A direct assessment of mycotoxin biomarkers in human urine samples by LC-MS/MS

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ABSTRACT

Detection of mycotoxin biomarkers in urine of humans and animals provides a direct approach for assessing exposure to these mycotoxins as opposed to the indirect approach of food analysis, which in most cases is affected by the heterogeneity of the toxin in the food samples. Seven (7) mycotoxins and their metabolites (total 18 analytes) were selected and an LC-MS/MS method for their determination in human urine was developed and validated. The method consisted of direct analysis of two mycotoxin conjugates, deoxynivalenol-glucuronide and zearalenone-glucuronide without beta glucuronidase digestion of the urine samples. Since high method sensitivity is of utmost importance in such study, critical factors which could improve the analyte recovery and method sensitivity were investigated by a D-optimal experimental design. Urine samples (10 mL) were first extracted with 15 mL ethyl acetate/formic acid (99/1, v/v) followed by SAX SPE clean-up of the acidified aqueous fraction. Both extracts were combined and analyzed using an LC-MS/MS system operated in the positive ionization mode. A total run time of 28 minutes was adopted with all the 18 analytes eluting within 15 minutes.

The method was validated by taking into consideration the guidelines specified in Commission Decision 2002/657/EC and 401/2006/EC. Forty samples obtained from volunteers within the laboratory research group were analyzed as part of a pilot study. All results were expressed per mg creatinine. A total of 9 samples were found contaminated with one or more of the following analytes; DON, OTA, OTα, 4-OH OTA, ZEN, CIT and β-ZOL. One-eighth (5/40) of the samples were contaminated with DON in the range of 3.7-67 ng mg^{-1} creatinine. Samples with detectable levels of DON did not show any co-occurrence of DON-3Glu. One sample was found to be contaminated with 4-OH OTA (<LOQ), co-occurring with only OTA (0.2 ng mg^{-1} creatinine). OTα (up to 4.4 ng mg^{-1} creatinine) was detected in three other samples co-occurring with low levels of OTA (up to 0.3 ng mg^{-1} creatinine) and no 4-

OH OTA detected. ZEN was detected in 10% (4/40) of the samples analyzed. Three samples were contaminated with β-ZOL (3.3-20 ng mg^{-1} creatinine), co-occurring with ZEN (<LOQ-10.8 ng mg^{-1} creatinine). The ratio of ZEN/β-ZOL varied for all the three samples. α-ZOL was not detected in any of the 40 samples. CIT was detected in one sample at 4.5 ng mg^{-1} creatinine. This is the first study carried out with a small group of the Belgian population to assess exposure to mycotoxins using biomakers.

Keywords: Mycotoxin, biomarkers, urine, glucuronide, experimental design, stability study

1. INTRODUCTION

The classical approach for exposure assessment to mycotoxins has, for the past decades been based on detecting and quantifying the presence of this contaminant in foods. This approach is thought not to be reliable because it is flawed by inherent variability in (i) food contamination levels (ii) cooking (iii) individual consumption and individual toxicokinetics and toxicodynamics. On the other hand, detecting the presence of mycotoxin biomarkers in biological fluids such as blood and urine could be more useful and reliable in short-term and long-term exposure assessment as well to be able to predict future adverse health consequences. Deoxynivalenol (DON), aflatoxin B_1 (AFB_1), ochratoxin A (OTA), fumonisin B_1 (FB_1), T2 toxin and zearalenone (ZEN) are the most common mycotoxins which contaminate food and feedstuffs around the world. A thorough review on the metabolic pathway of the different mycotoxins in animals and humans and their possible transfer in urine has revealed a quite complex with sometimes conflicting outcomes for some analytes. Thus, for the purpose of this work, a review on the toxicokinetics and metabolism of each of these toxins would provide clues on the most frequently occurring metabolites in human urine which might be useful for exposure assessment.

DON and its detoxification metabolite DON-3-glucuronide (DON-3Glu) has often been reported in the urine of exposed humans. In a study carried out in the United Kingdom by Turner et al. [1] a strong correlation was found between these urinary metabolites (the sum of the free DON and DON-3Glu) and cereal intake of the study population. Still in a related study performed by the same group of authors, the mean transfer of DON to urine was estimated to be 72% [2]. For these reasons, detecting and or quantifying the sum of these metabolites (DON and DON-3-glucuronide) in human urine has been recommended for biomonitoring of this toxin

The increase in the sphinganine (Sa) to sphingosine (So) (Sa/So) ratio in urine and serum was proposed as a functional biomarker to evaluate exposure to FB_1 in exposed animals [3]. When this biomarker was investigated in serum and urine in several human studies, it resulted in inconclusive outcomes [4-7]. FB_1 does not appear to undergo any major metabolism as incubation with primary rat hepatocyte cultures and subcellular enzyme fractions failed to produce detectable metabolites [8]. Furthermore, FB_1 was recovered unaltered in the urine faeces and bile of dosed animals. For these reasons, recent studies have recommended the use of the parent analyte (FB_1) as an alternative biomarker [9]. Hydrolysis of the two tricarballylic acid ester groups of FB_1 has been reported to occur in the gut of vervet monkeys [10-11] and could possibly serve as an alternative biomarker. However, hydrolysis of FB1 has not yet been reported with human cell culture studies.

Biomarkers of exposure to OTA have been thoroughly investigated and documented in many scientific publications. Gilbert et al. [12], found a positive correlation between the urinary concentration of OTA and the consumption of OTA, while Munoz et al. [13] reported the presence of OTA and its major metabolite ochratoxin alpha (OTα) in 100% of the samples analyzed. OTα which is produced from the hydrolysis of OTA by the gut microflora in the intestine was the major metabolite detected in the urine of rats [14]. The hydroxylated form of OTA (4-OH OTA) was detected in urine of 96% of children under 5 years old in Sierra Leone at concentrations of 0.04-21 ng/mL [15]. Data on the formation of genotoxic products of OTA still remain inconclusive as *in vivo* experiments conducted with male Fischer-344 rats treated orally with high levels (1-2 mg/kg bodyweight) of ^{3}[H]OTA did not reveal significant detectable levels of OTA-DNA adduct [16-17].

ZEN, a mycoestrogen which is a frequent contaminant of cereals and especially breakfast cereals, was suspected to be a triggering factor for central precocious puberty observed in

adolescent females in the United States [18]. Review on the toxicity, occurrence, metabolism and detoxification of ZEN suggested two major biotransformation pathways in animals: (1) hydroxylation resulting in the formation of alpha zearalenol (α-ZOL) and beta zearalenol (β-ZOL) assumed to be catalyzed by 3α and 3β hydroxyl steroid dehydrogenase respectively and (2) conjugation of ZEN and its reduced metabolites with glucuronic acid [19]. Till date, no paper has reported the presence of α-ZOL-glucuronide and β-ZOL-glucuronide in human urine samples.

Human exposure to aflatoxins (AFs) is a concern worldwide because AFs are potent cancer-promoting agents, especially liver cancer (International Agency for Research on Cancer (IARC), 1993) [20]. Animal studies have shown that under normal conditions, 50% of the orally administered dose of AFB_1 is quickly absorbed from the duodenal region of the small intestine and enters the liver through the hepatic portal blood supply, where it is metabolized in several derivatives [21]. The metabolites of AFB_1 detected in human urine include aflatoxin P_1 (AFP_1), aflatoxin Q1 (AFQ_1), aflatoxin M_1 (AFM_1) and DNA-adduct (AFB_1-N7Guanine). The excretion rate of the different aflatoxin metabolites in human urine is not clearly defined. However, *in vitro* studies using primate and human liver microsomes have demonstrated that AFQ_1 is a major AFB_1 metabolite, with AFM_1, the hydroxylated metabolite constituting less than 10% of the total metabolized AFB_1 [22-23]. Accordingly, in a study carried out in China, the levels of urinary AFQ_1 were 60 fold higher than those of AFM_1. Levels of AFQ_1 were strongly correlated ($r = 0.673$) with detected levels of AFB_1-N7Guanine adduct (AFB_1-N7Gua), a strong biomarker for cancer effect. Consequently, AFQ_1 was suggested as a predictive marker for AFB_1 exposure [24]. However, the use of this metabolite is strongly compromised due to the lack of commercial standards.

Several acute and chronic toxic effects were observed in humans after consumption of food contaminated with T-2 toxin. T-2 toxin is rapidly metabolized by esterases, resulting in

several metabolites being detected *in vivo* and *in vitro* after ingestion. The spectrum and the ratios of T-2 metabolites in animals strongly depend on the investigated species [25]. The main biotransformation pathway is deacetylation of the C-4 acetyl group which leads to HT-2 toxin. In cell culture studies with human fibroblast cells and isolated microsomes from liver, kidney and spleen of various animals, HT-2 toxin was detected as the sole metabolite of T-2 toxin [26-29]. Other metabolites detected after incubation of T2 toxin with the Chinese hamster ovary cells and the African green monkey kidney cells included traces of T-2 triol and T-2 tetraol [27]. Johnson et al. [30] reported a possible metabolism of T-2 toxin to neosolaniol by carboxylesterase activity in human blood cells. In these cells, both metabolites (HT2 and neosolaniol) were produced in equal amounts.

Citrinin (CIT), a nephrotoxin, has been implicated in several disease outbreaks in animals and humans (IARC, 1986) [31]. Literature on the toxicokinetics and metabolism of CIT in humans is very scarce. However, Dunn et al. [32] isolated and successfully identified dihydrocitrone as the main urinary metabolite of CIT in rats. Low levels (2-5 ng/mL) of the un-metabolized toxin were also detected in the urine of humans [33], indicating a possible but low level of excretion. However, the analytical challenges associated with the detection of this analyte in biological and food matrices usually make this analyte escape routine surveillance.

Only a few analytical methods for multi-determination of mycotoxin biomarkers in human urine have been reported so far [34-36]. Most often, only a limited number of analytes are targeted due to the unavailability of commercial standards, which otherwise can be synthesized at the laboratory scale. Example of such analytes include the glucuronides DON-3GLu and ZEN-4Glu. Analysis has thus been based on detecting the presence of the parent analyte after β-glucuronidase digestion. Warth et al. [37] focused on the direct determination

of one of the conjugated forms (mycotoxin glucuronide, i.e (DON-3-Glu)) without β-glucuronidase digestion. This is the only direct assessment approach reported so far. No other analytes except DON were included in the method reported by Warth et al. [37].

In this study, a sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the simultaneous determination of DON, OTA, FB_1, AFB_1, ZEN, T-2 and CIT as well as their main metabolites in human urine was developed and validated. Direct determination of the conjugated forms namely DON-3Glu ZEN-4Glu without enzyme digestion of the urine samples was performed. Critical factors which could have an influence or effect on the analyte recovery and method sensitivity were investigated by a D-optimal experimental design. The optimized analytical protocol was used to analyze samples obtained from volunteers within the laboratory research group. Furthermore, the short term and long term stability of the different mycotoxin biomarkers spiked in urine sample was investigated as no literature was available at the time this work was been carried out.

2. MATERIALS AND METHODS

2.1 Reagents and Materials

LC-MS grade methanol, HPLC grade methanol and n-hexane were purchased from VWR International (Zaventem, Belgium). Dichloromethane and ethyl acetate were purchased from Acros Organics (Geel, Belgium). Ammonium acetate was supplied by Grauwmeer (Leuven, Belgium). Ammonium formate, picric acid (1.3%) and creatinine (Crea) standard were supplied by Sigma-Aldrich (Bornem, Belgium). Chloroform, methyl-ter-butyl ether, acetic acid, formic acid (FAc), hydrochloric acid (HCl) 37%, sodium hydroxide and sodium

carbonate (Na_2CO_3) were supplied by Merck (Darmstadt, Germany). Bond Elut strong anion exchange (SAX) SPE cartridges were obtained from Varian (Sint-Katelijne Waver, Belgium). Oasis HLB SPE cartridges were obtained from Waters (Zellik, Belgium). Ultrafree-MC centrifugal filter devices (0.22 μm) of Millipore (Millipore, Brussels, Belgium) were used. Trifluoroacetic acid (TFA) was obtained from Fluka (Buch, Switzerland). Water was purified on a Milli-Q Plus apparatus (Millipore, Brussels, Belgium).

2.1.1 Standards

Mycotoxin-reference standards and metabolites namely AFB_1, HT-2 toxin, OTA, ZEN, FB_1, deepoxy-deoxynivalenol (DOM), CIT, α-ZOL and β-ZOL, were purchased from Sigma-Aldrich (Bornem, Belgium). T-2 toxin was purchased from Biopure (Tulln, Austria). DON and AFM_1 were purchased from Fermentek (Jerusalem, Israel). OTα was obtained from Coring System Diagnostix (Gernsheim, Germany). Stock solutions of DON, AFM_1, AFB_1, HT-2 toxin, T-2 toxin, OTA, ZEN, FB_1, CIT, α-ZOL and β-ZOL were prepared in methanol at a concentration of 1 mg/mL. DOM and OTα were obtained as solutions, 100 μg/mL and 10.3 μg/mL respectively in acetonitrile. DON-3Glu was synthesized using the protocol described by Wu et al. [38] while AFB_1-N7Gua was prepared using the procedure described in Egner et al. [39]. Hydrolyzed FB_1 (HFB_1 or aminopentol) was synthesized by alkaline hydrolysis as described in Pagliuca et al. [40]. All stock solutions were stored at -20 °C. From the individual stock solutions, a standard mixture was prepared at the following concentrations: DON, DOM, β-ZOL, CIT and ZEN (5 ng/μL);, FB_1 and T-2 toxin (0.2 ng/μL); α-ZOL and AFB_1 (2 ng/μL); OTα and OTA (0.1 ng/μL); DON-3Glu (5 ng/μL); AFM_1, (0.05 ng/μL); 4-OH OTA, HT-2 toxin, HFB_1 and AFB_1-N7Gua (1 ng/μL). The standard mixture was prepared in methanol, stored at -20°C and renewed every 2 weeks.

2.2 Samples

Colleagues within the laboratory research group were kindly requested to provide urine samples including own samples. Samples from friends and family members of these colleagues were also welcomed. A written and approved informed consent was obtained from all the volunteers. The informed consent was in accordance with the Helsinki declaration on ethical principles for medical research involving human subjects. Since it is the most concentrated, first morning urine was obtained from each participating individual in urine recipients and stored at −20°C until analysis. In total 40 samples were collected. The participants were not subjected to any diet restriction before and during the sampling period and as such there were no inconveniences or health risk involved. Furthermore, all participants were asked to be anonymous but were required to indicate their sex M for male, F for female, C for children and or combinations of these. All samples were frozen within 6 hrs after collection. After confirmation with external standards, samples (other than the 40 samples collected) with undetectable levels of the target analytes were used for spiking and recovery studies.

2.3 Sample Preparation

Two sample preparation protocols were optimized with the aim of (1) obtaining satisfactory recoveries for the different analytes investigated and (2) eliminating as much matrix interferences as possible and hence reaching the low detection limits required for this study. The two protocols consisted of (1) liquid-liquid extraction (LLE) with ethyl acetate/FAc (99/1, v/v) followed by SAX-SPE clean-up and (2) using Oasis HLB SPE cartridges. Each of these sample preparation protocol had its strengths and weaknesses. Both approaches are described below.

2.3.1 LLE procedure

2.3.1.1 Experimental design for the optimization of the LLE procedure

The LLE procedure was optimized using a D-optimal design with star points. The experimental design consisted of 37 runs and was performed in one randomized batch. The response (analyte peak area) for each run was used to compute the statistical analysis. Five factors (one qualitative factor and four quantitative factors) with three levels each were considered for the experimental design. The factors included (1) extraction solvent (two polar aprotic solvents (ethyl acetate and methyl ter-butyl ether) and one non-polar aprotic solvent (chloroform)) with (2) varying percentages of FA (0.1%, 1.55% and 3%, v/v), (3) volume of extraction solvent (10 mL, 20 mL and 30 mL), (4) extraction time (10 min, 20 min and 30 mins) and (5) evaporation temperature (40°C, 50°C and 60°C). The statistical relationship between a response Y and the experimental variables X_i, X_j is of the following form: $Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ij} X_i X_j + \beta_{ii} X^2_i + \beta_{jj} X^2_j + \ldots \varepsilon$, where the βs are the regression coefficients and ε is the overall experimental error. The linear coefficients β_i and β_j describe the quantitative effect of the respective variables. The cross coefficient β_{ij} measures the interaction effect between the variables and the square terms $\beta_{ii} X^2_i$ and $\beta_{jj} X^2_j$ describe the nonlinear effects on the response.

2.3.1.2 Sample clean-up using LLE in combination with SAX SPE clean-up

The optimum conditions obtained from the experimental design described above were applied. Prior to the start of analysis, all urine samples were centrifuged at 4000g for 10 mins to sediment particulate matter. Samples with undetected levels of the mycotoxin biomarkers

were used for spiking experiments. A 10 mL portion of the centrifuged urine was then used for analysis. To the urine samples, 15 mL of extraction solvent ethyl acetate/FAc (99:1, v/v) were added followed by extraction on an orbital shaker for 30 mins. Centrifugation was later performed at 4000g for 10 mins. The ethyl acetate phase was aspirated into a new extraction tube and dried at 40°C under a gentle stream of nitrogen. The pH of the aqueous phase (acidified urine) was adjusted to pH between 6.5-7 with Na_2CO_3 (0.4 M). The pH-adjusted urine was diluted (1/5, v/v) in methanol and loaded on a pre-conditioned SAX SPE cartridge for sample clean-up. The SAX SPE cartridge was conditioned by passing 10 mL of methanol/water (85/15, v/v) followed by 10 mL of methanol. The loaded sample was allowed to flow-through at a flow rate of one drop per second. The SAX SPE cartridge was then washed with 1 mL water. The analytes were eluted with 5mL of acidified methanol (1% FAc). The eluate from the SAX SPE cartridge was combined with the residue obtained after LLE with ethyl acetate. The pooled extract was evaporated at 40°C. The residue of the combined fractions was reconstituted in 200 µL of injection solvent which consisted of water/methanol/FAc (61.8/37.9/0.3, v/v/v). Hexane (500 µL) was added and vortexed for 1 min. The content was brought into a centrifugal filter (Millipore corporation, Billerica, United States) and centrifuged for 15 mins at 14000g. A 150 µL aliquot of the aqueous phase was transferred into a vial for LC-MS/MS analysis. Figure 1 shows a schematic representation of the sample preparation protocol.

2.3.2 Sample clean-up using Oasis HLB cartridges

The SPE protocol consisted of diluting 10 mL urine sample with ultrapure water (1/1, v/v) followed by loading of the diluted urine on a pre-conditioned Oasis HLB cartridge. The cartridge was conditioned with 10 mL of dichloromethane/methanol (70/30, v/v) containing

50 mM HCl followed by 5 mL methanol. Acidified water (50 mM HCl), 20 mL, was used to activate the functional hydrophilic groups. The cartridges were then rinsed with 10 mL water. Samples were loaded and allowed to flow through at a flow rate of one drop per second. Interfering substances were washed off with 2 mL water without letting the cartridges go dry. Elution was performed with 10 mL dichloromethane/methanol (70/30, v/v) containing 50 mM HCl. The eluate consisted of two phases, a colored upper phase (which consisted of residual water after the washing step) and a transparent colorless lower phase (dichloromethane/methanol phase). The colored upper phase was aspirated separately into a clean test tube to which 5 mL of ethyl acetate/TFA (99/1, v/v) was added. The mixture was vortexed and centrifuged for 3000g for 3 mins. The ethyl acetate/TFA (99/1, v/v) phase was carefully aspirated and combined with the dichloromethane/methanol extract. This combined solution was then evaporated to dryness at 40°C. The final residue was re-dissolved in 200 µL of the injection solvent.

2.4 Synthesis, purification and characterization of DON-3-glucuronide, AFB_1-N7Gua adduct and HFB_1

As previously mentioned, DON-3Glu and AFB_1-N7Gua were synthesized using the protocol described in Wu et al. [38] and Egner et al. [39] respectively. Hydrolyzed FB_1 (HFB_1 or aminopentol) was synthesized by alkaline hydrolysis as described in Pagliuca et al. [40]. Slight modification in the synthetic protocols for DON-3Glu and AFB_1-N7Gua led to significant increases in the overall yields when compared to the original approaches described in the literature. These modifications are not described in this paper. Purification of the synthesized compounds DON-3Glu, AFB_1-N7Gua and HFB_1 was carried out on a Waters HPLC instrument coupled to a Waters Fraction Collector III (Waters, Zellik, Belgium).

Characterization of the synthesized compounds was performed by performing accurate mass measurements using an Exactive Orbitrap Mass Spectrometry (Thermo Scientific, Rockwood, USA). Quantification was carried out by spectrophotometric measurements as described in the original protocols. The synthesized metabolites were used for validation studies and for authentication of suspected peaks.

2.5 Chromatographic Conditions

The analytical column used was a 100 mm x 2.1 mm i.d., 3.5 μm, ZORBAX SB-C18, connected to a 10 mm x 2.1 mm i.d., 5 μm, ZORBAX Eclipse XDB-C8 guard column (Agilent, Diegem, Belgium). Two solvent mixtures were used as mobile phases, both containing 5mM ammonium formate. Solvent A consisted of water/FAc (99.7/0.3, v/v) while methanol/water/FAc (94.7/5/0.3,v/v/v) was used as solvent B. The sample injection volume was 20 μL. A solvent gradient (flow rate of 0.25 mL/min) was adopted for a total run time of 28 min, with all the 18 analytes eluting over 4-15 mins while the last 13 mins were used for column cleaning and regeneration. The solvent gradient was as follows: 0-1 min, 90% A; 1-5 min, 90-50% A, 5-10 min, 50-35% A; 10-15 min, 35-20% A; 15-25 min, 20-90% A; 25-28 min, 90% A.

2.6 Mass Spectrometry Conditions

Detection and quantification were performed with a Waters Acquity UPLC apparatus coupled to a Micromass Quattro Micro triple quadruple spectrometer (Waters, Milford, MA, USA). To obtain optimum sensitivity and selectivity, the mass spectrometer analyses were carried out using multiple reaction monitoring (MRM) mode. Ionization was performed in the positive

electrospray ionization (ESI) mode. The following instrumental settings were applied: source and desolvation temperatures 130°C and 350°C, respectively; capillary voltage 3.2 kV; cone and desolvation gas flows of 20 and 800 Lh¯1, respectively. Other instrumental parameters such as cone voltage and collision energy were optimized by direct infusion of 20 ng/µL freshly prepared standard solutions in methanol/ultrapure water (50/49.7, v/v) containing 5mM ammonium formate and 0.3% FAc at a flow rate of 10 µL/min for 0.5 min. The precursor ion for each analyte was mass-selected by the first quadruple and fragmented through a combination of cone voltages and collision energies to obtain the product ion of each analyte. Two product ion transitions for each analyte were selected in the final method and their collision energies further optimized. The primary product ion (first transition), which corresponds to the most abundant product ion, was used for quantification while the secondary product ion (second transition) was used for confirmation. The MS parameters for each analyte are shown in Table 1.

2.7 Method Validation.

Commission Decision 2002/657/EC [41] and 401/2006/EC [42]were used as guidelines for the validation studies. The intra-laboratory validation parameters consisted of limit of detection (LOD), limit of quantification (LOQ), apparent recovery, precision, selectivity/specificity and linearity. Each of the above-mentioned parameters was determined using blank urine samples. The apparent recovery (assessed by estimating the bias) and linearity (evaluated through the lack of fit test) were determined using one experimental design as follows. Six sets of samples were used. Five-point calibration curves were constructed in matrix-matched samples. The calibration range for each analyte is shown in Table 2. A blank sample (in triplicate) fortified with standards to 10 ng/mL for each analyte

was used to estimate the method bias (apparent recovery). A non-spiked blank sample always constituted one of the five points of the calibration curve. Analysis of each concentration level was performed in triplicate. The peak area of each analyte was plotted as a function of the spiked analyte concentration. From the established calibration curve, a lack of fit test was performed to assess the adequacy of the linear model in addition to the commonly reported regression coefficients (R2). LOD and LOQ were determined by serial dilution of spiked urine samples. They were also evaluated by using the signal-to-noise (S/N) ratio, which have been defined and set as 3:1 and 10:1 respectively by the International Union of Pure and Applied Chemistry (IUPAC) [43].

The precision of the analytical method was evaluated by injecting (3 times) the analytes spiked in blank urine samples at concentrations of 5 ng/mL and 10 ng/mL and determining the relative standard deviation (RSD) of the peak areas. Both intra-day RSD (RSDr) and inter-day RSD (RSDR) (resulting from three consecutive days of analysis) were computed.

2.8 Matrix effect and stability studies

2.8.1 Matrix effect

The influence of matrix components on the MS/MS signal can vary from sample to sample due to inter-individual variability in the urine samples. However, to have an idea on the possible matrix influence, the first morning void blank urine of 5 randomly selected individuals, 3 males and 2 females was separately evaluated. For each sample a five point calibration curve was constructed (calibration range, same as those used to determine the method linearity). The ratio of the slope obtained with fortified blank samples cleaned-up prior to the spiking to the slope of a calibration curve established in pure solvent, was

computed and expressed as a percentage of the signal recovered. Each concentration level was carried out in triplicate.

2.8.2 Stability studies

To have an insight into the stability of the different mycotoxins and their respective metabolites, a short term and long term stability study design was applied. For the short term stability test, 2 batches, each consisting of five sets of samples were used. A batch represented the specific storage temperature at which the samples were stored (4°C and 25°C for batch 1 and 2 respectively). Each of the five sample sets within each batch represented a specific storage period: 3 hrs, 6 hrs, 1 day, 3 days and 5 days. For each sample set, two series of analysis were performed concurrently: a begin spike and an end spike experiment. The experimental protocol was as follows. For the begin spike, samples were spiked to 10 ng/mL for each analyte (in five replicates) and kept at 4°C or 25°C for 3 hrs, 6 hrs, 1 day, 3 days and 5 days prior to analysis. To compensate for matrix variation during storage, another set of the same sample (end spike) was kept under the same storage temperature as the begin spike but was left un-spiked at time zero (T=0). After the desired storage period, the end spike samples were used to construct a five-point calibration curve. The ratio of the calculated recovered concentration (obtained from extrapolating with the calibration curve) to the theoretical spiked concentration was used to determine the percentage of the analyte degraded under the different treatment (time, temperature) conditions. For the long term stability, the protocol was the same like with the short term stability studies except for the fact that the storage temperature of the samples was -21°C for a period of two months.

2.9 Creatinine analysis

An in-house spectrophotometric method based on the principle of Jaffe's reaction was optimized for the determination of Crea in urine samples. In summary, 18 mM picric acid was reacted with 85 mM NaOH to form alkaline picrate. This solution was stored in the dark in an amber glass recipient. Alkaline picrate (2mL) was reacted with 1 mL of diluted urine (1/100, (v/v) in ultrapure water). The optical density was measured at 495 nm after 25 mins using a Philips PU 8620 spectrophotometer. Samples with a Crea concentration below 1.3 µg/mL (detection limit) were not to be considered for biomarker analysis.

3. RESULTS AND DISCUSSION

High method sensitivity is of utmost importance since the concentration of these analytes in urine samples is most often present in the low ng/mL range.

3.1 Sample preparation: preliminary investigations

The two SPE clean-up protocols developed in this work were compared with each other and then to two other so called "fast" sample preparation approaches such as (1) dilute, evaporate and shoot and (2) dilute and shoot. The latter approach gave very low signal intensity (high LOD, > 10ng/mL) for most of the analytes. Considering that these biomarkers are present in very low concentrations (low ng/mL) in urine samples, such a less sensitive approach was considered not suitable for routine monitoring of mycotoxin biomarkers. The most probable reason for this low sensitivity could be attributed to the relatively low sensitivity of the triple quadruple MS used in this work. Moreover, this approach might not be considered suitable

because it could lead to a shortened lifespan of the analytical column, due to increased deposition of matrix components on the analytical column. The second approach, dilute, evaporate and shoot was tested as follows. A 10 mL portion of urine was diluted with methanol (1/1, v/v) from which 10 mL was taken and evaporated at 50°C. Though this approach was practically very easy to execute, it resulted in a thick sedimentation after drying which could dissolve completely only in 1 mL of injection solvent. This resulted in very high analyte LODs. Furthermore, significant signal enhancement (SSE) was observed for ZEN (average of 50%) and FB_1 (average of 130%). This enhancement was inconsistent as it varied from sample to sample and between days. Because of the above mentioned reasons, this approach was not considered suitable and hence an alternative sample clean-up involving an SPE procedure was deemed necessary.

3.2 Comparison of SAX SPE and Oasis HLB SPE clean-up approaches

From the practical point of view, the procedure for sample clean-up with Oasis HLB cartridge was less labor-intensive than sample clean-up with SAX SPE cartridges. However, as previously mentioned in section 2.3.2, the eluate after SPE with Oasis HLB cartridges was subjected to further treatment (clean-up). The eluate consisted of two phases. Meanwhile, analysis of the complete eluate without further sample clean-up led to significant signal suppression for all the different analytes. Most of the matrix components were present in the upper phase of this eluate which could be seen visually through the intense coloration of this fraction. However, this upper phase (most probably a water phase) could not be discarded as it contained FB_1. FB_1 is more water soluble than in mixtures of dichloromethane and methanol. The colored upper phase was aspirated separately into a new test tube and evaporated to dryness. Addition of 5ml ethyl acetate/TFA (99.5/0.5, v/v) led to complete extraction of FB_1

(recovery 99%) into the ethyl acetate/TFA phase. This extract also contained a significant amount of co-extracted matrix components, which was due to the high acid strength of the TFA used. Lower percentages of TFA led to a reduction in the amount of co-extracted matrix components, but the recovery for FB_1 was greatly compromised.

As a further setback, DON-3Glu and AFB_1-N7Gua were not retained on the Oasis HLB cartridges, as a result the extraction recoveries for these analytes were less than 5% and 10% respectively. On the other hand LLE with acidified ethyl acetate (1% FAc) resulted in extraction recoveries of 57% and 76% for DON-3Glu and AFB_1-N7Gua respectively. However, the extraction recoveries for DON, DOM and OTα after Oasis HLB SPE clean-up were 70%, 85% and 89% respectively which were higher than those obtained with the SAX SPE approach, 55%, 48% and 62% for DON, DOM and OTα respectively. All the other analytes had very satisfactory extraction recoveries (greater than 72%) with both clean-up approaches. Data on apparent recovery are discussed in section 3.5.

In general, the LODs obtained with the SAX clean-up approach were 3-9 fold lower than those obtained with the Oasis HLB SPE cartridges. This could be attributed to a better sample clean-up (better elimination of matrix interferences) with the SAX approach than with the Oasis HLB cartridges. For these reason, extraction with acidified ethyl acetate followed by SAX SPE clean-up was selected as a suitable extraction and sample clean-up procedure

LLE of FB_1 from urine has been a challenge in the field of mycotoxin research. Less than 10% of FB_1 was extracted with the extraction solvent (ethyl acetate/FAc (99/1, v/v)). For this reason there was the need to perform SPE clean-up using SAX SPE cartridges with the aim of obtaining a satisfactory recovery for this analyte. Data from the literature revealed that prior to SAX SPE clean-up, complete ionization of FB_1 was achieved when the pH of the sample

extract (in this case, sweet pepper extract) was adjusted to between 5.8 to 6 [44]. However, in this study, complete ionization of FB_1 was achieved at pH 6.5-7, which highlights once more the influence of sample matrix on the ionization of FB_1 [45]. The strength of the base used for adjusting the pH was also found to have a significant influence in the degree of ionization and or hydrolysis of FB_1. Hydrolysis was observed by quantifying the presence of HFB_1 in the final extract. A strong base or alkali such as NaOH and triethylamine led to significant hydrolysis of FB_1. A milder inorganic base such as Na_2CO_3 gave complete ionization of FB_1 with no hydrolytic products. After adjusting the pH to 6.5-7 it was also necessary to dilute the urine samples before loading the extract on the SPE cartridges. Fivefold dilution with methanol or isopropanol (1/5, v/v) resulted in 100% retention of the ionized FB_1 on the SAX cartridges. Meanwhile, for a non-diluted sample, less than 30% of the ionized FB_1 was retained on the SAX cartridges.

3.3 Optimization of the LLE protocol

As previously mentioned, the LLE protocol was optimized by means of an experimental design. The factors included (1) extraction solvent (2) varying percentages of FA (3) volume of extraction solvent (4) extraction time and (5) evaporation temperature. The classical approach to estimate the effect of control factors on the relevant response is by the use of a standard central composite design. However, since qualitative factors (extraction solvent) were included in our design, this posed a very serious restriction in the use of this design. Moreover, central composite designs may also have the shortcoming of needing a predetermined number of runs which, in some cases, is considerably higher than what is strictly necessary [46]. A D-optimal design with 37 runs performed in one randomized batch (in duplicate measurement) was well suited to be used for this optimization. In general, if a

factor was found to be insignificant, the least time-consuming or the cheapest level of the factor was considered. Conversely, if a factor was considered critical, the level of the factor which gave the highest response was considered in the final experimental protocol. Should there be conflicting outcomes, the favorable level of a factor was decided by the number of analytes for which the highest response was obtained. From the regression coefficient plot (not shown), it was observed that the quantitative parameters i.e percentage of acid, volume of extraction solvent and extraction time in that order had the most influence on the analyte response (recovery). All interaction and quadratic effects were found insignificant since the error bars were much larger than the regression coefficients. Ranking of the qualitative factors in order of significance, revealed that ethyl acetate had the most significant influence (positive) on the analyte response followed by methyl-ter-butyl ether and chloroform. To better estimate the influence of the different quantitative parameters on the analyte signal, response surface plots were constructed. Figure 2 shows the different response plots for β-ZOL (as an example) for different combinations of the parameters investigated. The response surface plots for a majority of the other analytes were similar to that of β-ZOL. These response surface plots were used to select the best optimum conditions for sample preparation. The optimum conditions were chosen by taking into consideration data obtained from the regression coefficient plots and the response surface plots. The following conditions were chosen as optimum and subsequently used for the analysis of urine samples: extraction solvent ethyl acetate, percentage of acid 1%, extraction time 30mins, and extraction volume 15 mL. All statistical analysis were performed using MODDE 9.0 software (Umetrics, Malmo, Sweden).

3.4 Optimization of the LC-MS/MS conditions

Optimization of the LC separation parameters and MS detection conditions was performed with analytical standards spiked in pure solvent. Addition of FAc to the mobile phase did not only improve the analytical signal of most of the analytes, but also resulted in a more efficient separation of the two zearalenol isomers (α-ZOL and β-ZOL), without which both peaks overlapped with each other. Furthermore, the retention time of DON-3GLu was greatly influenced through the acidification of the mobile phases. Non acidification (pH 6) resulted in a retention time of 6.5 mins for DON-3GLu as opposed to 4.29 mins with an acidified mobile phase (pH 3). The retention time of 6.5 mins would have been preferred as it led to a better separation of this analyte from matrix interferences and hence a much better signal intensity for DON-3Glu. However, non-acidification was not considered because the signal intensity for most of the other analytes was greatly compromised. Thus acidification of the mobile phase was desirable. Adding 0.3% aliquot of FAc in the mobile phases gave optimal signal for most of the analytes.

All of the analytes except α-ZOL, β-ZOL, DON-3Glu and DON were most sensitive in the positive electrospray ionization mode (ESI+) than in the negative electrospray ionization mode (ESI-). For this reason, ESI+ was chosen and used in this work.

Addition of ammonium acetate has proven to be necessary to support the formation of $[M+NH4^+]$ adducts, which appear to be the most predominant ions in the spectra of DON-3Glu, T2 and HT2. For ZEN-4Glu, a sodium adduct was the predominant precursor ion $[M+Na^+]$. All the other analytes had protonated molecules as precursor ions (Table 1). Meanwhile the use of ammonium formate as an additive in the mobile phase gave a significantly much higher signal compared to ammonium acetate, for most of the analytes.

3.5 Validation results and matrix effect

Insufficient method specificity most often results from co-eluting interfering (matrix) peaks which could eventually lead to a high false positive rate. The specificity of the method was evaluated by measuring the relative ion intensities of the analytes in pure solvents and in spiked blank samples. A tolerance limit of 20% (based on the ion ratio) was used as recommended in Commission Decision 2002/657/EC. Should incase the tolerance limit in an alleged positive sample was greater than 20%, the product ion scan of the selected peak was obtained and the spectra compared with those of a standard solution. In case of non-conformity, the sample in question will be considered as false positive for this analyte. This situation was encountered with CIT in 70% of the samples analyzed whereby the ion ratio exceeded 20%.

The lack of fit test for linearity resulted in p value > 0.05 for all the analytes which illustrates the reliability of the chosen calibration range(s) for the quantification of the different analytes in real samples (Table 2). In addition, the coefficient of determination (R squared) was also determined and was between 0.9774-0.9994 for all the different analytes. The data shown in Table 2 also revealed LODs within the range of 0.01-2.88 ng/mL. The method LODs were in most cases less than 1ng/mL except for DON, DON-3Glu, CIT and ZEN which had LODs of 2.85 ng/mL, 2.25 ng/mL, 2.88 ng/mL and 1.24 ng/mL respectively. These four LOD values might seem to be a bit high, especially for biomarker studies, however, considering the concentration of these analytes which have been reported in urine samples from previous studies, the LODs reported herein were satisfactory enough to be used in this biomarker study.

The apparent recovery data evaluated at concentrations of 10 times the method LOQ for the different analytes were expressed as bias. The values ranged from 1-15% for all the compounds as shown in Table 2. The intra-day and inter-day precision values were in the range 5-19% and 8-29% respectively (Table 2). Because of the possible variability which could occur at the low concentration range of the analytes investigated, a statistical graphical (boxplot) test to detect or identify outliers was performed after checking for normality of the entire sample distribution. Outliers were excluded and the test performed again until all the other outliers were eliminated. The satisfactory RSD for the intra-day and inter-day repeatability could be attributed to the elimination of outliers from the dataset. The Cochran's "C" test revealed that the variation (population variance) on the different days were equal since the results obtained on the different days and different concentration levels were not significantly different.

Figure 3 shows the results of matrix effect. The polar analytes DON, DON-3Glu suffered the most signal suppression, likewise CIT. Almost no signal suppression was observed for AFB_1, AFM_1, FB_1 and OTA. For the rest of the other analytes, at least 60% of the original signal was recovered which was considered satisfactory.

3.6 Stability study

Data on the stability of mycotoxin biomarkers in human urine have not yet been reported. Such information could be very vital when carrying out field sampling during which urine samples are to be collected and stored temporary in a non-laboratory setting (room temperature and or refrigeration) and later transported to the laboratory for long term storage prior to analysis. Data (Figure 4a) obtained from the short term stability studies (3hrs and 6hrs

at 4°C and 25°C) revealed an almost insignificant degradation (<10%) of the different analytes at the different time-temperature combinations, except for CIT, for which less than 80% of the spiked analyte concentration was recovered after 6hrs at 25°C. Though no significant differences were found between samples stored at 4°C and 25°C, it is advisable to store samples at 4°C or in dry ice when carrying sampling on the field. The additional short term stability studies performed at 4°C and 25°C for 1 day, 3 days and 5 days resulted in a very interesting outcome. From the results (Figure 4b), it could be seen that all analytes were stable after day 1 irrespective of the storage temperature. Meanwhile for day 3 and day 5, significant losses were recorded. At least 50% of the initially spiked analyte concentrations was lost for samples kept at 25°C for 5 days. HT2, AFM_1, AFB_1, DOM and T2 toxins were the most degraded. Less than 30% of the initial concentration of T2 toxin was recovered after day 5 irrespective of the storage temperature. The non-polar analytes of the group (ZEN, β-ZOL, α-ZOL, OTA) were the least degraded irrespective of the time-temperature combinations when compared to the polar analytes such as DON, DON-3Glu, and DOM. For the long term stability test, an almost insignificant degradation (<5%) of the different analytes was observed after 2 months of storage at -20°C (data not shown).

3.7 Results of pilot study

The Crea results obtained with the in-house spectrophotometric method was in good agreement (inaccuracy 0-14%) with those obtained from two external accredited laboratories (data not shown). Quantitative results obtained from the biomarker analysis were expressed in mg Crea, thereby correcting for any possible dilution effect.

Analysis of all 40 urine samples was carried out without β-glucuronidase digestion (direct method of analysis). Table 3 shows the different biomarkers and their corresponding concentrations in the positive urine samples. In total 9 samples were found positive with one or more of the following analytes; DON, OTA, OTα, 4-OH OTA, ZEN, CIT and β-ZOL. One-eighth (5/40) of the samples were contaminated with DON (3.7-67 ng mg^{-1} Crea). In samples with quantifying levels of DON, DON-3Glu was not detected. However, not been able to detect DON-3Glu in subjects 5 and 9 with relatively high DON concentrations of 47 and 67 ng/mL is intriguing and warrants further investigation with a much larger population size

4-OH OTA (<LOQ) was detected in one sample, co-occurring with only OTA (0.2 ng mg^{-1} Crea). High levels of OTα (up to 4.4 ng mg^{-1} Crea) were detected in three samples co-occurring with very low levels of OTA (up to 0.3 ng mg^{-1} Crea). Figure 5 shows the chromatogram of positive sample 1 with detectable levels of DON, OTA and OTα. The concentration of OTα was always higher than those of OTA, which has also been reported by Munoz et al. [13]. These findings further confirm the fact that OTα is the principal metabolite of OTA detoxification in humans and not 4-OH OTA.

ZEN was detected in 10% (4/40) of the samples analyzed. A full MS scan performed for ZEN-4Glu did not show any detectable levels of this analyte which has been highlighted to be a possible biomarker for ZEN exposure [19]. In comparison to the findings of Massart et al. [18], who reported occurrence of α-ZOL and ZEN in the serum of female subjects affected by precocious puberty in the United States, we detected three samples co-contamination of β-ZOL (3.3-20 ng mg^{-1} Crea) and ZEN (<LOQ-10.8 ng mg^{-1} Crea). α-ZOL was not detected in any of the 40 samples. The ratio of ZEN/β-ZOL varied for all the three samples. Figure 6

shows the chromatogram of sample 6 with detectable levels of β-ZOL and ZEN. CIT was detected in one sample at 4.5 ng mg^{-1} Crea. All the identification criteria for peak confirmation were taken into account for the final confirmation of results.

4. Conclusion and recommendation

A sensitive LC-MS/MS method for the detection of mycotoxins and their metabolites (total 18 analytes) in human urine was successfully developed. LLE with acidified (1% FAc) ethyl acetate followed by SAX SPE clean-up showed very satisfactory apparent recovery values for all the analytes. Hence LLE followed by SAX SPE clean-up could be considered as a cheaper alternative for sample extraction and clean-up as compared to the use of immunoaffinity columns which had been widely reported by other authors. Direct determination of the glucuronides DON-3Glu and ZEN-4Glc was possible without enzyme digestion.

Occurrence of one or more of the following analytes DON, OTA and its metabolites, ZEN and its metabolites and CIT were detected in nine of the 40 samples analyzed. These results highlight the need to perform a more rigorous exposure assessment with different age groups within the Belgian population, especially children.

Most of the analytes were stable for up to 1 day at both refrigeration conditions (4°C) and at room temperature (25°C). Prolong storage for 3 days and 5 days resulted in significant degradation of these analytes, with T2 been the most affected. Thus, cold storage of urine samples during field sampling is strongly encouraged and all samples should be frozen within 24 hrs after collection.

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References

1. P.C. Turner, V.J. Burley, J.A. Rothwell, K.L.M. White, J.E. Cade, C.P. Wild. Food Add Contam. 25 (2008b) 864.

2. P.C. Turner, K.L.M. White, V.J. Burley, R.P. Hopton, A. Rajendram, J. Fisher, J. E. Cade, and C.P. Wild. Biomarkers. 15 (2010) 553.

3. G.S. Shephard, L. Van Der Westhuizen, V. Sewram. Food Add Contam. 24 (2007) 1196.

4. L. Van der Westhuizen, N.L. Brown, W.F. Marasas, S. Swanevelder, G.S. Shephard. Food Chem Toxicol. 37 (1999) 1153.

5. C.C. Abnet, C.B. Borkowf, Y.L. Qiao, P.S. Albert, E. Wang, A.H. Jr. Merrill, S.D. Mark, Z.W. Dong, P.R. Taylor, S.M. Dawsey. Cancer Causes Control. 12 (2001) 821.

6. M. Solfrizzo, S.N. Chulze, C. Mallmann, A. Visconti, A. De Girolamo, F. Rojo, A. Torres. Food Addit Contam. 21 (2004) 1090.

7. L.J. Silva, C.M. Lino, A. Pena. Toxicon. 54 (2009) 390.

8. M.E. Cawood, W.C.A. Gelderblom, J.F. Alberts, S.D. Snyman. Food and Chemical Toxicology. 32 (1994) 627.

9. Y.Y. Gong, L. Torres-Sanchez, L. Lopez-Carrillo, J.H. Peng, A.E. Sutcliffe, K.L. White, H-U Humpf, P.C. Turner, and C.P. Wild. Cancer Epidemiol Biomarkers Prev.17 (2008) 3.

10. G.S. Shephard, P.G. Thiel, E.W. Sydenham, J.F. Alberts. Toxicol. 32 (1994a) 489.

11. G.S Shephard, P.G Thiel, E.W Sydenham, M.E Savard. Nat. Toxins. 3 (1995) 145.

12. J. Gilbert, P. Brereton, S. MacDonald. Food Addit. Contam. 18 (2001) 1088.

13. K. Munoz, M. Blaszkewicz, G.H. J Degen. Chromatography B. 878 (2010) 2623.

14. H. Zepnik, W. Volkel, W. Dekant. Appl. Pharmacol. 192 (2003) 36.

15. F.E. Jonsyn-Ellis. Mycopathologia. 152 (2000) 35.

16. A. Mally, H. Zepnik, P. Wanek, E. Eder, K. Dingley, H. Ihmels, W. Volkel and W. Dekant. Chem. Res. Toxicol. 17 (2004) 234.

17. J-C. Guatier, J. Richoz, D.H. Welti, J. Markovic, E. Gremaud, F.P. Guengerich and R.J. Turesky. Chem Res. Toxicol. 14 (2001) 34.

18. F. Massart, V. Meucci, G. Saggese, and G. Soldani. J Pediatr. 152 (2008) 690.

19. A. Zinedine, J.M. Soriano, J.C. Moltó, J. Manes. Food Chem. Toxicol. 45 (2007) 1.

20. IARC (1993). Some Naturally Occurring Substances: Food items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins In Monographs of the Evaluation of the Carcinogenic Risk of Chemical to Human. IARC, Lyon, France. 56, 1–151.

21. R. Wilson, R. Ziprin, S. Ragsdale, D. Busbee. Toxicol Lett. 29 (1985) 169.

22. G.E. Neal, D.L. Eaton, D.J. Judah, A. Verma. Toxicol Appl Pharmacol. 151 (1998) 152.

23. H.S. Ramsdell, A. Parkinson, A.C. Eddy, D.L Eaton. Toxicol Appl Pharmacol. 108 (1991) 436.

24. H. Mykkanen, H. Zhu, E. Salminen, R.O. Juvonen, W. Ling, J. Ma, N. Polychronaki, H. Kemilainen, O. Mykkanen, S. Salminen, and H. El-Nezami. Int. J. Cancer. 115 (2005) 879.

25. B. Yagen, M. Bialer. Drug Metab. Rev. 25 (1993) 281.

26. R.A. Ellison, F.N. Kotsonis. Appl. Microbiol. 27 (1974) 423.

27. L.R. Trusal. Toxicon. 24 (1986) 597.

28. M. Ohta, K. Ishii, Y.J. Ueno. Biochem. 82 (1977) 1591.

29. EU, 2001. Opinion of the Scientific Committee On Food on Fusarium Toxins Part 5: T-2 Toxin and HT-2 Toxin. Scientific Committee On Food (SCF). 1983, 2009.

30. H. Johnsen, E. Odden, B.A. Johnsen, F. Fonnum. Biochem. Pharmacol. 37 (1988) 3193.

31. IARC (1986). Some Naturally Occurring and synthetic food components,coumarins ultraviolet radiation In Monographs of the Evaluation of the Carcinogenic Risk of Chemical to Human. IARC, Lyon, France. 40, 83–98.

32. B.B. Dunn, M.E. Stack, D.L. Park, A. Joshi, L. Friedman and R.L. King. (1983). Isolation and identification of dihydrocitrinone, a urinary metabolite of citrinin in rats. J Toxicol Environ Health. 12, 283-289.

33. R.D. Phillips, A.W. Hayes, W.O. Berndt. J. Chromatography. 190 (1980) 419.

34. J. Rubert, J.M. Soriano, J. Manes, C. Soler. Food Chem.Toxicol. 49 (2011) 2299.

35. J. Ahn, D. Kim, H. Kim, and K.Y. Jahng. Food Addit. Contam. 27 (2010) 1674.

36. M. Solfrizzo, L. Gambacorta, V.M.T. Lattanzio, S. Powers, A. Visconti. Anal Bioanal Chem. 401 (2011) 2831.

37. B. Warth, M. Sulyok, F. Berthiller, R. Schuhmacher, P. Fruhmann, C. Hametner, G. Adam, J. Fröhlich and R. Krska. Anal Bioanal Chem. 401 (2011) 195.

38. X. Wu, P. Murphy, J. Cunnick, S. Hendrich. Food Chem. Toxicol. 45 (2007) 1846.

39. P.A. Egner, J.D. Groopman, J-S. Wang, T.W. Kensler, M.D. Friesen. Chem. Res. Toxicol. 19 (2006) 1191.

40. G. Pagliuca, E. Zironi, A. Ceccolini, R. Matera, G.P. Serrazanetti and A. J Piva. Chromatography B. 819 (2005) 97.

41. Commission E (2002) Commission Decision 2002/657/EC of 12 August 2002. Off J Eur Union L221:8–36.

42. European Commission (2006) Commission Regulation of 23 February 2006. Off J Eur Union L70:12–34.

43. IUPAC (1978). Spectrochim. Acta B. 33B (1978) 242

44. S. Monbaliu, C. Van Poucke, C. Van Peteghem, K. Van Poucke, K. Heungens, S. De Saeger. Rapid. Commun. Mass Spectrom. 23 (2009) 3.

45. M.J. Hinojo, M.J. Hinojoa, A. Medinaa, F.M. Valle-Algarrab, J.V. Gimeno-Adelantadob, M. Jime´neza, R. Mateob.. Food Microbiology. 23 (2006) 119.

46. R.H. Myers, Y. Kim, K.L. Griffiths. Response surface methods and the use of noise variables. J. Qual. Technol. 29 (1997) 429.

Tables and Figures

Table 1: MS/MS parameters of the selected analytes

Analyte	Retention time (min)	Precursor ion (m/z)	Cone Voltage (V)	Product ions (m/z)	Collision energy (eV)
DON-Glu	4.26	490 [M+NH4$^+$]	15	297* 243	10 15
DON	4.50	297.10 [M+H$^+$]	18	249.20* 203.10	12 16
DOM	5.73	281.10 [M+H$^+$]	18	109.20* 233.20	17 10
AFB_1-N7Gua	6.34	481 [M+H$^+$]	22	152* 329	28 18
AFM_1	7.25	329 [M+H$^+$]	34	273.10* 229.10	25 40
OTα	7.68	257 [M+H$^+$]	12	239* 221	15 25
AFB_1	8.73	313 [M+H$^+$]	30	285.10* 241.10	20 35
CIT	9.13	251.50 [M+H$^+$]	25	90* 233.40	40 20
HFB_1	9.68	405.90 [M+H$^+$]	32	370* 388	20 20
HT-2	11.06	442.10 [M+NH4$^+$]	16	263.10* 215.20	15 15
ZEN-14Glu	10.22	517 [M+Na$^+$]	35	340.80* 323.0	25 25
β-ZOL	11.39	321,10 [M+H$^+$]	15	285.20* 303.20	10 13
FB_1	12.39	722,3 [M+H$^+$]	45	334.20* 352.20	35 35
T-2	12.75	484.20 [M+NH4$^+$]	18	305.20* 215.10	15 19
α-ZOL	13.87	321,10 [M+H$^+$]	10	285.20* 303.20	10 13
ZEN	13.32	319,10 [M+H$^+$]	15	301.10* 283.20	10 15
4-OH OTA	14.47	419 [M+H$^+$]	20	255* 271	15 10
OTA	14.63	404 [M+H$^+$]	20	239* 358	25 10

*Most abundant product ion

Table 2: Linearity, apparent recovery, LOD, LOQ and repeatability of the different

Analytes	Concentration range (ng/mL)	Lack of fit (p-value)	R2	Apparent recovery (bias, %)	LOD (ng/mL)	LOQ (ng/mL)	[a]RSDr (%)	[a]RSDR (%)
DON-3Glu	5-50	0.095	0.9882	11	2.25	4.5	19	29
DON	5-50	0.062	0.9892	8	2.85	5.7	16	19
DOM	5-100	0.412	0.9870	11	0.65	1.30	7	15
AFB_1-N7Gua	1-50	0.150	0.9923	6	0.85	1.70	9	12
AFM_1	0.05-10	0.123	0.9980	5	0.01	0.02	8	8
OTα	0.1-20	0.992	0.9991	4	0.03	0.06	11	13
AFB_1	2-20	0.080	0.9774	2	0.83	1.66	11	15
CIT	5-50	0.327	0.9936	12	2.88	5.76	13	27
HFB_1	1-50	0.236	0.9994	3	0.51	1.02	10	18
HT-2	1-20	0.096	0.9976	7	0.42	0.84	7	21
β-ZOL	5-20	0.178	0.9904	6	1.1	2.2	8	11
FB_1	0.2-20	0.215	0.9946	8	0.05	0.1	5	11
T-2	0.2-20	0.369	0.9992	5	0.05	0.1	9	11
α-ZOL	2-20	0.053	0.9845	9	0.61	1.22	8	10
ZEN	5-50	0.625	0.9933	15	1.24	2.48	8	9
ZEN-4Glu	1-50	0.123	0.9985	14	3.65	7.3	13	23
4-OH OTA	1-20	0.263	0.9875	12	0.12	0.24	14	22
OTA	0.1-10	0.479	0.9991	1	0.03	0.06	5	15

R2: coefficient of determination. RSD: relative standard deviation. RSDr: intra-day precision. . RSDR: inter-day precision. [a]RSD: data were obtained from 10 ng/mL spiked concentration levels

Table 3. Mycotoxin contamination levels for the different urine samples

	Analytes concentration (ng mg^{-1} creatinine)							Un-corrected analyte concentration (ng/mL)						
Sample(s)	OTA	OTα	4-OH OTA	ZEN	β-ZOL	DON	CIT	OTA	OTα	4-OH OTA	ZEN	β-ZOL	DON	CIT
1	0.3	2.5	nd	nd	nd	15.2	nd	0.61	5.1	nd	nd	nd	31	nd
2	0.04	6	nd	<LOQ	3.3	15.2	nd	0.1	15	nd	<LOQ	8.3	38.1	nd
3	nd	nd	nd	3.2	12	nd	nd	nd	nd	nd	4.3	16.1	nd	nd
4	0.1	4.4	nd	nd	2,5	3.7	nd	0.16	7.0	nd	nd	4	5.9	nd
5	nd	nd	nd	nd	nd	47	nd	nd	nd	nd	nd	nd	62.5	nd
6	nd	nd	nd	10.8	20	nd	nd	nd	nd	nd	12.6	24.8	nd	nd
7	0.2	nd	<LOQ	nd	nd	nd	nd	0.6	nd	<LOQ	nd	nd	nd	nd
8	nd	nd	nd	nd	nd	nd	4.5	nd	nd	nd	nd	nd	nd	6.8
9	nd	nd	nd	<LOQ	nd	67	nd	nd	nd	nd	<LOQ	nd	68.3	nd

nd: not detected. <LOQ: less than limit of detection

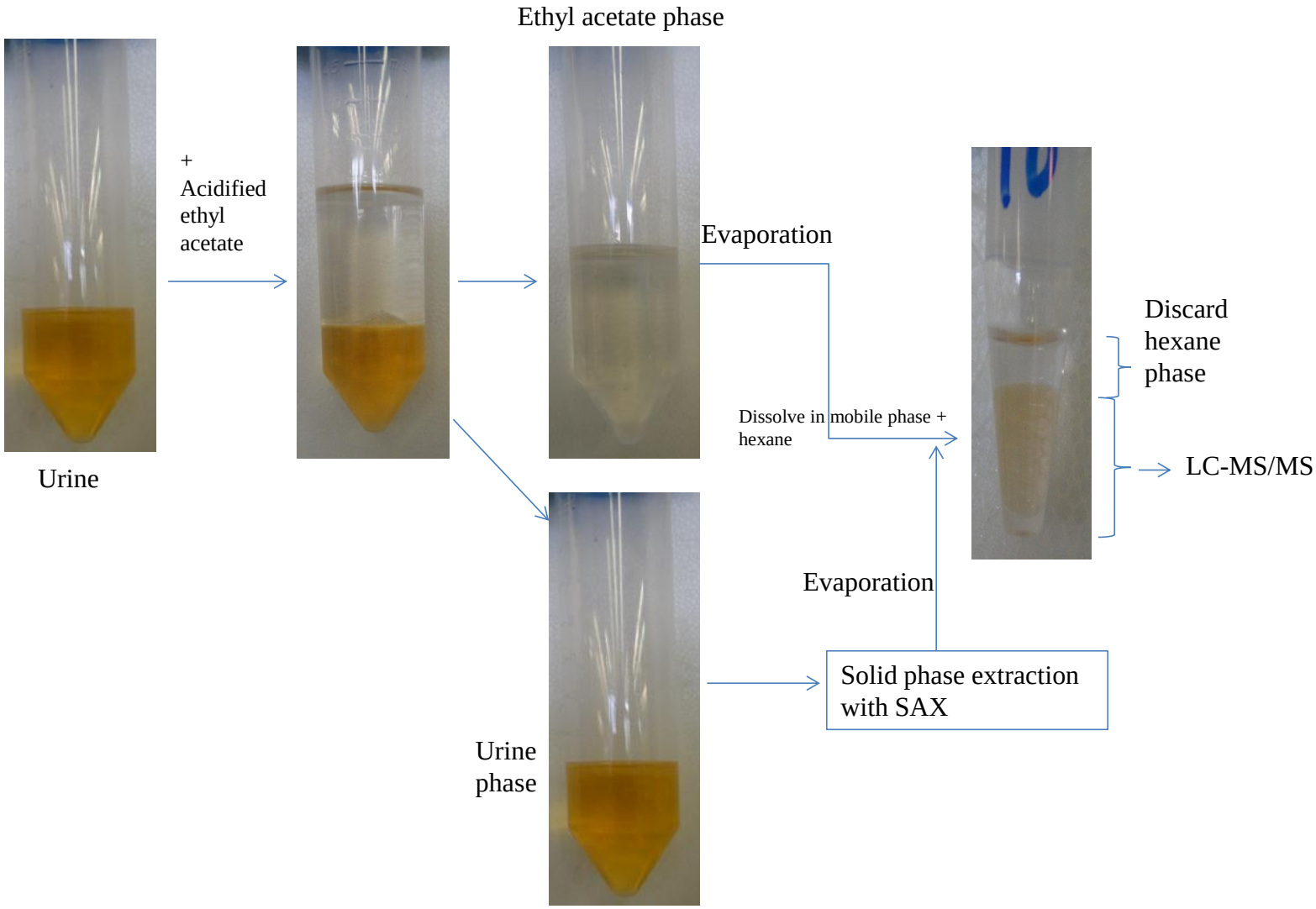
Ethyl acetate phase
+
Acidified
ethyl
acetate
Evaporation
Urine
Dissolve in mobile phase +
hexane
Discard
hexane
phase
LC-MS/MS
Evaporation
Solid phase extraction
with SAX
Urine
phase

Figure 1

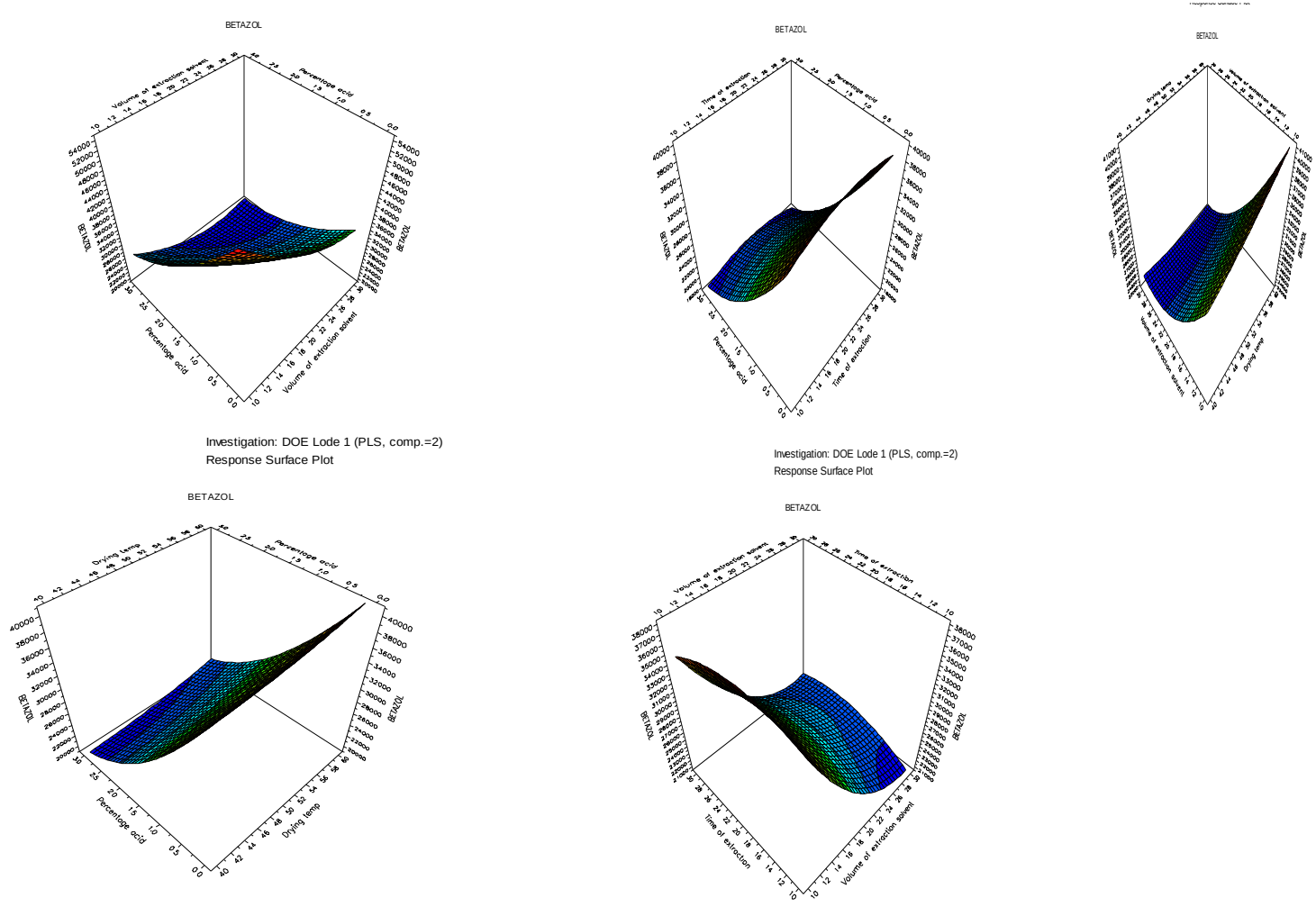
BETAZOL
Volume of extraction solvent
Percentage acid
BETAZOL
Investigation: DOE Lode 1 (PLS, comp.=2)
Response Surface Plot
BETAZOL
Time of extraction
Percentage acid
BETAZOL
Investigation: DOE Lode 1 (PLS, comp.=2)
Response Surface Plot
BETAZOL
Drying time
Volume of extraction solvent
BETAZOL
BETAZOL
Drying temp
Percentage acid
BETAZOL
BETAZOL
Volume of extraction solvent
Time of extraction
BETAZOL

Figure 2

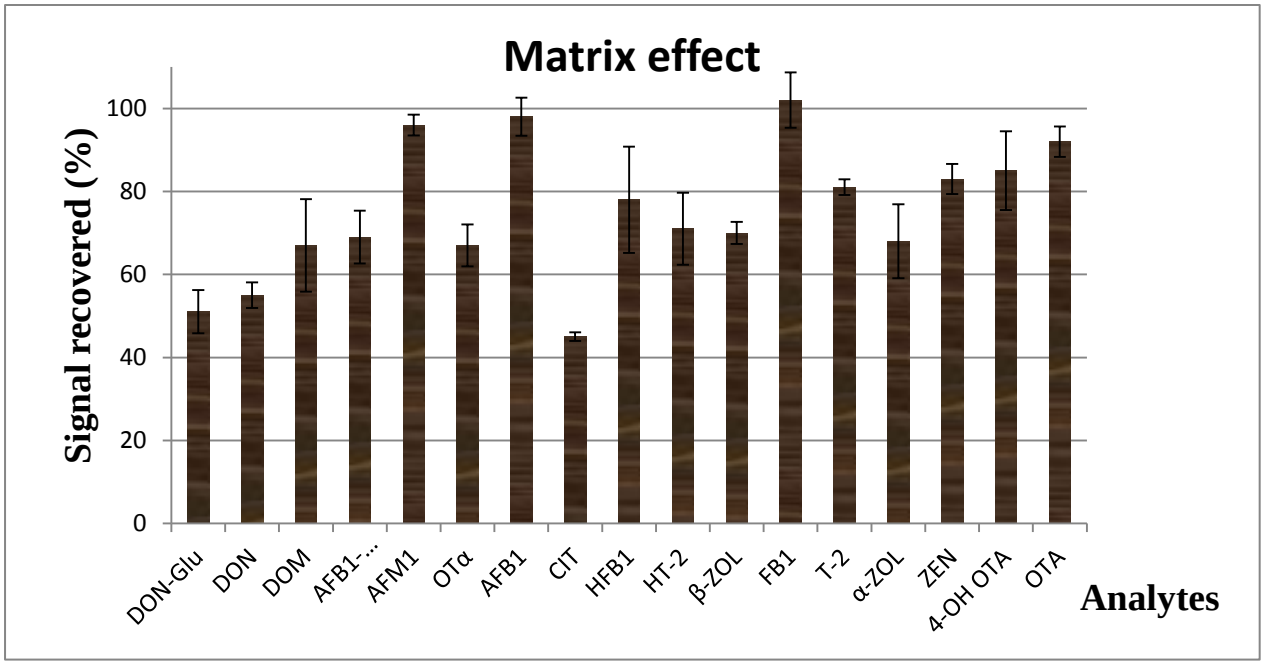

Figure 3

The error bars represent the standard deviations obtained from the five different individual samples. Calibration range, same as those used to determine the method linearity (see Table 2).

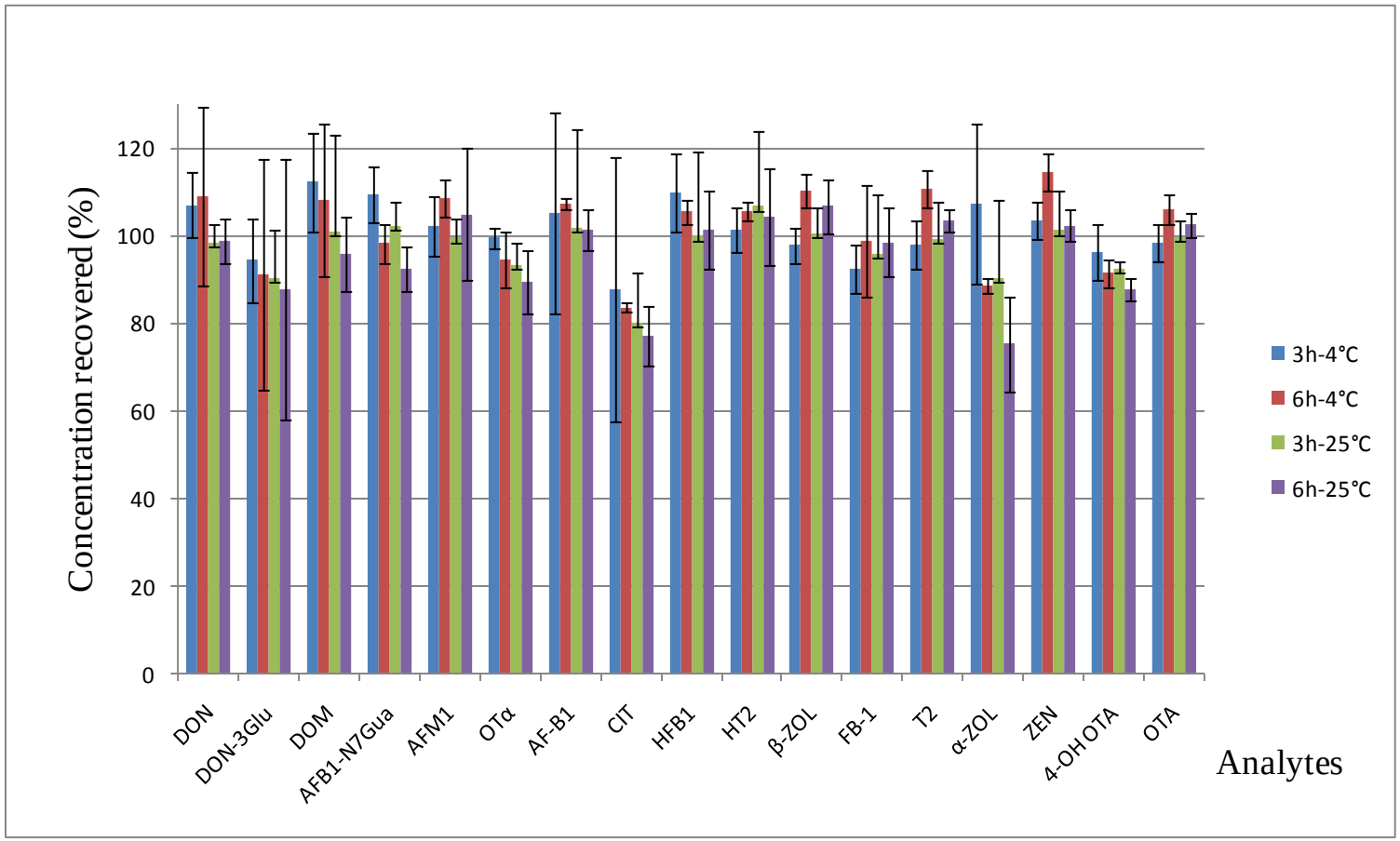
Concentration recovered (%)
120
100
80
60
40
20
0
DON
DON-3Glu
DOM
AFB1-N7Gua
AFM1
OTα
AF-B1
CIT
HFB1
HT2
β-ZOL
FB-1
T2
α-ZOL
ZEN
4-OH OTA
OTA
Analytes
3h-4°C
6h-4°C
3h-25°C
6h-25°C

Figure 4a

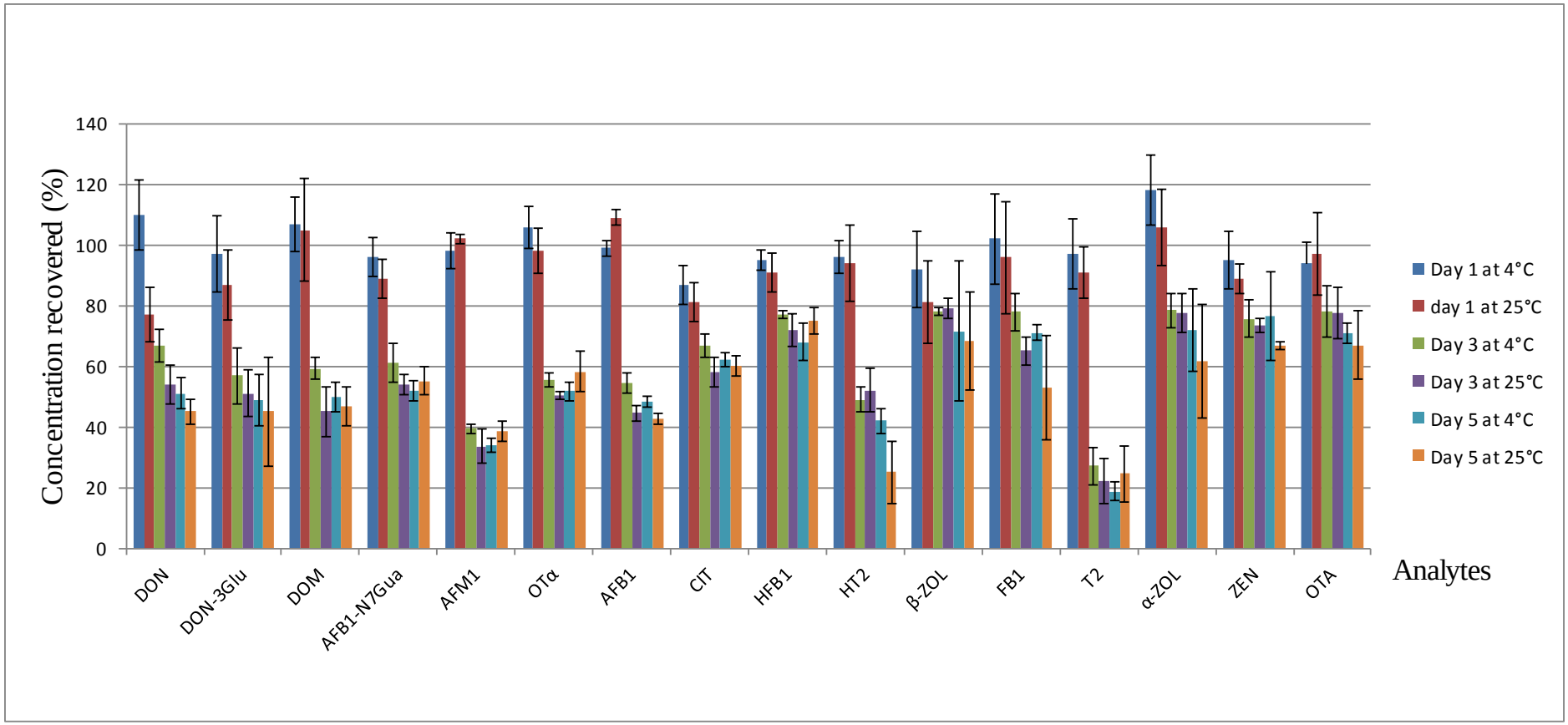
Concentration recovered (%)
140
120
100
80
60
40
20
0
DON
DON-3Glu
DOM
AFB1-N7Gua
AFM1
OTα
AFB1
CIT
HFB1
HT2
β-ZOL
FB1
T2
α-ZOL
ZEN
OTA
Analytes
Day 1 at 4°C
day 1 at 25°C
Day 3 at 4°C
Day 3 at 25°C
Day 5 at 4°C
Day 5 at 25°C

Figure 4b

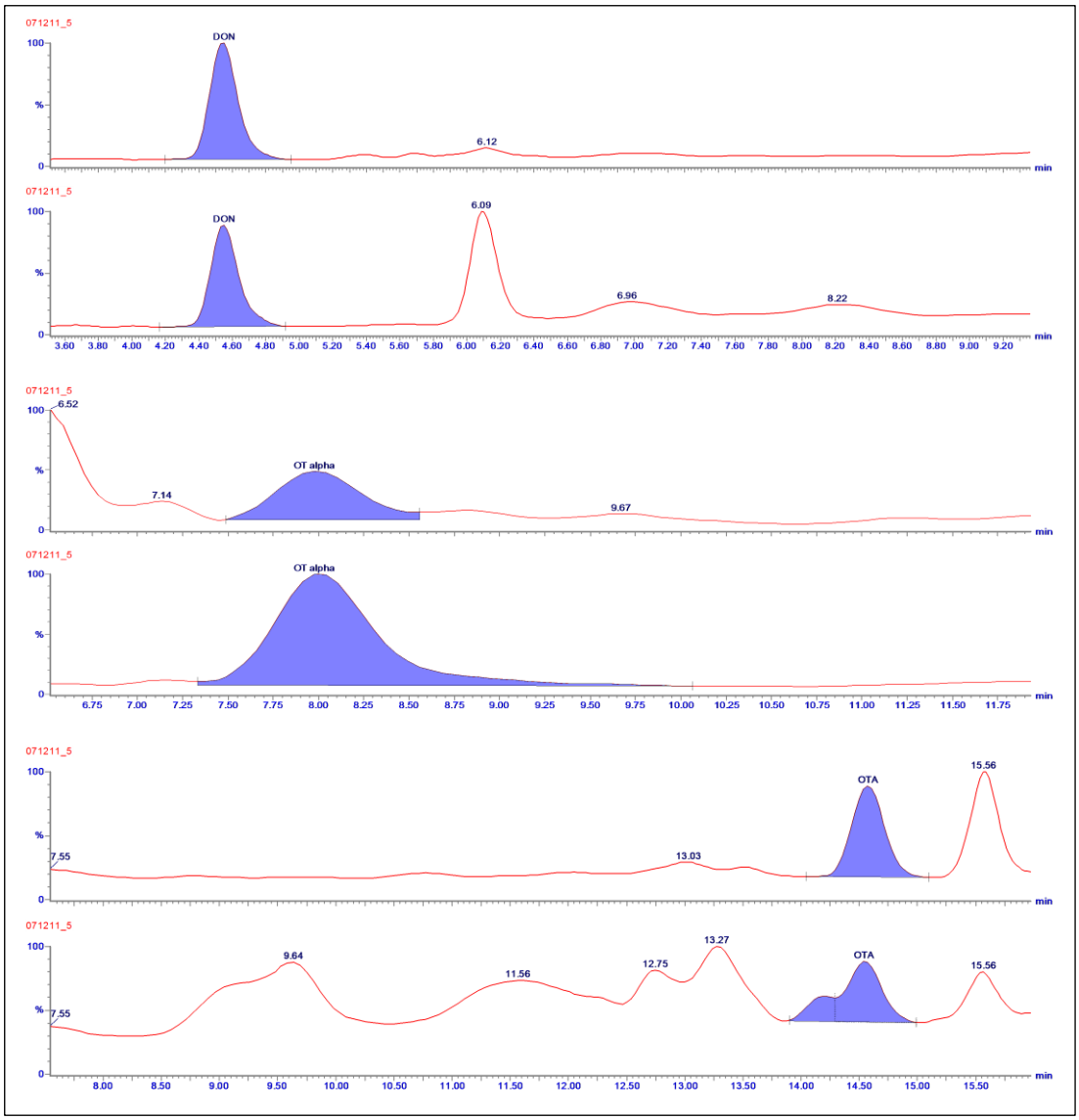
071211_5
DON
6.12
071211_5
DON
6.09
6.96
8.22
071211_5
6.52
OT alpha
7.14
9.67
071211_5
OT alpha
071211_5
OTA
15.56
7.55
13.03
071211_5
9.64
11.56
12.75
13.27
OTA
15.56
7.55
min

Figure 5

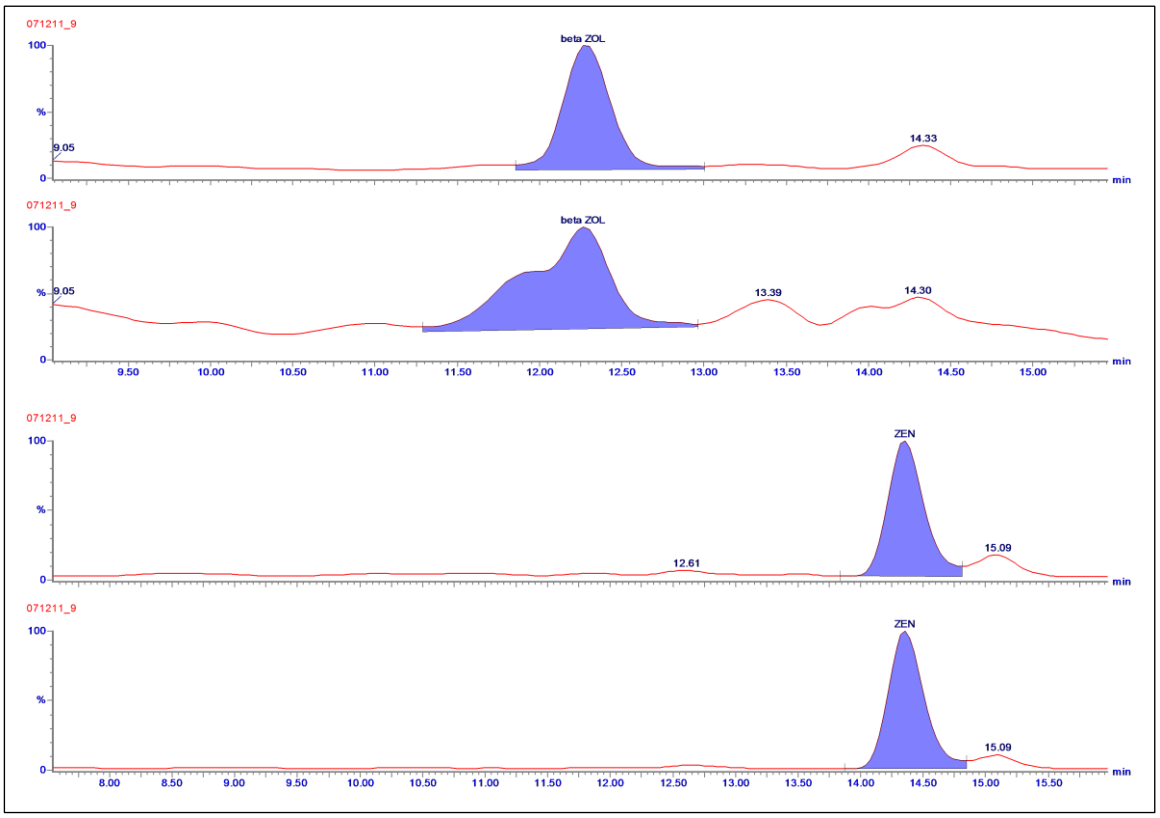
071211_9
100
%
0
beta ZOL
9.05
14.33
min
071211_9
100
%
0
beta ZOL
9.05
13.39
14.30
9.50
10.00
10.50
11.00
11.50
12.00
12.50
13.00
13.50
14.00
14.50
15.00
min
071211_9
100
%
0
ZEN
12.61
15.09
min
071211_9
100
%
0
ZEN
15.09
8.00
8.50
9.00
9.50
10.00
10.50
11.00
11.50
12.00
12.50
13.00
13.50
14.00
14.50
15.00
15.50
min

Figure 6

Figure captions

Figure 1: Schematic overview of the sample preparation protocol

Figure 2: Response surface plot for β-ZOL

Figure 3: Matrix effect for the different analytes

Figure 4a: Short term stability study at 4°C and 25°C for 3 hrs and 6 hrs storage time

Figure 4b: Short term stability study at 4°C and 25°C for 3 days and 5 days storage period

Figure 5: Chromatogram of sample 1 showing co-occurrence of DON, OTA and OTα

Figure 6: Chromatogram of sample 6 showing co-occurrence of ZEN and β-ZOL