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Research Article

Application of non-targeted analysis for the identification of uncommon or unreported chlorinated contaminants in human milk

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Abstract

Background: Many known halogenated substances have been shown to accumulate in the human body and transfer into maternal milk, with potential adverse health consequences for the breastfeeding infant. However, few studies have applied non-targeted analysis techniques to identify additional, possibly unknown, halogenated contaminants in human milk.

Methods: Using HaloSeeker, a powerful non-targeted data-mining software, several chlorinated-related features of interest were extracted from data obtained for 594 human milk samples collected in Canada (Montreal) and South Africa (Vhembe and Pretoria) during 2018–2019.

Results: In total, six compounds were confirmed at confidence level 1 using analytical standards: two antimicrobials (chloroxylenol and triclocarban), two pesticide-related chemicals (propanil and 4-hydroxy-2,5,6-trichloroisophthalonitrile), one ultraviolet filter (bumetrizole), and one antiretroviral metabolite (8-hydroxyefavirenz).

Conclusions: These findings demonstrate the effectiveness of non-targeted approaches in identifying uncommon or previously unreported chlorinated contaminants and their metabolites in human milk, and underscore the need for further research to assess their potential health risks.

Key words: Halogenated compounds; emerging chlorinated contaminants; liquid chromatography–mass spectrometry; non-targeted analysis; human milk; biomonitoring.

Introduction

According to the World Health Organization (WHO), human milk is the best source of nourishment for infants[1] as it provides essential nutrients that support growth and development and antibodies that protect against infections. However, human milk can also act as a vector for various contaminants, which may pose health risks to breastfeeding infants.[2] Extensive research has been conducted on the presence of halogenated flame retardants such as polychlorinated biphenyls, organochlorine pesticides and per- and polyfluoroalkyl substances (PFAS) in human milk.[3-10] However, a significant gap in the literature still persists concerning the presence of other lesser known halogenated chemicals[11] or their metabolites.[12-14]

Various chlorinated compounds used as pesticides, antimicrobials, or in medications, can enter the body and may be transferred to human milk.[15-18] The limited research on these chlorinated species indicates a need for enhanced detection efforts to improve current human milk biomonitoring practices. While traditional targeted analytical approaches are effective in identifying known contaminants, they are limited in their capability to detect novel or unreported compounds.[19] In contrast, non-targeted analysis (NTA) offers a powerful method for detecting and identifying a wide range of unknown contaminants across different matrices, thereby allowing for the comprehensive screening of chlorinated unknowns in human milk.[19,20] Over the last decade, the advancement in non-targeted tools, such as HaloSeeker, have significantly improved the data mining capability of halogenated features.[21] Multiple studies have demonstrated the efficacy of HaloSeeker in identifying halogenated contaminants.[20,22-25] Following data mining, chemical formula

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suggestions and structure elucidation can further assist in confirming the identities of detected suspects.[26]

Our previous studies focused on identifying widely used plastic-related and other synthetic phenolic contaminants in human milk collected between 2018–2019 from Canada (Montreal) and South Africa (Vhembe and Pretoria).[27-29] Detected compounds included bisphenol analogues (D8, D90, and TGSA), phenolic preservatives (methyl, ethyl, propyl, butyl, phenyl, and isooctyl parabens), as well as per- and polyfluoroalkyl substances such as perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA), and perfluorohexane sulfonic acid (PFHxS). Building on these works, using data collected from our previous studies, we developed a robust non-targeted approach for the detection and identification of different uncommon and unreported chlorinated contaminants in the milk samples.

Methodology

Chemicals

Chemicals

For non-targeted analysis, analytical standards used for confirmation in this study included 4-hydroxy-2,5,6-trichloroisophthalonitrile, 8-hydroxyefavirenz, bumetrizole, chloroxylenol, propanil and triclocarban. Additional details about the standards and reagents employed in this work can be found in the Supplementary Information (SI).

Sample collection and storage

The details of human milk sample collection from South Africa (Vhembe and Pretoria) and Canada (Montreal) have been described in our previous study.[27] In South Africa, mother-infant pairs were recruited from the maternity wards and vaccination clinics of Tshilidzini Hospital, located in the rural Vhembe district of Limpopo Province, and Tshwane Hospital in the urban area of Pretoria, Gauteng. In Montreal, participants were recruited following childbirth at the Royal Victoria and St Mary's Hospitals. Additional information on the collection procedures can be found in the SI. 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 (REB # A06-M19-17B and MP-37 - 2018–3730).

Sample preparation and analysis

Human milk sample preparation and analysis have been reported earlier and involved a QuEChERS extraction method optimized for the detection of a wide range of contaminants followed by analysis with an Agilent 1290 Infinity II LC system coupled to a 6545-quadrupole time-of-flight (Q-TOF) MS (Agilent Technologies, Santa Clara, USA).[27] The QTOF-MS system was operated with electrospray ionization in the negative mode (ESI-). 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 present work, the collected data for samples without enzyme treatment were further analyzed using a non-targeted framework with a focus on chlorinated compounds. A more detailed description about the sample preparation is provided in the SI.

Quality control/quality assurance

For sample collection and sample preparation, care was taken to avoid sample contact with materials known to contain halogenated flame retardants, bisphenols, plasticizers and pharmaceuticals. Milk samples were collected in polypropylene containers free of bisphenols, pharmaceuticals, and halogenated flame retardants. The storage bags used for the samples were also free of these contaminants. In addition, the glass jars and cardboard boxes used to store freeze-dried milk samples were tested for residual contamination. Similarly, all polypropylene and glass tubes used during sample preparation were tested to ensure they did not contain traces of these substances.

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 included controlling background contamination, monitoring mass accuracy, intensity and retention time (RT) shifts, signal stability and signal drift. Three procedural blanks were analyzed with each batch to monitor for analyte contamination. During sample analysis, an acetonitrile solvent blank was injected after every 10 samples to minimize possible carryover effects from the instrument.

Data analysis of halogenated suspects

For file conversion, we employed the open-access software MSConvert (ProteoWizard), which is integrated into HaloSeeker, to convert raw data into the mzXML format before processing. Data processing for the collected LC-HRMS datasets of 594 human milk samples was conducted using HaloSeeker 2.0, a tool initially developed by Léon et al.,[21] designed specifically to screen for halogenated compounds based on mass differences between chlorine and bromine isotopes, and isotopic ratios. These included previously collected datasets of 194 samples from Vhembe (South Africa), 193 samples from Pretoria (South Africa) and 207 samples from Montreal.

The isotopic data filtering is categorized into four levels: F0 to F2+. Level F0 includes all molecular features identified through peak picking. Level F1 includes paired features based on mass differences corresponding to Cl and Br isotopes. Levels F2 and F2+ correspond to halogenated and poly-halogenated clusters, respectively, filtered based on theoretical Cl and Br isotopic ratios.

Peak picking parameters were set with a *m/z* tolerance of 10 ppm and peak width ranging from 5 to 60 s. For the pre-filtering steps, an intensity threshold of 10000 was applied with a signal-to-noise threshold of 3. In halogen-pairing parameters, retention time tolerance was set to 1 s, and *m/z* tolerance was 0.5 mDa. Blank subtraction was achieved using a filter set to the sum of the average signal intensity of the features plus three times the standard deviation of procedural blanks ($n = 3$). Data files were analyzed in batches, each batch consisting of different sets of blanks ($n = 3$) and human milk samples ($n = 30$). An interactive H/Cl-scale mass defect plot was utilized to visualize HRMS data. Chlorinated features with at least three detectable isotopologs were selected as potential candidates for further analysis.

Following HaloSeeker analysis, the *m/z* values corresponding to the three most abundant isotopologs of the selected chlorinated features were extracted using MassHunter (Agilent Technologies) to monitor their corresponding peaks. Features whose peak intensities matched with the results from HaloSeeker were selected for further investigation. The proposed chemical formulas of these candidates were generated using MassHunter's chemical formula search and generation tools. Targeted MS/MS analysis was then conducted using the LC-Q-ToF-MS/MS ('Sample preparation and analysis' section) to obtain MS/MS spectra for each selected chlorinated candidate using the isotopologue with the highest abundance, prior to further analysis with SIRIUS.

Application of SIRIUS for compound identification

SIRIUS software was employed to generate structural representations of all selected candidates based on their fragmentation patterns obtained from targeted MS/MS analysis. SIRIUS uses fragmented parts of the suspect and provides a list of possible parent compounds, offering an overall matching score by comparing against databases such as PubChem, KEGG and ChEBI.[30] The MS/MS spectra were acquired for all selected halogenated candidates from the human milk samples after the use of HaloSeeker and MassHunter, and were subsequently analyzed using SIRIUS-CSI: FingerID.[31] The generated chemical formulas for these features were then compared with the ones generated using MassHunter (Table S1) to further validate the proposed structures and increase confidence in the identified suspects.

Confirmation of the identify of selected halogenated compounds

The analytical standards for the suspects with SIRIUS matching score (>80%) that were available at a reasonable cost were purchased to confirm their identity at confidence level 1 using the Schymanski scale.[32] For compound identification, a tolerance of ±0.1 min was allowed for the relative retention time between the suspects in the milk samples and their corresponding analytical standards. The MS/MS spectra of the pure standards were then compared with those of the tentative contaminants. The identity of the compound was confirmed if at least one fragment ion was present in both the standard and the compound, with a mass measurement error below 5 ppm and a retention time difference of no more than 0.1 min, in accordance with the European Commission guideline SANTE/11312/2021.[33] In case no standard was available at the time of their confirmation, compounds were annotated with confidence level 2 b if SIRIUS matching scores exceeded 85%, and if their MS/MS spectra matched either experimental spectra in online repositories such as MassBank or predicted spectra found in the Human Metabolome Database. The annotation was downgraded to confidence level 3 if the matching score fell below 85%, considering that alternative structures could not be excluded.

Results and discussion

Identified chlorinated compounds in human milk

Following HaloSeeker, a chemical formula for all halogenated suspects was generated using MassHunter as shown in Table S1. In total, targeted MS/MS of 38 tentative chlorinated suspects was conducted and their MS/MS spectra were imported into SIRIUS for structure elucidation, with their most probable identity (highest SIRIUS matching score) shown in Table S2. Different chlorinated suspects related to pharmaceuticals, antibacterial agents, plastic production or metabolites of organochlorine pesticides were detected. Following SIRIUS, analytical standards were purchased to confirm the presence of selected chemicals of interest including propanil, chloroxylenol, triclocarban, bumetrizole, 8-hydroxyefavirenz and 4-hydroxy-2,5,6-trichloroisophthalonitrile (Table 1 and Figures S1-S6, A and B). The detected clusters using HaloSeeker corresponding to chloroxylenol, triclocarban, bumetrizole and 4-hydroxy-2,5,6-trichloroisophthalonitrile in one batch of human milk are also highlighted in a H/Cl-scale mass defect plot (Figure 1). Other chlorinated suspects with confidence levels 2 b are shown in Figures S7, S8A, B and S9 as well as Table S2.

Propanil is an herbicide widely used for the treatment of various grasses, broad-leaved weeds, potatoes, rice, and wheat. It can be absorbed through ingestion or inhalation.[34] In the body, propanil is generally metabolized into 3,4-dichloroaniline or 3,4-dichloro phenyl hydroxylamine, which are then excreted.[35,36] Propanil was detected exclusively in South African human milk. The detection of propanil in human milk is significant as it suggests that residues of the herbicide can be transferred to human milk without undergoing metabolism. In terms of toxicity, a study using an *in vitro* kidney model reported that propanil can lead to nephrotoxicity.[37] Concerning human health, while other studies suggest that acute exposure to propanil may lead to central nervous system depression and methemoglobinemia, further research is needed to specifically assess its impact on the health of breastfeeding infants.[38,39] To our knowledge, neither propanil nor its metabolites have been reported in previous human milk studies highlighting the need for their regular monitoring to ensure the safety of both mothers and breastfeeding infants.

Chloroxylenol, which is a biocide against bacteria, algae and fungus, was identified in both South African and Montreal human milk. Chloroxylenol is used in cleaning fluids in medical facilities and institutions, and also in soaps, skin disinfectants and various topical medications.[40] Chloroxylenol is metabolized mainly in the liver and can undergo glucuronidation or sulfation leading to its excretion.[41] While certain studies have suggested that chloroxylenol may be toxic to aquatic lives and mammalian testes,[40,42-44] other studies have suggested that human exposure to a small amount of chloroxylenol may not lead to observable adverse effects.[41,45] Nonetheless, additional toxicity assessments are warranted to evaluate the safety of chloroxylenol and its conjugated forms in human milk, given the limited understanding of their presence and potential impacts on both mothers and breastfeeding infants.

Similar to chloroxylenol, triclocarban (TCC) is an antibacterial agent used in bar and liquid soaps and body washes. To our knowledge, only one study from China has reported the presence of triclocarban in human milk.[46] High doses of triclocarban have been associated with liver and kidney toxicity, as well as immune system dysfunction.[47-49] An *in vivo* study in rats demonstrated that prolonged exposure to triclocarban during pregnancy and lactation can result in developmental toxicity in the offspring.[50] Furthermore, triclocarban may exhibit endocrine-disrupting activity by enhancing androgen receptor-mediated transcriptional activity induced by native androgens, which may potentially affect the health of breastfeeding infants.[51,52] TCC can undergo hydroxylation in the body, and be converted into its hydroxylated forms, namely, 2'-hydroxytriclocarban (2′-OH-TCC), 3′-hydroxytriclocarban (3′-OH-TCC) and 6-hydroxytriclocarban (6-OH-TCC).[47] Following hydroxylation, TCC can undergo glucuronidation or sulfation and be converted into TCC-N-glucuronide or sulfate conjugates of 2′-OH-TCC or 3′-OH-TCC.[53,54] In our results, a peak corresponding to the tentative sulfated 2′-OH-TCC was detected in South African samples (Table S2 and Figure S10A), with its MS/MS spectra along with its SIRIUS score shown in Figure S10B. In the absence of analytical standard, further investigations are needed to fully confirm the identity of this conjugated TCC suspect.

Bumetrizole (UV-326), which is a chlorinated ultraviolet filter, is incorporated into various plastics, including polyvinyl chloride, polystyrene and polycarbonate.[55] Bumetrizole was identified in human milk from South Korea with concentrations ranging from below the limit of quantification up to 53.1 ng/g lipid weight.[56] While studies have suggested that exposure to UV filters such as bumetrizole may affect lipid metabolism, disrupt hormonal

Table 1. Confirmed chlorinated contaminants in human milk samples from Vhembe ($n = 194$), Pretoria ($n = 193$) and Montreal ($n = 207$) (confidence level 1).

Highest abundant isotope ($[M - H]^-$, m/z)	Formula	Human milk (Detected in Vhembe, Pretoria or Montreal)	Detection frequency % (HaloSeeker)	Confirmed Identity	CAS registry number	Previously reported in human milk elsewhere
215.998	$C_9H_9Cl_2NO$	Vhembe and Pretoria	Vhembe: 1 Pretoria: 1 Montreal: ND	Propanil	709-98-8	NA
155.026	C_8H_9ClO	All	Vhembe: 12 Pretoria: 3 Montreal: 1	Chloroxylenol	88-04-0	NA
312.9702	$C_{13}H_9Cl_3N_2O$	Vhembe and Pretoria	Vhembe: 3 Pretoria: 1 Montreal: ND	Triclocarban (TCC)	101-20-2	China[46]
314.1060	$C_{17}H_{18}ClN_3O$	Vhembe	Vhembe: 1 Pretoria: ND Montreal: ND	Bumetrizole (UV-326)	3896-11-5	South Korea[56]
330.0145	$C_{14}H_9ClF_3NO_3$	Vhembe and Pretoria	Vhembe: 6 Pretoria: 4 Montreal: ND	8-Hydroxyefavirenz	205754-33-2	NA
244.908	$C_8HCl_3N_2O$	All	Vhembe: 47 Pretoria: 50 Montreal: 64	4-Hydroxy-2,5,6-trichloroisophthalonitrile	28343-61-5	France[20]

ND, non-detected; NA, not available.

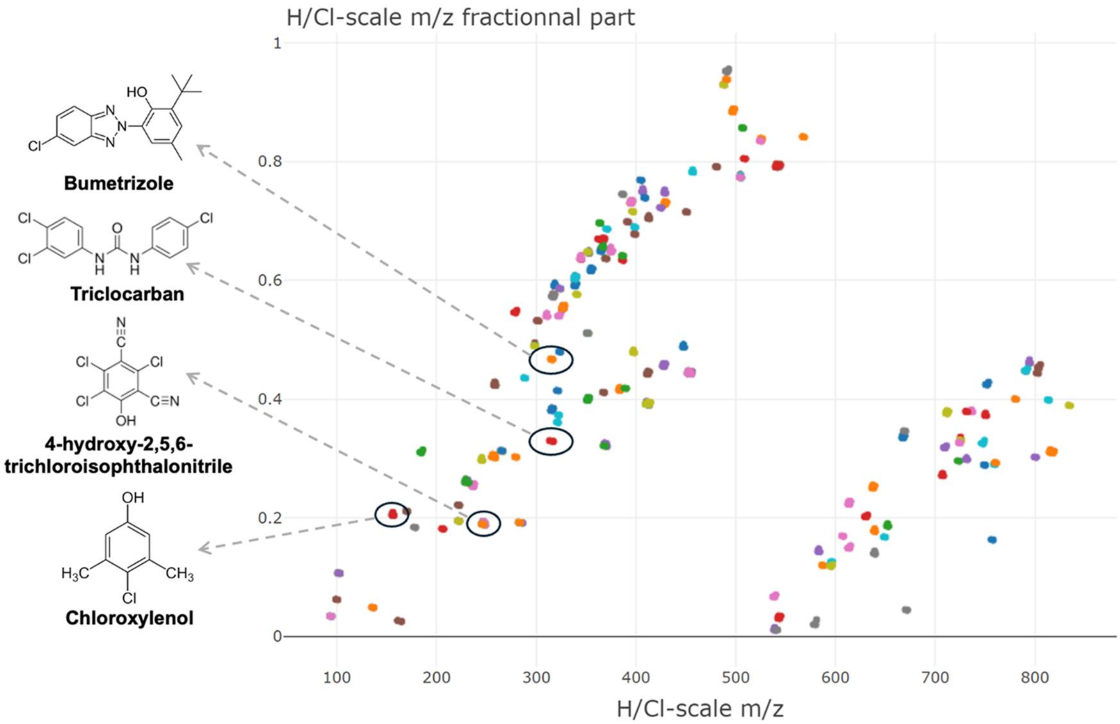

Figure 1. Example of H/Cl-scale mass defect plot (HaloSeeker) of a selected human milk batch with the highlighted clusters for 4 identified chlorinated contaminants. Blank filter set to the sum of the average signal intensity of the features plus 3 times the standard deviation of procedural blanks ($n = 3$)

balance, induce apoptosis and lead to inflammations, additional studies are needed to establish conclusive evidence regarding human health.[57,58] The presence of other ultraviolet filters was not investigated in this study.

Among the identified suspects, 4-hydroxy-2,5,6-trichloroisophthalonitrile (R182281) was detected in both South African and Montreal human milk samples. 4-Hydroxy-2,5,6-trichloroisophthalonitrile is the major metabolite of chlorothalonil, a fungicide commonly used on vegetables, fruits, and ornamental crops.[59] Studies suggest that 4-hydroxy-2,5,6-trichloroisophthalonitrile may exhibit greater environmental persistence and toxicity than its parent compound.[60-62] Further investigations are necessary to evaluate its potential health risks, especially in relation to infant exposure through breastfeeding.

One compound exclusively identified in South African human milk was 8-hydroxyefavirenz, the main metabolite of efavirenz. Efavirenz is an antiretroviral drug used in combination with other medications to treat human immunodeficiency virus infection (HIV). Metabolism of efavirenz in the human liver first involves oxidation into different hydroxylated metabolites such as 7- and 8-hydroxyefavirenz, followed by glucuronidation or sulfation of these species.[63,64] Additionally, 7- and 8-hydroxyefavirenz can undergo further oxidation to form dihydroxylated derivatives.[65] To our knowledge, while efavirenz has been previously detected in human milk,[66,67] reports on the presence of its identified metabolites in human milk are limited. The identification of 8-hydroxyefavirenz in milk samples from 2018–2019 suggests the use of efavirenz by South African mothers during or prior to these years. Studies have indicated that metabolites such as 8-hydroxyefavirenz are significantly more neurotoxic (up to 10 times more toxic in neuron cultures) than the parent compound.[68-70] Although efavirenz has largely been replaced by Dolutegravir since 2019, reducing the likelihood of its detection in current human milk samples, ongoing monitoring remains crucial due to reports of efavirenz being smoked in South Africa for its hallucinogenic and relaxing effects.[71]

Conclusion

In this study, different chlorinated contaminants used as pesticides, antibacterials, UV filters, or medications were detected in human milk using non-targeted analysis. The presence of previously unreported compounds, such as propanil, chloroxylenol and 8-hydroxyefavirenz, highlights the need for further investigation into other unknown halogenated contaminants. Expanding the scope of human milk biomonitoring is crucial to enhance our understanding of chemical exposure and its potential health implications.

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Author contributions

Zhi Hao Chi (Data curation [equal], Formal analysis [equal], Investigation [equal], Methodology [equal], Writing—original draft [equal], Writing—review & editing [equal]), Ronan Cariou (Conceptualization [equal], Data curation [equal], Funding acquisition [equal], Investigation [equal], Methodology [equal], Software [equal], Writing—review & editing [equal]), Solene Motteau (Data curation [equal], Software [equal], Writing—review & editing [equal]), Bruno Le Bizec (Project administration [equal], Resources [equal], Writing—review & editing [equal]), Gaud Dervilly (Project administration [equal], Resources [equal], Writing—review & editing [equal]), Lan Liu (Formal analysis [equal], Methodology [equal], Writing—review & editing [equal]), Jingyun Zheng (Formal analysis [equal], Methodology [equal], Writing—review & editing [equal]), Lei Tian (Formal analysis [equal], Methodology [equal], Writing—review & editing [equal]), Jonathan Chevrier (Conceptualization [equal], Funding acquisition [equal], Project administration [equal], Resources [equal], Writing—review & editing [equal]), Riana Bornman (Funding acquisition [equal], Methodology [equal], Project administration [equal], Resources [equal], Writing—review & editing [equal]), Muvhulawa Obida (Investigation [equal], Resources [equal], Writing—review & editing [equal]), Cindy Goodyer (Conceptualization [equal], Methodology [equal], Project administration [equal], Resources [equal], Writing—review & editing [equal]), Bernard Robaire (Funding acquisition [equal], Methodology [equal], Resources [equal], Writing—review & editing [equal]), and Barbara F. Hales (Funding acquisition [equal], Methodology [equal], Project administration [equal], Resources [equal], Supervision [equal], Writing—review & editing [equal]), Stephane Bayen (Conceptualization [equal], Funding acquisition [equal], Methodology [equal], Project administration [equal], Supervision [equal], Writing—review & editing [equal])

Supplementary material

Supplementary material is available at *Exposome* online.

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Conflicts of interest

None declared.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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