Optimization and Improvement of Chromatographic Techniques for Bisphenol A Extraction and Characterization from Infant Bottles

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Chemicals with high manufacturing volumes, such as bisphenol A (BPA), find widespread use in thermal paper, epoxy resins, and polycarbonate plastics. However, due to its endocrine-disrupting properties, several consumer items have restricted or banned its use. As a result, BPA has been replaced by other chemical substances with similar structures in consumer goods. This study aims to enhance and validate a chromatographic method for detecting BPA in baby bottles using chemometrics tools. A Box-Behnken experimental design was employed to optimize the laboratory conditions for the maximum release of BPA through forced degradation. The study utilized three parameters as independent variables, including incubation temperature (35 °C, 40 °C, and 60 °C), time (12 hours, 24 hours, and 36 hours), and solvent acetic acid concentration (2%, 3%, and 4%). A second-order polynomial model was proposed, and a total of 15 experiments with two replicates were conducted to determine the optimal extraction conditions. The high-performance liquid chromatography (HPLC) partitioning method was used to determine peak areas of the different extracts, serving as responses. By applying the individual desirability approach, the optimal extraction conditions were found to be a temperature of 35 °C, an extraction time of 36 hours, and an acetic acid concentration of 4% (pH = 2.72). The method was validated according to the conditions recommended by the International Conference on Harmonization (ICH) and SFSTP (Société Française des Sciences et Techniques Pharmaceutiques), ensuring high linearity, accuracy, precision, sensitivity, and robustness compared to existing liquid chromatographic methods. The improved method was applied to assess BPA levels in commercially available baby bottles, with 3 out of 7 samples testing positive for BPA, ranging from 0.615 ppb to 3.802 ppb. Notably, the enhanced method demonstrated a 12% increase in yield compared to the ISO EN 14350-2:2004 method. This study provides a reliable and effective approach for detecting BPA in baby bottles, addressing critical concerns related to consumer safety and regulatory compliance.

Keywords: bisphenol A, design of experiment, HPLC, response surface methodology, optimization

A chemical with a high rate of production known as BPA is widely employed in the production of polycarbonate plastics, which are used in a variety of food and beverage packaging applications [1]. Additionally, a lot of plastic products, including infant bottles and accessories, are composed of polycarbonate, which is often a BPA polymer [2]. Endocrine disruption and toxicity have piqued the interest of numerous governmental bodies due to their enormous exposure to the environment and individuals [3, 4]. The U.S. Food and Drug Administration (FDA) shared the views of the National Toxicology Program (NTP) in 2010, which prohibits the use of BPA in the manufacture of polycarbonate baby bottles [5]. Baby bottles constructed of BPA polymers come into contact with any nutrients, which could have negative effects on health if released [2]. The potential for interaction is determined by the type of material, the physicochemical qualities of the milk, and the child care item, in our case polycarbonate baby bottles [6]. The evaluation of this interaction is based on a good knowledge of the material and its manufacturing process [7]. These elements are completely under the control of the supplier. Material data combined with nutrient information allows for an evaluation of the container-content relationship and the likelihood of interaction [8]. When a nutrient is in contact with a plastic material for a long time or not, the two most critical aspects are quality and safety [9]. Once analyzing the presence of BPA, so-called extreme

laboratory migration tests are feasible to evaluate the safety of use of these baby products. These tests will assist the control authority in following the legal circuit of the feeding bottles and in identifying potential fakes [10, 11].

Our work's primary goal is to adapt the ISO EN 14350-2: 2004 method for use in hot climate zones through the development of an HPLC-FD method [12], This takes into account the local climate in the nations where this method was created. The Box-Behnken experimental design model has been used to optimize the extraction conditions.

Materials and methods

Standards and reagents. All reagents were of analytical quality. Reference Standard BPA ≥ 99 % Sigma-Aldrich, Methanol, 99.9 % for HPLC, was obtained from Sigma-Aldrich (France) and Acetic acid 99.8-100.5 % was supplied by Sigma-Aldrich (Germany), Purified water was obtained using a Milli-Q Plus® system (Millipore, Merck, France). Seven polycarbonate baby bottles named from A to G were purchased from local pharmacies and supermarkets (several brands with a nominal volume of 260 mL).

Preparation of standard solution

Preparation of the different concentrations of acetic acid: All three concentrations are prepared from the 100.15 % stock solution.

Preparation of the BPA standard solution: The standard solution is 10 ppb. 20 mg of BPA standard solution was weighed into a 200 mL flask. Then, 10 mL of methanol was added to solubilize the BPA, and the mixture was made up to the mark with 3% acetic acid (solution a). 1mL of solution (a) was taken and placed in a 100 mL flask. It was made up to the mark with 3 % acetic acid, resulting in solution b. Then, 1ml of solution b was taken in a 100 mL flask and made up to the mark with 3 % acetic acid.

P*reparation of the validation standards*

Eight concentration levels corresponding to 2.5, 5, 25, 50, 100, 150, 200 and 250 % of BPA standard (respectively 0.5, 1, 5, 10, 20, 30, 40, and 50 ppb) have been prepared (table 2).

Instrumentation and chromatographic conditions

A Waters high-performance liquid chromatography system (model 2695) equipped with the quaternary solvent manager, auto sampler, and fluorescence detector (FD) controlled by Empower 2.

The Separation was performed on a reversed-phase column, Symmetry C18 (150×4.6 mm, 3.5µm particle size). The elution mode is isocratic, consisting of a mixture of methanol/water (65:35; v/v). The mobile phase was filtered through a 0.45 µm Millipore filter, degassed under vacuum before use, delivered at a flow rate of 0.6 ml/min and the injection volume was 100 µl at a column temperature of 25 °C. The fluorescence detector was set at a wavelength of 275 nm (excitation) and 313 nm (emission).

The column was equilibrated with the mobile phase to saturate the stationary phase prior to sample injection.

Preparation of the samples

100 mL 3 % (v/v) acetic acid is transferred to the bottle. The filled bottle is incubated for 24 hours at various temperatures in an oven. 1 ml of the solution in the bottle is then taken for HPLC analysis.

Table 1. Presentation of the different bottle samples bases in the study.

Sample name	Volume (mL)	Weight (g)	Composite material
Baby Bottle (J)	120	3.50	Plastic
Baby Bottle (F)	120	3.31	Plastic
Baby Bottle (P)	120	3.28	Plastic
Baby Bottle (B)	120	3.39	Plastic
Baby Bottle (N)	120	3.23	Plastic
Baby Bottle (H)	120	3.42	Plastic
Baby Bottle (C)	120	3.25	Plastic

Optimization of BPA extraction using the Box-Behnken design of experiments (DOE)

The Box-Behnken method of experimental design is a statistical approach to planning experiments efficiently by modeling the relationships between independent variables and a dependent variable. It uses an experimental design based on a surface response design, thus reducing the number of trials needed while allowing optimization of factors to obtain desired results. It was generated using NemrodW version 2017 software.

To determine the most extreme circumstances for the stability of the bottles, this extraction was carried out using the surface response methodology. The parameters influencing the extraction were chosen using a 3-factor matrix with three levels for each factor. The low, medium and high levels were 35 °C, 40 °C and 60 °C for incubation temperature, 12 h, 24 h and 36 h for incubation time, 2 % (pH = 2.88), 3 % (pH = 2.81) and 4 % (pH = 2.72) for extraction solvent (acetic acid) respectively. The Experimental Design type Box-Behnken was used for the optimization of this phenomenon of interaction container-content.

The statistical second order polynomial model used for this design is of the following form (Eq 1).

Where Y is the selected answer, which is computed by the model, X_1, X_2 and X_3 are coded variables, corresponding respectively to the incubation temperature, extraction solvent and incubation time, b_1, b_2 and b_3 are linear effects, b_{12}, b_{13} and b_{23} are interaction effects, and b_{11}, b_{22} and b_{33} are quadratic effects of X_1, X_2 and X_3 on the response [13].

$$\hat{Y} = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad \textbf{Eq 1}$$

The p-value of the term is compared to the significance level (alpha) of 0.05 to assess association between the term and the response. The model was chosen based on the adjusted R^2 coefficient of determination [14]. The normality of the residuals and the homoscedasticity of the model were verified for the overall model and re-verified for the selected model. A model misfit test was also performed to test the adequacy of the model [15].

The selection of the optimal conditions of BPA extraction

The application of the idea of total desirability, introduced by Derringer and Suich allows optimizing the amount of BPA extracted. In this way for each response Yi(x), the desirability functions di (Yi) varies between 0 and 1 di (Yi) = 0 representing a totally undesirable value of Yi and di (Yi) = 1 representing the desirable or ideal response value. The desirability (di) of a response variable (Yi) may increase or decrease with the increase of (Yi), under certain conditions, the relationship between di and Yi may be parabolic in nature [16, 17]. In our study, the objective is to target a maximum release value; the following equation gives the desirability function for this response (Eq 2):

$$di = (Ui - Li)/(Ti - Li), \quad \textbf{Eq 2}$$

where: Li, Ui and Ti respectively are the desired lower, upper and target values for BPA release, with Li ≤ Ti ≤ Ui. Ti is considered as a sufficiently large value for the response.

Validation of the analytical method

Validation studies were performed according to ICH guidelines [18, 19] and SFSTP [20, 21]. Eight standard solutions of BPA were prepared at different concentrations (0.5 - 50 ppb) from the stock solution (50 ppb methanol). The calibration curve, at these different concentrations, as defined by the best-fit line equation Y = ax + b. In addition, the trueness and precision our method and the reference method (EN-ISO 14350-2:2004) were evaluated at nine determinations covering the range specified for the procedure (3 concentrations/3 replicates each). The Lower Limit of Quantitation (LOQ) was determined using the signal-to-noise (S/N) ratio.

Results and discussion

Identification and quantification of BPA by the reference method from the bottle (A)

The following criteria were used to determine BPA using the EN-ISO 14350-2:2004 French standard technique as a starting point: Incubation temperature 40 °C, Incubation time 24h and Extraction solvent Acetic acid 3 % (pH = 2.81). The choice of bottle A for the experiments was random. In order to quantify BPA, the internal normalization method was used. Given that we can see that BPA is present in the bottles of brand A from the chromatograms in Figure 1, this sample will be used to meet the requirements of the experimental design. We discovered a 329999575-peak area (2.6 ppb) for quantification.

Optimization of the method of extraction of BPA from the bottle A

Generation of the mathematical model

The NemrodW software was used to process the data from the experimental runs, which are carried out in accordance with the experimental design, and uses it to do statistical analysis. The experimental design and actual response data are shown in Table 2. According to the ANOVA table, the quadratic model seems to be the most suitable model. This model was highly significant (R^2 Adjusted = 0.755, the value of F = $5.8*10^8$) with a non-significant lack of fit. Terms with a significant p-value less than 0.05 were included in the model equation. Thus, the model was re- generated after removing the non-significant model terms (Eq 3).

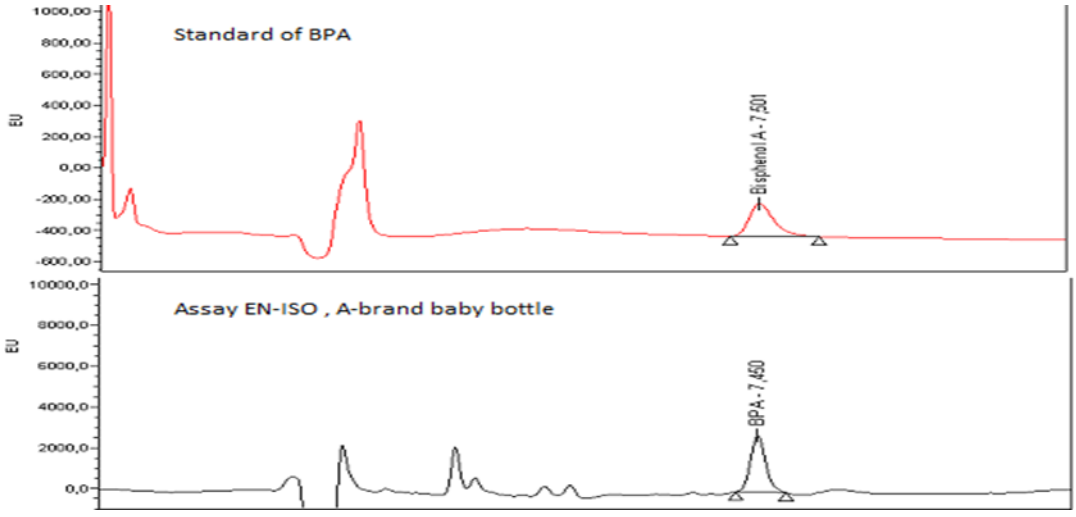

Figure 1. Comparison of the chromatogram of the BPA standard and that of BPA in the Brand A bottle according to the EN-ISO method.

$$\hat{Y} = 145683 + 16643.219X_1 + 156809.606X_2 + 31436.987X_3 + 335049.162X_1X_2 + 605.275X_1X_3 - 119263.750X_2X_3 + 170179.969X_1^2 + 329857.294X_2^2 + 222203.044X_3^2 \quad \text{Eq 3}$$

The normal residuals plot was used to study the diagnostic statistics and satisfy the normal error distribution before moving on to the response surface plots [22, 23]. Equations for regression were used to create the response curves [24]. In the designed space, the concentration of solvent and monomer fluctuates, and the % swelling is used as the response.

Optimization of the extraction process

Figure 2 displays the response surface plots. Using a desire diagram, the numerical optimization was performed. The range of the independent variables and the maximum release rate were both determined. The optimal process parameters were 35 °C, 4 % acetic acid (pH = 2.72), and a 36-hour incubation period. These results were achieved using a random sample. 371903186 was discovered to be the greatest peak area under ideal circumstances. Validation tests conducted under the revised conditions suggested were used to confirm the results. When the experimental design was optimized, a peak area increases of almost 12 % (371903186 vs. 329999575) was seen. Therefore, the environmental circumstances of infant bottle storage and use in hot climate zones are better represented by the new optimized approach because to its improved BPA extraction capabilities.

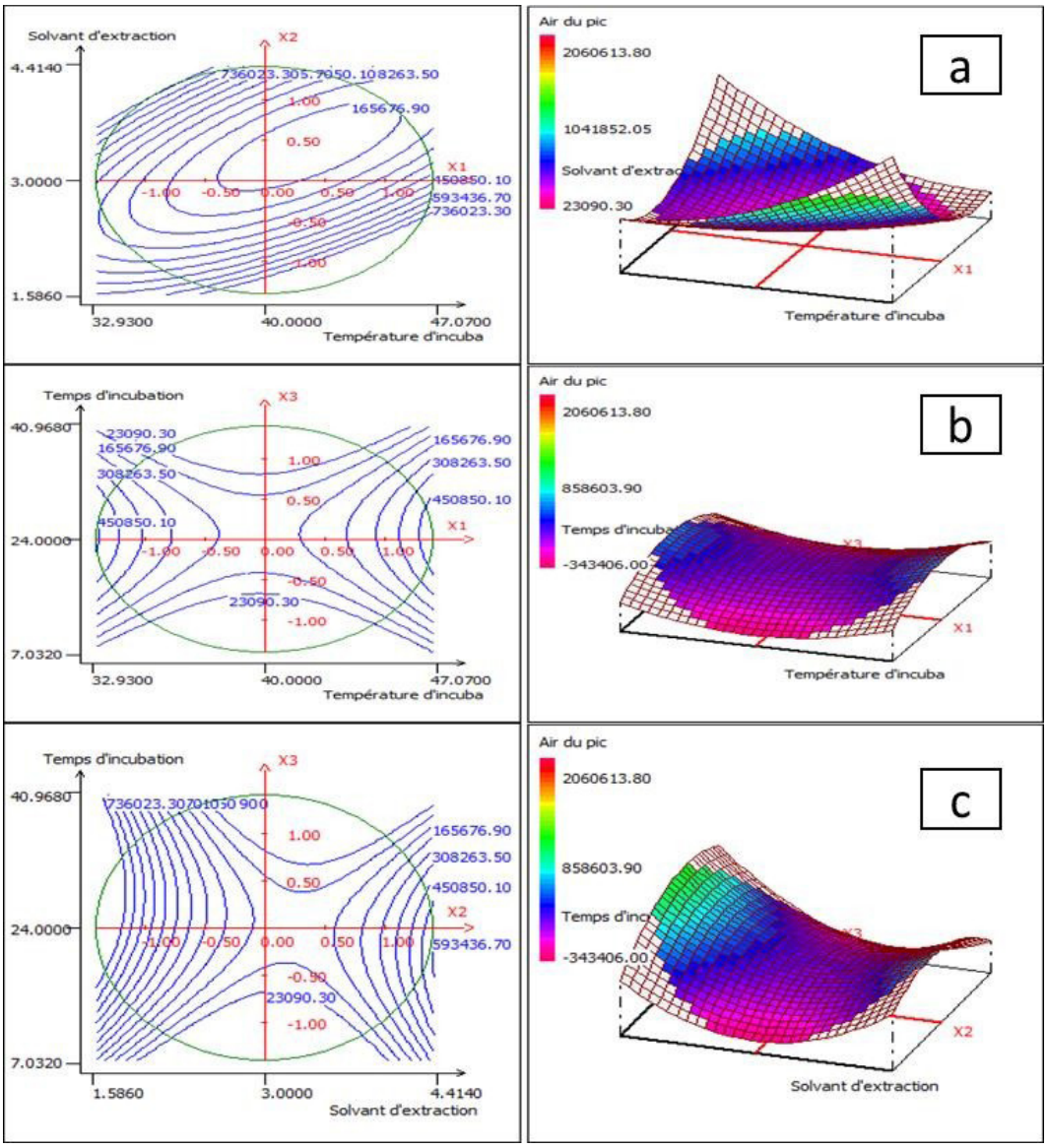

Figure 2. 2D graphic and 3D graphic of desirability variation in the plan a) fixed factor is the Incubation time of 36 h, b) fixed factor is the Extraction solvent = 4 % and c) fixed factor is the Incubation temperature = 35 °C.

Table 2. Box-Behnken design of experiment with Measured Responses.

Run	Incubation T °C	Extraction solvent %	Time incubation h	Pic area
1	35	2	24	458191
2	45	2	24	462682
3	35	4	24	807317
4	45	4	24	163151
5	35	3	12	123589
6	45	3	12	163019
7	35	3	36	23090
8	45	3	36	64941
9	40	2	12	174688
10	40	4	12	106950
11	40	2	36	638252
12	40	4	36	93459
13	40	3	24	145673
14	40	3	24	145681
15	40	3	24	145695

Validation of the method of BPA assaying

Following the optimization of the extraction conditions to obtain a maximum release of BPA, the performance of the devised method was validated with special emphasis to selectivity, linearity, fidelity, and limit of quantification after the extraction conditions were optimized to achieve a maximal release of BPA. The mobile phase's composition and the column's design were based on the experimental circumstances mentioned in the Materials and Methods section in order to validate the process.

Specificity. According to the chromatograms shown in Figure 3, there is no interference in the retention period of BPA between the peaks of methanol (MeOH) and acetic acid.

Linearity. Regression analysis was done on eight calibration standards (BPA alone) and eight validation standards (BPA in matrix) to determine the method's linearity. On the basis of three replications of the five standard solutions, it was discovered that the calibration curves were linear over a concentration range of 0.5 to 50 ppb. High correlation coefficients of 1 were attained, demonstrating the suggested method's high linearity over the examined range. Accordingly, the calibration and validation linear regression lines shown in Figure 4 were produced by applying the least squares linear regression technique to the data in Table 3 and are shown there. The slopes were significant and the fit was reliable, according to a statistical examination of the linear regression lines D1 and D2. These two lines were compared, and the results demonstrated that there was no matrix impact in the BPA assay.

Table 3. Overview of the method validation parameters: accuracy results.

Concentration Level (%)	Quantity Introduced (ppb)	Quantity Found (ppb)	Recovery %	S_i^2
2.5	0.500	0.536	107.201	17.77
	0.500	0.494	98.781	
	0.500	0.517	103.347	
5	1.004	1.027	102.689	5.87
	1.008	0.979	97.845	
	1.006	1.005	100.402	
25	5.002	5.012	100.198	0.01
	5.004	5.023	100.387	
	5.003	5.011	100.168	
50	10.004	9.963	99.594	0.26
	10.008	9.949	99.407	
	10.006	10.043	100.373	
100	20.008	19.852	99.218	0.81
	20.016	20.100	100.422	
	20.012	20.208	100.981	
150	30.012	30.044	100.107	0.03
	30.024	30.146	100.405	
	30.018	30.056	100.127	
200	40.016	39.872	99.642	0.05
	40.032	40.013	99.952	
	40.024	40.048	100.061	
250	50.02	49.947	99.854	0.29
	50.04	50.368	100.655	
	50.03	50.465	100.869	

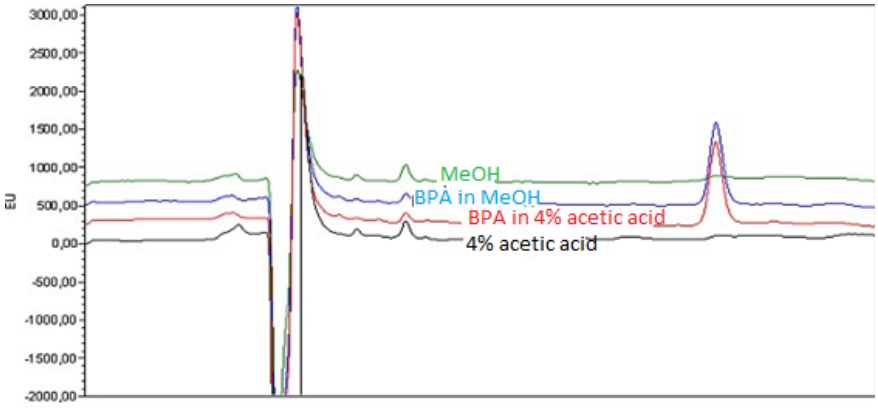

Figure 3. Chromatograms of the mobile phase, dilution solvent, BPA in the mobile phase and BPA in the dilution phase.

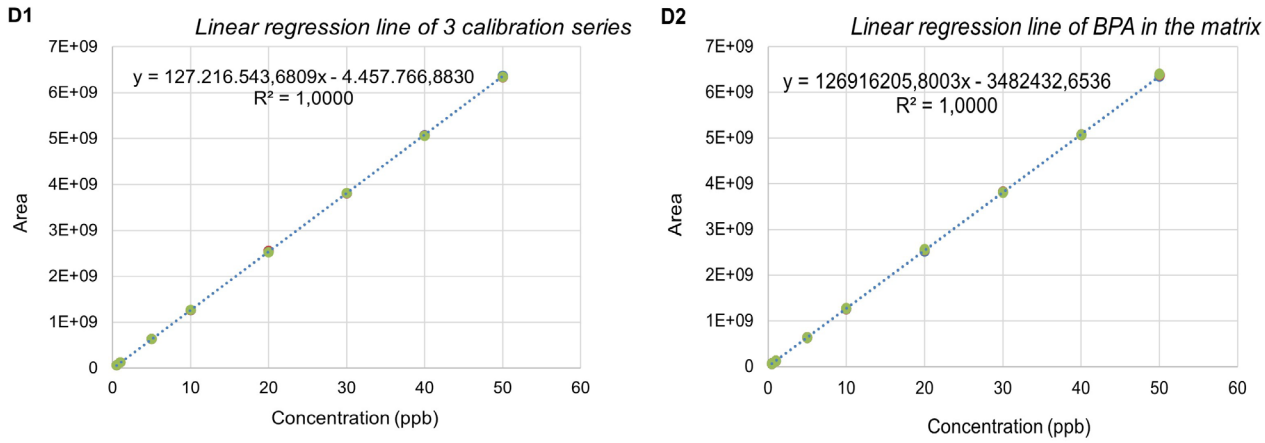

Figure 4. (D1) Linear regression plot of standard calibration. (D2) Linear regression plot of BPA in the matrix.

Accuracy. In eight concentration levels between 2.5 % and 250 %, the study assessed the method's accuracy. The typical recovery confidence interval, encompassing 100 % and spanning 99.84 % to 101.22 %. Table 3 presents the complete outcomes and statistical calculations.

Precision. Table 4 summarizes the fidelity values. For each analyte at each concentration level, the relative biases obtained are less than 2 %. The maximum Horwitz ratio for repeatability and intermediate precision is under 2, and the coefficients of variation for these metrics do not exceed 2 %. These numbers show the homogeneity of the validation samples, proving an appropriate level of fidelity.

Table 4. Overview of method validation parameters: Precision results.

Séries	Test	Quantity introduced, (ppb)	Quantity found, (ppb)	Recovery, %	Average	Variance	RSD, Repeatability,%	RSD, Intermediate precision, %
Day 1	1/1	24.04	23.96	99.65	100.53	0.39	0.62	0.087
	2/1	24.04	24.23	100.78				
	3/1	24.04	24.42	101.59				
	4/1	24.04	24.05	100.03				
	5/1	24.04	24.30	101.10				
	6/1	24.04	24.05	100.05				
Day 2	1/2	24.65	24.98	101.33	101.16			
	2/2	24.65	24.88	100.94				
	3/2	24.65	24.73	100.32				
	4/2	24.65	25.07	101.68				
	5/2	24.65	24.89	100.97				
	6/2	24.65	25.07	101.69				
Day 3	1/3	24.88	24.78	99.61	99.85			
	2/3	24.88	24.61	98.93				
	3/3	24.88	24.88	100.00				
	4/3	24.88	24.82	99.77				
	5/3	24.88	24.90	100.08				
	6/3	24.88	25.06	100.73				

Limit of quantification. The signal-to-noise ratio S/N is equal to 19. The LOQ value is 0.5 ppb.

Applications of method of dosing BPA in baby bottles

We discovered bisphenol A in the J, F, and P brand bottles, as illustrated in Figure 5. The B, N, H, and C brand bottles do not have this problem. We are unable to determine if the absence of BPA peaks on the chromatogram for these last three brands is a result of the monomer being completely absent from these bottles or a result of the detection threshold. Compared to the other brands (F and P), bottles of the brand (J) contain a sizable amount of BPA, as indicated in Table 5.

Table 5. Presentation of the different bottle samples and their peak areas.

Sample name	Retention time, min	Conc. of BPA, ppb
Baby Bottle (J)	7.447	3.802
Baby Bottle (F)	7.443	1.619
Baby Bottle (P)	7.221	0.615
Baby Bottle (B)	ND	ND
Baby Bottle (N)	ND	ND
Baby Bottle (H)	ND	ND
Baby Bottle (C)	ND	ND

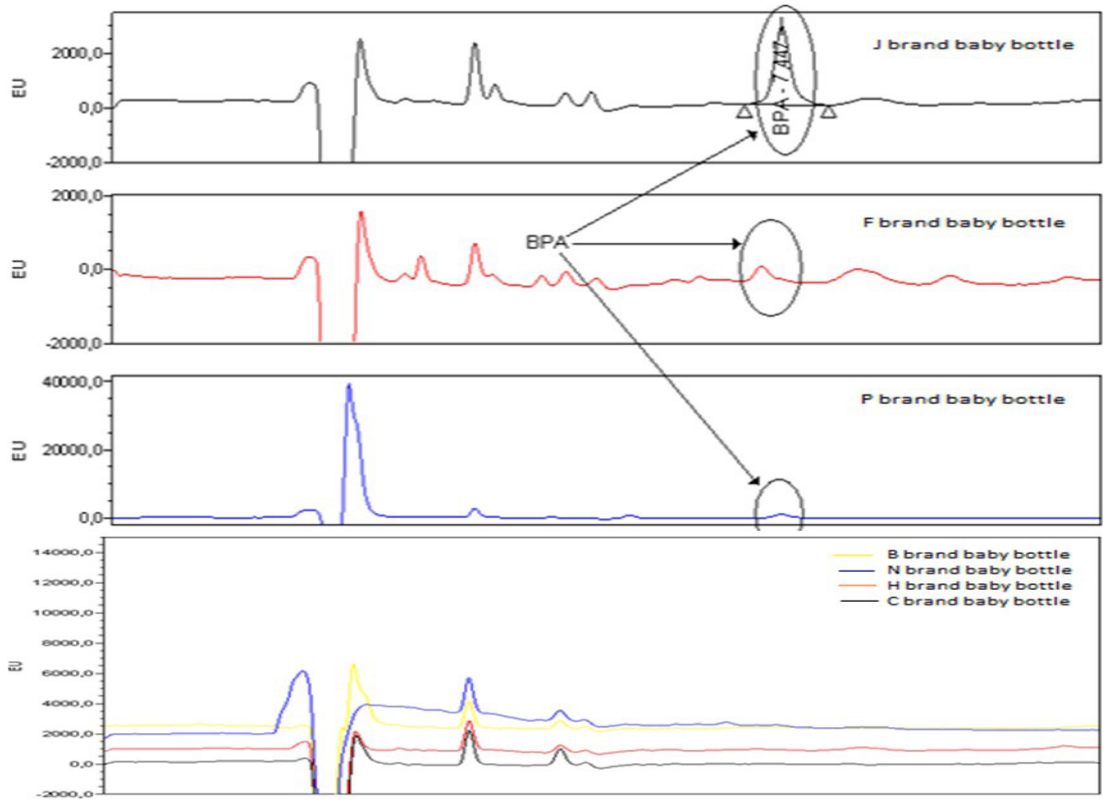

Figure 5. BPA chromatogram of J, F, P, B, N, H and C samples.

Conclusion

In conclusion, experimental designs have demonstrated to be a critical tactic for enhancing the conditions for extraction investigations. The operational conditions were enhanced by using the Box-Behnken design. Derringer's desirability function, a multi-criteria decision support tool, was used to maximize the extraction to the highest degree achievable. Following the optimization of the extraction process, this approach was assessed for its specificity, linearity, accuracy, and precision and was determined to be workable and efficient for baby bottle quality control at the distribution channel level. The market for material vigilance was being watched, and it was discovered that infant bottles made with bisphenol are still in use. Over the ISO standard, the enhanced approach had a 12 % higher yield.

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