National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 968e

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

This Standard Reference Material (SRM) 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 968e consists of three vials, each containing 1 mL of frozen human serum at one of three different concentrations levels.

Certified Values: The certified mass concentration and amount-of-substance concentration [1] values of retinol, selected tocopherols and carotenoids, cholesterol, and 25-hydroxyvitamin D_3 [25(OH)D_3] in SRM 968e 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 [2]. The certified value for cholesterol was determined from measurements using the NIST reference method, gas chromatography-isotope dilution mass spectrometry (GC-IDMS). The certified value for 25(OH)D_3 was determined from measurements using the NIST reference method, liquid chromatography-isotope dilution tandem mass spectrometry (LC-IDMS/MS). 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. A listing of these institutions is provided in Appendix A.

Reference Values: Reference mass concentration and amount-of-substance concentration [1] values for additional carotenoids 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 [2]. The reference values 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 Values: Information mass concentration and amount-of-substance concentration [1] values for additional analytes are provided in Table 3. 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 [2]. Information values cannot be used to establish metrological traceability.

Expiration of Certification: The certification of **SRM 968e** is valid, within the measurement uncertainty specified, until **30 April 2020**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Storage and Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

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

Overall direction and coordination of the preparation and analytical measurements leading to the certification of this SRM were performed by J.B. Thomas of the NIST Chemical Sciences Division.

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

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Certificate Issue Date: 23 November 2015
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Analytical measurements at NIST were performed by I.O. Mugenya, L.T. Sniegoski, S.S.-C. Tai, J.B. Thomas, and M.J. Welch of the NIST Chemical Sciences Division. Collaborating laboratories that performed analyses contributing to value assignment are listed in Appendix A.

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

NOTICE AND WARNING TO USERS

Warning: SRM 968e IS INTENDED FOR RESEARCH USE. 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 [3].

INSTRUCTIONS FOR STORAGE AND USE

Storage: Until required for use, SRM 968e 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 [4–7].

Use: SRM 968e is provided as a set of three vials of frozen serum that should be allowed to thaw at room temperature for at least 30 min under subdued light. The contents of a 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.

SOURCE, PREPARATION, AND ANALYSIS[(1)]

Source and Preparation: SRM 968e was prepared from source plasma obtained from Interstate Blood Bank, Inc., (Memphis, TN, USA). All units were tested and found negative for HBsAg, HIV, HCV, and HIV-1Ag prior to shipment to NIST. Levels of retinol, γ- and α-tocopherol, and carotenoids were measured at NIST in tubes of plasma obtained from the individual units at the time of plasmapheresis, and blending protocols were specified to result in three materials with different concentration 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. The serum was pooled, blended, bottled in 1-mL aliquots, and stored at –80 °C prior to shipment back to NIST. Analyte concentrations were *not* adjusted by spiking.

Analytical Approach for Determination of Retinol, Tocopherols, and Carotenoids: The assigned values for selected fat-soluble vitamins and carotenoids in this SRM were derived from results of analyses performed by NIST and 31 collaborating institutions (listed in Appendix A). Because the maintenance of pure and stable primary reference compounds for these analytes is technically 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. Proteins in the plasma were precipitated with ethanol containing an internal standard as has been previously described [8-10]. Analytes were extracted into hexane, which was evaporated. The reconstituted extracts were then analyzed by liquid chromatography with absorbance detection (LC-UV). Two different LC techniques were used at NIST for the determination of the fat-soluble vitamins and carotenoids in the SRM [8–10]: 1) a polymeric [11] C_{18} column with UV/visible absorbance detection [8,10] and 2) a C_{18} column with different selectivity and absorbance detection [9,10].

Retinol and selected tocopherols and carotenoids were measured in 2 extracts from each of 11 vials of each level of SRM 968e on one day using a 5-µm polymeric [11] C_{18} column (Vydac 201TP, 4.6 mm × 250 mm, Separations Group, Hesperia, CA, USA). A ternary solvent mixture consisting of methanol, butanol, and water was used [8]. UV/visible absorbance detection using a deuterium lamp at the following wavelengths was used: 325 nm for retinol, 292 nm for the tocopherols and tocol (the internal standard), and 450 nm for the carotenoids. This method was also used to assess the homogeneity of the three levels.

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

Retinol and selected tocopherols and carotenoids were measured in two extracts from each of three vials of each level of the SRM on one day using a C_{18} (Bakerbond C_{18} column, 4.6 mm × 250 mm; J.T. Baker, Phillipsburg, NJ, USA) column that exhibits selectivity intermediate to monomeric and polymeric C_{18} columns [11]. A ternary solvent method consisting of acetonitrile, methanol containing 0.05 mol/L ammonium acetate, and ethyl acetate was used [9]. Each of the three solvents contained a volume fraction of 0.05 % triethylamine (TEA) to enhance carotenoid recovery [9]. 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. *Trans*-β-apo-10'-carotenal oxime [12,13] was used as the internal standard for the quantification of retinol and the carotenoids. Tocol was used as the internal standard for the tocopherols.

Retinol, tocopherols, carotenoids, vitamin K, and coenzyme Q_{10} in SRM 968e were also 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.

Analytical Approach for Determination of 25-Hydroxyvitamin D_3: Concentrations of $25(OH)D_3$ were determined using the NIST LC-IDMS/MS reference method [14]. This method is approved by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) as a higher-order reference measurement procedure [15]. 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 spiked with an isotopically labeled internal standard, 25-hydroxyvitamin D_3-d_3. After equilibration for one hour at room temperature, the pH was adjusted with pH 9.8 carbonate buffer, and the sample was extracted 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 for LC-MS/MS. 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.

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

Analytical Approach for Determination of Cholesterol: Cholesterol concentrations were determined using the NIST GC-IDMS reference measurement procedure [16,17]. Three sets of samples, each consisting of two vials of each level of the SRM, were analyzed. Two aliquots from each vial were analyzed using an 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 [17]. 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-$^{13}C_3$.

Homogeneity Assessment: The homogeneity of retinol and selected tocopherols 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 certified values for retinol, tocopherols, and carotenoids. The GC-IDMS mean was used to assign certified values for cholesterol. The LC-IDMS/MS mean was used to assign certified values for $25(OH)D_3$. Reference values are based on the median of the laboratory means from the interlaboratory comparison exercise or on the mean of the interlaboratory median with the NIST method means available for that analyte.

Table 1. Certified Values for Selected Fat-Soluble Vitamins, Carotenoids, and Cholesterol in SRM 968e[(a)]

	Level 1		Level 2		Level 3	
	(μg/mL)	(μmol/L)	(μg/mL)	(μmol/L)	(μg/mL)	(μmol/L)
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-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
Total 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
Total 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
Total 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
	(ng/mL)[(e)]	(nmol/L)[(f)]	(ng/mL)	(nmol/L)	(ng/mL)	(nmol/L)
25-Hydroxyvitamin D_3[(d)]	7.09 ± 0.14	17.7 ± 0.4	12.9 ± 0.3	32.2 ± 0.7	19.9 ± 0.4	49.6 ± 1.0

[(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, unless otherwise noted. The results for total retinol include *cis*- plus *trans*-retinol. *Trans*-retinol was not separately 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, consistent with the ISO/JCGM Guides and its Supplement 1 [18–20]. The expanded uncertainty is calculated as ku_c, where u_c is the combined uncertainty, and $k = 2$ is a coverage factor corresponding to approximately 95 % confidence for each analyte [18]. The measurand is the concentration value for each analyte listed in Table 1. Metrological traceability is to the SI derived unit for mass concentration (expressed as micrograms per milliliter) and amount-of-substance concentration (expressed as micromoles per liter).

[(b)] Includes β-tocopherol.

[(c)] The certified concentration value for cholesterol was derived from measurements from three sets of samples using the NIST GC-IDMS method 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/JCGM Guide [18]. The uncertainty incorporates within-method uncertainty and Type B uncertainty components related to the analysis. The expanded uncertainty is calculated as ku_c, where u_c is the combined uncertainty, and $k = 2$ is a coverage factor corresponding to approximately 95 % confidence for each analyte [18]. The measurand is the concentration value for each analyte listed in Table 1. Metrological traceability is to the SI derived unit for mass concentration (expressed as micrograms per milliliter) and amount-of-substance concentration (expressed as micromoles per liter).

[(d)] The certified concentration value for 25-hydroxyvitamin D_3 was derived from measurements using the NIST LC-IDMS/MS method described above. The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurands with approximately 95 % confidence, consistent with the ISO/JCGM Guide [18]. The uncertainty incorporates within-method and Type B uncertainty components related to the analysis. The expanded uncertainty is calculated as ku_c, where u_c is the combined uncertainty, and $k = 2$ is a coverage factor corresponding to approximately 95 % confidence for each analyte [18].

[(e)] Mass concentrations were calculated based using the following measured serum densities: Level 1, 1.02118 g/mL, Level 2, 1.02080 g/mL, and Level 3, 1.02099 g/mL. The uncertainty in the serum density measurements was incorporated in values that are reported relative to units of volume.

[(f)] Molar concentration levels were calculated from mass concentration levels using the relative molecular mass 400.64 g/mol.

Table 2. Reference Values for Selected Carotenoids in SRM 968e[(a)]

	Level 1		Level 2		Level 3	
	(μg/mL)	(μmol/L)	(μg/mL)	(μmol/L)	(μg/mL)	(μmol/L)
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 ± 0.010	0.164 ± 0.018	0.203 ± 0.020	0.378 ± 0.036	0.363 ± 0.038	0.676 ± 0.070

[(a)] The reference concentration values are equally weighted means of the means from the two NIST LC/absorbance methods available for that analyte 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, consistent with the ISO/JCGM Guides and its Supplement 1 [18–20]. The expanded uncertainty is calculated as ku_c, where u_c is the combined uncertainty, and $k = 2$ is a coverage factor corresponding to approximately 95 % confidence for each analyte [18]. The measurand is the concentration value for each analyte listed in Table 2 as determined by the method or methods indicated. Metrological traceability is to the SI derived unit for mass concentration (expressed as micrograms per milliliter) and amount-of-substance concentration (expressed as micromoles per liter).

Table 3. Information Values for Additional Compounds SRM 968e[(a)]

	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
	(ng/mL)	(μmol/L)	(ng/mL)	(μmol/L)	(ng/mL)	(μmol/L)
Phylloquinone (vitamin K_1)	0.4	0.9	0.5	1.1	2.8	6.3

[(a)] These are noncertified values with no reported uncertainties as there is insufficient information to assess uncertainties [2]. The information values are derived from the median of results reported by fewer than six collaborating laboratories.

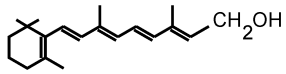
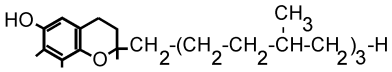
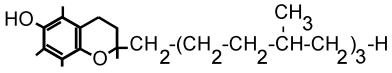
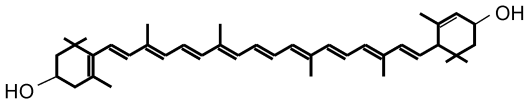
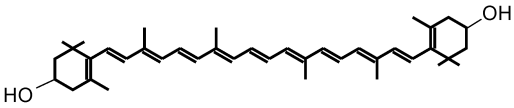
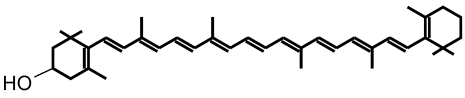
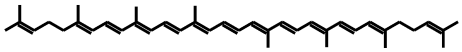
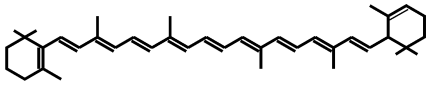
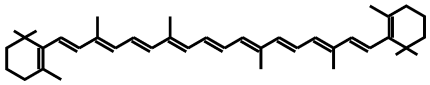
COMPOUND	STRUCTURE	λ_{max}	ABSORPTIVITY
trans-retinol		325 nm	1843 dL/g·cm in ethanol
γ-tocopherol		298 nm	91.4 dL/g·cm in ethanol
α-tocopherol		292 nm	75.8 dL/g·cm in ethanol
trans-lutein		445 nm	2550 dL/g·cm in ethanol
trans-zeaxanthin		452 nm	2540 dL/g·cm in ethanol
trans-β-cryptoxanthin		452 nm	2356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

Figure l. Wavelength maxima and absorptivities used for calibration of retinol, tocopherol, and carotenoid analyses at NIST [21–26].

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Certificate Revision History: 23 November 2015 (Editorial changes); 12 June 2012 (Editorial changes); 25 August 2011 (Added certified values for 25-hydroxyvitamin D_3; editorial changes); 30 September 2010 (Original certificate issue date).

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

APPENDIX A

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