National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 1950

Metabolites in Frozen Human Plasma

This Standard Reference Material (SRM) is intended primarily for validation of methods for determining metabolites such as fatty acids, electrolytes, vitamins, hormones, and amino acids in human plasma and similar materials. This SRM can also be used for comparison of measurement technologies used in metabolomic studies and for quality assurance when assigning values to in-house reference materials. This SRM is intended to represent "normal" human plasma. A unit of SRM 1950 consists of five vials, each containing approximately 1.0 mL of plasma.

The development of SRM 1950 was a collaboration between the National Institute of Standards and Technology (NIST) and the National Institutes of Health (NIH) National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Analyses for value assignment were performed by NIST and the Centers for Disease Control and Prevention (CDC) (Atlanta, GA). All certified and reference values, with the exception of vitamin D-binding protein, are based on the agreement between the results from NIST methods and from the CDC, where available. The reference value for vitamin D-binding protein is based on the agreement between the results from a NIST method and University of Washington method. The associated uncertainties are expressed at the 95 % level of confidence [1–3]. Values are reported in mass fraction, mass concentration, and amount-of-substance concentration units as appropriate [4].

Certified Values: The certified values of cholesterol and total glycerides, selected fatty acids, amino acids, vitamins and carotenoids, clinical markers and hormones, and electrolytes in SRM 1950 are provided in Tables 1, 2, 4, 6, 9, and 10, respectively. 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 [5].

Reference Values: Reference values are provided for fatty acids in Table 3, amino acids in Table 5, vitamins and carotenoids in Table 7, trace elements, selenium species, bilirubin, total protein, and vitamin D-binding protein in Table 11, and perfluorinated compounds in Table 12. A NIST reference value is a noncertified value that is the best estimate of the true value based on available data; however, the value does not meet the NIST criteria for certification and is provided with associated uncertainties that may reflect only measurement reproducibility, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods [5].

Information Values: Information concentration values are provided for selected vitamers in Table 8. A NIST information value is a value that may be of interest to the SRM user, but insufficient information is available to assess the uncertainty associated with the value. Information values cannot be used to establish metrological traceability.

Expiration of Certification: The certification of **SRM 1950** is valid, within the measurement uncertainty specified, until **30 September 2023**, 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.

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Overall direction and coordination of the technical measurements leading to the certification of this SRM were performed by K.W. Phinney of the NIST Biomolecular Measurement Division and S.A. Wise of the NIST Chemical Sciences Division.

Acquisition and preparation of the SRM were coordinated by K.E. Sharpless formerly of the NIST Chemical Sciences Division.

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

NOTICE AND WARNING TO USERS

SRM 1950 IS INTENDED FOR RESEARCH USE. THIS IS A HUMAN SOURCE MATERIAL. HANDLE THE PRODUCT AS A BIOHAZARDOUS MATERIAL CAPABLE OF TRANSMITTING INFECTIOUS DISEASE. The supplier has reported that each donor unit of plasma used in the preparation of this product was tested by Food and Drug Administration licensed tests and found to be negative for human immunodeficiency virus (HIV), HIV-1 antigen, hepatitis B surface antigen, and hepatitis C. However, no known test method can offer complete assurance that hepatitis B virus, hepatitis C virus, HIV, or other infectious agents are absent from this material. Accordingly, this human blood-based product should be handled at the Biosafety Level 2 or higher as recommended for any POTENTIALLY INFECTIOUS HUMAN SERUM OR BLOOD SPECIMEN in the CDC/NIH Manual [6].

INSTRUCTIONS FOR STORAGE AND USE

Storage: The SRM should be stored at –60 °C or lower in the original unopened vials. The certified values do not apply to contents of previously opened vials as the stabilities of the analytes have not been investigated.

Use: SRM 1950 is provided as frozen plasma that should be allowed to thaw at room temperature for at least 30 min under subdued light prior to use. After the material is thawed, it should be used immediately. The contents of the vial should be gently mixed prior to removal of a test portion for analysis. Precautions should be taken to avoid exposure to strong UV light and direct sunlight.

SOURCE, PREPARATION, AND ANALYSIS[1]

Source and Preparation: SRM 1950 is designed to represent "normal" human plasma. Plasma was obtained from 100 individuals who had undergone an overnight fast prior to blood draw by Bioreclamation (Hicksville, NY). Lithium heparin was used as the anticoagulant. A rapid glucose oxidase test was used to exclude individuals who did not adhere to the fasting requirement. Plasma from an equal number of men and women in a narrow adult age range (40 to 50 years) was used. Racial distribution of the donors reflected the distribution in the U.S. population, i.e., approximately 77 % white, 12 % African American or black, 2 % American Indian or Alaskan Native, 4 % Asian, 5 % "other," with approximately 15 % of the total taken from individuals of Hispanic origin. Plasma was not obtained from individuals (1) who were extreme exercisers (e.g., marathon runners), (2) with body-mass indices outside the 95th percentile, or (3) who adhered to extreme dietary regimens. Individuals should not have taken medication for at least 72 h prior to blood draw and were free from overt conditions (e.g., diabetes), diseases, and disorders.

This SRM was developed after an appropriate human subjects research determination by NIST.

[1] Certain commercial equipment, instruments, or materials are identified in this certificate adequately to 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.

Homogeneity Assessment: The homogeneity of all measurands for which certified and reference values are provided was assessed at NIST using the methods and test portion sizes described below; analysis of variance did not show statistically significant heterogeneity. The reported analytes have been treated as though they are homogeneously distributed in the material; the homogeneity of the other analytes present in the material and not reported by NIST and/or CDC was not assessed.

Value Assignment: Means of data sets from individual methods were combined to provide assigned values. The measured plasma density is 1.020 86 g/mL with a standard deviation of 0.000 20 g/mL; this uncertainty was incorporated in values that are reported relative to units of volume.

Analytical Approach for Determination of Cholesterol, Fatty Acids, and Total Glycerides: The cholesterol mass fraction was determined using the NIST isotope dilution (ID) gas chromatography (GC) mass spectrometry (MS) (ID-GC-MS) definitive method [7,8]. This method is an approved higher-order reference measurement procedure according to the Joint Committee for Traceability in Laboratory Medicine (JCTLM) [9]. This procedure employs hydrolysis of cholesterol esters using potassium hydroxide (KOH) in ethanol, followed by extraction with hexane, and derivatization of cholesterol using *bis*(trimethylsilyl)acetamide. Cholesterol-25,26,27-$^{13}C_3$ was used as the internal standard. Certified values are listed in Table 1.

Value assignment of the mass concentration of total glycerides (as triolein) was based upon the NIST ID-GC-MS definitive method [10]. This method is recognized as a higher-order reference measurement procedure by the JCTLM. The method involves hydrolysis of triglycerides with ethanolic KOH, deionization, reaction with butylboronic acid in pyridine, and derivatization with N-methyl-N-trimethylsilyltrifluoroacetamide. Tripalmitin-1,2,3-$^{13}C_3$ was used as the internal standard. Certified values for total glycerides are listed in Table 1.

Determination of Fatty Acids (NIST): Mass fractions of fatty acids in SRM 1950 were determined using two different extraction procedures and two analytical methods (see Table 2 for certified values and Table 3 for reference values). An internal standard solution containing stearic-d_{35} acid and myristic-d_{27} acid was used. One set of samples was saponified in methanolic KOH and esterified using sulfuric acid in methanol. A second set was treated with sodium methoxide in methanol followed by boron trifluoride [11]. Both sets of samples were analyzed by GC with flame ionization detection (FID) and GC-MS. GC-FID was performed using a 0.25 mm × 100 m biscyanopropyl polysiloxane fused silica capillary column. GC-MS was performed using a 0.25 mm × 60 m fused silica capillary column containing a 50 % cyanopropyl + 50 % phenylpolysiloxane (mole fraction) phase.

Determination of Fatty Acids (CDC): Total fatty acids were determined using ID-GC-MS based on Langerstedt's method [12] (see Table 2 for certified values and Table 3 for reference values). This procedure employs hydrolysis of fatty acids from cholesteryl esters, triglycerides, and phospholipids using sequential addition of acetonitrile:hydrochloric acid and methanol:sodium hydroxide in the presence of heat. Total fatty acids were extracted in hexane, concentrated, derivatized using pentafluorobenzyl bromide (PFB), and reconstituted in hexane. Twenty-four fatty acids were quantified using 10 isotopically labeled internal standards. Fragmentation of fatty acid-PFB esters by negative chemical ionization resulted in a reproducible loss of the PFB moiety giving a stable carboxylate anion on a capillary column (30 m × 0.25 mm × 0.25 μm) with helium as the carrier gas.

Analytical Approach for Determination of Amino Acids: Amino acids were quantified using ID liquid chromatography (LC) tandem MS (ID-LC-MS/MS), GC coupled to time-of-flight (TOF) MS (GC-TOF-MS), with two different types of derivatization, and two-dimensional GC×GC-TOF-MS, as described in reference 13. Certified values are listed in Table 4, and reference values are listed in Table 5. Cysteine thiols have been shown to oxidize in plasma forming cystine dimers or to form other disulfide bonds such as those of glutathione [14–16]. No specific reducing agents were added to prevent thiol reactions in this study; however, the experimental approach was optimized to minimize potential thiol reactions by keeping plasma samples at or below 4 °C at all times during LC analyses or until the derivatization step was reached during GC-MS analyses.

For the LC-MS/MS, GC-MS, and GC×GC-TOF-MS methods, amino acids were enriched from plasma using standard methanol precipitation and spiked with isotopically labeled amino acid analogs. For LC-MS/MS, analytes were separated on a mixed mode (ion-exclusion and reversed-phase) analytical column, and multiple reaction monitoring (MRM) was performed in a triple quadrupole mass spectrometer for two distinct fragmentation transitions from each ion. For GC-TOF-MS and GC×GC-TOF-MS, the methanol layer was evaporated to dryness and derivatized with N-(*t*-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA). The derivatized sample was injected into the GC and separated on two columns in series: a 5 % diphenyl/95 % dimethyl polysiloxane (mole fraction) column and a 50 % diphenyl/50 % dimethyl polysiloxane (mole fraction) column. GC×GC-TOF-MS was a two-dimensional separation with cryotrapping between the two columns and a modulation period of 3 s. GC-TOF-MS analysis was performed in one dimension without cryotrapping.

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For the second GC-TOF-MS method, plasma was spiked with isotopically labeled amino acid analogs. Derivatization was performed in the plasma matrix with propyl chloroformate (PCF)/propanol. Derivatized products were extracted using chloroform for injection into the GC and separated on two 5 % diphenyl/95 % dimethyl polysiloxane (mole fraction) columns in series, in one dimension without cryotrapping between the columns.

Analytical Approach for Determination of Fat-Soluble Vitamins and Carotenoids: Value assignment of the mass fractions of the vitamins and carotenoids in SRM 1950 was based on the combination of results provided from several different analytical methods at NIST and CDC. NIST provided measurements by using a combination of different LC methods with different detection modes as described below. Certified values are listed in Table 6 and reference values are listed in Table 7.

Determination of Vitamins A and E and Carotenoids (NIST): Retinol and carotenoids were measured at NIST by using combinations of two LC methods with absorbance detection: (1) a polymeric C_{18} column [17] with UV/visible absorbance detection [18,19] and (2) a C_{18} column with different selectivity and absorbance detection of retinol and carotenoids and fluorescence detection of tocopherols [19,20]. Proteins in the plasma were precipitated with ethanol containing an internal standard. Analytes were extracted into hexane, which was evaporated. The reconstituted extracts were then analyzed by LC with absorbance and/or fluorescence detection.

Determination of Vitamins A and E and Carotenoids (CDC): Fat-soluble micronutrients were measured at CDC using reversed-phase LC with photodiode array detection [21]. Plasma was mixed with an ethanol solution containing two internal standards, retinyl butyrate and β-apo-8′-carotenal. Micronutrients were extracted from the aqueous phase into hexane and dried under vacuum. The extract was redissolved in ethanol and acetonitrile and filtered to remove any insoluble material. An aliquot of the filtrate was injected onto a high carbon load C_{18} column (150 mm × 4.6 mm × 3 µm particle size) and eluted with a gradient consisting of ethanol and acetonitrile. Absorbance was monitored at 300 nm for vitamin E, 325 nm for vitamin A, and 450 nm for carotenoids.

Analytical Approach for Determination of Vitamin D Metabolites: The vitamin D metabolites, 25-hydroxyvitamin D_2, [25(OH)D_2], and 25-hydroxyvitamin D_3, [25(OH)D_3], were measured at NIST using ID-LC-MS and ID-LC-MS/MS [22], each with two different types of chromatographic separations. The certified values for 25(OH)D_3 are listed in Table 6 and reference values for 25(OH)D_2 are listed in Table 7.

Determination of 25(OH)D_3 and 25(OH)D_2 by ID-LC-MS: Plasma and an internal standard solution containing $^2H_6$-25(OH)D_3 and $^2H_3$-25(OH)D_2 were combined in glass tubes, proteins were precipitated, and the metabolites were extracted into hexane twice. The hexane phases were combined and evaporated to dryness at 40 °C under nitrogen. The residues were reconstituted and were further clarified using centrifuge filters. Extracts were analyzed by LC-MS using: (1) a deactivated C_{18} stationary phase and (2) a cyanopropyl stationary phase. Atmospheric pressure chemical ionization (APCI) MS detection with positive polarity was used for both chromatographic methods. The $[M - H_2O + H]^+$ ions were monitored for all species and were used for quantitation. The ions monitored included *m/z* 383 for 25(OH)D_3, *m/z* 395 for 25(OH)D_2, *m/z* 389 for $^2H_6$-25(OH)D_3 and *m/z* 398 for $^2H_3$-25(OH)D_2.

Determination of 25(OH)D_3 and 25(OH)D_2 by ID-LC-MS/MS: Two grams to 2.5 g of plasma were combined with water (to avoid protein precipitation when samples were spiked with internal standard solutions), and an internal standard solution containing $^2H_3$-25(OH)D_3 and $^2H_3$-25(OH)D_2. After equilibration at room temperature for 1 h, the pH of each sample was adjusted to pH 9.8 ± 0.2 with carbonate buffer. Analytes were extracted from the plasma matrix with a mixture of hexane and ethyl acetate. The combined extracts were dried under nitrogen at 45 °C, and the residues were reconstituted with methanol for LC-MS/MS analysis. APCI in the positive-ion mode and MRM mode were used. The transitions at *m/z* 401 → *m/z* 383 for 25(OH)D_3, *m/z* 404 → *m/z* 386 for $^2H_3$-25(OH)D_3, *m/z* 413 → *m/z* 395 for 25(OH)D_2, and *m/z* 416 → *m/z* 398 for $^2H_3$-25(OH)D_2 were monitored.

Analytical Approach for Determination of Water-Soluble Vitamins: Water-soluble vitamins were measured by NIST and CDC using combinations of LC methods with fluorescence or tandem mass spectrometric detection as described below. Certified values are listed in Table 6, reference values are listed in Table 7, and information values are listed in Table 8.

Determination of Folic Acid and 5-Methyltetrahydrofolate (NIST): Folic acid and 5-methyltetrahydrofolate were measured in SRM 1950 using a JCTLM-approved ID-LC-MS/MS method using $^{13}C_5$-folic acid and $^{13}C_5$-5-methyltetrahydrofolate as internal standards [23]. A gradient LC method with a water/methanol/formic acid mobile phase and a pentafluorophenyl column were used for the positive ion mode LC-MS/MS determination. For folic acid, the transitions at *m/z* 442 → *m/z* 295 (unlabeled) and *m/z* 447 → *m/z* 295 (labeled) were monitored; for 5-methyltetrahydrofolate, the transitions at *m/z* 460 → *m/z* 313 (unlabeled) and *m/z* 465 → *m/z* 313 (labeled) were monitored.

Determination of 5-Methyltetrahydrofolate and Folic Acid (CDC): The folate vitamers 5-methyltetrahydrofolate and folic acid were measured by ID-LC-MS/MS using $^{13}C_5$-5-methyltetrahydrofolate and $^{13}C_5$-folic acid as internal standards [24,25]. This is a JCTLM-approved method. Folate species were isolated from plasma by solid-phase extraction (SPE) with phenyl cartridges. The folate vitamers were separated on a C_8 column under isocratic conditions with an organic mobile phase containing acetic acid. The transitions monitored for 5-methyltetrahydrofolate and folic acid were the same as those listed above for the NIST determination.

Folate was also measured by a microbiological assay using a 96-well plate microtiter method [26,27]. Diluted serum was added to an assay medium inoculated with *Lactobacillus casei* (NCIB 10463) and containing all of the nutrients except folic acid necessary for growth of *L. casei.* The microtiter plate was incubated for 42 h at 37 °C. Because the growth of *L. casei* is proportional to the amount of total folate present in the serum sample, the folate concentration was quantified by measuring the turbidity of the inoculated assay medium at 590 nm in a micro plate reader. The assay was calibrated with 5-methyltetrahydrofolate.

Determination of Vitamin B_6 (NIST): Vitamin B_6 was determined as pyridoxal 5'-phosphate by ID-LC-MS/MS. A labeled internal standard (pyridoxal-[$^2H_3$]-5'-phosphate) was added to the plasma and allowed to equilibrate for 30 min. Plasma proteins were precipitated by the addition of aqueous trichloroacetic acid followed by incubation at room temperature. After centrifugation, supernatants were analyzed by LC-MS/MS. The transitions at *m/z* 248 → *m/z* 150 (unlabeled) and *m/z* 251 → *m/z* 153 (labeled) were monitored.

Determination of Vitamin B_6 (CDC): The vitamin B_6 vitamers pyridoxal 5'-phosphate and 4-pyridoxic acid were determined by LC with chlorite post-column derivatization and fluorescence detection after protein precipitation with metaphosphoric acid and sample filtration [28,29]. The B_6 vitamers were separated under isocratic conditions on a C_{18} column with a mobile phase comprised of aqueous phosphate buffer (with 0.2 % acetonitrile) and methanol. The initial mobile phase was comprised of 100 % aqueous buffer, and a linear gradient from 0 % to 30 % methanol was employed after elution of the B_6 vitamers to facilitate column cleanup between injections.

Analytical Approach for Determination of Glucose: Value assignment of the glucose mass fraction (see Table 9) was based on a modification of the NIST reference method for glucose, which involves ID-GC-MS and conversion of glucose into a dibutylboronate acetate derivative [30,31]. This method is an approved higher-order reference measurement procedure according to the JCTLM [9]. For certification of SRM 1950 this procedure was modified in that the serum was not passed through an ion-exchange resin prior to concentration, freeze-drying, and derivatization.

Analytical Approach for Determination of Creatinine: Creatinine was determined using an ID-LC-MS method [32] that is similar to a method developed at the Laboratory of the Government Chemist [33] and is approved by the JCTLM as a higher-order reference measurement procedure (see Table 9).

Analytical Approach for Determination of Urea and Uric Acid: Urea was determined using a modification of the ID-GC-MS method described in reference 34, approved by JCTLM, in which the plasma was spiked with urea-^{18}O, passed through an SPE cartridge, concentrated, then derivatized to 6-methyluracil overnight. Uric acid was determined using a modification of the ID-GC-MS method described in reference 35, approved by the JCTLM, in which plasma samples were spiked with uric acid-$^{15}N_2$, mixed with 0.001 mol/L ammonium hydroxide, passed through a strong anion exchange resin, eluted from the column with 1 mol/L acetic acid, freeze-dried, and derivatized with MTBSTFA. Certified values are provided in Table 9.

Analytical Approach for Determination of Homocysteine: Homocysteine was determined using an ID-GC-MS method similar to a method developed at the University of Colorado Health Sciences Center [36,37]. Plasma was spiked with homocystine-d_8, hydrolyzed with dithiothreitol in sodium hydroxide solution to break disulfide bonds and release homocysteine and homocysteine-d_4, which were isolated on an anion exchange resin, concentrated, derivatized, and analyzed by GC-MS with selective ion monitoring at *m/z* 420 and *m/z* 424 with confirmation made by monitoring *m/z* 318 and *m/z* 322 [38]. This method is approved by the JCTLM as a higher-order reference measurement procedure. Certified values are provided in Table 9.

Analytical Approach for Determination of Hormones: The value assignments for the cortisol, progesterone, and testosterone mass fractions were based upon the LC-MS/MS analytical approaches described below and the certified values are provided in Table 9.

Determination of Cortisol: Value assignment of the cortisol mass fraction was based on the NIST reference method (JCTLM-approved) for cortisol [39], which involves spiking the plasma with cortisol-d_3, acidifying the sample, putting the sample through an SPE C_{18} cartridge, liquid-liquid extraction, and analysis by LC-MS/MS using a C_{18} column and monitoring two transitions each for the unlabeled forms: *m/z* 363 → *m/z* 327 and *m/z* 363 → *m/z* 121 and labeled forms: *m/z* 366 → *m/z* 330 and *m/z* 366 → *m/z* 121.

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Determination of Progesterone: Value assignment of the progesterone mass fraction was based on the NIST reference method (JCTLM-approved), for progesterone [40,41], which involves spiking the plasma with progesterone-$^{13}C_2$, a liquid-liquid extraction, and analysis using LC-MS/MS with a C_{18} column and monitoring the transitions for the unlabeled form, *m/z* 315 → *m/z* 97 and the labeled form, *m/z* 317 → *m/z* 99.

Determination of Testosterone: Value assignment of the testosterone mass fraction was based on the NIST reference method (JCTLM-approved) for testosterone [42], which involves spiking the plasma with testosterone-d_3, SPE, and analysis using LC-MS/MS with a C_{18} column and monitoring the transitions for the unlabeled form, *m/z* 289 → *m/z* 97, and the labeled form, *m/z* 292 → *m/z* 97.

Analytical Approach for Determination of Elements: Calcium, copper, magnesium, potassium, selenium, and sodium were determined using a single method [43]. The certified mass fractions for calcium, magnesium, and potassium are based on measurements using isotope dilution collision cell technology (CCT) inductively coupled plasma mass spectrometry, (ID-CCT-ICP-MS) [44–46], which is a method approved by the JCTLM. The certified mass fraction for sodium is based on measurements using NIST's gravimetric definitive method [46,47], a JCTLM-approved method, without ion exchange. The reference mass fraction for selenium is based upon measurements using ID-ICP-MS. The reference mass fraction for copper is based on measurements using ICP-MS with standard additions. Certified values are listed in Table 10 and reference values are listed in Table 11.

Analytical Approach for Determination of Selenium Species: The selenium species selenoprotein P, glutathione peroxidase, and seleno-albumin were determined by ID-ICP-MS. Separation of selenoproteins was performed by affinity chromatography on heparin-sepharose and blue-sepharose stationary phases that had been packed into PEEK columns (4 mm × 50 mm). Proteins were eluted with 1.5 mol/L ammonium acetate. Quantitation was based upon ID with ^{77}Se. Reference values are listed in Table 11.

Analytical Approach for Determination of Total Protein: Total protein mass concentration (Table 11) was determined using a biuret method [48]. Spectrophotometric measurements were calibrated using SRM 927d Bovine Serum Albumin (7 % Solution).

Analytical Approach for Determination of Bilirubin: The mass concentration of bilirubin was determined using a spectrophotometric reference method developed by Doumas [49] and is provided in Table 11.

Analytical Approach for Determination of Vitamin D-Binding Protein: Vitamin D-binding protein (VDBP) was measured by NIST and the University of Washington (UW) with the LC-MS/MS methods as described below. Reference values listed in Table 11 are the mean of the mean measurements made at NIST and UW.

Determination of VDBP by NIST: Seven vials of SRM 1950 were randomly selected and analyzed by ID-LC-MS/MS in triplicate on different days. Samples were prepared gravimetrically using trifluoroethanol to denature the proteins as previously described [50]. Following tryptic digestion for 19 hours, peptides containing one Leucine residue with $^{13}C_6$ and ^{15}N were added to each sample. The two transitions with the greatest intensity for peptides TSALSAK and VLEPTLK were monitored during ID-LC-MS/MS. The total protein concentration was calculated as the mean of the two peptides. Concentrations were calculated in units milligrams per liter using the mean of the range of molecular masses of the common isoforms with or without glycosylation (51 200 g/mol and 51 900 g/mol).

Determination of VDBP by UW: Seven vials of SRM 1950 were randomly selected and sent to UW for analysis by ID-LC-MS/MS. The digestion procedure used at UW was similar to the one used at NIST [51]. However, peptides ELPEHTVK and VLEPTLK were used during LC-ID-MS/MS. Also, the labeled peptide standards were added to the samples at the beginning of the sample processing and the total digestion time was 30 minutes.

Analytical Approach for Determination of Perfluorinated Compounds (PFCs): Perfluorinated compounds were determined by LC-MS/MS in the negative-ion mode with ^{13}C-labeled PFCs as internal standards. Reference values are provided in Table 12. In the first method, samples were spiked with the internal standards and mixed with 50 % formic acid in water (volume fractions). The PFCs were isolated by SPE with weak anion exchange cartridges. Chromatographic separation was achieved on either a C_8 or a pentafluorophenyl column with gradient elution, and the mobile phases were comprised of methanol and 20 mmol/L ammonium acetate. In the second method, samples were spiked with the internal standards and plasma proteins were precipitated with acetonitrile. After centrifugation and a solvent exchange to methanol, PFCs were isolated from the supernatants by SPE with graphitized carbon cartridges. Chromatographic separation was achieved on either a C_8 or a pentafluorophenyl column with gradient elution, and the mobile phases were comprised of methanol and 20 mmol/L ammonium acetate.

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Certified Values for Cholesterol and Total Glycerides: Each certified value is the mean of NIST measurements using higher-order reference methods. The measurand is the certified value for cholesterol and total glycerides listed in Table 1. 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 within-method uncertainty and Type B components related to the analysis, consistent with the ISO/JCGM Guide [1]. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the effects of the combined components of uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence for each analyte. For the certified values shown below, $k = 2$. Metrological traceability is to the derived SI unit for mass concentration (expressed as milligrams per deciliter) and amount-of-substance concentration (expressed as millimoles per liter).

Table 1. Certified Values for Cholesterol and Total Glycerides

	Mass Concentration (mg/dL)	Amount-of-Substance Concentration (mmol/L)
Cholesterol	151.4 ± 3.3	3.917 ± 0.085
Total Glycerides (as triolein)	99.0 ± 2.1	1.12 ± 0.02

Certified Values for Fatty Acids: Certified values for fatty acids are weighted means of the results from analyses at NIST using GC-MS and GC-FID and from CDC using GC-MS [50,51] when available. The value for linoleic acid (C18:2 n-6) was based upon the NIST GC-FID and CDC GC-MS results. The uncertainty provided with each value is an expanded uncertainty about the mean [52,53], with coverage factor $k = 2$ (approximately 95 % confidence), calculated by combining a pooled within-method variance with a between-method variance following the ISO/JCGM Guides [1,2,54]. The measurand is the certified value for each fatty acid listed in Table 2. Metrological traceability is to the derived SI unit for mass faction (expressed as micrograms per gram) and amount-of-substance concentration (expressed as micromoles per liter).

Table 2. Certified Values for Fatty Acids

Lipid Name	Chemical Name (Common Name)	Mass Fraction (µg/g)	Amount-of-Substance Concentration (µmol/L)
C12:0	Dodecanoic Acid (Lauric Acid)	1.86 ± 0.11	9.47 ± 0.57
C16:0	Hexadecanoic Acid (Palmitic Acid)	594 ± 19	2364 ± 77
C16:1 n-7	(Z)-9-Hexadecenoic Acid (Palmitoleic Acid)	53.5 ± 6.4	215 ± 26
C18:0	Octadecanoic Acid (Stearic Acid)	179 ± 12	644 ± 41
C18:3 n-3	(Z,Z,Z)-9,12,15-Octadecatrienoic Acid (α-Linolenic Acid)	14.9 ± 1.0	54.6 ± 3.6
C18:1 n-9	(Z)-9-Octadecenoic Acid (Oleic Acid)	447 ± 43	1614 ± 154
C18:2 n-6	(Z,Z)-9,12-Octadecadienoic Acid (Linoleic Acid)	780 ± 39	2838 ± 143
C22:0	Docosanoic Acid (Behenic Acid)	15.9 ± 1.5	47.8 ± 4.6

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Reference Values for Fatty Acids: Reference values for fatty acids are provided below. The methods and uncertainties used in the calculations of the values are delineated in the footnotes. The measurand is the reference value for each fatty acid listed in Table 3 as determined by the indicated methods. Values are metrologically traceable to the measurement procedures as indicated in the footnotes.

Table 3. Reference Values for Fatty Acids

Lipid Name	Chemical Name (Common Name)	Mass Fraction (μg/g)	Amount-of-Substance Concentration (μmol/L)
C14:0	Tetradecanoic Acid (Myristic Acid)[a,b]	17.9 ± 3.8	80.1 ± 17.0
C14:1	(Z)-9-Tetradecenoic Acid (Myristoleic Acid)[c,d]	1.57 ± 0.03	7.1 ± 0.1
C15:0	Pentadecanoic Acid[b,e]	1.08 ± 0.01	4.56 ± 0.04
C17:0	Heptadecanoic Acid (Margaric Acid)[d,e]	4.7 ± 0.2	17.6 ± 0.7
C18:3 n-6	(Z,Z,Z)-6,9,12-Octadecatrienoic Acid (γ-Linolenic Acid)[a,b]	10.9 ± 2.3	39.9 ± 8.5
C18:1 n-7	(Z)-11-Octadecenoic Acid (Vaccenic Acid)[c,d]	37.7 ± 0.9	136 ± 3
C20:0	Eicosanoic Acid (Arachidic Acid)[c,d]	5.5 ± 0.2	18.0 ± 0.5
C20:1	(Z)-11-Eicosenoic Acid (Gondolic Acid)[c,d]	3.5 ± 0.1	11.5 ± 0.5
C20:2	(Z,Z)-1,14-Eicosadienoic Acid[c,d]	5.7 ± 0.2	18.8 ± 0.6
C20:3 n-6	(Z,Z,Z)-8,11,14-Eicosatrienoic Acid (Dihomo-γ-Linolenic Acid)[c,d]	41.8 ± 1.1	139 ± 4
C20:4 n-6	(Z,Z,Z,Z)-5,8,11,14-Eicosatetraenoic Acid (Arachidonic Acid)[a,b]	293 ± 54	984 ± 180
C20:5 n-3	(Z,Z,Z,Z,Z) -5,8,11,14,17-Eicosapentaenoic Acid (EPA)[c,d]	11.4 ± 0.1	38.6 ± 0.5
C22:1	(Z)-13-Docosenoic Acid (Erucic Acid)[c,d]	1.1 ± 0.4	3.4 ± 1.3
C22:4 n-6	(Z,Z,Z,Z)-7,10,13,16-Docosatetraenoic Acid[c,d]	8.3 ± 0.2	25.5 ± 0.6
C22:5 n-3	(Z,Z,Z,Z,Z)-7,10,13,16,19-Docosapentaenoic Acid (DPA)[c,d]	12.5 ± 0.2	38.5 ± 0.7
C22:5 n-6	(Z,Z,Z,Z,Z)-4,7,10,13,16-Docosapentaenoic Acid[c,d]	6.3 ± 0.1	19.5 ± 0.4
C22:6 n-3	(Z,Z,Z,Z,Z,Z)-4,7,10,13,16,19-Docosahexaenoic Acid (DHA)[a,b]	37.9 ± 6.8	118 ± 21
C24:0	Tetracosanoic Acid (Lignoceric Acid)[c,d]	16.8 ± 0.9	46.6 ± 2.6
C24:1	(Z)-15-Tetracosenoic Acid (Nervonic Acid)[c,d]	25.6 ± 1.2	71.3 ± 3.2

[a] Values are weighted means of the results from analyses at NIST using GC-MS and GC-FID and from CDC using GC-MS [52,53].

[b] The expanded uncertainty about the mean is calculated by combining a pooled within-method variance with a between-method variance and a coverage factor $k = 2$ (approximately 95 % confidence) following the ISO/JCGM Guides [1,2,52–54].

[c] Values are from analyses at the CDC using GC-MS.

[d] The expanded uncertainty U is calculated as $U = ku_c$, where u_c is one standard deviation of the analyte mean and k is determined from the Student's t-distribution corresponding to the associated degrees of freedom and 95 % confidence level for each analyte. For the reference values shown above, $k = 2$.

[e] Values which are from NIST GC-FID analyses.

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Certified Values for Amino Acids: Each certified value is the mean from the combination of the mean results from LC-MS/MS, GC-TOF-MS (MTBSTFA), and GC-TOF-MS (PCF), where available. The measurand is the certified value for each amino acid listed in Table 4. 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, in addition to Type B components related to purity of the standards used, consistent with the ISO/JCGM Guide and its Supplement 1 [1–3]. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the effects of the combined components of uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence for each analyte. For the certified values shown below, $k = 2$. Metrological traceability is to the derived SI unit for mass faction (expressed as milligrams per kilogram) and amount-of-substance concentration (expressed as micromoles per liter).

Table 4. Certified Values for Amino Acids

	Mass Fraction (mg/kg)	Amount-of-substance Concentration (µmol/L)
Alanine	26.2 ± 2.2	300 ± 26
Glycine	18.0 ± 1.2	245 ± 16
Histidine	11.04 ± 0.55	72.6 ± 3.6
Isoleucine	7.13 ± 0.42	55.5 ± 3.4
Leucine	12.90 ± 0.82	100.4 ± 6.3
Lysine	20.0 ± 1.9	140 ± 14
Methionine	3.26 ± 0.26	22.3 ± 1.8
Proline	19.9 ± 1.1	177 ± 9
Serine	9.87 ± 0.44	95.9 ± 4.3
Threonine	13.94 ± 0.70	119.5 ± 6.1
Tyrosine	10.17 ± 0.53	57.3 ± 3.0
Valine	20.9 ± 1.2	182.2 ± 10.4

Reference Values for Amino Acids: The reference values for arginine, cysteine, and cystine are based on the LC-MS/MS method means. The results for phenylalanine are based on three methods. The uncertainty provided with each value was calculated as described for the certified values, provided in Table 4. Type B components included were related to the analysis, consistent with the ISO/JCGM Guide and its Supplement 1 [1–3]. For the reference values shown below, $k = 2$. The measurand is the reference value for each amino acid listed in Table 5 as determined by the indicated methods. Values are metrologically traceable to the measurement procedures as indicated above.

Table 5. Reference Values for Amino Acids

	Mass Fraction (mg/kg)	Amount-of-substance Concentration (µmol/L)
Arginine	13.89 ± 0.40	81.4 ± 2.3
Cysteine	5.26 ± 0.81	44.3 ± 6.9
Cystine	1.83 ± 0.08	7.8 ± 0.4
Phenylalanine	8.2 ± 1.1	51 ± 7

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Certified Values for Selected Vitamins and Carotenoids: The certified values for carotenoids, retinol, tocopherol, 5-methyltetrahydrofolate, and pyridoxal 5'-phosphate are the means of the method means from measurements performed by NIST and CDC. The certified value for 25-hydroxyvitamin D_3 is the mean of the NIST LC-MS and NIST LC-MS/MS method means. The measurand is the certified value for each selected vitamin and carotenoid listed in Table 6. 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, in addition to Type B components, consistent with the ISO/JCGM Guide and its Supplement 1 [1–3]. For carotenoids, the Type B components included sample preparation and purity of the standards; for vitamins, the Type B components included were related to the analysis. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the effects of the combined components of uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence for each analyte. For the certified values shown below, $k = 2$. Metrological traceability is to the derived SI unit for mass faction (expressed as milligrams per kilogram or nanograms per gram) and mass concentration (expressed as micrograms per milliliter or nanograms per milliliter).

Table 6. Certified Values for Selected Vitamins and Carotenoids

	Mass Fraction (mg/kg)	Mass Concentration (μg/mL)
Retinol	0.396 ± 0.034	0.404 ± 0.035
α-Tocopherol	8.01 ± 0.22	8.18 ± 0.22
γ- + β-Tocopherol	1.67 ± 0.16	1.71 ± 0.17
Lutein	0.067 ± 0.022	0.069 ± 0.023
Zeaxanthin	0.021 ± 0.005	0.022 ± 0.005
β-Cryptoxanthin	0.038 ± 0.003	0.039 ± 0.003
Total α-Carotene	0.025 ± 0.005	0.026 ± 0.005
Total β-Carotene	0.077 ± 0.004	0.079 ± 0.004
	Mass Fraction (ng/g)	**Mass Concentration (ng/mL)**
25-Hydroxyvitamin D_3	24.27 ± 0.75	24.78 ± 0.77
5-Methyltetrahydrofolate	12.11 ± 0.31	12.36 ± 0.32
Pyridoxal 5'-phosphate	8.02 ± 0.45	8.19 ± 0.46

Reference Values for Vitamins and Carotenoids: The uncertainty provided with each value was calculated as described in Table 6 by using Type B components related to analysis [1–3]. Reference values for carotenoids are based upon the mean of measurements performed by CDC. The reference value for 25-hydroxyvitamin D_2 is the mean of the NIST LC-MS and NIST LC-MS/MS method means. The reference value for folic acid is the mean of the NIST and CDC method means. The measurand is the reference value for each vitamin and carotenoid listed in Table 7 as determined by the indicated methods. Values are metrologically traceable to the measurement procedures as indicated above.

Table 7. Reference Values for Vitamins and Carotenoids

	Mass Fraction (mg/kg)	Mass Concentration (μg/mL)	Coverage Factor, k
Trans-Lycopene	0.14 ± 0.01	0.14 ± 0.01	2.36
Total Lycopene	0.32 ± 0.02	0.33 ± 0.02	2.35
Trans-β-Carotene	0.071 ± 0.005	0.072 ± 0.005	2.38
Cis-β-Carotene	0.0040 ± 0.0003	0.0041 ± 0.0003	2.39
	Mass Fraction (ng/g)	**Mass Concentration (ng/mL)**	**Coverage Factor, k**
25-Hydroxyvitamin D_2	0.51 ± 0.17	0.52 ± 0.17	2
Folic acid	1.48 ± 0.44	1.51 ± 0.45	2

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Information Values for Additional Vitamers: Information values are the means of results provided by CDC. These values are provided without uncertainties because insufficient information is available to assess them.

Table 8. Information Values for Additional Vitamers

	Amount-of-substance Concentration (nmol/L)
Total folate[(a)]	30.6
4-Pyridoxic acid	28.7

[(a)] Microbiological analysis.

Certified Values for Selected Clinical Markers, Electrolytes, and Hormones: Each certified value is the mean of NIST measurements using higher-order reference methods. The measurand is the certified value for each selected clinical marker listed in Table 9 and each electrolyte listed in Table 10. 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 within-method uncertainty and Type B components related to the analysis, consistent with the ISO/JCGM Guide [1]. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the effects of the combined components of uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence for each analyte. For the certified values shown below, $k = 2$ unless otherwise noted. Metrological traceability is to the derived SI unit for mass fraction (expressed as nanograms per gram); and/or mass concentration (expressed as milligrams per deciliter, milligrams per liter, and/or nanograms per milliliter) and amount-of-substance concentration (expressed as millimoles per liter or micromoles per liter).

Table 9. Certified Values for Clinical Markers and Hormones

	Mass Concentration (mg/dL)	Amount-of-substance Concentration (mmol/L)
Creatinine	0.6789 ± 0.0108	0.0600 ± 0.0009
Glucose	82.16 ± 1.00	4.560 ± 0.056
Urea	23.45 ± 0.49	3.90 ± 0.08
Uric Acid	4.274 ± 0.089	0.254 ± 0.005

	Mass Concentration (mg/L)	Amount-of-substance Concentration (µmol/L)
Homocysteine	1.150 ± 0.026	8.50 ± 0.20

	Mass Fraction (ng/g)	Mass Concentration (ng/mL)
Cortisol	82.2 ± 1.7	83.9 ± 1.7
Progesterone	1.452 ± 0.037	1.482 ± 0.038
Testosterone	2.169 ± 0.046	2.214 ± 0.047

Table 10. Certified Values for Electrolytes

	Amount-of-substance Concentration (mmol/L)	Coverage Factor, k
Calcium	1.936 ± 0.024	2.19
Magnesium	0.696 ± 0.004	1.98
Potassium	3.665 ± 0.025	2.064
Sodium	141.76 ± 0.31	1.972

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Reference Values for Trace Elements, Selenium Species, Total Protein, Bilirubin, and Vitamin D-Binding Protein: Each reference value is the mean of NIST measurements using a single method, except for Vitamin D-Binding Protein, which is the mean of means of measurements from NIST and UW. The measurand is the reference value for each trace element, selenium species, total protein, bilirubin, and vitamin D-binding protein listed in Table 11 as determined by the indicated methods. 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 within-method uncertainty and Type B components related to the analysis, consistent with the ISO/JCGM Guide [1]. The uncertainty for vitamin D-binding protein also incorporates the observed difference between the results from the methods [1–3]. The expanded uncertainty is calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the effects of the combined components of uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence for each analyte. Values are metrologically traceable to the measurement procedures as indicated above.

Table 11. Reference Values for Trace Elements, Selenium Species, Total Protein, Bilirubin, and Vitamin D-Binding Protein

	Mass Fraction (mg/kg)			Coverage Factor, k
Copper	1.008	±	0.008	2.09
Selenium	0.1055	±	0.0038	2.2
	Mass Fraction (µg/kg)			**Coverage Factor, k**
Selenoprotein P	50.2	±	4.3	2.6
Glutathione Peroxidase	23.6	±	1.3	2.5
Seleno-Albumin	28.2	±	2.6	2.6
	Mass Concentration			**Coverage Factor, k**
Total Protein (g/L)	59.1	±	1.7	2
Bilirubin (mg/dL)	0.344	±	0.023	2.11
Vitamin D-Binding Protein (mg/L)	175	±	18	2.0

Reference Values for Perfluorinated Compounds (PFCs): Reference values are weighted means of the results from analyses at NIST using LC-MS/MS with two different sample preparation schemes and different chromatographic separations [52,53]. The concentrations reflect the total of both branched and linear forms of the analytes. The uncertainty provided with each value is an expanded uncertainty about the mean [54], with coverage factor $k = 2$ (approximately 95 % confidence), calculated by combining a pooled within-method variance with a between-method variance following the ISO/JCGM Guides [1,2,54]. The measurand is the reference value for each PFC listed in Table 12 as determined by the indicated methods. Values are metrologically traceable to the measurement procedures as indicated above.

Table 12. Reference Values for PFCs

	Mass Fraction (ng/g)			Mass Concentration (ng/mL)		
Perfluorooctanoic Acid (PFOA)	3.21	±	0.06	3.27	±	0.06
Perfluorononanoic Acid (PFNA)	0.705	±	0.028	0.720	±	0.028
Perfluorodecanoic Acid (PFDA)	0.315	±	0.006	0.322	±	0.007
Perfluoroundecanoic Acid (PFUnA)	0.182	±	0.003	0.186	±	0.003
Perfluorohexansulfonate (PFHxS)	3.19	±	0.08	3.25	±	0.08
Perfluorooctanesulfonic Acid (PFOS)	10.43	±	0.12	10.64	±	0.13

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