

Cornell University Certified Reference Material

Certificate of Analysis for Reference Material CU/PCC 42-1 (Cholestane) High Volume Steroid Carbon Isotope Ratio Standard Reference Material Batch 1 December 2014

1. Description of Reference Material

The reference material (RM) is comprised of dry cholestane in a 2ml flame sealed glass ampoule for use in calibration of Gas Chromatography Combustion Isotope Ratio Mass Spectrometry (GCC-IRMS) for carbon isotope ratio (CIR) measurements. The steroid material must be reconstituted in an appropriate solvent, such as 2-propanol, before use.

2. Steroid Content and Carbon Isotope Ratio Reference Values

The steroid CIR reference values, along with the CAS number and approximate mass in the ampoule are listed in the Table 1 below.

The CIR values were measured by GCC-IRMS and are reported in δ notation with respect to an international standard Vienna Pee Dee Belemnite (VPDB, $R_{VPDB}=0.011180$) [1], expressed in units of parts per thousand (‰) according to the equation:

$$\delta^{13}C_{VPDB} = (R_{SPL} - R_{VPDB}) / (R_{VPDB}) \times 1000$$

Table 1. Details of Contents per Ampoule for the Steroid and the CIR reference values.

<i>IUPAC Name</i>	<i>CAS #</i>	<i>Mass (mg)</i>	$\delta^{13}C_{VPDB}$ (‰)	\pm s.d. (‰)
5 α -cholestane	481-21-0	19.19	-25.03	0.01

* n=2; The $\delta^{13}C_{VPDB}$ value in Table 1 is the average of 2 data sets, each set containing 4 replicates for each of 8 randomly selected ampoules from the production batch (64 measurements made in total). Values are traceable to VPDB using CH₄ and C₂H₆ in the NIST RM8559 gas [2]. A standard deviation of the measurements is calculated for the final average numbers between 2 data sets. Within set precisions are discussed in section 8. Analytical procedures are described below and in reference [3].

3. Expiration, Storage and Stability of Reference Material

An expiration date has not been determined for the RM; however, the steroids in a dry state should be very stable, do not require refrigeration and are not light sensitive.

Storage of ampoules in a cool, dry, dark place should be sufficient to maintain their integrity. Similar standards containing cholestane (i.e. CU/USADA 33-1) have been robust over many years (>8yrs). Reconstituted RM should be stored at 4°C in a sealed glass container and should be robust over many months or more if properly handled.

4. Intended Use.

The RM is intended for use in calibration of Gas Chromatography Combustion Isotope Ratio Mass Spectrometry (GCC-IRMS) systems for carbon isotope ratio (CIR) measurements of steroids in human urine of interest in anti-doping, and other testing, in order to allow comparability of CIR data obtained by different laboratories and standardize measurement traceability to an international standard (VPDB). It is not for clinical or medical diagnostic use.

5. Instructions for use

This RM was prepared in December 2014 and characterized shortly thereafter. In order to prepare material for analysis, follow the steps below.

- Verify the score region at the neck of the amber glass ampoule is clean by rinsing with solvent. Carefully crack open the ampoule at the score.
- Fill the amber glass ampoule with 2 ml of appropriate solvent (i.e. 2-propanol) and let the steroids dissolve at room temperature over at least three hours.
- A clean glass pipet can be used to remove the solution from the amber glass ampoule into a clean working glass container.
- For this high volume standard, at least two additional solvation steps of adding and removing 2 ml of solvent as described above should be performed for the same ampoule to ensure complete removal of all the steroid material.
- Dilute the steroid solution according to the requirements of use.

6. Production of the Reference Materials

The RM was prepared in the Brenna/Tobias Laboratory at Cornell University, Ithaca NY according to published methods [3]. All the steroids were obtained from Steraloids Inc at >98% purity. Purity was verified by GC, and they were used without further purification. All-glass containers were used to prevent possible contamination due to solvent extraction of polymeric materials associated with plastic containers. Containers were solvent rinsed with 2-propanol and dried before use. Approximately 5g of the steroid was dissolved into a single volume of 500mL 2-propanol master solution to create ampoules with ~20mg steroid. Using a repipet, 2mL aliquots of the master solution were dispensed into 2mL amber glass ampoules. The solvent was evaporated from the ampoules, leaving crystallized standard in the container. The ampoules were then flame-sealed for safe and convenient storage and shipment.

7. Analytical Method used for Characterization of CIR

Eight randomly selected ampoules of the batch were analyzed for their steroid $\delta^{13}\text{C}$ isotope ratio. Two sets of data were acquired on two separate days. The RM was characterized by GCC-IRMS by measuring steroid $\delta^{13}\text{C}$ values that are traceable to the

international standard VPDB. This was performed using the methane and ethane contained in the NIST RM8559 natural gas reference material [2] to calibrate for the apparent isotope ratio of a CO₂ gas source, that in turn was used for CO₂ reference gas pulses during the beginning and end of each steroid analysis run. The procedure followed what is described in more detail in reference [3].

8. Homogeneity

The protocol used to prepare the steroid isotopic standard in ampoule containers maintained good isotopic integrity. The absolute $\delta^{13}\text{C}$ measured for the cholestane was -25.03‰ with an average standard deviation $\text{SD}(\delta^{13}\text{C}) = 0.08\text{‰}$ within ampoules (n=4) and $\text{SD}(\delta^{13}\text{C}) = 0.08\text{‰}$ between ampoules (n=8). The uniformity of steroid isotopic composition among the ampoule batch was confirmed. In addition to GCC-IRMS characterization, a dried aliquot of the master solution of CU/PCC 40-1 was analyzed by EA-IRMS (n=4) resulting in the measurement of a $\delta^{13}\text{C}_{\text{VPDB}}$ of $-25.21 \pm 0.08\text{‰}$, with a difference of -0.17‰ from the ampoule average. This is in good agreement with GCC-IRMS measurements.

9. Metrological Traceability of Reference Values

The carbon isotope ratio reference values are traceable to the international standard VPDB via the methane and ethane in the NIST RM 8559 natural gas reference material [2].

10. Legal Notice

Neither Cornell University, the distributor National Measurement Institute (NMI, as a representative of the commonwealth of Australia), nor any person acting on their behalf assumes any liability with respect to the use of, or damages resulting from the use of this reference material or information contained here.

11. Funding

Funding for the creation of these materials was provided by the Partnership for Clean Competition (PCC, <<http://www.cleancompetition.org/>>).

12. References

- [1] Coplen, T.B.; et. al. "Isotope-Abundance Variations of Selected Elements (IUPAC Technical Report)" *Pure Appl. Chem*, 2002, 74, 1987–2017.
- [2] National Institute of Standards & Technology, Gaithersburg, MD 20899. 2004; <http://www.cstl.nist.gov/div837/837.01/outputs/standards/NGS.pdf>
- [3] Zhang, Y.; Tobias, H.J.; Brenna, J.T. "Steroid Isotopic Standards for Gas Chromatography Isotope Ratio Mass Spectrometry (GCC-IRMS)", *Steroids*, 2009, 74, 369-378.