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RESEARCH PAPER

Abstract

The multi-biomarker approach was used to validate urinary biomarkers in piglets administered boluses contaminated with mixtures of deoxynivalenol (DON), aflatoxin B_1 (AFB_1), fumonisin B_1 (FB_1), zearalenone (ZEA) and ochratoxin A (OTA) at different concentrations. Boluses contaminated with mycotoxins were prepared by slurrying and freeze-drying feed material fortified with culture extracts of selected toxigenic fungi. Piglets were individually placed in metabolic cages to collect urine before gavage and 24 h post dose. Urine samples were hydrolysed with β-glucuronidase and analysed by a multi-biomarker LC-MS/MS method developed and validated to identify and measure biomarkers of FB_1, OTA, DON, ZEA and AFB_1. Urinary levels of FB_1, OTA, DON + de-epoxy-deoxynivalenol, ZEA + alpha-zearalenol and aflatoxin M_1 were selected as biomarkers of FB_1, OTA, DON, ZEA and AFB_1, respectively. Mean percentages of dietary mycotoxins excreted as biomarkers in 24 h post dose urine were 36.8% for ZEA, 28.5% for DON, 2.6% FB_1, 2.6% for OTA and 2.5% for AFB_1. A good correlation was observed between the amount of mycotoxins ingested and the amount of relevant biomarkers excreted in 24 h post dose urine. Linear dose-response correlation coefficients ranged between 0.68 and 0.78 for the tested couples of mycotoxin/biomarker. The good sensitivity of the LC-MS/MS method and the good dose-response correlations observed in this study permitted to validate the selected mycotoxin biomarkers in piglets at dietary levels close to the maximum permitted levels reported in Commission Directive 2003/100/EC for AFB_1 and the guidance values reported in Commission Recommendation 2006/576/EC for DON, ZEA, OTA and FB_1.

Keywords: bioavailability, urine, excretion, dose-response

1. Introduction

Deoxynivalenol (DON), aflatoxin B_1 (AFB_1), fumonisin B_1 (FB_1), zearalenone (ZEA) and ochratoxin A (OTA) are mycotoxins frequently occurring in cereals and cereal-based feeds. Farm animals are often exposed to these mycotoxins alone or in combinations (Grenier and Oswald, 2011; Streit *et al.,* 2012). Levels of these contaminants in cereals and cereal-based feeds should be kept as low as possible to protect not only animal health but also human health, since some mycotoxins or their toxic metabolites can be released in animal products such as milk, meat and offal. In the European Union, maximum permitted levels for AFB_1 in feed materials range from 0.005 to 0.02 mg/kg. Guidance values are reported in Commission Recommendation 2006/576/EC (EC, 2006) for DON (0.9-12 mg/kg), ZEA (0.1-3 mg/kg), OTA (0.05-0.25 mg/kg) and FB_1 + fumonisin B_2 (5-60 mg/kg). An indirect approach to reduce the negative impact of mycotoxins on animal health is the use of mycotoxin detoxifying-agents leading to a decreased bioavailability of mycotoxins (Boudergue *et al.*, 2009). Several types of mycotoxin-detoxifying agents are commercially available and largely used by farmers (Avantaggiato *et al.,* 2005). Some of them have been shown to efficiently work *in vitro,* but only a few have been tested *in vivo* (Boudergue *et al.*, 2009; Meissonnier *et al.* 2009;

Solfrizzo *et al.,* 2001a,b). Moreover, data on the efficacy of commercial binders to adsorb mixtures of mycotoxins are very scarce or absent. In recent guidelines, the European Food Safety Authority (EFSA) has stated that *in vitro* tests do not fully prove the efficacy of mycotoxin-detoxifying agents and that *in vivo* trials should be performed (EFSA, 2010). Moreover, *in vivo* trials have to be performed at mycotoxin levels below those reported in Directive 2003/100/EC for AFB_1 (EC, 2003) and Recommendation 2006/576/EC for DON, ZEA, OTA and FB_1 (EC, 2006).

The measurement of urinary mycotoxin biomarkers is a promising approach to assess the *in vivo* efficacy of binders, since a portion of ingested mycotoxin is released into urine as parent toxin or phase I and II metabolites. Therefore, their measurement could give an estimate of the ingested dose. Recently, EFSA has confirmed the validity of the biomarker approach for demonstration of the efficacy of substances that reduce the mycotoxin contamination of feed (EFSA, 2010). Mycotoxin biomarkers are present in biological fluids and urine is becoming the choice for the measurement of mycotoxin biomarkers (De Andrés *et al.,* 2008; Polychronaki *et al.,* 2008; Scott, 2005; Shephard *et al.,* 2007; Turner *et al.,* 2010). Urinary biomarkers for FB_1, OTA, DON, ZEA and AFB_1 could be FB_1, OTA, DON + de-epoxy-deoxynivalenol (DOM-1), ZEA+alpha-zearalenol (α-ZOL) + beta-zearalenol (β-ZOL) and aflatoxin M_1 (AFM_1), respectively. Recent developments in the field of analytical chemistry of mycotoxins have demonstrated the power of liquid chromatography tandem mass spectrometry (LC-MS/MS) in the simultaneous determination of mycotoxin biomarkers in urine (Ediage *et al.*, 2012; Solfrizzo *et al.*, 2011; Warth *et al.*, 2012). There is a need of validated multi-mycotoxin biomarkers that can be used in animal studies to evaluate the efficacy of mycotoxin detoxifying agents capable to reduce the bioavailability of mycotoxin mixtures.

The objective of this study was to evaluate the dose-response relationship between the simultaneous oral ingestion of FB_1, OTA, DON, ZEA and AFB_1 and urinary excretion of the relevant biomarkers within 24 h by using a mass balance approach.

2. Material and methods

Chemicals and reagents

Calibrant solutions of DON (100 µg/ml), DOM-1 (50 µg/ml), AFB_1 + aflatoxin B_2 + aflatoxin G_1 + aflatoxin G_2 (2.02 µg/ml, 0.5 µg/ml, 2.03 µg/ml and 0.5 µg/ml, respectively), AFM_1 (0.5 µg/ml), α-ZOL (10 µg/ml), β-ZOL (10 µg/ml), ZEA (100 µg/ml), OTA (10 µg/ml) prepared in acetonitrile (ACN) and a calibrant solution of FB_1 (50 µg/ml) prepared in ACN:water (50:50, v/v) were purchased from Romer Labs Diagnostic (Tulln, Austria). β-glucuronidase/sulfatase type H-2 from *Helix pomatia* (specific activity 130,200 units/ml β-glucuronidase, 709 units/ml sulfatase), chromatography-grade methanol (MeOH), acetonitrile, acetone, glacial acetic acid and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich (Milan, Italy). PBS solution at pH 7.4 was prepared by dissolving PBS tablets in an appropriate volume of distilled water. Sodium hydrogen carbonate was purchased from J.T. Backer (Deventer, the Netherlands). Ultrapure water was produced by use of the Milli-Q system (Millipore, Bedford, MA, USA). Myco6in1, DONtest™ Wide Bore (WB), AflaTest™ WB, FumoniTest™ WB, ZearaleTest™ WB and OchraTest™ WB immunoaffinity columns were purchased from Vicam L.P. (Watertown, MA, USA). Oasis HLB cartridges (60 mg, 3 ml) were purchased from Waters (Milford, MA, USA) and regenerated cellulose filters (0.45 µm) were purchased from Sartorius Stedim Biotech (Goettingen, Germany). No. 4 and GF/A paper and glass microfiber filters were obtained from Whatman (Maidstone, UK).

Design of the *in vivo* experiments with piglets

In vivo experiments with piglets were performed to check the feasibility of using urinary multi-biomarker determination as a suitable approach to estimate the ingestion of mixtures of DON, AFB_1, FB_1, ZEA and OTA at different levels in feed boluses. Sixteen 4-week-old weaned piglets (Pietrain/Duroc/Large-white) weighing 10.56±1.88 kg at the beginning of the experiment were obtained locally. Animals were acclimatised for 1 week in the animal facility of the INRA ToxAlim Unit (Toulouse, France) prior to being used in experimental protocols. During the acclimation and experimental periods, animals were given free access to water. Except during the urine collection periods, animals were fed a commercial diet *ad libitum*. Four groups of piglets (four piglets per group) were administered boluses contaminated with mixtures of DON, AFB_1, FB_1, ZEA and OTA at different levels (for mycotoxin intake, Table 1). Each piglet was fed the bolus and then housed in a metabolic cage. Urine samples were collected 3 times at regular intervals within 24 h and their volumes were measured. Control urine samples were collected from the same piglet the day before giving the contaminated bolus. Urine samples were frozen and shipped to ISPA (Bari, Italy) for determination of DON, DOM-1, AFM_1, ZEA, α-ZOL, β-ZOL, FB_1 and OTA.

Preparation of contaminated feed boluses

About 2 kg of commercial complete feed for piglets was homogenized, analysed for mycotoxins and used for preparation of mycotoxin-contaminated boluses as well as for recovery experiments of DON, AFB_1, FB_1, ZEA and OTA at ISPA. For the preparation of contaminated boluses, an aliquot of feed was ground and fortified with culture extracts of mycotoxigenic species of *Fusarium graminearum*, *Aspergillus ochraceus*, *Aspergillus parasiticus*

Table 1. Mycotoxin intakes in four groups of piglets that received a bolus containing a mixture of deoxynivalenol, aflatoxin B_1, fumonisin B_1, zearalenone and ochratoxin A.

Mycotoxin	Bolus 1 (n=4)[1]		Bolus 2 (n=4)[2]		Bolus 3 (n=4)[3]		Bolus 4 (n=4)[4]	
	µg/kg bw	µg/animal	µg/kg bw	µg/animal	µg/kg bw	µg/animal	µg/kg bw	µg/animal
Deoxynivalenol	7.16	63.61	20.44	191.12	24.14	315.05	57.38	630.03
Aflatoxin B_1	0.16	1.40	0.45	4.20	0.54	7.03	1.28	14.01
Fumonisin B_1	3.71	32.96	10.60	99.14	63.19	824.60	150.19	1,649.12
Zearalenone	0.68	6.05	1.94	18.15	2.38	31.08	5.66	62.15
Ochratoxin A	0.16	1.45	0.46	4.35	0.56	7.25	1.32	14.50

[1] Mean body weight (bw) of 4 piglets ± standard deviation: 8.88±1.26 kg.
[2] Mean bw of 4 piglets: 9.35±0.47 kg.
[3] Mean bw of 4 piglets: 13.05±0.58 kg.
[4] Mean bw of 4 piglets: 10.98±1.14 kg.

and *Fusarium verticillioides* (deposited at ISPA Collection, http://www.ispa.cnr.it/Collection) producing DON and ZEA, OTA, aflatoxins and fumonisins, respectively, cultured on cereals. Each fungal culture was dried, ground and separately extracted with water or mixtures of water and organic solvent. In particular, fumonisins were extracted with water, OTA with a mixture of water and ACN (40:60, v/v) and aflatoxins with a mixture of water and acetone (15:85, v/v). DON + ZEA were extracted from two portions of cereal culture with water and with a mixture of water and ACN (10:90, v/v) to obtain two different cultural extracts containing the necessary concentrations of DON + ZEA. The determination of each mycotoxin in these culture extracts was performed by HPLC-UV/FLD as described below. The concentration of 3-acetyl-DON (3-ADON) in extracts of *F. graminearum* was also measured by HPLC-UV, however, as the amount of 3-ADON measured in these extracts was <2% than that of DON, it was not considered for the mass balance. For the preparation of boluses contaminated with four different mixtures of DON, AFB_1, FB_1, ZEA and OTA, four aliquots of ground feed were contaminated with appropriate volumes of culture extracts necessary to reach the level of each mycotoxin as reported in Table 1. These four aliquots were separately slurried to ensure within sample homogenisation, then divided into 4×4 boluses that were separately freeze-dried and delivered to INRA for *in vivo* experiment with piglets. These *in vivo* experiments were performed during two different periods. The first experiment was performed with 2 groups of 4 piglets that were administered the two boluses (50 g) containing higher mycotoxin concentrations (bolus 3 and bolus 4; Table 1). The second experiment was performed with the other 2 groups of 4 piglets that were administered the two boluses (20 g) containing lower mycotoxin concentrations (bolus 1 and bolus 2; Table 1). A reduced weight of the bolus was used in the second experiment in order to decrease the time necessary to administer the bolus to the piglet. The feed was removed the evening before the experiment, thus the animal had no access to feed overnight. The animal keeper stayed with the animals while they were eating the bolus and verified that all the bolus was taken up by the animal. Depending on the animal, the bolus was eaten within 1 to 2 h.

Mycotoxin analysis in feed and culture extracts

The concentrations of DON, aflatoxins, ZEA, OTA and fumonisins in the feed used for the *in vivo* experiments were measured by analysing representative aliquots of ground feed with the relevant HPLC-based standard methods. In particular, DON, aflatoxins, ZEA, OTA and fumonisins were analysed by using EN 15791:2009, EN/ISO 17375:2006, EN 15792:2009, EN 16007:2012 and EN 16006:2012, respectively (CEN, 2006, 2009a,b, 2012a,b). Method performances were checked with recovery experiments of duplicate feed samples spiked at the following levels: 0.162 µg/g DON, 0.0136 µg/g AFB_1, 4.416 µg/g FB_1, 0.058 µg/g ZEA and 0.0044 µg/g OTA. Spiked samples were left overnight at room temperature to allow solvent evaporation and equilibration between analytes and matrix. Percentage recovery and repeatability of results were acceptable for all tested mycotoxins, i.e. 105±3.6% DON, 102±1.7% AFB_1, 88±1.9% FB_1, 95±0.5% ZEA and 95±2% OTA. Analysis of the feed used for *in vivo* experiments showed the absence of AFB_1 and FB_1 and low levels of DON (104 µg/kg), ZEA (8 µg/kg) and OTA (0.17 µg/kg). These low levels were considered acceptable as blank control samples. Therefore, an aliquot of this feed was used as a blank control diet whereas other aliquots were spiked with culture extracts to produce boluses contaminated with different concentrations of each mycotoxin.

The determination of DON, ZEA, OTA or AFB_1 in each culture extract was performed by direct injection of culture

extracts into HPLC-UV/FLD after appropriate dilution with water and filtration. For the determination of FB_1 in the water culture extract of *F. verticillioides*, it was necessary to purify an aliquot of culture extract through an immunoaffinity column before HPLC-FLD analysis. The sample clean-up protocol and HPLC conditions were the same as those described above for the analysis of feed (EN 16006:2012; CEN, 2012a). Direct analysis of diluted culture extract was not possible due to the presence of an interfering peak co-eluting with FB_1.

Biomarker analysis in urine

Three urine samples of each piglet collected during 24 h were pooled and analysed according to the multi-biomarker method based on multi-antibody immunoaffinity column (Myco6in1) and LC-MS/MS determination as described elsewhere (Solfrizzo *et al.*, 2011) with some modifications. In particular, ZEA was added in the panel of biomarkers. Briefly, centrifuged urine samples (6 ml) were incubated with 300 µl of β-glucuronidase/sulphatase solution (37 °C for 18 h), diluted with 6 ml water and cleaned up on a Myco6in1 immunoaffinity column (IAC) (Vicam) and an OASIS HLB solid phase extraction (SPE) column (Waters) connected in tandem. After sample application, the two columns were separated; the IAC column was washed with 4 ml water and biomarkers were eluted with 3 ml methanol and 2 ml water. The OASIS column was washed with 1 ml methanol:water (20:80, v/v) and DON and DOM-1 passed through the IAC and collected on the SPE column were eluted with 1 ml methanol:water (40:60, v/v). The separate eluates from the two columns were combined, dried down and reconstituted in 200 µl methanol:water (20:80, v/v) with 0.5% acetic acid. 5-points matrix-assisted calibration curves were prepared in the following concentration range for each biomarker: 2-1,254 ng/ml DON, 1-250 ng/ml DOM-1, 0.2-25 ng/ml AFM_1, 2-315 ng/ml ZEA, 2-250 ng/ml α- and β-ZOL, 0.4-157 ng/ml FB_1 and 0.02-13 ng/ml OTA. In particular, aliquots of control urine samples were pooled and sub-aliquots were used to prepare the purified extracts for the 5-points matrix-assisted calibration curves. Table 2 shows the results of matrix effect determination (% signal suppression enhancement (SSE)) for each biomarker in piglet urine, which are compared to results previously obtained for human urine. The %SSE values were calculated as the ratio between the slope of the matrix-matched calibration curve and the slope of the calibration curve in LC mobile phase multiplied by 100. Signal suppression was observed for all biomarkers in piglets and human urine except for FB_1, for which signal enhancement was observed. Piglet urine showed a stronger signal suppression of DON, DOM-1, AFM_1, α-ZOL, ZEA and OTA than human urine. The signal suppression of β-ZOL as well as the signal enhancement of FB_1 were lower in piglets.

Equipment

DON, AFB_1, ZEA and OTA determinations in feed and culture extracts were performed with an Agilent 1100 series HPLC apparatus equipped with a G1312A binary pump, G1313 Autosampler, G1316A column thermostat set at 30 °C, G1315B UV-visible DAD, G1321A spectrofluorometric detector, Agilent Chemstation G2170AA Windows 2000 operating system (Agilent, Waldbronn, Germany) and a post-column photochemical derivatizator (UVE Derivatizer, LC Tech, Dorfen, Germany). FB_1 and FB_2 determination was performed with a Varian HPLC system equipped with a ternary pump (ProStar 230),

Table 2. Comparison results from determination of matrix effects for each biomarker between piglet and human urine.

Biomarker[1]	Matrix-matched calibration curve in piglet urine[2]			Matrix-matched calibration curve in human urine[2]			Matrix effect in piglet urine (SSE %)[3]	Matrix effect in human urine (SSE %)
	a	b	r^2	a	b	r^2		
DON	591	2,510	0.9990	1,942	2,565	0.9968	12	43
DOM-1	1,846	4,623	0.9996	3,147	-4	0.9786	18	76
AFM_1	740	84	0.9995	1,928	-66	0.9966	33	81
FB_1	144	-74	0.9877	1,468	-9	0.9938	152	270
α-ZOL	114	375	0.9964	918	-320	0.9939	14	28
β-ZOL	113	1,668	0.9884	292	122	0.9994	29	15
ZEA	842	-393	0.9902	3,753	-881	0.9962	44	48
OTA	2,940	241	0.9961	9,396	116	0.9905	42	77

[1] DON = deoxynivalenol; DOM-1 = de-epoxy-deoxynivalenol; AFM_1= aflatoxin M_1; FB_1 = fumonisin B_1; α-ZOL = α-zearalenol; β-ZOL = β-zearalenol; ZEA = zearalenone; OTA = ochratoxin A.

[2] a and b are the slope and the intercept of the calibration curve, respectively; r is the coefficient of correlation.

[3] SSE = signal suppression enhancement.

autosampler (ProStar 410), fluorescence detector (ProStar 363) and Varian Star Data System 6.20 (Varian Inc., Palo Alto, CA, USA).

LC-MS/MS of urinary biomarkers was performed with a QTrap MS/MS system (Applied Biosystems, Foster City, CA, USA), equipped with an ESI interface, an 1100 series micro-LC system comprising a binary pump, a microautosampler (Agilent Tecnhnologies) and Analyst 1.4 acquisition data system (Applied Biosystems).

Linear regression analyses were performed using SigmaPlot for Windows Version 12.0 statistical software package (Sistat Software, Inc., Chicago, IL, USA).

3. Results and discussion

The sensitivity of the LC-MS/MS method used in this study was adequate to detect and quantify the mycotoxin biomarkers excreted in urine of piglets administered boluses contaminated at reasonably low levels of AFB_1, OTA, FB_1, DON and ZEA. The values of mycotoxin intake in the four groups of piglets used in this study are reported in Table 1. For DON and ZEA, the intake values were comparable with those used by Dänicke *et al.* (2005). For FB_1, they were much lower than those used in previous studies with pigs (Dilkin *et al.*, 2010; Fodor *et al.*, 2008). For AFB_1, they were lower than those used previously in studies with pigs (Dilkin *et al.*, 2003; Ho, 1987; Thieu and Pettersson, 2009). For OTA, they were much lower than those used by Stoev *et al.* (2002) and comparable or slightly lower than those used by Stoev *et al.* (2001). The values of mycotoxin intake used in this study were chosen considering the maximum permitted levels and guidance levels reported in Directive 2003/100/EC (EC, 2003) and Recommendation 2006/576/EC (EC, 2006), respectively. Considering that the piglets used in this study consumed about 350-400 g feed/day, the values of mycotoxin intake were below the limits for all tested doses of OTA and FB_1. For AFB_1, DON and ZEA, these values were below the limits for three out of the four doses and about twice the limits for the highest dose.

Table 3 shows the mean biomarker values (concentration and absolute amount of each biomarker excreted) found in the 24 h post dose urine samples collected from the four groups of piglets administered mixtures of five mycotoxins at four levels. All mycotoxin biomarkers were detected and measured in all urine samples with the exception of β-ZOL, which was detected and measured only in urines of piglets administered the highest dose of ZEA (5.66 μg/kg bw). The presence of low concentrations of β-ZOL, compared to α-ZOL, in urine of pigs fed with diet contaminated with ZEA was also reported by Dänicke *et al.* (2005). In particular, for urines containing both β-ZOL and α-ZOL, the ratio α-ZOL/β-ZOL ranged between 17.5-73.7, compared to 36.5 obtained in the present study. The mean percentage of mycotoxin ingested that was excreted in 24 h urine as biomarker(s) is shown in Figure 1 for AFB_1, OTA, FB_1, DON and ZEA. The measurement of urine volume from each piglet and the mass balance of each analyte in the boluses and in the urine permitted to calculate the relevant percentage of ingested mycotoxin that was excreted as

Table 3. Urinary excretion of mycotoxin biomarkers. Piglets received a bolus containing a mycotoxin mixture[1] and urinary excretion was measured during the next 24 h. Results are reported as mean ± standard deviation.

Biomarker[2]	Bolus 1 (n=4)[3]		Bolus 2 (n=4)[4]		Bolus 3 (n=4)[5]		Bolus 4 (n=4)[6]	
	ng/ml	μg/animal	ng/ml	μg/animal	ng/ml	μg/animal	ng/ml	μg/animal
DON	80.55±28.96	23.35±12.96	125.06±41.64	34.57±15.09	305.94±143.17	128.65±59.04	218.18±33.35	101.32±49.97
DOM-1	1.19±1.38	0.25±0.29	3.36±1.35	0.90±0.40	3.44±4.14	1.42±1.64	16.33±5.95	7.65±5.24
AFM_1	0.14±0.12	0.03±0.02	0.36±0.31	0.09±0.07	0.50±0.31	0.21±0.12	0.88±0.35	0.43±0.33
FB_1	1.55±0.21	0.48±0.36	4.82±2.72	1.22±0.43	77.37±43.62	32.47±17.57	117.78±71.91	62.69±54.53
ZEA	11.63±7.52	2.83±1.17	23.06±11.42	5.86±1.40	15.76±9.62	6.55±3.74	17.08±4.15	8.44±5.45
α-ZOL	3.60±3.70	0.74±0.74	6.76±4.43	1.69±0.74	5.24±3.29	2.18±1.30	6.58±2.36	2.96±1.49
β-ZOL[7]	nd	nd	nd	nd	nd	nd	0.39±0.36	0.17±0.16
OTA	0.12±0.05	0.04±0.02	0.65±0.22	0.17±0.02	0.52±0.15	0.22±0.06	0.36±0.12	0.15 ± 0.01

[1] Mycotoxin mixture containing deoxynivalenol (DON), aflatoxin B_1, fumonisin B_1 (FB_1), zearalenone (ZEA) and ochratoxin (OTA); see Table 1 for details.
[2] DOM-1 = de-epoxy-deoxynivalenol, AFM_1= aflatoxin M_1, α-ZOL = α-zearalenol, β-ZOL = β-zearalenol.
[3] Mean volume of 24 h urine 296.75±173.47 ml.
[4] Mean volume of 24 h urine 275.25±59.19 ml.
[5] Mean volume of 24 h urine 421.25±19.31 ml.
[6] Mean volume of 24 h urine 462.50±188.70 ml.
[7] nd = not detected.

biomarker(s) in the 24 h post dose urine. As shown in Table 3 and Figure 1, the amount of each biomarker excreted in the 24 h post dose urine was correlated with the amount of mycotoxin intake and the percentage of ingested mycotoxin that was excreted as biomarker in urine varied depending of the mycotoxin considered. The highest mean percentage of ingested mycotoxin excreted as biomarker in the 24 h post dose urine was observed for ZEA (28.4%) followed by DON (27.9%), α-ZOL (8.3%), FB_1 (2.6%), OTA (2.6%), AFM_1 (2.5%) and DOM-1 (0.6%) (Figure 1). These results fall within those obtained in studies previously conducted with pigs fed diets contaminated with a single or a combination of mycotoxins. For example, 14-15.6% of ingested ZEA was excreted as ZEA+α-ZOL in pig urine collected during 8 h post dose; β-ZOL was not detected (Olsen *et al.*, 1985; Zöllner *et al.*, 2002). A lower urinary excretion of ZEA was reported by MacDougald *et al.* (1990); only 5.3% of the ingested dose was excreted in the 8 h urine following ingestion of ZEA. The higher values of excretion observed in our study could be related to the longer period (24 h) of urine collection.

Our results of urinary excretion of DON (27.9%) and DOM-1 (0.6%) are in agreement with those reported in other studies wherein a range of 23-60% of ingested DON or 3-ADON was eliminated as DON, whereas a range of 0-3% was excreted as DOM-1 (Eriksen *et al.*, 2003; Dänicke *et al.*, 2004a,b,c, 2005; Goyarts and Dänicke, 2006). Higher excretion of DON in pig 24 h urine (54-82%) was reported by Prelusky *et al.* (1988) after intragastrical administration of radioactive-labelled DON. The mean percentage of urinary excretion observed in this study for FB_1 (2.6%) is comparable with the range of 0.7-2.5% reported in previous studies conducted with pigs using similar or different experimental conditions (Dilkin *et al.*, 2003, 2010; Fodor *et al.*, 2006, 2008; Szabó-Fodor *et al.*, 2008; Prelusky *et al.*, 1994). Few animal studies with pigs reported the urinary excretion of OTA after oral administration of the toxin. Blank and Wolffram (2004) reported that daily excretion of OTA in pig urine was 5.5% after a single ingested dose of 2,423 µg OTA equivalent to 66 µg/kg body weight. In our study, a lower percentage of OTA excretion was observed (2.6%) following a lower OTA ingestion (0.16-1.32 µg/kg body weight). Other studies performed with pigs only reported urinary concentrations of OTA following ingestion of feed naturally contaminated with OTA. Oral exposure to diets contaminated with 38-552 and 130-790 µg/kg of OTA resulted in urinary concentrations of 7.1-19.4 and 52.4 ng/ml (mean), respectively (Stoev *et al.* 1998, 2001). The mean percentage of ingested AFB_1 excreted as urinary AFM_1 (2.5%) found in this study was within the percentages reported in previous studies with pigs, ranging between 0.05 and 4% (Thieu, 2010).

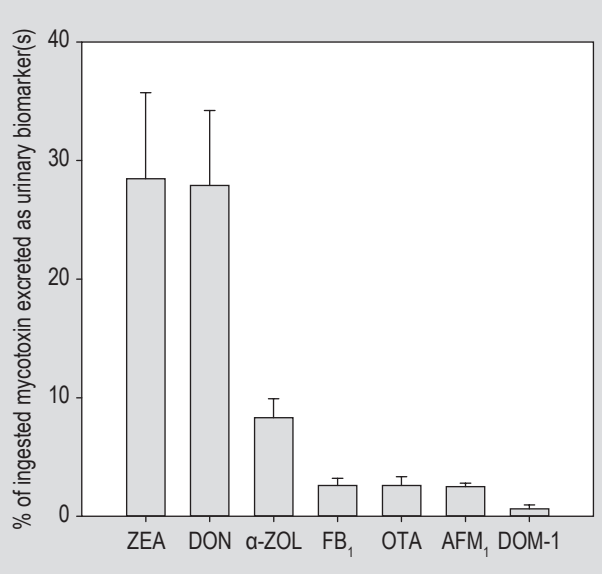

Figure 1. Mean percentage of ingested mycotoxins excreted as biomarker in 24 h urine. Each result is the mean ± standard error of 16 measurements in urine from 16 piglets (values of ingested mycotoxin and excreted biomarker are given in Tables 2 and 3, respectively). ZEA = zearalenone, DON = deoxynivalenol, α-ZOL = α-zearalenol, FB_1 = fumonisin B_1, OTA = ochratoxin A, AFM_1= aflatoxin M_1, DOM-1 = de-epoxy-deoxynivalenol.

Figure 2 shows the relationship between mycotoxin dose and urinary excretion of the relevant biomarker(s) over 24 h following oral administration for each mycotoxin/biomarker couple. The mycotoxin doses in the contaminated boluses and in the control diet consumed by the piglets the day before administration of the boluses were used in the graphs. The amount of each biomarker excreted in control urine collected during 24 h before administration of the boluses is also reported in these graphs. The lines were not forced through the origin. Linear regression analyses revealed correlation coefficients of 0.71 (P=0.0001), 0.78 (P<0.0001), 0.76 (P<0.0001), 0.76 (P<0.0001), 0.77 (P<0.0001) and 0.71 (P=0.0001) for the mycotoxin/biomarker couples DON/DON, DON/DOM-1, AFB_1/AFM_1, FB_1/FB_1, ZEA/α-ZOL and ZEA/ZEA, respectively. A lower correlation coefficient (r=0.68, P=0.0002) was observed for OTA due to the low excretion at the highest dose. A better correlation coefficient (r=0.95, P<0.0001) was obtained by eliminating the highest dose from the graph. A slight improvement was also observed for DON/DON, AFB_1/AFM_1, FB_1/FB_1, ZEA/α-ZOL and ZEA/ZEA by eliminating the highest dose from the graph (data not shown). These results suggest that there is a lower biomarker excretion at the highest dose, which is probably connected with a reduced and poorly reproducible absorption at the gastrointestinal level. This effect was particularly evident for OTA, although a good reproducibility of OTA excreted was observed at the highest dose (Figure 2). A better correlation was observed for DON/DOM-1 (r=0.78, P<0.0001) compared to DON/DON (r=0.71, P=0.0001), suggesting that DOM-1 could be a better exposure biomarker for DON in pigs. However, the

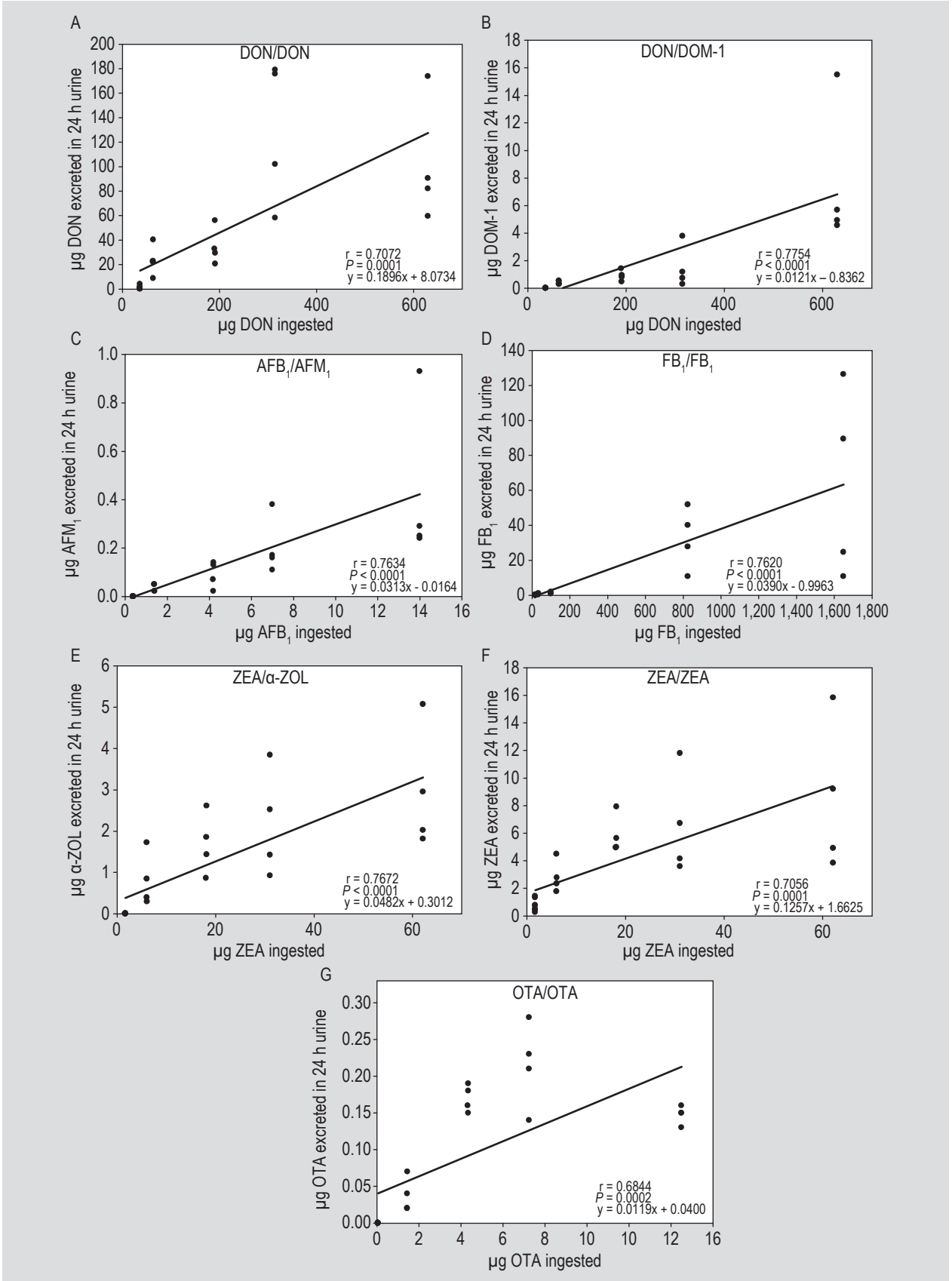

Figure 2. Linear regression analysis of ingested mycotoxin dose compared to the relevant urinary biomarker(s) excreted in 24 h post dose for each mycotoxin/biomarker couple. (A) Deoxynivalenol (DON) / DON, (B) DON / de-epoxy-deoxynivalenol (DOM-1), (C) aflatoxin B_1 (AFB_1) / aflatoxin M_1 (AFM_1), (D) fumonisin B_1 (FB_1) / FB_1, (E) zearalenone (ZEA) / α-zearalenol (α-ZOL), (F) ZEA / ZEA, and (G) ochratoxin A (OTA) / OTA.

urinary concentrations of DOM-1 were on average about 30 times lower than those of DON (Table 3). These results suggest that the proposed method for DON is also robust at low concentrations using DOM-1 as urinary biomarker, although DON should be preferred as biomarker when low exposure levels need to be monitored or a less sensitive analytical method is available. The same situation was observed for ZEA/α-ZOL (r=0.77, $P<0.0001$) and ZEA/ZEA (r=0.71, $P=0.0001$) and again the mean urinary concentrations of ZEA were 3 times higher than those of α-ZOL (Table 3), which makes ZEA a more sensitive biomarker for piglets. In dose-response experiments performed by Dänicke *et al.* (2005) with adult gilts, mean urinary concentrations of α-ZOL were about 30% higher than those of ZEA. The use of piglets in our experiment compared to adult gilts used by Dänicke *et al.* (2005) should explain this difference. Adults gilts have a mature metabolism system that should be capable to transform higher amounts of ZEA into α-ZOL compared to piglets.

Urinary AFM_1, FB_1, OTA and DON are recognised as good biomarkers of exposure to AFB_1, FB_1, OTA and DON, respectively, for humans, with adjusted correlation coefficients ranging from 0.52 to 0.91 (Turner *et al.*, 2012). The results reported in this study fall within this range including those obtained for ZEA, α-ZOL and DOM-1. Figure 2 also shows a difference between excreted amounts of biomarkers of individual animals receiving the same dose. For some biomarkers or dose, a high variability of results was observed. However, a high variability is common for *in vivo* studies and our results with piglets are comparable to or lower than those reported by other authors (Blank and Wolffram, 2004; Dänicke *et al.*, 2005; Fodor *et al.*, 2006; Stoev *et al.*, 2001; Thieu and Pettersson, 2009). We cannot exclude that matrix effects may have influenced the precision of results obtained in this study. However, we are confident that the variability of the biomarker levels observed in individual animals receiving the same contaminated bolus is animal dependent according to the results reported by other authors that were obtained with methods (HPLC-UV/FD) that do not suffer from unpredictable signal suppression or enhancement (Blank and Wolffram, 2004; Dänicke *et al.*, 2005; Thieu and Pettersson, 2009).

4. Conclusions

This is the first *in vivo* study that evaluated the dose-response between simultaneous oral ingestion of FB_1, OTA, DON, ZEA and AFB_1 and urinary excretion of the relevant biomarkers within 24 h after dosage by using a mass balance approach. The good sensitivity of the LC-MS/MS method used in this study permitted to detect and quantify low levels of most of the investigated biomarkers in control urine samples collected before administration of contaminated boluses. In particular, low levels of DON, DOM-1, FB_1, ZEA, α-ZOL and OTA were measured in 62, 44, 56, 100, 19 and 44% of control urines, respectively. The method also permitted to detect and quantify urinary biomarkers of mycotoxins at the doses investigated. The mass balance and the multi-mycotoxin approaches allowed to assess the simultaneous excretion rate of five mycotoxins in urine of piglets as specific biomarkers. A positive dose-response relationship for the tested combinations of mycotoxin/biomarker has been demonstrated, which suggest possible ranges of mycotoxin doses for future studies. The good dose-response correlations make the urinary multi-biomarker approach a good tool to assess *in vivo* the efficacy of mycotoxin detoxifying agents in reducing the bioavailability of mixtures of DON, FB_1, AFB_1, OTA and ZEA.

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