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# Investigation of the feeding effect on the $^{13}C/^{12}C$ isotope ratio of the hormones in bovine urine using gas chromatography/combustion isotope ratio mass spectrometry

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**Abstract**

The effect of the feeding on the $^{13}C/^{12}C$ isotope ratio of four endogenous steroid hormones testosterone (T), epi-testosterone (epi-T), dehydroepiandrosterone (DHEA) and etiocholanolone (ETIO) in bovine urine was investigated. An analytical method to determine the accurate isotope ratio was developed including an extensive clean up followed by enrichment of the analytes in two steps of HPLC fractionation. Feeding experiments with four young animals were performed using $C_3$ and $C_4$ plants (grass, maize silage, hay, etc.) over a time period of about 280 days. One cattle was used as a control animal with no change of its diet over the full period. The detection of the $^{13}C/^{12}C$ isotope ratio of the acetylated extracts was performed by gas chromatography/combustion isotope ratio mass spectrometry. After the first change of the feeding from $C_4$ to $C_3$ plants significant changes of the $\delta$ $^{13}C$ ‰ values were observed from the −19 to −23‰ level to the −24 to −32‰ level for etiocholanolone and epi-testosterone in urine of three animals, whereas the DHEA values remained under the level of the two metabolites. Testosterone could not be detected with GC–C–IRMS due to its low concentration in young animals. After the second change of the diet from $C_3$ to $C_4$ plants (after 222 days), the measured $\delta$ $^{13}C$ ‰ values have been stabilised at the original level. The results show that in case of the feeding with only $C_3$ plants the endogenous $\delta$ values of −32‰ can be reached. In this case the contribution of exogenous material with a $\delta$ value of −32‰ could not be detected independently of the concentration. If the diet contains $C_4$ plants the difference or the ratio of the $\delta$ $^{13}C$ ‰ values becomes the determinant in the discriminatory power. For validation of the method a human and a cattle were treated with testosterone and the $\delta$ $^{13}C$ ‰ values were measured in incurred human and cattle urine.


*Keywords:* Hormone; Carbon isotope ratio mass spectrometry; Cattle; Urine; $C_3$ plants; $C_4$ plants; Feeding

## 1. Introduction

The use of endogenous hormones as growth promoters is prohibited in the European Union. For the differentiation between the exogenous and the endogenous origin of hormone residues, several methods have been published in human [1–6] and in bovine urine samples [7]. These methods are based on the measurement of the relative difference between the $^{13}C/^{12}C$ ratio of endogenous steroid hormones in comparison to the $^{13}C/^{12}C$ ratio of the corresponding synthetic compound, using gas chromatography and isotope ratio mass spectrometry (GC–C–IRMS).

All published clean up procedures use careful purification to avoid interferences with the matrix. Unfortunately, in literature only few GC–C–IRMS chromatograms of real urine samples were published showing the separation of the analyte from interferences. The combination of solid phase extraction and HPLC for the sample preparation of hormone residues in urine samples has been considered by Daeseleire et al. [8]. The combination of HPLC with isotope mass spectrometry for the determination of testosterone (T) and its metabolites in cattle urine has been published by Ferchaud et al. [7,9]. Before starting the analysis by gas chromatography, a derivatisation step (acetylation) of the purified extracts is unavoidable. However, Mason et al. [10] have reported about the determination of androstanediol as major metabolite of testosterone and cholesterol as

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endogenous reference compound in bile of cattle without derivatisation.

The $^{13}C/^{12}C$ ratio of the endogenous hormones in cattle depends on the ratio of light and heavy carbon isotopes in the food. This has as consequence that the feeding has direct influence to their ratio during the biosynthesis of the endocrine hormones.

The carbon isotope fractionation in plants is a well known phenomenon. Plants with $C_3$ and $C_4$ photosynthetic pathways show different discrimination against $^{13}C$ during photosynthesis. The effects of the overall isotope discrimination during the photosynthesis are the diffusion of $CO_2$, the incorporation of $CO_2$ by phosphoenolpyruvate carboxylase or ribulose bisphosphate carboxylase and the respiration [11].

In $C_3$ plants, the $CO_2$ incorporation follows the Calvin-cycle and is limited mainly by ribulose bisphosphate carboxylase. In $C_4$ plants, where the $CO_2$ fixation takes place over the Hatch–Slack-cycle, the carbon incorporation is limited by the rate of $CO_2$ diffusion into the leaf [11], resulting lower discrimination power against $^{13}C$ ‰ and thus higher $^{13}C/^{12}C$ isotope ratio in the organic compounds of the plant.

Several papers were published on the determination and quantification of the physiological level of hormones in cattle, usually by GC/MS methods. The discussion and the presentation of the residue studies would go beyond the scope of this paper, and they are not relevant to this presentation, because the isotope ratios of the endogenous steroids are independent of their concentrations.

The influence of cattle feeding was not systematically investigated till now. Ferchaud et al. reported about the influence of the feeding on a 15-year-old cow after treatment with testosterone enanthate and with various diets [9]. However, the investigation of the influence of the feeding is particularly important in young animals both because of the low rate of steroid hormone production in the first year of their life, and also because young animals are often treated with endogenous hormones. In this paper we will report about the results of a study with four young bulls fed with $C_3$ and $C_4$ plants.

## 2. Experimental

### 2.1. Standards and chemicals

All solvents were of analytical grade unless stated otherwise. Demineralized water was used in all experiments (Milli-Q Gradient, Millipore). Testosterone (T), epi-testosterone (epi-T), etiocholanolone (ETIO) and dehydroepiandrosterone (DHEA) were purchased from Sigma (Taufkirchen, D), testosterone was also obtained from Acros Organics (Geel, B). Sep-Pak $C_{18}$ cartridges were purchased from Waters (Eschborn, D) and ß-glucuronidase (*Escherichia coli*) from Roche Diagnostics (Mannheim, D). For human administration a testosterone enanthate was purchased from Jenapharm (Jena, D).

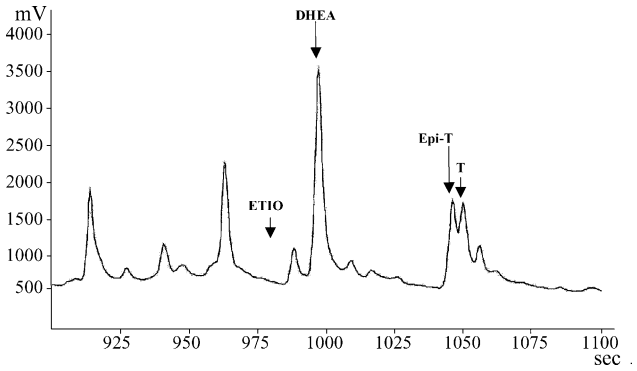


Fig. 1. A typical GC–C–IRMS chromatogram of cattle urine sample after solid phase extraction and one-step HPLC purification shows some interferences with the matrix.

### 2.2. Standard solutions

Stock solutions were prepared with methanol containing 1 mg/ml of the steroids T, epi-T, DHEA and ETIO. The working standard solution contained 2–10 μg/ml of each analyte dissolved in methanol.

### 2.3. Animals

Four young bulls (type: fattened cattle crossbred with milk cattle), about 1-year-old, were kept on a farm, owned and maintained by the Federal Institute for Risk Assessment in Berlin and were held during a period of about 9 months under controlled conditions.

### 2.4. Sample preparation

The main problem with using isotope mass spectrometry is the elimination of interferences, which are potentially able to disturb the signal specificity, especially in the case of the urine of cattle.

Fig. 1 shows a typical GC–C–IRMS chromatogram of a cattle urine after solid phase extraction and one-step HPLC fractionation. The chromatogram shows the overlap of the testosterone and epi-T with other interfering compounds of the matrix. Therefore, the application of a further clean up procedure is inevitable to get a better purification of the extracts using a second (preparative) HPLC as we have reported in our previous work [12].

Samples of 20 ml of urine were hydrolysed with *E. coli* (pH 4.8, 37 °C, 16 h) after centrifugation. Solid phase extraction was performed using Sep-Pak $C_{18}$ cartridges (washed with $2 \times 5$ ml $H_2O$, eluted with 5 ml methanol/ethyl acetate, 30:70%, v/v). The obtained fraction was evaporated under nitrogen at 40 °C to dryness.

For the deconjugation, 5 ml ethyl acetate and sulphuric acid (2.2 M) were given to the dry residue and incubated at 55 °C for 2 h. After neutralisation with 5 ml of 10% sodium carbonate and centrifugation, the ethyl acetate phase was selected, then the neutralisation step was repeated twice. The

organic phase was dried under nitrogen at 40 °C. The clean up was continued by fractionation with HPLC-I system using automatic valve switching, as described by Daeseleire et al. [8].

*2.4.1. HPLC-I*

The first HPLC system that we used comprised two pumps for gradient elution (Waters 515) and one pump for backflush from the pre-column. An autosampler (Waters 717), a UV-detector (Waters 490 at 254 and 280 nm wavelength), an automatic valve switching system (WAVS, Waters) and a fraction collector (Waters FC II) completed the system.

The mobile phase was methanol–water (65:35), the flow rate was 1.2 ml/min, the detector was set at 254 and 280 nm. The column was a Resolve $C_{18}$ (5 μm, 3.9 mm × 150 mm, Waters) protected by a $C_{18}$ guard column. Three minutes after injection of 70 μl of sample, the guard column was backflushed with methanol/water (65:35%) for 5 min. Afterwards, the valves were switched to the initial position and the analytes were eluated from the analytical column with gradient elution (from 65:35% methanol–water to 100% methanol in 20 min). Three fractions, containing epi-T, T, DHEA and ETIO were collected. Since DHEA and ETIO are non-UV active, the corresponding fractions were checked with LC/MS/MS in the case of standard solution.

*2.4.2. HPLC-II*

For a better purification, we injected the full fraction from the first HPLC (ca. 2 ml) manually into the second (preparative) HPLC. This HPLC purification was performed with the preparative gradient system (Waters 600 EF) and a photodiode-array detector (Waters 996) with a cell volume of 100 μl. The fraction collector Waters FC II was used. The gradient was started at 40% B and ended at 70% B. The run time was 20 min. Eluent A was water with 10% methanol and eluent B was methanol with 10% water. The photodiode-array detector was used at 254 nm as monitoring wavelength. The column we used was an XTerra $RP_{18}$, 7 μm, 19 mm × 150 mm (Waters).

The fractions were collected over 1 or 2 min then extracted with 2 × 5 ml of chloroform. After evaporation under $N_2$ (40 °C) the dry residue was derivatised as described below.

*2.4.3. Derivatisation*

Before the determination of the isotope ratio using GC–C–IRMS, the residues had to be acetylated with 100 μl acetic acid anhydride (60 min, 80 °C) then dried under $N_2$ and dissolved in 100 μl ethyl acetate and 10 μl were injected into GC–C–IRMS.

*2.5. Gas chromatograph–combustion–isotope ratio mass spectrometer*

The gas chromatograph model 6890 (Agilent) coupled with a DeltaPlus isotope ratio mass spectrometer with GC combustion III interface (ThermoFinnigan, Bremen, Germany) was used with a Combi-PAL large-volume auto sampler (CTC Analytics, Zwingen, CH) and UNIS 2000 programmed temperature vaporizer (Chromtech, Idstein, D).

The GC column was a fused silica capillary column BPX-50; 30 m × 0.25 mm i.d.; film thickness, 0.25 μm (SGE). The carrier gas was helium. The injector allowed the injection of large volumes (10–100 μl) of the samples to increase the sensitivity. The samples were injected into a 50 °C liner that was vented after 3.1 min to the atmosphere. Then the injector was heated rapidly to 350 °C. After separation the analytes were converted into $CO_2$ and $H_2O$ using a combustion oven functioning at 950 °C. After the water was removed, the $CO_2$ molecules was ionized and the masses of $CO_2$ (*m/z* 44, 45 and 46) were analysed by magnetic field mass spectrometry. The measured $^{13}C/^{12}C$ ratio was corrected with $^{17}O$ according to Craig [13] and converted to the $\delta$ $^{13}C$ ‰ notation, i.e. the relative difference between the $^{13}C/^{12}C$ of the sample and the $^{13}C/^{12}C$ of the international carbonate standard Pee Dee Belemnite (PDB) formation, according to the following equation:

$$\delta^{13}\,\mathrm{C}(‰) = \left[\frac{(^{13}\mathrm{C}/^{12}\mathrm{C})_{\mathrm{sample}}}{(^{13}\mathrm{C}/^{12}\mathrm{C})_{\mathrm{standard\,(PDB)}}} - 1\right] \times 10^3$$

## 3. Results and discussion

*3.1. GC–C–IRMS*

The measured $\delta$ $^{13}C$ ‰ values of the commercially available testosterone (e.g. Acros) showed good correlation with published data [7]: for underivatised T, $-29.8 \pm 0.42$‰ and for acetylated T, $-32.7 \pm 0.79$‰ ($n = 10$). The comparison between chromatographic behaviour of underivatised and derivatised (acetylated) standards shows, that acetylation produce more improved peaks.

To increase the sensitivity of the system, a PTV injector with Peltier cooled insert liner was installed onto the GC. The injector was operated in the solvent vent mode. The injection volume and the injection parameter were optimised to get high sensitivity and adequate separation. The optimum we found for the injection volume was at 10 μl. The variation of the program of the injector increased the sensitivity but the noise ratio as well, and therefore, we used two different temperature programs for high and low sensitivity depending on the concentration of the analytes in the samples. The closing times were, respectively, 2.5 min for the low and 3.1 min for the high sensitivity. Closing the venting of the injector at 2.5 min produced about three times higher peaks, but it caused a higher background noise, as well. The optimum we found for acetylated hormones was the closing of the venting valve at 3.1 min.

For the derivatised samples, a fused silica GC column with the stationary phase of 50% phenyl-polysilphenylene-

Table 1
Analytical mean and variation of $\delta$ $^{13}$C ‰ with and without sample preparation, and the biological mean and variation of $\delta$ $^{13}$C ‰ values of steroids in urine samples from four animals during the first period of the feeding (1–138 days) mainly with $C_4$ plant

| | Standard solution (mean ± S.D.) | Urine + standard solution (mean ± S.D.) | Four animals during the first period of feeding (mean ± S.D.) |
|---|---|---|---|
| DHEA | −33.11 ± 1.27 ($n$ = 24) | 30.90 ± 1.32 ($n$ = 15) | −21.23 ± 1.85 ($n$ = 15) |
| ETIO | −31.17 ± 0.47 ($n$ = 24) | 29.13 ± 0.71 ($n$ = 11) | −22.40 ± 1.06 ($n$ = 16) |
| epi-T | −36.66 ± 1.09 ($n$ = 24) | 32.19 ± 2.1 ($n$ = 14) | −20.57 ± 1.30 ($n$ = 11) |
| T | −30.66 ± 0.97 ($n$ = 25) | 29.38 ± 1.9 ($n$ = 14) | – |

siloxane showed a good separation. A length of 30 m is necessary for sufficient separation of epi-T and T. For checking the stability of the $\delta$ $^{13}$C ‰ values, $CO_2$ as a reference calibration gas was added four times in the first part of the chromatogram and once before the epi-T was eluated, because at this time no disturbing peaks could be observed in the chromatogram.

The main problem in using the isotope mass spectrometry is the elimination of interferences which are potentially able to disturb the signal specificity, especially in the case of the urine of cattle. The purity of the extracts was checked by comparing blank and spiked urine samples (from untreated cow). Even by using two HPLC fractionations an interfering compound at the retention time of DHEA in the fraction of testosterone was found. The origin of this peak is not clear. The $\delta$ $^{13}$C ‰ values of the artefact peak were found at −21‰, which is different from the DHEA standard of −32‰. Further investigations with GC/MS are necessary to identify this compound.

The isotope fractionation was checked by injection of standard solution (10 ng/μl) and by injection of spiked urine samples in range of 20–100 ng/ml. The $\delta$ $^{13}$C ‰ values for DHEA, epi-T, T and ETIO are shown in Table 1 and indicate, that no isotopic discrimination occurred during the sample clean up. The differences of the mean values of standard solution and spiked urine samples can be attributed to the natural endogenous steroid hormone content of the “control” animal.

### 3.2. Validation

The stability of the $\delta$ $^{13}$C ‰ values was checked with the daily injection of the standard solutions. Standard curves for each analyte were generated by analyzing “negative” urine samples from a cow, spiked with standard solution (in duplicate) of each of the four analytes at 20, 30, 40, 50, 60, 80, 90 and 100 ng/ml concentration level, corresponding to the expected concentration level in urine samples. The quantification of each compound was made by linear regression of the peak area versus spiked standard concentration. The limit of detection (LOD) and the limit of quantification (LOQ) were statistically calculated applying the method of Funk et al. [14]. The LOD values were about 30 ng/ml, the LOQ values between 40 and 90 ng/ml for the four analytes. They allow us to conclude, that for quantification purposes the GC–C–IRMS is not the best choice, and it may even be unsuitable.

However, since the $^{13}$C/$^{12}$C isotope ratio is independent of the quantity of the analyte, the presented method is suitable for the investigation of $\delta$ $^{13}$C ‰ changes caused by different feeding. Therefore, the more sensitive GC/MS methods should be used for kinetic studies.

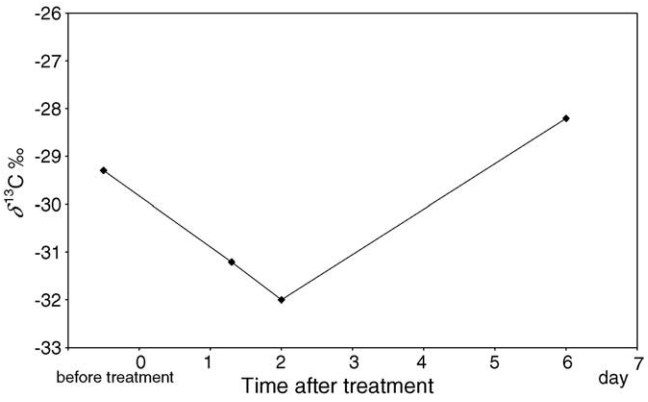


Fig. 2. $\delta$ $^{13}$C ‰ values of testosterone in human urine after treatment with testosterone enanthate.

The analytical variation was determined with and without sample preparation using standard calibration curves and spiked urine calibration curves. The obtained data are presented in Table 1. The $\delta$ $^{13}$C ‰ values of all four analytes were stable. They show no dependence of the injected amounts between 20 and 100 ng when standard solution were examined at the described conditions.

Spiked calibration curves show higher standard deviation of the $\delta$ $^{13}$C ‰ values mainly in low concentrations due to the endogenous steroid level of the “blank” urine.

For the determination of the recoveries blank urine samples were spiked with standard solutions at concentrations

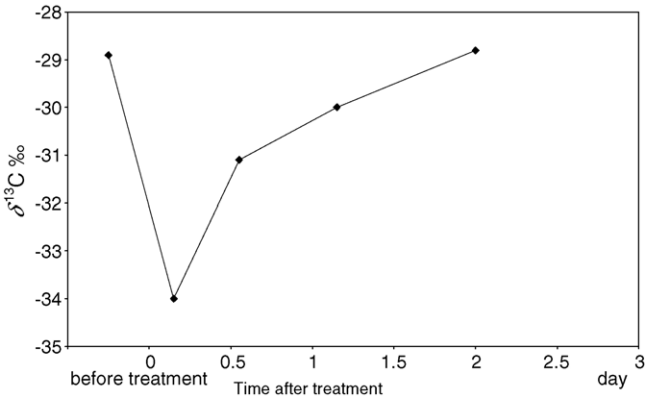


Fig. 3. $\delta$ $^{13}$C ‰ values of testosterone in cattle urine after treatment with testosterone esters.

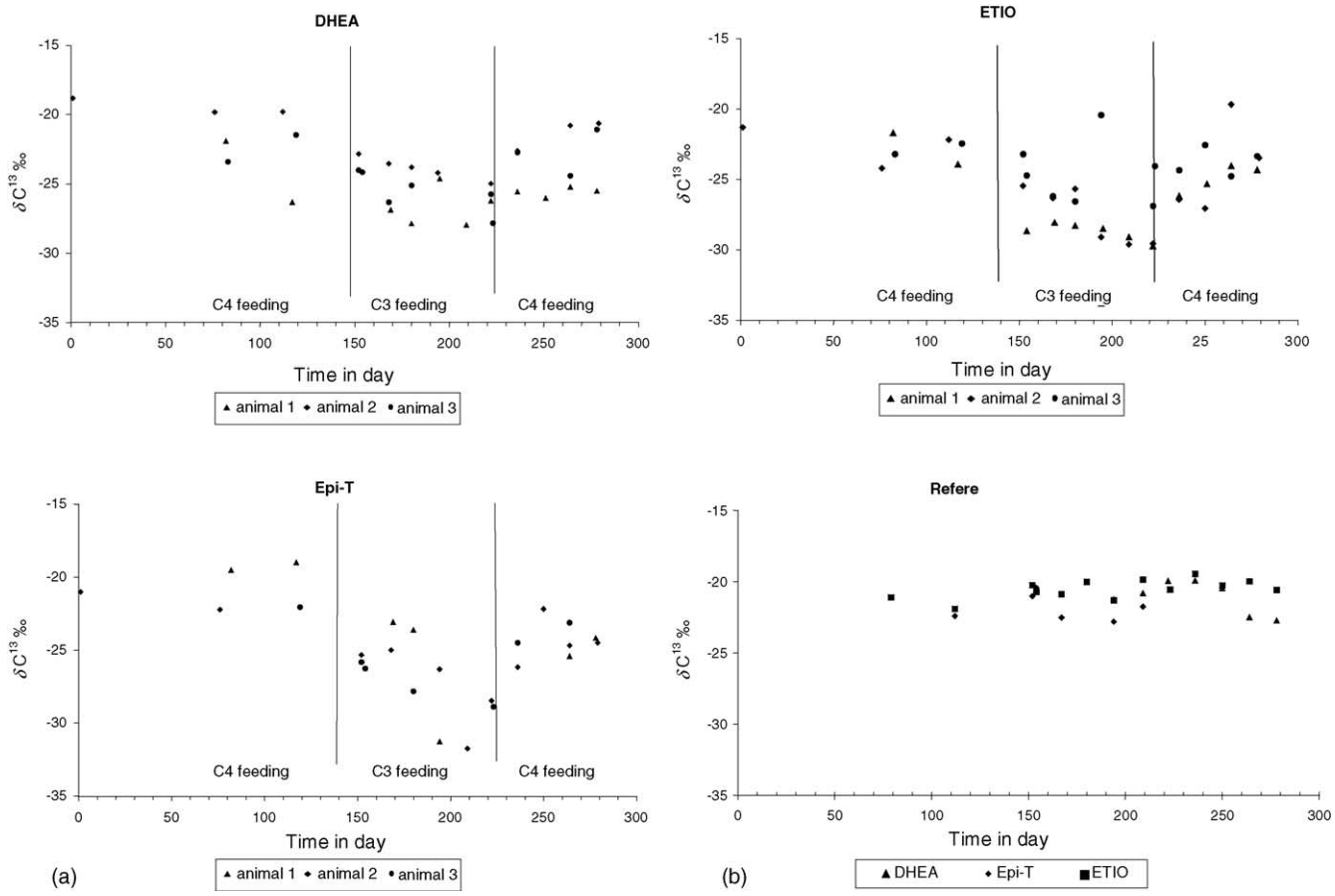


Fig. 4. Dependence of the $\delta$ $^{13}$C ‰ values of DHEA, epi-T and ETIO on the feeding in urine of three bulls. Bull 4 was used as a control animal, the feeding of which remained the same all along the experiment.

between 20 and 100 ng/ml in 10 ppb steps. The recovery data were obtained by comparing the peak heights or areas of the spiked samples and the standard solution by GC–C–IRMS. Due to the several purification steps, the recoveries were quite low and show high variation. For DHEA, the range of values were between 32% and 88%, for ETIO 23% and 68%, for epi-T between 18% and 56%. For testosterone, the recovery values were about 20%.

### 3.2.1. Human administration

The effect of testosterone administration to the $\delta$ $^{13}$C ‰ values was investigated by the treatment of a man with testosterone ester. Testosterone enanthate (250 mg/ml) was administered intramuscularly for a healthy male volunteer. The administration was carried out under medical control.

Untreated urine was taken on 2 days before he was treated and incurred urine samples were collected after the treatment over a period of 28 days.

The exact $\delta$ $^{13}$C ‰ values of testosterone enanthate preparate that was administered was unknown. However, we observed the change of $\delta$ $^{13}$C ‰ values of T after administration in the human urine from $-29.3 \pm 0.59$‰ to $-32.0 \pm 0.27$‰ ($n = 4$) as shown in Fig. 2. After 6 days, the $\delta$ $^{13}$C ‰ values returned to the original level.

### 3.2.2. Animal administration

The influence of testosterone administration to the $\delta$ $^{13}$C ‰ values in cattle was investigated, as well. Untreated urine samples were collected from a 5-year-old cow on a farm owned and maintained by the Federal Institute for Risk Assessment, Berlin. The urine samples were collected over a period of 6 days after treatment of the cow by intramuscularly injection with the mixture of testosterone 3.23 g (Acros), testosterone acetate 0.96 g (Sigma) testosterone benzoate 0.98 g (Sigma) and testosterone propionate 1.0 g (Sigma), dissolved in 150 ml of peanut oil. The measured $\delta$ $^{13}$C ‰ values of the (acetylated) testosterone and testosterone esters after fast saponification (Table 2) show a mean value of $-35.11 \pm 0.03$‰ ($n = 3$). According to this, a signifi-

Table 2
$\delta$ $^{13}$C ‰ values of acetylated testosterone used for the treatment of a cattle after fast saponification

| | $\delta$ $^{13}$C ‰ (mean ± S.D.) |
|---|---|
| Testosteron (T) | −34.45 ± 0.27 ($n = 3$) |
| T-acetate | −34.35 ± 0.59 ($n = 9$) |
| T-benzoate | −34.66 ± 0.32 ($n = 3$) |
| T-propionate | −33.08 ± 0.07 ($n = 3$) |
| Mixed for the administration | −35.11 ± 0.03 ($n = 3$) |

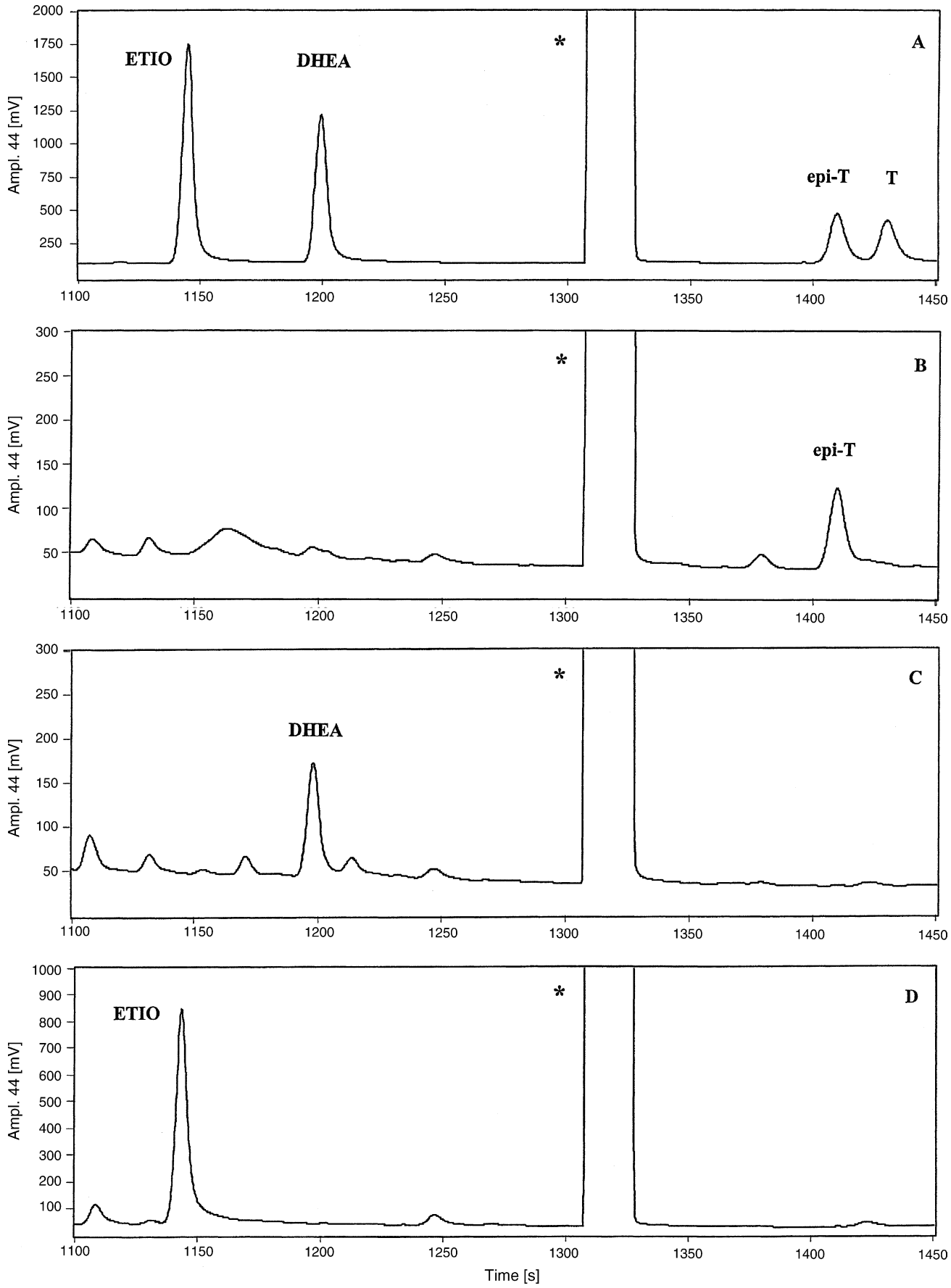


Fig. 5. A typical set of GC–C–IRMS chromatograms of standard solution (10 ng/μl in 10 μl injection volume, A) and the three fractions (B, C, D) of the bull urine samples, after two HPLC steps purification. (*) $CO_2$ as reference gas.

Table 3
Identification number (I.D.) and the age of the bulls used for feeding experiment

| I.D. of the animal | Age at the start of the experiment (month) | Remarks |
|---|---|---|
| 1 | 15 | |
| 2 | 16 | |
| 3 | 13 | |
| 4 | 12 | Control animal |

The feeding was not changed for the control animal (no. 4).

cant decrease of the $\delta$ $^{13}$C ‰ values from $-28.9 \pm 0.21$ to $-34.0 \pm 1.1$‰ ($n = 5$) was observed for T, in the treated animal, which returned to the base level 2 days after administration (see Fig. 3).

The administration was carried out with the permission of the ethical committee.

## 4. Influence of feeding

Four young bulls, about 1-year-old, were held during a period of about 9 months under controlled conditions. Table 3 shows the age of the cattle at the start of the experiment. For three animals, the feed was changed twice on 139th and on the 222nd day, as shown in Table 4. The fourth bull had the same feed during the full period of the experiment. The feeding plan is shown in Table 4. The animals were fed at the start of the experiment with a mixture of $C_3$ and $C_4$ plants (hay, maize silage, etc.). The feeding was changed after 138 days for three animals to $C_3$ plants. After 222 days, the feeding was changed back to the original diet (mainly $C_4$ plants). The fourth bull (i.e. the control animal) had the same food (hay, maize silage, etc.) over the full period of 9 months than the three other animals in the first feeding period. Urine samples were collected every 14 days over the period of about 9 months.

Table 4
Feeding plan

| Duration of the feeding | Amount/day/animal (kg) | Plant[a] | Way of the $CO_2$ fixation |
|---|---|---|---|
| Days 1–138 | 2 | Hay | $C_3$ |
| | 2 | Beet | $C_3$ |
| | 6.5 | Maize silage | $C_4$ |
| | 0.5 | Sugar beet | $C_3$ |
| | 0.3 | Soya | $C_3$ |
| | 0.3 | Feed additives | – |
| First change of the feeding | | | |
| Days 139–221 | Ad libitum | Hay | $C_3$ |
| | 6 | Beet | $C_3$ |
| | 1 | Sugar beet | $C_3$ |
| | 0.3 | Soya | $C_3$ |
| | 0.5 | Feed additives | – |
| Second change of the feeding | | | |
| Days 222–280 | 2 | Hay | $C_3$ |
| | 0.3 | Rape | $C_3$ |
| | 9.0 | Maize silage | $C_4$ |
| | 0.6 | Sugar beet | $C_3$ |
| | 0.8 | Soya | $C_3$ |
| | 0.3 | Feed additives | – |

[a] Straw: ad libitum.

At the start of the feeding experiment with (mainly) $C_4$ plants mean values of $\delta$ $^{13}$C ‰ between $-19$ and $-23$‰ were measured for epi-T, ETIO and DHEA in all four animals. After the first change of the feeding at the 139th day (from $C_4$ to $C_3$ plants) the $\delta$ $^{13}$C ‰ values decreased and remained stable between $-25$ and $-32$‰, whereas the DHEA values remained under the level of the two metabolites.

Concerning the $\delta$ $^{13}$C ‰ values of the control animal, fed mainly with $C_4$ plants during the full period of the experiment, no significant changes could be observed. The biological variation has been determined as the differences in the individual variation of the $\delta$ $^{13}$C ‰ values for all four animals during the first feeding period (138 days, mainly $C_4$ feed, Table 1).

The results of the GC–C–IRMS determination for the isotope ratio of the three steroids in urine samples during the full feeding experiment are presented in Fig. 4. These figures show the "time versus $\delta$ $^{13}$C ‰" curves of the metabolites and the precursor (DHEA) of testosterone for all animals during the experiment. The concentration of testosterone was too low for the accurate determination of the $\delta$ $^{13}$C ‰ value by IRMS. This is presumably due to the low own production of young animals and also to the low sensitivity of the IRMS. After the second modification of the feeding after 222 days (back to mainly $C_4$ plants), the $\delta$ $^{13}$C ‰ values were stabilised at the original level. Fig. 5 shows a GC–C–IRMS chromatogram of a standard solution and a typical set of the three fractions of cattle urine samples.

## 5. Conclusions

This study gives a valuable contribution for controlling the misuse of endogenous hormones as growth promoters for food producing animals, with the prediction about the carbon stable isotope ratio of steroid hormones. The results show the dependence of the $^{13}$C/$^{12}$C ratio of the two metabolites of testosterone and the precursor dehydroepiandrosterone on the variation of the feeding with $C_3$ and/or $C_4$ plants. In case of the feeding with only $C_3$ plants endogenous $\delta$ values of $-32$‰ can be reached, which means that the contribution of exogenous material with a $\delta$ value of $-32$‰ cannot be detected independent of the concentration. If the diet contains $C_4$ plants the difference or the ratio of the $\delta$ $^{13}$C ‰ values between DHEA and the metabolites becomes the determinant in the discriminatory power. Consequently, it is easier to prove the fact of administration in young bulls, which had been partially fed with $C_4$ plants than with only $C_3$ plants.

Future work will concentrate on the extension of the data set for treated animals during different feeding experiments.

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