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Concentration Levels of Zearalenone and Its Metabolites in Urine, Muscle Tissue, and Liver Samples of Pigs Fed with Mycotoxin-Contaminated Oats

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The content of zearalenone and its metabolites in urine and tissue samples from pigs fed zearalenone-contaminated oats was established by analytical methods combining solid-phase extraction cleanup of the samples with highly selective liquid chromatography-mass spectrometry (LC-MS)/MS detection. Investigation of the urine samples revealed that approximately 60% of zearalenone was transformed in vivo to α-zearalenol and its epimer β-zearalenol in a mean ratio of 3:1. Zeranol and taleranol as further metabolites could only be detected in trace amounts. Zearalanone was identified at considerable concentrations, though only in a couple of samples. In contrast, liver samples contained predominantly α-zearalenol, and to a minor extent β-zearalenol and zearalenone, with a mean ratio of α-/β-zearalenol of 2.5:1, while zeranol, taleranol, or zearalanone could not be identified in any of the investigated samples. The degree of glucoronidation was established for zearalenone as 27% in urine and 62% in liver; for α-zearalenol as 88% in urine and 77% in liver; and for β-zearalenol as 94% in urine and 29% in liver. Analyses of muscle tissue revealed relatively high amounts of nonglucuronidated zeranol and α-zearalenol together with traces of taleranol and zearalenone, indicating that the metabolism of zearalenone and its metabolites is not restricted to hepatic and gastrointestinal metabolic pathways.

KEYWORDS: Zearalenone; metabolism; zeranol; zearalanol; zearalenol; zearalanone; pigs; feeding study

INTRODUCTION

Zearalenone (**Figure 1**) is a nonsteroidal estrogenic mycotoxin produced by *Fusarium* species, which colonizes several grains including maize, oat, barley, wheat, and sorghum (*1*, *2*). Despite its low acute toxicity and carcinogenicity (*3*−*5*), zearalenone exhibits, due to its agonistic effect on the estrogen receptor (*1*, *6*), distinct estrogenic and anabolic properties in several animal species with pigs the most sensitive, resulting in severe effects on the reproductive system (*3*, *7*−*9*). To avoid any of these symptoms of hyperestrogenism, a guideline level of 200 μg/kg zearalenone in feed has recently been proposed (*10*). Scientific data about the effects of zearalenone in humans are limited to a few investigations, which are mainly based on a small number of individuals (*11*). In two cases, zearalenone was suspected to be the causative agent in epidemics of premature thelarche in children (*12*, *13*). Though zearalenone was unambiguously detected in serum and plasma samples, it was not possible to obtain clear scientific evidence for this assumption due to the incompleteness of the available data. Nonetheless, unambiguousness of the results of numerous animal studies concerning zearalenone toxicity and its estrogenic effects led to the establishment of tolerance levels throughout the world ranging between 30 and 1000 μg/kg in grains.

Zeranol (α-zearalanol, **Figure 1**) has been widely adopted as a growth stimulant in the U.S.A. since 1969 to improve fattening rates of cattle. Its use has been banned in the European Union since 1985 (*14*) together with a variety of other growth hormones, such as 17β-estradiol, progesterone, testosterone, trenbolone acetate, and melengestrol acetate (*15*). This also includes a ban on imported meat and meat products derived from cattle given these hormones other than for veterinary reasons. The EU legislation relies on the opinion that especially long-term health effects for humans of eating beef products produced using growth hormons are particularly uncertain, since only a few long-term investigations have been performed with

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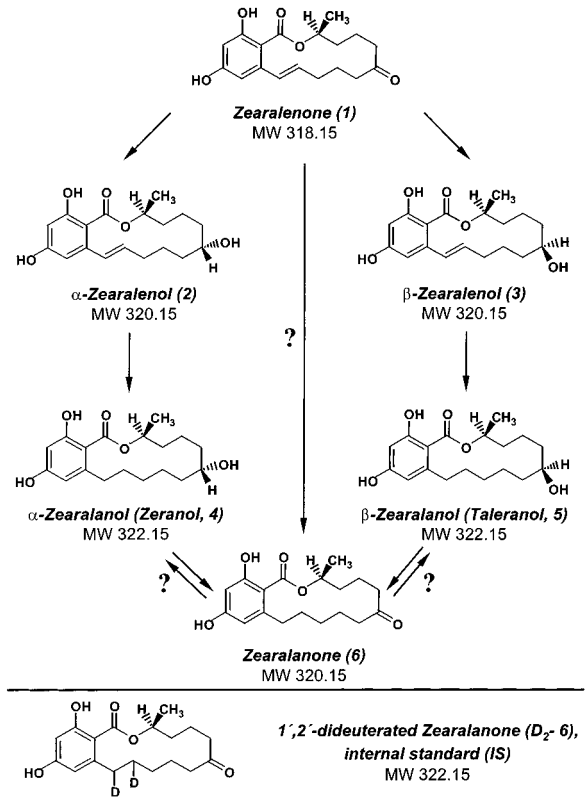

Figure 1. Zearalenone and its major in vivo metabolites in pigs and cattle (conjugated forms are not depicted).

rats, dogs, and monkeys. These studies indicate that zeranol is a weak estrogen, predominantly causing changes in mammary glands and organs of the reproductive system (*16*). Furthermore, results of investigations concerning the mutagenic, teratogenic, and cancerogenic properties of zeranol conflict (*16, 17*). In view of these scientific data, but in contrast to the European Commission's point of view, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) proposed in 1987 a maximum acceptable intake of 0.5 μg/kg body weight corresponding to maximum residue levels in liver of 10 μg/kg and in muscle of 2 μg/kg, which are based on the no hormonal effect level of zeranol (*16*). Recently, the Food and Drug Administration (FDA) established safe concentration levels for total zeranol residues in uncooked edible tissues of cattle as high as 150 μg/kg in muscle, 300 μg/kg in liver, 450 μg/kg in kidney, and 600 μg/kg in fat (*18*).

The in vivo metabolism of zearalenone has been investigated in several animal species and in humans. It has been shown that zearalenone is predominantly transformed into α- and β-zearalenol (**Figure 1**) (*1, 7, 19*). Concentration ratios of the metabolites, the parent compound, and their respective glucuronides vary strikingly with the animal species, e.g., a significant fraction of zearalenone was found in pig to be in the form of α-zearalenol (*7, 20*), while cows predominatly metabolize zearalenone to β-zearalenol (*21*). More recently, it was demonstrated that a further reduction of α-zearalenol and β-zearalenol may occur in deer, goats, sheep, cattle, and horses resulting in partly significant concentrations of zeranol (α-zearalanol, **Figure 1**) and taleranol (β-zearalanol, **Figure 1**) in urine (*22*). Further evidence for the natural formation of the growth promoter zeranol from the mycotoxin zearalenone was achieved when considerable concentrations were found in bile and urine of sheep and cattle that were most probably not treated with zeranol (*23*−*25*).

As shown in several studies, zearalenone and its metabolites are excreted mainly via feces and urine (*1, 26*). In contrast to zeranol, few attempts have been made to detect zearalenone and its metabolites in animal tissues or to determine respective residue rates (*1, 27*). In view of the above-mentioned EU regulations and the hormone conflict between the EU and the U.S.A., we performed an animal feeding study with pigs that are known to be very sensitive to the estrogenic properties of this mycotoxin. The purpose of this study was to investigate in detail the metabolic profile of zearalenone in this species and to clarify whether zeranol and/or zearalanone (**Figure 1**) are also formed from zearalenone in pigs, which to our knowledge has not been described before. A further focus of this study was to investigate whether zearalenone and metabolites, especially zeranol, are incorporated into animal tissues that are intended to be used for human nutrition purposes. Within this study, analyte identification and quantification was mainly based on a previously published liquid chromatography (LC)-tandem mass spectrometric (MS/MS) method, which combines high sensitivity of analyte detection (ppt to low ppb range) with an unequivocal analyte identification (*21*).

MATERIALS AND METHODS

Chemicals and Solvents. α-Zearalanol (zeranol), β-zearalanol (taleranol), α-zearalenol, β-zearalenol, zearalenone, and zearalanone were purchased from Sigma (Deisenhofen, Germany). Methanolic stock solutions with concentrations of 1 and 10 μg/mL were stored at 4 °C under exclusion of light. Hyflo Super Cel and *meta*-phosphoric acid (~65%) were provided from Fluka (Buchs, Switzerland). Ammonium acetate (p.a.), deuterated ethanol, platinum(IV) oxide, glucuronidase/arylsulfatase from *Helix pomatia* (30/60 U/mL), and high-performance liquid chromatography (HPLC) grade methanol and HPLC grade acetonitrile were purchased from Merck (Darmstadt, Germany). HPLC water was prepared by an Elgastat water purification system (Bucks, U.K.). RP-18 solid-phase extraction (SPE) columns with 100 mg of adsorbent and 1 mL of reservoir volume were obtained from Phenomenex (Torrence, CA). Oasis HLB SPE columns with 60 mg of adsorbent and 3 mL of reservoir volume were from Waters (Milford, MA).

The internal standard (IS), 1′,2′-dideuterated zearalanone (D_2-zearalanone), was synthesized by catalytic deuteration of zearalenone; compound purity (>99.5%), deuterium content (two deuterium atoms per molecule), and structure were established by electrospray mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. In brief, 5 mg of platinum(IV) oxide was suspended in 2 mL of deuterated ethanol. Three times, this solution was frozen with liquid nitrogen and degassed under vacuum for 10 min. A solution of 5 mg of zearalenone in 1 mL of deuterated ethanol was added, and the reaction mixture was stirred for 3 h under a deuterium atmosphere (1 atm) at 0 °C. The solution was then filtered, and the solvent was removed. The residue was dissolved in ethyl acetate and further purified with a silica column eluted with ethyl acetate. After the solvent was removed, the product was dried in a vacuum. To make sure that no phenolic hydrogen was replaced by deuterium, the final product was dissolved in 2 mL of ethanol with one drop of acetic acid and stirred overnight at room temperature. Finally, the solvent was removed, and the solid residue was dried in high vacuum. A methanolic stock solution of D_2-zearalanone with a concentration of 934 μg/L was stored at 4 °C under exclusion of light.

^{1}H NMR (400.13 MHz, $CDCl_3$): δ 1.34 (d, J = 6.1 Hz, 3H), 1.20−2.20 (m, 11H), 2.64 (ddd, J = 3.0, 5.8, 11.6 Hz, 1H), 2.92 (ddd, J = 2.5, 13.1, 16.2 Hz, 1H), 3.09 (d, J = 2.8 Hz, 1H), 5.17 (m, 1H), 5.62 (broad s, 1H), 6.191 and 6.194 (2d, J = 2.8 Hz, 1H), 6.27 (d, J = 2.8 Hz, 1H), 12.05 (s, 1H). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ 21.26, 22.50, 22.50, 22.64, 27.27, 31.04, 31.15, 35.02, 36.64, 43.98, 72.71, 101.63, 104.99, 105.01, 110.80, 110.85, 148.69, 160.44, 165.91, 171.50, 212.60.

Instrumentation (*21*). LC-MS/MS analyses were performed on a PE Sciex API 365 LC-MS/MS system (Perkin-Elmer Sciex Instruments, Thornhill, Ontario, Canada) equipped with an atmospheric pressure

Table 1. Method Validation Data for the Determination of Zearalenone and Its Metabolites in Pig Liver[a]

	zearalenone	α-zearalenol	β-zearalenol	zeranol	taleranol	zearalanone
recovery at 5 μg/kg (%)	80	74	68	85	55	75
standard deviation (%), $n = 3$	2	2	3	10	9	4
limit of detection (μg/kg)	0.1	0.1	0.3	0.1	1	0.1
limit of quantification (μg/kg)	0.5	0.5	1.0	0.5	3	0.5
linear range (μg/kg)	1–100	1–100	1–100	1–100	3–100	1–100

[a] Limit of detection ≡ 3 × baseline noise; limit of quantification ≡ 10 × baseline noise.

chemical ionization interface (APCI) (Perkin-Elmer Sciex Instruments) and a HP1100 HPLC system from Hewlett-Packard (Waldbronn, Germany). Chromatographic separation was achieved on a 125 mm × 3 mm i.d. Superspher 100 RP-18e column (Merck) at 35 °C using acetonitrile/methanol/water (10:45:45, v/v/v) with a concentration of 15 mM ammonium acetate. The flow rate was set at 0.5 mL/min.

The APCI interface was used in the negative ion mode at 400 °C with a needle current of 4 μA. For multiple reaction monitoring, the deprotonated molecular species of zeranol, taleranol, and D_2-zearalanone (*m*/*z* 321.15), of α/β-zearalenol and zearalanone (*m*/*z* 319.15), and of zearalenone (*m*/*z* 317.15) were selected as precursor ions. Ions at *m*/*z* 277.1/303.1 (zeranol/taleranol), *m*/*z* 207.1 (D_2-zearalanone), *m*/*z* 160.1/174.1 (α/β-zearalenol), *m*/*z* 205.1/275.1 (zearalanone), and *m*/*z* 131.1/175.1 (zearalenone) were selected as product ions. The collisional energy was adjusted by variation of the voltage between the entrance quadrupole (Q0) and the collisional cell quadrupole (RO2). A value of 30 eV gave the highest sensitivity for all analytes. Nitrogen was used as the collisional gas.

Sample Preparation. Mixed methanolic standard solutions of all analytes and of the IS ranging from 10 μg L^{-1} to 10 mg L^{-1} were prepared and stored at 4 °C under exclusion of light. To establish calibration curves, mycotoxin free urine, liver, and muscle samples were spiked with different concentrations of the analytes and the IS D_2-zearalanone. Sample preparation of muscle tissue and urine samples was performed according to a previously described method (*21*) while analyses of liver samples were based on a method of Horie and Nakazawa (*28*) that was significantly modified in our laboratory.

Urine Analysis According to Jodlbauer et al. (*21*). A 5 mL amount of pig urine was mixed with 25 μL of a D_2-zearalanone stock solution (934 μg/L; final concentration: 4.7 μg/L) and 25 mL of ammonium acetate buffer (0.05 M, pH 4.8). This solution was treated for 15 h at 37 °C with 150 μL of glucuronidase/arylsulfatase solution to cleave the respective conjugates. Afterward, this solution was adjusted with acetic acid to pH 4.0 and 10 mL was applied to a RP-18 SPE column, which was preconditioned with 5 mL of methanol followed by 5 mL of water. After the sample was loaded, the SPE column was washed with 2 mL of water and dried for 3 min by applying vacuum. Elution of analytes was carried out with 1.25 mL of methanol. The eluate was evaporated to dryness under a stream of nitrogen, and the residue was redissolved in 250 μL of the HPLC mobile phase. A 50 μL amount of this solution was injected into the LC-MS/MS system. Samples were centrifuged prior to LC-MS analysis.

Limit of detection (3 × baseline noise), 0.1−0.5 μg/kg; limit of quantification (10 × baseline noise), 0.5−1.0 μg/kg; linear range, 0.5/1−300 μg/kg; recovery, 94−105%; standard deviation ($n = 6$), 1.6−8.0%.

Liver Analysis According to a Modified Method of Horie and Nakazawa (*28*). A 5 g amount of liver and 25 μL of D_2-zearalanone stock solution (934 μg/L, final concentration: 4.7 μg/kg) were homogenized at 16 000 rpm for 90 s with 100 mL of 0.2% *meta*-phosphoric acid−methanol (4:6, v/v) using an Ultra Turrax T25 (IKA Labortechnik, Staufen, Germany). After the addition of 2 g of Hyflo Super Cel, the extraction solution was filtered and washed twice with 10 mL of methanol. The filtrate was evaporated under reduced pressure at 40 °C. The remaining solution (~10 mL) was adjusted with ammonium acetate buffer (0.05 M) to pH 4.8 and hydrolyzed for 15 h at 37 °C with 150 μL of glucuronidase/arylsulfatase solution. Afterward, the enzymatic digest was adjusted with acetic acid to pH 4.0 and applied to an Oasis HLB column, which was preconditioned with 5 mL of methanol and 5 mL of water. After the whole sample was loaded, the SPE column was washed with 5 mL of acetonitrile−water−acetic acid (20:77:3, v/v/v, pH 3.0). Elution was carried out with 5 mL of ethyl acetate. The eluate was evaporated to dryness under a stream of nitrogen, and the residue was redissolved in 500 μL of the HPLC mobile phase. After ultrasonication, 50 μL was injected into the LC-MS/MS system.

Table 2. Zearalenone, α-Zearalenol, and β-Zearalenol Concentrations in Liver Samples of Each Pig (Experimental Group Fed with Zearalenone-Contaminated Oats)[a]

pigs of the experimental group	zearalenone (μg/kg)	α-zearalenol (μg/kg)	β-zearalenol (μg/kg)
1	1.9	9.9	3.9
2	traces[b]	3.6	1.9
3	1.2	5.4	2.8
4	1.1	6.4	2.7
5	1.1	5.4	2.8
6	2.1	8.6	3.3
7	3.1	12.0	4.8

[a] Zeranol, taleranol, and zearalanone were not detected in any of the investigated liver samples above the limits of detection. [b] Traces ≡ concentrations between 0.1 (limit of detection) and 1.0 μg/kg.

Limit of detection (3 × baseline noise), 0.1−1.0 μg/kg; limit of quantification (10 × baseline noise), 0.5−3.0 μg/kg; linear range, 3−100 μg/kg (taleranol), 1−100 μg/kg (all other analytes); recovery, 55−85%; standard deviation ($n = 3$), 2.0−9.8% (see also **Table 1**).

Muscle Tissue According to Jodlbauer et al. (*21*). A 10 g amount of muscle tissue and 250 μL of D_2-zearalanone stock solution (934 μg/L; final concentration: 23.4 μg/kg) were homogenized at 24 000 rpm for 90 s with 100 mL of methanol using an Ultra Turrax T25 (IKA Labortechnik). After filtration through a filter paper 595 (Schleicher & Schuell, Dassel, Germany), 10 mL of the extract was diluted with 90 mL of water and adjusted to pH 4.0 with acetic acid. A 20 mL amount of this solution was applied to a RP-18 SPE column, which was conditioned with 5 mL of methanol followed by 5 mL of water. All of the following steps were carried out as described above for the cleanup of urine samples.

Limit of detection (3 × baseline noise), 0.5 μg/kg; limit of quantification (10 × baseline noise), 1.0 μg/kg; linear range, 1−100 μg/kg; recovery, 86−91%; standard deviation ($n = 6$), 3.7−5.6%.

Animal Feeding Study. The feeding study was conducted at the Federal Office for Animal Diseases (Mödling, Austria) under the supervision of T. Kuhn and W. Hochsteiner. The oats, either "mycotoxin free" or naturally contaminated with *Fusarium* toxins, were thoroughly homogenized, and several samples were taken and analyzed by the II. Medical Clinic for Ruminants and Swines of the Veterinary University of Vienna (Vienna, Austria) and by Biomin GmbH (Herzogenburg, Austria): 1370 μg/kg zearalenone, 267 μg/kg nivalenol, 2230 μg/kg 3/15-acetyldesoxynivalenol, and 23 400 μg/kg desoxynivalenol as the mean concentration values of three samples were found in the contaminated samples, and 79 μg/kg zearalenone, 358 μg/kg nivalenol, and 278 μg/kg desoxynivalenol as the mean concentration values of three samples were found in the so-called "noncontaminated" oats. No other mycotoxins were present in significant concentrations in the samples.

Fourteen female pigs (hybrids of *Deutsches Edelschwein* and *Pietrain*), known to be free from feeding with zearalenone-contaminated feed stuff, at the age of 3 months and a mean body weight of 58.6 kg

were randomly divided into two separate groups, each with seven animals, and housed on straw and fed with noncontaminated oats (daily zearalenone intake of both groups: 60 μg per animal) along with swine fodder and water. Both groups were allowed to familiarize with their new surroundings and the unusual feed (oats) for 6 days before commencing the study.

During the animal feeding study (18 consecutive days), one group of animals was fed with contaminated oats (experimental group) while the control group received the same amount of "blank" oats (daily zearalenone intake of control group: 60 μg per animal). The daily intake of 0.8 kg of zearalenone-contaminated oats of the experimental group (1.1 mg of zearalenone intake per animal per day) was diluted (mixed) with the same amount of mycotoxin free oats (0.8 kg) after 2 days, since the animals refused the feed, presumably due to the high deoxynivelanol content (23 400 μg/kg). Accordingly, the animals of the control group received additionally 0.8 kg of mycotoxin free oats. After 18 days of controlled feeding, all 14 animals, with a mean increase of weight of 10.5 kg (experimental group) and 5.4 kg (control group), were slaughtered and tissue samples (back, femoral region, and liver) were collected during postmortem examinations. Except on the weekend, urine samples were collected daily over the whole period of 24 days in the early morning before feeding; however, it proved impossible to aquire samples from all pigs on every day. All tissue and urine samples were stored at −20 °C until analysis.

RESULTS AND DISCUSSION

All 14 pigs were selected from a herd that was under permanent veterinary supervision of the Medical Clinic for Ruminants and Swines of the Veterinary University of Vienna. Urine samples of the pigs were investigated for zearalenone and its metabolites to ensure that they were mycotoxin free (concentrations in urine below 1.5 μg/L) at the beginning of the controlled study. It proved to be impossible to aquire a complete set of urine samples from all pigs over the whole period of the study, although female pigs were selected without exception to simplify collecting of urine samples. Nevertheless, a minimum mean number of three samples per day could be obtained for the experimental as well as the control group. After 2 days of feeding the highly contaminated oats, it was necessary to dilute the ration with mycotoxin free oats, since the animals of the experimental group refused the mycotoxin-contaminated feed, presumably due to its additional high desoxynivelanol content (23 400 μg/kg). This measure proved to be sufficient to raise the fodder and daily intake per animal to the original level of 1.1 mg of zearalenone and to run the study without further problems. The mean increase of weight was established close to slaughtering with 10.5 kg for the experimental group and 5.4 kg for the control group, giving clear evidence for the anabolic effect of zearalenone and its metabolites. Furthermore, histological investigations after slaughtering revealed in animals of the experimental but not of the control group significant changes of the genital tract, caused by the estrogenic effects of zearalenone (hyperestrogenism). A detailed survey of the veterinary and histological data of this study will be published elsewhere.

In contrast to previous studies (*7*, *29*−*31*) with extremely high zearalenone concentrations in feed between 15 and 40 mg/kg, the present study deals with distinctly lower zearalenone concentrations making it more appropriate to monitor the situation in pig fattening for two reasons.

(A) Natural zearalenone concentrations of 10 mg/kg and more are not likely to occur in feed, especially under Austrian field conditions. If present, they very often lead to feed refusal due to high concentrations of other accompanying mycotoxins, such as desoxynivalenol. This has also been observed in this study even at distinctly lower zearalenone concentrations.

(B) It has repeatedly been reported that symptoms of hyperestrogenism occur in several animal species, but especially in pigs, even at concentration levels below the 1 mg/kg level (*4*, *5*, *32*). Because hyperestrogenism also decreases the reproductive rates in swine, highly mycotoxin-contaminated feed will for economic reasons be either avoided or diluted with mycotoxin free stuff to reach more appropriate zearalenone concentrations.

Urine Samples. Because low amounts of zearalenone were also present in the mycotoxin free oats (79 μg/kg), some pig urine samples of the control group already contained low amounts of zearalenone (<1.5 μg/L) and traces of α-zearalenol (<0.5 μg/L). Other metabolites could not be detected.

The investigation of the urine samples of the experimental group revealed that approximately 60% of zearalenone was transformed in vivo to α-zearalenol (50−200 μg/L) and to its epimer β-zearalenol (15−60 μg/L) in a mean ratio of 3:1, while zeranol and taleranol as further metabolites could only be detected in trace amounts of less than 1 μg/L. Zearalanone was unambiguously identified at considerable concentrations (20−70 μg/L), though only in a few samples. Zearalanone has not previously been reported as a metabolite of zearalenone. However, the reason for its rare occurrence in only some of the samples remains unclear. The degree of glucuronidation of the analytes in urine was established by parallel investigations of contaminated urine samples with and without enzymatic (glucoronidase) digest, as 27% glucuronidation for zearalenone, 88% glucuronidation for α-zearalenol, and 94% glucuronidation for β-zearalenol. These results are in agreement with previous studies (*19*). A typical LC-MS chromatogram of a urine sample is depicted in **Figure 2A** with extracted ion chromatograms of zeranol/taleranol (**Figure 2B**) and of zearalanone (**Figure 2C**). Peaks marked with asterisks in **Figure 2B**,**C** are derived from α/β-zearalenol and zearalenone since all three analytes also influence the fragmentation pathways of zeranol, taleranol, and zearalanone.

The time−concentration course of zearalenone and the two major metabolites α- and β-zearalenol is an important and accurate marker for the overall progress of the study, since urine levels were found to increase and decrease very rapidly, dependent on the daily intake of zearalenone. Depending on the day, each point is the mean concentration value of a minimum of three and a maximum of seven animals of the experimental group (**Figure 3A**). The beginning of feeding mycotoxin-contaminated oats is clearly indicated by an immediate increase of the concentrations of all three analytes (days 0 and 1) followed by a distinct decrease of the concentration values almost to the starting level of the days −6 to 0, due to the refusal of oats feeding rations by all seven pigs of the experimental group (days 2 and 3). After dilution of the mycotoxin-contaminated oats ration fodder intake and consequently the zearalenone, α- and β-zearalenol concentration levels immediately rose again to 50 (β-zearalenol), 180 (α-zearalenol), and 275 μg/L (zearalenone), the highest levels found during the whole study (day 4). Surprisingly, concentration levels of all three analytes decreased continuously from that day until slaughtering of the animals (around and below 50 μg/L) although the feed intake was controlled and kept constant. A possible explanation for this unexpected concentration course might be the observation that the pigs increasingly ate straw (days 5−17) resulting in a kind of natural mycotoxin decontamination process by a possibly strong adsorption of zearalenone on the straw matrix, which consequently decreased the gastrointestinal resorption of zearelenone in the animal body. However, further

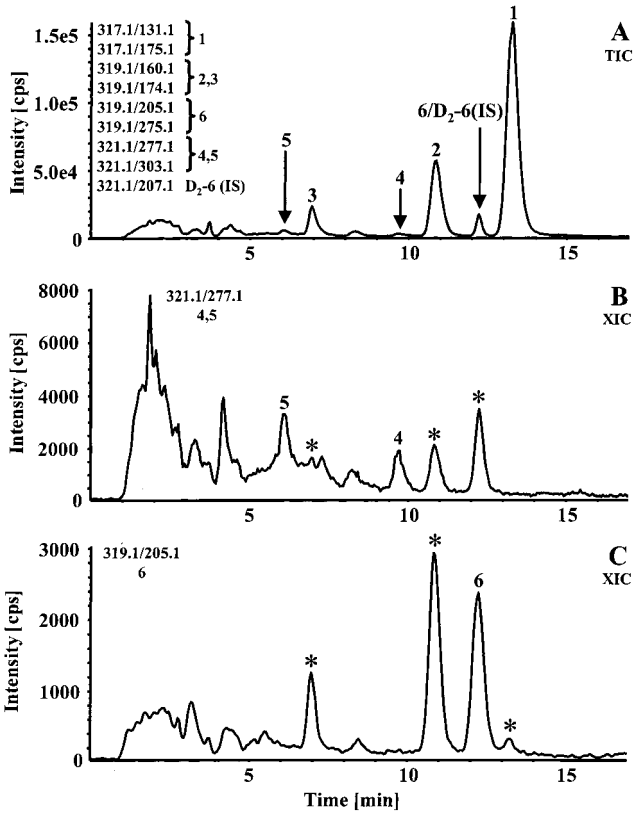

Figure 2. LC-MS/MS chromatogram of a pig urine sample of the experimental group contaminated with 155.4 μg/L of zearalenone (**1**), 68.7 μg/L of α-zearalenol (**2**), 23.8 μg/L of β-zearalenol (**3**), 53.1 μg/L of zearalanone (**6**), and traces of zeranol (**4**) and taleranol (**5**) (<1 μg/L) and spiked with 4.7 μg/L of D_2-zearalanone (IS) (D_2-6). (**A**) Total ion chromatogram. (**B**) Extracted ion chromatogram of zeranol and taleranol: 321.1 ⟹ 277.1. Peaks derived from zearalenols and D_2-zearalanone (IS) are marked with asterisks. (**C**) Extracted ion chromatogram of zearalanone: 319.1 ⟹ 275.1. Peaks derived from zearalenols and zearalenone are marked with asterisks.

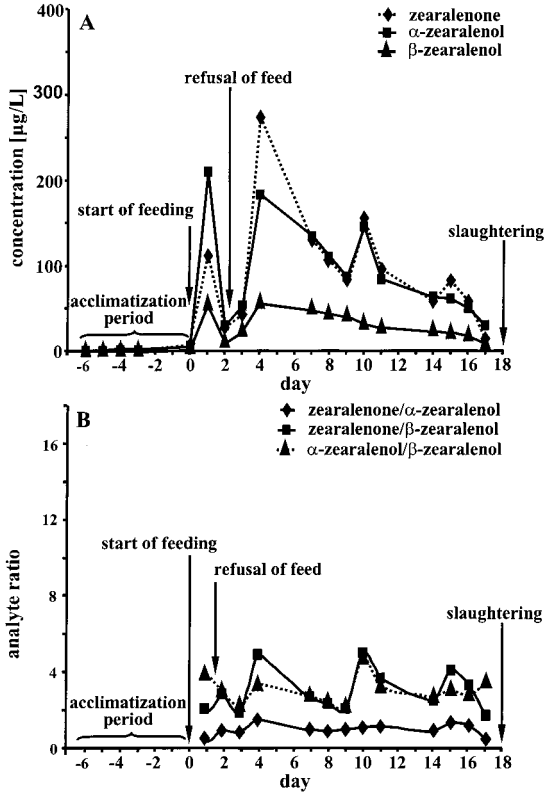

Figure 3. (**A**) Time course of zearalenone, α-zearalenol, and β-zearalenol concentrations in urine. Mean value of 3–7 animals fed with zearalenone-contaminated oats (experimental group). (**B**) Time course of analyte ratios.

detailed investigations of feces in the context of a feeding study would be necessary to give further evidence for or against this assumption.

However, the analyte ratios of zearalenone, α-zearalenol, and β-zearalenol were more or less constant during the whole feeding study (days 1−17) with an average ratio for zearalenone/α-zearalenol of 1:1, for zearalenone/β-zearalenol of 3:1, and for α/β-zearalenol of 3:1 (**Figure 3B**). These values are in agreement with the literature (*33*). The analyte ratios were not influenced by the feed refusal on days 2 and 3. Before the beginning of the feeding study (days −6 to 0) α- and β-zearalenol concentrations were at the low levels of the control group, below the quantification limits (α- and β-zearalenol < 0.5 μg/L; zearalenone < 1.5 μg/L). Thus, calculation of analyte ratios was not possible for this period of the study.

Liver Samples. The analysis of liver samples was mainly based on a sample preparation method dedicated to the analysis of zeranol in bovine liver (*28*). The method was modified according to the requirements of an effective multianalyte detection method in the very complex pig liver matrix. For this purpose, an enzymatic cleavage step of the conjugates with glururonidase/sulfatase was inserted, since preliminary experiments with naturally contaminated pig liver samples revealed that the major part of the analytes that are incorporated in the liver are bound to glucuronic acid (zearalenone, 62%; α-zearalenol, 77%; and β-zearalenol, 29%). Also, a large loss of analytes (recoveries: 35−60%), especially of β-zearalenol and taleranol (recoveries: 35−40%), was observed during the washing step of the SPE procedure. To inhibit partial deprotonation of the analytes, which may be the reason for these losses, 3% acetic acid was added to the washing solvent (pH 3) resulting in a significant increase of recoveries to 55% for taleranol and 85% for zeranol. Furthermore, acetonitrile was replaced by ethyl acetate as elution solvent, to enable a more complete elution of all analytes from the SPE columns. For final LC-MS/MS detection, the method used for urine and muscle tissue samples was also applied for the analysis of liver samples.

As depicted in **Figure 4A** for liver sample spiked with all analytes after sample cleanup, all analytes can be separated from each other and from matrix compounds. High abundant matrix compounds, especially at the beginning of LC-MS chromatograms, reflect the low selectivity and efficiency of SPE sample cleanup, which is, however, compensated for by the selective and efficient tandem MS detection, enabling unambiguous analyte identification and quantification. Nevertheless, it is apparent from a comparison of the LC-MS chromatograms of mixtures containing each analyte with a final concentration of 50 μg/kg, with and without the liver matrix (**Figure 4A**,**B**), that the ionization efficiency especially of taleranol and β-zearalenol is negatively influenced by coeluting matrix compounds, resulting in a distinct decrease of taleranol and β-zearalenol peak intensities relative to all other analytes. These observations along with a number of previous other reports (*34*−*37*) support the necessity in LC-MS analysis of complex biological matrixes, to always establish calibration curves in the presence of the sample matrix and not from (simple) aqueous standard solutions. Furthermore, it is recommended that appropriate IS protocols, either for the whole method or just for the MS detection, should be set up to compensate for these ion suppression phenonema

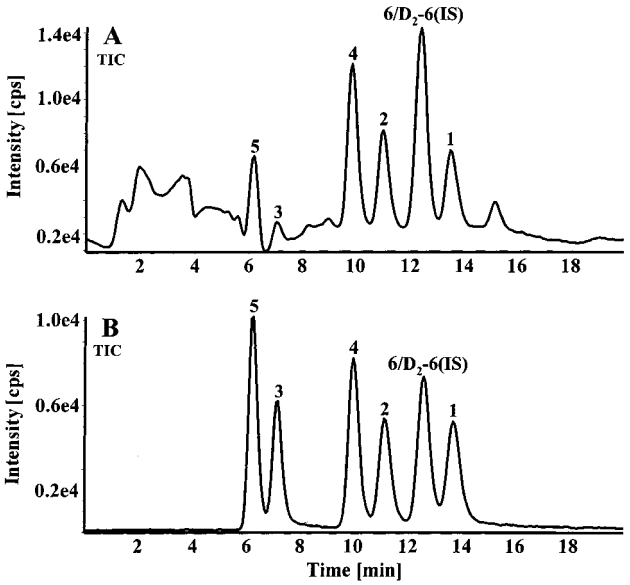

Figure 4. (**A**) LC-MS/MS chromatogram of a blank pig liver sample spiked with 5 μg/kg of each analyte. Final concentration in the injection solution: 50 μg/L of each analyte. (**B**) LC-MS/MS chromatogram of a matrix free standard solution of a mixture of 50 μg/L of each analyte.

(matrix effects) that distinctly decreased overall method sensitivity for β-zearalenol and taleranol (limit of detection: 0.3 and 1 μg/kg) as compared to the other analytes (limit of detection: 0.1 μg/kg). Summarized data are given in **Table 1**.

In all liver samples of the experimental group, α-zearalenol (3.6−12.0 μg/kg), along with smaller amounts of β-zearalenol (1.9−4.8 μg/kg) and zearalenone (<1.0−3.1 μg/kg), could be identified with analyte concentrations that were distinctly lower than those observed in urine samples (**Table 2**). Neither zeranol and taleranol nor zearalanone were present in any of the investigated samples, which is somewhat surprising as these metabolites could be detected in urine samples. Parallel analysis of outer and inner parts of a liver revealed that zearalenone, α-zearalenol, and β-zearalenol seem to be homogeneously distributed in this organ. Liver samples of the control group that had been fed with "zearalenone free" oats, were free of any of the analytes indicating that a higher daily intake of zearalenone is necessary to effect incorporation of this mycotoxin and its major metabolites in liver tissues. Analyte ratios of α/β-zearalenol were found to be comparable to values found in urine samples (1.90−2.62), while zearalenone/α-zearalenol (0.17−0.25) and zearalenone/β-zearalenol ratios (0.39−0.64) were distinctly lower. A typical LC-MS chromatogram of a liver sample of the experimental group is given in **Figure 5A** with extracted ion chromatograms of α/β-zearalenol (**Figure 5B**) and of zearalenone (**Figure 5C**).

Muscle Tissue Samples. In all muscle tissue samples, zeranol was detected with concentrations of up to 13.3 μg/kg along with α-zearalenol (up to 14.5 μg/kg) and traces of zearalenone and taleranol. β-Zearalenol and zearalanone could not be identifed in any of the investigated muscle samples. No indication of the incorporation of respective analyte glucuronides could be established, since a parallel experiment with an enzymatic deglucuronidation digest did not increase the concentration values found in the muscle tissues. The occurrence of the individual analytes was strikingly depedent on the type of muscle, since samples taken from the femoral region contained exclusively zeranol at low concentrations (0.5−2.1 μg/kg), while samples from the back zeranol (0.5−13.3 μg/kg) were also

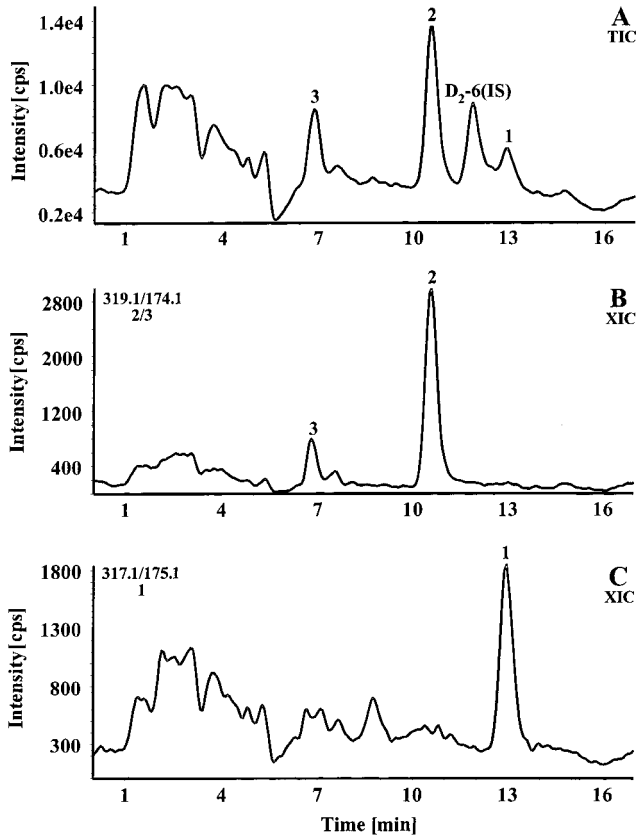

Figure 5. LC-MS/MS chromatogram of a pig liver sample of the experimental group contaminated with 2.1 μg/kg of zearalenone (**1**), 8.6 μg/kg of α-zearalenol (**2**), and 3.3 μg/kg of β-zearalenol (**3**) and spiked with 4.7 μg/kg of D_2-zearalanone (IS) (D_2-6). (**A**) Total ion chromatogram. (**B**) Extracted ion chromatogram of α-/β-zearalenol: 319.1 ⟹ 174.1. (**C**) Extracted ion chromatogram of zearalenone: 317.1 ⟹ 175.1.

accompanied by relatively high amounts of α-zearalenol (0.5−14.5 μg/kg) and occasionally traces of zearalenone and taleranol (0.5−1 μg/kg). A possible explanation for this observation might be the fact that muscle tissue from the back is better supplied with blood, which can be regarded as the most important carrier system for the analytes in the animal body. Thus, higher amounts of analytes were directly transported to this tissue and, consequently, could be more likely incorporated in this compartment.

A typical LC-MS chromatogram of the muscle tissue sample from the back with an zeranol content of 13.3 μg/kg is depicted in **Figure 6A** with extracted ion chromatograms of zeranol/taleranol (**Figure 6B**) and of α-zearalenol (**Figure 6C**). The peak marked with an asterisk in **Figure 6B** is derived from D_2-zearalanone, because the IS also influences the fragmentation pathways of zeranol and taleranol. As expected, in none of the muscle tissue samples taken from pigs of the control group that have been fed with zearalenone free oats were any of the analytes identified, giving evidence that incorporation of zearalenone metabolites is only possible at a higher daily intake of zearalenone.

Taking the concentration data of urine and liver analyses into account, where only trace amounts of zeranol could be detected, the results of muscle tissue analyses are completely unexpected, indicating that the metabolism of zearalenone and its metabolites may not be restricted to hepatic and gastrointestinal transformation pathways in the animal body. Along this line, it should be considered, that zeranol, and to a lesser extent α-zearalenol, has a pronounced estrogenic/anabolic effect and thus may bind

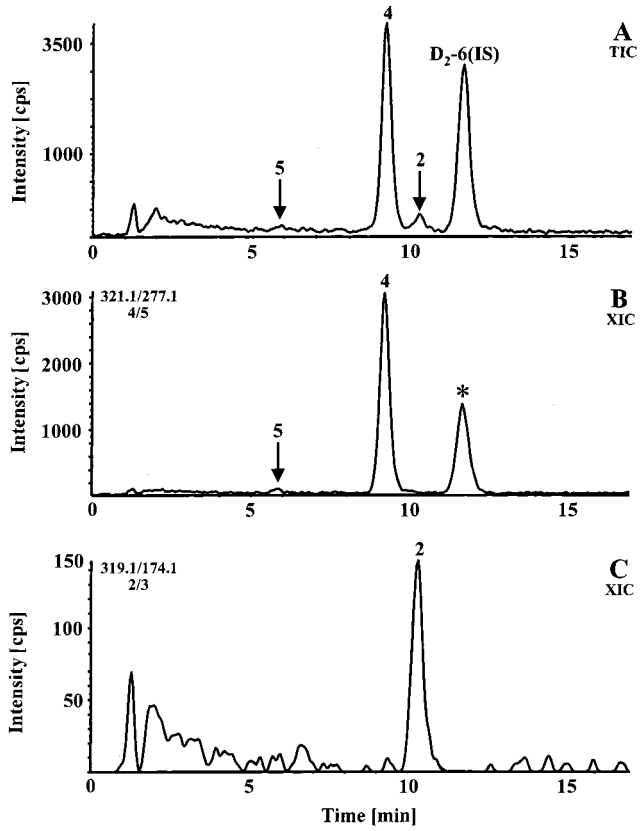

Figure 6. LC-MS/MS chromatogram of a mucle tissue sample of the back (experimental group) contaminated with 13.3 μg/kg of zeranol (**4**), 2.8 μg/kg of α-zearalenol (**2**), and traces of taleranol (**5**) (<1 μg/L) and spiked with 23.4 μg/kg D_2-zearalanone (IS) (D_2-6). (**A**) Total ion chromatogram. (**B**) Extracted ion chromatogram of zeranol and taleranol: 321.1 ⟹ 277.1. A peak derived from D_2-zearalanone (IS) is marked with an asterisk. (**C**) Extracted ion chromatogram of zearalenols: 319.1 ⟹ 174.1.

relatively strongly to possible binding sites in the muscle tissue. Consequently, the elimination rate of α-zearalenol but especially zeranol should be distinctly decreased, resulting in the accumulation of both compounds in the muscle tissues. However, further detailed investigation of respective blood samples representing the system of "drug" transport, in the context of a feeding study, would be necessary to give further evidence for or against the above-mentioned assumptions.

The present animal feeding study of pigs revealed that approximately 60% of the uptaken and gastrointestinally resorbed zearalenone is transformed into α- and β-zearalenol and to a distinctly lower degree into zeranol and taleranol. Zearalanone could be identified as a further hitherto not reported metabolite in swine. Considerable amounts of conjugated zearalenone, α-zearalenol, and β-zearalenol were found in liver and urine, while into muscle tissues only nonglucuronidated/sulfated analytes were incorporated. Taking an average daily urine volume of 3 L per animal into account, it can be roughly estimated that approximately 42% of intaken zearalenone was excreted via the urinary system either unchanged or as metabolites. Feces has been reported as a further major excretion pathway (*1*, *26*), which, however, was not analyzed in the present study. The observed decreasing analyte (zearalenone and metabolites) concentrations in urine samples toward the end of the controlled animal feeding study indicate that increasing amounts of the orally administered (fed) zearalenone may have been excreted via this pathway, since the pigs increasingly ate straw resulting in a kind of natural mycotoxin decontamination process by strong adsorption of zearalenone on the straw matrix possibly accompanied also by an enhancement of feces production.

Only minor amounts of zearalenone and its metabolites were incorporated into liver (approximately 0.07%) and muscle tissue (approximately 0.05%). In contrast to liver and urine samples, where only traces of zeranol could be identified, zeranol could be detected at considerable concentration levels in all muscle tissue samples (up to 13.3 μg/kg). This unexpected observation indicates that zearalenone and/or its metabolites may be taken up and stored in certain compartments in the course of the hepatic and gastrointestinal transformation pathways. For example, the enterohepatic system may effect a further metabolization step in the gut followed by readsorption of the respective zearalenone metabolites. Further detailed investigations of blood samples representing the common drug transport system, combined with the analysis of other organs of the digestion system, especially of bile and kidneys, would be necessary in the course of feeding studies to give further evidence for or against the above-mentioned hypothesis.

Further detailed studies are, nevertheless, necessary to get more insight into the metabolic pathways and distributions of zearalenone and its metabolites in pigs but also in other species, e.g., in cattle. Finally, it is clear from the present study that a risk assessment concerning the overall estrogenic and anabolic effect of zearalenone in food may not be restricted to zearalenone but should also include the analysis of all its possible metabolites.

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