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TRENDS

LC-MS/MS-based multibiomarker approaches for the assessment of human exposure to mycotoxins

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Abstract Mycotoxins are toxic fungal secondary metabolites that frequently contaminate food and feed worldwide, and hence represent a major hazard for food and feed safety. To estimate human exposure arising from contaminated food, so-called biomarker approaches have been developed as a complementary biomonitoring tool besides traditional food analysis. The first methods based on radioimmunoassays and enzyme-linked immunosorbent assays as well as on liquid chromatography were developed in the late 1980s and early 1990s for the carcinogenic aflatoxins and in the last two decades further tailor-made methods for some major mycotoxins have been published. Since 2010, there has been a clear trend towards the development and application of multianalyte methods based on liquid chromatography–electrospray ionization tandem mass spectrometry for assessment of mycotoxin exposure made possible by the increased sensitivity and selectivity of modern mass spectrometry instrumentation and sophisticated sample cleanup approaches. With use of these advanced methods, traces of mycotoxins and relevant breakdown and conjugation products can be quantified simultaneously in human urine as so-called biomarkers and can be used to precisely describe the real exposure, toxicokinetics, and bioavailability of the toxins present. In this article, a short overview and comparison of published multibiomarker methods focusing on the determination of mycotoxins and relevant excretion products in human urine is presented. Special attention is paid to the main challenges when analyzing these toxic food contaminants in urine, i.e., very low analyte concentrations, appropriate sample preparation, matrix effects, and a lack of authentic, NMR-confirmed calibrants and reference materials. Finally, the progress in human exposure assessment studies facilitated by these analytical methods is described and an outlook on probable developments and possibilities is presented.

Keywords Liquid chromatography–tandem mass spectrometry · Mycotoxin · Biomarker · Exposure assessment · Human urine · Glucuronide conjugate

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Introduction

Toxic fungal secondary metabolites, so-called mycotoxins, are a global hazard for food safety by frequently contaminating food and feed. To estimate the risk of exposed populations, traditional exposure assessment comprises the analysis of foodstuff and evaluation of dietary recalls or the estimation of average consumption patterns. To overcome the disadvantages of this indirect approach, such as a lack of information on individual exposure, toxicokinetics, and bioavailability, biomarker approaches were developed as a biomonitoring tool for some major mycotoxins (Fig. 1). Baldwin et al. [1] reviewed biomarker research for the commercially most important mycotoxins and defined biomarkers as measurable biochemical or molecular indicators of either exposure (exposure biomarker) or biological response (effect biomarker) to a mycotoxin that can be specifically linked to the proximate cause. Typical biomarkers of exposure are the parent toxins themselves, protein or DNA adducts, and/or major phase I or phase II metabolites (e.g. glucuronide conjugates), which are measured in biological fluids such as urine or plasma/serum, and are related to the actual intake of the toxin through contaminated food. In an excellent review, the role of biomarkers in the evaluation of human health concerns caused by mycotoxins was published recently. Here a biomarker of exposure was defined as a biological measure which is correlated with the quantity of the xenobiotic ingested, resulting in improved

exposure classification over more traditional approaches [2]. It was highlighted that validation of such a biomarker requires demonstration of (a) assay robustness, (b) intake versus biomarker level, and (c) stability of stored samples.

Biomarker research for human exposure assessment entered the mycotoxin research arena in the late 1980s and early 1990s when extensive studies on the carcinogenic aflatoxins were conducted [3–5]. They have been essential for the establishment of the etiologic role of aflatoxins in human disease through better estimates of exposure, expanded knowledge of the mechanisms of disease pathogenesis, and as tools for implementing and evaluating preventive interventions [5]. Three aflatoxin biomarkers were validated by the establishment of a dose–response relationship: in urine the level of the hydroxylated metabolite aflatoxin M_1 (AFM_1) was between 1.2 and 2.2 % of that of ingested aflatoxin B_1 (AFB_1) [6], while the level of the aflatoxin–N^7-guanine adduct ranged from 0.05 to 3.25 μg/L, with approximately 0.2 % of ingested AFB_1 excreted during a 3-day period [7]. AFM_1 was analyzed by a competitive direct enzyme-linked immunosorbent assay (ELISA) whereas aflatoxin–N^7-guanine was measured by high-performance liquid chromatography (HPLC) following elution from an antibody affinity column. In serum the aflatoxin–lysine adduct can be obtained through digestion of the aflatoxin–albumin adduct [8]. Later in the 1990s work on ochratoxin A (OTA) [9] and the fumonisins [10] was conducted mainly based on HPLC with fluorescence detection. However, occasionally radioimmunoassays, ELISA, and liquid chromatography–tandem mass spectrometry (LC-MS/MS) have been applied as well. Excretion of fumonisin B_1 (FB_1) in urine was recently estimated to be on average 0.075 % of the FB_1 intake in South African women (n=22) [11], whereas the estimates were significantly higher (0.5 %) in a US study (n=8) [12]. Despite this very low excretion rate and issues associated with interindividual variability and rapid clearance, urinary FB_1 was recommended as a valuable biomarker for fumonisin exposure and risk assessment. Most fumonisin biomarker research conducted within the last two decades was related to the inhibition of the sphinganine *N*-acetyltransferase (ceramide synthase) and subsequent sphingolipid biosynthesis disruption initiated by fumonisins. A correlation between fumonisin intake and the sphinganine-to-sphingosine ratio or an elevated sphinganine level was found to be useful in animals but not in humans and constitutes a typical biomarker of effect [10]. The first biomarker research on the trichothecene deoxynivalenol (DON, vomitoxin) was initiated in 2003 when Meky et al. [13] developed an LC-MS-based assay to measure the sum of free DON and DON glucuronides (DON-GlcAs) combined after enzymatic hydrolysis and use of an immunoaffinity column (IAC) as a sum parameter in human and rat urine. Further LC-MS/MS methods were developed for the determination of DON and DON-GlcA using either a synthetically produced authentic reference standard [14] or the hypothetical mass [15] for the detection of the glucuronide(s). A major limitation of proper exposure assessment including ideally all relevant mycotoxins and their biotransformation products was the lack of sufficient sensitivity and selectivity.

As a result of the advent of the latest generation of high-performance LC-MS/MS instruments, a clear trend towards the development and application of multianalyte methods in mycotoxin biomarker research can be observed. Purification of the analytes is often achieved by using sophisticated sample cleanup approaches with subsequent separation by liquid chromatography and detection using triple-quadrupole analyzers coupled via an electrospray ionization (ESI) interface. However, the latest studies have also successfully applied the so-called dilute and shoot approach by omitting any cleanup

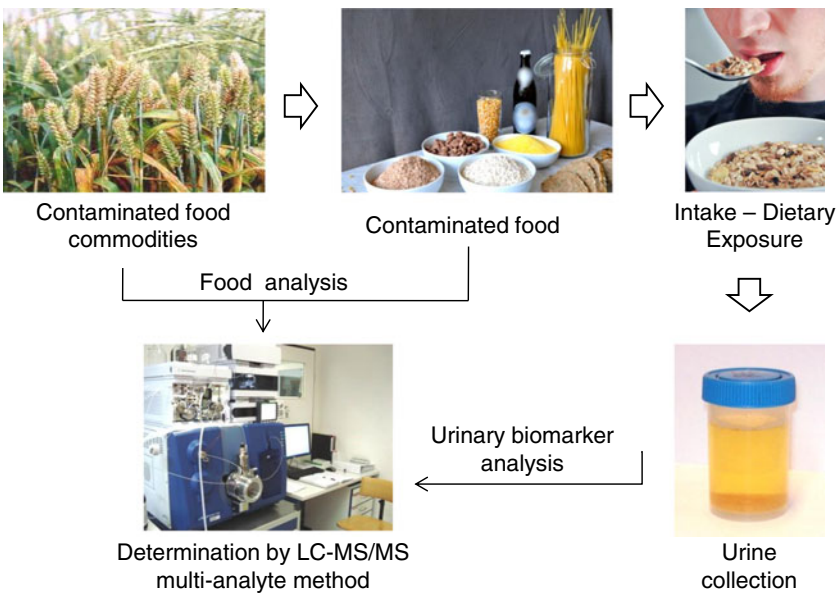

Fig. 1 Mycotoxin exposure assessment: traditional food analysis compared with the innovative, complementary biomarker approach

step [16]. This article provides a short overview and comparison of published multibiomarker methods, discusses challenges associated with very low analyte concentrations, sample preparation, matrix effects, and a lack of calibrants and certified reference materials, and describes the progress in human exposure assessment studies facilitated by these methods.

LC-MS/MS-based multibiomarker methods

The first method described for the determination of various mycotoxin biomarkers in human urine was developed by Ahn et al. [17]. To achieve sufficient sensitivity and selectivity, AFM_1, OTA, FB_1, and fumonisin B_2 were concentrated using three separate IACs. The eluates were pooled, dried under a stream of nitrogen, and resolved in a mixture of acetonitrile and water . Also two other published multibiomarker methods used the selectivity of antibodies by applying a novel multi-IAC column (Myco6in1™, Vicam) which comprises antibodies specific for aflatoxins, OTA, fumonisins, DON, zearalenone (ZEN), T-2 toxin, and HT-2 toxin [18, 19]. The first method did not include AFM_1, but instead included the aflatoxins B_1, B_2, G_1 and G_2, for which no correlation with food intake had been achieved in the past [2]. In addition, no enzymatic hydrolysis was performed despite the extensive glucuronidation of DON [13] and ZEN [20] one can expect in such studies. In constrast, the method of Solfrizzo et al. [19] used β-glucuronidase-assisted hydrolysis, resulting in increased levels of the parent toxins. Besides the IAC enrichment, a second step of sample preparation using solid-phase extraction (SPE; Oasis HLB, Waters) was conducted to overcome issues associated with low DON and deepoxy-DON recoveries. The advanced clean-up procedure resulted in lower limits of detection (LODs) of this method compared with that of Rubert et al. [18] although a less sensitive mass spectrometer was used (Table 1). Our group chose a time- and cost-effective "dilute and shoot" approach for sample preparation, where the urine sample is simply diluted 1:10 with acetonitrile/water (10:90) and injected directly into the LC-MS/MS system, to facilitate the quantification of 15 analytes [16]. A chromatogram of a blank urine sample spiked with reference standards is illustrated in Fig. 2. Besides the simplification, the advantage of this workflow is the full recovery of the polar conjugates such as glucuronides which are frequently lost during sample cleanup [21]. By implementation of these key excretion metabolites in a method using authentic reference standards, it is possible to investigate the metabolism of a certain mycotoxin as successfully exemplified for DON in vitro [22] and in vivo [23, 24]. The disadvantage of the dilute and shoot approach is the prerequisite of the latest state-of-the art triple-quadrupole mass analyzer to achieve the very low LODs required. Even when these highly advanced instruments are used, it is moderate to high exposure rather than very low background traces that is detectable. A method developed by Njumbe Ediage et al. [25] covers seven mycotoxins and several important conjugation and breakdown products (in total 18 analytes). Sample cleanup was optimized in a progressive procedure where urine samples were extracted with ethyl acetate/formic acid (99:1, v/v) followed by strong anion exchange (SAX) SPE cleanup of the acidified aqueous fraction. The combined extracts of the evaporated organic phase and the SAX eluate were injected into the LC-MS/MS system. Owing to the high concentration factor, the reported recovery was between approximately 45 and 100 %. In contrast to results obtained by various groups [15, 23, 26, 27], no DON-GlcA was detected in urine samples naturally contaminated with DON. This might indicate a loss of those conjugates during cleanup despite successful validation. However, this could also be because DON-3-GlcA was analyzed exclusively rather than DON-15-GlcA which was recently suggested as the human main excretion product [23]. The analytes included and the performance characteristics of the five multibiomarker methods described above are compared in Table 1. For quantitative analysis of urine samples, all methods were performed in selected reaction monitoring (SRM) mode. Methods 3 and 4 recently showed good agreement for most of the investigated analytes in a mini interlaboratory comparison [28]. Although in all the methods developed urine was the matrix of choice, there are limitations related to this approach, e.g., differing urine excretion owing to different fluid intakes. This can be overcome partially by normalization for the creatinine concentration of a urine sample. In exposure studies it is recommended to collect 24-h urine instead of first morning or spot urine samples if possible as spot samples are usually not representative of the excretion throughout a day [24]. In addition, urinary excretion mainly represents recent mycotoxin intake, whereas measurements in plasma/serum are more likely to represent long-term exposure.

Analytical challenges

Sample preparation

A major challenge in mycotoxin biomarker research are the extremely low analyte concentrations present in biological fluids following dietary exposure. Hence, appropriate sample preparation protocols are crucial to obtain acceptable LODs. This is, however, hampered by the great chemical diversity of analytes typically included in multibiomarker methods. This issue becomes even more complex once polar conjugates such as glucuronides are included as they are frequently lost during common cleanup approaches such as SPE or IAC procedures [16, 21]. The five methods presented in the previous section and in Table 1 illustrate different

Table 1 Performance characteristics of five liquid chromatography–tandem mass spectrometry (LC-MS/MS)-based multibiomarker methods developed for the determination of mycotoxins and relevant metabolites in human urine

No. of analytes	Analytes included in the method	Sample preparation and cleanup	Instrument	Total chromatographic run time (min)	Injection volume (μL)[a]	LOD range (μg/L)	Country of pilot study and no. of participants	References
4	AFM_1, OTA, FB_1, FB_2	IAC (AflaMPrep, Ochraprep, Fumonitest) + SIDA (OTA and FB_1) + β-glucuronidase	QTrap 3200 (AB Sciex)	22	50 (500)	0.001-0.045	Korea, n=12	Ahn et al. [17]
11	AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, DON, T-2, HT-2, ZEN	IAC (Myco6in1)	QTrap 3200 (AB Sciex)	20	20 (200)	0.4-10	Spain, n=27	Rubert et al. [18]
7	AFM_1, OTA, FB_1, DON, DOM-1, α-ZEL, β-ZEL	IAC (Myco6in1) + C_{18} SPE + β-glucuronidase	QTrap 2000 (AB Sciex)	38	20 (600)	0.01-2.2	Italy, n=10 South Africa, n=54	Solfrizzo et al. [19]
15	AFM_1, OTA, FB_1, FB_2, DON, DON-3-GlcA, DON-15-GlcA, DOM-1, T-2, HT-2, NIV, ZEN, ZEN-14-GlcA, α-ZEL, β-ZEL	None, "dilute and shoot"	QTrap 5500 (AB Sciex)	18	5 (0.5)	0.05-20	Austria, n=27 Cameroon, n=175 South Africa, n=54	Warth et al. [16]
18	AFM_1, AFB_1, AFB_1-N^7-Gua, OTA, OTα, 4-OH-OTA, FB_1, HFB_1, DON, DON-3-GlcA, DOM-1, T-2, HT-2, ZEN, ZEN-14-GlcA, α-ZEL, β-ZEL, CIT	Liquid–liquid extraction + SAX SPE	QqQ (Micromass Quattro Micro, Waters)	28	20 (1,000)	0.01-3.65	Belgium, n=40	Njumbe Ediage et al. [25]

AFB_1 aflatoxin B_1, *AFB_2* aflatoxin B_2, *AFG_1* aflatoxin G_1, *AFG_2* aflatoxin G_2, *AFM_1* aflatoxin M_1, *CIT* citrinin, *DOM* de-epoxy deoxynivalenol, *DON* deoxynivalenol, *FB_1* fumonisin B_1, *FB_2* fumonisin B_2, *GlcA* glucuronide, *Gua* guanine, *IAC* immunoaffinity column, *LOD* limit of detection, *NIV* nivalenol, *OTA* ochratoxin A, *OTα* ochratoxin α, QqQ triple quadrupole, *SAX* strong anion exchange, *SIDA* stable-isotope standard-dilution assay, *SPE* solid-phase extraction, *ZEN* zearalenone, *ZEL* zearalenol

[a] Values in *parentheses* represent the amount of urine injected taking the sample enrichment/dilution into account.

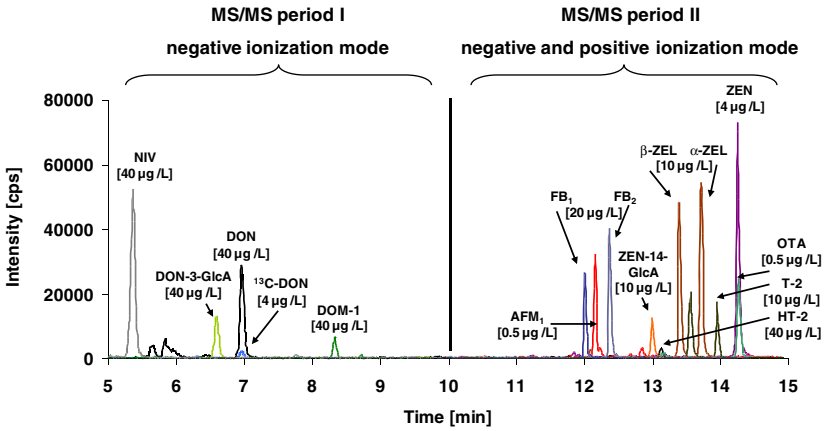

Fig. 2 Chromatogram from selected reaction monitoring (SRM) of a blank urine sample spiked with reference standards. Between 5 and 10 min, the analytes were monitored in negative ionization mode only (*period I*), whereas between 10 and 15 min both polarity modes were measured simultaneously using fast polarity switching (*period II*). *AFM*$_1$ aflatoxin M_1, *FB*$_1$ fumonisin B_1, *FB*$_2$ fumonisin B_2, *DON* deoxynivalenol, *DOM* de-epoxy deoxynivalenol, *GlcA* glucuronide, *NIV* nivalenol, *OTA* ochratoxin A, *ZEL* zearalenol, *ZEN* zearalenone. (Adapted from [16])

concepts in an excellent way. The great advantage of the methods using IAC cleanup is the specific retention of the target compounds only. Thereby, high enrichment factors are obtained without concentrating also potentially interfering matrix compounds as they are removed efficiently. The major disadvantage is the preselection of analytes by the column chosen depending on the antibodies used. Therefore, usually no conjugates or other biomarkers/analytes of interest can be included in a method. Furthermore, enzymatic hydrolysis should be performed to include conjugates, and the overall procedure is time-consuming and costly and requires a labor-intensive sample preparation. This is in contrast to the dilute and shoot approach, where a urine sample is centrifuged, diluted, and analyzed without further pretreatment. However, to overcome matrix effects and interfering matrix peaks, eluents, the chromatographic gradient, and the dilution factor need to be carefully optimized [14, 16]. Njumbe Ediage et al. [25] investigated different procedures including dilute and shoot, dilute, evaporate, and shoot, liquid–liquid extraction, and two different SPE cartridges (SAX and Oasis HLB). They concluded that the LODs obtained with SAX columns were threefold to ninefold lower compared with those obtained with Oasis HLB columns, whereas the approaches based on sample dilution yielded unfeasibly high LODs and significant signal enhancement for ZEN and FB_1. Various SPE cartridges (Oasis HLB and MAX, Sigma Supel-Select HLB, Sequant ZIC-HILIC, Bakerbond Polar Plus) have also been tested during method development of the established dilute and shoot method but failed to retain the polar glucuronide conjugates, with the exception of the Oasis HLB [16] and the ZIC-HILIC cartridges when using optimized protocols.

Matrix effects and peaks

Co-eluting matrix components can negatively influence the accuracy of quantitative methods through ion suppression or enhancement in the ion source. This is particularly true for ESI, where the competition for electrical charges or the effect on the evaporation of ESI droplets can lead to significant ion suppression [29]. Hence, it is of great importance to thoroughly investigate these effects during method development and validation. Ion suppression can be reduced efficiently by careful optimization of the eluents and gradient. However, this is not trivial and is a particular issue in multianalyte methods, where compromises are unavoidable. Matrix effects can be controlled by using matrix-matched calibration [19], inclusion of internal standards [17, 30], or correction of results with the apparent recovery [16]. However, when matrix-matched calibration or apparent recovery for the correction of results is used, it still needs to be considered that urine samples can differ in their concentration, thereby influencing matrix effects. This depends largely on the volume of drinks consumed by an individual prior to sample donation. Therefore, the blank urine which is used for preparation of matrix-matched standards or the spiked samples, respectively, needs to be chosen with the greatest care and the effect of differing urine sample concentrations should be investigated during validation.

Another major issue is the frequent co-elution of matrix compounds. This requires careful selection of SRM transitions in order to minimize background noise as well as interfering peaks that might trigger false-positive results. Descriptive examples are illustrated for an AFM_1 interference by Ahn et al. [17] and for zearalenone-14-glucuronide (ZEN-14-GlcA) in Fig. 3. During common tandem mass spectrometric compound optimization, usually the two most

abundant fragment ions are chosen as quantifier and qualifier ions, respectively. However, in challenging biomarker applications, one should consider several SRM candidates in order to select specific fragment ions. This evaluation must include the injection of spiked matrix samples to identify potential interferences and is particularly required if no proper sample cleanup was performed. This issue is visualized in Fig. 3.

Lack of authentic reference standards and certified reference materials

In the past, most biomarker methods focused on parent mycotoxins rather than on conjugated forms as no (certified) calibrants are commercially available for these metabolites. Despite this caveat, considerable progress has been achieved in the direct quantification of mycotoxin conjugates without the need for enzymatic hydrolysis. By application of this direct approach, problems such as the loss of information on the analyte's structure and its detoxification potential, but also incomplete hydrolysis and the time-consuming sample preparation can be overcome. Glucuronide conjugates have been synthesized either using chemical synthesis as in the case of DON-3-GlcA [31] and ZEN-14-GlcA [32] or by in vitro assays using liver microsomes. With use of this approach, GlcAs of DON [27, 33], ZEN and metabolites [34], and T-2 toxin and HT-2 toxin [35] were obtained in small quantities. An important quality control measure is the use of certified reference materials including well-characterized calibrants to monitor the performance of a certain laboratory. However, for mycotoxin biomarkers, i.e., mycotoxins and their conjugates, there is no matrix reference material available that would make it possible to assess the measurement performance in the analysis of biologically important matrices such as human or animal urine, plasma/serum, or feces. This is critical especially in view of the complex biological matrices and makes efforts such as a recent interlaboratory comparison [28] even more important to ensure analytical accuracy. The preliminary results obtained in this study which determined up to eight mycotoxin biomarkers in human urine showed good agreement between most analytes. The overall rate of satisfactory z scores [36] ($|z|\leq 2$) was 85 % (68 of 80 results), with unsatisfactory or questionable z scores obtained for FB_1, OTA, and α-zearalenol.

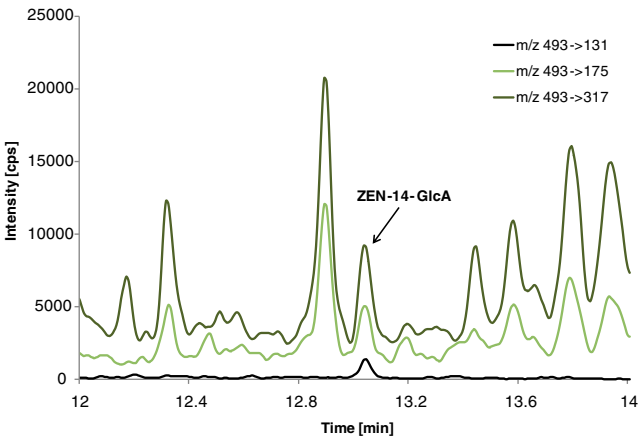

Fig. 3 SRM chromatogram of a blank urine sample spiked at a level of 12.5 µg/L ZEN-14-GlcA. It is obvious that the transition m/z 493 $[M-H]^- \rightarrow 131$ results in a far better signal-to-noise ratio than the other product ions despite its lower absolute abundance. Hence, this fragment should be chosen as a quantifier ion. In addition, the more intense transitions comprise many interferences which mimic ZEN-14-GlcA and thus potentially may lead to false-positive results

Application of LC-MS/MS methods in exposure studies

The multibiomarker methods presented have been applied in several pilot studies to prove their applicability and to estimate mycotoxin exposure in the populations/individuals tested. In general, the application of these methods resulted in advanced data on exposure patterns and revealed new findings on co-exposure to the mycotoxin combinations reported in Table 2. This is a significant advancement compared with the results presented in the only reported co-exposure study in which three separate methods based on ELISA, HPLC with fluorescence detection, and LC-MS/MS were applied to reveal exposure to aflatoxin and DON in pregnant women from Egypt [37]. An example of the relevance of the reported new exposure data is the extent of co-exposure observed in samples from Cameroonian individuals [16]. Overall, in 110 samples (63 %, $n=175$) at least one analyte was detected, with a maximum of six analytes (AFM_1, FB_1, OTA, DON, DON-15-GlcA, nivalenol) detected in a single individual simultaneously, a severe co-exposure that had never been reported before (see also Table 2). In this study additionally the first quantification of ZEN-14-GlcA and nivalenol in naturally contaminated human urine was described. In a very recent South African survey among women living in a rural, high esophageal cancer region, two different multibiomarker methods and, in addition, two single-target LC-MS/MS methods were used and indicated frequent mycotoxin co-exposure for the first time in South Africa. Furthermore, the first finding of urinary DON, ZEN, their conjugates, and OTA in this region and an advanced understanding of toxicokinetic patterns by direct determination of conjugation and hydroxylation products of DON and ZEA was achieved [38]. In an Austrian pilot survey, the structure of DON-15-GlcA was tentatively

Table 2 Human exposure to mycotoxins: results and characteristics of pilot studies conducted using the novel LC-MS/MS multibiomarker methods

Country	No. of subjects	Individuals investigated	No of positive samples (%)	Analytes detected	Co-exposure in a single individual[a]	References
Korea	12	11 adults, 1 child	12 (100 %)	AFM_1, OTA	AFM_1–OTA	Ahn et al. [17]
Spain	27	Adults	Not stated	AFG_2, OTA, DON	Not stated	Rubert et al. [18]
Italy	10	Adults	10 (100 %)	OTA, DON	OTA–DON	Solfrizzo et al. [19]
Austria	27	Adults	26 (96 %)	DON, DON-3-GlcA, DON-15-GlcA	DON–DON-3-GlcA–DON-15-GlcA	Warth et al. [23]
Cameroon	175	145 HIV-positive adults 30 HIV-negative adults	110 (63 %)	AFM_1, OTA, FB_1, FB_2, DON, DON-3-GlcA, DON-15-GlcA, NIV, ZEN, ZEN-14-GlcA, α-ZEL	AFM_1–OTA–FB_1–DON–DON-15-GlcA–NIV	Warth et al. [16]
Belgium	40	Adults	9 (23 %)	DON, OTA, OTα, 4-OH-OTA, ZEN, CIT, β-ZEL	OTA–OTα–DON–ZEN–β-ZEL	Njumbe Ediage et al. [25]
South Africa	53	Adult women	53 (100 %)	OTA, FB_1, DON, DON-3-GlcA, DON-15-GlcA, NIV, ZEN, ZEN-14-GlcA, α-ZEL, β-ZEL	OTA–FB_1–DON–DON-3-GlcA–DON-15-GlcA–ZEN–ZEN-14-GlcA–α-ZEL–β-ZEL	Shephard et al. [38] and unpublished results

[a] Only the most severe co-contamination is reported

elucidated and identified as the major conjugation product in human urine. Furthermore, it was estimated that a significant number of study participants exceeded the tolerable daily intake established for DON [23]

Outlook

The current trend of multianalyte methods in mycotoxin biomarker research will certainly continue. We expect these methods to be optimized and validated for even more challenging matrices such as feces and plasma as done for single-target methods in the past [2]. The methods developed will significantly contribute to improved exposure assessment. Thereby, they offer a new innovative and complementary way of quantifying the risks associated with mycotoxins, and will be of increasing importance besides traditional food analysis.

Driven by the increasing sensitivity of modern mass spectrometers, more detailed in vivo toxicokinetic studies will be performed directly in humans following low toxin intake via naturally contaminated food. These experiments have mainly been restricted to animals in the past because of high doses. Thereby, metabolism and detoxification routes will be discovered as recently demonstrated for DON and ZEN [24] to support advanced risk assessment. Furthermore, it is expected that more biomarkers of mycotoxin exposure will be validated using these methods by means of a dose–response relationship.

We also expect more laboratories to be involved in efforts to synthesize novel mycotoxin conjugates such as α-zearalenol glucuronide, β-zearalenol glucuronide, OTA glucuronide, and ochratoxin α glucuronide as calibrants and implement them in multianalyte methods. This includes regulated toxins but also mycotoxins which have rarely or not been addressed yet by biomarker research, such as T-2/HT-2 toxin, nivalenol, citrinin, *Alternaria* toxins, and moniliformin. The quest for new key metabolites will be supported by high-resolution mass spectrometry and increasingly sensitive triple-quadrupole analyzers.

Ultimately, the multibiomarker approach could serve in the identification of what are some of the most important mycotoxin mysteries: the role of mycotoxins in chronic disease caused by low-dose long-term background exposure through the intake of contaminated food and the toxicological risks posed by combinations of mycotoxins of frequent natural occurrence.

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