# WADA Technical Document – TD2019NA

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## HARMONIZATION OF ANALYSIS AND REPORTING OF 19-NORSTEROIDS RELATED TO NANDROLONE

## 1.0 Introduction

This document has been established to harmonize the Confirmation Procedure for the analysis and reporting of findings for 19-norsteroids related to nandrolone by Laboratories.

The detection of the *Use* of nandrolone (19-nortestosterone) and other 19-norsteroids (*e.g.* 19-norandrostenedione, 19-norandrostenediol) is based primarily upon the identification of the main urinary *Metabolite*, 19-norandrosterone (19-NA). More than one *Metabolite* of administered 19-norsteroids may be detected in urine *Samples* and reported [*e.g.* 19-noretiocholanolone (19-NE)]; however, the identification of 19-NA, including the demonstration, when required, that the 19-NA is not of endogenous origin [1], is sufficient to report an *Adverse Analytical Finding* (*AAF*).

Under specific circumstances, as described below, additional Analytical Testing and reporting may be required.

## 2.0 Initial Testing Procedure

The initial test must detect the presence of 19-NA in urine *Samples* at levels greater than 1 ng/mL and also provide its estimated concentration when lower or equal to 15 ng/mL in order to guide the Confirmation Procedure. The Initial Testing Procedure shall include the following characteristics:

- A single calibration point at 15 ng/mL;
- An appropriate deuterated internal standard;
- The use of a negative and a positive quality control (QC) samples.

## 3.0 Confirmation Procedures

In addition to meeting the identification criteria described in the IDCR Technical Document [1], the Laboratory shall confirm the estimated concentration of 19-NA and/or perform GC/C/IRMS analysis to establish the origin (endogenous [1] or exogenous) of the 19-NA detected.

[1] In the context of this Technical Document, "endogenous" origins of 19-NA include i) trace amounts normally present in males and females; ii) pregnancy; iii) *in-situ* microbial degradation of androsterone (A) to 19-NA; iv) consumption of the offal of intact, non-castrated pigs.

## 3.1 Identification and Estimation of Concentration

The Confirmation Procedure to estimate the concentration of 19-NA in the *Sample* shall include the following characteristics:

- A single calibration point at 15 ng/mL, preferably with the 19-NA concentration based on/traceable to a Certified Reference Material;
- An appropriate deuterated internal standard (*e.g.* 19-NA-$d_4$-glucuronide);
- The use of a negative QC sample (at less than 2.5 ng/mL) and a positive QC sample (at greater than 15 ng/mL).

## 3.2 GC/C/IRMS Analysis

The GC/C/IRMS method to establish the origin of the 19-NA detected shall include the following characteristics (also refer to the TDIRMS [2] for general method characteristics):

- Each sequence of analysis by GC/C/IRMS shall include:
  - a negative QC urine: $\delta^{13}C$ values of 19-NA and endogenous reference compound (ERC) in a normal endogenous range (*i.e.* between -16‰ and -26‰) **[2]**, with an absolute difference in $\delta^{13}C$ values ($\Delta\delta$) between ERC and 19-NA not greater than ($\leq$) 3‰; and
  - a positive QC urine: $\delta^{13}C$ value of ERC in a normal endogenous range (*i.e.* between -16‰ and -26‰), with an absolute $\Delta\delta$ between ERC and 19-NA greater than 3‰.

  These controls shall be subjected to the same sample preparation procedure as the *Sample* Aliquot.
- The GC/C/IRMS analysis shall include the confirmation of the 19-NA peak identity[3].

[2] Range of $\delta^{13}C$ isotopic signatures around the world; the QC samples will reflect the geographical location of the Laboratory and do not have to cover the entire possible range of $\delta^{13}C$ values.

[3] For example, confirmation by GC/MS analysis performed under comparable chromatographic conditions. The purpose is to produce a chromatogram with similar peak profiles so that the spectra can be used to identify the peak(s) of interest. Minor differences in retention time between the two techniques are expected.

GC/C/IRMS analysis shall be performed in the following cases [4]:

- *Samples* in which the concentration of 19-NA is estimated between 2.5 and 15 ng/mL [5], except in cases of pregnancy or in the presence of tetrahydronorethisterone;
- In cases of pregnancy, when the estimated 19-NA concentration is greater than 15 ng/mL [5, 6].

Furthermore, GC/C/IRMS analysis may be performed on *Samples* containing 19-NA at estimated concentrations not greater than 2.5 ng/mL. In such cases, a positive GC/C/IRMS analysis showing the presence of 19-NA of exogenous origin is sufficient evidence to report an *AAF*.

Laboratories that do not have the analytical capacity to perform GC/C/IRMS analysis for 19-NA shall have *Samples* transferred to and analyzed by another Laboratory that has such analytical capacity.

Due to the occurrence of preparations of 19-norsteroids with a carbon isotopic signature ($^{13}C/^{12}C$) close to that of endogenous human urinary steroids (*e.g.* $\delta_{19\text{-NA}}$ =-20 ‰ to -24 ‰), the result of the GC/C/IRMS analysis of the produced 19-NA may not readily indicate its exogenous origin in some populations of *Athletes*. Therefore, in *Samples* from males and non-pregnant females, when the estimated concentration of 19-NA is greater than 2.5 ng/mL and the result of the GC/C/IRMS analysis is negative (*i.e.* not consistent with an exogenous origin of 19-NA) or inconclusive, the Laboratory shall determine the ratio of 19-NA to 19-NE based on

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[4] To reject the hypothesis of endogenous or *in-situ* 19-NA formation the following criteria, based on the application of GC/C/IRMS analysis, shall be met simultaneously:

i- The absolute Δδ value between the endogenous reference compound (ERC) [*e.g.* A or pregnanediol (PD)] and 19-NA, *i.e.* $|\Delta\delta| = |\delta_{ERC} - \delta_{19\text{-NA}}|$ , is greater than 3 ‰, and

ii- The standard combined uncertainty ($u_c$) associated with the determination of $\delta^{13}C$ values, as estimated by the Laboratory during the GC/C/IRMS method validation, is not greater than 1.0 ‰ ($u_{c\_Max}$).

[5] After adjustment for the urine specific gravity (SG), if $SG_{Sample} > 1.018$, according to:

$$\mathbf{Conc_{1.020}} = \frac{(1.020 - 1)}{(SG_{sample\_Max} - 1)} \cdot \mathbf{Conc_{measured}}$$

[Refer to the effective TD DL for instructions on calculating $SG_{Sample\_Max}$].

[6] In cases of pregnancy, when the estimated concentration of 19-NA in a urine *Sample* is between 2.5 and 15 ng/mL, the GC/C/IRMS analysis may also be performed to ascertain the endogenous origin of 19-NA.

the relative signals from the GC/MS analysis [7]. This ratio may serve as a possible indicator of the administration of 19-norsteroids by excluding the *in-situ* formation of 19-NA [3].

## 3.3 Additional Tests

### 3.3.1 Test for Norethisterone and Pregnancy

19-NA is excreted during pregnancy and as a minor *Metabolite* of norethisterone [4], a progestogen agent of permitted use present in some oral contraceptives. Therefore, when the estimated concentration of 19-NA exceeds 2.5 ng/mL in the urine *Sample* of a female *Athlete,* the Laboratory shall perform:

- an analysis for the use of norethisterone-based contraceptives (*e.g.* detection of tetrahydronorethisterone), and if negative
- an analysis for pregnancy [*e.g.* based on the measurement of urinary human Chorionic Gonadotrophin (hCG)].

### 3.3.2 Test for demethylation

In addition, but rarely, 19-NA may be produced in urine *Samples*, in small concentrations, by *in-situ* 19-demethylation of androsterone (A) [5]. The reaction being more efficient with the 5β-isomer (*i.e.* 19-NE), such *Samples* show a lower than usual ratio of 19-NA to 19-NE (*i.e.* $\leq$ 3), which is also less than the ratio of their respective urinary precursors androsterone(A)/etiocholanolone(Etio) [8]. This possible *in-situ* formation of 19-NA can be verified by GC/C/IRMS analysis [3, 6].

## 3.3 "B" *Sample* Confirmation Procedure

- In cases when the *AAF* for the "A" *Sample* is based on the results of a GC/C/IRMS analysis, the "B" *Sample* Confirmation Procedure also requires the GC/C/IRMS analysis (and identification of 19-NA);
- In cases when the estimated concentration of 19-NA is shown to be greater than 15 ng/mL in a *Sample* collected from a male or a non-pregnant female *Athlete*, the "B" *Sample* Confirmation Procedure requires the identification of 19-NA only.

[7] The response of 19-NA and 19-NE is assumed to be sufficiently similar.

[8] In the absence of inhibitors of 5α-reductase (*e.g.* finasteride).

## 4.0 Reporting

The Laboratory shall report 19-NA detected in a *Sample* from a male or a female *Athlete* as defined below:

### A. *Samples* from pregnant female *Athletes*

**No reference to the pregnancy status of an *Athlete* shall be reported in any case.**

- *Adverse Analytical Finding (AAF):*

  ○ *Samples* for which the results of the GC/C/IRMS analysis are consistent with the exogenous origin of 19-NA (see section 3.2 above) [4].

  [The estimated concentration of 19-NA [9] and the results of the GC/C/IRMS analysis [10] shall be included in the Test Report].

- *Atypical Finding* (*ATF*):

  ○ *Samples* for which the estimated 19-NA concentration is greater than (>) 15 ng/mL [5] and the results of the mandatory GC/C/IRMS analysis are inconclusive or consistent with an endogenous origin of 19-NA (see section 3.2 above) [4].

  [The estimated concentration of 19-NA [9] and the results of the GC/C/IRMS analysis [10] shall be included in the Test Report].

- "No *Prohibited Substance* or *Method* on the test menu was detected":

  ○ No other *Prohibited Substance* or *Prohibited Method* has been confirmed in the *Sample*, and

  ○ *Samples* for which the estimated 19-NA concentration is equal to or less than (≤) 15 ng/mL [5] and the GC/C/IRMS analysis was either not performed or the results are inconclusive/consistent with an endogenous origin of 19-NA (see section 3.2 above) [4].

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[9] No strict quantification (and, therefore, no Measurement Uncertainty estimation) is required in the Confirmation Procedure for 19-NA. The application of a one-point calibrator at 15 ng/mL and appropriate QC samples is sufficient to confirm the estimated 19-NA concentration. The result shall be expressed as "≤ 15 ng/mL" or ">15 ng/mL", as applicable.

[10] The Test Report for the GC/C/IRMS analysis shall include a comment indicating whether or not the GC/C/IRMS finding is consistent with an exogenous origin of 19-NA, the $\delta^{13}C$ values for 19-NA and ERC as well as the associated $u_c$, expressed in ‰ units.

## B. *Samples* from female *Athletes* using norethisterone

- *Atypical Finding* (*ATF*):

  ○ *Samples* for which the 19-NA concentration is estimated to be greater than (>) 10 ng/mL [5].

  [The estimated concentration of 19-NA [9] shall be included in the Test Report. In addition, a comment shall be added describing the finding that demonstrates the use of norethisterone [11] (*e.g.* "19-norandrosterone (19-NA) was found in the *Sample* at an estimated concentration greater than (>) 10 ng/mL. Tetrahydronorethisterone, a *Metabolite* of norethisterone, was also detected in the *Sample*)].

- "No *Prohibited Substance* or *Method* on the test menu was detected":

  ○ No other *Prohibited Substance* or *Prohibited Method* has been confirmed in the *Sample*, and

  ○ *Samples* for which the 19-NA concentration is equal to or less than (≤) 10 ng/mL [5].

  [**In this case, no reference to the use of norethisterone shall be included in the Test Report**]

## C. *Samples* from male or female *Athletes* (neither pregnant nor using norethisterone)

- *Adverse Analytical Finding (AAF):*

  ○ *Samples* for which the estimated 19-NA concentration is greater than (>) 15 ng/mL [5].

  [The estimated concentration of 19-NA [9] shall be included in the Test Report. In addition, for female *Athletes*, a comment shall be added explaining that pregnancy and the use of norethisterone were excluded (*e.g. "the 19-NA finding is not consistent with pregnancy or the use of norethisterone"*);

  ○ *Samples* for which the estimated 19-NA concentration is equal to or less than (≤) 15 ng/mL [5] and the results of the GC/C/IRMS analysis are consistent with an exogenous origin of 19-NA (see section 3.2 above) [4].

[11] Or of any other substance that is converted to norethisterone and further metabolized to tetrahydronorethisterone.

[The estimated concentration of 19-NA [9] and the results of the GC/C/IRMS analysis [10] shall be included in the Test Report. In addition, for female *Athletes*, a comment shall be added explaining that the use of norethisterone was excluded (*e.g. "the 19-NA finding is not consistent with the use of norethisterone"*)].

- *Atypical Finding* (*ATF*):

  ○ *Samples* for which the estimated 19-NA concentration is equal to or less than (≤) 15 ng/mL [5] and the results of the GC/C/IRMS analysis are inconclusive or consistent with an endogenous origin of 19-NA (see section 3.2 above) [4], and the ratio of 19-NA to 19-NE is greater (>) than 3.

  [The estimated concentration of 19-NA [9], the results of the GC/C/IRMS analysis [10] and the ratio of 19-NA to 19-NE shall be included in the Test Report. A comment shall be added explaining that the results of the GC/C/IRMS analysis were inconclusive (*e.g.* due to the presence of interfering compound(s) or any other factor preventing a reliable GC/C/IRMS measurement) or consistent with an endogenous origin of 19-NA. In addition, for female *Athletes*, a comment shall be added explaining that pregnancy was excluded (*e.g. "the 19-NA finding is not consistent with pregnancy"*)].

- "No *Prohibited Substance* or *Method* on the test menu was detected":

  ○ *No* other *Prohibited Substance* or *Prohibited Method* has been confirmed in the *Sample*, and

  ○ *Samples* for which the estimated 19-NA concentration is equal to or less than (≤) 2.5 ng/mL [5] (and too low to perform GC/C/IRMS analysis);

  ○ *Samples* for which the estimated 19-NA concentration is greater than (>) 2.5 ng/mL but not exceeding (≤) 15 ng/mL [5], and the ratio of 19-NA to 19-NE is not greater than (≤) 3, and the results of the GC/C/IRMS analysis are consistent with an endogenous origin (*i.e. in-situ* formation) of 19-NA (see section 3.2 above) [4].

## 5.0 References


1. *WADA* Technical Document TDIDCR: Minimum Criteria for Chromatographic-Mass Spectrometric Confirmation of the Identity of Analytes for Doping Control Purposes.

   https://www.wada-ama.org/en/what-we-do/science-medical/laboratories

2. *WADA* Technical Document TDIRMS: Detection of synthetic forms of Endogenous Anabolic Androgenic Steroids by GC/C/IRMS.
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3. Guay C, Goudreault D, Schänzer W, Flenker U, Ayotte C. Excretion of norsteroids' phase II metabolites of different origin in human. *Steroids* 2009; 74: 350-8.

4. Walker CJ, Cowan DA, James VHT, Lau JCY, Kicman AT. Doping in sport—1. Excretion of 19-norandrosterone by healthy women, including those using contraceptives containing norethisterone. *Steroids* 2009; 74: 329-35.

5. Grosse J, Anielski P, Hemmersbach P, Lund H, Mueller RK, Rautenberg C, Thieme D. Formation of 19-norsteroids by *in situ* demethylations of endogenous steroids in stored urine samples. *Steroids* 2005; 70: 499-506.

6. Hebestreit M, Flenker U, Fusshöller G, Geyer H, Güntner U, Mareck U, Piper T, Thevis M, Ayotte C, Schänzer W. Determination of the origin of urinary norandrosterone traces by gas chromatography combustion isotope ratio mass spectrometry. *Analyst* 2006; 131:1021-6.

## Annex A – Flowchart for 19-NA findings