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Detection of Synthetic Forms of Endogenous Anabolic Androgenic Steroids by GC/C/IRMS

1.0 Introduction

This Technical Document describes the analytical method to detect the presence of synthetic forms of Endogenous Anabolic Androgenic Steroids (EAAS) by Gas Chromatography / Combustion / Isotope Ratio Mass Spectrometry (GC/C/IRMS) in urine *Samples*.

Consideration is also given to boldenone, boldenone *Metabolite*(s) and formestane[1], which may be found naturally in urine *Samples* at low concentrations.

19-Norandrosterone (19-NA) and 19-noretiocholanolone (19-NE) are considered in a separate Technical Document, TD19NA [1], and the technical recommendations and requirements described herein shall not be applied to their analyses.

1.1 Application of GC/C/IRMS

1.1.1 GC/C/IRMS analysis as a Confirmation Procedure for the exogenous administration of EAAS

GC/C/IRMS analysis shall be conducted as a Confirmation Procedure when the Laboratory receives an "*Atypical Passport Finding* (*ATPF*) Confirmation Procedure Request" or a "Suspicious Steroid Profile (SSP) Confirmation Procedure Request" notification through *ADAMS*, as described in the Technical Document on the Measurement and Reporting of EAAS (TDEAAS) [2].

In addition, even if the *Markers* of the "steroid profile" are within the normal ranges, the *Testing* Authority, the *Athlete* Passport Management Unit (APMU), or *WADA* may request GC/C/IRMS analysis to be performed on any urine *Sample*.

[1] Formestane (4-hydroxyandrost-4-en-3,17-dione) is an aromatase inhibitor but its structure is similar to EAAS and it may also be found naturally in urine *Samples*; therefore, it requires an Analytical Testing approach similar to that described in the TDEAAS [2].

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Furthermore, the Laboratory may at any time advise[2] the *Testing* Authority to perform (or not) the GC/C/IRMS analysis based upon its expertise, for example in the presence of any other *Marker(s)* of administration of EAAS such as 6α-hydroxyandrostenedione, 3α,5-cyclo-5α-androstan-6β-ol-17-one, 6β-hydroxyandrosterone or 6β-hydroxyepiandrosterone (sulfates), or an altered ratio of 7β-hydroxydehydroepiandrosterone to 16α-hydroxyandrosterone (sulfates).

1.1.2 GC/C/IRMS analysis for formestane, boldenone or boldenone *Metabolite(s)*

In *Samples* containing formestane, boldenone or boldenone *Metabolite*(s), the GC/C/IRMS analysis for these compounds shall be conducted before reporting an *Adverse Analytical Finding* when their estimated specific gravity (SG)-adjusted[3] concentrations are determined as follows:

- Concentration of formestane between 50 ng/mL and 150 ng/mL.

  [Findings for formestane below 50 ng/mL (SG-adjusted[3], if needed) are to be considered as negative, unless the result of the GC/C/IRMS analysis, if performed (depending on Laboratory's analytical capacity and following consultation with the *Testing* Authority), conclusively establishes the exogenous origin of the substance (*Adverse Analytical Finding*).

  Findings for formestane above 150 ng/mL (SG-adjusted[3]) shall be considered as *Adverse Analytical Findings*].

- Concentration of boldenone and/or its *Metabolite*(s) between 5 ng/mL and 30 ng/mL.

  [Findings for boldenone and/or its *Metabolite*(s) at concentrations estimated below 5 ng/mL (SG-adjusted[3], if needed) are to be considered as *Atypical Findings*, unless the results of the GC/C/IRMS analysis, if performed (depending on Laboratory's analytical capacity and following consultation with

[2] Or as covered by agreement between the Laboratory and the *Testing* Authority.

[3] When the SG of the urine *Sample* is greater than 1.020, the concentrations are adjusted to a SG of 1.020 based on the following equation (free and hydrolyzed glucuroconjugated steroids).

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

the *Testing* Authority), conclusively establish the exogenous origin of the substance (*Adverse Analytical Finding*).

Findings for boldenone and/or its *Metabolite*(s) above 30 ng/mL should be considered as *Adverse Analytical Findings* without the need for GC/C/IRMS analysis, unless it is determined that the *Sample* is substantially degraded due to a high level of microbial contamination (in which case the *Sample* shall be reported as an *Atypical Finding*)].

Laboratories that do not have the analytical capacity to perform GC/C/IRMS analysis for formestane, boldenone or its *Metabolite*(s) shall have the *Sample* analyzed by another Laboratory that has such analytical capability.

1.1.3 "B" *Sample* Confirmation Procedure

When an *Adverse Analytical Finding* is reported for the *Marker*(s) of the "steroid profile" or for Non-Threshold Substances such as formestane, boldenone or its *Metabolite*(s) based on the results of a GC/C/IRMS analysis performed on the "A" *Sample*, only the GC/C/IRMS analysis shall be repeated during the "B" *Sample* Confirmation Procedure, if applicable.

2.0 GC/C/IRMS analysis

The application of GC/C/IRMS is based on the following:

- The determination of the $\delta^{13}C$ value of the Target Compound(s) (TCs), *i.e.* the urinary *Metabolite(s)* or *Marker(s)* of the "steroid profile", *e.g.* androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αAdiol), 5β-androstane-3α,17β-diol (5βAdiol), testosterone (T), epitestosterone (E), and/or the Non-Threshold Substance(s), *e.g.* boldenone, boldenone *Metabolite*(s), formestane;
- The determination of the $\delta^{13}C$ value of the Endogenous Reference Compound(s) (ERC), *e.g.* pregnanediol (PD), 5α-androst-16-en-3α-ol (16-en), 11β-hydroxyandrosterone (11-OH-A), 11-keto-etiocholanolone (11-oxo-Etio); and
- The calculation of the difference in $\delta^{13}C$ values, *i.e.* the $\Delta\delta^{13}C$ value, between the ERC(s) and the TC(s).

The GC/C/IRMS analysis shall be conducted on a single *Sample* Aliquot.

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2.1. GC/C/IRMS Method Characteristics

Laboratories shall implement the following into the GC/C/IRMS methodology:

- The system shall be calibrated periodically against a steroid Certified Reference Material (CRM) [*e.g.* CU/USADA-33, CU/USADA-34, or other mixture of certified steroid(s)[4]] that is traceable to the assigned values of the recognized international Reference Material (RM). Major revisions of the system (*e.g.* change of reference gas, cleaning of the ion source) shall require calibration of the system.
- As part of the method validation, the Laboratory shall determine:
    - the linearity of the ion source using CO_2 pulses of different peak heights/intensities;
    - the linearity of the instrument by the injection of different amounts of RM(s) of underivatized or acetylated, as appropriate, steroid standards (TC(s) and ERC(s)). The instrumental linearity is defined as the range of intensities (expressed in mV or nA, as appropriate) and/or as amounts (ng) of injected steroids that give a consistent $\delta^{13}C$ value (within 0.5 ‰ of the mean $\delta^{13}C$ value, determined within 6 to 10 different peak heights/intensities);
    - the linearity of the method, *i.e.* the range of TC and ERC concentrations in urine (ng/mL) producing consistent $\delta^{13}C$ values within the signal linearity range of the instrument;
    - the Limit of Quantification (LOQ) for each TC and ERC as the lowest concentration in urine (ng/mL) which produces a measurable signal in the linear range of the instrument with a standard deviation (SD) < 1.0 ‰, n ≥ 3;
    - the estimated combined standard Measurement Uncertainty (u_c) for the determination of the $\delta^{13}C$ values of each TC and ERC. In each case, the u_c shall be not greater than 1.0 $^0/_{00}$ (u_{c_Max}).
    - the $\Delta\delta^{13}C$ values for each ERC-TC pair (including, at least, A, Etio, 5αAdiol, 5βAdiol and T as TCs and using at least two

[4] The use of a mixture of certified steroids covering the range of $\delta^{13}C$ values normally found for TCs and ERCs in urine (*e.g.* -17 ‰ to -34 ‰) is recommended.

ERCs, one of which shall be PD) analyzed in volunteer urine samples and/or *Athlete* negative *Samples* (a minimum of 20 male and 20 female urine samples), covering the range of steroid concentrations usually found in *Doping Control Samples* [5]. The analysis of these negative urine samples shall serve to evaluate method performance by the Laboratory and shall meet the following criteria:

- the mean $\Delta\delta^{13}C$ + 2 SD value for ERC-TC combinations containing A, T or the Adiols as TCs shall be not higher than 3 $^{0}/_{00}$;
- the mean $\Delta\delta^{13}C$ + 2 SD value for ERC-TC combinations containing Etio or E as TCs shall be not higher than 4 $^{0}/_{00}$; and
- for each ERC-TC combination, the SD of all $\Delta\delta^{13}C$ values shall not be greater than 1.2 $^{0}/_{00}$.

- The stability of CO_2 pulses shall be tested before the analysis of each batch of *Samples*. The linearity of the signal shall be checked regularly, *e.g.* monthly.
- The urinary TC(s) and ERC(s) once hydrolyzed shall be further purified by High Performance Liquid Chromatography (HPLC) (recommended), Solid Phase Extraction (SPE) or other equivalent purification step prior to the GC/C/IRMS analysis.
- A negative[6] (QCN) and a positive (QCP) urine quality control sample containing all relevant TC(s) and ERC(s) shall be included in each batch of *Samples* analyzed and subjected to the same *Sample* preparation procedure (including hydrolysis of glucuronide conjugates and analyte derivatization, if applicable). The relevant TC(s) shall meet the positivity criteria in the positive QC sample. The consistency of the $\delta^{13}C$ determinations of the QCN and QCP shall be monitored through the use of QC-charts.

[5] The requirement to have measurements from a minimum of 20 urine reference samples from females does not apply to T and E determinations. However, a minimum total number of 40 reference determinations for T is required.

[6] This does not apply to GC-C-IRMS determinations for formestane, boldenone or boldenone *Metabolite(s)*.

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- An appropriate RM of the relevant free (unconjugated) steroids, underivatized or acetylated TC(s) and ERC(s), as appropriate, with traceable $\delta^{13}C$ value(s), shall be injected prior to the analysis of each batch of *Samples*. It is recommended that this RM is also injected at the end of each sequence (*e.g.* after 25-30 injections).
- Laboratories shall be capable of performing GC/C/IRMS analyses on A, Etio, T, 5αAdiol and 5βAdiol. When the concentration is sufficient, the TC(s) should be selected/prioritized depending on the *variable*(s) of the "steroid profile" that prompted the GC/C/IRMS analysis.
    - T, 5αAdiol and/or 5βAdiol are the preferred TC(s) to detect the administration of T.
    - Laboratories shall perform GC/C/IRMS analyses on E on those *Samples* in which the concentration of E is abnormally high, *i.e.* if greater than 200 ng/mL in males or greater than 50 ng/mL in females (SG-adjusted [3]).
- The analysis should be based on the use of PD as the principal ERC. However, another ERC, either 16-en, 11-OH-A or 11-oxo-Etio, should also be routinely used and shall replace PD when the signal is suppressed, affected by poor chromatography or by the administration of a precursor, *e.g.* pregnenolone. The same ERC shall be used for the determination of all the $\Delta\delta^{13}C$ values.
- No value obtained from any peaks of intensity below or above the range of linearity or in the presence of significant co-eluting peaks shall be considered or reported.
- The steroids may be analyzed underivatized or after acetylation, but only values equivalent to underivatized compounds shall be used to determine the $\Delta\delta^{13}C$ value of the ERC-TC pair. The following mass balance equation for adjustment of the measured $\delta^{13}C$ values from acetates back to the free form shall be used:

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

where n: number of carbon atoms; s: native steroid (underivatized form); d: derivative group (*e.g.* acetyl), and cd: derivatized compound.

As δC_d is not known, δC_{dcorr} is estimated empirically by consecutive measurements of a non-acetylated and acetylated steroid (*e.g.* 16-en or 5α-androstanol).

2.2 Identification of urinary *Metabolite*s prior to reporting an *Adverse Analytical Finding*

- A GC-MS analysis is required to ensure the identity of the peaks of the relevant TC(s) and ERC and the absence of significant interference prior to reporting an *Adverse Analytical Finding* or an *Atypical Finding* based on GC/C/IRMS results. This is not necessary when the GC/C/IRMS results are negative.
- The same mixtures shall be analyzed by GC-MS^n under similar chromatographic conditions. Minor differences in retention times (RT) between the two techniques are expected. The provisions of the Technical Document on Identification Criteria (TDIDCR) shall be followed [3]. In addition, a full scan spectrum shall be obtained over the complete width of the steroid chromatographic peak(s) of interest to document the lack of interference.

2.3 Interpretation of GC/C/IRMS results

The results of the GC/C/IRMS analyses shall be interpreted as follows:

2.3.1 Positive

When $\Delta\delta^{13}C$ value(s) are consistent with the exogenous origin of the TC(s), *i.e.* if one of the following sets of criteria is fulfilled [7] (**Appendix 1**):

i. The $\Delta\delta^{13}C$ value of the ERC-T pair and of one of the ERC-5αAdiol or ERC-5βAdiol pairs are both greater than 3 $^0/_{00}$;

ii. The $\Delta\delta^{13}C$ values of the ERC-5αAdiol and ERC-5βAdiol pairs are both greater than 3 $^0/_{00}$;

iii. The concentration of E is greater than 50 ng/mL in females or greater than 200 ng/mL in males (SG-adjusted) and the $\Delta\delta^{13}C$ value of the ERC-E pair is greater than 4 $^0/_{00}$;

iv. The $\Delta\delta^{13}C$ value of the ERC-A pair is greater than 3 $^0/_{00}$ or the $\Delta\delta^{13}C$ value of the ERC-Etio pair is greater than 4 $^0/_{00}$;

[7] It is not expected that all TCs will be affected to the same extent. Decisions based on the $\Delta\delta^{13}C$ criteria specified in 2.3.1 i) to vii) and Appendix 1 take into account the Measurement Uncertainty associated with the contributing $\delta^{13}C$ values.

v. The $\Delta\delta^{13}C$ value of the ERC-A pair is between 2 $^{0}/_{00}$ and 3 $^{0}/_{00}$ or the $\Delta\delta^{13}C$ value of the ERC-Etio pair is between 3 $^{0}/_{00}$ and 4 $^{0}/_{00}$, and the $\Delta\delta^{13}C$ value of one of the ERC-5αAdiol or ERC-5βAdiol pairs is greater than 3 $^{0}/_{00}$;

vi. The $\Delta\delta^{13}C$ value of the ERC-5αAdiol pair is greater than 4 $^{0}/_{00}$ and the $\delta^{13}C$ value of 5αAdiol is equal to or less than -27 $^{0}/_{00}$ (*e.g.* DHT administration);

vii. The $\Delta\delta^{13}C$ value of either the ERC-formestane, ERC-boldenone or ERC-boldenone *Metabolite*(s) pairs is greater than 4 $^{0}/_{00}$.

2.3.2 Negative

When $\Delta\delta^{13}C$ values do not confirm the exogenous origin of the TC(s), *i.e.* when the $\Delta\delta^{13}C$ values of the ERC-TC pairs do not meet any of the criteria specified in section 2.3.1 above.

2.3.3 Inconclusive

i) When only one of the combined criteria specified in points i), ii), v) or vi) above is met (*e.g.* the $\Delta\delta^{13}C$ value for the ERC-T pair is greater than 3 $^{0}/_{00}$ but the $\Delta\delta^{13}C$ values for the ERC-Adiol pairs are both less than 3 $^{0}/_{00}$).

ii) Due to technical limitations, *e.g.* when there is insufficient *Sample* volume or very low concentrations of TC(s) or ERC(s), or in the presence of interfering compounds or any other factor preventing a reliable measurement of the relevant diagnostic *Metabolite* or ERC-TC pair.

iii) The Laboratory may interpret the results as inconclusive when the criteria for reporting an *Adverse Analytical Finding* are not met but, in its opinion, are neither consistent with the endogenous origin of the urinary *Metabolites* (*e.g.* ERC $\delta^{13}C$ value at -24.5 $^{0}/_{00}$ and TC at -27.0 $^{0}/_{00}$).

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3.0 Reporting GC/C/IRMS Results

The Laboratory shall report the results of the GC/C/IRMS analyses as follows:

3.1 *Adverse Analytical Finding*

Samples for which the results of the GC/C/IRMS analysis were positive:

- Each *Sample* for which an *Adverse Analytical Finding* is reported shall be reported individually.
- The Test Report shall include:
  - A comment indicating that the GC/C/IRMS finding is consistent with the exogenous administration of steroids specifying the identity of the relevant TC(s) that produced a positive GC/C/IRMS finding.
  - The $\delta^{13}C$ value of the relevant TC(s) (which produced a positive GC/C/IRMS finding) and ERC, and each associated u_c, expressed in $^0/_{00}$.
  - The confirmed values (*e.g.* concentrations, T/E) of the *Marker(s)* of the "steroid profile", and the associated u_c, expressed in units (see example below; refer to TD EAAS [2]) [8].

Reporting example for the Test Report:

GC/C/IRMS results are consistent with the exogenous origin of T and 5βAdiol ($\delta^{13}C$ values: T= -27.5 $^o/_{oo}$, u_c = 0.9 $^o/_{oo}$; 5βAdiol= -25.2 $^o/_{oo}$, u_c = 0.8 $^o/_{oo}$; PD= -20.2 $^o/_{oo}$; u_c = 0.6 $^o/_{oo}$). T = 90 ng/mL, u_c = 12 ng/mL; E = 20 ng/mL, u_c = 3 ng/mL; T/E = 4.5, u_c = 0.5.

Provision of a Second Opinion for GC/C/IRMS

When the results of the GC/C/IRMS analysis indicate an *Adverse Analytical Finding* for a *Sample*, the Laboratory should seek the opinion, in writing, of an expert from a second Laboratory before reporting the *Adverse Analytical Finding*. If obtained, the second opinion shall be recorded in the Laboratory Documentation Package.

[8] When the GC-C-IRMS Confirmation Procedure is applied to formestane, boldenone or boldenone *Metabolite*(s) only, the Laboratory does not need to perform the quantitative confirmation of these substances or report confirmed values of the *Markers* of the "steroid profile".

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3.2 *Atypical Finding*

Samples for which the results of the GC/C/IRMS analysis were inconclusive:

- Each *Sample* for which an *Atypical Finding* is reported shall be reported individually.
- The Test Report shall include:
  - A comment indicating that the GC/C/IRMS finding is inconclusive specifying the identity of each relevant TC that produced an inconclusive GC/C/IRMS finding;
  - The $\delta^{13}C$ value of all TC(s) and ERC analyzed and each associated u_c, expressed in $^0/_{00}$;
  - The confirmed values (*e.g.* concentrations, T/E) of the abnormal *Marker(s)* of the "steroid profile", and the associated u_c, expressed in units [2] (see example below) [8].

Reporting example for the Test Report:

The results of the GC/C/IRMS analyses for T and the Adiols are inconclusive ($\delta^{13}C$ values: A = -25.3 $^o/_{oo}$, u_c = 0.6 $^o/_{oo}$; Etio = -26.2 $^o/_{oo}$, u_c = 0.7 $^o/_{oo}$; T = -27.6 $^o/_{oo}$, u_c = 0.9 $^o/_{oo}$; 5αAdiol= -26.6 $^o/_{oo}$, u_c = 0.8 $^o/_{oo}$; 5βAdiol= -26.2 $^o/_{oo}$, u_c = 0.7 $^o/_{oo}$; PD= -24.5 $^o/_{oo}$; u_c = 0.8 $^o/_{oo}$). T = 110 ng/mL, u_c = 16 ng/mL; E = 10.8 ng/mL, u_c = 1.8 ng/mL; T/E = 10.2, u_c = 0.8.

3.3 No *Prohibited Substance*(s) or *Metabolite*(s) or *Marker*(s) of a *Prohibited Method*(s) on the test menu were detected

Samples for which the results of the GC/C/IRMS analysis were negative:

- Each *Sample* shall be reported individually.
- The Test Report shall include:
  - A comment that the GC/C/IRMS results do not indicate an exogenous administration of steroids;
  - The $\delta^{13}C$ value of all TC(s) and ERC analyzed and each associated u_c, expressed in $^0/_{00}$;
  - The confirmed values (concentrations, T/E) of the abnormal *Marker(s)* of the "steroid profile" that triggered the GC/C/IRMS analysis, and the associated u_c, expressed in units [2] (see example below) [8].

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Reporting example for the Test Report:

GC/C/IRMS results do not confirm the exogenous origin of steroids $\delta^{13}C$ values: A = -24.3 °/₀₀, u_c = 0.7 °/₀₀; Etio = -23.8 °/₀₀, u_c = 0.8 °/₀₀; T = -25.9 °/₀₀, u_c = 0.8 °/₀₀; 5αAdiol= -25.8 °/₀₀, u_c = 0.8; 5βAdiol= -26.0 °/₀₀, u_c = 0.9 °/₀₀; PD= -23.5 °/₀₀; u_c = 0.6 °/₀₀). T = 43.4 ng/mL, u_c = 7.5 ng/mL; E = 7 ng/mL, u_c = 1.0 ng/mL; T/E = 6.2, u_c = 0.5.

4.0 Interpretation

- The GC/C/IRMS and GC-MS^n confirmation methods provide independent and complementary information, but their results must be considered together to arrive at a conclusion that is supported by the scientific literature and knowledge.
- The urinary "steroid profile" may show no major anomaly whilst being excreted following the administration of an EAAS; in such a case, the results of the GC/C/IRMS analysis indicating a synthetic origin of the steroid *Metabolites* shall prevail.
- Conversely, values for *Marker*(s) of the "steroid profile" may be outside the subject-based longitudinal reference range while still being of endogenous origin (*e.g.* heavy ethanol drinking leading to an increased urinary excretion of T and 5βAdiol, microbial formation of free T, or intense, prolonged exercise increasing the excretion of A).
- The "steroid profile" may be altered by the administration of a preparation of a steroid related to testosterone having a relatively enriched $\delta^{13}C$ value, which therefore may not be detected by GC/C/IRMS. In such cases, the provisions of the Technical Document on Results Management Requirements for the *Athlete Biological Passport* (TDRMR) shall be followed [4].

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5.0 References

https://www.wada-ama.org/en/resources/search?f[0]=field_resource_collections%3A30

1. *WADA* Technical Document TD19NA (current version): Harmonization of Analysis and Reporting of 19-Norsteroids related to Nandrolone.

2. *WADA* Technical Document TDEAAS (current version): Endogenous Anabolic Androgenous Steroids: Measurement and Reporting.

3. *WADA* Technical Document TDIDCR (current version): Minimum Criteria for Chromatographic-Mass Spectrometric Confirmation of the Identity of Analytes for Doping Control Purposes.

4. *WADA* Technical Document TDRMR (current version): Results Management Requirements for the *Athlete* Biological Passport. Appendix E to the "Athlete Biological Passport Operating Guidelines".

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Appendix 1. Interpretation criteria for GC/C/IRMS positive test

Positive Criteria Section 2.3	**$\Delta\delta$ ERC-TC**					
	T	**E**[#]	**A**	**Etio**	**5αAdiol, 5βAdiol**	**Formestane, Boldenone, Boldenone *Metabolites***
i.	**> 3 $^{0}/_{00}$**				**> 3 $^{0}/_{00}$** (either Adiol)	
ii.					**> 3 $^{0}/_{00}$** (both Adiols)	
iii.		**> 4 $^{0}/_{00}$**				
iv.			**> 3 $^{0}/_{00}$**			
				> 4 $^{0}/_{00}$		
v.			**2-3 $^{0}/_{00}$**		**> 3 $^{0}/_{00}$ (either Adiol)**	
				3-4 $^{0}/_{00}$	**> 3 $^{0}/_{00}$ (either Adiol)**	
vi.					**$\Delta\delta$(ERC-5α) > 4 $^{0}/_{00}$** and **δ(5α) ≤ -27 $^{0}/_{00}$**	
vii.						**> 4 $^{0}/_{00}$**

[#] Concentration (SG-adjusted [3]) greater than 50 ng/mL in females or greater than 200 ng/mL in males.