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Online solid phase extraction-LC-MS/MS with two step peak focusing for sensitive multi-analyte analysis of mycotoxins in urine

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ABSTRACT

Mycotoxins are food contaminants that may pose a risk to human health. Biomonitoring of mycotoxins is a valuable tool to assess this potential risk and to evaluate the effect of mitigation strategies against mycotoxin exposure. Comprehensive exposure assessment requires sensitive multi-analyte methods that achieve low limits of detection (LOD) in physiological samples, while not becoming laborious with the high sample numbers required for high-quality biomonitoring studies. Online solid phase extraction (SPE) coupled to liquid chromatography with tandem mass spectrometry (LC-MS/MS) is a technique that can meet these demands, but requires careful optimization. We have developed and validated an online SPE-LC-MS/MS method covering 36 relevant mycotoxin biomarkers of exposure, mycotoxins, and mycotoxin metabolites. Optimized sample loading and washing on the reversed-phase polymeric SPE column ensured matrix removal and enrichment of structurally diverse analytes. In two separate steps, first the highly polar deoxynivalenol along with its glucuronides, followed by the remaining less polar mycotoxins, were transferred and refocused on the head of the analytical column by flow dilution using a mixing tee. LODs in the low pg/mL to low ng/mL urine range were reached, greatly improving sensitivity over established dilute-and-shoot methods. Stable isotope-labelled internal standards assured accurate quantification for 14 analytes, while the remaining analytes were quantified using matrix calibration. Reliable measurements were possible with intra- and inter-day precision values at three spiking levels $\leq$20 % for 21 analytes. The group of enniatins (A, A_1, B, B_1) and beauvericin exerted highly variable matrix effects among urine samples, allowing for only estimated quantitative results. Applying the method to a cohort of 50 pregnant women from Bangladesh demonstrated improved performance, with a 304 % increase in positive detections compared to a commonly used dilute-and-shoot approach.

1. Introduction

Human biomonitoring has become an important tool for exposure assessment of environmental toxins, enabling the estimation of associated health risks and the development of mitigation strategies [1]. Mycotoxins are naturally occurring contaminants that are produced by crop-infecting filamentous fungi. Human exposure to mycotoxins therefore primarily occurs through ingestion of contaminated foodstuffs, with contamination potentially occurring during cultivation, post-harvest storage, or transportation. Owing to their remarkable thermal stability, mycotoxins can persist through processing and remain detectable in finished products [2,3]. Additionally, dermal uptake or inhalation can also be significant routes of exposure in certain scenarios [4]. Thus, human exposure to mycotoxins is unavoidable, but patterns and levels of exposure differ between countries, depending on climatic conditions, agricultural practice, dietary habits, and regulatory measures, among other factors [5]. Together with the numerous fungal species producing these secondary metabolites, the chemical diversity of mycotoxins is high, resulting in a broad range of polarities (Fig. S1, Supporting Information). From the very polar cyclic sesquiterpene deoxynivalenol (DON; calculated log*P* -0.7) and its glucuronic acid conjugates (DON-GlcA; calculated log*P* -2.1) to the nonpolar cyclic depsipeptide structure of enniatine A (ENA; calculated log*P* 7.6). Other prevalent mycotoxins with calculated log*P* values in between are the

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group of aflatoxins (AF; B_1, B_2, G_1, G_2, M_1), ochratoxin A (OTA), citrinin (CIT), fumonisins ($FB_{1/2}$), the trichothecenes T-2 toxin, HT-2 toxin, as well as zearalenone (ZEN) [6]. Furthermore, so-called "emerging mycotoxins" such as sterigmatocystin (STG) or the toxins of *Alternaria* species gain in relevance due to their increasing detection frequency [7]. Toxicity occurs mainly with long-term exposure and ranges from immunosuppressive and cytotoxic effects to pronounced nephrotoxicity or even cancer-inducing effects [8]. Thereby, lower amounts over time can already pose a risk, making sensitive detection a necessity for reliable risk assessment. Although not all of these mycotoxins have validated biomarkers for exposure assessment, monitoring them or their metabolites can still help to identify previously overlooked risks [9]. Although analysis of both urine and blood samples is needed to detect most of the mycotoxins or their metabolites, urine has the advantage of non-invasive sampling. At the same time, concentrations of the more polar biomarkers are relatively high. Nonetheless, strong matrix effects in LC-ESI-MS/MS measurements, the standard technique for quantitative and selective analysis, impede sensitive detection [10]. Consequently, comprehensive biomonitoring of mycotoxin exposure requires sensitive methods accommodating a variety of structures and, at the same time, reducing the practical burden to analyse large sample cohorts that represent the exposure of a population. Different approaches to urine sample preparation have been applied to minimize interfering matrix, often with drawbacks in analyte diversity or throughput. Antibody-based immuno-affinity columns (IAC) offer very effective matrix removal at the cost of analyte diversity and time efficiency. Their application has been reported mainly in smaller cohort studies analyzing up to eleven mycotoxins [11–13]. A common approach for the reduction of urinary matrix effects without analyte loss is a quick and simple dilute-and-shoot (DaS) procedure [14]. Diluted urine samples are directly subjected to LC-MS/MS measurement, whereby the effectiveness of matrix removal is solely dependent on the chromatographic separation. Therefore, no enrichment of mycotoxins is achieved, and the limits of detection (LOD) are typically high [15,16]. Solid phase extraction (SPE) protocols with reversed-phase materials applicable to a broad analyte spectrum can be used to improve sensitivity [17,18]. This approach becomes laborious when performed hands-on for a large number of samples. Online SPE setups, typically consisting of two LC pumps dedicated to the SPE column and a downstream analytical column (AC), reduce sample preparation time while providing effective sample clean-up for a wide range of compounds. However, preventing analyte loss and achieving good matrix separation along with adequate chromatography on the AC can be challenging [19]. Generally, the retention of analytes on the SPE column should be weaker than on the AC, ensuring a focused analyte band at the AC head after transfer and thus better separation from coeluting matrix components [20]. This proves to be difficult when the range of analyte structures and polarities is large. In the field of mycotoxin analysis, online-SPE methods have mainly been applied to food matrices [21–24], and only more recently to urine samples [25,26]. However, a first report on the analysis of CIT in urine by online SPE-HPLC with fluorescence detection was already published in 1986 [27]. Both of the recent publications utilizing online SPE described difficulties in implementing polar analytes like DON for the above-mentioned reasons, which limited the analyte spectrum [25,26]. Peak focusing strategies using mixing tees to dilute the SPE eluate before transfer to the AC have already been established in online SPE approaches where physicochemical properties of analytes require it [28–30].

Here, we developed an online SPE-LC-MS/MS method using two separate transfer and dilution steps to reduce the organic content of the SPE eluates and achieve peak focusing of 36 analytes at the AC head. The validated method was applied to 50 urine samples from Bangladesh to underline the importance of sensitive multi-analyte methods for human biomonitoring studies.

2. Experimental section

2.1. Chemicals and standards

Acetonitrile (LC-MS grade) was purchased from Fisher Scientific (Schwerdte, Germany). Water ASTM type I (>18 MΩ) was produced with a Purelab Flex system (ELGA, Bielefeld, Germany). Formic acid was purchased from Merck KGaA (Darmstadt, Germany). AFM_1, CIT, beauvericin (BEA), enniatins (EN; ENA, ENA_1, ENB, ENB_1), and $^{13}C_{34}$-FB_1 were purchased from Sigma-Aldrich (Taufkirchen, Germany). Dihydrocitrinone (DH-CIT) was from AnalytiCon Discovery GmbH (Potsdam, Germany). Paxilline (PAX) and α-cyclopiazonic acid (α-CPA) were obtained from Enzo Life Sciences (Lausen, Germany). Sterigmatocystin (STG) was purchased from Cayman Chemicals (Ann Arbor, Michigan, USA). U-[$^{13}C_{17}$]-AFM_1 was purchased from Romer Labs Deutschland GmbH (Butzbach, Germany). U-[$^{13}C_{18}$]-STG was purchased from Fianovis (Vindry-sur-Turdine, France). Fungal cultures were used to isolate and purify OTA, ochratoxin α (OTα), 10-hydroxy-ochratoxin a (10-OH-OTA), T-2, HT-2, DON, FB_1, FB_2, ZEN, alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), and penitrem A (PENA) [31–37]. d_5-OTA, d_5–2′*R*-OTA, $^{13}C_2$-OTα, $^{13}C_3$-CIT, $^{13}C_3$-DH-CIT, tenuazonic acid (TEA), $^{13}C_2$-TEA, d_3-T-2, d_9-HT-2, d_1-DON, and d_2-ZEN were chemically synthesized [31,33,34,38,39]. 2′*R*-OTA was produced by thermal isomerization of OTA as described in Cramer *et al* [40]. Ochratoxin-*N*-acetyl-L-cysteine (OTB-NAC) and its isotope-labelled standard d_5-OTB-NAC were produced *via* photoreaction as described in Sueck *et al* [41]. α- and β-zearalenol (α−/ β-ZEL) were produced by chemical reduction of ZEN [42]. Mycotoxin glucuronides deoxynivalenol-3-glucuronide (DON-3-GlcA), HT-2-3-GlcA, HT-2-4-GlcA, zearalenone-14-glucuronide (ZEN-14-GlcA), zearalenone-14-glucuronide (ZAN-14-GlcA), α−/ β-zearalenol-14-glucuronide (α−/ β-ZEL-GlcA) were enzymatically synthesized using the parent compound and liver microsomes of rat and pig according to Welsch *et al* [43]. Three different multi-stock solutions in ACN together containing all mycotoxins at 20- to 200-fold concentration of the highest calibration point were stored at −20 °C. From these, three working solutions in ACN/H_2O were freshly prepared for calibration on each day of analysis. A mix of all internal standards (IS) was used at a 100-fold concentration of the final concentration needed in the urine sample.

2.2. Urine samples

Individual blank urine samples were collected from one female and three male German volunteers. Prior to urine collection, all volunteers abstained from cereals and cereal-based food for approximately 36 h to lower or eliminate mycotoxin concentrations in the urine samples. The single urine samples were pooled in equal amounts, and this pooled blank urine was used for further matrix calibration and method validation. DH-CIT, TEA, and small amounts of OTA could still be detected in some individual urines and also in the pooled urine (see 2.3 and 2.5). In addition to the determination of limits of detection (LODs) and limits of quantification (LOQs) in the pooled urine sample, these experiments were also done in the single urine samples to evaluate varying matrix effects of different urine samples (see 2.5). For the method application, 50 urine samples were randomly selected from a set of 447 urine samples of 439 pregnant women in Bangladesh. The samples were collected as part of a previously conducted cohort study focused on *Maternal Exposure to Mycotoxins and Adverse Pregnancy Outcomes* (MEMAPO) [44,45].

2.3. Sample preparation

Thawed urine samples were vortexed, and 297 μL were transferred into a 96-well plate with a conical bottom, followed by the addition of 3 μL of the IS mix. The eight-point matrix calibration was done using the pooled blank urine and the freshly prepared working solutions. Because

the blank urine still contained amounts of DH-CIT and TEA, a calibration in water was done for the quantification of both analytes. The IS mix was added to the calibrations in the same amount as in the samples. The whole plate was then put on an orbital shaker (Matrix Orbital Delta Plus, IKA GmbH, Staufen, Germany) at 550 rpm for 10 min. Before injection into the online SPE-LC-MS/MS, the whole 96-well plate was centrifuged for 10 min at 3200 ×*g* to prevent solids from clogging the LC system. For each analysis, a spiked urine sample, stored at −20 °C, was used as quality control.

2.4. Online SPE-LC-MS/MS method

The instrumental setup consisted of a 1260 Infinity II Bio-inert LC system, with two quaternary pumps and an additional 1290 Infinity Flexible Cube module with two ten-port valves (all Agilent, Waldbronn, Germany) coupled to a Sciex 7500 QTRAP-activated mass spectrometer (Sciex, Darmstadt, Germany). For the online solid phase extraction (SPE), two ten-port valves were used to enable the 2-way transfer and dilution *via* a stainless-steel mixing tee. The pump (Pump 2) dedicated for loading and washing the sample on the SPE column used water (A) and acetonitrile (B) both containing 1 % formic acid (FA), while the pump (Pump 1) for dilution and gradient elution from the analytical column (AC) used water (A) and acetonitrile (B) both containing 0.1 % FA. For the online sample clean-up, an Oasis HLB column (2.1 × 20 mm, 5 µm, Waters GmbH, Eschborn, Germany) was used, and further gradient separation was performed on a Nucleodur Phenyl-Hexyl column (2 × 100 mm, 3 µm) with a guard column of the same material (2 × 4 mm, both Macherey-Nagel, Düren, Germany). The column oven, which held both the SPE column and the AC, was set at a temperature of 45 °C. The needle position for injection was optimized to avoid the aspiration of particles from the bottom of the well. The following method steps are also visualized in Fig. 1: After aspiration of 100 µL of the urine sample into the sample loop, Pump 2 transfers the sample to the SPE column with 0 % B at 1 mL/min, while Pump 1 conditions the AC at 2 % B at 0.6 mL/min for 4 min (Fig. 1A). With the flow still in the loading direction, Pump 2 then increases the organic solvent proportion to 10 % B at 0.1 mL/min to remove the matrix from the SPE column. For the very polar analytes DON and DON-GlcA, the proportion of 10 % B is already sufficient to elute them from the SPE and transfer them towards the AC (Fig. 1B). During transfer, the eluate from the SPE column is diluted by the solvent coming from Pump 1 *via* the mixing tee. Pump 1 delivers 2 % B at a flow rate of 0.35 mL/min. After 6 min, the remaining analytes on the SPE are further washed by solvent containing 15 % B delivered at 0.5 mL/min from Pump 2 (Fig. 1C). At the same time, the focused analytes on the AC are eluted with the following linear gradient at 0.5 mL/min from Pump 1: 0 min 2 % B, 2 min 2 % B, 5 min 25 % B. From 12 to 15.5 min, the transfer of the remaining analytes on the SPE is done in backflush flow by Pump 2 at 60 % B and 0.1 mL/min (Fig. 1D). The eluate is again diluted with a 0.35 mL/min flow and an increase in organic from 2 to 20 % B from Pump 1, producing a dilution gradient. Lastly, analytes transferred to the AC are again chromatographically separated by gradient elution with the following gradient at 0.5 mL/min: 0 min 20 % B, 7.5 min 95 % B, 9.5 min 95 % B (Fig. 1E). During this step, Pump 2 removes the remaining matrix from the SPE column with 95 % B at 0.5 mL/min for 4.5 min, followed by 5 min of equilibration at 0 % B and 0.5 mL/min to prepare for the next sample. Detailed information on each step, together with valve switching times, can be found in Table S1 (Supporting Information). To reduce analysis time, the flow rate before each transfer and dilution was held at 0.5 mL/min and reduced to 0.1 mL/min just before the analytes left the SPE column. The first 4 min of the run were discarded by switching of the diverter valve on the mass spectrometer. Total run time of the analysis amounted to 25 min. The online SPE columns and AC tested during method development can be found in Table S2 (Supporting Information). The LC parameters used for the conventional backflush online SPE-LC-MS/MS method are summarized in Table S3 (Supporting Information).

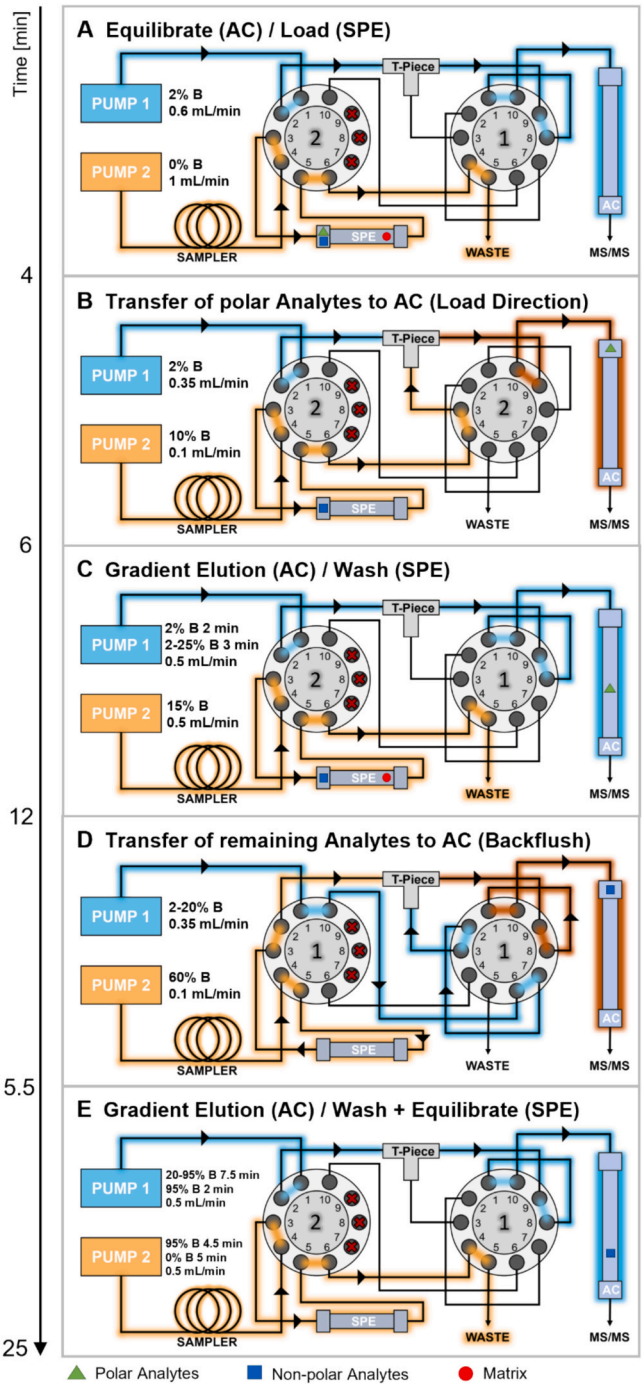

Fig. 1. Online solid phase extraction (SPE)-LC-MS/MS setup with peak focusing. Consistent of two quaternary pumps, two 10-port valves, one SPE column (reversed-phase, polymeric), one analytical column (AC, phenyl-hexyl phase), and a mixing tee. Pump 1 uses water (A) and acetonitrile (B), both containing 0.1 % FA, while pump 2 uses the same solvents, only with 1 % FA added.

Analytes were ionized by electrospray ionization with switching between positive and negative modes in one run. Spray voltages were set to ±3000 V at a source temperature of 450 °C. Curtain gas, nebulizer gas (gas 1), and drying gas (gas 2) were set to 50 psi, 65 psi, and 50 psi, respectively. Gas for collision-induced fragmentation was set to an instrument value of 9 arbitrary units. Measurements were performed in scheduled multiple reaction monitoring (sMRM) mode with a target cycle time of 400 ms and retention time windows of 30 to 60 s. For

quantification and qualification of each compound, at least two MRM transitions were selected based on signal-to-noise (S/N) ratio and the absence of interfering signals in the matrix. Optimized collision energies and collision cell exit potentials, together with more details on the used MRM parameters, can be found in Table S4 (Supporting Information). Data recording and analysis were performed with Sciex OS Version 3.1.

2.5. Method validation

Method validation included determination of LOD, LOQ, setting of working range, and selecting a regression model for matrix calibration, as well as calculating recovery, and intra−/inter-day accuracy and precision. The LODs and LOQs were estimated by spiking the pooled blank urine at 12 concentration levels covering the expected values ($n = 3$). LOD was determined at a S/N ratio of 3 and LOQ at a S/N ratio of 10 using the quantifier MRM transitions with the best S/N ratio. As OTA, DH-CIT, and TEA were found in the pooled urine, LOD and LOQ of OTA were set equal to the values of 2′*R*-OTA, and respective values for DH-CIT and TEA were determined in the single urines free of the analytes. Further validation was done using the pooled blank urine sample. The working range was set covering one to two decimal powers from the respective LOQ at eight calibration points. For analytes with available isotopically labelled IS, the area ratios of analyte to IS were used for calibration. Next to ZEN, d_2-ZEN was used for α- and β-ZEL, ZEN-14-GlcA, α- and β-ZEL-GlcA, and ZAN-14-GlcA as IS. Models for calibration regression were selected considering the regression coefficients. For the above-mentioned analytes using d_2-ZEN as an IS, a quadratic regression model was used [33]. For all other analytes, a linear regression model was preferred. All calibration functions were weighted by 1/x for higher accuracy at the lower working range. Apparent recovery R_A or respective matrix effect was calculated as the ratio of slopes from matrix calibration ($n = 3$) to calibration in water ($n = 3$). Spiking experiments as described for the LOD/LOQ determination were performed in four different blank urines to evaluate variability in matrix effect, called relative matrix effect by Matuszewski *et al* [46]. Calculations were based on relative standard deviation of peak areas in the four urines at a concentration level of LOQ or above. Accuracy and precision of intra- and inter-day measurements were determined at three spiking levels (low, medium, high) of the pooled blank urine covering the respective working range of each analyte. Intra-day repeatability consisted of 10 repeated injections of each spiking level, and inter-day measurements were done on three different days in quadruplicate. Accuracy and precision were calculated as the percentage recovered of the spiked concentration and the relative standard deviation of the accuracy. TEA and its isomer *allo*-TEA could not be chromatographically separated and were measured as a sum parameter. Likewise, DON-3-GlcA and DON-15-GlcA were not chromatographically separated. DON-15-GlcA is the major metabolite in humans, but since only the DON-3-GlcA standard was available, quantification of the metabolites in human urine was performed by correcting the signal in the samples with a response factor of 1.88, as described by Warth *et al* [47].

3. Results and discussion

3.1. Method development

In order to implement various mycotoxins in one method and to achieve effective matrix removal for a sensitive analysis, various aspects must be taken into account and optimized. The effectiveness of common online sample clean-up by solid phase extraction (SPE) coupled to LC-MS is dependent on the combination of the used SPE material and the downstream analytical column (AC). Polar analytes require sufficient retention on the SPE to avoid losses by breakthrough, while an AC with too little retention for the analytes after SPE elution leads to peak broadening and inefficient matrix removal. Thus, multi-analyte approaches are difficult to implement for mycotoxins due to their diverse chemical structures and broad polarity range.

DON and its glucuronic acid conjugates are frequently detected in human urine samples, especially in Europe [48], but their high polarity makes it challenging to include them in a multi-analyte method [16,25]. To address this aspect, we first tested different ACs (Nucleodur Gravity SB, Pyramid, Phenylhexyl, Biphenyl-propyl; and Poroshell EC-18; Table S2, Supporting Information) without prior clean-up steps, to achieve optimal retention for most analytes, but especially for DON and its glucuronides. Also, peak shape and signal intensities in matrix were taken into account. From these tests, the Nucleodur Phenyl-Hexyl column (2 × 100 mm, 3 µm) was selected as most suitable. From literature and prior experiments, it was known that the Oasis HLB material is suitable for preconcentration of various mycotoxins and was therefore used as online SPE column [26,29]. However, also turbulent flow chromatography columns of different materials (polymeric reversed phase, silica-based fluorinated alkyl phase; Table S2, Supporting Information) were tested for comparison. All showed little retention for the highly polar analytes and were therefore excluded from further method development. Organic mobile phase modifiers methanol (MeOH) and acetonitrile (ACN) were tested together with both 0.1 % and 1 % formic acid (FA) as additives. When tested with the AC only, MeOH gave better signal intensities for many analytes compared to ACN. But implementing the SPE clean-up led to broader peaks using MeOH, hence, ACN was chosen as the organic modifier. The addition of 0.1 % FA resulted in overall better signal intensities, although improved peak shape and signal intensity were observed for CIT with 1 % FA. Nevertheless, 1 % FA was required as an additive for loading the urine samples onto the SPE column. This ensured that varying urine pH values did not affect chromatographic parameters such as retention or peak shape of analytes like DH-CIT or TEA. The SPE column and the AC were tested for the breakthrough of analytes by stepwise increasing the organic content as described in Wrobel *et al* [49]. For the elution of DON and its glucuronides from the SPE, an organic content of 10 % ACN was required, a concentration that resulted in insufficient retention on the AC (Fig. S2, Supporting Information). Thus, unwanted polar matrix components would elute alongside the analytes, potentially interfering with the MS measurement and hindering the reliable identification of DON and its glucuronides. In a recently published online SPE sample clean-up method, DON was excluded during method development because interfering matrix compounds could not be separated sufficiently [26]. The use of online TurboFlow™ sample clean-up has also been described as causing peak broadening of DON due to its insufficient retention on the AC after backflush elution from the online cartridge [25].

To obtain sufficient retention of the polar analytes on the AC, we therefore decided to dilute the SPE eluate (10 % ACN) with aqueous solvent before reaching the AC. It was required to perform this first transfer in loading direction of the online SPE, due to unwanted washout of other analytes occurring in backflush. Eluate dilution was achieved by installing a mixing tee in the tubing that connects the online SPE with the AC, as shown in Fig. 1 (“T-piece”). Consequently, to the eluate delivered by Pump 2 and containing the analytes, a highly aqueous solvent was added by Pump 1 (98 % water), resulting in an overall lower organic solvent content and thus better retention of the polar analytes on the AC. When compared to a conventional backflush online SPE setup, in which analytes are eluted in the opposite direction of loading, the peak focusing setup developed here resulted in less interfering matrix and a two- to five-fold higher signal intensity for DON and DON-3-GlcA, respectively (Fig. 2). Similar strategies have been used for different online SPE applications to overcome difficulties with chromatography or achieve successful sample enrichment [28,30,50,51]. Organic content and flow rates of both pumps were optimized to reduce the organic content of the SPE eluate by a factor of about 2.6, which was proven to be sufficient for DON and DON glucuronide retention. At this stage, besides DON and DON glucuronides, no other mycotoxins or mycotoxin metabolites eluted from the online SPE. Subsequently, the SPE column was washed with 15 % ACN before the

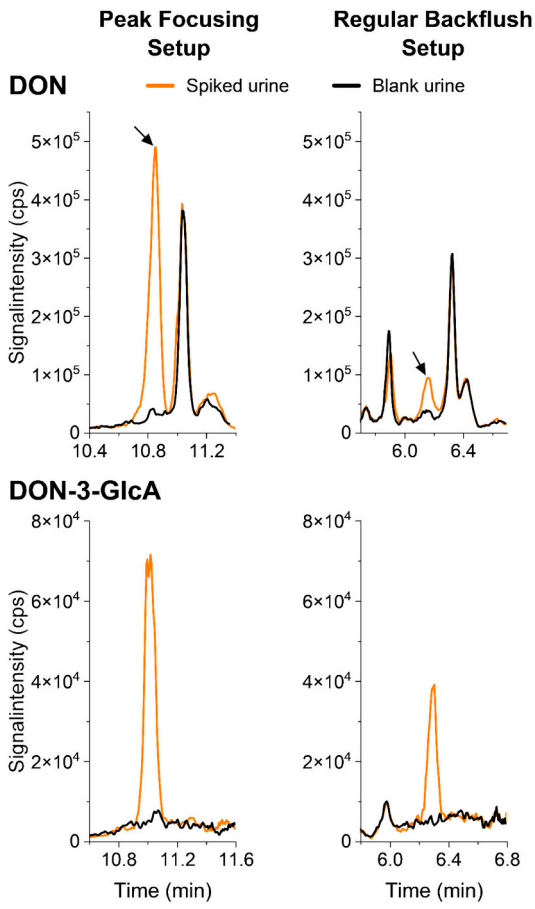

Fig. 2. Comparison of the developed peak focusing online SPE setup with a conventional backflush online SPE setup. Extracted ion chromatograms of a urine sample spiked with deoxynivalenol (DON) and its glucuronide (DON-3-GlcA).

second transfer, in which elution of all remaining compounds of interest from the SPE was done with 60 % ACN. This concentration of ACN was sufficient to quantitatively elute all compounds. To minimize peak broadening and accelerate elution of highly nonpolar substances, this was done in backflush mode. After the first transfer, analytes focused at the head of the AC were chromatographically separated by a short gradient from Pump 1. A longer gradient was applied for the remaining analytes after the second transfer step. During this period, the SPE was washed and then equilibrated for the next run. To encounter the comparably high delay volume of the low-pressure gradient system in combination with the installed valves and tubing, flow rates were optimized to shorten equilibration and run times. Transfer steps were accelerated by reducing the flow rate for dilution just before the analytes leave the SPE column. Equilibration of the AC was done during loading of the next sample, also saving analysis time. Injection volumes from 20 to 100 µL were tested, and 100 µL was selected for giving the best overall analyte S/N ratio in matrix. A summary of key parameters and optimization experiments can be found in Table S5 (Supporting information).

3.2. Method validation

Next to implementing a variety of analytes into one automated analysis method, the goal was to improve the method's sensitivity compared to common DaS approaches. LOD and LOQ values were determined in spiked pooled blank urine at S/N values of 3 and 10. Lowest LODs were achieved for FB_1 and STG with values of 0.8 and 0.7 pg/mL (Table 1). Other analytes like DH-CIT, OTA, ZEN, or AME have LODs ranging in the low pg/mL range (1.4–5.3 pg/mL), while the highest values were reached for HT-2 and ZAN-14-GlcA with 0.83 and 0.67 ng/mL, respectively. Representative chromatograms of FB_1, HT-2, AME, and ZEN in urine matrix at the respective LOQ level can be found in Fig. S3 (Supporting Information). Starting from the determined LOQ, the working range was set to range over two decimal powers for most analytes. For some analytes, the working range was limited due to either detector saturation (*e.g.*, ZAN-14-GlcA) or a presumable overloading of the online SPE, leading to a loss of the very polar DON glucuronides. Working range was also limited, *e.g.*, α−/β-ZEL-14-GlcA and HT-2 to 100 ng/mL because higher values were not expected in real samples. Linearity was confirmed by the calculation of the regression coefficient R_2. Only for ZEN, ZEN-14-GlcA, α−/β-ZEL, α−/β-ZEL-14-GlcA, and ZAN-14-GlcA, all using d_2-ZEN as IS, a quadratic regression model was used, attributed to the naturally occurring $^{13}C_2$-isomer of ZEN [33]. Regression coefficients ranged from 0.952 to 0.996. Apparent recovery R_A was calculated from the ratio of slopes in water and urine calibration. For all analytes with analogue stable isotopically-labelled IS, values were between 88 % and 113 % showing good compensation of matrix effects. For the ZEN metabolites mentioned above, higher recoveries were obtained when d_2-ZEN was used as an IS for matrix effect compensation. In the case of analytes without IS, the calculation of R_A represents the existing matrix effect. Strongest signal suppression could be observed for ALT, HT-2-4-GlcA, and AOH with 15, 16, and 17 % while 10-OH-OTA still showed 77 % of its signal in the matrix. Only for α-CPA, a strong signal enhancement of 389 % was noticeable. In a very recently published DaS approach, the observed matrix effects for AOH and AME were less pronounced, at about 45 % and 55 %, respectively [52]. The stronger matrix effects can be explained by a higher matrix load resulting from the undiluted urine used in the presented method. For PENA, PAX, BEA, ENA, ENA_1, ENB, and ENB_1, no apparent recovery or matrix effect could be calculated because no reasonable calibration curve in water could be established. It can be assumed that those analytes experience strong signal enhancement in matrix, leading to unsatisfactory results in pure water. Matrix calibration in the pooled blank urine was applied to compensate for the matrix effects of analytes without IS. To evaluate the variability of matrix effects, spiking experiments in four different blank urines were done (Table S6, Supporting Information). Relative matrix effect given as the relative standard deviation of measured peak areas at a given concentration ranged from 16 % and 20 % for α-CPA and DON-3-GlcA to about 80 % for both HT-2 glucuronides. All ZEN metabolites showed similar values of around 70 % whereas the group of enniatins exceeded these values with almost 200 % relative matrix effect. While for lower values of up to 20 % this variability is within an acceptable precision range, analytes with higher relative matrix effects are difficult to quantify correctly when the urine sample is much different from the used blank urine. Without isotope-labelled standards being commercially available for these analytes, and standard addition not being feasible for large sample numbers, this error has to be accepted and considered. Because the enniatins (BEA, ENA, ENA_1, ENB, ENB_1) showed pronounced variations in the strength of signal enhancement between urines, no further method parameters were determined, and a semi-quantitative measurement was applied, providing only an estimate of the concentration. Nevertheless, for all other analytes, accuracy and precision during intra- and inter-day measurements were recorded at low, medium and high spiking levels to assess method performance (Table 1: low and medium spiking level; Table S7, Supporting Information: high spiking level). Intra-day accuracies were lowest for QC_{Medium} of PAX and PENA, with 44 % and 51 %, respectively. Other values ranged between 74 % and 127 % for all analytes and spiking levels, showing good agreement with added standard concentrations over the whole calibration range. A slightly higher accuracy range of 40 % to 149 % was observed for inter-day measurements. Good intra-day precision <20 % was observed for 28 out of 31 analytes at all spiking levels (Table 1; Table S7, Supporting Information). The inter-day precision resulted in higher values with 22 out of 31 analytes ≤20 % RSD. Both α-ZEL-14-GlcA and TEA showed the highest

Table 1
Method validation data of the developed online SPE-LC-MS/MS method including intra- and inter-day accuracy and precision at the low and medium spiking level.

Analyte	LOD[a] / LOQ[b] [ng/mL urine]	Working range [ng/mL urine]	R^2 [c]	R_A^d / ME[e] [%]	Intra-day[f]		Inter-day[g]	
					Accuracy [%]	Precision [%]	Accuracy [%]	Precision [%]
AFM_1*	0.01 / 0.06	0.06–6	0.987	89	100 / 92	11 / 15	95 / 85	15 / 16
STG*	0.001 / 0.002	0.002–0.2	0.989	94	90 / 98	12 / 10	97 / 94	10 / 12
FB_1*	0.001 / 0.005	0.005–0.5	0.983	94	106 / 100	8 / 7	110 / 106	13 / 8
FB_2	0.003 / 0.010	0.010–1	0.993	– [h]	106 / 125	9 / 10	122 / 117	17 / 14
CIT*	0.062 / 0.125	0.125–12.5	0.996	92	102 / 93	8 / 3	111 / 112	10 / 18
DH-CIT*	0.003 / 0.016	0.016–1.6	0.992	104	111 / 107	23 / 11	98 / 105	17 / 10
OTA*	0.002 / 0.008	0.008–0.8	0.991	98	92 / 100	13 / 13	106 / 126	18 / 24
2'*R*-OTA*	0.002 / 0.008	0.008–0.8	0.989	88	99 / 105	12 / 15	121 / 119	21 / 15
OTα*	0.02 / 0.12	0.12–12	0.991	98	92 / 99	10 / 9	105 / 93	19 / 8
10-OH-OTA	0.004 / 0.020	0.020–2	0.994	77	110 / 97	6 / 4	112 / 100	14 / 3
OTB-NAC*	0.008 / 0.040	0.040–4	0.992	88	96 / 97	13 / 10	106 / 98	18 / 7
ZEN*	0.005 / 0.027	0.027–2.7	0.991	108	77 / 100	10 / 10	104 / 99	20 / 10
ZEN-14-GlcA	0.40 / 0.67	0.67–67	0.968	42	115 / 113	9 / 12	105 / 94	14 / 18
α-ZEL	0.053 / 0.107	0.107–10.7	0.992	56	81 / 105	12 / 16	86 / 94	25 / 18
β-ZEL	0.070 / 0.117	0.117–11.7	0.986	39	87 / 107	19 / 13	92 / 95	21 / 16
α-ZEL-14-GlcA	0.267 / 1.33	1.33–100	0.983	35	119 / 125	17 / 12	96 / 101	28 / 22
β-ZEL-14-GlcA	0.533 / 2.67	2.67–100	0.978	50	106 / 120	9 / 10	103 / 98	11 / 24
ZAN-14-GlcA	0.667 / 2.67	2.67–47	0.985	47	103 / 101	18 / 10	93 / 84	17 / 20
T-2*	0.020 / 0.080	0.080–8	0.981	113	94 / 106	14 / 14	111 / 94	19 / 15
HT-2*	0.833 / 5.0	5.0–100	0.990	95	103 / 92	18 / 15	103 / 105	16 / 8
HT-2-3-GlcA	0.007 / 0.02	0.02–2	0.992	31	105 / 100	8 / 6	89 / 90	16 / 12
HT-2-4-GlcA	0.005 / 0.016	0.016–1.6	0.985	16	115 / 108	6 / 5	107 / 92	13 / 13
DON*	0.10 / 0.67	0.67–67	0.995	99	86 / 116	11 / 5	89 / 105	13 / 13
DON-3-GlcA	0.33 / 1.3	1.3–47	0.993	62	106 / 112	17 / 5	98 / 101	11 / 8
ALT	0.34 / 2.0	2.0–40	0.964	15	82 / 99	9 / 7	102 / 97	19 / 8
AME	0.001 / 0.004	0.004–0.4	0.980	33	100 / 91	13 / 9	102 / 97	15 / 7
AOH	0.026 / 0.132	0.132–13.2	0.991	17	101 / 95	11 / 6	102 / 96	16 / 10
TEA*	0.064 / 0.192	0.192–19.2	0.985	92	127 / 115	21 / 8	130 / 104	28 / 8
α-CPA	0.003 / 0.008	0.008–0.8	0.990	389	101 / 93	7 / 5	149 / 119	26 / 17
PENA	0.005 / 0.027	0.027–2.7	0.975	– [h]	78 / 51	4 / 14	90 / 53	12 / 14
PAX	0.10 / 0.17	0.17–17	0.963	– [h]	76 / 44	3 / 3	68 / 40	11 / 10
BEA	0.033 / 0.067	0.067–1.3	0.985	– [h]	– [i]	– [i]	– [i]	– [i]
ENA	0.004 / 0.012	0.012–0.12	0.996	– [h]	– [i]	– [i]	– [i]	– [i]
ENA_1	0.007 / 0.012	0.012–0.23	0.997	– [h]	– [i]	– [i]	– [i]	– [i]
ENB	0.001 / 0.002	0.002–0.2	0.958	– [h]	– [i]	– [i]	– [i]	– [i]
ENB_1	0.002 / 0.010	0.010–1	0.952	– [h]	– [i]	– [i]	– [i]	– [i]

[a] Limit of detection at S/N 3; [b] Limit of quantification at S/N 10; [c] Regression coefficient; [d] Apparent recovery for analytes with internal standard; [e] Matrix effect (ME) for analytes without internal Standard; [f] Lowest and medium calibration point, $n = 10$, one day; [g] Lowest and medium calibration point, $n = 4$, three days; [h] No calibration in water could be established; [i] Semi-quantitative measurements due to strongly varying ME; * Stable isotope-labelled analogue standard available.

variation with 28 % at the lowest calibration level. The integration of 36 analytes required a complex setup, with multiple equilibration steps and valve switches that can cause greater variability between measurements. A conventional, less complex online SPE-LC-MS/MS setup showed better values for the intra-day precision, ranging only from 4 to 16 % at the lowest calibration point [26].

Commonly used DaS methods have no matrix reduction steps and usually low injection volumes. Accordingly, the LODs of several DaS methods found in literature are higher than that of the online SPE-LC-MS/MS method presented here [16,44,53]. The reported LODs of Gerding et al. [15,44] are at least three times higher, and even up to 1250-fold higher for FB_1. A similar improvement in sensitivity was observed for the DaS method by Warth et al. [16], although not all of the analytes measured here were included. The use of a QuEChERS-based liquid-liquid extraction (LLE) protocol to analyse 37 analytes in urine resulted in similar or higher LODs for most analytes [54]. The combination of LLE and strong anion-exchange SPE also showed mostly higher LOD values, but in line with the QuEChERS-based procedure, sensitivity for HT-2 was slightly better (0.42 ng/mL) [55]. For AFM_1 and CIT, lower LODs (2 and 1 pg/mL, respectively) were achieved using an IAC clean-up with separate columns for each analyte [11]. Šarkanj et al. [18] used Oasis PRiME HLB columns for offline SPE clean-up for highly sensitive analysis with LODs in the low pg/mL range for 12 mycotoxins and their metabolites in urine. The respective LODs for AFM_1, CIT, and α−/ β-ZEL were 21- to 53-times lower compared to the online SPE clean-up presented here. In a recently published online SPE-LC-MS/MS approach, slightly lower LOD values were reported for AFM_1, ALT, and α−/β-ZEL with 0.007, 0.21, and 0.05 ng/mL, respectively [26]. However, the LODs for OTA, FB_1, AOH, AME, and DH-CIT were 2- to 23-times higher. Furthermore, only 11 analytes were measured, whereas the method presented here includes 36 different mycotoxins and mycotoxin metabolites. It has to be mentioned that the method presented here uses a triple quadrupole mass spectrometer of the newest generation, which, at least in parts, enables more sensitive analysis compared to older instruments. In the same way, different blank urines used for method validation can lead to changes in method performance caused by matrix effects.

3.3. Method application

The newly developed method was used to analyse urine samples ($n = 50$) of pregnant women in rural Bangladesh. The aim of the cohort study, already published by Kyei et al. [45], was to link individual mycotoxin exposure to adverse pregnancy outcomes. Here, we applied the newly developed online SPE-LC-MS/MS method to evaluate its applicability for an improved biomonitoring analysis. The results are summarized in Table 2. All samples were contaminated with at least one mycotoxin or a mycotoxin metabolite, while one-third of all samples contained four different analytes. Most positives were detected for OTA with 98 %, followed by TEA + *allo*-TEA, DH-CIT, and STG with 86, 78, and 76 %. OTA and CIT are both mycotoxins produced mainly by *Aspergillus* and *Penicillium* fungi species, which primarily infest harvested crops like wheat, barley, or rice. Both are known to be nephrotoxic, and OTA is classified as possibly carcinogenic to humans [56–58].

Table 2
Results of the online SPE-LC-MS/MS analysis of urine samples ($n = 50$) from pregnant women in Bangladesh.

Analyte	Positives n (%)	n > LOQ	LOQ > n < LOD	95th Percentile [ng/mL]	Maximum [ng/mL]	Mean [ng/mL]	Median [ng/mL]
CIT	20 (40)	12	8	2.60	4.62	0.72	0.34
DH-CIT	39 (78)	38	1	0.82	6.11	0.30	0.060
OTA	49 (98)	36	13	0.509	2.37	0.21	0.092
OTα	4 (8)	0	4	–	–	–	–
T-2	5 (10)	0	5	–	–	–	–
DON	2 (4)	1	1	–	1.05	–	–
DON-GlcA	6 (12)	6	0	10.9	11.0	6.40	5.31
STG	38 (76)	11	27	0.032	0.033	0.012	0.007
ZEN	1 (2)	0	1	–	–	–	–
ZAN-14-GlcA	1 (2)	1	0	–	9.43	–	–
AOH	1 (2)	0	1	–	–	–	–
AME	7 (14)	2	5	–	0.009	–	–
TEA + *allo*-TEA	43 (86)	43	0	4.32	7.69	1.57	0.79
ENA_1	4 (8)	4	0	0.034	0.034	0.033	0.033
ENB	2 (4)	2	0	–	0.005	–	–

Median concentrations for OTA and CIT were 0.092 and 0.34 ng/mL urine. The main urinary metabolite DH-CIT was detected almost twice as much as its precursor, but at a lower median concentration of 0.060 ng/mL. In contrast, in Belgian adults, the prevalence of DH-CIT detections was only about a fifth compared to CIT, which is probably explained by the ten times higher LOD for DH-CIT compared to CIT in that study [59]. The high occurrence rate for TEA + *allo*-TEA is comparable with that of European cohorts in Germany (100 %) and Sweden (98.9 %), but the average concentration found here was about four times lower [60,61]. Although STG shows genotoxic and carcinogenic effects, it is rarely investigated in humans, which is probably due to its more potent biogenic relative, aflatoxin B_1 [62]. Nonetheless, with a very low LOD, it was detected in 38 of 50 samples, with up to 0.033 ng/mL urine.

This sample subset was previously measured using an established DaS method [15,44]. This allowed for a comparison to the newly developed online SPE method. Only OTA, CIT, and DH-CIT were detected with the DaS approach, while the here presented method detected 15 different mycotoxins and mycotoxin metabolites (Table 2). In total 304 % more positive detections were observed, clearly showing the effect of improved sensitivity for a multitude of mycotoxins. Of the analytes detected, STG and TEA were not included in the DaS approach, yet they showed high prevalence in the online SPE method. Fig. 3 depicts a direct comparison of positive detections for OTA, CIT, and DH-CIT using DaS and online SPE. Total positive OTA findings were only 8 % more frequent with the online SPE sample treatment compared to DaS, owing to the already low LOD of 0.02 ng/mL of the DaS method [44]. However, an increase in positive samples of 24 % and 38 % was observed for CIT and DH-CIT, respectively, for samples measured with the newly developed online SPE method compared to DaS. Of the positive CIT detections, 50 % were above the LOQ with DaS and reached 60 % with the online SPE technique. This increase in quantifiable samples was more pronounced for DH-CIT, with 25 % using DaS and 97 % using online SPE. The results clearly show how combining high sensitivity with a large analyte spectrum can improve human biomonitoring data and thus further risk assessment.

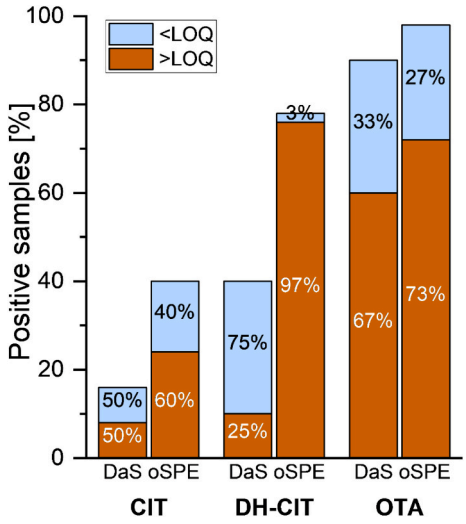

Fig. 3. Comparison of positive samples and respective percentages of positive samples above or below the limit of quantification (LOQ). Urine samples of Bangladeshi women ($n = 50$) were measured with the developed online solid phase extraction (oSPE) clean-up and a commonly used dilute-and-shoot (DaS) approach [15,44].

4. Conclusion

Creating meaningful results from human biomonitoring of mycotoxins relies on a sensitive analysis of a wide range of analyte structures and polarities. This challenges conventional analytical approaches, often resulting in a compromise between sensitivity, analyte spectrum, and throughput. Good specifications in these terms were achieved by careful optimization of an online SPE-LC-MS/MS approach with two-step peak focusing, as presented here. The analyte spectrum of the method covers confirmed biomarkers of exposure such as AFM_1, CIT, DH-CIT, DON, DON-glucuronides, OTA, and TEA, as well as other mycotoxins and mycotoxin metabolites that are not yet confirmed biomarkers. The analysis of these compounds in urine samples can help to identify potential biomarkers of exposure, although their applicability for exposure assessment still needs validation. The high sensitivity reduces uncertainties of exposure calculations due to a lower number of left-censored values. At the same time, the rapid sample preparation enables the analysis of large sample cohorts at high throughput. While reliable quantitative results were obtained for 21 analytes, BEA, ENA, ENA_1, ENB, and ENB_1 showed strong and highly variable matrix effects allowing only a semi-quantitative assessment. Overall, the developed online SPE-LC-MS/MS method enables more comprehensive human biomonitoring of mycotoxins. Future work will need to focus on developing strategies for reliable automated data analysis, as this aspect is becoming an increasingly significant bottleneck.

Author contribution

Michael Kuhn: Writing – original draft, investigation, methodology, validation, visualization; Nicholas N. A. Kyei: Writing – review & editing, resources (human samples); Benedikt Cramer: Writing – review & editing, supervision, conceptualization, project administration; Hans-Ulrich Humpf: Writing – review & editing, resources, supervision, conceptualization, funding acquisition, project administration.

CRediT authorship contribution statement

Michael Kuhn: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation. **Nicholas N.A. Kyei:** Writing – review & editing, Resources. **Benedikt Cramer:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Hans-Ulrich Humpf:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2025.115821.

Data availability

Data will be made available on request.

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