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ORIGINAL PAPER

Preparation and value assignment of standard reference material 968e fat-soluble vitamins, carotenoids, and cholesterol in human serum

Jeanice B. Thomas · David L. Duewer · Isaac O. Mugenya · Karen W. Phinney · Lane C. Sander · Katherine E. Sharpless · Lorna T. Sniegoski · Susan S. Tai · Michael J. Welch · James H. Yen

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Abstract Standard Reference Material 968e Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum provides certified values for total retinol, γ- and α-tocopherol, total lutein, total zeaxanthin, total β-cryptoxanthin, total β-carotene, 25-hydroxyvitamin D_3, and cholesterol. Reference and information values are also reported for nine additional compounds including total α-cryptoxanthin, trans- and total lycopene, total α-carotene, *trans*-β-carotene, and coenzyme Q_{10}. The certified values for the fat-soluble vitamins and carotenoids in SRM 968e were based on the agreement of results from the means of two liquid chromatographic methods used at the National Institute of Standards and Technology (NIST) and from the median of results of an interlaboratory comparison exercise among institutions that participate in the NIST Micronutrients Measurement Quality Assurance Program. The assigned values for cholesterol and 25-hydroxyvitamin D_3 in the SRM are the means of results obtained using the NIST reference method based upon gas chromatography-isotope dilution mass spectrometry and liquid chromatography-isotope dilution tandem mass spectrometry, respectively. SRM 968e is currently one of two available health-related NIST reference materials with concentration values assigned for selected fat-soluble vitamins, carotenoids, and cholesterol in human serum matrix. This SRM is used extensively by laboratories worldwide primarily to validate methods for determining these analytes in human serum and plasma and for assigning values to in-house control materials. The value assignment of the analytes in this SRM will help support measurement accuracy and traceability for laboratories performing health-related measurements in the clinical and nutritional communities.

Keywords Standard reference material · Fat-soluble vitamins · Carotenoids · Frozen human serum · Cholesterol · Value assignment

J. B. Thomas (✉) · D. L. Duewer · K. W. Phinney · L. C. Sander · K. E. Sharpless · L. T. Sniegoski · S. S. Tai · M. J. Welch
Analytical Chemistry Division,
National Institute of Standards and Technology,
100 Bureau Drive, Stop 8392,
Gaithersburg, MD 20899-8392, USA
e-mail: jbthomas@nist.gov

J. H. Yen
Statistical Engineering Division,
National Institute of Standards and Technology,
100 Bureau Drive,
Gaithersburg, MD 20899, USA

I. O. Mugenya
Testing Services Department,
Food and Agriculture Laboratories, Kenya Bureau of Standards,
Nairobi, Kenya

Introduction

In 1989 the National Institute of Standards and Technology (NIST) developed Standard Reference Material (SRM) 968 Fat-Soluble Vitamins in Human Serum [1] to help address the need for well-characterized performance standards and health-related reference materials for use by the clinical and epidemiological communities. Due to the popularity and increased usefulness of SRM 968, it has been re-issued five times as SRM 968a, SRM 968b, SRM 968c, SRM 968d, and as the newly developed material, SRM 968e Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum.

Released in the fall of 2010, SRM 968e consists of three concentration levels of vitamins in human serum that have been characterized for eighteen constituents. This SRM is intended for use in validating methods for determining total retinol, γ- and α-tocopherol, total lutein, total zeaxanthin, total β-cryptoxanthin, total β-carotene, 25-hydroxyvitamin D_3, and cholesterol. Values are also assigned for nine additional analytes including *trans*- and total lycopene, total α-carotene, and *trans*- β-carotene. Along with method validation, SRM 968e can also be used for quality assurance when assigning values to in-house control materials.

Assigned values for retinol, tocopherols, and carotenoids in SRM 968e were based on the agreement of results from two different liquid chromatographic (LC) methods used at NIST and the LC methods employed by collaborating institutions that participated in an interlaboratory comparison study as part of the NIST Micronutrients Measurement Quality Assurance (QA) Program [2]. One of the modes traditionally used by NIST for characterization of certified reference materials employs two or more chemically independent, critically evaluated analytical techniques [3]. The results from these techniques, if in agreement, are used to assign values to the measured constituents. This approach is based on the assumption that combined results from independent techniques with potentially different sources of errors or biases provide a good estimation of the "true value." Other NIST value-assignment modes use two or more sets of independent measurements made through a collaborative effort between NIST and outside laboratories, or value assignment (of reference or information values) may be based solely on the use of data obtained in a well-defined interlaboratory study. Values for retinol, tocopherols, and carotenoids in SRM 968e were assigned using one of these three modes.

Another certification mode involves the use of a 'definitive method.' As in the previous five issues of this SRM [1, 4], value assignment of cholesterol in this SRM is based on the mean results obtained by using the NIST reference method for cholesterol based upon gas chromatography-isotope dilution mass spectrometry (GC-IDMS) [5, 6]. Assigned values for 25-hydroxyvitamin D_3 in SRM 968e using isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) is described by Tai et al. [7].

In this paper the preparation of SRM 968e and the analytical procedures used at NIST for the characterization of SRM 968e are described. Also described is the value assignment of the concentrations of the above compounds, including a comparison of NIST results with results from the interlaboratory comparison exercise and a comparison of this reference material with previous issues.

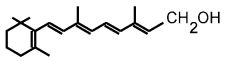
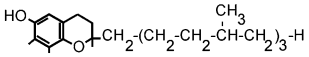
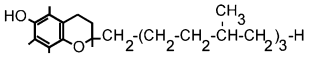
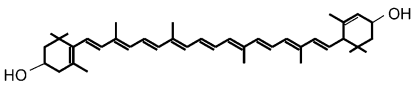
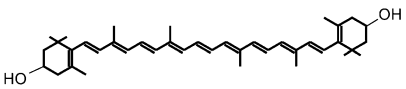
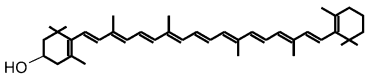
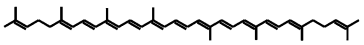
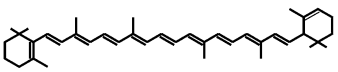
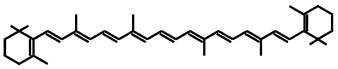
Materials and methods[1]

Reagents/Standards Stock solutions of retinol (CAS 68-26-8), δ-tocopherol (CAS 119-13-1), α-tocopherol (CAS 59-02-9), γ-tocopherol (CAS 7616-22-0), lutein (CAS 127-40-2), zeaxanthin (CAS 144-68-3), β-cryptoxanthin (CAS 472-70-8), *trans*-α-carotene (CAS 7488-99-5), *trans*-β-carotene (CAS 7235-40-7), and *trans*-lycopene (CAS 502-65-8) were prepared by dissolving each compound in absolute ethanol that contained 30 mg/L butylated hydroxytoluene (BHT; added to prevent analyte oxidation). All compounds were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tocol (25 μg/mL in ethanol containing 30 μg/mL BHT), from Eisai Co., Ltd. (Tokyo, Japan), was used as the internal standard. *Trans*-β-apo-10′-carotenal, from Hoffman-LaRoche (Nutley, NJ, USA), was used for the synthesis of the internal standard *trans*-β-apo-10′-carotenal oxime (0.05 μg/mL in ethanol containing 30 μg/mL BHT) used for the quantification of retinol and the carotenoids in one of the LC methods used at NIST [8, 9]. HPLC-grade solvents were used without further purification. The 25-hydroxyvitamin D_3 (as monohydrate) reference compound was obtained from the United States Pharmacopeia (USP, Rockville, MD, USA). The 25-hydroxyvitamin D_2 reference compound and the isotopically labeled 25-hydroxyvitamin D_3-d_3 were obtained from IsoSciences (King of Prussia, PA, USA). Labeled cholesterol-$^{13}C_3$, the internal standard for the measurement of cholesterol in SRM 968e, was obtained from Isotec (Miamisburg, OH, USA).

Calibration and quality control Because the maintenance of pure and stable primary reference compounds for retinol, the tocopherols, and the carotenoids is difficult, the concentrations of these analytes in stock solutions were determined spectrophotometrically using Beer's Law [10], and calibration standards were prepared by diluting the stock solutions. Concentrations were corrected for analyte purity as determined by LC. NIST concentration calculations for the fat-soluble vitamins and carotenoids were based on the absorptivities and wavelengths provided in Fig. 1 [11–16]. All measurements are traceable to the amount-of-substance units of SI based on the extinction coefficients, masses of measurands, purity assessments, and appropriate uncertainties provided herein. Compounds of the following purities (mass fractions) were used in this study: *trans*-retinol (96%), δ-tocopherol (97%), γ-tocopherol (>99%), α-tocopherol (96%), lutein (91%), zeaxanthin (98%), β-cryptoxanthin (96%), *trans*-α-caro-

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

Fig. 1 Wavelength maxima and absorptivities used for calibration at NIST [11–16]

COMPOUND	STRUCTURE	λ_{max}	ABSORPTIVITY
trans-retinol		325 nm	1843 dL/g·cm in ethanol
γ-tocopherol		298 nm	91.4 dL/g·cm in ethanol
α-tocopherol		292 nm	75.8 dL/g·cm in ethanol
trans-lutein		445 nm	2550 dL/g·cm in ethanol
trans-zeaxanthin		452 nm	2540 dL/g·cm in ethanol
trans-β-cryptoxanthin		452 nm	2356 dL/g·cm in ethanol
trans-lycopene		472 nm	3450 dL/g·cm in hexane
trans-α-carotene		444 nm	2800 dL/g·cm in hexane
trans-β-carotene		452 nm	2592 dL/g·cm in hexane

tene (96%), *trans*-β-carotene (90%), and *trans*-lycopene (83%). Standard uncertainties for the purity measurements represent the standard deviation of the mean values and were less than one percent for all compounds. A three-point calibration curve for the above analytes using the ratio of analyte-to-internal-standard peak areas was constructed by employing calibration standards that encompassed a known low, middle, and high level of each analyte relative to the physiological levels expected in human serum. SRMs 968c and 968d were used for quality assurance at NIST for the measurement of the selected vitamins and carotenoids.

For the determination of 25-hydroxyvitamin D_3 in SRM 968e, six calibrants with mass ratios of unlabeled to labeled 25-hydroxyvitamin D_3 were gravimetrically prepared; SRM 972 (Level 1) Vitamin D in Human Serum was used for quality control [7, 17]. SRM 911c Cholesterol was used as the primary calibration standard (certified to be 99.2%± 0.4% pure) for the determination of cholesterol in SRM 968e; SRM 1950 Metabolites in Human Plasma was used for quality assurance [18, 19]. Traceability to amount-of-substance units of the SI is based on masses of cholesterol and the 25-hydroxyvitamin reference compound, purity assessments, and appropriate uncertainties.

Preparation of serum pools for SRM 968e Three concentration levels of SRM 968e were prepared from source plasma units (3.6 L per level) obtained from Interstate Blood Bank, Inc. (Memphis, TN, and Chicago, IL, USA). All units were tested and found non-reactive for hepatitis B surface antigen (HBsAg), human immunodeficiency virus (HIV), hepatitis C virus (HCV), and human immunodeficiency virus 1 antigen (HIV-1Ag) prior to shipment to NIST. Over 100 units were screened to identify appropriate candidates for inclusion in SRM 968e to obtain target levels of retinol, γ- and α-tocopherol, and β-carotene. Target values were based on the relative physiological levels of these analytes in human serum. Table 1 summarizes the target values, the measurement results for the individual units and their volume-weighted averages, and the resulting certified values for the pools used to produce the three SRM 968e levels. The plasma was shipped by NIST to Solomon Park Research Laboratories (Kirkland, WA, USA), where it was frozen at −80°C, thawed, and filtered through Whatman 541 filter paper twice to convert it to serum. NIST provided Solomon Park with the blending protocols to reach the desired target levels. Unlike previous issues, no ethanolic solutions containing retinol

Table 1 Blending protocol used for SRM 968e

	Pool for Level 1 [a]					Pool for Level 2 [a]					Pool for Level 3 [a]				
	Vol	TR	αT	γ/βT	TβC	mL	TR	αT	γ/βT	TβC	mL	TR	αT	γ/βT	TβC
Target [b]	4000	0.30	7.0	1.5	0.09	4000	0.50	12	2.0	0.2	4000	0.90	18	4.0	0.40
Unit 1 [c]	824	0.43	4.4	2.1	0.06	690	0.48	9.9	1.9	0.23	880	0.50	9.1	2.3	0.32
Unit 2 [c]	880	0.27	7.2	1.3	0.07	725	0.47	7.3	1.4	0.35	824	0.82	27.9	2.6	0.35
Unit 3 [c]	880	0.29	5.2	1.8	0.06	824	0.48	8.1	2.3	0.12	825	0.93	26.4	1.8	0.42
Unit 4 [c]	880	0.35	4.9	0.9	0.14	824	0.53	18.7	2.0	0.23	824	0.93	24.5	2.6	0.33
Unit 5 [c]	800	0.37	6.7	2.8	0.08	880	0.56	11.1	2.1	0.04	822	0.73	20.1	2.6	0.43
Calculated [d]	4264	0.35	5.7	1.8	0.08	3943	0.50	11.2	2.0	0.19	4175	0.78	21.4	2.4	0.37
Certified [e]		0.34	6.5	1.9	0.10		0.48	15.2	1.4	0.23		0.65	19.4	2.3	0.41

[a] "Vol" is the nominal volume of plasma in mL, "TR" is Total Retinol in μg/mL, "αT" is α-Tocopherol in μg/mL, "γ/βT" is γ/β-Tocopherol in μg/mL, and "TβC" is Total β-Carotene in μg/mL

[b] The targeted quantity values

[c] The nominal volume of plasma indicated on each unit and the NIST-determined levels for the four target analytes

[d] The calculated total volume and volume-weighted average analyte levels in each pool

[e] The certified values for the four target analytes in the SRM 968e materials produced from each pool.

or tocopherols were spiked into SRM 968e. The serum was pooled, blended, bottled in 1-mL aliquots, and stored at −80°C prior to shipment back to NIST. Prior to shipment to NIST, Solomon Park analyzed four test portions from 15 random samples of each level for cholesterol to assess homogeneity. Vials for certification analysis were selected using a stratified random sampling scheme that enabled examination of production- and fill-order effects.

Preparation of serum extract for LC analysis The sample preparation protocol used by NIST (and most of the collaborating laboratories) for the extraction of the fat-soluble vitamins and carotenoids from the SRM involved protein precipitation followed by an organic solvent extraction. This extraction approach provides an extract suitable for reversed-phase LC analysis. Prior to extraction, the serum samples were equilibrated to room temperature. For the NIST analyses, samples were placed in an ultrasonic water bath for at least 3 min and vortex mixed for 10 s. Aliquots of serum (200 μL) were pipetted into amber glass tubes and were combined with an equal volume of ethanol containing the internal standard(s) and BHT (30 mg/L). The samples were vortex-mixed for 15 s to precipitate proteins. One milliliter of hexane was added, and the samples were vortex-mixed for at least 45 s. The samples were then centrifuged at 263 rad/s (2500 rpm) for about 1 min, and supernatants were removed and placed in glass vials. This extraction process was repeated, and the supernatants were removed and combined with those of the first extraction. The combined extracts were evaporated under a stream of nitrogen under subdued fluorescent light to minimize possible degradation of the analytes, and were reconstituted in either 100 μL ethanol or 50/50 (volume fraction) ethyl acetate/ethanol containing 30 mg/L BHT, depending on the LC procedure used. The reconstituted extracts were then vortex-mixed and ultrasonically agitated for approximately 30 s to ensure dissolution and placed in autosampler vials for LC analysis with absorbance detection.

Description of analytical methods used for value assignment of selected fat-soluble vitamins and carotenoids in SRM 968e The assigned values for selected fat-soluble vitamins and carotenoids in SRM 968e were derived from results of analyses performed by NIST and 31 collaborating institutions (listed in Table 2). Two different LC techniques were used at NIST for the determination of the fat-soluble vitamins and carotenoids in the SRM: 1) a polymeric C_{18} column with UV/visible absorbance detection and 2) a C_{18} column with different selectivity and absorbance detection [4, 20–23]. Details of the two LC methods are provided below. The composition of the solvent mixtures described in these methods is volume fraction expressed as percent [24]. Chromatograms from the analysis of SRM 968e using the two NIST methods are provided in Fig. 2.

LC method 1: Determination of retinol, tocopherols, and carotenoids using a polymeric C_{18} stationary phase Retinol, selected tocopherols, and carotenoids were measured in two extracts from each of 11 vials of each level of SRM 968e on 1 day using a 5-μm polymeric C_{18} column (4.6× 250 mm; Vydac 201TP; Separations Group, Hesperia, CA,

Table 2 Analysts at the institutions listed below performed measurements that contributed to the value assignment of constituents in SRM 968e

ARUP Laboratories, Salt Lake City, UT, USA
Bio-Reference Laboratories, Elmwood Park, NJ, USA
Cancer Research Center of Hawaii, University of Hawaii at Manoa, Honolulu, HI, USA
Centers for Disease Control and Prevention, Atlanta, GA, USA
Centro Nacional de Alimentación-CENAN, Instituto Nacional de Salud, Lima. Peru
Biochemical Genetics Laboratory, Duke University, Research Triangle Park, NC, USA
Biochemical Genetics Laboratory, Mayo Clinic, Rochester, MN, USA
Biochemical Genetics Laboratory, University of Pittsburgh Medical Center, Pittsburgh, PA, USA
Children's Hospital and Regional Medical Center, Seattle, WA, USA
Children's Hospital National Medical Center, Washington, DC, USA
Départment de Biologie Intégree, Grenoble, France
Department of Human Nutrition, University of Stellenbosch, Tygerberg Campus, Tygerberg, South Africa
Department of Laboratory Medicine and Pathology, University of Alberta Hospital, Alberta, Canada
Department of Nutrition, Harvard School of Public Health, Boston, MA, USA
Division of Nutritional Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, USA
Fred Hutchinson Cancer Research Center, Seattle, WA, USA
Global Central Laboratory, Highland Heights, KY, USA
Harborview Medical Center, University of Washington, Seattle, WA, USA
Human Nutrition Unit, National Institute for Food and Nutritional Research, Rome, Italy
International Centre for Diarrhoeal Diseases Research, Dhaka, Bangladesh
Kronos Science Laboratory, Phoenix, AZ, USA
Laboratoire de Biochimie, Hôpital Purpan, Toulouse, France
MetaMetrix Medical Laboratory, Duluth, GA, USA
MRC Laboratory for Human Nutrition Research, Cambridge, England
Neonatal Nutrition Research Laboratory, University of Louisville, Louisville, KY, USA
Nutrition Research Laboratory, University of California at San Diego, La Jolla, CA, USA
Pediatric CTRC CORE Laboratory, University of Colorado Health Sciences Center, Denver, CO, USA
Quest Diagnostics, Inc., Chantilly, VA, USA
R&D Analytical Research Center, DSM Nutritional Products, Ltd., Kaiseraugst, Switzerland
Rowett Research Institute, Aberdeen, Scotland
Servicio de Bioquímica Clínica, Hospital Universitario Puerta de Hierro, Madrid, Spain

USA). A ternary solvent mixture was used in this method. Solvent A was 60% methanol/10% butanol/30% water. Solvent B was 89.5% methanol/10% butanol/0.5% water. All solvent compositions are provided as volume fractions. An initial 5 min isocratic hold of 75% solvent A followed by a 40 min linear gradient from 75% solvent A to 90% solvent B with a flow rate of 1.5 mL/min was used to isolate the analytes in the SRM. UV/visible absorbance detection using a deuterium lamp at the following wavelengths was used: 325 nm for retinol, 292 nm for the tocopherols, and 450 nm for the carotenoids. Tocol (the internal standard) was monitored at 292 nm. This method was also used to assess the homogeneity of the three levels, described below.

LC method 2: Determination of retinol, tocopherols, and carotenoids using a C_{18} stationary phase with different selectivity Retinol, selected tocopherols, and carotenoids were measured in two extracts from each of three vials of each level of the SRM using a Bakerbond C_{18} column (4.6 mm x 250 mm; J.T.Baker, Phillipsburg, NJ, USA). This column exhibits selectivity intermediate to monomeric and polymeric C_{18} columns [23]. A ternary solvent method was used to separate the analytes: solvent A was acetonitrile, solvent B was methanol containing 0.05 mol/L ammonium acetate, and solvent C was ethyl acetate. Each of the three solvents contained a volume fraction of 0.05% triethylamine (TEA) to enhance carotenoid recovery [21]. The method consisted of two linear gradients and an isocratic component with flow rates of 1.5 mL/min. The first gradient ran from 92% solvent A/8% solvent B to 75% solvent A/18% solvent B/7% solvent C in 10 min. A second linear gradient ran from this composition to 68% solvent A/25% solvent B/7% solvent C in 5 min. The final composition was held for 15 min longer, then the system was returned to initial conditions of 92% solvent A/8% solvent B over 5 min and re-equilibrated for 5 min. A programmable UV/visible absorbance detector with a deuterium lamp was used for measurement of retinol, the tocopherols, and the carotenoids at 325 nm, 292 nm and 450 nm, respectively. Two separate sets of analyses for the determination of retinol and the tocopherols and for the determination of the carotenoids in the SRM were performed. *Trans*-β-apo-10′-carotenal oxime was used as the internal standard for the quantification of the carotenoids; tocol was used as the internal standard for retinol and the tocopherols.

Methods used by collaborating laboratories for the analysis of SRM 968e Retinol, retinyl palmitate, tocopherols, carotenoids, vitamin K_1, 25-hydroxyvitamin D, and coenzyme Q_{10} in SRM 968e were measured by collaborating institutions (listed in Table 2) that participated in an interlaboratory comparison exercise in which blind samples of the SRM were distributed as part of the NIST Micronutrients Measurement Quality Assurance Program [2].

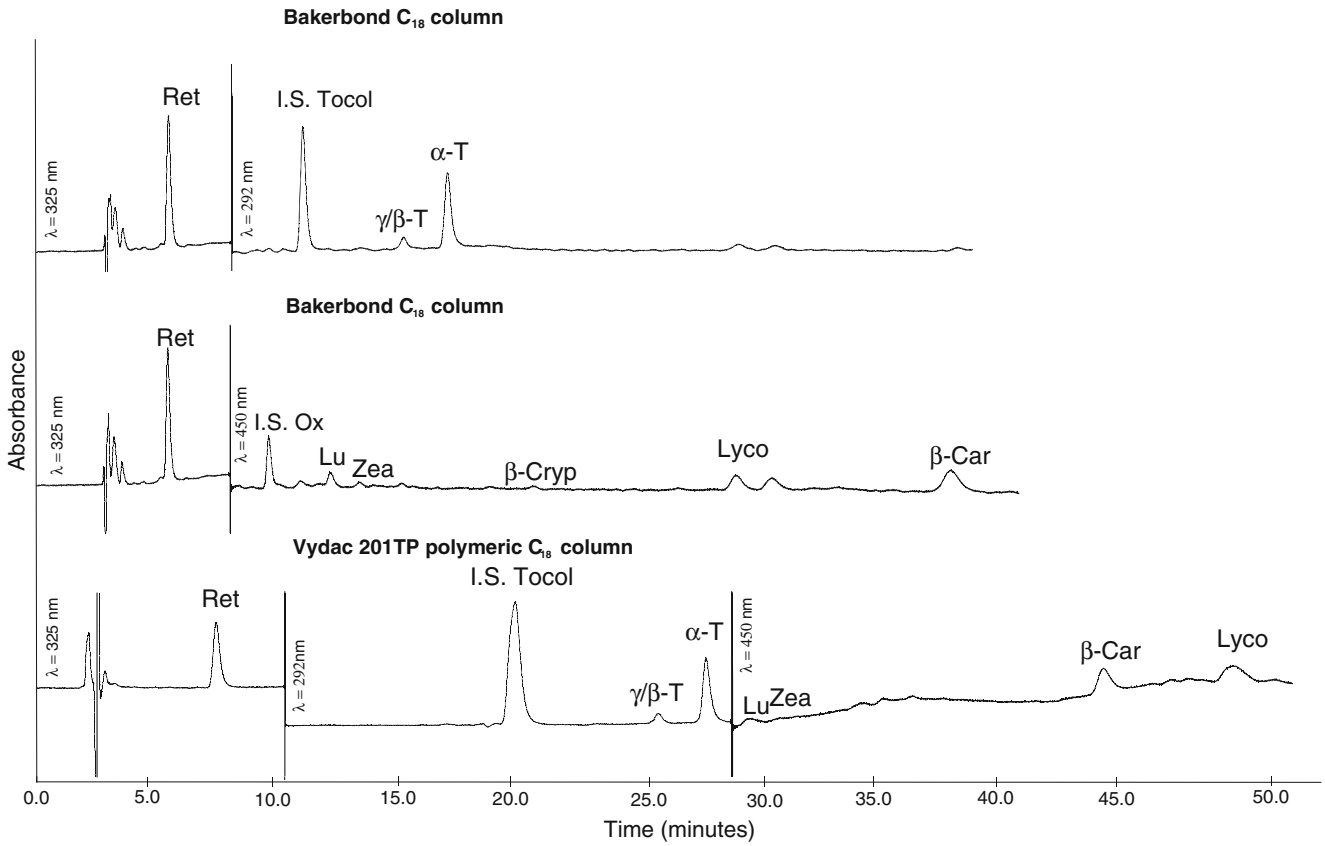


Fig. 2 Chromatograms from the analyses of Level 3 of SRM 968e using two different LC methods performed at NIST. Chromatographic conditions are described in the text

Analyses typically involved precipitation of serum proteins with ethanol followed by extraction of the supernatant with a lipophilic solvent (e.g., hexane or petroleum ether). The extracts were then analyzed by LC using various stationary phase and mobile phase combinations, detectors, and internal standards.

Measurement of 25-hydroxyvitamin D_3 in SRM 968e 25-Hydroxyvitamin D_3 concentrations were determined using the NIST LC-IDMS/MS reference measurement procedure (RMP) [7]. A total of three sets of samples, each set consisting of three to four samples for each of the three levels of SRM 968e, were analyzed. Each sample (2 g from combined contents of two vials) was analyzed using the RMP, which employs equilibration at room temperature for 1 h with an isotopically labeled internal standard 25-hydroxyvitamin D_3-d_3, followed by alkalinization with pH 9.8 carbonate buffer and extraction with hexane-ethyl acetate (50:50, volume fraction) prior to reversed-phase LC-MS/MS. Atmospheric pressure chemical ionization in the positive ion mode and multiple reaction monitoring (MRM) were used, The transitions at m/z 401→m/z 383 and m/z 404→m/z 386 for 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_3-d_3, respectively, were monitored. Duplicate injections of each sample and each standard were made in each set. Instrumental response was determined from a linear regression fit of the calibration data using a $y = mx + b$ regression model.

During preliminary measurements, a small amount of 25(OH)D_2 was detected, and an attempt was made to measure the concentrations of 25-hydroxyvitamin D_2 in SRM 968e using the previously described LC-IDMS/MS RMP. The limit of quantitation for this method at a signal-to-noise ratio of≈10 is approximately 0.5 ng/g. The concentrations of 25-hydroxyvitamin D_2 in SRM 968e estimated in this experiment were below 0.5 ng/g for all three levels, and therefore were not measured.

Measurement of cholesterol in SRM 968e Cholesterol concentrations were determined using the NIST GC-IDMS reference method [5, 6]. Three sets of samples, each consisting of two vials from each level of the SRM, were analyzed. Two aliquots from each vial were analyzed using a previously established procedure that employs hydrolysis of cholesterol esters using potassium hydroxide in ethanol, followed by extraction with hexane, and derivatization of cholesterol using

bis(trimethylsilyl)acetamide [6]. Cholesterol-25,26,27-$^{13}C_3$ was used as the internal standard and was added prior to hydrolysis. Duplicate injections of each sample and each standard were made in each set. Quantitation of cholesterol was achieved by the use of a standard curve obtained by measurement of standards of weighed mixtures of SRM 911c Cholesterol and cholesterol-25,26,27-$^{13}C_3$.

Homogeneity assessment The homogeneity of retinol, selected tocopherols, and carotenoids was assessed at NIST by using the reversed-phase polymeric C_{18} LC method described above. Two aliquots (200 μL each) from each of 11 different vials from the 3 levels of SRM 968e were extracted and analyzed. All 11 samples from each level of the SRM were prepared and analyzed on the same day to determine the variability associated with inhomogeneity throughout each level. Level 2 of SRM 968c Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum was prepared using the above extraction protocol and analyzed for quality assurance. An analysis of variance did not show inhomogeneity for the test portions analyzed. All measurands were treated as though they were homogeneously distributed, although homogeneity of all measurands was not assessed.

Stability of fat-soluble vitamins and carotenoids in SRM 968e

The stability of selected fat-soluble vitamins and carotenoids in SRM 968e has been monitored at NIST since the material was prepared (spring of 2009). Based on the results obtained from three interlaboratory comparison exercises (fall of 2009, spring and fall of 2010) in which blind samples of SRM 968e were distributed to participants in the NIST Micronutrients Measurement QA Program (MMQAP), all of the constituents that were value-assigned in the SRM have remained stable. Many of the MMQAP participants reported results in all three of these studies: no systematic differences from the certified values have been observed for any of these participants for any analyte. This indicates that the SRM 968e materials are commutable across the methods used by these laboratories.

Data from a 10-year stability study of fat-soluble vitamins and carotenoids in human serum at NIST [25] demonstrated that retinol and α-tocopherol concentration levels in frozen human serum samples stored in the dark at −80°C were stable for at least 5 years. β-Carotene levels in frozen serum stored at −80°C were stable for at least 3 years. Beginning in 2000, sixteen MMQAP studies have involved pairs of lyophilized and liquid frozen samples prepared from the same serum pool. No systematic changes in the consensus level or variability have been observed for any analyte in either matrix. Further, other than expected differences in concentration attributable to reconstitution volume, there have been no participant-specific between-matrix differences in the results for any analyte.

SRM 968e should be stored in the dark at or between −20°C and −80°C until required for use. The material should be stored at or below −70°C in the dark if carotenoids are to be measured since carotenoids appear to be less stable than the retinoids and tocopherols at −20°C [26–28]. The expiration date for this material is provided on the Certificate of Analysis for the SRM as April 30, 2020 and represents a conservative estimate of the period for which the material is stable [29]. Stability will be monitored over this period of certification. If changes are observed that affect the certification, NIST will notify the purchaser. Currently, there is no available data for the stability of cholesterol and 25-hydroxyvitamin D_3 in SRM 968e. However, cholesterol and 25-hydroxyvitamin D concentration levels have been monitored in similar serum-based SRMs (SRM 1951 Lipids in Frozen Human Serum for cholesterol and SRM 972 Vitamin D in Human Serum for 25-hydroxyvitamin D) over time [30, 31]. These measurands have been found to be stable for at least 2 years in these materials.

Results and discussion

Based on current practices at NIST for value assigning SRMs and reference materials, three data quality descriptors are used: certified values, reference values, and information values [3]. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias in the measurements have been fully investigated or taken into account. NIST reference values are noncertified values that are the best estimate of the true values based on available data. 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. A NIST information value is considered to be a value that will be of interest to the SRM user, but insufficient information is available to adequately assess the uncertainty associated with the value.

Table 3 summarizes the results for the analysis of selected fat-soluble vitamins and carotenoids in SRM 968e from the different analytical procedures used at NIST and the results obtained by the collaborating laboratories that participated in the interlaboratory comparison study. Results were in good agreement for most analytes. The assigned values for the analytes in the three levels of SRM 968e are provided in Tables 4, 5 and 6. The sets of results

Table 3 Summary of results from the analyses of SRM 968e fat-soluble vitamins, carotenoids, and cholesterol in human serum performed by NIST and by collaborating institutions that participated in an interlaboratory comparison study. Mass concentrations (μg/mL) are the mean results from the number of reported data (indicated as n). The uncertainties, shown in parentheses, are expressed as one standard deviation of the mean

Analyte	NIST LC-UV Methods		NIST LC-IDMS/MS	Interlaboratory Comparison	
	Polymeric C_{18}, n=11	C_{18} with Different Selectivity, n=6	n=10	Median	n
Level 1					
Total Retinol	0.346 (0.016)	0.326 (0.008)		0.351	33
trans-Retinol				0.330[a]	5
Retinyl Palmitate				0.008[a]	11
δ-Tocopherol				0.091	4
γ/β-Tocopherol	2.03 (0.10)	1.84 (0.03)		1.72	21
α-Tocopherol	6.96 (0.34)	5.84 (0.10)		6.75	34
Total Lutein[b]	0.069 (0.004)	0.059 (0.003)		0.072	10
Total Zeaxanthin[b]	0.029 (0.003)	0.029 (0.001)		0.037	9
Total α-Cryptoxanthin[b]				0.016	4
Total β-Cryptoxanthin[b]	0.041 (0.003)	0.035 (0.003)		0.047	2
trans-Lycopene	0.156 (0.005)			0.115	9
Total Lycopene[c]	0.173 (0.004)	0.294 (0.008)		0.236	19
Total α-Carotene[b]		0.013 (0.001)		0.008	18
trans-β-Carotene	0.093 (0.004)			0.083	9
Total β-Carotene[b]	0.114 (0.004)	0.093 (0.004)		0.090	24
Total *cis*-β-Carotene[b]				0.005	5
Coenzyme Q_{10}				0.9	5
Phylloquinone (vitamin K_1)[d]				0.4	2
25-Hydroxyvitamin D_3[d]			6.95 (0.03)	7[a]	1
Level 2					
Total Retinol	0.491 (0.011)	0.454 (0.009)		0.428	33
trans-Retinol				0.471[a]	5
Retinyl Palmitate				0.014[a]	9
δ-Tocopherol				0.072	4
γ/β-Tocopherol	1.49 (0.07)	1.47 (0.08)		1.34	21
α-Tocopherol	10.25 (0.20)	10.33 (0.25)		10.30	34
Total Lutein[b]	0.100 (0.006)	0.102 (0.003)		0.88	10
Total Zeaxanthin[b]	0.029 (0.002)	0.028 (0.001)		0.032	9
Total α-Cryptoxanthin[b]				0.021	4
Total β-Cryptoxanthin[b]	0.036 (0.003)	0.035 (0.001)		0.049	20
trans-Lycopene	0.326 (0.021)			0.287	9
Total Lycopene[c]	0.393 (0.015)	0.576 (0.020)		0.594	19
Total α-Carotene[b]	0.034 (0.003)	0.029 (0.003)		0.030	20
trans-β-Carotene	0.193 (0.016)			0.213	9
Total β-Carotene[b]	0.247 (0.019)	0.218 (0.008)		0.241	24
Total *cis*-β-Carotene[b]				0.013	8
Coenzyme Q_{10}				1.0	5
Phylloquinone (vitamin K_1)[d]				0.5	2
25-Hydroxyvitamin D_3[d]			12.65 (0.06)	15[a]	2

Table 3 (continued)

Analyte	NIST LC-UV Methods		NIST LC-IDMS/MS	Interlaboratory Comparison	
	Polymeric C_{18}, n=11	C_{18} with Different Selectivity, n=6	n=10	Median	n
Level 3					
Total Retinol	0.657 (0.017)	0.630 (0.016)		0.497	33
trans-Retinol				0.549[a]	5
Retinyl Palmitate				0.083[a]	15
δ-Tocopherol				0.202	5
γ/β-Tocopherol	2.30 (0.014)	2.44 (0.07)		2.08	21
α-Tocopherol	19.52 (0.43)	20.02 (0.46)		18.48	34
Total Lutein[b]	0.131 (0.006)	0.125 (0.002)		0.115	10
Total Zeaxanthin[b]	0.027 (0.004)	0.029 (0.002)		0.034	9
Total α-Cryptoxanthin[b]				0.015	4
Total β-Cryptoxanthin[b]	0.017 (0.001)	0.017 (0.002)		0.029	20
trans-Lycopene	0.602 (0.025)			0.374	9
Total Lycopene[c]	0.717 (0.029)	0.910 (0.062)		0.965	19
Total α-Carotene[b]		0.015 (0.002)		0.016	19
trans-β-Carotene	0.382 (0.017)			0.344	9
Total β-Carotene[b]	0.431 (0.020)	0.415 (0.010)		0.386	24
Total *cis*-β-Carotene[b]				0.016	8
Coenzyme Q_{10}				1.4	5
Phylloquinone (vitamin K_1)[d]				2.8	2
25-Hydroxyvitamin D_3[d]			19.46 (0.08)	21[a]	2

[a] Not used in value assignment

[b] Includes *cis* and *trans* isomers

[c] Includes the *cis* and *trans* isomers of lycopene; may include other carotenoid compounds

[d] Concentration expressed as ng/mL

for each analyte in the three levels of the SRM provided by the NIST methods were characterized as independent normal distributions. The equally weighted mean of the two NIST method means and the median of the collaborating laboratories' means comparison exercise were used to calculate certified values for total retinol, γ- and α-tocopherol, lutein, zeaxanthin, β-cryptoxanthin, and total β-carotene. The certified value for total retinol includes both *cis*- and *trans*-retinol. *Trans*-retinol in the SRM was not determined by either method employed at NIST. The certified values for 25-hydroxyvitamin D_3 are from the mean of the measurements from one NIST method and do not include the results from the interlaboratory comparison exercises. The reference values (*trans*- and total lycopene, total α-carotene, and *trans*-β-carotene) are based on the agreement of results from the two analytical methods performed at NIST and the median of results from the interlaboratory comparison exercise. Values for total α-carotene and *trans*-β-carotene are designated as reference values because the identity of components present in the measured chromatographic peak is less certain.

The cholesterol was measured in SRM 968e using the GC-IDMS definitive method described above. The certified concentration value for cholesterol was derived from measurements from three sets of samples.

The uncertainty associated with each certified or reference value is expressed as a 95% confidence expanded uncertainty; the value±the expanded uncertainty is expected to include the true value of the measurand with approximately 95% confidence. It expresses both the observed difference between the results from the methods and their respective uncertainties [32–34]. The relative expanded uncertainties for the fat-soluble vitamins and carotenoids in SRM 968e for which certified values are provided range from 1% for α-tocopherol

Table 4 Certified values for selected fat-soluble vitamins, carotenoids, and cholesterol in SRM 968e[a]

Analyte	Level 1				Level 2				Level 3			
	μg/mL		μmol/L		μg/mL		μmol/L		μg/mL		μmol/L	
	Value	U_{95}	Value	U_{95}	Value	U_{95}	Value	U_{95}	Value	U_{95}	Value	U_{95}
Total Retinol	0.341	0.016	1.19	0.06	0.482	0.030	1.68	0.10	0.647	0.021	2.26	0.073
γ/β-Tocopherol [b]	1.86	0.16	4.47	0.38	1.432	0.081	3.44	0.19	2.27	0.17	5.45	0.41
α-Tocopherol	6.53	0.86	15.2	2.0	10.33	0.14	23.98	0.34	19.37	0.63	45.0	1.5
Lutein	0.067	0.008	0.117	0.014	0.097	0.007	0.170	0.013	0.124	0.010	0.218	0.017
Zeaxanthin	0.031	0.005	0.055	0.008	0.029	0.006	0.052	0.010	0.029	0.005	0.052	0.009
β-Cryptoxanthin	0.041	0.006	0.074	0.011	0.040	0.006	0.072	0.011	0.021	0.004	0.037	0.007
Total β-Carotene	0.099	0.018	0.184	0.033	0.234	0.023	0.436	0.042	0.411	0.022	0.765	0.041
Cholesterol[c]	1467	8	3794	20	1585	8	4099	21	1811	10	4683	25
25-Hydroxy D_3[c]	0.00709	0.00014	0.01770	0.00036	0.01292	0.00026	0.03224	0.00065	0.01987	0.00040	0.0496	0.0010

[a] Each certified concentration value is an equally weighted mean of the means from the two NIST LC methods and the median of the individual laboratory means from the interlaboratory comparison exercise. The results for total retinol include *cis*- plus *trans*-retinol. *Trans*-retinol was not determined in the SRM by either method employed at NIST. The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurand with approximately 95% confidence: it expresses both the observed difference between the results from the methods and their respective uncertainties, consistently with the ISO Guide and its Supplement 1 [32–34].

[b] Includes γ- and β-tocopherol

[c] The certified concentration values for cholesterol and 25-hydroxyvitamin D_3 were derived from measurements from three sets of samples each using the NIST GC-IDMS and the NIST LC-IDMS/MS methods respectively described above. The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurand with approximately 95% confidence, consistent with the ISO Guide [32]. The uncertainty incorporates within-method uncertainty and Type B uncertainty components related to the analysis.

Table 5 Reference values for selected carotenoids in SRM 968e

Analyte	Level 1				Level 2				Level 3			
	μg/mL		μmol/L		μg/mL		μmol/L		μg/mL		μmol/L	
	Value	U_{95}	Value	U_{95}	Value	U_{95}	Value	U_{95}	Value	U_{95}	Value	U_{95}
trans-Lycopene	0.135	0.040	0.252	0.075	0.307	0.039	0.571	0.072	0.49	0.23	0.676	0.070
Total Lycopene	0.234	0.095	0.44	0.18	0.52	0.15	0.97	0.28	0.86	0.17	1.60	0.31
Total α-Carotene	0.011	0.005	0.020	0.009	0.031	0.004	0.058	0.008	0.015	0.002	0.028	0.004
trans-β-Carotene	0.088	0.01	0.164	0.018	0.203	0.020	0.378	0.036	0.363	0.038	0.676	0.070

The reference concentration values are equally weighted means of the means from the two NIST LC/absorbance methods and the medians of the laboratory means from the interlaboratory comparison exercise. The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurand with approximately 95% confidence: it expresses both the observed difference between the results from the methods and their respective uncertainties, consistently with the ISO Guide and its Supplement 1 [32–34].

to 19% for zeaxanthin and β-cryptoxanthin. The relative expanded uncertainty for cholesterol in all three levels of the SRM was 1%.

The information values for δ-tocopherol, total α-cryptoxanthin, total *cis*-β-carotene, coenzyme Q_{10}, and phylloquinone (vitamin K_1) were derived from the median of results reported by fewer than six collaborating laboratories. These are noncertified values with no reported uncertainties because there is insufficient information to assess uncertainties.

Comparison of SRM 968e to previous issues All of the SRM 968 issues have been intended for use in the validation of methods for determining selected retinoids, tocopherols, and carotenoids at low- to high-normal levels. To achieve the desired levels of *trans*-retinol and the tocopherols, some of the plasma pools used in previous SRM 968 issues were supplemented (spiked) with ethanolic solutions of one or more of these analytes. SRM 968d, which was issued as a single serum, was originally intended to have three components. After failing to obtain plasma with target analyte levels and following a successful small-scale demonstration of spiking capability, a contractor prepared plasma pools intended to provide mid- and high-levels by spiking retinol, the tocopherols, and β-carotene. (This was the first time we attempted to produce an SRM containing a spike of β-carotene .) The within- and between-vial heterogeneity of the spiked β-carotene in the resulting materials proved unacceptable; only the low-level material, which had been prepared without spiking, was acceptable with regard to homogeneity. As an interim measure, SRM 968d was released containing just this single level until SRM 968e could be prepared as a replacement. To ensure the homogeneity of all levels in SRM 968e and to address concerns that spiking could make some of the SRM components behave differently than in native serum, SRM 968e was produced without supplementing any analyte.

To achieve the desired target analyte values, the three SRM 968e materials were produced by blending individual units of source plasma into pools with targeted levels of retinol, γ- and α-tocopherol, and total β-carotene (Table 1). The levels of these analytes in more than 100 units of plasma were characterized at NIST over a period of several months. Screening-quality measurements were made on the plasma in sample-tubes that had been collected at the time of plasmapheresis. For each of the three sets of target values, five units of plasma were selected from the 100 candidates that provided the best match between the concentration levels expected for the blend of the materials and the target concentrations. The units were identified by comparing the mean of the relative differences between the

Table 6 Information values for additional compounds SRM 968e

These are noncertified values with no reported uncertainties as there is insufficient information to assess uncertainties [3]. The information values are derived from the median of results reported by fewer than six collaborating laboratories.

Analyte	Level 1		Level 2		Level 3	
	μg/mL	μmol/L	μg/mL	μmol/L	μg/mL	μmol/L
δ-Tocopherol	0.09	0.2	0.07	0.2	0.20	0.5
Total α-Cryptoxanthin	0.016	0.03	0.02	0.04	0.015	0.03
Total *cis*-β-Carotene	0.005	0.009	0.013	0.02	0.016	0.03
Coenzyme Q_{10}	0.9	1.0	1.0	1.1	1.4	1.7
Phylloquinone (vitamin K_1)	0.0004	0.0009	0.0005	0.001	0.003	0.006

target and the volume-weighted levels estimated from the screening measurement values for every possible set of five units. The agreement between the target and certified values is fit-for-purpose.

SRM 968e is provided with assigned values (see Tables 3 through 6) for 17 selected fat-soluble vitamins and carotenoids as well as cholesterol. The earlier issues (SRM 968 and 968a) were provided with certified values for only retinol, α-tocopherol, and total β-carotene (*trans* plus *cis* isomers). SRM 968b was provided with certified values for eight analytes: retinol, retinyl palmitate, α-tocopherol, *trans*- and total β-carotene, total α-carotene, lutein, and cholesterol. Improvement in the measurement capability for fat-soluble vitamins and carotenoids at NIST and among the collaborating laboratories is evident in the latter issues of SRM 968 where concentrations values were assigned for 14 to 22 analytes in each material. Due to the recent development of a reference measurement procedure at NIST [7], SRM 968e is the only issue that is provided with certified values for 25-hydroxyvitamin D_3.

The number of collaborating laboratories that helped to value-assign the constituents in these materials increased from seven laboratories in 1989 to more than 50 laboratories in 1999 for SRM 968c when many laboratories were investigating the potential cancer chemopreventive effects of carotenoids. More than 30 laboratories helped to value assign the constituents in the most recent issues SRM 968d and SRM 968e. Table 7 lists all of the SRM 968 issues along with the value-assigned constituents in each. The descriptive nomenclature for NIST SRMs has changed since 1989. Values in the early issues of this SRM (SRM 968, SRM 968a, and SRM 968b) were designated as

Table 7 Comparison of SRM 968e to previous issues. An explanation of the evolution of NIST nomenclature is provided in the text as well as in the footnotes below

Analyte	1989 SRM 968 3 Levels	1991 SRM 968a 3 Levels	1995 SRM 968b 3 Levels	1999 SRM 968c 2 Levels	2008 SRM 968d 1 Levels	2010 SRM 968e 3 Levels
Retinol	Certified	Certified	Certified	Certified	Certified	Certified
α-Tocopherol	Certified	Certified	Certified	Certified	Certified	Certified
γ-Tocopherol	Information	Information	Non-certified	Certified	Certified	Certified
δ-Tocopherol			Non-certified	Certified		Information
Trans-β-Carotene	Information	Information	Certified	Certified		Reference
Total β-Carotene	Certified	Certified	Certified	Certified	Certified	Certified
Total *cis*-β-Carotene						Information
Trans-α-Carotene			Non-certified	Reference		
Total α-Carotene		Information	Certified	Reference	Reference	Reference
Total α-Cryptoxanthin				Information		Information
Total β-Cryptoxanthin		Information	Non-certified	Reference	Reference	Certified
Trans-Lutein				Reference		
Total Lutein		Information	Certified	Reference	Reference	Certified
Total Zeaxanthin		Information	Non-certified	Reference	Reference	Certified
Trans-Lycopene		Information	Non-certified	Reference	Information	Reference
Total Lycopene		Information	Non-certified	Reference	Reference	Reference
Phylloquinone (Vitamin K_1)						Information
Retinyl Palmitate			Certified	Information		
Coenzyme Q_{10}					Information	Information
25-Hydroxyvitamin D				Reference		
25-Hydroxyvitamin D_2				Information		
25-Hydroxyvitamin D_3				Information		Certified
Cholesterol		Information	Certified	Certified	Certified	Certified

A NIST 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 [3].

Reference values are the best estimate of the true values based on available data; 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 [3].

A NIST information or noncertified value is considered to be a value that is of interest to the SRM user, but insufficient information is available to adequately assess the uncertainty associated with the value [3].

certified and noncertified concentration values; values in SRM 968c, SRM 968 d, and SRM 968e are designated as certified, reference, and information values.

The relative uncertainties associated with the certified values for most analytes are much the same in all of the SRM 968 issues: 3% to 10% for retinol and α-tocopherol, 3% to 15% for total β-carotene. The relative uniformity of these uncertainty values reflects the presence of small measurement biases among the results from the independent methods used in the value-assignment process, as well as similar-magnitude allowances for measurement precision and between-vial heterogeneity.

SRM 968e is now one of two health-related NIST reference materials with concentration values assigned for selected retinol, tocopherols, carotenoids, and cholesterol in a human serum matrix, the other being SRM 1950 Metabolites in Human Plasma. SRM 968e is used extensively by laboratories worldwide primarily to validate methods for determining these analytes in human serum and plasma and for assigning values to in-house control materials. The value assignment of the analytes in this SRM will help support measurement accuracy and traceability for laboratories performing health-related measurements in the clinical and nutritional communities.

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Disclaimer Certain commercial products are identified to specify adequately the experimental procedure. Such identification does not imply endorsement or recommendation by the National Institute of Standards and Technology, nor does it imply that the materials identified are necessarily the best available for the purpose.

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