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Application of Gas Chromatography–Mass Spectrometry/Combustion/Isotope Ratio Mass Spectrometry (GC-MS/C/IRMS) To Detect the Abuse of 17β-Estradiol in Cattle

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ABSTRACT: Although the ability to differentiate between endogenous steroids and synthetic homologues on the basis of their $^{13}C/^{12}C$ isotopic ratio has been known for over a decade, this technique has been scarcely implemented for food safety purposes. In this study, a method was developed using gas chromatography–mass spectrometry/combustion/isotope ratio mass spectrometry (GC-MS/C/IRMS) to demonstrate the abuse of 17β-estradiol in cattle, by comparison of the $^{13}C/^{12}C$ ratios of the main metabolite 17α-estradiol and an endogenous reference compound (ERC), 5-androstene-3β,17α-diol, in bovine urine. The intermediate precisions were determined as 0.46 and 0.26‰ for 5-androstene-3β,17α-diol and 17α-estradiol, respectively. This is, to the authors' knowledge, the first reported use of GC-MS/C/IRMS for the analysis of steroid compounds for food safety issues.

KEYWORDS: *GC-MS/C/IRMS, steroids, natural hormones, estradiol, urine, bovine, food safety, abuse*

INTRODUCTION

The influence of steroid hormones on muscle/meat building has been known for over 70 years, which led to widespread use in both sports and stock farming, respectively. Whereas their immediate effect on animals from the farmer's point of view is clear, risk assessment was subjected to debates because of divergent opinions at the international level, for example, Codex Committee on Residue of Veterinary Drugs in Foods (CCRVDF) and Scientific Committee on Veterinary Measures relating to Public Health (SCVPH). Although hormones are licensed in various countries worldwide, they have been banned for use as growth promoters in the European Union since 1988.[1] As a result, monitoring the abuse of steroid hormones in large-scale surveillance programs for food safety reasons is mandatory for all member states.[2] When looking at the results of these monitoring plans from 2000 up to 2010,[3] an annual average of approximately 8% of the noncompliances for steroids (group A3) are attributable to 17β-estradiol. However, these monitoring programs are still based on the classical approach using either GC-MS or LC-MS, which are unable to provide unambiguous results when it comes to the detection of synthetic analogues of naturally occurring steroid hormones in urine. In the case of estradiol, a population study on the presence of natural steroids in bovine urine in the United Kingdom showed that when a concentration threshold value for 17α-estradiol in bovine urine is set to indicate 17β-estradiol abuse, a confirmatory analysis is required because there is an overlap in the concentration of 17α-estradiol between treated and nontreated animals.[4] Since it became clear during the late 1990s that a distinction could be made between endogenous steroids and exogenous homologues based on their carbon isotopic composition ($^{13}C/^{12}C$), reported as $\delta^{13}C$ values (‰), analyses using gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) have been adopted in the field of sports doping control[5] and food safety.[6−14] Exogenous steroids, which are synthesized from plant material, are enriched in ^{12}C compared to endogenously produced steroids because the source material originates from plant species that are naturally low in ^{13}C content. The $\delta^{13}C$ values of exogenous steroids are usually lower than −30‰, whereas $\delta^{13}C$ values of endogenous steroids reflect the diet and are usually above −28‰. Because of the individual variability of the $\delta^{13}C$ values, mostly caused by differences in diet, both precursors, also called endogenous reference compounds (ERCs), and metabolites of the targeted steroid hormone are measured. Only the $\delta^{13}C$ values of the metabolites are influenced by the administration of the exogenous steroid and, therefore, the difference between the $\delta^{13}C$ value of the ERC and the metabolite, expressed as $\Delta^{13}C$ (‰), provides proof of administration. Still, in the field of food safety, the use of GC/C/IRMS to differentiate between endogenous steroid hormones and synthetic homologues in cattle has been applied only scarcely until now,[6] and the number of published methods is slowly growing.[7−14]

A method for the detection of 17β-estradiol administration to cattle is presented in this paper using gas chromatography–mass spectrometry/combustion/isotope ratio mass spectrometry (GC-MS/C/IRMS) for the measurement in urine of 5-

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androstene-3β,17α-diol as ERC and 17α-estradiol as metabolite. Sample preparation was based on the previously published method,[14] with minor adaptations to adjust to the current laboratory situation and to allow further automation.

MATERIALS AND METHODS

Urine Samples. *Noncompliant Samples.* One male and one female bovine, aged between 18 and 24 months and weighing approximately 400 kg, were treated with a single intramuscular injection containing 1 mg/kg body weight testosterone (administered as 1.194 mg/kg testosterone propionate) and 0.2 mg/kg body weight estradiol (administered as 0.276 mg/kg 17β-estradiol-3-benzoate). Urine samples were collected before and during the first 28 days after administration and stored frozen at −21 °C. Afterward, the samples were stored at −85 °C until assay.

Compliant Samples. Twenty-nine urine samples of pregnant cows were collected at different farms to be used as reference population samples. The samples were stored at −85 °C until assay.

Spiked Samples. Routine samples in which no 17α-estradiol could be detected and with concentrations of 5-androstene-3β,17α-diol below 5 ppb were used for the preparation of samples spiked with 17α-estradiol and 5-androstene-3β,17α-diol, to be employed for validation and quality control.

Reagents and Chemicals. All reagents and solvents were of analytical grade and were provided by Sigma-Aldrich (Bornem, Belgium). The solvents for liquid chromatography were of LC and HPLC grade from Biosolve (Valkenswaard, The Netherlands). 17β-Testosterone acetate was supplied by Sigma-Aldrich. Other steroids were obtained from Steraloids (Wilton, NY, USA). SPE C_{18} cartridges were obtained from Achrom (Zulte, Belgium). β-Glucuronidase was from *Escherichia coli* from Roche Diagnostics GmbH (Mannheim, Germany). Pyridine and acetic anhydride used in derivatization reactions were from Sigma-Aldrich.

Apparatus. *HPLC/UV.* During sample preparation, two subsequent HPLC purifications were performed. The first system used was a Waters Alliance 2690 equipped with a UV detector (diode array detector, DAD), operated between 205 and 235 nm, and an automated fraction collector. The system was set up with a C_{18} functionalized precolumn (Grace Alltima C18; 7.5 mm × 3 mm; 5 μm) and a C_{18} functionalized column (Grace Alltima C18; 250 mm × 3 mm; 5 μm). An isocratic method was used with a rinsing phase at the end of the run. A mobile phase made of H_2O/MeOH (95:5; v/v) (solvent A) and MeOH (solvent B) was used, held at a constant composition (A:B; v/v) of 37:63. The flow rate was set at 0.6 mL/min and column temperature at 40 °C, and the injected volume was 100 μL. The second Waters Alliance 2690 system was equipped with two diol functionalized columns (LiChrospher Diol; 250 mm × 4 mm; 5 μm) in series. An isocratic method was used with a mobile phase of isooctane/isopropanol (85:15; v/v) and a rinsing phase at the end of the run. The flow rate was set at 1 mL/min and column temperature at 40 °C, and the injected volume was 100 μL.

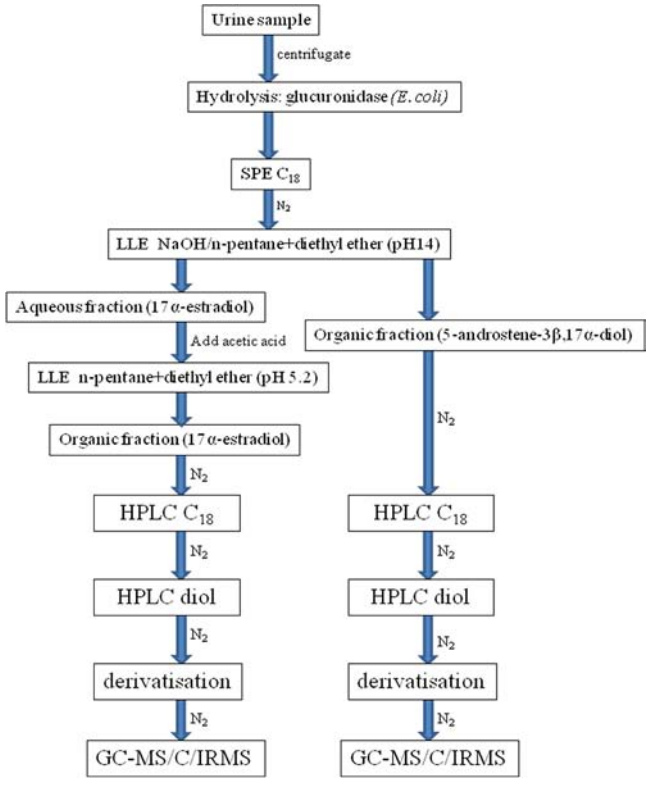
GC-MS/C/IRMS. The samples were analyzed with a Thermo Trace GC Ultra gas chromatograph, equipped with an RXI 5 SIL MS column (Restek −30 m; 0.25 mm i.d.; 0.25 μm df) and a Thermo Scientific TriPlus autosampler. After GC, the sample was split by means of a T-piece, which was coupled to a Thermo DSQ II single-quadrupole mass spectrometer at one end and to a Thermo MAT 253 isotope ratio mass spectrometer, via the Thermo Scientific GC Isolink, at the other end. Gas flows were regulated using the Thermo Scientific Conflo IV interface. Eight microliters was injected at 20 μL/s into the injector in programmed temperature vaporizer mode. The initial injector temperature was 100 °C, which was held for 0.05 min with a vent flow of 20 mL/min. The temperature was raised at 8 °C/min and held at 280 °C for 2 min during sample transfer on column. The initial GC oven temperature was 110 °C, which was held for 1.5 min. The temperature was then raised to 280 °C at 8 °C/min and held for 2 min. Finally, the temperature was raised to 320 °C at 50 °C/min and held for 3 min. The carrier gas was helium at a constant flow rate of 1.5 mL/min. The temperature of the transfer line was set at 300 °C. The steroids were detected on MS using full-scan mode (m/z 50−400). The combustion furnace was set at 950 °C and was oxidized prior to each series of analyses for 1 h. The combustion gases were passed through a Nafion membrane filter for water removal. After ionization, the ions with m/z 44, 45, and 46 were magnetically separated and simultaneously measured in three Faraday collectors. The CO_2 reference gas was calibrated with a mixture of steroids with certified $\delta^{13}C$ values, measured with an elemental analyzer (5-androstene-3β,17α-diol monoacetate, β-testosterone acetate, and dehydroepiandrosterone (DHEA) acetate). Carbon isotope ratios of the compounds were expressed relative to Vienna Pee Dee Belemnite (VPDB). The shift of the $\delta^{13}C_{VPDB}$ value due to the formation of acetates is corrected as follows:

$$D_{OH} = D_{OAc} + 2m(D_{OAc} - D_{Ac})/n$$

D_{OH} is the $\delta^{13}C_{VPDB}$ value of the underivatized steroids, D_{OAc} the $\delta^{13}C_{VPDB}$ value of the acetylated steroids, D_{Ac} the $\delta^{13}C_{VPDB}$ value of the acetylating reagent, n the number of carbon atoms in a molecule, and m the number of hydroxyl groups to be acetylated.[12]

Sample Preparation. A schematic overview of the analytical strategy is presented in Scheme 1. The urine samples were centrifuged (15 min, 3113g) prior to analysis, to avoid obstruction of the solid phase extraction (SPE) cartridges in a later stage. To 10 mL of the centrifuged urine sample were added 3 mL of phosphate buffer 0.8 M (pH 6.8) and 50 μL of glucuronidase from *E. coli*. Hydrolysis was performed for 15 h at 37 °C. Then, the sample was brought onto a C_{18} SPE column, subsequently washed with 6 mL of H_2O and 5 mL of H_2O/acetonitrile (ACN) (80:20; v/v), and eluted using 8 mL of H_2O/ACN (10:90; v/v). The eluted sample was evaporated to approximately 300 μL under a nitrogen stream at 40 °C, and 0.5 mL of 1 M sodium hydroxide was added. Next, a liquid−liquid extraction (LLE) was performed using 4 mL of *n*-pentane/diethyl ether

Scheme 1. Analytical Strategy for the Extraction and Purification of 17α-Estradiol and the Endogenous Reference Compound 5-Androstene-3β,17α-diol[a]



[a]LLE, liquid−liquid extraction.

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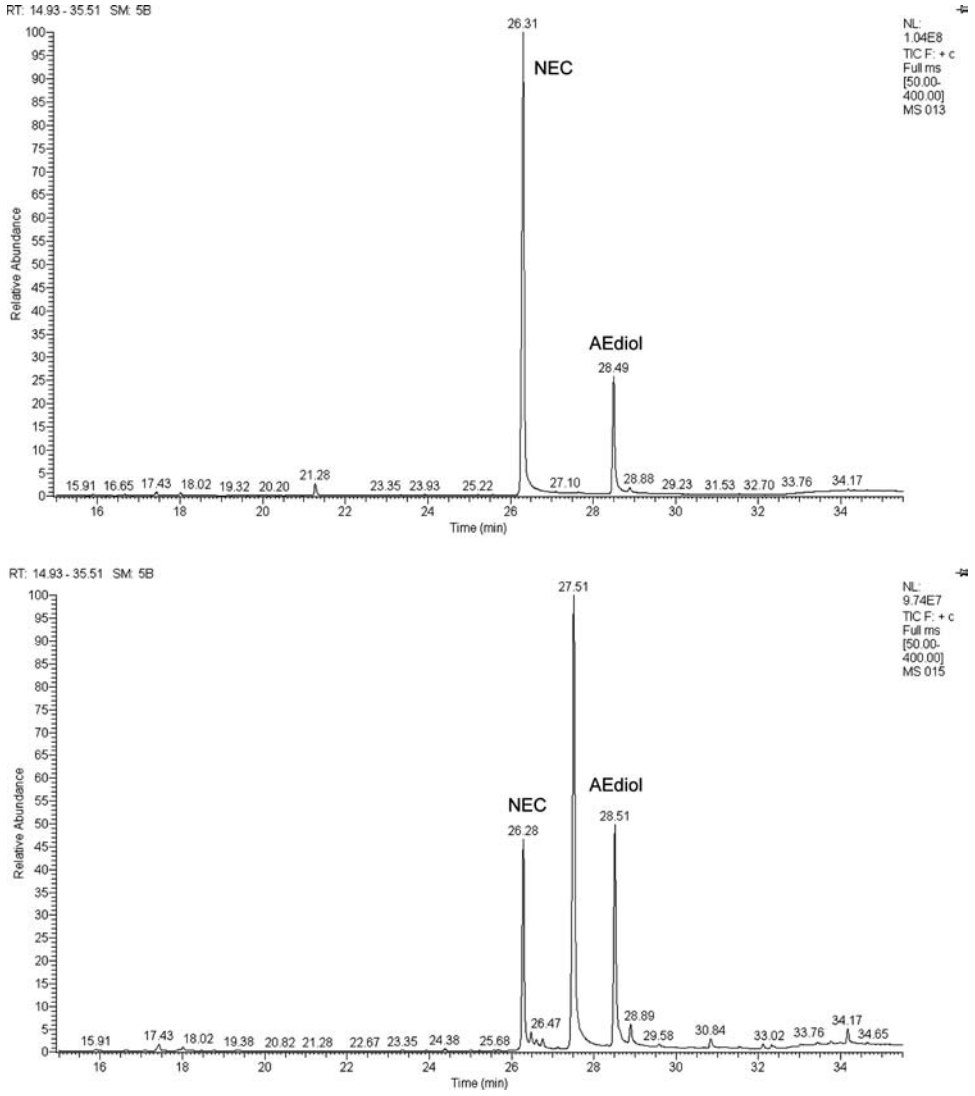

Figure 1. MS chromatograms of the androgen fraction of a urine sample of a pregnant cow after the complete sample preparation procedure (top) and after sample preparation without straight phase HPLC purification (bottom), showing the internal standard noretiocholanolone acetate (NEC) and 5-androstene-3β,17α-diol (AEdiol). The data were produced in full scan mode (m/z 50−400).

(92.5:7.5; v/v). The organic layer was kept and labeled "fraction A", containing androgenic steroids, among which was the targeted ERC 5-androstene-3β,17α-diol. Next, the pH of the aqueous layer was adjusted using glacial acetic acid and a second LLE using 4 mL of *n*-pentane/diethyl ether (92.5:7.5; v/v) was performed. The organic layer was kept and labeled "fraction E", containing estrogenic steroids, among which was the target metabolite 17α-estradiol. Both fractions were evaporated under a nitrogen stream at 37 °C after the addition of 100 μL of glycerol solution (10% in methanol) to serve as a keeper solution and mixed with 70 μL of water and 40 μL of fluoxymesterone in methanol (50 ng/μL) as internal standard. Prior to injection onto the first HPLC system, the fractionation windows for the ERC and metabolite were determined through the three-fold injection of a standard solution containing fluoxymesterone, 17α-estradiol, 5-androstene-3β,17α-diol, and 17β-testosterone. The collected fractions A and E, containing 5-androstene-3β,17α-diol and 17α-estradiol, respectively, were evaporated under a nitrogen stream at 37 °C and dissolved in 120 μL of isopropanol/isooctane (10:90; v/v). An aliquot of 20 μL of a medroxyprogesterone standard solution (100 ng/μL) was added as internal standard, used for both verification of the retention time and estimation of the analyte concentration during the second HPLC purification. The samples were injected after calculation of the fractionation windows with the three-fold injection of a standard solution containing medroxyprogesterone, 17α-estradiol, 5-androstene-3β,17α-diol, and 17β-testosterone. The collected fractions were evaporated to dryness under a nitrogen stream at 40 °C, and 25 μL of both acetic anhydride and pyridine were added. The derivatization was done overnight at room temperature in a closed vial. Afterward, the sample was evaporated to dryness under a gentle nitrogen stream at 40 °C, and the residue was dissolved in the appropriate volume of isooctane to provide measurement within the linear range of the IRMS apparatus. Finally, after the addition of noretiocholanolone acetate (4 ng/μL) as external standard, the two fractions were injected onto GC-MS/C/IRMS for further characterization of $\delta^{13}C_{VPDB}$ of 5-androstene-3β,17α-diol diacetate and 17α-estradiol diacetate.

RESULTS AND DISCUSSION

Sample Preparation and Analysis. The presented sample preparation method is based on the previously published method by Buisson et al.,[14] with a number of adaptations to accelerate the process or to allow further automation in the future. First, only 5-androstene-3β,17α-diol was measured as ERC. By not measuring dehydroepiandrosterone (DHEA), the necessity for the time-consuming analysis of the sulfoconjugated steroids was eliminated.

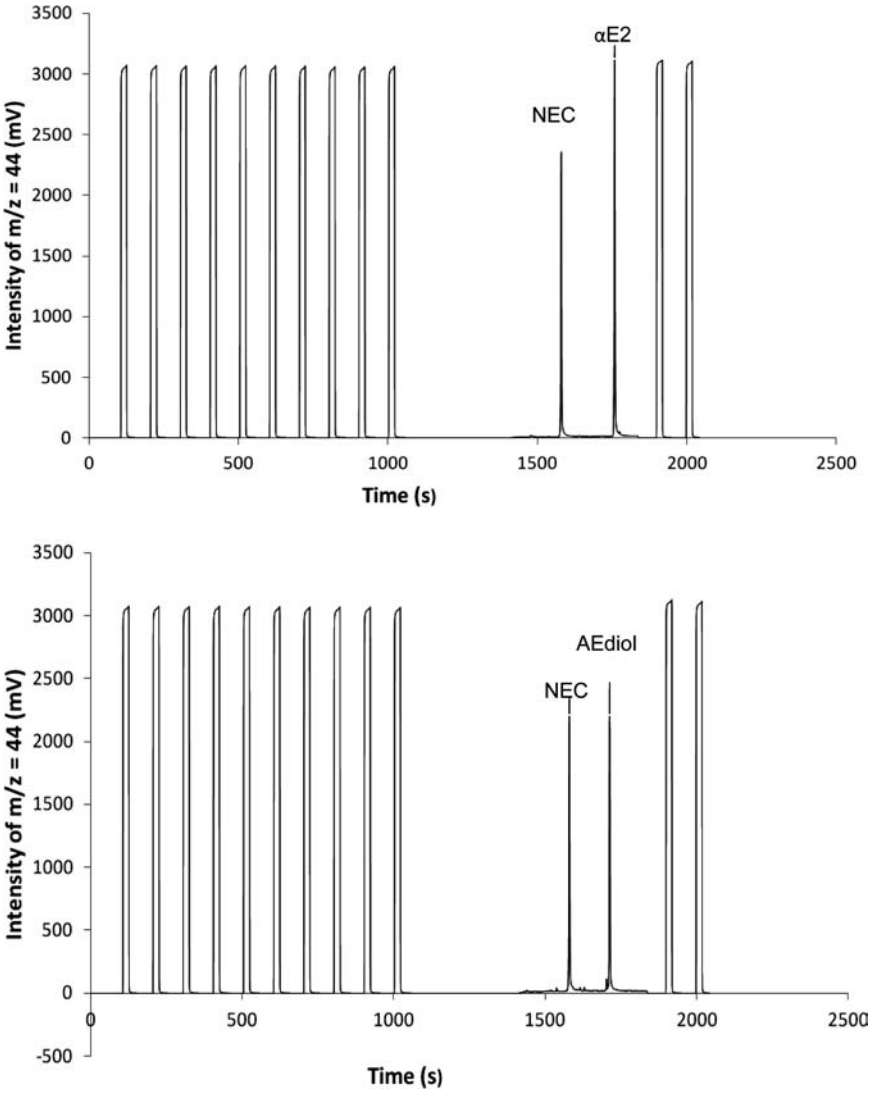

Figure 2. IRMS chromatograms (m/z 44) of a positive bovine urine sample, showing the internal standard noretiocholanolone acetate (NEC), the metabolite 17α-estradiol diacetate (αE2) (top), and the ERC 5-androstene-3β,17α-diol (AEdiol) (bottom).

Table 1. Intermediate Precision of the $\delta^{13}C_{VPDB}$ Values (Not Corrected for the Acetate Moiety) of a Spiked Urine Sample [200 ppb of 17α-Estradiol (αE2) and 5-Androstene-3β,17α-diol (AEdiol)], Analyzed on Three Different Days

	$\delta^{13}C_{VPDB}$ AEdiol (mean ± SD) (‰)	$\delta^{13}C_{VPDB}$ αE2 (mean ± SD) (‰)
series 1 (n = 6)	−32.86 ± 0.27	−31.53 ± 0.15
series 2 (n = 2)	−33.16 ± 0.01	−32.02 ± 0.24
series 3 (n = 4)	−33.75 ± 0.18	−31.92 ± 0.07
mean (n = 12)	−33.26	−31.82
SD (n = 12)	0.46	0.26

Second, one SPE purification step and the two preparative HPLC steps were replaced by just two subsequent HPLC purifications prior to derivatization. Because the use of a 3-(dimethylamino)propyl-functionalized silica gel column resulted in unstable retention times (after a sudden shift, the retention times of the analytes differed significantly), a diol-functionalized stationary phase was selected for the second HPLC purification. Even though a cleanup procedure without the straight phase HPLC purification provided accurate results, the addition of the second HPLC preparation step was preferred to further reduce the risk of impurities coeluting with the analytes. The additional effect of this HPLC step on the sample cleanup is illustrated in Figure 1.

Third, by replacing the previously used splitless injection by programmed temperature vaporization (PTV)−injection, the required sample volume was successfully reduced from 20 to 10 mL. Finally, the separate GC-MS analysis, which was until now required prior to each GC/C/IRMS analysis of steroids, could be eliminated. By estimating the concentration of the analytes by means of UV detection during the final HPLC step, the dilution factor of the sample could be successfully determined to obtain measurements well within the linear working range of the C/IRMS apparatus. Additionally, by means of the parallel coupled MS in the GC-MS/C/IRMS setup, the analytes could be successfully identified and controlled for purity simultaneously with the isotope ratio measurement, thus avoiding possible criticism of GC/C/IRMS that identification is not done during the same injection as isotope ratio measurement.[15] The resulting IRMS chromatograms of a urine sample of a treated animal after sample preparation are shown in Figure 2. The resulting chromatograms are clean, and the analyte peaks are baseline separated and free of any coelutions, demonstrating

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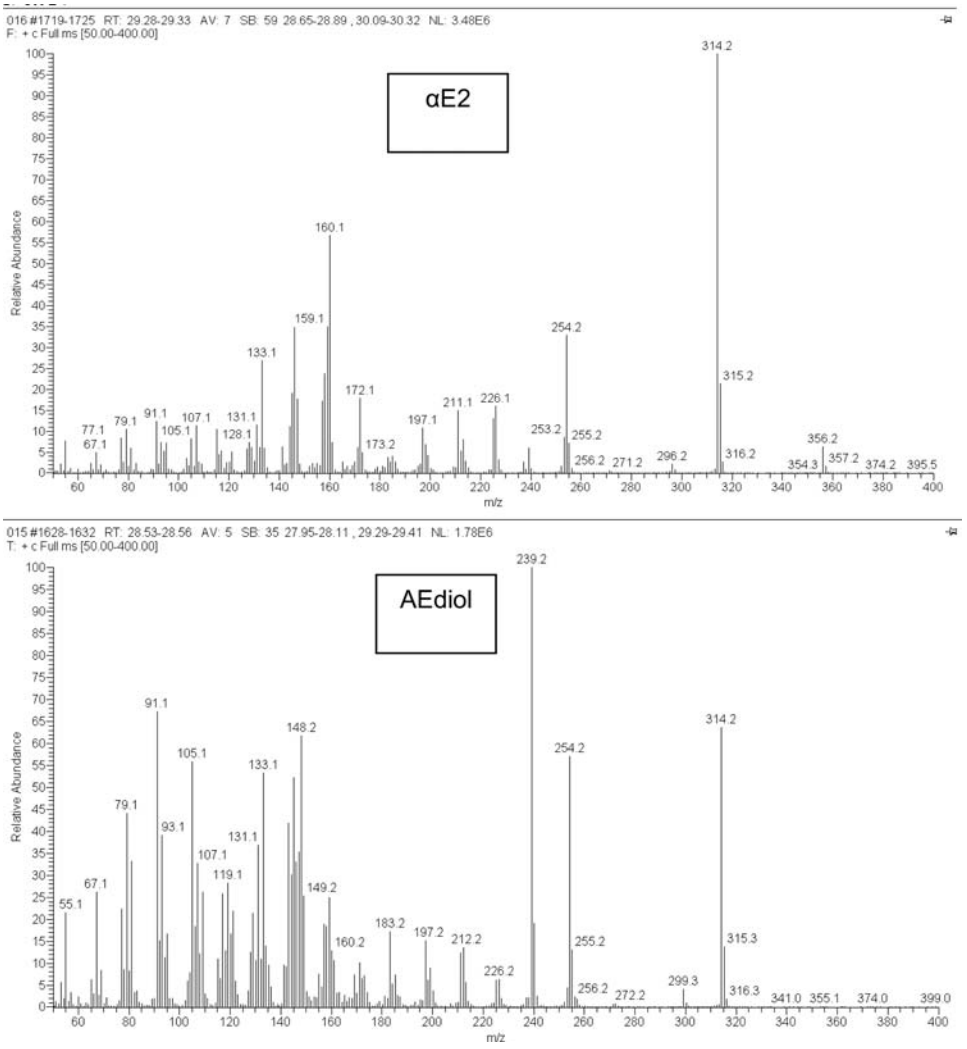

Figure 3. MS spectra of 17α-estradiol diacetate (αE2) (top) and 5-androstene-3β,17α-diol (AEdiol) (bottom). Fragmentation was done by electron ionization with an ion source temperature of 250 °C. The data were produced in full-scan mode (m/z 50−400).

Table 2. $\delta^{13}C_{VPDB}$ Values of 17α-Estradiol and 5-Androstene-3β,17α-diol in Six Noncompliant Urine Samples of Bovines Treated with 17β-Estradiol

sample	$\delta^{13}C_{VPDB}$ AEdiol (‰)	$\delta^{13}C_{VPDB}$ αE2 (‰)	$\lvert\Delta^{13}C_{VPDB}$ (αE2 − AEdiol)$\rvert$ (‰)
1	−15.57	−30.23	14.66
2	−15.64	−30.15	14.51
3	−15.26	−29.99	14.73
4	−15.45	−30.24	14.79
5	−15.17	−30.11	14.94
6	−15.38	−30.08	14.70

the performance of the purification method. However, due to the necessary extensive sample preparation, the yield of the analytes is highly variable. The recovery of the sample preparation was calculated under reproducibility conditions at different concentrations and ranged from 40 to 80% for 5-androstene-3β,17α-diol and from 40 to 76% for 17α-estradiol.

Method Validation. GC/C/IRMS has been accepted as the confirmation method for the differentiation between endogenous steroid hormones and synthetic homologues. Still, official guidelines for the validation of IRMS analysis are currently lacking. However, the sample cleanup procedures remain complex, with many different purification steps involved. Because every cleanup step introduces a risk of isotopic fractionation, the robustness assessment was mandatory.

Intermediate Precision. To determine the intermediate precision, a blank urine sample was spiked with 5-androstene-3β,17α-diol and 17α-estradiol at 200 μg L^{-1}. The sample was divided into 12 subsamples, which were analyzed by three different operators on three different dates over a time span of 2 months. The first series consisted of six subsamples, the second series of two subsamples, and the third series of four subsamples. The results are presented in Table 1. The sample standard deviations (SD) (n = 12) of the $\delta^{13}C_{VPDB}$ were 0.46 and 0.26‰ for 5-androstene-3β,17α-diol and 17α-estradiol, respectively. Standard deviations below 0.5‰ are considered to be acceptable.[10,16,17]

Isotope Fractionation. The $\delta^{13}C_{VPDB}$ values after sample preparation of six water samples spiked at 200 μg L^{-1} with 5-androstene-3β,17α-diol and 17α-estradiol were compared with those of four standard injections, not subjected to sample preparation, to assess the isotope fractionation occurring during sample preparation. The difference between the mean $\delta^{13}C_{VPDB}$ values was 0.33‰ for 5-androstene-3β,17α-diol and 0.04‰ for 17α-estradiol. In comparison with results from the literature for

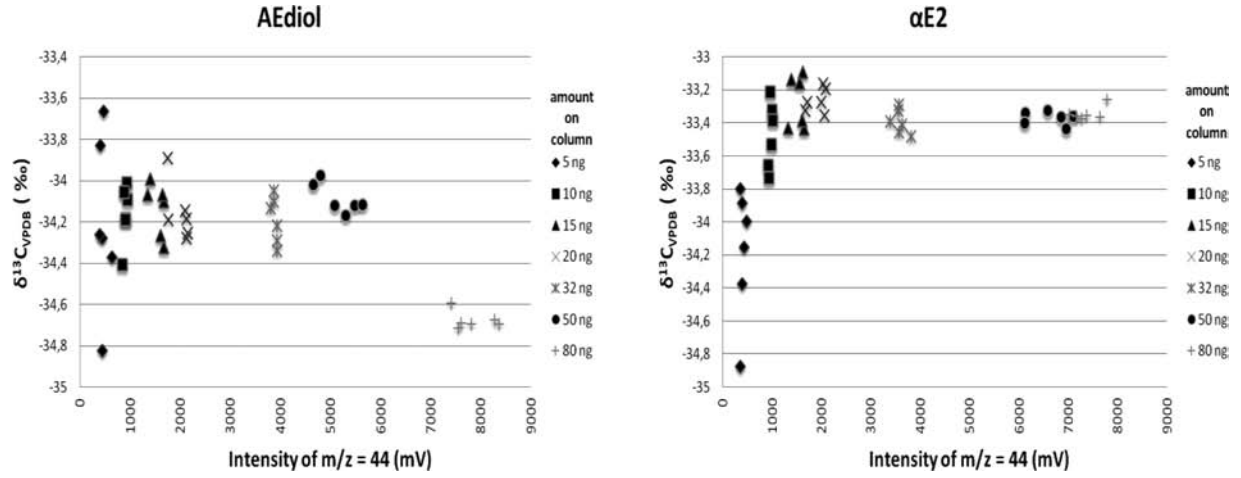

Figure 4. $\delta^{13}C_{VPDB}$ values (expressed in ‰) of 17α-estradiol diacetate (αE2) (right) and 5-androstene-3β,17α-diol diacetate (AEdiol) (left) and the corresponding peak intensities (expressed in mV) for the injected amounts of 5, 10, 15, 20, 32, 50, and 80 ng on column (six injections for each amount).

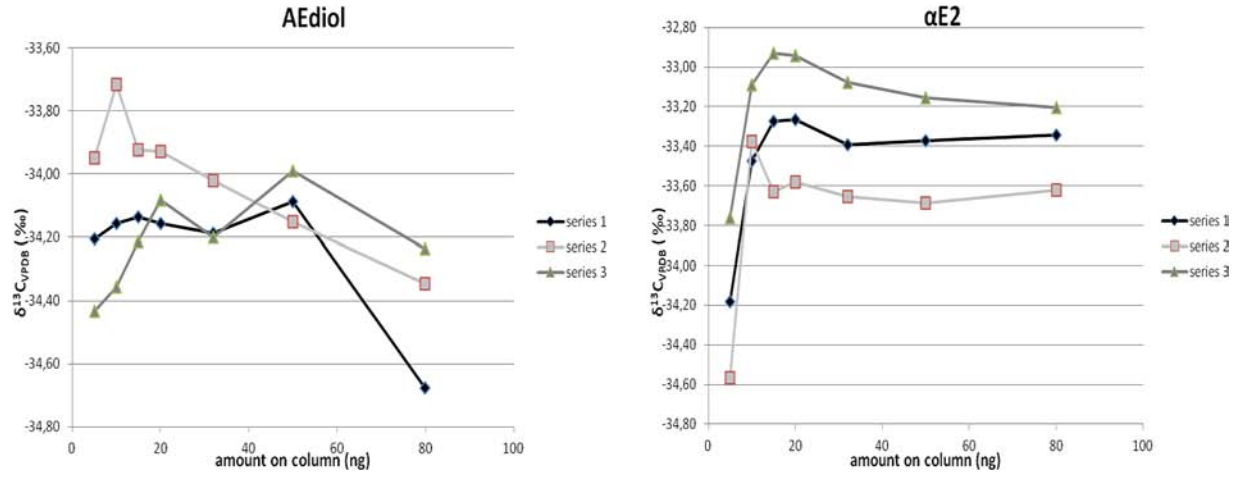

Figure 5. $\delta^{13}C_{VPDB}$ values (expressed in ‰) of 17α-estradiol diacetate (αE2) (right) and 5-androstene-3β,17α-diol diacetate (AEdiol) (left) and the corresponding amounts (expressed in ng) for the injected amounts of 5, 10, 15, 20, 32, 50, and 80 ng on column (mean values of six injections for each amount). Series 1 and 3 were performed with a GC-MS/C/IRMS setup and series 2 with a GC/C/IRMS setup.

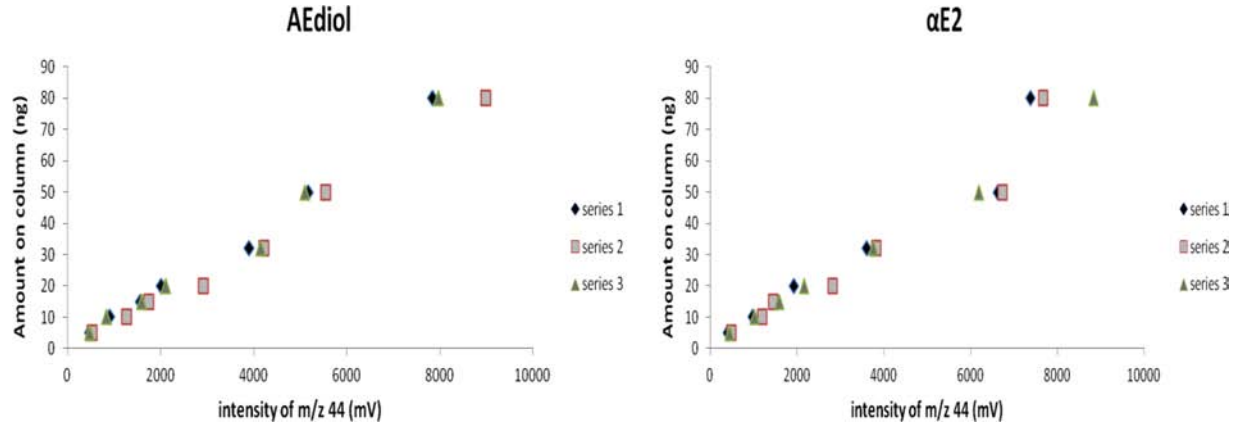

Figure 6. Average peak intensities (expressed in mV) and the corresponding amounts on column (expressed in ng) of 17α-estradiol diacetate (αE2) (right) and 5-androstene-3β,17α-diol diacetate (AEdiol) (left). Series 1 and 3 were performed with a GC-MS/C/IRMS setup and series 2 with a GC/C/IRMS setup.

a similar experiment with other steroids,[11] these differences are significantly lower than previously reported values, leading to the conclusion that the isotope fractionation, if any, is acceptable.

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Specificity. Ten reference standards, 10 spiked water samples, 16 spiked urine samples, and 15 urine samples collected from pregnant cows were analyzed according to the described method. For all samples, the identification of 5-androstene-3β,17α-diol and 17α-estradiol was done according to the legal criteria,[18] by comparison of the abundance of six fragment ion ratios of the analytes with those of a standard injected in the same series. The typical MS spectra of 5-androstene-3β,17α-diol and 17α-estradiol are depicted in Figure 3. For all of the samples, the analytes could be correctly identified, and no impurities or coelutions were observed.

In addition, the MS data were evaluated using AMDIS software. This program employs specific algorithms on the MS data to detect interfering peaks hidden beneath others. Also, AMDIS compared the MS data of the analytes with those of a standard injection in the same series, to provide a "Net Match" factor and a "Purity" factor for all samples. The objective was to study if these factors could be used as a criterion for the evaluation of both the identity and the purity of the analytes. In the AMDIS data, the Net Match factor ranged from 92 to 100 and the Purity factor from 88 to 97. For future analysis, the use of fragment ion abundance ratios will be required only if the Net Match factor is below a threshold value of 92 and the Purity factor below a threshold value of 88.

Noncompliant Threshold Value. For doping control purposes, the World Anti Doping Agency (WADA) uses a threshold value of $|\Delta^{13}C| > 3‰$ for noncompliant samples. This criterion was assessed to be adequate in the field of food safety as well. To verify if this value is suited for the used setup, $\Delta^{13}C$ values were determined in a compliant control population of 29 pregnant cows. Pregnant cows' urine is preferred for this, because the concentrations of 17α-estradiol in regular samples are often too low for reliable measurement with C/IRMS. The data were evaluated using a one-sided Student *t* test and, as such, a threshold value of 1.8‰ was calculated. To verify the adequacy of the developed method and the determined threshold value, six urine samples collected from treated animals were analyzed according to the described procedure. The resulting $\delta^{13}C_{VPDB}$ values are presented in Table 2. All $\Delta^{13}C$ values are above 14‰, clearly illustrating the capability of the method to detect positive samples, as well as the validity of the used threshold value.

Linear Working Range. The range in which the isotope ratio mass spectrometer produces accurate measurements of $\delta^{13}C_{VPDB}$ values was determined by a series of injections of 17α-estradiol diacetate and 5-androstene-3β,17α-diol diacetate in increasing amounts, from 2.5 to 80 ng on column. The resulting peak intensities and $\delta^{13}C_{VPDB}$ values are shown in Figure 4. The injections of 5 ng, corresponding with peak intensities just below 500 mV, clearly show deviating $\delta^{13}C_{VPDB}$ values and a larger spreading of the results; hence, the lower limit of the linear range lies between 500 and 1000 mV, corresponding with approximately 7 ng of the steroids on column. For the injection of 80 ng of 5-androstene-3β,17α-diol diacetate, lower $\delta^{13}C_{VPDB}$ values are observed, which might be due to peak fronting. The injection of 2.5 ng did not yield measurable results. The measured standard deviations within the linear domain were 0.23 and 0.13‰ for 5-androstene-3β,17α-diol diacetate and 17α-estradiol diacetate, respectively.

MS Detector Influence on Isotope Ratio Measurement. To evaluate the influence of the parallel coupled MS on the isotope ratio mass spectrometers' measurements, the experiment performed to determine the linear range, as described above, was repeated twice, once after uncoupling the mass spectrometer and again after recoupling. The results for 17α-estradiol diacetate and 5-androstene-3β,17α-diol diacetate of the three carbon isotope ratio measurement series are presented in Figure 5. No differences in $\delta^{13}C_{VPDB}$ values between the three series, other than those caused by natural spreading of the results, could be observed. The repeatability of the standard injections at different concentrations without and with the MS coupled in the setup revealed no significant differences in either precision or accuracy.

Because the same sample amount is split between the MS and the IRMS in the GC-MS/C/IRMS setup, the measured peak intensities for the GC/C/IRMS setup generally were slightly higher. However, the difference is limited and causes no significant disadvantages within the regular range of the measurements, between 10 and 40 ng on column, as illustrated in Figure 6.

GC-MS/C/IRMS offers a powerful tool for the detection of steroid abuse. It is clear, however, that the described procedure needs to be extended in the future to include a number of additional metabolites and ERCs to allow simultaneous detection of abuse of a broader range of steroids. Still, analysis with GC-MS/C/IRMS is relatively complex, the sensitivity of the apparatus is limited, and the sample preparation remains laborious and time-consuming, making the application for routine analyses limited to experienced laboratories. Therefore, adequate screening procedures need to be worked out to complement the C/IRMS confirmatory analyses. Publications on using profiles of direct metabolites of steroids for screening of steroid abuse[19−21] indicate that applicable strategies will be available soon, and research into the use of indirect biomarkers[20−27] holds interesting promises for future application. GC-MS and LC-MS analyses based on both targeted and untargeted profiling would provide rapid and powerful screening methods to be used in combination with GC-MS/C/IRMS confirmation methods.

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Notes

The authors declare no competing financial interest.

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