Zearalenone (ZEN) and ZEN metabolites in feed, urine and bile of sows: Analysis, determination of the metabolic profile and evaluation of the binding forms

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Abstract

A new alkaline-based method was developed for extracting zearalenone (ZEN) from feedstuffs. The procedure was shown to increase the extraction efficiency by 7 to 56 %. The addition of β-glucosidase did not increase the analyzed ZEN concentrationssuggesting ZEN-β-glucosides being of minor importance.

Urine and bile samples from sows were analyzed by HPLC-FLD after treating the aqueous solution with β-glucuronidase/arylsulfatase and cleaning by liquid/liquid partition on a Kieselgur (Extrelut®) column and IA-chromatography. A recovery of 82 to 99 % for ZEN, α- and β-zearalenol (α- and β-ZEL) was determined.

ZEN and α-ZEL concentrations in bile samples from an experiment with sows fed with ZEN contaminated diets increased significantly with the ZEN concentration of the feed. In urine, such a dependency was found only for α-ZEL. The proportion of α-ZEL of the sum of ZEN and its metabolites (α- and β-ZEL) increased from 42 to 53 % in bile and from 27 to 49 % in urine (without control groups) with dietary ZEN concentrations, respectively.

ZEN and its metabolite α-ZEL were nearly exclusively conjugated to glucuronic acid or sulfuric acid in bile and urine. The percentage of the free, ZEN and α-ZEL varied between 1 and 6 %. The proportion of glucuronidated conjugates of ZEN in bile as well as in urine of sows was >95 % and no sulfated conjugates were present. The relation of glucuronidated to sulfated conjugates of α-ZEL was 82 to 17 % in bile, whereas in urine the sulfated (62 %) form of α-ZEL was predominant to the glucuronidated (33 %) form.

Keywords: *zearalenone, metabolites, glucuronic acid, sulfuric acid, conjugates, feedstuff, extraction*

Zusammenfassung

Zearalenon (ZEN) sowie ZEN-Metaboliten in Futter, Urin und Galle von Sauen: Analytik, Bestimmung von Metabolitenprofilen und Abschätzung der Bindungsformen

Zur Extraktion von Zearalenon (ZEN) aus Futter wurde eine neue Methode auf alkalischer Grundlage entwickelt. Damit wurde die Effektivität der Extraktion um 7 bis 56 % gesteigert. Die Zugabe von β-Glucosidase erhöhte nicht die analysierten ZEN-Konzentrationen, was auf zu vernachlässigende Konzentrationen von ZEN-β-Glucosiden hinweist.

Harn- und Galleproben von Sauen wurden mit HPLC-FLD analysiert, nachdem die wässrige Lösung mit β-Glucuronidase/Arylsulfatase behandelt wurde und mittels flüssig-flüssig-Verteilung über eine Kieselgursäule (Extrelut®) und IA-Chromatographie gereinigt wurde. Die Wiederfindungen betrugen 82 bis 99 % für ZEN, α- und β-Zearalenol (α- und β-ZEL).

Zwischen der ZEN-Konzentration im Futter und den ZEN- und α-ZEL-Konzentrationen der Galleproben, die aus einem Versuch mit Sauen stammen, die mit ZEN-kontaminiertem Futter (bis zu 358 ng/g) gefüttert wurden, besteht ein hoch signifikanter Zusammenhang. Im Harn wurde solch eine Abhängigkeit nur für α-ZEL festgestellt.

In Galle und Harn waren ZEN und sein Metabolit α-ZEL fast ausschließlich mit Glucuronsäure oder Schwefelsäure konjugiert. Der prozentuale Anteil an freiem ZEN und α-ZEL variierte zwischen 1 und 6 %. Der Anteil an glucuronidiertem ZEN war sowohl in der Galle als auch im Harn der Sauen >95 % und es waren keine sulfatierten Konjugate vorhanden. In der Galle war das Verhältnis von glucuronidiertem zu sulfatiertem α-ZEL 82 zu 17 %, wohingegen im Harn die sulfatierte Form (62 %) gegenüber der glucuronidierten Form (33 %) von α-ZEL vorherrschte.

Schlüsselwörter: *Zearalenon, Metabolite, Glucuronsäure, Schwefelsäure, Konjugate, Futtermittel, Extraktion*

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1 Introduction

The *Fusarium* fungi form several toxic secondary metabolic products which are harmful for humans and animals. ZEN belongs to these mycotoxins and can frequently be found in agricultural commodities which are used as feedstuffs for farm animals. Under unfavourable conditions ZEN concentrations in feed might exceed critical concentrations which might result in reproductive disorders of sensitive animals such as pigs (European Food Safety Authority, 2004).

Evaluation of toxic effects of ZEN on animals requires appropriate analytical methods, both for feed and for physiological specimens in order to assign the internal exposure of animals (e.g., concentrations of ZEN and its metabolites in urine as indicator) to a certain feed contamination as a prerequisite for risk evaluation and management. Critical steps in feed analysis include especially the extraction from the solid feed matrix with total extraction still being a challenge. In addition, ZEN and other mycotoxins in feedstuffs have been reported to exist both in free form and conjugated with glucose (Rychlik et al., 2014). As the free form can be rapidly converted from the conjugated form in the digestive tract, the knowledge of the concentrations of the glucosidic bound ZEN is of toxicological relevance (Dänicke and Brezina, 2013).

For physiological specimens such as urine and bile there are additional challenges which arise from the fact that ZEN undergoes metabolic alterations including reduction to its main metabolites α- and β-zearalenol (α- and β-ZEL) and conjugation of ZEN and its metabolites with glucuronic acid or sulfuric acid. As microbes are also capable of forming α- and β-ZEL, these metabolites can also be detected in feed (Keese et al., 2008; Seidel et al., 1993).

Thus, the aim of the present investigations was to optimize the extraction efficiency for ZEN (and α- and β-ZEL) from the feed matrix and to evaluate the proportions of glucuronic acid and sulfuric acid conjugates of ZEN and α- and β-ZEL in urine and bile collected from sows exposed to graded dietary levels of ZEN.

2 Materials and methods

2.1 Experiments for improving extraction efficiency for ZEN (and α- and β-ZEL) from the feed matrix

The methods published by the Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA) (Seibold et al., 1983) for feed analysis were modified because the addition of buffer for enzymatic cleavage of possible zearalenone-β-glucosides (Gareis et al., 1990) often formed emulsions which were difficult to handle or gave impure extracts. Furthermore, other extraction solvents and combinations of purifying steps were tested for the analysis of various feedstuffs.

2.1.1 Determination of ZEN and α- and β-ZEL in feedstuffs

The modified analytical procedure for feed analysis based upon the combination of two methods published by VDLUFA (Seibold et al., 1983). A total of 5 g feedstuffs was homogenized with 20 ml of 0.05 mol/l acetate buffer pH 4.7 and 2 units β-glucosidase from almonds (Sigma No. G0395) and shaken overnight at 40 °C. The samples were shaken for 45 min after the addition of 100 ml of alkaline extraction medium (25 % NH_3/methanol/ethyl acetate/dichloromethane; 4/20/100/200 *v/v/v/v*) and 4 g sodium chloride. The organic phase was evaporated to dryness under nitrogen and the residue was dissolved with approximately 10 ml chloroform. Ten ml of 1 mol/l sodium hydroxide was added to clean the extract by base-acid treatment, the mixture was well shaken and centrifuged to clearness. 1 drop of 1 % phenolphthalein (in ethanol) was added to 8 ml of the upper alkaline phase, and then phosphoric acid (2 mol/l) was added drop wise until colour change of the indicator. After mixing with 10 ml toluene and centrifuging, 8 ml of the solvent phase was evaporated to dryness under nitrogen and dissolved in 3 ml acetonitrile (MeCN). The mixture was diluted with 17 ml phosphate buffered saline (PBS)-solution (see instruction of IA-columns) and loaded onto an IA-column (Easi-Extract® Zearalenone, R-Biopharm, Darmstadt, Germany), which was prepared earlier by washing with 20 ml PBS-solution. The column was washed with 15 ml water to remove matrix and the analytes were eluted with 1.8 ml MeCN. The eluate was evaporated to dryness with nitrogen and redissolved in 0.5 ml mobile phase.

HPLC conditions: 0.8 x 0.4 cm precolumn with 25 x 0.4 cm C-18 column (5 µm, Pyramid® HPLC column No. 161803.40, Macherey-Nagel, Düren, Germany), mobile phase consisted of MeCN/H_2O 45/55 *v/v* and 2 ml 85 % phosphoric acid/1000 ml, 0.9 ml/min, 50 µl sample loop, 35 °C column temperature, detection was done by HPLC connected to a fluorescence detector (HPLC-FLD): λ_{ex} = 273 nm and λ_{em} = 455 nm, 40 µl flow cell, UV-detector at 238 nm in series. The limit of detection (3-fold the detector noise) in feedstuffs was 1 ng/g estimated for ZEN and α-ZEL and 4 ng/g for ß-ZEL.

2.1.2 Attempts for the optimization of the analytical procedure for feedstuffs

The analytical procedures followed the modified method above with the exceptions indicated.

The reference method (No. 1-3) consists of the addition (No. 2), or non-addition of β-glucosidase (No. 1) to the acetate buffer, or without buffer and β-glucosidase (No. 3), extraction with 100 ml chloroform, base-acid treatment and IA-chromatography.

The alkaline method (No. 4-6) uses 100 ml of a mixture of 25 % NH_3/methanol/ethyl acetate/dichloromethane (4/20/100/200 *v/v/v/v*) for extraction. Previously the sample was treated with β-glucosidase in acetate buffer (No. 5), only with buffer (No. 4) or without buffer (No. 6). Base-acid treatment and IA-chromatography followed.

The Extrelut® procedure (No. 8-12): The sample was mixed with 20 ml acetate buffer and β-glucosidase (No. 10-12) or only with buffer (No. 8-9). Extraction was carried out with 20 ml methanol + 0.26 ml 25 % NH_3, pH approx. 9.0 (No. 9 and 11) or with 20 ml methanol + 0.4 g tartaric acid, pH approx. 4.0 (No. 10 and 12) or just with 20 ml methanol (No. 8). 5 ml were loaded onto a column filled with 5 g Extrelut® (Merck, Darmstadt, Germany) and eluted with 35 ml dichloromethane followed by IA-chromatography. The extract was evaporated to dryness with nitrogen and reconstituted in mobile phase.

Other methods (No. 7 and 13): The sample was homogenized with 20 ml acetate buffer and extracted with 100 ml dichloromethane/ethyl acetate (2/1 *v/v*), base-acid treatment and IA-chromatography (No. 7). After pre-treatment with β-glucosidase in 20 ml acetate buffer, 30 ml acetonitrile were added and the pH adjusted from 7.0 to 7.5. The mixture was shaken, filtrated, an aliquot was taken, concentrated and 17 ml PBS-solution (pH 7.2 to 7.5) were added. Subsequently the sample solution was cleaned by IA-chromatography (No. 13).

All methods used 4 g sodium chloride for better phase separation after incubating over night with or without enzyme and addition of the extraction solvent.

Total ZEN, α- and β-ZEL

3 to 5 g bile fluid or urine were diluted with 0.1 mol/l acetate buffer pH 5.3 + 0.1 ml β-glucuronidase/arylsulfatase from *Helix pomatia* and filled with buffer to 15 ml (about 3 U β-glucuronidase, measured with nitrophenyl-β-glucuronide, 38 °C, pH 6.2; about 6 U arylsulfatase, measured with nitrophenylsulfate, 38 °C, pH 6.2; No. 104114.0002, VWR, Darmstadt, Germany). Incubation was done overnight by shaking at 37 °C. Five ml were loaded onto a column filled with 5 g Extrelut®. After 15 min the column was eluted with 35 ml dichloromethane. The extract was evaporated to dryness with nitrogen, 3 ml acetonitrile were added and diluted with 17 ml PBS-solution. The mixture was cleaned by IA-chromatography and after concentration the eluate was measured by HPLC-FLD as described in 2.1.1.

Free and glucuronic acid conjugated ZEN, α-and β-ZEL

The same procedure as for total mycotoxin determination has been carried out, but without enzyme addition (determination of free mycotoxins) or using 0.02 ml β-glucuronidase from *E. coli* instead (No. 03707598001, Roche, Mannheim, Germany; about 2 U, measured with ß-nitrophenyl-glucuronide, 37 °C, pH 7.0) for determination of ß-glucuronidated ZEN, α- and ß-ZEL.

2.2 Evaluation of the proportions of glucuronic acid and sulfuric acid conjugates of ZEN and α- and β-ZEL in urine and bile

2.2.1 Experimental design and diets

The urine and bile samples were taken from an experiment with 36 sows ranged into 4 groups (Groups 1 to 4) of 9 animals each. The animals received diets with increasing ZEN concentrations, as uncontaminated wheat was substituted by *Fusarium* toxin-contaminated wheat. The diet of Group 1 (control) contained 0 g contaminated wheat, while 133.5, 266.5 and 400 g contaminated wheat/kg diet were incorporated into the diets of Groups 2, 3 and 4. The analyzed ZEN content of the diets of Groups 1 to 4 amounted to: 4, 88, 235 and 358 µg/kg diet. Barley, dried chips, soybean meal, premix and L-lysine-HCl (284, 100, 175, 10, 30 and 0.7 g/kg) completed the diet of the four groups (Dänicke et al., 2007). Pooled samples from 3 animals (3 samples/group) with 3 treatments/sample (without addition of enzymes, with β-glucuronidase and with β-glucuronidase + arylsulfatase) were analyzed for ZEN and α- and β-ZEL using the Extrelut® method (see below), whereby all 4 x 3 x 3 = 36 bile and as many urine samples were measured with 4 replications.

2.2.2 Determination of ZEN, α- and β-ZEL in bile and urine using Extrelut®

Analytical methods as described earlier (Ueberschär, 1999; Dänicke et al., 2001) were adapted for analysing ZEN and its metabolites in urine and bile of sows. The modified method described here rendered cleaner extracts, which makes the measurement by HPLC-FLD more reliable.

2.3 Calculations and statistics

Efficiency of extraction procedure was expressed as percentage of detected ZEN concentration of test procedure compared to the standard procedure No. 1. Variation coefficient (relative standard deviation, RSD) was used as variance parameter.

For physiological specimens, the metabolite profile was defined as the ratio between a particular metabolite (ZEN, α-ZEL or β-ZEL) of the sum of all residues (ZEN + α-ZEL + β-ZEL), expressed as percentage. The diet ratio was calculated as the sum of ZEN + α-ZEL + β-ZEL concentration in the physiological sample divided by the dietary ZEN concentration.

Free ZEN residues (not conjugated) were determined without any enzymatic treatment of the samples prior to HPLC analysis. Glucuronic acid bound ZEN residues were obtained by the difference between the toxin levels obtained after incubating the samples with ß-glucuronidase (*E. coli*) and the free toxin contents. The sulfuric acid conjugates were calculated as the difference between total residues – as determined after treating the samples both with β-glucuronidase and arylsulfatase (*H. pomatia*) – and the sum of free and glucuronic acid bound toxins.

Data were analysed according to a complete 4 x 2 two-factorial design of analysis of variance (ANOVA) with diet ZEN level and enzymatic treatment of the physiological specimens and their interaction being the fixed factors:

$$y_{ijk} = \mu + a_i + b_j + axb_{(ij)} + e_{ijk}$$

where y_{ijk} = k^{th} observation on diet ZEN level i and enzyme treatment j; μ = overall mean; a_i = effect of diet ZEN level

(4, 88, 235 and 358 µg/kg diet); b_j = effect of enzyme treatment (without, with treatment of β-glucuronidase/arylsulfatase); $axb_{(ij)}$ = interactions between diet ZEN level and enzyme treatment; e_{ijk} = residual error.

In addition, linear and quadratic effects were estimated for the dietary ZEN level.

All statistics were performed using Statistica for the Windows Operating System (StatSoft, 1995).

3 Results

3.1 Analysing ZEN in feedstuffs

Method No. 1 (Table 1) using buffer, chloroform as extraction solvent, base-acid treatment and IA-chromatography was chosen as reference. A 30 % higher gain was obtained by soaking the sample in buffer overnight in comparison to method No. 3 (without buffer addition). Relatively less efficient was the addition of buffer using the alkaline method as shown in Nos. 6 and 4, which could be caused by the water content in the ammonia solvent. As suggested earlier, the 10 % water addition to the extraction solvent was advantageous (Seidel et al., 1993). Other authors used acetonitrile with 15 % water (Llorens et al., 2002). In comparison to the reference, the use of dichloromethane/ethyl acetate (No. 7) as extraction solvent was 14 % less or equally suitable for the analysis of hen's feed or triticale. Chromatography on Extrelut® with the addition of NH_3 or tartaric acid (No. 9 to 12) was comparable to the reference excepting the chromatography without additions (No. 8). The purification of an extract of compound feedstuff with only IA-chromatography (No. 13) led to a 36 % reduction of the analysed mycotoxin content of the sample.

3.2 ZEN-glucosides in feedstuffs

The addition of β-glucosidase to the buffer had no significant influence (t-test: p = 0.11 or p = 0.34) on the analytical results using the reference (No. 1, No. 2) or the alkaline method (No. 4 and 5) for the analysis of hen's feed. The results must be interpreted with caution due to the small basis of data. Therefore, many more feed samples and compound feedstuffs need to be analyzed. To be on the safe side, β-glucosidase should be added to the buffer as described in the experimental section of feed analysis.

The application of the alkaline extraction solvent mixture (25 % NH_3/methanol/ethyl acetate/dichloromethane; 4/20/100/200 *v/v/v/v*) together with base-acid clean up and IA-chromatography was much more effective than the reference method (140 to 156 %) when hen's feed was analyzed

Table 1

Experiments to optimize the analyzing procedure of zearalenone (ZEN) in feedstuffs (200 to 470 µg ZEN/kg) or compound feedstuffs (3 to 370 µg ZEN/kg). The efficiency of the procedures is shown in relation to the reference method (No.1)

Feedstuff / Method	(No.)		n	Efficiency (%)	RSD (%)
Hen´s feed					
Reference method	(1)	buffer + $CHCl_3$ + B/A[1] + IA[2]	9	**100**	9
	(2)	buffer/β-gluc.[3] + B/A[1] + IA[2]	10	120	24
	(3)	+ B/A[1] + IA[2]	6	70	7
Alkaline method	(4)	buffer + NH_3-solvent + B/A[1] + IA[2]	7	156	8
	(5)	buffer/β-gluc.[3] + NH_3-solvent + B/A[1] + IA[2]	5	140	15
	(6)	+ NH_3-solvent + B/A[1] + IA[2]	3	137	4
Extrelut® method	(8)	buffer + methanol + Extrelut® + IA[2]	4	60	17
	(9)	buffer + methanol/NH_3 + Extrelut®	3	112	5
Other method	(7)	buffer + CH_2Cl_2/ethyl acetate + B/A[1] + IA[2]	3	86	10
Triticale					
Reference method	(1)	buffer + $CHCl_3$ + B/A[1] + IA[2]	7	**100**	10
Alkaline method	(4)	buffer + NH_3-solvent + B/A[1] + IA[2]	3	107	15
Extrelut® method	(8)	buffer + methanol + Extrelut® + IA[2]	7	82	21
	(10)	buffer/β-gluc.[3] + methanol/tartaric acid + Extrelut®	4	99	2
Other method	(7)	buffer + CH_2Cl_2/ethyl acetate + B/A[1] + IA[2]	3	103	5
Compound feedstuffs					R^2
Reference method	(1)	buffer + $CHCl_3$ + B/A[1] + IA[2]	20	**100**	
Alkaline method	(4)	buffer + NH_3-solvent + B/A[1] + IA[2]	10	139	0.94
Extrelut® method	(11)	buffer/β-gluc.[3] + methanol/NH_3 + Extrelut® + IA[2]	13	114	0.97
	(12)	buffer/β-gluc.[3] + methanol/tartaric acid + Extrelut® + IA[2]	20	96	0.88
Other method	(13)	buffer/β-gluc.[3] + acetonitrile + IA[2]	5	64	0.96

[1] base-acid treatment
[2] immunoaffinity chromatography
[3] β-glucosidase

(Table 1). Acidic solvents, above all diluted phosphoric acid, influence the results negatively as zearalenone is less stable under acidic conditions. ZEN in naturally contaminated feed is more resistant than when added artificially as a crystalline substance to the feed (Valenta, 1999). No effect was found for the analysis of triticale, whereas for compound feedstuff (hen's feed) markedly higher mycotoxin contents were obtained using the alkaline extraction solvent with the base-acid cleaning step and IA-chromatography (139 % efficiency). To test the various methods for analysing compound feedstuffs, up to 20 data entries over the whole range of 3.0 to 370 µg ZEN/kg feed were available. These feedstuff samples were analysed by the reference and test-methods. It could be shown that a linear relation exists between the reference method (No. 1) and the test-method (No. 4, 11, 12 or 13) for a range of mycotoxin concentrations. All regression lines (plots not shown) were characterised by the coefficient of determination $R^2 > 0.88$ (Table 1).

3.3 ZEN, α- and β-ZEL in bile and urine using Extrelut®

The optimized analysis method for feedstuffs using the NH_3-containing extraction solvent did not work with physiological samples such as bile or urine due to the formation of emulsions after the base-acid clean up step. Therefore, a new method was developed for these types of samples to obtain sufficiently clear extracts and to attain the limits of detection of 4 µg/kg for β-ZEL and 1 µg/kg for ZEN and α-ZEL. For these reasons the Extrelut® method was tested as described above. The mean recoveries for the three toxins were in the range of 82 to 99 % and relative standard deviations (RSD) of 10 to 20 % for the analyzed matrices (Table 2). Additions of 30 to 200 ng at sample weights of 2.5 to 5 g were made. Relative standard deviations (RSD) were 10 to 20 % for urine at concentrations up to 50 µg/kg. Relative standard deviations (RSD) were 30 to 40 % for bile at concentrations up to 300 ng/g (Table 3). For urine at concentrations up to 50 ng/g RSD-values of 30 to 50 % were determined (Table 4).

Table 2
Extrelut®-Method: Recovery (%) and relative standard deviation (RSD) of zearalenone (ZEN), α- and β-zearalenol (α- and β-ZEL)

Matrix		Addition (ng)	Recovery (%), RSD (%) α-ZEL	β-ZEL	ZEN
Control	(n = 8)	40 - 60	105 (22)	75 (33)	98 (8)
Urine, sow *	(n = 6)	30 - 120	91 (21)	83 (12)	93 (15)
Bile, sow *	(n = 7)	60 - 200	96 (10)	82 (10)	89 (12)

Control: 4x without enzymes; 2x with ß-glucuronidase;
2x with ß-glucuronidase/arylsulfatse
* 2.5-5 g sample amount

3.3.1 Residue levels in bile and urine

Tables 3 and 4 show the effects of the addition of *Fusarium*-contaminated wheat with zearalenone contents up to 358 µg/kg feedstuff on the contents of ZEN and its metabolites in bile and urine of sows. β-Zearalenol concentrations were always lower than the limit of detection of 4 ng/g, therefore, such values were considered with the detection limit for further calculations.

Concentrations of ZEN and α-ZEL in bile ($p_{linear} < 0.001$) or α-ZEL in urine ($p_{linear} = 0.003$) increased in linear fashion with dietary ZEN concentrations, whereas ZEN in urine remained at a dietary ZEN-independent level ($p = 0.206$) with variable values ranging from 29 to 59 ng/g (Table 4). The proportion of α-ZEL in the sum of ZEN and its metabolites (α- and β-ZEL) increased from 42 to 53 % in bile and from 27 to 49 % in urine (without control groups) with dietary ZEN concentrations, respectively ($p_{linear} < 0.001$). The proportion of ZEN in bile amounted from 47 to 55 % or from 47 to 64 % in urine (control groups were not considered). Both were inversely related to dietary mycotoxin concentrations.

3.3.2 Metabolic profile in bile and urine

The influence of the enzymatic sample treatment (addition of glucuronidase/arylsulfatase) was evaluated by the ANOVA procedure. The α-ZEL content in bile fluid and urine increased slightly and significantly, respectively ($p = 0.054$ or $p < 0.001$). This is also reflected in the metabolic profile where significantly higher proportions were found for α-ZEL ($p = 0.033$ and $p < 0.001$) after the addition of glucuronidase/arylsulfatase. However, no influence of this enzyme on the ZEN concentrations was noticed in bile fluid or urine ($p = 0.729$ or $p = 0.966$). The concentrations were nearly the same with or without addition of enzyme (Tables 3 and 4). Also, the metabolic profile of ZEN in bile remained unaffected ($p = 0.07$), but a significant influence was found for urine ($p = 0.003$).

No significant interaction was found between the ZEN-concentration of the diet and enzymatic treatment for both ZEN and α-ZEL concentrations in bile and urine and their metabolic profiles in these fluids ($p_{myco\ x\ treatment} >> 0.05$).

The last column in Tables 3 and 4 shows the diet ratio, which specifies the ratio of all mycotoxin concentrations (ZEN + β-ZEL + α-ZEL) in bile or urine to the dietary ZEN concentrations. The ratios were distinctly higher in bile with values of 1.3 to 2.0 compared to urine with values of 0.22 to 0.6. No dependency of the ratios on the mycotoxin concentrations of the diet was found ($p = 0.287$ or $p = 0.096$) for urine.

3.3.3 Binding forms of ZEN and α-ZEL

The calculation of the proportion of glucuronic and sulfuric acid conjugates of ZEN and its metabolite α-ZEL follows treatment of the samples without enzymes, with ß-glucuronidase or ß-glucuronidase/arylsulfatase prior to the mycotoxin analysis. ZEN is mostly (96 %) found as glucuronide conjugate as well in bile as in urine with nearly no sulfate conjugates, whereas the opposite applies for α-ZEL in urine where 17 % or even 62 % were bound in bile or in urine as sulfates, respectively, and 82 % were glucuronides in bile and a smaller portion of 32 % in urine (Table 5). Negligible amounts of 1 to 6 % in both fluids are analysed as free, i.e., unbound ZEN or α-ZEL.

Table 3

Effect of mycotoxin-contaminated feed and β-glucuronidase or β-glucuronidase/arylsulfatase treatment on the concentration of zearalenone (ZEN) and its metabolites β-zearalenol (β-ZEL) and α-zearalenol (α-ZEL) in bile of sows (n = 3)

Treatment	Zearalenone	Concentration (ng/g)				Metabolite profile (% of the sum of β-ZEL + α-ZEL + ZEN)			Diet ratio
	(ng ZEN/g feed)	β-ZEL	α-ZEL	ZEN	β-ZEL + α-ZEL + ZEN	β-ZEL	α-ZEL	ZEN	
1 (-)	4.0	4.0	26.9	73.9	105	5.4	27.4	67.2	
1 (+)	4.0	4.0	24.1	52.1	80.2	6.4	31.0	62.6	n.d.
2 (-)	88.0	4.0	65.2	104	173	2.6	37.4	60.0	
2 (+)	88.0	4.0	75.3	99.9	179	2.6	42.0	55.3	2.0
3 (-)	235	4.0	121	141	266	1.6	46.0	52.4	
3 (+)	235	4.0	153	149	306	1.4	49.8	48.8	1.3
4 (-)	358	4.0	291	311	606	0.7	48.0	51.3	
4 (+)	358	4.0	347	308	659	0.6	52.6	46.8	1.8
ANOVA (probabilities)									
ZEN			< 0.001	< 0.001	< 0.001	0.001	< 0.001	< 0.001	0.287
Linear			< 0.001	< 0.001	< 0.001	0.008	< 0.001	0.001	
Quadratic			< 0.001	< 0.001	< 0.001	0.013	< 0.001	0.003	
Treatment			0.054	0.729	0.437	0.798	0.033	0.07	
ZEN x treatment			0.323	0.923	0.643	0.919	0.995	0.999	
PSEM			24	21	44	0.5	1.9	1.7	0.2

Diet ratio: (ZEN + α-ZEL + β-ZEL) concentration in sample/ dietary ZEN concentration
Treatment: (-) with addition of β-glucuronidase (*E.coli*); (+) with addition of β-glucuronidase/arylsulfatase (*H. pomatia*)
n.d. = not determined
PSEM = pooled standard error means

Table 4

Effect of mycotoxin-contaminated feed and β-glucuronidase or β-glucuronidase/arylsulfatase treatment on the concentration of zearalenone (ZEN) and its metabolites β-zearalenol (β-ZEL) and α-zearalenol (α-ZEL) in urine of sows (n = 3)

Treatment	Zearalenone	Concentration (ng/g)				Metabolite profile (% of the sum of β-ZEL + α-ZEL + ZEN)			Diet ratio
	(ng ZEN/g feed)	β-ZEL	α-ZEL	ZEN	β-ZEL + α-ZEL + ZEN	β-ZEL	α-ZEL	ZEN	
1 (-)	4.0	4.0	4.0	58.5	66.5	6.8	6.4	86.8	
1 (+)	4.0	4.0	10.2	56.2	70.4	6.2	15.4	78.4	n.d.
2 (-)	88.0	4.0	4.7	30.0	38.7	14.0	12.7	73.3	
2 (+)	88.0	4.0	13.3	38.7	56.0	8.9	26.7	64.4	0.6
3 (-)	235	4.0	8.2	31.3	43.5	9.6	19.3	71.1	
3 (+)	235	4.0	19.3	28.5	51.8	7.9	37.3	54.8	0.22
4 (-)	358	4.0	16.8	53.3	74.1	7.6	24.9	67.5	
4 (+)	358	4.0	48.0	51.4	103	4.9	48.5	46.6	0.29
ANOVA (probabilities)									
ZEN			< 0.001	0.206	0.095	0.244	< 0.001	0.002	0.096
Linear			0.003	0.77	0.280	0.484	< 0.001	0.002	
Quadratic			0.003	0.33	0.139	0.282	0.002	0.009	
Treatment			< 0.001	0.966	0.252	0.218	< 0.001	0.003	
ZEN x treatment			0.060	0.974	0.892	0.850	0.338	0.639	
PSEM			3.1	4.9	6.6	1.0	2.9	3.0	0.09

Diet ratio: (ZEN + α-ZEL + β-ZEL) concentration in sample/ dietary ZEN concentration
Treatment: (-) with addition of ß-glucuronidase (*E. coli*); (+) with addition of β-glucuronidase/arylsulfatase (*H. pomatia*)
n.d. = not determined
PSEM = pooled standard error means

Table 5
Binding form of zearalenone and α-zearalenol in bile (n = 9) and urine of sows (n = 12) (Overall mean values and range (n = 3), given in brackets)

	Binding form of zearalenone and α-zearalenol (% of total recovered amount)		
	Free (not conjugated)	Conjugated with glucuronic acid	Conjugated with sulfuric acid
Bile*			
zearalenone	4 (3 - 7)	96 (93 - 98)	0 (-4 - 5)
α-zearalenol	1 (1 - 2)	82 (78 - 84)	17 (13 - 21)
Urine			
zearalenone	2 (2 - 4)	96 (75 - 106)	1 (-4 - 22)
α-zearalenol	6 (2 - 10)	32 (28 - 37)	62 (58 - 65)

*Means of groups 2-4

4 Discussion

The analysis of ZEN in feedstuffs has up to now been an issue in several papers (Krska and Josephs, 2001), whereby the extraction solvent and especially the pH-value play an important role. Therefore, several acid and basic extraction solvents were tested. As discussed earlier (Valenta, 1999; Seidel et al., 1993), a long lasting treatment at acid pH values, and above all the addition of phosphoric acid to the extraction solvent, may lead to lower extraction efficiencies. However, a quickly performed base-acid treatment was found to be an efficient purification step and is part of the proposed analysis method for ZEN in feedstuffs. In comparison with pure methanol, acetone, acetonitrile or mixtures of these, water containing solvents have a positive influence. Therefore, a pre-treatment of feed samples with buffer is important to render the feed in soaked form, which makes the analyte more easily extractable. The buffer enables the addition of glucosidase to cleave ZEN-glucosides which may occur in feedstuffs (Gareis et al., 1990; Schneweis et al., 2002). In our experiments, the analysed feedstuffs gave no hints for the existence of ZEN-glucosides (data not shown). However, to recover possibly yet existing conjugates in some charges or different types of feedstuffs, glucosidase was always added to the buffer with an overnight incubation time.

In a former feeding experiment with ZEN-contaminated feed, the objective was to examine the placental transfer (Dänicke et al., 2007). In the present study the mycotoxin transfer into the physiological fluids bile and urine, the metabolic profiles and the kind and quantification of binding status of mycotoxin conjugates in the samples were analyzed. Because of deviating and variable values, no controls were considered for the calculation of the metabolic profiles. The concentration of β-ZEL was not higher than the detection limit of 4 µg/kg, which was assumed for the calculation of not detectable concentrations. Bile, and at a significantly lower level, urine, contained the highest ZEN concentrations as was also shown in other, earlier, experiments (Biehl et al., 1993; Dänicke et al., 2005). The metabolic profiles agree with former results demonstrating that ZEN and its main reduction product α-ZEL are the prevailing metabolites in the pig (Biehl et al., 1993; Olsen et al., 1986). In the present experiment, the proportion of α-ZEL in the sum of ZEN and its metabolites (α- and β-ZEL) increased with dietary ZEN concentrations from 42 to 53 % in bile and from 27 to 49 % in urine (without control groups), respectively. In the bile of hens and Pekin ducks the main product is ZEN (75 and 80 %) (Dänicke et al., 2002; Dänicke et al., 2004). The proportion of the metabolites α- and β-ZEL is, in comparison to sows, fairly low (15 and 16 % α-ZEL; 10 and 4 % β-ZEL).

The diet ratio of bile fluid varied from 1.3 to 2.0 and was up to 10-fold higher than that of urine (0.2 to 0.6). ZEN and its metabolites accumulate in bile in relation to the ZEN concentration of the diet by up to two-fold. Nearly no accumulation (diet ratio about 1:2) takes place in the bile of hens (9). At first glance, because of its easy accessibility, urine of sows could serve as a simple alternative sample to trace ZEN exposure of pigs if the corresponding feed is no longer available for analysis. However, there is no reliable chance to consider urine or bile to trace a contamination and to do a risk assessment due to the fact that rapid metabolism and elimination of the toxin and high individual variability are the main obstacles.

β-ZEL was found in neither urine nor bile. Also, only traces of this ZEN metabolite were detected in the liver of turkeys despite a high dose of 800 mg ZEN/kg feed (Olsen et al., 1986). In the bile and urine of sows, ZEN and its metabolite α-ZEL are nearly exclusively conjugated to glucuronic acid or sulfuric acid. The percentage of the free, not conjugated ZEN and α-ZEL is minimal: 1 to 6 % for bile and urine of sows, respectively. In contrast, the proportions of free ZEN and α-ZEL in bile and livers of hens were approximately 70 % and 40 %, respectively (Dänicke et al., 2002). The proportion of glucuronic acid conjugates of ZEN in bile as well as in urine of sows is >95 % and no sulfuric acid conjugates were present. Also in bile of hens, the conjugation to glucuronic acid is the preferred binding form of ZEN (20 %), whereas 10 % were conjugated to sulfuric acid (Dänicke et al., 2002). Therefore, in contrast to glucuronic acid, sulfuric acid does not react or only reacts to a lower extent with the phenolic OH-groups of ZEN. The proportions of sulfuric and glucuronic acid conjugates of α-ZEL in bile of sows are 17 and 82 %. In urine the sulfated form is predominant, with 62 % and about half of that is the proportion of α-ZEL bound to glucuronic acid. For hens, α-ZEL in bile is mainly bound with sulfate (27 %) and only 4 % are conjugated with glucuronic acid. To a lower extent chemical reactivity in the respective matrix, but mainly due to different animal species, the enzymatic specificity and elimination kinetics may explain that the epimeric OH group of α-ZEL is the favoured reaction target for sulfuric acid in urine. This contradicts to the expected 0.67 to 0.33 ratio of phenolic to epimeric hydroxyl groups in α-ZEL, based just on chemical reactivity and molar concentration only. Conjugation of the mycotoxin with glucuronic and sulfuric acid increases the polarity of the molecule and renders it more suitable for biliary renal excretion, which is important

for the elimination of toxic substances in sensitive animals as like sows and pigs (European Food Safety Authority, 2004). The higher proportions of conjugated ZEN and α-ZEL in bile of sows in comparison to the lower conjugation proportion of the more ZEN insensitive hens are remarkable. Further experiments with other species of animals should prove whether a relationship exists between sensitivity for toxins and the capability to form conjugates, with a possible altered toxicity.

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