Application Note GCMS-04

Analysis of Trenbolone at low levels in animal tissues using the EVOQ GC-TQ

Introduction

Trenbolone, administered as trenbolone esters, is a steroid presenting strong anabolic properties. In rearing animals, it is known to improve weight gain and feed conversion efficiency, resulting in faster growth of the animals and subsequent reduction of production costs.

The administration of natural and synthetic steroids as growth promoters for food-producing animals has been banned within the European Union since 1988, while some hormones are still licensed in other countries around the world. For instance, estradiol, progesterone, testosterone, melengesterol acetate, trenbolone acetate, and zeranol are banned within the EU in cattle farming and should not be present in meat (either imported or produced within the EU).

The European Scientific Committee on Veterinary Measures Relating To Public Health (SCVPH) concluded that for all six hormones endocrine, developmental, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects could be envisaged, but the available data do not enable a quantitative estimate of the risk. Even exposure to small levels of residues in meat and meat products carries risks and no threshold levels can be established for any of the six substances. Of the various risk groups, pre-pubertal children are the group of greatest concern.

Authors

- Marc Bourgeois, Bruno Veyrand, Philippe Marchand, Fabrice Monteau, Bruno Le Bizec.
LUNAM Université, ONIRIS, Ecole Nationale Vétérinaire, Agroalimentaire et de l'Alimentation Nantes-Atlantique, Laboratoire d'Etude des Résidus et Contaminants dans les Aliments (LABERCA), Atlanpôle-La Chantrerie, BP 50707, Nantes F-44307, France.

- Alexandre Collgros, application engineer.
Bruker Daltonique France, Chemical & Applied Market

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In order to avoid consumers' exposure to these residues, efficient monitoring of the banned chemicals is necessary. Searching for illicit use of hormones is carried out under the terms of EU directive 96/23/EC. Since the ban in 1988, the constant evolution in analytical chemistry enables the detection and quantification of molecules at lower and lower levels. Current MS/MS techniques permit the detection of steroids in meat at the sub-ppb level (ng/g).

After intra-muscular administration to the animal, trenbolone esters are metabolized into, among other things, 17α and 17β-trenbolone. The 17β-epimer is the most biologically potent compound, while the 17α-epimer only possesses about 5% of the biological activity of the 17β-epimer. Furthermore, as trenbolone is strictly a xenobiotic, detecting its metabolites is sufficient to confirm its use.

A sensitive method was developed using a EVOQ GC-TQ for both target molecules in animal tissues. Moreover, very specific diagnostic signals were optimized with Selected Reaction Monitoring (SRM), using a special derivatization with MSTFA / I_2. According to the regulatory requirements in Commission Decision 2002/657/EC, two SRM transitions were required for each analyte, providing unambiguous identification.

Instrument

Gas chromatography (436 GC™, Bruker) coupled to a triple quadrupole mass spectrometer (EVOQ GC-TQ™, Bruker).

Material and reagents

For this study, 17α-trenbolone and 17β-trenbolone were purchased from Roussel-Uclaf (France), and 17β-trenbolone-d3 from the National Institute for Public Health and the Environment (RIVM, the Netherlands). Standard solutions were prepared in ethanol and were kept in a freezer at -10°C. Derivatization reagent was prepared with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) from Fluka, with Iodine (I2) as catalyzer.

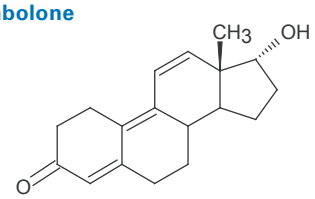
Sample preparation

15 g of fresh muscle tissue were freeze-dried and further ground into a fine powder. All samples were fortified with deuterated trenbolone as an internal standard in order to obtain a final concentration of 1.0 ng/g. The spiked sample, used as quality control, was also spiked with target analytes, 17α-Trenbolone 17β-Trenbolone, at the level of 0.5 ng/g. A specific derivatization protocol developed in the LABERCA (Maume *et al., Analyst*, 1998) was used.

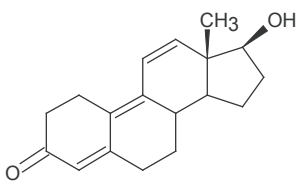
Sample preparation was performed as follows and illustrated below. After liquid/solid extraction of the meat powder with a mixture of methanol / acetate buffer 0.2M

Chemical structure of Trenbolone

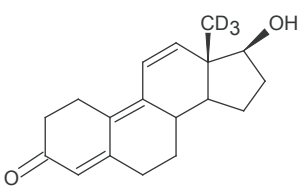
17α-Trenbolone



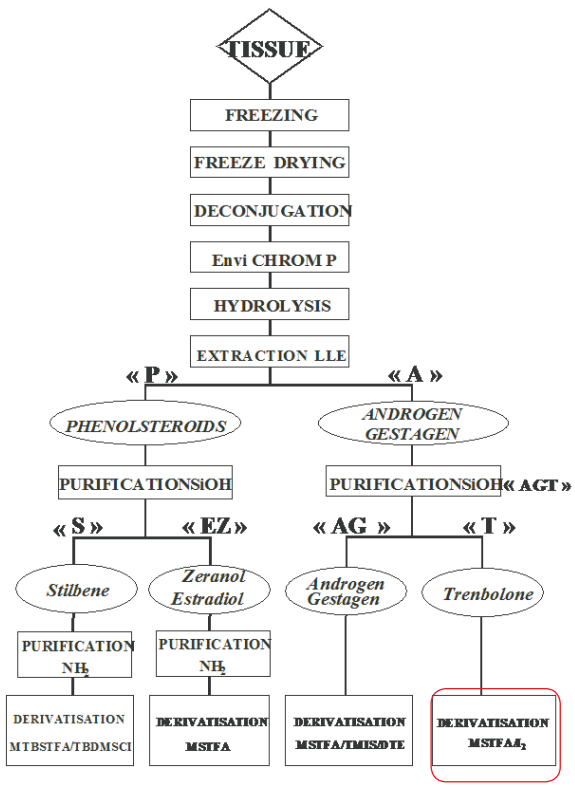
17β-Trenbolone



17β-Trenbolone-d3 (Internal standard)



Synthetic overview of the applied procedure



and centrifugation (2500 g, 20 min), the supernatant was collected. A deconjugation step (enzymatic hydrolysis) was then performed using *Helix pomatia* extract during 15h at 50°C. After centrifugation the supernatant was applied on an Envi-ChromP solid phase extraction (SPE) column. The column was washed with hexane and the analytes of interest were eluted with a mixture of hexane / diethylether (70/30), evaporated, and then further hydrolyzed with 1% sodium methylate (50°C / 30 min).

The "phenol-steroids" group and the "androgen – progestagen" group were separated with a liquid / liquid extraction: 1 mL of sodium hydroxide 1 M and 8 mL of hexane / diethylether mix (70/30, v/v) were added and the sample vortexed. The "androgen – progestagen" fraction was purified on SPE SiOH cartridge, and analytes were eluted with a mixture of hexane / ethyl acetate (60/40). Derivatisation with MSTFA / I_2 reagent was done on the final dry residue before injection.

GC-MS/MS Conditions

Samples and calibration solutions were injected with the following GC and MS/MS parameters. For ionization, filament current was set to 80 µA. Argon was used as collision gas at a pressure of 2.0 mTorr.

For data acquisition, windows of 3 minutes width were centered at the specified retention time, and scan times were set by the software at 100 ms for each compound, adjusted for peak width (in sec), desired number of data points per peak, and number of transitions simultaneously monitored.

Chromatographic conditions

Column	DB5 MS 30 m x 0.25 mm x 0.25 µm
Gas	Helium, 1.0 mL/min constant flow
Injector	250°C, Splitless
Injection volume	2 µL

Oven temperature program

Rate (°C/min)	Temperature(°C)	Hold (min)
-	120	2
15	300	7

Full scan and product-scan ion mode acquisitions were used to optimize transitions and collision energies of target analytes. Selected transitions are described in the next table.

MS Conditions

Ionisation mode	Electron Impact, 70eV
Source temperature	250°C
Transfer Line Temperature	310°C
Acquisition mode	SRM
Quadrupoles Resolutions	Standard on Q1 (i.e. 2.0 amu width) Unit on Q3 (i.e. 0.7 amu width)

Results:

Each batch of analysis included as quality controls a spiked sample (0.5 ng/g), a blank (compliant) sample and a mixture of analytical standards.

SRM Transitions monitored for each target analyte

Compound	Transition 1 (CE, V)	Transition 2 (CE, V)
17α - Trenbolone	380.3>323.3 10 V	449.3>307.3 20V
17β - Trenbolone	380.3>323.3 10V	442.3>311.3 20V
17β – Trenbolone-d3	445.3>310.3 25V	445.3>311.3 25V

Ion chromatograms of spiked equine muscle are shown in figure 1.

The quantifier transition (most intense signal) is common for 17α and 17β trenbolone. However, the two qualifiers transitions (illustrated on figure 1) are specific of each compound.

Chromatographic resolution obtained between 17α-trenbolone and 17β-trenbolone was superior to 1.

About 10% of the total trenbolone spiked in the tissue could be recovered in the final sample. The two main reasons were the recovery and the fact that 50% of the trenbolone was also retrieved in another fraction (androgen-progestagen fraction as described above) and then submitted to another kind of derivatization. However, this multi residue strategy enables detection of 20 steroids with the protocol described above.
Limits of quantification for both compounds were evaluated at 0,1 ng/g in meat using the EVOQ GC-TQ.

Diagnostic ion chromatograms obtained for an unknown equine sample are shown in Figure 2. Specific transitions monitored for trenbolone showed no interference and allowed unambiguous results. This extract of meat was therefore declared compliant regarding to European criteria.

Conclusion

An efficient GC-MS/MS method has been developed for both screening and confirmation of trenbolone in animal tissue. The sensitivity of the EVOQ GC-TQ system, combined with the specificity of SRM acquisition allow the detection of 17α and 17β-trenbolone at 0.1 ng/g level which is in accordance with concentrations observed in incurred samples.

Ion Chromatograms of equine muscle sample spiked with 0.5 ng/g of 17α and 17β-Trenbolone.

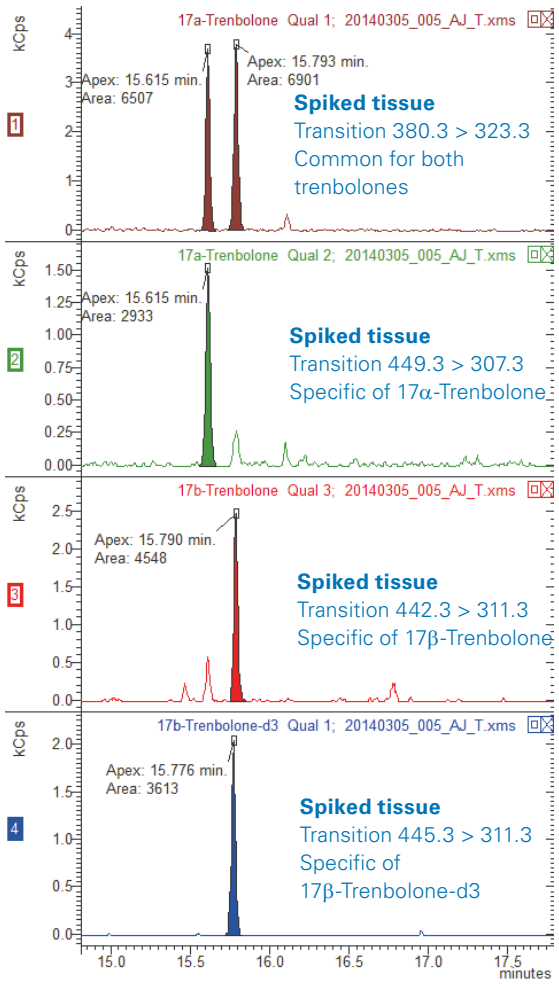


Figure 1: spiked equine muscle, with a common transition (m/z 380.3>323.3) for α and β-trenbolones (quantifier transition in, brown), a specific transition for each one (qualifiers in green and red), and the internal standard (m/z 445.3>311.3, in blue).

Chromatogram: equine muscle, sample "303T"

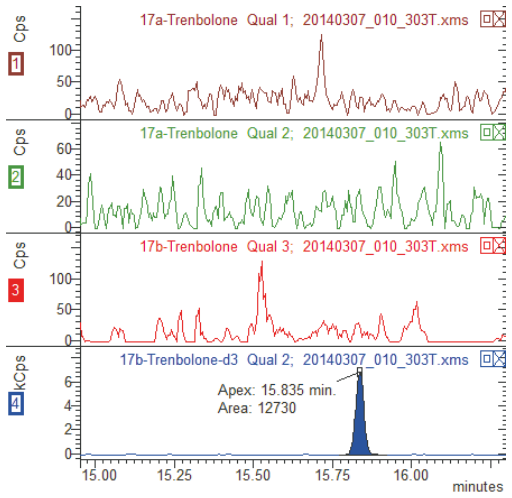


Figure 2: Diagnostic ion chromatograms obtained for an unknown equine muscle sample. Transitions and colors of chromatograms correspond to description of fig. 1 above.

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References

- Maume, Le Bizec, Marchand, Montrade and Andre. N-Methyl-N-alkylsilylfluoroacetamide – I2 as a new derivatization reagent for anabolic steroid control. Analyst, 123 (1998) 2645-2648.

- Marchand, Le Bizec, Gade, Monteau, Andre. Ultra trace detection of a wide range of anabolic steroids in meat by gas chromatography coupled to mass spectrometry. Journal of Chromatography A, 867 (2000) 219–2331

- EU COMMISSION DECISION 2002/657/EC N° L221/8 of 12 August 2002, Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.

- COUNCIL DIRECTIVE 96/23/EC N° L125/10 of 29 April 1996 on measures to monitor certain substances and residues thereof 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.

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Bruker Daltonik GmbH

Bremen · Germany
Phone +49 (0)421-2205-0
Fax +49 (0)421-2205-103

Bruker Daltonics Inc.

Billerica, MA · USA
Phone +1 (978) 663-3660
Fax +1 (978) 667-5993

ms.sales.bdal@bruker.com - www.bruker.com