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Simplified QuEChERS technique followed by UHPLC-MS/MS analysis for the determination bisphenol A in whole and powdered milk

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Abstract

Bisphenol A (BPA), an important endocrine disruptor, is employed in the manufacturing of many materials such as food packaging. The ingestion of contaminated food is considered the most relevant form of exposure to this compound. Data concerning the presence of this contaminant in milk in Brazil, however, are still lacking. In this context, an analytical method for the determination of BPA in ultrahigh temperature (UHT) milk, pasteurised milk, and milk powder was developed. A modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method was used for sample treatment, and BPA was determined by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). The method was selective for BPA in all investigated milk samples. No matrix effects were observed, thus indicating that calibration curves could be prepared in solvent for routine analysis. The method presented satisfactory accuracy, with recovery values between 78 and 94%. Relative standard deviation values obtained in the repeatability (11%) and intermediate precision (4.8%) studies were also satisfactory ($HorRat_r$ and $HorRat_R$ values less than 2). The analyte limits of detection and quantification were 0.12 and 0.36 ng/g for while milk, and 0.40 and 1.20 ng/g for reconstituted milk powder (1:10), respectively, and the sensitivity of the analytical method was considered adequate for the purpose of the present work.

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Introduction

The development of synthetic chemicals in the last century occurred exponentially (Wang *et al.*, 2020). Their increasing production, use, and lack of control policies based on toxicological and environmental criteria, however, have led to numerous teratogenic and carcinogenic substances applied to industrial, agricultural, and domestic uses involving both animals and humans (Björvang and Damdimopoulou, 2020). Bisphenols are part of a class consisting of diphenylalkane substances such as 2,2-bis (4-hydroxyphenyl) propane; 2,2-bis (4,4'-dihydroxyphenyl) propane or 4,4'-isopropylenediphenol, CAS No. 80-05-7, popularly known as bisphenol A (BPA), currently the most important representative of this class. *In vitro* studies have demonstrated that BPA displays the ability to activate the differentiation of 3T3-L1 fibroblasts into adipocytes, and accelerate the differentiation of terminal adipocytes, while *in vivo* studies have associated BPA with increases in type 2 diabetes mellitus rates, as well as in body mass index and waist circumference, thus leading to the development of obesity, hyperlipidaemia, DNA damage, and ultimately cancer (Masuno *et al.*, 2005; Shankar and Teppala, 2011; Jalal *et al.*, 2019; Wan *et al.*, 2021).

BPA is used mainly in the manufacture of resistant polycarbonate plastics and epoxy resins employed as coatings for metallic food and beverage packaging. Overall, food is the predominant source of BPA exposure, as BPA in food packaging can migrate to food through direct contact with both plastic and metallic packaging following temperature or pH changes. Studies have demonstrated that migration in even trace amounts could lead to human health risks (Trasande *et al.*, 2012; Ong *et al.*, 2020). BPA has been detected in a variety of animal products such as milk, dairy products, eggs, fish, and meat, as well as other food items, *i.e.*, vegetable oils, cereals, snacks,

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soy, canned products, and water (Liao and Kannan, 2014).

Due to the adverse effects of BPA, several countries have increasingly taken safety measures to limit BPA exposure, and researchers have developed new analytical methods to determine BPA in different foods, contributing to exposure level assessments. However, most methods are expensive and time consuming. Therefore, new techniques aiming at more sensitive, selective, cheaper, and fast BPA determination have been increasingly developed (Ballesteros-Gómes *et al.*, 2009; Chen e*t al.*, 2019). These include HPLC-FLD and HPLC-DAD employing different sorbents such as the new Z-Sep, Z-Sep Plus, EMR-lipid, and chitin, and other widely applied compounds like PSA and C_{18}, used during the dispersive solid phase extraction (d-SPE) clean-up step combined with solid phase extraction (SPE) (Tuzimski and Szubartowski, 2019; 2021; 2022; Tuzimski *et al.*, 2020).

Ultra-efficient liquid chromatography coupled with sequential mass spectrometry (UHPLC-MS/MS) combines the advantages of both chromatography and mass spectrometry alongside sequential mass spectrometry as an identification and confirmation tool. This method reduces the need for excessive sample preparation steps, while contributing to less false positive and/or negative results in complex samples, thus allowing for analyte identification at ultra-trace levels. The application of the QuEChERS method (Quick, Easy, Cheap, Effective, Rugged, and Safe) allows for quick and simultaneous interferent and residual water removal, making the method even quicker and more efficient (Cunha and Fernandes, 2013; Dualde *et al.*, 2019).

Although many studies have investigated the presence of BPA in processed foods, BPA contamination in whole milk and whole milk powder packed in different types of packaging have not been well-investigated. To the best of our knowledge, this is the first optimised and validated analytical method developed for BPA determination combining the advantages of the miniaturised QuEChERS extraction method with UHPLC-MS/MS applied to whole milk packaged in glass containers, poly(terephthalate of ethylene) (PET), polyethylene (PE), poly(vinylidene chloride) (PVDC), and Tetra Pak® cartons, as well as whole milk powder packaged in metallic cans and metallic polyester-polyethylene (laminated film) sold in the city of Rio de Janeiro, south-eastern Brazil.

Material and methods

Samples

Fifty-one whole milk (ultra-high temperature (UHT) and pasteurised) and whole powdered milk samples from different manufacturing batches were obtained from retail stores in the city of Rio de Janeiro between April and July 2019, totalling 19 powdered milk, 27 UHT milk, and five pasteurised milk samples.

The samples were stored at the National Institute for Quality Control in Health (INCQS) at the Oswaldo Cruz Foundation (Fiocruz) in their original packaging following manufacturer storage recommendations before and after opening.

Chemicals and reagents

Bisphenol A standard (purity > 99%) was purchased from Sigma-Aldrich (Pennsylvania, USA). Acetonitrile (HPLC-grade), sodium chloride (purity > 99%), anhydrous magnesium sulphate (purity > 98%), and ammonium hydroxide 25% (for analysis) were purchased from Merck (Darmstadt, Germany). Methanol (HPLC-grade) was purchased from Tedia (Darmstadt, Germany). Hexane (purity > 96%) was purchased from J.T. Baker (Pennsylvania, USA). Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA).

Standard solutions and samples

The BPA standard stock solution (1,000 µg/mL) was prepared by dissolving 10 ± 0.1 mg of BPA standard in methanol (MeOH), made up to 10 mL in a volumetric flask, and stored in screw capped glass tubes at -18°C in the dark. Working solutions used to prepare the calibration and sample spike solutions were prepared weekly by serially diluting the stock solution with MeOH to 5 ng/mL, and stored at 5°C.

Three grams of whole milk and/or 0.3 g of powdered milk were weighed (Sartorius, LP 620P) in 15 mL Falcon tubes. In the case of powdered milk, 3 mL of ultrapure Milli-Q water were added, and the Falcon tubes was vortexed for 30 s (Marconi, MA 162), followed by the addition of 3 mL of acetonitrile (ACN) and 2 mL of filtered hexane, and was further vortexed for 30 s. Subsequently, 1.2 g of anhydrous magnesium sulphate ($MgSO_4$) and 0.3 g of sodium chloride (NaCl) were added, and the Falcon tubes were vortexed again for 2 min. The samples were then

subjected to centrifugation at 3,000 rpm for 7 min at 20°C using a 5804R Eppendorf centrifuge, followed by separation of the organic phase (hexane) extract in ACN, salts, and water. The supernatant phase (hexane) was discarded, and only the intermediate layer (ACN extract) was conserved. One-mL aliquots of the ACN extract were then transferred to glass vials, and evaporated (Reacti-Therm III, 18935/Reacti-Vap III, 18785) until dryness under a gentle N_2 gas flow. The dry extracts were then resuspended in 1 mL of MeOH/H_2O (80:20, v/v) with 0.1% ammonium hydroxide, submitted to an ultrasound treatment (Branson, 2510) for 5 min, and filtered through a disposable filtration membrane (Millex-FG, 0.2 µm, hydrophobic PTFE) with the aid of a glass syringe. The filtered extracts were collected directly into vials for the instrumental UHPLC-MS/MS analysis.

UHPLC-MS/MS analysis

An ultra-efficient liquid chromatograph (UHPLC) UPLC™ I-Class ACQUITY™ (Waters, USA) was employed coupled to an Acquity™ UPLC BEH C_{18} column (100 × 2.1 mm d.i., particle size of 1.7 µm) as the stationary phase. Column temperature was maintained at 35°C, and the mobile phase flow rate was adjusted to 0.3 mL/min. A MeOH/H_2O solution (70:30, v/v) was used as the mobile phase at an isocratic elution gradient, followed by washing with ACN:MeOH:isopropanol:H_2O (1:1:1:1, v/v/v/v) at the end of the run for 5.0 min, and a 5-min stabilisation step in initial conditions. A sample injection volume of 5 µL was used. Analyte detection was performed employing a tandem quadrupole mass spectrometer (Waters, Xevo® TQ-S) equipped with an electrospray ionisation source (ESI). The optimised source parameters were capillary voltage of 2 kV, desolvation temperature of 400°C, and source temperature of 150°C. Argon was used as the collision gas at a 0.15 mL/min flow rate. Nitrogen was used as the desolvation gas at 750 L/h, and the cone gas was maintained at 150 L/h. Collision energies of 15 and 20 V were used for the quantification (*Q* - *m/z* 227 > 212) and qualification (*q* - *m/z* 227 > 133) transitions, respectively.

Method validation

Different extraction conditions were optimised during method development. The QuEChERS procedure was applied for the analysis of fortified milk samples at two levels, 0.5 and 1.0 ng/g for whole milk, and 1.7 and 3.3 ng/g for milk powder, respectively. After establishing optimum sample preparation and UHPLC-MS/MS conditions, validation was performed by evaluating several analytical performance parameters namely selectivity, matrix-effect, linearity, trueness (recovery), precision (intra-day and inter-day repeatability), limit of detection (LOD), and limit of quantification (LOQ).

Selectivity

Method selectivity was evaluated by analysing whole milk powder and fortified whole milk powder blank samples up to 0.5 ng/mL of BPA. No interference signals eluting at the same retention time as BPA were verified for any of the samples. This was confirmed by comparing the signal intensity ratios of the two ion transitions (*Q* - *m/z* 227 > 212 and *q* - *m/z* 227 > 133) of each analyte with those obtained using the standard solution.

Matrix-effect and linearity

Matrix interferences were investigated in whole milk powder. Two analytical curves were prepared, one in the matrix using a final blank whole milk powder matrix extract dissolved in MeOH:H_2O (80:20, v/v, 0.1% ammonium hydroxide), and another in MeOH:H_2O (80:20, v/v, 0.1% ammonium hydroxide) solution only. Calibration curves for the target compound the matrix extracts and in MeOH:H_2O (80:20, v/v, 0.1% ammonium hydroxide) were prepared at concentration levels ranging from 0.5 to 2.0 ng/mL, and each solution was analysed in triplicate. Calibration curve slopes and intersections were compared based on the *F*-test at a 95% confidence level. The homogeneities of the variances of the residues from two curves were then evaluated and compared by the *t*-test. The ordinary least squares method was applied to the calibration curves. Linearity was assessed according to Souza and Junqueira (2005). Homoscedasticity and the independency and normality of the regression residuals were checked as assumptions for the regression analysis. Outliers were successively investigated by the Jacknife standardised residuals test (Belsley *et al.*, 1980) until no further outlier detection, or a maximum exclusion of 22.2% from the original results (Horwitz, 1995) were obtained. The homoscedasticity of residues was verified by a modified Levene test (Brown and Forsythe, 1974) and the independency of residuals by the Durbin-

Watson's statistical test (Durbin and Watson, 1951). Residual normality was checked by Ryan-Joiner test (Ryan and Joiner, 1976). Regression significance and lack-of-fit were verified by an analysis of variance (ANOVA) test.

Bias (recovery)

Bias was evaluated through recovery values, calculated from the ratio between the chromatographic peak areas obtained from reconstituted (1:10) blank milk powder samples fortified with BPA at two concentration levels, 0.5 ng/mL (n = 3) and 1.0 ng/mL (n = 4), and the chromatographic peak areas obtained with a BPA solution at 0.5 ng/mL (n = 3) and 1.0 ng/mL (n = 4).

Precision (intra- and inter-day repeatability)

Intra-day precision was evaluated through reconstituted spiked blank milk powder samples (1:10) at a BPA level of 0.5 ng/mL (n = 3). Precision was estimated at this concentration and expressed as relative standard deviation (%RSDr). Inter-day precision was assessed on three different days employing reconstituted powdered milk (1:10) spiked with BPA at 0.5 ng/mL, comprising three replicates. Inter-day precision was estimated at this concentration, and expressed as relative standard deviation of intermediate precision (%RSD$_R$).

Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (signal-to-noise ratio > 3) and limit of quantification (signal-to-noise ratio > 10) were determined from reconstituted spiked blank milk powder samples (1:10), and confirmed through the final analytical curves.

Precautions to minimise background contamination

Precautions were taken prior to the sample preparation procedure to eliminate cross contamination. A previous contaminant analysis indicated BPA residues only in the hexane, $MgSO_4$, and NaCl reagents. The salts used in the QuEChERS method ($MgSO_4$ and NaCl) were muffled overnight at 400°C, and maintained in closed glass bottles prior to use. Hexane was filtered through an ENVI^{TM} membrane (18 DSK 47 mm, Sigma-Aldrich) employing a vacuum filtration system. No contamination issues were observed in the blanks following these procedures.

Results and discussion

UPLC-MS/MS optimisation

Mass spectrometer conditions were optimised by injecting a standard BPA solution (50 ng/mL) prepared in MeOH/H_2O (1:1, v/v). Detections were carried out in the positive and negative ionisation ESI modes, and the negative mode was selected based on chemical BPA properties. Appropriate cone voltages were optimised to obtain maximum intensity signals for each ion from selected precursor ions (Shao *et al.*, 2005; 2007; Rodríguez-Gómez *et al.*, 2015). Protonated [M + H]- ions were selected for the target substance. Capillary energy was evaluated from 0 to 4 kV. Desolvation gas (N_2) temperature was investigated from 200 to 500°C at a source temperature set at 150°C. The desolvation gas (N_2) flow was assessed from 300 to 1,200 L/h, while the cone gas flow (N_2) was evaluated from 150 to 300 L/h. Collision energies of 15 and 20 V were applied for the quantification and qualification transitions, respectively. The two most intense ion transitions were selected employing the signal-to-noise ratio of the detected chromatographic peaks.

The best BPA responses were obtained at 2 kV. Temperatures above 400°C did not offer any signal gain, so this temperature was selected alongside a source temperature of 150°C for all analyses. A desolvation gas (N_2) flow of 750 L/h was selected since higher flows did not offer any BPA signal gain. The cone gas flow (N_2) flow was maintained at 150 L/h, determined as the best BPA sensitivity condition. Collision energies of 15 and 20 V were used for the quantification [Q (m/z 227 > 212)] and qualification [q (m/z 227 > 133)] transitions, respectively. Transitions were monitored in the ESI mode (-) through the multiple reaction monitoring (MRM) acquisition mode of the standard BPA solution at 0.5 ng/mL. Two mobile phase systems were evaluated, ACN/H_2O (55:45, 60:40, 65:35, and 70:30, v/v) and MeOH/H_2O (55:45, 60:40, 65:35, and 70:30, v/v), to assess the best analytical response, and the best ratio between the two solvents, always under isocratic conditions. The mobile phase OF choice was based on the comparison between the mean BPA chromatographic peaks heights, with the MeOH/H_2O system (70:30, v/v) chosen as the most suitable eluent at 0.3 mL/min, as a flow change from 0.3 to 0.35 mL/min was accompanied by a 21.2% loss in sensitivity.

Different injection volumes (1 to 10 µL) were also tested using the standard 1.0 ng/mL BPA solution. A 5 µL injection volume led to the highest sensitivity (signal-to-noise ratio) and the best chromatographic peak symmetry, and thus selected as the standard injection volume for all analyses. Depending on the chromatographic responses to the eluent, the influence of the mobile phase flow based on the best sensitivity condition was also evaluated. The addition of ammonium hydroxide to the mobile phase has been reported in the literature for other studies (Young and Mallet, 2012). Therefore, the effect of adding 0.1% ammonium hydroxide to the mobile phase system was also evaluated, leading to a 61% loss in sensitivity. The carryover effect was evaluated following solvent injection after the introduction of the standard 5 ng/mL BPA solution (*n* = 3), and no memory effect was detected.

Different solvents and washing times were tested using the 5 ng/mL BPA solution (*n* = 3), comprising (a) different running times in the isocratic mode using MeOH/H_2O (70:30, v/v) from 1.5 to 5.0 min; (b) changing the cleaning solvent to 100% MeOH after a 5.0 min run; (c) the use ACN:MeOH:isopropyl alcohol:H_2O (1:1:1:1, v/v/v/v) as the washing solvent at the end of the run for 5.0 min. At the end of each run, the system was rinsed for 5.0 min with ACN:MeOH:isopropyl alcohol:H_2O (1:1:1:1, v/v/v/v) followed by stabilisation at initial conditions [methanol: water (70 : 30, v/v)] for another 5.0 min.

The sample cleaning step was first carried out using PSA and C_{18} columns to decrease the load of unwanted substances in the extract, and consequently, eliminate signal increase effects. A signal increase effect decrease was observed after the first injection using PSA and C_{18} but did not solve the repeatability problem. Therefore, the clean-up step was discarded for the sample treatment step.

QuEChERS optimisation for sample treatment conditions

A sample treatment using QuEChERS for free BPA analysis was based on two methods. The first, described by Przybylski and Segard (2009), employed extraction with hexane and 1% acetic acid in ACN, drying the extract with magnesium sulphate and sodium acetate, centrifugation, removing the hexane layer, and another centrifugation. In sequence, 1 mL of the extracted supernatant was treated with PSA (clean-up step), and 200 µL of the supernatant layer was dried and resuspended in 20 µL with 0.1% acetic acid in ACN without applying a filtration step. The second method, described by Sartori *et al.* (2015), on the other hand, used hexane and 1% acetic acid in ACN for extraction, adding $MgSO_4$ and NaCl to the extract, followed by a centrifugation step, hexane layer removal, 5 mL evaporation from the ACN phase to dryness (under heating at 50°C), followed by resuspension with 1 mL MeOH/H_2O (1:1, v/v), and filtration. An adapted QuEChERS method was applied herein, where hexane and ACN were maintained in the extraction step, but no acetic acid in ACN was added. Regarding salts, $MgSO_4$ and NaCl were added, followed by centrifugation. However, the amount of salts used was ¼ than that applied by Sartori *et al.* (2015). A clean-up step was not carried outd and evaporation was carried out at room temperature. During this last step, 1 mL of the ACN extract phase was dried, resuspended in 1 mL of the employed solvent (80:20, v/v) containing 0.1% ammonium hydroxide, and filtered. The efficiency of the sample treatment method applied in the present work was initially assessed by analysing six BPA-fortified milk powder samples at 50 ng/mL (1:10 reconstituted milk). The method displayed an average recovery of 93.8% and a DPR_r of 8.3%, and thus selected for validation. The results indicated that ACN was efficient for BPA extraction in this type of matrix, also precipitating proteins, thus improving the cleaning step. Hexane, a non-polar solvent, was used alongside ACN to extract BPA from high-fat content samples, as well as to improve the cleaning step. Regarding the resuspension solvent, a mixture of ACN/H_2O was used as the final solvent for sample injection, and consequently, as the resuspension solvent to adapt to the initial mobile phase (ACN/H_2O), as reported in the literature (Yi *et al.*, 2010; Khedr, 2013). A mobile phase change, however, was established, to MeOH/H_2O (70:30, v/v), as improved chromatographic BPA responses were obtained. In this context, different solvent mixtures based on MeOH/H_2O were also tested for sample resuspension. The comparison of the chromatographic peak heights obtained from a 5 ng/mL BPA standard solution resuspended in different MeOH/H_2O compositions (50:50, 70:30, 75:25, 80:20, 90:10, and 100:0, v/v) indicated that the MeOH/H_2O (80:20, v/v) mixture was the most adequate. Despite chromatographic response increases using MeOH:H_2O (80:20, v/v), the influence of the addition of 0.1% formic acid and

0.1% ammonium hydroxide in the resuspension solvent was also analysed. The addition of 0.1% formic acid suppressed resuspension responses, but the addition of 0.1% ammonium hydroxide increased resuspension responses, as depicted in Figure 1.

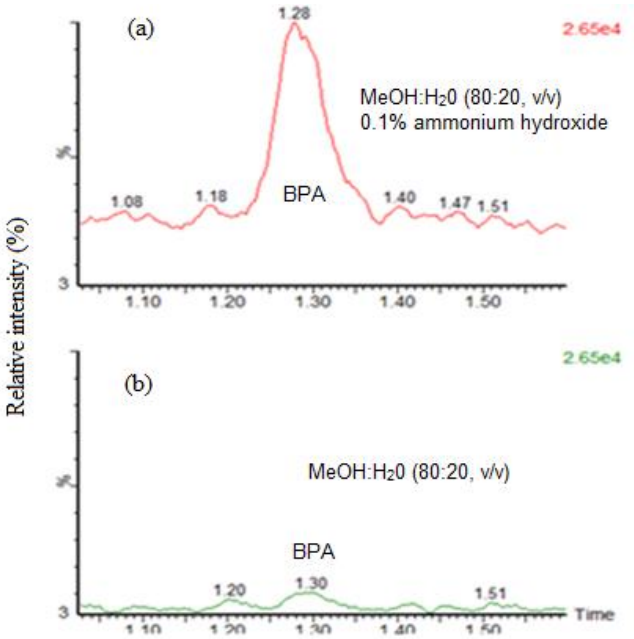

Figure 1. Chromatograms depicting the influence of the addition of 0.1% ammonium hydroxide in the final solvent used in resuspension, at a BPA matrix level of 1 ng/mL. **(a)** Chromatogram depicting 1 ng/mL of matrix BPA with the addition of 0.1% ammonium hydroxide in the final solvent used for resuspension. **(b)** Chromatogram depicting 1 ng/mL of matrix BPA without the addition of 0.1% ammonium hydroxide in the final solvent used for resuspension.

Internal quality control

Certain measures were taken to guarantee the validity of the obtained results. The first involved the evaluation of reconstituted powdered milk (1:10) fortified at 0.5 ng/mL BPA throughout sample batches, yielding a 76% recovery. Another internal control comprised assessments on the absence of interferences and potential BPA contamination in the reagents/solvents used in the routine analyses. The reagent blank was also analysed during a sample batch analyses, confirming the absence of interferences and reagent/solvent cross contamination. A reconstituted powdered milk sample (1:10) fortified at 0.5 ng/mL BPA was subsequently analysed, confirming the absence of reagent/solvent contamination (Figure 2).

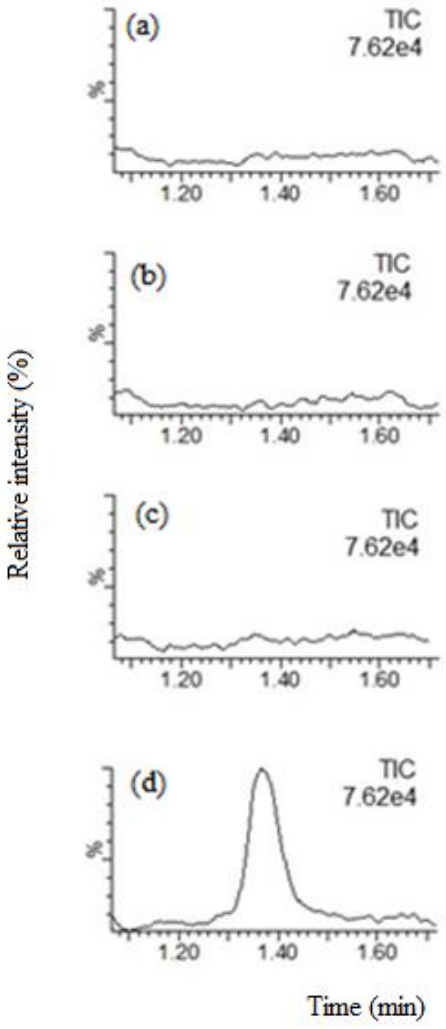

Figure 2. Chromatograms depicting no contamination of the reagent/solvents used in routine analyses. **(a)**, **(b)**, and **(c)** reagent blank analysis in comparison to **(d)** control powdered milk sample fortified with 0.5 ng/mL BPA.

Method validation

Method selectivity was evaluated by analysing blank matrix whole powdered milk samples. No BPA interference signals eluting at same retention time as BPA were verified for all evaluated matrices. Figure 3 displays a chromatogram of a whole powdered milk sample reconstituted with water (1:10) spiked with BPA at 0.5 ng/mL, and a non-spiked whole powdered milk sample also reconstituted with water (1:10). Confirmation was obtained by comparing the signal intensity ratios of the two ion transitions (*Q* - *m/z* 227 > 212 and *q* - *m/z* 227 > 133) of each sample analyte with those obtained using the standard solution.

BPA calibration curves were constructed from 0.5 to 2.0 ng/mL, with whole milk and powered milk equivalents ranging from 0.5 to 2.0 and 1.7 to 6.6

ng/mL, respectively. Good linearity was achieved, with correlation coefficients (R^2) over 0.990. Matrix effects were verified by comparing the slopes obtained with the matrix-matched calibration to those obtained with the standard solution calibrations, with the slope and intersection ratios of the matrix/solvent calculated for BPA. The slopes and intersections of the analytical curves prepared in the solvent and matrix were equivalent at a 95% confidence level, thus indicating no matrix effect.

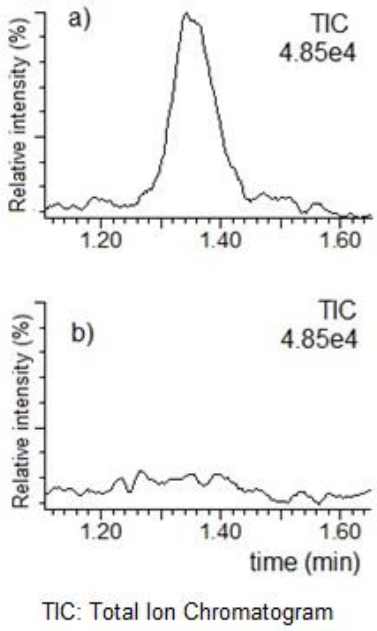

Figure 3. Chromatograms depicting the absence of interferences in the BPA retention time (tR 1.35 min). **(a)** Reconstituted (1:10) powdered whole milk sample fortified with BPA (0.5 ng/mL). **(b)** Non-fortified reconstituted (1:10) powdered whole milk sample.

The index matrix was calculated as EM% = 100 × (Cad/Cp), where Cad was the concentration determined by the matrix curve, and Cp was the concentration determined by the solvent curve. An acceptable EM range varies from 70 to 120%. Table 1 displays the index matrix determination obtained in the present work.

Table 1. Index matrix (EM%).

Concentration (mg/L)	EM%
0.5	108
1.0	102
1.5	94
2.0	105

The limits of detection (LOD, S/N = 3) and limits of quantification (LOQ, S/N = 10) for BPA in the investigated samples were 0.12 and 0.36 ng/g for whole milk, and 0.40 and 1.20 ng/g for milk powder, respectively. Bias/recovery were analysed by spiking blank samples with the BPA standard at two levels, 0.5 ng/g (n = 3) and 1.0 ng/mL (n = 4), expressed by recovery values $\bar{R}$ (%), determined as 70.8 and 93.6%, respectively. These results were compared against the Association of Official Analytical Chemists (AOAC, 2016) and European Commission (EC, 2002) guidelines which establish ranges from 40 to 120% and 50 to 120%, respectively, for ≤ 1 µg/kg of the analyte.

The intra- and inter-day precision for BPA were also applied to evaluate the blank sample spiked with BPA at 0.5 ng/mL (n = 3), expressed by the relative standard deviation %RSD$_r$ and intermediate relative standard deviation %RSD$_R$, determined as 11 and 22%, respectively. The %RSD$_r$ and %RSD$_R$ were compared against the Association of Official Analytical Chemists (AOAC, 2016) guideline which established values below 20 and 30%, respectively, for ≤ 1 µg/kg of the analyte. *HorRat*$_r$ values from 0.730 to 0.996 and *HorRat*$_R$ values of 0.217, both less than 2, were obtained, thus indicating adequate repeatability and intermediate precision, respectively. Therefore, the method displayed good bias and precision for the evaluated concentration levels.

Application of the developed method to whole and powered milk

The 51 samples investigated herein comprised 27 different brands purchased in six different packages, namely glass, poly (ethylene terephthalate) (PET), polyethylene (PE), and poly (vinylidene chloride) (PVDC), as well as Tetra Pak® cartons for whole milk. The whole powdered milk samples were packaged in metal cans and metallised polyester-polyethylene (laminated film). Figure 4 shows packaging category percentages for the analysed milk samples.

Of the 51 analysed samples, only two (3.9%) contained BPA levels above the limit of quantification (0.36 ng/g), comprising whole milk and powdered milk samples (1.20 ng/g). The BPA concentrations for reconstituted whole milk powder (1:10) and whole milk were 1.75 and 0.50 ng/g, respectively. The remaining 44 samples (86,3%) contained BPA levels below the LOD, and only five (9.8%) above the LOD, but below the LOQ. Figure 5

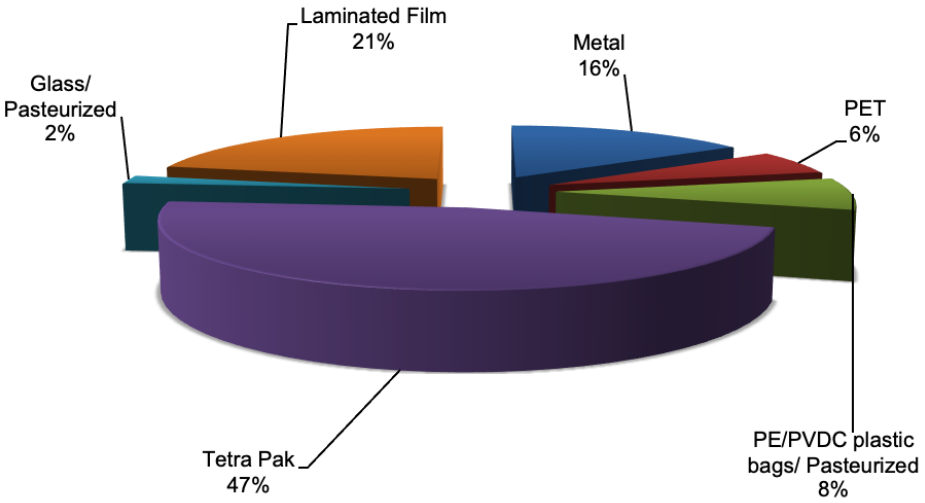

Figure 4. Milk packaging sample categories analysed in the present work.

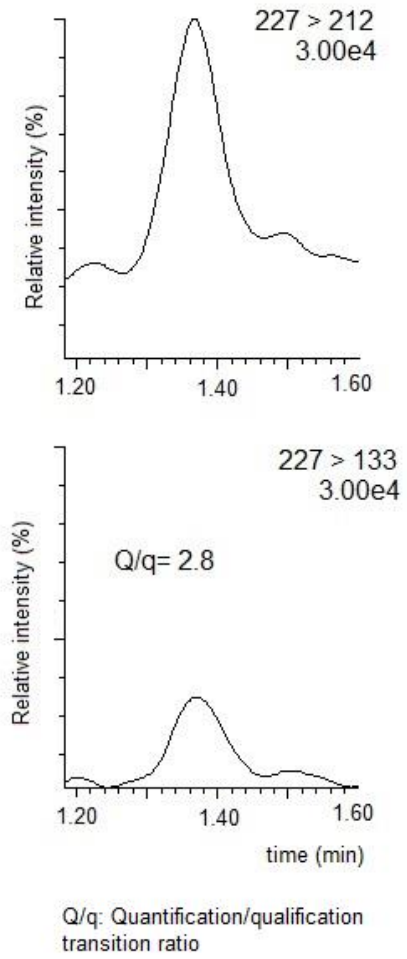

Figure 5. Chromatogram of powdered milk sample.

displays the chromatogram of a reconstituted powdered milk sample contaminated with BPA.

Among the 19 powdered milk samples evaluated in the present work, BPA was detected in three whole powdered milk samples, two samples packed in laminated film packaging (metallised polyester-polyethylene) and one sample packed in a metallic can. One sample packed in laminated film presented a BPA concentration of 1.75 ng/g. For the 32 whole milk samples, BPA was detected in two UHT samples packaged in PET-poli (ethylene terephthalate) packaging and Tetra Pak® carton packaging. One pasteurised whole milk sample packed in polyethylene (PE) and poly (vinylidene chloride) (PVDC) packaging yielded a BPA concentration of 0.50 ng/g. To the best of our knowledge, no studies evaluating the presence of PA in whole powdered milk and UHT and pasteurised whole milk are available in Brazil. Studies in several countries have been carried out to assess BPA milk contamination, reporting very variable results. Kang and Kondo (2003) and Niu *et al.* (2015), for example, did not detect BPA in milk, while Liao and Kannan (2014) detected an average value of 1.47 ng/g BPA when evaluating different foods, including milk. Liu *et al.* (2008) detected BPA in milk levels ranging from 1.6 to 2.6 ng/mL, while Grumetto *et al.* (2013) detected BPA in four samples, ranging from 15 to 481 ng/g, out of a total of 68 commercial milk samples. Mercogliano *et al.* (2021), when evaluating BPA contamination in the milk chain, observed the highest BPA contamination levels in raw milk at an average of 0.265 µg/L (0.265 ng/mL). The BPA levels detected herein in reconstituted whole milk powder (1:10) (0.53 ng/mL or 0.53 ng/g, considering a density equal to 1) and pasteurised whole milk (0.50 ng/mL) were close to the 0.49 and 0.216 ng/g BPA values reported by Shao *et al.* (2007) in China, and Bemrah *et al.* (2014) in France, respectively. Casajuana and Lacorte (2004) reported mean BPA

values between 0.99 to 2.64 ng/mL for UHT milk, and 1.17 to 1.29 ng/mL for sterilised milk. In the present work, pasteurised whole milk samples presented BPA contamination at 0.50 ng/g. Different contact times between the milk samples and the packaging during heat treatment may be responsible for these different BPA levels. Liu *et al*. (2008) also analysed whole milk packaged in different types of packaging, and reported BPA values of 2.6 and 1.6 ng/mL in polyethylene and high-density polyethylene packaging, respectively. The authors, however, reported that they did not detect BPA in samples packaged in Tetra Pak® cartons, probably due to the fact that BPA migration increases with increasing temperatures and packaging exposure times, and UHT milk is exposed to the packaging during sterilisation for only 2 to 4 s at 130 and 150°C, while regular milk is exposed to the packaging from 20 to 40 min at 110 and 130°C. Yoshida *et al*. (2001) assessed 14 canned food samples, and detected varying BPA levels in the solid and aqueous portions of the same foods. Hoekstra and Simoneau (2013) pointed out that BPA releases from polycarbonate packaging for liquid foods may be linked to the diffusion of residual BPA present in the polycarbonate or to polymer hydrolysis. Diffusion is the only relevant process, however, for dry foods. It is important to note that the milk contamination does not occur only due to migration *via* packaging, as BPA can enter the milk chain via multiple paths such as animal feed, farm environments, various points during milk production (*i.e.*, tubes used during milk processing, milk transfer to storage locations, and equipment used for filling, among others), in addition to the duration of the technological processing applied in the final production chain stage. Mercogliano *et al.* (2021), for example, reported the highest BPA contamination levels in raw milk from storage tanks when compared to pasteurised milk and packaged milk.

Despite BPA being the most abundant endocrine disruptor, studies have recognised that 16 BPA analogues have been used for various commercial applications (Chen *et al*., 2016) such as bisphenol S (BPS), bisphenol F (BPF), bisphenol E (BPE), bisphenol P (BPP), bisphenol C (BPC), bisphenol AF (BPAF), and bisphenol A diglycidyl ether (BADGE) (Eladak *et al*., 2015; García-Córcoles *et al*., 2018; McDonough *et al*., 2021). However, many *in vitro* and *in vivo* studies have shown that these analogues lead to similar or even more significant endocrine disruptive effects as compared to BPA including cytotoxicity, genotoxicity, adipogenicity, reproductive toxicity, and neurotoxicity (Mokra *et al*., 2018; Shi *et al*., 2019; Ramírez *et al*., 2021). As these compounds are not yet regulated, many are used without restriction, and few studies examining their potential toxicity are available. Therefore, further research is required to elucidate the occurrence of BPA analogues in Brazil.

Conclusion

The optimised method developed herein combining sample treatment by a modified QuEChERS method and UHPLC-MS/MS analysis was applied to determine BPA in 51 milk samples acquired in the city of Rio de Janeiro, Brazil. The results demonstrated quick, sensitive, and effective BPA determination at trace levels. The modified QuEChERS method led to the elimination of co-extraction of analytes in the final extraction solvent, thus resulting in a faster alternative for sample preparation with an adequate environmental footprint, due to the consumption of less inputs. Adequate sensitivity to the purpose of the study was obtained, comparable to other published methods. Of the 51 analysed samples, only two (3.9%) contained BPA levels above the LOQ. The others 44 samples (86.3%) were below the method LOD, and only five (9.8%) were above the LOD but below the LOQ. Overall, the developed method displayed significant potential for BPA assessments at trace levels in organic compounds with complex matrices, like whole milk.

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