassay: TEXTB assessment

MCF-7 cells were used in the test of estrogenicity according to a technique slightly modified (Villalobos et al., 1995) from that originally described elsewhere (Soto et al., 1992). Briefly, 0.5 ml of each baby food extract including “starting” infant formulae (Mf, Sf and Hf) and pooled “follow-on” infant formulae and solid foods and beverages (fMf+SFB5, fMf+SFB9, fSf+SFB5, fSf+SFB9, fHaf+SFB5, and fHaf+SFB9) were evaporated to dryness under a gentle nitrogen stream. Then, the extracts were resuspended in 10 ml of experimental medium (10% CD-FBS-supplemented phenol red-free DMEM), vigorously shaken and left at rest for 30 min, then filtered through a 0.22 µm filter and tested on MCF-7 cells at dilutions of 1:1 to 1:10,000. In each experiment, a dose-response curve (0.1 pM-1000 pM) of 17β-estradiol (E₂), and a negative control (cell treated only with hormone-free medium) were included. The bioassay was terminated on day 6 (late exponential phase) by removing the media from the wells, fixing the cells and staining them with sulphorhodamine-B (SRB). The

proliferative effect (PE) was calculated as the ratio between the highest cell yield obtained with 100 pM of E₂ and the proliferation of hormone-free control cells. The PEs obtained with 1:1 to 1:10,000 dilutions of individual extracts were converted to estradiol equivalents (Eeq) units by interpolation on the estradiol dose-response curve and expressed as Eeq per gram of sample, as an estimation of the total effective xenoestrogen burden (TEXB) (Rivas et al., 2001).

Determination of BPA and its chlorinated derivatives in baby food samples

An accurate and reproducible gas chromatographic-mass spectrometric (GC-MS) method was developed to quantify BPA and its chlorinated derivatives in infant formulae. Sample of 0.4 g of fw infant formula was extracted involving a protein precipitation step with acetonitrile and a solid-phase extraction (SPE) step using Isolute C₁₈ cartridges. After isolation, the solvent was evaporated with nitrogen stream and a silylation step was carried out using N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchloro silane (TMCS) (99:1,v/v). The silylated compounds were identified and quantified by GC-MS using a ZB-5 MS column and selected-ion monitoring (SIM) mode. Bisphenol F was used as internal standard.

Extraction of 0.5 g of freeze dried solid foods and beverages was carried out on ASE using *n*-hexane, followed by a liquid-liquid extraction with acetonitrile and consequent cleaning with *n*-hexane. The extracted sample was taken to dryness with a gentle stream of nitrogen and reconstituted with deionised water. A SPE step was carried out using isolute C₁₈ cartridges in order to clean up the sample. The solvent was removed and a silylation step was performed using BSTFA and TMCS. Finally, the silylated compounds were identified and quantified by GC-MS.

The linearity of the calibration curves was tested according to the Analytical Methods Committee (AMC, 1994). The results for the intercept (a), slope (b), correlation coefficient (R²), and probability level of the lack-of-fit test and for the linearity, precision, accuracy, sensitivity, and selectivity of the overall assay have been presented elsewhere (Del Olmo et al., 2005; Zafra et al., 2003; Ballesteros et al., 2006). The limit of detection (LOD) and the LOQ for BPA and its chlorinated derivatives in infant formula samples ranged from 0.8 to 1.7 and 2.6 to 5.8 ng/g, respectively and from 1.5 to 3.3 and 4.9 to 10.9 ng/g, respectively, for solid foods and beverages samples. The methodology was validated using recovery studies with spiked samples. Recoveries ranged from 85.3 to 108.4%, with mean values of 99.0% (BPA), 101.2% (ClBPA), 92.9%, (Cl₂BPA), 93.3% (Cl₃BPA), and 93.5% (Cl₄BPA).

Elaboration of the monthly diets for EU babies for the first 9 months of life

The mean daily dietary exposure to PCDD/F, PCB, OCP, and BPA was calculated based on the estimated average amount of infant formulae (ml/day) and solid foods and beverages (g/day) consumed by an “average EU baby”. The values thus obtained were 25.2, 22.9, 21.0, and 18.3 g

(fw)/kg bw for the “starting” formulae consumed by infants 0-1, 1-2, 2-3, and 3-4 months of age, respectively; 12.8, 8.0, 4.6, 4.4 and 4.2 g (fw)/kg bw for the “follow-on” formulae consumed by infants 4-5, 5-6, 6-7, 7-8 and 8-9 months of age, respectively; and 28.8, 54.7, 72.6, 70.2 and 71.9 g (fw)/kg bw for the solid foods and beverages consumed by infants 4-5, 5-6, 6-7, 7-8 and 8-9 months of age, respectively. Further details have been reported elsewhere (Piccinelli et al., 2010).

The results of compound determination were converted into daily dietary exposure based on the daily consumption of the baby foods and bw of infant at a certain month, respectively. In order to establish a meaningful comparison the calculated values were additionally expressed in the same unit as the tolerable daily intakes established by international expert committees.

Results and Discussion

Levels of PCDD/F and dioxin-like PCB and TE-EROD value baby foods

Table 2 presents the LB and UB for the levels of PCB and PCDD/F in infant formulae and solid foods and beverages based on chemical analysis.

(Table 2 here)

No or low levels of PCDD/F or PCB were detected in the solid foods and beverages and negligible PCB levels were detected in the infant formulae. With regard to infant formulae a LB range of 0.01 to 0.05 PCDD/F (WHO-TEQ) pg/g fw that correspond to 0.04 to 0.2 (WHO-TEQ) pg/g lipid (the package labelled lipid content of approximately 25 g per 100 g fw) was estimated. For comparison, slightly higher median value of total WHO-TEQ of 0.07 pg/g fw were suggested in infant formulae from the Swedish market (Broman et al., 2009). However, elevated PCDD/F level of 0.11 PCDD/F (WHO-TEQ) pg/g fw (0.44 PCDD/F (WHO-TEQ) pg/g lipid) was found for the “starting” hypoallergenic infant formula (HAf). It was also noted similar PCDD/F UB level was detected for both hypoallergenic infant formulae, i.e. HAf and fHAf. In fact, all these concentrations were lower than reported 0.7 ± 0.2 pg and 0.98 (WHO-TEQ) pg/g lipid in formula milk from southern Taiwan (Hsu et al., 2007) and Slovak Republic (Chovancová et al., 2005) markets, respectively and far lower than the total median TEQ₉₈ of 15 pg/g lipid in mother milk from randomly recruited primiparas (N=183) who lived in Uppsala Country and delivered between 1996 and 2006 (Lignell et al., 2009). Recent EFSA report on monitoring of dioxin level in food and feed including “Infant and baby food” group suggested mean total dioxins equal to 0.42 pg TEQ WHO₉₈/g lipid (EFSA, 2010).

The induction of EROD activity was used as a well-established biomarker for assessing the exposure to stressors such as mixture of contaminants able to elicit a response at the AhR level. However, no TE levels higher than the LOD (0.5 pg/g fw) and LOQ (3.5 pg/g fw) were observed. Therefore, the present study can not use the EROD data for further calculations of dietary exposure to dioxin-like compounds. However, the use of a bioanalytical tool is important since it allows to investigate

possible interactions between different chemicals present in the sample. These possible effects are not considered when single analytical determinations are performed.

Level of organochlorine pesticides (OCP) in baby foods

Among of the different isomers of HCH, only α -HCH in Sf and fHAf and only β -HCH in fHAf were detected in infant formulae (Table 3). The α -, β -, and γ -isomers of HCH were present in all solid foods and beverages samples, with a monthly stepwise increase in the content of the α -HCH level as the diet became more and more varied. Since the nervous system is among the vulnerable targets for lindane exposure and perinatal exposure to lindane has been associated with the development of Parkinson's disease later in life (Heusinkveld et al., 2010) the γ -HCH isomer (lindane) is the most important one within the HCHs. This compound was found predominantly in the solid foods and beverages samples, although still at levels much lower than the maximum residue limits (MRL) proposed for lindane i.e. 0.010 mg/kg lipid (FAO/WHO, 2006). (Table 3 here)

As also shown in Table 3, in almost all of the baby food samples analysed, DDTs were present at levels above the detection limit. In addition, in both types of baby food (infant formulae and solid foods and beverages) the levels of 4,4'-DDT, 2,4'-DDT, 4,4'-DDD, 2,4'-DDD and 2,4'-DDE were relatively similar. Out of the six DDTs analysed, 4,4'-DDE was present at highest levels (20.9 pg/g fw) in the milk-based formulae and in solid foods and beverages where the corresponding levels of SFB8 and SFB9 were 45.5 and 37.0 pg/g fw, respectively. On a lipid basis, Mf value was 0.08 ng/g, which is far lower than the level of p,p'-DDE in breast milk reported Czech Republic 600 ng/g lipid (Cerna et al., 2010).

Heptachlor, cis-chlordane, trans-heptachlorepoxyde and endrin were not detected in any of the samples analysed (Table 3). However, trans-chlordane, oxy-chlordane and aldrin were present at negligible concentrations in solid foods and beverages (100, 60 and 40%, respectively), in particular food designed for 8- and 9-month-old infants. Although cis-heptachlorepoxyde was found in all of the baby foods, with the highest level of 0.023 ng/kg fw (0.092 ng/kg lipid) in fMf, its levels were well below the MRL of 6 μ g/kg lipid (FAO/WHO, 2006).

Among the OCP, endosulfan isomers were mostly dominant in investigated milk- and hypoallergenic-based infant formulae. The determined concentrations were 139, 132 and 148 pg/g of fw for Mf, HAf and fHAf, respectively which is below the MRL (0.1 mg/kg lipid) of endosulfan in milk (FAO/WHO, 2006). Although industrialized nations have restricted or banned many organochlorine pesticides (e.g., endosulfans), according to the available information, Spain is the main consumer of endosulfans within the European Union, accounting for almost half of the total consumption (Cerrillo et al., 2005).

In fact, 20% of samples of milk-based infant formulas from Spain were positive for this contaminant, which is identified as an endocrine disruptor (Mezcua et al., 2007). Also, reports on human exposure to persistent OCP have indicated considerable exposure to endosulfans, as the fact that they have been detected in placenta, umbilical cord blood and breast milk of women as well as in adipose tissue of children living in Southern Spain (Lopez-Espinosa et al., 2008).

The result of the study revealed that out of total 26 OCP investigated only 6 were found below the LOD. Although in all baby food samples investigated the detected OCP levels were much lower than the MRL, nevertheless, there is need of continuous monitor from infant food safety point of view and also time tendency after restriction and ban of some OCP. It indicates also the high degree of dispersion of these substances in the environment as well as the distribution of OCP in the soil-plant system.

Estrogenicity of baby food extracts in the E-Screen bioassay

In order to estimate the estrogenicity of the samples, a total of nine baby food extracts of “starting” infant formula (Mf, Sf, HAF) and pooled “follow-on” infant formulae and solid foods and beverages (fMf+SFB5, fMf+SFB9, fSf+SFB5, fSf+SFB9, fHAF+SFB5, and fHAF+SFB9) were tested using a MCF-7 cell based proliferation assay (E-Screen bioassay). The biomarker of estrogenicity (TEXB) was developed to measure the cumulative estrogenicity of chemicals using the E-Screen bioassay. Table 4 lists the TEXB values (mean and standard deviation) expressed as Eeq/g sample. Because all tested samples were toxic for MCF-7 cells at 1:1 dilution, due to solvent toxicity, we tested serial dilutions up to 1:10,000 in order to obtain measurable estrogenicity of the fractions. TEXB levels ranged from undetectable (<1 pM Eeq) to 73.60 pM Eeq/g, the maximal estrogenicity observed. (Table 4 here)

Estrogenicity was <1 pM Eeq/g in Mf and HAF samples, whereas the highest TEXB values corresponded to the soy-based formula samples. These findings are in agreement with recent findings by Riu et al. (2008) on the estrogenicity of the same soy-based formula samples, using a reporter gene assay based on MELN and HELN bioluminescent cells. They observed a preference for transactivation of ER β versus ER α , a type of transactivation signal profile previously described for estrogenic compounds such as phytoestrogens (Escande et al., 2006). Interestingly, they confirmed the presence of several phytoestrogens identified as aglycones (daidzein, glycitein, and genistein) using ER α -based affinity columns. Therefore, the estrogenicity of soy-based formula samples can be assumed to derive mostly from natural chemicals (e.g. phytoestrogens) rather than from man-made xenoestrogens.

Concerns have been raised by the scientific community about the potential risks to infant health of soy-based formula consumption, due to its phytoestrogen content. Tuohy (2003), in a review of the conflicting evidence on the risks and benefits of soy phytoestrogens, denounced the absence of

adequate scientific research that quantifies the level of risk for infants. He suggested the need for a precautionary approach to be taken in relation to the potential developmental effects of the consumption of pharmacologically active compounds in infancy and childhood.

Levels of BPA and its chlorinated derivatives in baby foods

Eleven baby foods including infant formulae (Mf, Sf, HAf, fMf, fSf and fHAf,) and solid foods and beverages (SFB5, SFB6, SFB7, SFB8, and SFB9) were analysed for BPA and its derivatives and the results were presented in ng/g of fw. No detectable levels were found in infant formulae samples, with the exception of the BPA concentration (3.60 ng/g of sample) being detected in HAf. This result is in agreement with previous studies that report BPA concentration in canned infant formula samples (powdered and whole milk) ranging from <1.7 to 15.2 ng/g (Maragou et al., 2006). In addition, the concentrations reported in the present work fall within the range of previously published measurements of BPA in canned liquid infant formula from the United States (Ackerman et al., 2010) as well as Canada (Cao et al., 2008) and are much lower than the BPA levels reported in powdered infant formula purchased from the local Taiwan market that ranged from 45 to 113 ng/g (Kuo and Ding, 2004).

Regarding solid foods and beverages samples investigated the BPA levels were below the determined LOQ level of 1.5 ng/g. However, a concentration of 17.35 ng/g of sample was detected in the food designed for 9 month-old infants (SFB9). To date, several studies have been dedicated to determine BPA levels in food and via food packaging (Muncke, 2009). In general, concentrations of BPA in canned food products differ considerably among food types. For example, BPA values range from 7.8 to 24.4 ng/g in canned fruits and from 55 to 103 ng/g in canned vegetables from the Spanish market (Garcia-Prieto et al., 2008). By contrast, the mean of BPA concentrations in canned vegetables from Canadian (Cao et al., 2010) and Italian markets (Grumetto et al., 2008) were relatively low (less than 10 ng/g).

BPA chlorinated derivatives (ClBPA, Cl₂BPA, Cl₃BPA and Cl₄BPA) were not detectable in SFB5, SFB6, SFB7, while these compounds were below LOQ (4.9 ng/g) in SFB8 sample, and subsequently Cl₃BPA and Cl₄BPA were quantified at concentrations 12.45 and 5.11 ng/g of sample, respectively, in SFB9. In general, a tendency to higher concentrations of BPA and its derivatives was found in industrial solid foods and beverages with increasing months.

Daily intake of PCDD/F, dioxin-like PCB, OCP and BPA of non-breasted infants (0-9 month aged) fed with baby foods

The dietary exposure to PCDD/F and PCB (pg WHO-TEQ/kg bw/day) of 0-9 month-old infants was estimated considering the LB levels in “starting” Mf, Sf, HAf (for infants 0-4 months), “follow-on”

fMf, fSf, fHaf infant formula, respectively, and solid foods and beverages (SFB5-SFB9) and their foods consumption (Table 5). The study does not consider the additional drinking water exposure as well as the specific exposure at month 6th and 9th that may arise during crawling and soil ingestion. (Table 5 here)

PCDD/F and PCB exposure appeared to decrease from month to month, due to the reduced consumption of formula per kg bw. Moreover, exposure to dioxin-like PCB via the six typologies of infant formulae during the first 9 months of life was far lower than the exposure to PCDD/F (Table 5). Exposure of 5-9 month-old infants to PCB via solid foods and beverages investigated was estimated to be between 0 and 0.73 pg WHO-TEQ/kg bw/day, which is higher than the corresponding value for PCDD/F from fifth to ninth month of postnatal life.

The highest estimated exposure to PCDD/F of more than 2 pg WHO-TEQ/kg bw/day was exhibited by infants consuming HAF who had an average body weight of less than 6.4 kg (unique LB and UB PCDD/F concentration levels). In general, HAF is occasionally recommended by pediatricians for babies who have a severe problem digesting cow's milk based formula or soy formula. HAF require more intensive processing than Mf and Sf and more processing tend to introduce more contamination. Hypoallergenic formulae are processed by enzymatic hydrolysis of different protein sources such as bovine casein/whey and soy followed by further processing such as heat treatment and/or ultrafiltration, or they are based on amino acid mixtures (Høst and Halken, 2004). Exposure to dioxins of infants up to two months of age through HAF is of potential concern. Furthermore, consideration of UB PCDD/F and PCB values in Mf and Sf may suggest critical dietary exposure for same bw group infants such as 2.42 ± 0.32 and 1.99 ± 0.26 pg WHO-TEQ/kg bw/day, respectively. Although the similar UB levels for PCDD/F in HAF and fHAF, lower than 2 pg WHO-TEQ/kg bw/day exposure was estimated during the weaning period due to differences in the pattern of consumption.

In general, excluding hypoallergenic formula the baby food items examined may expose 0-4- and 4-9-month-old non-breast-fed infants to approximately 1 and 0.7 pg WHO-TEQ/kg bw/day, respectively. Similarly, the estimated chronic exposure to PCDD/F and dioxin-like PCB was 1.1 pg WHO-TEQ/kg bw/day for infants 5 months of age who consume infant formulae and vegetables available on the Dutch market (Weijs et al., 2006). Overall, the dietary exposures estimated here do not exceed the maximum TDI of 4 pg WHO-TEQ/kg bw/day and moreover, are orders of magnitude lower than reported total TEQ daily intake for breast-fed infants from Czech Republic 117- and 271 pg/kg bw/day (Bencko et al., 2004), Turkey 37- and 70 pg/kg bw/day (Cok et al., 2009) and Spain 49.6 pg/kg bw/day (Bordajandi et al., 2008).

BPA level was detected only in the HAF out of the six infant formulae samples analysed. Consequently, the estimated dietary exposure to BPA of 0.09, 0.08, 0.08 and 0.07 $\mu\text{g/kg bw/day}$ for 0-1, 1-2, 2-3 and 3-4 month aged infants are referred only to infants fed with this particularly infant formulae. These dietary exposures are lower than the established TDI (50 $\mu\text{g/kg bw/day}$) for BPA. Regarding dietary exposure during the weaning period, a value of 1.7 $\mu\text{g/kg bw/day}$ for 8-9 months of age infants was estimated. For solid foods and beverages consumed in the fifth and eighth months of life, the BPA and chlorinated derivatives levels were lower than the determined LOQ range.

In general, only low levels of OCP were detected in the samples of baby food investigated. The highest DDT daily exposure 0.0001 $\mu\text{g/kg bw}$ was estimated for 4-5- month-old infants fed fMf, a value below the limit of 10 $\mu\text{g/kg bw/day}$ (FAO/WHO, 2000). Indeed, the daily dietary exposure to DDT varies considerably among regions. The lowest dietary exposure has been observed in countries where restriction and ban of DDT had been executed earliest (Sweden, Germany and Norway). Due to later termination of production and restricted use of DDT, higher dietary exposure was reported in Poland, the Czech Republic and Russia. In this regard, our results seems to be comparable with the Canadian data (Hoover, 1999) for infants consuming breast milk, but lower than the dietary exposures of 8 $\mu\text{g/kg bw}$ recently estimated in Poland (Szyrwińska and Lulek, 2007) or Czech Republic (Cerna et al., 2010) daily.

With regard to α -, β - and γ - HCH, the worst-case scenario would involve exposure to 0.6, 0.02 and 0.6 ng/kg bw/day for infants consuming solid foods and beverages during the eighth month (BFB8), fHAF during the fourth month and solid foods and beverages during the fifth month of life (BFB5), respectively. However, all of these values are also very low compared to the Tolerable Daily Intakes (TDI) of 5, 1 and 12.5 $\mu\text{g/kg bw/day}$, respectively (DFG, 1982).

Conclusions

The present assessment of levels of four classes of endocrine disrupting compounds: PCDD/F, PCB, OCP and BPA and its derivatives in formulae and solid foods and beverages designed for infants focused on the products with the largest shares of the market in 22 EU countries. In addition, the dietary exposure to these contaminants in the first 9 months of life of non-breast-fed infants was estimated. The results were compared with established tolerable daily intakes. The established special regulations for infants and children nutrition were also considered whereas here presented scientific results could be used to improve our current knowledge on potential exposure to nuclear receptor modulators through calculation in the different scenarios. Although direct analyses in human biological samples (e.g. urine, blood) are known to be the best estimators of total exposure to some of these contaminants, the aim of the present study was to identify and quantify potential sources of exposure in infant diet. In sum the following findings were found:

- a. Using chemical target analysis the levels of PCDD/F and PCB in baby foods were low, with highest value of 0.11 pg/g (0.44 pg/g lipid) being detected in HAF. The sensitivity of the EROD bioassay was not high enough to calculate possible AhR inducer intakes at these low levels.
- b. Due to infant high food consumption per kg bw of the infants, the determined low PCDD/F and PCB concentrations in baby foods may contribute to a dietary exposure of 0.14 to 1.27 pg WHO-TEQ/kg bw/day for 0-9 months non-breastfed infants. The study notified that 0-4 months aged infants consuming HAF may have dietary exposure of more than 2 pg WHO-TEQ/kg bw.
- c. Only 6 of 26 OCP studied were found below the LOD, implying a wide exposure of this susceptible population, even within the MRL.
- d. TEXB assessment resulted in an estrogenicity in the range from undetectable (<1 pM Eeq/g) to 73.60 pM Eeq/g (maximal estrogenic activity), the latter for soy-based formula samples, therefore attributable to phytoestrogens content.
- e. Among investigated baby foods BPA was only found in HAF (3.60 ng/g) and in solid foods and beverages designed for 9 month-old infants (17.35 ng/g). In addition, the Cl₃BPA and Cl₄BPA concentrations were only detected in solid foods and beverages designed for 9 month-old infants at levels of 12.45 and 5.11 ng/g of sample, respectively.

Finally, the levels of contaminants detected in major commercial baby food products sold in the EU and the estimated dietary exposure by non-breast-fed infants are lower in magnitudes than those reported for nursing infants. However, and more importantly, low exposure to environmental chemicals, especially in the first months of life, must also take account of possible additive effects of mixture of chemicals. Indeed combinations may cause an effect even at concentrations of each individual chemical that alone would not produce measurable consequences. Further research is warranted to investigate and interpret the levels of endocrine disruptors. These results should provide a basis for recommendations concerning the risks posed by commercial baby foods to the health of young children.

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Table 1. Infant age for inclusion of “infant formulae” and “baby foods and beverages”

Foods	Months								
	1	2	3	4	5	6	7	8	9
“Infant formulae”									
“Starting” infant formula	X	X	X						
“Follow - on” infant formula				X	X	X	X	X	X
“Baby foods and beverages” (BFB)									
Vegetables / potatoes					X	X	X	X	X
Fruit / fruit juice					X	X	X	X	X
Cereals:									
Infant cereals without gluten					X	X	X	X	X
Infant cereals with gluten						X	X	X	X
Infant cereals with milk						X	X	X	X
Pasta							X	X	X
Biscuits								X	X
Meats					X	X	X	X	X
Pulses						X	X	X	X
Dairy based dessert with fruit							X	X	X
Fish								X	X
Dairy based dessert with chocolate									X

Table 2. Lower bound (LB) and upper bound (UB) concentration levels of PCDD/F and PCB (WHO-TEQ) pg/g fw in “infant formulae” and “baby foods and beverages” samples

Type baby food		Mf	Sf	HAf	fMf	fSf	fHAf	Baby foods and beverages				
Infant age (month)		0 - 4 th month			up to the 5 th month			5 th	6 th	7 th	8 th	9 th
PCDD/F (WHO-TEQ) pg/g fw	Lower bound (LB)	0.04	0.05	0.11	0.04	0.02	0.01	n.d.	0.0035	0.00001	0.00002	0.0005
	Upper bound (UB)	0.09	0.09	0.11	0.09	0.07	0.12	0.03	0.0436	0.0384	0.0640	0.0693
PCB (WHO-TEQ) pg/g fw	Lower bound (LB)	0.0010	0.0003	0.0005	0.0030	0.0001	0.0006	0.0000	0.01	0.01	0.01	0.01
	Upper bound (UB)	0.0021	0.0013	0.0006	0.0031	0.0013	0.0017	0.17	0.01	0.01	0.01	0.01

fw: fresh weight; n.d.: not detectable; Mf: “starting” infant formulae of milk-based; Sf: “starting” infant formulae of soy-based; HAf: “starting” infant formulae of hypoallergenic-based; fMf: “follow on” infant formulae of milk-based; fSf: “follow on” infant formulae soy-based; fHAf: “follow on” infant formulae hypoallergenic-based

Table 3. OCP concentration level (pg/g fw) in “infant formulae” and “baby foods and beverages” samples

Substance	Mf	Sf	HAF	fMf	fSf	fHAF	BFB5	BFB6	BFB7	BFB8	BFB9	LOD (mean±STD)	LOQ (mean±STD)
<i>α</i> -HCH	n.d.	2.7	n.d.	n.d.	n.d.	2.3	4.3	6.6	6.9	8.0	7.6	1.0±0.9	2.0±1.8
<i>β</i> -HCH	n.d.	n.d.	n.d.	n.d.	n.d.	1.7	1.1	0.9	1.5	1.4	1.2	2.0±2.6	4.0±5.2
<i>γ</i> -HCH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	22.7	10.7	13.3	17.4	13.4	1.6±1.8	2.9±3.6
<i>δ</i> -HCH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.1±2.7	4.1±5.4
<i>ε</i> -HCH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.3±0.8	2.3±1.7
Pentachlorobenzene	n.d.	4.2	7.3	n.d.	n.d.	6.4	0.28	3.7	8.2	27.4	122	0.2±0.2	0.4±0.4
Hexachlorobenzene	n.d.	69.9	60.8	n.d.	n.d.	45.9	n.d.	n.d.	n.d.	0.52	8.9	1.8±1.8	3.5±3.5
4,4'-DDT	n.d.	5.8	1.3	7.6	8.6	1.8	2.1	1.9	2.2	5.6	3	0.6±0.6	1.2±1.2
2,4'-DDT	2.9	1.6	n.d.	2.7	1	0.84	0.9	0.8	0.9	1.9	1.1	0.6±0.6	1.2±1.2
4,4'-DDD	4.2	3.2	5.2	10.8	5.3	3.1	0.7	0.7	1.0	4.6	3.9	0.3±0.3	0.5±0.5
2,4'-DDD	2.4	0.84	1.5	2.4	1.7	0.84	0.1	0.1	0.1	1.3	0.9	0.2±0.2	0.5±0.5
4,4'-DDE	20.9	1.9	10.1	16.5	4.9	11.2	8.6	10.1	9.4	45.5	37.0	0.7±0.8	1.4±1.5
2,4'-DDE	4.0	0.67	0.61	n.d.	0.72	0.84	n.d.	n.d.	n.d.	0.3	0.2	0.6±0.7	1.1±1.3
<i>trans</i> -Chlordan	n.d.	1.8	n.d.	n.d.	n.d.	n.d.	0.7	0.3	0.3	0.3	0.4	0.2±0.1	0.4±0.3
<i>cis</i> -Chlordan	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.2±0.1	0.4±0.3
<i>oxy</i> -Chlordan	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.4	0.4	0.3	1.0±1.4	1.9±2.9
Heptachlor	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.5±0.6	0.9±1.2
<i>cis</i> -Heptachlorepoxyd	10.9	9.7	2.2	23	11.2	1.5	1.3	1.6	1.5	1.4	1.3	0.4±0.6	0.8±1.2
<i>trans</i> -Heptachlorepoxyd	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.5±2.2	3.0±4.4
Aldrin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.2	0.2	0.9±1.3	1.8±2.6
Dieldrin	8.5	27	3.9	18	25.2	6.4	5.7	8.1	7.0	4.7	4.2	1.0±1.0	2.0±2.0
Endrin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.6±2.4	5.1±4.8
Endosulfan-I	74.5	34.3	101.0	89.9	33.9	116	15.3	22.4	17.8	19.5	16	2.2±2.6	4.5±5.3
Endosulfan-II	139.0	39.6	132.0	83.9	61.8	148	35.5	37.0	27.4	33.2	24.9	2.1±2.0	4.2±3.9
Methoxychlor	n.d.	0.82	n.d.	n.d.	1.4	n.d.	0.4	0.3	0.5	0.6	0.5	0.8±0.5	1.6±1.0
Mirex	n.d.	n.d.	1.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.2±0.2	1.5±0.3

fw: fresh weight; n.d.: not detectable; STD: standard deviation; Mf: “starting” infant formulae of milk-based; Sf: “starting” infant formulae of soy-based; HAF: “starting” infant formulae of hypoallergenic-based; fMf: “follow on” infant formulae of milk-based; fSf: “follow on” infant formulae soy-based; fHAF: “follow on” infant formulae hypoallergenic-based; BFB5–BFB6–BFB7–BFB8–BFB9: baby foods and beverages available for month 5, 6, 7, 8, and 9 respectively

Table 4. The amount of the total effective xenoestrogen burden (TEXB) of baby food extracts in E-Screen bioassay

	TEXB (pM Eeq/g of sample)
Mf	0.25 ± 0.11
HAf	0.44 ± 0.22
Sf	70.13 ± 9.81
fMf+ BFB5	0.22 ± 0.15
fHAf+ BFB5	0.31 ± 0.18
fSf+ BFB5	73.60 ± 6.22
fMf+ BFB9	0.29 ± 0.15
fHAf+ BFB9	0.85 ± 0.31
fSf+ BFB9	69.25 ± 8.21

Mf: “starting” infant formulae of milk-based; Sf: “starting” infant formulae of soy-based; HAf: “starting” infant formulae of hypoallergenic-based; fMf: “follow on” infant formulae of milk-based; fSf: “follow on” infant formulae soy-based; fHAf: “follow on” infant formulae hypoallergenic-based; BFB5 and BFB9: baby foods and beverages available for month 5 and 9, respectively

Table 5: Estimated dietary exposure to PCDD/F and PCB (pg WHO-TEQ/ kg bw/day) for non-breasted infants 0-9 months of age fed with baby foods available on the EU market

Type baby foods	Infant age (month)								
	0-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9
PCDD/F TEQ (pg/kg bw)/day									
Milk infant formula	1.00	0.92	0.84	0.73	0.51	0.32	0.18	0.18	0.17
Soy infant formula	1.26	1.15	1.05	0.91	0.26	0.16	0.09	0.09	0.09
Hypoallergenic infant formula	2.78	2.52	2.31	2.01	0.13	0.08	0.05	0.04	0.04
Baby foods and beverages					0.00	0.19	0.00	0.00	0.04
PCB TEQ (pg/kg bw)/day									
Milk infant formula	0.03	0.02	0.02	0.02	0.04	0.02	0.01	0.01	0.01
Soy infant formula	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00
Hypoallergenic infant formula	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00
Baby foods and beverages					0.00	0.55	0.73	0.70	0.72
Total TEQ (pg/kg bw)/day									
Milk infant formula	1.03	0.94	0.86	0.75	0.55	1.08	0.92	0.89	0.94
Soy infant formula	1.27	1.16	1.06	0.92	0.26	0.90	0.82	0.79	0.85
Hypoallergenic infant formula	2.79	2.53	2.32	2.02	0.14	0.82	0.78	0.74	0.80