NIST Special Publication 260-210

Certification of Standard Reference Material®s 2969 and 2970:

Vitamin D Metabolites in Frozen Human Serum (Total 25-Hydroxyvitamin D Low Level) and (25-Hydroxyvitamin D_2 High Level)

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Abstract

Standard Reference Materials (SRMs) 2969 and 2970 are intended for 1) use in validating methods for determining concentrations of 25-hydroxyvitamin D_2 ($25(OH)D_2$) and 25-hydroxyvitamin D_3 ($25(OH)D_3$) in human serum and plasma and 2) value assigning in-house produced control materials analyzed using those methods. A unit of SRM 2969 consists of two vials of frozen serum with a low total 25-hydroxyvitamin D (25(OH)D, the sum of $25(OH)D_2$ plus $25(OH)D_3$) concentrations. A unit of SRM 2970 consists of two vials of frozen serum with a relatively high $25(OH)D_2$ concentration. This publication documents the production, analytical methods, and statistical evaluations involved in characterization of these products.

Keywords

25-hydroxyvitamin D_2; 25-hydroxyvitamin D_3; 25(OH)D; $25(OH)D_2$; $25(OH)D_3$; human serum; Standard Reference Material (SRM); total 25-hydroxyvitamin D.

Technical Information Contact for this SRM

Please address technical questions you may have about this SRM to srms@nist.gov where they will be assigned to the appropriate Technical Project Leader responsible for support of this material. For sales and customer service inquiries, please contact srminfo@nist.gov.

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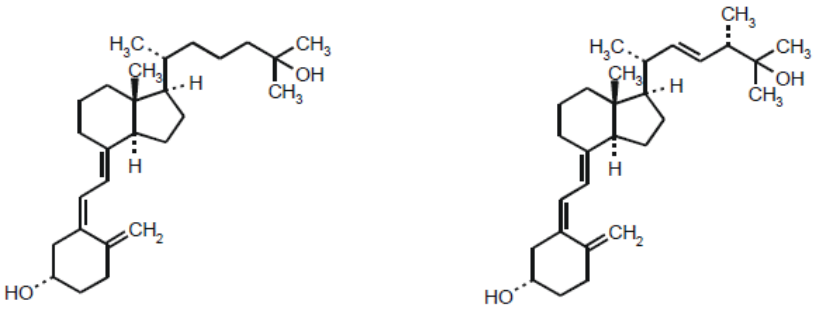
Glossary

[25(OH)D_2]	concentration of 25(OH)D_2
[25(OH)D_3]	concentration of 25(OH)D_3
^{1}H-^{13}C HSQC	^{1}H-^{13}C heteronuclear single quantum correlation NMR
^{1}H-qNMR	^{1}H quantitative NMR
^{1}H-qNMR$_{IS}$	^{1}H-qNMR using an internal standard
24R,25(OH)$_2$$D_3$	24R,25-dihydroxyvitamin D_3
24R,25(OH)$_2$$D_3$-$d_6$	isotopically labelled 24R,25(OH)$_2$$D_3$
25(OH)D	total 25-hydroxyvitamin D, the sum of 25(OH)D_2 and 25(OH)D_3
25(OH)D_2	25-hydroxyvitamin D_2
25(OH)D_2-d_3	isotopically labelled 25(OH)D_2
25(OH)D_3	25-hydroxyvitamin D_3
25(OH)D_3-d_6	isotopically labelled 25(OH)D_3
CLSI	Clinical and Laboratory Standards Institute
ID-LC-MS/MS	isotope-dilution LC-MS/MS
HPLC	High-performance liquid chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
NIST	National Institute of Standards and Technology
NMR	nuclear magnetic resonance spectroscopy
PVDF	polyvinylidene difluoride
SRM®	Standard Reference Material®
TPOC	Technical point of contact

1. Introduction

Vitamin D is a group of fat-soluble steroid-related analytes with many biological activities critical to human health. For humans the most important compounds in this group are vitamin D_3 and vitamin D_2. These compounds are converted in the liver to 25-hydroxyvitamin D_3 ($25(OH)D_3$) and 25-hydroxyvitamin D_2 ($25(OH)D_2$). A second hydroxylation occurs in the kidneys to produce 1,25-dihydroxyvitamin D, the physiologically active form, which participates in many biological processes including bone growth and reduction of inflammation. Serum concentrations of $25(OH)D_3$ and $25(OH)D_2$ are currently the main indicators of vitamin D status due to their higher concentration and longer half-life compared to 1,25-dihydroxyvitamin D [1]. **Figure 1** displays the chemical structures of these vitamin D metabolites.



25-hydroxyvitamin D_3
$25(OH)D_3$, 400.64 g/mol

25-hydroxyvitamin D_2
$25(OH)D_2$, 412.66 g/mol

Figure 1. Chemical Structures of $25(OH)D_3$ and $25(OH)D_2$

The National Institute of Standards and Technology (NIST) in collaboration with the National Institutes of Health's Office of Dietary Supplements has developed several Standard Reference Materials (SRMs) to support the accurate measurement of these metabolites in human serum. The four-component SRM 972 Vitamin D in Human Serum was released for sale in 2009 and was sold out in 2012. Its replacement, the four-component SRM 972a Vitamin D Metabolites in Frozen Human Serum, was released in 2014. A specialty single-component material, SRM 2973 Vitamin D Metabolites in Frozen Human Serum (High Level), was released in 2016. **Figure 2** displays the sales history of these materials.

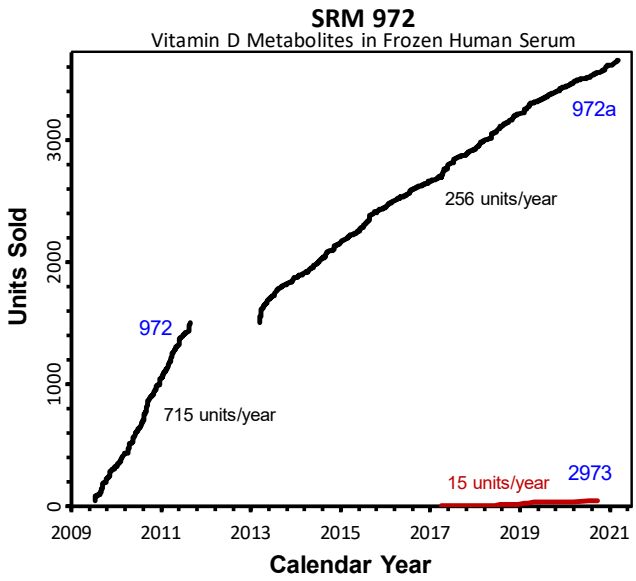


Figure 2. Sales History of the Vitamin D Metabolites in Human Serum SRMs

The total 25-hydroxyvitamin D [25(OH)D, the sum of 25(OH)D_3 and 25(OH)D_2] levels currently available in SRM 972a and SRM 2973 do not encompass the 30 nmol/L (≈ 12 ng/g) level associated with the vitamin D deficient cut-off nor the > 50 nmol/L (≈ 21 ng/g) high 25(OH)D_2 range. These levels were covered by components of the sold-out SRM 972. However, these levels were initially achieved in the original material through dilution with horse serum or spiking with exogenous compounds. These manipulations resulted in commutability issues with several assays.

In order to provide the desired commutable standards, NIST procured pooled human serum materials that contain endogenous vitamin D metabolites at or near the desired levels. These sera have been used to produce two new vitamin D metabolite standards, SRM 2969 Vitamin D Metabolites in Frozen Human Serum (Total 25-Hydroxyvitamin D Low Level) and SRM 2970 Vitamin D Metabolites in Frozen Human Serum (25-Hydroxyvitamin D_2 High Level). **Figure 3** displays the 25(OH)D_3 and 25(OH)D_2 compositions of the past, current, and just-developed vitamin D metabolite SRMs.

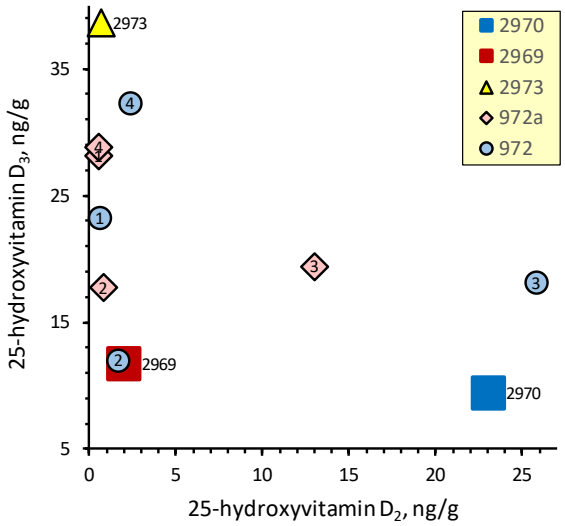


Figure 3. Composition of Vitamin D Metabolite SRM Components

This document describes the production, measurements, and data analysis used to provide the nutritional and clinical communities with the new SRMs 2969 and 2970.

2. Production

An open solicitation for quotations was issued for the preparation of 2,000 vials each of SRM 2969 Vitamin D Metabolites in Frozen Human Serum (Total 25-Hydroxyvitamin D Low Level) and SRM 2970 Vitamin D Metabolites in Frozen Human Serum (25-Hydroxyvitamin D_2 High Level). The following Sections describe the Statement of Work put out to bid.

2.1. Statement of Work

2.1.1. Specifications

The successful contractor shall produce 2,000 vials each of two serum materials at the concentrations specified below, with each vial containing 1.0 mL of serum. The contractor shall acquire a minimum of 2,000 mL of serum pooled at each level from donors using the Clinical and Laboratory Standards Institute (CLSI) C37-A protocol for a total of approximately 4 L of serum. All vitamin D levels must be native and not achieved through dilution or fortification of any of the serum samples. The vitamin D metabolite target value ranges for the materials should be as follows:

- SRM 2969: Pooled human serum containing 25(OH)D_2 > 2 nmol/L and 25(OH)D_3 > 2 nmol/L, with total 25(OH)D = 25(OH)D_2 + 25(OH)D_3 = (25 to 30) nmol/L
- SRM 2970: Pooled human serum containing 25(OH)D_2 > 50 nmol/L; any level of 25(OH)D_3

Each unit of single-donor serum shall be analyzed to ascertain the 25(OH)D_2, 25(OH)D_3, and total 25(OH)D concentrations, using a method that can distinguish between 25(OH)D_2 and 25(OH)D_3, to determine serum donor units suitable to fulfill the specified range. Acceptable methods for these analyses include liquid chromatography with UV/visible absorbance

detection, liquid chromatography-mass spectrometry, or liquid chromatography-tandem mass spectrometry. The contractor is responsible for this analysis, but the analysis can be subcontracted out. The results of these analyses must be submitted to NIST. Measured values for $25(OH)D_2$ and $25(OH)D_3$ shall be reported in units of nmol/L.

The contractor shall test the serum units for biosafety. All sera shall be demonstrated to be nonreactive when tested for hepatitis B surface antigen, hepatitis C virus, human immunodeficiency virus, and human immunodeficiency virus antigen 1 by tests licensed by the U.S. Food and Drug Administration. The contractor will provide written documentation stating the negative results of all donor units utilized for SRM 2969 and SRM 2970 preparation. Donor units testing positive for any of the stated infectious agents will not be included in the serum pools.

After the donor units are selected, they shall be pooled. The contractor shall thoroughly blend and filter the pools (0.22 μm polyvinylidene difluoride (PVDF)). The contractor shall dispense the serum into labeled amber glass serum vials capable of withstanding ultracold temperatures (-80 °C), each containing nominally 1 mL of serum (1.1 mL dispensed with an accuracy better than 0.1 mL), and seal under nitrogen with a butyl rubber stopper and an aluminum crimp cap. The vials must be 3 mL amber serum vials, 17 × 37 mm, 13 mm crimp finish. The contractor shall source vials from Voigt Global Distribution[1] (catalogue #62413U-3). The contractor must stress test the lot of serum vials prior to filling by picking 10 vials randomly from the lot and subjecting them to 5 freeze and thaw cycles at -80 °C to ensure that there is no breakage.

Prior to filling, the contractor shall label vials with labels that are appropriate for use at low temperature; these labels will be provided to the contractor by NIST within 60 days after award. The contractor shall use two different color aluminum crimp caps to differentiate between the two specified serums for easy identification. Vials shall be transferred, in fill order, from the bottling equipment to a box in a "Z" pattern, filling each row left to right. The location of the first vial in each box shall be noted on each side of the outside corner of the box, and boxes will be numbered sequentially. Boxes shall be labeled to indicate their contents. Materials shall be stored frozen (-80 °C) prior to overnight shipment on dry ice to NIST. Overnight shipments should not be sent on Friday/Saturday or before a Monday Federal Holiday. Delivery is expected within 365 days after award.

The Contractor shall provide NIST with details of the steps involved in material preparation not later than 365 days after award of this contract to ensure documentation of any deviations as well as to provide information that shall be utilized in the NIST material acquisition Report of Analysis.

2.1.2. Protection of Human Subjects

(a) Research involving human subjects is not permitted under this award unless expressly authorized in writing by the Contracting Officer. Such authorization will specify the

[1] Certain commercial entities, equipment, or materials may be identified in this document in order to describe an experimental procedure or concept adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the entities, materials, or equipment are necessarily the best available for the purpose.

details of the approved research involving human subjects and will be incorporated by reference into this contract.

(b) The Federal Policy for the Protection of Human Subjects (the "Common Rule"), adopted by the Department of Commerce at 15 CFR part 27, requires contractors to maintain appropriate policies and procedures for the protection of human subjects in research. The Common Rule defines a "human subject" as a living individual about whom an investigator conducting research obtains data through intervention or interaction with the individual, or identifiable private information. The term "research" means a systematic investigation, including research development, testing and evaluation, designed to develop or contribute to generalizable knowledge. The Common Rule also sets forth categories of research that may be considered exempt from 15 CFR part 27. These categories may be found at 15 CFR 27.101(b).

(c) In the event that human subjects research involves pregnant women, prisoners, or children, the contractor is also required to follow the guidelines set forth at 45 CFR part 46 subpart B, C and D, as appropriate, for the protection of members of a protected class.

(d) Should research involving human subjects be included in the proposal, prior to issuance of an award, the contractor shall submit the following documentation to the Contracting Officer:

(1) Documentation to verify that contractor has established a relationship with an appropriate Institutional Review Board ("cognizant IRB"). An appropriate IRB is one that is located within the United States and within the community in which the human subjects research will be conducted;

(2) Documentation to verify that the cognizant IRB possesses a valid registration with the United States Department of Health and Human Services' Office for Human Research Protections ("OHRP");

(3) Documentation to verify that contractor has a valid Federal-Wide Assurance (FWA) issued by OHRP.

(e) Prior to starting any research involving human subjects, the contractor shall submit appropriate documentation to the Contracting Officer for institutional review and approval. This documentation may include:

(1) Copies of the human subjects research protocol, all questionnaires, surveys, advertisements, and informed consent forms approved by the cognizant IRB;

(2) Documentation of approval for the human subjects research protocol, questionnaires, surveys, advertisements, and informed consent forms by the cognizant IRB;

(3) Documentation of continuing IRB approval by the cognizant IRB at appropriate intervals as designated by the IRB, but not less than annually; and/or

(4) Documentation to support an exemption for the project from the Common Rule [Note: this option is not available for activities that fall under 45 CFR part 46 subpart C].

(f) In addition, if the contractor modifies a human subjects research protocol, questionnaire, survey, advertisement, or informed consent form approved by the cognizant IRB, the contractor shall submit a copy of all modified material along with documentation of approval for said modification by the cognizant IRB to the Contracting Officer for institutional review and approval. The contractor shall not implement any IRB approved-modification without written approval by the Contracting Officer.

(g) No work involving human subjects may be undertaken, conducted, or costs incurred and/or charged to the project, until the Contracting Officer approves the required appropriate documentation in writing.

2.1.3. Acceptable Quality Level

The vials of all pooled serum with the associated physical and chemical properties specified in section III of this Statement of Work shall be suitable for use as reference materials. If deficiencies or inconsistencies between the material and the documentation (defined in sections II and III) are found, or if less than the stated number of vials are received intact, the contractor has 30 days to correct the deficiency at no additional cost to the Government.

2.1.4. Monitoring Method

The NIST TPOC will verify that the materials were successfully prepared and acceptable. Acceptance will be based upon the delivery of a 2,000 vials of each pooled serum material intact and unbroken, accompanied by screening results for 25(OH)D_2 and 25(OH)D_3. This will be completed no later than 30 days after receipt and acceptance of the vials.

2.2. Acceptance

The vitamin D metabolite screening results are close to or within the specifications provided in the solicitation.

Solomon Park screening results indicate likely successful preparation of SRM 2969 and SRM 2970. Both materials will undergo additional analysis for vitamin D metabolites by ID-LC-MS/MS Reference Measurement Procedures at NIST for certified value assignment.

For SRM 2969, Solomon Park reported a total 25(OH)D screening value of 41.1 nmol/L, which is higher than the specified a total 25(OH)D of 25 nmol/L to 30 nmol/L. However, a total 25(OH)D of ≈40 nmol/L is still significantly lower than the lowest level of the current SRM 972a (47.1 nmol/L). For SRM 2970, Solomon Park reported a 25(OH)D_2 screening value of 61.9 nmol/L, which is well above the specified > 50 nmol/L specification.

3. Screening Values for 24R,25-Dihydroxyvitamin D_3

This section reports preliminary isotope-dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) measurements of 24R,25-dihydroxyvitamin D_3 in SRM 2969 and SRM 2970. Mass fractions of 24R,25(OH)$_2D_3$ were determined using the NIST

Reference Measurement Procedure (RMP) [2] ID-LC-MS/MS in positive atmospheric pressure chemical ionization mode.

3.1. Materials

A custom synthesis of 24R,25(OH)$_2$D$_3$ was acquired in two batches from IsoSciences with purity assessments of (94.1 ± 0.8) % and (93.2 ± 0.8) %. These purity values were used to correct the ID-LC-MS/MS measurements provided in this report. Isotopically labeled compound 24R,25(OH)$_2$D$_3$-*d*$_6$ was obtained from IsoSciences. No purity assessments were performed for this compound. High-performance liquid chromatography (HPLC) grade methanol and water were obtained from JT Baker.

3.2. Standard Preparation

Approximately 2 mL of a 20 ng/µL isotopically labeled 24R,25(OH)$_2$D$_3$-*d*$_6$ stock solution was diluted with 98 mL of anhydrous ethanol to gravimetrically prepare a 507 ng/g 24R,25(OH)$_2$D$_3$-*d*$_6$ stock solution. 19.5 ng/g 24R,25(OH)$_2$D$_3$-*d*$_6$ working solution was gravimetrically prepared by diluting 2 mL of the 507 ng/g stock solution with 50 mL of anhydrous ethanol.

Three standard stock solutions for 24R,25(OH)$_2$D$_3$ were gravimetrically prepared for ID-LC-MS/MS analysis. The 24R,25(OH)$_2$D$_3$ reference materials were removed from -20 °C storage and allowed to equilibrate to room temperature before accurately weighing approximately 1 mg of compound in an aluminum foil cup. The cup was placed into a 100 mL volumetric flask, the flask was stoppered and tared, then approximately 100 mL of anhydrous ethanol was added to the flask and weighed. The first batch of 24R,25(OH)$_2$D$_3$ was used to prepare stocks 1 and 2; the second batch was used for stock 3. The mass fractions of 24R,25(OH)$_2$D$_3$ in the three stock solutions ranged from 12 µg/g to 13 µg/g.

A working solution was gravimetrically prepared from each stock solution by diluting approximately 1 mL of the stock solution with approximately 150 mL of anhydrous ethanol. Mass fractions of 24R,25(OH)$_2$D$_3$ in each working solution ranged from 86 ng/g to 88 ng/g. Standard stock and working solutions were stored in opaque 20 mL to 50 mL tubes in a -20 °C freezer when not in use.

Eight calibrants were gravimetrically prepared from 24R,25(OH)$_2$D$_3$ working solutions to produce mass ratios ranging from 0.3 to 2.0 of unlabeled to labeled compound. Two to three aliquots (35 µL to 244 µL) from each 24R,25(OH)$_2$D$_3$ working solution were spiked with 500 µL of the 19.5 ng/g 24R,25(OH)$_2$D$_3$-*d*$_6$ working solution. All labeled and unlabeled solutions were removed from -20 °C storage and allowed to equilibrate to room temperature prior to weighing. The mixtures were dried under nitrogen at approximately 45 °C, reconstituted with 150 µL of methanol, and transferred to autosampler vials for ID-LC-MS/MS analysis. Calibration solutions were prepared the same day as sample preparation.

3.3. Sample Preparation

Sample vials (four vials total of SRM 972a Level 1; six vials total each of SRM 2969 and SRM 2970) were removed from -80 °C freezer and allowed to equilibrate to room temperature for 1 h prior to weighing. Each sample (approximately 2 g from combined

contents of 2 vials) was accurately weighed into a 50-mL glass centrifuge tube. Each sample was spiked gravimetrically with aliquots (90 µL to 338 µL) of 24R,25(OH)$_2$D$_3$-d_6 working solution to get an approximately 1:1 mass ratio of analyte to internal standard. After equilibration at room temperature for 1 h, the pH of each sample was adjusted to pH (9.8 ± 0.2) with 0.1 g/mL pH 9.8 carbonate buffer (approximately 200 µL buffer per mL of liquid). 24R,25(OH)$_2$D$_3$ was extracted from the serum matrix with 8 mL of hexane-ethyl acetate (50:50, volume fraction). Each sample was shaken vigorously for 10 min using a mechanical shaker to allow complete mixing. The upper hexane-ethyl acetate layer was transferred to another 50-mL centrifuge tube. Hexane-ethyl acetate extraction was repeated once more with another 8 mL of solvent by shaking for 3 min. The combined extract was dried under nitrogen at 45 °C, and the residue was reconstituted with 100 µL of methanol for ID-LC-MS/MS analysis.

All samples and calibrants were stored at -20 °C prior to ID-LC-MS/MS analysis, and remained at 5 °C in a temperature-controlled autosampler once analysis was initiated. After analysis, all calibrants and samples were removed from the autosampler and placed back at -20 °C for storage.

3.4. Instrumental method

Liquid chromatographic separation was achieved using an Ascentis Express C18 column (15 cm × 4.6 mm, 2.7 µm particle diameter) at a flow rate of 0.75 mL/min and 35 ºC column temperature control. An isocratic method of 30:70 (volume fraction) water: methanol was applied for 40 min, followed by a 15 min column rinse with 100 % methanol, and 12 min re-equilibration to initial conditions. The injection volume was 10 µL. The autosampler tray temperature was set at 5 ºC. The analyses were performed on an Applied Biosystems API 5000 LC-MS/MS system equipped with an Agilent 1260 Series LC system. Positive atmospheric pressure chemical ionization in positive ion mode and multiple reaction monitoring (MRM) mode was used for LC-MS/MS. Transitions at *m/z* 417 → *m/z* 381 and *m/z* 423 → *m/z* 387 were monitored for 24R,25(OH)$_2$D$_3$ and 24R,25(OH)$_2$D$_3$-d_6, respectively. The curtain gas and collision gas were nitrogen at settings of 90 kPa (13 psi) and 34 kPa (5 psi). The ion source gas 1 was air at a setting of 621 kPa (90 psi). The needle current was set at 4 µA and the temperature was maintained at 325 ºC. The declustering potential, entrance potential, collision energy, and collision exit potential were set at 80 V, 10 V, 177 V, and 27 V, respectively.

3.5. Quantitation

Eight calibrants were analyzed along with the samples. First each of the calibrants was analyzed followed by a single analysis of the first preparation of each sample followed by the second preparation of each sample. The entire series was analyzed again in reverse order. Blank injections of methanol were included at the beginning and end of the sequence, and at the start of the analysis in reverse order. By combining the data of calibrants run before and after the samples, a linear regression was calculated using the $y = mx + b$ model that converts the measured intensity ratios of analyte to mass ratios. The mass ratios were then used along with the amounts of the internal standard added to calculate analyte concentrations.

3.6. Results

The overall results of the 24R,25(OH)$_2$D$_3$ ID-LC-MS/MS measurements in all SRMs are shown in **Table 1**. Calibration solutions prepared from the 24R,25(OH)$_2$D$_3$ reference compound produced a consistent mean response factor of (1.17 ± 0.005) and was fit for use in the preparation of calibration solutions. The correlation coefficient of the 8-point linear regression line was 0.9993. Measured values have been adjusted for purity of the calibrants.

Table 1. Summary of 24R,25(OH)$_2$D$_3$ Measurement Results

SRM	Number of Measurements	(Mean ± SD) ng/g	CV, %
972a Level 1	4	2.5 ± 0.10	0.92
2969	6	0.56 ± 0.01	1.49
2970	6	0.71 ± 0.01	0.82

Measurements for 24R,25(OH)$_2$D$_3$ in the 972a Level 1 material were within the certified 95 % confidence range of (2.60 ± 0.10) ng/g with a coefficient of variation (CV) of less than 1 %. The mean screening values can be used as target values for future value assignment measurements.

4. Serum Density

Density values are needed to express mass concentrations of 25(OH)D in SRMs 2969 and 2970 with appropriate mass/volume units. Density values were determined by the Lang-Levy pipet method using approximately 500 µL of sample volume [3].

4.1. Materials

HPLC-grade water was used for calibration and pipet rinsing and ethanol (200 proof) was used for pipet rinsing.

4.2. Sample Preparation

A 500 µL Lang-Levy pipet was calibrated with water at ambient room temperature of 22.8 °C. The dry pipet was wiped with a lint-free cloth and weighed on a metal stand on a semi-micro balance having a readability of 0.01 mg and a repeatability precision of 0.015 mg. The mass of the empty pipet was tared, the pipet was filled to the mark with water, wiped with a lint-free cloth, and weighed. The pipet was then rinsed by attaching it to a vacuum trap and pulling through several mL of water followed by ethanol. The pipet remained attached to the vacuum trap and air was pulled through the pipet until it was dried. The water weighing was performed in triplicate. The volume of the pipet was calculated from the weight of water and the 0.99756 g/mL density of water at 23 °C. Corrections for observed temperature displayed on a balance in the weighing room (22.8 °C) were made by the volumetric expansion formula:

$$V_{23} = V_{obsd} (1 - (T_{obsd} - 23)(0.00021)$$

where V_{23} is the volume at 23 °C, V_{obsd} is the observed volume, and T_{obsd} is the observed temperature in °C [3].

Three vials each of SRM 2969 (Boxes 4, 8, and 26) and SRM 2970 (Boxes 10, 33, and 37) were removed from -80 °C storage and were left undisturbed to thaw at room temperature for one hour. The three vials of each SRM were combined into individual 15-mL Falcon tubes. The weighing procedure was repeated for each serum pool in triplicate. Between each weighing, the pipet was rinsed with water, then ethanol, and dried.

4.3. Quantitation

The volume of the pipet was first calibrated with triplicate fillings and masses of water at 22.8 °C. This was followed by triplicate fillings and masses of SRM 2969 serum and then triplicate fillings and masses of SRM 2970 serum. The calculated mean volume of the water was used as the volume for the pipet in subsequent calculations of density for serum. The masses of serum were divided by the mean pipet volume to calculate density of serum. All measurements were obtained over an approximate two-hour time window. A minor shift in room temperature from 22.8 °C to 22.7 °C was observed during this time.

4.4. Results

The volume calibration measurements of the nominal 500 µL Lang-Levy pipet at 22.8 °C are shown in **Table 2**. The (mean ± standard deviation) volume at 22.8 °C was determined to be (0.49959 ± 0.00014) mL.

Table 2. Calibration of Lang-Levy Pipet Volume with Water

Sample	Mass g	Density at 23 °C g/mL	Volume at 23 °C mL	Volume at 22.8 °C mL	(Mean ± standard deviation) Volume at 22.8 °C mL	CV %
Water-1	0.49845	0.99756	0.49967	0.49965		
Water-2	0.49823	0.99756	0.49945	0.49943	0.49959 ± 0.00014	0.0288
Water-3	0.49850	0.99756	0.49972	0.49970		

The density measurements for SRMs 2969 and 2970 are shown in **Table 3**. The calculated mean density of (1.02353 ± 0.00013) g/mL for SRM 2969 and (1.02229 ± 0.00077) g/mL for SRM 2970 are consistent with the nominal 1.02 g/mL density expected of human serum.

Table 3. Determination of Density for SRMs 2969 and 2970

Sample	Mass g	Volume mL	Density g/mL	(Mean ± standard deviation) Density g/mL	CV %
SRM 2969-1	0.51129	0.49959	1.02342		
SRM 2969-2	0.51133	0.49959	1.02350	1.02353 ± 0.00013	0.013
SRM 2969-3	0.51142	0.49959	1.02368		
SRM 2970-1	0.51075	0.49959	1.02234		
SRM 2970-2	0.51033	0.49959	1.02149	1.02229 ± 0.00077	0.076
SRM 2970-3	0.51110	0.49959	1.02304		

5. Calibrant Purity

Purity assays were conducted via quantitative ^{1}H nuclear magnetic resonance spectroscopy (qNMR) using an internal standard prior to use of these calibration standards for certification of SRM 2969 and SRM 2970. The chemical purity of these materials had been determined in 2014, however subsequent measurements made in 2016 indicated that the 25(OH)D_2 content had changed substantially and re-investigation of mass purity was warranted for contemporary use. The results are traceable to the International System of Units through the determination of chemical structure and use of NIST PS1 Primary Standard for qNMR (Benzoic Acid) [4,5,6]

5.1. Materials

Chemical purity was determined for the following materials, stored at -20 °C in a glass jar desiccator prior to and after the purity analysis:

Calcifediol (25-hydroxyvitamin D_3); USP Lot G1E064
Ercalcidiol (25-hydroxyvitamin D_2); IsoSciences Lot RT-4-2013-062A1

A previously opened vial of the 25(OH)D_2 material and an unopened vial of the 25(OH)D_3 material were assayed for this study and used for certification of SRM 2969 and SRM 2970. The NIST PS1 Primary Standard for qNMR (Benzoic Acid) was used as an internal standard and is stored at room temperature in a desiccator. Samples of neat chemical materials were diluted with methanol-d_4; 99.8 % D atom purity, Cambridge Isotope Laboratories.

5.2. Sample Preparation

Four qNMR sample replicates and a control were evaluated for each vitamin D metabolite material. Sample preparation was performed under incandescent light (single white incandescent bulb) to reduce photodegradation of the vitamin D metabolites. Glassware used during sample preparation was rinsed with acetone, ethanol, methanol, and distilled water, baked in a furnace at 450 ˚C, and stored in a desiccator. Clean Bruker 600 MHz NMR tubes (5 mm internal diameter, 7-inch length) were stored in a desiccator prior to use. Neat material masses were determined using an ultramicrobalance. Approximately 0.7 mL of methanol-d_4 was used to dilute the samples. To facilitate total dissolution, samples were sonicated and vortexed. Care was taken to ensure complete dissolution and that no crystals of the neat materials adhered to the weigh bottle walls. All samples were diluted individually and immediately analyzed.

5.3. Analysis

Experimental NMR data was acquired by a Bruker Avance II 600 MHz spectrometer equipped with a 5-mm broadband inverse detection probe and operating with Topspin (Version 3.2) software. Experiments were performed at a temperature of 298 K with 96 scans, spectral sweep width was set to 20.0276 ppm, and the transmitter frequency offset was set to 6.175 ppm. 90-degree excitation pulse widths were used for these analyses and globally optimized alternating phase rectangular pulse ^{13}C decoupling was executed during free induction decay acquisition. Transmitter frequency offset of the carbon channel was 90 ppm. Data acquisition time was 5.452595 2 s for each scan to generate 131,072 data points. The spin lattice relaxation time (T_1) for all analyzed resonances was determined using a magnetization inversion recovery NMR experiment. Identity of each 25-hydroxyvitamn D

species was confirmed using ^{1}H NMR and ^{1}H-^{13}C heteronuclear single quantum correlation (^{1}H-^{13}C HSQC) NMR experiments. The recycle delay was set to 65 s for samples with 25(OH)D_2 and 55 s for samples with 25(OH)D_3 and was at least eleven times longer than the greatest T_1 of analyzed resonances in the respective samples.

Mass fraction purity (g/g), P, via ^{1}H-qNMR$_{IS}$ was derived using an estimation model based on the following measurement function:

$$P = \left(\frac{N_{\mathrm{I}}}{N_{\mathrm{P}}}\right)\left(\frac{M_{\mathrm{P}}}{M_{\mathrm{I}}}\right)\left(\frac{A_{\mathrm{P}}}{A_{\mathrm{I}}}\right)\left(\frac{m_{\mathrm{I}}}{m_{\mathrm{C}}}\right)P_{\mathrm{I}}$$

where:

N_P = multiplicity (H per peak) of the primary chemical component spectral peak
N_I = multiplicity (H per peak) of the internal standard peak
M_P = relative molar mass (molecular weight, g/mol) of the primary chemical component
M_I = relative molar mass (molecular weight, g/mol) of the internal standard
A_P = integrated area of the primary component peak
A_I = integrated area of the internal standard peak
m_C= mass (g) of the composite material
m_I = mass (g) of the internal standard
P_I = purity (g/g) of the internal standard.

The measurand, mass purity of the respective 25(OH)D, was calculated using a hierarchical Bayesian procedure modeled on observation equations based on the above equation [7,8,9].

5.4. Results

A crimp-sealed vial of USP Lot G1E064 25(OH)D_3 was opened and sampled for the first time during this analysis. The 25(OH)D_2 material, IsoSciences Lot RT-4-2013-062A1**,** was analyzed a third time. The chemical identity of the primary component of each neat material analyzed was confirmed via ^{1}H and ^{1}H-^{13}C NMR. These spectra and peak assignments are shown in **Figure 4** and **Figure 5**. The ^{1}H-qNMR$_{IS}$ spectra are shown in **Figure 6**. For purity assessments, distinct ^{1}H spectral regions were analyzed to determine integrals for the primary chemical component of each material and internal standard. A summary of these integrals is presented in **Table 4**.

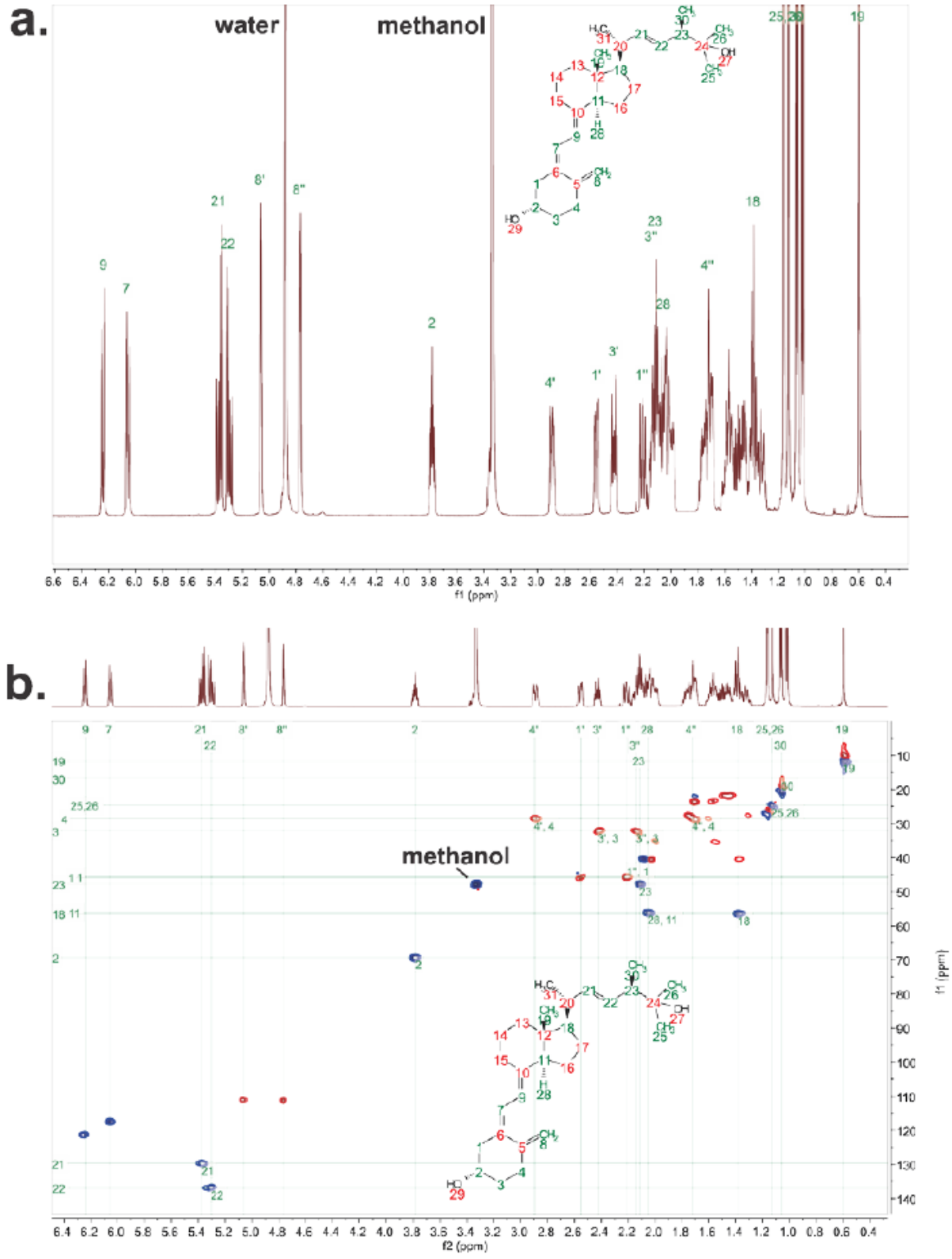


Figure 4. ^{1}H-NMR and ^{1}H-^{13}C HSQC Spectra of 25(OH)D$_2$ Calibrant in Methanol-*d*$_4$
a) ^{1}H-NMR spectra; b) ^{1}H-^{13}C HSQC spectra with ^{1}H moiety peak assignments

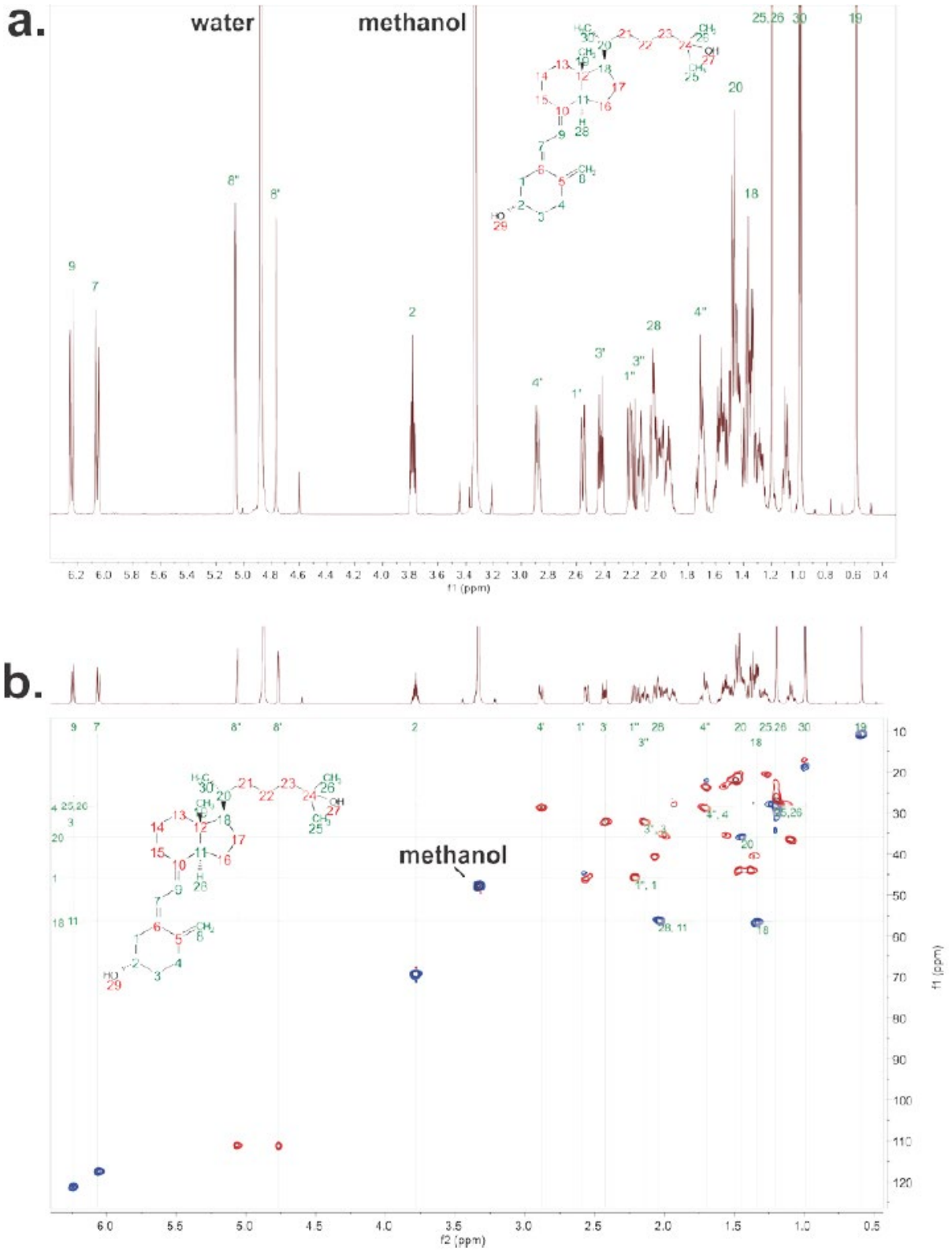


Figure 5. ^{1}H-NMR and ^{1}H-^{13}C HSQC Spectra of 25(OH)D$_3$ Calibrant in Methanol-d_4 a) ^{1}H-NMR spectra; b) ^{1}H-^{13}C HSQC spectra with ^{1}H moiety peak assignments

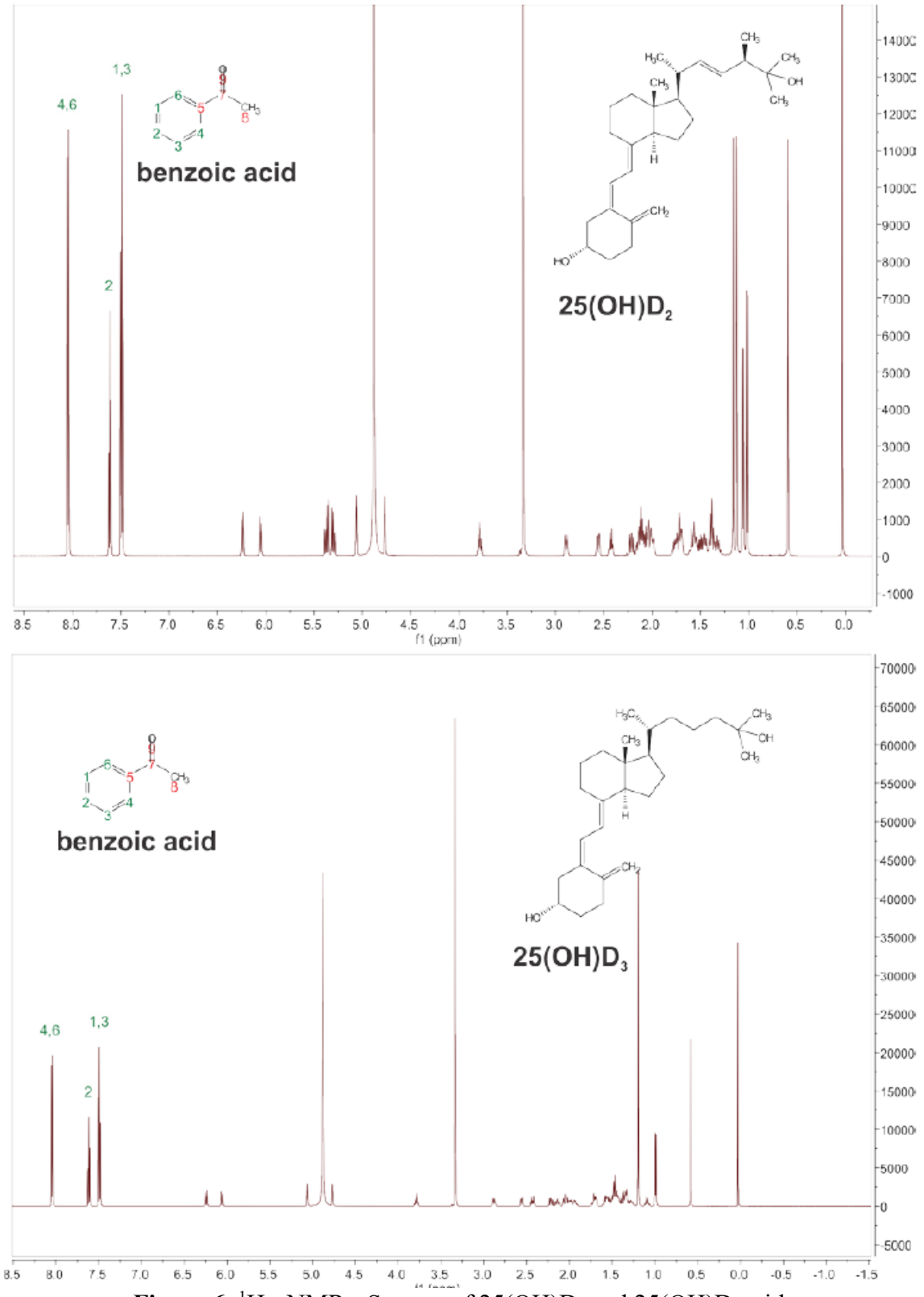


Figure 6. ^{1}H-qNMR$_{IS}$ Spectra of 25(OH)D$_2$ and 25(OH)D$_3$ with NIST PS1 Benzoic Acid Internal Standard in Methanol-*d*$_4$

Table 4. ^{1}H-NMR Integral Regions Evaluated for ^{1}H-qNMR$_{IS}$ Purity Assessment

Analyte	Chemical Shift (ppm)	Peak and Multiplet Type	^{1}H Structural Moiety [a]	^{1}H Multiplicity
25(OH)D$_2$	2.9	doublet	4'	1
	6.1	2 doublets	7,9	2
25(OH)D$_3$	0.5	singlet	19	3
benzoic acid	7.5	2 triplets	1,2,3	3
	7.7	2 triplets, doublet	1,2,3,4,6	5
	8.1	doublet	4,6	2

[a]The ^{1}H chemical structure moiety numbering scheme for 25(OH)D$_2$ is shown in Figure 4, the scheme for 25(OH)D$_3$ in Figure 5, and that for benzoic acid in Figure 6.

Several impurity peaks are observed in the ^{1}H-NMR spectra of the 25(OH)D$_2$, especially those in the regions of aliphatic resonances (Figure 6). The two doublets (7,9) at (5.8 and 6.4) ppm and the doublet (4') at 2.9 ppm had little or no peak overlap with other 25(OH)D$_2$ moieties and impurities, and thus were integrated to quantify the primary analyte. The methyl resonance peak at 0.5 ppm was integrated for quantification of 25(OH)D$_3$. This single peak was evaluated for this assay because the resulting integral could be adjusted to account for suspected impurity interference bias. Other peaks in the ^{1}H spectrum had significant overlap with impurity and/or 25(OH)D$_3$ moieties and corrections for the resulting bias could not be confidently made.

The uncertainty associated with variables of Eq. 1 were evaluated as follows:

- standard uncertainty of the primary component peak integral (A_P) for 25(OH)D$_2$ was the larger of Type B 0.1 % relative uncertainty and the standard deviation of the ^{1}H multiplicity-normalized integrals;
- standard uncertainty of the primary component peak integral (A_P) for 25(OH)D$_3$ is 0.35 % relative Type B uncertainty, whereby this larger uncertainty is attributable to the significant interference bias adjustment and quantification using a single resonance;
- the uncertainty of the NIST PS1 benzoic acid internal standard integral (A_I) was assigned a Type B relative standard uncertainty of 0.05 %, which is larger than the standard deviation of the three ^{1}H multiplicity-normalized integrals;
- masses of composite material (m_C) and internal standard material (m_I) were assigned a Type B uncertainty of 0.5 µg;
- the standard uncertainty of the internal standard (P_I) and the corresponding relative molecular mass (M_I) are provided on the NIST PS1 Certificate of Analysis as (-0.004, +0.002) % and 0.00019 g/mol. The asymmetric uncertainty of P_I was increased to ±0.01 % for this analysis;
- the uncertainty of the relative molecular mass of the 25(OH)D species (M_P) is 0.015 g/mol, calculated according to IUPAC Guidelines provided by the Commission on Isotopic Abundances and Atomic Weights (CIAAW), using a web-based molecular weight calculator [10];
- no uncertainty was considered for the ^{1}H multiplicities of the primary component (N_P) and internal standard (N_I).

A summary of the 25(OH)D purity results for the neat chemical reference materials used as calibrants for certification of SRM 2969 and SRM 2970 is given in **Table 5**. The results were estimated using a Bayesian observation equation approach [8,10], for which the probability density plots are shown in Figure 7. The OpenBUGS code and data for both calibrants are provided in Section 5.5.

Table 5. Purity (g/g) Results for 25(OH)D_2 and 25(OH)D_3 via ^{1}H-qNMR$_{IS}$

		Purity (g/g)		
Chemical	Lot	Value [a]	u(value)[b]	$U_{95\ \%}$(value)[c]
25(OH)D_2	IsoSciences, RT 4-2013-062A1	0.8594	0.0039	[0.8515, 0.8667]
25(OH)D_3	USP, G1E064	0.9472	0.0032	[0.9407, 0.9528]

a Purity expressed as the mean value of the Bayes posterior distribution
b Standard uncertainty expressed as the standard deviation of Bayes posterior distribution
c The 95 % confidence interval determined from the 95 % coverage interval of the posterior distribution

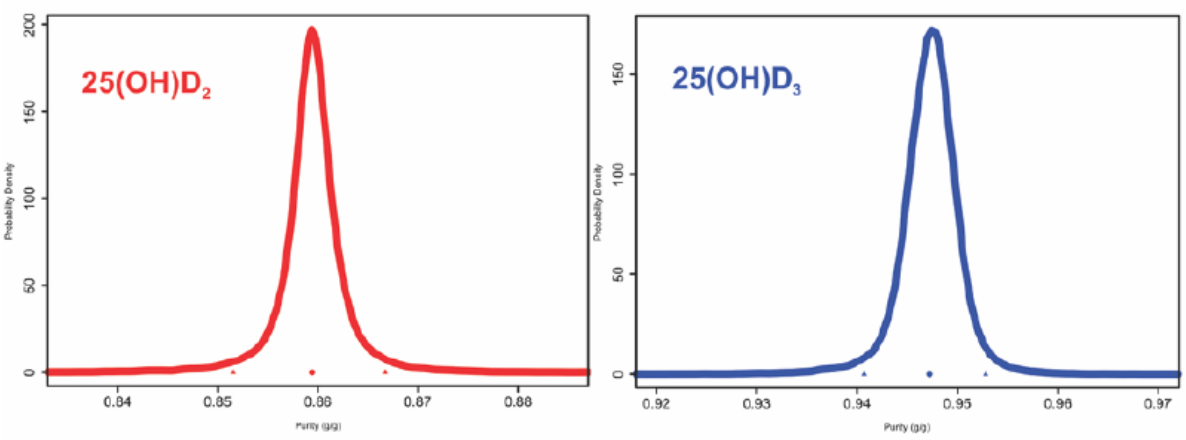


Figure 7. Posterior Distributions for ^{1}H-qNMR$_{IS}$ Purity Determinations.

5.5. OpenBUGS Evaluation of Mass Purity

The Bayesian model used to estimate the purity of the 25(OH)D_2 and 25(OH)D_3 calibrants was implemented using the OpenBUGS system [11]. The OpenBUGS code and data for the two calibrants are provided in the following Sections.

5.5.1. 25(OH)D_2:

```
{
mws1~dnorm(122.12204,27700831) # M_I
mwb1~dnorm(412.652, 4444) # M_P
px1<-1/(umx1*umx1)
c<-mu/(sd*sd)
d<-(1-mu)/(sd*sd)
mu~dunif(0.8,1)
sd~dunif(0,.1)
ps1~dnorm(0.9999,100000000)
for(i in 1:4)  {pb1[i]~dbeta(c,d)}
for(i in 1:4)  {ms1[i]~dnorm(meanms1[i],px1)
                mb1[i]~dnorm(meanmb1[i],px1)}
for(i in 1:4)  {k1[i]~dunif(0,0.01)
                meanas1[i]<-ps1*ms1[i]/mws1/k1[i]
                pxa1[i]<-1/(uareas1[i]*uareas1[i])}
for(i in 1:4)  {areas1[i]~dt(meanas1[i],pxa1[i],2)}
for(i in 1:4)  {k.cut1[i]<-cut(k1[i])
                meanab1[i]<-pb1[i]*mb1[i]/mwb1/k.cut1[i]
                precareab1[i]<-1/(uareab1[i]*uareab1[i])}
for(i in 1:4)  {y1[i]~dnorm(meanab1[i],precareab1[i])}
}

list(meanms1=c(0.0035608,0.0020873,0.0024618,0.0026274), # m_I
meanmb1=c(0.0020148,0.0021851,0.0020439,0.002171),umx1=0.0000005, # u(m_I), u(m_c)
areas1=c(1.43637,0.81315,0.96048,0.872709866), # A_I
uareas1=c(0.00072,0.00041,0.00048,0.000436355), # u(A_I)
y1=c(0.20606,0.21692,0.2033,0.183553402), # A_P
uareab1=c(0.00021,0.00022,0.00077,0.000183553)) # u(A_P)
```

5.5.2. 25(OH)D_3:

```
{
mws1~dnorm(122.12204,27700831) # M_I
mwb1~dnorm(400.643, 4444) # M_P
px1<-1/(umx1*umx1)
c<-mu/(sd*sd)
d<-(1-mu)/(sd*sd)
mu~dunif(0.8,1)
sd~dunif(0,.1)
ps1~dnorm(0.9999,100000000)
for(i in 1:4)   { pb1[i]~dbeta(c,d)}
for(i in 1:4)   {ms1[i]~dnorm(meanms1[i],px1)
                mb1[i]~dnorm(meanmb1[i],px1)}
for(i in 1:4)   {k1[i]~dunif(0,0.01)
                meanas1[i]<-ps1*ms1[i]/mws1/k1[i]
                pxa1[i]<-1/(uareas1[i]*uareas1[i])}
for(i in 1:4)   {areas1[i]~dt(meanas1[i],pxa1[i],2)}
for(i in 1:4)   {k.cut1[i]<-cut(k1[i])
                meanab1[i]<-pb1[i]*mb1[i]/mwb1/k.cut1[i]
                precareab1[i]<-1/(uareab1[i]*uareab1[i])}
for(i in 1:4)   {y1[i]~dnorm(meanab1[i],precareab1[i])}
}

list(meanms1=c(0.0020256,0.0021626,0.0014179,0.0022081), # m_I
meanmb1=c(0.0020560,0.0021712,0.0020529,0.0020791),umx1=0.0000005, # u(m_I), u(m_c)
areas1=c(1.269956682,1.154563449,0.739515492,1.21379566), # A_I
uareas1=c(0.000634978,0.000577282,0.000369758,0.000606898),  # u(A_I)
y1=c(0.372618747,0.335144735,0.309187317,0.329592054), # A_P
uareab1=c(0.001304166,0.001173007,0.001082156,0.001153572)) # u(A_P)
```

6. Certification Measurements

The value assignment measurements for 25(OH)D_2 and 25(OH)D_3 detailed in this Section were performed using 1 mL of serum and the NIST RMP for Vitamin D metabolite determination in human serum by ID-LC-MS/MS in positive atmospheric pressure chemical ionization mode [12].

6.1. Materials

Twelve samples each of SRM 2969 and SRM 2970 were randomly selected out of a total of 41 sample vial boxes. Six samples of each SRM were analyzed on two separate dates.

SRM 972a Vitamin D Metabolites in Frozen Human Serum Level 2 and Level 3 were selected as controls for the determination of 25(OH)D in SRM 2969 and SRM 2970, respectively.

The neat 25(OH)D_3 and 25(OH)D_2 reference materials described in Section 5 were used as calibrants to establish metrologically traceable results. The chemical purity of the 25(OH)D_3 was (0.9472 ± 0.0032) g/g; that for the 25(OH)D_2 was (0.8594 ± 0.0039) g/g. These calibrants were stored at -20 °C in screw-cap glass jars containing desiccant.

Isotopically labeled 25(OH)D_3-d_6 in ethanol was obtained from Cerilliant (Round Rock, TX) with a stated concentration of 50.00 µg/mL. Isotopically labeled 25(OH)D_2-d_3 in ethanol was obtained from IsoSciences with a stated concentration of 48.06 µg/mL. Ampoules containing each isotopically labeled standard in ethanol solution were stored at -20 °C. No NIST purity assessments were performed for these compounds.

HPLC-grade methanol and water were obtained from J.T. Baker. Ethyl alcohol, U.S.P, anhydrous, was obtained from the Warner Graham Company.

6.2. Standard Solution Preparation

Three standard stock solutions were gravimetrically prepared for each 25(OH)D_3 and 25(OH)D_2 compound. The neat reference materials were removed from -20 °C storage and allowed to equilibrate to room temperature before accurately weighing 1 mg of compound in an aluminum foil cup. The cup was placed into a 100 mL volumetric flask, the flask was stoppered and tared, and ≈100 mL of anhydrous ethanol was quickly poured into the flask to weigh. Each solution was sonicated for about 10 min to completely dissolve the compound before transferring to 8-ounce amber glass bottles for -20 °C storage.

The 25(OH)D_3 stock solutions were prepared on the same day and ranged from (13.9 to 14.9) µg/g mass fraction. Stock solutions 2 to 4 for 25(OH)D_2 ranged from (13.6 to 14.4) µg/g mass fraction. Stock solution 4 was prepared 17 days after stock solutions 2 and 3. After correcting for purity, the final mass fractions of the stock solutions were (13.2 to 14.0) µg/g for 25(OH)D_3 and (11.7 to 12.4) µg/g for 25(OH)D_2.

A working solution was prepared from each 25(OH)D_3 and 25(OH)D_2 stock solution for a total of six working solutions. One milliliter of the stock solution was transferred into a 200 mL glass volumetric flask and accurately weighed. Then ≈120 mL of anhydrous ethanol was

added to the flask and weighed. Mass fractions in the three 25(OH)D_3 working solutions ranged from (295.3 to 313.8) ng/g; mass fractions in the three 25(OH)D_2 working solutions ranged from (94.2 to 95.5) ng/g. All working solutions were prepared the same day as the matching stock solutions.

6.3. Isotopically Labeled Solution Preparation

Approximately 1 mL of the isotopically labeled ethanolic solution was diluted with 3 mL of anhydrous ethanol to gravimetrically prepare a 14.2 µg/g stock solution of 25(OH)D_2-d_3. A 15.5 µg/g stock solution of 25(OH)D_3-d_6 was prepared in the same manner. Isotopically labeled stock solutions were weighed in 25 mL amber glass vials and stored at -20 °C. Approximately 1 mL of each isotopically labeled stock was diluted with ≈60 mL to ≈89 mL of anhydrous ethanol to gravimetrically prepare a 174.0 ng/g working solution for 25(OH)D_3-d_6 and a 240.2 ng/g working solution for 25(OH)D_2-d_3. An additional 174.6 ng/g working solution for 25(OH)D_3-d_6 was later prepared. Approximately 5 mL of the 240.2 ng/g working solution was diluted with ≈51 mL of anhydrous ethanol to produce a 21.9 ng/g working solution for 25(OH)D_2-d_3. All solutions were gravimetrically prepared in 4-ounce amber glass bottles and stored at -20 °C.

6.4. Calibration Solution Preparation

Six calibration solutions with (0.7 to 1.2) mass ratios were gravimetrically prepared to produce calibration curves for 25(OH)D_3 and 25(OH)D_2. The first day analysis for 25(OH)D_3 consisted only of five calibration solutions. The aliquot of 25(OH)D_3 neat standard solution needed in Calibrant 5 was combined into Calibrant 4, which resulted in Calibrant 4 having a mass ratio of 2.0 instead of the target 1.2 and the exclusion of Calibrant 5.

Prior to weighing, all neat and labeled internal standard solutions were removed from -20 °C storage and allowed to equilibrate to room temperature. Aliquots from one of three neat working standard solutions were spiked with aliquots from one or two internal standard working solutions and accurately weighed in autosampler vials. For the 25(OH)D_2 calibration solutions, (93 to 281) µL aliquots from one of three 25(OH)D_2 neat standard working solutions were spiked with (100 to 500) µL aliquots from one of two 25(OH)D_2-d_3 internal standard working solutions, yielding working solutions of 21.9 ng/g on Day 1 and 240.2 ng/g on Day 2. In a similar manner, (118 to 200) µL aliquots of 25(OH)D_3 neat standard working solutions were spiked with 300 µL aliquots of 25(OH)D_3-d_6 internal standard working solution, yielding working solutions of 174.0 ng/g on Day 1 and 174.6 ng/g on Day 2.

All mixtures were dried under nitrogen at approximately 45 °C, reconstituted with 300 µL of methanol, and transferred to amber autosampler vials containing 300-µL glass polyspring inserts for ID-LC-MS/MS analysis. Calibration solutions were prepared the same day as samples. Calibration solutions were gravimetrically prepared using a balance.

6.5. Sample Preparation

Twelve sample vials each of SRM 2969 and SRM 2970 were randomly selected from 41 sample boxes. Samples were organized into two sample sets to prepare on two separate days. Each sample set consisted of six samples of SRM 2969 and six samples of SRM 2970, as well as two vials of SRM 972a Level 2 and two vials of SRM 972a Level 3 as controls.

Sample vials were removed from -80 °C and allowed to equilibrate to room temperature for 1 h prior to weighing. One milliliter of each sample was transferred into a 50-mL glass centrifuge tube, capped, and accurately weighed. Next, each serum sample was spiked with fixed aliquots of the 25(OH)D_3-d_6 and 25(OH)D_2-d_3 internal standard solutions to achieve a 1:1 mass ratio of analyte to internal standard. The 174.0 ng/g working solution for 25(OH)D_3-d_6 was used for spiking into the Day 1 sample set; the 174.6 ng/g working solution was used for spiking into the Day 2 sample set. The spiking volumes of 25(OH)D_3-d_6 working solution were as follows: 129 µL into SRM 972a Level 2 controls, 141 µL into SRM 972a Level 3 controls, 86 µL into SRM 2969 samples, and 68 µL into SRM 2970 samples. For 25(OH)D_2-d_3, the 21.9 ng/g working solution was used to spike 46 µL into SRM 972a Level 2 controls and 113 µL into SRM 2969 samples; the 240.2 ng/g working solution was used to spike 69 µL into SRM 972a Level 3 controls and 115 µL into SRM 2970 samples.

Internal standard spiking volumes were fixed to be less than 20 % of the serum volume to prevent the precipitation of proteins and to eliminate the addition of water. After weighing the spiking volumes of each internal standard solution, the glass centrifuge tube was gently rolled horizontally to fully incorporate any remaining internal standard solution into the serum sample. The samples were left undisturbed for 1 h to equilibrate at room temperature. Approximately 245 µL of 0.1 g/mL carbonate buffer (pH 9.8) was added to each sample to adjust the pH to 9.8 ± 0.2. Eight milliliters of hexane-ethyl acetate (50:50, volume fraction) was added to each sample tube to simultaneously extract 25(OH)D_3 and 25(OH)D_2 from the serum matrix. Each sample was shaken vigorously for 10 min using a mechanical shaker. Then, the upper hexane-ethyl acetate layer was carefully transferred to individual 50-mL glass centrifuge tubes using a disposable pipette. An additional 8 mL of the hexane-ethyl acetate solvent was added to each sample and shaken vigorously for an additional 3 min. The upper hexane-ethyl acetate layer was transferred and combined with the first in the same 50-mL centrifuge tubes. The combined extract was dried under nitrogen at 45 °C and the residue reconstituted with 125 µL of methanol. Each sample volume was divided into two individual autosampler vials containing 300-µL glass polyspring inserts to be analyzed simultaneously for 25(OH)D_3 and 25(OH)D_2 on two separate LC-MS/MS systems.

All samples and calibrants were stored at -20 °C prior to ID-LC-MS/MS analysis, and remained at 5 °C in a temperature-controlled autosampler once analysis was initiated. After analysis, all calibrants and samples were removed from the autosampler and stored at -20 °C.

6.6. Instrumental method for 25(OH)D_3

Liquid chromatographic separation was achieved using a Zorbax SB-CN column (4.6 mm × 150 mm, 3.5 µm particle diameter, Agilent Technologies) at a flow rate of 0.75 mL/min and 30 ºC column temperature control. An isocratic method of 33:67 (volume fraction) water: methanol was applied for 30 min, followed by a 10 min column rinse with 100 % methanol, and 12 min re-equilibration to initial conditions. Volume fractions of 33:67 water and methanol were combined and applied through Pump A; Pump B consisted of 100 % methanol. The injection volume was 5 µL and 10 µL for calibrants and samples, respectively. The autosampler tray temperature was set at 5 ºC.

Analysis was performed on an Agilent 1260 Series LC system coupled to an Applied Biosystems API 5000 LC-MS/MS system with atmospheric pressure chemical ionization in positive ion mode. Multiple reaction monitoring (MRM) mode was used for data acquisition from 5 min to 30 min of the analysis time. Sample flow was diverted to waste after 30 min to minimize contamination of the system over time. The transitions at *m/z* 401 → *m/z* 383 and at *m/z* 407 → *m/z* 389 were monitored for 25(OH)D_3 and 25(OH)D_3-d_6, respectively. The dwell times were 0.2 s for each MRM. The curtain gas and collision gas were nitrogen at settings of 345 kPa (50 psi) and 34 kPa (5 psi), respectively. The ion source gas 1 was air at a setting of 345 kPa (50 psi). The needle current was set at 5 μA and the temperature was maintained at 325 ºC. The declustering potential, entrance potential, collision energy, and collision exit potential were set at 90 V, 7 V, 10 V, and 20 V, respectively.

6.7. Instrumental method for 25(OH)D_2

Liquid chromatographic separation was achieved using an Ascentis Express F5 column (15 cm × 4.6 mm, 2.7 μm particle diameter, from Sigma) at a flow rate of 0.75 mL/min and 30 ºC column temperature control. An isocratic method of 27:73 (volume fraction) water: methanol was applied for 30 min, followed by an 8 min column rinse with 100 % methanol, and 12 min re-equilibration to initial conditions. Volume fractions of 27:73 water and methanol were combined and applied through Pump A; Pump B consisted of 100 % methanol. The injection volume was 5 μL and 10 μL for calibrants and samples, respectively. The autosampler tray temperature was set at 5 ºC.

Analysis was performed on an Agilent 1290 Infinity II Series LC system coupled to a SCIEX QTRAP 6500+ LC-MS/MS system with atmospheric pressure chemical ionization in positive ion mode. MRM mode was used for data acquisition from 5 min to 30 min of the analysis time. Sample flow was diverted to waste after 30 min to minimize contamination of the system over time. The transitions at *m/z* 413 → *m/z* 395 and *m/z* 416 → *m/z* 398 were monitored for 25(OH)D_2 and 25(OH)D_2-d_3, respectively. The dwell times were 0.2 s for each MRM. The curtain gas and collision gas were nitrogen at settings of (138 kPa (20 psi) and LOW, respectively. The ion source gas 1 was air at a setting of 552 kPa (80 psi). The needle current was set at 2 μA and the temperature was maintained at 350 ºC. The declustering potential, entrance potential, collision energy, and collision exit potential were set at 80 V, 6 V, 12 V, and 20 V, respectively.

6.8. Results

Summary results for 25(OH)D_3 and 25(OH)D_2 in SRM 972a Level 2 and Level 3 are provided in **Table 6**. The measured values for both control materials were within the certified ranges with acceptably small differences between injections and between measurement dates. The relatively high CV for SRM 972a Level 2 of (1.5 to 2.0) % is attributable to the low level of 25(OH)D_2 in that material. The agreement of the control results with their certified values validates the measurement process for 25(OH)D_3 and 25(OH)D_2 in SRM 2969 and SRM 2970.

The overall ID-LC-MS/MS results for the determination of 25(OH)D_3 and 25(OH)D_2 mass fraction values in SRM 2969 are provided in **Table 7**; those for SRM 2970 are in **Table 8**.

Table 6. Summary Results for $25(OH)D_3$ and $25(OH)D_2$ in the Controls

Analyte	Control	Certified Value ng/g	Day	Mean ± SD ng/g	CV %
$25(OH)D_3$	SRM 972a L2	17.7 ± 0.4	1	17.4 ± 0.09	0.5
			2	17.3 ± 0.03	0.2
	SRM 972a L3	19.4 ± 0.4	1	19.2 ± 0.09	0.5
			2	19.2 ± 0.05	0.3
$25(OH)D_2$	SRM 972a L2	0.80 ± 0.06	1	0.79 ± 0.02	2.0
			2	0.77 ± 0.01	1.5
	SRM 972a L3	13.0 ± 0.3	1	12.9 ± 0.05	0.4
			2	13.0 ± 0.1	0.5

Table 7. Summary Results for $25(OH)D_3$ and $25(OH)D_2$ SRM 2969

Day	Run	Box	Injection	$[25(OH)_2D_3]$ ng/g	$[25(OH)_2D_2]$ ng/g
1	17	1	1	11.59	1.976
1	41	1	2	11.64	2.007
1	18	7	1	11.83	1.945
1	40	7	2	11.82	1.972
1	19	12	1	11.58	1.950
1	39	12	2	11.62	1.959
1	20	14	1	11.74	1.975
1	38	14	2	11.84	1.948
1	21	15	1	11.66	1.966
1	37	15	2	11.57	1.978
1	22	19	1	11.81	1.988
1	36	19	2	11.70	1.973
2	17	30	1	11.77	1.967
2	41	30	2	11.59	1.947
2	18	32	1	11.71	1.984
2	40	32	2	11.68	1.977
2	19	33	1	11.52	1.943
2	39	33	2	11.58	1.951
2	20	35	1	11.51	1.957
2	38	35	2	11.40	1.947
2	21	36	1	11.69	1.942
2	37	36	2	11.70	1.937
2	22	41	1	11.60	1.954
2	36	41	2	11.46	1.960

Table 8. Summary Results for 25(OH)D_3 and 25(OH)D_2 SRM 2970

Day	Run	Box	Injection	[25(OH)$_2$D$_3$] ng/g	[25(OH)$_2$D$_2$] ng/g
1	23	1	1	9.304	23.325
1	35	1	2	9.304	23.017
1	24	4	1	9.526	22.916
1	34	4	2	9.481	22.987
1	25	5	1	9.448	22.945
1	33	5	2	9.461	23.081
1	26	8	1	9.435	22.963
1	32	8	2	9.541	22.832
1	27	16	1	9.482	22.791
1	31	16	2	9.523	22.778
1	28	19	1	9.279	22.902
1	30	19	2	9.430	23.045
2	23	22	1	9.318	23.377
2	35	22	2	9.414	23.079
2	24	24	1	9.298	22.808
2	34	24	2	9.446	23.214
2	25	27	1	9.247	22.911
2	33	27	2	9.205	22.764
2	26	34	1	9.474	23.150
2	32	34	2	9.339	22.894
2	27	39	1	9.389	23.331
2	31	39	2	9.490	23.130
2	28	41	1	9.277	23.045
2	30	41	2	9.386	22.959

Figure 8 displays the results for 25(OH)D3 and 25(OH)D2 for both SRMs as functions of packaging order (Box 1 to Box 41), analysis set (Day 1 or Day 2), and injection (1 or 2). The chromatographic run order is a function of the Box and Injection variables. Given the differences between injections separated in time by approximately 1 day, no substantial trends are apparent.

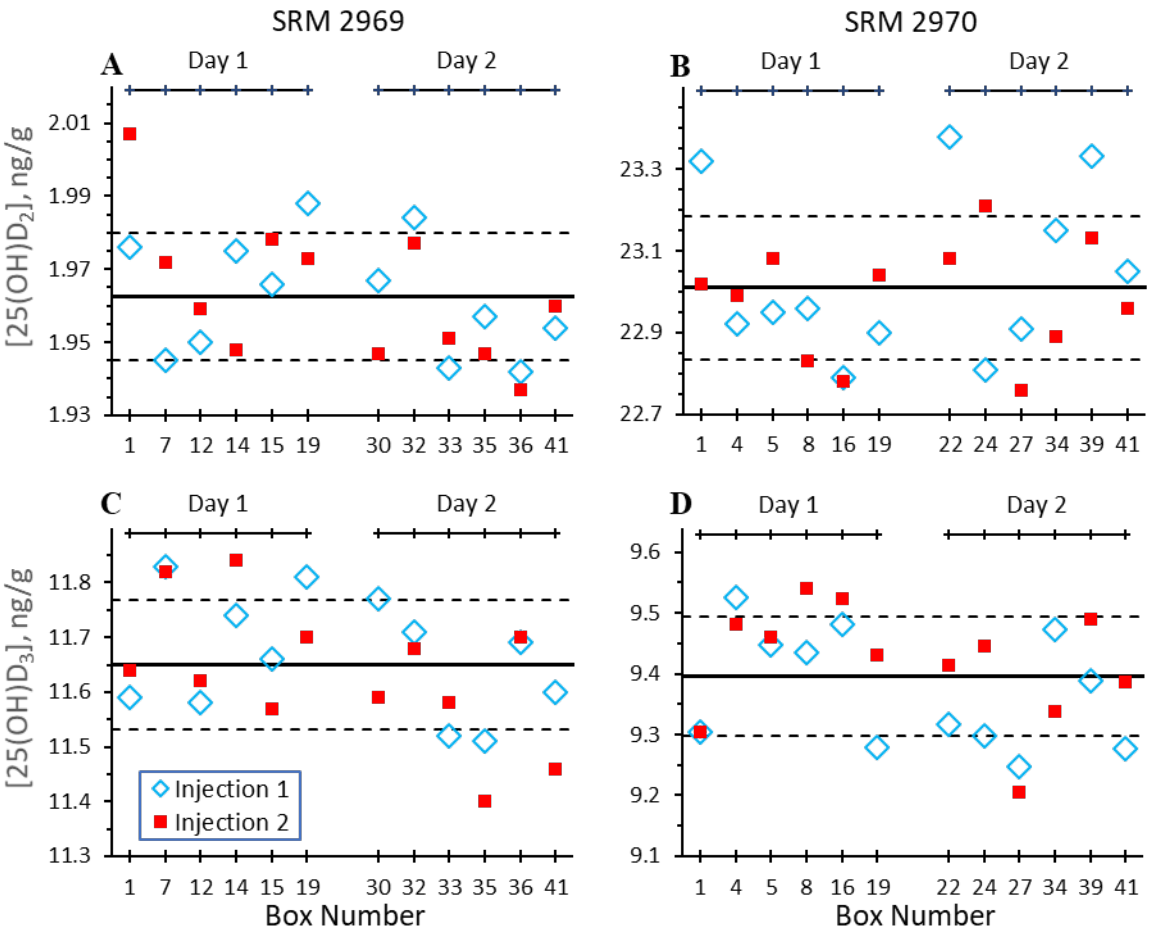


Figure 8. Results as a Function of Box, Day, and Injection Order
Solid lines represent the mean value, dashed lines the mean value ± one standard deviation, open diamonds results for the first injection, and solid squares results for the second injection.

Table 9 lists the mean mass fraction values (w), standard uncertainty (u), and 95 % confidence interval (U_{95}) for 25(OH)D$_2$ and 25(OH)D$_3$ based on the ID-LC-MS/MS measurements and evaluated using the ABACUS Linear Regression Analysis App [9,13]. The uncertainties in the masses of internal standard working solutions, standard working solutions, and samples were the manufacturer-specified repeatability values of 0.015 mg and 0.0001 mg, which were used to propagate uncertainties associated with preparation of standard stock solutions. Data inputs into and outputs from the ABACUS Linear Regression Analysis App are detailed in Section 6.9.

Table 9. ABACUS 25(OH)D_3 and 25(OH)D_2 Results for SRM 2969 and 2970

Analyte	SRM	Day	$w \pm w(u)$ ng/g	U_{95} ng/g
25(OH)D_3	2969	1	11.73 ± 0.12	[11.48, 11.96]
		2	11.60 ± 0.08	[11.45, 11.76]
	2970	1	9.471 ± 0.097	[9.284, 9.666]
		2	9.365 ± 0.067	[9.23, 9.49]
25(OH)D_2	2969	1	1.970 ± 0.019	[1.933, 2.008]
		2	1.955 ± 0.023	[1.908, 2.001]
	2970	1	22.94 ± 0.11	[22.70, 23.15]
		2	23.05 ± 0.18	[22.68, 23.44]

Table 10 presents consensus values for the two sets of ABACUS results calculated using the Hierarchical Bayes and Linear Pool models in the NIST Consensus Builder [14]. For the Hierarchical Bayes approach, the number of iterations, length of burn in, and thinning size used to generate data were 250000, 50000, and 25, respectively. For the Linear Pooling approach, the default weights (weights equal to 1) and sample size of 100000 were used. A coverage probability of 0.95 and a random number generator seed of 5 were used for both methods of analysis. The degrees of freedom were not specified for any of the data sets.

Table 10. Consensus Builder 25(OH)D_3 and 25(OH)D_2 Results SRM 2969 and 2970

SRM	Analyte	Method	$w \pm w(u)$ ng/g	U_{95} ng/g
2969	25(OH)D_3	Bayes	**11.65 ± 0.14**	**[11.40, 11.91]**
		Linear Pool	11.66 ± 0.12	[11.46, 11.93]
	25(OH)D_2	Bayes	**1.964 ± 0.023**	**[1.925, 2.003]**
		Linear Pool	1.962 ± 0.022	[1.916, 2.004]
2970	25(OH)D_3	Bayes	**9.40 ± 0.12**	**[9.21, 9.62]**
		Linear Pool	9.42 ± 0.10	[9.25, 9.63]
	25(OH)D_2	Bayes	**22.98 ± 0.15**	**[22.72, 23.27]**
		Linear Pool	22.99 ± 0.16	[22.71, 23.34]

The Hierarchical Bayes method and the Linear Pooling method generated very similar results for 25(OH)D_3 and 25(OH)D_2 in both SRM 2969 and SRM 2970, indicating that the results are robust to the model assumptions. The results provided by the Hierarchical Bayes model are preferred as their $w(u)$ estimates tend to be slightly larger (and therefore slightly more conservative) than those from the Linear Pool.

6.9. Quantitation

Six (five on Day 1 for 25(OH)D_3) calibrants were analyzed contemporaneously along with the samples in duplicate injections. Each calibrant, control, and sample was injected once with the order reversed for the second injection. Blank injections of methanol were measured at the beginning and end of the batch sequence, at the start of the analysis in reverse order, and between the last calibration solution (Calibrant 6) and first sample analysis (SRM 972a Level 2 control) within the batch sequence.

The 5-point and 6-point linear regression for 25(OH)D_3 produced adequate linearity with a correlation coefficient of 0.9993 for the first analysis and 0.9973 for the second analysis. The 6-point linear regressions for 25(OH)D_2 also produced adequate linearity with correlation coefficients of 0.9945 and 0.9986.

Figure 9 presents example chromatograms of the metabolite transition signals and those of their isotopically labeled internal standards for injections of blank methanol, **Figure 10** of Calibration solution 1, **Figure 11** of the 972a control materials, and **Figure 12** of SRM 2969 and 2970.

Agilent 1260 Series LC
Applied Biosystems API 5000 LC-MS/MS
System Used for 25(OH)D_3

Agilent 1290 Infinity II LC
Sciex QTRAP 6500+ LC-MS/MS
System Used for 25(OH)D_2

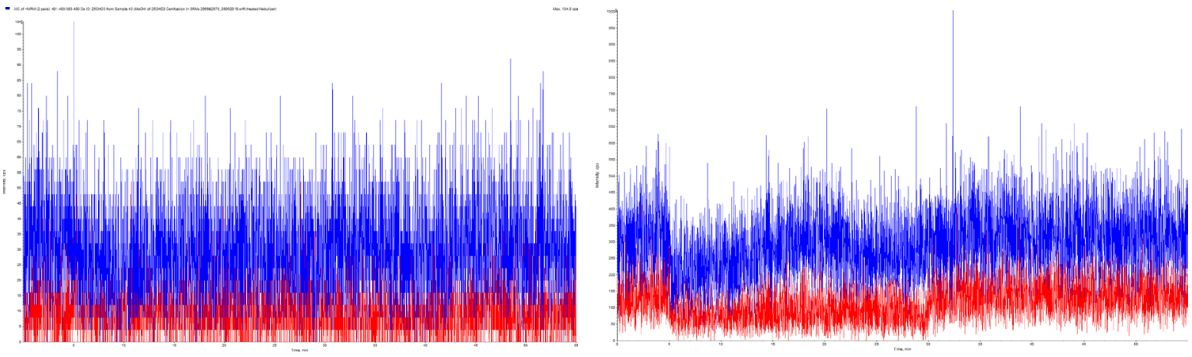


Figure 9. Example Chromatograms for Methanol Blank

Chromatograms for the LC-MS/MS system used for 25(OH)D_2 analysis are to the left, for 25(OH)D_3 analysis are to the right. Data for the unlabeled 25(OH)D metabolite are in blue, data for its isotopically labeled internal standard are in red.

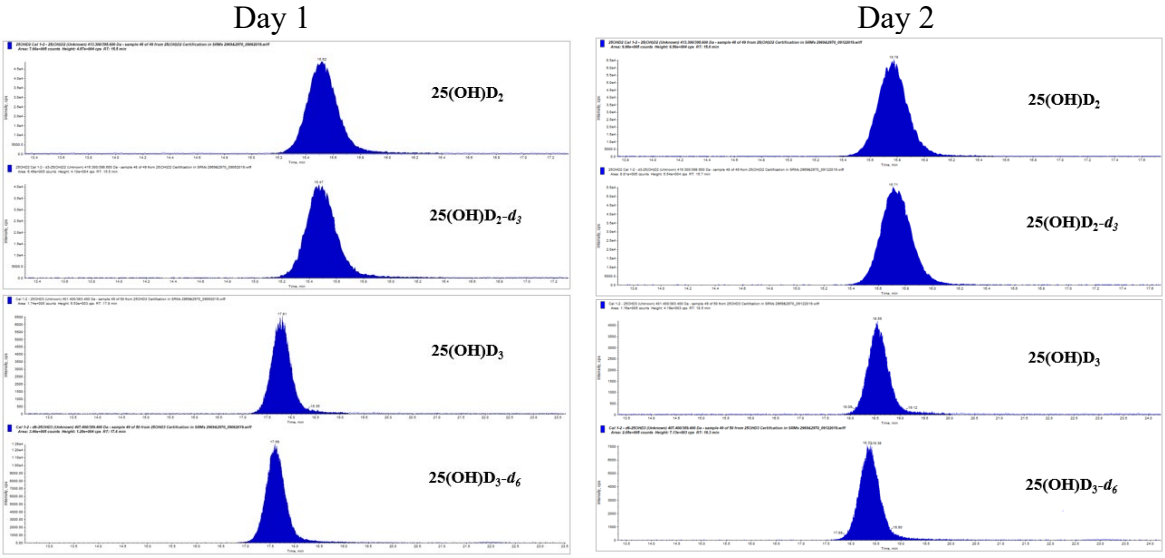


Figure 10. Example Chromatograms for Calibrant 1

Chromatograms for SRM 2969 are to the left, for SRM 2970 are to the right. Chromatograms are stacked in descending order: 25(OH)D_2, 25(OH)D_2-d_3, 25(OH)D_3, and 25(OH)D_3-d_6. All chromatograms are from the second injection of Box 1 samples.

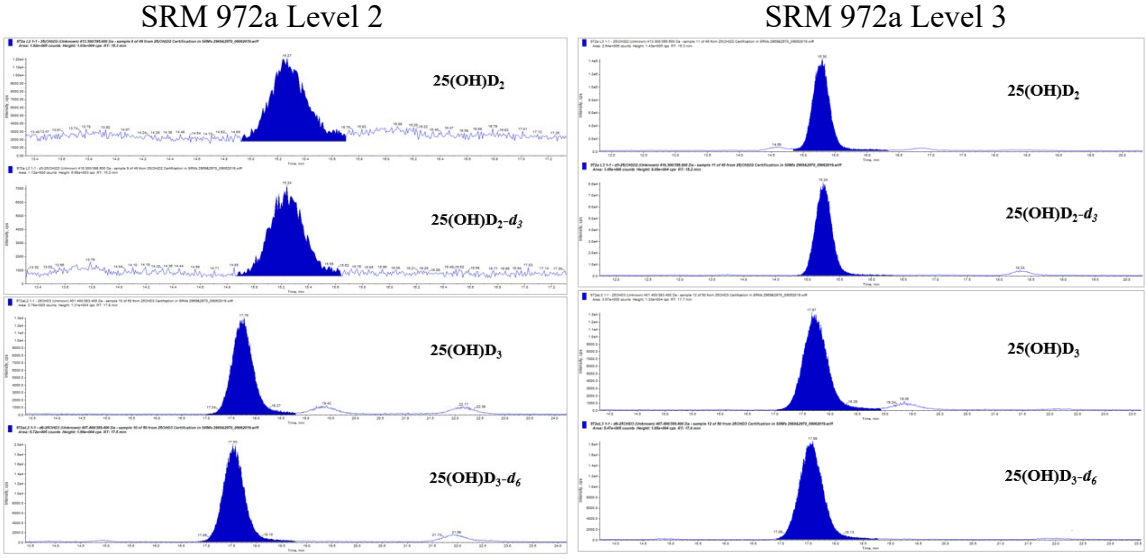


Figure 11. Example Chromatograms for the SRM 972a Controls

Chromatograms for SRM 2969 are to the left, for SRM 2970 are to the right. Chromatograms are stacked in descending order: 25(OH)D_2, 25(OH)D_2-d_3, 25(OH)D_3, and 25(OH)D_3-d_6. All chromatograms are from the first injection of the first sample preparation of the control on Day 1.

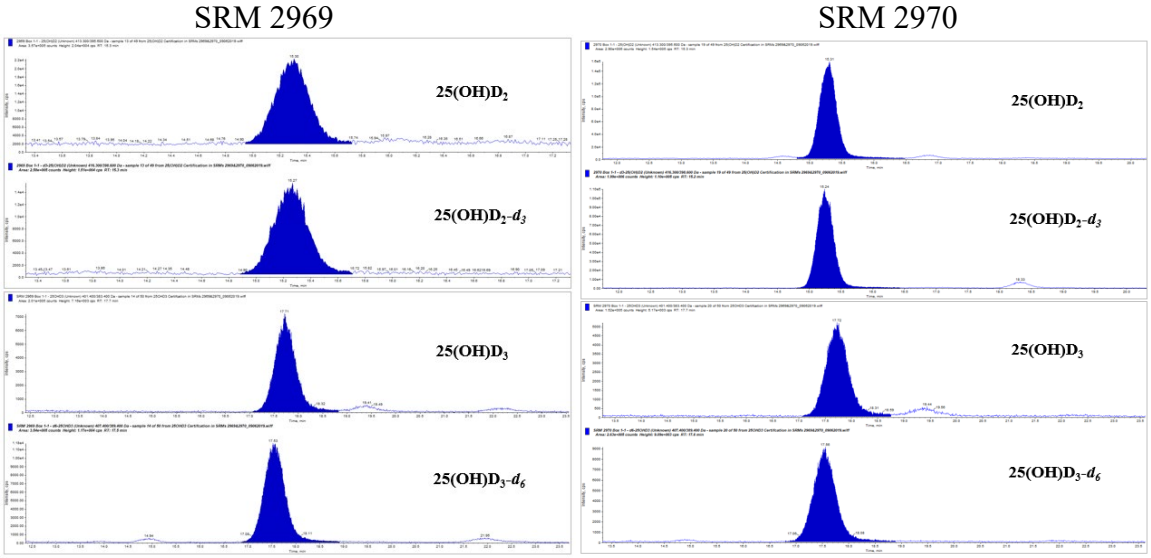


Figure 12. Example Chromatograms for SRM 2969 and SRM 2970

Chromatograms for SRM 2969 are to the left, for SRM 2970 are to the right. Chromatograms are stacked in descending order: 25(OH)D_2, 25(OH)D_2-d_3, 25(OH)D_3, and 25(OH)D_3-d_6. All chromatograms are from the first injection of Box 1 samples.

Measurement data from the duplicate injections of each calibrant were combined to generate linear regression $y = mx + b$ calibration functions for both of the 25(OH)D metabolites for the Day1 and Day 2 analyses. These functions were used to convert the measured peak area ratios of analyte and internal standard in samples into mass ratios. These mass ratios and respective masses of internal standard were used to calculate mass fractions of 25(OH)D_3 and 25(OH)D_2 in SRM 2969 and SRM 2970 using the NIST ABACUS: Chemical Measurement by Linear Calibration with Internal Standard Shiny application software [9,13].

6.10. ABACUS Analysis

This section provides the input values for the ABACUS: Chemical Measurement by Linear Calibration with Internal Standard Shiny Application Software.

6.10.1. Basic Parameters

Data Input Sizes	**Values**
Number of Calibrants	6 or 5
Max Number of Calibrant Repeats	2
Number of Samples	6
Max Number of Sample Repeats	2
Number of Analyte Standard Solutions	3
Calibration/ Inputs for 25(OH)D_2	
Concentrations of analyte in standard solutions (ng/g)	94.2, 95.5, 94.5
Uncertainties in concentrations of analyte standard solutions (ng/g)	0.427, 0.434, 0.429
Calibration/ Inputs for 25(OH)D3	
Concentrations of analyte in standard solutions (ng/g)	309.7, 295.3, 313.8
Uncertainties in concentrations of analyte standard solutions (ng/g)	1.047, 0.998, 1.060
Calibration/ Sample Inputs	
Uncertainty in masses of internal standard added to calibrants (g)	0.000015
Uncertainty in masses of the working standard solution added to calibrants (g)	0.000015
Uncertainty in masses of internal standard working solution spiked into samples (g)	0.000015
Uncertainty in the sampled mass of the measured substance (g)	0.000015
Data Fitting Setting	
Coverage Probability 0.95	0.95
Number of decimal places for reporting results 2	2
Markov chain Monte Carlo Settings	
Total number of iterations	10000 or 20000
Length of burn in	5000

6.10.2. Inputs and Outputs for 25(OH)D_3 in SRM 2969 on Day 1

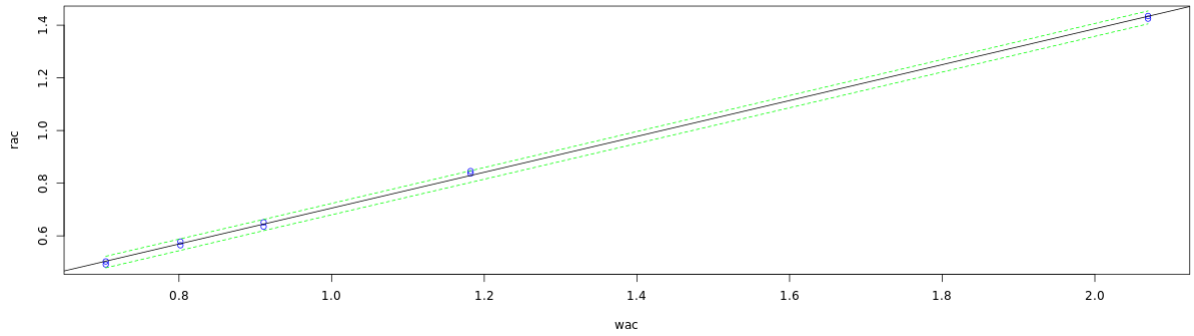
Calibration Data

mid	mad	wsol	rac1	rac2
40.05774	0.09125	1	0.491525	0.502890
40.40047	0.10971	2	0.564286	0.578348
41.17292	0.11932	3	0.635088	0.651558
41.52261	0.15615	3	0.836957	0.845921
41.90362	0.28057	1	1.424749	1.434783

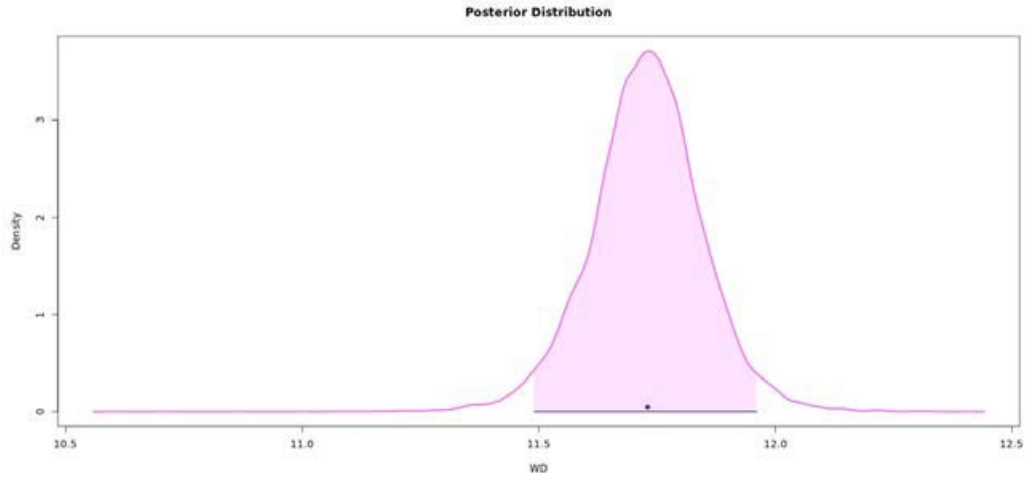
Sample Data

mids	mdi	ras1	ras2
12.34179	0.85230	0.567797	0.570248
12.11214	0.97314	0.674419	0.673410
12.40442	0.92957	0.615584	0.617801
11.96948	0.98312	0.684066	0.689840
12.21652	0.94563	0.640103	0.635135
11.98166	0.94821	0.663239	0.656642

mid (mass internal standard in ng); mad (mass analyte solution in g); wsol (# of working solution); rac1 (analyte/internal standard area ratio injection 1 calibrant); rac2 (analyte/internal standard area ratio injection 2 calibrant); mids (mass internal standard added to sample in ng); midi (mass serum in g); ras1 (analyte/internal standard area ratio injection 1 of serum); ras2 (analyte/internal standard area ratio injection 2 of serum)



R-squared: 0.9993
Intercept Mean: 0.02451
Intercept sd: 0.00996
Slope Mean: 0.68096
Slope sd: 0.00850



The posterior mean is: 11.73
The standard uncertainty is: 0.12
The posterior median is: 11.73
The 95 % credible interval ranges from: 11.48 to 11.96

6.10.3. Inputs and Outputs for 25(OH)D_3 in SRM 2969 on Day 2

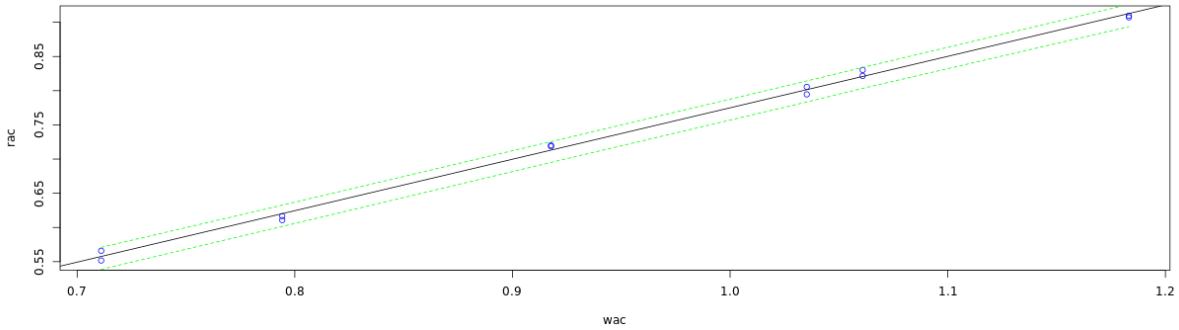
Calibration Data

mid	mad	wsol	rac1	rac2
39.62470	0.09099	1	0.551685	0.565854
40.74042	0.10956	2	0.610778	0.616580
41.04598	0.12002	3	0.720000	0.718631
40.00883	0.13376	1	0.794643	0.805556
42.11630	0.15129	2	0.830303	0.821622
41.56979	0.15672	3	0.907285	0.909677

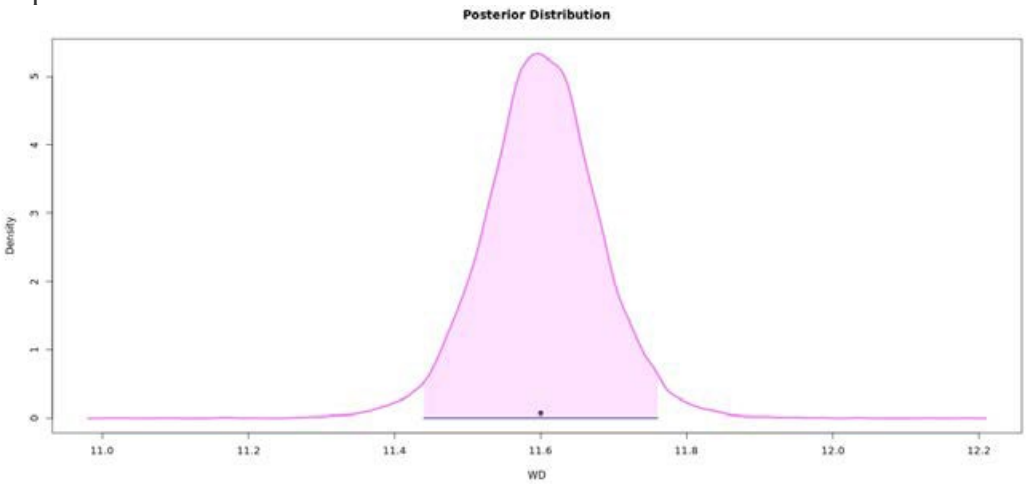
Sample Data

mids	mdi	ras1	ras2
12.21357	0.96418	0.721774	0.711297
12.28341	0.99108	0.733607	0.732000
12.14722	0.92339	0.681481	0.685039
12.18563	1.00853	0.739623	0.732824
12.03198	0.96024	0.724409	0.725100
12.27992	0.98854	0.725100	0.716846

mid (mass internal standard in ng); mad (mass analyte solution in g); wsol (# of working solution); rac1 (analyte/internal standard area ratio injection 1 calibrant); rac2 (analyte/internal standard area ratio injection 2 calibrant); mids (mass internal standard added to sample in ng); midi (mass serum in g); ras1 (analyte/internal standard area ratio injection 1 of serum); ras2 (analyte/internal standard area ratio injection 2 of serum)



R-squared: 0.9973
Intercept Mean: 0.02264
Intercept sd: 0.01464
Slope Mean: 0.75238
Slope sd: 0.01521



The posterior mean is: 11.6
The standard uncertainty is: 0.078
The posterior median is: 11.6
The 95 % credible interval ranges from: 11.45 to 11.76

6.10.4. Inputs and Outputs for 25(OH)D_2 in SRM 2969 on Day 1

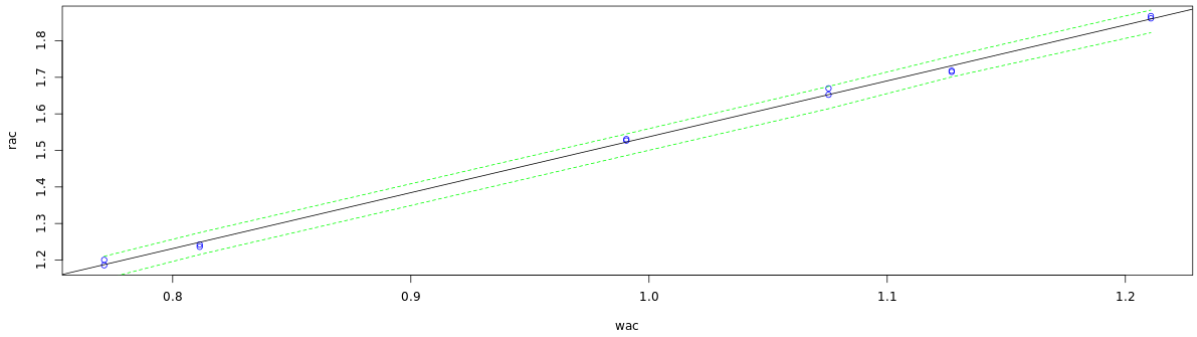
Calibration Data

mid	mad	wsol	rac1	rac2
18.595770	0.15155	1	1.200306	1.185759
8.641400	0.07406	2	1.236152	1.242236
18.828720	0.19657	3	1.526946	1.530758
8.740407	0.09932	1	1.652299	1.669725
19.513190	0.23231	2	1.714697	1.718121
8.835481	0.11275	3	1.861290	1.867550

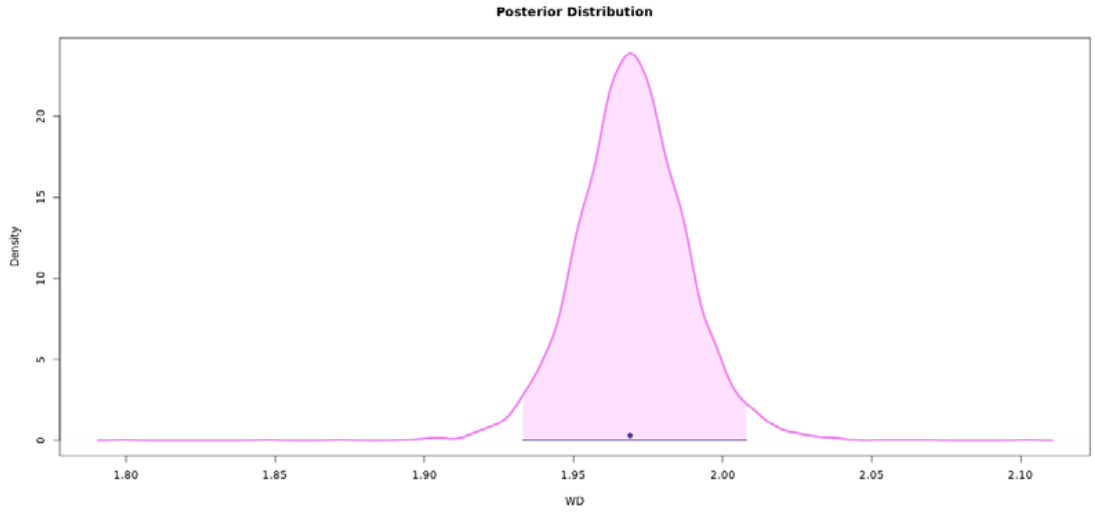
Sample Data

mids	mdi	ras1	ras2
1.873272	0.85230	1.383721	1.405594
1.903652	0.97314	1.528226	1.548736
1.890757	0.92957	1.473913	1.480565
1.892506	0.98312	1.575510	1.554770
1.895565	0.94563	1.507634	1.516245
1.896440	0.94821	1.527881	1.515901

mid (mass internal standard in ng); mad (mass analyte solution in g); wsol (# of working solution); rac1 (analyte/internal standard area ratio injection 1 calibrant); rac2 (analyte/internal standard area ratio injection 2 calibrant); mids (mass internal standard added to sample in ng); midi (mass serum in g); ras1 (analyte/internal standard area ratio injection 1 of serum); ras2 (analyte/internal standard area ratio injection 2 of serum)



R-squared: 0.9982
Intercept Mean: 0.00759
Intercept sd: 0.03085
Slope Mean: 1.52978
Slope sd: 0.03090



The posterior mean is: 1.97
The standard uncertainty is: 0.019
The posterior median is: 1.969
The 95 % credible interval ranges from: 1.933 to 2.008

6.10.5. Inputs and Outputs for 25(OH)D_2 in SRM 2969 on Day 2

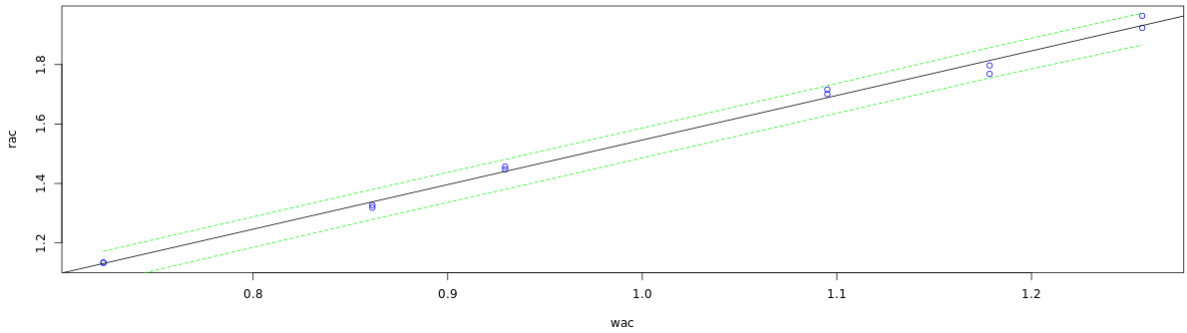
Calibration Data

mid	mad	wsol	rac1	rac2
19.525900	0.14952	1	1.134307	1.132804
8.469701	0.07679	2	1.318452	1.327273
19.273720	0.18911	3	1.447197	1.456876
8.429703	0.09776	1	1.700658	1.715909
18.966310	0.23528	2	1.796982	1.768632
8.687394	0.11526	3	1.963855	1.923274

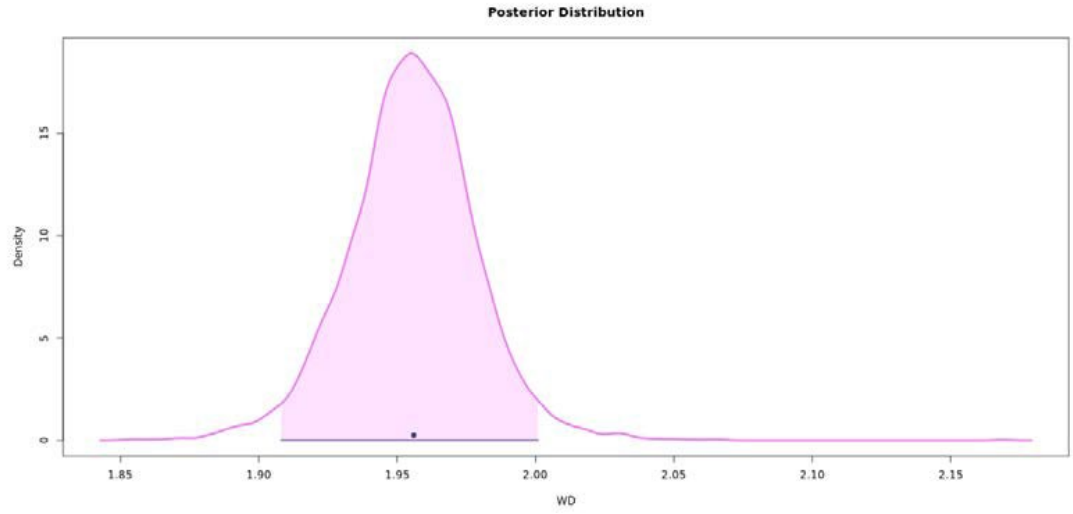
Sample Data

mids	mdi	ras1	ras2
1.832839	0.96418	1.597561	1.582090
1.835462	0.99108	1.651822	1.646341
1.857973	0.92339	1.494071	1.500000
1.829998	1.00853	1.662963	1.654545
1.835462	0.96024	1.569767	1.565693
1.876114	0.98854	1.590000	1.594595

mid (mass internal standard in ng); mad (mass analyte solution in g); wsol (# of working solution); rac1 (analyte/internal standard area ratio injection 1 calibrant); rac2 (analyte/internal standard area ratio injection 2 calibrant); mids (mass internal standard added to sample in ng); midi (mass serum in g); ras1 (analyte/internal standard area ratio injection 1 of serum); ras2 (analyte/internal standard area ratio injection 2 of serum)



R-squared: 0.9945
Intercept Mean: 0.04625
Intercept sd: 0.04378
Slope Mean: 1.49992
Slope sd: 0.04292



The posterior mean is: 1.955
The standard uncertainty is: 0.023
The posterior median is: 1.956
The 95 % credible interval ranges from: 1.908 to 2.001

6.10.6. Inputs and Outputs for 25(OH)D_3 in SRM 2970 on Day 1

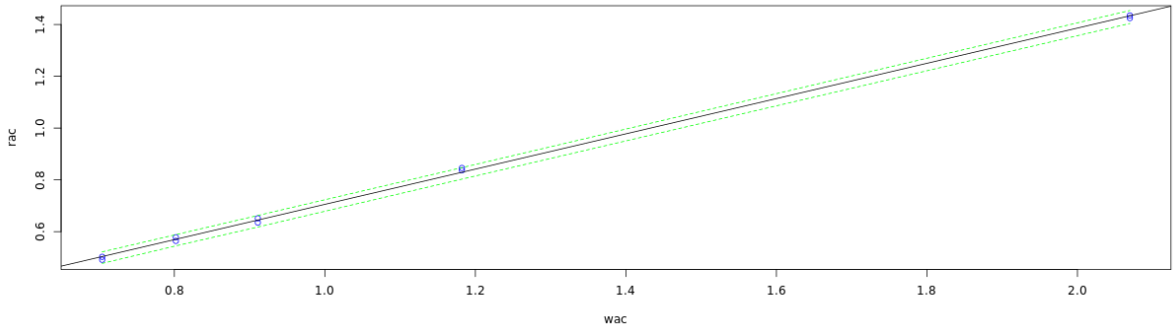
Calibration Data

mid	mad	wsol	rac1	rac2
40.05774	0.09125	1	0.491525	0.502890
40.40047	0.10971	2	0.564286	0.578348
41.17292	0.11932	3	0.635088	0.651558
41.52261	0.15615	3	0.836957	0.845921
41.90362	0.28057	1	1.424749	1.434783

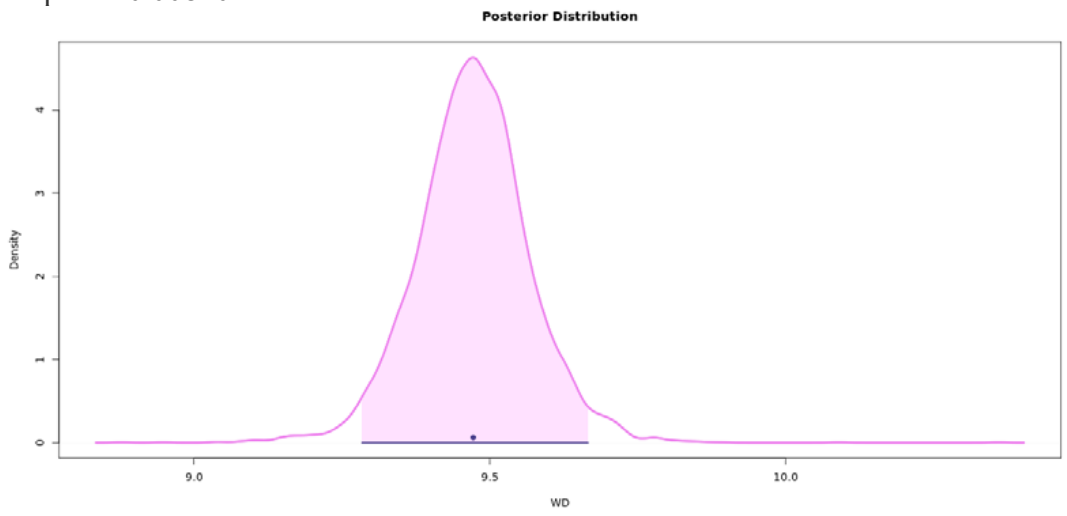
Sample Data

mids	mdi	ras1	ras2
9.64691	0.84440	0.577947	0.577947
9.43640	0.98121	0.702479	0.699187
9.59472	1.04400	0.729008	0.730038
9.57558	0.88257	0.617021	0.623932
9.62778	0.96632	0.675000	0.677966
9.90614	0.96785	0.643110	0.653571

mid (mass internal standard in ng); mad (mass analyte solution in g); wsol (# of working solution); rac1 (analyte/internal standard area ratio injection 1 calibrant); rac2 (analyte/internal standard area ratio injection 2 calibrant); mids (mass internal standard added to sample in ng); midi (mass serum in g); ras1 (analyte/internal standard area ratio injection 1 of serum); ras2 (analyte/internal standard area ratio injection 2 of serum)



R-squared: 0.9993
Intercept Mean: 0.02462
Intercept sd: 0.01003
Slope Mean: 0.68086
Slope sd: 0.00840



The posterior mean is: 9.471
The standard uncertainty is: 0.097
The posterior median is: 9.472
The 95 % credible interval ranges from: 9.284 to 9.666025

6.10.7. Inputs and Outputs for 25(OH)D_3 in SRM 2970 on Day 2

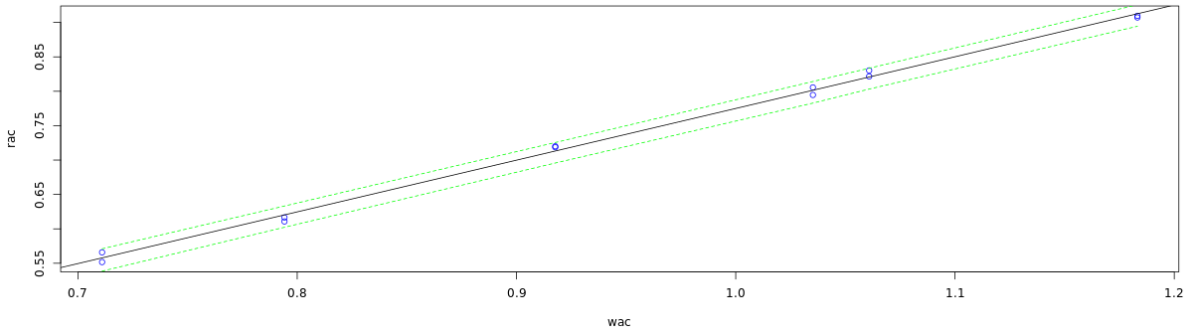
Calibration Data

mid	mad	wsol	rac1	rac2
39.624700	0.09099	1	0.551685	0.565854
40.740420	0.10956	2	0.610778	0.616580
41.045980	0.12002	3	0.720000	0.718631
40.008830	0.13376	1	0.794643	0.805556
42.116300	0.15129	2	0.830303	0.821622
41.569790	0.15672	3	0.907285	0.909677

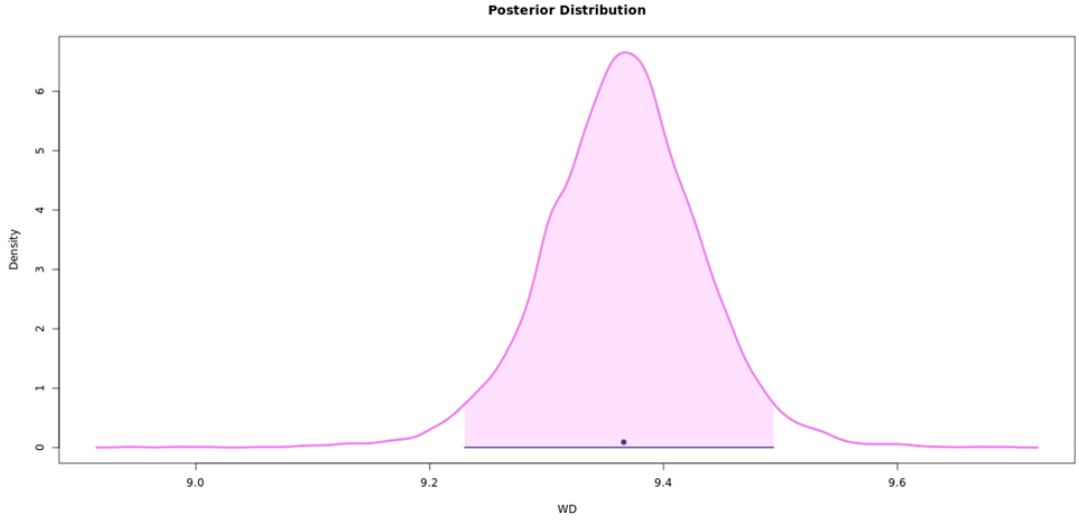
Sample Data

mids	mdi	ras1	ras2
9.10387	0.87500	0.696552	0.703448
9.11784	0.87435	0.693571	0.704255
9.93673	0.83607	0.608075	0.605405
9.11434	0.98905	0.796262	0.785185
9.31689	1.06572	0.830769	0.839416
9.06371	0.95125	0.755245	0.763889

mid (mass internal standard in ng); mad (mass analyte solution in g); wsol (# of working solution); rac1 (analyte/internal standard area ratio injection 1 calibrant); rac2 (analyte/internal standard area ratio injection 2 calibrant); mids (mass internal standard added to sample in ng); midi (mass serum in g); ras1 (analyte/internal standard area ratio injection 1 of serum); ras2 (analyte/internal standard area ratio injection 2 of serum)



R-squared: 0.9973
Intercept Mean: 0.02337
Intercept sd: 0.01432
Slope Mean: 0.75167
Slope sd: 0.01502



The posterior mean is: 9.365
The standard uncertainty is: 0.067
The posterior median is: 9.366
The 95 % credible interval ranges from: 9.23 to 9.494025

6.10.8. Inputs and Outputs for 25(OH)D_2 in SRM 2970 on Day 1

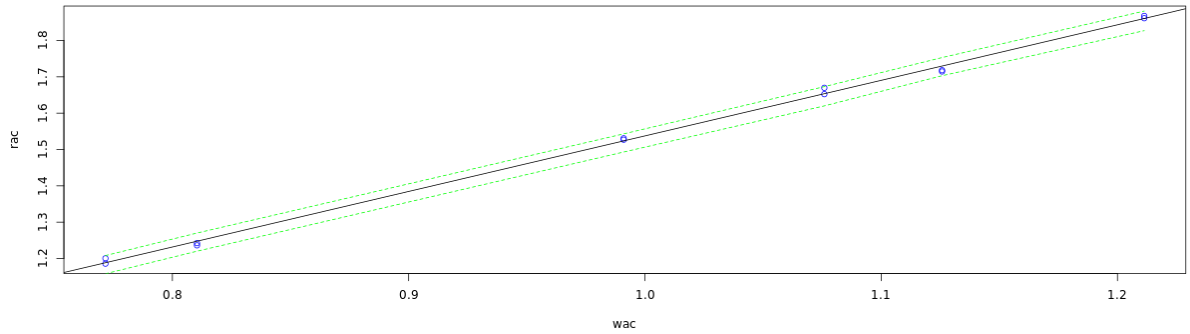
Calibration Data

mid	mad	wsol	rac1	rac2
18.595770	0.15155	1	1.200306	1.185759
8.641400	0.07406	2	1.236152	1.242236
18.828720	0.19657	3	1.526946	1.530758
8.740407	0.09932	1	1.652299	1.669725
19.513190	0.23231	2	1.714697	1.718121
8.835481	0.11275	3	1.861290	1.867550

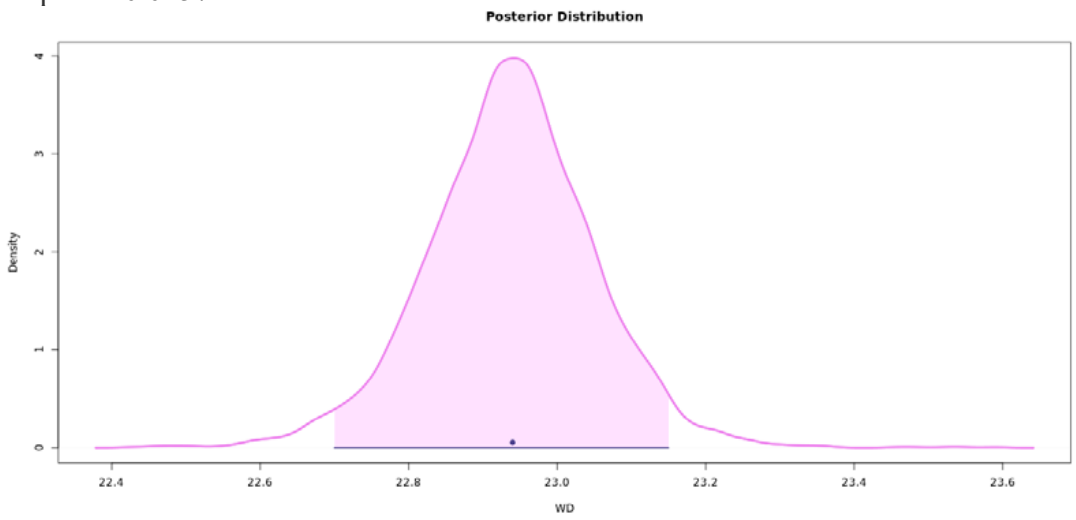
Sample Data

mids	mdi	ras1	ras2
20.78605	0.84440	1.457286	1.438424
20.96857	0.98121	1.645933	1.650943
20.95176	1.04400	1.753247	1.763485
21.11027	0.88257	1.476190	1.467890
21.13668	0.96632	1.600000	1.599078
20.88692	0.96785	1.629108	1.639130

mid (mass internal standard in ng); mad (mass analyte solution in g); wsol (# of working solution); rac1 (analyte/internal standard area ratio injection 1 calibrant); rac2 (analyte/internal standard area ratio injection 2 calibrant); mids (mass internal standard added to sample in ng); midi (mass serum in g); ras1 (analyte/internal standard area ratio injection 1 of serum); ras2 (analyte/internal standard area ratio injection 2 of serum)



R-squared: 0.9986
Intercept Mean: 0.00842
Intercept sd: 0.02554
Slope Mean: 1.52906
Slope sd: 0.02571



The posterior mean is: 22.94
The standard uncertainty is: 0.11
The posterior median is: 22.94
The 95 % credible interval ranges from: 22.7 to 23.15

6.10.9. Inputs and Outputs for 25(OH)D_2 in SRM 2970 on Day 2

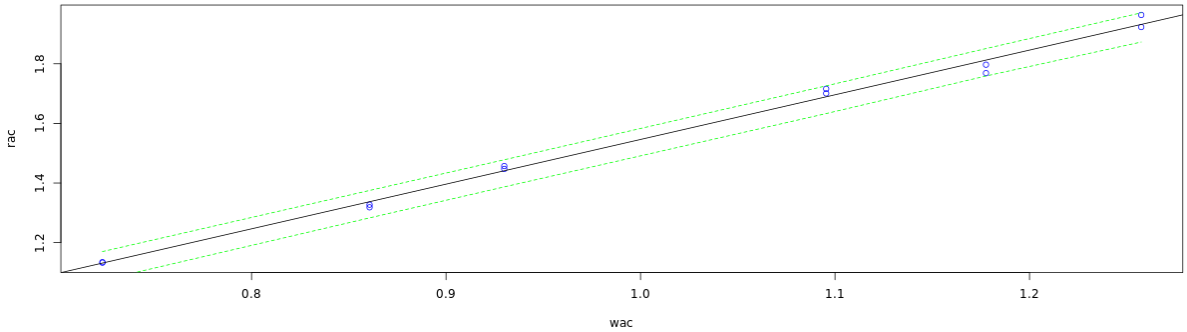
Calibration Data

mid	mad	wsol	rac1	rac2
19.525900	0.14952	1	1.134307	1.132804
8.469701	0.07679	2	1.318452	1.327273
19.273720	0.18911	3	1.447197	1.456876
8.429703	0.09776	1	1.700658	1.715909
18.966310	0.23528	2	1.796982	1.768632
8.687394	0.11526	3	1.963855	1.923274

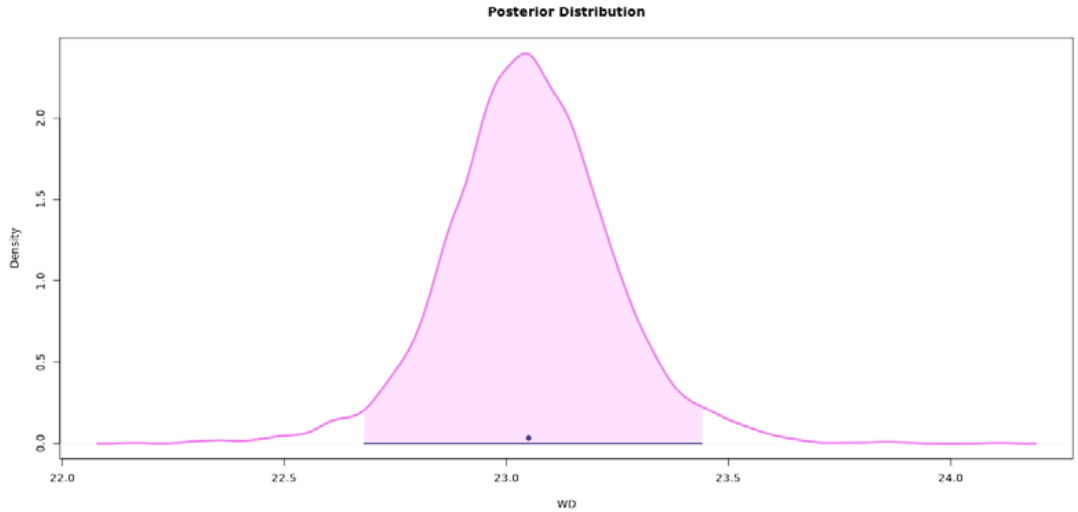
Sample Data

mids	mdi	ras1	ras2
20.94455	0.87500	1.510753	1.492147
20.93735	0.87435	1.474747	1.500000
20.35135	0.83607	1.457983	1.448980
20.32253	0.98905	1.734463	1.715847
20.38978	1.06572	1.872832	1.857143
20.41380	0.95125	1.655738	1.649746

mid (mass internal standard in ng); mad (mass analyte solution in g); wsol (# of working solution); rac1 (analyte/internal standard area ratio injection 1 calibrant); rac2 (analyte/internal standard area ratio injection 2 calibrant); mids (mass internal standard added to sample in ng); midi (mass serum in g); ras1 (analyte/internal standard area ratio injection 1 of serum); ras2 (analyte/internal standard area ratio injection 2 of serum)



R-squared: 0.9949
Intercept Mean: 0.04710
Intercept sd: 0.04094
Slope Mean: 1.49912
Slope sd: 0.04031



The posterior mean is: 23.05
The standard uncertainty is: 0.18
The posterior median is: 23.05
The 95 % credible interval ranges from: 22.68 to 23.44

7. Statistician's Report for SRM 2969 and 2970

Because for each SRM there were two different data sets with separate calibration runs, results described in Section 6.10 were obtained using a two-step procedure. First, the ABACUS App [9] was run separately for each of the two data sets and then in the second step the results were combined using the NIST Consensus Builder [14]. Because this process did not fully account for correlations between the two sets of results, it was important to validate the results of the two-step procedure. This was done by showing that a Bayesian hierarchical model, which directly accounted for the correlation, yielded the same results. The implementation of the Bayesian model was in OpenBUGS and the code is given in Section 7.2.

7.1. Results

7.1.1. Values in ng/g

The results obtained in Section 6.10 and verified by the Bayesian analysis were as follows:

- For 25(OH)D_2 in SRM 2969, the estimated mean value was 1.962 ng/g with a standard uncertainty of 0.023 ng/g and a 95 % confidence interval of [1.922, 2.000] ng/g.
- For 25(OH)D_3 in SRM 2969, the estimated mean value was 11.66 ng/g with a standard uncertainty of 0.136 ng/g and a 95 % confidence interval of [11.46, 11.92] ng/g.
- For 25(OH)D_2 in SRM 2970, the estimated mean value was 22.98 ng/g with a standard uncertainty of 0.153 ng/g and a 95 % confidence interval of [22.71, 23.27] ng/g.
- For 25(OH)D_3 in SRM 2970, the mean value was 9.416 ng/g with a standard uncertainty of 0.121 ng/g and a 95 % confidence interval of [9.214, 9.618] ng/g.

7.1.2. Values in ng/g for Total 25[OH]D

The 25(OH)D = 25(OH)D_2 + 25(OH)D_3 is needed for each SRM.

- For SRM 2969, the total is 13.62 ng/g with standard uncertainty of 0.14 ng/g and 95 % uncertainty interval of [13.35, 13.89] ng/g.
- For SRM 2970, the total is 32.40 ng/g with standard uncertainty of 0.20 ng/ and 95 % uncertainty interval of [32.01, 32.78] ng/g.

7.1.3. Values in ng/mL

The certified values are to be in ng/mL as well as ng/g. This transformation is obtained by multiplication of the ng/g value by the density in g/mL.

For SRM 2969 the density is 1.02353 g/mL with standard uncertainty of 0.00013 g/mL. Using the NIST Uncertainty Machine [15]:

- the 25(OH)D_2 in SRM 2969 is 2.008 ng/mL with standard uncertainty of 0.024 ng/mL and 95 % uncertainty interval of [1.962, 2.054] ng/mL.
- the 25(OH)D_3 in SRM 2969 is 11.93 ng/mL with standard uncertainty of 0.14 ng/mL and 95 % uncertainty interval of [11.66, 12.21] ng/mL.
- The 25(OH)D in SRM 2969 is 13.94 ng/mL with standard uncertainty of 0.14 ng/mL and 95 % uncertainty interval of [13.66, 14.21] ng/mL.

For SRM 2970 the density is 1.02229 g/mL with standard uncertainty of 0.00077 g/mL. Using the NIST Uncertainty Machine [15]:

- The 25(OH)D_2 in SRM 2970 is 23.49 ng/mL with standard uncertainty of 0.16 ng/mL and 95 % uncertainty interval of [23.19, 23.80] ng/mL.
- The 25(OH)D_3 in SRM 2970 is 9.626 ng/mL with standard uncertainty of 0.157 ng/mL and 95 % uncertainty interval of [9.318, 9.933] ng/mL.
- The 25(OH)D in SRM 2970 is 33.12 ng/mL with standard uncertainty of 0.22 ng/mL and 95 % uncertainty interval of [32.68, 33.55] ng/mL.

7.1.4. Values in nmol/L

The certified values are to be in nmol/L as well as ng/g. For 25(OH)D_3 and 25(OH)D_2, this transformation is obtained by division of the ng/mL value by the molar mass of the metabolite expressed in grams per nanomole. The molar mass of 25(OH)D_3 is (0.400636 ± 0.000016) g/nmol, that of 25(OH)D_2 is (0.412647 ± 0.000016) g/nmol [10]. The values for 25(OH)D are obtained by summing the nmol/L results of 25(OH)D_3 and 25(OH)D_2.

Using the NIST Uncertainty Machine [15]:

- the 25(OH)D_2 in SRM 2969 is 4.866 nmol/L with standard uncertainty of 0.057 nmol/L and 95 % uncertainty interval of [4.754, 4.978] nmol/L.
- the 25(OH)D_3 in SRM 2969 is 29.78 nmol/L with standard uncertainty of 0.35 nmol/L and 95 % uncertainty interval of [29.10, 30.46] nmol/L.
- The 25(OH)D in SRM 2969 is 34.65 nmol/L with standard uncertainty of 0.35 nmol/L and 95 % uncertainty interval of [33.95, 35.34] nmol/L.
- The 25(OH)D_2 in SRM 2970 is 56.93 nmol/L with standard uncertainty of 0.38 nmol/L and 95 % uncertainty interval of [56.17, 57.68] nmol/L.
- The 25(OH)D_3 in SRM 2970 is 24.03 nmol/L with standard uncertainty of 0.39 nmol/L and 95 % uncertainty interval of [23.26, 24.80] nmol/L.
- The 25(OH)D in SRM 2970 is 80.96 nmol/L with standard uncertainty of 0.55 nmol/L and 95 % uncertainty interval of [79.88, 82.04] nmol/L.

7.2. OpenBUGS Bayesian Hierarchical Model

The following code and data sets analyzes two sets of data with their own calibration runs to produce a consensus value in ng/g for each of the four analytes.

7.2.1. Code

```
{#calculate known mass ratios wac for N calibrants.
midsprec1<-1/(umids1*umids1); madsprec1<-1/(umads1*umads1)

for(i in 1:NWS1){wadprec1[i]<-1/(uwad1[i]*uwad1[i]); wad1[i]~dnorm(wadm1[i],wadprec1[i])}

for(i in 1:N1){mids1[i]~dnorm(midsm1[i],midsprec1); mads1[i]~dnorm(madsm1[i],madsprec1)}
for(i in 1:N1){wac1[i]<-wad1[wsol1[i]]*mads1[i]/mids1[i]}

# Calibration equation
a1~dnorm(0,1.0E-5); b1~dnorm(0,1.0E-5)

xins1~dnorm(0,0.0016)I(0.001,); chsqns1~dgamma(0.5,0.5); fitprec1<-xins1/sqrt(chsqns1)

for(i in 1:N1){mean1[i]<-a1+b1*wac1[i]}
for(i in 1:NT1){rac1[i]~dnorm(mean1[sol1[i]],fitprec1)}

# Compute the mass fraction wd
a.cut1<-cut(a1); b.cut1<-cut(b1)
sigras1~dgamma(1.0E-5,1.0E-5); wdsig1~dgamma(1.0E-3,1.0E-3)
```

```
wd[1]~dnorm(0,1.0E-5)
for(i in 1:M1){wdm1[i]~dnorm(wd[1],wdsig1)
    rasmean1[i]<-a.cut1+b.cut1*wdm1[i]*md1[i]/midsi1[i]
    rasmeanp1[i]~dnorm(rasmean1[i],fitprec1)}
for(i in 1:MT1){ras1[i]~dnorm(rasmeanp1[sampl1[i]],sigras1)}

#######################################################################
midsprec2<-1/(umids2*umids2); madsprec2<-1/(umads2*umads2)
for(i in 1:NWS2){wadprec2[i]<-1/(uwad2[i]*uwad2[i]); wad2[i]~dnorm(wadm2[i],wadprec2[i])}

for(i in 1:N2){mids2[i]~dnorm(midsm2[i],midsprec2); mads2[i]~dnorm(madsm2[i],madsprec2)}
for(i in 1:N2){wac2[i]<-wad1[wsol2[i]]*mads2[i]/mids2[i]}

#calibration equation
a2~dnorm(0,1.0E-5); b2~dnorm(0,1.0E-5)

xins2~dnorm(0,0.0016)I(0.001,); chsqns2~dgamma(0.5,0.5); fitprec2<-xins2/sqrt(chsqns2)

for(i in 1:N2){mean2[i]<-a2+b2*wac2[i]}
for(i in 1:NT2){rac2[i]~dnorm(mean2[sol2[i]],fitprec2)}

# Compute the mass fraction wd
a.cut2<-cut(a2); b.cut2<-cut(b2)
sigras2~dgamma(1.0E-5,1.0E-5); wdsig2~dgamma(1.0E-3,1.0E-3)

wd[2]~dnorm(0,1.0E-5)
for(i in 1:M2){wdm2[i]~dnorm(wd[2],wdsig2)
    rasmean2[i]<-a.cut2+b.cut2*wdm2[i]*md2[i]/midsi2[i]
    rasmeanp2[i]~dnorm(rasmean2[i],fitprec2)}
for(i in 1:MT2){ras2[i]~dnorm(rasmeanp2[sampl2[i]],sigras2)}

#######################################################################
dns~dnorm(1.02353,236686390); T~dcat(P[]); P[1:2]~ddirich(alpha[])
for(i in 1:2){alpha[i]<-1}
mumeanfin<-wd[T]; cmumean<-dns*mumeanfin}

list(sigras1=1,wdsig1=1,a1=0,b1=1,sigras2=1,a2=0,b2=1,wdsig2=1)
```

7.2.2. Data for 25(OH)D_2 in SRM 2969

```
list(
midsm1=c(18.59577,8.6414,18.82872,8.740407,19.51319,8.835481),
umids1=0.000015,madsm1=c(0.15155,0.07406,0.19657,0.09932,0.23231,0.11275),umads1=0.000015,
rac1=c(1.200306,1.236152,1.526946,1.652299,1.714697,1.86129,1.185759,1.242236,1.530758,1.669725,1.718121,1.86755),
sol1=c(1,2,3,4,5,6,1,2,3,4,5,6), sampl1=c(1,2,3,4,5,6,1,2,3,4,5,6),
ras1=c(1.383721,1.528226,1.473913,1.57551,1.507634,1.527881,1.405594,1.548736,1.480565,1.55477,1.516245,1.515901),
midsi1=c(1.873272,1.903652,1.890757,1.892506,1.895565,1.89644),md1=c(0.8523,0.97314,0.92957,0.98312,0.94563,0.94821),
wadm1=c(94.2,95.5,94.5),uwad1=c(0.427,0.434,0.429),wsol1=c(1,2,3,1,2,3),NWS1=3,N1=6,M1=6,MT1=12,NT1=12,

midsm2=c(19.5259,8.469701,19.27372,8.429703,18.96631,8.687394),
umids2=0.000015,madsm2=c(0.14952,0.07679,0.18911,0.09776,0.23528,0.11526),umads2=0.000015,
rac2=c(1.134307,1.318452,1.447197,1.700658,1.796982,1.963855,1.132804,1.327273,1.456876,1.715909,1.768632,1.923274),
sol2=c(1,2,3,4,5,6,1,2,3,4,5,6), sampl2=c(1,2,3,4,5,6,1,2,3,4,5,6),
ras2=c(1.597561,1.651822,1.494071,1.662963,1.569767,1.59,1.58209,1.646341,1.5,1.654545,1.565693,1.594595),
midsi2=c(1.832839,1.835462,1.857973,1.829998,1.835462,1.876114),md2=c(0.96418,0.99108,0.92339,1.00853,0.96024,0.98854),
wadm2=c(94.2,95.5,94.5),uwad2=c(0.427,0.434,0.429),wsol2=c(1,2,3,1,2,3),NWS2=3,N2=6,M2=6,MT2=12,NT2=12
)
```

7.2.3. Data for 25(OH)D_3 in SRM 2969

```
list(
midsm1=c(40.05774,40.40047,41.17292,41.52261,41.90362),
umids1=0.000015,madsm1=c(0.09125,0.10971,0.11932,0.15615,0.28057),umads1=0.000015,
rac1=c(0.491525,0.564286,0.635088,0.836957,1.424749,0.50289,0.578348,0.651558,0.845921,1.434783),
sol1=c(1,2,3,4,5,1,2,3,4,5), sampl1=c(1,2,3,4,5,6,1,2,3,4,5,6),
ras1=c(0.567797,0.674419,0.615584,0.684066,0.640103,0.663239,0.570248,0.67341,0.617801,0.68984,0.635135,0.656642),
midsi1=c(12.34179,12.11214,12.40442,11.96948,12.21652,11.98166),md1=c(0.8523,0.97314,0.92957,0.98312,0.94563,0.94821),
wadm1=c(309.7,295.3,313.8),uwad1=c(1.047,0.998,1.06),wsol1=c(1,2,3,3,1),NWS1=3,N1=5,M1=6,MT1=12,NT1=10,

midsm2=c(39.6247,40.74042,41.04598,40.00883,42.1163,41.56979),
umids2=0.000015,madsm2=c(0.09099,0.10956,0.12002,0.13376,0.15129,0.15672),umads2=0.000015,
rac2=c(0.551685,0.610778,0.72,0.794643,0.830303,0.907285,0.565854,0.61658,0.718631,0.805556,0.821622,0.909677),
sol2=c(1,2,3,4,5,6,1,2,3,4,5,6), sampl2=c(1,2,3,4,5,6,1,2,3,4,5,6),
ras2=c(0.721774,0.733607,0.681481,0.739623,0.724409,0.7251,0.711297,0.732,0.685039,0.732824,0.7251,0.716846),
midsi2=c(12.21357,12.28341,12.14722,12.18563,12.03198,12.27992),md2=c(0.96418,0.99108,0.92339,1.00853,0.96024,0.98854),
wadm2=c(309.7,295.3,313.8),uwad2=c(1.047,0.998,1.06),wsol2=c(1,2,3,1,2,3),NWS2=3,N2=6,M2=6,MT2=12,NT2=12
)
```

7.2.4. Data for 25(OH)D_2 in SRM 2970

```
list(
midsm1=c(18.59577,8.6414,18.82872,8.740407,19.51319,8.835481),
umids1=0.000015,madsm1=c(0.15155,0.07406,0.19657,0.09932,0.23231,0.11275),umads1=0.000015,
rac1=c(1.200306,1.236152,1.526946,1.652299,1.714697,1.86129,1.185759,1.242236,1.530758,1.669725,1.718121,1.86755),
sol1=c(1,2,3,4,5,6,1,2,3,4,5,6), sampl1=c(1,2,3,4,5,6,1,2,3,4,5,6),
ras1=c(1.457286,1.645933,1.753247,1.47619,1.6,1.629108,1.438424,1.650943,1.763485,1.46789,1.599078,1.63913),
midsi1=c(20.78605,20.96857,20.95176,21.11027,21.13668,20.88692),md1=c(0.8444,0.98121,1.044,0.88257,0.96632,0.96785),
wadm1=c(94.2,95.5,94.5),uwad1=c(0.427,0.434,0.429),wsol1=c(1,2,3,1,2,3),NWS1=3,N1=6,M1=6,MT1=12,NT1=12,

midsm2=c(19.5259,8.469701,19.27372,8.429703,18.96631,8.687394),
umids2=0.000015,madsm2=c(0.14952,0.07679,0.18911,0.09776,0.23528,0.11526),umads2=0.000015,
rac2=c(1.134307,1.318452,1.447197,1.700658,1.796982,1.963855,1.132804,1.327273,1.456876,1.715909,1.768632,1.923274),
sol2=c(1,2,3,4,5,6,1,2,3,4,5,6), sampl2=c(1,2,3,4,5,6,1,2,3,4,5,6),
ras2=c(1.510753,1.474747,1.457983,1.734463,1.872832,1.655738,1.492147,1.5,1.44898,1.715847,1.857143,1.649746),
midsi2=c(20.94455,20.93735,20.35135,20.32253,20.38978,20.4138),md2=c(0.875,0.87435,0.83607,0.98905,1.06572,0.95125),
wadm2=c(94.2,95.5,94.5),uwad2=c(0.427,0.434,0.429),wsol2=c(1,2,3,1,2,3),NWS2=3,N2=6,M2=6,MT2=12,NT2=12
)
```

7.2.5. Data for 25(OH)D_3 in SRM 2970

```
list(
midsm1=c(40.05774,40.40047,41.17292,41.52261,41.90362),
umids1=0.000015,madsm1=c(0.09125,0.10971,0.11932,0.15615,0.28057),umads1=0.000015,
rac1=c(0.491525,0.564286,0.635088,0.836957,1.424749,0.50289,0.578348,0.651558,0.845921,1.434783),
sol1=c(1,2,3,4,5,1,2,3,4,5), sampl1=c(1,2,3,4,5,6,1,2,3,4,5,6),
ras1=c(0.577947,0.702479,0.729008,0.617021,0.675,0.64311,0.577947,0.699187,0.730038,0.623932,0.677966,0.653571),
midsi1=c(9.646913,9.436404,9.594721,9.575584,9.627776,9.906136),md1=c(0.8444,0.98121,1.044,0.88257,0.96632,0.96785),
wadm1=c(309.7,295.3,313.8),uwad1=c(1.047,0.998,1.06),wsol1=c(1,2,3,3,1),NWS1=3,N1=5,M1=6,MT1=12,NT1=10,

midsm2=c(39.6247,40.74042,41.04598,40.00883,42.1163,41.56979),
umids2=0.000015,madsm2=c(0.09099,0.10956,0.12002,0.13376,0.15129,0.15672),umads2=0.000015,
rac2=c(0.551685,0.610778,0.72,0.794643,0.830303,0.907285,0.565854,0.61658,0.718631,0.805556,0.821622,0.909677),
sol2=c(1,2,3,4,5,6,1,2,3,4,5,6), sampl2=c(1,2,3,4,5,6,1,2,3,4,5,6),
ras2=c(0.696552,0.693571,0.608075,0.796262,0.830769,0.755245,0.703448,0.704255,0.605405,0.785185,0.839416,0.763889),
midsi2=c(9.103868,9.117837,9.936731,9.114344,9.316885,9.063709),md2=c(0.875,0.87435,0.83607,0.98905,1.06572,0.95125),
wadm2=c(309.7,295.3,313.8),uwad2=c(1.047,0.998,1.06),wsol2=c(1,2,3,1,2,3),NWS2=3,N2=6,M2=6,MT2=12,NT2=12
)
```

8. Certifiable Values

Table 11 summarizes the certifiable values for 25(OH)D_3, 25(OH)D_2, and 25(OH)D in SRMs 2969 and 2970. These values echo the results presented in Section 7.1. These are not the final certified values, which are only available in the current Certificate of Analysis available on the NIST SRM website.

Table 11. Certifiable 25(OH)D_3 and 25(OH)D_2 Results for SRM 2969 and 2970

SRM	Analyte	Units	$w \pm w(u)$	U_{95}
2969	25(OH)D_3	ng/g	11.66 ± 0.14	[11.46, 11.92]
		ng/mL	11.93 ± 0.14	[11.66, 12.21]
		nmol/L	29.78 ± 0.35	[29.10, 30.46]
	25(OH)D_2	ng/g	1.962 ± 0.023	[1.922, 2.000]
		ng/mL	2.008 ± 0.024	[1.962, 2.054]
		nmol/L	4.866 ± 0.057	[4.754, 4.978]
	25(OH)D	ng/g	13.62 ± 0.14	[13.35, 13.89]
		ng/mL	13.94 ± 0.14	[13.66, 14.21]
		nmol/L	34.65 ± 0.35	[33.95, 35.34]
2970	25(OH)D_3	ng/g	9.416 ± 0.121	[9.214, 9.618]
		ng/mL	9.626 ± 0.157	[9.318, 9.933]
		nmol/L	24.03 ± 0.39	[23.26, 24.80]
	25(OH)D_2	ng/g	22.98 ± 0.15	[22.71, 23.27]
		ng/mL	23.49 ± 0.16	[23.19, 23.80]
		nmol/L	56.93 ± 0.38	[56.17, 57.68]
	25(OH)D	ng/g	32.40 ± 0.20	[32.01, 32.78]
		ng/mL	33.12 ± 0.22	[32.68, 33.55]
		nmol/L	80.96 ± 0.55	[79.88, 82.04]

Table 12 summarizes the certifiable values for density in SRMs 2969 and 2970.

Table 12. Certifiable Density Results for SRM 2969 and 2970

SRM	Measurand	Units	$x \pm U_{95}(x)$
2969	Density at 23 °C	g/mL	1.0235 ± 0.0003
2970	Density at 23 °C	g/mL	1.0223 ± 0.0015

9. Data

Electronic files containing certified values and their uncertainties, and the data used to assign those values are available to registered users of the SRMs. To register, visit **https://www.nist.gov/srm**.

The following files contain inputs to the ABACUS app that were used to calculate the certified values as shown in section 6.10 and assigned values.

SRM 2969 24OHD2 SED_01232020.xlsx (This file contains the data shown in sections 6.10.1-6.10.3)
SRM 2969 24OHD3 SED_01232020.xlsx (This file contains the data shown in sections 6.10.4-6.10.5)
Assigned_values_for_SRM_2969.xlsx

SRM 2970 25OHD2 SED_01232020.xlsx (This file contains the data shown in sections 6.10.6-6.10.7)
SRM 2970 25OHD3 SED_01232020.xlsx (This file contains the data shown in sections 6.10.8-6.10.9)
Assigned_values_for_SRM_2970.xlsx

Section 7 discusses how the calculations done by the ABACUS app in section 6.10 were confirmed using a separate statistical model using different software (OpenBUGS) described in 7.2. The same data as shown in the above Excel files was reformatted to be used by this software and is given in 7.2.2-7.2.5.

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