RESEARCH ARTICLE

Determination of Zeranol in Caprine Urine by ELISA in the Republic of Albania

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Abstract

Zeranol was a widely used in animals as growth promoters to increase body weight. Zeranol (α-zearalanol) is a non-steroidal oestrogenic growth promoter that increases live-weight gain in food animals following implantation. But, the use of zeranol for growth promotion in food animals has been banned in the European Union (EU). As no data exist on the occurrence of Zeranol in caprine urine, in Republic of Albania we have analyzed them by ELISA method. The samples were taken from different regions of Albania. All samples were collected using official sampling methods. The method is based on a competitive enzyme immunoassay ELISA for the quantitative analysis of Zeranol in urine. The urine samples were spiked at the level 0.5 ng/ml. The Screening Target Concentration (Cut-Off FM) value for Zeranol was determined 0.404 ng/ml. All the concentration found above this value were considered suspected and were analyzed with UPLC-MS/MS confirmatory method. The overall recovery was 95%. Fourty six (n=46) caprine urine samples were taken in the study during the period 2021 until now. Of which forty three (n= 43) were found to be compliant below the Cut-Off FM value. Three (n=3) samples were found to be suspect with the ELISA method above the Cut-Off FM value. After analyzing the suspect samples with the UPLC-MS/MS confirmatory method, caprine urine samples were compliant below decision limit for confirmation (CCα). It is concluded that ELISA method relatively economical and rapid for screening Zeranol residues in urine, so it could be used as an alternative for the UPLC-MS/MS method. To investigate the Zeranol screening of caprine urine samples.

Keywords: Zeranol, Growth promotor, Caprine urine, ELISA.

1. Introduction

Mycotoxins, which are produced by fungi, are secondary metabolites found in food and feed at all stages of the food chain. Mycotoxin-contaminated cereal grain and animal feed are frequently found throughout the world [26], [22]. Zearalenone it is mainly formed pre harvest but its synthesis might continue under poor storage conditions. The climatic conditions during plant development prior to harvest are the major determinants for the ZEA contamination level of feed [11].

ZEA is typically detected in high levels in samples of natural animal feed, because of their improper storage and lead to Zearalenone accumulation before harvest time [29], [14].

The fusarium toxin ZEA is of concern due to its pronounced estrogenic effects in mammalian species. ZEA contaminates various grain-based food and cereal along with modified forms which contribute to overall

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mycoestrogen exposure [1]. These compounds are heat resistant and are difficult to inactivate and remove during cooking or processing [27]. Zeranol (α-zearalanol) is synthetic oestrogenic derivative of the mycotoxin ZEA and a resorcyclic acid lactone. Zearanol and its metabolites are shown in the figure below.

These compounds are resistant to heat and are difficult to inactivate and remove during cooking or processing [27].

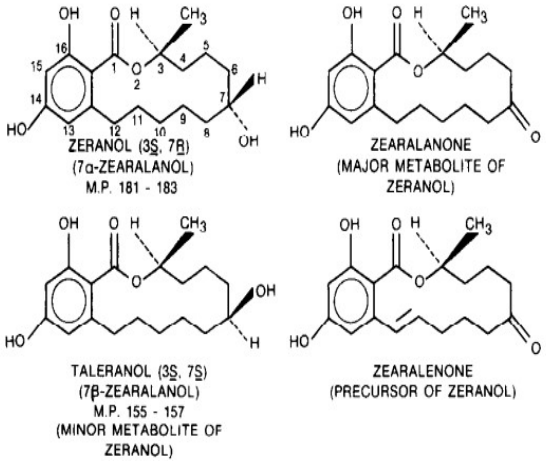

Figure 1 Chemical configurations of zeranol and related compounds, with the Chemical Abstracts numbering system [30].

ZEA was known to be a toxic substance of extensive concern to livestock. Decades later it was found that zearalenone is an oestrogen agonist [13].

In the literature, a wide variety of clinical effects attributed to zearalenone have been described. Abnormal estrus cycles, swollen vulvas, reduced milk production, decreased fertility, vaginitis and mammary gland enlargement are the most common findings reported in mammalian species. Single or multiple effects have been observed from the aforementioned changes. A change in the estrus cycle can manifest itself in various forms. Irregular, prolonged or skipped heats are commonly associated with zearalenone effects. While these abnormal estrus changes are not exclusively specific to zearalenone toxicity, one should investigate feed related causes when increases in abnormal estrus cycles are observed on farm [16], [21].

The Fusarium mycotoxin ZEA is of concern because of its lifelong estrogenic effects in animals [28], [25].

Zeranol (α-zearalanol) is a non-steroidal oestrogenic growth promoter that increases live-weight gain in food animals following implantation. But, the use of zeranol for growth promotion in animals that produce products for human consumption has been banned in the European Union (EU) [10], [17], [19] .

The determination of the banned anabolic substance zeranol (α-zearalanol) and the metabolites taleranol and zearalanone in urine is complicated because the occurrence of the structurally-related mycotoxin zearalenone. The corresponding α- and β-zearalenol metabolites which possess similar estrogenic properties [4], [3].

To contribute to the knowledge disposition of zeranol and other RALs in animals, and to give an accurate method for their detection after possible illegal use or contamination, we describe in this paper a procedure for the analysis of Zeranol in urine and report the levels of these substance in caprine urine.

The objective of this study was to assess the risk of zeranol exposure posed to Albania livestock. Random samples (n=46) of caprine urine were collected and analyzed for zeranol.

Immunoassay methods are more cost-efficient and easy to use with sufficient specificity and sensitivity. This method can satisfy the requirements for rapid detection. ELISA is the most commonly developed immunoassay method for detecting mycotoxins and veterinary drugs [20], [9].

As no data exist on the occurrence of zeranol in urine in Albania we have analyzed this compound by ELISA method, in urines from goat. The method is based on a competitive enzyme immunoassay ELISA for the quantitative analysis of zeranol in urine. The overall recoverie of the analysis was 95%.

2. Material and Methods

2.1 Study area

Fourty six urine samples from goat were collected respectively during a time period 2021-2022. Were randomly sampled, collected using official sampling methods. The samples were taken from different regions of Albania as Berat, Diber, Durres, Elbasan, Fier, Gjirokaster, Korça, Lezhe, Shkoder, Tirana, Vlora.

Figure 2. The map of coordinates' from which the samples were taken

The distribution of the samples is presented in the Table 2:

Table 2. Sampling by location

Region	2021	2022
Diber		1
Durres		1
Elbasan	3	3
Fier	2	3
Gjirokastra	1	3
Korça	2	4
Lezhe		3
Shkoder	1	2
Tirana	1	4
Vlora	9	2
Berat	1	
Total	20	26

2.2 Sampling

The urine samples were packed in sterile plastic containers, transported in refrigerated boxes and were labeled with a specific code, then a form was filled out, which contains all the data about the animal from which the sample was taken, such as age, registration number, sampling date, sampling time, sampling method, reason for sampling, transport conditions and farm address. The samples were stored at -20°C until the analysis was performed.

2.3 Reagents and Standards

Standards and reagents are provided by the kit (I' screen Zeranol). Reagents not provided by the kit: Helix Pomatia β-glucuronidase (Sigma Aldrich) Methanol (Sigma Aldrich)

2.4 Sample Extraction

0.5 ml of urine sample was dilute with 2.5 ml of sodium acetate buffer 50 mM pH 4.8. Helix Pomatia β-glucuronidase 10 µl was added. Incubation for 2 h at 37°C was performed. Solid Phase Extraction (SPE) procedure was done for purification of urine samples as follow. C18 columns were equilibrate with 3 ml methanol, 2 ml Tris HCl / methanol. After that samples of urine were applied through the column. Columns were washed 2 ml Tris HCl / methanol and 2 ml of methanol 40%. Columns were completely dry for 2 minutes, then elute with 1 ml of 80% methanol.

Eluate was evaporating to dryness under a stream of nitrogen/air. Residues were dissolved in 0.5 ml of Dilution Buffer.

2.5 Zeranol analysis

Urine samples were analyzed using an Enzyme immunoassay ELISA kit (I' screen Zeranol), following exactly the instructions of the manual included in the kit.

Calibration curve in solvent in the range 0 - 3 ng/ml was used to calculate the concentration in ng/ml of zeranol as shown in the figure below.

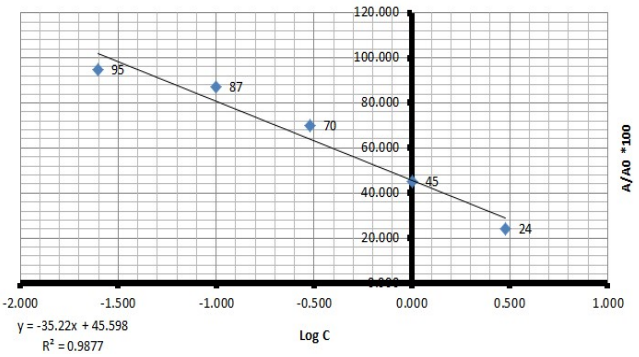

Figure 3. The standard curve in a semi-logarithmic system.

2.6 Statistical analyses

The data were statistically analyzed using Excel 2013, the T-test was performed to compare values, and Paired Two Sample for comparison of variances was utilized. Analysis of variance, and simple regression analysis were also applied.

2.7 Validation procedure

The acceptance criteria of the method are as follows:

Table 3. Acceptance criteria

Criterion	Range
Mean A_0 absorbance (per manufacturer)	≥ 0.7 OD 450nm
A/A0 50% (per manufacturer)	0.22-0.64 ng/ml
Detection limit (per manufacturer)	0.25 ppb

Data were accepted only if criteria were met. To calculate threshold value T and the Cut-Off Fm, were analyzed 20 negative quality control samples (QC) and 20 positive quality control samples (QC) spiked at ½ minimum method performance requirements (MMPRs). [5]

T= mean concentration negative QC+1.64*SD
Cut Off FM= mean concentration positive QC-1.64*SD
Cut-Off Fm (Screening Target Concentration)
QC (Quality control)
SD (Standard deviation)
The urine sample were spiked at the level 0.5 ng/ml for zeranol (α-zearalanol), following the EURL Guidance on Minimum Method Performance Requirements (MMPRs) [12].

Table 4. Analyzes of Zeranol by enzyme immunoassay.

Item	Zeranol
Spike level	0.5 ng/ml
Mean calculated	0.492 ng/ml
Recovery	95%
SD	0.054
Cut off Fm	0.404 ng/ml

All concentration found above the Cut-Off Fm value, were declared suspected samples. All the suspected samples were transported to CER Group to be analysed with RALs confirmatory method using UPLC-MS/MS (Ultra-high performance liquid chromatography-tandem mass spectrometry).

3. Results and Discussion

Results (table 5) show that the zeranol concentration for twenty samples analyzed in 2021, have turned out to be smaller than the Cut off FM value for eighteen samples which are considered as compliant samples. The zeranol concentration for two samples have turned out to be higher than Cut off FM value which are considered as suspected samples and were transported to CER Group to be analysed with RALs confirmatory method using UPLC-MS/MS (Ultra-high performance liquid chromatography-tandem mass spectrometry).

Table 5. Reveals the zeranol concentration results in caprine urine during 2021.

Analyte	Zeranol
Cut off FM **(ng/ml)**	0.404
Tested samples	20
Compliant samples (≤ Cut off FM)	18
Suspected Samples (≥ Cut off FM)	2

Results (table 6) show the zeranol concentration for twenty six samples analyzed in 2022, have turned out to be smaller than the Cut-Off FM value for twenty five samples which are considered as compliant samples.

Table 6. Reveals the zeranol concentration in caprine urine during 2022.

Analyte	Zeranol
Cut off FM **(ng/ml)**	0.404
Tested samples	26
Compliant samples (≤ Cut off FM)	25
Suspected Samples (≥ Cut off FM)	1

The zeranol concentration for one sample has turned out to be higher than Cut-Off FM value which was considered as suspected sample and was transported to CER Group to be analyzed with RALs confirmatory method using UPLC-MS/MS.
The results of table 5 and 6 are presented with graphics below. During 2021 twenty caprine urine samples were analyzed and their concentrations are reflected in the figure below.

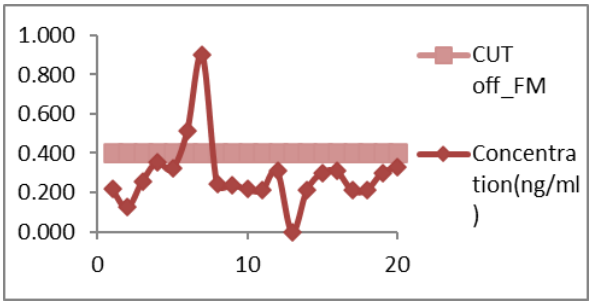

Figure 4. Zeranol concentration in caprine urine during 2021

During 2022 twenty six caprine urine samples were analyzed and their concentrations are reflected in the figure below.

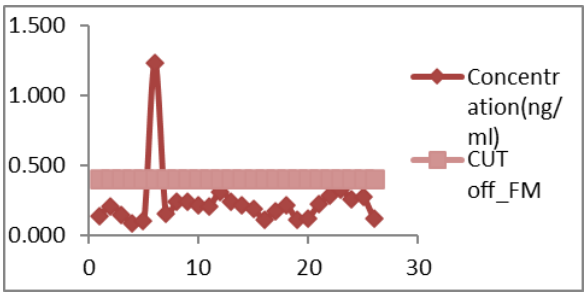

Figure 5. Zeranol concentration in caprine urine during 2022

After analyzing the suspect samples with the UPLC-MS/MS confirmatory method, caprine urine samples were compliant below decision limit for confirmation (CCα).

Table 7. Reveals the CC alpha value for Zeranol

Analyte	***CCα (µg/kg)***
Zeranol	0.38

4. Conclusions

Fourty six caprine urine samples were taken in the study during the period 2021-2022.

To protect consumers from these contaminants, most international organizations and countries have set regulations for permissible levels in cattle origin foods [23],[15]. While zeranol is banned for use in livestock and must not be detected in cattle origin foods in the EU. Zeranol (α-zearalanol) is a synthetic oestrogenic derivative of the mycotoxin ZEA and a resorcyclic acid lactone. The use of this compound in animals as a growth promoter is another alternative that causes zeranol residues in animal products. Furthermore, results of examinations of endocrine-disrupting potentials of zeranol were incongruous (Directive of The European Parliament and of the Council 96/22/EC 1996) [8]. The main oestrogenic anabolic compounds that might be used (illegally) as growth promoters in meat-producing animals are zeranol (a-zearalanol), 17b-oestradiol, ethynyloestradiol (EE2) and diethylstilbestrol (DES) [2], [18]. The use of zeranol as an anabolic agent in animals has only an insignificant effect on the overall potential human exposure to estrogenic compounds naturally present in our food supply. Due to the very low hormonal activity of zeranol, total dietary exposure does not produce adverse effects on human health [25].

It is concluded that ELISA method relatively economical and rapid for screening zeranol (a-zearalanol) residues in urine, so it could be used as an alternative for the UPLC-MS/MS method. To investigate the zeranol in routine, screening in caprine urine samples.

The concentrations of zeranol (α-Zeralanol) found in caprine urine are not sufficient to prove that abuse has occurred. Further studies should be conducted to contribute to the knowledge disposition of zeranol and other RALs in animals to give an accurate method for their detection after possible contamination or illegal use.

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