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Monitoring of Trenbolone Residues on Bovine Meat in Kosovo

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Running title: **Evaluation of TBA Residues as Synthetic Anabolic Substances**

Abstract
The aim of this study is to present the monitoring results of synthetic growth promoting hormones (GPH), as prohibited substances which can cause serious public health consequences, according to European Scientific Committee on Veterinary Measures. The lack of a unique policy framework and standards among different countries represents an enormous drawback regarding the prevention of human exposure to growth-promoting substances. Kosovo Competent Authority adopted the National Plan for Residue Monitoring, in compliance with European Union rules, which strictly prohibits the use of Group A substances respectively Trenbolone parameter in animals used for food production, as well as consumption of meat, according to the Directive 96/22/EC and 96/23. In order to assess the occurrence of residue levels in the above- mentioned parameter of fresh bovine meat, samples from 25 animals were collected in five different regions in Kosovo, in various slaughterhouses. Samples were tested by ELISA as competitive enzyme immunoassay for the quantitative analysis, as a screening method. Out of 25 samples, two (8%) of them resulted as suspicious because of non-compliance with the screening method. Findings of this research show that it is necessary to increase the number of official controls and the samples mainly in the farm level. In addition, it would be of interest to promote ultra-sensitive methods to detect GPH residues through further validation of the current methods.

Practical applications
This monitoring programme provides data on the incidence residues for growth promoting substances which is intended to be possible in animal tissues. Trenbolone residues can be determined by several methods whith extremely procedures such GC-MC, but definitively competitive enzyme immunoassay can be used which is simple, rapid, sensitive and lower cost effective.

Key words: GPH, Elisa, meat, residue, Trenbolone

Introduction

According to the Scientific Committee on Risk Analysis meat originating from animals previously treated with illegal banned substances for growth promoting purposes " Growth Promotant Hormones" (GPH) results in high risk for consumers (ref: EFSA Journal (2007) 510,1-62). Potential target is YOPI (young, old, pregnant, immunodeficient). The European Community countries strictly prohibit GPH. In this regard the Competent Authorities have set barriers through legislations and official control measures (ref: Work Programmes on Food Audits ang Analyses, DG-Sante, European Commission / EC ec.europa.eu/dgs/health_food-safety). The use of hormonal promoters in farm animals can increase the production of veal and beef significantly up to 15%. However, in the different parts of the world the regulation regarding the use of such hormones differs sharply. In the European Union there exists a total ban on such use in contrast to the United States of America where the use of some hormones is authorized under strict conditions. (ref: Stephany, R.W., (2001). Doubtless, stakeholders such as farmers, veterinarians, veterinary pharmacies have a vital role to play in whole process. According to the scientific literature and other related publications, there exists a positive correlation between red meat consumption prostate and breast cancer incidence (Bahrke,Yesalis, 2004; Maravelias *et al.,* 2005)., and some alterations in the immune system (Quinn *et al.,* 2007b). One of the obstacles may be that not all countries possess a unique food safety policy regarding this problem such as EU and other third countries (USA, Canada, Australia, etc). The use of steroid hormones and other substances in various combinations which aim to improve weight, is common legal practice in some non-EU countries (ref: EFSA Journal (2007) 510, 1-62). EU adopted Directive 96/22/EC and 96/23/EC, as amended by Directive 2003/74/EC which prohibited the administration of GPH. Experts claim that consumption of the meat containing GPH residues could cause serious problems in human health (ref: SCVPH Reports (EC 1999; 2000; 2002;
200
7)

Group A3 (steroids) which is the subject of this publication cover Trenbolone parameter as synthetic substances. Trenbolone has synthetic anabolic properties, 8-10 times as potent as testosterone, which has a binding affinity for the androgen receptor (Bouffault and Willemart, 1983). Synthetic hormones including GPHs have been shown to bind steroid hormones receptors which have equal or higher affinity than the most potent natural hormones (Bauer et al., 2000; Wilson et al., 2002; Ankley et al., 2003; Perry et al., 2005).

Materials and methods

Sampling

The samples were collected at a slaughterhouse in Kosovo. The selected slaughterhouses were located in five regions in Kosovo. Samples have been taken from muscular tissue and offals (liver and kidney). Each sample was divided in two parallel units, which possessed similar characteristics from the same animal. The slaughtered animals were of domestic origin. One unit (subsample) of each sample was analyzed as target and other parallel unit was treated and conserved for the further necessity analysis as suspected (Decision 2002/657/EC). Sampling procedure was carried out using the standard operating procedures and protocols. In order to trace the product, during the sampling process we kept the documentation with relevant and additional information. Sample bags were identified by serial code and approval number of slaughterhouses. Samples were placed in cool box and were stored at 4-8 C. Each sample was divided in two subsamples. The samples for this purpose were collected during the year 2015.

REGION		SLAUGHTER HOUSE CODE	NO. SAMPLE
Pristina	01	RKS 064	5
Mitrovica	02	RKS 061	5
Peja	03	RKS 052	5
Prizren	04	RKS 047	5
Ferizaj	05	RKS 041	5

Table 1. Sampling place and number of samples

SUBSTANCES	PARAMETER	NO. SAMPLE
Steroids A3	Trenbolone	25

Table 2. Analyzed substances and specific parameter

ELISA Testing Method

-Procedure overview

The method was based on the competitive colorimetric ELISA assay. The drug of interest (Trenbolone-TBA) has been coated in the plate wells. During the analysis sample was added along with the primary antibody specific for the target drug. Afterwards the secondary antibody was tagged with peroxidase enzyme in order to target the primary antibody.

-Kit datasheets

ELISA Test Kit had the capacity for 96 wells plate determinations or testing of 42 samples in duplicate including 12 wells for the standards in duplicate as well. Also Kit contents: standards; trenbolone antibody #1; 100xHRP-conjugated antibody #2; diluents; wash solution; stop buffer; TMB substrate; 10xPBS; extraction buffer. KIT specificity (cross reactivity) was 100 % trenbolone (TBA) parameter as target analytes.

Trenbolone standards (*LOT Kit 11255*):

- 0 ppt (0 standard);
- 25 ppt (0.02^{5}µg/kg);
- 50 ppt (0.05µg/kg);
- 100 ppt (0.1µg/kg);
- 200 ppt (0.2µg/kg);
- 400 ppt (0.4µg/kg);

S.con.	OD 1	OD 1	OD avg	OD sd
S_0.00	1,097	1,116	**1,107**	0,013
S_25.0	0,996	0,954	**0,975**	0,030
S_50.0	0,828	0,848	**0,838**	0,014
S_100.0	0,715	0,679	**0,697**	0,025
S_200.0	0,598	0,556	**0,577**	0,030
S_400.0	0,434	0,409	**0,422**	0,018

Table 3. Standards Trenbolone Lot Kit

Legend: table 3 and 4:

-STD = standards
-OD 1 = first optical density
-OD 2 = second optical density
-OD avg = average optical density
-OD sd = optical density standard deviation
-B 1&2 = relative absorbance
-B avg = average relative absorbance

STD_Name	STD_Conc	%B_1&2	%B_avg	%B_sd
S_25.0	25,0	90,0%	**88,1%**	2,7%
S_50.0	50,0	74,8%	**75,7%**	1,3%
S_100.0	100,0	64,6%	**63,0%**	2,3%
S_200.0	200,0	54,0%	**52,1%**	2,7%
S_400.0	400,0	39,2%	**38,1%**	1,6%
S_25.0	25,0	86,2%		
S_50.0	50,0	76,6%		
S_100.0	100,0	61,4%		
S_200.0	200,0	50,2%		
S_400.0	400,0	37,0%		

Table 4. Standards Interpretation

y = a * lnX + b	
a=	-0,17836
b=	145,660

ln(STD_Conc)	%B_avg
3,219	88,1%
3,912	75,7%
4,605	63,0%
5,298	52,1%
5,991	38,1%

Table 5 / 6. Calculation curbe formulae / Relative absorbance in standards concetration

-Usage materials

Microtiter plate reader 450 nm; incubator; mixer; evaporator; mixer; pipette. This material was not provided with Kit.

-Sample preparation (meat tissue)

_i. Preparation of 1xPBS: Mixed 1 vol. of the 10xPBS with 9 vol. of distilled water; ii. Preparation of 1 PBS-Methanol Solution: Mixed 3 vol. of the 1xPBS with 2 vol. of methanol; iii. Preparation of 1xTissue Extraction Buffer: Took all of the powder from the

Concentrate of Tissue Extraction Buffer bag put to a 200-mL bottle, added 180 mL of distilled water, vortexed it 2 min., left the solution at room temp., for 20 min. Weighted 2 gr homogenized meat sample, added 6 mL of acetonitrile and 2 mL of 1xTissue Extraction Buffer. Vortexed it for 3 min. Centrifuged for 10 min., at room temp., and transferred 3 mL of the supernatant to a new tube. Added 300 mg Tissue and vortexed it for 30 sec., left it at room temp., for 5 min. Centrifuged it for 10 min at room temp. Transferred 2.4 mL of supernatant to a new tube. Used an evaporator to dry the sample. Added 300 uL of 1xPBS/Methanol, vortexed it for 30 sec. Used 50 mL of the sample for the assay.

-ELISA Testing Protocol

1. Added 50 mL of each Trenbolone Standards in duplicate into different wells; 2. Added 50 mL of each sample in duplicate into different sample wells; 3. Added 100 mL of antibody #1 and mixed it well by plate manually for 1 min; 4.Incubated the plate for 30 min. at room temp; 5.Washed the plate 3 times with 250 mL of 1x Wash Solution. After the last wash, the plate was dried on paper towels; 6. Added 150 mLof 1x antibody #2 solution. Incubated the plate for 30 min. at room temp; 7. Washed the plate 3 times with 250 of mL 1xWash Solution. After the last wash, the plate was dried on paper towels; 8. Added 100 mL of

TMB substrate. Timed the reaction immediately after adding the substrate. Mixed the solution by the plate manually for 1 min. while incubating; 9. After incubating for 15 min. at room temp. added 100 mL of Stop Buffer to stop the enzyme reaction (RIDASCREEN Trenbolone Enzyme Immunoassay for the quantitative analysis of trenbolone Art. NO.: R2601, R-Biopharm AG Darmstadt, Germany).

-Trenbolone TBA concentration calculations

A standard curve constructed by the mean relative absorbance (%) obtained from each standard against its concentration on logarithmic curve.

-Validation ELISA screening method

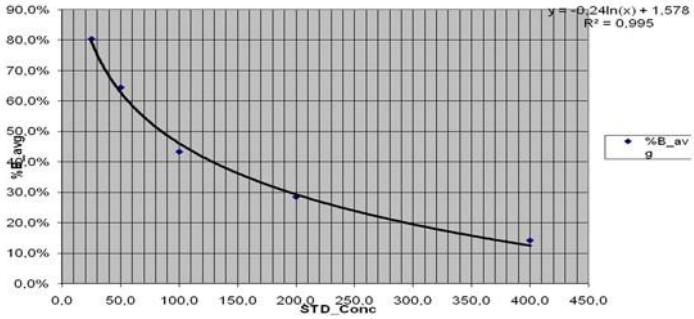

Figure1.Calibration curve on 25-400 ppt

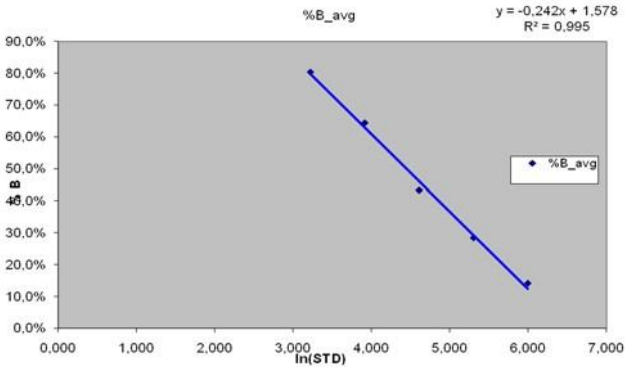

Figure2.View of logarithmic scale

Limit detection calculated through this form:

$LOD=X_{sr}+3xSD$.

Average value 65.378 ppt (0.06[54]µg/kg) between internal proves. LOD = 65.378 ppt + 3 x 7.932.

Limit detection = 89.170 ppt (0.08[92] µg/kg).

The Minimum required performance limits (MRPLs) for Trenbolone in muscle was 1µg/kg, meanwhile Lab. detection capability (CCβ-permitted limit) less/equal to respective MRPLs., as followed:

CCβ = w(MRPL) +1.64*SD.

CCβ average values 439.151 ppt (0.44µg/kg) between internal proves.
CCβ = 439.151 ppt + 1.64 x 37.228.
Final CCβ = 500.200 ppt (0.51µg/kg).

Results and Discussions

The reading of the program through the optical density detection shows that among all 25 samples tested in Trenbolone parameter, two of them resulted as low optical density samples. The detection results were translated by Excel Program. Two (8%) of them exceeded the threshold allowance, and as such were suspected of non-compliance since they have reached the suspection limit (ref: Community Reference Laboratories CRL Guidance Paper Dec - 07, 2007; Trenbolone, matrix muscle recommended concentration 1 µg/kg). According the table 7, samples no:. 23 and 24 resulted as suspected toward non-compliance respectively 0.51 µg/kg and 0.55 µg/kg (lower optical density-higher analyse), where they yield the minimal limit (threshold limit). The sampling place, animals identity as well as the meat origin suspected of containing these substances are traceable as a result of the implementation of the traceability system in the slaughterhouses.

Conclusions

This would result in concrete actions, such as increasing residue monitoring at all levels of the food chain, starting from the feed 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 among all stakeholders related to the legislation in force.

The Competent Authority itself must continue with the staff training, laboratories should be further enriched with equipment in order to detect hormonal levels in low limits, and more work needs to be done in 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 are of paramount importance.

Acknowledgements

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Schorarly articles for alterations in the immune system (Quinn et al., 2007b).

Directives 96/22/EC; 96/23/EC; 2003/74/EC; European Commission, Brussel.

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Synthetic hormones including GPHs affinity (Bauer et al., 2000; Wilson et al., 2002; Ankley et al., 2003; Perry et al., 2005).

Decision 2002/657/EC: Criteria and procedures to validate analytical methods, ensuring the quality and comparability of the results of official laboratories.

RIDASCREEN Trenbolone Enzyme Immunoassay for the quantitative analysis of trenbolone Art. NO.: R2601, R- Biopharm AG Darmstadt, Germany.

Community Reference Laboratories CRL Guidance Paper DEC/07/2017.

ANEX I

No. Sample	First Optical Density	Second Optical Density	Optical Density average	Concentrations Calculation ppt	
-	0.651	0.656	0.654	**256.813**	**blank**
-	0.427	0.363	0.395	**951.590**	**spike (recovery 95.2%)**
1	0.632	0.657	0.645	**268.796**	
2	0.710	0.676	0.693	**210.231**	
3	0.777	0.591	0.684	**220.040**	
4	0.538	0.635	0.587	**360.622**	
5	0.804	0.724	0.764	**146.710**	
6	0.791	0.767	0.779	**135.973**	
7	0.636	0.629	0.631	**287.825**	
8	0.690	0.646	0.668	**238.622**	
9	0.609	0.624	0.617	**309.768**	
10	0.823	0.890	0.857	**91.815**	
11	0.691	0.662	0.677	**228.563**	
12	0.653	0.709	0.681	**223.411**	
13	0.870	0.867	0.869	**86.399**	
14	0.807	0.765	0.786	**131.235**	
15	0.668	0.676	0.672	**233.834**	
16	0.567	0.564	0.566	**401.109**	
17	0.832	0.861	0.847	**96.587**	
18	0.824	0.737	0.781	**134.944**	
19	0.762	0.760	0.761	**148.958**	
20	0.599	0.621	0.610	**320.140**	
21	0.737	0.715	0.726	**177.861**	
22	0.671	0.686	0.679	**226.259**	
23	**0.556**	**0.484**	**0.520**	**505.110**	
24	**0.524**	**0.481**	**0.503**	**551.944**	
25	0.820	0.835	0.828	**106.321**	

Table 7. Final results according program reader

(Sample number 23 and 24 which are marked by red color, results as suspected for non-compliance samples,505.110 ppt converted to >0.51 µg/kg [no.23] and 551.944 ppt converted to >0.55 µg/kg [no.24]).

ANEX II

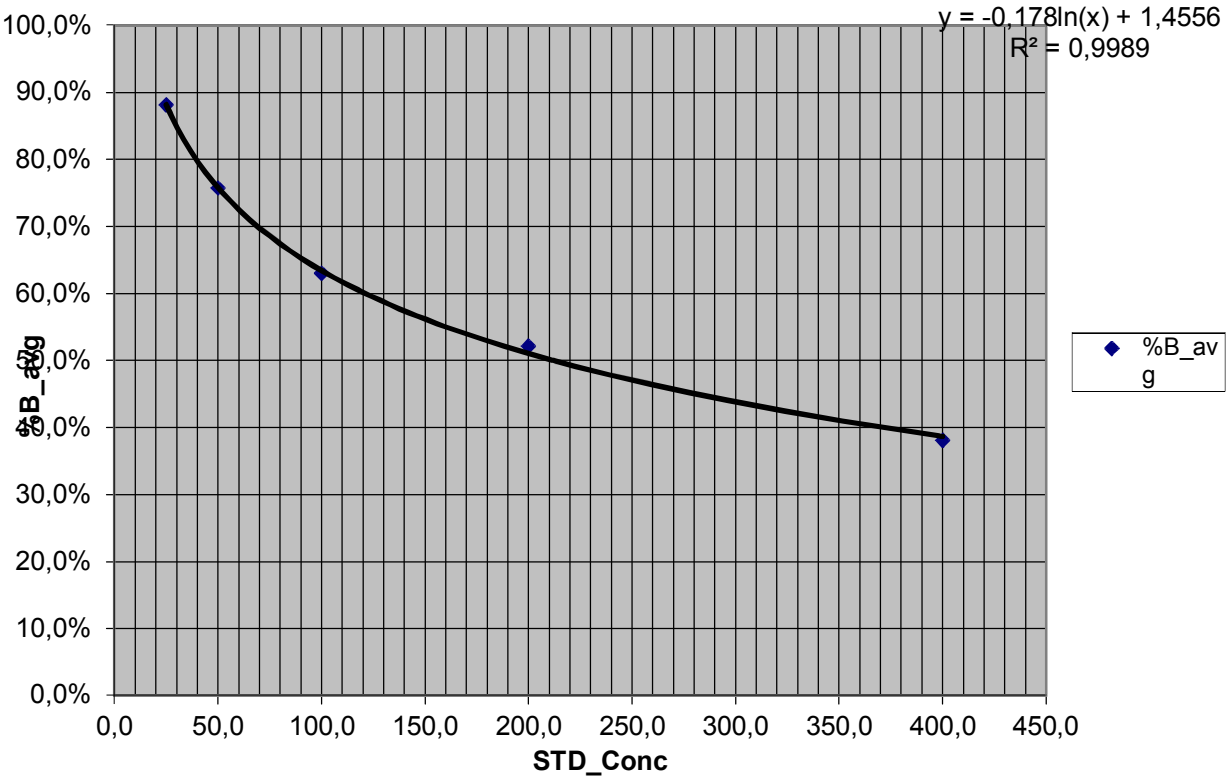

Figure3. Standard curve constructed by plotting the mean relative absorbance (%) obtained from each reference standards against its concentration (linked table 4,5,6 inside the manuscript)