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Endogenous Anabolic Androgenic Steroids
Testing, Reporting and Interpretive Guidance

1.0 Introduction

The purpose of this Technical Document is to further harmonize the approaches to the detection and confirmation of administration of endogenous anabolic androgenic steroids (AAS)[1].

Following the recommendations and requirements of this Technical Document, the Laboratories can report, in a uniform way, *Adverse Analytical Findings* and/or the presence of abnormal profiles of urinary steroids resulting from the administration of endogenous AAS, in particular of testosterone or its precursors, including for example androstenediol, androstenedione, prasterone (dehydroepiandrosterone or DHEA), dihydrotestosterone (DHT, a testosterone metabolite) or epitestosterone.

Each urine *Sample* shall be analyzed to determine its "steroid profile".

For the purposes of this Technical Document, the "steroid profile" from the Initial Testing Procedure is based on the following steroids (obtained from hydrolysis of the glucuronides):

- Testosterone (T);
- Epitestosterone (E);
- Androsterone (A);
- Etiocholanolone (Etio);
- 5α-androstane-3α,17β-diol (5αAdiol); and
- 5β-androstane-3α,17β-diol (5βAdiol).

Either elevated or suppressed concentrations of the urinary metabolites which are part of the "steroid profile" are not consistent with normal endogenous steroid production. Altered ratios of specific pairs of steroid metabolites can be indicative of the exogenous administration of these steroids or their precursors. The relevant ratios include:

- testosterone / epitestosterone (T/E);
- androsterone / etiocholanolone (A/Etio);
- 5α-androstane-3α,17β-diol / 5β-androstane-3α,17β-diol (5αAdiol /5βAdiol);
- androsterone / testosterone (A/T); and
- 5α-androstane-3α,17β-diol / epitestosterone (5αAdiol /E).

[1] In seeking to further harmonize the analysis and reporting of endogenous AAS, this document is in no way intended to imply that the various other methods previously or currently used by the Laboratories are not fit for purpose or have not produced reliable results.

Alteration of the "steroid profile" can also occur as a result of the abuse of other anabolic steroids or other doping substances (e.g. probenecid, diuretics)[2]. It is important, therefore, to have a method fit for the purpose of comparing results for either increases or decreases from the population- or subject-based reference concentration ranges.

Norandrosterone and noretiocholanolone are considered in a separate Technical Document and the following technical recommendations and requirements shall not be applied to their analysis.

2.0 Initial Testing Procedure for the measurements of the Endogenous Anabolic Androgenic Steroids

The Laboratory shall use a method that is fit for the purpose of estimation of the concentrations of endogenous AAS for comparison to population- or subject-based reference ranges. Appropriate internal standard(s) shall be included in the method to allow the estimation of the concentrations of the variable(s) of the "steroid profile", as well as other endogenous AAS such as DHEA. It is recommended that deuterated internal standard(s) are utilized to estimate the concentrations of the variable(s) of the "steroid profile".

The Initial Testing Procedure is normally conducted on a single Aliquot.

2.1 Assay

2.1.1 Method Characteristics

- Purified β-glucuronidase from *E. coli* shall be used for enzymatic hydrolysis (*H. pomatia* mixtures are not acceptable).
- The limit of quantitation (LOQ) for both T and E should be not higher than 2 ng/mL;
- The LOQs for 5αAdiol and 5βAdiol, A and Etio should be not higher than 10 ng/mL.

2.1.1.1 Routine verification of method performance

- To assess the completeness of the steroid glucuronide hydrolysis an appropriate set of deuterated internal standards (e.g. measurement of the ratio of d_4-androsterone - from d_4-androsterone-glucuronide - to d_5-etiocholanolone is recommended) shall be included;
- The effectiveness of derivatization shall be monitored. An appropriate compound (e.g. the mono-TMS derivative of A in the case of enolTMS derivatives) shall be monitored to ensure that the derivatization process is complete.

[2] A steroid profile acquired for a *Sample* that was found to contain another AAS should not be utilized in longitudinal studies.

2.1.1.2 *Quality Control*

- The batch of *Samples* analyzed in the Initial Testing Procedure shall include a control sample that has a T/E ratio close to 4 and a control sample with a T/E ratio much less than 4. The concentrations of the "steroid profile" analytes in these control samples shall be monitored over time and used for quality control purposes.

2.1.1.3 *Steroid Measurement*

- The ratios of endogenous AAS included in the "steroid profile" shall be determined from the estimated concentrations of the relevant AAS. In cases where the estimated concentration of the AAS included in the ratio denominator (e.g. E for calculation of T/E ratio) is below the LOQ of the particular AAS, a concentration value corresponding to the LOQ for this steroid should be used for the estimation of the ratio;
- In the specific case of the T/E ratio it is acceptable to measure the ratio of the chromatographic peak heights or areas of the two steroids within a *Sample*. The peak height or area ratio shall be corrected according to the peak height or area ratio, respectively, obtained for a known T/E ratio in a natural or simulated urine matrix assayed contemporaneously;
- The *Sample* measurement should be repeated using an alternative procedure in every case where matrix interference hampers the estimation of the concentration of the variables of the "steroid profile".

2.1.1.4 *Microbial Degradation*

- An elevated level of androstanediones is an indicator of microbial degradation. Consequently, the Laboratory shall monitor at least one diagnostic ion for 5α- and 5β-androstanedione as an indicator of microbial degradation;
- Concentrations of 5α- or 5β-androstanedione greater than 2% of the concentrations of A or Etio, respectively, may be indicative of microbial degradation;
- The presence of signs of microbial degradation is an indication that the results for the "steroid profile" should be reviewed with caution.

2.1.2 Additional testing

GC/C/IRMS analysis is an independent test that may be conducted at any time. The Laboratory should perform the GC/C/IRMS confirmation analysis upon request by the relevant Testing Authority or *WADA.*

As guidance, a urine *Sample* in which any one of the following criteria from the Initial Testing Procedure is met should be submitted to the GC/C/IRMS analysis:

- T/E ratio greater than 4;
- Concentration of T or E greater than 200 ng/mL in males or greater than 100 ng/mL in females;
- Concentration of A or Etio greater than 10,000 ng/mL in either sex;
- Concentration of DHEA (in the combined free and glucuronide fraction) greater than 200 ng/mL;
- Ratio of A / Etio less than 0.4 in males or greater than 4 in either sex;
- Ratio of 5αAdiol /5βAdiol greater than 2;
- Ratio of 5αAdiol /E greater than 10;
- Concentration of 5αAdiol greater than 250 ng/mL for males and 150 ng/mL for females;
- Concentration of formestane less than 100 ng/mL;
- Concentration of main boldenone metabolite less than 20 ng/mL;
- Values of variable(s) of the 'steroid profile' outside the Laboratory's population reference.

The concentrations mentioned in the above list refer to estimated concentrations adjusted to a specific gravity (SG) of 1.020 based on the equation in Section 2.2, applicable to steroids extracted from the urine glucuronide fraction.

2.2 Specific Gravity Adjustment

- Individual concentration values of endogenous AAS shall be adjusted to a SG value of 1.020 using the equation:

$$\mathbf{Conc_{corr} = Conc_{measured} * (1.020 - 1)/(SG - 1)}$$

2.3 Reporting results from the Initial Testing Procedure

- Measurement Uncertainty (MU): The MU shall not be applied when reporting the results from the Initial Testing Procedure;
- The Laboratory shall provide estimated concentrations for T, E, A, Etio, 5αAdiol, 5βAdiol and the relevant ratios (derived from the Initial Testing Procedure of all *Sample*s analysed) to the responsible Testing Authority;
- The Laboratory shall report the values of pH and SG of the *Sample*;
- The Laboratory may also report the adjusted concentration values of endogenous AAS if requested by the client. AAS concentrations that have been adjusted for SG should be clearly marked as such;

- A *Sample* which has a variable(s) outside the Laboratory's population reference range or meet the criteria listed in section 2.1.2 shall be reported as a Presumptive Analytical Finding based on the Initial Testing Procedure. A comment may be included in the test report indicating that the concentration(s) or ratio(s) exceeded normal physiological limit(s) and recommending that Confirmation Procedure(s) (possibly including GC/C/IRMS confirmation analysis) be performed on this *Sample*. The Confirmation Procedure(s) shall be performed upon request by the relevant Testing Authority or *WADA*[3];
- If 5α- or 5β-androstanedione is present in the *Sample* at a concentration greater than 2% of A or Etio, respectively, the Laboratory shall indicate it in the test report. In such cases, a comment or opinion should be added by the Laboratory indicating that the *Sample* presents signs of microbial degradation and therefore the 'steroid profile' should be interpreted with caution;
- Likewise, the Laboratory should advise a careful interpretation of the 'steroid profile' for *Samples* showing pH values above 8.0.

3.0 Confirmation Procedure for Endogenous Anabolic Androgenic Steroids

- If requested by the relevant Testing Authority, variables of the "steroid profile" at any concentration above the documented LOQ of the Laboratory shall be confirmed and/or quantitatively determined in compliance with this Technical Document and the IDCR Technical Document.
- The Laboratory shall utilize a method that is fit-for-purpose for the quantitative confirmation of the concentrations of endogenous AAS;
- The quantitative Confirmation Procedure shall employ three Aliquots of *Sample.* If there is insufficient *Sample* volume to complete the GC/MS Confirmation Procedure and the GC/C/IRMS Confirmation Procedure, the Laboratory may use less than three Aliquots. Please refer to provision 5.2.4.3.1.6 of the ISL;
- If the *Sample* volume is such that the Laboratory determines that it cannot perform the quantitative Confirmation Procedure, then this situation should be communicated to the relevant *Anti-Doping Organization* and documented prior to any further action;
- Laboratories shall determine the assay MU associated with the quantification of any endogenous AAS in accordance with the provisions of DL Technical Document.

[3] According to the Technical Document on Decision Limits, E constitutes a Threshold Substance. Therefore, Initial Testing Procedure findings of E concentrations above the 200ng/mL threshold shall be submitted to confirmation analysis and reported according to Section 4.0 of this Technical Document.

3.1 *Assay*

3.1.1 Method characteristics

- The GC/MS or GC/MS/MS Confirmation Procedure provides independent information that a doping violation has occurred. As such, the results of the GC/MS or GC/MS/MS Confirmation Procedure can be evaluated without reference to the results of the GC/C/IRMS Confirmation Procedure. The Laboratory shall, therefore, confirm a Presumptive Analytical Finding (e.g. T/E ratio greater than 4) by using GC/MS or GC/MS/MS. GC/C/IRMS and GC/MS procedures are independent but can provide complementary information;
- The GC/MS or GC/MS/MS Confirmation Procedure should incorporate one or more different analytical steps (sample clean-up, chromatographic conditions such as temperature program, etc) than the Initial Testing Procedure;
- The Laboratory should use the information from the Initial Testing Procedure to ensure the quality of results (e.g. use of a larger *Sample* volume) in its Confirmation Procedure;
- The LOQ of both T and E in the Confirmation Procedure should be not higher than 0.5 ng/mL.

3.1.2 Identification of Endogenous Anabolic Androgenic Steroids

All Confirmation Procedure analyses for endogenous AAS analytes shall meet the compound identification criteria according to the IDCR Technical Document.

3.1.2.1 *Testosterone and Epitestosterone*

- In cases where a full scan spectrum of T or E (or its derivative(s)) is not possible for identification purposes, a minimum of three diagnostic ions shall be collected in the selected ion monitoring (SIM) mode. However, in order to ensure that no co-eluting substance contributes to the diagnostic ion intensity and interferes in the quantitative analysis, a full scan spectrum shall be acquired at the retention time of the peak(s) of interest. The acquisition of a full scan may require analysis of an additional Aliquot of *Sample* to which the internal standards are not added. This full scan spectrum shall be included in the documentation package;
- In the case of low E concentrations precluding its identification in a T/E ratio case, only the identification of T shall be required;
- In the case of low T and/or low E concentrations in a T/E ratio case, the chromatogram shall be carefully inspected in the region of the expected T and/or E retention time(s) to ensure that there is no significant interference for the ion(s) used in the T/E ratio determination. The results of this search should be documented.

3.1.3 Quantification of ratios of endogenous anabolic steroids

- The ratios of endogenous AAS included in the "steroid profile" shall be determined from the estimated concentrations of the relevant AAS. In cases where the estimated concentration of the AAS included in the ratio denominator (e.g. E for calculation of T/E ratio) is below the LOQ of the particular AAS, a concentration value corresponding to the LOQ for this steroid should be used for the estimation of the ratio;
- In the specific case of the T/E ratio it is acceptable to measure the ratio of the chromatographic peak heights or areas of the two steroids within a *Sample*. The peak height or area ratio shall be corrected according to the peak height or area ratio, respectively, obtained for a known T/E ratio in a natural or simulated urine matrix assayed contemporaneously;
- The Confirmation Procedure shall employ appropriate standard(s) to determine the ratios of endogenous AAS.

3.2 Interpretation

3.2.1 Other variables

Other variables such as LH may be used to provide additional information to help in determining the use of some substances, especially injected T and many of its esters. A high T/LH ratio or suppressed LH level may be used as ancillary evidence.

3.2.2 Microbial degradation

In addition to the measurement of 5α- and 5β-androstanedione, performed during the Initial Testing Procedure, the Laboratory shall test for the presence of unconjugated (or free) steroids in the *Sample* Confirmation Procedure, e.g. T.

- The presence of more than 5% free T in the *Sample* should be considered to be indicative of microbial degradation;
- The presence of signs of microbial degradation is an indication that the results for the "steroid profile" should be reviewed with caution;
- However, microbial degradation does not invalidate an *Adverse Analytical Finding* reported for an endogenous AAS when supported by results from the GC/C/IRMS analysis.

3.2.3 The effect of ethanol consumption on the T/E ratio

- It has been demonstrated that the ingestion of large doses of ethanol may produce significant effects on the "steroid profile". The excretion of T glucuronide is increased, thus increasing the T/E ratio in some individuals while the A/T ratio may be decreased;
- The appearance of ethyl glucuronide and ethyl sulfate in the urine follows the appearance and elimination of testosterone glucuronide. Based on unpublished data available to date, it appears that concentrations of ethyl

glucuronide greater than 100 ng/mL are consistent with elevated T/E ratios in some individuals;

- The ethyl sulfate concentration may be of assistance in interpreting the source of ethyl glucuronide;
- The Laboratories shall have a procedure to quantify the presence of ethyl glucuronide and ethyl sulfate in urine and perform such procedure for cases of elevated T/E ratios (T/E > 4);
- The Laboratories should consider also performing the procedure in cases of abnormal longitudinal "steroid profiles" with elevated values of T and/or T/E or decreased values of A/T.

3.3 Reporting

- Each *Sample* for which an *Adverse Analytical Finding* is reported shall be reported on its own test report form;
- For each *Sample* for which an *Adverse Analytical Finding* is reported, the Laboratory shall include on the test report the mean values of concentrations (adjusted for SG) and/or ratio(s) of the AAS and the expanded MU (95% confidence limits, k = 2);
- The Laboratory shall also report the values of pH and SG of the *Sample*;
- The Laboratory shall include in every Confirmation Procedure *Sample* test report (to be provided to the responsible *Anti-Doping Organization* and to *WADA*) if 5α- or 5β-androstanedione were present at a concentration greater than 2% of A or Etio, respectively, or if the percentage of free T was greater than 5%. In such cases, a comment or opinion should be added by the Laboratory indicating that the *Sample* presents signs of microbial degradation and therefore the 'steroid profile' should be interpreted with caution;
- The Laboratory should also indicate in the test report if the presence of 5% free T in the *Sample* could not be determined (e.g. when this concentration value is below the LOQ for T) and recommend in such cases that the 'steroid profile' be interpreted with caution;
- Likewise, the Laboratory should advise a careful interpretation of the 'steroid profile' for *Samples* showing pH values above 8.0;
- The results of the ethanol analysis (concentrations of ethyl glucuronide and ethyl sulfate), when performed, shall be included in the test report.

4.0 Confirmation Procedure (GC or HPLC interfaced with MS or tandem MS) for elevated epitestosterone concentration

4.1 Analytical guidance

- The Confirmation Procedure shall employ three Aliquots of *Sample*. If there is insufficient *Sample* volume to analyze three Aliquots in the Confirmation Procedure, the Laboratory may use less than three Aliquots as per provision 5.2.4.3.1.6 of the ISL;

- It is recommended that a deuterated internal standard be used for quantification;
- In the case of E concentrations greater than 200 ng/mL, a full scan shall be obtained. This may require that an additional *Sample* Aliquot without the addition of the deuterated internal standard be analyzed.

4.2 Adjusted Threshold

Only in the case of urine *Sample*s measured with a SG above 1.020 (in the Laboratory), an adjustment to the threshold shall be made to take into account the SG of the *Sample*, using the following formula:

$$\text{Threshold}_{\text{adjusted}} = [(\text{SG of the } \textit{Sample} - 1) / (1.020 - 1)] \times 200 \text{ ng/mL}$$

4.3 Decision limit for epitestosterone

The DL for E is established in the DL Technical Document. In cases where the SG is greater than 1.020, the guard band represented by the difference between the value of the DL and the value of the threshold, as specified for E in the DL Technical Document, shall be added to the adjusted threshold to determine the DL for an individual E test result[4]. The DL (after adjustment of the threshold for SG) shall be included on the Laboratory test report. The DL shall be used to determine whether an *Adverse Analytical Finding* is reported for the *Sample*.

4.4 Reporting

An *Adverse Analytical Finding* for E shall be reported if the concentration of E in the urine *Sample* is greater than the Decision Limit (after adjustment of the threshold for SG if necessary) in accordance with the Technical Document on Decision Limits.

5.0 Confirmation Procedure (GC or HPLC interfaced with MS or tandem MS) for the presence of metabolites of other endogenous steroids

Administration of some naturally-occurring steroids results in the formation of unique metabolites not observed in normal urine *Samples*. Examples are listed in the following table. The identification of these metabolites (see TD IDCR) may help identify the administered steroid.

[4] Thus the Decision Limit applicable to a test result for E for a *Sample* with a SG of 1.020 or less is 240 ng/mL. When the SG of a *Sample* is 1.030, for example, the adjusted threshold is 300 ng/mL and DL shall be 340 ng/mL (according to TD2010DL).

Table 2. Urinary Metabolites of Administered Endogenous Steroids

Steroid Administered	Steroid found in Urine	Conjugate Type
Androstenedione	6α-hydroxyandrostenedione	Glucuronide
	6β-hydroxyandrosterone	Glucuronide
	6β -hydroxyetiocholanolone	Glucuronide
	6β -hydroxyepiandrosterone	Sulfate
Dehydroepiandrosterone	Dehydroepiandrosterone[2]	Glucuronide
	7β-hydroxydehydroepiandrosterone	Sulfate
	16α-hydroxyandrosterone	Sulfate
	3α,5-cyclo-5α-androstan-6β-ol-17-one	Free
7-ketodehydroepiandrosterone	7-ketodehydroepiandrosterone	Free
	7ζ-hydroxydehydroepiandrosterone	Free

[2] Dehydroepiandrosterone sulfate can be easily hydrolyzed. The Laboratory shall take appropriate steps to ensure that the DHEA detected in the glucuronide fraction does not arise from free DHEA.

In some cases, it may be useful to evaluate the concentrations of some steroid metabolites in the sulfate fraction. The Laboratory should have methods in place for evaluating concentrations of sulfate conjugates of endogenous AAS and their metabolites. It is recommended that solvolysis and not enzymatic hydrolysis with aryl sulfatase be used for cleavage of sulfate conjugates due to the positional cleavage selectivity of the aryl sulfatase.

It is recommended that a urine *Sample* in which any one of the metabolites of androstenedione or DHEA listed in Table 2 shows a value normally not found in urine be submitted to the GC/C/IRMS analysis.

6.0 Compound-specific isotope ratio analysis (CSIRA) Confirmation Procedure for determining the administration of natural steroids related to testosterone

- The CSIRA analysis, which is currently based on the GC/C/IRMS determination of the carbon ($^{13}C/^{12}C$) isotope ratio of specific steroids, is an independent test for the detection of doping with naturally-occurring steroids and their precursors. As such, the results of the GC/C/IRMS test can be evaluated without reference to the results of the Initial Testing Procedure or the Confirmation Procedure for the "steroid profile". A Presumptive Analytical Finding from the Initial Testing Procedure for steroids is not a necessary pre-requisite to GC/C/IRMS testing;
- The application of GC/C/IRMS in doping control is based on the difference between the carbon isotope ratio ($^{13}C/^{12}C$) of one or more specific steroid metabolites (A, Etio, 5αAdiol, 5βAdiol, T and others) compared to an endogenous reference compound (ERC) from another metabolic pathway unaffected by the administration of endogenous anabolic androgenic steroids (or their precursors) and therefore used to define the basal carbon isotope ratio value of the person;

- For the purpose of harmonizing the application of the GC/C/IRMS analysis, pregnandiol (PD) is recommended as the first-choice ERC. Alternatively, 11β-hydroxyandrosterone may be used. Decision limits have been established for the differences in δ value (Δδ values) between AAS and these ERCs (see below). For the use of any other ERC, the Laboratory must have scientific data to support their decision limit;
- For GC/C/IRMS determination of the administration of endogenous AAS, the metabolism of steroids shall be considered in order to select the most appropriate target analytes.

  For example, to determine the administration of T or its precursors, the 5αAdiol and 5βAdiol are the preferential analytes. In addition, A and Etio should be analyzed if necessary; T should also be analyzed if in sufficient concentration;
- For *Samples* where E has been implicated, the measurands shall include E;
- The steroids may be analyzed underivatized or after derivatization with acetic anhydride. An appropriate mass balance equation (Docherty *et al*) for adjustment of the measured GC/C/IRMS δ values from acetate derivatives back to the underivatised form shall be used when reporting δ values.

$$\delta^{13}C_s = (n_{cd}\delta^{13}C_{cd} - n_d\delta^{13}C_{dcorr}) / n_s$$

where n: number of moles of carbon; s: native steroid (underivitised form); d: derivative group (e.g. Acetic Anhydride), and cd: derivatised compound.

Example for calculation of $\delta^{13}C$ pregnanediol (PD) measured as diacetate (PD-diac):

$$\delta^{13}C_{PD} = (25\ \delta^{13}C_{\text{PD-diac}} - 4\ \delta^{13}C_{\text{acetate corr}}) / 21$$

The δ value for the acetate group $\delta^{13}C_{dcorr}$ should be estimated following the measurement of a non acetylated and acetylated steroid (for example 5αAdiol, 5ßAdiol or PD).

$$\delta^{13}C_{dcorr} = (n_{cd}\delta^{13}C_{cd} - n_s\delta^{13}C_s) / n_d$$

Example for calculation of the δ value of the introduced acetate group following the measurement of non acetylated (PD) and diacetylated pregnanediol (PD-diac):

$$\delta^{13}C_{\text{acetate corr}} = (25\ \delta^{13}C_{PD} - 21\ \delta^{13}C_{\text{PD-diac}}) / 4)$$

The calculation of Δδ values shall be performed on the δ value of the steroid itself, not including the $\delta^{13}C$ contribution from the derivatizing reagent.

6.1 Analytical Conditions

The GC/C/IRMS Confirmation Procedure may be run on one Aliquot of urine.

6.1.1 Method characteristics

It is recommended that the following are incorporated into the GC/C/IRMS method:

- The calibration gas used in each chromatogram to calculate the δ values be calibrated against a standard of known δ value;
- Both the m/z 44 trace and the ratio trace of m/z 45 to m/z 44 be examined for peak overlap;
- Each target compound is included in a reference standard to verify retention times;
- A negative control (δ value of all steroids in a natural range) and a positive control (δ value of target steroids different from natural steroids or ERC) be included in each batch of *Samples* analyzed (e.g. a positive control from a T or precursor administration study).

6.1.2 Identification of analytes

- GC/C/IRMS only produces a retention time value in the chromatogram since all the compounds are converted to carbon dioxide. Thus an alternative GC/MS Confirmation Procedure is required to ensure identity of each compound measured;
- The contents of the same vial prepared for GC/C/IRMS shall be analyzed in a GC/MS system. The same column (phase; film thickness; diameter; and length) and temperature program should be used. The purpose is to produce a chromatogram with similar peak profiles and overlaps so that the spectra can be used to both identify and document the absence of significant contamination of the analyte peak. Minor differences in retention time between the two techniques is expected;
- Full scan mass spectra shall be produced and compared to spectra obtained from reference materials run contemporaneously or from a library generated at the Laboratory. Both the retention times (from either the library or the reference material) and spectra should be comparable (see IDCR Technical Document).

6.2 Interpretation

6.2.1 Compound Specific Isotope Ratio Analysis Measurements

- A difference of Δδ values greater than the Decision Limit(s) as noted in Table 1 below between the measured δ value of the analyte and the ERC (after adjustment for the respective contribution of the derivatizing agent) shall be deemed an *Adverse Analytical Finding*;

Table 1. Decision Limits for GC/C/IRMS Δδ values when usi*ng Pregnandiol (PD) or 11β*-hydroxyandrosterone (OHA) as the ERC.

	PD-A	**PD-Etio**	**PD-T**	**PD-5αAdiol**	**PD-5βAdiol**	**PD-E**
Decision Limit	2.3	3.3	5.1	4.3	2.9	4.7
	OHA-A	**OHA-Etio**	**OHA-T**	**OHA-5αAdiol**	**OHA-5βAdiol**	
Decision Limit	2.2	4.3	5.7	5.0	3.8	

- In the case of an elevated T/E ratio, GC/C/IRMS results for T, 5αAdiol and 5βAdiol which are consistent with endogenous production may conclude the analysis on this individual *Sample.* A GC/C/IRMS result for A and Etio consistent with endogenous production, on the other hand, should NOT be used to conclude an investigation into an elevated T/E ratio because of the potential for these specific steroids to give a false negative conclusion;
- Any GC/C/IRMS result should be considered along with other information (e.g., T concentration, non-analytical information) in order for the responsible Testing Authority to decide whether or not the *Athlete* should be charged with an anti-doping rule violation (ADRV);
- A difference in GC/C/IRMS Δδ values that indicates an exogenous origin does not require an abnormal concentration or ratio of the relevant target analyte(s) (such as a T/E ratio greater than 4 or a deviation from the individual longitudinal reference range) in order to be considered an *Adverse Analytical Finding;*
- Microbial degradation does not affect the results of the GC/C/IRMS results and therefore is not a concern when this technique detects an *Adverse Analytical Finding*.

6.3 Reporting of GC/C/IRMS results

- The test report to the responsible Testing Authority shall contain the name of each AAS analyzed, the name of each associated ERC compound, the δ value (corrected for any ^{13}C contribution by the derivatizing reagent) of each steroid and ERC and the Δδ values for each relevant steroid – ERC pair;

- The Laboratory shall also include on the test report the values of the MU (95% confidence limits, *k*=2) associated with the measurement of the Δδ values for each relevant steroid – ERC pair;[5]
- If the GC/C/IRMS analysis determines the exogenous origin of the substance(s) (Δδ values for one or more of the ERC-steroid combinations higher than the corresponding DL shown in Table 1), then the *Sample* shall be reported as an *Adverse Analytical Finding* for such substance(s);
- A δ value more negative that -28‰ for any AAS should be reported as an *Atypical Finding*. A comment may be included in the test report recommending that the Testing Authority collect additional *Sample(s)* from the *Athlete*;
- *Samples* which based on the Confirmation Procedure have a variable(s) outside the Laboratory's normal reference range or meet the criteria listed in section 2.1.2 (e.g. T/E > 4), but for which the results of GC/C/IRMS analysis do not confirm the exogenous origin of the substance, shall be reported as an *Atypical Finding*. A comment may be included in the test report indicating that the concentration(s) or ratio(s) exceeded normal physiological limit(s) and recommending that the Testing Authority collect further *Sample(s)* from the *Athlete* concerned.
- A GC/C/IRMS result not confirming the exogenous origin of the steroid(s) does not invalidate an *Adverse Analytical Finding* that may otherwise result from a significant deviation of the concentration(s) and/or ratio(s) of the "steroid profile" variable(s) from the established subject-based longitudinal profile value(s).

7.0 Analysis of Boldenone and Formestane

- Bacteria in the gastrointestinal tract have been shown in rare circumstances to produce small amounts of boldenone which is then reabsorbed in the lower intestine, metabolized in the liver, and excreted in the urine as boldenone metabolites. In such circumstances, the concentration of boldenone metabolites in the urine has been observed to be less than 20 ng/mL;
- Small amounts of 4-hydroxyandrostenedione (formestane) have also been reported to be produced endogenously.

7.1 Reporting

- Confirmed results for boldenone metabolites above 20 ng/mL (after adjustment to a SG of 1.020) shall be reported as an *Adverse Analytical Finding* for boldenone;

[5] The Laboratory estimate of MU is not applied for *Adverse Analytical Finding* decisions since the measured δ values used to establish the population statistics giving rise to the Decision Limits already include the components of MU.

- When boldenone metabolites are present at a concentration of less than 20 ng/mL (after adjustment to a SG of 1.020), the Laboratory should report the result as an *Atypical Finding* and include an opinion that the result may be consistent with bacterial production. The responsible Testing Authority should be advised to collect at least one additional *Sample.* When possible, a GC/C/IRMS analysis should be done on the boldenone metabolite to determine whether the compound is from exogenous origin;
- Formestane concentrations of up to 40 ng/mL have been associated with endogenous production and should not be considered an *Adverse Analytical Finding* unless the GC/C/IRMS result indicates an exogenous origin;
- A GC/C/IRMS difference in δ values (Δδ) between formestane or boldenone metabolite(s) and either PD or 11β-hydroxyandrosterone of three per mil ($3^0/_{00}$) or greater shall be reported as an *Adverse Analytical Finding*.

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8.0 References

Ayotte C, Goudreault D, Charlebois A. (1996) Testing for natural and synthetic anabolic agents in human urine. J Chromatogr B 687:3-25.

Mareck-Engelke U, Geyer H, Donike M. (1993) Stability of steroid profiles. In M Donike, H Geyer, A Gotzmann, U Mareck-Engelke, S Rauth (eds.) 10th Cologne Workshop On Dope Analysis. Sport und Buch Strauß, Köln, pp 87-8

Geyer H, Schanzer W, Mareck-Engleke U, Donike M. (1996) Factors influencing the steroid profile. In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (3). Sport und Buch Strauß, Köln, 95-113

Queraltó JM, Boyd JC, Harris EK. (1993) On the calculation of reference change values with examples from a long-term study. Clin Chem 39:1398-403.

Sharpe K, Ashenden MJ, Schumacher YO. (2006) A third generation approach to detect erythropoietin abuse in athletes. Haematologica 91:356-63.

Sottas PE, Baume N, Saudan C, Schweizer C, Kamber M, Saugy M. (2007) Bayesian detection of abnormal values in longitudinal biomarkers with an application to T/E ratio. Biostatistics 8:285-96.

Sottas PE, Saudan C, Schweizer C, Baume N, Mangin P, Saugy M. (2008) From population- to subject-based limits of T/E ratio to detect testosterone abuse in elite sports. Forensic Sci Intl. 174:166-72.

Cawley AT, Trout GJ, Kazlauskas R, George AV. The detection of androstenedione abuse in sport: a mass spectrometry strategy to identify the 4-hydroxyandrostenedione metabolite. Rapid Commun Mass Spectrom. 2008;22:4147-57.