National Institute of Standards and Technology
U.S. Department of Commerce

Date of Issue:
26 May 2022

Standard Reference Material® 2972a
25-Hydroxyvitamin D Calibration Solutions
CERTIFICATE OF ANALYSIS

Purpose: This Standard Reference Material (SRM) and its associated certified values are intended primarily for use in calibration of instruments and techniques used for the determination of these vitamin D metabolites.

Description: A unit of SRM 2972a consists of twenty, two-milliliter ampoules, of four separate ethanolic solutions of vitamin D metabolites, five ampoules of each solution. Each two-milliliter ampoule contains approximately 1.2 mL of solution. The four separate ethanolic solutions of vitamin D metabolites are described below:

25-Hydroxyvitamin D_3 Calibrant in Ethanol Level 1 [25(OH)D_3 in Ethanol Level 1]
25-Hydroxyvitamin D_3 Calibrant in Ethanol Level 2 [25(OH)D_3 in Ethanol Level 2]
25-Hydroxyvitamin D_2 Calibrant in Ethanol [25(OH)D_2 in Ethanol]
3-Epi-25-hydroxyvitamin D_3 Calibrant in Ethanol [3-epi-25(OH)D_3 in Ethanol]

The 25-Hydroxyvitamin D_3 Calibrant in Ethanol Level 1 and 25-Hydroxyvitamin D_2 Calibrant in Ethanol solutions in SRM 2972a are the same two solutions that were in the former SRM 2972. Development of SRM 2972a was through collaboration between the National Institute of Standards and Technology (NIST) and the National Institutes of Health, Office of Dietary Supplements.

Certified Values: The certified values for 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2, and 3-epi-25-hydroxyvitamin D_3 in Table 1 are based on the analytical results determined using isotope dilution liquid chromatography with mass spectrometric detection (ID-LC-MS), LC with absorbance detection (LC-absorbance), and gravimetric preparation (for 3-epi-25-hydroxyvitamin D_3 only). A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [1]. The measurand is the total amount-of-substance of each analyte, expressed with either mass fraction (ng/g) or concentration units (nmol/L), in Table 1 [2]. Metrological traceability is to the International System of Units (SI) unit of mass.

The certified amount-of-substance mass fraction values and concentration values for vitamin D metabolites are provided in Table 1. The certified amount-of-substance concentration values listed in Table 1 apply only to aliquots removed at 16 °C to 30 °C (see "Storage and Use").

Additional Information: Additional information is provided in the appendices.

Period of Validity: The certified values delivered by **SRM 2972a** are valid within the measurement uncertainty specified until **31 August 2028**. The certified values are nullified if the material is stored or used improperly, damaged, contaminated, or otherwise modified.

Maintenance of Certified Values: NIST will monitor this SRM over the period of its validity. If substantive technical changes occur that affect the certification, NIST will issue an amended certificate through the NIST SRM website (https://www.nist.gov/srm) and notify registered users. SRM users can register online from a link available on the NIST SRM website or fill out the user registration form that is supplied with the SRM. Registration will facilitate notification. Before making use of any of the values delivered by this material, users should verify they have the most recent version of this documentation, available through the NIST SRM website (https://www.nist.gov/srm).

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Table 1. Certified Values for Vitamin D Metabolites in SRM 2972a

Metabolite	Mass Fraction[(a)] (ng/g)	Concentration[(b)] (nmol/L)
25-Hydroxyvitamin D_3 (Level 1)	410.0 ± 14.9	806.2 ± 32.4
25-Hydroxyvitamin D_3 (Level 2)	812.0 ± 29.2	1596.5 ± 64.1
25-Hydroxyvitamin D_2	293.6 ± 9.1	560.4 ± 19.9
3-Epi-25-hydroxyvitamin D_3	293.4 ± 13.5	577.0 ± 28.5

[(a)] The uncertainty provided with each value is an expanded uncertainty about the weighted mean to cover the measurand with approximately 95 % confidence. The expanded uncertainty is calculated as $U = ku_c$, where u_c incorporates the observed difference between the results from the methods and their respective uncertainties, as well as uncertainties related to purity estimation and possible degradation of the solution over time, consistently with the ISO/JCGM Guide and with its Supplement 1, and k is the coverage factor ($k = 2$) corresponding to approximately 95 % confidence [3–7].

[(b)] The amount-of-substance concentrations (nmol/L) were obtained by multiplying the certified values in mass fraction units by the density of ethanol at 22 °C and dividing by the relative molecular masses of 412.65 g/mol for 25-hydroxyvitamin D_2, and 400.64 g/mol for 25-hydroxyvitamin D_3 and 3-epi-25-hydroxyvitamin D_3. These concentrations are for use in the temperature range of 16 °C to 30 °C, and an allowance for the change in density over this temperature range is included in the uncertainties.

Safety: These solutions contain primarily ethanol, which is a flammable solvent. Open flames and sources of spark should be avoided while using this SRM. Use proper methods for disposal of flammable, potentially hazardous waste. Consult the Safety Data Sheet (SDS), enclosed with the SRM shipment, for health and safety information.

Storage: Sealed ampoules, as received, should be stored immediately in the dark at temperatures of –20 °C or below because of analyte instability at higher temperatures [8].

Use: Ampoules should be removed from the freezer and allowed to equilibrate to room temperature before weighing or volumetrically transferring. Due to the instability of the analytes at temperatures greater than –20 °C, the total amount of time at room temperature for equilibrating and processing should be minimized to less than 3 h. Precautions should be taken to avoid exposure of ampoules and test portions to strong UV light and direct sunlight.

Test portions for use should be withdrawn immediately after opening the ampoules and should be processed or diluted without delay for the certified concentration to be valid within the stated uncertainty. Because of the volatility of ethanol, the certified concentration value is NOT applicable to material stored in ampoules that have been opened for more than 2 min, even if they are resealed. The certified concentration values listed in Table 1 apply only to aliquots removed at 16 °C to 30 °C. If possible, samples should be placed in thermostatted compartments at 4 °C or colder during analysis.

Guidance for diluting the SRM 2972a solutions is provided in Appendix A.

Metrological Traceability

Metrological traceability of measurement results to a given reference must be established through an unbroken chain of calibrations and/or comparisons, each having stated uncertainties [9], using measurement standards that are appropriate for the property measured. The certified values in this calibration SRM are traceable to the International System of Units (SI) through such chains. These chains include: confirmation of the chemical identity and determination of the purity of primary standards, fitness evaluation of the solvent used to prepare each SRM solution, calibration of the devices used to determine mass, control of known influence factors such as temperature and ultraviolet (UV) radiation, and evaluation of the homogeneity and stability of each certified property as delivered in the SRM units.

Approaches to establishing the traceability of other measurement results of the certified property include calibration of a measurement process using the SRM as-is or by preparing in-house solutions though dilution of this SRM. The property values of in-house solutions can be made traceable to the SRM's certified value and through it to the SI by properly evaluating the uncertainties involved in procedures used in their preparation. Gravimetric and volumetric methods for preparing such in-house solutions are described in Appendix A Guidance for Diluting SRM 2972a 25-Hydroxyvitamin D Calibration Solutions. The property value uncertainties assigned to the in-house solutions must include the uncertainty of the SRM's certified value appropriately combined with the uncertainties of the preparative process.

The SI traceability of measurement results made using a measurement process calibrated to the SRM directly or to in-house solutions prepared from this SRM can then be established by properly evaluating the uncertainty of the

calibration function resulting from the calibration process. The property value uncertainties assigned to measurement results from the calibrated process must properly combine the calibration function uncertainty with the measurement process imprecision appropriate to the sample analyzed.

Guidance on evaluating and combining uncertainties is provided in reference 9.

Preparation and Analysis

The solutions were prepared gravimetrically at NIST from anhydrous ethanol and primary standards for 25-hydroxyvitamin D_2 and 3-epi-25-hydroxyvitamin D_3 obtained from IsoSciences (King of Prussia, PA) and 25-hydroxyvitamin D_3 obtained from the United States Pharmacopeia (Rockville, MD). The solutions of 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 were mixed overnight (a minimum of 16 h), whereas the solution of 3-epi-25-hydroxyvitamin D_3 was stirred for 3 h after preparation and then stored at 4 °C overnight. The morning following preparation, the solutions were chilled completely with ice and then aliquoted into 2 mL amber glass ampoules that had been purged with argon prior to addition of the solution. The ampoules were then flame-sealed. The masses of the primary standards and the total masses of the solutions were used to calculate the gravimetric concentrations.

NIST Analysis of Vitamin D Metabolites Using ID-LC-MS: Mass fractions of the vitamin D metabolites were measured at NIST using ID-LC-MS. Separate calibrants were prepared gravimetrically for each of the analytes at mass fractions intended to approximate the levels of the vitamin D metabolites in each solution of the SRM. Stable-isotope labeled internal standard solutions were used for each analyte and were added to the calibrants and the SRM 2972a samples. The internal standards consisted of separate solutions containing $^2H_6$-25-hydroxyvitamin D_3, $^2H_3$-25-hydroxyvitamin D_2, and $^2H_3$-3-epi-25-hydroxyvitamin D_3. Test portions ranged from approximately 200 mg to 500 mg, depending on the mass fraction of the metabolite being measured. For all SRM solutions, duplicate test portions from each of 10 ampoules selected using a stratified random sampling scheme were accurately weighed into 2 mL amber autosampler vials. An aliquot of the internal standard solution corresponding to the metabolite being measured was added, followed by mixing and injection. Details of the separation and a typical chromatogram for each of the three metabolites are provided in Figure 1. MS detection with selected ion monitoring was used for quantitation. 25-Hydroxyvitamin D_3 and $^2H_6$-25-hydroxyvitamin D_3 were monitored at *m/z* 383 and *m/z* 389, respectively. 25-Hydroxyvitamin D_2 and $^2H_3$-25-hydroxyvitamin D_2 were monitored at *m/z* 395 and *m/z* 398, respectively. 3-Epi-25-hydroxyvitamin D_3 and $^2H_3$-3-epi-25-hydroxyvitamin D_3 were monitored at *m/z* 383 and *m/z* 386, respectively.

NIST Analysis of Vitamin D Metabolites Using LC-absorbance: Mass fraction values of the vitamin D metabolites were measured at NIST using LC-absorbance at 265 nm. Separate calibrants were prepared gravimetrically for each of the analytes at mass fractions intended to approximate the levels of the vitamin D metabolites in each solution of the SRM. The LC-absorbance measurements were calibrated using an external standard approach and hence no internal standard was used. Aliquots from 10 ampoules, selected using a stratified random sampling scheme, were analyzed with LC-absorbance using both a C_{18} and a pentafluorophenylpropyl (PFP) column. Representative chromatograms and the separation conditions for each of the three metabolites are presented in Figure 2.

Purity Assessment: The concentrations determined by gravimetric preparation, ID-LC-MS, and LC-absorbance were adjusted for the purity estimates of the primary standards, which were determined using LC-absorbance and three different stationary phases, LC-MS, thermogravimetric analysis, Karl Fischer titration, and quantitative nuclear magnetic resonance spectroscopy with an internal standard.

Homogeneity Assessment: The homogeneity of vitamin D metabolites in this SRM was assessed at NIST using the methods and test portion sizes described above, graphical analyses, and analyses of variance at the 5 % significance level. No significant inhomogeneity was observed for any of the vitamin D metabolites.

Value Assignment: The weighted mean of NIST results provided by ID-LC-MS and LC-absorbance were used to calculate assigned values for 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2; the assigned value for 3-epi-25-hydroxyvitamin D_3 is the weighted mean of the NIST results from gravimetric preparation, ID-LC-MS, and LC-absorbance.

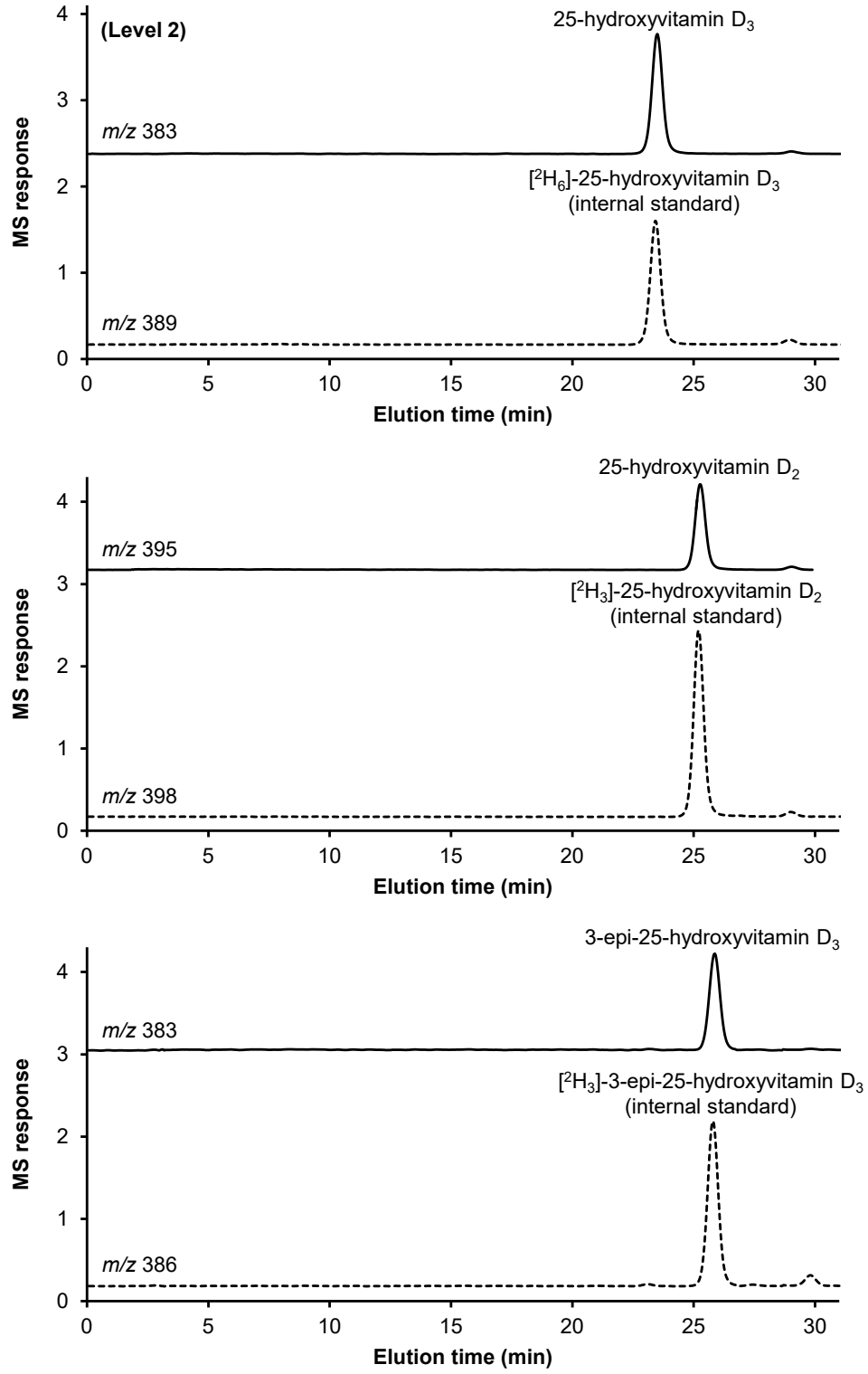

Figure 1. ID-LC-MS chromatograms of SRM 2972a 25-Hydroxyvitamin D Calibration Solutions. A pentafluorophenylpropyl column with dimensions of 150 mm × 4.6 mm ID and containing 2.7 µm diameter particles and an isocratic mobile phase of 78 % methanol, 22 % water were used with a column temperature of 15 °C. MS detection was achieved using selected ion monitoring at the indicated ions. The chromatograms for Level 1 of the 25-hydroxyvitamin D_3 solutions are similar to those shown for Level 2.

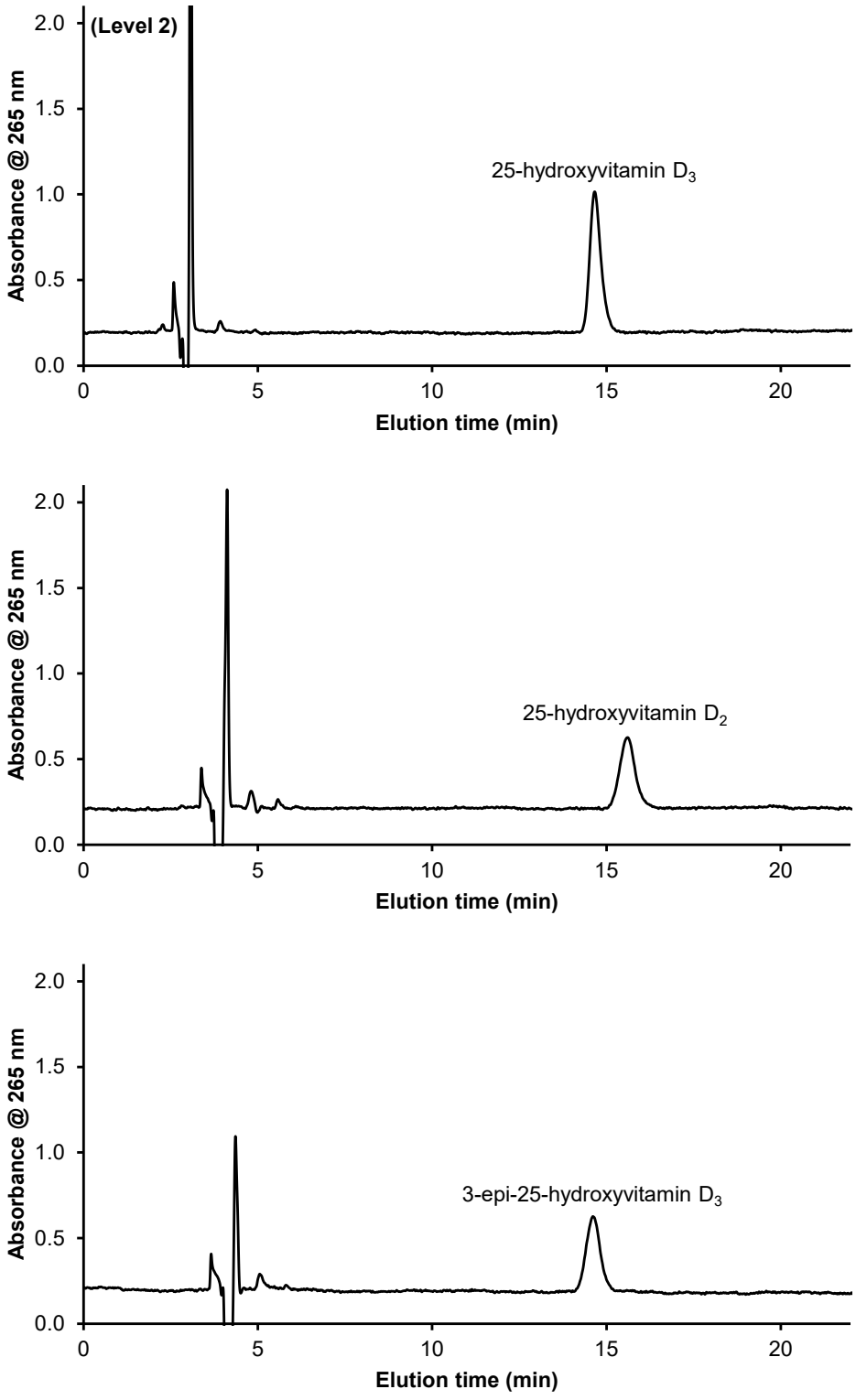

Figure 2. LC-Absorbance chromatograms of SRM 2972a 25-Hydroxyvitamin D Calibration Solutions. A C_{18} column with dimensions of 250 mm × 4.6 mm ID and containing 5 µm particles and an isocratic mobile phase of 86 % methanol, 14 % water were used with a column temperature of 45 °C. Absorbance detection was achieved using 265 nm. The chromatograms for Level 1 of the 25-hydroxyvitamin D_3 solutions are similar to those shown for Level 2.

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Certificate Revision History: 26 May 2022 (Change of period of validity; updated format; editorial changes); 14 February 2017 (Change of expiration date; editorial changes); 21 November 2014 (Original certificate date)

Certain commercial equipment, instruments, or materials may be identified in this Certificate of Analysis to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the Office of Reference Materials 100 Bureau Drive, Stop 2300, Gaithersburg, MD 20899-2300; telephone (301) 975-2200; e-mail srminfo@nist.gov; or the Internet at https://www.nist.gov/srm.

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APPENDIX A

Guidance for Diluting SRM 2972a 25-Hydroxyvitamin D Calibration Solutions

The ethanolic calibration solutions of SRM 2972a for 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2, and 3-epi-25-hydroxyvitamin D_3 are of higher mass fraction and concentration than is typically encountered in human serum/plasma samples. Therefore, dilution of the calibration solutions may be required for analysis by many of the common vitamin D metabolite assays. Recommendations for dilution of the calibration solutions are as follows:

1) The solutions should be allowed to reach room temperature and be thoroughly mixed prior to opening the ampoules for dilution. However, due to the instability of the vitamin D metabolites at room temperature, care should be taken to minimize the total time at room temperature for equilibrating, diluting and analyzing to less than 3 h.

2) The most accurate results for dilution will be obtained using gravimetry with a calibrated analytical balance. Both the masses of the SRM 2972a solution and the total amount of solution after dilution are required to calculate the new mass fraction or concentration.

3) For assays that utilize volumetric measurements, use of either a gas-tight syringe or a positive displacement pipette (PDP) is recommended for solution transfer. If using a PDP, ensure all solution is delivered from the capillary (touching the tip of the capillary to the wall of the container may be required to fully deliver the correct volume). The best results for a volume dilution will be obtained if a volumetric flask is used to achieve the desired total volume.

4) If a positive displacement pipette is not available, an air-displacement pipette can be used. However, the errors in the amount of the ethanolic solution dispensed and the mass fraction or concentration of the diluted solution will be greater. Also, in both (3) and (4), use of pipettes that are out of calibration will result in additional error.

5) The choice of diluent does not matter, as long as the ethanol/analytes are soluble. Dilution with organic solvents such as alcohols or acetonitrile is preferable, but water can be used as the diluent to minimize solvent losses due to evaporation. All diluted SRM 2972a solutions should be stored in the dark in a sealed container (e.g., amber threaded bottle with a lined screw cap) to minimize mass fraction or concentration changes that could occur from evaporative losses. Solutions diluted with organic solvents should be stored at –20 °C until ready for analysis (up to 2 months) to minimize metabolite degradation. The viability of solutions that have been diluted with water, stored at –20 °C, and then equilibrated to room temperature for analysis has not been investigated at NIST. Therefore, it is recommended that samples diluted with water be analyzed without delay and discarded after 3 h.

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APPENDIX B

Coordination of the technical measurements leading to the certification of this SRM were performed by M. Bedner and L.C. Sander of the NIST Chemical Sciences Division and K.W. Phinney of the NIST Biomolecular Measurement Division. Analytical measurements at NIST were performed by M. Bedner, K.A. Lippa, and M.A. Nelson of the NIST Chemical Sciences Division and B.E. Lang of the Biosystems and Biomaterials Division.

Statistical analysis was provided by J.H. Yen of the NIST Statistical Engineering Division.

Support aspects involved in the issuance of this SRM were coordinated through the NIST Office of Reference Materials.

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