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# 5α-Estrane-3β,17β-diol and 5β-estrane-3α,17β-diol: Definitive screening biomarkers to sign nandrolone abuse in cattle?

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ABSTRACT

17β-Nandrolone (17β-NT) is one of the most frequently misused anabolic steroids in meat producing animals. As a result of its extensive metabolism combined with the possibility of interferences with other endogenous compounds, detection of its illegal use often turns out to be a difficult issue. In recent years, proving the illegal administration of 17β-NT became even more challenging since the presence of endogenous presence of 17β-NT or some of its metabolite in different species was demonstrated. In bovines, 17α-NT can occur naturally in the urine of pregnant cows and recent findings reported that both forms can be detected in injured animals. Because efficient control must both take into account metabolic patterns and associated kinetics of elimination, the purpose of the present study was to investigate further some estranediols (5α-estrane-3β,17β-diol (*abb*), 5β-estrane-3α,17β-diol (*bab*), 5α-estrane-3β,17α-diol (*aba*), 5α-estrane-3α,17β-diol (*aab*) and 5β-estrane-3α,17α-diol (*baa*)) as particular metabolites of 17β-NT on a large number of injured ($n = 65$) or pregnant ($n = 40$) bovines. Whereas the metabolites *abb*, *bab*, *aba* and *baa* have previously been detected in urine up to several days after 17β-NT administration, the present study showed that some of the isomers *abb* (5α-estrane-3β,17β-diol) and *bab* (5β-estrane-3α,17β-diol) could not be detected in injured or pregnant animals, even at very low levels. This result may open a new way for the screening of anabolic steroid administration considering these 2 estranediols as biomarkers to indicate nandrolone abuse in cattle.



## 1. Introduction

The use of anabolic compounds is prohibited in food producing animals in the European Union [1]. Amongst other steroids, 17β-nandrolone (17β-19-nortestosterone, 17β-hydroxyestr-4-en-3-one) (17β-NT) may be used for its anabolic activity comparable to that of testosterone with less androgenic associated side effects [2]. Detection of anabolic steroid abuse is performed through effective monitoring, detection, identification and confirmation methods. In this context, gas chromatography coupled to mass spectrometry (GC–MS) is the technique commonly used for the analysis of urine samples by anti-doping laboratories [3–9]. Improved analytical sensitivity, leading to increased periods of detection post drug administration, can be achieved in urine using MS/MS approaches either on GC [10–13] or LC [14–19] systems. Efficient control of the illegal use of anabolic steroids must both take into account metabolic patterns and associated kinetics of elimination [6,12,20] to target analytes of interest and appropriate detection windows. Regarding 17β-NT metabolism, studies have mainly been reported on human subjects [6,12,13,21–25] but also on horses [26], pigs and bovine animals [27–30], but in particular calves [27–35]. In horses [36–38], main reported metabolites observed after 17β-NT administrations are 17α-nortestosterone (17α-NT), 19-noretiocholanolone (NE) but also 19-norandrosterone (NA), which has rarely been observed in cattle. These metabolic pathways are slightly different from that observed in human [6,12,21,24,26] and pig [38–40] where no epimerization exists and main observed metabolites are NA and NE. In cattle, the major pathway is C17 epimerization, 17α-NT being the most abundant metabolite after 17β-NT administration [27–30,34,41]. Most previous studies have also reported estranediols as important metabolites resulting from the complete reduction of Δ4-3-oxo group, in different animal species. The isomers 5β-estrane-3α,17β-diol (*bab*) and 5α-estrane-3β,17β-diol (*abb*) have been identified in pigs after 17β-nandrolone laureate ester administration [38–40], and in horses the isomers *aba* and *abb* have been detected as main

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**Table 1**
Nandrolone and estranediols concentrations in urine samples collected on 40 pregnant cows (gestation stage is indicated in months).

| | Nandrolone and estranediols concentrations ($\mu g\,L^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | β-NT | α-NT | *baa* | *aab* | *aba* | *bab* | *abb* |
| 4 months | <LOD | 4.3 | 1.2 | <LOD | <LOD | <LOD | <LOD |
| 4 | <LOD | 9.2 | 1.8 | <LOD | 6.6 | <LOD | <LOD |
| 4 | <LOD | 2.7 | <LOD | <LOD | 3.3 | <LOD | <LOD |
| 4 | <LOD | <LOD | <LOD | <LOD | 0.6 | <LOD | <LOD |
| 5 | <LOD | 4.8 | 1.1 | <LOD | 4.6 | <LOD | <LOD |
| 5 | <LOD | 7.6 | 1.4 | <LOD | 2.4 | <LOD | <LOD |
| 5 | <LOD | 0.0 | 1.1 | <LOD | 1.2 | <LOD | <LOD |
| 5 | <LOD | 12.2 | 2.5 | <LOD | 6.2 | <LOD | <LOD |
| 5 | <LOD | <LOD | <LOD | <LOD | 1.5 | <LOD | <LOD |
| 5 | <LOD | 1.5 | <LOD | <LOD | 1.7 | <LOD | <LOD |
| 5 | <LOD | 0.0 | 0.8 | <LOD | 2.2 | <LOD | <LOD |
| 5 | <LOD | 0.8 | <LOD | <LOD | 0.4 | <LOD | <LOD |
| 5 | <LOD | <LOD | <LOD | <LOD | 0.7 | <LOD | <LOD |
| 6 | 2.5 | 6.5 | 2.3 | <LOD | 7.4 | <LOD | <LOD |
| 6 | <LOD | <LOD | <LOD | <LOD | 5.9 | <LOD | <LOD |
| 6 | <LOD | 2.3 | <LOD | <LOD | 8.7 | <LOD | <LOD |
| 6 | <LOD | 1.8 | <LOD | <LOD | <LOD | <LOD | <LOD |
| 6 | <LOD | 4.1 | 1.0 | <LOD | 2.2 | <LOD | <LOD |
| 6 | <LOD | <LOD | <LOD | <LOD | 0.7 | <LOD | <LOD |
| 6 | <LOD | <LOD | <LOD | <LOD | 0.9 | <LOD | <LOD |
| 6 | <LOD | 1.5 | <LOD | <LOD | 0.6 | <LOD | <LOD |
| 6 | <LOD | <LOD | <LOD | <LOD | 1.4 | <LOD | <LOD |
| 6 | <LOD | <LOD | <LOD | <LOD | 0.5 | <LOD | <LOD |
| 7 | <LOD | 3.5 | <LOD | <LOD | <LOD | <LOD | <LOD |
| 7 | <LOD | 6.9 | <LOD | <LOD | 46.8 | <LOD | <LOD |
| 7 | <LOD | 2.1 | <LOD | <LOD | <LOD | <LOD | <LOD |
| 7 | <LOD | 2.5 | <LOD | <LOD | 1.4 | <LOD | <LOD |
| 7 | <LOD | 5.1 | <LOD | <LOD | 18.5 | <LOD | <LOD |
| 7 | <LOD | 5.1 | 3.2 | <LOD | 10.3 | <LOD | <LOD |
| 7 | <LOD | 0.5 | <LOD | <LOD | 1.6 | <LOD | <LOD |
| 7 | <LOD | <LOD | 1.7 | <LOD | 3.4 | <LOD | <LOD |
| 7 | <LOD | 1.4 | <LOD | <LOD | 1.3 | <LOD | <LOD |
| 8 | <LOD | 3.6 | <LOD | <LOD | <LOD | <LOD | <LOD |
| 8 | <LOD | 16.3 | <LOD | <LOD | 26.4 | <LOD | <LOD |
| 8 | <LOD | 2.4 | <LOD | <LOD | <LOD | <LOD | <LOD |
| 8 | <LOD | 4.2 | <LOD | <LOD | 18.1 | <LOD | <LOD |
| 8 | <LOD | 3.4 | <LOD | <LOD | 9.7 | <LOD | <LOD |
| 8 | <LOD | 5.7 | 1.1 | <LOD | 18.8 | <LOD | <LOD |
| 8 | <LOD | <LOD | 1.5 | <LOD | 3.0 | <LOD | <LOD |
| 8 | 2.0 | <LOD | 1.8 | <LOD | 5.2 | <LOD | <LOD |

metabolites [36,37]. In bovines, a recent study reported quantitative data on estranediols profiles after the administration of 17β-nortestosterone laureate to calves focussing on five isomers which were selected and monitored including 5α-estrane-3β,17β-diol (*abb*), 5β-estrane-3α,17β-diol (*bab*), 5α-estrane-3β,17α-diol (*aba*), 5α-estrane-3α,17β-diol (*aab*) and 5β-estrane-3α,17α-diol (*baa*) [35]. The metabolites *aba*, *baa*, *bab* and *abb* could be detected in the samples, the configuration *aba* being the major one as already highlighted [27,28,30,42].

This result may open a new way for the control of anabolic steroid administration by global steroid profiling and therefore extend the field of investigation. Indeed, and mainly as a result of this extensive metabolism combined with the possibility of interferences with other endogenous compounds, detection of the illegal use of 17β-NT often turns out to be a difficult issue. In recent years, proving the illegal administration of 17β-NT became even more challenging since different authors reported data to demonstrate the endogenous presence of 17β-NT or some of its metabolites in different species [23,43–46]. In bovines particularly, it has been known for a long time that 17α-NT can occur naturally in the urine of pregnant cows [47], it was generally accepted that neither 17α-NT nor 17β-NT could occur naturally in the urine of steers or bulls [48]. Nevertheless, some recent findings reported that both forms can be detected in injured animals [49] and that 17α-NT together with *aba*-estranediol can be present at very low concentration in the urine of un-injured steers [50] which will most likely represent a difficulty for EU member states that currently rely measurement of 17α-NT and 17β-NT to monitor and control the abuse of this popular drug. In this context, the purpose of the present work was to extend the previous estranediol study [35] to pregnant cows and injured animals. The main objective was the establishment and comparison of estranediols profiles in the different populations of interest.

## 2. Materials and methods

### 2.1. Biological samples

Urine samples have been collected from pregnant cows ($n = 40$) (Breed: Limousine) presenting different gestation states ranging from 4 to 8 months, and at the slaughterhouse from mixed breed injured steers ($n = 65$). In the EU, carcases of injured animals slaughtered on-farm for welfare reasons, can enter the food chain. Such animals must have had a satisfactory *ante mortem* veterinary inspection before slaughter. Animals must also be transported within a time limit to abattoirs for processing and *post mortem* inspection. Injuries may also be detected/reported during *ante mortem* inspection of live animals considered fit to travel for slaughter. Apart from the

**Table 2**
Nandrolone and estranediols concentrations in urine samples collected on 65 injured mixed breed steers.

| Nandrolone and estranediols concentrations ($\mu g\,L^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|
| β-NT | α-NT | *baa* | *aab* | *aba* | *bab* | *abb* |
| <LOD | 0.4 | 0.0 | <LOD | 0.2 | <LOD | <LOD |
| <LOD | 5.3 | 5.5 | <LOD | 23.4 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 3.5 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 0.3 | <LOD | <LOD |
| <LOD | <LOD | 1.9 | <LOD | 12.3 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 4.6 | <LOD | <LOD |
| <LOD | 4.4 | 1.7 | <LOD | 0.6 | <LOD | <LOD |
| <LOD | <LOD | 1.2 | <LOD | 0.6 | <LOD | <LOD |
| <LOD | <LOD | 3.2 | <LOD | 0.6 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 0.2 | <LOD | <LOD |
| <LOD | <LOD | 0.3 | <LOD | 1.3 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 4.3 | <LOD | <LOD |
| <LOD | 0.2 | 0.4 | <LOD | 1.6 | <LOD | <LOD |
| 0.1 | 1.2 | 0.4 | <LOD | 0.5 | <LOD | <LOD |
| <LOD | 1.4 | 0.6 | <LOD | 1.8 | <LOD | <LOD |
| <LOD | 0.8 | 1.0 | <LOD | 5.7 | <LOD | <LOD |
| <LOD | <LOD | 0.4 | <LOD | 2.8 | <LOD | <LOD |
| <LOD | 0.5 | 0.4 | <LOD | 1.3 | <LOD | <LOD |
| <LOD | 0.1 | 0.4 | <LOD | 0.4 | <LOD | <LOD |
| <LOD | 0.2 | 0.2 | <LOD | 0.5 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 0.1 | <LOD | <LOD |
| <LOD | 0.6 | 0.1 | <LOD | 0.4 | <LOD | <LOD |
| <LOD | 0.4 | <LOD | <LOD | 0.3 | <LOD | <LOD |
| <LOD | 0.1 | <LOD | <LOD | 0.2 | <LOD | <LOD |
| <LOD | 0.4 | <LOD | <LOD | 3.3 | <LOD | <LOD |
| <LOD | 0.2 | <LOD | <LOD | 0.5 | <LOD | <LOD |
| <LOD | 0.3 | <LOD | <LOD | 0.2 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 0.0 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 1.4 | <LOD | <LOD |
| <LOD | <LOD | 1.4 | <LOD | 5.2 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 1.1 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 0.3 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 0.2 | <LOD | <LOD |
| <LOD | <LOD | 2.9 | <LOD | 1.1 | <LOD | <LOD |
| <LOD | <LOD | 2.6 | <LOD | 7.1 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| <LOD | <LOD | 0.2 | <LOD | 0.8 | <LOD | <LOD |
| <LOD | <LOD | 0.3 | <LOD | 1.4 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| <LOD | <LOD | 0.2 | <LOD | 0.3 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 2.3 | <LOD | <LOD |
| <LOD | <LOD | 5.1 | <LOD | 15.4 | <LOD | <LOD |
| <LOD | <LOD | 0.4 | <LOD | 2.3 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| <LOD | <LOD | 3.3 | <LOD | <LOD | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 0.9 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 0.6 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| <LOD | <LOD | 1.5 | <LOD | 2.4 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| <LOD | <LOD | 3.6 | <LOD | 1.8 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 0.4 | <LOD | <LOD |
| <LOD | <LOD | 0.8 | <LOD | 0.3 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 0.2 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 0.8 | <LOD | <LOD |
| <LOD | 2.1 | 11.8 | <LOD | 30.7 | <LOD | <LOD |
| <LOD | <LOD | 2.7 | <LOD | 3.3 | <LOD | <LOD |
| <LOD | 0.6 | 7.8 | <LOD | 6.9 | <LOD | <LOD |
| <LOD | <LOD | <LOD | <LOD | 1.1 | <LOD | <LOD |
| <LOD | <LOD | 4.2 | <LOD | 1.9 | <LOD | <LOD |
| <LOD | <LOD | 6.9 | <LOD | 4.0 | <LOD | <LOD |
| <LOD | <LOD | 3.3 | <LOD | 0.9 | <LOD | <LOD |

acute injury, in both cases the animals should otherwise be healthy to be eligible for human consumption. Most animals presented as casualties have limb fractures (about 50%) with lameness and back injuries accounting most of the remainder (35%).

Some urine samples collected on nandrolone (17β-nortestosterone laureate (Decadurabolin®, 50 mg/mL, Intervet, Boxmeer, The Netherlands)) treated calves have also been involved in the present study; full protocol has been published previously [34,35].

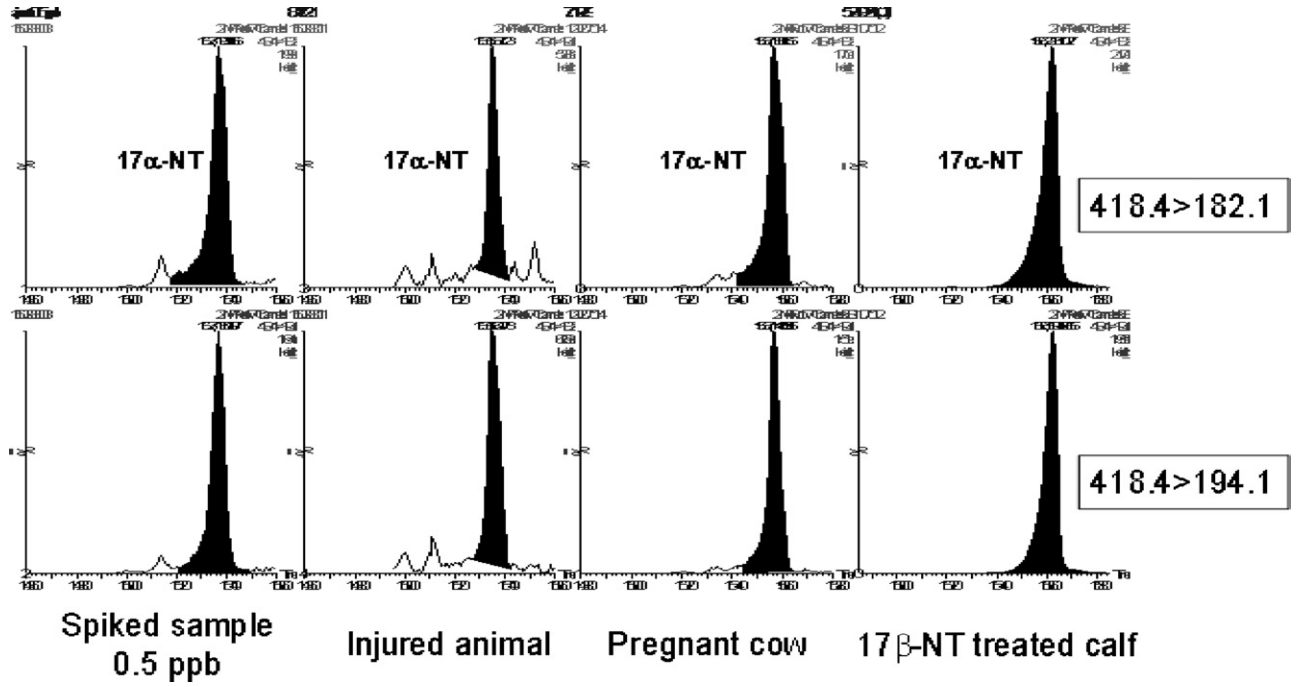


**Fig. 1.** Example of ion chromatograms of spiked urine sample as well as urine samples collected on three animals: injured bovine, pregnant cow and 17β-NT treated calf. Monitored signals (418.4 > 182.1 and 418.4 > 194.1) correspond to 17α-NT. GC–EI(+)-MS/MS analysis and SRM acquisition mode.

### 2.2. Chemicals

Most of the reagents and solvents were of analytical grade quality and provided by Carlo Erba Réactifs SDS (Val de Rueil, France). Envi-ChromP and silica (0.5 g and 1 g stationary phase, respectively) solid-phase extraction (SPE) cartridges were purchased from Carlo Erba Réactifs SDS (Val de Rueil, France). Derivatisation reagents N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), dithiothreitol (DTE) and trimethyliodosilane (TMIS) were purchased from Sigma–Aldrich (St. Quentin Fallavier, France). The reference steroids were from Interchim (Montluçon, France), Sigma–Aldrich (St. Quentin Fallavier, France), NARL reference materials (Pymble, Australia) and RIVM (Bilthoven, The Netherlands). The internal standards used were 17β-nandrolone-$d_3$ (17β-NT-$d_3$), 19-norandrosterone-$d_4$ (NA-$d_4$) and 19-noretiocholanolone-$d_3$ (NE-$d_3$) NARL reference materials (Pymble, Australia).

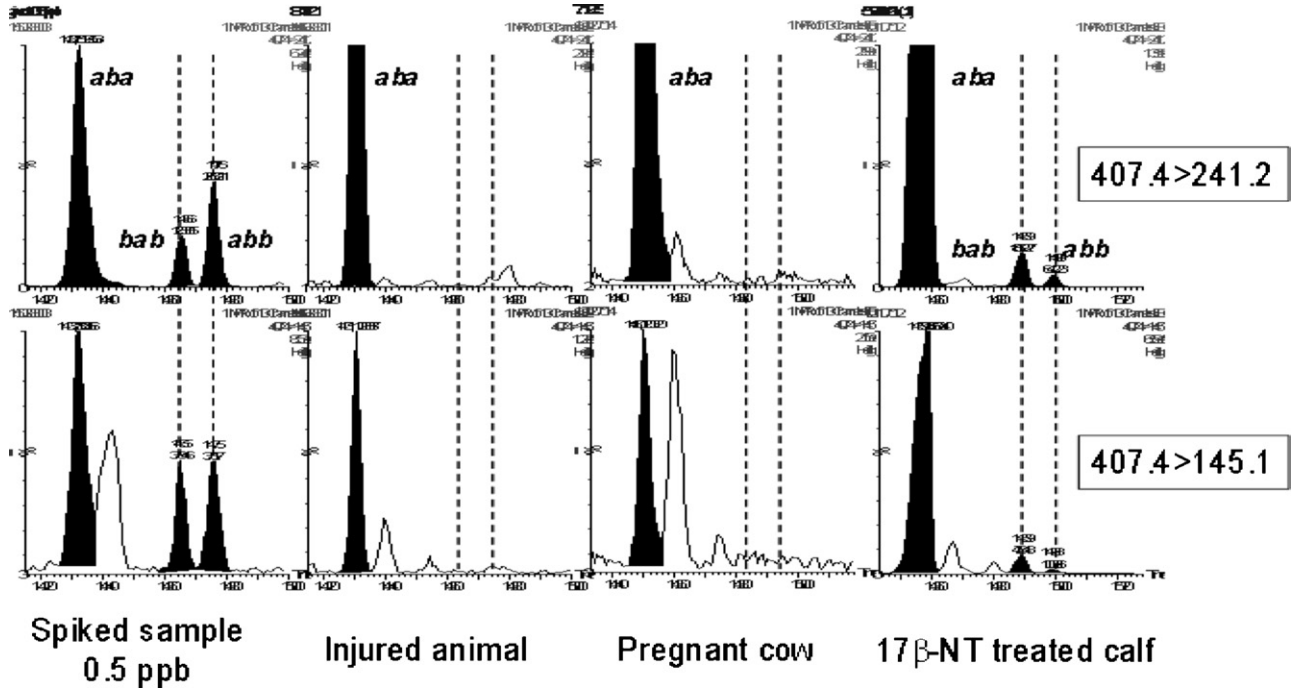


**Fig. 2.** Example of ion chromatograms of spiked urine sample as well as urine samples collected on three animals: injured bovine, pregnant cow and 17β-NT treated calf. Monitored signals correspond to *aba*, *bab* and *abb*. GC–EI(+)-MS/MS analysis and SRM acquisition mode.

### 2.3. Sample preparation

Samples have been prepared as already described [35]. Briefly, 10 mL of urine samples was added with 10 ng 17β-NT-$d_3$, NA-$d_4$ and NE-$d_3$, 1 mL acetate buffer (2 M, pH 5.2) and 200 μL β-glucuronidase from purified *Helix pomatia* (Sigma–Aldrich, St. Quentin Fallavier, France). Hydrolysis was performed over 15 h at 52 °C. Urine samples were centrifuged (10 min, 1000 × *g*) before purification onto SPE Envi-ChromP. Cartridges were conditioned with 6 mL ethyl acetate, 6 mL methanol then 6 mL water. The extract was applied onto the column. The phase was washed with 3 mL water then 2 mL hexane. High vacuum was applied before and after each washing. Steroids were eluted with 14 mL hexane/diethyl ether (70:30, v/v) which were evaporated to dryness under a gentle stream of nitrogen. After hydrolysis, 1 mL NaOH 1 N was added. Liquid/liquid extractions in alkaline medium phase performed with 4 mL hexane/diethyl ether (70:30, v/v) permitted to extract nortestosterone's metabolites.

After evaporation, the dry residue was dissolved in 500 μL hexane/dichloromethane (60:40, v/v) and applied onto a SPE silica column conditioned with hexane. The column was washed with 3 mL hexane/ethyl acetate (75:25, v/v) then 8 mL hexane/ethyl acetate (85:15, v/v). Analytes were eluted with 20 mL hexane/ethyl acetate (60:40, v/v) which were evaporated to dryness under a gentle stream of nitrogen. After the addition of 7.5 ng norgestrel (Sigma–Aldrich) as external standard, the samples were derivatised 40 min at 60 °C with 20 μL MSTFA–TMIS–DTE (1000:5:5, v/v/w). Of this extract, 2 μL was injected onto the GC-column.

### 2.4. Gas chromatography–mass spectrometry measurements

Steroids of interest have been analysed as previously described [35] and as follows: an HP 6890 gas chromatograph was coupled to a VG Quattro II (Micromass, Manchester, UK) triple quadrupole mass spectrometer and a OV-1 (OHIO VALLEY, 30 m × 0.25 mm, 0.25 μm) was used. Helium was used as carrier gas at a flow rate of 1 mL/min in the constant pressure mode and the transfer line was maintained at 320 °C. Pulsed splitless injection was operated at 250 °C and 60 kPa during 1 min. The initial oven temperature was 120 °C for 2 min and increased to 250 °C at 15 °C/min then to 300 °C at 5 °C/min and hold for 8 min. The mass spectrometer was operated in the selected reaction monitoring (SRM) acquisition mode after electron ionisation of the analytes. In the collision cell, argon was used as collision gas at $4.5 \times 10^{-4}$ mbar. Retention times ($R_T$ values) and optimised acquisition parameters for transitions and collision energies for each compound of interest have been published previously [35].

## 3. Results and discussion

The GC–MS/MS detection method allowed the measurement of 17β- and 17α-nortestosterone as well as the 5 following estranediol isomers (5α-estrane-3β,17β-diol (*abb*), 5β-estrane-3α,17β-diol (*bab*), 5α-estrane-3β,17α-diol (*aba*), 5α-estrane-3α,17β-diol (*aab*) and 5β-estrane-3α,17α-diol (*baa*)) in urine samples taken from pregnant cows and injured bovines (Tables 1 and 2).

Regarding pregnant cows, α-NT could be identified in more than 67% of urine samples, especially during the latest stages of pregnancy, where higher concentration values were also observed, which is expected and in accordance with previous data on the natural occurrence in pregnant cows [47].

The analysis of urine samples collected on injured animals allowed identification of both 17α-NT and 17β-NT as recently described [49] and confirming to a larger extent the possible natural occurrence of nandrolone in male cattle when injured. The 17α-NT form was detected in about 30% of the samples in the range 0.1–5.3 μg $L^{-1}$, whereas 17β-NT was found in only 1 animal out of 65, in which the 17α-NT form was also present. These findings highlight the potential difficulty for EU member states to monitor and control the abuse of this popular drug, since its identification in urine samples is possible in either nandrolone treated animals or in particular cases such as gestation or after bone injuries (Fig. 1), its finding can therefore no longer be ascribed exclusively to abuse of 17β-NT.

From the five estranediols which have been analysed in the urine samples taken from injured bovines and pregnant cows, only the isomers 5β-estrane-3α,17α-diol (*baa*) and 5α-estrane-3β,17α-diol (*aba*) could be detected and quantified, *aba* being the major one, as already described after 17β-NT administration in bovines [27,28,30,35,42] (Fig. 2). Based on this particular metabolite, a threshold approach strategy has recently been proposed to screen for 17β-NT abuse in cattle [50]. The present study focuses on other estranediol isomers: indeed, and whereas the isomers 5α-estrane-3β,17β-diol (*abb*) and 5β-estrane-3α,17β-diol (*bab*) could be identified in a previous study [35] up to several days after 17β-NT administration (Fig. 2), they could never be detected in the present study (Fig. 2), neither in pregnant cows nor in injured animals (Tables 1 and 2). This may be explained by the fact that these compounds, which present a 17β-diol function, are direct metabolites from the 17β-NT form administered, and are therefore produced only in this particular case of administration [35]. This result is of importance since it allows discriminating fraud situations from physiological ones. In this context the identification of 5α-estrane-3β,17β-diol (*abb*) and/or 5β-estrane-3α,17β-diol (*bab*) in urine sample would allow classifying the sample as suspicious regarding nandrolone administration. The present results arise from the analysis of a large number of samples, 40 pregnant cows and 65 injured bovines in the present study as well as more than 100 urine samples collected on 17β-NT treated bovines in the frame of a previous study [35], which enforces the statistical power of the results. It should be noticed that applying those criteria to the samples of the whole study, no false suspicious results neither false compliant have arisen, which is of prime importance for any screening criteria.

## 4. Conclusion

Nandrolone has been reported as natural occurring compound, for some time in pregnant cows and more recently in injured bovines, which creates a problem in the of control 17β-NT abuse in cattle. The present study focused on both populations at a large scale involving more than 100 animals with the aim of finding alternative criteria to detect the abuse of nandrolone. Estranediols have been investigated in a large number of urine samples and profiles compared to those obtained in urine samples collected from 17β-NT treated animals in a previous study. Amongst the 5 estranediol isomers investigated, two of them (5α-estrane-3β,17β-diol (*abb*) and 5β-estrane-3α,17β-diol (*bab*)) were not detected in pregnant cows and injured animals urine samples (LOD < 0.1 μg $L^{-1}$) whereas they could always be identified after 17β-NT treatment (even several days after administration) which permits their consideration as suitable candidates for screening purposes of nandrolone abuse in cattle. The robustness of the proposed criteria will be further assessed by increasing the number of animals involved in the study. Emphasis will also be given to better understanding the natural occurrence of nandrolone in injured animals since how this happens is not yet clear. Nandrolone is known for its healing and recovery properties toward

the muscle tissue [51]; its ability to restore the loss in carbonate in bone has also been reported, which is in particular investigated in human medicine to treat osteoporosis where accelerated loss of bone mass is generally observed during menopause [52,53]. A potential way of investigation regarding the present injured animal issue may lie in this particular property of nandrolone, and one could imagine that following a fracture, or similar acute injury, the animal would produce nandrolone to restore the damaged bone.

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