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Biomonitoring of Mycotoxins in Human Breast Milk: Current State and Future Perspectives

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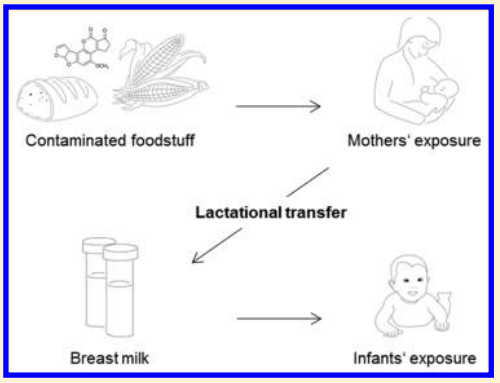
ABSTRACT: Human breast milk is considered as the best and ideal form of nutrition for infants. However, food contaminants such as mycotoxins, which may be transferred from maternal blood to milk, are poorly described. Mycotoxins are a major group of natural toxins frequently detected in foods. Here, we review the current state-of-the-art in the monitoring of mycotoxins in human breast milk, i.e., knowledge on occurrence, metabolism, and analytical assays utilized for their quantification. We highlight that most of the data captured to date have not been verified with the precision now capable utilizing LC-MS/MS and LC-HRMS approaches. One concern is that some studies may overestimate individual measures, and most cannot capture the patterns and levels of mycotoxin mixtures. We propose accurate assessment as a priority, especially for aflatoxins, fumonisins, ochratoxin A, zearalenone, and deoxynivalenol as well as their major metabolites. However, also so-called emerging toxins such as citrinin, the enniatins, beauvericin, aurofusarin, or *Alternaria* toxins should be considered to evaluate their potential relevance. Key requirements for analytical quality assurance are identified and discussed to guide future developments in this area. Moreover, research needs including investigations of lactational transfer rates, the role of human metabolism for bioactivation or detoxification, and an evaluation of potential combinatory effects of different mycotoxins are pointed out. It is hoped that LC-MS based multianalyte methods will enable more accurate, rapid and affordable human biomonitoring approaches that support informed decisions for maternal and infant health.



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INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by a variety of fungal genera such as *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* that contaminate crops and foodstuff worldwide.

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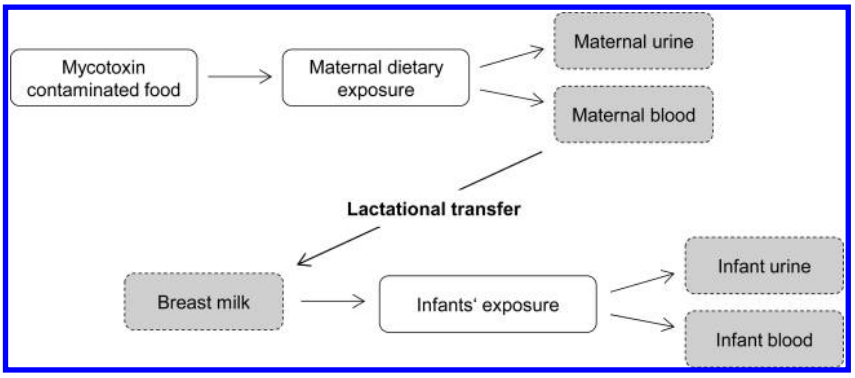

Figure 1. Scheme illustrating maternal and infants' exposure toward mycotoxin contaminated food. Potential sampling points to obtain biological fluids for biomonitoring purpose are indicated by dotted boxes. The scheme was created on the basis of a previously published concept by Muñoz et al.[46]

Several hundred mycotoxins have been described since the aflatoxins (AFs) were first discovered around 1960.[1−3] Occurrence of these major food contaminants depends on many factors including climate and poor agricultural practice, both pre and postharvest; thus, wealth may be a significant predictor of the level of food contamination.[4] Climate change is predicted to influence future mycotoxin contamination patterns and levels on a global scale, with consequences for national and international trade, and levels to which consumers may be exposed through their diet.[5,6] Regions with poorly developed regulatory structures and practices are likely to be most affected by international trade issues around contamination, and populations in such regions will more frequently have higher levels in their diets.

Mycotoxins are diverse with respect to both their chemical structure and properties, and also their effects on human health. Because of their toxicity and frequency as contaminants, AFs, fumonisins (FBs), zearalenone (ZEN), ochratoxin A (OTA), and deoxynivalenol (DON) are of particular concern for public health. Ingestion of these mycotoxins causes animal toxicities including cancer, immune-modulation, inflammation, kidney toxicity, stunted growth, or endocrine changes.[3,7,8] AFs are proven human liver carcinogens and implicated in immune-modulation and impaired child growth.[1,7,9,10] FBs interfere with sphingolipid synthesis in animals and humans,[11] are suspected human esophageal carcinogens,[12] and have been implicated in the etiology of both neural tube defects and stunting.[13] OTA is carcinogenic in rodents and nephrotoxic in many animal species, though its role in human disease remains controversial.[14,15] DON, also referred to as vomitoxin, is the major type-B trichothecene occurring frequently in moderate climate regions. Its primary mode of action is the inhibition of protein translation,[16] whereas ZEN, another *Fusarium* toxin, and its phase-I metabolites exert potent estrogenic effects.[8,17] Mixtures of *Fusarium* toxins in the diet have been implicated in dozens of acute poisoning incidences, on occasion affecting tens of thousands of individuals.[6] However, the most often reported mycotoxicoses relate to AFs which can even lead to acute death, for example, in a severe outbreak in Kenya 2004 resulting in 125 deaths.[7,18]

While the Food and Agriculture Organization (FAO) has estimated about two decades ago that 25% of the world's agricultural crops contain mycotoxins,[19] a large survey measuring the presences of AFs, FBs, DON, ZEN, and OTA in cereal-based feed report both a high frequency of contamination (72% with at least one mycotoxin, n > 19,000 samples) and frequent cocontamination with other mycotoxins.[20] Similar frequencies have been reported for cereal based foods, albeit typically at lower levels at least in highly developed world regions.[19,21] Representative data for most African regions were notably absent at that time, though it was predicted that contamination of particularly AFs and FBs is frequent and at high levels compared to more developed regions.[7] The application of multianalyte LC-MS/MS methods confirmed frequent cocontamination of cereals and foodstuff in several sub-Saharan countries.[22−27] Thus, there remains a concern that individuals, particularly in developing regions, are frequently exposed to mixtures of mycotoxins through their regular diet. Recently, these predicted coexposures have been demonstrated in several populations and world regions by innovative biomeasurements of urine.[28−36]

With respect to infant feeding, there are strict regulatory limits in place for complementary or weaning food in the European Union.[37] The maximum residual concentration levels in infant formula, follow-on formula, dietary foods for special medical purposes intended specifically for infants or processed cereal-based foods and baby foods for infants and young children, respectively, have been defined as follows: AFB_1 (0.1 ng/mL), AFM_1 (0.025 ng/mL), OTA (0.5 ng/mL), FB_1 (200 ng/mL), patulin (10 ng/mL), ZEN (20 ng/mL), and DON (200 ng/mL). While these regulations are not based on any clearly established risk from mycotoxins at such low levels in these items, food analysis and exposure assessment showed that baby foods in Europe can be contaminated by multiple mycotoxins and that infants are typically the most exposed population group.[21,38−41] For newborns and infants, breast milk is regarded as the ideal form of nutrition. In addition, breastfeeding supports health benefits for both mother and child, and it is recommended by the World Health Organization to exclusively breastfeed for 6 months.[42,43] However, food contaminants, including some mycotoxins, may be transferred from the maternal diet to breast milk (see Figure 1).

It is predicted that the health benefits of exclusive breastfeeding likely far surpass the putative health risk from modest levels of transfer, especially in highly regulated world regions. However, while the limits of these toxins in infant food are very restrictive and controlled by surveillance programs, breast milk is comparably rarely evaluated. Accurate assessment of toxins or toxicants in breast milk, however, is complex. Key aspects include the timing within any given feed, time of day of feed, duration of breastfeeding, birth order, stability of toxins, and the analytical accuracy of measurement tools.[44] Despite this, several studies report data on levels and frequency of contamination (see Table 1) though not all provide sufficient details related to the accuracy of collection and importantly the

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Table 1. Summary of Case Studies and Analytical Methods Used for the Determination of Aflatoxins, Ochratoxin A, Zearalenone, and Fumonisin B_1 in Breast Milk 2006−2016[a]

country	mycotoxin	milk type	dietary information	sample number: positive/total (%)	concentration range (ng/L)	mean conc. (ng/L)	LOD/LOQ (ng/L)	sample preparation	analytical method	ref
Brazil	AFM_1[b]	NI[c]	NI[c]	2/100 (2)	LOD − LOQ[a]		0.3/0.8	IAC[d]	LC-FD[e]	96
Brazil	OTA[f]	NI	NI	66/100 (66)	LOQ - 21		0.3/0.8	IAC	LC-FD	96
Cameroon	AFM_1	mature	FQ[g]	3/62 (5)	5−625		NI[c]	LLE[h]	LC-FD	97
Chile	OTA	All	FQ	27/37 (73)	LOD - 186	86 ± 59[i]	10/30	LLE	LC-FD	46
Chile	OTA	colostrum	NI	9/9 (100)	44−184	106 ± 45	10/30	LLE	LC-FD	98
Chile	OTα[j]	colostrum	NI	9/9 (100)	LOQ - 100	40 ± 30	20/40	LLE	LC-FD	98
Columbia	AFM_1	NI	FQ	45/50 (90)	LOD - 19	5.2 ± 2.1	1/2	IAC	LC-FD	88
Egypt	AFB_1[k]	mature	NI	24/50 (48)	NI	1900 ± 600	NI	SkM[l]	ELISA[m]	47
Egypt	AFM_1	all	FQ	138/388 (36)	6−5131		4/NI	SPE[n]	LC-FD	48
Egypt	AFM_1	NI	FQ	248/443 (56)	6−497[o]		4/NI	SPE	LC-FD	99
Egypt	AFM_1	NI	FQ	248/443 (56)	LOD − 108[p]		4/NI	SPE	LC-FD	99
Egypt	AFM_1	mature	NI	98/150 (65)	200−19000	7100 ± 500	NI	SkM	ELISA	100
Egypt	AFM_1	all	FQ	87/125 (70)	7−328	74 ± 7	NI	SkM	ELISA	101
Egypt	OTA	mature	NI	36/50 (72)	NI	1890 ± 980	NI	SkM	ELISA	102
Germany	OTA	all	FQ	45/90 (50)	LOD - 100		10/30	LLE	LC-MS[q]	55
Iran	AFM_1	NI	FQ	157/160 (98)	LOD - 27	8 ± 5	5/NI	SkM	ELISA	103
Iran	AFM_1	NI	FQ	8/132 (6)	7−10	9.5 ± 1.5	5/NI	SkM	ELISA	104
Iran	AFM_1	mature	FQ	20/182 (11)	LOD - 8	7 ± 1	5/NI	SkM	ELISA	105
Iran	AFM_1	NI	FQ	85/85 (100)	2−10	5.9 ± 2	NI	SkM	ELISA	106
Iran	AFM_1	mature	FQ	1/80 (1)	6.8		5/NI	SkM	ELISA	107
Iran	AFM_1	NI	NI	24/87 (28)	0.13−5	0.56 ± 1.23	NI	SkM	ELISA	108
Iran	AFM_1	mature	FQ	1/136 (1)	20		NI	LLE	LC-FD	109
Iran	OTA	mature	FQ	2/136 (3)	90−140		NI	LLE	LC-FD	109
Iran	OTA	NI	NI	84/87 (97)	2−60	25 ± 14	NI	SkM	ELISA	110
Italy	AFM_1	mature	FQ	4/82 (5)	7−140	55 ± 59	3/7	IAC	LC-FD	90
Italy	OTA	mature	FQ	61/82 (74)	5−405	30 ± 67	2/5	IAC	LC-FD	90
Italy	OTA	colostrum	FQ	45/57 (79)	1−75	10 ± 16	0.5/1	IAC	LC-FD	89
Italy	ZEN[s]	mature	FQ	47/47 (100)	260−1780	1130 ± 340	60/NI	IAC	ELISA and LC-FD	53
Jordan	AFM_1	mature	NI	80/80 (100)	10−137	68 ± 5	NI	SkM	ELISA	111
Kuwait	AFM_1	NI	NI	5/12 (42)	9−15	9.7	5/NI	SkM	ELISA	112
Nigeria	AFM_1	NI	FQ	41/50 (82)	4−92		10/50	IAC	LC-FD	113
Nigeria	AFM_1	NI	NI	5/28 (18)	LOD - 4000		2000	NI	TLC[r]	114
Nigeria	AFM_1	NI	NI	17/120 (14)	2000−187000	44000	NI	LLE	LC-FD	115
Poland	OTA	colostrum	NI	5/13 (38)	6−17		5/15	IAC	LC-FD	91
Serbia	AFM_1	mature	NI	6/10 (60)	5−50		1.5/5	SkM	ELISA	116
Slovakia	OTA	mature	NI	23/76 (30)	LOQ - 60		5/14	LLE, IAC	LC-FD	117
Sudan	AFM_1	NI	NI	51/94 (54)	LOD - 2561	401 ± 525	13/NI	LLE	LC-FD	118
Tanzania	FB_1[t]	colostrum/ transitory	NI	58/131 (44)	6570−47105		5500/19500	LLE, SAX[u]	LC-FD	51
Tanzania	AFM_1	all	FQ	143/143 (100)	10−550		5/NI		LC-FD	119
Turkey	AFB_1	NI	NI	75/75 (100)	95−4123		5/NI	LLE	LC-FD	120
Turkey	AFM_1	NI	NI	75/75 (100)	61−300		5/NI	LLE	LC-FD	120
Turkey	AFM_1	NI	FQ	18/73 (25)	1−6	3 ± 1.4	10/NI	SkM	ELISA	121
Turkey	AFM_1	NI	NI	8/61 (13)	5−7	5.7 ± 0.6	NI	IAC	LC-FD	122
Turkey	AFM_1	mature		9/70 (13)		5730 ± 740			ELISA	123
Turkey	OTA	mature		34/70 (49)		140 ± 30			ELISA	123
Turkey	OTA	NI	NI	75/75 (100)	620−13111	>1500	10/NI	LLE	LC-FD	124

[a]LOD, limit of detection; LOQ, limit of quantification. [b]Aflatoxin M_1. [c]No information provided. [d]Immuno-affinity cartridge. [e]Liquid chromatography with fluorescence detector. [f]Ochratoxin A. [g]Food questionnaire. [h]Liquid−liquid extraction. [i]In colostrum. [j]Ochratoxin α. [k]Aflatoxin B_1. [l]SkM, skimmed milk. Centrifugation and analysis of aqueous phase. [m]Enzyme-linked immunosorbent assay. [n]Solid phase extraction. [o]Summer month. [p]Winter month. [q]Liquid chromatography mass spectrometry. [r]Thin layer chromatography. [s]Zearalenone. [t]Fumonisin B_1. [u]Strong anion exchange.

demonstrated accuracy of the analytical methodology. This remains a concern, particularly if inaccurate data discourage breastfeeding of infants. Here, we propose that more accurate tools to better understand patterns, levels, and frequency of contamination are required.

CURRENT STATE OF THE ART

Lactational Transfer of Mycotoxins. There are significant knowledge gaps regarding the uptake and transfer of mycotoxins and their metabolites to breast milk. The transfer of mycotoxins

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from food to breast milk may depend on dietary diversity and maternal hydration, physicochemical properties of the toxicant for both uptake and distribution, frequency of infant feeding, and probably breast infections that may occur as a consequence of breast milk production and feeding. However, our knowledge of these processes in humans is scarce or nonexistent with respect to mycotoxins. The few studies that have been conducted to determine lactational transfer rates for AFs and OTA from blood to breast milk were previously reviewed by Degen et al.[45] and may vary through different stages of breastfeeding. Muñoz et al.[46] demonstrated that milk to blood plasma (M/P) ratios for OTA changed from 0.40 ± 0.26 during the first days (colostrum) to 0.15 ± 0.26 in the later stages (15 days−four months). For AFM_1, a M/P ratio of 0.21 was derived from levels in milk and blood of nursing Egyptian mothers,[47] though AFM_1 levels in plasma tend to be transient and thus less informative than OTA. It was also shown that AFM_1 concentrations in breast milk are higher at the early stages of lactation.[45,48] Moreover, estimates of the percentage of aflatoxins in the diet excreted as AFM_1 in breast milk ranged from 0.09 to 0.43% in a study from The Gambia.[49] It should also be noted that besides the major metabolite, AFM_1, the parent toxin AFB_1 as well as other AFs and their metabolites may be present in breast milk, although no transfer rates are published to the best of our knowledge.

Since knowledge on transfer rates in humans is limited, the carry-over from feed to milk in dairy cattle can give a rough approximation of what to expect.[45] However, it is important to consider that (a) bacterial conversion in ruminants is different from that in monogastric species and that this may heavily impact the disposition of a given mycotoxin and that (b) the milk production rates/kg bw in dairy herds far exceeds that of a breastfeeding mother, significantly modifying the distribution of systemically available toxicants. With those caveats, in brief, for FB_1, DON, and NIV, very low carryover to dairy milk is described (<0.1%) and is sometimes nondetectable, whereas about 0.7% of ZEN and its metabolites are transferred. Carryover of AFB_1 as AFM_1 is reported at about 2−6% and of OTA and metabolites at about 6%.[50] However, new reported data on FB_1 concentrations in human breast milk (max. 471 ng/mL)[51] obtained from Tanzanian mothers raises significant concern. This observation may reflect (i) very high transfer rates in humans that would be counter to the physicochemical properties of FB_1, (ii) extraordinary high FB_1 exposure in the mothers' diet, or (iii) issues around peak identification or quantification in the HPLC-FD methodology with FB_1-conjugates. The physicochemical properties of FBs mean that they are poorly taken up from the gut of humans[10] and at least in dairy herds have suggested transfers to milk of only up to 0.05%.[52]

Clearly data on transfer rates are limited on the families of mycotoxins of public health concern. In addition to lactational transfer rates, the knowledge on potential correlations between mycotoxin concentrations in breast milk and in the urine of both the mother and/or the infant would be useful. Sample collection of infants' urine could enable a combined exposure assessment for both the infant and the mother where contributions from complementary food may also be relevant. Where exclusive breastfeeding is conducted, it could be argued that breast milk measures may be easier and more informative of infant exposures compared to estimates from urinary biomeasures of the infant. For the latter, clear dose−response relationships between infant exposure and the urinary measure are lacking, compared to the direct measure from breast milk. More research efforts are required for correlating maternal dietary exposure, blood plasma, and breast milk levels in a proper way utilizing advanced analytical methodologies and robust experimental designs including proper sampling procedures[44] and longitudinal data collection.

Single Toxin Analysis. In order to assess putative risks related to mycotoxin exposure of infants via breast milk, many groups have established analytical methods to determine AFs and OTA in breast milk, starting in the 1980s,[9] with more recent contributions for FB_1[51] and ZEN.[53,54] The current state of the art is still the monitoring of single or a single class of mycotoxins (e.g., different AFs) in human breast milk.[45] There is particular concern where some of these methods lack the specificity of LC-MS identification of analytes but are reporting high levels of toxins in breast milk, with known low uptake and transfer rates,[50] as recently reported for FBs.[51] To date, around 98% of all data generated do not involve the specificity of the LC-MS techniques in primary measurements or as a confirmatory tool for data generated by more rapid approaches that can remain useful in primary screening. An overview of methods, case studies, and the analytical methodologies employed in studies published during the last 10 years is presented in Table 1.

Most of the generated data reported concentrations of AFM_1 and OTA in breast milk. When looking at the geographical variation, especially for AFM_1, exposure is apparently very low or negligible in most European countries (low risk countries), whereas critical concentrations can be reached in sub-Saharan Africa and other tropical or subtropical regions as well as in Egypt and Turkey. OTA shows a wide range of concentrations in samples from various countries, with more than 100-fold higher levels in breast milk from Egypt, Turkey, and Sierra Leone than in European or South American countries (Table 1). The extremely limited data on Fusarium toxins FB_1 and ZEN do not allow for a conclusion regarding breast milk contamination.

Analytical Methods and Sample Preparation Approaches. Published methods used to detect mycotoxins after sample preparation are mainly based on liquid chromatography coupled to a fluorescence detector (LC-FD) or by enzyme-linked immunosorbent assays (ELISA). These methods enabled very low detection limits even before the advent of highly sensitive triple quadrupole mass spectrometers during the last 15 years. However, published LC-FD methods may require tedious sample preparation, some with derivatization steps, while the disadvantage of ELISA methods is the potential cross-reactivity with metabolites of the target compounds or with matrix components. LC-MS/MS based methods have been introduced in this field just recently.[55] Liquid−liquid extraction (LLE), immune-affinity cartridges (IAC), and solid phase extraction (SPE) are the most widely used cleanup procedures that have been applied in the past (see Table 1). Moreover, when formation of phase-II metabolites (i.e., glucuronides and sulfates) is expected, an enzymatic hydrolysis with β-glucuronidase/arylsulfatase prior to sample cleanup is recommended (unless reference compounds for all conjugated forms are available) to cover both conjugated and parent forms of a mycotoxin.

Toward Multianalyte Methods. Development of multi-mycotoxin analytical tools has been a priority in recent years to better capture potential mixtures in a single assay. Approaches include extraction of toxins from different matrices, e.g., various foodstuffs, feed, and biological fluids.[5,20,34,56] Such multimethods are also available for measuring mycotoxins in cow's milk.[57,58] However, only very few methods measuring more than one mycotoxin species in parallel in human breast milk have been

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reported to date. Andrade et al.[59] optimized a method analyzing AFB_1, B_2, G_1, G_2, M_1, and OTA by LC-FD with recoveries from 73 to 100% and limits of quantification (LOQ) ranging from 5 to 30 ng/L. Human breast milk samples from Brazil were tested with only 2 out of 224 samples positive for AFB_2, the toxin with the lowest LOQ in this assay.[59] A screening method using LC coupled to high resolution mass spectrometry (LC-HRMS) was reported by Rubert et al.[60] Thirty-five human mature milk samples (i.e., 30 days after birth) from Spain were analyzed in a pilot survey. ZEN was found in 37% of the samples in concentrations ranging from 2−14 ng/mL; its metabolites (α- and β-zearalenol) were only found in one sample in which the parent compound was not detected.[60] Of concern was the fact that some other toxins were reported at surprisingly high concentrations (HT-2 toxin, neosolaniol, enniatins, and nivalenol (NIV)). From a biological point of view, these findings remain controversial as T2/HT-2 toxin (produced mainly by *Fusarium sporotrichioides* and *F. langsethiae*) is typically not a frequent contaminant in Spanish food and undergoes fast and abundant metabolism in humans.[61] This group used an LC-HRMS approach employing Orbitrap technology and, in contrast to many other surveys in Europe (see Table 1), did not detect OTA in any sample. Also, the reported[60] high concentrations of ZEN (2−14 ng/mL) require urgent confirmation by more targeted methods which have been cross-validated between different laboratories.

FUTURE PERSPECTIVES AND RESEARCH NEEDS

Targeted and Untargeted Multianalyte Methods and Inclusion of Key Metabolites. A clear trend toward the development and application of multianalyte methods with high end LC-MS instruments is obvious in the area of food and feed safety[62−67] and, more specifically, in the field of human biomonitoring.[31,35,56,68] We propose that this trend will also take off in the analysis for mycotoxins in human breast milk utilizing advanced LC-MS/MS and LC-HRMS instrumentation. Besides the regulated, major mycotoxins, this will be particularly true for representatives of the group of so-called "emerging mycotoxins" such as beauvericin, the enniatins, aurofusarin, or *Alternaria* toxins. Also other toxins which have received little or no attention in breast milk so far (e.g., nivalenol or citrinin) will be of importance. Citrinin and its main metabolite, for example, were frequently detected in urine samples from Germany and Bangladesh recently.[69,70] Yet, the main sources of exposure are unknown and may differ between countries. Moreover, multimethods may additionally allow evaluating the occurrence and metabolism of key metabolites. The chemical or biochemical synthesis of glucuronide and/or sulfate conjugates of ZEN,[71−73] zearalenols,[71,74] DON,[75−78] or T-2/HT-2[76,79] enabled already the development of targeted analytical methods and subsequently a better insight into the metabolism of mycotoxins in *in vitro* studies[80−83] and biomonitoring in human urine.[28,29,31,32,84−87] Integration of these metabolites into multianalyte methods for the analysis of breast milk may aid in a better understanding of exposure and metabolite pattern. Also, other (commercially available) mycotoxin metabolites which may be of relevance in breast milk, such as aflatoxicol, ochratoxin-α, dihydrocitrinone, α- and β-zearalenol, and deepoxy-DON, should be preliminarily evaluated to inform if any of those might occur in relevant concentrations. Furthermore, co-occurring secondary metabolites which might not be toxic per se but may potentially modulate the toxicokinetics of a mycotoxin by, e.g., competing for detoxification cosubstrates could be included in these multimethods.

Here, we suggest that for routine surveillance fast and simple high throughput approaches such as ELISA or lateral flow devices (once validated for breast milk) are valuable but also that highly accurate LC-MS/MS measurements will be valuable tools to support such biomonitoring approaches. While LC-MS/MS offers great promise in terms of specificity and accuracy of analyte identification, with additional capabilities of multianalyte and metabolite quantification, there are still outstanding issues. To date, often highly specific but expensive IAC cleanup was required in order to reach acceptable sensitivity.[88−91] These antibody-based sample preparation approaches can be discriminative when it comes to metabolic products. For example, several IACs were reported to not quantitatively retain glucuronides of DON and ZEN.[35] When using the latest state-of-the-art mass spectrometers, it seems even realistic to omit time and cost intensive sample preparation and just "defat and dilute" the breast milk samples. This would not only speed up analysis time but also avoid the loss of metabolites during the cleanup process. This will be important once high resolution mass spectrometers (HR-MS) find their way into this field of research as a routine technique. It is speculated that during the next decade more laboratories will utilize HR-MS instruments such as TOFs or Orbitraps in the full scan mode for biomonitoring studies instead of triple quadrupole instruments which are the leading type in the field currently. This is caused by the increasing sensitivity of new HR-MS instruments, their superb mass accuracy, and the great advantage that the acquired data can always be evaluated on new compounds retrospectively. This approach is already applied in other areas of food safety,[62−64,66] and we believe that the number of HR-MS applications in the biomarker/food safety arena will continuously increase.

Analytical Quality Issues. Breast milk is a complex biological matrix, and it is part of the nature of any multianalyte method to accept compromises during sample extraction and cleanup, chromatographic separation, and setting ionization and detector parameters. Furthermore, the way and timing of sample collection will greatly influence contaminant levels in breast milk. To overcome analytical issues and ensure the generation of comparable, high-quality data sets, the following analytical needs have been identified: (1) Stability testing of analytes in breast milk and robust sampling procedures including proper documentation will be of crucial importance. Milk can be obtained either by a mechanic or electric pump (complete breast-milk expression) or by hand expression. The removal of all milk from the breasts at one time point utilizing a pump has been the preferred method in several studies.[44] While this gives no information about the milk which the baby would have consumed, this approach supports uniform sampling as the condition of the milk changes during one breastfeed. As the fat content is typically much higher during a later time point (hind milk), this might have an effect on the contaminant concentration according to its lipophilicity. Large variations in fat content might also considerably influence matrix effects during electrospray ionization. For exposure assessment, it is suggested to collect several spot samples over a time of, e.g., 24 h or even beyond and combine to pooled samples. In addition, transport and storage conditions of breast milk should be considered and reported accordingly. We are aware of course that this ideal desired collection may not always be possible in many situations, especially for low-income populations and because of cultural and nutritional issues.

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(2) The establishment and thorough validation of robust sample preparation protocols minimizing both analyte losses during extraction and cleanup as well as matrix effects. Priority should be given to reduce the contamination of the chromatographic column and the mass spectrometer interface in order to ensure constant performance throughout long sequences and to reduce the cost of maintenance. Innovative concepts such as stable isotope dilution assays (SIDA), multitoxin IACs, QuEChERS, or new SPE materials should be considered and evaluated carefully. It is important to keep in mind that the composition of the milk varies through different stages of breastfeeding (i.e., colostrum versus mature milk) and also within a specific feed (fore milk vs hind milk). Consequently, proper validation should include an in-depth evaluation of possible effects caused by the varying matrix on the methods' performance.

(3) For accurate quantification, especially in challenging biological matrices, the use of suitable, ideally isotope-labeled (^{2}H or ^{13}C), internal standards is highly recommended. In recent years, ^{13}C-labeled reference standards have become commercially available for all regulated mycotoxins. However, only for very few key metabolites (e.g., AFM_1) internal standards are available. It is our hope that many of these metabolite standards will become routinely available. Also, recent achievements in the synthesis of nonlabeled reference standards of phase II metabolites should be continued. It is likely that some of these conjugates such as ZEN-14-glucuronide also occur in breast milk in the case of maternal exposure to the parent toxin and thus could aid in the characterization of infant exposure.

(4) Lastly, the critical evaluation of analytical performance parameters of single as well as multianalyte methods using well-defined reference materials and interlaboratory comparisons will be of high relevance. Yet, these initiatives are not established in the mycotoxin biomonitoring field in general. Only one study comparing two multibiomarker and two single biomarker methods in human urine was published by Solfrizzo et al.[92] The results showed feasible z-score values for most analytes but also highlighted several issues leading the authors to the recommendation of further studies in this area.

Biomonitoring and Toxicological Aspects. There is limited information on either the pattern of mycotoxins or their metabolites that will occur in breast milk. Even for the AFs, the class for which by far most data is available, little is known besides the classical representatives AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1. However, even if a toxicological relevance of other metabolites such as aflatoxicol or sterigmatocystin seems unlikely, an evaluation of their potential impact and other co-occurring mycotoxins through plausible toxicological pathways should be considered. Hence, we recommend investigating typical patterns of mycotoxins and key metabolites beyond AFs and OTA in different populations (e.g., West African vs European vs Asian) and mycotoxin susceptible subgroups such as vegetarians. In parallel, it will be crucial to investigate mycotoxin exposure in infants who are not breastfed and thus consume postweaning food which is likely to result in far higher contaminant exposures as compared to exclusively breastfed infants.[45]

Once the proposed multibiomarker methods for breast milk are established, we further suggest their application to correlate maternal dietary exposure to plasma and breast milk levels. To date, such dose−response relationships remain basically unexplored. Furthermore, innovative biomonitoring in breast milk alongside urine and/or plasma analysis may support evaluation of intervention strategies to reduce the mycotoxin burden in the most exposed rural settings in Africa or Asia.

When looking at the potential impact of mycotoxins and their metabolites in breast milk, several aspects require attention. First, an improved characterization of the mycotoxin patterns and levels in breast milk samples from various continents is required. From what is known already from food contaminant analysis, it is predicted that the pattern and levels will vary considerably also in human breast milk samples from different countries. Once such information is available, it could provide some guidance for studies focusing on combined effects of naturally occurring mixtures.[93−95]

■ CONCLUDING REMARKS

To date, we are not aware of any reported adverse health outcome related to maternal transfer of mycotoxins to infants, though some reported levels would exceed recommended TDIs for some toxins. However, it remains important that potential sources of contamination are measured and evaluated accurately. There is no doubt that breast milk is the ideal initial diet for infants from both nutrition and immune standpoints. This may be particularly true for populations in economically less developed regions where alternatives will lack essential nutrients found in breast milk; and importantly, these breast milk alternatives may be less hygienic, contain fewer non-nutrient beneficial components, and likely be contaminated with mycotoxins at significantly higher levels than breast milk. To date, little is known about the pattern of mycotoxins and their metabolites in breast milk as well as lactational transfer rates or potential combinatory effects. In this perspective, we propose that recent advances in mass spectrometry and the toxicological evaluation of contaminant mixtures will shed light on these issues. Such data will improve risk communication and inform policy makers. It is hoped that this outline of anticipated research activities will support the scientific community to aid those who are most susceptible toward the negative effects of mycotoxins and other contaminants, and where encouragement of methods to protect mothers and infants is crucial.

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Notes

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Paul C. Turner, Ph.D. is an Assistant Professor at the University of Maryland, College Park. He is an applied environmental toxicologist involved in developing programs and tools to assess and intervene in environmental causes of disease. His focus is the development and use of exposure biomarkers for mycotoxins, with a global research portfolio including parts of Africa, South America, Asia, and Europe. Dr. Turner has >50 publications, including ground breaking research from West Africa demonstrating links between early life exposure to aflatoxin and stunting. Recent initiatives seek better understanding of the relationship between infant growth, mycotoxin exposure, nutrition, and hygiene to inform and develop targeted interventions.

Dr. Gisela H. Degen, trained as a Food Chemist, graduated in Toxicology (Ph.D.) at the University of Würzburg, Germany, then worked as Postdoctoral Fellow at the NIEHS (USA) in the Laboratories of Reproductive and Developmental Toxicology. Back in Germany, she completed her "Habilitation", received formal admission as lecturer in 1990, and in 1997 the title apl. Prof. in Biochemical Toxicology. She has been Head of Research Unit "Chemical Risks" at Leibniz-Institut für Arbeitsforschung an der TU Dortmund since 1992 and is now retired. Dr. Degen is the author of numerous original research papers and some reviews or book chapters on mycotoxins and other topics.

Doris Marko is Professor for Food Chemistry and head of the Department of Food Chemistry and Toxicology at the University of Vienna, Austria. Educated in food chemistry and toxicology, she is accredited as Registered European Toxicologist (EUROTOX) serving in several national and international commissions on food safety. Doris Marko is known for innovative studies on molecular mechanisms of food constituents. She has >120 publications including ground-breaking studies on genotoxic mechanisms of emerging mycotoxins. In interdisciplinary approaches combining food science, state-of-the art analytics and toxicology, current studies aim to unravel combinatory effects of naturally occurring mycotoxin mixtures on mammalian cells.

■ ABBREVIATIONS

AFs, aflatoxins; DON, deoxynivalenol; ELISA, enzyme-linked immunosorbent assay; FAO, Food and Agriculture Organization; FBs, fumonisins; HPLC-FD, high pressure liquid chromatography coupled to a fluorescence detector; IAC, immune-affinity cartridges; LC-FD, liquid chromatography coupled to a fluorescence detector; LC-HRMS, liquid chromatography coupled to high resolution mass spectrometry; LCL, lower calibration limit; LC-MS/MS, liquid chromatography tandem mass spectrometry; LC-MS, liquid chromatography−mass spectrometry; LLE, liquid−liquid extraction; LOD, limits of detection; LOQ, limits of quantification; M/P, milk to plasma; ND, not detected; NIV, nivalenol; OTA, ochratoxin A; QuEChERS, quick, easy, cheap, effective, rugged and safe; SAX, strong anion exchange; SIDA, stable isotope dilution assay; SPE, solid phase extraction; TLC, thin layer chromatography; TOF, time-of-flight; ZEL, zearalenol; ZEN, zearalenone

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