National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 968d

Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum

Standard Reference Material (SRM) 968d is intended for use in validating methods for determining fat-soluble vitamins, carotenoids, and cholesterol in human serum and plasma. This SRM can also be used for quality assurance when assigning values to in-house control materials for these constituents. A unit of SRM 968d consists of two vials of frozen human serum at a single concentration level.

Certified Concentration Values: The certified concentration values of total retinol, γ-tocopherol, α-tocopherol, total β-carotene, and cholesterol in SRM 968d are provided in Table 1. 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 [1]. The certified value for cholesterol was determined from measurements using the NIST definitive method, gas chromatography-isotope dilution mass spectrometry (GC-IDMS). The certified concentration values for the fat-soluble vitamins and carotenoids are based on the agreement of results from two different liquid chromatography (LC) procedures performed at NIST and the median of results from an interlaboratory comparison exercise among institutions that participate in the NIST Micronutrients Measurement Quality Assurance Program. An alphabetized listing of these institutions is provided in Appendix A.

Reference Concentration Values: Reference concentration values for total lutein, total zeaxanthin, total lutein plus zeaxanthin, total β-cryptoxanthin, total α-carotene, and total lycopene are provided in Table 2. 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 [1]. The reference values for the carotenoids are based on the agreement of results from analytical methods performed at NIST and the median of results from an interlaboratory comparison exercise. Values for some carotenoids are designated as reference values because the identity of components present in the measured chromatographic peak is less certain.

Information Concentration Values: Information values for *trans*-lycopene and coenzyme Q_{10} are provided in Table 3. An information value is considered to be a value that may be of interest to the SRM user, but insufficient information is available to assess the uncertainty associated with the value or only a limited number of analyses were performed [1].

Expiration of Certification: The certified values of this SRM lot are valid until **30 September 2013**, within the measurement uncertainties specified, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Use"). However, the certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Maintenance of SRM Certificate: 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) will facilitate notification.

The overall direction and coordination of the preparation and analytical measurements leading to the certification of this SRM were performed by K.E. Sharpless and J.B. Thomas of the NIST Analytical Chemistry Division.

Stephen A. Wise, Chief
Analytical Chemistry Division

Gaithersburg, MD 20899
Certificate Issue Date: 06 October 2008

Robert L. Watters, Jr., Chief
Measurement Services Division

Analytical measurements at NIST were performed by C.A. Rimmer, L.T. Sniegoski, J.B. Thomas, and M.J. Welch of the NIST Analytical Chemistry Division. Collaborating laboratories that performed analytical measurements are listed in Appendix A.

Statistical consultation was provided by J.H. Yen of the NIST Statistical Engineering Division and D.L. Duewer of the NIST Analytical Chemistry Division.

The support aspects involved in the issuance of this SRM were coordinated through the NIST Measurement Services Division.

NOTICE AND WARNING TO USERS

Warning: SRM 968d IS INTENDED FOR IN-VITRO DIAGNOSTIC USE ONLY. THIS IS A HUMAN-SOURCE MATERIAL. HANDLE PRODUCT AS A BIOHAZARDOUS MATERIAL CAPABLE OF TRANSMITTING INFECTIOUS DISEASE. The supplier of the source materials used to prepare this product found the materials to be non-reactive when tested for hepatitis B surface antigen (HBsAg), human immunodeficiency virus (HIV), hepatitis C virus (HCV), and human immunodeficiency virus 1 antigen (HIV-1Ag) by Food and Drug Administration (FDA) licensed tests. However, because no test method can offer complete assurance that HIV, hepatitis viruses, or other infectious agents are absent, this SRM should be handled at the Biosafety Level 2 for any potentially infectious human serum or blood specimen [2].

Storage: Until required for use, SRM 968d should be stored in the dark at or between –20 °C and −80 °C. If carotenoids are to be measured, the unit should be stored at or below –70 °C in the dark. Carotenoids appear to be less stable than the retinoids and the tocopherols at –20 °C [3-6].

Instructions for Use: SRM 968d is provided as a set of two vials of frozen serum that should be allowed to thaw at room temperature for at least 30 min under subdued light. The contents of the vial should then be gently mixed prior to removal of a test portion for analysis. Precautions should be taken to avoid exposure to strong ultraviolet (UV) light and direct sunlight.

PREPARATION AND ANALYSIS[1]

Preparation of SRM Serum Pools: SRM 968d was prepared from source plasma obtained from Interstate Blood Bank, Inc., Memphis, TN. All units were tested and found negative for HBsAg, HIV, HCV, and HIV-1Ag prior to shipment to NIST. Units were stored at –80 °C until use. Bovine thrombin and calcium chloride were added to convert the plasma to serum. The serum was dialyzed to remove bovine thrombin, calcium chloride, and anticoagulants. Salts were added back into the serum. The serum was pooled along with isotonic saline, blended, bottled in 1 mL aliquots, and stored at –80 °C.

Description of Analytical Measurements Used for Value Assignment

A. Measurement of the Fat-Soluble Vitamins and Carotenoids in SRM 968d

The assigned values for selected fat-soluble vitamins and carotenoids in this SRM were derived from results of analyses performed by NIST and 34 collaborating institutions (listed in Appendix A). Because the maintenance of pure and stable primary reference compounds for these analytes is difficult, detector responses were calibrated against solutions whose concentrations were determined by spectrophotometry with corrections made for purity as determined by LC. NIST analyses were based on the absorptivities provided in Figure 1. Two different LC techniques (chromatograms shown in Figure 2) were used at NIST for the determination of the fat-soluble vitamins and carotenoids in the SRM [7-10]. Details of the two LC methods are provided below. (**NOTE:** The compositions of the solvent mixtures described in these methods are expressed as volume fractions in percent [11].) SRM 968c Fat-Soluble Vitamins and Cholesterol in Human Serum was analyzed for quality assurance at NIST during the certification analyses of SRM 968d.

[1]Certain commercial products are identified in this certificate to adequately describe 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 products identified are necessarily the best available for this purpose.

Preparation of Serum Extract for LC Analysis

Aliquots of serum (200 µL) were pipetted into glass test tubes and were combined with equal volumes of ethanol containing the internal standard(s) and butylated hydroxytoluene (BHT, an antioxidant). The samples were vortex-mixed for 15 s. One milliliter of hexane was added to each and the samples were vortex-mixed for at least 45 s. The samples were then centrifuged 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 ethyl acetate/ethanol containing 30 mg/L BHT, depending on the LC procedure used. The reconstituted extracts were vortex-mixed and ultrasonically agitated for approximately 30 s to ensure dissolution and then placed in glass inserts in autosampler vials.

Reversed-Phase LC Using a Polymeric C_{18} Stationary Phase

Total retinol, γ-tocopherol, α-tocopherol, total lutein, total zeaxanthin, total β-cryptoxanthin, and total β-carotene were measured in 2 extracts from each of 14 vials of SRM 968d over 2 days using a 5-µm polymeric [12] C_{18} column (4.6 mm × 250 mm; Vydac 201TP; Separations Group, Hesperia, CA, USA). This method was also used to assess the homogeneity of the SRM material. A ternary solvent mixture was used in this method. Solvent A was 60 % methanol/10 % butanol/30 % water (volume fractions). Solvent B was 89.5 % methanol/10 % butanol/0.5 % water (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 was used to sequentially determine these 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 452 nm for the carotenoids. Tocol (the internal standard) was monitored at 292 nm. A typical separation is shown in Figure 2 (top).

Reversed-Phase LC Using a C_{18} Stationary Phase with Different Selectivity

Total retinol, γ-tocopherol, α-tocopherol, total lutein, total zeaxanthin, total β-cryptoxanthin, and total β-carotene were measured in two extracts from each of seven vials of the SRM on one day using a Bakerbond C_{18} column (4.6 mm × 250 mm; J.T. Baker, Phillipsburg, NJ, USA). This column exhibits selectivity intermediate to monomeric and polymeric C_{18} columns [12]. A ternary solvent method was used to isolate the analytes from the serum extract. 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 [8]. The method consisted of two linear gradients and an isocratic component. The first gradient ran from 98 % solvent A/2 % 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. The system was then returned to initial conditions of 98 % solvent A/2 % solvent B over 5 min and re-equilibrated for 5 min. In this method, a programmable UV/visible absorbance detector with a tungsten lamp was used for measurement of retinol and the carotenoids at 325 nm and 450 nm, respectively. *Trans*-β-apo-10'-carotenal oxime [13,14] was used as the internal standard for the quantification of retinol and the carotenoids. A fluorescence spectrometer was used to measure the tocopherols and tocol (the internal standard) using an excitation wavelength of 295 nm and an emission wavelength of 335 nm. Signals from both detectors were recorded simultaneously. Typical separations with absorbance and fluorescence detection are shown in Figure 2 (bottom).

Interlaboratory Methods Used for the Analysis of SRM 968d

Retinol, tocopherols, carotenoids, and coenzyme Q_{10} in SRM 968d were measured by collaborating institutions 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. 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.

B. Measurement of Cholesterol in SRM 968d

Cholesterol concentrations were determined using the NIST GC-IDMS definitive method [15,16]. Three sets of samples, each consisting of four vials 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 [16]. Cholesterol-25,26,27-$^{13}C_3$ was used as the internal standard. 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-$^{13}C_3$. Two aliquots from one vial of SRM 1951b-Level I Lipids in Frozen Human Serum were run as controls in each set. Two aliquots from one vial each of SRM 1951b-Level II and SRM 1950 Metabolites in Human Plasma were also run as controls in sets two and three, respectively.

Homogeneity Assessment: The homogeneity of fat-soluble vitamins and carotenoids was assessed at NIST by using the reversed-phase polymeric C_{18} LC method described above. 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.

Value Assignment: The equally weighted mean of the two NIST method means and the median of the laboratory means from the interlaboratory comparison exercise were used to calculate each certified concentration value. The reference concentration value is based on the median of the laboratory means from the interlaboratory comparison exercise or on the mean of the interlaboratory median with the two NIST method means.

Table 1. Certified Concentration Values for Fat-Soluble Vitamins, β-Carotene, and Cholesterol in SRM 968d[(a)]

	µg/mL	µmol/L
Total Retinol	0.311 ± 0.050	1.09 ± 0.17
γ-Tocopherol[(b)]	1.39 ± 0.12	3.34 ± 0.30
α-Tocopherol	5.93 ± 0.55	13.77 ± 1.28
Total β-Carotene[(c)]	0.078 ± 0.007	0.145 ± 0.013
Cholesterol[(d)]	1335 ± 4	3453 ± 10

[(a)] Each certified concentration value, expressed as mass fractions, for total retinol, γ-tocopherol, α-tocopherol, and total β-carotene 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. No *cis* isomers for β-carotene were detected, therefore, *trans*- and total β-carotene are considered equivalent. The uncertainty in the certified value, calculated according to the method described in the ISO Guide [17, 18], is expressed as an expanded uncertainty, *U*. 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-laboratory and within-laboratory components of uncertainty. The coverage factor (k) is determined from the Student's *t*-distribution corresponding to the appropriate associated degrees of freedom and approximately 95 % confidence for each analyte [18-20].

[(b)] Includes β-tocopherol.

[(c)] No *cis* isomers for β-carotene were detected, therefore *trans*- and total β-carotene are considered equivalent.

[(d)] The certified concentration value for cholesterol was derived from measurements from three sets of samples using the NIST GC-IDMS method described above.

Table 2. Reference Concentration Values for Carotenoids in SRM 968d[a]

	µg/mL	µmol/L
Total Lutein[b]	0.049 ± 0.016	0.085 ± 0.028
Total Zeaxanthin[b]	0.023 ± 0.005	0.041 ± 0.009
Total Lutein and Zeaxanthin[b]	0.088 ± 0.011	0.155 ± 0.019
Total β-Cryptoxanthin[b]	0.028 ± 0.013	0.050 ± 0.024
Total Lycopene[b,c]	0.278 ± 0.034	0.517 ± 0.063
Total α-Carotene[b]	0.009 ± 0.002	0.018 ± 0.004

[a] The reference concentration values, expressed as mass fractions, for total lutein, total zeaxanthin, and total β-cryptoxanthin 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 reference concentration values for total α-carotene, total lycopene, and total lutein plus zeaxanthin are from the medians of the laboratory means from the interlaboratory comparison exercise [17, 18]. 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 within-laboratory and between-laboratory components of uncertainty. The coverage factor (k) is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and approximately 95 % confidence for each analyte [18-20].

[b] May include *cis* and *trans* isomers.

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

Table 3. Information Concentration Values for *Trans*-Lycopene and Coenzyme Q_{10} in SRM 968d[a]

	µg/mL	µmol/L
Trans-Lycopene	0.11	0.21
Coenzyme Q_{10}	0.64	0.75

[a] These are noncertified values with no reported uncertainties as there is insufficient information to assess uncertainties [1]. The information concentration values are derived from the median of results reported by fewer than 10 collaborating laboratories.

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Users of this SRM should ensure that the certificate in their possession is current. This can be accomplished by contacting the SRM Program at: telephone (301) 975-2200; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.

APPENDIX A

Analysts at the institutions listed below performed measurements that contributed to the value assignment of constituents in SRM 968d.

ARUP Laboratories, Salt Lake City, UT, USA
Bio-Reference Laboratories, Elmwood Park, NJ, USA
Cancer Geriatrics Center, University of Michigan, Ann Arbor, MI, USA
Cancer Research Center of Hawaii, University of Hawaii at Manoa, Honolulu, HI, USA
Centers for Disease Control and Prevention, Atlanta, GA, USA
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
Department of Human Nutrition, University of Illinois at Chicago, Chicago, IL, USA
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
ESA Laboratories, Inc., Chelmsford, MA, 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
Life Sciences Group, Wyle Laboratories, Inc., Houston, TX, USA
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
Services Economiques, Département de Biologie Intégree, Grenoble, France
Servicio de Endocrinologia y Nutricion, Hospital Universitario Puerta de Hierro, Madrid, Spain

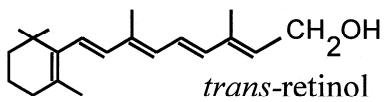
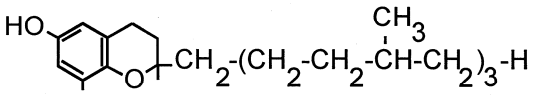
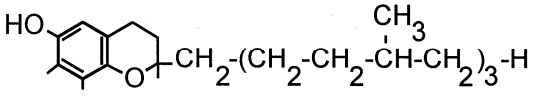
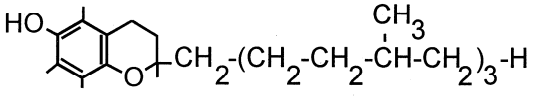
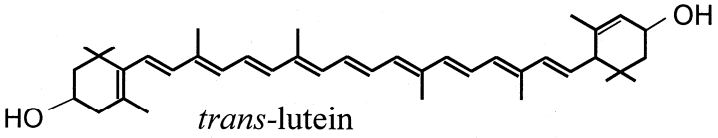
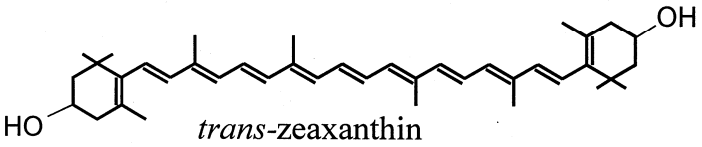
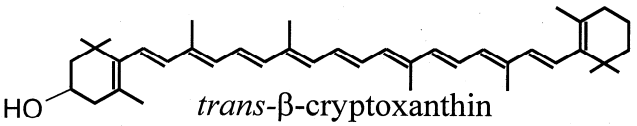
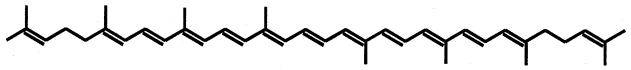
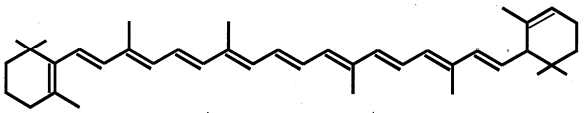
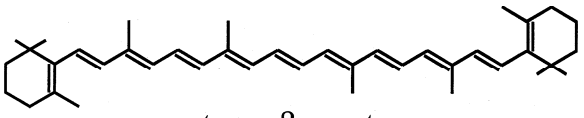
COMPOUND	λ_{max}	ABSORPTIVITY
 *trans*-retinol	325 nm	1843 dL/g·cm in ethanol
 δ-tocopherol	297 nm	91.2 dL/g·cm in ethanol
 γ-tocopherol	298 nm	91.4 dL/g·cm in ethanol
 α-tocopherol	292 nm	75.8 dL/g·cm in ethanol
 *trans*-lutein	445 nm	2550 dL/g·cm in ethanol
 *trans*-zeaxanthin	452 nm	2540 dL/g·cm in 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

Figure l. Wavelength maxima and absorptivites used for calibration at NIST [21-26].

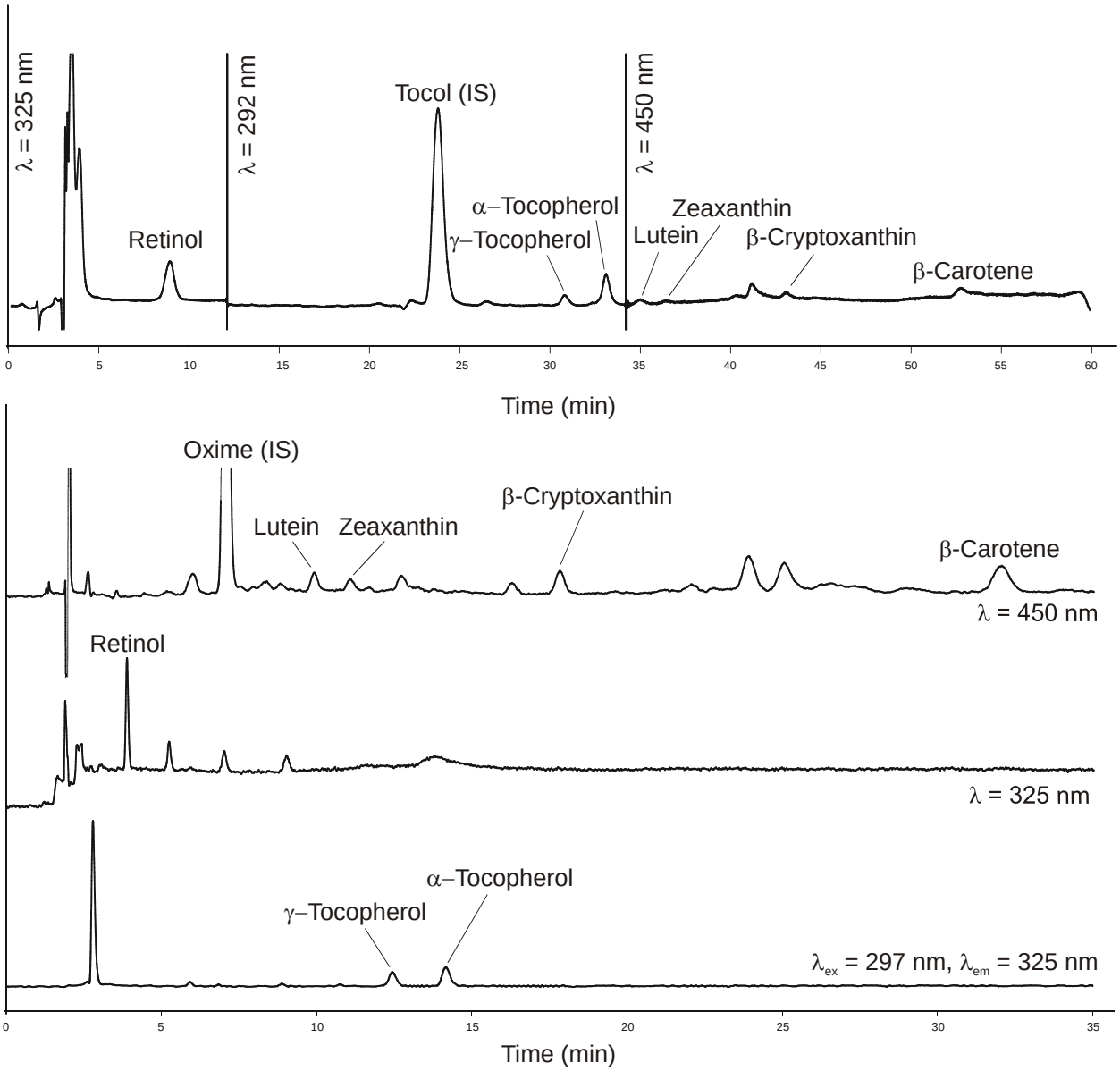


Figure 2. Chromatograms from NIST's analyses of SRM 968d using two LC methods. Chromatographic conditions are described in the text.