

## ***“Addition of 19-Norandrosterone in a new certified reference material for human urinary steroids”***

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### **Project Overview**

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Detection of the use of the anabolic steroid nandrolone is based primarily upon the identification of the main urinary metabolite, 19-norandrosterone. Laboratories use the sophisticated technique of gas chromatography mass spectrometry to detect 19-norandrosterone in athletes' urine. WADA have recently taken steps to harmonize the analysis of nandrolone, issuing WADA Technical Document TD2016NA in 2016.

Reference materials are a vital tool for validating analytical testing methods and for ongoing laboratory quality assurance. International best practice specifies that CRMs be used as part of routine laboratory quality control. However, CRMs for the measurement of steroid metabolites in human urine are not readily available. The project involves certification of the stable carbon isotope delta value of 19-norandrosterone (19-NA) in CRM MX017, currently being prepared for WADA project 15J04JM. Laboratories will reconstitute this material with water, and then analyse it alongside samples of urine taken from athletes for doping control. The testing laboratory can then compare the measured values for the CRM to those certified, providing assurance of the validity of the testing procedure.

### **Results and Conclusions:**

A new freeze-dried human urine NMIA MX017 certified reference material (CRM) has been prepared by NMIA, under the funding from WADA, to replace the depleted MX005 CRM. Approximately 2400 units had been produced and made available to WADA laboratories since April 2019. This material will assist WADA laboratories to demonstrate comparability of measurements for the Athlete Biological Passport introduced in 2014. The replacement material will continue to support the longitudinal profiling measurements for detection of testosterone abuse and to provide SI -traceable values for the mass fraction and mass concentration of the glucuronides of six steroids specified in the WADA technical document TD2018EAAS: testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (5 $\alpha$ -Adiol) and 5 $\beta$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (5 $\beta$ -Adiol).

In February 2020, the carbon isotope delta value ( $\delta^{13}\text{C}_{\text{VPDB}}$ ) of 19-norandrosterone (19-NA) has been added to the MX017 certificate. The new property value ( $\delta^{13}\text{C}_{\text{VPDB}}$  of 19-NA) is traceable to the VPDB reference via the NMIA MX018 steroid carbon isotopic reference materials normalised by two secondary isotopic reference materials IAEA-CH-6 (sucrose) and IAEA CH 7

(polyethylene). The reference value,  $\delta^{13}\text{C}_{\text{VPDB}} -29.82 \pm 0.41$ , was determined using 36 bottles of MX017 analysed in five batches of experiments. The combined standard uncertainties of the reference value was expanded with a coverage factor of 2.0 to provide a 95% level of confidence.

Analysis was performed on 20 mL of reconstituted urine CRM (MX017) after hydrolysis with  $\beta$ -glucuronidase (*E.coli*). Free steroids were extracted into hexane and purified by two-dimensional HPLC. In-house quality control samples including water spikes, matrix spikes and solvent standards were included to monitor fractionation due to sample transformation procedures. The purified fractions were dried and reconstituted in cyclohexane/2-propanol (4:1) for analysis by GC-C-IRMS. The identical treatment principle was followed to ensure samples and calibration solutions were combusted and transferred the same way into the IRMS. Each sample was injected twice in a randomised order bracketed by calibration standards. Frequent bracketing of the test sample with calibration solutions allowed any drift in the instrument to be fully captured. An approximate  $\delta$ -value of the internal working gas was used to calculate all raw  $\delta$ -values for samples and standards. A multi-point isotopic bracketing calibration approach was adopted to normalise all the measured  $\delta$ -values of samples using steroid isotopic CRM mixtures NMIA MX018-1 and MX018-3. A linear regression line ( $R^2 > 0.99$ ) fitted through the measured and the reference delta values from the eight steroid compounds, in the two calibration mixtures, allowed the measured  $\delta$ -values of 19-norandrosterone in the MX017 to be normalised to  $\delta^{13}\text{C}_{\text{VPDB}}$  the VPDB scale (Figure 1). The multi-point steroid isotopic calibration approach has allowed the property value to be assigned with lower uncertainty than the measurement approach employing calibrated reference gas.

**Figure 1: Calibration of GC-C-IRMS using MX018 steroid mixtures**

