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Detection of resorcyclic acid lactones in hair of cattle fed corn contaminated with *Fusarium* spp. toxins and implanted with Zeranol

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ABSTRACT

Nonsteroidal resorcyclic acid lactones (RALs) include compounds such as Zearalenone (ZEN), which is produced by *Fusarium* spp. fungi, α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL), zearalanone (ZAN), and α- and β-zearalanol (Zeranol and taleranol), which exhibit hormonal activity. Zeranol, whether naturally occurring or synthetically produced, acts as an anabolic agent and is banned in some countries due to potential health risks. This study aimed to evaluate the presence and persistence of RALs in the hair of cattle fed with *Fusarium* spp. toxins contaminated corn (A), implanted with Zeranol (B) and both fed contaminated corn and implanted with Zeranol (C), to determine whether hair could serve as a matrix to distinguish natural contamination from illegal growth promoter use. It was also tested by the application of the mathematical equation developed by the European Union Reference Laboratory for Growth Promoters (EURL). Twenty-four crossbred Zebu cattle were allocated into three treatment groups and monitored over time. Hair samples were analyzed using LC–MS/MS. RALs were not observed on day 0 (control groups). From day 2 onward ZEN, α-ZEL, β-ZEL, ZAN and Taleranol were not detected or recorded in low concentrations. Zeranol were detected in higher concentrations than the other RALs and the highest values were 17.2 μg kg^{-1} in group A, 28.35 μg kg^{-1} in group B and 42.88 μg kg^{-1} in group C, all recorded on day 2. It remained detectable up to 120 days. It was concluded that RALs may be detected in bovine hair. However, the EURL equation, developed for urine samples, was not able to distinguish between Zeranol abuse and dietary exposure to *Fusarium* spp. toxins.

1. Introduction

Zearalenone (ZEN) is a mycotoxin chemically classified as a nonsteroidal resorcyclic acid lactone (RAL), produced mainly by *Fusarium* species such as *F. graminearum*, *F. culmorum*, *F. equiseti*, and *F. cerealis* [1]. This mycotoxin is globally distributed and frequently found in grains that contaminate the feed of livestock, including cattle, swine, and poultry. ZEN production occurs in grains, hay, silage, grasses, and other forages contaminated by the fungus [2].

After ingestion by ruminants, ruminal protozoa, enterocytes, and hepatocytes metabolize ZEN in successive stages, forming various products [3,4]. These metabolites include α-zearalenol (α-ZEL), its isomer β-zearalenol (β-ZEL), α-zearalanol (α-ZAL or Zeranol), β-zearalanol (β-ZAL or taleranol), and zearalanone (ZAN). ZEN, α-ZEL and β-ZEL are called *Fusarium* spp. toxins [5]. Due to their structural similarity to estrogen, RALs compete with estrogen for the same receptors in animal tissues [6,7].

Zeranol, both naturally occurring and synthetically produced, has estrogenic effects and is considered an anabolic agent allowed in some countries to promote growth in livestock. However, its use has been banned in the European Union and Brazil for decades due to potential human health risks [8–10]. Zeranol functions as an endocrine disruptor

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that may contribute to reproductive disorders, congenital disabilities, and certain cancers in humans due to its hormone-mimicking activity. Additionally, its use in beef cattle can indirectly contaminate the environment, water, and the food chain [11,12]. Nevertheless, the presence of Zeranol residues in animal-derived samples is not always conclusive evidence of illicit use.

Therefore, identifying suitable biological matrices for detecting RALs is essential to establish criteria that can distinguish between Zeranol abuse and contamination from *Fusarium* toxins in feed.

In vivo studies in cattle have demonstrated that hair is a more suitable matrix than urine for detecting β-agonists and certain steroids, leading to its inclusion in European monitoring programs for controlling illicit hormone use [13]. Hair is particularly useful because such substances remain detectable long after administration, even when no longer measurable in urine [14]. However, no published studies have yet investigated the presence or depletion of RALs in bovine hair following the ingestion of corn contaminated by *Fusarium* spp. toxins or Zeranol implantation. Given those both *Fusarium* spp. toxins and Zeranol are rapidly metabolized and excreted in urine [15]. Hence, hair may represent an alternative matrix if these substances persist longer in it.

Considering those facts, compared to previously published studies on RALs, the importance of this study lies in the evaluation of these substances in bovine hair from naturally exposed animals under real field conditions, including simultaneous exposure to mycotoxins (zearalenone) and the use of zeranol. This design allows for a more realistic assessment of the behavior of RALs in regulatory monitoring scenarios. Thus, this study aimed to evaluate the behavior of RALs in bovine hair collected during and after exposure to contaminated feed and/or Zeranol implants, and to assess whether hair analysis can discriminate between natural dietary contamination and illegal growth promoter administration. Considering that the European Union (EU) developed an equation to differentiate between exogenous administration and natural occurrence of RALs in urine of bovine [16], we also evaluated if this mathematical model maybe used for bovine hair.

2. Materials and methods

2.1. Experimental conditions

The experimental trial was conducted in a Zebu crossbred cattle farm. There were selected 12 males and 12 females for this study. All animals were approximately 18 months old, had similar body weights (average of 180 kg for females and 200 kg for males). They were separated by sex and treatment and housed in different barns. A 15-day acclimatization period preceded the start of the experiment.

The animals were randomly allocated into three experimental groups, each comprising four males and four females: bovines in group A were fed with ZEN-contaminated corn; group B implanted with Zeranol and group C were fed with ZEN-contaminated corn + implanted with Zeranol.

In group A, animals received contaminated feed for 12 days; in group B, animals received implants releasing Zeranol for 90 days, as specified by the manufacturer; and in group C, animals were both fed contaminated corn for 12 days and implanted with Zeranol, which was removed after 30 days.

Baseline hair samples were collected on day 0 (control) from all animals before treatment began. On day 1, animals received both the implants and contaminated feed. Soon after, hair samples were collected from all groups on days 2, 7, 15, 30, 40, 60 and 120, except in Group A in which samples were not collected on day 60. Hair samples were individually collected using disposable blades, stored at room temperature, and shipped to the European Union Reference Laboratory for Growth Promoters at Wageningen University & Research, The Netherlands, for analysis.

A daily diet composed of 15 to 20 kg of silage, 0.5 kg of corn and mineral salt without protein supplementation was fed *ad libitum*. Corn and grass silage were analyzed for the presence of ZEN before being fed to the animals, using a validated LC–MS/MS method in accordance with Commission Decision 2023/2782/EU [17]. *Fusarium* spp. toxins (ZEN, α-ZEL, and β-ZEL) were not detected. Some corn was contaminated with ZEN, homogenized, and analyzed to determine the toxin concentration, which reached 4,200 ± 400 μg kg^{-1}. Thus, animals were fed approximately 2,100 ± 200 μg of ZEN per day.

Three implants containing 12,000 μg of Zeranol were inserted subcutaneously between the skin and cartilage on the backside of the ear, below the midline, on day 1 of the experimental period. The Zeranol concentration declared by the manufacturer (Ralgro, Merck, Rahway, NJ, USA) was verified by LC–MS/MS.

This research complied fully with the animal welfare international guidelines adopted by the National Council for the Control of Animal Experimentation (CONCEA). The experimental protocol n° 258/2018 was approved by the Animal Ethics Committee of the Federal University of Minas Gerais (UFMG).

2.2. Detection and quantification of RALs

2.2.1. Laboratory analyses

The presence and concentrations of RALs (ZEN, α-ZEL, β-ZEL, Zeranol, taleranol, and ZAN) in hair samples were determined through methanol extraction, immunoaffinity column (IAC) cleanup, and LC–MS/MS analysis. The method employed in this study was previously validated for Zeranol, taleranol and ZAN in the bovine hair matrix by the European Union Reference Laboratory for Growth Promoters (EURL-GP, Wageningen Food Safety & Research, Netherlands), where the analyses of this study were conducted. The method was fully validated in accordance with Commission Implementing Regulation (EU) 2021/808. The validation data are shown in the Tables 1–3.

Other RALs, such as ZEN, α-ZEL and β-ZEL, were also included in the method. However, quantitative validation for these compounds was not fully performed because these analytes were detected in nearly all bovine hair samples available during the validation study. This was attributed to the natural occurrence of these mycotoxins in animal feed and grazing pastures, since all hair samples available at that time originated from cattle raised in the Netherlands, where exposure to *Fusarium* spp. toxins is common. Consequently, suitable blank samples were not available for conventional validation experiments.

Based on previous findings [15], we already knew that urine samples from the treatment B which had received Zeranol implant, as well as all samples collected on Day 0 (Control), did not contain ZEN, α-ZEL and β-ZEL residues. Therefore, a similar behavior was expected for the hair samples. However, the amount of Brazilian bovine hair material from Day 0 available in the Netherlands at the time of analyses was insufficient to perform a complete re-validation for these analytes prior to

Table 1
Performance parameters of the method for RALs detection in bovine hair by LC-MS/MS.

RALs	Level	μg/kg	Precision (%)	RSD_r (%)	RSD_{RL} (%)
Zeranol	0.5*RW	0.5	103	4.3	9.5
	1.0*RW	1.0	106	3.3	3.5
	1.5*RW	1.5	105	2.6	3.6
Taleranol	0.5*RW	0.5	98	7.8	10.1
	1.0*RW	1.0	107	3.7	4.1
	1.5*RW	1.5	107	6.9	7.9
Zearalanone (ZAN)	0.5*RW	0.5	105	13.1	15.7
	1.0*RW	1.0	113	11.4	11.9
	1.5*RW	1.5	108	10.2	13.4

RALs = Resorcyclic Acid Lactones.
RSD_r: Relative Standard Deviation – repeatability.
RSD_{RL}: Relative Standard Deviation – reproducibility limit.
RW: Within-laboratory Reproducibility.

Table 2
Repeatability of RALs from seven blank bovine hair samples measured in three days.

RALs	Level - RW	μg/kg	RSD_r (%)
Zeranol	1.0*RW	1.0	5.4
Taleranol	1.0*RW	1.0	6.4
Zearalanone (ZAN)	1.0*RW	1.0	9.5

RALs = Resorcyclic Acid Lactones.
RSD_r = Relative Standard Deviation – repeatability.
RW = Within-laboratory Reproducibility.

Table 3
Values of decision limits (CCα) and detection capability (CCβ) of RALs in bovine hair.

RALs	CCα (μg/kg)	CCβ (μg/kg)
Zeranol	0.11	0.23
Taleranol	0.27	0.53
Zearalanone (ZAN)	0.48	0.95

RALs = Resorcyclic Acid Lactones.

sample analyses. All available blank hair material (free from RALs) had already been combined into a pooled sample and fully allocated for the preparation of calibration curves and quality control samples used in the analytical batches. Subsequently, an overall assessment of the calibration performance obtained during the analytical batches in which the study samples were analyzed was conducted using data generated over three independent analytical days. The results are presented in the Table 4.

Additionally, seven fortified blank samples at the lowest calibration level were included in the analyses. The standard deviation obtained from these results was subsequently used for the estimation of CCα values for all analytes included in the method (Table 5).

These findings support the suitability of the method for the quantification of both the previously validated analytes and the mycotoxins, whose determination provided relevant information for the bovine hair study.

2.2.2. Chemicals and reagents

The analytical standards (Certified Reference Material - CRM) Zeranol, and Taleranol were obtained from National Measurement Institute (Canberra, Australia), ZEN, α-ZEL, β-ZEL from Sigma-Aldrich (St. Louis, MO, USA), and ZAN from LGC Standards (Teddington, UK). The internal standards α/ß-zearalanol-d4, α-zearalenol-d4, and ß-zearalenol-d4 were obtained from Wageningen Food Safety Research (Wageningen, Netherlands), zearalanone-d6 and zearalenone-d6 from Toronto Research Chemicals (Toronto, Canada).

Acetonitrile and methanol Ultra LC-MS (Actu-All Chemicals (Oss, The Netherlands), ammonium formate 97% and phosphate buffered saline (PBS) (Sigma-Aldrich) were used.

2.2.3. Instrumentation

Waters Technologies ACQUITY UPLC I-Class system coupled to Xevo TQ Absolute Triple Quadrupole mass spectrometer (Waters, Milford, MA, USA) was used to carry out the analyses. An Acquity UPLC CSH, (2.1 × 100 mm, 1.7 μm) (Waters) was used for the chromatographic separation at 50 °C. Mobile phase A consisted of ammonium formate (1M), formic acid and water 2/0.5/1000 (v/v/v), and mobile phase B used Ammonium formate (1M), formic acid and acetonitrile 2/0.5/900/100 (v/v/v/v) at a flow rate of 0.5 mL min^{-1}. The chromatographic gradient was programmed as follows: initial conditions at 80% A; decreased to 50% in 2 min, to 30% in 6 min, and to 0% in 7 min; held at 0% for 1 min; returned to 80% in 9 min and maintained until 10 min. A volume of 10 μL was injected.

The direct infusion of the standards was used to determine the operational conditions of the mass spectrometer. One ion product was monitored for quantitation and other for confirmation each analyte. Electrospray ionization (ESI) was performed in the negative ion mode. Nitrogen was used as desolvation and cone gas at flow rate of 800 L h^{-1} and 150 L h^{-1}, respectively. The desolvation temperature was kept at 600 °C, the capillary voltage was set at −2.5 kV and the nebulizer gas flow at 7 bar. Argon flow was set at 0.22 mL min^{-1} as collision gas.

Table 4
Values of Coefficients of Determination (R^2) of RALs measured in three days in bovine hair.

RALs	R^2 Day 1	R^2 Day 2	R^2 Day 3
Zearalenone (ZEN)	0.998	0.999	0.999
α-Zearalenol (α-ZEL)	0.998	0.998	0.997
β-Zearalenol (β-ZEL)	1.000	0.997	0.997
Zeranol	0.988	0.999	0.997
Taleranol	0.998	0.999	0.993
Zearalanone (ZAN)	0.996	0.999	0.997

RALs = Resorcyclic Acid Lactones.

Table 5
Values of lowest calibration level (LCL), mean, standard deviation (SD) and Decision Limits (CCα) for RALs in bovine hair.

RALs	LCL (μg/kg)	Mean (μg/kg)	SD	CCα
Zearalenone (ZEN)	0.25	0.225	0.034	0.31
α-Zearalenol (α-ZEL)	0.25	0.316	0.036	0.31
β-Zearalenol (β-ZEL)	0.25	0.293	0.043	0.32
Zeranol	0.25	0.259	0.039	0.31
Taleranol	0.25	0.254	0.035	0.31
Zearalanone (ZAN)	0.25	0.290	0.077	0.38

RALs = Resorcyclic Acid Lactones.

2.3. Samples and extraction procedure

The hair samples were not washed, since the study was conducted under controlled experimental conditions minimizing the risk of uncontrolled external contamination. As the hair is susceptible to external contamination, particularly in farm environments, washing protocols are often applied to minimize this risk. However, washing may remove not only externally deposited compounds but also incorporated analytes, potentially leading to underestimation and inconsistent results. Given that our objective was to assess whether hair could serve as a matrix for exposure under controlled conditions, washing was omitted to avoid altering the total analyte amount.

Hair samples were homogenized without prior washing using a ball mill (Retsch). Approximately 500–1000 mg of hair was placed into the grinding jar together with five zirconium oxide balls (10 mm each) and ground for 4 min at 25 Hz. The pulverized material was transferred to a polypropylene tube. Then, 200 mg of the homogenized hair were weighed into a 15 mL polypropylene centrifuge tube, and 10 μL of an internal standard solution (0.1 mg/L) containing β-zearalanol-d5, α-zearalanol-d5, zearalanone-d6, β-zearalenol-d5, α-zearalenol-d5, and zearalenone-d6 were added.

For matrix-fortified standards (calibration curve), appropriate amounts of analyte standards were added at this stage to obtain final concentrations ranging from 0.25 to 5.0 μg/kg.

After the addition of internal standards, the samples were homogenized and allowed to equilibrate for 15 min. Then, 2 mL of methanol (MeOH) were added, and the tubes were vortex-mixed for 30 s. Samples were sonicated for 1 h in an ultrasonic bath and centrifuged at 3,600 × g for 5 min. The supernatant was transferred to a clean 50 mL polypropylene tube, 11 mL of phosphate-buffered saline (PBS) solution were added, and the mixture was vortexed.

2.3.1. Sample cleanup using immunoaffinity column

Immunoaffinity columns were placed on an SPE manifold (Alltech).

Columns were first allowed to drain and then conditioned with 3 mL of PBS solution. The diluted sample extract was loaded onto the column, which was subsequently washed with 10 mL of PBS followed by 10 mL of ultrapure water. After applying vacuum for a few seconds to remove residual liquid, the collection tubes were positioned, and 2 mL of acetonitrile (ACN) were added for elution. The valve was opened until approximately five drops of ACN had passed through, then closed, and the column was left to stand for 15 min before reopening to collect the remaining eluate.

The eluate was evaporated to dryness at 55 °C under a gentle stream of nitrogen. The residue was reconstituted in 120 μL of a methanol/water (40:60, v/v) solution, transferred to an HPLC vial, and stored for LC–MS/MS analysis.

2.4. Discrimination between abuse and contamination using the statistical model

The equation proposed by the EURL, originally designed for application in animal urine matrices, to differentiate the abusive use of the growth promoter zeranol from natural contamination by *Fusarium* spp. toxins was applied to interpret the results. This model establishes a relationship between the sum of zeranol and taleranol concentrations (response variable) and the sum of *Fusarium* spp. toxins concentrations (explanatory variable), which were transformed using base-10 logarithms [16].

The prediction interval was calculated using the following equation:

$$\alpha + \beta x_0 \pm t(d.f)\sqrt{S^2\left(\frac{(x_0-\overline{x})^2}{S_{xx}}+\frac{1}{n}+1\right)}$$

where n represents the number of observations and t(d.f.) corresponds to the selected percentile of the t distribution. Thus, for a prediction interval of, for example, 99% and a given value of x_0, the equation provides two limits within which the true y value is expected to lie with 99% confidence for that x value.

The estimated regression coefficients and their associated standard errors were used to calculate a 99% prediction interval for the sum of zeranol and taleranol concentrations for any given value of the sum of ZEN, α-ZEL, and β-ZEL, following the approach described by McConway [18]. The equation derived by the EURL considers 0.6000 as the intercept (α) and 0.5318 as the slope (β).

2.5. Statistical analyses

The experiment was conducted in a completely randomized split-plot design, with treatments assigned to the main plots and hair collection days assigned to the subplots. R language was used to perform statistical analysis [19]. Each treatment had eight replicates of one bovine, since no differences were observed between the sexes.

The behavior of metabolite concentrations in hair, as influenced by treatments and sampling days, was evaluated using a mixed effect Generalized Additive Model (GAM) [20]. Diagnostic analysis of the residuals revealed a pronounced and progressively increasing heteroscedastic pattern, forming a characteristic funnel-shaped distribution ("funnel plot"), indicative of structural misspecification of the model.

This heteroscedasticity, reflected by a non-random dispersion of residuals and a substantial increase in variability at intermediate and high fitted values, resulted from a combination of outliers observed in specific sampling periods and a high frequency of non-detected (zero) values, which represented the majority of samples across the time series. In some cases, isolated high concentrations were detected in a single animal, further increasing data dispersion and compromising model stability.

These features of the dataset made it impossible to identify an analytical model capable of adequately representing the event, as the distribution of observations did not follow the assumptions required for parametric modeling. Consequently, the results are presented in a descriptive manner, highlighting the observed trends rather than relying on inferential statistical modeling.

3. Results

3.1. Detection and quantification of RALs

No RALs were detected in the hair samples collected on day 0 (control) from any of the experimental groups. Starting from day 2, however, RALs began to appear at quantifiable levels in all groups. Fig. 1 shows the chromatograms obtained from an analyte-free sample (day 0), a working standard solution for all analytes, and a naturally contaminated sample.

All RALs were detected and quantified in the hair of animals in group A, which received contaminated corn for 12 days (Fig. 2). ZEN was quantified at low concentrations up to day 15, peaking on day 7 (mean 2.08 μg kg^{-1}). α-ZEL was observed in only two animals - one on day 7 (0.28 μg kg^{-1}) and another on day 15 (0.83 μg kg^{-1}). β-ZEL was quantified between days 2 and 15, at low concentrations, with a peak on day 15 (mean 1.08 μg kg^{-1}). Zeranol was found up to 120 days, with a peak on day 2 (mean 8.49 μg kg^{-1}). Taleranol was recorded in low concentrations until day 30, peaking on day 7 (mean 0.2 μg kg^{-1}). ZAN was quantified up to 120 days, also at low levels, peaking on day 7 (mean 0.58 μg kg^{-1}).

In group B, which received only Zeranol implants, RALs were found up to 120 days (Fig. 3). The highest mean value (15.05 μg kg^{-1}) was recorded for Zeranol on day 2. Taleranol and ZAN were quantified at low concentrations, peaking on day 15, with mean values of 0.24 μg kg^{-1} and 0.82 μg kg^{-1}, respectively.

In group C, which received both *Fusarium* spp. toxins contaminated corn and Zeranol implants, ZEN was quantified up to day 15, peaking on day 7 (mean 2.20 μg kg^{-1}). α-ZEL was detected in three animals at low concentrations (one on day 2 and two on day 7). As in group A, β-ZEL was found at low concentrations up to day 15, with a peak on day 7 (mean 0.70 μg kg^{-1}). As observed in the other treatments, Zeranol was detected up to 120 days, peaking on day 2 (mean 20.94 μg kg^{-1}). Taleranol and ZAN were found up to day 40, both peaking on day 15 (mean 0.25 μg kg^{-1} and 0.73 μg kg^{-1}, respectively) (Fig. 4).

In all Groups, Zeranol showed numerically the highest concentrations and the longest persistence, remaining detectable up to 120 days after administration, with early peaks at day 2.

3.2. Application of the EURL equation for bovine hair

The discrimination between the illegal use of zeranol and the consumption of feed contaminated with *Fusarium* spp. toxins was evaluated by applying the EURL mathematical equation to bovine hair samples, based on the relationship between the sum of zeranol and taleranol concentrations and the sum of ZEN, α-ZEL, and β-ZEL concentrations. The quantitative outputs of the EURL equation, including the calculated 99% prediction intervals and the position of each sample relative to these intervals, are presented in Fig. 5.

Unlike what was observed for urine samples, the application of the EURL equation to bovine hair did not allow clear discrimination between treatment groups. Results from animals of all treatments showed substantial overlap within and around the 99% prediction interval. No consistent separation of samples relative to the EURL equation threshold line was observed.

4. Discussion

The absence of RALs in hair samples on day 0 (control) confirmed that the animals were not exposed to *Fusarium* spp. toxins contaminated feed and had no circulating residues of these substances prior to the experiment. The quantification of RALs from day 2 onward indicates

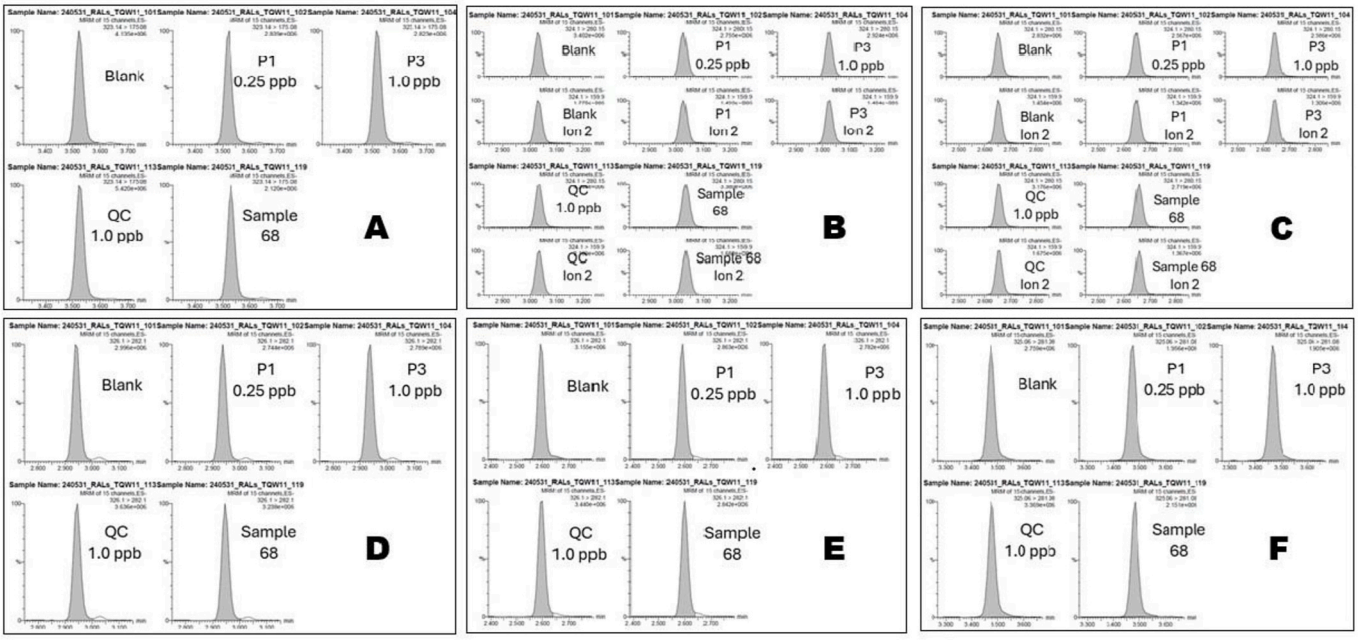

Fig. 1. Chromatograms with Internal Standards for blank samples, working standard solutions, and naturally contaminated samples with all analytes.
A = Zearalenone (ZEN); B = α-Zearalenone (α-ZEL); C = β-Zearalenone (β-ZEL); D = Zeranol; E = Taleranol; F = Zearalanone (ZAN); QC = Quality Control; Sample 68 = naturally contaminated sample.

their systemic absorption and subsequent incorporation into the hair matrix.

Variation in RALs concentrations among animals receiving the same treatment may be attributed to individual differences in hepatic metabolism, deposition rate of lipophilic compounds in keratin, and matrix incorporation efficiency. After oral administration, RALs can be detected in serum, liver, bile, muscle tissue, milk, and urine [15,21–24].

In Group A (feed contaminated with *Fusarium* spp. toxins), the sporadic detection of RALs suggests the conversion of ZEN into estrogenic metabolites and their deposition in hair, although not uniformly. This finding is relevant from a sanitary control perspective, as it shows that the presence of residues in hair does not necessarily indicate illicit anabolic use, since it may result from natural dietary contamination.

In Groups B (Zeranol implant) and C (*Fusarium* spp. toxins contaminated feed + Zeranol implant), a continuous release of the hormone was expected for approximately 90 days according to the manufacturer. However, a clear accumulation or maintenance trend in RALs levels over time was not observed. Instead, periods with undetectable levels occurred, indicating that systemic presence of Zeranol does not always lead to proportional hair incorporation. This suggests that hair may not reflect illegal exposure levels in a linear manner.

Nonetheless, hair can serve as a biological matrix that accumulates substances over time, reflecting chronic rather than acute exposure, as observed in keratinized tissues [25]. Previous studies on cortisol [26] and other hormones [27] have shown that hair records long-term exposure rather than single short-term events, in which the recommended matrix would be urine [16]. Moreover, hair represents an easily accessible biological matrix that can be collected from live animals and, when protected from external contamination, requires no special storage conditions. These features underscore its considerable potential for monitoring anabolic compound use [14].

Among the RALs analyzed, Zeranol showed numerically the highest concentrations and longest persistence, remaining detectable up to 120 days after administration, with early peaks at day 2. This finding on synthetic anabolic hormones shows that lipophilic compounds accumulate in keratinized tissues (such as hair and mane) long after exposure.

Lipophilic compounds generally exhibit greater incorporation efficiency and persistence in keratinized tissues due to their affinity for lipid-rich cellular structures and melanin-associated components present in the hair shaft. Hair incorporation occurs mainly during active hair growth through passive diffusion of circulating compounds from the bloodstream into the hair follicle during keratinization, allowing analytes to become entrapped within the keratin matrix. However, incorporation into hair is considered a multifactorial process that may also involve deposition from sweat and sebaceous secretions after hair formation, as well as adsorption from the external environment [28]. This mechanism may explain the longer persistence and higher concentrations observed for Zeranol compared with ZEN and its more hydrophilic metabolites, which are rapidly metabolized and predominantly excreted in urine.

External deposition should also be considered when interpreting hair residues, particularly under field conditions where animals may be exposed to contaminated feed particles, dust, urine, feces, sweat, or sebaceous secretions. Reviews on hair toxicology emphasize that keratinized matrices reflect not only systemic exposure but also environmental contact, making the interpretation of residue origin inherently complex [28,29]. Nevertheless, in the present study, the risk of uncontrolled external contamination was minimized because animals from each experimental group were housed separately under controlled experimental conditions throughout the study. Furthermore, the absence of detectable RALs in all day-0 samples supports that the residues quantified during the experimental period were predominantly associated with exposure occurring after treatment administration.

Conversely, ZEN, α-ZEL, and β-ZEL were found at low concentrations and for a limited duration (up to day 15), consistent with their rapid hepatic metabolism and predominant urinary excretion. The low affinity of these molecules for the keratin matrix confirms that measured levels reflect chronic exposure to more stable and lipophilic compounds (Zeranol, taleranol, and ZAN) [30] rather than hydrophilic metabolites that are quickly eliminated.

It was expected that animals in Groups B and C would exhibit consistent and detectable RALs levels throughout the experimental period, but this was not observed. Some cattle showed high levels while others displayed minimal or undetectable values, suggesting that RALs incorporation into hair does not directly mirror systemic availability.

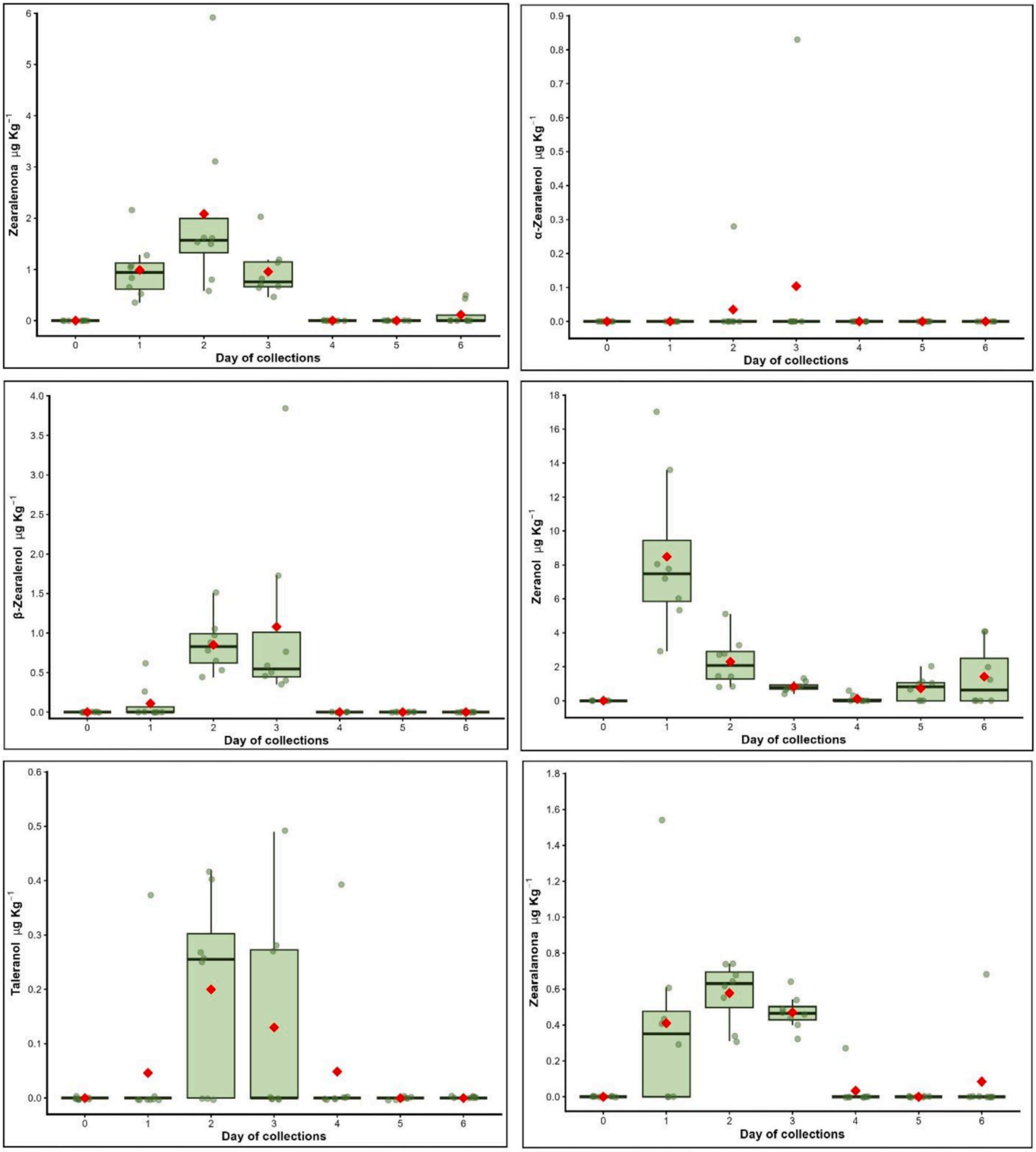

Fig. 2. Zearalenone, α-ZEL, β-ZEL, Zeranol, taleranol and ZAN concentrations of residues in the hair of cattle fed ZEN-contaminated corn at a concentration of 2100 ± 200 µg per day for 12 days (Group A). Days of collection: 0 = day 0 (control); 1 = day 2; 2 = day 7; 3 = day 15; 4 = day 30; 5 = day 40; and 6 = day 120. = mean.

Similar variability was reported by Duvivier [31] and Tallo-Parra [32], who noted that physiological and environmental factors influence hormone deposition in keratinized structures.

Considering the results of Groups A and C, it is evident that the concentration of β-ZEL in the hair samples was numerically more elevated than that of α-ZEL throughout the collection period. This finding can be explained by the bovine organism metabolizing ZEN preferentially into β-ZEL rather than α-ZEL, as reported by Gomes [15], who analyzed the concentrations of RALs in the urine of cattle fed corn contaminated with *Fusarium* spp. toxins and implanted with Zeranol.

Environmental or dietary exposure to *Fusarium* spp. mycotoxins, which can be biotransformed into estrogenic compounds, should also be

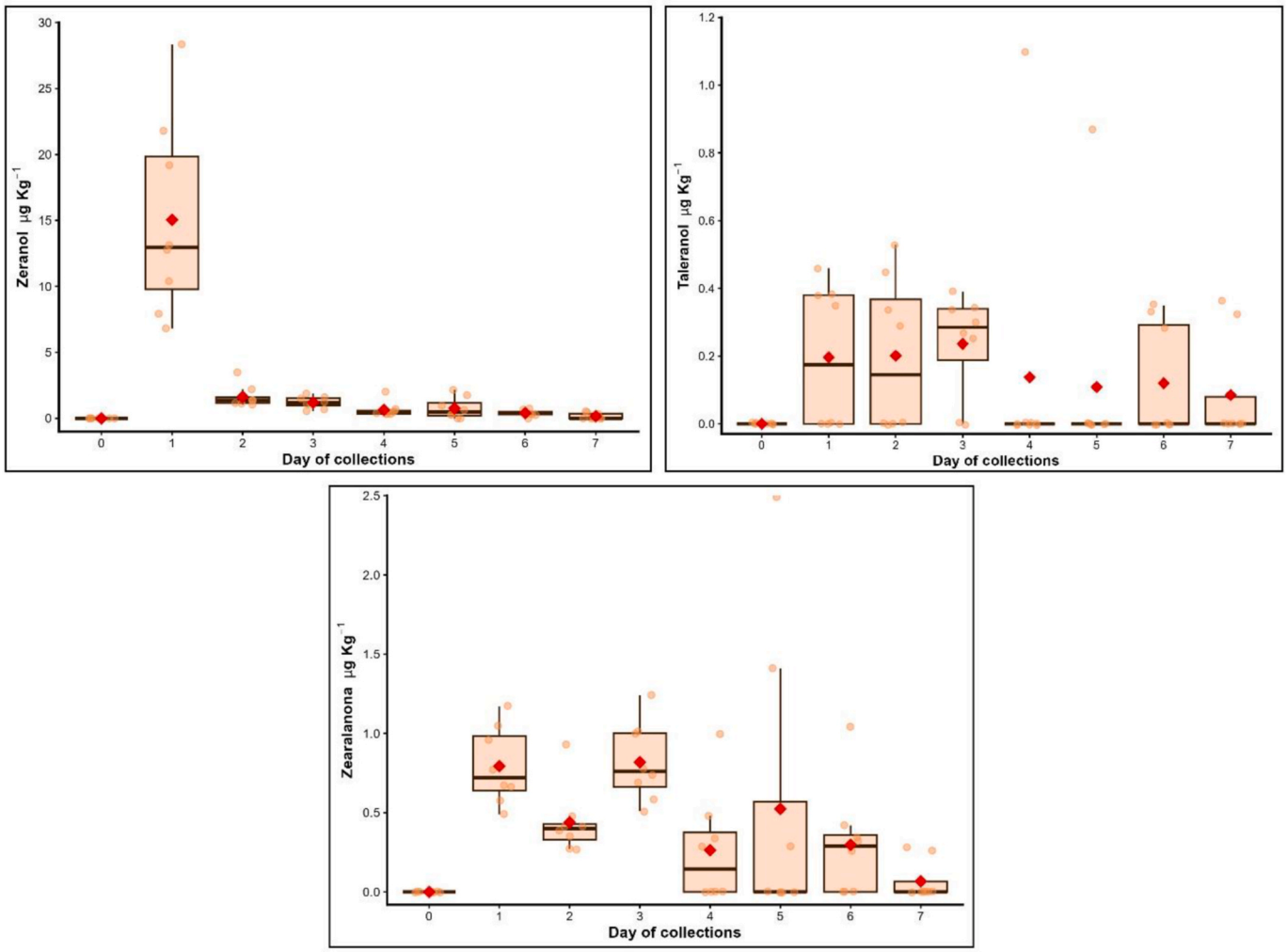

Fig. 3. Zeranol, taleranol and ZAN concentrations in the hair of cattle that received Zeranol implants used as a growth promoter at a concentration of 36,000 µg of Zeranol for a period of 120 days. (Group B). Days of collection: 0 = day 0 (control); 1 = day 2; 2 = day 7; 3 = day 15; 4 = day 30; 5 = day 40; 6 = day 60; and 7 = day 120. = mean.

considered. This overlap between natural contamination and illicit administration represents one of the greatest challenges for food safety and regulatory control. The present study demonstrates that detecting RALs in bovine hair does not allow reliable differentiation between illegal Zeranol use and contamination from *Fusarium* spp. toxins.

Some hair samples from animals fed contaminated corn showed elevated ZAN concentrations, which resulted in positive classifications when the EURL equation was applied to this matrix, despite the absence of illegal zeranol administration. When the equation was tested on samples with higher ZAN levels, several results fell within or above the 99% prediction interval, indicating potential misclassification.

These findings highlight an important limitation of directly applying the EURL equation, originally developed and validated for animal urine, to bovine hair. Unlike urine, hair is a cumulative matrix that integrates exposure over extended periods, which may amplify dietary-derived ZAN levels and obscure the distinction between natural contamination and hormonal treatment. In addition, differences in compound incorporation, persistence in the hair shaft, and possible external contamination may further contribute to the observed overlap between treatment groups.

Therefore, while the EURL equation remains a robust and useful control tool for urine analysis, its use for hair samples should be approached with caution. Matrix-specific models or complementary criteria may be required to reliably discriminate the origin of resorcylic acid lactones in bovine hair.

5. Conclusions

This study demonstrated that bovine hair is a suitable biological matrix for the detection and quantification of RALs. However, under the evaluated conditions and using the currently available discrimination models, RALs analysis in hair does not support a reliable distinction between illegal zeranol implantation and dietary exposure to *Fusarium* spp. derived mycotoxins. Consequently, bovine hair should not be used for origin discrimination purposes unless matrix-specific models are developed and validated.

Ethical statement

The animal study protocol was approved by the National Council for the Control of Animal Experimentation (CONCEA) at the Brazilian Ministry of Science and Technology and Innovation (MCTI). The protocol was approved by the Ethics Committee in Animal Experimentation at the Federal University of Minas Gerais State (UFMG) (Protocol Number: 258/2018).

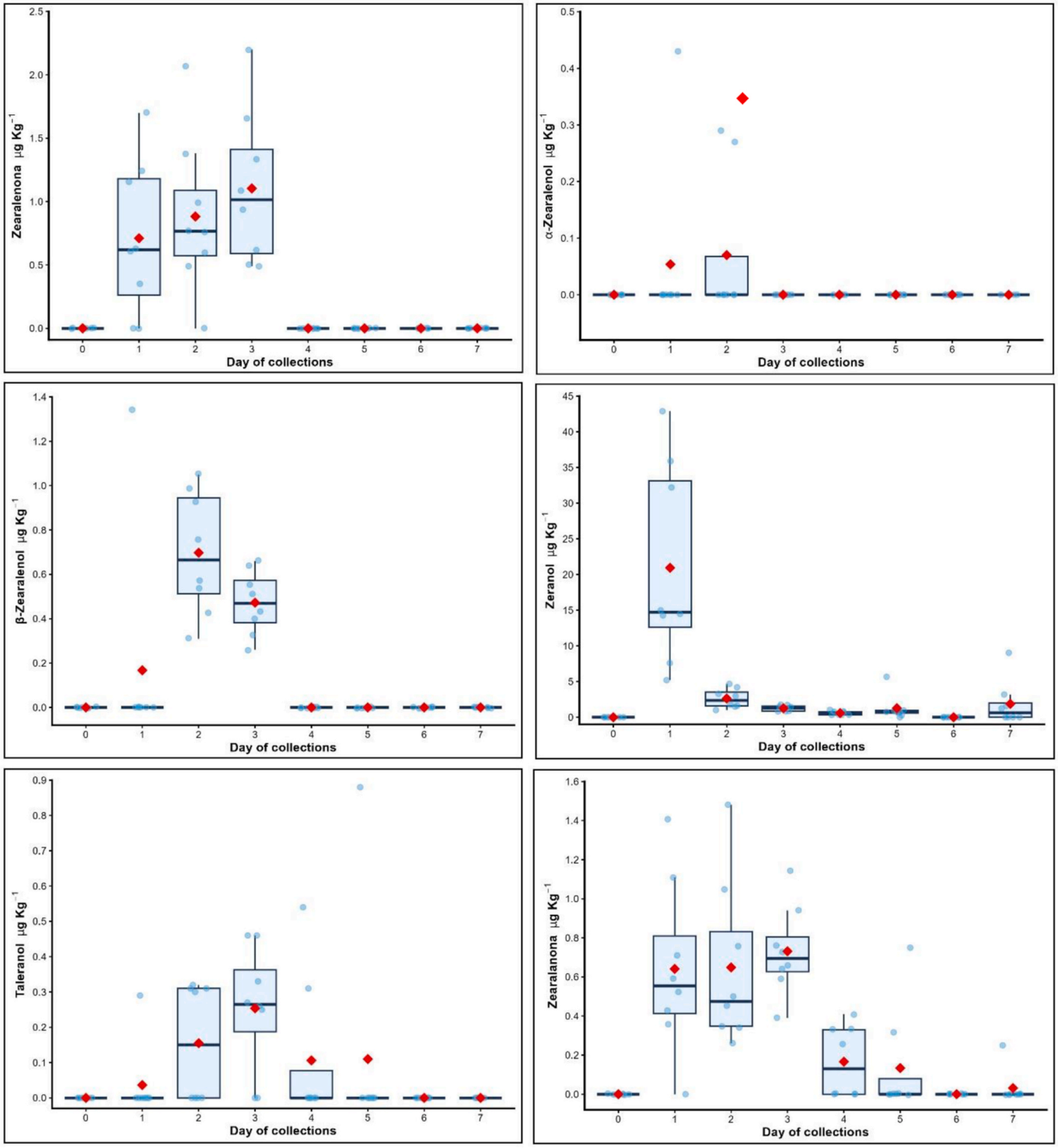

Fig. 4. Zearalenone, α-ZEL, β-ZEL, Zeranol, taleranol and ZAN concentrations in the hair of cattle that received Zeranol implants and were fed with ZEN-contaminated corn at a concentration of 2100 ± 200 µg per day for 12 days (treatment C). Days of collection: 0 = day 0 (control); 1 = day 2; 2 = day 7; 3 = day 15; 4 = day 30; 5 = day 40; 6 = day 60; and 7 = ◆ day 120. = mean.

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CRediT authorship contribution statement

Vanessa Gonçalves dos Santos: Formal analysis, Methodology. **Rafael Silva Gomes:** Investigation. **Carlos Juliano da Silva:** Data curation. **Marco Blokland:** Conceptualization, Methodology. **Ane Arrizabalaga-Larrañaga:** Formal analysis, Methodology. **Dieke van Doorn:** Formal analysis. **Rafael Romero Nicolino:** Data curation. **Marcelo Resende de Souza:** Writing – original draft, Writing – review & editing. **Tadeu Chaves de Figueiredo:** Data curation, Investigation, Writing – original draft. **Saskia Sterk:** Conceptualization, Supervision. **Silvana de Vasconcelos Cançado:** Conceptualization, Investigation,

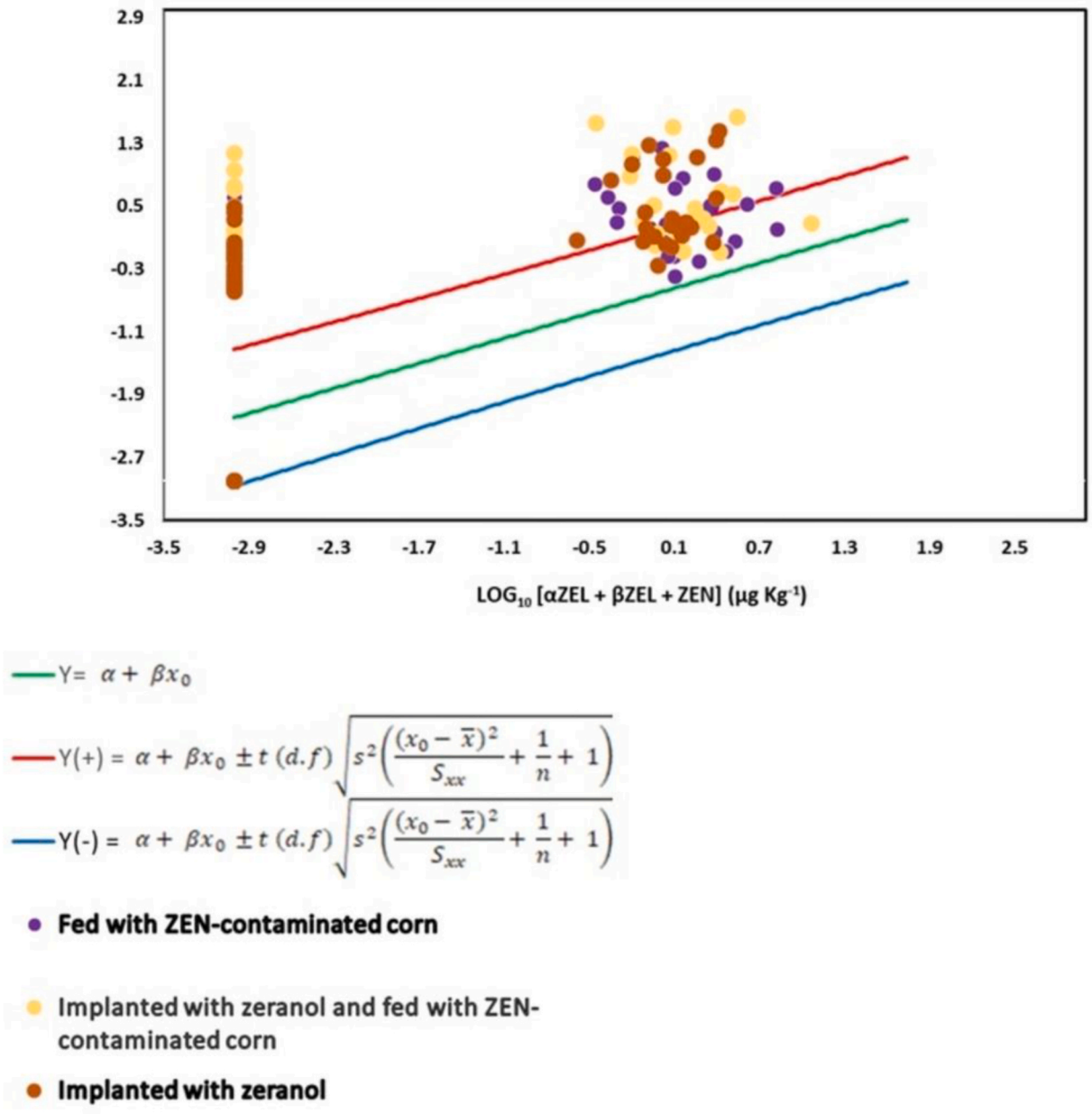

Fig. 5. Distribution of *Fusarium* spp. toxin metabolites and zeranol-related compounds in bovine hair from animals implanted with zeranol, fed *Fusarium* spp. contaminated corn, or subjected to both treatments, according to the EURL equation considering a 99% confidence interval.

Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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