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Application of stable carbon isotope analysis to the detection of 17β-estradiol administration to cattle

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Abstract

The use of anabolic agents in food producing animals is prohibited within the EU since 1988 (96/22/EC directive). The control of the illegal use of natural steroid hormones in cattle is still an exciting analytical challenge as far as no definitive method and non-ambiguous analytical criteria are available. The ability of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) to demonstrate the administration of 17β-estradiol to bovine has been investigated in this paper. By comparison of $^{13}C/^{12}C$ isotopic ratio of main urinary estradiol metabolite, i.e. 17α-estradiol, with two endogenous reference compounds (ERCs), i.e. dehydroepiandrosterone (DHEA) and 5-androstene-3β,17α-diol, the differentiation of estradiol metabolite origin, either endogenous or exogenous, has been proved to be achievable. After treatment, the $\delta^{13}C_{VPDB}$-values of 17α-estradiol reached −27‰ to −29‰, whereas $\delta^{13}C_{VPDB}$-values of DHEA remained between −13‰ and −20‰ depending on the diet, maize and grass, respectively. A significant difference of $\delta^{13}C_{VPDB}$ between ERCs and 17α-estradiol was measurable over a period of 2 weeks after estradiol ester administration to the animal.

Keywords: GC/C/IRMS; Steroid; Cattle; Natural hormone; DHEA; Estradiol; Urine

1. Introduction

The use of anabolic agents for livestock fattening has been widely documented since the early 1950s. The EEC issued a Directive prohibiting the use, in livestock farming, of certain substances having a hormonal action on animals bred within the European Union (88/146/EEC). The last revision of this Directive (96/22/EC) stated that member states shall prohibit import of meat from animals from third countries, to which substances with a thyrostatic, estrogenic, androgenic or gestagenic action have been administered. The analytical methods currently used by member states are based on mass spectrometry, at least for confirmatory analysis, and are generally efficient for most synthetic anabolic steroids. However, the detection of misuse associated with natural hormones is much more problematic since these steroids are naturally produced by food producing animals. In fact, the classical approach based either on GC/MS, or LC/MS whatever the mass analyser and the acquisition technique, traditionally lead to confusing conclusions, and finally to disputes in court. There is actually no recognised unambiguous criterion to distinguish endogenous and exogenous origin of natural hormones; neither official concentrations threshold nor discriminative metabolites have been accepted by the scientific community. This is because the estrogen, androgen and progestagen profiles depend too much on sex, age, feeding or season.

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One of the unequivocal approaches actually used by some laboratories either in the antidoping [1–3] or food safety field [4] is to demonstrate the presence of the injected steroid ester in hair. This approach has been applied to estradiol benzoate in bovine hair [4], and testosterone esters in human hair [1]. An additional hyphenated approach based on the steroid $^{13}C/^{12}C$ ratio measurement by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) proved to be promising as well. Indeed, the administration of natural hormones (testosterone, estradiol, progesterone) to cattle leads to an alteration of the $^{13}C/^{12}C$ ratio of their metabolites whereas precursor steroids in the biosynthetic pathway remain unchanged in their isotopic composition. A significant difference of the isotopic composition between these so called endogenous reference compounds (ERC) and metabolites highlights an illegal administration of natural hormones. The few papers dealing with this approach in food producing animals are focused onto androgens and mainly testosterone administration [5–7], but estrogens were never studied. In this paper, a method dedicated to both androgen (precursors) and estrogen (estradiol metabolites) purification of urine samples and GC/C/IRMS measurements is presented and the applicability illustrated with several control and estradiol treated animals. The applicability of this method for surveillance of the illegal use of natural hormone in cattle is discussed.

2. Experimental

2.1. Animals

Pharmaceutical preparations of testosterone enanthate (250 mg) and estradiol valerate (10 mg) were injected into young and adult females, steers and bulls at dose levels of 0.5 mg/kg bw for testosterone and 0.05 mg/kg bw for estradiol (BfR, Berlin). Two different feeding regimens were prepared for these animals: diet 1 mainly based on maize (fresh weight: 76% maize silage + 23% hay + minerals and vitamins), diet 2 relying upon grasses (hay and grass made up of different plants + rye straw + minerals and vitamins) alone. Urine samples were collected before and after intramuscular injections and stored frozen below −16 °C.

2.2. Apparatus

GC/MS analysis was performed on a HP 6890 gas chromatograph coupled to a HP 5973 quadrupole mass analyser (Hewlett-Packard, Palo Alto, CA, USA). A DB-5MS (30 m × 0.25 mm i.d., 0.25 μm, Agilent, Palo Alto, CA, USA) column was used. For steroid separation, the following conditions were applied: injector 250 °C, splitless injection (1 min), source temperature (230 °C). The column flow rate was 1.5 mL/min (constant flow). The oven temperature was increased from 60 °C (1.5 min) to 220 °C at 40 °C/min, then to 240 °C (1 min) at 1 °C/min, then to 300 °C (1 min) at 20 °C/min. Mass spectra were recorded in the SCAN mode (m/z 50–550 mass range). The temperature of the transfer line was 280 °C. The HPLC system used was a Hewlett-Packard HP-1100 system equipped with a fraction collector and UV-detector (DAD, diode array detector) programmed from 200 to 280 nm. A 3-(dimethylamino)propyl-functionalized silica gel column (EC-Nucleosil 100-5 $N(CH_3)_2$-propyl, 250 mm × 4 mm, 5 μm) from Macherey-Nagel (Hoerdt, France) held at 50 °C was used for semi-preparative purification. The mobile phase was made of *n*-hexane (solvent A) and isopropanol (solvent B). Flow rate was set at 1 mL/min and injected volume was 50 μL. Mobile phase composition (A:B; v/v) was 96:4 at 0 min. Two different gradients were applied depending on the analytes. For precursors (DHEA and 5-androstene-3β,17α-diol), the initial mobile phase composition was kept during 15 min, then linearly changed to reach 20:80 at 19 min and maintained until 28 min. For estrogens, the mobile phase composition A:B was linearly changed from 0 to 15 min from 96:4 to 85:15, then modified so that the mobile phase reached 20:80 at 20 min, and finally kept unchanged until 25 min. To monitor the fraction collection, a DAD was used. When interferences were observed in GC/MS so that the GC/C/IRMS was considered unfeasible as such, a second semi-preparative HPLC purification was operated. The stationary phase used was a reversed phase C_{18}, LiChrospher 100 column (250 mm × 4 mm, 5 μm) from Merck (Darmstadt, Germany). The mobile phase consisted of water (C) and acetonitrile (D). Flow rate was set at 1 mL/min and injected volume was 50 μL. Mobile phase composition (C:D; v/v) was 70:30 at 0 min, and linearly modified to reach 0:100 in 25 min and maintained as such during 10 min.

GC/C/IRMS measurements were performed on a HP 6890 gas chromatograph coupled to an IsoPrime isotope ratio mass spectrometer via a GC-V Combustion interface (GV, Manchester, UK). The separated analytes were heart-cut into a combustion furnace filled with copper oxide wires (Elemental Microanalysis Limited, UK) held at 850 °C. The combustion gases were passed through a liquid nitrogen water removal trap; the remaining CO_2 was introduced in an electron ionisation source operating at 100 eV. Ions (m/z 44, 45, and 46) were separated on a magnet and detected by three Faraday collectors. The calibration of the reference gas was performed with a mixture of different alkanes (15 analytes from C-16 to C-30) which $\delta^{13}C_{VPDB}$-values were previously calibrated (Biogeochemical Laboratories, Indiana University, Indiana, USA). Carbon isotope ratios of each compound were expressed relatively to Vienna-Pee Dee Belemnite (VPDB) [8,9]. The negative shifts of the $\delta^{13}C_{VPDB}$-value due to the formation of acetate were corrected as follows:

$$D_{OH} = D_{OAc} + 2m\frac{D_{OAc} - D_{Ac}}{n}$$

where D_{OH} is the $\delta^{13}C_{VPDB}$-value for the underivatised steroids, D_{OAc} the $\delta^{13}C_{VPDB}$-value for the acetylated steroids, D_{Ac} the $\delta^{13}C_{VPDB}$-value for the acetylating reagent,

n the number of carbon atom in a molecule and *m* is the number of hydroxyl groups to be acetylated. All subsequent $\delta^{13}C_{VPDB}$-values have been corrected for this negative shift. The D_{Ac} value of our acetylating reagent was measured at −50.0‰.

2.3. Chemicals

The reagents and solvents used were of analytical-grade quality from SDS (Peypin, France). The solvents for liquid chromatography were of HPLC-grade from SDS (Peypin, France). The β-glucuronidase from *Escherichia coli* used was from Roche Diagnostics GmbH (Mannheim, Germany). The solid-phase extraction (SPE) columns (SiOH: 1000 mg/6 mL, C_{18}: 2000 mg/15 mL) were from UCT (Bristol, PA, USA). The derivatisation reagent pyridine and acetic anhydride were from Aldrich (Steinheim, Germany). Reference steroids were from Research Plus (Bayonne, NJ, USA), Steraloids (Wilton, NY, USA).

2.4. Method

The whole analytical strategy is described in Fig. 1. To hydrolyse steroid glucuronide conjugates, 6 mL of 0.8 M phosphate buffer (pH 6.8) were added to the urine samples (20 mL). After an overnight incubation at 37 °C with 100 μL β-glucuronidase from *E. coli*, samples were centrifuged at 1200 × *g* (5 °C) for at least 10 min. The supernatant was then applied onto a C_{18} SPE column (2000 mg) and the steroids were purified by washing with 10 mL ultra pure water and 10 mL *n*-hexane and eluted with 5 mL methanol:ethyl acetate (30:70, v/v). The eluted fraction was evaporated to dryness under a gentle stream of nitrogen at 45 °C. This residue was dissolved in 2 mL of 1 M sodium hydroxide and

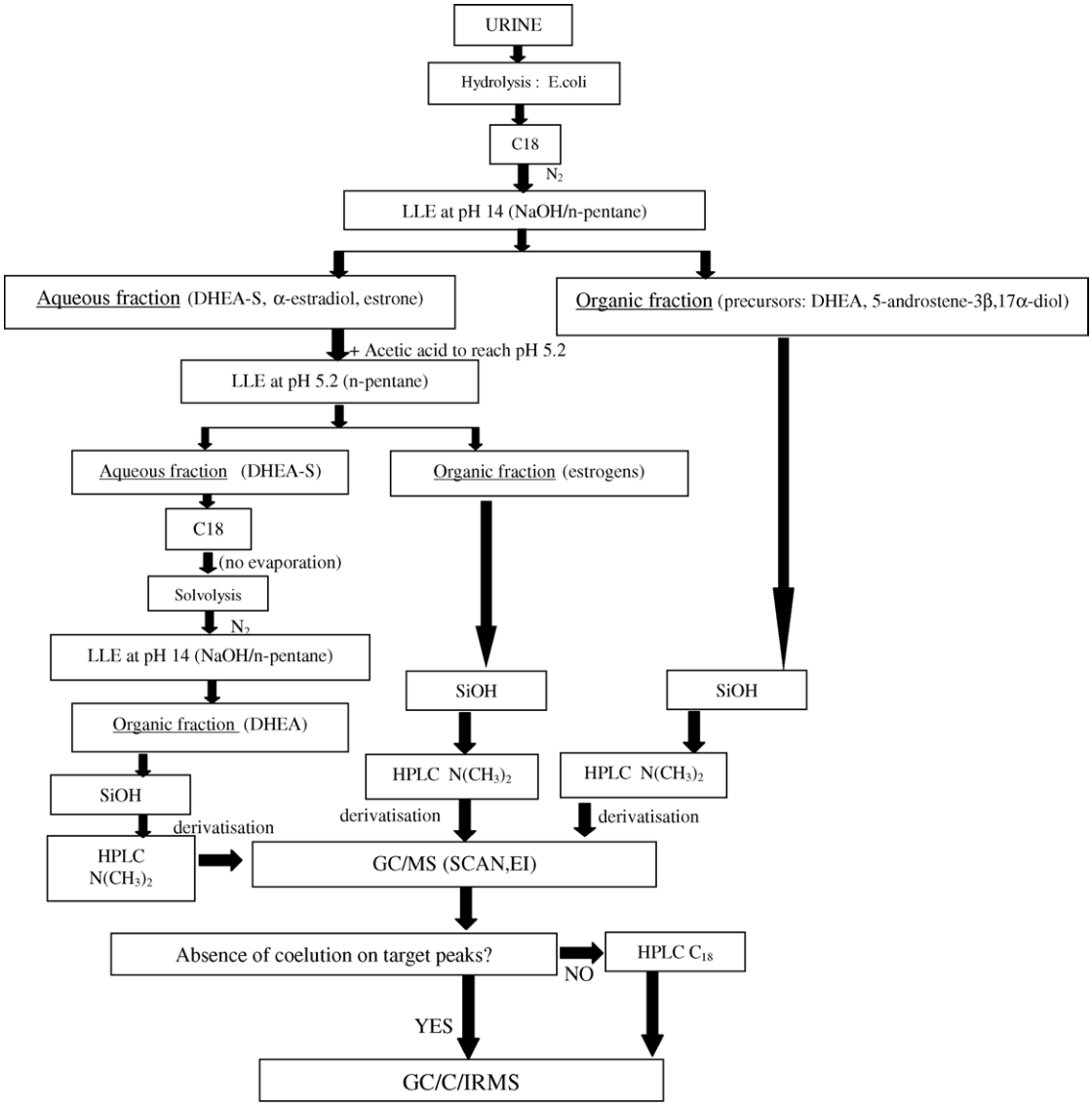

Fig. 1. Analytical strategy for the extraction and purification of estrogens and their endogenous reference compounds. Estrogens are separated from androgens by LLE at pH 14, and the analytes are then purified by SPE SiOH and HPLC before derivatisation and GC/MS–GC/C/IRMS measurements.

a liquid–liquid extraction was performed at pH 14 twice with 5 mL *n*-pentane. The organic layer containing the free and aglycone androgens was kept in a glass tube labelled "A" and evaporated to dryness. The pH of the aqueous layer was then adjusted to 5.2 with glacial acetic acid (150 μL) and a second liquid–liquid extraction was carried out using twice 5 mL *n*-pentane. The organic layer containing the estrogens was kept in a glass tube labelled "E" and evaporated to dryness. The aqueous layer containing the sulphate androgens was labelled "S" and applied onto a C_{18} SPE column (2000 mg). The steroids were purified with the same conditions described above but the SPE cartridges were dried over P_2O_5 before elution step and the eluate was not evaporated. The chemical solvolysis of the sulphate steroids was done with a mixture of ethyl acetate:sulphuric acid (200 mg of concentrated H_2SO_4 in 100 mL of ethyl acetate) in a water bath (55 °C for 1 h). The reaction was stopped by adding 0.75 mL of 1 M sodium hydroxide to the mixture. The organic solvents were evaporated under a nitrogen stream and a liquid–liquid extraction was performed twice with *n*-pentane (5 mL). The organic layer was kept in a glass tube labelled "S" and evaporated to dryness.

Residue "A", "E" and "S" were dissolved in ethyl acetate (75 μL) and *n*-hexane (425 μL). Each dissolved residue was applied onto a SiOH column (1000 mg) conditioned with 18 mL *n*-hexane. The androgen column was washed with 9 mL *n*-hexane:ethyl acetate (85:15, v/v) and eluted with 13 mL *n*-hexane:ethyl acetate (60:40, v/v). The estrogen column was washed with 3 mL *n*-hexane:ethyl acetate (85:15, v/v) and eluted with 11 mL *n*-hexane:ethyl acetate (60:40, v/v). The eluted fractions were evaporated to dryness under a nitrogen stream.

The dried residues "A" and "S" were dissolved in 50 μL *n*-hexane:isopropanol (90:10, v/v) and the residue "E" in 50 μL *n*-hexane:isopropanol (86:14, v/v), and injected onto the HPLC system (3-(dimethylamino)propyl-functionalized silica gel column). The collected fractions were evaporated to dryness under a nitrogen stream. Acetylation of the extracts was carried out with 100 μL of pyridine and 100 μL of acetic anhydride at 60 °C for 45 min. The derivatisation reagent was evaporated to dryness under a nitrogen stream and the residue was redissolved in 50 μL cyclohexane. The samples were first injected into the GC/MS for sample extract characterisation (identification, interference scrutiny, concentration assessment) and then in the GC/C/IRMS for $^{13}C/^{12}C$ ratio determination.

3. Results and discussion

3.1. Hydrolysis of steroid conjugates

The enzymatic and chemical hydrolysis steps of sulpho- and glucuro-conjugates of androgens and estrogens were widely discussed in previous papers [6,10–12]. The most critical steroid conjugates to hydrolyse by the enzymatic way are well known to be the sulpho-conjugates. In this study, a special attention has been paid to DHEA, which is known to be mainly sulpho-conjugated [13], the other target steroids being known to be mainly bonded in bovine urine to glucuronic acid.

The different strategies of deconjugation either chemically or enzymatically (with different sources of enzyme, e.g. *Helix pomatia* and *E. coli*) were assessed for their hydrolysis efficiency. Indeed, *E. coli* was found to be the most efficient and specific way to deconjugate steroid glucuroconjugates and chemical solvolysis the most appropriate way to hydrolyse DHEA–SO_3H [14]. From a strategic point of view, and because the relative non-specificity of the solvolysis step, the chemical hydrolysis was performed after two SPE and two LLE steps to minimise the production of interfering compounds. During the second LLE step, the estrogens were separated from the aqueous layer so that only sulphate compounds underwent the chemical hydrolysis. In fact, the solvolysis is not suitable for estrogens because of degradation of estradiol during this step (about 30% of loss).

3.2. Sample purification

Because of their radical difference in term of chemical behaviour, estrogens and androgens were fractionated between sodium hydroxide and pentane at pH 14. At this pH, phenolic steroids are converted into their phenolate form, their pK_a value (estradiol and estrone) being 10.7 ± 0.1 [15]. Phenolates were neutralised by addition of acetate buffer (pH 5.2), for further extraction into organic solvents.

The purification by semi-preparative HPLC is an essential step before any GC/C/IRMS measurement of steroids at the low ng/mL level in urine samples. The need for such a strategy has been already reported in some articles [10,11,15,16]. Prevost et al. [16] based their purification on a semi-preparative aminopropyle column (250 mm × 4 mm, 10 μm, Macherey-Nagel). This column was used as a first approach in our laboratory, but some unexpected chromatographic issues due mainly to peak tailing and memory effects were observed with Δ^4-3-one androgens resulting in the need to assess another stationary phase. A system involving a 3-(dimethylamino)propyl-functionalized silica gel column was tested; demonstration of the purification efficiency is shown on Fig. 2. This step clearly permitted a powerful clean-up of the wide number of interferences occurring in the neighbourhood of DHEA and 5-androstene-3β,17α-diol retention time; the GC/C/IRMS measurement which is very demanding in term of peak purity cannot be performed without this step. A second HPLC based on a reversed phase C_{18} column was developed for further purification of the acetylated analytes. This step is applied when necessary to the androgenic fraction for purification of the precursor 5-androstene-3β,17α-diol. The efficiency of this process is shown in Fig. 3. The GC/C/IRMS measurement of the 5-androstene-3β,17α-diol

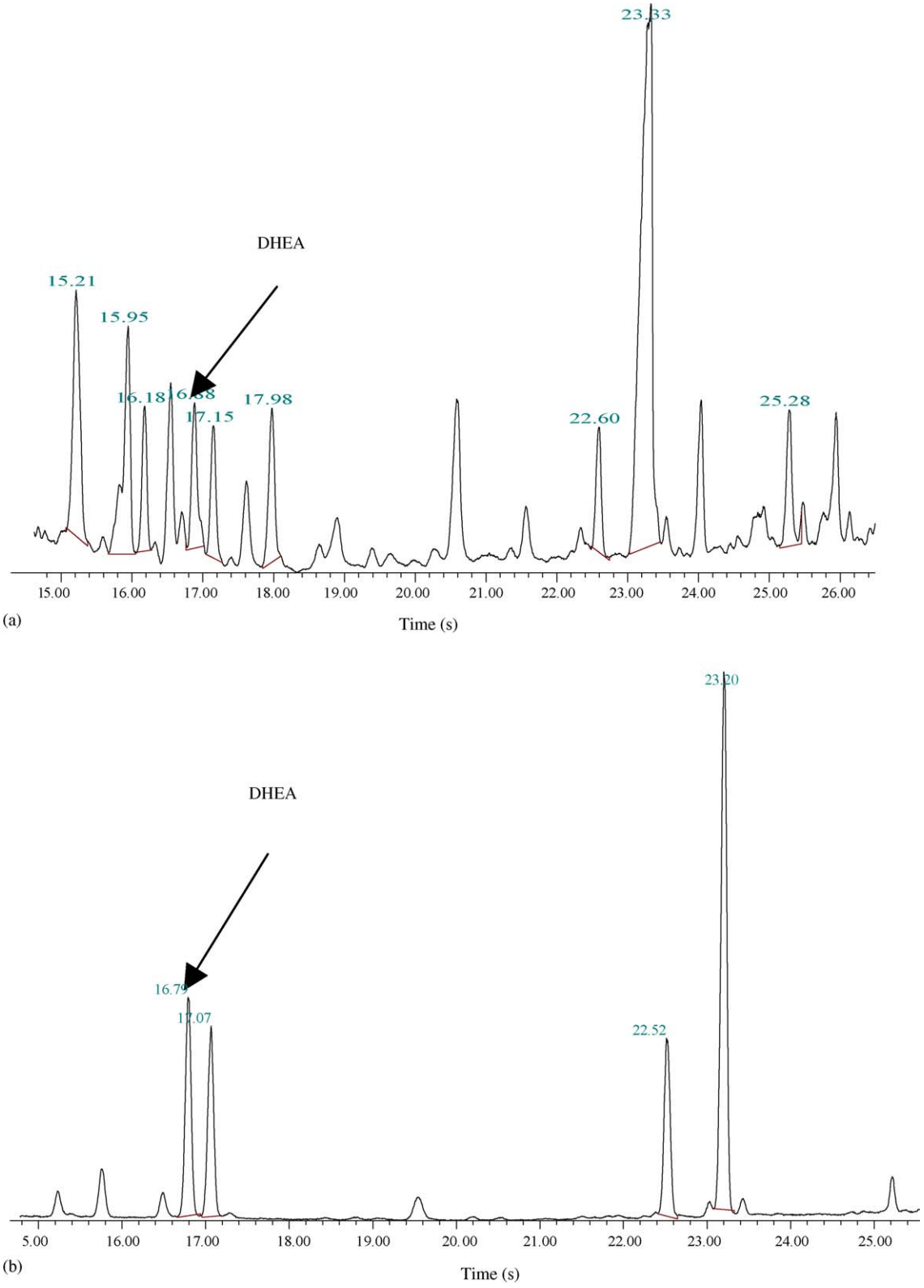

Fig. 2. GC/MS chromatograms (TIC) of incurred DHEA in bovine urine sample observed without (a) and with (b) $N(CH_3)_2$-propyl semi-preparative HPLC. The GC/C/IRMS measurement is only possible after HPLC purification.

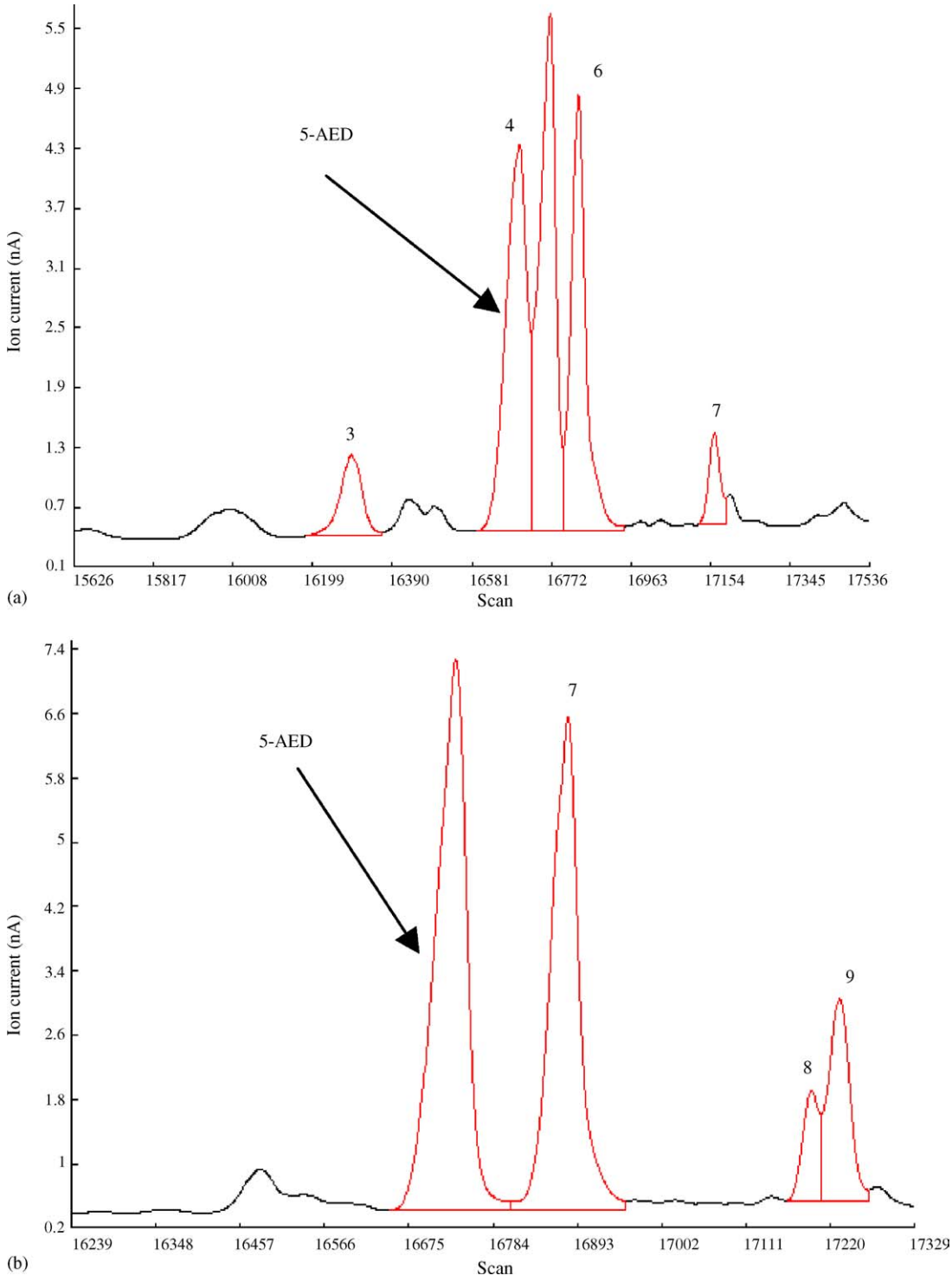

Fig. 3. GC/C/IRMS chromatograms (*m*/*z* 44) of incurred 5-androstene-3β,17α-diol (5-AED) in bovine urine sample observed without (a) and with (b) a second semi-preparative HPLC (LiChroCART 250 mm × 4 mm, LiChrospher 100 RP-18C_{18} column). In this sample, the GC/C/IRMS measurement is only possible after HPLC purification.

is obviously not possible (because of the co-eluting analytes) without the second C_{18}-HPLC purification. This second C_{18}-HPLC purification is not necessary for the estrogenic and sulphate fractions as these extracts were free of interferences.

3.3. Derivatisation

The first derivatisation approach developed in the study relied upon trimethylsilylation using MSTFA/NH_4I/DTE because this is widely used in the field of steroid analysis

and has proven to be robust [16]. The main advantage of the technique was above all the good chromatographic behaviour of the derivatised analytes, a single synthesised product and the high derivatisation yield. The main disadvantage was due to the high (six) number of carbon introduced in the molecule disturbing the $^{13}C/^{12}C$ measurement, and affecting negatively the difference of isotopic deviation between precursors and metabolites. At the end, acetylation was chosen as the first advantage of this approach is the introduction of only two carbons to each alcohol function [17–19]. In addition, acetylated steroids are stable over weeks in solvents such as cyclohexane, and can be further purified when necessary. The use of non-derivatised steroids should have been in theory a better choice for GC/C/IRMS measurements as the steroid $^{13}C/^{12}C$ ratio is not affected; but non-protected steroids clearly show a worse chromatographic behaviour, and this can be critical in some cases, e.g. for estrogens. Underivatised steroids showed significant increase of peak tailing, width and asymmetry when compared to acetylated steroids. The derivatisation leads to reduced peak tailing, allowing the GC/C/IRMS integration software to better characterise the chromatographic peak, with a more efficient determination of the start and the end of the signal. This improves the repeatability and the precision of the $^{13}C/^{12}C$ measurement.

3.4. Chromatography and isotopic fractionation

Isotopic fractionation is recurrently mentioned as a major potential pitfall in GC/C/IRMS analysis. This phenomenon can occur all along the chromatographic processes including the SPE, HPLC or GC steps [20–22]. In this study the actual isotopic fractionation was assessed for the two HPLC purification stages. The elution of non-derivatised steroids from the dimethylamino-grafted silica (used in normal phase) was studied. The injection of steroid reference standards onto the HPLC system, followed by collection of multiple sub-fractions of the HPLC peak, showed in GC/C/IRMS a significant difference in term of $^{13}C/^{12}C$ ratio from the start to the end of the peak. Indeed, depending on the class of steroid, the variation of $^{13}C/^{12}C$ ratio was found to increase or decrease. Two steroids, DHEA and 17α-estradiol, were injected on a normal ($N(Me)_2$) and on a reversed phase (C_{18}). On normal phase column, as shown in Fig. 4, lower $\delta^{13}C_{VPDB}$-values were observed for DHEA at the beginning of the elution (−36‰, uncorrected value, derivatisation after fractionation) whereas the isotopic deviation was found constant at the end of the elution (−33‰, uncorrected value, derivatisation after fractionation); heavier isotopomers eluted afterwards in this case. For 17α-estradiol, the highest $\delta^{13}C$ values were recorded at the beginning of the elution with a significant difference of 4‰ between start and end. On the tested reversed phase column C_{18}, higher $\delta^{13}C$ values were obtained at the beginning of the elution for both steroids (Fig. 5). Peak start-end differences reached 17‰ for DHEA (derivatisation before the injection in HPLC). This data corroborates with previous results observed by Kenig et al. [20]

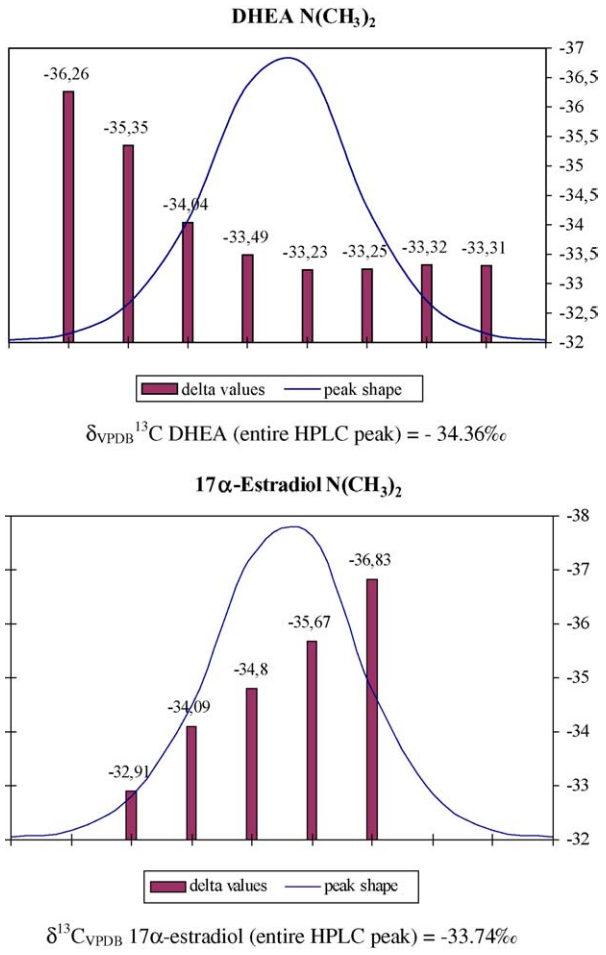

Fig. 4. Observation of steroid isotopic fractionation on a dimethylamino grafted silica (normal phase). $\delta^{13}C_{VPDB}$-values were measured after acetylation of the collected fractions (non-corrected values).

where a maximum ^{13}C isotopic fractionation across a peak was 18‰ on a HPLC reversed phase. As a consequence, the fraction collection has to be definitely considered as a critical step of the purification step regarding measurement accuracy. To guarantee a robust peak collection of the target steroids, a DAD system was coupled on-line to the collector. The collection windows of steroids was determined by signal monitoring at 205 nm for androgens and 280 nm for estrogens, on the basis of retention time determination in a standard mixture.

Coelutions on target peaks can affect $\delta^{13}C_{VPDB}$ accuracy [23]. Consequently a complete baseline separation of two adjacent peaks is mandatory. This is the case for 5-androstene-3β,17α-diol (ERC) and 5α-androstane-3β,17α-diol (testosterone metabolite), which are characterised by very close GC retention times. On non-polar GC stationary phases, heavier isotopomers steroids elute at the front of the chromatographic peak while ^{13}C depleted counterparts eluted towards the tail [24], and a poor integration of overlapping peaks can lead to depleted values for the later eluting analyte. An optimisation of chromatographic conditions (stationary phase, temperature programme) was necessary to separate

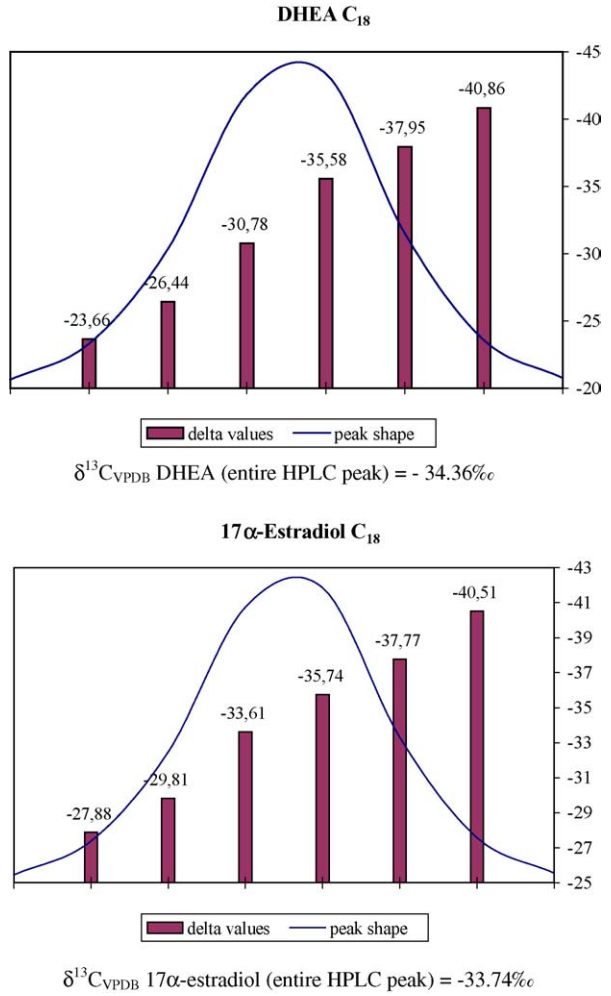

Fig. 5. Observation of steroid isotopic fractionation on a RP-C_{18} grafted silica. $\delta^{13}C_{VPDB}$-values corresponded to acetylated steroids (non-corrected values).

the two steroids as best as possible and to permit an unbiased IRMS measurement of both analytes.

3.5. Analytical strategy before any GC/C/IRMS measurement

The first step prior to any IRMS measurement is the control of the instrument's ability to accurately measure the steroid $^{13}C/^{12}C$ ratio. Several points have to be checked: the stability, the linearity, the accuracy and the reproducibility. The stability and the linearity were controlled by introducing pulses of reference carbon dioxide gas into the ion source. An internal laboratory mixture of acetylated steroids (testosterone, DHEA, estrone and 17β-estradiol) was injected several times daily to check for any drift in the delta values and to ensure the fit-for-purpose sensitivity of the instrument [25,26].

Sample extracts were injected into GC/MS before GC/C/IRMS to prove unambiguously the analyte identity, assess the peak purity and evaluate the concentration for eventual further dilution/concentration. Peak purity is a

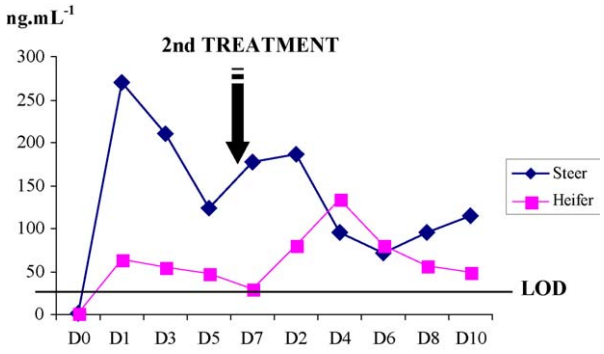

Fig. 6. Elimination kinetic of 17α-estradiol in urine after i.m. administration of estradiol valerate (0.05 mg/kg bw) to a steer and to a heifer. Second injection performed at day 7.

mandatory stage because a hidden analyte underneath the target steroid can lead to an erroneous estimation of the isotopic composition. Concentration estimation of target analytes is necessary as well, because the linear range of the GC/C/IRMS is given to be 2×10^{-9} to 1×10^{-8} A on the *m/z* 44 channel; it corresponds roughly to 15–70 ng of steroid on column. Outside this range, accuracy and precision are significantly affected [27].

3.6. Application to incurred samples

Various animals were injected intramuscularly with estradiol valerate. GC/MS/MS was used (EI ionisation, SRM acquisition) to assess the efficiency of the administration and to determine the pharmacokinetics of the main urinary metabolites. As shown in Fig. 6, 17α-estradiol concentrations

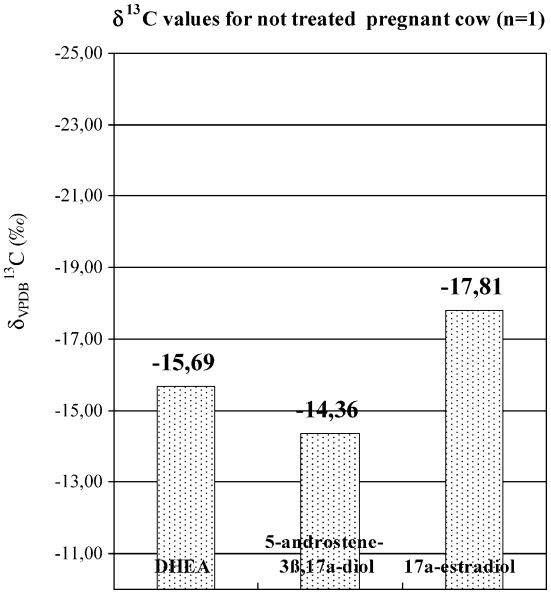

Fig. 7. $\delta^{13}C_{VPDB}$-values of DHEA, 5-androstene-3ß,17α-diol and 17α-estradiol in urine sample collected in non-treated pregnant cow. In this sample, the 17α-estradiol is measurable in GC/C/IRMS; its value gives an overview of the endogenous delta value of estrogens (with the feeding: maize silage 56% of fresh weight).

significantly increased in steer and heifer urine samples during 2 weeks, making them compatible with the GC/C/IRMS approach.

The $\delta^{13}C_{VPDB}$-values of DHEA, 5-androstene-3β,17α-diol and 17α-estradiol were measured in urine samples collected in treated (estradiol valerate) and non-treated animals of different sexes (cows, bulls or steers), age classes (sexually mature and immature) and feedings (grass or maize). All animal characteristics are shown in Table 1 and measured $\delta^{13}C_{VPDB}$ results are shown in Figs. 7–9. These diagrams show isotopic deviations observed for cows fed with maize with and without estradiol treatment, calves fed with maize

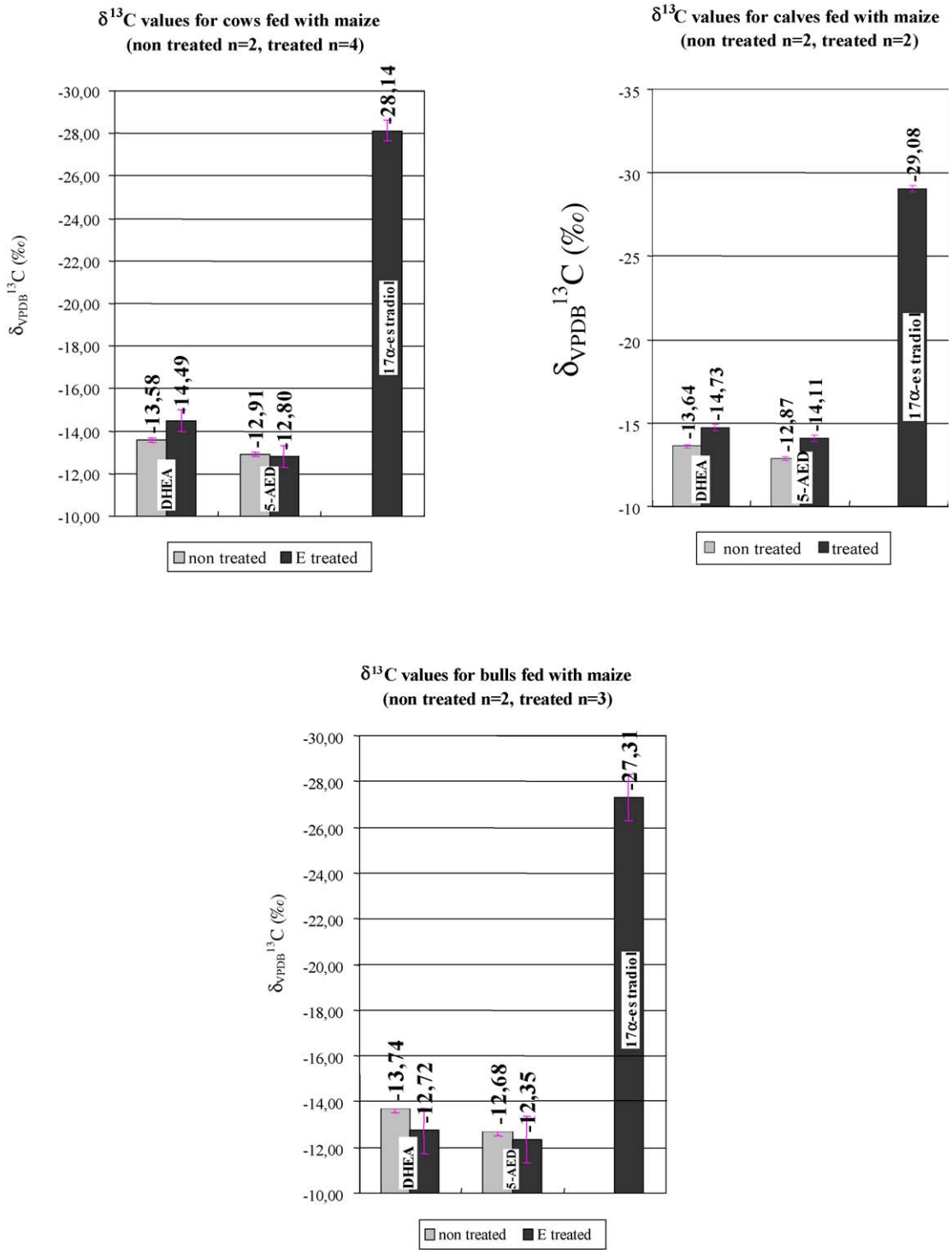

Fig. 8. $\delta^{13}C_{VPDB}$-values of DHEA, 5-androstene-3ß,17α-diol (5-AED) and 17α-estradiol in urine sample collected in treated (estradiol valerate) animals of different sexes (male, male castrated, female), ages (sexually mature: cows, bulls and immature: calves) and a feeding with maize (76% of fresh weight).

Table 1
Collected urine sample, feeding, animal's details, and $\delta^{13}C_{VPDB}$-values of DHEA, AED and 17α-estradiol in urine samples

Sample number	Day	Animal	Maize 76%	Grass	Treatment	DHEA Sulphur δ-values	Acetate corrected values	5-Androstene-3ß,17α-androstendiol	Acetate corrected values	17α-estradiol	Corrected values
20	D-4	298 adult cow	X		No	−16.98	−13.50	−19.36	−12.91	nm	nm
38	D2	298 adult cow	X		E + T	−17.43	−14.00	−19.4	−12.96	−31.19	−27.01
66	D11	298 adult cow	X		E + T	−17.53	−14.11	−18.83	−12.27	−32.8	−28.98
31	D1	297 adult cow	X		No	−17.12	−13.66	nm	nm	nm	nm
139	Mixed	Pregnant cow	56%		No	−18.96	−15.69	−20.56	−14.36	−23.66	−17.81
Control		Mix pregnant cow + bull			No	−18.09	−14.73	−20.74	−14.58	−22.94	−16.93
30	D1	306 female calf	X		E	−18.22	−14.87	−20.23	−13.96	nm	nm
48	D7	306 female calf	X		E	−17.96	−14.59	−20.47	−14.25	−32.88	−29.08
149	D3	699 adult bull		X	E	−23.08	−20.25	Coeluted	Coeluted	−31.93	−27.91
366	D7	699 adult bull	X		E	−15.83	−12.23	−17.73	−10.94	−30.37	−26.01
275	D14	698 adult bull	X		No	−17.24	−13.79	−19.35	−12.90	nm	nm
257	D9	698 adult bull	X		No	−17.14	−13.68	−18.99	−12.46	nm	nm
154	D4	699 adult bull	X		E	−16.41	−12.87	−19.41	−12.97	−31.82	−27.78
756	Mixed	298 adult cow	X		E	−18.14	−14.79	−19.57	−13.16	−32.13	−28.16
535	Mixed	298 adult cow	X		E	−18.39	−15.06	nm	nm	−32.33	−28.40
709	Mixed	303 calf	X		T	−17.01	−13.54	−19.35	−12.90	nm	nm
500	Mixed	303 calf	X		T	−17.19	−13.74	−19.31	−12.85	nm	nm
998	Mixed	699 adult bull	X		E	−16.58	−13.06	−19.54	−13.13	−32.11	−28.13
605	Mixed	773 steer	X		T	−16.00	−12.42	−19.70	−13.32	nm	nm
803	Mixed	773 steer	X		T	−17.26	−13.81	−19.25	−12.78	nm	nm

nm, not measurable; AED, 5-androstene-3ß,17α-diol.

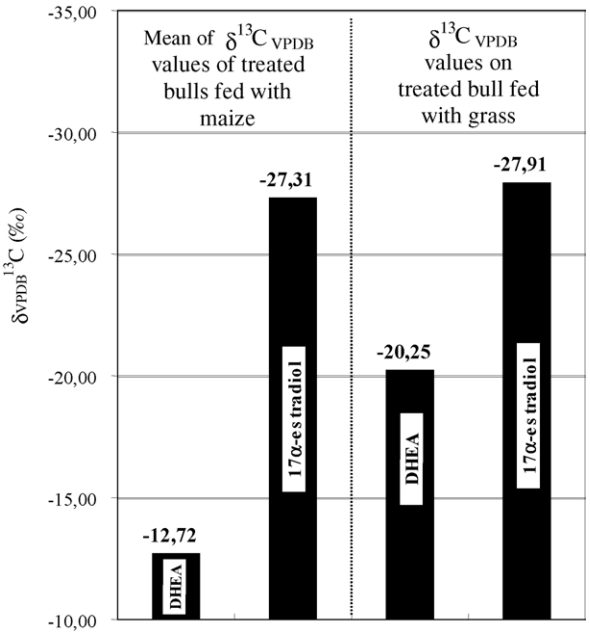

Fig. 9. Comparison of $\delta^{13}C_{VPDB}$-values of DHEA and 17α-estradiol in urine sample collected from treated (estradiol valerate) bull under maize ($n = 3$) or grass ($n = 1$) feed.

with and without estradiol treatment, bulls fed with maize or grasses with and without treatment, and one pregnant cow. Two endogenous reference compounds, DHEA (ERC_1) and 5-androstene-3β,17α-diol (ERC_2), and the main estradiol metabolite, i.e. 17α-estradiol, were monitored in each urine sample. 17α-estradiol was found difficult to measure in all samples from untreated animals except pregnant cow because of low endogenous concentrations. For the ERCs, as expected, we observed that the $^{13}C/^{12}C$ isotopic ratios of DHEA and 5-androstene-3β,17α-diol were not affected by the treatment of the animal with estradiol valerate; so that they can be considered as a valuable reference. The corrected $\delta^{13}C_{VPDB}$ mean value was -13.52 ± 0.27‰ ($n = 8$) for DHEA and -12.88 ± 0.15‰ ($n = 7$) for 5-androstene-3β,17α-diol in urine samples from non-estradiol-treated animals fed with maize (76%). In urine samples from estradiol-treated animals fed with maize, the $\delta^{13}C_{VPDB}$ mean value was -13.98 ± 1.10‰ ($n = 9$) for DHEA and –13.08±0.91‰ ($n = 8$) for 5-androstene-3β,17α-diol. The $\delta^{13}C_{VPDB}$-value is very repeatable from one animal to another when the feeding composition is kept the same; this observation is again in favour of their utilisation as endogenous reference compound. The difference between treated and untreated animals was 0.64‰ for DHEA, and 0.20‰ for 5-androstene-3β,17α-diol and this demonstrates the $^{13}C/^{12}C$ composition of these steroids is not affected by administration.

Concerning estrogens, the $\delta^{13}C_{VPDB}$-values of 17α-estradiol in untreated pregnant cow fed with less maize (56% instead of 76% fresh weight, proportion of grasses increased correspongly) were found to be −17.81‰. In non-treated calves, castrated males and non-gravid females, the concentration of 17α-estradiol is usually at the low μg/L level, so that the $\delta^{13}C_{VPDB}$ determination is not possible using only 20 mL of urine sample. After treatment, the $\delta^{13}C_{VPDB}$ mean value for 17α-estradiol was assessable; measured values for animals fed with maize were -28.17 ± 0.88‰ ($n = 8$).

The $\Delta(\delta‰)$, i.e. the difference between the $\delta^{13}C_{VPDB}$-value of the ERC and 17α-estradiol after treatment is higher than 14‰. This difference is substantial regarding the measurement uncertainty to allow unambiguous differentiation between treated and non-treated animal. The measured $^{13}C/^{12}C$ isotopic composition of 17α-estradiol is nearly that of injected β-estradiol, the endogenous 17α-estradiol being only at trace level. The $^{13}C/^{12}C$ isotopic ratio of the pharmaceutical preparation was measured by GC/C/IRMS after saponification, purification and acetylation of the solution. The $\delta^{13}C_{VPDB}$-value was -27.01 ± 0.71‰ ($n = 4$).

The influence of the feeding (maize versus grasses) was assessed on a treated bull (Fig. 9). The $\delta^{13}C_{VPDB}$-value of DHEA for bulls fed with maize was -12.72 ± 0.43‰ ($n = 3$), whereas $\delta^{13}C_{VPDB}$ was −20.25‰ during grass diet. On the other hand, the $\delta^{13}C_{VPDB}$-value measured for 17α-estradiol whatever the feeding (grass or maize) and with the same treatment was almost identical: -27.31 ± 1.14‰ ($n = 3$) with maize feeding and −27.91‰ with grass feeding. This depleted value confirms that the measured $\delta^{13}C_{VPDB}$ of 17α-estradiol is almost only correlated to estradiol originating from the exogenous administration. However, depending on the diet composition, the difference between ERC_1 and estradiol metabolite is affected; a decrease of the $\Delta(\delta‰)$ is clearly observed from maize (14‰) to grass (8‰), but it does not alter the discrimination capability of the technique.

Ferchaud et al. [6] previously studied the influence of the feeding on DHEA $\delta^{13}C_{VPDB}$. The authors observed a clear enrichment in the ^{13}C isotopic composition when the feeding was changed from grass to maize.

4. Conclusion

The ability of GC/C/IRMS to demonstrate exogenous administration of 17α-estradiol has been shown. The strategy relies upon an efficient hydrolysis both for sulphate and glucuro conjugates, a specific multi-stage purification, and derivatisation of the analytes of interest. The determination of endogenous reference compounds is of prime importance; the selection of DHEA and 5-androstene-3β,17α-diol proved to be a reliable strategy as the $\delta^{13}C_{VPDB}$-values remain homogeneous for a group of bovine subjected to the same maize-based diet (-13.52 ± 0.27‰ ($n = 8$) for DHEA and -12.88 ± 0.15‰ ($n = 7$) for 5-androstene-3β,17α-diol), and because their $^{13}C/^{12}C$ isotopic ratio is not affected by estradiol ester administration (-13.98 ± 1.10‰ ($n = 9$) for DHEA and -13.08 ± 0.91) ‰ ($n = 8$) for 5-androstene-3β,17α-diol). Comparison of $\delta^{13}C$ values of 17α-estradiol in treated and non treated animals (maize diet) showed substantial difference -28.17 ± 0.88‰ ($n = 8$) and −17.81‰. When

the diet is changed (maize to grass), no difference occurred concerning the $\delta^{13}C$ value of 17α-estradiol after treatment. The $\delta^{13}C_{VPDB}$ difference between ERCs and metabolite differs depending on the diet, with $\Delta(\delta‰)$ of 14‰ (maize) and 8‰ (grass) for DHEA. A significant difference of isotopic deviations can be observed during at least 2 weeks after the treatment. Nevertheless, no threshold level in terms of $\Delta(\delta‰)$ has been decided yet. Moreover, no criterion devoted to the interpretation of GC/C/IRMS analytical data are available in any official analytical document such as 2002/657/EC decision.

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