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Development and application of salting-out assisted liquid/liquid extraction for multi-mycotoxin biomarkers analysis in pig urine with high performance liquid chromatography/tandem mass spectrometry

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Abstract

Direct determination of urinary mycotoxins is a better approach to assess individual's exposure than the indirect estimation from average dietary intakes. In this study, a new analytical method was developed and validated for simultaneous analysis of aflatoxin B_1, deoxynivalenol, fumonisin B_1, ochratoxin A, zearalenone and T2 toxin and their metabolites in pig urine. In total 12 analytes were selected. A salting-out assisted liquid-liquid extraction procedure was used for sample preparation. High performance liquid chromatography tandem mass spectrometry was used for the separation and detection of all the analytes. The extraction recoveries were in a range of 70% to 108%, with the intra-day relative standard deviation and inter-day relative standard deviation of lower than 25% for most of the compounds at 3 different concentration levels. Meanwhile method bias for all the analytes did not exceed 20%. The method limits of quantification ranged from 0.07 ng mL^{-1} for ochratoxin A to 3.3 ng mL^{-1} for deoxynivalenol. Matrix effect was evaluated in this study and matrix-matched calibration was used for quantification. The developed method was also validated for human urine as an extension of its application. Finally, the developed method was applied in a pilot study to analyze 28 pig urine samples. Deoxynivalenol, aflatoxin B_1, fumonisin B_1 and ochratoxin A were detected in these samples.

1. Introduction

Mycotoxins, a diverse group of secondary metabolites produced by fungi, can contaminate a wide range of plants, thus compromising the safety of food or feed supplies and adversely affecting the health of humans as well as animals [1]. Mycotoxins have a wide range of adverse effects such as carcinogenic, nephrotoxic, hepatotoxic, neurotoxic, mutagenic, estrogenic and immunosuppressive effects [2-4], and hence may lead to great economic losses to farm husbandry [5]. In addition to mycotoxicoses which is caused by direct consumption of contaminated food and feed, the effect of "carry over" of mycotoxins and their metabolites into animal tissues, milk and eggs should not be neglected.

Among the mycotoxins that have been found in food, aflatoxin B_1 (AFB_1), deoxynivalenol (DON), fumonisin B_1 (FB_1), ochratoxin A (OTA), zearalenone (ZEN) and T2 toxin (T2) have been reported as the most frequently occurring toxins [6]. These mycotoxins often co-occurred naturally in cereals since one kind of crop can be infected by different toxigenic moulds and also each mould can produce several kinds of mycotoxins simultaneously [5]. Therefore, the actions of these co-occurring mycotoxins on human or animal can be antagonistic, additive or synergistic. Additionally, in order to understand the possible links between mycotoxins and human disease/animal toxicosis, it is necessary to measure the exposure of a population to the multiple toxins.

Traditional evaluation of human and animal exposure to mycotoxins is based on direct analysis of food and feed or more generally based on occurrence data combined with consumption data [7, 8]. However, this approach has some unavoidable shortcomings. Firstly, there are some other routes for mycotoxins exposure such as dermal contact and inhalation. Secondly, during disease outbreak or toxicosis implicating mycotoxins, the feed or food is already destroyed before it can be analyzed [1]. Last but not the least, the traditional evaluation method is only suitable to assess the exposure of populations to some toxins or to identify the risk group; it cannot reflect accurate information of individual intake of mycotoxins.

To circumvent all these shortcomings, biomarkers have been proposed as suitable targets to assess mycotoxin exposure. Different from the analysis of food and feed, the measurement of biomarkers of exposure can account for variations in food contamination levels. All the factors, such as food consumption, exposure routes, diet composition and food preparation techniques, metabolism and excretion of the toxin can be integrated into the formation of one

indicator (biomarker), which will, out of question, greatly compromise all sources not being taken into account and simplify the analytical procedure [9, 10]. Therefore, biomarkers allow for more accurate and objective assessment of exposure at the individual level. So far, advances in analytical techniques in the fields of molecular biology and biochemistry have allowed the development and usage of various biomarkers in human and animal tissue or body fluids, and thus can provide definitive identification of a specific mycotoxicosis [11-13]. Generally, the candidate biomarkers include the excreted toxin or its metabolites, as well as the products of interaction between the toxin and macromolecules such as protein or nucleic acid. However, the choice of biomarkers are subjected to the commercial availability of these compounds, the convenience of its use, and the scientific question to be solved.

Nowadays, liquid chromatography (LC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) has been widely used as a confirmatory method for multi-mycotoxin detection with high selectivity and sensitivity [14-16]. However, due to the wide range of properties of the different mycotoxins, sample preparation method still remains the bottleneck in the entire protocol. A wide variety of sample preparation has been reported in literature for mycotoxins, such as liquid-liquid extraction (LLE), solid phase extraction (SPE) and dilute-and-shoot (DAS) [17, 18]. SPE is widely used in sample preparation now days. However, this technique still has some limitation, such as high requirement for clean sample, not applicable for solid sample and high variability in results [19]. Although the application of the newly developed materials has greatly improved their performance, these commercial SPE columns are relatively expensive which hinder their uses in high through-put analysis. In addition, for multi-mycotoxin analysis, with SPE and LLE, it is difficult to obtain satisfactory recoveries for all compounds in one step [20]. Different from SPE or LLE, the DAS approach is a promising sample preparation method for multi-residue detection in urine and plasma especially when the concentration levels of target analytes are relatively high and the matrix components do not co-elute or interfere with ionization of the target analytes [21]. However the wide spectra of DAS are based on the sacrifice of the sensitivity.

In LLE, the nonpolar, water-immiscible organic solutions are relatively poor for the extraction of polar compounds due to their low dielectric constants. Although the more polar organic solvents, such as ethanol, methanol, acetone or acetonitrile, can provide solubility for the related compounds, they are water-miscible which cannot be adopted for conventional LLE method. It is also well known that these kind of polar organic solvents are miscible with water at any proportion. However, the addition of salts can reduce the mutual miscibility, and

can even lead to phase separation [22]. Therefore, with the help of salt, the polar analytes which existed in the aqueous phase can selectively move into the polar organic phase. This technique is called salting-out assisted liquid/liquid extraction (SALLE). So far, sample preparation using SALLE for multi-mycotoxin analysis from biological fluids has not yet been reported. Most of the applications reported with this method are limited to sample analysis in plasma [22-24]. In this paper, we describe the development of a cost-effective, time-efficient and easy-to-use sample preparation method based on SALLE for the simultaneous extraction of the 12 most important mycotoxins and/or their metabolites from pig urine samples. Coupled with an optimized LC-MS/MS method, this method was successfully applied for the determination of mycotoxins and their metabolites in pig urine samples.

2. Experimental

2.1 Reagents and chemicals

Methanol and acetonitrile were of HPLC and HPLC-MS grade (VWR International, Zaventem, Belgium). Ethyl acetate was purchased from Acros Organics (Geel, Belgium). Ammonium acetate and ammonium formate were supplied by Grauwmeer (Leuven, Belgium) and Sigma-Aldrich (Bornem, Belgium) respectively. Formic acid (FA), magnesium sulfate and ammonium sulfate were supplied by Merck (Darmstadt, Germany). Ultrafree-MC centrifugal filter devices (0.22 μm) of Millipore (Millipore, Brussels, Belgium) were used. Water was purified on a Milli-Q Plus apparatus (Millipore, Brussels, Belgium).

Mycotoxin standards neosolaniol (NEO), aflatoxin B_1 (AFB_1), HT2 toxin (HT2), ochratoxin A (OTA), zearalenone (ZEN), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL) and fumonisin B_1 (FB_1) were purchased from Sigma-Aldrich (Bornem, Belgium). T2 toxin (T2) was obtained from Romer Labs (Tulln, Austria). Deoxynivalenol (DON) and aflatoxin M_1 (AFM_1) were purchased from Fermentek (Jerusalem, Israel). Ochratoxin α (OTα) was obtained from Coring System Diagnostix (Gernsheim, Germany). OTα and NEO were obtained as solutions, at 10.3 μg mL^{-1} and 104.7 μg mL^{-1} respectively in acetonitrile. Stock solutions of DON, AFM_1, and AFB_1, HT2, T2, OTA, ZEN, FB_1, α-ZEL and β-ZEL were prepared in methanol (1 mg mL^{-1}) and stored at -20°C. From the individual stock standard solutions, a standard mixture was prepared in methanol and stored at -20°C. Fresh standard mixture was prepared every month at the following concentrations: DON (50 ng μL^{-1}); OTα

(25 ng μL^{-1}); NEO (20 ng μL^{-1}); T2 and AFB_1 (2 ng μL^{-1}); AFM_1, α-ZEL, β-ZEL and ZEN (10 ng μL^{-1}); HT2 and FB_1 (5 ng μL^{-1}); OTA (1 ng μL^{-1}). The structures of selected biomarkers are shown in Fig. 1.

2.2 Instruments and analytical conditions

A Waters Acquity ultra-performance liquid chromatography (UPLC) system coupled to a Micromass Quatro Micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA) was used to analyze the samples. The analytical column was a Symmetry C_{18}, 5 μm, 2.1×150 mm (Waters, Milford, MA, USA), the guard column was a Waters Sentry C_{18}, 3.5 μm 2.1×10mm (Waters, Milford, MA, USA). The column was kept at room temperature.

A mobile phase consisting of eluent A (water, 0.3% formic acid, 5mM ammonium formate) and eluent B (methanol, 0.3% formic acid, 5mM ammonium formate) was used at a flow rate of 0.25 mL min^{-1}. The gradient elution program applied was as follows: 0-1 min, 5% B; 1-5 min, 5-25% B; 5-7 min, 25-60% B; 7-15 min, 60-80% B; 15-16 min, 80-100% B; 18-22 min, 100% B; 22-25 min, 5% B. The sample injection volume was set at 20 μL. All the compounds were eluted within the first 15 min. The last 10 minutes were used for column cleaning and re-equilibration.

The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode and the mass spectrometer analyses were carried out using multiple reaction monitoring (MRM) mode to get the best sensitivity and selectivity. MS parameters were set as follows: ESI source block and desolvation temperatures, 120°C and 350°C respectively. Capillary voltage, 3.2 kV; cone and desolvation gas flows of 20 and 800 L h^{-1}, respectively. After selection of the precursor ion for each analyte, product ions were obtained through a combination of cone voltages and collision energies.

The optimum MS/MS conditions of the mycotoxins were obtained by direct injection of 10 ng μL^{-1} (20 ng μL^{-1} for DON and OTα) of freshly prepared standard solutions in methanol/ultrapure water containing 5mM ammonium formate (50/50, v/v) and 0.3% FA with a flow rate of 10 μL min^{-1} for 30 s. Full scan and daughter scan mass spectra were acquired in order to obtain at least one precursor and two product ions for each compound for both identification and quantification purposes. The most abundant product ion was selected for quantification while the second product ion for confirmation.

2.3 Samples and Sample preparation

Three batches of pig urine samples (28 samples in total) were obtained from three different animal farms in Belgium. All samples were stored at -20°C until analysis. Because certified blank urine samples were not available, samples with undetected levels of the analytes of interest were chosen as "blank" and used in spiking and recovery studies. Prior to analysis, urine samples were thawed completely at room temperature and centrifuged at 4000 g for 10 min to sediment particulate matter. Three different sample preparation approaches were evaluated in this study, namely, SALLE, DAS and "dilute-evaporate-and-shoot" (DES). For both DAS and DES approaches, different dilution factors were tested. Meanwhile the factor of 2 was chosen as a compromise in terms of sensitivity and matrix effects.

SALLE

A 5 mL urine was transferred into a 50 mL Gosselin tube, followed by addition of 10 mL of $MgSO_4$ (2 M). A 5 mL ethyl acetate/FA (99/1, v/v) extraction solvent was added to each of the tubes and extraction was performed for 15 min on an overhead shaker, followed by centrifugation for 15 min at 4000 g. The ethyl acetate phase was aspirated into a new extraction tube. Then 5 mL of acetonitrile/FA (99/1, v/v) was added to the remaining aqueous phase, and extraction was repeated as previously described. After extraction, the acetonitrile phase was combined with the ethyl acetate phase and evaporated at 60°C under a gentle stream of nitrogen. Before analysis, a 500 µL of injection solvent, which contained 50% each of mobile phase A and B was used to reconstitute the residue. After filtration through a centrifugal filter (Millipore Corporation, Billerica, United States) for 5 min at 10000 g, 200 µL volume of this filtrate was brought into vials and used for analysis.

DAS

A simple DAS approach was evaluated for the analysis of urine sample based on the method developed by Warth et al. [25]. Concisely, 5 mL of urine was mixed with an equal volume of methanol. After filtration, 20 µL volume of this diluted urine was injected into the LC-MS/MS.

DES

The DES approach was modified based on the DAS method. After mixing the urine samples with the same volume of methanol, the mixture was centrifuged for 15 min at 4000 g. Afterwards the supernatant was evaporated at 60°C under a gentle stream of nitrogen. The reconstitution step and injection sequence were the same as described in SALLE.

2.4 Method validation

The evaluated performance characteristics of this multi-mycotoxin method included linearity, apparent recovery (R_A, expressed by bias), extraction recovery (R), repeatability (intra-day relative standard deviation, RSD_r), intra-laboratory reproducibility (inter-day relative standard deviation, RSD_R), limit of detection (LOD) and quantitation (LOQ), matrix effects, selectivity and expanded measurement uncertainty. Commission Decision 2002/657/EC and 401/2006/EC were used as guidelines for the validation studies. All the parameters were determined with spiked blank samples.

Method linearity was assessed by spiking blank urine samples at 5 concentration levels for each analyte. Calibration curves were obtained by plotting the peak area versus the analyte concentration. The coefficient of determination (R^2) was determined by means of the least square approach for each analyte. The method bias was estimated by fortifying blank urine samples with standards at 3 different concentrations. Meanwhile the peak area ratio of the sample spiked before extraction to sample spiked after extraction were used to calculate the extraction recovery of the entire sample preparation procedure [26]. The repeatability was evaluated at 3 concentration levels on the same day. Meanwhile for intra-laboratory reproducibility, the three concentrations were analyzed in three different days. A fresh solution was prepared daily for the inter-day precision. The concentration levels used for the different analytes were presented in Table 3.

LODs and LOQs were determined as the lowest concentration of the selected compounds that produce chromatographic peaks with signal to noise ratio (S/N) of 3 and 10 respectively [16]. Data from this approach were justified by calculating 3 times or 6 times the standard error of the intercept divided by the slope of the calibration curve for the LOD and LOQ respectively [15].

Matrix effect was determined by constructing calibration curves in blank extract and in the pure solvent. The effects were expressed in terms of signal suppression/enhancement (SSE) and calculated as follows: SSE=100×slope of spiked extract/slope of pure standard [27]. The selectivity was evaluated by analyzing 6 different blank urine samples. The signal interference between different MRMs was checked. The identification of the target mycotoxins was carried out by searching the characteristic transitions of the analytes in the appropriate retention time windows (RTW), which were obtained by mean retention time ± three times the standard deviation of the retention time of 6 blank samples.

The expanded measurement uncertainty (*U*) was obtained using the top down approach as

described by Ediage et. al. [28], in which, the intra-laboratory reproducibility standard deviation (S_{RW}), the uncertainty associated with the mean recovery (U_{bias}) as well as the uncertainty due to the purity of the standards ($U(C_{ref})$) were taken into account. Briefly, the expanded measurement uncertainty was estimated by multiplying the combined uncertainty (U_c) by the coverage factor 2 (corresponding to a confidence interval of approximately 95%). The equation used to calculated the U_c was as follows: $U_c{}^2 = (S_{RW})^2 + (U(C_{ref}))^2 + (U_{bias})^2$.

All the experiments were repeated at least 3 times at each concentration level.

3. Results and discussion

3.1 Optimization of the LC-MS/MS conditions

The concentrations of the mycotoxins and/or metabolites often occur in low ng mL^{-1} in urine samples, hence it is important to optimize the sensitivity of the method. For this purpose, the MS conditions were thoroughly optimized. Firstly, the appropriate precursor and product ions were obtained by infusion of individual standard solutions. The results showed that all the 12 analytes except DON gave better response in ESI+ than in negative electrospray ionization mode (ESI-). Therefore, as a compromise, the ESI+ mode was applied for all the analytes. With full scan and product ion scan, the precursor ions of T2, HT2 and NEO were ammonium adducts $[M+NH4]^+$, while the others were protonated adducts $[M+H]^+$. The influence of the matrix towards the peak shape of the quantification ions was also considered. The optimized MS/MS parameters are listed in Table 1.

In order to obtain an acceptable HPLC separation due to the wide range of polarity of the different analytes, different LC separation parameters was optimized. Acid is usually used to improve the chromatographic peak shape and provide a source of protons in reverse phase LC-MS. In this study, we compared the different effects of FA and acetic acid on the chromatographic behavior. The results demonstrated that addition of 0.3% aliquot of formic acid gave better signal intensity; moreover, it also helped in the separation of the two ZEL isomers (α-ZEL and β-ZEL). As reported in the literature, both ammonium acetate and ammonium formate can be used to avoid the formation of stable sodium adducts [29]. In this study, ammonium formate gave much better response than ammonium acetate for all the compounds investigated. More importantly, without addition of ammonium ion into the mobile phase, no response can be detected for T2, HT2 and NEO. Other LC parameters, such as the flow rate and gradient designs were also tested. Based on these experiments, water with 0.3% FA and 5mM ammonium formate was used as mobile phase A, while methanol with 0.3% FA and 5mM ammonium formate was used as mobile phase B. All the biomarkers were

eluted between 7 min and 15 min. The total run time was 25 min including column re-equilibration (Fig. 2A).

3.2 Salting out assisted liquid-liquid extraction

For multi-residue analysis, the sample preparation procedure constitutes one of the critical steps, especially when biological matrices, such as blood and urine, are analyzed. The partition coefficient (the ratio of concentrations of the compound in water and in organic solvent octanol, Log*P*) is often used to measure how hydrophilic or hydrophobic a chemical substance is. The postulated Log*P* values of the biomarkers are demonstrated in Table 1. The data were taken from PubChem Public Chemical Database (http://pubchem.ncbi.nlm.nih.gov/). As shown in these data, some biomarkers, such as DON, NEO and FB_1, are more hydrophilic (Log*P*<0). Hence it is difficult to get satisfactory recoveries for these analytes compared to the other low polarity analytes (Log*P*>0), especially when LLE is performed with water-immiscible organic solvent as the extraction solvent. For the same reason, few papers have been published for multi-analyte preparation from urine samples using SPE [14]. The use of immuno-affinity columns can solve these problems, however, these columns are sometimes very expensive. Furthermore, it is not generic material and not applicable for muti-analyte detection [30-32].

LLE with water-miscible organic solvents had so far been reported for analysis of biological samples [23, 24, 33]. It can improve the recovery and applicability and greatly reduce the extraction time. However, all the reports were targeted at specific analytes so far. The chemical properties of these analyses were within a narrow range and the matrices were confined to blood. Therefore, SALLE method was developed for the extraction of 12 mycotoxin biomarkers with widely ranged properties in urine samples in this research.

Although acetone, methanol, ethanol and acetonitrile (with the polarity index of 5.1, 5.1, 5.2 and 5.8) are candidates for SALLE, acetonitrile was selected in this study with regard to its more close polarity to that of water. Firstly, the effects of different salts towards the recovery of each biomarker were compared. Inorganic and organic salts such as $MgSO_4$, $(NH_4)_2SO_4$ and NH_4Ac were evaluated. For convenience, high concentration salt solutions were evaluated in this study. The optimized results are listed in Fig. 3. It has been suggested that NH_4Ac was a better salting-out agent [24], because of its compatibility with spectrometry. However the recoveries for all the target analytes were very low (<75%), with FB_1 being the least recovered analyte (recovery, <1%). Further modifications in the

concentration of NH_4Ac could not enhance the recoveries. On the contrary, all the other salt solutions gave very satisfactory results, the recoveries for all the 12 biomarkers were more than 70%. The possible reason the high recoveries is that $MgSO_4$ has more ionic strength (4 mol L^{-1}) per unit concentration in aqueous phase than the other salts, which will facilitate the phase separation and also improve the analyte recovery [22, 34]. Obtaining satisfactory recovery for FB_1 has been one major challenge in the field of mycotoxin research; however, with the SALLE protocol, its recovery was more than 80%. Given that the extraction recoveries for all the analytes were similar for both 2 M $MgSO_4$ and a saturated solution, a 2 M concentration of $MgSO_4$ was preferred for economical reason.

Due to the fact that the SALLE approach comprised of two consecutive LLE steps, (1) with ethyl acetate and (2) with acetonitrile, the efficiency of extraction of each of these steps were evaluated separately. The results indicated that all the analytes gave much lower recoveries when ethyl acetate was used for extraction (Fig. 4). Interesting to mention is that the recoveries for FB_1 changed apparently from 0% with only ethyl acetate extraction to about 100% when two LLE steps were performed. Other parameters, such as the required volume of urine, volume of $MgSO_4$ solution and volume of extraction solvent, were optimized during the extraction. The best parameters were as follows: 5 mL of urine, 10 mL of 2 M $MgSO_4$ solution, 5 mL of ethyl acetate and 5 mL of acetonitrile. Fig. 2B shows the typical chromatogram of a blank pig urine sample spiked with 12 of the targeted mycotoxins.

3.3 Comparison of SALLE, DAS and DES

DAS and DES are sometimes preferred to LLE and SPE because of their simplicity and high sample throughput..

We compared SALLE with DAS and DES in this study. For each of these approaches, calibration curves were constructed in blank urine samples. From these calibration curves, the slopes and LODs were computed for comparison. As shown in Table 2, SALLE had the highest slope values for all the compounds in pig urine, which indicated that the matrix effect was minimal. Meanwhile for the other two approaches, much more serious signal suppression was observed. In addition, although DES had better sensitivity and response than DAS because of the concentration step (evaporation), both the approaches required high concentration of the analytes for a significant signal to be seen. In some extreme cases (AFM_1), the LODs were 8 and 20 times higher with DES and DAS than with SALLE. Normally, DAS and DES are applicable when the LODs fulfill the aim of the study. However,

in this research, it was not advisable to inject sample directly into the LC-MS/MS using these two approaches. The main reason was that the sample extract from DAS and DES were still very dirty, which could shorten the HPLC column life.

3.4 Validation of the SALLE approach

Next to pig urine, the developed method was also validated for human urine as an extension of its application.

The method performance characteristics were obtained by spiking the urine samples at 5 levels and quantified by use of matrix-matched calibration curves. The results were shown in table 3. The coefficients of determination (R^2) for the different analytes were higher than 0.98 which indicated good linearity of the analytical method, except for AFB_1 and OTA which had R^2 of 0.97. As demonstrated in Table 3, the biases for different analytes both in pig urine and human urine was within 20% at all spiked concentrations, which confirmed the suitability of the proposed extraction procedure for the simultaneous extraction of 12 mycotoxins from pig and human urine samples.

The extraction process could be regarded as a pre-concentration process, hence it is desirable to evaluate the yield of the extraction step for all the compounds. As shown in Table 3, the extraction recoveries of the different analytes from pig urine were between 70% and 108%. It is worth to point out that FB_1, the most difficult compound in sample preparation, also had high recoveries (more than 80%). To the best of our knowledge, only a few papers reported extraction yields of FB_1 of more than 80% in food or feed [35]. For biofluid matrices, this is the first report in which high recoveries for FB_1 were obtained without the use of SPE. Meanwhile the method RSD_r was lower than 20% for both pig and human urine samples for all the analytes except for OTα (<31%). Meanwhile the RSD_R was lower than 25%, for both animal and human urine, again except for OTα (<29%). The LOQ ranged from 0.07 ng mL^{-1} (OTA) to 3.3 ng mL^{-1} (DON) for pig urine, while for human urine the LOQ ranged from 0.03 ng mL^{-1} (OTA) to 1.7 ng mL^{-1} (DON). No co-eluting peaks were observed at the RTW for the different analytes, which thus confirms the good selectivity of the analytical method (Fig. 2B). The expanded measurement uncertainty (*U*) is a criterion for the integral acceptability of a analytical method. In this study, most of the calculated expanded measurement uncertainties were below 40%, except for OTα (50% and 41% for pig urine and human urine samples respectively). The high *U* values for OTα could have been greatly influenced by the high intra-laboratory reproducibility levels.

It is well established that, with LC-MS/MS, the presence of matrix can influence the ionization of the target analytes, and thus could result in signal suppression or enhancement. As can be seen in Table 3, all the mycotoxins were subject to signal suppression. For most of the compounds, the signal recovered was low both in pig urine and human urine (<30%), except for T2, FB_1 and OTA, for which, the signal recovered was higher than 30% and 40% in pig urine and human urine respectively.

4. Application of the validated method

Once the method was validated, it was applied to investigate the occurrence of the 12 analytes in 28 pig urine samples. To ensure the reliability of the results, matrix-matched calibration samples, together with blank urine and pure solvent control were analysed.

As shown in Table 4, only DON, AFB_1, FB_1, and OTA were detected in pig urine samples. In total, 68% (19/28) of the samples were contaminated with one or more mycotoxins. Of these 19 samples, one sample was contaminated with 3 mycotoxins (sample 19); 5 samples were found with two mycotoxins at the same time (sample 2, 5, 8, 16 and 18). Thirteen samples were found positive for only one kind of mycotoxin. In these contaminated samples, DON had the highest frequency of occurrence (50%), with concentrations ranging from less than LOQ to 302 ng mL^{-1}. Eighteen percent of the samples were found contaminated with OTA (less than LOQ to 0.32 ng mL^{-1}), with no co-occurrence of OTα. In addition, 4 samples were found contaminated with FB_1, the concentration ranged from less than LOQ to 0.74 ng mL^{-1}. AFB_1 was detected in three samples, without co-occurrence of AFM_1. A typical chromatogram of sample 19 is shown in Fig. 5A, which was co-contaminated with DON (18.7 ng mL^{-1}) (Fig. 5B), AFB_1 (0.32 ng mL^{-1}) (Fig. 5C) and OTA (0.32 ng mL^{-1}) (Fig. 5D).

The contamination data from the three different animal farms were also compared. The frequency of occurrence for DON was 30% (5/16), 100% (7/7) and 40% (2/5) for samples obtained from farm 1, 2 and 3 respectively. Except for two samples (14 and 15), all the other samples from farm 2 had DON concentration greater than 17.4 ng mL^{-1}. It can therefore be speculated that all the animal feed from the 3 farms were contaminated with DON, with the situation in farm 2 been the most serious. AFB_1 was detected only in the urine of farm 3 animals, while FB_1 was detected only in urine of animals from farm 1. OTA was also detected in the urine of pigs from the three farms. All these indicated that the selected compounds were good indicators to reflect the contamination of feed from different farms. Hence the direct determination of urinary mycotoxins and/or metabolites is a approach to assess individual's

exposure. Furthermore, for the samples from the same farm, the concentration of particular biomarkers varied a lot, which demonstrated that the feed ingestion was different across the different pigs, so biomarkers are more applicable to evaluate individual exposure.

5. Conclusion and outlook

A high-throughput method based on SALLE with acetonitrile as extraction solvent was developed for the simultaneous detection of DON, AFB_1, FB_1, T2, OTA and ZEN and their possible metabolites in this work. After evaluation of the performance characteristics, this method was successfully applied to the analysis of pig urine samples. The whole SALLE sample preparation procedure included 2 steps of LLE with ethyl acetate and acetonitrile respectively. With regard to other multi-mycotoxin determination methodologies, this method was fast and easy to perform. $MgSO_4$ proved to be very efficient for phase separation of urine and water-miscible acetonitrile. The SALLE method showed very good extraction of the polar compounds, such as DON and FB_1. It must be highlighted that, although 12 analytes could be simultaneously detected, this method can be easily expanded for detection of more compounds with a wide range of physiochemical properties after validation.

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References

[1] S.O. Sherif, E.E. Salama, M.A. Abdel-Wahhab, Int. J. Hyg. Environ. Health 212 (2009) 347.
[2] P. Zollner, B. Mayer-Helm, J. Chromatogr. A 1136 (2006) 123.
[3] J.W. Bennett, M. Klich, Clin. Microbiol. Rev. 16 (2003) 497.
[4] C.P. Wild, Y.Y. Gong, Carcinogenesis 31 (2010) 71.
[5] CAST, Council for Agricultural Science and Technology, Ames,Iowa,USA, 2003 p. 1.
[6] R.T. Riley, J. Pestka, In: D.E. Diaz (Ed.), The Mycotoxin Blue Book, Nottingham University Press, 2005, 2794.
[7] M. N. Routledge, Y. Y. Gong, in: S. De Saeger (Ed.), Determining mycotoxins and mycotoxigenic fungi in food and feed, Woodhead Publishing , 2011, pp. 225.
[8] C. Brera, F. Debegnach, V. Minardi, E. Prantera, E. Pannunzi, S. Faleo, B. De Santis, M. Miraglia, J. Agric. Food Chem. 56 (2008) 10611.
[9] R. Montesano, P. Hainaut, J. Hall, IARC Sci. Publ. 142 (1997) 291.
[10] P.C. Turner, P. Nikiema, C.P. Wild, Mutat. Res. 443 (1999) 81.
[11] T.T. Baldwin, R.T. Riley, N.C. Zitomer, K.A. Voss, R.A. Coulombe, J.J. Pestka, D.E. Williams, A.E. Glenn, World Mycotoxin J. 4 (2011) 257.
[12] S.C. Duarte, A. Pena, C.M. Lino, Crit. Rev. Toxicol. 41 (2011) 187.

[13] F.A. Meky, P.C. Turner, A.E. Ashcroft, J.D. Miller, Y.L. Qiao, M.J. Roth, C.P. Wild, Food Chem. Toxicol. 41 (2003) 265.
[14] M. Solfrizzo, L. Gambacorta, V.M. Lattanzio, S. Powers, A. Visconti, Anal. Bioanal. Chem. 401 (2011) 2831.
[15] S. Monbaliu, A. Wu, D. Zhang, C. Van Peteghem, S. De Saeger, J. Agric. Food Chem. 58 (2010) 12664.
[16] E.N. Ediage, J.D. Di Mavungu, S. Monbaliu, C. Van Peteghem, S. De Saeger, J. Agric. Food Chem. 59 (2011) 5173.
[17] R. Krska, P. Schubert-Ullrich, A. Molinelli, M. Sulyok, S. Macdonald, C. Crews, Food Addit. Contam. A 25 (2008) 152.
[18] B. Warth, M. Sulyok, P. Fruhmann, H. Mikula, F. Berthiller, R. Schuhmacher, C. Hametner, W.A. Abia, G. Adam, J. Frohlich, R. Krska, Rapid Commun. Mass Spectrom. 26 (2012) 1533.
[19] M.C. Hennion, J. Chromatogr. A 856 (1999) 3.
[20] E. Van Pamel, A. Verbeken, G. Vlaemynck, J. De Boever, E. Daeseleire, J. Agric. Food Chem. 59 (2011) 9747.
[21] J. Henion, E. Brewer, G. Rule, Anal. Chem. 70 (1998) 650a.
[22] J. Zhang, H.Q. Wu, E. Kim, T.A. El-Shourbagy, Biomed. Chromatogr. 23 (2009) 419.
[23] J. Zhang, R. Rodila, E. Gage, M. Hautman, L.M. Fan, L.L. King, H.Q. Wu, T.A. El-Shourbagy, Anal. Chim. Acta 661 (2010) 167.
[24] H.Q. Wu, J. Zhang, K. Norem, T.A. El-Shourbagy, J. Pharm. Biomed. Anal. 48 (2008) 1243.
[25] B. Warth, M. Sulyok, F. Berthiller, R. Schuhmacher, P. Fruhmann, C. Hametner, G. Adam, J. Frohlich, R. Krska, Anal. Bioanal.Chem. 401 (2011) 195.
[26] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[27] M. Sulyok, F. Berthiller, R. Krska, R. Schuhmacher, Rapid Commun. Mass Spectrom. 20 (2006) 2649.
[28] E.N. Ediage, J.D. Di Mavungu, M.L. Scippo, Y.J. Schneider, Y. Larondelle, A. Callebaut, J. Robbens, C. Van Peteghem, S. De Saeger, J. Chromatogr. A 1218 (2011) 4395.
[29] A.G. Frenich, J.L.M. Vidal, R. Romero-Gonzalez, M.D. Aguilera-Luiz, Food Chem. 117 (2009) 705.
[30] J. Ahn, D. Kim, H. Kim, K.Y. Jahng, Food Addit. Contam. A 27 (2010) 1674.
[31] N. Thieu, H. Pettersson, Mycotox. Res. 25 (2009) 59.
[32] J. Rubert, J.M. Soriano, J. Manes, C. Soler, Food Chem. Toxicol. 49 (2011) 2299.
[33] F. Myasein, E. Kim, J. Zhang, H.Q. Wu, T.A. El-Shourbagy, Anal Chim Acta 651 (2009) 112.
[34] G.M. Nikolic, J.M. Perovic, Facta Universitatis 2 (2003) 293.
[35] C. Cavaliere, P. Foglia, E. Pastorini, R. Samperi, A. Lagana, Rapid Commun. Mass Spectrom. 19 (2005) 2085.

Figure and table captions

Fig. 1.

Structures of selected mycotoxin biomarkers.

Fig. 2.

Chromatograms of 12 compounds in standard solution (A) and in pig urine after SALLE (B).

The concentration of each compound was as follows: DON (5 ng mL^{-1}), NEO (2 ng mL^{-1}), AFM_1 (1 ng mL^{-1}), AFB_1 (0.2 ng mL^{-1}), OTα (2.5 ng mL^{-1}), FB_1 (0.5 ng mL^{-1}), HT2 (0.5 ng mL^{-1}), T2 (0.2 ng mL^{-1}), β-ZEL (1 ng mL^{-1}), α-ZEL (1 ng mL^{-1}), ZEN (1 ng mL^{-1}), OTA (0.1 ng mL^{-1}).

Fig. 3.

The effects of different salts towards the recovery of each analytes.

The concentration of each compound was as follows: DON (100 ng mL^{-1}), NEO (40 ng mL^{-1}), AFM_1 (20 ng mL^{-1}), AFB_1 (4 ng mL^{-1}), OTα (50 ng mL^{-1}), FB_1 (10 ng mL^{-1}), HT2 (10 ng mL^{-1}), T2 (4 ng mL^{-1}), β-ZEL (20 ng mL^{-1}), α-ZEL (20 ng mL^{-1}), ZEN (20 ng mL^{-1}), OTA (2 ng mL^{-1}).

Fig. 4.

The extraction yields of LLE with only ethyl acetate and with SALLE for 12 mycotoxins biomarkers in pig urine.

The concentration of each compound was as follows: DON (100 ng mL^{-1}), NEO (40 ng mL^{-1}), AFM_1 (20 ng mL^{-1}), AFB_1 (4 ng mL^{-1}), OTα (50 ng mL^{-1}), FB_1 (10 ng mL^{-1}), HT2 (10 ng mL^{-1}), T2 (4 ng mL^{-1}), β-ZEL (20 ng mL^{-1}), α-ZEL (20 ng mL^{-1}), ZEN (20 ng mL^{-1}), OTA (2 ng mL^{-1}).

Fig. 5.

The chromatogams of a contaminated pig urine sample.

(A) the TIC chromatogram; (B) the extracted ion chromatogram of DON (18.7 ng mL^{-1}); (C) the extracted ion chromatogram of AFB_1 (0.32 ng mL^{-1}); (D) the extracted ion chromatogram of OTA (0.32 ng mL^{-1}).

Table 1.

Optimized ESI+ MS/MS parameters, partition coefficient values (Log*P*) and retention time window (RTW) of the analytes.

[a] Log*P*, source: PubChem Public Chemical Database (http://pubchem.ncbi.nlm.nih.gov/).

[b] CE=collision energy (eV)

Table 2.

Comparison of salting-out assisted liquid-liquid extraction (SALLE), dilute-evaporate-and-shoot (DES), and dilute-and-shoot (DAS) approaches for sample preparation.

Table 3.

Overview of the apparent recovery (R_A), recovery (R), repeatability (RSD_r), reproducibility (RSD_R), limits of detection and quantitation (LOD and LOQ), signal suppression/enhancement (SSE) and expanded measurement uncertainty for each mycotoxin analytes.

[a] The validation parameters are determined at medium concentration.

Table 4.

Mycotoxins detected in pig urine samples as analyzed with the SALLE-based LC-MS/MS method.