Journal of Chromatography A 1635 (2021) 461757

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Multivariate optimization of low-temperature cleanup followed by dispersive solid-phase extraction for detection of Bisphenol A and benzophenones in infant formula

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ARTICLE INFO

Article history:
Received 7 August 2020
Revised 21 November 2020
Accepted 23 November 2020
Available online 26 November 2020

Keywords:
Endocrine disruptor
Bisphenol A, Benzophenones
Plackett-Burman
LTP-dSPE
Infant formula

ABSTRACT

A simple and effective analytical method to determine six contaminants, including five benzophenones (BP, BP-1, BP-3, BP-8, and BP-12) and bisphenol A (BPA) in infant formulas was developed in this study. For this, a sequential experimental design was used to optimize the extraction and cleanup method using low temperature partition (LTP) combined with dispersive solid phase extraction (dSPE). The effect of primary secondary amine (PSA), sodium chloride (NaCl), graphitized carbon black (GCB), octadecyl (C18), strong anion exchanger (SAX), water, acetonitrile (ACN) and, ultrasound (US) time were evaluated using a sequential design of experiments including a Plackett-Burman, a central composite rotatable design, and the Derringer and Suich's tool. The method was validated, and it showed a limit of quantification varying from 0.06 to 2 mg.kg^{-1}, good precision ($<$ 20% RSD), and recovery (52-106%). The method proposed was applied to twenty-five samples of commercial infant formulas.

1. Introduction

Bisphenol A (2,2-bis (4-hydroxyphenyl) propane, BPA) is a monomer used in the production of polycarbonate, epoxy resins, and other polymeric materials. The BPA is widely used in can linings and in metal lids for food and beverage containers [1,2].

Benzophenone (BP) and its derivatives are aromatic ketones used as photo initiators for printing inks and varnishes cured with ultraviolet light (UV). BPs are also widely used as UV absorbers in personal care products, such as lotions and sunscreens [3]. Currently, BP-3 was prohibited in sunscreens, due to its relation with degradation of corals and part of marine vegetation, described as a major emerging contaminant [4].

BPA and BP are endocrine disruptors and are arising also as emerging contaminants gaining notoriety among researchers and regulatory agencies around the world. They can also migrate from food contact materials exposing the population to their toxic effects [5]. The limit of these substances in foodstuffs is regulated by many agencies such as the European Food Safety Authority (EFSA) and the Brazilian Health Regulatory Agency (ANVISA) [6]. In Brazil, ANVISA established a specific migration limit of 0.6 mg.kg^{-1} for BPA and 0.6 mg.kg^{-1} for BP used in food contact material [7].

Traditionally, the instrumentation and detection systems used for analysis of bisphenol A and BPs are based on liquid chromatography (LC) coupled to a fluorescence detector (FLD) or diode array (DAD) [8-10]. Gas chromatography (GC) coupled to mass spectrometry can also be used, resulting in greater sensitivity; however for using GC, a derivatization step of these compounds is required, making the analysis with GC more time-consuming and expensive than the one with LC [11]. Furthermore, the different polarities of BP compounds make their simultaneous determination more suitable by reversed phase liquid chromatography [12].

Researchers have been working to develop faster and more efficient analytical methods and to reduce the use of toxic solvents. Among these techniques, low temperature partition (LTP), combined with dispersive solid phase extraction (dSPE) has been efficiently used for the extraction of contaminants in fatty matrices prior to instrumental analysis. LTP reduces the concentration of fatty interferent, improving the cleanup made with dSPE [13,14]. dSPE can be used for the extraction of target compounds or sample cleanup. Both commercial or synthesized adsorbents from different materials and properties can be used for this purpose [15-18].

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https://doi.org/10.1016/j.chroma.2020.461757

The use of multivariate optimization methods, such as Plackett-Burman (PB) and central composite rotatable design (CCRD), to develop analytical methods, has increased in the past years. The main advantages of these techniques are the simultaneous optimization of many responses with a reduced number of experiments [19,20]. In view of this, the use of multivariate optimization and adequate sample preparation methods can reduce the required time, decrease the use of toxic reagents and decrease the costs during the method development step [19,21,22].

Infant formula is a breast-milk substitute used when human milk is not available or breastfeeding is not appropriate, such as for the HIV positive individuals [23]. Infant formula may contain BPA and BP derived from food packaging or from raw materials used in food production. This contamination should be monitored to ensure no health concerns for infants.

According to the consulted literature, to date, no studies focusing on the development of analytical methods for the simultaneous determination of BPA, BP, and BP derivatives by LTP-dSPE have been reported. Therefore, the objective of this study was to develop a method using liquid chromatography for the simultaneous determination of BPA, BP, and BP derivatives in infant formulas using LTP-dSPE followed by HPLC-FLD-DAD. For this purpose, multivariate techniques were used to optimize the extraction method. The method was validated and used to evaluate the incidence of BPA and BPs in 25 samples of infant formula commercialized in Campinas, São Paulo, Brazil.

2. Material and methods

2.1. Samples

Twenty-five samples of infant formulas (IF) in metal packaging were evaluated. The samples were randomly collected from different markets in Campinas, São Paulo, Brazil. The samples were stored at room temperature in their original packaging until the analysis.

2.2. Chemicals

Standards of BP (99%, CAS 119-61-9, Log P = 3.18), benzophenone-1 (BP-1, 99%, CAS 131-56-6, Log P = 2.96), benzophenone-3 (BP-3, 98%, CAS 131-57-7, Log P = 3.79), benzophenone-8, (BP-8, 98%, CAS 131-53-3, Log P = 3.82), benzophenone-12 (BP-12, 98%, CAS 1843-05-6, Log P = 6.96), 1-hydroxycyclohexyl phenyl ketone (1-HPK, 99%, CAS 947-19-3, Log P = 2.17) and BPA (99%, CAS 80-05-7, Log P = 3.32) were purchased from Sigma-Aldrich (St. Louis, USA). Primary secondary amine (PSA; particle size 50 μm), strong anion exchanger (SAX, particle size 50µm), C18-bonded silica (particle size 50µm) and GCB (particle size 120-400 mesh) were purchased from Supelco Inc (Bellefonte, PA, USA). Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany), NaCl from Synth (São Paulo, Brazil), trichloroacetic acid (TCA) from Êxodo científica (São Paulo, Brazil). Syringe filters (0.22 µm) were purchased from GVS (Sanford, USA). Ultrapure water was obtained from Milli-Q system (Bangalore, India).

The compounds were quantified using 1-HPK as internal standard (IS) previously prepared in acetonitrile (MeCN). For this, a stock solution containing 1000 µg.mL^{-1} of each standard prepared in MeCN was used. Subsequently, a working solution was prepared in MeCN containing all analytes at 25 mg.kg^{-1} and stored at 6°C. The calibration curve was constructed spiking an IF with the highest content of lipids due to its high interference in the chromatographic step. The sample was spiked in the limit of quantification (LOQ), 2, 4, 6, 8, 10, and 12 mg.kg^{-1}.

2.3. Sample preparation

2.3.1. Multivariate optimization

Sequential experimental designs were used to optimize the extraction of the compounds from infant formulas by LTP-dSPE. For this purpose, it was evaluated the effect of primary secondary amine (PSA), NaCl, graphitized carbon black (GCB), C18, water, acetonitrile (MeCN) and SAX, as well as the ultrasound (US) time (min) using a Plackett-Burman design. The variables were evaluated using 10% of significance, to avoid the exclusion of any important variables in the next step.

Subsequently, variables that showed a significant effect were optimized through a central composite rotatable design ($p<0.05$). The area of each compound was used as an answer for the optimization. Finally, the tool proposed by [Derringer and Suich [24]], was used in obtaining the best response to extract all compounds evaluated.

This tool is based on the desirability function for each response evaluated in the interval 0 to 1, in which 0 and 1 means a less and a more desirable value, respectively. It is worth mentioning that the Derringer and Suich's tool supplies the multi-response of the general desirability, formed by the individual response through the maximization of each compound extraction. The individual desirability was set to reduce the amount of organic solvent, salt, or adsorbent during the extraction process. The condition provided by the algorithm was validated through the extraction of the analytes from the infant formula spiked in triplicate. Next, the extraction process optimized was applied to detect BPA, BP, and BP derivatives in infant formula samples.

Plackett-Burman and central composition designs were evaluated by Statistica 6.0 (Statsoft Inc., 2001). The best condition proposed by the Derringer and Suich's tool was determined using the Design Expert 6.0 software (Stat-Ease, Minneapolis, USA).

2.3.2. Sample Extraction

The samples (2g) were weighted into vials of 40 mL and spiked with internal standard (IS) (5 mg.kg^{-1}). Next, 7.1 mL of water was added, followed by vortexing for 30 seconds. Then, 6 mL of ACN was added, followed by vortexing (30s). For salting out effect, 2.1 g of NaCl was added with subsequent stirring (30s). Finally, the effect of TCA on the precipitation of soluble proteins were evaluated using the method proposed by Koontz with minor modifications [25]. For this, 120 µL of saturated TCA was added and the sample was kept at rest for 5 min, followed by stirring for 5 min. After being centrifuged for 5 min, 3000 rpm at 7°C, the extract was frozen for approximately 12 h at -22°C. This procedure was used to reduce the concentration of fat in the extract and, the amounts co-extractives. After, the upper phase was recovered for dSPE cleanup step. For this, 2 mL of the supernatant was collected in a Falcon tube, 90 mg of PSA were added, followed by stirring (30s) and centrifugation (3500 rpm/5 min). The supernatant was transferred to glass tubes, dried in a stream of nitrogen at room temperature and resuspended with 500 µL of acetonitrile: isopropanol (1:1). The extract was sonicated for 5 min, filtered, and injected into an HPLC-DAD-FL system (Fig. 1).

2.4. Instrumental analysis and method validation

The incidence of BPA, BP, and BP derivatives were evaluated in an HPLC Agilent (1100) equipped with an automatic injector and fluorescence and diode array detectors (HPLC-FLD-DAD).

The separation was performed using an ACE 3 C18 column (4.6 mm x 15 cm x 3 µm) with a flow rate of 1 mL.min^{-1}. The system was operated at 40°C using water (A) and acetonitrile (B) as the mobile phase. The initial composition was 40% solvent A and 60% solvent B for 5 min, followed by a linear increase of solvent

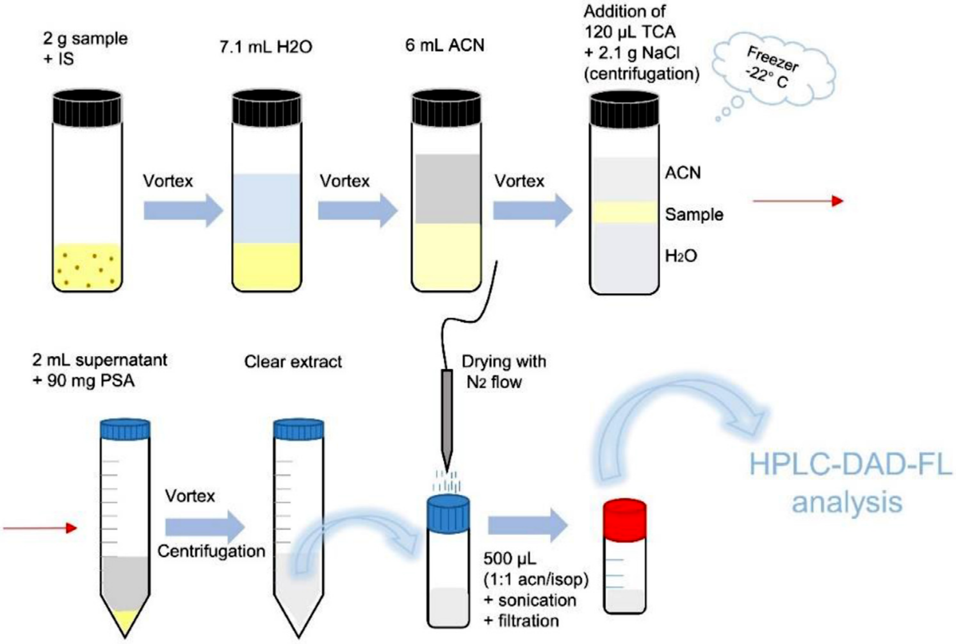

Fig. 1. Sample preparation for determination of BPs and BPA in infant formula.

B to 100% until 17 min. Subsequently, at 17.1 min, the system returned to the initial condition, and it remained there until reach 20 min. The injection volume was 10 µL. The fluorescence detection system was used to monitor BPA at an excitation wavelength of 270 nm and ate an emission of 305 nm. BPs were monitored using diode array detector with wavelengths of 250 nm for BP and internal standard, and 290 nm for BP-1, BP-3, BP-8, and BP-12.

The method was validated in terms of limit of detection (LOD), limit of quantification (LOQ), linearity, precision, and recovery. The LOD was determined by successive dilution of the work solution in the matrix until obtaining a three-fold signal-to-noise ratio. The LOQ was calculated as three times the LOD value.

The linearity of the method was evaluated using a seven-points calibration curve (LOQ, 2, 4, 6, 8, 10, and 12 mg.kg^{-1}) injected in triplicate. The lack of fit and linearity were evaluated through analysis of variance (ANOVA).

Recovery, intra and inter-day precision were determined at three points of the calibration curves (LOQ, middle, and upper point of the curve). Intra-day precision was determined by successive measurements using 10 replicates evaluated on the same day, while for inter-day precision, three replicates were evaluated in three different days. Precision was expressed as relative standard deviation (RSD). For recovery, the matrix was spiked with the work solution and subjected to the extraction procedure. The analyte loss was determined by injecting an aliquot of blank extract spiked with the same concentration as was used before the HPLC-DAD-FLD analysis [26]. The method validation was performed in the matrix.

3. Results and discussion

3.1. Multivariate optimization

The conditions of sample preparation for BPA and BPs extraction, from infant formula or baby food, have been established by univariate optimization [12,27-31]. However, the univariate optimization of analytical methods requires a long time, large number of experiments, high costs with solvents and do not allow adequate statistical treatment, such as the evaluation of interaction effect of the factors involved in the process, compromising the reliability of the results. Multivariate statistic tools can be used to overcome these problems [19].

In view of this situation, a method for the simultaneous detection of BPA and BPs was optimized by multivariate statistic tools using LTP-dSPE prior to HPLC-DAD-FL analysis. For this, preliminary analyzes were performed to find the best solvent and adsorbent salt for the extraction of the analytes in infant formulas samples. Then, a Plackett-Burman and a CCRD design, followed by the optimization proposed by the Derringer and Suich were used to improve the extraction of the analytes by LTP-dSPE.

Acetonitrile presented best performance in the preliminary tests with LTP (Table S1). Dichloromethane has low polarity and higher toxicity than acetonitrile which increases the extraction of fatty acids from infant formula. On the other hand, hexane formed a single-phase with the fat from the sample, resulting in low recovery of the solvent and co-extractives than acetonitrile, making the d-SPE step unfeasible. This is a drawback of the use of a highly compatible solvent with a fatty matrix for the extraction of non-polar analytes in low concentrations. Choosing the best extraction solvent becomes a crucial step in the development of multi-residue methods, in which some aspects must be noticed, such as polarity, safety, selectivity in the extraction stage, compatibility with the technique used, among others. The contaminants evaluated in this study, have Log P ranging from 2.17 to 6.96, which is similar to hexane (Log P = 3.90). However, acetonitrile (Log P = -0.34) proved to be the best solvent, even compared with dichloromethane (Log P = 1.25) due to low extraction of co-extractives and high recovery of the solvent, which allowed the cleanup step. Furthermore, acetonitrile has been reported as a good extractor for a wide range of contaminants in food [32].

Infant formulas have different chemical compounds in their formulation, being designated as a complex matrix. With this, the cleanup step is necessary to reduce interferences in the analyte detection, which are found in very low concentrations. Losses of analyte may occur during the cleanup step, compromising the result of the chromatographic analysis, thus, this step becomes crucial during the development of a method.

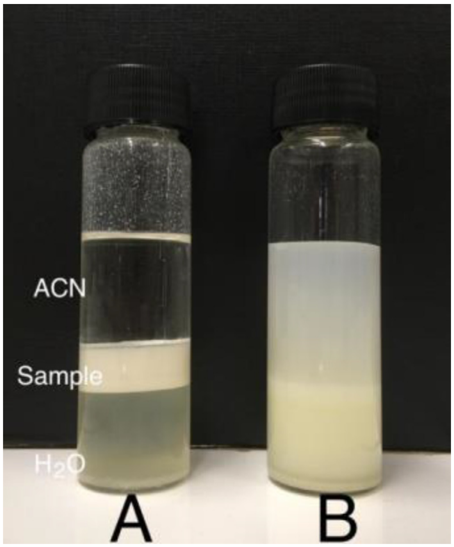

Fig. 2. Comparison of samples submitted to extraction (A) after solvents, NaCl and TCA addition and (B) without TCA addition.

For this, a previous step was carried out to analyze the effect of PSA, GCB, and C18 content in the recovery of the analytes. Each adsorbent was evaluated in triplicate using an amount of 100 mg. The test was performed in a solvent and the results (Table S2) were used to determine the experimental domain applied to the PB design. PSA and C18 showed better performance compared to GCB adsorbent salt. This fact can be explained by the ability of the GCB to retain planar compounds, such as BP-12. C18 was used to clean samples with high fat content. PSA can also retain lipid compounds, in particular, free fatty acids in addition to several polar compounds [17,33]. Given the composition of infant formulas, PSA became the best adsorbent salt to be used in the d-SPE cleaning step. To confirm these results, adsorbent salts were added to the PB design.

Finally, the effect of TCA in the cleanup of a sample not submitted to LTP was evaluated (Fig. 2). The addition of TCA forms a protein aggregate, compact, flexible, and in a partially folded state, named molten globule. This effect, mainly on whey proteins, increases the cleanup and selectivity of the method reducing the content of interferents [34-36]. Hence, this step was included in the extraction process before freezing.

3.1.1. Plackett-Burman design

PB design is a saturated statistical design that does not evaluate the interaction between variables studied. The PB was performed with fifteen experiments, including a triplicate of the central point (Table S3). The impact of each variable on the extraction by LTP-dSPE was tested at two experimental levels: a low level (−) and a high level (+). All the factors showed a significant effect ($p < 0.1$) on the extraction process, except for US time and SAX (Fig. 3). For this design, the increase of ACN concentration had a negative impact ($p < 0.1$) on the extraction of all compounds, except for BP-1. The content of ACN showed relatively high percentages in the recovery of residues, contaminants, and low concentrations of lipophilic co-extractives; it also acted on protein denaturation [33]. On the other hand, the amount of water positively affected the extraction of the same compounds. This result can be attributed to the increase of the extraction of analytes with high contents of water and the dilution effect when the compounds are transferred to ACN in the salting out step. Therefore, these factors were included in the second stage of the optimization process.

Among the adsorbent salts analyzed, PSA showed a significant effect ($p < 0.1$) on extraction of BPA, BP, BP-3, and BP-8. PSA is the most widely used anion exchanger for retention of free fatty acids, which are widely found in infant formulas. Commercial PSA is a silica compound functionalized with ethylenediamine-N-propyl groups which results in a bidentate chemical structure with a high chelation effect [37]. The primary and secondary amines form intermolecular hydrogen bonds because they can act as both hydrogen bond donors and acceptors [38]. Hence, PSA shows a strong retention level towards free fatty acids and other polar compounds from the matrix, such as organic acids, sugars, and co-extractives which can form hydrogen bonds. In this work, PSA did not affect the recovery of most analytes; and this result is due to the nonpolar characteristics of the evaluated compounds (Log P varying from 2.17 to 6.96) [21,39,40]. PSA was also included in the subsequent optimization step.

GCB had a negative impact ($p < 0.1$) for all compounds, except for BP-1. Even at the low level (-1) (10mg), the complete adsorption of some analytes was observed. GCB can completely remove planar compounds such as BP-12, resulting in low recoveries. For this reason, the GCB was excluded from the optimization stage.

C18 had a significant effect ($p < 0.1$) for BP-8 and BP-12. C18 has been used to cleanup fatty samples [41,42]. However, in view of the kind of lipid material in the sample (fatty acids) and the best effect of PSA, C18 was not considered for the optimization step.

Regarding NaCl, a positive effect was observed on the extraction of BP-3 and BP-12. The presence of salt in the MeCN:H_2O mixture promotes immiscibility through thermodynamic changes. Consequently, the compounds with low polarity are directed to the organic phase (MeCN). In addition, NaCl improve the salting out effect decreasing the solubility of proteins that may interfere with the analysis [32,43,44]. However, considering the low coefficients obtained for NaCl in PB design and, the requirement for salt in the extraction process, we opted to use the central point concentration (2.1g) and to not include it in the optimization step.

The US time evaluated in the PB design ranged from 2 to 20 min. This procedure is a simple and rapid pretreatment used to extract the analytes from the sample. However, the US time was not significant ($p < 0.1$) for the extraction of any compound. In view of this, the US time was not evaluated in the CCRD.

Finally, the curvature was evaluated using the central points. The evaluation was performed to check if the significance of any factor was masked by the p-value increasing in view of the standard error increasing. This procedure is recommended in the evaluation of all factors when using a saturated design, such as PB. The curvature had a negative effect ($p<0.1$) for BPA, BP, and BP-8. However, the factors that had an impact on the extraction process of these compounds were in consonance with the other analytes, such as water, MeCN, and PSA. Moreover, R^2 higher than 0.88 showed a low error associated with a screening experiment. For this reason, only water, MeCN, and PSA were evaluated in the next step.

3.1.2. Central composite rotatable design (CCRD) and Derringer and Suich's tool

After selecting the factors that interfered with the extraction by LTP-dSPE, a CCRD ($p < 0.05$) was carried out (Table 1) to evaluate the volume of acetonitrile (mL) and water (mL), as well as the amount of PSA (mg) necessary for the method.

A prediction model without lack of fit was obtained for all evaluated compounds, with the exception of BP-8 (Table S4). Due to the different extraction conditions obtained, with linear and quadratic models, the Derringer and Suich's tool was used to find the best extraction condition for all the compounds evaluated, using smaller amounts of acetonitrile and PSA.

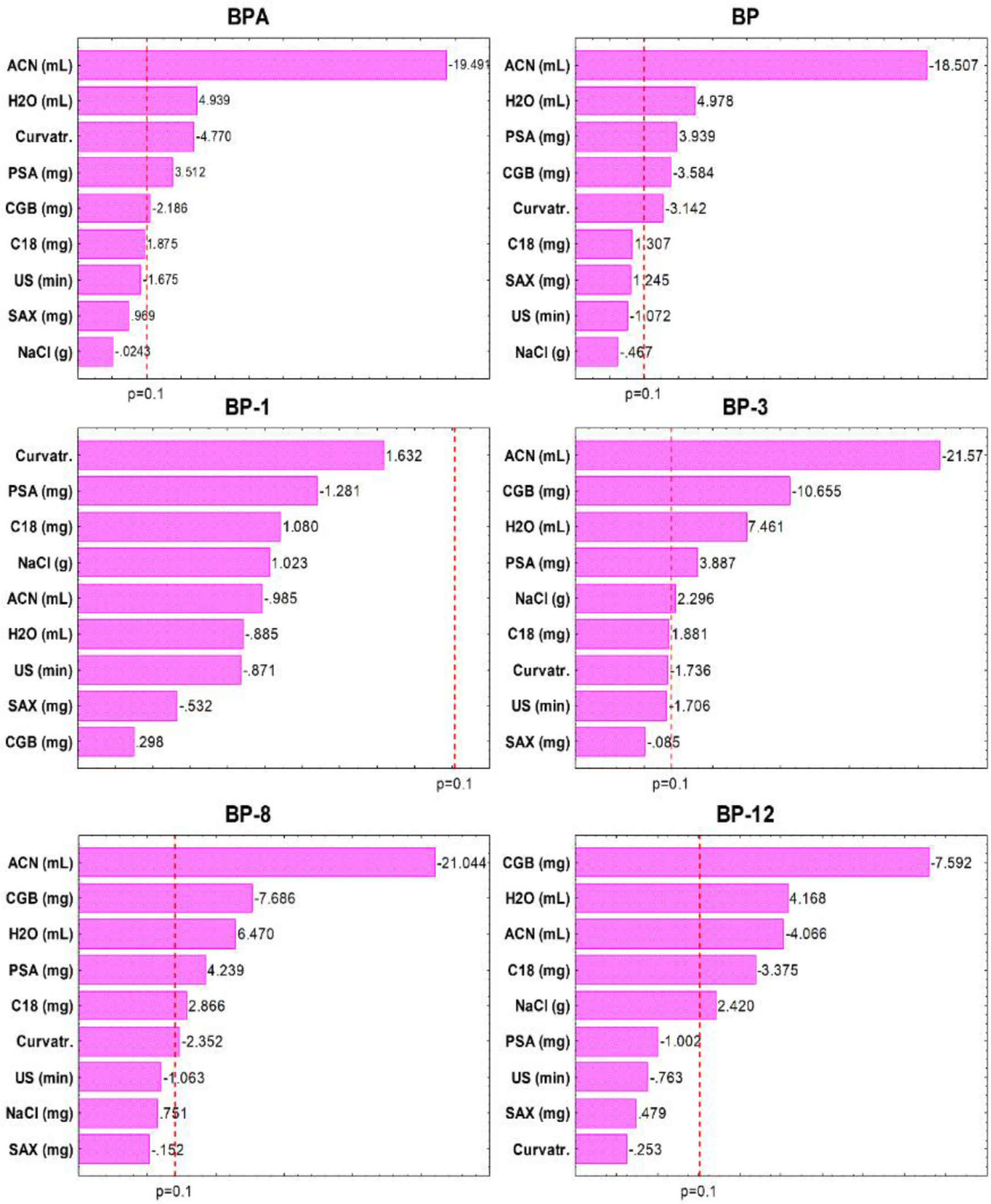

Fig. 3. Pareto diagram corresponding to the selected responses in Plackett-Burman design.

Table 1
Variables evaluated by the Central Composite Rotatable Design.

	Variables	-1.68	-1	0	1	+1.68
X_1	PSA (mg)	49.1	90	150	210	250.9
X_2	ACN (mL)	4.6	6	8	10	11.4
X_3	H_20 (mL)	4.6	6	8	10	11.4

The significance of the factors was evaluated by ANOVA ($p<0.05$).

The condition found, showed desirability of 0.789. For that point, the extraction required 7.1 mL of water, 90 mg of PSA, and 6 mL of ACN. Many studies have been using higher adsorbents in dSPE step than this work [41,42,45,46]. Conversely, the Derringer and Suich's tool showed an optimized condition for the simultaneous extraction of the compounds using less solvents and adsorbents.

The condition proposed by the algorithm was validated and the data obtained is shown in Table 2. Three confirmatory tests were performed to evaluate the best condition. The predicted values based on the peak area confirmed all compounds that were obtained experimentally, except for BP-1 which showed a peak area above the predicted value. This situation is common in the development of multi-residue extraction methods for analytes with a wide polarity range. In view of this, the overall quality of the data should be considered to reach satisfactory sensitivity for all compounds evaluated.

The improvement of the recoveries obtained after LTP-dSPE optimization can be observed in Fig. 4. In this method, BPA and BPs

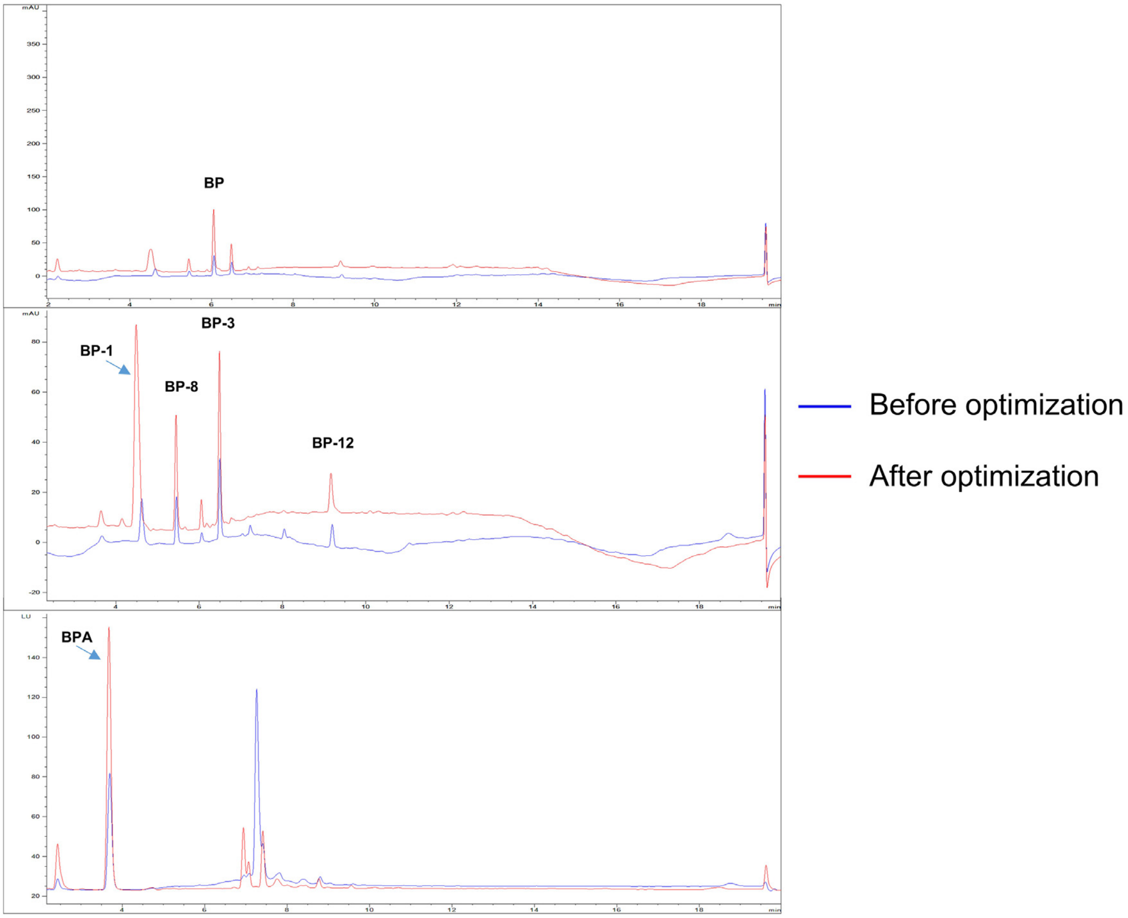

Fig. 4. Chromatogram of a representative sample of infant formula spiked at 5 mg.kg^{-1} level.
Chromatograms obtained from the critical point of the CCRD; and the desirability point, with λ = 290 for BP-1, BP -3, BP -8 and BP-12, λ = 250 for BP and λex = 270 and λem = 305 for BPA.

Table 2
Values predicted by the Derringer and Suich tool and obtained experimentally.

Variable	Predict Values Lower limits	Upper limits	SE Pred	Observed values
PSA	-1	1	-	-
Water	-1	1	-	-
Acetonitrile	-1	1	-	-
BPA	451.84	852.00	91.83	890.17±27.62
BP	90.17	338.19	58.18	322.57±12.92
BP-1	88.74	194.89	23.46	512.47±81.26
BP-3	71.07	235.88	37.72	232.88±7.61
BP-8	52.04	175.43	27.27	163.60±10.15
BP-12	41.10	102.63	14.44	71.95±3.99

*SE Pred: Standard error predicted

showed good separation between the peaks, without visual coelution with the interfering peaks. The desirability function assists in the responses, showing the best methodology for extraction of several compounds and consequently, reducing the number of experiments to be carried out.

Retention times (Rt) of 6.1 for BP at 250 nm, 4.4, 5.4, 6.4, and 9.1min for BP-1, BP-8, BP-3, and BP-12, respectively, presented at 290 nm wavelength; and 3.6 min for BPA at 270 nm of excitation and 305 nm of emission, showed the low peak signal (Figure), due to a greater concentration of water and acetonitrile solvents; about 10 mL of acetonitrile as used in the original technique of QuECheRS. However, this amount of solvent caused a dilution of the compounds, and because of it, was appointed the amount of solvents as a critical point in the CCRD. Comparatively, in Fig. 4, the peak signals of the compounds spiked in the sample at a 5 mg.kg^{-1} level, are clearly higher due to the application of the Derringer and Suich's tool. Thus, the result showing the combination of sequential experimental designs and the maximization proposed by the Derringer and Suich's tool is an important approach for fast optimization of analytical methods.

Many researchers used different methods for the detection and confirmation of these compounds in the matrix, such as the mass spectrometer [47-50]. However, liquid chromatographic coupled with a diode array and fluorescence detectors are routinely used to analyze the same compounds due to the simplicity and easiness of the technique [8,51].

3.2. Method validation

The method was successfully validated, and the data is described in Table 3. LTP-dSPE is a simple, easy, selective, and effective technique for the simultaneous determination of endocrine disruptors, as of this writing, not evaluated in Brazil. For optimization of the LTP-dSPE technique, a blank infant formula sample was spiked with a working solution and an internal standard. The method showed good limits of detection and quantification as other studies have shown [12,52] in addition of being easy to perform, showing low operating costs compared with other solid-phase extraction processes. Some improvements still might be reached for non-fatty matrices, such as the reduction of LTP time, but, for IF, the time established (12 h) was necessary when taking into consideration the high content of fatty acids.

Table 3
Method validation parameters in infant formulas.

Rt (min)	Compound	RE	LOD (mg.kg^{-1})	LOQ (mg.kg^{-1})	Concentration(mg.kg^{-1})	REC (%) *(n=3)*	Intra-day precision *(n=10)*	Inter-day precision *(n=9)*
3.6	BPA	$y = 1.5948x + 0.049$	0.02	0.06	0.06	99.57	17.68	16.96
					6	100.74	6.81	8.02
					12	106.45	9.66	7.59
4.4	BP-1	$y = 0.4796x - 0.0444$	0.5	2	2	96.01	9.68	10.03
					6	84.5	8.71	3.63
					12	99.67	7.91	6.57
6.1	BP	$y = 1.5948x + 0.049$	0.1	0.3	0.3	94.25	16.99	8.43
					6	95.76	5.53	8.27
					12	97.93	10.72	8.91
5.4	BP-8	$y = 0.2639x + 0.0111$	0.1	0.3	0.3	96.12	14.45	15.22
					6	101.58	6.82	7.52
					12	100.88	8.54	7.35
6.4	BP-3	$y = 0.3237x - 0.015$	0.1	0.3	0.3	101.13	9.44	14.27
					6	98.07	6.12	9.16
					12	104.08	9.54	7.75
9.1	BP-12	$y = 0.1476x + 0.0054$	0.1	0.3	0.3	58.9	9.88	12.46
					6	54.59	6.96	13.26
					12	52.38	10.26	9.22

RE: regression equation; LOD: limit of detection; LOQ: limit of quantification; REC: recovery; Rt: retention time

Linearity was evaluated using analytical curves obtained for each compound. The curves were evaluated using ANOVA ($p < 0.05$) through observing R^2, lack of fit, and residual graph, for each compound. The obtained data showed regression coefficients (R^2) greater than 0.990, homoscedastic behavior, and no lack of fit.

The LOQs were 0.06 mg.kg $^{-1}$ for BPA, 0.3 mg.kg^{-1}for BP, BP-3, BP-8, BP-12, and 2 mg.kg^{-1} for BP-1. The limits for BP-1 were higher than for other compounds, probably because the factors evaluated did not impact on the recovery of this compound, as emphasized in the PB design.

According to ANVISA, the specific migration values from food contact materials for bisphenol A and benzophenone must be less than 0.6 mg.kg^{-1}. However, for some derivatives from BP contained in this research, such as 2,2-Dihydroxy-4-methoxybenzophenone, 2-Hydroxy-4-methoxybenzophenone, 2,4-Dihydroxybenzophenone (BP-1), 2-Hydroxy-4-n-octyloxybenzophenone, and specially 2-Hydroxy-4-n-octyloxybenzophenone (BP-12), the specific migration total limit are 6 mg.kg^{-1} [7,53], which makes the method suitable for the evaluation of these compounds.

The precision of the method was evaluated through intra and inter-day measurements in three levels of the curves (LOQ, middle, and upper point). Experimental results showed intra-day ranging from 5.53 to17.68%, while the inter-day values were between 3.63 and 16.96%. Hence, the good reproducibility of the method was as recommended for contaminants analysis below 20%.

For recovery, the method showed values varying from 52.38 to 106.45%. The lower percentage of recovery was observed for BP-12. Probably, this result is due to the strong interaction of the octyl chain of the BP-12 with the fatty acids presented in the infant formula, reducing the compounds extraction. Moreover, BP-12 has lower polarity (Log P = 6.96) than MeCN (Log P = -0.34) which results in a low recovery. This is common in extraction methods for compounds with different polarities. A change in the solvent polarity could improve the BP-12 recovery and it could impact the extraction of fatty matter. For this reason, the changes were not performed considering the overall quality and the method sensitivity. Furthermore, since the method was developed using a spiked matrix, the losses of analytes were taken into consideration in the calibration curve, making it possible to use the method for the BP-12 quantification.

Methods for the simultaneous determination of bisphenol A and benzophenones in fatty foodstuffs such as infant formula are scarce. Reports conducted on both compounds were carried out as migration studies using food simulants or non-fatty matrices [41,42,54,55]. Hence, this method represents an advance in monitoring these groups of substances and in the mitigation of any early exposure of infants.

3.3. *Occurrence*

The validated method was applied to the evaluation of BPA, BP, and BP derivatives in twenty-five samples of infant formulas commercialized in the city of Campinas, São Paulo, Brazil. However, there was no occurrence of BPA or BPs in any of the studied samples. A similar fact was already described in infant formulas evaluated by salting out assisted liquid-liquid extraction followed by SPE for BPA extraction [29]. Other study analyzed seventeen compounds, including BP and its derivatives, in baby food. The authors did not identify BP, BP-1, or BP-12 compounds in food samples using dSPE before the UHPLC-MS/MS method [12].

These data are important because they ratify the quality of infant formulas sold in the Brazilian market regarding the incidence of BPA and benzophenones. In addition, the developed method will serve as a support for regulatory agencies.

4. Conclusion

For the first time, a method to simultaneously quantify BPA and BP by LTP-dSPE was optimized using a sequential experimental design. Suitable performance characteristics were reached using less solvent and adsorbent. Moreover, co-extractive removal was achieved using only low temperature and one adsorbent (PSA), reducing the method cost. After validation in HPLC-FLD-DAD, 25 samples of infant formulas were evaluated. The incidence of BPA and BP were not detected in the analyzed samples. However, the procedure has shown potential for use as a monitoring method taking into account its easy application, low cost, and low waste generation. This is the first report on these contaminants in Brazilian infant formula.

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.

Author Contributions

Helena Teixeira Godoy and Wellington da Silva Oliveira designed and supervised the study. Marcella Vitoria Galindo and Wellington da Silva Oliveira performed the experiments, analyzed the data, and prepared the manuscript. Wellington da Silva Oliveira revised and edited the manuscript. All authors have read the final version of the manuscript and approved its submission for publication.

Funding

This work was supported by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES) [88882.329526/2019-01] through a scholarship given to the first author; and by The São Paulo Research Foundation (FAPESP) [2017/11635-8].

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461757.

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