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Dried Milk Spots: A viable approach for assessing the chemical exposome in mothers and their infants by targeted LC-MS/MS

verfasst von | submitted by

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Abstract

The chemical exposome, i.e., the sum of environmental exposures, affects human health from conception onwards. As the primary source of nutrition and immune protections for newborns, human breast milk is a valuable matrix for monitoring early-life exposure to environmental contaminations following maternal exposure. Dried milk spots (DMS), similar to dried blood spots, offer a practical alternative to liquid milk sampling, with advantages including minimal sample volume, ease of collection, transport and storage, and the potential for improved analyte stability. Similarly, new microsampling devices like volumetric absorptive microsampling (VAMS) tips additionally offer the possibility for quantitative sample collection. This thesis aimed to optimize an LC-MS/MS assay for the quantification of >200 xenobiotics in DMS samples. Two extraction solutions were evaluated, yielding comparable results with approximately half of the compounds detected within the acceptance range for matrix effects (60-140%), and about 80% meeting the criteria for recovery (42-134%). Given the minimal difference between both extraction solutions, the solution consisting of ACN/MeOH/H2O (40:40:20; v/v) was selected for a proof-of-principle study, in which compound concentrations were compared between pooled breast milk samples from Austria and the NIST Standard Reference Material (SRM) 1954 (USA). A total of 30 compounds were detected in SRM 1954, 22 of which were also found in the Austrian pooled sample. These included 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). A stability assessment was conducted by storing DMS samples at -80, -20, 4, 18 and 37°C for two days, two weeks and two months. No significant changes were observed for the full set of compounds at -20°C throughout all storage durations and for 80% of compounds at 4, 18 and 37°C for up to 2 days. Finally, a comparison between DMS and Mitra tips revealed overall good agreement in number of detectable compounds, SSE and recovery. The main differences between the two methods were related to background contamination in terms of the number and concentration of contaminants.
In this study the suitability of DMS for an LC-MS/MS based exposure assessment in both mothers and infants was demonstrated. The workflow revealed the presence of various xenobiotics in breast milk samples from the US and Austria, albeit at very low concentrations. The preliminary stability study revealed good stability for most analytes even at extended storage temperatures and durations, highlighting the potential for sample preparation even in low-resource settings. Furthermore, the comparison between DMS and Mitra tips showed similar performances, making Mitra tips a promising device for at-home sampling and therefore possibly simplifying large-scale studies.

Zusammenfassung

Das Exposom umfasst die Gesamtheit aller Umweltexpositionen, welche die menschliche Gesundheit bereits ab der Empfängnis beeinflussen, wobei Muttermilch als wichtigste Nahrungsquelle für Neugeborene eine wertvolle Matrix zur Überwachung frühkindlicher Umweltbelastungen darstellt. *Dried Milk Spots* (DMS), auf Filterpapier getrocknete Milchproben, bieten analog zu *Dried Blood Spots* eine praktische Alternative zur Analyse von flüssiger Milch. Zu ihren Vorteilen zählen ein minimales Probenvolumen, eine einfache Probennahme und Lagerung sowie potenziell erhöhte Stabilität der Analyten. Neue Microsampling-Technologien wie *volumetric absorptive microsampling* (VAMS) bieten zusätzlich die Möglichkeit einer simplen quantitativen Arbeitsweise. Das Ziel dieser Arbeit war die Optimierung einer LC-MS/MS-Methode zur Quantifizierung von >200 Xenobiotika in DMS-Proben. Es wurden zwei Extraktionslösungen bewertet, die vergleichbare Ergebnisse lieferten, wobei etwa die Hälfte der nachgewiesenen Verbindungen innerhalb des Akzeptanzbereichs für Matrixeffekte (60–140%) lagen und etwa 80% die Kriterien für die Wiederfindung (42–134%) erfüllten. Da beide Lösungen ähnliche Ergebnisse lieferten, wurde die Lösung bestehend aus ACN/MeOH/H2O (40:40:20; v/v) für eine *Proof-of-Principle*-Studie ausgewählt. Analysiert wurden sowohl das Standardreferenzmaterial (SRM) 1954 als auch österreichische Muttermilchproben. Insgesamt wurden 30 Verbindungen in SRM 1954 nachgewiesen, von denen 22 auch in den österreichischen Proben gefunden wurden. Dazu gehörten Luftschadstoffe (Cotinin), kunststoffbezogene Chemikalien (Phthalate und Bisphenole), Flammschutzmittel (TBBPA und TCBPA), Per- und polyfluorierte Alkylverbindungen (PFOA und PFOS), Inhaltsstoffe von Körperpflegeprodukten (Parabene) und Arzneimittelrückstände (Acetaminophen und Fluconazol). Darüber hinaus wurde eine Stabilitätsstudie durchgeführt, indem DMS-Proben zwei Tage, zwei Wochen und zwei Monate lang bei -80, -20, 4, 18 und 37°C gelagert wurden. Bei einer Lagerungstemperatur von -20°C zeigten die Analyten weder temperatur- noch zeitbedingten Veränderungen. Auch bei höheren Temperaturen blieben rund 80 % der Substanzen über einen Zeitraum von bis zu zwei Tagen stabil. Ein Vergleich zwischen DMS und Mitra VAMS ergab eine insgesamt gute Übereinstimmung hinsichtlich der berechneten Matrixeffekte und Wiederfindungsraten. Der wesentliche Unterschied betraf die Konzentration von Hintergrundkontaminanten, die bei Mitra-VAMS geringer ausfiel.

Die vorliegende Studie zeigte, dass DMS sich gut eignen, um die Exposition von Säuglingen ausgehend von der Muttermilch zu analysieren. Das durchgeführte Protokoll zeigte außerdem das Vorhandensein verschiedener Xenobiotika in Muttermilchproben aus den USA und Österreich, wenn auch in sehr geringen Konzentrationen. Die vorläufige Stabilitätsstudie ergab eine gute Stabilität für die meisten Analyten, selbst bei erhöhten Temperaturen und längerer Lagerung, was das Potenzial für eine Probenvorbereitung in schlecht ausgestatteten Gebieten hervorhebt. Der Vergleich zwischen DMS und Mitra VAMS ergab eine vergleichbare Leistung, womit Mitra VAMS eine vielversprechende Option für die häusliche Probenahme darstellen und groß angelegte Studien erleichtern könnten.

List of abbreviations

Abbreviation	Full word
DBS	Dried blood spots
DC	Direct current voltage
DOHaD	Developmental Origins of Health and Disease
ESI	Electrospray ionization
EWAS	Exposure-wide association studies
GWAS	Genome-wide association studies
HBM	Human biomonitoring
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
LRMS	Low-resolution mass spectrometry
m/z	Mass-to-charge ratio
MRM	Multiple reaction monitoring
RF	Radio frequency
RPLC	Reversed-phase liquid chromatography
TOF	Time-of-flight
UHPLC	Ultra-high-performance liquid chromatography
VAMS	Volumetric absorptive microsampling
WHO	World Health Organization

Contents

1. Introduction

1.1. Breast milk

Breast milk serves as the primary source of nutrition for newborns and is specially adapted to the physiological needs of neonates. The World Health Organization (WHO) recommends exclusive breastfeeding for the first 6 months of life, as breast milk provides all essential nutrients required for optimal growth and development during this period.[1] Its composition is predominantly water, followed by lactose as the main carbohydrate, along with fats and proteins.[2] Notably, the composition of breast milk is dynamic and varies depending on several factors, including whether the infant is born preterm or at term.[3] It also fluctuates throughout a single feeding session, across different times of the day, and over the course of lactation. Additionally, maternal factors such as nutrition, genetic background, and lifestyle strongly influence the overall composition of breast milk.[4]

Several studies have shown a correlation between the duration of breastfeeding and improved cognitive and motor development in children.[5,6] For instance, research by Huang *et al.* found that the developmental advantages associated with breastfeeding persist throughout childhood despite schooling.[6] However, it is not only the presence or duration of breastfeeding that influences infant development, but also the nutritional status of the mother. Maternal deficiencies in vitamins such as B6, B12, and riboflavin have been shown to result in the corresponding deficiencies breast milk, thereby compromising the infants nutrient supply.[7] Beyond providing essential macro – and micronutrients, human milk also supplies the newborn with immunological protection through antibodies. These play a crucial role, as the baby's immune system is still developing when it is born.[8] For instance, Rogier *et al.* found that immunoglobulin A (IgA) antibodies present in breast milk act as a barrier against enterobacteria that can cause diarrheal diseases.[9]

In addition to its endogenous components, breast milk may contain exogenous substances like food contaminants and additional xenobiotics originating from maternal exposure. A study by Memiş *et al.*[10] demonstrated that breast milk may be contaminated with mycotoxins, posing potential health risks to the infant. Notably, the risk of contamination with ochratoxin A increased significantly when the mother was also exposed to cigarette smoke. Beyond mycotoxins, research by Jamnik *et al.*[11] found additional xenobiotics in human milk, including plasticizers, pharmaceuticals and phytoestrogens that may impact neonates health.

1.2. Early life exposure & exposomics

The concept of the Developmental Origins of Health and Disease (DOHaD) originated from the work of Barker and colleagues in the 1980s and revealed a link between low birthweight and an increased risk of coronary heart disease in adulthood.[12] This research laid the foundation for the idea that environmental factors such as nutrition during critical time windows of development can have profound and lasting effects on health across a lifespan. The prenatal and postnatal periods are thus increasingly recognized as critical windows of susceptibility as key organ systems are still undergoing maturation, making them particularly, vulnerable to disruption.[13,14] Exposure to environmental contaminants can occur through maternal transfer, both via the placenta during pregnancy[15], and later through breast milk. A growing body of research on early-life exposure has demonstrated associations between exposure to environmental pollutants and adverse health outcomes, such as preterm birth[16], low birth weight[17] or altered gut microbiota[18].

These early disturbances may set the stage for long-term health consequences. For instance, low birth weight has been linked to a higher risk of developing depression, type 2 diabetes, attention-related disorders or cardiovascular diseases later in life, representing a significant public health burden.[19–22]

Human biomonitoring (HBM) is a key tool in exposure assessment, enabling the direct measurement of environmental chemicals and their metabolites in human biological matrices such as blood, urine or breast milk, reflecting internal exposure from all exposure paths. Traditional exposure assessment relied on environmental measurements and behavioral data. In contrast, HBM provides a more accurate and individualized measure of the internal dose absorbed/metabolized by the body and can be applied not only for assessing persistent chemicals that accumulate in the body over time (such as cadmium, for instance) but also to short-term exposure (such as cotinine, as a smoking marker).[23]

The exposome, first proposed by C. Wild in 2005[24], further extends this concept by aiming to capture the totality of environmental exposures over a life course. Like genome-wide association studies (GWAS), exposure-wide association studies (ExWAS) are a hypothesis-free approach that aims to systematically examine the relationship between a wide range of environmental exposures and specific health outcomes. In contrast to the human biomonitoring strategy, EWAS provides a broader understanding of multiple exposures that occur in parallel.[25–27] Within this context, HBM data from breast milk can be integrated with omics technologies (e.g. metabolomics, exposomics) to better understand the maternal exposome, the infants exposure and finally how early-life exposures influence long-term health outcomes.

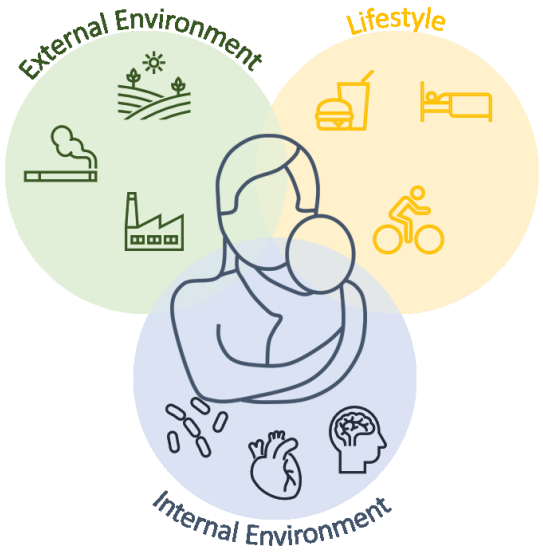

Figure 1: The concept of the exposome, comprising exposures from the external environment, internal environment, and lifestyle.

1.3. Dried Matrix Spots

In the dried matrix spot technique, biological fluids are applied to a filter paper and allowed to dry. Once dried, the spots are cut out, and the analytes of interest are extracted. Due to limited sample volume and the complexity of the biological matrices employed, highly sensitive and selective analytical instruments, such as mass spectrometers, are typically used for analysis.[28–30] The origins of dried matrix spots can be traced back to 1913, when dried blood spots (DBS) were first used to determine blood glucose levels.[31]

However, widespread application began in the 1960s, when Guthrie introduced DBS for neonatal screening programs. In this procedure, a heel-prick blood sample from a newborn is collected on filter paper and analyzed for metabolic disorders like phenylketonuria, a practice that remains standard in many countries today. [28–30]

To date, DBS remains the most commonly used form of dried matrix spots.[32] Their versatility has led to widespread application in various scientific and clinical fields, including metabolomics[33–35] , therapeutic drug monitoring[36–38] and metal analysis[39,40]. Beyond blood, other biological fluids have also been explored for dried matrix spots sampling, such as saliva[41–43], urine[44–46] and breast milk[47–49]. The ease of collection is one of the major benefits of the dried matrix spot technique. Collecting small volumes of biological fluids onto filter paper is minimally invasive, often requiring only a finger prick or small volume of milk, saliva or urine, depending on the matrix. This makes this method especially suitable for vulnerable populations such as newborns, infants, or patients for whom venipuncture may be challenging or impossible. In many cases, samples can even be collected at home by individuals without specialized training, using simple instructions and standardized collection kits.[50–52] This reduces the burden on healthcare facilities and enables broader participation in population-based studies. Additionally, there is the chance to lower overall costs due to home sampling.[53] Finally, the ability to store and transport dried samples without cold chain logistic, makes them particularly well-suited for biobanking, large-scale research or for low-resource settings. Furthermore, the limited amount of biological material lowers the risk of biohazard exposure during collection, handling and transport. When combined with the drying process, which further reduces the viability of infectious agents, dried matrix spots offer a safer alternative to traditional liquid samples.[54,55]

Another key advantage of dried matrix spots is the enhanced stability of analytes. Once the biological fluid is dried, enzymatic activity is halted, and the risk of microbiological degradation is reduced. This preservation effect allows many analytes to remain stable for extended periods, even at ambient temperatures. Various studies have demonstrated that analytes stored in dried spots can remain stable for weeks to months without the need of refrigeration or freezing, depending on the matrix and the analyte of interest.[56–60] This advantage also offers the opportunity for historical exposure assessment. For example, in the study by Blomberg et al, PFAS levels where successfully measured in DBS samples collected between 1985 and 2013.[61] Factors like temperature, humidity, light exposure and the choice of filter paper can still influence stability and must be considered when developing such methods.[57,62,63]

Despite their many advantages, dried matrix spots also present certain limitations. One common challenge is the variability in spot volume and analyte distribution, where factors like hematocrit can influence the spread of blood on filter paper.[64,65] Moreover, the small sample volume limits the number of analyses that can be performed from a single spot as well as the absolute amount of each analyte available, which might limit their detection/quantification reliably.

In recent years, microsampling technologies have emerged as innovative solutions to address the limitations of traditional dried matrix spots, particularly in the field of quantitative bioanalysis. Volumetric absorptive microsampling (VAMS) is one of the widely adopted advancements in this area. Devices like Mitra sampler collect a fixed volume of blood using an absorptive polymer tip that can hold a precise amount of fluid. Sampler sizes are available in 10, 20 and 30 µL formats.[66] In the context of blood analysis, it has already been shown that a precise amount of blood can be absorbed, regardless of the hematocrit.[67,68]

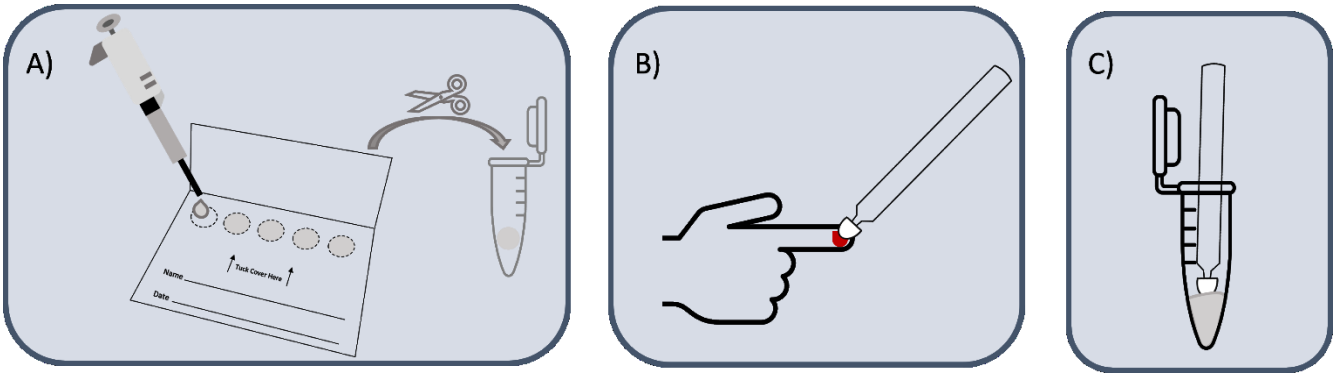

Figure 2: Scheme of A) DMS preparation and transfer to sample tube and of Mitra VAMS application B) directly on the finger and C) in sample tube.

An alternative approach is found in capillary microsampling systems such as hemaPEN or Capitainer. These devices use integrated microfluid channels to collect and isolate precise volumes.[69,70] Some of these systems can even generate plasma-like fractions on-device, which may further improve matrix consistency.[71]

While the majority of these new microsampling technologies are focused on blood collection, particularly due to the quantitative challenges posed by heel- or finger-prick sampling, they also offer promising applications for other biological matrices. In addition to providing standardized sample volumes, these technologies eliminate the need for manual punching of dried spots. The simplification may not only improve reproducibility but could also enhance compatibility with automated, high-throughput systems.

1.4. Liquid chromatography

Due to its high sensitivity and broad coverage of various compounds, liquid chromatography-mass spectrometry (LC-MS) is the preferred instrument platform for analytical exposome research. High-performance liquid chromatography (HPLC) is a widely used technique for the separation of compounds that have low volatility. The primary requirement is that the analytes are soluble in the eluent. Due to its versatility, HPLC is applicable to a broad range of chemical substances and is employed for both qualitative and quantitative analysis.

Separation in HPLC is based on the interaction of analytes with the stationary phase within the chromatographic column, which is packed with highly porous adsorbent particles, typically ranging in size from 1.8 to 5 µm.[72] Different separation mechanisms can be achieved by varying the stationary phase and the eluents (mobile phase). In normal-phase chromatography, the stationary phase is polar and silica gel is usually used as column packing material, while a non-polar mobile phase is used. Under these conditions, more hydrophilic compounds interact more strongly with the stationary phase and are thus eluted later. In contrast, reversed-phase liquid chromatography (RPLC) employs a non-polar stationary phase, typically silica gel modified with alkyl chains. A polar mobile phase is used in this setup, resulting in stronger retention of hydrophobic compounds. This makes RPLC suitable for a wide range of biological and environmental analytes.[73]

To improve chromatographic separation, a two- or multi-component mobile phase is often employed. In RPLC, typically water and a more polar organic solvent, such as methanol or acetonitrile are used as mobile phase. The choice and ratio of solvents influence both retention time and resolution of analytes. When the composition of the mobile phase remains constant throughout the separation, it is referred to as isocratic elution. In contrast, if the solvent composition changes over time, the method is known as gradient elution. Gradient elution is particularly useful for separating complex mixtures with analytes of varying polarity, as it allows for better resolution and shorter run times.[74]

In recent years, ultra-high-performance liquid chromatography (UHPLC) has further advanced the capabilities of traditional HPLC. UHPLC employs columns packed with particles smaller than 2 µm and therefore operates at higher system pressures (600-1400 bar), resulting in increased chromatographic efficiency and improved resolution. Furthermore, with typical injection volumes of 0.5-2.0 µL, UHPLC requires up to 100 times less sample than conventional HPLC. These improvements reduce analysis time to a few minutes but also make UHPLC valuable for high-throughput analyses and precise quantification in research.[75]

1.5. Mass spectrometry

In mass spectrometry, ions are detected based on their mass-to-charge ratio (*m/z*). Typically, the compounds of interest are first separated using liquid or gas chromatography, after which they undergo ionization. The resulting ions are then introduced into a mass analyzer, which separates them according to their *m/z*, and finally reach the detector. In this work, a triple quadrupole mass spectrometer coupled with electrospray ionization (ESI) was used to perform multiple reaction monitoring (MRM) experiments, as further detailed in the next sections.

1.5.1. Ionization

In order to accelerate analyte molecules through an electric field, deflect them in a magnetic field, and ultimately detect them based on their *m/z*, the molecules must first be ionized. This ionization occurs in the ion source of the mass spectrometer and can be achieved through various approaches. For gaseous analytes, methods such as electron ionization or chemical ionization directly ionize the molecules in the gas phase. For liquid samples, techniques like ESI or atmospheric pressure chemical ionization transfer the analyte molecules into the gas phase and then introduce them from atmospheric pressure into the high vacuum of the mass spectrometer.[76]

In ESI, the analyte solution first enters the ion source, via direct infusion or previous chromatographic separation, and is then converted into a fine spray, as it passes through a capillary under high-pressure nitrogen and an applied voltage. The potential difference between the capillary and the counter electrode creates an electric field, causing ions from the solution to migrate to the liquid surface depending on their charge. A so-called Taylor cone is formed from which droplets are emitted. The formation and stability of the Taylor-cone are counteracted by the surface tension of the solvent, which is largely dependent on its composition. The addition of organic solvents helps reduce surface tension, thereby facilitating droplet formation. The charged droplets are then desolvated using a heated stream of nitrogen gas. As the solvent evaporates, the charge density on the shrinking droplets increases until it reaches the Rayleigh limit, leading to a Coulomb explosion that ultimately releases individual ions in the gas phase.[77]

ESI can be used in positive and negative mode, creating mostly protonated ($[M-H]^+$) and deprotonated ($[M-H]^-$) ion species, respectively, depending on the mobile phase used. Based on the physicochemical properties of each analyte, one specific ionization mode can be favored (or even unique), which is the reason why a combination of both ESI+ and ESI- is typically needed for analyzing a diverse range of analytes. Ionization efficiency in positive mode can be enhanced by adding weak acids to the mobile phase. The same principle is valid for negative mode, in which a weak base can be added to assist proton withdrawn.[78] In this work, ammonium fluoride was used for this purpose, as it has been shown to improve ionization in negative mode of analysis.[79] A wide variety of solvents can be used in ESI, including more polar solvents such as water or methanol to less polar solvents like dichloromethane and diethyl ether or mixtures such as methanol/water or acetonitrile/water. The choice of solvent impacts both ionization efficiency and chromatographic separation.[77]

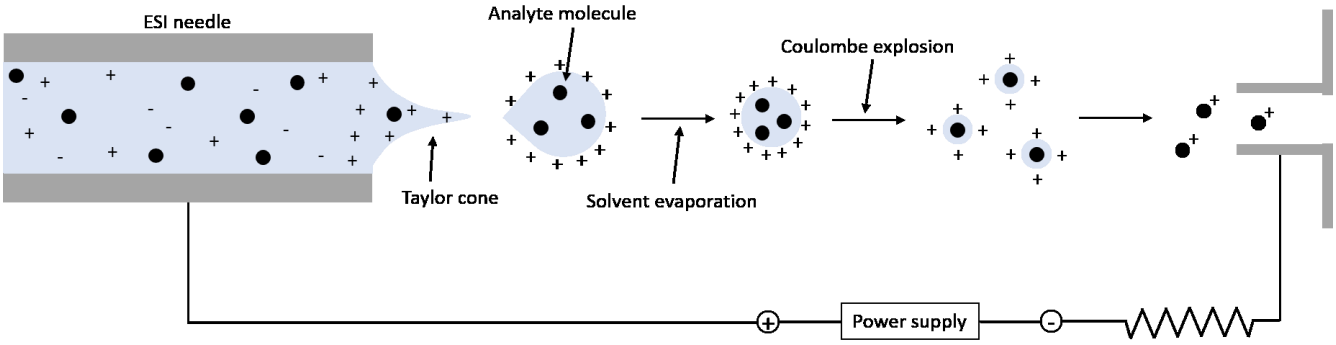

Figure 3: Scheme of electro spray ionization. A droplet is emitted from the Taylor cone and after solvent evaporation and Coulombe explosion, single charged analyte ions remain, that can enter the mass spectrometer. Adapted from Banerjee *et al*.[80]

1.5.2. Mass analyzer

As a critical component, the mass analyzer is responsible for separating ions based on their *m/z* and therefore enabling their detection and characterization. Depending on the mass analyzer used, one can distinguish between low-resolution and high-resolution mass spectrometry (LRMS and HRMS). HRMS instruments like time-of-flight or orbitrap analyzers can measure *m/z* values with high mass accuracy, typically to four or more decimal places, allowing good molecular identification and differentiation of isobaric species. This makes them particularly suitable for untargeted approaches, where a broad range of small molecules, including unknown or unexpected xenobiotics, are being analyzed.[78,81] LRMS instruments in contrast, measure *m/z* ratios at unit mass resolution, meaning it cannot distinguish between isobars, which are molecules with the same nominal mass but different exact mass. Despite these limitations, LRMS offers high sensitivity and specificity, making it ideal for targeted approaches, where the focus is on the precise quantification of predefined chemicals.[81,82]

A quadrupole mass analyzer is a low-resolution instrument and consists of four rod-shaped electrodes arranged parallel to each other. A combination of direct current voltage (DC) and radio frequency (RF) is applied to these electrodes. This creates an electric field that influences the trajectory of ions passing through the quadrupole. As ions enter the field, they are induced to oscillate. The stability of these oscillations depends on their *m/z* as well as the applied voltage parameters.

Only ions with specific *m/z* values will maintain stable trajectories and pass through the quadrupole to reach the detector, while others are destabilized and filtered out. By systematically adjusting the voltages, the quadrupole can selectively transmit ions of interest.[76,81]

This characteristic is utilized for triple quadrupoles in tandem mass spectrometry. In tandem spectrometry two or more mass analyzers are arranged successively. A commonly used configuration is the triple quadrupole, consisting of three quadrupoles (Q1, Q2 and Q3). Q_1 serves as a mass filter, selecting precursor (parent) ions of specific *m/z*. These ions then enter Q2, which functions as a collision cell. Here, the ions collide with an inert collision gas (e.g. nitrogen or argon), causing them to fragment into product ions. Finally, Q3 acts as a second mass filter, transmitting only selected fragment ions to the detector.[76]

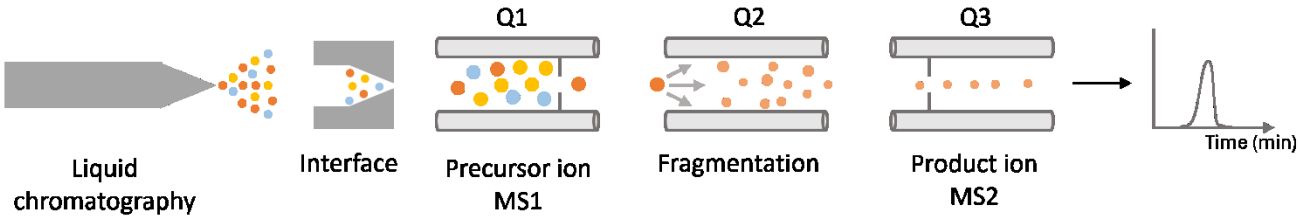

Figure 4: Scheme of triple quadrupole with multiple reaction monitoring. The ions are chromatographically separated before entering the first quadrupole Q1, where one precursor ion is selected. In Q2 fragmentation takes place and in Q3 specific product ions are select, that are detected afterwards. Adapted from Boja *et al.*[83]

Triple quadrupole instruments support several scan modes, each serving different analytical purposes[76]:

- Multiple reaction monitoring (MRM): Q1 and Q3 are set to specific *m/z* values to monitor defined precursor-product ion transitions.
- Product ion scan: Q1 is set to a specific *m/z* value and Q3 scans a range of *m/z* values to detect all resulting product ions.
- Precursor ion scan: Q3 is set to a specific *m/z* value and Q1 scans for all precursor ions that fragment to produce it.
- Neutral loss scan: Both Q1 and Q3 scan in parallel, looking for precursor-product ion pairs that differ by a specific neutral mass.

In terms of specificity and sensitivity, MRM experiments provide advantages over other scan modes. By applying two successive stages of mass filtering and monitoring a defined precursor ion and a characteristic fragment ion, background noise is reduced and specificity is enhanced. Furthermore, monitoring multiple transitions per compound (qualifier and quantifier ions) further increases selectivity.[84]

2. Aims and structure of the thesis

The specific aims of this thesis can be summarized as follows:

1. Optimization of a sample preparation protocol for targeted LC-MS/MS analysis of >200 xenobiotics in DMS.
2. Evaluation of analytical figures of merit of a LC-MS/MS workflow including extraction recovery, matrix effects, limits of detections and compound stability.
3. Application of the optimized method to pooled breast milk from Austria and USA samples as a proof-of-concept study.
4. Analytical performance comparison between conventional paper-based dried milk spots (DMS) and the novel Mitra volumetric absorptive microsampling (VAMS) device.

This thesis is written as a cumulative work that includes an original research article submitted for peer-review and published on a pre-print server.

3. Original work

Status	Pre-print
Title	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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Year	2025 (submitted)
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Keywords	Exposomics, human biomonitoring, public & environmental health, sample stability, early life exposure, volumetric absorptive microsampling (VAMS)
Contribution	Katharina Pfundt optimized the sample extraction procedure, applied the method in the pilot study, evaluated and interpreted the results together with the supervisors, and wrote the initial draft of the manuscript.

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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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-)quantitively assessing >200 xenobiotics in DMS were optimized and thoroughly 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 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 detected in SRM 1954, 22 of which were also found in the Austrian pooled 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 two 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 (VAMS) devices showed comparable performance but differences in background contamination. The developed assay is fit-for-purpose enabling broad exposome-type population studies for investigating early-life exposure patterns.

Keywords: exposomics, human biomonitoring, public & environmental health, sample stability, early life exposure, volumetric absorptive microsampling (VAMS)

1. 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] Exposures mostly occur 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 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] 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 alternative solutions to the limitations presented by dried matrix spots, particularly for quantitative purposes. Mitra microsampling devices are one example based on the VAMS technology (volumetric absorptive microsampling) for the quantitative collection of biofluids, increasing accuracy and consistency in sample collection when compared to traditional filter paper substrates. Different works 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 a targeted LC-MS/MS workflow that includes >200 highly diverse xenobiotics including mycotoxins, bisphenols, PFAS chemicals and other substances. 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 (2d, 2w, 2m). 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.

2. Material and Methods

2.1. 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] 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.

2.2. Samples preparation optimization

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 the tested extraction solutions was added (ACN/MeOH/H_2O 40:40:20 v/v vs. 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 x 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.

2.3. LC-MS/MS instrumentation

An Agilent 1290 Infinity II LC connected to a SCIEX Triple Quad 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 can be found 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**.

2.4. 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).

2.5. Preliminary stability assessment

DMS were spiked before extraction and prepared 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 3 samples and 1 blank, 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).

2.6. 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 5 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 three hours at room temperature and protected from direct light. The final extraction solution (ACN/MeOH/H2O 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.

2.7. 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.

Pre-spiked samples 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 (included all along the analytical acquisition sequence) were excluded in order to ensure data reliability, retaining a total of 137 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 (2d, 2w, 2m), 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 were performed. Compounds presenting a p-value < 0.05 after false discovery rate (FDR) correction in both tests were considered to be significant (i.e., 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.

3. Results and discussion

3.1. Optimization of extraction solution

3.1.1. Extraction recovery

Extraction recoveries could be evaluated for 198 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 is performed against it.

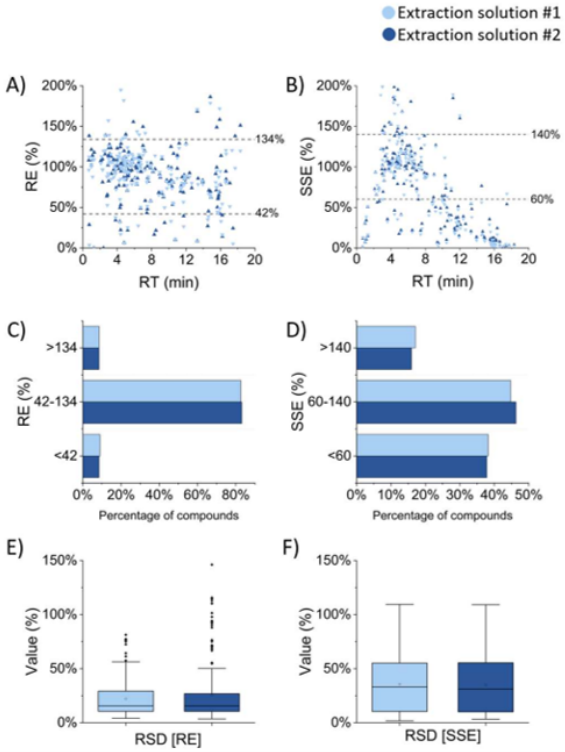

Figure 1: Comparison of two extraction solutions: Extraction solution #1 (ACN/MeOH/H2O 40:40:20 v/v) vs. extraction solution #2 (ACN/MeOH/MTBE/H2O 30:30:20:20 v/v). A) Recovery (RE, %) and B) signal suppression and enhancement (SSE, %) in dependence of chromatographic retention time for >200 highly diverse 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 (%).

To the best of our knowledge, this is the first study to assess the analytical performance of DMS for exposomics 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 x 81 ± 20% at 3 ng/mL), and α-zearalanol (α-ZAL, 68 ± 8% at 1.15 ng/mL x 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).

3.1.2. Matrix effects

Matrix effects could be evaluated for 197 compounds 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/H2O (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.* 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).[40] This comparison suggests that the use of filter paper can amplify the matrix effects, possibly contributing to the observed signal enhancement.

3.1.3. 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 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 of 0.01-0.1 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.

3.1.4. 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 an undesirable source 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), 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] 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**.

3.2. 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 (

Two pharmaceutical drugs were identified, namely acetaminophen and fluconazole. Acetaminophen was detected in the SRM 1954 at a concentration approximately 1,000 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 2 hours after a single 150 mg oral dose.[46] It remains the most frequently prescribed outpatient antifungal in the U.S. (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.*, 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[52], 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 TBBA and its derivates in food, establishing a tolerable daily intake (TDI) of 0.7 µg/mL body weight per day.[53] 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.

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 58 ng/mL and 54 ng/mL.[54,55] 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 US[52], 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.[56] 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**

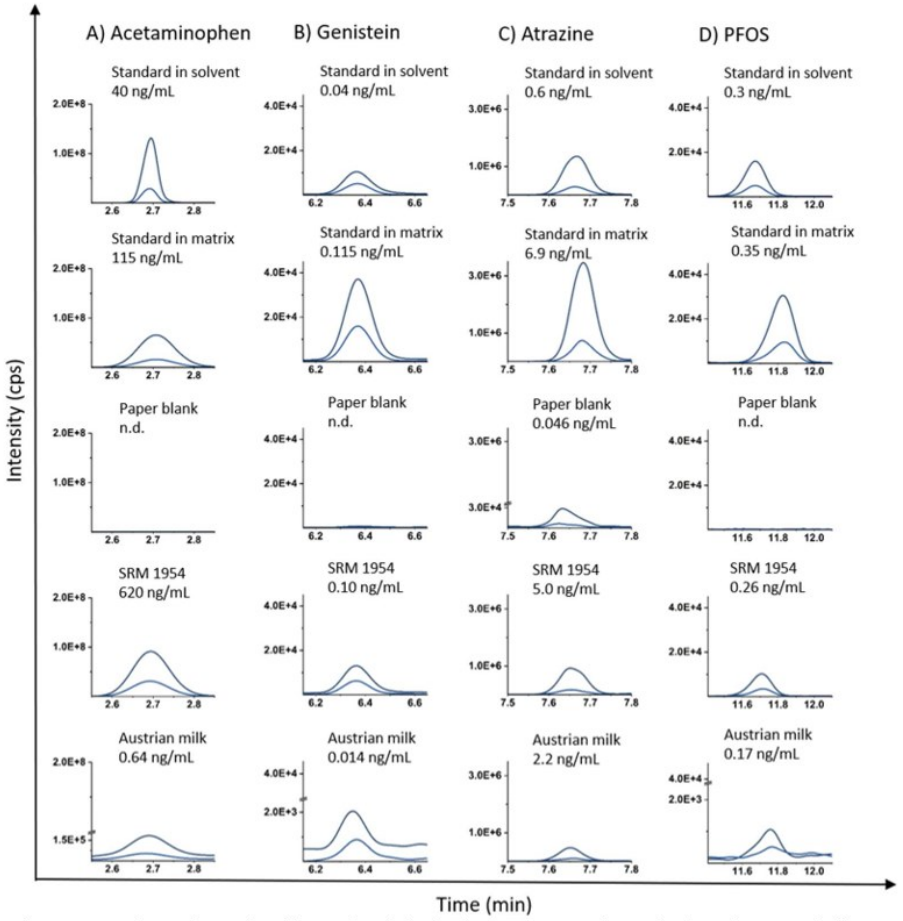

Figure 2. MRM-chromatograms (quantifier and qualifier ions) and calculated concentrations of a standard in solvent, standard in matrix, blank paper substrate, SRM 1954 pooled milk and Austrian pooled milk samples for selected analytes: (A) acetaminophen, (B) genistein, (C) atrazine and (D) PFOS.

Table 1. Detected xenobiotics in SRM 1954 (n=30) and Austrian pooled milk samples (n=22) out of 216 analyzed compounds. Results are reported as mean of 5 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]

Compound	CAS	Quantification confidence level*	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*	6.5 ± 3.8	n.d.
TCBPA	79-95-8	5*	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*	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*	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	5/2/9001	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	2/3/4247	5*	0.041 ± 0.0039	n.d.
4-Methylbenzophenone	134-84-9	2	12 ± 10	19 ± 16
Triclosan	3380-34-5	5*	17 ± 11	n.d.
Plastic components				
Benzylbutyl phthalate	85-68-7	5*	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

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

3.3. 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, 135 (100%), 97 (71%), 103 (75%) and 90 (66%) 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 (i.e., 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 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 04°C, 18°C and 37°C for long-chain parabens.

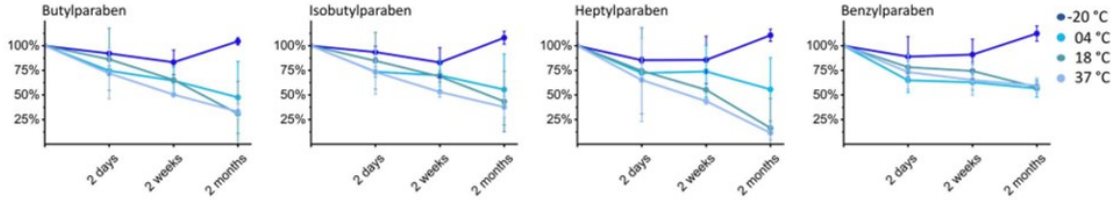

Figure 3. Stability patterns of long-chain parabens over time. Peak areas were normalized to the -80°C samples as a reference (100%) and plotted against storage durations of 2 days, 2 weeks, and 2 months. Short-chain parabens (methyl-, ethyl and propylparaben) remained stable across all conditions, while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

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).[58] A complete overview of the results obtained in the performed statistical analysis is provided in **Table S8**.

3.4. 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 vs. 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). Overall, 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 (BPA, 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% x 97 ± 18%), PFOA (118 ± 10% x 107 ± 19%),

PFHxA (94 ± 7% x 95 ± 16%) and PFNA (109 ± 16% x 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 heterogenous behavior 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 effected 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.

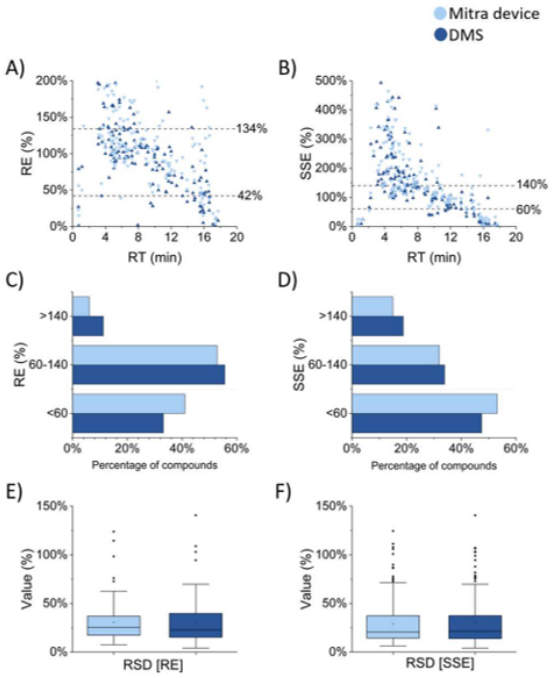

Figure 4. Comparison of two microsampling approaches: Dried milk spots (DMS) and Mitra device. A) Recovery (RE, %) and B) signal suppression and enhancement (SSE, %) in dependence of chromatographic retention time for >200 highly diverse analytes. An overview of method performance is given in C) RE (%), 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.

Background levels of contaminants were assessed as in the first experiment (see section 3.1.4) 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**. Reference value reported by Hernandes *et al.*[1]

4. Limitations

Despite the systematic optimization and extensive assessment of the fit-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 only refers to blood.

5. Conclusion

The presented LC-MS/MS workflow demonstrates the viability of dried milk spots (DMS) as a matrix for multiclass 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. A preliminary stability assessment has shown that

analyte stability was generally maintained at -20 °C for all compounds and for 80% of the analytes during short-term storage at higher temperatures (up to 2 days). Extended storage at 4 °C, 18°C, or 37 °C, however, resulted in significant changes for several compounds, emphasizing the need for proper storage to ensure data reliability. A comparative evaluation of DMS and Mitra VAMS sampling approaches revealed overall good agreement, with the main distinction being differences in 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[59] 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.

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Supplementary data

Supplementary material is available at Exposome online.

7. Authors contribution

Katharina Pfundt (Data curation, Formal analysis, Investigation, Visualization, Writing – original draft), Vinicius V. Hernandes (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing), Benedikt Warth (Conceptualization, Funding Acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing).

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Supplementary Information

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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Supplementary material is available online at
https://chemrxiv.org/engage/chemrxiv/article-details/68dfeddcdfd0d042d1d8e3c1

4. Conclusion

This thesis demonstrated for the first time the feasibility of DMS for multi-class exposure assessment in mothers and infants through a targeted LC-MS/MS workflow. Despite the typically extremely low concentrations of these analytes, the method showed satisfactory performance with respect to SSE, recovery and LODs for a high number of analytes. A proof-of-principle study was conducted using both SRM 1954 and Austrian breast milk samples. In total 30 compounds were detected in SRM 1954 and 22 compounds in the Austrian sample.

The stability of analytes was tested across a range of temperatures from -80°C to 37°C for up to two months. While -20°C and short-term storage of 2 days at higher temperatures showed no to minimal significant changes, extended storage at 4°C, 18°C, and 37°C led to significant effects. This makes the method applicable for field work in the global south and low-resource settings.

A comparative analysis with Mitra VAMS revealed a generally good agreement between the two sampling methods, with notable differences observed only for background contamination. Mitra VAMS offers practical advantages, including ease of use and volumetric accuracy without the need for pipetting, making it a user-friendly option for field and at-home sampling. These benefits are counterbalanced by higher costs and the environmental footprint of single-use plastic devices.

In summary, this work established a solid foundation for using DMS in large-scale human exposure studies and highlighted key considerations for future method development and validation.

Statement

During the preparation of this work the author used ChatGTP in order to polish the language of minor sections. After using this tool/service, the author reviewed and edited the content as needed and takes full responsibility for the content of the published thesis.

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A. Appendix

SOP – Preparation of dried breast milk spots for LC-MS/MS analysis on the Triple Quad™ 7500

Author	Katharina Pfundt
Reviewer	Vinicius Verri Hernandes
Date	06.10.2025
Version	2

Instruments used:

Instrument	**Brand**
Centrifuge	Hettich Mikro 200 R
LC-MS/MS	See Table 1
SpeedVac	CentriVap Vacuum Concentrator and Cold Trap
Thermo shaker	Bosan TS-100
Ultrasonic bath	Bandelin Sonorex RK100
Vortexer	Vortex Genie 2

Working solutions:

ExoMix Cal 1000	Follow preparation draft for ExoMix found in: Z:\12 AK Benedikt Warth\2 Lab Management\2.2 SOPs & Protocols\Under development\240401_ExoMix223
Extraction solvent	ACN/MeOH/H_2O, 40/40/20
Reconstitution solvent	H_2O/ACN, 90/10
Extraction solution	84.4 µL ZEN IS (16 ng/mL) added to 44.9156 mL of extraction solvent
Reconstitution solution 1	60 µL BPA IS (200 ng/mL) added to 5.94 mL of reconstitution solvent
Reconstitution solution 2	18 µL BPA IS (200 ng/mL) and 36 µL ExoMix (Cal1000) added to 1746 µL of reconstitution solvent

Calibration curve:

Calibration point	**Quantity of solution**	**Quantity of solvent (H_2O/ACN, 90/10)**
Cal 02	37.5 µL of Cal 08	112.5 µL
Cal 08	60 µL of Cal 2	90 µL
Cal 2	37.5 µL of Cal 8	112.5 µL
Cal 8	60 µL of Cal 20	90 µL
Cal 20	37.5 µL of Cal 80	112.5 µL
Cal 80	60 µL of Cal 200	90 µL
Cal 200	40 µL of Cal 1000 ExoMix	160 µL

1. Preparation of dried breast milk spots

1.1. Gently thaw an aliquot of breast milk and vortex briefly to homogenize
1.2. Add 40 µL of milk to filter paper (Whatman 903 Proteinsaver card)
1.3. Let filter paper dry, keeping the spots secured from contacting any surface.
1.4. Clean puncher and tweezer with ACN before cutting
1.5. Cut out the whole sample spots using a 1.5cm paper puncher and transfer with tweezer to 1.5 mL Eppendorf tube.
1.6. Ensure the spot to be at the bottom of the tube so it is completely covered by the extraction solvent.

2. Extraction of dried breast milk spots

2.1. Add 1 mL extraction solution
2.2. Shake samples for 5 min at 1200 rpm
2.3. Sonicate for 10 min
2.4. Shake samples for 5 min at 1200 rpm
2.5. Centrifuge at 4°C and 10000 rpm for 10 min
2.6. Transfer 800 µL supernatant to new 1.5 mL tube
2.7. Dry until complete dryness in vacuum centrifuge at 20°C (~24 h)
2.8. Add 80 µL reconstitution solution 1
2.9. Shake samples for 10 min at 1200 rpm
2.10. Centrifuge at 4°C and 10000 rpm for 10 min
2.11. Transfer samples to LC glass vial with a 300 µL glass insert

3. Spiking of dried breast milk spots (Cal 20)

3.1. Pre-spiked: Add 75 µL of ExoMix (Cal1000) to 1425 µL of breast milk, vortex briefly and continue at 1.2
3.2. Post-spiked: Continue at 2.7 and add reconstitution solution 2, proceed with general extraction protocol

LC-MS/MS measurements

Table 1: LC-MS/MS measurements

Instrument	SCIEX Triple Quad™ 7500 LowMass	
Acquisition method	240813_XtremeXeno_Uniformed found in D:\SCIEX OS Data\2024 Dried Milk Spots - VV&KP\Acquisition Methods (on instrument computer)	
Injection volume	5 µL	
Autosampler temperature	7°C	
Column temperature	40°C	
Column	Acquity HSST3 (100mm×2.1mm, 1.8µm)	
Precolumn	Waters VanGuard Acquity HSST3 (5×2.1mm, 1.8µm)	
Flow rate	0.4 mL/min	
Needle wash	Acetonitrile/Water/Methanol/Isopropanol (25/25/25/25 v/v)	
Eluent A	Water + 0.3mmol/L ammonium fluoride	
Eluent B	Acetonitrile	
Gradient	Time (min)	A%
	1	95
	1.8	82
	4.2	65
	13	52
	15.8	10
	15.81	2
	17.6	2
	17.7	95
	20	95
Curtain gas	45	
Gas 1 (psi)	50	
Gas 2 (psi)	50	
Spray voltage (V)	2500 /-2500	
Scan Mode	MRM	

Sequence design

Abbreviation	Sample type	Reason for acquisition
ExtSolv	Extraction solvent	Analysis of background contamination in working solutions
RecSolv	Reconstitution solvent	
Proc_Blank	Process blank	Analysis of background contamination in equipment
PPRBLK	Paper blank	Analysis of background contamination in filter paper
SOLSTD	Standards in solvent	Creating an external calibration curve for quantification
QC	Quality control (non-spiked DMS)	Analysis of system stability throughout the sequence
PRSDMS	Pre-spiked DMS	Calculation of recovery
PTSDMS	Post-spiked DMS	Calculation of matrix effects
SRM1954	SRM 1954 DMS	Analysis of present analytes
NSPDMS	Non-spiked DMS (Austrian milk)	

List of analytical LC-MS sequences

Table 2: Overview of LC-MS/MS measured sequences

Date	Batch name	Sample	Number of injections	Purpose of experiment	Method name	Instrument
06.09.24	240906_DMS_ExtOpt_KP	Human breast milk	157	Method optimization xenobiotics in DMS	240813_XtremeXeno_Uniformed	Triple Qua 7500
23.01.25	250123_DMS_Stability_Mitra_Munich	Human breast milk	267	Stability of xenobiotics in DMS, Comparison DMS/Mitra	240813_XtremeXeno_Uniformed	Triple Quad 7500