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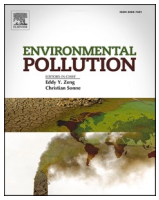

Biomonitoring of bisphenol A (BPA) and bisphenol analogues in human milk from South Africa and Canada using a modified QuEChERS extraction method☆

Zhi Hao Chi [a], Lan Liu [a], Jingyun Zheng [a], Lei Tian [a], Jonathan Chevrier [b], Riana Bornman [c], Muvhulawa Obida [c], Cynthia Gates Goodyer [d], Barbara F. Hales [e], Stéphane Bayen [a,*]

[a] Department of Food Science and Agricultural Chemistry, McGill University, Montreal, QC, Canada
[b] Department of Epidemiology, Biostatistics and Occupational Health, McGill University, Montreal, QC, Canada
[c] University of Pretoria, Pretoria, South Africa
[d] McGill University Health Centre Research Institute, Montreal, QC, Canada
[e] Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada

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ABSTRACT

A sensitive modified QuEChERS extraction method was developed to assess the levels of free and conjugated bisphenols (BPs) in human milk collected between 2018 and 2019 from two regions of South Africa (the Limpopo Province Vhembe district, n = 194; Pretoria, n = 193) and Canada (Montreal, n = 207). Total BPA (free and conjugated) and BPS were the predominant bisphenols detected in samples from Vhembe and Pretoria, whereas total BPS was the predominant bisphenol detected in Montreal samples. The levels of total BPA in samples from Vhembe and Pretoria ranged between < MDL-18.61 and <MDL-19.38 ng/mL, with medians of 1.03 ng/mL and 0.69 ng/mL and detection frequencies of 73% and 68%, respectively. The speciation analysis of BPA revealed a predominantly conjugated form in South African samples. In contrast, total BPA was detected in only one milk sample from Montreal. Total BPS levels were lower than BPA in South Africa, with detection frequencies of 57% and 21% in Vhembe and Pretoria, respectively. In contrast, total BPS was the major BP detected (42%) in Montreal (up to 4.42 ng/mL). BPAF was found exclusively in South Africa, with detection frequencies for total BPAF of 40% and 9% in Vhembe (<MDL-12.41 ng/mL) and Pretoria (<MDL-0.11 ng/mL), respectively. To our knowledge, this is one of the first studies to detect bisphenols in human milk from data-scarce countries such as South Africa and to highlight the notable disparities in the types and levels of bisphenols detected across two distinct countries (Canada and South Africa).

1. Introduction

For many years, Bisphenol A (BPA) was utilized extensively in the global production of polycarbonate and epoxy resins used to manufacture food containers, such as bottles for water and milk; its annual production is estimated to have reached as high as 6 million metric tons in 2023 (Rogers, 2021; Kumar et al., 2023). Because of incomplete polymerization, monomers such as BPA can leach out from these matrices (Bittner et al., 2014); humans are continuously exposed to BPA, primarily through dietary intake (Vandenberg et al., 2007; Geens et al., 2012). Importantly, studies have demonstrated that, due to its lipophilic nature, BPA may be transferred into breast milk in lactating women within a few hours after ingestion (Çiftçi et al., 2021). During the past two decades BPA has been widely recognized to act as an endocrine-disrupting chemical (Rubin, 2011); the possible adverse health effects associated with exposure to BPA include obesity, cardiovascular disease, the impairment of brain development in infants and abnormal prostate gland development (Vom Saal et al., 2021). This has led governmental agencies in various countries to impose regulations on its use in specific products (Resnik et al., 2015). Recently, the European Union has introduced regulations on the use of BPA in food contact materials (Krivohlavek et al., 2023). Specifically, these regulations

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* Corresponding author. Department of Food Science and Agricultural Chemistry McGill University, 21111 Lakeshore, Ste-Anne-de-Bellevue Quebec, H9X 3V9, Canada.
E-mail address: stephane.bayen@mcgill.ca (S. Bayen).

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impose lower migration limits (0.05 mg of BPA per kg of food), extend the ban on the use of BPA in baby bottles, and prohibit the migration of BPA from varnishes or coatings applied to materials intended for contact with food for infants and children aged 0–3 years (European Commission, 2018).

The production of BPA has decreased as a result of regulations and it has been replaced by other chemicals in many products; these alternatives are frequently bisphenol analogues (Qadeer et al., 2022). Specifically, exposure to BPA alternatives such as BPS and BPF has increased during the past decade (Alharbi et al., 2022). There is limited information on the extent to which some of these bisphenols are transferred to maternal milk or may have an adverse impact on infant health. Numerous studies have analyzed the levels of certain bisphenol analogues in human milk from countries such as the United States, China, Korea, Japan and Spain (Dualde et al., 2019; Niu et al., 2021; Ye et al., 2008; Tateoka, 2015; Lee et al., 2018). Interestingly, these studies have reported the presence of diverse bisphenols, such as BPA, BPS, and BPF; BPA was detected at notably high concentrations, in the range of 43 ng/mL, in milk from Korea (Lee et al., 2018).

Although the existing data on bisphenol levels in human milk across different countries contribute to advancing current human biomonitoring, a significant data gap persists, particularly concerning the presence of bisphenol A and its analogues in some regions, such as West Asia and Africa (Chi et al., 2023). There is a need to assess the levels of bisphenols in these data-poor countries.

BPA is extensively metabolized by glucuronidation and sulfation in humans, prior to its elimination predominantly via the urine or bile (Street et al., 2017; Abdulhameed et al., 2022; Michałowicz, 2014). Some studies have suggested that conjugated forms of BPA have little estrogenic activity (Matthews et al., 2001), but a more recent study provided evidence that some conjugates, such as BPA-glucuronide, induce adipocyte differentiation and alter adipogenesis (Boucher et al., 2015). Thus, differences in the toxicity of free and conjugated BPA are still not fully understood. Further, deconjugation may occur once metabolites are transferred to the breastfeeding infant, thus exposing infants to the bioactivities of the parent compound (Arbuckle et al., 2015). To date, there is limited information on the potential bioactivities of many bisphenols or their metabolites. Thus, assessing the levels of both free and conjugated bisphenols in human milk is crucial to understanding their fate and potential impact following transfer from the mother to the infant during breastfeeding.

In recent years, the use of QuEChERS extraction has been considered a promising method for the extraction and identification of environmental contaminants with a wide range of polarities, such as bisphenols and phthalates (Melo et al., 2019; Beser et al., 2019; Tuzimski et al., 2019). In this present study, we: (i) developed and validated a robust QuEChERS extraction method for the identification and quantification (free, conjugated and total concentrations) of nine selected bisphenols in human milk; (ii) applied this method to human milk samples collected from South Africa and Canada to determine their bisphenol levels; and (iii) compared the types and levels of detected bisphenols between South African and Canadian samples. By developing an effective extraction method and analyzing samples collected from these different geographical locations, we hope to improve our understanding of exposure to bisphenols and their potential implications for maternal and infant health, especially in the data-poor regions of South Africa. These findings will contribute to ongoing efforts in human milk biomonitoring.

2. Material and methods

2.1. Chemicals

LC-MS grade Ammonium acetate (NH_4Ac), acetic acid, and HPLC-grade solvents (water, acetonitrile, methanol) were purchased from Fisher Scientific. Magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) were obtained from Sigma-Aldrich. Agilent Technologies supplied C18 Endcapped SPE Bulk Sorbent and PSA SPE Bulk Sorbent.

Analytical standards (purity >98%) of BPA, BPF, BPS, BPAF, BPE, BPC, BPB, BPAP, and BPBP (Fig. 1) were purchased from Sigma-Aldrich. β-glucuronidase type H1 (≥500,000 units/g) and arylsulfatase type H1 (>10,000 units/g) were also supplied by Sigma-Aldrich. BPA-d_4 (≥98%) came from CDN isotopes. BPAF-$^{13}C_{12}$, and BPS-$^{13}C_{12}$ were obtained from Toronto Research Chemicals, and BPF-$^{13}C_{12}$ from Cambridge Isotope Laboratory.

Individual bisphenol stock solutions (100 mg L^{-1}) were prepared in methanol. A monthly mixture of these solutions, along with four isotope-labelled internal standards (ILIS), was prepared at 1 mg L^{-1} in methanol. All stock solutions were stored at −20 °C in amber glass vials. A 1.0 M ammonium acetate buffer solution was created by dissolving 5.39 g of ammonium acetate in 66 mL of HPLC-grade water. The pH was adjusted to 5.5 ± 0.1 with acetic acid, reaching a total volume of 70 mL. The enzymatic solution was made by dissolving β-glucuronidase/sulfatase powder in 35 mL of the prepared buffer (pH = 5.5) to yield a solution of 3500 U/mL.

2.2. Sample collection, storage and determination of moisture content

In South Africa, mother-infant pairs were recruited from the maternity wards and vaccine clinics of Tshilidzini Hospital, located in the Vhembe district of rural Limpopo Province, and Tshwane Hospital, located in urban Pretoria, Gauteng. Participants were eligible if they were at least 18 years of age, spoke English or Tshivenda (the main language spoken in the Vhembe district), expected or gave birth to a live singleton and (if recruited from the maternity ward) expected to be able to return one month post-delivery to respond to a short questionnaire and provide a breastmilk sample.

In a similar fashion, participants from Montreal were recruited from the Royal Victoria and St. Mary's hospitals, within maternity wards following childbirth. Eligible participants were required to be at least 18 years old, proficient in either French or English, and willing to participate in two follow-up sessions. The first session involved an explanation of the manual breast milk collection process, accompanied by a questionnaire, while the second session focused on the actual collection of samples.

Milk samples were collected between the 4th and 8th week after delivery from study participants in the three locations (Vhembe, Pretoria and Montreal) over a period of 1.5 years (2018–2019) via manual expression into BPA-free polypropylene containers and were stored in cryovials at −80 °C until shipment on dry ice to the analytical laboratory. A total of 594 human milk samples from different mothers were analyzed for their bisphenol levels (n = 194 from Vhembe, n = 193 from Pretoria and n = 207 from Montreal). The Montreal samples were stored in a −80 °C freezer until they were freeze-dried in a lyophilizer (FreeZone Cascade Benchtop Freeze Dry Systems, Labconco, Kansas City, KA, USA); the South African samples were freeze-dried using a different lyophilizer (Freeze dryer ALPHA 1–2 LD+, Osterode am Harz, Germany). All samples were freeze-dried with a vacuum of 0.09 Torrs at −80 °C for 3 days. The initial and final masses were noted to calculate the % moisture and solid contents (total minus the moisture content) for each milk sample.

All freeze-dried sample aliquots were stored in amber glass vials at −80 °C until analysis. This study was approved by ethics committees at McGill University, the Research Institute of the McGill University Health Centre (#MP-37-2018-3730), the University of Pretoria and the Limpopo Department of Health and Social Services.

2.3. Sample preparation

An optimization step was performed for QuEChERS extraction to determine the appropriate quantities of salts and cleanup powders for analyzing bisphenols in human milk, as described in Section 1 of the supplementary information.

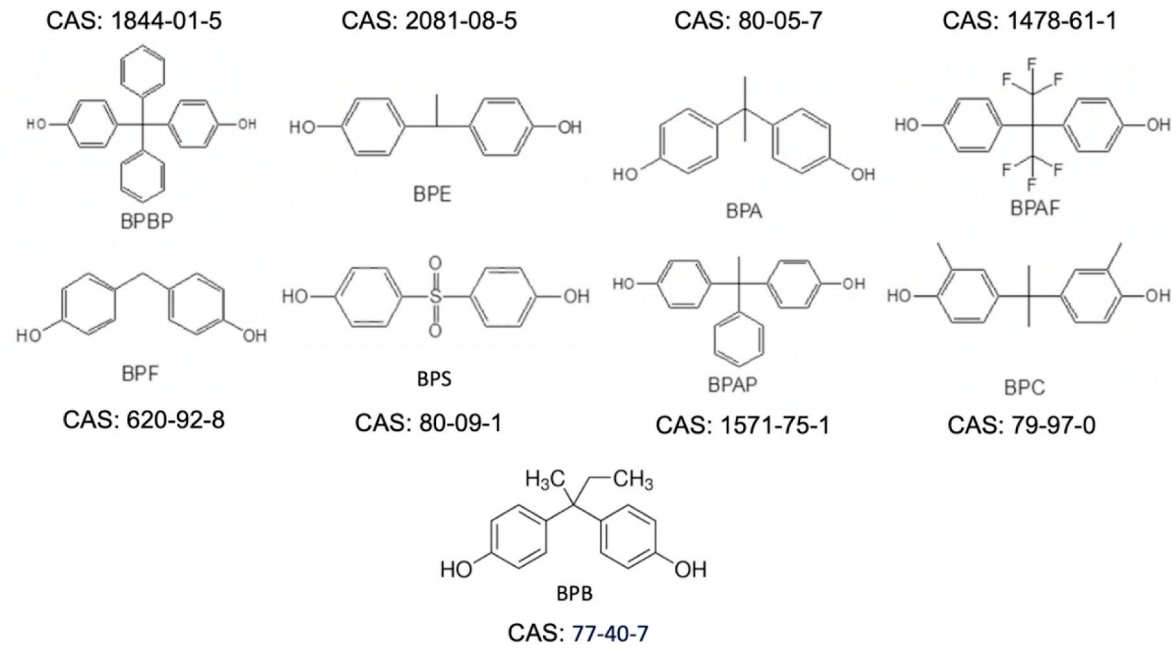

Fig. 1. Structures and CAS numbers of BPA and bisphenol analogues.

For the analyses of the free bisphenol compounds, approximately 0.2 g of freeze-dried milk powder was added to a 50 mL polypropylene tube, diluted in 2 mL of HPLC-grade water, and spiked with 30 μg/L of the previously prepared labelled bisphenol mixture. Next, 1 mL of the prepared 1 M ammonium acetate buffer solution was added to the sample followed by vortexing for 10 s. Subsequently, 10 mL of acetonitrile was added to each milk sample and the sample was subjected to ultrasonication for 10 min in a sonicator bath of 100 W at 40 Hz. One gram of $MgSO_4$ and 2 g of NaCl were then added to the sonicated sample. The mixture was vortexed for 4 min, shaken for 1 min and centrifuged at 4000 RCF for 10 min. The supernatant was carefully transferred to a 15 mL polypropylene tube containing 0.2 g PSA (primary secondary amine), 0.1 g C_{18} sorbent and 0.25 g $MgSO_4$. The sample was shaken vigorously for 45 s and then centrifuged at 3000 RCF for 10 min. After centrifugation, the supernatant (roughly 7.5 mL) was transferred to a 15 mL glass tube and evaporated to dryness under a nitrogen stream. The residue was reconstituted in 1 mL of acetonitrile (ACN) and water (1:1) prior to the LC-Q-TOF-MS analysis.

For the analysis of free and conjugated (total) analytes, a deconjugation step was implemented prior to the extraction step. This involved the addition of the ILIS solution and 1 mL of the prepared enzymatic solution (β-glucuronidase and arylsulfatase) to the sample. The mixture was vortexed and then incubated for 20 h at 37 °C. Each collected human milk sample underwent testing twice: once without enzymatic treatment and another time following deconjugation.

2.4. Instrumental analysis

Samples were analyzed using an Agilent 1290 Infinity II LC system (Agilent Technologies, Santa Clara, CA, USA) coupled to a 6545 quadrupole time-of-flight (Q-TOF) MS. The LC separation was conducted on a Poroshell 120 phenyl hexyl column (Agilent Technologies; 100 mm × 3.0 mm, particle size 2.7 μm) connected to a Poroshell 120 phenyl hexyl guard column (Agilent Technologies; 5 mm × 3.0 mm, particle size 2.7 μm) with a gradient elution at a flow rate of 0.2 mL/min. Elution was performed in gradient mode using A = water and B = Acetonitrile: Methanol (1:1), both containing 5 mM NH_4Ac; 5% B (0–1 min), linear increase to 100% B (1–15 min), 100% B (15–20 min) and restore to 5% B for 5 min (20–25 min). The injection volume was 20 μL and the column temperature was maintained at 30 °C. The 6545 Q-TOF-MS system was operated in negative electrospray ionization (ESI-) mode for analysis of the 9 BPs. The flow rate of the drying gas (nitrogen, 325 °C) was set at 5 L min^{-1}. Full scan mode with fragmentor energies of 125 V was used to collect the data in both centroid and profile modes in the mass-to-charge ratio (m/z) ranging from 50 to 1700. BPS was rerun in all samples using the same method, but using 100% methanol for mobile phase B with 5 mM NH_4Ac.

LC-Q-TOF-MS data treatment

LC-Q-TOF-MS data were analyzed using Agilent MassHunter Quantitative analysis (B.07.01) software to quantify nine targeted bisphenol analogues in breast milk samples, sample blanks and procedural blanks. The most abundant isotopes of $[M\text{-}H]^-$ were used as quantifiers for the nine bisphenols. The following m/z were extracted from the total ion chromatogram for quantification in calibration solvents and milk samples: 227.1072 for BPA, 199.0759 for BPF, 335.0506 for BPAF, 249.0222 for BPS, 255.1385 for BPC, 241.1229 for BPB, 213.0916 for BPE, 289.1229 for BPAP, and 351.1385 for BPBP. The following qualifier ions (m/z) were used to confirm the identity of the detected compound: 212.0839 and 133.0653 (BPA), 93.0344 and 105.0341 (BPF), 265.0479 and 315.0446 (BPAF), 108.0215 and 155.9883 (BPS), 240.1154 and 147.0814 (BPC), 212.0837 and 147.0808 (BPB), 198.068 and 119.0498 (BPE), 248.0143 and 274.0995 (BPAP), and 274.0989 and 258.1043 (BPBP) (Figs. S2–S10).

Compound identifications and confirmations were based on SANTE/11813/2021 (SANTE/11312/2021 and Analytical quality control and, 2021) requiring 2 product ions (one used for quantification and another for confirmation), with the ion ratio from sample extracts within ±30% (relative) of the average of the calibration standards, and a tolerance of ±0.1 min for relative retention time (RRT) between the suspected bisphenol in the milk sample and the corresponding bisphenol in the spiked samples.

The chromatographic peaks for all 9 spiked bisphenols in human milk are shown in Fig. S11. The total ion chromatogram of a human milk sample and a calibration solvent is shown in Fig. S12. The chromatogram extraction window was set at ± 10 ppm for mass and ±0.1 min for retention time (RT). The relative intensities of qualifier ions (% of base peak) of the BPs in pure solvent and in milk were also compared with each other (Tables S1 and S2).

2.5. Quality assurance/quality control

Prior to sample collection, a preliminary test was conducted to test for any bisphenol contamination in the glass and polypropylene vials and jars, as well as the cardboard containers used to store the human milk; the four major bisphenols (BPA, BPS, BPF, BPAF) were not detected.

Before sample analysis, measures were taken to avoid any possible contamination of bisphenol A and other analogues from the surrounding environment to the milk samples. These measures included the testing of the polypropylene and glass tubes for any bisphenol residue. Additionally, aluminum film was set up on each workbench to mitigate any risk of contamination during the sample preparation.

Quality assurance for the analyses included the control of background contamination, the monitoring of mass accuracy, intensity and RT shifts and signal drift. This was achieved through the repeated analysis of calibration standards, and by regularly evaluating the recovery of quality control samples (QCs) every 10 injections. Additionally, solvent blanks were subjected to the extraction method in triplicate to identify any possible analyte contamination. In some extractions, very low concentrations (<MDL) of BPA and BPS were detected in the blanks. The concentrations of BPA and BPS detected in these blanks, along with any other possible bisphenols present, were subtracted from the corresponding concentrations found in the milk samples to account for possible contamination. During sample analysis, an acetonitrile solvent blank was injected after every 10 samples to minimize possible carry over effects from the instrument.

One breast milk homogenate was created as a pool of $n = 40$ individual samples to be used for the recovery tests. The recoveries for all target compounds in breast milk were calculated using the internal standard method to correct for the matrix effect (Bienvenu et al., 2017). The matrix effects (ME%) for all 9 bisphenols were calculated using the equation below and are shown in Table S3:

$$ME\% = \left(\frac{peak\ area\ of\ analyte\ in\ matrix}{peak\ area\ of\ analyte\ in\ solvent}\right) * 100$$

The recoveries for the bisphenols that did not have corresponding ILIS standards were assessed using labelled $^{13}C_{12}$-BPF and $^{13}C_{12}$-BPAF. Labelled BPF was matched together with BPBP and BPC while labelled BPAF was matched with BPE, BPB and BPAP. The relative standard deviation (RSD) for the inter-day precision was calculated based on the analysis of three replicates using homogenate human milk on different days. An inter-day precision (RSD) lower than 20% was judged acceptable (SANTE/11312/2021 and Analytical quality control and, 2021). The spiking level was first evaluated at 100 ng/mL under different conditions (varying amounts of PSA and C18 during the clean-up step and the addition of ammonium buffer pH 5.5) for the selection of the most optimal amount of PSA and C18 for subsequent use. The selected amounts of C18 and PSA were then further evaluated at a final spiking level of 30 ng/mL, reflecting the levels of BPA present in South African human milk samples based on preliminary test results. The same spiking level was applied to Montreal samples to maintain consistency in the method used. For the quantification of BPS in human milk, the samples were re-analyzed using pure methanol as mobile phase B, due to better signal intensity (Fig. S13) compared to 1:1 ACN/methanol. Aliquots of the native bisphenol standard mixture solution and the labelled standard mixture solution (also representing about 30 ng for each compound) were spiked before the extraction into the prepared homogenate sample to assess the validity of the method. The isotope labelled bisphenols were used to monitor the recoveries of the extraction method and the quantification of bisphenols in human milk samples. To validate the performance of the instruments for the target compounds, 10 calibration points (0.5–100 ng/mL) of the target analytes, 30 ng/mL for the mass labelled surrogates) were selected based on the normal range of BPs in human milk reported from previous studies. The linearity of the instrument response ($R^2 > 0.98$) for all BPs was assessed using the response factor of the bisphenol standards in spiked human milk prepared in ACN:H_2O (1:1) (Table S3). The method detection limit (MDL) was calculated as three times the standard deviation of procedural blanks (US EPA, 2016). If the analyte was absent in all procedural blanks, the MDL was determined as the lowest concentration of the target analyte in breast milk extracts that yielded a signal-to-noise ratio above three. The limit of quantification (LOQ) was determined by multiplying the standard deviation of the lowest detectable concentrations of the bisphenols in procedural blanks (if any) or milk samples by 10 (Rice et al., 2012).

2.6. Statistics

Analysis of variance (ANOVA) was employed to assess differences in solid content among all three regions (Vhembe, Pretoria, and Montreal). Following the methodology described by Hornung and Reed (Hornung et al., 1990), values below the MDL were replaced by the MDL divided by the square root of 2. The % free bisphenol was calculated by dividing the contaminant level in the absence of enzymatic treatment by the level obtained with enzymatic hydrolysis (Dualde et al., 2019). Samples in which bisphenol levels were slightly higher than or equal to the non-enzymatic samples, compared to the enzymatic samples, were considered to be 100% in their free form. Conversely, samples that exhibited detectable levels of free and conjugated bisphenol after enzymatic hydrolysis, but had non-detectable levels in their non-enzymatic counterparts (0%), were treated as representing 100% conjugation. After calculating the % free bisphenol, the percentage of conjugated bisphenol was determined by subtracting a value of 1 (100% free form) from the percentage of free bisphenol values.

To test for the significance of the percentage of conjugated BP in terms of their conjugation potential, a one-sample *t*-test was conducted by comparing the calculated percentage of conjugated BP with a value of 0% (0% conjugation) for all detectable BPs in all three regions. Samples without any detectable free and total bisphenol were excluded from the analysis. Statistical analyses were performed using IBM SPSS Statistics (version 29, IBM Corporation, New York, NY).

3. Results and discussion

3.1. Solid content determination in sampled human milk

The levels of bisphenols in human milk were expressed in ng/mL using the solid content %. The average solid contents for Vhembe, Pretoria and Montreal human milk were 15.6 ± 4.5%, 16.1 ± 6.2% and 18.1 ± 3.7%, respectively. There was a significant difference (ANOVA, $p < 0.001$) in solid content between Vhembe and Pretoria when compared to Montreal (Table S4). No statistically significant difference was observed between Vhembe and Pretoria, implying that the main difference in solid content of breast milk samples was between South Africa (Vhembe and Pretoria) and Canada (Montreal). Differences in the solid content may result from variations in milk composition, such as in oligosaccharides, fatty acids or other trace elements (Erney et al., 2000; Yamawaki et al., 2005; Kumar et al., 2016). Factors influencing these compositions are considered to be genetic or environmental (Azad et al., 2018). However, it is important to note that despite these variations, the differences in solid content means for both regions were relatively small. Accordingly, the same extraction method was applied for both the South African and Montreal human milk samples.

3.2. Method validation

The variations between the measured *m/z* values and the theoretical values for all BPs were below 3 ppm, as presented in Table S1, and no major differences were observed among the values. The retention time consistency between the solvent standards and the actual samples for all

BPs exhibited a difference of 0.05 min; this difference is within the acceptable tolerance of 0.1 min (SANTE/11312/2021 and Analytical quality control and, 2021). The retention times of spiked BPs for both enzymatic and non-enzymatic samples showed negligible differences (within 0.05 min). The response of the instrument was deemed linear ($r^2 > 0.98$) for all target compounds, as determined through calibration standards (Table S3). This linearity was considered acceptable for establishing the calibration curve and is comparable to the linearity reported by Dualde et al. for bisphenol analogues (Dualde et al., 2019).

Matrix effect analysis revealed that eight bisphenols exhibited a "medium" matrix effect (20%–50%), while BPBP exhibited a "strong" matrix effect (>50%) (Table S3) (Kim et al., 2023). Among these, BPE, BPB, BPAP, and BPBP exhibited matrix effects exceeding 30%, suggesting persistent signal suppression despite the cleanup process. The high matrix effect may be attributed to potential interferences from remaining natural components, such as proteins, lipids, and carbohydrates in the human milk extracts. Despite the elevated matrix effect for certain bisphenols, the optimized sample preparation was employed for analysis. This decision acknowledges the necessity for a compromise, as non-targeted analysis will follow the targeted bisphenol analysis in a future study, aiming to detect other plastic-related unknowns in human milk. In this context, excessive removal of interferences during the targeted analysis may potentially impact the results of the subsequent non-targeted analysis.

From the previous QuEChERS optimization, by employing matrix-matched calibration, the mean recoveries of the 9 bisphenol compounds spiked at 100 ng/mL using the most optimal sample preparation, ranged between 75% and 95%, which is within the acceptable range of 70–120% (Table S5) (Steiner et al., 2020). With the exception of BPA, BPS and BPAF, the levels of the bisphenol analogues in homogenized human milk samples were below the method detection limit.

For the recovery test at 30 ng/mL, all non-detected bisphenols, except for BPF, were excluded. Thus, we focused on the four bisphenols (BPA, BPS, BPAF and BPF) with a higher likelihood of being present in all of the collected human milk samples, based on the reports from other human milk studies (Table 1).

Further inter-day precision of BPA, BPS, BPAF and BPF at a spiking level of 30 ng/mL was conducted prior to analyses of the South African and Montreal human milk samples (Table S5) and the recoveries for each major BP were comparable with the intra-day recoveries. The variation in MDLs for each recovery test (intra-day and inter-day) was lower than 0.1 ng/mL milk for all target compounds, indicating that this is a promising method for the quantification of selected BPs present at low concentrations in human milk.

In terms of precision, the intra-day relative standard deviation was below 10% (n = 3, ranging between 3.2 and 6.5%), which demonstrates satisfactory precision in the analysis (Viswanathan et al., 2007). Other studies of the BPs in human milk (Dualde et al., 2019; Niu et al., 2021; Lee et al., 2018; Cao et al., 2015; Zimmers et al., 2014; Ye et al., 2006; Jin et al., 2020; Czarczyńska-Gośliń et al., 2021) have reported comparable recoveries for all bisphenols, their corresponding RSD%, as well as their MDLs. Table S6 presents the average MDL and LOQ values for all spiked bisphenols, along with the ranges of MDL values observed across all sample batches for the four major bisphenols.

Three distinct samples from Vhembe, which had previously undergone analysis, were subjected to re-extraction and re-analysis to further assess the precision of the employed extraction method (Table S7). These results indicate the presence of only BPA in both the first and second extraction, displaying minimal variation in their levels. The other major BPs were below the MDL.

Examples of chromatographic peaks for all 3 bisphenols (BPA, BPS, BPAF) detected in unspiked human milk samples, as well as the blanks and calibration solvents, are shown in Figs. S14–16. In summary, the current method demonstrates sensitivity, acceptable precision, and accuracy.

3.3. Bisphenol levels in South African and Montreal human milk

Six bisphenols (BPF, BPC, BPE, BPB, BPBP and BPAP) were below the method detection limit (MDL) in all samples. BPA, BPS and BPAF were detected in breast milk samples from both the Vhembe and Pretoria regions in South Africa (Fig. 2, Table 1): the detection frequency and concentration levels of BPA were notably higher than those for BPS and BPAF. BPAF was detectable in samples that were primarily from Vhembe. In contrast to South Africa, only one Montreal milk sample contained detectable levels of BPA. BPS was the predominant bisphenol found. BPAF was not detectable. Our analysis revealed substantial variations in the % conjugation of all detected bisphenols (BPA, BPS, and BPAF) across all three regions ($p < 0.001$), but overall, these bisphenols were predominantly present in their conjugated forms (Table S8). Details on individual bisphenol levels are discussed below.

3.4. BPA levels in South African human milk

The concentrations of total (free and conjugated) and free BPA in human milk from Vhembe ranged between <MDL-18.61 ng/mL and <MDL-7.83 ng/mL, respectively. The GMs for total BPA and free BPA in Vhembe were 0.55 ng/mL and 0.15 ng/mL, with medians of 1.03 ng/mL and 0.10 ng/mL, respectively. The detection frequency was 73% for total BPA and 53% for free BPA.

For Pretoria, the levels detected were comparable to those in the Vhembe district, with total BPA ranging between < MDL-19.38 ng/mL and free BPA from <MDL-7.78 ng/mL. The detection frequencies for total and free BPA in Pretoria were similar to those in Vhembe: 68% and 54%, respectively. The GMs for total and free BPA in Pretoria were 0.42 ng/mL and 0.20 ng/mL, with medians of 0.69 ng/mL and 0.17 ng/mL, respectively.

The levels for total BPA detected in the Vhembe district and Pretoria were in the same range as those reported elsewhere in the literature (Table 1): the medians were lower compared to Poland, but similar compared to the medians reported in the US and Korea and higher than the BPA levels reported in Spain, China and Canada. It is also important to note that the year when the human milk samples are collected will inevitably influence the levels of bisphenols that can be detected. To the best of our knowledge, the latest study for bisphenol A in US human milk was conducted by Hartle et al. with samples collected in 2015 (Hartle et al., 2018). The difference in time periods may lead to variations in BPA exposure across countries, making direct comparisons of BPA levels extremely challenging, if not impossible.

Given that free BPA is considered to be most biologically active (Ougier et al., 2021), the amount of free BPA that is present in human milk may be of biological significance in breastfeeding infants (Nachman et al., 2014). Free BPA % was above the limit of detection in 78% of the breast milk samples from Vhembe and 72% of the samples from Pretoria. The percent ratio of free to total BPA determined for each sample collected from the Vhembe district and Pretoria ranged between 0 and 100% (Fig. 3). The geometric means (GM) were 12% and 21%, and the medians were 22% and 52%, respectively (Table S9). In other words, BPA was mostly present in its conjugated form in South African samples.

To the best of our knowledge, only 2 papers have calculated the % free BPA content in human milk, a previous study conducted in Canada and one from Spain (Dualde et al., 2019; Cao et al., 2015). The GM and medians for % free BPA in Vhembe and Pretoria samples were relatively low compared to the values reported in human milk from both Spain and Canada (Table S9). Country differences in the amount of free BPA detected may be due to many factors, such as the diet, environment and life style of the mothers (Ougier et al., 2021; Anderson et al., 2012). For instance, Dualde et al. reported that human milk from mothers who dyed their hair a week prior to the sampling had significantly higher free BPA levels compared to mothers who dyed their hair between a week and one month prior to the sampling (Dualde et al., 2019). Çiftçi et al. also reported higher BPA levels in mothers who consumed fast food at least

Table 1
Comparison of BPA, BPS and BPAF concentrations (ng/mL) in human milk (this study) with other papers.

Country	Sampling year	Instrument (Method)	MDL LOD (LOQ) (ng/mL)	Free bisphenol				Total (free and conjugated) bisphenol				Study and publication year
				Detection frequency (DF, %)	Range (ng/mL)	GM (ng/mL)	Median (ng/mL)	DF (%)	Range (ng/mL)	GM (ng/mL)	Median (ng/mL)	
BPA												
South Africa (Vhembe) n=194	2018–2019	HPLC-Q-TOF-MS/MS	MDL: 0.037 (0.123)	53	<MDL-7.83	0.15	0.10	73	<MDL-18.61	0.55	1.03	This study
South Africa (Pretoria) n=193	2018–2019	HPLC-Q-TOF-MS/MS	MDL: 0.037 (0.123)	54	<MDL-7.78	0.20	0.17	68	<MDL-19.38	0.42	0.69	This study
Canada (Montreal) n=207	2018–2019	HPLC-Q-TOF-MS/MS	MDL: 0.037 (0.123)	ND	ND	ND	ND	0.5	<MDL-0.34	<LOQ	<LOQ	This study
Spain n = 120	2015	HPLC-quadrupole-MS/MS	(0.10)	77.4	<LOQ-41	0.15	0.1	83	<LOQ-42	0.29	0.26	Dualde et al., 2019
Canada n = 278	2009–2011	GC-MS	MDL: 0.21[c]	16.5	0.036-2.3 [b]	0.11 [b]	0.10 [b]	25.9	0.036-2.5 [b]	0.13[b]	0.11 [b]	Cao et al., 2015
USA n = 20	NA	HPLC-quadrupole-MS/MS	LOD: 0.28	60	<LOD-6.3	1.3 [c]	0.40	90	<LOD-7.3	1.9[c]	1.1	Ye et al., 2006
USA n = 21	2015	GC-MS	LOD: 0.1	100	0.80-42.2[b]	9.60 [c] ng/g milk	6.5[b]	NA	NA	NA	NA	Hartle et al., 2018
Korea n = 127	2011–2012	HPLC- MS/MS	LOD: 0.30	NA	NA	NA	NA	80	<LOD-43.2	0.85	0.74	Lee et al., 2018
China n = 181	2014	UHPLC- quadrupole-MS/MS	LOD: 0.017 (0.05)	NA	NA	NA	NA	72.9	<LOD-5.86	0.44	0.26	Niu et al., 2021
Poland n = 20	NA	LCMS- quadrupole	LOD: 0.19 (0.64)	71.4	<LOQ-4.85	1.62 [c]	1.48	76.2	<LOQ-4.86	1.91 [c]	1.90	Czarczyńska-Goślińet al., 2021
China n = 190	2018–2019	UPLC- quadrupole-MS/MS	LOD: 0.20	NA	NA	NA	NA	53	<LOD-15	2.5	53	Jin et al., 2020
BPS												
South Africa (Vhembe) N=194	2018–2019	HPLC-Q-TOF-MS/MS	MDL: 0.002 (0.007)	53	<MDL-0.53	NA	NA	57	<MDL-1.62	NA[a] (Not applicable)	<LOQ	This study
South Africa (Pretoria) n=193	2018–2019	HPLC-Q-TOF-MS/MS	MDL: 0.002 (0.007)	20	<MDL-0.48	<LOQ	<LOQ	21	<MDL-0.40	NA[a]	<LOQ	This study
Canada (Montreal) n=207	2018–2019	HPLC-Q-TOF-MS/MS	MDL: 0.002 (0.007)	42	<MDL-1.42	<LOQ	<LOQ	42	<MDL-4.42	NA[a]	<LOQ	This study
Spain n = 120	2015	HPLC- quadrupole-MS/MS	(0.25)	NA	NA	NA	NA	1.1	<LOQ-0.37	NA	NA	Dualde et al. (2019)
China n = 181	2014	UPLC- quadrupole-MS/MS	LOD: 0.003 (0.010)	NA	NA	NA	NA	46.4	<LOQ-0.453	0.027 [c]	<LOQ	Niu et al. (2021)
Poland n = 20	NA	LCMS- quadrupole	LOD: 0.01 (0.03)	95.2	<LOQ-0.40	0.08 [c]	0.07	100	<LOQ-0.84	0.06	0.05	Czarczyńska-Goślińet al., 2021
China n = 190	2018–2019	UPLC- quadrupole-MS/MS	LOD: 0.10	NA	NA	NA	NA	44	<LOD-1.30	0.19	<LOD	Jin et al. (2020)
BPAF												
South Africa (Vhembe) N=194	2018–2019	HPLC-Q-TOF-MS/MS	MDL: 0.0035 (0.012)	30	<MDL-1.79	NA[a]	<LOQ	40	<MDL-12.41	NA[a]	<LOQ	This study
South Africa (Pretoria) n=193	2018–2019	HPLC-Q-TOF-MS/MS	MDL: 0.0035 (0.012)	8	<MDL-0.11	NA[a]	<LOQ	8	<MDL-0.11	NA[a]	<LOQ	This study
Canada (Montreal) n=207	2018–2019	HPLC-Q-TOF-MS/MS	MDL: 0.0035 (0.012)	ND	<LOQ	<LOQ	<LOQ	ND	<LOQ	<LOQ	<LOQ	This study
China n = 181	2014	UPLC- quadrupole-MS/MS	LOD: 0.003 (0.010)	NA	NA	NA	NA	8.8	<LOQ-0.615	NA	<LOQ	Niu et al. (2021)
Poland n = 20	NA	LCMS- quadrupole	LOD: 0.03 (0.10)	33.3	<LOQ-0.12	0.07 [c]	0.05	38.1	<LOQ-0.14	0.06 [c]	0.05	Czarczyńska-Goślińet al., 2021
China n = 190	2018–2019	UPLC- quadrupole-MS/MS	LOD: 0.060	NA	NA	NA	NA	21	<LOD-0.58	0.092	<LOD	Jin et al., 2020

[a] The geometric mean for total (free and conjugated) BPS and BPAF for Vhembe and Pretoria was not calculated since the overall detection frequency was below 60%.
[b] *(ng/g milk).*
[c] Mean bisphenol value (ng/mL). LOD: Limit of detection. LOQ: Limit of quantification. MDL: Method detection limit. NA: Not applicable. ND: Not detected.

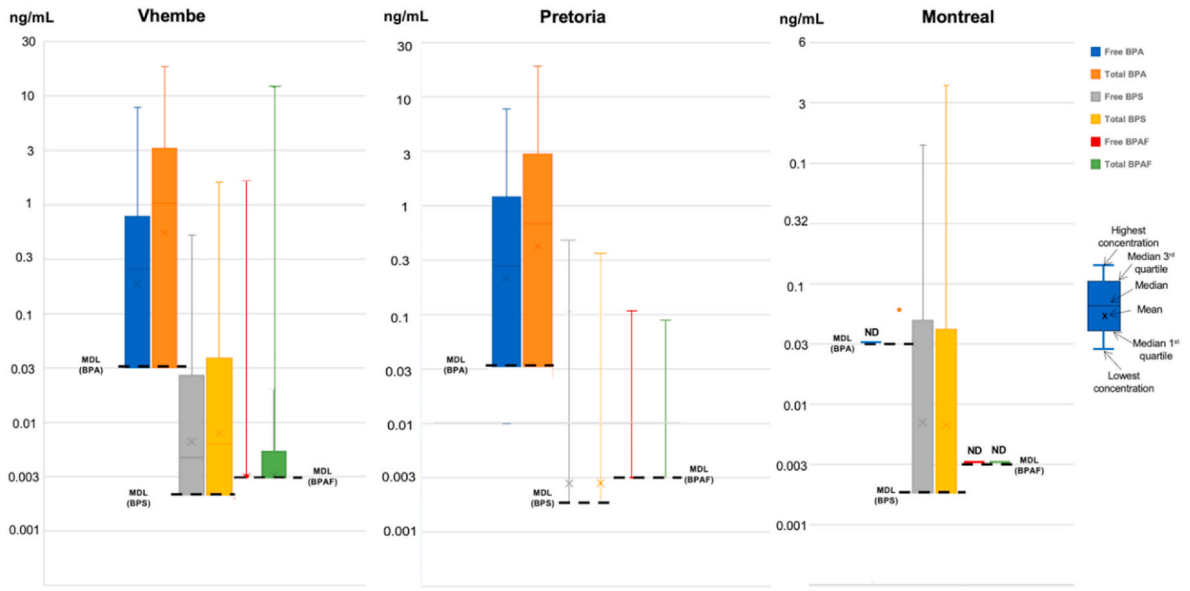

Fig. 2. Levels of bisphenols in human milk from South Africa (Vhembe and Pretoria) and Montreal (ng/mL)*
*Values below the MDL are not reported in Fig. 2. Total levels refer to the sum of free and conjugated bisphenols.

once a month compared to those who did not consume fast food (Çiftçi et al., 2021). Additional factors may include variability in the levels of drug metabolizing enzymes, such as the UDP-glucuronosyltransferases, or the milk collection period of breastfeeding mothers (Jalal et al., 2018; Yang et al., 2017; Yalcin et al., 2016). Additional investigations are warranted to better understand the difference in free BPA content observed among countries.

3.5. BPS levels in South African and Montreal human milk

BPS residues were detected in samples from Vhembe, Pretoria and Montreal. The levels of free BPS detected in Montreal, with concentrations ranging from <MDL to 1.42 ng/mL, were higher than the levels detected in Vhembe and Pretoria, ranging from <MDL to 0.53 ng/mL and <MDL to 0.48 ng/mL, respectively. The levels of free and conjugated (total) BPS detected in Montreal, ranging from <MDL to 4.42 ng/mL, were also higher than those detected in Vhembe and Pretoria, which ranged from <MDL to 1.62 ng/mL and <MDL-0.40 ng/mL, respectively. The medians for free and total BPS were below the LOQ in all three regions. The detection frequencyfor free and total BPS in Vhembe was comparable to that in Montreal samples; samples from Pretoria had the lowest detection frequency.

Given that the overall detection frequency was below 60% across all three regions, a calculation of the geometric mean for BPS levels was not done. This choice aligns with the approach adopted by the methodology of the NHANES (National Health and Nutrition Examination Survey) for geometric mean computation (CDC, 2022).

A comparison with other studies (Table 1) shows that the levels of free and conjugated (total) BPS detected in Vhembe and Montreal were higher than those reported in most papers, with Montreal having the highest detected levels. A recent study conducted by Jin et al. reported detection frequencies and BPS concentrations in Hangzhou, China, similar to those in Vhembe, but lower than those detected in Montreal (Jin et al., 2020). Conversely, the levels of BPS detected in Pretoria were similar to those previously reported in studies from China (9 Provinces), Spain, and Poland (Dualde et al., 2019; Niu et al., 2021; Czarczyńska-Goślińet al., 2021).

Similar to BPA, BPS is used in the production of food containers and packaging and can migrate into food and accumulate in the body through dietary exposure (Thoene et al., 2020). Studies have reported that the estrogenic effects of BPS are similar to those of BPA, highlighting its importance as a bisphenol analogue to include in future biomonitoring studies (Moreman et al., 2017).

3.6. Levels of BPAF and other bisphenols in South African and Montreal human milk

The free and total (free and conjugated) BPAF concentrations for Vhembe ranged between < MDL to 1.79 ng/mL and

<MDL to 12.41 ng/mL, respectively. Free and total BPAF were detected in 30% and 40% of the samples from Vhembe, respectively, with medians below the LOQ. In Pretoria, the levels of free and total BPAF were much lower, ranging between < MDL to 0.11 ng/mL, with detection frequencies of 8% for both. BPAF levels were below the limit of detection for all Montreal human milk samples. The geometric mean was not calculated since the low overall detection frequency in all 3 regions was lower than 60%.

A comparison of the levels of total BPAF reported here with those in other studies showed that the total BPAF levels in Vhembe milk were higher than those reported in China and Poland (Table 1). In contrast, the total BPAF levels detected in Pretoria samples were lower than the levels reported in China and similar to the total BPAF levels reported in Poland. The identification of BPAF in South African human milk samples emphasizes the importance of monitoring for unexpected bisphenol-related compounds. Further research and surveillance are necessary to better understand the sources, exposure pathways and potential health effects associated with BPAF exposure in South Africa, China and Poland.

None of the other six bisphenols analyzed in the present study were detected in either South African or Montreal human milk. However, studies in China (Hangzhou) and Spain have reported levels of BPF in their milk samples (Dualde et al., 2019; Jin et al., 2020); one study on human milk collected from 9 different provinces in China in 2014 reported traces of BPE and BPAP at maximum levels of 0.025 ng/mL and 0.10 ng/mL, respectively (Niu et al., 2021). It is possible that the production and usage of these and other BP analogues has increased in subsequent years, highlighting the need for continuous monitoring of these compounds in human milk.

3.7. Research implications

Our study is the first to detect BPA levels in South African human

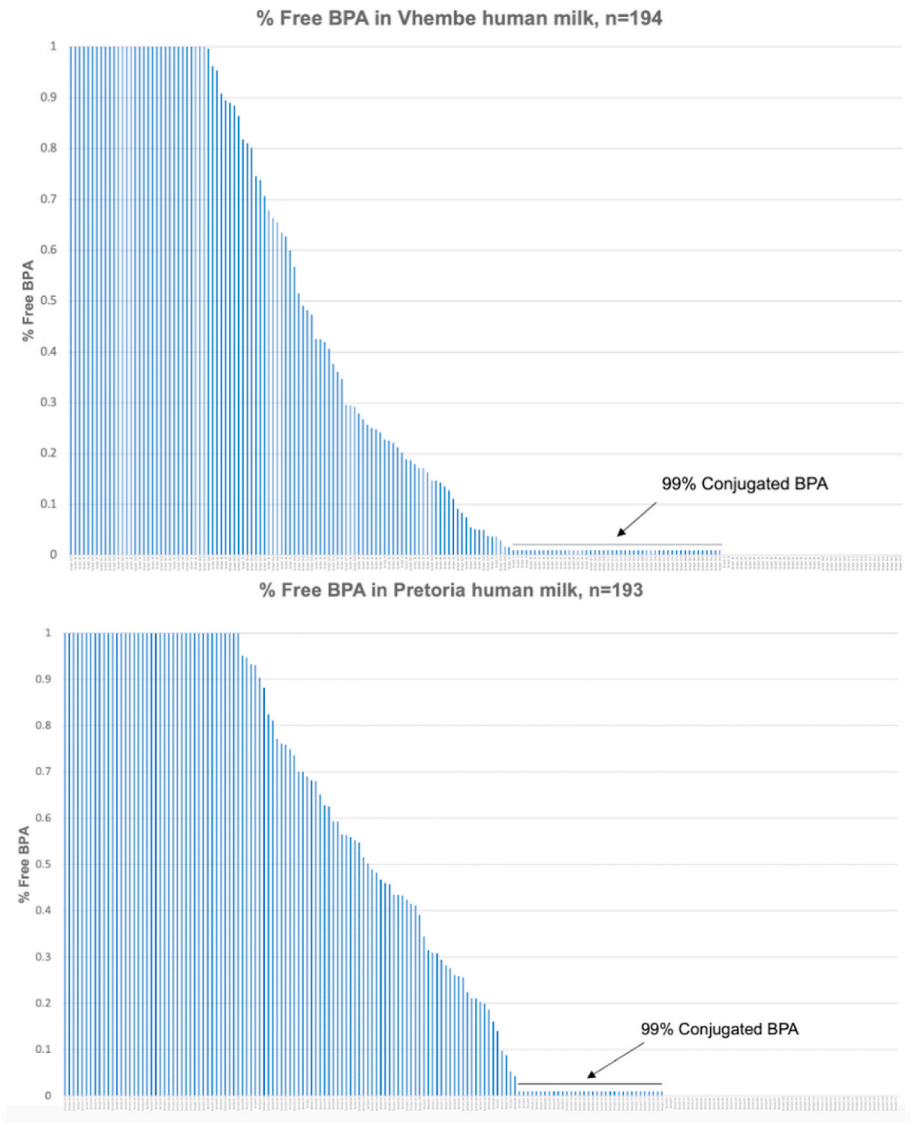

Fig. 3. % Free BPA distribution in human milk from Vhembe and Pretoria.

milk, the first to report a noticeable decrease in BPA and the presence of BPS in Canadian milk samples, and one of the few studies to identify BPAF in human milk. Our results highlight the importance of regular monitoring and assessment of BPA and its replacements, particularly in countries with limited data availability. Our findings from South Africa and Montreal also confirm variations in the levels of bisphenols detected in human milk samples among different countries. The observed decrease in BPA usage in Canada that our data suggest is further supported by a recent study from Health Canada which reported a significant decrease of 43% in BPA concentrations in urine samples collected from the Canadian population between 2007-2009 and 2017–2018 (Health Canada et al., 2021). Another study by Cao et al. reported low levels of BPA in Canadian human milk samples collected between 2009 and 2011 (Cao et al., 2015). Interestingly, a recent study reported no traces of any type of bisphenol in Montreal drinking water (Struzina et al., 2022).

The presence of BPS in Montreal human milk may indicate a more extensive usage of BPS in products that are available on the Canadian market. This suggestion is reinforced by the data from a recent Montreal study in which Tian et al. (2022) reported that BPS was prevalent in food samples. A subsequent study revealed that a variety of bisphenol-related chemicals, including BPS, were present in thermal labels and could be transferred to food (Xu et al., 2023); other major bisphenols (BPF, BPAF) appeared to be used less frequently than BPS based on these analyses.

In contrast to Montreal, the high levels of BPA in South African human milk suggest that BPA is still used there in large quantities. Detection of BPS and BPAF in South Africa may indicate that different bisphenol analogues are in use there currently. A number of studies have reported high levels of BPA in water samples collected in South Africa, from surface, treated drinking water, water in storage containers, as well as water from wastewater treatment plants (Rotimi et al., 2021). BPA has also been detected in South African foods, most notably in packaged vegetable composites and canned tuna (Tian et al., 2022). Thus, multiple sources of BPA may contribute to the high levels detected in South African human milk. Further investigations are required to better understand the different sources contributing to the high levels of BPA in South Africa.

4. Conclusion

In conclusion, plastic-related contaminants, such as bisphenols, pose potential health risks to both mothers and infants through exposure. The present study aimed to evaluate and compare the levels of bisphenols detected in human milk samples from South Africa and Montreal, with the objective of enhancing current human milk biomonitoring programs. Our findings revealed that BPA and BPAF were predominantly

detected in South African human milk samples, while BPS was the only detectable bisphenol in Montreal human milk, indicating observable differences in the types and levels of bisphenols in the respective populations. These variations may be attributed to factors such as environmental influences and maternal diet. These results highlight the importance of regular monitoring and assessment of BPA and its replacements, particularly in countries with limited data availability. They also call for the ongoing need for research and surveillance to safeguard the well-being of both breastfeeding mothers and breastfeeding infants. In future studies, we plan to correlate the levels of detected bisphenols with the sociodemographic, lifestyle and dietary habits of the donors. Our goal is to identify specific sources that may contribute to the accumulation of different bisphenols in human milk by conducting statistical analyses based on questionnaires provided to the participating mothers.

CRediT authorship contribution statement

Zhi Hao Chi: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lan Liu:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Jingyun Zheng:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Lei Tian:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Jonathan Chevrier:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Riana Bornman:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Muvhulawa Obida:** Writing – review & editing, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Cynthia Gates Goodyer:** Writing – review & editing, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Barbara F. Hales:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Stéphane Bayen:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.123730.

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