analytical chemistry

Article

Cite This: Anal. Chem. 2018, 90, 14569−14577

pubs.acs.org/ac

Monitoring Early Life Mycotoxin Exposures via LC-MS/MS Breast Milk Analysis

Dominik Braun,[†] Chibundu N. Ezekiel,[‡] Wilfred A. Abia,[§] Lukas Wisgrill,[∥] Gisela H. Degen,[⊥] Paul C. Turner,[#] Doris Marko,[†] and Benedikt Warth*[,†,▽]

[†]University of Vienna, Faculty of Chemistry, Department of Food Chemistry and Toxicology, Währingerstraße 38, 1090 Vienna, Austria

[‡]Department of Microbiology, Babcock University, Ilishan Remo, Ogun State, Nigeria

[§]Laboratory of Pharmacology and Toxicology, Department of Biochemistry, Faculty of Science, University of Yaounde I, P.O. Box 812, Yaounde, Cameroon

[∥]Division of Neonatology, Pediatric Intensive Care and Neuropediatrics, Department of Pediatrics and Adolescent Medicine Medical University of Vienna, 1090 Vienna, Austria

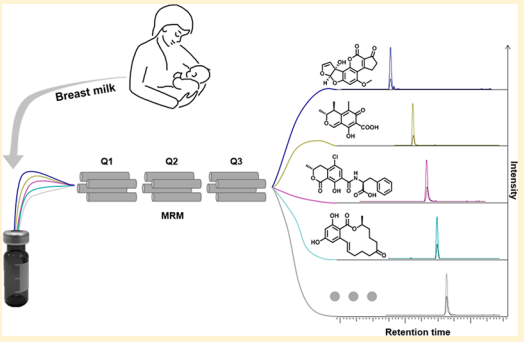
[⊥]Leibniz-Research Centre for Working Environment and Human Factors (IfADo), Ardeystraße 67, D-44139 Dortmund, Germany

[#]MIAEH, School of Public Health, University of Maryland, College Park, Maryland 20742, United States

[▽]Vienna Metabolomics Center (VIME), University of Vienna, 1090 Vienna, Austria

Supporting Information

ABSTRACT: Infants are particularly susceptible toward the toxic effects of food contaminants, including mycotoxins. However, multimycotoxin exposure assessment in breast milk has received very limited attention so far, resulting in a poor understanding of coexposures during early life. Here, we present the development and 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 protocol combined with a freeze-out step was validated in-house. The limits of quantification varied between 0.009 and 2.9 ng/mL, and for most analytes extraction recovery (74−116%) and intermediate precision (2−20%) were satisfactory. The method was applied to examine multiple breast milk samples obtained from 22 women (n = 75 in total) from Ogun State, Nigeria. Most samples were either entirely free of mycotoxins or contaminated to a minimal extent with beauvericin (56%), enniatin B (9%), ochratoxin A (15%), and aflatoxin M_1 (1%). 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. On the basis of 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.



Human breast milk is generally considered an ideal 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 the literature: e.g., lower risks of being overweight and developing type II diabetes and obesity.[1,2] However, food contaminants, including several mycotoxins, may be transferred to some extent to human breast milk due to exposure of the mother to contaminated foodstuffs.[3,4]

Mycotoxins are secondary metabolites produced by several molds, including *Aspergillus*, *Fusarium* and *Penicillium* species that contaminate many agricultural crops.[5] While global contamination of agricultural products was estimated to be higher than 25%, climate changes and globalization of trade clearly influence contamination and exposure patterns.[3,6,7] The 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, and AFG_2 frequently contaminate maize and groundnuts but can occur in a broad spectrum of foods, especially in (sub)tropical countries. AFs contribute to stunting, modulation of intestinal function, and hepatomegaly in children.[4,8,9] FBs are a group of toxins (primarily FB_1, FB_2, and FB_3) produced by *Fusarium* species that commonly contaminate maize. They interfere with sphingolipid homeo-

Received: October 5, 2018
Accepted: November 18, 2018
Published: November 18, 2018

DOI: 10.1021/acs.analchem.8b04576
Anal. Chem. 2018, 90, 14569−14577

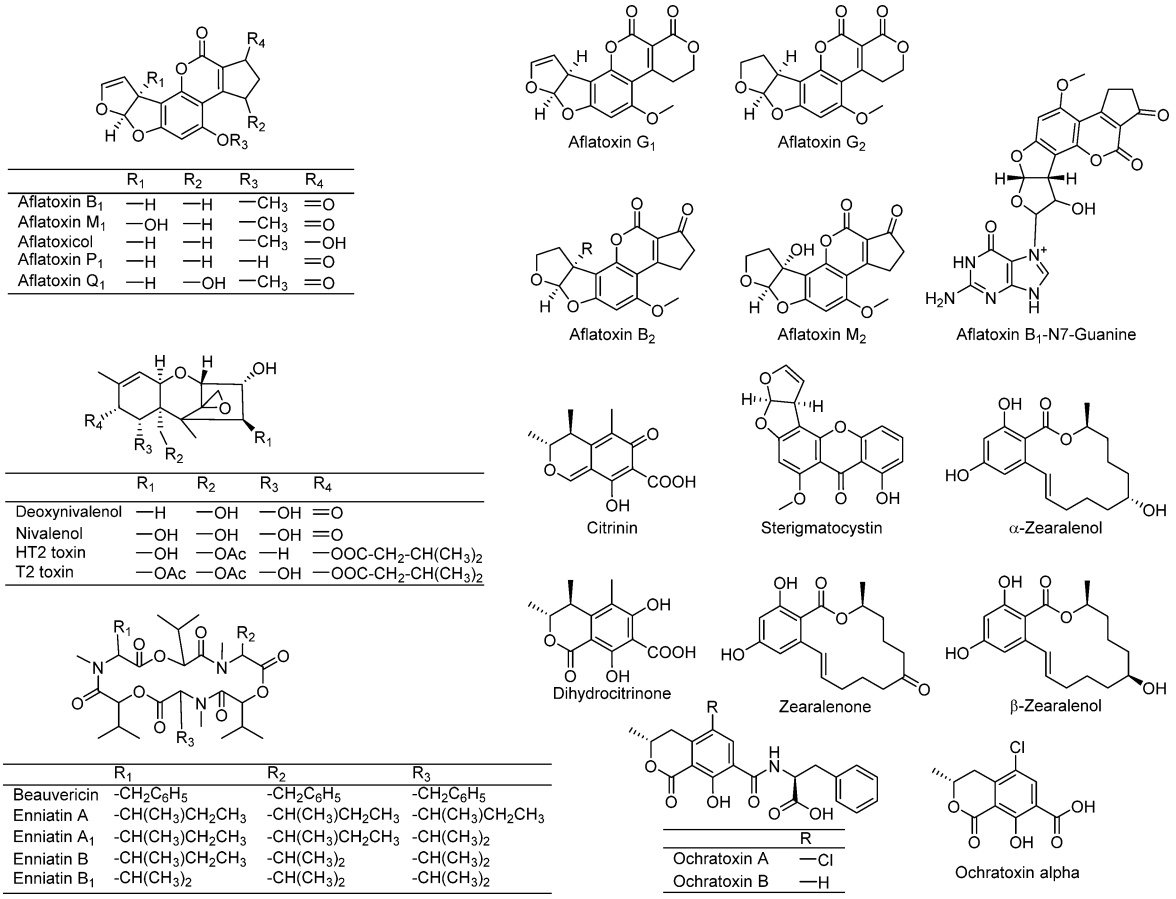


Figure 1. Chemical structures of the 28 investigated mycotoxins.

stasis and have been implicated in neural tube defects, stunting, and esophageal cancer.[10] OTA is mainly found in cereals and coffee and can cause kidney toxicity.[5,11] ZEN occurs frequently in cereals globally, is known as a potent endocrine disruptor with high affinity for the estrogen receptor, and has been controversially discussed in the context of breast cancer and its therapy.[5,12,13] Trichothecenes such as deoxynivalenol (DON) are produced by *Fusarium* species on wheat and maize and are associated with gastrointestinal effects and immune suppression.[14] 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.[15] So-called "emerging mycotoxins" such as beauvericin (BEA) and enniatins (ENNs) have been reported in food, in part due to advancement in analytical techniques, and have gained interest in recent years.[16]

It has been demonstrated by monitoring both food and urine that humans are typically exposed to diverse mixtures of mycotoxins.[6,17,18] Therefore, it is generally accepted that coexposures are the rule and not the exception and may lead to combinatory effects.[19,20] Numerous ingested toxins, especially fat-soluble compounds, can be transferred from consumed food of the mother to her breast milk.[21] Exposures of the nursing mothers to mycotoxins may vary largely due to seasonal changes or regional and individual dietary habits. Furthermore, transfer rates during all stages of lactation can differ.[22] Since neonates rely on breast milk as the dominant food source and are more susceptible to the adverse effects of environmental toxins,[23] exposure during the early stages of life may have both immediate effects and effects on health later in life.[4,24,25]

The occurrence of mycotoxins in human breast milk has been previously described, mainly for AFM_1 and OTA. Several studies reported AFM_1 in breast milk, including those in Brazil, Cameroon, Italy, Nigeria, and Tanzania, with significant variations in concentrations ranging up to 187 ng/mL.[26−30] OTA was determined in samples of similar regional origin (Brazil, Germany, Italy, and Sierra Leone) with concentrations up to 337 ng/mL.[26,27,31,32] Only one study from Italy described the occurrence of ZEN in human breast milk with concentrations between 0.26 and 1.78 ng/mL.[33] The assessment of mycotoxins was commonly based on single-analyte methods using either an enzyme linked immunosorbent assay (ELISA) or high-pressure liquid chromatography with fluorescence detection (LC-FD).[26] One method explored high-resolution mass spectrometry,[34] and two others assessed AFs and OTA together by LC-FD.[32,35]

While there is a clear trend toward the employment of multianalyte[26] and exposome-scale methods[36,37] in the assessment of food contaminants, no triple-quadrupole multimycotoxin method has been applied to mycotoxins in breast milk to date. Here we report the development and validation of a highly sensitive LC-MS/MS tool to simultaneously measure 28 mycotoxins/metabolites in breast milk. The methods' performance and feasibility to assess mycotoxins in this complex biofluid was demonstrated by utilizing samples from Nigerian mothers. These data were then used to estimate infant exposure in an area of predicted high mycotoxin risk and

DOI: 10.1021/acs.analchem.8b04576
Anal. Chem. 2018, 90, 14569−14577

subsequently compared to exposure estimates based on infant food MTLs established in the EU.

EXPERIMENTAL SECTION

Chemicals and Reagents. All chemicals, reagents, reference materials, and ^{13}C-labeled standards, which were used during the development, validation, and pilot application of the method, are described in the Supporting Information.

Sample Preparation Protocol. During the developmental stage several sample cleanup approaches were tested and optimized. Sample cleanup was performed utilizing liquid−liquid extraction with hexane, chloroform, or combinations thereof. Extracts were either directly analyzed or further purified by applying solid-phase extraction (Oasis PRiME HLB, Waters; see the Supporting Information). For method validation the following protocol was finally chosen for sample extraction and cleanup: an aliquot of 2 mL of 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 of anhydrous magnesium sulfate and 0.2 g of sodium chloride were added, followed by a further vortexing step (3 min). The sample was then centrifuged for 10 min (4750*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 microreaction tube, chilled, and kept at −20 °C for 2 h. Thereafter, another centrifugation step was performed (15 min at 14000*g*, 4 °C), the supernatant was filtered (PTFE, 0.22 μm, Carl Roth, Karlsruhe, Germany), and 90 μL was spiked with 10 μL of internal standard mix; then 3 μL was injected into 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) was subjected to enzymatic deconjugation. A mix of 250 μL of 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, 2.1 × 100 mm, Waters, Vienna, Austria) guarded by a VanGuard precolumn (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. LC gradient and MS operation parameters as well as optimized MS and MS/MS parameters (Table S-1) are reported in the 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).

Validation and Quality Control. In-house validation was carried out according to the guidelines of Eurachem[38,39] and the EU commission decision 2002/657/EC[40] 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), signal suppression or enhancement (SSE), and expanded measurement uncertainty. 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 the Supporting Information.

Breast Milk Samples. Anonymized and blended breast milk aliquots (1−3 mL) 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 Ilishan Remo, Ogun State. Samples (0.5−3 mL) were obtained in the morning and the evening on two consecutive days from most women. Detailed information on study subjects is provided in the Table S-2 in the Supporting Information. 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).

Exposure Assessment. The estimated daily intake of mycotoxins for Nigerian infants was calculated through a deterministic method assuming an upper bound worst case scenario. For regulated analytes not detected within our study, we considered LOD values as maximum concentrations. LOQ values or the respective maximum concentrations were used for analytes determined in the samples (see Table S-3 in the Supporting Information). The mean daily breast milk intake of 151 mL/kg body weight was determined on the basis of the quantity of milk intake multiplied by the frequency of breastfeeding per day divided by the average infant body weight (see Table S-2 in the Supporting Information). The quantity of milk intake was assessed one time for each infant, as more measurements were not feasible. For this purpose a typical volume of breast milk was self-expressed by the mother into a 150 mL graduated bottle. The estimated daily maximum mycotoxin exposure via breast milk (Table 2) was then calculated by multiplying the hypothetical upper bound exposure with the estimated milk intake. In comparison, a hypothetical worst case scenario for infant food contaminated at the MTL was calculated. Here, the equal daily intake (151 mL/kg body weight) of commercially available breast milk substitute was assumed.

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 availability of mycotoxin reference standards.[41−43] MS optimization was

DOI: 10.1021/acs.analchem.8b04576
Anal. Chem. 2018, 90, 14569−14577

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), Limits of Quantification (LOQ) and Expanded Measurement Uncertainty (U)

				$R_E \pm RSD_R$ (%)								
analyte	concn range (ng/mL)	regression coeff R^2	spiking level[a] (ng/mL)	level 1[b]	level 2	level 3	level 4	RSD_r[c] (%)	SSE[d] (%)	LOD (ng/mL)	LOQ (ng/mL)	U (%)
aflatoxin B_1	0.05−30	0.995	0.24/0.6/1.5/3	99 ± 16	85 ± 9	93 ± 6	92 ± 3	17/9/9/5	108	0.040	0.080	27
aflatoxin B_2	0.05−30	0.998	0.24/0.6/1.5/3	96 ± 18	93 ± 10	103 ± 6	102 ± 4	8/7/4/5	98	0.042	0.085	29
AFB_1-N7-Gua[e]	0.25−150	0.998	1.2/3/7.5/15	28 ± 19	33 ± 22	41 ± 7	40 ± 15	25/19/14/16	87	0.20	0.40	-
aflatoxin G_1	0.05−30	0.997	0.24/0.6/1.5/3	98 ± 12	116 ± 7	122 ± 5	116 ± 2	8/7/6/4	80	0.043	0.086	35
aflatoxin G_2	0.1−30	0.998	0.24/0.6/1.5/3	109 ± 19	102 ± 16	106 ± 12	102 ± 4	19/15/15/5	88	0.079	0.16	28
aflatoxicol	0.25−150	0.999	1.2/3/7.5/15	92 ± 12	74 ± 3	77 ± 5	79 ± 3	14/11/6/3	108	0.15	0.31	41
aflatoxin M_1	0.05−30	0.998	0.24/0.6/1.5/3	94 ± 19	91 ± 13	97 ± 7	95 ± 4	16/12/10/4	98	0.043	0.087	26
aflatoxin M_2	0.1−30	0.997	0.24/0.6/1.5/3	86 ± 17	91 ± 16	92 ± 7	96 ± 8	11/16/4/9	94	0.076	0.15	32
aflatoxin P_1	0.1−30	0.997	0.24/0.6/1.5/3	90 ± 20	92 ± 8	91 ± 7	87 ± 7	19/19/10/6	100	0.068	0.14	30
aflatoxin Q_1	0.1−30	0.997	0.24/0.6/1.5/3	99 ± 17	104 ± 6	104 ± 6	105 ± 8	19/14/6/8	89	0.063	0.13	28
beauvericin	0.01−6	0.996	0.048/0.12/0.3/0.6	99 ± 12	108 ± 4	110 ± 5	108 ± 2	12/6/6/3	100	0.006	0.011	20
citrinin[f]	0.05−30	0.995	0.24/0.6/1.5/3	98 ± 17	85 ± 6	92 ± 3	91 ± 3	13/3/2/6	129	0.025	0.049	31
dihydrocitrinone	0.1−60	0.996	0.48/1.2/3/6	100 ± 20	92 ± 9	107 ± 4	104 ± 3	9/10/7/7	133	0.092	0.18	27
deoxynivalenol[e]	1.5−450	0.995	3.6/9/22.5/45	51 ± 10	37 ± 14	64 ± 12	74 ± 10	11/30/8/8	91	0.77	1.5	-
enniatin A	0.01−6	0.997	0.048/0.12/0.3/0.6	97 ± 11	71 ± 6	69 ± 2	67 ± 3	13/6/3/3	103	0.005	0.009	54
enniatin A_1	0.01−6	0.998	0.048/0.12/0.3/0.6	110 ± 12	99 ± 10	91 ± 6	88 ± 3	14/15/6/4	102	0.012	0.023	24
enniatin B	0.01−6	0.999	0.048/0.12/0.3/0.6	89 ± 19	88 ± 4	94 ± 4	91 ± 3	15/5/3/3	99	0.004	0.009	25
enniatin B_1	0.01−6	0.998	0.048/0.12/0.3/0.6	100 ± 18	94 ± 6	94 ± 4	91 ± 3	19/6/5/3	101	0.006	0.012	22
HT-2 toxin	1.5−450	0.996	3.6/9/22.5/45	83 ± 18	85 ± 7	94 ± 9	91 ± 4	24/27/14/4	122	1.4	2.9	37
nivalenol[e]	1.333−800	0.997	6.4/16/40/80	22 ± 5	18 ± 6	16 ± 11	19 ± 6	5/11/16/12	97	0.254	0.51	-
ochratoxin A	0.1−60	0.998	0.48/1.2/3/6	90 ± 13	96 ± 5	99 ± 5	96 ± 2	14/5/6/4	93	0.048	0.096	14
ochratoxin B	0.1−60	0.999	0.48/1.2/3/6	87 ± 14	93 ± 7	94 ± 4	96 ± 2	10/6/3/3	103	0.063	0.13	23
ochratoxin α	0.333−100	0.996	0.8/2/5/10	108 ± 8	97 ± 13	114 ± 5	113 ± 5	7/16/9/10	96	0.21	0.42	26
sterigmatocystin	0.025−15	0.998	0.12/0.3/0.75/1.5	79 ± 10	78 ± 8	82 ± 3	81 ± 3	11/5/4/4	108	0.013	0.026	41
T-2 toxin	0.2−60	0.997	0.48/1.2/3/6	87 ± 16	88 ± 18	94 ± 9	98 ± 9	18/23/20/9	97	0.18	0.36	32
zearalenone	0.2−60	0.999	0.48/1.2/3/6	92 ± 16	97 ± 10	106 ± 5	101 ± 2	9/7/6/3	95	0.093	0.19	19
α-zearalenol	0.133−80	0.999	0.64/1.6/4/8	90 ± 13	99 ± 6	99 ± 3	97 ± 2	11/5/3/3	89	0.073	0.15	14
β-zearalenol	0.133−80	0.999	0.64/1.6/4/8	82 ± 17	100 ± 3	99 ± 6	99 ± 4	14/6/4/3	88	0.068	0.14	17

[a]Spiking levels reported in the following order: level 1/level 2/level 3/level 4. [b]Carried out two times for DON and NIV at this level. [c]RSD_r values reported in the following order: level 1/level 2/level 3/level 4. [d]SSE calculated as the slope of calibration in the matrix divided by the slope of calibration in solution expressed in percent. [e]U was not calculated, as the toxin was not considered as successfully validated. [f]Measured as the methanol adduct (m/z 281.1).

DOI: 10.1021/acs.analchem.8b04576
Anal. Chem. 2018, 90, 14569−14577

carried out in positive and negative ionization modes to determine preferential parameters for all analytes and is described in detail in the Supporting Information. The development of a quantitative multianalyte 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 mycotoxins (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%, and 1% acetic acid and ammonium acetate) were tested. Overall, chromatographic separation improved using MeOH instead of ACN, due to favorable peak widths and faster elution of ENNs. Moreover, the observed intensities for citrinin (CIT) were significantly higher when measuring the MeOH adduct described above. The acidification of the aqueous eluent showed a positive effect on overall peak width and shape. The concentration of 0.1% acetic acid was deemed most suitable, as higher concentrations resulted in broader CIT, dihydrocitrinone (DH-CIT), and ochratoxin α (OTα) peaks (>30 s). Ammonium acetate (5 mM) was added to avoid formation of sodium adducts and additionally resulted in higher signal intensities, especially for BEA and ENNs. In contrast, the early eluting compounds NIV and DON showed decreased intensities. 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. The bioavailability of FBs from food is estimated at less than 0.1%,[44] and the lactational transfer, on the basis of physicochemical properties, is additionally expected to be minute. Hence, these toxins may only occur in low concentrations in breast milk, except for regions with high FB contamination of food. Due to these factors, we excluded FBs from the method.

Optimization of the 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 for their feasibility (see the Supporting Information).[45] 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,[34,46] this approach was further investigated to overcome the observed extraction losses during liquid−liquid extractions in combination with solid-phase extraction (LLE-SPE). Spiked breast milk samples were extracted using an adapted and thoroughly optimized protocol (see the Experimental Section). 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 cleanup/enrichment. However, the same analytes as for the LLE-SPE protocol described in the Supporting Information (BEA, CIT, ENNs, sterigmatocystin, α/β-zearalenol (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 multimycotoxin 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[38,39] and the European commission decision 2002/657/EC[40] by evaluating sensitivity, selectivity, repeatability, intermediate precision, linearity, extraction recovery, matrix effects, and expanded measurement uncertainty. 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, which were assessed utilizing the EuraChem guideline, 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 and 0.012 ng/mL. LODs of other mycotoxins were <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 for DON and HT-2 are 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 (factors of 5 to 100) in comparison to the only publication reporting on the simultaneous measurement of more than one class of mycotoxins in human breast milk.[34] The selectivity of the method was assessed by comparing extracted blank samples with spiked samples. No interfering peaks ($S/N \geq 3$) within a time frame of ±0.15 min were detected for any analyte, ensuring proper quantification. Identification was based on four criteria: retention time, quantifier and qualifier ions, 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.[40] 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 the Figure S-1 in the Supporting Information.

Extraction recoveries as stated in Table 1 were in good agreement with the EC Decision 2002/657/EC[40] except for DON (<64%), NIV (<22%), and AFB_1-N7-guanine adduct (AFB_1-N7-Gua, < 41%). 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. As we focused on more lipophilic contaminants, we accepted this compromise. However, since results were generally corrected for extraction losses and these were sufficiently stable for all spiking levels (RSD < 18% 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 (80−110% for spiking levels above 10 ng/mL; 70−110% between 1 and 10 ng/mL; 50−120% below 1 ng/mL) with minor exceptions for AFG_1 and OTα. Repeatability (intraday RSD; RSD_r) and intermediate precision (interday RSD; RSD_R) ranged from 2 to 30% and from 2 to 25% for all analytes, respectively. Except for the lowest spiking levels of HT-2 and AFB_1-N7-Gua and the second lowest spiking levels 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

DOI: 10.1021/acs.analchem.8b04576
Anal. Chem. 2018, 90, 14569−14577

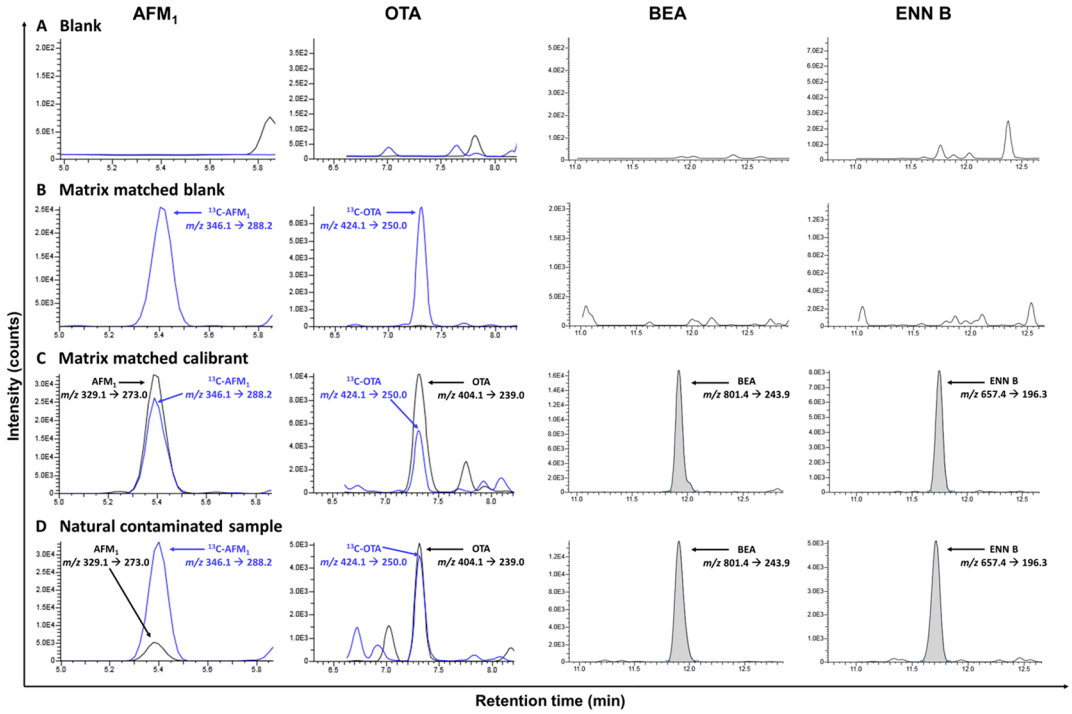


Figure 2. MRM chromatograms of a solvent blank (A), a matrix-matched blank sample (B), a matrix-matched calibrant (C), and a naturally contaminated breast milk sample (D) of AFM_1, OTA, BEA, and ENN B, respectively. For AFM_1 and OTA ^{13}C-labeled internal reference standards were included for confirmation purposes, while for BEA and ENN B no labeled standards were available.

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 by 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 signal enhancement.

The mean value of the expanded measurement uncertainties (U) is reported in Table 1. In general, lower U values were obtained for BEA, OTA, ZEN, α-ZEL, and β-ZEL (14−20%). The bias of the analytical method had a high contribution to U for all spiking levels, leading to high values especially for aflatoxicol, sterigmatocystin, and ENN A (41−54%). For the more polar toxins DON, NIV, and AFB_1-N7-Gua, U was not calculated, as the extraction recovery of these analytes did not fulfill the requirements. Generally, U was in the range of 14−54%, which is in compliance with several analytical methods measuring mycotoxins in other complex matrices.[47,48] Overall, the method can be used as a screening tool for the rather polar mycotoxins DON, NIV, and AFB_1-N7-Gua. All of the other 25 analytes were successfully validated.

Due to interindividual 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 interindividual 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 proved to be for the 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 multianalyte methods, some minor compromises in the method performance had to be accepted.[49,50]

Application of the Developed Method to Human Breast Milk Samples. To evaluate the applicability of the method, multiple human breast milk samples (n = 75) of a Nigerian cohort (n = 22) 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 only minor levels (close to or below the LOQ). Overall, in 42 samples (56%) BEA was detected, while quantification was only possible in six samples with concentrations up to 0.019 ng/mL. Seven samples (9%) showed trace amounts of ENN B up to a concentration of 0.009 ng/mL. OTA was present in 11 samples (15%) below LOQ and, except for AFM_1 in a single sample (below LOQ), neither aflatoxins nor their metabolites were observed (Table S-3). In general, not only validation criteria but also the S/N ratio had to be greater than 3 for positive evaluation. In addition, all experimental samples with detectable levels of OTA and AFM_1 were confirmed by ^{13}C-labeled reference standards. To the best of our knowledge, no data on BEA in natural contaminated human breast milk have been published to date. 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.

DOI: 10.1021/acs.analchem.8b04576
Anal. Chem. 2018, 90, 14569−14577

Table 2. 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 (ng/mL)	max concn (ng/mL)	max estimated daily intake via breast milk[a] (ng/kg bw per day)	TDI for adults (ng/kg bw per day)	infant corrected TDI[b] (ng/kg bw per day)	MTL in infant food[c] (ng/g)	Theoretical intake via infant food at MTL[d] (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

[a]For calculation, either LOD or, if available, maximum concentration in breast milk (as reported in Table 1) was multiplied by 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).[24] [c]According to EC 1881/2006[15] 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 the LOQ level. [f]According to EFSA, 2012.[53] [g]According to EFSA, 2013.[54] [h]According to EFSA, 2013.[55] [i]TDI calculated as 120 ng/kg bw per week[11] divided by 7. [j]According to EFSA, 2011.[56]

To further confirm the identity of the detected analytes (AFM_1, BEA, ENN B, and OTA) we additionally enriched selected samples <LOQ, by concentration of the filtered extract by a factor of 5, and reanalyzed 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 14 samples, while merely two mycotoxins were present.

The potential existence of phase II metabolites was investigated by the measurement of five selected samples for the possible presence of glucuronide and sulfate conjugates. After treatment with a mixture of glucuronidase and sulfatase followed by the established sample preparation 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 the literature, showing that phase II metabolites are more likely to be eliminated through the kidney and may thus not be relevant for lactational transfer.[51,52]

Since the volume of the Austrian samples was rather limited and was entirely used as a pooled sample for method development and validation, we were not able to assess individual exposures. However, since no mycotoxin was detected in the pooled sample, this suggests no abundant exposures via breast milk in the Austrian population. Still, it is likely that some samples might have been contaminated at low concentration but were diluted out. We plan to confirm this in subsequent large-scale biomonitoring studies. While OTA was frequently determined in samples obtained from German mothers,[31] 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 are tested in Austria.

Implications for Exposure Assessment. Since infants are more susceptible toward 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 the tolerable daily intake (TDI) by a factor of 3 for infants for the first 16 weeks of life, a key window for early life exposures.[24] In addition, MTLs for mycotoxins in infant food, as outlined by the EU commission regulation 1881/2006/EC,[15] are therefore lower than for other foodstuffs.

To relate our breast milk estimates to an exposure scenario from infant food, the MTLs of regulated mycotoxins and the results gained within this biomonitoring study were compared (Table 2). We assumed that a contamination above the LOD would have been detected. The calculated values constitute the upper bound scenario and real exposures are most likely lower in a majority of samples. As an example, an MTL of 0.5 ng/g was set for OTA in infant food.[15] By comparison of this regulatory value with the LOQ of the developed method for OTA (<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).

The estimated daily exposure was subsequently calculated and compared for both breast milk and commercial breast milk substitutes, assuming a worst case scenario. Here, the infant exposure via breast milk is significantly lower than the calculated theoretical intake at the MTL, pointing out the high value of breast milk even in areas of high mycotoxin risk. However, the exposure calculation is based on a worst case scenario, assuming a contamination of infant food at the MTL. On the basis of the high sensitivity of the analytical method it was possible to derive that established TDI values were not exceeded for mycotoxins by breastfeeding in the reported pilot survey.

If a contamination of OTA at the LOQ level is assumed for experimental samples above the LOD but below the LOQ, and subsequently correlated to the TDI corrected for infants, the milk of seven mothers would have exceeded this guidance value.[24] However, it has to be highlighted that the low number of quantified mycotoxins within this pilot study constitutes a factor of uncertainty in exposure estimates. Despite this limitation the assessment suggests that a sample in which no regulated mycotoxin can be detected with the developed method is, in addition to its unmatched nutritional and immunological value, safe from a mycotoxin food safety perspective. Importantly, appropriate alternatives in regions with poor infrastructure and diminished access to purified or

DOI: 10.1021/acs.analchem.8b04576
Anal. Chem. 2018, 90, 14569−14577

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. 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.

CONCLUSION AND OUTLOOK

In this paper we report the development and successful application of a novel targeted LC-MS/MS method for assessing early life mycotoxin exposures via contaminated breast milk. The method was successfully validated for 25 mycotoxins and key metabolites. The rather polar toxins DON, NIV, and AFB_1-N7-Gua did not fulfill all required validation parameters; however, our method can be used as a screening tool for these toxins until a tailored approach for their quantification is available. On the basis of our results and their comparison with maximum permitted levels in infant food, 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 BEA, 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. It will be valuable to generate data on intake and further biomarkers in blood and urine along with breast milk measures to better understand the toxicokinetics including lactational transfer rates. Such studies should include countries of various regions with differing exposure patterns to better understand global occurrence patterns and the potentially associated risks. The developed methodology could help in risk assessments for both mothers and their infants, by providing comprehensive analytical data: e.g., on the success of efforts intended to minimize mycotoxin exposures as much as possible during this critical window of susceptibility.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b04576.

Additional text, figures, and tables describing used chemicals and reagents, LC-MS/MS parameters, in-house validation, quality control measures, information about study subjects, generated results, and additional discussion (PDF)

AUTHOR INFORMATION

Corresponding Author

*B.W.: tel, +431427770806; e-mail, benedikt.warth@univie.ac.at.

ORCID

Dominik Braun: 0000-0002-9232-4285
Chibundu N. Ezekiel: 0000-0002-2113-2948
Paul C. Turner: 0000-0001-7744-891X
Doris Marko: 0000-0001-6568-2944
Benedikt Warth: 0000-0002-6104-0706

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors want to gratefully thank all participants who provided breast milk samples. Additionally, we acknowledge the Semmelweis Women's Clinic of Vienna (Dr. Michaela Riegler-Keil and Astrid Veprek) for providing anonymized breast milk samples. We also appreciate Beatrice Mark and Emmanuel Michael-Chikezie for their immense support during sampling in Nigeria. Moreover, we extend our gratitude toward Dr. Michael Sulyok (BOKU University, Vienna) for providing reference material and our colleagues at the University of Vienna for constructive discussions. For technical support and advice we thank the staff of the Mass Spectrometry Centre of the University of Vienna, where LC-MS/MS measurements were performed. This work was financed by the University of Vienna and the *MycoMarker* project (BOKU Research Funding).

REFERENCES

(1) Horta, B. L., Bahl, R., Martines, J. C., Victora, C. G. *World Health Organization* 2007, 1−57; http://apps.who.int/iris/bitstream/handle/10665/43623/9789241595230_eng.pdf, Accessed 07.11.2018.

(2) Victora, C. G.; Bahl, R.; Barros, A. J. D.; França, G. V. A.; Horton, S.; Krasevec, J.; Murch, S.; Sankar, M. J.; Walker, N.; Rollins, N. C. *Lancet* **2016**, *387*, 475−490.

(3) CAST. *Task Force Report No. 139* **2003**, 13−85.

(4) Gong, Y.; Hounsa, A.; Egal, S.; Turner, P. C.; Sutcliffe, A. E.; Hall, A. J.; Cardwell, K.; Wild, C. P. *Environ. Health Perspect.* **2004**, *112*, 1334−1338.

(5) Bennett, J. W.; Klich, M. *Clin. Microbiol. Rev.* **2003**, *16*, 497−516.

(6) Schatzmayr, G.; Streit, E. *World Mycotoxin J.* **2013**, *6*, 213−222.

(7) Uhlig, S.; Eriksen, G. S.; Hofgaard, I. S.; Krska, R.; Beltran, E.; Sulyok, M. *Toxins* **2013**, *5*, 1682−1697.

(8) Turner, P. C.; Moore, S. E.; Hall, A. J.; Prentice, A. M.; Wild, C. P. *Environ. Health Perspect.* **2003**, *111*, 217−220.

(9) Gong, Y. Y.; Wilson, S.; Mwatha, J. K.; Routledge, M. N.; Castelino, J. M.; Zhao, B.; Kimani, G.; Kariuki, H. C.; Vennervald, B. J.; Dunne, D. W.; Wild, C. P. *Environ. Health Perspect.* **2012**, *120*, 893−896.

(10) Shirima, C. P.; Kimanya, M. E.; Routledge, M. N.; Srey, C.; Kinabo, J. L.; Humpf, H.-U.; Wild, C. P.; Tu, Y.-K.; Gong, Y. Y. *Environ. Health Perspect.* **2015**, *123*, 173−178.

(11) EFSA. *EFSA J.* **2006**, *4*, 365.

(12) Warth, B.; Raffeiner, P.; Granados, A.; Huan, T.; Fang, M.; Forsberg, E. M.; Benton, H. P.; Goetz, L.; Johnson, C. H.; Siuzdak, G. *Cell Chemical Biology* **2018**, *25*, 291−300.

(13) Belhassen, H.; Jiménez-Díaz, I.; Arrebola, J. P.; Ghali, R.; Ghorbel, H.; Olea, N.; Hedili, A. *Chemosphere* **2015**, *128*, 1−6.

(14) Turner, P. C.; Flannery, B.; Isitt, C.; Ali, M.; Pestka, J. *Nutr. Res. Rev.* **2012**, *25*, 162−179.

(15) EC. European Union Commission. *Off. J. Eur. Communities L* **2006**, *173*, 5−24.

(16) Gruber-Dorninger, C.; Novak, B.; Nagl, V.; Berthiller, F. *J. Agric. Food Chem.* **2017**, *65*, 7052−7070.

(17) Ezekiel, C. N.; Warth, B.; Ogara, I. M.; Abia, W. A.; Ezekiel, V. C.; Atehnkeng, J.; Sulyok, M.; Turner, P. C.; Tayo, G. O.; Krska, R.; Bandyopadhyay, R. *Environ. Int.* **2014**, *66*, 138−145.

(18) Šarkanj, B.; Ezekiel, C. N.; Turner, P. C.; Abia, W. A.; Rychlik, M.; Krska, R.; Sulyok, M.; Warth, B. *Anal. Chim. Acta* **2018**, *1019*, 84−92.

(19) Vejdovszky, K.; Hahn, K.; Braun, D.; Warth, B.; Marko, D. *Arch. Toxicol.* **2017**, *91*, 1447−1460.

(20) Speijers, G. J.; Speijers, M. H. *Toxicol. Lett.* **2004**, *153*, 91−98.

(21) LaKind, J. S.; Berlin, C. M., Jr.; Sjodin, A.; Turner, W.; Wang, R. Y.; Needham, L. L.; Paul, I. M.; Stokes, J. L.; Naiman, D. Q.; Patterson, D. G., Jr. *Environ. Health Perspect.* **2009**, *117*, 1625−1631.

(22) Degen, G.; Muñoz, K.; Hengstler, J. In *Handbook of dietary and nutritional aspects of human breast milk*; Zibadi, S., Watson, R. R.,

DOI: 10.1021/acs.analchem.8b04576
Anal. Chem. 2018, 90, 14569−14577

Preedy, V. R., Eds.; Wageningen Academic: Wageningen, The Netherlands, 2013; pp 813−831.
(23) Degen, G. H.; Partosch, F.; Muñoz, K.; Gundert-Remy, U. *Toxicol. Lett.* **2017**, *277*, 69−75.
(24) Hardy, A. *EFSA J.* **2017**, *15*, No. e04849.
(25) Johnson, C. H.; Athersuch, T. J.; Collman, G. W.; Dhungana, S.; Grant, D. F.; Jones, D. P.; Patel, C. J.; Vasiliou, V. *Hum. Genomics* **2017**, *11*, 32.
(26) Warth, B.; Braun, D.; Ezekiel, C. N.; Turner, P. C.; Degen, G. H.; Marko, D. *Chem. Res. Toxicol.* **2016**, *29*, 1087−1097.
(27) Iha, M. H.; Barbosa, C. B.; Heck, A. R.; Trucksess, M. W. *Food Control* **2014**, *40*, 310−313.
(28) Oluwafemi, F. T. *Archives of Clinical Microbiology* **2012**, *3*, 1−6.
(29) Magoha, H.; Kimanya, M.; Meulenaer, B. D.; Roberfroid, D.; Lachat, C.; Kolsteren, P. *World Mycotoxin J.* **2014**, *7*, 277−284.
(30) Bogalho, F.; Duarte, S.; Cardoso, M.; Almeida, A.; Cabeças, R.; Lino, C.; Pena, A. *Food Control* **2018**, *90*, 140−145.
(31) Muñoz, K.; Wollin, K.; Kalhoff, H.; Degen, G. *Gesundheitswesen* **2013**, *75*, 194−197.
(32) Jonsyn, F. E.; Maxwell, S. M.; Hendrickse, R. G. *Mycopathologia* **1995**, *131*, 121−126.
(33) Massart, F.; Micillo, F.; Rivezzi, G.; Perrone, L.; Baggiani, A.; Miccoli, M.; Meucci, V. *Toxicol. Environ. Chem.* **2016**, *98*, 128−136.
(34) Rubert, J.; Leon, N.; Saez, C.; Martins, C. P. B.; Godula, M.; Yusa, V.; Manes, J.; Soriano, J. M.; Soler, C. *Anal. Chim. Acta* **2014**, *820*, 39−46.
(35) Andrade, P. D.; Gomes da Silva, J. L.; Caldas, E. D. *J. Chromatogr. A* **2013**, *1304*, 61−68.
(36) Dennis, K. K.; Marder, E.; Balshaw, D. M.; Cui, Y.; Lynes, M. A.; Patti, G. J.; Rappaport, S. M.; Shaughnessy, D. T.; Vrijheid, M.; Barr, D. B. *Environ. Health Perspect.* **2017**, *125*, 502.
(37) Warth, B.; Spangler, S.; Fang, M.; Johnson, C. H.; Forsberg, E. M.; Granados, A.; Martin, R. L.; Domingo-Almenara, X.; Huan, T.; Rinehart, D.; Montenegro-Burke, J. R.; Hilmers, B.; Aisporna, A.; Hoang, L. T.; Uritboonthai, W.; Benton, H. P.; Richardson, S. D.; Williams, A. J.; Siuzdak, G. *Anal. Chem.* **2017**, *89*, 11505−11513.
(38) Magnusson, B. The fitness for purpose of analytical methods: A laboratory guide to method validation and related topics. *Eurachem* **2014**, 57; https://www.eurachem.org/images/stories/Guides/pdf/MV_guide_2nd_ed_EN.pdf, Accessed 07.11.2018.
(39) Magnusson, B.; Näykki, T.; Hovind, H.; Krysell, M. Handbook for Calculation of Measurement Uncertainty in Environmental Laboratories. *Eurachem*, **2011**, 52; http://www.nordtest.info/images/documents/nt-technical-reports/nt_tr_537_ed3_1_English_Handbook%20for%20Calculation%20of%20Measurement%20uncertainty%20in%20environmental%20laboratories.pdf, accessed 07.11.2018.
(40) EC. European Union Commission. *Off. J. Eur. Communities L* **2002**, *221*, 8−36.
(41) Mally, A.; Solfrizzo, M.; Degen, G. H. *Arch. Toxicol.* **2016**, *90*, 1281−1292.
(42) EFSA. *EFSA J.* **2014**, *12*, 3802.
(43) Waseem, A.; Shah, S. S. A.; Sajjad, A.; Siddiqi, A.; Nafees, M. 2014, *36*, 1196−1214.
(44) Van Der Westhuizen, L.; Shephard, G. S.; Gelderblom, W. C. A.; Torres, O.; Riley, R. T. *World Mycotoxin J.* **2013**, *6*, 223−232.
(45) Krska, R.; Schubert-Ullrich, P.; Molinelli, A.; Sulyok, M.; MacDonald, S.; Crews, C. *Food Addit. Contam., Part A* **2008**, *25*, 152−163.
(46) Paya, P.; Anastassiades, M.; Mack, D.; Sigalova, I.; Tasdelen, B.; Oliva, J.; Barba, A. *Anal. Bioanal. Chem.* **2007**, *389*, 1697−1714.
(47) Stadler, D.; Sulyok, M.; Schuhmacher, R.; Berthiller, F.; Krska, R. *Anal. Bioanal. Chem.* **2018**, *410*, 4409−4418.
(48) Njumbe Ediage, E.; Van Poucke, C.; De Saeger, S. *Food Chem.* **2015**, *177*, 397−404.
(49) Sulyok, M.; Krska, R.; Schuhmacher, R. *Anal. Bioanal. Chem.* **2007**, *389*, 1505−1523.
(50) Malachova, A.; Stranska, M.; Vaclavikova, M.; Elliott, C. T.; Black, C.; Meneely, J.; Hajslova, J.; Ezekiel, C. N.; Schuhmacher, R.; Krska, R. *Anal. Bioanal. Chem.* **2018**, *410*, 801−825.
(51) Frederiksen, H.; Skakkebaek, N. E.; Andersson, A.-M. *Mol. Nutr. Food Res.* **2007**, *51*, 899−911.
(52) Notarianni, L. J.; Oldham, H. G.; Bennett, P. N. *Br. J. Clin. Pharmacol.* **1987**, *24*, 63−67.
(53) EFSA. *EFSA J.* **2012**, *10*, 2605.
(54) EFSA. *EFSA J.* **2013**, *11*, 3379.
(55) EFSA. *EFSA J.* **2013**, *11*, 3262.
(56) EFSA. *EFSA J.* **2011**, *9*, 2197.

DOI: 10.1021/acs.analchem.8b04576
Anal. Chem. 2018, 90, 14569−14577