Exposome, 2026, **6(1)**, osag001
https://doi.org/10.1093/exposome/osag001
Advance Access Publication Date: 9 February 2026
Research Article

Dried milk spots: a viable approach for assessing the chemical exposome in mothers and their infants by targeted LC-MS/MS

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Abstract

Breast milk is a key matrix for assessing early-life exposure. Dried milk spots (DMS) and microsampling devices are convenient low-volume sampling alternatives. Here, a sample preparation protocol and LC-MS/MS method for (semi-)quantitatively assessing 216 xenobiotics in DMS were optimized and evaluated. Two extraction solutions were compared. Both approaches performed similarly, with about 50% of analytes falling within the assigned acceptance range for matrix effects (60%–140%), and about 80% fulfilling the proposed extraction recovery criteria (42%–134%). In a proof-of-principle study, the method was applied to a pooled Austrian milk sample as well as to the NIST standard reference material SRM 1954 (pooled breast milk from US donors). A total of 30 exposure compounds were identified in SRM 1954, 22 of which were also determined in the Austrian pooled milk sample. Compounds were mostly detected at very-low trace levels and included air pollutants (cotinine), plastics-related chemicals (phthalates, bisphenols), flame retardants (TBBPA, TCBPA), perfluoroalkyl substances (PFOA, PFOS), personal care products ingredients (parabens) and pharmaceuticals (acetaminophen, fluconazole). The stability of analytes was assessed in DMS at −20, 4, 18 and 37°C for up to 2 months. No significant changes were observed during storage at −20°C regardless of storage time, while short-term stability was confirmed for approximately 80% of all tested exposure chemicals even at more elevated temperatures. A comparison between DMS and Mitra volumetric absorptive microsampling devices showed similar performance but differences in background contamination. Of the 24 compounds detected in the paper blank, 19 were also present in the Mitra tips, though at concentrations up to ten times lower. The developed assay is fit-for-purpose, enabling broad exposome-type population studies for investigating early-life exposure patterns.

Key words: exposomics; human biomonitoring; public & environmental health; sample stability; early life exposure.

Highlights

- A sample preparation protocol for 216 xenobiotics in breast milk was optimized.
- A total of 30 compounds were reliably detected and quantified in DMS-based NIST SRM 1954 including flame retardants, PFAS and air pollutants.
- Satisfactory compound stability across three time points and four temperature conditions could be shown for most analytes.
- Volumetric absorptive microsampling device was compared to conventional paper spots, demonstrating comparable method performance.

Introduction

In 2005, C. Wild introduced the concept of the exposome, defined as the totality of endogenous and exogenous environmental influences acting on an individual from conception onwards.[1] Compared to the genome, which remains relatively stable over a person's lifetime, the exposome is highly dynamic regarding both its chemical composition and concentration levels.[2] Exposure to natural and synthetic chemicals mostly occurs via ingestion, dermal contact or inhalation, with common sources including diet, use of prescription drugs and personal care products and exposure to ambient air pollution, among others.[3] By assessing the internal exposure, conclusions can be drawn about the presence of chemicals or their metabolites of various sources.[4,5]

The importance of early-life developmental factors shaping long-term health outcomes has also been emphasized by the

Received: October 3, 2025; **Revised:** December 18, 2025; **Accepted:** January 6, 2026

Developmental Origins of Health and Disease (DOHaD) concept, introduced by Barker and colleagues.[6] While DOHaD does not directly address chemical exposure, it links prenatal and early-life conditions, such as low birth weight, to increased risk of diseases later in life, including coronary heart disease. In the same direction, different studies have linked early life chemical exposure to adverse health effects in later stages of development. For instance, previous works have reported, the presence of per- and polyfluoroalkyl substances (PFAS),[7] pesticides,[8] mycotoxins,[9] parabens,[10,11] plasticizers,[12,13] pharmaceuticals and phytoestrogens[14,15] in human breast milk, all with potential implications on neonates' health. While many of these substances have been associated with adverse developmental outcomes, such as preterm birth[16] or low birth weight,[17] their presence in breast milk raises concerns about postnatal effects, including impacts on the infant gut microbiota[18] and possible long-term effects such as increased risks for depression or diabetes.[19,20] Importantly, exposure to these chemicals is typically lower in breast milk than in alternative food sources.

Breast milk should be the primary source of nutrients for newborns, at least during the first 6 months, for which the World Health Organization (WHO) recommends exclusive breastfeeding.[21,22] It is a complex matrix whose composition is not only influenced by genetic background but also dependent on external factors such as nutrition and lifestyle, with significant differences observed on a daily-basis.[23,24] Traditionally, a liquid aliquot of breast milk is collected for LC-MS/MS applications. This sampling method typically requires a cooling chain for storage and transportation, which might limit its use in remote conditions. Dried matrix spots, especially dried blood spots (DBS), are already widely used in various fields, including metabolomics[25,26] and exposomics[27,28] and partially overcome such limitations. They are prepared by placing a few drops of a liquid matrix (e.g., whole blood) directly onto a filter paper, followed by transportation, storage and extraction after sample drying. This method offers advantages such as a low sample volume, good stability of the analytes and the possibility of sampling in low resource settings.[29] However, potential limitations include variable spot size (i.e., sample volume) and uneven analyte distribution across the dried matrix spot, negatively impacting quantification precision. Dried milk spots (DMS), more specifically, are still an emerging approach, and while first applications have been reported in the field of lipidomics[30,31] and in analyzing pharmaceuticals,[32,33] their use in broader metabolomics and exposomics-contexts remains unexplored.

In addition, novel microsampling devices have emerged in recent years as alternatives to dried matrix spots, addressing several of their limitations, particularly for quantitative purposes. Mitra microsampling devices are one example based on the volumetric absorptive microsampling (VAMS) technology for the quantitative collection of biofluids, increasing accuracy and consistency in sample collection when compared to traditional filter paper substrates. Different experiments have shown the potential of such devices in metabolomics,[34,35] or for exposure assessment,[36] mostly using blood collection. To the best of our knowledge, no previous study has reported the use of Mitra microsampling for breast milk analysis.

To investigate the suitability of DMS for exposomics research, we optimized a sample preparation protocol for an LC-MS/MS workflow that includes 216 xenobiotics including mycotoxins, bisphenols, PFAS chemicals and other substances, aiming to assemble a target panel that is representative of a broad spectrum of chemical classes. Although the selection of analytes was not made specifically for breast milk, previous work has already demonstrated their presence in breast milk and thus their relevance for biomonitoring of breast milk.[14] Recovery and matrix effects were assessed for selecting the most suitable protocol, which was then applied in a proof-of-concept study focused on comparing detected compounds from pooled Austrian and American breast milk samples. In addition, compound stability was assessed for all compounds under four storage conditions (−80°C, −20°C, 4°C, 18°C and 37°C) at three timepoints (2 days, 2 weeks, 2 months). Finally, a preliminary comparison on the analytical performance (based on recovery and matrix effects) was conducted between conventional DMS paper substrate and microsampling device (Mitra tips), showcasing for the first time the suitability of these devices for breast milk analysis.

Material and methods

Chemicals, reagents and solvents

LC-MS grade solvents were employed throughout all experimental steps. Serial dilution of a multicomponent stock solution of analytical standards containing 216 analytes was conducted for building a calibration curve covering seven concentration levels. Concentration ranges were compound-dependent and were previously optimized by Jamnik et al.[15] and Gu et al.,[37] hence, we refer to concentration levels rather than to actual concentration values in this work. An overview on the analytes included, alongside their working concentrations are reported in Table S1. Labeled bisphenol A (bisphenol-A-diphenyl-$^{13}C_{12}$), was purchased from Sigma-Aldrich, labeled zearalenone (U-[$^{13}C_{18}$]-zearalenone), from Romer Labs. All chemicals and solutions were stored at −20°C until use. Detailed information on the chemical suppliers is provided by Gu et al.[37]

Sample extraction

Breast milk samples from Austria were provided by the Semmelweis Women's Clinic milk bank in Vienna. Samples of more than 150 women were collected in 2015, pooled and stored at −20°C.[9] For preparing the DMS, 40 µL of pooled breast milk sample were pipetted onto 903 Whatman protein saver cards and allowed to dry for 3 h at room temperature. The dried spots were then prepared using a 1.2 cm diameter punch, ensuring the entire spot was utilized. The spots were put into 1.5 mL tubes and 1 mL of two tested extraction solutions was added (ACN/MeOH/H_2O 40:40:20 v/v versus ACN/MeOH/MTBE/H_2O 30:30:20:20 v/v). The samples were extracted at 1200 rpm for 5 min at room temperature on a thermo mixer, followed by sonication for 10 min at room temperature and additional shaking for 5 min under the same conditions. After centrifugation at 9,500*g* and 4°C for 5 min, 800 µL of the supernatant were transferred to another tube. The samples were placed into a vacuum concentrator set at 20°C until complete dryness. The remaining residue was reconstituted in 80 µL of H_2O/ACN (90:10 v/v). After shaking for 10 min, the samples were centrifuged for 5 min, the supernatant transferred to glass vials and measured afterwards. For evaluating extraction recoveries and matrix effects, spiked samples were utilized. Samples spiked before extraction ("pre-spiked") were prepared by spiking an aliquot of the pooled breast milk with the working solution of standards prior to spotting in the cards. A medium concentration spiking level was chosen, aiming at obtaining a final extract at theoretical concentration Level 23" (see Table S1), assuming 100% recovery. Post-spiked samples were prepared by reconstituting the residue of non-spiked DMS samples in a spiked reconstitution solution prepared with the same mix of

compounds to achieve the same nominal concentration level. For each extraction solution tested, five replicates of both pre- and post-spiked samples were used. In addition to the pooled breast milk samples, standard reference material (SRM 1954) from the National Institute of Standards and Technology (NIST, USA) were spotted and processed in parallel following the same protocol. Five replicates were extracted for each sample type.

LC-MS/MS instrumentation

An Agilent 1290 Infinity II LC connected to a SCIEX QTrap 7500 system with a heated electrospray ionization source (ESI, OptiFlow Pro) was used. The LC-MS method was recently developed and validated in-house and detailed information is available at Gu et al.[37] In short, data was obtained in scheduled multiple reaction monitoring mode (sMRM) using a previously validated method. MRM transitions and RT are described in Table S2. Chromatographic separation was achieved using a Waters Acquity HSST3 (100 mm × 2.1 mm, 1.8 μm) paired with a Waters VanGuard Acquity HSST3 precolumn (50 × 2.1 mm, 1.8 μm). The column compartment was kept at a temperature of 40°C, and the autosampler was maintained at 7°C. The injection volume was 5 μL and the flow rate was set to 0.4 mL/min. The mobile phases used were water + 0.3 mmol/L NH_4F (eluent A) and acetonitrile (eluent B). Details of the settings, including chromatographic gradient and ion source settings can be found in Table S3.

Quality control

Several quality control (QC) measures were applied: isotopically labeled internal standards were added to all extraction solutions ($^{13}C_{18}$-zearalenone, 0.3 ng/mL) and to the reconstitution solution ($^{13}C_{12}$-bisphenol A, 2 ng/mL) for controlling procedural/instrumental variability (Table S4). QC samples were prepared by extracting additional non-spiked samples, pooled after reconstitution. For the method optimization, five samples of each extraction solutions tested were merged. For both the stability study and the comparison between DMS and Mitra samples, a new set of five freshly prepared DMS samples was employed. Background contaminant levels were evaluated for working solutions (extraction and reconstitution), process blanks (extraction protocol without any matrix) and paper blanks (filter papers without breast milk undergone extraction protocol).

Preliminary stability assessment

Pre-spiked DMS were prepared and extracted in the same manner as described above. In summary, 2450 μL of milk were spiked with 50 μL of the stock solution mix to produce DMS spiked at concentration "Level 20". Twelve independent sample sets (paper cards), each containing three samples and one blank spot were prepared on the same day. Four of these sets were allocated to each tested temperature condition for evaluation at the 2-month time point. The remaining sets were stored at -80°C until needed. To ensure all samples were extracted and analyzed simultaneously, additional sets were moved from -80°C to the respective test temperatures either 2 weeks or 2 days before the end of the 2-month period. This allowed all samples to complete the intended duration by the final time point. In addition, one reference sample set with a larger number of replicates (2 paper cards containing 8 samples and 2 blanks) was stored continuously at −80°C for 2 months. In total, 14 paper cards were prepared, containing 34 samples and 14 blank spots, and used for the stability assessment. Extraction of the DMS was carried out using the final extraction solution ACN/MeOH/H_2O (40:40:20 v/v).

Comparison of dried milk spots and mitra tips

The performance of DMS and Mitra tips (Trajan Scientific Americas Inc.) was compared in terms of extraction recovery, matrix effects (SSE, signal suppression/enhancement), and blank contamination. A total of five pre-spiked 10 μL-Mitra sticks and 10 μL-DMS were prepared using pre-spiked milk. The same number of replicates were prepared for post-spiked samples by reconstituting the residue of non-spiked pooled samples in a spiked reconstitution solution at the same nominal concentration (Level 8, see Table S1). The Mitra sticks were prepared by briefly contacting the surface of the milk with the sampler tip for 8 s without complete submersion, as recommended by the vendor. The DMS were prepared by pipetting 10 μL of pooled human milk onto the Whatman cards. Both sample types were allowed to dry for 3 h at room temperature and protected from direct light. The final extraction solution (ACN/MeOH/H_2O 40:40:20 v/v) was used for sample preparation. For Mitra sticks, the tip of the sampler was inserted into tubes and treated as previously described.

Data analysis

Data analysis was performed using SCIEX OS (v3.0). First, automatic peak integration was performed using AutoPeak algorithm, followed by visual inspection of possible missing peaks, wrong peak assignment or peak integration of false positives. Next, calibration curves were built based on linear regression with 1/x weighting. Average Peak areas of samples spiked before and after extraction were used to calculate analyte recovery and matrix effect.[38] Compound recoveries were assessed based on the ratio of average peak area of pre- and post-spiked samples ($n = 5$ each). SSE was calculated as the ratio of average areas between post-spiked average and solvent standard at same spiking level. Average peak areas of non-spiked samples were subtracted from both pre- and post-spiked samples to minimize the effects of previously present xenobiotics. All figures of merit were calculated in Excel 16.0.

Concentrations of detected xenobiotics in both pooled Austrian breast milk and SRM 1954 were calculated by external calibration in solvent. For compounds detected in the reconstitution solution (used for sample reconstitution and serial dilution of the calibration curve) standard addition approach was used to calculate concentration. Next, the theoretical concentrations for the calibration curve were obtained by summing the concentration of the reconstitution solution to the original values. Compound concentrations were then calculated based on the corrected calibration curve. Finally, compound concentrations were corrected for previously estimated recoveries and matrix effects.

Samples spiked before extraction were used for the estimation of the methods limit of detection (LOD). The choice of pre-spiked samples was intended to accurately represent the method performance, i.e., considering recoveries and matrix effects. Signal-to-noise ratios (S/N) were obtained using SCIEX OS software with MQ4 algorithm for peak integration and Peak to Peak algorithm for computing S/N. For this purpose, a noise region was defined for every analyte as the remaining region within the MRM window that did not contain the peak of interest. Mean S/N were determined to calculate the ratio corresponding to a S/N of 3 to estimate the LOD. The respective concentrations of the individual compounds were then used to determine the actual concentrations at which an S/N of 3 was reached.

For a preliminary stability assessment, compounds with RSD > 30% in the −80°C reference samples (measured throughout data acquisition) were excluded in order to ensure data

reliability, retaining a total of 137 out of 216 compounds for subsequent statistical analysis. This approach was favored over employing RSD calculations on QC samples since only a few compounds were detected in the non-spiked QC samples used in this study, limiting RSD calculations for all compounds. Initially, principal component analysis (PCA) was performed for exploratory data visualization using autoscaled data. For assessing compound stability, across all storage temperatures (−20°C, 4°C, 18°C and 37°C) and time points (2 days, 2 weeks, 2 months), independent temperature-based one-way ANOVA tests were performed, which included the reference group (−80°C) against all time points, for each temperature. The data was log10-transformed (no scaling) and both parametric and non-parametric one-way ANOVA (Kruskal–Wallis test) were performed. Compounds presenting a P-value < 0.05 after false discovery rate (FDR) correction in both tests were considered to be significant (ie, not fully stable). This approach was taken in order to ensure robustness of results in a low-sample scenario. Finally, a spearman rank correlation between peak areas and time profile within each tested condition was performed in order to assess the most prominent time-dependent patterns (correlation coefficient > 0.9, with either positive or negative correlation). All analyses were performed in MetaboAnalyst 6.0 using raw peak areas as input.

Results and discussion

Optimization of extraction solution

Extraction recovery

Extraction recoveries could be evaluated for 198 out of 216 compounds. Evaluation criteria followed the approach by Gu et al.[37] in which an acceptable recovery range based on empirical validation data from large-scale exposomics multi-analyte assay was proposed (42%–134%). For both tested extraction solutions more than 80% of the reported compounds were found to be within this range. For 17 analytes, recovery assessment was not possible due to the absence of detectable peaks in pre- and/or post-spiked samples or due to inconclusive chromatograms resulting, for instance, from retention time shifts. Detailed information can be found in Table S5. In general, recovery values were largely comparable, with only 22 compounds (10%) presenting a difference higher than 30% between both extraction methods. Since the extraction solution consisting of ACN/MeOH/H2O (40:40:20 v/v) was ultimately selected due to a simpler composition, subsequent comparisons with previously reported methodologies are performed against it.

To the best of our knowledge, this is the first study to assess the analytical performance of DMS for exposomic studies and, therefore, liquid breast milk analysis is used as a point of comparison. Overall, all aflatoxins, zearalenone (ZEN) and its derivates, bisphenols and parabens (except for methylparaben) fell within the acceptance range. Several recovery values can be compared with those reported by Jamnik et al.,[14] who optimized an LC-MS/MS method for a variety of xenobiotics in breast milk. In general parabens, bisphenols and ZEN derivates showed improved performance in our study compared to the liquid milk approach used by Jamnik et al. For instance, recoveries were higher in our work for ethylparaben (87 ± 14% at 0.4 ng/mL x 71 ± 17% at 0.3 ng/mL), bisphenol A (BPA, 94 ± 10% at 2.3 ng/mL × 81 ± 20% at 3 ng/mL), and α-zearalanol (α-ZAL, 68 ± 8% at 1.15 ng/mL × 54 ± 20% at 1.5 ng/mL). From a total of seven PFAS analyzed, five showed satisfactory performance, including perfluorooctanoic acid (PFOA, 101 ± 5%, 0.12 ng/mL), perfluorohexanoic acid (PFHxA, 101 ± 9%, 0.8 ng/mL), perfluorononanoate (PFNA, 110 ± 5%, 2.4 ng/mL), perfluorodecanoic acid (PFDA, 129 ± 10%, 1.6 ng/mL), perfluorooctanesulfonic acid (PFOS, 129 ± 11%, 0.12 ng/mL). Similar recovery values were reported by Vela-Soria et al.[39] for a HPLC-MS/MS method as 88.5% (PFOA, 0.1 ng/mL), 102.5% (PFHxA, 0.1 ng/mL), 110.8% (PFNA, 0.5 ng/mL), 110.0% (PFDA, 0.5 ng/mL) and 93.7 (PFOS, 0.1 ng/mL).

Matrix effects

Matrix effects could be evaluated for 197 compounds out of 216 and were found to be comparable between both extraction solutions with about half of the compounds falling within the acceptance range of 60%–140%. It was observed that about 60% of the compounds were affected by signal suppression compared to signal enhancement. More lipophilic analytes (later eluting compounds) were more prone to signal suppression (see Figure 1), likely caused by coelution with lipid species naturally present in breast milk. A full account of the results is available in Table S5. Overall, SSE values showed good agreement between the two extraction protocols, with differences exceeding 30% observed for only 35 compounds (18%). Comparisons with previously reported methods are again conducted using the results of extraction solution consisting of ACN/MeOH/H_2O (40:40:20 v/v).

A total of four PFAS showed satisfactory performance, including PFHxA (117 ± 11%, 0.8 ng/mL), PFOA (123 ± 7%, 0.12 ng/mL), perfluorobutanesulfonic acid (PFBS) (126 ± 10%, 0.16 ng/mL) and PFNA (127 ± 18%, 2.4 ng/mL). In contrast, PFOS showed poorer performance with 160 ± 59% at 0.12 ng/mL. Similar to the recovery results, most parabens and ZEN derivates demonstrated satisfactory results for matrix effects, which were largely consistent with the values reported by Jamnik et al.[14] However, methylparaben and heptyl paraben exhibited strong signal suppression with 17 ± 3% and 9 ± 3%, respectively. Of the 12 bisphenols analyzed, half of them were within the acceptance range, including BPA (91 ± 9%), BPE (103 ± 11%), and BPS (79 ± 6%). Six bisphenols could be directly compared with the values reported by Jamnik et al.,[14] with overall good agreement, except for BPF, which showed poorer performance in our study. Aflatoxins exhibited matrix effects of 115 ± 58% for aflatoxin B_1 (AFB_1), 149 ± 59% for aflatoxin G_1 (AFG_1), 210 ± 133% for aflatoxin M_1 (AFM_1), 110 ± 57% for aflatoxin P_1 (AFP_1), 750 ± 495% for aflatoxin Q_1 (AFQ_1) and 216 ± 133% for AflatoxinB_1-N7-guanine (AFB_1-N7-guanine). Braun et al.[40] reported matrix effects for aflatoxins in breast milk calculated based on ratio of the slopes between pure solvent and matrix-matched calibration curves as 62% (AFB_1), 70% (AFG_1), 89% (AFM_1), 49% (AFP_1), 75% (AFQ_1) and 90% (AFB_1-N7-guanine). This comparison suggests that the use of filter paper can amplify the matrix effects, possibly contributing to the observed signal enhancement.

Limit of detection

The methods limit of detection (LOD) was estimated by the average S/N of the peak observed for the samples spiked before extraction ($n = 5$). Detailed results are reported in Table S5. The LOD could be estimated for about 196 compounds from which more than 50% were <0.1 ng/mL (102 out of 197). About 30% fell into the range of 0.1–1 ng/mL (58 out of 197) and 20% were estimated to be higher 1 ng/mL (37 out of 197). The achieved sensitivity in general proved to be feasible for monitoring very low exposure levels in an early-life context. For example, aflatoxins generally demonstrated high sensitivity. LODs for AFB_1, AFB_2 and AFG_1 ranged between 0.001 and 0.01 ng/mL, while AFM_1, AFQ_1 and AFP_1 demonstrated LODs between 0.01 and 0.1 ng/mL. AFG_2

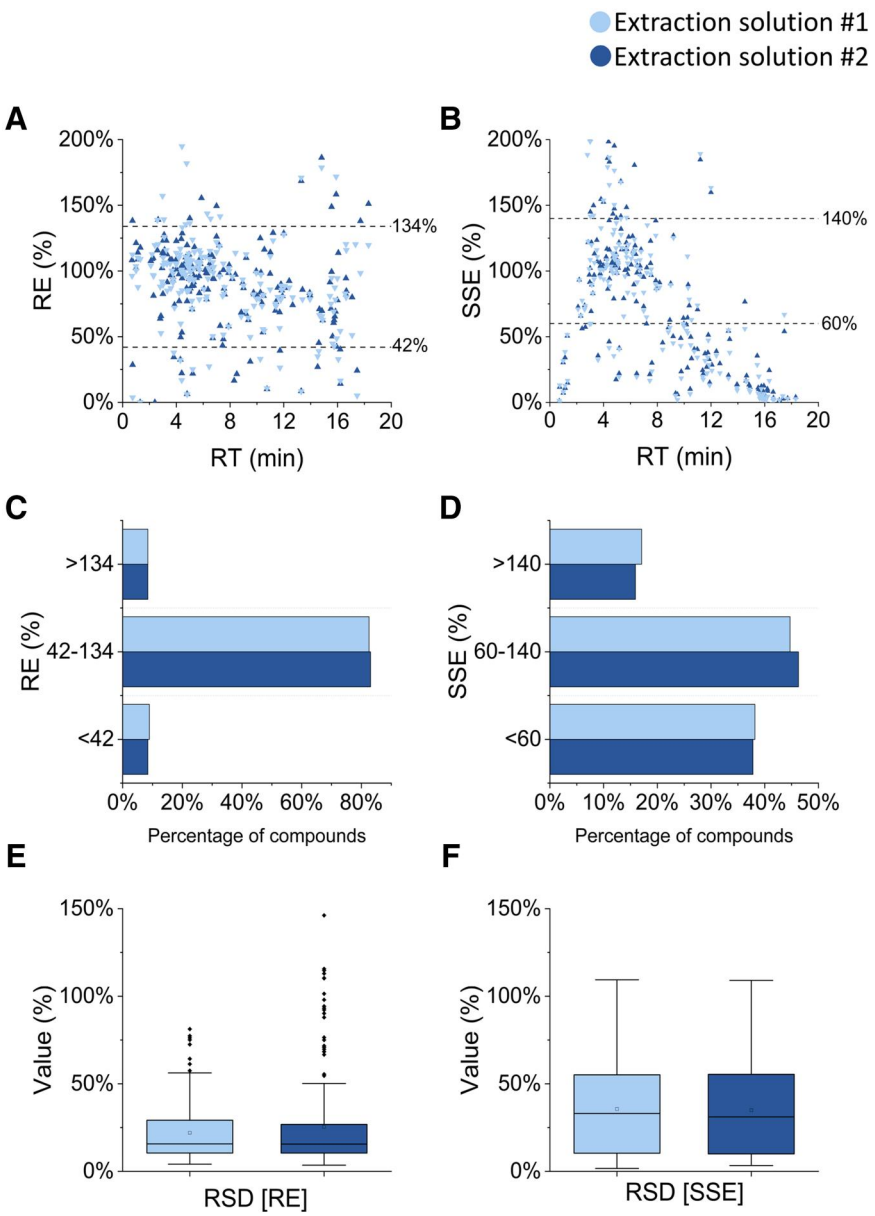

Figure 1. Comparison of two extraction solutions: Extraction solution #1 (ACN/MeOH/H_2O 40:40:20 v/v) versus extraction solution #2 (ACN/MeOH/MTBE/H_2O 30:30:20:20 v/v). (A) Recovery (RE, %) and (B) signal suppression and enhancement (SSE, %) in dependence of chromatographic retention time for 198 out of 216 highly diverse analytes in dried milk spots. An overview of method performance is given in (C) RE (%), (D) SSE (%), (E) RSD of RE (%) and (F) RSD of SSE (%).

was the only variant with an LOD exceeding 1 ng/mL, measured at 1.3 ng/mL. These results are broadly consistent with those reported by Braun et al.,[9] who reported LODs between 0.01 and 0.1 ng/mL for the same analytes in breast milk. The pattern remains consistent across both datasets: AFM_1, AFQ_1, AFP_1 and AFQ_2 showed higher LODs than the other aflatoxins. Given their high toxicity and proven link to liver cancer, aflatoxins are considered critical contaminants in food safety monitoring.[41] Similarly, PFAS, another critical exposure group in early life, are persistent environmental pollutants that are associated with immunotoxicity, endocrine disruption, and developmental effects.[42] Six out of the seven PFAS measured, demonstrated LODs between 0.01 and 0.03 ng/mL, an approximate two to five times higher value when compared to those reported by Vela-Soria et al.,[39] with LODs of 0.006 ng/mL for all PFAS analyzed. The only exception was PFBS, which exhibited an even lower LOD of 0.004 ng/mL.

Background contamination in paper substrate

Background contamination is a key component in any analytical assay but especially in the context of exposomics/human biomonitoring. Laboratory consumables, instrument parts or airborne dust, for instance, can be undesirable sources of analytes of interest and should be considered for ensuring data reliability. In this study the filter paper used for sample collection and storage is a potential source for pre-analytical contamination.

In total 29 compounds were detected in the paper blank. Notably, 14 of them were also detected in the working solutions at similar concentration levels, indicating a contamination source other than the paper substrate. Among the detected

compounds, five had previously been reported as background contamination in labware by Krauss et al.[43] Phthalates were the predominant chemical class detected in the analysis, with five out of 22 phthalates identified, followed by phosphates with four compounds detected. Additionally, three of the seven parabens analyzed were detected. Among the seven PFAS compounds tested, only PFBS was found. Detailed results and concentrations can be found in Table S6.

Overview of class-specific analytical performance

The results presented highlight the use of breast milk as a suitable matrix for multi-class exposure assessment. Despite the large chemical diversity covered by this assay (LogP = −4.8 to 9.6), adequate analytical performance was observed for both lipophilic and hydrophilic compounds. For drugs and drug metabolites, for instance, ibuprofen (LogP = 3.5) and the polar conjugate SN-38-glucuronide (LogP = −0.1) were reliably detected, showing recoveries and SSE values close to 100%. Among mycotoxins, aflatoxins (LogP = 0.2–1.8) generally showed recoveries and SSE values between 100–150%, with the exception of aflatoxin M_1 and Q_1, which exhibited more pronounced matrix effects. PFAS (LogP = 2.3–6.9) displayed recoveries and matrix effects largely within 100%–160%; higher values were observed for the longer chain compounds perfluorodecanoic acid and perfluoroundecanoic acid. For phthalates, performance was more variable: 12 of the 20 analytes showed recoveries and matrix effects close to 100%, whereas the remaining compounds exhibited reduced recoveries (mostly <60%) or substantial signal enhancement. Phytoestrogens and their metabolites (LogP = 0.9–4.3) were largely performing well, with 11 of 15 analytes showing recoveries and matrix effects around 100%, while the remaining four showed lower values (~50%). Finally, bisphenols (LogP = 1.9–6.5) showed recoveries between 70% and 100%, although matrix effects were dominated by signal suppression, ranging from 6% to 60%.

Proof-of-principle study

A proof-of-principle analysis was performed to demonstrate the suitability of the method for the detection of a variety of xenobiotics in DMS. In total, 30 compounds were detected in all replicates of the SRM 1954 pooled samples, while 22 of those were present in the Austrian pooled sample (Table 1), including air pollutants (cotinine), plastic-related chemicals (phthalates and bisphenols), flame retardants (TBBPA and TCBPA), perfluoroalkyl substances (PFOA and PFOS), personal care products ingredients (parabens) and drug-related compounds (acetaminophen and fluconazole). Reported concentrations were corrected for recovery and matrix effects and blank subtracted if necessary. Both sample types showed similar detection profiles for plastic components, though concentrations varied. The majority of these compounds were phthalates with diethyl phthalate (DEP) being the most abundant in both samples. In addition to the phthalates, N-butylbenzenesulfonamide was also consistently detected, while BPS was only present in the Austrian pooled milk samples. When compared to the ranges reported by Jamnik et al.,[14] BPS concentrations in this study are approximately tenfold higher, whereas the N-butylbenzenesulfonamide levels fall within the reported ranges.

Personal care product related compounds were the second most abundant group in both milk types. Among these, five parabens were quantified with methylparaben showing the highest concentrations: 11 ng/mL in SRM 1954 and 9.7 ng/mL in the Austrian pooled milk. Overall, paraben levels were found to be higher in the SRM 1954 samples. This observation is supported by the work of Iribarne-Durán et al.,[44] who reported that parabens concentrations tend to be higher in human milk samples from the United States compared to those from Europe. In addition to the parabens, 4-methylbenzophenone was detected in both sample types and triclosan was exclusively detected in SRM 1954.

For phytoestrogens, genistein was present in both samples, while daidzein and glycitein were only detected in SRM 1954. Since phytoestrogens are secondary plant metabolites, their levels in the human body are strongly diet-related, explaining the varying ranges detected.[45]

Two pharmaceutical drugs were identified, namely acetaminophen and fluconazole. Acetaminophen was detected in the SRM 1954 at a concentration approximately 1000 times higher than in the Austrian pooled milk. For the Austrian samples, the detected concentration was below the lowest standard, so the value needs to be interpreted with caution. Acetaminophen glucuronide, a major metabolite, was only detected in the SRM 1954, which is consistent with the substantially higher concentration of the parent compound.

In SRM 1954 samples, fluconazole levels exceeded the upper limit of the calibration curve (6 ng/mL), preventing accurate quantification, whereas the concentration of the Austrian pooled milk sample was calculated with 0.075 ng/mL. Fluconazole is a widely used antifungal, with reported breast milk concentration up to 2.9 ng/mL in breast milk 2h after a single 150 mg oral dose.[46] It remains the most frequently prescribed outpatient antifungal in the USA (over 17 million prescriptions annually)[47] and is similarly prevalent in Europe.[48] While usage data for lactating women are limited, there is documented evidence of fluconazole being used in this population to treat thrush infections.[49,50]

Two out of seven analyzed PFAS could be detected in both sample types, namely PFOS and PFOA. For both substances, concentrations were higher in the SRM 1954 samples, with concentrations at 0.14 ± 0.013 ng/mL and 0.26 ± 0.10 ng/mL for PFOA and PFOS, respectively. The PFOA concentration aligns closely with the consensus values reported by Keller et al. (0.13 ± 0.04 ng/mg), whereas the PFOS concentration is somewhat elevated compared to the reported consensus (0.16 ± 0.03 ng/mg), however, the values overlap within the respective standard deviations.[51] In the Austrian milk pool, concentrations of 0.080 ± 0.010 ng/mL (PFOA) and 0.17 ± 0.064 ng/mL (PFOS) were determined. These levels fall within the ranges reported by Hartmann et al.[52] who analyzed PFAS in 40 Austrian breast milk samples collected between 2013 and 2016, with concentrations up to 0.08 ng/mL for PFOA and 0.31 ng/mL for PFOS.

Among the analyzed flame retardants, tetrabromobisphenol A (TBBPA, 6.5 ± 3.8 ng/mL) and tetrachlorobisphenol A (TCBPA, 3.7 ± 1.6 ng/mL) were detected exclusively in the SRM 1954 samples. Notably, 12 halogenated phenolic compounds were spiked into SRM 1954 at a concentration of 0.5 ng/mL,[53] which may have included TBBPA and TCBPA. However, the measured concentrations significantly exceeded this spiking level, suggesting additional exposure sources. These elevated concentrations may reflect regional differences in environmental exposure, industrial application or regulatory policies concerning flame retardants. In 2024, the European Food Safety Authority (EFSA) published an updated scientific opinion on TBBPA and its derivates in food, establishing a tolerable daily intake (TDI) of 0.7 μg/mL body weight per day.[54] Although this TDI is not legally applicable in the United States, it provides a health-based benchmark for assessing potential risks. Based on this value, the TBBPA concentration in SRM 1954 would not be expected to result in infant exposure exceeding the TDI.

Table 1. Detected xenobiotics in SRM 1954 ($n = 30$) and Austrian pooled milk samples ($n = 22$) out of 216 analyzed compounds.

Compound	CAS	Quantification confidence level[a]	Pooled US milk samples (SRM 1954) [ng/mL] (mean ± RSD)	Pooled Austrian milk samples [ng/mL] (mean ± RSD)
Per- and polyfluoroalkyl substances				
PFOA	335-67-1	3	0.14±0.013	0.080±0.010
PFOS	1763-23-1	4	0.26±0.10	0.17±0.064
Flame retardants				
TBBPA	79 - 94-7	5[a]	6.5±3.8	N.D.
TCBPA	79 - 95-8	5[a]	3.7±1.6	N.D.
Drugs and drugs metabolites				
Acetaminophen	103-90-2	4	610±180	0.64±0.33
Acetaminophen glucuronide	16110-10-4	4	33±16	n.d.
Fluconazole	86386-73-4	4	>6.0	0.075±0.049
Pesticides				
Atrazine	1912-24-9	4	5.0±1.9	2.2±0.86
Metribuzin	21087-64-9	3	0.41±0.19	0.28±0.15
N, N-Dimethylbenzamide	611-74-5	2	0.77±0.38	1.1±0.62
Phytoestrogens				
Daidzein	486-66-8	2	0.030±0.0074	n.d.
Genistein	446-72-0	2	0.10±0.012	0.014±0.0040
Glycitein	40957-83-3	5[a]	0.084±0.0038	n.d.
Air Pollutants				
Cotinine	486-56-6	2	0.58±0.23	0.13±0.049
Trans-3-hydroxycotinine	34834-67-8	5[a]	0.15±0.076	n.d.
Industrial side products				
2-Phenylphenol	90-43-7	2	16±9.0	70±42
Personal care product-related chemicals				
Methylparaben	99-76-3	2	11±4.3	9.7±4.9
Ethylparaben	120-47-8	2	0.11±0.019	0.077±0.028
Propylparaben	94-13-3	3	0.23±0.083	0.13±0.076
Butylparaben	94-26-8	3	0.10±0.013	0.035±0.0042
Isobutylparaben	4247-02-3	5[a]	0.041±0.0039	n.d.
4-Methylbenzophenone	134-84-9	2	12±10	19±16
Triclosan	3380-34-5	5[a]	17±11	n.d.
Plastic components				
Benzylbutyl phthalate	85-68-7	5[a]	57±35	53±32
Diethyl phthalate	84-66-2	4	150±100	350±270
Monobenzyl phthalate	2528-16-7	2	0.27±0.042	0.15±0.041
Monobutyl-/ Monoisobutyl phthalate	131-70-4/30833-53-5	2	0.54±0.15	1.9±0.98
Monomethyl phthalate	4376-18-5	2	1.3±0.16	17±6.1
Bisphenol S	80-09-1	4	detected	0.094±0.071
n-Butylbenzolsulfonamide	3622-84-2	2	13±1.9	9.4±1.8

Results are reported as mean of five independent technical measurements. Data is blank subtracted and corrected for recovery and matrix effects. Quantification confidence levels were defined according to SRM 1954 results as proposed by Petrick et al.[57] Note that most concentrations are at extremely low levels compared to other biospecimen or alternative food sources.

[a]Level 5 was attributed to compounds not detected in QC samples as no RSD calculation was possible.

Cotinine, a biomarker of nicotine exposure, was detected at 0.58 ng/mL in SRM 1954 and 0.13 ng/mL in the Austrian pooled milk, while its metabolite trans-3-hydroxycotinine was only found in SRM 1954. Cotinine levels for passive smokers were previously reported as around 17 ng/mL and 54 ng/mL.[55,56] The cotinine levels observed in our study are approximately two orders of magnitude lower than those reported for passive smokers, suggesting minimal environmental tobacco exposure among the milk donors. Considering that the SRM 1954 was obtained from six milk banks across the USA,[53] our finding is consistent with the donor screening policies of the Human Milk Banking Association North America, which excludes individuals who smoke or use tobacco products.[57] Concentrations for all detected compounds are displayed in Table 1, with illustrative MRM chromatograms depicted in Figure 2 Detailed results on the calculation of concentrations for each compound is depicted in Table S7.

Preliminary stability assessment

To assess analyte stability under different storage conditions, samples stored at –80°C for 2 months were used as reference, since all breast milk samples were initially kept at this temperature before transfer to the respective test conditions. In addition to –80°C, storage at –20°C, 4°C, and 18°C was evaluated, as these conditions are more practical for routine use. Demonstrating sufficient stability under such conditions would reduce reliance on ultra-low freezers and increase the feasibility of large-scale studies. To mimic further extreme conditions for in-field sample collection and storage, an additional set of samples was stored at 37°C.

Compounds were considered to be fully stable if FDR-corrected P-value > 0.05 for both parametric and non-parametric one-way ANOVA, aiming to ensure the robustness of results. In total, 137 (100%), 96 (70%), 101 (74%) and 86 (63%) compounds fulfilled these criteria for –20°C, 4°C, 18°C and 37°C, respectively, first demonstrating no significant degradation at –20°C for the entire compound panel at all storage times tested. PCA analysis revealed a trend towards more pronounced separation from –80°C samples (ie, larger differences in either PC1 or PC2) with increasing temperature, especially for 2 months at non-refrigerated conditions (18°C and 37°C), as observed in Figure S8.1. To determine which groups in specific presented a significant difference

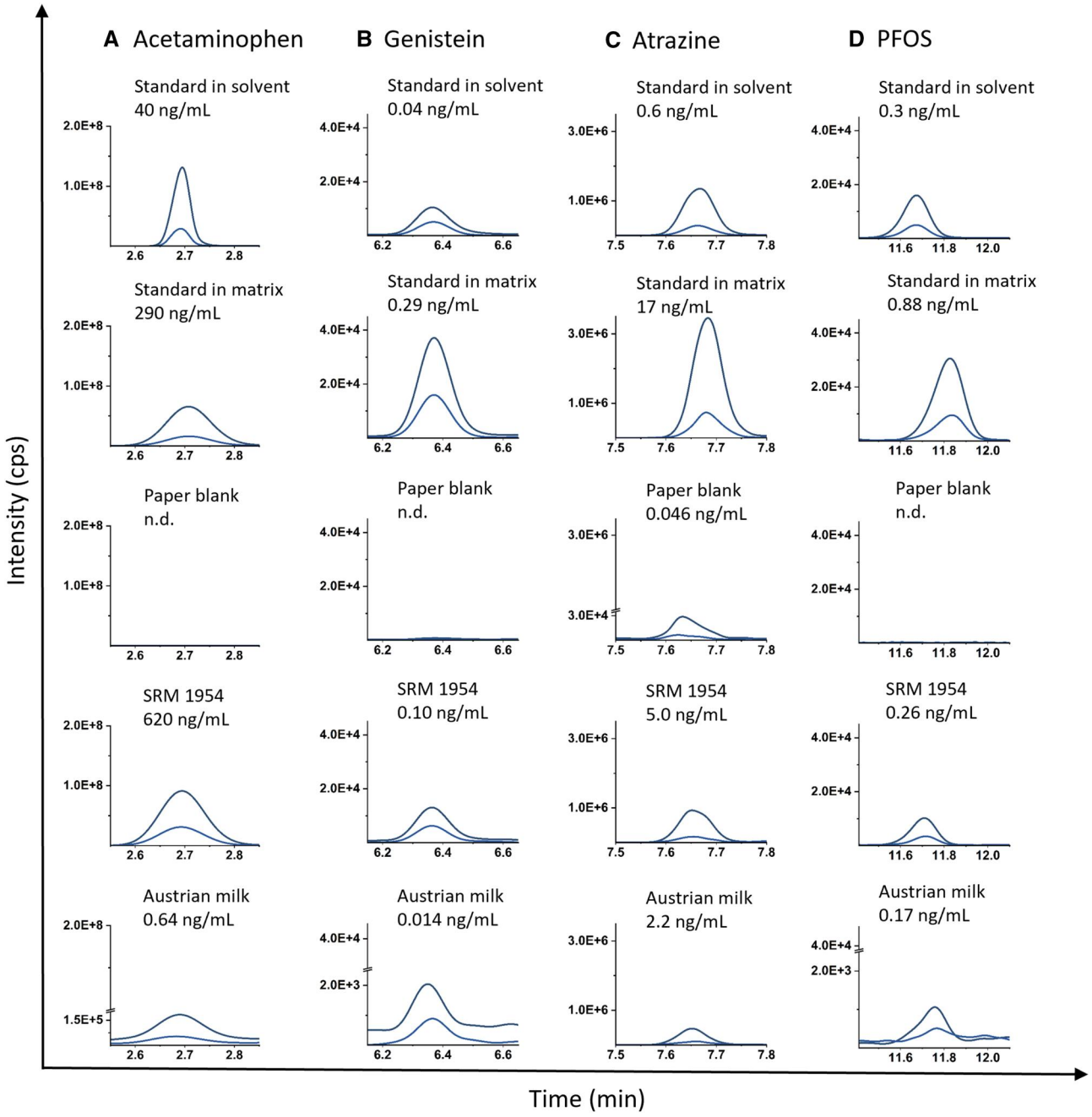

Figure 2. MRM-chromatograms (quantifier and qualifier ions) and calculated concentrations of a standard in solvent, standard in matrix, blank paper substrate, SRM 1954 pooled milk and Austrian pooled milk samples for selected analytes: (A) acetaminophen, (B) genistein, (C) atrazine and (D) PFOS.

against −80°C-samples, a Tukey's HSD post-hoc test was conducted for the parametric ANOVA. Around 80% of all compounds stored at 4°C, 18°C and 37°C remained stable when stored for up to 2 days, while up to 70% of the compounds remained stable after 2 months at 37°C.

In order to highlight the more robust findings (considering the relatively small sample size used in the statistical analysis), we next focused on key compounds/classes based on the overlap between the 10 lowest raw *P*-values and spearman rank correlation >0.9 against the time profile for 18°C (with either positive or negative correlation). This temperature was chosen as the most typical storage/transport condition for dried matrix spots. Parabens were the most prevalent class considering both criteria, with isobutyl-, benzyl- and heptylparaben, alongside with mono-2-ethylhexyl phthalate. Butylparaben also presented a high correlation (and significance as 12th lowest *P*-value), while short-chain parabens showed no significant changes over time or across temperature. Figure 3 highlights the consistent decreasing trend for storage at 4°C, 18°C and 37°C for long-chain parabens.

Mono-2-ethylhexyl phthalate, on the other hand, demonstrated an increasing trend, possibly due to migration from the packaging material or due to degradation of its parent compound di-2-ethylhexyl phthalate (DEHP).[59] Apart from the parabens, no structural patterns could be observed with regard to chemical class or functional groups. To further investigate potential predictors of instability, we evaluated the available information on

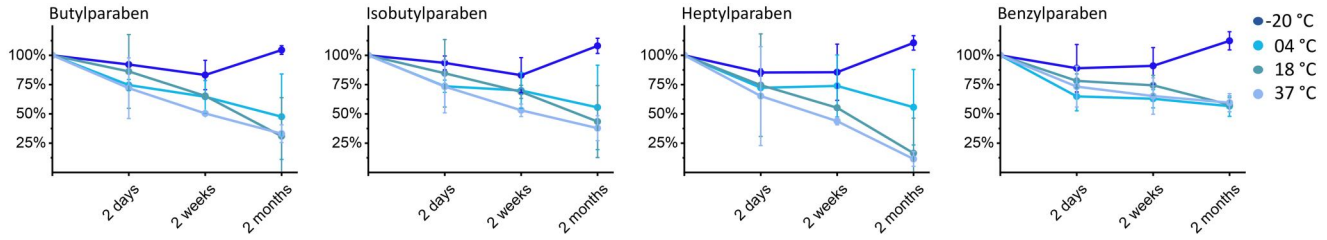

Figure 3. Stability patterns of long-chain parabens over time. Peak areas were normalized to the -80°C samples as a reference (100%) and plotted against storage durations of 2 days, 2 weeks, and 2 months. Short-chain parabens (methyl-, ethyl and propylparaben) remained stable across all conditions, while long-chain parabens (butyl-, isobutyl-, heptyl- and benzylparaben) showed a degradation trend at 4°C, 18°C and 37°C.

biological half-life for several analytes. Urinary half-lives have been reported for methyl-, ethyl-, and propylparaben as well as triclosan,[60] among these, triclosan has the longest half-life but was found to be unstable. Similarly, TBBPA and avobenzone were classified as unstable in DMS, despite having longer biological half-lives than the previously mentioned analytes.[61,62] Together, these findings indicate that in-vivo half-life is not a suitable predictor of stability in DMS. A complete overview of the results obtained in the performed statistical analysis is provided in Table S8.

Comparison with mitra tips as alternative minimal sampling devices

To the best of our knowledge, no previous work has evaluated the feasibility of Mitra devices for the analysis of human breast milk. Their analytical performance was compared to DMS by assessing recovery and matrix effects at a lower spiking level (nominal 8 versus 23 in the previous experiment). For consistency, 10 µL of milk was applied to both Mitra tips and DMS.

Analyte recovery could be assessed for 196 (DMS) and 195 (Mitra) compounds. For both sampling methods, nearly 50% of the reported compounds were found to be within an acceptable recovery range of 42%–134%, compared to 80% in the previous experiment, likely due to the reduced volumes and concentrations employed (see Table S9 for detailed results). As shown in Figure 4, recoveries were similar across the two sampling methods and varied primarily in an analyte-dependent manner. For aflatoxins, differences in recovery between filter paper and Mitra tips, were generally within 20%, with the exception of AFP_1 and AFQ_1, which showed greater variability. A similar trend was observed for bisphenols, with recovery differences not exceeding 20% for the majority of compounds (BPB, BPE and BPF). In contrast, BPFL, BPM and BPS exhibited poor recovery and greater variability. PFAS recoveries were likewise consistent across both sampling types (DMS x Mitra): PFOS (103 ± 18% × 97 ± 18%), PFOA (118 ± 10% × 107 ± 19%), PFHxA (94 ± 7% × 95 ± 16%) and PFNA (109 ± 16% × 108 ± 17%). Phthalates were largely comparable and showed satisfactory recovery results across both sampling types. For diethyl phthalate and monobenzyl phthalate, DMS showed better performance with recoveries of 106 ± 53% and 114 ± 20%, respectively, compared to 200 ± 57% and 181 ± 27% obtained with Mitra tips. Matrix effects were evaluated for 192 (DMS) and 194 (Mitra) compounds. About 30% of the compounds were within the acceptance range of 60%–140%, compared to 50% in the previous experiment. Aflatoxins consistently exceeded the acceptance range for both DMS and Mitra tips, with partly high variance, particularly for AFG_2, AFM_1 and AFQ_1. Bisphenols showed a heterogeneous behaviour with BPA, BPAF, BPB and BPF being within the acceptance range for both DMS and Mitra tips. In contrast, BPFL and BPM showed notable signal suppression, while BPS was more affected by signal enhancement. All PFAS, except for PFBS, fell within the acceptance range, including PFOS (107 ± 15% x 106 ± 14%), PFOA (135 ± 15% x 141 ± 20%) and PFDA (103 ± 12% x 109 ± 13%), for DMS and Mitra tips, respectively. Background levels of contaminants were assessed as in the first experiment (see section *Background contamination in paper substrate*) for blank paper substrate, working solutions (extraction- and reconstitution solution), as well as for blank Mitra tips. In general, there was a good agreement in calculated concentrations between the first and second experiment for the paper substrate background. The majority of compounds that presented substantial concentration difference in the paper blank between both experiments (mostly lower in second experiment) were mainly originating from the extraction process and/or analytical system rather than the paper substrate itself, evidenced by the fact that a similar trend was observed in the process blanks levels. A total of 19 compounds were detected in the Mitra tips, which largely demonstrated lower concentrations compared to filter paper. Compounds such as methylparaben, and triphenyl phosphate were present at significantly higher levels in the filter paper, with concentrations up to 10x greater than in the Mitra tips. Results are summarized in detail in Table S10 along with previously reported values by Hernandes et al.[28]

Limitations

Despite the systematic optimization and extensive assessment of the fitness-for-purpose of the described methodology for the potential use of DMS in exposomics research, there are several aspects in which the presented protocol could be further refined. Only two different extraction solutions were compared, leaving room for exploring alternative solvent compositions and adjusting parameters such as extraction time. Moreover, the composition of the breast milk itself was not characterized in this study. Since lipid content may influence the methods performance, this aspect could be tested in future works. LOD values were estimated, and the tailored criteria used to evaluate extraction recoveries were developed for other biological matrices and not breast milk. Furthermore, the use of a single spiking level limits the conclusions about recovery and matrix effects. The quantification confidence was primarily influenced by the absence of compounds in the QC samples, a limitation that could be addressed by incorporating spiked QC samples. While Mitra VAMS devices showed high potential for sample collection, the accuracy of the collected sample volume for breast milk remains unverified, as current validation data provided by the producer is only available for other biofluids. Similarly, for future in-field applications of DMS, sample volume variability will likely be a

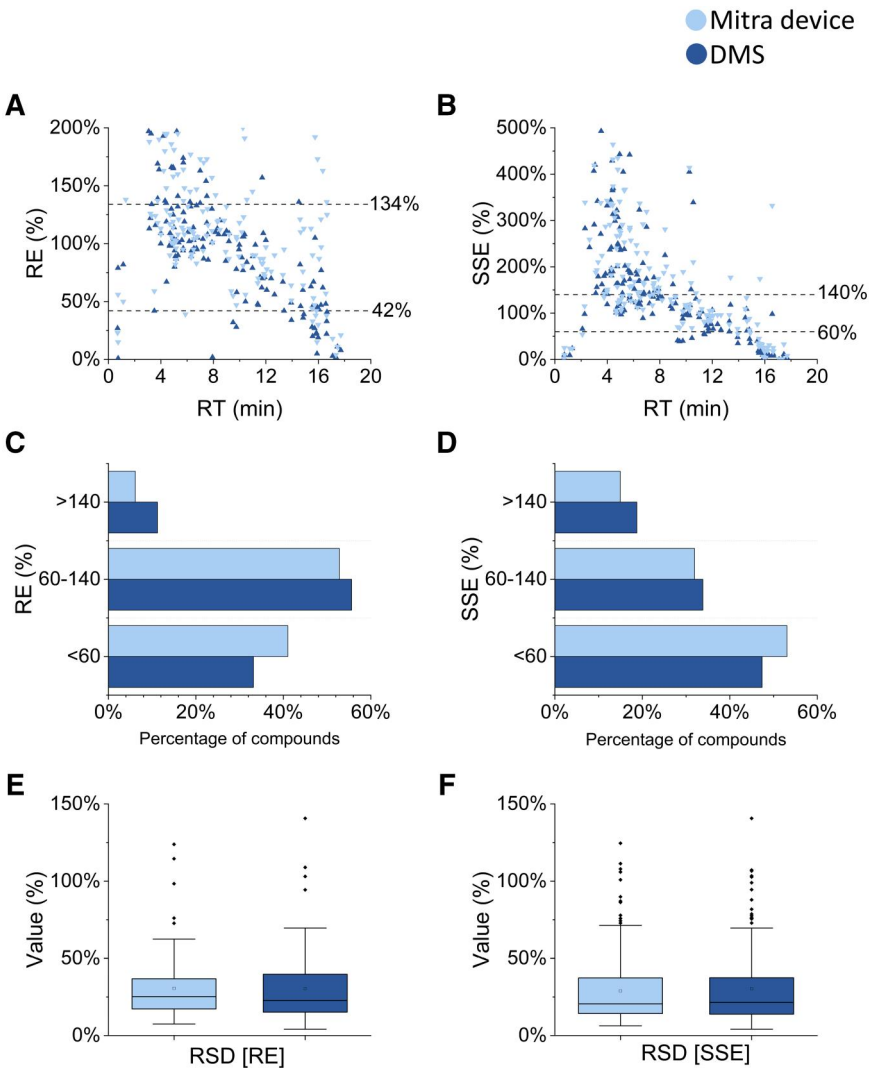

Figure 4. Comparison of two microsampling approaches: Dried milk spots (DMS) and Mitra device. (A) Recovery (RE, %) and (B) signal suppression and enhancement (SSE, %) in dependence of chromatographic retention time for 195 out of 216 highly diverse analytes. An overview of method performance is given in (C) RE (%), (D) SSE (%), (E) RSD of RE (%) and (F) RSD of SSE (%). Please note that, in contrast to the first experiment, a lower volume of milk (10 against 40 μL) was employed to evaluate the performance of the method.

major factor impacting on quantification precision, which in turn may be addressed by the development of a reference normalization parameter such as hemoglobin for dried blood spot analysis.

Conclusion

The presented LC-MS/MS workflow demonstrates the feasibility of dried milk spots as a matrix for multi-class exposure assessment in both mother and infant. Despite the typically extremely low concentrations of such markers of exposure, the method achieved satisfactory performance in terms of matrix effects, recovery and LOD for a large portion of compounds evaluated. Even when considering the broad range of polarities (LogP values) included in our assay, the results confirmed the method's fitness for purpose for many lipophilic (e.g., perfluorononanoic acid and monohexylphthalate) and hydrophilic compounds (e.g., acetaminophen glucuronide and cotinine), with performance for both SSE and recovery between 80 and 130%. A preliminary stability assessment has shown that analyte stability was maintained for the complete set of compounds evaluated at −20 °C. Nevertheless, more than 75% of analytes still presented a stable profile even after long-term storage (2 months) at room temperature (18°C). These results highlight the overall compound stability in DMS even at room temperature, while also pointing to a compound-dependent behavior that should be more systematically evaluated in future studies. A comparative evaluation of DMS and Mitra VAMS sampling approaches revealed a fair agreement in terms of analytical performance while with the main distinction being related to the concentration of background contamination, overall less pronounced in Mitra tips. Additionally, Mitra tips demonstrated advantages in terms of ease of use and quantitative sampling without pipetting, making it a user-friendly alternative for field and at-home sampling. However, these benefits come with trade-offs, including higher costs[63] and the environmental impact of single-use plastics. Despite the presence of low levels of food and environmental contaminants detected in this work, it is essential to highlight that breast milk remains by far the safest and most beneficial source of nutrition for newborns from an exposomics perspective. Our findings should not be interpreted to discourage breastfeeding by any means.

Acknowledgments

The authors would like to thank all members of the Warth laboratory for their valuable support. Appreciation is also extended to Maximilian Zeyda and Lukas Wisgrill from the Medical University of Vienna as well as to Stefan Rakete and Stephan Böse-O'Reilly from LMU Munich for their ideas and the fruitful discussions. The authors would also like to express their gratitude to the Mass Spectrometry Center of the Faculty of Chemistry, University of Vienna for technical support.

Author contributions

Katharina Pfundt (Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing—original draft), and Vinicius Verri Hernandes (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Visualization, Writing—review & editing), Benedikt Warth (Conceptualization , Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing—review & editing)

Supplementary data

Supplementary material is available at *Exposome* online.

Funding

This work was supported by the University of Vienna via the Exposome/EIRENE Austria Research Infrastructure, the Austrian Federal Ministry of Women, Science and Research (BMFWF; project DigiOmics4AT), the Austrian Federal Ministry for Agriculture and Forestry, Climate and Environmental Protection, Regions and Water Management (BMLUK) and the Austrian Federal Ministry for Innovation, Mobility, and Infrastructure (BMIMI).

Disclosure statement

Benedikt Warth holds the position of Associate Editor for Exposome and has not peer reviewed or made any editorial decisions for this article.

Conflicts of interest

None declared.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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Exposome, 2026, 6, 1–13
https://doi.org/10.1093/exposome/osag001
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