Supporting information

Implementation and performance of the gas chromatography/combustion/isotope ratio mass spectrometry-based method for the confirmatory analysis of endogenous anabolic steroids during the Rio de Janeiro Olympic and Paralympic Games 2016

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1. HPLC purification of testosterone

The optimization of the collection windows during HPLC purification steps made it possible to remove the interferent that coelutes perfectly during GC analysis from the testosterone (T) fraction. Figure S1 presents a peak of T acetate (T_Ac) that coeluted perfectly with the interferent, obtained by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). The S-shape without any distortion suggests that the peak is pure. Figure S2 presents the mass spectrum of the same peak, obtained by gas chromatography-mass spectrum (GC-MS) in comparison with the mass spectrum of a pure standard, as well as the spectrum of T from the same urine after the optimization of the collection windows during HPLC purification.

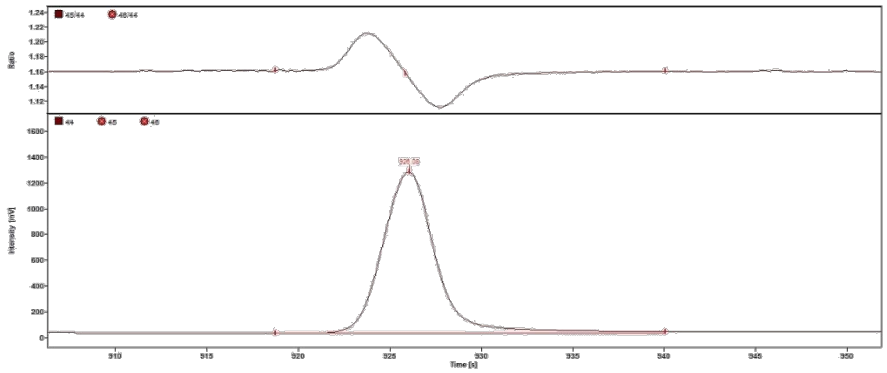


Figure S1. Zoomed chromatogram of urinary T_Ac overlapped with endogenous interferent. The perfect coelution produces a peak and an S-shape without any distortion, which suggests that the peak is pure.

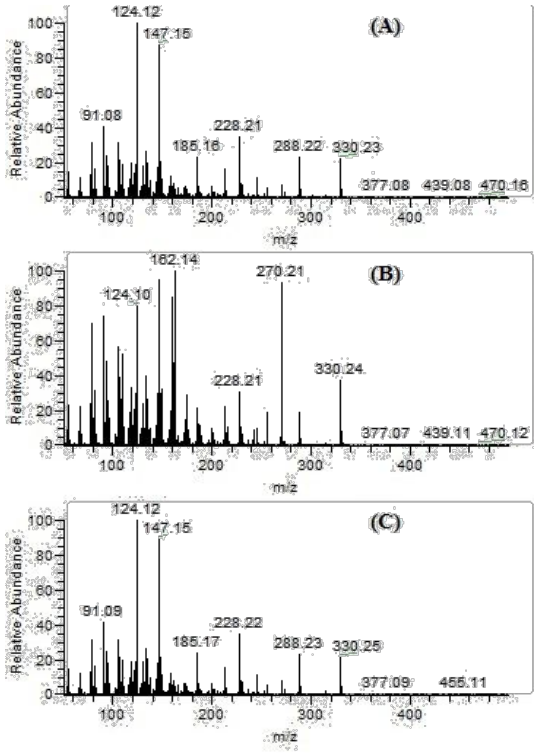


Figure S2. Mass spectra showing the elimination of interferent within the T_Ac fraction by the application of a convenient collection window in HPLC purification steps. (A) Previously acetylated pure T standard. (B) Urinary T_Ac collected in a 1.50 min collection window in both HPLC steps and overlaid with the interferent. (C) Urinary T_Ac properly purified by a 0.80 min shortened collection window in both HPLC steps.

During validation, the fractions before and after each steroid collection were taken to confirm the absence of partial losses. These "blank" fractions were analyzed by GC-MS and proved the efficiency of the HPLC purification.

2. Gas calibration

The assembly of the combustion reactor is fundamental to ensuring the elution of the compounds into the hot zone and adequate conversion into CO_2[1,2]. In the Brazilian Doping Control Laboratory (Laboratório Brasileiro de Controle de Dopagem, LBCD),

approximately 1 cm of the capillary tip was burned to remove the polyimide coating, thus preventing ulterior bleeding during analysis that would affect the CO_2 background. The capillary after the backflush splitter was installed through the connector and inside the ceramic tube of the reactor with the glassy tip at a distance of 1 mm from the metal wires. The centering of the wire braid inside the ceramic tube was checked by measuring the distance between the edge of the connector and the tip of the capillary, which must be between 21 and 23 mm. The reactor was positioned inside the GC oven with the edge of the connection nut at 1 mm of the reactor furnace.

CU/USADA 33-1 was the unique CRM available containing a mixture of standards of acetylated steroids from its creation in 2009[3] until 2016, too close to the Games for implementation, when new preparations were produced by the National Measurements Institute from Australia.

Table S1 presents the carbon isotope ratio (CIR) results of a five-fold analysis of CU/USADA 33-1 obtained in June 17th 2016. The standard deviations (SD) between the five results for each analyte were lower than 0.40 ‰, and the offset between the mean values and the respective certified values were lower than 0.50 ‰. If any offset was greater than the limit, the mean offset would be used to correct the reference gas CIR value.

Table S1. Results of a sequence of analyses of CU/USADA 33-1 obtained in the hybrid system on June 17th 2016.

	$\delta^{13}C$ values, or CIR values, in CU/USADA 33-1 (‰)			
Injection	3β-OH_Ac	5α-chol	A_Ac	11K_Ac
1	-30.88	-24.98	-32.58	-16.80
2	-30.83	-24.95	-32.73	-16.58
3	-30.89	-25.15	-32.68	-16.59
4	-30.73	-25.07	-32.60	-16.80
5	-30.60	-24.96	-32.77	-16.79
Average	-30.79	-25.02	-32.67	-16.71
SD	0.12	0.09	0.08	0.12
Certified value	-30.61	-24.77	-33.04	-16.69
Offset	0.18	0.25	-0.37	0.02
Mean offset	0.02			

3β-OH_Ac: 5α-androstan-3β-ol acetate; 5α-chol: 5α-cholestane; A_Ac: androsterone acetate; 11K_Ac: 11-ketoetiocholanolone acetate.

3. Quality control charts

Quality control (QC) Shewhart charts were built following the Westgard approach[4] to monitor the QCs, encompassing in-house RMs, QCNs and QCPs. A single-rule QC chart was built to monitor CU/USADA 33-1 considering a 0.50 ‰ deviation from the certified $\delta^{13}C$ values of the steroids.

4. Internal standard evaluation

In each sample cleanup sequence by HPLC, the retention time (t_R) of trenbolone acetate (Tren_Ac) was monitored as an internal standard (IS) to ensure system stability and, consequently, proper sample purification (exempt from isotopic fractionation). The stability was guaranteed when the t_R varied neither more than 0.05 min between the three consecutive standard mix injections nor more than 0.10 min within the full sequence.

Similarly, in each sample analysis sequence by GC/C/IRMS, the system stability was monitored by the analysis of 3β-OH_Ac as an IS, not only regarding the t_R but also with respect to the CIR measurements. System stability was guaranteed when the t_R of IS did not vary more than 0.10 min in a sequence, whereas the CIR values did not vary more than 1.00 ‰ and their SD did not exceed 0.40 ‰.

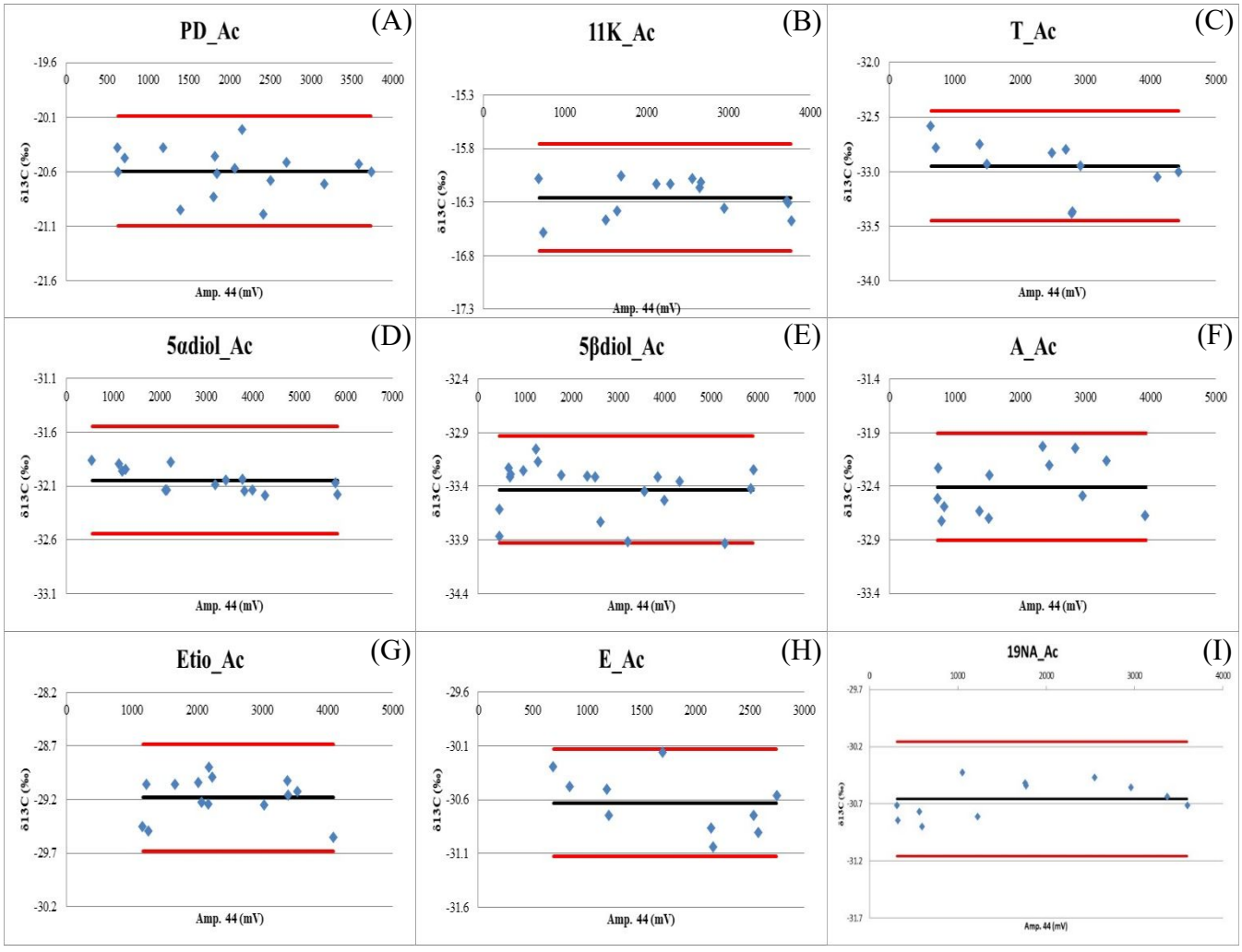
Table S2 summarizes the evaluation of IS in a typical batch of samples in the two HPLC steps and GC/C/IRMS. The first HPLC sequence encompasses three injections of the standard mix followed by QCN_T, four samples and QCP_T. The second HPLC sequence encompasses three injections of the standard mix followed by QCN_T, four samples and QCP_T of fractions FII, FIII and FIV. The IRMS sequence encompasses the injection of the RMs of each steroid included in the sequence, followed by the fractions PD_Ac, T_Ac, 5α-diol_Ac and 5β-diol_Ac from QCN_T, four samples and QCP_T.

Table S2. Summary of the evaluation of IS in the two steps of HPLC and GC/C/IRMS sequences.

	1st HPLC	2nd HPLC	GC/C/IRMS	
IS	Tren_Ac	Tren_Ac	3β-OH_Ac	
Parameter	t_R (min)	t_R (min)	t_R (min)	$\delta^{13}C$ (‰)
n	9	21	28	28
Mean value	17.37	7.29	10.92	-32.38
SD	0.02	0.01	0.003	0.15
Minimum value	17.33	7.27	10.92	-32.64
Maximum value	17.39	7.30	10.93	-31.94

5. Linearity of the instrument

The points were properly distributed all-over the peak intensity ranges to test the linearity of the instrument. The homogeneous distribution of the points over the respective ranges are presented in Figure S3.



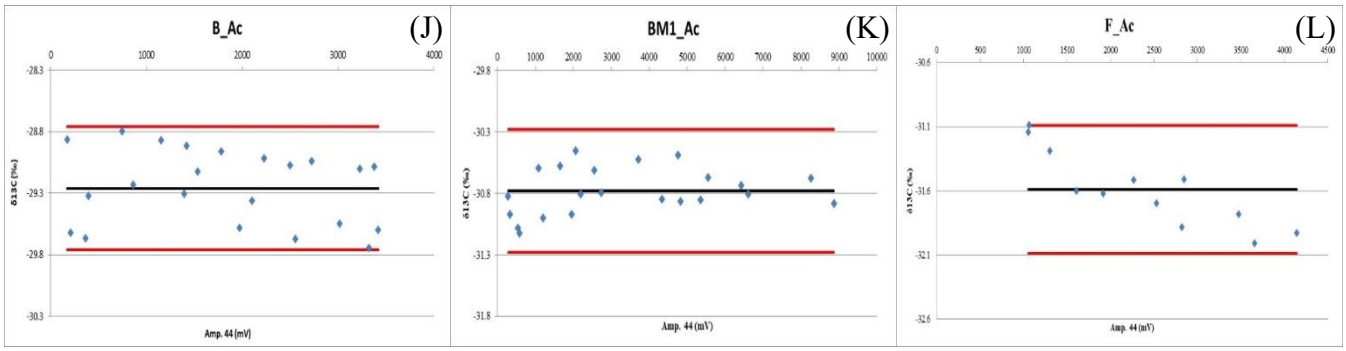


Figure S3. Distribution of the points over the respective peak intensity ranges. (A) PD_Ac. (B) 11K_Ac. (C) T_Ac. (D) 5α-diol_Ac. (E) 5β-diol_Ac. (F) A_Ac. (G) Etio_Ac. (H) E_Ac. (I) 19NA_Ac. (J) B_Ac. (K) BM1_Ac. (L) F_Ac. The solid black lines are the mean values; the solid red lines are the ranges established as the mean values ± 0.50 ‰.

6. Androsterone bias

The bias was determined by means of linear mixing models (LMMs). For androsterone (A), the bias was close to the maximum combined uncertainty required by WADA[5]. However, it is reasonable to consider that a slightly inaccurate concentration of A in the negative quality control for T (QCN_T) determined by a single point quantification is the main reason for the high bias obtained for this analyte, as highlighted by Piper *et al.*[6,7]. Table S2 presents the mean CIR values obtained for A and the respective c_e/c_m ratios for each level of the LMM, calculated based on the concentration (5520 ng/mL) previously estimated by a single point quantification. In support of the author's hypothesis, the c_e/c_m ratios calculated based on a supposed concentration equal to 5800 ng/mL (which represents a reasonable offset between a single point estimation and a quantification based on a calibration curve) are also presented in Table S3. Figure S4 presents both curves, showing that the best fit curve is not jeopardized by the hypothetical concentration. As the measured CIR value for the A standard was -32.4 ‰, the intercept of the line of best fit experimentally obtained from an endogenous concentration equal to 5800 ng/mL would lead to a lower bias (from 0.9 ‰ to 0.6 ‰). Thus, this hypothesis should be investigated.

Table S3. Linear mixing model for androsterone, with c_e/c_m ratios based on the estimated concentration and on a hypothetical concentration.

	c_e/c_m (based on the estimated concentration, 5520 ng/mL)	c_e/c_m (based on a hypothetic concentration, 5800 ng/mL)	CIR value (‰)
P0	1.00	1.00	-20.4
P1	0.70	0.71	-23.5
P2	0.54	0.55	-25.4
P3	0.44	0.45	-26.7
P4	0.37	0.38	-27.5
P5	0.32	0.33	-28.0

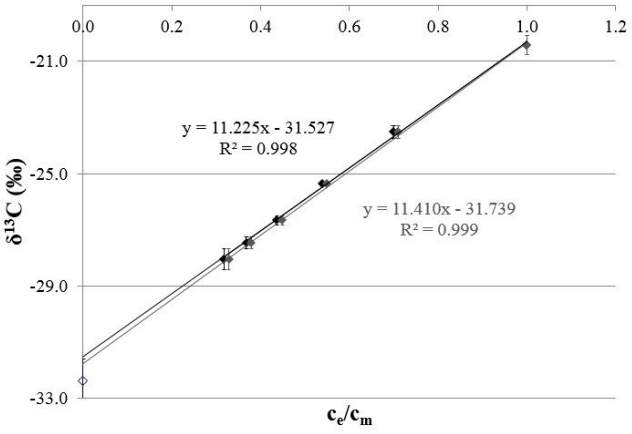


Figure S4. Comparison between the best fit curve obtained from the estimated concentration (5520 ng/mL) of endogenous A, based on a single point calibration (black trace), and the curve obtained from a hypothetical concentration equal to 5800 ng/mL (gray trace).

7. Reference population

While only $\Delta\delta^{13}C$ values are useful for doping control, the comparison of the absolute CIR values presented in Table 5 with the literature allows us to understand how the carbon isotopes are distributed in steroids as a consequence of the dietary habits of Brazilian individuals. Moreover, the comparison between the different steroids in the T pathway, supported by the literature, supports the consistency of the results obtained.

This experiment corroborates previous studies[8] showing that the South American (in that study, Brazilian and Colombian) population presents CIR values comparable to those of South African individuals and more enriched values than Caucasian and Asian populations.

Additionally, relevant notes from other reports[1,2,9] were observed in this experiment, as follows: a) very similar CIR values to those of PD and A, and Etio presenting ± 1 ‰ more depleted values; and b) very similar CIR values to Etio, 5α-diol and 5β-diol. On the other hand, 11K presented a CIR value comparable to PD, while those papers reported significant differences between the two ERCs.

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(7) Piper, T.; Thevis, M. Applications of isotope ratio mass spectrometry in sports drug testing accounting for isotope fractionation in analysis of biological samples. *Methods Enzymol*. **2017**, *596*, 403–432.

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