Unraveling early-life mycotoxin exposures via LC-MS/MS breast milk analysis

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ABSTRACT

Infants are particularly susceptible towards the toxic effects of food contaminants including mycotoxins. However, multi-mycotoxin exposure assessment in breast milk has received very limited attention so far, resulting in a poor understanding of exposure during the early months of life. Here, we present the development and first application of a highly sensitive, specific and quantitative assay assessing up to 28 mycotoxins including regulated (aflatoxins, ochratoxin A, deoxynivalenol, zearalenone) and emerging mycotoxins as well as key metabolites by LC-MS/MS. After careful optimization of the sample preparation procedure, a QuEChERS (quick, easy, cheap, effective, rugged and safe) protocol combined with a freeze-out step was utilized for method validation after spiking blank breast milk matrix. The limits of quantification varied between 0.009 and 2.9 ng/mL, for most analytes extraction recovery (74-116%) and intermediate precision (2-25%) were satisfactory. To assess multi-mycotoxin exposure for the first time in breast milk, the method was applied to examine contamination in 75 samples from Ogun State, Nigeria. Most of the samples were either entirely free of mycotoxins or contaminated to a minimal extent. Interestingly, the most abundant mycotoxin was beauvericin, which was not reported in this biological fluid before, with concentrations up to 0.019 ng/mL. In conclusion, the method demonstrated to be fit for purpose to determine and quantify low background contaminations in human breast milk. Based on the high sensitivity of the novel analytical method, it was possible to deduce that tolerable daily intake values were not exceeded by breastfeeding in the examined infants.

KEYWORDS

Biomonitoring, exposome/exposomics, food safety, infant and public health, emerging/modified mycotoxins, environmental contaminants, exposure assessment

Human breast milk is generally considered a safe and complete diet for infants, and breastfeeding provides abundant health benefits to both, mother and child. Numerous positive effects associated with the ingestion of breast milk have been described in literature, e.g. reduction of total cholesterol and blood pressure, lower risk of being overweight, developing type II diabetes, and obesity.[1-3] However, food contaminants, such as mycotoxins, may be transferred to some extent to human breast milk due to exposure of the mother to contaminated foodstuffs.[4,5]

Mycotoxins are secondary metabolites produced by several moulds, including *Aspergillus*, *Fusarium* and *Penicillium* species that contaminate many agricultural crops.[6] Globally, contamination of agricultural products was estimated by the Council for Agricultural Science and Technology[4] to be about 25%, but more recent reports using modern analytical methodology indicate far higher contamination levels.[7] Food crops cannot only be contaminated in the field, but some also occur post-harvest during inadequate storage or handling. In addition, climate changes and globalization of trade influence contamination patterns.[4,8] Contamination of food and feed with mycotoxins is varied and can cause diverse diseases in humans and animals.[6,9] Main mycotoxins of public health interest are aflatoxins (AFs), fumonisins (FBs), ochratoxin A (OTA), zearalenone (ZEN) and trichothecenes (Figure 1). The four major aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2), frequently contaminate maize and groundnuts, but can occur in a broad spectrum of foods in tropical countries. They contribute to stunting, modulation of intestinal function and hepatomegaly in children.[5,10,11] AFB_1 is a potent liver carcinogen, causes immune suppression, and acute high dose exposures lead to death through liver failure.[12,13] Fumonisins (FBs) are a group of toxins (primarily, FB_1, FB_2, FB_3) produced by *Fusarium* species that commonly contaminate maize. They interfere with sphingolipid homeostasis and have been implicated in neural tube defects, stunting and esophageal cancer.[14-16] OTA is mainly found in cereals and coffee and can cause kidney toxicity.[6,17] ZEN occurs frequently in cereals globally, is known as a potent endocrine disruptor with a high affinity towards the estrogen receptor, and has been controversially discussed in the context of breast cancer and its therapy.[6,18,19] Trichothecenes such as deoxynivalenol (DON) are produced by *Fusarium* species on wheat and maize, and are associated with gastrointestinal effects and immune suppression[15]. For some mycotoxins maximum tolerated limits (MTLs) are established in many food types, including also complementary infant food as outlined by the EU commission regulation 1881/2006/EC[20]. Emerging mycotoxins, such as beauvericin (BEA) and enniatins (ENNs), whose occurrence in food have been reported due to advancement in analytical techniques, gained more interest in recent years.[21]

Please insert Figure 1 here

It has been verified by monitoring food and urine that humans are typically exposed to diverse mixtures of mycotoxins.[7,22-24] Therefore, it is generally accepted that co-exposures are the rule and not the exception, and may lead to combinatory effects.[25-27] Numerous ingested toxins, especially fat soluble compounds, can be transferred from ingested food of the mother to infant food in the form of breast milk.[28] Consequently, the determination of co-exposure patterns and resulting effects from breast milk are a priority. Exposures of the nursing mothers to mycotoxins may vary largely due to seasonal changes that affect contamination levels, regional and individual dietary habits, and the transfer rate during different stages of lactation.[29] Furthermore, the variable protein content and the mobilization of lipids out of adipose tissue may influence mycotoxin transfer.[30] Since neonates are considered to be more susceptible to the adverse effects of environmental toxins than adults[31], exposure during the early stages of life may have both immediate effects and impact on health later in life.[5,32,33]

The occurrence of mycotoxins in human breast milk was previously described, mainly for AFM_1 and OTA. Several studies reported AFM_1 in breast milk, including Brazil, Cameroon, Italy, Nigeria and Tanzania with significant variations in concentrations ranging up to 187 ng/mL.[34-39] OTA was determined in samples of similar regional origin (Brazil, Germany, Italy and Sierra Leone) with concentrations up to 337 ng/mL.[35,40-43] Only one study from Italy described the occurrence of ZEN in human breast milk with concentrations between 0.26 to 1.78 ng/mL.[44] The assessment of mycotoxins was commonly based on single analyte methods using either enzyme linked immunosorbent assay (ELISA) or high pressure liquid chromatography with fluorescence detection (LC-FD).[34] One method explored high-resolution mass spectrometry[45] and two others assessed AFs and OTA together by LC-FD.[43,46]

While there is a clear trend towards the employment of multi-analyte[34] and exposome-scale methods[47,48] in the assessment of food contaminants, no targeted multi-mycotoxin method has been applied to mycotoxins in breast milk. Here we report a highly sensitive LC-MS/MS tool to simultaneously measure 28 mycotoxins/metabolites in breast milk from Nigerian mothers. The data was subsequently used to estimate infant exposure in an area of high mycotoxin risk.

EXPERIMENTAL SECTION

Sample preparation protocol

Several sample clean-up approaches were tested and optimized (see results section). The following protocol was finally chosen for sample extraction and clean-up: An aliquot of 2 mL human breast milk was shaken using a vortex mixer and 2 mL of acidified ACN (1% formic acid) was added and thoroughly mixed for 3 min. Subsequently, 0.8 g anhydrous magnesium sulfate and 0.2 g sodium chloride were added, followed by a further vortexing step (3 min). The sample was then centrifuged for 10 min (4750 x g, 10 °C) in order to concentrate the analytes of interest in the upper layer (ACN). A volume of 1.5 mL of this ACN extract was transferred to a new micro-reaction tube, chilled and kept at -20°C for 2 h. Thereafter, another centrifugation step was performed (15 min at 14000 x g, 4 °C), the supernatant filtered (PTFE, 0.22 µm, Carl Roth, Karlsruhe, Germany) and 3 µL injected to the LC-MS/MS system. To evaluate the possible occurrence of glucuronides or sulfate conjugates as phase II metabolites, a small set of naturally contaminated breast milk samples (n=5) were subjected to enzymatic deconjugation. A mix of 250 µL β-glucuronidase/sulfatase (250 U/mL, 0.2 U/mL in PBS) was added and subsequently incubated under shaking conditions at 37 °C overnight. The breast milk samples were then processed as described above.

LC-MS instrumentation and parameters

The LC-MS/MS system consisted of a Dionex Ultimate 3000 UHPLC coupled to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, Vienna, Austria) equipped with a heated electrospray ionization (ESI) interface. Chromatographic separation was performed on an Acquity UPLC® HSS T3 column (1.8 µm, 2.1x100 mm, Waters, Vienna, Austria) guarded by a VanGuard pre-column (1.8 µm, Waters, Vienna, Austria). The Autosampler was set to 10 °C and the column oven temperature maintained at 40 °C. The mobile phase was composed of solvents A (water / ammonium acetate (5 mM) / acetic acid (0.1%)) and B (methanol) at a flow rate of 0.25 mL/min. For the first 0.5 min the methanol content was kept constant at 10%. Then, eluent B was raised to 35% until 1.0 min and further to 60% (3.0 min) and 97% (10.0 min). The latter was held for 6.0 min before starting conditions were reached within 0.1 min and consequently the column was re-equilibrated at starting conditions (10% B) for 2.9 min. The overall runtime was 19 min. The column effluent was transferred either to the mass spectrometer (min 2 and 19) or to the waste via a six port valve. MS/MS measurements were performed in multiple reaction monitoring (MRM) mode in both, positive and negative polarity, using fast polarity switching. MS operation parameters as well as optimized MS and MS/MS parameters (Table S1) are reported in Supporting Information.

External calibration (1/x weighted) was conducted using at least five matrix matched standards to compensate for matrix effects. These standards were produced by spiking blank breast milk extracts (prepared as described above) with different volumes of working standard solution. Results were corrected for analyte specific extraction recoveries as obtained during method validation. Data acquisition was performed using Xcalibur (version 3.1) and quantification was conducted by the TraceFinder software package (version 3.3).

In-house validation and quality control

In-house validation was carried out according to the guidelines of Eurachem (second edition)[49] and the EU commission decision 2002/657/EC[50] concerning the performance of analytical methods by evaluating the following parameters: sensitivity, selectivity, repeatability (intraday precision, RSD_r), intermediate precision (interday precision, RSD_R), linearity, extraction recovery (R_E) and signal suppression or enhancement (SSE). Since no matrix reference material was available, breast milk samples with no detectable mycotoxins were pooled and considered as blank matrix. Details concerning in-house validation and quality control measures are reported in Supporting Information.

Breast milk samples

Anonymized breast milk aliquots for method development and validation were kindly provided by the Semmelweis Women's Clinic in Vienna, Austria. Samples from more than 150 women were collected in 2015 and stored immediately at -20°C. Subsequently, samples were pooled, aliquoted and stored at -20 °C. Nigerian samples (n=75) were collected between January and February 2016 from 22 volunteers within a larger, ongoing human biomonitoring study in Ogun state. Samples were obtained in the morning and the evening on two consecutive days from most women. Detailed information on study subjects is provided in Supporting Information (Table S2). Participants maintained their regular diet before sample donation. Hand expressing was used to collect breast milk samples into sterile 25 mL tubes. After collection, samples were immediately frozen at -20 °C until analysis. Prior to breast milk donation written informed consent was obtained from all volunteers. The studies were permitted by ethics committees in Austria (University of Vienna, No 00157) and Nigeria (Babcock University, No BUHREC294/16).

RESULTS AND DISCUSSION

LC-MS/MS method development

The selection of analytes for this targeted biomonitoring assay was based on general occurrence, toxicological relevance and the availability of reference material.[51-53] MS optimization was carried out in positive and negative ionization mode to determine preferential parameters for all analytes. Optimization of the MS system was performed by flow injection analysis (FIA, 5 µL/min), with eluents A and B mixed at 50/50 (v/v) at a flow rate of 0.2 mL/min. Analyte concentrations for analyte specific MS parameter optimization were in the range of 0.2 to 5 µg/mL. After a stable ion beam was established, a full spectrum was recorded to select the most abundant precursor ion. These were in line with most literature reports[54-56] with the exception of citrinin (CIT) which is normally measured as protonated ion (*m/z* 251.0)[57] or after deprotonation (*m/z* 249.1).[58] We and others[59] have observed the formation of a methanol adduct $[M+MeOH-H]^-$. This species (*m/z* 281.1) had a five times higher intensity and was consequently selected (Table S1). Furthermore, AFL did not form the same precursor ion as the other aflatoxins, but predominantly an $[M-H_2O+H]^+$ ion at *m/z* 297.1. AFM_1, AFG_1 and AFQ_1 $[M+H]^+$ (*m/z* 329.1) as well as AFM_2 and AFG_2 $[M+H]^+$ (*m/z* 331.1) share the same precursor masses, however, chromatographic separation and distinction by at least one specific product ion enabled selectivity and accurate quantification. The isomeric compounds α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) were successfully baseline separated.

MS parameters were ramped for best gas pressure, spay voltage, vaporizer and capillary temperatures in both ionization modes. S-Lens and collision energies were optimized using the instruments automatic compound optimization tool. Collision energies were optimized for the eight most intensive product ions. MRM transitions were evaluated for signal to noise (S/N) ratios and the two ions with the highest S/N ratio were recorded as quantifier and qualifier ions in the final method, respectively. Fast polarity switching was utilized to allow for most efficient ionization. To guarantee appropriate acquisition, the LC run was divided into four segments (see Supporting Information).

The development of a quantitative multi-analyte LC-MS/MS method targeting highly diverse molecules (Figure 1) is a complex task. The selection of appropriate mobile and stationary phases is critical to retain both, very polar and lipophilic analytes. The utilized column (Acquity HSS T3) demonstrated excellent interaction even with highly polar toxins (nivalenol, NIV; DON) which often elute close to the void volume of other reversed phase materials. Water, MeOH and ACN combined with different organic modifiers (0.1%, 0.5%, 1% HAc and ammonium acetate) were tested. Overall, chromatographic separation improved using MeOH instead of ACN, due to favorable peak widths and faster elution of

enniatins. Moreover, the observed intensities for CIT were significantly higher measuring the MeOH adduct described above. The acidification of the aqueous eluent showed a positive impact on overall peak width and shape. The concentration of 0.1% HAc was deemed most suitable as higher concentrations resulted in broader CIT, dihydrocitrinone (DH-CIT) and ochratoxin α (OTα) peaks (>30 sec). Ammonium acetate (5 mM) was added to avoid formation of sodium adducts and additionally resulted in higher signal intensities especially for beauvericin and enniatins. The early eluting compounds NIV and DON showed decreased intensities. Fumonisins (FB_1 and FB_2) were initially included during method development, however poor performance with the selected chromatographic conditions and generally low signal intensities impaired proper measurement. Since (maternal) bioavailability of FBs is very low[60] and the lactational transfer, based on physico-chemical properties, is expected to be minute, these toxins may only be measured in very low concentrations in breast milk. Due to these factors we excluded them from the method.

Optimization sample preparation protocol

Due to different polarities of the target analytes, extraction is not possible without either analyte loss or extraction of interfering matrix components. Therefore, several sample preparation methods were tested on their feasibility [61]. As a starting point a time and cost effective 'dilute and shoot' protocol was chosen. Straight-forward procedures like this approach are frequently employed for diverse analyte mixtures.[55,62,63] However, abundant matrix effects and interfering signals may diminish the chance of such an approach. This was also the case in our experiments, where centrifugation (10 min at 14,000 x g, 20°C), subsequent dilution of the supernatant up to 20-fold and additional filtration (PTFE, 0.22 µm, Carl Roth, Karlsruhe, Germany) did not yield in the required sensitivity and selectivity, due to severe matrix interferences. Therefore, several liquid-liquid extractions (LLE) were assessed in combination with solid phase extraction (SPE). Factors subjected for optimization were liquid-liquid partitioning, extraction solvents, SPE solvents and reconstitution solvent. The milk fat was mainly removed using either hexane or chloroform.[64] As extraction solvents, acidified methanol or acetonitrile (up to 2% of formic acid or acetic acid) were tested. Extracts were evaporated to dryness using a vacuum concentrator (Labconco, Missouri, USA). Vacuum dried samples were reconstituted in water or aqueous MeOH or ACN solutions (up to 10%). Then, samples were loaded onto a C18 SPE cartridge (Oasis HLB or HLB Prime, 1cc, Waters, Vienna, Austria), and several washing solutions were examined in order to maximize analyte recoveries while minimizing matrix interferences. Finally, mycotoxins were eluted with pure MeOH or ACN. After evaporating the eluate, samples were reconstituted in mobile phase (starting conditions) prior to analysis. This protocol resulted in enhanced sensitivities for many analytes; however, BEA, CIT, ENNs, sterigmatocystin (STC), α/β-ZEL, and ZEN suffered significant losses (extraction recoveries below 50%).

Since the QuEChERS method (quick, easy, cheap, effective, rugged and safe) was applied in many food matrices with high fat content to sufficiently extract lipophilic analytes before[45,65], this approach was further investigated to overcome the observed extraction losses during LLE-SPE. Spiked breast milk samples were extracted using an adapted and thoroughly optimized protocol (see materials and methods). An important step was the implementation of a freezing step (2 h at -20 °C) to precipitate proteins followed by centrifugation and filtration. When this extract was directly injected onto the LC-MS it clearly resulted in reduced matrix effects and interferences. In addition, we further tested SPE clean-up/enrichment. However, the same analytes as for the LLE-SPE protocol described above (BEA, CIT, ENNs, STC, α/β-ZEL, and ZEN) were again not extracted quantitatively. Since the sensitivity and selectivity obtained by injection of extracts generated by the modified QuEChERS/freeze-out method were demonstrated to be sufficient for accurate multi-mycotoxin trace level quantification (see Table 1), we consequently selected this protocol for method validation.

Validation experiments

In-house validation of the method was performed according to the EuraChem guideline[49] and the European commission decision 2002/657/EC[50] by evaluating sensitivity, selectivity, repeatability, intermediate precision, linearity, extraction recovery and matrix effects. Overall, the validation was successful, and results are reported in Table 1.

The newly developed method allowed the determination of 27 of the 28 selected mycotoxins in the parts per trillion (ng/L) range. LOD and LOQ values ranged from 0.004 to 1.4 ng/mL and from 0.009 to 2.9 ng/mL, respectively. Very low LODs were achieved for the four ENNs and BEA between 0.004-0.012 ng/mL. Detection limits of other mycotoxins were below 0.3 ng/mL, except for the rather polar trichothecenes DON and HT-2 with values of 0.77 and 1.4 ng/mL, respectively. However, these slightly higher values are clearly sufficient to quantify potential 'carry-over' from the mother to breast milk. The MTL for DON in processed baby food is 200 ng/g, which is more than a factor of 100 higher than our LOQ. The LOQ values obtained demonstrate that this method is able to quantify most analytes at lower levels (factor 5 to 100) compared to the only publication reporting on the simultaneous measurement of more than one class of mycotoxins in human breast milk.[45] Selectivity of the method was assessed by comparing extracted blank samples with spiked samples. No interfering peaks ($S/N \geq 3$) within a timeframe of ± 0.15 min were detected for any analyte, ensuring proper quantification. Identification was based on four criteria: retention time, quantifier and qualifier ion and their respective ratio. Ion ratios were calculated from matrix matched calibration standards (average of five concentrations measured in triplicate) and spiked samples proven to be within the tolerance limit according to Commission Decision 2002/657/EC.[50] Weighted linear regression analysis

(1/x) showed linearity for the concentration ranges used with regression coefficients ranging from 0.995 to 0.999. MRM chromatograms of breast milk samples spiked at a low level are shown in Supporting Information (Figure S1).

Extraction recoveries were in good agreement with the EC Decision 2002/657/EC[50] except for DON, NIV and AFB_1-N7-guanine adduct (AFB_1-N7-Gua). The latter toxins have a relatively polar character and may remain to a certain extent in the aqueous phase during the extraction step with organic solvent. Since we rather focused on lipophilic contaminants, we accepted this compromise. However, since results were generally corrected for extraction losses and these were sufficiently stable (RSDs <14% for the trichothecenes), quantification was still deemed feasible although sensitivity was slightly impaired (see above). The 25 more lipophilic analytes were within the tolerated range (80-120% for spiking levels above 10 ng/mL; 70-110% between 1-10 ng/mL; 50-110% below 1 ng/mL) with minor exceptions for AFG_1 and OTα. Repeatability (intra-day RSDs; RSD_r) and intermediate precision (inter-day RSDs; RSD_R) ranged from 2-30% and 2-25% for all analytes, respectively. Except for the lowest concentration of DON, HT-2, T-2 and AFB_1-N7-Gua, all analytes were below the EU commission decision criteria of 20% standard deviation for both, RSD_r and RSD_R. As discussed above, more polar compounds tend to remain in the aqueous phase, thus extraction may not be as efficient and variation is more likely to occur. No significant differences were observed between repeatability and intermediate precision. SSE was assessed comparing the calibration slopes of matrix matched and solvent standard calibrants throughout the whole validation procedure and are reported as average values. Overall, SSE was within 80-120% for all analytes, except CIT (129%), DH-CIT (133%) and HT-2 (122%), which exhibited some signal enhancement.

Please insert Table 1 here

Due to inter-individual variability, the MS/MS signal may vary from sample to sample through the influence of the matrix. As a proof-of-principle experiment, five Nigerian samples were randomly selected after ensuring the absence of measurable mycotoxin contamination and spiked before the extraction step to compare inter-individual effects on the extraction efficiency. For 27 analytes the values matched those obtained during validation, with the exception of AFB_1-N7-Gua which exhibited higher recoveries (81%, RSD 20%). Overall, the method performance was highly satisfying and proved to be fit for purpose to determine and quantify low background contaminations in human breast milk. Importantly, this was achieved without expensive or time-consuming procedures through a smartly modified extraction protocol and careful optimization of chromatographic and mass spectrometric parameters. Due to the generic sample preparation protocol, which is required for broad multi-analyte methods, some minor compromises in the method performance had to be accepted.[63,66,67]

Application of the developed method to human breast milk samples

To evaluate the applicability of the method, human breast milk samples (n=75) of a Nigerian cohort were analyzed to determine potential mycotoxin contamination. Generally, it can be stated that in most samples no mycotoxins were detectable or samples were contaminated by minor levels only. In the analyzed samples mainly three mycotoxins (BEA, ENN B and OTA) were found. Overall, in 42 samples (56%) BEA was detected. Seven samples (9%) showed trace amounts of ENN B and in eleven samples (15%) OTA was present. Here, not only validation criteria such as retention time, quantifier, qualifier ion and their respective ratio, but moreover the S/N ratio had to be greater than 3 for positive evaluation. Since most positive samples were below the LOQ of the respective analyte, quantification was only possible for BEA in six samples with concentrations up to 0.019 ng/mL and for ENN B in one sample with a concentration of 0.009 ng/mL. To the best of our knowledge, no data on BEA in natural contaminated human breast milk was published to date. Except AFM_1 in a single sample (below LOQ), neither aflatoxins nor their metabolites were observed (Table 2). The contamination pattern in samples obtained from the same individual were variable, reflecting the heterogeneity of dietary mycotoxin contamination. Figure 2 shows MRM-chromatograms of mycotoxin contamination in comparison to blank and matrix-matched samples.

Please insert Table 2 here

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To further confirm the identity of the detected analytes (AFM_1, BEA, ENN B and OTA) we additionally enriched selected samples <LOQ, by concentrating the filtered extract by a factor of five, and re-analyzed them. This resulted in higher peak intensities, however, we did not use these measurements for quantification since the method was not validated for this enrichment. Co-occurrence of these mycotoxins was observed in overall 14 samples while merely two mycotoxins were present. Main transfer of common chemicals into breast milk occurs via passive diffusion, where molecular weight (<800 Da), degree of ionization (pKa) and chemical structure are crucial factors.[28,68] Active transport may, however, facilitate the transfer of more polar mycotoxins.

Therefore, the potential existence of phase II metabolites was investigated by the measurement of five samples for possible glucuronide and sulfate conjugates. After treatment with a mixture of glucuronidase and sulfatase followed by the established ‘clean-up’ procedure, no increase in signal intensities was observed. This indicates that no phase II metabolites were transferred to maternal milk in concentrations detectable with this method. This is in line with literature, showing that phase II

metabolites are more likely to be eliminated through the kidney and may thus not be relevant for lactational transfer.[69,70]

Since the volume of the Austrian samples was rather limited and entirely used as a pooled sample for method development and validation, we were unfortunately not able to assess individual exposures. However, since no mycotoxin was detected in the pooled sample, this indicates no abundant exposures via breast milk in the Austrian population. However, it is likely that some samples might have been contaminated at low concentration but diluted out. We plan to confirm this in subsequent large-scale biomonitoring studies. While OTA was frequently determined in samples obtained from German mothers[40], this pooled sample from Austria did not indicate the presence of this toxin. This is most likely due to the higher sensitivity of the tailored single analyte assay employed in Germany and may change once individual samples will be tested in Austria.

Implications for exposure assessment

Since infants are more susceptible towards the toxic effects of food contaminants, it is mandatory to minimize exposure to an acceptable level whenever possible. This is reflected by a very recent report of the EFSA proposing to reduce TDIs by a factor of three for infants for the first 16 weeks of life, a key window for early life exposures.[32] Also MTLs for mycotoxins in infant food, as outlined by the EU commission regulation 1881/2006/EC[20] are therefore lower than for other foodstuffs. As an example, an MTL of 0.5 ng/g was set for OTA in infant food.[20] Since the LOQ of the developed method for OTA is below 0.1 ng/mL, it can be derived that breast milk contaminated by a level exceeding the tolerated concentration for commercial breast milk substitutes would be easily quantified. The same is true for most other regulated mycotoxins (AFB_1, DON, HT-2, OTA, T-2 and ZEN). This suggests that a sample in which no regulated mycotoxin can be detected is, besides its unmatched nutritional and immunological value, very safe from a mycotoxin food safety perspective. Importantly, appropriate alternatives in regions with poor infrastructure and diminished access to purified or boiled water for the proper preparation of complementary infant food are frequently missing. Therefore, the potential presence of mycotoxins or other contaminants in maternal milk should not be a factor leading to avoid breastfeeding. The beneficial effect of mother's milk as the optimal food source for newborns typically clearly prevail the risk of a potential mycotoxin contamination.

Occurrence of mycotoxins in Nigerian foods is a severe but still under recognized public health issue. In particular, the frequency and levels of aflatoxins can be critical.[36,71-75] AFM_1 contamination in breast milk in African countries analyzed with LC-FD ranged from 0.004 to 187 ng/mL.[34,38,39] No study on the occurrence of OTA in breast milk from Nigeria was reported so far. However, in other world regions OTA was found in varying concentrations, with up to 337 ng/mL in Sierra Leone.[29,43] ZEN was examined in breast milk in only one study from Italy in which unexpectedly all samples (n=47) were tested

positive by an ELISA that was not validated for this complex matrix.[44] In addition, the reported mean concentration of 1.1 ng/mL seems unrealistic, and more specific methodology such as LC-MS/MS is needed for confirmation. The samples tested within this present study were all negative for ZEN, despite the fact that the LOD value is ten times lower than the average concentration from the Italian study. Recent occurrence data in food from African countries suggest frequent ZEN contamination.[71,72,76] DON is also frequently reported in Sub-Saharan Africa, although typically at lower concentrations than in temperate climate regions.[76-78] For example a study quantified DON in fermented food in concentrations up to 118 ng/g.[71] A recent publication predicted high lactational DON transfer, based on an algorithm which captures (only) distribution processes depending on physicochemical properties.[31] However, none of the analyzed Nigerian samples were contaminated with DON. Possibly, this is due to the generally rather low exposure of the Nigerian mothers or the fast metabolism and excretion of DON which could not be accounted for by the prediction model.[31]

Based on the results obtained in this multi-analyte study, which is the first of its kind, exposure to mycotoxins is far more likely via cereal or maize-based infant food or infant formula compared to human breast milk. In the cereal-based alternatives to breast milk often much higher concentrations were reported.[79,80] This was also the conclusion of Ishikawa *et al.* (2016) who reported AFM_1 with average concentrations of 0.003 ng/g in breast milk (5% positive, n=94) and 0.011 ng/g in infant powdered milk (44% positive, n=16), respectively. In the latter, 19% of samples exceeded the established MTL in the EU.[81] A study on Tanzanian maize flour samples intended as complementary food for infants revealed that aflatoxin contaminated samples resulted in an exposure from 0.14 to 120 ng/kg body weight per day. These concentrations were above the health concern level of 0.017 ng/kg body weight per day established by EFSA.[82] In a recent study conducted in the US, milk- and soy-based infant formula as well as infant cereal products were evaluated for their OTA contamination: Infant formula did not reveal any OTA contamination, whereas in cereal based products 0.6 to 22.1 ng/g OTA were found – all above the MTL established by the European Commission (0.5 ng/g).[83]

The low abundance and concentrations of mycotoxins in the 75 measured breast milk samples, obtained from 22 volunteers, suggest the relative safety of breast milk with regard to mycotoxins for the investigated women during the duration of the study. Based on the high sensitivity of the analytical method it was possible to derive that TDI values were not exceeded for mycotoxins by breastfeeding in the reported pilot survey. Hypothetical daily maximum exposures were estimated based on the LODs, for analytes not detected, and the LOQ or respective maximum concentrations for analytes determined in the samples (see Table 2). Moreover, the mean daily breast milk intake of 151 mL/kg

body weight was calculated from the quantity of milk intake multiplied with the frequency of breastfeeding per day and divided by the averaged infant weight (see Table S2) as reported in Table 3. We assumed that a contamination above the LOD would have been detected, thus the calculated values constitute the upper bound scenario and real exposures are most likely lower in a majority of samples. Following this logic, exceedance of the infant corrected TDI[32] was only found for OTA after assuming contamination at LOQ level. In addition, MTLs established in infant formula were used to compare a theoretical upper bound intake via complementary infant food with upper bound breast milk estimates. This includes the carcinogenic aflatoxins for which no TDI can be established. Finally, it could be derived that all analyzed samples were below the maximum limits established for commercial infant food, again pointing at the high value of breast feeding also from this food safety perspective.

Please insert Table 3 here

CONCLUSION AND OUTLOOK

In this paper we report the development and successful application of the first targeted LC-MS/MS method for assessing early-life mycotoxin exposures via contaminated breast milk. Based on our results and their comparison with maximum permitted levels in infant formula, breast milk samples from the cohort of Nigerian mothers can be considered as generally safe regarding this class of food contaminants. The high frequency of beauvericin, a cyclic hexadepsipeptide, not reported before in human breast milk, and the partially observed co-occurrence of mycotoxins highlight the need for large-scale follow-up biomonitoring studies. These should include countries of different world regions to better understand global occurrence patterns in this biological fluid and the potentially associated risks. The developed methodology may also serve to improve our knowledge regarding lactational transfer, once a similar method is developed for quantifying mycotoxins accurately in blood. Thus, this would enable a combined exposure assessment of mothers and their infants. Overall, these analytical efforts are intended to minimize mycotoxin exposures as much as possible during this critical window of susceptibility.

Conflict of Interest Disclosure

The authors declare no competing financial interest.

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TABLES AND FIGURES

Table 1: In-house validation results including concentration range of matrix matched standard calibration, regression coefficients (R^2), spiking levels, recoveries of the extraction step (R_E), intermediate precision (RSD_R), repeatability (RSD_r), signal suppression/enhancement (SSE), limits of detection (LOD) and limits of quantification (LOQ)

Analyte	Concentration range	Regression coefficients	Spiking level[a]	$R_E \pm RSD_R$ low level	$R_E \pm RSD_R$ medium level	$R_E \pm RSD_R$ high level	RSD_r[b]	SSE[c]	LOD	LOQ
	[ng/mL]	R^2	[ng/mL]	[%]	[%]	[%]	[%]	[%]	[ng/mL]	[ng/mL]
Aflatoxin B_1	0.05 – 30	0.995	0.6/1.5/3	85 ± 9	93 ± 6	92 ± 3	9/9/5	108	0.040	0.080
Aflatoxin B_2	0.05 – 30	0.998	0.6/1.5/3	93 ± 10	103 ± 6	102 ± 4	7/4/5	98	0.042	0.085
AFB_1-N7-Gua	0.25 – 150	0.998	3/7.5/15	33 ± 22	41 ± 7	40 ± 15	19/14/16	87	0.20	0.40
Aflatoxin G_1	0.05 – 30	0.997	0.6/1.5/3	116 ± 7	122 ± 5	116 ± 2	7/6/4	80	0.043	0.086
Aflatoxin G_2	0.1 – 30	0.998	0.6/1.5/3	102 ± 16	106 ± 12	102 ± 4	15/15/5	88	0.079	0.16
Aflatoxicol	0.25 – 150	0.999	3/7.5/15	74 ± 3	77 ± 5	79 ± 3	11/6/3	108	0.15	0.31
Aflatoxin M_1	0.05 – 30	0.998	0.6/1.5/3	91 ± 13	97 ± 7	95 ± 4	12/10/4	98	0.043	0.087
Aflatoxin M_2	0.1 – 30	0.997	0.6/1.5/3	91 ± 16	92 ± 7	96 ± 8	16/4/9	94	0.076	0.15
Aflatoxin P_1	0.1 – 30	0.997	0.6/1.5/3	92 ± 8	91 ± 7	87 ± 7	19/10/6	100	0.068	0.14
Aflatoxin Q_1	0.1 – 30	0.997	0.6/1.5/3	104 ± 6	104 ± 6	105 ± 8	14/6/8	89	0.063	0.13
Beauvericin	0.01 – 6	0.996	0.12/0.3/0.6	108 ± 4	110 ± 5	108 ± 2	6/6/3	100	0.006	0.011
Citrinin[d]	0.05 – 30	0.995	0.6/1.5/3	85 ± 6	92 ± 3	91 ± 3	3/2/6	129	0.025	0.049
Dihydrocitrinone	0.1 – 60	0.996	1.2/3/6	92 ± 9	107 ± 4	104 ± 3	10/7/7	133	0.092	0.18
Deoxynivalenol	1.5 – 450	0.995	9/22.5/45	37 ± 14	64 ± 12	74 ± 10	30/8/8	91	0.77	1.5
Enniatin A	0.01 – 6	0.997	0.12/0.3/0.6	71 ± 6	69 ± 2	67 ± 3	6/3/3	103	0.005	0.009
Enniatin A_1	0.02 – 6	0.998	0.12/0.3/0.6	99 ± 10	91 ± 6	88 ± 3	15/6/4	102	0.012	0.023
Enniatin B	0.01 – 6	0.999	0.12/0.3/0.6	88 ± 4	94 ± 4	91 ± 3	5/3/3	99	0.004	0.009
Enniatin B_1	0.01 – 6	0.998	0.12/0.3/0.6	94 ± 6	94 ± 4	91 ± 3	6/5/3	101	0.006	0.012
HT-2 toxin	1.5 – 450	0.996	9/22.5/45	85 ± 7	94 ± 9	91 ± 4	27/14/4	122	1.4	2.9
Nivalenol	1.333 – 800	0.997	16/40/80	18 ± 6	16 ± 11	19 ± 6	11/16/12	97	0.254	0.51
Ochratoxin A	0.1 – 60	0.998	1.2/3/6	96 ± 5	99 ± 5	96 ± 2	5/6/4	93	0.048	0.096
Ochratoxin B	0.1 – 60	0.999	1.2/3/6	93 ± 7	94 ± 4	96 ± 2	6/3/3	103	0.063	0.13
Ochratoxin α	0.333 – 100	0.996	2/5/10	97 ± 13	114 ± 5	113 ± 5	16/9/10	96	0.21	0.42
Sterigmatocystin	0.025 – 15	0.998	0.3/0.75/1.5	78 ± 8	82 ± 3	81 ± 3	5/4/4	108	0.013	0.026
T-2 toxin	0.2 – 60	0.997	1.2/3/6	88 ± 18	94 ± 9	98 ± 9	23/20/9	97	0.18	0.36
Zearalenone	0.2 – 60	0.999	1.2/3/6	97 ± 10	106 ± 5	101 ± 2	7/6/3	95	0.093	0.19
α-Zearalenol	0.133 – 80	0.999	1.6/4/8	99 ± 6	99 ± 3	97 ± 2	5/3/3	89	0.073	0.15
β-Zearalenol	0.133 – 80	0.999	1.6/4/8	100 ± 3	99 ± 6	99 ± 4	6/4/3	88	0.068	0.14

[a] Spiking levels reported in the following order: low/medium/high.

[b] RSD_r values reported in the following order: low/medium/high spiking level.

[c] SSE calculated as slope of calibration in matrix / slope of calibration in solution expressed in percent.

[d] measured as methanol adduct (*m/z* 281.1).

460 **Table 2:** Mycotoxins detected in human breast milk samples (n=75) obtained from a cohort in Nigeria

Analyte	Positive samples (%)	Samples ≥LOQ	Range [ng/mL]	Mean concentration [ng/mL]
Beauvericin	42 (56)	6	<LOQ[a] to 0.019	0.010[b]
Enniatin B	7 (9)	1	<LOQ to 0.009	–
Ochratoxin A	11 (15)	-	<LOQ	–
Aflatoxin M_1	1[c] (1)	-	<LOQ	–

461 [a] <LOQ are samples with detectable traces of analytes, ranging from LOD to LOQ.

462 [b] The mean values reported were calculated for positive samples by considering half LOQ (LOQ/2) for less than
463 LOQ values.

464 [c] Traces above LOD found in a five times concentrated sample.

Table 3: Upper bound case scenario of infant exposure compared with infant corrected tolerable daily intake (TDI) and exposure at maximum tolerated limits (MTL) in infant food.

Analyte	LOD	Maximum concentration	**Maximum estimated daily intake via breast milk[a]**	TDI for adults	Infant corrected TDI[b]	MTL in infant food[c]	Theoretical intake via infant food at MTL[d]
	[ng/mL]	[ng/mL]	**[ng/kg bw per day]**	[ng/kg bw per day]	[ng/kg bw per day]	[ng/g]	[ng/kg bw per day]
Aflatoxin B_1	0.040	-	**6**	-	-	0.1	15
Aflatoxin M_1	0.043	0.087[e]	**13**	-	-	0.025	4
Beauvericin	0.006	0.019	**3**	-	-	-	-
Citrinin	0.025	-	**4**	200[f]	67	-	-
Deoxynivalenol	0.770	-	**116**	1000[g]	333	200	30200
Enniatin B	0.004	0.009	**1**	-	-	-	-
Nivalenol	0.254	-	**38**	1200[h]	400	-	-
Ochratoxin A	0.048	0.096[e]	**14**	17.4[i]	6	0.5	76
Zearalenone	0.093	-	**14**	250[j]	83	20	3020

Body weight (bw)

[a] For calculation, either LOD or, if available, maximum concentration in breast milk (as reported in Table 1) was multiplied with the averaged value of daily intake (151 mL/kg bw).

[b] TDI was age corrected according to the EFSA guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age (infant corrected TDI = TDI/3).[32]

[c] According to EC 1881/2006[20] for infant formula, including follow-on milk (AFs), processed cereal-based foods and baby foods for infants (OTA, DON and ZEN).

[d] Calculated as MTL multiplied by the infant daily intake of 151 mL/kg bw.

[e] Assuming upper bound exposure, values <LOQ were estimated at LOQ level.

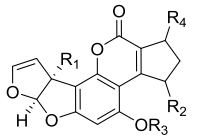
[f] According to EFSA, 2012.[84]

[g] According to EFSA, 2013.[85]

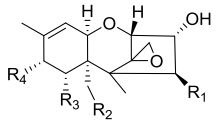
[h] According to EFSA, 2013.[86]

[i] TDI calculated as 120 ng/kg bw per week[17] divided by 7.

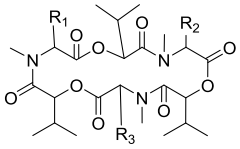
[j] According to EFSA, 2011.[87]



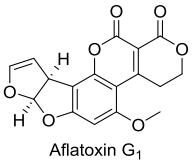
	R_1	R_2	R_3	R_4
Aflatoxin B_1	—H	—H	—CH_3	=O
Aflatoxin M_1	—OH	—H	—CH_3	=O
Aflatoxicol	—H	—H	—CH_3	—OH
Aflatoxin P_1	—H	—H	—H	=O
Aflatoxin Q_1	—H	—OH	—CH_3	=O

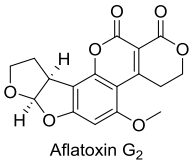


	R_1	R_2	R_3	R_4
Deoxynivalenol	—H	—OH	—OH	=O
Nivalenol	—OH	—OH	—OH	=O
HT2 toxin	—OH	—OAc	—H	—OOC-CH_2-$CH(CH_3)_2$
T2 toxin	—OAc	—OAc	—OH	—OOC-CH_2-$CH(CH_3)_2$



	R_1	R_2	R_3
Beauvericin	-$CH_2C_6H_5$	-$CH_2C_6H_5$	-$CH_2C_6H_5$
Enniatin A	-$CH(CH_3)CH_2CH_3$	-$CH(CH_3)CH_2CH_3$	-$CH(CH_3)CH_2CH_3$
Enniatin A_1	-$CH(CH_3)CH_2CH_3$	-$CH(CH_3)CH_2CH_3$	-$CH(CH_3)_2$
Enniatin B	-$CH(CH_3)CH_2CH_3$	-$CH(CH_3)_2$	-$CH(CH_3)_2$
Enniatin B_1	-$CH(CH_3)_2$	-$CH(CH_3)_2$	-$CH(CH_3)_2$


Aflatoxin G_1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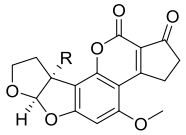
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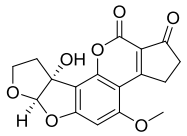
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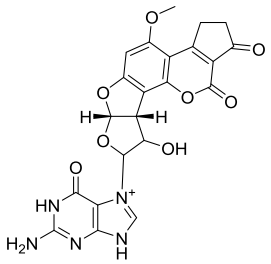
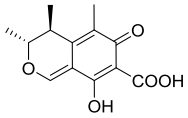
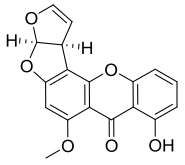
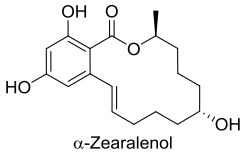
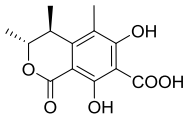
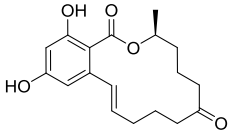

Aflatoxin B_1-N7-Guanine

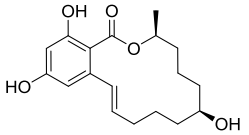

Citrinin

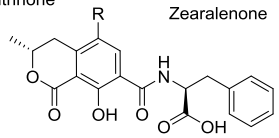

Sterigmatocystin


α-Zearalenol


Dihydrocitrinone


Zearalenone


β-Zearalenol



	R
Ochratoxin A	—Cl
Ochratoxin B	—H

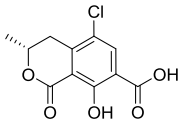

Ochratoxin alpha

Figure 1: Chemical structures of the 28 investigated mycotoxins

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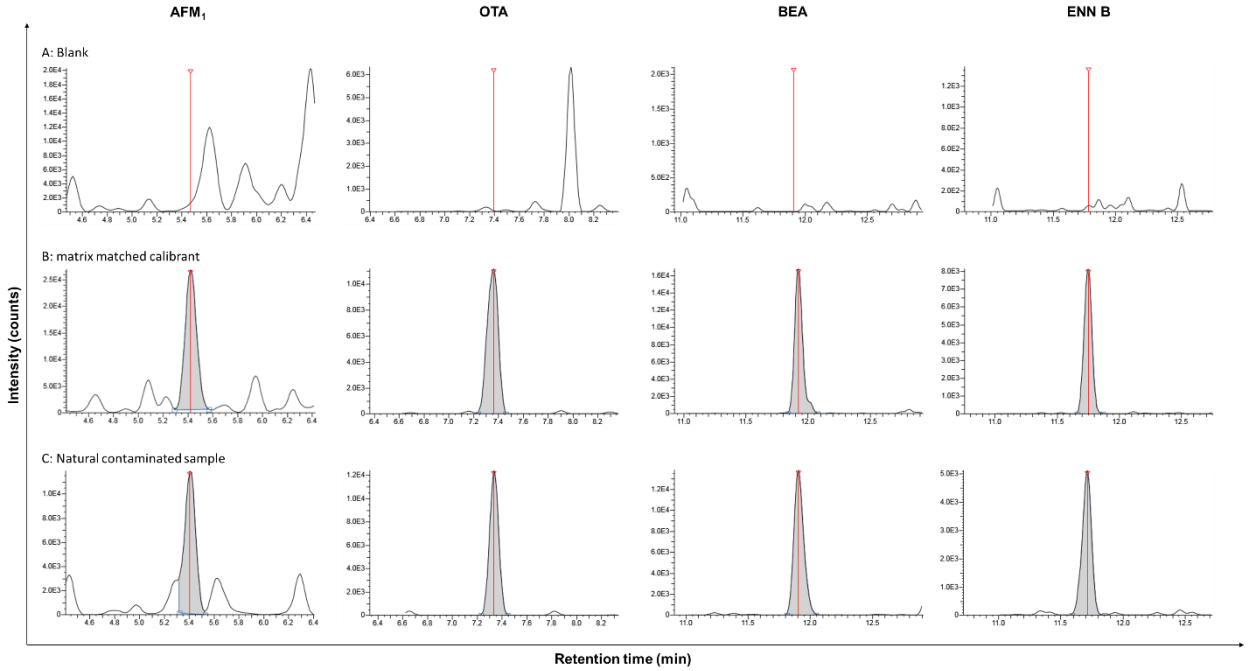

Figure 2: MRM-chromatograms of a blank (A), lowest matrix matched calibrant (B) and natural contaminated breast milk sample (C) of AFM_1, OTA, BEA and ENN B, respectively.

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