Australian Government
Department of Industry, Science, Energy and Resources

National Measurement Institute

NATA ACCREDITED FOR TECHNICAL COMPETENCE

CERTIFIED REFERENCE MATERIAL
CERTIFICATE OF ANALYSIS

NMIA MX017: Steroid Metabolites in Freeze-Dried Human Urine

Certificate II: Carbon Isotope Delta Values

Certified values

Steroid	CAS No.	$\delta^{13}C_{VPDB}$ / ‰	Coverage Factor (k)	V_{eff}
19-Norandrosterone	1225-01-0	-29.82 ± 0.41	2.0	35
†Etiocholanolone	53-42-9	-23.60 ± 0.51	2.1	23
†Androsterone	53-41-8	-22.27 ± 0.57	2.1	18
†Testosterone	58-22-0	-27.48 ± 0.73	2.1	15
†Epitestosterone	481-30-1	-23.74 ± 0.80	2.1	23
†5α-androstane-3α,17β-diol	1852-53-5	-23.83 ± 0.90	2.3	9
†5β-androstane-3α,17β-diol	1851-23-6	-23.76 ± 0.61	2.1	17
11-oxoetiocholanolone	739-27-5	-22.23 ± 0.48	2.0	37
11β-hydroxyandrosterone	57-61-4	-22.38 ± 0.64	2.1	14
Pregnanediol	80-92-2	-22.79 ± 0.77	2.0	33
16-androstenol	1153-51-1	-22.51 ± 0.60	2.0	32

The measurands are defined as the carbon isotope delta values of the stated free steroids in reconstituted CRM after hydrolysis of steroid glucuronide conjugates. The certified value is reported as relative to the primary reference Vienna Pee Dee Belemnite (VPDB), normalised by consensus $\delta^{13}C_{VPDB}$ values of +1.95 ‰ (NBS 19) and -46.6 ‰ (LSVEC).[a] The uncertainties are expanded to provide a level of confidence of 95%.

Expiry: 1 September 2023 **Batch No.:** 2016.12

Description: This reference material consists of freeze-dried human urine contained in a sealed glass bottle with a crimped rubber septum. In addition to natural levels of steroid metabolites, the material was fortified with 19-norandrosterone glucuronide and testosterone glucuronide. The material must be reconstituted with 20 mL of water as described in this certificate prior to use.

Intended use: The reference material is intended to be used to validate analytical methods and act as quality control or matrix calibration standard for measurement of carbon isotope ratios of the stated steroids in human urine in anti-doping analysis.[1,2]

† These steroids have been certified for mass fraction and concentration and the values can be found in MX017 Certificate I.

Instructions for IRMS analysis: The recommended minimum sample size is 20 mL for carbon isotope delta measurement of 19-norandrosterone and epitestosterone, and 10 mL for all other stated steroids. The volume can be reduced if derivatisation is employed but the minimal volume to obtain optimum instrument response has to be verified by end-user.

Reconstitution protocol

1. Remove the freeze-dried urine bottle from cold storage and equilibrate to room temperature.
2. Remove the aluminium crimp cap and septum, add 20.0 mL purified water.
3. Replace septum and seal the bottle with a crimp cap. Invert gently to dissolve all solid material around the sides of the bottle and the rubber stopper.
4. Heat the bottle at 40 °C for 30 minutes, equilibrate to room temperature before taking subsamples of the reconstituted material.

Storage: Store at -20 °C out of direct light in the closed container as issued. Exposure of the material to elevated temperatures should be avoided.

Metrological traceability: The certified carbon isotope delta values are 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)[3], in accordance with IUPAC recommendation.[4]

Stability: The stability of the material under the recommended storage conditions has been verified and will continue to be monitored. Transport stability was assessed at room temperature (23 ± 3 °C) over a period of a month with no evidence of change in the certified values.

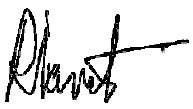
Homogeneity: At least seven bottles were selected using randomized sampling and tested in a single batch under repeatability conditions using the GC-C-IRMS. No significant inhomogeneity was detected.

Safety: CRM NMIA MX017 is intended for in-vitro diagnostic analysis only. Handle product as a biohazardous material potentially capable of transmitting disease.

Production: Preparation of the material was completed in December 2016 using urine collected from healthy volunteers. Specimens from individual donors were screened for potential analytical interferences before use. Selected urine specimens were centrifuged and combined such that the pooled urine contained an approximate epitestosterone concentration of 22 ng/mL. After treatment with sodium azide (0.5 mg/g), the pooled material was filtered through 0.65 µm and 0.2 µm filters. The filtered urine was fortified with testosterone glucuronide to provide a T/E ratio of 4 and with 19-norandrosterone glucuronide to give an approximate concentration of 8 ng/mL. After stirring overnight the bulk material was accurately dispensed in 20 mL aliquots into 50 mL clear glass bottles. The bottled urine was freeze-dried and then stored at -20 °C. The pH of the reconstituted material is 6.9 measured at 20 °C using a calibrated pH meter. The average mass of freeze-dried material in each bottle is 0.60 g.

Analytical method: Analysis was performed on the subsampled reconstituted urine after hydrolysis with β-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 δ-value of the internal working gas was used to calculate all raw δ-values for samples and standards. A multi-point isotopic bracketing calibration approach was adopted to normalise all the measured δ-values of samples using NMIA MX018 CRM steroid isotopic mixtures. All keto-steroids were normalised using MX18-1 and MX018-3 while diols were normalised with MX018-1 and MX018-2. In each case, a linear regression line fitted through the measured and the reference delta values allowed the measured δ-values of each target steroid to be normalised to the VPDB scale.

Measurement uncertainty: Standard uncertainties were estimated and combined as described in the JCGM Guide to the Expression of Uncertainty in Measurement[6]. The major contributions to the combined measurement standard uncertainty were between-unit inhomogeneity, measurement precision and reproducibility, potential instability due to transport conditions and total bias relating to sample transformation, matrix effects and normalisation procedure. The combined standard uncertainties were expanded with coverage factors calculated from degrees of freedom obtained from the Welch-Satterthwaite equation.



Raluca Iavetz
Manager Chemical Reference Values
15 December 2021

Accreditation No.198

The property values specified in this report supersedes any issued prior to 15 December 2021.

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References:

1. WADA *Technical Document – TD2021NA: Harmonisation of analysis and reporting of 19-norsteroids related to nandrolone, Version 2.0*
2. WADA *Technical Document – TD2021IRMS: Detection of synthetic forms of prohibited substances by GC/C/IRMS, Version 1.0*
3. IUPAC *Technical Report: Assessment of international reference materials for isotope-ratio analysis,* Pure Appl Chem, 2014, 86, 425-467
4. IUPAC *Standard atomic weights of 14 chemical elements revised,* Chem Intl, 2018, 40(4), 23-24
5. W. Meier-Augenstein, A. Schimmelmann, *A guide for proper utilisation of stable isotope reference materials,* Isotopes in Environmental and Health, 2019, 55 (2), 113-128
6. JCGM *Evaluation of measurement data – Guide to the expression of uncertainty in measurement*. JCGM100:2008

[a] **IUPAC CIAWW Notice:** In 2017, IUPAC advised that LSVEC is no longer suitable for normalization of the VPDB scale but all carbon isotope delta measurements are to be normalized to VPDB using at least two appropriate international reference materials *(online: https://www.ciaaw.org/carbon-references.htm)*

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