Early-life intake of major trace elements, bisphenol A, tetrabromobisphenol A and fatty acids: Comparing human milk and commercial infant formulas

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HIGHLIGHTS

- Levels of BPA and some elements were higher in infant formulas than in human milk
- Chemical concentrations were below EFSA thresholds in both breast and formula milks
- Fatty acid profiling revealed major differences according to the kind of milk
- Our results reinforce breastfeeding as the first option in early life

ABSTRACT

In the present study, the presence of a wide spectrum of major and trace elements (As, Ag, Al, Ba, Cd, Co, Cr, Cu, Hg, Mn, Ni, Sr, Sb, Se, Sn, Pb, V, and Zn), fatty acids, as well as some pollutants like free and total BPA and tetrabromobisphenol A (TBBPA), was analysed in human milk (n=53) and infant formula (n=50) samples. In addition, the infant exposure to these chemicals was assessed. The content of free BPA and several elements (Al, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Sn, Sr, and Zn) was higher ($p<0.01$) in infant formula samples. Furthermore, human milk contained levels of BPA and elements that, in almost all cases, were well below their respective EFSA and/or WHO thresholds, being also independent of the maternal characteristics (e.g., age, BMI or breastfeeding period). The fatty acid profiling also revealed major differences between human milk and infant formulas, which should be taken in account in the development of new formulas as well as in specific recommendations for the diet of breastfeeding mothers. Anyway, the results of this study reinforce that breastfeeding should be always the first feeding option in early life.

Key words: Bisphenol A, chemical elements, human milk, formula milk, fatty acids

1. Introduction

Since it is adapted to the nutritional requirements of babies, human milk is the gold standard for infant nutrition during the first months of life (Ballard and Morrow, 2013). Breast milk contains a wide spectrum of biologically active components, including immunoglobulins, chemokines, growth factors, cytokines, bioactive lipids, oligosaccharides, microRNAs, hormones, immune cells and microorganisms, among others compounds (Hennet and Borsig, 2016; Oftedal, 2012). The concentrations of human milk components can vary among individuals, being also dependent of several factors, such as mother's genotype, geographical location, gestational age, maternal health status, diet and time of lactation (Gómez-Gallego et al., 2017; Inthavong et al., 2017; Shi et al., 2009).

Globally, the complex and dynamic composition of breast milk promotes a healthy infant growth and development (Mosca and Giannì, 2017). Therefore, the World Health Organization (WHO) recommends exclusive breast-feeding during the first six months of life; thereafter, infants should receive nutritionally adequate and safe complementary foods while breastfeeding continues for up to two years of age or beyond (WHO, 2003). In European countries, the exclusive breastfeeding at six months of age ranged from 0.7% to 37.0% in Greece and Hungary, respectively, whereas in Spain was 28.5% (WHO, 2013). Despite the short- and long-term health benefits that breastfeeding provides to mother-infant pairs, many breastfed neonates are, exclusively or partly, fed with cow's milk-derived infant formulas. As a consequence, a wide range of infant powdered milks have been developed over the last few years, with a great variety in terms of nutritional content, taste, digestibility of digestion and energy (O'Connor, 2009).

Unfortunately, neither human milk nor infant formula are pristine, and they can contain chemical contaminants depending on mother's diet and lifestyle, including persistent organic pollutants (POPs), pesticides, heavy metals and other well-known

endocrine disruptors, such as bisphenol A (BPA) (Cardoso et al., 2014; Mead, 2008; Mendonca et al., 2015; Soleimani et al., 2014; Vela-Soria et al., 2016). These pollutants can be easily transferred during infant feeding (Klein et al., 2017). The confluence of different abiotic contaminants in human milk and in infant formula milk, and its potential impact on the infant's health, has been largely investigated (Cruz et al., 2009; Winiarska-Mieczan and Tupaj, 2009; Soleimani et al., 2014). BPA is a raw material for many manufactured goods, including food and beverages packaging materials and medical devices. In fact, diet is considered the major source of BPA exposure (Mendonca et al., 2015). Prenatal exposure to BPA has been associated with obesity and diabetes diseases in childhood, as well as reproductive, behavioral and neurodevelopment problems (Martínez et al., 2017). Brominated Flame Retardants (BFR) include a variety of substances frequently applied to industrial and household products to make them less flammable. Among the large group of BFR, tetrabromobisphenol A (TBBPA) is known to be produced in high amounts, representing around 60% of the total BFR market (Vandermeersch et al., 2015). TBBPA exposure can have adverse health effects, affecting thyroid hormones, the neurological function, and the reproductive system (Cruz et al., 2009). Cadmium (Cd), lead (Pb) or mercury (Hg), among other heavy metals, are widely dispersed in the environment and have bioaccumulative features, being also described as neurotoxic substances (Karri et al., 2017; Mead, 2008; Tchounwou et al., 2012). Therefore, the presence/absence of these chemicals in human milk and infant formulas must be a priority to assure that the intake of milk in early-life does not mean an additional exposure to pollutants, whose effects are sometimes not observed until long-term.

The main polyunsaturated fatty acids are arachidonic, eicosapentaenoic, and docosahexaenoic acids. They are important for regulating growth, inflammatory responses, immune function, playing key role in neural tissue structure and function, cell membrane structure, cognitive development, and motor systems in newborns

(Barreiro et al., 2018). For that reason, the WHO joint expert committee published the intake recommendations for these fatty acids and linolenic acid, considering human milk as the reference (WHO, 2018). A correct diet of the mother is also important to provide these essential fatty acids, first to the fetus, and later to the newborn through breast milk (Martin et al., 2016).

This study was firstly aimed at analyzing the presence of a wide spectrum of components (e.g., major and trace elements, fatty acids, free and total BPA and TBBPA) in human milk and infant formula samples. Subsequently, the newborn exposure to these chemicals through the intake of milk, either breast or formula, was evaluated.

2. Materials and methods

2.1 Participating women, collection and preparation of the samples

Human milk samples (n=53) were collected from healthy Spanish mothers (with healthy infants) at La Paz University Hospital (Madrid). Samples were immediately placed on ice until their arrival to the laboratory where they were frozen (-20 °C). Mothers' age ranged from 25 to 43 years old, being classified into 3 groups: (a) <30 years old (n=6); (b) from 30 to 35 years old (n=20); and (c) >35 years old (n=23). The mean body mass index (BMI) was 24.5 kg/m^2 and, according to this parameter, women were also grouped into 3 categories: (a) <18.5 kg/m^2 (n=1); (b) between 18.5 and 25 kg/m^2 (n=27); and (c) >25 kg/m^2 (n=22). Depending on the months of breastfeeding (ranging between 1 and 18 months), women were divided into 3 different groups: (a) first month (n=18); (b) 1 to 6 months (n=20); and (c) >6 months (n=5). Data relative to some specific samples were missing. All volunteers gave written informed consent to the protocol (C.P.-C.I. 10/017-E), which had been previously approved by the Ethical Committee of Clinical Research of La Paz University Hospital (Madrid, Spain).

149 Several samples of infant formula milk (n=50) of different commercial brands
150 and types, including first infant (n=25), follow-on (n=14), and growing-up (n=11)
151 formulas, were purchased in pharmacies and supermarkets from Spain. Infant formula
152 samples were prepared according to manufacturer instructions in clean and BPA-free
153 materials, using bottled water (Aquarel®), whose content of BPA and trace elements
154 was also determined.

155

156 *2.2 BPA and TBBPA analysis*

157 *2.2.1. Standards and reagents for BPA and TBBPA*

158 BPA (99% purity) and TBBPA (99% purity) were purchased from Sigma-Aldrich
159 (West Chester, PA, USA). Tetrabromobisphenol A ring-$^{13}C_{12}$ (TBBPA$^{13}C_{12}$; 99% purity)
160 and d16-bisphenol A ($BPAd_{16}$; 98 atom % D), used as internal standards (I.S.), were
161 purchased from Sigma-Aldrich and Cambridge Isotope Laboratories, Inc. (Tewksbury,
162 MA, USA) respectively. Individual standard solutions and internal standards were
163 prepared in methanol (HPLC grade from Sigma-Aldrich) at concentrations of 2000
164 µg/L. Acetonitrile (MeCN, gradient grade for HPLC), acetic anhydride (AA; >99%
165 purity), trichloroacetic acid (TCA, >99% purity), tetrachloroethylene (T4CE, >99%
166 purity), anhydrous magnesium sulfate (anhydrous $MgSO_4$; 99.5% purity) and β-
167 glucuronidase (Type 1 from Helix pomatia, ≥3000,000 U/g solid glucuronidase and
168 ≥10,000 U/g solid sulfatase) were purchased from Sigma-Aldrich. MeOH (MeOH, for
169 HPLC LC-MS grade), hydrochloric acid (HCl, 32%) and potassium carbonate (K_2CO_3,
170 analytical grade) were purchased from Merck (Darmstadt, Germany). Sodium chloride
171 (NaCl; 99.5% purity), sodium hydroxide (NaOH) and ammonium acetate ($C_2H_3O_2NH_4$,
172 97% purity) were purchased from AppliChem Panreac ITW Companies (Barcelona,
173 Spain). Ultra-pure Milli-Q water was obtained using a Millipore Milli-Q system (Millipore,
174 Bedford, MA, USA). Ultra-high-purity helium (99.999%) for GC-MS was obtained from
175 Gasin (Maia, Portugal).

176

177 *2.2.2. Instrument and analytical conditions for analysis of TBBPA*

178 A high-performance liquid chromatography (HPLC) system Waters Alliance
179 2695 (Waters, Milford) was interfaced to a Quattro Micro triple quadrupole mass
180 spectrometer (Waters, Manchester, UK). The chromatographic separation was
181 achieved using a Kinetex C18 2.6 µ particle size analytical column (150 × 4.6 mm) with
182 a Phenomenex pre-column (Tecnocroma, Portugal), at a flow-rate of 200 µL/min. The
183 column was kept at 30ºC and the sample manager was kept at ambient temperature (±
184 25ºC). The mobile phase consisted of methanol (90%) and an aqueous solution of 5
185 mM ammonium acetate (pH <5), isocratic (10%). Total run time was 15 min, while the
186 sample injection volume was 20 µL.

187 MS/MS acquisition was operated in negative-ion mode with multiple reaction
188 monitoring (MRM); the collision gas was Argon 99.995% (Gasin, Portugal) with a
189 pressure of 2.9 $\times 10^{-3}$ mbar in the collision cell. Capillary voltages of 3.0 KV were used
190 in the negative ionization mode. Nitrogen was used as desolvation gas and cone gas
191 being the flows of 350 and 60 L/h, respectively. The desolvation temperature was set to
192 350ºC and the source temperature to 150ºC. Dwell times of 0.1 s/scan were selected.
193 The data were collected using the software programme MassLynx4.1.

194 For each analyte, two transitions were selected for identification, and the
195 corresponding cone voltage and collision energy were optimized for maximum intensity
196 as described (Cunha et al., 2016). The quantification was made in multiple reaction
197 monitoring (MRM), 524.87 >419.87, 542.87 > 446 for TBBPA and 554.92 > 428.84 and
198 554.92 > 457.92 for TBBPA$^{13}C_{12.}$

199

200 *2.2.3. Instrument and analytical conditions for analysis of BPA*

A gas chromatograph 6890 (Agilent, Little Falls, DE, USA) equipped with a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland) and an electronically controlled split/splitless injection port, was interfaced to a single quadrupole inert mass selective detector (5975B, Agilent) with electron ionization (EI) chamber, was used. GC separation was performed on a DB-5MS column (30 m × 0.25 mm I.D. × 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA). Helium was the carrier gas with a constant flow of 1 mL min^{-1}. The injection was made in splitless mode (purge-off time, 60 s) at 280°C. The oven temperature program was as follows: 100°C held for 1 min, ramped to 280°C at 30°C min^{-1} held for 5.0 min. Total run time was 12 min. The MS transfer line was held at 280°C. Mass spectrometric parameters were set as follows: electron ionization with 70 eV energy; ion source temperature, 230°C and MS quadrupole temperature, 150°C. Agilent ChemStation (version D.0200SP1) was used for data collection/processing and GC–MS control. The quantification was made in selected ion monitoring (SIM), m/z 213, 228 and 270 for BPA and 224, 242 and 284 for $BPAd_{16}$. The ion m/z 213 and 224 was used for quantification of BPA and $BPAd_{16}$, respectively, and the others for confirmation.

2.2.4. Sample extraction for TBBPA analysis

Sample preparation for extraction of TBBPA entailed the following steps: 1 g of homogenized sample (or 1 g of sample previous hydrolyzed with 20 µL β-glucuronidase solution -20,000 U/ml in 1 M ammonium acetate buffer pH 5.0, overnight at 37°C - Total TBBPA) spiked with 25 µL of TBBPA$^{13}C_{12}$ (IS, 1000 µg/L) was added with 10 µL of NaOH 2.5 M until pH ≥10. The mixture was added with 1 mL of hexane shake by hand 1 min centrifuge at 4736 *g* for 1 min. The supernatant was discarded and repeat the previous step again. Then, add 20 µL of HCl 3N until pH<5 followed by 2.5 mL of MeCN, 1 g of anhydrous $MgSO_4$ and 0.25 g of NaCl, shake vigorously by hand for 5 and centrifuge the tube at 4736 *g* for 3 min. One point five mL of extract was

228 evaporated until dryness using a gentle nitrogen stream at room temperature. Finally,
229 the dry extract was re-dissolved in 100 µL of mobile phase and 20 µL was injected in
230 the LC-MS/MS system.

231

232 *2.2.5. Sample extraction for BPA analysis*

233 BPA extraction was performed according to Cunha et al. (2011), with some
234 minor modifications adopted to human milk. Briefly, 2 g of homogenized sample (or 2 g
235 of sample previous hydrolyzed with 40 µL β-glucuronidase solution -20,000 U/ml in 1 M
236 ammonium acetate buffer pH 5.0, overnight at 37°C - Total BPA) was spiked with 40 µL
237 of BPAd (250 µg L^{-1}) and mixed with 5 mL TCA solution (10% in MeOH) in a vortex for
238 2 min. The sample was centrifuged at 2,750 *g* for 5 min and the uplayer was added
239 with 5% K_2CO_3 solution until pH >10. Then 4 ml of the extract were transferred to a
240 tube with a conical bottom and a mixture of MeCN (210 mL), T4CE (60 µL) and AA (60
241 µl) was rapidly injected. The tube was closed and hand-shaken gently for 1 min. After
242 that it was centrifuged at 2,750 *g* for 5 min and 50 µL of the sedimented phase were
243 transferred for a vial and 1 µL of the extract was injected in the GC-MS system.

244 *2.2.6. Quality assurance and control for TBBPA and BPA*

245 In order to avoid any kind of contamination, nitrile plastic gloves were used
246 throughout the analytical work and the use of plastic materials was avoided. Amber
247 glass vials were heated (400°C) overnight prior to use. Using these precautions, no
248 problems concerning levels in analytical blank samples were observed.

249 Due to the extent matrix effects exhibited by these particular samples, matrix-matched
250 calibration curves were performed using powered infant formula free of TBBPA and
251 BPA. Linearity was evaluated in the range of 1 to 100 µg/L for TBBPA and 0.1 to 100
252 µg/L range for BPA, using 7 calibration points for both analytes. Good correlation
253 coefficients (>0.998) were obtained in both matrix-matched curves, confirming method

reliability. The precision of the method was determined by repeatability (intraday precision) on a positive sample, using three replicates on each day. The relative standard deviations for TBBPA and BPA were lower than 18%. Recovery studies were performed by adding two known concentrations of TBBPA (5 and 25 µg/L) and BPA (0.5 and 10 µg/L) to a negative sample before the extraction and purification steps. Average recoveries of 88.5% (± 15.8) for TBBPA and 88% for BPA (± 5.1) were achieved, supporting the efficiency of the method. The detection and quantification limits (LOD and LOQ, respectively) were determined as the amount corresponding to signal-to-noise ratios of 3 and 10, respectively, from the analysis of a blank powered infant formula sample. LOQ was 1 µg/L and 0.05 µg/L TBBPA and BPA, respectively. LOD were 0.04 µg/L and 0.02 µg/L for TBBPA and BPA, respectively.

2.3 Main and trace elements analysis

Samples were submitted to a pre-treatment as follows: 0.50 mL of the milk sample was treated with 5 mL of 65% nitric acid (Suprapur, E. Merck, Darmstadt, Germany) in hermetic Teflon. Digestion was firstly performed at room temperature for 8 h and, then, at 80ºC for an additional period of 8 h. After cooling, the extracts were filtered and made up to 25 mL with MiliQ water.

The content of most elements (As, Ag, Al, Ba, Cd, Co, Cr, Cu, Hg, Mn, Ni, Sr, Sb, Se, Sn, Pb, V, and Zn) was determined by induction plasma coupled to a mass detector (ICP-MS, Perkin Elmer Elan 6000). Rhodium was used as internal standard. In turn, the levels of Ca, Fe, K, Mg and Na were determined by induction coupled plasma optical detector (ICP-OES, Perkin Elmer Optima 3200RL). LOD were: 0.03 mg/L Ba, Cd, Co, Cu, Mn, Sr, Se, Sn and Zn; 0.05 mg/L for As, Hg, Ni, Sb, and Pb; 0.13 mg/L for Ag, Cr and V; 0.25 mg/L for Al; 1.25 mg/L for Fe; 12.5 mg/L for Ca; 25.0 mg/L for Na; 50.0 mg/L for Mg and 125 mg/L for K.

For quality control, duplicate samples and blanks were also analysed. Three reference patterns were also used: whole milk powder (WMP), lobster hepatopancreas (TORT-2) and trace elements in spinach leaves (TES 1), obtaining recoveries ranging from 75% to 101%.

2.4 Fat and fatty acids analysis

For FA analysis, triundecanoin was used as internal standard for quantification (Larodan; Sweden). The fatty acid methyl esters standards used for quantitative and identification purposes were from diverse suppliers (Nu-Chek Prep, USA; Matreya, USA; and Supelco Inc., USA). Heptane, 2-propanol and cyclohexane, all <99%, were from Carl Roth (Germany). Boron trifluoride solution (14% in methanol) was purchased from Sigma-Aldrich. Dichloromethane (DCM, >99.5%) and anhydrous sodium sulphate (Na_2SO_4, analytical grade) were purchased from Merck (Darmstadt, Germany). Sodium chloride (NaCl; 99.5% purity), and potassium hydroxide (KOH) were purchased from AppliChem Panreac ITW Companies (Barcelona, Spain).

Fat extraction was achieved by liquid extraction, following the method described by Feng et al. (2004) with minor modifications, and the combined derivatization method (López-López et al., 2002), also with minor adjustments. Briefly, an accurate amount of 500 microliters of homogenised milk was spiked with 100 µL of internal standard solution (triundecanoin, 10 mg/ml) and mixed sequentially with 1.6 mL of 2-propanol, 2ml of cyclohexane and 2.2 mL of NaCl aqueous solution (1%), with 1 min. vortex mixture between steps. After centrifugation (2,750 *g* for 5 min), the upper layer was transferred to a second vial and the extraction repeated with 2 ml of cyclohexane. The two organic phases were combined, dehydrated with anhydrous sodium sulphate, and taken to dryness under a gentle nitrogen stream at 40ºC. The extracted fat was dissolved in dichloromethane and the fatty acids were converted into their methyl esters first with hot alkaline derivatization with KOH (0.5 M in methanol; 80ºC) followed

by addition the BF3 reagent. After cooling, phase separation was achieved by addition of heptane and NaCl aqueous solution (1%), with the upper layer collected for GC analysis. This derivatization method was previously compared with plain alkaline derivatization and a global increase of recovered lipids mass was achieved with combined derivatization, without an increase or alteration of the trans fatty acids.

The fatty acid composition was determined by gas chromatography on a Chrompack (CP 9001), equipped with a FAME CP-Select CB column (100 m x 0.25 mm x 0.2 µm; JW), with helium as carrier gas at 0.7 ml/min, and a temperature gradient from 100 ºC to 240 ºC, in a total of 60 min. Injection port was at 250 ºC, with a 1:100 split ratio, and the detector was at 270 ºC. Each peak was identified using known standards of fatty acid methyl esters (FAME, Nu-Chek Prep, Elysian, MN, USA; Matreya, Pleasant Gap, PA, USA; and Supelco 37 Component FAME mix, Supelco Inc.). A total of 80 fatty acids, from 6 to 24 carbon atoms, were quantified. Fatty acids contents were recorded as % weight of total fatty acids after external calibration with individual standards, and on a milk basis (mg/100 mL) using triundecanoin as internal standard that is used also to estimate the milk fat content (g/100 mL).

2.5 Exposure assessment

Equation 1 (see below) was used to establish the daily intake of BPA and elements. Three periods of exposure were considered: (a) <1-month-old; (b) between 1- and 6-month-old; and (c) between 6 and 12-month-old infants fed exclusively with either human milk or infant formula.

$$DI_{i,p} = C_{i,p} \cdot I_{milk,\,p}. \quad \text{(Equation 1)}$$

Where $DI_{i,p}$ is the daily intake of the chemical *i* in the period *p* (in µg/kg_{bw}/day), $C_{i,p}$ is the concentration of the chemical *i* in milk in the feeding period (*p*) (in µg/mL), and $I_{milk,p}$ is the daily amount of milk ingested by body weight in each period (mL/day/kg_{bw}). Similar

333 milk intakes between the breastfed and the formula-fed groups were assumed. Data on
334 milk intake were obtained from the US EPA exposure handbook (EPA, 2011) with
335 monthly temporal resolution.

336

337 *2.6 Statistics*

338 For the statistical analysis of results, the items with values below the detection
339 limit (LD), were assumed to be equal to one-half of that limit (ND = ½ LD). Statistical
340 significance was established using firstly the Levene test to establish whether the data
341 showed parametric distribution, or not. Subsequently, the ANOVA test for data
342 following a parametric distribution, or the Kruskal-Wallis for non-parametric data were
343 applied. A difference was considered as statistically significant when the probability
344 was lower than 0.05 ($p<0.05$). Principal component analysis (PCA) was applied to
345 reduce the number of variables extracting as much information as possible. PCA was
346 performed with fatty acid relative contribution. For statistical analysis and PCA, IBM
347 SPSS Statistics was used.

348

349 **3. Results and discussion**

350 Mean levels of BPA (free and total), TBBPA, elements and fatty acids in the
351 human milk and infant formula samples analysed in this study are shown in Table 1.
352 Tables 2 to 5 present the levels of the same chemicals, according to different
353 parameters: feeding-period, mother's BMI and maternal age.

354

355 *3.1. Free and total BPA content*

356 Free and total BPA (free plus conjugated BPA) were respectively detected in 38
357 and 76% of the samples of breast milk, with mean levels of 0.26 and 1.30 µg/L,
358 respectively. In turn, the concentration of free BPA in infant formula was 0.88 µg/L,

359 while that of total BPA was 3.85 µg/L (Table 1). The concentrations of both free BPA
360 and total BPA in formula samples were significantly higher (p<0.001) than those in
361 human milk (Table 1). When the ratios between the concentrations of free or total BPA
362 and the fat content were calculated, means of 23.5 and 106 ng/g of fat, respectively,
363 were found for formula samples, while values in breast milk were 6.4 and 59.0 ng/g of
364 fat, respectively, Although both parameters were lower in human milk samples, only the
365 ratio BPA:fat content showed a statistically significant (p<0.001) difference (Table 1)
366 according to the kind of milk. No differences (p>0.05) in the BPA (free or total) levels
367 were observed according to formula type, breastfeeding period, maternal age or
368 mother's BMI (Tables 2 to 5). In the scientific literature, a wide range of values
369 regarding BPA levels in human milk have been reported, ranging from 0.7 µg/L (Otaka
370 et al., 2003) to as high as 42.6 µg/L (Yi et al., 2010). However, important differences
371 between the studies have been also noted, including the fact of monitoring only free or
372 total (free and conjugated) BPA. More recently, Cao et al. (2015) analysed the
373 presence of both free and total BPA in human milk samples, observing similar results
374 to those observed in our study: free BPA was detected in fewer samples than total BPA
375 (16.5% *vs.* 25.9%), with amounts ranging from <0.036 to 2.3 ng/g. In agreement to our
376 findings, the same authors also stated that the dietary exposure to BPA for breastfed
377 infants was expected to be lower compared to that of formula-fed infants (Cao et al.,
378 2015). However, the presence of BPA in infant formulas could have decreased in the
379 last years due to the general tendency of using BPA-free coatings for canned formulas
380 (Adesman et al., 2017; Cao et al., 2015). As for the presence of BPA in human milk, it
381 is surely associated with the mother's ingestion of contaminated foods (Martinez et al.
382 2017, 2018).

383

384 *3.2. Concentrations of TBBPA*

In this study, only few samples (5 out of 50 among formula samples, and 3 out of 53 among human milk ones) presented TBBPA levels above the detection limit (0.50 µg/L). In those cases, the mean levels were 0.57 µg/L (14.6 ng/g of fat) and 0.58 µg/L (18.7 ng/g of fat) for formula and human milk samples, respectively, being the difference not significant (p>0.05; Table 1). Moreover, TBBPA concentrations were not statistically significant (p>0.05) according to the maternal characteristics (age, BMI, and breastfeeding period) (Tables 2 to 5). TBBPA was only detected in some samples corresponding to mothers aged >35 years, while it was not detected in the group with more than 6 months of breastfeeding. In any case, TBBPA concentrations were similar to those observed in other studies performed in France (Inthavong et al., 2017). In the Czech Republic, Lankova et al. (2013) found that TBBPA and α-hexabromocyclododecane (α-HBCD) were the only brominated flame retardants detected in human milk samples. However, they could be only detected in a low percentage of such samples and in none of the tested infant formulas samples (Lankova et al., 2013). These results should be taken with caution since TBBPA is usually detected and quantified either by gas or liquid chromatography coupled to mass spectrometry (MS) (Cariou et al., 2008), and the acidification and chloroformate derivatization steps required for GC-MS analysis may be responsible for a low recovery rate of this compound (Covaci et al., 2009).

3.3 Content of main and trace elements in the samples

The levels of Ag, As, Sb and V in all the samples and the levels of Cd and Co in the formula samples were below their respective LOD (Table 1). The levels of Al, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Sn, Sr, and Zn were significantly higher (p<0.05) in the infant formula samples, while Cd was the only element with a significantly higher concentration in breast milk (p<0.05) (Table 1). In relation to the formula type, the levels of Ca, Fe, K, Mg, Na, and Sn in first infant formulas were statistically lower

412 (p<0.05) than in follow-on and growing-up milk samples (Table 2). In relation to the
413 breastfeeding period, the levels of Cu were higher among women during their first
414 month (0.40 mg/L) than in the other women groups (0.25 and 0.18 mg/L for >1-6 and
415 >6 month of breastfeeding, respectively) (Tables 2 to 5). Mercury (Hg) was detected
416 only in a single human milk sample from a woman within the >6-months group. No
417 statistically significant (p>0.05) differences were found for the levels of any element
418 depending on maternal BMI or age (Tables 4 and 5, respectively).

419 Minerals and trace elements accounting for about 4% of total human body mass
420 play an important role in bone structure, regulate certain body functions, and help
421 maintain the body's water balance (WHO and FAO, 2004). It is known that apart from
422 essential elements, human milk can also transfer potentially toxic metals, such as Pb,
423 As or Cd, with varied concentrations depending on the environmental exposure, the
424 diet or bad habits (Klein et al., 2017). Rapid growth and development may put infants at
425 risk of deficiencies of certain essential minerals in vulnerable populations. Micronutrient
426 deficiencies are associated with a higher frequency of infections in the short-term and
427 increased rates of chronic diseases. However, excessive amounts of these elements
428 can also be detrimental for health (Klein et al., 2017). For example, high levels of iron
429 in formulas may increase the infant risk of infection by increasing nutrient bioavailability
430 to pathogenic bacteria (Quinn, 2014), while high Mn exposure in children has been
431 associated with impaired cognitive development and motor coordination (O'Neal and
432 Zheng, 2015). Therefore, it is essential that infant formula and milk products intended
433 for use by infants contain minerals in amounts that satisfy their nutritional requirements
434 without leading to adverse effects (Poitevin, 2016).

435

436 3.4. Fat and fatty acid content in the samples

437 The fat content was similar among the human milk and the infant formula
438 samples (3.86 and 3.88%, respectively) (Table 1). For this parameter, no statistically

439 significant differences (p>0.05) were found according to the formula milk type (first
440 infant, follow-on, and growing-up formula), breastfeeding period (<1, 1-6, and >6
441 month), maternal age (>30, 30-35, and >35 years old) or mother's BMI (18.5-25 kg/m^2
442 and >25 kg/m^2) (Tables 2 to 6). However, it is known that breastfed infants absorb fat
443 better than formula-fed infants due to the presence of lipases in human milk that are
444 not present in cow's milk (Fields and Demerath, 2012). It is generally considered that a
445 breastfed infant consumes less calories (around 85 kcal/kg body weight/day) during the
446 first months of life than a formula-fed infant (100 kcal/kg/day) (Committee on the
447 Evaluation of the Addition of and Ingredients New to Infant Formula, 2004). The
448 breastfed infant has a lower total energy expenditure and a slower rate of weight gain
449 (Butte et al., 1990). In addition, the gastro-esophageal reflux is usually lower in
450 breastfed infants, most likely due to a more rapid gastric emptying time, resulting in
451 lower loss of ingested food (Committee on the Evaluation of the Addition of and
452 Ingredients New to Infant Formula, 2004).

453

454 Non-identified fatty acids ranged from 2.9 to 24.8% with a mean of 6.4% for all samples
455 analysed. Globally, saturated fatty acids (SFA) contribution was higher in formula milks
456 than in human milk, while the opposite was observed for unsaturated fatty acids (Table
457 1). The fatty acids that contributed the most to the total content were oleic acid (18:1),
458 palmitic acid (16:0), linoleic acid (18:2), lauric acid (12:0), myristic acid (14:0), and
459 stearic acid (18:0), with an overall contribution of 87.0% and 84.2% in formula and
460 human milk samples, respectively (Table S1). Palmitic acid (16:0), γ-linolenic acid
461 (18:3n-6), gondoic acid (20:1n-9), octadecatetraenoic acid (18:4n-3) and
462 eicosapentaenoic acid (EPA) (C20:5n-3) showed a similar (p>0.05) contribution (in %,
463 g/100 g of fat) between human milk and formula samples (Table S1). When levels (g/L
464 of milk) of fatty acids were taken into account, decanoic acid (10:0), lauric acid (12:0),
465 erucis acid (22:1n-9), and tetracosenoic acid (24:1n-9) presented also similar (p<0.05)

levels between formula and human milk (Table S1). In contrast, the comparison of the composition of fatty acid between formulas and human milk revealed several differences. The main differences in the fat composition were a higher ($p<0.05$) contents of octanoic acid (8:0) lauric acid (12:0), linolenic acid (LA) (18:2n-6) and α linolenic acid (18:3n-3) (ALA) in the formula samples (Table S1), in opposition to a higher content ($p<0.05$) of myristic (14:0), palmitoleic (16:1), and stearic (18:0) in the human milk ones. In addition, human milk samples contained a higher ($p<0.05$) percentage of total SFA (35.9 vs 38.5 %), omega 7 fatty acids (0.3 vs. 2.2%), eicosatetraenoic acid (AA) (0.19 vs. 046%), docosahexaenoic acid (DHA) (0.22 vs. 0.41%) and conjugated linoleic acid (CLA) (0.01 vs 0.10%) and a lower ($p<0.05$) contributions of total MUFA (40.1 vs 37.8%) and polyunsaturated fatty acids (PUFA) (17.7 vs. 15.2 %), including omega 3 (2.38 vs. 1.13%) and omega 6 (15.3 vs. 14.0%) fatty acids, when they are compared to formula samples (Table 1). Similar significant differences in fatty levels (in g/L) were noted, except for SFA and MUFA that not reach the significance between formula and human milk.

In relation to the type of formula (Table 2), lower ($p<0.05$) levels of SFA were found in growing up formula than Follow –on which shown similar ($p>0.05$) levels than first infant formulas. Levels of AA and DHA were higher ($p<0.05$) in first formula levels than growing up milk formula. Regarding contribution of each fatty acid, differences were found regarding the percentages of low-chain FA (8:0 to 14:0), docosahexaenoic acid (DHA) and araquidonic acid (AA) with higher ($p<0.05$) in first formulas than in growing-up formulas, while the percentages of palmitic (16:0) and γ-linolenic were lower ($p<0.05$). Finally, higher ($p<0.05$) percentages of omega 7 and 9 were found in growing-up formulas (>12-month formula milk) when compared to the other formula milks (first infant: 0-6 month; follow-on: >6 month).

Regarding to the breastfeeding period (Table 3), no differences in the content and levels of all the fatty acids classes, both saturated and unsaturated, were found.

493 Similar findings were reported from López-López et al., 2002. Only some unsaturated
494 FA with low representativeness (>0.5%) were higher during the first period (<1 month)
495 than after 6-months of breastfeeding.

496 Only minor significant difference observed in relation to the maternal BMI (Table
497 4), higher levels of MUFA and ω-3 PUFA were higher ($p<0.05$) in women with BMI>25
498 kg/m^2 compared with the group of BMI18.5-25 kg/m^2. Regarding individual fatty acids,
499 few differences were noted; higher ($p<0.05$) amounts of 18:0 and 20:3n-6 in the milk fat
500 of the group of women with a BMI>25 kg/m^2 when compared to the group comprising
501 women with a BMI ranging from 18.5 to 25 kg/m^2.

502 No clear tendencies were found according to the maternal age (Table 5).
503 Samples from mothers below 30 years old had a higher ($p<0.05$) levels of PUFA and
504 total omega-6 fatty acids than those from mothers between 30-35 years old but, in
505 contrast, their content in such fatty acids were similar ($p>0.05$) to that found among
506 women >35 years-old (Table 5). Additionally, some individual FA presented higher
507 contributions in the mothers whose age was below 30 years ($p<0.05$) as linoleic acid,
508 C18:3t, C18:2n6c, C20:2n6 and C22:2 than those older than 35.

509 Data on the fatty acid composition of the samples (in g/100mg of fat) were
510 submitted to PCA (Figure 1). The results are represented as a graph with two principal
511 components (PC) that explain 50 and 23 % of the data variance. The first PC (PC1)
512 showed a highly positive correlation with MUFA and highly negative correlation with
513 SFA, while PC2 was highly positively correlated with ω-6 PUFA and negatively
514 correlated with TFA. In addition, PC1 and PC2 were positively correlated with ω-6
515 PUFA. PCA results revealed two main clusters that were differentiated through PC1
516 according the milk type (infant formula or human milk). This difference seems to be
517 more relevant if PCA was conducted with fatty acids contributions instead of
518 categorizing fatty acids (Figure S1). However, the human milk cluster showed bigger
519 differences among samples (Figure 1), while formula milk samples appeared as a more

520 homogeneous cluster, regardless of the type of formula (first infant, follow-on and
521 growing-up formula).

522 Although the development of infant formulas can be traced to the nineteenth
523 century, the addition of nucleotides in 1999 and long-chain polyunsaturated fatty acids
524 in 2002 marked a new era in infant formula (Barreiro et al., 2018). In this work, first-
525 stage formulas showed a lipid profile closer to that of the human milk samples, in terms
526 of monounsaturated acids. New born do not have a fully developed ability to convert
527 essential fatty acids into their long-chain metabolites and, for this reason,
528 supplementation with DHA is critical in infant formulas (Barreiro et al., 2018). PCA
529 performed in this work clearly showed that human milk is not a static fluid and changes
530 over time; in contrast, infant formulas seem to be uniform with no clear fatty acid
531 differences despite being marketed as intended for different stages of the infant
532 development. It is important to mention that human milk fat includes medium-chained
533 fatty acids (MCFA) and triacylglycerols emulsified by a sphingomyelin-rich phospholipid
534 membrane (milk phospholipids, MPL) while the sphingomyelin is lacking in infant
535 formulas. Both the sphingomyelin content and the saturated level of phospholipids
536 affect gut lipase activity, which alters the concentrations of lipid hydrolysis products in
537 the ileum and colon, as a consequence, differences in phospholipid and fatty acid
538 composition may modulate the acquisition and development of the infant gut microbiota
539 (Nejrup et al., 2017).

540

541 *3.6 Exposure assessment*

542 In general terms, the exposure to the compounds analysed in this study was
543 estimated to be higher in formula-fed infants than in breastfed infants. Furthermore, the
544 intake of BPA and most trace elements showed a similar decreasing trend in the
545 different feeding period groups (< 1 month, 1-6 months and >6-12 months) (Table 6).
546 Such decreasing trend may be due to the fact that the milk intake:body weight ratio

547 decreases with age. As abovementioned, the levels of BPA and elements were
548 generally higher in infant formula samples than in human milk. Free BPA intake was far
549 below the TDI threshold set by EFSA (4 µg/kg/day) for both formula-fed and breastfed
550 infants. Our results on BPA exposure are in agreement with those obtained by
551 Sarigiannis et al. (2016) for breastfed infants. However, they are lower for formula-fed
552 infants (Sarigiannis et al., 2016). The fact that only BPA-free material was used in this
553 study may account for the lower BPA exposure values estimated for formula-fed
554 infants. The exposure to Al, Cr, Cu and Sn was below the respective tolerable intake
555 values (2 mg/kg/week, 300 µg/kg/day, 500 µg/kg/day, and 14 mg/kg/week for Al, Cr,
556 Cu and Sn, respectively) set by EFSA (2015a, 2015b, 2014, 2011, 2010ab) or WHO
557 (2018). On the other hand, the Pb exposure (4.50 µg/kg/day) was higher than the
558 provisional tolerable weekly intake (PTWI) set at 25 µg/kg (EFSA, 2010a) in the first
559 period group (<1-month-old). This could be due to the low weight of the babies in their
560 first weeks of life. Similarly, Ni exposure was higher than its corresponding TDI (2.80
561 µg/kg/day) (EFSA, 2015a) in all periods and milk types except for the group of infants
562 who had been breastfed from 6 to 12 months. It should be noted that a conservative
563 scenario was here considered by assuming non-detected values as one-half of the
564 LOD.

565 Dietary reference values for fatty acids were referenced as energy ingested.
566 Only fatty acid with a reference value by quantity is DHA for children between 6 and 24
567 month. These reference values are set between 10-12 mg/kg/day by WHO (WHO,
568 2008) and 100 mg/day by EFSA (EFSA, 2010b) (Table 6). These reference intakes
569 were reached by breast feeding infants older than 6 months (11.8 mg/kg/day (109
570 mg/day)), but not by formula feeding infants of same age (6.62 mg/kg/day (60.9
571 mg/day)). Essential fatty acids (LA and ALA) shown higher intake levels, especially for
572 LA in <1 month group and ALA in the three ages groups.

573

4. Conclusions

In this study, human milk samples contained levels of BPA and well below the EFSA/WHO thresholds, being also irrespective of the maternal characteristics (i.e., age, BMI and breastfeeding period). Actually, the concentration of free BPA was significantly higher in infant formula samples than in breast milk, which also contained significantly lower values of some essential elements, such as Al, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Sn, Sr, and Zn. The fatty acid profiling also revealed major differences between human milk and infant formulas, which should be taken in account in the development of new formulas a well as in specific recommendation for the diet of breastfeeding mothers. DHA acceptable intake limits set by EFSA and WHO were not reached by formula feeding infants in 6-12 months group. Anyway, the results of this study reinforces that breastfeeding should be always the first feeding option in early life.

Acknowledgements

This research was supported by the “HEALS” project (FP7-603946), Health and environmental-wide associations based on large population surveys, as well as by the Spanish Ministry of Economy and Competitiveness through grants AGL2016-75476-R and AGL2016-78942-R. J. Rovira received funds from Health Department of Catalonia Government trough "Pla Estratègic de Recerca i Innovació en Salut" (PERIS 2016-2020). Sara C. Cunha. Susana Casal and José O. Fernandes thanks REQUIMTE, FCT (Fundação para a Ciência e a Tecnologia) and FEDER through the project UID/QUI/50006/2013 – POCI/01/0145/FEDER/007265 with financial support from FCT/MEC through national funds and co-financed by FEDER, under the Partnership Agreement PT2020. Sara C. Cunha acknowledges FCT for the IF/01616/2015 contract.

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783

784 **Table 1.** Levels of BPA (free and total), TBBPA, elements and fat content in samples of
785 human milk and infant formulas.

		By volume of milk			By value of fat		
	% detected	Formula (n=50)	Human (n=53)	*p-value*	Formula (n=50)	Human (n=53)	*p-value*
BPA (free)	38	0.88 ± 1.01	0.26 ± 0.81	**<0.001**	23.5 ± 29.5	6.38 ± 24.0	**<0.001**
Total BPA	76	3.85 ± 4.19	1.30 ± 4.24	**0.003**	106 ± 127	59.0 ± 270	0.270
TBBPA	8	0.57 ± 0.27	0.58 ± 0.34	0.970	14.6 ± 8.62	18.7 ± 16.7	0.129
Elements							
Ag	0	<0.13	<0.13	-	-	-	-
Al	17	0.64 ± 1.85	0.28 ± 0.67	**0.015**	16.8 ± 46.3	5.71 ± 8.98	0.212
As	0	<0.05	<0.05	-	-	-	-
Ba	25	0.04 ± 0.06	0.02 ± 0.03	0.167	0.76 ± 1.62	0.42 ± 0.80	0.494
Ca	100	511 ± 177	273 ± 73.9	**<0.001**	13457 ± 5087	8648 ± 6132	**<0.001**
Cd	8	<0.03	0.02 ± 0.03	**0.004**	-	0.60 ± 1.19	**<0.001**
Co	1	<0.03	0.02 ± 0.09	0.334	-	0.17 ± 0.43	**0.017**
Cr	100	0.38 ± 0.13	0.32 ± 0.05	**0.001**	9.98 ± 3.92	9.48 ± 4.15	0.534
Cu	98	0.49 ± 0.29	0.34 ± 0.24	**0.005**	13.1 ± 8.19	10.3 ± 8.04	0.086
Fe	53	7.50 ± 3.01	1.63 ± 5.84	**<0.001**	197 ± 84.0	43.5 ± 133	**<0.001**
Hg	2	0.03 ± 0.00	0.03 ± 0.01	0.614	0.96 ± 0.20	0.94 ± 0.61	0.379
K	100	727 ± 208	509 ± 88.0	**<0.001**	19792 ± 8818	16136 ± 9617	**0.048**
Mg	40	55.1 ± 18.9	26.1 ± 5.43	**<0.001**	1483 ± 684	800 ± 494	**<0.001**
Mn	51	0.15 ± 0.09	0.02 ± 0.02	**<0.001**	3.98 ± 2.53	0.27 ± 0.60	**<0.001**
Na	100	208± 69.1	172 ± 144	**<0.001**	5696 ± 2934	5323 ± 5279	**0.006**
Ni	44	1.22 ± 4.56	0.04 ± 0.04	**<0.001**	34.6 ± 138	1.13 ± 1.07	**<0.001**
Pb	4	0.03 ± 0.01	0.03 ± 0.01	0.992	1.00 ± 0.29	1.00 ± 0.82	0.199
Sb	0	<0.05	<0.05	-	-	-	-
Se	51	0.03 ± 0.03	0.07 ± 0.16	0.151	0.72 ± 1.11	2.33 ± 7.06	0.115
Sn	12	0.02 ± 0.04	0.01 ± 0.00	**0.001**	0.40 ± 1.21	0.17 ± 0.43	0.358
Sr	93	0.25 ± 0.09	0.05 ± 0.04	**<0.001**	6.58 ± 2.59	1.65 ± 1.36	**<0.001**
V	0	<0.13	<0.13	-	-	-	-
Zn	83	5.73 ± 3.00	2.30 ± 3.54	**<0.001**	154 ± 86.6	62.1 ± 86.9	**<0.001**
% Fat	100	3.88 ± 0.74	3.86 ± 1.63	0.319	-	-	
Fatty acids							
SFA		14.0 ± 3.56	15.2 ± 6.97	0.748	35.9 ± 5.30	38.5 ± 4.18	**0.006**
MUFA		15.5 ± 3.20	14.6 ± 6.25	0.061	40.1 ± 4.68	37.8 ± 3.56	**0.005**
PUFA		6.97 ± 1.87	6.06 ± 3.30	**0.005**	17.7 ± 2.08	15.2 ± 2.98	**<0.001**
ω-3 PUFA		0.93 ± 0.24	0.42 ± 0.20	**<0.001**	2.38 ± 0.47	1.13 ± 0.52	**<0.001**
ω-6 PUFA		6.04 ± 1.71	5.60 ± 3.20	**0.040**	15.3 ± 2.08	14.0 ± 3.11	**0.012**
TFA		0.12 ± 0.07	0.43 ± 0.20	**<0.001**	0.33 ± 0.18	0.80 ± 0.31	**<0.001**
LA		5.90 ± 1.68	5.05 ± 2.95	**0.002**	15.1 ± 2.08	12.6 ± 2.99	**<0.001**
ALA		0.81 ± 0.22	0.20 ± 0.11	**<0.001**	2.08 ± 0.40	0.53 ± 0.25	**<0.001**
AA		0.08 ± 0.09	0.18 ± 0.09	**<0.001**	0.19 ± 0.20	0.46 ± 0.12	**0.001**
EPA		0.02 ± 0.03	0.02 ± 0.02	0.946	0.04 ± 0.05	0.07 ± 0.06	0.303
DHA		0.08 ± 0.09	0.16 ± 0.09	**<0.001**	0.22 ± 0.14	0.41 ± 0.26	**<0.001**
CLA		0.00 ± 0.01	0.04 ± 0.02	**<0.001**	0.01 ± 0.03	0.10 ± 0.05	**<0.001**

Results presented as mean ± standard deviation. SFA: Saturated fatty acids; MUSFA: Monounsaturated fatty acids; PUFA: Poliunsaturated fatty acids; TFA: Trans fatty acids; EPA: Eicosapentaenoic acid (20:5n-3); DHA: Docosahexaenoic acid (22:6 n-3); CLA: 9c,11t-octadecadienoic acid; AA: Arachidonic acid (eicosatetraenoic acid (20:4 n-6)).
Units (by volume of milk): Total BPA, Free BPA and TBBPA, in µg/L; elements, in mg/L; % fat in g/100 mL; Fatty acids in g/L
Units (by content of fat): Total BPA, Free BPA and TBBPA, in ng/g of fat; elements, in µg/g of fat; Fatty acids in g/100 g of fat

786

787 **Table 2.** Levels of BPA (free and total), TBBPA, elements and fat content in formula
788 milk according to the feeding period. Results are shown by volume of milk.

	Formula milk		
	First infant **<1-6months (n=25)**	***Follow-on*** **>6 month (n=14)**	***Growing-up*** **>12 month (n=11)**
BPA (free)	0.98 ± 1.03	0.82 ± 1.03	0.77 ± 1.01
Total BPA	4.13 ± 4.01	3.01 ± 3.11	4.33 ± 5.93
TBBPA	0.50 ± 0.02	0.71 ± 0.46	0.56 ± 0.20
Elements			
Ag	<0.13	<0.13	<0.13
Al	0.74 ± 2.54	0.69 ± 0.89	0.38 ± 0.49
As	<0.05	<0.05	<0.05
Ba	0.04 ± 0.07	0.04 ± 0.06	0.04 ± 0.04
Ca	433 ± 162 [a]	555 ± 163 [b]	631 ± 148 [b]
Cd	<0.03	<0.03	<0.03
Co	<0.03	<0.03	<0.03
Cr	0.38 ± 0.12	0.40 ± 0.13	0.33 ± 0.13
Cu	0.52 ± 0.35	0.51 ± 0.22	0.41 ± 0.25
Fe	5.85 ± 3.11 [a]	9.35 ± 1.93 [b]	8.89 ± 1.67 [b]
Hg	0.03 ± 0.01	<0.05	<0.05
K	646 ± 182 [a]	730 ± 91.9 [b]	910 ± 260 [c]
Mg	49.7 ± 20.3 [a]	57.8 ± 19.2 [ab]	64.0 ± 10.8 [b]
Mn	0.15 ± 0.08	0.15 ± 0.09	0.14 ± 0.11
Na	183 ± 61.0 [a]	218 ± 43.7 [b]	251 ± 90.9 [b]
Ni	1.73 ± 6.43	0.83 ± 0.93	0.55 ± 0.50
Pb	0.03 ± 0.01	0.03 ± 0.01	<0.05
Sb	<0.05	<0.05	<0.05
Se	0.04 ± 0.04	0.03 ± 0.03	0.02 ± 0.02
Sn	0.01 ± 0.00 [a]	0.05 ± 0.08 [b]	0.02 ± 0.01 [b]
Sr	0.25 ± 0.10	0.25 ± 0.07	0.26 ± 0.09
V	<0.13	<0.13	<0.13
Zn	5.26 ± 2.68	6.6 ± 3.45	5.69 ± 3.12
% Fat	3.84 ± 0.86	4.08 ± 0.56	3.69 ± 0.67
Fatty acids			
SFA	14.2 ±4.06 [ab]	15.3 ± 2.37 [a]	11.7 ± 2.66 [b]
MUFA	15.2 ± 3.59	15.7 ± 2.31	15.9 ± 3.41
PUFA	6.69 ± 1.85	7.41 ± 1.51	7.06 ± 2.33
ω-3 PUFA	0.91 ±0.24	0.97 ± 0.18	0.93 ± 0.32
ω-6 PUFA	5.78 ±1.68	6.43 ± 1.42	6.13 ± 1.71
TFA	0.11 ± 0.05	0.15 ±0.09	0.13 ± 0.08
LA	5.63 ± 1.62	6.29 ± 1.41	6.05 ±2.11
ALA	0.77 ± 0.22	0.83 ± 0.14	0.85 ±0.29
AA	0.09 ± 0.09 [a]	0.09 ± 0.11 [ab]	0.02 ± 0.03 [b]
EPA	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.03
DHA	0.10 ± 0.04 [a]	0.09 ± 0.07 [ab]	0.05 ± 0.07 [b]
CLA	0.00 ± 0.00	0.01 ± 0.02	0.00 ± 0.01

Results presented as mean ± standard deviation.
Units: Total BPA, Free BPA and TBBPA, in µg/L; elements, in mg/L; fat, in g/100 mL; fatty acids, in g/L
Significant differences at $p<0.05$ are indicated with different superscripts

789

790 **Table 3.** Levels of BPA (free and total), TBBPA, elements and fat content in human
791 milk according to the time of breastfeeding. Results are shown by volume of milk.

	Human feeding period		
	<1month n=18	**1-6 month n=20**	**> 6 month n=5**
BPA (free)	0.37 ± 1.08	0.30 ± 0.84	<0.05
Total BPA	3.01 ± 7.20	0.36 ± 0.36	0.69 ± 1.31
TBBPA	0.6 ± 0.42	0.61 ± 0.39	<1.00
Elements			
Ag	<0.13	<0.13	<0.13
Al	0.35 ± 0.73	0.32 ± 0.86	0.20 ± 0.15
As	<0.05	<0.05	<0.05
Ba	0.02 ± 0.04	0.03 ± 0.03	0.02 ± 0.01
Ca	266 ± 63.1	273 ± 87.3	283 ± 59.2
Cd	0.02 ± 0.03	0.03 ± 0.04	<0.03
Co	0.05 ± 0.15	<0.03	<0.03
Cr	0.31 ± 0.04	0.31 ± 0.06	0.33 ± 0.08
Cu	0.40 ± 0.19 [a]	0.25 ± 0.24 [b]	0.18 ± 0.11 [b]
Fe	3.32 ± 9.96	0.81 ± 0.79	0.88 ± 0.55
Hg	<0.05 [a]	<0.05 [a]	0.04 ± 0.03 [b]
K	543 ± 93.3	491 ± 93.1	492 ± 73.9
Mg	26.6 ± 6.85	26.4 ± 6.12	<50.0
Mn	0.02 ± 0.02	0.01 ± 0.01	<0.02
Na	215 ± 146	136 ± 155	102 ± 15.5
Ni	0.05 ± 0.06	0.03 ± 0.02	<0.05
Pb	0.03 ± 0.01	<0.05	<0.05
Sb	<0.05	<0.05	<0.05
Se	0.12 ± 0.27	0.04 ± 0.04	0.01 ± 0.00
Sn	<0.01	0.01 ± 0.00	<0.01
Sr	0.04 ± 0.02	0.06 ± 0.04	0.06 ± 0.02
V	<0.13	<0.13	<0.13
Zn	2.77 ± 2.47	1.60 ± 3.95	0.24 ± 0.32
% Fat	3.54 ± 1.37	3.99 ± 2.02	4.48 ± 1.11
Fatty acids			
SFA	14.1 ± 5.71	15.3 ± 8.67	18.8 ± 6.25
MUFA	13.4 ± 5.66	15.3 ± 7.70	15.9 ± 2.46
PUFA	5.16 ± 2.36	6.35 ± 3.94	7.23 ± 3.11
ω-3 PUFA	0.41 ± 0.19	0.48 ± 0.25	0.45 ± 0.09
ω-6 PUFA	4.71 ± 2.27	5.82 ± 3.78	6.75 ± 3.18
TFA	0.32 ± 0.16	0.33 ± 0.22	0.34 ±0.13
LA	4.13 ± 2.03	5.29 ± 3.46	6.22 ±2.99
ALA	0.18 ± 0.08	0.23 ± 0.13	0.20 ±0.07
AA	0.18 ± 0.08	0.18 ± 0.12	0.17 ± 0.04
EPA	0.02 ± 0.02	0.03 ± 0.02	0.03 ± 0.02
DHA	0.16 ± 0.10	0.16 ± 0.09	0.16 ± 0.07
CLA	0.04 ± 0.02	0.04 ± 0.04	0.04 ± 0.02

Results presented as mean ± standard deviation.
Units: Total BPA, Free BPA and TBBPA, in µg/L; elements, in mg/L; fat, in g/100 mL; fatty acids, in g/L
Significant differences at $p<0.05$ are indicated with different superscripts

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Table 4. Levels of BPA (free and total), TBBPA, elements and fat content in human milk according to the body mass index (BMI) of the mother. Results are shown by volume of milk.

	BMI		
	<18.5 kg/m^2	**18.5-25 kg/m^2**	**>25 kg/m^2**
	n=1	**n=27**	**n=22**
BPA (free)	<0.05	0.38 ± 1.08	0.13 ± 0.24
Total BPA	<0.05	1.11 ± 3.30	1.75 ± 5.57
TBBPA	<1.00	0.58 ± 0.34	0.58 ± 0.38
Elements			
Ag	<0.13	<0.13	<0.13
Al	3.98	0.14 ± 0.07	0.31 ± 0.66
As	<0.05	<0.05	<0.05
Ba	<0.05	0.02 ± 0.02	0.03 ± 0.04
Ca	186	282 ± 64.7	272 ± 85.5
Cd	<0.03	0.02 ± 0.02	0.03 ± 0.04
Co	<0.03	<0.03	0.04 ± 0.14
Cr	0.37	0.31 ± 0.05	0.33 ± 0.05
Cu	0.23	0.31 ± 0.24	0.38 ± 0.26
Fe	4.17	2.28 ± 8.16	0.86 ± 0.8
Hg	<0.05	<0.05	0.03 ± 0.01
K	470	511 ± 79.3	505 ± 105
Mg	52.4	<50.0	26.3 ± 6.20
Mn	0.05	0.02 ± 0.02	0.01 ± 0.00
Na	741	143 ± 81.4	168 ± 139
Ni	0.08	0.03 ± 0.02	0.04 ± 0.05
Pb	<0.05	<0.05	0.03 ± 0.01
Sb	<0.05	<0.05	<0.05
Se	<0.03	0.08 ± 0.23	0.05 ± 0.04
Sn	<0.03	<0.03	<0.03
Sr	<0.13	0.06 ± 0.04	0.05 ± 0.04
V	<0.13	<0.13	<0.13
Zn	4.98	2.29 ± 3.33	2.45 ± 4.04
% Fat	6.57	3.48 ± 1.10	4.41 ± 1.98
Fatty acids			
SFA	24.7	13.8 ± 5.30	17.2 ± 8.34
MUFA	24.8	13.1 ± 3.75^a	16.8 ± 7.82^b
PUFA	13.1	5.22 ± 2.31	7.10 ±3.86
ω-3 PUFA	0.83	0.38 ± 0.15^a	0.49 ± 0.23^b
ω-6 PUFA	12.3	4.80 ± 2.28	6.56 ± 3.75
TFA	0.36	0.30 ± 0.14	0.36 ± 0.21
LA	1.16	4.34 ± 2.16	5.88 ± 3.42
ALA	0.34	0.18 ± 0.07	0.23 ±0.13
AA	0.24	0.15 ± 0.06	0.21 ± 0.12
EPA	0.04	0.02 ± 0.02	0.03 ± 0.02
DHA	0.36	0.13 ± 0.09	0.16 ± 0.08
CLA	0.04	0.04 ± 0.02	0.05 ± 0.03

Results presented as mean ± standard deviation.
Units: Total BPA, Free BPA and TBBPA, in µg/L; elements, in mg/L; fat, in g/100 mL; fatty acids, in g/L
Significant differences at p<0.05 are indicated with different superscripts

Table 5. Levels of BPA (free and total), TBBPA, elements and fat content in human milk according to the maternal age. Results are shown by volume of milk.

	<30 years n=6	>30-35 years n=20	>35 years n=23
BPA (free)	0.75 ± 1.62	0.12 ± 0.26	0.28 ± 0.89
Total BPA	0.66 ± 1.32	1.71 ± 5.64	1.23 ± 3.60
TBBPA	<1.00	<1.00	0.67 ± 0.51
Elements			
Ag	<0.13	<0.13	<0.13
Al	<0.25	0.15 ± 0.08	0.43 ± 1.00
As	<0.05	<0.05	<0.05
Ba	0.03 ± 0.04	0.02 ± 0.01	0.03 ± 0.04
Ca	279 ± 54.0	270 ± 68.1	273 ± 81.2
Cd	<0.03	0.03 ± 0.04	0.02 ± 0.03
Co	<0.03	<0.03	0.04 ± 0.14
Cr	0.33 ± 0.03	0.31 ± 0.05	0.33 ± 0.06
Cu	0.36 ± 0.16	0.36 ± 0.34	0.30 ± 0.16
Fe	7.83 ± 17.3	0.78 ± 0.48	0.93 ± 0.99
Hg	<0.05	<0.05	0.03 ± 0.01
K	500 ± 64.3	506 ± 98.6	505 ± 86.4
Mg	<50.0	<50.0	27.5 ± 8.13
Mn	0.03 ± 0.04	0.01 ± 0.00	0.02 ± 0.01
Na	141 ± 82.1	171 ± 144	165 ± 148
Ni	<0.05	0.03 ± 0.02	0.04 ± 0.05
Pb	<0.05	0.03 ± 0.01	<0.05
Sb	<0.05	<0.05	<0.05
Se	0.04 ± 0.03	0.05 ± 0.04	0.09 ± 0.25
Sn	<0.03	<0.03	0.01 ± 0.00
Sr	0.05 ± 0.02	0.06 ± 0.04	0.05 ± 0.04
V	<0.13	<0.13	<0.13
Zn	2.59 ± 3.45	1.93 ± 3.35	2.46 ± 3.71
% Fat	4.33 ± 1.01	3.40 ± 1.37	4.33 ± 1.90
Fatty acids			
SFA	17.3 ±5.91	13.2 ± 6.38	17.1 ± 7.62
MUFA	15.4 ± 2.46	13.0 ± 5.09	16.4 ± 7.58
PUFA	7.61 ± 2.40^{a}	5.00 ± 2.26^{b}	6.82 ± 4.09^{ab}
ω-3 PUFA	0.37 ± 0.11	0.42 ± 0.24	0.47 ± 0.19
ω-6 PUFA	7.20 ± 2.38^{a}	4.55 ± 2.15^{b}	6.30 ± 3.98^{ab}
TFA	0.35 ±0.10	0.27 ± 0.14	0.37 ± 0.21
LA	6.52 ± 2.23^{a}	4.12 ± 1.99^{b}	5.67 ± 3.67^{ab}
ALA	0.19 ± 0.07	0.20 ± 0.13	0.21 ± 0.09
AA	0.22 ± 0.08	0.15 ± 0.07	0.20 ± 0.11
EPA	0.01 ± 0.01	0.03 ± 0.02	0.03 ± 0.02
DHA	0.11 ± 0.04	0.14 ± 0.08	0.17 ± 0.11
CLA	0.04 ± 0.02	0.03 ± 0.02	0.05 ± 0.03

Results presented as mean ± standard deviation.

Units: Total BPA, Free BPA and TBBPA, in µg/L; elements, in mg/L; fat, in g/100 mL; fatty acids, in g/L

Significant differences at p<0.05 are indicated with different superscripts

801 **Table 6.** Mean exposure to BPA (free and total), TBBPA, and elements through
802 exclusive formula and breast feeding scenarios, and tolerable daily intake thresholds.

	<1 month		1-6month		>6-12month		TDI	TDI
µg/kg/day	**Formula**	**Human**	**Formula**	**Human**	**Formula**	**Human**	**WHO**	**EFSA**
BPA (free)	0.15	0.06	0.12	0.04	0.06	NA		4
Total BPA	0.62	0.45	0.51	0.04	0.22	0.05		
TBBPA	0.08	0.09	0.06	0.07	0.05	NA		
µg/kg/day								
Al	111	52.5	90.5	39.1	50.7	14.7	286[a]	
Ba	6.00	3.00	4.89	3.67	2.94	1.47		
Cd	NA	3.00	NA	3.67	NA	NA	0.83[a]	0.36[a]
Co	NA	7.50	NA	1.22	NA	NA		
Cr	57.0	46.5	46.5	37.9	29.4	24.3		300[b]
Cu	78.0	60.0	63.6	30.6	37.5	13.2	500	
Hg	4.50	NA	NA	NA	NA	2.94		
Mn	22.5	3.00	18.4	1.22	11.0	0.74		
Ni	86.6	7.50	70.6	3.67	61.0	2.21		2.80
Pb	4.50	4.50	3.67	NA	2.21	NA	3.57[a]	3.57[a]
Se	6.00	18.00	4.89	4.89	2.21	0.74		
Sn	NA	NA	NA	NA	3.68	NA	2000[a]	
Sr	37.5	6.00	30.6	7.34	18.4	4.41		
mg/kg/day								
Ca	64.9	39.9	53.0	33.4	40.8	20.8		
K	96.9	81.5	79.0	60.1	53.7	36.2		
Fe	0.88	0.50	0.72	0.10	0.69	0.06		
Mg	7.46	3.99	6.08	3.23	4.25	1.84		
Na	27.5	32.3	22.4	16.6	16.0	7.50		
Zn	0.79	0.42	0.64	0.20	0.49	0.02		
g/kg/day								
SFA	2.13	2.12	1.74	1.87	1.12	1.38		ALAP
MUFA	2.28	2.01	1.86	1.87	1.15	1.17		
PUFA	1.00	0.77	0.82	0.78	0.54	0.53		
ω-3 PUFA	0.14	0.06	0.11	0.06	0.07	0.03		
ω-6 PUFA	0.87	0.71	0.71	0.71	0.47	0.50		
TFA	0.02	0.05	0.01	0.04	0.01	0.02		ALAP
mg/kg/day								
LA	845	620	689	647	462	457		
ALA	116	27.0	94.2	28.1	61.0	14.7		
AA	13.5	27.0	11.0	22.0	6.62	12.5		
EPA	3.00	3.00	2.45	3.67	1.47	2.21		
DHA	15.0	24.0	12.2	19.6	6.62	11.8	10-12[c]	100[d]
CLA	0.00	6.00	0.00	4.89	0.74	2.94		

Exposure expressed in µg/kg/day except for Ca, K, Fe, Mg, Na and Zn expressed in *mg/kg/day*. NA: Not assessed due all samples were below detection limit. ALAP: as low as possible

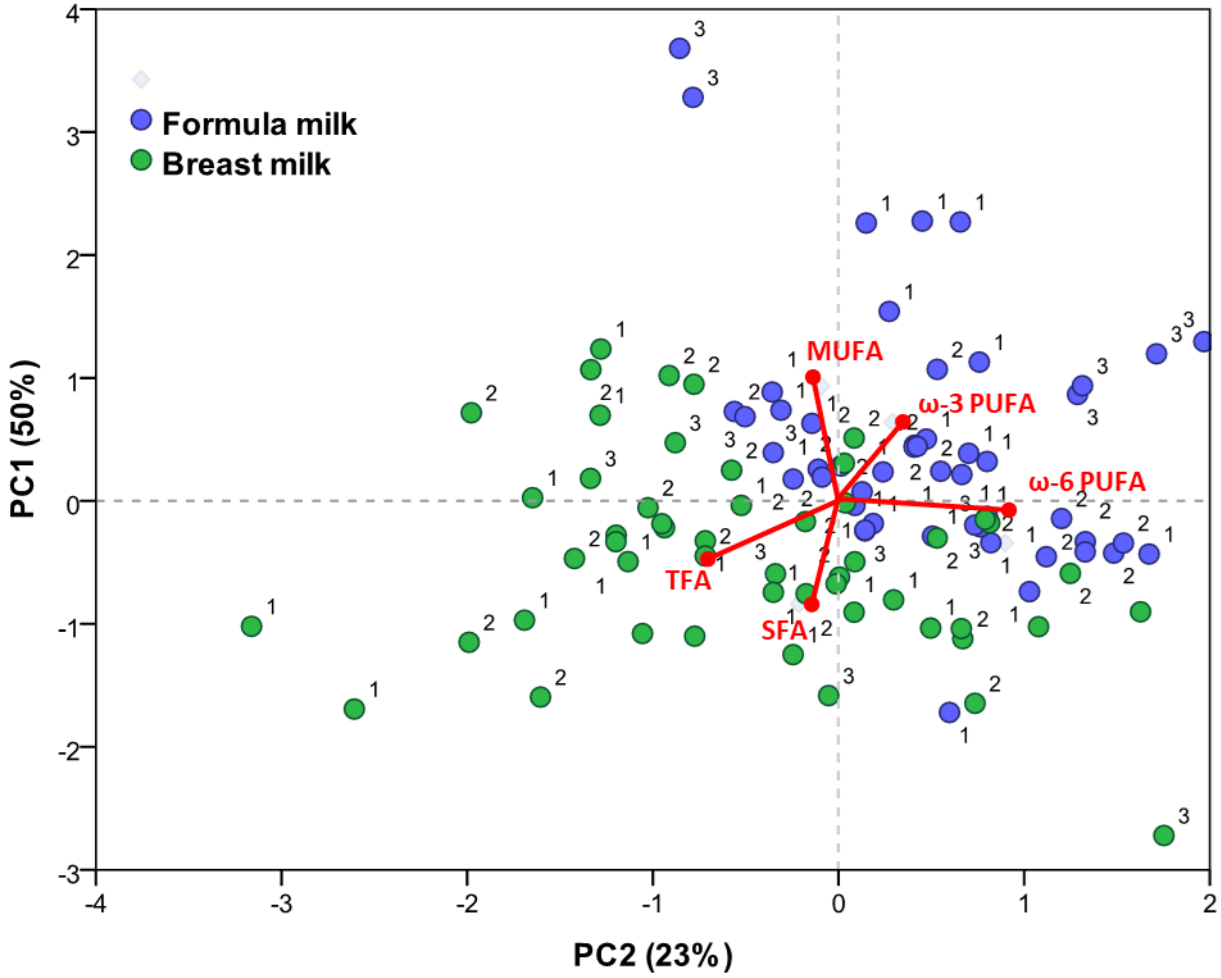
Ag, As, Sb, and V not assessed due all samples were below their respective detection limits.

TDI: Tolerable daily intake. [a] Derived from provisional weekly or monthly intake (PWTI or PMTI).

[b]Expressed as Cr(III). [c] month recommended daily intake 10-12 mg/kg/day for children between 6 and 24 month. [d]Acceptable intake 100 mg/d for >6 month <24 month children

TDI were obtained from WHO (2008, 2018) and EFSA (2010a, 2010b, 2011, 2014, 2015a, 2015b)

803



804

805 **Figure 1.** Principal component analysis (PC1 vs. PC2) of the fatty acid content. Each
806 number represent the period of breast feeding (1: <1 month; 2: 1-6 month; and 3: >6
807 month) or type of formula milk (1: first infant; 2: follow-on; and 3: growing-up
808 formula)

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