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Bisphenol A and its structural analogues in infant formulas available in the Brazilian market: Optimisation of a UPLC-MS/MS method, occurrence, and dietary exposure assessment

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ABSTRACT

For the first time, structural analogues to bisphenol A were investigated in infant formulas marketed in Brazil. A fast and high throughput UPLC-MS/MS method was established for simultaneous analysis of bisphenol A, B, E, F, and S in complex infant formula matrices. The influence of mobile phase composition on electrospray ionization response in negative mode was studied to improve the detectability of the method. As also, the main sample preparation variables that could affect the extraction and cleanup were screened by the Plackett-Burman design. The method performance characteristics were adequate, including reliable limits of detection (5–10 μg kg^{-1}) and quantification (10–20 μg kg^{-1}) with suitable recoveries (84.2–108.9 %) and precision (≤18 %). Sixty-one infant formulas were analyzed, and 36 % of total samples contained at least one bisphenol analogue, whose levels ranged between 10.9 and 198.9 μg kg^{-1}. Based on a deterministic approach, the estimated daily intakes for babies up to 6 months old, fed exclusively with infant formula, were below the temporary tolerable daily intake of 4 μg kg^{-1} body weight set for bisphenol A by the European Food Safety Authority.

1. Introduction

Bisphenol analogues comprise industrial chemicals containing two hydroxyphenyl functionalities in their structure (Table 1), which are often employed as an additive or monomer in the manufacture of polymeric materials including epoxy resins and polycarbonate plastics, and certain paper products (EFSA, 2015; Lin et al., 2021). Particularly, bisphenol A (BPA) provides rigidity, transparency, and resistance to polycarbonate plastics, which are used in food contact materials such as bottles, tableware, cookware, microwaves ovenware, reservoirs for water dispensers, and storage containers; whereas, BPA-based epoxy resins have been employed as protective linings for food and beverage cans (EFSA, 2015). Besides, BPA has been also used in a large number of non-food-related applications such as paints, medical devices, surface coatings, printing inks, flame retardants, and consumer products including toys, electronic equipment, and others, thus contributing to distinct human exposure sources to BPA (EFSA, 2015).

In recent years, BPA has been at the center of the discussion of food safety authorities and governmental and non-governmental organizations in several countries. For instance, the use of BPA in the manufacture of polycarbonate infant feeding bottles was banned by the European Commission (EC, 2011). The specific migration limit (SML) of BPA, from varnishes or coating applied to food contact materials, was reduced from 600 to 50 μg kg^{-1} (EC, 2018). Besides, no migration of BPA from materials specifically addressed to come into contact with infant formula, follow-on formula, and other products intended for infants and young children has been permitted (EC, 2018). Consequently, there is evidence that structural analogues to BPA of emerging concern such as bisphenol B (BPB), bisphenol F (BPF), bisphenol S (BPS), and others, have been used for the purpose of replacing (Liao & Kannan, 2013, 2014; Shaaban et al., 2022).

A temporary tolerable daily intake (*t*-TDI) of 4 μg kg^{-1} body weight (bw) per day was established for BPA by the European Food Safety Authority (EFSA), which represents the estimated substance quantity that can be ingested daily over a lifetime without any appreciable risks to human health (EFSA, 2015). Nonetheless, a lower TDI of 0.04 ng kg^{-1} bw per day has been suggested for BPA in a recent EFSA draft scientific opinion, which was open for public consultation until February 2022 (EFSA, 2021). This severe reduction in TDI of BPA was based on studies that have emerged in the literature from 2013 until 2018, particularly

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those conducted in animals that indicate adverse effects of BPA on the immune system such as the development of allergic lung inflammation (EFSA, 2021). With respect to the harmful effects of bisphenols on human health, there is evidence that exposure to the BPA during pregnancy may be associated with disturbed fetal growth; maternal and infant decreased thyroid function; altered child behavior in a sex-dependent manner; and effects on brain development (EFSA, 2015). In addition, there are indications of exposure to BPA and immunological outcomes in humans (EFSA, 2015). In animals, metabolic effects, evidenced by glucose or insulin regulation or lipogenesis and body weight gain, were also linked to pre-and postnatal exposure to BPA; as also a possible role of BPA in increasing the susceptibility to mammary gland carcinogenesis (EFSA, 2015). Recently, associations between decline in semen quality and BPA exposure, based on animal and epidemiological studies, were presented (Kortenkamp, Martin, Ermler, Baig, & Scholze, 2022).

Similar qualitative effects on estrogen and androgen receptor activities, as well as on steroid hormone syntheses such as increased progestogen and estrogen levels and decreased androgen levels, were observed for both BPA and its structural analogues BPB, bisphenol E (BPE), BPF, and BPS using *in vitro* bioassays (Rosenmai et al., 2014). Specifically, BPS showed the lowest estrogenic and anti-androgenic effects, nonetheless, it had the largest efficacy on increased progestagen levels, when compared to others (Rosenmai et al., 2014). In a recent study *in vitro*, nine alternative substitutes for BPA were evaluated regarding their endocrine-disrupting potential, in which all tested bisphenols, including BPB, BPF, and BPS, showed endocrine toxicity by distinct mechanisms of action such as interfering or/and altering nuclear receptor signaling, gene expression of some steroidogenic enzymes, and steroid hormone balance (Lin et al., 2021). Compared to BPA, BPF showed lower estrogenic activity, whereas BPB and bisphenol Z (BPZ) had both higher ability on estrogenic activity and steroid hormone disturbance than the BPA (Lin et al., 2021).

Because of its high separation power for complex mixtures and distinct selectivity, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been nowadays one of the most used analytical techniques in the analysis of bisphenols in food matrices (Tan et al., 2018), particularly, reversed-phase liquid chromatography interfaced to triple quadrupole mass spectrometer with electrospray ionization (ESI) operated in negative mode. Alternatively, atmospheric pressure chemical ionization (APCI), as well as ESI operated in positive mode after pyridine 3-sulfonyl chloride derivatization reaction has contributed to an efficient determination of bisphenol compounds with low limits of detection (Vilarinho, Sendón, van der Kellen, Vaz, & Silva, 2019). In addition, gas chromatography-mass spectrometry comprises an attractive technique for high sensitivity analysis of bisphenols after derivatization, including silylation using BSTFA or MTBSTFA, or acetylation with acetic anhydride (Vilarinho et al., 2019). Dependent on the characteristics of the matrix, various sample preparation approaches have been successfully applied for extraction and concentration of analytes, and cleanup of extracts, which stands out DLLME (dispersive liquid–liquid microextraction), QuEChERS (quick, easy, cheap, effective, rugged, and safe), SBSE (stir bar sorptive extraction), SPE (solid-phase extraction), SPME (solid-phase microextraction), due to their green features in terms of reduced amount of chemicals and organic solvents required, and low production of waste (Vilarinho et al., 2019).

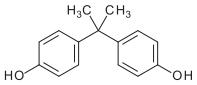
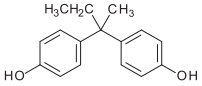
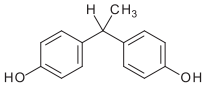
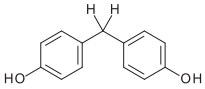
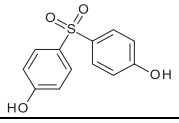
In the literature, studies on dietary exposure to bisphenols in early childhood are limited. The vast majority of them have been focused exclusively on BPA (Ackerman et al., 2010; Biles, McNeal, & Begley, 1997; Bomfim, Silvestre, Zamith, & Abrantes, 2015; Cao et al., 2008, 2015; Cirillo et al., 2015; Ferrer et al., 2011; Schecter et al., 2010). Thus, investigation of structural analogues to BPA in infant formulas is still little explored (Cunha, Almeida, Mendes, & Fernandes, 2011; Karsauliya, Bhateria, Sonker, & Singh, 2021; Li, Feng, Schepdael, & Wang, 2022). To the best of our knowledge, this is the first study evaluating simultaneously BPA and its structural analogues B, E, F, and S in infant formulas available in the Brazilian market. The study also included: (i) establishment and validation of an ultra performance liquid chromatography-tandem mass spectrometry method for fast analysis of bisphenol analogues; (ii) effect of the mobile phase composition on the electrospray ionization response, (iii) optimisation of an easy and simple sample preparation method by Plackett-Burman experimental design; and (iv) assessment of dietary exposure to the bisphenol analogues through the intake of infant formulas by babies up to 6 months old.

2. Material and methods

2.1. Standards and chemicals

Analytical standards of bisphenol A (2,2-bis(4-hydroxyphenyl)propane; CAS number: 80-05-7; 99 % purity), bisphenol B (2,2-bis(4-hydroxyphenyl)butane; CAS number: 77-40-7; 98.4 % purity), bisphenol

Table 1
Chemical structures, physicochemical proprieties, and UPLC-MS/MS parameters for simultaneous analysis of bisphenols in infant formulas.

Analyte	Chemical structure	Monoisotopic mass (Da)	p*Ka* [a]	Log *Kow* [a]	Retention time (min)	SMR transitions, precursor → product ion (*m*/*z*) Quantification	CE (V) [b]	Confirmation	CE (V) [b]
Bisphenol A		228.1150	10.29–10.93	3.64	4.0	227 → 212	−20	227 → 133	−30
Bisphenol B		242.1307	10.27–10.91	4.13	4.2	241 → 212	−19	241 → 147	−22
Bisphenol E		214.0994	9.91–10.64	3.19	3.9	213 → 198	−20	213 → 197	−25
Bisphenol F		200.0837	9.91–10.54	3.06	3.6	199 → 93	−23	199 → 105	−23
Bisphenol S		250.0300	7.64–8.23	1.65	0.9	249 → 108	−27	249 → 156	−23

[a] Wang et al. (2021).
[b] Collision energy.

E (1,1-bis(4-hydroxyphenyl)ethane; CAS number: 2081-08-5; 99.9 % purity), bisphenol F (bis(4-hydroxyphenyl)methane; CAS Number: 620-92-8; 99.1 % purity), and bisphenol S (bis(4-hydroxyphenyl)sulfone; CAS number: 80-09-1; 99.4 % purity) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Individual stock solutions were prepared in methanol at concentrations between 1013.52 μg mL^{-1} (bisphenol B) and 1720 μg mL^{-1} (bisphenol A). From these stock solutions, a multi-analyte solution was prepared in acetonitrile at 100 μg mL^{-1}. Then, working standard solutions at 10, 5, 1, 0.5, and 0.1 μg mL^{-1} were prepared weekly by diluting the multi-analyte solution with acetonitrile. All standard solutions were stocked in amber glass vials and protected from light at –18 °C.

Acetonitrile and methanol, all HPLC-grade, were acquired from J.T. Baker® (Avantor Performance Materials, Inc. C.V. Xalostoc, Mexico). Ammonium hydroxide and glacial acetic acid were obtained from Synth (Diadema, SP, Brazil). Bondesil-C_{18} bulk sorbent (40 μm) was purchased from Agilent (Santa Clara, CA, USA), primary secondary amine (PSA) sorbent from Supelco (Bellefonte, PA, USA), and PVDF syringe filters (13 mm, diameter; 0.22 μm, pore) were supplied by Analítica (São Paulo, SP, Brazil). Ultra-pure deionized water was obtained from the Milli-Q water purifier system (Direct 8, Millipore, Bedford, MO, USA).

2.2. Infant formula samples

Powdered infant formulas from different manufacturing companies, including milk- and soy-based infant formula samples, were purchased in the city of Campinas, SP, located in the Southeastern region of Brazil. A total of sixty-one samples were randomly collected from retail markets and drugstores, between October 2020 and January 2021. According to the packaging label, many of these products were produced in other counties (Table 5). All canned samples were maintained in their original packaging (300, 354, 400, or 800 g each) at 20 °C and protected from the light until analysis.

2.3. Determination of bisphenols by UPLC-MS/MS

2.3.1. Sample preparation approach

Two grams of powdered infant formula were weighed into a 15 mL glass centrifuge tube and 5 mL of acetonitrile was added to the tube, followed by vigorous vortex agitation for 1 min. The mixture was centrifuged at 2500 rpm for 15 min at 20 °C (Centrifuge 5804R, Eppendorf, Hamburg, Germany). Then, 2 mL of extract was transferred to another 15 mL glass centrifuge tube containing 40 mg of C_{18} cleanup sorbent, followed by fast vortex agitation. After centrifugation at 2500 rpm for 5 min at 20 °C, the extract was filtered through a PVDF syringe filter (0.22 μm) and then injected into UPLC-MS/MS system.

2.3.1.1. Plackett-Burman screening design. A Plackett-Burman experimental design was used to evaluate the main effects of sample preparation variables on the bisphenol extraction, according to Rodrigues and Iemma (2014). Four independent variables were evaluated at two levels, high (+1) and low (−1), whose coded and real values are presented in Table 2. The design matrix included eight trials (four trials more than the number of variables to ensure sufficient degrees of freedom for calculating the standard error), plus 3 central points (to evaluate the conditions in the central region of the studied range as also the repeatability of the analyses), resulting in 11 independent trials (Table 2) (Rodrigues & Iemma, 2014). The data were processed with STATISTICA 8.0 software (Statsoft Inc., Tulsa, OK, USA), and a significance level of 5 % was preferably used to evaluate the influence of sample preparation variables on the bisphenols recovery (Table 3).

2.3.2. UPLC-MS/MS analysis

ACQUITY ultra performance liquid chromatography (UPLC®) system coupled to Xevo® TQD triple quadrupole mass spectrometer (Waters, Milford, MA, USA) was employed in the analysis of bisphenols in infant formulas. The chromatographic separation was established on a reversed-phase analytical column (50 mm × 2.1 mm i.d., 1.7 μm particle size; ACQUITY UPLC® BEH C18, Waters, Ireland), which was maintained at 40 °C. The mobile phase consisted of 0.1 % ammonium hydroxide aqueous solution (solvent A) and methanol (solvent B) with the following linear gradient: from 5 % to 95 % of solvent B over 4 min, held for 1 min at 95 % of solvent B; then, returned to the initial condition (5 % of solvent B) at 5.1 min and held for 3 min, totaling 8 min of the chromatographic run. The flow rate was 0.2 mL min^{-1} and the injection volume was 5 μL. The UPLC-MS/MS system was operated under MassLynx 4.1 software and the data acquisition was performed in selected reaction monitoring (SRM) mode. The electrospray ionization source was operated in negative mode (ESI−), whose optimal conditions were: −3.5 kV, capillary voltage; −35 V, cone voltage; 150 °C, source temperature; 350 °C, desolvation temperature; 50 L h^{-1}, cone gas flow; and 550 L h^{-1}, desolvation gas flow. Nitrogen was used as desolvation and nebulizing gas, and argon as collision gas. The collision energy (CE) was optimized for each analyte which is reported in Table 1.

Table 2
Plackett-Burman design with the coded (and real) conditions evaluated for four sample preparation variables.

	X_1 (vortex agitation time, *sec*)	X_2 (ultrasonic extraction time, min) [a]	X_3 (PSA sorbent amount, mg) [b]	X_4 (C_{18} sorbent amount, mg) [b]
1	1 (90)	−1 (0)	−1 (0)	1 (40)
2	1 (90)	1 (10)	−1 (0)	−1 (0)
3	1 (90)	1 (10)	1 (20)	−1 (0)
4	−1 (30)	1 (10)	1 (20)	1 (40)
5	1 (90)	−1 (0)	1 (20)	1 (40)
6	−1 (30)	1 (10)	−1 (0)	1 (40)
7	−1 (30)	−1 (0)	1 (20)	−1 (0)
8	−1 (30)	−1 (0)	−1 (0)	−1 (0)
CP 1	0 (60)	0 (5)	0 (10)	0 (20)
CP 2	0 (60)	0 (5)	0 (10)	0 (20)
CP 3	0 (60)	0 (5)	0 (10)	0 (20)

CP: central point.
[a] Ultrasonic water bath at room temperature.
[b] Amount used for 2 mL of acetonitrile extract.

2.3.3. Identification criteria and quantification

For identification and confirmation purposes, the criteria set by the Commission Decision 2002/657/EC (EC, 2002) were considered including (i) two characteristic ion transitions (precursor → product ion) for each analyte, being the most intense used for quantification and another for confirmation purposes (Table 1); (ii) relative ion intensities within the tolerance of ± 30 % to those obtained with spiked samples; and (iii) retention time equal to that observed in spiked samples within the maximum tolerance of ± 2.5 %. External standard calibration was employed for the quantification of the analytes in the samples. The analytical curves were prepared in blank matrix extracts which were obtained by plotting analyte peak area versus mass fraction (μg kg^{-1}). The obtained results were not corrected for the recovery.

2.4. In-house validation

A commercial infant formula sample intended for infants between 0 and 6 months of life, containing 55 % of carbohydrates, 28 % of lipids, 9 % of proteins, and others, according to the package label, was used as a representative matrix for the in-house validation procedure. The sample was analyzed in triplicate and the target analytes were not detected. Thus, 2 g of "free-bisphenol sample" was weighed into a 15 mL glass centrifuge tube and spiked with a multi-analyte standard solution (1 μg mL^{-1} in acetonitrile) at 10, 20, and 50 μg kg^{-1}, followed by vortex

Table 3
Main effect of sample preparation variables on the recovery of bisphenols estimated from Packett-Burman screening design.

Variables (range)		Bisphenol				
		A	B	E	F	S
Vortex agitation time (30–90 *sec*)	Effect (%)	2.7	4.1	11.6	−2.1	4.4
	Standard error	12.2	5.3	4.6	7.3	5.0
	t (5)	0.2	0.8	2.5	−0.3	0.9
	p-value	0.831289	0.472857	0.054664 *	0.787071	0.414979
Ultrasonic extraction time (0–10 min)	Effect (%)	−1.2	0.8	0.8	−14.3	4.0
	Standard error	12.2	5.3	4.6	7.3	5.0
	t (5)	−0.1	0.1	0.2	−1.9	0.8
	p-value	0.926206	0.886576	0.861993	0.107656	0.458707
PSA sorbent amount (0–20 mg)	Effect (%)	−10.2	5.2	−1.7	9.3	−29.6
	Standard error	12.2	5.3	4.6	7.3	5.0
	t (5)	−0.8	1.0	−0.4	1.3	−6.0
	p-value	0.442184	0.369859	0.729299	0.257560	0.001901 *
C_{18} sorbent amount (0–40 mg)	Effect (%)	8.8	13.9	−10.4	19.8	9.9
	Standard error	12.2	5.3	4.6	7.3	5.0
	t (5)	0.7	2.6	−2.2	2.7	2.0
	p-value	0.502420	0.046475 *	0.075518	0.042575 *	0.101506

* Significant factor: $p \leq 0.05$.

agitation for 1 min. The spiked samples were maintained at room temperature and protected from light at least 1 h before the extraction step. The precision expressed as coefficient of variation (CV) under repeatability and within-laboratory reproducibility conditions, as well as the recovery experiments were obtained from independent replicates of the spiked samples that were analyzed on two different days (5 replicates at each level per day) by the same analyst under the same chromatographic conditions. Linearity was evaluated in extract-based analytical curves at least seven concentration levels each, being the limit of quantification as the first level. The Commission Decision 2002/657/EC was used as a guideline regarding the method performance criteria (EC, 2002).

2.5. Quality assurance

To avoid cross-contamination some precautions were taken, such as the minimal use of plastic materials in the sample preparation. The standard solutions were prepared and stored in amber glass flasks, and the entire extraction process was carried out using glass centrifuge tubes, including the extract filtration step on 1 mL glass syringes (Artiglass, Italy). Before use, the glassware (flasks, centrifuge tubes, syringes, and vials) as also the disposable pipettes were rinsed with methanol, followed by acetone and, finally, hexane, all HPLC-grade. A procedural blank containing only reagents and processed as a sample, including the filtration step through PVDF syringe filter (0.22 μm), was injected before each set of samples and the target analytes were not detected in any blank.

2.6. Dietary exposure assessment

The dietary exposure to bisphenols was estimated by combining the content found in infant formula samples with the corresponding consumption data, according to WHO and FAO (2009). Mean and 95th percentile bisphenol contents were used to estimate the average and high dietary exposure, respectively, which were stratified according to the age of babies (up to 2 weeks to 2 months, 2 to 3 months, 3 to 5 months, and 6 months). The dietary exposure assessment was focused on babies up to 6 months old fed exclusively with infant formulas (non-breastfed) since other foodstuffs are gradually introduced into the diet from the sixth month of life, which may be a possible source of bisphenols and lead to underestimations. Concerning the undetected analytes, namely, concentrations below the limit of detection (LoD), two scenarios were considered: concentrations lower than LoD were assumed as zero numerical value (scenario 1 – lower-bound dietary exposure) or equal to the LoD (scenario 2 – upper-bound dietary exposure) (EFSA, 2010; WHO & FAO, 2009).

The estimated daily intake (EDI) of individual bisphenols was calculated as follows: EDI (μg kg^{-1} bw per day) = C × F / bw; where *C* is the content of bisphenol in the sample (μg g^{-1}), *F* is the infant formula consumption per day (g), and *bw* is body weight (kg). Child Growth Standards of the World Health Organization, particularly 50th percentile weight, were used to obtain the average body weight for age in consecutive months of life (WHO, 2018), such as 4.4 kg (up to 2 weeks to 2 months), 5.8 kg (2 to 3 months), 6.7 kg (3 to 5 months), and 7.6 kg (6 months). The infant formula consumption was based on the daily needs of non-breastfed babies in line with the energy requirements of different age groups, including quantities between 540 and 975 mL of reconstituted infant formula per day (Trust, 2021), which corresponds to an average amount of 83 g (up to 2 weeks to 2 months), 124 g (2 to 3 months), 150 g (3 to 5 months), and 139 g (6 months) of powdered infant formula per day according to the preparation instructions stated in the label of products analyzed.

3. Results and discussion

3.1. Influence of the mobile phase composition on the electrospray ionization response

Since the eluent has a strong influence on the efficiency of electrospray ionization (ESI) in liquid chromatography-mass spectrometry (LC-MS/MS) (Kostiainen & Kauppila, 2009), mobile phases of distinct composition were explored to enhance the analytical response in negative mode and, consequently, to improve the method detectability at trace levels. In this way, common additives with good volatility and compatibility with LC-MS/MS, such as acetic acid or ammonium hydroxide at 0.1 % in water, as well as acetonitrile or methanol as an organic phase, were evaluated and then compared regarding their effects on the ESI response (Fig. 1).

Ammonium hydroxide solution at 0.1 % as an aqueous mobile phase provided the highest ESI response for most analytes, particularly those with high pk_a values such as BPA, BPB, BPE, and BPF (Table 1), compared with an acetic acid solution at 0.1 % or water (Fig. 1A). The great signal of bisphenols under alkaline conditions is primarily attributed to an acid-base reaction in which the acidic analytes are deprotonated enhancing the sensitivity in negative ESI mode (Chan, Bolgar, Dalpathado, & Lloyd, 2012; Henriksen, Juhler, Svensmark, & Cech, 2005; Tan et al., 2018). Additionally, considering the strong basicity of ammonium hydroxide, bisphenols with high pK_a values can easily dissociate in their anionic form contributing to the analytical response (Tan et al., 2018). Although pure or acidified water has been suggested as an aqueous mobile phase in bisphenols analysis by negative ESI mode,

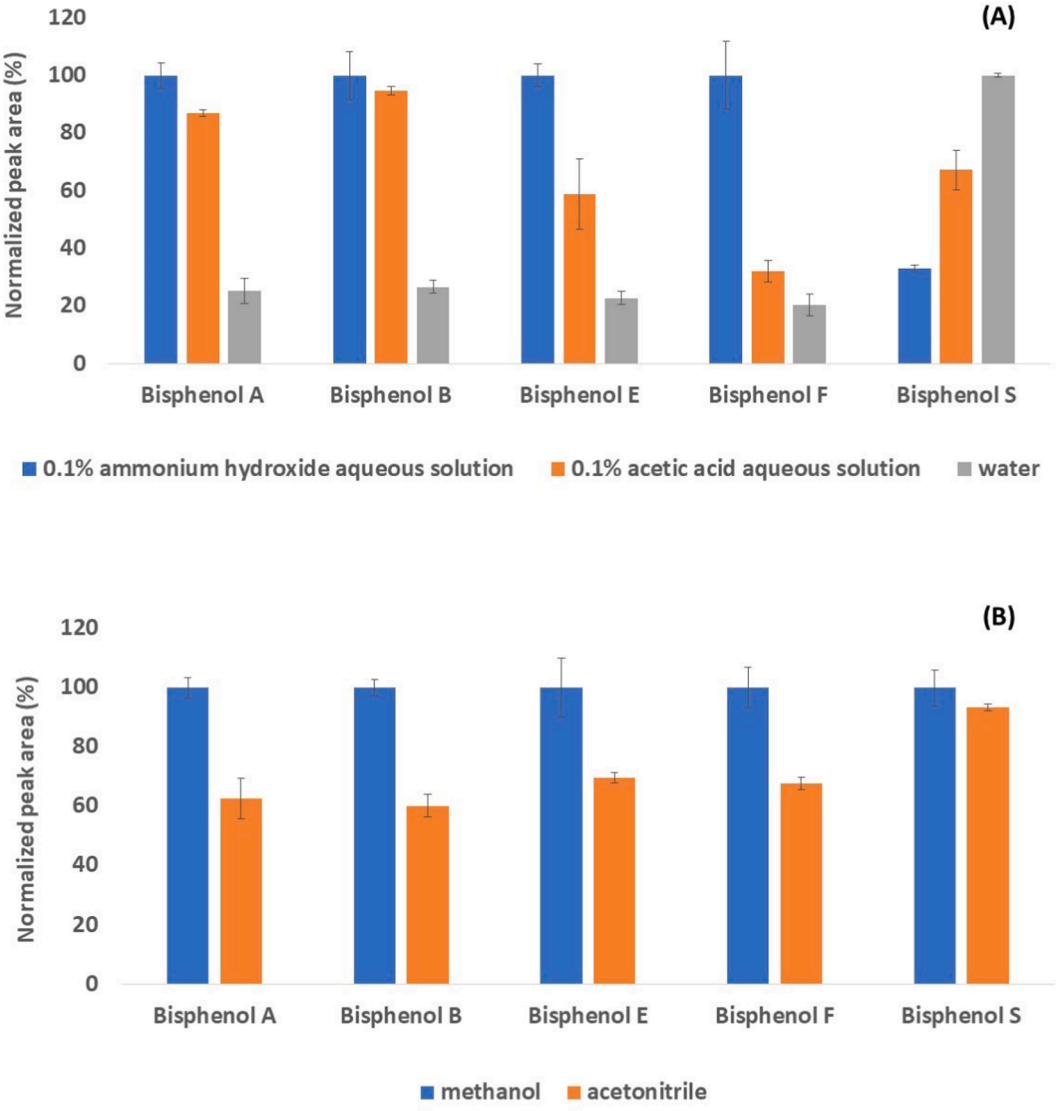


Fig. 1. Influence of the mobile phase composition on the electrospray ionization response in negative mode (mean ± standard deviation, $n = 3$; standard solution at 0.1 μg mL^{-1}). (A) Mobile phase composed of solvent A (0.1 % ammonium hydroxide aqueous solution or 0.1 % acetic acid aqueous solution or water) and solvent B (methanol). (B) Mobile phase composed of solvent A (0.1 % ammonium hydroxide aqueous solution) and solvent B (acetonitrile or methanol).

relatively weak responses were observed for the target analytes (Fig. 1A). Our findings support previous reports that alkaline additives such as ammonium hydroxide are a suitable choice for the mobile phase to improve ESI response and method detectability of bisphenols (Owczarek et al., 2018; Tan et al., 2018; Wang et al., 2021; Xian et al., 2017; Xiao, Wang, Suo, Li, & Su, 2020).

Irrespective of the composition of the mobile phase, the signal intensity of BPS was much greater than other bisphenols. Similar findings were observed in the analysis of serum (Tan et al., 2018) and egg (Xiao et al., 2020) extracts in negative ESI mode. Notwithstanding the similarity in molecular structures (Table 1), BPS contains additional electronegative atoms and hence a better sensitivity can be attained in negative ESI mode (Xiao et al., 2020). Particularly, BPS showed a performance contrary to the other bisphenols, being the highest and lowest analytical responses obtained with water and 0.1 % ammonium hydroxide solution, respectively (Fig. 1A). The reason can be attributed to the distinct pK_a values, being BPS is the most acidic compound with the smaller pK_a (7.64–8.23) among the bisphenols studied (Table 1). Evidence has been presented that ESI response in negative mode is inversely proportional to the pk_a of the analyte (Henriksen et al., 2005). In this way, considering the good repeatability and improved ESI response achieved for the majority of analytes (Fig. 1A), 0.1 % ammonium hydroxide solution was chosen as the aqueous mobile phase without compromising the detection of the BPS.

In addition to the additives used in the aqueous mobile phase, the positive effect of the organic phase (acetonitrile or methanol) on the ESI response was also verified. The signal was much higher in methanol (protic solvent) than acetonitrile (aprotic solvent) for all analytes (Fig. 1B). Deprotonated molecules of acidic analytes are expected to be more greatly solvated in methanol instead of acetonitrile due to the high ability of polar protic solvents to form strong hydrogen bonds with simple anions (Cox, 2013), thus achieving better bisphenol responses in protic solvents as eluent (Henriksen et al., 2005). Although acetonitrile has been commonly used in the bisphenols analysis, the results confirm previous studies that methanol is presumed to be a more suitable solvent for this analytes group in negative ESI mode (Henriksen et al., 2005; Owczarek et al., 2018; Xiao et al., 2020). Therefore, methanol was fixed as the organic mobile phase, in combination with 0.1 % ammonium hydroxide solution, for the determination of bisphenols in infant formulas.

3.2. A simplified sample preparation method by Plackett-Burman design

A straightforward sample preparation method based on the solid–liquid extraction (SLE) and dispersive solid-phase extraction (d-SPE) techniques was established for the extraction of bisphenols and cleanup of extracts. Four sample preparation variables that could influence the bisphenols extraction were evaluated through the Plackett-Burman screening design (Table 2). For this purpose, a commercial infant formula was spiked at 50 μg kg^{-1} with a multi-analyte standard solution, and then the sample was extracted according to the conditions fixed for each trial of the Plackett-Burman design (Table 2). From the recoveries obtained, the effect (%) of each sample preparation variable on the extraction efficiency was calculated, which ranged from −29.6 % to 19.8 % depending on the time (vortex agitation and ultrasonic extraction) and the amount of sorbent (PSA and C_{18}) used in the cleanup step (Table 3).

Infant formulas constitute complex matrices due to the high content of carbohydrates (37–69 %), lipids (17–29 %), proteins (8–27 %), and others in their composition, according to the packaging label of samples analyzed. Based on the low solubility in acetonitrile of both highly non-polar fats and highly polar proteins (Koesukwiwat, Lehotay, Mastovska, Dorweiler, & Leepipatpiboon, 2010), this solvent was preferably used in the extraction step. Suitable homogenization between the matrix and extraction solvent was achieved at a 1:2.5 w/v sample (g) to extraction solvent (mL) ratio, under constant vortex agitation. Variations in the agitation vortex from 30 to 90 *sec* had a significant positive influence ($p \leq 0.05$) on the recovery of the BPE (Table 3). After vortex agitation, an additional extraction step using an ultrasonic water bath was tested to maximize the extraction of analytes; however, no significant effects on the recoveries were observed (Table 3). Therefore, the extraction step was based on only vortex agitation for 1 min, contributing to a fast and simple procedure without impairing the bisphenols recovery.

The d-SPE technique presents attractive features for the cleanup step. The technique stands out for its easy and simple execution, as well as the low amount (usually 10–50 mg per mL of extract) and the combination of distinct chemical sorbents. Besides, no additional organic solvents are required in the procedure, which consists of the addition of a solid sorbent to the sample extract, followed by quick agitation and centrifugation. The dispersion of the sorbent as fine particles throughout the sample extract provides effectively the retention of matrix co-extractives due to the high contact surface. In this way, the sorbents PSA and C_{18}, with distinct action mechanisms, were evaluated. PSA, a primary and secondary amine sorbent, has been used to remove sugars, organic acids, and free fatty acids from sample extracts; whereas C_{18}, a hydrophobic reversed-phase sorbent, retains mainly fats and some natural pigments (Rutkowska, Lozowicka, & Kaczynski, 2018).

An increment in the amount of PSA, from 0 to 20 mg, resulted in a statistically significant negative effect ($p \leq 0.05$) on the recovery of BPS (Table 3). Among the analytes studied, BPS presents the lowest log *Kow* value (1.65), being the more polar of them. PSA acts as both a polar phase and weak anion exchanger (Rutkowska et al., 2018); thus, the retention of acidic analytes might be expected as a result of its interaction with the primary and secondary amine groups of the sorbent (Rodríguez-Gómez et al., 2015). On the other hand, variation in the amount of C_{18} sorbent, from 0 to 40 mg, resulted in statistically significant positive effects ($p \leq 0.05$) on the recovery of BPB and BPF (Table 3). Although the hydrophobic long-chain C_{18} sorbent presents extreme retentive nature for lipids and non-polar matrix co-extractives (Rutkowska et al., 2018), the results indicated that C_{18} does not adversely bind lipophilic bisphenols such as BPB (log *Kow* = 4.13). Similar to our findings, both negative effect of PSA and positive effect of C_{18} on the extraction of bisphenols and parabens were reported by Rodríguez-Gómez et al. (2015) in the analysis of breast milk samples. Therefore, 40 mg of C_{18} sorbent (20 mg for each mL of acetonitrile extract) was fixed for the d-SPE cleanup step.

3.3. In-house validation

The proposed method provides suitable performance characteristics for simultaneous determination of the bisphenols A, B, E, F, and S in infant formulas (Table 4). Their ability to identify and quantify the target analytes in complex infant formula samples, without interferences from matrix components, was verified under the optimized UPLC-MS/MS conditions. In the selected reaction monitoring (SRM) mode, two diagnostic ion transitions were addressed for each analyte (Table 1), which allowed distinguishing between the target analytes and other co-extractives, confirming the selectivity of the method.

Reliable limits of detection (LoD) and limits of quantification (LoQ) were established based on the recovery and precision experiments using a spiked infant formula sample. The LoD was defined as the lowest analyte concentration detected in the sample but not necessarily quantified, whose values were 5 μg kg^{-1} (BPA, BPB, BPE, and BPS) and 10 μg kg^{-1} (BPF). Whereas, the LoQ, between 10 (BPA, BPB, BPE, and BPS) and 20 μg kg^{-1} (BPF), was set as the smallest analyte concentration in infant formula that can be detected and quantified with acceptable recovery (87–109 %) and precision (relative standard deviation values ≤ 16 %), under repeatability and within-laboratory reproducibility conditions (Table 4). Particularly, a specific migration limit (SML) of 50 μg kg^{-1} has been fixed for BPA from food contact materials (EC, 2018). Therefore, the LoDs and LoQs were low enough for monitoring the BPA and its analogues in infant formula samples.

Low (−20 % < ME < 20 %) or medium (−50 % < ME < −20 % or 20 % > ME > 50 %) matrix effects (ME) (Economou, Botitsi, Antoniou, & Tsipi, 2009) were observed for all analytes, demonstrating the great efficiency of d-SPE cleanup on matrix co-extractives removal (Table 4). Signal suppression was prevalent, such phenomenon has been supported by different theories as detailed by Stahnke and Alder (2015). In summary, co-eluted endogenous (matrix components) and exogenous (others introduced during sample preparation) substances can suppress the analytical signal by interfering with the addition of charge to the analyte in the liquid phase and/or transfer of ions from the droplet surface to the gas phase during the ionization process stages (Furey, Moriarty, Bane, Kinsella, & Lehane, 2013; Panuwet et al., 2016).

To compensate for the matrix effects, analytical curves were prepared in infant formula extracts, which included at least seven concentration levels each with the LoQ being the first level. For this purpose, adequate aliquots of working standard solution were added to blank extracts to obtain 10, 20, 30, 40, 50, 60, 70, and 80 μg kg^{-1} of standard equivalent in the sample. Specifically, for BPF, a wider linear range from 50 to 300 μg kg^{-1} was also provided to quantify those samples with high BPF content. The analytical curves were injected into UPLC-MS/MS for each set of samples analyzed, resulting in adequate linearity for all analytes with coefficients of determination (R^2) ≥ 0.994.

The fitness for purpose of the developed method was supported by recovery and precision experiments using a commercial infant formula spiked at 10, 20, and 50 μg kg^{-1} (Table 4). All obtained results comply with performance criteria set by the Commission Decision 2002/657/EC for a quantitative method of analysis (EC, 2002), including average recoveries within the ranges of 70–110 % (for mass fractions between 1 and 10 μg kg^{-1}) and 80–110 % (for mass fractions higher or equal to 10 μg kg^{-1}). Under repeatability conditions, the precision (expressed in terms of coefficient of variation – CV) ranged from 1 % (10 μg kg^{-1}) to 10 % (50 μg kg^{-1}), whereas under within-laboratory reproducibility conditions the CV values varied between 4 % (10 μg kg^{-1}) and 18 % (50 μg kg^{-1}). According to Commission Decision 2002/657/EC, the CV values shall be as low as possible for fraction mass lower than 100 μg kg^{-1} (EC, 2002).

3.4. Bisphenol contents in infant formula and dietary exposure assessment

The occurrence of the target bisphenols was investigated in sixty-one

Table 4
Method performance characteristics using a representative infant formula matrix [a].

Bisphenol	LoD μg kg^{-1}	LoQ μg kg^{-1}	Recovery %, $n = 5$			Repeatability %, $n = 5$ (within-laboratory reproducibility %, $n = 10$) [b]			Matrix effect (%) [c]
			10 μg kg^{-1}	20 μg kg^{-1}	50 μg kg^{-1}	10 μg kg^{-1}	20 μg kg^{-1}	50 μg kg^{-1}	
A	5	10	96.3	102.4	94.9	7.7 (10.9)	6.8 (15.9)	10.1 (18.1)	–22.2
B	5	10	101.6	95.6	97.9	7.3 (12.9)	6.9 (11.7)	7.8 (15.4)	–10.6
E	5	10	87.4	93.1	84.2	1.1 (4.0)	6.9 (10.9)	10.3 (12.6)	–38.8
F	10	20	n.a.	108.9	98.2	n.a.	6.5 (9.8)	5.3 (14.0)	–14.3
S	5	10	89.8	92.8	92.1	6.6 (12.3)	4.6 (11.5)	7.7 (14.4)	–24.8

LoD: limit of detection; LoQ: limit of quantification; n.a.: not applicable because the spiked level is lower than the LoQ established for the analyte.
[a] Commercial sample of powdered infant formula based on milk, whey, and vegetable oils, containing 9 % of proteins, 28 % of fats, and 55 % of carbohydrates, among others, intended for infants between 0 and 6 months of life.
[b] Precision expressed as relative standard deviation (RSD).
[c] Matrix effect (%) = [(peak area in sample extract − peak area in standard solution)/peak area in standard solution] × 100.

infant formulas marketed in Brazil, although many of them were produced in other countries. Twenty-two out of the total samples contained at least one bisphenol at concentrations higher or equal to the LoQ. BPF and BPA were the analytes more frequently found, being present in approximately 20 % and 11.5 % of the samples, respectively. BPB was not detected in any sample considering an LoD of 5 μg kg^{-1}, as well as more than one bisphenol was observed in none of them. The bisphenol contents found in infant formula samples are summarized in Table 5.

Among the five bisphenols, the highest mean contents were verified for BPF, which varied between 46.4 and 198.9 μg kg^{-1} (Tabel 5). Data on the BPF in infant formulas are scarce. For instance, BPF contents of 0.13 μg kg^{-1} (mean) and 0.79 μg kg^{-1} (95th percentile) were found in dairy products from the United States of America (USA), including infant formula samples (Liao & Kannan, 2013). Recently, BPF contents of 0.27 μg kg^{-1} (mean) and 1.13 μg kg^{-1} (95th percentile) were reported in infant formulas from China (Li et al., 2022). In another study with samples from China, a frequency of occurrence of 52.9 % was related to BPF in milk and milk products such as infant formulas (Liao & Kannan, 2014). In general, BPF has been identified as one of the most prevalent bisphenols in foodstuffs, accounting for 17 % (Liao & Kannan, 2013), 11 % (Shaaban et al., 2022), and 10 % (Liao & Kannan, 2014) of the total bisphenol contents. These data suggest that the restrictions on the use of BPA have led to utilization of alternative substitutes such as BPF in food contact materials, although with toxic properties similar to the BPA (Lin et al., 2021; Rochester & Bolden, 2015; Rosenmai et al., 2014; Usman, Ikhlas, & Ahmad, 2019).

Table 5
Content of bisphenols in infant formulas marketed in Brazil ($n = 61$).

Sample	Manufacturing country	Bisphenol (μg kg^{-1}) [a]			
		A	E	F	S
IF01	Brazil	n.d.	n.d.	88.3 ± 7.9	n.d.
IF16	Netherlands	n.d.	n.d.	180.0 ± 34.6	n.d.
IF17	Germany	14.1 ± 0.8	n.d.	n.d.	n.d.
IF18	Germany	18.3 ± 1.6	n.d.	n.d.	n.d.
IF21	USA	n.d.	n.d.	105.0 ± 28.6	n.d.
IF23	USA	n.d.	n.d.	111.8 ± 3.9	n.d.
IF24	USA	n.d.	n.d.	46.4 ± 5.0	n.d.
IF25	England	n.d.	n.d.	56.0 ± 12.1	n.d.
IF26	England	n.d.	14.8 ± 3.0	n.d.	n.d.
IF28	Netherlands	18.7 ± 2.7	n.d.	n.d.	n.d.
IF30	Singapore	n.d.	n.d.	75.7 ± 10.7	n.d.
IF31	Singapore	n.d.	n.d.	n.d.	10.9 ± 1.0
IF39	Argentine	11.3 ± 0.7	n.d.	n.d.	n.d.
IF40	Argentine	n.d.	n.d.	198.9 ± 20.7	n.d.
IF41	Argentine	15.4 ± 0.9	n.d.	n.d.	n.d.
IF42	Brazil	15.9 ± 1.2	n.d.	n.d.	n.d.
IF43	Brazil	n.d.	n.d.	103.7 ± 5.4	n.d.
IF46	Brazil	n.d.	n.d.	48.4 ± 0.7	n.d.
IF49	USA	n.d.	n.d.	107.2 ± 6.8	n.d.
IF55	Brazil	n.d.	11.5 ± 0.3	n.d.	n.d.
IF58	Brazil	n.d.	n.d.	140.8 ± 17.9	n.d.
IF60	Brazil	11.9 ± 1.0	n.d.	n.d.	n.d.

n.d.: not detected.
[a] mean ± standard deviation ($n = 3$).

BPA was detected in seven samples at levels varying from 11.3 to 18.7 μg kg^{-1}, whose contents are below the specific migration limit (SML) of 50 μg kg^{-1} fixed for the analyte (EC, 2018). As also, BPE was found in two samples at 11.5 and 14.8 μg kg^{-1}, and BPS was observed in one sample at 10.9 μg kg^{-1} (Table 5). A wide variation in the BPA contents has been observed in infant formulas marketed in different countries, in some instances exceeding the SML. In infant formulas collected in Rio de Janeiro, Brazil, the BPA contents ranged from 0.16 to 10.2 μg kg^{-1} (Bomfim et al., 2015). Levels of BPA between 0.1 and 13.2 μg kg^{-1} (Biles et al., 1997), from 0.97 to 1.24 μg kg^{-1} (Schecter et al., 2010), and between < 0.15 and 11 μg kg^{-1} (Ackerman et al., 2010) were related in infant formulas from the USA. In samples collected in Canada, the BPA contents varied between 2.27 and 10.23 μg kg^{-1} (Cao et al., 2008) and from < 0.2 to 5 μg kg^{-1} (Cao et al., 2015). Higher BPA contents, between 3 and 108 μg kg^{-1} (Cirillo et al., 2015) and from 70 to 1290 μg kg^{-1} (Ferrer et al., 2011), were reported in infant formulas from Italy and Spain. Whereas, the smallest BPA levels (0.23–0.40 μg L^{-1}) were found in samples from Portugal (Cunha et al., 2011). Besides BPA, BPS (0.58 μg kg^{-1}) and BPZ (1.64 μg kg^{-1}) were reported in infant formulas from India (Karsauliya et al., 2021), as also BPS (0.21 μg kg^{-1}) was found in samples from China (Li et al., 2022).

Based on the contents found in the samples, average and high dietary exposure to bisphenols was estimated for babies up to 6 months old fed exclusively with infant formula. A comparison of estimated daily intakes (EDI) between four age groups is presented in Table 6. Ideally, the temporary tolerable daily intake (*t*-TDI) of 4 μg kg^{-1} bw stated for BPA was employed as a reference value to evaluate the dietary exposure (EFSA, 2015). In cases where TDI values are not available for all of the compounds, the lowest available reference value (i.e., for the most toxic chemical in the assessment group) has been used, assuming that all of the compounds with missing reference values are equally potent (EFSA, 2019). The average dietary exposure (mean bisphenol contents) for BPA, BPE, and BPS was at least thirty times lower than the *t*-TDI, whereas for

Table 6
Estimated daily intake (EDI) of bisphenols for formula-fed babies.

			EDI (μg kg^{-1} body weight per day)			
		Age	Up to 2 weeks to 2 months	2 to 3 months	3 to 5 months	6 months
		Weight (kg) [a]	4.4	5.8	6.7	7.6
		Consumption (g per day) [b]	83	124	150	139
S1	Bisphenol A	Mean	0.0326	0.0370	0.0387	0.0317
		95th percentile	0.2905	0.3292	0.3448	0.2816
	Bisphenol E	Mean	0.0081	0.0092	0.0096	0.0079
		95th percentile	0.0000	0.0000	0.0000	0.0000
	Bisphenol F	Mean	0.3903	0.4424	0.4632	0.3784
		95th percentile	2.1089	2.3902	2.5029	2.0447
	Bisphenol S	Mean	0.0034	0.0038	0.0040	0.0033
		95th percentile	0.0000	0.0000	0.0000	0.0000
S2	Bisphenol A	Mean	0.1161	0.1316	0.1378	0.1126
		95th percentile	0.2905	0.3292	0.3448	0.2816
	Bisphenol E	Mean	0.0993	0.1126	0.1179	0.0963
		95th percentile	0.0943	0.1069	0.1119	0.0914
	Bisphenol F	Mean	0.5418	0.6141	0.6431	0.5253
		95th percentile	2.1089	2.3902	2.5029	2.0448
	Bisphenol S	Mean	0.0961	0.1089	0.1141	0.0932
		95th percentile	0.0943	0.1069	0.1119	0.0914

S1 (scenario one): concentration lower than LoD (limit of detection) = zero.
S2 (scenario two): concentration lower than LoD = LoD.
[a] Average weight based on the 50th percentile weight for age of WHO Child Growth Standards (WHO, 2018).
[b] Amount based on the recommendations for daily intake of infant formula for non-breastfed infants (Trust, 2021).

BPF the EDI was at least six times less than the reference value (Table 6). Besides, the estimated high dietary exposure (95th percentile bisphenol contents) was also smaller than the *t*-TDI in all age groups, with EDI values between 0 and 2.5 μg kg^{-1} bw per day (Table 6). Particularly for BPA, the daily intake was estimated between 0.03 and 0.34 μg kg^{-1} bw, whose results are in the same order of magnitude as those reported by EFSA for infants and toddlers (EFSA, 2015). In general, the highest daily intakes were estimated for BPF, between 0.38 and 2.5 μg kg^{-1} bw, not exceeding the *t*-TDI (Table 6).

It should be stressed that a proposal to reduce the TDI of BPA from 4 μg kg^{-1} bw per day to 0.04 ng kg^{-1} bw per day was presented in a recent EFSA draft scientific opinion, which was open to public consultation until a few months ago (EFSA, 2021). Although it is not conclusive, the suggested TDI could also be considered a probable future scenario for the dietary exposure assessment. Therefore, comparing the EDI values with the TDI of 0.04 ng kg^{-1} bw per day, both average and high dietary exposure to BPA and its analogues largely exceeded the proposed TDI in all age groups in most cases, indicating health concerns.

In the EFSA re-evaluation on BPA, the average and high exposures were 0.03 and 0.08 μg kg^{-1} bw per day, respectively, in the case of formula-fed infants aged 0–6 months; whereas for infants (6 to 12 months) and toddlers (12 to 36 months) the daily dietary exposure ranged from 0.29 to 0.38 μg kg^{-1} bw for the average exposure and from 0.81 to 0.86 μg kg^{-1} bw for the high exposure, respectively (EFSA, 2015). Exposure to BPA through infant formula products in Italy was also evaluated, whose EDI values varied from 0.12 to 1.24 μg kg^{-1} bw per day (Cirillo et al., 2015). Probable daily intake (PDI) of BPA, between 0.08 and 1.35 μg kg^{-1} bw, was estimated for different age groups of infants through consumption of canned liquid infant formulas in Canada (Cao et al., 2008). In addition to BPA, the dietary exposure to BPS and BPZ was assessed considering the infant formula intake in India, whose daily intake was estimated between 0.04 and 0.25 μg kg^{-1} bw for BPA, from 0.004 to 0.05 μg kg^{-1} bw for BPS, and between 0.01 and 0.14 μg kg^{-1} bw for BPZ (Karsauliya et al., 2021). Whereas, average dietary exposure of 0.006 μg kg^{-1} bw per day (BPA), 0.004 μg kg^{-1} bw per day (BPF), and 0.003 μg kg^{-1} bw per day (BPS) was estimated based on infant formula consumption in China (Li et al., 2022). It can be noted that all EDI values reported from different countries exceeded the suggested TDI of BPA of 0.04 ng kg^{-1} bw per day.

It is important to reinforce that the deterministic approach, a point evaluation, used here for the exposure assessment presents some limitations. It presupposes that all individuals within the same age group have the same body weight and intake the same amount of infant formula per day. Furthermore, it was assumed that the baby bottles and water used to reconstitute the infant formulas are free of bisphenols. Therefore, our results give an idea of dietary exposure to bisphenols in early childhood, as well as it can serve as a basis for planning future studies including other food items present in the infant diet to obtain a more accurate dietary exposure to bisphenols, and then a risk characterization.

4. Conclusions

An accurate analytical method with great advantages in the determination of bisphenol A and its structural analogues in complex infant formula matrices was established. It includes a simple and easy sample preparation based on solid–liquid extraction and dispersive SPE cleanup, followed by fast UPLC-MS/MS analysis over 8 min, resulting in low or medium matrix effects. The Plackett-Burman screening design appointed the main sample preparation variables that could affect the extraction of bisphenols, thus maximizing the recovery of the analytes and minimizing the time and the amount of chemicals involved in the approach. Besides, the method detectability was improved by exploring the effect of the mobile phase of distinct compositions on the electrospray ionization response in negative mode. In the face of little data available on infant exposure to endocrine-disrupting compounds, the current work contributes to expanding the knowledge of dietary exposure to bisphenols A, B, E, F, and S in early childhood. The high and average dietary exposure estimates were below the temporary TDI of 4 μg kg^{-1} bw per day established for bisphenol A. In contrast, considering a probable future scenario based on the TDI of 0.04 ng kg^{-1} bw per day of bisphenol A, both high and average exposures exceeded the suggested TDI in the majority of cases. Special attention should be given also to the bisphenol F which presented the highest incidence and contents among the target analytes. In summary, these data can serve as a basis for planning actions and/or policies to reduce human exposure to bisphenols.

CRediT authorship contribution statement

Mateus Henrique Petrarca: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Mary Angela Favaro Perez:** Methodology, Formal analysis, Writing – review & editing, Resources. **Silvia Amelia Verdiani Tfouni:** Resources, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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