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# Metabolic Profiles of the Mycotoxin Zearalenone and of the Growth Promoter Zeranol in Urine, Liver, and Muscle of Heifers

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A group of five heifers were fed for 84 days with 2 kg of zearalenone-contaminated oats (1370 $\mu$g/kg) resulting in an average daily intake of 2740 $\mu$g of zearalenone per animal. In a parallel experiment five heifers were implanted with two 25 mg zeranol pellets, one at the beginning of the study and one after 42 days, and fed with 2 kg of "blank" control oats (79 $\mu$g/kg, daily intake = 158 $\mu$g). A third group of five animals were also fed with 2 kg of "blank" oats and served as control. Urine samples of all animals were collected every 5−6 days during the whole period of the study. Animals of all three groups were killed 84 days after the beginning of the feeding study. Tissue samples (back, femoral region, liver, and residues of implanted pellets) were taken during post-mortem investigations. The content of zearalenone and zeranol and their metabolites in urine and tissue samples was established by an analytical method combining solid-phase extraction and high-performance liquid chromatography−tandem mass spectrometry. Urinary excretion rates of zeralenone and zeranol were calculated from these results.

**KEYWORDS: Zearalenone; zeranol; α-zearalanol; metabolism; heifer; cow; feeding study**

## INTRODUCTION

Zearalenone (**Figure 1**) is a nonsteroidal estrogenic mycotoxin produced by *Fusarium* species on several grains (*1*, *2*). Despite its low acute toxicity and carcinogenity (*3*−*5*), zearalenone exhibits, due to its agonistic effect on the estrogenic receptor (*1*, *6*), distinct estrogenic and anabolic properties in several animal species, resulting in severe effects on the reproductive system (*3*, *7*−*9*). Zearalenone contamination of food is caused either by direct contamination of grains and fruits and their products (*3*, *10*) or by "carry-over" of mycotoxins and their metabolites in animal tissues, milk, and eggs after intake of contaminated feedstuff (*10*−*12*). 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 and incomplete available data (*13*−*15*). Nevertheless, results of numerous animal studies concerning the zearalenone estrogenic effects led to the establishment of tolerance levels between 30 and 1000 $\mu$g/kg in grain throughout the world (*16*).

Zeranol (α-zearalanol, **Figure 1**), a resorcyl lactone derived from the mycotoxin zearalenone, has been widely used as a growth promoter in the United States since 1969 to improve fattening rates of cattle. Its application has been banned in the European Union (EU) since 1985 (*17*, *18*). This includes also a ban for imported meat derived from cattle given these hormones for other than veterinary reasons. Long-term investigations with rats, dogs, and monkeys indicate that zeranol is a weak estrogen, effecting predominantly changes in mammary glands and organs of the reproductive system (*19*). Furthermore, results of examinations concerning the mutagenic, teratogenic, and carcinogenic properties of zeranol are incongruous (*19*, *20*). In view of these scientific data, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) proposed maximum residue levels in liver of 10 $\mu$g/kg and in muscle of 2 $\mu$g/kg (*19*). Recently, the U.S. Food and Drug Administration (FDA) established safe concentration levels for total zeranol residues in uncooked edible tissues of cattle with values as high as 150 $\mu$g/kg in muscle, 300 $\mu$g/kg in liver, 450 $\mu$g/kg in kidney, and 600 $\mu$g/kg in fat (*21*).

The in vivo metabolism of zearalenone and zeranol has been investigated in several animal species and in humans. It has been shown that the anabolic agent zeranol is predominantly metabolized into its diastereoisomer $\beta$-zearalanol (taleranol) and to a minor extent into zearalanone (**Figure 1**) (*22*, *23*). The mycotoxin zearalenone is predominantly transformed into α- and $\beta$-zearalenol (**Figure 1**) (*1*, *7*, *24*−*26*), whereby the concentration ratios of the metabolites, the parent compound, and their respective glucuronides are strikingly dependent on the animal species. More recently, it was demonstrated that a further reduction of α- and $\beta$-zearalenol may occur in cattle and sheep, resulting in significant concentrations of zeranol and

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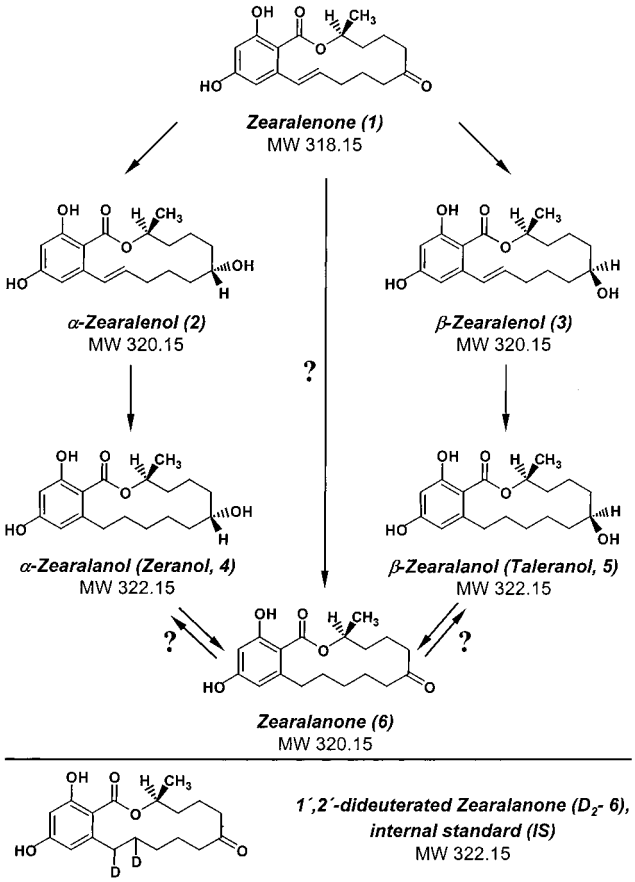


**Figure 1.** Zearalenone and its major in vivo metabolites in pigs and cattle (conjugated forms are not depicted).

taleranol (**Figure 1**) in urine (*11*, *12*, *27*, *28*). A feeding study has demonstrated that zeranol is also a zearalenone metabolite in pigs, where it could unambiguously be detected in urine and muscle tissue (*29*).

In contrast to zeranol (*22*, *23*, *29*, *30*), few attempts have been made to determine residue rates of zearalenone and of its metabolites in tissues and biological liquids of animals (*1*, *31*, *32*). In view of the above-mentioned EU regulations and the hormone conflict between the EU and the United States, which is predominantly focused on the application of zeranol in cattle fattening, and on the basis of the results of a similar experiment with pigs (*29*), we performed an animal feeding study with heifers. The purpose of this study was to establish in detail the metabolic profile of zearalenone, which is so far not well investigated in cattle, and of zeranol. A further focus was to investigate whether zeranol, zearalenone, and their metabolites are incorporated into animal tissues that are intended to be used for human nutrition purposes. The metabolism, distribution, and excretion of zearalenone in cattle were compared with the results of our previous zearalenone feeding study with pigs (*29*).

## 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. Ammonium acetate (p.a.), 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.).

The internal standard 1′,2′-dideuterated zearalanone (zearalanone-$d_2$) was synthesized as described previously by catalytic deuteration of zearalenone (*32*). Compound purity (>99.5%), deuterium content (two deuterium atoms per molecule), and structure were established by electrospray mass spectrometry and NMR spectroscopy.

**Instrumentation (*26*).** LC-MS/MS analyses were performed on a PE Sciex API 365 LC-MS/MS system (Perkin-Elmer Sciex Instruments, Thornhill, ON, Canada) equipped with an atmospheric pressure chemical ionization interface (APCI) and an 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, Darmstadt, Germany) 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 (MRM), the deprotonated molecular species of zeranol, of taleranol, and of zearalanone-$d_2$ (*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 (zearalanone-$d_2$), *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 highest sensitivity for all analytes. Nitrogen was used as collisional gas.

**Sample Preparation.** Mixed methanolic standard solutions of all analytes and of the internal standard 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 internal standard (IS) zearalanone-$d_2$. Sample preparation of muscle tissue, urine, and liver samples was performed according to previously described methods (*26*, *29*).

**Animal Feeding Study.** The feeding study approved by the Federal Ministry of Science and Transport (Vienna, Austria; GZ 68.205/20-Pr/4/2000) was conducted at the IInd Medical Clinic for Ruminants and Swine of the Veterinary University of Vienna (Vienna, Austria) under the supervision of W. Hochsteiner and H. Kahlbacher. The oats naturally contaminated with *Fusarium* toxins and the "blank" control oats were thorougly homogenized (mixed) and analyzed by the IInd Medical Clinic for Ruminants and Swine of the Veterinary University of Vienna with ELISA and by Biomin GmbH (Herzogenburg, Austria) with GC-MS: 1370 μg/kg zearalenone, 267 μg/kg nivalenol, 2230 μg/kg 3-/15-acetyldeoxynivalenol, and 23400 μg/kg deoxynivalenol were found in the contaminated samples and 79 μg/kg zearalenone, 358 μg/kg nivalenol, and 278 μg/kg deoxynivalenol were found in the blank control oats. No other typical *Fusarium* mycotoxins (fusarenone X, ochratoxin A, T-2, and HT-2 toxin) were detected in the samples. To exclude any further intake of mycotoxins, only hay and straw was used before and during the feeding experiment that offered no indication of *Fusarium* contamination (striking color, smell, moisture, and dust). The import of 25 mg zeranol pellets was approved by the Federal Ministry for Social Security and Generations (Vienna, Austria; GZ 382.026/3-IX/A/6/00).

Fifteen heifers (Simmendal breed), 1.5−2.0 years or age, known to be free from feeding with zearalenone-contaminated feedstuff, with a mean body weight of 392 kg were randomly divided into three separate groups, each with five animals, and housed on straw and fed with 2 kg of blank control oats (daily zearalenone intake of all three groups = 158 μg per animal) along with straw, hay, and water. All three groups were allowed to familiarize themselves with their new surroundings for 35 days before the study was begun.

During the animal feeding study (84 consecutive days) one group of animals (zearalenone group) was fed with 2 kg of contaminated oats per day (daily intake of zearalenone group = 2.74 mg of zearalenone per animal) while the control group and the third group received the same amount of blank control oats (daily zearalenone intake = 158 μg per animal). To avoid any cross-contamination, the zearalenone group was kept in a separate stable. The heifers of the third group were implanted successively on different sides of the neck with two 25 mg zeranol pellets (zeranol group), one at the beginning of the study and one after 42 days. Also, this group of animals was fed with 2 kg of

**Table 1.** Zearalenone, α/β-Zearalenol, Zeranol, Taleranol, and Zearalanone Concentrations in Heifer Urine and Liver Samples of the Zearalenone Group (Fed with Zearalenone-Contaminated Oats), of the Zeranol Group (Implanted with Zeranol Pellets), and of the Control Group (Fed with "Zearalenone-Free" Oats)[a]

| group | | zearalenone | α-zearalenol | β-zearalenol | zeranol | taleranol | zearalanone | increase of wt (kg) | dose of active compound |
|---|---|---|---|---|---|---|---|---|---|
| zearalenone | urine[b] (μg/L) | 5–8 | 3–5 | 20–65 | 2–3 | 2–3 | tr | 142.5 ± 10 | daily: 2740 μg of zearalenone |
| | liver[c] (μg/kg) | tr–1.2 | tr–1.2 | 5–11.5 | | | | | |
| zeranol | urine[b] (μg/L) | tr | tr | | 2–5 | 2–5 | tr | 115.5 ± 12 | 2 × 25 mg zeranol pellets + daily: 158 μg of zearalenone |
| | liver[c] (μg/kg) | | | | | | | | |
| control | urine[b] (μg/L) | tr | | tr | | | | 94.4 ± 12 | daily: 158 μg of zearalenone |
| | liver[c] (μg/kg) | | | tr | | | | | |

[a] None of the analytes were detected in any of the investigated muscle tissue samples. [b] Traces in urine ≡ concentrations between 0.1 and 0.5 μg/L (limits of detection) and between 0.5 and 1.0 μg/L (limits of quantification). [c] Traces in liver ≡ concentrations between 0.1 and 1.0 μg/kg (limits of detection) and between 0.5 and 3.0 μg/kg (limits of quantification).

blank oats per day (daily zearalenone intake = 158 μg per animal). One heifer of the zearalenone group was already pregnant before the beginning of the study and was, therefore, removed from this group. After 84 days of controlled feeding, the remaining 14 animals of the three test groups were weighed immediately before being killed with T 61 (a mixture of embutramide, mebezoniumiodide, and tetracain). Tissue samples (musculus psoas major of the back, musculus quadriceps femoris of the femoral region, liver, and skin of the neck with second implanted pellet) were collected during post-mortem examinations. The residues of the animal bodies were burned. Urine samples were collected every 5−6 days over the whole period of 84 days in the early morning before feeding. All tissue and urine samples were stored at −20 °C until analysis.

## RESULTS AND DISCUSSION

Heifers were used for this experiment to establish eventual pathological or histological changes of the reproductive system. All 15 animals were selected from a herd that was under permanent veterinary supervision of the Medical Clinic for Ruminants and Swine of the Veterinary University of Vienna. Urine samples were investigated for zearalenone, zeranol, and their metabolites to ensure that they were "mycotoxin-free" (concentrations of zearalenone and β-zearalenol in urine were <0.5 μg/L; other metabolites could not be detected) at the beginning of the controlled study. Despite the additional high deoxynivalenol content (23400 μg/kg), the animals of the zearalenone group did not refuse the mycotoxin-contaminated feed at any time during the feeding period. The mean increase of weight (**Table 1**) was established shortly before slaughter as 142.5 ± 10 kg for the zearalenone group, 115.5 ± 12 kg for the zeranol group, and 94.4 ± 12 kg for the control group, giving clear evidence for the anabolic effect of zearalenone, zeranol, and its metabolites. Clinical, pathological, and histological investigations during the feeding study and after slaughter revealed no striking changes of the reproductive system (hyperestrogenism), although no conclusion about the influence of zearalenone or zeranol on reproductive rates and milk production can be drawn from these results. However, decreased fertility and milk production of dairy cows by zearalenone intoxication has previously been reported (*33*, *34*). Blood and rumen juice samples taken at the beginning, in the middle, and at the end of the feeding period offered also no striking changes in values. A detailed survey about the veterinary and histological data of this study will be published elsewhere.

**Urine Samples.** *Control Group.* Because low amounts of zearalenone were also present in the "blank" control oats (79 μg/kg), some urine samples of the control group contained traces of zearalenone and of β-zearalenol (<0.5 μg/L, **Table 1**). Other metabolites could not be detected.

*Zearalenone Group.* Investigation of the urine samples of the experimental group revealed that ∼80% of zearalenone analyzed as the sum of the mother compound and its metabolites was transformed in vivo to α-zearalenol (3−5 μg/L) and to its epimer β-zearalenol (20−65 μg/L) in a ratio of 1:8, whereas zeranol and taleranol as further metabolites could be detected at lower concentration levels between 2 and 3 μg/L with a ratio of 1:1 (**Table 1**). Zearalanone, which has not been reported before as a metabolite of zearalenone, was identified in traces, although not in all samples. The degree of glucuronidation of the analytes in urine was established by parallel investigations of contaminated urine samples, with and without enzymatic (glucoronidase) digestion, and found to be almost 100% for all analytes. This is in distinct contrast to the glucuronidation rates in pigs, for which values between 27% (zearalenone) and 94% (β-zearalenol) have been found (*29*). A typical LC-MS chromatogram of a urine sample is depicted in **Figure 2A** with extracted ion chromatograms of zearalenone (**Figure 2B**), α/β-zearalenol (**Figure 2C**), zearalanone (**Figure 2D**), and zeranol/taleranol (**Figure 2E**). Peaks marked with asterisks in **Figure 2D**,**E** are derived from α/β-zearalenol and zearalanone-$d_2$ because all three analytes also influence the fragmentation pathways of zeranol, taleranol, and zearalanone.

The time−concentration course of zearalenone and its major metabolites α/β-zearalenol, zeranol, and taleranol was an important and accurate marker for the overall progress of the study. Depending on the day, the curve consisted of mean concentration values of a minimum of three and a maximum of five animals of the zearalenone group (**Figure 3**). The beginning of the feeding with mycotoxin-contaminated oats is clearly indicated by an immediate increase of the concentrations of all five analytes (days 0−14) to the highest levels found during the whole study (days 14−24). Afterward, concentration values decreased slightly and varied between 20 and 50 μg/L (β-zearalenol), 5 and 8 μg/L (zearalenone), 3 and 5 μg/L (α-zearalenol), and 2 and 3 μg/L (zeranol/taleranol) until the end of the study.

Except for days 0−7, analyte ratios of zearalenone and its metabolites α/β-zearalenol and zeranol were more or less

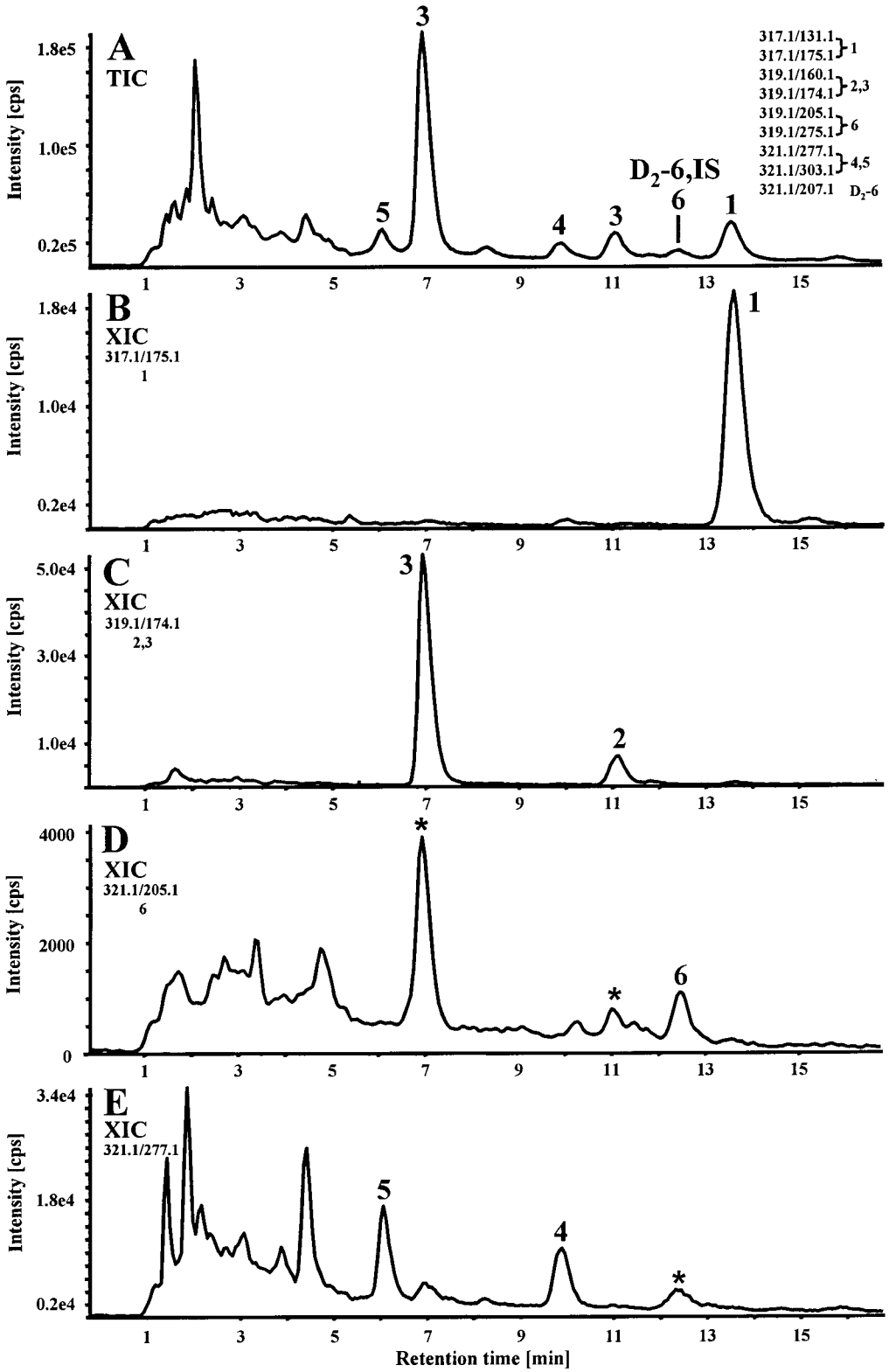


**Figure 2.** LC-MS/MS chromatograms of a heifer urine sample of the zearalenone group contaminated with 26.4 $\mu$g/L zearalenone, 18.4 $\mu$g/L α-zearalenol, 111.9 $\mu$g/L $\beta$-zearalenol, 5.8 $\mu$g/L zeranol, 5.9 $\mu$g/L taleranol, and 1.1 $\mu$g/L zearalanone and spiked with 5.0 $\mu$g/L zearalanone-$d_2$ (IS): (A) total ion chromatogram; (B) extracted ion chromatogram of zearalenone, 317.1 ⟹ 175.1; (C) extracted ion chromatogram of α-/$\beta$-zearalenol, 319.1 ⟹ 174.1; (D) extracted ion chromatogram of zearalanone, 319.1 ⟹ 205.1 (peaks derived from zearalenols are marked with asterisks); (E) extracted ion chromatogram of zeranol and taleranol, 321.1 ⟹ 277.1 [peak derived from zearalanone-$d_2$ (IS) is marked with an asterisk].

constant during the whole feeding study (days 8−84) with an average ratio for zearalenone/α-zearalenol of 1.5:1, for zearalenone/$\beta$-zearalenol of 1:5, for zearalenone/zeranol of 2.5:1, for zearalenone/taleranol of 2.5:1, for α-zearalenol/$\beta$-zearalenol of 1:8, and for zeranol/taleranol of 1:1. In principle, this metabolic profile is in agreement with earlier studies (*29*−*31*) and gives evidence that zearalenone metabolization exhibits significant differences between species, such as pigs, for example, where zearalenone is predominantly transformed into α-zearalenol (*24*, *29*).

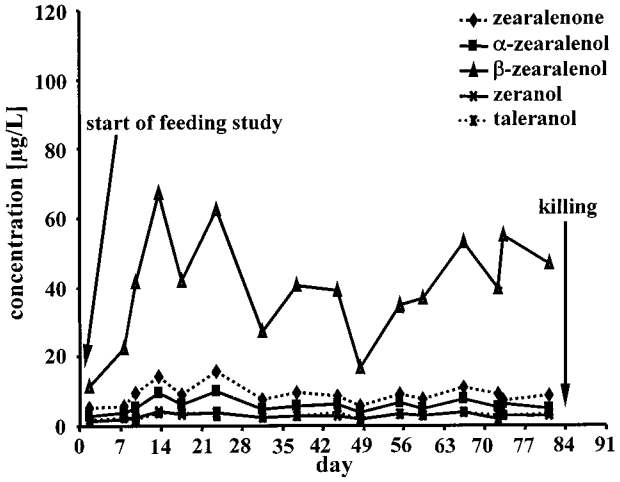


**Figure 3.** Time course of zearalenone and α/β-zearalenol concentrations from mean value of three to five animals fed with zearalenone-contaminated oats (zearalenone group).

*Zeranol Group.* Traces of zearalenone and its major metabolite β-zearalenol (both <0.5 µg/L) were found in all investigated urine samples of the implanted heifers (**Figure 4A**, extracted ion chromatograms not shown, and **Table 1**), revealing a certain degree of zearalenone contamination via the blank control oats (79 µg/kg). As mentioned above, traces of both analytes were also detected in the urine samples of the control group. Apart from that, analyte profiles in urine of the zeranol-implanted heifers are completely different from those of the animals fed with zearalenone-contaminated oats (**Figure 4A**). Zeranol and taleranol were found at low concentration levels between 2 and 5 µg/L in a ratio of 1:1 (**Figure 4C**), revealing metabolic epimerization of zeranol via oxidation of zeranol to zearalanone and further reduction to taleranol. Intact zearalanone as a stable metabolite was identified only in traces, although not in all samples (**Figure 4B**). These results are partially in contrast to an earlier investigation, during which distinctly higher concentrations of zearalanone (1−2.5 µg/L) were found in the urine of zeranol-implanted steers and a mean zeranol/taleranol ratio of 1:2.5 was established (*23*). A possible explanation for these different results might be a distinctly shorter experimental period (6 days) of the study of Chichila et al. and possible differences between the zeranol metabolism in steers and heifers. Furthermore, just one steer was used for these experiments; thus, the statistical significance of the data obtained is low compared to our experimental results obtained with five animals. In other species, such as rats, dogs, and rhesus monkeys, zearalanone has been reported as a major phase I metabolite and taleranol as the minor component, giving evidence that zeranol metabo-

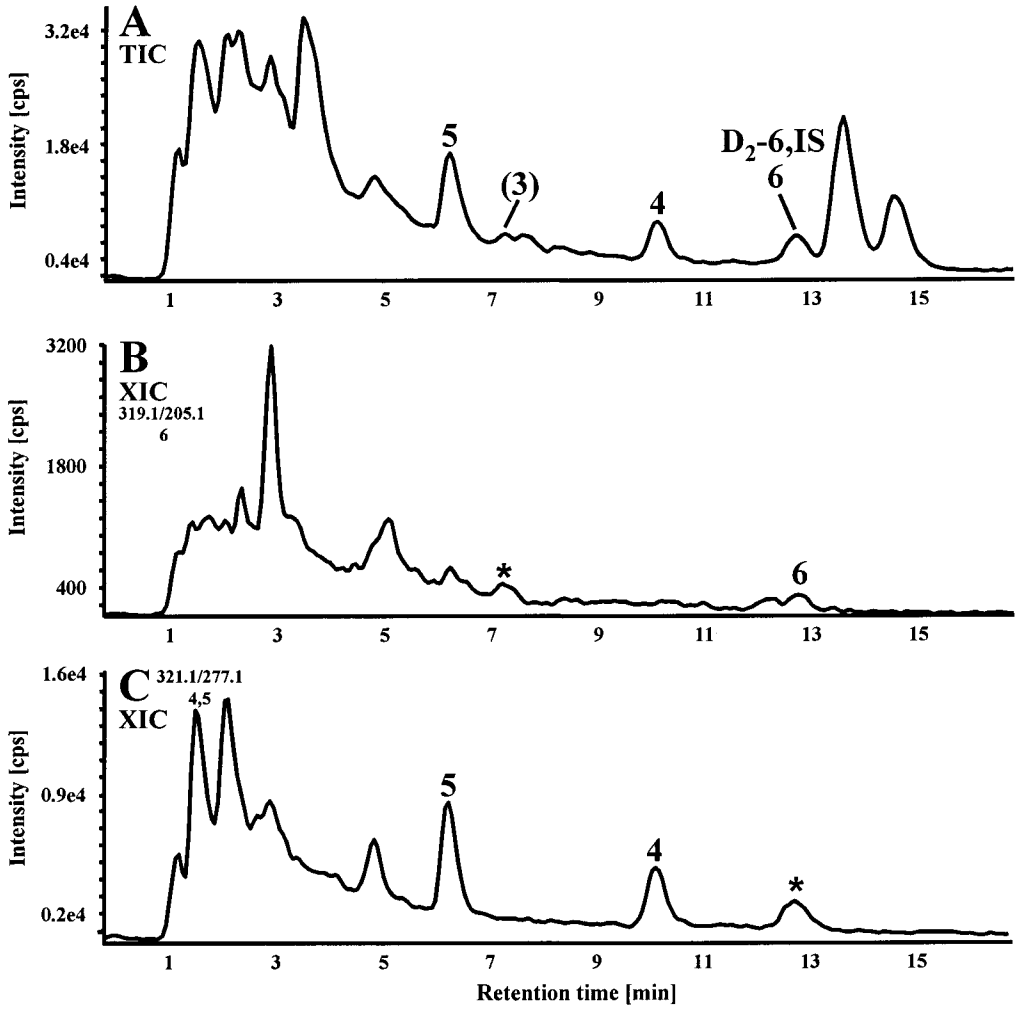


**Figure 4.** (A) LC-MS/MS chromatograms of a heifer urine sample of the zeranol group contaminated with 3.6 µg/L zeranol, 4.7 µg/L taleranol, and traces of zearalanone, zearalenone, and β-zearalenol and spiked with 5.0 µg/L zearalanone-$d_2$ (IS); (B) extracted ion chromatogram of zearalanone, 319.1 ⟹ 275.1 (peak derived from β-zearalenol is marked with an asterisk); (C) extracted ion chromatogram of zeranol and taleranol, 321.1 ⟹ 277.1 [peak derived from zearalanone-$d_2$ (IS) is marked with an asterisk].

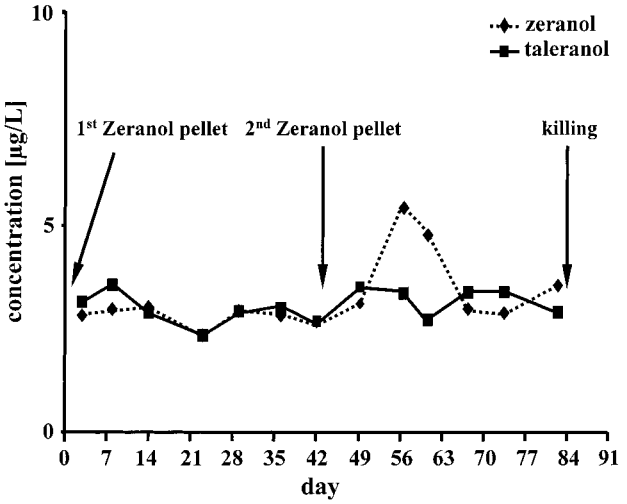


**Figure 5.** Time course of zeranol and taleranol concentrations in heifer urine from mean value of three to five animals implanted with zeranol (zeranol group).

lism also exhibits pronounced species-dependent differences (*30*).

As depicted in **Figure 5**, the concentration levels of zeranol (mean = 3 $\mu$g/L) and its major metabolite taleranol (mean = 3 $\mu$g/L) as well as their ratio (mean = 1:1) remained more or less constant over the whole period of this study, revealing a constant release of zeranol from the pellets. The pronounced increase of the zeranol concentrations (up to 5 $\mu$g/L) between days 50 and 60 was presumably caused by the application of the second pellet, resulting in the temporarily increased zeranol release from both pellets. Analytical investigation of the second pellet supports this assumption, because it could be shown that 60% of the dosed zeranol had not been released from the pellet after 42 days. Compared to earlier investigations of Brown, who has shown that ~96% of implanted zeranol was absorbed after 70 days (*35*), the release rates found in this study are lower. Unfortunately, the zeranol level of the first implant could not be established 84 days after implanting, because the respective pellets could not be detected and isolated during post-mortem examinations. From these findings, and by taking the low zeranol/taleranol concentrations in urine samples into account, the zeranol concentrations found seem to be essentially a function of the rate of drug release/absorption from the implant.

**Liver Samples.** *Control Group.* Liver samples of the control group that had been fed with blank control oats (79 $\mu$g/kg) contained only traces of $\beta$-zearalenol (<1 $\mu$g/kg), indicating that a higher daily intake of zearalenone is necessary to effect incorporation of this mycotoxin and its major metabolites in liver tissues.

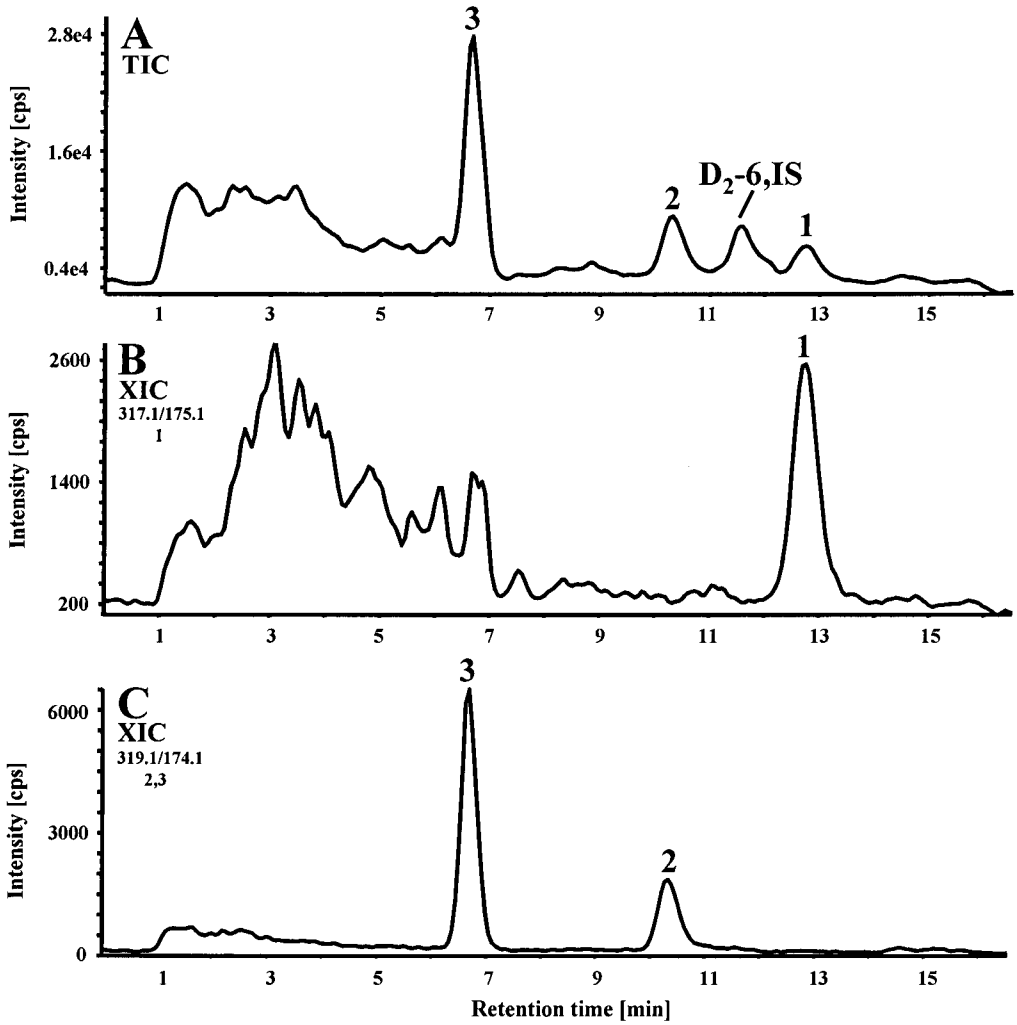


**Figure 6.** LC-MS/MS chromatogram of a liver tissue sample (zearalenone group) contaminated with 2.1 $\mu$g/kg zearalenone, 2.4 $\mu$g/kg $\alpha$-zearalenol, and 11.4 $\mu$g/kg $\beta$-zearalenol and spiked with 5.0 $\mu$g/kg zearalanone-$d_2$ (IS): (A) total ion chromatogram; (B) extracted ion chromatogram of zearalenone, 317.1 ⟹ 175.1; (C) extracted ion chromatogram of $\alpha$-/$\beta$-zearalenol, 319.1 ⟹ 174.1.

*Zearalenone Group.* In liver samples of the zearalenone group, β-zearalenol (5.0−11.5 μg/kg) along with smaller amounts of α-zearalenol (<1.0−2.4 μg/kg) and zearalenone (<1.0−2.1 μg/kg) could be identified with analyte concentrations being distinctly lower than those observed in urine samples (**Table 1**). The analyte ratio of zearalenone/β-zearalenol (1:5) was found to be comparable to the values in urine, whereas zearalenone/α-zearalenol (1:1) and α-zearalenol/β-zearalenol ratios (1:5) were slightly higher. Zeranol and taleranol could not be detected in any of the investigated samples, which is somewhat surprising as these metabolites were found in urine samples. A possible explanation might be that they are present at concentration levels below the limit of detection of the applied analytical method. Parallel analysis of outer and inner parts of a liver revealed that zearalenone and α/β-zearalenol seem to be homogeneously distributed in this organ. A typical LC-MS chromatogram of a liver sample of the zearalenone group is given in **Figure 6A**), along with extracted ion chromatograms of α/β-zearalenol (**Figure 6B**) and zearalenone (**Figure 6C**). Preliminary experiments with naturally contaminated heifer liver samples of the zearalenone group revealed that a considerable part of the analytes that are incorporated in the liver are glucuronidated/sulfated (zearalenone, 65%; α-zearalenol, 47%; β-zearalenol, 30%), which is comparable to the glucuronidation rates found previously in liver samples of pigs (*29*).

*Zeranol Group.* Except for traces of β-zearalenol and zearalenone most probably deriving from the blank control oats feed, in liver samples of the zeranol group (implanted with zeranol pellets), absolutely no traces of zeranol and/or its metabolites taleranol and zearalanone could be detected. This is in contrast to an earlier study (*23*) in which zeranol and taleranol could be found at low concentrations (low parts per billion range) just 6 days after implantation of zeranol pellets. However, this study was performed with steers, which might have different residue rates in this organ compared to heifers. In addition, the zeranol release of the pellets could have been much faster and may, therefore, not be fully comparable to this study.

**Muscle Tissue Samples.** In none of the analyzed muscle tissue samples (back; femoral region) of the zearalenone and zeranol groups could incorporation of analyte concentrations above the analytical detection limits be observed. This is surprising, because Chichila et al. were able to demonstrate that the application of zeranol pellets in steers resulted in zeranol and taleranol concentrations at the 0.1 μg/kg level 6 days after implantation (*23*); however, the same reasons as discussed above could be taken into account. Also, zeranol and α-zearalenol were found previously in the parts per billion range in tissue samples of pigs fed with zearalenone-contaminated oats (*29*). Although heifers are capable of metabolizing zearalenone into zeranol and α-zearalenol, none of these analytes could be detected in the investigated muscle tissue samples. By taking the comparable higher zeranol concentrations in urine into account (heifers compared to pigs), these results indicate that zeranol especially is more efficiently excreted via the urinary system (and via feces) by heifers than by pigs. Furthermore, incorporated concentrations levels, if present in muscle, might be below our detection limits (0.5 μg/kg). Thus, more sensitive analytical methods would be necessary to clarify this point.

To conclude, approximately 80% of the analyzed zearalenone in urine and tissue samples was shown to be transformed by heifers to β-zearalenol and to a distinctly lower degree to α-zearalenol, zeranol, taleranol, and zearalanone. The metabolic profile of zearalenone in the urine of heifers and pigs was found to be similar in principle, although the major metabolite in pigs is α-zearalenol and in heifers β-zearalenol. Furthermore, zeranol and taleranol concentrations in heifer urine samples were distinctly higher than those in pigs. Taking an average daily urine volume of 12 L per heifer into account, it can be roughly estimated that ∼33% of the zearalenone was excreted via the urinary system (sum of zearalenone, its metabolites, and the respective isomers). Only minor amounts of zearalenone and its metabolites were incorporated into liver (∼0.005% with an average liver weight of 6.2 kg), and none of the analytes could be detected in muscle tissue samples. Feces have been reported as a further major excretion pathway (*1*, *36*), which, however, was not analyzed in the present study.

Fifty percent of the implanted and absorbed zeranol is transformed in zeranol-implanted heifers into its epimer taleranol and to a minor extent to zearalanone. The keto compounds zearalenone and zearalanone seem to be metabolized (reduced) to the respective hydroxy compounds α-zearalenol, β-zearalenol, zeranol, and taleranol, because zearalenone and zearalanone levels in urine are relatively low. However, it cannot be ruled out that reoxidation of the hydroxy compounds to the keto compounds followed by a further reduction step could also occur to some extent, which consequently may then be responsible for the so-called epimerization reaction of zeranol and taleranol.

Fourteen percent of zeranol (sum of zeranol, taleranol, and zearalanone) is excreted via the urinary system of implanted heifers. In liver and muscle tissue none of the analytes could be detected. Another major part of the implanted zeranol seems to remain in the pellet, because analysis of the second pellet gives evidence that 60% of zeranol had not been released after 42 days. If one considers the release rate to be constant over the whole study, it can be estimated that 35% of the absorbed zeranol is excreted via the urinary system. Feces as a further major excretion pathway and other components of the digestive system, such as bile and kidney, were not analyzed in the present study.

For future investigations, it seems necessary to obtain also a more detailed knowledge about analyte concentrations in serum and in feces but also in other organs of the digestive system. In view of EU regulations and the hormone conflict between the United States and the EU, it is apparent from this study and the previous study with pigs that further statistically more relevant investigations are necessary to make an accurate risk assessment concerning the overall estrogenic and anabolic effects of natural zearalenone contamination in feed and the application of zeranol implants in livestock. Nevertheless, it is a fact that the zearalenone group gained the most weight, followed by the zeranol group and the control group, which indicates that overall zearalenone and its metabolites could be considered as an estrogenically active cocktail of "drugs", implying that the related regulatory issues may need to be reconsidered and reinterpreted.

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