Ultra-sensitive, stable isotope assisted quantification of multiple urinary mycotoxin exposure biomarkers

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HIGHLIGHTS

- An ultra-sensitive method for urinary biomarkers of mycotoxin exposure was established.
- Simultaneous biomonitoring of regulated and emerging mycotoxins at trace levels by a single analytical method.
- First multiple stable isotope assisted quantification method for mycotoxin exposure biomarkers validated.
- Applicability in realistic chronic low dose exposure to mycotoxins in large-scale cohort.

GRAPHICAL ABSTRACT
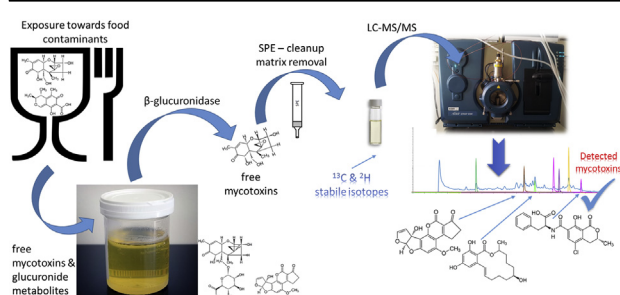
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ABSTRACT

There is a critical need to better understand the patterns, levels and combinatory effects of exposures we are facing through our diet and environment. Mycotoxin mixtures are of particular concern due to chronic low dose exposures caused by naturally contaminated food. To facilitate new insights into their role in chronic disease, mycotoxins and their metabolites are quantified in bio-fluids as biomarkers of exposure. Here, we describe a highly sensitive urinary assay based on ultra-high performance liquid chromatography - tandem mass spectrometer (UHPLC-MS/MS) and ^{13}C-labelled or deuterated internal standards covering the most relevant regulated and emerging mycotoxins. Utilizing enzymatic pre-treatment, solid phase extraction and UHPLC separation, the sensitivity of the method was significantly higher (10-160x lower LODs) than in a previously described method used for comparison purpose, and stable isotopes provided compensation for challenging matrix effects. This method was in-house validated and applied to re-assess mycotoxin exposure in urine samples obtained from Nigerian children, adolescent and adults, naturally exposed through their regular diet. Owing to the methods high sensitivity, biomarkers were detected in all samples. The mycoestrogen zearalenone was the most frequently detected contaminant (82%) but also ochratoxin A (76%), aflatoxin M$_1$ (73%) and fumonisin B$_1$ (71%) were quantified in a large share of urines. Overall, 57% of 120 urines were contaminated with both,

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Liquid chromatography tandem mass spectrometry

aflatoxin M_1 and fumonisin B_1, and other co-exposures were frequent. These results clearly demonstrate the advanced performance of the method to assess lowest background exposures (pg mL^{-1} range) using a single, highly robust assay that will allow for the systematic investigation of low dose effects on human health.

1. Introduction

Characterizing complex environmental exposures and their combined effects on toxicity and human health has become a priority recently and is often referred to in the context of the 'exposome' paradigm [1–3]. Mycotoxins are a major class of natural contaminants that humans are typically exposed to throughout their life. This chemically diverse group of toxic secondary metabolites are produced by filamentous fungi and frequently occur in our diet [4]. The patterns and concentrations largely depend on climatic conditions as well as the level of hygienic standards and economic wealth. Higher mycotoxin exposures are often evident in tropical and sub-tropical regions of third world countries [5,6], although the changing climate is also altering occurrence patterns and concentrations throughout the world [7]. The toxicities of several mycotoxins have necessitated their regulation in almost all countries world-wide [8], though less affluent regions often lack the capacity to effectively implement or control such guidelines to protect their population. The permitted concentrations of major mycotoxins in the European Union are regulated within EC 1881/2006 [9]. For some mycotoxins including the carcinogenic aflatoxins, permitted levels are typically very low(e.g. aflatoxin M_1 (AFM$_1$) in milk: 50 pg mL^{-1}). However, it is known from biomarker driven research that combined exposures to mycotoxins are common [10–13]. Moreover, there is growing scientific evidence that mixtures of co-occurring mycotoxins [14–16] and mycotoxins with other xenobiotics (bioactive food constituents, drugs etc.) [17,18] have the potential to cause an additional threat through combinatory effects that legislation does not take into account to date.

To assess multiple exposures to mycotoxins, LC-MS/MS-based methods have been successfully developed to measure the parent compound or their metabolite(s) in urine, and tested mostly in smaller pilot surveys [10,12,13,19]. However, typically they constitute a compromise on assay sensitivity compared to single-analyte methods utilizing tailored sample clean-up protocols. Published multi-mycotoxin LC-MS/MS methods include rapid and cost-effective but partially less sensitive dilute and shoot approaches [20,21], a (semi)-quantitative direct injection method [19], sample clean-up using highly specific but expensive immunoaffinity (IAC) columns [19,22] and the combination of IAC columns with solid phase extraction (SPE) columns [13] which is time- and cost intensive. Moreover, salting out assisted liquid/liquid extraction [23–25], and a combination of liquid/liquid extraction with SPE columns [26] was described. Urine is used as a non-invasive, easily obtainable material for estimation of exposure to mycotoxins. Since humans are exposed to mycotoxins mainly through diet (with some exceptions where they can be exposed also through dust [27]), the main absorption region is the small intestine and transfer to the liver [28,29]. Via their normal diet, humans are also exposed to modified ("masked") mycotoxins, which may have different adsorption patterns. The typical conjugation to sugars (mainly glucose) is making them more resistant to adsorption in the small intestine. When reaching the colon, the microbiota can hydrolyse the conjugated form [30,31], and the "parent" mycotoxin is released and may be adsorbed there, following the same route to liver by portal vein, and metabolism process. Modified mycotoxins are an issue since they are not regulated to date and not covered by most analytical methods, although they can significantly contribute to the overall mycotoxin exposure [30–32]. For some mycotoxins such as deoxynivalenol (DON), this process is highly efficient and glucuronides are the major metabolites found in urine [33]. Therefore, the measurement of parent toxins often resulted in insufficient correlations with dietary intake estimates and the direct assessment of conjugated forms [34] or pre-treatment with β-glucuronidase/arylsulfatase was suggested [35–37].

From an analytical perspective, urine is known as a challenging matrix particularly due to vast differences in composition and concentrations between individuals, which may depend on sex, age, health status, metabolism and predominantly diet [10]. For mycotoxin biomarkers urine, blood (plasma/serum), milk, and hair may be used, depending on the targeted mycotoxin, exposure timeframe, or available analytical technique [38]. Urine was used most often in the past to describe recent exposure [38], since most of mycotoxins are rapidly metabolised and excreted via urine [33]. Sample pre-treatment with beta-glucuronidase is important to reconvert conjugates of mycotoxins back to parent mycotoxins. There is limited availability of mycotoxin glucuronide standards on the market and as they are mostly synthetized in small quantities in-house, hence de-glucuronidation can help in analysing total exposures if reference standards are not available or too costly. However, for estimating total exposure both, the direct and the indirect assessment of metabolites proved their feasibility [34,39]. To effectively compensate for varying matrix effects, extraction losses and other potential issues during LC-MS/MS quantification of mycotoxins, stable isotope dilution assays are often used as the state-of-the-art technique for data quality assurance [40]. Despite its advantages, no stable isotope labelling (SIL) workflow was reported for multi-mycotoxin exposure assessment in human biofluids to date to the best of our knowledge.

Hence, the aim of this study was to develop a highly specific and robust method for multi-mycotoxin biomarker analysis using tailored sample clean-up and stable isotopologues. Its feasibility and performance to assess individual exposure levels was demonstrated in a well-defined sample collection with urines reflecting a wide range and diverse mix of mycotoxins, a set likely to cover typical ranges in large-scale epidemiological studies.

2. Materials and methods

2.1. Reagents and chemicals

Methanol (MeOH; LC gradient grade), acetonitrile (ACN; LC gradient grade), and glacial acetic acid (HAc; MS grade) were purchased from Merck (Darmstadt, Germany). Mycotoxin standards were purchased from Romer Labs Diagnostic GmbH Tulln, Austria: nivalenol (NIV), ^{13}C-NIV, deoxynivalenol (DON), ^{13}C-DON, deepoxy-DON (DOM-1), ochratoxin A (OTA), ^{13}C-OTA, aflatoxin M_1 (AFM$_1$), ^{13}C- AFM$_1$, citrinin (CIT), fumonisin B_1 (FB$_1$), ^{13}C-FB$_1$, ^{13}C-zearalenone (ZEN)) or Sigma, Vienna, Austria (ZEN, α- and β-zearalenol (ZEL)). The deuterated [^{2}H$_4$] alternariol (AOH) was synthesized in-

house [41]. Solid standard substances were dissolved in pure ACN (α- and β-ZEL; [²H₄] AOH). All other standards were delivered in either ACN or ACN/H₂O (FB₁) and stored at −20 °C. A combined multi-standard working solution for preparation of calibrants and spiking experiments was prepared in ACN containing: NIV (1500 ng mL⁻¹), DON (1500 ng mL⁻¹), DOM-1 (1500 ng mL⁻¹), FB₁ (300 ng mL⁻¹), α- and β-ZEL (300 ng mL⁻¹), ZEN (300 ng mL⁻¹), CIT (300 ng mL⁻¹), DHC (300 ng mL⁻¹), AOH (300 ng mL⁻¹), AFM₁ (30 ng mL⁻¹), OTA (30 ng mL⁻¹). Also a fresh mixture of ¹³C and deuterated [²H₄] stable isotope standards was prepared regularly containing: ¹³C-NIV (3.75 ng mL⁻¹), ¹³C-DON (3.75 ng mL⁻¹), ¹³C-OTA (0.04 ng mL⁻¹), ¹³C-AFM₁ (0.008 ng mL⁻¹), ¹³C-FB₁ (0.38 ng mL⁻¹), ¹³C-ZEN (0.38 ng mL⁻¹); and deuterated [²H₄] AOH (3.33 ng mL⁻¹).

2.2. Samples

Blank urine for spiking experiments and quality control samples was obtained from a 30-year-old male volunteer from Austria used in a previous study [33], who avoided the consumption of presumably mycotoxin contaminated foodstuffs such as cereal-based products for two days prior to 24 h urine sample collection. The blank urine sample was re-evaluated, and only traces of OTA were detected (below LOQ, 0.002 ng mL⁻¹), while the other mycotoxins were <LOD. The urine samples from Nigeria were residual aliquots, which were part of a previously published study [11], stored at −20 °C. These were re-analysed to evaluate the new method's performance and enable a comparison with previously published data, based on a dilute and shoot approach [11,21]. Ethical approval was permitted by the responsible ethical commissions (State Ministry of Health (MOH/OFF/237/VOL.1)) and written consents of all volunteers were obtained prior to urine donation.

2.3. Equipment

Method development and sample analysis was performed using a Sciex QTrap®6500+ LC-MS/MS system (Foster City, CA) equipped with a Turbo V electrospray ionization (ESI) source interfaced with an Agilent 1290 series UHPLC system (Waldbronn, Germany). For data evaluation the Analyst (version 1.6.3.) and Multiquant® 3.0.2. software programs were applied.

2.4. Sample preparation

Urine samples were allowed to reach room temperature, and centrifuged for 3 min at 5600 × g. Five hundred μL of the supernatant was incubated with 500 μL PBS (200 mM, pH = 7.4) containing 3000 U of β-glucuronidase from E. coli Type IX-A (Sigma-Aldrich, G7396-2MU) [42] for 16 h at 37 °C to allow deglucuronidation of mycotoxin-glucuronides. Following hydrolysis 1 mL was passed through Oasis PRiME HLB® SPE columns (Waters, Milford, MA), pre-equilibrated with 1 mL MeOH, and 1 mL H₂O. After washing twice with 500 μL H₂O, mycotoxins were eluted with 200 μL ACN, three times. Extracts were evaporated under a gentle stream of nitrogen at room temperature, reconstituted with 470 μL dilution solvent (10% ACN, 0.1% HAc) and fortified with 30 μL of the IS mixture.

2.5. LC-MS/MS conditions

Analytes of interest were separated on an Acquity HSS T3 column (2.1 × 100 mm; Waters, Wexford, Ireland) with 1.8 μm particle size. Eluent A was water while eluent B was ACN, both acidified with 0.1% HAc. After an initial period of 2.0 min at 90% A, the percentage of B was linearly raised to 50% until minute 15.0. Then,

eluent B was raised to 95% until min 18.0 followed by a hold-time of 4.0 min and subsequent 3 min column re-equilibration at 90% A. The flow rate was set to 100 μL min⁻¹. After injection of 10 μL the needle was washed for 20 sec to minimize carry-over. The column effluent was transferred either to the mass spectrometer (minutes 5 to 22.5) or to the waste via a six-port valve. The column was operated at 35 °C.

ESI-MS/MS was performed in scheduled multiple reaction monitoring (sMRM) mode, with a 180 sec detection windows. At least two individual transitions were monitored for each analyte. One chromatographic run consisted of two MS/MS experiments where both ionization modes run simultaneously using fast polarity switching. All measurements were conducted using: source temperature 550 °C, curtain gas 30psi (69 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 80 psi (345 kPa of nitrogen), ion source gas 2 (drying gas) 80 psi (345 kPa of nitrogen), collision gas (nitrogen) high. Ion spray voltage was −4500 V in negative mode while it was set to 4500 V in positive mode. The analyte dependent MS/MS parameters were optimized via direct infusion of reference standards and are displayed in Table S1.

2.6. Validation experiments

In-house validation was conducted following EU Commission Decision 2002/657/EC [43] with minor modifications. The parameters investigated included limit of detection (LOD), limit of quantification (LOQ), repeatability, within-laboratory reproducibility, trueness and linearity. Recovery experiments were performed by spiking the blank urines with mycotoxin standard mixture. The recovery was investigated in more detail than stipulated by using six different concentrations (1x, 1.5x, 2 x LOQ, and additionally 30x, 100x, and 300x LOQ) to be in a useful range of expected concentrations in moderate and highly exposed populations. The spike concentrations were selected according to the calibration range and the LOQ of each analyte. Since no suitable reference material was available, the trueness and selectivity was estimated by using recovery according to the EC 657/2002 directions [43]. The measurements were repeated on three days with six determinations per concentration level. The calibration curve (1/x weighted) for external calibration was generated for each mycotoxin based on at least five concentration levels. Multi-mycotoxin calibrants were obtained through dilution 1:100 (v/v) with the dilution solvent. LOD and LOQ values were calculated based on a signal to noise ratio of 3:1 and 10:1 from spiked urine chromatograms by using the Analyst® S-to-N-script. For additional confirmation, the quantifier to qualifier ion ratio was used with maximum permitted tolerances of 50% when the relative intensity of the base peak was ≤10% [43]. The relative retention time of the tested mycotoxin and the internal standards were required to be within of 0.1 min to that of the calibration solution [43]. For the mycotoxins without the ISs (DOM-1, DHC, α- and β- ZEL) all calculations were performed by using the peak area, and not the peak area ratio as for the mycotoxins for which ISs were available. For the calculation of the extraction efficiency, matrix effect (signal suppression of enhancement (SSE)) and apparent recovery (R_A) following formula were used [44]:

$$EE(\%) = \frac{average\ area\ (spiked\ samples)}{average\ area\ (matrix\ matched\ standard)} \times 100 \qquad (1)$$

$$R_A(\%) = \frac{average\ area\ (spiked\ samples)}{average\ area\ (eluent\ diluted\ standard)} \times 100 \qquad (2)$$

$$SSE(\%) = \frac{average\ area\ (matrix\ matched\ standard)}{average\ area\ (eluent\ diluted\ standard)} \times 100 \quad (3)$$

2.7. Statistical analysis

For the raw data box-plot diagrams presented in Fig. 2, and supplementary material 2, Statistica software (Dell Statistica, ver. 12, Dell Inc., Tulsa, Oklahoma, USA) was used. Excel (Microsoft, Redmond, Washington, USA) was used for the calculation of validation parameters.

3. Results and discussion

3.1. Development of clean-up protocol

Based on their broad analyte coverage allowing for potential addition of analytes in the future, we focused on SPE columns for urine clean-up rather than highly specific and cost-intensive IAC columns. During optimization Oasis® HLB and PRiME HLB [45] columns were compared for extraction efficiency (EE) and matrix reduction obtained in MS/MS chromatograms. Since the newly available PRiME HLB exhibited better EEs, S/N ratios and a faster processing time, this column type was chosen (Fig. S1). The column was tested with and without equilibration [45], however, the absence of equilibration resulted in a slower clean-up. Therefore, an equilibration step was used for subsequent sample preparation. The elution was tested with different eluents including 100% MeOH, 100% ACN, 50% MeOH/50% ACN, and acidified versions with 1% HAc. The best EE was obtained when pure ACN was used and elution was carried applying three times a volume of 200 μL. The chosen SPE columns resulted in sufficient EEs for both types of mycotoxins, polar ones such as NIV and DON, and nonpolar ones like ZEN, and OTA. A slightly lower efficiency on mid-polar range analytes such as FB₁, AOH, or α-/β- ZEL was accepted (Table 1).

3.2. Optimization of LC and MS/MS parameters

For each analyte five different MRM transitions were optimized. The two transitions with the greatest S/N ratio were selected for monitoring in the scheduled MRM mode. For DON three transitions were recorded since one was the highly sensitive but rather noisy transition m/z 355.1→59.2 used also in other studies [13,46]. The source conditions were also optimised to protect the interface and the ion path from contaminations, therefore relatively high curtain gas 1 and 2 settings were used combined with high source

temperature, and maximal distance from the source entrance. The provided blank urine was spiked with the analyte and then tested for the mycotoxin retention times and matrix associated noise. In the 'blank' urine only traces of OTA were observed, and two intense peaks interfered with AFM₁ transitions. Hence, nine different MRM transitions were tested additionally. Interestingly, an interfering peak was observed on all investigated MRM traces for AFM₁, which is a rather rare phenomenon. Therefore, the LC gradient was optimized to enable proper quantification, and the exclusion of false positive peaks. The second problem with AFM₁ interferences is that they are rarely appearing in the urines suggesting that those interferences are highly specific and coming from the diet, or due to specific individual physiological properties of the subject, and it is correlated with the specific exposome of the subject. The exact properties and source of the interferences will be investigated in future studies.

During the LC optimization MeOH and ACN were tested as eluent B. The S/N ratios were higher for most of the included components when the ACN was used. The most relevant exception of this behaviour was observed for CIT, which can be measured as a highly sensitive methanol adduct (factor of 3) that was described before [19]. Due to interferences in many AFM₁ chromatograms, a longer holding time of the aqueous eluent was required to separate the AFM₁ peak from these peaks. Moreover, a relatively low flow rate was favourable as faster flow and an eluent containing higher amounts of water caused overlap of the AFM₁ transition with the matrix interferences. Also for CIT high background noise was an issue which has been described before [44,47]. Because the obtained S/N ratio and the resulting detection limit were deemed sensitive enough, the $[M+H]^+$ ion was chosen and background noise accepted.

3.3. Stable isotope dilution assay

To the best of our knowledge, this is the first stable isotope dilution assay (SIDA) method reported for the simultaneous quantification of biomarkers in urine which can be used to assess the exposure of humans to multiple co-occurring mycotoxins. There are numerous advantages of applying IS in LC-MS/MS analysis of matrices prone to matrix effects such as urine. The main problem when analysing urine by LC-MS/MS are the severe differences in matrix composition and concentrations between individual subjects. To cope with compromised ionization caused by matrix effects, ISs can be added prior to final analysis by LC-MS/MS. The addition of IS could also be done prior to enzymatic treatment and SPE clean up to compensate for all losses during sample preparation, if the EE is low. However, since the EEs were in an

Table 1
Performance characteristics of the developed method as obtained during in-house validation.

Analyte	Calibration range [ng mL⁻¹]	Relative intensity[a]	EE	RSD intraday	RSD interday	LOD matrix [ng mL⁻¹]	LOQ matrix [ng mL⁻¹]
Nivalenol	0.015–15	5.2	91%	5%	15%	0.05	0.10
Deoxynivalenol	0.015–15	1.1	96%	5%	17%	0.05	0.15
Deepoxy-deoxynivalenol	0.015–15	0.8	89%	10%	13%	0.30	0.50
Aflatoxin M₁	0.0003–0.3	2.2	95%	7%	18%	0.0003	0.001
Fumonisin B₁	0.003–3.0	1.1	84%	20%	18%	0.001	0.01
Dihydrocitrinone	0.003–3.0	3.7	98%	6%	9%	0.003	0.01
Alternariol	0.003–3.0	1.3	70%	16%	18%	0.01	0.03
Citrinine	0.003–3.0	10.4	92%	8%	17%	0.003	0.01
β-Zearalenol	0.003–3.0	1.7	77%	11%	26%	0.001	0.003
α-Zearalenol	0.003–3.0	4.0	74%	20%	33%	0.003	0.01
Ochratoxin A	0.0003–0.3	1.8	90%	14%	19%	0.0003	0.001
Zearalenone	0.003–3.0	1.5	89%	11%	15%	0.001	0.003

EE — extraction efficiency; RSD — relative standard deviation; LOD — limit of detection; LOQ — limit of quantification.
[a] Intensity of the quantifier transition/intensity of the qualifier transition in spiked blank urine sample.

acceptable range (Table 1), the amount of expensive labeled IS was minimized by addition just before injection to the LC-MS/MS system to yield an overall price per sample which is more affordable even in large cohort studies with 1000 + samples. The overall sensitivity of the method further enabled the usage of minimal concentrations of IS to accomplish the task of affordability. According to Hewavitharana [48], matrix matched calibration and matrix effect estimation is not required when using stable isotopologues of the analytes under study as IS. Van Eeckhaut et al. [49] evaluated the matrix effects of biological fluids, and concluded that SIDA should be used in LC-MS/MS methods if feasible. An additional advantage of the method hinges on the utilisation of β-

glucuronidase pre-treatment prior the sample purification, which aids the quantification of increased amounts of parent mycotoxins. When comparing the results with other methods not employing this enzymatic hydrolysis, this fact needs to be considered since most mycotoxins undergo glucuronidation in the liver prior to urinary excretion.

3.4. In-house validation

The validation of the method was performed as recommended by EU directive 657/2002 concerning the performance of analytical methods and the interpretation of results [43]. For some
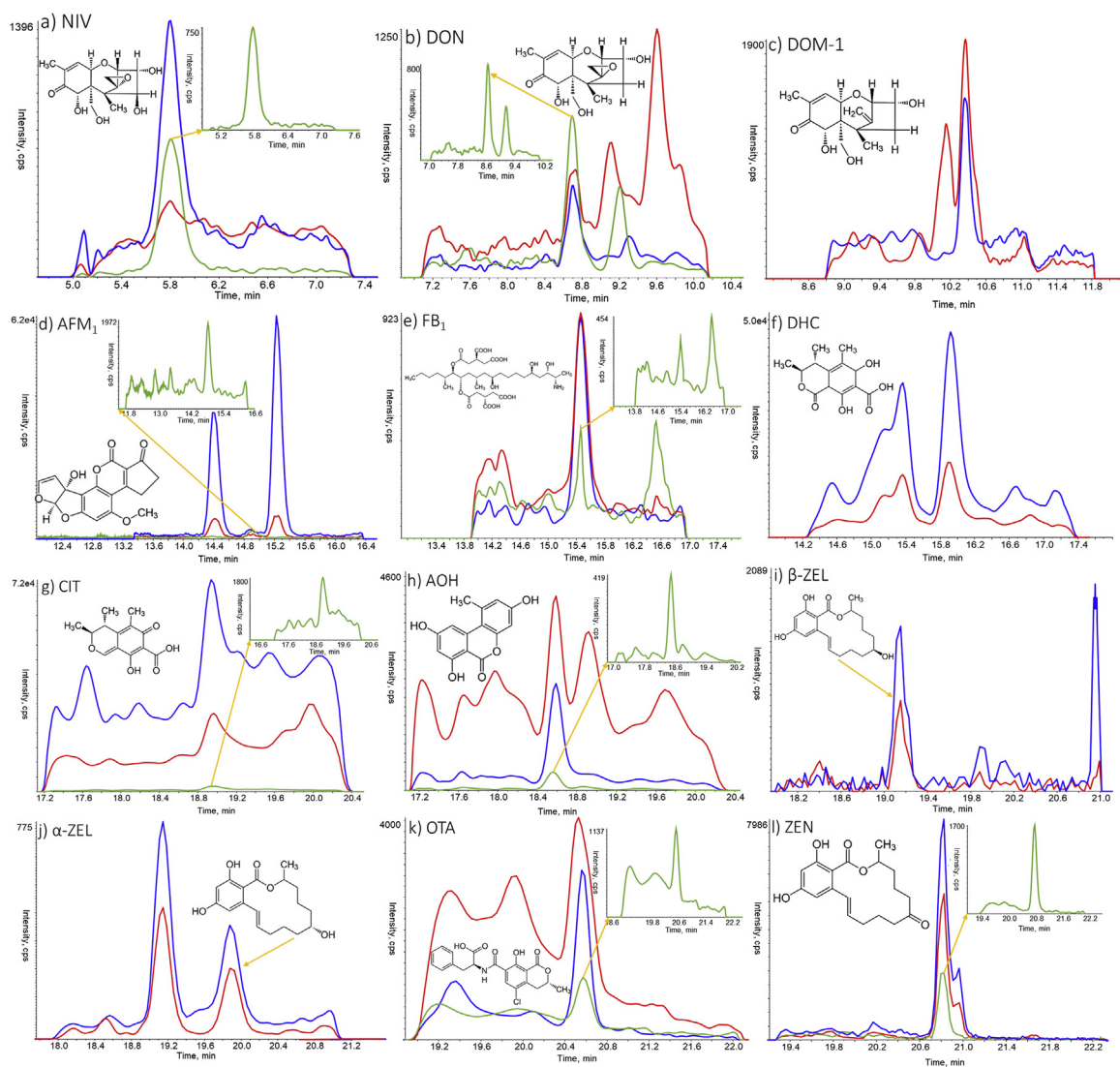

Fig. 1. MRM-chromatograms of a blank urine sample spiked with a multi-standard solution and stable isotopic standards. The individual concentrations were: a) NIV 0.1 ng mL^{-1}; b) DON 0.3 ng mL^{-1}; c) DOM-1 0.5 ng mL^{-1}; d) AFM$_1$ 0.003 ng mL^{-1}; e)FB$_1$ 0.01 ng mL^{-1}; f) DHC 0.03 ng mL^{-1}; g) CIT 0.01 ng mL^{-1}; h) AOH 0.03 ng mL^{-1}; i) β-ZEL 0.003 ng mL^{-1}; j) α-ZEL 0.01 ng mL^{-1}; k) OTA 0.001 ng mL^{-1}; l) ZEN 0.003 ng mL^{-1}. The blue line represents the quantifier ion, while the red and green lines indicate qualifier ion and the internal standard, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

parameters additional experiments were performed. In order to evaluate the performance criteria, the EE was checked on six levels for differences between low levels at LOQ, 1.5 x LOQ and 2 x LOQ, and additionally at 30 x, 100 x, and 300 x LOQ. The results in Table 1 are expressed as means of the tested range while sMRM chromatograms of a blank urine sample spiked with the multi-standard spiking solution are shown in Fig. 1. The EE was generally above 70% for all analytes. Moreover, all analytes showed linear response within the tested range. Intra- and interday relative standard deviations ranged from 5% for DON and NIV, to a maximum 33% for α-ZEL.

The LOD and LOQ values were calculated based on S/N ratios (3:1 and 10:1) from spiked blank urine. The LOD levels ranged from ppq (300 fg mL^{-1} for OTA) to ppt levels (300 pg mL^{-1} for DOM-1). A comparison with other published methods suggests a higher sensitivity for most compounds by the newly developed method despite the fact that some other approaches involve highly specific IAC columns. NIV was not reported in other methods before with the exception of Warth et al. [21] where a 10 x higher LOQ value was reported. For DON many different methods were published, and the LOQ values ranged from 0.5 ng mL^{-1} [19] to 4 ng mL^{-1} [21]; the glucuronide metabolites of DON were also included in those direct methods. Since a de-glucuronidation step is included here, an LOQ of 0.15 ng mL^{-1} includes the sum of parent DON and DON-glucuronides found in urine. Of all included mycotoxins, DOM-1 had the highest LOQ level (0.5 ng mL^{-1} in urine matrix), while Huybrechts et al. [19] reported a slightly lower LOQ level of 0.3 ng mL^{-1} due to usage of IAC for pre-concentrating the sample [19]. AFM$_1$ and OTA had the LOQ values of 0.001 ng mL^{-1}, allowing for the monitoring of lowest background exposures. Other methods had either significantly higher LOQs (0.17 ng mL^{-1} for both AFM$_1$ and OTA) [21] or similar levels (0.003 ng mL^{-1} for OTA and 0.005 ng mL^{-1} for AFM$_1$) [19]. The LOQ for FB$_1$ was 0.01 ng mL^{-1} which was slightly lower than the 0.0125 ng mL^{-1} limit reported before by Gerding et al. [20]. The LOD values for CIT and DHC were 0.01 ng mL^{-1} which are in line what Gerding et al. [20], and Ali et al. [50] reported. The ZEN family (ZEN, α-/β-ZEL) also had low LOQ

values (0.003/0.01/0.003 ng mL^{-1}) comparable to other published methods [19,20]. Alternariol which has never been previously reported in human bio-fluids as potential biomarker of exposure was added to the methods due to its frequent occurrence in cereal [51,52], tomato [53], or apple [54] products and beverages [41]. Based on a recent exposure estimate [52], it is predicted to be observed in urine, and may be a useful biomarker of exposure. Due to a lack of certified reference materials for mycotoxins in urine, spiked blank samples were used estimate trueness according to the EC 657/2002 recommendations.

3.5. Application and critical performance evaluation

A comparison of the sensitivity of the presented method to that published by Warth et al. [21] was performed by re-analysing a set of 120 urine samples from Nigerian individuals naturally contaminated with numerous mycotoxins [11]. The results clearly demonstrate the enhanced performance, indicated by a higher number of positive samples (samples with concentrations > LOD) with the new method reported in this study (Table 2). Importantly, the number of quantified analytes (samples with mycotoxin concentrations > LOQ) was greatly increased, enabling a far more accurate exposure and risk assessment. Ezekiel et al. [11] reported 17 (14.2%) positive samples for AFM$_1$ of which only seven had AFM$_1$ values > LOQ while the present study yielded 87 (73%) positive samples, all above the LOQ. This is also the case for other key analytes (OTA and ZEN) quantified. The developed method has shown the capacity to assess realistic chronic exposures due to the very low LOQ values for the majority of the analytes, also because the data points > LOQ are more in number compared to the <LOD values; a fact contrasted by our former analysis [11].

The prevalence and concentrations of CIT (incidence: 66%; max: 241 ng mL^{-1}), DHC (incidence: 58%; max: 17 ng mL^{-1}), FB$_1$ (incidence: 71%; max: 15 ng mL^{-1}) and ZEN (incidence: 82%; max: 20 ng mL^{-1}) were very high compared to the previous study [11] (Table 2) while based on mean values (Fig. 2) the highest urinary levels were obtained for CIT (5.96 ng mL^{-1}), DHC (2.39 ng mL^{-1}),

Table 2

Urinary biomarker concentrations in human urines obtained from volunteers in northern Nigeria (n = 120). To enable the direct comparison with a less sensitive method (Warth et al. [18]) applied before to this sample set, results from the previous analysis (Ezekiel et al. [8]) and the newly developed method are reported below each other. Results from (Ezekiel et al. [8]) are indicated by italic font; only a subset of analytes was assessed previously.

Mycotoxin	LOQ[b] (ng mL^{-1})	Number (%) quantified[c]	Number (%) positive[d]	Concentration (ng mL^{-1})				
				Minimum	Maximum	Mean[e]	Std Dev.	Median
Aflatoxin M$_1$ old[a]	*0.15*	*7 (5.8)*	*17 (14.2)*	*0.08*	*1.54*	*0.34*	*0.45*	*0.08*
Aflatoxin M$_1$	0.001	87 (72.5)	87 (72.5)	0.001	0.62	0.04	0.08	0.01
Alternariol[g]	0.03	8 (6.7)	8 (6.7)	0.03	0.20	0.06	0.06	0.03
Citrinin[g]	0.01	78 (65.0)	79 (65.8)	0.015	241.46	5.96	27.43	0.84
Dihydrocitrinone[g]	0.01	69 (57.5)	69 (57.5)	0.05	16.89	2.39	3.56	1.00
Deoxynivalenol old[a]	*4.0*	*3 (2.5)*	*6 (5.0)*	*0.94*	*6.84*	*2.56*	*2.31*	*1.67*
Deoxynivalenol	0.15	21 (17.5)	23 (19.2)	0.08	6.22	2.37	1.88	1.79
Fumonisin B$_1$ old[a]	*2.0*	*16 (13.3)*	*16 (13.3)*	*2.08*	*12.77*	*4.56*	*2.82*	*3.84*
Fumonisin B$_1$	0.01	71 (59.2)	85 (70.8)	0.08	14.88	1.09	2.04	0.48
Nivalenol[f]	0.1	40 (33.3)	40 (33.3)	0.24	3.02	0.95	0.60	0.75
Ochratoxin A old[a]	*0.15*	*16 (13.3)*	*34 (28.3)*	*0.08*	*0.56*	*0.15*	*0.11*	*0.08*
Ochratoxin A	0.001	94 (78.3)	94 (78.3)	0.003	0.31	0.05	0.06	0.04
Zearalenone old[a]	*0.6*	*8 (6.7)*	*13 (10.8)*	*0.94*	*6.84*	*3.13*	*2.28*	*2.40*
Zearalenone	0.003	98 (81.7)	98 (81.7)	0.03	19.99	0.75	2.59	0.20
α-Zearalenol[f]	0.01	5 (4.2)	5 (4.2)	0.52	2.52	1.27	0.87	0.87
β-Zearalenol[f]	0.003	7 (5.8)	7 (5.8)	0.06	2.74	0.88	1.08	0.33

[a] Data as published by Ezekiel et al. [8].
[b] Limit of quantitation.
[c] Number (percentage) of samples with analyte concentrations above the LOQ.
[d] Number (percentage) of samples with analyte concentrations above the LOQ and those less than the LOQ but higher than the LOD.
[e] Means were calculated for positive samples by considering half LOQ (LOQ/2) for samples below the LOQ value.
[f] Analytes sought for by Ezekiel et al. [8] but not detected.
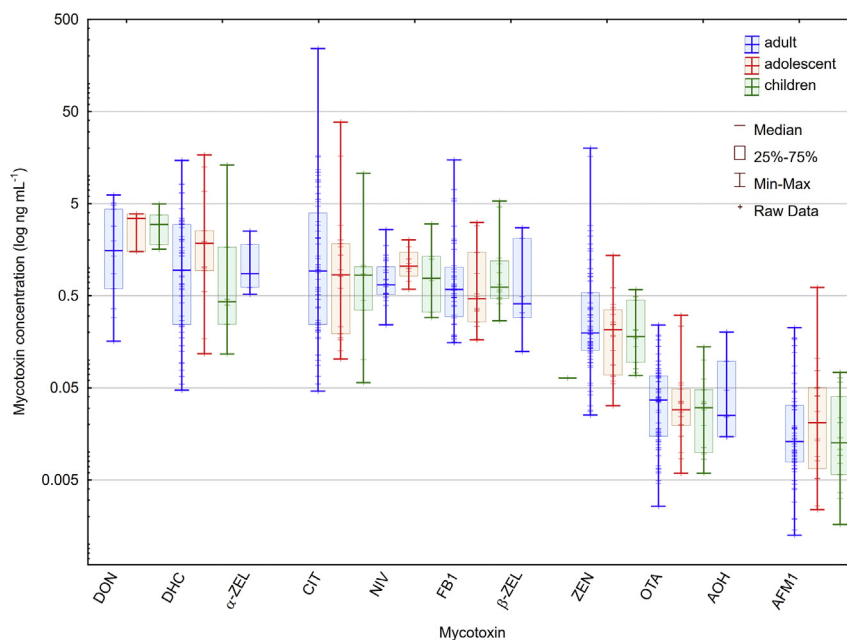[g] Analytes sought for only by the newly developed method.

Fig. 2. Distribution of urinary mycotoxins in individuals (adults, adolescents and children) from northern Nigeria arranged form the highest to lowest medium concentration.
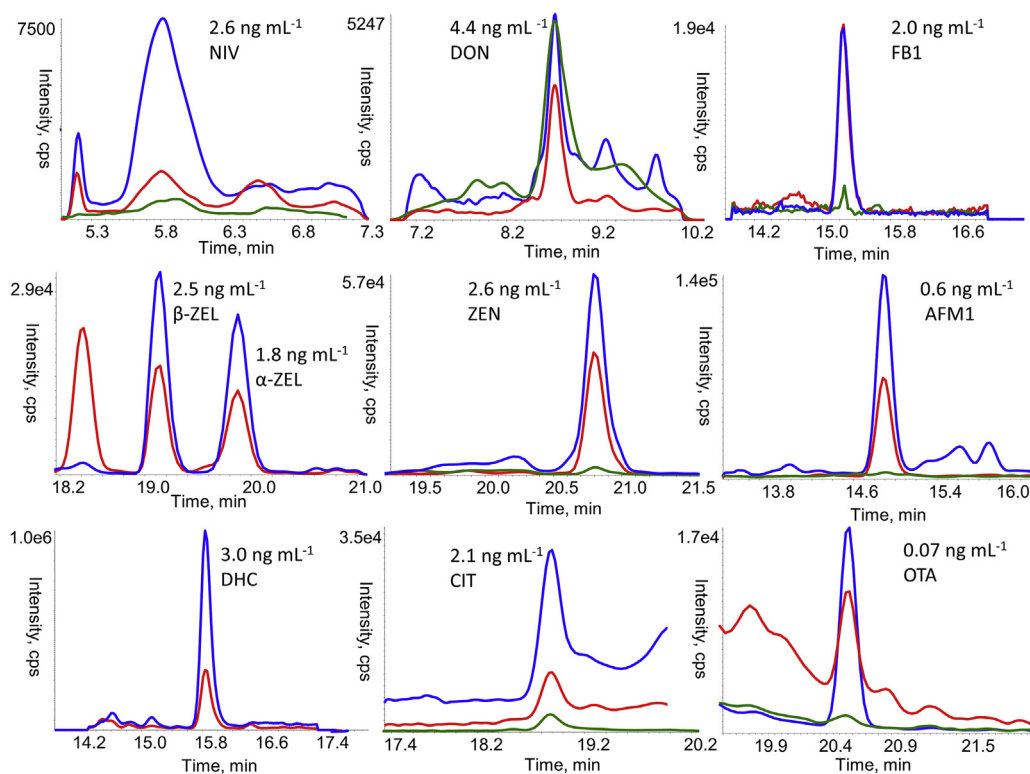

Fig. 3. Examples of naturally contaminated human urine samples for NIV, DON, FB$_1$, β-ZEL, α-ZEL, ZEN, AFM$_1$, DHC, CIT, OTA, with their respective concentrations.

DON (2.37 ng mL^{-1}), α-ZEL (1.27 ng mL^{-1}) and FB$_1$ (1.09 ng mL^{-1}). Unexpectedly and similar to our previous analyses [11], the hydroxylated metabolites of ZEN hardly occurred. For the first time, AOH was found in human urine at concentrations reaching 0.2 ng mL^{-1} (mean 0.06 ng mL^{-1}) in eight samples. The prevalence of AFM$_1$ was 72%, mean level (0.04 ng mL^{-1}). With respect to exposure patterns observed across age groups (Fig. 2), higher AFM$_1$, DON, DHC, NIV and ZEN median levels were found in adolescent urines than in adult and children urines while CIT, FB$_1$ and OTA median levels were higher in adult urines than in urines from other age groups.

When applying the new analytical method to previously analysed samples, it could be revealed that about 98% of the urine samples contained more than one mycotoxin (Supplementary Table S2), and 25% contained as many as five co-occurring mycotoxins, while another one third contained at least six mycotoxins and up to nine different mycotoxin combinations; these are in contrast to the fewer co-occurrence patterns reported in our 2012 measurements [11]. The discovered mixtures included several mycotoxins which are regulated and these mixtures may exert unknown synergistic effects on the exposed populations. Combinatory exposure effects have recently been reported in cell lines and animal models [15,55,56], thus making it imperative to develop highly sensitive targeted and untargeted workflows to efficiently monitor background human exposures and create appropriate interventions among the affected populations. The data provided by this new method (example in Fig. 3) further suggest the need for *in vitro* testing of mycotoxin co-occurrence patterns in realistic, real-life scenarios (very low to high concentration ranges).

4. Conclusions and outlook

This paper reports on the development, validation, and critical performance evaluation of the first SIDA-based UHPLC-MS/MS method for urinary multi-mycotoxin exposure assessment. Through the unique combination of a more general clean-up (HLB), the use of internal standards, enzymatic hydrolysis of conjugated toxins, and optimized chromatographic separation, the ultrasensitive quantification of biomarkers of exposure to multiple co-occurring mycotoxins is now possible. The application of this method in large-scale cohort exposure assessment studies is encouraged in order to obtain realistic individual exposure data. Importantly, this approach is also feasible for expansion to investigate exposure patterns beyond mycotoxins (potentially towards capturing the 'exposome' and mycotoxin-drug interactions) based on the non-discriminative sample preparation protocol. Applying this method to link dietary mycotoxin exposure to the susceptibility, aetiology and outcomes of specific diseases such as exposure-related cancers (e.g. breast, liver, colon, and oesophagus), stunting or HIV/AIDS will be a highly important future endeavour.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgement

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.02.036.

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