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Study of Endocrine Disrupting Chemicals in Infant Formulas and Baby Bottles: Data from the European LIFE-MILCH PROJECT

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Abstract: Exposure to endocrine-disrupting chemicals (EDCs) is inevitable, and growing scientific evidence indicates that even very low doses can negatively impact human health, particularly during pregnancy and the neonatal period. As part of the European project LIFE18 ENV/IT/00460, this study aims to identify the presence of EDCs in 20 infant formulas (both powdered and liquid) and the release from baby bottles and teats. Particularly, sensitization of young people and future parents towards the potential harmful effects of EDCs could significantly help to reduce exposure. Seven different UPLC-MS/MS methodologies and one ICP-AES were set-up to quantify already assessed and suspected EDCs among 85 different chemicals (bisphenols, parabens, phthalates, pesticides, herbicides, and their main metabolites, PFAS, and metals). Results showed that in 2 out of 14 baby bottles only anthracene and phenanthrene of the group of PAHs were released (10.68-10.81 ng/mL). Phthalates such as mono-ethyl phthalate (MEP) were found in 9 of 14 samples (0.054–0.140 ng/mL), while mono(2-ethyl-5-oxohexyl) phthalate (MeOHP) appeared in 2 samples (0.870–0.930 ng/mL). In accordance with current EU regulations, other chemicals were not detected in baby bottles and teats. However, Bisphenols, Parabens, PAHs, Phthalates, PFAS and Metals were detected in infant formula, emphasizing the need for continued monitoring and public health interventions.

Keywords: endocrine disrupting chemicals; biomarkers; infant formula; phthalates; bisphenols; baby bottles; heavy metals

1. Introduction

Exclusive breastfeeding during the first 6 months of life is highly recommended by all pediatric scientific societies and by the World Health Organization (WHO), and breastfeeding is encouraged also while introducing complementary foods, and up to 2 years of age or longer [1,2]. Infant formula

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(IF) or combination feeding becomes necessary if maternal milk is insufficient, or maternal circumstances do not allow breastfeeding.

A few studies have shown that pollutants are detectable in both breast and formula milk, posing questions on the possible consequences of exposures in utero, and early childhood exposures can have lifelong consequences on health and disease [3–5]. Diet and lifestyle are important factors related with exposure to environmental pollutants, among these endocrine-disrupting chemicals.

In 2018, an European Food Safety Authority (EFSA) and European Chemicals Agency (ECHA) guidance document described the substances with endocrine disrupting properties in pesticides and biocides [6]. According to this latest document, an endocrine disrupting chemical (EDC) is a substance showing an adverse effect in an intact organism, having an endocrine mode of action, i.e., capable of altering the function(s) of the endocrine system, and the adverse effect is a consequence of the endocrine mode of action. In this context, monitoring the substances that might represent a health threat, and that interfere with hormonal pathways, and that are related with the environment, food, and consumer products, is a growing need. The group of molecules identified as endocrine disruptors is highly heterogeneous. Synthetic chemicals used in industrial processes as plastics (bisphenols), plasticizers (phthalates), pesticides (chlorpyrifos, glyphosate) insecticides (pyrethroids), and preservatives of cosmetics and pharmaceutical products (parabens) are among the possible released compounds [7]. Moreover, other EDCs like polycyclic aromatic hydrocarbons (PAHs) occur naturally in crude oil and gasoline or are produced when coal, oil, gas, wood, garbage, and tobacco are burned. Perfluoroalkyl substances (PFASs) are a numerous group of composts characterized by a chemical resistance difficulty degradable used in variety industrial application and consumer products. They are under study for ED assessment and the European commission ask to scientific community to collect the data on PFAS in consumed food due the lacking of information on these.

Since infant formulas are intended to serve as a substitute for breast milk in infants who cannot be fed at the breast, their composition should meet specific nutritional requirements to promote regular growth and development of the babies. Thus, infant formulas should only contain components in such amounts that provide a nutritional purpose or another benefit. The inclusion of unnecessary components, or unnecessary amounts of components, may alter metabolic and/or other physiologic functions of the infant. For this reason, minimum and maximum levels of nutrient contents in infant formulas are suggested aiming to provide safe and nutritionally adequate infant formula products. Metals and metalloids are present in infant formulas both because they are added on purpose for nutritional purposes and as inorganic pollutants. The metal uptake by newly born children depends on bioavailability from milk diets and can affect physical and cognitive development irreversibly [8]. Thus, they represent a concern, although studies at this regard are still relatively scarce [9]. Specific regulations impose minimum and maximum values for some essential elements (Na, K, Cl, Ca, P, Mg, Fe, Zn, Cu, I, Se, Mn, and F) and maximum levels for some inorganic contaminants (e.g., As, Cd, Pb) [10]. In terms of health hazards, Cd, Cr, Mn, Ni, Pb, and Zn are particularly relevant because of bio-accumulation in vital organs, long-term persistence in the human body, and possible serious negative effects on infants' health [11]. Although current legislation devoted to infant formulas imposes limits for the mentioned metals, to day there are no guidelines concerning Al and Exley (2013) discussed into detail the inadequacy of such published recommendations. A number of works (f.i. [12,13]) clearly elucidate the contamination with Al whose origin is still unknown, possibly due to ingredients, packaging and processing [14].

Although most toxic agents are hazardous in high doses, the human health risks associated with EDCs concerns continuative low dose exposures. Maternal exposure to EDCs during pregnancy results in foetal exposure through placenta and neonatal exposure, can continue through breast milk and/or formula milk. Exposure to EDCs is unavoidable and increasing scientific evidence has shown that they have negative effects on growth, bone, adipose tissue, metabolism, puberty and fertility, and predisposition to developing cancer. Infant formulas can potentially contain harmful contaminants and residues considering the source of the raw material, production sites [**Error! Bookmark not defined.**], and possible contaminants from the packaging. Although external contaminants are continuously monitored and certified and regulated by legislations that have

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established maximum limits and guide values for the safety of newborns, an increasing number of environmental chemicals have been measured in formula besides breast milk [15], eliciting a need for improved methodologies for assays, and a continuous biomonitoring, and adjustment of safety limits of exposure.

Furthermore, in this context, increased public awareness is essential for the perception of the risk of health damage from exposure to EDCs. Particularly, sensitization of young people and future parents towards the potential harmful effects of EDCs could significantly help reduce exposure. The LIFE18 ENV/IT/00460—Life MILCH project entitled "Mother and Infant dyads: Lowering the impact of endocrine disrupting chemicals in milk for a Healthy Life" , supported by the European Community aims at evaluating exposure to a number of EDCs and their metabolites in the serum and urine of mothers at the end of pregnancy, and in their newborns with a subsequent follow-up at 1, 3, 6 and 12 months of life with a special focus on breast milk, and relationships among the possible sources of EDCs and neurodevelopment, growth, distribution of adipose tissue, and pubertal stages.

The current study aimed at evaluating the amount of EDCs present in the infant formulas that were used when needed. Eight different methodologies to quantify 85 different chemicals (bisphenols, parabens, phthalates, pesticides, herbicides, and their main metabolites, PFAS, and metals) were set-up. We moved on from the existing methods in the literature, improving and validating the modified protocols. Moreover, besides the infant formulas we evaluated the release of EDCs from the infant formula containers, from the bottles and teats.

2. Results and Discussion

EDCs can be present in infant formula and/or its containers. The presence of EDCs in infant formula was widely documented in literature, but often such studies focus on a single group or at most two groups of EDCs. Herein we reported a systematic study to quantify 8 different groups of EDCs to establish a wide exposure risk for infant's growth. With this aim the present study consist of i) the development of the analytical method for each EDCs group quantification including the sample extraction procedure optimization; ii) the analysis of background considering that these chemicals are ubiquitous; and iii) obtain an overview of exposure of child during feeding time.

Considering that EDCs can be present not only in infant formula but also in their containers, 7 of the most commonly available baby bottles, and 8 feeding bottles shipped directly from Intensive Natal therapy units (with and without their corresponding teats) were analyzed. Their release of molecules of the different EDCs groups was evaluated.

2.1. Analytical Methods for the Simultaneous Determination of EDCs

Of the preliminary activities set-up to obtain sensitive and reliable analytical methods for the simultaneous determination of EDCs, in particular, the analytical methods for the identification and quantification of 4 different bisphenols, 11 different polycyclic aromatic hydrocarbons (PAHs), 14 phthalates (diesters and corresponding monoesters), 7 parabens, 3 polar pesticides, 2 pyrethroids and chlorpyrifos, and 27 PFAS were optimized independently by UPLC-MS/MS using the specific standards.

2.1.1. Bisphenols

Bisphenol A (BPA) is used as additive to make clear and hard polycarbonate plastics, as well as in thermal papers. It is traditionally found in many clear plastic bottles and sippy cups. BPA used in plastic containers can migrate to food particularly at high temperatures [16]. For this reason currently BPA is prohibited both in formula milk and in baby food containers [17,18], and the European Food Safety Authority (EFSA) has decreased the tolerance daily intake (TDI, the amount of a substance that can be consumed daily over a lifetime without presenting an appreciable risk to health) at 4 ug/Kg in 2015, and successively restricted to 0.2 ng/Kg in 2023 [19]. But these restrictions open the way to other similar structural analogues such as bisphenol S (BPS), and bisphenol F (BPF). For this reason, in this study, we evaluated not only BPA presence but also BPS and BPF which are not

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regulated by EU. Moreover, given that in the literature lack of information on the toxicity levels and their combined effects information on new bisphenol derivatives, bisphenol F diglycidyl ether (BPFDGE) was also monitored.

To separate 4 bisphenols an UPLC–MS-MS analysis was set-up starting from the previous method reported by van der Meer et al. [20]. The analytical method is a linear gradient of 10 minutes supplied biphenyl column with acetonitrile/water mobile phase containing 0.1 % acetic acid. Electrospray ionization in negative mode was used. LODs and LOQs for each chemical are presented in the Supplementary section (Table S2). No significant matrix effect differences were observed for analysis of bisphenols.

2.1.2. Parabens

Parabens are extensively used as preservatives in cosmetics, pharmaceuticals, food products, including beverages. Their use became important because they stop mold and bacteria growth in baby skin care products that potentially harm the baby. Likewise, without preservatives like parabens, baby skin care products would have a short shelf life. The former EC Scientific Committee for Food evaluated the parabens in 1994 and allocated their Acceptable Daily Intake at 0-10 mg/kg b.w. (milligrams/kilogram body weight), for the sum of methyl, ethyl and propyl p-hydroxybenzoic acid esters and their sodium salts, this limit was further confirmed by EFSA in 2004 [21].

Although the occurrence of parabens in humans has been reported, few investigations about milk and infant formulae are available [22]. In order to quantify the level of parabens in the infant formula we proposed to separate 7 parabens with an efficient LC—MS-MS method starting from the previous method reported by Dualde et al. [23].

The UPLC-MS/MS analytical method to separate 7 parabens is a linear gradient of 10 mins with water and acetonitrile (ACN) with 0.1% of HCOOH as eluent. The electrospray ionization in negative mode was employed. LODs and LOQs and the rest of analytical parameters for each chemical are presented in Supplementary section (Table S3). No significant matrix effect differences were observed for analysis of Parabens.

2.1.3. PAHs

In nature, more than 100 PAHs exist; in the present study he 11 listed by Commission Regulation (EC) No. 1881/2006 listed in Table 1 were selected. Some of which: benzo (a) pyrene, dibenzo (a, h) anthracene benzo (a) anthracene, benzo (b) fluoranthene, benzo (k) fluoranthene, chrysene and indene (1,2,3-cd) perylene have been classified by the Agency for Research on Cancer (IARC) as carcinogenic to humans, or possibly carcinogenic to humans. The level of contamination of infant formula by PAHs is likely to depend on the geographic conditions where the milk was collected and the condition of the farm animals [24,25].

Table 1. Assessed or suspected endocrine disrupting chemicals selected for the study.

Group	Chemical compound	Abbreviations	CAS number
Bisphenols	Bisphenol A 4,4′-(1-Methylethylidene)bisphenol	BPA	80-05-7
	Bisphenol S 4,4′-Sulfonylbisphenol	BPS	80-09-1
	Bisphenol F 4,4′-Methylenebisphenol	BPF	620-92-8
	Bisphenol F Bis(3-chloro-2-hydroxypropyl)ether 1-chloro-3-[4-[4-(3-chloro-2-hydroxypropoxy)phenyl]methyl]phenoxy]propan-2-ol	BPFDGE	374772-79-9
Parabens	Methylparaben Methyl 4-hydroxybenzoate	MePB	99-76-3
	Ethylparaben	EtPB	120-47-8

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	Ethyl 4-hydroxybenzoate		
	n-Propylparaben Propyl 4-hydroxybenzoate	PrPB	94-13-3
	iso-Propylparaben Propan-2-yl 4-hydroxybenzoate	iPrPB	4191-73-5
	n-Butylparaben Butyl 4-hydroxybenzoate	BuPB	94-26-8
	iso-Butylparaben 2-Methylpropyl 4-hydroxybenzoate	iBuPB	4247-02-3
	Benzylparaben Propyl 4-hydroxybenzoate	BzPB	94-18-8
Polycyclic aromatic hydrocarbons (PAHs)	Anthracene Anthracene	ANTHR	120-12-7
	Pyrene Pyrene	PYR	129-00-0
	Phenanthrene Phenanthrene	PHEN	85-02-8
	Chrysene Chrysene	CHRY	218-02-9
	Benz[a]antracene Tetraphene	BAA	56-55-3
	Benzo[b]fluoranthene Benzo[e]acephenanthrylene	BKF	205-99-2
	Benzo[k]fluoranthene	BBF	207-08-9
	Benzo[a]pyrene Benzo[pqr]tetraphene	BAP	50-32-8
	Benzo[ghi]perylene Benzo[ghi]perylene	BGHIP	191-24-2
	Dibenz[a,h]anthracene Benzo[k]tetraphene	DAA	53-70-3
	Indeno [1,2,3-cd]pyrene Indeno [1,2,3-cd]pyrene	IND	193-39-5
Polar pesticides	Glyphosate 2-(Phosphonomethylamino)acetic acid	GLY	1071-83-6
	Glufosinate 2-Amino-4-[hydroxy(methyl)phosphoryl]butanoic acid	GLUF	51276-47-2
	AMPA (Aminomethyl)phosphonic acid	AMPA	1066-51-9
Pyrethroids and chlorpyrifos	Cypermethrin [cyano-(3-phenoxyphenyl)methyl] 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate	CP	52315-07-8
	Cyfluthrin [cyano-(4-fluoro-3-phenoxyphenyl)methyl] 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate	CYFL	68359-37-5
	Chlorpyrifos Diethoxy-sulfanylidene-(3,5,6-trichloropyridin-2-yl)oxy-λ5-phosphane	CPS	2921-88-2
Phthalates	Dimethyl phthalate Dimethyl benzene-1,2-dicarboxylate	DMP	131-11-3
	* Monomethyl phthalate	MMP	4376-18-5

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	2-(Methoxycarbonyl)benzoic acid		
	Diethyl phthalate Diethyl benzene-1,2-dicarboxylate	DEP	84-66-2
	* Monoethyl phthalate 2-(Ethoxycarbonyl)benzoic acid	MEP	2306-33-4
	Dibutyl phthalate Dibutyl benzene-1,2-dicarboxylate	DBP	84-74-2
	# Mono-n-butyl phthalate 2-(Butoxycarbonyl)benzoic acid	MBP	131-70-4
	Butylbenzyl phthalate 2-O-benzyl 1-O-butyl benzene-1,2-dicarboxylate	BBP	85-68-7
	* Monobenzyl phthalate 2-[(Benzyloxy)carbonyl]benzoic acid	MBzP	2528-16-7
	Di(2-ethylexyl) phthalate Di(2-ethylhexyl)benzene-1,2-dicarboxylate	DEHP	117-81-7
	* Mono(2-ethylexyl) phthalate 2-(2-Ethylhexoxycarbonyl)benzoic acid	MEHP	4376-20-9
	* Mono(2-ethyl-5-hydroxyhexyl) phthalate 2-(2-Ethyl-5-hydroxyhexoxycarbonyl)benzoic acid	MEHHP	40321-99-1
	* Mono(2-ethyl-5-oxohexyl) phthalate 2-(2-Ethyl-5-oxohexoxycarbonyl)benzoic acid	MEOHP	40321-98-0
	Di-n-octyl phthalate Dioctyl benzene-1,2-dicarboxylate	DNOP	117-84-0
	* Monooctyl phthalate *2-(Octoxycarbonyl)benzoic acid*	MnOP	5393-19-1
Perfluoroalkyl substances (PFAS)	2,2,3,3,4,4,4-heptafluorobutanoic acid Perfluoro-n-butanoic acid	PFBA	375-22-4
	2,2,3,3,4,4,5,5,5-nonafluoropentanoic acid Perfluoro-n-pentanoic acid	PFPeA	2706-90-3
	2,2,3,3,4,4,5,5,6,6,6-undecafluorohexanoic acid Perfluoro-n-hexanoic acid	PFHxA	307-24-4
	2,2,3,3,4,4,5,5,6,6,7,7,7-tridecafluoroheptanoic acid Perfluoro-n-heptanoic acid	PFPHpA	375-85-9
	2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluorooctanoic acid Perfluoro-n-octanoic acid	PFOA	335-67-1
	2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,9-heptadecafluorononanoic acid Perfluoro-n-nonanoic acid	PFNA	375-95-1
	2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-nonadecafluorodecanoic acid Perfluoro-n-decanoic acid	PFDA	335-76-2
	2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-henicosafluoroundecanoic acid Perfluoro-n-undecanoic acid	PFUnDA	2058-94-8
	2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,12,12,12-tricosafluorododecanoic acid Perfluoro-n-dodecanoic acid	PFDoDA	307-55-1
	2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,12,12,13,13,13-pentacosafluorotridecanoic acid Perfluoro-n-tridecanoic acid	PFTrDA	72629-94-8

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	2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,12,12,13,13,14,14,14-heptacosafluorotetradecanoic acid Perfluoro-n-tetradecanoic acid	PFTreDA	376-06-7
	1,1,2,2,3,3,4,4,4-nonafluorobutane-1-sulfonic acid Perfluoro-1 butanesulphonamide	PFBS	375-73-5
	1,1,2,2,3,3,4,4,5,5,5-undecafluoropentane-1-sulfonic acid Perfluoropentanesulphonic acid	PFPeS	2706-91-4
	1,1,2,2,3,3,4,4,5,5,6,6,6-tridecafluorohexane-1-sulfonic acid Perfluorohexanesulphonic acid	PFHxS	355-46-4
	1,1,2,2,3,3,4,4,5,5,6,6,7,7,7-pentadecafluoroheptane-1-sulfonic acid Perfluoroheptanesulphonic acid	PFHpS	375-92-8
	1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-heptadecafluorooctane-1-sulfonic acid Perfluorooctanesulphonic acid	PFOS	1763-23-1
	1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,9-nonadecafluorononane-1-sulfonic acid Perfluorononanesulfonic acid	PFNS	68259-
	1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,12,12,12-pentacosafluorododecane-1-sulfonic acid Perfluorododecanenesulfonic acid	PFDS	79780-39-5
	2,3,3,3-tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)propanoic acid 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoic acid	GenX (HFPO-DA)	13252-13-6
	2,2,3-trifluoro-3-[1,1,2,2,3,3-hexafluoro-3-(trifluoromethoxy)propoxy]propanoic acid 4,8-Dioxa-3H-perfluorononanoic acid	ADONA	919005-14-4
	2-(6-chloro-1,1,2,2,3,3,4,4,5,5,6,6-dodecafluorohexoxy)-1,1,2,2-tetrafluoroethanesulfonic acid Perfluoro(2-((6-chlorohexyl)oxy)ethanesulfonic acid)	9Cl-PF3ONS	756426-58-1
	2-(8-chloro-1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8-hexadecafluorooctoxy)-1,1,2,2-tetrafluoroethanesulfonic acid 11-chloroeicosafluoro-3-oxaundecane-1-sulfonic acid	11Cl-PF3OUdS	763051-92-9
	1H,1H,2H,2H-Perfluorohexanesulphonic acid 3,3,4,4,5,5,6,6,6-nonafluorohexane-1-sulfonic acid	4 :2 FTS	757124-72-4
	1H,1H,2H,2H-perfluorooctanesulfonic acid 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctane-1-sulfonic acid	6:2 FTS	27619-97-2
	31H,1H,2H,2H-Perfluorodecanesulfonic acid 3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-heptadecafluorodecane-1-sulfonic acid	8 :2 FTS	39108-34-4
	1,1,2,2,3,3,4,4,4-nonafluorobutane-1-sulfonamide Perfluorobutane Sulfonamide	FBSA	30334-69-1
	Perfluorooctanesulfonamide 1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-heptadecafluorooctane-1-sulfonamide	FOSA	754-91-6
Metals	Aluminum	Al	7429-90-5
	Arsenic	As	7440-38-2
	Barium	Ba	7440-39-3
	Bismuth	Bi	7440-69-9
	Cadmium	Cd	7440-43-9
	Cobalt	Co	7440-48-4

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	Chromium	Cr	7440-47-3
	Copper	Cu	7440-50-8
	Manganese	Mn	7439-96-5
	Molybdenum	Mo	7439-98-7
	Nickel	Ni	7440-02-0
	Lead	Pb	7439-92-1
	Titanium	Ti	7440-32-6
	Thallium	Tl	7440-28-0
	Vanadium	V	7440-62-2
	Zinc	Zn	7440-66-6

According to the EU Scientific Committee on Food, benzo(a)pyrene can be used as a marker of occurrence and effect of carcinogenic PAH in food, including also benz(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)-perylene, chrysene, dibenz(a,h)anthracene. Further analyses of the relative proportions of these PAH in food would be necessary to inform a future review of the suitability of maintaining benzo(a)pyrene as a marker [26].

The quantitative analysis of 11 polycyclic aromatic hydrocarbons was carried out using an UPLC–MS/MS system based on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) system and coupled to a Waters XEVO TQ-S triple quadrupole system using electrospray ionization with positive ionization. Using the biphenyl column and Water/ACN as system eluent 12 PAHs were eluted separately with a short analytical method (10 mins). On the EFSA suggested, the quantification of BBF, BKF, BAP was reported as sum of concentrations of the 3 analytes (ΣPAHs). LODs and LOQs and analytical parameters for each chemical are presented in the Supporting section (Table S4). No significant matrix effect differences were observed for analysis of PAHs.

2.1.4. Glyphosate and Its Metabolites

Glyphosate is widely used in herbicide products and its use has been authorised for 10 years by the European Commission in extensive agriculture following a safety evaluation.

Many laboratories are studying an efficient method to separate and identify at trace levels glyphosate, its major metabolite AMPA and glufosinate ammonium considering their dramatic increase as herbicides [27]. We propose a new fast and simple extraction method to remove all products present in the milk matrix that interference on the analysis, followed by liquid chromatography coupled with the determination of triple quadrupole mass spectrometry to quantify glyphosate, glufosinate and AMPA. Moreover, we also propose a fast and efficient chromatographic separation protocol in only 8 mins without molecules derivatization, overcoming the previously described protocols which reported a chromatographic separation using derivatization methodologies. Thanks to a new column Raptor Polar X characterized by unique hybrid phase that balances HILIC and ion exchange retention modes we can separated the polar compounds as glyphosate and its metabolites applying the classical mobile phase water/ACN with formic acid. The eluent for separation was H_2O/ACN with 0.5% of HCOOH and the ESI-MS is in MRM negative mode.

In order to avoid ionization suppression a passivation procedure of LC system was performed before real samples analyses. 10 full loop injections of the 10 mM medronic acid were performed without column, with the mobile phases directed to waste instead of the mass spectrometer and 5 full loop injections (2µL) with column was performed using analytical methods conditions improving peak shape. After this process all polar phosphorylated pesticides exhibited sharp and symmetrical peaks for the subsequent injections.

With all these precautions the described method allowed to obtain calibration curve plots independently for glyphosate, glufosinate, and AMPA with optimum correlation coefficients ($R^2 > 0.99$) generated with 10 serial dilutions of the stock solution. The intra-assay coefficients of variability (CV) were ≤10% and interassay CV were ≤12% for all analytes at two QC levels. Recoveries ranged from 84 to 104%. LODs and LOQs for each chemical are summarized in the Supporting section (Table

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S5). Herein a rapid and sensitive method without derivatization was described for quantification of glyphosate and its metabolites.

2.1.5. Phthalates (Diesters and Corresponding Monoesters)

Phthalates are used to make plastics soft and durable, as well as to bind fragrances. Phthalates are classified as endocrine-disrupting chemicals and have been linked to adverse health effects particularly in relation to early life exposures. Inhalation, ingestion, and skin contact are the major via to exposure, even if, recent studies demonstrate that a majority of exposure is probably food related [28] and correlate with metabolic disorders [29,30]. Moreover, diesters can migrate into food from plasticized PVC materials such as tubing typically used in the milking process, lid gaskets, food-packaging films and gloves used in the preparation of foods [31].

Among all the possible phthalates described in the literature in this study, 14 compounds were selected: 6 diesters and their respective 8 monoester metabolites. Monoesters phthalates are originated when the corresponding di-esters enter the organism and are hydrolysed and then further oxidized through complex pathways.

To separate 14 phthalates LC—MS-MS analysis was set-up starting from our previously described method [32] and the one reported by van der Meer et al. [33]. Modifications to this methodology were applied. Particularly, the analytical method developed to separate and quantify 14 different phthalates is a 22 minutes method, longer than those described by Van der Meer et al., but in a single injection. Using the biphenyl column, the di- and monoesters were well separated, even those with similar characteristics DEHP and DnOP or MEHP and MnOP. Electrospray ionization with negative switching mode was adopted. The method developed provides another column. In fact, one of the most difficult problems in accurately quantifying trace level of phthalates in sample is a background contamination. Since phthalates, given their extensive use and their persistence, are ubiquitous environmental contaminants, they are present as contaminants in almost all laboratory equipment, solvents, and laboratory air. To overcome the contaminations of phthalates and have a good separation between the background phthalate response from the phthalates in the sample, a second column (Isolator Column) was inserted in the method. Time of elution in analytical method is longer than the others, but background phthalates are retained on the Isolator Column until the gradient elutes them through the analytical column. In this way, the phthalates from the sample elute later than the background phthalates. Hence the quantification of phthalates in the sample is more accurate and the determination is more sensitive.

Analytical parameters for each chemical are presented in Table S6. The problem of matrix effect observed for analysis of phthalates was discussed in the paragraph control of contamination.

2.1.6. Pyrethroids and Chlorpyrifos

The broad use of insecticides in intensive agriculture has increasingly sparked the interest and the need to develop analytical methods capable of also detecting intricacies of these substances in food and in particular in baby food.

The insecticide family, characterized by polar molecules containing halogens, phosphorus, sulphur or nitrogen, is vast. Pyrethroids constitutes one of the major group of insecticides, largely applied because they present non-systemic effect in plants. They are also found to be effective as a contact insecticide and stomach poison because of its anti-feeding action towards insects and arachnids. In this study, cypermethrin and cyfluthrin pyrethroids were chosen and an efficient method was developed to determine their concentration in formula milk. Moreover, the herbicide chlorpyriphos has been included in the pyrethroid mixture in order to develop an efficient protocol for their quantification. The proposed protocol involves the use of the liquid chromatography with biphenyl column coupled with triple quadrupole mass spectrometry (UPLC-MS/MS) in MRM positive mode using as eluent H_2O/ACN. UPLC–MS-MS analysis was set-up starting from the previous method reported by Tran et al. [34]. LODs and LOQs for each chemical are summarized in the Supporting section (Table S7).

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2.1.7. Perfluoroalkyl Substance (PFAS)

Perfluoroalkyl substances (PFASs) are a very large class of compounds (>30,000) and used in various consumer and industrial product sectors thanks to their characteristics of high resistance to thermal, chemical, and organic. Over the years they have bioaccumulated in the environment and humans and animals have assimilated these substances mainly through water and air. In fact, unlike other compounds, PFAS bind with high affinity to proteins so their half-time release is very long (5-8 years) [35–37]. In recent years, the European Commission has shown considerable interest in these fluorinated compounds and its particular interest is aimed at studying their effects on human health [38]. In particular, the scientific community is investigating their possible classification as EDcs [39,40]. In particular, EFSA has classified PFOS as such [41].

In 2022 the European Union recommended monitoring the presence of 4 PFAS in foods: PFOS, PFOA, PFNA, PFHxS, also determining the limits allowed in foods for infants.

On this occasion the European committee strongly advised to monitor the presence of other fluorinated compounds in foods [42]. In fact, EU commission commits to phasing out all PFAS, allowing their use only in the irreplaceable cases essential to society [43]. Many recent studies are highlighting that infants can be contaminated with PFAS through breast milk. [44,45] The values found today in breast milk are slightly higher than in formula milk [46].

Herein we proposed to investigate the presence of 27 PFAS in infant formula following the extraction and HPLC-MS/MS methodologies as previously described [47]. Analytical data are summarised in (Table S8)

2.2. Control of Contamination

After the setting up a sensitive and reliable analytical method to separate and identify each compound in the 7 EDCs groups, we investigated with all the methodologies employed the background contamination due to the system, solvents, reagents, glassware and microfilters, plastic containers, and consumables for sample storage. In order to avoid and check the contamination due mainly to phthalates, parabens and bisphenols, some preliminary procedures were taken before starting the quantification process and during sample collection and treatment.

The first step was based on the identification and evaluation of the potential contamination sources from the different labware materials market, selecting which with minor release of EDCs. In particular potential origin of contaminations in: reagents, solvents, and plastic containers used during all the quantification process. Moreover, the analysis of the entire laboratory stuff, i.e., gloves, plastic containers, and consumables for sample storage have been tested to evaluate eventual EDCs release. Contamination from solvents and reagents during the analysis was monitored through the area response of reagent blank, procedural blank and mobile phase injections.

The second step was based on the identification of the best consumables for sample storage. At this purpose, pure water has been stored for 3 months in different plastic containers to quantify the contamination. After repeated quality controls, the best storage-containers were selected, all in Polypropylene (PP) or Polyethylene (PE) composition.

The third step was based on removal of some particular phthalates and PFAS contributing to a background contamination during phthalates and PFAS evaluation. In this regard, we placed in-line the ACQUITY UPLC Isolator Column (p/n 186004476) (Waters, Acquity, Midfold, Massachusetts, USA), between the solvent mixer and the injector, to isolate the background contamination and to delay interferences with sample containing phthalates being able to get low detection limits [48].

2.3. Sample Pre-Treatment

Milk is a very complex and variable mixture of proteins, fats, lactose, and minerals in water. Therefore, an exhaustive clean-up of the sample extract is necessary before the UPLC-MS/MS analysis. For this reason, four specific and sensitive protocols for milk samples pre-treatment have been set-up and applied (Figure 1). To remove the protein content (about 3.5%) acetonitrile was used in the extraction procedure as extractant solvent thanks to its excellent ability for protein precipitation

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[49]. The content of lipids (about 4-6%), mostly represented by esters of fatty acids, is another main interference which can decrease the column lifetime and may bring non-negligible matrix effects. Before the UPLC-MS/MS of formula milk sample, remaining proteins, fats, and other components interfering with the analysis and quantification of the different bisphenols, PAHs, phthalates (diesters and the corresponding monoesters), parabens, pyrethroids, and chlorpyrifos a standard QuEChERS method was applied. Then, the efficiency based on recoveries of extracted compounds was also evaluated.

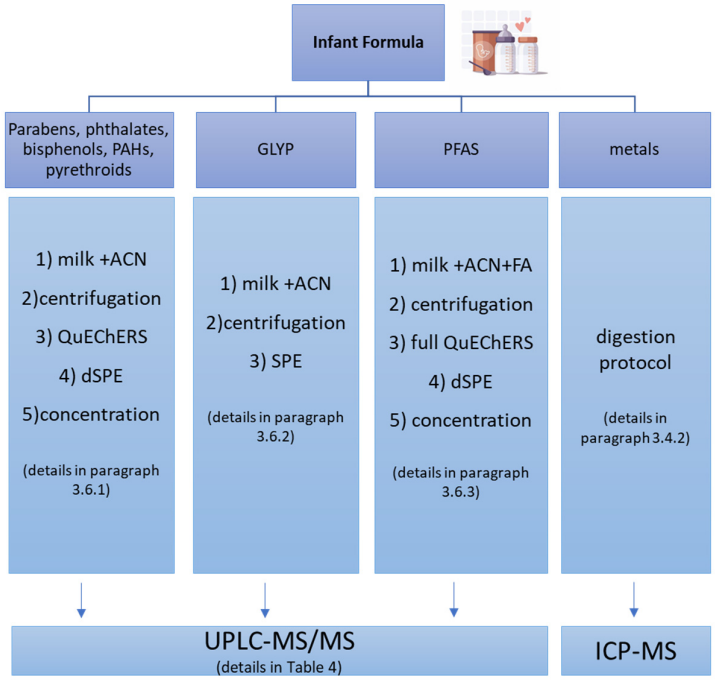

Figure 1. Pre-treatments of formula milk samples.

On the contrary, in the particular case of glyphosate and its metabolites, the recommended and validated method by UE (EURL-SRM version 8.1, 2015) for extraction based on QuPPe-Method showed low recoveries when applied to the infant formula. For this reason, herein set-up a new protocol filtering the aqueous solution after protein precipitation with ACN through a STRATA-X SPE plate to clean up the lipid content from sample. Thanks to this new protocol we obtained high recoveries for glyphosate, glufosinate and AMPA (ranging from 70 to 110%). The new protocol selected in this study presented several advantages such as reduced time for extraction, low cost, and simplicity.

The full QuEChERS extraction and dSPE cleanup for PFAS compounds is outlined in the described protocol [44] and summarized in the experimental part.

2.4. Quantification of Assessed and Suspected EDCs in Baby Bottles

Results showed that no EDCs of bisphenols, parabens, pesticides, PFAS and pyrethroids were detectable in baby bottles and teats. These results are in accordance with the presence of BPA-free, PVC-free, and DEHP-free labels in most part of the baby bottles tested.

Anthracene and phenanthrene within the PAHs Group were detected in 2 out of 14 samples (14% of samples; levels from 10.68 to 10.81 ng/mL, mean 10.75 ng/mL). Moreover, the analyses of phthalates showed the presence of mono-ethyl phthalate (MEP) in 9 out of 14 samples (64% of samples; levels from 0.054 to 0.14 ng/mL, mean 0.073 ng/mL) and MeOHP in 2 out of 14 samples (14% of samples; levels from 0.87 to 0.93 ng/mL, mean 0.90 ng/mL). On the other hand, samples flowed

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through their corresponding teats did not presented increased concentrations, then we can conclude that teats did not contribute to increase the release of EDCs.

The EDCs detected were found in the in commercial bottles only, and not in sterile bottles used in the neonatal Intensive care units participating in the MILCH project.

Additionally, the usured baby bottle with its nipple daily used by a mother to breastfeed her baby was also tested. No trace of EDCs was found in the bottle used repeatedly for breastfeeding, results in line with a previous study by Siddique et al. in which the repeated use of the baby bottles did not increased the leaching of chemicals [50]. This result confirms the suitability and strength of the material used after repeated uses of the baby bottle. Moreover, no chemical compounds were found in the kit of manual breast pump.

2.5. Quantification of Assessed and Suspected EDCs in Infant Formula

Applying the previously described methodologies, we selected 20 commercially available infant milk including 11 formula type 1 and 9 formula type 2 and the findings are summarized in Table 2 and in Figure 2.

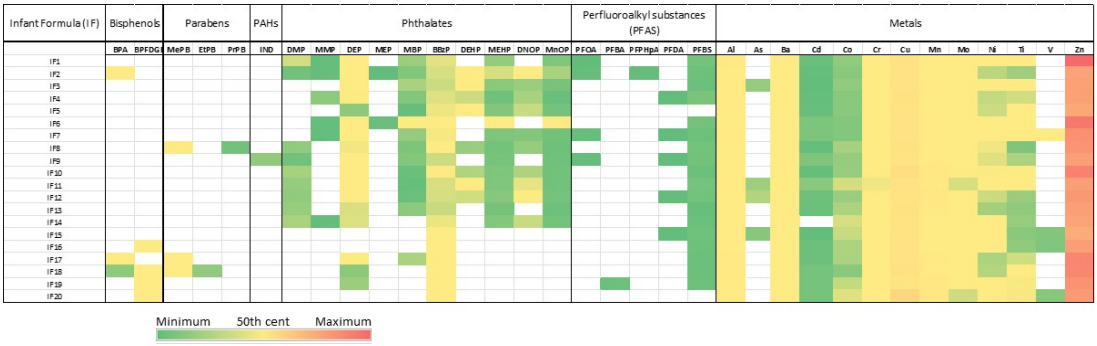

Figure 2. Concentrations expressed in ng/mL of assessed and suspected EDCs in infant formula milk. Using the color spectrum, the green color indicates minimum concentration, the yellow color indicates the 50th percentile and the red color the maximum concentration.

Table 2. Assessed and suspected EDCs concentrations in ng/g dry weight[a] of infant formula (N=20).

EDC group	Compound	Frequency (%)	Mean±sd	Median	Min-Max
Bisphenol	BPA	3/20 (15)	1.76±1.28	2.25	0.31-2.73
	BPFDGE	4/20 (20)	5.43±1.97	6.35	2.48-6.54
Parabens	MePB	3/20 (15)	2.9±2.95	1.36	1.04-6.29
	EtPB	1/20 (5)	0.32	0.32	
	PrPB	1/20 (5)	0.12	0.12	
Polycyclic aromatic hydrocarbons (PAHs)	IND	1/20 (5)	0.32	0.32	0.32–0.32
Phthalates	DMP	9/20 (45)	0.37±0.19	0.34	0.11-0.75
	MMP	6/20 (30)	0.07±0.11	0.03	0.02-0.29
	DEP	17/20 (85)	1.51±1.56	1.27	0.28-7.21
	MEP	2/20 (10)	0.03±0.03	0.03	0.01-0.05
	MBP	14/20 (70)	0.48±0.93	0.22	0.04-3.65
	BBzP	20/20 (100)	1.27±0.91	0.94	0.68-3.45
	DEHP	8/20 (40)	1.09±0.76	0.89	0.38-2.79
	MEHP	14/20 (70)	0.40±0.61	0.21	0.09-2.40
	DNOP	10/20 (50)	0.68±0.40	0.60	0.23-1.52
	MnOP	14/20 (70)	0.24±0.35	0.12	0.05-1.46

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Perfluoroalkyl substances (PFAS)	PFOA	4/20 (20)	0.02±0.01	0.03	0.01-0.03
	PFBA	1/20 (5)	0.09	0.09	
	PFPHpA	1/20 (5)	0.02	0.02	
	PFDA	5/20 (25)	0.01±0.001	0.01	0.009-0.011
	PFBS	18/20 (90)	0.096±0.03	0.09	0.04-0.13
Metals	Al	20/20(100)	546±254	528	192-1005
	As	4/20(20)	11±3.7	11	7.3-16.3
	Ba	20/20(100)	178±101.8	156.5	60.6-458.2
	Cd	19/20(95)	3±2.1	2	0.4-7.7
	Co	20/20(100)	13±5.9	10.8	6.5-24.7
	Cr	20/20(100)	81±47.3	71.1	28-252
	Cu	20/20(100)	4168±1172	3953	3085-8649
	Mn	20/20(100)	768±538	571	173-1820
	Mo	20/20(100)	116±72.1	100.8	21.3-269.3
	Ni	20/20(100)	32±15.1	30.6	13.1-65.9
	Ti	20/20(100)	35±31.8	25.8	5.8-104.0
	V	4/20(20)	13±13.3	6.9	6-33.3
	Zn	20/20(100)	34740±6942.4	32042.7	25883.2-52680.8

[a]Mean ± SD, Median and Range.

Among bisphenols, detectable levels of BPA and BFDGE were observed. In particular, BPA was found in 3 out of 20 samples (15%) and BFDGE was found in 4 out of 20 samples (20%).

Previously published Italian studies on infant formulas reported the presence of BPA. In particular, Cirillo et al. declared to detect BPA levels in 60% of milk samples (levels between 3 and 375 pg/mL, median 15 pg/mL) [51]. Also E. Ferrer et al. declared to find BPA levels on 2 out of 5 infant formulas (levels: from 0.07 to 1.29 mg kg^{-1}) in a comparative Spanish-Italian study on infant formulas [52]. In the meantime, regulation on BPA has become more restricted: the EU Commission Regulation 2018/213 established that no migration of BPA shall be permitted from varnishes or coatings applied to materials and articles specifically intended to come into contact with infant formula, follow-on formula, baby food of infants and young children or milk-based drinks and similar products specifically intended for young children. No further regulation has been found for other substances in the class of Bisphenol-based molecules despite other bisphenols are under investigation.

Regarding Parabens, MePB was detected in 3 out of 20 samples (15%), EtPB was detected in 1 out of 20 samples (0.5%), and PrPB was detected in 1 out of 20 samples (0.5%).

PAHs were not present except indenol (IND) that was quantifiable in 1 out of 20 samples (0.5%).

In a previous study focused in Southern Italy, Santonicola et al. reported that the levels of PAHs detected in commercial milk were 53.68 mg kg^{-1}. In some case the ΣPAHs level exceeded the allowed limit of 1 mg kg^{-1} that could be linked to the presence of petrogenic and pyrolytic environmental sources, i.e., the incineration of waste present in the area surrounding the residence This is the main source of exposure for infants during breastfeeding, through the exposure of mothers residing in some areas of Southern Italy [53].

Among phthalates, DMP was detected in 9 out of 20 samples (45%), DEP was detected in 17 out of 20 samples (85%), BBP was detected in 20 out of 20 samples (100%), DnOP was detected in 10 out of 20 samples (50%), DEHP was detected in 8 out of 20 samples (40%), MMP was detected in 6 out of 20 samples (30%), MEP was detected in 2 out of 20 samples (10%), MBP was detected in 14 out of 20 samples (70%), MnOP was detected in 14 out of 20 samples (70%), and MEHP was detected in 14 out of 20 samples (70%).

By analyzing the data obtained in this study it can be confirmed that there is a phthalate pollution in the milk used for feeding, in fact most of the diesters and some monoesters selected for the study have been quantified. Breaking it down: BBP and DEP are the most frequently encountered analytes followed by DEHP and DnOP. In addition, hydrolysed monoesters were also identified:

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MMP, MEP, MBP, MEHP and MnOP. The oxidative monoesters MEHHP e MEOHP in infant formula were not detected.

Other Italian studies of infant formula have shown contamination of phthalate diesters and monoesters highlighting an extent of phthalate diester contamination, consideration supported in the broad data reported in literature on infant formulas [54,55]. In particular, we confirmed that long-chain alkyl phthalates (DEHP and DnOP) and their respective metabolites (MEHP and MnOP) were detected in most of the samples, as evidenced in other studies.

No residues of chlorpyrifos, glyphosate or its main metabolites glufosinate and AMPA, and pyrethroids were detected in the 20 infant formulae tested.

Concerning PFAS in infant formula, among the 27 PFAS studied detectable levels of PFOA, PFBA, PFPHpA, PFDA and PFBS were founded. Only perfluorooctanoic acid (PFOA) of the 4 EU regulated compounds was detected in 4 out of 20 samples (20%). Moreover, three perfluoroalkyl carboxylic acids were detected, PFBA in 1 out of 20 samples (5%), PFPHpA in 1 out of 20 samples (5%), and PFDA in 5 out of 20 samples (25%). Perfluoroalkane sulfonic acid PFBS was detected in 18 out of 20 samples (90%). Lakind et al. reviewed publications reporting PFAS in infant formula concluding that PFAS measurement data for infant formula were sparse and the reported mean PFOA concentrations were slightly above the children's drinking water screening values [**Error! Bookmark not defined.**]. In fact, no PFAS residues were found in the European studies reported in the review. To be best of our knowledge just target analytes PFOS and PFOA were previously detected in Italian cow milk with measured contamination up to 97 ng/L and 32 ng/L respectively [**Error! Bookmark not defined.**].

As concerning metals, Bi, Pb and Tl showed concentrations below the reported LOQ in all the analysed samples. As and V were observed at concentrations above the LOQ only in 4 samples out of 20 and only one of those they both exceeded the LOQ simultaneously. All the other measured metals (Al, Ba, Cd, Co, Cr, Cu, Mn, Mo, Ni, Ti, Zn) showed concentrations well above the LOQ (Table S16) in all the samples.

Among the elements detected, Cu was present at the highest concentrations, followed by Mn and Al, with the latter showing comparable mean and median values. The lowest concentrations were observed for Cd, As and Co. Cu and Mn are both essential nutrients for infants' growth and toxic elements. Cu is needed for cellular metabolism in enzymatic and non-enzymatic systems [56] and its deficiency may cause growth impairment, neutropenia, anemia, and increased risk of infection [57]. Nevertheless, excessive chronic exposure to Cu may cause acute gastrointestinal symptoms, such as abdominal pain, vomiting, and diarrhea [58]. As concerning Mn, serious concerns have recently been raised about relatively high Mn exposures and possible adverse effects on child neurodevelopment, with a particular attention to attention deficit hyperactivity disorder (ADHD) [59].

The presence of Al and other metals such as As, and Cd (although to a lesser extent) in milk can be ascribed to environmental contamination (soil or grasslands), milk production or storage and shipment to industrial plants [**Error! Bookmark not defined.**].

2.6. Estimated Dietary Exposure in Infants at 1 Month of Age

To better evaluate the exposure of infants to EDCs due to daily consumption of the powdered infant formula type 1, the estimated daily intake (EDI) was calculated for each group of EDCs at 30 days after birth. The newborn body weight was assumed considering the WHO growth curves [60]. The equivalent intake of EDCs was calculated and reported in Table 3. This was done to compare the hypothetical exposure with the regulatory limits currently available for EDCs.

Table 3. Estimated daily intake (EDI, ng/Kg/b.w./day) for each compound (y) in infant formula type 1, using the WHO curves to estimate mean weight at days of life. Tolerable daily intake (TDI, ng/Kg/b.w./day) is according to EFSA.

EDCs	Chemical	Frequency (%)	Mean±sd	Median	Min–Max	EDI for infant's weight (kg) at the 50th percentile (average for age)	TDI values reported in the literature
Bisphenols	BPA	1/11 (9.1)	2.73[a]	2.73	2.73–2.73	59.74	0.2 (ng/Kg/b.w./day) [**Error! Bookmark not defined.**]
			0.3±0.86[b]	0.02	0.49-59.74	6.46	
			0.25±0.82[c]	0	0-59.74	5.43	
	BPFDGE	1/11 (9.1)	6.39	6.39	6.39–6.39	139.84	Maximum PDI of BPFDGE < 3.4 µg/kg body weight/day [61]
Parabens	MePB	1/11 (9.1)	1.04	1.04	1.04–1.04	22.7	Σ of parabens in food for EFSA 0-10 mg/kg b.w. [**Error! Bookmark not defined.**]
	PrPB	1/11 (9.1)	0.12	0.12	0.12–0.12	2.62	ADI for PrPB at 1.25 mg /Kg/b.w./day) [62–64].
Phthalates	DMP	7/11 (64)	0.41±0.43	0.36	0.11-0.75	8.92	0.9–7.2 and 1.6–11.7 µg/kg b.w. for DBP, BBP, 50 µg/kg b.w. per day for DEHP [65]
	MMP	5/11 (45)	0.08±0.09	0.03	0.02-0.29	1.73	
	DEP	9/11 (82)	1.91±2.02	1.27	0.8-7.21	42.23	
	MEP	2/11 (18)	0.03±0.02	0.03	0.01-0.05	1.11	
	MBP	9/11 (82)	0.57±1.15	0.22	0.04-3.65	12.64	
	BBzP	11/11 (100)	1.09±0.46	0.91	0.68-2.32	24.14	
	DEHP	5/11 (45)	1.15±0.89	0.71	0.38-2.79	25.54	
	MEHP	9/11 (82)	0.50±0.74	0.9	0.09-2.44	11.16	
	DNOP	6/11 (62)	0.67±0.4	0.6	0.34-1.12	14.81	
	MnOP	9/11 (82)	0.3±0.45	0.12	0.05-1.46	6.65	
PFAS	PFOA	2/11(18)	0.07		0.03-0.11	0.3	TWI: Σ of PFAS for EFSA 4.4 ng/Kg body weight/week [**Error! Bookmark not defined.**]
	PFPHpA	1/11 (9.1)	0.102	0.102	0.102	0.02	
	PFDA	2/11(18)	0.05		0.04-0.06	0.01	
	PFBS	11/11 (100)	0.46		0.17-0.74	0.07	
Metals	Al	11/11(100)	493±252	390	193-851	10778	PTWI of 7 mg/kg for FAO Expert Committee [66] Sub-chronic oral Reference Dose of 1mg/kg per day for ATSDR [67,68]
	As	2/11(18)	10±3	10	7-12	209	PTWI of 0,015 mg/kg of body weight

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						Sub-chronic oral Reference Dose of 0.0003 mg/kg per day for [**Error! Bookmark not defined.**]
Ba	11/11(100)	151±99	133	61-405	3315	
Cd	10/11(91)	2±2	1	1-8	52	PTWI of 2.5 μg kg−1 b.w. by [69] Sub-chronic oral Reference Dose of 0.001 mg/kg per day by [**Error! Bookmark not defined.**]
Co	11/11(100)	15±7	14	7-25	320	
Cr	11/11(100)	64±26	59	28-131	1399	300 mg/kg b.w. PTDI [70]
Cu	11/11(100)	4510±1490	4006	3319-8649	98679	0.20 mg/day for infant 0-6 months old [71]
Mn	11/11(100)	741±561	597	185-1774	16212	0.0504 and 5.04 μg g^{-1} in formula as minimum and maximum content [72] upon [73]
Mo	11/11(100)	95±68	86	21-244	2072	
Ni	11/11(100)	32±15	31	13-65	707	2.8 mg/kg b.w./ day PTDI [74]
Ti	11/11(100)	31±32	22	6-104	686	
V	2/11(18)	7±1	7	6-7	152	
Zn	11/11(100)	35252±8504	31194	25883-52681	771407	a Tolerable Upper Limit of 7 mg/day for SCF [75]

Mean values of BPA calculated with the following statistical methods to handle left-censored observations: [a]neglecting non-detected value; using substitution method for results reported to be below the LOD, when the non-detected value was imputed: [b]LOD (upper bound), [c]as zero (lower bound). Abbreviations: ADI: Acceptable Daily Intake; ATSDR: Agency for toxic substances and disease registry; PDI: Probable daily intakes; PTDI: Provisional tolerable daily intake; PTWI; Provisional tolerable weekly intake; SCF: Scientific committee on food; TWI: Tolerable weekly intake.

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The mean values reported in Table 3 were statistically calculated handling left-censored observations by neglecting non-detectable values, aware that this decision can overestimate the values. In any case all calculated EDI showed values under the regulatory limits currently available, except for BPA. In fact, EDI value for this bisphenol has been calculated also using two different substitution methods for the results reported to be below the LOD, when the non-detectable value was imputed as LOD (upper bound) and as zero (lower bound). Anyhow, the three obtained EDI values for BPA were above 0.2 ng/Kg/b.w./day, the limit established as tolerable daily intake (EDI) by EFSA in April 2023 [**Error! Bookmark not defined.**]. To be note that this EFSA recommendation is recent, and before this period the reference value was 4 μg/kg body weight (b.w.)/day and our calculated EDI values were well within this parameter.

Special attention needs to be directed towards PrPB (before classified as additive E216) used as antimicrobial preservative in veterinary medicinal products. PrPB and its sodium salt, if allowed as an antimicrobial preservative in the intensive livestock farms, can pass into the animal's milk and therefore be the route of PrPB transmission to the child. In 2015 European Medicines Agency (EMA) suggested the ADI value reported in table [**Error! Bookmark not defined.**].

Usually, phthalates monoesters are used as urinary markers of phthalate diesters, i.e., high concentrations of MEHP correlate with exposure to the parent chemical DEHP [76]. In our particular case, the presence of the monoesters phthalates in infant formula cannot be used to estimate EDI of phthalate diesters because the relationship between the diester intake and the amount of monoesters delivered to the milk is unknown. In any case, as all the infant formula herein tested were based on cows' milk, this can be a possible via for phthalate monoesters pollution. In fact, phthalates and their metabolites can be incorporated to infant formula and transferred to the nursing child from consumer milk [77].

3. Conclusions

The composition of infant formula milk is strictly regulated in Europe and in Italy by precise directives (Commission Regulation (EU) 2006/141 and subsequent amendments; in Italy it is regulated by Ministerial Decree (DM) no. 82 of 9 April 2009 and subsequent modifications, based on the rules established by the Codex Alimentarius produced by the WHO and by the Food and Agriculture Organization (FAO) of the United Nations, which express fundamental non-derogable principles, such as the impossibility of adding ingredients to milky formulas only because they are present in breast milk. Despite these documents are clear regarding the ban on the use of pesticides, no information regarding other EDCs is released. With the idea in mind to evidence potential risks in infant diet associated with contamination of formula milk by EDCs, in the present study 85 chemical compound defined as EDCs or suspected EDCs, were quantified in different formula milks and containers. Eight different groups of chemicals were considered, and corresponding sensitive and consistent analytical methods were developed for the quantitation of 4 bisphenols, 11 PAHs, 14 phthalates (diesters and corresponding monoesters), 7 parabens, 3 polar pesticides, 2 pyrethroids and chlorpyrifos, 27 PFAS, and 16 metals.

The release of EDCs in 5 commercial baby bottles with their teats, in8 feedings bottles used in the neonatal Intensive care units, and 1 kit of a manual breast pump was lower compared to infant formulas. This is probably due to the normative restrictions proposed by the Scientific Committee for Food applied to plastic food contact materials, especially for childcare. Moreover, the present migration study also suggested that the repeated use of the baby bottles did not increase the leaking of EDCs.

In infant formula, at variance, bisphenols, parabens, phthalates, and PFAS were largely present. Among PAHs, IND was found in one sample only.

Based on the data herein reported, particular caution is recommended during the inspection of the investigated formulas by public health authorities regarding contamination by toxic metals, especially Al, consistently identified in most infant formulas.

Concluding, this study, by analysing the data obtained it has shown that formula milk can be polluted. To understand the possible sources of contamination, the whole raw material (milk of

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animal origin), the manufacturing processes for obtaining powdered milk and their packaging will need to be taken into consideration. For example, a possible release of phthalates from the PVC tubes used for milking in the big farm or the bags containing animal feed could be potential sources of contamination. Another source of contamination could be closely linked to the urban or rural environmental pollutants in which the production process is located considering the volatile properties of PAHs.

Finally, the chemical risk assessment was traditionally done on a chemical-by-chemical basis, thus neglecting possible combined effects. Focusing only on food exposure and not on other possible contaminants, newborns are also exposed to many different chemical substances that can have the same toxicological effect. Herein we described the child exposure to different chemical compounds found in formula milk, although individually low exposure is observed for each substance, we cannot overlook the combined effects of the substances found. The presence of EDCs in amounts below those reported for the legal limits doesn't exclude effects from their interaction in mixture. Many of these compounds are assessed or suspected of impacting human health, and their combination could exacerbate their harmful effects. In this context, the EU commission indicates that the exposure analysis should be focalized depending on the modes of action. If chemicals have similar modes of action there is a potential for cumulative effects when such chemicals are present together in a mixture (even when the concentration of each substance is below its "safe level") and then, the concentration/dose addition approach is preferred in order to assure an adequate level of protection. On the other hand, in the case of chemicals with independent modes of action, the establishment of "safe levels" based on the assessment of individual substances appears, in relation to human health, to provide a sufficient safeguard against possible negative effects from mixtures/combinations [78].

4. Materials and Methods

4.1. Reagents and Chemicals

Ultra-pure water was produced with a Milli-Q system (Sartorius Arium 611 VF, Varedo MB, Italy). The solvents acetonitrile (ACN), methanol and formic acid (UPLC-MS grade) were supplied by Carlo Erba (Milano, Italy). QuEChERS (Quick Easy Cheap Effective Rugged Safe) Extract Pouches-EN method (salt packet containing 4 g $MgSO_4$, 1 g NaCl), for QuEChERS extraction and QuEChERS fatty dispersive-SPE AOAC kit, 15 mL polypropylene tube containing 400 mg PSA, 400 mg C18EC and 1200 mg $MgSO_4$ were obtained from DTO Services Srl (Venezia, Italy). Strata-X® SPE plate Polimeric Reverse phase (30 mg/well, 96-Well Plates, 33µM Phenomenex, Castelmaggiore, BO, Italy) beta-Glucuronidase from E. Coli K 12 was obtained from Merck (Milano, Italy). Vacuum Extraction Plate Manifold for Oasis 96-Well Plates (Waters, Acquity, Milford, MA, USA). LC passivation solution containing 10 M medronic acid (1,760 µg/mL, Methanol/Water (50:50)) was from Restek Corp. (Bellefonte, PA, USA).

A selection of bisphenols, parabens, di-esters phthalates with its metabolites, mono-ester phthalates, polycyclic aromatic hydrocarbons (PAHs), polar pesticides: glyphosate and its metabolites glufosinate and aminomethylphosphonic acid (AMPA), pyrethroids (cypermethrin and cyfluthrin) and chlorpyrifos standards were selected and are summarized in Table 1. These were all obtained from MERCK-Sigma Aldrich (Milano, Italy). Native PFAS Precision and Recovery Standard Solution was from Waters S.p.A. (Milano, Italy).

4.2. Preparation of Standard Solutions.

Each stock solution (1 µg/mL) containing a specific EDC group (4 different bisphenols, 11 polycyclic aromatic hydrocarbons, 14 phthalates, 7 parabens, 3 pesticides polar (Glyphosate and its metabolites), and 3 pyrethroids and chlorpyrifos, respectively) was prepared by dissolving a weighed amount of substance or by dilution of a commercial standard solution in ACN and stored at -20°C. Standard solutions (ng/mL) were prepared by dilution of the respective stock solutions with water: ACN (1:1, v/v).

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4.3. Sampling Collection

Five commercial baby bottles selected among the most popular conventional brands available in the market with their teats were bought from the local pharmacies in Florence (Italy). Before use, sterilization of the baby bottles was performed following the package leaflet instruction, bottles were boiled in water for 5 minutes. In addition, one baby bottle with the daily used teat was provided by one mother, and 8 new feeding bottles were shipped directly from the neonatal Intensive Care Units of Parma and Reggio Emilia, and one kit for breast pumping was also collected and evaluated. Food-grade polypropylene (PP) was the plastic material used for the feeding bottles and for for breast pumping, but for one bottle in polyethylene. Silicone are the material used for teats. In this study only two teats were in latex rubber, the rest were in silicone. Moreover, all baby bottles presented the BPA-free label.

In order to evaluate if the baby bottles released any of the selected EDCs, we carried out experiments to simulate the contact between baby bottles and water. For this purpose, all baby and feeding bottles were filled with milli-Q water that was left in contact for at least 48 hours, and then collected for the analyses. These solutions were evaluated also after being flowed through their corresponding teats.

When using a pump, breast milk is usually collected by means of a "catheter" connected to the pump that delivers breast milk directly into the baby bottle. Milli-Q water was flowed over the catheter and further stored in the corresponding baby bottle at -20 °C until the analyses were performed.

Twenty different samples of infant formula were purchased. These were all included in the Italian National Register and commercialized in the pharmacies of Parma and Reggio Emilia, and were from the most widely used brands that were selected from the questionnaires filled in by the mothers enrolled in the LIFE MILCH Project.

Selected samples included: 9 powder infant formulas type 1 used from birth up to 6 months; 8 powder infant formulas type 2 used after 6 months; 3 liquid infant formulas (2 infant formulas type 1 and 1 infant formula type 2).

Liquid formulas were packed in Tetrapack or high-density polyethylene (HDPE), whereas milk powders were stored in Al, Poliethylen terephthalate (PET), and Poliethylen (PE) containers.

Each sample of Infant formula was prepared freshly before analysis following the instructions reported in the infant formula container. The powder sample was accurately weighed (4.5 g) using an analytical balance (Fisherbrand™ Bilance 220g, Fisher Scientific Italia, Rodano, Milano, Italy) and was reconstituted with Milli-Q boiled water (30mL, ~37ºC) in a polyethylene tube. A simplified solid-phase extraction (SPE) or dispersive SPE procedure was employed to extract all EDCs possibly present in the Infant Formula.

4.4. Instrumentation

4.4.1. General Procedure UPLC-MS/MS Analysis

Ultra performance liquid chromatography UPLC (Waters, Acquity, Midfold, MA, USA) coupled to a Waters XEVO TQ-S triple quadrupole using electrospray ionization instrumentation was employed. The instrument was equipped with a Raptor biphenyl column (1.8 µm, 2.1 mm x 100 mm, Restek Srl, Milano, Italy) for phthalates, bisphenols, parabens, PAHs, and pyrethroids analysis, with a Raptor Polar X (2.7um 30 x 2.1mm, Restek Srl, Milano, Italy) for glyphosate and its metabolites and with a ACQUITY UPLC® HSS T3 (1.8 µm, 2.1 mm x 100 mm, Waters, Acquity, Midfold, MA, USA). In any case, the Ultrashield UPLC pre-column filter 0,2µm frit was inserted. Column temperatures and flow rates are reported for each analyte in Table S1. Injection volumes: 10 µL. Used solvent systems and gradients are reported for each analysed compound. All reagents were of at least UPLC reagent grade. Calibration curve ranges for each group of EDCs are reported in the Supporting information. (Tables S2-S8).

MS/MS Parameters: Bisphenols, parabens, glyphosate and its metabolites, and monoesters phthalates were analysed in negative electrospray ionization while polycyclic aromatic hydrocarbons

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(PAHs), phthalate diesters, and pyrethroids and chlorpyrifos were analyzed in positive electrospray ionization.

For compounds, two MRM transitions were acquired for quantification and confirmation purposes. By direct infusion of standard solution (500 ng/mL) MRM data were optimized. The details of each MRM transition used for the MS/MS analysis detection are reported in the Supporting Information (Tables S9-S15). Data were acquired and processed using MassLynx™ software version 4.2 (Waters, Midfold, MA, USA) including the TargetLynx XS software.

Preparation of standard solutions. The stock solution containing the various analytes were prepared at 1 µg/mL in ACN; ten working standard solutions (0.05-2000 pg/mL for PFAS group and 0.01-500 ng/mL for the rest of compounds) were prepared for calibration curve plotting by diluting from stock solution with H_2O/ACN (1:1) and the addiction of 2 mM of ammonium acetate for PFAS.

4.4.2. ICP-AES Analysis

An amount of approximately 0.5 g of infant formula was accurately weighted in PFA vessels and digested using an acidic solution of 2 mL suprapure HNO_3 (obtained by sub-boiling distillation) and 0.5 mL of suprapure HCl (30%). The sample digestion was carried out by using a microwave digester (CEM Mars Xpress, CEM Corporation, Matthews, NC; USA) with a protocol including an initial 10 min ramp to 170°C followed by a 20 min hold and a 40 min cool down. After the digestion, the samples were transferred to 25 mL vials and were diluted to ca. 10 mL with ultrapure water (UHQ—resistivity 18 MΩ cm—Milli-Q system by Millipore, Billerica, MA, USA) before analysis. The determination of heavy metal concentrations in the samples was performed in triplicate by a Varian 720-ES axial Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) (© Agilent Technologies, Inc, Santa Clara, CA, USA); 5 mL of each sample was spiked with 1.0 ppm of Ge used as an internal standard prior the analysis. The introduction system consisted of a concentric pneumatic nebulizer and a cyclonic spray chamber. Calibration standards were prepared by gravimetric serial dilution from commercial stock standard solution at 100 mg/L. The operating conditions were optimized to obtain maximum signal intensity, and between each sample, a rinse solution of 2% v/v HNO_3 was used. All details are reported in Table S16.

4.5. Analytical procedures

Stock standard solutions were prepared in ACN : H_2O (50:50, v/v) or ACN : H_2O (1:1) containing 2mM ammonium acetate at a concentration of 1 ug/mL stored at -20°C. Ten standard solutions (ranging from 0,01 to 500 ng/mL or from 0.05 to 2000 pg/mL for PFAS) were prepared for all the metabolites and analyzed for calibration curve plotting. Curves with correlation coefficients (R^2) greater than 0.999 were generated. One blank and two control samples were included in each batch of samples. The controls were prepared by spiking the standard mixture in the blank at low (10 ng/mL) and high concentration (100 ng/mL) of the calibration curve and were subjected to the same extraction and analysis procedures as real samples and calibration curve points. Calibration curve plots for all target compounds with correlation coefficients ($R^2 > 0.99$) were generated with 10 serial dilutions of the stock solution. The intra-assay coefficients of variability (CV) were =10% and interassay CV were =12% for all analytes at two QC levels. In each methodology the recovery was estimated by spiking the pool of blank formula milk at a fixed concentration, estimating a Recovery percentage acceptable within the range 100% ± 20%. Inter- and intra-day precision was also evaluated by measuring replicates of each concentration daily and during at least five different days, respectively.

The limit of quantification (LOQ) for each analyte was determined by analyzing ten different standard concentrations with progressively lower concentrations. The LOQ was set where the imprecision was CV ≤ 20% and the signal to noise ratio was >10 on all 6 days. For each analyte the LOD was set using a signal-to-noise ratio > 3 on all 6 days.

Intra-assay imprecision was determined by analyzing two urine pools on the same day in 10 replicates. Inter-assay imprecision was assessed by measuring two urine pools on 10 different days.

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4.6. Formula Milk Samples Pre-Treatment

4.6.1. QuEChERS for Phthalates, Bisphenols, PAHs, Parabens, and Pyrethroid Extractions

A simplified QuEChERS procedure was employed to extract chemicals from Infant Formula. Each infant formula (30 mL) was prepared according to the indications given in the feeding table. In polypropylene tubes for the hydrolysis of conjugated species, the β-glucuronidase solution (1 mL,) was added to infant formula (10 mL). The enzymatic solution was prepared dissolving the enzyme purified powder in ammonium acetate 1M (pH=5) to obtain a solution of 3500 U/mL. The mixture was incubated overnight at 37 °C in an C24 incubator shaker (New Brunswick Scientific, Edison, NJ, USA) to finalize the deconjugation of samples. ACN (10 mL) was added to the mixture, and the solution was shaken for 1 min using a Vortex and then put in an ice bath. QuEChERS salts (4 g anhydrous $MgSO_4$, 1 g NaCl) were added to the mixture, vigorously vortexed, and replaced in the ice bath. The falcon with salts and milk was centrifuged at 4000 rpm at 4 °C for 20 min (Heraeus® Megafuge®, Kendro Laboratory Products GmbH., Hanau, Germany). The supernatant was transferred into a 15 mL QuEChERS fatty dispersive-SPE polypropylene tube (Agilent dispersive SPE, 15 mL, Association of Official Analytical Chemists (AOAC) method, ©Agilent Technologies, Inc, Santa Clara, CA, USA). The mixture was shaken for 1 min and centrifuged for 15 min at 4000 rpm at 4 °C. The cleaned supernatant was transferred into a glass tube (15 mL) and evaporated to dryness. The dry residue was then dissolved in 300 µL of ACN: water (50:50, v/v) and placed into an eppendorf and ultra-centrifuged. The final supernatant was transferred into an injection vial and analysed on the UPLC–MS/MS system.

4.6.2. Milk Sample Filtration for Glyphosate and Its Metabolite Extractions

Each infant formula was prepared according to the indications given in the feeding table. The β-glucuronidase solution (1 mL) was added to the infant formula (1 mL) in polypropylene tubes for the hydrolysis of the conjugated species, and the suspension was incubated overnight at 37 °C in a C24 incubator shaker (New Brunswick Scientific, Edison NJ, USA). ACN (2 mL), the mixture was shaken for 1 min with a Vortex and put in an ice bath to precipitate the proteins. In the meantime STRATA-X® SPE plate (33 µm, 30 mg/well, 96-well plates, Phenomenex, Torrance, CA, USA) was preconditioned with 2 mL ACN : water (50:50, v/v). After centrifugation (20 min, 4000 rpm at 4°C, 30 min), the aqueous layer was filtered through to SPE column Strata-X® SPE clean 96-well plate using a vacuum Extraction Plate Manifold for Oasis 96-Well Plates (Waters, Acquity, Milford, MA, USA). The plate was then dried under vacuum for 1 min and each eluate collected into the clean 96-well plate for analysis by LC-MS/MS.

4.6.3. QuEChERS for PFAS

The full QuEChERS extraction and dSPE cleanup for PFAS compounds is fully outlined in the described protocol [**Error! Bookmark not defined.**]. Briefly, 5 mL of pure water were added to 10 mL of infant formula. Then 10 mL of acetonitrile and 150 uL of formic acid and shake for 1 min. Then AOAC QuEChERs salts (6 g $MgSO_4$ and 1.5 g of sodium acetate) were added and mixed for 5 min and then centrifuged for 5 min at 4000 rpm. 5 mL of supernatant were transferred to a 15 mL dSPE (1200 mg $MgSO_4$, 400 mg PSA, and 400 mg C18), shake for 1 min and centrifugated for 5 min at 4000 rpm. Solution was diluted 1:1 in 2 mM ammonium acetate.

4.7. Overall Dietary Exposure to Single EDCs in the Infants

EDCs quantification in infant formula samples and in the baby bottles were used to evaluate the overall dietary exposure of infants by calculating the estimated dietary intake (EDI). The concentrations of EDCs which were found equal to or greater than their respective LOQ in the infant formula were considered for estimating the daily intake. EDI was calculated combining the EDCs mean concentrations found in samples with the corresponding average milk consumption at the arbitrary age of 1 month.

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The 50th percentile average intakes, respectively, for formula milk, calculated for the age of 1 month were calculated as follows:

$$\text{EDI } (\mu g\ kg^{-1}\ \text{b.w. per day}) = \text{EDCs mean Conc} \times \text{IF} / \text{b.w.};$$

where EDCs Conc ($\mu g\ g^{-1}$) is the mean concentration of each EDC in the sample, IF is the daily Infant Formula consumption per day (g) at 1 month of age (93g dry weight/day, 50th percentile), and b.w. is average body weight at 1 month (4.25 kg, 50th percentile) in accordance with the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) and with the help of weight growth charts by WHO. [**Error! Bookmark not defined.**] EDI values were calculated using the means of the EDCs in the different type 1 formulas (N=11).

Supplementary Materials: The following supporting information can ebe downloaded at the website of this paper posted on Preprints.org. Tables S1-S16.

Author Contributions:

F.N. and F.R.F. performing EDCs analyses and revising data; R.F. and M.S. performing metal analyses and revising data; F.N., F.R.F., M.S., R.T. writing the original draft; A.M.P. and P.R. supervision; F.N., F.R.F., V.F., M.E.S., P.P., P.R. and A.M.P. writing and editing the final version of the manuscript; V.F., P.P., M.E.S., A.M.P. LIFE18 ENV/IT/00460—Life MILCH administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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