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EVALUATION OF ZERANOL PRESENCES ON BOVINE MEAT RESIDUES IN KOSOVO

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ABSTRACT

Aim of this publication is to collect the all information about the possible administration of prohibited substances as Growth Promoter Hormones on the animals for comercial purposes in Kosovo. The matter of this publication included the substances of Group A4 Resorcylic Acyd Lactones within representative parameter Zeranol in bovine meat residues. Since 1 January 1989, according to Directive 96/22/EC, the EC prohibits the administering to a farm animal substances having a oestrogenic or gestagenic action for growth promotion purposes. The use of synthetic hormones regarding this topic si prohibited. Some third countries permit the use of these hormones in cattle for growth promotion purposes. The monitored activity means a conducting a planned sequence of observations, or measurements with the view to obtaining an overview of the state of compliance with the Food and Feed Law, Animal Health and Welfare rules. All monitored results were servired the Competent Authority for food safety and veterinary issues to set the appropriate barriers and to take the necessary measures in accountability of Public Health and to well informed the consumers, farmers, veterinarians about the human health risks.

Keywords: Elisa, Growth Promoter, Meat, Slaughterhouse, Public Health.

1. INTRODUCTION

According to the Scientific Committee on Risk Analyses, meat consumption originating from animals treated whith banned substances for comercial purpose as growth promoters Growth Promotant Hormones (GPH), results in high risk for consumers (EFSA,2007) [1]. The European Union countries and EU aspirants strictly prohibit GHP except for the therapeutic and zootechnical purposes, for some specific substances of steroids. In this regard the Competent Authorities have set barriers through legislation and official control measures (DG-Sante) [2]. Doubtless, stakeholders such as farmers, veterinarians, veterinary pharmacies have a vital role to play in whole process. EU adopted Directives on measures to monitor certain substances and

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residues thereof in live animals and animal products, and on concerning the prohibition on the use in stockfarming of certain substances which having a hormonal action regarded the administration of GPH (European Commission, 1996, 2003) [3]. Whithin this, Kosovo adopted the legal rules in compliance with community directive as well (RKS-MAFRID, 2016) [4]. Experts claim that meat consumption containing residues GPH can cause serious problems in human health (SCVPH, 1999, 2000, 2002) [5]. Group A substances which is matter of this publication include the Resorcylic Acyd Lactones (RAL) which classified in A4 substances and has been tested Zeranol as synthetic hormone, which has estrogen affinity (Leffers, 2001; Le Guevel, 2001; Takemura, 2007; Yuri, 2006) [6]. Zeranol is naturaly mycoestrogen derived from zearalenone. Zearalenone originated from diferent kinds of Fusarium molds species. Zeranol has influence in the estrogen target organs which may aquired accidentally or by purpose usage as synthetic substance (Journal ISSN: 0377-8401, 2012) [7]. Zeranol binding affinity is similar to that of Diethylstilbestrol as the main GroupA1-Stilbenes (Fitzpatrick DW, 1989) [8]. According to risk assessment regarding GPH, JEFCA established Allowance Daily Intake (ADI) for zeranol 0-0.5 µg/kg body weight equaly daily intake of zeranol of 35 µg for a 70 kg person consuming 500 gr meat. This set the Acceptable Residue Level for zeranol at 10 mg/kg in bovine liver and 2 µg/kg in bovine muscle (JEFCA-CAC, 2014) [9].

2. METHODOLOGY

2.1. Sampling

The samples were collected from 25 carcases at slaughterhouse in Kosovo. Selected slaughterhouses are located in five regions. Samples have been taken from muscle tissue and liver. Each sample contains two parallel subsample (units) whith the same characteristics from the same animal. The slaughtered animals particulary were domestic. One unit of each sample was tested for analysis and considered as target sample and other one parallel unit was treated and conserved for the necessity testing or for any additional confirmatory analysis (European Commission, 2010) [10]. Sampling procedure was carried out using the standard protocols. Sample plastic bags were marked by identification code and were placed in cool box, stored at the 4 C. Samples were collected and tested in 2015 up to 2016 years.

Table 1: Tested substances and parametres

Substance Group	Parameter	Samples No
Resorcyl Acid Lactones - A 4	Zeranole ZER	25 x 2 unit

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Table 2: Sampling place, address, number, matrix

Sampling process		
Slaughterhouses location / code	No. Samples	Matrix
PR 01 / RKS 064	5 x 2 unit	Muscle, Liver
MI 02 / RKS 061	5 x 2 unit	Muscle, Liver
PE 03 / RKS 052	5 x 2 unit	Muscle, Liver
PZ 04 / RKS 047	5 x 2 unit	Muscle, Liver
FE 05 / RKS 041	5 x 2 unit	Muscle, Liver
TOTALLY	25 sample x 2 unit	

2.2. Testing method ELISA

The method was competitive enzyme immunoassay for the quantitative analysis of zeranole-ELISA Test. The principle is based the test of the antigen-antibody reaction. The microtiter wells were coated whith capture antibodies directed against anti-zeranol antibodies. Standards, sample solution, zeranol enzyme conjugate and anti-zeranol antibodies are added. Free and enzyme conjugated zeranol, compete for the antibody binding. The same time anti-zeranol antibodies are also bound by the imobilized antibodies. Unbound enzyme conjugate removed by washing. Bound enzime converts by chromogen in blue, after this stop solution change into yellow. The plate reader 450 nm has been read. The calculation has been made from the standard curve by finding the average of relative absorbance obtained from diferent standards to its concentration the Excell Programe.

2.3. KIT-data sheets

Each KIT contains: microtiter plate 96 wells; zeranol standards; conjugated peroxidase; anti-zeranole antibody; substrate (urea peroxide); chromogen; stop solution; buffer; materials (microtiter plate, incubator, tissue mixer, vortex, evaporator, nitrogen gas, automatic and multichanel pipette in diferent size and reagents.

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Zeranol LOT Kit 11005, - Reference Standards

0 ppt (0.0 µg/kg) ;
100 ppt (0.1 µg/kg) ;
300 ppt (0.3 µg/kg) ;
900 ppt (0.9 µg/kg) ;
2700 ppt (2.7 µg/kg) ;
8100 ppt (8.1 µg/kg) ;

Table 3: Zeranol Standards Lot Kit

	Std. conc.	***OD 1***	***OD 2***	***OD avg***
S	0	1.453	1.411	1,432
0				
S	0.1	1.106	1.087	1.097
0.1				
S	0.3	0.837	0.861	0.849
0.3				
S	0.9	0.593	0.612	0.603
0.9				
S	2.7	0.365	0.373	0.369
2.7				
S	8.1	0.127	0.113	0.12
8.1				

Std-Standards;

OD1-First Optical Density

OD2-Second Optical Density

OD avg-Average Optical Density

2.4. Sample preparation (meat and offal tissue)

a). Preparation of 1XPBS: mixed 1 volume of the 10X PBS with 9 volumes of distilled water., b). Preparation of 1X PBS-Methanol Solution: mixed 3 volume of the 1X PBS with 2 volumes of methanol., c). Preparation of 1XTissue Extraction Buffer: Took all of the powder from the

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Concentrate of Tissue Extraction Buffer bag to a 200-mL bottle, added 180 mL of distilled water, vortexed 2 minutes manually, leave the solution at room temperature for 20 minutes.

Weighted 2 gr homogenized meat sample, added 6 mL of acetonitrile and 2 mL of 1XTissue Extraction Buffer. Vortexed for 3 minutes at maximum speed or shake for 20 minutes at a multi-tube vortexer . Centrifuged for 10 minutes at room temperature (20 - 25 C), transfered 3 mL of the supernatant to a new tube. Added 300 mg Tissue Cleaned Up Mix and vortexed for 30 seconds, leaved at room temp for 5 mins. Centrifuged for 10 minutes at room temperature (20 - 25 C), Transfered 2.4 mL of supernatant to a new tube. Used a rotary evaporator to dryed the sample in a 50 - 60 C water bath under reduced pressure. Alternatively, the sample can be dried by blowing nitrogen gas in a 50 - 60 C water bath. Added 300 uL of 1XPBS/Methanol, vortexed rigorously for 30 seconds at maximum speed. Used 50 mL of the sample for the assay. Regarding the Elisa testing protocol added 50 ml of each zeranole standards in duplicate different wells; added 50 ml of each sample. in duplicate in diferent sample wells; added 100 ml antibody #1 and mixed well manually for 1 minute; incubated the plate for 30 minute at room temperature; washed the plate 3 times with 250 ml in 1xWash Solution; after the last wash, the plate was dried on paper towels; added 150 ml of 1x antibody solution #2. Incubated the plate for 30 minute at room temperature; washed the plate 3 times with 250 ml 1 x Wash Solution. After the last wash, the plate was dried on paper towels; added 100 ml of TMB substrate and timed the reaction immediately after adding the substrate, mixed while incubating for 15 minute at room temperature ; added 100 ml stop buffer to stop the enzyme reaction (Ridascreen Zeranol, NO.:R3301) [11]. A standard curve constructed by the mean relative absorbance (%) obtained from each standard against its concentration on logarithmic curve.

3. RESULTS AND DISCUSSION

The reading of the programme through the optical density detection shows that one tested sample in Zeranol parameter, resulted as low optical density samples among all 30 samples. The detection results were translated by Excel Programme. One sample (3.3%) of them being 0.52 µg/kg, which exceeding the threshold allowance, and can considered as suspection limit such Community Reference recommended concentration for muscul matrix is 1 ppb (CRL,2007) [12].

In table 4 apeared standard interpretation according the KIT datashets and method validation.

According table 5, sample number 25 resulted as suspected for non compliance. The sampling place, animals identity and meat origin which contain suspected substances will be auditing subject, when the documentation and the implementation of the food traceability system in the slaughterhouse level are being provided. These results will be presented to the competent

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authority of food safety in order to take the necessary measures in the farm animals, planning of official controls and the food residue monitoring.

Table 4: Standard Interpretations (Zeranol)

STD Name	STD Conc.	% B1	% B avg	% B sd	% B sv	C	C Calc.
S 0.100	S 0.100	77.20%	76.60%	0.90%	1.20%		**0.098**
S 0.300	S 0.300	58.40%	59.30%	1.20%	2.00%		**0.301**
S 0.900	S 0.900	41.40%	42.10%	0.90%	2.20%		**0.915**
S 2.700	S 2.700	25.50%	25.80%	0.40%	1.50%		**2.627**
S 8.100	S 8.100	8.90%	8.40%	0.70%	8,2 %		**8.088**
				Log STD			
				STD Conc.			% B avg
S 0.100	S 0.100	75.90%		-2.303			76.60%
S 0.300	S 0.300	60.10%		-1.204			59.30%
S 0.900	S 0.900	42.70%		-0.105			42.10%
S 2.700	S 2.700	26.00%		0.993			25.80%
S 8.100	S 8.100	7.90%		2.092			8.40%

Legend:

- *OD 1 (first optical density)*
- *OD 2 (second optical density)*
- *OD avg (average optical density)*
- *OD sd,sv (deviation)*
- *B (absorbance)*
- *C (results calculation).*

Table 5: Optic Density reading results

1	1,307	1,315	1,311	0,006	0,4%	91,6%	**0,075**
2	0,912	0,918	0,915	0,004	0,5%	63,9%	**0,447**
3	0,759	0,777	0,768	0,013	1,7%	53,6%	**0,867**
4	0,883	0,946	0,915	0,045	4,9%	63,9%	**0,448**
5	1,016	0,989	1,003	0,019	1,9%	70,0%	**0,301**
6	0,907	0,931	0,919	0,017	1,8%	64,2%	**0,439**
7	1,133	1,143	1,138	0,007	0,6%	79,5%	**0,163**
8	1,296	1,262	1,279	0,024	1,9%	89,3%	**0,086**
9	1,183	1,169	1,176	0,010	0,8%	82,1%	**0,137**
10	0,955	0,933	0,944	0,016	1,6%	65,9%	**0,392**
11	1,035	0,997	1,016	0,027	2,6%	70,9%	**0,283**
12	0,923	0,905	0,914	0,013	1,4%	63,8%	**0,449**
13	1,183	1,143	1,163	0,028	2,4%	81,2%	**0,146**
14	1,097	1,068	1,083	0,021	1,9%	75,6%	**0,210**
15	1,194	1,141	1,168	0,037	3,2%	81,5%	**0,143**
16	1,032	1,011	1,022	0,015	1,5%	71,3%	**0,276**
17	1,083	1,064	1,074	0,013	1,3%	75,0%	**0,218**
18	1,231	1,225	1,228	0,004	0,3%	85,8%	**0,109**
19	1,154	1,151	1,153	0,002	0,2%	80,5%	**0,153**
20	1,096	1,016	1,056	0,057	5,4%	73,7%	**0,236**
21	0,998	0,958	0,978	0,028	2,9%	68,3%	**0,336**
22	1,107	1,130	1,119	0,016	1,5%	78,1%	**0,178**
23	0,914	0,916	0,915	0,001	0,2%	63,9%	**0,447**
24	0,949	0,915	0,932	0,024	2,6%	65,1%	**0,414**
25	**0,886**	**0,880**	**0,883**	**0,004**	**0,5%**	**61,7%**	**0,516**
26	0,978	0,959	0,969	0,013	1,4%	67,6%	**0,351**
27	1,165	1,175	1,170	0,007	0,6%	81,7%	**0,141**
28	0,983	0,955	0,969	0,020	2,0%	67,7%	**0,350**

4. CONCLUSION

The lower optical density, the higher analyte concentration where the minimal limit (threshold-limit) 0.52 µg/kg. The possibility of a error on the flushing solution according to the KIT manual is not excluded. In this case, further testing will be conducted through confirmatory analysis. This would result in concrete actions, such as residue monitoring in all levels of the food chain,

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starting from the animals food up to the slaughterhouse. The previous results of this study indicate that a great deal of attention should be paid to the control of suppliers and warehouses with medical and veterinary equipment, as well as to the production veterinarians, farmers, and in general the awareness building of all stakeholders related to the legislation in force. The Competent Authority itself must continue with the staff training, laboratories, enrichment with equipment to detect hormonal levels in low limits, the accreditation of laboratories and validation of methods. We conclude that the data exchange with the competent authorities of the countries from which we import meat as well as medicinal and veterinary products is of paramount importance.

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