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Organic Contaminants in Human Breast Milk Identified by Non-Targeted Analysis

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Abstract

Understanding the health implications of human exposure to mixtures of chemical contaminants is aided by analytical methods that can screen for a broad range of both expected and unexpected compounds. We performed a proof-of-concept analysis combining human breast milk, a biomonitoring matrix for determining contaminant exposure to mothers and infants, with a non-targeted method based on comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC/TOF-MS). A total of 172 presumably anthropogenic halogenated compounds and non-halogenated cyclic and aromatic compounds were tentatively identified in breast milk from San Diego, California through mass spectral database searches. Forty of the compounds were prioritized for confirmation based on halogenation or 100% frequency of detection, and the identities of 30 were verified using authentic standards. Thirty-four (85%) of the prioritized contaminants are not typically monitored in breast milk surveys, and 31 (77%) are regulated in at least one market worldwide, indicating breast milk may be a useful biomonitoring matrix for non-targeted analysis and the assessment of human exposure to future emerging or undiscovered contaminants.

Graphical Abstract

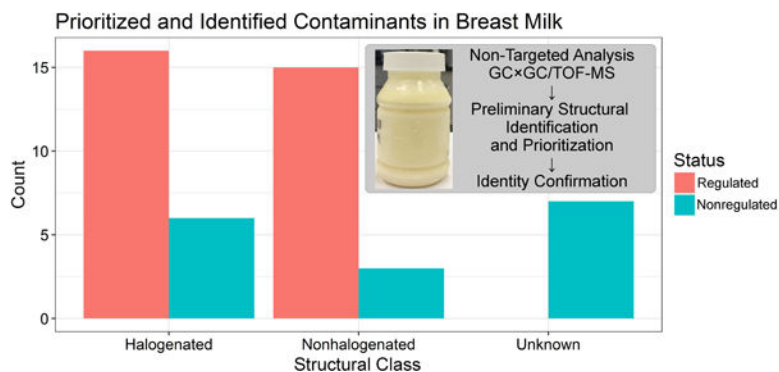
The picture was taken by Nathan Dodder.

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The authors declare no competing financial interest.



Keywords

Organic contaminants; human breast milk; non-targeted analysis; GC×GC/TOF-MS; biomonitoring

INTRODUCTION

Measurements of the human exposome aim to assess environmental exposure and associated risks in their entirety¹. Within this context, the uptake of exogenous lipophilic chemicals by lactating women will result in contaminant elimination via the fatty portion of milk, and since the 1950s breast milk has been used as a biomonitoring matrix to assess human contaminant exposure, the mother's internal dose, pre-natal exposure, and transfer to the infant through breast feeding^{2–4}. Although breast-feeding has well-established benefits (for both mothers and infants) and is recommended^{5,6}, breast milk contaminants, including biotransformation products, are concerns for infant and children's health^{7–9}. Contaminant detection in breast milk also serves as an indicator of general human exposure¹⁰. Prior breast milk contaminant surveys have detected multiple classes of compounds, including persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) and organochlorine pesticides^{11,12}; components of personal care products⁴ including cosmetic UV filters, phthalates, fragrances and parabens; bisphenol A¹³; and polycyclic aromatic hydrocarbons (PAHs)¹¹.

Historically, the analysis of breast milk has been key to the investigation of unexpected environmental contaminants. In 1973–1974, cattle feed in Michigan was erroneously mixed with the flame retardant polybrominated biphenyl (PBB), severely affecting the health of cattle and subsequently contaminating meat and dairy products from exposed farms¹⁴. The farms were quarantined, and it was initially thought human exposure was limited to individuals living on the farms or directly receiving their products. However, during a pesticide screening of breast milk in 1976, it was discovered that mothers from the general population of Michigan were exposed to PBB¹⁵, and subsequent work confirmed PBB had entered the food supply^{16,17}. In another case, temporal trend surveys of Swedish breast milk collected between 1972–1997 were among the first studies to indicate widespread and increasing human exposure to another brominated flame retardant, polybrominated diphenyl ether (PBDE)^{18,19}. These PBDE breast milk studies were among the initial investigations

that lead to a multitude of occurrence and toxicological studies, and the eventual reductions of PBDE production and use approximately 10 years later²⁰.

Breast milk contaminant analyses are typically targeted (the compounds of interest are pre-determined)², and will miss unexpected compounds regardless of their abundance²¹. Unexpected contaminants in this and other matrices are usually identified in an ad hoc manner, which may result in the widespread environmental occurrence and increasing environmental concentration of compounds missed by routine targeted contaminant screening. In contrast, non-targeted analysis is a relatively new class of full-scan mass spectrometry based methods that aim to systematically identify both known and unknown compounds. For example, non-targeted analysis has been used to identify contaminants in inland waters^{22,23} and marine mammal blubber^{24–28}. It has also been integrated with toxicological investigations to identify and prioritize chemicals of interest^{29–31}. Mass spectrometry based non-targeted analysis of environmental contaminants has been developed using different types of instruments^{23,29}. In the present study, we apply non-targeted analysis based on comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC/TOF-MS) to determine the number and identity of unexpected contaminants in human breast milk. GC×GC provides improved chromatographic separation compared to one-dimensional GC, and is advantageous for the analysis of complex matrices³². The current study is a proof-of-concept test of non-targeted mass spectrometry for contaminant biomonitoring.

METHODS

Breast Milk Collection.

Breast milk was collected from three individuals who gave birth at the University of California San Diego hospital in November 2011. Upon discharge, the breast milk was donated for scientific research by the mother and stored at $-20\text{ }^{\circ}\text{C}$. The milk was received frozen with no identifying information and was stored at $-20\text{ }^{\circ}\text{C}$ until sample preparation.

Sample Preparation.

The extraction and cleanup procedure is summarized in Figure S1, and details are provided in supporting information-1 (SI-1). Each milk sample was 16 mL, and was first separated into lipid and water portions. Each portion underwent a specific cleanup procedure, resulting in two lipid fractions with differing extraction polarity (via silica solid phase extraction with hexane/dichloromethane, and dichloromethane) and one water extract. Each of the three final fractions per sample were individually analyzed. In total, three breast milk samples were processed to give 9 fractions. Each breast milk sample was coupled with a procedural blank.

Instrumental Analysis and Initial Compound Identifications.

Non-targeted analysis was performed using a Pegasus® 4D GC×GC/TOF-MS (LECO, St. Joseph, MI, USA) with the instrumental parameters described in Table S1. Compounds were tentatively identified using LECO® ChromaTOF® software (version 4.43.3.0) through comparison of the experimental spectra against the 2011 NIST Mass Spectral Library and a

series of screening criteria, as outlined in Figure 1. First, peaks with a signal to noise ratio (S/N) ≥ 50 and a 1st dimension retention time that of naphthalene (806 s) were selected and searched against the mass spectral library. Compounds identified as halogenated were manually reviewed for spectral similarity to the library match; in particular, the presence of halogenated isotopic patterns and fragments due to halogen loss. This chemical information is not incorporated in the similarity score³³, and for this reason a strict similarity score threshold was not used for halogenated compounds. Spectra matching a non-halogenated compound with an aromatic or cyclic structure were also selected for further evaluation using two criteria: 1) the spectral similarity or reverse similarity was ≥ 700 out of 999; and 2) the spectrum contained at least three identifying ions of relatively high intensity that matched the mass spectral library hit, with at least one ≥ 100 m/z. The average similarity and reverse similarity (the match factor when excluding experimental spectrum peaks that were not in the library spectrum) scores for all halogenated and non-halogenated compounds meeting these criteria were 849 and 871, respectively. Spectra initially identified as halogenated that did not have a matching library record were classified as halogenated unknowns. Finally, the intensity of the identified compounds (including unknowns) had to be at least three times greater than that found in the corresponding procedural blank.

Identification Uncertainty and Confirmation with Authentic Standards.

Tentatively identified compounds were prioritized for confirmation by authentic standards. Using system previously described in Hoh et al. (2012)²⁴, we categorized identifications within one of four categories, with the category names and number of compounds in brackets: 1) The experimental mass spectrum and retention times (within a modulation time of 3.5 s in the 1st GC dimension and ± 0.05 s in the 2nd) were matched to those of a reference standard analyzed under the same conditions [authentic MS RT]. 2) The experimental spectrum, but not the retention times, was matched to a reference standard, indicating the experimental spectrum is that of an isomer [authentic MS]. 3) The experimental spectrum was matched to one within the NIST Electron Ionization (EI) Mass Spectral Library [reference database MS] (the 2011 NIST EI Mass Spectral Library contains 220,460 spectra of 192,108 unique compounds). 4) The experimental spectrum was identified as belonging to a halogenated compound, but the chemical structure could not be further identified [unknown].

Detection limits were estimated by analyzing standard solutions at 0.1, 1, 10, 100, and 1000 ng/mL. The nine standard compounds represented three classes. 1) Benzophenone, 2-methylindole, and ethyl 4-ethoxybenzoate were non-halogenated aromatic compounds at relatively high concentrations in all three mothers' samples. 2) BDE-47, *p,p'*-DDE, and PCB-153 were legacy halogenated persistent organic pollutants (DDE = dichlorodiphenyldichloroethylene and PCB = polychlorinated biphenyl). 3) 4,4'-Dichlorodiphenylsulfide, N-(4-chlorophenyl)formamide, and 4-chloro-N-methylaniline were non-legacy halogenated organic compounds at relatively high concentration in the mothers' samples. The lowest concentration at which the compounds gave a sufficient mass spectrum for identification was 10 ng/mL, except for 2-methylindole and N-(4-chlorophenyl)formamide, for which the lowest concentration was 100 ng/mL. These values

correspond to original sample concentrations of approximately 0.2 ng/mL whole milk and 2 ng/mL whole milk, respectively.

The log K_{OW} and water solubility for each compound was determined using the Estimations Programs Interface for Windows (EPI Suite) software³⁴. Regulatory information was obtained using SciFinder's >347,000 compound Regulated Chemicals Listing³⁵, a database of international lists such as high production volume chemicals, priority chemicals, and pollutant release inventories. Detailed description can be found in SI-1.

RESULTS & DISCUSSION

We used a non-targeted analytical method to identify 1) halogenated compounds and 2) non-halogenated cyclic and aromatic compounds in human breast milk collected in San Diego, California. Halogenated compounds are typically the most common targets in breast milk contamination surveys due to their production volumes, environmental persistence, lipophilic nature, and concerns regarding health impacts³⁶. However, it has been proposed that current targeted methods may be missing uninvestigated anthropogenic halogenated contaminants³⁷. Non-halogenated cyclic and aromatic contaminants in breast milk are currently less frequently investigated, however the targeted detection of non-halogenated UV-filters, parabens, and musks in breast milk⁴ suggested that non-targeted analysis may identify other non-halogenated contaminants. Our data analysis procedure excluded non-halogenated aliphatic compounds because they are likely endogenous, and their electron impact fragmentation frequently results in non-specific mass spectra.

In total, 172 presumed anthropogenic contaminants were preliminarily identified among the three breast milk samples. The identifications across all samples and fractions are listed in SI-2. Twenty-four compounds were halogenated organic compounds, 141 were non-halogenated cyclic or aromatic organic compounds, and 7 were unknown halogenated compounds (the mass spectrum indicated the compound was halogenated, but a match was not found in the mass spectral library and the structure could not be determined manually). Sixteen of the tentatively identified compounds had a total of 37 isomers. The experimental and matching reference library spectra for all tentative identifications is provided in SI-3. Eight tentatively identified phthalic anhydrides were excluded from the count of 172 because they may rapidly hydrolyze in water³⁸, and are therefore unlikely to exist in breast milk. Unknown reagents may have transformed to the anhydrides in the GC injection port.

The 172 tentatively identified compounds (that were matched only to a reference library spectrum) were prioritized for confirmation by authentic standards using two criteria. 1) All tentatively identified halogenated compounds were selected. 2) Non-halogenated cyclic or aromatic compounds that were present in all three breast milk samples were selected. In total, 22 halogenated and 18 non-halogenated cyclic or aromatic compounds met the prioritization criteria. Table 1 shows all prioritized compounds with the column definitions as follows: CAS is the Chemical Abstracts Service Registry Number; Regulatory Status indicates if the compound is on a regulatory list catalogued by SciFinder; Additional Isomers indicates the additional number of compounds that share the same mass spectrum as the listed chemical, but have different retention times; ID Category is the chemical

identification category specified in the Methods Section; Breast Milk Sample is the number of detects among the three milk samples; Lipid Fraction is the combined number of detects in the two lipid fractions (3 mothers * 2 lipid fractions = 6 samples total); and Water Fraction is the number of detects in the water fraction (3 samples total). 1,7-Dimethylnaphthalene and 1,4,5-trimethylnaphthalene were prioritized because one of their isomers were detected in all three breast milk samples (see below).

Thirty-two authentic standards were used to verify the identities of 30 of the 40 prioritized compounds (Table 1 ID Category = 1 [authentic MS RT]); a verification success rate of 94%. The remaining 2 authentic standards matched the mass spectra but not the retention time of the corresponding breast milk contaminants, indicating they were isomers or a different chemical structure with the same mass spectrum. (Table 1 ID Category = 2 [authentic MS]). Authentic standards could not be obtained for 8 of the prioritized compounds (Table 1 ID Category = 3 [reference database MS]). Seven additional mass spectra that were halogenated based on the presence of characteristic bromine and/or chlorine isotopic patterns, but did not have a matching spectrum in the NIST EI Library, were classified as unknown halogenated compounds. Their mass spectra are provided in SI-3. Two of the unknown halogenated compounds were found in two of three mothers' samples (Unknown # 2 and Unknown #5), the other unknown halogenated compounds were found in one of three mothers' samples.

We detected the common breast milk targets *p,p'*-DDT, *p,p'*-DDE, PCB-153, BDE-47, HCB, and *beta*-HCH (DDT = dichlorodiphenyltrichloroethane, HCB = hexachlorobenzene, and HCH = hexachlorocyclohexane). We did not, however, detect other legacy or pharmaceutical and personal care product contaminants that have been measured in targeted studies, such as multiple PCB and PBDE congeners, and other organochlorine pesticides^{4,11,12,39}. This is likely due to the detection limit of the non-targeted method. Assuming a whole milk density of 1.031 g/mL⁴⁰, and an average lipid content of 3.5%⁴¹, our estimated range of detection limits was approximately 5 to 50 ng/g lipid. This range is higher than the concentration of many individual compounds typically quantified in breast milk surveys^{4,11,12,39}. It also indicates the concentrations of compounds detected by the non-targeted analysis may be higher than those of the typically monitored contaminants.

We applied the non-targeted GC×GC-TOF/MS method to the analysis of contaminants in the lipid and water fractions of breast milk, where lipid fraction refers to the combined hexane/DCM and DCM silica SPE extracts of the lipid portion of whole milk. The frequency of detection in each fraction is shown in Table 1. A majority of the 40 prioritized detections were in the lipid phase only (n = 19), perhaps due a greater proportion of lipophilic vs. water soluble compounds in whole milk, and/or instrumental detection bias towards non-polar compounds. The other compounds were detected in the both the lipid and water phases (n = 17) and water phase only (n = 4). Of the 172 total contaminants detected (prioritized plus non-prioritized), 44 were detected in the water phase only (26%). This indicates the water phase may be a significant matrix for breast milk contaminant measurements, perhaps with the addition of derivatization methods for GC based analysis, or liquid chromatography/tandem mass spectrometry. This finding was reinforced by comparison among the predicted or measured log K_{OW} of the contaminants identified in each fraction (Figure 2), where smaller log K_{OW} values indicates greater water solubility

(Figure 3). The maximum log K_{OW} among contaminants detected in the lipid fraction only was 7.6, in both the lipid and water fraction was 5.6, and in the water fraction only was 3.2.

The six persistent organic pollutants *p,p'*-DDT, *p,p'*-DDE, PCB-153, BDE-47, HCB, and *beta*-HCH were the only contaminants regularly measured in breast milk surveys. The other 34 prioritized contaminants (85%) are not typically monitored. Thirty-one of the 40 prioritized chemicals (77%) are regulated in at least one market worldwide. Presence on a regulatory listing indicates the compound is produced in significant quantities and has risk-associated properties. However, the exact source of the chemicals to the subjects in this study is unknown, and we cannot exclude the possibility that a chemical may have an alternate natural source or is a transformation product. Since chemical concentrations were not quantified by the non-targeted identification method, we did not further assess the risk of these compounds.

Two PAH identifications were verified with authentic standards: 1,7-dimethylnaphthalene and 1,4,5-trimethylnaphthalene (Table 1). Apparent isomers of both compounds (with identical mass spectra but differing retention times) were also observed. In total, dimethylnaphthalene had three isomers and trimethylnaphthalene had four isomers. Exposure to PAHs is usually determined through measurement of urinary phase 1 hydroxy-PAH metabolites⁴²; however, targeted measurements have found unmetabolized PAHs in human blood³⁴ and breast milk^{11,43–45}. The two PAHs we identified were not reported in these prior targeted analyses.

In conclusion, results of this study indicate GC×GC-TOF/MS non-targeted analysis of breast milk is capable of comprehensively identifying unexpected exogenous chemical exposure to the mother and infant. Non-targeted analysis is an initial step in assessing a broad range of contaminant exposure. Future related work is to 1) further increase the sensitivity of detection ; 2) evaluate the risk of exposure through expanded occurrence measurements and toxicological assessment; and 3) determine if the unknown spectra frequently occur in larger sample sets, and if so, identify and further investigate these compounds as emerging contaminants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- breast milk as a useful biomonitoring matrix for non-targeted analysis
- successful screening, prioritization, and identification via nontargeted analysis
- A total of 172 anthropogenic halogenated and non-halogenated cyclic and aromatic compounds identified in breast milk by non-targeted analysis
- 85% of 40 prioritized contaminants are not typically monitored in breast milk surveys
- Thirty compounds out of 32 prioritized compounds were matched with their corresponding authentic standards (at 94% verification success rate achieved)

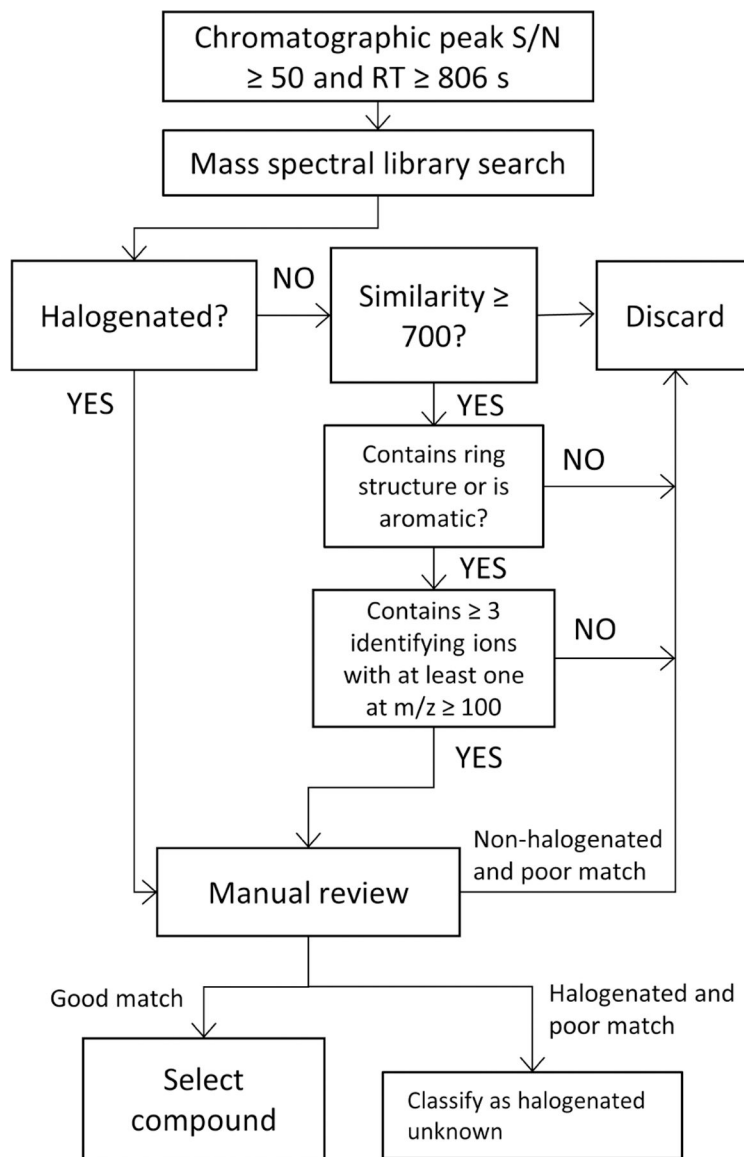


Figure 1. Initial contaminant identification criteria. RT = retention time. Identities of selected compounds were then confirmed with authentic standards. *Good match = 1) For halogenated compounds, the halogenated isotopic clusters should match those in the corresponding library mass spectrum, and loss of halogen must be observed in the mass spectrum. For non-halogenated compounds, the pattern (m/z values and relative abundances) of the 3 most prominent ions should match that of the corresponding library mass spectrum.*

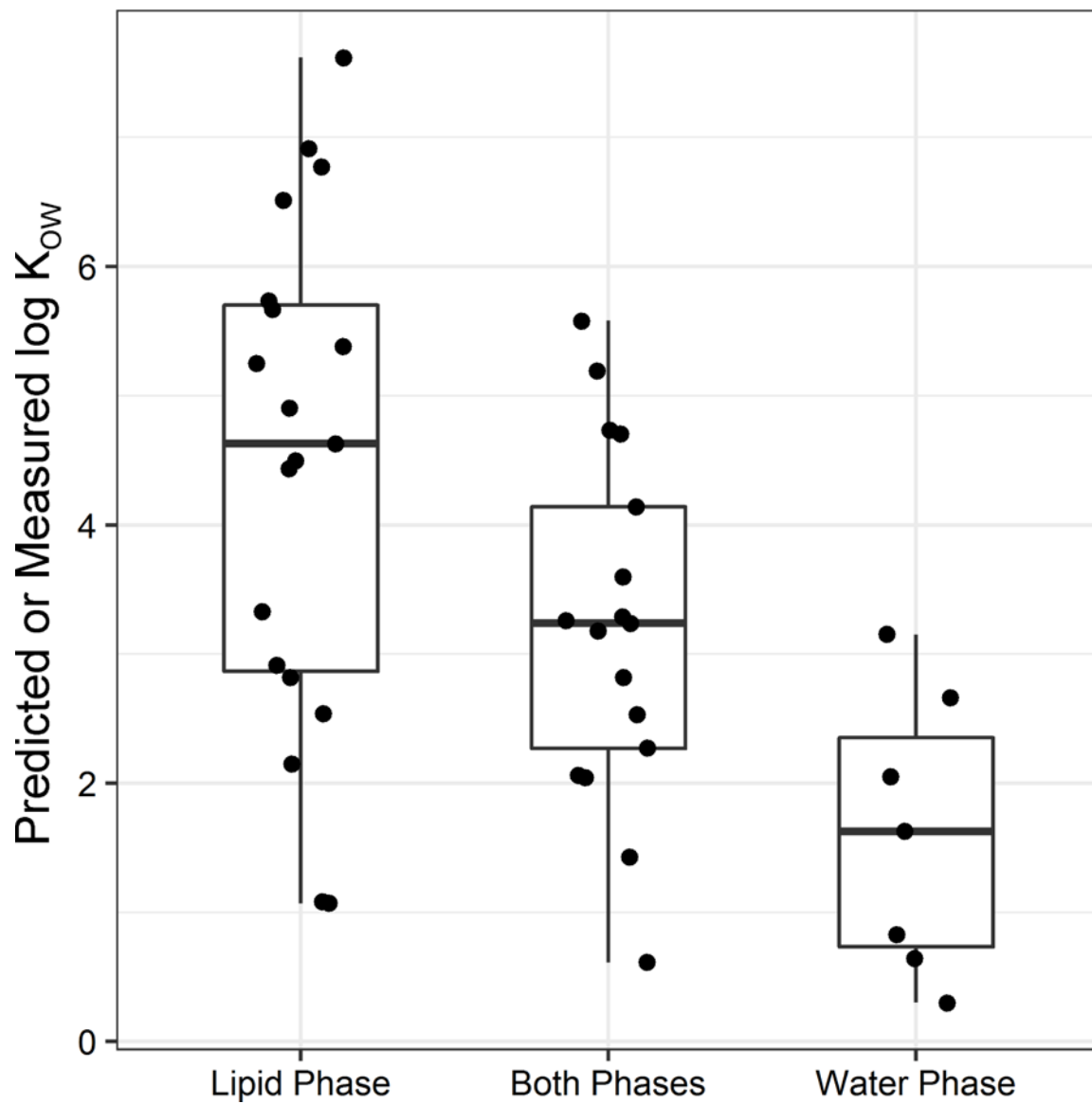


Figure 2. Logarithmic octanol-water partition coefficients ($\log K_{OW}$) for all prioritized breast milk contaminants listed in Table 1, plus the three additional water phase compounds identified in 2 of 3 samples (to increase from $n = 3$ to $n = 6$ in this category).

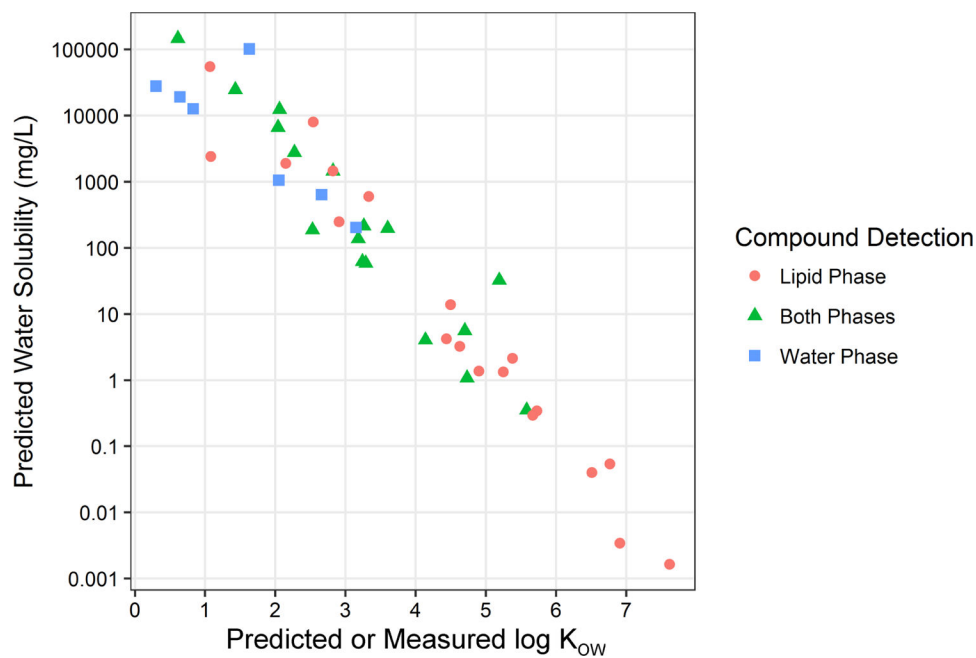


Figure 3. Logarithmic octanol-water partition coefficient ($\log K_{ow}$) vs. water solubility (mg/L) at 25 °C for the contaminants listed in Table 1, plus three additional water phase compounds as discussed in the text. The point shape and color indicates the phase in which the compound was detected (see legend).

Table 1.

Contaminants identified in human breast milk by non-targeted analysis. Det. Freq. = Detection Frequency.

Name	CAS	Regulatory Status	Additional Isomers	ID Category	Breast Milk Sample Det. Freq. (Out of 3)	Lipid Fraction 1 Det. Freq. (Out of 3)	Lipid Fraction 2 Det. Freq. (Out of 3)	Water Fraction Det. Freq. (Out of 3)
Halogenated Compounds								
1-Chloro-3-dimethylaminobenzene	6848-13-1	Regulated		1 [authentic MS RT]	3	0	2	1
4-Chloro-N-methylaniline	932-96-7	Regulated	1	1 [authentic MS RT]	3	3	2	2
N-(4-Chlorophenyl)formamide	2617-79-0	Not regulated		1 [authentic MS RT]	3	2	1	3
p-Chlorophenyl methyl sulfoxide	934-73-6	Regulated		3 [reference database MS]	3	0	3	0
1-(4-Chlorophenyl)pyrrole	5044-38-2	Not regulated		1 [authentic MS RT]	2	1	1	1
4,4'-Dichlorodiphenylether	2444-89-5	Regulated		1 [authentic MS RT]	2	1	1	0
4,4'-Dichlorodiphenylsulphide	5181-10-2	Regulated		1 [authentic MS RT]	2	1	2	1
4-Chlorodiphenyl ether	7005-72-3	Regulated		1 [authentic MS RT]	2	1	1	1
4-Chlorothioanisole	123-09-1	Regulated		1 [authentic MS RT]	2	1	2	1
HCB	118-74-1	Regulated		1 [authentic MS RT]	2	2	0	0
<i>beta</i> -HCH	319-85-7	Regulated		1 [authentic MS RT]	1	1	0	1
BDE-47	5436-43-1	Regulated		1 [authentic MS RT]	1	1	0	0
PCB-153	35065-27-1	Regulated		1 [authentic MS RT]	1	1	0	0
3'-Chloroacetanilide	588-07-8	Regulated		1 [authentic MS RT]	1	1	0	0
4-Chlorobutyrophenone	939-52-6	Regulated		1 [authentic MS RT]	1	0	1	0
7-Chloroquinaldine	4965-33-7	Regulated		1 [authentic MS RT]	1	0	1	0
<i>p,p'</i> -DDE	72-55-9	Regulated		1 [authentic MS RT]	1	1	0	0
2-Chloro-N,N-dimethylaniline	698-01-1	Not regulated		2 [authentic MS]	1	1	0	0
<i>p,p'</i> -DDT	50-29-3	Regulated		1 [authentic MS RT]	1	1	0	0
3-Chloro-2,6-dimethylpyridine	2405-06-3	Not regulated	1	3 [reference database MS]	1	1	0	0
4-Formylphenyl 3-chloropropanoate	NA	Not regulated		3 [reference database MS]	1	0	0	1

Name	CAS	Regulatory Status	Additional Isomers	ID Category	Breast Milk Sample Det. Freq. (Out of 3)	Lipid Fraction 1 Det. Freq. (Out of 3)	Lipid Fraction 2 Det. Freq. (Out of 3)	Water Fraction Det. Freq. (Out of 3)
Thiophene-2-carbonitrile, 5-tert-butyl-3-(4-chlorobenzylideneamino)-	NA	Not regulated		3 [reference database MS]	1	0	1	0
Non-Halogenated Aromatic or Cyclic Compounds								
1,3-Diacetylbenzene	6781-42-6	Regulated	1	1 [authentic MS RT]	3	3	2	1
2,4-Di-tert-butylphenol	96-76-4	Regulated	1	1 [authentic MS RT]	3	1	1	1
2-Cyanobenzoic acid	3839-22-3	Not regulated		1 [authentic MS RT]	3	1	2	0
2-Hydroxymethylbenzoic acid	612-20-4	Regulated		1 [authentic MS RT]	3	2	2	1
2-Methylindole	95-20-5	Regulated		1 [authentic MS RT]	3	2	0	3
4-Methylbiphenyl	644-08-6	Regulated	2	1 [authentic MS RT]	3	3	2	0
Benzophenone	119-61-9	Regulated		1 [authentic MS RT]	3	2	2	3
Benzyl butyl phthalate	85-68-7	Regulated		1 [authentic MS RT]	3	3	0	1
Ethyl 4-ethoxybenzoate	23676-09-7	Regulated		1 [authentic MS RT]	3	2	3	2
Isatin	91-56-5	Regulated		1 [authentic MS RT]	3	0	0	3
2,4-Dimethylpropiophenone	35031-55-1	Regulated		2 [authentic MS]	3	1	1	1
1,1,6-Trimethyltetralin	475-03-6	Regulated		3 [reference database MS]	3	3	0	0
5H-1-Pyridine	270-91-7	Not regulated		3 [reference database MS]	3	3	0	3
5-Methyltetralin	2809-64-5	Not regulated	1	3 [reference database MS]	3	2	3	0
N-(2-acetylphenyl)formamide	5257-06-7	Regulated		3 [reference database MS]	3	0	0	3
Phenylamide	55-21-0	Regulated		1 [authentic MS RT]	3	0	0	3
1,7-Dimethylnaphthalene	575-37-1	Regulated	2	1 [authentic MS RT]	2	2	2	0
1,4,5-Trimethylnaphthalene	2131-41-1	Regulated	3	1 [authentic MS RT]	1	0	1	0