Supporting Information for:

Development of a Standard Reference Material for Metabolomics Research

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Method information for analytes determined in Standard Reference Material (SRM 1950) Metabolites in Human Plasma

This section provides experimental details and supplementary references for the methods used in the value assignment of SRM 1950.

Determination of Cholesterol and Total Glycerides: The cholesterol mass fraction was determined using the NIST ID-GC-MS definitive method.[1,2] This method is an approved higher-order reference measurement procedure according to the Joint Committee for Traceability in Laboratory Medicine (JCTLM). 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. Value assignment of the mass concentration of total glycerides (as triolein) was based upon the NIST ID-GC-MS definitive method.[3] 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.

Determination of Glucose and Creatinine: Value assignment of the glucose mass fraction 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.[4,5] This method is an approved higher-order reference measurement procedure according to the JCTLM. 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. Creatinine was determined using an ID-LC-MS method[6] that is similar to a method developed at the Laboratory of the Government Chemist[7] and is approved by the JCTLM as a higher-order reference measurement procedure.

Determination of Urea and Uric Acid: Urea was determined using a modification of the NIST ID-GC-MS method, approved by the JCTLM, in which the plasma was spiked with urea-^{18}O, passed through an SPE cartridge, concentrated, then derivatized to 6-methyluracil overnight.[8] Uric acid was determined using a modification of the NIST ID-GC-MS method, 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.[9]

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.[10,11] 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.[12] This method is approved by the JCTLM as a higher-order reference measurement procedure.

Determination of Hormones: Value assignment of the cortisol mass fraction was based on the NIST reference method (JCTLM-approved) for cortisol,[13] which involves spiking the plasma with cortisol-d_3, acidifying the sample, putting the sample through an SPE 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. Value assignment of the progesterone mass fraction was based on the NIST reference method (JCTLM-approved), for progesterone,[14] 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. Value assignment of the testosterone mass fraction was based on the NIST reference method (JCTLM-approved) for testosterone,[15] 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.

Determination of Bilirubin and Total Protein: The mass concentration of bilirubin was determined using a spectrophotometric reference method developed by Doumas.[16] Total protein mass concentration was determined using a biuret method.[17] Spectrophotometric measurements were calibrated using SRM 927d Bovine Serum Albumin (7 % Solution).

Determination of Elements*:* Calcium, copper, magnesium, potassium, selenium, sodium, and zinc were determined using a single method.[18] 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), which is a method approved by the JCTLM.[19-21] The certified mass fraction for sodium is based on measurements using NIST's gravimetric definitive method,[21,22] a JCTLM-approved method, without ion exchange. The reference mass fraction for selenium is based upon measurements using ID-ICP-MS. The reference mass fractions for copper and zinc are based on measurements using ICP-MS with standard additions.

Determination of Amino Acids: Amino acids were quantified using ID-LC-MS/MS, GC-TOF-MS with two different types of derivatization, and two-dimensional GC×GC-TOF-MS.[23] Cysteine thiols have been shown to oxidize in plasma forming cystine dimers or to form other disulfide bonds such as those of glutathione.[24-26] 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. 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.

Determination of Fatty Acids (NIST): Mass fractions of fatty acids in SRM 1950 were determined using two different extraction procedures and two analytical methods. 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.[27] 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 Lagerstedt's method.[28] 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 (PFBBr) in the presence of triethylamine, and reconstituted in hexane. Twenty-four fatty acids (6 saturated-, 7-monounsaturated-, and 11 polyunsaturated-) were quantified using selected ion monitoring based on the recovery of 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 0.25 mm x 60 m (0.25 μm film thickness) 50% cyanopropyl + 50% methylpolysiloxane phase capillary column with helium as the carrier gas.

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[29] with UV/visible absorbance detection[30,31] and (2) a C_{18} column with different selectivity and absorbance detection of retinol and carotenoids and fluorescence detection of tocopherols.[31,32] 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.[33] 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.

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[34] and ID-LC-MS/MS,[35] each with two different types of chromatographic separations. For 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 – H2O + 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. For ID-LC-MS/MS, 2 g 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.

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.[36] 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 Folate, 5-Methyltetrahydrofolate and Folic Acid (CDC): The folate forms 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.[37,38] This is a JCTLM-approved method. Folate forms were isolated from plasma by SPE with phenyl cartridges. The folate forms 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.[39,40] 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.[41,42] 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.

Determination of Selenium Species: The selenium species selenoprotein P, glutathione peroxidase, and seleno-albumin were determined by ID-ICP-MS.[43] 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.

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.[44] 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.

Supplementary References

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