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Application of Stable Carbon Isotope Analysis to the Detection of Testosterone Administration to Cattle

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The use of anabolic substances is prohibited in food-producing animals throughout the European Union. No method is available to reliably detect the misuse of natural hormones in cattle. A method was developed to detect the abuse of testosterone in cattle fattening. Synthesized testosterone is rather depleted in the $^{13}C/^{12}C$ ratio. Hence, the method is based on gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) analysis of urine. To select testosterone metabolites and endogenous reference compounds (ERC), the concentration of urinary steroids of cattle was investigated. Dehydroepiandrosterone and androst-5ene-3β,17α-diol were chosen as ERCs to show endogenous $^{13}C/^{12}C$ ratios. Etiocholanolone and 5α-androstane-3β,17α-diol were chosen as the most important testosterone metabolites. Other metabolites known from literature like epitestosterone were less promising. In principle, GC/C/IRMS is a nonspecific method because finally carbon dioxide is analyzed. Therefore, a dedicated cleanup procedure for the selected steroids was developed. By means of proposed confidence intervals in the isotopic composition of ERCs and metabolites, the administration of testosterone to cattle could be detected reliably. Differences of up to 11‰ on the δ-scale between ERC and testosterone metabolites were found after testosterone administration, whereas endogenous differences did not exceed 2‰.

KEYWORDS: IRMS; testosterone; steroids; stable carbon isotopes; natural hormones; bovine; cattle; urine

INTRODUCTION

Throughout the European Union (EU), the use of steroids as growth promoters is prohibited in food-producing animals (Directive 96/22/EC), as they may be dangerous for consumers and may also affect the quality of the respective foodstuff. Additionally, the import of animals, meat, or products obtained from animals from countries outside the EU is restricted, if hormonal growth promoters were administered. The abuse of xenobiotic hormonal growth promoters (e.g., Clenbuterol) can be verified quite easily by analyzing the parent compound or their metabolites by mass spectrometry coupled to gas or liquid chromatographs. In contrast, the detection of the abuse of synthesized naturally occurring hormones needs another strategy. For screening purposes, the use of discriminant analysis considering changes in the steroid profile is conceivable. To decide if a sample is suspicious concerning an abuse with natural hormones, it is a good tool. However, a definite decision, whether an animal was treated with natural hormones or not, is quite impossible on the basis of an unusual steroid profile since endogenous values depend on age, gender, reproductive status, breed, and feeding. Individual factors outside accepted norms can especially influence the steroid profile and could cause false positive results. To discriminate between endogenous and synthesized natural steroids unambiguously, the method of choice is gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). This strategy was presented for doping control in sports in 1994 (*1*). Synthesized steroids are normally made from *Dioscorea* spp. or soy (*2*), which are C_3 plants and more depleted in ^{13}C as compared to C_4 plants. Endogenously produced steroids derive from the diet, which is normally a C_3/C_4 mixture. Consequently, administered steroids

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and subsequently their metabolites are depleted in the $^{13}C/^{12}C$ ratios (expressed as $\delta^{13}C_{VPDB}$ values). The isotopic composition of precursors of administered anabolic androgens as well as steroids on a different metabolic pathway (e.g., corticosteroids) remains unchanged and can be used as endogenous reference compounds (ERCs). If the difference between the $\delta^{13}C_{VPDB}$ values of an anabolic steroid or its metabolite and the ERC exceeds a given limit, this is considered as evidence for the presence of exogenous steroids. The analytical challenge to measure $^{13}C/^{12}C$ ratios of steroids in urine of cattle is given by a quite complex matrix in combination with rather low concentrations of target analytes. The procedure developed must strike a balance between necessary sample volume, sufficient purification to obtain baseline separation obligatory for GC/C/IRMS (*3*), and avoidance of significant losses during the cleanup. Ferchaud et al. presented a cleanup method in 1998 (*4*), which was modified twice (*5*, *6*). The procedure presented here still generally encompasses the same steps. However, significant modifications and substantial add-ons were performed in order to achieve sufficient purity, yield, and specificity.

MATERIALS AND METHODS

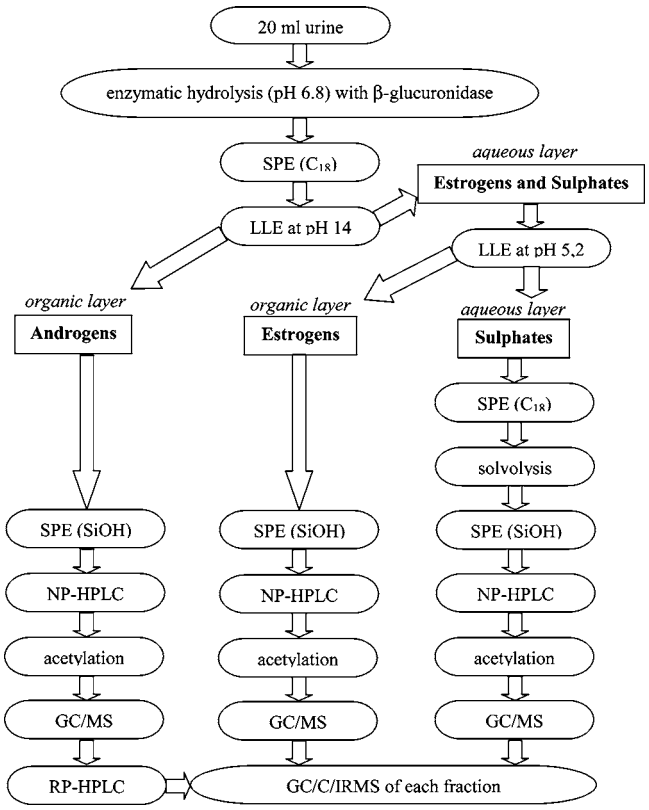
Materials. Unless stated otherwise, all reagents and solvents were of analytical grade. Acetic anhydride was distilled 1 week after adding 1% water (v/v) to eliminate byproducts and to remove acetic acid (HOAc). β-Glucuronidase from *Escherichia coli* was purchased from Roche Diagnostics (Mannheim, Germany). The steroids etiocholanolone, epitestosterone, dehydroepiandrosterone (DHEA), epiandrosterone, cholestane, and 5α-androstan-3β-ol were purchased from Sigma-Aldrich (Steinheim, Germany), whereas 5α-androstane-3β,17α-diol and androst-5ene-3β,17α-diol were purchased from Steraloids (Newport, United States). Reversed phase (RP) solid phase extraction (SPE) cartridges (2 g, 12 mL) on C_{18} material and the normal phase (NP) silica SPE cartridges (1 g, 6 mL) were from UCT (Bristol, United Kingdom). Pharmaceutical preparations of testosterone enanthate and testosterone propionate dissolved in peanut oil were purchased from Jenapharm (Jena, Germany). For administration purposes, they were mixed in a ratio of 4:1.

Purification was performed on an Agilent 1100 (Agilent Technologies, Palo Alto, CA) high-performance liquid chromatography (HPLC) system, equipped with degasser, quaternary pump, autosampler, column oven, and UV detector. Fractions were collected by an automatic fraction collector (Foxy 200) from Isco (Lincoln, NE). The NP-HPLC column from Macherey Nagel (Düren, Germany) (EC 250/4 Nucleosil 100-5 $N(CH_3)_2$) was equipped with a guard column [CC 8/4 Nucleosil 100-5 $N(CH_3)_2$]. The RP_{18} column [LiChroCART 250-4 LiChrospher 100 RP_{18} EC (5 μm)] was purchased from Merck (Darmstadt, Germany) and equipped with a precolumn from Merck [LiChroCart 25-4 LiChrospher 100 RP_{18} (5 μm)]. A GC 6890 (Agilent Technologies) was coupled to a Delta plus XP gas isotope ratio mass spectrometer from ThermoElectron (Bremen, Germany) by a combustion interface II (ThermoElectron). The GC column was a J&W HP-5MS (Agilent Technologies). Dimensions of the column were 30 m length and 0.25 mm inner diameter. The film thickness was 0.25 μm. A retention gap (deactivated fused silica, 2 m length, 0.32 mm inner diameter) was connected to the analytical column to improve splitless conditions. Helium (purity, 5.0−99.999%) was the carrier gas at a constant flow of 2 mL/min. An autosampler (A200SE, CTC Analytics, Zwingen, Switzerland) was used, which operated at an injection speed of 5.5 μL/s. $^{13}C/^{12}C$ ratios are expressed as $\delta^{13}C_{VPDB}$, where the working standard (carbon dioxide, $\delta^{13}C_{VPDB} = -3.0‰$) was calibrated vs a *n*-alkane mixture (*7*).

Semiquantification was performed by GC/MS. A GC 6890 (Agilent Technologies) was coupled to a 5973 N mass selective detector (Agilent Technologies). Ionization was performed at 70 eV by electron impact (EI) ionization. The GC and injection parameters were the same as described above.

General maintenance, handling, and killing of the animals at the Federal Institute for Risk Assessment in Berlin as well as the design and performance of the animal trial (number of animals, treatment, sampling, duration of the trial, etc.) were in accordance with the German animal welfare regulations (Animal Welfare Law and relating decrees). They were approved and supervised by the responsible Federal State authority (Federal State Department of Animal Welfare, LaGetSi Berlin, animal trial permission no. G 0060/02) and the institute's Animal Welfare Office.

Scheme 1. Flow Chart of the Cleanup Procedure for GC/C/IRMS



Selection of Target Analytes for GC/C/IRMS. Quantification of steroids in urine of cattle was performed by GC/MS using a method that had been developed previously for human antidoping (*8*). Results for two separated fractions were obtained. The first one contained free steroids and hydrolyzed glucuronic acid conjugates, whereas the second one contained hydrolyzed steroid sulfates.

Sample Preparation for GC/C/IRMS. A flow scheme of the method is presented in **Scheme 1**.

Enzymatic Hydrolysis of Steroid Glucuronides. Six milliliters of 0.8 M phosphate buffer (pH 6.8) was added to 20 mL of urine. After 100 μL of β-glucuronidase was added, samples were incubated at 37 °C for at least 5 h, preferably overnight.

SPE (C_{18}). The RP_{18} SPE cartridges were conditioned with 10 mL of methanol followed by 10 mL of water. The centrifuged urine (10 min at 1200 G) was placed on the column and washed consecutively with first 10 mL of water and then 10 mL of *n*-hexane. Analytes were eluted with 5 mL of a mixture of methanol and ethyl acetate (EtOAc) (30/70; v/v).

Liquid/Liquid Extraction (LLE): Partitioning of Androgens, Estrogens, and Sulfate Conjugates. After the eluate was dried, the pH was adjusted to 14 with 2 mL of 1 M sodium hydroxide. Androgens were extracted twice with 5 mL of *n*-pentane. After the pH of the aqueous layer was adjusted to 5.2 with 150 μL of HOAc, estrogens were extracted twice with *n*-pentane (5 mL). Both organic extracts were evaporated to dryness. The further cleanup of the estrogens was described by Buisson et al. (*9*). The remaining aqueous layer contained the steroid sulfate conjugates.

Chemical Solvolysis of the Sulfate Conjugates. The RP_{18} SPE was repeated using the aqueous layer. However, before the elution step, the cartridges were dried under vacuum over phosphorus pentoxide in a desiccator for at least 3 h to completely eliminate any water residues.

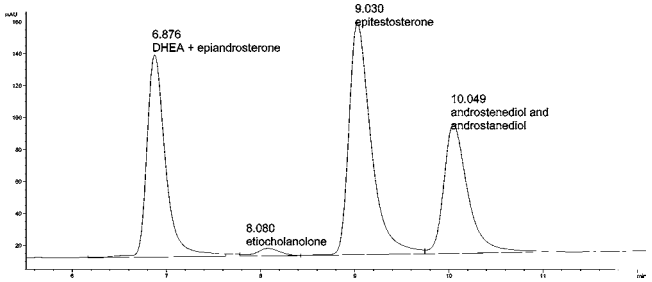


Figure 1. NP-HPLC/UV chromatogram at 200 nm of 5 μg of each of the following: DHEA, epiandrosterone, etiocholanolone, epitestosterone, androst-5ene-3β,17α-diol, and 5α-androstane-3β,17α-diol [*x*-axis = time (min); *y*-axis = UV absorbance].

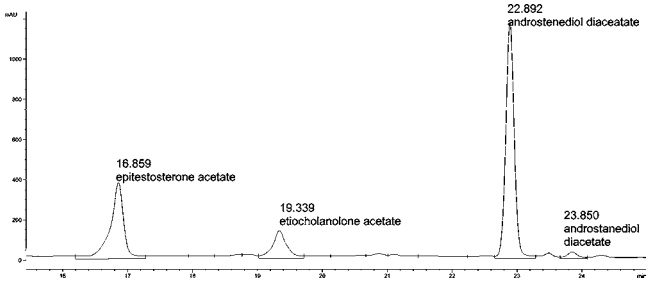


Figure 2. RP-HPLC/UV chromatogram at 200 nm of 5 μg of each of the following: epitestosterone acetate, etiocholanolone acetate, androst-5ene-3β,17α-diol diacetate, and 5α-androstane-3β,17α-diol diacetate [*x*-axis = time (min); *y*-axis = UV absorbance].

After elution of the analytes, 2 mL of EtOAc/sulfuric acid (concentrated) (100 mL/200 mg) was added to the eluate. The mixture was incubated for 1 h at 55 °C to hydrolyze steroid sulfates. The reaction was stopped by adding 0.75 mL of 1 M sodium hydroxide. After the organic solvents (about 0.75 mL aqueous residue) were evaporated, 1 mL of sodium hydroxide solution was added to adjust to alkaline pH. Solvolyzed androgens were extracted twice with 5 mL of *n*-pentane and evaporated to dryness.

NP Silica SPE. The silica SPE cartridges were conditioned with 18 mL of *n*-hexane. The two subdivisions of androgens (hydrolyzed glucuronides and sulfates) were purified separately. Each subdivision was dissolved in 75 μL of EtOAc. After vortexing, 425 μL of *n*-hexane was added to each subdivison and shaken again. The dissolved extracts were loaded on a conditioned SPE cartridge. Then, the test tubes were rinsed with 9 mL of *n*-hexane/EtOAc (85/15; v/v) and also applied onto the silica column. Each androgen subdivision was eluted with 13 mL of *n*-hexane/EtOAc (60/40; v/v). Extracts were evaporated to dryness and transferred twice with 100 μL of methanol into HPLC vials equipped with a microinsert. Methanol was removed under vacuum.

NP-HPLC on $(CH_3)_2$N-Propyl Column. For determination of the HPLC fraction limits, the retention time of each analyte was determined. For these purposes, a mixed standard of 5 μg of each target analyte, dissolved in 50 μL of *n*-hexane/2-propanol (90/10; v/v) was injected at least twice before the biological samples into the HPLC system. The mobile phase was 96% *n*-hexane and 4% 2-propanol for 15 min followed by 9 min of column washing with 80% 2-propanol and 20% *n*-hexane. Reequilibration of the HPLC column was achieved by isocratically washing for 9 min with the initial mixture prior to subsequent injections. The temperature of the column was set to 50 °C to reduce the required pressure during the washing step. The flow rate was set to 1 mL/min, and detection was performed at 200 nm. **Figure 1** shows an UV chromatogram of a mixed standard of DHEA + epiandrosterone (contemporaneous elution), etiocholanolone, epitestosterone, and androst-5ene-3β,17α-diol + 5α-androstane-3β,17α-diol (also contemporaneous elution). The dried extracts of the two subdivisions were dissolved in 50 μL of *n*-hexane/2-propanol (90/10; v/v) and injected separately. DHEA from the hydrolyzed sulfate conjugates containing subdivision was collected from 6:24 to 8:30 min. From the extract containing the free and hydrolyzed steroid glucuronides, etiocholanolone, epitestosterone, androst-5ene-3β,17α-diol, and 5α-androstane-3β,17α-diol were collected from 7:30 to 12 min. Fraction limits were determined with a wide safety margin before and after the analytes because retention times using NP-HPLC were less constant as compared to RP-HPLC and the influence of the biological matrix on the retention times cannot be monitored "online" due to the large background. Additionally, to exclude isotopic shifts induced by the cleanup procedure, analytes had to be collected completely because of isotopic discrimination from the beginning to the end of HPLC peaks (*9*−*11*).

Acetylation. Collected fractions were evaporated to dryness. Acetylation of hydroxy groups was achieved by heating for 45 min at 60 °C after adding 100 μL of pyridine and 100 μL of acetic anhydride. Derivatization reagents were then evaporated.

GC/MS: Semiquantification of Analytes. The acetylated extracts were transferred into autosampler vials with screw caps and microinserts with two washings of 50 μL of cyclohexane. One microliter was injected into GC/MS in scan mode (e.g., *m*/*z* 40−500) comparable to the GC/C/IRMS conditions. Semiquantification was performed by external standardization.

GC/MS is necessary to estimate the concentrations of the analytes, if no preliminary quantification of steroids in urine was carried out, because the dynamic linear range of the IRMS is only about one decade of substance. It is also recommended to check the collection pattern of the NP-HPLC. For the same reasons and furthermore to check the purity of the final extracts, GC/MS can also be applied after RP-HPLC.

RP-HPLC on C_{18} Column. A second RP_{18} HPLC cleanup step was applied to the subdivision containing the acetylated free and hydrolyzed androgen glucuronides. The mobile phase was 70% water and 30% acetonitrile. A gradient was run, within 20 min, up to 100% acetonitrile holding for 10 min to elute 5α-androstane-3β,17α-diol diacetate and androst-5ene-3β,17α-diol diacetate and to flush the column. The HPLC column was reequilibrated with the initial solvent mixture for 5 min prior to the next injection. The temperature of the column was set to 24 °C, the flow rate was 1 mL/min, and detection was performed at 200 nm. The fraction limits were determined as described above with a mixed standard of 5 μg of each in 50 μL of acetonitrile/2-propanol (50/50; v/v), which was injected into the HPLC system. **Figure 2** shows a typical HPLC chromatogram of epitestosterone acetate, etiocholanolone acetate, androst-5ene-3β,17α-diol diacetate, and 5α-androstane-3β,17α-diol diacetate. The dried extracts of the acetylated free and hydrolyzed androgen glucuronides were redissolved in 50 μL of acetonitrile/2-propanol (50/50; v/v) and injected into HPLC, and fractions were collected. The androgens were separated into four fractions: epitestosterone acetate (16:12−17:24 min), etiocholanolone acetate (18:42−19:54 min), androst-5ene-3β,17α-diol diacetate (22:12−23:30 min), and 5α-androstane-3β,17α-diol diacetate (23:30−25:00 min). Each fraction was evaporated to dryness and transferred into an autosampler vial rinsing twice with 50 μL of cyclohexane and dried again.

GC/C/IRMS. On the basis of the concentrations determined by GC/MS, the volumes of cyclohexane were calculated to add up to approximately 100 ng of each analyte per injection. The final extracts (DHEA acetate, epitestosterone acetate, etiocholanolone acetate, androst-5ene-3β,17α-diol diacetate, and 5α-androstane-3β,17α-diol diacetate) were dissolved in the determined volumes of cyclohexane (for practical reasons at least 10 μL). The use of isotopically characterized reference standards such as androstanol and cholestane dissolved in the cyclohexane (each 100 μg/mL) is recommended to control chromatographic conditions and the validity of the calculated isotope ratios. Measurement of $^{13}C/^{12}C$ ratios was performed by GC/C/IRMS. One microliter of final sample extract was injected in splitless mode (splitless time, 1:30 min) at an injector temperature of 250 °C. The initial oven temperature was set to 60 °C for 1:30 min, followed by an increase of 40 °C/min up to 225 °C. A second ramp of 3 °C/min until 260 °C followed during which separation of the relevant compounds was achieved. A third ramp of 40 °C was used until 300 °C and held for 1:30 min.

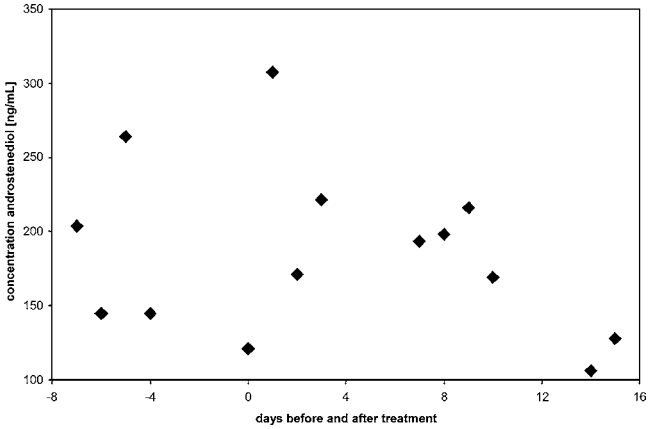


Figure 3. Concentration of the ERC androst-5ene-3β,17α-diol in the free and glucuronide fraction before and after testosterone injection.

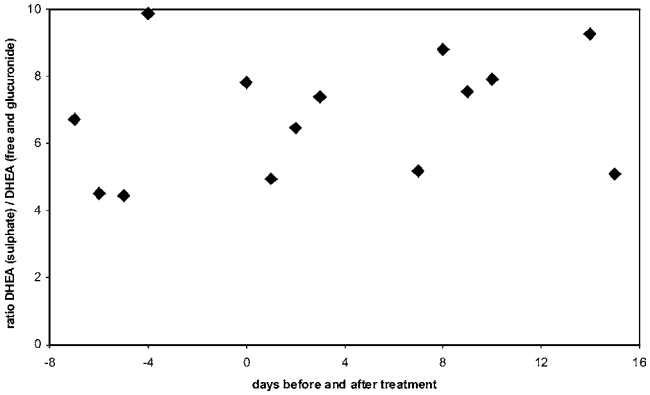


Figure 4. Relative amount of DHEA in the steroid sulfates containing subdivision vs DHEA in the free and steroid glucuronides containing subdivision before and after testosterone injection.

RESULTS AND DISCUSSION

Selection of Target Analytes for GC/C/IRMS. In the literature, several metabolites of a testosterone application to cattle are described. Samuels et al. (*12*) found etiocholanolone and three isomers of androstanediol as major metabolites. Epitestosterone and etiocholanolone were used as metabolites for the first IRMS experiments by Ferchaud et al. (*4*). DHEA was used as the ERC, which was not affected by testosterone application. Prévost et al. (*5*) detected 5α-androstane-3β,17α-diol and epiandrosterone as two further metabolites of testosterone and androst-5ene-3β,17α-diol as another ERC. Nevertheless, little information seems to be known concerning concentrations of steroids in urine of cattle. This caused difficulties in selection of suited target analytes for GC/C/IRMS. Therefore, several excretion studies were performed. In **Figures 3**−**5**, the excretion curves of different analytes after testosterone treatment of a 6 month old female Holstein−Friesian are presented. The concentrations obtained from two heifers and two steers showed a similiar pattern. In these studies, 0.6 mg testosterone/kg body weight was injected as a mixture of enanthate and propionate. Urine was collected beginning 7 days before injection until 15 days after application. In **Figure 3**, the values of androst-5ene-3β,17α-diol in urine are given. Concentrations between 100 and 300 ng/mL urine were found in the fraction containing free steroids and steroid glucuronides. Considerably less was found in the fraction containing steroid sulfates. **Figure 4** shows the ratio of hydrolyzed DHEA sulfate over combined free and hydrolyzed DHEA glucuronide. In contrast to androst-5ene-3β,-17α-diol, DHEA as a second possible ERC was mainly excreted

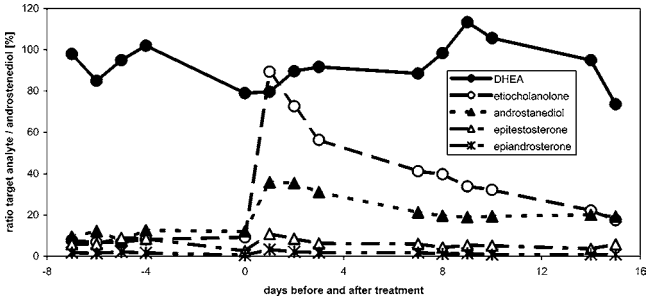


Figure 5. Relative amounts of target analytes vs androst-5ene-3β,17α-diol before and after testosterone injection.

as sulfate conjugate. Normally, the concentration of the sulfate conjugate was 5−10 times higher than the glucuroconjugate. In **Figure 5**, the ratios of the concentration of selected analytes to androst-5ene-3β,17α-diol are shown to demonstrate the influence of a testosterone administration. From these experiments, as expected, no effect on the DHEA concentration could be observed. Moreover, the concentrations of epiandrosterone and epitestosterone were normally too low to be measured by GC/C/IRMS. Epiandrosterone did not exceed a value of 10 ng/mL and was found on an average level between 1 and 5 ng/mL. The concentration of epitestosterone in urine normally ranged between 3 and 15 ng/mL and did not exceed 35 ng/mL. Furthermore, the changes of the ratios of these two steroids to androst-5ene-3β,17α-diol were not very pronounced. However, the concentrations of etiocholanolone and 5α-androstane-3β,-17α-diol were found to be sufficient for GC/C/IRMS measurements. Naturally occurring levels of etiocholanolone and 5α-androstane-3β,17α-diol ranged between 10 and 50 ng/mL. After testosterone treatment, the levels for etiocholanolone rose to 300 ng/mL and the levels for 5α-androstane-3β,17α-diol rose to 110 ng/mL, but the ratio of 5α-androstane-3β,17α-diol to Androst-5ene-3β,17α-diol showed a slower decline than that of etiocholanolone. So this compound might exhibit a depleted δ-value for a longer time than etiocholanolone. Both parameters did not reach baseline ratios even 15 days after testosterone application, which was also promising for the purpose of a long-time detection of testosterone abuse by GC/C/IRMS. Other steroids such as epietiocholanolone [also mentioned by Samuels et al. (*12*)] or 17β-hydroxy-steroids were not detected in significant amounts. In the urine of mature animals or pregnant cows, much higher concentrations of endogenous steroids were detected. However, these animals are only of small interest because an abuse of growth-promoting hormones to mature cows is not very probable since the largest anabolic effects can be achieved for yearling cattle (*13*). Androst-5ene-3β,17α-diol and DHEA were selected as ERCs, and etiocholanolone and 5α-androstane-3β,17α-diol were selected as the most promising metabolites for GC/C/IRMS measurements. Epitestosterone and epiandrosterone often showed too low concentrations to be suitable for GC/C/IRMS. Nevertheless, they were also included in the method development.

Sample Preparation. *Hydrolysis of Steroid Conjugates.* Different methods for hydrolysis of steroid conjugates are described in the literature. They are mainly based on glucuronidase, sulfatase, or chemical solvolysis. To find the most efficient method for hydrolysis of steroid conjugates, different methods were tested. One method was based on a combined enzymatic hydrolysis of steroid glucuronides and sulfate conjugates with *Helix pomatia* (*4*). This type of enzymatic hydrolysis was tested by 11 different urine samples (from four different animals, treated and not treated with hormones). After enzymatic hydrolysis, free and hydrolyzed steroid conjugates

were separated by LLE. The remaining aqueous layer was checked by chemical solvolysis (*8*) for steroid sulfates not hydrolyzed. In a second experiment with the same samples, only steroid glucuronides were hydrolyzed with β-glucuronidase from *E. coli* (*8*). After LLE solvolysis was applied to the remaining aqueous layer as mentioned above, DHEA was quantified by GC/MS in both experiments. Setting the quantity of DHEA−sulfate after hydrolysis with β-glucuronidase from *E. coli* to 100%, 80.9% ($\pm$7.2%; $n = 11$) of the DHEA−sulfate remained in the aqueous layer if hydrolysis was performed with *H. pomatia*. Only 19.1% of the DHEA−sulfate was hydrolyzed by the sulfatase activity ot this enzyme. As known from the quantification experiments, DHEA in urine from cattle mainly occurs as a sulfate conjugate. For this reason and furthermore because some estrogens degrade under solvolysis conditions, a separated hydrolysis of steroid glucuronides and steroid sulfates was established as follows: Glucuronides were hydrolyzed by β-glucuronidase from *E. coli* whereas sulfates were hydrolyzed chemically and purified separately. Besides the improved efficiency of the hydrolysis, another advantage is the very clean extract of the sulfates with regard to GC/C/IRMS purposes.

NP-HPLC Cleanup. In the literature, the further HPLC cleanup of urine samples is either based on RP_{18} columns (*11*, *14*) or on NH_2-propyl columns (*5*). Different types of RP columns (C_8 and C_{18}) were tested but discarded at this step. Because of more or less irreversible contamination with nonpolar matrix compounds, retention times of the analytes did not stay constant after few samples. Moreover, the cleanup was not sufficient. Better cleanup was achieved under NP conditions with NH_2-propyl columns. They could also be reequilibrated much easier. Some analytes, however, showed very low recoveries and peak tailing, and ghost peaks of target analytes occurred thus making fraction limits difficult to determine. This phenomenon was previously investigated (*15*) and explained by a reaction of the keto groups especially of epitestosterone and etiocholanolone with the primary amino groups of the stationary phase. This resulted in formation of Schiff's bases and influences the $\delta^{13}C_{VPDB}$ values of at least epitestosterone significantly. According to this, the use of columns with primary amino groups was also discarded. Because the purification of the extracts by NP columns was rather good, quite a similar column was chosen. Using the $(CH_3)_2N$-propyl columns, the possible formation of Schiff's bases is circumvented. In consequence, recoveries were almost complete for every analyte, the peak shape was good, ghost peaks were never observed, and the cleanup was comparable to the NH_2-propyl column.

Derivatization. Several methods were described to measure $\delta^{13}C_{VPDB}$ values of steroids by GC/C/IRMS. They can be measured following silylation (*5*) or acetylation (*16*), and several steroids can be measured underivatized (*11*). Silylation exhibits excellent gas chromatographic behavior. Unfortunately, trimethylsilyl (TMS) derivatives are not suited well for the following RP-HPLC. These compounds would hydrolyze at least partially under the given conditions. Moreover, TMS derivatives are not suited well for all combustion interfaces as the formed SiO_2 can deteriorate the oxidation reactor (*17*, *18*) and the end of the column or the backflush capillary might be blocked. We experienced that, if the derivatization reagent was injected into the GC, the column cannot be used for any other compounds but TMS derivatives any more. This would severely limit the practicability of this method. For these reasons and because estradiol cannot be measured underivatized [see Buisson et al. (*9*)], acetylation was chosen as the derivatization technique since no contamination of the GC/C/IRMS equipment, good chromatographic properties, and stability of the acetates during the further RP-HPLC were observed. The major disadvantage of acetylation generally is the change in the isotopic signature caused by the added carbon atoms. However, as will be described later, no $\delta^{13}C$ correction is necessary if etiocholanolone and epitestosterone are compared to DHEA and androstanediol to androstenediol as the number of acetates are kept the same in each case and if acetylation is carried out in a consistent fashion with the same batch of acetic anhydride.

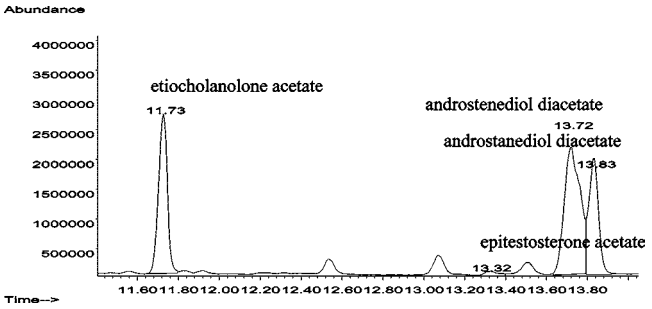


Figure 6. GC/MS chromatogram (scan mode, EI ionization) of the free and hydrolyzed androgen glucuronides containing subdivision without RP_{18} HPLC cleanup.

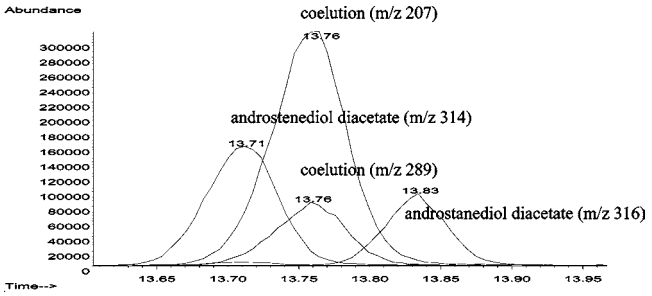


Figure 7. Selected ion gas chromatogram of the free and hydrolyzed androgen glucuronides containing subdivision revealing the coelution between androst-5ene-3β,17α-diol diacetate and 5α-androstane-3β,17α-diol diacetate.

RP-HPLC Cleanup. The GC/MS spectra, obtained after NP-HPLC and acetylation of the sample extracts, revealed sufficient cleanup for DHEA in the sulfate fraction. In contrast, the acetylated fraction of the free and hydrolyzed androgen glucuronides showed some confounding coelution between androst-5ene-3β,17α-diol diacetate and 5α-androstane-3β,17α-diol diacetate. As chromatographic baseline separation is mandatory (*3*), these analytes are not yet suitable to be measured by GC/C/IRMS. Only etiocholanolone acetate was cleaned up sufficiently and did not require further cleanup. However, androst-5ene-3β,17α-diol diacetate and 5α-androstane-3β,17α-diol diacetate were influenced by a more or less abundant coelution, which made valid GC/C/IRMS measurements impossible without further cleanup. **Figure 6** shows a typical GC/MS chromatogram (scan mode; EI ionization) of a purified sample extract after NP-HPLC and acetylation. The responses of etiocholanolone, androstenediol, and androstanediol are quite pronounced whereas epitestosterone is almost absent. The purity of etiocholanolone acetate is sufficient, but a coeluting substance inbetween the diols is present. **Figure 7** shows the main ions of the coelution (*m*/*z* 207 and *m*/*z* 289) between androst-5ene-3β,17α-diol diacetate (*m*/*z* 314) and 5α-androstane-3β,17α-diol diacetate (*m*/*z* 316). Identification of the confounding compound by standard MS libraries failed. Because the acetates are sufficiently stable, a further cleanup step of the acetylated androgen fraction following the NP-HPLC was employed. A RP_{18} HPLC step described by Flenker et al. (*11*) was slightly

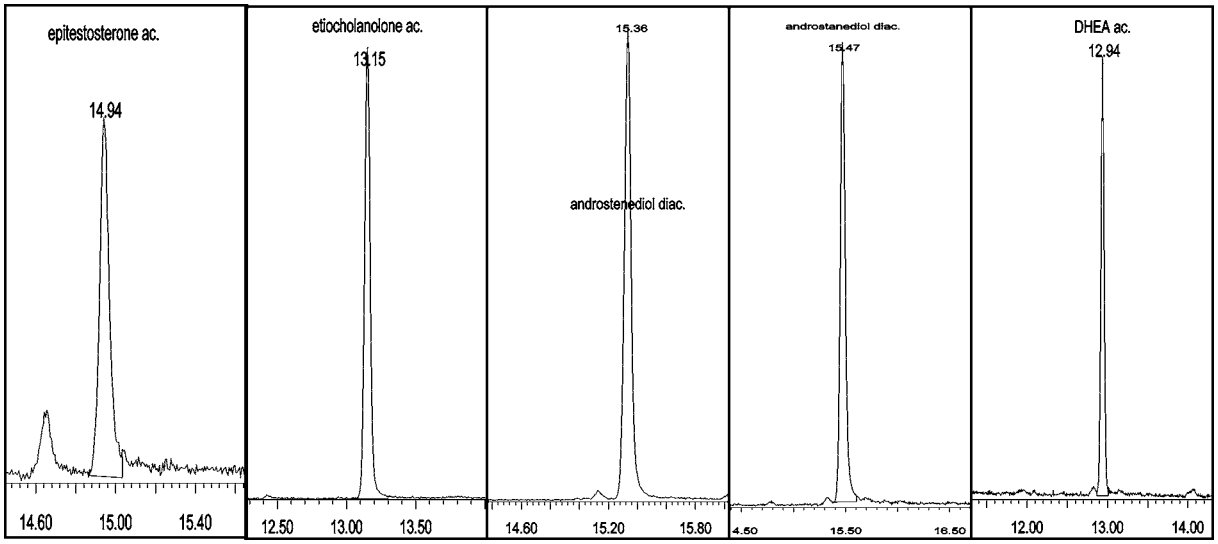


Figure 8. GC/MS chromatograms [scan mode, EI ionization; *x*-axis = time (min), *y*-axis = abundance] of the final biological extracts.

modified for the analysis of steroid acetates and introduced for further cleanup of the corresponding subdivision. Fractions containing only one analyte each were collected. The purity of the extracts was confirmed by GC/MS, and finally, the requirements for valid GC/C/IRMS measurements were met.

GC/MS. **Figure 8** shows the GC/MS chromatograms (scan mode; EI ionization) of a purified urine sample of all final fractions. From the free and steroid glucuronides containing subdivision epitestosterone acetate, etiocholanolone acetate, androst-5ene-3β,17α-diol diacetate, and 5α-androstane-3β,17α-diol diacetate and from the steroid sulfates containing subdivision DHEA acetate are included in the final method. The chromatograms demonstrate the sufficient performance of the cleanup concerning GC/C/IRMS measurements since no disturbing coelutions could be detected for any of the analytes. The main coeluting compound between the two diols was separated by RP-HPLC and retrieved in the etiocholanolone acetate-containing fraction.

GC/C/IRMS. **Table 1** gives an overview of the 24 urine samples from Holstein−Friesian cattle of different age and sex and under different feeding regimens used in this trial. Ten of them were derived from cattle treated with natural hormones. Besides testosterone-treated animals, also 17β-estradiol [see Buisson et al. (*9*)] or progesterone was injected. The 14 samples from untreated cattle generally served as a reference population. Because progesterone and estradiol (*9*) are not supposed to influence the testosterone metabolites, the 18 samples from nontestosterone-treated cattle served as a reference population for the purpose of the detection of testosterone abuse. Animal feedings were based either mainly on maize (C_4 plant, thus enriched ^{13}C amount; samples 1−17) or on grasses (C_3 plants, thus depleted ^{13}C amount; samples 18−24). In some animals, the feeding was changed from one to the other during the sampling period. Urine was collected at specific days, and $\delta^{13}C_{VPDB}$ values of the steroids were measured as described.

In **Figure 9**, the $\delta^{13}C_{VPDB}$ values of both ERCs are plotted against each other with DHEA acetate on the *x*-axis and androst-5ene-3β,17α-diol diacetate on the *y*-axis. $\delta^{13}C_{VPDB}$ values of DHEA acetate and androst-5ene-3β,17α-diol diacetate ranged randomly around the best fit. The difference of roughly 4‰ between both analytes is mainly caused by the acetylation. Two carbon atoms were added to DHEA whereas four carbon atoms were added to androst-5ene-3β,17α-diol and the $\delta^{13}C_{VPDB}$ value of the added carbons was approximately −55‰ (estimated by comparing free and acetylated standards). Statistical evaluation supported the assumption that $\delta^{13}C_{VPDB}$ values of the ERCs were independent from age, gender, and hormone treatment; they

Table 1. Collection Pattern of Urine Samples

sample no.	gender (animal)	treatment[a]	sampling date	feeding (days after changing the diet)
1	female calf (304)	no	23.06.2003	maize
2	female calf (303)	no	31.03.2003	maize
3	female calf (304)	17β-ED + PG, 1st day	01.04.2003	maize
4	male calf (685)	no	16.07.2003	maize (21 days)
5	male calf (685)	no	06.08.2003	maize (42 days)
6	adult bull (699)	no	22.08.2003	maize
7	adult steer (773)	no	22.08.2003	maize (63 days)
8	adult bull (699)	no	25.08.2003	maize
9	young steer (696)	no	26.08.2003	maize
10	young steer (697)	17β-ED + PG, 1st day	26.08.2003	maize
11	young steer (696)	no	01.09.2003	maize
12	young steer (697)	17β-ED + PG, 7th day	01.09.2003	maize
13	adult bull (699)	17β-ED, 16th day	01.09.2003	maize
14	female calf (303)	T, 2nd day	02.04.2003	maize
15	female calf (303)	T, 10th day	10.04.2003	maize
16	adult steer (773)	T, 44th day	16.07.2003	maize (26 days)
17	adult steer (773)	T, 7th day	01.09.2003	maize
18	female calf (305)	no	23.04.2003	grasses (7 days)
19	female calf (305)	no	21.05.2003	grasses (35 days)
20	adult bull (699)	no	02.06.2003	grasses
21	female calf (305)	no	17.06.2003	grasses (63 days)
22	female calf (303)	no	23.06.2003	grasses
23	adult steer (773)	T, 2nd day	04.06.2003	grasses
24	female calf (303)	T, 2nd day	25.06.2003	grasses

[a] Testosterone (T), 17β-estradiol (17β-ED), and progesterone (PG).

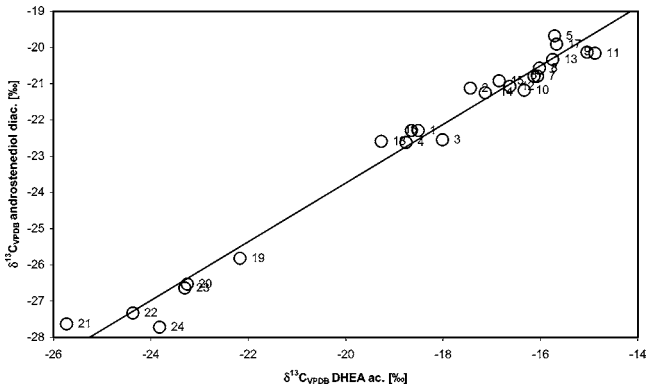


Figure 9. $\delta^{13}C_{VPDB}$ of both ERCs (DHEA acetate and androst-5ene-3β,17α-diol diacetate) of the test samples.

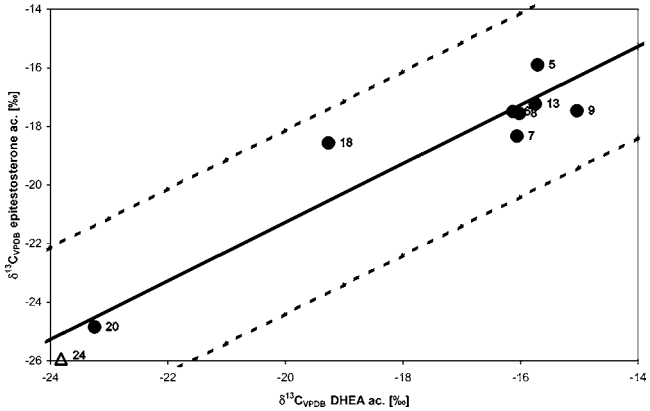


Figure 10. $\delta^{13}C_{VPDB}$ values of epitestosterone acetate vs DHEA acetate of urine samples of treated animals (△) or reference animals (●), the mean $\Delta\delta$ value (solid line; –1.3 ‰, $n = 8$), and the confidence interval (dashed line; ± the 3-fold standard deviation of 1.04 ‰).

were, as expected, dependent on the feeding. A linear mixed effects model was fitted to the data (*19*). $\delta^{13}C_{VPDB}$ was the dependent variable. The type of compound, the type of diet, and the administration of synthetic steroids served as independent variables. Random effects were grouped by individuals. Different $\delta^{13}C_{VPDB}$ values have to be assumed for different compounds ($p < 0.001$), which is due to differing states of derivatization. Application of synthetic steroids shows no effect ($p = 0.77$). In contrast, maize-based diets cause an average increase in $\delta^{13}C_{VPDB}$ of 6.24‰, which is highly significant ($p < 0.001$). The results also indicated that during a change in the composition of the feeding, the differences in the $\delta^{13}C_{VPDB}$ values (expressed as $\Delta\delta$-values) between the ERCs change parallel over the time. The two feeding groups (maize or grasses) were clearly separated. Samples 1−17 were based on maize feeding, and $\delta^{13}C_{VPDB}$ values ranged between −15 and −19‰ for DHEA acetate and between −19 and −23‰ for androst-5ene-3β,17α-diol diacetate. Samples 19−24 were based on grass feeding, and $\delta^{13}C_{VPDB}$ values were measured between −22 and −26‰ for DHEA acetate and between −26 and −28‰ for androst-5ene-3β,17α-diol diacetate. Sample 18 was taken from a female cow 7 days after changing the feeding from maize to grass. The measured ^{13}C ratios showed that the main carbon source of the ERCs reflected the former maize diet.

The method delivered $\delta^{13}C_{VPDB}$ ratios of acetates of androgenic steroids with either one or two hydroxy groups. A further correction of the $\delta^{13}C_{VPDB}$ values for acetylation was avoided as etiocholanolone acetate and epitestosterone acetate were compared with DHEA acetate, while 5α-androstane-3β,17α-diol diacetate was compared with androst-5ene-3β,17α-diol diacetate.

Figure 10 shows the $\delta^{13}C_{VPDB}$ values of epitestosterone acetate on the *y*-axis vs DHEA acetate on the *x*-axis. The mean $\Delta\delta$-value of −1.3‰ between epitestosterone acetate and DHEA acetate is shown with a solid line, and the confidence interval of the 3-fold standard deviation of 1.04‰ is shown with a dashed line. Because of generally low concentrations, epitestosterone could only be measured in nine of the 24 samples. Hence, the reference population of only eight samples from untreated cattle was small and the confidence interval for untreated cattle was quite large as a testosterone abuse could only be confirmed if the $\Delta\delta$-value between epitestosterone acetate and DHEA acetate was larger than 1.9‰ or lower than −4.4‰. In fact, the ^{13}C amount of epitestosterone acetate of sample 24 is only 2.1‰ depleted in contrast to DHEA acetate and the testosterone treatment could not be confirmed. These data finally supported the assumption that epitestosterone is not well-suited for GC/C/IRMS as a metabolite after testosterone application.

In **Figure 11** the $\delta^{13}C_{VPDB}$ values of etiocholanolone acetate are plotted against the corresponding values of DHEA acetate obtained from 23 of the 24 samples. The $\delta^{13}C_{VPDB}$ values of this metabolite of the reference population ($n = 17$) were 0.9‰ more depleted than the ERC on average. The standard deviation of these $\Delta\delta$-values was 0.77‰. Every sample could be classified correctly, the "positive" samples by falling outside the confidence interval from −3.2 to 1.4‰ (3-fold standard deviation). From five samples of the treated animals, almost constant $\delta^{13}C_{VPDB}$ values of etiocholanolone acetate (−28 to −27 ‰) were measured for at least 10 days after application. Hence, these values were almost independent from the feeding. Obviously, the endogenous production of etiocholanolone was suppressed for quite a long time because the metabolite exhibited the isotopic signature of the administered compound for more than 10 days. Furthermore, the testosterone treatment could be confirmed even 44 days after application (sample 16), which was not expected. Of course, $\Delta\delta$-values between metabolite and ERC are much larger when maize was used (about 10‰; samples 14, 15, and 17) than when grass feed was used (about 4‰; samples 23 and 24).

Figure 12 shows the $\delta^{13}C_{VPDB}$ values of 5α-androstane-3β,-17α-diol diacetate plotted against androst-5ene-3β,17α-diol diacetate of all 24 samples. Averaged, 5α-androstane-3β,17α-diol diacetate of the reference population ($n = 18$) was 1.2‰ more depleted in ^{13}C than androst-5ene-3β,17α-diol diacetate. The standard deviation of the $\Delta\delta$-values was 0.82‰, and the corresponding confidence interval of the 3-fold standard deviation ranged from −3.6 to 1.3‰. Every negative sample could be classified correctly falling inside this interval. However, for two "positive" samples, the testosterone treatment could not be confirmed. Sample 16 still showed a depleted $\delta^{13}C_{VPDB}$ value for the metabolite 44 days after testosterone application, but the $\Delta\delta$-value was −3.2‰ and no longer lower than −3.6‰. Sample 24 showed a $\Delta\delta$-value between the diols of −3.6‰ 2 days after testosterone application. The sample could not be classified "positive" on the basis of this pair despite the lowest ^{13}C amount of the metabolite of all samples because the $\delta^{13}C_{VPDB}$ value of the ERC was too depleted due to the grass feeding. In contrast to etiocholanolone acetate, 5α-androstane-3β,17α-diol diacetate did not show quite constant $^{13}C/^{12}C$ ratios for 10 days or even longer after testosterone application. The ^{13}C amount of 5α-androstane-3β,17α-diol diacetate of samples 15 and 17 (day 7 and 10) was significantly less depleted than of samples 14, 23, and 24 (day 2). This shows that the endogenous production of 5α-androstane-3β,17α-diol is suppressed for a shorter time and etiocholanolone is the better suited longtime metabolite due to slower endogenous "dilution".

Figures 10−12 also show that the changes in the diet of the animals could not induce $\Delta\delta$-values between metabolite and ERC, leading to false positive results. The finding that testosterone metabolites are generally more depleted than the ERCs can be explained by the fact that light isotopomers usually react faster than heavy ones (*20*); metabolites are more depleted in the ^{13}C amount than ERCs (if contemporaneously excreted) because they are positioned lower in the metabolic pathway.

Commercially available standards and also the testosterone of the medications generally showed $\delta^{13}C_{VPDB}$ values of −28 to −27‰. Hence, the upper limits of the confidence intervals (positive $\Delta\delta$-values) are, until now, not of practical interest; nevertheless, things may change in the future and both limits

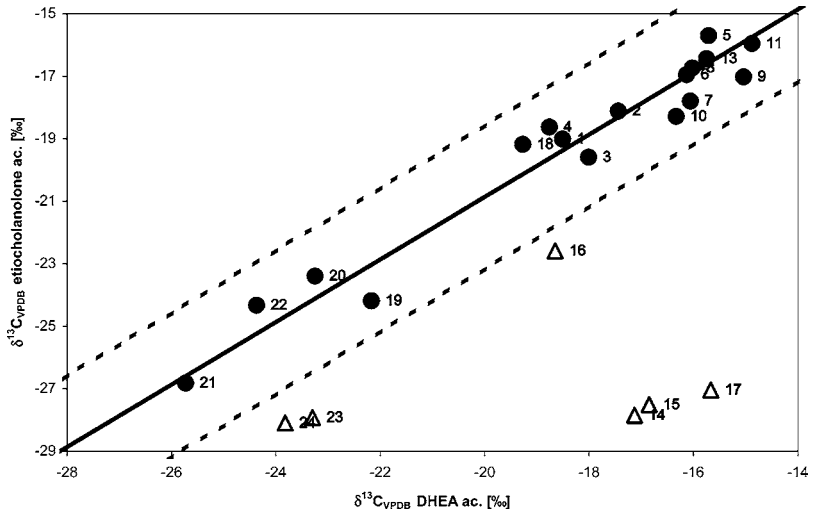


Figure 11. $\delta^{13}C_{VPDB}$ of etiocholanolone acetate vs DHEA acetate of urine samples of treated animals (△) or reference animals (●), the mean $\Delta\delta$ value (solid line; –0.9 ‰, $n = 17$), and the confidence interval (dashed line; ± the 3-fold standard deviation of 0.77 ‰).

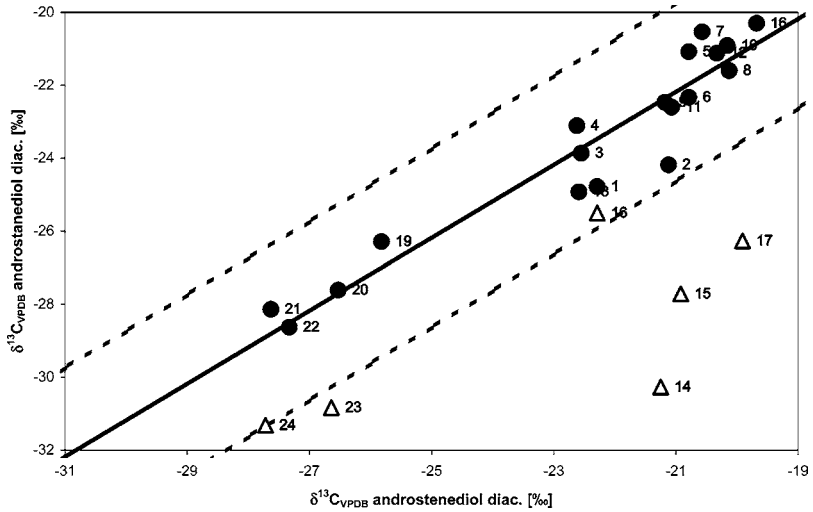


Figure 12. $\delta^{13}C_{VPDB}$ of 5α-androstane-3β,17α-diol diacetate vs androst-5ene-3β,17α-diol diacetate of urine samples of treated animals (△) or reference animals (●), the mean $\Delta\delta$ value (solid line; –1.2 ‰, $n = 18$), and the confidence interval (dashed line; ± the 3-fold standard deviation of 0.82 ‰).

of the confidence interval are needed. The confidence intervals are proposals based on 24 or less samples. The reference population has to be enlarged to verify or to adapt the intervals. Finally, with the developed method, the application of testosterone to the tested animals was detected at least 10 days and, in one case, even 44 days after treatment. GC/C/IRMS is suitable for the detection of misuse of natural hormones in the urine of cattle.

ABBREVIATIONS USED

EU, European Union; GC/C/IRMS, gas chromatography/combustion/isotope ratio mass spectrometry; ERC, endogenous reference compound; HOAc, acetic acid; DHEA, dehydroepiandrosterone; RP, reversed phase; SPE, solid phase extraction; NP, normal phase; HPLC, high-performance liquid chromatography; LLE, liquid/liquid extraction; EtOAc, ethyl acetate; TMS, trimethylsilyl; EI electron impact.

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