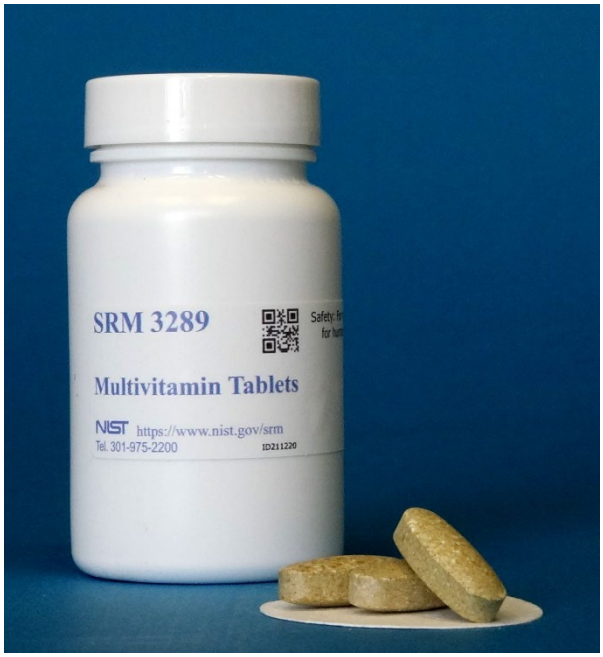
NIST Special Publication 260-220

Certification of Standard Reference Material® 3289
Multivitamin Tablets



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March 2022

U.S. Department of Commerce
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National Institute of Standards and Technology
James K. Olthoff, Performing the Non-Exclusive Functions and Duties of the Under Secretary of Commerce for Standards and Technology & Director, National Institute of Standards and Technology

National Institute of Standards and Technology Special Publication 260-220
Natl. Inst. Stand. Technol. Spec. Publ. 260-220, 64 pages (March 2022)
CODEN: NSPUE2

This publication is available free of charge from:
https://doi.org/10.6028/NIST.SP.260-220

Abstract

Standard Reference Material® (SRM®) 3289 Multivitamin Tablets was developed as part of a collaborative effort between the National Institute for Standards and Technology (NIST) and the National Institutes of Health Office of Dietary Supplements (NIH-ODS) as a partial replacement for SRM 3280 Multivitamin/Multielement Tablets. SRM 3289 was formulated with both vitamins and elements to replicate all analytical challenges associated with the measurement of vitamins in dietary supplement matrices. The material was purchased pre-packaged from Gemini Pharmaceuticals (Commack, NY), an experienced contract manufacturer. Certified values for fat-soluble and water-soluble vitamin-related measurands have been assigned based upon data obtained from NIST, manufacturer, and interlaboratory comparison measurements. A description of the material, sample preparations, results, and data analysis are discussed in the following report.

Keywords

Dietary Supplements;
Fat-Soluble Vitamins;
Reference Material;
Vitamin Tablets,
Water-Soluble Vitamins

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

CNCbl	cyanocobalamin
COSY	^{1}H-^{1}H correlation (COSY) NMR spectroscopy
D1	NMR pulse recycle delay
DMMA	dimethylmalonic acid
DMSO	dimethyl sulfone
EDTA	ethylenediamine tetraacetic acid disodium salt dihydrate
GARP	globally-optimized, alternating-phase, rectangular pulse
HAMQAP	Health Assessment Measurement Quality Assurance Program
HSQC	heteronuclear single quantum coherence
HNO_3	nitric acid
HPLC	high performance liquid chromatography
HR-MS	high-resolution mass spectrometry
ICP-MS	inductively coupled plasma mass spectrometer
ID-LC-MS/MS	isotope dilution liquid chromatography tandem mass spectrometry
IS	internal standard
KHP	potassium hydrogen phthalate
LC	liquid chromatography
LC-ICP-MS	liquid chromatography tandem inductively coupled plasma mass spectrometry
LDPE	low density polyethylene
NIH-ODS	National Institutes of Health Office of Dietary Supplements
NIST	National Institute of Standards and Technology
MRM	multiple reaction monitoring
NMR	nuclear magnetic resonance spectroscopy
^{1}H-NMR$_{IS}$	quantitative ^{1}H-NMR using an internal standard
SD	standard deviation
SI	International System of Units (Système International d'unités)
SOP	standard operating procedures
SRM	Standard Reference Material
T1	NMR spin lattice relaxation time
USP	United States Pharmacopeia

1 Introduction

SRM 3289 Multivitamin Tablets was developed as part of a collaborative effort between the National Institute of Standards and Technology (NIST) and the National Institutes of Health Office of Dietary Supplements (NIH-ODS). SRM 3289 is a partial replacement for SRM 3280 Multivitamin/Multielement Tablets [1,2] which has sold about 50 units/year since it was released in 2009 (Figure 1). Slightly more than half of these sales have been within the U.S. (Figure 2).

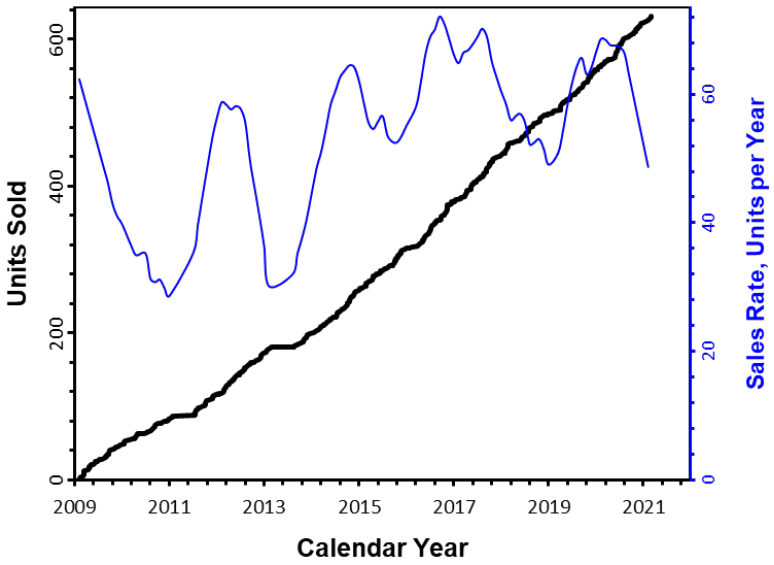

Figure 1. Sales History of SRM 3280 Multivitamin/Multielement Tablets.

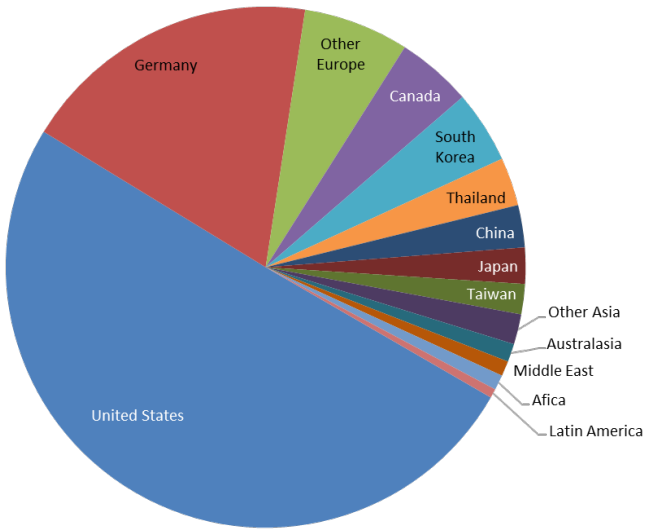

Figure 2. Customer Locations for SRM 3280 Multivitamin/Multielement Tablets.

However, vitamins in dietary supplement tablets have finite stability. Due to degradation of some of the vitamin-related measurands and increasingly difficult extraction of most, sale of SRM 3280 was halted in March 2021. While formulated with both vitamins and elements to replicate all analytical challenges associated with the measurement of vitamins in dietary supplement matrices, SRM 3289 provides certified values for just the vitamin-related measurands.

2 Material

2.1 Acquisition & Packaging

Two hundred fifty thousand (250 000) SRM 3289 tablets were purchased from Gemini Pharmaceuticals (Commack, NY). All tablets are from the same batch. The tablets have a microcrystalline cellulose matrix, are oval with dimension 0.927 cm by 2.223 cm (0.365 in by 0.875 in), beige in color, have a clear hydroxypropyl methylcellulose coating, and have a mean weight of (1.897 ± 0.005) gram per tablet.

The tablets were packed by the manufacturer into round, white, 100 mL, high-density polyethylene bottles, each containing 30 tablets. The bottles were capped with smooth, white, 38 mm lids with imprinted foam/foil liners. No cotton was included in the bottles. Each unit of SRM 3289 consists of five of these bottles.

The bottles were packed in 348 boxes, each box containing 24 bottles. The boxes were labeled sequentially from 1 to 348 in the order that the bottles were filled.

2.2 Storage

The SRM 3289 bottles have been stored at room temperature (18 °C to 22 °C) at NIST since their receipt.

2.3 Manufacturer's Analysis

Figure 3 reproduces the manufacturer's Certificate of Analysis. This table includes the acronyms: DFE = dietary folate equivalents, IU = International Unit, NE = niacin equivalents, and RAE = retinol activity equivalents.

Note: One microgram RAE is equivalent to 1 µg retinol and 2 µg supplemental beta-carotene [3]; 1 µg retinol is equivalent to (328.5 g/mol retinyl acetate)/(286.45 g/mol retinol)=1.147 µg retinyl acetate.

87 MODULAR AVENUE, COMMACK, NY 11725
631-543-3334 - 631-543-3335 –www.geminipharm.com

CERTIFICATE OF ANALYSIS

PRODUCT NAME:	Multivitamin Tablets
LOT NUMBER:	53970
DATE OF MANUFACTURE:	06/18
QUALITY ASSURANCE RELEASE DATE:	06/28/18

CHARACTERISTIC	SPECIFICATION	RESULT
Identification:	0.375" x 0.875" Oval Beige Tablet Film Coated Clear	Conforms
Average Weight:	1860mg – 2046mg	1897.0mg

COMPONENT	Label Claim: Per Tablet	Specification	% Label Claim
Vitamin A (as Retinyl Acetate)	1200 mcg RAE (4000IU)	80.0%-200.0%	130.5%
Vitamin A (as Beta Carotene)	420 mcg RAE (1400IU)	80.0%-200.0%	150.4%
Vitamin C (Ascorbic Acid)	70 mg	80.0%-200.0%	123.5%
Vitamin D2 (Ergocalciferol)	15 mcg (600IU)	80.0%-200.0%	134.7%
Vitamin D3 (Cholecalciferol)	15 mcg (600IU)	80.0%-200.0%	136.7%
Vitamin E (as dl-alpha Tocopheryl Acetate)	27 mg (30IU)	80.0%-200.0%	126.6%
Vitamin K1 (Phytonadione)	30 mcg	80.0%-200.0%	103.7%
Vitamin B1 (Thiamine from Thiamine HCl)	2 mg	80.0%-200.0%	117.4%
Vitamin B2 (Riboflavin)	2 mg	80.0%-200.0%	118.2%
Vitamin B3 (as Niacin)	20 mg NE (20mg)	80.0%-200.0%	116.9%
Vitamin B6 (Pyridoxine from Pyridoxine HCl)	2 mg	80.0%-200.0%	127.4%
Folate	1000 mcg DFE (600 mcg Folic Acid)	80.0%-200.0%	141.8%
Vitamin B12 (as Cyanocobalamin)	9 mcg	80.0%-200.0%	117.8%
Biotin (as D-Biotin)	40 mcg	80.0%-200.0%	101.5%
Vitamin B5 (Pantothenic Acid from d-Calcium Pantothenate)	10 mg	80.0%-200.0%	138.3%
Calcium (from DiCalcium Phosphate & Calcium Carbonate)	200 mg	80.0%-200.0%	115.5%
Iron (from Ferrous Fumarate)	20 mg	80.0%-200.0%	102.5%
Phosphorus (from Dicalcium Phosphate)	100 mg	80.0%-200.0%	113.0%
Iodine (from Potassium Iodide)	200 mcg	80.0%-200.0%	151.0%
Magnesium (from Magnesium Oxide)	100 mg	80.0%-200.0%	96.0%
Zinc (from Zinc Oxide)	20 mg	80.0%-200.0%	107.8%
Selenium (from Sodium Selenate)	30 mcg	80.0%-200.0%	81.2%
Copper (from Cupric Oxide)	2 mg	80.0%-200.0%	113.6%
Manganese (from Manganese Ascorbate)	2 mg	80.0%-200.0%	119.1%
Chromium (from Chromium Citrate)	140 mcg	80.0%-200.0%	125.3%
Molybdenum (from Sodium Molybdenum)	100 mcg	80.0%-200.0%	112.4%
Chloride (from Potassium Chloride)	80 mcg	80.0%-200.0%	107.5%
Potassium (from Potassium Chloride)	90 mcg	80.0%-200.0%	160.1%
Nickel (from Nickel Sulfate)	10 mcg	80.0%-200.0%	171.0%
Tin (from Tin Chelate)	10 mcg	80.0%-200.0%	114.0%
Vanadium (from Vanadyl Sulfate)	10 mcg	80.0%-200.0%	93.5%
Silicon (from Silicon Dioxide)	3 mg	80.0%-200.0%	165.3%
Lutein	350 mcg	80.0%-200.0%	109.7%

Figure 3. Manufacturer's Certificate of Analysis

3 NIST Measurements of Vitamins B_1, B_2, B_3, B_5, B_6, and B_7

The mass fractions of thiamine hydrochloride (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxine hydrochloride (B_6), and biotin (B_7) were determined by isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) [4].

3.1 Materials

One bottle from each of ten boxes of SRM 3289 (numbers 10, 42, 88, 111, 151, 185, 230, 260, 293, and 318) were selected based on a stratified random sampling scheme. The bottles were labeled with the box number. One bottle of SRM 3280 Multivitamin/Multielement Tablets was used as control.

Table 1 lists the calibrants used in the analysis of these B vitamins. The identity of these compounds was confirmed and their purity assessed using nuclear magnetic resonance spectroscopy (NMR) techniques (see Section 3.7.1). Table 2 lists the isotopically labeled standards. All solvents used were high-performance liquid chromatography (HPLC) grade. All other salts and acids used in sample and mobile phase preparation were reagent grade.

Table 1. Vitamin B_1, B_2, B_3, B_5, B_6, and B_7 Calibration Materials

Compound		Source	Lot Number	Assessed Purity, % Coverage Interval
B_1	Thiamine hydrochloride	U.S. Pharmacopeia (Rockville, MD)	#O1F236	93.38 to 97.34
B_2	Riboflavin	U.S. Pharmacopeia (Rockville, MD)	#N0C021	91.8 to 94.2
B_3	Niacin (Nicotinic acid)	U.S. Pharmacopeia (Rockville, MD)	#J0J235	99.12 to 99.97
B_5	Calcium pantothenate	U.S. Pharmacopeia (Rockville, MD)	#O1H081	94.37 to 95.12
B_6	Pyridoxine hydrochloride	U.S. Pharmacopeia (Rockville, MD)	#Q0G409	98.83 to 99.74
B_7	Biotin	Sigma-Aldrich (St. Louis, MO)	#073K07115	96.49 to 98.80

Table 2. Vitamin B_1, B_2, B_3, B_5, B_6, and B_7 Isotopically Labeled Standards

Labeled Compound		Source	Lot Number
B_1	Thiamine chloride ($^{13}C_4$)	Isosciences (King of Prussia, PA)	NM1-2019-241A1
B_2	Riboflavin ($^{13}C_4$, $^{15}N_2$)	Cambridge Isotope Laboratories (Andover, MA)	#I-24053F
B_3	Niacin ($^{2}H_4$)	Isosciences (King of Prussia, PA)	#RS2-2004-126A
B_5	Calcium pantothenate monohydrate ($^{13}C_6$,$^{15}N_2$)	Isosciences (King of Prussia, PA)	#RS9-2018-233A1
B_6	Pyridoxine hydrochloride (4,5-bis(hydroxymethyl)-$^{13}C_4$)	Cambridge Isotope Laboratories (Andover, MA)	#M-1270
B_7	Biotin ($^{2}H_2$)	Isosciences (King of Prussia, PA)	#SL3-2005-147A1

3.2 Equipment

All tablet samples were ground using a Retsch RM-100 (Newtown, PA) automated mortar grinder. Samples were analyzed using an Agilent Series 1290 LC equipped with an Agilent Series 6410 Triple Quadrupole MS with electrospray ionization in the positive ion mode. The system was composed of a mobile phase degasser, binary pump, autosampler, and mass selective detector.

3.3 Preparation

All samples were analyzed in as-received condition. All sample, stock calibrant, and internal standard solutions were prepared in 1% (volume fraction) acetic acid in water (i.e., one volume glacial acetic acid plus 99 volumes water). All solution preparation was conducted under reduced lighting to minimize potential vitamin degradation.

Four independent gravimetrically prepared stock solutions for each of the vitamin calibrants were prepared. The calibrants were prepared to reflect the mass fraction of each vitamin in SRM 3289. Four independent gravimetrically prepared internal standard (IS) solutions were prepared from the six isotopically labeled standards. Table 3 lists the composition of the four IS stock solutions. Four working calibration solutions were gravimetrically prepared by combining the calibrant and IS stocks.

Table 3. Vitamin B_1, B_2, B_3, B_5, B_6, and B_7 Internal Standard Stock Solutions

	Mass, mg			
Components	IS_1	IS_2	IS_3	IS_4
Thiamine chloride ($^{13}C_4$)	3.8833			
Riboflavin ($^{13}C_4$, $^{15}N_2$)		1.5982		
Niacin ($^{2}H_4$)			20.5883	
Calcium pantothenate monohydrate ($^{13}C_6$,$^{15}N_2$)			9.5346	
Pyridoxine hydrochloride (4,5-bis(hydroxymethyl)-$^{13}C_4$)	2.1084			
Biotin ($^{2}H_2$)				0.5291
1 % v/v acetic acid solution	15073.5	15061.4	15070.3	15081.9

Thirty tablets from each bottle were ground for 10 min with the automated mortar grinder. The ground material was transferred back to the SRM bottles. Two subsamples were analyzed from each SRM bottle. For analysis, a known mass of about 0.2 g was added to a 50 mL polypropylene centrifuge tube with 0.4 g of the isotopically labeled stocks IS_1, IS_2, and IS_4 and 0.2 g of IS_3. The extraction solvent (24 mL of 1% acetic acid) was added to the centrifuge tube and vortexed for 30 s. The samples were placed in an ultrasonic bath and sonicated for 30 min with no added heat. The samples were then centrifuged at 314 rad/s (3000 RPM) for 15 min. Roughly 5 mL of the supernatant was withdrawn with a disposable syringe and transferred to an autosampler vial through a 0.45 μm nylon filter.

Three subsamples of the SRM 3280 control were prepared using the above procedure.

3.4 Analysis

The SRM 3289 and SRM 3280 samples were analyzed by ID-LC-MS/MS using the parameters listed in Table 4, the gradient profile listed in Table 5, and the multiple reaction monitoring (MRM) conditions listed in Table 6. Mass fractions of B_1, B_2, B_3, B_5, B_6, and B_7 in the samples were bracketed with mass fractions in the calibration solutions. A response factor was calculated for each transition in each injection. Figure 4 displays typical chromatograms.

Table 4. LC-MS/MS Instrument Settings

System	Parameter	Value
Triple Quadrupole MS	Nebulizer pressure	103 kPa (15 psig)
	Drying gas flow	11 L/min
	Drying gas temperature	300 ˚C
	Capillary voltage	4000 V
	Dwell time	100 ms
LC	Column	Imtakt Cadenza CD-C18 column (250×4.6 mm i.d., 3 µm particles)
	Injection volume	10 µL
	Flow rate	0.8 mL/min
	Mobile phase A	20 mmol/L ammonium formate in water adjusted to pH 3.0 with formic acid
	Mobile phase B	methanol

Table 5. LC Gradient Profile Used for Analysis of Vitamin B_1, B_2, B_3, B_5, B_6, and B_7

Time (min)	% A	% B
0 to 6	100	0
6.1 to 20	50	50
20.1 to 25	0	100
25.1 to 40	100	0

Table 6. Multiple Reaction Monitoring Conditions for Vitamin B_1, B_2, B_3, B_5, B_6, and B_7

Time (min)	Compound (Abbreviation)	Precursor Ion (*m*/*z*)	Product Ion (*m*/*z*)	IS Precursor Ion (*m*/*z*)	IS Product Ion (*m*/*z*)	Fragmentor (V)	Collision Energy (eV)
8.0	Niacin (B_3)	124.0	52.1	128.0	53.0	120	30
			53.0		56.1		30
			78.0		81.0		22
			80.0		84.0		20
11.0	Thiamine (B_1)	266.1	42.1	270.1	42.1	110	52
			81.0		81.1		30
			123.1		123.1		10
13.5	Pyridoxine (B_6)	170.1	77.0	174.1	81.1	120	38
			80.1		83.1		40
			134.0		138.0		18
			152.1		156.1		10
16.5	Pantothenic Acid (B_5)	220.0	41.1	224.0	41.1	110	48
			43.1		43.1		30
			72.1		76.0		16
			90.1		94.1		10
20.0	Biotin (B_7)	245.1	96.9	247.1	99.0	130	36
			227.1		229.1		16
22.5	Riboflavin (B_2)	377.2	43.1	383.2	43.1	146	38
			172.1		175.1		38
			198.0		202.1		38
			243.1		249.1		18

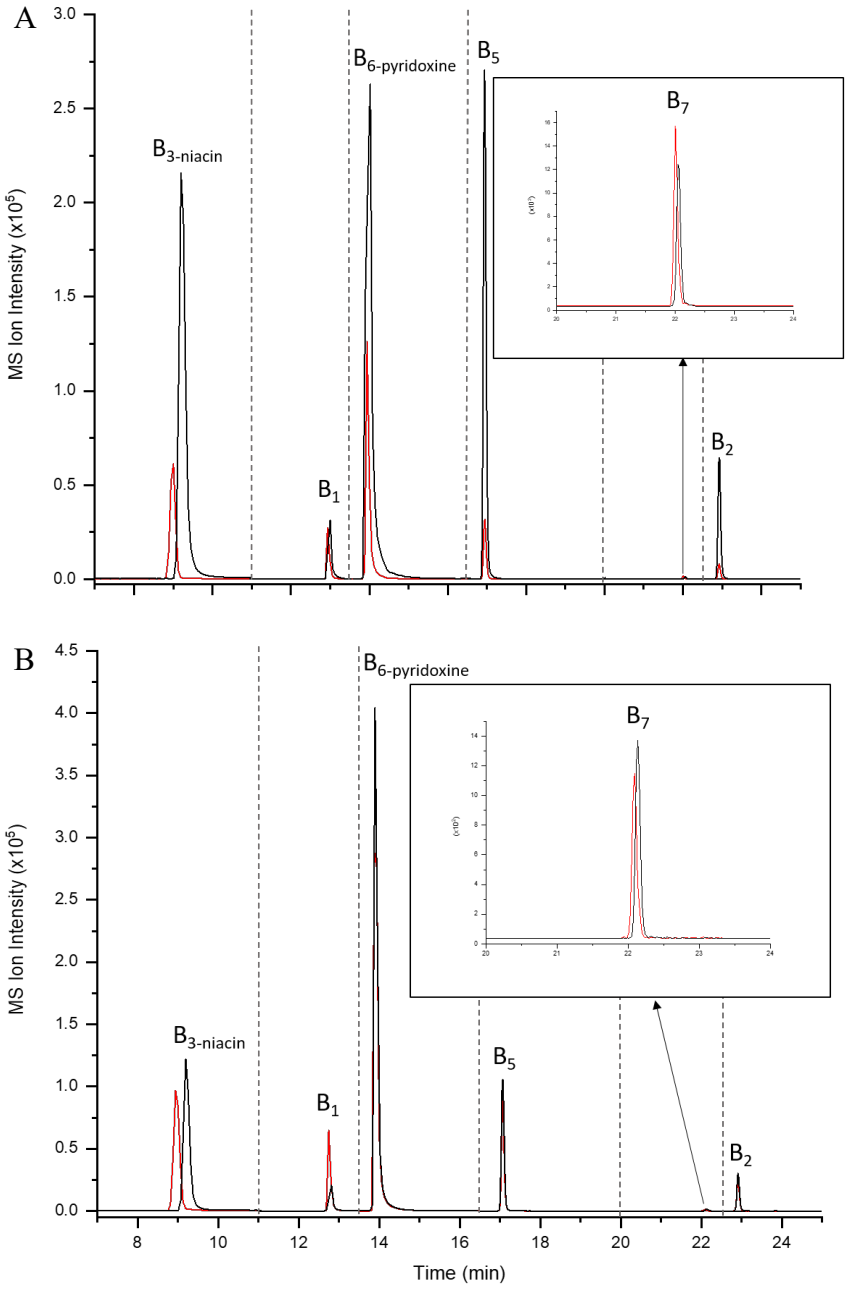

Figure 4. Exemplar Chromatograms

Typical extracted ion chromatograms using ID-LC-MS/MS with multiple reaction monitoring (MRM) for A) SRM 3289 Multivitamin Tablets extract and B) calibration solution prepared in 1% acetic acid in water. Transitions for vitamin ions are shown in black, transitions for isotopically labeled internal standards are shown in red. Only traces for most intense transitions are displayed.

Average mass fraction values for B_1, B_2, B_5, B_6, and B_7 from the SRM 3280 control samples were consistent with the certified values (Figure 5). However, B_3 as niacin was not present in the control where B_3 is present as nicotinamide.

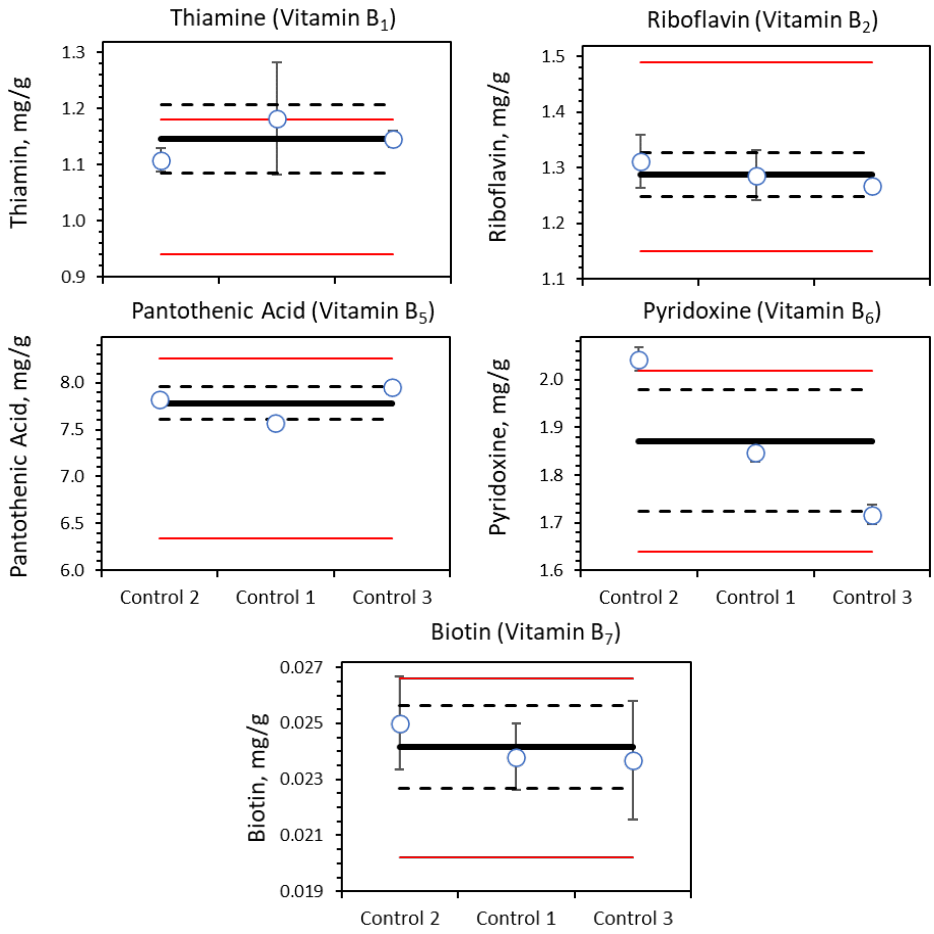

Figure 5. Mass Fraction Analytes in SRM 3280 Control as Functions of Analysis Order
Open circles represent results of the analysis of the three SRM 3280 control preparations. Solid black lines represents the mean value; dashed lines bound one standard deviation (SD) above and below the mean. Error bars represent the SD for the three transitions averaged to determine each value. Red solid lines bound the approximate 95 % level of confidence expanded uncertainty intervals for the analytes stated in the SRM 3280 Certificate of Analysis [1].

3.5 Re-analysis of Vitamin B_3

The results for B_1, B_2, B_5, B_6, and B_7 in SRM 3289 agreed well with the manufacturer's values. However, the ID-LC-MS/MS results for B_3 were significantly lower than expected. Combined with the absence of niacin in the SRM 3280 control material, a re-analysis of vitamin B_3 as niacin was performed.

The same materials and equipment were used as described in Sections 3.1 and 3.2. However, given that the concentration of niacin in the SRM 3289 tablets is much higher than that of the other analytes, matching that concentration in the multi-component IS required making a fairly concentrated labeled-standard stock. To avoid possible saturation of the solution, two stock IS solutions for niacin were prepared at about half the concentration used previously. Table 7 lists the composition of the IS stock solutions. Four working calibration solutions were gravimetrically prepared using a combination of the unlabeled and labeled stocks.

Table 7. Vitamin B_3 as Niacin Internal Standard Stock Solutions

	Mass, mg	
Components	IS_5	IS_6
Niacin ($^2H_4$)	9.9381	20.2228
1 % acetic acid solution	14019.075	29542.52

The niacin assays were completed in two parts. Three bottles (151, 293, and 318) were analyzed in duplicate during the first part with the remaining seven bottles (10, 42, 77, 111, 185, 230, and 260) were analyzed in duplicate during the second part. Both assays provided similar results. These results agreed well with expectations based on the manufacturer's COA.

3.6 Bias Assessments

Systematic bias was evaluated as a function of packaging order, sample preparation order, and chromatographic run order for vitamins B_1, B_2, B_3, B_5, B_6, and B_7. Biases in B_3 (niacin) were evaluated for results from both the original and the re-analysis.

3.6.1 Packing-Order Homogeneity

Figure 6 displays results as a function of box number, which is isomorphic with the order in which the tablets were bottled and packed. There are no apparent trends indicating packing order inhomogeneity.

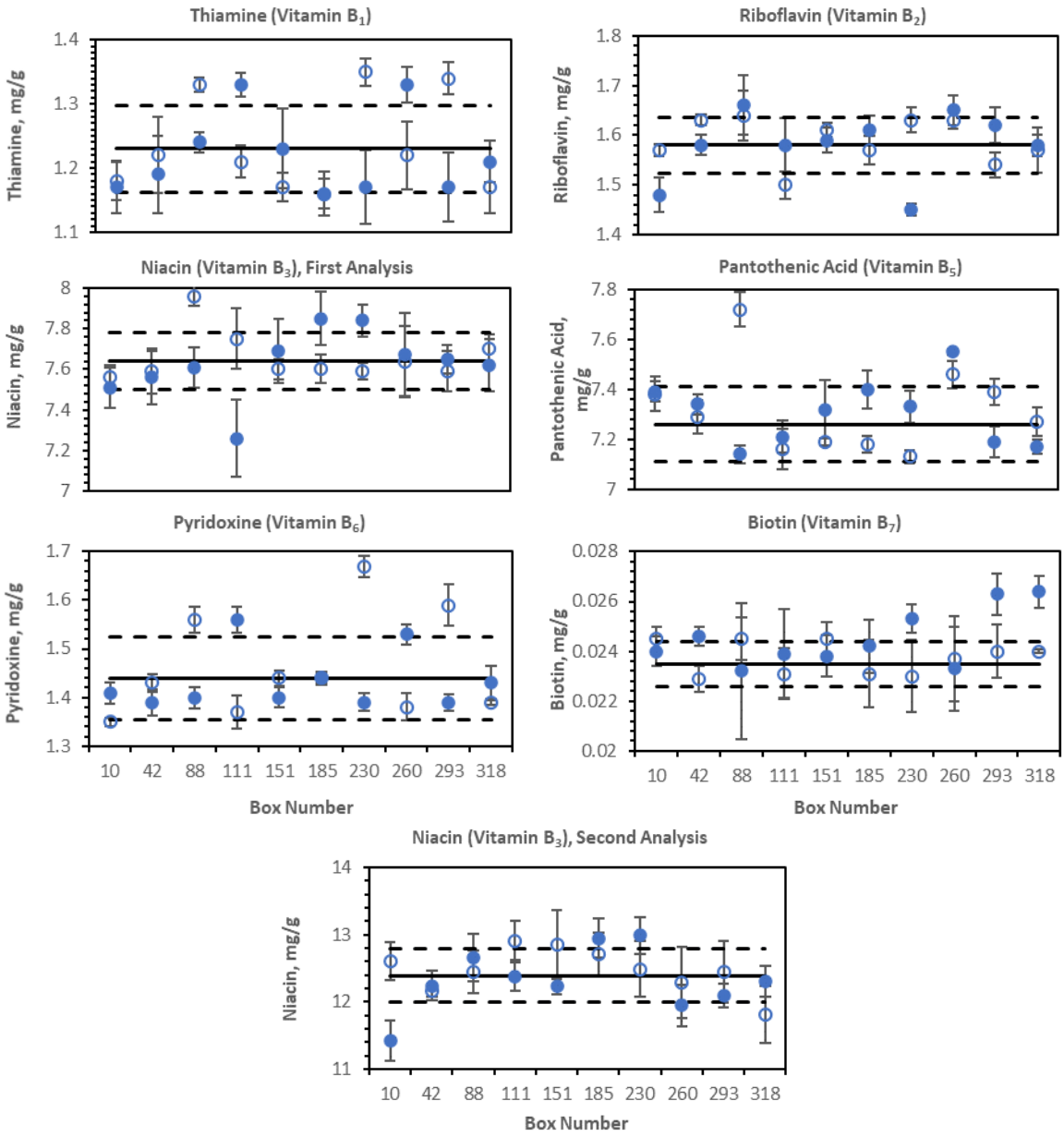

Figure 6. Mass Fractions of Analytes in SRM 3289 as Functions of Packing Order

Blue circles represent the result of the analysis of the first preparation from each bottle, hollow circles represent the results of the second preparation. Solid lines denote the mean value; dashed lines bound one standard deviation (SD) above and below the mean. Error bars represent the SD for the three transitions averaged to determine the value.

3.6.2 Sample Preparation

Figure 7 displays results as a function of the sample code arranged in sample preparation order. There are no apparent trends indicating systematic changes in the sample-preparation process over time.

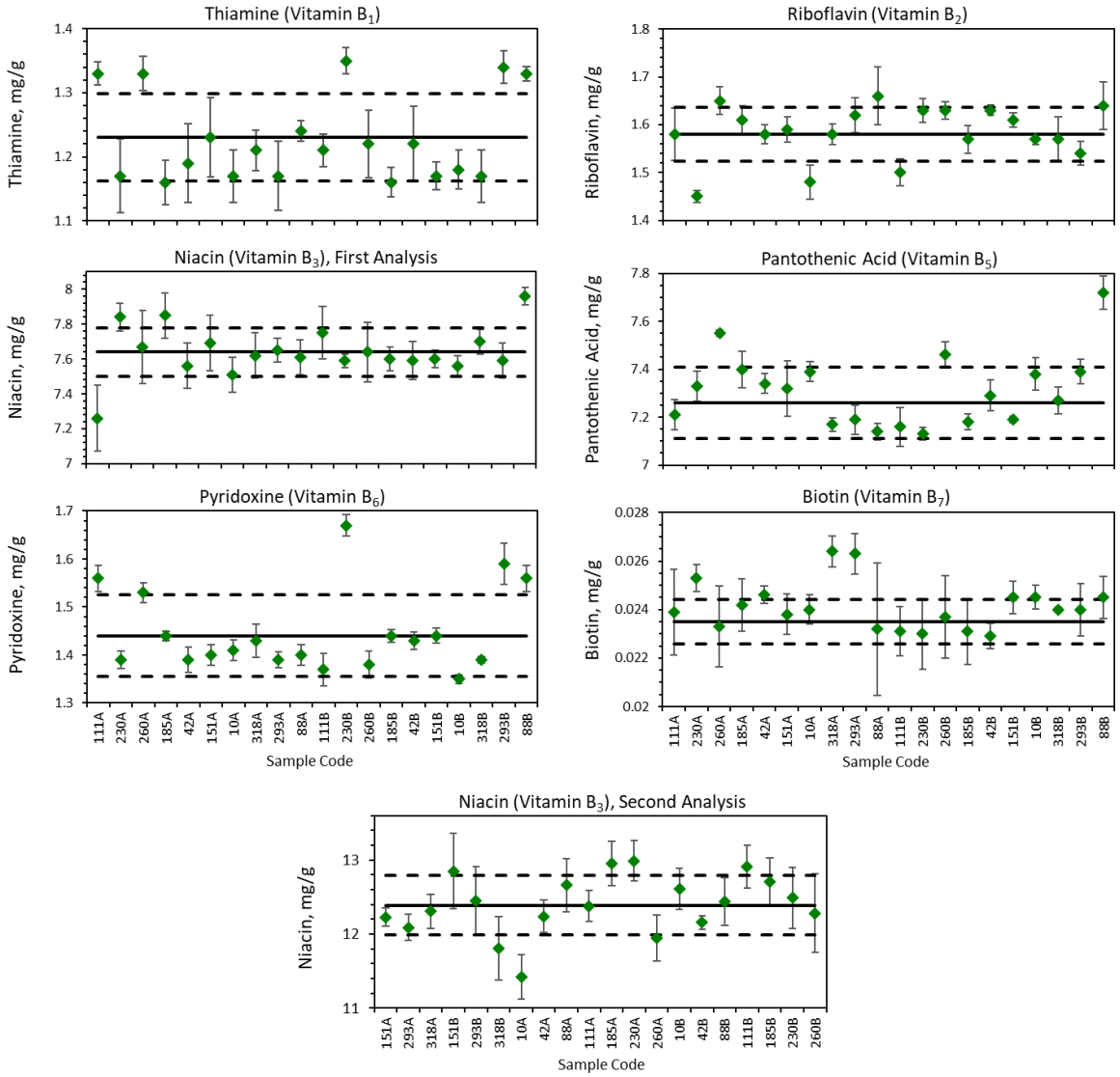

Figure 7. Mass Fractions of Analytes in SRM 3289 as Functions of Sample Preparation Order

Green diamonds represent results of the analysis of one of the two SRM 3289 preparations from each bottle. Solid lines denote the mean value; dashed lines bound one standard deviation (SD) above and below the mean. Error bars represent the SD for the three transitions averaged to determine the value.

3.6.3 Sample analysis

Figure 8 displays results as a function of the sample code arranged in chromatographic run order. There are no apparent trends for vitamins B_2, B_3, B_6, and B_7. However, the first five results for B_1 (thiamin) and B_6 (pyridoxine) are relatively larger and the peak areas were considerably higher than those of the following 15 results.

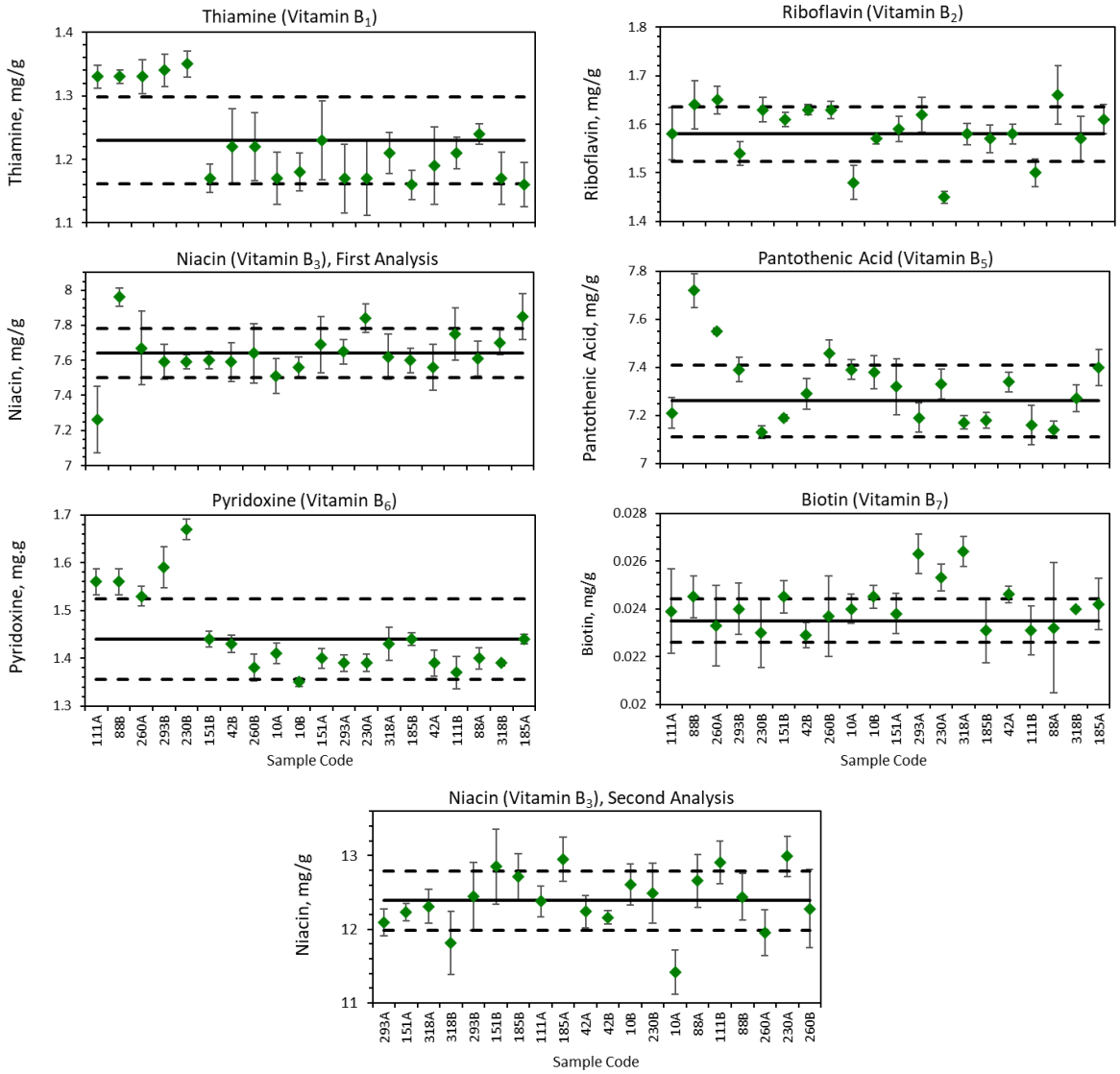

Figure 8. Mass Fractions of Analytes in SRM 3289 as Functions of Analysis Order
Green diamonds represent results of the analysis of one of the two SRM 3289 preparations from each bottle. Solid lines denote the mean value; dashed lines bound one standard deviation (SD) above and below the mean. Error bars represent the SD for the three transitions averaged to determine the value.

Thiamine and pyridoxine neighbor each other in the chromatographic separation (Figure 4) and closely straddle a change in selected ion monitoring window. Since the sample preparation and extractions were consistent for all samples, controls, and calibrants, the differences in peak areas from these samples suggest that an instrumental bias may be present with respect to the mass fragmentation transitions for these particular samples.

3.7 Calibrant Identity and Purity Determinations

Analyte identity confirmation and purity determinations were accomplished via quantitative ^{1}H-NMR using an internal standard (^{1}H-NMR$_{IS}$), a primary ratio method for evaluating the chemical purity of neat organic materials that contain at least one non-exchangeable ^{1}H atom. The resulting purity estimates are metrologically traceable to the International System of Units (SI) unit of mass, expressed as mass percentage of analyte in neat calibrant material, through linkage of the purity values of the ^{1}H-NMR internal standards used to that of the NIST PS1 Primary Standard for ^{1}H-NMR (benzoic acid) [5,6].

3.7.1 NMR Spectroscopy

Experimental NMR data were 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.

3.7.1.1 Sample Preparation

All sample preparation was performed under incandescent light with the lamp pointed away from the materials and samples. Glassware was cleaned with distilled water and organic solvents, baked in a furnace at 450 °C, and stored in a desiccator. Clean Bruker 600 MHz NMR tubes (5 mm internal diameter, 17.8 cm length) were stored in a desiccator prior to use. All calibrant standards were stored with desiccant at -20 °C. All internal standards were stored at room temperature in a desiccator. Deuterated solvents from Cambridge Isotopes Laboratory with ≥ 99.8 % D-atom purity were used for all analyses. Samples were diluted with approximately 1.4 mL of solvent withdrawn from ampoules by cleaned glass Pasteur pipettes. Samples were sonicated and vortexed several times to facilitate total dissolution. Care was taken to ensure complete dissolution and that no crystals of the neat materials adhered to the weighing bottle walls.

Sample mass determinations and preparations for ^{1}H-NMR analysis were performed in accordance with balance use and sample preparation Standard Operating Procedures (SOPs). However, due to limited laboratory access during periods of 2020 and 2021, masses of some of the calibrants were determined using an analytical balance with 0.01 mg readability rather than an ultra-microbalance with 0.1 µg readability usually used for these types of analyses. To compensate for the lower precision of the analytical balance, larger amounts of the calibrant and IS were used to achieve higher confidence in mass determinations. Due to the difficulty in adding large amounts of material to the weigh boats, some materials were added directly to tared glass vials without weigh boats.

3.7.1.2 Instrumental Parameters

One-dimensional ^{1}H-NMR spectroscopy experiments were conducted at 298 K with 20.0276 ppm spectral sweep width. The transmitter frequency offset for ^{1}H was set to 6.175 ppm. 90-degree ^{1}H excitation pulse widths were used. When necessary to achieve adequate selectivity with NMR spectral features, some experiments were conducted with globally-optimized, alternating-phase, rectangular pulse (GARP) composite pulse ^{13}C decoupling during acquisition of the free induction decay signal (FID). Spin lattice relaxation time (T1) inversion recovery experiments were performed to establish the time required for net magnetization of all analyzed resonances to return to practically 100 % of the equilibrium

value between 90-degree excitation pulses. A recycle delay (D1) of 55 s to 60 s was typically used to stabilize temperature fluctuations during GARP ^{13}C decoupling. Data acquisition time was either 5.453 s per scan to generate an FID with 131 072 data points or 5.62 s to generate an FID with 135168 data points. Experiments were performed using 64 to 80 scans. Apodization was performed using an exponential window function to achieve 0.3 Hz line broadening.

Two-dimensional multiplicity-edited ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) NMR experiments were conducted at 298 K. One thousand twenty-four data points were collected in the ^{1}H dimension having a spectral width of 13.018 ppm, centered at 6.012 ppm; 256 data points were collected in the ^{13}C dimension having a 165 ppm spectral width centered at 90 ppm. Typically, 8 scans, preceded by16 dummy scans for which no data was acquired, were performed using a 64 μs dwell time.

3.7.1.3 Purity Measurement Model

The chemical mass fraction purity (%) of the primary component, *P*p, of a calibrant sample is determined using the following ^{1}H-NMR equation:

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

where N_{P} = multiplicity (# H/peak) of the primary chemical component spectral peak
N_{I} = multiplicity (# H/peak) of the internal standard peak
M_{P} = relative molar mass, g/mol, of the primary chemical component
M_{I} = relative molar mass, 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 sample material
m_{I} = mass (g) of the internal standard
P_{I} = mass fraction purity (%) of the internal standard

The proton multiplicities and relative molar masses (g/mol) of primary components and internal standards are determined by their respective chemical structures. The multiplicities, N_{P} and N_{I}, are considered to be exact without uncertainty. The uncertainty of the relative molar masses (g/mol) are determined with a web-based molecular weight calculator [7] that applies the International Union of Pure and Applied Chemistry Guidelines provided by the Commission on Isotopic Abundances and Atomic Weights. The peak integrals (A_{P} and A_{I}) were determined through spectral analysis of multiple peaks for each compound. Their standard uncertainties were estimated as the standard deviation of the respective impurity-adjusted, proton multiplicity-normalized peak areas. The mass of the sample material and internal standard are determined by weighing, with an assigned uncertainty based on observed balance performance. The purities of the internal standards and their uncertainties are linked via ^{1}H-NMR_{IS} to the value for purity of the NIST PS1.

3.7.1.4 Calculations

The evaluation programs used to estimate the standard uncertainty, $u(P\text{p})$, and the approximate 95 % uncertainty interval about Pp have evolved over time. All of the programs that were used for the vitamin B calibrants were implemented using Monte Carlo approaches.

The earliest method was a parametric bootstrap [8] wherein all of the above model's input variables were varied randomly using a bespoke Matlab program. During 100,000 iterations, Gaussian kernel "pseudo values" for each of the inputs were defined using the Matlab "randn" random number generation function and each input variable's value and standard uncertainty. The value of Pp was calculated using these pseudo values and the result recorded. The uncertainties were estimated from the distribution of the 100,000 calculated Pp results. Since each of the inputs was varied independently and did not consider degrees of freedom nor covariances between the inputs, the estimated uncertainties tended to be conservative (in the sense of providing large uncertainty estimates). However, the method can be used when results from only three samples are available.

When measurement data for four or more independently prepared samples are available, a hierarchical Bayesian method can make fuller use of all of the information implicitly available in the results and the experimental design [9]. The method was first implemented as bespoke OpenBUGS [10] programs. It has since been implemented as the NIST ABACUS ^{1}H-NMR Shiny app for chemical purity assessment [11].

3.7.2 Vitamin B_1 (Thiamine)

One vial of thiamine hydrochloride (Lot # O1F326, USP) was evaluated using potassium hydrogen phthalate (KHP, NIST SRM 84k) as internal standard and D_2O as solvent. Analysis of control samples indicated that the material was stable over time.

Due to the larger masses required for accurate weighing using the analytical balance, only three samples were prepared and analyzed. The longest T1 was 4.1 s. Figure 9 presents the ^{1}H-NMR- spectra with labeled peak assignments. Table 8 lists descriptions of the integrated peaks.

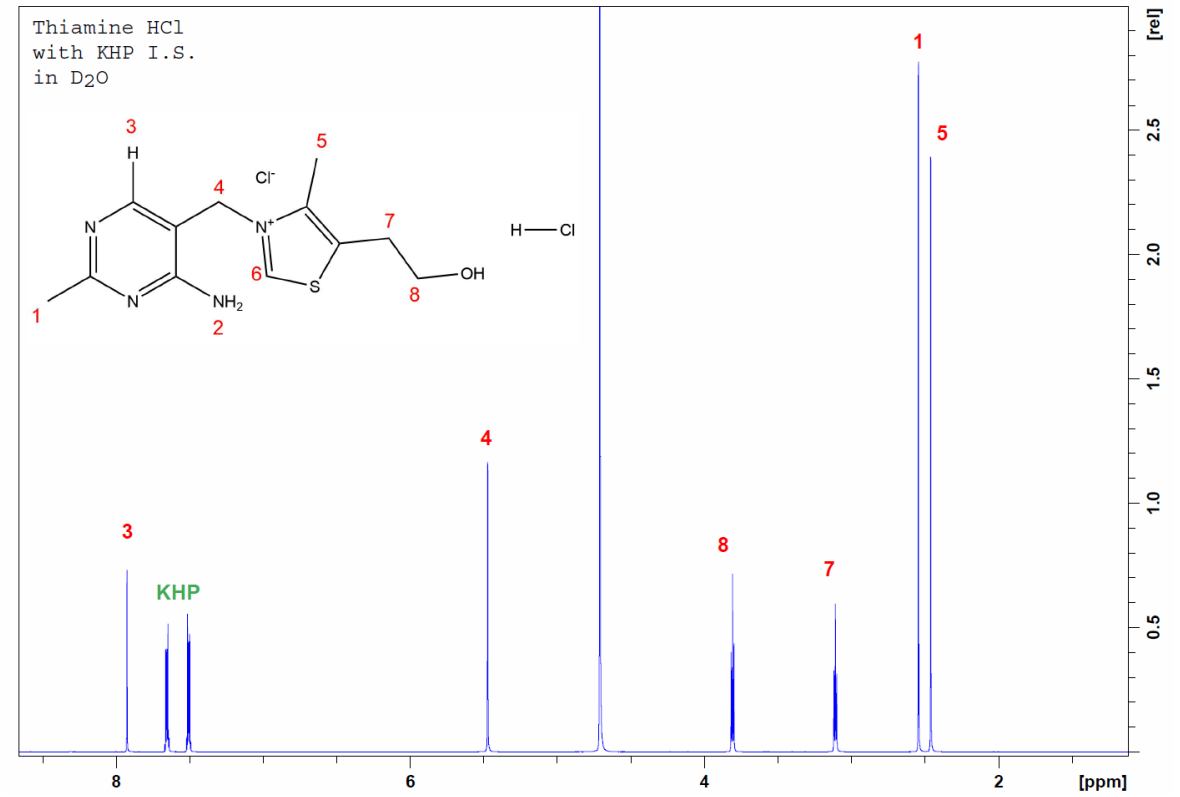

Figure 9. ^{1}H-NMR Spectrum of Thiamine + KHP in D_2O

Table 8. ^{1}H-NMR Integration Regions for Thiamine Purity Assessment

Analyte	Chemical Shift (ppm)	Multiplet Type	Proton Moiety	Proton Multiplicity
Thiamine Hydrochloride	5.5	Singlet	4	2
	3.8	Triplet	8	2
Potassium Hydrogen Phthalate	7.6	2× Multiplets	Aromatic	4

Since data for more than three samples are needed for the more rigorous Bayesian approach, the purity result was calculated using the parametric bootstrap method. The purity of thiamine hydrochloride was assessed as (95.36 ±1.01) % with a 95 % level of confidence expanded uncertainty interval of (93.38 to 97.34) %.

3.7.3 Vitamin B_2 (Riboflavin)

One 500 mg bottle of the riboflavin (USP Lot #N0C021) calibrant was characterized using dimethylmalonic acid (DMMA, Fluka TraceCERT Lot #BCBL6998V) as internal standard. Previous studies established that concentrations of riboflavin in D_2O that are great enough for accurate ^{1}H-NMR measurements are not achievable at neutral pH. A solution of ≈ 0.3 mol/L NaOD in D_2O was found suitable for dissolving riboflavin at mass concentrations > 1mg/mL. However, an initial experiment demonstrated that riboflavin significantly degrades with time in the NaOD/D_2O solvent, with the degree of degradation correlated to the amount of time between sample dilution and the NMR experiment.

A set of five samples were prepared and each sample was individually diluted just prior to the NMR experiment to minimize degradation prior to analysis. The longest T1 lasted approximately 1.1 s. Figure 10 presents the ^{1}H-NMR- spectra with labeled peak assignments. Table 8 lists descriptions of the integrated peaks.

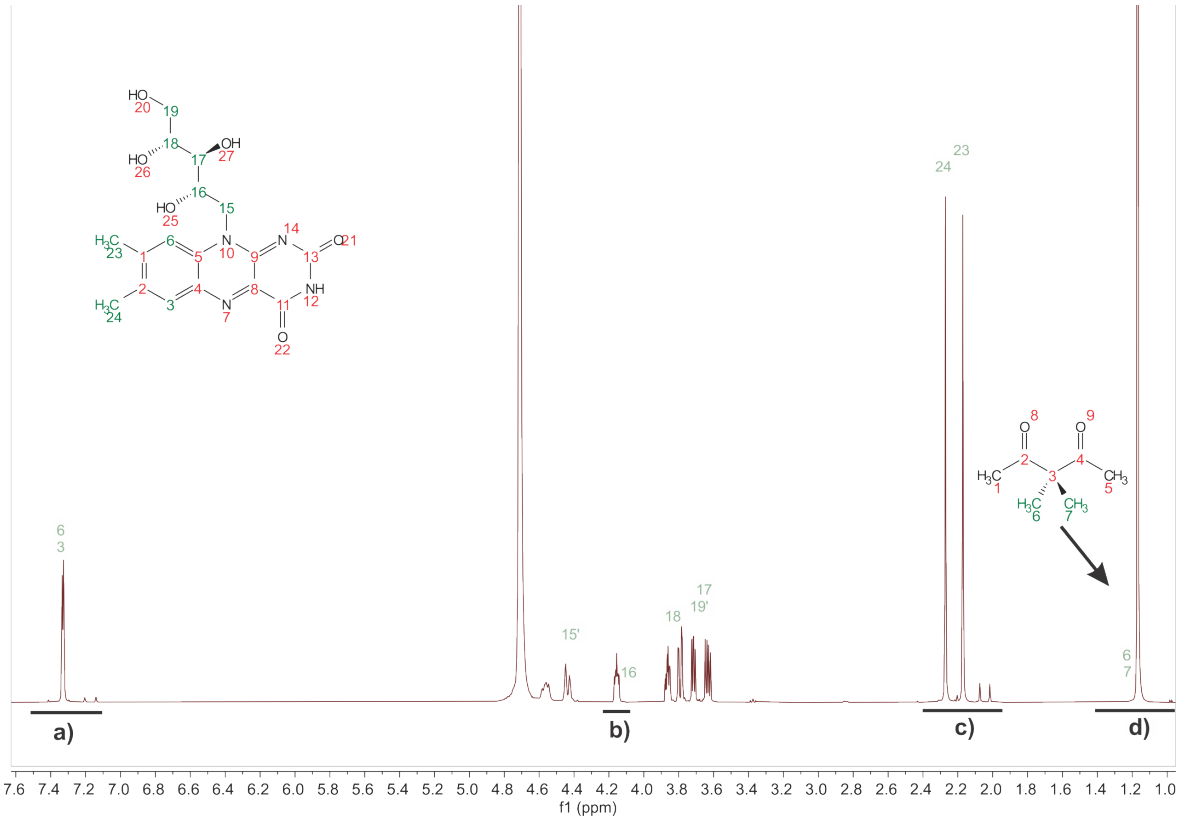

Figure 10. ^{1}H-NMR Spectrum of Riboflavin + DMMA in ≈ 0.3 mol/L NaOD/D_2O

Table 9. ^{1}H-NMR Integration Regions for Calcium Pantothenate Purity Assessment

Analyte	Chemical Shift (ppm)	Multiplet Type	Proton Moiety	Proton Multiplicity
Riboflavin	a) 7.3	2 Overlapping Singlets	3,6	2
	b) 4.2	Multiplet	16	1
	c) 2.1	2 Overlapping Singlets	24,23	6
Dimethylmalonic Acid	d) 1.3	Singlet	2× (-CH_3)	6

Due to the presence of overlapping impurity peaks, only the integral region around the peak at 4.2 ppm was used to estimate riboflavin purity. This integral also contained impurity peaks, but they were smaller and more readily quantified than those of the other regions. The interfering peaks are believed to have arisen from impurities that structurally related to riboflavin.

A bespoke OpenBUGS implementation of the hierarchical Bayesian procedure was used to estimate riboflavin purity. The purity was assessed as (93.1 ±0.6) % with a 95 % level of confidence expanded uncertainty interval of (91.8 to 94.2) %.

3.7.4 Vitamin B_3 (Niacin)

One vial of the niacin (USP Lot #J0J235, Rockville, MD) calibrant was characterized using maleic acid (Sigma Lot #BCBM8127V) as internal standard and perdeuterated dimethyl sulfone (DMSO-d_6) as solvent. Earlier studies had noted difficulties in dissolving niacin in D_2O. Analysis of control samples indicated that the material was stable over time.

Four replicate samples were prepared. The longest T1 was 4.5 s, however a long D1 of 105s was used to limit effects from temperature fluctuations due to ^{13}C decoupling. Figure 11 presents the ^{1}H-NMR- spectra with labeled peak assignments. Table 10 lists descriptions of the peaks integrated for quantitation.

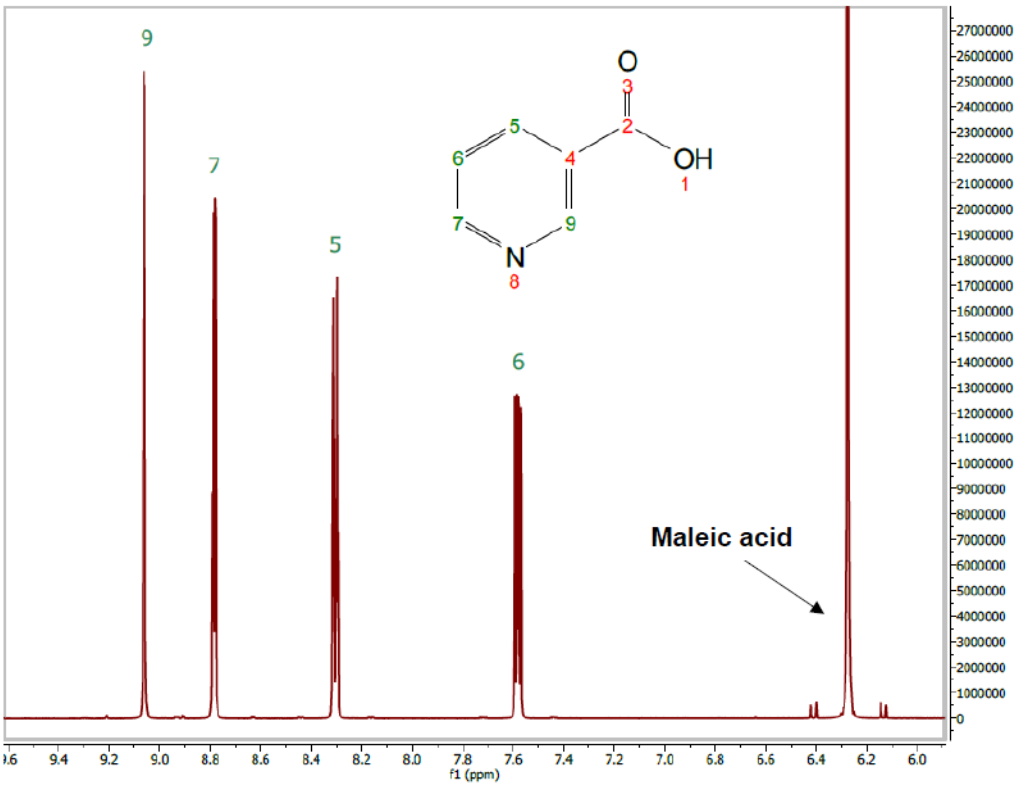

Figure 11. ^{1}H-NMR Spectrum of Niacin + Maleic Acid in DMSO-d_6

Table 10. ^{1}H-NMR Integration Regions for Niacin Purity Assessment

Analyte	Chemical Shift (ppm)	Peak and Multiplet Type	Proton Moiety	Proton Multiplicity
Niacin	7.5	Triplet	6	1
	8.3	Doublet	5	1
	9.0	Doublet (8.9) + Singlet (9.1)	7,9	2
Maleic Acid	6.3	Singlet		2×(=CH)

The aromatic peaks at 9.06 ppm and 8.78 ppm were evaluated as a combined integral since they were very close to each other.

Estimates of purity for niacin were determined using the NIST ABACUS ^{1}H-NMR Shiny app for chemical purity assessment. The purity was assessed as (99.68 ±0.23) % with a 95 % level of confidence expanded uncertainty interval of (99.12 to 99.97) %.

3.7.5 Vitamin B_5 (Calcium pantothenate)

One vial of calcium pantothenate (Lot #O1H081) was evaluated using potassium hydrogen phthalate (KHP, SRM 84k) as internal standard and D_2O as solvent. Analysis of control samples indicated that the material was stable over time.

Five replicate samples were prepared and analyzed. The longest T1 was 4.5 s. Figure 12 presents the ^{1}H-NMR- spectra with labeled peak assignments. Table 11 lists descriptions of the integrated peaks.

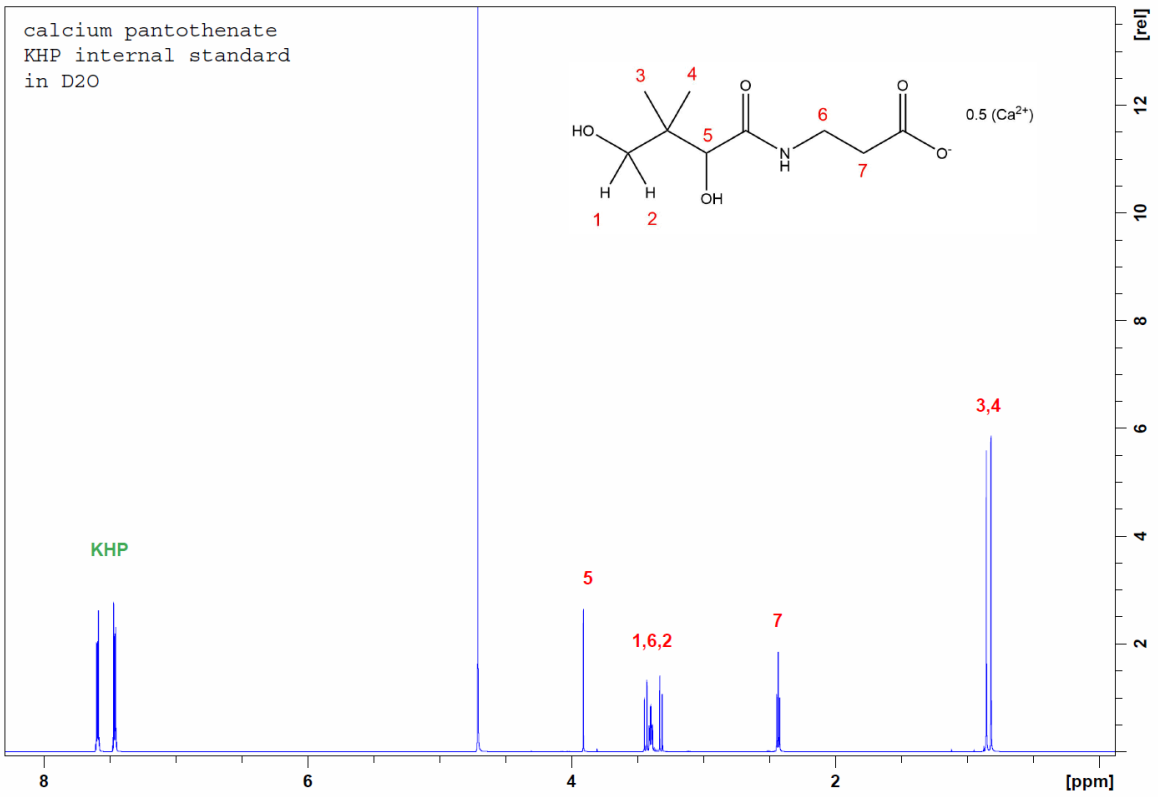

Figure 12. ^{1}H-NMR Spectrum of Calcium Pantothenate + KHP in D_2O

Table 11. ^{1}H-NMR Integration Regions for Calcium Pantothenate Purity Assessment

Analyte	Chemical Shift (ppm)	Multiplet Type	Proton Moiety	Proton Multiplicity
Calcium Pantothenate	3.9	Singlet	5	1
	2.4	Triplet	7	2
Potassium Hydrogen Phthalate	7.6	2× Multiplets	Aromatic	4

Estimates of purity for calcium pantothenate were determined with the hierarchical Bayesian procedure implemented via the NIST ABACUS ^{1}H-NMR Shiny app for chemical purity assessment. The purity of calcium pantothenate was assessed as (94.77 ±0.20) % with a 95 % level of confidence expanded uncertainty interval of (94.37 to 95.12) %.

3.7.6 Vitamin B_6 (Pyridoxine Hydrochloride)

One vial of pyridoxine hydrochloride (Lot # Q0G409) was evaluated using dimethylmalonic acid (DMMA, Sigma Lot # BCBG455V) as internal standard and D_2O as solvent. No solubility issues were encountered. Analysis of control samples indicated that the material was stable over time.

Due to the larger masses required for accurate weighing using the analytical balance, only three samples were prepared and analyzed. The longest T1 was 1.5 s. Figure 13 presents the ^{1}H-NMR- spectra with labeled peak assignments. Table 12 lists descriptions of the integrated peaks.

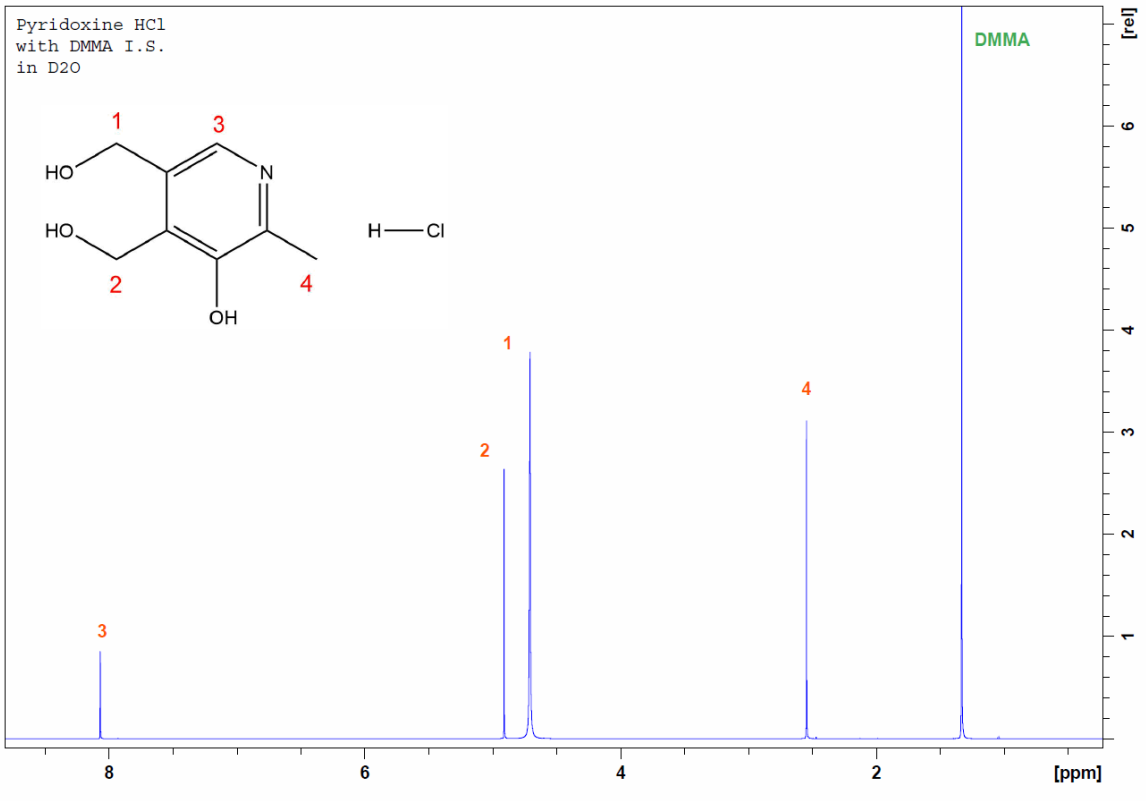

Figure 13. ^{1}H-NMR Spectrum of Pyridoxine + DMMA in D_2O

Table 12. ^{1}H-NMR Integration Regions for Pyridoxine Purity Assessment

Analyte	Chemical Shift (ppm)	Multiplet Type	Proton Moiety	Proton Multiplicity
Pyridoxine Hydrochloride	8	Singlet	3	1
Dimethylmalonic Acid	1.3	Singlet	2× (-CH_3)	6

Only one peak (8.0 ppm) was selected for integration since the peaks for moieties 1 and 2 (Figure 3) partially overlapped with the D_2O peak.

Since more than three samples are needed for the more rigorous Bayesian approach, the purity result was calculated using the parametric bootstrap method. The purity of pyridoxine hydrochloride was assessed as (99.50 ±0.67) % with a 95 % level of confidence expanded uncertainty interval of (98.20 to 100.00) %. While the symmetric distribution defined by the (mean ±SD) is computationally convenient, the asymmetric distribution (99.50 +0.24, -0.67) % is likely a better representation.

3.7.7 Vitamin B_7 (Biotin)

One vial of the D (+) enantiomer of biotin (Sigma Aldrich Lot #073K07115) calibrant was characterized using 1,2,4,5-tetrachloro-3-nitrobenzene (tecnazene, Sigma Aldrich Lot # BCBC2607V) as internal standard and DMSO-d_6 as solvent.

Five biotin replicate samples were prepared. ^{13}C decoupling was not used during the FID acquisition. Figure 14 presents the ^{1}H-NMR spectrum of biotin with labeled peak assignments; Figure 15 presents the ^{1}H-NMR spectrum of tecnazene with labeled peak assignment. Table 13 lists descriptions of the integrated peaks.

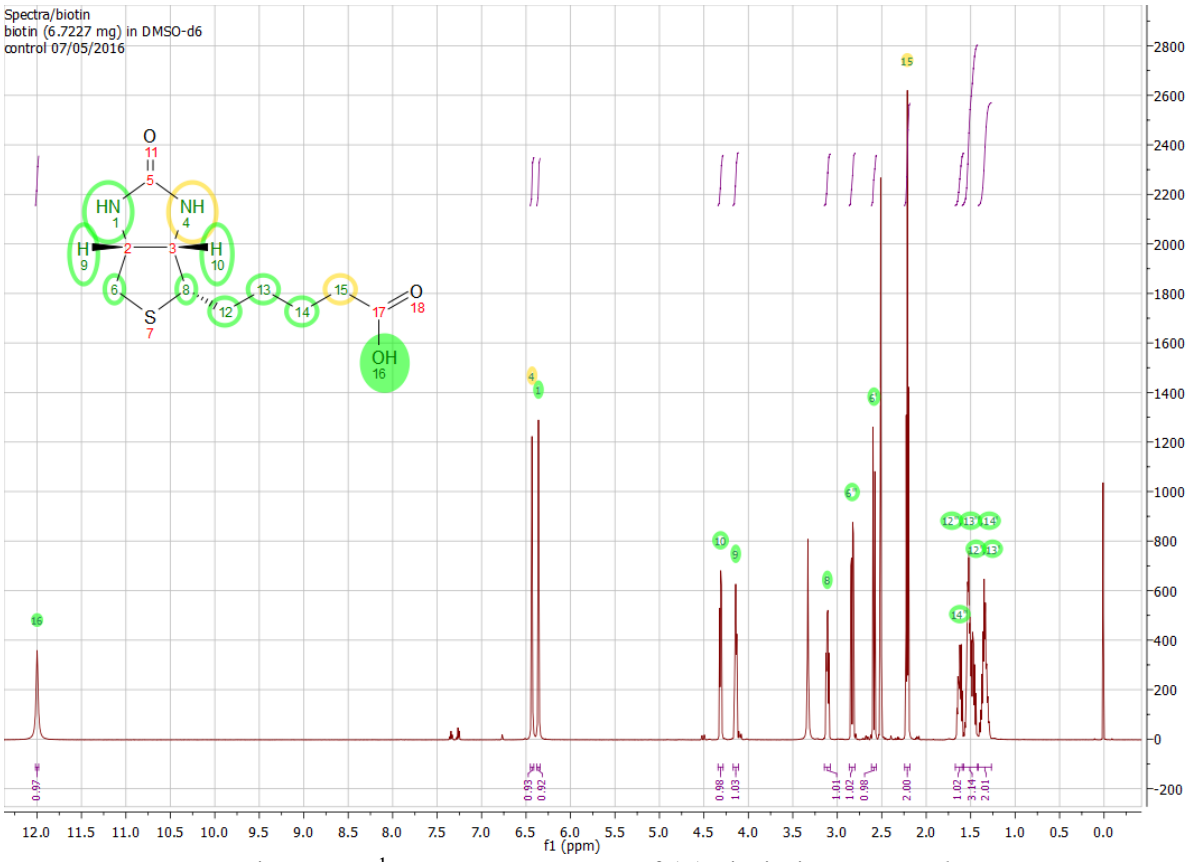

Figure 14. ^{1}H-NMR Spectrum of (+)Biotin in DMSO-d_6

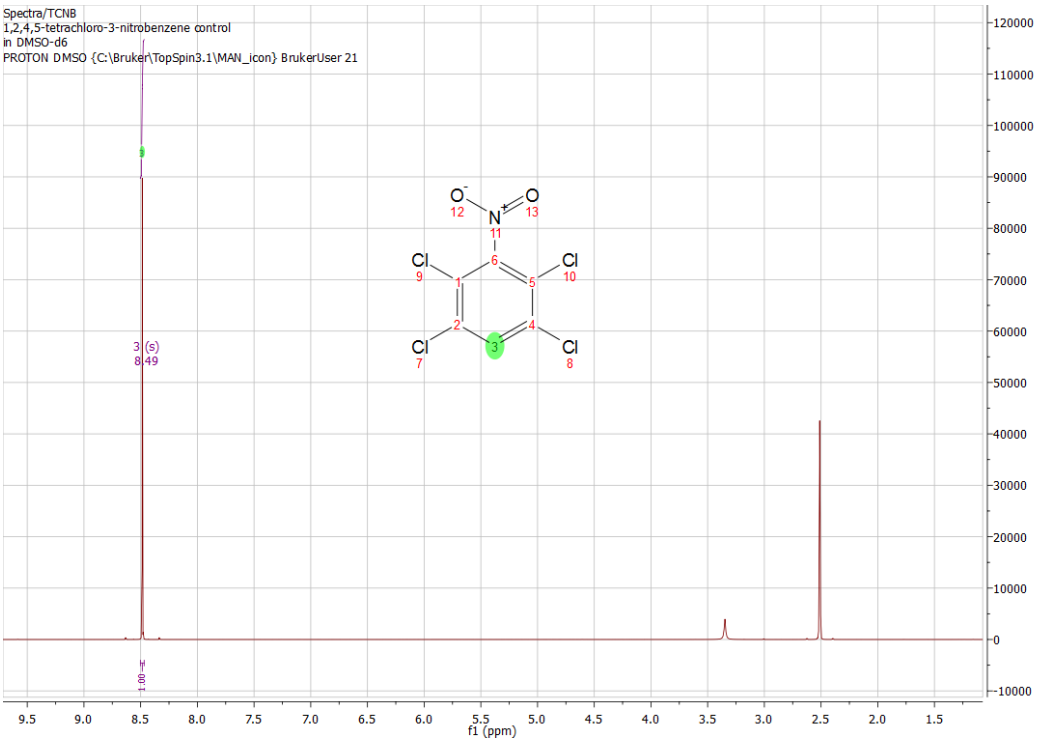

Figure 15. ^{1}H-NMR Spectrum of Tecnazene in DMSO-*d*

Table 13. ^{1}H-NMR Integration Regions for Biotin Purity Assessment.

Analyte	Chemical Shift	Multiplet Type	Proton Moiety	Proton Multiplicity
Biotin	4.2 3.1 2.2	Triplet and Double Triplet Multiplet Triplet	10 & 9 8 15	2 1 2
Tecnazene	8.4	Singlet	3	1

Of the many ^{1}H peaks in the biotin spectrum, only three were assessed to be of suitable quality for ^{1}H-NMR. Broad peaks in crowded spectral regions (δ1.8 ppm to δ1.1 ppm) were not selected due to the difficulty in determining reliable integral regions and the potential for significant underlying impurity components. The peak at δ2.5 ppm was not selected due to overlap with the solvent impurity peak, nor was the non-equivalent resonance peak of the same proton moiety (6) at δ2.9 ppm. Additionally, the peaks of the exchangeable -NH and -OH moieties were not selected for quantification. Tecnazene has only one suitable ^{1}H.

The purity of biotin was assessed as (97.64 ±0.59) % with a 95 % level of confidence expanded uncertainty interval of (96.49 to 98.80) % using a parametric bootstrap method. The biotin uncertainty largely reflects variability among the multiplicity-normalized integrals of the three biotin regions used for quantification.

4 NIST Measurement of Vitamin B_{12} (Cyancobalamin)

The mass fraction of cyanocobalamin (CNCbl) in SRM 3289 was determined using liquid chromatography tandem inductively coupled plasma mass spectrometry (LC-ICP-MS) [12] and single-point standard addition with use of an internal standard.

4.1 Materials

One bottle from each of ten boxes of SRM 3289 (numbers 7, 39, 84, 108, 148, 179, 221, 255, 285 and 318) was obtained for analysis. One bottle of SRM 3280 was obtained for use as a control.

The 18 MΩ-cm deionized water used as solvent was locally generated. Optima-grade nitric acid (HNO_3), HPLC-grade methanol, HPLC-grade acetonitrile, and reagent-grade ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA) were obtained from Thermo Fisher Scientific. US Pharmacopeia (USP, Rockville, MD) Reference Standard Cyanocobalamin lot# F07440 was purchased from USP.

4.2 Equipment

An Agilent 1260 Infinity LC system coupled to an Agilent 8800 inductively coupled plasma mass spectrometer (ICP-MS) was used for the determination of cobalt (Co) and CNCbl. The LC system consisted of an autosampler and a quaternary pump. Separation of CNCbl from Co was accomplished by using an Atlantis T3 column. An analytical balance was used in the preparation of samples and standards. The balance is serviced and calibrated annually. Prior to use, calibration of the balance was verified using standard masses ranging from 20 g to 100 g. A Retsch model RM 100 automated mortar-and-pestle grinder, a dual action shaker, an ultrasonic bath, and a centrifuge were used in sample preparation. A model DMA 35 density meter was used to measure the density of sample solutions in the extraction and derivatization process.

4.3 Preparation

All samples were analyzed in as-received condition. Sample preparations were conducted in a dark room with red lamps because aqueous solutions of CNCbl are light-sensitive.

Fifteen tablets from each bottle of SRM 3289 and SRM 3280 were ground for 15 min and 10 min, respectively, to generate homogenous samples. The ground samples were placed in 50 mL Falcon tubes that were covered on the outside with aluminum foil to protect the contents from exposure to light.

Duplicate 0.5 g portions were accurately weighted from each of the ten SRM 3289 samples into two 50 mL centrifuge tubes. An aliquot of 40 mL of de-ionized water was added and the contents were shaken to wet all the powder. The Falcon tubes with the samples were shaken in the shaker for 15 min at 60 cycles/min, and then were sonicated in a water bath for 15 min. Each sample was quantitatively transferred into a pre-weighed 100 mL volumetric flask, diluted to volume with water, and weighed. The contents were transferred to two 50 mL Falcon tubes and centrifuged for 15 min at 314 rad/s (3000 RPM). The supernatant from both Falcon tubes was filtered through a Whatman 2V filter paper into a 125 mL Erlenmeyer flask.

A pre-qualified S*PURE Maxi-Clean C18 900 mg SPE cartridge (Part #20942/5122344) was attached to a 20 mL syringe. The SPE cartridge was conditioned and rinsed by allowing 20 mL acetonitrile and then 10 mL water to pass through the cartridge by gently pressing the piston of the syringe. The conditioned SPE cartridge with the 20 mL syringe barrel was inserted onto the stopcock of the vacuum manifold. A 20 mL aliquot of the sample filtrate was passed through the cartridge to collect the analyte. The residual effluent was monitored to exit the SPE cartridge at approximately 47 drops per minute to prevent a loss of the analyte at excessive flow rate. The filtrate was discarded. After all the sample filtrate passed through the cartridge, the cartridge was air-dried by pulling vacuum until no more effluent was observed. The stopcock was then closed.

A 5 mL volumetric flask was placed under the cartridge, and a 4.5 mL aliquot of 30 % acetonitrile in water (volume fractions) was added to the syringe. CNCbl in the SPE cartridge was then eluted into the volumetric flask, assisted by gently pressing the piston. The sample was then diluted to volume with water. The contents of the volumetric flask were filtered through a 0.45 µm nylon filter. A 1 mL aliquot of the filtrate was collected for the determination of density, and the rest was used for the determination of CNCbl by LC-ICP-MS.

An aliquot of 200 mg of a solution containing 90 µg/kg Co was added as an internal standard to 2 g of the filtrate. The resulting solution was homogenized by gentle shaking. A 1 g aliquot subsample was transferred into a 2 mL amber vial. A 120 mg aliquot of a solution containing 71 µg/kg Co as CNCbl was added to the vial to constitute a spiked sample for the purpose of quantification by the method of standard additions (see Sections 4.5.3 and 4.5.4).

Four blanks and four SRM 3280 controls were prepared similarly.

4.4 Analysis

SRM 3289, SRM 3280, and procedural blanks were analyzed by LC-ICP-MS using the separation and spectrometric parameters listed in Table 14. Free Co from the internal standard and Co from CNCbl were measured at 59 *m*/*z* in the single-quadrupole no-gas mode. The measurement of all samples was completed in two days. Figure 16 displays a typical chromatogram.

Table 14. LC-ICP-MS Parameters for the Determination of Cyanocobalamin

System	Component	Description
LC	Column	150 mm x 2.1 mm i.d.
	Mobile Phase	10 mmol/L EDTA in 25:75 methanol:water
	Flow Rate	200 µL/min, isocratic
	Injection Volume	10 µL
Triple Quadrupole ICP-MS	RF Power	1550 W
	Nebulizer Gas Flow	1 mL/min
	Make Up Gas Flow	0.1 L/min
	Sample Introduction	PFA microflow nebulizer PFA Scott type spray chamber

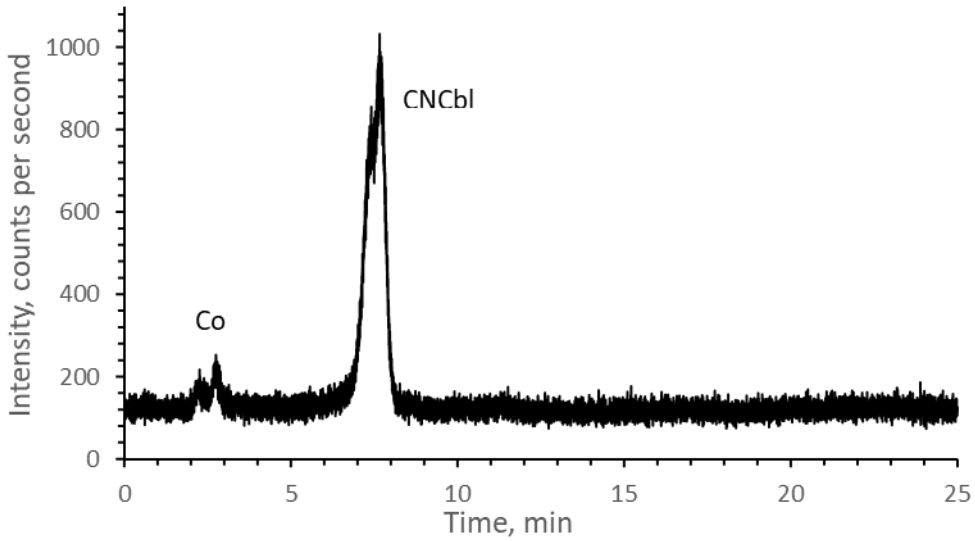

Figure 16. Exemplar LC-ICP-MS Chromatogram for Cyanocobalamin in SRM 3289.

There was no detectable CNCbl in the procedural blanks. The (mean ± SD) result of four replicate analyses of the SRM 3280 control was (4.63 ± 0.26) µg/g. This agrees well with the certified value and its 95 % expanded uncertainty of (4.80 ± 1.00) µg/g (Figure 17). These results suggest that there is no detectable measurement bias.

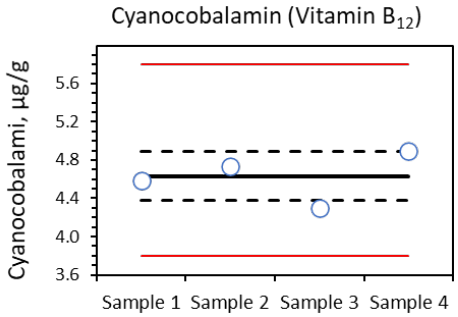

Figure 17. Mass Fraction Cyanocobalamin in SRM 3280 Control

Open circles represent results of the analysis of four SRM 3280 control preparations. The solid black line represents the mean value; the dashed lines bound one standard deviation above and below the mean. Red solid lines bound the approximate 95 % level of confidence expanded uncertainty interval for cyanocobalamin stated in the SRM 3280 Certificate of Analysis [1].

The standard uncertainty of these LC-ICP-MS measurements is estimated using:

$$u = \sqrt{u_{\text{rep}}^2 + \beta_1^2 + \beta_2^2 + \beta_3^2 + \beta_4^2 + \beta_5^2}\ .$$

These uncertainty components are described and their estimated values are provided in Table 15.

Table 15. Cyanocobalamin Uncertainty Components and Estimated Values

Component	Description	Degrees of Freedom	Value
u_{rep}	Standard uncertainty of replicate LC-ICP-MS measurements	19	0.069
β_1, Calibrant	Experiment-based CNCbl characterization standard uncertainty	large	0.022
β_2, Weighing	Balance calibration specification, converted to standard uncertainty	large	0.0026
β_3 Volumetry	Volumetric flask and syringe specifications, converted to standard uncertainty	large	0.01
β_4, Density	Density meter readability, converted to standard uncertainty	large	0.004
β_5, Recovery	Experiment-based method recovery standard uncertainty	large	0.048
u	Combined standard uncertainty:	49	0.088

4.5 Preparation of the Standard Additions Spiking Solution

Analyte identity confirmation for the USP Vitamin B_{12} standard used to produce the CNCbl spiking solution was confirmed using high-resolution mass spectrometry (HR-MS) and NMR spectroscopy. The mass fraction of Co contributed by CNCbl to the spiking solution was established using ICP-MS and LC-ICP-MS.

4.5.1 Identity by HR-MS

A 2.4 mg sample of the USP CNCbl standard was dissolved in 50:50 water/acetonitrile (volume fractions) to prepare a solution with mass fraction 240 µg/g. The HR-MS measurement was performed with a Thermo Q-Exactive via direct infusion at 500 µL/min into an electrospray ionization (ESI) source operated in positive mode. The mass spectrum was acquired between 150 *m*/*z* to 2000 *m*/*z* at a resolving power of 70 000. Prior to analysis the instrument was calibrated with a relative mass accuracy of 0.000 021 %. Table 16 summarizes the instrument settings.

Table 16. Instrument Parameters for Q-Exactive HR-MS of Cyancobalamin Standard

Parameter	Value
Sheath Gas Flow Rate	60 L/min
Auxiliary Gas Flow Rate	20 L/min
Spray Voltage	3.00 kV
Capillary Temperature	380 °C
Auxiliary Gas Temperature	350 °C

Figure 18 displays the HR-MS spectrum of the USP material. Table 17 compares the theoretical [**Error! Bookmark not defined.**] and measured mass to charge ratios (*m*/*z*) of CNCbl for the $[M+H]^+$ and $[M+2H]^{2+}$ ions. The measured values are consistent with those expected for CNCbl.

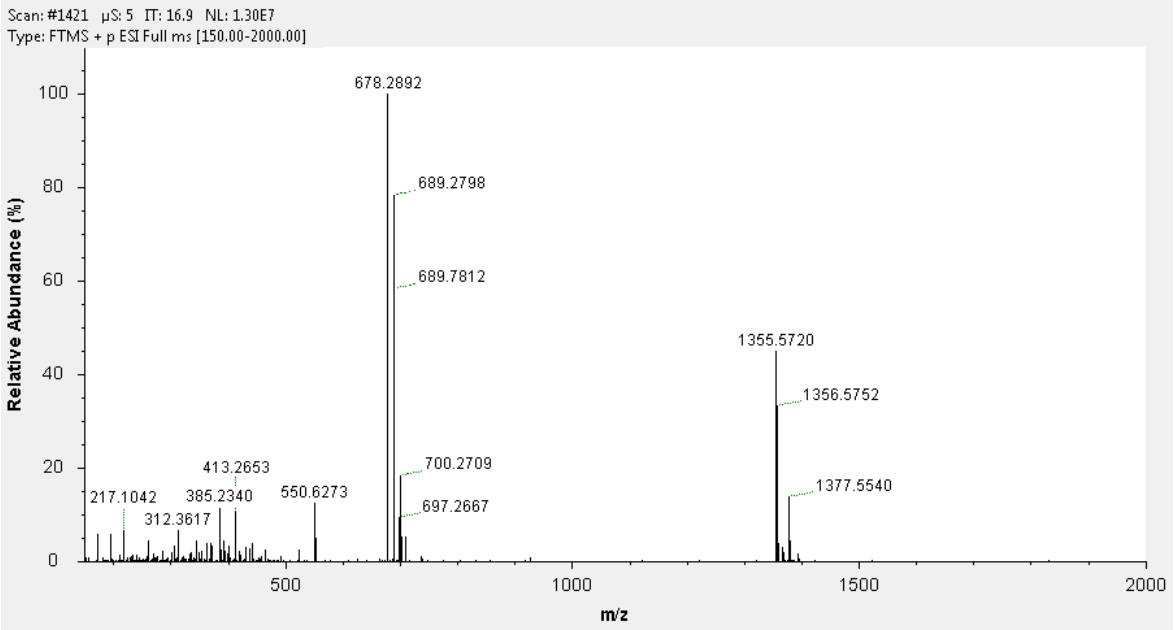

Figure 18. HR-MS Spectrum of the Cyanocobalamin Standard

Table 17. Theoretical and Measured *m/z* for the Cyanocobalamin Standard

Ion	Mass to Charge Ratio, *m/z*		
	Theoretical	Measured	Difference
$[M+H]^+$	1355.5747	1355.5720	0.0027
$[M+2H]^{2+}$	678.2910	678.2892	0.0018

4.5.2 Identity by NMR

One vial of the USP CNCbl standard was assessed with various NMR techniques using D_2O as solvent. One dimensional ^1H, two-dimensional ^1H-^{13}C HSQC with sensitivity enhancement using adiabatic shaped pulses, and ^1H-^1H correlation (COSY) NMR spectroscopy experiments were conducted at 298 K. Figure 19 displays the multiplicity-edited ^1H-^{13}C HSQC spectrum of CNCbl in D_2O with chemical structure assignments of correlation cross sections. Figure 20 and Figure 21 display the ^1H and ^1H--^1H COSY spectra used to support the HSQC assessment. These assignments are in full accordance with published literature [13,14].

There are no major signals in the NMR spectra (Figures 19-21) not attributable to CNCbl or water. Peaks that are suspected to be organic impurity components are observed in the ^1H spectrum (Figure 20); however, the relative areas of these peaks are <5 % of those for CNCbl.

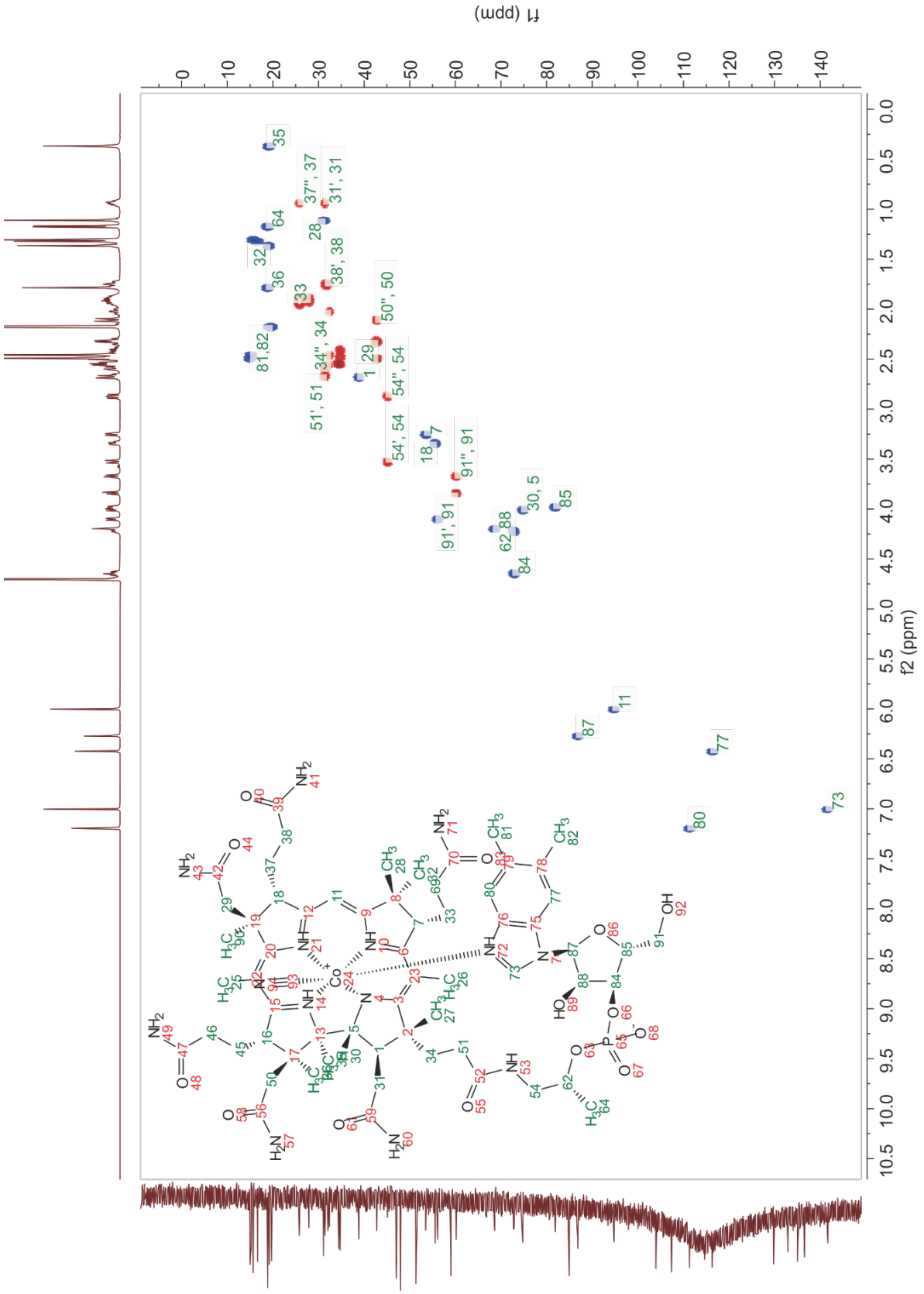

Figure 19. Multiplicity-Edited ^{1}H-^{13}C HSQC Spectrum of Cyanocobalamin Standard in D_2O

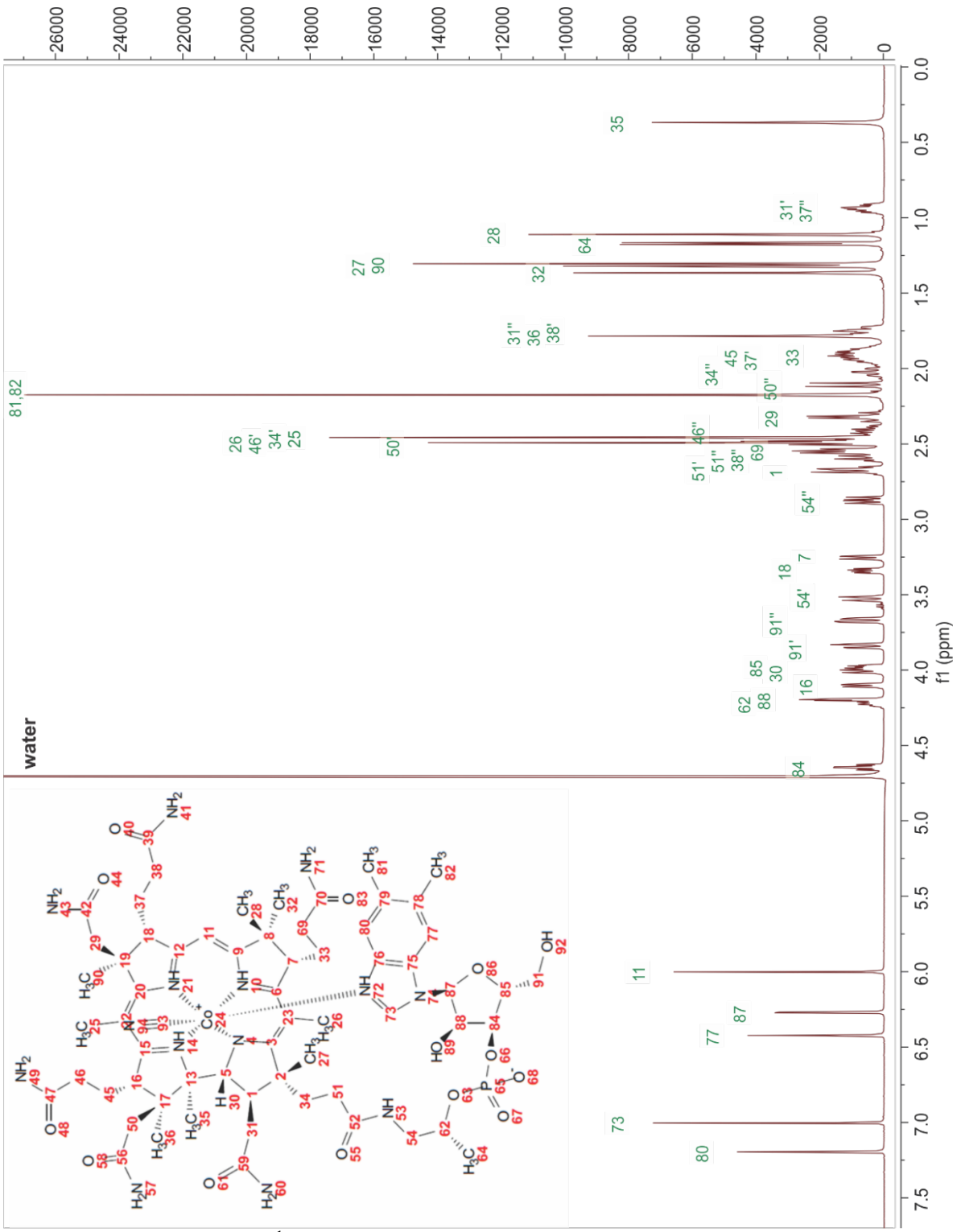

Figure 20. ^{1}H Spectrum of Cyanocobalamin Standard in D_2O.

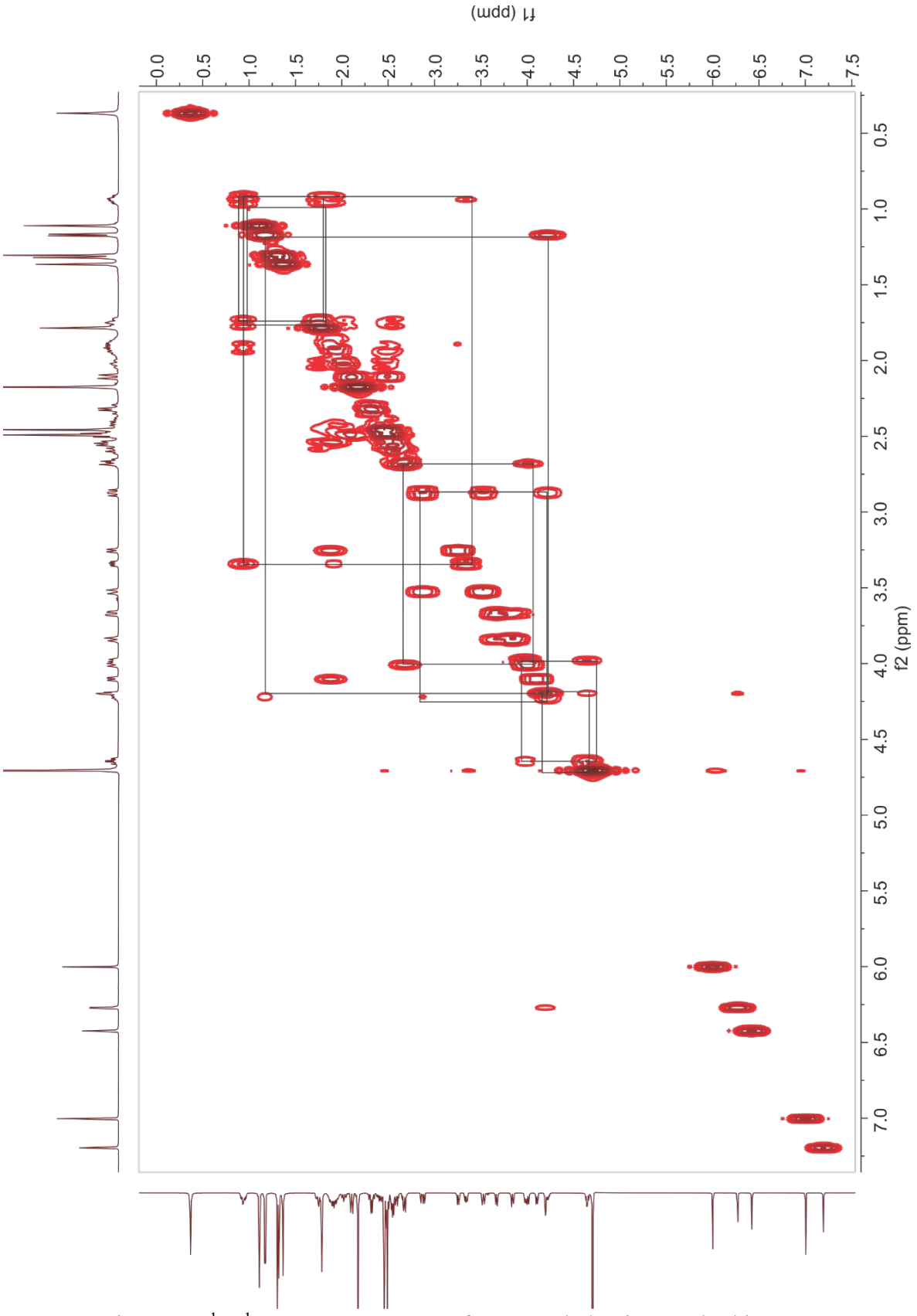

Figure 21. ^{1}H-^{1}H COSY Spectrum of Cyanocobalamin Standard in D_2O

4.5.3 Characterization of the Standard Additions Spiking Solution

The amount content of Co in the spiking solution attributable to CNCbl was established in two stages: 1) using ICP-MS to determine the total Co mass and 2) using LC-ICP-MS to determine the fraction of total Co coming from impurities. The mass of Co from CNCbl was calculated by subtracting the cobalt of the impurities from the total cobalt.

A stock solution containing nominally 123 µg/g of Co as CNCbl was prepared by dissolving 141 mg of the as-received CNCbl standard in 50 g water. Eight aliquots of 0.2 g of the solution were weighed into eight microwave vessels. Eight milliliters of HNO_3 were added to each vessel and the contents microwave digested using two cycles with: power, 1600 W; ramp time, 25 min; temperature, 220 °C; and hold time, 15 min. Four blanks were prepared similarly. Each digested sample was transferred to a 60 mL low-density polyethylene (LDPE) bottle, and the contents were diluted to 50 g with water. A 0.2 g aliquot of each sample was weighed into a 60 mL LDPE bottle, into which 0.2 g of a solution containing 501 µg/kg rhodium (Rh) was added as an internal standard. The contents were diluted to 50 g with 1.5 % HNO_3 in water.

A 25 g subsample of each replicate was weighed into a 30 mL LDPE bottle and combined with a 0.5 g aliquot of a solution containing a Co mass fraction of 218 µg/kg, producing a spiked sample for the purpose of quantification by single-point standard additions using ICP-MS. The Co spike was prepared from SRM 3113 Cobalt (Co) Standard Solution, lot #000630. The result of the measurement of total Co in the stock solution was (114.8 ± 0.46) µg/g based on the eight replicates.

Separately, six samples were prepared from the stock solution for assessment of Co-containing impurities therein. Each sample was prepared by diluting the stock solution with water to contain approximately 200 µg/kg Co as CNCbl. The samples were measured for Co species by LC-ICP-MS using the instrumental parameters described in Table 14. Figure 22 displays a typical chromatogram of the CNCbl stock solution.

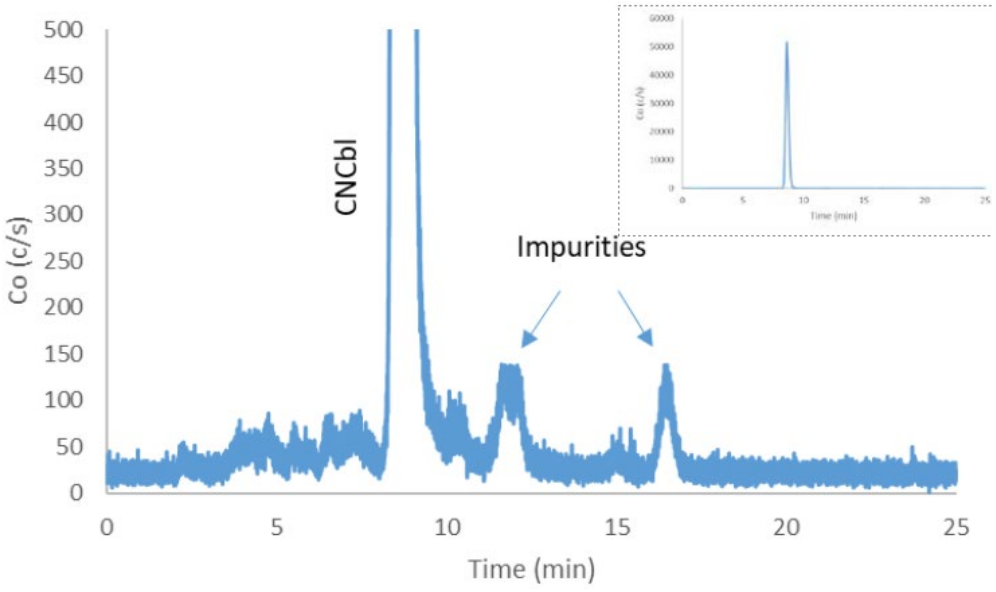

Figure 22. Typical Chromatogram of Cyanocobalamin Standard Stock Solution

There are some barely visible small peaks along with the dominant CNCbl peak. Given that the unknown peaks have retention time greater than the retention time of free Co, which is about 2 min, the impurities are most likely organic Co complexes. Assuming these Co-containing impurities have the same ICP-MS detector response factor as CNCbl, the purity of the CNCbl calibrant can be estimated from the ratio of CNCbl peak area to the total area of the Co signal. The fraction of Co in the CNCbl calibrant contributed by CNCbl was (0.9917 ± 0.0002).

The amount of Co from CNCbl in the stock solution is calculated as the product of the total Co amount and the fraction of Co as CNCbl, (113.9 ±0.5) µg/g. This result is traceable to the SI thru the SRM 3113 certified value and the LC-ICP-MS determination of the impurity content. The estimated standard uncertainty is almost entirely accounted for by the repeatability of the total Co measurement using the standard additions method.

4.5.4 Standard Additions Method

The method of standard additions refers to the calibration of an analytical instrument by measuring the increase in the analytical signal that occurs when a known amount of the analyte is added to the sample. It avoids multiplicative types of matrix interferences (enhancements or suppressions) since the calibrant is present with the same matrix as the sample. It can be used in any situation where an analyte and an internal standard can be quantitatively and homogeneously spiked into the sample.

The mass fraction of the analyte in the sample (F_{sample}) is calculated as:

$$F_{\text{sample}} = R_{\text{u}} \left(\frac{\left(m_{\text{sp}} F_{\text{sp}} \Big/ m_{\text{spsolu}} \right)}{R_{\text{sp}} - R_{\text{u}}} \right) \left(\frac{m_{\text{solu}}}{m_{\text{sample}}} \right)$$

where: F_{sp} mass fraction of the analyte in the spiking solution
m_{sample} mass of sample that is present in the solution to be analyzed
m_{solu} total mass of the sample solution after addition of the IS spike
m_{sp} mass of the analyte spiking solution delivered to the solution
m_{spsolu} mass of the solution that will be spiked
R_{sp} analyte/IS signal ratios for the spiked solution
R_{u} analyte/IS signal ratios for the unspiked solution.

5 HAMQAP

The Health Assessment Measurement Quality Assurance Program (HAMQAP) was launched in collaboration with the NIH ODS in 2017. HAMQAP was established to enable laboratories to improve the accuracy of measurements in samples that represent human intake (e.g., foods, dietary supplements, tobacco) and samples that represent human metabolism (e.g., blood, serum, plasma, urine) for demonstration of proficiency and/or compliance with various regulations. Participation in HAMQAP Exercises is voluntary and anonymous.

As of Fall 2021, SRM 3289 tablets have been distributed in four HAMQAP Exercises. The relevant measurands of interest were:

- Exercise 3, Spring 2019, prefix "C": folic acid (vitamin B_9), β-carotene (provitamin A), and lutein [16].
- Exercise 4, Summer 2019, prefix "D": cyanocobalamin (vitamin B_{12}) and phylloquinone (vitamin K_1) [17].
- Exercise 5, Spring 2020, prefix "E": thiamine (vitamin B_1), riboflavin (vitamin B_2), niacin (vitamin B_3), pantothenic acid (vitamin B_5), pyridoxine (vitamin B_6), ergocalciferol (vitamin D_2), and cholecalciferol (vitamin D_3) [15].
- Exercise 6, Spring 2021, prefix "F": biotin (vitamin B_7), retinyl acetate (vitamin A), ascorbic acid (vitamin C), and α-tocopherol (vitamin E) [18].

Laboratory participants in each exercise are identified with a unique code consisting of an alphabetic exercise-specific prefix and a numeric index reflecting the sign-up order.

While all results are provided as-received in the publicly accessible Final Reports of each exercise, the HAMQAP results presented in the following Section have been lightly screened using standard "outlier" detection methods to eliminate technically suspect values. The most common discrepancies apparently arise from misstating or miscalculating the units of measurement by factors of 10 to 1000.

6 Measurement Results

6.1 Vitamin B_1 (Thiamine)

Table 18 lists the thiamine (vitamin B_1) measurement results from the NIST ID-LC-MS/MS, analysis of 10 bottles, the manufacturer's HPLC-UV analysis of 10 samples as provided by their COA, and the 17 accepted results from HAMQAP Exercise 5. Figure 23 displays these values. See Section 3 of [15] for information regarding the HAMQAP results and summary statistics.

Table 18. Measurement Results for Thiamine (Vitamin B_1), mg/g

NIST

Bottle	A	B	Mean	SD
10	1.12	1.13	1.125	0.007
42	1.14	1.17	1.155	0.021
88	1.19	1.28	1.235	0.064
111	1.27	1.16	1.215	0.078
151	1.17	1.12	1.145	0.035
185	1.11	1.11	1.110	0.000
230	1.12	1.29	1.205	0.120
260	1.27	1.16	1.215	0.078
293	1.12	1.28	1.200	0.113
318	1.16	1.12	1.140	0.028
		Mean:	1.175	
		SD:	0.044	
		N:	10	

Manufacturer

Sam	%Lbl	mg/g
1	90.5	0.954
2	105.6	1.113
3	101.5	1.070
4	101.1	1.066
5	106.5	1.123
6	111.2	1.172
7	108.2	1.141
8	98.2	1.035
9	111.3	1.173
10	103.0	1.086
Mean:		1.093
SD:		0.067
N:		10

HAMQAP Exercise 5

Lab	A	B	C	Mean	SD
E001	1.540	1.550	1.640	1.577	0.055
E002	1.374	1.486	1.457	1.439	0.058
E004	1.171	1.148	1.153	1.157	0.012
E005	1.280	1.240	1.250	1.257	0.021
E006	1.440	1.340	1.420	1.400	0.053
E007	1.440	1.450	1.421	1.437	0.015
E010	1.366	1.328	1.358	1.351	0.020
E013	0.740	0.765	0.739	0.748	0.015
E016	0.872	0.905	0.821	0.866	0.042
E023	1.090	1.010	1.060	1.053	0.040
E025	1.179	1.118	1.116	1.138	0.036
E030	1.236	1.298	1.291	1.275	0.034
E033	0.730	0.740		0.735	0.007
E040	1.220	1.205	1.208	1.211	0.008
E041	1.315	1.330	1.340	1.328	0.013
E043	1.295	1.253	1.296	1.281	0.025
E044	1.167	1.297	1.309	1.258	0.079
			Consensus Mean:	1.240	
			Consensus SD:	0.064	
			Accepted *N*:	17	

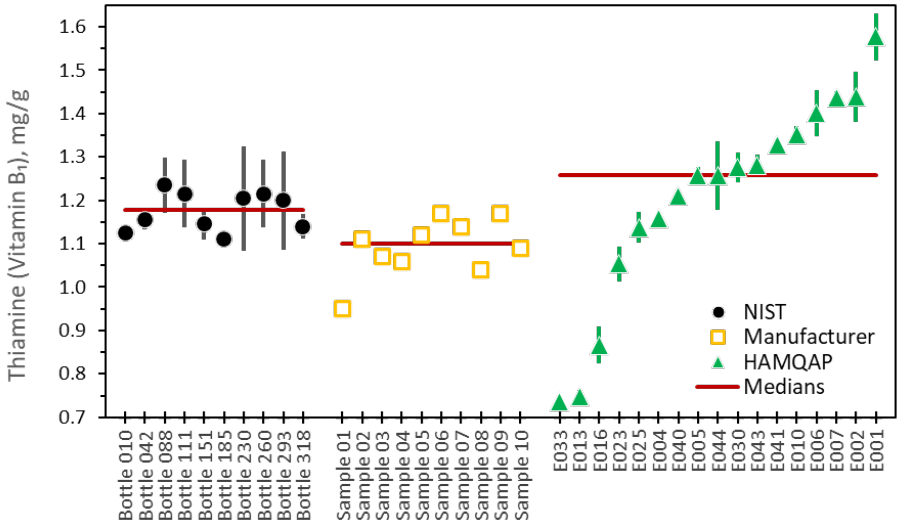

Figure 23. Measurement Results for Thiamine (Vitamin B1), mg/g
Symbols represent mean values; error bars represent one standard deviation.

6.2 Vitamin B_2 (Riboflavin)

Table 19 lists the riboflavin (vitamin B_2) measurement results from the NIST ID-LC-MS/MS, analysis of 10 bottles, the manufacturer's HPLC-UV analysis of 10 samples as provided by their COA, and the 16 accepted results from HAMQAP Exercise 5. Figure 24 displays these values. See Section 3 of [15] for information regarding the HAMQAP results and summary statistics.

Table 19. Measurement Results for Riboflavin (Vitamin B_2), mg/g

NIST

Bottle	A	B	Mean	SD
10	1.38	1.46	1.420	0.057
42	1.47	1.52	1.495	0.035
88	1.54	1.52	1.530	0.014
111	1.47	1.40	1.435	0.049
151	1.48	1.50	1.490	0.014
185	1.50	1.47	1.485	0.021
230	1.35	1.52	1.435	0.120
260	1.54	1.52	1.530	0.014
293	1.41	1.43	1.420	0.014
318	1.48	1.46	1.470	0.014
		Mean:	1.471	
		SD:	0.042	
		N:	10	

Manufacturer

Sam	%Lbl	mg/g
1	118.4	1.248
2	119.3	1.258
3	111.3	1.173
4	114.6	1.208
5	114.2	1.204
6	123.6	1.303
7	127.1	1.340
8	127.6	1.345
9	130.8	1.379
10	128.3	1.353
Mean:		1.281
SD:		0.072
N:		10

HAMQAP Exercise 5

Lab	A	B	C	Mean	SD
E001	1.540	1.550	1.640	1.577	0.055
E002	1.374	1.486	1.457	1.439	0.058
E004	1.171	1.148	1.153	1.157	0.012
E005	1.280	1.240	1.250	1.257	0.021
E006	1.440	1.340	1.420	1.400	0.053
E007	1.440	1.450	1.421	1.437	0.015
E010	1.366	1.328	1.358	1.351	0.020
E013	0.740	0.765	0.739	0.748	0.015
E016	0.872	0.905	0.821	0.866	0.042
E023	1.090	1.010	1.060	1.053	0.040
E030	1.236	1.298	1.291	1.275	0.034
E033	0.730	0.740		0.735	0.007
E040	1.220	1.205	1.208	1.211	0.008
E041	1.315	1.330	1.340	1.328	0.013
E043	1.295	1.253	1.296	1.281	0.025
E044	1.167	1.297	1.309	1.258	0.079
			Consensus Mean:	1.327	
			Consensus SD:	0.058	
			Accepted *N*:	16	

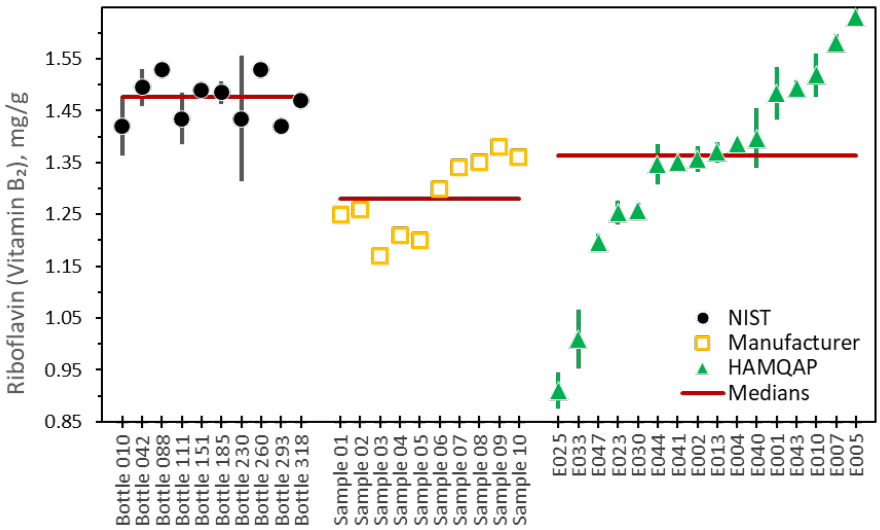

Figure 24. Measurement Results for Riboflavin (Vitamin B_2), mg/g
Symbols represent mean values; error bars represent one standard deviation.

6.3 Vitamin B_3 (Niacin)

Table 20 lists the niacin (vitamin B_3) measurement results from the NIST ID-LC-MS/MS, analysis of 10 bottles, the manufacturer's HPLC-UV analysis of 10 samples as provided by their COA, and the 13 accepted results from HAMQAP Exercise 5. Figure 25 displays these values. See Section 3 of [15] for information regarding the HAMQAP results and summary statistics.

Table 20. Measurement Results for Niacin (Vitamin B_3), mg/g

NIST

Bottle	A	B	Mean	SD
10	11.38	12.57	11.98	0.84
42	12.20	12.12	12.16	0.06
88	12.62	12.40	12.51	0.16
111	12.34	12.87	12.60	0.37
151	12.20	12.81	12.50	0.43
185	12.91	12.67	12.79	0.17
230	12.95	12.45	12.70	0.35
260	11.91	12.24	12.08	0.24
293	12.05	12.42	12.23	0.26
318	12.27	11.78	12.02	0.35
		Mean:	12.36	
		SD:	0.30	
		N:	10	

Manufacturer

Sam	%Lbl	mg/g
1	117.6	12.40
2	118.7	12.51
3	114.7	12.09
4	116.6	12.29
5	114.4	12.06
6	116.5	12.28
7	118.6	12.50
8	115.6	12.19
9	117.7	12.41
10	117.7	12.41
Mean:		12.32
SD:		0.16
N:		10

HAMQAP Exercise 5

Lab	A	B	C	Mean	SD
E001	11.20	11.30	11.00	11.17	0.15
E002	12.36	12.37	12.36	12.36	0.01
E004	12.58	12.71	12.69	12.66	0.07
E006	12.50	13.10	12.40	12.67	0.38
E007	12.31	12.14	12.39	12.28	0.12
E010	13.32	13.01	13.19	13.17	0.16
E030	12.19	12.37	12.67	12.41	0.25
E033	11.14	11.67		11.41	0.37
E040	12.46	12.98	12.35	12.60	0.34
E043	12.20	11.95	12.15	12.10	0.13
E044	11.64	11.98	11.79	11.80	0.17
E046	12.37	12.16	12.27	12.26	0.11
E047	11.55	11.36	11.37	11.43	0.11
			Consensus Mean:	12.18	
			Consensus SD:	0.22	
			Accepted *N*:	13	

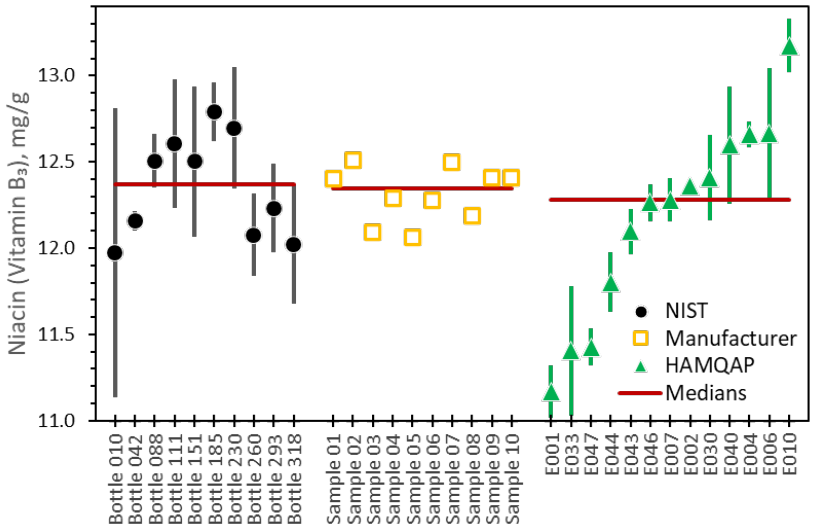

Figure 25. Measurement Results for Niacin (Vitamin B_3), mg/g
Symbols represent mean values; error bars represent one standard deviation.

6.4 Vitamin B_5 (Pantothenic Acid)

Table 21 lists the pantothenic (vitamin B_5) measurement results from the NIST ID-LC-MS/MS, analysis of 10 bottles as provided by their COA, the manufacturer's HPLC-UV analysis of 10 samples, and the 13 accepted results from HAMQAP Exercise 5. Figure 26 displays these values. See Section 3 of [15] for information regarding the HAMQAP results and summary statistics.

Table 21. Measurement Results for Pantothenic Acid (Vitamin B_5), mg/g

NIST

Bottle	A	B	Mean	SD
10	7.00	7.00	7.00	0.00
42	6.95	6.91	6.93	0.03
88	6.77	7.31	7.04	0.38
111	6.90	6.78	6.84	0.08
151	6.94	6.81	6.88	0.09
185	7.01	6.80	6.91	0.15
230	6.95	6.76	6.86	0.13
260	7.16	7.07	7.12	0.06
293	6.81	7.01	6.91	0.14
318	6.80	6.89	6.85	0.06
		Mean:	6.93	
		SD:	0.09	
		N:	10	

Manufacturer

Sam	%Lbl	mg/g
1	123.4	6.51
2	125.9	6.64
3	151.0	7.96
4	141.4	7.45
5	118.0	6.22
6	122.9	6.48
7	138.8	7.32
8	124.0	6.54
9	136.6	7.20
10	129.1	6.81
Mean:		6.91
SD:		0.55
N:		10

HAMQAP Exercise 5

Lab	A	B	C	Mean	SD
E001	7.36	7.44	7.25	7.35	0.10
E002	7.01	7.09	7.04	7.05	0.04
E004	7.11	7.00	7.12	7.08	0.07
E005	7.51	7.57	7.28	7.45	0.15
E006	6.64	6.72	6.48	6.61	0.12
E007	7.19	7.28	7.10	7.19	0.09
E010	6.44	6.62	6.85	6.64	0.21
E025	6.92	6.90	6.85	6.89	0.04
E030	7.34	7.26	7.36	7.32	0.05
E033	6.90	6.70		6.80	0.14
E041	7.42	7.76	7.03	7.04	7.77
E043	6.92	6.70	6.92	6.72	6.97
E047	7.10	7.16	6.95	6.96	7.18
			Consensus Mean:	6.99	
			Consensus SD:	0.13	
			Accepted *N*:	13	

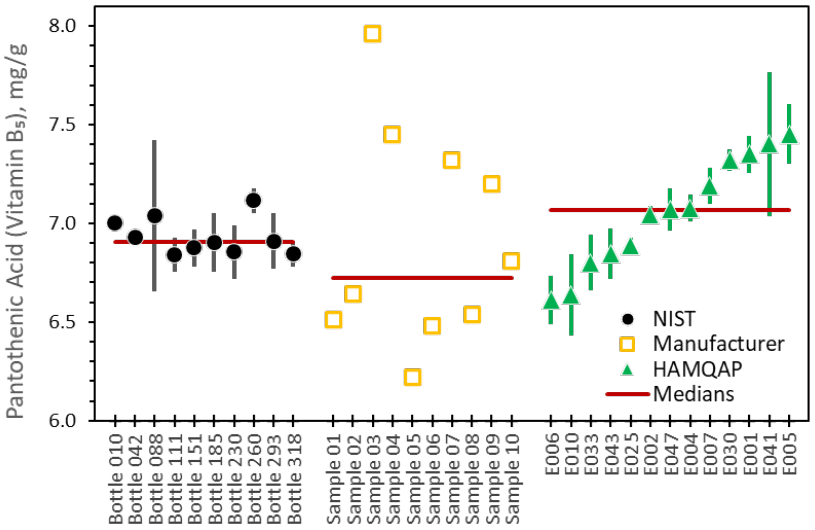

Figure 26. Measurement Results for Pantothenic Acid (Vitamin B_5), mg/g
Symbols represent mean values; error bars represent one standard deviation.

6.5 Vitamin B_6 (Pyridoxine)

Table 22 lists the pyridoxine (vitamin B_6) measurement results from the NIST ID-LC-MS/MS, analysis of 10 bottles, the manufacturer's HPLC-UV analysis of 10 samples as provided by their COA, and the 11 accepted results from HAMQAP Exercise 5. Figure 27 displays these values. See Section 3 of [15] for information regarding the HAMQAP results and summary statistics.

Table 22. Measurement Results for Pyridoxine Acid (Vitamin B_6), mg/g

NIST

Bottle	A	B	Mean	SD
10	1.41	1.34	1.375	0.049
42	1.38	1.43	1.405	0.035
88	1.39	1.55	1.470	0.113
111	1.55	1.36	1.455	0.134
151	1.40	1.44	1.420	0.028
185	1.43	1.43	1.430	0.000
230	1.38	1.67	1.525	0.205
260	1.53	1.37	1.450	0.113
293	1.38	1.58	1.480	0.141
318	1.42	1.38	1.400	0.028
		Mean:	1.441	
		SD:	0.044	
		N:	10	

Manufacturer

Sam	%Lbl	mg/g
1	123.0	1.303
2	130.5	1.319
3	123.6	1.348
4	125.1	1.337
5	127.9	1.322
6	126.8	1.333
7	125.4	1.327
8	126.4	1.377
9	125.9	1.303
10	130.6	1.319
Mean:		1.334
SD:		0.027
N:		10

HAMQAP Exercise 5

Lab	A	B	C	Mean	SD
E002	1.484	1.488	1.453	1.475	0.019
E004	1.308	1.296	1.353	1.319	0.030
E005	1.270	1.310	1.360	1.313	0.045
E006	1.340	1.440	1.300	1.360	0.072
E010	1.496	1.349	1.365	1.403	0.081
E023	1.350	1.320	1.280	1.317	0.035
E030	1.304	1.367	1.382	1.351	0.041
E040	1.366	1.472	1.326	1.388	0.076
E041	1.321	1.307	1.354	1.327	0.024
E043	1.344	1.342	1.303	1.329	0.023
E044	1.363	1.357	1.335	1.351	0.015
Consensus Mean:				1.370	
Consensus SD:				0.024	
Accepted *N*:				11	

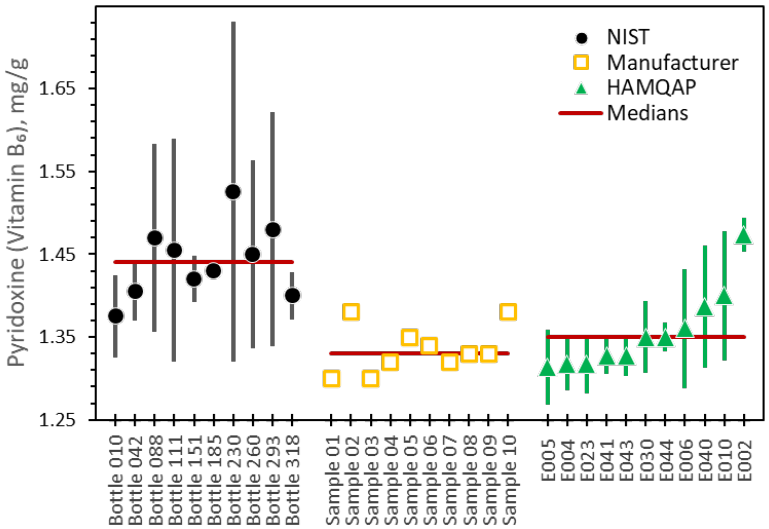

Figure 27. Measurement Results for Pyridoxine Acid (Vitamin B_6), mg/g

Symbols represent mean values; error bars represent one standard deviation.

6.6 Vitamin B_7 (Biotin)

Table 23 lists the biotin (vitamin B_7) measurement results from the NIST ID-LC-MS/MS, analysis of 10 bottles, the manufacturer's HPLC-UV analysis of 10 samples as provided by their COA, and the 12 accepted results from HAMQAP Exercise 6. Figure 28 displays these values. See Section 3 of [18] for information regarding the HAMQAP results and summary statistics.

Table 23. Measurement Results for Biotin (Vitamin B_7), µg/g

NIST

Bottle	A	B	Mean	SD
10	23.45	23.92	23.69	0.33
42	24.05	22.41	23.23	1.16
88	22.68	23.93	23.31	0.88
111	23.36	22.54	22.95	0.58
151	23.21	23.92	23.57	0.50
185	23.60	22.55	23.08	0.74
230	24.69	22.49	23.59	1.56
260	22.71	23.15	22.93	0.31
293	25.69	23.48	24.59	1.56
318	25.81	23.45	24.63	1.67
		Mean:	23.56	
		SD:	0.61	
		N:	10	

Manufacturer

Sam	%Lbl	µg/g
1	103.3	21.78
2	111.0	23.41
3	103.8	21.89
4	103.3	21.78
5	111.0	23.41
6	106.8	22.52
7	103.8	21.89
8	101.0	21.30
9	102.3	21.57
10	99.3	20.94
Mean:		22.05
SD:		0.83
N:		10

HAMQAP Exercise 6

Lab	A	B	C	Mean	SD
F017	19.80	21.60	24.30	21.90	2.26
F026	35.05			35.05	
F030	20.53	19.35	21.30	20.39	0.98
F034	42.80	44.90	44.40	44.03	1.10
F036	17.93	20.55	20.53	19.67	1.51
F039	13.50	14.20	12.50	13.40	0.85
F040	31.70	29.70	30.50	30.63	1.01
F046	28.58	24.75	24.80	26.04	2.20
F059	18.60	19.50	21.40	19.83	1.43
F073	28.20	27.40	28.50	28.03	0.57
F075	18.60	23.80	18.80	20.40	2.95
F080	16.66	16.89	16.81	16.79	0.12
			Consensus Mean:	23.5	
			Consensus SD:	9.2	
			Accepted *N*:	12	

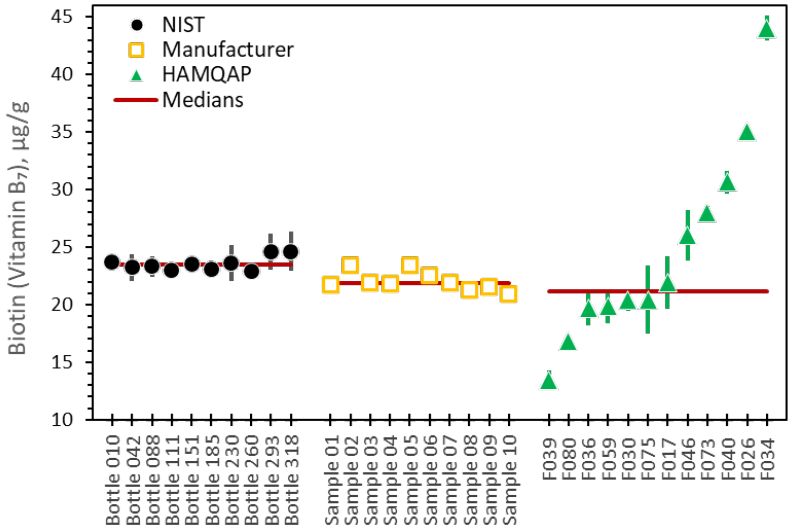

Figure 28. Measurement Results for Biotin (Vitamin B_7), µg/g
Symbols represent mean values; error bars represent one standard deviation.

6.7 Vitamin B_9 (Folic Acid)

Table 24 lists the folic acid (vitamin B_9) measurement results from the manufacturer's HPLC-UV analysis of 10 samples as provided by their COA and the 16 accepted results from HAMQAP Exercise 3. Figure 29 displays these values. See Section 3 of [16] for information regarding the HAMQAP results and summary statistics.

Table 24. Measurement Results for Folic Acid (Vitamin B_9), µg/g

Manufacturer		
Sam	%Lbl	µg/g
1	147.6	466.8
2	148.0	468.1
3	146.5	463.4
4	149.2	471.9
5	140.5	444.4
6	149.1	471.6
7	146.6	463.7
8	147.4	466.2
9	147.1	465.3
10	148.2	468.7
Mean:		465.0
SD:		7.8
N:		10

HAMQAP Exercise 3					
Lab	A	B	C	Mean	SD
C008	387.5	393.0	395.7	392.1	4.2
C010	406.0	443.0	435.0	428.0	19.5
C013	432.0	483.0	487.0	467.3	30.7
C014	352.0	350.0	362.0	354.7	6.4
C020	385.7	384.8	390.2	386.9	2.9
C021	400.0	425.0	406.0	410.3	13.1
C022	447.9	443.8	432.5	441.4	8.0
C023	311.2	316.9	300.8	309.6	8.2
C027	461.0	512.0	481.0	484.7	25.7
C028	417.0	401.0	395.0	404.3	11.4
C029	417.5	418.6	417.9	418.0	0.6
C033	415.0	412.9	417.3	415.1	2.2
C035	394.0	383.0	400.0	392.3	8.6
C039	464.0	427.0	442.0	444.3	18.6
C044	353.0	359.0	353.0	355.0	3.5
C053	476.0	501.0	452.0	476.3	24.5
Consensus Mean:				412	
Consensus SD:				60	
Accepted *N*:				16	

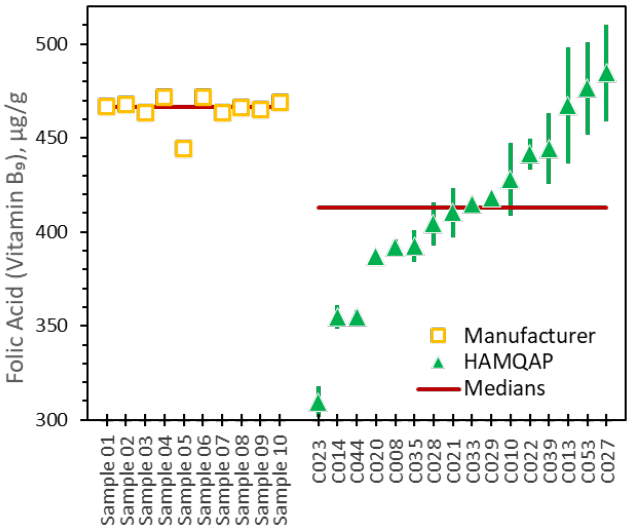

Figure 29. Measurement Results for Folic Acid (Vitamin B_9), µg/g
Symbols represent mean values; error bars represent one standard deviation.

6.8 Vitamin B_{12} (Cyanocobalamin)

Table 25 lists the cyanocobalamin (vitamin B_{12}) measurement results from the NIST ID-LC-MS/MS, analysis of 10 bottles, the manufacturer's HPLC-UV analysis of 10 samples as provided by their COA, and the 13 accepted results from HAMQAP Exercise 4. Figure 30 displays these values. See Section 3 of [17] for information regarding the HAMQAP results and summary statistics.

Table 25. Measurement Results for Cyanocobalamin (Vitamin B_{12}), µg/g

NIST

Bottle	A	B	Mean	SD
7	4.41	4.58	4.50	0.12
39	4.92	4.08	4.50	0.59
84	4.29	4.49	4.39	0.14
108	4.26	4.43	4.35	0.12
148	4.88	4.89	4.89	0.01
179	4.66	5.04	4.85	0.27
221	4.96	4.80	4.88	0.11
255	4.11	4.24	4.18	0.09
285	4.93	4.92	4.93	0.01
318	4.44	4.49	4.47	0.04
Mean:			4.59	
SD:			0.27	
N:			10	

Manufacturer

Sam	%Lbl	µg/g
1	122.2	5.80
2	118.9	5.64
3	120.0	5.69
4	123.3	5.85
5	118.9	5.64
6	123.3	5.85
7	121.1	5.75
8	121.4	5.76
9	125.6	5.96
10	124.4	5.90
Mean:		5.78
SD:		0.11
N:		10

HAMQAP Exercise 4

Lab	A	B	C	Mean	SD
D001	5.51	5.22	6.50	5.74	0.67
D009	6.75	6.17	5.99	6.30	0.40
D010	7.16	9.40	8.45	8.34	1.12
D014	8.80	8.02	7.48	8.10	0.66
D019	4.63	5.19	4.90	4.91	0.28
D021	4.22	4.50	4.82	4.51	0.30
D024	7.09	7.20	6.64	6.98	0.30
D026	3.75	4.64	4.81	4.40	0.57
D031	4.37	4.17	4.57	4.37	0.20
D036	4.36	4.44	4.39	4.40	0.04
D048	4.64	4.44	4.42	4.50	0.12
D049	3.88	3.67	3.60	3.72	0.15
D050	5.34	5.34	5.53	5.40	0.11
Consensus Mean:				5.47	
Consensus SD:				0.43	
Accepted *N*:				13	

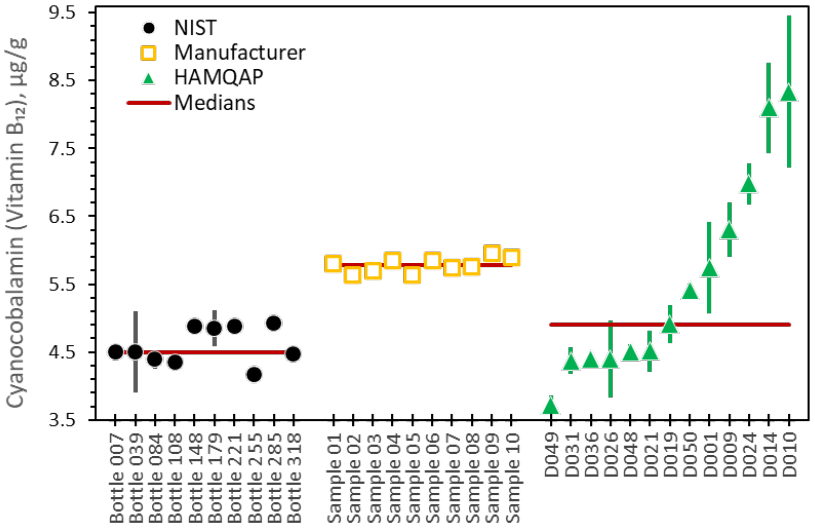

Figure 30. Measurement Results for Cyanocobalamin (Vitamin B_{12}), µg/g
Symbols represent mean values; error bars represent one standard deviation.

6.9 Vitamin A (as Retinyl Acetate)

Table 26 lists the retinyl acetate (vitamin A) measurement results from the manufacturer's HPLC-UV analysis of 10 samples as provided by their COA and the 11 accepted results from HAMQAP Exercise 6. Figure 31 displays these values. See Section 3 of [18] for information regarding the HAMQAP results and summary statistics.

Table 26. Measurement Results for Retinyl Acetate (Vitamin A), µg/g

Manufacturer

Sam	%Lbl	µg/g
1	126.5	917.7
2	121.6	882.2
3	126.1	914.8
4	120.3	872.7
5	122.8	890.9
6	122.6	889.4
7	128.1	929.3
8	119.4	866.2
9	126.6	918.4
10	124.4	902.5
Mean:		898
SD:		21
N:		10

HAMQAP Exercise 6

Lab	A	B	C	Mean	SD
F005	486.1	525.2	521.4	510.9	21.6
F011	746.4	818.2	758.6	774.4	38.4
F022	774.0	757.0	722.0	751.0	26.5
F034	789.0	830.0	787.0	802.0	24.3
F039	682.0	718.0	744.0	714.7	31.1
F059	607.0	583.0	623.0	604.3	20.1
F060	731.3	748.9	740.6	740.3	8.8
F061	590.0	599.0	615.0	601.3	12.7
F069	666.0	638.0	658.0	654.0	14.4
F075	700.0	689.0	757.0	715.3	36.5
F088	737.8	762.1	561.4	687.1	109.5
Consensus Mean:				704	
Consensus SD:				108	
Accepted *N*:				11	

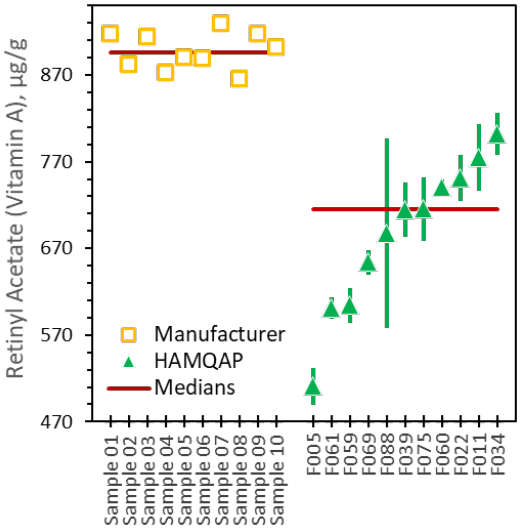

Figure 31. Measurement Results for Retinyl Acetate (Vitamin A), µg/g
Symbols represent mean values; error bars represent one standard deviation.

6.10 Provitamin A (β-Carotene)

Table 27 lists the β-carotene (provitamin A) measurement results from the manufacturer's HPLC-UV analysis of 10 samples as provided by their COA and the 14 accepted results from HAMQAP Exercise 3. Figure 32 displays these values. See Section 3 of [16] for information regarding the HAMQAP results and summary statistics.

Table 27. Measurement Results for β-Carotene (Provitamin A), µg/g

Manufacturer		
Sam	%Lbl	µg/g
1	137.7	609.7
2	149.0	659.8
3	162.9	721.3
4	142.6	631.4
5	161.3	714.2
6	159.0	704.1
7	145.5	644.3
8	151.4	670.4
9	172.6	764.3
10	149.6	662.4
Mean:		678
SD:		47
N:		10

HAMQAP Exercise 3					
Lab	A	B	C	Mean	SD
C005	514	613	526	551	54
C007	574	568	579	574	6
C010	573	499	565	546	41
C013	274	281	281	279	4
C014	554	562	541	552	11
C016	812	785	816	804	17
C020	606	639	596	614	23
C021	512	547	514	524	20
C028	592	587	589	589	3
C029	584	646	685	638	51
C036	481	490	468	480	11
C038	372	299	233	301	70
C039	640	566	648	618	45
C044	600	653	627	627	27
Consensus Mean:				532	
Consensus SD:				153	
Accepted *N*:				14	

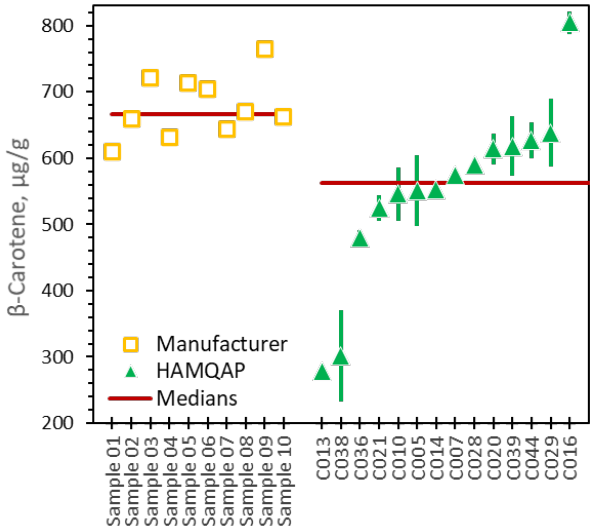

Figure 32. Measurement Results for β-Carotene (Provitamin A), µg/g
Symbols represent mean values; error bars represent one standard deviation.

6.11 Lutein

Lutein is a "non-provitamin A" carotenoid believed to have protective health effects [19]. Table 28 lists the lutein measurement results from the manufacturer's HPLC-UV analysis of 10 samples as provided by their COA and the 10 accepted results from HAMQAP Exercise 3. Figure 33 displays these values. See Section 3 of [16] for information regarding the HAMQAP results and summary statistics.

Table 28. Measurement Results for Lutein, µg/g

Manufacturer			HAMQAP Exercise 3					
Sam	%Lbl	µg/g	Lab	A	B	C	Mean	SD
1	100.6	186	C005	166.6	187.7	205.4	187	19
2	115.4	213	C007	195.0	205.0	221.0	207	13
3	103.1	190	C021	165.2	178.0	153.0	165	13
4	93.7	173	C025	209.0	200.0	213.0	207	7
5	113.1	209	C028	210.0	190.0	200.0	200	10
6	102.6	189	C029	218.0	227.0	225.0	223	5
7	104.6	193	C036	200.3	195.9	215.5	204	10
8	112.6	208	C039	186.5	205.6	195.4	196	10
9	110.9	205	C042	128.7	166.0	189.5	161	31
10	113.7	210	C044	187.0	175.0	188.0	183	7
Mean:		197	Consensus Mean:				193	
SD:		13	Consensus SD:				33	
N:		10	Accepted *N*:				10	

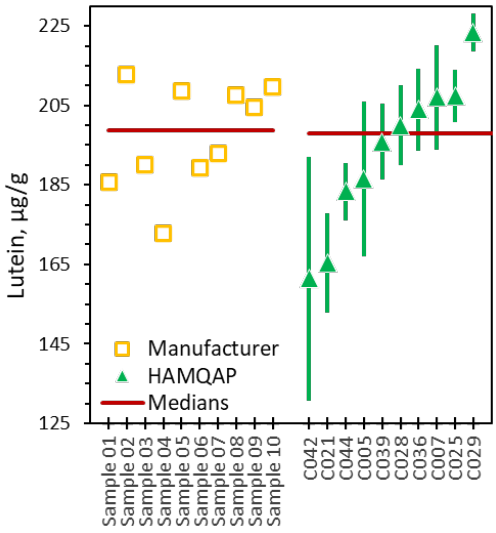

Figure 33. Measurement Results for Lutein, µg/g

Symbols represent mean values; error bars represent one standard deviation.

6.12 Vitamin C (Ascorbic Acid)

Table 29 lists the ascorbic acid (vitamin C) measurement results from the manufacturer's HPLC-UV analysis of 10 samples as provided by their COA and the 23 accepted results from HAMQAP Exercise 6. Figure 34 displays these values. See Section 3 of [15] for information regarding the HAMQAP results and summary statistics.

Table 29. Measurement Results for Ascorbic Acid (Vitamin C), mg/g

Manufacturer		
Sam	%Lbl	mg/g
1	126.5	46.68
2	128.4	47.38
3	127.1	46.90
4	129.2	47.68
5	124.9	46.09
6	125.1	46.16
7	117.9	43.51
8	128.4	47.38
9	125.2	46.20
10	131.5	48.52
Mean:		46.6
SD:		1.3
N:		10

HAMQAP Exercise 6					
Lab	A	B	C	Mean	SD
F011	45.20	45.74	45.49	45.48	0.27
F013	48.08	49.82	48.68	48.86	0.89
F014	46.60	44.40	48.70	46.57	2.15
F017	48.00	45.00	47.90	46.97	1.70
F022	42.37	41.79	38.56	40.91	2.05
F026	43.87			43.87	
F030	48.90	47.20	47.50	47.87	0.91
F031	46.18	45.98	42.38	44.85	2.14
F034	38.62	38.89	38.02	38.51	0.45
F036	46.02	42.28	42.35	43.55	2.14
F039	44.60	44.90	43.80	44.43	0.57
F040	41.06	42.80	40.70	41.52	1.12
F046	47.10	42.96	43.96	44.67	2.16
F057	36.77	37.84	36.94	37.19	0.58
F059	44.30	44.80	45.00	44.70	0.36
F060	49.78	49.33	48.19	49.10	0.82
F069	40.09	41.71	45.56	42.45	2.81
F070	36.41	34.63	33.75	34.93	1.36
F073	45.09	43.87	44.18	44.38	0.64
F074	36.10	37.90	38.30	37.43	1.17
F075	44.20	42.30	42.10	42.87	1.16
F079	46.60	42.00	43.20	43.93	2.39
F080	34.99	34.83	34.55	34.79	0.22
Consensus Mean:				42.8	
Consensus SD:				5.0	
Accepted *N*:				23	

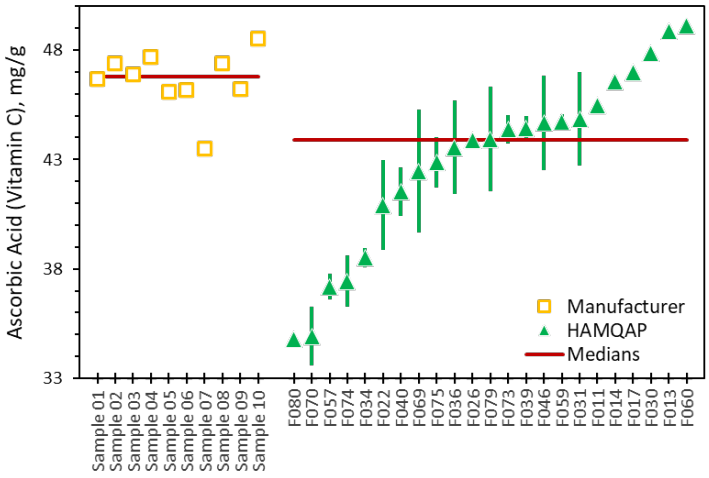

Figure 34. Measurement Results for Ascorbic Acid (Vitamin C), mg/g
Symbols represent mean values; error bars represent one standard deviation.

6.13 Vitamin D_2 (Ergocalciferol)

Table 30 lists the ergocalciferol (vitamin D_2) measurement results from the manufacturer's HPLC-MS/MS analysis of 10 samples as provided by their COA and the 14 accepted results from HAMQAP Exercise 5. Figure 35 displays these values. See Section 3 of [18] for information regarding the HAMQAP results and summary statistics.

Table 30. Measurement Results for Ergocalciferol (Vitamin D_2), µg/g

Manufacturer		
Sam	%Lbl	µg/g
1	138.0	11.01
2	139.3	10.65
3	134.7	10.75
4	136.0	10.65
5	134.7	10.38
6	131.3	10.86
7	137.3	10.75
8	136.0	10.65
9	134.7	10.65
10	134.7	11.01
Mean:		10.73
SD:		0.18
N:		10

HAMQAP Exercise 5					
Lab	A	B	C	Mean	SD
E001	8.21	8.31	7.80	8.11	0.27
E002	16.51	16.59	18.14	17.08	0.92
E003	3.68	3.47	3.69	3.61	0.12
E005	5.73	5.74	6.11	5.86	0.22
E010	6.18	7.91	8.49	7.53	1.20
E012	15.00	15.00	15.30	15.10	0.17
E014	11.13	8.53	9.34	9.67	1.33
E015	1.23	1.32	1.29	1.28	0.05
E016	0.12	0.13	0.11	0.12	0.01
E023	7.55	7.82	7.80	7.72	0.15
E030	6.77	6.70	7.05	6.84	0.19
E033	6.10	5.50		5.80	0.42
E040	7.98	7.17	7.35	7.50	0.43
E043	8.15	8.03	8.00	8.06	0.08
Consensus Mean:				7.07	
Consensus SD:				0.94	
Accepted *N*:				14	

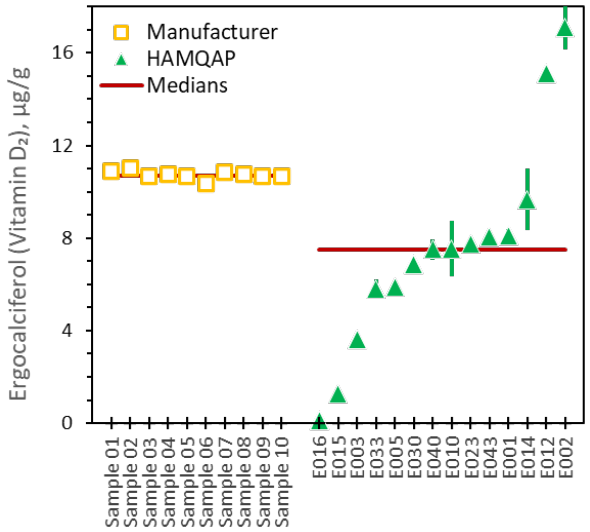

Figure 35. Measurement Results for Ergocalciferol (Vitamin D_2), µg/g

Symbols represent mean values; error bars represent one standard deviation.

6.14 Vitamin D_3 (Cholecalciferol)

Table 31 lists the cholecalciferol (vitamin D_3) measurement results from the manufacturer's HPLC-MS/MS analysis of 10 samples as provided by their COA and the 18 accepted results from HAMQAP Exercise 5. Figure 36 displays these values. See Section 3 of [18] for information regarding the HAMQAP results and summary statistics.

Table 31. Measurement Results for Cholecalciferol (Vitamin D_3), µg/g

Manufacturer		
Sam	%Lbl	µg/g
1	140.7	11.13
2	142.0	11.23
3	134.0	10.60
4	137.3	10.86
5	127.3	10.07
6	132.0	10.44
7	136.0	10.75
8	140.7	11.13
9	132.7	10.49
10	138.7	10.97
Mean:		10.76
SD:		0.37
N:		10

HAMQAP Exercise 5					
Lab	A	B	C	Mean	SD
E001	7.69	8.05	7.24	7.66	0.41
E002	11.07	9.98	9.90	10.32	0.65
E003	8.99	8.58	9.10	8.89	0.27
E005	5.59	5.53	5.49	5.54	0.05
E007	12.70	12.80	13.00	12.83	0.15
E012	16.10	16.00	16.24	16.11	0.12
E014	7.72	7.49	7.65	7.62	0.12
E015	0.31	0.38	0.34	0.34	0.04
E016	0.13	0.15	0.12	0.13	0.02
E023	14.05	14.97	13.97	14.33	0.56
E030	8.54	8.39	7.96	8.30	0.30
E032	9.30	9.30	9.30	9.30	0.00
E033	15.90	14.60		15.25	0.92
E040	8.82	8.12	7.55	8.16	0.64
E041	9.95	8.73	5.98	8.22	2.03
E043	9.13	9.00	9.21	9.11	0.11
E044	12.04	13.09	12.15	12.43	0.58
E047	3.70	3.10	3.10	3.30	0.35
Consensus Mean:				9.0	
Consensus SD:				1.0	
Accepted *N*:				18	

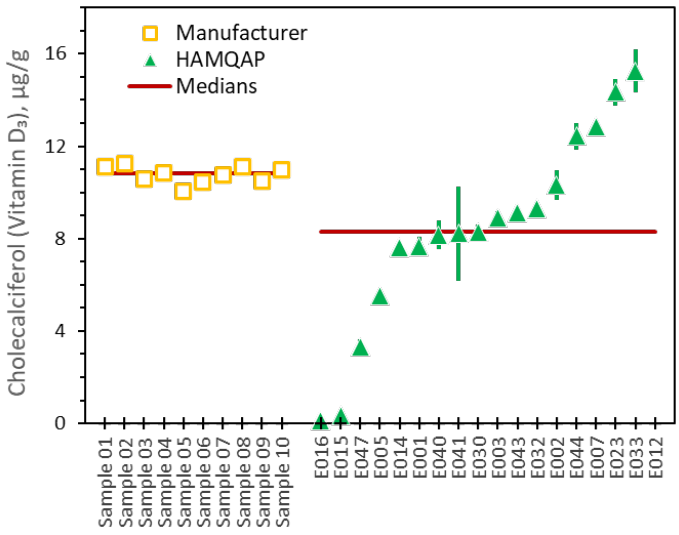

Figure 36. Measurement Results for Cholecalciferol (Vitamin D_3), µg/g
Symbols represent mean values; error bars represent one standard deviation.

6.15 Vitamin E (as α-Tocopherol Acetate)

Table 32 lists the α-tocopherol acetate (vitamin E) measurement results from the manufacturer's HPLC-UV analysis of 10 samples as provided by their COA and the 12 accepted results from HAMQAP Exercise 6. Figure 37 displays these values. See Section 3 of [18] for information regarding the HAMQAP results and summary statistics.

Table 32. Measurement Results for α-Tocopherol Acetate (Vitamin E), mg/g

Manufacturer		
Sam	%Lbl	mg/g
1	127.4	19.90
2	125.4	19.59
3	126.3	19.73
4	124.1	19.39
5	124.6	19.47
6	124.8	19.50
7	125.1	19.54
8	128.2	20.03
9	125.6	19.62
10	128.3	20.04
Mean:		19.68
SD:		0.24
N:		10

HAMQAP Exercise 6					
Lab	A	B	C	Mean	SD
F005	18.95	19.04	19.22	19.07	0.14
F011	19.94	21.58	20.60	20.71	0.83
F013	19.50	19.54	19.43	19.49	0.06
F017	18.70	18.91	20.11	19.24	0.76
F034	21.30	21.35	20.98	21.21	0.20
F039	18.80	20.00	19.10	19.30	0.62
F046	19.42	19.29	19.87	19.53	0.30
F056	20.49	20.67	19.90	20.35	0.40
F057	18.32	18.09	18.10	18.17	0.13
F059	18.40	18.20	18.90	18.50	0.36
F060	18.69	18.48	18.91	18.69	0.21
F075	19.70	19.60	19.60	19.63	0.06
Consensus Mean:				19.49	
Consensus SD:				1.62	
Accepted *N*:				12	

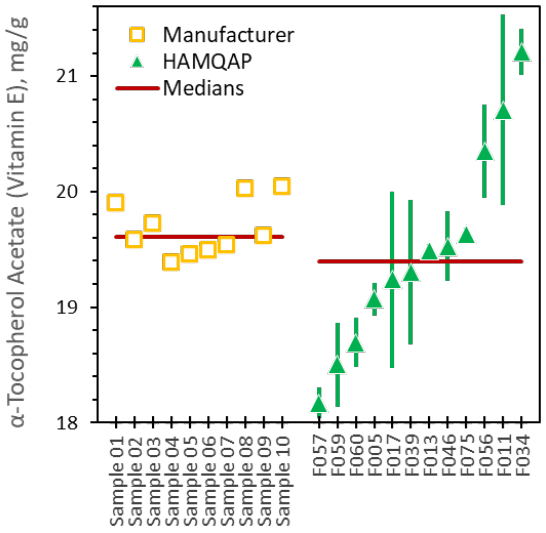

Figure 37. Measurement Results for α-Tocopherol Acetate (Vitamin E), mg/g
Symbols represent mean values; error bars represent one standard deviation.

6.16 Vitamin K_1 (Phylloquinone)

Table 33 lists the phylloquinone (vitamin K_1) measurement results from the manufacturer's HPLC-fluorescence analysis of 10 samples as provided by their COA and the 8 accepted results from HAMQAP Exercise 4. Figure 38 displays these values. See Section 3 of [17] for information regarding the HAMQAP results and summary statistics.

Table 33. Measurement Results for Phylloquinone (Vitamin K_1), µg/g

Manufacturer

Sam	%Lbl	µg/g
1	103.7	16.40
2	103.7	16.40
3	105.3	16.65
4	103.0	16.29
5	102.7	16.24
6	102.0	16.13
7	101.7	16.08
8	102.7	16.24
9	103.3	16.34
10	104.3	16.49
	Mean:	16.33
	SD:	0.17
	N:	10

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Lab	A	B	C	Mean	SD
D005	10.91	11.13	11.49	11.18	0.29
D009	10.68	10.33	10.52	10.51	0.18
D010	11.10	11.90	11.30	11.43	0.42
D019	20.96	22.23	22.31	21.83	0.76
D021	28.00	27.30	27.50	27.60	0.36
D049	15.70	15.90	15.70	15.77	0.12
D050	11.21	11.14	11.17	11.17	0.04
D055	16.20	16.62	16.54	16.45	0.22
			Consensus Mean:	17.9	
			Consensus SD:	3.6	
			Accepted *N*:	8	

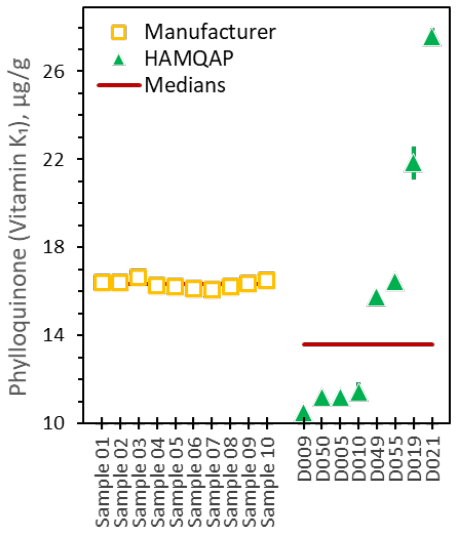

Figure 38. Measurement Results for Phylloquinone (Vitamin K_1), µg/g
Symbols represent mean values; error bars represent one standard deviation.

7 Statistical Analysis

Average mass fractions and associated uncertainties were calculated for each source of data: the mean result for individual methods used by NIST, the mean result of the manufacturer's analyses, and the median of HAMQAP analyses as described below.

7.1 Single-Laboratory Methods

There are measurements from the manufacturer for all analytes. NIST provided ID-LC-MS/MS measurements for six B vitamins (B_1, B_2, B_3, B_5, B_6, and B_7) and LC-ICP-MS measurements for B_{12}. For each analyte, a separate mean is calculated for the results obtained using each method. The uncertainty of each such mean is the standard error of that mean.

7.2 Interlaboratory Studies

For each analyte, the method estimate is the weighted median of the individual laboratory means for that analyte, where the weights are based on a Laplace random effects model [20]. For this SRM, the weighted median is equal to the unweighted median of laboratory means for all analytes in the exercise. The uncertainty of the weighted median is estimated using a bootstrap procedure based on a Laplace random effects model for the between-lab and within-lab effects [8,20,21,22,23].

7.3 Assignment of Values and Uncertainties

For analytes for which there are measurements from NIST, the manufacturer, and HAMQAP, the certified or reference value is the mean of the NIST method mean and the HAMQAP-manufacturer combined mean. The estimate is equal to a weighted mean with the NIST method estimate having 50 % weight. Weights were recommended by the analyst due to NIST using established ID-LC-MS/MS and LC-ICP-MS methods with analyte identity confirmation and purity determinations which are metrologically traceable to the SI unit of mass.

For Vitamin B12, it was determined by the analyst to exclude the HAMQAP data (which is extremely variable), so the estimate is the mean of the NIST and manufacturer means.

For analytes with only measurements from HAMQAP and the manufacturer, the estimated value is the mean of the HAMQAP and manufacturer results.

For vitamin A acetate, which was only measured by the manufacturer, the estimate is the manufacturer mean with the uncertainty calculated as the standard error of that mean.

When the value is based on more than one method, the uncertainty of the combined mean is estimated using a bootstrap procedure based on a Gaussian random effects model for the between-method effects [8,21,22,23].

7.4 Homogeneity Assessment

To address issues of possible inhomogeneity of the SRM, analyses of variance with 5% significance level were run on NIST data where bottle information was available. There was no evidence of significant bottle effects.

7.5 Analysis Results

The results of the statistical analyses are presented in the Certificate of Analysis for SRM 3289. For the most current version of this document, please visit: https://www-s.nist.gov/srmors/view_detail.cfm?srm=3289

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