GC-MS/C/IRMS AS A CONFIRMATORY ANALYSIS FOR THE ADMINISTRATION OF NATURAL STEROID HORMONES IN BOVINES

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Introduction

Abuse of hormonal substances is actively monitored for food safety reasons in the European Union, using GC/MS and LC/MS. However, the abuse of natural steroid hormones remains undetected through this approach. Evaluating the difference in $\delta^{13}C_{VPDB}$ values between a metabolite (M) and an endogenous reference compound (ERC), $\Delta^{13}C_{VPDB}$, allows to differentiate between endogenous and exogenous steroids (figure 1). Here, the measurement of a combination of different metabolites and one reference compound in urine samples, allows the simultaneous detection of abuse of natural estrogens,[2] androgens,[3] and progestagens.[4] (figure 2)

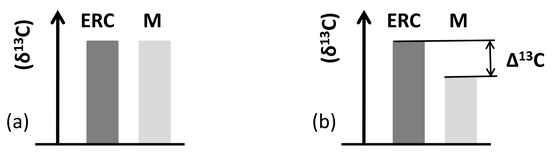
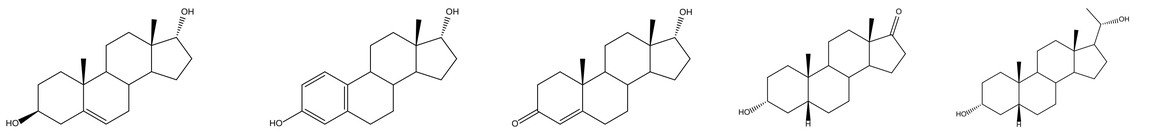


Figure 1: Visualization of $\Delta^{13}C$ value and the effect of (a) non-administration, (b) administration of exogenous steroid hormones[1]



5-Androstene-3β,17α-diol (AEdiol) ERC | 17α-Estradiol (αE2) EM | 17α-Testosterone (αT) AM | Etiocholanolone (Etio) AM | 5β-pregnane-3α,20α-diol (PD) PM

Figure 2: Structure of the endogenous reference compound (ERC), estrogen (EM), androgen (AM) and progestagen metabolites (PM)

Sample preparation

An extensive sample preparation protocol was developed (figure 3), consisting of a hydrolysis, a solid phase extraction (SPE), two subsequent liquid-liquid extractions (LLEs), and two subsequent HPLC-purification steps. After acetylation, the five target analytes, isolated into different fractions, are analyzed using gas chromatography coupled to both mass spectrometry and combustion/isotope ratio mass spectrometry (GC-MS/C/IRMS) in parallel.

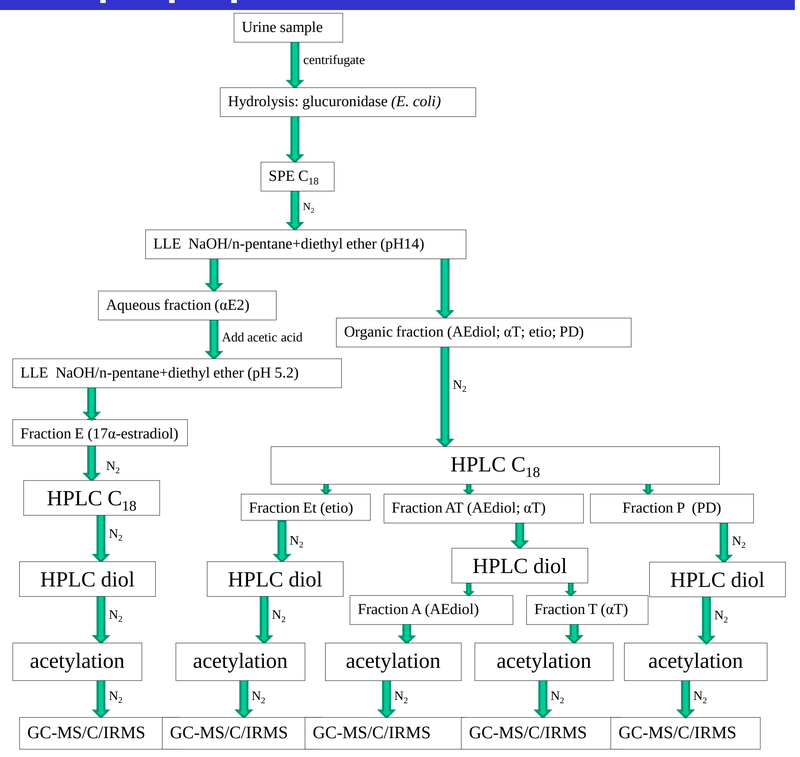


Figure 3. Analytical strategy for the extraction and purification of the samples

Chromatography

- The use of GC-MS/C/IRMS allows simultaneous determination of the $\delta^{13}C_{VPDB}$ values and evaluation of the identity and purity of the extracted analytes, eliminating the need for additional GC-MS analysis.
- The obtained fractions are sufficiently clean to provide accurate IRMS measurements (figure 4).

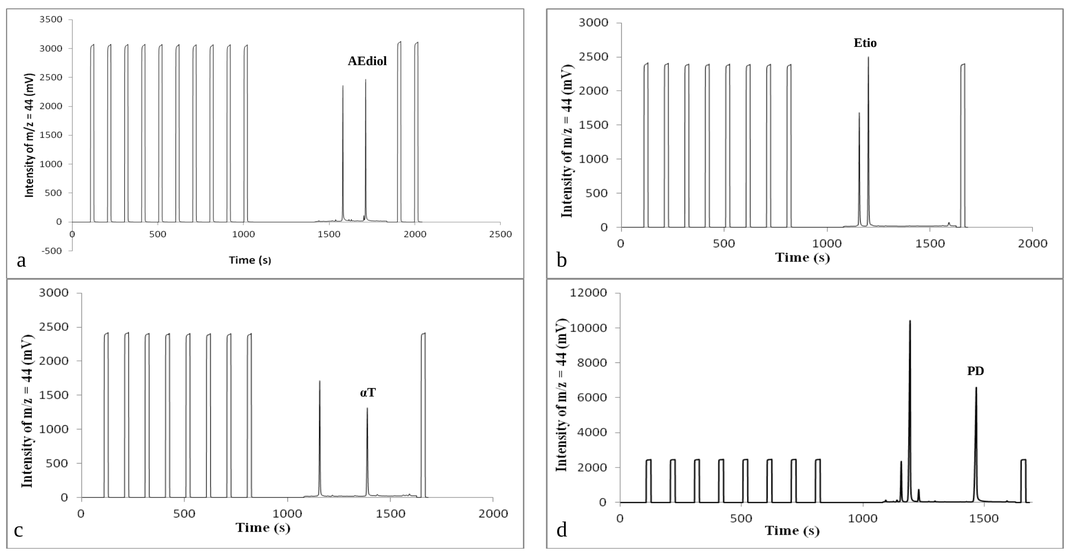


Figure 4. Examples of IRMS chromatograms of fraction A (a), fraction Et (b) fraction T (c) and fraction P (d) in compliant urine samples

Results compliant control population

$\Delta^{13}C_{VPDB}$ values of a control population were measured and threshold values for compliance could be determined (figure 5).

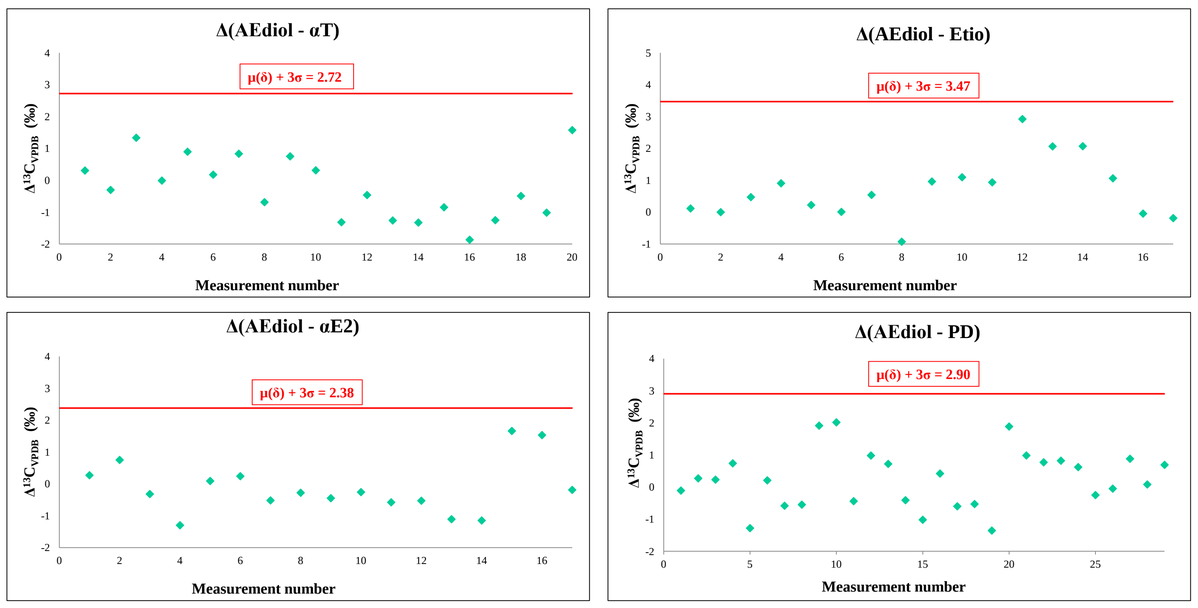


Figure 5. Plot of the $\Delta^{13}C$ values in bovine urine samples from a compliant control population

Results progesterone treated animals

Three cows were treated intramuscularly with progesterone (PG). Cow A and B were on a hay-based feeding regime, and received 2 injections of 200 mg PG, 24 h apart. Cow C was on a corn-based diet, and received 3 injections of 1 g PG, 24 h apart. Even though the impact of the injections on both the levels and $\delta^{13}C_{VPDB}$ values of PD differed significantly between the animals, it was possible to demonstrate the treatment in all 3 of them (figure 6).

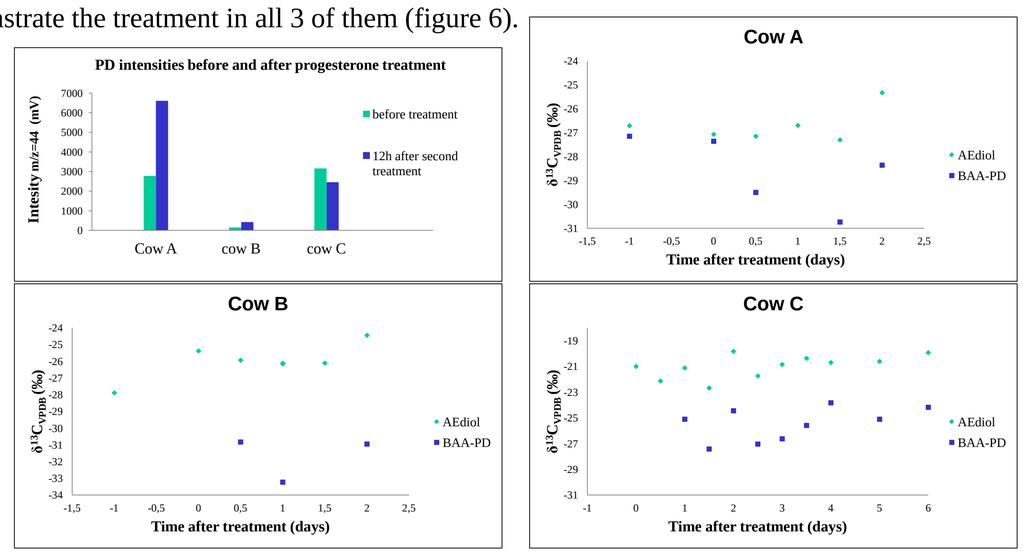


Figure 6. Effect of progesterone treatment on abundance (upper left) and $\Delta^{13}C$ values of pregnanediol in urine samples of a three cows

Results testosterone/estradiol treated animals

In urine samples from a bull and a heifer, treated with a single intramuscular injection containing testosterone propionate and estradiol benzoate, this treatment could be confirmed. Unexpectedly, this treatment had a significant influence on the $\Delta^{13}C_{VPDB}$ values of PD as well (figure 7).

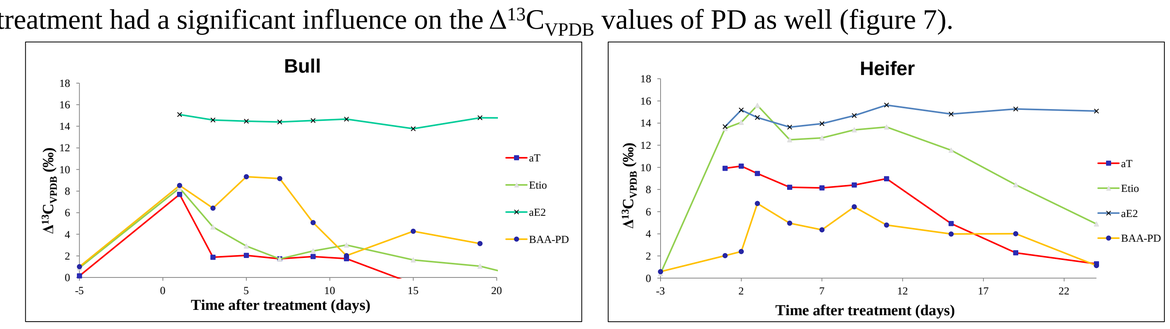


Figure 7. $\Delta^{13}C$ values in urine samples of a bull (left) and a heifer (right) after treatment with estradiol benzoate and testosterone propionate

Conclusions

- A method for the simultaneous detection of abuse of testosterone, estradiol and progesterone in bovines was developed and validated.
- The method allowed to successfully differentiate between samples from treated and untreated animals.
- Although not specific for progesterone treatment, PD can be successfully used as a steroid metabolite.

References: [1] Janssens G.; Courtheyn D.; Mangelinckx S.; Prévost S.; Bichon E.; Monteau F.; De Poorter G.; De Kimpe N.; Le Bizec B. *Anal. Chim. Acta* **2013**,*772*, 1-15. [2] Janssens G.; Mangelinckx S.; Courtheyn D.; Prévost S.; De Poorter G.; De Kimpe N.; Le Bizec B. *J. Agric. Food Chem.* **2013**, *61*, 7242-7249. [3] Janssens G.; Mangelinckx S.; Courtheyn D.; De Kimpe N.; Matthijs B.; Le Bizec B. *J. Agric. Food Chem.* **2015**, *63*, 7574-7581. [4] Janssens G.; Mangelinckx S.; Courtheyn D.; De Kimpe N.; Matthijs B.; Le Bizec B. *J. Chromatogr. A* **2016**, *1449*, 129-140.