Scholars Journal of Agriculture and Veterinary Sciences OPEN ACCESS
Abbreviated Key Title: Sch J Agric Vet Sci
ISSN 2348–8883 (Print) | ISSN 2348–1854 (Online)
Journal homepage: https://saspublishers.com

Resorcylic Acid Lactones Interpretation, Discrimination Abuse or Contamination through the Statistical Model

Suela Teqja[1*], Jani Mavromati[2]

[1]Laboratory of Toxicology and Veterinary Drug Residues, Department of Toxicology / Food Safety and Veterinary Institute, Tirana, Albania
[2]Department of Veterinary Public Health, Faculty of Veterinary Medicine / Agricultural University of Tirana, Albania

DOI: https://doi.org/10.36347/sjavs.2024.v11i06.001 | **Received:** 24.07.2024 | **Accepted:** 30.08.2024 | **Published:** 03.09.2024

***Corresponding author:** Suela Teqja
Laboratory of Toxicology and Veterinary Drug Residues, Department of Toxicology / Food Safety and Veterinary Institute, Tirana, Albania

Abstract **Original Research Article**

Growth-promoting hormones are produced naturally in the animal body and can also be artificially synthesized and supplemented. It is challenging for control laboratories to monitor them and verify if the presence of certain RALs is due to an illegal practice or natural occurrence. Resorcylic acid lactones (RALs) are Zeranol (α-zearalanol) and its primary metabolite taleranol (β-zearalanol), which also include α- and β-zearalenol, zearalenone and zearalenone. Administration of zeranol, a non-steroidal oestrogenic growth-promoting compound, to animals raised as food is banned in the EU due to the potential risk to human health. Commonly found in animal feed is zearalenone also known as the Fusarium spp. toxin. The purpose of this study was to monitor the farms from which urine samples were taken, if zeranol or taleranol were used as a growth promoter for weight gain or if their presence would be a consequence of feed contamination with zearalenone. Through the statistical model we can distinguish illegal use of zeranol from consumption of food contaminated with Fusarium spp. toxin based on comparing the sum of zeranol and taleranol mass concentrations with the sum of zearalenone and its two major metabolites, α and β-zearalenol. We have analyzed urine samples using a confirmatory method by LC-MS/MS. The samples were taken from different regions of Albania. During the year 2023 from January to December, forty eight (n=48) urine samples from sheep, goat, swine were taken in the study. After analysis, all the urine samples resulted compliant lower than the decision limit for confirmation CCα for all compounds except three (n=3) urine samples, two from sheep and one from swine. Based on statistical model we can conclude that the feed was contaminated by mycotoxin Zearalenone. But further investigation should be conduct, to indicate the origin of the finding in order to protect animal health and public health.

Keywords: Zeranol, resorcylic acid lactones (RALs), urine, abuse, contamination, LC-MS/MS.

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 (Changwon *et al*., 2020). The climatic conditions during plant development prior to harvest are the major determinants for the zearalenone contamination feed levels. Zearalenone it is mainly formed pre harvest but its synthesis might continue under poor storage conditions (Dänicke *et al*., 2015). Zearalenone is also known as the Fusarium spp. toxin and is commonly found in animal feed (Blokland *et al*., 2006; Launay *et al*., 2004).

Growth-promoting hormones are produced naturally in the animal body and can also be artificially synthesized and supplemented (D, Mukherjee *et al*., 2018). Part of the growth-promoter compounds in animals are Resorcylic acid lactones (RALs). The main oestrogenic anabolic compounds that might be used (illegally) as growth promoters in meat-producing animals are α-zearalanol, 17b-oestradiol, ethynyloestradiol (EE2) and diethylstilbestrol (DES). Zeranol (α-zearalanol) is a synthetic oestrogenic derivative of the mycotoxin zearalenone, which is produced by Fusarium moulds and a resorcylic acid lactone (Lafayette, 2023).

These compounds are heat resistant and are difficult to inactivate and remove during cooking or processing (Yin *et al*., 2020). Therefore, it is challenging for control laboratories to monitor them and verify if the presence of certain RALs is due to an illegal practice or

natural occurrence (Larrañaga *et al*., 2023). Resorcylic acid lactones (RALs) are a class of mycotoxins isolated from various strains of fungi and are defined by the presence of a b-resorcylic acid ring (Jana *et al*., 2018). RALs include Zeranol (α-zearalanol) and its primary metabolite, taleranol (β-zearalanol) also α- and β-zearalenol, zearalanone, and zearalenone. Administration of zeranol, a non-steroidal oestrogenic growth-promoter compound, which increases live-weight gain in food-producing animals, is banned in the EU due to the potential risk to human health by Council Directive 96/22/EC.

The objective of this study was to assess the risk of mycotoxin zearalenone exposure posed to Albania livestock. We have analyzed urine samples using a confirmatory method by LC-MS/MS. The samples were taken from different regions of Albania. During the year 2023 from January to December, forty eight (n=48) urine samples from sheep, goat, swine were taken in the study. After analysis, all the urine samples resulted compliant lower than the decision limit for confirmation CCα for all compounds except three (n=3) urine samples, two from sheep and one from swine.

A statistical model based on the metabolite pattern in 2004 (Launay *et al*., 2004) was developed using the concentrations of all RALs in cow's urine. The purpose of this study is to distinguish illegal use of zeranol from consumption of food contaminated with Fusarium spp. toxin Zearalenone through the statistical model in urine samples from sheep, goat and swine analyzed by LC-MS/MS. To evaluate if this established criteria, the obtained results were used for discriminating the presence of zeranol between illegal treatment and contaminated feed in cows is also applicable to sheep, goat and pigs (Larrañaga *et al*., 2023).

RALs chemical structures and its metabolites are shown in the figure below.

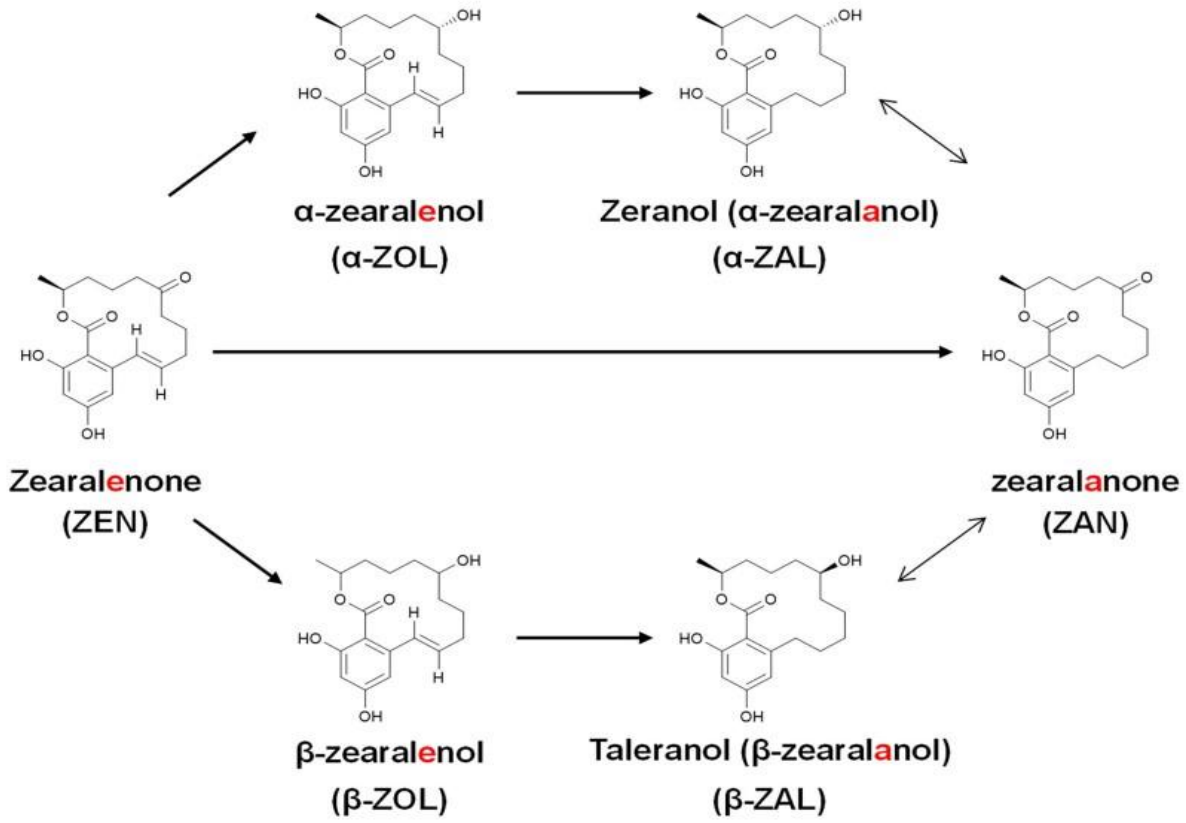

Figure 1: Chemical configurations of RALs (Larrañaga *et al*., 2023)

2. MATERIAL AND METHODS

2.1 Study area

During the year 2023 from January to December, forty eight (n=48) urine samples were collected, twenty seven (n=27) from sheep, thirteen (n=13) from goat and eight (n=8) from swine.

The samples were taken from different regions of Albania as Berat, Diber, Durres, Elbasan, Fier, Gjirokaster, Korça, Lezhe, Shkoder, Tirana, Vlora. The distribution of the samples is presented in the Table 1.

Table 1: Sampling by location

Region	Year 2023
Diber	2
Durres	2
Elbasan	4
Fier	7
Gjirokastra	4
Korça	7
Lezhe	3
Shkoder	7
Tirana	1
Vlora	7
Berat	2
Kukes	2
Total	***48***

2.2 Sampling

Sampling is done by official veterinarians, according to established standards. Sampling is based on Order no. 24, dated 30.1.2013 "On the unification of procedures, methods and documentation of the operation of laboratories, Instruction no. 14 dated 20.6.2011 "For completing the levels and frequencies of sampling provided for in Regulation no. 1, dated 17.3.2000 "On measures for monitoring certain substances and residues in live animals and animal products", in Republic of Albania.

2.3 Reagents and Standards

alpha-Zearalanol CAS No.26538-44-3
alpha-Zearalenol CAS No.36455-72-8
beta-Zearalenol CAS No.71030-11-0
beta-Zearalanol CAS No.42422-68-4
Zearalanone CAS No.5975-78-0
Zearalenone CAS No.17924-92-4
Sodium acetate
Acetic acid
Methanol
Ethyl acetate

2.4 Sample Extraction

Pipette 5 ml of centrifuged blank urine into a 50 ml vial. 2 aliquots of 5 ml of blank urine are pipette into 50 ml vials and fortified at the level of 0.5 µg/ml for α-zearalanol, β-zearalanol and at the level of 1 µg/ml for α-Zearalenol, β-Zearalenol, Zearalanone, Zearalenone. 2ml of 2M sodium acetate/acetic acid buffer solution was added (check pH 5.2 ± 0.2). Centrifuge for 10 min at 2000 rpm. Then the samples were purified in the C18 and NH_2, SPE column. The samples were dried under a gentle stream of nitrogen and dissolved with 50µl methanol and 50 µl of water.

2.5 Chromatographic analysis

Flow: 0.5 ml/min.
Column temperature: 50°C.
Injection volume: 10µl.
Injector temperature 10°C.
Eluent A: 0.05mM ammonium acetate:MeOH
Eluent B: Methanol LC-MS.
Polarity: Negative

2.6 Validation procedure

Determination of validation parameters such as linearity, recovery, decision limit for confirmation CCα, quantification limit is done according (EU) 2021/808 of 22 March 2021. The minimum method performance requirements (MMPRs) for these substances have been set under EURL Guidance September 2020 (EURL_MMPR_guidance-paper).

Table 2: Validation parameters for RALs

Item	Zearalenone	Zearalanone	α-zearalenol	β- zearalenol	α-zearalanol	β-zearalanol
LOQ (µg/kg)	1	1	1	1	0.5	0.5
CCα (µg/kg)	1.07	1.16	1.07	1.18	0.52	0.53

2.7 Statistical model

Through the statistical model we can distinguish illegal use of zeranol from consumption of feed contaminated with Fusarium spp. toxin based on comparing the sum of zeranol and taleranol mass concentrations with the sum of zearalenone and its two major metabolites, α and β-zearalenol (Larrañaga *et al*., 2023; Blokland *et al*., 2006).

The differences in the metabolite pattern of α/β-zearalanol (zeranol and taleranol) + zearalanone versus α/β-zearalenol + zearalenone can lead us to the conclusion that a natural contamination or abuse has happened. When the combined concentrations of α/β-zearalanol + zearalanone are higher than those of α/β-zearalenol + zearalenone it is an indication of illegal use. The discrimination is possible with a significant solid difference in C12/C13 between endogenous and synthetic forms of a molecule; in this case combustion-

Isotope Ratio Mass Spectrometry is used for confirmation. Confirmation is based on statistical solid differences between the analyte isolated from a biological sample and an endogenous compound (Larrañaga *et al*., 2022). 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.

3. RESULTS

The purpose of this study was to monitor the farms from which urine samples were taken, if zeranol or taleranol were used as a growth promoter for weight gain or if their presence would be a consequence of contamination with the mycotoxin zearalenone in feed. Forty eight urine samples from sheep, goat and swine were analyzed by LC-MS/MS during the year 2023 from January to December. After analysis, all the urine samples resulted compliant lower than the decision limit for confirmation presented in table 2, for all compounds. Except three (n=3) urine samples two from sheep and one from swine, respectively the concentration found are presented in Table 3.

Table 3: RALs concentration found in urine samples

Sample	Region	Concentration (µg/kg) α-zearalenol	Concentration (µg/kg) β- zearalenol
Swine urine	Korce	3	
Sheep urine	Korce		1
Sheep urine	Berat	1	3

Based on the EURL-recommendations, we used the statistical model comparing the sum of zeranol and taleranol mass concentrations with the zearalenone and its two major metabolites, α- and β-zearalenol. In practice, the ratio between the sum of zeranol and taleranol (indicating treatment) to the sum of α- and β-zearalenol (indicating feed contamination) is calculed.

In swine urine from Korce, α-zearalenol was the only compound detected, indicating a potential feed contamination and not an illegal treatment as shown in Table 4.

Table 4: RALs concentration found in swine urine, from Korce

Component	Concentration (µg/kg)
Zeranol	0.0
Taleranol	0.0
α-Zearalenol	3.0
β- zearalenol	0.0
Zearalenone	0.0

In sheep urine from Korce, β-zearalenol was detected, indicating a potential feed contamination and not an illegal treatment as shown in Table 5.

Table 5: RALs concentration found in sheep urine, from Korce

Component	Concentration (µg/kg)
Zeranol	0.0
Taleranol	0.0
α-Zearalenol	0.0
β- zearalenol	1.0
Zearalenone	0.0

In sheep urine from Berat, was detected α-zearalenol and β-zearalenol, indicating a potential feed contamination and not an illegal treatment as shown in table 6.

Table 6: RALs concentration found in sheep urine, from Berat

Component	Concentration (µg/kg)
Zeranol	0.0
Taleranol	0.0
α-Zearalenol	1.0
β- zearalenol	3.0
Zearalenone	0.0

4. DISCUSSION

To protect consumers from these contaminants, most international organizations and countries have set regulations for permissible levels in cattle origin foods while zeranol is banned for use in livestock and must not be detected in cattle origin foods in the EU. Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding have set zearalenone guidance value 2 mg/kg for cereal and cereal products, 3 mg/kg for maize by-products (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32006H0576#).

For prohibited or unauthorized pharmacologically active substances for which no RPA has been set in food, minimum method performance requirements (MMPRs) in food and non-food are established. For zeranol and taleranol in urine 1 µg/kg and for zearalenone and their metabolite α and β-zearalenol 2 µg/kg (https://eurl-residues.eu/wp-content/uploads/2020/12/EURL_MMPR_guidance-paper_final.pdf).

In our study forty eight urine samples from sheep, goat and swine were analyzed by LC-MS/MS during the year 2023 from January to December. After analysis, all the urine samples resulted compliant lower than the decision limit for confirmation CCα presented in table 2, for all compounds. Except three (n=3) urine

samples two from sheep and one from swine, respectively the concentration found are presented in table 3. The results found above the CCα limit were reported to the Ministry of Agriculture and Rural Development of Albania for follow-up actions. To go back to the farm and bring to the laboratory feed samples to determine if those contain zearalenone toxin. We expect that this study will contribute to effectively controlling the abuse of "Natural growth-promoting substances in biological samples" or feed natural contamination with zearalenone in Republic of Albania.

5. CONCLUSIONS

Based on the fact that all urine samples were found to be compliant below the decision limit for confirmation for all compounds, except three samples evaluated through the statistical model we can conclude that zeranol was not used as growth promoter in sheep, goat and swine as an illegal treatment to gain weight but a potential feed contamination was possible.α-Zearalenol and β-Zearalenol concentrations found in urine indicate a zearalenone contamination of animal feed. This comes as a consequence of poor animal feed storage and leads to the accumulation of zearalenone before harvest time. Animal feed need to be stored in a better way to avoid zearalenone contamination. Further investigation should be conduct, to indicate the origin of the finding in order to protect animal health and public health.

REFERENCES

- Blokland, H., Sterk, S., Stephany, W., Launay, M., Kennedy, G., & Van Ginkel, A. (2006). Determination of resorcylic acid lactones in biological samples by GC–MS. Discrimination between illegal use and contamination with fusarium toxins. *Analytical and Bioanalytical Chemistry*, *384*(5), 1221-1227.
- Commission Implementing Regulation (EU) 2021/808 of 22 March 2021 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC. https://eur-lex.europa.eu/eli/reg_impl/2021/808/oj
- Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32006H0576#
- Council Directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyreostatic action and of beta-agonists 1996. Off J Eur Commun L125, 3-9.
- Dänicke, S., & Winkler, J. (2015). Invited review: Diagnosis of zearalenone (ZEN) exposure of farm animals and transfer of its residues into edible tissues (carry over). *Food and Chemical Toxicology*, 84, 225-249.
- EURL Guidance on Minimum Method Performance Requirements (MMPRs) for Specific Pharmacologically Active Substances in Specific Animal Matrices. https://eurl-residues.eu/wp-content/uploads/2020/12/EURL_MMPR_guidance-paper_final.pdf(September 2020,date last accessed)
- Jana, N., & Nanda, S. (2018). Resorcylic acid lactones (RALs) and their structural congeners: recent advances in their biosynthesis, chemical synthesis and biology. New Journal of Chemistry.
- Lafayette. (2003). The Presence, Effect, and Diagnosis of Zearalenone in Dairy Cattle West. "Winter Newsletter." West Lafayette:406 S. University West Lafayette, IN 47907.
- Launay, M., Ribeiro, L., Alves, P., Vozikis, V., Tsitsamis, S., Alfredsson, G., & Kennedy, G. (2004). Prevalence of zeranol, taleranol andFusariumspp. toxins in urine: implications for the control of zeranol abuse in the European Union. *Food Additives and Contaminants*, *21*(9), 833–839.
- Larrañaga, A. A., Linders, R., Blokland, M. H., & Sterk, S. (2023). Occurrence of resorcyclic acid lactones in porcine urine: discriminationbetween illegal use and contamination, Food Additives & Contaminants.
- Larrañaga, A. A., Groot, M., Blokland, M., Barbu, I., Smits, N., & Sterk, S. (2022). Natural growth promoting substances in biological samples. Wageningen Food Safety Research.
- Linders, R., Blokland, M., & Sterk, S. (2023). Occurrence of resorcyclic acid lactones in porcine urine: discriminationbetween illegal use and contamination. Food Additives & Contaminants.
- Mukherjee, D., Royce, S. G., Alexander, J. A., Buckley, B., Isukapalli, S. S., Bandera, E. V., & Georgopoulos, P. G. (2014). Physiologically-Based Toxicokinetic Modeling of Zearalenone and Its Metabolites, Application to the Jersey Girl Study. *PLoS ONE*, *9*(12), e113632.
- Rai, A., Das, M., & Tripathi, A. (2019). Occurrence and toxicity of a fusarium mycotoxin, zearalenone. Critical Reviews in Food Science and Nutrition. 1-20.
- Yang, C. H., Song, G., & Lim, W. (2020). Effects of Mycotoxin-Contaminated Feed on Farm Animals. *Journal of Hazardous Materials*. 122087.
- Yin, M., Hu, Xi., Sun, Y., Xing, Y., Chai, Sh., Xing, G., Yang, Y., Teng, M., Li, Q., Wang, Y., Deng, R., & Zhang, G. (2020). The broad-spectrum and ultra-sensitive detection of zeranol and its analogues by an enzyme-linked immunosorbent assay in cattle origin samples. *RSC Advances*, *10*(35), 20809-20816.