Supplementary Information for:

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

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Supplementary information
This file contains Supplementary Figures S1-S17 and Tables S1-S6.

Table of Contents

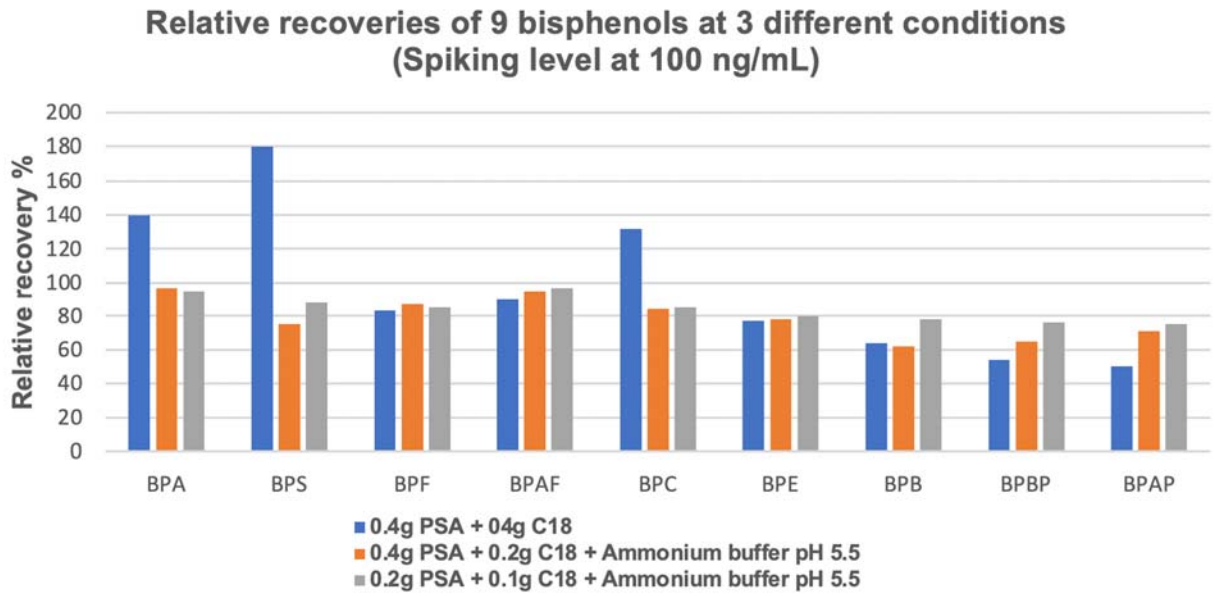
Section 1. QuEChERS Extraction Optimization

The recoveries of the analytes during QuEChERS extraction can be influenced by different factors including the amount of sample, the type and volume of the solvent, as well as the nature and quantity of the salt employed [1]. Another crucial factor when dealing with intricate matrices such as human milk is the clean-up step. This step involves the removal of undesirable interferences, including proteins, fatty acids, and other non-polar substances, by using common adsorbents such as PSA and C18 [2].

In the optimization of QuEChERS extraction for this study, two primary parameters were examined to assess variations in the recoveries of selected bisphenol compounds. This involved comparing recoveries with different amounts of PSA and C18 during the cleanup step, as well as assessing recoveries after the addition of an ammonium acetate buffer solution. Ammonium-buffered QuEChERS, with a pH of approximately 5, is recognized for its effectiveness in extracting pesticide residues across various matrices [3-5]. In this study, a pH of 5.5 was selected for the ammonium buffer solution to align with the optimum pH conditions for the enzymes used in this study (glucuronidase and sulfatase). This pH also corresponds to the optimal pH used in previous QuEChERS studies for a wide range of analytes, including pesticides and pharmaceuticals [4, 6]. Other parameters, such as the amount of human milk, NaCl and MgSO4, and the type of solvent, remained constant.

An amount of 0.2 g of human milk was selected to avoid too much undesired interference during analysis. Two grams of NaCl was selected to enhance protein precipitation while one gram of $MgSO_4$ was selected to avoid major loss of acetonitrile during the extraction. Acetonitrile was chosen for its ability to extract analytes with varying polarities, its high selectivity, and its compatibility with liquid chromatography [7].

Among the two main parameters tested, three conditions were selected; bisphenol recoveries for each condition are presented in Figure S1.

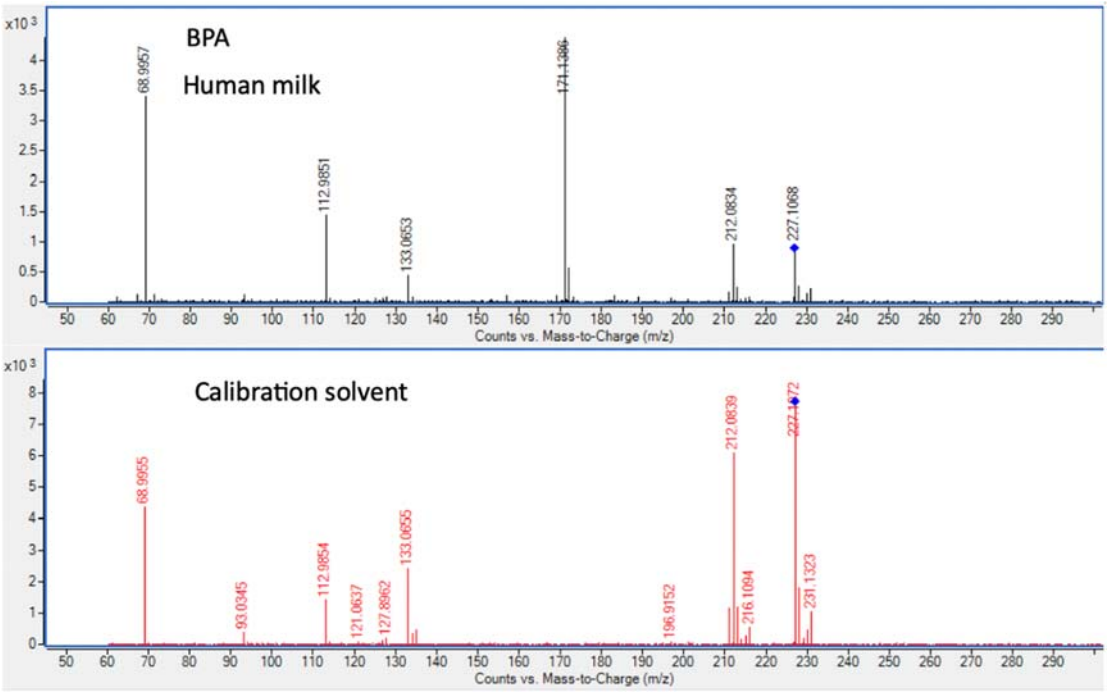


**Relative recoveries of BPC and BPBP obtained using $^{13}C_{12}$-BPF; Relative recoveries of BPE, BPB and BPAP obtained using $^{13}C_{12}$-BPAF*

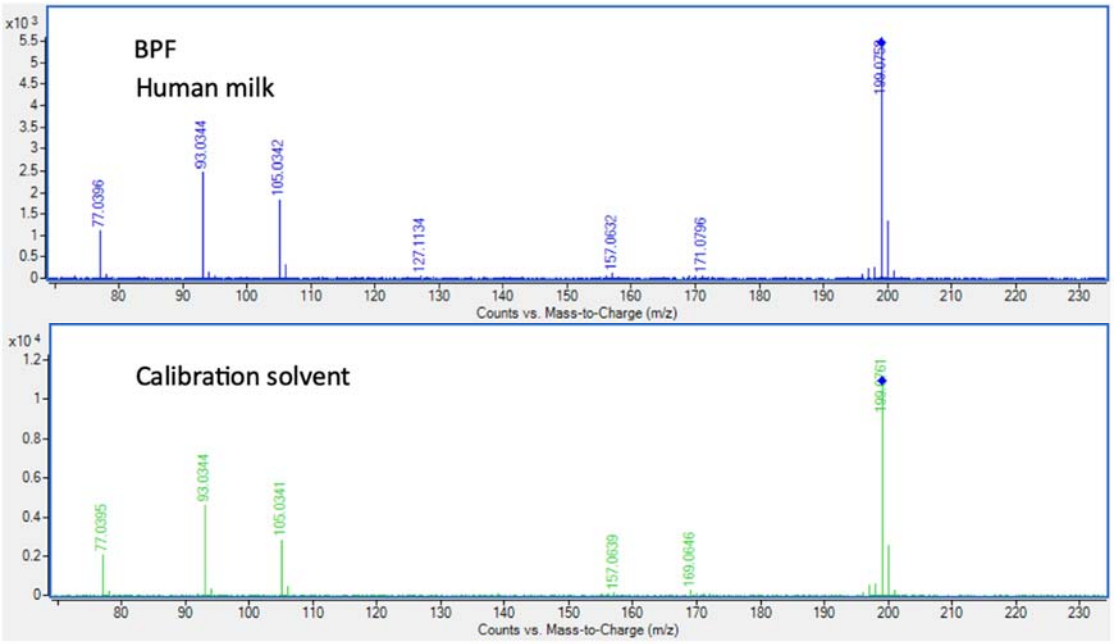
Figure S1. Relative recoveries of 9 selected bisphenol in human milk from three different conditions

Initially, using 0.4 g of PSA and C18 in the QuEChERS cleanup step did not yield satisfactory recoveries for bisphenols (ranging from 50% for BPAP to 180% for BPS) as shown in Figure S1. High relative recoveries were observed for BPA and BPS, while low recoveries were noted for compounds such as BPBP and BPAP. Subsequent modifications in the extraction were made to reduce the amount of C18 along with the addition of ammonium acetate buffer solution (pH 5.5). These adjustments resulted in improved bisphenol recoveries, ranging between 62% for BPB to 97% for BPA, demonstrating satisfactory results for seven out of nine bisphenols (Figure S1). Comparative analysis highlighted that the addition of ammonium acetate buffer enhanced the stability of many bisphenols within the acetonitrile phase, including BPA, BPS, and BPC, contributing to overall improved recoveries for all selected bisphenols in milk. For further

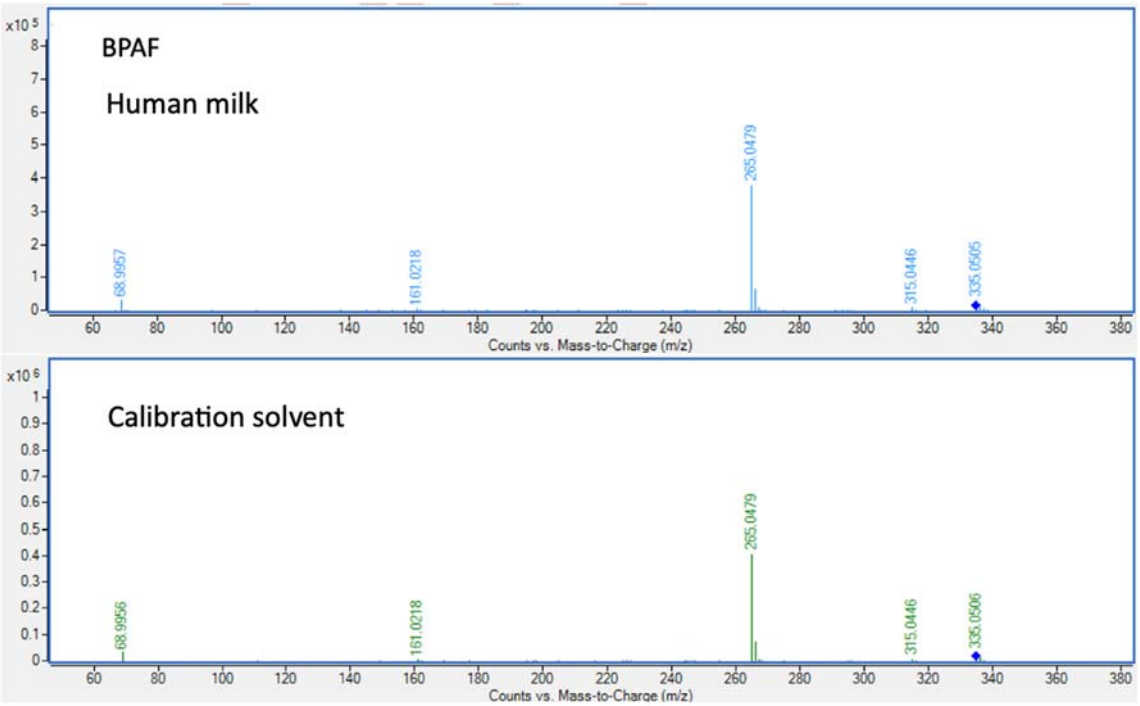
refinement, a third modification involved reducing the amount of PSA and C18 to 0.2 g and 0.1 g, respectively, to prevent excessive adsorption of bisphenols to cleanup powders. Results for this adjustment ranged between 95% to 75%, demonstrating satisfactory recoveries for all nine spiked bisphenols in milk. Consequently, the use of 0.2 g PSA and 0.1 g of C18, combined with the addition of ammonium buffer (pH 5.5), was determined to be the optimal extraction method for bisphenol analysis in human milk samples.



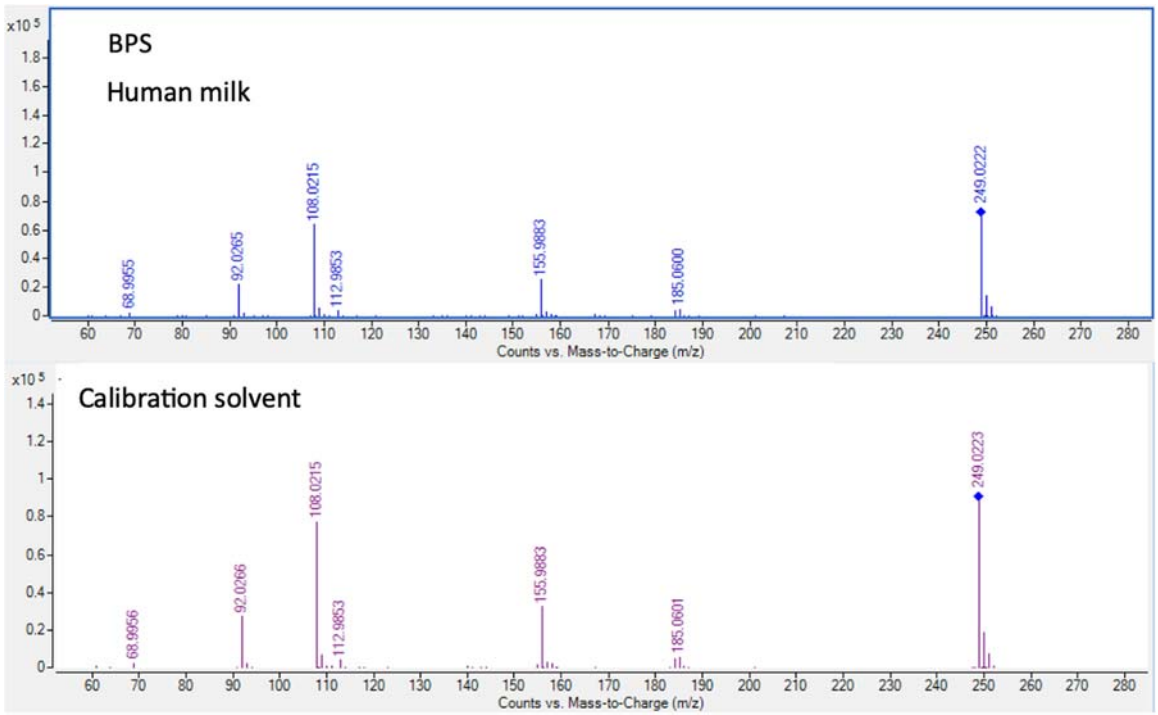
Supplemental Fig S2. Targeted MS/MS of BPA in human milk and pure calibration solvent



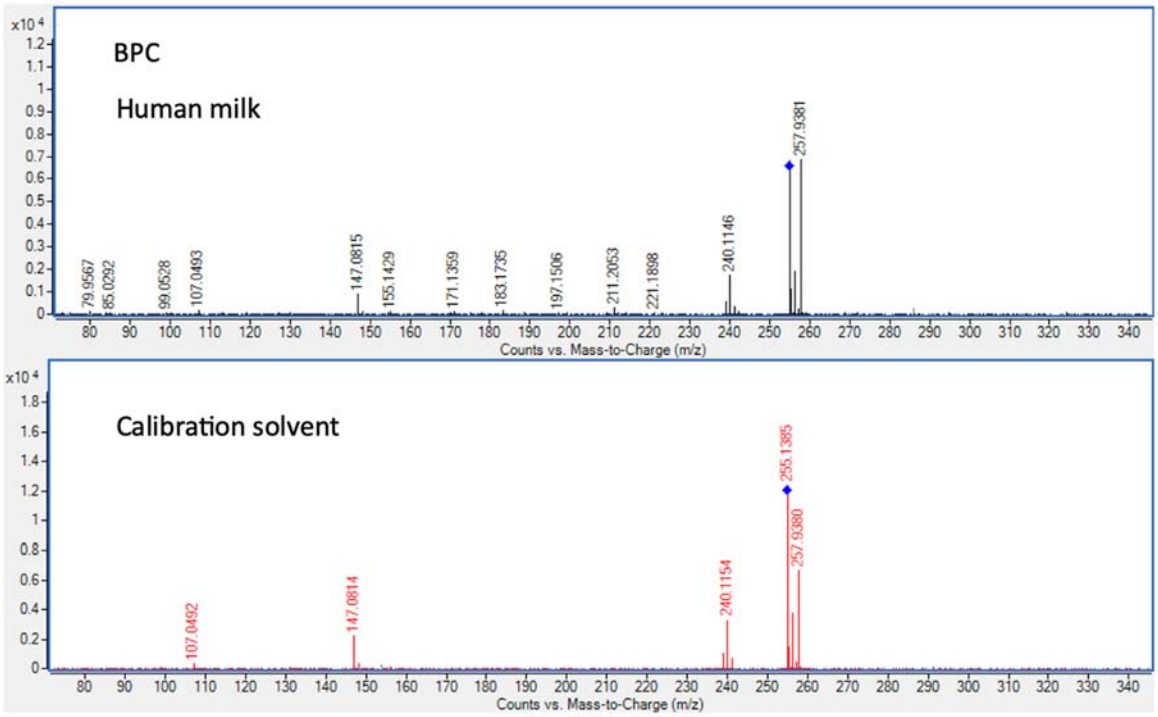
Supplemental Fig S3. Targeted MS/MS of BPF in human milk and pure calibration solvent



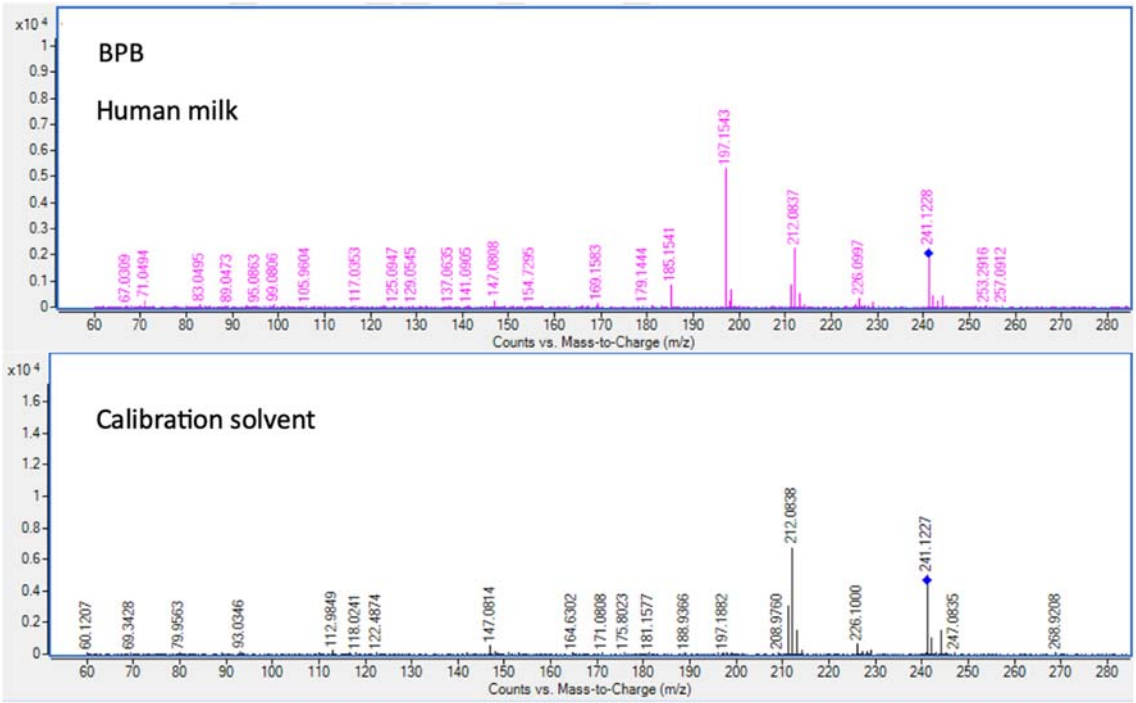
Supplemental Fig S4. Targeted MS/MS of BPAF in human milk and pure calibration solvent



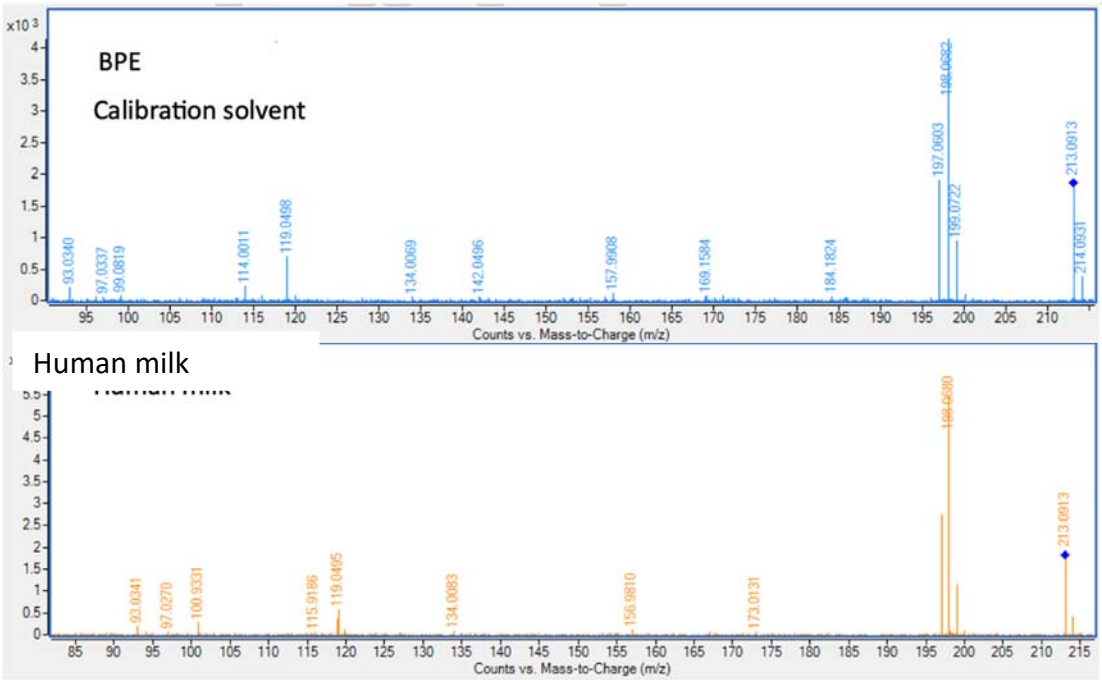
Supplemental Fig S5. Targeted MS/MS of BPS in human milk and pure calibration solvent



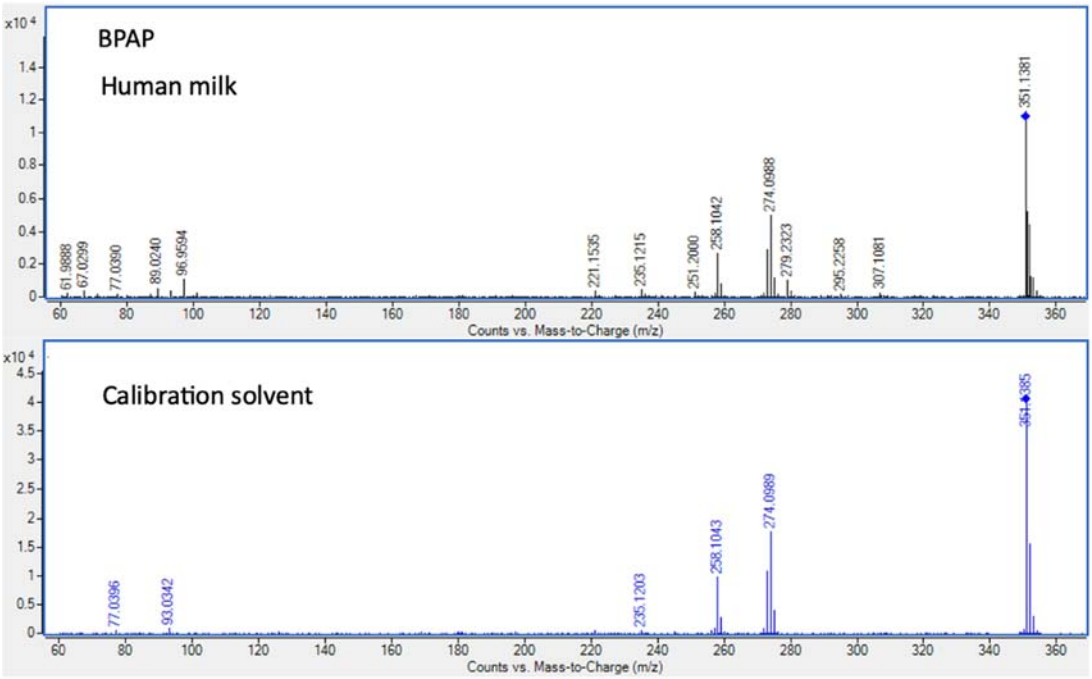
Supplemental Fig S6. Targeted MS/MS of BPC in human milk and pure calibration solvent



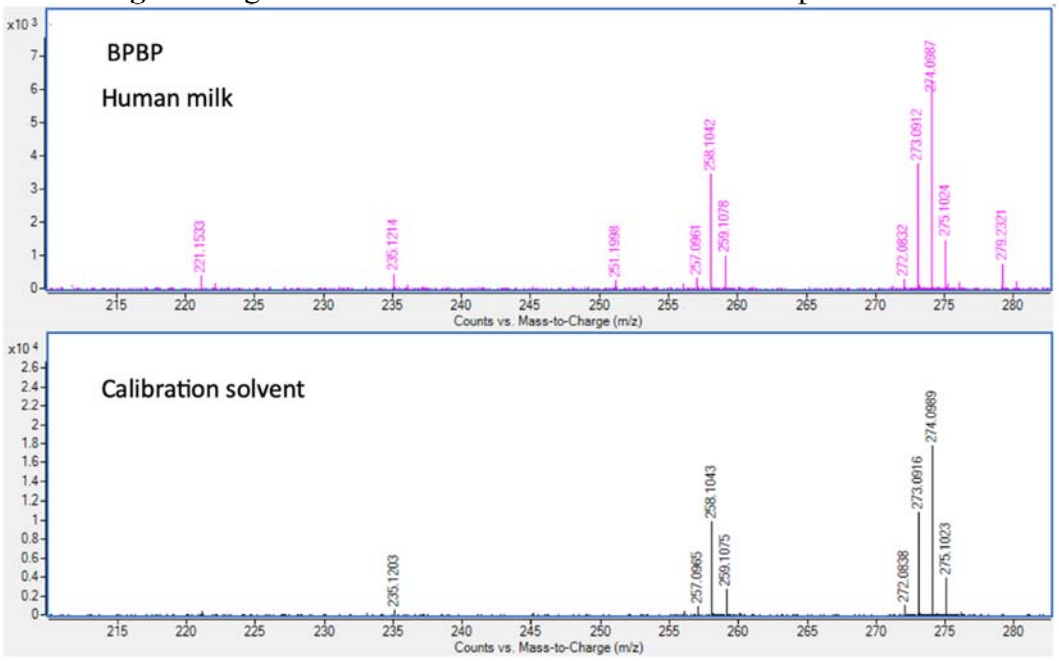
Supplemental Fig S7. Targeted MS/MS of BPB in human milk and pure calibration solvent



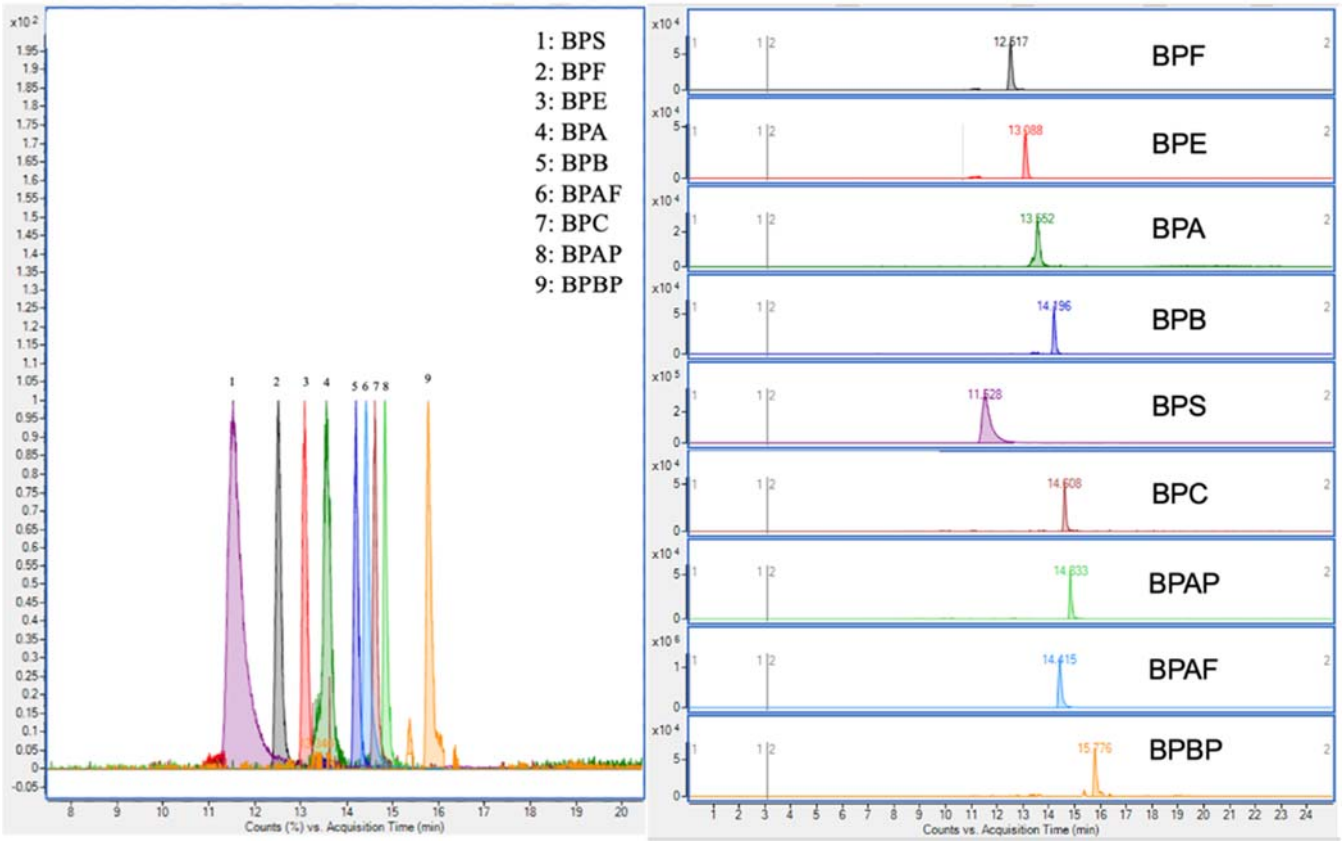
Supplemental Fig S8. Targeted MS/MS of BPE in human milk and pure calibration solvent



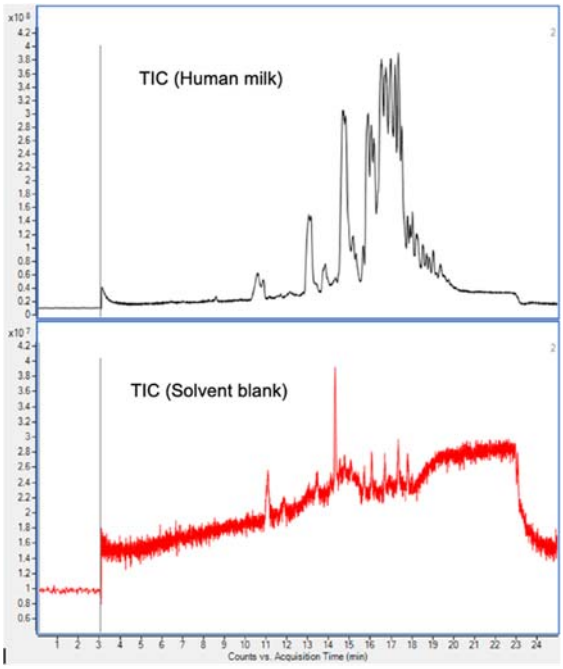
Supplemental Fig S9. Targeted MS/MS of BPAP in human milk and pure calibration solvent



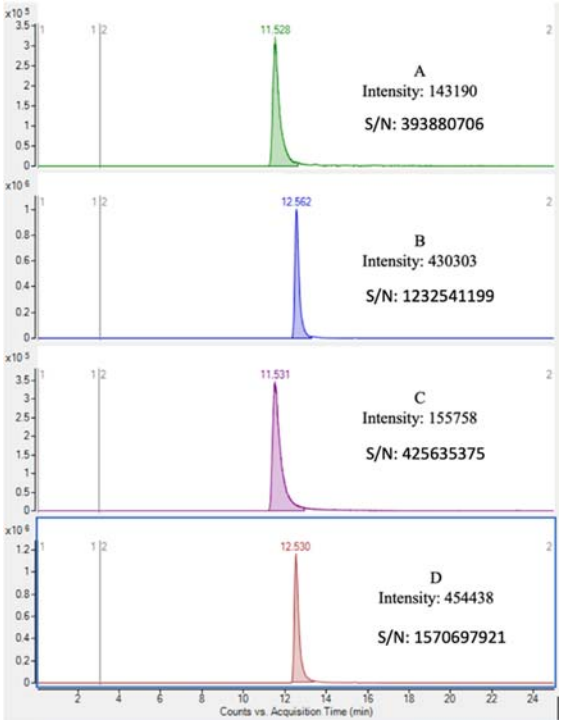
Supplemental Fig S10. Targeted MS/MS of tested analyte BPBP in human milk and pure calibration solvent



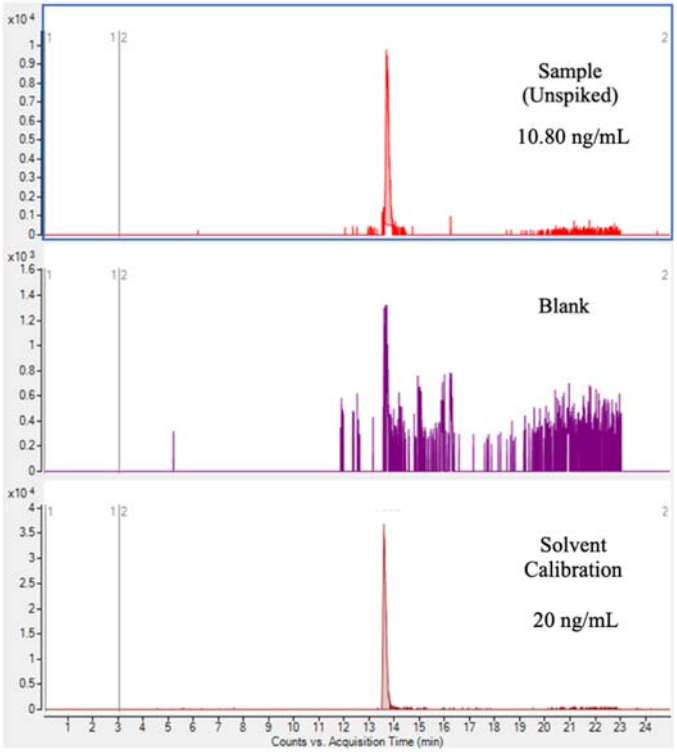
Supplemental Fig S11. Chromatographic peaks with normalized peak height and separated peaks for all 9 spiked bisphenols in human milk matrix at 30 ng/mL



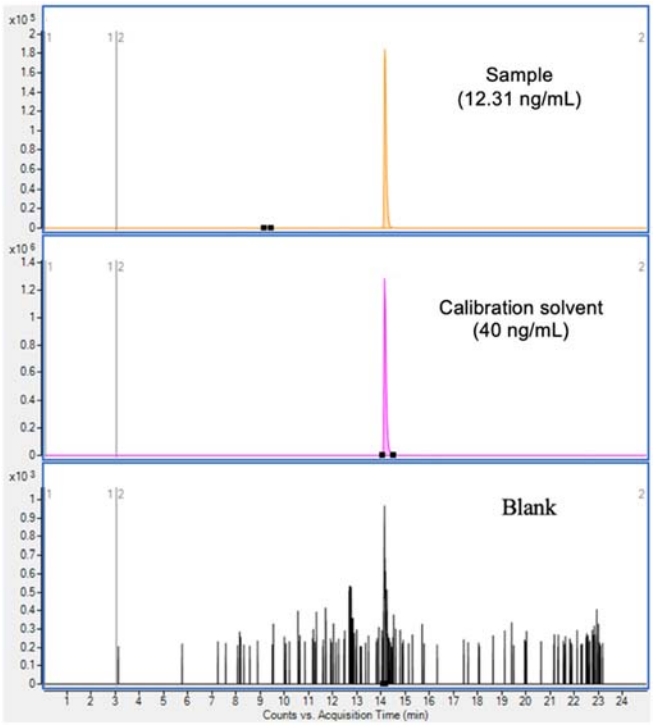
Supplemental Figure S12. Total ion chromatograms (TIC) for human milk and solvent blank (50% acetonitrile and water) with 20 µL injection volume



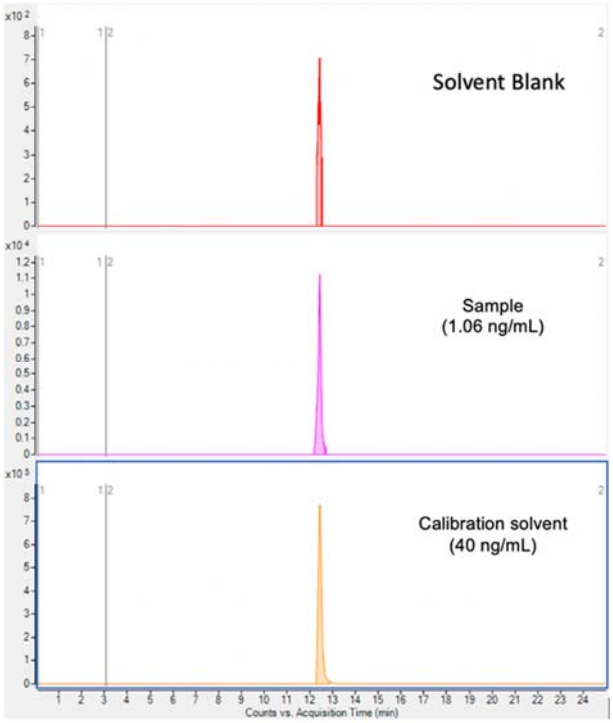
Supplemental Figure S13. Chromatographic peaks for the intensity of BPS in human milk matrix and solvent calibration in 2 different mobile phases at 30 ppb. A: 30 ppb spiked human milk sample using ACN and MeOH (1:1) as mobile phase B. B: 30 ppb spiked human milk sample using pure MeOH as mobile phase B. C: 30 ppb spiked calibration solvent using ACN and MeOH (1:1) as mobile phase B. D: 30 ppb spiked calibration solvent using MeOH as mobile phase B.



Supplemental Figure S14: Chromatographic peak for BPA in blank, unspiked human milk and calibration solvent with injection volume of 20 µL



Supplemental Figure S15: Chromatographic peak for BPAF in blank, unspiked human milk and calibration solvent



Supplemental Figure S16: Chromatographic peak for BPS in blank, unspiked human milk and calibration solvent

Table S1: Mean mass measurement errors (ppm) for six analytes in pure solvent and sample matrices

Compound	***m/z***	**Mass measurement error (ppm) of standards in pure solvent (n=6)**	**Mass measurement error (ppm) in human milk matrix (n=6)**
BPA	227.1072	2.20 ±0.9	2.79±0.7
BPF	199.0759	1.93±0.5	1.93±0.5
BPS	249.0222	0.80±0.3	0.63±0.8
BPAF	335.0506	0.89±0.6	1.04±0.7
BPC	255.1385	1.10±0.8	1.18±0.5
BPE	213.0916	1.96±0.2	2.11±0.3
BPAP	289.1229	1.15±0.2	1.21±0.5
BPB	241.1229	1.45±0.6	1.79±0.2
BPBP	351.1385	0.28±0.9	0.33±0.8

Table S2. The relative intensities of qualifier to quantifier ions for the 9 bisphenols in pure solvent and human milk matrix

Compound	Matrix	Base peak $[M-H]^-$ (m/z)	Qualifier 1			Qualifier 2			CE (V)
			m/z	Relative intensity %	Relative difference %*	m/z	Relative intensity %	Relative difference %*	
BPA	Solvent	227.1068	133.0653	31.0	-	212.0839	77.1%		20
	Milk			40.0	29.0		100.06%	29.8	
BPF	Solvent	199.0761	93.0344	41.8	-	105.0341	25.8		20
	Milk			41.4	-1.00		30.8	18.97	
BPS	Solvent	249.0222	108.0215	82.9	-	155.9883	34.7		20
	Milk			84.3	1.65		33.0	-4.73	
BPAF	Solvent	335.0506	265.0479	1252.64	-	315.0446	25.88	-	20
	Milk			1265.37	-1.00		24.75	-4.37	
BPC	Solvent	255.1380	240.1154	26.75	-	147.0814	18.28	-	20
	Milk			25.41	-4.98		13.33	-27.08	
BPE	Solvent	213.0913	198.068	281.94	-	119.0498	30.40	-	20
	Milk			292.71	3.82		36.31	19.44	
BPAP	Solvent	289.1227	248.0143	653.30	-	274.0995	224.44	-	20
	Milk			670.30	-2.55		240.09	6.98	
BPB	Solvent	241.1228	212.0837	110.28	-	147.0808	13.00	-	20
	Milk			135.32	-18.50		11.38	14.18	
BPBP	Solvent	351.1385	274.0989	42.85	-	258.1043	23.80	-	20
	Milk			44.25	3.28		24.00	0.57	

*Relative difference was calculated using the difference between relative intensity obtained in milk matrix and solvent divided by the relative intensity of the solvent

Table S3. Method performance including linearity (r^2 for the linear fit) using matrix-matched calibration (10 points) ranging between 0.5 to 100 (ng/mL) and matrix effect (n=3)

Compounds	Method validation human milk	
	Linearity r^2	Matrix effects
BPA	0.99	20%
BPF	0.99	29%
BPAF	0.99	19%
BPS	0.99	21%
BPE	0.99	33%
BPB	0.99	39%
BPAP	0.99	33%
BPBP	0.98	52%
BPC	0.99	29%

Table S4: ANOVA for South African and Montreal human milk solid content %

Regions

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.075	2	0.037	16.142	<.001
Within Groups	1.372	591	0.002		
Total	1.447	593			

	(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.
Bonferroni	Vhembe	Pretoria	-0.00337	0.00489	1
		Montreal	-.02512*	0.00482	<.001
	Pretoria	Vhembe	0.00337	0.00489	1
		Montreal	-.02174*	0.00482	<.001
	Montreal	Vhembe	.02512*	0.00482	<.001
		Pretoria	.02174*	0.00482	<.001

Table S5. Intra-day and inter-day relative recovery of all spiked BPs in homogenate human milk (n=3)

Analyte	Average intra-day relative recovery % (100 ng/mL)	Relative Standard Deviation (RSD%)	Average Intra-day relative recovery % (30 ng/mL)	RSD%	Average inter-day relative recovery % (30 ng/mL)	RSD%	Retention time difference: sample vs. solvent (min)
BPA	95	3.2	94	2.8	96	3.2	<0.05
BPS[a]	88	4.8	102	3.7	98	4.8	<0.05
BPF	85	4.7	97	2.2	91	1.2	<0.05
BPAF	97	1.3	96	1.0	98	1.3	<0.05
BPC[b]	85	3.4	-	-	-	-	<0.05
BPE[c]	80	5.5	-	-	-	-	<0.05
BPB[c]	78	4.8	-	-	-	-	<0.05
BPBP[b]	76	5.8	-	-	-	-	<0.05
BPAP[c]	75	6.5	-	-	-	-	<0.05

[a.] Relative recovery with *pure methanol in mobile phase B; [b.] Relative recovery using $^{13}C_{12}$-BPF; [c.] Relative recovery using $^{13}C_{12}$-BPAF*

Table S6: Average MDL and LOQ values for all 9 bisphenols including ranges for BPA, BPF, BPS and BPAF across each batch of human milk (n=20)

Bisphenols	Average MDL values (ng/mL)	MDL range (ng/mL)	Average LOQ values (ng/mL)	LOQ range (ng/mL)
BPA	0.037	0.030-0.045	0.123	0.10-0.15
BPF	0.032	0.028-0.055	0.108	0.093-0.183
BPAF	0.0035	0.002-0.006	0.012	0.0067-0.02
BPS	0.002	0.0012-0.012	0.007	0.004-0.04
BPC	0.042	-	0.14	-
BPE	0.079	-	0.26	-
BPB	0.017	-	0.06	-
BPBP	0.026	-	0.09	-
BPAP	0.049	-	0.16	-

Table S7. BPA levels (ng/mL) detected in three Vhembe milk samples with 1st and 2nd extractions conducted with a period of 1 year apart

Vhembe Sample (HSN)	No enzyme (1st extraction)	No enzyme (2nd extraction)	Enzyme (1st extraction)	Enzyme (2nd extraction)
S3 (141009)	0	0.10	8.53	8.04
S20 (141043)	6.07	7.21	5.67	7.09
S27 (141054)	7.83	8.64	9.57	10.62

Table S8. One sample t-test for % conjugated bisphenols (BPA, BPS and BPAF) for all 3 regions

One-Sample Statistics	N	Mean	Std. Deviation	Std. Error Mean
% Conjugated BPA Vhembe	152	0.6108	0.41034	0.03328
% Conjugated BPS Vhembe	145	0.4091	0.40616	0.03373
% Conjugated BPAF Vhembe	96	0.3537	0.38576	0.03937
% Conjugated BPA Pretoria	139	0.4797	0.40449	0.03431
% Conjugated BPS Pretoria	51	0.3241	0.42229	0.05913
% Conjugated BPAF Pretoria	40	0.3016	0.29341	0.04639
% Conjugated BPS Montreal	107	0.2905	0.38747	0.03746

One-Sample Test							
	Test Value = 0						
	t	df	Significance		Mean Difference	95% Confidence Interval of the Difference	
			One-Sided p	Two-Sided p		Lower	Upper
% Conjugated BPA Vhembe	18.351	151	<.001	<.001	0.61077	0.545	0.6765
% Conjugated BPS Vhembe	12.128	144	<.001	<.001	0.40909	0.3424	0.4758
% Conjugated BPAF Vhembe	8.983	95	<.001	<.001	0.35368	0.2755	0.4318
% Conjugated BPA Pretoria	13.981	138	<.001	<.001	0.47967	0.4118	0.5475
% Conjugated BPS Pretoria	5.481	50	<.001	<.001	0.32413	0.2054	0.4429
% Conjugated BPAF Pretoria	6.501	39	<.001	<.001	0.30161	0.2078	0.3954
% Conjugated BPS Montreal	7.754	106	<.001	<.001	0.29046	0.2162	0.3647

Table S9: % Free BPA in breast milk from Vhembe and Pretoria (South Africa) and other studies

Study	Country	Geometric mean %	Median %	Range %	Frequency of detection in total number of samples %	Number of samples analyzed
This study	*South Africa (Vhembe)*	12	22	0-100	78	194
This study	*South Africa (Pretoria)*	21	52	0-100	72	193
Dualde et al. (2019) [8]	*Spain*	57	70	16-100	83	120
Cao et al. (2015) [9]	*Canada*	57	70	7.9-100	25.9	278

References

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