Multi-analyte approach for detection of relevant mycotoxin biomarkers of exposure.

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1. Background

Mycotoxins 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 choices 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 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, 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 kinds 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 project report, 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. Objectives

The general aim of this project proposal is to develop a reliable, cost-effective and time-efficient analytical method based on liquid chromatography – tandem mass spectrometry (LC-MS/MS) for detection of relevant biomarkers of exposure. Given the co-occurrence of mycotoxins in food/feed a multi-analyte approach will be followed involving the simultaneous

detection of a number of diverse compounds with a single method.

The different objectives can be listed as follows:

- To select relevant mycotoxin biomarkers of exposure in animals and humans based on the established literature data. Due to the non-invasive character of urine sampling and the well-established urinary biomarkers, most attention will be paid to them.
- To develop a multi-analyte LC-MS/MS method for the simultaneous detection of relevant mycotoxin biomarkers of exposure representing several classes of mycotoxins.
- To develop a fast and reliable clean-up method for the urine matrix taking into account the multi-analyte approach.
- To validate the developed analytical method according to Commission Decision 2002/657/EC and to apply it to human and animal urine samples.

3. Methodologies

3.1 Selection of relevant biomarkers of exposure

Focus will be on the six regulated mycotoxins. Given the non-invasive character of urine sampling, our analytical method will be focused on this matrix. The stuctures of chosen targets and metabolites are shown in Fig. 1.

3.2 Sample preparation

For this study, three different sample preparation approaches were evaluated, namely SALLE, “dilute and shoot” (DAS) and “dilute, evaporate and shoot” (DES).

SALLE

Five mL of urine were transferred into a 50 mL extraction tube, followed by addition of 10 mL of magnesium sulfate (2 M). Five mL ethyl acetate/FA (99/1, v/v) were used as extraction solvent to extract for 15 min on an overhead shaker, followed by centrifuging for 15 min at 4500 rpm. The ethyl acetate phase was aspirated into a new extraction tube. Then 5mL of acetonitrile/FA (99/1, v/v) were added into 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 to 60°C under a gentle stream of nitrogen. Before analysis, a 500 µL volume 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 1000 rpm, 20 µL volume of this filtrate was injected into the LC-MS/MS system for analysis.

DAS

A simple DAS approach was evaluated for the analysis of urine sample based on the method developed by Warth et al. [24]. Concisely, five mL of urine were 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 based on the DAS method. After mixing the urine samples with the same volume of methanol, the mixture was centrifuged for 15 min at 4500 rpm, afterwards 5 mL of supernatant was taken and evaporated at 60°C under a gentle stream of nitrogen. The reconstitution step and injection sequence were the same as described in SALLE.

3.3 Method validation

The evaluated performance characteristics of this multi-mycotoxin method included linearity, apparent recovery (RA, expressed by method bias), extraction recovery (R), repeatability (intra-day relative standard deviation, RSDr), intra-laboratory reproducibility (inter-day relative standard deviation, RSDR), 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 (R2) 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 [25]. The repeatability (intra-day precision, expressed as RSDr) was evaluated at 3 concentration levels in the same day; for intra-laboratory reproducibility (inter-day precision, expressed as RSDR), the three concentrations were analyzed in three different days. A fresh solution was prepared daily for the intra- and inter- day precision.

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 verified and confirmed 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 [26]. The selectivity was evaluated by analyzing 6 different blank urine samples. The signal interference between the 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. [27], in which, the intra-laboratory reproducibility standard deviation (SRW), the uncertainty associated with the mean recovery (Ubias) as well as the uncertainty due to the purity of the standards (U(Cref)) were taken into account. Briefly, the expanded measurement uncertainty was estimated by multiplying the combined uncertainty (Uc) by the coverage factor 2 (corresponding to a confidence interval of approximately 95%). The equation used to calculated the Uc was as follows: Uc 2 =(SRW)2+(U(Cref))2+(Ubias)2.

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

4. Results and discussion

4.1 Optimization of the LC-MS/MS conditions

Because all the mycotoxins usually occur at low ng mL-1 in urine samples, it is most important to optimize the sensitivity of the method. For this purpose, the MS conditions and LC

parameters were thoroughly optimized. As a compromise, the ESI+ mode was applied for all the analytes. The optimized MS/MS parameters are listed in Table 1. In this study, different gradient designs with methanol and water containing different additives were tested as mobile phase. Based on the 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).

4.2 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, LogP) is often used to measure how hydrophilic or hydrophobic a chemical substance is. The postulated LogP values of the biomarkers are demonstrated in Table 1. The data were taken from PubChem Public Chemical Database (http://pubchem.ncbi.nlm.nih.gov/). Just as shown in these data, some biomarkers, such as DON, NEO and FB1, are more hydrophilic (LogP<0). Hence it is thus difficult to get satisfactory recoveries for these analytes compared to the other low polarity analytes (LogP>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 the problems, however, these columns are very expensive, furthermore, it is not generic material and not applicable for the envisaged muti-analyte detection [20, 29, 30].

LLE with water-miscible organic solvents had so far been reported for analysis of biological samples [22, 23, 31]. 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 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 is mostly adopted during sample analysis with regard to the more close polarity of acetonitrile to that of water. Therefore acetonitrile was selected for SALLE in this study. Firstly, the effects of different salts towards the recovery of each biomarker were compared. Inorganic and organic salts such as MgSO4, (NH4)2SO4 and NH4Ac 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 NH4Ac was a better salting-out agent [23], because of its compatiblility with spectrometry. The obtained results did not agree with this literature. The recoveries for all the target analytes were very low (<75%), with FB1 being the least recovered analyte (recovery, <1%). Further modifications in the concentration of NH4Ac could not enhance the recoveries. However, all the other salt solutions gave very satisfactory results, the recoveries for all the 12 biomarkers were more than 70%. Obtaining satisfactory recovery for FB1 has been one major challenge in the field of mycotoxin research. However with the SALLE protocol the recovery was more than 80%. MgSO4 has more ionic strength (4 mol L-1) per unit concentration in aqueous phase than the other salts [21], which will facilitate the phase separation and also improve the analyte recovery [32]. Given that the

extraction recoveries were similar for both 2 M MgSO4 and a saturated solution, a 2 M concentration of MgSO4 was preferred for economical reason.

The extraction efficiencies of SALLE with only ethyl acetate and SALLE with the combination of ethyl acetate and acetonitrile were evaluated separately. The results indicated that all the biomarkers gave much lower recoveries when extracted with ethyl acetate (Fig. 4). Interesting to mention is that the recoveries for FB1 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 MgSO4 solution and volume of extraction solvent, were optimized during extraction. The best parameters were as follows: 5 mL of urine, 10 mL of 2 M MgSO4 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.

4.3 Comparison of SALLE, DAS and DES

DAS and DES are sometimes preferred to LLE and SPE because of their simplicity and high sample throughput. For some reports in the literature, satisfactory analyte signals have been obtained with the DAS or DES approach especially when the concentration of the targeted analytes was relatively high and the matrix components did not co-elute or interfere with ionization of the analytes [33].

We compared SALLE with DAS and DES. 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 matrix effect was observed. In addition, although DES had better sensitivity and response than DAS because of the concentration step by evaporation, both the approaches could only detect the compounds at a much higher concentration level compared to SALLE. In some extreme cases (AFM1), the LODs were 8 and 20 times higher with DES and DAS than with SALLE. DAS and DES are applicable when the LODs fulfill the requirement of the legislation, however, it is not advisable to inject sample directly into the LC-MS/MS after processing by these two approaches. The main reason is that the sample extract from DAS and DES can be very dirty, which could shorten the HPLC column life.

4.4 Validation of the SALLE approach

The coefficients of determination (R2) for the different analytes were higher than 0.98 which indicated good linearity of the analytical method (Table 3). Only in pig urine, two compounds (AFB1 and OTA) showed R2 of 0.97. As demonstrated in Table 3, the method biases for pig urine urine ranged within 20% at all spiked concentrations. The method biases confirmed the suitability of the proposed extraction procedure for the simultaneous extraction of 12 mycotoxins from pig 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 values in pig urine were between 70% and 108% at three concentrations respectively. It is worth to point out that FB1, 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 FB1 of more than 80% in food or feed [34]. For biofluid matrices,

this is the first report in which high recoveries for FB1 were obtained without the use of SPE. Repeatability, expressed as RSDr, was lower than 20% for pig urine samples for all the analytes except for OTα. Meanwhile the RSDR was lower than 25%, again except for OTα. For pig urine, the LOQ values ranged from 0.07 ng mL-1 (OTA) to 3.3 ng mL-1 (DON). No co-eluting peaks were observed at the special RTW for the different analytes, which thus confirms the good selectivity of the analytical method (Fig. 1B). The expanded measurement uncertainty (U) is a criterion for the integral acceptability of the developed method. In this study, most of the calculated expanded measurement uncertainties were below 40%, except for OTα (50%) in pig urine. These high values were attributed to the high intra-laboratory reproducibility levels.

It is well known that, when analyzing with LC-MS/MS, the presence of matrix can influence the ionization of the target analytes, and thus result in signal suppression or enhancement. As can be seen in Table 3, all the mycotoxins are subject to signal suppression. For most of the compounds, the signal suppression was quite obvious both in pig urine (<30%), except for T2, FB1 and OTA, for which, the SSE was higher than 30% in pig urine. The SSE effect strongly encourages the use of matrix-matched calibration curves for quantification purposes. The use of isotope-labeled internal standards can greatly compensate for matrix effect. However, most of these isotope-labeled internal standards are rather expensive and in most cases not commercially available, which limit their use in multi-residue detection. In certain mycotoxin research, mycotoxin analogs such as de-epoxy deoxynivalenol (DOM) and zearalanone (ZAN), were used as internal standards [16, 35]. However for human or animal studies, there could be a possible occurrence of these metabolites in human or animal matrices. Because the broad range of the analytes in this study, it was impossible to find structural analogs which could be used as internal standards for all the compounds.

4.5 Application of the proposed method

Once the proposed method was optimized, it was applied to investigate the occurrence of the 12 mycotoxins 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, AFB1, FB1, and OTA were detected in pig urine samples. In total, 68% (19/28) of the samples were contaminated by at least one kind of analyte. Of these 19 samples, one sample was contaminated by 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, with no co-occurrence of OTα. In addition, 4 samples were found contaminated with FB1, the concentrations were from less than LOQ to 0.74 ng mL-1. This further proved the suitability of the method for FB1 determination. AFB1 was found in three samples, without any co-occurrence of AFM1. A typical chromatogram of sample 19 is shown in Fig. 5A, which was co-contaminated with DON (18.7 ng mL-1) (Fig. 5B), AFB1 (0.32 ng mL-1) (Fig.5C) and OTA (0.32 ng mL-1) (Fig. 5D).

We also compared the contamination data from three different animal farms. 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 sample 14 and sample 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 3 farms were contaminated by DON, while the situation in farm 2 was most serious. AFB1 was detected only in the urine of farm 3, while FB1 was detected only in urine of animals in farm 1. Besides, OTA was also found in pigs from the three farms. All this indicated that these compounds were good biomarkers to reflect the contamination of feed from different farms and direct determination of urinary mycotoxin biomarkers is a better 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 between individuals, so biomarkers are more applicable in individual exposure.

5 Conclusion

A high-throughput method based on SALLE with acetonitrile as extraction solvent was developed for the simultaneous detection of DON, AFB1, FB1, T2, OTA and ZEN and their possible biomarkers 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 only 2 steps of LLE with ethyl acetate and acetonitrile respectively, with regard to other multi-mycotoxin determination methodologies, this method was really fast and easy to perform. MgSO4 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 FB1. 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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Supplement

Figures and tables

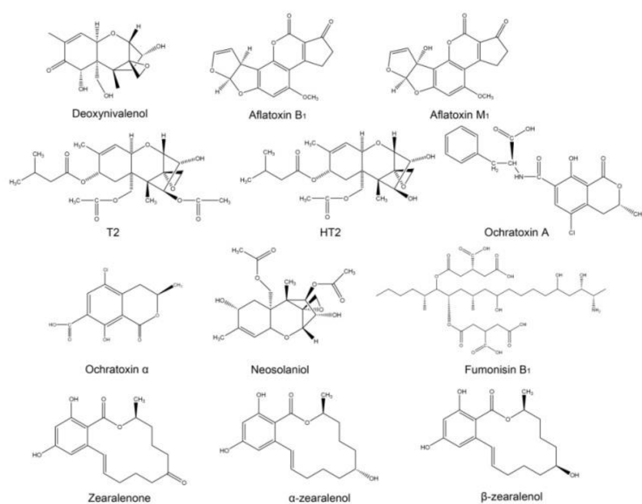

Fig. 1. Structures of selected mycotoxin biomarkers.

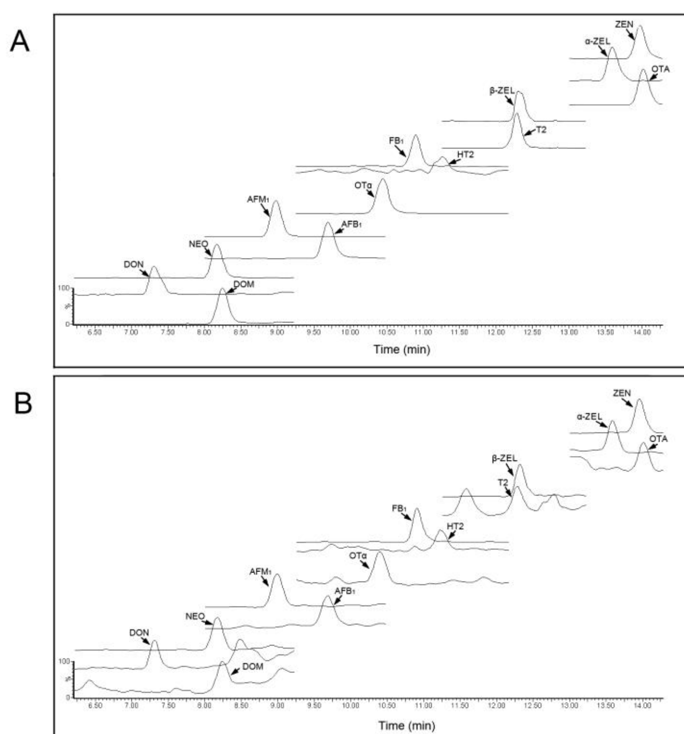

Fig. 2. Chromatograms of 12 compounds in standard solution (A) or in pig urine after SALLE (B). The concentration of each compound was as follows: DON (5 ng mL-1), NEO (2 ng mL-1), AFM1 (1 ng mL-1), AFB1 (0.2 ng mL-1), OTα (2.5 ng mL-1), FB1 (0.5 ng mL-1), HT2 (0.5 ng mL-1), T2 (0.2 ng mL-1), β-ZEL (1 ng mL-1), α-ZEL (1 ng mL-1), ZEN (1 ng mL-1), OTA (0.1 ng mL-1).

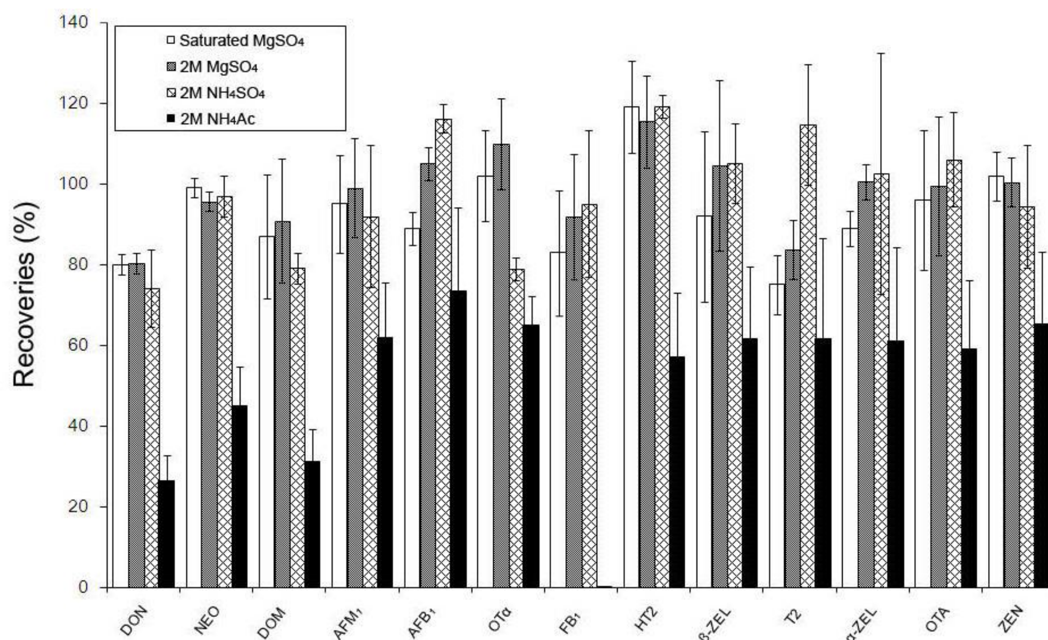

Fig. 3. The effects of different salts towards the recovery of each biomarker. The concentration of each compound was as follows: DON (100 ng mL-1), NEO (40 ng mL-1), AFM1 (20 ng mL-1), AFB1 (4 ng mL-1), OTα (50 ng mL-1), FB1 (10 ng mL-1), HT2 (10 ng mL-1), T2 (4 ng mL-1), β-ZEL (20 ng mL-1), α-ZEL (20 ng mL-1), ZEN (20 ng mL-1), OTA (2 ng mL-1).

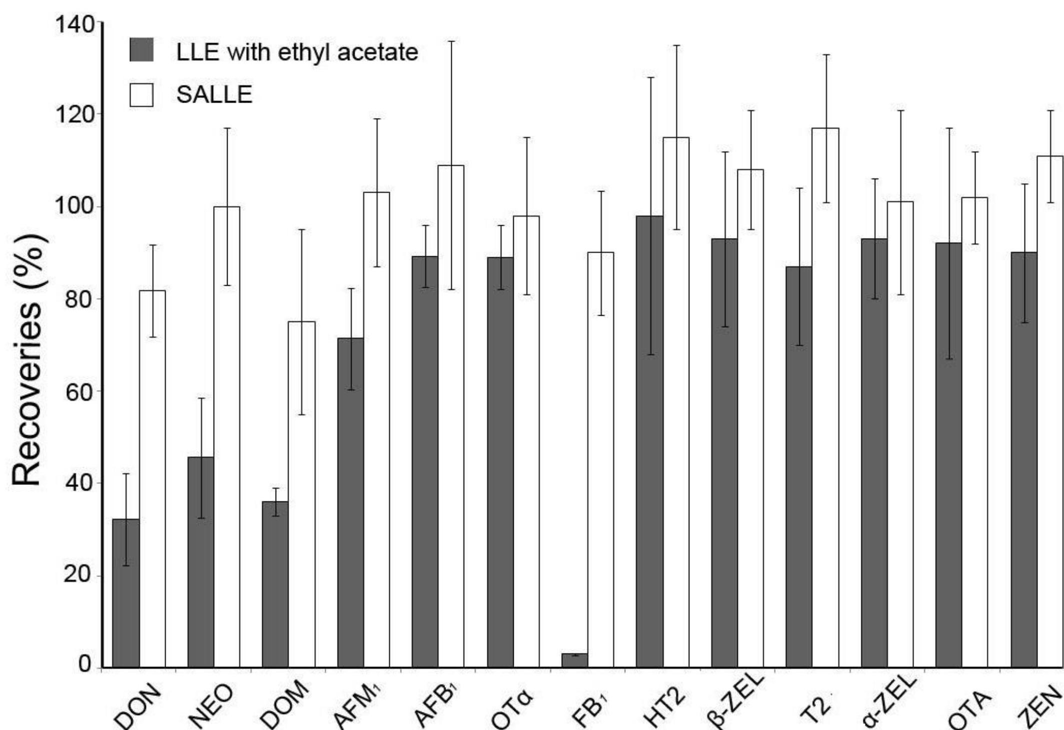

Fig. 4. The extraction yields of LLE with only ethyl acetate and with SALLE for 12 mycotoxins biomarkers in pig urine. The concentration of each compound was as follows: DON (100 ng mL-1), NEO (40 ng mL-1), AFM1 (20 ng mL-1), AFB1 (4 ng mL-1), OTα (50 ng mL-1), FB1 (10 ng mL-1), HT2 (10 ng mL-1), T2 (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).

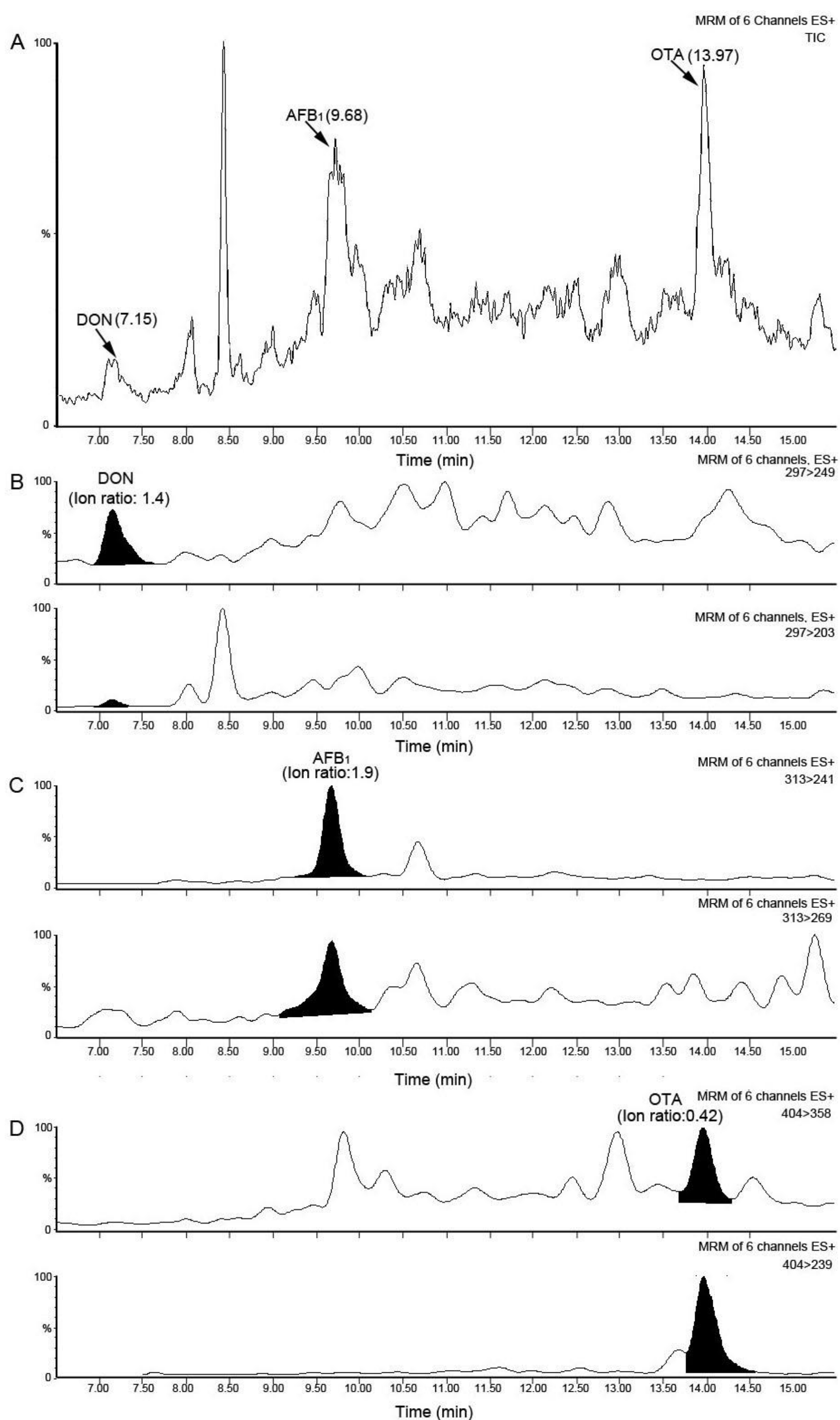

Fig. 5 The chromatogams of pig urine sample 19. (A) the TIC chromatogram; (B) the extracted ion chromatogram of DON (18.7 ng mL-1); (C) the extracted ion chromatogram of AFB1 (0.32 ng mL-1); (D) the extracted ion chromatogram of OTA (0.32 ng mL-1).

Table 1. Optimized ESI+ MS/MS parameters, partition coefficient values (LogP) and retention time window (RTW) of the analytes.

Mycotoxins	Proposed biomarkers	Log*P* [a]	RTW (min)	Quantitation transition (CE) [b]	Confirmation transition (CE) [b]	Cone voltage (V)
DON	DON	-0.7	7.06-7.29	297 > 249(10)	297 > 203 (15)	19
AFB$_1$	AFB$_1$	1.6	9.61-9.78	313 > 241(30)	313 > 269 (25)	35
	AFM$_1$	0.5	8.90-9.04	329 > 273 (20)	329 > 259 (20)	25
T2	T2	0.9	12.27-12.49	484 > 215 (10)	484 > 305 (15)	15
	HT2	0.4	11.15-11.38	442 > 215 (15)	442 > 263 (15)	12
	NEO	-0.9	8.11-8.29	400.1 > 305.3 (13)	400.1 > 365.1 (10)	20
FB$_1$	FB$_1$	-0.5	10.78-11.03	772.3 > 352.2 (35)	772.3 > 334.2 (35)	45
OTA	OTA	4.7	13.91-14.24	404 > 358 (10)	404 > 239 (25)	20
	OTα	3.4	10.34-10.52	257 > 221 (25)	257 > 239 (15)	12
ZEN	ZEN	3.6	13.96-14.29	319 > 283 (10)	319 > 301 (10)	12
	α-ZEL	4	13.53-13.84	321 > 285 (9)	321 > 303 (9)	10
	β-ZEL	4	12.20-12.54	321 > 285 (10)	321 > 303 (9)	10

a LogP, source: PubChem Public Chemical Database (http://pubchem.ncbi.nlm.nih.gov/). b CE=collision energy (eV)

Table 4. Mycotoxins detected in pig urine samples as analyzed with the SALLE-based LC-MS/MS method.

		Farm 1									Farm 2							Farm 3		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Biomarkers detected (ng mL^{-1})	DON	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ					77.8	20	162	302	<LOQ	5.7	17.4		16.9	18.7
	AFB$_1$																	<LOQ	<LOQ	0.33
	FB$_1$		<LOQ			0.74	<LOQ		0.3											
	OTA							0.07	<LOQ	<LOQ							0.13			0.32

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.

Proposed biomarkers	**Concentration range** ($ng\ mL^{-1}$)	**SALLE**		**DES**		**DAS**	
		Slope	LOD ($ng\ mL^{-1}$)	Slope	LOD ($ng\ mL^{-1}$)	Slope	LOD ($ng\ mL^{-1}$)
DON	1-100	45.6	1	14	6	5.7	18
AFB_1	0.2-20	1286	0.1	980	0.4	329.5	1
AFM_1	0.2-20	462.6	0.1	324.6	0.8	132.6	2
T2	0.04-4	885.7	0.04	561.4	0.2	43.9	0.8
HT2	0.1-10	273.4	0.3	122.5	2	15.2	5
NEO	0.4-4	77.4	1	40.2	2	15.8	4
FB_1	0.1-10	313.1	0.05	246.4	0.4	162.7	1
OTA	0.02-2	2095	0.02	1490.6	0.1	98.4	0.4
OTα	0.5-50	331.8	0.25	191.5	0.8	32.8	4
ZEN	0.2-20	384.8	0.4	175.2	2	21.4	4
α-ZEL	0.2-20	501.4	0.3	280.1	0.8	35	6
β-ZEL	0.2-20	245.5	0.3	142.7	2	14.9	4

Table 3. Overview of the apparent recovery (R_A), extraction recovery (R), repeatability (RSD_r), reproducibility (RSD_R), limits of detection and quantitation (LOD and LOQ), signal suppression/enhancement (SSE) and expanded measurement uncertainty (*U*) for each mycotoxin and/or metabolites.

Sample	Proposed biomarkers	Concentration (ng mL^{-1})	R^2	Low concentration				Medium concentration				High concentration				LOD (ng mL^{-1})	LOQ (ng mL^{-1})	SSE (%)	U[a] (%)
				R_A (bias, %)	R (%)	RSD_r (%)	RSD_R (%)	R_A (bias, %)	R (%)	RSD_r (%)	RSD_R (%)	R_A (bias, %)	R (%)	RSD_r (%)	RSD_R (%)				
Pig urine	DON	10-30-50	0.980	8	96	3	11	5	70	7	13	7	75	5	11	1	3.3	4	28
	AFB_1	0.4-1.2-10	0.973	8	88	13	16	0	86	8	16	2	100	19	12	0.1	0.33	5	32
	AFM_1	2-6-10	0.985	5	86	19	18	2	81	5	7	0	100	13	8	0.1	0.33	5	15
	T2	0.4-1.2-2	0.993	8	85	13	22	3	85	13	12	2	108	6	10	0.04	0.13	32	25
	HT2	1-3-5	0.993	20	86	19	20	8	92	16	14	1	96	2	8	0.3	1	17	32
	NEO	4-12-20	0.990	7	80	14	15	6	81	7	14	2	83	9	13	1	3.3	4	30
	FB_1	1-3-5	0.983	0	81	0.1	13	7	82	4	5	3	92	11	10	0.05	0.17	57	18
	OTA	0.2-0.6-1	0.973	7	93	15	22	6	83	10	8	3	85	13	13	0.02	0.07	46	20
	OTα	1-3-5	0.988	19	99	31	28	12	91	15	22	1	106	17	13	0.25	0.8	4	50
	ZEN	2-6-10	0.995	8	80	9.6	16	2	100	4	5	1	89	5	4.2	0.4	1.3	20	11
	α-ZEL	2-6-10	0.986	17	105	14	22	7	88	10	8	3	100	11	10	0.3	1	23	12
	β-ZEL	2-6-10	0.981	17	85	11	24	12	91	12	14	6	95	13	8	0.3	1	16	37

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