National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 916a

Bilirubin

This Standard Reference Material (SRM) consists of a sample of unconjugated bilirubin that is certified as a chemical of known purity. It is intended primarily for use in the calibration and standardization of procedures used for the determination of bilirubin in clinical samples and for routine evaluations of daily working standards used in these procedures. This material can also be used for quality assurance when assigning values to in-house control materials. A unit of SRM 916a consists of one bottle containing 100 mg of crystalline bilirubin.

Due to concerns that the molar absorptivities for bilirubin and its azopigments in the original June 1989 certificate were lower than those in a published study [1], an interlaboratory exercise to reassess the molar absorptivities was conducted. The resultant revised values are reported in Table 2 of this certificate; for comparison the original data generated in 1988 can be found under Supplemental Information.

Certified Purity and Uncertainty: The certified chemical purity is based upon the results from several analytical techniques used at NIST that are designed to measure impurities and on scientific judgement of these results. The certified chemical purity presented in Table 1 was determined by measuring the mass fractions of impurities, including water, and residue from ashing, summing the impurities, and subtracting this sum from 100 %. A certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account. The certified chemical purity and estimated uncertainty presented in Table 1 are based upon scientific judgment and evaluation of the numerous analytical tests applied to this SRM in the certification process. The uncertainty is meant to approximate two standard deviation limits for the certified value.

Reference Values and Uncertainties: Reference concentration values for the molar absorptivity in caffeine reagent [2] and of the blue and red azopigment products, obtained by the Reference Method for Total Bilirubin (developed by the Committee on Standards of the American Association for Clinical Chemistry [AACC]) [3] were derived from results reported by six collaborating laboratories in 1998 and are listed in Appendix A. The reference values for molar absorptivities are presented in Table 2. The molar absorptivity value of the red azopigment at 530 nm was obtained by omitting the addition of alkaline tartrate in the Reference Method. Reference values are noncertified values that are the best estimate of the true value; however, the values do not meet the NIST criteria for certification and are provided with associated uncertainties that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods.

Expiration of Certification: The certification of **SRM 916a** is valid, within the uncertainty specified, until **01 September 2017**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet or register online) will facilitate notification.

Analyses for the original certification of purity and characterization of SRM 916a in 1988 were performed by R.G. Christensen, A. Cohen, B. Coxon, M.J. Welch and D.A. Becker of NIST.

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

Carlos A. Gonzalez, Chief
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Gaithersburg, MD 20899
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Steven J. Choquette, Director
Office of Reference Materials

Overall direction and coordination of the technical measurements leading to the original certification of purity and characterization were provided by A. Cohen and E. White V of NIST. Coordination of the technical measurements leading to the establishment of the original absorptivity constants in 1988 was performed by B.T. Doumas of the Medical College of Wisconsin, Department of Pathology (Milwaukee, WI). Coordination of the technical measurements leading to the value assignment of the revised molar absorptivities for this SRM in 1998 were performed by B.T. Doumas and B.W. Perry of the Medical College of Wisconsin, Department of Pathology (Milwaukee, WI) and M.J. Welch.

Original statistical consultation was provided by R.C. Paule of NIST. The design and statistical analysis of the molar absorptivity measurements made in 1998 were performed by L.M. Gill of the NIST Statistical Engineering Division.

NOTICE AND WARNING TO USERS

SRM 916a IS INTENDED FOR RESEARCH USE.

Storage: The SRM is supplied in a vial that has been capped under argon. The vial is sealed in a foil-lined polyester film bag also purged with argon and shipped on dry ice. Upon receipt, the storage temperature of SRM 916a is conditional upon the expected time frame for use. A refrigerator temperature between 2 °C and 8 °C is acceptable for storage up to one week. If a longer storage time is anticipated, the material should be stored at or below –20 °C. Prior to use, the material should be allowed to come to room temperature (between 20 °C and 25 °C) before opening the container. After opening, the material should be kept in the tightly-closed vial, stored in the refrigerator between 2 °C and 8 °C, and protected from light.

INSTRUCTIONS FOR USE

The SRM 916a, consisting of unconjugated bilirubin, is virtually insoluble in water near physiological pH. It is soluble, however, in a mixture of dimethyl sulfoxide and sodium carbonate. A 0.2 g/L standard solution for the preparation of a calibration curve may be obtained as follows [3]. Weigh out 20.3 mg (provides correction for purity) of SRM 916a into a small plastic dish. Transfer the bilirubin quantitatively into a 100 mL volumetric flask with the aid of 1 mL of dimethyl sulfoxide and swirl the flask until the crystals are uniformly dispersed. Add 2.0 mL of 0.1 mol/L aqueous sodium carbonate and swirl the mixture until an optically clear, red-orange solution is obtained. Dilute the solution with a solution of bovine serum albumin (BSA, Cohn Fraction V, 40 g/L, previously adjusted to pH 7.4) or with an acceptable serum diluent for unconjugated bilirubin [4]. For a detailed description of the preparation and handling of the standard solution see reference [3]. Since bilirubin is light sensitive, the flask should be immediately wrapped with aluminum foil. It is further recommended that the solution preparation be carried out with low-intensity incandescent light and absolutely away from sunlight. Standard solutions of lower concentration may be prepared by dilution of appropriate aliquots with the serum albumin solution. It has been reported [5] that deterioration of the standard solution was about 1.5 % per month at –20 °C, and about 1 % in six months at –70 °C.

The molar absorptivity of SRM 916a in caffeine reagent [2], and of the blue and red azopigment products obtained by the Reference Method for Total Bilirubin [3] were determined in a round-robin collaborative study. The molar absorptivity value of the red azopigment at 530 nm was obtained by omitting the addition of alkaline tartrate in the reference method. The reference values for molar absorptivity are shown in Table 2.

Source, Preparation, and Analysis[(1)]

Source of Material: The bilirubin used for this SRM was supplied by Pfanstiehl Laboratories, Inc. (Waukegan, IL). It was prepared from material that was isolated from hog bile and crystallized as the acid. It was purified further by treatment in chloroform with sodium sulfate as described in reference [6] and recrystallization from chloroform.

[(1)]Certain commercial instruments, materials, or processes are identified in this certificate to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the instruments, materials, or processes identified are necessarily the best available for the purpose.

Analyses for Revised Molar Absorptivity Determinations: In the protocol to reassess the molar absorptivity of bilirubin and its azopigments, each of the six participating laboratories made up three solutions at 200 mg/L on three different days and made measurements at four wavelengths: 423 nm, 457 nm, 530 nm, and 598 nm. Each solution was measured on three different days and in triplicate each day, i.e. each laboratory made nine measurements for each wavelength.

Analyses Related to the Original Characterization of SRM 916a in 1988: SRM 916a consists of a mixture of three isomers of unconjugated bilirubin [7]. The relative amounts of the isomers were determined by two methods. The mole fractions of the bilirubin IIIα, IXα, and XIIIα isomers measured by thin-layer chromatography (TLC), using 1 % acetic acid in chloroform [7] on silica gel G were 5.3 %, 83.1 %, and 11.5 %, respectively; and measured by high performance liquid chromatography (HPLC) on silica using approximately 1 % acetic acid in dichloromethane were 7.5 %, 83.2 %, and 9.3 %, respectively. Recent LC measurements made in 1996 to assess the stability of the isomer concentrations found the relative isomer concentrations unchanged. The absorptivity of the material in chloroform (reagent grade containing 0.75 % ethanol stabilizer) at 453 nm was (104.8 ± 0.2) $L \cdot cm^{-1} \cdot g^{-1}$ at 20 °C. A separately prepared solution, whose volume and absorbance were determined at 25 °C gave (105.0 ± 0.1) $L \cdot cm^{-1} \cdot g^{-1}$. Both values are ncorrected for the 1.2 % chloroform, and other impurities in the crystals. The absorptivity reflects contributions from the three isomers of bilirubin present.

Samples of the SRM were dissolved in dimethyl sulfoxide-d_6 and measured by ^{1}H nuclear magnetic resonance (NMR). The amount of chloroform detected was 1.2 %. Neither biliverdine, an oxidation product of bilirubin, nor mesobilirubin, the diethyl analogue, were detected by NMR. The chloroform present is tightly retained in the crystals. The loss of weight at 60 °C for 71 h under vacuum was only 0.03 %. Since the chloroform is so firmly retained, we recommend that the material be used as supplied. Mesobilirubin was not detected by mass spectrometry. Biliverdine was not detected by HPLC (0.1 % limit of detection) or by visible absorption spectrophotometry (0.01 % limit of detection).

Impurities more acidic than bilirubin were determined quantitatively by extracting samples with 5 % sodium bicarbonate, washing with chloroform, acidifying, extracting with chloroform, washing with water, and concentrating the chloroform extract. The dry mass of the extracts was 0.20 %. Examination by thin-layer chromatography and by mass spectrometry after silylation indicated that some material similar to or identical to bilirubin had passed through the extraction procedure and was in the extract and thus the amount of impurity more acidic than bilirubin is less than 0.20 %. Nonacidic impurities were 0.013 %. They were determined by mixing a solution of 100 mg of bilirubin dissolved in chloroform with 0.1 mol/L sodium carbonate. Bilirubin forms a sodium salt that is water soluble. This solution was extracted with chloroform, the extract washed with water, the chloroform removed by evaporation and the mass of the residue determined.

An unidentified impurity was detected by thin-layer chromatography with polyamide as the adsorbent and 3:1 (v/v) methanol-aqueous ammonium hydroxide (3.3 %) as the developer [8]. One hundred micrograms of SRM 916a bilirubin dissolved in chloroform gave a single yellow-orange spot at R_f 0.63, but under 365 nm radiation, a pink fluorescent spot, which was barely detectable, developed above the bilirubin spot at R_f 0.76. The quantity of material was estimated by comparing the intensity of the spot with spots of known amounts of bilirubin after spraying with a reagent consisting of phosphomolybdic-phosphotungstic acid (Folin-Denis reagent) and exposure to ammonia vapor. The amount of the unknown material was estimated to be 0.2 %.

Table 1. Certified Mass Fraction Purity and Uncertainty[(a)] of Bilirubin

Purity	98.3 %	±	0.3 %

[(a)] The certified chemical purity and estimated uncertainty are based upon scientific judgment and evaluation of the numerous analytical tests applied to this SRM in the certification process. The uncertainty is meant to approximate two standard deviation limits for the certified value. The measurand is the total mass fraction purity value for bilirubin. Metrological traceability is the SI derived unit for mass fraction (expressed as a percent).

Table 2. Reference Values for Molar Absorptivities for SRM 916a Bilirubin and Its Azopigments at 200 mg/L

Wavelength (nm)	Molar Absorptivity[a,b] ($L \cdot mol^{-1} \cdot cm^{-1}$)	S. D.[c] ($L \cdot mol^{-1} \cdot cm^{-1}$)
Alkaline Azopigment at 598	76 641 ± 631	601
Neutral Azopigment at 530	57 079 ± 765	729
Bilirubin in Caffeine Reagent at 432	50 091 ± 891	849
Bilirubin in Caffeine Reagent at 457	49 241 ± 466	444

[a] Corrected for purity of bilirubin.

[b] The reference values are the equally weighted mean of results obtained at six participating laboratories. The uncertainty in the reference values is expressed as an expanded uncertainty, *U*, at the 95 % level of confidence, and is calculated according to the method described in the ISO/JCGM Guide [9]. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined effect of between-lab, within-lab and purity. The coverage factor, $k = 2.57$, is determined from the Student's *t*-distribution corresponding to 5 degrees of freedom and 95 % level of confidence for each wavelength. The measurand is the molar extinction coefficient as determined by the method indicated. Metrological traceability is to the SI units for volume, amount-of-substance, and length (expressed as $L \cdot mol^{-1} \cdot cm$).

[c] One standard deviation of a single measurement.

REFERENCES

[1] Doumas, B.T.; Perry, B.W.; McComb R.B.; Kessner, K.; Vader, H.L.; Vink, K.L.J.; Koedam, J.C.; Paule, R.C.; *Molar Absortivities of Bilirubin (NIST SRM 916a) and Its Neutral and Alkaline Azopigments*; Clin. Chem, Vol. 36, pp. 1698–1701 (1990).

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[3] Doumas, B.T.; Kwokcheung, P.P.; Perry, B.W.; Jendrzejczak, B.; McComb, R.B.; Schaffer, R.; Hause, L.L.; *Candidate Reference Method for Determination of Total Bilirubin in Serum: Development and Validation*; Clin. Chem., Vol. 31, pp. 1779–1789 (1985).

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[7] McDonagh, A.F.; Assisi, F.' *Commercial Bilirubin: a Trinity of Isomers*; FEBS Lett., Vol. 18, pp. 315–317 (1971).

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[9] JCGM 100:2008; *Evaluation of Measurement Data — Guide to the Expression of Uncertainty in Measurement* (GUM 1995 with Minor Corrections); Joint Committee for Guides in Metrology (2008); available at http://www.bipm.org/utils/common/documents/jcgm/JCGM_100_2008_E.pdf (accessed Aug 2016); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297; U.S. Government Printing Office: Washington, DC (1994); available at http://www.nist.gov/pml/pubs/tn1297/index.cfm (accessed Aug 2016).

Certificate Revision History: 22 August 2016 (Change of expiration date; editorial changes); 12 August 2014 (Extension of the certification period; editorial changes); 30 November 2009 (Updated storage instructions and extension of certification period); 30 December 2005 (This technical revision reports an extension of the certification period); 05 December 2001 (This technical revision reports the addition of revised reference values for molar absorptivity); 01 June 1989 (Original certificate date).

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.

APPENDIX A

Collaborating Laboratories for Revised Molar Absorptivities (1998). We gratefully acknowledge the contributions of the participants in this interlaboratory exercise.

Doumas, B.T.; Perry, B.W.; Dept. of Pathology, Medical College of Wisconsin (Milwaukee, WI, USA).
Hill, B.; Wisconsin State Laboratory of Hygiene (Madison, WI, USA).
Külpmann, W.R.; Institut fur Klinische Chemie I, Medizinische Hochschule Hannover (Hannover, Germany).
Nealon, D.A.; Ortho Clinical Diagnostics, Inc., Johnson and Johnson (Rochester, NY, USA).
Schlebusch, H.; der Universitats-Frauenklinik (Bonn, Germany).
Vader, H.L.; Clinical Laboratories, St. Joseph Hospital (Veldhoven, The Netherlands).

Supplemental Information

Molar Absorptivity Values Generated in the Original Round Robin of 1988

Wavelength (nm)	Molar Absorptivity[a,b] ($L \cdot mol^{-1} \cdot cm^{-1}$)
Alkaline Azopigment at 598	76 500
Neutral Azopigment at 530	56 700
Bilirubin in Caffeine Reagent at 432	50 100
Bilirubin in Caffeine Reagent at 457	49 000

[a] Corrected for purity of bilirubin.
[b] One standard deviation of the mean of the round-robin results is 300; one standard deviation of a single measurement is 700.

Collaborating Laboratories for Original Analyses (1988) for Molar Absorptivities. We gratefully acknowledge the contributions of the participants in the round robin study.

Doumas, B.T.; Perry, B.W.; Jendrzejczak, B.; Hubbard, L.; Medical College of Wisconsin (Milwaukee, WI, USA).
McComb, R.B.; Okorodudu, A.O.; Hartford Hospital (Hartfort CT, USA).
Navazi, W.; Kessner, A.; Beckman Instruments, Inc. (Brea, CA, USA).
Vander, H.L.; Vink, K.L.J.; St. Joseph Hospital (Eindhoven, The Netherlands).
Koedam, H.C.; Steentjes, G.M.; Wikkeling, R.H.; Phielix-Strubbe, C.J.; National Institute of Public Health and Environmental Protection (Bilthoven, Netherlands).