CONTROL OF ANABOLIC SUBSTANCES IN URINE BY LC-MS/MS

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INTRODUCTION

The European Union, in the Council Directive 96/22/EC[1], bans the use of substances with anabolic effect in stock farming. To control the use of these unauthorized additives (such as stilbenes, steroids, resorcylic acid lactones and mycotoxins) in the production process of animals and primary products of animal origin, the Council Directive 96/23/EC[2] regulates how the member states have to elaborate the monitoring plans for its detection. As this analytes doesn't have a maximum residue limit (MRL), the Community Reference Laboratories (CRL) published the Guidance paper (7 December 2007)[3]. Recommended maximum CCα levels for the substances depending on the matrix analysed are established in this document in order to improve and harmonize the performance of the methods used in the control plans.

The Agri-food Laboratory analyses anabolic substances in urine samples as part of the Spanish National Plan for Waste Research (PNIR). In order to differentiate the presence of natural substances to the ones that indicate the fraudulent use of an anabolic substance, the previous method used for this analysis was improved.

SAMPLE TREATMENT

Weight 8 mL of sample
↓
Centrifuge 5 min, 3000 rpm
↓
Weight 5 mL of sample
↓
Add 1.5 mL acetate buffer 2M pH 4.8
↓
Add 50 µL β-glucoronidase E.Coli
↓
Digest 1 hour, 55ºC
↓
Add 10 mL H_2O and 15 mL ACN
↓
Add QuEChERS AOAC 5982-6755
↓
Agytax
↓
Sonication 5 min
↓
Centrifuge 5 min, 3500 rpm
↓
Transfer 10 mL to AOAC 5982-5158
(repeat the previous three steps)
↓
Evaporate to dryness
↓
Reconstitute with 500 µL ACN/H_20 (30:70)
↓
LC-MS/MS

VALIDATION

- The validation was done according to Decision 2002/657/EC
- During three different days, three sample series were prepared. Each series consisted of three blank urine sample, each one spiked at 0, 0.5, 0.75, 1, 1.5, 2, 3, 4 and 5 µg/L
- These series were used to calculate trueness, reproducibility, calibration curves and to calculate the quantification limit (LQ), the decision limit (CCα) and the detection capability (CCβ)
- The following internal standards were established: Testosterone-d3 (β-trembolone, α-trembolone, hydroxystanozolol, stanozolol, β-boldenone, α-metilboldenone, α-boldenone and metiltestosterone), taleranol-d5 (taleranol), zeranol-d5 (β-zearalenol, zeranol, α-zearalenol, zearalanone, zearalenone), DES-d8 (DES), dienestrol-d4 (dienestrol) and hexestrol-d6 (hexestrol)

,

	CCα (µg/L)	CCβ (µg/L)	LQ (µg/L)	Linearity range (µg/L)	Recommended CRL (µg/L)
β-trembolone	1.00	2.00	-	-	2.0
α-trembolone	1.00	2.00	-	-	2.0
Hydroxystanozolol	1.00	2.00	-	-	2.0
Stanozolol	1.00	2.00	-	-	2.0
β-boldenone	1.00	2.00	-	-	1.0
α-metilboldenone	0.75	1.50	-	-	-
α-boldenone	0.50	1.00	-	-	-
Metiltestosterone	0.50	1.00	-	-	2.0
Taleranol	0.50	1.00	1.0	1-5	2.0
β-zearalenol	1.00	2.00	-	-	-
Zeranol	0.50	1.00	1.0	1-5	2.0
α-zearalenol	0.50	1.00	1.0	1-5	-
Zearalanone	0.50	1.00	1.0	1-4	-
Zearalenone	0.50	1.00	1.0	1-5	2.0
DES	0.50	1.00	1.0	1-5	1.0
Dienestrol	0.50	1.00	1.0	1-5	2.0
Hexestrol	0.50	1.00	1.0	1-5	2.0

LC-MS/MS ANALYSIS

Chromatographic conditions

- Column: Atlantis dC18 3µm 2.1*100mm
- Mobile phase A: ACN
- Mobile phase B: H_2O
- Injection volume: 5 µl
- Column temperature: 35°C
- Flow: 0.3 ml/min

Time (min)	%A	%B
0	30	70
17.00	40	60
17.10	50	50
25.00	90	10
26.00	90	10
26.10	30	70
27.50	30	70

Mass Spectrometer conditions

Ionization mode	ESI +/ESI -
Capillary (kV)	2.5
Source Temperature (°C)	150
Desolvation Temperature (°C)	650
Cone Gas Flow (L/Hr)	75
Desolvation Gas Flow (L/Hr)	950
Collision Cell Gas (Ar) Flow	3-3.5*10^{-3}

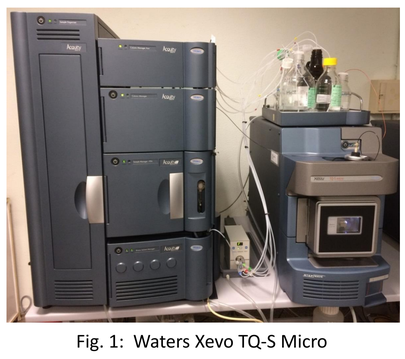
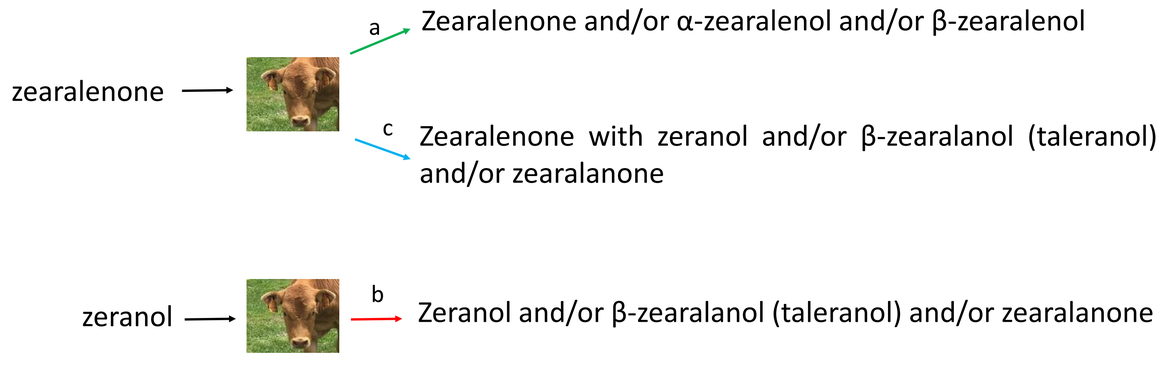


Fig. 1: Waters Xevo TQ-S Micro

CONTROL OF RAL'S AND MYCOTOXINS

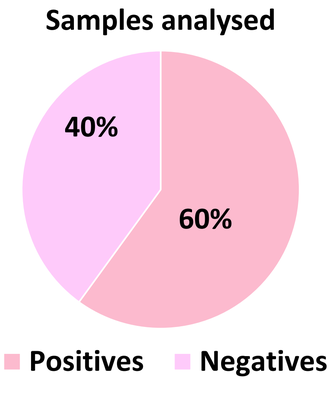
Evaluation of results in urine samples[4]

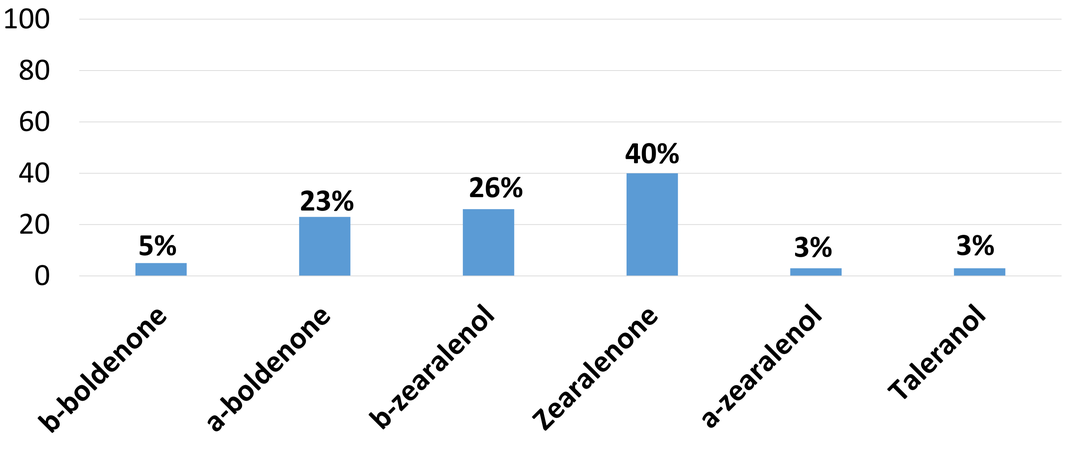
- The presence of **Zearalenone** and its metabolites **(α-zearalenol and β-zearalenol)** indicates that the **feed** was **contamined** (natural substances) **(a)**
- The presence of **zeranol** and/or **taleranol** and/or **zearalanone <u>without</u> the presence of zearalenone and its metabolites** indicates an **illegal treatment** with zeranol (b)
- The presence of **zeranol** and/or **taleranol** and/or **zearalanone <u>with</u> the presence of zearalenone and its metabolites** indicates that the **feed** was **contamined (c)**



ROUTINE OVERVIEW

During 2020, 62 samples were analysed





Total of positive samples	37
Steroids	15
RAL's	2
Mycotoxins	29

In some cases, the same sample is positive in more than one family of analytes

CONCLUSIONS

- This method is fit per porpoise for the analysis of 17 anabolic substances in urine
- Most of the positive samples found in routine were due to the presence of mycotoxins
- In the case of RAL's, the 3% of positive analytes in taleranol are also positive samples in zearalenone. This indicates that the presence of taleranol with the presence of zearalenone in the same sample, is probably due to the fact that the feed was contaminated (no confirmation of illegal treatment with zeranol).

BIBLIOGRAPHY

(1) Council Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists
(2) Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues there of in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC
(3) CRL Guidance Paper (7 December 2007)
(4) Comunicación CNA No 110: Zearalenona en el control del tratamiento ilegal con zeranol

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