

# APPLICATION NOTE



## *Detection of synthetic forms of endogenous anabolic androgenic steroids by GC-C-IRMS*



### *Introduction*

Compound-specific stable carbon isotope analysis of urinary extracts is a technique that forms part of the standard analysis for doping control as required by the World Anti-Doping Agency (WADA) and laid out in their Technical Document TD2016IRMS. First introduced in the 1996 Summer Olympic Games in Atlanta, USA and fully implemented for the Nagano Winter Olympic Games in 1998 in Japan.

Synthetic testosterone is usually synthesized from plant sterols and has a more negative  $\delta^{13}\text{C}$  value compared to testosterone produced naturally in the human body. By measuring the  $^{13}\text{C}/^{12}\text{C}$  isotopic ratios of testosterone and its metabolites from urinary extracts using a gas chromatograph coupled to an isotope ratio mass spectrometer (GC-IRMS), one can detect the use of synthetic forms of endogenous anabolic androgenic steroids.

WADA guidelines for the detection of testosterone misuse in sports requires IRMS measurements of testosterone (T) and four other metabolites, including  $5\alpha$ -androstane diol ( $5\alpha$ -adiol),  $5\beta$ -androstane diol ( $5\beta$ -adiol), androsterone (A) and etiocholanolone (Et) as well as endogenous reference compounds (ERC), e.g. pregnane diol (Pgdiol). ERC are not affected by testosterone administration or metabolism and provide a benchmark value for the isotopic composition of the athletes' naturally produced steroids ( $\delta^{13}\text{C}$  between  $-16$  and  $-24$  ‰ depending on diet and country of origin). Large departures in the  $\delta^{13}\text{C}$  (over 3 ‰) between the ERC and testosterone/metabolites are indicative of external administration.

### OVERVIEW

Analysis of steroids according to the WADA technical documents TD2016IRMS with excellent results



## Anthro**vis**ION technology

In the pursuit of fair competition, it is imperative that even the smallest amounts of performance enhancing steroids are detectable. This places the highest analytical demands on the application of compound-specific isotope ratio mass spectrometry. Anthro**vis**ION delivers the highest levels of detection, excellent chromatographic performance and long-term analytical robustness providing the best possible tools to ensure that sporting competition remains fair.

Anthro**vis**ION is a complete Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) solution which provides compliance with WADA Technical Document TD2016IRMS. The system comprises of the following components:

1. Agilent Technologies has been a leading provider of analytical instruments for drug testing in sports for nearly 40 years. We are a VAR partner of Agilent Technologies and we use the market leading Agilent **7890B Gas Chromatograph** as our front-end interface for separation of compounds of interest using a capillary column and helium as carrier gas. The Agilent 7890B is supplied with a split/splitless injector and flame ionization detector but can be upgraded to include other injection/detector technology including Q-MS, QQQ-MS or ToF-MS.
2. The **GC-5 Furnace Interface** is responsible for the conversion of the separated compounds to CO<sub>2</sub> prior to analysis by the IRMS. This process is critical to the ultimate system performance since the conversion must be free from isotopic fractionation (i.e. 100 % quantitative combustion of the compound to CO<sub>2</sub>) whilst maintaining the chromatographic integrity of the separated compounds. The GC-5 performance stands apart due to the microbore reactor technology with copper oxide furnace reactant and fully automated O<sub>2</sub> bleed and recharge providing exceptional performance for several months of analysis.
3. **vis**ION is an exceptional stable isotope analyzer which is responsible for generating the isotopic ratio of the separated compounds. **vis**ION offers completely automated instrument validation procedures including ion source tuning and linearity checking with different CO<sub>2</sub> gas pulses. The instrument also offers various access levels to methods and parameters so that user hierarchy can be set for greater auditing and SOP configurations. **vis**ION also has the smallest benchtop footprint of any commercially available instrument making the instrument ideal for even the busiest laboratory.
4. **IONOS** provides a completely new experience in stable isotope data processing and it offers a number of powerful features for anti-doping laboratories. All data processing is done within **IONOS**, not in external spreadsheet software, which means that all data processing can be traced back to the original instrument data. Our patented Peak Mapping algorithm is able to automatically detect isotope standards such as USADA-33 and USADA-34 and then automatically apply an isotope calibration to raw data so that all data is consistently calibrated. Finally, our Calculated Columns functionality is able to automatically calculate Endogenous Reference Compound (ERC) and Target Compound (TC) combinations and flag them as lying outside the permissible range as laid out in TD2016IRMS.



# Stable carbon isotope analysis of urinary metabolites of testosterone-related steroids

The diagnostic urinary metabolites and endogenous reference compounds (ERC) are present as conjugates in urine and thus must first be extracted, hydrolyzed and purified via solid phase extraction (SPE) or high performance liquid chromatography (HPLC). As stipulated by WADA, all extraction methods must be able to target T, A, Et, 5 $\alpha$ -adiol and 5 $\beta$ -adiol as well as ERC. Prior to analyses the extracts are typically derivatized (e.g. acetylated), however with this additional carbon added, a mass balance correction is also required.

Recently, the prestigious Laboratoire de contrôle du dopage, INRS, Canada, developed a simplified method to accurately measure  $\delta^{13}\text{C}$  values from urinary metabolites of testosterone-related steroids using GC-IRMS (Ouellet, et al., 2013). Briefly, an HPLC purification step is used to isolate seven diagnostic urinary metabolites, including T, dehydroepiandrosterone (DHEA), 5 $\alpha$ -adiol, epitestosterone

(E), 5 $\beta$ -adiol, A and Et, and two endogenous reference compounds, Pgdol and androstenol (16-enol). All the steroids were then pooled into three fractions with an external standard (androstanol, ESTD) and analyzed via GC-IRMS without any derivatization. To ensure the integrity of the data, a multi-level isotopic calibration approach was applied using the principal of identical treatment.

To evaluate their methodology, negative and positive control samples were produced and were analyzed along with a series of reference standards. The negative control was urine from a male volunteer that was not using any anabolic androgenic steroids (AAS) and the positive control was from another volunteer who was receiving testosterone enanthate for medical reasons. The long-term precision observed from these samples was excellent, with standard deviations being 0.5 ‰ or less for both the control samples and 0.2 ‰ or less for the reference standards (Table 1).

Table 1. Long-term precision of the negative and positive control samples and reference standards (Ouellet et al., 2013).

	NEGATIVE CONTROL		POSITIVE CONTROL		REFERENCE STANDARDS		
	$\delta^{13}\text{C}$ (‰)	SD (‰)	$\delta^{13}\text{C}$ (‰)	SD (‰)	$\delta^{13}\text{C}$ (‰)	SD (‰)	CERTIFIED $\delta^{13}\text{C}$ (‰)
T	-21.5	0.3	-28.5	0.3	-28.1	0.2	-28.3
E	-22.4	0.4	-	-	-33.1	0.2	-33.6
5 $\beta$ -adiol	-20.9	0.2	-27.5	0.3	-29.1	0.1	-29.0
5 $\alpha$ -adiol	-21.9	0.5	-27.4	0.3	-30.9	0.2	-30.9
DHEA	-19.7	0.2	-19.9	0.5	-33.3	0.1	-33.7
A	-20.6	0.2	-26.3	0.2	-30.7	0.1	-30.8
Et	-21.1	0.1	-26.6	0.2	-28.9	0.1	-29.0
16-enol	-20.9	0.3	-20.9	0.3	-27.2	0.2	-27.3
Pgdol	-20.9	0.2	-21.0	0.3	-16.8	0.1	-17.1

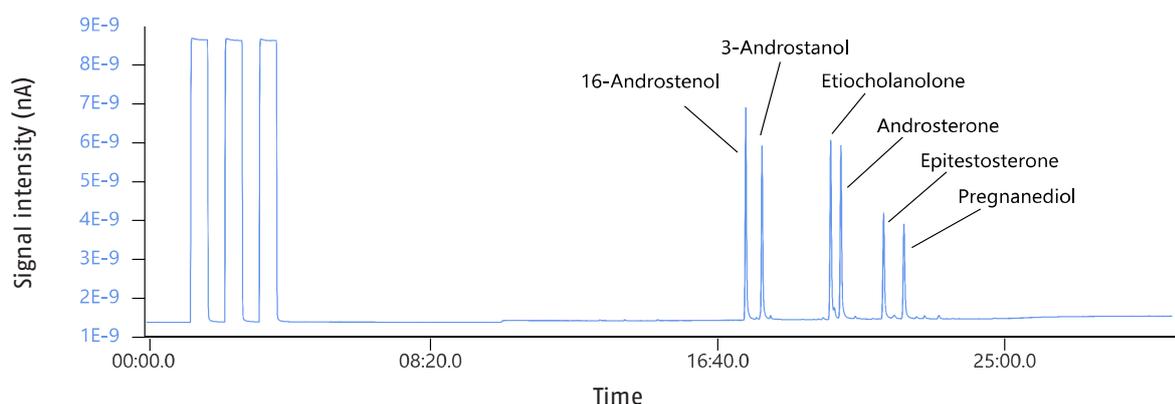


Figure 1. Example chromatogram of a standard mixture B from INRS. Analyzed at Isoprime House.

Table 2. Linearity of the instrument over a large range of sample concentrations (Ouellet et al., 2013)

	CERTIFIED	SIGNAL INTENSITY (0.1 < nA < 0.5)		SIGNAL INTENSITY (0.5 < nA < 17)	
	$\delta^{13}\text{C}$ (‰)	$\delta^{13}\text{C}$ (‰)	SD (‰) n = 3	$\delta^{13}\text{C}$ (‰)	SD (‰) n = 17
<b>STD A</b>					
5B-ADIOL	-29.02	-28.9	0.21	-29.1	0.08
DHEA	-33.49	-32.6	0.53	-33.2	0.12
5A-ADIOL	-30.88	-30.4	0.72	-30.8	0.16
T	-27.77	-26.9	0.62	-27.5	0.10
PGDIOL	-17.09	-16.2		-16.8	0.24
<b>STD B</b>					
16-ENOL	-27.27	-27.0	0.34	-27.1	0.14
ANDROSTANOL	-31.24	-31.6	0.67	-31.1	0.22
ET	-22.06	-21.5	0.78	-22.1	0.18
A	-20.88	-20.2	1.59	-20.9	0.14
E	-33.34	-34.1	0.28	-33.6	0.37
PGDIOL	-18.60	-18.2	0.26	-18.7	0.12

## References

Ouellet, A., N. LeBerre and C. Ayotte, A simplified and accurate method for the analysis of urinary metabolites of testosterone-related steroids using gas chromatography / combustion / isotope ratio mass spectrometry, Rapid Communications in Mass Spectrometry, 27, 1739–1750, 2013. <http://www.ncbi.nlm.nih.gov/pubmed/23821567>

WADA Technical Document (TD2016IRMS), Detection of synthetic forms of endogenous anabolic androgenic steroids by GC/IRMS. <https://www.wada-ama.org/en/resources/science-medicine/td2016-irms>

WADA Technical Document (TD2016EAAS), Endogenous anabolic androgenic steroids measurement and reporting <https://www.wada-ama.org/en/resources/science-medicine/td2016-eaas>

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Ouellet et al. evaluated the linearity (i.e. the independence of the result on the sample concentration) and the limit of isotopic quantification (LOiQ) through the analyses of reference standards at different concentrations (Table 2). With peak heights ranging from 0.1 to 17 nA, linearity was found to be excellent and although some steroids performed better, they set their LOiQ as 0.5 nA peak height, which based on their instrument setup equated to approximately 4 ng of compound. An example chromatogram for one of the standards is displayed in Figure 1.

## Summary

The overall sensitivity, accuracy, precision and its ease of use makes **AnthroVISION** the ideal choice for the fight against the use of illicit drugs in sport. Thanks to our VAR partnership with Agilent we can ensure a world leading chromatographic solution which we maintain through our GC-5 furnace interface where separated compounds are converted to CO<sub>2</sub> with a 100% yield. Coupled with our revolutionary **IONOS** software, the **VISION**, with integrated **centriON** unit, offers unrivaled automation. The **IONOS** software offers further advantages in data processing, such as automatic peak mapping, calibration and calculated columns.

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