National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material 968a

Fat-Soluble Vitamins in Human Serum

This certificate provides updated concentration values for SRM 968a (measurements for the initial certification of SRM 968a were completed in May, 1991). Due to concerns regarding the stability of fat-soluble vitamins and carotenoid compounds in serum, measurements have been continually made on this material since its initial certification. This certificate revision reflects the results of the stability measurements.

Standard Reference Material (SRM) 968a is intended for use in validating methods for determining retinol, α-tocopherol, and total ß-carotene in human serum and plasma. This SRM can also be used for assigning values to serum or plasma used as in-house control materials. A unit of SRM 968a consists of six vials of lyophilized human serum, two each of three different concentration levels for retinol, α-tocopherol, and total ß-carotene. Information values are also provided for cholesterol, γ-tocopherol, all-*trans*-ß-carotene, α-carotene, ß-cryptoxanthin, lutein, lycopene, and zeaxanthin in each of the three levels. The serum in each vial is to be reconstituted with 1.00 mL of HPLC-quality water.

Certified Concentrations: The **updated** certified concentrations of retinol, α-tocopherol, and total ß-carotene (all-*trans* plus *cis* isomers) in reconstituted SRM 968a are provided in Table 1. The certified concentration values were derived by pooling results from analyses performed at NIST and 33 collaborating institutions over a two-year period between May 1991 and June 1993. An alphabetized listing of these laboratories is provided in Appendix A. A summary of values and measured uncertainties derived from round-robin analyses of SRM 968a by more than 50 laboratories involved in the NIST Fat-Soluble Vitamin Quality Assurance Program is provided in Appendix B. Values are in good agreement with the NIST values, and the uncertainties expressed represent the current state-of-the-practice for these analytes for experienced practitioners. The certified concentrations and information values apply to solutions obtained after the serum has been reconstituted using the procedure described in "Instructions for Use."

Information Values: Information values determined at NIST for γ-tocopherol and several additional carotenoid compounds have also been updated. These values and information values for cholesterol are listed in Table 2.

Expiration of Certification: When the SRM is stored properly, this certification is valid within the specified uncertainty limits for one year from the date of shipment. In the event that the certification becomes invalid prior to that time, users will be notified by NIST. Please return the attached registration form to facilitate notification.

The overall direction and coordination of the preparation and technical measurements leading to the certification of this SRM were performed by J. Brown Thomas, N.E. Craft, and W.E. May of the NIST Organic Analytical Research Division and M.C. Kline of the NIST Biotechnology Division.

Analytical measurements at NIST were performed by J. Brown Thomas, A. Cohen, N.E. Craft, K.E. Sharpless, and L.T. Sniegoski of the NIST Organic Analytical Research Division.

Statistical consultation was provided by S.B. Schiller, of the NIST Statistical Engineering Division and D.L. Duewer of the NIST Organic Analytical Research Division.

The technical and support aspects involved with the certification and issuance of this SRM were coordinated through the Division of Cancer Prevention and Control, National Cancer Institute, by W. Malone and through the NIST Standard Reference Materials Program by J.C. Colbert and T.E. Gills.

Gaithersburg, MD 20899
November 17, 1993
(Revision of certificate 5-14-92)

Thomas E. Gills, Acting Chief
Standard Reference Materials Program

(over)

NOTICE TO USERS

Storage: Until required for use, the freeze-dried serum should be stored in the dark at a temperature between -20 and -80 °C.

Warning: The supplier of this serum has reported testing the source materials used to prepare this product and found them to be non-reactive when tested for Hepatitis B Surface Antigen (HB_sAg) and for human immunodeficiency virus (HIV) by FDA-required tests. However, because no test method can offer complete assurance that HIV, Hepatitis (Revision of certificate dated 3-27-92) B virus, or other infectious agents are absent, these specimens should be handled at the Biosafety Level 2 as recommended in the Center for Disease Control/National Institutes of Health Manual for any potentially infectious human serum or blood specimen. [1]

Instructions for Use: SRM 968a is provided as a set of two vials of lyophilized serum for each of three concentration levels for the certified analytes and must be reconstituted prior to use. Before reconstitution, the vials should be allowed to stand at room temperature for at least 30 min, but no longer than 16 h. Since the vials were sealed under argon at slight positive pressure, it is recommended that the vials be vented by insertion of an empty syringe needle prior to opening. To achieve the certified concentrations, the analyst must reconstitute the freeze-dried serum with 1.00 mL of HPLC-quality water. Dissolution should be facilitated by ultrasonic agitation for 3 to 5 min or by intermittent swirling for at least 15 min. Vigorous shaking or mechanical swirling may cause the formation of foam and should be avoided. After reconstitution, the contents should be used immediately or stored at refrigerator temperature ($\sim$4 °C) and used within 24 h for the certified values to be valid. Precautions should be taken to avoid exposure to strong UV light and direct sunlight.

Preparation of SRM Serum Pools: Plasma used to prepare three 1.9-L pools of materials used as SRM 968a was obtained from Interstate Blood Bank, Inc., Memphis, TN. Several serum sub-pools were obtained from the blood of volunteers whose diets had been supplemented with β-carotene. At NIST, plasma was converted to serum by several freeze-thaw clotting processes, followed by filtration through a microfiber borosilicate glass membrane to remove fibrin. Once processed, the plasma-converted serum was clot-free and sub-pools were blended. This blending process yielded the desired levels of ß-carotene for all pools and of retinol for the low and medium pools. The desired levels of retinol in the high pool and α-tocopherol in all pools were achieved by supplementing the serum pool with an ethanolic solution (less than 0.2 volume percent) containing retinol and/or α-tocopherol. Following supplementation, the pools were stirred for 1 h, equilibrated for 18 h, and again stirred for 1 h. The three serum pools were filtered and dispensed as 1.00-mL aliquots into 3.5-mL serum vials. The samples were then lyophilized, sealed under slight positive pressure with argon, and stored at -80 °C.

Analytical Measurements for Value Assignment: The certified concentrations of retinol, α-tocopherol, and total ß-carotene for this SRM represent the pooled results from analyses performed at NIST and 33 collaborating institutions. The collaborating institutions selected to assist in the certification of SRM 968a were those laboratories that had participated in the NIST Fat-Soluble Vitamin QA program for at least three years. Most of the analyses involved precipitation of protein with ethanol followed by extraction of the supernatant with a lipophilic solvent (e.g., hexane or petroleum ether). The extracts were then analyzed by liquid chromatography using various stationary phase-mobile phase combinations, detectors, and internal standards. Since the maintenance of pure and stable primary reference compounds for retinol, α-tocopherol and β-carotene is difficult, detector responses were calibrated against solutions whose concentrations were determined spectrophotometrically based on the following absorptivities in absolute ethanol: 1850 $dL \cdot g^{-1} \cdot cm^{-1}$ at 324 nm for retinol; 75.8 $dL \cdot g^{-1} \cdot cm^{-1}$ at 292 nm for α-tocopherol; 2560 $dL \cdot g^{-1} \cdot cm^{-1}$ at 452 nm for β-carotene.

Cholesterol concentrations were determined using the NIST gas chromatography-isotope dilution mass spectrometry (GC-IDMS) definitive method.[2,3] Two different procedures were used at NIST for the determination of retinol, α-tocopherol, and total ß-carotene in this SRM. In both procedures, the serum was extracted by precipitating the protein with ethanol and extracting the supernatant with hexane. NIST procedure A was based on gradient elution of the extract from a 30 nm (300-Å) pore diameter, polymerically bonded C_{18} column using a methanol:butanol:acetic acid mobile phase.[4] During the initial value-assignment exercise, randomly selected vials from each of the three levels were analyzed in duplicate (extraction of two aliquots from each vial; one injection per aliquot) using this method. NIST procedure B involved a gradient analysis of the extracts on a polymerically bonded C_{18} column using a mobile phase of acetonitrile:methanol containing 0.05M ammonium acetate:ethyl acetate. Triethylamine (0.05%) is also added to each solvent as a modifier in this procedure.[5] Retinol and α-tocopherol

were monitored at 300 nm and β-carotene was monitored at 450 nm. Twelve stratified randomly selected vials from each level were analyzed in duplicate to determine the variability associated with fill weights and inhomogeneity throughout each lot. No statistically significant vial-to-vial variability was detected.

REFERENCES

[1] U.S. Department of Health and Human Services. "Biosafety in Microbiological and Biomedical Laboratories." U.S. Government Printing Office, Washington, D.C., 1988.

[2] A. Cohen, H.S. Hertz, J. Mandel, R.C. Paule, R. Schaffer, L.T. Sniegoski, T. Sun, M.J. Welch, E. White V, Total Serum Cholesterol by Isotope Dilution Mass Spectrometry: a Candidate Definitive Method. Clin. Chem. 26, (1980), 854-860.

[3] P. Ellerbe, S. Meiselman, L.T. Sniegoski, M.J. Welch, E. White V, Determination of Serum Cholesterol by a Modification of the Isotope Dilution Mass Spectrometric Definitive Method. Anal. Chem. 61, (1989), 1718-23.

[4] W.A. MacCrehan, E. Schönberger, Determination of Retinol, α-Tocopherol, and ß-Carotene in Serum by Liquid Chromatography with Absorbance and Electrochemical Detection. Clin. Chem. 33, (1987), 1585-92.

[5] K.S. Epler, N.E. Craft, R.G. Ziegler, Liquid Chromatography Method for the Determination of Carotenoids, Retinoids, and Tocopherols in Human Serum and in Food. J. Chromatogr. Biomed. Applic. 619, (1993), 37-48.

Table 1. Certified Concentrations of Retinol, α-Tocopherol, and Total ß-Carotene in Reconstituted SRM 968a.

	Low Level		Medium Level		High Level	
Analyte	μg/mL	μmol/L	μg/mL	μmol/L	μg/mL	μmol/L
Retinol	0.191 ± 0.014	0.667 ± 0.049	0.505 ± 0.024	1.763 ± 0.084	0.664 ± 0.036	2.318 ± 0.126
α-Tocopherol	4.57 ± 0.16	10.61 ± 0.37	10.49 ± 0.42	24.36 ± 0.98	15.93 ± 0.53	36.99 ± 1.23
Total ß-Carotene	0.246 ± 0.023	0.458 ± 0.043	0.883 ± 0.083	1.65 ± 0.16	2.25 ± 0.25	4.19 ± 0.46

The uncertainties listed represent a 95% confidence interval for the mean of the measured concentrations and include allowances for analytical imprecision and imprecision associated with the reconstitution process. The uncertainties do not include possible unrecognized bias due to the extraction process and the purity assessment of primary reference compounds.

Table 2. Information values for cholesterol, γ-tocopherol, all-*trans*-β-carotene, α-carotene, ß-cryptoxanthin, lutein, all-*trans*-lycopene, total lycopene, and zeaxanthin in SRM 968a are provided below for information only. The values are derived from a limited number of analyses using methods developed and employed at NIST.

	Low Level		Medium Level		High Level	
Analyte	μg/mL	μmol/L	μg/mL	μmol/L	μg/mL	μmol/L
cholesterol[a]	820	2121	1733	4482	1662	4299
γ-tocopherol	0.9	2.1	2.9	6.9	3.7	8.8
all-*trans*-β-carotene[b]	0.2	0.37	0.8	1.5	2.0	3.7
α-carotene	0.02	0.037	0.06	0.111	0.1	0.19
ß-cryptoxanthin	0.02	0.036	0.05	0.090	0.04	0.07
lutein	0.04	0.072	0.09	0.158	0.08	0.141
all-*trans*-lycopene	0.07	0.13	0.2	0.37	0.2	0.37
total lycopene	0.2	0.37	0.5	0.93	0.4	0.75
zeaxanthin	0.02	0.035	0.04	0.070	0.03	0.053

[a] Performed on two vials in duplicate using GC-IDMS.

[b] Most commercially available HPLC columns do not separate all-*trans* β-carotene from its *cis* isomers. Two different columns that are capable of separating all-*trans*-β-carotene from its *cis* isomers were used at NIST to provide data for all-*trans*-β-carotene.

Appendix A

The investigators listed below performed measurements that contributed to the certification of SRM 968a.

Name	Analytes Measured
Dr. David S. Alberts Arizona Cancer Center Tucson, AZ	Retinol, α-Tocopherol, β-Carotene
Dr. Nancy W. Alcock University of Texas Medical Branch Galveston, TX	Retinol, α-Tocopherol, β-Carotene
Dr. Gary R. Beecher United States Department of Agriculture Beltsville, MD	Retinol, α-Tocopherol, β-Carotene
Dr. Phyllis E. Bowen University of Illinois Health Science Center Chicago, IL	Retinol, α-Tocopherol
Dr. Peter P. Chou American Medical Laboratories Chantilly, VA	Retinol, α-Tocopherol, β-Carotene
Dr. M. R. Clemens Universität Tubingen Medizinische Klinik Tubingen, Germany	Retinol, α-Tocopherol, β-Carotene
Ms. Liliana I. Clement Wilmer Eye Institute Baltimore, MD	Retinol, α-Tocopherol
Ms. Cari Countryman Fred Hutchinson Cancer Research Center Seattle, WA	Retinol, α-Tocopherol, β-Carotene
Dr. Nikolay Dimitrov Michigan State Department of Medicine East Lansing, MI	α-Tocopherol, β-Carotene
Dr. Alison Dinwoodie University of Alberta Hospitals Edmonton, Alberta	Retinol, α-Tocopherol
Dr. Show-Hong Duh University of Maryland Medical System Baltimore, MD	α-Tocopherol
Dr. Herbert A. Fritsche M.D. Anderson Cancer Institute Houston, TX	Retinol, α-Tocopherol, β-Carotene
Dr. Yu-Tang Gao Shanghai Cancer Institute Shanghai	Retinol, α-Tocopherol, β-Carotene

Mr. Carl-Gustaf Gref National Public Health Institute Helsinki, Finland	Retinol, α-Tocopherol, β-Carotene
Dr. Myron Gross University of Minnesota Division of Epidemiology Minneapolis, MN	Retinol, α-Tocopherol, β-Carotene
Dr. Jukka Marniemi Rehabilitation Research Centre of the Social Insurance Institution Turku, Finland	Retinol, α-Tocopherol, β-Carotene
Dr. Susan T. Mayne Yale University School of Medicine New Haven, CT	Retinol, α-Tocopherol, β-Carotene
Dr. Ralph W. McKee UCLA School of Public Health Los Angeles, CA	Retinol, α-Tocopherol
Dr. Jerry McLarty University of Texas Health Center Tyler, TX	Retinol, α-Tocopherol, β-Carotene
Ms. Judy Miller University of Cincinnati Medical Center Cincinnati, OH	Retinol, α-Tocopherol, β-Carotene
Dr. David Nierenberg Dartmouth-Hitchcock Medical Center Lebanon, NH	Retinol, α-Tocopherol, β-Carotene
Ms. Iris Osberg University of Colorado Health Sciences Center Denver, CO	Retinol, α-Tocopherol, β-Carotene
Ms. Paula Radmacher University of Louisville Neonatal Research Center Louisville, KY	Retinol, α-Tocopherol
Dr. Eugene J. Rogers University of Lowell Lowell, MA	Retinol, α-Tocopherol, β-Carotene
Dr. Willy Schüep F. Hoffmann-La Roche & Co. Basel, Switzerland	Retinol, α-Tocopherol, β-Carotene
Dr. Morton K. Schwartz Memorial Sloan-Kettering Cancer Center New York, NY	Retinol, α-Tocopherol
Dr. Anne Sowell Centers for Disease Control Atlanta, GA	Retinol, α-Tocopherol, β-Carotene

Dr. Yu-Hai Sun Cancer Institute, CAMS Panjia-Yao, Beijing	Retinol, α-Tocopherol, β-Carotene
Professor Tsuguyoshi Suzuki University of Tokyo Tokyo, Japan	α-Tocopherol, β-Carotene
Dr. Charles A. Thomas, Jr. Helicon Foundation San Diego, CA	Retinol, α-Tocopherol, β-Carotene
Dr. David I. Thurnham University of Ulster at Coleraine Coleraine, Northern Ireland	Retinol, α-Tocopherol, β-Carotene
Dr. Govind T. Vatassery Veterans Administration Hospital South Minneapolis, MN	α-Tocopherol
Ms. Megan Veldee University of Washington Seattle, WA	Retinol, α-Tocopherol

Appendix B

SRM 968a was distributed to 53 laboratories around the world for analysis as part of a Fat-Soluble Vitamin in Serum Measurement Quality Assurance program organized and coordinated by NIST and sponsored by the National Cancer Institute, Division of Cancer Prevention and Control. The values provided below represent a summary of data (excluding outliers) submitted by those laboratories. The low level SRM was distributed once in a round robin study since the original certification of SRM 968a; the medium and high levels were distributed twice.

Interlaboratory Determination of Retinol, α-Tocopherol, γ-Tocopherol, and Total ß-Carotene in SRM 968a

	Low Level		Medium Level		High Level	
Analyte	μg/mL	μmol/L	μg/mL	μmol/L	μg/mL	μmol/L
Retinol	0.20 ± 0.04[a]	0.70 ± 0.13	0.506 ± 0.039	1.77 ± 0.14	0.673 ± 0.046	2.35 ± 0.16
α-Tocopherol	4.85 ± 0.69	11.26 ± 1.60	10.6 ± 0.8	24.5 ± 1.7	15.8 ± 1.46	36.6 ± 3.4
γ-Tocopherol	0.83 ± 0.07	1.99 ± 0.17	2.84 ± 0.31	6.82 ± 0.74	3.72 ± 0.23	8.93 ± 0.55
Total ß-Carotene	0.26 ± 0.03	0.48 ± 0.05	0.89 ± 0.11	1.66 ± 0.20	2.28 ± 0.53	4.25 ± 0.99

[a] Uncertainties represent one standard deviation of the reported laboratory means. This standard deviation incorporates both within-and between-laboratory measurement imprecision.