Analysis of plastic-related chemical contaminants in human milk using targeted and non-targeted screening

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Abstract

Human milk, which is vital for infant growth and development, may contain various xenobiotics, highlighting the need for its comprehensive biomonitoring on a regular basis. While traditional targeted approaches are widely employed for monitoring contaminants in biological samples around the world, numerous unknown chemicals are often overlooked. Plastic-related contaminants (PRCs), ubiquitous in the environment, represent one of the most extensive contaminants detected in human milk; bisphenols are one of the dominant classes of PRCs that have been studied. Despite the abundance of studies, however, data are lacking with respect to the levels of these contaminants in certain matrices and geographical regions. Furthermore, the use of targeted analysis (TA) limits the detection of previously underreported or unknown PRCs (i.e. preservatives, UV filters, synthetic antioxidants), suggesting the need to develop innovative tools such as non-targeted analysis (NTA) coupled with high-resolution mass spectrometry to detect these substances.

The purpose of this study was to detect and identify the presence of classes of PRCs, focusing on bisphenols and parabens, that may be present in human milk. Specifically, the goal was to quantitatively assess and compare bisphenol types and levels across regions, to identify related unknowns along with other common and unusual parabens, as well as to evaluate their conjugation potential in human milk by employing both TA and NTA. There is a detailed review of all detected environmental contaminants in human milk, as well as highlights of all previous studies conducted on PRCs in Chapter 2. Limitations of the predominant use of TA, obstructing risk assessment and toxicity evaluation for chemicals present in human milk in terms of chemical mixtures, are also described. Evidently, there is a need for further data to fill data gaps with respect to the PRCs, such as bisphenols, in specific countries. To address this, bisphenols were detected in human milk from

Canada and South Africa (Vhembe and Pretoria), a country where data are scarce (Chapter 3). An efficient QuEChERS extraction method was developed for the TA of 9 selected bisphenols in human milk. BPA was the predominant bisphenol detected in South African human milk, followed by BPS and BPAF. BPS was the exclusive bisphenol detectable in milk from Montreal, suggesting differences in exposure to bisphenols in these two countries and providing crucial insights for future investigations by health officials. The TA of human milk in Chapter 3 also reinforced the need for NTA as a valuable emerging tool to detect and identify different unknown contaminants. A customized database library for the detection of bisphenol-related unknowns using NTA is introduced in Chapter 4. Successful workflow implementation, with the extracted data using the same extraction method as in Chapter 3, was used to identify different bisphenol S related-unknowns that are used in thermal labels, along with different synthetic antioxidants and UV absorbers. Among these compounds, 2 synthetic antioxidants-related unknowns (including 1 metabolite) have not been reported previously in human milk studies.

In Chapter 5, the use of NTA is extended for the identification of common and unusual parabens, along with other PRCs of interest. Seven parabens, various phthalate metabolites, and per- and polyfluoroalkyl substances were detected in human milk, including a unique paraben exclusive to South Africa. The detection of these different and unexpected PRCs highlights the usefulness of applying NTA in human milk biomonitoring.

Together, this research has demonstrated that integrating TA with NTA in human milk biomonitoring can facilitate the detection of unexpected contaminants and is both cost effective and time efficient. This research also emphasizes the significance of applying NTA for the detection of family-specific contaminants, providing regulatory agencies with essential information on their presence for regular human milk biomonitoring.

Résumé

Le lait maternel, essentiel à la croissance et au développement des nourrissons, peut contenir divers xénobiotiques, soulignant la nécessité d'une biosurveillance complète à intervalles réguliers. Alors que les approches ciblées traditionnelles sont largement utilisées pour surveiller les contaminants dans les échantillons biologiques à travers le monde, de nombreux produits chimiques inconnus sont souvent négligés. Les contaminants liés aux plastiques (CLPs), omniprésents dans l'environnement, représentent l'un des contaminants les plus étendus détectés dans le lait maternel incluant les bisphénols qui sont l'une des classes dominantes des CLPs étudiées. Cependant, malgré l'abondance des études, il y a un manque de données en ce qui concerne les niveaux de ces contaminants dans certaines matrices et régions géographiques. De plus, l'utilisation de l'analyse ciblée limite la détection des CLPs précédemment sous-rapportés ou inconnus (c'est-à-dire les conservateurs, les filtres UV, les antioxydants synthétiques), ce qui suggère la nécessité de développer des outils innovants tels que l'analyse non-ciblée (ANC) couplée à la spectrométrie de masse à haute résolution pour détecter ces substances chimiques.

Le but de cette étude était de détecter et d'identifier la présence de classes spécifiques de CLPs, en particulier les bisphénols et les parabènes, qui peuvent être présents dans le lait maternel. Plus précisément, l'objectif était d'évaluer quantitativement et de comparer les types et les niveaux de bisphénols à travers différentes régions, d'identifier les composés inconnus associés aux bisphénols ainsi que d'autres parabènes courants et inhabituels, et d'évaluer leur potentiel de conjugaison dans le lait maternel en utilisant à la fois l'analyse ciblée et l'ANC.

Le chapitre 2 contient une revue détaillée de tous les contaminants environnementaux détectés dans le lait maternel mettant en évidence toutes les études précédentes menées sur les CLPs. Les limitations concernant l'utilisation prédominante de l'analyse ciblée obstruant l'évaluation des

risques et l'évaluation de la toxicité pour les produits chimiques présents dans le lait maternel en termes de mélanges chimiques sont également décrites. Il est évident qu'il est nécessaire d'obtenir de données supplémentaires pour combler les lacunes concernant les CLPs, tels que les bisphénols, dans certains pays. Pour ce faire, les bisphénols ont été détectés dans le lait maternel du Canada (Montréal) et d'Afrique du Sud (Vhembe et Pretoria), un pays où les données sur les bisphénols sont rares (chapitre 3). Une méthode d'extraction QuEChERS efficace a été développée pour l'analyse ciblée de 9 bisphénols dans le lait maternel. BPA était le bisphénol prédominant détecté dans le lait maternel sud-africain, suivi du BPS et du BPAF. Le BPS était le bisphénol exclusif détectable dans le lait venant de Montréal, suggérant des différences à propos de l'utilisation des bisphénols entre ces deux pays et fournissant, en même temps, des informations cruciales pour les futures enquêtes qui vont être menées par les responsables de la santé. L'analyse ciblée effectuée sur le lait maternel au Chapitre 3 a renforcé également la nécessité d'utiliser l'ANC en tant qu'outil émergent pour détecter et identifier différents contaminants inconnus.

Le Chapitre 4 introduit l'utilisation d'une bibliothèque de base de données personnalisée pour la détection des inconnus liés aux bisphénols en utilisant l'ANC. La mise en œuvre réussie du flux de travail, avec les données extraites en utilisant la même méthode d'extraction que dans le chapitre 3, a été utilisée pour identifier différents inconnus liés au bisphénol S qui sont utilisés dans les étiquettes thermiques, ainsi que différents antioxydants synthétiques et absorbeurs UV. Parmi ces composés, 2 inconnus liés aux antioxydants synthétiques (dont 1 métabolite) n'ont pas été rapportés dans les études antérieures sur le lait maternel.

Dans le chapitre 5, l'utilisation de l'ANC est étendue pour l'identification des parabènes courants et inhabituels, ainsi que d'autres CLPs d'intérêt. Sept parabènes, divers métabolites de phtalate, et différentes substances per- et polyfluoroalkylées ont été détectés dans le lait maternel, y compris

un parabène exclusif à l'Afrique du Sud. La détection de ces différents CLPs inattendus souligne l'importance d'utiliser l'ANC dans le cadre de la biosurveillance sur le lait maternel. Ensemble, cette recherche a démontré que l'intégration de l'analyse ciblée avec l'ANC dans les études sur le lait maternel peut faciliter la détection des CLPs inconnus ou inattendus, et est considérée comme efficace en termes de temps et de coût. Cette étude souligne également l'importance d'appliquer l'ANC pour la détection des familles spécifiques de contaminants dans le cadre de la biosurveillance régulière sur le lait maternel afin de fournir aux agences de réglementation des informations cruciales sur leur présence.

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Contribution of authors

This thesis is presented in a manuscript form and comprises of six chapters. Chapter 1 is a general introduction of different xenobiotics present in human milk and the importance for monitoring certain xenobiotics such as PRCs (plastic-related contaminants) using both targeted and non-targeted analysis. Chapter 2 is presented in the form of a literature review which summarizes the current findings of different families of chemical contaminants and knowledge gaps of surrounding human milk biomonitoring. A connecting text is made to Chapter 3 which describes the current knowledge gaps regarding current human milk biomonitoring and the importance of analyzing PRCs in milk. Then, Chapter 3 to Chapter 5 are prepared in a manuscript form and are also arranged sequentially through connecting texts. The review article for Chapter 2 (Characterization of different contaminants and current knowledge for defining chemical mixtures in human milk: A review) was published in the journal *Environment International.* A manuscript reporting the data in Chapter 3 (Biomonitoring of bisphenol A and bisphenol analogues in human milk from South Africa and Canada using a modified QuEChERS extraction method) was published in the journal of *Environmental Pollution*. Chapter 4 (Non-targeted analysis of bisphenol A (BPA) structural analogues and functional alternatives with semi-quantification of bisphenol-related unknowns in human milk from Canada and South Africa) was submitted for publication in the journal of *Environmental Science and Technology.* Chapter 5 (Investigation of plastic-related contaminants in human milk: identification of common and unreported parabens using non-targeted strategies) will be submitted for publication in the journal of *Science of the Total Environment*. Finally, Chapter 6 presents an overall conclusion of the thesis as well as some recommendations for future research.

The present author was responsible for the concepts, the design of experiments, experimental work, data acquisition, data treatment and manuscript preparation for all the manuscripts. Drs. Stéphane Bayen & Barbara Hales, the thesis supervisors and the co-authors for all the manuscripts, had direct advisories for all the experimental design and the manuscripts. Drs. Jonathan Chevrier, Cindy Goodyer and Barbara F. Hales were responsible for the supervision on the ethic and research side as well as the collection, packaging of all human milk samples collected in Montreal (Canada) and South Africa (Vhembe and Pretoria) in Chapter 3: Biomonitoring of bisphenol A (BPA) and bisphenol analogues in human milk from South Africa and Canada using a modified QuEChERS extraction method, Chapter 4 (Non-targeted analysis of bisphenol A (BPA) structural analogues and functional alternatives with semi-quantification of bisphenol-related unknowns in human milk from Canada and South Africa) and Chapter 5 (Investigation of plastic-related contaminants in human milk: identification of common and unreported parabens using non-targeted strategies).

Jing Yun Zheng collected, and tested the amber glass jars for any contamination, and freeze-dried every collected human milk sample, then transferred the milk to those glass jars, and is a co-author of Chapter 3, Chapter 4 and Chapter 5. Dr. Hales and Dr. Goodyer contributed to the editing and correction of Chapter 2.

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Publications

- Chi, Z.H., et al., ***Characterization of different contaminants and current knowledge for defining chemical mixtures in human milk: A review***. Environment International, 2023. **171**: p. 107717.

- Chi, Z.H., et al., ***Biomonitoring of bisphenol A (BPA) and bisphenol analogues in human milk from South Africa and Canada using a modified QuEChERS extraction method***. Environmental Pollution, 2024. **348**: p. 123730.

Conference publications

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Abbreviations

ACN Acetonitrile

ANOVA Analysis of variance

BP Bisphenol

BPA Bisphenol A

BPAF Bisphenol AF

BPAP Bisphenol AP

BPB Bisphenol B

BPBP Bisphenol BP

BPC Bisphenol C

BPE Bisphenol E

BPF Bisphenol F

BPS Bisphenol S

EU European Union

ESI Electrospray ionization

GC gas chromatography

LC liquid chromatography

LOD limit of detection

LOQ limit of quantification

MBP Monobutyl phthalate

MDL Method detection limit

MEHP Mono-2-ethylhexyl phthalate

MeOH Methanol

NA Not available

ND Not detected

NTA Non-targeted analysis

OCPs Organochlorine pesticides

OPPs Organophosphate pesticides

OPEs Organophosphate esters

PAHs Polycyclic aromatic hydrocarbons

PBDEs Polybrominated diphenyl ethers

PCBs Polychlorinated biphenyls

PFOA Perfluorooctanoic acid

PFOSA Perfluorooctanesulfonamide

POPs Persistent Organic Pollutants

PFHxS Perfluorohexanesulfonic acid

PFOS Perfluorooctanesulfonic acid

PPCPs Pharmaceutical and personal care products

PRCs Plastic-related contaminants

HRMS High-resolution mass spectrometry

QA Quality assurance

QC Quality control

QuEChERS Quick, easy, cheap, effective, rugged, and safe

THRMS Tandem high-resolution mass spectrometry

UV Ultraviolet

WHO World Health Organization

Ch 1. Introduction

1.1 General Introduction

Human milk is the gold standard for the nutrition of newborn babies thanks to its variable composition of nutrients for the infant's development and contribution to the protection against pathogenic bacteria or viruses [1]. Mothers are, however, exposed to a wide range of man-made chemicals through different routes such as diet, dermal and inhalation. As a result, a range of xenobiotics (chemicals foreign to the body) have been detected in human milk [2]. Some of these compounds, such as organochlorine pesticides, polychlorinated biphenyls (PCBs), dioxins are known to be toxic to human and the environment. Studies have already explored the impact of these residues in milk on the human health and the development of infants [3-8]. The list of chemical residues detected in human milk keeps on lengthening, including contaminants of emerging concern, personal care products or other substances used in the modern time reflecting our current lifestyle [9, 10]. Among the large range of chemicals that can be transferred to human milk, the bisphenols, a dominant class of plastic-related chemicals, are considered one of the most studied types of contaminants for human milk analysis. These compounds are already known as endocrine disruptors warranting the need for regular monitoring of their levels in human milk across countries [11-14].

Furthermore, outside of commonly used bisphenols, there are currently arising concerns that these compounds are being replaced by chemicals equally, if not more, hazardous [15-17]. However, there is still little understanding concerning the presence of bisphenol replacements in human milk. It is also important to note that other emerging plastic-related chemicals in human milk are also underreported. These include potential plasticizers, preservatives, color additives, ultraviolet filters and absorbers, synthetic antioxidants as well as per- and polyfluoroalkyl substances from

fluorinated plastics, highlighting the need to detect and investigate their presence for the safety of both the mothers and breastfeeding infants.

Moreover, while studies have assessed the levels of bisphenols across many East Asian, North American, and European countries, there is currently a lack of data from certain African or West Asian countries due to the lack of monitoring [10]. Unexpected bisphenol analogues can accumulate in human milk, and despite industry assurances about their safe usage and non-toxic nature, these unfamiliar chemicals still pose potential risks to the health of both the mother and breastfeeding infant [18, 19]. Additionally, a few available studies have demonstrated that some plastic-related contaminants present in human milk can undergo enzymatic conjugation [20, 21]. These conjugated compounds can be transferred to the breastfeeding infant, where they may undergo deconjugation due to the presence of glucuronidase or sulfatase, potentially reactivating their endocrine-disrupting activity [22]. However, the proportions of these various species remain poorly described or understood due to limitations in current analytical tools [23].

Collectively, existing literature suggests that breast milk contains complex mixtures of xenobiotics, including numerous plastic-related ones that are yet to be characterized. To date, biomonitoring of chemical contaminants in human milk has primarily relied on targeted analysis, quantifying and reporting the levels of a limited set of different types of targeted chemical residues (usually no more than 50) [24]. As sample preparation and instrumental parameters are optimized for specific compounds in targeted analysis, they provide little to no information on emerging, unexpected, or unknown compounds in samples [25].

Recently, non-targeted analysis (NTA) has been developed and can be employed across multiple matrices using mass spectrometry for the broader characterization of the chemical exposome [25]. This novel approach should be considered for its potential to uncover unknown xenobiotics in

human milk. To date, no study has applied NTA to human milk specifically for plastic-related chemicals, highlighting the need for their detection and identification for the safety of both mothers and breastfeeding infants. The integration of both targeted and non-targeted screening could yield detailed information on the levels of these various contaminants in human milk, thereby enhancing current biomonitoring efforts by detecting both the selected analytes of interest as well as the emerging contaminants present, thus overcoming some of the current limitations surrounding human milk biomonitoring.

1.2. Research hypotheses

The present study was conducted with the hypothesis that:

Hypothesis 1: The QuEChERS method is effective for the extraction and analysis of major PRCs such as bisphenols in human milk, with variations in the types and levels of detected bisphenols across different countries.

Hypothesis 2: In addition to conventional bisphenols, other underreported or unknown bisphenol-related compounds, including synthetic phenolic antioxidants and UV absorbers and filters, are present in human milk and can be identified using non-targeted analysis.

Hypothesis 3: Various parabens and other underreported PRCs are present in human milk, and non-targeted analysis will be able to detect these compounds including the common and unreported parabens.

1.3. General objectives

The primary aim of this study was **to quantitatively assess and compare the types and levels of bisphenols in human milk across different regions, while also identifying bisphenol-related unknowns and other underreported plastic-related contaminants using non-targeted analysis.**

Specific Objectives:

(i) Comparative analysis of major bisphenol analogues: Identify and quantify major bisphenol analogues, such as bisphenol A, S, F, and AF. Compare the types and levels of these bisphenols in human milk between South Africa (data-lacking country) and Canada (Montreal) (Chapter 3).

(ii) Detection and identification of bisphenol-related unknowns: Utilize a customized database library and employ structure elucidation through non-targeted analysis (NTA) software to identify bisphenol-related contaminants (Chapter 4).

(iii) Detection and identification of parabens and other plastic-related unknowns: Extend the application of NTA to identify parabens used in food-related products as well as other surrounding plastic-related unknowns of interest used alongside bisphenols (Chapter 5).

Chapter 2. Literature Review

Chapter 2.0: Characterization of different contaminants and current knowledge for defining chemical mixtures in human milk: a review

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2.1. Abstract

Hundreds of xenobiotics, with very diverse origins, have been detected in human milk, including contaminants of emerging concern, personal care products and other current-use substances reflecting lifestyle. The routes of exposure to these chemicals include dermal absorption, ingestion and inhalation. Specific families of chemicals are dominant among human milk monitoring studies (e.g., organochlorine pesticides, bisphenol A, dioxins), even though other understudied families may be equally toxicologically relevant (e.g., food-processing chemicals, current-use plasticizers and flame retardants, mycotoxins). Importantly, the lack of reliable human milk monitoring data for some individual chemicals and, especially, for complex mixtures, is a major factor hindering risk assessment. Non-targeted screening can be used as an effective tool to identify unknown contaminants of concern in human milk. This approach, in combination with novel methods to conduct risk assessments on the chemical mixtures detected in human milk, will assist in elucidating exposures that may have adverse effects on the development of breastfeeding infants.

Key words*: endocrine disrupting chemicals; non-targeted analysis; dietary exposure; exposome; contaminants of emerging concern*

List of abbreviations

AFs Aflatoxins

BP Bisphenols and other phenols

DDE Dichlorodiphenyldichloroethylene

DDT Dichlorodiphenyltrichloroethane

DON Deoxynivalenol

FBs Fumonisin

HAAs Heterocyclic aromatic amines

HBCDD Hexabromocyclododecane

HCH Hexachlorocyclohexane

HpCDD Heptachlorodibenzo-para-dioxin

HRMS High-resolution mass spectrometry

HxCDD Hexachlorodibenzo-p-dioxin

MCPDs Monochloro-1,2-propanediol

OCPs Organochlorine pesticides

OPEs Organophosphate esters

OPPs Organophosphate pesticides

OTA Ochratoxin A

PAHs Polycyclic aromatic hydrocarbons

PAPs Polyfluorinated alkyl phosphate ester surfactants

PBDEs Polybrominated diphenyl ethers

PCBs Polychlorinated biphenyls

PCDDs Polychlorinated dibenzodioxins

PCDFs Polychlorinated dibenzofurans

PCN Polychlorinated naphthalene

PCP Pentachlorophenol

PCTs Polychlorinated terphenyls

PeCDD Pentachlorodibenzodioxin

PFAS Perfluoroalkyl and polyfluoroalkyl substances

PFOA Perfluorooctanoic acid

PFOS Perfluorooctanesulfonic acid

PFOSA Perfluorooctanesulfonamide

POPs Persistent Organic Pollutants

PPCPs Pharmaceutical and personal care products

THRMS Tandem high-resolution mass spectrometry

Zen Zearalenone

2.2. Introduction

We are exposed to an increasingly long list of man-made chemical substances in our everyday life. For instance, more than 100 000 chemicals are registered by the European Chemicals Agency (ECHA); 30 000 to 70 000 are synthetically produced by industries and used for industrial applications or individual needs [1, 2]. Some residues of these man-made chemical substances, in personal care products, disinfectants, plasticizers, detergents, flame retardants and pesticides, are detected in the human body, with accumulation observed in muscle and fatty tissues, blood and even human milk [3]. Human milk contains endogenous constituents, such as macronutrients (protein, fat) [4], minerals and vitamins [5], and is considered to be a gold standard for infant nutrition [6]. The World Health Organization (WHO) recommends mothers worldwide to breastfeed infants for a minimum of the first six months to achieve optimal growth, development and health [7]. However, breastfeeding is also a source of exposure of newborns to chemical contaminants and other xenobiotics (Figure 2.1) [8]. Infants may exhibit unique susceptibilities to the toxic effects of exposure to chemicals during development. Infants also consume large quantities of breast milk and, thus, may be subjected to high levels of exposure to specific chemicals [9]. As a result, human milk is considered to be a key matrix for biomonitoring as it provides information on chemical exposures in both mothers and infants.

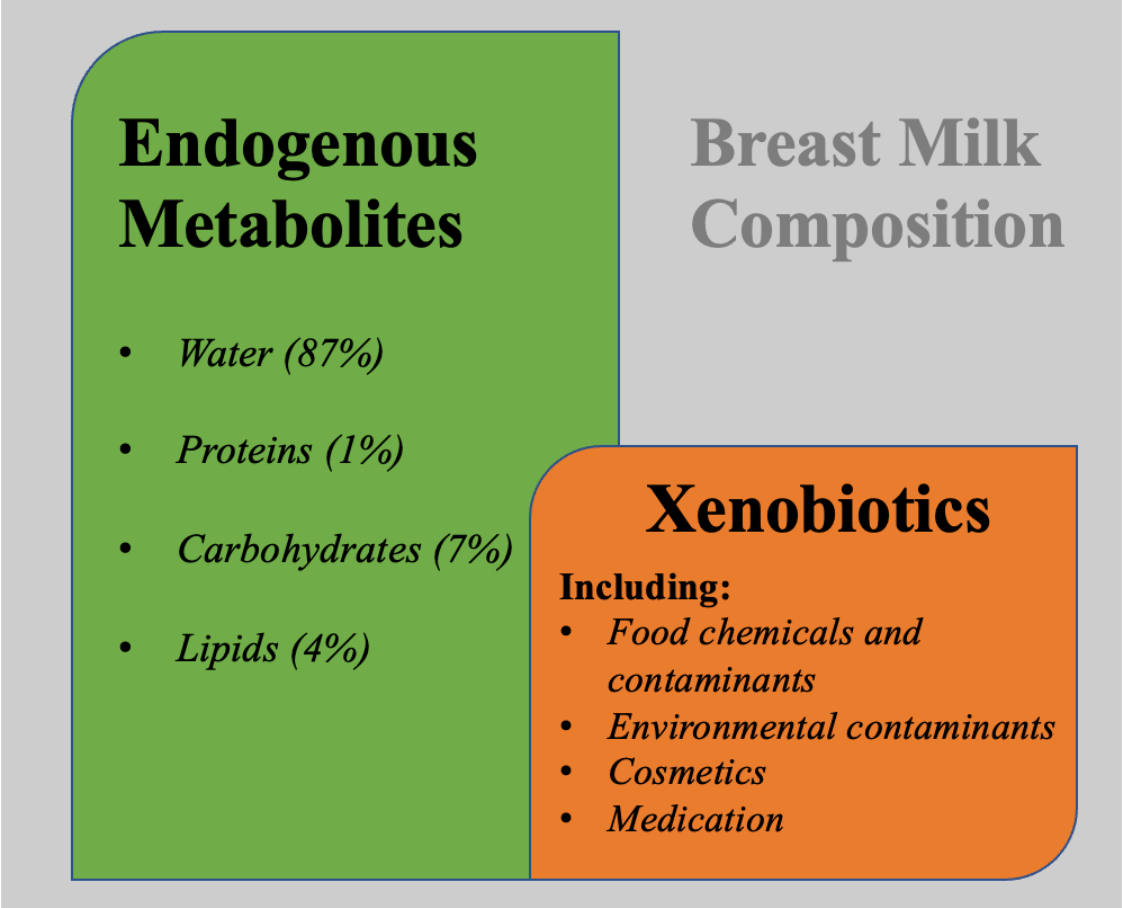

Figure 2.1. Breast milk composition [10]

Human milk biomonitoring studies have been conducted for many contaminants across the world for over 70 years. The literature reviews published to date have each tackled aspects of these analyses such as the occurrence, temporal trends, spatial distribution, and the potential risks of specific chemical residues in human milk (e.g., [11]). Key reviews have discussed the occurrence of legacy persistent organic pollutants (POPs), such as organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and dioxins [3, 12-19], toxic metals [17, 20, 21], organic solvents [17], bisphenol A [22] and mycotoxins [23, 24]. One of the key reviews, conducted by Lehman et al. in 2018, summarized the literature on a broad range of environmental contaminants in human milk in the United States [25].

The possibility that chemicals found in milk, such as OCPs, and dioxins, may have an adverse impact on the health and development of infants is a concern. Correlations have been reported between exposure to levels of certain OCPs in breast milk and different aspects of infant development [26]. A Norwegian study reported that concentrations of hexachlorocyclohexane (β-HCH) in breast milk were associated with an increase in the odds ratio for hyperactivity disorders in the offspring [27]. Another study from Croatia reported that exposure to higher levels of dichlorodiphenyldichloroethylene (*p,p′*-DDE) in breast milk was correlated with impaired neurodevelopmental competencies [28]. Together, these studies suggest that OCP exposure through breastfeeding may exert a negative influence on infant development. However, studies relating the exposure of different types of chemicals and their impacts on the development of the infant are limited. It is clear that there is a need for additional research focused on a comprehensive strategy to identify potentially harmful compounds in milk. It is also important to note that the levels and patterns of accumulated chemicals vary among individuals, according to their age, obesity, occupation, and where they live, among other factors [29]. For instance, a study reported that the mean levels of total PAHs (polycyclic aromatic hydrocarbons) in human milk detected in the body mass index (BMI) >30 obese group were approximately four times greater than in the BMI 18.5-24.9 normal range group [30].

To date, risk assessments related to xenobiotics in milk generally have been conducted for individual compounds or families. For example, Santonicola et al. estimated the intake of benzo[a]pyrene (BaP, a marker for PAHs) from human milk, and compared it with the Joint Expert Committee on Food Additives (JECFA) provisional daily intake value of 10 ng/kg bw/day; 53% of the tested breast milk samples in Italy exceeded this value [31]. For various other PAHs, a margin of exposure was calculated to assess potential risks based on the benchmark dose lower

limit [31]. However, real life exposures are to chemical mixtures, and individual components of a mixture may have additive, synergistic or antagonistic effects [32]. Recently, researchers and policymakers have highlighted an urgent need to develop strategies for risk assessment of chemical mixtures [32]. Several approaches have been proposed in the literature, including the use of a hazard index, a binary weight-of-evidence determination, and a toxic equivalency factor (TEF) [33]. Risk assessments for chemical mixtures have already been developed for other fields, e.g., the consumption of untreated water [34] or ecological assessments [35].

The exposome, defined as "the cumulative measure of environmental influences and associated biological responses throughout the lifespan, including exposures from the environment, diet, behavior, and endogenous processes", is a relatively recent concept [36]. In addition to supporting chemical risk assessments, characterizing chemical mixtures has emerged as one important step to better understand the exposome, to identify co-contamination patterns, to better characterize/manage sources, and to identify patterns and trends for use as early warning systems (e.g., for new chemical hazards).

The specific objectives of the present review are to: (i) summarize the global trend for human milk biomonitoring; (ii) provide a general list of the different types of chemicals reported in human milk; (iii) describe the potential sources of exposure to different contaminants for both mother and infant; (iv) discuss the current trends and knowledge gaps in terms of characterization of chemical mixtures in human milk; and (v) introduce the concept of non-targeted analysis (NTA) to improve the description of the chemical exposome by better characterizing chemical mixtures in human milk.

2.3. Human milk biomonitoring studies

The number of studies of human milk contaminants has increased since the 1950s (Figure 2.2.). In 1951, DDT was the first environmental pollutant detected in human milk and, since then, DDT and its metabolites have been detected in essentially all human milk samples tested worldwide [37]. Subsequent chemical surveillance of human milk has focused on PCBs, dioxins, OCPs, organophosphate pesticides (OPPs), bisphenols and PAHs, all persistent lipophilic chemicals that are preferentially stored in maternal adipose tissues (Figure 2.2.) [3]. The biomonitoring of contaminants in human milk was deployed in many countries when POPs emerged as a global issue [38, 39], enabling the description of temporal trends for legacy POPs, such as OCPs [16, 40]. Over recent years, human milk analyses have also increased for other families of chemicals, including those found in pharmaceutical and personal care products, perfluoroalkyl and polyfluoroalkyl substances (PFAS), chlorinated paraffins, and other pesticides in current use. In 2018, Lehmann et al. compiled data on environmental chemicals in human milk in the United States and highlighted that these data are, indeed, mostly available for persistent, lipophilic chemicals in human milk [25]. Only a few studies have investigated compounds such as heterocyclic aromatic amines (HAAs), chloroethers, polychlorinated naphthalenes (PCNs), polychlorinated terphenyls (PCTs), siloxanes, nitrates and related chemicals, volatile organic compounds (VOCs), and monochloro-1,2-propanediol (MCPD) fatty acid esters in human milk. If we consider the exposome from a broader perspective, including not only environmental contaminants but also other xenobiotics originating from foodstuffs, packaging materials and personal care products, there is an obvious imbalance in the data collection process, with many data-poor contaminants. Furthermore, while there are numerous studies conducted for specific families of contaminants, such as PCBs, OCPs, and plasticizers (e.g., phthalates and bisphenols),

currently there is a relative lack of monitoring data pertaining to their related replacements or their metabolites.

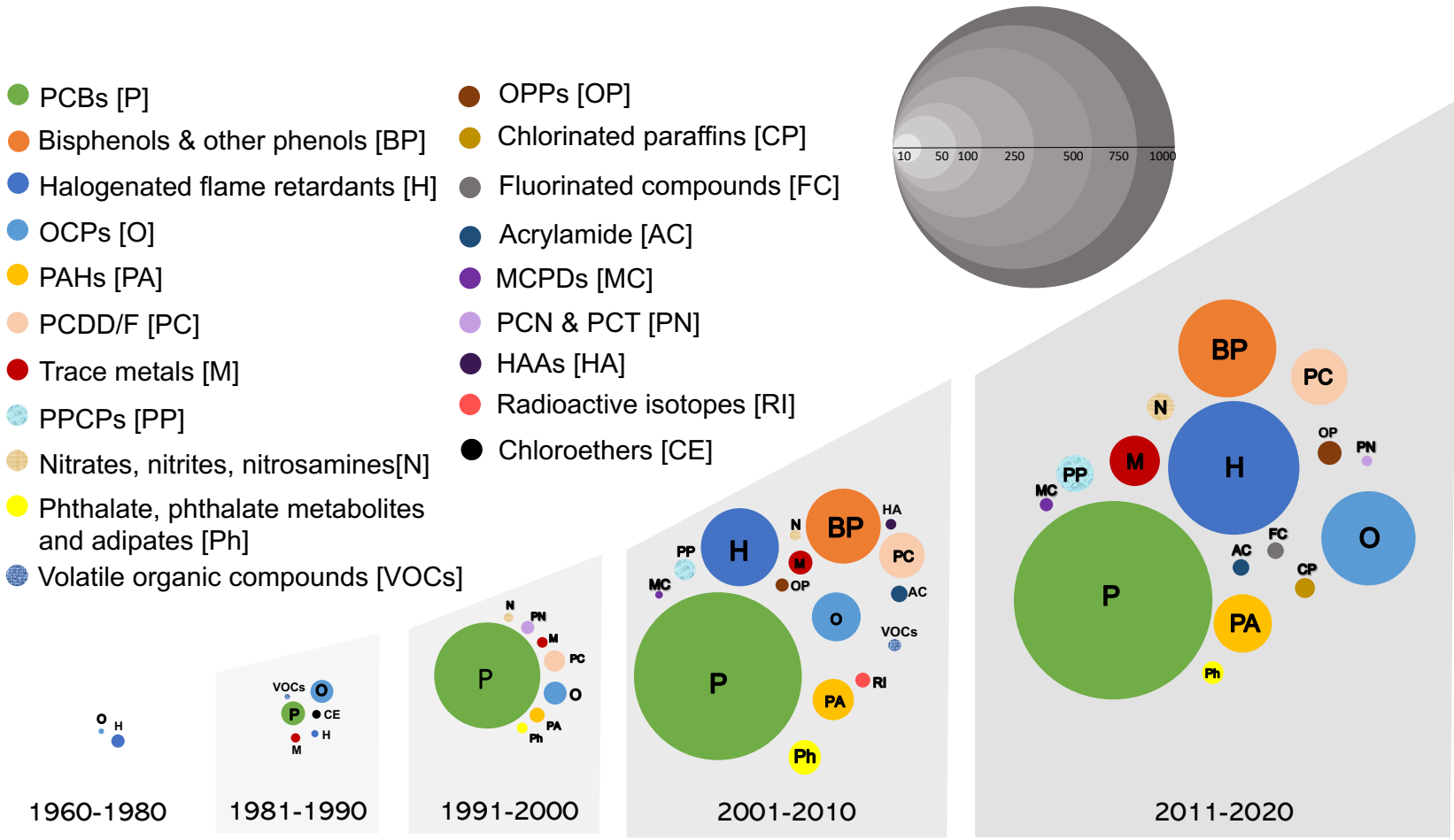

Figure 2.2. Numbers of unique studies from 1960-2020 on specific families of contaminants detected in human milk

2.3.1. Types and origins of xenobiotics detected in human milk

A list of different chemicals reported in human milk in studies published from 1960-2020 were generated by browsing websites, such as the Web of Science, Wiley Online Library, and different journals using ScienceDirect (the key words included “colostrum”, “human milk”, and the specific chemical family); results were compiled in Figure 2.2. Compounds were classified into different families of contaminants. Studies that included 2 or more different types of contaminants were counted as once for these specific families. The list of studies was then curated manually to avoid duplicate or unrelated studies that were not based on detection in human milk. Some key selected papers are presented in Table 2.2. to provide an overview of the different types of chemicals detected in human milk. A wide range of chemical contaminants has been detected in human milk, although POPs are among the most commonly studied compounds [41].

Table 1 summarizes the contaminants reported in human milk along with their respective concentrations. A comprehensive description of these xenobiotics is presented as supplementary information (SI). The sources of these chemical residues are diverse; they include environmental pollutants (e.g., by-products of waste incineration, chemical manufacturing, petroleum refining, fuel combustion and wood burning), naturally occurring mycotoxins, contaminants generated during the processing of food and water, and man-made substances used in our daily lives, such as pesticides, flame retardants, building blocks or additives in materials/fluids/polymers/inks, food additives, pharmaceuticals and personal care products. Many of these chemicals are currently reported to be high production volume chemicals; the use and production of others are being phased-out but some residues are so persistent that they can still be detected decades later [42].

With the exception of a few substances (e.g., nitrates and some pesticides), individual chemicals are present at sub ppm levels in human milk, often in the nanogram or picogram per mL range

(Table 2.1.; SI). Some families of chemicals that are considered ubiquitous, such as the bisphenols and phthalates, have been analyzed frequently in human milk studies. In addition, OCPs, PAHs, pyrethroids, PCBs and OPPs have been detected in human milk in many countries at relatively high concentrations (ng/mL) (Table 1). Other chemicals, such as nitrates and chlorinated paraffins, have been less commonly studied, leading to an underrepresentation among human milk studies for certain contaminants (Figure 2). In addition, our current understanding concerning the conjugation and speciation for certain chemicals remains limited due to the lack of novel analytical tools allowing for their detection in human milk. This is apparent for chemicals such as esterified 3-MCPDs (3-chloropropane-1,2-diol) with fatty acids (bound 3-MCPDs), where only two papers have reported their presence in human milk [43, 44]. While the levels of free 3-MCPDs in human milk are considered below the limit of quantification, bound 3-MCPDs have been reported in relatively high levels and can be hydrolyzed to their free form. Although the impact of 3-MCPD on human health is currently not fully understood, one study reported that exposure of rats to MCPD induced neoplasia by a mechanism that did not involve DNA damage or require exposure above a threshold dose [43, 45].

In most studies, the levels of the contaminants that were reported, when interpreted individually, have not been associated with adverse health impacts. However, in some studies the overall calculated estimated daily intake (EDI) for certain families of chemicals, such as the PCDD/Fs and PCBs, did exceed the tolerable daily intake (TDI). Thus, our understanding concerning the overall health impacts of these chemicals on the breastfeeding infant remains limited [46, 47]. In addition, current studies on human milk analysis generally focus on a limited number of xenobiotics. Since typical exposures are to complex mixtures, there is a need for the improvement of current risk

assessments for mixtures. Furthermore, the absence of toxicological data for some of the chemicals identified in these mixtures complicates their assessment.

Table 2.1. Main types of xenobiotics detected in human milk in various countries and their concentrations. (lw: lipid weight. dw: dry weight basis. ND: not detected. N/A: Not available)[1]

Type	Compounds detected	Sampling time	Collection period (postpartum)	Country	Concentrations	Ref.
OCPs	*p,p'*-DDT; *p,p'*-DDE; dieldrin; HCHs	1997-2001	4-8 weeks	Finland	*Means (ng/g lw): p,p'*-DDT:4.35; *p,p'*-DDE:77.26; dieldrin:2.84; β-HCH:11.63; α-HCH:0.19; γ-HCH:0.61; δ-HCH:0.04	[48]
	o,p'-DDT; *p,p'*-DDD	2007-2008	4-8 weeks	China, Vietnam, Japan, South Korea	*Means with all 4 countries combined (range of country means, ng/g lw*): *o,p'*-DDT : 0.84 (nd–4.4); *p,p'*-DDD:1.4 (0.41–4.0)	[49]
	β-HCH; oxychlordane; *p,p'*-DDE; HCB	2002-2006	1-18 weeks	Norway	*Medians (range, ng/g lw*): β-HCH: 4.7 (0.9-	[50]

[1] **Additional details about the different types of contaminants in human milk are summarized in the supplementary information (SI)**

					37); oxychlordane: 2.8 (0.5-16); *p,p'*-DDE: 41 (5.4-492); HCB : 11 (3.6-24)	
	γ-HCH; α-HCH	1997-2009	2-8 weeks	Russia	*Medians (ng/g lw)*: γ-HCH: 3.7 ; α-HCH: 0.6;	[51]
	Alpha-endosulfan; dieldrin; oxychlordane; aldrin; heptaclor	1997-2001	4-12 weeks	Finland, Denmark	*Medians (range, ng/g lw):* Alpha-endosulfan : 6.95 (1.83–17.84) ; dieldrin: 4.06 (0.77–35.50) ; oxychlordane : 4.52 (0.91–8.93) ; aldrin : (below LOQ) ; heptaclor : (below LOQ)	[52]
	Toxaphene	1978-1979	N/A	Sweden	*Median (µg/g lw)*: 0.1	[53]
Pyrethroids	Tetramethrin; bifenthrin; alpha-cyhalothrin; deltamethrin; esfenvalerate	2009-2010	2-20 weeks	Brazil	*Medians (range, ng/g lw):* tetramethrin: 0.36 (nd-0.82); bifenthrin: 1.44 (nd-7.48); alpha-	[54]

					cyhalothrin:1.99 (nd-8.77); deltamethrin: 0.50 (0.2-0.92); esfenvalerate: 0.20 (0.06-0.38)	
	Tetramethrin; bifenthrin; alpha-cyhalothrin; deltamethrin; esfenvalerate	2009-2010	2-20 weeks	Spain	*Medians (range, ng/g lw):* *t*etramethrin: 0.56 (nd.-4.54); bifenthrin: 0.12 (nd-0.47); alpha-cyhalothrin: 0.14 (0.04-0.85); deltamethrin: 0.40 (0.09-0.71); esfenvalerate: 0.04 (nd-0.33)	[54]
	Tetramethrin; bifenthrin; alpha-cyhalothrin; deltamethrin; esfenvalerate	2009-2010	2-20 weeks	Columbia	*Medians (range, ng/g lw):* tetramethrin: 0.83 (nd.-14.8); bifenthrin: (nd.-0.48); alpha-cyhalothrin: 0.6 (0.05-4.49); deltamethrin: negligible. (nd.-1.86); esfenvalerate: 0.22 (nd.-3.11)	[54]

	Cyfluthrin; fenvalerate; cypermethrin	N/A	N/A	India	*Medians (range, ng/g lw):* cyfluthrin : 105 (ND-4100.39); fenvalerate: 742.54 (ND-1172.87); cypermethrin: 105.9 (ND-276.35)	[55]
Neonicotinoids	Imidacloprid; thiacloprid; acetamiprid; thiamethoxam; clothianidin	N/A	N/A	Switzerland	*Median (total neonicotinoids)*: 2 pg/mL	[56]
	Imidacloprid; acetamiprid; acetamiprid-N-desmethyl (ACE-DE); thiamethoxam; clothianidin	2017-2019	3-8 weeks	China	*Means (range, ng/L)*: imidacloprid: 41 (ND-191); acetamiprid : 19.1 (ND-230); ACE-DE: 161 (8.30-1479); thiamethoxam: 20.3 (ND-105); clothianidin : 13 (ND-51.6)	[57]
OPPs	Azinphos-methyl, chlorpyrifos; diazinon	2002-2011	N/A	United States	*Medians (range, µg/mL)*: azinphos-Methyl: 0.39; clorpyrifos: 0.06; diazinon: 0.04	[25]

	Chlorpyrifos; chlorpyrifos-methyl; ethion; dimethoate	2006	NA	India	*Means (mature milk ng/g lw):* dimethoate:26.752 ; ethion: 744.925; chlorpyrifos: 37.274	[58]
	Malathion; chlorpyrifos; methyl-parathion	N/A	1-4 weeks	India	*Means (range, mg/L)*: malathion: 0.043 (n.d.-0.086) ; chlorpyrifos: 0.23 (0.085-0.355) ; methyl-parathion : 0.001 (n.d.-0.009)	[59]
	Profenophos; chlorpyriphos; monocrotophos; phosalone	N/A	N/A	India	*Medians (range, ng/g)*: profenophos: 169.45 (ND-297.25); chlorpyriphos: 21.91(ND-160.48); monocrotophos: 207.81 (ND-207.81); phosalone: 36.13(ND-37.02)	[60]
Other Pesticides	Atrazine	N/A	0-12 weeks	United States	*Mean (nM):* 0.404 ±0.089	[61]

	Procymidone	2002-2005	3-5 days post-partum	France	*Median (ng/g fat)*: 0.9	[62]
	Lufeneron	2018-2019	N/A	Lebanon	*Mean (range, ug/L):* 5.8754 (5.1208-12.0447)	[63]
PCBs	PCB congeners 105, 118, 128, 138, 146, 153, 156, 167, 170, 180, 183, and 187 PCB metabolites 4-OH-CB-146 and 4-OH-CB-187	1987, 1994 and 1999	3-5 days post-partum	Denmark	All cohorts: *Mean total concentration ΣPCBs (range, ng/g lw):* 1800 (690–4600)	[64]
	PCB congeners 28, 52, 77, 81, 101, 105, 114, 118, 123, 126, 138, 153, 156, 157, 167, 169, 170, 180 and 189	2007	1-12 weeks	Hungary	*Mean total concentration ΣPCBs (ng/g fat):* 34.45	[65]
PCDDs	2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD; 1,2,3,6,7,8-HxCDD; 1,2,3,7,8,9-HxCDD; 1,2,3,4,6,7,8-HpCDD	N/A	1-4 weeks	Japan	*Means (range, pg/g lw):* 2,3,7,8-TCDD: 0.81 (0.10-4.34); 1,2,3,7,8-PeCDD: 4.19 (0.56-11.28); 1,2,3,4,7,8-HxCDD: 1.44 (0.15-5.01); 1,2,3,6,7,8-HxCDD: 13.67 (1.98-37.55);	[66]

					1,2,3,7,8,9-HxCDD: 1.94 (0.82-7.82); 1,2,3,4,6,7,8-HpCDD: 6.78 (1.24-25.36)	
	2,3,7,8-TCDD; 1,2,3,7,8-PeCDD; 1,2,3,4,7,8-HxCDD; 1,2,3,6,7,8-HxCDD; 1,2,3,7,8,9-HxCDD; 1,2,3,4,6,7,8-HpCDD	2007	1-12 weeks	Hungary	*Means (pg/g lw):* 2,3,7,8-TCDD: 0.34; 1,2,3,7,8-PeCDD: 0.74; 1,2,3,4,7,8-HxCDD: 0.96; 1,2,3,6,7,8-HxCDD: 3.32; 1,2,3,7,8,9-HxCDD: 1.12; 1,2,3,4,6,7,8-HpCDD: 3.71	[65]
PCDFs	2,3,7,8-TCDF 1,2,3,7,8-PeCDF 2,3,4,7,8-PeCDF 1,2,3,4,7,8-HxCDF 1,2,3,6,7,8-HxCDF 2,3,4,6,7,8-HxCDF 1,2,3,7,8,9-HxCDF 1,2,3,4,6,7,8-HpCDF 1,2,3,4,7,8,9-HpCDF	N/A	1-4 weeks	Japan	*Means (range, pg/g lw):* 2,3,7,8-TCDF: 1.02 (0.10-3.76); 1,2,3,7,8-PeCDF: 0.29 (nd-1.64); 2,3,4,7,8-PeCDF: 8.14 (1.08-20.99); 1,2,3,4,7,8-HxCDF: 2.25 (0.31-12.62);	[66]

				1,2,3,6,7,8-HxCDF: 2.27 (0.25-7.91); 2,3,4,6,7,8-HxCDF: 1.46 (0.11-7.05); 1,2,3,7,8,9-HxCDF: 0.03 (nd-0.77); 1,2,3,4,6,7,8-HpCDF: 1.19 (nd-7.63); 1,2,3,4,7,8,9-HpCDF: 0.04 (nd-1.57)	
2,3,7,8-TCDF; 2,3,4,7,8-PECDF; 1,2,3,4,7,8-HXCDF; 1,2,3,6,7,8-HXCDF; 2,3,4,6,7,8-HXCDF; 1,2,3,4,6,7,8-HPCDF; OCDF	1989-1990	N/A	Canada (Quebec)	*Means (pg/g lw):* 2,3,7,8-TCDF: 6.1; 2,3,4,7,8-PECDF: 5.2; 1,2,3,4,7,8-HXCDF: 3.3; 1,2,3,6,7,8-HXCDF: 2.3; 2,3,4,6,7,8-HXCDF: 1.1; 1,2,3,4,6,7,8-HPCDF: 4.5; OCDF: 1.1	[67]
OCDD, 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, 2,3,4,7,8-PeCDF, 1,2,3,4,7,8-HxCDF	2007	1-12 weeks	Hungary	*Mean total concentration*: 2.70 ± 1.57 pg-TEQ (Toxic	[65]

					equivalency)/g fat	
PBDFs (Polybrominated dibenzofurans)	2,3,7,8-TeBDF	2008	N/A	Vietnam	Below limit of detection (LOD);LOD: 50 pg/g lw	[68]
PCNs (Polychlorinated naphthalene)	CN-27, CN-42, CN-52, CN-67, CN-73, and CN-7	2017-2019	N/A	China	*Range (ng/g lw):* 0.21107-2.49743	[69]
	CN-52	1972-1985 1988-1994 1996-1997	1-12 weeks	Sweden	All cohorts *Range (ng/g lw):* ND-1	[70]
Chlorinated paraffins	SCCPs; MCCPs	2007 and 2011	N/A	China	*Medians (ng/g lw):* SCCPs: 303 and MCCPs: 35.7 in 2007; SCCPs: 360 and MCCPs: 45.4 in 2011	[71]
	SCCPs; MCCPs; LCCPs	2010-2016	N/A	China	*Medians (ng/g lw):* SCCPs: 35.0; MCCPs: 78.8; LCCPs: 8.80	[72]
	LCCPs; MCCPs; SCCPs	2010-2016	N/A	Norway, Sweden	*Medians (countries combined, ng/g lw):* SCCPs: 14.0; MCCPs: 29.6; LCCPs: 4.17	[72]

PAHs	Acenaphthylene; anthracene; fluorene; phenanthrene; fluoranthene; pyrene; benzo(*a*)anthracene; chrysene; benzo(*b*)fluoranthene; benzo(k)fluoranthene; benzo(*a*)pyrene; dibenzo(ah)anthracene; benzo(ghi)perylene; indeno(*cd*)pyrene	N/A	4-10 days	Italy	*Mean total concentration ΣPAHs (range, ng/g lw):* 114.93 (40.97–259.93)	[31]
	PAHs OH-PAHs	2019-2020	N/A	Portugal	*Medians (ng/g lw):* ∑PAHs: 11; ∑OH-PAHs: 23	[73]
	Naphthalene; benzo[k]fluoranthene; benzo[a]pyrene; Benzo[g,h,i]-perylene; Dibenzo[a,h]anthracene	2009	1-4 weeks	Turkey	*Mean total concentration ΣPAHs (ng/g lw):* 9.91	[41]
PBDEs	BDE congeners 28,47, 49, 66, 85, 99, 100, 138, 153, 154, 183 and 209	2011-2012	N/A	United Kingdom	*Lower Median total concentration ΣPBDEs (range, ng/g lw)* 5.59 (1.28-22.02)	[74]
	BDE congeners 17, 28, 47, 66, 85, 99, 100, 138, 153, 154, 183 and 209	2000-2001	1-2 weeks	Taiwan	*Mean total concentration ΣPBDEs (range,*	[75]

					ng/g lw): 3.93 ± 1.74	
	BDE congeners 99, 100, 153, 47, 28, 85, 154, 209,71, 66, 183, 32	N/A	N/A	United States and Canada	*Mean total concentration ΣPBDEs (range, ng/g lw)*: 96 (6-321)	[76]
Other halogenated flame retardants	α, β and γ-HBCDD	2004	N/A	Sweden, Norway	*Median total concentration ΣHBCDs (ng/g lw):* Sweden: 0.35; Norway: 0.60	[77]
	α, β and γ-HBCDD	2004-2005	2-8 weeks	United States	*Mean total concentration ΣHBCDs (range, pg/g lw):* 1020 (360-8100)	[78]
	TBBPA	2011-2014	1-12 weeks	China	*Median (ng/g lw):* 1.57	[79]
	TBBPA	2004-2005	2-8 weeks	United States	Below LOQ LOQ: 30 pg/g lw	[78]
	TBBPA	N/A	N/A	United Kingdom	*Mean (ng/g lw):* 0.06	[80]
Organophosphate esters	TEP TNBP TBOEP	2019	N/A	United States		[81]

	TEHP TCIPP TCEP TPHP EHDP				*Median total concentration* Σtri-OPEs *(ng/mL*) 3.85	
	DNBP BBOEP BDCIPP BCIPP DPHP				*Median total concentration* Σdi-OPEs *(ng/mL)* 8.32	
PPCPs	Triclosan	2003-2004	6-12 weeks	Sweden	*Range (ng/g lw):* < 0.018 to 0.95	[82]
	Parabens (methyl-parabens; n-propyl paraben; ethyl paraben	2009-2010	1-12 weeks	Canada	*Means (range, µg/L):* methyl-paraben: 0.991(n.d.-16.325) ; n-propyl paraben: 0.334 (ND-4.588); ethyl paraben: 0.123 (ND- 2.183)	[83]
	AHTN HHCB HHCB-lactone OTNE Musk Ketone	2009	N/A	China	*Medians (ng/g lw):* AHTN: 16.5; HHCB: 11.5; HHCB-lactone:7.85; OTNE: <1.5; Musk Ketone: <1.4	[84]

	UV filters: (Ethylhexyl-methoxy cinnamate; 4-methylbenzylidene camphor; homosalate and octocrylene)	2004-2006	1-4 weeks	Germany	*Means (range, ng/g lipid)*: Ethylhexyl-methoxy cinnamate 27.50 (2.10–79.85); Octocrylene 30.18 (4.70–134.95); 4-Methyl-benzylidene camphor 22.12 (6.70–48.37); Homosalate 29.37 (11.40–61.20); Benzophenone-3 52.23 (7.30–121.40)	[85]
	UV filters: EHMC; Octocrylene; 4-MBC; 3-BC (3-Benzylidene camphor); OD-PABA	2004-2006	1-4 weeks	Germany	*Medians* (*range, ng/g lipid*): EHMC / OMC 25 (2.1 - 78.1); Octocrylene 12.5 (4.7 - 77.5); Bp-3 19.8 (7.3 - 121.4); 4-MBC 18.4 (6.7 - 19.0); OD-PABA 50 (n.d.-50)	[86]

	FTOH (fluorotomer alcohol); PFOA; PFOS; PFOSA; PAPs (Polyfluorinated alkyl phosphate ester surfactants)	2015	N/A	Spain	*Range* ΣFluorinated compounds (ng/mL)*:* 0.066-0.356	[87]
Perfluoroalkyl and polyfluoroalkyl substances	PFBuS (Perfluorobutanesulfonic acid); PFHxS (Perfluorohexanesulphonic acid); PFOS (Perfluorooctanesulfonic Acid); THPFOS (Tridecafluorooctanesulfonic Acid); PFDS (Perfluorodecanesulfonic Acid); PFHxA (Perfluorohexanoic Acid); PFHpA (Perfluoroheptanoic Acid); PFOA (Perfluorooctanoic Acid); PFNA (Perfluorononanoic Acid);	2004	3rd week after delivery	Sweden	*Means (ng/L):* PFOS:0.201; PFHxS:0.085; ΣFluorinated compounds: 0.34	[88]

	PFDA (Perfluorodecanoic Acid); PFUnDA (Perfluoroundecanoic Acid); PFDoDA (Perfluorododecanoic Acid); PFOSA (Perfluorooctanesulfonamide);					
	PFOS branched isomers: Br-PFOS; L-PFOS; PFOA; PFNA; PFDA; PFHxS	2010	N/A	Czech Republic	*Mean total fluorinated compounds concentrations (range, pg/mL):* 115 (38–279)	[89]
Siloxanes	D4, D5 and D6	2005	N/A	Sweden	*Maximum concentrations: D4:10 µg/L; D5: 4.5 µg/L; D6: 4.8 µg/L*	[90]
Chlorobenzenes	Hexachlorobenzene; pentachlorobenzene	Czech Republic: NA Denmark: 1997-2001	Czech Republic: NA Denmark: 1-12 weeks	Czech Republic; Denmark	*Means (ng/g lw):* hexachlorobenzene:56 (Czech Republic); pentachlorobenz	[13]

					ene: 0.32 (Denmark)	
VOCs	MTBE; chloroform; benzene; toluene	2005	N/A	United States	*Medians (ng/mL):* MTBE: 0.09; chloroform: 0.55; benzene: 0.12; toluene:0.46	[91]
Plastic additives and monomer residues	BPA; BPS; BPF	2015	2-8 weeks	Spain	*Means (ng/mL*): BPA: 0.29; BPS, BPF: below LOQ	[92]
	Nonylphenol; octylphenol; BPA	2013	1-2 weeks	South Korea	*Medians (ng/mL*): BPA: 7.8; octylphenol: below LOQ; nonylphenol: below LOQ	[93]
	BPA; BPS; BPF; BPB; BADGE (Bisphenol A diglycidyl ether)	2018	1-2 weeks	Poland	*Means (ng/mL*): BPA: 0.35; BPS: 0.45 ; BPF: 0.25 ; BPB: below LOQ	[94]
	BPA BPA analogues: BPS; BPF; BPAF; BPE; BPAP	N/A	Within 1 week	China	*Means (pg/mL)*: BPA: 225 (77.1-1010); ∑BPA analogues: 121 (34.5-1420);	[95]

	BPS derivatives: 2-4 BPS; BPSIP; BPS-MAE; BPS-MPE; TGSA; D-90; DDS				∑BPS derivatives: 90.3 (8.45-943)	
	Synthetic Phenolic antioxidants (SPAs): BHT; DBP; AO 246; DTPSBP; BHA; D*t*AP; 4-*t*OP; AO 2246; Irganox 1076; Irganox 1135 Transformation products of BHT (TPs): BHT-OH; BHT-CHO; BHT-Q; BHT-quinol	2018-2019	N/A	China	*Means (ng/mL)*: ∑SPAs: 20.1 (1.96-64.9); ∑TPs: 2.21 (0.21-5.55)	[96]
	Phthalate monoesters: MMp; MEP; MBP; MBzP; MEHP; MiNP	1997-2001	1-12 weeks	Denmark	All cohorts: *Medians (range, µg/mL)*: MMp: 0.11 (<0.01-1.49); MEP: 0.95 (0.07-15.4); MBP: 3.5 (0.6-10900); MBzP: 0.8 (0.2-10); MEHP: 9.5	[97]

				(2.7-72); MiNP: 101 (27-382)	
Phthalate diesters and monoesters: DiBP; DBP; BzBP; DEHP; DiNP; MiBP; MBP; MBzP; MEHP; MiNP	N/A	N/A	Italy	*Means (range, µg/kg*): DiBP: 37 (11-77); DBP: 7.1 (3.5-19); BzBP: 1.7 (<2.0-3.2); DEHP: 34 (<13-94); DiNP: 20 (6.3-51); MiBP: 9.9 (2.3-25); MBP: 4.0 (2.1-6.1); MBzP: 0.80 (0.15-1.2); MEHP: 10 (4.1-18); MiNP: 7.6 (1.5-29)	[98]
Phthalate diesters: DEHP; DnBP; DiBP; DiNP Phthalate metabolites: MEHP; MiBP; MnBP	2007-2008	4-8 weeks	Germany	*Median Concentrations (ng/g):* DiBP: 1.2 (<0.1-5.3); DnBP: 0.8 (<0.1-7.4); DEHP: 3.9 (<0.5-23.5) *Median concentrations (ug/L)*: MiBP: 11.8 (4.4-43.8); MnBP: 2.1 (0.4-	[99]

					18.1); MeHP: 6.6 (27.4-2.3)	
	DEHA; DMP; DEP; DBP; DiBP; DEHP	2009-2011	2-10 weeks	Canada	*Range (ng/g lw)*: 30.4–237	[100]
Mycotoxins	Aflatoxins; beauvericin; enniatin; ochratoxin A	2016	N/A	Nigeria	*Ranges (ng/mL)*: aflatoxin M1: 0.043-0.087; beauvericin: 0.006-0.019; enniatin B: 0.004-0.009; ochratoxin A: 0.048-0.096	[101]
Nitrate, nitrite and Nitrosamines	Nitrate; nitrite	N/A	1-3 days	United States	*Means (mg/ 100mL)*: nitrite: 0.001; nitrate: 0.3 *Means (*µg*/L)*: nitrite: 100 000; nitrate: 30000000	[102]
	Nitrosoamines	N/A	2-36 weeks	United States	*Mean Σnitrosoamines (ng/mL)*: <0.2 *(*µg*/L)*: <0.2	[103]
	PhIP (2-amino-1-methyl-6-	N/A	4-6 weeks	United States	Below LOQ	[104, 105]

	phenylimidazo[4,5-b]pyridine)				LOD: N/A	
Heterocyclic Aromatic Amines (HAAs)	PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine)	N/A	N/A	Canada	*Mean (range, pg/mL)*: 23 (ND-59)	[105]
Acrylamide	Acrylamide	2000-2004	N/A	Sweden	*Mean total concentration*: <0.5 µg/kg lw	[106]
3-MCPD fatty acid esters	3-MCPD fatty acid esters	N/A	2-11 weeks	Czech Republic	*Mean total concentration after hydrolysis Σ3-MCPD (range, µg/kg lw)*:1014 (300 - 2195)	[43]
2-MCPD fatty acid esters 3-MCPD fatty acid esters	2-MCPD fatty acid esters 3-MCPD fatty acid esters	2006	2-10 weeks	Canada	2-MCPD fatty acid esters: (below detection limit: 1 ng/g); 3-MCPD fatty acid esters: (below detection limit: 2 ng/g);	[44]
Chloroethers	Bis(2,3,3,3-tetrachloropropyl) ether	1979-1980	N/A	Japan	*Mean (range, ng/ mL whole milk)*: 0.5 (nd.-0.9)	[107]

Heavy metals	Arsenic; cadmium; mercury; lead	N/A	N/A	United Arab Emirates	*Means (range, µg/L*): arsenic: 0.196 (0.022-0.65); cadmium: 0.27 (0.023-0.19); mercury: 0.115 (0.04-0.187); lead:1.51 (0.025-2.41)	[108]
	Arsenic; lead	2012-2013	N/A	Argentina, Namibia, Poland, United States	*Means (range, µg/L*): total arsenic: 4.18 (2.4-11.2); total lead: 0.91 (0.21-2.48)	[109]
Methylmercury	Methylmercury	N/A	1-4 weeks	Japan	*Mean (range, ng/g*): 0.45 (0.06-1.20)	[110]
Radioactive isotopes	Cerium	2007-2009	1-51 weeks	Spain and Germany	*Means (range, ng/L*): Germany: <10 (<10–12.7) ; Spain: 38.8 (21.6-70.3)	[111]
	Rubidium	2011	1-2 weeks	Iran	*Mean (range, ppm dw*): 32.176 (8.660-107.210) *Mean (range, µg/mL dw)* 32.176 (8.660-107.210)	[112]

	^{137}Cesium	1989	N/A	Italy	*Mean activity (range, d/L):* 0.22 (0.06-0.41)	[113]

2.3.2. Routes of exposure and the accumulation of chemicals in human milk

Xenobiotics detected in human milk enter the human body primarily through dermal absorption, by ingestion or by inhalation (Figure 2.3) [114, 115]. Table 2.2 provides examples of routes of exposure for women for each of the families of xenobiotics detected in human milk. Dermal exposure and adsorption through the skin occurs for many lipophilic chemicals, such as halogenated flame retardants, synthetic musks, UV filters and some heavy metals; these chemicals enter the circulation and then may bioaccumulate in fatty tissues [116]. Oral exposure includes the ingestion of dust; chemicals such as PBDEs and HBCDDs enter the body through the accidental ingestion of settled dust [117]. Exposure to contaminants in drinking water, food or food contact materials is also oral; residues such as bisphenols or PFAS can migrate from food contact materials into food matrices [118]. The inhalation of air or vapor that is contaminated with toxicants contributes to exposure to volatile compounds [119] or chemicals associated with air particulates. For instance, POPs, such as PBDEs and PCBs, may enter the body through inhalation [120].

For many substances, all three routes of exposure (dermal absorption, ingestion and inhalation) contribute to the total exposure [121]; however, ingestion of contaminated foods appears to be a common route of exposure for many of the xenobiotics detected in human milk, as summarized in Table 2.2. For many persistent organic chemicals, such as dioxins, PAHs, PCNs, PFOS, PCBs, OCPs, PCDD/Fs and HCB, diet is a major contributor to the contaminant burden recorded in human milk since such lipophilic compounds bioaccumulate in milk fat [122]. Fish consumption often plays a significant role [17]. In fish-eating populations from countries such as Japan, high traces of methylmercury and PCBs were detected in human milk [110, 123]. Another study reported elevated levels of DDTs in women living in coastal cities of China due to their high sea food intake [124].

Diet has an impact on the composition of chemical mixtures detected in human milk. Since both dietary and contamination patterns vary globally and temporally, complex time trends and regional variability may be expected among chemical mixture patterns globally. Factors such as the timing of the sample collection relative to exposure events, as well as the inter-individual variation in chemical toxicokinetics, could also influence the different levels of detected chemicals in human milk [125]. Thus, assessment of the basis for the variability of different levels of contaminants in human milk remains difficult.

To date, only a few studies have compared the changes in human milk contaminant levels over time or regionally. The last review that addressed this, by Solomon et al. (2002), described a decrease in DDT between 1968 and 1984 and a decrease in dieldrin between 1965 and 1998 in Sweden [17]. This study also described the variability of DDT levels in human milk across different European countries, as well as the decrease in HCB in Swedish human milk samples between 1970 and 2000 [17]. There is an obvious need to assess the changes in levels of not only POPs but also plastic-related contaminants and pesticides in human milk in recent years.

Differences in the levels of certain contaminants have been observed across different regions. For example, it has been noted that in Asian countries, such as China, there were higher concentrations of fluoroalkyl substances (PFOA, PFOS, and F53-B) compared to European countries, such as Sweden, especially for F53-B (6:2 chlorinated polyfluorinated ether sulfonate) [126]. Geographical differences between the reported concentration levels of PCDDs/DFs and PCBs have also been reported: relatively higher concentrations of these chemicals were observed in human milk in most European countries and some Asian countries, like China and India, compared to two North American countries, Canada and Mexico, and certain African countries [127]. For DDT related compounds, the levels of DDTs, DDEs and DDDs across two North American countries,

Canada and the United States, were considered low in the last decade [127]. A new review paper on DDEs in human milk, published in 2021, reported that China had the highest concentration levels of *p,p*'-DDE, while European countries, such as Belgium and France, had the lowest reported levels [46]. Altogether, the different patterns in the levels of chemicals detected in different geographical regions suggest that aggregating human milk biomonitoring data from multiple countries/years may not provide an accurate description of the specific chemicals to which any group of infants is exposed.

Figure 2.3. Summary of main routes of human exposure for environmental chemicals that may be detected in milk

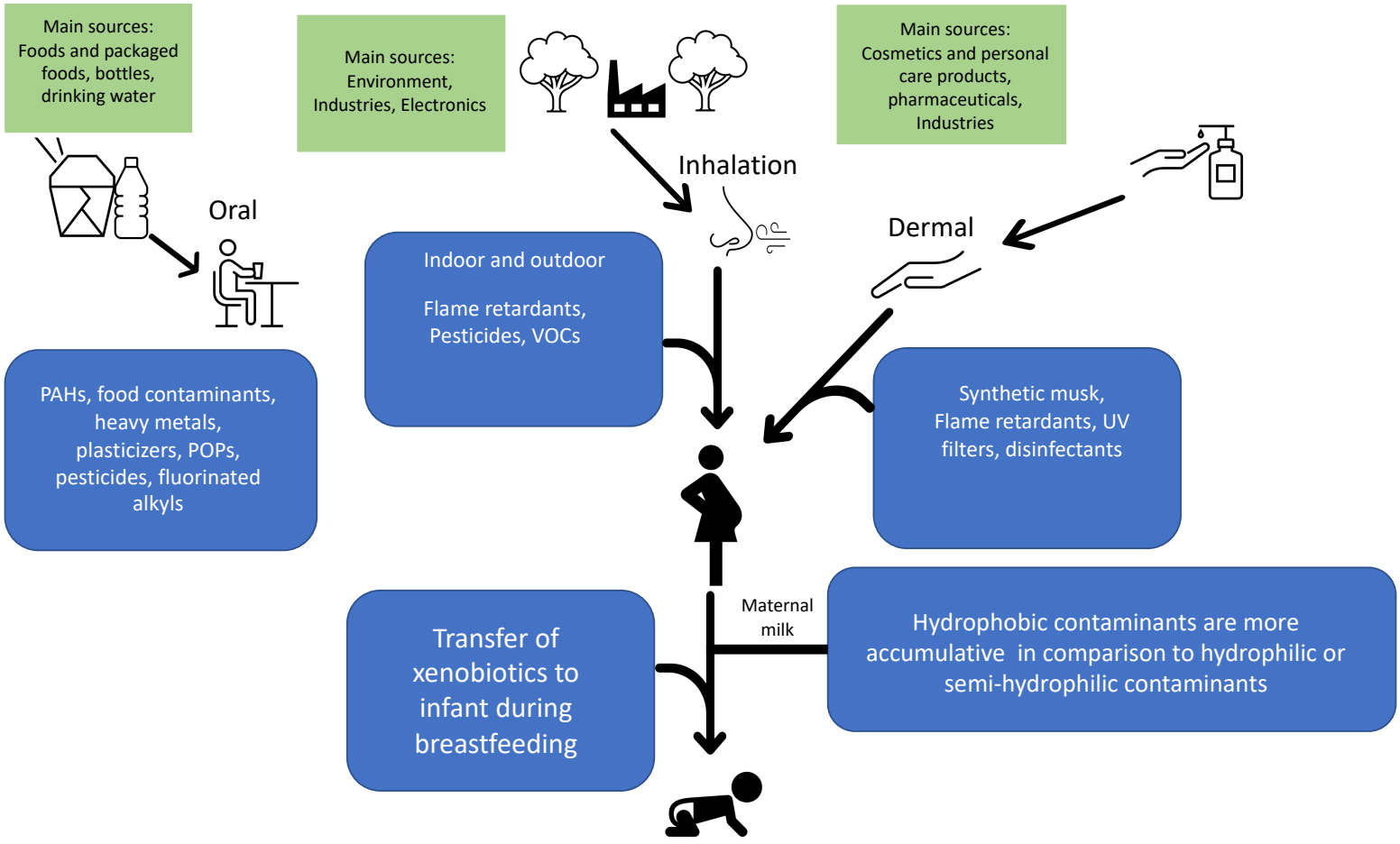

Table 2.2. Examples of main routes of exposure to xenobiotics for breast feeding mothers

	Inhalation		Ingestion	Ingestion	Dermal	Ref.
Xenobiotic	**Indoor**	**Outdoor**	**Food/Beverage**	**Dust**	**Personal care products or accidental contact**	
OCPs	×	×	×	×		**[128, 129]**
Pyrethroids	×	×	×	×		**[130]**
Neonicotinoids	×	×	×	×		**[131]**
OPPs	×	×	×	×		**[132, 133]**
PCBs	×	×	×	×	×	**[134, 135]**
PCDDs	×	×	×	×		**[136, 137]**
PCDFs	×	×	×	×		**[136, 137]**
PCNs	×	×	×			**[138]**
Chlorinated paraffins	×	×	×	×	×	**[139-141]**
PAHs	×	×	×	×	×	**[142]**
PBDEs	×	×	×	×	×	**[143, 144]**
Other halogenated flame retardants	×	×	×	×	×	**[145, 146]**
Triclosan	×	×	×		×	**[147]**
Synthetic Musk	×	×	×	×	×	**[148, 149]**
Per and polyfluoroalkyl	×	×	×	×	×	**[150, 151]**
Chlorobenzenes	×	×			×	**[152, 153]**
VOCs	×	×	×		×	**[154-156]**
Plastic additives and monomers	×	×	×	×	×	**[157]**
Mycotoxins			×	×		**[158, 159]**
Nitrate, nitrite and Nitrosamines			×			**[160]**
Heterocyclic Aromatic Amines (HAAs)			×			**[161]**
Acrylamide			×			**[162]**
MCPD fatty acid esters			×			**[163]**
Chloroethers			×	×	×	**[107]**
Heavy metals	×	×	×	×	×	**[164]**
Methylmercury			×			**[165]**
Radioactive isotopes	×	×	×	×	×	**[114]**

2.4. Characterizing health risks associated with chemical mixtures in human milk: current challenges and emerging approaches

Health risks associated with chemicals can be assessed for the mixtures as a whole or be based on individual components [166]. A key consideration in the assessment of chemical mixtures is to decide which chemical substances to consider together from a hazard perspective [167]. Grouping the mixture components can be based on structural similarities or on similarities in toxicological or biological responses/effects. Various guidance documents have been proposed in the last decade to harmonize methodologies for human health risk assessment of combined exposure to multiple chemicals [32, 167-170]. This section describes the current challenges and applications of emerging approaches for the analyses of chemical mixtures in human milk.

2.4.1. Exposure to chemical mixtures in human milk

Most biomonitoring studies of human milk were designed (in terms of sampling, sample preparation, instrumental analysis) to target specific substances, as depicted in Figure 2. Using this approach, known (target) compounds were detected and quantified. Although a wide variety of chemicals are found in human milk, concentrations in human milk (e.g., means, medians, ranges) are most often reported and interpreted for individual chemicals. In addition, chemical speciation and the metabolism or conjugation of contaminants in the body add complexity to the chemical mixtures that are present in human milk that may affect the overall toxicity of the mixture [171, 172].

The term “exposome” is used to describe the environmental exposures of an individual during their lifetime, i.e. from conception to death. Deciphering how this exposome may impact the health of

mothers and their infants is a major challenge. There is a need for the development of novel approaches and tools.

2.4.2. Speciation and conjugation of chemicals in milk

The characterization of contaminant speciation, i.e. the interaction of chemicals with molecules in milk, and the formation of conjugates are important aspect of human milk analyses. For example, the zinc in milk is bound predominantly to low-molecular-weight compounds such as citrate [173]. Although an exposure may be to inorganic mercury, organic mercury (ethylmercury or methylmercury) may be responsible for effects on the central nervous system of the infant at low doses [174]. In addition, studies have reported that binding of copper (II) to albumin renders it unavailable for cell uptake, and that the bioavailability of nickel (II) is reduced by the presence of certain organic ligands, such as histidine and cystine [175]. Methods have also been developed to investigate cadmium speciation in human milk and the interaction of this metal with proteins [176] . Even though the speciation of trace metals is related to their bioavailability and, eventually, their toxicity [173], data are available mostly for total metal concentrations in milk.

Many organic compounds and metabolites of parent compounds can be present either in their free and/or conjugated forms in human milk. Conjugation (e.g., glucuronidation, sulfation) may alter the bioavailability and decrease the potential toxicity of these chemicals [177]. Compounds such as bisphenols, phthalates, triclosan and parabens can undergo conjugation in the body and their conjugated forms may be less toxic than the parent compounds [178]. Although conjugated compounds are generally thought to be biologically inactive and are excreted by the body, one study has reported that glucuronidated bisphenol A induced adipocyte differentiation and altered adipogenesis [179], suggesting that differences in toxicity between free and conjugated

xenobiotics are still not fully understood. Conjugated xenobiotics such as bisphenols and phthalate metabolites can also undergo deconjugation which can prolong their endocrine disrupting activity in the body [180]. As such, measurement of their levels is crucial to improve current human milk biomonitoring. During the analysis, enzyme treatment (e.g., using β-glucuronidase/sulfatase) is generally necessary to quantify the total level of organic contaminants in milk, such as bisphenols, parabens and phthalate metabolites. A systematic comparison of the contaminant levels obtained when adding or omitting this step can be used to investigate the fractions of free and conjugated contaminants. A study of 120 breastfeeding women from Spain reported that the geometric mean for the ratio of unconjugated BPA to total-BPA in human milk was 0.54 (range from 0.16 to 1.0) [92]. In contrast, as reported in another study, the levels of conjugated parabens were generally higher than their free forms: the concentrations of free parabens ranged between <LOQ-31 ng/mL compared to total parabens ranging between <LOQ-49 ng/mL [181]. Characterization of the multiple species and fractions of a xenobiotic in milk will be an important contribution to our understanding of possible impacts on the child and mother.

2.5. Non-targeted analysis as a tool to improve characterization of chemical mixtures in human milk

Current biomonitoring studies have relied mostly on target analysis, which provides little information on other emerging, unexpected or unknown xenobiotics in the samples. NTA is a novel analytical approach that can be used to characterize the chemical exposome in multiple matrices [182]. NTA, using high-resolution mass spectrometry (HRMS), can systematically identify both known and unknown chemicals in a sample; these data can then be integrated with toxicological investigations to identify and prioritize chemicals of interest [183]. In particular,

suspect screening is applied when compound-specific information, such as the molecular formula, the chemical structure and the physicochemical properties of the suspects, are known. However, when reference standards for the suspects are not available, quantification of the analytes may not be possible [184].

To date, only a few studies have pursued a non-targeted screening approach to analyze known and unknown xenobiotics in human milk. Tran et al. reported the use of two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC/TOF-MS) for the non-targeted screening of halogenated compounds and non-halogenated cyclic and aromatic compounds in human milk [185]. A total of 172 compounds were identified tentatively, including 34 compounds which are not typically monitored in breast milk surveys, highlighting the capability of NTA to detect unexpected chemicals. Pourchet et al. [186] integrated both GC and LC-HRMS for the non-targeted screening of halogenated compounds in human milk. The resulting workflow was successful in identifying both legacy contaminants, such as HCB and *p,p'*-DDE, and some emerging halogenated compounds, such as 4-hydroxychlorothalonil, a metabolite of the pesticide chlorothalonil. Musatadi et al. [187] reported a method for the suspect screening of relatively polar compounds by LC-HRMS: a range of xenobiotics, such as 2-(8-hydroxy-4a,8-dimethyldecahydro-2-naphthalenyl)acrylic acid (sesquiterpene), 2,5-di-tert-butylhydroquinone (industrial chemical), 4-indolecarbaldehyde (algal metabolite), avobenzone (UV filter), piperine (alkaloid), saccharin (artificial sweetener) and shogaol (plant component), were identified in the milk of four donors from Spain. These first non-targeted studies on human milk illustrate the diversity of xenobiotics in human milk. In addition, data obtained through NTA may contain information about endogenous compounds/metabolites in milk [188].

With the integration of both targeted and non-targeted analysis, it is possible to obtain a more comprehensive characterization of chemical mixtures in complex matrices [189]. The importance of new approaches to assess the impact of combined exposures on risk is widely recognized today [190]. While NTA still faces challenges in terms of identification and quantification, strategies are being developed to tackle these issues. For example, Pourchet et al. [186] have proposed the spiking of a reference mix of 30 compounds to assess the performances of a non-targeted workflow for human milk. NTA working groups, such as the BP4NTA (Benchmarking and Publications for Non-Targeted Analysis Working Group), have also put into place standards to optimize the use of NTA across different matrices [191]. Overall, the use of NTA in human milk analysis could improve current human milk biomonitoring by detecting potentially unknown chemicals of concern, one aspect which the conventional targeted approach is unable to do.

2.6. Hazard assessment approaches and chemical mixtures

Human milk biomonitoring studies face particular challenges related to sample size, collection process, the representation of selected donors within a specific geographic region, and the harmonisation of analytical methods [14]. In the context of chemical mixtures, biomonitoring studies need to be large enough, so that statistics can be applied to the study results to generate probabilities of co-exposure for different combinations of substances and to interpret health outcomes [155].

2.6.1. Main different interactions of chemicals within a mixture

Generally, the term additivity is used to describe the interaction of components in a chemical mixture when the effects of the total mixture equal the sum of the individual chemicals [192].

However, other interactions are also possible [193]; these include potentiation, synergism and antagonism [192]. Potentiation occurs when the effect/toxicity of one chemical is enhanced in the presence of another chemical which alone does not have this effect [166]. Synergism is considered when the effect caused during exposure to two or more chemicals results in effects that are greater than the sum of the effects of the individual chemicals [193]. Lastly, antagonism is considered when two or more chemicals in a mixture have an overall effect that is less than the sum of their individual effects [194]. In one *in vivo* study that investigated the effects of exposure to endosulfan and cypermethrin on motor coordination in rats, impairment was observed after exposure to either chemical individually. However, exposure to a mixture of these two pesticides increased motor activity, suggesting that in combination they were had a synergistic effect [195]. The effects of chemical mixtures may also vary depending on the type of exposure. For instance, Yang et al. assessed the combined effect of endocrine disruptors such as bisphenol A and heavy metals on bioluminescent bacteria, and reported that BPA and heavy metal mixtures showed antagonism and additivity in the context of acute exposure, but displayed synergism and additivity in the context of chronic exposure [196]. Overall, despite the many *in vitro* and *in vivo* studies that have already been conducted on chemical mixtures, our understanding of the overall effects of multiple compounds for real life exposure remains limited, suggesting the need to develop new methods and to assess the impacts of chemical mixtures on the health of the breastfeeding infants.

2.6.2. Analysis of associations between exposure to chemical mixtures and infant development in biomonitoring studies

It is clear that there is a need to understand the effects of the complex chemical mixtures found in media such as human milk, the possible molecular interactions between individual components

(e.g., synergistic [194] or antagonistic effects), and their possible interaction(s) with non-chemical stressors. Univariate or multivariate analysis, as well as machine learning, have been applied to compare contaminant profiles in milk and to identify possible associations with the health and development of infants. For example, correlation analysis was applied to identify possible associations between the concentrations of PFAS detected in human milk and measures of infant development [197]. In a Norwegian study, linear regression and logistic regression analyses revealed that there was no correlation between the presence of PBDE flame retardants in human milk and the levels of thyroid simulating hormones in newborn infants [198]. Forns et al. used principal component analysis, elastic net and Bayesian model averaging to analyze the relationship between the concentrations of 24 different POPs (PCBs, PBDEs and DDT) in human milk and early child behavioral problems; these investigators reported that only *p,p'*-DDT concentrations were associated with behavioral problems [199]. In a study by Santonicola et al., a margin of exposure approach was used to assess the risks associated with the presence of several PAHs in breast milk, based on the sum concentrations of specific PAH markers (PAH8, PAH4 and PAH2) [31]. Summation of levels was also used to assess the risks associated with the organochlorine pesticides (a grouping of DDT isomers and metabolites and chlordane isomers) detected in human milk in Saudi Arabia [200]. TEF was applied to estimate the additional dose of dioxins in breast-fed infants [201]. Recently, Crépet et al. have suggested a step-by-step approach (including uncertainty analysis) to perform chemical risk assessment when relevant comprehensive data concerning specific effects and modes of action of the mixture components are not available [202]. This integrated approach was applied to assess the risk of neurodevelopmental and thyroid effects for infants associated with the occurrence of 19 substances in human milk.

2.6.3. Current hazard assessment approaches

The "hazard" associated with exposure to a chemical mixture can be evaluated as a whole or on the basis of the individual components of the mixture [166]. Several methods may be applicable for the assessment of chemical mixtures in human milk; these include grouping the mixture components based on structural similarities using a QSAR-based approach, grouping of the components by their toxicological effects by identifying chemicals having similar end-points and common toxic effects, or with dose addition approaches using hazard indices [166, 203]. These methods have been suggested by different governmental agencies, such as the Environmental Protection Agency and the Directorate-General for Health and Food Safety (DG SANTE) [166, 204].

For example, hazard indices, based on the available data for individual components, are summed to evaluate the non-cancer health risks associated with a chemical mixture [33]. Assuming dose additivity, hazard values (i.e. the ratio between exposure and the reference value), such as the acceptable daily intake for each component in the mixture, are added [33]. The common assumption for some chemicals, at least for certain government agencies, is that these chemicals have similar mechanisms of action. This approach has been used to assess the toxicity of a complex mixture involving dioxins and related compounds [205]. Each compound is assigned a relative potency factor, or Toxic Equivalency Factor (TEF), based on a comparison with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most toxic compound among the polyaromatic hydrocarbons that target the Aryl Hydrocarbon Receptor [206]. This premise was, however, challenged recently by an analysis of a mixture constituting of 8 compounds that did show dose additivity with respect to their effects on one endpoint (craniofacial malformations in zebrafish embryos) but had different mechanisms of action [207].

Aggregated and cumulative exposure from all sources to multiple chemicals must also be considered during the assessment [166]. Exposure assessment regarding chemical mixtures is often complicated and must often rely on simplifications and assumptions in cases where exposure data are not available [203]. The uncertainty in chemical composition of the mixture and the variation of certain components over time and environmental conditions further increases the difficulty in the exposure assessment of mixtures. The current limitations in characterizing and evaluating the risks associated to chemical mixtures suggest the need for further improvements of current risk assessment strategies.

2.6.4. Emerging hazard assessment approaches

Clearly, new hazard assessment approaches are needed to establish the toxicological profiles of the infinite number of chemical combinations present in real-world mixtures; it is not feasible to do this with traditional approaches, such as standardized animal toxicity tests. Tools such as high throughput *in vitro* toxicity tests and *in silico* assessments (e.g., quantitative structure-activity relationship (QSAR) models [208]) have emerged as alternatives to animal testing methods, and may be applied to mixtures. For example, QSARs have been developed to predict toxicities for binary mixtures [209]. Tools at the interface of hazard and exposure assessments (e.g., effect directed analysis) can also provide further toxicological understanding regarding the impact of real-world mixtures, as highlighted for the assessment of endocrine disrupting chemicals [210]. Paired with non-targeted screening, QSAR may provide insight into the possible risks associated with exposure to chemical mixtures [211]. Indeed, some applications of QSARs have been used to predict chemical transfer into breast milk [212, 213],

Novel strategies based on multi-omics appear to be particularly promising and were applied recently to biomonitoring. For example, Misra et al. [214] combined untargeted metabolomics, metagenomics and targeted protein/contaminant analyses to decipher the molecular link between chronic exposure to pollution and human skin dysfunction. Interestingly, a multi-omics approach was used recently to analyze the interactions among diet, the fecal metabolome and microbiota associated with breastfeeding in mother-infant dyads [215]. This approach has yet to be used to analyze the health risks associated with the chemical mixtures in human milk.

2.7. Conclusions

Hundreds of xenobiotics, with very diverse origins, are detected in human milk. The list of chemicals detected in milk includes contaminants of emerging concern, personal care products and other current-use substances reflecting lifestyles and it is growing. While some families of contaminants have been widely analyzed for human milk biomonitoring studies (e.g., POPs), there are other understudied types of chemicals (e.g., food-processing-induced chemicals and mycotoxins) that may be equally toxicologically relevant.

Furthermore, human milk contains relatively complex mixtures of xenobiotics, many of which have not been characterized. Knowledge gaps, with respect to both the individual chemicals and chemical mixtures, limit our understanding of the major routes of exposure to these chemicals and their possible impact on infant health and development. New methods, such as NTA to enable characterization of the human milk exposome in different populations, multi-omics approaches and other novel methods to assess the risk associated with contaminant mixtures in human milk will provide critical information, and could be game changers in assessing the impact of chemical mixtures on the health of both breastfeeding infants and their mothers.

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2.8. References

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2.9. Supplementary Information for:

Characterization of different contaminants and current knowledge for defining chemical mixtures in human milk: a review

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Table of Contents

This supplementary file contains a comprehensive description of the chemicals that have been reported in human milk.

1. Pesticides

Various types of pesticides are used globally, including organochlorine pesticides (OCPs), organophosphate pesticides (OPPs), synthetic pyrethroids, atrazine, lufenuron and, recently, neonicotinoids [1]. While the usage of pesticides is systematically regulated in developed countries to control pesticide residues in food and the environment, the indiscriminate usage of pesticides remains a problem in many developing countries [2].

Among the pesticides, OCPs are highly stable compounds used to control crop insect infestation [3]. Various OCPs are listed in the Stockholm Convention as persistent organic pollutants (see www.pops.int/). Although their usage has been restricted in many countries since the early 1980s, due to health and environmental concerns, OCPs are still present in the environment due to their persistence [4]. Because of the lipophilic nature of OCPs, they accumulate in human fat and milk, potentially leading to adverse health effects in both the mother and the infant [5].

Among OCPs, DDT was widely used as an insecticide until its restrictions in the 1970s in most countries [6]. However, in some developing nations, DDT is still used as an insecticide to control malaria since it is inexpensive [7]. DDT can be transformed through microbial or UV light degradation, or by metabolism into DDD (dichlorodiphenyldichloroethane) and DDE (dichlorodiphenyldichloroethylene) [7]. DDT, DDD and DDE can be absorbed by both plants and animals, leading to their accumulation in the tissue or fluids of living organisms [7]. For humans, exposure to DDT and its metabolites is primarily due to the consumption of meat, fish, poultry and dairy products [7]. While the use of DDT has been banned in most countries, and greatly reduced in others, residues of DDT and DDE are still commonly detected in food products due to their extreme persistence. The usage of other OCPs, such as heptachlor, dieldrin and aldrin, endosulfan and hexachlorobenzene, for pest control has also resulted in their widespread accumulation within

the environment and in living organisms [8]. Analysis and quantification of OCPs and their metabolites in breast milk is considered important due to their potential toxicity for both mother and breastfeeding infant [9].

OPPs, such as chlorpyrifos, chlorpyrifos-methyl, ethion, and dimethoate, are non-polar compounds and are used worldwide as insecticides, mostly in developing nations [10]. Their widespread presence in the environment may lead to the bioaccumulation of these compounds in the fatty tissues of various living organisms. Studies have shown that OPPs can be detected in human milk [11]. The metabolites of OPPs, such as diethyl thiophosphonate and diethyl phosphate, can also enter the human body via different routes of exposure (Table 2) [12].

Neonicotinoids are insecticides derived from nicotine which bind strongly to nicotinic acetylcholine receptors in the central nervous system of insects, causing overstimulation of their nerve cells, paralysis and death [13]. This relatively new class of pesticide is currently among the most widely used pesticides globally [14]. Neonicotinoids are water soluble, leading to their migration from treated plants into food sources, and their estimated half-lives in the environment can range between 7 to 1000 days [15]. They are primarily used for seed and soil treatment or on plant foliage [14]. Neonicotinoids, such as thiamethoxam, clothianidin, imidacloprid, acetamiprid and thiacloprid, have been detected in human milk, raising concerns that their accumulation may affect infant development during breastfeeding [13].

Pyrethroids, another type of insecticide, are derived from pyrethrins, insecticidal substances found in natural pyrethrum extracted from chrysanthemum flowers [1]. Synthetic pyrethroids, such as bifenthrin, cypermethrin, esfenvalerate, lambda-cyhalothrin, permethrin and tetramethrin, were detected in human milk [16]. Voltage sensitive sodium channels are considered the primary target

site for the toxicity of these chemicals in mammals [17]. The presence of pyrethroids in treated fields can lead to their accumulation in human milk, primarily through the diet [18].

High levels of neonicotinoids and pyrethroids were reported in human milk in countries with an increasing usage in pyrethroids over recent years, such as the United States and Brazil. In other countries with infrequent use of pyrethroids, such as India, relatively low or non-detectable levels of these compounds were reported [19]. The usage of both pyrethroids and neonicotinoids is currently increasing worldwide, especially in countries in which pyrethroids are used as a replacement for organophosphate pesticides [1].

Glyphosate (N-(phosphonomethyl)glycine) is used as an herbicide for pre-emergence weed control as well as for pre-harvest desiccation treatment [20]. Residues of glyphosate have been reported in foods containing barley, oats, sorghum, sunflower seeds, soybeans, wheat, rye, linseed, rapeseed, mustard seed, cotton seed, lentils, peas and lupins [21]. These residues in food can be absorbed by the human body and can potentially accumulate in human milk [22]. To date, however, studies on glyphosate and its major metabolite, aminomethylphosphoric acid (AMPA), have reported levels below the detection limit; the conclusion from these studies is that it is unlikely for these chemicals to accumulate in animal tissues and their presence is rarely reported in human milk [23].

Carbamates, such as N-methyl carbamates, are widely used as insecticides [24]. Carbamates inhibit acetylcholinesterases, which are responsible for hydrolyzing (deactivating) acetylcholine in the human nervous system [25]. Acetylcholinesterase inhibiting pesticides can lead to acute or chronic health issues. To date, studies have reported that levels of carbamates in human milk are below the detection level, possibly due to biodegradation of carbamates in the maternal intestine. Thus, carbamates are unlikely to impact on the development of breastfeeding infants [26]. Other pesticides such as atrazine (a triazine pesticide) have been detected in human milk [27]. Atrazine

is commonly used as a herbicide in countries such as the United States [28]. Atrazine is considered to be an endocrine disruptor as its exposure to humans may affect the hormonal systems [29]. Lufenuron is used in the control of flea infestations. A study on albino rats reported that exposure to lufenuron can lead to reproductive toxicity and genotoxic effects [30]. However, due to the low levels of lufenuron in human milk that were reported in a case study of its accidental ingestion by a mother, there is currently limited information as to its possible health effects in humans, including infants exposed to lufenuron during breastfeeding [31].

2. Polychlorinated biphenyls (PCBs)

The polychlorinated biphenyls, a suite of 209 chemicals, are another major class of POPs [32] that are persistent in the environment [33]. PCBs were used as dielectrics in transformers and capacitors in industrial activities. They have been also used as paint additives, heat exchange fluids, plastics and inks [34]. PCBs can enter the body through the consumption of contaminated foods, such as fish, or through skin contact with old electrical or laboratory equipment and have been detected in human milk worldwide [35]. Exposure to PCBs is associated with an increase in cancer, birth defects, growth impairment and hormonal imbalances [36]. Although PCBs may be conjugated, studies have shown that there is no, or a very limited, transfer of the glucuronide, sulfate or other conjugated forms of PCBs from mothers to their nursing children [37]. The absence or low levels of PCBs reported in human milk may be due to their excretion or elimination after undergoing enzymatic conjugation as these metabolites are commonly detected in the urine and plasma [38]. Although the Stockholm Convention prohibits any new production and use of PCBs, residues of these contaminants are still present in the environment today; a chronic diet of contaminated fish and wildlife may lead to potential adverse health effects [36].

3. Polyhalogenated dibenzodioxins and dibenzofurans

PCDDs/DFs, another major class of POPs, are found in soil, air, sediments and biota. They are by-products of various human activities, such as waste incineration, chemical manufacturing, petroleum refining, fuel combustion and wood burning [39]. Once released in the environment, they biomagnify in food webs due to their hydrophobicity [40]. These chemicals, through various exposure routes, can accumulate in human milk [9] (Table 2).

In addition to PCDDs, brominated dioxins (PBDD/DFs), such as 2,3,7,8-TBDF, have also been detected in human milk [41]. The presence of PBDD/DF residues was reported in soil, dust and air in e-waste recycling sites. The occurrence of PBDD/DFs in e-waste recycling sites has been associated with the high content of brominated flame retardants [42]. Studies have reported that PBDD/DFs are impurities in the technical formulations for PBDEs. The generation of PBDDs and PBDFs also occurs during waste incineration, or through degradation reactions under natural sunlight; these chemicals enter the body mainly through food intake or ingestion of house dust [41]. Due to the dioxin-like toxic effects of PBDD/DFs, affecting the aryl-hydrocarbon receptor, they have been recommended to be included in the WHO Toxicity Equivalency Factor (TEF) Program for the evaluation of their potential risks regarding human health, including that of breastfeeding infants [43].

4. Polyaromatic hydrocarbons (PAHs)

PAHs are ubiquitous contaminants formed from the incomplete combustion of organic materials from either manmade or natural sources. Human activities contribute to more than half of the annual global emissions of PAHs [44]. PAHs may be released from a wide range of anthropogenic sources, including coal plants, residential heating, coal gasification and liquefying plants, carbon

black, coal-tar pitch and asphalt production, coke and aluminum production [45]. PAHs can also be formed as a result of forest fires. PAHs are nonpolar and accumulate in the fatty tissues of living organisms, especially marine animals [46]. The atmospheric partitioning of PAH compounds between the particulate and the gaseous phases strongly influences their fate and transport in the atmosphere and the routes of exposure by which they enter the human body (Table 2) [44]. Hydroxylated PAHs have been identified as PAH metabolites in breast milk and may induce cellular damage due to the formation of epoxide intermediates and reactive oxygen species [47]. Since PAHs can cross the placenta, prenatal or early postnatal exposure may be associated with developmental toxicity [44].

5. Plastic additives and monomer residues

Bisphenols are used in the production of polycarbonate plastics and epoxy resins. Bisphenol A (BPA), an extensively studied bisphenol, has been used in the production of plastics, food can linings, dentistry sealants and thermal paper [48]. BPA is considered to be an endocrine disruptor, and its presence in human milk makes it an emerging contaminant of concern [49]. Analogs of BPA, such as BPS and BPF, and halogenated bisphenols, such as TBBPA and TCBA (Tris(2-hydroxyethyl) isocyanurate based charring agent), have also been detected in human milk. Epidemiological studies suggest that exposure to BPA and some of its analogs during early childhood development may lead to adverse health outcomes [50]. In addition to bisphenols, studies on human milk have detected glucuronidated, sulfated and oxidative BPA metabolites [49]. Studies with rats and cell bioassays have shown that these metabolites can also act as endocrine disruptors; indeed oxidative metabolites of BPA may possess enhanced endocrine activity [51]. In addition, oxidative forms of BPA, such as BPA *ortho*-quinone, can form covalent adducts with

DNA [51]. Furthermore, glucuronide metabolites of BPA may have other biological effects, such as on adipogenesis [52].

Plasticizers are low molecular weight additives used to increase the plasticity or decrease the viscosity of a given polymer solution [53]. During plastic production, plasticizers make polymer solutions more suitable for film coating. Plasticizers are classified according to their function or structure, and can be considered to be either primary or secondary [54]. Plasticizers can be released from products via volatilization (evaporation), extraction (into contacting liquids) or migration (into contacting solids), and enter the human body. Exposure can also occur through medications formulated with phthalates [55]. A wide range of chemical structures are used as plasticizers, including dipates, azelates, citrates, benzoates, ortho-phthalates, phthalates, terephthalates, sebacates and trimellitates [56]. Phthalates are one of the main classes of plasticizers used in the manufacture of common consumer products, such as food wrapping, personal care products and cleaning materials [57]. High production volume phthalates, such as di(2-ethylhexyl) phthalate (DEHP), di-n-butyl phthalate (DnBP), di-iso-nonyl phthalate (DiNP) and di-isodecyl phthalate (DiDP), are found in a variety of applications and products, such as PVC, wiring insulation, personal care products and toys [58]. Since phthalates are not chemically bound to polymers, they are easily released by evaporation or by abrasion, and migrate into the human body though different exposure routes (Table 2) [59]. Phthalates may be metabolized to their monoester forms and then further to oxidative products. Metabolites of phthalates, such as MEHP, MnBPn and MiBP, can accumulate in human milk [59]. Some papers have reported that the relatively more hydrophobic phthalates and their monoester metabolites may be more abundant in milk than in urine [58]. Di-n-butyl phthalate (DBP), DEHP and DiNP, along with their monoester metabolites, are among the more abundant phthalates in human milk [60]. Other plasticizers, such as adipates

(mostly DEHA), have also been detected in human milk [61]. Some phthalates were reported to have endocrine-disrupting effects in animal studies [62] so their presence in human milk in high concentrations may be of concern.

6. Synthetic phenolic antioxidants

Anthropogenic antioxidants, such as the synthetic phenolic antioxidants (SPAs) used in foodstuffs, food packaging materials or personal care products, have also been detected in human milk [63]. 2,6-Di-*tert*-butyl-hydroxytoluene (BHT), 2-*tert*-butyl-4-hydroxyanisole (BHA), 2,4-di-*tert*-butylphenol (DBP) and 2,2′-methylenebis(4-methyl-6-*tert*-butylphenol) are among the main anthropogenic antioxidants in use [63]. Degradation of synthetic antioxidants such as BHT, either through biotic or abiotic transformation, can lead to the formation of toxic products such as 2,6-di-*tert*-butyl-4-(hydroxymethyl) phenol (BHT-OH), 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde (BHT-CHO), 2,6-di-*tert*-butyl-1,4-benzoquinone (BHT-Q) and 2,6-di-*tert*-butyl-4-hydroxy-4-methyl-2,5-cyclohexadienone (BHT-quinol) [64]. The widespread occurrence of these synthetic antioxidants has become a growing concern due to their toxicity in fish and rats [65]. SPAs were detected in human milk, raising the question of risks to breastfeeding newborns [66].

7. Alkylphenols

Nonylphenol and octylphenol are non-ionic surfactants used in industrial applications, personal care products and household applications [67]. The release of nonylphenol ethoxylates from surface materials, such as during volatilization, can lead to human exposure through air, water and food [68]. Both nonylphenol and octylphenol were detected in human milk [67]. While

alkylphenols are known to be estrogen, androgen and thyroid agonists, there is currently only a limited understanding of their overall impact on the development of breastfeeding infants [57, 69].

8. Triclosan, Veterinary drugs, Ortho-Phenylphenol and Synthetic Musks

Residues of pharmaceutical compounds, antibiotics and personal care products (e.g. shampoos, moisturizers, perfumes, etc.) were also detected in human milk. Triclosan is added as an antimicrobial agent to some consumer products, including antibacterial soaps, toothpastes, body washes, detergents, toys, cosmetics and surgical cleaning treatments [70]. Triclosan residues have been reported in soil, surface waters and several wastewater treatment plants [71]. Exposure to various sources of triclosan can lead to bioaccumulation within fatty tissues [72]. Another compound detected in human milk is ortho-phenylphenol; this chemical acts as a fungicide and antibacterial agent to control fungal and bacteria growth on fruits and vegetables and is used commercially in food industries [73].

Antibiotics are used in veterinary and agricultural practices to treat infections and prevent diseases [71]. They can also be used at sub-therapeutic levels to increase feed efficiency and to promote growth in food producing animals [74]. The presence of beta-lactam, quinolone, aminoglycoside sulphonamide and macrolide residues has been reported in human milk samples [75]. Residues of anti-inflammatory drugs, such as tolfenamic acid, meloxicam and metamizole, have also been detected in human milk [75]. The use of veterinary and agricultural drugs in animals should be supervised to prevent any potential health impacts concerning the development of the breastfeeding infant [75].

Synthetic musks, including nitro, polycyclic and macrocyclic musks, are chemicals used as personal care product fragrances [76]. Nitro musks, such as musk xylene and musk ketone, have been widely used in detergents and cosmetics, but their production in Europe has declined since

the 1980s [77]. Currently, polycyclic musks, such as 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB) and 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (AHTN), are widely used, comprising about 95% of the European market and 90% of the United States market in the past decades [76]. Synthetic musks can accumulate in adipose tissues and have been detected in human milk [78]. Transfer of these compounds to the breastfeeding infants suggests the need for further investigations to determine if there is a risk associated with this exposure.

9. Perfluoroalkyl and Polyfluoroalkyl Substances (PFAS)

Perfluoroalkyl and polyfluoroalkyl substances are characterized by a fluorinated hydrophobic linear carbon chain attached to one or more hydrophilic heads. PFAS repel both water and oil, making them ideal chemicals for surface treatment. These compounds are used in various industrial products, as stain repellents and in textiles, paints, adhesives, polishes and electronics [57]. The use and disposal of PFAS has led to their ubiquity in the environment. It is also important to note that once they are in the environment, per- and poly-fluorinated compounds can be converted into by-products [79] that may accumulate in human tissues, leading to a variety of adverse health effects [80]. For example, fluorotelomer alcohols (FTOHs), a major type of PFCs, undergo reactions to produce compounds such as PFOS, PFOA and perfluorocarboxylic acids (PFCAs). Metabolites of neutral PFCs include fluorotelomer alcohols (PFTOHs), perfluorinated sulfonamides (PFASAs) and perfluorinated sulfonamide ethanols (PFASEs) [80]. Another degradation pathway was observed for *N*-alkyl perfluorooctane sulfonamidoethanols (e.g. N-EtFOSA or N-MeFOSA), which are used in paper-protecting applications or are reacted with acrylic or methacrylic acid to form the respective polymers [81]. It was reported that N-EtFOSE may be converted into N-EtFOSA or N-MeFOSA during biodegradation [82]. PFOS and PFOA

are the final metabolites produced from N-EtFOSA, N-MeFOSA or N-EtFOSE by several oxidation and N-dealkylation steps and cannot be further degraded during a hydroxyl radical-initiated oxidation [83]. PFAS can accumulate in food products of animal origin; their accumulation in fish is considered to be the main source of PFAS in the human diet. Greaseproof packaging, food contact papers and fast food packaging may also introduce PFAS into food matrices, leading to the accumulation of PFAS in the human body and transfer to human milk [84]. Studies in animals suggest that some of these fluorinated compounds act as endocrine disruptors, reduce immune function and cause adverse health effects in the liver and pancreas [85]. Furthermore, epidemiological studies suggest that exposure to these fluorinated compounds may be associated with adverse effects on fetal and postnatal growth and on immune function [86]. Some studies have also suggested that fluoroalkyl substances may affect neurodevelopmental endpoints [86].

10. Flame retardants: OPEs, PBBs, PBDEs and HBCDD

Flame retardants are compounds added to manufactured materials, such as plastics, textiles, surface finishes and coatings, to suppress or delay the production of flames and prevent the spread of fire [57]. The use of halogenated (chlorinated and brominated) flame retardants was extensive due to their effectiveness and cost efficiency [87]. Other types of flame retardants include inorganic flame retardants, nitrogen based flame retardants and phosphorus containing compounds, such as the organophosphate esters (OPEs) [88].

Halogenated flame retardants are widely detected in the environment, household dust and people [89] and bioaccumulate. Exposure to brominated and chlorinated flame retardants in high amounts may lead to adverse health issues as they may have endocrine disrupting and immunosuppressive

effects [90]. Brominated flame retardants, including polybrominated biphenyls (PBBs) and polybrominated diphenyl ethers (PBDEs), are used to reduce the flammability of a wide range of products, such as paints, plastics, textiles and electronics [91]. These contaminants are relatively stable and resistant to biodegradation [92]. The primary exposure pathway to these chemicals for humans is thought to be the ingestion of dust [93]; high levels of exposure have been reported among workers involved in e-waste recycling [94]. Hexabromocyclododecane (HBCDD) is another flame retardant that has been used mainly as an additive in textiles and polystyrene products. Technical HBCDD consists of approximately 12% α-HBCDD, 6% β-HBCDD and 80% γ-HBCDD [95]. With a log K_{OW} value of 5.6, technical HBCDD has a high bioaccumulative potential [96]. An increase in HBCDD concentrations in human milk was observed over the past few decades due to its widespread use [97].

Organophosphorus flame retardants, such as the organophosphate esters (OPEs), are used increasingly in household and industrial products, such as electronic and electrical devices, building and decorative materials, furniture, plastics, vehicle parts, lubricants and hydraulic fluids [98]. OPEs are also used as plasticizers and antifoaming agents in industrial processes [99]. Since OPEs are not chemically bound to products, they can easily migrate into the environment and have become ubiquitous in water, soil, dust, indoor air and foodstuffs. After entering the human body, OPEs can be metabolized to their respective diester forms (di-OPEs) or hydroxylated metabolites (OH-OPEs) [99]. Studies have shown that OPEs accumulate in the fatty tissue of nursing mothers and can be transferred to human milk [100]. Accurate determination of exposure levels of different types of OPEs in human milk will be necessary to determine their potential health risks.

11. Mycotoxins

Mycotoxins are toxic secondary metabolites produced by a wide variety of fungi species, such as *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria,* that contaminate crops and foodstuffs worldwide [101]. Consumer exposure to mycotoxins is a major public health issue in many countries, as it has been associated with a range of adverse health effects, including liver cancer, immune-modulation, inflammation, kidney toxicity, stunted growth and endocrine changes [102]. Mycotoxins are generally heat resistant compounds and are also resistant to weak acids, but they can be degraded under pressure cooking, oxidation and alkali conditions [103]. Aflatoxin M1 may be used as a biomarker to evaluate aflatoxin exposure in diets for both animals and humans [104]. Mycotoxins, such as aflatoxins, fumonisins, zearalenone, ochratoxin A and deoxynivalenol, have been detected in human milk, and reported levels may exceed recommended tolerable daily intakes for some of these toxins [105].

12. Other chlorinated compounds such as chloroethers, PCNs and chlorinated paraffins (CPs)

Chloroethers, such as bis(2,3,3,3-tetrachloropropyl)ether, were used in Japan in the early 1970s as synergists in pyrethrum insecticides. Residues of this chemical were detected in human milk in certain Japanese populations during the 1980s [106]; follow up publications as to their possible adverse health effects have been limited.

Polychlorinated naphthalenes (PCNs) were used as insulating materials for cables and in lubricants or other commercial goods starting in the 1910s and until their restriction in the 2000s [107]; these restrictions were accompanied by the introduction of PCBs. Several PCN congeners have physicochemical and toxic properties similar to the dioxins [107, 108]. Human exposure to PCNs is through inhalation, dietary intake or other pathways. Due to evidence that these chemicals are

toxic, persistent and bioaccumulative, PCNs were listed as POPs in the Stockholm Convention in 2015. The health impact of PCN exposure in breastfeeding infants remains unclear since studies of their levels in milk are scarce [109].

Chlorinated paraffins (CPs) are complex mixtures of polychlorinated *n*-alkanes and commercial CP products that can be divided into three groups: short-chain CPs (SCCPs, $C_{10–13}$), medium-chain CPs (MCCPs, $C_{14–17}$) and long-chain CPs (LCCPs, $C_{18–30}$) [110]. CPs are extensively used around the world as metal-cutting fluids, flame retardants, plasticizers and sealants [110]. Since CPs are highly lipophilic (log K_{OW} 4–12), they bioaccumulate in humans and wildlife. CPs have been identified as potential hazardous chemicals: articles on the effects of CP on the environment, wildlife, and humans were published starting in the early 1980s [111]. However, the complexity of the structure of CPs has been an obstacle to quantitative analyses. Detection and quantification of CPs, especially in complex biological matrices such as human milk, have become easier in recent years due to advances in analytical methods and instrumentation [110]. Levels of CPs that are now quantifiable in human milk should lead to a better understanding of their potential health impact in breastfeeding infants.

13. Food processing associated contaminants

Food processing induces chemical changes in food materials. These processes, either at an industrial scale or in the domestic kitchen, can lead to the formation of undesired by-products that may be associated with health concerns [112]. Several process-induced contaminants have been identified, including acrylamide, glyoxal, furans or methylfurans, monochloropropanediol esters (MCPDEs) and glycidyl esters [113]. Some chemicals, such as the heterocyclic aromatic amines formed from proteinaceous foods during heat processing or flavor forming, are absorbed by the human body. These heterocyclic aromatic amines may be mutagenic and carcinogenic [114]. The

formation of nitrosamines in foods has also been observed [115]. Nitrosamines such as N-nitrosodimethylamine (NDMA) are known as carcinogens. Exposure to nitrosamine can also occur through medications [116]. Although food industries use mitigation strategies to reduce the formation of these compounds in processed foods, residues of these by-products have been detected in human milk, most notably acrylamide, MCPDEs and heterocyclic amines [113]. The management of these heat-induced contaminants remains an important task in food processing.

14. Metal residues

Traces of metal residues can enter the body through the diet and have been detected in human milk. The levels of metal residues detected in human milk vary across regions [117]. For instance, a study reported that the milk from women living in an industrial/mining zone had higher levels of aluminum, zinc, arsenic, lead, mercury and nickel compared to women living in agricultural zones, who had higher levels of manganese, chromium and iron [117]. High exposure levels of heavy metals such as mercury, lead, cadmium and arsenic can lead to significant dose-related toxicological implications [118]. High levels of these heavy metals can impact the circulatory, respiratory, endocrine, immune, nervous, urinary and reproductive systems [118]. These metals are also known as carcinogens [119] and can be transferred to the breastfeeding infant [120]. A better understanding of the potential health impacts that heavy metals may have on the early breastfeeding infants is an important task for improvement of current human milk biomonitoring programs [120].

15. Other xenobiotics (medications, plant-based residues and food-related compounds) in human milk

Other contaminants that have been detected in human milk include various types of chemicals used in everyday life. The residues of medications, drugs and alcoholic drinks are infrequently reported in human milk and are generally present in small quantities [121]. Concerning the prescription or non-prescription drugs taken by the mother, antibiotics and residues of analgesics, antihistamines and sedatives have all been detected in human milk; their impact on the development of the breastfeeding infant is not clear [122]. Within these categories, several drugs may affect the volume of milk that is produced; these include levodopa, phenelzine tranylcypromine, ergocryptine, barbiturates and apomorphine [123].

Plant-based chemicals, such as tobacco metabolites (nicotine and cotinine), have also been reported in human milk obtained from both smoker and non-smoker donors [124]. Other plant-based xenobiotics originating from the diet may be present in human milk. For instance, fennel seed, coriander seed and chamomile flower contain volatile oils, anisic acid, coriandrol and bitter glycoside, all of which have been detected in human milk [125]. The constituents of other herbs, including lemongrass, borage leaf, blessed thistle leaf, star anise, comfrey leaf, fenugreek seed, as well as coffee plant and blue cohosh, may also be transferred to milk through maternal oral intake [126].

The presence of food-related compounds, such as caffeine, alcohol [124] and nitrites/nitrates [127], has been reported in human milk. Various food additives known as non-nutritive sweeteners (sucralose, saccharin, aspartame, acesulfame-k) can also be transferred to human milk [128]. Piperine can also be found in breast milk after maternal ingestion of peppers [129]. However, studies detecting these chemicals in human milk remain scarce. To date, saccharin, sucralose and acesulfame-potassium have been detected in human milk samples, suggesting the need for further

studies to determine whether early exposure of non-nutritive sweeteners or other food-related chemicals via breast milk to breastfeeding infants may have clinical implications [128].

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Connecting paragraph

Following this extensive review, plastic-related contaminants were chosen as the family of contaminants to be studied in human milk for this research, specifically bisphenol A and its main analogues. The decision to investigate these compounds came from a noticeable gap in data, particularly in most African countries where limited information on their levels exists. Furthermore, few to no studies have assessed differences in bisphenol levels for human milk across different regions, highlighting the need for further investigation. To enhance our understanding of bisphenol levels in human milk from data-poor countries and improve current human milk biomonitoring, Chapter 3 covers the analysis of bisphenol A and its 8 selected analogues, and the comparison of their detected levels in Canada (Montreal) and South Africa (Pretoria and Vhembe). This chapter has been published in *Environmental Pollution* as "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, Lan LIU, Jingyun ZHENG, Lei TIAN, Jonathan CHEVRIER, Riana BORNMAN, Muvhulawa OBIDA, Cynthia Gates GOODYER, Barbara F. HALES, Stéphane BAYEN, date of publication on March 03, 2024).

3.0. 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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3.1. Abstract

A sensitive modified QuEChERS extraction method was developed to assess the levels of free and conjugated bisphenols 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 those for BPA in South Africa, with detection frequencies of 57% and 21% in Vhembe and Pretoria, respectively. In contrast, total BPS was the major bisphenol 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).

Key words: bisphenols, human milk, biomonitoring, liquid chromatography–mass spectrometry, South Africa, Canada

3.2. 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 [1, 2]. Because of incomplete polymerization, monomers such as BPA can leach out from these matrices [3]; humans are continuously exposed to BPA, primarily through dietary intake [4, 5]. 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 [6]. During the past two decades BPA has been widely recognized to act as an endocrine-disrupting chemical [7]; 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 [8]. This has led governmental agencies in various countries to impose regulations on its use in specific products [9]. Recently, the European Union has introduced regulations on the use of BPA in food contact materials [10]. Specifically, these regulations 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 [11].

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 [12]. Specifically, exposure to BPA alternatives such as BPS and BPF has increased during the past decade [13]. 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 [14-18]. 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 [18].

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 [19]. 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 [20-22]. Some studies have suggested that conjugated forms of BPA have little estrogenic activity [23], but a more recent study provided evidence that some conjugates, such as BPA-glucuronide, induce adipocyte differentiation and alter adipogenesis [24]. 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 [25]. 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 [26-28]. In this present study, we: (i) developed and validated a robust QuEChERS extraction method for the identification and quantification 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.

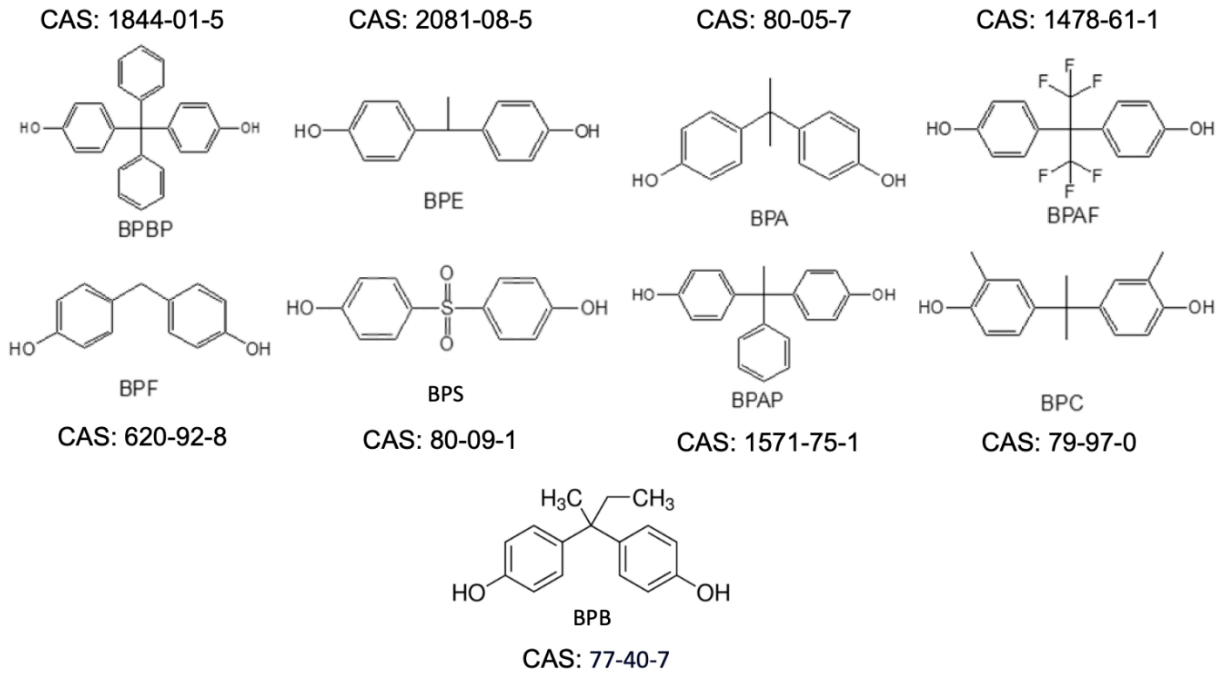
3.3. Material and methods

3.3.1. Chemicals

LC-MS grade Ammonium acetate (NH4Ac), acetic acid, and HPLC-grade solvents (water, acetonitrile, methanol) were purchased from Fisher Scientific. Magnesium sulfate (MgSO4) 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 (Figure 3.1) 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-d4 (≥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.

Figure 3.1. Structures and CAS numbers of BPA and bisphenol analogues



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.

3.3.2. 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 Limpopo Province, and Tshwane Hospital, located in 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 4^{th} and 8^{th} 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.

3.3.3. 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.

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, one milliliter of the prepared 1 M ammonium acetate buffer solution was added to the sample followed by vortexing for 10 seconds. Subsequently, 10 mL of acetonitrile was added to each milk sample and the sample was subjected to ultrasonication for 10 minutes 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 sec 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 human milk sample underwent testing twice: once without enzymatic treatment and another time following deconjugation.

3.3.4. 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; 10 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 (ESI-) electrospray ionization mode for analysis of the 9 BPs. The flow rate of the drying gas (nitrogen, 325 °C) was set at 5 L/min. 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 $[M-H]^-$ 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) (Figures S3.2–S3.10).

Compound identifications and confirmations were based on SANTE/11813/2021 [29] 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 chromatograph peaks for all 9 spiked bisphenols in human milk are shown in Figure S3.11. The total ion chromatograph of a human milk sample and a calibration solvent is shown in Figure S3.12. 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 S3.1. and S3.2).

3.3.5. 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 include 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 [30]. The matrix effects (ME%) for all 9 bisphenols were calculated using the equation below and are shown in Table S3.3:

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

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 [29]. 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 (Figure S3.13) compared to 1:1 ACN/methanol. Aliquots of the native bisphenol standard mixture solution and the labeled 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 to 100 ng/mL) of the target analytes, 30 ng/mL for the mass labeled 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 S3.3). The method detection limit (MDL) was calculated as three times the standard deviation of procedural blanks [31]. 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 [32].

3.3.6. 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 [33], 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 [14]. 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 bisphenols 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 bisphenols in terms of their conjugation potential, a one-sample t-test was conducted by comparing the calculated percentage of conjugated bisphenols with a value of 0% (0% conjugation) for all detectable bisphenols 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).

3.4. Results and discussion:

3.4.1. 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 S3.4). 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 [34-36]. Factors influencing these compositions are considered to be genetic or environmental [37]. 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.

3.4.2. Method validation

The variations between the measured *m/z* values and the theoretical values for all bisphenols were below 3 ppm, as presented in Table S3.1, and no major differences were observed among the values. The retention time consistency between the solvent standards and the actual samples for all bisphenols exhibited a difference of 0.05 min; this difference is within the acceptable tolerance of 0.1 min [29]. The retention times of spiked bisphenols 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 S3.3). This linearity was considered acceptable for establishing the calibration curve and is comparable to the linearity reported by Dualde et al. for bisphenol analogues [14].

Matrix effect analysis revealed that eight bisphenols exhibited a "medium" matrix effect (20%-50%), while BPBP exhibited a "strong" matrix effect (>50%) (Table S3.3) [38]. 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 interference 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, with the goal of detecting 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, employing matrix-matched calibration and optimal sample preparation, the mean recoveries of the 9 bisphenol compounds spiked at 100 ng/mL ranged between 75% to 95%, which is within the acceptable range of 70-120% (Table S3.5) [39]. 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 3.1).

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 S3.5) and the recoveries for each major bisphenol 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 bisphenols 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 to 6.5%), which demonstrates satisfactory precision in the analysis [40]. Other studies of the bisphenols in human milk [14, 15, 18, 41-45] have reported comparable recoveries for all bisphenols, their corresponding RSD%, as well as their MDLs. Table S3.6 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.

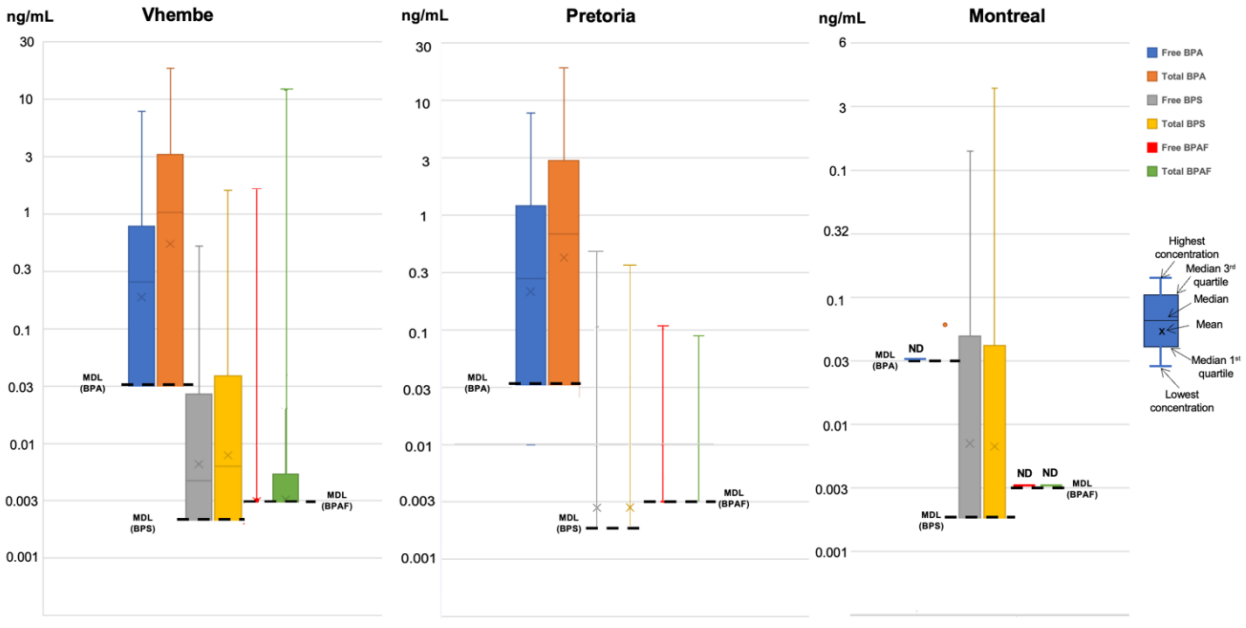
Three distinct samples from Vhembe, which had previously undergone analysis, were subjected to re-extraction and re-analysis to further assess the precision of the employed extraction method (Table S3.7). These results indicate the presence of only BPA in both the first and second extraction, displaying minimal variation in their levels. The other major bisphenols 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 Figures S3.14-3.16. In summary, the current method demonstrates sensitivity, acceptable precision, and accuracy.

3.4.3. Bisphenol levels in South African and Montreal human milk

Six bisphenols (BPF, BPC, BPE, BPB, BPBP and BPAP) were below the method detection limit in all samples. BPA, BPS and BPAF were detected in breast milk samples from both the Vhembe and Pretoria regions in South Africa (Figure 3.2, Table 3.1): 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 S3.8). Details on individual bisphenol levels are discussed below.

Figure 3.2. Levels of bisphenols in human milk from South Africa (Vhembe and Pretoria) and Montreal (ng/mL)*



**Values below the MDL are not reported in Figure 3.2. Total levels refer to the sum of free and conjugated bisphenols.*

3.4.4. 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 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.

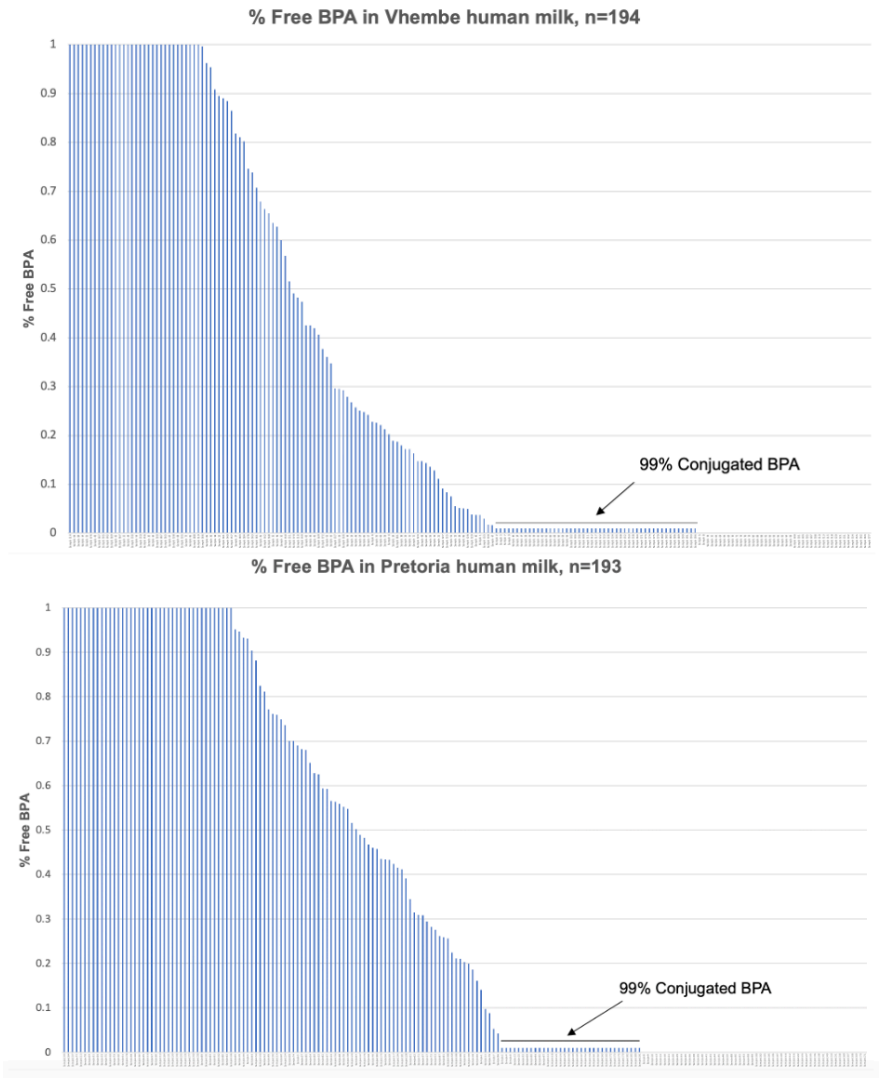
For Pretoria, the levels detected were comparable to those in the Vhembe district, with total BPA ranging between <MDL-19.38 ng/mL and free BPA from <MDL-7.78 ng/mL. The detection frequencies for total and free BPA in Pretoria were similar to those in Vhembe: 68% and 54%, respectively. The GMs for total 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 3.1): 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(s) 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 [46]. 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 [47], the amount of free BPA that is present in human milk may be of biological significance in breastfeeding infants [48]. 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% (Figure 3.3). The geometric means (GM) were 12% and 21%, and the medians were 22% and 52%, respectively (Table S3.9). In other words, BPA was mostly present in its conjugated form in South African samples.

Figure 3.3. % Free BPA distribution in human milk from Vhembe and Pretoria



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 [14, 41]. 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 S3.9). Country differences in the

amount of free BPA detected may be due to many factors, such as the diet, environment, and lifestyle of the mothers [47, 49]. 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 [14]. Çiftçi et al. also reported higher BPA levels in mothers who consumed fast food at least once a month compared to those who did not consume fast food [6]. 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 [50-52]. Additional investigations are warranted to better understand the difference in free BPA content observed among countries.

Table 3.1. 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
				DF (Detection frequency) (%)	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) [14]
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) [41]
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) [43]
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) [46]
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) [18]
China n=181	2014	UPLC-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) [15]
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-Goślińska et al. (2021) [45]

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 . [44]
Country	**Sampling year**	**Instrument (Method)**	**MDL LOD (LOQ)**	**Free BPS**				**Total (free and conjugated) BPS**				**Study and publication year**
				DF (%)	**Range (ng/mL)**	**GM (ng/mL)**	**Median (ng/mL)**	**DF (%)**	**Range (ng/mL)**	**GM (ng/mL)**	**Median (ng/mL)**	
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) [14]
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) [15]
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-Goślińska et al. (2021) [45]
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) [44]

Country	Sampling year	Instrument (Method)	MDL LOD (LOQ)	Free BPAF				Total (free and conjugated) BPAF				Study and publication year
				DF (%)	Range (ng/mL)	GM (ng/mL)	Median (ng/mL)	DF (%)	Range (ng/mL)	GM (ng/mL)	Median (ng/mL)	
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) [15]
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-Goślińska et al. (2021) [45]
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. (2021) [44]

[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

3.4.5. 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 frequency for 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 [53].

A comparison with other studies (Table 3.1) 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 [44]. Conversely, the levels of BPS detected in Pretoria were similar to those previously reported in studies from China (9 Provinces), Spain, and Poland [14, 15, 45].

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 [54]. 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 [55].

3.4.6. 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 3.1). 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 [14, 44]; 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 [15]. 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.

3.4.7. Research implications

Our study is the first to detect BPA levels in South African human 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. This 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 [56]. Another study by Cao et al. reported low levels of BPA in Canadian human milk samples collected between 2009-2011 [41]. Interestingly, a recent study reported no traces of any type of bisphenol in Montreal drinking water [57].

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. [58] 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 [59]; 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 [60]. BPA has also been detected in South African foods, most notably in packaged vegetable composites and canned tuna [58]. 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.

3.4.8. Conclusion

In conclusion, exposure to plastic-related contaminants, such as bisphenols, pose potential health risks to both mothers and infants. 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.

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3.6. Supplementary information

Section 3.6.1. QuEChERS Extraction Optimization

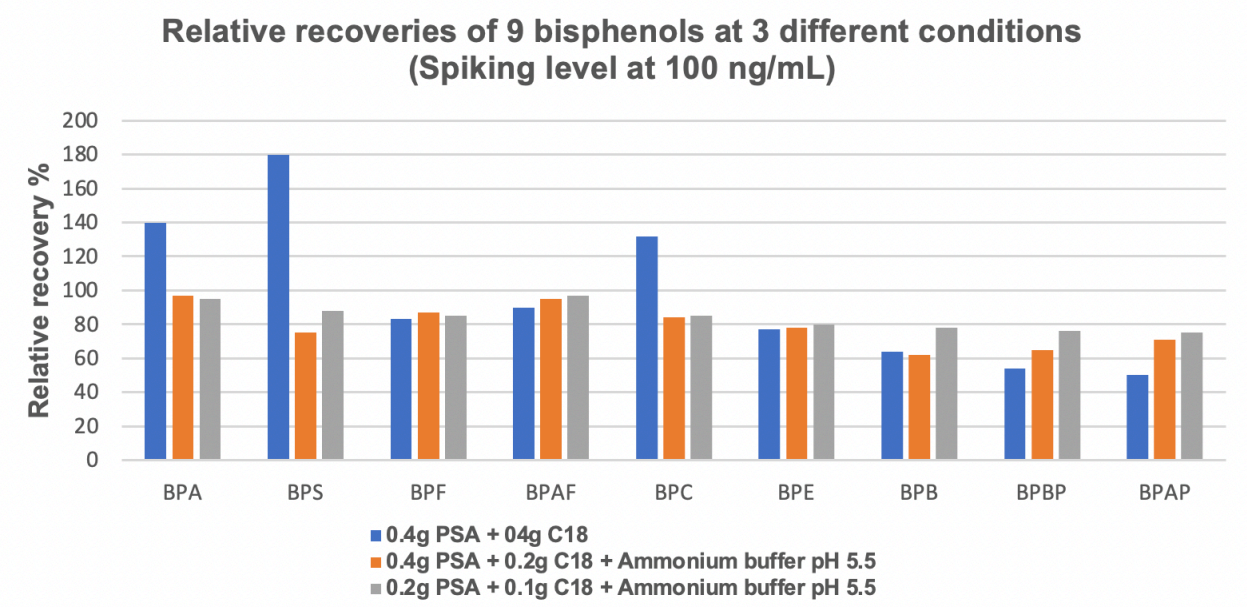
The recoveries of the analytes during QuEChERS extraction can be influenced by different factors including the amount of sample, the type and volume of the solvent, as well as the nature and quantity of the salt employed [1]. Another crucial factor when dealing with intricate matrices such as human milk 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 S3.0.

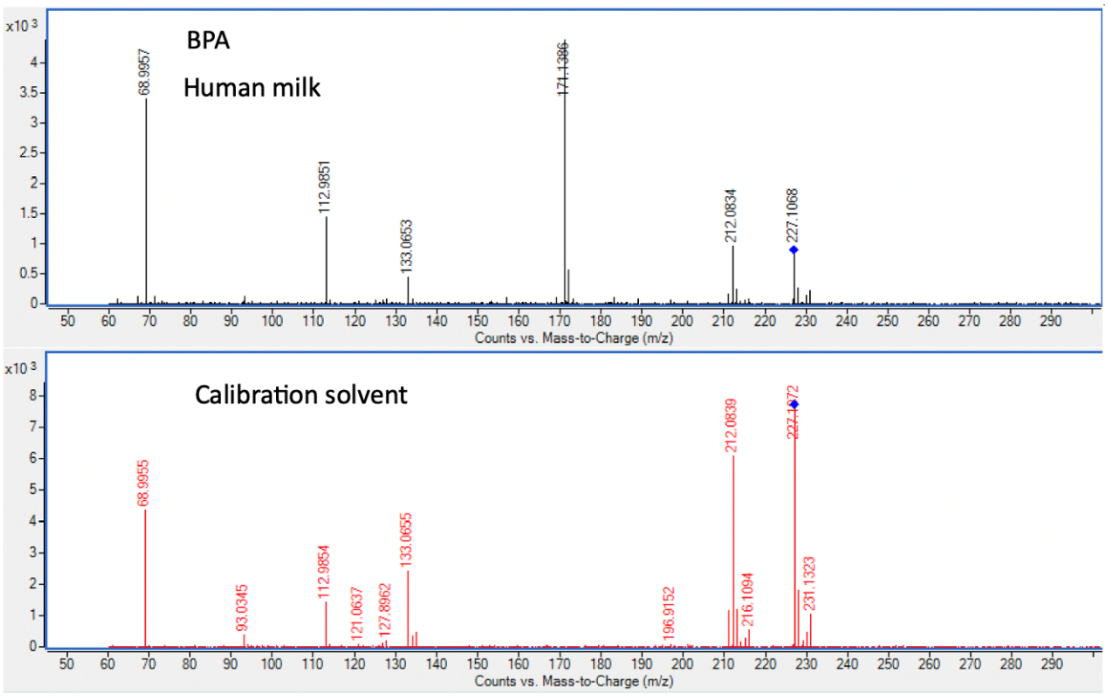


**Relative recoveries of BPC and BPBP obtained using $^{13}C_{12}$-BPF; Relative recoveries of BPE, BPB and BPAP obtained using $^{13}C_{12}$-BPAF*

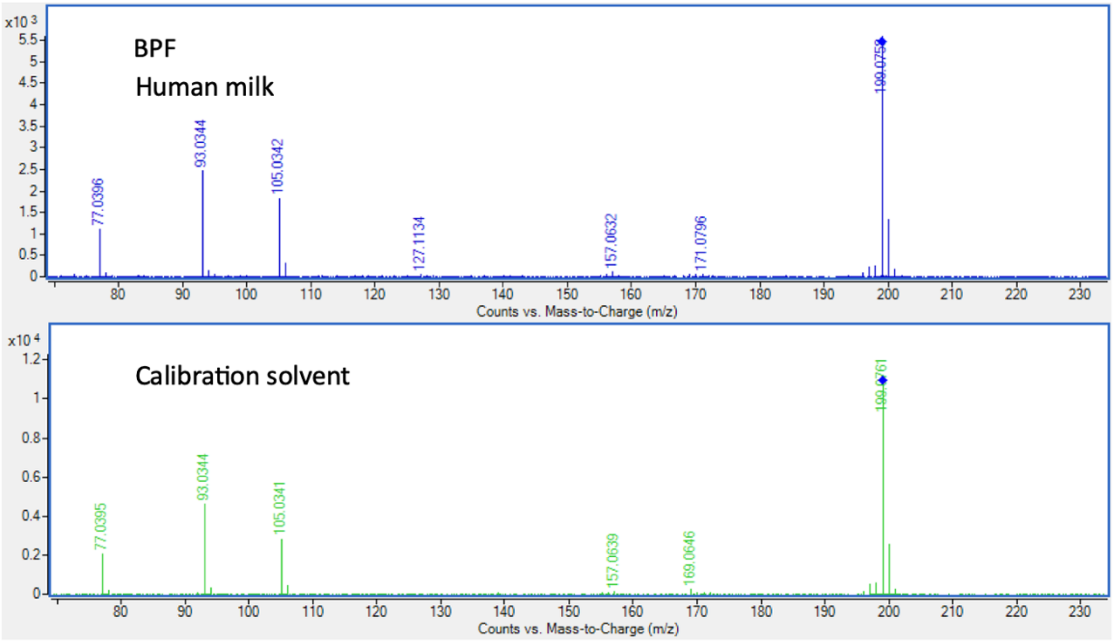
Figure S3.0 Relative recoveries of 9 selected bisphenol in human milk from three different conditions

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 S3.0 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 S3.0).

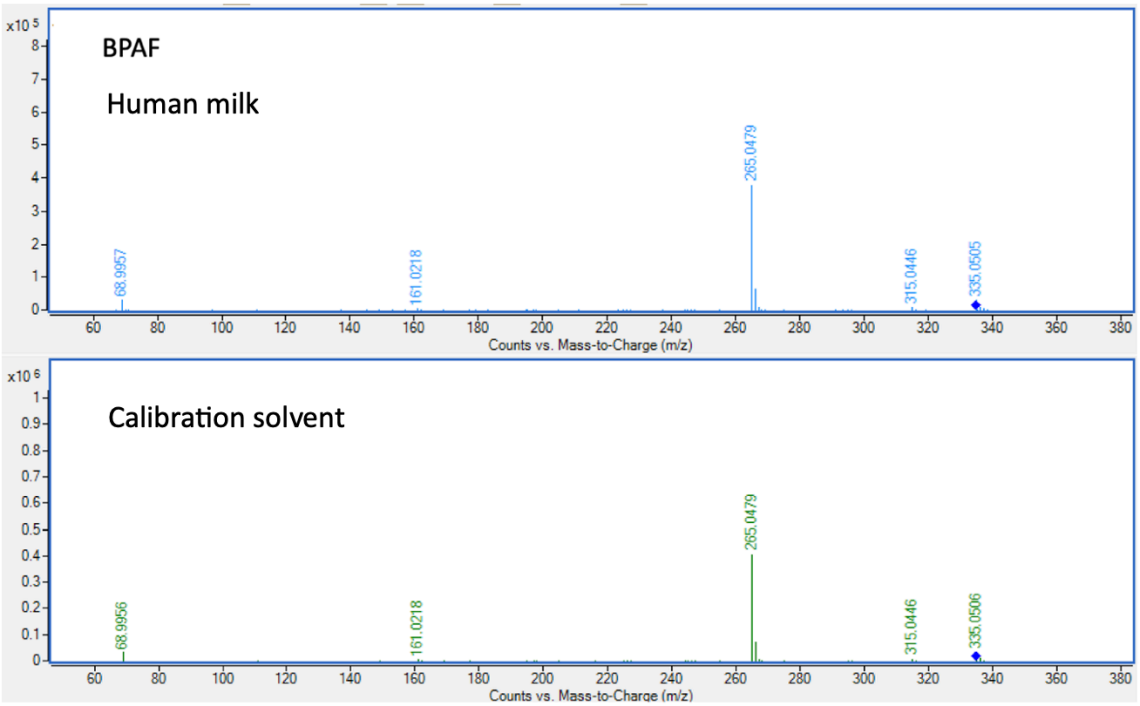
Comparative analysis highlighted that the addition of ammonium acetate buffer enhanced the stability of many bisphenols within the acetonitrile phase, including BPA, BPS, and BPC, contributing to overall improved recoveries for all selected bisphenols in milk. For further refinement, a third modification 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.



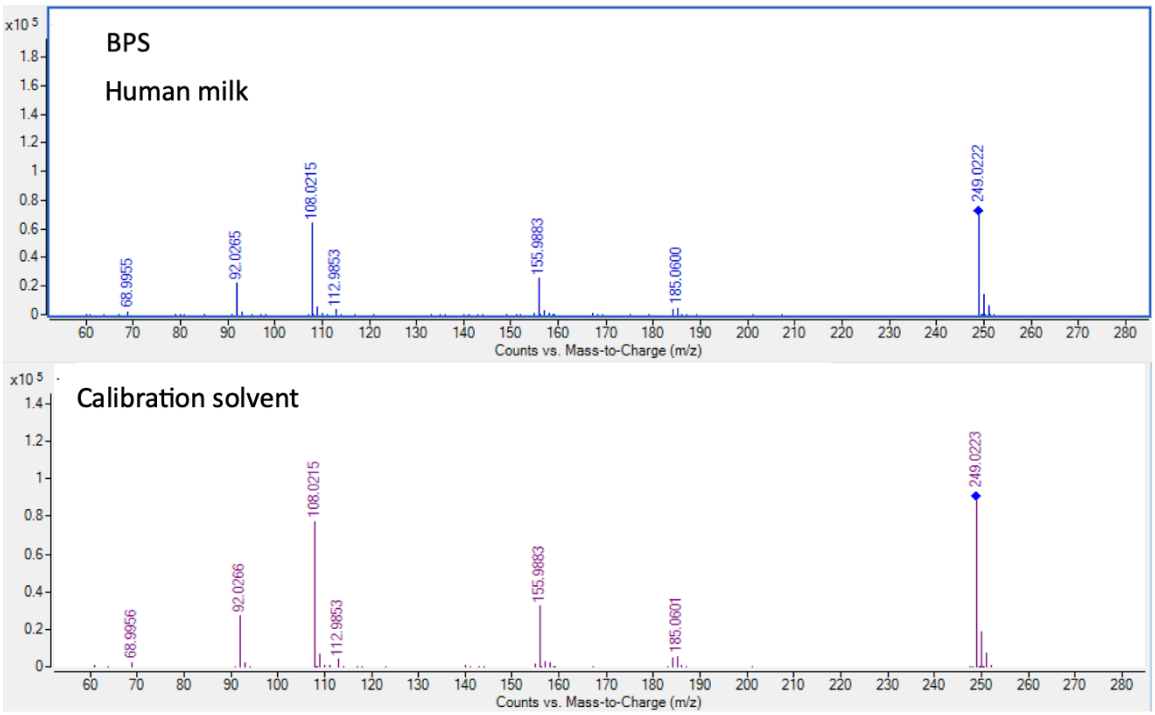
Supplemental Fig S3.1. Targeted MS/MS of BPA in human milk and pure calibration solvent



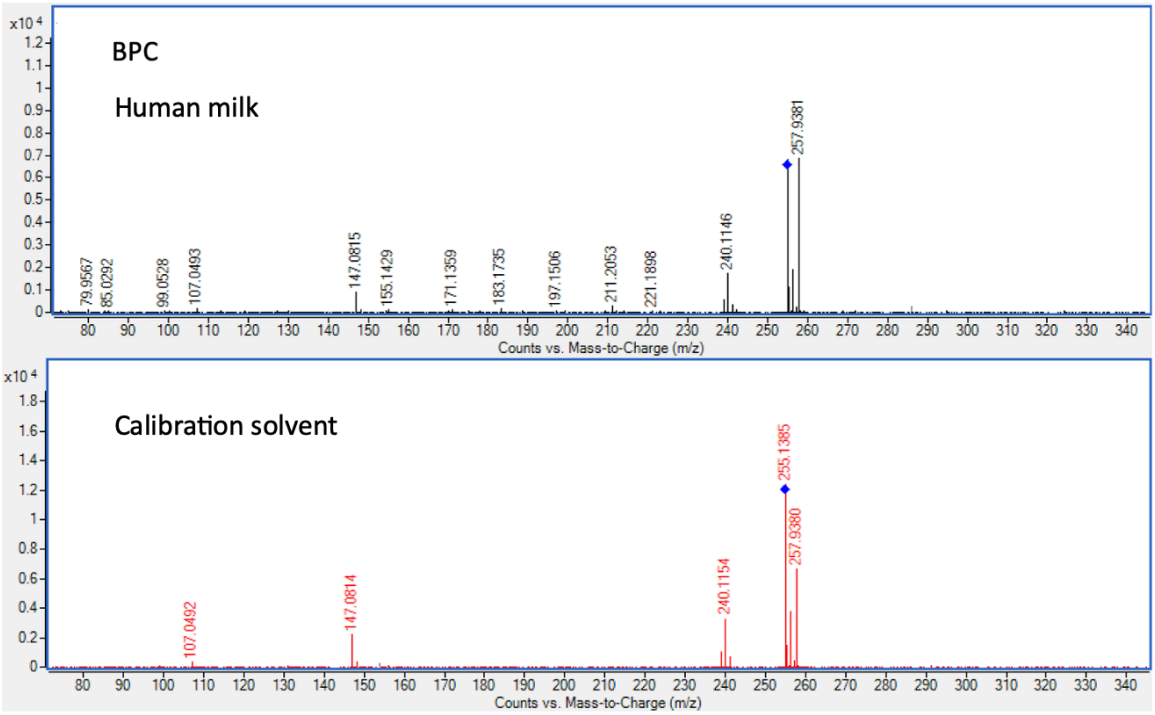
Supplemental Fig S3.2. Targeted MS/MS of BPF in human milk and pure calibration solvent



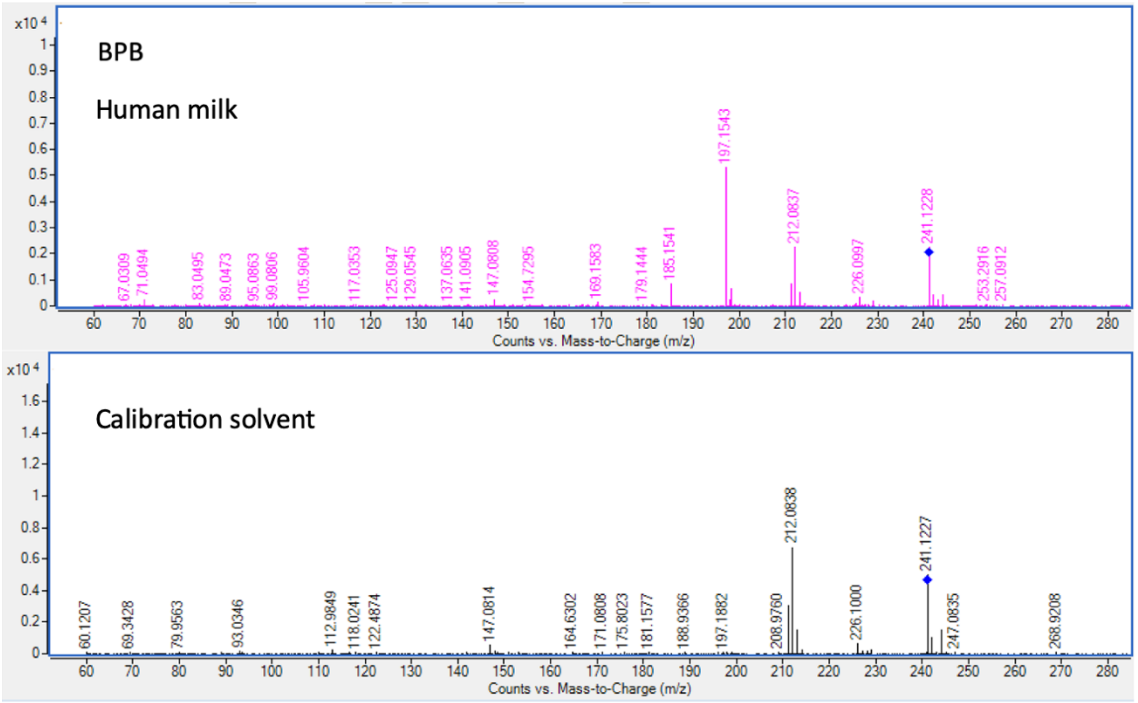
Supplemental Fig S3.3. Targeted MS/MS of BPAF in human milk and pure calibration solvent



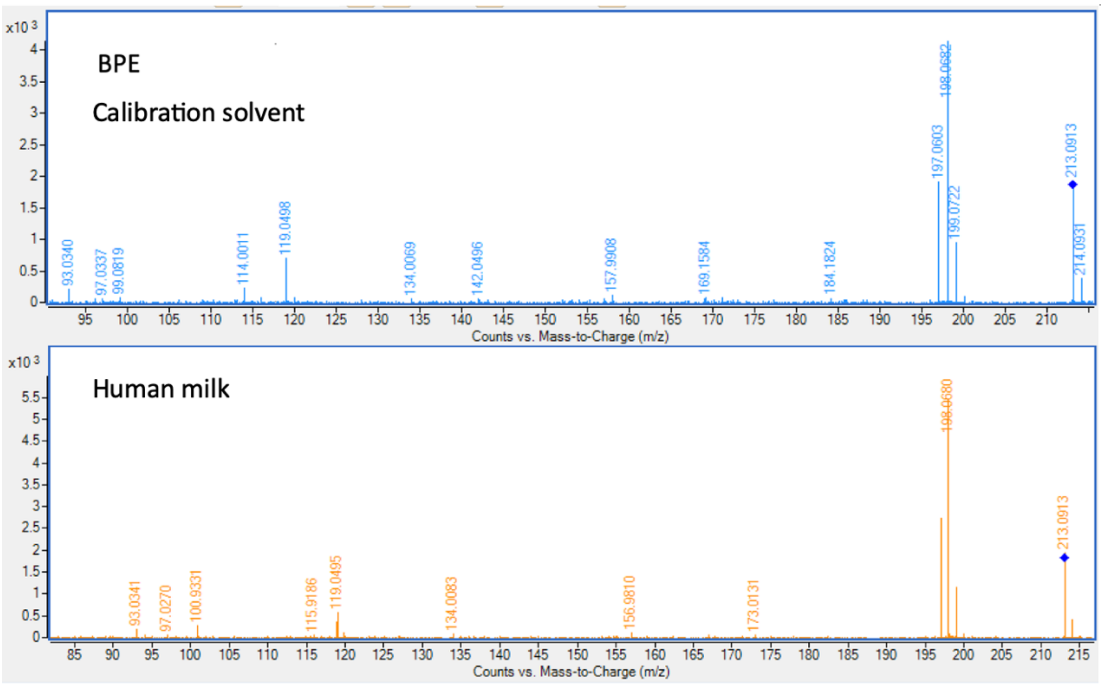
Supplemental Fig S3.4. Targeted MS/MS of BPS in human milk and pure calibration solvent



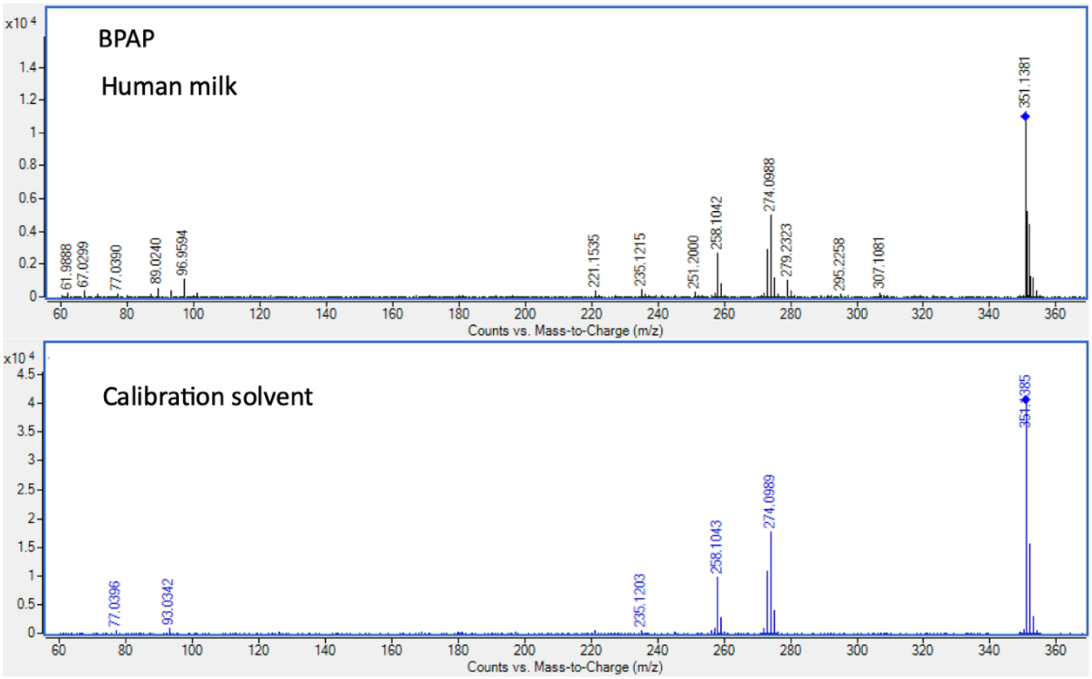
Supplemental Fig S3.5. Targeted MS/MS of BPC in human milk and pure calibration solvent



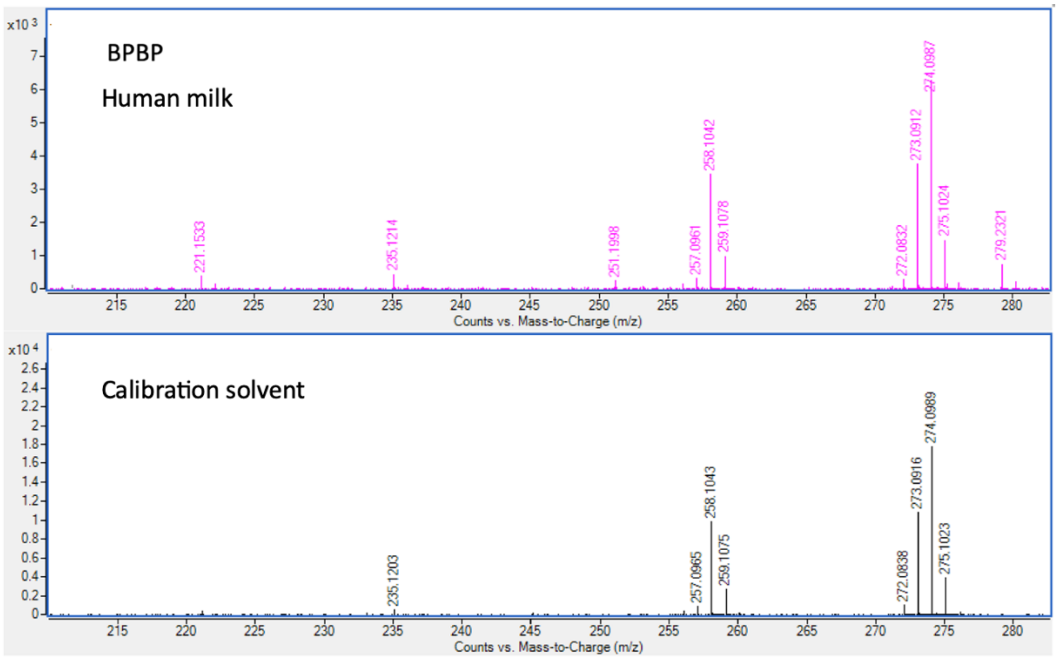
Supplemental Fig S3.6. Targeted MS/MS of BPB in human milk and pure calibration solvent



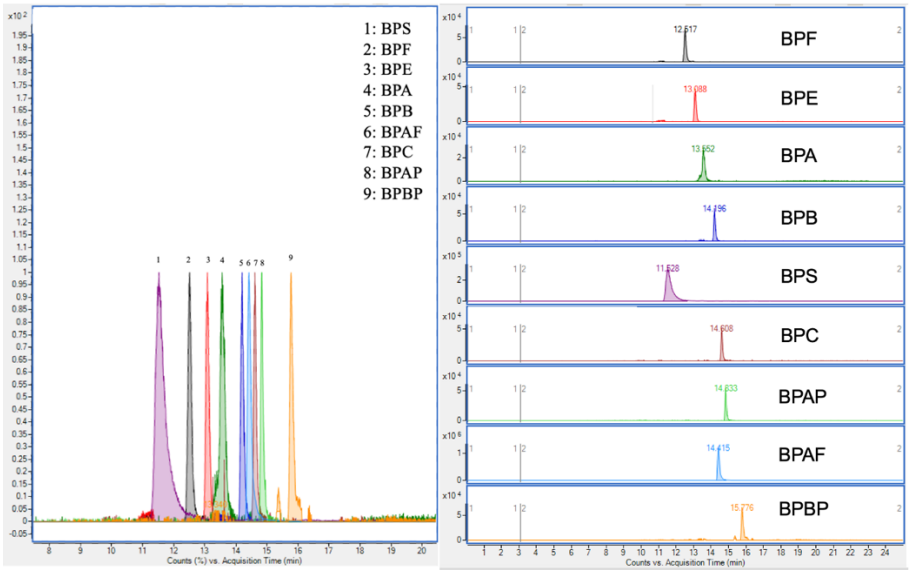
Supplemental Fig S3.7. Targeted MS/MS of BPE in human milk and pure calibration solvent



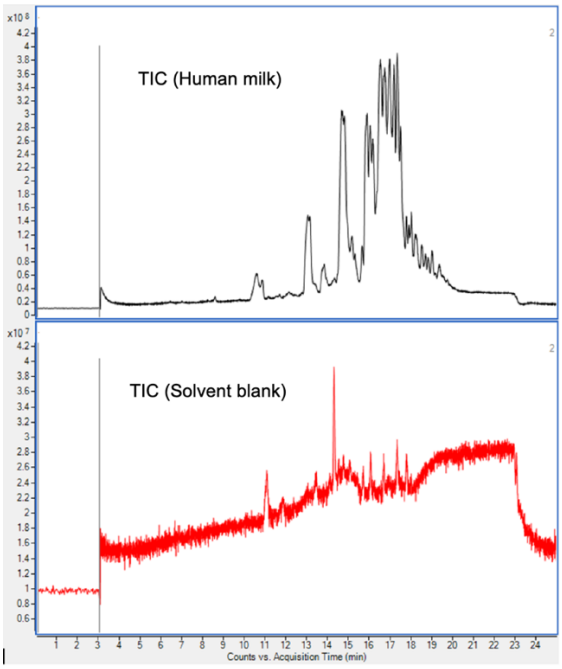
Supplemental Fig S3.8. Targeted MS/MS of BPAP in human milk and pure calibration solvent



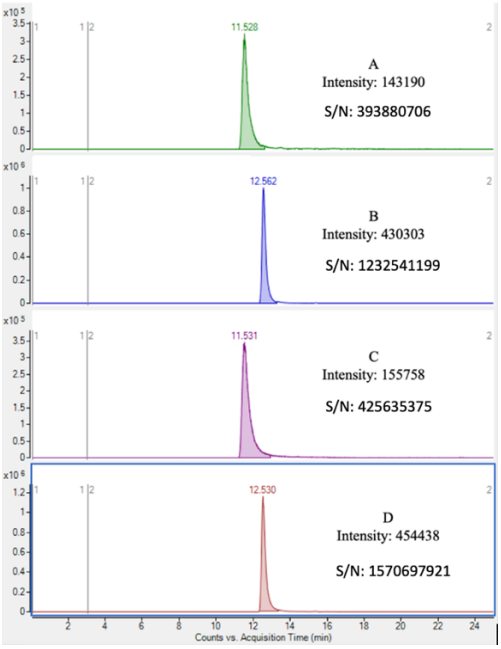
Supplemental Fig S3.9. Targeted MS/MS of tested analyte BPBP in human milk and pure calibration solvent



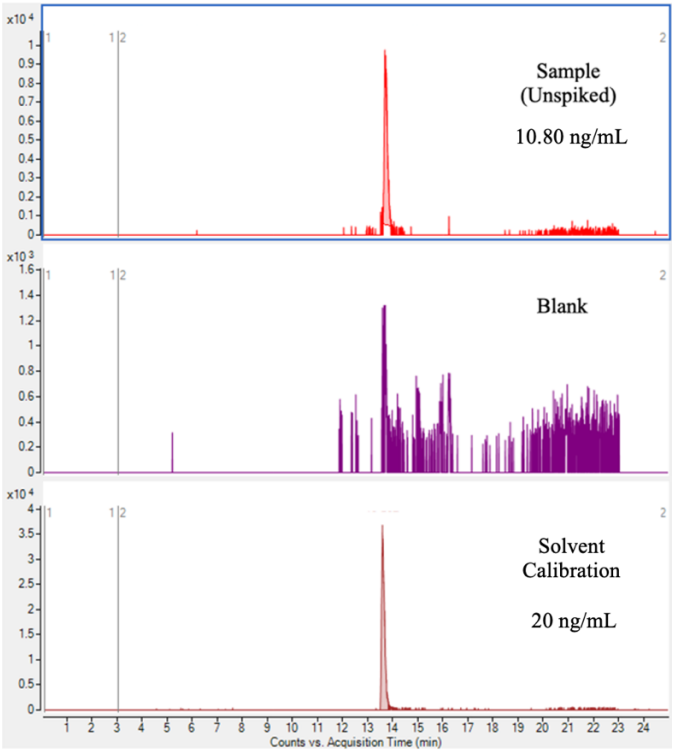
Supplemental Fig S3.10 Chromatographic peaks with normalized peak height and separated peaks for all 9 spiked bisphenols in human milk matrix at 30 ppb



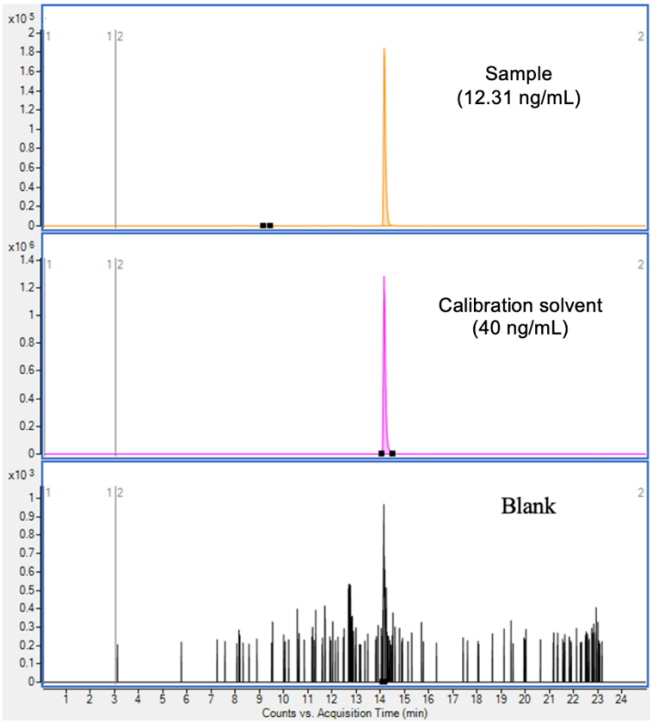
Supplemental Figure S3.11: Total ion chromatograms (TIC) for human milk and solvent blank (50% acetonitrile and water) with 20 µL injection volume



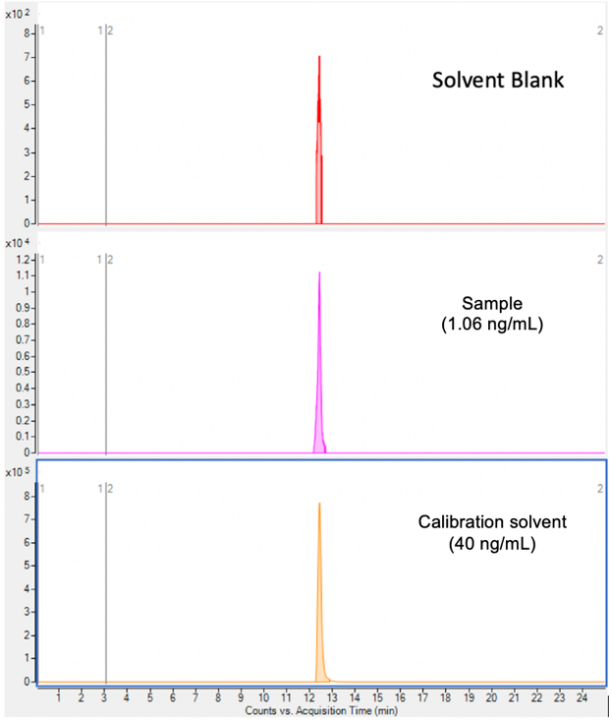
Supplemental Figure S3.12. Chromatographic peaks for the intensity of BPS in human milk matrix and solvent calibration in 2 different mobile phases at 30 ppb. A: 30 ppb spiked human milk sample using ACN and MeOH (1:1) as mobile phase B. B: 30 ppb spiked human milk sample using pure MeOH as mobile phase 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 S3.13: Chromatographic peak for BPA in blank, unspiked human milk and calibration solvent with injection volume of 20 µL



Supplemental Figure S3.14: Chromatographic peak for BPAF in blank, unspiked human milk and calibration solvent



Supplemental Figure S3.15: Chromatographic peak for BPS in blank, unspiked human milk and calibration solvent

Table S3.1: 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 S3.2: 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 S3.3: 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 S3.4: 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 S3.5. 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 S3.6: 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 S3.7. 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 S3.8. 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 S3.9: % 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) [26]	*Spain*	57	70	16-100	83	120
Cao et al. (2015) [27]	*Canada*	57	70	7.9-100	25.9	278

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Connecting paragraph

The data in Chapter 3 showed that a suitable extraction method is required for the extraction and quantification of bisphenols in complex matrices such as human milk. The selected QuEChERS extraction for the different bisphenol compounds underwent several modifications to yield satisfactory results in terms of recovery and reproducibility. A first-time insight into the levels of bisphenols in regions with limited available data, notably South Africa, were presented in Chapter 3. These data also revealed regional disparities in the types and levels of bisphenols detected in two different countries, Canada and South Africa.

Chapter 4 presents an in-depth investigation using non-targeted analysis to identify other bisphenol-related contaminants that may be present in human milk but are not commonly reported. The same QuEChERS extraction method employed in Chapter 3 was utilized for the detection and identification of these bisphenol-related unknowns.

Chapter 4. Non-targeted analysis of bisphenol A (BPA) structural analogues and functional alternatives with semi-quantification of bisphenol-related unknowns in human milk from Canada and South Africa

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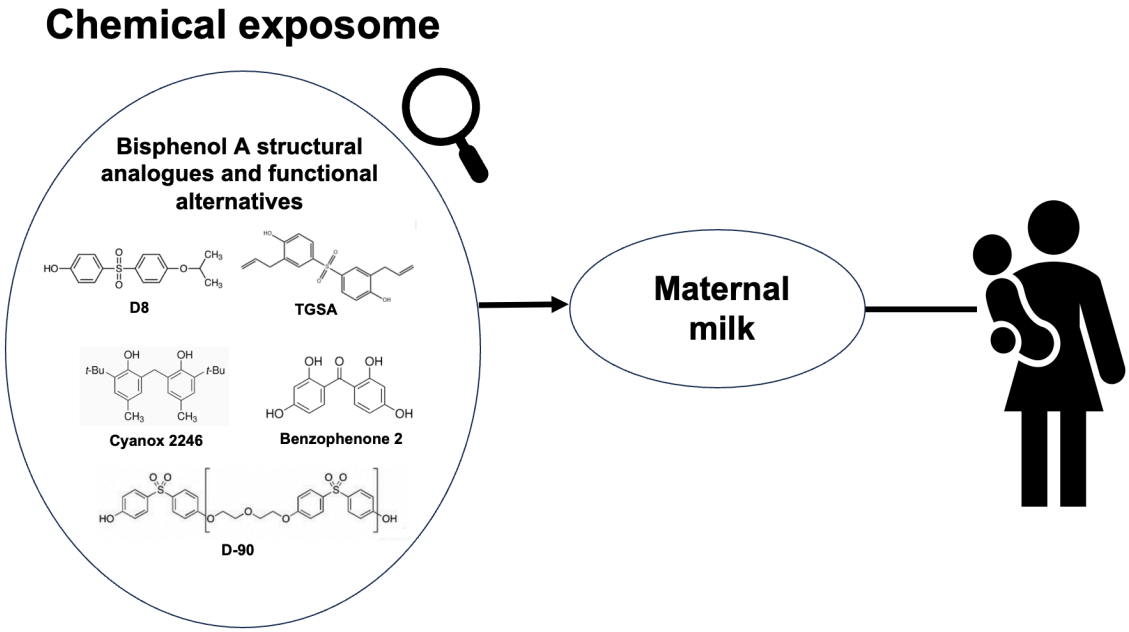
4.1. Abstract

The objective of this study was to investigate structural analogues and functional alternatives of bisphenol A (BPA) in 594 human milk samples collected in 2018-2019 from Canada (Montreal) and South Africa (Vhembe and Pretoria) using non-targeted analysis (NTA). A total of eleven compounds were identified in human milk: these included four compounds structurally similar to bisphenol S that are used in thermal labels, four ultraviolet absorber-related compounds, and three synthetic antioxidant-related chemicals, including two compounds not previously reported in human milk (Irganox 1010 and BHT-COOH). Semi-quantification was applied for three of the bisphenol S-related compounds (D8, D90 and TGSA) in human milk samples. D8, D90 and TGSA were detected levels at levels up to 1.24, 1.98 and 0.72 ng/mL, respectively. This study highlights the importance of using NTA in human milk biomonitoring studies to identify emerging contaminants to which breastfeeding infants are exposed.

Graphical Abstract



Key words: bisphenol A substitutes, plastic-related contaminants, liquid chromatography–mass spectrometry, non-targeted analysis, human milk, biomonitoring, exposome

4.2. Introduction:

Human milk, which is considered the optimal nourishment for infants by the World Health Organization (WHO), contains a myriad of endogenous components crucial for the health and growth of the infant [1]. Rich in growth factors, antimicrobial agents, immune boosters, oligosaccharides, and a diverse array of beneficial bacteria, human milk provides unparalleled nutrition and protection to breastfeeding infants, supporting their growth and shielding them from various illnesses and diseases [2-4]. Beyond its nutritional and protective roles, human milk also serves as an invaluable matrix for assessing the human exposome through the biomonitoring of chemical contaminants [5, 6]. More specifically, human milk is reflective of the maternal environment and it can harbor chemical contaminants that the mother has ingested, inhaled or dermally absorbed; importantly, these chemicals will be transferred to the breastfeeding infant [7].

While the traditional targeted analysis methods used in most studies have enabled the surveillance of a wide variety of contaminants in human milk, they fall short in identifying the presence of all the potentially important compounds that may be present in this complex matrix [8]. In recent years, the advent of non-targeted analysis (NTA) has offered a promising approach for detecting multiple unknown contaminants across diverse matrices [9], complementing targeted screening methods. This advancement has enabled the development of analytical workflows that can characterize human exposures in multiple environmental media (air, water, dust, soil) and human samples through the detection of a broad spectrum of unknown compounds with varying polarities [8, 10]. By utilizing different data processing techniques, such as feature extraction and alignment and statistical analyses, non-targeted analysis employs a comprehensive approach capable of detecting an unlimited number of different compounds in samples, making it an appealing technique to better characterize the human exposome [6]. Additionally, through the

analysis of accurate mass data and fragmentation patterns obtained from high-resolution mass spectrometry (HRMS), researchers can generate molecular formulas and propose structures for selected unknowns. The numbers of molecular formulas that are generated will be influenced by the different parameters and algorithms used during the data mining process [11]. Furthermore, subsequent confirmation of their identities can be achieved by comparing their MS/MS spectra with those of their corresponding pure analytical standards.

Only a few studies have employed non-targeted analysis (NTA) in the assessment of chemicals in human milk; these have focussed mostly on the detection of halogenated compounds as well as various organic contaminants [6, 8, 12, 13]. To the best of our knowledge, there have been no studies to date that have employed NTA with a specific focus on detecting the presence of plastic-related contaminants (PRCs) in human milk. These contaminants, ubiquitous in the environment, may affect the health of both mothers and their breastfeeding infants as various phthalates and bisphenols, which are the dominant classes of PRCs, may act as endocrine disruptors [14-17]. The existing data gap regarding unidentified plastic-related substances highlights the importance of investigating their possible presence in human milk from different regions, particularly in data-scarce countries like South Africa, where limited information exists on the types of PRCs present in maternal milk.

Our previous study reported high levels of bisphenol A (BPA) as well as residues of bisphenol S (BPS) and bisphenol AF (BPAF) in South African milk samples, suggesting the possible presence of other types of bisphenol-related contaminants [18]. Beyond the detection of conventional bisphenols, a recent report from the Government of Canada highlighted a surge in the utilization of BPA alternatives and provided a list of 343 substances that share similar structural and/or functional properties with BPA [19]. Humans are exposed to some of these bisphenol

alternatives, used as color developers in thermal papers and as synthetic antioxidants, and this can lead to their transfer to human milk, raising concerns with respect to their potential health impact on breastfeeding infants [20, 21].

Studies conducted in various countries, including Canada, China, Korea, Japan, the United States, and European countries have focused on the targeted analysis of BPA and its major analogues in human milk [5, 22-28], while other bisphenol-related contaminants are often overlooked. There is a need to investigate the presence of these bisphenol-related chemicals in breast milk due to the fact that these compounds may possess endocrine disrupting properties that are similar to those of known bisphenols [21, 29-32]. While non-targeted analysis allows for the detection of various unknown or unexpected contaminants, it remains difficult to quantify their levels in comparison to the traditional targeted approach [33]. To date, only a small number of non-targeted studies have attempted retrospective quantification or estimation of the levels of their detected unknowns. One general approach is the use of semi-quantification to estimate the levels of different unknowns by using standards that have structural similarities to the identified unknowns [34]. However, the major drawback of this approach is the potentially large inaccuracies in the levels of identified unknowns that can occur during the semi-quantification process [33]. Thus, in addition to the necessity to investigate the presence of bisphenol-related compounds in human milk, there is a need to explore new semi-quantitative approaches that may provide more precise estimations of the levels of these unknowns.

The aim of this study was to determine the presence of infrequently reported or as yet unknown bisphenol-related contaminants in human milk from women residing in Canada and South Africa. To do so, we: i) developed a suitable and rapid non-targeted approach for the tentative identification of bisphenol-related unknowns using a customized database library; ii) utilized NTA

software to elucidate the structure of these unknown compounds using their MS/MS spectra and to confirm their identities through pure analytical standards; and iii) developed a semi-quantitative approach for the estimation of the levels of certain detected unknowns, based on structural similarities with known bisphenol standards.

4.3. Materials and methods:

4.3.1. Chemicals

Ammonium acetate (NH_4Ac) (LC-MS grade), acetic acid (LC-MS grade), HPLC-grade solvents (water, acetonitrile, methanol) and 2,2',4,4'-tetrahydroxybenzophenone (benzophenone-2, purity ≥ 98%) were purchased from Fisher Scientific (Hampton, VA, USA). Analytical standards for BPA (≥ 99%), BPF (≥ 98%), BPS (≥ 98%), BPAF (≥ 99%), bisphenol E (BPE, ≥ 98%), bisphenol C (BPC, ≥ 99%), bisphenol B (BPB, ≥ 99%), bisphenol AP (BPAP, ≥ 99%), bisphenol BP (BPBP, ≥ 98%), 4,4′-dihydroxybenzophenone (≥ 99%), 2,4-dihydroxybenzophenone (benzophenone-1, ≥ 99%), oxybenzone (benzophenone-3, ≥ 98%), pentaerythritol tetrakis (3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate) (Irganox 1010, ≥ 97%), 2,2'-methylenebis(4-methyl-6-tert-butylphenol) (Cyanox 2246 , ≥ 96%) and 3,5-di-tert-butyl-4-hydroxybenzoic acid (BHT-COOH, ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

D-8 (≥95%, CAS number: 95235-30-6), TGSA (≥ 98%, CAS number: 41481-66-7), D-90 (copolymer, n=1) (≥99%, CAS number: 191680-83-8) and 2,4-BPS (≥ 96%, CAS number: 5397-34-2) were obtained from Toronto Research Chemicals (Toronto, Canada).

Stock solutions of the individual bisphenols, D8, D90, 2-4 BPS, and TGSA, were prepared in methanol (100 mg L^{-1}). A monthly mixture of stock solutions containing the nine native bisphenols,

D8, D90 and TGSA was prepared at a concentration of 1 mg L^{-1} in methanol. All stock solutions were stored in amber glass vials in a freezer (-20 °C) prior to analysis.

4.3.2. Sample collection and storage

Procedures for the collection of breast milk samples from South Africa (Vhembe and Pretoria) and Canada (Montreal) were the same as for the previous study using a targeted analysis to identify known bisphenol analogues [18]. In South Africa, mother-infant pairs were recruited from the maternity wards and vaccine clinics of Tshilidzini Hospital, located in the Vhembe district of Limpopo Province (a rural area), and of Tshwane Hospital, located in Pretoria, Gauteng (an urban area). Eligible participants, aged 18 and above, were required to speak either English or Tshivenda, the primary language spoken in the Vhembe district. Additionally, they were required to have given birth to or expecting a live singleton and, if recruited from the maternity ward, able to return one month post-delivery for a brief questionnaire and provision of a breastmilk sample.

Similarly, participants from Montreal were recruited from the Royal Victoria and St. Mary's hospitals following childbirth. Eligible participants, also aged 18 and above, were proficient in either French or English and willing to participate in two follow-up sessions. The first session included an explanation of the manual breast milk collection process along with a questionnaire, while the second session focused on actual sample collection.

Milk samples were collected between the 4th and 8th week post-delivery from participants in the three locations (Vhembe, Pretoria, and Montreal) over a 1.5-year period (2018–2019) via manual expression into polypropylene containers (free of bisphenol A and the 8 analogues investigated in the previous study) [18]. These samples were then stored in cryovials at -80 °C until shipment on dry ice to the analytical laboratory. Montreal samples were stored in a -80 °C

freezer until they underwent freeze-drying in a lyophilizer (FreeZone Cascade Benchtop Freeze Dry Systems, Labconco, Kansas City, KA, USA); South African samples were freeze-dried using a different lyophilizer (Freeze dryer ALPHA 1-2 LD+, Osterode am Harz, Germany). All samples followed an identical freeze-drying protocol, using a vacuum of 0.09 Torrs at -80 °C for 3 days. Freeze-dried sample aliquots were stored in amber glass vials at -80°C until analysis. A total of 594 human milk samples from different mothers were analyzed for the non-targeted analysis of bisphenol-related compounds (n=194 from Vhembe, n=193 from Pretoria and n=207 from Montreal).

This study was approved by ethics committees at McGill University, the Research Institute of the McGill University Health Centre, the University of Pretoria, and the Limpopo Department of Health and Social Services.

4.3.3. Sample Preparation

Sample preparation was previously optimized for the quantification of different major bisphenol analogues in human milk [18]. Briefly, for the extraction process, approximately 0.2 g of freeze-dried milk powder was mixed with 2 mL of HPLC-grade water. This mixture was spiked with 30 µg/L of a bisphenol labeled mixture. Subsequently, 1 mL of a 1 M ammonium acetate buffer solution and 10 mL acetonitrile was added, and the milk sample underwent ultrasonication for 10 minutes. Following sonication, 1 g of MgSO4 and 2 g of NaCl were added to the milk, the mixture was vortexed and then centrifuged at 4000 RCF for 10 minutes. The resulting supernatant was transferred to a 15 mL polypropylene tube containing 0.2 g PSA, 0.1 g C18 sorbent, and 0.25 g MgSO4. The sample was vigorously shaken for 45 seconds and then centrifuged at 3000 RCF for 10 minutes. After centrifugation, the supernatant was moved to a glass tube and evaporated

under a nitrogen stream until dryness. The residue was reconstituted in a 1:1 ratio of acetonitrile and water before analysis using LC-QTOF-MS.

4.3.4. Instrumental analysis

Non-targeted analysis was performed using an Agilent 1290 Infinity II LC system (Agilent Technologies, Santa Clara, USA) coupled to a 6545-quadrupole time-of-flight (Q-TOF) MS (Agilent Technologies, Santa Clara, USA). 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 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 contained 5 mM ammonium acetate 5%; B (0–1 min), with a linear increase to 100% B (1–15 min), 100% B (15–20 min), restored 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 QTOF-MS system was operated in negative (ESI-) electrospray ionization mode for the detection of bisphenol-related contaminants. 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. For the semi-quantification of identified bisphenol S-related compounds, the samples were further rerun with the same method, but using 100% methanol for mobile phase B with 5 mM ammonium acetate. D8, D90 and TGSA in human milk samples were analyzed using pure methanol as mobile phase B due to better signal intensity compared to 1:1 ACN/methanol (Figures S4.1-S4.3).

4.3.5. Quality assurance/ Quality control

For sample collection and analysis, care was taken to avoid sample contact with materials known to contain bisphenols and plasticizers. These measures included the testing of the polypropylene and glass tubes for any bisphenol residue. Additionally, aluminum films were set up on each workbench and in every fume hood to mitigate any risk of contamination during the sample preparation.

Quality assurance for the non-targeted analysis included controlling background contamination, monitoring mass accuracy, intensity and retention time (RT) shifts, and signal drift. To ensure signal stability during the analysis, retention time and mass accuracy of all 9 selected spiked bisphenols in the calibration standards were monitored through repeated analysis; in addition, 6 quality control samples of human milk were spiked with all 9 bisphenols at 30 ng/mL (BPA, BPF, BPS, BPAF, BPC, BPE, BPB, BPBP and BPAP). Solvent blanks were subjected to the extraction method in triplicate to identify any possible analyte contamination. During sample analysis, an acetonitrile solvent blank was injected after every 10 samples to minimize possible carryover effects from the instrument.

LC-QTOF-MS data treatment and tentative bisphenol-related compound identifications

Data alignment and molecular feature extraction were done using Agilent MassHunter Profinder (B.10.00) with the specified data processing parameters (Table S4.1). To identify bisphenol-related features, a personalized library was constructed using Agilent PCDL Manager (B08.00). This library incorporated the bisphenols listed in Health Canada's consultation document [19], along with 18 additional compounds. These compounds were added to the list because they were previously reported or mentioned in other relevant literature and share similar structure or

functional properties to bisphenols (Table S4.2) [19, 35-38]. In total, a list of 361 compounds was used for the non-targeted analysis of human milk samples from Montreal and South Africa.

The data files were analyzed in batches, with each batch consisting of different sets of blanks, non-spiked samples, and spiked samples containing all 9 bisphenol analogues in the previous study [18]. The purpose of this step was twofold: (i) to successfully identify all 9 bisphenols in each spiked sample in the analyzed batch with an acceptable score of >90%, and (ii) to identify the conditions that yield the maximum number of features, specifically bisphenol-related unknowns, from the customized library.

The software for non-targeted analysis (NTA) was optimized and validated by determining its ability to detect and identify unknown bisphenol replacements present at trace levels in human milk. The primary objective was to challenge the data treatment by blindly detecting the nine previously spiked bisphenols at levels of 30 ng/mL in the quality control (QC) samples [18]. Multiple batches were tested using different parameters to assess the accuracy of Profinder in detecting all nine bisphenols in the QCs. For each parameter, one value was changed at a time while keeping the remaining parameters at their default settings. The following three parameter values were evaluated: peak filter height (300 and 1000 counts), mass window (±5 and ±10.00 ppm), and post-processing peak absolute height (>300 and >1000 counts). The number of extracted features and the accurate detection of bisphenols were then compared for different sets of data processing conditions. During the preliminary test, all parameter sets successfully detected all nine spiked bisphenols, with a score exceeding 90% in every tested batch, and revealed a significant number of features. Substantial differences in the scores were not observed among the custom parameters; the parameters with lower values were selected for the non-targeted analysis of both

Montreal and South African human milk samples to maximize the number of features, as presented in Table S4.1.

For data treatment, all samples were processed using the "Targeted Feature Extraction" mode, which extracts all features in the samples and compares their mass and mass spectra information with the customized library database. Features that exhibited a high matching score (>90%) and satisfactory average height (>3000) were considered potential candidates for unknown bisphenol-related compounds. In cases where features were also found in the blank samples, the average intensity in the blanks (triplicate) was added to three times the standard deviation of the blank signals [39]. This combined value was then compared to the intensity of the corresponding feature observed in the samples. If the intensity of the feature in the samples exceeded that of the blanks, it was considered a potential candidate in the analyzed human milk samples.

4.3.6. Application of SIRIUS and proof-of-concept

SIRIUS software was utilized to generate structural representations of the compounds based on their fragmentation patterns. SIRIUS utilizes fragmented parts of the suspected compounds and provides a list of possible parent compounds, offering an overall accuracy score. For proof-of-concept, MS/MS spectra were acquired for all the selected unknown compounds in human samples using Profinder. The obtained MS/MS spectra were subsequently analyzed using Sirius-CSI:FingerID [40]. Features were deemed as bisphenol suspects if their elemental formula matched 100% and their candidate match score exceeded 70% when compared to compound databases such as PubChem, KEGG, and CheBI. These features were then compiled into a list, and pure analytical standards were purchased to validate potential candidates. The MS/MS spectra of pure analytical standards and the tentative unknowns were compared with each other. The

identity of tentative compounds was confirmed if at least one fragment ion was present in both the standard and the compound, within a mass error of 5 ppm in accordance with the European Commission guideline SANTE/11312/2021 [41].

4.3.7. Semi-quantification of three detected bisphenol-related unknowns with similar structures to BPS

For bisphenol S-related compounds, including D8, D90 and TGSA, the chromatogram extraction window was established at ± 10 ppm for mass and ± 0.1 min for retention time (RT). A comparison was made between the relative intensities of qualifier ions (% of base peak) of these compounds in both pure solvent and milk samples to assess any variations (Table S4.3). The recovery test for D8, D90 and TGSA was done using a pool of *n*=40 individual human milk samples by spiking isotope-labelled BPS ($^{13}C_{12}$-BPS). BPS was selected due to its structural similarity compared to D8, D90 and TGSA. The semi-quantitative recovery test used the internal standard method which can correct for the matrix effect [42]. The matrix effect (ME%) for all 3 bisphenol-related unknowns was calculated using the equation below and is shown in Table S4.5:

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

The relative standard deviation (RSD) was calculated based on the analysis of 3 replicates using the pool of human milk samples. An RSD lower than 15% was judged acceptable [43]. The spiking level was evaluated at 30 ng/mL which was the same spiking level used for the 4 major bisphenols (BPA, BPF, BPAF and BPS) in our previous study [18].

To validate the performance of the instruments for the target compounds, 10 calibration points (0.5 to 100 μg L^{-1} of the target analytes, 30 μg L^{-1} for the $^{13}C_{12}$-BPS) were selected for the normal range of D8, D90 and TGSA in human milk. The linearity of the instrument response

(R^2>0.98) for all bisphenol-related compounds was assessed using their relative response factor (RRF) in spiked human milk (Table S4.5), calculated using the equation provided below.

$$RRF = \left(\frac{(peak\ area\ of\ native\ compound)/(peak\ area\ of\ isotope-labelled\ internal\ standard)}{(concentration\ of\ of\ native\ compound)/(concentration\ of\ isotope-labelled\ internal\ standard)}\right)$$

The method detection limit (MDL) was calculated as three times the standard deviation of procedural blanks [44]. If the analyte was absent in all procedure 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 compounds in procedural blanks (if any) or milk samples by 10 [45].

Subsequent to the initial analysis, a re-extraction was performed on a set of previously analyzed milk samples (n=17) to compare the concentrations obtained from the first and second extractions (Table S4.7). These included an assessment of D8, D90, TGSA as well as BPS and BPA levels for 14 selected samples from the first extraction and the corresponding levels of these bisphenol-related compounds after their 2nd extraction. In addition to the 14 samples, the bisphenol A levels in the 1st and 2nd extractions of the 3 samples from the previous study [18] were also compared with each other. To improve robustness, the concentrations for each compound for both 1st and 2nd extractions (excluding non-detects) shown in Table S4.7 were then log scaled, and samples that had non-detectable levels in either the 1st or 2nd extraction were then excluded for the assessment of any difference between the 2 extractions.

A paired sample t-test was conducted to determine significant differences (p-value <0.05) for detected levels of each compound between the 1st and 2nd extraction (Table S4.8). The data for

the first extraction were plotted against the second extraction and a linear regression curve was generated. 95% confidence intervals and prediction intervals were calculated to assess method precision by evaluating points within the prediction intervals (Figure S4.16).

4.4. Results and discussion:

4.4.1. Selection of features relating to tentative bisphenol-related compounds

The settings selected for Profinder (Table S4.1) yielded the highest number of bisphenol-related features. A total of over 150 features were observed across every batch of human milk samples. From this extensive list, features with an average score above 90% were chosen as potential candidates. The selection of this threshold was based on the scores obtained using Profinder for the 9 spiked bisphenols, which consistently exceeded 90% in all analyzed batches. The potential suspects, along with the regions where they were detected, were compiled and are presented in Table S4.4: overall, 42 tentative compounds at confidence level 3 using the Schymanski scale were selected for further analysis regarding their potential chemical structures [46].

Among the candidates, a significant proportion of the listed suspects are plastic-related contaminants that are commonly employed as colour additives, UV filters and absorbers or synthetic antioxidants [21]. Similar to BPA, these compounds have been used in various products, including those related to commercial food and other industrial applications [36, 47], and can enter the human body through diet as the primary source of exposure [36, 48, 49]. Interestingly, some of these candidates have rarely been reported or have not previously been detected in human milk. However, it is important to note that the actual identity of these suspects may differ from their

suggested identity. Only compounds that were identified and confirmed using analytical standards are discussed further in the following section [46].

4.4.2. Identification of detected bisphenol unknowns using SIRIUS and pure analytical standards

The identities of the above 42 suspects were further studied using a re-analysis in targeted MS/MS and structural prediction *in silico*. The MS/MS spectra were first compared with the ones found in online databases, when available. Reference standards were subsequently obtained to confirm the unknowns with a SIRIUS score higher than 70%. In total, the identities and structures of four bisphenol replacements, four UV-related absorbers, two synthetic antioxidants, and one metabolite of the synthetic antioxidant butylated hydroxytoluene (BHT) were confirmed using pure analytical standards (Table 4.1: Figures S4.4-4.14).

Table 4.1. List of 11 identified bisphenol-related compounds using pure analytical standards

Feature ([M-H]-,m/z)	Formula	Human milk (Vhembe, Pretoria or Montreal)	% detection in human milk samples	Confirmed Identity	CAS registry number	Previously reported in another country
291.0691	$C_{15}H_{16}O_4S$	All	Vhembe: 17 Pretoria:4 Montreal: 2	D-8 (4-hydroxyphenyl 4-isopropoxyphenyl sulfone)	95235-30-6	China [20]
569.0940	$C_{12}H_{10}O_4S[C_{16}H_{16}O_5S]_n$ n=1	All	Vhembe: 7 Pretoria:4 Montreal: 2	D-90 (Phenol, 4,4'-sulfonylbis-, polymer with 1,1'-oxybis[2-chloroethane])	191680-83-8	China [20]
329.0848	$C_{18}H_{18}O_4S$	All	Vhembe: 4 Pretoria: 3 Montreal: 1	TGSA (4,4′-sulfonylbis(2-allylphenol))	41481-66-7	China [20]
249.0224	$C_{12}H_{10}O_4S$	Montreal	<1	2,4- BPS	5397-34-2	China [20]
213.0552	$C_{13}H_{10}O_3$	All	Vhembe: 6	Benzophenone-1	131-56-6	Spain [50]

			Pretoria: 8 Montreal: 7			
245.0450	$C_{13}H_{10}O_5$	Montreal	3	Benzophenone-2	131-55-5	Spain [50]
227.0708	$C_{14}H_{12}O_3$	Montreal	1	Benzophenone-3	131-57-7	Spain [51]
213.0552	$C_{13}H_{10}O_3$	All	Vhembe: 6 Pretoria: 9 Montreal: 7	4,4'-Dihydroxybenzophenone	611-99-4	Spain [50]
1175.780	$C_{73}H_{108}O_{12}$	All	Vhembe: 20 Pretoria: 31 Montreal: 41	Irganox 1010 (Pentaerythritol tetrakis(3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate)	6683-19-8	NR*
339.2324	$C_{23}H_{32}O_2$	South Africa	Vhembe: 1 Pretoria: <1	Cyanox 2246 (Antioxidant 2246) 2,2'-Methylenebis(4-methyl-6-tert-butylphenol)	119-47-1	China [36]
249.1490	$C_{15}H_{22}O_3$	All	Vhembe: 26 Pretoria: 45 Montreal: 11	BHT-COOH (3,5-di-tert-butyl-4-hydroxybenzoic acid)	1421-49-4	NR*

*NR: Not reported in other human milk studies

D-8, D-90, TGSA 2, and 4-BPS are used as BPA replacements (Table 4.1); studies have indicated they are extensively employed as BPA alternatives in the production of thermal papers and are utilized as colour developers in thermal labels for food packaging [21, 52-56].

Previously, a study reported the presence of these compounds in human milk samples collected in China [20]. Government agencies, including the EPA, have classified these compounds as chemicals of concern, warranting further monitoring to better comprehend their impact on human health [57]. Limited data are available regarding exposure and toxicity of these chemicals in animals or in humans [29, 55, 58, 59]. Research is still needed to evaluate whether these chemicals have endocrine-disrupting properties similar to those of other bisphenols, which may exhibit developmental toxicity, reproductive toxicity, or carcinogenicity [30, 60-62].

The presence of UV absorbers, including benzophenones 1, 2, and 3, as well as 4,4'-dihydroxybenzophenone, was also reported in a few studies of human milk collected in Spain [50]. These UV absorbers are frequently found in personal care product formulations, including shampoos, toothpastes, body washes lotions and sunscreens [63]. Beyond personal care products, benzophenones and their derivatives are also used as additives in plastic food-related packaging to prevent degradation of the food from exposure to sunlight and UV radiation and to increase the shelf life [64] . These chemicals can migrate from plastic materials into food, consequently leading to human exposure, and can be transferred to human milk [50, 65, 66]. There is evidence that benzophenones may act as endocrine disruptors, and lead to reproductive and developmental effects [67-69]. Epidemiological studies on benzophenones also suggest possible reduced fertility, fetal birth outcomes, endometriosis, and possible kidney diseases [69-73].

Irganox 1010 is a synthetic antioxidant used as an additive in many plastic packaging materials. Its usage in food-related packaging can lead to its migration into food products and entry into the human body [74]. Similar to Irganox 1076, which was previously detected in brown bread, Irganox 1010 has been used in low density polypropylene products such as food packaging where it can migrate into food [75-77]. Information on the possible health impact of exposure to Irganox 1010 is very limited [21].

Cyanox 2246 (Antioxidant 2246) is also an antioxidant that is used in polypropylene food packaging [77, 78]. To date, there is little information about its usage or its potential toxicity concerning human health. To the best of our knowledge, only one other study on human milk in China has reported the presence of Cyanox 2246 [36].

A metabolite of BHT, 3,5-di-tert-butyl-4-hydroxybenzoic acid (BHT-COOH), was also detected in our human milk samples. BHT-COOH was previously detected in human urine, and

has been considered as a potential urinary biomarker of exposure to BHT [35]. One previous study on human milk in China reported the presence of other BHT metabolites, such as 2,6-di-tert-butyl-4-(hydroxymethyl)phenol (BHT-OH), 3,5-di-tert-butyl-4-hydroxybenzaldehyde (BHT-CHO), 2,6-di-tert-butyl-p-benzoquinone (BHT-Q) and 2,6-di-tert-butyl-4-hydroxy-4-methyl-2,5-cyclohexadienone (BHT-quinol) [36]. The presence of BHT-COOH, along with the other metabolites discussed above, in human milk strongly suggests the need for further investigations regarding their potential health effects on both the mother and breastfeeding infant.

This study is the first to investigate bisphenol-related unknowns in human milk. Our findings emphasize the importance of employing non-targeted analysis for the detection of underreported or unknown contaminants in human milk; these compounds would likely not be detected by relying on the traditional targeted approach.

It is possible that there are additional bisphenol-related unknowns among the remaining tentative compounds (Table S4.4). However, confirmation of their actual identities was not pursued in the present study due to their low signal intensity, poor MS/MS spectra scores when imported into SIRIUS, or the difficulty of obtaining their corresponding standard. Future investigations will explore these substances.

4.4.3. Semi-quantification and estimated levels of D8, D90 and TGSA detected in Montreal and South African human milk

One of the current challenges of non-targeted analysis lies in the retrospective semi-quantification of the newly identified compounds in data files without reanalysis. In our previous study, data were collected using LC-HRMS for 594 human milk samples during the simultaneous targeted analysis of BPA, BPF, BPS and BPAF in 20 separate sample batches over 2 years [18]. Since datasets acquired in different batches can be affected by instrument variability over time, we

explored the possibility of revisiting these datasets to semi-quantify newly identified bisphenol-related unknowns, namely D8, D90 and TGSA, which share similar chemical properties with BPS [79].

To account for any variability in the datasets collected across all of the batches, and to obtain a more precise estimation of the levels of the three compounds (D8, D90 and TGSA), retrospective semi-quantification was first attempted in the present study by calculating their concentrations using their specific relative response versus $^{13}C_{12}$-BPS initially spiked in each of the milk samples that were analyzed (Figures S4.15-4.16, Tables S4.3, S4.5-S4.8). The validity of this semi-quantification approach is supported by the minimal variations in analytical performance [18, 80] and, most importantly, by the stability of the RRFs obtained for BPA, BPF, BPS and BPAF in human milk from the previous study across all analyzed batches (RSD<15%; Figure S4.15) over the course of two years.

Since the RRF for BPS showed limited variability (RSD < 8.89%), it was hypothesized that the RRFs for D8, D90 and TGSA obtained using the matrix-matched calibration would also display similar stability across the analyzed batches. Consequently, the RRFs from the matrix-matched calibration for D8, D90 and TGSA were employed to calculate the recoveries of these three compounds and were compared to the values of BPS obtained from the previous study [18], as shown in Table S4.5. The measured *m/z* values for all three compounds showed variations below 3 ppm compared to theoretical values (Table S4.6), with no major differences observed between the values in milk and solvent standard (Table S4.3). The recoveries for D8, D90, and TGSA fell within the range of 80-110% with a relative standard deviation (RSD%) lower than 10% (Table S4.5). These values are considered acceptable compared to the recommended range of 70-120% and RSD of 15% [81]. The method detection limits (MDL) for D8, D90, and TGSA were

determined to be 0.0014 ng/mL, 0.0067 ng/mL, and 0.0041 ng/mL, respectively (Table S4.5). The limits of quantification (LOQ) were established as 0.005, 0.024, and 0.014 ng/mL, respectively (Table S4.5).

Concentrations of D8, D90, TGSA, BPS and BPA for the 1st and 2nd extraction are shown in Table S4.7 and were log-scaled for further analysis. Differences were observed in the detected levels between the 1st and 2nd extractions across certain samples. These differences may be influenced by the sample preparation, which can have a serious effect on the quantification accuracy [34].

After excluding the non-detects in either extraction, a paired sample t-test was conducted between data collected for the 1st and 2nd extractions (Table S4.8). P-values for all of the compounds (D8, D90, TGSA, BPS and BPA) were higher than 0.05, indicating no significant difference in the detected levels of these compounds between the 1st and 2nd extractions. The variations in concentrations for BPS, D8, D90, TGSA and BPA between the initial and subsequent extractions are also illustrated in a linear regression curve, as shown in Figure S4.16. All but one of the points fall within the prediction intervals suggesting that detected levels for future extractions would also fall within a similar range to the detected levels for the 1st and 2nd extractions. This observation further emphasizes that the extraction method was sufficiently precise for the semi-quantification of the BPS-related unknowns D8, D90 and TGSA.

A common approach in non-targeted analysis involves semi-quantification using structurally similar standards compared to the unknown compounds. The concentration of the unknown is typically obtained by dividing its peak area with the response factor (RF) of a similar standard [34]. However, one major limitation occurs when the use of structurally similar surrogate standards leads to high inaccuracies (up to several hundred-fold for drugs in biological samples) [33]. In the absence of a standard, ionization efficiency (IE) can be used to predict the

concentration of an analyte using its predicted ionization efficiency [33]. The predicted IE of various compounds can be obtained through different prediction models. The IE varies depending on the properties of each compound, and can be influenced by the sample matrix as well as the eluent used [34].

In the present study, we aimed to enhance the precision of our semi-quantification by using isotope-labeled internal standards for our selected compounds. Unlike many other non-targeted studies, we also explored retrospective semi-quantification using a set of challenging criteria (Table 4.2.); this method is often overlooked and involves revisiting previously collected data. This approach can help mitigate inaccuracies that may arise during the semi-quantification process. To the best of our knowledge, only one previous non-targeted study was done to investigate the use of retrospective semi-quantification for green tea samples, highlighting the need to develop more robust semi-quantitative methods that can reduce potential inaccuracies and contribute new data for risk assessments and the investigation of temporal trends [34, 82].

Table 4.2. Criteria assessed for implementation of the retrospective semi-quantification of bisphenol-related compounds newly discovered in human milk using NTA

Assessment of past batches	*a posteriori* validation of the method performance for the new compounds
• Quality assurance: method fully validated for the targeted analysis of 9 bisphenol compounds [18] • Stable isotope-labeled internal standards spiked in all the samples. Assessment of relative response factor (RRF) for the targeted bisphenols	• Quality assurance: full method validation for the newly discovered bisphenol-related compounds • Determination of the relative response factor (RRF) using one of the structurally similarly internal standards initially spiked in all samples

across batches over the course of two years (low relative standard deviation) • Quality control: procedural blanks (n≥3) and QC sample (spiking with targeted bisphenols) with every batch. • Assessment of the signal-to-noise ratios and/or method detection limits across the batches	• Assessment of the maximum mass measurement errors and retention time shifts across the past batches. Visual inspection of peak shapes • Re-analysis of selected samples across the past batches. Comparison of the semi-quantification and re-analysis results

Table 4.3. Semi-quantified levels of D8, D90 and TGSA in human milk from South Africa, Canada and China

Study	Country	Sampling year	Instrument (Method)	MDL and LOD (ng/mL)	Compound	Frequency of detection (DF) %	Median	Range (ng/mL)
This study n=194	*South Africa (Vhembe)*	2018-2019	HPLC-q-TOF-MS/MS	*0.0014	D8	17	<LOQ	<MDL-0.60
				*0.0067	D90	7	<LOQ	<MDL-1.68
				*0.0041	TGSA	3	<LOQ	<MDL-0.72
This study n=193	*South Africa (Pretoria)*	2018-2019	HPLC-q-TOF-MS/MS	*0.0014	D8	4	<LOQ	<MDL-0.15
				*0.0067	D90	4	<LOQ	<MDL-0.66
				*0.0041	TGSA	3	<LOQ	<MDL-0.41
This study n=207	*Canada (Montreal)*	2018-2019	HPLC-q-TOF-MS/MS	*0.0014	D8	2	<LOQ	<MDL-1.24
				*0.0067	D90	2	<LOQ	<MDL-1.98
				*0.0041	TGSA	1	<LOQ	<MDL-0.19
Luo et al. [20] n=60	*China*	2017-2018	HPLC-triple-quad-MS/MS	**0.0004	D8	100	0.00256	0.00050-0.0581
				**0.004	D90	16.7	<LOD	<LOD-0.378
				**0.0004	TGSA	26.7	<LOD	<LOD-0.0166

***MDL: Method detection limit**
****LOD: Limit of detection**
LOQ: Limit of quantification

The estimated MDL values (method detection limits) for D8, D90 and TGSA in our study (Table 4.3) were higher than the reported LOD (limit of detection) values in the only previous targeted study on human milk from China [20] that identified these compounds. It is possible that this difference may arise from the variation in instrumental analysis since liquid chromatography coupled to a triple quadrupole, used in their study, is known for its higher sensitivity and lower detection limits compared to LC-QTOF-MS/MS [83]. Based on the overall performance of the method used here, detected concentrations of D8, D90 and TGSA were calculated using the semi-quantification approach for each region (Table 4.3). In Vhembe, estimated concentrations for the levels of D8, D90 and TGSA ranged from <MDL to 0.60 ng/mL, <MDL to 1.68 ng/mL and <MDL to 0.72 ng/mL, with detection frequencies of 17%, 7% and 3%, respectively. Levels in Pretoria ranged from <MDL to 0.15 ng/mL for D8, <MDL to 0.66 ng/mL for D90, and <MDL to 0.41 ng/mL for TGSA, with detection frequencies of 4% for both D8 and D90 and 3% for TGSA. For Montreal, the estimated concentrations for D8, D90 and TGSA ranged from <MDL to 1.24 ng/mL, <MDL to 1.98 ng/mL and < MDL to 0.19 ng/mL, respectively, with detection frequencies lower than Vhembe and Pretoria: 2% for both D8 and D90 and 1% for TGSA. The median values for D8 in all three regions fell below the limit of quantification (LOQ) in this study and were comparable to reported levels of D8, D90 and TGSA in China, where the median for D8 was 0.00256 ng/mL, while D90 and TGSA fell below the limit of detection.

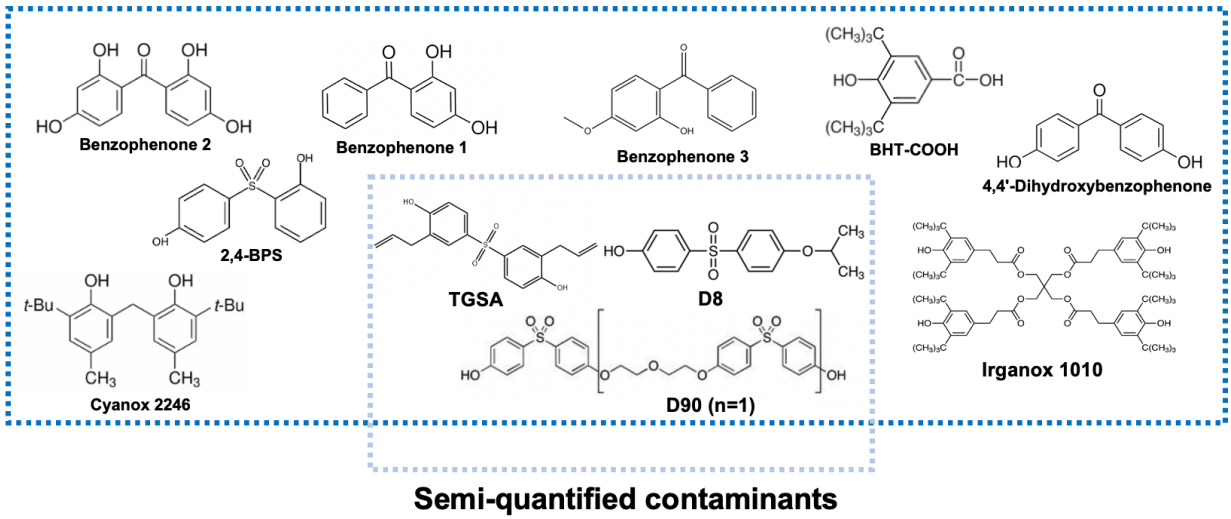
The findings of the present study provide evidence that lesser-known BPS derivatives, commonly employed as colour developers in thermal labels for food packaging, are detected in human milk from Canada (Montreal) and South Africa (Vhembe and Pretoria). In 2023, the European Food Safety Authority (ESFA) concluded that the tolerable daily intake (TDI) value for BPA should be lowered to 0.2 ng/kg of bw/day [84]. To the best of our knowledge, tolerable daily intakes have

not been established for other bisphenols [85]. While the levels of BPS and its derivatives in human milk have generally been reported to be lower than those for BPA in studies from different countries, such as France, Spain, and China, their possible health implications deserve some consideration [23, 24, 27, 86].

4.5. Research implications

The environmental chemicals to which mothers are exposed through diet, personal care products, household products, pharmaceuticals, and their work environment can be transferred to their milk during lactation [87]. Early life exposure to these contaminants can have health implications, as infancy represents a critical window of susceptibility to their toxic effects [88]. It is crucial for researchers to explore a greater portion of the chemical exposome by detecting and identifying compounds that are beyond those that are commonly reported across different countries [10]. The present study demonstrates the utility of employing non-targeted screening for the exclusive investigation of bisphenol-related compounds in human milk (Figure 4.1).

Figure 4.1. Bisphenol-related compounds identified using non-targeted analysis in human milk from Canada and South Africa



In addition to the bisphenol unknowns, it is important to recognize that other chemicals of concern may also be present in human milk, highlighting the need to broaden the scope for future non-targeted studies to detect a wider range of contaminants from multiple families.

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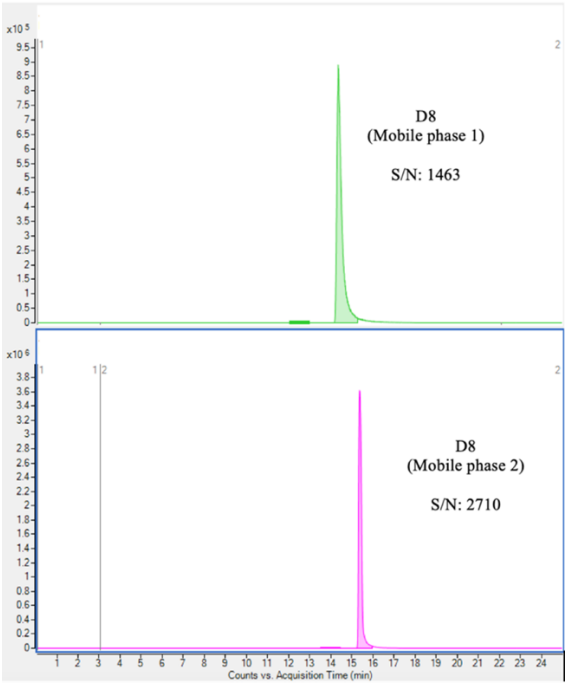
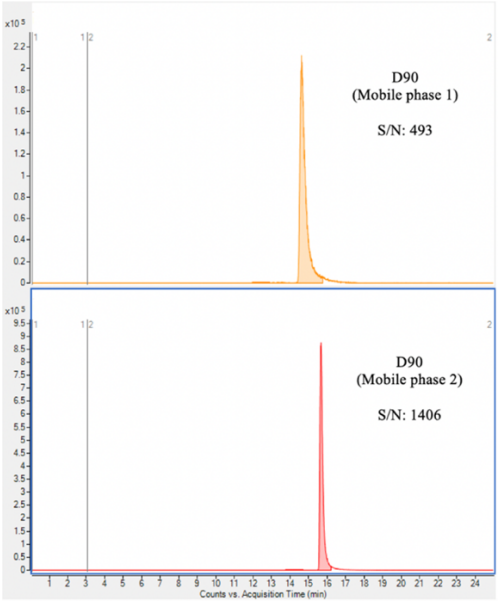
4.7. Supplementary information

Figure S4.1: Signal intensities of D8 (30 ng/mL) in calibration solvent in 2 different mobile phases (S/N: Signal-to-noise ratio)



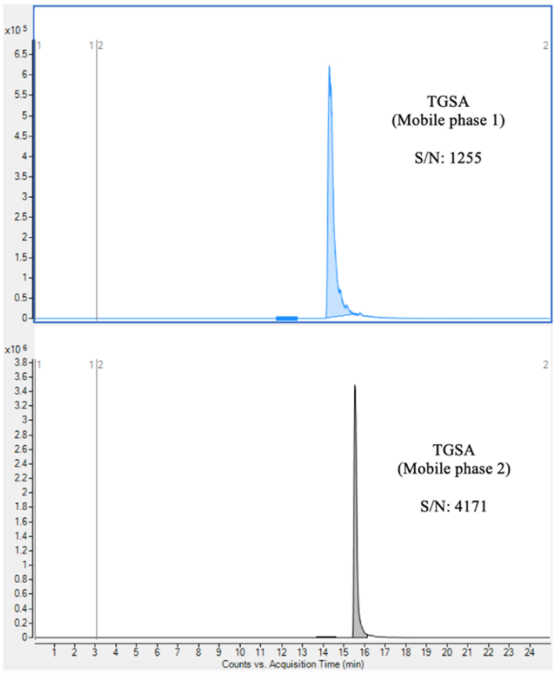
Mobile phase 1: ACN and MeOH (1:1)
Mobile phase 2: MeOH

Figure S4.2: Signal intensities of D90 (30 ng/mL) in calibration solvent in 2 different mobile phases



Mobile phase 1: ACN and MeOH (1:1)
Mobile phase 2: MeOH

Figure S4.3: Signal intensities of TGSA (30 ng/mL) in calibration solvent in 2 different mobile phases



Mobile phase 1: ACN and MeOH (1:1)
Mobile phase 2: MeOH

Figure S4.4: MS/MS spectra and chromatograph peak of 2,4-BPS in an unspiked human milk sample and pure analytical standard (20 V) (Fragmented parent ion at m/z of 249.02)

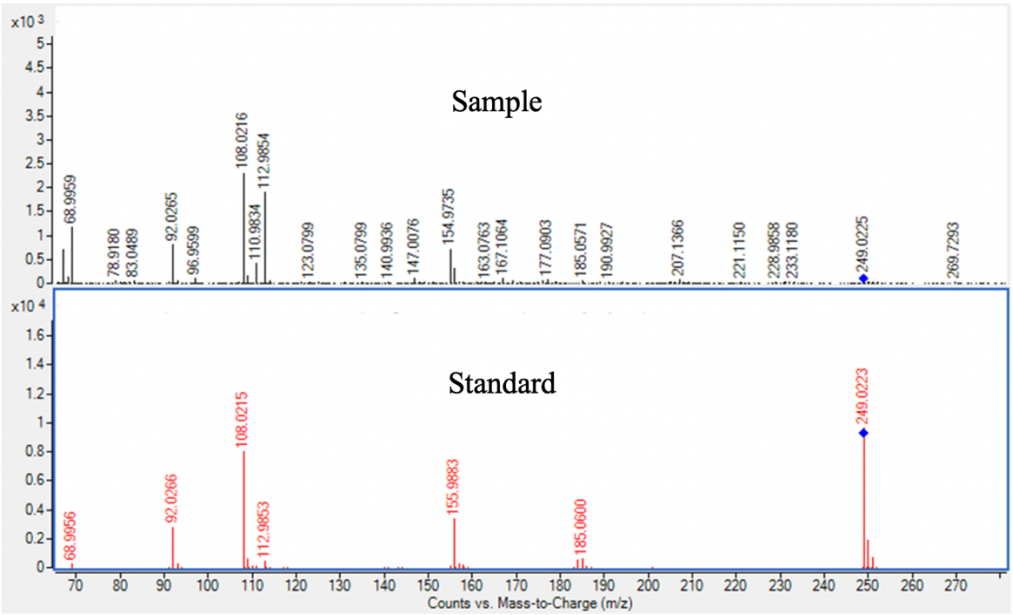


Figure S4.5: MS/MS spectra of benzophenone-1 in an unspiked human milk sample and pure analytical standard (20 V) (Fragmented parent ion at m/z of 213.06)

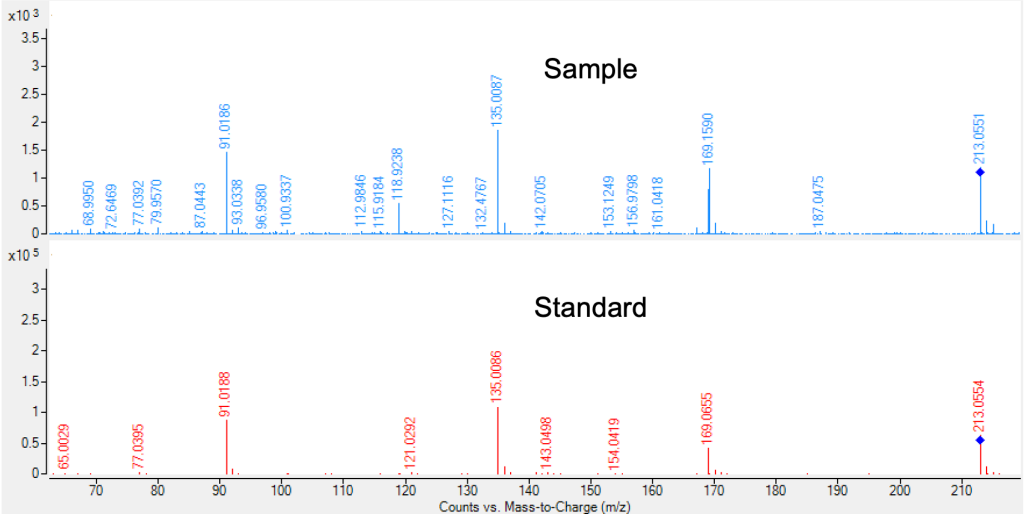


Figure S4.6: MS/MS spectra of benzophenone-2 in an unspiked human milk sample and pure analytical standard (20 V) (MS1 value at 245.05 not visible)

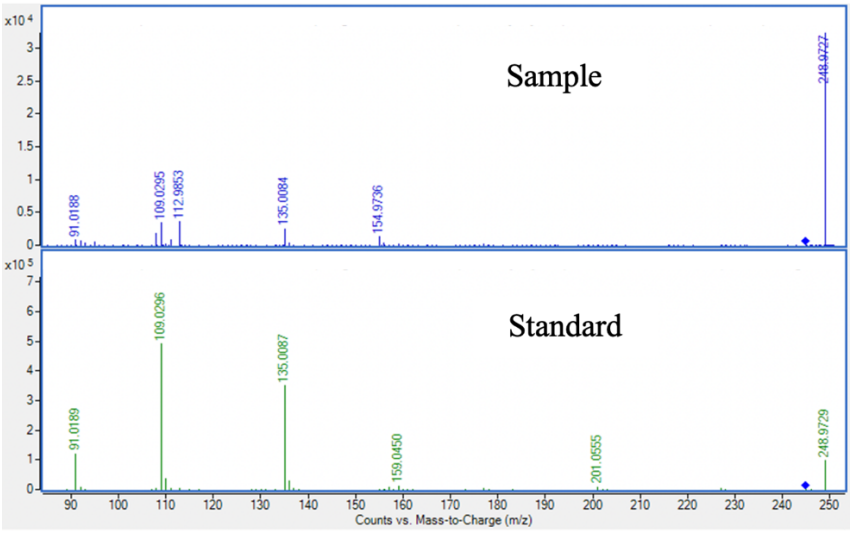


Figure S4.7: MS/MS spectra of benzophenone-3 in an unspiked human milk sample and pure analytical standard (20 V) (Fragmented parent ion at m/z of 227.07)

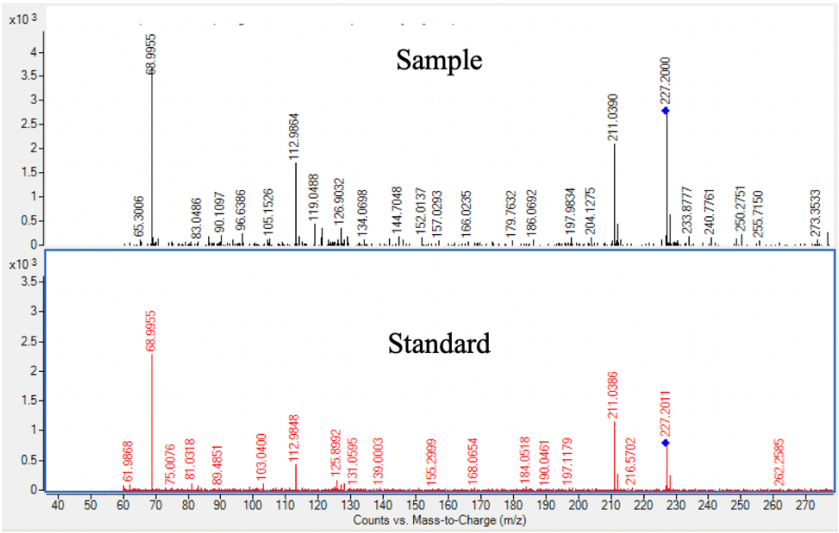


Figure S4.8: MS/MS spectra of 4,4'-dihydroxybenzophenone in an unspiked human milk sample and pure analytical standard (20 V) (Fragmented parent ion at m/z of 213.06)

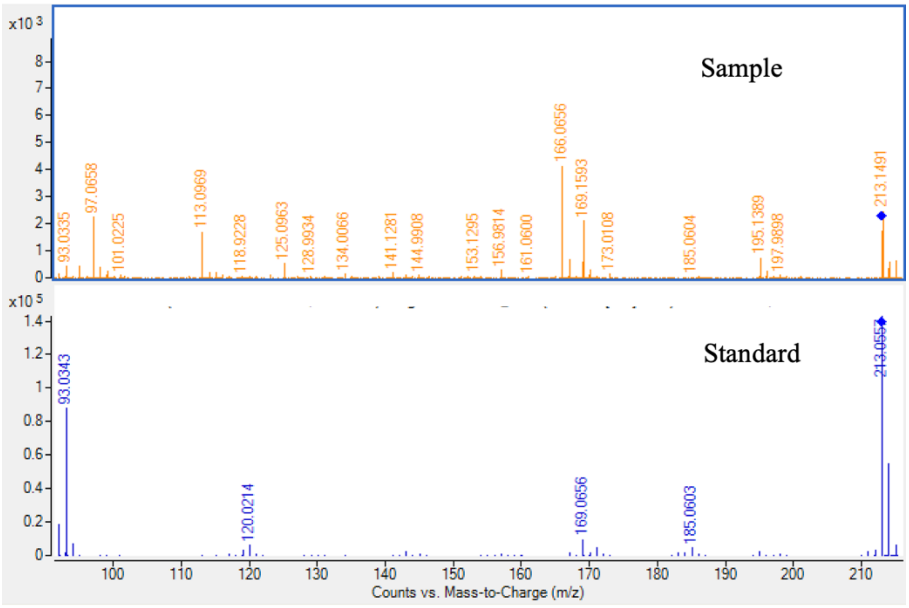


Figure S4.9: MS/MS spectra of D8 in an unspiked human milk sample and pure analytical standard (20 V) (Fragmented parent ion at m/z of 291.07)

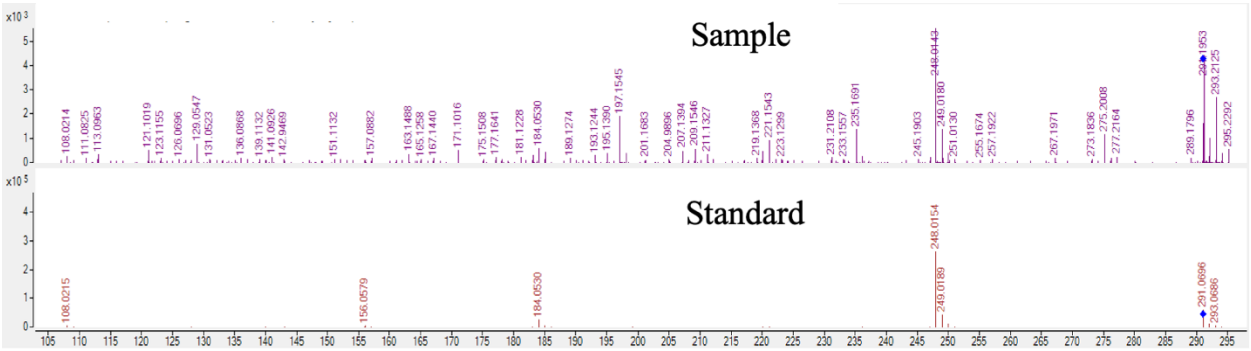


Figure S4.10: MS/MS spectra of D90 in an unspiked human milk sample and pure analytical standard (35 V) (Fragmented parent ion at m/z of 569.09)

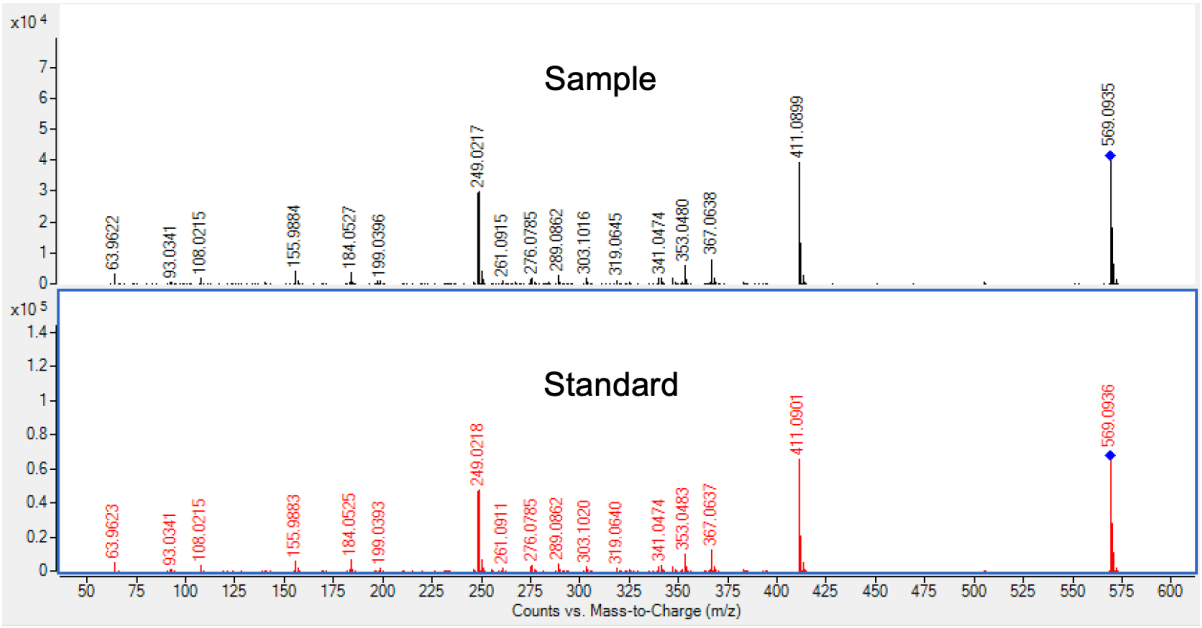


Figure S4.11: MS/MS spectra of TGSA in an unspiked human milk sample and pure analytical standard (20 V) (Fragmented parent ion at m/z of 329.09)

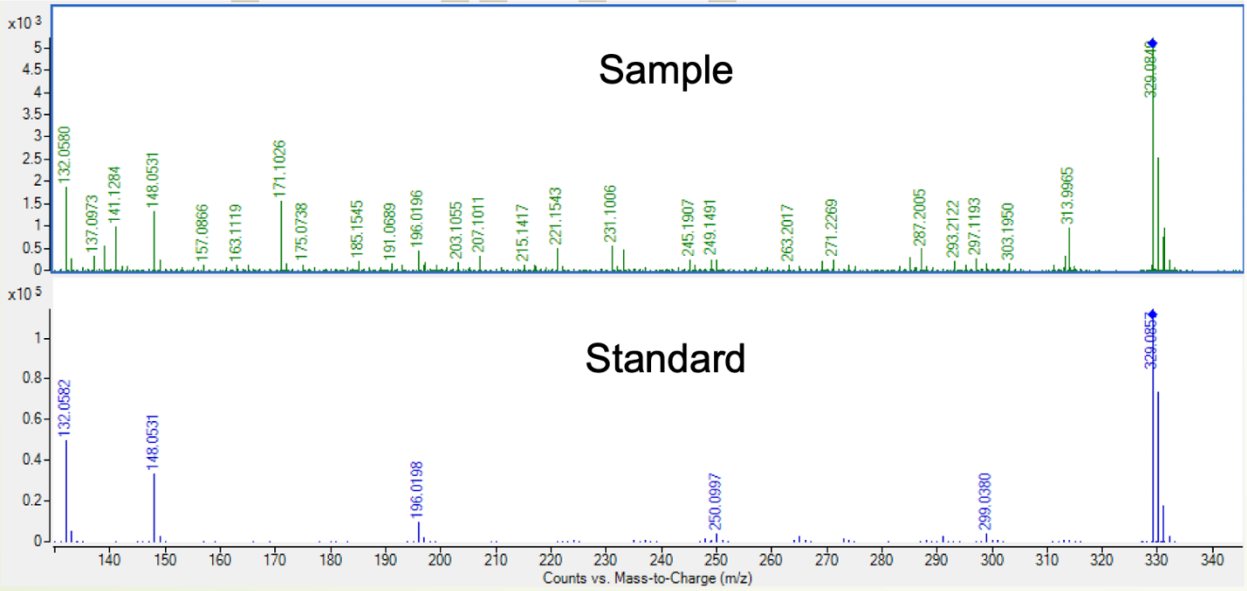


Figure S4.12: MS/MS spectra of Irganox 1010 in an unspiked human milk sample and pure analytical standard (40 V) (MS1 value at 1175.78 not visible)

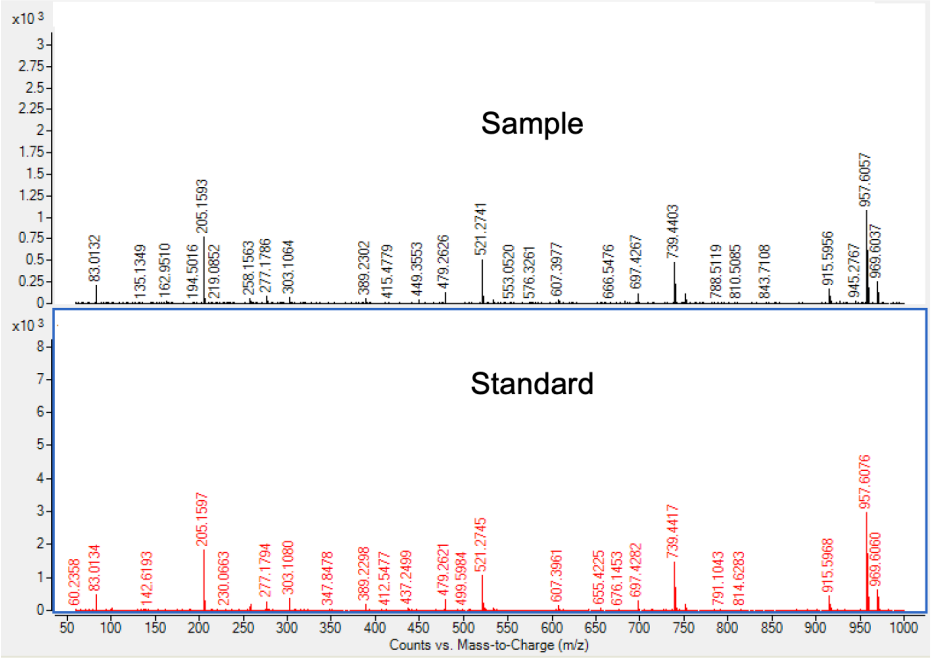


Figure S4.13: MS/MS spectra of Antioxidant 2246 in an unspiked human milk sample and pure analytical standard (40 V) (MS1 value at 339.20 not visible)

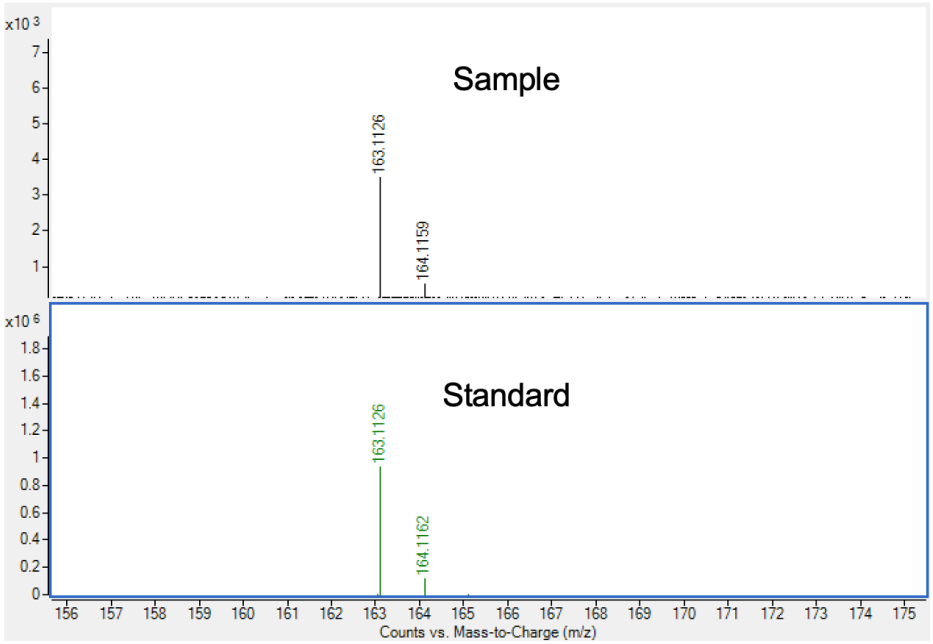


Figure S4.14: MS/MS spectra of BHT-COOH in an unspiked human milk sample and pure analytical standard (20 V) (Fragmented parent ion at m/z of 249.1)

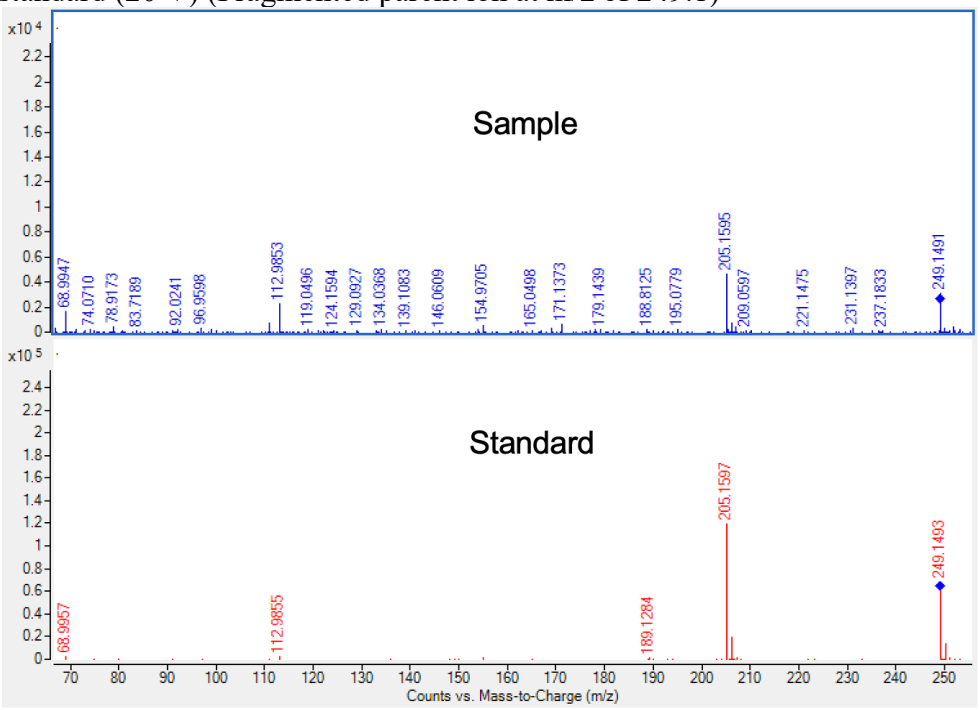


Figure S4.15: Relative response factors (RRFs) for BPA, BPF, BPAF and BPS versus their respective $^{13}C_{12}$ and d_4 labelled surrogates across all 20 analyzed batches and the date of their analysis (RRF for BPS was constant with relative standard deviation of 8.89 %)

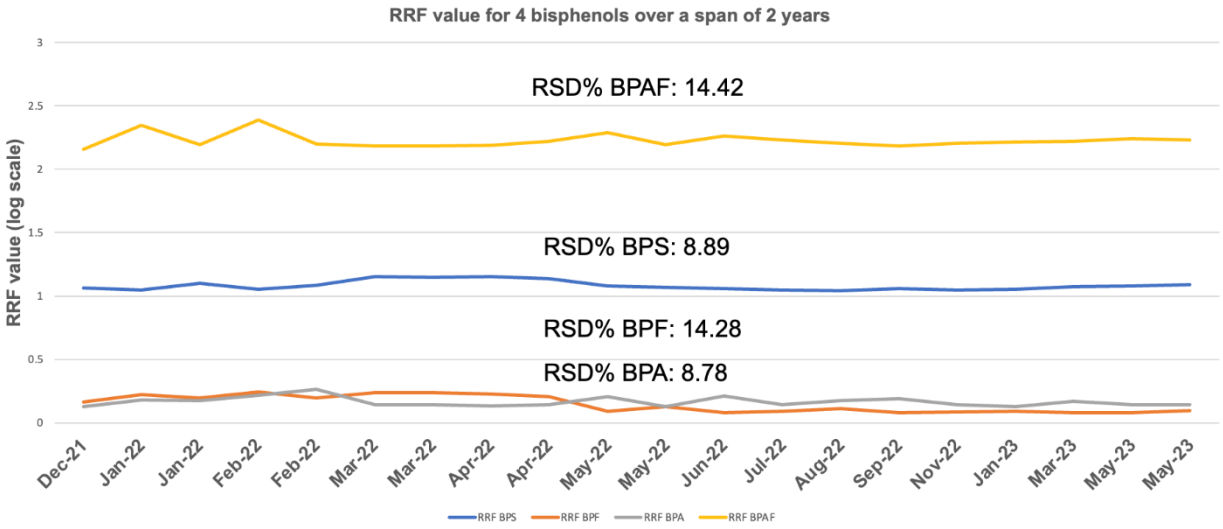
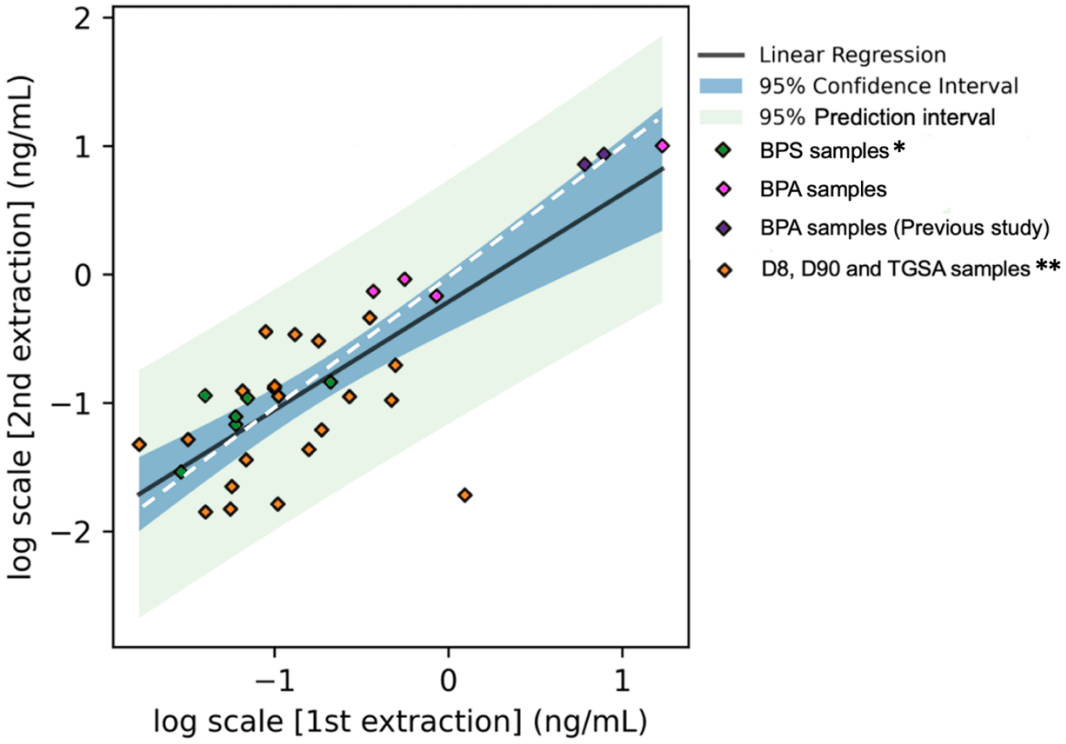


Figure S4.16: Linear regression plot of BPS, D8, D90, TGSA and BPA for 1st and 2nd extraction (The dashed white line represents the 1:1 line)



***BPS and BPA for 1st and 2nd extractions were quantified using $^{13}C_{12}$-BPS and D_4-BPA, respectively**
****D8, D90 and TGSA for 1st and 2nd extractions were semi-quantified using $^{13}C_{12}$-BPS**

Table S4.1: Non-targeted analysis selected parameters (Profinder)

Parameters	Values
Match tolerance mass	10 ppm
Expansion values for chromatogram extraction (*m/z*) (+/-)	50 ppm
Isotope abundance score	60%
Retention time window	±0.3 min
Peak filter (absolute height)	≥ 300 counts
Integrator method	Agile 2
Peak spectra: spectra to include how much percent of average scan	>10%
TOF spectra: exclude if above how much saturation	20%
Post processing: Find by formula peak filter (absolute height)	≥ 300 counts

Table S4.2: Additional bisphenol-related compounds added to the database library (n=18)

Name	Formula	CAS RN
4,4'-Dihydroxybenzophenone	$C_{13}H_{10}O_3$	611-99-4
Benzophenone 1	$C_{13}H_{10}O_3$	131-56-6
Benzophenone 3	$C_{14}H_{12}O_3$	131-57-7
Butylated hydroxyanisole (BHA)	$C_{11}H_{16}O_2$	25013-16-5
Butylated Hydroxytoluene (BHT)	$C_{15}H_{24}O$	128-37-0
3,5-di-tert-butyl-4-hydroxybenzaldehyde (BHT-CHO)	$C_{15}H_{22}O_2$	1620-98-0
2,6-di-*tert*-butyl-4-(hydroxymethyl) phenol (BHT-OH)	$C_{15}H_{24}O_2$	88-26-6
2,6-di-*tert*-butyl-1,4-benzoquinone (BHT-Quinone)	$C_{14}H_{20}O_2$	719-22-2
3,5-Di-tert-butyl-4-hydroxybenzoic acid (BHT-COOH)	$C_{15}H_{22}O_3$	1421-49-4

2,6-di-*tert*-butyl-4-hydroxy-4-methyl-2,5-cyclohexadienone (BHT-quinol)	$C_{15}H_{24}O_2$	10396-80-2
Octyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-propionate (Irganox 1135)	$C_{25}H_{42}O_3$	13417-12-4
Octadecyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-propionate (Irganox 1076)	$C_{35}H_{62}O_3$	2082-79-3
Pentaerythritol tetrakis(3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate) (Irganox 1010)	$C_{73}H_{108}O_{12}$	6683-19-8
4-tert-Octylphenol	$C_{14}H_{22}O$	140-66-9
4-Nonylphenol	$C_{15}H_{24}O$	104-40-5
2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	96-76-4
2,4-di-tert-amylphenol	$C_{16}H_{26}O$	120-95-6
4-sec-Butyl-2,6-di-tert-butylphenol	$C_{18}H_{30}O$	17540-75-9

Table S4.3. The relative intensities of qualifier to quantifier ions for BPS, D8, D90 and TGSA in pure solvent and human milk matrix.

Compound	Matrix	Base peak [M-H]- (m/z)	Qualifier 1 (m/z)	Relative Intensity %	Relative difference %*	Qualifier 2 (m/z)	Relative intensity %	Relative difference %*	Collision energy (V)
BPS**	Solvent	249.0222	108.0215	82.9	-	155.9883	34.7	-	20
	Milk			84.3	1.65		33	-4.73	
D8	Solvent	291.0691	248.0144	535.8	-	184.0527	60.1	-	20
	Milk			515.8	-3.74		58.9	2.00	
D90	Solvent	569.094	411.0901	91.1	-	249.0217	69.0	-	35
	Milk			92.42	-1.46		67.3	2.40	
TGSA	Solvent	329.0848	132.0579	17.7	-	148.0529	11.94	-	20
	Milk			17.1	3.57		11.53	3.57	

*Relative difference was calculated as the ratio using the difference between the relative intensities obtained in milk matrix and solvent

**BPS values obtained from previous study

Table S4.4. Non-targeted identification of 42 selected bisphenol-related candidates in breast milk from Montreal, Vhembe and Pretoria (Profinder score >90%)

ESI- m/z [M-H]-	*Retention time (min)*	*Region (Montreal, Vhembe or Pretoria)*	*Formula*	*Suspected identity (Confidence level 3-Schymanski scale)* **[1]**	*Further identification*
291.0691	14.40	All	$C_{15}H_{16}O_4S$	4-(4-propan-2-yloxyphenyl) sulfonylphenol (D8)	Confirmed (level 1) [1]
569.0940	14.65	All	$C_{12}H_{10}O_4S[C_{16}H_{16}O_5S]_n$ (n=1)	Phenol, 4,4'-sulfonylbis-, polymer with 1,1'-oxybis[2-chloroethane] (D90)	Confirmed (level 1)
329.0848	14.55	All	$C_{18}H_{18}O_4S$	4-(4-hydroxy-3-prop-2-enylphenyl)sulfonyl-2-prop-2-enylphenol (TGSA)	Confirmed (level 1)
249.0224	12.20	Montreal	$C_{12}H_{10}O_4S$	2,4- bisphenol S	Confirmed (level 1)
245.0450	12.11	Montreal	$C_{13}H_{10}O_5$	2,2',4,4'-Tetrahydroxybenzophenone (Benzophenone 2)	Confirmed (level 1)
227.0708	14.64	Montreal	$C_{14}H_{12}O_3$	Oxybenzone (Benzophenone 3)	Confirmed (level 1)
257.081	14.16	All	$C_{15}H_{14}O_4$	Methyl bis(4-hydroxyphenyl)acetate (MBHA)	Inconclusive (suggested identity by SIRIUS to be Davidigenin; standard was not purchased)
379.1698	13.84	All	$C_{27}H_{24}O_2$	2,2-Bis(2-hydroxy-5-biphenylyl) propane (BPPH)	Inconclusive (Poor SIRIUS score)
287.0556	12.57	All	$C_{15}H_{12}O_6$	3,3'-Methylenedisalicylic acid	Inconclusive (Poor SIRIUS score)
201.0552	11.49	All	$C_{12}H_{10}O_3$	p-(p-Hydroxyphenoxy)phenol	Inconclusive (Poor SIRIUS score)
109.0290	9.41	All	$C_6H_6O_2$	Hydroquinone	Incorrect identity using standard

434.1604	15.03	All	$C_{25}H_{25}NO_6$	3,3'-bis(6-hydroxy-5-methylbenzoic acid)	Inconclusive (Poor SIRIUS score)
305.1178	15.18	All	$C_{20}H_{18}O_3$	1,1,1-Tris(4-hydroxyphenyl)ethane	Inconclusive (Poor SIRIUS score)
459.3263	18.32	All	$C_{32}H_{44}O_2$	2,2'-(Octahydro-4,7-methano-1H-indenediyl)bis[6-tert-butyl-p-cresol]	Inconclusive (Poor SIRIUS score)
507.1573	15.67	All	$C_{28}H_{31}O_7P$	Thymolphthalein monophosphate ([4-[1-(4-hydroxy-2-methyl-5-propan-2-ylphenyl)-3-oxo-2-benzofuran-1-yl]-5-methyl-2-propan-2-ylphenyl] phosphate)	Inconclusive (Poor SIRIUS score)
544.3942	17.85	All	$C_{37}H_{52}O_3$	4,4',4"-(Butane-1,1,3-triyl)tris(2-tert-butyl-5-methylphenol)	Inconclusive (Poor SIRIUS score)
464.1657	14.84	Montreal	$C_{27}H_{29}O_5S$	Thymol blue (4-[3-(4-hydroxy-2-methyl-5-propan-2-ylphenyl)-1,1-dioxo-2,1λ6-benzoxathiol-3-yl]-5-methyl-2-propan-2-ylphenol)	Inconclusive (Poor SIRIUS score)
621.4308	18.75	Vhembe and Pretoria	$C_{43}H_{58}O_3$	1,1,3-Tris(5-cyclohexyl-4-hydroxy-o-tolyl)butane	Inconclusive (Poor SIRIUS score)
341.2230	16.72	Vhembe and Pretoria	$C_{21}H_{30}N_2O_2$	2,2'-methylenebis[5-(diethylamino)phenol]	Inconclusive (Poor SIRIUS score)
339.2324	17.10	Vhembe and Pretoria	$C_{23}H_{32}O_2$	2,2'-Methylenebis(4-methyl-6-tert-butylphenol) (Cyanox 2246)	Confirmed (level 1)
269.1542	15.04	Montreal	$C_{18}H_{22}O_2$	4,4'-(1,3-Dimethylbutylidene)diphenol	Inconclusive (Poor SIRIUS score)
309.1854	17.29	All	$C_{21}H_{26}O_2$	(4-[1-(4-hydroxyphenyl)-3,3,5-	Same retention time as the standard, and several MS/MS fragments also

				trimethylcyclohexyl]phenol) (Bisphenol TMC)	matching with the standard but missing important fragment of bisphenol TMC at m/z value at 215.14
381.2794	17.22	All	$C_{26}H_{38}O_2$	(4,4'-(Butane-1,1-diyl)bis(2-(tert-butyl)-5-methylphenol) (Santowhite)	Inconclusive (Poor SIRIUS score)
423.3263	18.28	Vhembe and Pretoria	$C_{29}H_{44}O_2$	4,4'-Dihydroxy,3,3'-5,5'-tetra-t-butyldiphenylmethane (Ionox 220)	Inconclusive (Poor SIRIUS score)
365.0725	14.70	Vhembe and Pretoria	$C_{15}H_{12}F_6N_2O_2$	2-Amino-4-[2-(3-amino-4-hydroxyphenyl)-1,1,1,3,3,3-hexafluoro-2-propanyl]phenol	Inconclusive (Poor SIRIUS score)
657.3917	21.71	Vhembe and Pretoria	$C_{41}H_{50}N_6O_2$	Bisoctrizole (2-(benzotriazol-2-yl)-6-[[3-(benzotriazol-2-yl)-2-hydroxy-5-(2,4,4-trimethylpentan-2-yl)phenyl]methyl]-4-(2,4,4-trimethylpentan-2-yl)phenol)	Inconclusive (Poor SIRIUS score)
219.1749	16.68	All	$C_{15}H_{24}O$	2,4-Di-tert-butyl-5-methylphenol	Inconclusive (Poor SIRIUS score)
177.1279	10.32	All	$C_{12}H_{18}O$	5-tert-butyl-2,3-dimethylphenol	Inconclusive (Poor SIRIUS score)
273.0763	11.05	Montreal	$C_{15}H_{14}O_5$	2,2'-Dihydroxy-4,4'-dimethoxybenzophenone (Benzophenone-6)	Incorrect identity using standard
311.2011	17.10	All	$C_{21}H_{28}O_2$	4,4'-(Propane-2,2-diyl)bis(2-isopropylphenol (Bisphenol G)	Inconclusive (Poor SIRIUS score)
243.0658	12.95	All	$C_{14}H_{12}O_4$	Bis(4-hydroxyphenyl)acetic acid	Inconclusive (Poor SIRIUS score)

1175.7763	18.10	All	$C_{73}H_{108}O_{12}$	[3-[3-(3,5-ditert-butyl-4-hydroxyphenyl)propanoyloxy]-2,2-bis[3-(3,5-ditert-butyl-4-hydroxyphenyl)propanoyloxymethyl]propyl] 3-(3,5-ditert-butyl-4-hydroxyphenyl)propanoate) (Irganox 1010)	Confirmed (level 1)
287.0556	14.63	All	$C_{15}H_{12}O_6$	5,5'-Methylenedisalicylic acid	Inconclusive (Poor SIRIUS score)
807.5481	18.32	All	$C_{53}H_{77}O_4P$	Phosphorous acid, 2-(1,1-dimethylethyl)-4-[1-[3-(1,1-dimethylethyl)-4-hydroxyphenyl]-1-methylethyl]phenyl bis(4-nonylphenyl) ester	Inconclusive (Poor SIRIUS score)
213.0552	11.53	Montreal	$C_{13}H_{10}O_3$	4,4'-Dihydroxybenzophenone	Confirmed (level 1)
213.0552	11.21	Montreal	$C_{13}H_{10}O_3$	2,4-Dihydroxybenzophenone (Benzophenone 1)	Confirmed (level 1)
219.1749	15.09	All	$C_{15}H_{24}O$	Butylated Hydroxytoluene (BHT)	Inconclusive (Poor SIRIUS score)
249.1490	14.37	All	$C_{15}H_{22}O_3$	3,5-di-tert-butyl-4-hydroxybenzoic acid (BHT-COOH)	Inconclusive (Poor SIRIUS score)
219.1385	14.61	All	$C_{14}H_{20}O_2$	2,6-di-tert-butyl-p-benzoquinone (BHT-quinone)	Inconclusive (Poor SIRIUS score)
233.1542	14.20	All	$C_{15}H_{22}O_2$	3,5-di-tert-butyl-4-hydroxybenzaldehyde BHT-CHO	Inconclusive (Poor SIRIUS score)
179.1072	12.32	All	$C_{11}H_{16}O_2$	Butylated hydroxyanisole (BHA)	Inconclusive (Poor SIRIUS score)
205.1593	16.31	Vhembe and Pretoria	$C_{14}H_{22}O$	4-tert-Octylphenol	Inconclusive- acceptable SIRIUS score, but retention time different

	than standard (presence of possible isomers)

Table S4.5: Relative recovery of spiked D8, D90 and TGSA in pooled human milk (n=3 replicate)

Analyte	Relative response factor (RRF)	Linearity r^2	Matrix effects	Average intraday relative recovery % (30 ppb)	Relative standard deviation (RSD%)	Method detection limit (MDL) (ng/mL milk)	Limit of quantification (LOQ) (ng/mL milk)
BPS*	12.07**	0.99*	21%*	102*	3.7*	0.0020*	0.007*
D8	13.99	0.99	46%	100	8.9	0.0014	0.005
D90	3.40	0.99	47%	86	4.9	0.0067	0.024
TGSA	11.74	0.99	47%	103	5.2	0.0041	0.014

**BPS values obtained from previous study*
***Average RRF obtained within a span of 2 years throughout 20 batches*

Table S4.6: Mean mass measurement errors (ppm) for D8, D90 and TGSA in pure solvent and sample matrices

Compound	*m/z*	Mass measurement error (ppm) of standards in pure solvent (n=3)	Mass measurement error (ppm) in human milk matrix (n=3)
D8	291.0691	0.34 ±0.27	1.00±0.34
D90	569.0940	0.11±0.52	0.18±0.77
TGSA	329.0849	0.45±0.18	0.90±0.54

Table S4.7: Concentrations (ng/mL) of BPS, D8, D90, TGSA and BPA for 1st and 2nd extractions (ND: not-detected)

Sample	1st extraction data												2nd extraction data											
	BPS	BPS (log-scaled)	D8	D8 (log-scaled)	D90	D90 (log-scaled)	TGSA	TGSA (log-scaled)	BPA	BPA (log-scaled)	BPA [2]	BPA (log-scaled) [2]	BPS	BPS (log-scaled)	D8	D8 (log-scaled)	D90	D90 (log-scaled)	TGSA	TGSA (log-scaled)	BPA	BPA (log-scaled)	BPA [2]	BPA (log-scaled) [2]
Sample 1	ND	ND	ND	ND	ND	ND	ND	ND	0.85	-0.071	ND	ND	0.043	-1.37	ND	ND	ND	ND	0.13	-0.89	0.68	-0.17	0.10	-1.00
Sample 2	ND	ND	ND	ND	ND	ND	ND	ND	0.37	-0.43	6.07	0.78	0.068	-1.17	ND	ND	ND	ND	0.16	-0.80	0.74	-0.13	7.21	0.86
Sample 3	ND	ND	ND	ND	ND	ND	ND	ND	16.98	1.23	7.83	0.89	0.038	-1.42	ND	ND	ND	ND	0.095	-1.02	10.06	1.00	8.64	0.94
Sample 4	0.025	-1.60	ND	ND	ND	ND	0.03	-1.50	0.56	-0.25	-		ND	ND	ND	ND	ND	ND	0.052	-1.28	0.92	-0.036	-	
Sample 5	0.060	-1.22	ND	ND	ND	ND	0.27	-0.57	0.06	-1.22	-		0.07	-1.17	ND	ND	ND	ND	0.11	-0.95	ND	ND	-	
Sample 6	ND	ND	ND	ND	ND	ND	0.49	-0.31	2.75	0.44	-		0.06	-1.22	ND	ND	ND	ND	0.20	-0.71	ND	ND	-	
Sample 7	0.06	-1.22	ND	ND	ND	ND	0.35	-0.45	ND	ND	-		0.08	-1.11	0.023	-1.64	ND	ND	0.46	-0.34	ND	ND	-	
Sample 8	0.03	-1.54	0.040	-1.40	ND	ND	0.47	-0.33	ND	ND	-		0.03	-1.53	0.014	-1.85	0.34	-0.47	0.11	-0.96	ND	ND	-	
Sample 9	0.04	-1.40	0.056	-1.25	0.18	-0.75	0.02	-1.78	ND	ND	-		0.11	-0.94	0.015	-1.83	0.30	-0.52	0.048	-1.32	ND	ND	-	
Sample 10	0.07	-1.15	0.056	-1.25	0.10	-1.00	0.19	-0.73	ND	ND	-		0.11	-0.96	0.022	-1.65	0.13	-0.89	0.062	-1.21	ND	ND	-	
Sample 11	0.21	-0.68	0.066	-1.18	0.13	-0.88	0.16	-0.80	ND	ND	-		0.14	-0.84	0.12	-0.91	0.34	-0.47	0.044	-1.36	ND	ND	-	
Sample 12	ND	ND	0.089	-1.05	0.07	-1.16	0.1	-1.00	ND	ND	-		ND	ND	0.36	-0.44	0.04	-1.44	0.14	-0.87	ND	ND	-	
Sample 13	ND	ND	0.11	-0.98	0.10	-0.98	ND	ND	ND	ND	-		ND	ND	0.016	-1.79	0.11	-0.95	ND	ND	ND	ND	-	
Sample 14	ND	ND	1.24	0.094	ND	ND	ND	ND	ND	ND	-		ND	ND	0.019	-1.72	0.26	-0.58	ND	ND	ND	ND	-	

Table S4.8: Paired sample t-test for BPS, D8, D90, TGSA and BPA between 1st and 2nd extractions excluding non-detects in either extraction

Analyte	t-value	df	Significance (p-value)
BPS	1.31	5	0.25
D8	-1.54	6	0.18
D90	0.89	4	0.43
TGSA	-1.3	8	0.23
BPA	0.39	3	0.73
BPA [2]	4.33	1	0.14

References

1. Schymanski, E.L., et al., *Non-target screening with high-resolution mass spectrometry: critical review using a collaborative trial on water analysis.* Analytical and Bioanalytical Chemistry, 2015. **407**(21): p. 6237-6255.
2. Chi, Z.H., et al., *Biomonitoring of bisphenol A (BPA) and bisphenol analogues in human milk from South Africa and Canada using a modified QuEChERS extraction method.* Environ Pollut, 2024: p. 123730.

Connecting paragraph

The data in Chapter 4 highlighted the advantage of using NTA to detect the presence of different emerging bisphenol-related unknowns that would likely not have been detected using traditional targeted analysis. The results revealed the presence of various bisphenol S derivatives employed as color developers in thermal papers, distinct UV absorbers, and synthetic antioxidants within human milk. The presence of these compounds suggests the need to further explore other potential plastic-related unknowns that may bear equal significance to these identified bisphenol-related contaminants.

Aside from bisphenols, with diet being recognized as one of the main routes for human exposure, many other PRCs used in food-related products may enter the body and accumulate in human milk. In Chapter 5, an emphasis was placed on the need to investigate commonly used preservatives, specifically parabens, another group of phenolic compounds, that have been known for their widespread use in food-related products. For this chapter, non-targeted strategies were applied to collected human milk data to identify the presence of common and unusual parabens along with other possible surrounding PRCs of interest.

Chapter 5. Investigation of plastic-related contaminants in human milk: identification of common and unreported parabens using non-targeted strategies

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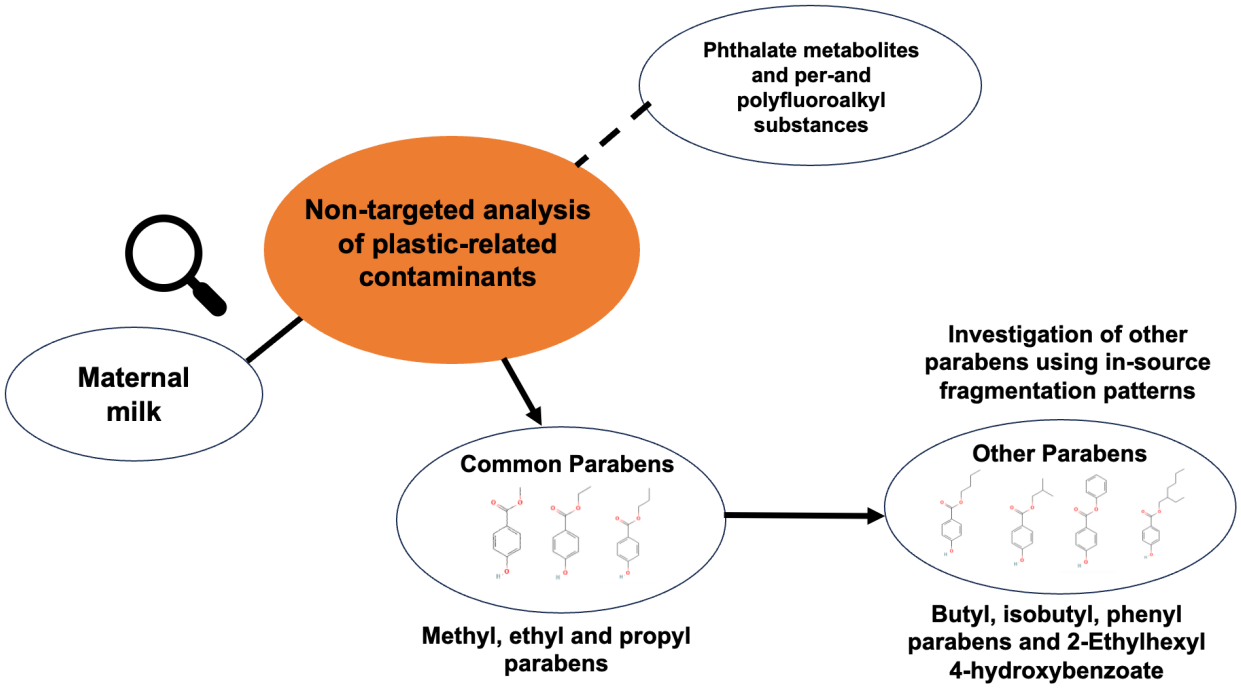
5.1. Abstract

Studies of plastic-related contaminants (PRCs) in human milk mainly utilize a targeted approach to screen and quantify a limited number of contaminants. While targeted analysis allows health officials to quantify the levels of these chemicals in human milk, it fails to detect the presence of other plastic-related unknowns that may be of equal importance. Hence, the objective of the present study was to apply non-targeted screening for the detection and identification of both common and unknown plastic-related contaminants in human milk, with a specific focus on paraben-related compounds. Extracts of 594 human milk samples collected in Canada (Montreal) and South Africa (Vhembe and Pretoria) in 2018-2019 were analyzed and the presence of methyl, ethyl and propyl parabens was confirmed. An additional investigation step, using in-source fragmentation, identified the presence of four other paraben-related compounds in human milk, including 2-EHHB, an unusual paraben exclusive to South African samples. Other PRCs detected included several phthalate metabolites, per- and poly-fluoroalkyl substances (PFAS) and 1,3 diphenyl guanidine, a tire-related chemical. This is the first study to have used different non-targeted analytical strategies for the detection and confirmation of several common and unusual parabens as well as other PRCs in human milk.

Graphical Abstract



Key words: Plastic-related chemicals, non-targeted analysis, liquid chromatography-mass spectrometry, in-source fragmentation, human milk, parabens, phthalates, PFAS, biomonitoring, exposome

5.2. Introduction

Human milk, an essential food source for the growth and development of infants, is rich in vital endogenous metabolites, growth factors, antimicrobial agents, immune boosters and oligosaccharides [1-3]. While the significance of these endogenous components for infant growth is well-established, concerns have emerged regarding the presence of chemical contaminants that may pose a risk to both maternal and infant health [3, 4].

Plastic-related contaminants (PRCs), which are used in food-related materials, cosmetics, pharmaceuticals and many industrial applications, can find their way into the human body and subsequently accumulate in human milk [4-6]. While there are numerous studies that have analyzed PRCs, they have focused primarily on a targeted approach, quantifying a limited spectrum of contaminants, mainly common types of bisphenols, phthalates, parabens and several ultraviolet filters [4, 7-18]. This approach leaves a significant gap in our understanding, as numerous other plastic-related unknowns with potential health implications remain unexplored.

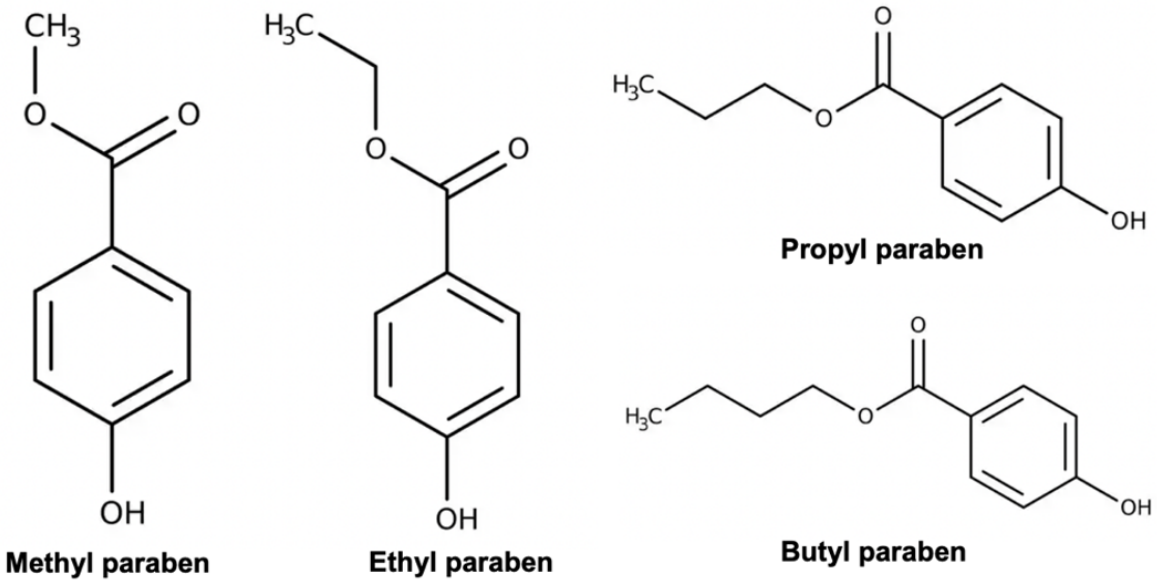
In our previous investigations, we initially analyzed the presence of major bisphenol analogues (bisphenol A, S and AF) in human milk samples from Canada as well as two regions in South Africa with limited data [19]. Subsequently, we used a non-targeted approach (NTA) to identify previously underreported or unknown bisphenol-related compounds (Chapter 4). In the present study, our focus has shifted to the detection of parabens in human milk due to rising concerns about the extensive use of parabens as preservatives in food-related products, cosmetics and pharmaceuticals in the last decade [20]. This has led to their ubiquitous presence in water sources, air, and soil, as well as in human fluids and tissues [21].

Since research has indicated that parabens may act as endocrine disruptors, they are currently considered to be chemicals of emerging concern [20, 22]. Previous studies have found a possible

relationship between exposure to parabens and estrogenic activities, as well as the development of overweight children [23-26]. Parabens have also been shown to possess androgen antagonist activity [27, 28]. Thus, there is a need to investigate the presence of parabens in human milk, including those not commonly reported in previous human biomonitoring studies. While several human milk studies have used a targeted approach to quantify the levels of common parabens (Figure 5.1), uncommon as well as unexpected parabens remain unexplored.

Parabens can undergo conjugation (mainly glucuronidation or sulfation) or hydrolysis reactions into p-hydroxybenzoic acid and other metabolites in the body; free and conjugated parabens can then be transferred to human milk [18, 29, 30]. Conjugated species of parabens have been reported previously in studies on human urine [31-33]. The conjugation potential of parabens deserves investigation because studies have suggested that other conjugated PRCs, such as conjugated bisphenols, may undergo deconjugation in the body, thereby reactivating their endocrine disrupting potential [34-37].

Figure 5.1. Parabens commonly reported in previous human milk studies [30, 38-41]



Unlike traditional targeted screening, NTA offers a comprehensive view of emerging or unexpected families of contaminants (Chapter 4) [42, 43]. Non-targeted screening utilizes data processing techniques, such as feature extraction, peak alignment and statistical analysis, that are capable of detecting an unlimited number of different compounds in samples, making it an appealing technique to better characterize the human exposome [42]. Additionally, through the analysis of accurate mass data and fragmentation patterns obtained from high-resolution mass spectrometry (HRMS), researchers can generate molecular formulas and propose structures for selected unknown compounds [42]. Subsequent confirmation of their identities can be achieved by comparing their MS/MS spectra with those of their corresponding pure analytical standards.

In the present study, we applied non-targeted strategies to detect and identify the presence of various plastic-related components in human milk, with a focus on parabens. We further explored the in-source fragmentation pattern of parabens to identify the presence of unexpected or unknown parabens that share similar patterns to those commonly reported in previous human milk studies. In summary, the objectives of this study were: i) to develop a robust approach utilizing NTA software for the detection and identification of different paraben-related compounds as well as other PRCs in human milk; ii) to explore the conjugation potential of detected parabens; and iii) to investigate the fragmentation pattern of parabens in order to detect other as yet unknown parabens in human milk.

5.3. Materials and methods

5.3.1. Chemicals

Ammonium acetate (NH_4Ac) (LC-MS grade), acetic acid (LC-MS grade), HPLC-grade solvents (water, acetonitrile, and methanol), phenyl paraben (purity ≥ 99%), heptyl paraben (purity ≥ 99%), octyl paraben (purity ≥ 99%) and 2-ethylhexyl 4-hydroxybenzoate (2-EHHB) (purity ≥ 99%) were

purchased from Fisher Scientific (Hampton, VA, USA). Magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). C18 endcapped bulk sorbent and PSA (primary secondary amine) came from Agilent Technologies (Santa Clara, CA, USA). β-glucuronidase type H1 from *Helix Pometia*, (≥500,000 units/g) was purchased from Sigma-Aldrich (Milwaukee, WI, USA). Analytical standards for 1,3-diphenylguanidine (purity≥ 98%), mono-2-ethylhexyl phthalate (purity≥ 98%), monobutyl phthalate (purity≥ 96%), monoisobutyl phthalate (purity≥ 96%), methyl paraben (purity ≥ 99%), ethyl paraben (purity ≥ 99%), propyl paraben (purity ≥ 99%), butyl paraben (purity ≥ 99%), iso-butyl paraben (purity ≥ 99%) and arylsulfatase type H1 from *Helix Pomatia* (>10000 units/g) were purchased from Sigma-Aldrich. A standard solution mixture (PFAC-MXA) with purities of ≥ 98% containing native perfluorohexanoic acid (PFHxA), perfluoroctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), perfluorododecanoic acid (PFDoA), perfluorohexanesulfonate (PFHxS) and perfluorooctanesulfonate (PFOS) was purchased from Wellington Laboratories (Guelph, Canada). Pentyl paraben (purity ≥ 98%) and hexyl paraben (purity ≥ 98%) were purchased from Ambeed, Inc. (Arlington, VA, USA).

Monthly mixtures of stock solutions containing the 4 major parabens (methyl, ethyl, propyl and butyl paraben) were prepared at a concentration of 1 mg L^{-1} in methanol. All stock solutions were stored in amber glass vials in a freezer (-20 °C) prior to analysis.

5.3.2. Sample collection and storage

Procedures for the collection of human milk samples from Canada (Montreal) and South Africa (Vhembe and Pretoria) were the same as for two previous studies (Chapter 3 and 4). In South Africa, mother-infant pairs were recruited from the maternity wards and vaccine clinics of

Tshilidzini Hospital, located in the Vhembe district of Limpopo Province (a rural area), and Tshwane Hospital, located in Pretoria, Gauteng (an urban area). Eligible participants, aged 18 and above, were required to speak either English or Tshivenda, the primary language in the Vhembe district. Additionally, they were expected to have given birth to, or were expecting a live singleton, and, if recruited from the maternity ward, to be able to return one month post-delivery for a brief questionnaire and provision of a human milk sample.

Similarly, participants from Montreal were recruited from the Royal Victoria and St. Mary's Hospitals following childbirth. Eligible participants, also aged 18 and older, were required to be proficient in either French or English and willing to participate in two follow-up sessions. The first session included an explanation of the manual breast milk collection process along with a questionnaire, while the second session focused on actual sample collection.

Milk samples were collected between the 4^{th} and 8^{th} week post-delivery from participants in the three locations (Vhembe, Pretoria, and Montreal) over a 1.5-year period (2018–2019) via manual expression into polypropylene containers (tested to be free of methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl and phenyl parabens). These samples were then stored in cryovials at -80 °C until shipment on dry ice to the analytical laboratory.

Montreal samples were stored in a -80 °C freezer until they underwent freeze-drying in a lyophilizer (FreeZone Cascade Benchtop Freeze Dry Systems, Labconco, Kansas City, KA, USA), while South African samples were freeze-dried using a different lyophilizer (Freeze dryer ALPHA 1-2 LD+, Osterode am Harz, Germany). All samples underwent freeze-drying at a vacuum of 0.09 Torrs at -80 °C for 3 days. Freeze-dried sample aliquots were stored in amber glass vials at -80°C until analysis. A total of 594 human milk samples from different mothers were analyzed for

bisphenol-related compounds using non-targeted analysis (n=194 from Vhembe, n=193 from Pretoria, and n=207 from Montreal).

This study was approved by ethics committees at McGill University, the Research Institute of the McGill University Health Centre, the University of Pretoria, and the Limpopo Department of Health and Social Services.

5.3.3. Sample Preparation

Sample preparation was previously optimized for the quantification of nine major bisphenol analogues in human milk [19]. For the present study the collected data were further analyzed using a non-targeted approach with a focus on parabens.

Briefly, for the extraction process, approximately 0.2 g of freeze-dried milk powder was mixed with 2 mL of HPLC-grade water. This mixture was spiked with 30 µg/L of a bisphenol labeled mixture. Subsequently, 1 mL of a 1 M ammonium acetate buffer solution and 10 mL acetonitrile were added, and the milk sample underwent ultrasonication for 10 minutes. Following sonication, 1 g of MgSO4 and 2 g of NaCl were added to the milk, and the mixture was vortexed, and then centrifuged at 4000 RCF for 10 minutes. The resulting supernatant was transferred to a 15 mL polypropylene tube containing 0.2 g PSA, 0.1 g C18 sorbent, and 0.25 g MgSO4. The sample was vigorously shaken for 45 seconds and then centrifuged at 3000 RCF for 10 minutes. After centrifugation, the supernatant was moved to a glass tube and evaporated under a nitrogen stream until dryness. The residue was reconstituted in a 1:1 ratio of acetonitrile and water before analysis using LC-QTOF-MS.

For the analysis of conjugated + free analytes (total), a deconjugation step was implemented prior to the extraction step. This step involved the addition of the internal labeled solution and 1 mL of the prepared 1M enzymatic solution to the sample. The mixture was vortexed and then incubated for 20 h at 37°C prior to sample extraction.

To prepare the 1 M ammonium acetate buffer solution, 5.39 g of ammonium acetate was dissolved in 66 mL of HPLC-grade water. The pH was then adjusted to 5.5 ± 0.1 using acetic acid, bringing the total volume to 70 mL. For the enzymatic solution, β-glucuronidase/sulfatase powder was dissolved in 35 mL of the prepared buffer (pH 5.5) to achieve a final concentration of 3500 U/mL [19].

5.3.4. Instrumental analysis

NTA was done using an Agilent 1290 Infinity II LC system (Agilent Technologies, Santa Clara, USA) coupled to a 6545 quadrupole time-of-flight (Q-TOF) MS (Agilent Technologies, Santa Clara, USA). 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 gradient elution at a flow rate of 0.2 mL/min. Elution was performed in gradient mode using A = water and B = methanol, both containing 5 mM ammonium acetate 5%; B (0–1 min), linear increase to 100% B (1–15 min), 100% B (15–20 min), 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 QTOF-MS system was operated in negative (ESI-) electrospray ionization mode. 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.

5.3.5. Quality assurance/quality control

Quality assurance for the non-targeted analysis followed the established procedures outlined in the preceding study of bisphenol analogues in human milk [19]. This included controlling background

contamination, monitoring mass accuracy, intensity and retention time (RT) shifts, and signal drift. Briefly, the retention time and mass accuracy of 9 previously spiked bisphenols in the calibration standards were monitored through repeated analysis in addition to the use of 6 quality control (QC) samples of human milk previously spiked with all 9 bisphenols at 30 ng/mL (3 enzyme-treated and 3 non-enzyme treated samples) [19]. Solvent blanks were subjected to the extraction method in triplicate to filter out any possible analyte contamination during data treatment. During sample analysis, an acetonitrile solvent blank was injected after every 10 samples to minimize possible carryover effects from the instrument.

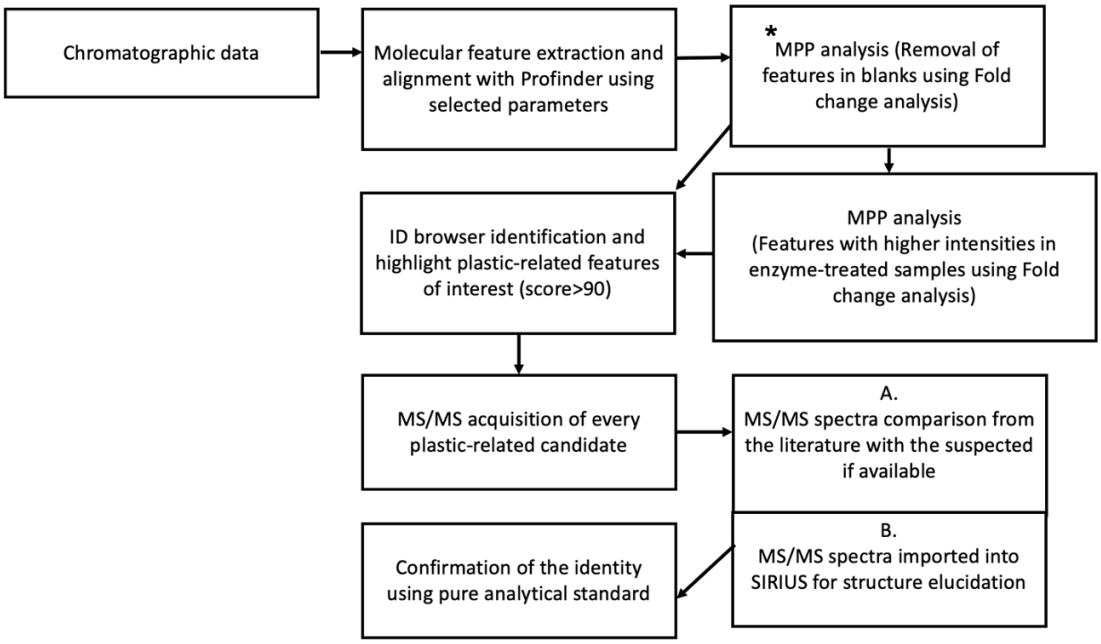
5.3.6. Non-targeted screening of plastic-related contaminants and assessment of conjugated parabens

To explore plastic-related unknowns present in human milk and obtain a better understanding of their conjugation potential, the data files were analyzed in batches, with each batch consisting of different sets of blanks, enzyme-treated and non-enzyme treated samples. In total, we selected 6 batches out of the 20 previously analyzed batches (2 each from Pretoria, Vhembe, and Montreal) for analysis of potential plastic-related contaminants in human milk with a focus on parabens. It was hypothesized that these 6 batches would be representative of the entire 20 batches of human milk samples. The paraben-related compounds identified in the 6 analyzed batches were then further assessed throughout all 20 batches (n=594 samples) to determine their detection frequencies.

MS/MS data alignment and molecular feature extraction were performed using Agilent MassHunter Profinder (B.10.00), incorporating the specific data processing parameters shown in Table S5.1. These parameters were chosen in alignment with the default settings in Profinder

software, with the exception of a shortened mass window (10 ppm) and an elevated selected molecular feature extraction score of 90%. The purpose for this adjustment was to enhance the removal of undesired features and to optimize the identification of relevant molecular characteristics. The workflow for identifying potential plastic-related contaminants is illustrated in Figure 5.2.

Figure 5.2. Non-targeted workflow for the identification of parabens and other PRCs.



**MPP: Mass Profiler Professional*

Molecular features were then exported as .pfa files and imported into Mass Profiler Professional (MPP) (v 16.1). Human milk samples were categorized as enzyme treated (sample + enzyme), non-enzyme treated (sample), blanks (blanks) and unique samples spiked with the 9 bisphenol standards as quality controls (QCs).

Initially, a Principal Component Analysis (PCA) plot was generated to assess common and unique components among sample groups, QCs and blanks. The PCA plots shown in Figures S5.1-A,B

and C depict differences between enzyme treated and non-enzyme treated samples for one batch, and highlight components 1, 2 and 3 that explain (52.08%) of this variation. A "fold change" analysis in Mass Profiler Professional was then applied to the feature abundance to identify molecular features more concentrated in sample groups than in blanks (fold change cut-off: 2.0, p-value cut-off: 0.05 with multiple testing correction: Benjamini-Hochberg). Fold change analysis has been documented as an efficient way to discriminate features in previous non-targeted analytical investigations [44], and resulted in the generation of a list of molecular formulas of interest. These formulas (higher numbers in sample groups than in blanks) were based on exact mass and isotopic patterns.

An ID browser analysis was subsequently conducted to compare selected features with a reference library for the identification of potential plastic-related candidates using the “Extractables and Leachables” database library from Agilent Technologies. This comprehensive library encompasses 1006 different plastic-related compounds, including common parabens, such as methyl, ethyl and propyl paraben, various other plasticizers, epoxy resins, synthetic antioxidants and UV filters. The mass accuracy was set at 5 ppm, and compounds with a matching score exceeding 90% were marked as potential candidates for further investigation. A score threshold of 90% was selected as a more stringent criterion for the identification of PRCs in human milk with the goal of eliminating an excessive number of unwanted features (which may correspond to natural milk components) in order to focus solely on the more relevant ones.

To assess the conjugation potential of detected parabens and other PRCs, an additional fold change analysis was conducted after blank correction (fold change cut-off: 2.0, p-value cut-off: 0.05 with multiple testing correction: Benjamini-Hochberg). The purpose was to identify molecular features with higher intensity in the enzyme treated groups compared to non-enzyme treated groups. Such

features may likely correspond to deglucuronidated or desulfated compounds, including different deconjugated parabens following enzyme treatment.

Formulas for molecular features higher in the enzyme treated groups were generated using the "Extractables & Leachables" database library, and those with a matching score higher than 90% were selected as potentially conjugated candidates.

To validate the extracted features, as well as their conjugation potential after fold change analysis, a volcano plot was generated to show key m/z values representing the extracted candidates for high conjugation (parabens) and low conjugation (PFAS).

5.3.7. Application of SIRIUS and proof-of-concept

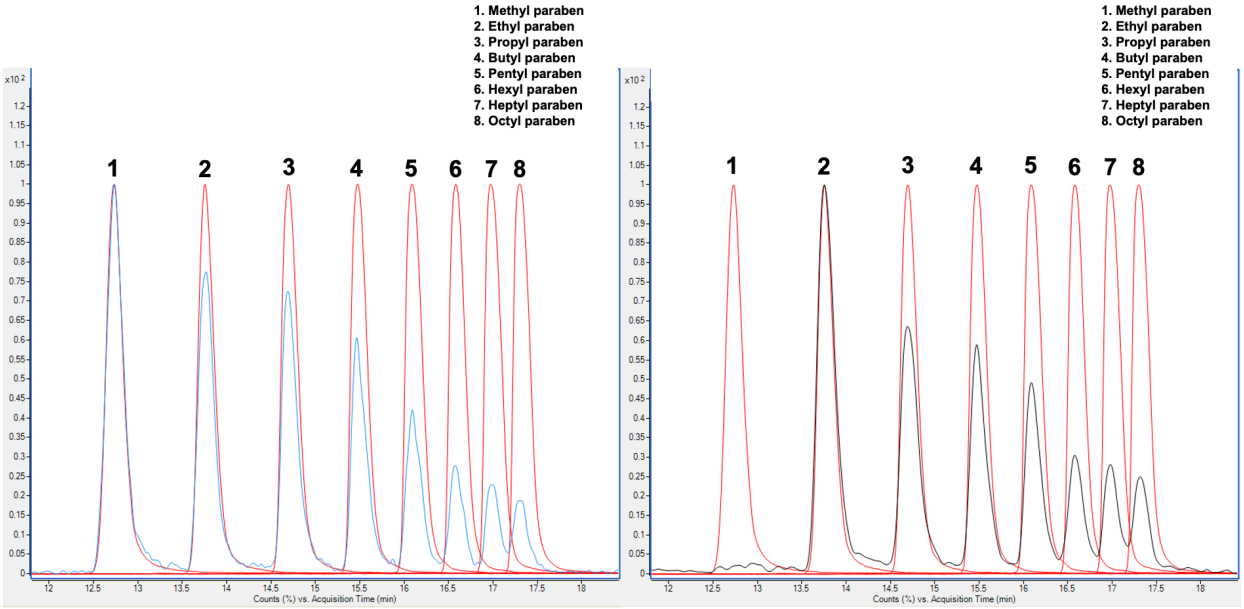
To establish proof-of-concept, MS/MS spectra were systematically acquired for all highlighted parabens and other plastic-related features. Subsequent analysis of the spectra was conducted utilizing Sirius-CSI:FingerID [45]. Compounds were categorized as plastic-related if their elemental formula exhibited a 100% match and their candidate matching score surpassed 90% in comparison to compound databases such as PubChem, KEGG and CheBI. To validate these potential candidates, analytical standards were purchased. A comparative assessment was then executed between the MS/MS spectra of the acquired pure analytical standards and the tentative unknowns. Confirmation of the identity of the tentative compounds was considered acceptable with the presence of at least one fragment ion shared between the standard and the compound, with a mass error not exceeding 5 ppm, in accordance with the European Commission guideline SANTE/11312/2021 [46].

5.3.8. Investigation of other paraben-related compounds in human milk using in-source fragmentation

An additional investigative step was undertaken to identify potential parabens in human milk beyond those identified using the database library. This involved determining the primary fragmentation pattern of four commonly used parabens (methyl, ethyl, propyl and butyl paraben) by conducting targeted MS/MS acquisitions at collision energies of 10, 20 and 30 V. Common m/z values appearing for all four spiked parabens were identified from the targeted MS/MS data of the parabens using (B.10.00) MassHunter Qualitative Analysis (Agilent Technologies), specifically focusing on two m/z values of high intensity: 92.027 and 93.034 (Figures S5.2-A,B,C and D). The chemical formulas of $C_6H_4O^{-\bullet}$, corresponding to a distonic dehydrophenoxide radical anion, and $C_6H_5O^-$, corresponding to an even-electron dehydrophenoxide anion, were considered for paraben identification at the *m/z* values of 92 and 93, respectively. These phenoxide radical ions were reported in a previous study that explored the formation of radical ions under electrospray ionization (ESI) [47].

Following this selection, the m/z values of 92.027 and 93.034 were extracted from a mixture of 8 spiked parabens, including longer chain parabens such as pentyl, hexyl, heptyl and octyl paraben (Figure 5.3). The m/z value of 92.027 was consistently observed for all spiked parabens, while the m/z value of 93.034 was not detectable for methyl paraben, suggesting the generation of the distonic dehydrophenoxide radical anion from methyl paraben under ESI [47]. An overlap for the m/z values of 92.027 and 93.034 (except for methyl paraben) with the actual extracted m/z values of each spiked paraben revealed that they shared the same retention time. Methyl paraben, possessing the shortest chain, had the shortest observed retention time among the other spiked parabens (Figure 5.3).

Figure 5.3. Extracted m/z values of 92.027 (left) and 93.034 (right) overlapping with the actual m/z of 8 spiked parabens with normalized peak (50 ng/mL)*



***93.034 not visible for methyl paraben**

Peaks corresponding to either the m/z values of 92.027 or 93.034 were then extracted in all human milk samples (n=594) to highlight paraben-related peaks. The actual m/z values that are after the retention time of methyl paraben, aligning with the retention time of the fragmented m/z values (92.027 and/or 93.034), were highlighted as potential parabens. Investigations were then conducted to identify their actual m/z values based on current parabens that were mentioned in the literature [48-50]. Their MS/MS spectra were then acquired and imported into SIRIUS for structure elucidation and comparison with existing spectra from online databases, such as the European MassBank. Analytical standards were acquired to confirm the identity of each accurately matched peak.

5.3.9. Assessment of instrumental signal stability for the detection frequency of identified parabens

Minimal variation for the relative response factors (RRFs), with a relative standard deviation (RSD) below 15% across all batches, was previously assessed for all four labelled bisphenols, suggesting instrumental signal stability throughout a span of 2 years for bisphenol-related compounds (Chapter 4). Based on the stability of the RRFs, it was hypothesized that signal detection for other phenolic-related compounds, such as parabens, would also be comparable across batches. To further validate signal stability of the detected parabens across batches and their detection frequencies in milk samples from all 3 regions (Montreal, Vhembe and Pretoria), the signal-to-noise ratio (S/N) of the previously spiked $^{13}C_{12}$-BPAF was assessed for all QCs across 20 batches (6 QCs per batch) (Figure S5.3). The signal to-noise-ratio is defined as a measure that compares the level of a desired signal to the level of background noise: the higher the S/N, the higher the detection power of the instrument will be [51]. S/N values for all QCs across all batches were log-scaled, and the relative standard deviation was calculated to assess any variations across batches. In this study, we selected $^{13}C_{12}$-BPAF by monitoring its S/N across all QC samples due to its stability and comparable intensity to that of the detected parabens. Additionally, its retention time is similar to the retention time for butyl paraben; this is considered a mid-range retention time between the retention time for short chain parabens (methyl, ethyl and propyl parabens) and long chain parabens (pentyl, hexyl, heptyl and octyl parabens).

As shown in Figure S5.3, the S/N ratios for $^{13}C_{12}$-BPAF in 14 out of the 20 batches were comparable to each other, with a relative standard deviation (RSD) of 12%. Very high detection power from the instrument (S/N values >2000000) was observed for the remaining 6 batches which primarily included Montreal human milk samples. Despite the high detection power of the

instrument (indicated by higher S/N values) in multiple Montreal batches, lower overall detection frequencies for all parabens were observed for Montreal samples compared to the South African human milk samples. From the signal intensity of phenolic-related compounds, it was deemed reasonable that the detection frequencies of each paraben in Canadian (Montreal) and South African (Vhembe and Pretoria) human milk were considered comparable to each other. This decision was supported by the minimal variation of the RRFs for all four labelled bisphenols assessed in the previous study (Chapter 4), as well as the consistent S/N values for $^{13}C_{12}$-BPAF across the remaining 14 batches, despite the high S/N values observed in multiple Montreal batches in which detection frequencies for all detected parabens were lower in comparison to the South African human milk samples.

5.4. Results and discussion

5.4.1. Confirmation of plastic-related compounds and conjugated unknowns

Compounds with features with a >90% matching score in the ID browser analysis in the Mass Profiler Professional (MPP) were designated as potential candidates for parabens or other plastic-related contaminants. Table S5.2 is a comprehensive list of the tentative suspects, categorized by their confidence level 3 based on the Schymanski scale [52]. Extracted paraben-related features were then highlighted for further analysis.

Aside from parabens, numerous tentative phthalate metabolites were detected, as well as a diverse range of other plastic-related additives such as preservatives, UV filters, absorbers and synthetic antioxidants. Subsequently, acquisition of the MS/MS spectra for detected tentative parabens, phthalate metabolites and PFAS was conducted, and these spectra were imported into SIRIUS for structure elucidation. Analytical standards were purchased for candidates demonstrating a strong

matching score, and their identities were then confirmed (Figures S5.4-S5.13). These plastic-related candidates, including their conjugation potential after fold change analysis (MPP), are presented in Table 5.1. In summary, this approach identified three major parabens, three distinct phthalate metabolites, three different PFAS, and one tire-related chemical, 1,3-diphenylguanidine (DPG).

To verify the conjugation potential of the extracted plastic-related features (focussing specifically on paraben-related ones), the features corresponding to three identified parabens as well as two PFAS (PFOA and PFOS) of a selected single batch were highlighted in a volcano plot as shown in Figure S5.14. These key features were highlighted because they were detected in multiple human milk samples (over 30% within a batch for the parabens, PFOA and PFOS). The features corresponding to the three parabens were observed among the red datapoints indicating higher levels in the enzyme treated group. In contrast, features corresponding to PFOA and PFOS exhibited little to no conjugation potential, as shown by the grey datapoints. Additionally, features corresponding to infrequently detected phthalate metabolites, such as mono-2-ethylhexyl phthalate, monobutyl phthalate, and monoisobutyl phthalate, were also observed among the red datapoints. Other infrequently detected features related to DPG and perfluorohexanesulfonic acid (PFHxS) were found among the grey datapoints.

The blue datapoints, representing features with lower intensity in the enzyme-treated groups compared to the untreated groups, suggest a decrease in various conjugated compounds in human milk following deglucuronidation or desulfation due to enzyme treatment.

Table 5.1. PRCs in Canadian and South African human milk identified using analytical standards along with their conjugation potential.

Feature ([M-H]-,m/z)	Retention time (min)	Formula	Region (South Africa or Montreal)	Conjugation potential	Final identity using MS/MS matching and analytical standard
151.0395	12.75	$C_8H_8O_3$	All	Yes	Methyl paraben
165.0552	13.76	$C_9H_{10}O_3$	All	Yes	Ethyl paraben
179.0708	14.71	$C_{10}H_{12}O_3$	All	Yes	Propyl paraben
221.0814	11.94	$C_{12}H_{14}O_4$	All	Yes	Monobutyl phthalate
221.0814	11.67	$C_{12}H_{14}O_4$	All	Yes	Monoisobutyl phthalate
277.1440	15.03	$C_{16}H_{22}O_4$	All	Yes	Mono(2-ethylhexyl) phthalate (MEHP)
498.9297	14.61	$C_8HF_{17}O_3S$	All	No	Perfluorooctanesulfonic acid (PFOS)
412.9659	14.10	$C_8HF_{15}O_2$	All	No	Perfluorooctanoic acid (PFOA)
398.9360	13.74	$C_6HF_{13}O_3S$	All	No	Perfluorohexane sulfonic acid (PFHxS)
210.1031	11.85	$C_{13}H_{13}N_3$	SA	No	1,3-diphenylguanidine (DPG)

Methyl, ethyl and propyl parabens are commonly used as preservatives in cosmetics and pharmaceuticals, and as additives in food-related materials [53]. Among the other PRCs detected, monobutyl, monoisobutyl and mono(2-ethylhexyl) phthalates are recognized as metabolites of their parent compounds (dibutyl, di-isobutyl and di(2-ethylhexyl) phthalates, respectively), which are commonly used as plasticizers. Previous human milk studies have reported the presence of

these metabolites that, similar to their parent compounds, can act as endocrine disruptors, potentially leading to adverse health effects [54, 55].

The three long chain PFAS detected (PFOA, PFOS and PFHxS) are used to manufacture stain/water-resistant coatings for various consumer products and are also used as coatings in the production of food packaging, especially for fast-foods [56, 57]. Epidemiological studies examining PFAS have identified links between exposure to certain PFAS and a range of health impacts, such as changes in immune and thyroid function, liver conditions, disturbances in lipid and insulin levels, kidney problems, adverse effects on reproduction and development, and cancer [58-62].

1,3-diphenylguanidine (DPG) is used as an accelerator in rubber vulcanization during tire, rubber glove or synthetic equipment production [63-65]. Leaching of DPG from rubber products into the environment over time may lead to human exposure [63, 66]. One study demonstrated that DPG and its chlorinated by-products can cause cell toxicity and potential genotoxic effects *in vitro* [67]. The EPA's ToxCast program predicted that DPG may have neurotoxic and endocrine-disrupting effects [68].

The parabens and phthalate metabolites identified in human milk in the present study were observed to have high conjugation potential, in contrast to the PFAS or DPG. To the best of our knowledge, there is currently no study that has assessed the toxicity potential of conjugated PRCs, such as parabens or phthalate monoesters. Conjugation of parabens mainly involves glucuronidation or sulfation, while phthalate monoesters mainly undergo glucuronidation [69, 70]. There is concern that these compounds, similar to glucuronidated or sulfated forms of bisphenols, may undergo deconjugation in the body leading to potential health implications [34-37]. Therefore,

evaluating the levels of both free and conjugated parabens and phthalates should prove useful for future risk assessment studies.

Because the main focus of the present study was to identify common and unusual parabens, the non-targeted analysis of these contaminants was conducted exclusively using negative ion mode electrospray ionization (ESI). Further assessment involving targeted analyses of phthalate diesters, phthalate monoesters, parabens, and non-targeted analyses of other potential plastic-related compounds was not carried out in this study.

5.4.2. Detection of unusual parabens using in-source fragmentation

Given our limited understanding of the occurrence of parabens (especially in human milk collected from South Africa), we decided to extend our present investigation to explore the possible presence of other unidentified parabens. During liquid chromatography under ESI in-source fragmentation arises due to inherent weaknesses in chemical bonds within the target analytes [71]. This phenomenon has been observed previously in other studies with pharmaceuticals and their metabolites as well as pesticides [71, 72]. Another study assessed the formation of phenoxide radical anions using different nitrobenzoic acids, methylphenols, nitrophenols, hydroxybenzaldehydes and hydroxyacetophenones [47]. Based on this study, we hypothesized that, under ESI, synthetic phenolic compounds, such as parabens, would also yield different phenoxide anions having m/z values that are similar to, if not exactly the same, as the previously reported m/z values of 92 and 93.

To investigate the in-source fragmentation patterns of various parabens, we first selected four primary parabens (three previously detected, with the addition of butyl paraben) and compiled their fragmented m/z charge values to determine their possible correspondence to the

aforementioned phenoxide radical ions (Figures S5.2-A,B,C and D). Two major m/z values of 92.0267 and 93.0343 were selected for the detection of paraben-related compounds in human milk. A peak related to a suspect closely resembling octyl paraben was successfully detected by extracting these m/z values in human milk samples, (Figure S5.15). Further investigations of detected peaks overlapping with these m/z values led to the detection of phenyl, iso-butyl and butyl parabens. The MS/MS spectra of these tentative parabens were subsequently imported into SIRIUS and the identities of phenyl, iso-butyl and butyl parabens were confirmed using analytical standards (Figures S5.16-S5.18). Additional searches in the literature for the octyl paraben-related suspect confirmed the presence of 2-ethylhexyl 4-hydroxybenzoate (2-EHHB), an isomer of octyl paraben, in human milk from South Africa (Figure S5.19). A comparison between the signal intensity of a standard solution (30 ng/mL) and the signal intensity of 2-EHHB in one South African human milk sample is shown in Figure S5.20. In total, seven parabens were identified, including four new parabens, following the use of the database library across the 6 batches of samples (Tables 5.2 and S5.3). To our knowledge, this is the first study to report the presence of phenyl paraben and 2-EHHB in human milk, highlighting the need to explore the presence of other parabens reported in the literature that may be present in human milk in different countries [48-50].

5.4.3. Comparison of the seven identified parabens in Canadian (Montreal) and South African (Pretoria and Vhembe) human milk

Table 5.2 compares the detection frequencies of the seven parabens in human milk samples from Montreal, Pretoria and Vhembe. In addition, it provides information from the EU and Canada as to the most likely sources of exposure (food products, cosmetics and/or pharmaceuticals) and whether their use is regulated. To the best of our knowledge, the presence of parabens in food-

related products in South Africa is not yet regulated. The South African National Standards' Self-Regulatory Code of Practice stands alone as the sole instance of cosmetics self-regulation globally within industry regulations [73]. In terms of self-regulatory practices, many South African industries, particularly in the cosmetic sector, align their practices with regulations imposed by other major organizations such as the European Union. This is exemplified by various cosmetic industries in South Africa, which have formed an association called the "Cosmetic, Toiletry, and Fragrance Association of South Africa" (CTFA) and have opted to use the European Regulation (EC 1223/2009) as a framework for the self-regulation of parabens [74, 75].

Table 5.2. Parabens detected in Canadian and South African human milk samples, including detection frequencies (DFs), EU and Canadian-based usage and regulations.

Parabens	Canada	South Africa		Status and regulations (European Union) [22, 74, 76-78][a]	Status and Regulations (Government of Canada) [33, 79-81]
	Montreal (n=207)	Pretoria (n=193)	Vhembe (n=194)		
	DF (%)	DF (%)	DF (%)		
Methyl paraben	96	100	100	Used as food additive: - Acceptable daily intake of 10 mg/kg body weight and maximum amount of 300 mg paraben/kg in food as total mixtures of methyl and/or ethylparaben. Used in cosmetics and pharmaceuticals -[b]Maximum concentration of 0.4% and total maximum concentration of 0.8% for mixtures of other paraben esters.	Not directly added to foods but used as preservatives in: - Antifoaming preparations with maximum limit of 5000 ppm. - Enzyme preparations with maximum limit of 5000 ppm. - Aqueous colour formulations with maximum limit of 20 ppm for chewing gum or unstandardized confectionery and maximum limit of 40 ppm for marinades. Used in natural health products - Oral upper limit of 10 mg/kg bw/day exposure for the sum of methyl, ethyl, and propyl paraben. Used in cosmetics and pharmaceuticals: - No concentration restriction for the use of parabens in cosmetic products in Canada. - No information available for maximum concentration for pharmaceuticals.
Ethyl paraben	57	93	82	Used as food additive: - Acceptable daily intake of 10 mg/kg body weight and maximum amount of 300 mg paraben/kg in food as total mixtures of methyl and/or ethylparaben.	Not used in food-related products, but can be present as incidental additive in food processing plants Used in natural health products

				Used in cosmetics and pharmaceuticals - [b]Maximum concentration of 0.4% and total maximum concentration of 0.8% for mixtures of other paraben esters.	-Oral upper limit of 10 mg/kg bw/day exposure for the sum of methyl-, ethyl-, and propylparaben. Used in cosmetics and pharmaceuticals: -No concentration restriction for the use of parabens in cosmetic products in Canada. -No information available for maximum concentration for pharmaceuticals. Used in topical products: - [b]Maximum concentration of 0.4% and total maximum concentration of 0.8% for mixtures of other paraben esters.
Propyl paraben	35	89	48	Restricted (banned) in food Used in cosmetics and pharmaceuticals - [c]Maximum concentration of 0.80% for paraben mixtures where combined concentration of propyl and butyl paraben alone cannot exceed 0.14%.	Not directly added to foods but used as preservatives in: -Antifoaming preparations (maximum limit of 5000 ppm). -Enzyme preparations with maximum limit of 5000 ppm. -Aqueous colour formulations with maximum limit of 20 ppm for chewing gum or unstandardized confectionery and maximum limit of 40 ppm for marinades. Used in natural health products -Oral upper limit of 10 mg/kg bw/day exposure for the sum of methyl-, ethyl-, and propylparaben. Used in cosmetics and pharmaceuticals: -No concentration restriction for the use of parabens in cosmetic products in Canada. -No information available for maximum concentration for pharmaceuticals.
Butyl paraben	<1	7	3	Restricted (banned) in food	[d]Not used as food additive

				Used in cosmetics and pharmaceuticals - [c]Maximum concentration of 0.80% for paraben mixtures where combined concentration of propyl and butyl paraben alone cannot exceed 0.14%.	-No information available for oral upper limit. Used in natural health products, cosmetics, and pharmaceuticals -No information available for maximum concentration for natural health products. -No concentration restriction for the use of parabens in cosmetic products in Canada. -No information available for maximum concentration for pharmaceuticals.
Isobutyl paraben	Not detected	3	1	Restricted in both food, cosmetics and pharmaceuticals.	[d]Not used as food additive -No information available for oral upper limit. Used in natural health products, cosmetics, and pharmaceuticals -No information available for maximum concentration for natural health products. -No concentration restriction for the use of parabens in cosmetic products in Canada. -No information available for maximum concentration for pharmaceuticals.
Phenyl paraben	10	22	18	Restricted in both food, cosmetics and pharmaceuticals.	No information available for food, cosmetics or pharmaceuticals.
2-ethylhexyl 4-hydroxybenzoate (2-EHHB)	Not detected	80	39	No information available for food, cosmetics or pharmaceuticals.	No information available for food, cosmetics or pharmaceuticals.

a. Cosmetic industries in South Africa are reliant on EU regulations.

b. Can also be interpreted as a maximum limit of 4 g/kg product for individual parabens and 8 g/kg product for mixtures of other parabens.

c. Can also be interpreted as a maximum limit of 8 g/kg product for paraben mixtures where concentrations of propyl and butyl parabens do not exceed 1.4 g/kg product.

d. Butyl and isobutyl parabens are generally not added as preservatives to food, but there is currently no regulation or restriction on their usage in food in Canada.

In the European Union (EU), methyl and ethyl parabens are commonly utilized as preservatives in food-related products with maximum acceptable daily intakes (ADI) of 10 mg/kg body weight for both parabens. However, the use of propyl paraben as a preservative in food has been banned since 2006 while butyl paraben is used solely as an antimicrobial preservative in cosmetics [82]. The use of both propyl and butyl paraben in cosmetics has recently become a subject for tighter control by the EU; the maximum allowed concentration when used individually was decreased from 0.4% to 0.14% [21, 83].

In Canada, parabens are not permitted to be added directly to foods. However, certain compounds, like methyl and propyl parabens, can be used as preservatives in specific applications, such as antifoaming preparations and enzyme preparations, with a maximum limit of 5000 ppm [80, 81]. They are also allowed in aqueous color formulations with a maximum limit of 20 ppm for chewing gum and unstandardized confectionery, and 40 ppm for marinades (Table 5.2) [79, 80]. For natural health products, Health Canada has established a maximum oral limit for the total exposure to methyl, ethyl, and propyl parabens at 10 mg/kg body weight per day. No information is available regarding the maximum limits for butyl and iso-butyl parabens [33]. To our knowledge, while the maximum concentration for ethyl paraben in topical products is set at 0.4%, there are currently no restrictions on the use of parabens in cosmetics and pharmaceuticals in Canada [33, 80].

Methyl paraben was detected in 96% of the Canadian human milk samples, followed by ethyl paraben (57%) and propyl paraben (35%). The higher detection frequency observed for methyl paraben (96%) suggests there may be a relatively high usage in food-related products, cosmetics and pharmaceuticals in comparison to the other detected parabens (Table 5.2).

Unlike methyl and propyl paraben, ethyl paraben is not used as a food additive or in food packaging materials in Canada, yet there was a relatively high detection frequency (57%) in human milk

samples from Montreal; this surpassed that of propyl paraben, which is used in categories, that include personal care products, natural health products and drugs, as well as food and beverages [33]. One possibility is that incidental addition of ethyl paraben to foods may occur. For administrative purposes, ethyl paraben can be considered an incidental additive in food processing since it is not directly added to food. Residues of ethyl paraben in foods may be the result of exposure from cleaners, packaging or sanitizers [33, 84].

Phenyl paraben, an uncommon paraben used in cosmetics and pharmaceuticals, was also detectable in Montreal human milk, but with a lower frequency of 10%. Butyl paraben was detectable in only two Montreal human milk samples. No information was available for the maximum oral upper limit for the exposure of phenyl and butyl parabens.

In contrast to Canada, South African milk samples exhibited a high parabens detection frequency (DF), with both Vhembe and Pretoria having a 100% DF for methyl paraben. Ethyl paraben was also notably high in both regions: 93% DF for Pretoria and 82% for Vhembe. Propyl paraben showed the highest detection frequency in Pretoria (89%), whereas Vhembe was lower at 48%. Phenyl paraben had a detection frequency of 22% for Pretoria, comparable to the 18% found in Vhembe samples. Lower detection frequencies were observed for butyl paraben and isobutyl paraben, with 7% and 3%, respectively, for Pretoria and 3% and 1%, respectively, for Vhembe. Interestingly, two other parabens, isobutyl paraben and 2-EHHB, an isomer of octyl paraben, were detected only in South African human milk. 2-EHHB exposure was quite high with 80% DF in Pretoria samples and 39% for Vhembe. The higher detection frequencies for 2-EHHB in urban Pretoria suggests higher usage compared to rural Vhembe. However, limited information is available regarding the usage of this "unusual" paraben. Only one study described the use of 2-EHHB as a preservative in different food products as well as in cosmetics and pharmaceuticals

[85]. Similarly, studies on its toxicity potential are limited [85, 86]. The high presence of 2-EHHB in South African human milk emphasizes the need for further investigations into its different applications as well as its toxicity potential for both mothers and breastfeeding infants.

Overall, lower detection frequencies were observed for the detected parabens (methyl, ethyl, propyl, butyl, and phenyl parabens) in Canadian human milk samples compared to those in South African milk samples. The higher detection frequencies for parabens in South African samples, along with the exclusive presence of 2-EHHB, suggest differences in the usage of parabens across different countries.

5.5. Conclusions

Although numerous previous studies of human milk have investigated plastic-related contaminants, they have predominantly used a targeted approach, limiting our knowledge of what is present to commonly recognized compounds. In this study, we explored the use of non-targeted analyses to detect the presence of multiple paraben-related contaminants, including restricted and uncommon parabens. We also confirmed the presence in human milk of different phthalate metabolites, several PFAS and a tire-related chemical. These findings highlight the significance of employing non-targeted analysis as a robust tool in biomonitoring studies of human milk, to improve current strategies, especially in countries where data are limited. Moreover, they suggest a need for further investigation into the usage and toxicity potential of previously unreported parabens, such as 2-EHHB.

5.6. References

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5.7. Supplementary information for Investigation of plastic-related contaminants in human milk: identification of common and unreported parabens using non-targeted strategies

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Supplementary information
This file contains Supplementary Figures S5.1-S5.20 and Tables S5.1-S5.3

Figure S5.1-A. 3D Principal component analysis of enzyme treated and non-enzyme treated samples for one human milk batch.

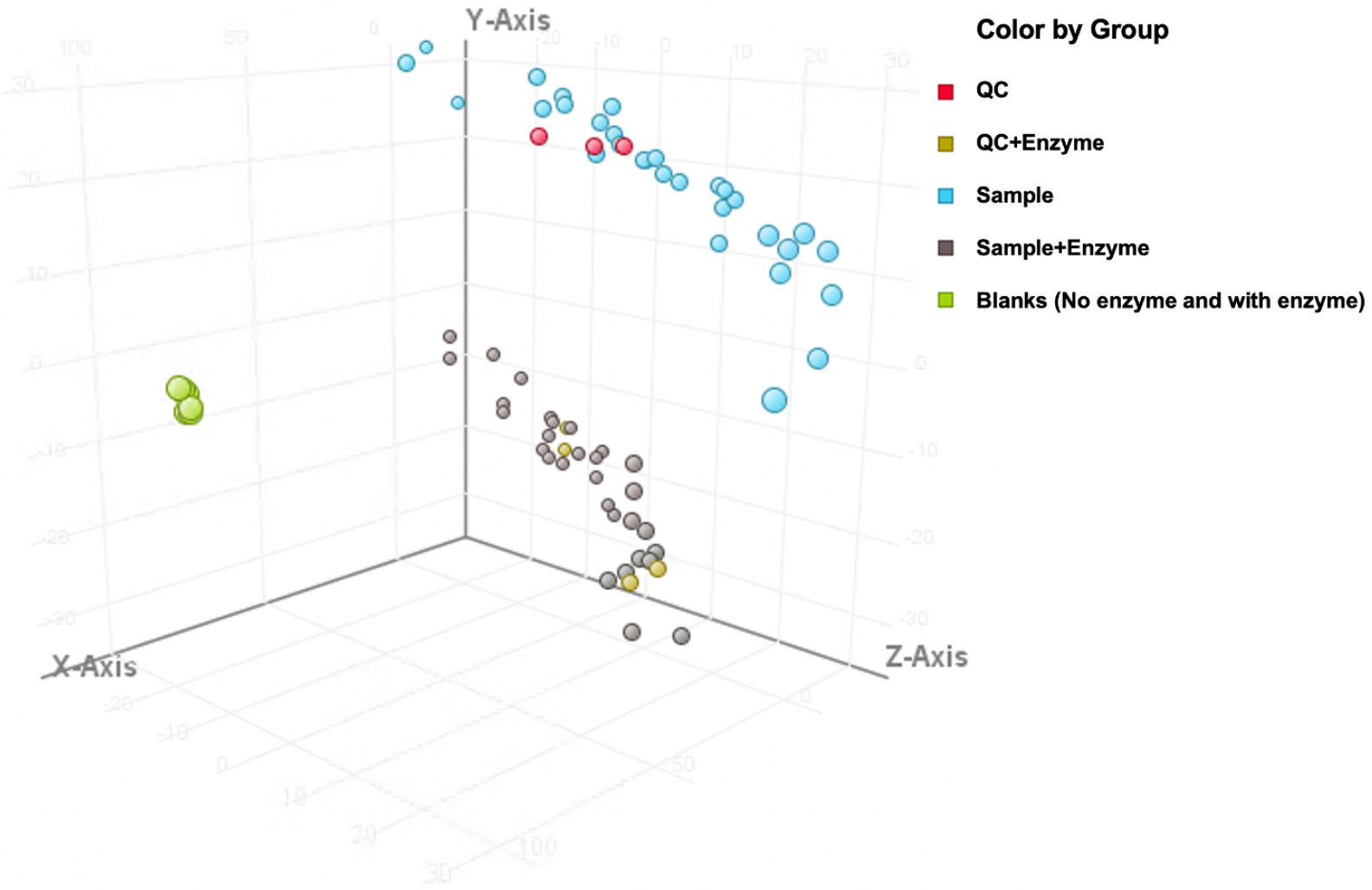
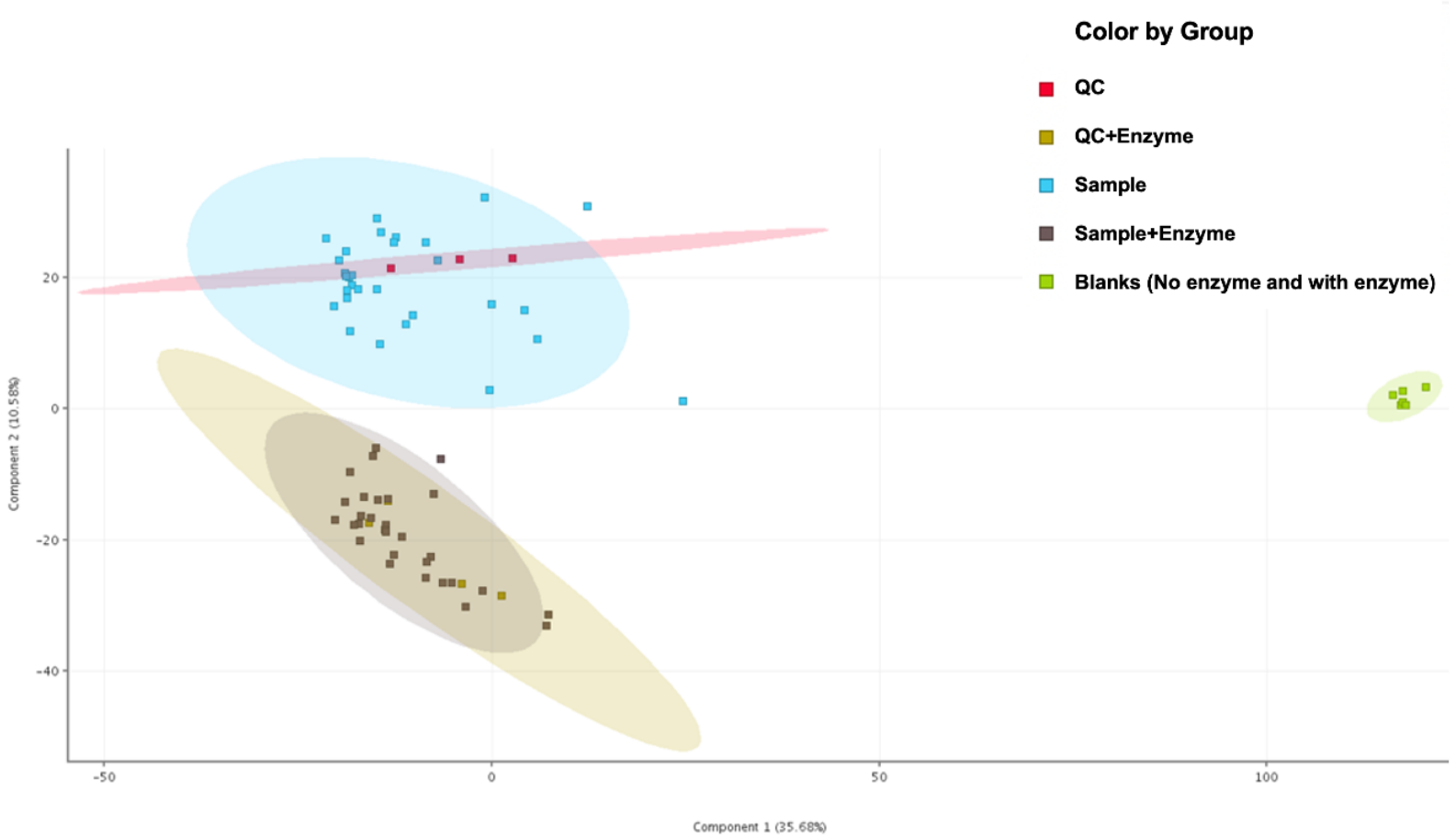
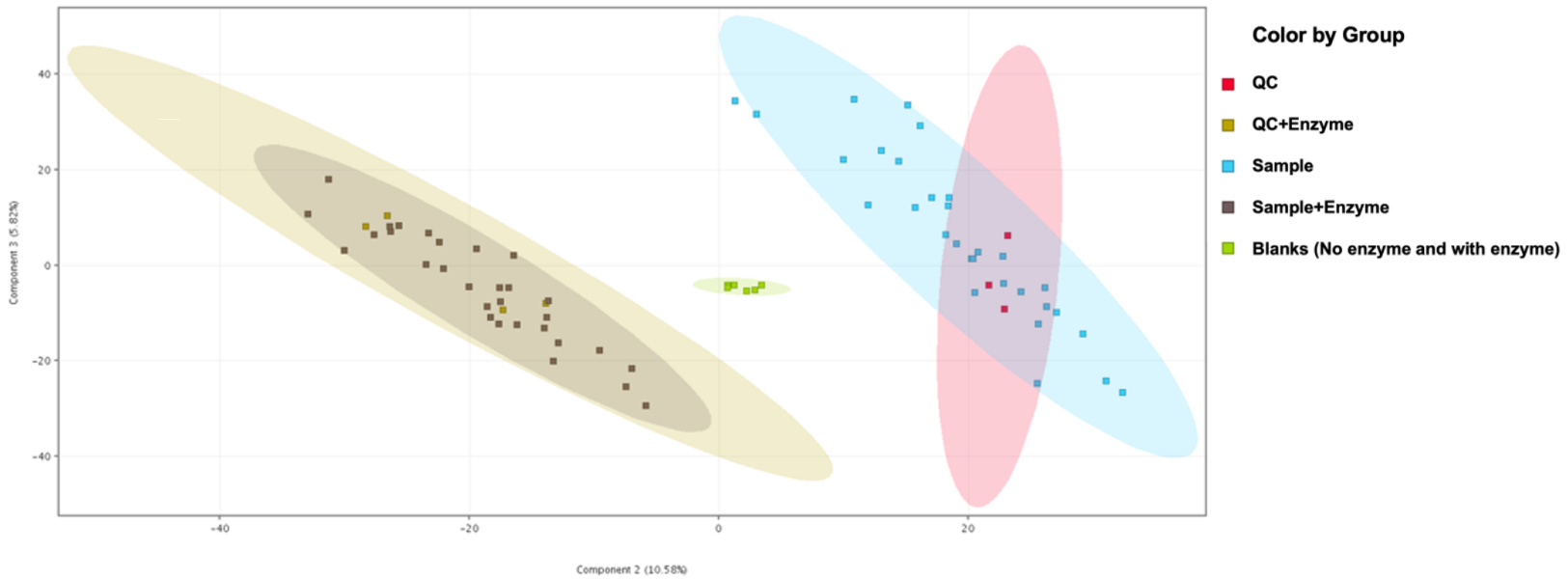


Figure S5.1-B. 2D Principal component analysis of enzyme treated and non-enzyme treated samples for one human milk batch (Component 1 and Component 2)



X-Axis Component 1 (35.68%)
Y-Axis Component 2 (10.58%)

Figure S5.1-C. 2D Principal component analysis of enzyme treated and non-enzyme treated samples for one human milk batch (Component 2 and Component 3)



X-Axis Component 2 (10.58%)
Y-Axis Component 3 (5.82)

Figure S5.2-A. Targeted MS/MS of methyl paraben and its common fragmented *m/z* values at 10 V, 20 V and 30 V in solvent at 30 ng/mL

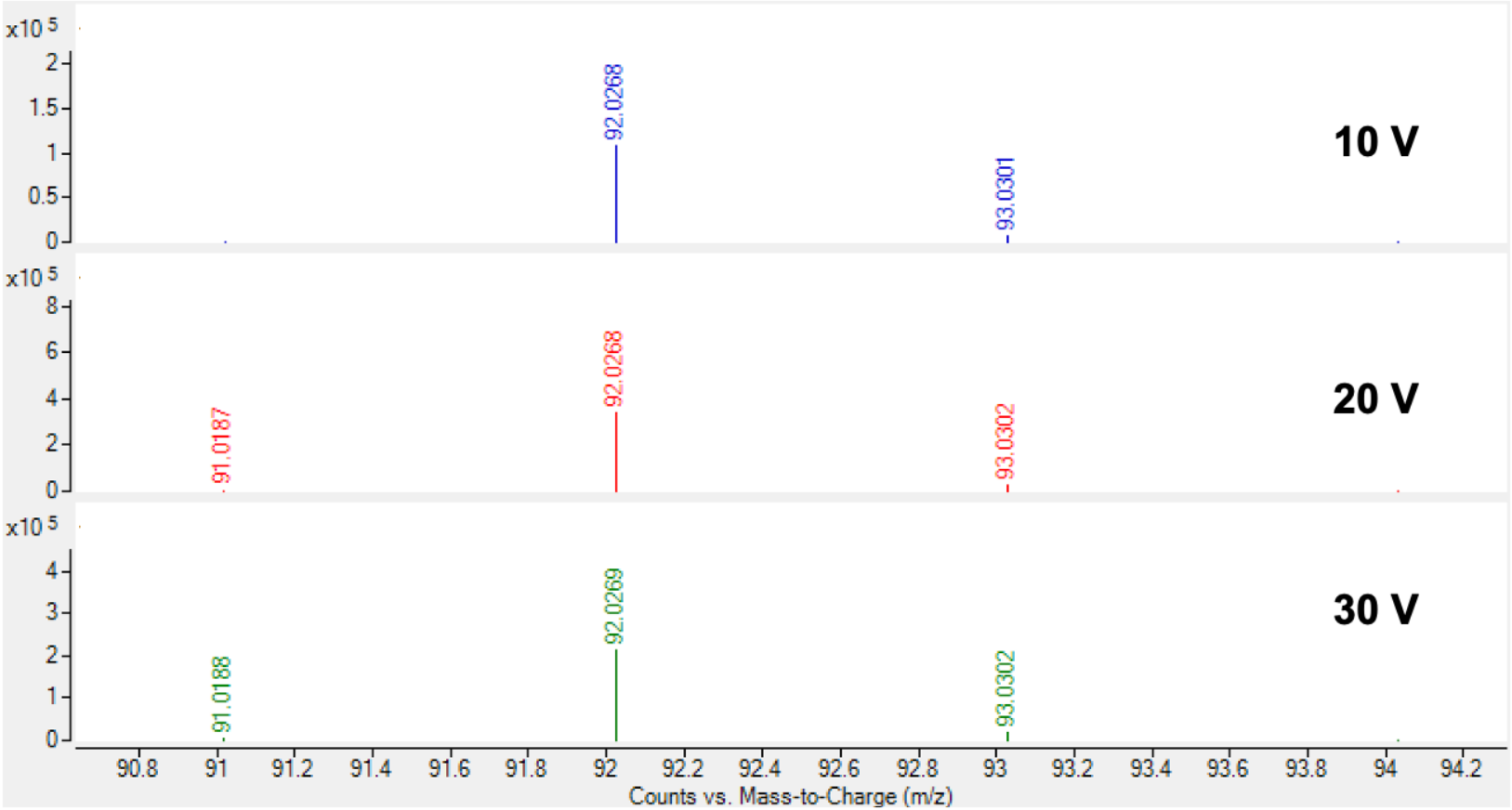


Figure S5.2-B. Targeted MS/MS of ethyl paraben and its common fragmented m/z values at 10 V, 20 V and 30 V in solvent at 30 ng/mL

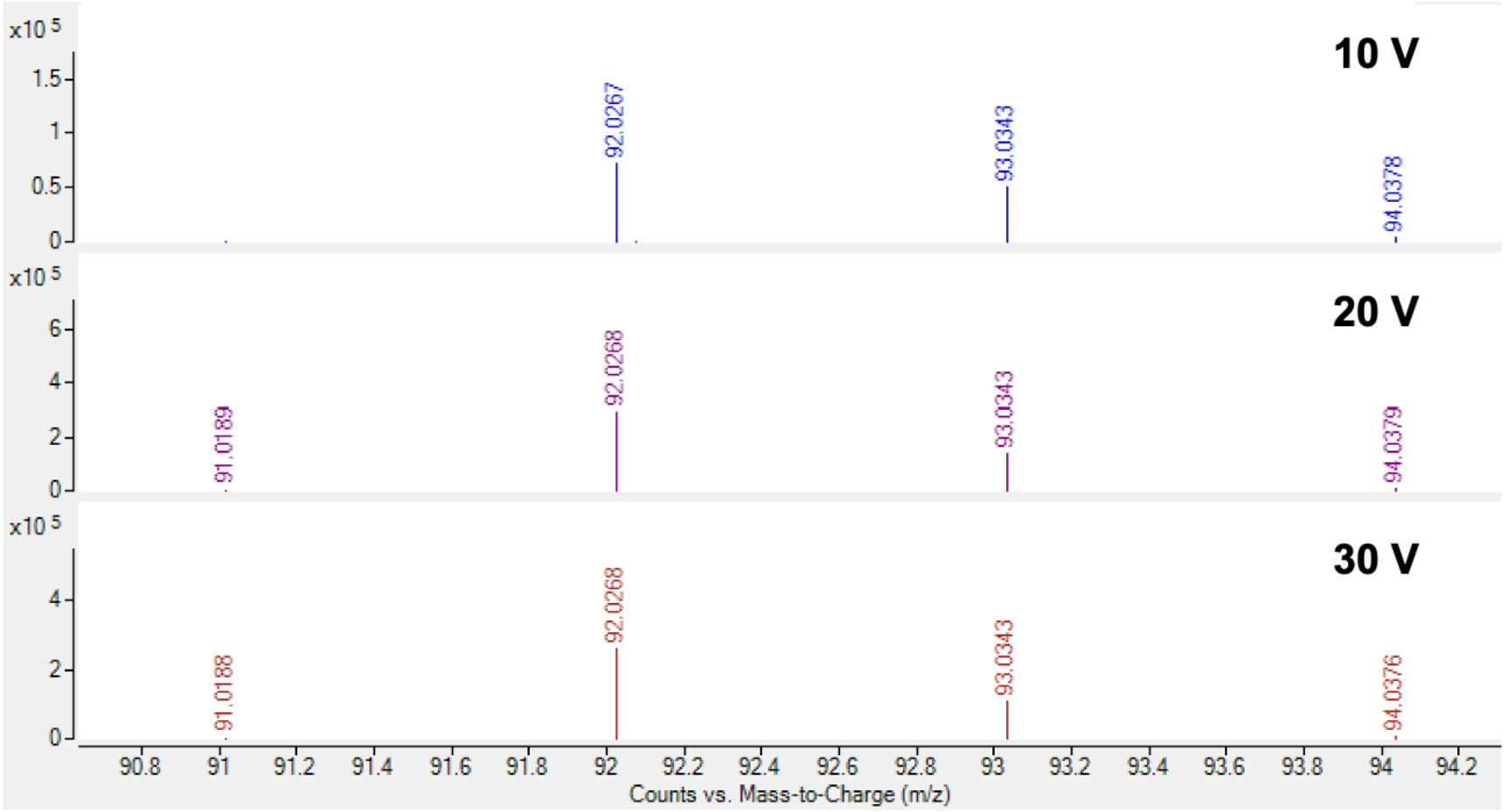


Figure S5.2-C. Targeted MS/MS of propyl paraben and its common fragmented m/z values at 10 V, 20 V and 30 V in solvent at 30 ng/mL

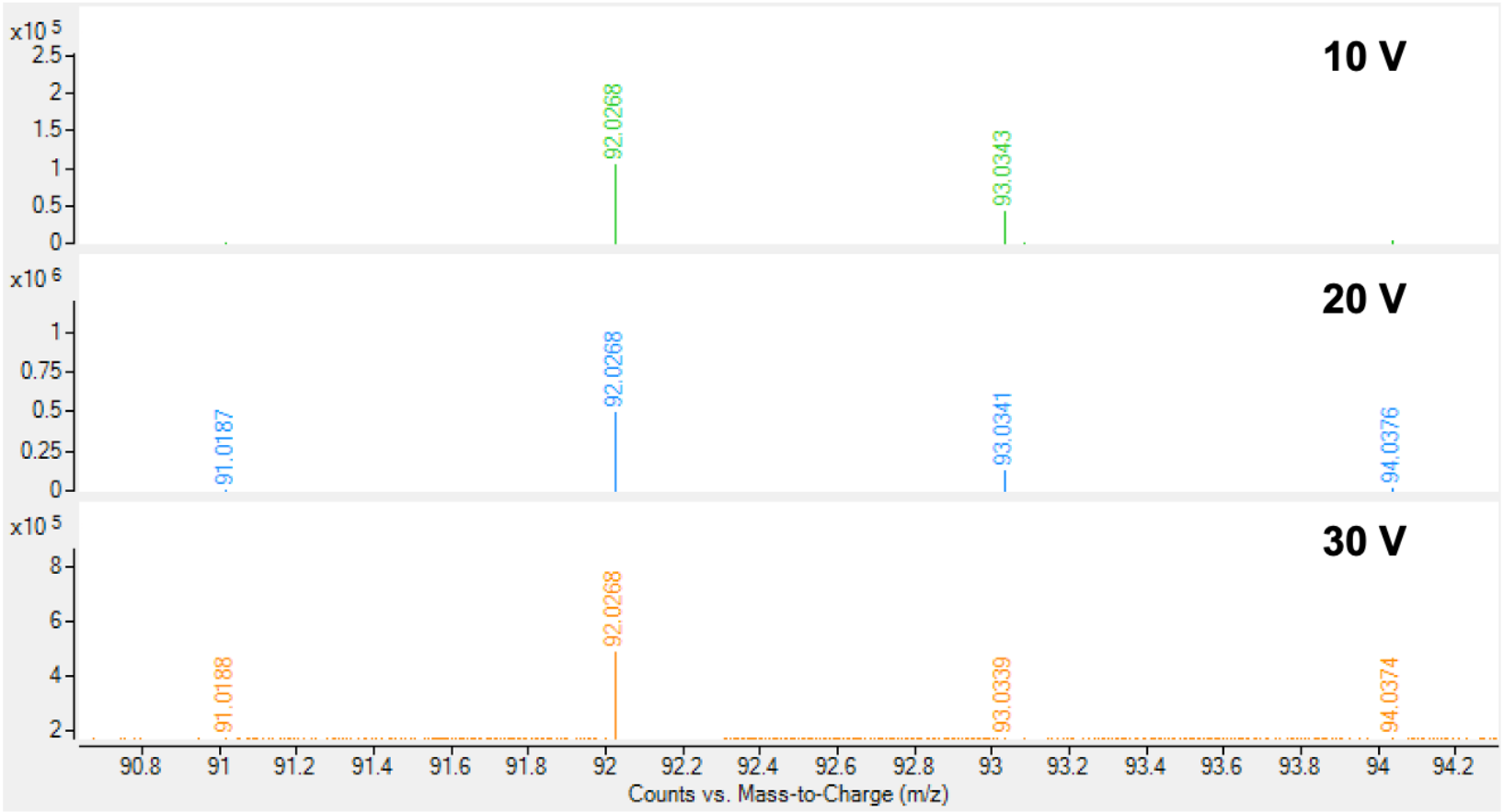


Figure S5.2-D. Targeted MS/MS of butyl paraben and its common fragmented m/z values at 10 V, 20 V and 30 V in solvent at 30 ng/mL

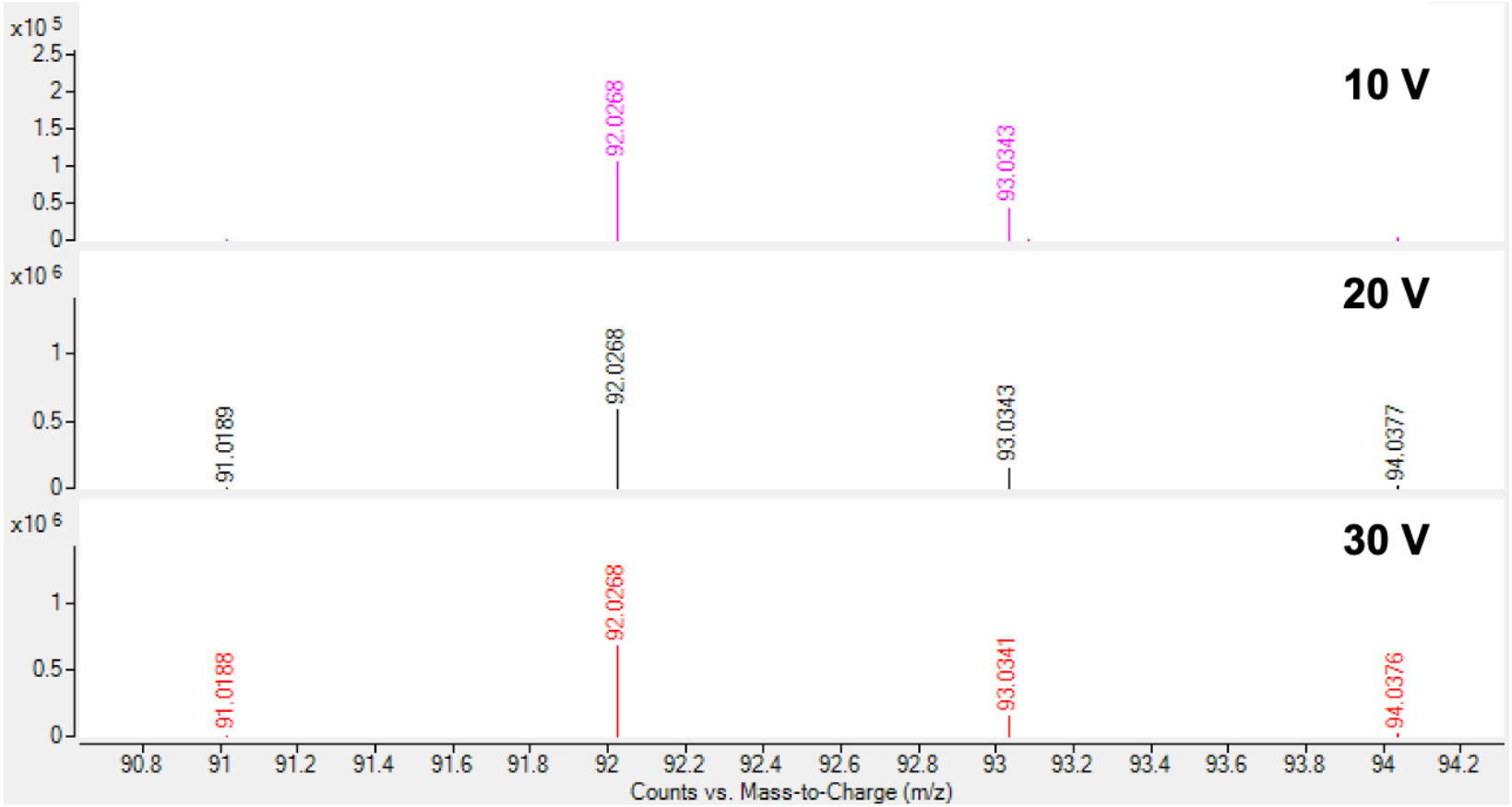
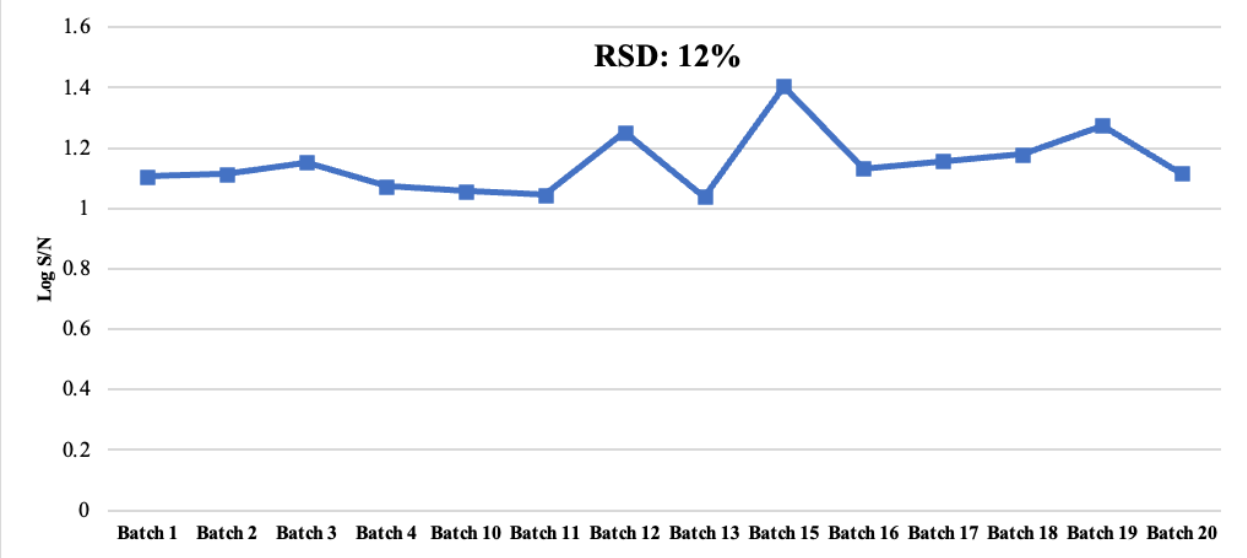


Figure S5.3. log S/N ratio for internal labelled BPAF in all QCs across 20 batches



S/N ratio: Signal-to-noise ratio
(S/N ratio values for $^{13}C_{12}$ BPAF of Batch 5 to 9 and batch 14 are >2000000)

Figure S5.4. Targeted MS/MS of monobutyl phthalate in an unspiked human milk sample and pure calibration solvent (10 V). Fragmented product ion at *m/z* 221.1.

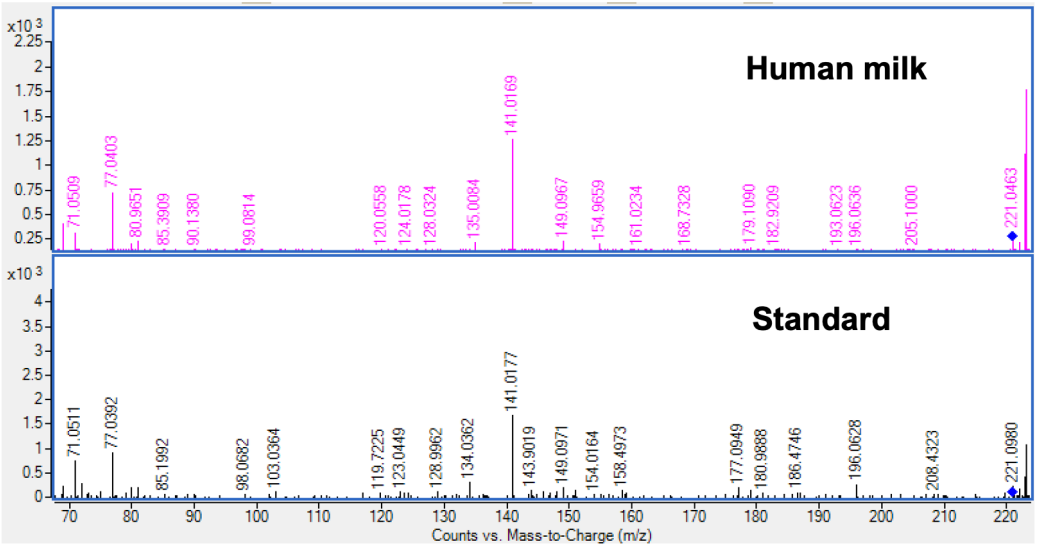


Figure S5.5. Targeted MS/MS of mono isobutyl phthalate in an unspiked human milk sample and pure calibration solvent (10 V). Fragmented product ion at m/z 221.1

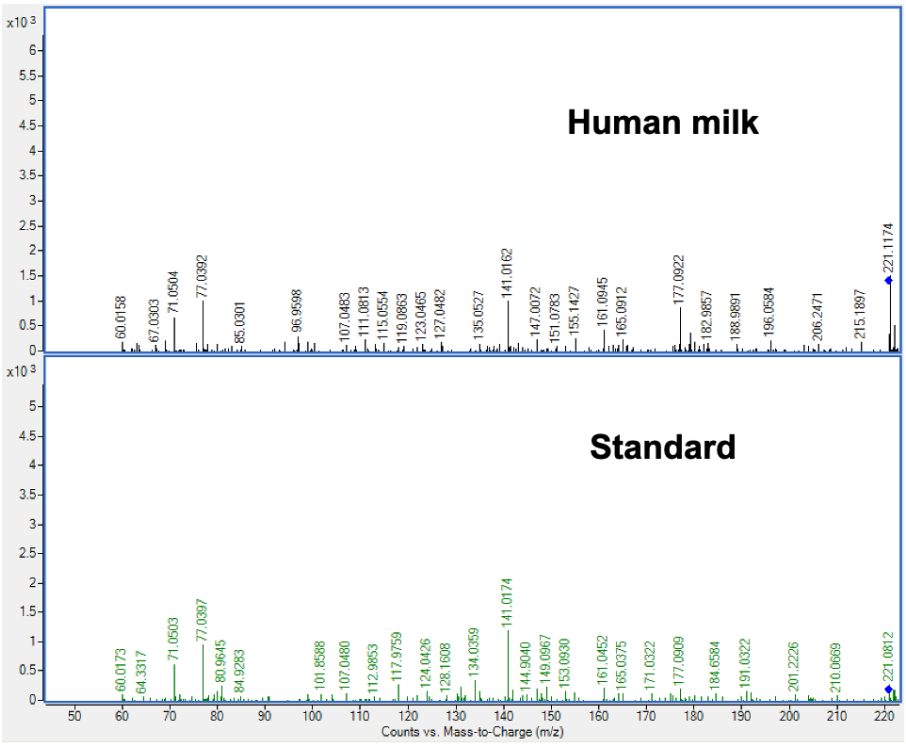


Figure S5.6. Targeted MS/MS of mono(ethylhexyl) phthalate in an unspiked human milk sample and pure calibration solvent (10 V). Fragmented product ion at m/z 277.14

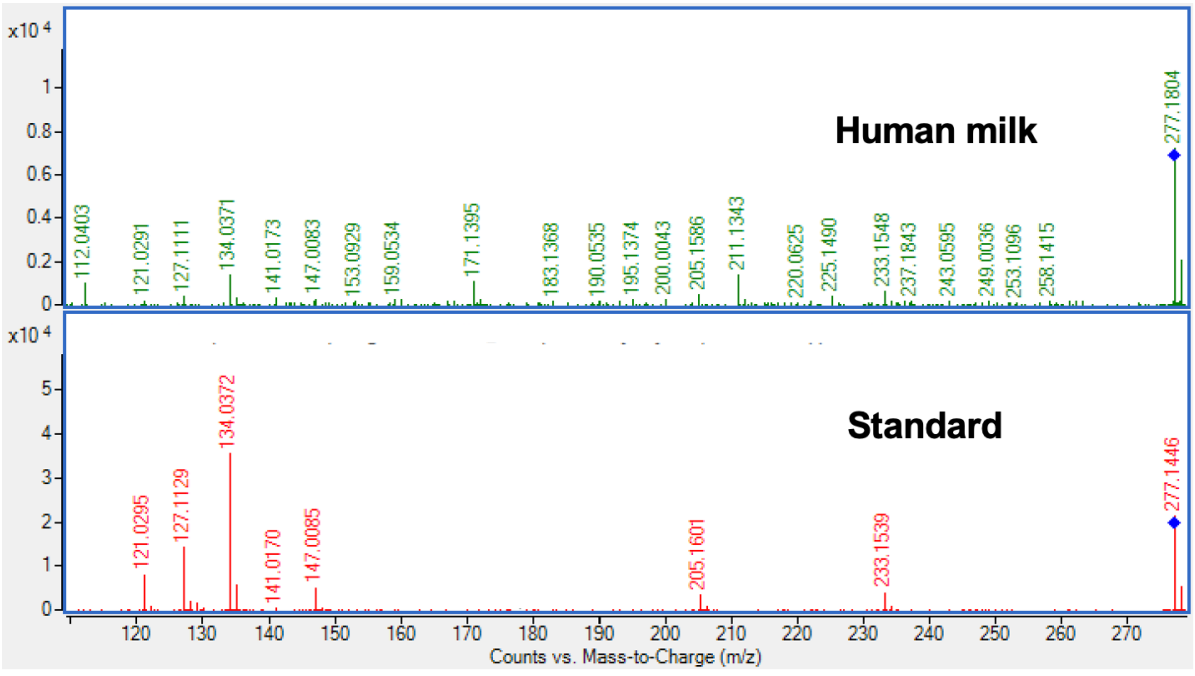


Figure S5.7. Targeted MS/MS of perfluorooctanoic acid in an unspiked human milk sample and pure calibration solvent (20 V). MS1 at m/z 412.97 was not visible

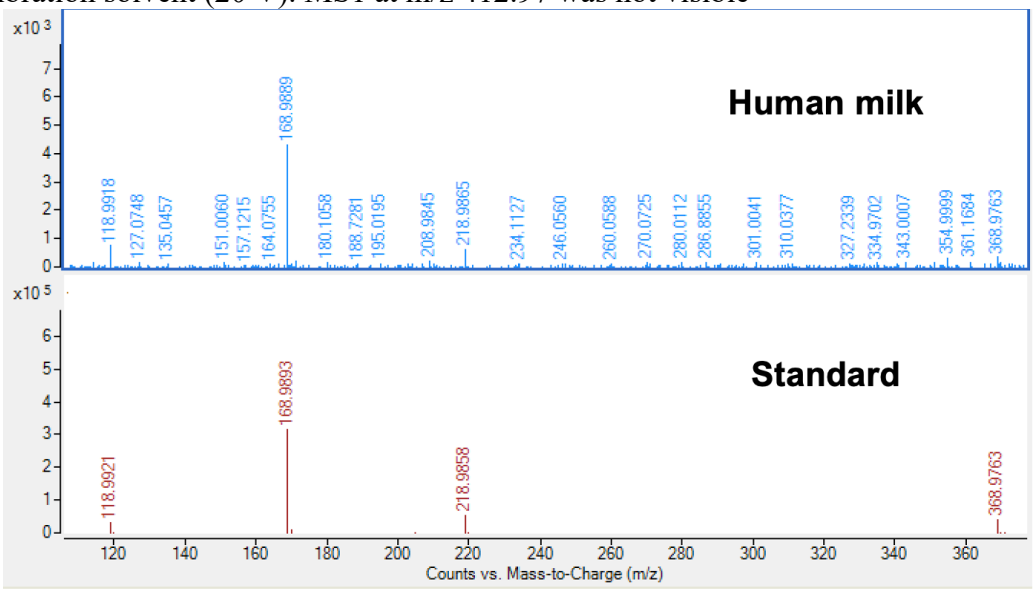


Figure S5.8. Targeted MS/MS of perfluorooctanesulfonic acid in an unspiked human milk sample and pure calibration solvent (40 V). Parent ion at *m/z* 498.93 (No fragmentation visible)

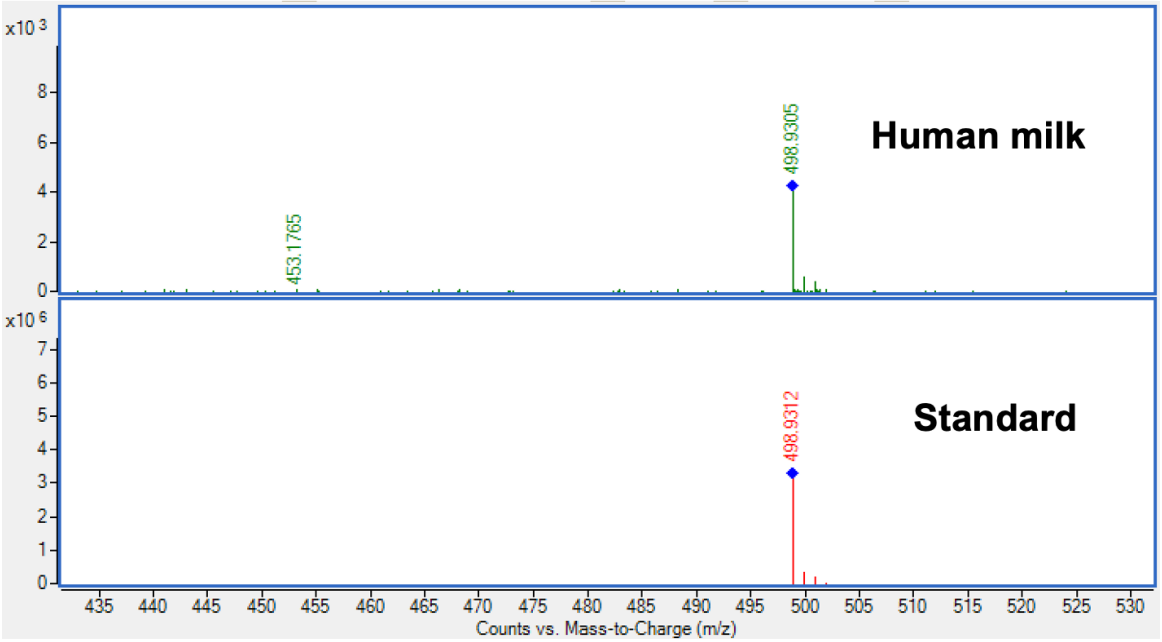


Figure S5.9. Targeted MS/MS of perfluorohexanesulfonic acid in an unspiked human milk sample and pure calibration solvent (40 V). Parent ion at 398.9 (No fragmentation visible)

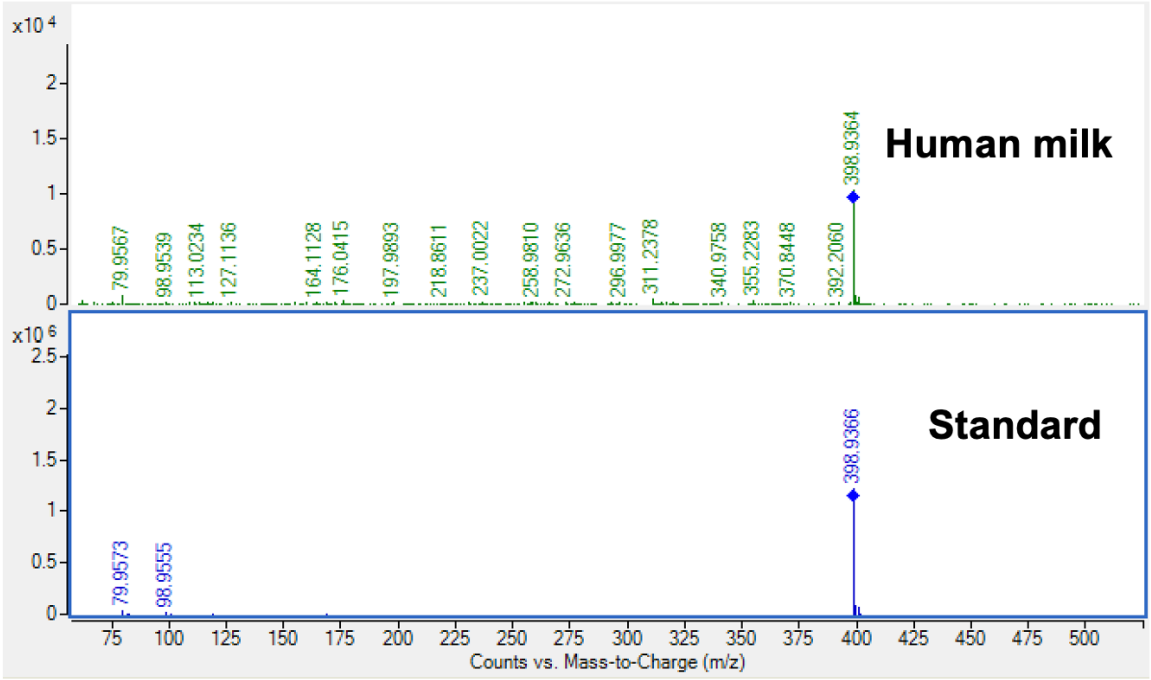


Figure S5.10. Targeted MS/MS of methyl paraben in an unspiked human milk sample and pure calibration solvent (20 V). Fragmented product ion at *m/z* 151.04

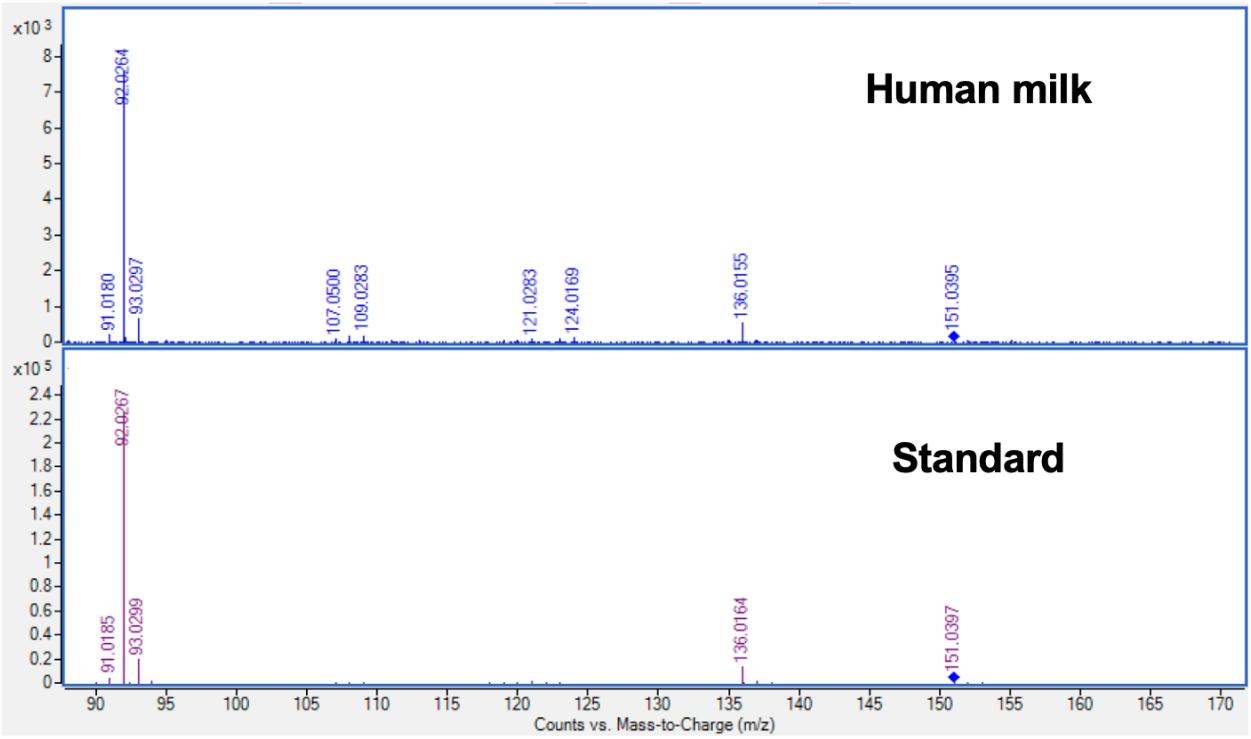


Figure S5.11. Targeted MS/MS of ethyl paraben in an unspiked human milk sample and pure calibration solvent (20 V). Fragmented product ion at *m/z* 165.06

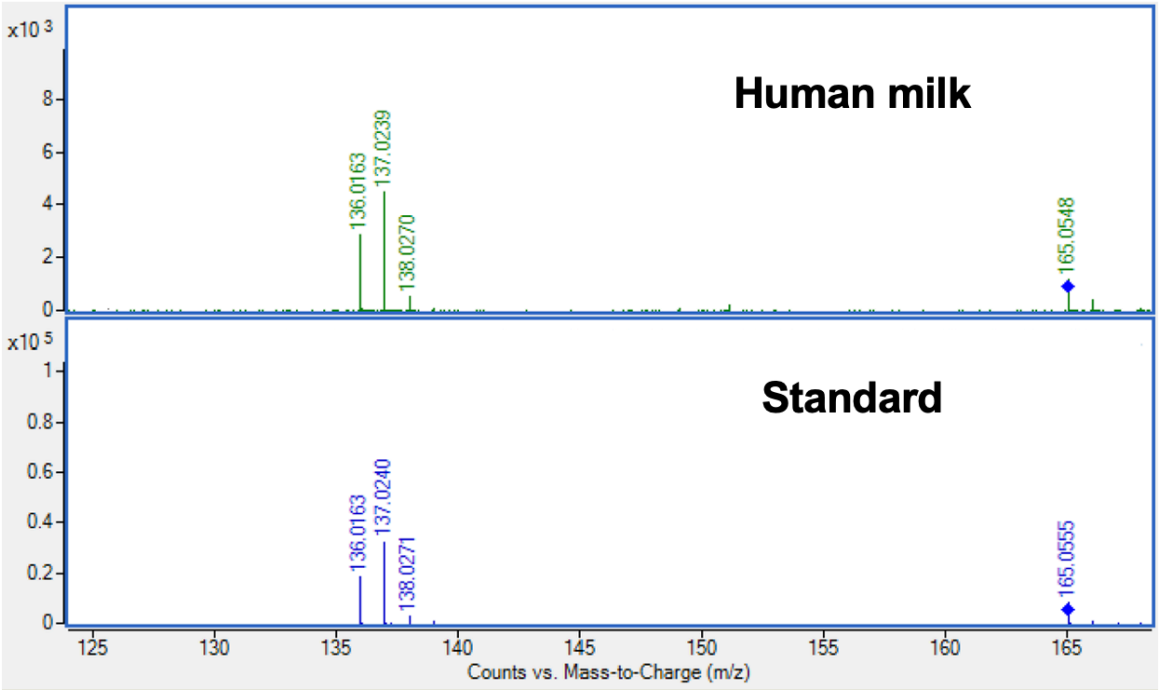


Figure S5.12. Targeted MS/MS of propyl paraben in an unspiked human milk sample and pure calibration solvent (20 V). MS1 at *m/z* 179.1 was not visible.

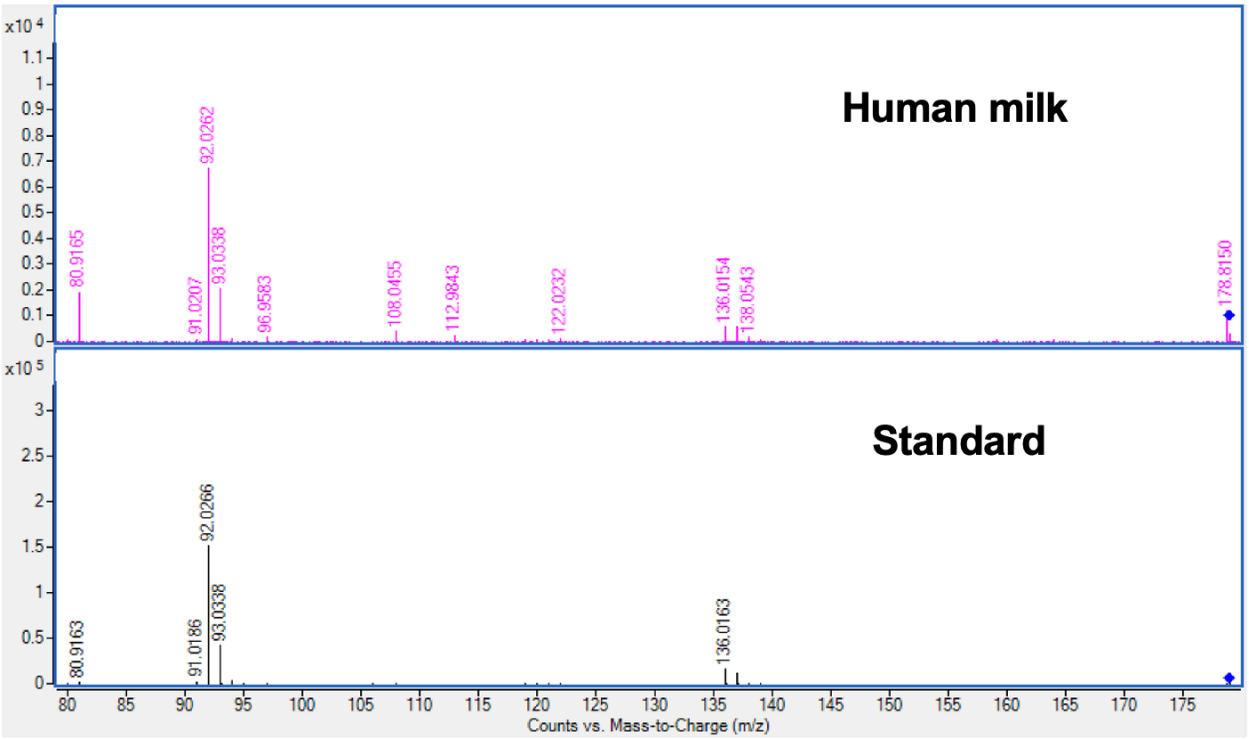


Figure S5.13. Targeted MS/MS of 1,3 diphenyl guanidine in an unspiked human milk sample and pure calibration solvent (20 V). MS1 at *m/z* of 210.1 was not visible.

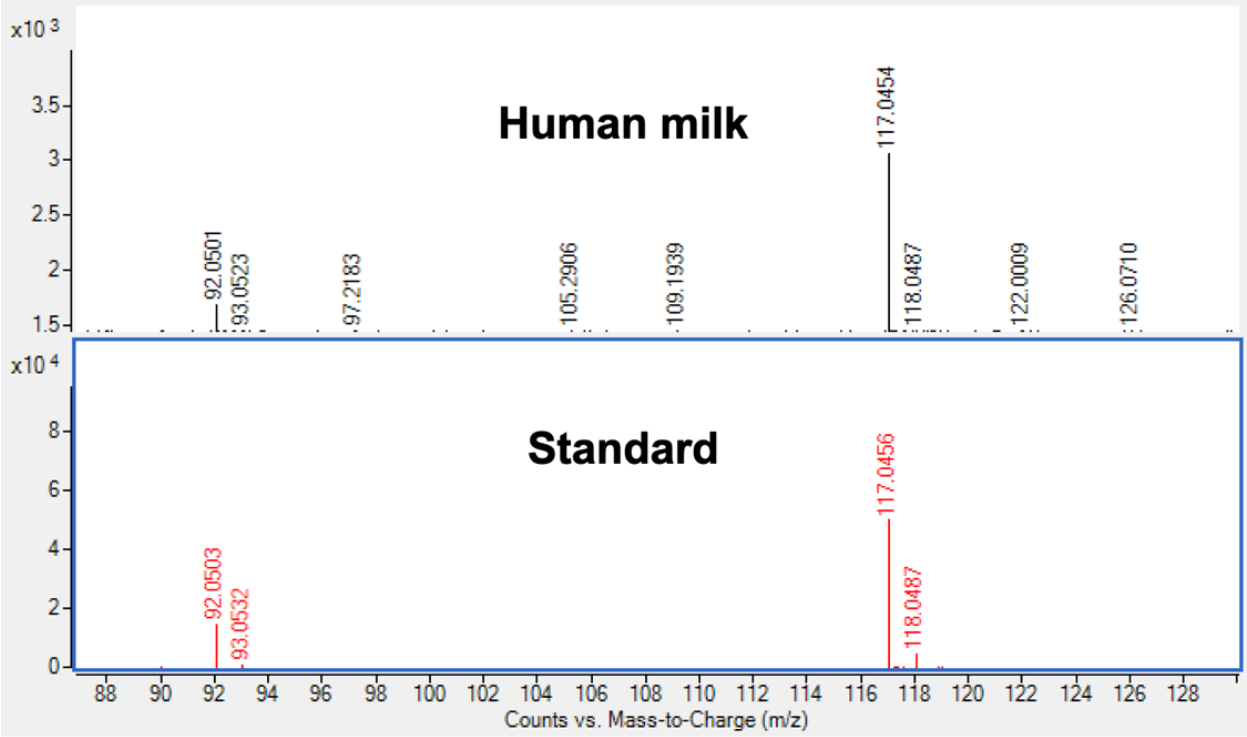


Figure S5.14. Generated volcano plot with the highlighted features representing identified methyl, ethyl, propyl parabens (high conjugation potential) and 2 PFAS (PFOS and PFOA) with low conjugation potential for one batch.

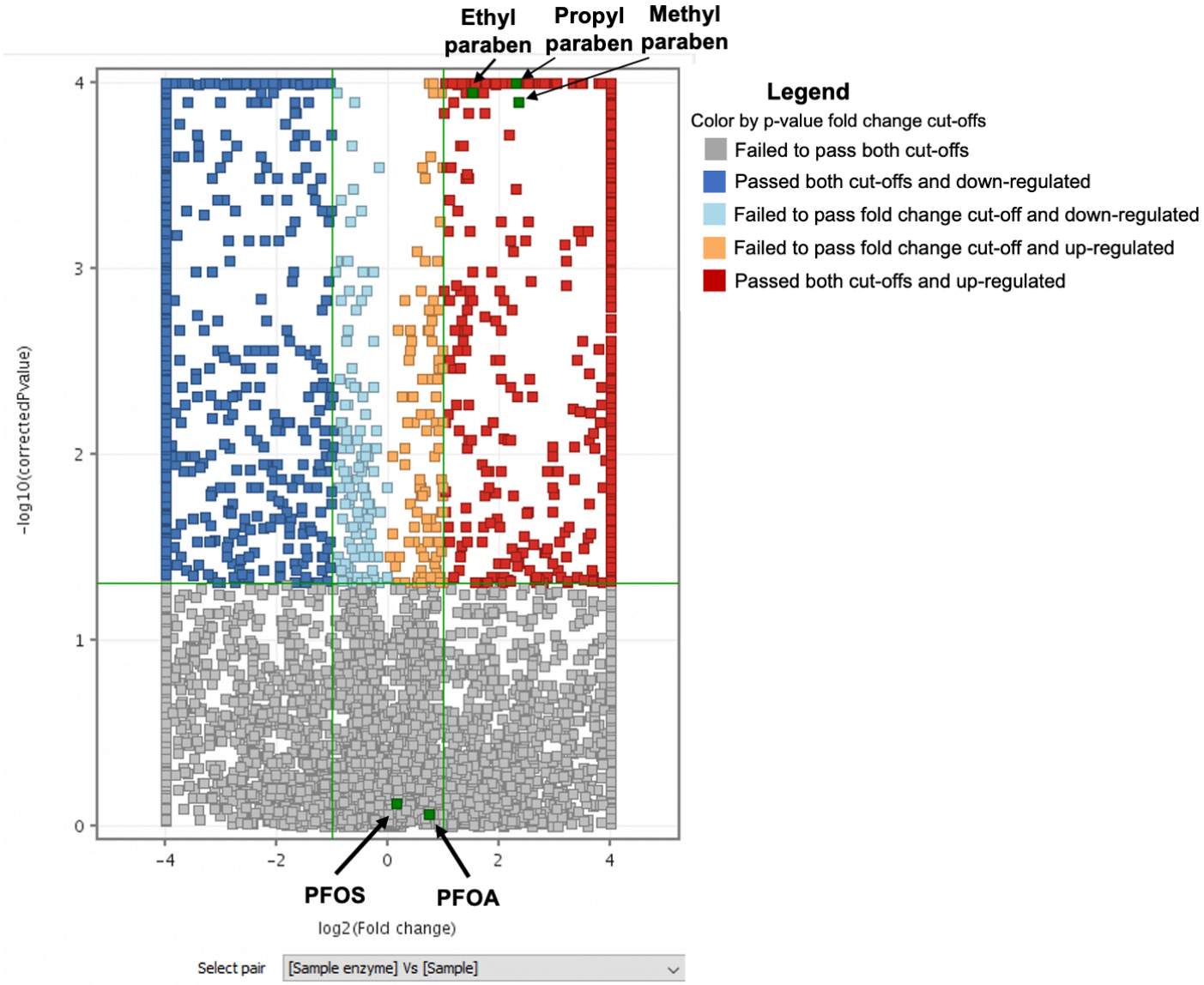


Figure S5.15. Extracted *m/z* values of 92.027, 93.034 overlapping with the retention time (17.1 min) of the tentative octyl paraben (m/z of 249.1491)

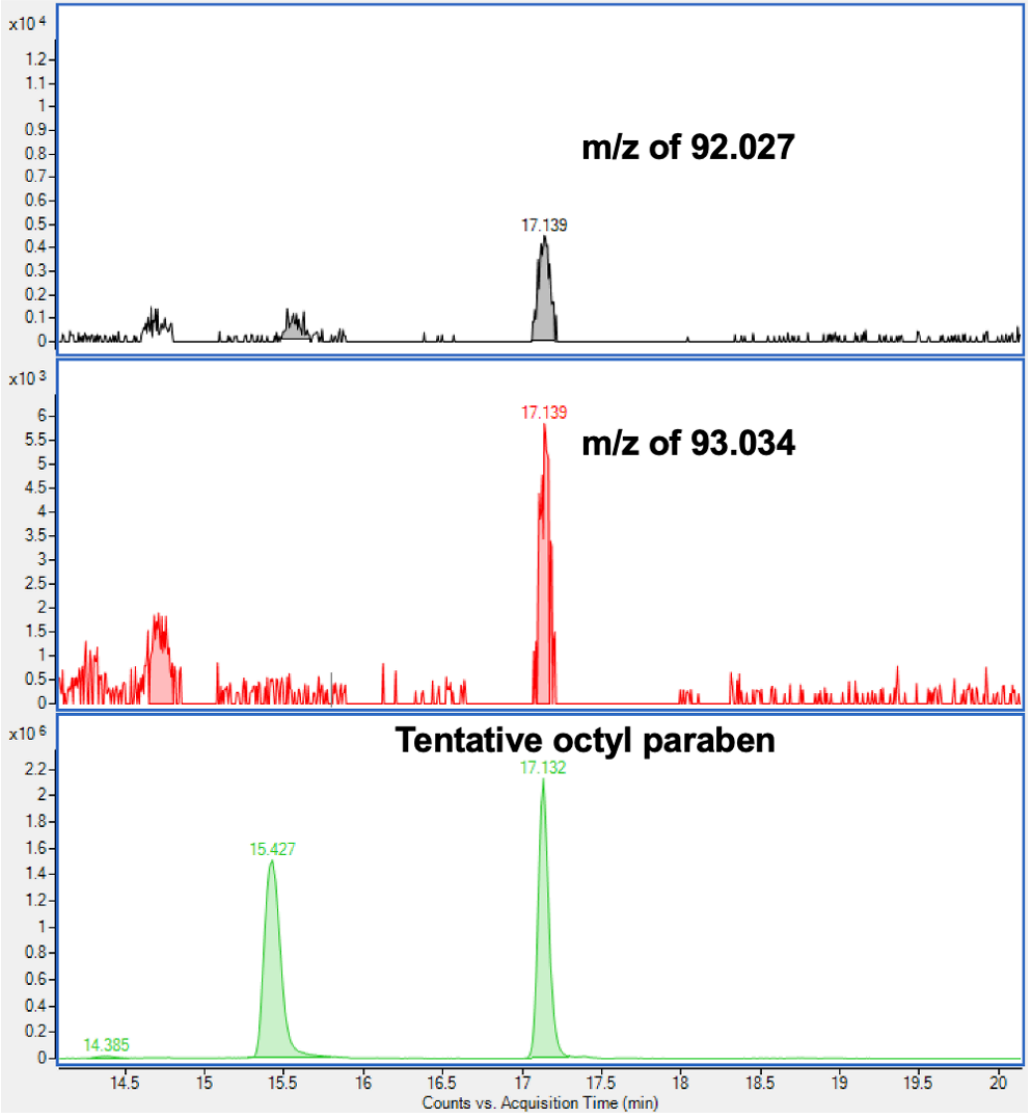


Figure S5.16. Targeted MS/MS of butyl paraben in an unspiked human milk sample and pure calibration solvent (20 V). Fragmented parent ion at *m/z* 193.09

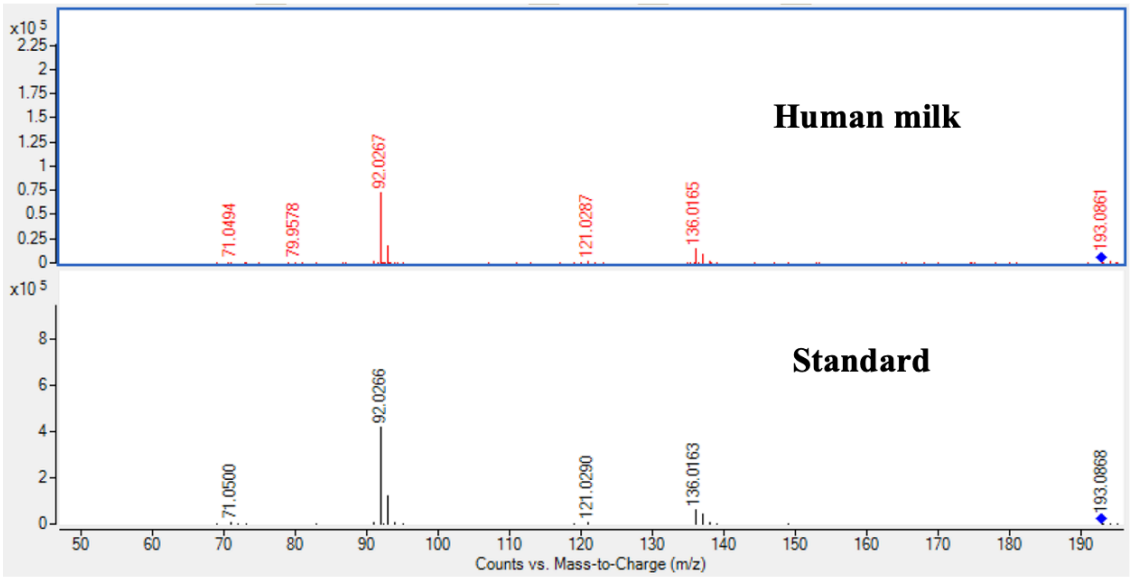


Figure S5.17. Targeted MS/MS of isobutyl paraben in an unspiked human milk sample and pure calibration solvent (20 V). Fragmented parent ion at m/z 193.09

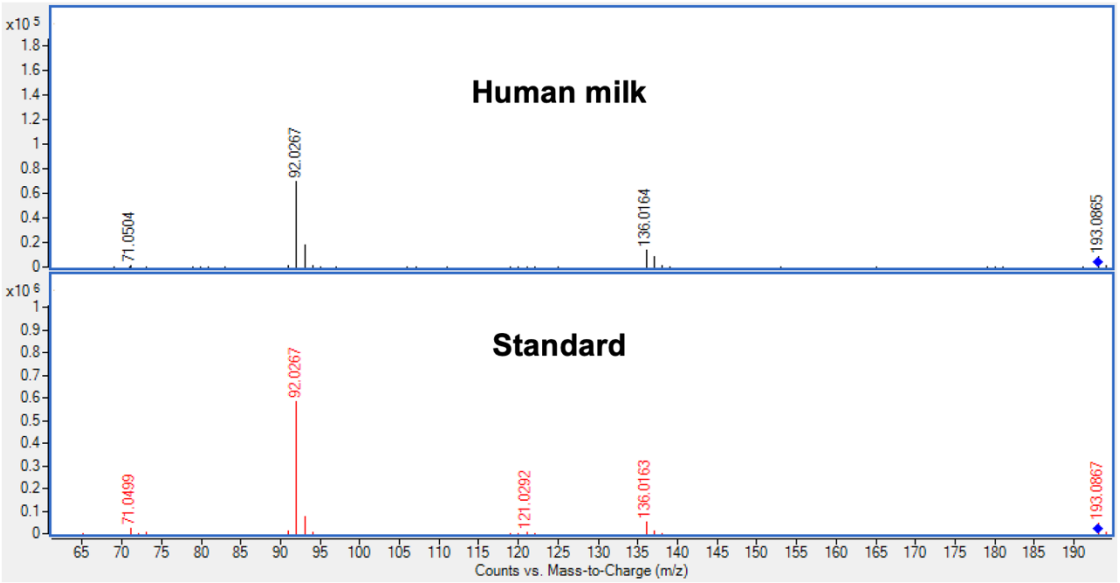


Figure S5.18. Targeted MS/MS of phenyl paraben in an unspiked human milk sample and pure calibration solvent (20 V). MS1 at m/z value of 213.06 was not visible

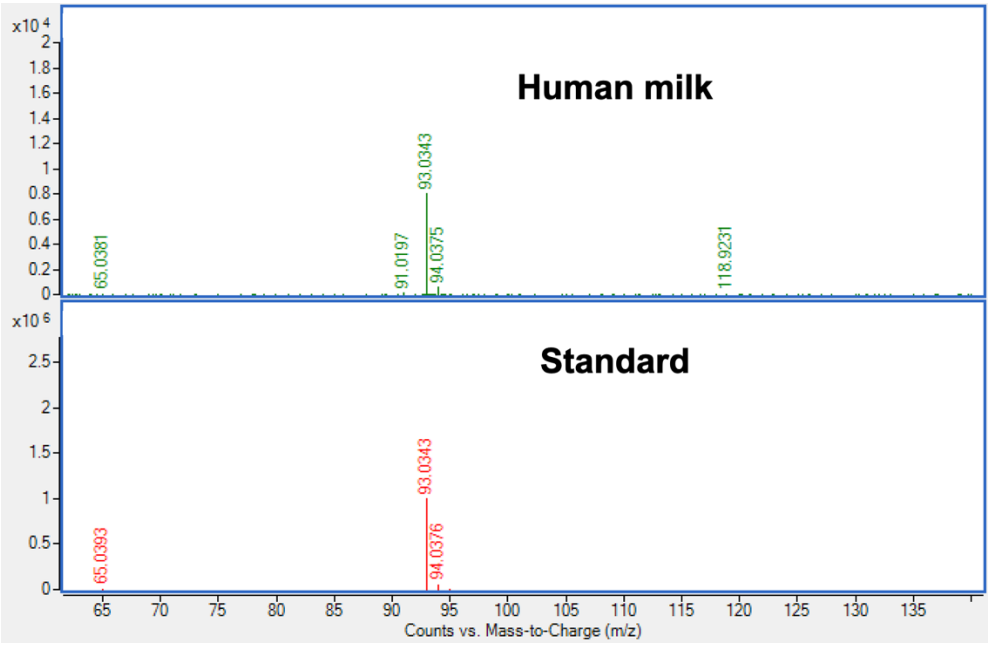


Figure S5.19. Targeted MS/MS of 2-ethylhexyl 4-hydroxybenzoate in an unspiked human milk sample and pure calibration solvent (20 V). Fragmented product ion at m/z 249.15

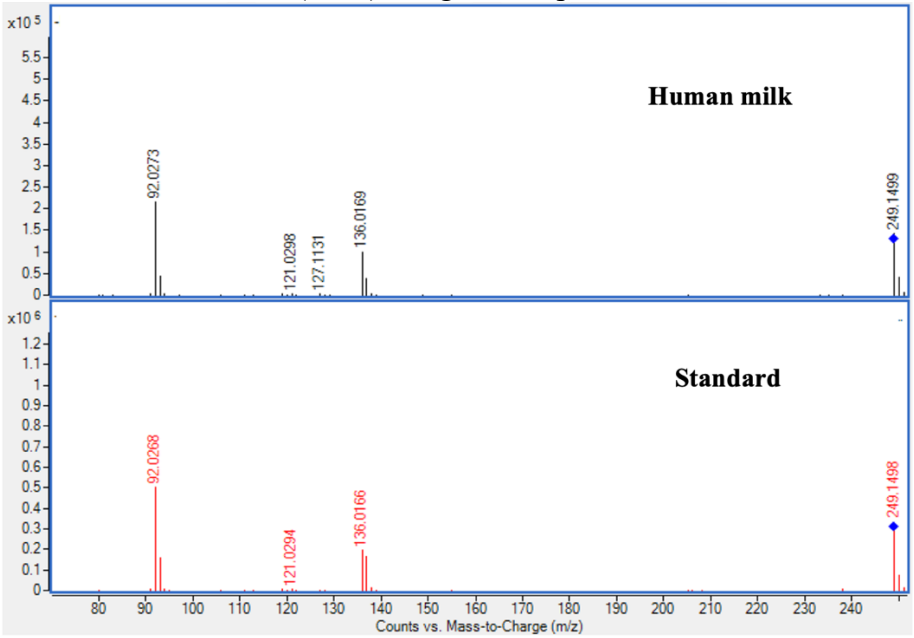


Figure S5.20. Peak comparison between a standard of 2-EHHB (30 ng/mL) and 2-EHHB in an unspiked human milk sample

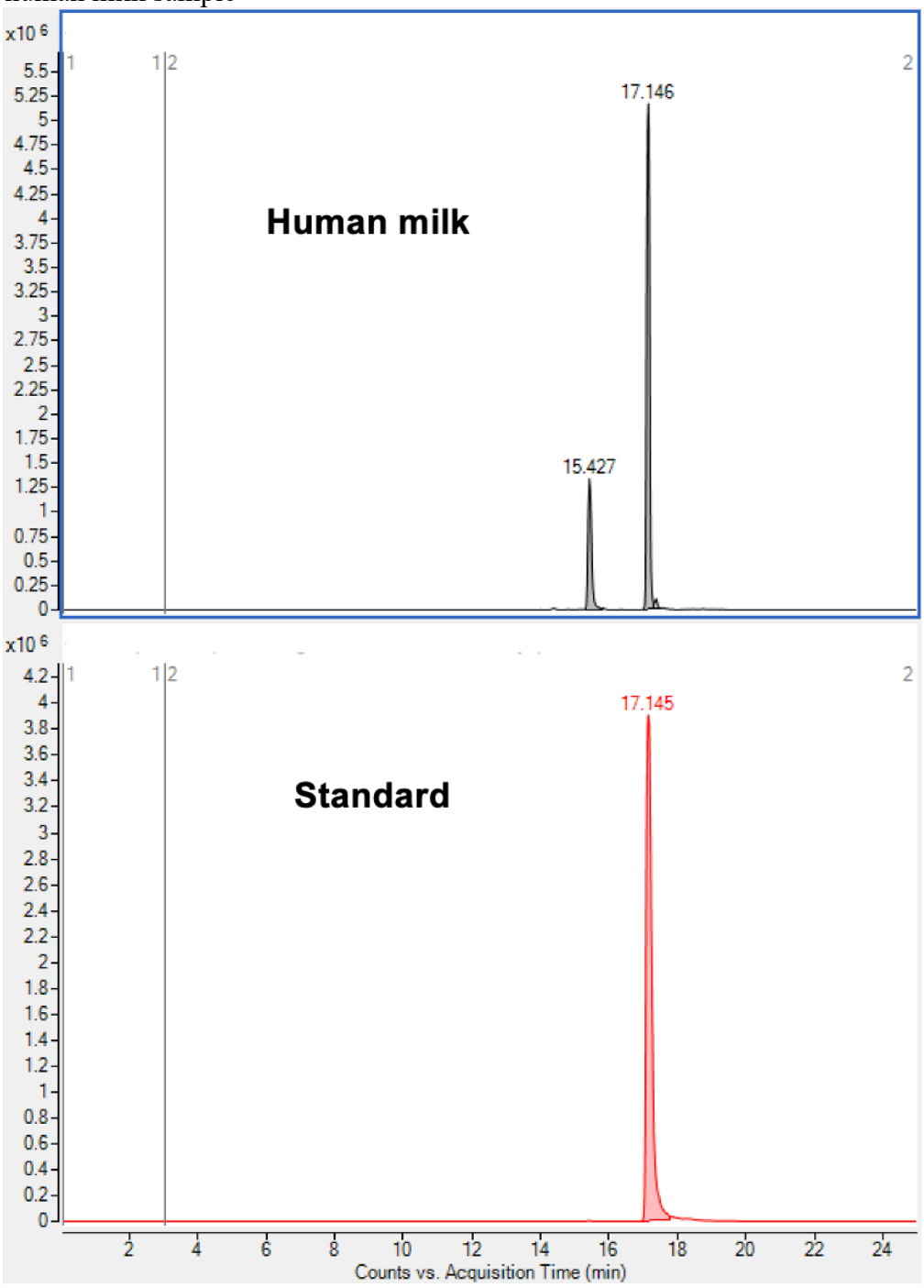


Table S5.1: Non-targeted analysis selected parameters (Profinder).

Parameters	Values
Mass window	**10 ppm**
Retention time window	**±0.2 min**
Peak filter (absolute height)	**≥ 200 counts**
Integrator method	**Agile 2**
Peak spectra: spectra to include how much percent of average scan	**>10%**
TOF spectra: exclude if above how much saturation	**20%**
Post processing: Find by formula peak filter (absolute height)	**≥ 1000 counts**
Molecular feature extraction score	**90%**

Table S5.2. List of plastic-related candidates along with their conjugation potential (confidence level 3 and ID score > 90%) detected in 6 batches of Canadian (Montreal) and South African (Vhembe and Pretoria) human milk.

ESI- m/z [M-H]⁻	***Retention time (min)***	***Region (Montreal, Vhembe or Pretoria)***	***Formula***	***Conjugation Potential after fold change analysis***	***Suspected identity (Confidence level 3)***
151.0395	12.75	All	$C_8H_8O_3$	Yes	Methyl paraben
165.0552	13.76	All	$C_9H_{10}O_3$	Yes	Ethyl paraben
179.0708	14.71	All	$C_{10}H_{12}O_3$	Yes	Propyl Paraben
221.0814	11.94	All	$C_{12}H_{14}O_4$	Yes	Monobutyl phthalate (MBP)
221.0814	11.67	All	$C_{12}H_{14}O_4$	Yes	Monoisobutyl phthalate
277.1440	15.03	All	$C_{16}H_{22}O_4$	Yes	Mono(2-ethylhexyl) phthalate (MEHP)
498.9297	14.61	All	$C_{15}H_{12}O_4$	No	Perfluorooctanesulfonic acid (PFOS)
412.9659	14.10	All	$C_8HF_{17}O_3S$	No	Perfluorooctanoic acid (PFOA)
398.9360	13.74	All	C_8HF15O_2	No	Perfluorohexane sulfonic acid

210.1031	11.85	Vhembe and Pretoria	$C_{13}H_{13}N_3$	No	1,3-diphenylguanidine
233.0198	12.97	All	$C_{10}H_6N_2O_5$	No	Acid yellow 24
337.2379	16.80	All	$C_{20}H_{34}O_4$	No	1,4-Bis-2-[(2-methyl-2-propanyl)peroxy]-2-propanyl benzene
155.1436	16.53	Vhembe and Pretoria	$C_{10}H_{20}O$	Yes	Cyclohexanol, 4-(1,1-dimethylethyl)-
157.1229	12.48	All	$C_9H_{18}O_2$	No	Pelargonic acid
163.0759	13.46	All	$C_{10}H_{12}O_2$	No	2-Hydroxy-2-methylpropiophenone (Darocur 1173)
223.0971	14.21	All	$C_{12}H_{16}O_4$	Yes	2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone
163.1123	12.40	All	$C_{11}H_{16}O$	Yes	4-tert-amylphenol
165.0188	12.71	All	$C_8H_6O_4$	No	Phthalic acid
293.1389	14.86	All	$C_{16}H_{22}O_5$	Yes	Mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP)
207.0657	11.89	All	$C_{11}H_{12}O_4$	Yes	Monopropyl phthalate (MPP)
255.0657	15.46	All	$C_{15}H_{12}O_4$	Yes	Monobenzyl phthalate (MBzP)
165.9785	19.34	All	$C_7H_5NS_2$	Yes	2-Mercaptobenzothiazole
167.1072	12.36	All	$C_{10}H_{16}O_2$	No	Bicyclo[2.2.1]heptan-2-one, 3-hydroxy-4,7,7-trimethyl, exo-
215.1647	12.70	All	$C_{12}H_{24}O_3$	No	Texanol
211.0607	13.15	All	$C_{10}H_{12}O_5$	No	Propyl gallate
217.1593	13.83	All	$C_{15}H_{22}O$	No	2,4-Dimethyl-6-(2-methylcyclohexyl)phenol
221.1542	16.79	All	$C_{14}H_{22}O_2$	No	2,5-Di-tert-butylhydroquinone
224.0824	14.54	All	$C_{13}H_{11}N_3O$	No	2-(2H-Benzotriazol-2-yl)-p-cresol
225.1855	16.26	All	$C_{14}H_{26}O_2$	No	Tetramethyl decynediol
235.1698	15.49	All	$C_{15}H_{24}O_2$	No	3,5-di-tert-Butyl-4-hydroxymethylphenol
249.1855	16.23	All	$C_{16}H_{26}O_2$	No	2,5-Di-tert-pentylbenzene-1,4-diol
248.1651	15.36	All	$C_{15}H_{23}NO_2$	No	N-(4-hydroxyphenyl)nonan-1-amide
357.3005	14.90	All	$C_{21}H_{42}O_4$	No	Glyceryl mono-stearate
399.3111	17.01	All	$C_{23}H_{44}O_5$	No	Pentaerythritol monooleate
219.1749	16.09	All	$C_{15}H_{24}O$	No	Butylated hydroxytoluene (BHT)

179.1072	14.30	All	$C_{11}H_{16}O_2$	No	Butylated hydroxyanisole (BHA)
413.1965	16.41	All	$C_{24}H_{30}O_6$	No	Di-(p-ethylbenzylidene)sorbitol
437.3056	17.88	All	$C_{29}H_{42}O_3$	No	Tinuvin 120 (2,4-Di-tert-butylphenyl 3,5-di-tert-butyl-4-hydroxybenzoate)
446.2232	16.25	Montreal	$C_{30}H_{29}N_3O$	No	Tinuvin 234
314.1060	19.61	Montreal	$C_{17}H_{18}ClN_3O$	No	Tinuvin 326
350.2232	16.19	Montreal	$C_{22}H_{29}N_3O$	No	Tinuvin 328
469.3140	18.19	All	$C_{30}H_{46}O_2S$	No	Bis(3,5-di-tert-butyl-4-hydroxy benzyl)sulfide
429.3733	19.75	All	$C_{29}H_{50}O_2$	No	Irganox 201 (3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6-ol)
203.1072	15.22	All	$C_{13}H_{16}O_2$	No	1-Hydroxycyclohexyl Phenyl Ketone (Irgacure 184)

Table S5.3. List of additional identified parabens in Canadian (Montreal) and/or South African (Vhembe and Pretoria) human milk.

ESI- m/z [M-H]-	***Retention time (min)***	***Region (Montreal, Vhembe or Pretoria)***	***Formula***	***Confirmed identity using analytical standard***
193.0865	15.37	Vhembe and Pretoria	$C_{11}H_{14}O_3$	Iso-butyl Paraben
193.0865	15.49	Vhembe and Pretoria	$C_{11}H_{14}O_3$	Butyl paraben
213.0552	15.61	All	$C_{13}H_{10}O_3$	Phenyl paraben
249.1491	17.10	Vhembe and Pretoria	$C_{15}H_{22}O_3$	2-ethylhexyl 4-hydroxybenzoate (2-EHHB)

6. General conclusions

6.1.Conclusions

This work developed a suitable extraction method for the detection and analysis of various plastic-related contaminants present in the human milk matrix using high-resolution mass spectrometry. The data that were generated provide a general overview of the presence of multiple families of contaminants that can potentially accumulate in the body and be transferred to human milk; further, data-scarce contaminants that are infrequently reported in human milk studies were identified. The data generated by this research also highlighted major data gaps for certain PRCs, particularly bisphenols and parabens; both targeted and non-targeted approaches that allow for the detection and identification of various types of bisphenols, as well as different common and unusual parabens and other PRCs in human milk, were employed.

The first step included a literature search of all possible contaminants that may accumulate in human milk while assessing the weaknesses and limitations in current human milk biomonitoring. It was observed that current human milk studies predominantly used targeted analysis to quantify levels for a limited number of contaminants. While PRCs are widely studied contaminants, the dominant classes for the PRCs that are analyzed in milk mostly include bisphenols and phthalates. Despite the worldwide analyses of bisphenols and phthalates, data on their levels are still missing in certain parts of the world, specifically in most African countries, highlighting the need to explore their levels in data-poor countries. Also, despite the many studies conducted for these contaminants in human milk, other PRCs are often underreported. There was an evident lack of data for reported PRCs used as preservatives, UV absorbers and filters, synthetic antioxidants, and color additives, in which the use of targeted analysis limits their detection.

To overcome these limitations, an efficient extraction method for PRCs that combined both targeted and non-targeted approaches for the quantification of major bisphenol analogues, as well

as the detection of other infrequently reported bisphenol and paraben-related compounds was developed.

An initial step was taken to quantify the different types and levels of bisphenols in data-lacking countries such as South Africa and compare their levels with the bisphenols from Canadian human milk to assess regional differences. The findings demonstrated that using an efficient QuEChERS extraction led to the recoveries of 9 selected bisphenols. From the results, it was observed that only BPS could be detected in Canadian (Montreal) human milk, while BPA, BPS and BPAF were present in South African human milk (Vhembe and Pretoria). Notably, this was the first study to have reported bisphenols levels in human milk from South Africa. Our findings suggest differences in the types of levels observed across countries as well as an observable decrease in BPA usage for Canada over the last decade compared to South Africa. In South African human milk samples, BPA was frequently detected and observed to have high conjugation potential, though many samples had BPA levels below the detection limit. Consequently, geometric means for bisphenol A (BPA) in both Vhembe and Pretoria were calculated using the Hornung and Reed method to account for non-detectable values, yielding values near the lower limit of the calibration curve. If the geometric means had been calculated only from samples with detectable BPA, the resulting values would have been higher. The calibration range for this study was based on detectable bisphenol levels reported from other human milk studies prior to the analysis of South African and Montreal human samples. To improve the quantification of bisphenols close to their detection limits, a lower calibration range should be selected in future work.

Following the analysis of targeted bisphenols, non-targeted screening was applied for the collected human milk to identify other bisphenol-related unknowns. Using a non-targeted workflow along with a customized database library derived from the Technical Consultation List

of Health Canada, 11 bisphenol-related unknowns were identified [19]. These included the presence of various bisphenol S derivatives (D8, D90 and TGSA) used as color developers in thermal paper production. Other identified unknowns included the presence of UV absorbers, such as benzophenone 1,2 and 3, and synthetic antioxidants-related contaminants, such as Irganox 1010, Cyanox 2246 and BHT-COOH. The identification of these contaminants highlighted the effectiveness of using NTA to detect other PRCs that would not likely have been detected by only employing a targeted approach.

Aside from D8, D90 and TGSA, since this work focused on the non-targeted analysis of bisphenol-related unknowns, targeted or semi-quantification for the levels of other identified contaminants were not pursued. It is important to note that lower detection limits could be achieved if the LC-MS triple quadrupole was employed for the targeted analysis of these contaminants in a future study. However, since HRMS was needed for the non-targeted analysis of these bisphenol suspects, a compromise was made to conduct semi-quantification or any targeted analyses using the HPLC–QTOF-MS/MS. This approach, though less sensitive than the LC-MS triple quadrupole, allowed for the broader detection capabilities required for this study.

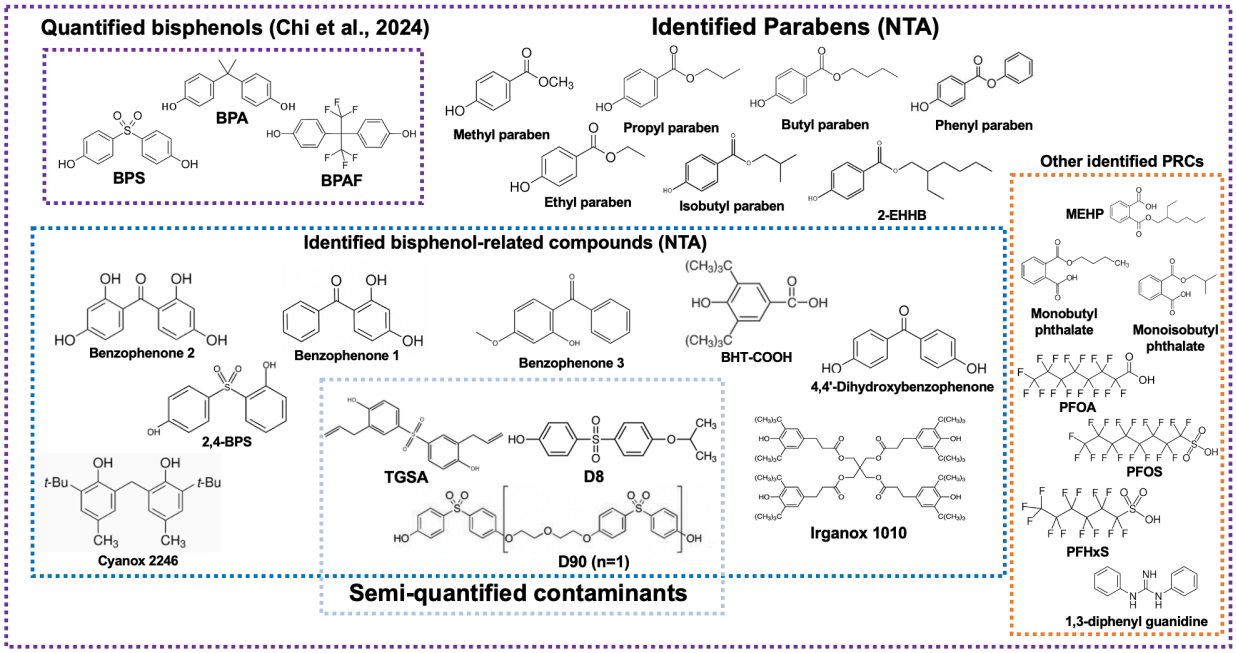
Lastly, non-targeted strategies were applied for the identification of other PRCs, specifically parabens, as well as the assessment of their conjugation potential in human milk. By comparing differences between each enzyme treated sample and their non-enzyme treated counterparts through statistical analysis, paraben-related features were selected along with other plastic-related candidates of interest. From the tentative PRCs, 3 common parabens, 2 phthalate metabolites, 3 PFAS, one tire-related chemical (1,3 diphenyl guanidine) were identified using analytical standards. Further investigations of other uncommon parabens in human milk using in-source fragmentation under electrospray ionization identified the presence of butyl, isobutyl and phenyl

paraben, as well as the presence of 2-ethylhexyl 4-hydroxybenzoate, a unique paraben from South Africa. While an attempt was made to semi-quantify the levels of the common parabens using internal labeled bisphenol S similar to Chapter 4, their recoveries exceeded 120%, indicating potential inaccuracies. Consequently, only the detection frequencies of the identified parabens were discussed in this study. Future research should focus on the targeted analysis of all identified parabens using an instrument with higher sensitivity to achieve more accurate quantification of their levels. Additionally, there should be a quantitative assessment of their conjugation potential in human milk using different statistical analyses.

The analysis of environmental contaminants in human milk plays a crucial role in ongoing human milk biomonitoring efforts, ensuring the safety of maternal-infant health. Additionally, investigating these contaminants would help in the advancement of current human exposome studies. One of the goals of this work was to improve current limitations surrounding human milk biomonitoring by quantifying levels of major PRCs in human milk from different countries (including data scarce countries) to assess regional differences. Furthermore, the research has enhanced our current understanding of the chemical exposome concerning both mothers and breastfeeding infants by identifying different uncommon bisphenol and paraben-related compounds alongside other PRCs using NTA (Figure 6.1). This research has also explored the conjugation potential (glucuronidation or sulfonation) of different chemical contaminants where compounds such as bisphenols, parabens or phthalate metabolites could become conjugated in the body prior to their transfer in milk. Altogether, the results of this work indicated regional differences in detected bisphenol residues, with BPA, BPS, and BPAF present in South African human milk samples, and BPS being exclusively detected in Canadian samples. Further analysis revealed the presence of other underreported bisphenol-related unknowns used as color developers,

UV filters as well as synthetic antioxidants including 2 compounds (BHT-COOH and Irganox 1010) that have never been reported in previous human milk studies. Our results also showed the presence of commonly used and unreported parabens such as phenyl paraben and 2-EHHB, alongside other phthalate metabolites, PFAS residues and a tire-related contaminant in human milk highlighting the potential of NTA in detecting a broad range of contaminants. Overall, our findings demonstrated the usefulness of integrating targeted and non-targeted analysis to detect both the specific bisphenols of interest as well as the surrounding unknown contaminants that are of equal importance in improving current human milk biomonitoring.

Figure 6.1. Identified plastic-related contaminants in South African (Vhembe and Pretoria) and Canadian (Montreal) human milk



Beyond the scope of this work, numerous other persistent and emerging contaminants reported in previous studies on water and soil, such as flame retardants, pesticides, and various PFAS, may be present in human milk but this remains unexplored [28-31]. Detection of these compounds could be challenging with the use of the same analytical workflow employed in this

study, primarily due to limitations associated with the extraction method and the instrumentation employed for their analysis. For instance, the use of LC-Q-TOF-MS/MS in this work may raise questions on what other types of contaminants might have been detected if gas chromatography or Fourier transform infrared spectroscopy were operated instead [32, 33].

Another important consideration is the use of ESI positive mode which would have enabled the detection of a broader range of compounds in human milk compared to the ESI negative mode, prompting further investigations into the detection and identification of other types of contaminants of interest that might not have been detected using the ESI negative mode [34].

The application of QuEChERS for the extraction of PRCs in this work also prompts us to consider what other contaminants of concern might have been detected with the use of a different sample preparation, and whether there exists a more efficient extraction method than QuEChERS. For human milk analysis, we should also explore the possibility of combining another extraction method together with the current approach. For instance, the use of a different type of solvent (i.e. hexane) alongside QuEChERS would enable the detection of a broader range of contaminants, specifically the lipophilic contaminants such as brominated, chlorinated flame retardants, as well as other halogenated compounds [35-37].

Lastly, it is important to note that levels of certain contaminants in human milk may vary depending on the collection period. Studies have found that contaminant levels in colostrum, transition, and mature milk differ from each other, with respect to compounds such as PFAS, parabens and lipophilic flame retardants; for example, levels are generally higher in colostrum compared to mature milk [38-40]. This raises the question of whether the collection period should be considered a critical factor in human milk biomonitoring, as contaminant levels may vary throughout the sampling process.

6.2. Contribution to knowledge

The research presented in this thesis contributed the following novel aspects, both in terms of health safety, environmental applications, and analytical point of view:

- Assessment and summary of all current reported environment contaminants in previous human milk studies while highlighting the limitations surrounding current human milk biomonitoring

- Development of an efficient extraction method allowing the application of both targeted and non-targeted analysis of PRCs in human milk

- Assessment of bisphenol (types and levels) in data-lacking countries, such as South Africa, and differences in detected bisphenols across 2 countries (Canada and South Africa)

- Application of non-targeted analysis for the identification of overlooked or unreported bisphenol-related unknowns in human milk to enhance our current understanding on chemical exposome

- Application of non-targeted strategies for the identification of common and unreported parabens together with other PRCs of interest in human milk

6.3. Recommendations to future research

Based on the results obtained in this thesis, the following topics are recommended for future research:

- To investigate the levels of common phthalate diester and monoesters as well as parabens in human milk using targeted analysis in data-scarce country such as South Africa. This research aims to enhance human milk biomonitoring and quantitatively assess the conjugation potential of these compounds using statistical analyses, such as ANOVA.

- To investigate the presence of other plastic-related unknowns using the positive ESI mode of the HPLC-QTOF-MS/MS using non-targeted analysis. This would assist health officials in better characterizing the chemical exposome by providing a deeper understanding of other contaminants present in human milk, including phthalate diesters.

- To confirm the identities of the other tentative bisphenol-related compounds detected in human milk from Chapter 4. The accumulation potential of these suspects in human milk still needs to be assessed by employing different strategies to evaluate their MS/MS spectra or by purchasing and analyzing their corresponding standards.

- To apply additional data mining software for the detection and identification of halogenated compounds in the collected human milk dataset. This approach will enhance our understanding of the presence of various pharmaceuticals, antimicrobial agents, and

metabolites from organochlorine pesticides, thereby expanding the scope of non-targeted analysis.

- To detect and identify other lipophilic contaminants such as organochlorine pesticides and flame retardants in human milk through non-targeted screening using a modified QuEChERS sample extraction, followed by gas chromatography mass spectrometry for their analysis. During sample preparation, hexane or other organic solvent should be used for the extraction and analysis of lipophilic contaminants such as OCPs, PCBs and other halogenated flame retardants. These contaminants have not been explored in this research, and determining their levels using gas chromatography, an appropriate technique for their analysis, would prove useful for data-scarce countries such as South Africa.

- To conduct statistical analyses for the detected levels of specific families of contaminants (i.e., PRCs, flame retardants, pharmaceuticals, and pesticides) after targeted analysis between colostrum and mature human milk. This would allow health experts to monitor changes in the levels of different contaminants over different time periods for the safety of the mother and breastfeeding infant.

- To explore the presence of different plastic-related contaminants in other human matrices such as serum and urine using targeted and non-targeted screening. Identifying and assessing the levels of these contaminants in matrices other than milk, especially in data-scarce countries, would significantly enhance overall human biomonitoring efforts.

Every year, approximately 2,500 new chemicals are introduced to the market, adding to the roughly 85,000 chemicals currently in commerce [41, 42]. Given that humans are exposed to hundreds, if not thousands, of these chemicals daily, it is crucial to develop efficient less time-consuming and more cost-effective methods for detecting substances of concern that may impact on the health of both mothers and breastfeeding infants. The QuEChERS approach, coupled high-resolution mass spectrometry, should be recognized as an overall effective method for investigating PRCs together with various other families of contaminants. Future research should prioritize developing robust sample preparation techniques that enable the extraction and analysis of a wide range of contaminants in human milk biomonitoring.

General Reference List Note:

In accordance with the Guidelines for Thesis Preparation, each of the manuscript chapters (i.e., Chapters 2-5) contain their own reference list. Hence, the following list corresponds to the references included in the remaining chapters of the thesis (i.e., Chapters 1 and 6).

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