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Certification of Standard Reference Material® 916b: Bilirubin

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Abstract

Standard Reference Material (SRM) 916b Bilirubin comprises neat unconjugated bilirubin that is certified as a chemical substance of known purity. It is intended for use in the calibration and standardization of measurement procedures for the determination of bilirubin in clinical samples and for assignment of bilirubin quantity values to in-house control materials. A unit of SRM 916b consists of 100 mg of bilirubin. This publication documents the production, analytical methods, and computations involved in characterizing this product.

Keywords

Azopigment;
Bilirubin;
Caffeine Reagent;
Quantitative Proton Nuclear Magnetic Resonance Spectroscopy With Internal Standard (q^1H-NMR_{IS});
Standard Reference Material (SRM)

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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Acronyms

^{1}H-^{13}C HSQC	^{1}H-^{13}C heteronuclear single quantum correlation NMR
COA	Certificate of Analysis
CWRSL	Children's Wisconsin Reference Standards Laboratory
NIST	National Institute of Standards and Technology
NMR	nuclear magnetic resonance spectroscopy
q^{1}H-NMR$_{IS}$	^{1}H quantitative NMR using an internal standard
SOP	standard operating procedures
SD	standard deviation
SI	International System of Units
SRM®	Standard Reference Material®

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1. Introduction

Bilirubin is an orange-yellow compound that is produced by vertebrates during the normal catabolic breakdown of heme. Bilirubin measurements are used in the diagnosis of health conditions like jaundice, anemia, and liver diseases; serum bilirubin may also have chemopreventive effects [1,2]. Figure 1 displays the chemical structure of the predominant form of bilirubin in SRM 916b, the IX-α isomer.

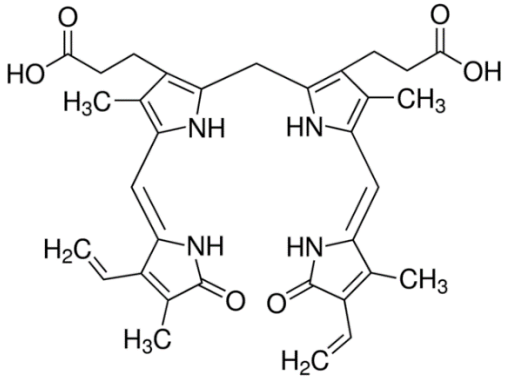

Figure 1. Chemical Structure of Bilirubin (IX-α)

In 1962 a joint committee of clinical health scientists representing the American Academy of Pediatrics, the College of American Pathologists, the American Association for Clinical Chemistry (AACC), and the National Institutes of Health recommended that a uniform bilirubin standard be developed having a molar absorptivity between (59 100 and 62 300) L mol^{-1} cm^{-1} in chloroform at a wave length of 453 nm and at 25 °C [3]. This range is congruent with a mass fraction purity of (94.9 to 100.0) %.

To meet this call, in 1971 NIST produced Standard Reference Material® (SRM®) 916 Bilirubin, having a certified purity of 99.0 % [4]. SRM 916a was issued in 1989 with a certified purity of (98.3 ± 0.3) % [5,6]. Figure 2 displays the sales history of SRM 916a from 1990 to the last sale in 2010. Approximately 82 units of SRM 916a were sold per year.

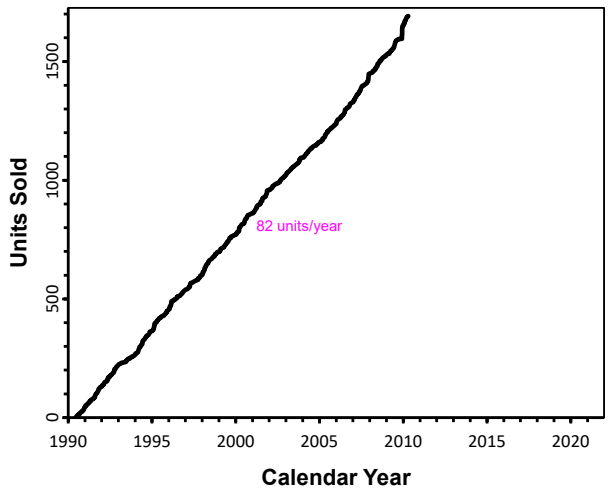

Figure 2. Sales History of SRM 916a

Since the depletion of SRM 916a stock, a replacement material has been sought to provide a certified value for high-purity (≥ 98 %) bilirubin content. Several candidate materials were analyzed and found to have a purity considerably lower than this target.

Given the unavailability of high-purity bilirubin materials, a preliminary study was conducted in 2019 to assess the feasibility of providing a clinically useful SRM of lower purity. Based on the results of this study, the highest purity candidate (≈95%) was assessed as likely suitable for use with the reference methods for diagnostic bilirubin measurements currently employed by the clinical community. A later batch of the same product was acquired in suitable bulk and its purity assessed as somewhat better than that of the material studied. The new material was accepted for use as SRM 916b Bilirubin and packaged into units of 100 mg.

Certification of the candidate SRM 916b Bilirubin material required metrological diligence to ensure that it was suitable for accurate clinical measurements. Characterization of the chemical impurity profile ensured that constituents other than bilirubin do significantly affect results determined through use of SRM 916b as a calibrant.

A mass fraction purity value that is metrologically traceable to the International System of Units (SI) has been determined. Comparison of this result to results from spectrophotometric assay demonstrates that the methods are consistent and that the material's impurities do not give rise to significant bias in results for the determination of bilirubin in clinical samples.

Comparison of molar absorptivity values calculated from the spectrophotometric assay results provides evidence for the continuity of the reference system initially established for bilirubin using SRM 916 and SRM 916a. Given that the 95 % coverage interval of the certified value overlaps with the range established in 1962 [3], the SI-traceable purity of SRM 916b is adequate for use as a primary bilirubin standard.

The following sections of this document describe the SRM 916b Bilirubin material, how this material was determined to be fit for clinical purposes, and how its purity has been determined.

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2. SRM 916b Material Identification and Production

Several candidate bilirubin materials were screened from 2011 to 2019. Mass purity determinations for these materials were made at NIST via quantitative ^{1}H nuclear magnetic resonance spectroscopy using an internal standard (q^1H-NMR$_{IS}$) [7,8,9,10,11,12] and compared to spectrophotometric results that are metrologically traceable to the reference absorptivity value of SRM 916a [5,6,13]. None of the materials that were available in sufficient quantity were of the targeted ≥ 98 % purity.

2.1. Evaluation of Clinical Utility

To move forward, three neat bilirubin materials of known lower purity were evaluated for potential clinical utility: Sigma-Aldrich (St. Louis, MO USA) Product B4126 Lot 098K1365, Sigma-Aldrich B14126, Lot SLBX1739, and Lee BioSolutions, Inc. (Maryland Heights, MO, USA) Product 127-12 Lot 07B2672. Samples of the three materials were sent to three laboratories in a blind study.

Each laboratory was sent three sets of samples of each of the three candidate bilirubin materials:

- Candidate A was Sigma-Aldrich Lot 098K1365. This material was purchased in 2011 and packaged at NIST for possible use as a SRM 916a replacement material. Vials were randomly selected from three different boxes of 144 vials per box.
- Candidate B consisted of vials prepared from two bottles, each containing 1 g of bilirubin, of the Lee BioSolutions material.
- Candidate C consisted of vials prepared from a 5 g unit of Sigma-Aldrich Lot SLBX1739.

Each set consisted of six vials that contained 90 mg to 100 mg of bilirubin. Each vial was coded by Set and replicate number. The laboratories were asked to prepare and analyze the samples using their normal laboratory protocol. To make sure that results among the assays were comparable, the laboratories were asked to use the same measurement procedure and reference standard for all three Sets. Figure 3 displays the letter of instruction that was provided to the laboratories.

November 26, 2018

Dear Colleague,

Thank you for agreeing to assist us with testing candidate materials for the next NIST Standard Reference Material (SRM) 916b Bilirubin. Enclosed are three (3) individual sets of samples for analysis. Each set consists of 6 vials. We recommend that these samples be stored at -20 °C and in the dark until use. Please prepare and analyze the samples using your normal laboratory protocol. To make sure that results among the assays are comparable, please use the same measurement procedure and reference standard for all three assays.

Note that each sample vial is coded within each set (e.g., Set A: A1-A6, Set B: B1-B6; Set C: C1-C6). Please report results according to the code on each vial. A data sheet for reporting your results is provided for your convenience. Please report the % purity of each assay to three (3) significant figures (e.g., 99.5 %, 95.4 %, 94.6%, etc.). We would also appreciate any practical observations/comments that you have about the samples as you assay them.

Please return your results to Technical Project Leader Dr. Michael Nelson at michael.nelson@nist.gov or fax: 301-977-0685 **on or before December 14, 2018**. If this schedule is problematic for your laboratory or if you have questions, please feel free to email me at jbthomas@nist.gov or Michael at michael.nelson@nist.gov. I can also be reached at phone: 301-975-3120.

Thank you once again for your assistance.

Figure 3. Letter of Instruction Sent to Participants

Only the Children's Wisconsin Reference Standards Laboratory (CWRSL), Milwaukee, WI USA provided fit-for-purpose results. Their spectrophotometric procedure results are traceable to molar absorptivity reference values for SRM 916a in caffeine reagent [14] and blue and red bilirubin azopigments [13] obtained using the Reference Method for Total Bilirubin developed by the Committee on Standards of the AACC.

For each of the three candidate materials, CWRSL provided triplicate determinations using four methods: alkaline azopigment at 598 nm, neutral azopigment at 530 nm, and caffeine reagent at 432 nm and 457 nm. Table 1 lists the derived spectrophotometric purity estimates for the materials.

Table 1. Spectrophotometric Results for Bilirubin Candidate Materials, %

	Candidate A			Candidate B			Candidate C		
Measurement Method	Set A	Set B	Set C	Set A	Set B	Set C	Set A	Set B	Set C
Alkaline Azopigment	94.53	94.57	94.76	92.33	92.18	91.57	93.29	92.78	93.08
598 nm	94.34	94.91	95.11	92.63	92.44	91.80	93.57	93.08	93.66
	94.84	95.04	94.90	92.85	92.32	91.50	93.70	93.25	93.63
Neutral Azopigment	93.90	93.91	93.73	92.02	91.99	90.97	94.21	93.52	93.87
530 nm	94.08	93.99	93.89	92.22	91.93	91.00	93.72	93.31	93.45
	93.84	94.14	93.96	92.25	91.85	91.09	93.41	92.91	93.24
Caffeine Reagent	94.83	94.37	94.21	92.40	92.18	91.17	94.37	93.80	94.09
432 nm	94.52	94.23	94.18	92.54	92.38	91.10	93.84	93.40	93.56
	94.23	94.17	94.11	92.13	92.02	91.17	93.54	93.20	93.31
Caffeine Reagent	94.91	94.45	94.16	92.46	92.09	91.04	94.09	93.48	93.83
457 nm	94.74	94.46	94.33	92.65	92.50	91.08	93.81	93.45	93.60
	94.51	94.49	94.41	92.45	92.25	91.37	93.82	93.67	93.56
Mean	94.44	94.39	94.31	92.41	92.18	91.24	93.78	93.32	93.57
Standard Deviation (SD)	0.37	0.34	0.42	0.24	0.21	0.26	0.32	0.30	0.28
Grand Mean		94.38			91.94			93.56	
2 × Grand SD		0.74			1.13			0.70	

Figure 4 compares the results from the four spectrophotometric methods.

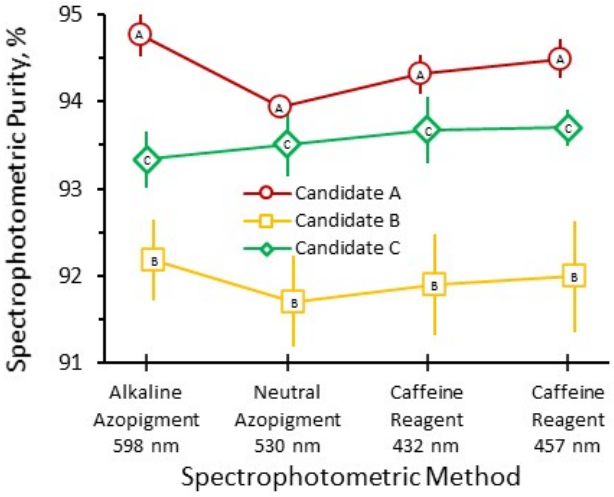

Figure 4. Comparison of Results from Four Spectrophotometric Assays
Each symbol represents the mean of 9 results; error bars represent standard deviations.

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Table 2 summarizes NIST's q^1H-NMR$_{IS}$ results and the purity estimates derived from CWRSL's 36 independent spectrophotometric results for each material. All values are expressed in percent; uncertainties are approximate 95 % level of confidence coverage intervals. Figure 5 compares the q^1H-NMR and over-all spectrophotometric results in dot-and-bar format.

Table 2. Summary of Purity Results for Bilirubin Candidate Materials, %

Candidate	q^1H-NMR$_{IS}$	Spectrophotometric
A	92.63 ±1.3 [a]	94.38 ±0.74 [b]
B	93.97 ±1.2 [a]	91.94 $^{-1.08}_{+0.82}$ [c]
C	94.93 ±1.4 [a]	93.56± 0.70 [b]

a The symmetric 95% coverage interval includes sample replication and the variable terms of the q^1H-NMR$_{IS}$ measurement function [15].
b The symmetric 95% coverage interval is expressed as twice the standard deviation of 36 measurements.
c The asymmetric 95% coverage interval was determined using linear pooling [16] of the mean and standard deviation reported for the three Sets.

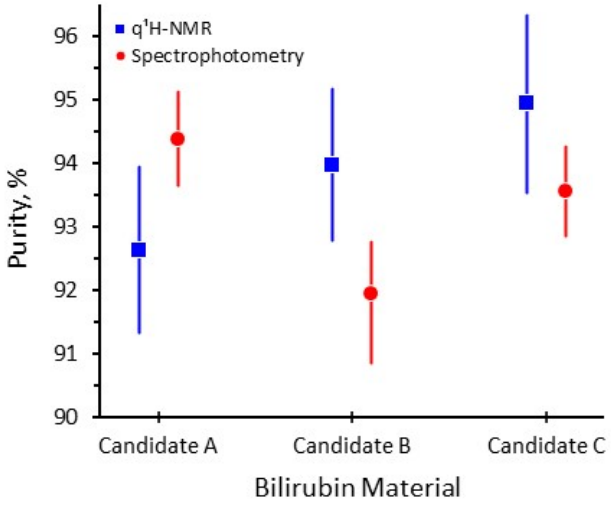

Figure 5. Comparison of Bilirubin Material Results by q^1H-NMR$_{IS}$ and Spectrophotometry
Each symbol represents a mean result; error bars represent approximate 95 % level of confidence intervals on the respective measurement populations.

The purity results among the four spectrophotometric assays for the candidate A material are more variable than for candidates B and C; the mean over all four assays is greater than the corresponding q^1H-NMR$_{IS}$ result. These observations agree with spectrophotometric observations made at NIST in 2011. These results suggest that there are impurities in Candidate A that contribute to positive bias of the spectrophotometric procedure.

The spectrophotometric purity result for the candidate B material is the lowest of the materials studied and is significantly less than the corresponding q^1H-NMR$_{IS}$ result. The differences among results for the three Sets may indicate some within-bottle heterogeneity.

The candidate C material provided a spectrophotometric result that was the most precise and most consistent with the respective q^1H-NMR$_{IS}$ result and was determined by q^1H-NMR$_{IS}$ to have the highest purity. However, no additional product with this lot number was available.

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2.2. Material Acquisition and Preliminary Analysis

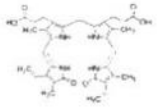
In early 2019 170 g of Sigma-Aldrich B14126 Lot SLCB2802 was acquired. This bilirubin material was delivered as a single bulk unit in an amber glass jar with a tightly-closed screw cap. Upon receipt, the jar was stored in a freezer at approximately -20 °C. Figure 6 displays the Certificate of Analysis (COA) that was provided with this material.

SIGMA-ALDRICH sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Bilirubin - ≥98% (EmM/453 = 60), powder



Product Number:	**B4126**
Batch Number:	**SLCB2802**
Brand:	SIGMA
CAS Number:	635-65-4
MDL Number:	MFCD00005499
Formula:	C33H36N4O6
Formula Weight:	584,66 g/mol
Storage Temperature:	Store at -20 °C
Quality Release Date:	14 DEC 2018

Test	Specification	Result
Appearance (Color)	Faint Brown to Brown and Faint Orange to Dark Orange	Dark Orange
Appearance (Form)	Powder	Powder
Solubility (Color)	Faint Yellow to Dark Yellow and Faint Orange to Orange	Yellow
Solubility (Turbidity) 0.1 mg/ml, CHCl3	Clear to Hazy	Clear
Purity by UV based on EmM = 60 at 453 nm, CHCl3	≥ 98 %	100 %
HPLC Identity	Conforms	Conforms
1H NMR Spectrum	Conforms to Structure	Conforms

Sigma-Aldrich warrants, that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current Specification sheet may be available at Sigma-Aldrich.com. For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.

Version Number: 1

Page 1 of 1

Figure 6. Sigma-Aldrich Certificate of Analysis

This bulk material was preliminarily assayed at NIST prior to packaging to confirm its chemical identity and evaluate its purity. Identity was confirmed using both 1- and 2-dimensional NMR experiments. Its purity was determined using q^1H-NMR$_{IS}$ as 95.4 % with a slightly asymmetric approximate 95 % level of confidence interval from 94.3 % to 96.3 %. This is somewhat higher purity than the candidate C (Lot SLBX1739).

The decision was made to pursue development of SRM 916b using this material.

2.3. Production

The Sigma-Aldrich B14126 Lot SLCB2802 material was distributed at NIST into 1523 amber glass screw-cap vials. These vials, Gewindeflasche mit Inneneinzug 1 ml (52 × 22/1,2 mm), were purchased from PharMediPack Direkt (Part 521814100, Stolberg, DE) and have a 1 mL capacity

V-shaped internal bottom. Special large vials with small containment volume capacity were used to simplify unit bottling as well as label design and application.

Bilirubin was manually dispensed into vials (100 mg ± ≈ 10 mg), weighed, and capped under yellow incandescent light. Aliquots of the bulk were collected for each filling session in order to keep the remainder of the bulk stored at -20 °C. The filling sequence of boxes with vials was consistent with the order of filling the respective vials with bilirubin. Boxes were stored at -20 °C and protected from light after filling.

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3. Characterization of SRM 916b Bilirubin

3.1. Bilirubin

The estimate of purity used for certification of SRM 916b is based on measurement data from twelve vials, sampled at regular intervals across the entire production lot. These samples were stored at -20 °C; they were allowed to reach ambient laboratory temperature (approximately 21 °C) prior to use in experiments. A unit of SRM 916 Bilirubin was measured for validation of the q^1H-NMR$_{IS}$ procedures.

The NIST PS3 Caffeine, NIST PS1 Primary Standard for qNMR (Benzoic Acid), and dimethyl terephthalate were used as internal standards for q^1H-NMR$_{IS}$. These materials were stored at ambient laboratory temperature (approximately 21 °C) prior to use.

Samples were dissolved using perdeuterated dimethyl sulfoxide (DMSO-d_6), followed by addition of two drops of deuterium oxide (D_2O). These solvents were purchased from Cambridge Isotope Laboratories (Tewksbury, MA) and characterized as having at least 99.9 % D atom purity. Perdeuterated chloroform and methylene chloride were also tested, however solubilities of bilirubin and internal standard materials were not suitable.

3.1.1. Sample Preparation

Table 3 summarizes the series of q^1H-NMR$_{IS}$ experiments conducted for this investigation. The purposes of these experiments include stability monitoring of bilirubin solutions, development of a suitable q^1H-NMR$_{IS}$ procedure, and purity measurements for characterization of candidate SRM 916b Bilirubin. All sample preparation was conducted under low-intensity incandescent light. Once prepared, samples were protected from visible and ultraviolet light.

Table 3. Sample Sets for q^1H-NMR$_{IS}$ Purity Experiments

Material	Internal Standard	Solvent	# Samples	Purpose
SRM 916	PS1 Benzoic Acid	2 mM DTT in DMSO-d_6 w/2 drops D2O	3	procedure validation
SRM 916b	PS1 Benzoic Acid	DMSO-d_6 w/2 drops D_2O	2	stability monitoring
SRM 916b	PS1 Benzoic Acid	2 mM DTT in DMSO-d_6 w/2 drops D_2O	2	stability monitoring
SRM 916b	PS1 Benzoic Acid	DMSO-d_6 w/2 drops D_2O	14	procedure development and chloroform measurement
SRM 916b	dimethyl terephthalate	DMSO-d_6 w/2 drops D_2O	5	procedure development
SRM 916b	PS3 Caffeine	DMSO-d_6 w/2 drops D_2O	14	procedure development and purity characterization

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 used for all NMR measurements. Sample mass determinations were performed in accordance with established balance use and sample preparation standard operating procedures (SOPs). An ultra-microbalance (Mettler Toledo XPR2U, Columbus, OH USA) was used for these determinations. Approximately 0.7 mL of DMSO-d_6 solvent was added to samples approximately one hour immediately prior to the q^1H-NMR$_{IS}$ experiment. During this

time, samples were sonicated for approximately 15 min and vortexed in attempt to achieve complete dissolution of neat materials. Two drops of D_2O were added after the first 10 minutes of sonication, whereby supersaturation of bilirubin at the DMSO-d_6/D_2O boundary was apparent, however the bilirubin was redissolved during subsequent sonication and vortex action. No undissolved material was apparent in the solutions prior to transfer to NMR tubes. NMR experiments were manually initiated for each sample an hour after the initial addition of DMSO-d_6 to ensure that changes in bilirubin concentration due to sample instability were insignificant.

3.1.2. Analysis

Experimental NMR data was acquired with a Bruker Avance II 600 MHz spectrometer equipped with a 5-mm diameter double resonance broadband inverse (BBI) detection probe optimized for ^{1}H observation and operated via Topspin (Version 3.2) software. The q^{1}H-NMR$_{IS}$ experiments, subsequent data processing and purity calculations were performed according to the established SOP. q^{1}H-NMR$_{IS}$ experiments were conducted at a temperature of 298 K, spectral sweep width was set to 12,019.2 Hz (20.0276 ppm), and the transmitter frequency offset for ^{1}H (O1) was set to 3,706.0 Hz (6.175 ppm). 90-degree excitation pulse widths were used and globally optimized, alternating phase, rectangular pulse (i.e., GARP) ^{13}C decoupling was executed during FID acquisition. Transmitter frequency offset of the carbon channel for ^{1}H experiments with ^{13}C decoupling (^{1}H{^{13}C}) was 10,563.2 Hz (70 ppm). Data acquisition time was 5.453 s to generate an FID with 131,072 data points. Experiments were performed using 64 scans for data collection, preceded by 8 'dummy' scans for which no data was collected. The spin lattice relaxation time (T1) for all analyzed resonances was determined using magnetization inversion recovery NMR experiments (Table 4). The recycle delay was set to 60 s, which is greater than twelve (12) times the longest relevant T1. Experiment duration for each sample was 79 min.

Table 4. ^{1}H-NMR Spectrum Integral Regions Evaluated for Bilirubin in DMSO-d_6

P	chemical shift (ppm)	peak type	^{1}H structural moiety [a]	proton multiplicity	T1 (s)
bilirubin	6.8	doublet of doublets (dd)	6	1	3.2
	6.6	dd	41	1	3.0
benzoic acid	8.0	doublet	4,6	2	3.7
	7.6	triplet	2	1	4.5
	7.5	triplet	1,3	2	3.4
caffeine	8.0	singlet	8	1	3.6
dimethyl terephthalate	8.1	singlet	1,2,4,5	1	3.3

a ^{1}H chemical structure moiety numbering schemes are shown in Figure 15 to Figure 17.

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The mass fraction (g/g) of bilirubin, w_P, via q^{1}H-NMR$_{IS}$ was derived using an estimation model based on the following measurement function:

$$w_p = \left(\frac{N_I}{N_P}\right) \times \left(\frac{M_P}{M_I}\right) \times \left(\frac{A_P}{A_I}\right) \times \left(\frac{m_I}{m_C}\right) \times P_I \quad (1)$$

where: N_P = ^{1}H multiplicity (# H/peak) of the integrated bilirubin peak,
N_I = ^{1}H multiplicity (# H/ peak) of the integrated internal standard peak,
M_P = relative molar mass (g/mol) of the primary component, bilirubin,
M_I = relative molar mass (g/mol) of the internal standard,
A_P = integral of the bilirubin ^{1}H peak,
A_I = integral of the internal standard ^{1}H peak,
m_C = mass (g) of sampled neat bilirubin material,
m_I = mass (g) of the internal standard, and
P_I = purity (g/g) of the internal standard.

For each NMR spectrum, ratios of bilirubin and internal standard peak integrals normalized to the respective values of N, $(A_P N_I)/(A_I N_P)$, were determined in triplicate replication of the ^{1}H spectral data processing procedure. To accommodate data inputs in this ratio form for each measured sample, the purity estimation model is based on a reduced form of Eq. 1:

$$w_p = \left(\frac{M_P}{M_I}\right) \times (R_A) \times (R_m) \times P_I, \quad (2)$$

where: R_A = $(A_P N_I)/(A_I N_P)$, determined through repeated processing of the ^{1}H-NMR spectrum and
$R_m = m_I/m_C$.

The measurand, w_p, was calculated using a Bayesian procedure modeled on "observation equations" via Markov Chain Monte Carlo (MCMC) [15,17]. Code for the model used to calculate the purity result is provided in Section 4.1.1.

3.1.3. Chemical Identity

The chemical identity of the primary bilirubin component of candidate SRM 916b was confirmed through ^{1}H-NMR (Figure 7), ^{1}H-^{1}H correlation spectroscopy (COSY), and ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) NMR (Figure 8) experiments.

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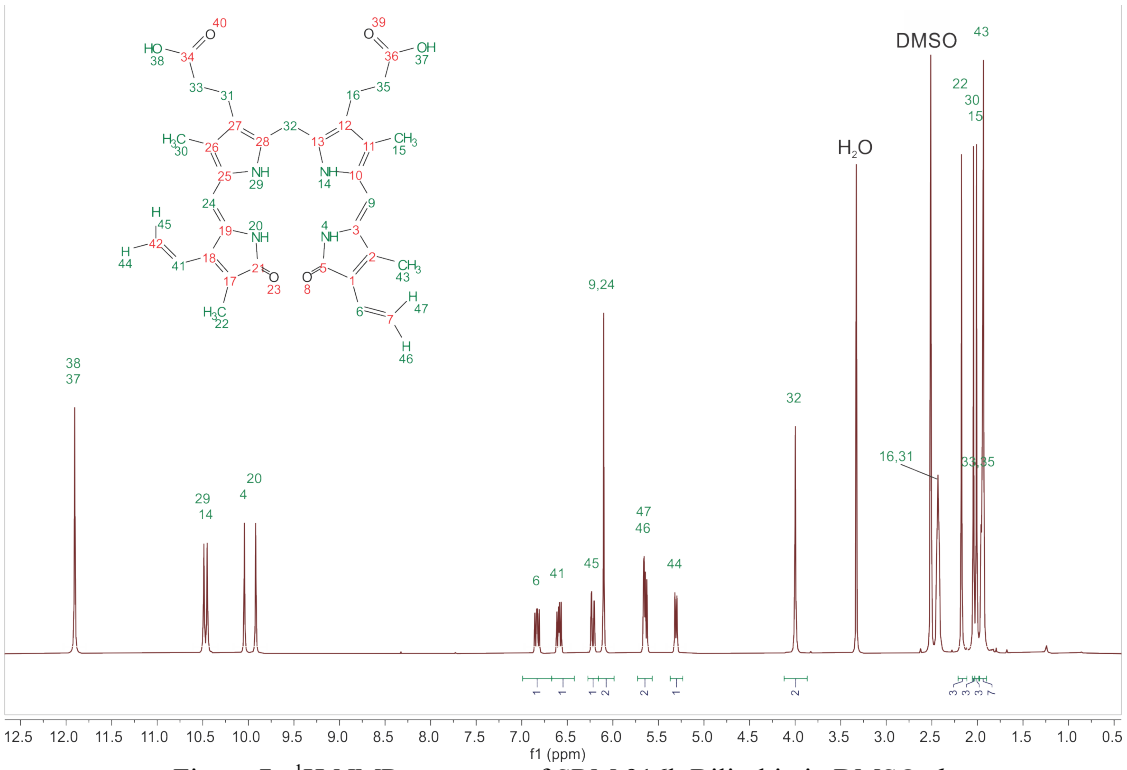

Figure 7. ^{1}H-NMR spectrum of SRM 916b Bilirubin in DMSO-d_6

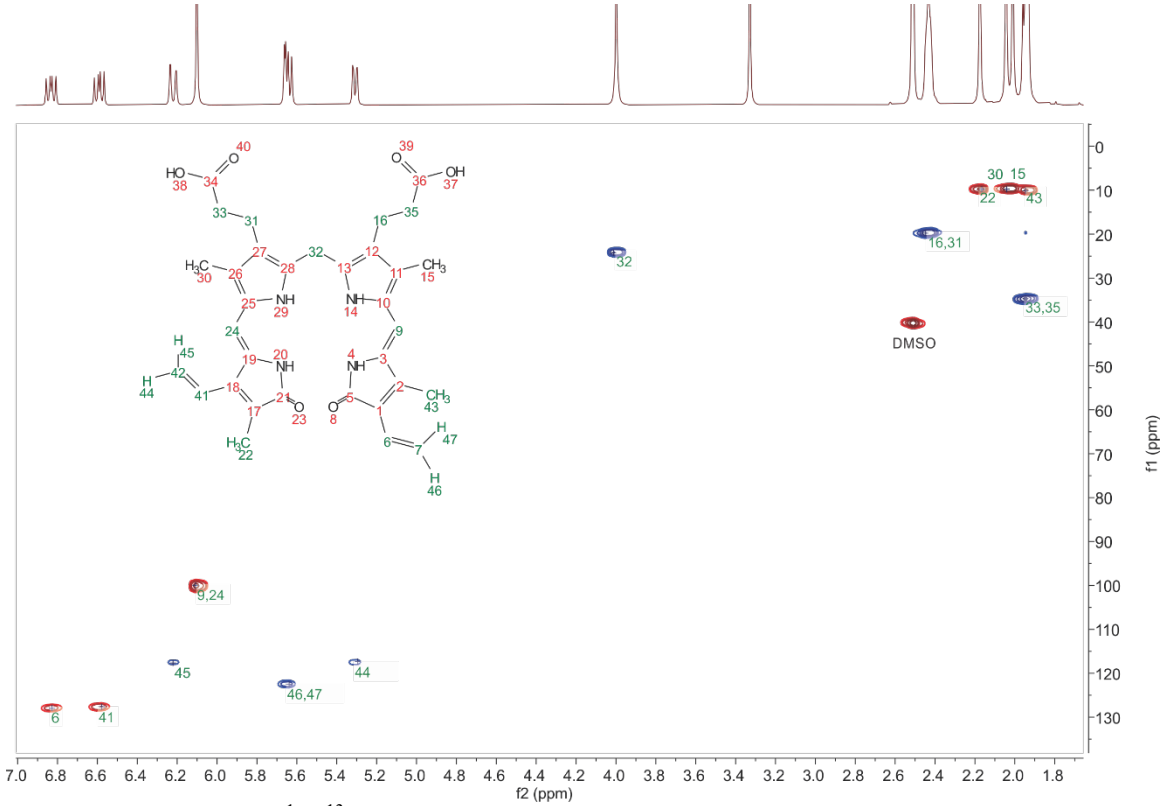

Figure 8. ^{1}H-^{13}C HSQC spectrum of SRM 916b Bilirubin in DMSO-d_6

The assignment of the peaks in both the ^{1}H- and ^{1}H-^{13}C spectra was done manually.

For q^{1}H-NMR, distinct spectral regions were analyzed to determine integrals for bilirubin and the internal standards. A quantitative analysis of all ^{13}C-coupled ^{1}H bilirubin signals was conducted using samples of SRM 916 Bilirubin. It was determined that the peaks of olefinic moieties 6 (δ_{ppm} = 6.6) and 41(δ_{ppm} = 6.8) could be integrated most reliably and repeatably. All other signals were affected by significant peak overlap or there was greater variability in integral magnitudes from repeated determinations and experiments. Adjustments of integrals were made to correct for small proportions (≈ 0.1 % or less) of area attributable to apparent impurity peak overlap. These adjustments were performed for each spectrum through manual baseline adjustment and integration of the impurity peaks.

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3.1.4. Sample Stability

During this study, apparent solution properties of bilirubin in DMSO-d_6 evolved from translucent and orange to opaque and dark green as the solutions aged over several (> 7) days (Figure 9).

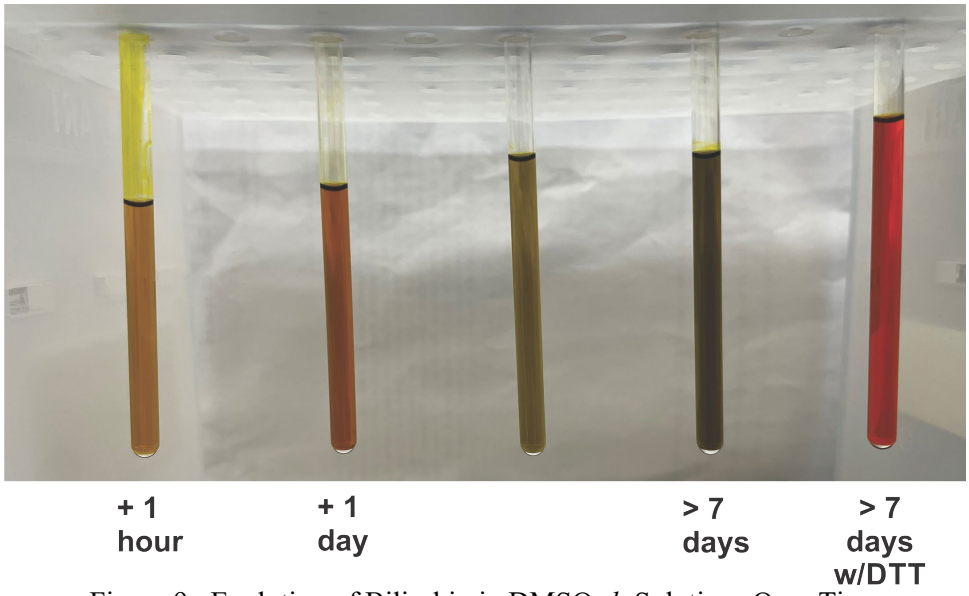

Figure 9. Evolution of Bilirubin in DMSO-d_6 Solutions Over Time
Dithiothreitol was added to the solution in the right-most position, denoted "w/DTT", which did not undergo the same evolution color, but was a brighter red-orange color and remained translucent.

Evolution of color became apparent after one day, indicating that bilirubin is unstable in DMSO-d_6 and suggesting the possibility that a significant proportion of bilirubin degrades during the period between sample dissolution and completion of a q^1H-NMR$_{IS}$ experiment. To investigate this possibility and understand the rate of bilirubin sample degradation, a study was conducted to measure the mass ratio of bilirubin:neat SRM 916b sample as a function of time. Dithiothreitol (DTT), a strong acidic antioxidant, was added to selected solutions to explore the effect on sample stability. Potential correlations between bilirubin reactivity and the admixture of internal standard was also explored. Three samples containing NIST PS1 benzoic acid as internal standard were measured: 1) with DTT and an SRM 916b:NIST PS1 mass ratio of ≈1; 2) with DTT and an SRM 916b:NIST PS1 mass ratio of ≈2; and 3) without DTT and an SRM 916b:NIST PS1 mass ratio of ≈1. Two more bilirubin samples were prepared to assess the suitability of dimethyl terephthalate and NIST PS3 Caffeine for use as internal standards.

The one-day changes in bilirubin content as determined using q^1H-NMR$_{IS}$ are shown in Figure 10. Addition of DTT significantly increased the rate of bilirubin degradation in both solutions having different levels of acidity. The solutions without DTT were also labile, however the rate of change in bilirubin concentration was substantially less.

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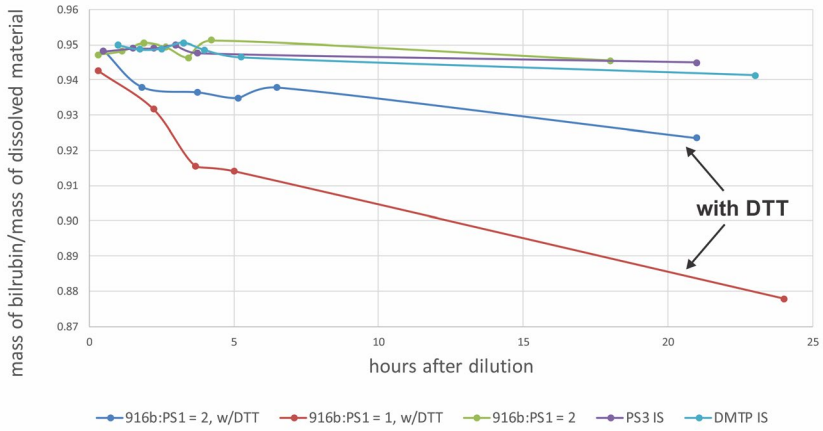

Figure 10. One-Day Stability of SRM 916b Bilirubin in DMSO-d_6
The mass ratio of SRM 916b:NIST PS1 is indicated for samples with benzoic acid internal standard.

Figure 11 displays at higher resolution the first four-hours of results for the three samples containing different internal standards, but not containing DTT. There is no apparent negative trend of bilirubin/SRM 916b mass ratio during this period; dispersion of datapoints is indicative of measurement repeatability of the respective samples. Solutions with any of the three internal standards appear sufficiently stable. All three internal standard substances are chemically viable and samples are adequately stable for the duration of the q^1H-NMR$_{IS}$ procedure.

All q^1H-NMR$_{IS}$ experiments for purity measurement of candidate SRM 916b were completed in less than 4 hours.

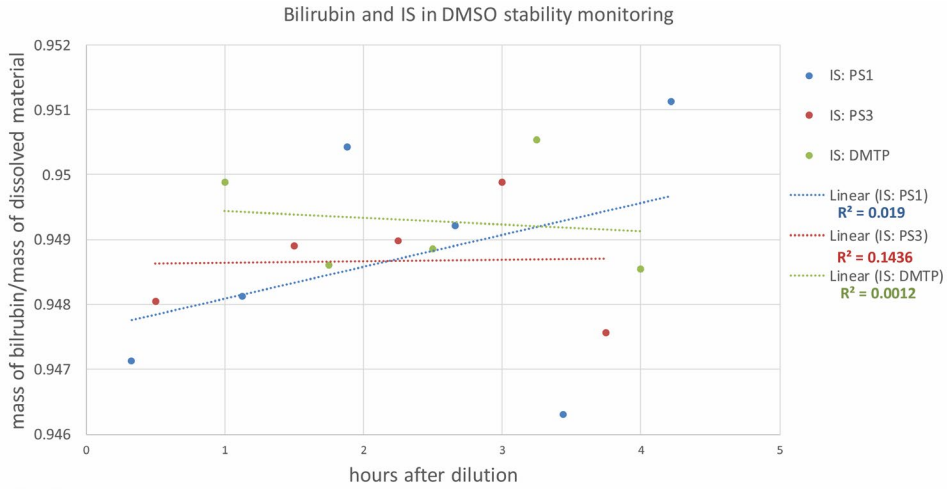

Figure 11. Four-Hour Stability of SRM 916b Bilirubin in DMSO-d_6 in Samples Without DTT

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3.1.5. Degradation Products

Chemical products of the degradation of bilirubin in DMSO-d_6 could not be confidently identified. Analysis of spectra collected for bilirubin solutions several weeks after dissolution did not clearly indicate that substantial proportions of biliverdin or mesobilirubin had evolved. However, several peaks indicating the evolution of degradation products were apparent in the $^{1}H\{^{13}C\}$-NMR spectrum collected approximately 30 days after dissolution of bilirubin (Figure 12).

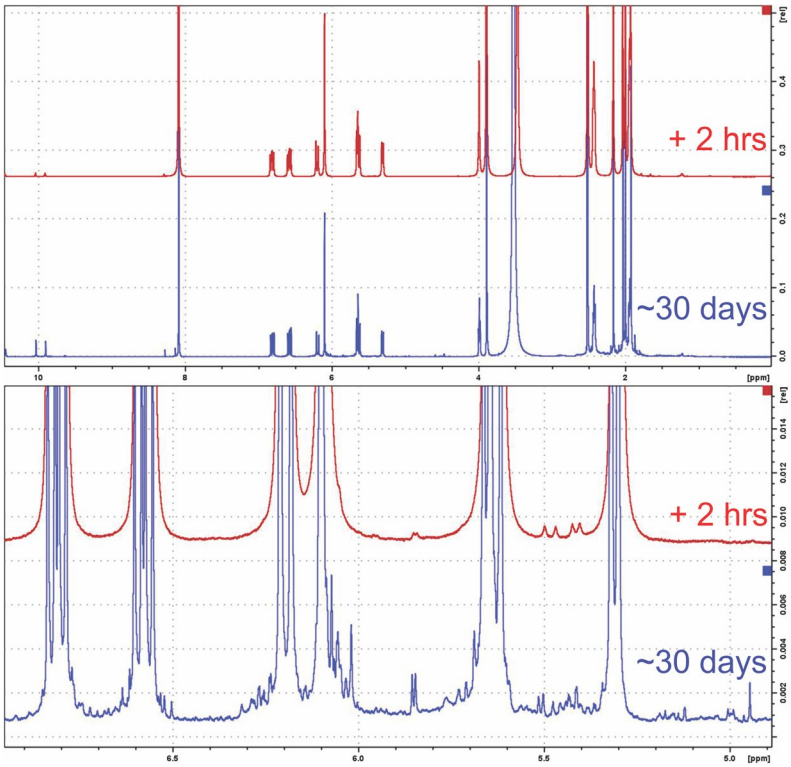

Figure 12. Comparison of $^{1}H\{^{13}C\}$-NMR Spectra at 2 H and 30 D Post Dissolution
The lower panel displays the spectral window from 6.9 ppm to 4.9 ppm at high resolution.

3.1.6. Isomers

Bilirubin has a number of isomers [18], with bilirubin IX-α predominant in SRM 916b. The difference in relative amounts of bilirubin III-α and XIII-α isomers as a proportion of total bilirubin in candidate SRM 916b was determined through the difference in integrals evaluated for $^{1}H\{^{13}C\}$-NMR peaks of bilirubin IX-α moieties 6 and 41 (Figure 13). Bilirubin III-α content is estimated to be 3 % (g/g, mol/kg) greater than that of the XIII-α isomer. This difference in isomer composition is consistent with that observed for SRM 916a [13].

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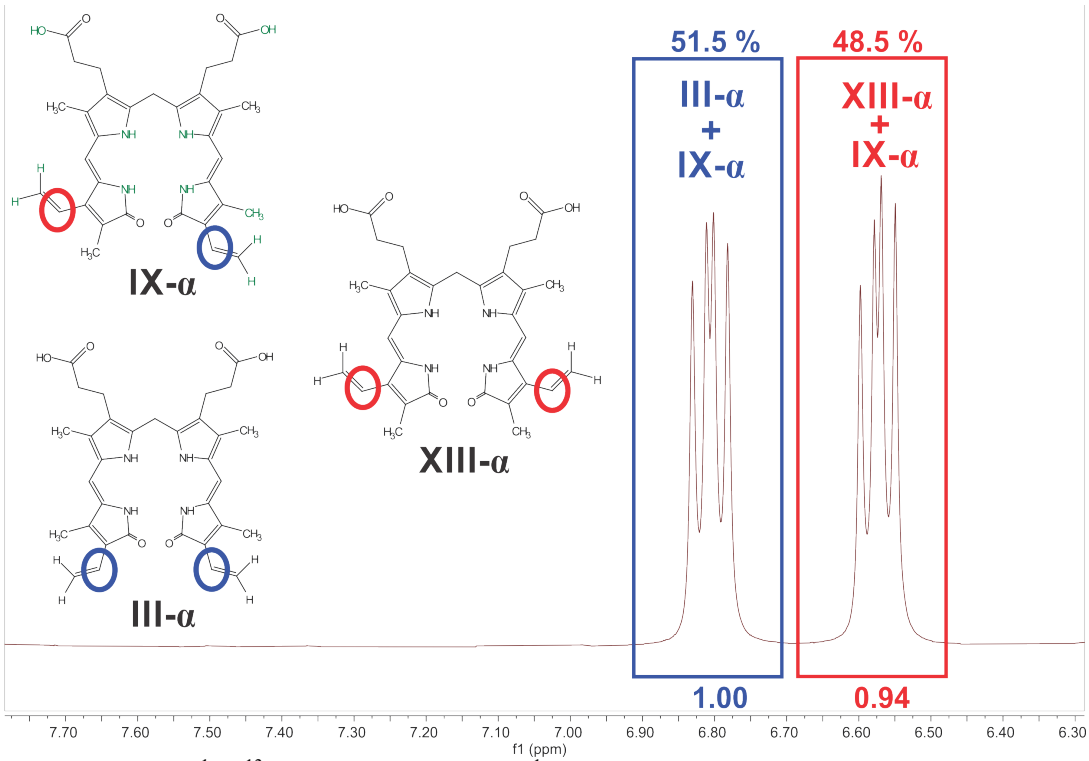

Figure 13. $^{1}H\{^{13}C\}$-NMR Integrals for ^{1}H Moieties 6 and 41 in Bilirubin Isomers

Organic chemical impurities with structures like that of bilirubin were not confidently identified in this study. There was no clear indication of significant proportions of biliverdin or mesobilirubin in candidate SRM 916b.

Figure 14 compares $^{1}H\{^{13}C\}$-NMR spectra for the SRM 916b and SRM 916 materials, both in DMSO-d_6 and containing the benzoic acid internal standard. The spectral resolution of peaks from signals of SRM 916 is clearly better than that of SRM 916b. Consistently broader lines for both bilirubin and benzoic acid internal standard in the samples indicates that the broadening effect is not attributable to incomplete dissolution of bilirubin. The effect is also not attributable to poor magnetic field homogeneity, i.e., 'poor shimming', during the measurement; line broadening effects were observed for every sample of SRM 916b measured over several months, while adequate field homogeneity was consistently achieved, as evidenced by spectra collected from measurement of SRM 916 during the same period.

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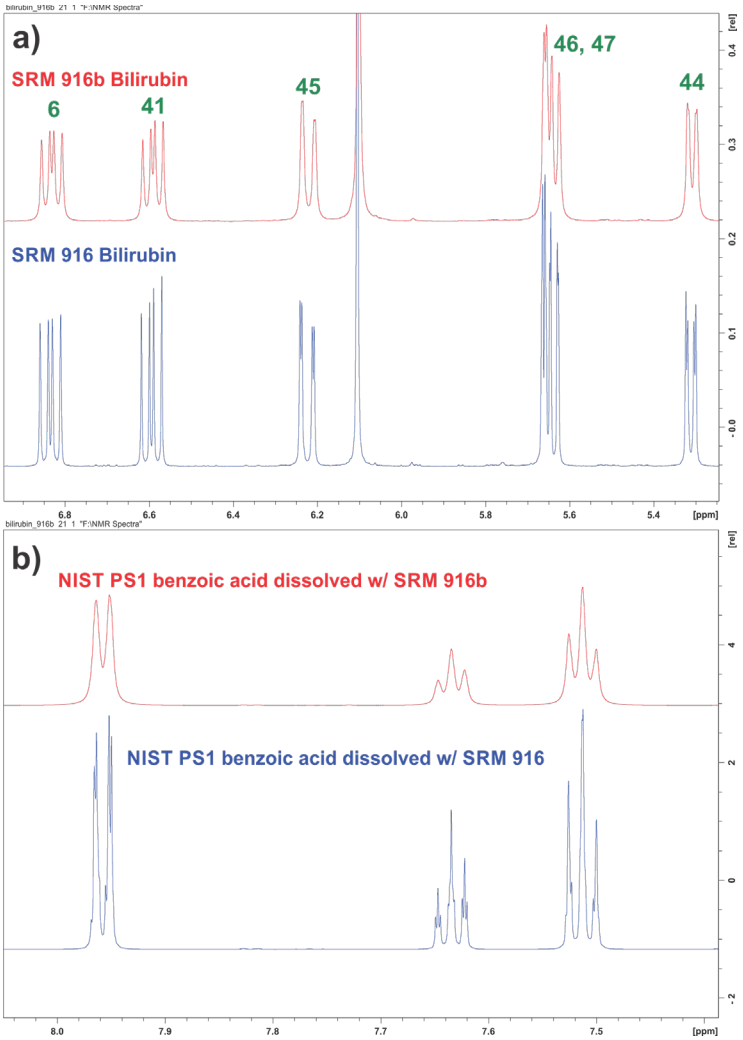

Figure 14. Comparison of $^{1}H\{^{13}C\}$-NMR Spectra of SRM 916 and 916b
Both samples contain NIST PS1 benzoic acid. a) Spectra range 6.9 ppm to 5.3 ppm. b) Spectral range 8.1 ppm to 7.4 ppm.

These observations suggested that the SRM 916b material contains a greater quantity of paramagnetic substances than is present in SRM 916. An analysis of paramagnetic elements in candidate SRM 916b was conducted via inductively coupled plasma mass spectrometry (ICP-MS). The total content of paramagnetic and ferromagnetic elements, which are known to give rise to effects like those observed in the SRM 916b spectra, is approximately 0.1 % (g/g). The total mass fraction of all elements readily attributable to impurity components, determined by semiquantitative ICP-MS and X-ray fluorescence spectroscopy methods, is approximately 0.6 %. Combustion analyses and elemental microanalysis suggest that the unidentified impurities are largely of organic nature, possibly some that there are macromolecular species or larger organic particles not readily observable via ^{1}H-NMR. These analyses are described in detail in Sections 3.4 and 3.5.2.

3.1.7. Chloroform

Based on q^{1}H-NMR$_{IS}$ data collected during measurement of bilirubin samples, the mass fraction of residual chloroform (CCl_3H) impurity is estimated to be (0.4 ± 0.1) %.

3.1.8. Spectral Resolution Anomaly

It was observed that the spectral resolution for the SRM 916b material in DMSO-d_6 solution was better at 30 d than that of the fresh 2 h solution. Several hypotheses, as yet unexplored, have been proposed to account for this observation: deagglomeration of large particles in solution, changes in DMSO/D_2O solvent effects, or settling of macromolecular or paramagnetic particles.

3.1.9. Internal Standards

Table 5 summarizes the bilirubin purity results from the q^{1}H-NMR$_{IS}$ experiments described in Table 3.

Table 5. ^{1}H-NMR$_{IS}$ Bilirubin Purity for SRM 916 and for SRM 916b

Material	Internal Standard	w_P,%	$u(w_P)$, %	# Samples
SRM 916	NIST PS1 Benzoic Acid	97.61	0.48	2
SRM 916b	NIST PS1 Benzoic Acid	94.65	0.81	12
SRM 916b	dimethyl terephthalate	94.66	0.53	5
SRM 916b	PS3 Caffeine	94.72	0.34	12

The purity of two (2) samples of SRM 916 Bilirubin were measured using the NIST PS1 Primary Standard for qNMR (Benzoic Acid) as an internal standard. The result of this measurement is consistent with the certified value of purity of SRM 916. This analysis substantiates the validity of the q^{1}H-NMR$_{IS}$ procedures implemented in this investigation for measuring purity of neat bilirubin samples.

Three results determined from experiments using three different internal standards were mutually consistent. Complete dissolution of samples containing dimethyl terephthalate internal standard was not readily achieved. Figure 15, Figure 16, and Figure 17 present exemplar ^{1}H-NMR spectra of SRM 916b bilirubin in DMSO-d_6 using the benzoic acid, dimethyl terephthalate, and caffeine internal standards. The structure assignments of the integrated peaks are provided in each Figure.

The q^{1}H-NMR$_{IS}$ procedure using NIST PS3 Caffeine as internal standard yielded the result held with the highest degree of confidence. Complete dissolution was easily achieved for samples containing caffeine, the solutions were sufficiently stable during ^{1}H-NMR experiments, and spectrum processing, including peak integration, was adequately repeatable.

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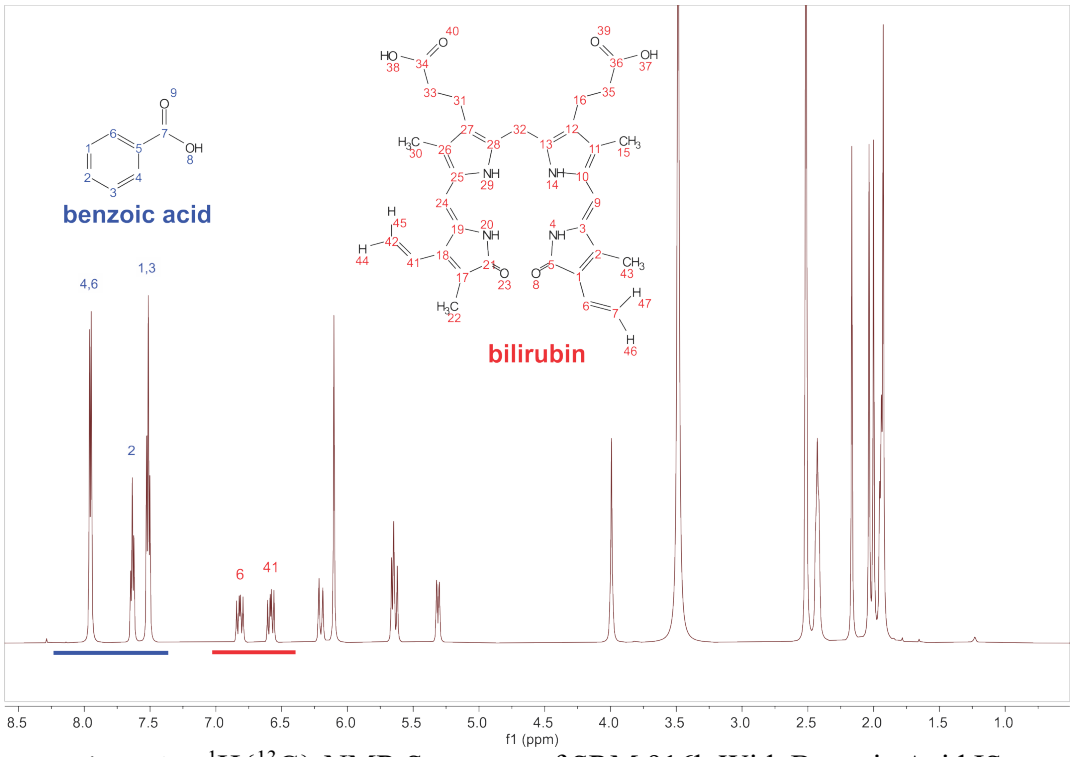

Figure 15. $^{1}H\{^{13}C\}$-NMR Spectrum of SRM 916b With Benzoic Acid IS

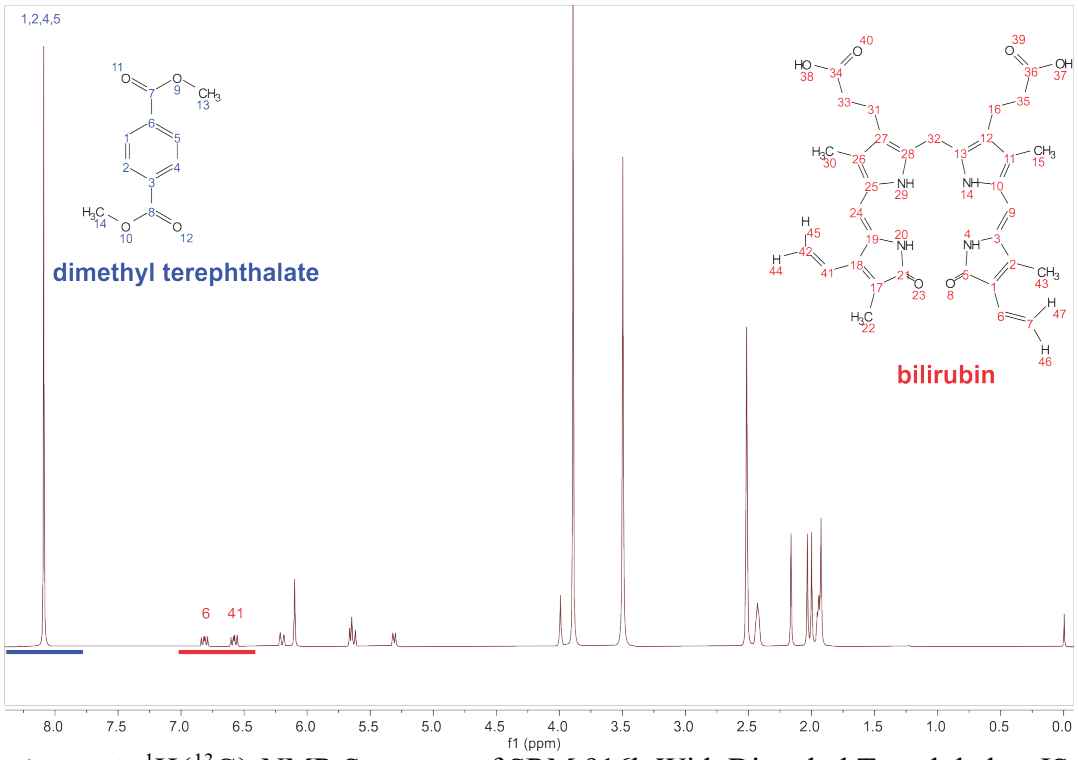

Figure 16. $^{1}H\{^{13}C\}$-NMR Spectrum of SRM 916b With Dimethyl Terephthalate IS

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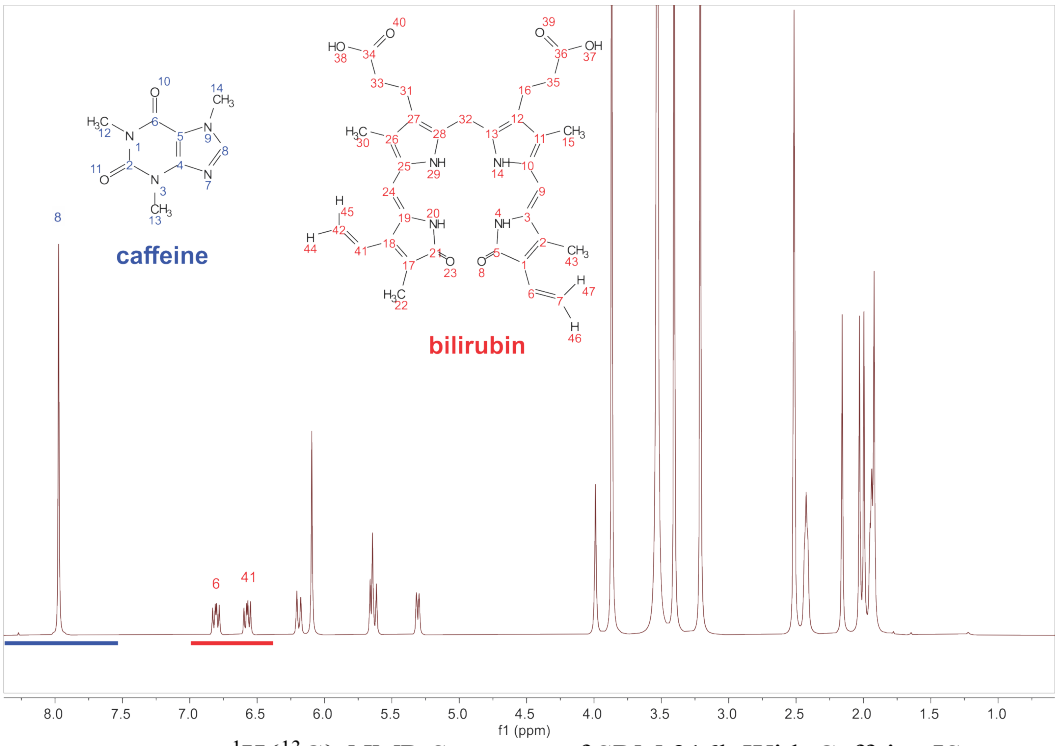

Figure 17. $^{1}H\{^{13}C\}$-NMR Spectrum of SRM 916b With Caffeine IS

3.1.10. Homogeneity

Figure 18 displays the purities estimated using the caffeine IS for the 12 SRM 916b samples. There is no apparent trend of bilirubin purity with respect to filling order of units. The production lot was assessed as sufficiently homogenous.

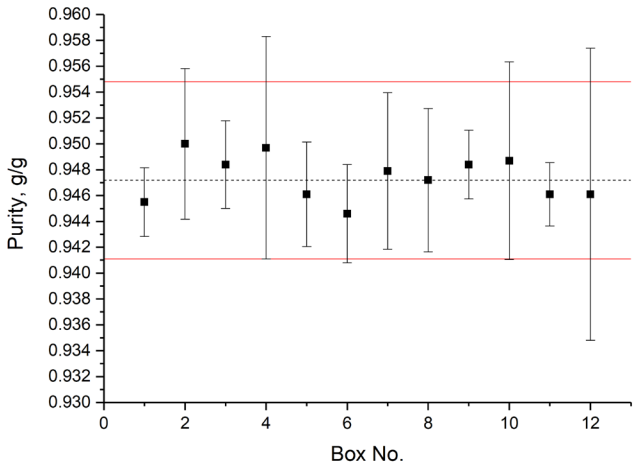

Figure 18. Purity Estimates as a function of Box Number

Symbols represent the measured bilirubin purity values; error bars represent standard uncertainties. The dotted horizontal line represents the mean. The horizontal dashed lines bound an approximate 95 % level of confidence interval. Purities are expressed as mass fraction in units of g/g rather than %.

3.2. Ferromagnetic Impurities By ICP-MS

NMR results suggested the presence of paramagnetic impurities. A red-colored residue left after ashing of SRM 916b indicated the presence of iron oxide. Semiquantitative measurements were made using ICP-MS to assess the ferromagnetic and paramagnetic impurities in the SRM 916b material.

An Agilent model 7500cs ICP-MS was used for elemental measurements. A Mettler model AT 261 DeltaRange analytical balance was used for weighing during standard preparation. Before use, calibration of the balance was verified using standard masses ranging from 1 g to 50 g.

Optima grade nitric acid (HNO_3) was purchased from Fisher Scientific. The ICP-MS Multi-Component Calibration Standard was purchased from HighPurity Standards. Locally prepared sub-boiling distilled water was used as the solvent for the preparation of all solutions.

Two samples of bilirubin each weighing approximately 0.1 g were transferred into 50 mL Falcon tubes. A 1 mL aliquot of HNO_3 was added to each tube. Loosely capped, the tube was transferred to an oven preheated to 95 °C. The tube was taken out of the oven after 4 h and the contents were allowed to cool to room temperature in the hood. The contents were diluted with water to 50 g for measurements. Three procedural blanks were prepared similarly.

All samples were measured using the semi-quantitative analysis mode of the ICP-MS instrument without the use of a collision gas. The SemiQuant mode is capable of quantifying all elements in the mass range from (2 to 250) u. The instrument response curve was calibrated using a solution nominally containing 20 µg/kg of each of 31 elements (Li, Be, B, Na, Mg, Al, Ca, Sc, V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Sr, Mo, Ag, Cd, Sb, Ba, La, Eu, Ho, Yb, Tl, Pb, Th, and U) prepared by diluting the calibration standard with 1.5 % HNO_3. SRM 1643f Trace Elements in Water was measured in concert with the test samples as a quality control material. Because the instrument had recently been used for the measurement of high-purity iron samples, the instrument was extensively cleaned. After the cleaning, samples of SRM 916b were measured twice, nine days apart. The results of the two measurements were statistically equivalent.

Table 6 lists the mass fraction of the elements with ferromagnetic, antiferromagnetic, or paramagnetic properties in the SRM 916b material. Measurement of Ca and K were affected by background that caused these two elements to routinely fail quality assurance criteria. The mean mass fraction of Ca and K indicated that they were major contributors to the impurities in the sample; therefore, an effort was made to estimate the measurement uncertainty. A multiplier of 5-times and 2-times the mean for Ca and K respectively were applied to arrive at an approximate 95 % level of confidence coverage interval. The factors of 5 and 2 were chosen to produce a standard uncertainty that results in an absolute Z-score of approximately 1 for the two elements in the quality assurance sample, as the value for most analytes was below 1 for the quality assurance sample.

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Table 6. Semiquantitative Elemental Contents in Candidate SRM 916b
All values are in mg/kg

Element	Property [a]	Value	U_{95}	Element	Property [a]	Value	U_{95}
Li	Para	0.01	0.008	Ba	Para	5	3
Na	Para	200	100	La	Para	0.03	0.02
Mg	Para	60	30	Ce	Para	0.06	0.03
Al	Para	10	8	Pr	Para	0.007	0.004
K [b]	Para	*30*	*(0 to 100)*	Nd	Para	0.03	0.02
Ca [b]	Para	*300*	*(0 to 2300)*	Sm	Para	0.006	0.003
Sc	Para	0.1	0.06	Eu	Para	0.004	0.002
Ti	Para	1	0.8	Gd	Fero	0.006	0.003
V	Para	0.05	0.03	Tb	Para	0.0007	0.0004
Cr	Antiferro	1	0.8	Dy	Para	0.004	0.002
Mn	Para	7	4	Ho	Para	0.001	0.0008
Fe	Ferro	600	300	Er	Para	0.002	0.001
Co	Ferro	0.08	0.04	Tm	Para	<0.0007	
Ni	Ferro	0.5	0.3	Yb	Para	0.003	0.002
Rb	Para	0.1	0.06	Lu	Para	0.0005	0.0003
Sr	Para	3	2	Hf	Para	0.002	0.001
Y	Para	0.03	0.02	Ta	Para	<0.0008	
Zr	Para	0.05	0.03	W	Para	0.008	0.005
Nb	Para	0.005	0.003	Re	Para	<0.002	
Mo	Para	<0.08		Os	Para	<0.004	
Ru	Para	<0.007		Ir	Para	<0.002	
Rh	Para	<0.001		Pt	Para	<0.004	
Pd	Para	0.008	0.005	Th	Para	0.01	0.007
Cs	Para	0.003	0.002	U	Para	0.03	0.02

a Antiferro – antiferromagnetic, Ferro = ferromagnetic, Para = paramagnetic
b Elements do not meet quality assurance criteria, uncertainty expressed as an interval.

Table 7 lists the measured and certified values of trace elements in SRM 1643f. The uncertainty of the measured value, except those for Ca and K, is assumed to be 50 % of the measured value, uniformly distributed. For a presumed uniform distribution, the uncertainty is normalized by dividing by $\sqrt{3}$ with infinite degrees of freedom. The normalized standard uncertainty is multiplied by a coverage factor of 2 to produce the approximate 95 % level of confidence expanded uncertainty. The absolute *Z*-scores are used to evaluate whether the measured value agrees with the certified value. Except for B, K, and Ca, the values for all analytes are below 2, suggesting that there is no statistically significant detectable bias in the measurement of these analytes.

Table 7. Certified and Measured Values in Control SRM 1643f
All values except |*Z*|-scores are in mg/kg

	Measured		Certified		
Element	x	U_{95}	x	U_{95}	|Z| [a]
Li	0.015	0.009	0.01642	0.00035	0.33
Be	0.011	0.006	0.01353	0.00011	0.8
B	*0.084*	*(0 to 0.18)*	0.1508	0.0066	**2.73**
Na	16	9	18.64	0.24	0.57
Mg	6.5	3.7	7.38	0.058	0.47
Al	0.12	0.07	0.1325	0.0012	0.36
K [b]	*0.87*	*(0 to 2.9)*	1.9133	0.009	**4.15**
Ca [b]	*8.4*	*(0 to 57)*	29.14	0.32	**8.53**
V	0.035	0.020	0.03571	0.00027	0.07
Cr	0.018	0.010	0.01832	0.0001	0.06
Mn	0.037	0.021	0.03677	0.00058	0.02
Fe	0.1	0.06	0.09251	0.00077	0.26
Co	0.023	0.013	0.02505	0.00017	0.31
Ni	0.053	0.031	0.0592	0.0014	0.4
Cu	0.018	0.010	0.02144	0.0007	0.66
Zn	0.059	0.034	0.0737	0.0017	0.86
As	0.051	0.029	0.05685	0.00037	0.4
Rb	0.011	0.006	0.01251	0.00012	0.48
Sr	0.3	0.2	0.311	0.018	0.13
Mo	0.11	0.06	0.1142	0.0017	0.13
Ag	0.00081	0.0005	0.0009606	0.0000053	0.64
Cd	0.0054	0.0031	0.00583	0.00013	0.28
Sb	0.051	0.029	0.0549	0.00039	0.26
Te	0.00089	0.0005	0.0009672	0.0000082	0.3
Ba	0.49	0.28	0.5131	0.0073	0.16
Tl	0.0063	0.0036	0.006823	0.000034	0.29
Pb	0.016	0.009	0.0183	0.000081	0.5
Bi	0.01	0.006	0.0125	0.0001	0.87

a $|Z| = 2\times|\text{Measured} - \text{Certified}|/(U_{95}(\text{Measured})^2 + U_{95}(\text{Certified})^2)^{½}$
b Elements do not meet quality assurance criteria, uncertainty expressed as an interval.

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3.3. Inorganic Impurities by WDXRF

Semiquantitative analysis of potential impurity elements was performed using wavelength dispersive X-ray Fluorescence (WDXRF) spectroscopy. One 48.19 mg sample of the SRM 916b material and a 50.19 mg sample of SRM 1566b Oyster Tissue were analyzed using the 'Omnion' (Malvern Panalytical, Almeo, NL) semiquantitative WDXRF method. The SRM 1566b material was analyzed as a control sample. Table 8 lists the results of the WDXRF analysis.

Table 8. WDXRF Spectroscopy Results for Selected Elements
All values are in mg/kg

	SRM916b Bilirubin		SRM 1566b Oyster Tissue				
Element	Measured	U_{95} [a]	Measured	U_{95} [a]	Certificate	U_{95}	|Z| [b]
Na	160	64	3200	1300	3297	53	0.15
Mg	60	24	970	390	1085	23	0.59
Al	60	24	210	84	197.2	6.0	0.30
Si	60	24	720	290			
P	220	88	7100	2800			
S	430	170	6000	2400	6890	140	0.74
Cl	2900	1200	4300	1700	5140	100	0.99
K	40	16	5500	2200	6520	90	0.93
Ca	770	310	680	270	838	20	1.17
Ti	330	130			12.24	0.39	
Mn			30	12	18.5	0.2	1.92
Fe	470	190	230	92	205.8	6.8	0.52
Cu	30	12	90	36	71.6	1.6	1.02
Zn	110	44	1300	520	1424	46	0.48
Sum	5640	2260	30330	12100	25699	10300	0.58

a Based on expert knowledge and experience, theses expanded uncertainties are estimated as 40% of the measured value.
b $|Z| = 2\times|\text{Measured} - \text{Certified}|/(U_{95}(\text{Measured})^2 + U_{95}(\text{Certified})^2)^{½}$

Comparison of the measurement results for SRM 1566b and the respective certified values provides evidence that the semi-quantitative estimates of element mass fractions are suitably accurate. The most abundant element attributable to impurity components is chlorine, with an approximate mass fraction of 0.29 %. This is compatible with the q^1H-NMR$_{IS}$-estimated (0.4 ± 0.1) % chloroform content (Section 3.1.7).

The results for Na, Mg, Al, and Fe are similar to those obtained for candidate SRM 916b by ICP-MS (Section 3.2). The ICP-MS Ca and Mg results did not meet quality assurance criteria and have very large uncertainties. The semi-quantitative assessment of the Ca and Mg results via WDXRF are likely to be more accurate. Expressed in percent, the sum of estimates for all impurity elements in Table 4 is 0.57 % ± 0.23 %.

3.4. Microchemical C, H, N Elemental Analysis

Mass fractions of carbon (C), hydrogen (H), and nitrogen (N) were determined by Atlantic Microlab, Norcross, GA USA (Atlantic) and at NIST.

3.4.1. Atlantic Microlab Analysis

Three samples of the SRM 916b material were delivered to the laboratory. A pure acetanilide standard, with metrological traceability to SRM 141d Acetanilide, was used as quality assurance control for C, H, and N analyses. Masses of samples submitted for microanalysis were determined using a Mettler Toledo Sartorius Genius analytical semi-micro balance. Samples were shipped in a cool shipping package containing approximately 2 kg of dry ice.

Elemental microanalysis was performed using sample combustion and detection of CO_2, H_2O, and N_2 with thermal conductivity analyzers (TCD) to determine the C, H, and N compositions.

3.4.2. NIST Analysis

Seven units of the packaged SRM 916b material were analyzed. The units include the first produced; the remaining six were randomly selected. A vario MACRO cube CHNOS elemental analyzer (Elementar Americas Inc., Ronkonkoma, NY USA) was used for the analysis. The instrument was operated in the CHNS mode, using a TCD with helium as a carrier gas and oxygen to aid the combustion of the sample. The combustion tube was controlled at 1150 °C for the analysis and the reduction tube was controlled at 950 °C. A calibrated Mettler XPR2U analytical balance was used for mass determination in the preparation of samples and standards. The method was calibrated using SRM 143d Cystine [19]. SRM 141e Acetanilide was used as control [20].

Three nominal 5 mg test portions were taken from each of the seven units. A known mass of each test portion was added to a tared tin foil boat. After the test portion was added, the foil boat was folded and sealed to minimize entrapment of air and prevent sample loss during further handling. Three analytical samples were taken from each unit in order to have sufficient sampling and to account for inaccurate data that can often arise because of the inherent challenges of the measurement technique. Samples and controls were used as is without drying because the material is stable and has minimal water content.

Calibration samples of SRM 143d Cystine were prepared by transferring a known mass of the SRM into tared tin foil boats of known mass. Nominal test portion masses of the calibrant needed were pre-determined so that the mass range of a given element in the standards would bracket the average mass of the element in the test portions. Calibration masses ranged from 1 mg to 20 mg.

After enough blanks were run to ensure a minimal and constant signal measurement for every element, samples were analyzed so that carryover between samples was either minimized or accounted for. Standards of SRM 143d were analyzed in order of increasing mass, followed by two blanks. The analytical samples were then run in a random order, beginning with a conditioning sample of the same mass to compensate for column carryover and followed by control samples. After running at least two blanks to minimize carryover, a second set of standard samples was run.

Numerical analysis of the instrument data determined a consensus mass fraction of each element and its uncertainty. Uncertainty components were quantified and propagated using parametric bootstrap [21] and Monte Carlo [22] approaches.

A preliminary analysis was performed for each element by first fitting the calibration data to both a first and second order polynomial, to ensure that the calibration data is fit-for-use and to remove

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suspected erroneous data points. These calibration points are removed because elemental analysis is prone to the occasional spurious data point (most likely resulting from contamination or mechanical sample loss), and spurious results at the high and low ends of the detector range can skew the calibration. Calibration points may be rejected based on analysis of the residuals of the first and second order polynomial fits. Elemental mass fractions for the analytical samples and controls are then calculated based on the first or second order polynomial (generally the second order polynomial is used).

After the preliminary analysis is complete, the raw data from the calibrants and analytical samples for each element are exported for processing through the parametric bootstrap method. After importing the data for an element, the best fit for the calibration data is found using an errors-in-variables model and maximum likelihood estimation. The errors-in-variables model is used to account for random effects in both the x-axis (element mass) and y-axis (detector signal). The model fits a range of polynomials to the calibration data and calculates the best polynomial degree as the one having the lowest value of the Bayesian information criterion [23]. In general, there is agreement between the results from the preliminary analysis and the bootstrap method.

The uncertainty in these determinations has been minimized by carefully controlled sample preparation and mass determination, as well as an experimental design in which the sample test portions all have the same nominal mass. Minimizing the variation in the test portion masses can help decrease the uncertainty driven by sample carryover. Careful sample preparation and accurate mass determination also helps to minimize uncertainty contributions from other sources of uncertainty. The components of known uncertainty are listed in Table 9.

Table 9. Components of standard uncertainty for each daily run

Source	Type	% Contribution [a]	Notes
Instrument signal	A	1.35 to 19.97	Replication uncertainty estimated for repeating the measurement with a new set of samples from different bottles. Uses a random coefficients model to predict machine signal (area) based on the test portion mass.
Calibration *X*	B	0.46 to 2.59	Estimated from the uncertainties for CHNS from calibrant and the uncertainty of the mass determination for the calibration standards.
Calibration *Y*	B	56.08 to 96.06	Estimated from the imperfect fit of the calibration curve arising from misfit between the curve and the observations and/or noise in the *Y* calibration values.
Element mass	B	≤ 0.01	Transformation of the Monte Carlo sampling of the calibration curve and the machine signals.
Test portion mass	B	< 0.01 to 0.04	Estimated from the standard uncertainty of the mass determination of the analytical test portions.
Residual	B	<0.01 %	Uncertainty not accounted for in the linear decomposition of uncertainty components.

a Proportion of the total variance for each uncertainty source for each element in each set of samples run. The values shown here are the ranges for all of the elements.

Figure 19 displays the carbon, hydrogen, and nitrogen elemental compositions for the seven units. There is no evidence of between-unit heterogeneity.

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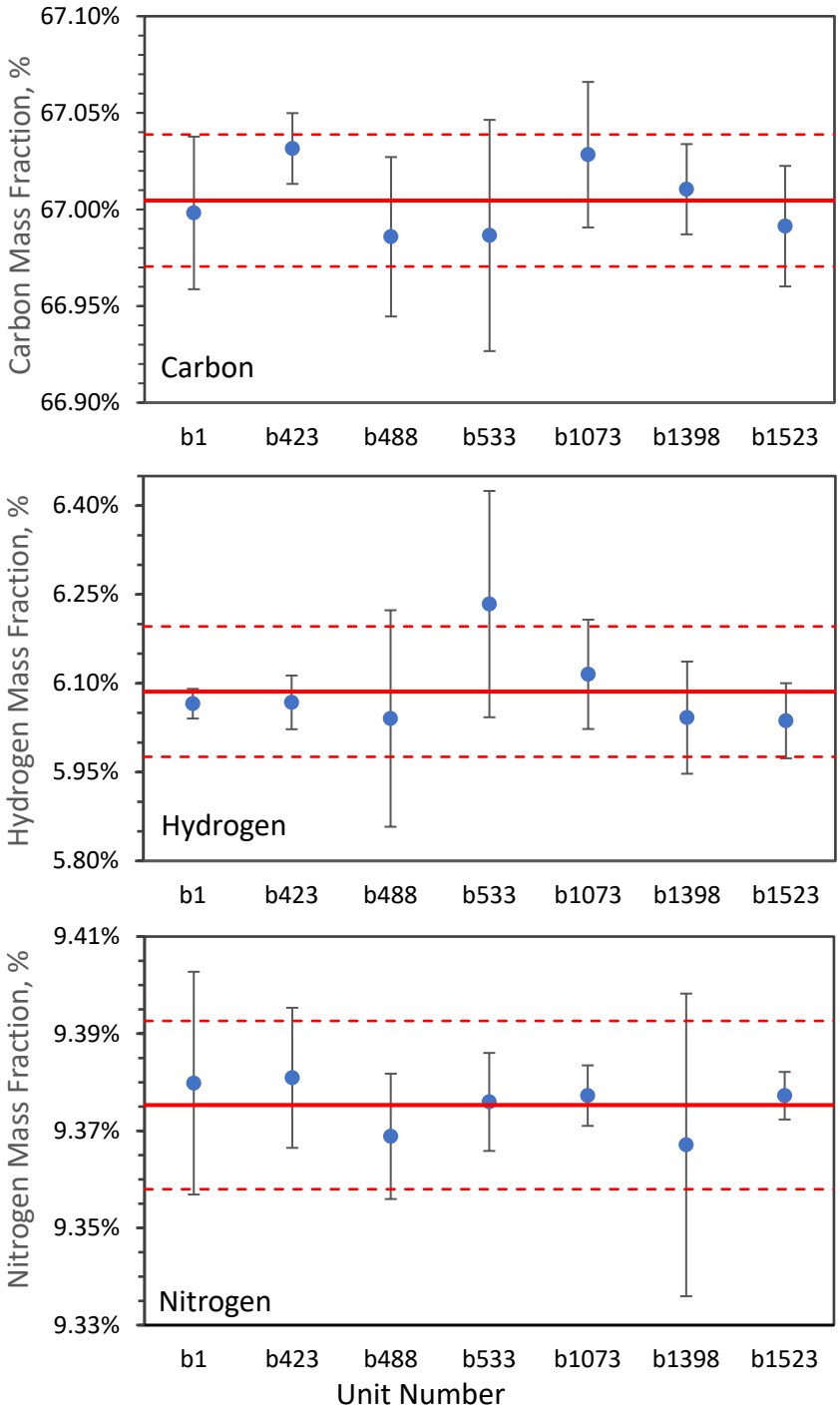

Figure 19. Elemental Compositions as Functions of Box Number

Each symbol represents the average mass faction for one unit; error bars represent one standard deviation.
The solid horizontal line represents the consensus value as calculated by the bootstrap method.
The dashed lines bound the 95 % level of confidence interval.

3.4.3. Summary of Results

Table 10 summarizes the results provided by Atlantic Microlab and the NIST analysis.

Table 10. Measured and Theoretical C, H, N Composition of SRMs 916b[a]

	Bilirubin								
	C			H			N		
	x,%	*s,%*	%Δ	*x,%*	*s,%*	%Δ	*x,%*	*s,%*	%Δ
Atlantic	67.02	0.07	-1.1	6.19	0.03	-0.3	9.41	0.02	-1.8
NIST	67.004	0.034	-1.2	6.09	0.11	-1.9	9.375	0.017	-2.1
Theoretical	67.79			6.21			9.58		

a *x,%* = mean of replicate analysis, expressed as % of total mass;
s,% = standard deviation of the analyses, expressed as % of total mass;
%Δ = percent bias from the theoretical composition, 100×(*x*(Measured)/*x*(Theoretical) - 1)

The (-1 to -2) % biases between the measured and theoretical content suggests that the impurity content in this material includes organic constituents with structures that are substantially different from bilirubin.

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3.5. Water and Ash

The water content of SRM 916b has been determined with Karl Fischer titration (KF) and thermogravimetric analysis (TGA). TGA also provided estimates of the mass fraction of total inorganic content (ash). Ion chromatography identified the major cation content of the ash.

3.5.1. Coulometric Karl Fischer Titration

Samples from eight randomly selected vials of SRM 916b Bilirubin were evaluated without modification. Prior to analysis, the material in each vial was weighed out into a 6 mL glass headspace vial that had been previously dried in an oven at 175 °C for 4 h. After weighing, the vials were hermetically sealed with a septum cap to prevent uptake of water from the atmosphere.

The mass of water (in µg) in each sample is determined by the amount of electricity (in coulombs) required to convert I^- into I_2 at the generator electrode [24]. The percent mass fraction of water in the sample, w_{H2O}, is calculated:

$$w_{\mathrm{H2O}} = 1000\left(\frac{V_\mathrm{s}-V_\mathrm{b}-tR_\mathrm{d}}{m_i}\right)F \quad (3)$$

where: V_s is the volume of titrant consumed titrating the sample,
V_b is the volume of titrant consumed titrating a blank,
t is the titration time
R_d is the drift rate,
m is the sample mass, and
F is a calibration factor determined from titrating samples of known water content.

The analysis of water in SRM 916b Bilirubin was made on a coulometric Karl Fischer system with attached Karl Fischer oven. The coulometric Karl Fischer system was set up using a generator electrode without a diaphragm and was filled with Hydranal Coulomat AG Oven. Liquid standards were added to the apparatus using a gas-tight syringe via a silicone septum. Solid samples in headspace vials were inserted into the Karl Fischer oven that was maintained at 160 °C to release the moisture from the samples. The resulting water vapor was transferred into the Karl Fischer (KF) titration cell using dry nitrogen as the carrier gas, flowing at a rate of 30 ml/min. The transfer is made possible by piercing the septum of the headspace vials with a needle assembly that allows the carrier gas to flow in from the supply and out to the KF cell. The stirring rate in the KF cell was set relatively high to help maximize the uptake of the water vapor into the solvent in the KF cell. Preliminary testing of bilirubin at 160 °C did not indicate the material was breaking down to yield water or materials that would interfere with the Karl Fischer analysis.

On the first day of measurements, the system suitability was checked using SRM 2890 Water Saturated 1-Octaonal) and an in house standard of water saturated octanol (WSO). The WSO was prepared in 2010 and stored at 22 °C, where the organic phase is used for the calibration. The WSO solution is periodically checked against gravimetrically prepared water in octanol solutions, and against SRM 2890 to establish traceability [25]. The system test consisted of five measurements of WSO and six measurements of SRM 2890. Each measurement of WSO and SRM 2890 was made by injecting 50 µL (40 mg nominal) of material into the Karl Fischer titration vessel through a silicone septum via a gas-tight syringe. Samples of the test solutions were weighed out on an analytical balance having 0.01 mg readability. The amount of all solutions injected into the Karl Fischer cell was determined by weighing the syringe before and after the injection on an analytical balance. Measurements for both test solutions were consistent with previous measurements demonstrating that the system was working properly.

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At the start of each day's analysis, two measurements of WSO were performed to check the system. Following the WSO check runs, one of the vials containing candidate SRM 916b Bilirubin was placed into the KF oven. The titration was started, then the septum cap was pierced with the oven's needle to start the transfer of evolved water vapor to KF cell. At the completion of the titration, the old sample vial was removed from the oven and a new sample was placed in the oven for the next titration. Following the runs of the samples and blank vials, water saturated octanol was run in the KF oven to help estimate the uncertainty of the water loss during transit from the oven to the KF titration cell. The control runs typically used 50 µL of WSO (nominally 40 mg) sealed in a sample vial and were conducted in the exact same manner as measurement of the bilirubin test samples.

All titrations were run for a set length of time rather than only according to electrochemical potential of the cell (40 minutes for liquid samples and 50 minutes for samples). The drift of the instrument was calculated at the conclusion of every run over two successive 10-minute intervals to check for consistency in the baseline and to correct the final Karl Fischer signal. After every second measurement, a titration was run using an empty vial to determine the atmospheric water contribution. On average, the blank correction for the Karl Fischer analysis is (154 ± 20) µg of water.

Samples were run in a random order. Table 11 lists the results for the eight samples.

Table 11. Water Content of SRM 916b by Karl Fischer Titration

Unit	Sample mass, g	w_{H2O} %	$u(w_{H2O})$ %
1203	0.08110	0.2755	0.0368
1463	0.10976	0.2649	0.0303
33	0.07984	0.2420	0.0352
1008	0.09673	0.2611	0.0320
228	0.11003	0.2444	0.0284
1138	0.09711	0.2636	0.0312
618	0.10721	0.2352	0.0281
943	0.10369	0.2578	0.0296
	$\bar{x}$ =	0.2557	
	s =	0.0137	
	$\overline{u(w_{H2O})}$ =		0.0288
	u =	0.0113	
	k_{95} =	2.365	
	U_{95} =	0.027	

Figure 20 shows the water content for each tested unit. There might be a slight trend in water mass fraction across the filling order of the units, but all of the individual sample uncertainty intervals overlap and the potential trend is not significant with respect to the certified value of bilirubin purity for SRM 916b.

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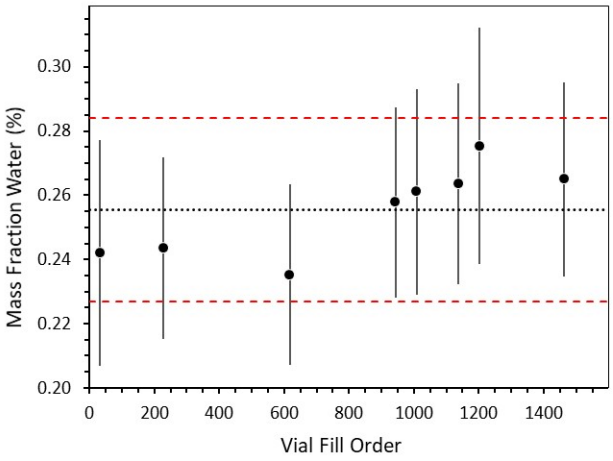

Figure 20. Water Content as a function of Vial Number

Symbols represent the measured water content; error bars represent standard uncertainties. The dotted horizontal line represents the mean. The horizontal dashed lines bound an approximate 95 % level of confidence interval.

3.5.2. Thermogravimetric Analysis

Ten samples were analyzed by TGA, five nominally 0.6 g samples of the bulk bilirubin used to prepare the SRM and five nominally 0.2 g samples prepared by combing the contents of two SRM 916b units. Three sets of gold wires served as controls.

Both the percent water and ash content are determined by gravimetric mass loss after drying in a thermogravimetric oven [26]. Test portions were heated in a LECO Thermogravimetric Analyzer 701 in an air atmosphere. The analyzer consists of an electronics unit for furnace control and data management and a furnace that allows sequential analysis of multiple samples. The furnace holds 20 crucibles, one of which is designated as an empty reference crucible. After an analysis profile was created and selected, empty crucibles were loaded into the furnace carousel and tare weights were obtained. Two runs on the TGA were performed. The first used the nominal 0.6 g test portions of the bulk sample bottles, the second the nominal 0.2 g samples of SRM 916b. Each crucible containing a test portion was transferred to the TGA to determine and record an initial mass. The mass loss of each sample was monitored by the TGA and recorded at approximately 4 min intervals. The samples were first heated to 107 °C and held for 4 h, then to 300 °C and held for 1 h, then to 500 °C and held for 1 h and finally to 750 °C and held for 2 h.

The accuracy and precision of the LECO TGA 701 instrument was monitored in real time using surrogate samples of high-purity gold wire. Three sets of gold wires, weighing a total of about 1 g, were added to three different crucibles. After the initial mass of a set was recorded, the large piece of wire was removed, creating a known mass loss for that sample, which could be compared to that determined by the instrument. Any gain or loss in mass of the gold wire serves as a measure of the high temperature buoyancy correction. The difference between the room temperature mass of gold, $m_{Au,rt}$, and the mass of gold at 750 °C, $m_{Au,750}$, was used to determine the buoyancy correction for the thermogravimetric analyzer: $c_b = m_{Au,750} - m_{Au,rt}$. This correction is only marginally significant for these samples.

The water and ash contents, w_{H2O} and w_{ash}, are calculated as percent from the masses at the end of the 107 °C and 750 °C treatments, $m_{s,107}$ and $m_{s,750}$, the buoyancy correction, and initial sample mass at room temperature, $m_{s,rt}$:

$$w_{\mathrm{H2O}} = 100\left(\frac{m_{\mathrm{s,rt}} - m_{\mathrm{s},107} - c_{\mathrm{b}}}{m_{\mathrm{s,rt}}}\right) \text{ and } w_{\mathrm{ash}} = 100\left(\frac{m_{\mathrm{s},750} - c_{\mathrm{b}}}{m_{\mathrm{s,rt}}}\right) \quad (4)$$

Table 12 lists the TGA-estimated water and ash contents of the SRM 916b Bilirubin. The listed summary uncertainty estimates reflect only measurement replication.

Table 12. Water Content and Ash of SRM 916b by Thermal Gravimetric Analysis

	Mass, g			Content, %	
Sample	≈20 °C	107 °C	750 °C	Water	Ash
Bulk 1	0.53776	0.53674	0.00246	0.1905	0.4579
Bulk 2	0.62518	0.62375	0.00284	0.2286	0.4549
Bulk 3	0.77383	0.77231	0.00369	0.1965	0.4768
Bulk 4	0.67731	0.67600	0.00316	0.1933	0.4667
Bulk 5	0.65255	0.65103	0.00317	0.2331	0.4860
98+163	0.19836	0.19762	0.00089	0.3738	0.4462
1333+1268	0.21961	0.21920	0.00083	0.1865	0.3801
293+358	0.20910	0.20877	0.00098	0.1578	0.4689
683+748	0.19263	0.19212	0.00091	0.2673	0.4747
813+878	0.19821	0.19761	0.00107	0.3015	0.5420
			$\bar{x}$ =	0.233	0.465
			s =	0.065	0.040
			u =	0.021	0.013
			k_{95} =	2.2622	2.2622
			U_{95} =	0.047	0.029

The Karl Fischer and TGA estimates for w_{H2O} are in good agreement: (0.256 ± 0.027) % and (0.233 ± 0.047) %.

Considering the semi-quantitative nature of the WDXRF values and the likely volatile-loss of much of the material's chlorine content, the WDXRF and TGA estimates of inorganic content are also in good agreement: (0.56 ± 0.23) % and (0.456 ± 0.040) %. Qualitative analysis of the ash determined that the cation component of the ash was primarily sodium and potassium. The color of the ash was reddish orange, indicating the presence of iron oxide as well.

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3.6. Molar Absorptivity

The effective purity of candidate SRM 916b Bilirubin for use in spectrophotometric applications was determined through reference of molar absorptivities measured via the procedures described in [13] and [14] to the respective purity-adjusted Reference Values for 200 mg/L bilirubin solutions provided on the Certificate of Analysis (COA) for SRM 916a Bilirubin [5]. The spectrophotometric measurements were made by Children's Wisconsin Reference Standards Laboratory (CWRSL), Milwaukee, WI USA. Dr. Stanley Lo, Director of the Reference Standards Laboratory, arranged for measurements to be made in this laboratory.

Four units of candidate SRM 916b Bilirubin, along with a single unit of SRM 136f Potassium Dichromate and one 100 g bottle of Bovine Serum Albumin (Sigma Aldrich, St. Louis, MO USA), were delivered to CWRSL. The SRM 916b units were stored at -20 °C in individual unit packaging, including the primary containment vial, sealed polymer bag, and a box. A cooler shipping container was used for bilirubin and bovine serum albumin, packed with dry ice. Potassium dichromate was shipped separately without ice and in compliance with requirements for hazardous materials.

Measurements of candidate SRM 916b molar absorptivity values followed the protocol described in Section 3.6.1. Three solutions (50 mg/L, 100 mg/L, and 200 mg/L of the SRM material) were prepared and evaluated for each of the four vials. Each vial was processed individually on a separate day. All measurements were accomplished during an 8-day interval. Table 13 summarizes the molar absorptivity results for each solution measured using each method.

Table 13. Molar Absorptivity

		Molar Absorptivity, L mol^{-1} cm^{-1}					
Method	Solution	$Vial_1$	$Vial_2$	$Vial_3$	$Vial_4$	Mean	SD
Alkaline Azopigment, 598 nm	50 mg/L	72 389	72 422	72 922	72 681	72 604	249
	100 mg/L	72 818	72 745	72 884	72 683	72 783	87
	200 mg/L	72 734	73 090	73 565	72 732	73 030	394
Neutral Azopigment, 530 nm	50 mg/L	53 563	53 965	54 041	53 895	53 866	211
	100 mg/L	53 929	53 972	53 821	53 890	53 903	64
	200 mg/L	53 791	54 001	54 018	54 024	53 959	112
Caffeine Reagent, 432 nm	50 mg/L	47 009	47 032	47 238	47 177	47 114	111
	100 mg/L	47 284	47 239	47 298	46 968	47 197	155
	200 mg/L	47 225	47 490	47 461	47 260	47 359	136
Caffeine Reagent, 457 nm	50 mg/L	46 043	46 097	46 315	46 176	46 158	118
	100 mg/L	46 426	46 398	46 454	46 122	46 350	154
	200 mg/L	46 481	46 780	46 733	46 545	46 635	144

The SRM 916a COA provides non-certified "reference" molar absorptivity values only for 200 mg/L solutions of the SRM 916a material. Therefore, only the 200 mg/L SRM 916b results are directly comparable to those in [5]. Table 14 lists the molar absorptivities for the 200 mg/L solutions of both materials and the derived SRM 916b % purity estimates for the four methods. Combining the four results using the DerSimonian-Laird consensus estimator as implemented in the NIST Consensus Builder [16], the uncertainty-weighted 95 % level of confidence interval is (94.79 ± 0.64) %. Figure 21 displays these spectrophotometric estimates relative to the $q^1H\text{-}NMR_{IS}$ value (Section 4).

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Table 14. Spectrophotometric % Purity of SRM 916b by Four Methods

	Molar Absorptivities				% Purity	
	SRM 916a		SRM 916b		SRM 916b	
Spectrophotometric Method	x	$U_{95}/2$	x	s	x	u
Alkaline Azopigment, 598 nm	76 641	316	73 030	394	95.29	0.65
Neutral Azopigment, 530 nm	57 079	383	53 959	112	94.53	0.66
Caffeine Reagent, 432 nm	50 091	446	47 359	136	94.55	0.88
Caffeine Reagent, 457 nm	49 241	233	46 635	144	94.71	0.54

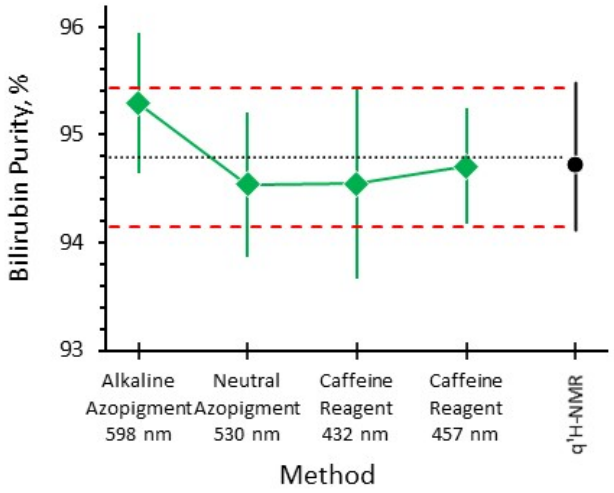

Figure 21. Comparison of Results from the Spectrophotometric Assays and q^{1}H-NMR
Each diamond symbol represents the mean of 4 spectrophotometric-derived results; error bars represent standard deviations (SDs). The dotted horizontal line represents the DerSimonian-Laird consensus mean; the dashed horizontal lines bound the 95 %level of confidence interval around that mean. The circular symbol represents the q^{1}H-NMR$_{IS}$ purity estimate; its error bars represent an approximate 95 % level of confidence interval.

3.6.1. Spectrophotometric Measurement Protocol for SRM 916b Characterization

A laboratory protocol for spectrophotometric measurement of molar absorptivities was provided by NIST. These procedure instructions are provided in 3.6.1.1 – 3.6.1.5, as received by the analytical laboratory.

3.6.1.1. Equipment and Specifications

Pipettes	Use Class A volumetric pipettes for pipetting all solutions. Mechanical pipettors may be used if their accuracy is verified.
Glassware	Use Class A volumetric flasks for preparing all solutions.
Analytical Balance	A balance sensitive to 0.01 mg is required. The accuracy of the balance should be verified.
Spectrophotometer	Use the best instrument you have available that has bandpass of 2 nm or less. Record the manufacturer and model number (if any) of your spectrophotometer.

Wavelength Calibration Use one of the following procedures:

1. Holmium Oxide glass filter. Some of its characteristic peaks are located at 279.4, 360.9, 453.2 and 536.2 nm.
2. Spectral lines of the H_2 or D_2 lamp. Hydrogen exhibits emission lines at 486.1 and 656.3

nm. The D_2 lines are of slightly longer wavelength (0.3 nm). Record the method used for wavelength calibration.

Photometric Accuracy Use one of the following.

1. Glass filters from NIST, SRM 930, or from another source if they are traceable to NIST. Record both measured and certified absorbances for the filters.
2. Potassium dichromate. Prepare the following Blank Solution from SRM 136f $K_2Cr_2O_7$. Dilute 0.6 mL of concentrated H_2SO_4 to 2 liters with distilled or deionized water. The water used for the preparation of the Blank Solution should be free from materials absorbing at 350 nm. Do not use water that has been stored in plastic containers.
   (a) 50 mg/L. Weigh 0.05000 g $K_2Cr_2O_7$. Dissolve and dilute to 1 liter with Blank Solution.
   (b) 100 mg/L. Weigh 0.10000 g $K_2Cr_2O_7$. Dissolve and dilute to 1 liter with Blank Solution.
   Store solutions (a), (b) and Blank Solution in glass containers or preferably in well-stoppered volumetric flasks in the dark at room temperature.

   NOTE: It is somewhat difficult to weigh exactly 0.05000 g and 0.10000 g of $K_2Cr_2O_7$• Weigh approximately the amount required, record the exact weight and correct the absorbance values. For example: If solution (a) contains 0.05050 g of K2Cr2O7 instead of 0.05000 g, the corrected absorbance, A_1, is determined by using the following equation, where A_2 is the observed absorbance value

$$A_1 = \frac{A_2 \times 0.0500\ \text{g}}{0.05050\ \text{g}}.$$

Absorbance Measurements

1. Set spectrophotometer to zero absorbance at 350 nm with Blank Solution.
2. Measure the absorbance of solutions (a) and (b) in duplicate.
3. If necessary, correct the absorbance, as described above, if the weight of the $K_2Cr_2O_7$ is not exactly 0.05000 g or 0.10000 g.
4. Record the data. If the weight of the $K_2Cr_2O_7$ was not exactly 0.05000 g or 0.10000 g, record the corrected absorbances. The reported mean absorbance values at 350 nm for solutions (a) and (b) are 0.535 and l.072, respectively.

Photometric Linearity: Cyanmethemoglobin Solutions

1. Cyanmethemoglobin Reagent Dissolve in succession, 200 mg of $K_3Fe(CN)_6$, 50 mg of KCN and 140 mg of KH_2PO_4 (anhydrous) in water and dilute to 1 liter. Store in a dark, glassbottle at room temperature.
2. Obtain about 5 mL of blood collected with EDTA as anticoagulant. Centrifuge the blood for 10 minutes at 1200 *g*. Remove and discard the plasma.
3. Add 1 mL of red blood cells to 100 mL of cyanmethemoglobin reagent in an Erlenmeyer flask. Mix well by swirling and let stand for 10 min. Centrifuge about 50 mL of this solution at 1500 *g* for 20 minutes to remove the "stroma". Transfer the supernatant into a clean flask. (Do not disturb the sediment.)
4. Measure the absorbance of the supernatant at 540 nm against the cyanmethemoglobin reagent. Adjust the absorbance of this solution to about 1.5 A by diluting with the reagent. This is your Stock Solution.
5. Working Cyanmethemoglobin Solutions Label four 25 mL volumetric flasks 1 through 4. Using volumetric pipets, add the volumes of stock solution listed below to the flasks. Dilute the contents of each flask to volume with the cyanmethemoglobin reagent.

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Flask No.	mL of Stock Solution
1	5.0
2	10.0
3	15.0
4	20.0

6. Measure the absorbance of the Stock and Working cyanmethemoglobin solutions at 540 nm against the cyanmethemoglobin reagent.

Criteria for Acceptable Linearity:

1. Pearson's coefficient (r^2) should be > 0.999.
2. Using the regression equation, calculate the concentration of each standard. All calculated concentrations should be within ±1 % of the nominal values. An example is given below.

Concentration (*X*)	Absorbance, 540 nm (Y)
1	0.2990
2	0.5976
3	0.8962
4	l.1943
5 (Stock)	1.5005

Linear regression analysis of the above data provided the following values:
Slope (a)= 0.3000; Intercept (b) = -0.0024; r^2 = 1.000

The regression equation is $Y = aX + b$. Rearranging, $X = (Y–b)/a$. In this example:

$$X = (Y - -0.0024)/0.3000$$

The Values of *X* calculated from this equation are:

Calculated Concentration	Nominal Concentration	% Difference
1.005	1.000	0.47
2.000	2.000	0.00
2.995	3.000	-0.16
3.989	4.000	-0.28
5.010	5.000	0.19

In this case, the criteria for linearity are met because r^2 is > 0.999 and calculated concentrations are within ± 1% of the nominal values.

3. Record absorbances and criteria for acceptable linearity

Photometric Drift Photometric drift should not exceed 2 mA per hour and can be checked as follows: after spectrophotometer has been turned on, allow it to "warm up" according to the manufacturer's instructions. Zero the spectrophotometer with air and record the absorbance at 0, 15 and 30 minutes after zeroing.

Do Not proceed with the next steps of this protocol if the instrument response is not linear or photometric drift exceeds 2 mA per hour.

3.6.1.2. Procedure for Preparation of Bilirubin Solutions

Prepare 200 mg/L, 100 mg/L, and 50 mg/L bilirubin solutions.

A total of four (4) Stock Bilirubin Standard solutions should be prepared and analyzed. Each Stock Bilirubin Standard Solution shall be prepared using an aliquot from a separate vial of candidate SRM 916b Bilirubin. A suggested procedure for preparing a Stock (200 mg/L) bilirubin solution is described below.

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Tris Buffer, 0.1 mol/L, pH 7.35

Dissolve 12.1 g of Tris Base (tris (hydroxymethyl)-aminomethane) in 800 mL of water. Adjust to pH 7.35 ± 0.05 with HCl and dilute to 1 liter with water. Store at 4° C if not used immediately. Stable for 6 months.

Bovine Serum Albumin (BSA), 40 g/L

Dissolve 20 g of BSA in about 400 mL of tris buffer. Check the pH and adjust, if necessary, to 7.35 ± 0.05 with dilute HCl or NaOH. Dilute to 500 mL with tris buffer and store at 4° C if not used immediately. If the solution is not to be used within 2-3 days following preparation, it should be kept frozen at -20° C.

200 mg/L Stock Bilirubin Solution

- Work in subdued or Red light.
- Use a plastic, micro-weighboat to weigh the bilirubin.
- It is not necessary to weigh exactly 20.00 mg of bilirubin. Weigh 20.00 ± 1.00 mg of bilirubin and record the exact weight to 0.01 mg.

Place the weighboat on the balance pan and wait until you obtain a constant weight. Weigh the bilirubin in one comer as quickly as possible. Record the exact amount of bilirubin.

Quantitatively transfer the bilirubin into a 100 mL volumetric flask. While keeping the weighboat on the opening of the flask, "pinch" two of its corners and tap gently to facilitate the transfer ofbilirubin. Tap the bottom of the flask on the counter to help bring all of the bilirubin to the bottomof the flask.

While keeping the weighboat "pinched" and in the vertical position over the opening of the flask, wash as much as possible of the remaining bilirubin into the flask with 1.0 mL of DMSO. Add the DMSO dropwise and make sure any bilirubin stuck to the neck of the flask is also washed down. Swirl the flask until all of the bilirubin is finely dispersed.

Wash the weighboat and neck of the flask with 2.0 mL of 0.1 M sodium carbonate solution. Swirl the flask gently until a clear, ruby red solution is obtained. This may take as long as 5 minutes. However, all bilirubin should be in solution 10 minutes after the addition of sodium carbonate.

NOTE: It is very important that all of the bilirubin has dissolved because any not in solution at this point will not dissolve after the addition of the BSA.

Add about 80 mL of the 4 g/dL BSA solution, pH 7.35, and gently swirl. Add more BSA until thelevel of the solution is just below the volume mark in the neck of the flask. Any foam that has formed during mixing should be removed: dip one end of a wooden applicator stick into caprylic alcohol, wipe the stick with a lint-free tissue and carefully place the moistened end over the foam. Move the stick in a circular motion against the neck of the flask until all the bubbles have disappeared.

Carefully dilute the solution to a final volume of 100 mL with the BSA. Cap and mix gently butthoroughly by inversion.

Standard Blank

The preparation of the Standard Blank solution is identical to that of the Stock Bilirubin Standard solution except that the bilirubin is omitted. Prepare 200 mL. Freeze in amounts appropriate for use each day.

50 mg/L and 100 mg/L Bilirubin Solutions

Dilute the Stock Bilirubin Standard Solutions with the Standard Blank according to the specifications in thetable below. Use Class A volumetric pipettes and prepare the diluted standards immediately before use.

Concentration, mg/L	mL of Stock Bilirubin Solution	mL of Blank Solution
50	3	9
100	6	6

3.6.1.3. Procedure for Determining Molar Absorptivity of the Alkaline Azopigment at 598 nm. Reference Method [13]

Reagent Preparation

Water	Use freshly distilled or deionized water to prepare all solutions.
Caffeine Reagent	In this order, dissolve 1.0 g of disodium EDTA (gentle warming may be necessary to dissolve the EDTA), 56.0 g of anhydrous sodium acetate (or 93 g of $CH_3COONa \cdot 3H_2O$) and 56.0 g of sodium benzoate in about 700 mL of water. Add 37.5 g of caffeine and stir until completely dissolved. Dilute to 1 liter with water. This reagent is usually slightly turbid and must be clarified by filtration with Whatman #1 filter paper. Store in a well stoppered glass bottle at room temperature. Stable for 6 months.
Alkaline Tartrate	Dissolve 320 g of sodium potassium tartrate ($NaKC_4H_6O_6 \cdot 4H_2O$) and 75.0 g of NaOH in water and dilute to one liter. This reagent is occasionally turbid and if it is, should be clarified by filtration with Whatman #1 filter paper. Store in a well stoppered plastic bottle at room temperature. Stable for 6 months.
Sodium Nitrite, 5.0 g/L	Dissolve 0.50 g of $NaNO_2$ in 100 mL of water. Store at 4°C in a well stoppered glass bottle. Stable for 2 months.
Sulfanilic Acid, 5.0 g/L	Dissolve 5.0 g of sulfanilic acid in 500 mL of water. Add 15.0 mL of concentrated HCl and dilute to 1 liter with water. Store at room temperature in a well stoppered glass bottle. Stable for 6 months.
Diazo Reagent	Combine 0.50 mL of 5.0 g/L sodium nitrite and 20 mL of 5.0 g/L sulfanilic acid in aflask, mix well. Prepare fresh for each analysis.

Analytical Procedure

- Work in subdued light or Red light.
- Use Class A volumetric pipets to deliver sample and reagents.
- Use clean 16 × 100 mm disposable borosilicate glass tubes.
- Use the same cuvette for all measurements. Keep the cuvette in the same orientation with respect to the light beam throughout the run.
- Flow-through cuvettes may be used instead of regular quartz cuvettes if they have a pathlength of 10.0 mm (1 cm) and carry-over is eliminated by thorough rinsing between samples.
- Analyze Reagent Blank and Standard Blank in duplicate and all Bilirubin Standard Solutions in triplicate.
- All mixing in Steps 1 through 5 should be done using a vortex mixer.

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1. Pipet 4.0 mL of caffeine reagent into appropriately labeled tubes.
2. Add 0.5 mL of sample to the first tube and mix well using a vortex mixer. Start a stopwatch.
3. At timed intervals (e.g., 30 or 45 sec) add sample to caffeine reagent in the remaining tubes and mix well.
4. At 10 minutes on the stopwatch add 1.0 mL of DSA to the first tube and mix well using a vortexmixer.
5. At the same timed intervals established in Step 1 add DSA to the remaining tubes and mix well.
6. At 20 minutes on the stopwatch add 3.0 mL of Alkaline Tartrate reagent to the firsttube, cover with Parafilm and mix gently by inversion, about 10 times.
7. At the same timed intervals established in Step 1 add alkaline tartrate reagent to the remaining tubes, cover with Parafilm and mix well by gentle inversion, about 10 times, and let stand at room temperature for 10 minutes.

Absorbance Measurements

1. Set the spectrophotometer at zero absorbance (0.000 A) at 598 nm with air in both the sample and reference beams (i.e., with no cuvettes in the cuvette holder).
2. Read all solutions vs air in the reference beam starting with the Reagent Blank and record the absorbances. Rinse the cuvette twice with small aliquots of each solution to be read to avoid carryover. Make sure to wipe off fingerprints with lint-free tissue. If a flow-through cuvette is used, fill the cuvette with water and set the absorbance at zero. Aspirate solution 2 to 3 times before recording the absorbance.

3.6.1.4. Determining Molar Absorptivity of the Neutral Azopigment at 530 nm

Analyze the Bilirubin Standard Solutions and Standard Blank as outlined above but use 5.0 mL of caffeine reagent (instead of 4.0 mL) and do not add alkaline tartrate. Measure the absorbance at 530 nm and record the data.

3.6.1.5. Determining Molar Absorptivity of Bilirubin in Caffeine Reagent at (432 and 457) nm [14]

Preparation of "Vink's" Caffeine Reagent

Note: This reagent is 1.34 times stronger than that used with the Reference Method.

In this order, dissolve 1.0 g of disodium EDTA (gentle warming may be necessary to dissolve the EDTA), 75 g of anhydrous sodium acetate (or 125 g of $CH_3COONa \cdot 3H_20$) and 75 g of sodium benzoate in about 600 mL of water. Add 50 g of caffeine and stir until completely dissolved. Dilute to 1 liter with water. This reagent is usually slightly turbid and must be clarified by filtration with Whatman #1 filter paper. Store in a well stoppered glass bottle at room temperature. Stable for 6 months.

Analytical Procedure

- Work in subdued light.
- Use Class A volumetric pipets to deliver sample and reagents.
- Use clean, rinsed 16 x 100 mm disposable borosilicate glass tubes.
- Analyze Reagent Blank and Standard Blank in duplicate and all Bilirubin Standard Solutions in triplicate.

1. Pipet 10.0 mL of caffeine reagent into appropriately labeled tubes.
2. Add 0.5 mL of sample to the first tube, cover with Parafilm and mix gently by inversion, about 10times. Start a stopwatch.
3. Add sample to caffeine reagent in the remaining tubes, cover with Parafilm, mix well.

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4. Set the spectrophotometer at zero absorbance (0.000A) at 435 and 457 nm with air in both the sample and reference beams (i.e., with no cuvettes in the cuvette holder).
5. Read all solutions vs air in the reference beam within 15 minutes of making dilutions. Start with the Reagent Blank and record the absorbances in the appropriate table of the result reporting sheet. Rinse the cuvette twice with small aliquots of each solution to be read to avoid carryover. Make sure to wipe off fingerprints with lint-free tissue. If a flow-through cuvette is used, fill the cuvette with water and set the absorbance at zero. Aspirate solution 2 to 3 times before recording the absorbance.

4. Value Assignment

The measurand was determined via q^1H-NMR$_{IS}$ using a variant of routine computational approaches consistent with those described in [15,17]. The q^1H-NMR$_{IS}$ analysis is described in Section 3.1. Results were determined using measurement data collected for a sample from each of 12 units, selected uniformly across the production lot. The mass fraction of bilirubin, w_P, via q^1H-NMR$_{IS}$ was derived using an estimation model based on measurement Eq. 2 (Section 3.1.2):

The estimate of w_p was calculated using a Bayesian procedure modeled on "observation equations" via Markov Chain Monte Carlo (MCMC). Different from the approach described in [15], no hierarchy was placed on the estimate for purity; estimates for individual sample replicates were combined via linear pooling to calculate an estimate of w_p.

The prior distribution for the estimate of w_p was specified as being uniform along the interval [0.6 g/g/, 1.0 g/g]. The distribution of the purity of the internal standard, known to be 0.9983 g/g, u = 0.0007 g/g, was specified as $\mu_{P_I} \sim N(0.9983, 2040816)$, where the variability is in terms of precision ($1/u^2$). Table 15 lists the values of the model parameters and their standard uncertainties.

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Table 15. q^{1}H-NMR$_{IS}$ Parameters and Their Standard Uncertainties

Parameter	x	$u(x)$	Unit
M_P	584.67	0.019	g/mol
M_I	94.191	0.005	g/mol
P_I	0.9983	0.0007	g/g
N_P	1	0	(Count)
N_i	1	0	(Count)

Box	R_A [a]	$u(R_A)$	R_m	$u(R_m)$
4	0.323457	0.001431	1.023626	0.0007
9	0.643754	0.000746	2.040192	0.0014
2	0.275342	0.000791	0.871086	0.0006
7	0.359476	0.001100	1.139908	0.0008
8	0.297617	0.000815	0.944380	0.0007
12	0.441388	0.002608	1.402251	0.0010
1	0.402559	0.000438	1.279635	0.0009
3	0.302120	0.000444	0.957510	0.0007
5	0.323294	0.000612	1.027038	0.0007
6	0.347410	0.000615	1.105491	0.0008
10	0.326225	0.001277	1.033606	0.0007
11	0.310294	0.000261	0.985764	0.0007

[a] values of R_A are based on integrals normalized to the ^{1}H multiplicity of bilirubin moieties 6 and 41 and caffeine moiety 8.

The uncertainties associated with the parameters were evaluated as follows: standard uncertainties of the R_A, $u(R_A)$, were estimated as the standard deviation of values of R_A determined from three (3) repetitions of the ^{1}H-NMR$_{IS}$ data processing procedure; standard uncertainties of the R_m, $u(R_m)$, were assigned a Type B uncertainty of 0.07 %; the standard uncertainties of the purity of the NIST PS3 Caffeine internal standard, $u(P_I)$, is 0.0007 g/g; the standard uncertainties of the average relative molar masses (M_I, M_P) were calculated using the IUPAC Commission on Isotopic Abundances and Atomic Weights (CIAAW) Molecular Weight Calculator [27]; no uncertainty was considered for the ^{1}H atom multiplicities of the primary component (N_P) and internal standard (N_I), which are based on known chemical structures.

The value range expected to contain the true value of purity of bilirubin in the SRM 916b material with an approximate 95 % level of confidence was determined from the 95 % coverage interval of the posterior distribution for the measurand (Figure 22). The median value of this distribution is 94.72 %. The 95% coverage interval (94.11 to 95.48) % represents an asymmetric probability density distribution, however the bilirubin mass fraction can be practically treated, with at least a 95 % level of confidence, as the symmetric interval (94.7 ± 0.7) %.

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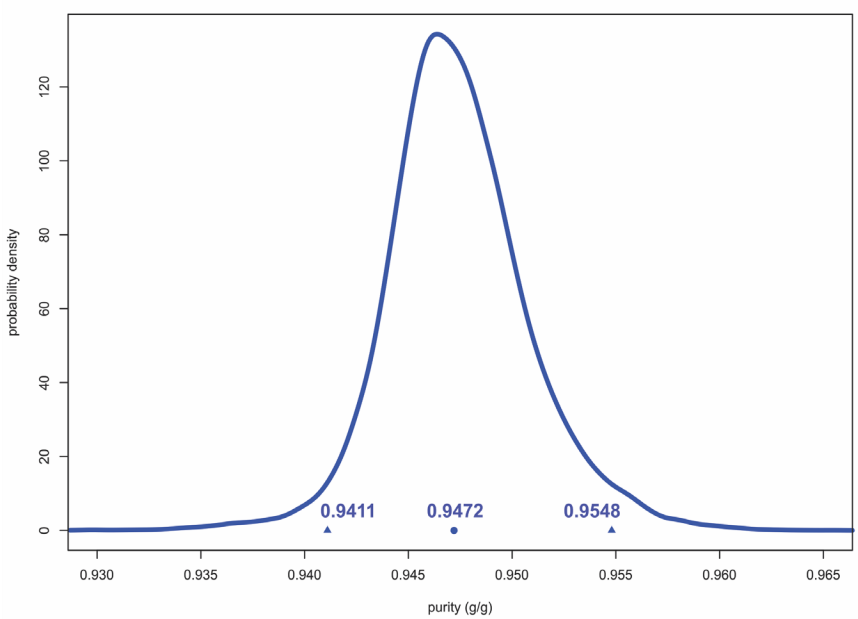

Figure 22. Posterior Distribution for the $q^{1}H\text{-}NMR_{IS}$ Purity Estimation
Purity is expressed as mass fraction in units of g/g rather than %.

4.1.1. OpenBUGS code and data tables for calculation of chemical purity

{MIS1~dnorm(194.191, 40000) **#** $\boldsymbol{M_{\mathrm{I}}}$
MPC1~dnorm(584.661, 2771) # $\boldsymbol{M_{\mathrm{P}}}$

##prior for the unknown purity mu
a<-mu/(sd*sd)
b<- (1-mu)/(sd*sd)
mu~dunif(0.6,1) # **prior distribution for the measurand,** $\boldsymbol{w_{\mathrm{p}}}$
sd~dunif(0,1)
uncertainty for the mass ratios
um<-0.0007 **#** $\boldsymbol{u(R_m)}$
precm<-1/(um*um)

muP1~dnorm(0.9983, 2040816) # $\boldsymbol{P_{\mathrm{I}}}$
muP1.cut<-cut(muP1)

#calculate twelve purity estimates based on observations for each of twelve sample replicates
for(i in 1:12){ pPC1[i]~dunif(0.7,1)
Mrat1[i]~dnorm(mrat1[i],precm)
Mrat1.cut[i]<-cut(Mrat1[i])
mu1[i]<-pPC1[i]/muP1.cut*Mrat1.cut[i]*MIS1/MPC1
preca1[i]<-1/(uareaR1[i]*uareaR1[i])
var1[i]<-1/preca1[i]
prec1[i]<-1/var1[i]
arat1[i]~dnorm(mu1[i],prec1[i])}

#linear pooling of all estimates for the twelve sample replicates, the pPC1[i],
T~dcat(P[])
P[1:12]~ddirich(alpha[])

alpha[1]<-1; alpha[2]<-1; alpha[3]<-1; alpha[4]<-1; alpha[5]<-1; alpha[6]<-1
alpha[7]<-1; alpha[8]<-1; alpha[9]<-1; alpha[10]<-1; alpha[11]<-1; alpha[12]<-1

mup<-pPC1[T]

mum<-cut(mup)/(MPC1/1000) **# estimate of** $\boldsymbol{w_{\mathrm{p}}}$
}

#q^{1}H-NMR$_{\mathrm{IS}}$ measurement data:
list(arat1=c(0.32345714,0.643754,0.275342,0.359476,0.297617,0.441388,0.402559,0.30212,0.323294,0.34741,0.326225, 0.31029), **#** $\boldsymbol{R_A}$
uareaR1=c(0.00143101,0.000746,0.000791,0.0011,0.000815,0.002608,0.000438,0.000444,0.000612,0.000615,0.001277,0.000261), **#** $\boldsymbol{u(R_A)}$
mrat1=c(1.02362607,2.040192,0.871086,1.139908,0.94438,1.402251,1.279635,0.95751,1.027038,1.105491,1.033606, 0.985764)) **#** $\boldsymbol{R_m}$

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