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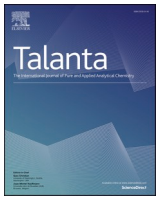

Reliable and sensitive analytical platform to assess dietary exposure of pigs to mycotoxins and explore potential urinary biomarkers

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ABSTRACT

A reliable and sensitive analytical platform is proposed for the assessment of pig exposure to mycotoxins through the consumption of commercial feed. A total of 48 naturally contaminated feed and 55 urine samples collected from eight Spanish farms were analyzed using a fast and simple methodology based on solid-liquid extraction (SLE) or liquid-liquid extraction (LLE) and dispersive liquid-liquid microextraction (DLLME). High-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) was used for the targeted analysis of 27 mycotoxins from different families in both matrices achieving limits of quantification in a range of 0.019–73.5 ng g^{-1} in feed and 0.011–31.7 ng mL^{-1} in urine. All feed samples showed contamination with at least 7 mycotoxins. Enniatins (A, A1, B and B1) and beauvericin were quantified in 100 % of feed samples. ENNB, tenuazonic acid (TeA) and deoxynivalenol (DON) were the mycotoxins with the highest mean total concentrations (1.0 ± 1.9 μg g^{-1}, 155 ± 209 ng g^{-1} and 81 ± 94 ng g^{-1}, respectively). In urine samples, DON, TeA, ENNB1 and ENNA were the most prevalent mycotoxins; and TeA, fumonisin B1 and alternariol had the highest mean total concentration (133 ± 199 ng mg^{-1}, 0.43 ± 1.3 μg mg^{-1} and 0.29 ± 1.3 μg mg^{-1} creatinine, respectively). Statistical tests revealed the correlation of DON and TeA occurrence in feed and urine. Untargeted analysis by HPLC coupled to quadrupole-time-of-flight mass spectrometer (Q-TOF-MS) yielded some urinary biomarkers of mycotoxin exposure and other relevant compounds such as certain antibiotic residues in urine.

1. Introduction

Mycotoxins frequently contaminate a wide variety of agricultural crops, being very common in grains (maize, wheat, barley, rye, oats or soybeans), nuts, and spices. Crops can be infected with multiple fungi at different stages of growth, and the resulting mycotoxins persist throughout the process, from harvest to packaging [1,2]. Mycotoxins constitute a major safety concern for the feed industry. More than 50 % of feed weight comes from cereals, which represents a threat of mycotoxin contamination due to their susceptibility to fungal growth [3]. In fact, over 70 % of feed worldwide is contaminated with at least one mycotoxin. The most frequent mycotoxins in animal feed are deoxynivalenol (DON), fumonisins (FBs) and aflatoxin B1 (AFB1) [4]. However, there are important regional variations; for example, in a study of 228 Spanish pig feed, 40 % of samples were contaminated with more than five mycotoxins and the most commonly found (considering samples with concentrations above the limits of quantification (LOQs)) were: enniatins (ENNs) (ENNB, 100 % samples; ENNB1, 53.5 %, ENNA1, 40.8 %), beauvericin (BEA) (93.4 %), and fumonisin B1 (FB1) (50.0 %) [5].

The European Commission established maximum permitted concentration levels for AFB1 (0.005–0.02 μg g^{-1}) and guideline values for DON (0.9–12 μg g^{-1}), the sum of FB1 and FB2 (5–60 μg g^{-1}), zearalenone (ZEN) (0.1–3 μg g^{-1}), the sum of T-2 and HT-2 toxins

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(250–2000 ng g^{-1}), and ochratoxin A (OTA) (0.05–0.25 μg g^{-1}) in products for animal feeding [6–8]. However, emerging mycotoxins, such as ENNs and BEA, are not regulated and are increasingly detected in animal feed. The European Food Safety Agency (EFSA) requires relevant data to determine their toxicity [9].

Feed analysis has been the traditional method for assessing animal exposure to mycotoxins, but this approach can be challenging due to the heterogeneity of mycotoxin distribution in feed. Besides that, the individual exposure is not assessed, so fluctuations due to differences in feed consumption or in the absorption, distribution, metabolism and excretion (ADME) processes between animals are not considered. In addition, the possible conversion of modified or conjugated mycotoxins to their parent compounds are also not evaluated by that approach, and may contribute to adverse effects associated with mycotoxin exposure [10,11]. Exposure biomarkers have been proposed as an effective alternative to assess mycotoxin exposure.

Pigs are highly exposed to mycotoxins due to their diet based on cereals. Most of the established tolerable daily intake (TDI) for humans are based on the observed negative effects in pigs [12]. Therefore, many studies have been conducted on the evaluation of biomarkers in these animals to assess their exposure to mycotoxins. Urine is the biological fluid commonly chosen to the biomarker analysis, although serum and feces have also been frequently used [12]. Among the mycotoxins detected in pig feed, the legislated mycotoxins (DON, ZEN, OTA, FB1 and AFB) and their metabolites have been the most studied. DON was identified as the most important urinary biomarker for the assessment of DON in diets, as a single biomarker [11,13,14] or in combination with de-epoxy-deoxynivalenol (DOM-1) [15–20]. Glucuronide metabolites of DON (DON-15-GlcA and DON-3-GlcA) have also been proposed as exposure biomarkers [13,21], as well as DON-3-sulfate [21]. The Joint FAO/WHO Expert Committee on Food Additives considered that 3-ADON and 15-ADON contribute to the total dietary exposure to DON [22]. Feed contamination with ZEN was assessed using ZEN as a biomarker of exposure in urine [23], and in combination with alpha-zearalenol (α-ZEL) [15], and also with β-ZEL [16,17,19]. Glucuronide metabolites of ZEN were also considered [11,14,21]. OTA [16,17] and its combination with ochratoxin alpha (OTα) [15] have been proposed as biomarkers of OTA exposure, AFM1 as a biomarker of AFB1 [16,17] and FB1 for FB1 exposure in pig [16,17].

Research on biomarkers of mycotoxin exposure in pigs by urine analysis are often dose-response studies, where contaminated feed at different concentrations is given to pigs to assess the behavior of mycotoxins in their organism. However, currently, these types of studies are controversial from the ethical perspective of animal welfare. In this study, an analytical platform is proposed that provides a global approach to the exposure of pigs to mycotoxins through the consumption of naturally contaminated feed. Specifically, a targeted approach was applied for the determination of 27 mycotoxins from different families from consumed feed and urine excreted by pigs from eight Spanish farms. The analysis was carried out by high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS), being the reference technique in recent years for mycotoxin analysis [24]. In addition, an untargeted analysis was also performed by an ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS) for the research of urinary metabolites and the assignment of biomarkers of exposure to these mycotoxins.

To date, a wide variety of sample treatments have been applied for mycotoxin extraction, ranging from traditional approaches based on solid-liquid extraction (SLE) [25,26] and liquid-liquid extraction (LLE) [11] to the development of novel magnetic materials for solid phase extraction (SPE) [27–29], immunoaffinity columns [30,31] and microextraction techniques [32,33]; being QuEChERS still the most widely used methodology nowadays [34].

The determination of multiple mycotoxins simultaneously remains a challenge due to the different chemical structures of the mycotoxins, the low concentrations and the complexity of food matrices. The proposed methodology is based on a combination of different sample pretreatment methods: SLE (feed) or LLE (urine) assisted by salt and dispersive liquid-liquid microextraction (DLLME). It is well known that the addition of salt facilitates the transfer of analytes to the organic extraction solvent providing a simple, fast and highly efficient method, while DLLME provides fast and simple extractions with high recovery and high enrichment factors with low organic reagent consumption. Thus, the proposed methodology provides simple, fast and efficient extraction of multiple mycotoxins from different families allowing a broad evaluation of pig exposure to these contaminants.

2. Materials and methods

2.1. Standards and reagents

AFs (G1, G2, B1, B2), ENNs (A, B, A1, B1), BEA, OTA and DON standards were obtained from Sigma Aldrich (St. Louis, MO, USA). Individual solutions of standards were prepared at 1000 μg mL^{-1} in acetonitrile (ACN). Standards solutions of ZEN, FBs (B1 and B2), T-2 and HT-2 toxin at 100 μg mL^{-1} in ACN were purchased from n'TOX (Saint Jean d'Illac, France). Fusarenon-X (FUS-X) (100.1 μg mL^{-1}), diacetoxyscirpenol (DAS) (101.4 μg mL^{-1}), 3-acetyldeoxynivalenol (3-ADON) (100 μg mL^{-1}), sterigmatocystin (STE) (50.5 μg mL^{-1}), cyclopiazonic acid (CPA) (100.3 μg mL^{-1}), ochratoxin B (OTB) (10 μg mL^{-1}), citrinin (CIT) (100.1 μg mL^{-1}), tenuazonic acid (TeA) (100.8 μg mL^{-1}), tentoxin (TEN) (100.2 μg mL^{-1}), altenuene (ALT) (10.08 μg mL^{-1}) and alternariol (AOH) (100.1 μg mL^{-1}) solutions in ACN were purchased from Romer Labs Division Holding GmbH (Getzersdorf, Austria). ENNs, BEA, AFs, DON, T-2, HT-2, ZEN FBs, OTA and STE solutions were stored at −20 °C, and the remaining solutions at 4 °C.

Methanol (MeOH), ACN and chloroform were supplied by Chem-Lab (Zedelgem, Belgium). Sodium chloride, formic acid (ACS reagent, ≥98 %) and ammonium formate were obtained from Sigma Aldrich. Magnesium sulfate anhydrous was obtained from Thermo Fisher Scientific (Waltham, MA, USA). A Millipore Milli-Q system (Bedford, MA, USA) was used to obtain ultrapure water.

2.2. Instrumentation and software

A 1200 high-performance liquid chromatograph coupled to a 6410 triple quadrupole mass spectrometer with an electrospray ionization (ESI) from Agilent Technologies (Santa Clara, CA, USA) was used for targeted analysis. Chromatographic separation was carried out using an InfinityLab Poroshell 120 EC-C18 column (4.6 × 100 mm x 2.7 μm), also from Agilent. MassHunter Quantitative Analysis (QqQ) software from Agilent was used to collect and analyze data, using the multiple reaction monitoring (MRM) mode. Untargeted experiments were performed on a 1290 Infinity II series ultra-high performance liquid chromatograph with a 6550 Q-TOF mass spectrometer (UHPLC-Q-TOF-MS) from Agilent. System was equipped with an ESI source operating in positive mode. The column, also from Agilent, was a Zorbax Eclipse Plus C-18 (2.1 × 100 mm x 1.8 μm). In this case, MassHunter Qualitative Analysis B.10.0 was used for the identification of mycotoxin metabolites. The MS/MS fragmentation spectra obtained was compared with the experimental spectra suggested in the Personal Compound Database and Library (PCDL) of mycotoxins and related metabolites from Agilent. This PCDL contains information on the name, formula, exact mass, structure and acquired MS/MS spectra of 455 mycotoxins and metabolites. SigmaPlot software version 13.1 (Systat Software, San Jose, CA, USA) was used for the statistical analysis.

An LLG-uniTEXER vortex from Heathrow Scientific (Illinois, USA), an EBA 20 centrifuge (Hettich, Tuttlingen, Germany), an XcelVap air-drying system from Horizon Technology (Salem, MA, USA) and nylon syringe filters of 0.2 μm × 13 mm and 0.45 μm × 25 mm from Agilent were used during sample treatment. Feed samples were ground using an IKA A11 basic analytical mill (Wilmington, USA). Creatinine

determination was performed using a UV–visible spectrophotometer (ATI Unicam UV2) equipped with the VISION V3.00 software package.

2.3. Pig feed and urine samples

A total of 48 feed and 55 urine samples were collected from eight Spanish farms from Murcia, Sevilla, Córdoba and Lérida, and sent directly to the laboratory. Specifically, the following samples were collected from each farm: Murcia ("farm 1": 6 feed and 10 urine; "farm 2": 4 feed and 2 urine), Sevilla ("farm 3": 2 feed and 11 urine; "farm 4": 10 feed and 2 urine), Córdoba ("farm 5": 7 feed and 12 urine; "farm 6": 7 feed and 2 urine; "farm 7": 4 feed and 7 urine) and Lérida ("farm 8": 8 feed and 9 urine).

Feed collection was carried out at different sampling points: 11 samples were collected from a silo, 23 from feeders and 14 from sacks. Samples (approximately 1 kg) were sent directly to the laboratory by the veterinarians responsible for the farms. First, samples were homogenized and manually divided into four equal parts. Then, two opposite parts were mixed diagonally and the other two were discarded. This process was repeated until a subsample of 125 g of feed was obtained. This subsample was pulverized, homogenized and stored in a dry place protected from light until analysis. Coinciding with each feed sampling, clean-catching midstream urine samples were collected from the sows (35 lactating and 20 pregnant) during spontaneous micturition immediately after rising from periods of rest. Urines were collected in 100 mL polypropylene flasks and stored at −20 °C until analysis. Stærk et al. proved that urine specimens collected by this clean-catch method are only minimally contaminated by cutaneous and vaginal commensals. Thus, this collection method represents an improvement in terms of animal welfare and allows a more frequent collection of urine specimens, which can enhance the scientific outcome of experimental animal studies in pigs [35].

2.4. Analytical procedure

2.4.1. Sample treatment

A scheme of the proposed sample treatment for feed and urine samples is shown in Fig. 1. Feed (2 g) was transferred to a 50 mL polypropylene tube and mixed with 6 mL of ultrapure water. After homogenization, 6 mL of ACN containing 5 % of formic acid was added and the mixture was vortexed for 2 min. Then, 4 g of magnesium sulfate and 1 g of sodium chloride were added, and the tube was agitated manually for 1 min. After centrifuging during 5 min at 4000 rpm, the supernatant obtained was collected, filtered using the 0.45 μm filter and divided into two 2 mL aliquots. One aliquot was dried using an air-drying system and reconstituted in 500 μL of MeOH:H_2O (50:50, *v/v*). The extract was filtered (0.2 μm) and injected into the system for the analysis of DON, FUS-X, CIT, AOH, ALT, TeA, FB1 and FB2. The other 2 mL of supernatant was subjected to dispersive liquid-liquid microextraction (DLLME), using 6 mL of ultrapure water and 600 μL of chloroform. The mixture was centrifuged for 3 min at 3500 rpm and the sedimented phase was collected and dried. It was reconstituted using the same conditions and analyzed for the determination of the remaining mycotoxins (Fig. 1a).

Urine samples were prepared by salting-out liquid-liquid extraction (SALLE) for the determination of DON, FUS-X, CIT, AOH, ALT, TeA, FB1 and FB2, and by DLLME for the rest of the mycotoxins. SALLE was carried out using 6 mL of urine, 2 mL of ACN, 2 g of magnesium sulfate and 1 g of sodium chloride. After shaking and centrifugation at 4000 rpm for 5 min, the supernatant (2 mL) was collected, filtered (0.45 μm) and dried using an airstream. On the other hand, 10 mL of urine, 1.75 mL of ACN and 800 μL of chloroform were used for DLLME extraction. The sedimented phase obtained after centrifugation at 3500 rpm for 3 min was collected and dried. The dried samples were reconstituted in 500 μL of MeOH:H_2O (50:50, *v/v*) and filtered (0.2 μm) for the chromatographic analysis (Fig. 1b).

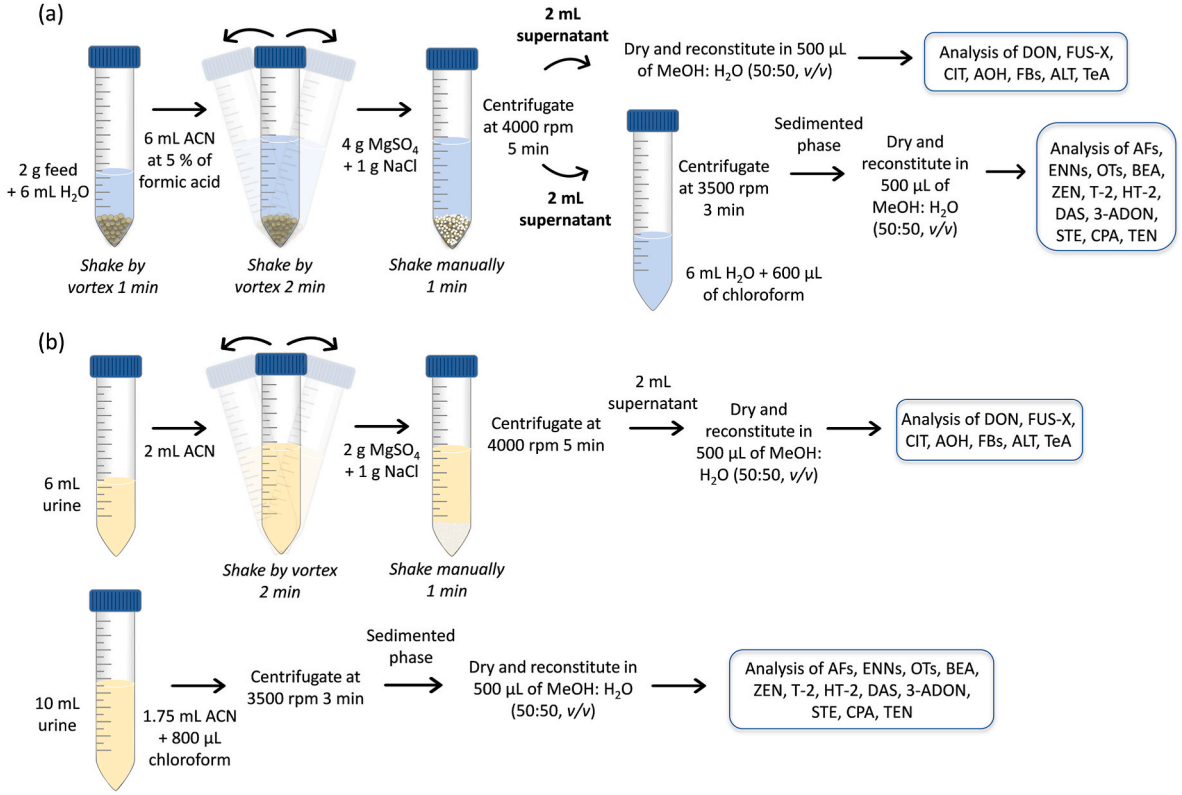

Fig. 1. Proposed sample treatment for the extraction of mycotoxins in feed (a) and urine (b).

2.4.2. Sample analysis

Feed and urine samples were analyzed in targeted mode by HPLC-QqQ-MS/MS under the following chromatographic conditions. Mobile phases were composed of an aqueous solution at 0.1 % of formic acid with 2 mM of ammonium formate (solvent A) and MeOH with 0.1 % of formic acid (solvent B). The elution program started at 30 % of solvent B and increased linearly to 99 % after 20 min, which was maintained until 35 min. Then, solvent B decreased gradually to reach 30 % again at 37 min. Finally, this ratio was maintained for 8 min. The flow rate was 0.5 mL min^{-1} and the volume of the sample injected was 20 μL. Regarding source parameters, gas temperature was 350 °C, gas flow was set at 9 L min^{-1}, nebulizer pressure was 40 psi and capillary voltage was 3000 V. Mycotoxins were determined in positive ESI mode using the MRM conditions shown in Table S1. The molecular ions monitored of the target mycotoxins were $[M+H]^+$, except for DAS, HT-2, T-2, ENNs and BEA, where the adducts $[M + NH_4]^+$ were more abundant than the protonated molecules.

Urine samples were also analyzed in untargeted mode by UHPLC-Q-TOF-MS for mycotoxin-related metabolites. In this case, mobile phases were: H_2O:MeOH (95:5, *v/v*) at 0.1 % of formic acid with 2 mM of ammonium formate (solvent A), and MeOH: H_2O (95:5, *v/v*) at 0.1 % of formic acid with 2 mM of ammonium formate (solvent B). Flow rate was set at 0.4 mL min^{-1}. The elution program started at 100 % of solvent A and progressively decreased until solvent B was 99 % at 20 min. Then, solvent A was increased again up to 100 % and held for up to 26 min. The sample volume injected was 20 μL. The instrumental parameters used were: 150 °C gas temperature, 16 L min^{-1} gas flow, 35 psi nebulizer pressure, 4000 V capillary voltage and 360 V fragmentor. MS data were acquired from 100 to 1000 *m/z* in Auto MS/MS mode at 40 V of collision energy.

The tentative identification of metabolites was carried out using the PCDL mycotoxin library and the MassHunter Qualitative Analysis software from Agilent. The "Compound discovery" workflow and "Find by Auto MS/MS" compound mining was applied for the compound search. Results were filtered by an absolute peak area of 100 counts, mass tolerance of ±5 ppm and LC/MS tolerance of 5 ppm + 2 mDa (for precursor ion *m/z*) and 7.5 ppm + 5.0 mDa (for fragment ion *m/z*).

2.5. Creatinine determination

For the determination of urinary creatinine levels, 500 μL of urine were mixed with 1250 μL of Milli-Q water and 250 μL of alkaline picrate solution (0.2 g of picric acid mixed with 250 mL of 1 M NaOH) in a 1 cm path length quartz cuvette. Calibration curves were obtained replacing urine by 500 μL of aqueous creatinine standard solutions in a 50–2000 μg mL^{-1} range and absorbance of the picrate–creatinine complex was monitored for 2 min at a wavelength of 500 nm. The reaction rate was used as the analytical signal. A certified reference material 'Organic contaminants in smokers' urine (frozen)' (Standard reference material, SRM 3672) obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) with a certified creatinine content of 734 ± 5 mg kg^{-1} was measured in triplicate to verify the accuracy of the method. The creatinine content obtained was 737 ± 6 mg kg^{-1} (n = 3).

3. Results and discussion

3.1. Optimization of sample treatment

During the first experiments for the extraction of mycotoxins from feed, DLLME was investigated after salt-assisted extraction. The combination of both extractions improved the isolation of most, but was less effective for some mycotoxins (DON, FUS-X, CIT, AOH, ALT, TeA, FB1 and FB2). For this reason, the sequence of both extractions was selected, and for mycotoxins whose extraction was not improved by the DLLME step, the sample was subjected only to salt-assisted extraction, dried and analyzed. The use of sodium chloride was investigated for DLLME step at 5 % (*m/v*). However, since it did not improve extraction, it was not used for sample treatment. In addition, several volumes of the extractant solvent in DLLME, chloroform, were tested (200–800 μL), with higher preconcentration values obtained with 600 μL (Fig. S1). In the urine samples, DLLME was first studied for mycotoxin isolation. As in the case of feed, this treatment was effective for the extraction of most mycotoxins except for DON, FUS-X, CIT, AOH, ALT, TeA, FB1 and FB2. Therefore, SALLE treatment was investigated for mycotoxins that were not isolated by DLLME. Two different sample treatments were definitely applied to urine samples: SALLE for the determination of DON, FUS-X, CIT, AOH, ALT, TeA, FB1 and FB2 and DLLME for the remaining mycotoxins. The volume of ACN used during SALLE step was studied in a range of 2–6 mL. An increase in the volume of extraction solvent did not increase the extraction efficiency, so 2 mL was selected. A multivariate optimization was performed for DLLME extraction. The investigated conditions were the volume of sample (5–10 mL) and of extractant (200–800 mL) and dispersant (1–2 mL) solvents. Finally, the optimal volumes were 10 mL of urine, 1.75 mL of ACN and 800 μL of chloroform (Fig. S2). The use of sodium chloride at 5 % (*m/v*) was also tested for DLLME without improving mycotoxin extraction. The volume of reconstitution solvent was investigated using 0.5 and 1 mL to evaluate the matrix effect in feed and urine samples. Similar results were obtained in both cases: from −99.3 to 5.2 and from −95.7 to 7.7 of matrix effect using 0.5 and 1 mL, respectively. Based on the results, 0.5 mL was selected as the optimum volume as it provided a higher preconcentration. In addition, it was the minimum volume that allowed adequate filtration of the obtained extract.

Extraction efficiency was calculated for both matrices to evaluate the proposed sample procedure (Table 1). Feed and urine samples were spiked before and after extraction at different concentrations: 5 ng g^{-1} or ng mL^{-1} for ENNs, OTs, AFs, ALT and STE, 10 ng g^{-1} or ng mL^{-1} for CIT, HT-2 and T-2, 25 ng g^{-1} or ng mL^{-1} for CPA, DAS, 3-ADON, TEN and ZEN and 100 ng g^{-1} or ng mL^{-1} for FBs, AOH, TeA, DON and FUS-X. Thus, the analyte peak areas of the spiked sample before and after the extraction process were compared. These experiments were prepared in triplicate. The efficiency obtained ranged from 79.8 to 98.8 % in feed and from 73.1 to 97.6 % in urine, demonstrating the effectiveness of the proposed sample procedure for the determination of multiple mycotoxins.

3.2. Validation of the analytical method

The proposed methodology was validated following the international guidelines [36]. Linearity, limit of detection (LOD) and LOQ,

Table 1
Extraction efficiency of mycotoxins in feed and urine samples.

Extraction efficiency (%)					
Mycotoxin	Feed	Urine	Mycotoxin	Feed	Urine
DON	83.8 ± 6.4	75.3 ± 7.4	AOH	92.7 ± 7.3	96.2 ± 3.2
FUS-X	93.7 ± 5.6	75.7 ± 9.5	OTB	95.9 ± 2.9	81.6 ± 9.5
3-ADON	88.9 ± 9.4	82.7 ± 6.6	T-2	88.5 ± 9.4	86.3 ± 0.3
AFG2	91.9 ± 7.4	82.9 ± 3.7	FB2	80.2 ± 7.9	91.5 ± 8.2
AFG1	88.5 ± 5.1	83.5 ± 5.4	OTA	93.2 ± 5.1	84.4 ± 8.1
AFB2	88.8 ± 7.4	90.4 ± 6.6	ZEN	91.6 ± 7.4	89.7 ± 7.1
DAS	92.2 ± 6.0	84.1 ± 5.1	STE	86.2 ± 8.9	92.0 ± 3.6
ALT	79.8 ± 9.2	93.1 ± 5.2	CPA	81.3 ± 9.6	84.8 ± 7.2
AFB1	96.9 ± 3.2	80.9 ± 9.7	ENNB	94.0 ± 5.6	93.7 ± 1.6
TeA	98.8 ± 1.1	73.1 ± 8.6	BEA	82.5 ± 3.1	80.0 ± 5.8
CIT	82.4 ± 7.4	93.5 ± 4.2	ENNB1	80.7 ± 8.2	97.6 ± 1.4
HT-2	82.7 ± 8.8	92.4 ± 4.0	ENNA1	89.7 ± 9.2	90.1 ± 6.8
TEN	82.0 ± 4.1	95.8 ± 3.1	ENNA	90.5 ± 9.1	86.3 ± 4.7
FB1	83.2 ± 9.1	78.3 ± 4.9			

Concentration level: 5 ng g^{-1} or ng mL^{-1} (ENNs, OTs, AFs, ALT and STE), 10 ng g^{-1} or ng mL^{-1} (CIT, HT-2 and T-2), 25 ng g^{-1} or ng mL^{-1} (CPA, DAS, 3-ADON, TEN and ZEN) and 100 ng g^{-1} or ng mL^{-1} (FBs, AOH, TeA, DON and FUS-X).

trueness, repeatability and matrix effect were evaluated. Table 2 summarizes the results achieved for each parameter. For trueness, precision and matrix effect experiments, samples were spiked at the same mycotoxin concentrations as for the extraction efficiency study (see section 3.1).

To study the matrix effect, three calibration curves in the 5–100 ng g^{-1} range were performed with three feed samples from different farms. An ANOVA statistical test was performed to compare the slopes of the calibration curves obtained for each mycotoxin in the different feed matrices, in order to verify that there were no significant differences. The same procedure was applied to the urine samples, and five calibration curves were performed for different farms including urine from

Table 2
Performance characteristics of the proposed method for mycotoxin determination.

Feed samples							
Mycotoxin	Linear range[a] (ng g^{-1})	R^2	LOD[b] (ng g^{-1})	LOQ[c] (ng g^{-1})	Matrix effect[d] (%)	Repeatability[d], %RSD[e] (n = 6)	Trueness[d] (%RSD)
DON	12.9–500	0.972	3.9	12.9	3.7	6.3	106 (2.4)
FUS-X	73.5–500	0.997	22.3	73.5	−72.1	7.6	103 (8.1)
3-ADON	3.9–250	0.996	1.17	3.9	1.5	8.4	117 (2.2)
AFG2	0.13–100	0.999	0.04	0.13	−38.9	9.3	100 (9.9)
AFG1	0.28–100	1	0.08	0.28	−58.0	5.1	99 (5.1)
AFB2	0.33–100	1	0.10	0.33	−34.0	7.4	96 (7.4)
DAS	1.6–250	0.999	0.49	1.6	−46.2	5.8	91 (9.3)
ALT	1.8–100	0.995	0.54	1.8	−29.3	9.2	103 (3.2)
AFB1	0.019–100	0.999	0.005	0.019	5.2	2.2	98 (8.3)
TeA	51.1–500	0.976	15.5	51.1	3.4	2.9	112 (3.1)
CIT	5.8–100	0.868	1.7	5.8	−8.4	6.4	94 (4.0)
HT-2	0.21–100	0.998	0.06	0.21	2.5	8.1	110 (7.4)
TEN	0.73–250	0.999	0.22	0.73	5.0	4.1	111 (4.5)
FB1	1.9–500	0.996	0.57	1.9	4.1	5.5	103 (6.3)
AOH	31.9–500	0.941	9.7	31.9	−94.4	6.1	99 (8.2)
OTB	0.70–100	0.997	0.21	0.70	−22.9	1.9	108 (1.1)
T-2	1.2–100	0.999	0.35	1.2	−40.3	8.3	112 (1.9)
FB2	3.3–500	0.983	0.98	3.3	1.7	7.9	99 (3.4)
OTA	0.40–100	0.997	0.12	0.40	0.1	5.2	101 (5.4)
ZEN	0.43–250	0.991	0.13	0.43	−29.4	7.6	112 (6.7)
STE	0.17–100	0.999	0.05	0.17	−38.8	8.9	101 (5.5)
CPA	1.10–250	0.997	0.33	1.10	−69.4	8.4	116 (2.7)
ENNB	0.04–100	0.999	0.01	0.04	−57.2	4.6	112 (5.2)
BEA	0.03–100	0.997	0.008	0.03	−48.2	3.1	100 (8.4)
ENNB1	0.24–100	0.999	0.07	0.24	−66.2	8.2	96 (7.8)
ENNA1	0.30–100	0.995	0.09	0.30	−82.2	7.2	92 (9.4)
ENNA	0.27–100	0.997	0.08	0.27	−77.9	8.1	108 (4.0)
Urine samples							
Mycotoxin	Linear range (ng mL^{-1})	R^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Matrix effect (%)	Repeatability, %RSD[e] (n = 6)	Trueness (%RSD)
DON	0.75–500	0.972	0.22	0.75	−48.8	7.4	102 (8.3)
FUS-X	31.7–500	0.993	9.5	31.7	−32.4	8.5	107 (2.9)
3-ADON	0.22–250	0.997	0.07	0.22	−44.9	5.6	96 (3.2)
AFG2	0.07–100	0.999	0.02	0.07	−0.8	3.7	96 (4.6)
AFG1	0.03–100	0.999	0.009	0.03	−13.1	4.4	99 (5.3)
AFB2	0.04–100	1	0.01	0.04	−43.6	6.6	111 (7.0)
DAS	0.21–250	0.999	0.06	0.21	1.5	5.0	95 (4.6)
ALT	2.9–100	0.999	0.88	2.9	2.7	3.1	116 (2.9)
AFB1	0.02–100	1	0.007	0.02	−28.1	9.6	94 (4.6)
TeA	27.4–500	0.994	8.2	27.4	3.9	7.6	95 (4.2)
CIT	4.0–100	0.999	1.2	4.0	−97.0	1.5	98 (3.2)
HT-2	0.35–100	1	0.10	0.35	4.4	4.0	97 (4.0)
TEN	0.30–250	0.999	0.09	0.30	−17.0	5.1	102 (5.1)
FB1	17.1–500	0.998	5.1	17.1	1.8	4.9	94 (9.7)
AOH	28.8–500	0.932	8.7	28.8	−99.3	4.7	113 (4.1)
OTB	0.63–100	0.996	0.19	0.63	0.2	6.9	93 (9.0)
T-2	0.40–100	0.999	0.12	0.40	1.9	0.3	106 (0.3)
FB2	2.5–500	0.998	0.76	2.5	4.2	8.6	91 (4.7)
OTA	0.03–100	0.995	0.009	0.03	−64.2	8.8	94 (7.8)
ZEN	0.27–250	0.999	0.08	0.27	−57.5	6.1	103 (3.0)
STE	0.02–100	1	0.005	0.02	−48.4	3.2	102 (3.4)
CPA	0.33–250	0.995	0.10	0.33	−75.3	6.2	98 (2.1)
ENNB	0.03–100	0.999	0.01	0.03	−54.1	1.6	95 (1.6)
BEA	0.011–100	0.999	0.003	0.011	−72.6	3.8	99 (5.9)
ENNB1	0.021–100	0.999	0.006	0.021	−47.4	1.4	108 (1.5)
ENNA1	0.020–100	0.998	0.005	0.020	−68.4	5.8	105 (7.6)
ENNA	0.017–100	0.998	0.005	0.017	−35.1	4.2	109 (5.2)

[a] Linearity: from 25 to 500 ng g^{-1} or ng mL^{-1} (for FBs, AOH, TeA, DON and FUS-X), from 1 to 250 ng g^{-1} or ng mL^{-1} (for CPA, DAS, 3-ADON, TEN and ZEN), from 0.5 to 100 ng g^{-1} or ng mL^{-1} (for CIT, HT-2 and T-2); and from 0.1 to 100 ng g^{-1} or ng mL^{-1} (for ENNs, OTs, AFs, ALT and STE).

[b] LOD: limit of detection.

[c] LOQ: limit of quantification.

[d] Mycotoxin concentrations: 5 ng g^{-1} or ng mL^{-1} (ENNs, OTs, AFs, ALT and STE), 10 ng g^{-1} or ng mL^{-1} (CIT, HT-2 and T-2), 25 ng g^{-1} or ng mL^{-1} (CPA, DAS, 3-ADON, TEN and ZEN) and 100 ng g^{-1} or ng mL^{-1} (FBs, AOH, TeA, DON and FUS-X).

[e] RSD: relative standard deviation.

lactating and pregnant sows, in the same range of concentrations as for the feed.

Matrix effect was calculated as [(analyte signal in spiked sample – analyte signal in standard solution)/analyte signal in standard solution] x 100 %. Almost no matrix effect was observed in the determination of some mycotoxins such as OTA, FB2, 3-ADON, HT-2 or TeA in feed and AFG2, OTB, or DAS in urine samples. However, generally the results obtained showed a significant response of suppression in the determination of most mycotoxins, especially in feed samples since the matrix is usually more complex.

Linearity was studied using matrix-matched calibration by analyzing a mycotoxin-free feed and a blank urine sample previously analyzed. In the case of feed samples, mycotoxins were added at five concentrations in different ranges depending on the mycotoxin: from 25 to 500 ng g^{-1} (for FBs, AOH, TeA, DON and FUS-X), from 1 to 250 ng g^{-1} (for CPA, DAS, 3-ADON, TEN and ZEN), from 0.5 to 100 ng g^{-1} (for CIT, HT-2 and T-2); and from 0.1 to 100 ng g^{-1} (for ENNs, OTs, AFs, ALT and STE). Linearity experiments of urines were carried out at the same concentrations as feed samples, calculated in ng mL^{-1}. Mycotoxins showed good linearities over the ranges studied, with high values of the coefficients of determination (R^2). LODs were calculated using the following formula based on the European guidance document on the estimation of LOD and LOQ for measurements in the field of contaminants in feed and food [37]: $LOD = 3.8 \times \frac{S_{y.x}}{b} \times \sqrt{1.1 + \frac{\bar{x}^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2}}$ where $S_{y.x}$ is the standard deviation of the residuals, b is the slope of the calibration curve, $\bar{x}$ is the mean calibration level, and x_i is the content value of the analyte at calibration level *i*. LOQs were calculated as 3.3 times the LODs. Very satisfactory limits were obtained, allowing the quantification of mycotoxins in urine and feed at very low concentrations. In general, lower LOQs were obtained in urine than in feed, reaching up to 0.011 ng mL^{-1} for BEA. The LOQ obtained for AFB1 was below the legislated maximum level in animal feed (<0.02 μg g^{-1}) [7], as were the LOQs for DON, ZEN, OTA, FBs, T-2 and HT-2, below the maximum recommended levels (DON <0.9 μg g^{-1}, ZEN <0.25 μg g^{-1}, OTA <0.05 μg g^{-1}, sum of FB1 and FB2 < 5 μg g^{-1}, and sum of T-2 and HT-2 < 0.25 μg g^{-1}) [6,8]. In addition, One-Sample Signed Rank tests were performed to compare the obtained concentrations of the legislated mycotoxins in feed with the maximum permissible values. The tests concluded that the median concentration obtained was significantly different from the maximum allowable value for each of the legislated mycotoxins (P = <0.001) and, therefore, the concentrations of the feed samples were significantly below the legal limit.

Repeatability was studied in feed and urine samples to evaluate the precision of the proposed methodology. For these experiments, both matrices were fortified in triplicate and the analysis was performed in duplicate on the same day. Results were expressed in terms of relative standard deviation (RSD) values. The variation in the analyses was not greater than 9.6 % for both matrices (Table 2).

Trueness was expressed as apparent recovery and was calculated as [measured concentration/actual (added) concentration] x 100 %. Experiments were performed in triplicate. The RSD values were also calculated for each mycotoxin. Recoveries ranged from 91 to 117 % for feed samples, and from 91 to 116 % for urine samples. The RSD values were below 9.7 % in all cases. Recoveries of AFs, OTA, DON, ZEN, FBs, T-2 and HT-2 in feed would comply with European Commission Regulation 401/2006, which establishes the laboratory control requirements for the determination of mycotoxins in foodstuffs [38].

3.3. Analysis of feed and urine samples

A total of 48 feed and 55 urine samples from eight Spanish pig farms were prepared by the proposed sample treatments and analyzed in a targeted mode by HPLC-MS/MS to explore the occurrence of mycotoxins in both types of samples. In the occurrence study, samples with concentrations above the LOD were considered positive and were included in the percentage of incidence. Tables S2 and S3 summarize the results of the concentrations obtained in the samples from each farm. For the calculation of the mean concentration, the corresponding LOD value was assigned to concentrations below the LOQ.

All feed samples were contaminated with at least 7 mycotoxins of the 27 mycotoxins studied. AOH, ALT and CPA were not detected. In contrast, ENNs and BEA were quantified in 100 % of the samples, with ENNB showing the highest concentrations, up to 11 μg g^{-1}. STE, HT-2, DON, T-2 and TEN also showed a high incidence, while AFG2, AFG1, AFB2 and FUS-X were the mycotoxins with the lowest occurrence in feed (Table S2). The mycotoxins with the highest mean total concentrations were ENNB (1.0 ± 1.9 μg g^{-1}), TeA (155 ± 209 ng g^{-1}), and DON (81 ± 94 ng g^{-1}) (Fig. 2). Regarding the differences between farms, the results showed that "farm 7" had a lower incidence of mycotoxins, containing only ENNs, BEA, STE, HT-2, FUS-X and DAS. The chromatogram of a real feed sample contaminated with multiple mycotoxins is shown in Fig. 3. Due to the high incidence of mycotoxins in animal feed, co-occurrence is a factor that should be evaluated, as it may have additive, synergistic or antagonistic effects on health [39]. The co-occurrence of 14 mycotoxins is the most observed, in the 25 % of feed samples. HT-2, STE, ENNs and BEA co-occur in all of them. Concerning the regulated mycotoxins, none of the analyzed samples contained AFB1 above the established maximum limits. Similar results were found for DON, OTA, the sum of FB1 and FB2, and the sum of HT-2 and T-2. However, two feed samples were found to contain ZEN exceeding the maximum limits, at 300 and 275 ng g^{-1}.

On the other hand, all mycotoxins were quantified in some of the sow urine samples analyzed. DON, TeA, ENNB1 and ENNA were the most prevalent, while the mycotoxins with the lowest incidence were 3-ADON, AFG2, AFB2, DAS, OTs and STE. Mycotoxin concentrations were corrected with urinary creatinine, therefore the final concentration was expressed as ng mycotoxin per mg creatinine. The highest mean total concentration in urine were found for TeA (133 ± 199 ng mg^{-1}), FB1 (0.43 ± 1.3 μg mg^{-1}) and AOH (0.29 ± 1.3 μg mg^{-1}) (Fig. 2). Results showed that sow urine from "farm 7" also contained less mycotoxins (Table S3), which suggests a relationship between the consumption of mycotoxin-contaminated feed and urinary mycotoxin excretion and highlights the utility of urinary biomarkers for exposure assessment. Thus, Fisher's exact tests were performed for each mycotoxin to evaluate the possible association between the two qualitative variables (feed and urine), and establish a possible relationship between the presence of mycotoxins in feed and the urine produced by pigs that have consumed this feed. This test assumes the null hypothesis that the two variables are independent, meaning that the values of one variable do not depend on the values of the other. What is more, DON and TeA were the most prevalent in urine, and TeA, FB1 and AOH had the highest mean total concentrations in urine; while ENNs and BEA were quantified in 100 % of the feed samples and ENNB, TeA and DON had the highest mean total concentrations in feed. With the purpose of studying the dependency between mycotoxins detected in urine and those detected in feed, 2 × 2 contingency tables were performed for all mycotoxins (Fig. S3). In the case of contingency tables in which one row contained only zeros, the z-test was performed. As a result, the two variables were found to be related for DON (P = 0.002) and TeA (P = 0.003). Prior studies have identified urine as the main route of DON excretion [40], which is consistent with its positive relation between feed and urine by Fisher's exact test. In the case of TeA, its presence in urine has been reported in previous works after exposure through consumption of naturally contaminated food. Urinary excretion is an important route for this mycotoxin, with up to 90 % of TeA being excreted in the first 24 h [41]. Based on these findings, DON and TeA show promise as potential biomarkers of DON and TeA exposure.

Furthermore, 3-ADON is rapidly hydrolyzed to DON when ingested [42], which explains why this modified form of DON was detected in feed but very infrequently in urine. The Joint FAO/WHO Expert

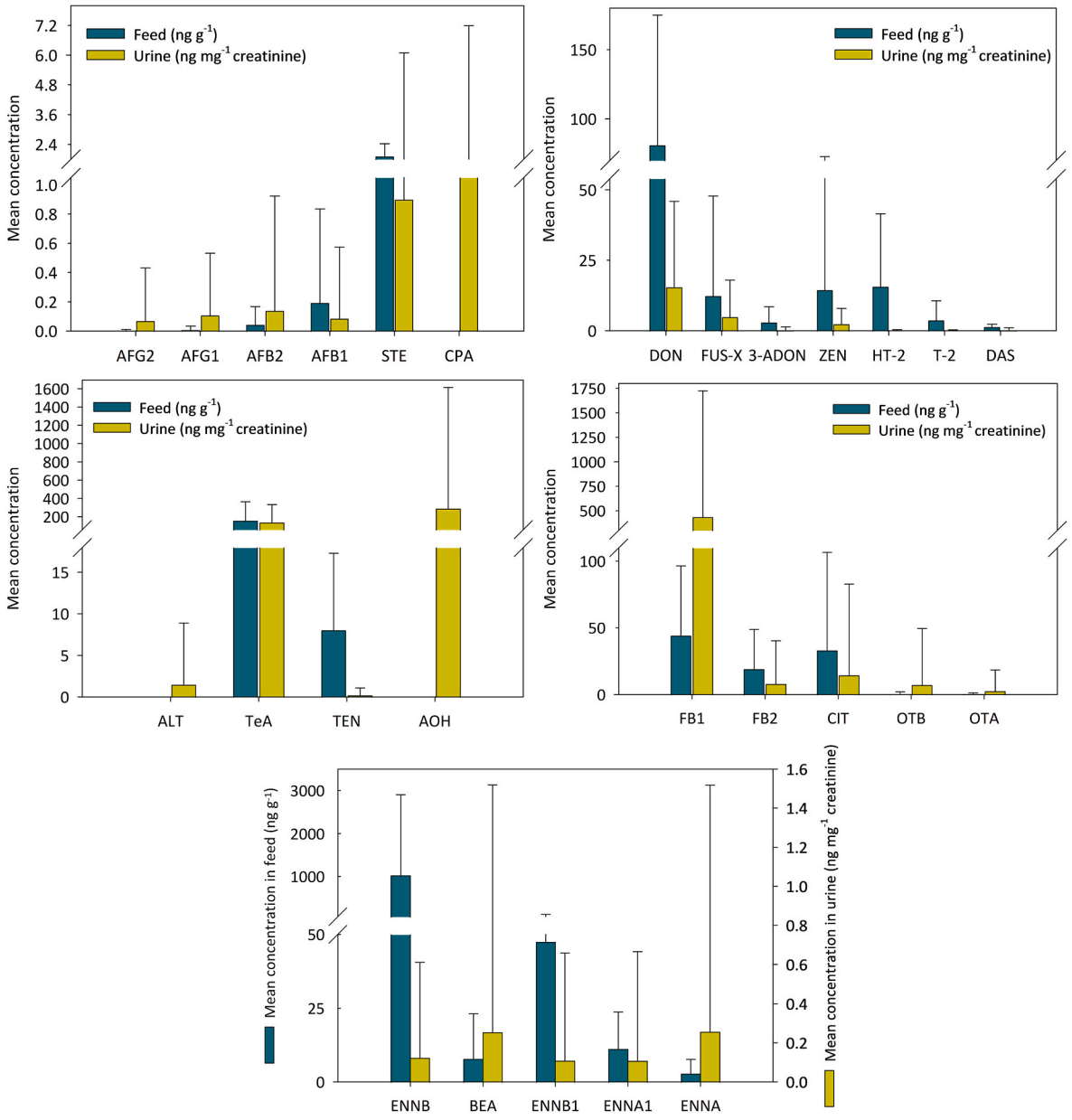

Fig. 2. Mean total concentrations of mycotoxins found in feed and urine samples with error bars of standard deviation values.

Committee on Food Additives considered 3-ADON and 15-ADON as an additional contributors to total dietary DON exposure [22]. Similarly, T-2 is rapidly metabolized to HT-2 during digestion in agreement with the obtained results. HT-2 and free T-2 can be considered as the main biomarkers of T-2 in urine [43]. On the other hand, some mycotoxins as FBs are excreted mainly through feces and very low levels are usually reported in urine. Free forms of FBs are established as possible biomarkers of FBs [1].

Regarding emerging *Fusarium* mycotoxins, ENNs and BEA were found in 100 % of the feed samples, while a lower incidence was observed in urine. This may suggest possible bioaccumulation in the sow's body or slower metabolism. Previous studies carried out in our laboratory demonstrated the accumulation of ENNs and BEA in human body, showing their occurrence in different organs. ENNB is the most prevalent mycotoxin in the body and accumulates mainly in the liver [44]. In addition to humans, this mycotoxin has also been determined in animal liver samples, including pigs, which is the species' focus of the current work [45]. On the other hand, AOH, ALT and CPA were detected in urine in some samples, specifically, in 9, 7 and 4 urines, respectively, of the 55 samples analyzed, while they were not found in feed. It is recognized that feed analysis does not provide exact data about the individual exposure because of differences in food consumption and ADME processes between the animals [12]. However, those urine mycotoxins could have a different origin from the animal's feed, be derived from other mycotoxins, or be of slower metabolization or come from a previously consumed diet.

3.4. Untargeted analysis of urine samples

Urine samples were analyzed in an untargeted mode for the tentative identification of mycotoxin metabolites. The PCDL database of 455 mycotoxins and related metabolites from Agilent Technologies (Santa Clara, CA, USA) was used for this purpose. Metabolites were identified based on the name, formula, accurate mass, structure and MS/MS fragmentation spectra available on the database. Results were filtered as detailed in section 2.4.2. As a result, a total of 4 compounds (breviamide F (BVD-F), emodin, lincomycin and oxytetracycline (OTC)) were detected in the urine samples, with score values > 75.4 % and mass

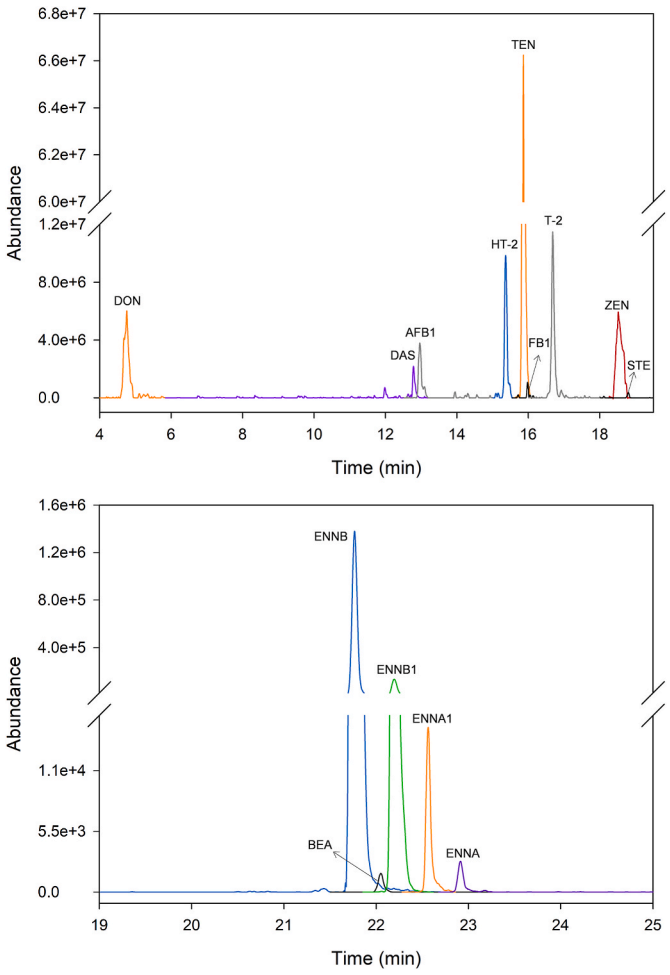

Fig. 3. Extracted ion chromatograms of a real feed sample contaminated with DON (59.4 ng g^{-1}), DAS (1.8 ng g^{-1}), AFB1 (0.04 ng g^{-1}), HT-2 (11.4 ng g^{-1}), TEN (7.3 ng g^{-1}), FB1 (28.2 ng g^{-1}), T-2 (2.4 ng g^{-1}), ZEN (1.2 ng g^{-1}), STE (1.7 ng g^{-1}), ENNB (2.3 μg g^{-1}), BEA (1.7 ng g^{-1}), ENNB1 (64.2 ng g^{-1}), ENNA1 (10.6 ng g^{-1}) and ENNA (1.9 ng g^{-1}).

errors < ±3.9 ppm. The identification results are summarized in Table S4.

The most abundant metabolite was BVD-F, found in the 40 % of urine samples. Emodin was detected in 10 samples (18.2 %) and lincomycin and OTC only were detected in one urine. The co-occurrence in urine of BVD-F and emodin with the investigated mycotoxins was studied. Fig. S4 shows the level of co-occurrence of metabolites and mycotoxins in the samples. Mycotoxins were divided into five groups: AFs, CPA and STE (Group 1), DON, FUS-X, 3-ADON, ZEN, HT-2, T-2 and DAS (Group 2), ALT, TeA, TEN and AOH (Group 3), FBs, CIT and OTs (Group 4), and ENNs and BEA (Group 5). BVD-F showed a higher frequency of co-occurrence with mycotoxins of groups 2 and 5. In particular, co-occurrence with DON was observed in 70.4 % of the urine samples, and in 62.9 % of the samples with TeA (Table S5). Emodin showed a higher frequency of co-occurrence with the mycotoxins that constitute Group 3. However, individually, the highest percentage of co-occurrence was obtained with DON and TeA (37 %). Both metabolites exhibited highest co-occurrence with DON compared to the other studied mycotoxins. These results may be in agreement with previous studies of co-occurrence with DON in feed [46], considering the lower mycotoxin concentrations detected in urine.

BVD-F is a mycotoxin metabolite precursor of a variety of prenylated fungal alkaloids such as the fumitremorgins [47]. The presence of this metabolite has been previously reported in animal feed [46,48], with a high presence (95 %) and co-occurrence with DON (96 %) in swine feed samples collected worldwide [46]. In addition, it has also been detected in cereals as fonio millet, sesame or wheat, and mango seeds [49–51].

Emodin is considered as an "emerging mycotoxin" produced by *Aspergillus* species. The contamination of foods as nuts [52], bread, fruits and vegetables [53] and seeds [49,51] by this metabolite has been reported. However, this genotoxic compound is also an endogenous metabolite of vegetables and herbs [54] and, therefore, it cannot be ruled out that, in some cases, its occurrence is not due to fungal infection. Although the co-occurrence of DON and emodin has been reported in swine diets, the individual toxicity of DON has not been exacerbated [46].

Using the proposed method, lincomycin and OTC were found in one sample from each of the 55 urines analyzed. Lincomycin was identified with a score of 96.58 %, at a retention time of 5.72 min and a mass error of 1.03 ppm. OTC showed a score of 80.55 %, at 7.16 min and 4.03 ppm mass error. Lincomycin and OTC are antibiotics used as veterinary drugs in farms. In general, more than 60 % of the initial administered dose of antibiotics in animals is excreted without changes allowing their detection in urine [55]. Lincomycin is a sulfur-containing pyranoside antibiotic that is primarily effective against Gram-positive bacteria by inhibiting protein synthesis [56]. This antibiotic has previously been detected in feces [55] and urine [56,57] from pigs. OTC is one of the predominant antibiotics for its broad spectrum and is effective for Gram-positive and Gram-negative bacteria [58]. This antibiotic has also been previously detected in feces [55] and urine [57,59] from pigs. Based on the results, in addition to assessing mycotoxins in biological samples, the proposed methodology also enables the assessment of other important metabolites such as antibiotic residues; which may be very useful on controls for reducing antibiotic use in pig farming [60].

4. Conclusion

In this study, different sample treatments were optimized and successfully applied for simple and rapid extraction of multiple mycotoxins in pig feed and urine. Subsequent targeted and untargeted analysis of the samples allowed an accurate assessment of the exposure of pigs to mycotoxins through the consumption of commercial feed. The relationship between both matrices was studied and biomarkers of exposure to mycotoxins were established. According to the results of Fisher's exact test, the occurrence of DON and TeA in feed and urine is related. In addition, DON and 3-ADON were identified as potential urinary biomarkers of DON exposure, TeA was identified as a biomarker of TeA exposure, HT-2 and T-2 for T-2, and free forms of FBs as biomarkers of FB2 and FB1exposure. The assessment of mycotoxin exposure by urine biomarkers is an effective alternative to traditional feed analysis, thus eliminating sample heterogeneity as a limiting factor. The proposed analytical method has been optimized and proven valid, as it allows the detection of very low levels of mycotoxins in both matrices. In addition, metabolites in urine previously detected in feed by literature, as well as residues of antibiotics used on farms for treatment or prevention of diseases have been identified by the untargeted approach.

CRediT authorship contribution statement

Ana Castell: Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Natalia Arroyo-Manzanares:** Writing – review & editing, Visualization, Supervision, Software, Methodology, Conceptualization. **Natalia Campillo:** Writing – review & editing, Supervision, Formal analysis. **Santos Sanz-Fernández:** Resources. **Vicente Rodríguez-Estévez:** Writing – review & editing, Resources, Methodology, Conceptualization. **Josep Roquet:** Resources, Conceptualization. **Antonio González:** Writing – review & editing, Resources, Methodology, Conceptualization. **José Fenoll:** Resources, Methodology. **Pilar Viñas:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition,

Conceptualization.

Declaration of competing interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2024.127441.

Data availability

No data was used for the research described in the article.

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