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Measuring Polyphenols in Breast Milk by LC-MS/MS

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Abstract

Polyphenols are a diverse group of naturally occurring compounds found in plants, known for their multiple health benefits. Despite their widespread occurrence in various foods and their potentially positive impact on human health, relatively little is known about the lactational transfer of polyphenols through breast milk to infants and their potential health impact on newborns. Exposure to polyphenols during crucial stages of infant development, particularly during the breastfeeding phase, may have a substantial impact on development and later life health outcomes. Existing methods for quantifying polyphenols in breast milk are limited, often focusing on a fraction of the analytes and not comprehensively investigating all main polyphenol classes.

In the scope of this thesis, a targeted LC-MS/MS assay was developed for the quantification of 86 analytes from various polyphenol groups in breast milk. To achieve this, a sample preparation procedure was optimized for the extraction of polyphenols from breast milk. Subsequently, the processed samples were measured using a previously established targeted LC-MS/MS method developed for assessing polyphenols in human urine, serum, and plasma [1]. The developed method was in-house validated according to Eurachem guidelines [2] and the EU Commission decision 2002/657/EC [3]. Out of the 86 analytes included, 59 polyphenols fulfilled all of the stringent criteria and a further nine analytes partially fulfilled the validation criteria. For all analytes, the mean recovery was 81% and the mean signal suppression or enhancement was 117%. Following validation, the method was applied in a pilot study that involved 30 breast milk samples obtained from twelve different Nigerian mothers. In total, 50 different polyphenols were identified in the samples of the pilot study.

The results from the method validation and pilot study were published in *Analytical and Bioanalytical Chemistry* [4]. In the future, this developed method can be used in larger cohort studies to further investigate the role of maternal diet on the polyphenol content of breast milk and the impact of polyphenol intake on infant health.

Zusammenfassung

Polyphenole sind eine vielfältige Gruppe von natürlich vorkommenden Verbindungen in Pflanzen, die für ihre zahlreichen positiven Auswirkungen auf die Gesundheit bekannt sind. Trotz ihres weitverbreiteten Vorkommens in verschiedenen Lebensmitteln und ihres potenziell positiven Einflusses auf die menschliche Gesundheit ist relativ wenig über die Übertragung von Polyphenolen durch die Muttermilch an Säuglinge und deren potenzielle gesundheitliche Auswirkungen auf Neugeborene bekannt. Die Exposition gegenüber Polyphenolen während entscheidender Entwicklungsphasen von Säuglingen, insbesondere während der Stillzeit, kann einen erheblichen Einfluss auf die Entwicklung und die Gesundheitsfolgen im späteren Leben haben. Bestehende Methoden zur Quantifizierung von Polyphenolen in Muttermilch sind begrenzt und konzentrieren sich oft nur auf einen Bruchteil der Analyten, ohne alle Hauptklassen der Polyphenole umfassend zu untersuchen.

Im Rahmen dieser Arbeit wurde ein gerichtetes LC-MS/MS-Verfahren zur Quantifizierung von 86 Analyten aus verschiedenen Polyphenolgruppen in Muttermilch entwickelt. Dazu wurde ein Probenvorbereitungsverfahren zur Extraktion von Polyphenolen aus Muttermilch optimiert. Anschließend wurden die verarbeiteten Proben mit einer zuvor etablierten gerichteten LC-MS/MS-Methode gemessen, die für die Bestimmung von Polyphenolen in menschlichem Urin, Serum und Plasma entwickelt wurde [1]. Die entwickelte Methode wurde gemäß den Eurachem-Richtlinien [2] und der EU-Kommissionsentscheidung 2002/657/EG [3] in-house validiert. Von den 86 einbezogenen Analyten erfüllten 59 Polyphenole alle strengen Kriterien und weitere neun Analyten erfüllten die Validierungskriterien teilweise. Für alle Analyten betrug die durchschnittliche Wiederfindung 81% und die durchschnittliche Signalunterdrückung oder -verstärkung 117%. Nach der Validierung wurde die Methode in einer Pilotstudie angewendet, die 30 Muttermilchproben von zwölf verschiedenen nigerianischen Müttern umfasste. Insgesamt wurden 50 verschiedene Polyphenole in den Proben der Pilotstudie identifiziert.

Die Ergebnisse der Validierung der Methode und der Pilotstudie wurden in *Analytical and Bioanalytical Chemistry* [4] veröffentlicht. Zukünftig kann die entwickelte Methode in Studien mit größeren Kohorten weiter verwendet werden, um die Rolle der Ernährung der Mutter auf den Polyphenolgehalt der Muttermilch und die Auswirkungen der Polyphenolaufnahme auf die Gesundheit von Säuglingen zu untersuchen.

List of abbreviations

Abbreviation	Definition
ACN	Acetonitrile
API	Atomic pressure ionisation
CI	Chemical ionisation
ESI	Electrospray ionisation
GC-MS	Gas chromatography-mass spectrometry
FA	Formic acid
FT-ICR	Fourier transform ion cyclotron resonance
FT-MS	Fourier transform mass spectrometry
HL	High level (spiking level)
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LL	Low level (spiking level)
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass to charge ratio
MeOH	Methanol
ML	Middle level (spiking level)
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NPC	Normal-phase chromatography
Q	Quadrupol
QuEChERS	Quick Easy Cheap Effective Rugged and Safe
R^2	Regression coefficient
RPC	Reversed-phase chromatography
RSD_R	Intermediate precision
RSD_r	Repeatability
SPE	Solid phase extraction
SSE	Signal supression or enhancement
TOF	Time-of-flight
TOF-MS	Time-of-flight mass spectrometry

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1. Introduction

Polyphenols are secondary plant metabolites that have received increasing attention in recent years due to their diverse biological activities and potential health benefits [5, 6]. They are known for their antioxidant capacity, which allows them to scavenge free radicals and chelate metal ions [7, 8]. Research has shown that they also possess anti-inflammatory and antibacterial properties [5, 9], as well as protective effects against various diseases such as cardiovascular disease [10], cancer [11], and neurodegenerative disorders [12, 13]. These naturally occurring phytochemicals can be found in a variety of plants and thus plant-based foods such as fruits, vegetables, nuts, tea, coffee and wine. Due to their widespread occurrence, they are ingested in large quantities through daily food consumption [14].

The structure of polyphenols is fundamentally diverse, ranging from simple phenolic molecules to highly polymerized compounds. The basic structure of all polyphenols contains at least one aromatic ring substituted with one hydroxyl group [15, 16]. In general, polyphenols can be categorized into flavonoids, which include e.g. flavonols, flavanols, flavones, flavanones, isoflavones, and anthocyanins, and into non-flavonoids, which include e.g. phenolic acids, lignans, and stilbenes.

Flavonoids are perhaps the most well-known class of polyphenols and are further divided into several subclasses. Flavonols, such as quercetin and kaempferol, can be found in onions, kale, and broccoli, and are known for their antioxidant properties. These compounds help neutralize free radicals and reduce oxidative stress [17, 18]. Flavanols, including catechins and epicatechins, are abundantly present in tea, cocoa, and grapes. They can be linked to an improved cardiovascular health, as they enhance endothelial function, lower blood pressure, and improve lipid profiles [19]. Isoflavones, e.g. daidzein and genistein, are mostly found in soy products and have been widely studied for their phytoestrogenic activity. These compounds mimic the activity of estrogen in the body, providing relief from symptoms associated with estrogen deficiency [20, 21]. Anthocyanins, which include compounds such as cyanidin and delphinidin, give berries, and red cabbage their vibrant colors. These potent antioxidants have been linked to improved cognitive function and eye health. Anthocyanins protect neural and retinal cells from oxidative stress and inflammation, potentially lowering the risk of neurodegenerative diseases and improving vision [22–24].

Non-flavonoid polyphenols also play significant roles in human health. Phenolic acids, such as caffeic and ferulic acid, are abundant in coffee and whole grains and have been shown to have anti-inflammatory and anticancer activities [25]. These compounds help to

modulate inflammatory pathways and induce apoptosis in various cancer cell lines [26, 27]. Lignans, such as matairesinol and secoisolariciresinol, are found in flaxseeds, sesame seeds, and whole grains. These polyphenols are metabolised by the intestinal microbiota into other compounds such as enterodiol and enterolactone, which exhibit anti-estrogenic properties and can modulate hormone metabolism. Enterolactone and enterodiol have also been shown to exhibit antioxidant, anti-inflammatory, and anticarcinogenic activities, contributing to their protective effects [28, 29]. Stilbenes, e.g. resveratrol, are present in red wine and grapes and have gained attention for their cardiovascular benefits and potential in cancer prevention. Resveratrol has been shown to exhibit antioxidant and anti-inflammatory properties, which contribute to its role in protecting against cardiovascular diseases and different forms of cancer [30–32].

A crucial factor for the health promoting effects of polyphenols is their bioavailability. Despite their high dietary intake, polyphenols often exhibit low bioavailability due to their extensive metabolism by the liver and gut microbiota as well their low level of absorbtion. These metabolic products can sometimes be more active than the parent compounds. Understanding the function and role of polyphenols and their metabolites in different biological matrices can provide valuable insights into their biological activities and potential health benefits [33–35].

Breast milk, for example, is a highly complex biofluid that nourishes and protects infants from disease while their own immune system matures [36, 37]. It is the primary and most ideal source of nutrition for infants, offering a complex and dynamic blend of nutrients, bioactive compounds, and immunological factors which is uniquely tailored to promote a healthy development and growth of infants. Breast milk contains lipids, proteins, carbohydrates, and a wide range of bioactive compounds including immunoglobulins, vitamins and lactoferrin, which are essential for infant health. The composition of breast milk is dynamic and changes depending on several factors such as the different stages of lactation, maternal diet, environmental factors or the storage of the milk [38].

To gain a better understanding of the presence of polyphenols in breast milk and their associated influence on infant health and development, accurate measurement of these compounds in breast milk is essential. A quantitative method to determine the polyphenol concentration in breast milk, would provide help provide insights into the transferability and metabolic transformation of dietary polyphenols from food through the mother into breast milk and from mother to child through lactation. This requires the use of precise and reliable analytical methods, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), known for its high sensitivity, specificity, and ability to simultaneously quantify multiple classes of polyphenols.

2. Methodological Background

2.1. Sample Preparation

2.1.1. Solid Phase Extraction

Solid Phase Extraction (SPE) is a sample preparation technique, used to concentrate and purify analytes from complex matrices, thereby improving the accuracy and sensitivity of the subsequent liquid chromatography (LC) analysis. SPE is a particularly useful for applications involving trace analysis, environmental monitoring, pharmaceutical testing, and biological sample analysis [39].

During the SPE process, the sample is loaded onto the cartridge containing a solid adsorbent material (stationary phase). The analytes of interest interact with the stationary phase and are retained, whereas the unwanted compounds are washed away. This step is followed by an elution phase, in which the retained analytes are desorbed using an appropriate solvent, resulting in a concentrated and purified extract (see Figure2.1) [40].

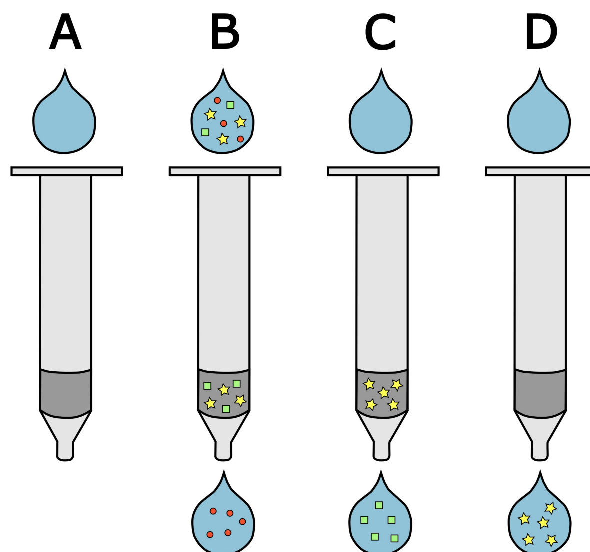

Figure 2.1.: Schematic separation of analytes via solid phase extraction. A: conditioning of the SPE cartridge; B: loading the sample which contains the analytes (stars) and other interfering compounds (squares, circles); C: washing to remove the interferences; D: elution of the analytes

The effectiveness of SPE depends on selecting the right stationary phase for the analytes of interest, and optimizing the conditions for loading, washing, and elution. Factors such as the polarity of the stationary phase, the pH of the sample, and the choice of solvents play a significant role in the efficiency of the extraction [39].

2.1.2. QuEChERS

The QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method is a widely used sample preparation technique. Originally developed for pesticide residue analysis in fruits and vegetables, QuEChERS has been adapted for a broad range of applications, including the analysis of pharmaceuticals, mycotoxins, polycyclic aromatic hydrocarbons, and other environmental contaminants in various biological and environmental samples [41].

The QuEChERS method involves an initial liquid-liquid extraction (LLE) using an organic extraction solvent like acetonitrile, followed by a salting-out step, with salts like magnesium sulfate and sodium chloride, to separate the aqueous and organic phases. The organic phase, that contains the analytes of interest, can then be further purified, for example with solid phase extraction, or directly analyzed by techniques such as gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) [42].

One of the main advantages of the QuEChERS method is its flexibility and adaptability. By adjusting the type of extraction solvent, salt mixtures, and following clean up procedures, the method can be optimized for different analytes and sample matrices, making it a versatile tool in analytical chemistry. Furthermore, QuEChERS are known for their high efficiency, low solvent use, and ability to be automated, rendering it more effective and less prone to human error [43].

2.2. Liquid Chromatography

Liquid chromatography is a versatile analytical technique used to separate and quantify components in a liquid solution. With the aid of a mobile phase (eluent), the liquid sample is passed through a stationary phase (column material). The separation occurs based on the distinct interactions and affinities of each analyte with the stationary and mobile phases, leading to their different elution times. The most commonly utilized LC method is reversed-phase chromatography (RPC), which was also used in this work. In RPC, a stationary phase consisting of non-polar material, e.g. modified silica particles with different alkyl chains (e.g. C18), is combined with a polar mobile phase such as a mixture of water and an organic solvent like acetonitrile. In contrast, normal-phase chromatography (NPC) involves a polar stationary phase, e.g. unmodified silica, and a

non-polar mobile phase, such as hexane [44].

The selection of the appropriate stationary and mobile phases significantly impacts the separation capability of the LC system. Analyte elution can be achieved using two different approaches: isocratic elution or gradient elution. During isocratic elution, the mobile phase remains unchanged throughout the entire LC measurement, whereas with gradient elution, the composition of the mobile phase changes over time, which can improve the separation of analytes with a wide range of polarities. Choosing the elution method depends on the complexity of the sample and the desired resolution. Isocratic elution is simpler and faster for samples with closely related compounds, while gradient elution is more effective for separating complex mixtures with a broad range of retention behaviors [44–46].

2.3. Mass Spectrometry

Mass spectrometry (MS) is an extremely powerful analytical technique that is widely used in various scientific disciplines. It enables the identification, characterization and quantification of molecules based on their mass-to-charge ratios (m/z). The basic principle of MS is to ionize analytes using an ion source and then separate them in a mass analyzer based on their m/z ratio in a magnetic or electric field. In general, there are various types of ionization like electrospray ionization (ESI), chemical ionization (CI), and atmospheric pressure ionization (API), and various types of mass spectrometers utilizing different mass analyzers, such as the quadrupole (Q), time-of-flight (TOF), ion trap, and Fourier transform ion cyclotron resonance (FT-ICR) [47].

2.3.1. Ionization

The electrospray ionisation, used in this work, is a prominent ionization technique often applied in the analysis of biological samples. ESI has the advantage that it is easily coupled with LC and has minimal fragmentation thus can generate intact gas-phase ions.

The principle of ESI is as follows: after the analytes have been separated in the LC, they are transferred to the tip of the ESI needle via the inlet capillary. The speed at which they arrive there is set beforehand by the determined flow rate (in the method used during this work, the flow rate was set at 0.6 mL/min). A high voltage applied to the tip of the ESI needle results in an excess of similarly charged ions. Repulsive forces and the formation of a Taylor cone then releases these ions as a fine aerosol, generating charged droplets containing the analytes. A nitrogen sheath gas flow, operating in a coaxial manner, helps with the nebulization and guides the charged droplets toward the MS. The solvent evaporates as a result of the increased temperature and nitrogen flow. This reduction in droplet size increases the surface charge density, leading to a destabilization. A Coulomb explosion occures, when the Coulomb repulsion surpasses the surface tension.

As a result of this Coulomb explosion smaller droplets are generated. This cycle repeats until all of the solvent is evaporated, and the charge from the ions is then transferred to the analytes (see Figure 2.2) [47].

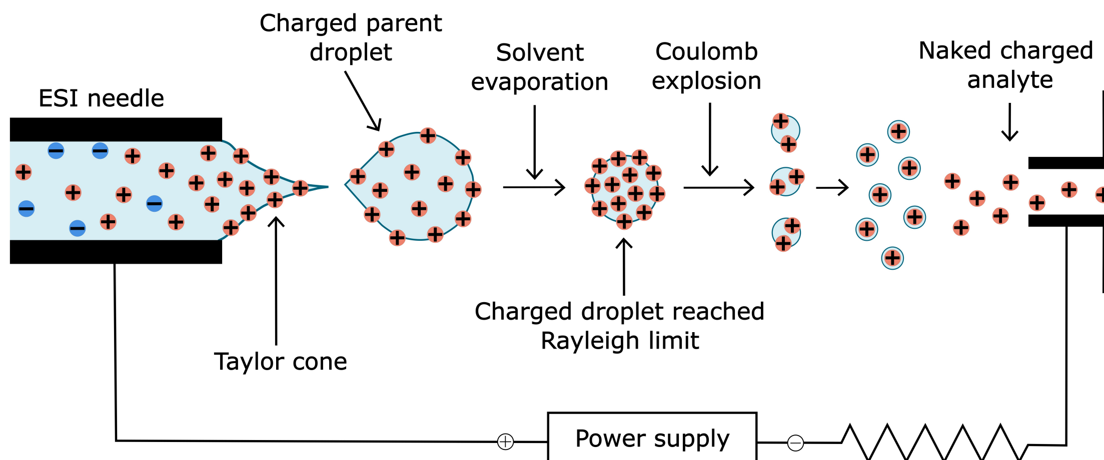

Figure 2.2.: Schematic of the process during electrospray ionization

2.3.2. Tandem Mass Spectrometry

Tandem mass spectrometry (MS/MS) is a type of MS in which two or more mass analyzers are combined. It is a standard instrument in analytical chemistry and is used in a wide range of applications such as metabolomics [48], environmental analytics [49, 50], food analytics [51], and drug discovery [52], due to its high sensitivity, selectivity and ability to identify and quantify molecules in complex samples. One of the most commonly used types of mass spectrometers for MS/MS are triple quadrupole devices, e.g. the QTrap 7500 (SCIEX), used for the experiments in this work. Triple quadrupoles are composed of three quadrupoles connected in series.

A quadrupole (Q) consists of four parallel rod electrodes arranged in a square configuration. Opposing electrodes are connected to each other and subjected to the same electrical voltage. One electrode pair is subjected to a direct current (DC), while the other electrode pair is subjected to a radiofrequency (RF) voltage. The ion beam enters the quadrupole along its axis. Ion separation occurs based on the stable oscillation trajectories of ions within the electric field created by the electrodes. Based on the voltages applied, only ions with a specific m/z-ratio have stable trajectories and can pass through the quadrupole, while other ions with unstable trajectories are deflected, collide with the electrodes, and are neutralized. By continuously changing the applied voltage, the quadrupole can filter and allow different ions to pass through (see Figure 2.3) [47].

The setup of the Qtrap and the multiple reaction monitoring (MRM) measurements was

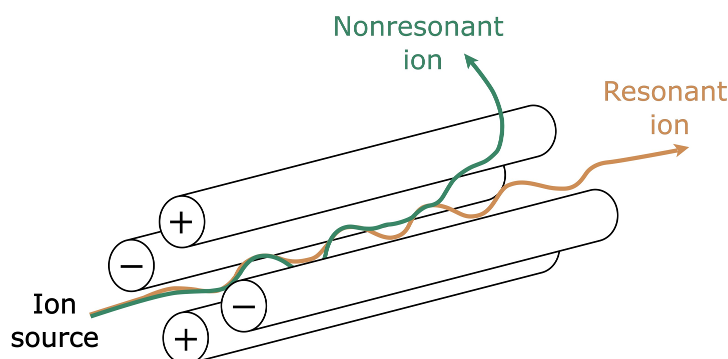

Figure 2.3.: Schematic of a quadrupole. The green arrow shows an ion that fails to meet stability criteria and is deflected. The orange path indicates an ion with a stable trajectory, passing through to the detector.

as follows (Figure 2.4): The ion beam entering the mass spectrometer is focused by the quadrupoles D jet and Q0 and efficiently transferred to Q1. The quadrupole Q1 filters the ion beam for a specifically selected m/z value as the parent ion ("precursor ion"). The parent ion is then fragmented by collision with gas molecules in the collision cell (Q2) and selected fragments ("product ions") are then selected in the third quadrupole (Q3). A significant advantage of MRM is that multiple transitions of several analytes can be measured simultaneously [53].

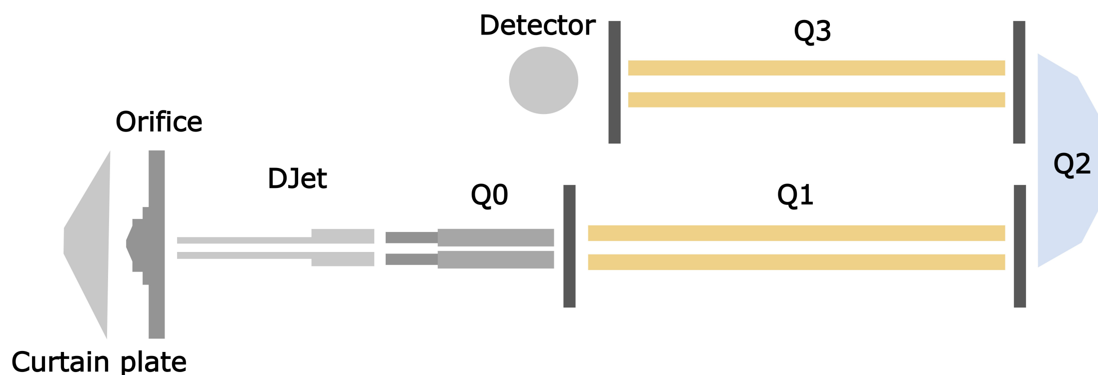

Figure 2.4.: Schematic representation of the QTrap 7500 mass spectrometer used in this work. The ions enter through the curtain plate, are focused in the DJet and Q0, filtered in Q1 for a specific parent ion and broken apart in the collision cell Q2. The emerging product ions are then filtered in the Q3 and detected with the detector.

3. Aims of the thesis

The overall aim of this thesis was to develop a sample preparation method to extract various polyphenols from different polyphenol classes in breast milk and to quantify them using a targeted LC-MS/MS method. The scope of this research can be briefly outlined as follows:

1) Development of sample preparation and transfer of the LC-MS/MS method. Different extraction methods such as liquid-liquid extraction, QuEChERS method, and SPE were tested. The obtained figures of merit were compared, and the most suitable method was selected. For the measurements of the extracts, an established LC-MS/MS method developed by *Oesterle et al.* [1] was transferred to a new mass spectrometer (from QTrap 6500+ to QTrap 7500) and adapted accordingly.

2) Validation of the developed workflow in-house, according to the Eurachem guidelines [2] and the EU Commission decision 2002/657/EC [3]. Therefore, pooled breast milk samples were spiked with a multi-standard mix containing 86 distinct polyphenol analytes, including biotransformation products, extracted using the optimized extraction method, and measured. This process was carried out at three different concentration levels (low, middle, high), in technical triplicates, on three different days over the course of several months.

3) Application of the developed method in a proof-of-principle study. In a pilot study, breast milk samples from twelve Nigerian mothers, collected at different lactation stages (one, six, and twelve months after childbirth), were analyzed for their polyphenol content.

4. Original Work

Status	Published
Title	Polyphenol exposure of mothers and infants assessed by LC-MS/MS-based biomonitoring in breast milk
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Contribution	Sabrina Berger optimized the sample preparation procedure, performed the validation experiments, applied the method in the pilot study, evaluated and interpreted the results together with the supervisors, and wrote the initial draft of the manuscript.

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RESEARCH PAPER

Polyphenol exposure of mothers and infants assessed by LC–MS/MS based biomonitoring in breast milk

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Abstract
Exposure to polyphenols is relevant throughout critical windows of infant development, including the breastfeeding phase. However, the quantitative assessment of polyphenols in human breast milk has received limited attention so far, though polyphenols may positively influence infant health. Therefore, a targeted LC–MS/MS assay was developed to investigate 86 analytes representing different polyphenol classes in human breast milk. The sample preparation consisted of liquid extraction, salting out, freeze-out, and a dilution step. Overall, nearly 70% of the chemically diverse polyphenols fulfilled all strict validation criteria for full quantitative assessment. The remaining analytes did not fulfill all criteria at every concentration level, but can still provide useful semi-quantitative insights into nutritional and biomedical research questions. The limits of detection for all analyzed polyphenols were in the range of 0.0041–87 ng*mL^{-1}, with a median of 0.17 ng*mL^{-1}. Moreover, the mean recovery was determined to be 82% and the mean signal suppression and enhancement effect was 117%. The developed assay was applied in a proof-of-principle study to investigate polyphenols in breast milk samples provided by twelve Nigerian mothers at three distinct time points post-delivery. In total, 50 polyphenol analytes were detected with almost half being phenolic acids. Phase II metabolites, including genistein-7-β-D-glucuronide, genistein-7-sulfate, and daidzein-7-β-D-glucuronide, were also detected in several samples. In conclusion, the developed method was demonstrated to be fit-for-purpose to simultaneously (semi-) quantify a wide variety of polyphenols in breast milk. It also demonstrated that various polyphenols including their biotransformation products were present in breast milk and therefore likely transferred to infants where they might impact microbiome development and infant health.

Keywords Polyphenols · Breast milk · Tandem mass spectrometry · Human biomonitoring · Exposome research

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Introduction

Polyphenols are secondary plant metabolites that contain a minimum of one aromatic ring substituted with at least one hydroxyl group [1, 2]. In general, they can be split into two major groups, flavonoids and non-flavonoids, that can be further divided into several classes (see Fig. S1). Examples of flavonoids are flavanones, flavones, flavonols, isoflavones, and proanthocyanidins, whereas non-flavonoids contain stilbenes, lignans, and phenolic acids such as hydroxybenzoic acids, hydroxycinnamic acids, and hydroxyphenylacetic acids. As polyphenols typically have functional groups, namely hydroxyl groups, they are frequently and abundantly conjugated by xenobiotic metabolizing enzymes in the human body. The resulting conjugates, mostly glucuronides and sulfates, are commonly found in human biofluids, especially in urine. The biotransformation of xenobiotics,

including polyphenols, impacts their chemical properties and bioavailability [3].

Polyphenols are widely studied due to various health benefits, including antibacterial, anti-inflammatory, and antioxidant properties [4–7]. Current research indicates that polyphenols may contribute to a reduced risk of noncommunicable diseases such as cancer [8], cardiovascular disease [9], and neurodegenerative disorders [10, 11]. Besides potentially beneficial effects, polyphenols may also exhibit adverse properties that depend on various factors, e.g., dosage and environmental interactions [12]. These adverse human effects of polyphenols include reducing iron absorption [13–15], interactions with drugs and other xenobiotics [16–19], inhibiting of digestive enzymes [12], and affecting the hormonal balance [20, 21]. For example, combinatory effects between polyphenols and mycotoxins may contribute to increased estrogenic effects of both the polyphenols and the mycotoxins [22]. In addition to their bioactive properties, polyphenols are of great interest as they are a class of molecules prevalent in numerous plant-based foods including fruits, vegetables, grains, tea, cocoa, and coffee [23–25].

Due to the health-promoting effects of polyphenols and their prevalence in foodstuff, significant research interest exists in evaluating uptake, distribution, metabolism, and excretion of polyphenols in humans. In particular, the question arises if polyphenols are present in breast milk and follow lactational transfer to infants, and if so, whether they have a positive or negative influence on infant health, such as aiding in preventing the development of chronic diseases [26] or modulating microbiome development [27]. In general, breast milk is considered the ideal food for infants. The World Health Organization (WHO) recommends exclusively breastfeeding infants for the first 6 months of life and to continue breastfeeding following the introduction of complementary foods for up to 2 years or longer [28, 29]. To determine the potential impact of polyphenols on infant development and health during this critical window of susceptibility, reliable quantification in breast milk is needed. This would allow investigation of the transfer and biotransformation of ingested polyphenols from the diet of the mothers to their breast milk and subsequently their infants. Moreover, this information would yield new insights to pediatricians and mothers, potentially allowing a tailored adjustment of their diet to positively impact their infant's health. For example, it could be investigated if the consumption of a polyphenol-rich diet may be an alternative to antibiotics for either treating or preventing (mild) urinary tract infections in susceptible neonates and infants.

Since polyphenols are an extensive family of diverse molecules containing many different classes, it is advantageous to quantify individual polyphenols rather than simply the total polyphenol content [30]. Therefore, a suitable sample preparation approach and a sensitive and specific analytical method are required for the comprehensive quantification of polyphenols. An essential technique in modern human biomonitoring is liquid chromatography coupled to triple quadrupole mass spectrometry (LC–MS/MS) using multiple reaction monitoring (MRM) mode [31]. Targeted LC–MS/MS allows to selectively detect and quantify specific analytes with a high sensitivity, specificity, and accuracy.

Therefore, the aim of this study was to develop and in-house validate a targeted LC–MS/MS method to quantify a comprehensive selection of analytes representing all main polyphenol classes in human breast milk. This involved transferring a previously published method for polyphenols in other human specimens (urine, serum, and plasma) [32] to the highly complex breast milk matrix. A sample preparation method was developed to extract 86 polyphenols representing 15 different chemical classes for broad coverage. After optimization, the method was validated and applied in a pilot study to prove its suitability and fit-for-purpose.

Materials and methods

Chemicals, reagents, and solvents

Information on the reference standards, reagents, and solvents used during method development, validation, and pilot study are available in Table S1 in the supplementary information (SI). Single standard stock solutions were prepared by dissolving the solid polyphenol standards in methanol (MeOH), as described by Oesterle et al. [32]. For optimization of the sample preparation and the method validation, individual stock solutions were mixed at different concentrations and diluted with MeOH to prepare multiple working solutions with concentrations between 0.2 and 130,000 ng*mL^{-1}. All working and individual standard solutions were stored at − 20 °C.

Sample preparation

As breast milk is a highly complex biological matrix, different sample preparation approaches were tested and optimized, including solid phase extraction (SPE) with *Waters* Oasis cartridges. The final optimized sample preparation protocol was established as follows: to an aliquot of 200 µL of human breast milk, 400 µL of acetonitrile (ACN) acidified with 1% v/v formic acid (FA) was added and thoroughly vortexed for 3 min. Subsequently, 80 mg anhydrous magnesium sulfate and 20 mg sodium chloride were added, and the sample was again vortexed for 3 min. The sample was then centrifuged for 10 min (2000 × g, 4 °C), and the supernatant was chilled for 2 h at − 20 °C. Following the freeze-out step,

the sample was centrifuged for 2 min (18,000 × g, 4 °C) and the supernatant diluted 1:1 with acidified water (1% v/v FA). The sample was then centrifuged for 5 min (18,000 × g, 4 °C) and the supernatant was transferred to an amber LC glass vial. Enzymatic deconjugation was not performed as several conjugated reference standards were included in the method for direct determination and because deconjugation enzymes are typically contaminated with a high number of xenobiotics, especially polyphenols [33].

LC–MS/MS instrumentation

The UHPLC-ESI-QTrap-MS/MS system used was composed of a 1290 Infinity II LC (*Agilent*) connected to a QTrap 7500 MS (*Sciex*), equipped with a heated electrospray ionization source (ESI). Data was acquired in scheduled multiple reaction monitoring (sMRM) mode using fast polarity switching. An optimized LC–MS/MS method that was previously developed for the measurement of polyphenols in other complex biological matrices, i.e., urine, serum, and plasma [32], was transferred from a QTrap 6500^+ to a QTrap 7500 system and used as the basis for the breast milk assay described here. The majority of the LC and MS parameters remained the same; however, some parameters, such as retention times, retention time windows, and declustering potential, were adjusted accordingly (Table S2). A VanGuard precolumn (1.8 µm, *Waters*) attached to an Acquity UPLC HSS T3 column (1.8 µm, 2.1 × 100 mm, *Waters*) was used to achieve chromatographic separation. The temperature of the column compartment was set to 30 °C and of the autosampler to 7 °C. The mobile phases used were 0.1% v/v FA in H_2O (eluent A) and 0.1% v/v FA in ACN (eluent B). The injection volume was 3 µL and the flow rate was set to 0.6 mL*min^{-1}. The gradient (Table S3) started with 5% eluent B and was held for 2 min. Afterwards, eluent B was raised linearly to 64% within 10 min and then increased to 95% for a 2 min hold. Eluent B was then immediately decreased to 5% for a final 2 min re-equilibration step. The following ESI parameters were used: curtain gas 35 arb, sheath gas 90 arb, drying gas 90 arb, collision gas set to medium, source temperature 550 °C, and entrance potential at 10 V in positive and − 10 V in negative mode. The voltage of the ion capillary was set to 5500 V in positive and − 4500 V in negative mode.

Validation experiments

The method was validated in-house following the guidelines set by Eurachem [34] and the EU Commission decision 2002/657/EC [35]. Analytical figures of merit including selectivity, repeatability (RSD_r), intermediate precision (RSD_R), regression coefficient (R^2), recovery (R_E), and signal suppression or enhancement (SSE) were evaluated at three concentration levels.

Due to a lack of matrix-matched reference material, multiple breast milk samples were pooled and used as "blank" breast milk for spiking experiments and for the matrix-matched calibration curves [36]. For spiking and creation of the calibration curves, a multi-standard working solution was prepared from the individual polyphenol stock solutions. This working solution was then serially diluted to create five additional multi-standard working solutions. With these six working solutions, a six-point neat solvent (ACN:H_2O:FA, 49.5:49.5:1) and a matrix-matched calibration curve (calibration ranges are reported in Table 1) were prepared. During method optimization, a multi-standard solution was measured to estimate the LOQs of the analytes. Based on these values, the calibration points for each analyte were set as 0.33, 1, 3, 10, 30, and 100 times their respective estimated LOQ. Matrix-matched samples were spiked at three different concentration levels: low, middle, and high (Table S4) before the sample preparation procedure (pre-spiked samples). For each validation experiment, triplicates of the pre-spiked samples were prepared at each spiking level. Overall, three individual validation experiments were performed over the course of 3 months, and one of the validation experiments included two additional re-measurements of the acquisition sequence on the same day to determine the intraday stability (RSD_r) of the method. To ensure the selectivity of the method, solvent and matrix-matched blanks and spiked samples were examined for any potential interfering signals throughout the validation procedure.

The recovery was calculated by dividing the measured concentration of the pre-spiked samples by the theoretical concentration spiked at each of the three different levels. For each spiking level, the overall recovery was calculated as the mean of all measurements ($n = 9$). Limit of detection (LOD) was evaluated by dividing the standard deviation of the measured concentration of the pre-spiked samples (low level) by the square root of the number of replicates of all measurements ($n = 9$) and multiplying it by three. The limit of quantification (LOQ) was defined as two times the LOD. Intermediate precision and repeatability were evaluated at each spiking level. The intermediate precision was defined as the relative standard deviation of the measured concentration of the nine pre-spiked samples from the three separate validation experiments, measured on different days. Intraday repeatability was defined as the relative standard deviation of the measured concentration of the nine pre-spiked samples from the validation experiment that was measured three times on the same day. The regression coefficient from each matrix-matched calibration curve was calculated. Signal suppression and enhancement (SSE) effect was calculated by dividing the slope of the matrix-matched calibration curve by the slope of the solvent calibration curve and expressed

Table 1 Range of the calibration curve, regression coefficient (R^2), signal suppression and enhancement (SSE), limit of detection (LOD), limit of quantification (LOQ), and the mean recovery (R_E) of the three spiking levels for each analyte as evaluated during in-house validation. Parameters that could not be determined are listed as n.d

Analyte	CAS number	Calibration range (ng*mL^{-1})	R^2	SSE (%)	LOD (ng*mL^{-1})	LOQ (ng*mL^{-1})	R_E (%)
Dihydrochalcones							
Phloretin	60–82-2	0.015–7.2	0.991	114	0.017	0.034	96
Hydroxybenzoic acids							
3,5-Dihydroxybenzoic acid	99–10-5	0.22–29[a]	0.903	120	0.41	0.82	44
3-Hydroxybenzoic acid	99–06-9	1.2–590	0.989	109	0.84	1.7	96
4-Hydroxybenzoic acid	99–96-7	0.08–35	0.988	109	0.19	0.38	88
Benzoic acid	65–85-0	3.5–1600	0.976	107	46[b]	92[b]	84
Ellagic acid	476–66-4	1.74–78[c]	0.991	163	8.9[b]	18[b]	8
Ethyl gallate	831–61-8	0.004–2.4	0.992	113	0.0024	0.0048	88
Gallic acid	149–91-7	0.023–3.0[a]	0.905	129	0.028	0.056	33
Protocatechuic acid	99–50-3	0.015–6.6	0.873	112	0.059	0.12	42
Salicylic acid	69–72-7	0.2–27[a]	0.994	124	0.48	0.96	86
Syringic acid	530–57-4	0.022–11	0.992	112	0.068	0.14	95
Vanillic acid	121–34-6	0.16–70	0.988	111	0.17	0.33	94
Hydroxycinnamic acids							
Caffeic acid	501–16-6	0.3–130	0.993	110	0.55	1.1	88
Caffeic acid-3-β-D-glucuronide	1093679–73-2	0.014–6.8	0.991	107	0.0085	0.017	69
Chlorogenic acid	327–97-9	0.29–38[a]	0.996	112	0.32	0.65	60
Cinnamic acid	621–82-9	1.5–650	0.994	113	2.1	4.3	90
Dihydrocaffeic acid	1078–61-1	0.082–36	0.996	110	0.16	0.33	88
Dihydroferulic acid	1135–23-5	0.11–49	0.989	114	0.35	0.71	100
Ferulic acid/Isoferulic acid	537–98-4/537–76-5	0.058–26	0.994	110	0.096	0.19	89
p-Coumaric acid	501–98-4	0.044–19	0.990	109	0.046	0.092	94
Sinapic acid	530–59-6	0.022–11	0.993	110	0.087	0.17	96
trans-m-Coumaric acid	588–30-7	0.25–110	0.992	107	0.69	1.4	86
trans-o-Coumaric acid	583–17-5	0.13–64	0.994	107	0.2	0.4	94
Hydroxyphenylacetic acids							
3-(3-Hydroxyphenyl)propionic acid	621–54-5	0.09–37	0.990	108	0.38	0.76	95
3-Hydroxyphenylacetic acid	621–37-4	2–890	0.990	107	4.5	9	98
4-Hydroxyphenylacetic acid	156–38-7	2.6–1100	0.991	104	5.9	12	94
Homoprotocatechuic acid	102–32-9	0.4–182	0.992	107	1.7	3.4	93
Homovanillic acid	306–08-1	0.73–331	0.991	109	0.64	1.3	97
Lignans							
Enterodiol	80226–00-2	0.005–2.1	0.987	109	0.017	0.034	88
Enterolactone	78473–71-9	0.014–6.8	0.992	117	0.018	0.036	89
Others							
2,6-Dimethoxyphenol	91–10-1	0.05–21	0.986	106	0.061	0.12	98
3,5-Dimethoxy-4-hydroxyphenylacetic acid	4385–56-2	0.31–140	0.993	109	0.43	0.86	97
3-Methylcatechol	488–17-5	0.15–67	0.992	109	0.16	0.32	94
4-Methylcatechol	452–86-8	0.33–150	0.993	110	0.24	0.48	94
Catechol	120–80-9	3.3–130[a]	0.759	110	5	10	57
Eugenol	97–53-0	2.2–990	0.985	103	4.7	9.4	91
Hydroxytyrosol	90–05-1	0.041–19	0.992	112	0.034	0.068	90
Pyrogallol	10597–60-1	0.42–190	0.985	127	1.4	2.8	91
Thymol	89–83-8	0.67–300	0.937	99	69[b]	140[b]	76
Urolithin A	1143–70-0	0.007–3.6	0.992	111	0.014	0.028	90

Table 1 (continued)

Analyte	CAS number	Calibration range (ng*mL^{-1})	R^2	SSE (%)	LOD (ng*mL^{-1})	LOQ (ng*mL^{-1})	R_E (%)
Stilbenes							
Dihydroresveratrol	58,436–28-5	0.035–16	0.991	109	0.054	0.11	91
Polydatin	65914–17-2	0.012–5	0.988	106	0.061	0.12	99
Pterostilbene	537–42-8	0.016–7	0.994	121	0.034	0.068	94
Resveratrol	501–36-0	0.03–14	0.992	113	0.043	0.086	89
Anthocyanins							
Cyanidin	87725–42-6	4.4–2000[a]	0.975	115	39[b]	78[b]	46
Cyanidin-3-O-glucoside	47705–70-4	0.06–8.1[a]	0.995	140	0.18[b]	0.36[b]	15
Cyanidin-3-O-rutinoside	28338–59-2	0.052–6.9[a]	0.995	136	0.26[b]	0.52[b]	21
Cyanidin-3-O-sambubioside	63535–17-1	0.1–15[a]	0.996	138	0.29[b]	0.58[b]	7
Delphinidin	528–53-0	5.3–703[a]	0.959	178	28[b]	56[b]	23
Delphinidin-3-O-glucoside	50986–17-9	2.1–300[a]	0.981	160	11[b]	22[b]	10
Catechins							
(-)-Epicatechin	490–46-0	0.2–90	0.992	107	0.24	0.48	90
(-)-Epicatechin gallate	1257–08-5	0.08–36	0.989	117	0.13	0.26	74
(-)-Epigallocatechin	970–74-1	1.4–620	0.968	123	5.3	11	92
(-)-Epigallocatechin gallate	989–51-5	1–440	0.970	135	4.2	8.4	74
(-)-Gallocatechin	3371–27-5	1.4–620	0.983	250	4.9	9.8	86
(+)-Catechin	154–23-4	0.12–53	0.993	113	0.17	0.34	83
Flavanones							
(+/-)-Naringenin	153–18-4	0.008–1.1[a]	0.992	121	0.036	0.072	86
8-Prenylnaringenin	53846–50-7	0.02–8.8	0.992	114	0.016	0.032	93
Hesperetin	520–33-2	0.009–3.8	0.993	114	0.013	0.026	96
Hesperidin	520–26-3	0.006–2.6	0.991	120	0.0094	0.019	93
Isoxanthohumol	521–48-2	0.004–1.8	0.994	115	0.0054	0.011	93
Naringin	10236–47-2	0.23–100	0.989	112	0.56	1.1	84
Neohesperidin	13241–33-3	0.3–140	0.992	110	0.6	1.2	85
Neohesperidin dihydrochalcone	20702–77-6	0.006–2.6	0.994	109	0.0085	0.017	85
Xanthohumol	6754–58-1	0.012–5.3	0.992	109	0.017	0.034	91
Flavones							
Apigenin	520–36-5	0.009–3.9	0.988	117	0.0047	0.0094	89
Diosmetin	520–34-3	0.005–2.9	0.990	112	0.015	0.03	93
Diosmin	520–27-4	0.024–11	0.982	119	0.069	0.14	100
Flavonols							
(+)-Rutin	480–41-1	0.03–13	0.988	106	0.031	0.062	67
Isorhamnetin	480–19-3	0.006–2.6	0.993	119	0.0089	0.018	79
Kaempferol	520–18-3	0.12–60	0.990	121	0.17	0.34	90
Kaempferol-3-O-glucuronide	22688–78-4	0.008–3.5	0.992	111	0.013	0.026	87
Quercetin	117–39-5	0.052–7.1[a]	0.991	129	0.12	0.24	63
Quercetin-7-O-β-D-glucuronide	38934–20-2	0.031–14	0.989	121	0.066	0.13	62
Isoflavones							
Biochanin A	491–80-5	0.009–3.9	0.994	117	0.014	0.028	90
Daidzein	486–66-8	0.01–4.8	0.992	109	0.034	0.068	89
Daidzein-7-β-D-glucuronide	38482–80-3	0.032–14	0.993	107	0.063	0.13	88
Genistein	446–72-0	0.01–4.3	0.977	119	0.0047	0.0094	79
Genistein-7-β-D-glucuronide	38482–81-4	0.04–16	0.993	108	0.054	0.11	92
Genistein-7-sulfate	182322–62-9	0.27–13[d]	0.930	105	0.18	0.36	59
S-Equol	531–95-3	0.42–190	0.986	106	2.2	4.4	88

Table 1 (continued)

Analyte	CAS number	Calibration range (ng*mL^{-1})	R^2	SSE (%)	LOD (ng*mL^{-1})	LOQ (ng*mL^{-1})	R_E (%)
Proanthocyanidins							
Procyanidin A2	41743–41-3	0.07–31	0.983	108	0.24	0.48	82
Procyanidin B1	20315–25-7	1.1–500	0.994	106	1.1	2.2	67
Procyanidin B2	29106–49-8	0.3–130	0.950	108	16[b]	33[b]	69
Procyanidin C1	37064–30-5	0.21–93	0.986	113	0.62	1.2	60

[a]The maximum concentrations of the calibration curve exceeded the range of linearity, thus the highest calibration point was excluded

[b]No chromatographic peak at the lowest spiking level thus, the standard deviation of the next highest spiking level with a chromatographic peak was used to calculate LOD and LOQ

[c]The two highest concentrations of the calibration curve exceeded the range of linearity, thus they were excluded

[d]The concentrations of the calibration curve were chosen too high, and the limit of linearity was reached. Therefore, the three highest calibration points were excluded

as percentage. Therefore, a SSE value below 100% indicates signal suppression, while a SSE value greater than 100% indicates signal enhancement [37]. The mean of the regression coefficients and the signal suppression and enhancement effect over the three validation experiments were calculated and reported. Ensuring the evaluation of these validation figures of merit for each analyte, the following criteria for validation requirements were used: a recovery between 50 and 120%, a regression coefficient of at least 0.95, and repeatability and intermediate precision below 45%, 30%, and 25% for low, middle, and high spiking levels, respectively. The repeatability and intermediate precision criteria were determined with the Horwitz equation [34].

Data analysis, peak integration, and concentration calculations were evaluated with *SCIEX OS (v3.0)*. All chromatographic peaks were smoothed with a low-grade filter. A 1/x weighting was applied to all calibration curves. Standard addition was applied to the calibration curves of analytes in which a signal was detected in the non-spiked matrix-matched samples. Calculations of the standard addition and the other validation figures of merit were performed in *Excel 16.0*.

Biological samples

The pooled breast milk used for method development and validation was kindly provided by the Semmelweis Women's Clinic in Vienna [36, 38]. The proof-of-principle experiments included aliquots of breast milk samples from a previous study conducted by Ayeni et al. [39] that explored mycotoxin exposure patterns in different biological matrices and a potential impact on gut microbiome development. Details of sample collection are reported in Ayeni et al. [39]. In brief, breast milk samples were collected from twelve Nigerian mothers from Ilishan-Remo, Ogun state. The mothers' age ranged between 25 and 40 years, and their diet consisted of various cereal-based foods (e.g., bread, rice, ogi), tubers (yam, cassava), legumes (e.g., beans), vegetables (e.g., okra, onion), fruits (e.g., tomatoes, oranges, apples, bananas), fish, and meat. The breast milk was expressed manually by the mothers and stored in a fridge overnight until they were collected by trained study personnel and stored at − 20 °C. The samples were transported on dry ice to the laboratory in Vienna for mass spectrometric analysis. Ethical approval was obtained from the Ethical Committee of Babcock University (BUHREC421/21R, BUHREC466/23). Prior to their inclusion in the studies, all mothers were informed and provided written consent.

For the positive identification of the polyphenol analytes in the biological samples, stringent criteria were defined. Analytes with a retention time deviation greater than 0.05 min compared to their respective matrix-matched calibration curve were excluded. Additionally, only analytes that had both the quantifier and qualifier ions present, with an ion ratio deviation of less than 20% compared to their respective matrix-matched calibration curve, were considered. For analytes that showed a chromatographic signal near the LOD, an ion ratio deviation of up to 50% was considered acceptable, since the background noise has a strong influence on the ion ratios at these low concentrations. For all positively identified analytes, the concentration was determined using the matrix-matched calibration curve and corrected with the recoveries calculated during method validation.

Results and discussion

Method optimization

Extracting a wide range of analytes from a complex biological matrix such as breast milk is a challenging task. In several studies, a QuEChERS approach (quick, easy, cheap,

effective, rugged, and safe) has been successfully utilized to extract analytes such as pesticides [38, 40, 41] and other xenobiotics [42–44] from foods with a high lipid content. Few studies [45, 46] investigated the quantification of polyphenols in breast milk, but these did not include as many analytes from multiple polyphenol classes. Moreover, the method presented here includes phase II metabolites of polyphenols such as sulfates and glucuronides, whereas previous studies from Song et al. [45] and Lu et al. [46] used β-glucuronidase/sulfatase treatment to deconjugate potential phase II metabolites. As a starting point, a method established for quantifying mycotoxins in breast milk was selected [36, 47]. This method combined a QuEChERS approach with a freeze-out step, a SPE cleanup, and an evaporation step. Here, in the first step, the procedure was scaled down in order to use a reduced volume of breast milk (200 µL instead of 1 mL) and improve the high-throughput feasibility. However, the results showed low recoveries and severe matrix interferences. Therefore, various extraction solvents, including ACN, MeOH, and hexane, both pure and acidified with up to 3% v/v FA, were tested at different extraction ratios (solvent to breast milk), e.g., 1:1 v/v, 2:1 v/v, and 3:1 v/v, for lipid removal. Different approaches were also tested to optimize the SPE step, including acidifying the ACN with up to 3% FA used to elute the analytes from the C18 SPE cartridges (Oasis HLB Prime, 1 cc, 30 mg, *Waters*). Protocols with and without the SPE step as well as procedures with and without the drying step with a vacuum concentrator were additionally tested.

The final, optimized sample preparation procedure, which yielded the overall best analyte recoveries with the least signal suppression/enhancement effects, is described in "Sample preparation." In brief, the procedure contained a liquid extraction step with acidified ACN, a salting-out step with anhydrous magnesium sulfate and sodium chloride, a freeze-out step, and finally a dilution step with acidified H_2O.

Validation experiments

Overall, the in-house validation was successful with 59 out of 86 (69%) of the polyphenol analytes fulfilling all validation criteria at all three spiking levels. An additional ten polyphenols (11%) passed all validation criteria for the medium and high spiking levels. A summary of the validation results is listed in Table 1 and the detailed results are reported in Tables S4–S5. In comprehensive multi-analyte human biomonitoring assays, pragmatic compromises are essential to keep a fine balance between covering as many analytes as possible while ensuring high sensitivity and minimal matrix interferences [48]. Consequently, it was not expected that all 86 analytes will perform ideally applying this method. For the polyphenols that did not fulfill all the strict validation figures of merit, semi-quantification is still possible and can be helpful in comprehensive exposome studies as well as for answering biological and nutrition-related questions. The selectivity of the method was evaluated by comparing the matrix-matched samples enriched with standards to the matrix-matched "blank" and solvent samples enriched with standards. No interferences were detected for the majority of the analytes. Due to a lack of available reference material, the biological matrix used was not a true "blank", thus, several analytes, e.g., (+/-)-naringenin, had a chromatographic peak present in the matrix-matched "blank" (Table S4). Consequently, standard addition was applied for these analytes. Moreover, despite having individual standards for the isomers ferulic acid and isoferulic acid, these two analytes co-eluted and were acquired as a sum parameter because the same MRM transitions were observed during MS parameter optimization.

The recovery, intermediate precisions, and repeatability of the method are listed in Table S4, with the mean recoveries also reported in Table 1. For 70% of all analytes, the mean recoveries, calculated from the three spiking levels of each analyte, were in the range of 80–120%. The intermediate precision of the low, middle, and high spiking levels was in the ranges of 5–61%, 4–56%, and 7–62%, respectively, and the repeatability for the three spiking levels was in the ranges of 4–87%, 3–59%, and 2–71%, respectively. These results demonstrate the overall stability of the workflow for most analytes, both intraday and interday when taking into account that the higher values were typically derived from very few analytes for which full quantitative assessment was not intended by design. The LOD and LOQ values, calibration range, regression coefficient, and SSE are reported in Table 1. The linear calibration curves of each analyte from one validation sequence are depicted in Table S7. It was observed that the regression coefficients for all analytes were between 0.76 and 0.996, with a median R^2 of 0.991. Moreover, 93% of all analytes had a regression coefficient greater than 0.95. For some analytes, the maximum concentrations chosen for calibration were too high and exceeded the linear range of the detector; thus, the highest points of the calibrations were removed (Table S4). As expected, the limits of detection varied greatly between the different analytes and the polyphenol classes. The LODs for all analytes ranged between 0.0041 and 87 ng*mL^{-1}, with a median LOD of 0.17 ng*mL^{-1}. Many of the included polyphenol classes showed very low LODs, such as flavanones, flavonols, hydroxycinnamic acids, isoflavones, and stilbenes with LODs ranging from 0.0069 to 0.48 ng*mL^{-1}, 0.015–0.15 ng*mL^{-1}, 0.014–2.5 ng*mL^{-1}, 0.0041–1.9 ng*mL^{-1}, and 0.039–0.069 ng*mL^{-1}, respectively. The SSE was evaluated throughout the validation procedure by comparing the slope of the matrix-matched calibration curve with that of the solvent calibration curve. The SSE was calculated in a manner that a value of 100%

indicates that there is no effect of the biological matrix on the ionization efficiency, while a value above 100% would indicate an enhanced signal and a value below 100% that the signal is decreased. Overall, the SSE for all the analytes was in the range of 99% (thymol) to 250% ((-)-gallocatechin). Furthermore, 91% of all analytes had a SSE between 99 and 130%. The two polyphenol classes that showed the highest average SSE were anthocyanins and catechins which were 145% and 141%, respectively. The signal enhancement of these two classes may be attributed to their structure, as e.g. anthocyanins have a positive charge unlike other polyphenol classes. Although breast milk is an extremely complex matrix, the optimized sample preparation resulted in minimal SSE, a high sensitivity, and decent recoveries for most analytes.

Since the presented assay is a comprehensive multi-analyte method, it was expected that some polyphenol classes performed better than others based on the accepted compromises during sample preparation, chromatographic separation, and mass spectrometric detection. However, the classes without superb performance were not excluded, to give a more holistic overview. The overall validation results and specific figures of merit that did not meet the validation criteria are shown in Table S5. For example, the anthocyanins did not fulfill all validation criteria. This could be attributed to their structure with a positive charge, which makes anthocyanins more polar than other polyphenols. Therefore, during sample preparation, anthocyanins may remain in the aqueous phase during the liquid–liquid extraction step with an organic solvent, leading to their lower recoveries. Moreover, carry-over was observed for anthocyanins in the LC–MS/MS method; thus, for a successful validation, different or more acidic chromatographic conditions would be needed [49, 50]. Also, several catechins, proanthocyanidins, and hydroxybenzoic acids were not successfully validated as some of these analytes showed carry-over. In addition, for the two hydroxybenzoic acids, benzoic acid and ellagic acid, only one MRM transition was available. On the contrary, for dihydrochalcones, flavanones, flavones, flavonols, hydroxycinnamic acids, isoflavones, lignans, and stilbenes, more than 70% of the included analytes fulfilled all stringent validation criteria. The analytical figures of merit evaluated during the method validation for all analytes, separated by polyphenol class, are displayed in Fig. 1 and Fig. S2. It can be observed that polyphenols from the same chemical class typically behave in a similar manner, as they show comparable recoveries, SSEs, intermediate precisions, and repeatability.

Comparing this novel workflow with previously published methods is challenging as only a limited number of methods have been published that were designed specifically for polyphenols in human breast milk. Many biomonitoring methods investigating xenobiotics in breast milk focused on toxicants, including mycotoxins [36, 47, 51], heavy metals [52, 53], persistent organic pollutants [54, 55], volatile organic compounds [56], phthalates [57], and perfluorinated compounds [58], to study their transfer and potential adverse health impact on infants. The methods that quantify polyphenols in breast milk commonly focus on a fraction of the number of analytes that were included in the method developed here and do not comprehensively investigate all the main polyphenol classes [38, 45, 46, 59]. A method published by Song et al. [45] measured eight flavonoids and several carotenoids in breast milk and reported LODs that were higher than those established in the present study for the majority of the analytes common between both methods. For example, the LODs determined for epicatechin gallate, hesperetin, and quercetin (2.7 ng*mL^{-1}, 6.7 ng*mL^{-1}, and 2.5 ng*mL^{-1}, respectively) were approximately 21, 516, and 21 times, respectively, higher than the LODs determined herein. The next-generation biomonitoring method developed by Jamnik et al. [38] for a wide range of xenobiotics in different biofluids showed LODs that were overall in a similar range as reported here for breast milk, e.g., for the analytes 8-prenylnaringenin, isoxanthohumol, and resveratrol, Jamnik et al. [38] reported LODs of 0.0075 ng*mL^{-1}, 0.0048 ng*mL^{-1}, and 0.15 ng*mL^{-1}, respectively, whereas the LODs reported here were at 0.016 ng*mL^{-1}, 0.0054 ng*mL^{-1}, and 0.043 ng*mL^{-1} respectively. However, unlike in this work, the included polyphenols, 8-prenylnaringenin, daidzein, enterodiol, enterolactone, genistein, isoxanthohumol, resveratrol, and xanthumol did not fulfill their defined validation criteria. Finally, Lu et al. [46] analyzed twelve polyphenols (six flavonoids and six non-flavonoids) in breast milk. Lu et al. [46] reported mainly higher LODs than the values achieved with the method presented here. For instance, their reported LODs for kampferol, quercetin, and daidzein (2.2 ng*mL^{-1}, 1.2 ng*mL^{-1}, and 0.5 ng*mL^{-1}, respectively) were approximately 15, 11, and 19 times, respectively, higher than the LODs determined with the workflow presented here. Considering the large quantity of positively validated analytes and their relatively low LODs, it can be concluded that, despite its broad chemical coverage and the quite generic sample preparation, the method performs favorably.

Application of the developed method to human breast milk samples

To show its applicability in real-life samples, the validated method was applied in a pilot study to comprehensively assess the polyphenol profiles in 30 breast milk samples from twelve Nigerian mothers obtained at months one, six, and twelve post-delivery. Since some mothers dropped out of the study, and others did not breastfeed until the twelfth month, not all samples were available for every time point.

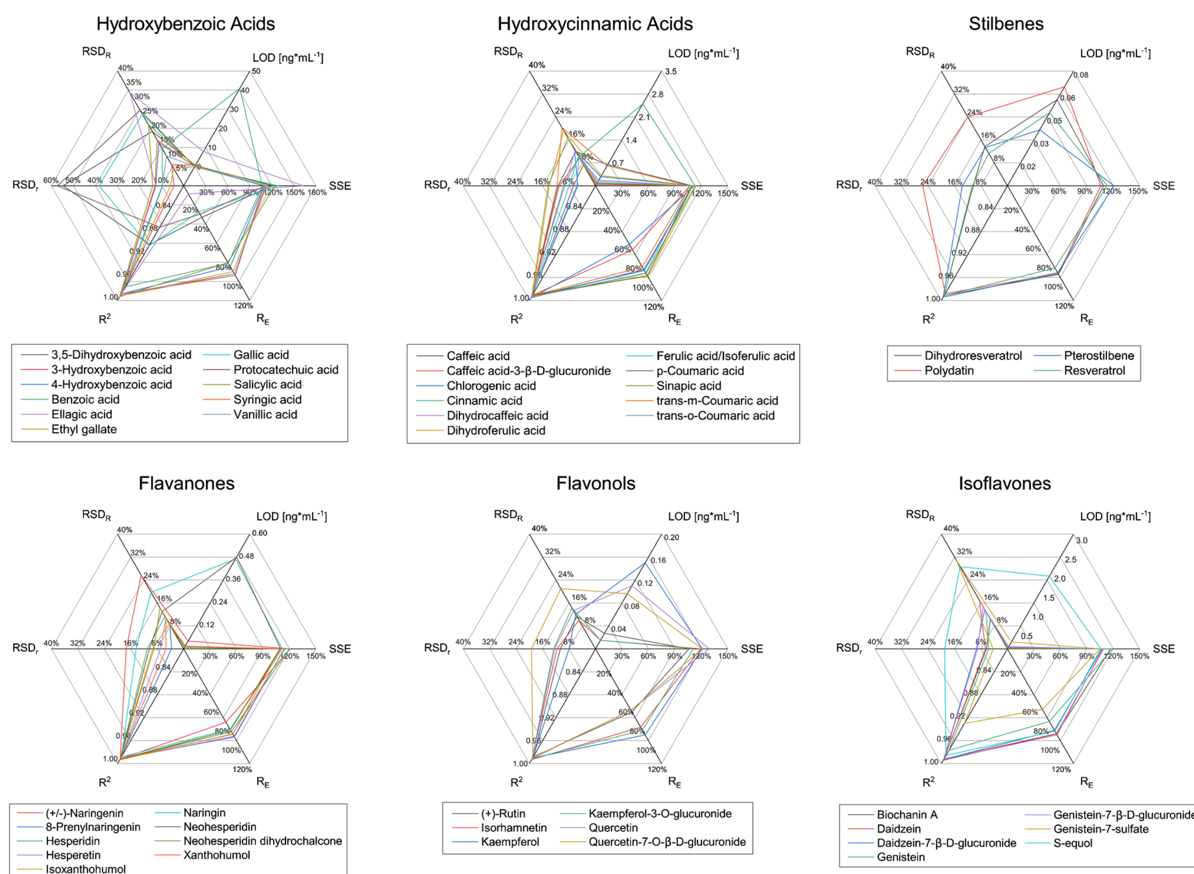

Fig. 1 Analytical figures of merit evaluated during method validation for six selected polyphenol classes (three flavonoid and three non-flavonoid classes). Detailed results for all analytes are reported in Table 1, S4, and S5. The recovery (R_E), intermediate precision (RSD_R), and repeatability (RSD_r) are displayed as the mean of the three spiking levels (low, middle, high). The limit of detection (LOD), calculated from the standard deviation of the lowest spiking level, and signal suppression and enhancement effect (SSE), calculated from the slopes of the calibration curves, are also displayed. For graphical representations of the remaining polyphenol classes, the interested reader is referred to the SI (Fig. S2)

From the 86 polyphenol analytes included in the method, a total of 50 polyphenols, including some metabolic products, were identified in the breast milk samples (Fig. 2a, Table 2). The majority of the detected polyphenols were phenolic acids, a class that includes numerous biotransformation products of larger polyphenols, such as proanthocyanidins [60–62]. Several analytes were detected in a high number of the samples including salicylic acid (found in all 30 samples), an abundant plant metabolite, (+/-)-naringenin (27 samples), a biomarker for citrus fruit consumption [63, 64], and protocatechuic acid (17 samples), a hydroxybenzoic acid present in many vegetables and fruits, and one of the main metabolites of anthocyanins and procyanidins [65, 66]. The polyphenol contents in breast milk can be significantly influenced by several factors. These include dietary habits and the metabolism of the mothers, as well as the polyphenol content of the consumed food, which can be influenced by geographic location and climatic conditions [67]. Examples of chromatographic peaks for polyphenols identified in the pilot study for selected analytes are illustrated in Fig. 2d and e. The quantification of polyphenols present in breast milk provides only a brief insight on the breast milks' current composition, and it is difficult to compare between different mothers and time points, especially as the sample size is relatively small.

As previously mentioned, comparing the polyphenol concentrations to other studies is not straightforward since only a few published reports focused on polyphenols in human breast milk. A previous study by Jamnik et al. [38] investigated xenobiotics in breast milk from one individual over the first 211 days after birth, including several polyphenols. In that study, 8-prenylnargingenin, daidzein, enterodiol, and enterolactone were quantified at mean concentrations of 0.11 ng*mL^{-1}, 0.032 ng*mL^{-1}, 0.013 ng*mL^{-1}, and < LOQ, respectively,

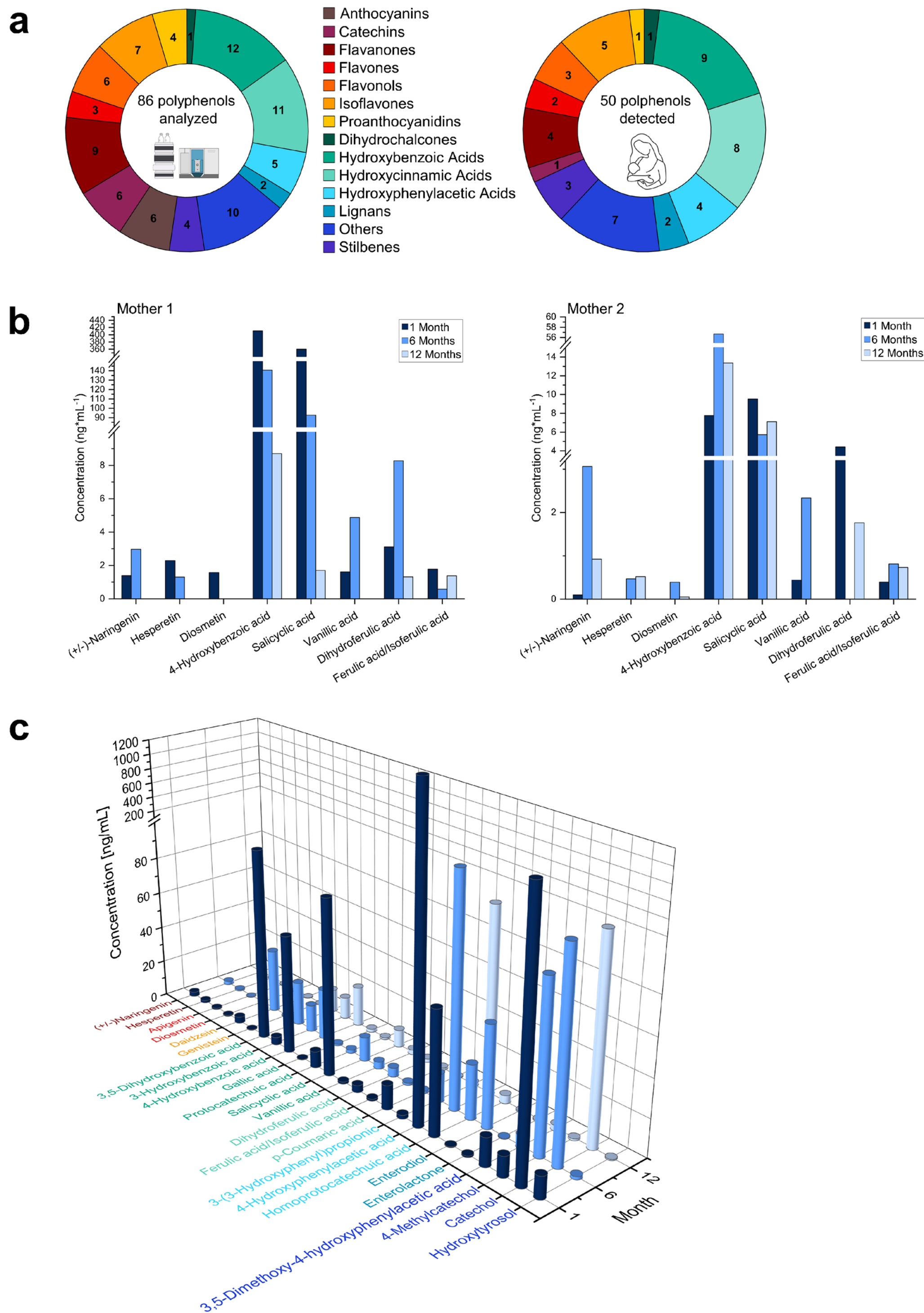

Fig. 2 **a** Pie charts showing the number of polyphenol analytes included in the method (left) and the number of polyphenol analytes detected in the pilot study (right) separated by their polyphenol class. **b** Boxplots of the concentrations for selected analytes at the three different sampling time points for two Nigerian mothers. Only analytes detected with concentrations over the LOQ are displayed. **c** A 3D boxplot of the average concentration between the twelve mothers for each analyte detected, separated by time points. Only the analytes that were detected at least once per time point and had a concentration over the LOQ are shown

Table 2 Minimum (min), maximum (max), and mean concentration[e] of the 50 detected polyphenols in the pilot study of breast milk samples from Nigerian mothers. In addition, the number of samples (*n*) in which the analyte was positively detected, out of 30 total samples, is listed. The limit of quantification (LOQ) for each detected polyphenol is also given

Analyte	LOQ (ng*mL^{-1})	Min (ng*mL^{-1})	Max (ng*mL^{-1})	Mean ± standard deviation (ng*mL^{-1})	*n*
Dihydrochalcones					
Phloretin	0.034	<LOQ	<LOQ	-	2
Hydroxybenzoic acids					
3,5-Dihydroxybenzoic acid	0.82	<LOQ	400	49 ± 110	16
3-Hydroxybenzoic acid	1.7	<LOQ	43	12 ± 12	21
4-Hydroxybenzoic acid	0.38	6.7	410	38 ± 76	30
Ethyl gallate	0.0048	0.05	0.05	0.05	1
Gallic acid	0.056	0.098	3.2	0.98 ± 1.2	6
Protocatechuic acid	0.12	<LOQ	32	3.9 ± 9	17
Salicylic acid	0.96	1.4	360	41 ± 93	30
Syringic acid	0.14	<LOQ	9.8	2.8 ± 4	7
Vanillic acid	0.32	0.44	4.9	2 ± 1.5	12
Hydroxycinnamic acids					
Caffeic acid	1.1	2.7	2.8	2.8 ± 0.05	2
Chlorogenic acid	0.65	<LOQ	8.1	4.2 ± 3.3	4
Cinnamic acid	4.3	16	24	20 ± 5.7	2
Dihydrocaffeic acid	0.33	<LOQ	290	88 ± 120	6
Dihydroferulic acid	0.71	1.2	8.3	3.4 ± 2.3	9
Ferulic acid/Isoferulic acid	0.19	<LOQ	5	1.3 ± 1.2	29
p-Coumaric acid	0.092	<LOQ	23	5.6 ± 7.8	17
Sinapic acid	0.17	<LOQ	2	1.2 ± 1.2	6
Hydroxyphenylacetic acids					
3-(3-Hydroxyphenyl)propionic acid	0.76	<LOQ	77	8.5 ± 20	18
4-Hydroxyphenylacetic acid	12	<LOQ	12000	970 ± 2800	23
Homovanillic acid	3.4	<LOQ	14	6.9 ± 4.2	11
Homoprotocatechuic acid	1.3	4.4	65	27 ± 28	5
Lignans					
Enterodiol	0.034	0.14	110	22 ± 42	6
Enterolactone	0.038	0.21	1.9	0.54 ± 0.5	11
Others					
2,6-Dimethoxyphenol	0.12	0.33	0.39	0.36 ± 0.04	2
3,5-Dimethoxy-4-hydroxyphenylacetic acid	0.86	2.9	24	8 ± 7.6	7
4-Methylcatechol	0.48	0.96	170	41 ± 72	5
Catechol	10	<LOQ	2100	410 ± 550	24
Hydroxytyrosol	0.068	0.16	23	4.3 ± 8.6	7
Pyrogallol	2.8	3.6	5.4	4.4 ± 0.84	5
Urolithin A	0.028	0.15	0.39	0.27 ± 0.17	2
Stilbenes					
Dihydroresveratrol	0.11	<LOQ	<LOQ	-	2
Polydatin	0.12	1	1	1	1
Pterostilbene	0.068	1.4	1.4	1.4	1
Catechins					
(-)-Epicatechin	0.48	1.2	3.5	2.4 ± 1.7	2
Flavanones					
(+/-)-Naringenin	0.072	<LOQ	13	2.1 ± 2.9	27
8-Prenylnaringenin	0.032	1.3	1.3	1.3	1
Hesperetin	0.026	0.4	2.3	0.96 ± 0.7	6

Table 2 (continued)

Analyte	LOQ (ng*mL^{-1})	Min (ng*mL^{-1})	Max (ng*mL^{-1})	Mean ± standard deviation (ng*mL^{-1})	*n*
Xanthohumol	0.034	< LOQ	< LOQ	-	1
Flavones					
Apigenin	0.0094	0.047	1.8	0.38 ± 0.59	8
Diosmetin	0.03	< LOQ	1.6	0.27 ± 0.41	20
Flavonols					
Isorhamnetin	0.018	< LOQ	0.34	0.1 ± 0.12	10
Kaempferol	0.34	< LOQ	0.64	0.6 ± 0.06	5
Kaempferol-3-O-glucuronide	0.026	0.21	0.82	0.51 ± 0.43	2
Isoflavones					
Daidzein	0.068	< LOQ	67	16 ± 25	15
Daidzein-7-β-D-glucuronide	0.13	0.13	0.59	0.42 ± 0.22	5
Genistein	0.0094	0.08	1.1	0.35 ± 0.4	10
Genistein-7-β-D-glucuronide	0.11	< LOQ	1.9	1.1 ± 0.89	5
Genistein-7-sulfate	0.36	< LOQ	< LOQ	-	11
Proanthocyanidins					
Procyanidin C1	1.2	< LOQ	< LOQ	-	6

[e]The concentrations were calculated using the matrix-matched calibration curve and corrected with the recovery determined during the method validation

which was lower than the values of 1.3 ng*mL^{-1}, 16 ng*mL^{-1}, 22 ng*mL^{-1}, and 0.54 ng*mL^{-1}, respectively, reported for the same analytes in the present study. Song et al. [45] investigated the phytochemical content in breast milk samples, collected at three different time points, from 17 mothers donated by the Cincinnati Children's Hospital Medical Center and reported epicatechin, (+/-)-naringenin, hesperetin, and kaempferol at higher average concentrations (42 ng*mL^{-1}, 60 ng*mL^{-1}, 120 ng*mL^{-1}, and 7 ng*mL^{-1}, respectively) compared to the values reported here (2.4 ng*mL^{-1}, 2.1 ng*mL^{-1}, 0.96 ng*mL^{-1}, and 0.6 ng*mL^{-1}, respectively). Furthermore, Song et al. [45] detected epigallocatechin, epigallocatechin gallate, and quercetin, which were not detected in the Nigerian samples. Lu et al. [46] detected twelve different polyphenols in 89 breast milk samples from Hong Kong women. Higher mean concentrations were reported for quercetin, (+/-)-naringenin, caffeic acid, and protocatechuic acid (41 ng*mL^{-1}, 110 ng*mL^{-1}, 30 ng*mL^{-1}, and 112 ng*mL^{-1}, respectively) compared to the values of 2.1 ng*mL^{-1}, 2.8 ng*mL^{-1}, and 3.9 ng*mL^{-1} for (+/-)-naringenin, caffeic acid, and protocatechuic acid, respectively, in the present study. However, chlorogenic acid, (-)-epicatechin, and daidzein had similar average concentrations of 2 ng*mL^{-1}, 9 ng*mL^{-1}, and 15 ng*mL^{-1}, respectively, compared to the present study. An increased consumption of e.g. tea, which is rich in flavanols, can lead to an increased quercetin concentration, which could explain the amount of quercetin found in Lu et al. [46], whereas an increased intake of legumes and seeds can lead to an increased enterodiol and enterlactone concentrations, as their parent molecule, matairesinol, is prevalent in legumes and seeds [68, 69]. The disparities in the type and concentrations of polyphenols found in the various studies can be attributed to several factors such as different diets of the mothers, differences in analytical sensitivities, and sample size, as well as seasonal and growth-related differences in polyphenol contents [67].

Polyphenols readily undergo phase II biotransformation in the small intestine and liver; hence, a higher concentration of glucuronidated, compared to unconjugated, metabolites are typically detected in urine [60, 70, 71]. Phase II conjugated metabolites, including daidzein-7-β-D-glucuronide and genistein-7-β-D-glucuronide, were detected in several breast milk samples albeit at low concentrations (0.42 ng*mL^{-1} and 1.1 ng/mL^{-1}, respectively). Overall, genistein and daidzein were present in more breast milk samples than their respective glucuronides. Interestingly, when a sample contained both, the parent compound and the glucuronidated compound, the glucuronide concentration was usually higher than the parent compound (Fig. 2e). It must be noted that as polyphenols have several hydroxyl groups, different positional isomers are possible and only one isomer was included in this method. Thus, different positional isomers of conjugated metabolites could be missed, especially as the LODs for most of the conjugated metabolites were similar or lower than their respective parent compound, e.g., caffeic acid-3-β-D-glucuronide had an LOD of 0.0085 ng*mL^{-1} and caffeic acid had 0.55 ng*mL^{-1}. To get a more complete picture of polyphenol biotransformation, additional analyses, for example, by untargeted workflows, would be beneficial [72]. Previous studies have also shown that phase II metabolites of other xenobiotics, such

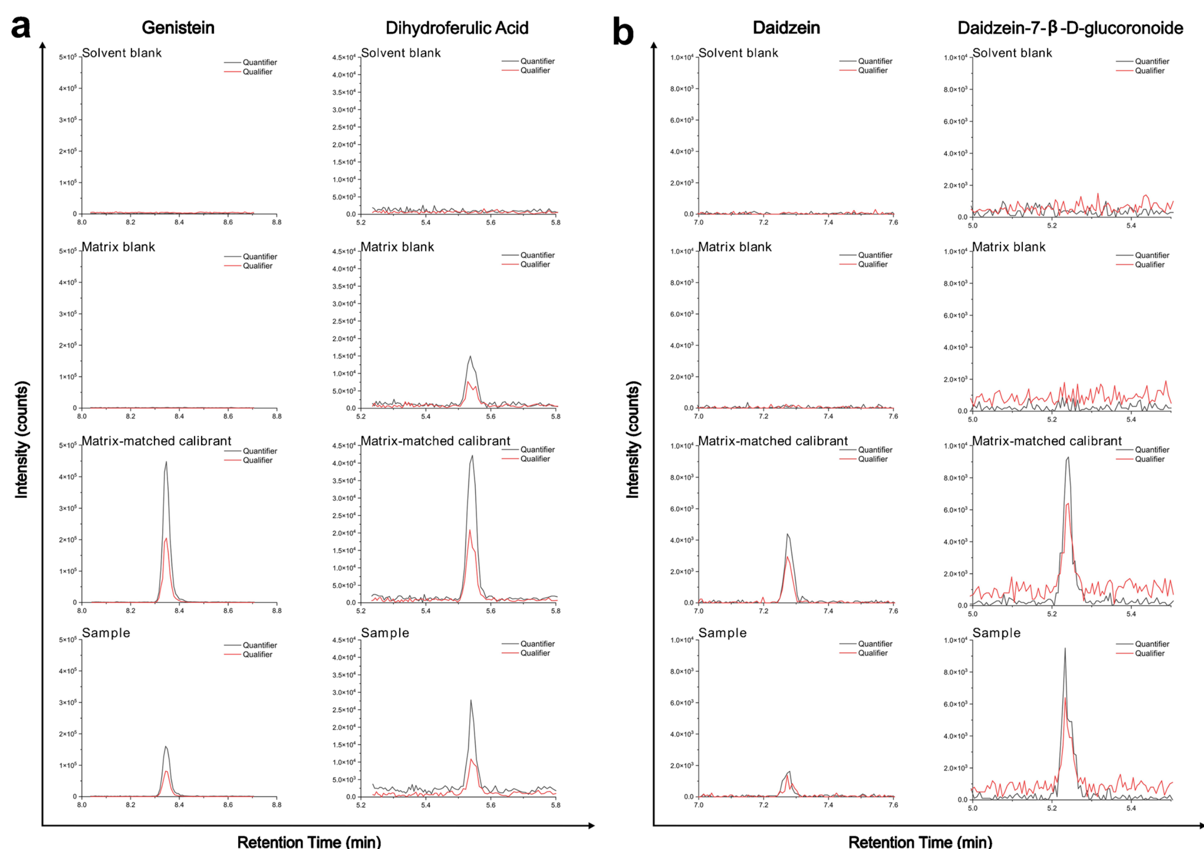

Fig. 3 **a** MRM chromatograms (quantifier and qualifier ions) of a solvent blank, a non-spiked breast milk "blank," a matrix-matched calibrant (0.43 ng*mL^{-1} for genistein and 1.5 ng*mL^{-1} for dihydroferulic acid), and a breast milk sample obtained from a Nigerian mother. **b** MRM chromatograms (quantifier and qualifier ions) of daidzein and daizein-7-β-D-glucuronide from the same mother and same timepoint, with the MRM chromatograms of a solvent blank, a matrix-matched breast milk "blank," and a matrix-matched calibrant (0.037 ng*mL^{-1} for daidzein and 0.11 ng*mL^{-1} for daizein-7-β-D-glucuronide)

as plasticizers, pesticides, and phytoestrogens, can be found in breast milk [33, 41, 57]. Further research is needed to study the pathways and presence of polyphenols and their biotransformation products in human milk.

A rough estimation of the exposure levels of infants to polyphenols was conducted. In order to exclude other possible polyphenol sources, e.g., from complementary foods, only breast milk sampled at month one after birth was used for this estimation. Analytes that were detected below the LOQ value were considered positive and the corresponding LOQ value was applied (i.e., upper bound scenario). An average infant body weight of 4 kg [73] and a daily consumption of 500 mL breast milk were assumed. Based on this estimation (individual, median, and mean daily intakes are reported in Table S6), it was derived that the approximate daily intake per polyphenol detected was in the lower microgram per kilogram body weight range, with the median analyte concentration ranging from 0.0044 µg*kg^{-1} body weight per day (phloretin) to 31 µg*kg^{-1} body weight per day (catechol). The most common analytes detected in the breast milk samples were 4-hydroxybenzoic acid, diosmetin, salicylic acid, ferulic acid, and (+/-)-naringenin, and had an estimated median daily intake of 1.9 µg*kg^{-1}, 0.022 µg* kg^{-1}, 0.54 µg* kg^{-1}, 0.094 µg* kg^{-1}, and 0.14 µg* kg^{-1} of body weight, respectively. Though numerous known polyphenols have not been included in this method, the sum of the investigated polyphenols detected yielded an estimated median daily intake of 57 µg*kg^{-1}. It must be noted that these estimations were calculated for only one sampling time point and should be interpreted with caution. However, the estimates provide rough insights into the exposure of infants towards a large panel of polyphenols. Therefore, to better ascertain the daily polyphenol exposure, further studies are needed that include a larger sample size and information on the polyphenol content of the food consumed by the mothers on the day of sampling (Fig. 3).

Conclusion and outlook

In conclusion, the successful optimization and in-house validation of an LC–MS/MS method targeting 86 polyphenols that are representatives of all major polyphenol classes in human breast milk are presented. Despite low sample volumes, a high-throughput sample preparation, and a wide variety of analytes, this approach demonstrated high sensitivity while retaining high recoveries and low signal suppression and enhancement effects. Moreover, the application of the method in a pilot study demonstrated its feasibility to be readily used in large cohort studies. Thus, it can be applied to investigate and better comprehend the transfer of ingested dietary polyphenols to breast milk, which would be beneficial in further nutritional intervention and prevention studies. Moreover, it can also be used to investigate human metabolism in vivo. Its application in large cohorts would also aid the advanced investigation of the impact of polyphenols in nutritional intervention studies. Finally, this method can also be applied, to better understand the transfer of polyphenols to newborns. Especially in the era of exposome-type research, it may reveal new insights on potential health benefits and polyphenol impact on microbiome development and of co-exposure and mixture of toxicological effects with other xenobiotics that infants are exposed to via their diet and environment.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00216-024-05179-y.

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Declarations

Ethics approval Ethical approval was obtained from the Ethical Committee of Babcock University (BUHREC421/21R, BUHREC466/23). Prior to their inclusion in the studies, all mothers were informed and provided written consent.

Competing interests The authors declare no competing interests.

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Supplementary Information

Polyphenol exposure of mothers and infants assessed by LC-MS/MS based biomonitoring in breast milk

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Figure S1: Molecular structures of the analytes included in this study……………………………...…….2

Table S1: Supplier information on the reagents, solvents, and chemicals used……...……………..…...….3

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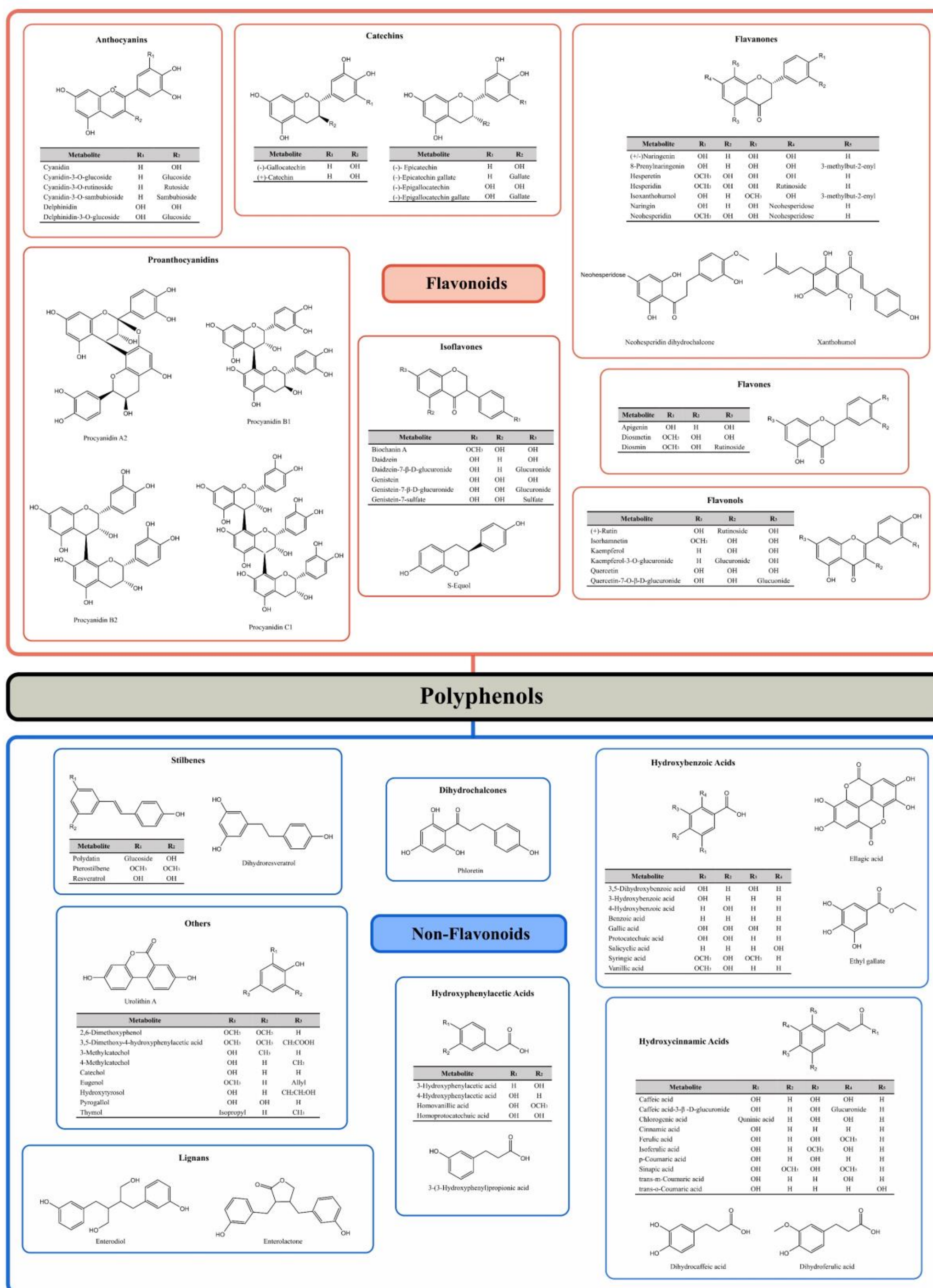


Figure S1. Molecular structures of the analytes included in this study, divided into their polyphenol classes [1].

Table S1. Supplier information on the reagents, solvents, and chemicals used. The polyphenol standards are the same as reported by Oesterle et al. [1]

Item	Supplier	Item Number	Lot Number	Purity
Dihydrochalcones				
Phloretin	Cayman Chemical Company	14452-1g	0447930-17	1
Hydroxybenzoic Acids				
Caffeic acid	Sigma	C0625	089K1114	0.993
3-Hydroxybenzoic acid	Sigma-Aldrich	36333-100mg	BCBV2381	0.998
4-Hydroxybenzoic acid	Sigma	240141-50g	BCCB8991	1
Benzoic acid	Sigma-Aldrich	242381	MKBG9391V	0.999
Ellagic acid	Sigma	E-2250	70K1240	0.99
Ethyl gallate	Phytolab	83080-100mg	5950	1
Gallic acid	Sigma	G7384-100g	SLBW1280	1
Protocatechuic acid	Sigma	P-5630	072K3446	0.998
Salicyclic acid	Sigma-Aldrich	247588	09712LE	0.998
Syringic acid	Sigma	S6881-5g	BCCB1235	0.995
Vanillic acid	Sigma	V-2250	50H7714	0.99
Hydroxycinnamic Acids				
Caffeic acid	Sigma	C0625	089K1114	99.3
Caffeic acid-3-β-D-glucuronide	Toronto Research Chemicals	C080015-1mg	1-KMR-141-3	0.9965
Chlorogenic acid	Aldrich	C3878	SLBL9959V	0.99
Cinnamic acid	Fluka	96340	408492/1	0.993
Dihydrocaffeic acid	Aldrich	10.260-1	1235963	0.98
Dihydroferulic acid	Aldrich	17803	BCBH4069V	1
Ferulic acid	Fluka	46278	357835/1	1.0004
Isoferulic acid	Toronto Research Chemicals	H946180-2.5mg	12-XJZ-152-1	0.98
p-Coumaric acid	Sigma	C9008-5g	095K1340	0.997
Sinapic acid	Sigma	D7927	0000071240	0.985
trans-m-Coumaric acid	Phytolab	83258-10mg	10022	1

Item	Supplier	Item Number	Lot Number	Purity
trans-o-Coumaric acid	Phytolab	82343-100mg	4341	0.9985
Hydroxyphenylacetic Acids				
3-(3-Hydroxyphenyl)propionic acid	Sigma-Aldrich	91779-10mg	BCCB6466	0.998
3-Hydroxyphenylacetic acid	Aldrich	H49901-5g	STBB5523	0.992
4-Hydroxyphenylacetic acid	Sigma	H50004-5g	BCCB4700	0.997
Homoprotocatechuic acid	Aldrich	85,021-7	S23418-404	0.994
Homovanillic acid	Sigma-Aldrich	69673-25mg	BCCC5315	0.996
Lignans				
Enterodiol	Phytolab	80436-10mg	11769	0.9846
Enterolactone	Phytolab	80437-10mg	5185	0.9907
Others				
2,6-Dimethoxyphenol	Aldrich	D135550	MKBG7714V	0.994
3,5-Dimethoxy-4-hydroxyphenylacetic acid	Aldrich	631310	07406JC	0.991
3-Methylcatechol	Aldrich	M34006	MKBB9900	0.991
4-Methylcatechol	Aldrich	M34200	MKBB9773	0.988
Catechol	Sigma	C-9510	96F-0536	0.99
Eugenol	Extrasynthese	6178 S	08-0921/0	0.9978
Hydroxytyrosol	Extrasynthese	4999 S	12-0520/0	1
Pyrogallol	Merck	612	1151574	0.995
Thymol	Sigma	16254	SZBB0460V	0.994
Urolithin A	Sigma-Aldrich	SML1791-5mg	0000076439	0.988
Stilbenes				
Dihydroresveratrol	Toronto Research Chemicals	D678960-1mg	2-MJJ-97-1	0.98
Polydatin	Extrasynthese	4974 S	02-0822/0	0.992
Pterostilbene	Toronto Research Chemicals	P839890-50mg	1-NYL-63-1	0.98
Resveratrol	Extrasynthese	4963 S	02-1219/0	0.997

Item	Supplier	Item Number	Lot Number	Purity
Anthocyanins				
Cyanidin chloride	Extrasynthese	0909 S	38-0723/0	0.98
Cyanidin-3-O-glucoside chloride	Extrasynthese	0915 S	45 – 1114/0	0.976
Cyanidin-3-O-rutinoside chloride	Extrasynthese	0914 S	31 – 0917/0	0.973
Cyanidin-3-O-sambubioside chloride	Extrasynthese	0949 S	04 – 0311/0	0.972
Delphinidin chloride	Extrasynthese	0904 S	63 – 0814/0	0.978
Delphinidin-3-O-glucoside chloride	Extrasynthese	0938 S	27 – 1023/0	0.995
Catechins				
(-)-Epicatechin	Sigma	E-1753	32H2519	0.98
(-)-Epicatechin gallate	Extrasynthese	0978 S	12-0721/0	0.993
(-)-Epigallocatechin	Extrasynthese	0979 S	11-0703/0	0.992
(-)-Epigallocatechin gallate	Sigma	E4143	031M1175V	0.95
(-)-Gallocatechin	Extrasynthese	0973 S	05-1014/0	0.989
(+)-Catechin	Extrasynthese	0976 S	25-0204/0	1
Flavanones				
(+/-)-Naringenin	Sigma	N5893-1g	BCBC1784	0.96
8-Prenylnaringenin	Sigma	75119-5mg	BCCB4227	1
Hesperetin	Cayman Chemical Company	Cat. 10006084	121938-5	0.984
Hesperidin	Fluka	52040	1117099	0.923
Isoxanthohumol	Extrasynthese	1367 S	01-1020/0	0.995
Naringin hydrate	Alfa Aesar	L10163	10126866	0.98
Neohesperidin	Extrasynthese	1132 S	09-0924/0	0.996
Neohesperidin dihydrochalcone	SAFC	W381101-25g-K	MKBD9742V	0.85
Xanthohumol	Extrasynthese	1346 S	03-0524/0	1
Flavones				
Apigenin	Sigma	10798-25mg	E445301/1V	0.954
Diosmetin	Extrasynthese	1108 S	15-0312/0	0.998

Item	Supplier	Item Number	Lot Number	Purity
Diosmin	Extrasynthese	1109 S	11-1021/0	0.996
Flavonols				
(+)-Rutin trihydrate	Aldrich	R230-3	10422EW	0.95
Isorhamnetin	Extrasynthese	1120 S	32-0215/0	0.993
Kaempferol	Sigma	60010	BCCC4134	0.997
Kaempferol-3-O-glucuronide	Extrasynthese	1356 S	03-0302/0	0.996
Quercetin	Sigma	Q4951-10g	060M1196V	0.98
Quercetin-7-O-β-D-glucuronide	Toronto Research Chemicals	Q509515-2.5mg	12-QFY-8-3	0.9539
Isoflavones				
Biochanin A	Extrasynthese	1349 S	01-0106/0	0.999
Daidzein	Extrasynthese	1370 S	03-0204/0	0.995
Daidzein-7-β-D-glucuronide potassium salt	Toronto Research Chemicals	D103510-5mg	3-LGA-24-2	0.95
Genistein	Extrasynthese	1372 S	01 – 0114/0	1
Genistein-7-β-D-glucuronide	Toronto Research Chemicals	G350015-2.5mg	3-JAE-172-1	0.95
Genistein-7-sulfate sodium salt	Toronto Research Chemicals	G350045-2.5mg	10-JLI-110-4	0.9989
S-equol	Sigma	SML2147-5mg	0000081122	0.97
Proanthocyanidins				
Procyanidin A2	Extrasynthese	0985 S	05-0128/0	0.999
Procyanidin B1	Phytolab	89764-5mg	14872	0.9739
Procyanidin B2	Toronto Research Chemicals	P755830-1mg	52-GHZ-187-1	0.9826
Procyanidin C1	Phytolab	89537-5mg	15436	0.974
Reagents				
Methanol	Honeywell	34966	Various	LC-MS grade
Acetonitrile	Honeywell	34967	Various	LC-MS grade
Water	VWR	63645.320	Various	LC-MS grade
Formic acid	Bartelt	SO9679B001	1373 811	UPLC-MS Optigrade
Hexane	Roth	7567.1	Various	≥ 96%

Item	Supplier	Item Number	Lot Number	Purity
Ethylacetate	Sigma-Aldrich	33211-1L-R	SZBD128SV	≥ 99.5%
2-Propanol	Honeywell	34965	Various	LC-MS grade
Magnesium sulfate, anhydrous	Acros Organics	4134850000	A0379632	97%
Sodium chloride	Roth	0962.2	390289120	≥ 99.8%

Table S2. Multiple reaction monitoring (MRM) parameters of the included analytes. All values that could not be determined are marked as n.d. (not determined). The delustering potential for all analytes was set to -10V.

Analyte	Retention Time (min)	Total Retention Time Window (s)	Parent Ion *m/z*	Product Ions (Quantifier/Qualifier) *m/z*	Collision Energy (V)	Cell Exit Potential (V)	Ion Ratio (%)
Dihydrochalcones							
Phloretin	8.3	20	272.982	166.9/123.2	-26/-30	-17/-47	22
Hydroxybenzoic Acids							
3,5-Dihydroxybenzoic acid	2.6	25	152.862	108.8/67	-28/-28	-17/-19	12
3-Hydroxybenzoic acid	4.7	20	136.873	92.9/64.9	-16/-32	-11/-35	9
4-Hydroxybenzoic acid	4.0	20	136.891	92.9/65.1	-18/-38	-23/-9	16
Benzoic acid	6.3	20	120.945	77.1	-18	-13	n.d.[a]
Ellagic acid	5.9	25	300.873	145.0/255.3	-50/-16	-9/-19	n.d.[a]
Ethyl gallate	5.4	20	196.970	123.9/168.9	-26/-24	-23/-11	58
Gallic acid	1.3	20	168.915	125.0/79.0	-20/-26	-9/-7	16
Protocatechuic acid	2.7	25	152.939	108.1/80.9	-34/-26	-11/-11	26
Salicylic acid	6.6	20	136.937	92.9/65.1	-24/-36	-13/-13	15
Syringic acid	4.8	20	196.943	181.9/121.1	-18/-22	-17/-23	15
Vanillic acid	4.6	20	166.926	152.0/108.1	-18/-26	-13/-7	64
Hydroxycinnamic Acids							
Caffeic acid	4.7	20	178.914	107.0/89.0	-30/-42	-7/-7	85
Caffeic acid-3-β-D-glucuronide	4.2	20	355.074	135.0/179.2	-36/-30	-21/-5	110
Chlorogenic acid	4.3	20	353.006	191.0/85.0	-20/-52	-13/-11	17
Cinnamic acid	7.7	20	146.936	77.0/103.0	-28/-14	-7/-17	660
Dihydrocaffeic acid	4.4	20	180.971	58.9/121.0	-22/-22	-9/-9	42
Dihydroferulic acid	5.6	20	194.954	136.0/121.1	-24/-34	-11/-9	48
Ferulic acid/Isoferulic acid	5.8	20	192.941	134.1/178.0	-22/-18	-9/-11	56
p-Coumaric acid	5.5	20	162.923	119.0/92.9	-22/-38	-13/-11	17

[a] No ion ratio could be determined since only one transition showed a chromatographic peak

Analyte	Retention Time (min)	Total Retention Time Window (s)	Parent Ion *m/z*	Product Ions (Quantifier/Qualifier) *m/z*	Collision Energy (V)	Cell Exit Potential (V)	Ion Ratio (%)
Sinapic acid	5.9	20	222.999	208.0/164.1	-18/-18	-13/-11	55
trans-m-Coumaric acid	6.0	20	162.864	119.1/91.0	-12/-32	-7/-33	18
trans-o-Coumaric acid	6.5	20	162.942	118.8/116.9	-18/-34	-47/-17	18
Hydroxyphenylacetic Acids							
3-(3-Hydroxyphenyl)propionic	5.7	20	164.973	106.1/121.0	-28/-16	-1/-23	310
3-Hydroxyphenylacetic acid	4.8	20	150.972	107.1/79.2	-12/-28	-11/-39	10
4-Hydroxyphenylacetic acid	4.4	20	150.937	79.1/107.1	-22/-10	-9/-7	350
Homovanillic acid	4.8	20	180.887	122.0/135.9	-24/-10	-19/-21	9
Homoprotocatechuic acid	3.4	20	166.928	123.0/121.9	-12/-30	-13/-7	14
Lignans							
Enterodiol	7.4	20	300.927	253.1/271.2	-30/-30	-11/-23	33
Enterolactone	8.6	20	296.944	253.1/107.1	-28/-32	-13/-17	53
Others							
2,6-Dimethoxyphenol	6.2	20	154.833	123.1/77.1	15/25	10/4	48
3,5-Dimethoxy-4-	5.0	20	211.02	167.1/136.9	-10/-32	-21/-9	55
3-Methylcatechol	5.8	20	122.97	107.8/95.0	-24/-22	-55/-7	24
4-Methylcatechol	5.4	20	122.935	108.0/104.9	-30/-28	-11/-11	51
Catechol	2.6	25	108.908	91.0/80.9	-26/-22	-13/-39	66
Eugenol	9.2	20	164.896	136.8/123.9	13/23	14/6	40
Hydroxytyrosol	2.7	25	152.936	123.1/122.5	-20/-30	-13/-9	10
Pyrogallol	1.5	25	124.932	69.0/79.0	-24/-26	-11/-19	180
Thymol	5.8	20	149.012	134.0/133.6	-20/-16	-11/-23	39
Urolithin A	7.3	20	226.838	197.8/181.8	-44/-38	-11/-13	44
Stilbenes							
Dihydroresveratrol	7.2	20	230.956	121.1/137.1	21/25	6/4	130
Polydatin	5.8	20	389.061	227.3/143.1	-16/-58	-13/-21	21
Pterostilbene	11.0	20	257.118	242.4/133.2	33/27	14/12	260

Analyte	Retention Time (min)	Total Retention Time Window (s)	Parent Ion *m/z*	Product Ions (Quantifier/Qualifier) *m/z*	Collision Energy (V)	Cell Exit Potential (V)	Ion Ratio (%)
Resveratrol	7.1	20	226.977	185.0/142.9	-26/-34	-29/-51	57
Anthocyanins							
Cyanidin	4.5	30	286.837	230.9/213.1	33/39	20/24	33
Cyanidin-3-O-glucoside	4.5	30	448.866	286.8/212.8	35/77	44/10	13
Cyanidin-3-O-rutinoside	4.6	30	595.127	287.0/449.1	31/29	24/22	16
Cyanidin-3-O-sambubioside	4.5	30	581.015	286.9/212.9	35/95	40/12	13
Delphinidin	5.1	20	302.813	229.0/201.2	37/37	14/18	29
Delphinidin-3-O-glucoside	4.2	30	465.163	303.1/228.9	35/71	24/18	16
Catechins							
(-)- Epicatechin	5.0	20	288.948	245.1/203.0	-20/-26	-17/-15	46
(-)-Epicatechin gallate	5.9	20	440.878	169.0/289.0	-22/-28	-27/-17	70
(-)-Epigallocatechin	4.3	25	304.943	124.9/179.0	-26/-20	-35/-9	57
(-)-Epigallocatechin gallate	5.1	20	457.091	168.9/125.0	-24/-48	-11/-13	41
(-)-Gallocatechin	2.6	25	304.962	124.9/219.0	-22/-20	-13/-37	31
(+)-Catechin	4.4	20	288.903	123.2/203.0	-42/-26	-5/-33	110
Flavanones							
(+/-)-Naringenin	8.3	20	271.004	151.0/119.1	-24/-34	-13/-3	56
8-Prenylnaringenin	10.6	20	339.074	219.0/119.0	-26/-40	-29/-7	58
Hesperetin	6.5	20	609.045	301.0/286.2	-34/-52	-25/-21	47
Hesperidin	8.6	20	300.976	163.9/135.9	-32/-38	-9/-7	29
Isoxanthohumol	9.4	20	352.998	118.9/233.1	-26/-26	-27/-55	89
Naringin	6.3	20	580.954	272.8/152.8	21/65	16/22	55
Neohesperidin	6.5	20	610.963	449.1/303.0	11/27	32/18	290
Neohesperidin dihydrochalcone	7.1	20	611.058	303.0/125.0	-46/-52	-19/-13	25
Xanthohumol	12.3	20	352.996	233.2/119.1	-24/-56	-29/-15	83
Flavones							
Apigenin	8.3	30	268.945	117.0/151.0	-44/-36	-9/-19	55

Analyte	Retention Time (min)	Total Retention Time Window (s)	Parent Ion *m/z*	Product Ions (Quantifier/Qualifier) *m/z*	Collision Energy (V)	Cell Exit Potential (V)	Ion Ratio (%)
Diosmetin	6.3	20	607.023	298.9/283.8	-36/-68	-43/-17	79
Diosmin	8.6	25	298.925	284.1/256.0	-32/-40	-19/-27	14
Flavonols							
(+)-Rutin	5.7	20	609.041	300.1/301.0	-44/-38	-9/-21	51
Isorhamnetin	8.6	25	314.919	299.8/151.0	-34/-34	-17/-5	22
Kaempferol	8.5	35	284.952	117.1/184.9	-54/-40	-13/-15	120
Kaempferol-3-O-glucuronide	6.3	20	460.963	284.9/112.9	-32/-20	-17/-17	35
Quercetin	7.6	25	300.911	151.0/178.9	-32/-26	-9/-27	47
Quercetin-7-O-β-D-glucuronide	5.8	20	477.062	300.8/150.9	-28/-48	-25/-11	17
Isoflavones							
Biochanin A	10.5	20	282.89	267.9/239.0	-26/-44	-21/-21	30
Daidzein	7.3	20	253.013	131.9/224.0	-50/-36	-19/-21	150
Daidzein-7-β-D-glucuronide	5.3	20	429.141	252.8/113.0	-32/-22	-23/-17	74
Genistein	8.4	20	268.962	133.0/132.1	-38/-54	-15/-13	46
Genistein-7-β-D-glucuronide	6.1	20	444.932	269.0/174.9	-40/-18	-45/-17	53
Genistein-7-sulfate	7.1	30	348.798	268.9/133.1	-38/-58	-53/-7	18
S-Equol	8.4	20	240.938	120.9/119.0	-20/-26	-19/-13	72
Proanthocyanidins							
Procyanidin A2	6.0	20	574.998	284.9/448.9	-38/-26	-35/-35	71
Procyanidin B1	4.1	20	579.296	409.2/288.9	27/19	26/18	83
Procyanidin B2	4.7	20	578.552	426.8/409.0	23/29	32/34	83
Procyanidin C1	5.1	20	867.202	579.1/577.2	21/21	26/36	66

Table S3. LC gradient applied in the final method.

Time [min]	Eluent A [%]	Eluent B [%]
0	95	5
2	95	5
12	36	64
12.01	5	95
14	5	95
14.01	95	5
16	95	5

Table S4. Concentration of the three different spiking levels: low level (LL), middle level (ML), and high level (HL), used during validation for each analyte. The calculated recovery (R_E), Intermediate precision (RSD_R) and interday repeatability (RSD_r) are given. Figures of merit which could not be determined are listed as n.d.

Analyte	Spiking Level (LL/ML/HL) (ng*mL^{-1})	$R_E \pm RSD_R$ (LL) (%)	$R_E \pm RSD_R$ (ML) (%)	$R_E \pm RSD_R$ (HL) (%)	RSD_r (LL/ML/HL) (%)
Dihydrochalcones					
Phloretin[b]	0.22/2.8/8.8	98 ± 10	96 ± 12	94 ± 12	7/10/7
Hydroxybenzoic Acids					
3,5-Dihydroxybenzoic acid	2.9/39/120	39 ± 36	47 ± 8	46 ± 13	85/36/43
3-Hydroxybenzoic acid	18/240/710	92 ± 7	99 ± 6	96 ± 9	4/5/4
4-Hydroxybenzoic acid[b]	1.1/14/42	75 ± 31	93 ± 6	95 ± 8	30/5/3
Benzoic acid[b]	47/630/1900	n.d.	80 ± 12	88 ± 14	n.d./10/9
Ellagic acid[b]	23/310/930	n.d.	10 ± 39	6 ± 26	n.d./19/6
Ethyl gallate	0.071/0.95/2.9	84 ± 5	90 ± 4	91 ± 9	8/7/3
Gallic acid	0.3/4.1/12	18 ± 53	40 ± 8	41 ± 14	50/28/37
Protocatechuic acid	0.2/2.6/7.9	42 ± 53	37 ± 12	46 ± 14	59/42/71
Salicylic acid[b]	2.7/36/110	74 ± 31	92 ± 14	91 ± 19	32/6/4
Syringic acid	0.32/4.3/13	104 ± 26	91 ± 10	91 ± 12	27/7/5
Vanillic acid	2.1/28/84	101 ± 10	91 ± 7	89 ± 14	15/7/7
Hydroxycinnamic Acids					
Caffeic acid	4/53/160	86 ± 21	91 ± 7	88 ± 8	26/5/3
Caffeic acid-3-β-D-glucuronide	0.2/2.7/8.2	63 ± 8	71 ± 9	72 ± 12	17/10/6
Chlorogenic acid	3.8/51/150	58 ± 19	59 ± 9	63 ± 10	5/8/3
Cinnamic acid	19/260/780	92 ± 15	89 ± 7	89 ± 8	17/7/5
Dihydrocaffeic acid	1.1/15/44	88 ± 22	89 ± 7	86 ± 8	17/7/4
Dihydroferulic acid[b]	1.5/20/59	113 ± 27	95 ± 16	92 ± 15	24/10/8
Ferulic acid/Isoferulic acid[b]	1/14/41	80 ± 15	95 ± 6	93 ± 10	13/7/4
p-Coumaric acid	0.58/7.8/23	90 ± 11	96 ± 4	95 ± 7	14/3/3

[b] Analyte with standard addition applied as a chromatographic peak was present in the matrix-matched blank.

Analyte	Spiking Level (LL/ML/HL) (ng*mL^{-1})	$R_E \pm RSD_R$ (LL) (%)	$R_E \pm RSD_R$ (ML) (%)	$R_E \pm RSD_R$ (HL) (%)	RSD_r (LL/ML/HL) (%)
Sinapic acid[b]	0.33/4.4/13	99 ± 34	95 ± 7	95 ± 9	30/10/3
trans-m-Coumaric acid	3.3/45/130	63 ± 42	99 ± 9	95 ± 10	27/4/4
trans-o-Coumaric acid	1.9/26/77	93 ± 14	94 ± 8	95 ± 8	7/6/4
Hydroxyphenylacetic Acids					
3-(3-Hydroxyphenyl)propionic acid[b]	1.1/15/44	91 ± 49	98 ± 7	95 ± 9	42/8/3
3-Hydroxyphenylacetic acid	27/360/1100	93 ± 23	104 ± 10	96 ± 9	46/5/3
4-Hydroxyphenylacetic acid	34/460/1400	101 ± 22	89 ± 17	92 ± 19	22/9/6
Homovanillic acid	9.9/130/400	97 ± 23	97 ± 9	96 ± 12	28/5/5
Homoprotocatechuic acid	5.5/73/220	88 ± 17	97 ± 8	93 ± 8	15/4/2
Lignans					
Enterodiol	0.064/0.85/2.6	87 ± 36	87 ± 8	91 ± 8	23/7/6
Enterolactone	0.2/2.7/8.1	84 ± 13	90 ± 13	92 ± 15	16/5/4
Others					
2,6-Dimethoxyphenol	0.62/8.3/25	100 ± 13	100 ± 11	95 ± 14	13/14/14
3,5-Dimethoxy-4 -hydroxyphenylacetic acid[b]	4.3/58/170	94 ± 14	102 ± 10	94 ± 12	19/7/4
3-Methylcatechol	2/27/80	90 ± 11	96 ± 6	95 ± 11	12/4/3
4-Methylcatechol	4.4/59/180	92 ± 7	94 ± 6	95 ± 10	9/5/3
Catechol[b]	13/170/520	85 ± 45	53 ± 36	34 ± 53	68/59/38
Eugenol	30/390/1200	87 ± 24	93 ± 15	92 ± 13	26/12/8
Hydroxytyrosol	0.56/7.5/23	87 ± 9	91 ± 5	91 ± 8	13/5/2
Pyrogallol	5.8/77/230	92 ± 33	92 ± 9	89 ± 13	45/23/14
Thymol	8.9/120/360	n.d.	n.d.	76 ± 32	n.d./n.d./44
Urolithin A	0.11/1.5/4.4	87 ± 20	90 ± 6	92 ± 10	16/7/4
Stilbenes					
Dihydroresveratrol	0.47/6.2/19	88 ± 17	92 ± 11	93 ± 13	18/5/8
Polydatin	0.15/2.1/6.2	121 ± 38	88 ± 21	87 ± 13	60/10/7
Pterostilbene[b]	0.21/2.9/8.6	99 ± 19	91 ± 11	91 ± 10	26/8/7

Analyte	Spiking Level (LL/ML/HL) (ng*mL^{-1})	$R_E \pm RSD_R$ (LL) (%)	$R_E \pm RSD_R$ (ML) (%)	$R_E \pm RSD_R$ (HL) (%)	RSD_r (LL/ML/HL) (%)
Resveratrol	0.42/5.6/17	86 ± 15	92 ± 8	89 ± 11	24/4/4
Anthocyanins					
Cyanidin	59/780/2400	n.d.	43 ± 15	48 ± 15	n.d./39/43
Cyanidin-3-O-glucoside	0.81/11/32	n.d.	16 ± 13	14 ± 14	n.d./20/6
Cyanidin-3-O-rutinoside	0.69/9.2/28	n.d.	20 ± 18	21 ± 20	n.d./22/9
Cyanidin-3-O-sambubioside	1.5/20/61	n.d.	7 ± 28	6 ± 19	n.d./37/13
Delphinidin	70/940/2800	n.d.	23 ± 17	22 ± 13	n.d./35/36
Delphinidin-3-O-glucoside	30/400/1200	n.d.	11 ± 31	8 ± 16	n.d./43/13
Catechins					
(-)- Epicatechin	2.7/36/110	94 ± 12	85 ± 9	91 ± 10	20/10/8
(-)-Epicatechin gallate	1.1/14/43	90 ± 17	65 ± 12	66 ± 8	32/5/6
(-)-Epigallocatechin	19/250/740	101 ± 37	89 ± 9	87 ± 14	46/27/11
(-)-Epigallocatechin gallate	13/180/530	123 ± 31	50 ± 19	49 ± 18	22/14/13
(-)-Gallocatechin	19/250/740	90 ± 35	84 ± 9	84 ± 12	87/17/12
(+)-Catechin	1.6/21/64	85 ± 16	78 ± 5	85 ± 13	16/4/6
Flavanones					
(+/-)-Naringenin[b]	0.11/1.4/4.3	78 ± 50	88 ± 11	91 ± 16	43/6/3
8-Prenylnaringenin	0.26/3.5/11	93 ± 8	95 ± 6	92 ± 11	4/4/3
Hesperetin[b]	0.11/1.5/4.6	96 ± 16	91 ± 10	92 ± 8	10/6/5
Hesperidin	0.079/1.1/3.2	99 ± 15	94 ± 10	95 ± 7	14/7/12
Isoxanthohumol[b]	0.054/0.73/2.2	96 ± 13	92 ± 6	92 ± 7	19/5/4
Naringin	3/40/120	85 ± 28	86 ± 14	80 ± 17	16/13/14
Neohesperidin	4/54/160	86 ± 22	84 ± 8	84 ± 9	14/11/6
Neohesperidin dihydrochalcone	0.079/1/3.1	86 ± 16	82 ± 15	87 ± 12	9/9/9
Xanthohumol[b]	0.16/2.1/6.3	92 ± 14	91 ± 10	90 ± 8	8/5/3
Flavones					
Apigenin	0.12/1.6/4.7	87 ± 6	86 ± 9	94 ± 11	6/6/3

Analyte	Spiking Level (LL/ML/HL) (ng*mL^{-1})	$R_E \pm RSD_R$ (LL) (%)	$R_E \pm RSD_R$ (ML) (%)	$R_E \pm RSD_R$ (HL) (%)	RSD_r (LL/ML/HL) (%)
Diosmetin	0.086/1.2/3.5	92 ± 25	92 ± 9	95 ± 11	7/6/3
Diosmin	0.34/4.5/14	120 ± 20	93 ± 20	79 ± 21	17/18/12
Flavonols					
(+)-Rutin[b]	0.39/5.2/16	78 ± 13	61 ± 9	62 ± 12	12/12/8
Isorhamnetin	0.079/1.1/3.2	95 ± 15	71 ± 5	72 ± 10	28/5/2
Kaempferol	1.8/24/72	93 ± 13	90 ± 10	88 ± 13	8/8/8
Kaempferol-3-O-glucuronide	0.1/1.4/4.2	91 ± 18	85 ± 9	84 ± 16	21/6/11
Quercetin[b]	0.71/9.5/28	97 ± 15	47 ± 12	44 ± 13	15/11/11
Quercetin-7-O-β-D-glucuronide	0.43/5.8/17	80 ± 22	55 ± 21	51 ± 20	34/13/11
Isoflavones					
Biochanin A	0.12/1.6/4.7	88 ± 18	91 ± 5	91 ± 8	12/4/3
Daidzein	0.15/2/5.9	84 ± 35	91 ± 7	93 ± 9	11/5/4
Daidzein-7-β-D-glucuronide	0.43/5.7/17	88 ± 22	88 ± 9	88 ± 10	16/9/3
Genistein	0.13/1.7/5.1	78 ± 6	65 ± 16	93 ± 17	5/7/5
Genistein-7-β-D-glucuronide	0.48/6.5/19	94 ± 15	91 ± 13	90 ± 16	12/10/5
Genistein-7-sulfate[b]	3.8/51/150	89 ± 7	59 ± 23	30 ± 62	6/4/3
S-Equol	5.8/77/230	77 ± 61	94 ± 13	92 ± 12	40/11/6
Proanthocyanidins					
Procyanidin A2	0.93/12/37	94 ± 34	78 ± 13	73 ± 11	38/14/17
Procyanidin B1	15/200/590	60 ± 16	71 ± 7	71 ± 8	12/4/3
Procyanidin B2	3.9/53/160	n.d.	70 ± 56	68 ± 22	n.d./21/8
Procyanidin C1	2.8/37/110	80 ± 31	53 ± 12	48 ± 12	51/12/6

Table S5. Method validation outcomes, the analytes where all figures of merit fit the validation criteria are check marked, while for the others the criteria that are out of acceptable range are listed. Evaluated figures of merit are regression coefficient (R^2), recovery (R_E), intermediate precision (RSD_R) and repeatability (RSD_r) at the low (LL), middle (ML) and high (HL) spiking level.

Analyte	Validation Outcome
Dihydrochalcones	
Phloretin	✓
Hydroxybenzoic Acids	
3,5-Dihydroxybenzoic acid	R_E LL/ML/HL, RSD_R LL, RSD_r LL/ML/HL
3-Hydroxybenzoic acid	✓
4-Hydroxybenzoic acid	✓
Benzoic acid	R_E LL, RSD_R LL, RSD_r LL
Ellagic acid	R_E LL/ML/HL, RSD_R LL, RSD_r LL
Ethyl gallate	✓
Gallic acid	R_E LL/ML/HL, RSD_R LL, RSD_r LL/HL
Protocatechuic acid	R_E LL/ML/HL, RSD_R LL, RSD_r LL/ML/HL, R^2
Salicylic acid	✓
Syringic acid	✓
Vanillic acid	✓
Hydroxycinnamic Acids	
Caffeic acid	✓
Caffeic acid-3-β-D-glucuronide	✓
Chlorogenic acid	✓
Cinnamic acid	✓
Dihydrocaffeic acid	✓
Dihydroferulic acid	✓
Ferulic acid/Isoferulic acid	✓
p-Coumaric acid	✓
Sinapic acid	✓
trans-m-Coumaric acid	✓
trans-o-Coumaric acid	✓
Hydroxyphenylacetic Acids	
3-(3-Hydroxyphenyl)propionic acid	RSD_R LL, RSD_r LL
3-Hydroxyphenylacetic acid	RSD_r LL
4-Hydroxyphenylacetic acid	✓
Homovanillic acid	✓
Homoprotocatechuic acid	✓
Lignans	
Enterodiol	✓

Analyte	Validation Outcome
Enterolactone	✓
Others	
2,6-Dimethoxyphenol	✓
3,5-Dimethoxy-4-hydroxyphenylacetic acid	✓
3-Methylcatechol	✓
4-Methylcatechol	✓
Catechol	R_E ML/HL, RSD_R LL/ML/HL, RSD_r LL/ML/HL, R^2
Eugenol	✓
Hydroxytyrosol	✓
Pyrogallol	R_E LL, RSD_r LL
Thymol	R_E LL/ML, RSD_R LL/ML/HL, RSD_r LL/ML/HL, R^2
Urolithin A	✓
Stilbenes	
Dihydroresveratrol	✓
Polydatin	RSD_r LL
Pterostilbene	✓
Resveratrol	✓
Anthocyanins	
Cyanidin	R_E LL/ML/HL, RSD_R LL, RSD_r LL/ML/HL
Cyanidin-3-O-glucoside	R_E LL/ML/HL, RSD_R LL, RSD_r LL
Cyanidin-3-O-rutinoside	R_E LL/ML/HL, RSD_R LL, RSD_r LL
Cyanidin-3-O-sambubioside	R_E LL/ML/HL, RSD_R LL, RSD_r LL/ML
Delphinidin	R_E LL/ML/HL, RSD_R LL, RSD_r LL
Delphinidin-3-O-glucoside	R_E LL/ML/HL, RSD_R LL/ML, RSD_r LL/ML
Catechins	
(-)-Epicatechin	✓
(-)-Epicatechin gallate	✓
(-)-Epigallocatechin	R_E LL, RSD_r LL
(-)-Epigallocatechin gallate	R_E LL
(-)-Gallocatechin	R_E LL, RSD_r LL
(+)-Catechin	✓
Flavanones	
(+/-)-Naringenin	RSD_R LL, RSD_r LL
8-Prenylnaringenin	✓
Hesperetin	✓
Hesperidin	✓
Isoxanthohumol	✓

Analyte	Validation Outcome
Naringin	✓
Neohesperidin	✓
Neohesperidin dihydrochalcone	✓
Xanthohumol	✓
Flavones	
Apigenin	✓
Diosmetin	✓
Diosmin	✓
Flavonols	
(+)-Rutin	✓
Isorhamnetin	✓
Kaempferol	✓
Kaempferol-3-O-glucuronide	✓
Quercetin	R_E HL
Quercetin-7-O-β-D-glucuronide	✓
Isoflavones	
Biochanin A	✓
Daidzein	✓
Daidzein-7-β-D-glucuronide	✓
Genistein	✓
Genistein-7-β-D-glucuronide	✓
Genistein-7-sulfate	RSD_R HL, R^2
S-Equol	RSD_R LL, RSD_r LL
Proanthocyanidins	
Procyanidin A2	✓
Procyanidin B1	✓
Procyanidin B2	R_E LL, RSD_R LL/ML, RSD_r LL
Procyanidin C1	R_E HL, RSD_r LL

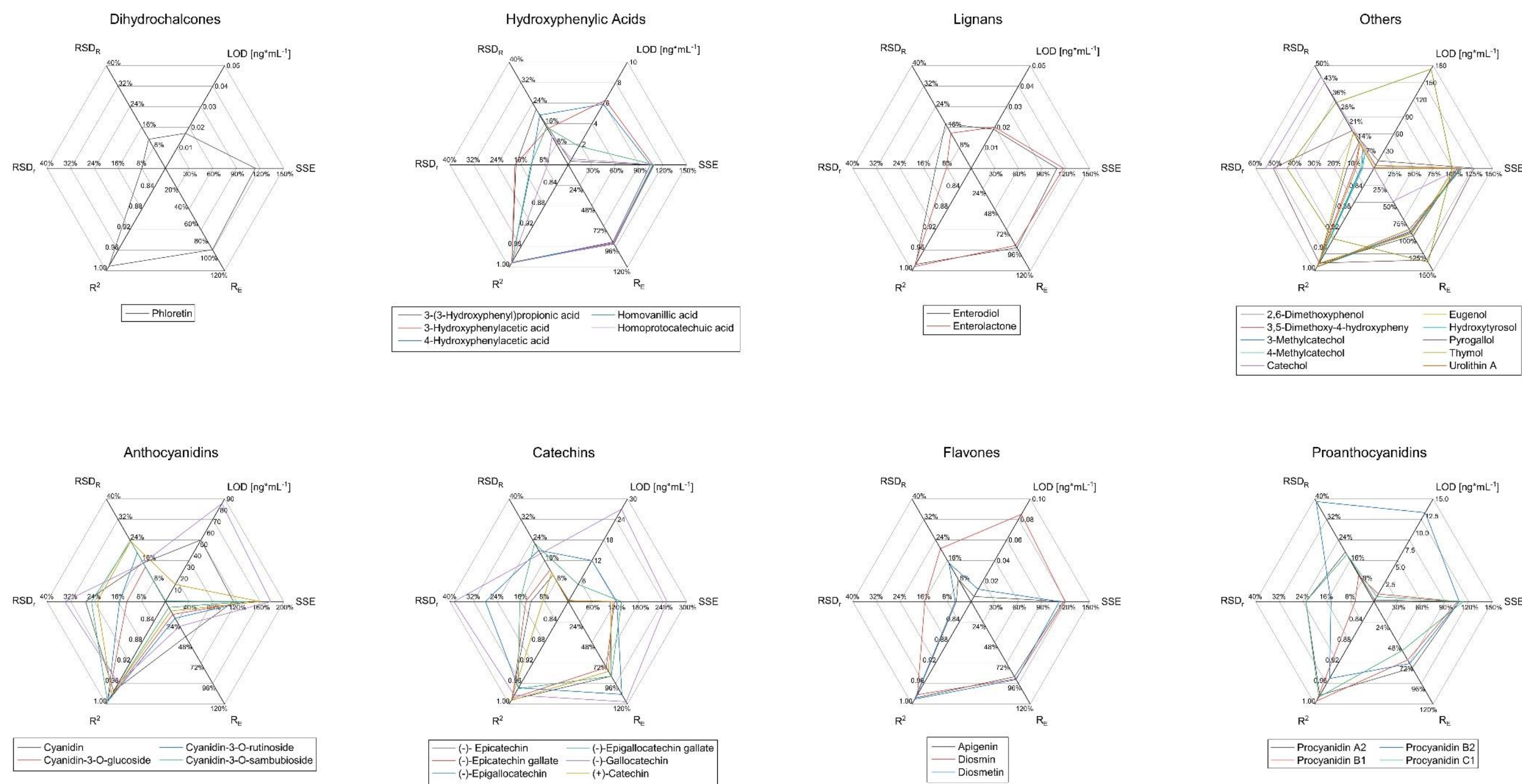


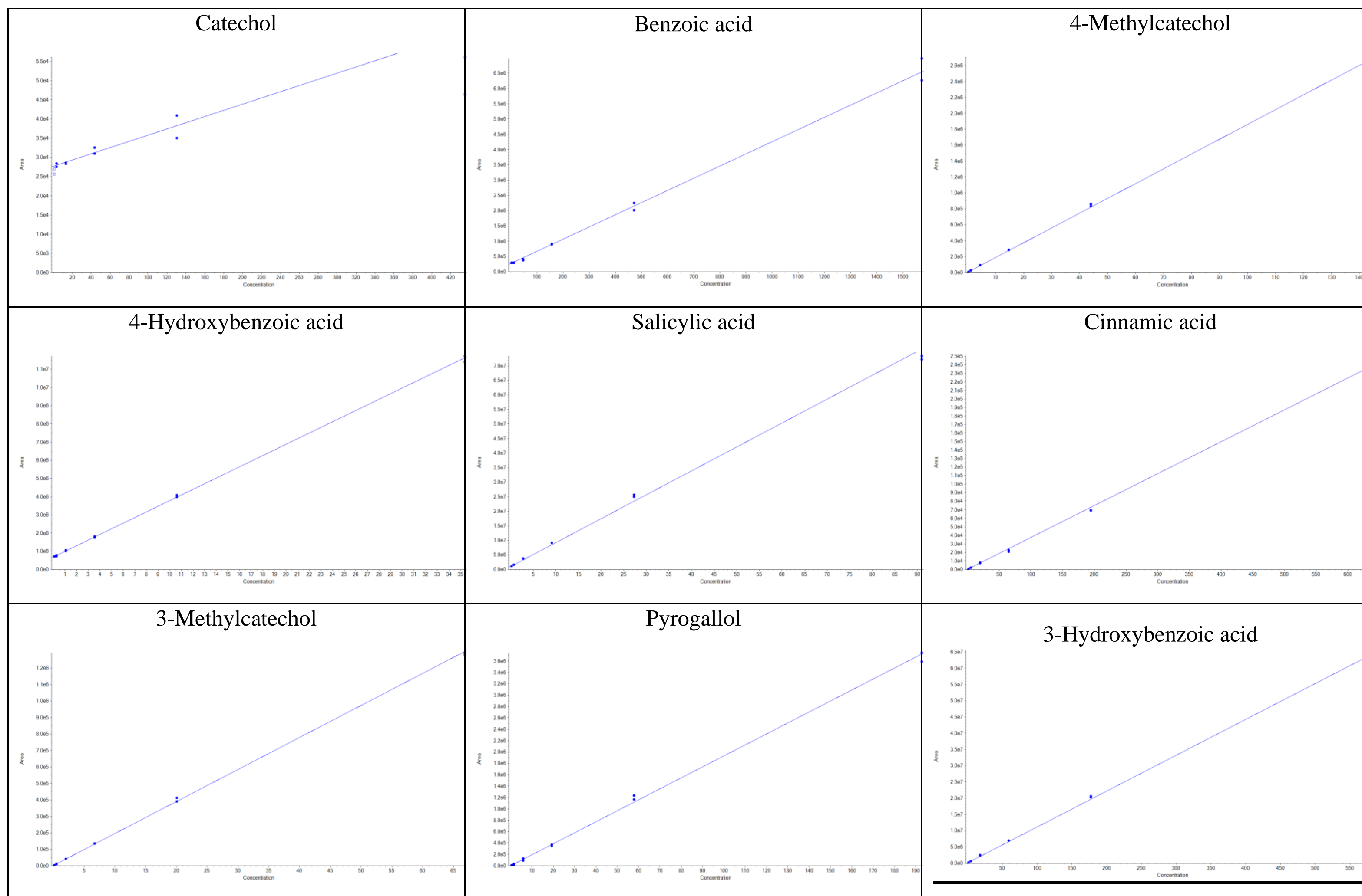
Figure S2. Analytical figures of merit evaluated during the method validation for remaining eight polyphenol classes (see Figure 1). The limit of quantification (LOQ), regression coefficient (R^2), average recovery (R^E), average intermediate precision (RSD_R) and average repeatability (RSD_r) are displayed.

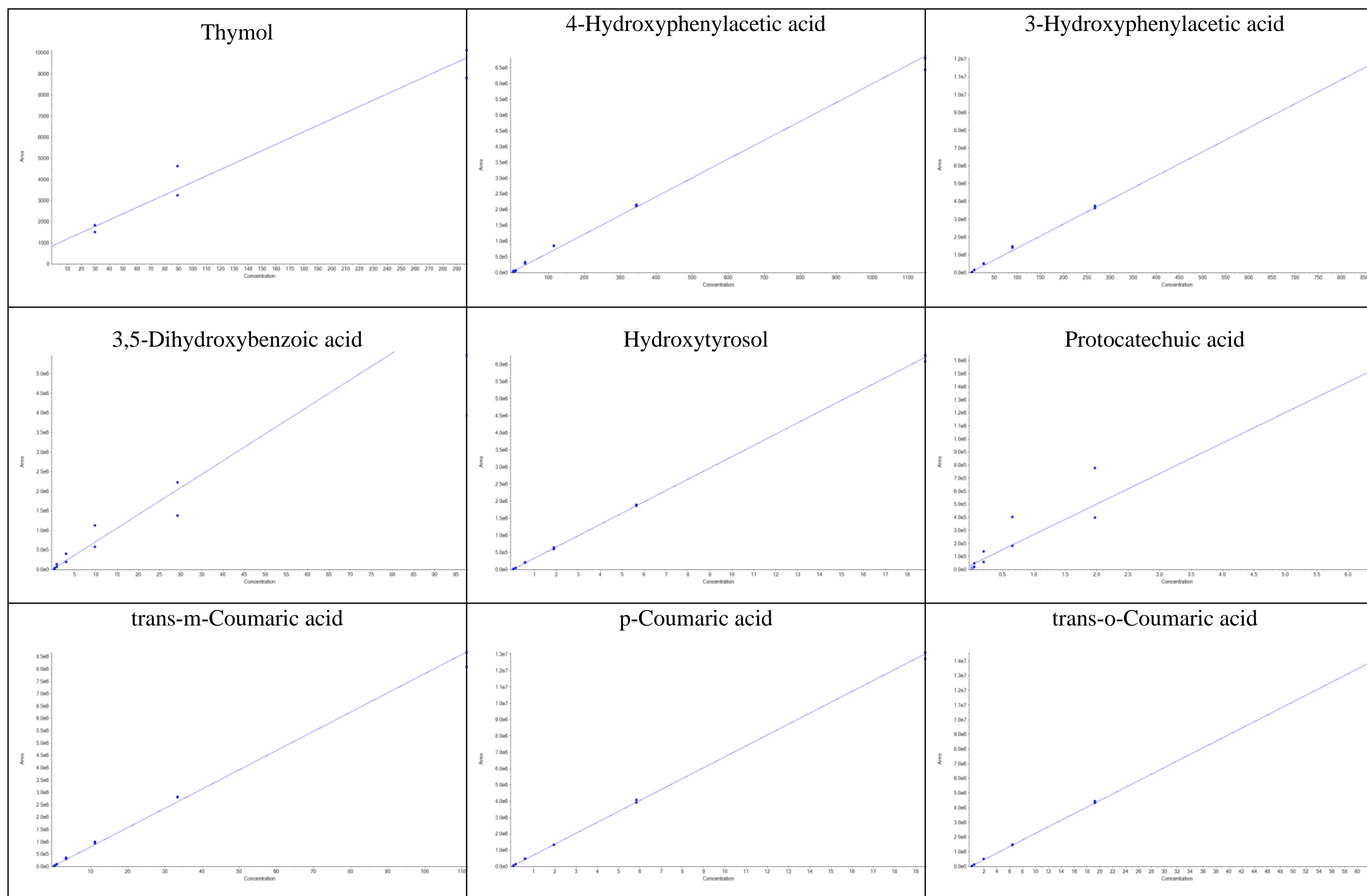
Table S6. Estimated daily polyphenol intake calculated for each breast milk sample (S1-S10) from one month after birth and calculated mean and median values. All values are given in microgram analyte per kilogram infant bodyweight per day ($\mu g*kg^{-1}$ bw day^{-1}).

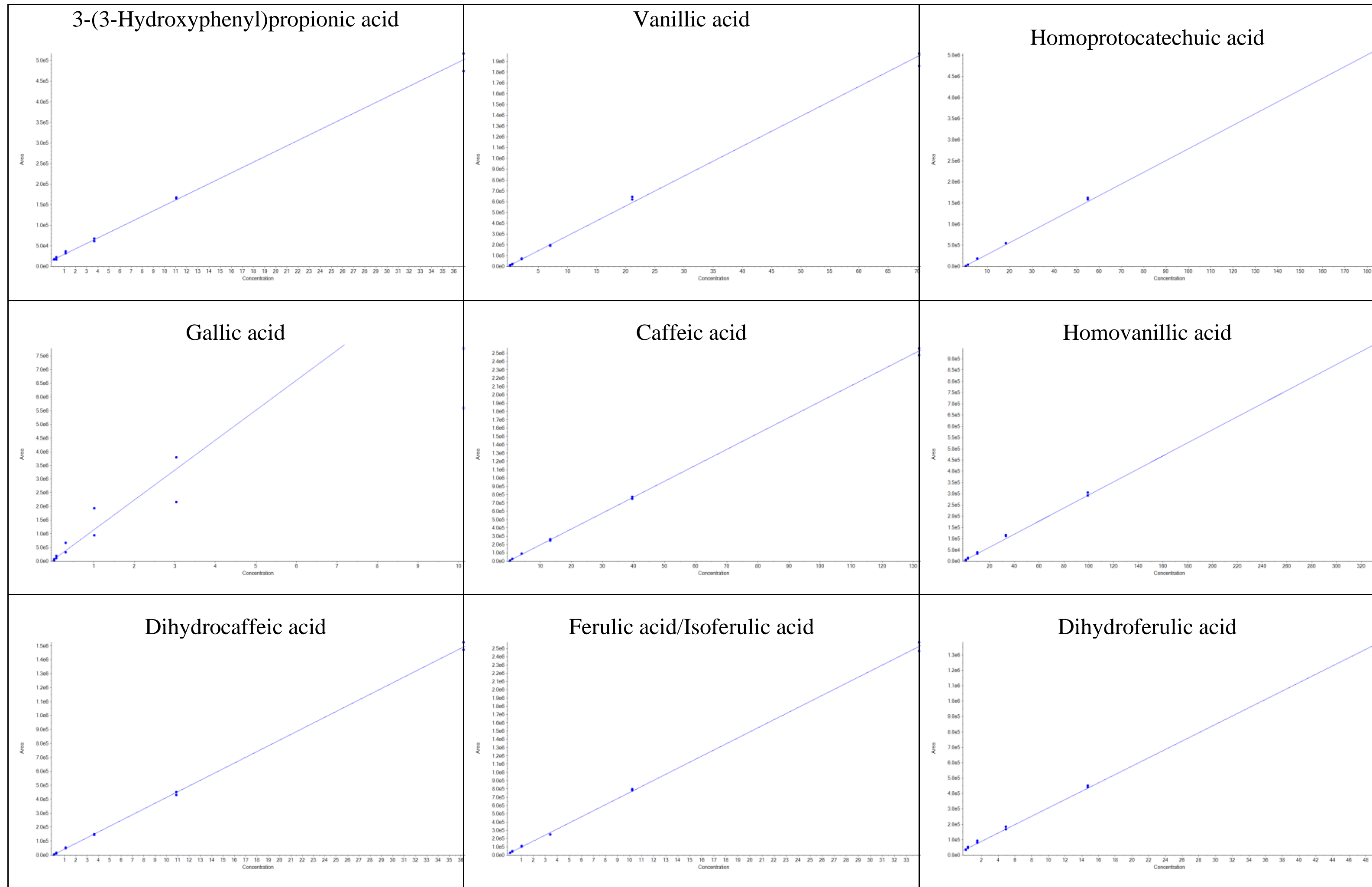
Analyte	Mean	Median	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Dihydrochalcones												
Phloretin	0.0044	0.0044	-	-	-	-	-	-	-	-	0.0044	-
Hydroxybenzoic Acids												
3,5-Dihydroxybenzoic acid	8.6	0.34	-	-	0.42	4.1	0.1	47	0.25	-	0.1	-
3-Hydroxybenzoic acid	0.46	0.44	-	-	0.44	0.53	0.25	0.88	0.23	-	0.66	0.21
4-Hydroxybenzoic acid	7.5	1.9	0.91	0.83	6	11	1.1	48	0.94	1.4	2.4	2.3
Gallic acid	0.032	0.032	-	-	-	-	-	0.032	-	-	-	-
Protocatechuic acid	0.72	0.024	0.015	-	0.038	0.39	-	4.5	0.024	-	0.015	0.015
Salicylic acid	11	0.54	1.1	0.32	0.51	28	0.5	42	36	0.16	0.49	0.56
Vanillic acid	0.14	0.15	0.056	-	0.11	0.2	0.087	0.21	0.19	-	-	-
Hydroxycinnamic Acids												
Chlorogenic acid	0.68	0.67	-	1	-	-	0.34	-	-	-	-	-
Dihydrocaffeic acid	1.3	0.16	-	-	-	0.16	-	3.8	0.041	-	-	-
Dihydroferulic acid	0.49	0.50	0.58	-	-	-	-	0.41	-	-	-	-
Ferulic acid/Isoferulic acid	0.12	0.094	0.046	0.091	0.39	0.06	0.096	0.21	0.026	0.11	0.076	0.11
p-Coumaric acid	1.1	0.79	-	-	0.08	1.9	-	1.5	2.8	0.012	-	0.012
Sinapic acid	0.021	0.021	-	0.021	-	-	-	-	-	-	-	-
Hydroxyphenylacetic Acids												
3-(3-Hydroxyphenyl)propionic acid	0.2	0.195	0.27	0.095	-	-	0.12	-	-	-	-	0.31
4-Hydroxyphenylacetic acid	180	5.5	1.5	-	58	19	2.9	1500	5.5	1.5	47	2
Homovanillic acid	0.64	0.66	-	-	0.81	-	-	0.46	-	-	-	0.66
Homoprotocatechuic acid	7.8	7.8	-	-	-	-	-	7.8	-	-	-	-
Lignans												
Enterodiol	0.054	0.054	-	-	-	-	-	0.091	-	-	0.017	-

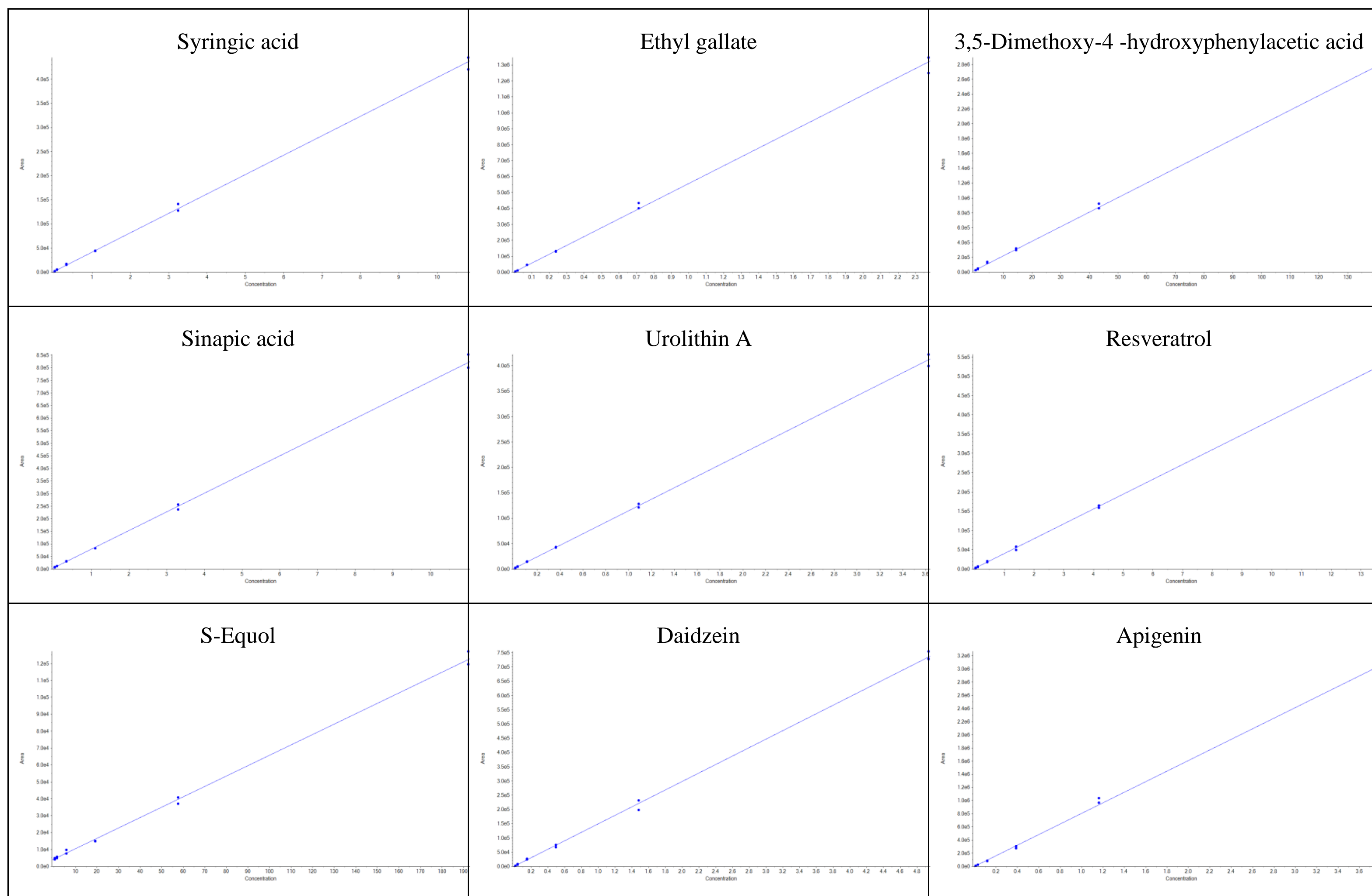
Analyte	Mean	Median	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Enterolactone	0.099	0.031	-	-	0.03	0.24	-	-	-	-	0.031	-
Others												
2,6-Dimethoxyphenol	0.04	0.04	-	-	-	-	-	0.04	-	-	-	-
3,5-Dimethoxy-4-hydroxyphenylacetic acid	1.7	1.7	-	-	-	0.66	-	2.8	-	-	-	-
4-Methylcatechol	1.4	1.4	-	-	-	-	-	1.4	-	-	-	-
Catechol	89	31	13	1.3	-	160	1.3	290	-	-	31	120
Hydroxytyrosol	1.5	1.5	-	-	-	-	-	2.9	-	-	0.019	-
Pyrogallol	0.47	0.47	0.5	0.44	-	-	-	-	-	-	-	-
Flavanones												
(+/-)-Naringenin	0.27	0.14	0.012	0.009	0.24	0.24	-	0.17	0.14	0.013	1.6	0.043
8-Prenylnaringenin	0.16	0.16	-	-	-	-	-	-	-	-	0.16	-
Hesperetin	0.17	0.17	-	-	-	-	-	0.29	0.052	-	-	-
Flavones												
Apigenin	0.093	0.037	-	-	0.009	0.037	-	0.23	-	-	-	-
Diosmetin	0.055	0.022	0.0038	0.011	0.04	0.1	-	0.2	-	-	0.022	0.008
Flavonols												
Isorhamnetin	0.0073	0.0045	0.0023	-	-	0.015	-	0.004	-	-	-	-
Kaempferol	0.043	0.043	-	-	-	0.043	-	-	-	-	0.043	-
Kaempferol-3-O-glucuronide	0.1	0.1	-	0.1	-	-	-	-	-	-	-	-
Isoflavones												
Daidzein	0.26	0.066	-	-	0.008	1.1	-	0.066	0.099	-	-	0.008
Genistein	0.043	0.018	-	-	0.018	0.14	-	0.014	0.026	-	-	0.013
Genistein-7-sulfate	0.045	0.045	-	-	-	0.045	-	-	0.045	0.045	-	0.045
Proanthocyanidins												
Procyanidin C1	0.15	0.15	0.16	-	0.15	-	0.15	0.15	-	-	-	-

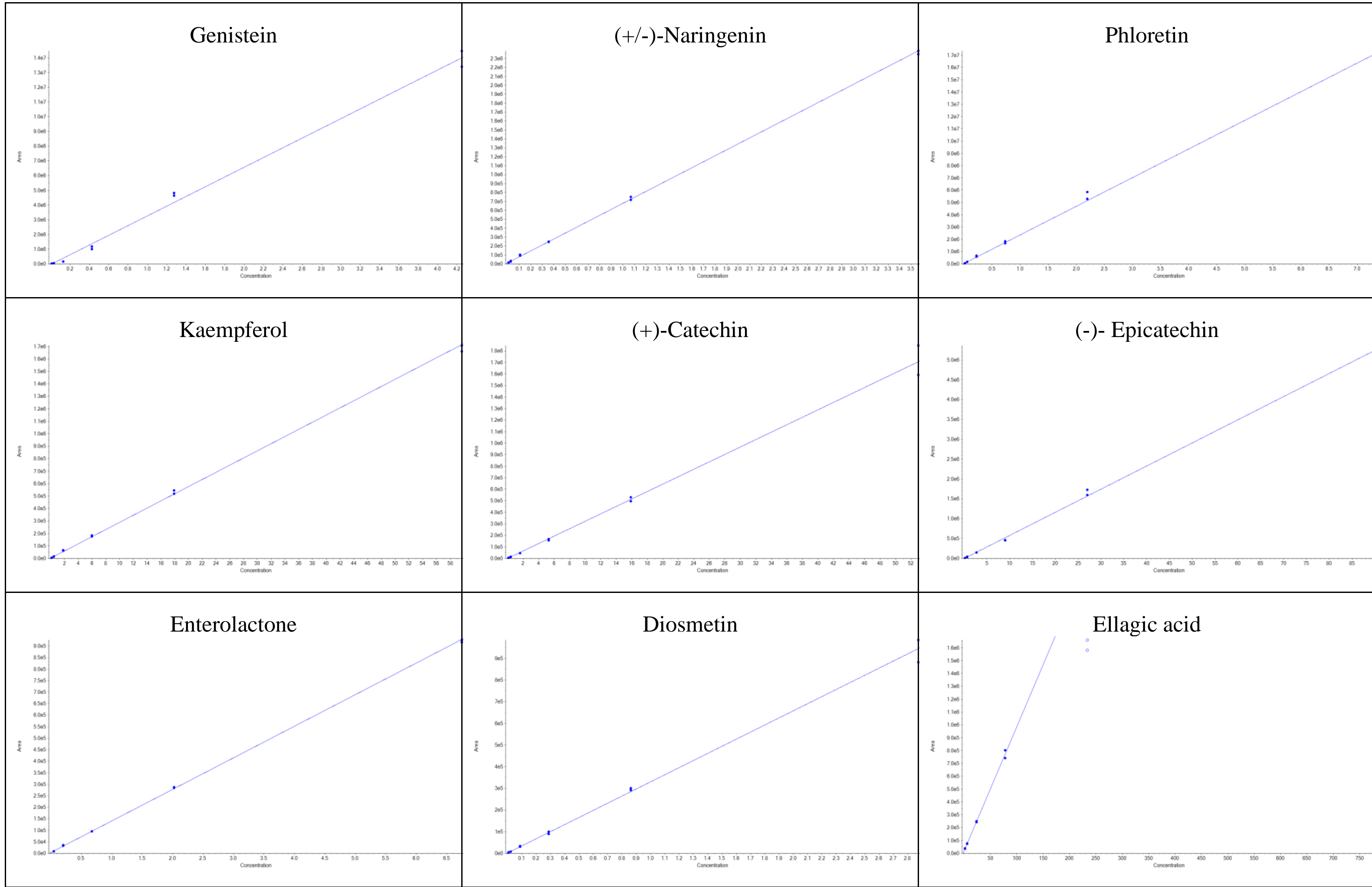
Table S7. Calibration curves for each analyte, with the analyte concentration [ng*mL^{-1}] on the x-axis and the peak area on the y-axis.

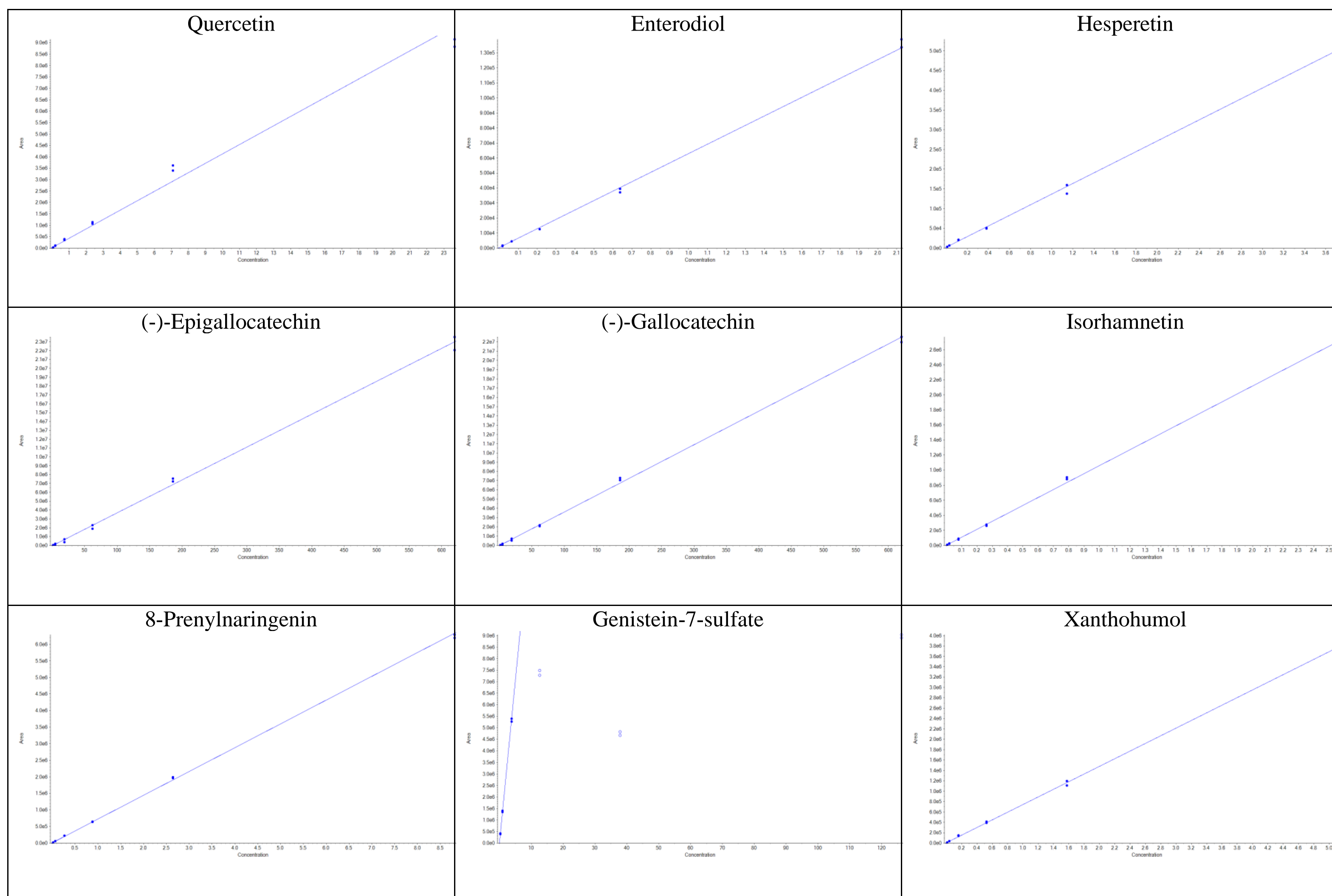


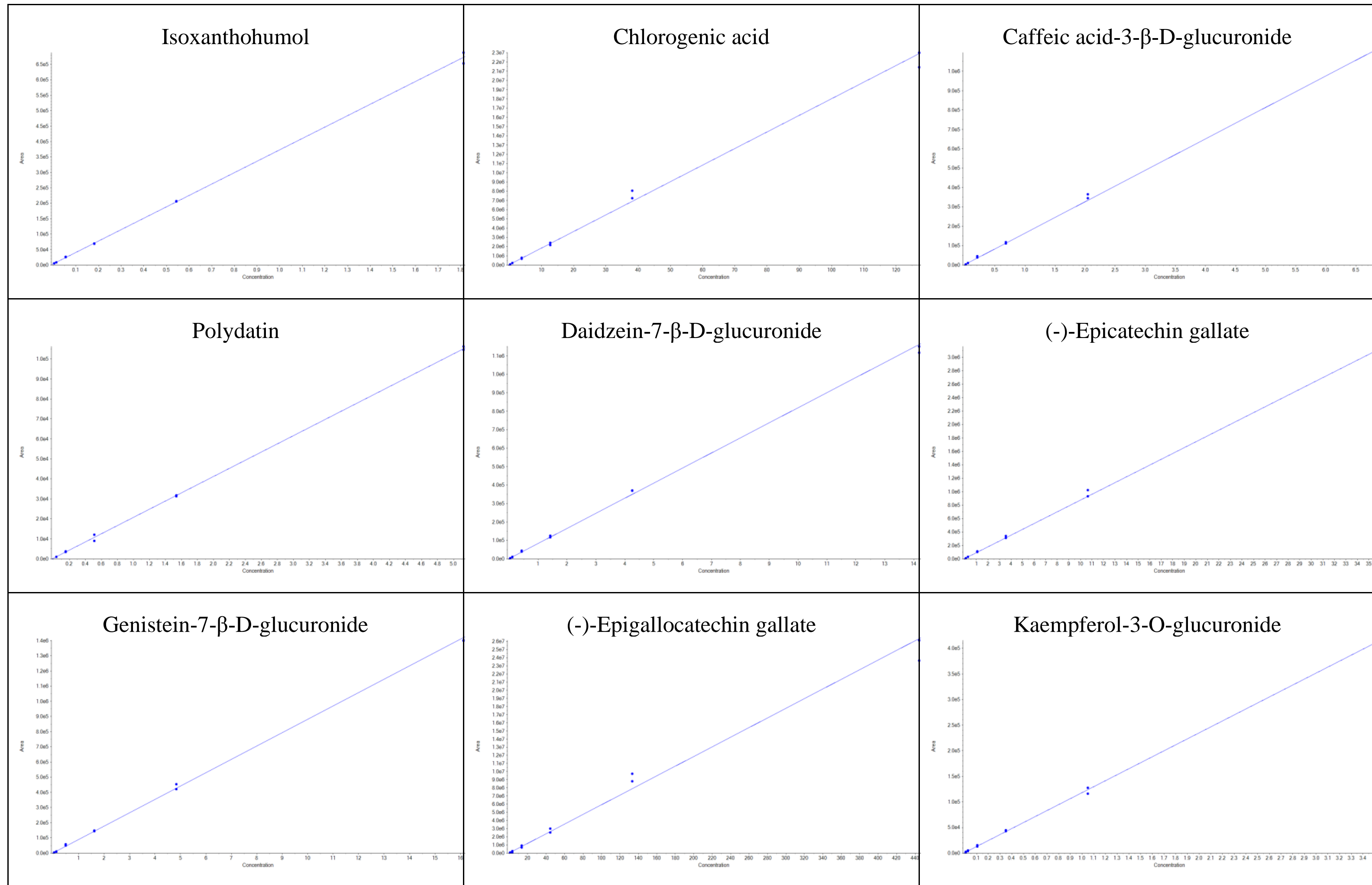

Thymol
4-Hydroxyphenylacetic acid
3-Hydroxyphenylacetic acid
3,5-Dihydroxybenzoic acid
Hydroxytyrosol
Protocatechuic acid
trans-m-Coumaric acid
p-Coumaric acid
trans-o-Coumaric acid


3-(3-Hydroxyphenyl)propionic acid
Vanillic acid
Homoprotocatechuic acid
Gallic acid
Caffeic acid
Homovanillic acid
Dihydrocaffeic acid
Ferulic acid/Isoferulic acid
Dihydroferulic acid

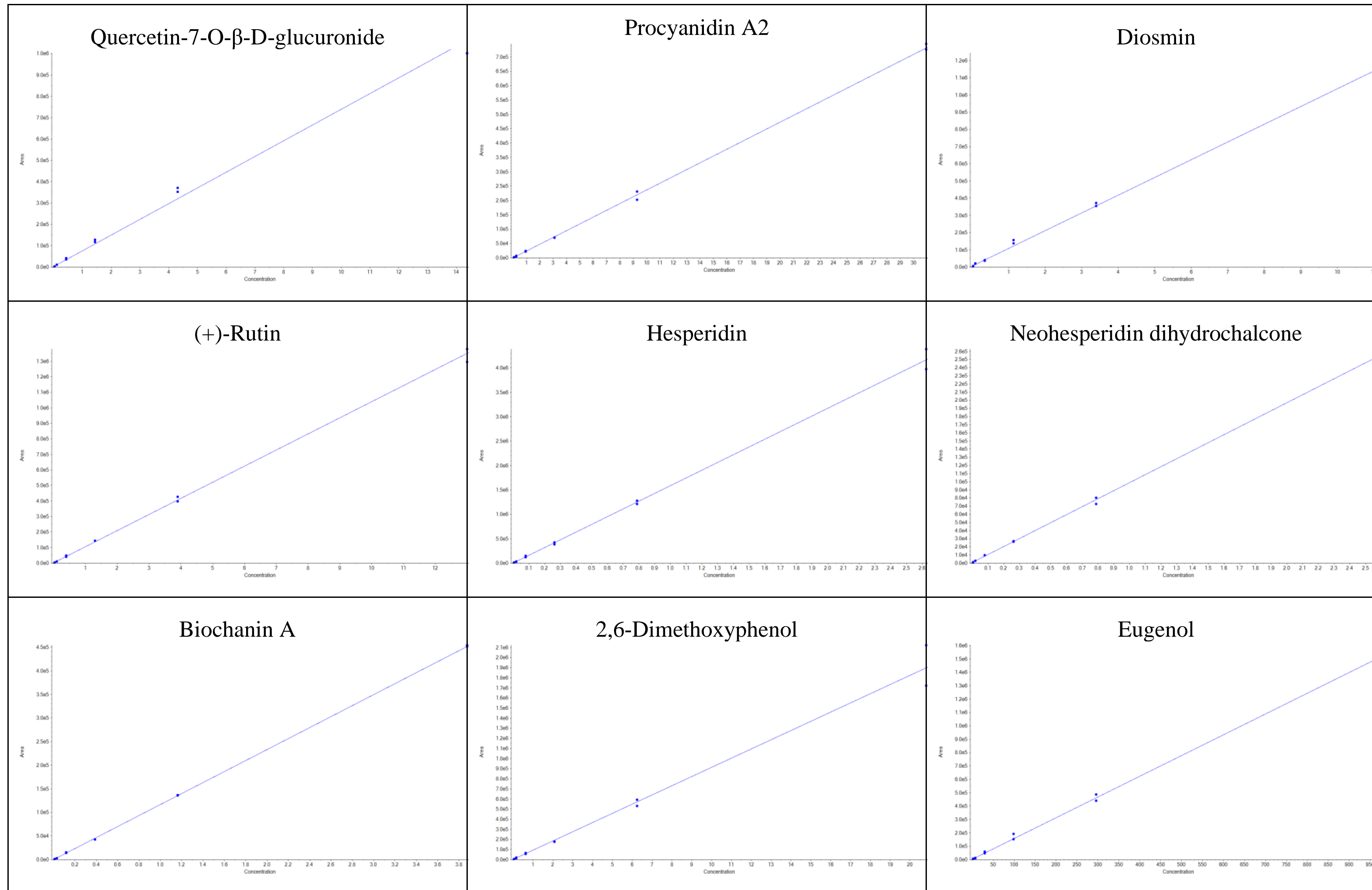

Syringic acid
Ethyl gallate
3,5-Dimethoxy-4 -hydroxyphenylacetic acid
Sinapic acid
Urolithin A
Resveratrol
S-Equol
Daidzein
Apigenin

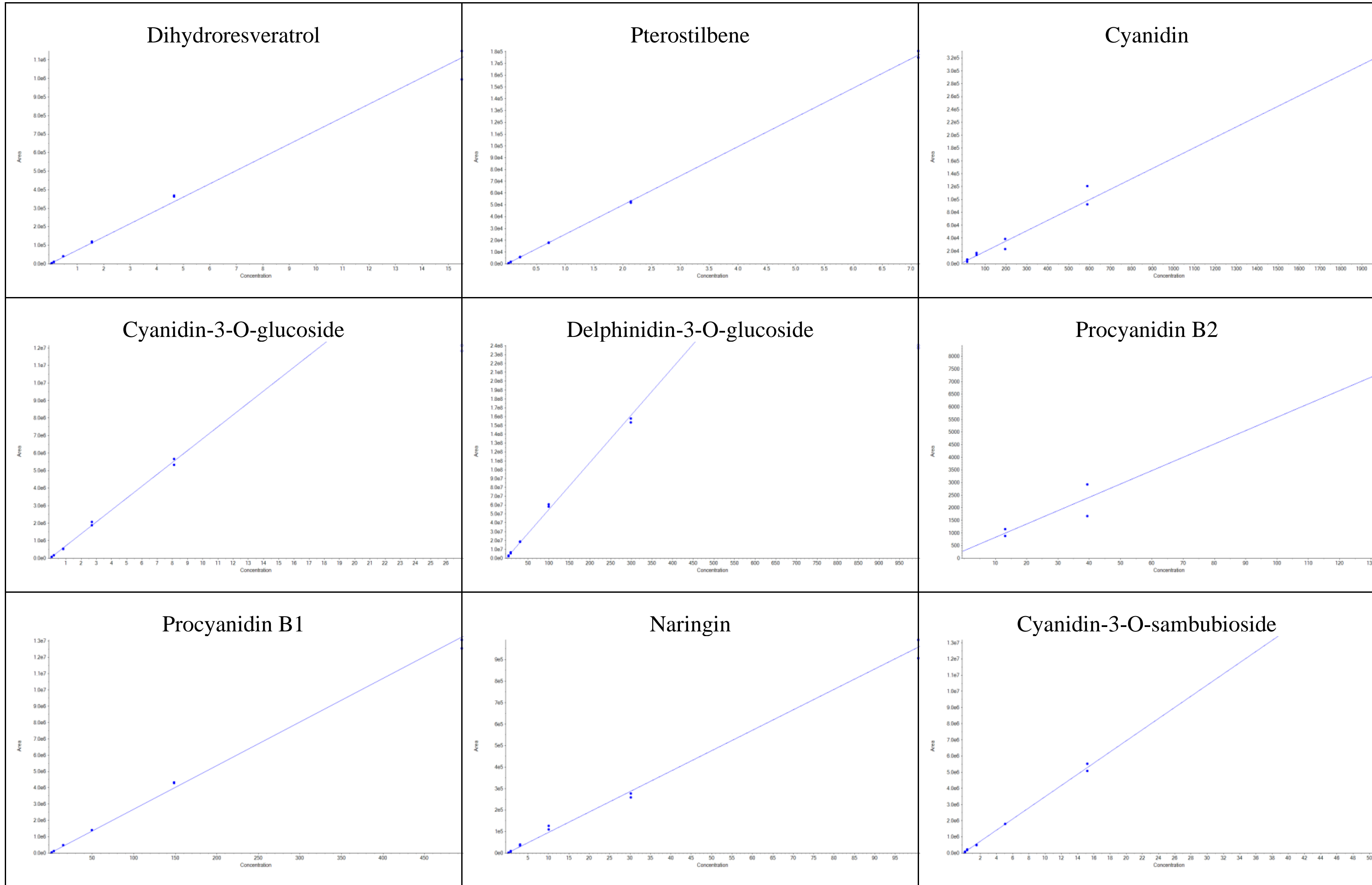

Genistein
(+/-)-Naringenin
Phloretin
Kaempferol
(+)-Catechin
(-)- Epicatechin
Enterolactone
Diosmetin
Ellagic acid

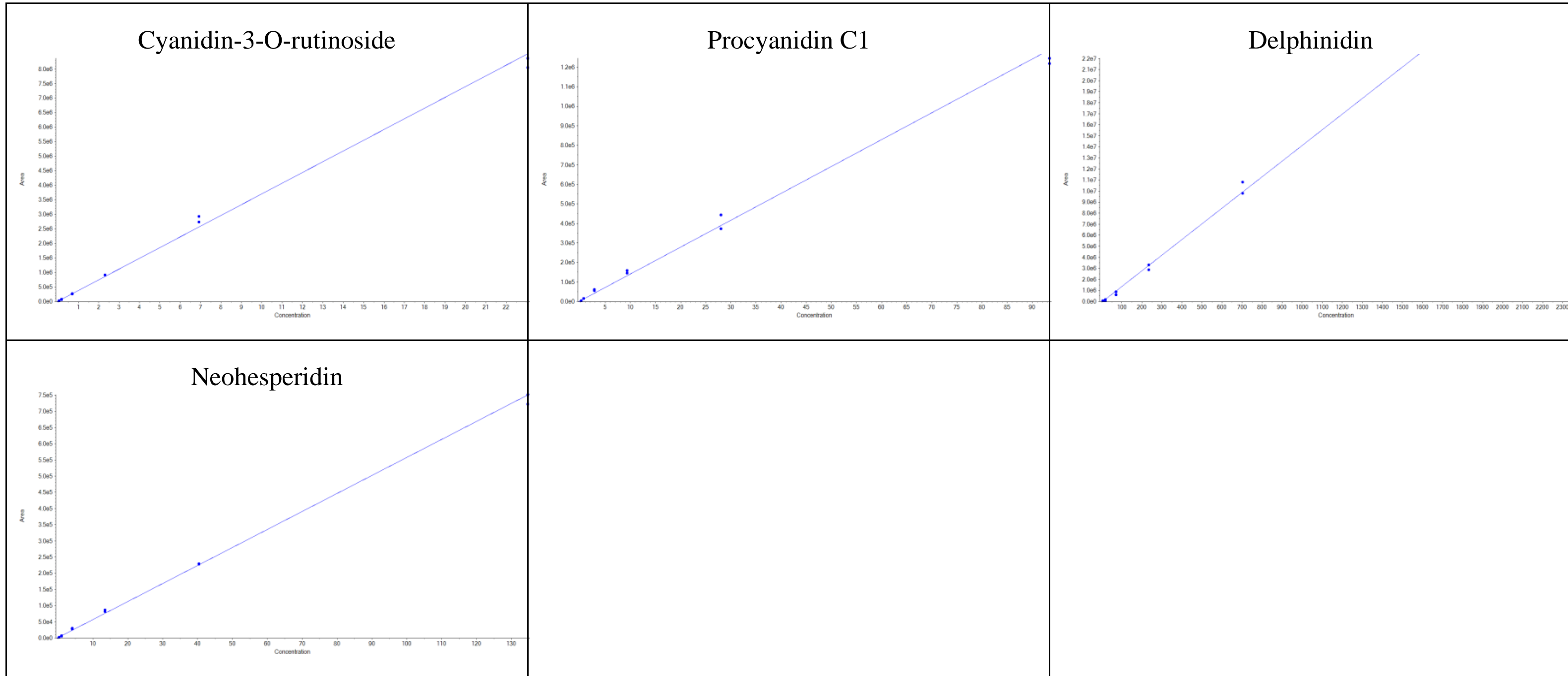

Quercetin
Enterodiol
Hesperetin
(-)-Epigallocatechin
(-)-Gallocatechin
Isorhamnetin
8-Prenylnaringenin
Genistein-7-sulfate
Xanthohumol



Isoxanthohumol
Chlorogenic acid
Caffeic acid-3-β-D-glucuronide
Polydatin
Daidzein-7-β-D-glucuronide
(-)-Epicatechin gallate
Genistein-7-β-D-glucuronide
(-)-Epigallocatechin gallate
Kaempferol-3-O-glucuronide


Quercetin-7-O-β-D-glucuronide
Procyanidin A2
Diosmin
(+)-Rutin
Hesperidin
Neohesperidin dihydrochalcone
Biochanin A
2,6-Dimethoxyphenol
Eugenol
Area
Concentration


Dihydroresveratrol
Pterostilbene
Cyanidin
Cyanidin-3-O-glucoside
Delphinidin-3-O-glucoside
Procyanidin B2
Procyanidin B1
Naringin
Cyanidin-3-O-sambubioside


Cyanidin-3-O-rutinoside
Procyanidin C1
Delphinidin
Neohesperidin
Area
Concentration

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5. Conclusion

To summarize, a sample preparation method for the extraction and subsequent quantification of 86 polyphenols from all major polyphenol classes in breast milk using LC-MS/MS was developed and validated in-house. Approximately 70% of these polyphenols fully met the stringent validation criteria. Although the remaining analytes did not meet all validation criteria at every concentration level, they still offer valuable semi-quantitative insights for nutritional and biomedical research. The validation process demonstrated a mean recovery of 81% and a mean signal suppression or enhancement effect of 117%. The limits of detection for all analytes ranged from 0.0041 to 87 ng*mL^{-1}. In the pilot study, 50 different polyphenols were identified in the breast milk samples from twelve Nigerian mothers, with phenolic acids being the most prevalent.

This high-throughput sample preparation technique demonstrated minimal matrix effects, a high sensitivity, and efficient analyte recoveries, despite the challenges posed by limited sample volumes and a range of diverse analytes. The developed method holds promise for future studies, potentially aiding in better understanding human metabolism. Its application in large cohort studies can enhance our understanding of how dietary polyphenols are transferred through breast milk to infants. Additionally, this method may prove valuable in future research, supporting nutritional interventions and prevention studies, as well as investigating the impact of polyphenols on infant health and microbiome development.

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A. Appendix

SOP - Extraction of Polyphenol in Human Breast Milk for LC-MS/MS Analysis on the QTrap 7500+

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Reviewer	Ian Oesterle
Date	20.09.2023
Version	1

- **Prepare freshly or use old standard reference mix:**
  - Prepare fresh or use old standard reference individual stock solutions at 1 mg/mL and then serially dilute them to make stocks with concentrations of: 100 µg/mL and 10 µg/mL
  - Use the individual stock solutions to prepare the mixes A and B (see file: *Working_Solutions_PoPhe_BM.xlsx*)

- **Prepare working mixes:**
  - Prepare working mix 6 by following the mixing scheme in *Working_Solutions_PoPhe_BM.xlsx*
  - Prepare the other working solutions by serial dilution as follows:

Working Mix	Quantity and Which Mix	Quantity of Methanol added
Working Mix 5	270 µL of working mix 6	630 µL
Working Mix 4	400 µL of working mix 5	800 µL
Working Mix 3	270 µL of working mix 4	630 µL
Working Mix 2	200 µL of working mix 3	600 µL
Working Mix 1	180 µL of working mix 2	420 µL

- **General extraction procedure:**
  1. Gently thaw a 1 mL aliquot of breast milk and vortex briefly to homogenize
  2. Transfer 200 µL to a 0.5 mL Eppendorf tube
  3. Add 400 µL of extraction solvent (acetonitrile with 1% v/v formic acid)
  4. Vortex for 3 min
  5. Weigh in 20 mg NaCl and 80 mg anhydrous $MgSO_4$
  6. Add extraction solution (step 4) to the salts (step 5) and vortex this solution for 3 min
  7. Centrifuge at 4°C and 2'000 x g for 10 min
  8. Transfer 300 µL supernatant to an new Eppendorf tube
  9. Place supernatant at -20°C for at least 2 h
  10. Centrifuge at 4°C and 18'000 x g for 2 min
  11. Transfer 250 µL of the supernatant to a new tube
  12. Dilute supernatant with 250 µL water acidified with 1% formic acid
  13. Centrifuge at 4°C and 18'000 x g for 5 min
  14. Transfer supernatant to a 1.5 mL amber LC glass vial with a 300 µL glass insert

- **Quality control (pre-spike):**
  - Low level: to the 200 µL of matrix (step 2), add 15 µL of working mix 2 and 385 µL extraction solvent (acetonitrile with 1,04% v/v formic acid)
  - Middle level: to the 200 µL of matrix (step 2), add 15 µL of working mix 4 and 385 µL extraction solvent (acetonitrile with 1,04% v/v formic acid)

- High level: to the 40 µL of matrix (step 2), add 15 µL of working mix 5 and 385 µL extraction solvent (acetonitrile with 1,04% v/v formic acid)

The samples then continued the general extraction procedure (starting at step 4).

- **Calibration curves:**
  - For matrix-matched calibration: use supernatant at the end of sample extraction (step 14) from a "blank matrix"
  - For neat solvent calibration: use acetonitrile: water (1:1) with 1% v/v formic acid

Calibration point	Quantity of supernatant (for matrix-matched calibration) or solvent (for solvent calibration)	Quantity and which working mix
Blank	157 µL	3 µL of Methanol
Standard level 1 (STD1)	157 µL	3 µL of working mix 1
Standard level 2 (STD2)	157 µL	3 µL of working mix 2
Standard level 3 (STD3)	157 µL	3 µL of working mix 3
Standard level 4 (STD4)	157 µL	3 µL of working mix 4
Standard level 5 (STD5)	157 µL	3 µL of working mix 5
Standard level 6 (STD6)	157 µL	3 µL of working mix 6

- **LC-MS/MS Measurements:**

Instrument	Agilent Infinity 1290 II UHPLC with SCIEX QTrap 6500+		
Acquisition method	230321_PoPhe_Final_Method_Transfered_3uL		
Injection volume	3 µL		
Autosampler temperature	7°C		
Column temperature	30°C		
Column	Acquity UPLC HSS T3 (2.1 x 100 mm, 1.8 µM, Waters) with a VanGuard precolumn (1.8 µM, Waters)		
Flow rate	0.6 mL/min		
Needle wash	Water: methanol: acetonitrile: isopropanol (1:1:1:1) with 1% v/v formic acid		
Eluent A	Water with 0.1% v/v formic acid		
Eluent B	Acetonitrile with 0.1% v/v formic acid		
Gradient	Time (min)	Eluent A (%)	Eluent B (%)
	0	95	5
	2	95	5
	12	36	64
	12.01	5	95
	14	5	95
	14.01	95	5
	16	95	5
Curtain gas	35 arb		
Ion source gas 1	90 arb		
Ion source gas 2	90 arb		
CAD gas	Medium		

Table 1: Overview of LC-MS/MS measured sequences

Date	Batch name	Sample	Number of injections	Purpose of experiment	Method name	Instrument
22.07.22	20220722_Test_stds	Human breast milk	20	Measuring PoPhe Standards	201022_PoPhe_QC_v4, 220307_PoPhe_final_method_noAS	Qtrap 6500+
03.08.22	220803_Expomas_Urine _PoPhe	Urine	76	Exposomas infant urine samples	201022_PoPhe_QC_v4, 220307_PoPhe_final_method, 220307_PoPhe_final_method_Blank	Qtrap 6500+
30.09.22	220930_BM_Test_v1	Human breast milk	91	Method optimization PoPhe in BM	220307_PoPhe_final_method, 201022_PoPhe_QC_v4	Qtrap 6500+
02.10.22	220930_BM_Test_v1	Human breast milk	91	Method optimization PoPhe in BM	220307_PoPhe_final_method, 201022_PoPhe_QC_v4	Qtrap 6500+
25.10.22	221021_BM_Test_v2	Human breast milk	82	Method optimization PoPhe in BM	220307_PoPhe_final_method, 201022_PoPhe_QC_v4	Qtrap 6500+
11.11.22	221111_QC_and_STDs_Test _v4	Human breast milk	3	Method optimization PoPhe in BM	220307_PoPhe_final_method, 201022_PoPhe_QC_v4, 220307_PoPhe_final_method_blank	Qtrap 6500+
24.11.22	221124_Contamination_Test	Solvent	10	System testing	220307_PoPhe_final_method, 201022_PoPhe_QC_v4	Qtrap 6500+
29.11.22	221129_Thermo_LC_Test	Solvent	6	System testing	220307_PoPhe_final_method, 201022_PoPhe_QC_v4	Qtrap 6500+
30.11.22	221130_Solv_Test	Solvent	10	System testing	-	Qtrap 6500+
30.11.22	221130_Test_newPump	Solvent	20	System testing	220307_PoPhe_final_method, 201022_PoPhe_QC_v4	Qtrap 6500+
02.12.22	221202_Test_Autosampler	Solvent	10	System testing	-	Qtrap 6500+
05.12.22	221205_Test_Bottles	Solvent	5	System testing	220307_PoPhe_final_method, 201022_PoPhe_QC_v4	Qtrap 6500+

05.12.22	221205_BM_Test_v3	Human breast milk	78	Method optimization PoPhe in BM	220307_PoPhe_final_method, 201022_PoPhe_QC_v4	Qtrap 6500+
21.12.22	221221_BM_Test_v5	Human breast milk	100	Method optimization PoPhe in BM	220307_PoPhe_final_method, 201022_PoPhe_QC_v4	Qtrap 6500+
09.02.23	230209_BM_v6	Human breast milk	75	Method optimization PoPhe in BM	220307_PoPhe_final_method, 201022_PoPhe_QC_v4	Qtrap 6500+
13.02.23	230213_Test_New_Capillary	Solvent	8	System testing	201022_PoPhe_QC_v4	Qtrap 6500+
13.02.23	230213_Test_Cal_Curve	Solvent	7	Method optimization PoPhe in BM	220307_PoPhe_final_method	Qtrap 6500+
21.02.23	230221_BM_v9	Human breast milk	66	Method optimization PoPhe in BM	20230221_PoPhe_Final_Method_Transfered, 230221_PoPhe_Final_Method_Transfered_changedRTs_V2	QTrap 7500
27.02.23	230221_BM_v9_remeasured	Human breast milk	21	Method optimization PoPhe in BM	20230221_PoPhe_Final_Method_Transfered, 230221_PoPhe_Final_Method_Transfered_changedRTs_V2	QTrap 7500
01.03.23	230301_BM_v10	Human breast milk	27	Method optimization PoPhe in BM	20230221_PoPhe_Final_Method_Transfered, 230221_PoPhe_Final_Method_Transfered_changedRTs_V2	QTrap 7500
07.03.23	230307_BM_v11	Human breast milk	18	Method optimization PoPhe in BM	20230221_PoPhe_Final_Method_Transfered, 230221_PoPhe_Final_Method_Transfered_changedRTs_V3	QTrap 7500
10.03.23	230310_BM_v12	Human breast milk	14	Method optimization PoPhe in BM	220307_PoPhe_final_method	Qtrap 6500+
14.03.23	230314_BM_Test_Guaiacol _HydroCinA_Tyrsl	Human breast milk	6	Method optimization PoPhe in BM	230221_PoPhe_Final_Method_Transfered_5uL, 230314_PoPhe_Final_Method_Transfered_Guaiacol _HydroCinA_Tyrsl_only, 230314_PoPhe_Final_Method_Transfered_Guaiacol _HydroCinA_Tyrsl_only_noRT	QTrap 7500

21.03.23	230321_BM_val_1	Human breast milk	147	Method Validation	20230221_PoPhe_Final_Method_Transfered_3uL, 230321_PoPhe_Method_BM_Val1	QTrap 7500
24.04.23	230424_BM_val_2	Human breast milk	107	Method Validation	20230221_PoPhe_Final_Method_Transfered_3uL, 230321_PoPhe_Method_BM_Val1	QTrap 7500
16.05.23	230516_BM_val_3	Human breast milk	54	Method Validation	20230221_PoPhe_Final_Method_Transfered, 230321_PoPhe_Method_BM_Val1	QTrap 7500
18.07.23	230718_BM_Exposomas_JK	Human breast milk	71	Exposomas breast milk samples	20230221_PoPhe_Final_Method_Transfered, 230321_PoPhe_Method_BM_Val1	QTrap 7500