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Definitive method certification of clinical analytes in lyophilized human serum: NIST Standard Reference Material (SRM) 909b

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Abstract The National Institute of Standards and Technology (NIST) has developed several Standard Reference Materials (SRMs) based on human serum. NIST SRM 909b, Human Serum, is a lyophilized human serum material with concentrations for seven organic and six inorganic analytes at two levels certified solely by definitive methods (DMs). This material provides the vehicle by which high precision, high accuracy measurements made with DMs at NIST can be transferred through the measurement hierarchy to other laboratories. Isotope dilution gas chromatographic-mass spectrometric (GC-IDMS) methods were applied to measure cholesterol, creatinine, glucose, urea, uric acid, triglycerides, and total glycerides. Thermal ionization isotope dilution mass spectrometry (TI-IDMS) was used for determination of lithium, magnesium, potassium, calcium, and chloride. In addition, chloride was determined by coulometry, providing a comparison between two DMs. Sodium, which lacks a stable isotope that would permit isotope dilution mass spectrometric (IDMS) measurement, was determined by gravimetry. SRM 909b includes certified values for total glycerides and triglycerides, which were not certified in the previous lot of this material (SRM 909a). Improvement in uniformity of vial fill weight in the production of SRM 909b resulted in smaller certified uncertainties over previous freeze-dried serum SRMs. Uncertainties at the 99% level of confidence for relative expanded uncertainty (%) for certification of the organic analytes on a mmol/L/g basis ranged from 0.44% for urea (level II) to 5.04% for glucose (level II). (In-house studies have shown glucose to be a relatively unstable analyte in similar lyophilized serum materials, degrading at about 1% per year.) Relative expanded uncertainties (99% C.I.) for certification of inorganic analytes on a mmol/L/g basis ranged from 0.25% for chloride (level I) to 0.49% for magnesium (level II).

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Introduction

For many years, NIST has developed and used definitive methods (DMs), based primarily on isotope dilution mass spectrometry (IDMS), to achieve measurements of clinically significant analytes that most closely approach true value. Analytical techniques that have shown high precision and for which potential sources of bias have been rigorously investigated are considered DMs [1]. DMs were used in the certification of SRM 909b, Human Serum, to provide the accuracy base for thirteen key analytes for the National Reference System for the Clinical Laboratory (NRSCL). SRM 909b is used primarily by standardization and calibration laboratories in the clinical laboratory community, and secondarily by clinical laboratories as a quality control tool for maintaining the accuracy of patient sample measurements [2]. Standard Reference Materials are generally not intended for use as working standards, nor are DMs intended for routine quality control during the analysis of patient samples. Rather, these materials are the vehicle by which high precision, high accuracy measurements made via DMs at NIST can be transferred to secondary reference laboratories. Additionally, these materials provide a well defined natural matrix sample for analytical method research and development.

SRM 909b is the most recent issue in a series of NIST freeze-dried human serum SRMs. SRM 909, issued in 1980, was followed by the issue of SRM 909a in 1991. Units of SRM 909b became available in February 1996. SRM 909b is issued with three vials each of low level (level I) and high level (level II) lyophilized serum, and six bottles of deionized, autoclaved water. The values for the certified concentrations are given in mmol/L, mg/dL, and mmol/L/g, and are based on reconstitution of the material with 10.00 ± 0.02 mL of the provided diluent. Each certified value represents the mean of DM measurements. Chloride was measured by two DMs, and is discussed as a separate case.

Uncertainty for the mmol/L and mg/dL results (based on a 95% confidence, 95% coverage statistical tolerance interval) reflects the combined effect of fill weight variation between vials and measurement imprecision. Nor-

malizing to the mass of freeze-dried serum in each vial (mmol/L/g) allows the certification to meet even greater replication and accuracy needs. Here, the uncertainty is based on a 99% confidence interval, which reflects only the measurement imprecision.

DMs using isotope dilution mass spectrometry (IDMS) were used to certify all of the organic analytes, and five of the six inorganic analytes. Isotope dilution is an ideal technique for definitive method analysis since the analytical determination is not dependent on sample recovery. After equilibration of the native and labeled materials, the results are not affected by analyte recovery since it is the ratio of unlabeled to labeled material that is measured [3]. Details have been published for the IDMS determination of organic [4–9] and inorganic [10–14] analytes in serum. Sodium, which is monoisotopic, and thus not amenable to IDMS analysis, was determined by an ion-exchange gravimetric technique [15]. Improvement of procedures applied to the preparation and certification of SRM 909b are outlined below.

Experimental

Serum pool preparation

Sufficient material was prepared to provide approximately 3,500 units of SRM 909b. The material was prepared in two serum pools, one for each concentration level. In contrast to the manufacture of pervious serum SRMs, material for SRM 909b was dispensed in liquid form into individual serum vials, and then subjected to lyophilization. Each serum pool was tested by the manufacturer for certain properties such as pH, percent moisture, microbial content, and reconstitution time. The processed material was also tested for reconstituted stability (over eight days) for selected analytes, vial to vial homogeneity, analyte recovery, and enzyme activity. The base serum used to manufacture these materials was also tested by an FDA-approved method and found non-reactive for HbsAg, Anti-HIV ½, and Anti-HCV. Information values are provided for orthophosphoric-monoester phosphohydrolase (ALP), lactate dehydrogenase (LDH), L-alanine:2-oxyglutarate aminotransferase (ALT), L-aspartate:2-oxyglutarate aminotransferase (AST), creatine kinase (CK), total bilirubin, and pH [16]. These values were not determined by NIST, but were determined by the manufacturer's quality assurance laboratory. Fill mass variability was checked at NIST by performing refractive index measurements on selected samples from each fill head. Moisture measurements were also performed as an additional quality assurance procedure.

Sampling procedure

A stratified random sample was taken from each of the 40 drying trays used to prepare the material. Three vials per tray were selected for each level. Determinations were performed on 6 to 9 vials per constituent, except sodium.

The dry serum was reconstituted with 10 mL of the diluent water provided. The exact volume of water added was calculated by measuring the mass of the added water and dividing by the density of water at the measured laboratory temperature. Concentrations were corrected by the derived dilution factor to represent the addition of exactly 10.00 mL of the diluent. This procedure was adopted to reduce addition of the diluent as a source of uncertainty in the certified values. In this manner diluent water was added to the dry serum with an uncertainty of ± 0.01% [17]. The solution was mixed by swirling and inverting the vials gently between periods of standing until all of the serum was in solution (~2 h) The reconstituted solution was sampled by withdrawing a quantity of the serum into a plastic syringe. Aliquot masses were weighed by difference on a semi-micro balance with a readability of ± 0.01 mg.

Table 1 Standards and Spike Materials used for Isotope Dilution Analyses

Primary Standard Materials	Source	Purity (%)
Magnesium Metal	Johnson Matthey	99.999%
Calcium Carbonate	NIST SRM 915	99.9+%
Potassium Chloride	NIST SRM 999	99.98%
Lithium Carbonate	NIST SRM 924/924a	100.05%/ 99.867%
Cholesterol	NIST SRM 911b	99.8%
Glucose	NIST SRM 917a	99.7%
Tripalmitin	NIST SRM 1595	99.5%
Creatinine	NIST SRM 914a	99.7%
Sodium Chloride	NIST SRM 919	99.9%
Urea	NIST SRM 912a	99.9%
Uric Acid	NIST SRM 913	99.7%
Enriched/Labeled Materials	**Source**	**Purity (atom %)**
^{6}Li	Oak Ridge Nat. Lab.	99.15 atom %
^{41}K	Oak Ridge Nat. Lab.	99.18 atom %
^{42}Ca	Oak Ridge Nat. Lab.	94.47 atom %
^{37}Cl	Oak Ridge Nat. Lab.	98.21 atom %
^{25}Mg	Oak Ridge Nat. Lab.	98.81 atom %[a]
^{26}Mg	Oak Ridge Nat. Lab.	99.16 atom %[a]
Cholesterol-3,4-$^{13}C_2$	MSD Isotopes	99.3 atom %
Creatinine-$^{13}C_2$	MSD Isotopes	99.4 atom %
Glucose-UL-$^{13}C_6$	MSD Isotpes	98.9 atom %
Tripalmitin-1,2,3-$^{13}C_3$	In-house synthesis	99.0 atom %
Creatinine-$^{13}C_2$	In-house synthesis	95.0 atom %
Urea-^{18}O	In-house synthesis	90.0 atom %
Uric Acid-1,3-$^{15}N_2$	MSD Isotopes	99.0 atom %

[a] Combined to form a spike mixture of composition ^{24}Mg = 0.7%, ^{25}Mg = 50.0 %, and ^{26}Mg = 49.3%

Calibration materials and standard solution preparation

The high purity primary standards used as IDMS calibration materials for each analyte are summarized in Table 1. Subsamples of the solid primary standard materials were weighed in tared, clean, metal boats (Pt boats were used for acid dissolutions). The mass of the boat plus sample was obtained on a microbalance with a readability of ± 0.001 mg and transferred to clean, tared glass flasks. The standard was dissolved with an appropriate, high purity reagent and subsequently diluted to the desired concentration. The exact mass of solution was determined by subtracting the mass of the flask and weigh boat.

Determination of organic analytes

Gas chromatography combined with IDMS was used to certify the concentration of cholesterol, creatinine, glucose, urea, uric acid, triglycerides, and total glycerides. Combined capillary gas chromatography and electron impact mass spectrometry were used to obtain the relative abundance ratio of the unlabeled and labeled quantification ions. The analytical instrumentation consists of a single focusing, magnetic sector mass spectrometer directly inter-

Table 2 Derivatives and Quantification Ions Used for GC-IDMS Measurements

Analyte	Derivative Measured	Quantification Ions Monitored
Cholesterol	Cholesterol-TMS	1°–458 and 461 2°–329 and 332
Creatinine	Ethyl ester of N-(4,6-dimethyl-2-pyrimidinyl)-N-methyl-glycine	1°–150 and 152 2°–223 and 225
Glucose[a]	α-D-glucofuranose cyclic-1,2 : 3,5-bis-(butylboronate)-6-acetate	1°–297 and 302 2°–355 and 361
Total Glyerides and Triglycerides	Butylboronic TMS ether of glycerol	1°–215 and 218 2°–185 and 187
Urea	6-Methyl-2,4-bis-[(trimethylsilyl)oxy]-pyrimidine	1°–255 and 257 2°–120 and 121
Uric Acid	Tetra[(1,1-dimethylethyl)-dimethylsilyl] derivative of uric acid	1°–567 and 569 2°–625 and 627

1° – Primary ion pair used for quantification
2° – Confirmatory measurement ion pair
[a] See Note added in proof and [31]

faced to a capillary column gas chromatograph (GC). Electric beam switching sweeps the ion beam across the collector slit to switch between masses selected for quantification (selected ion monitoring) in a rapid and precise fashion [3]. Principal measurements are generally made by monitoring a predominant electron impact (EI) ion from the target analyte and from its stable isotope labeled analog. To test for measurement bias, samples were remeasured by confirmatory analysis using alternate quantification ions generated by chemical ionization (vs. electron impact ionization). In some cases measurements were also repeated using chromatographic columns with a different polarity than the primary analytical column [18]. IDMS derivatives and primary and confirmatory IDMS quantification ion pairs used for the organic analytes are summarized in Table 2.

Quantification was achieved by determination of the abundance ratios between measurements of two standards whose ion intensity ratios for the unlabeled/labeled analyte pair (r/r*) bracket that of the sample. Each standard and sample was measured twice in succession. The two observed intensity ratios were considered acceptable if they agreed within 0.5%. If not, a third measurement was made, which had to agree within 0.5% of one of the other two. If they did, then the three were averaged to constitute one valid measurement. Each sample was measured on different days, and the calculated mass ratios had to agree within 0.5%, or the sample was remeasured on a third day. The average mass ratio of these two or three measurements was used to calculate the sample component concentration. The analyte concentration was calculated by linear interpolation of the measured ratio of the sample between the measured ratios of the standards. The primary (natural) and isotope enriched materials used for the preparation of these standards are listed in Table 1. Two stock solutions of labeled and unlabeled (native) materials were prepared independently, and a set of standards subsequently prepared from each. These standards were run against each other to check for errors, then interleaved for the calibration of sample measurements. The two sets of standards were intercompared and intracompared by IDMS, and any variation greater than 0.5% of the expected mass ratio was considered grounds for rejection of that standard. For each analyte measured, two or three sets of samples were prepared. Each set contained three vials of SRM 909b level I, three vials of SRM 909b level II, and one or two control vials of SRM 909a, level I and/or level II. Each sample represented one aliquot from one vial. For glucose, cholesterol, total glycerides, and triglycerides only, three sets of samples and three independent sets of standards, one for each set, were prepared.

Cholesterol

SRM 909b was analyzed for cholesterol by GC-IDMS, following the established definitive method [3]. The method consists of spiking a weighed aliquot of serum with a known mass of cholest-5-en-25,26,27-$^{13}C_3$-3-ol (cholesterol-$^{13}C_3$), and treating the sample with alcoholic KOH to hydrolyze the cholesterol esters. The mixture of labeled and unlabeled cholesterol is then extracted with hexane. This extract is dried, and the cholesterol is converted to the corresponding trimethylsilyl (TMS) ethers with bis(trimethylsilyl)acetamide (BSA). Primary measurements were performed using ions at m/z 458/461. Confirmatory measurements, run on a selected subset of samples by measuring different masses, i.e., m/z 329/332 under the same GC/MS conditions, were in excellent agreement with the principal measurements.

Creatinine

SRM 909b was analyzed for creatinine (2-amino-1,5-dihydro-1-methyl-4H-imidazol-4-one) by GC-IDMS. The procedure consists of spiking samples with a known mass of labeled creatinine (creatinine $^{13}C_2$), equilibration with the native material, chromatographic separation of interferences, and preparation of derivatives [5]. A closely related compound present in human serum, creatine (N-[aminoiminomethyl]-N-methylglycine), forms the same derivative, the ethyl ester of N-(4,6-dimethyl-2-pyrimidinyl)-N-methylglycine, as the analyte compound. Failure to remove creatine completely from the sample extract will result in a positive bias and inaccurate quantification. For this work, creatinine-^{14}C (3.52 mCi/mmol) and creatine-^{14}C (9.00 mCi/mmol) were used to develop column chromatography conditions that would ensure complete separation of the native and isotope-labeled creatinine from native creatine found in the reconstituted sera. The equilibrated serum samples were passed through a 5 mL column of Amberlite[1] IRC-50 resin (H+ form), slurry packed in 0.1 mol/L HCl in a 20 cm × 10 mm chromatography column. The creatine was eluted with 15 column volumes of water and this fraction was discarded. The creatinine was subsequently eluted with 15 column volumes of 1.0 mol/L ammonia. This fraction was shell frozen in round bottom flasks for lyophilization. The lyophilized material was dissolved in 95% ethanol, and reduced to minimum volume (~3 mL) in a rotary evaporator.

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

Derivatives for GC-IDMS analysis were prepared in absolute ethanol, using acetic acid with 2,4-pentanedione. The principal measurements were made on a non-polar (5%-phenyl dimethylpolysiloxane) GC column. The principal measurements were of the abundances of the $[M\text{-}COOC_2H_5]^+$ ions at m/z 150 and 152.

Glucose

Glucose was measured according to the published GC-IDMS definitive method [6]. A weighed aliquot of serum was spiked with uniformly labeled glucose-^{13}C, and allowed to equilibrate. The sample was deproteinized and deionized. D-glucose was converted into the dibutylboronate derivative of glucose by reaction with 1-butylboronic acid in anhydrous pyridine. Next, this derivative was acetylated with acetic anhydride to form α-D-glucofuranose cyclic 1,2 : 3,5-bis(butylboronate)-6-acetate (glucose BBA). The glucose-BBA peak was well separated from the other monosaccharide BBA derivatives formed. The $[M-57]^+$ EI ions at m/z 297 and m/z 302 were monitored for the primary IDMS measurements.

Urea

For each sample a weighed aliquot of serum was added to an appropriate amount of labeled urea-^{18}O solution. The solutions were mixed thoroughly and allowed to equilibrate at room temperature overnight. The liquid phase was then reduced by vacuum centrifugation. The subsequent sample preparation steps are described in detail in the established GC-IDMS definitive method [6]. Briefly, a measured amount of the supernatant, calculated to contain approximately 600 μg total urea, was then transferred to a micro-sublimation apparatus. The sublimed urea was carefully transferred to a small reaction vial and prepared for derivatization. Investigations are currently under way at NIST to replace the somewhat tedious micro-sublimation with solid phase extraction cartridges. Diketene and glacial acetic acid were added to the dried sample to form 6-methyluracil. This material was treated with 50 μL to 100 μL of BSA to form 6-methyl-2,4-bis[(trimethylsilyl)oxy]pyrimidine. Principal IDMS measurements were made at in the EI mode monitoring the $[M-15]^+$ ions at m/z 255 and 257.

Uric Acid

Uric acid was measured according to the established GC-IDMS definitive method [7]. For each sample a weighed aliquot of serum containing 77 μg–94 μg of native uric acid was added to an appropriate amount of 1,3-$^{15}N_2$ uric acid solution. It is essential to maintain careful control of the ratio of ammonium hydroxide : uric acid to avoid decomposition of the uric acid, yet still maintain its solubility [19]. The samples were mixed thoroughly and allowed to equilibrate at room temperature overnight. An ion-exchange column was used to isolate the uric acid. N-methyl-N-(*tert*-butyldimethylsilyl)-trifluoroacetamide with 1% imidazole was added to each vial to form the derivative for GC-IDMS analysis. Principal IDMS measurements were made in the EI mode by monitoring the ions at m/z 567 and 569. Confirmatory measurements were performed using chemical ionization (CI). Ammonia was used as the CI reagent gas for measurement of the $[M + H]^+$ ion of the uric acid derivatives (m/z 625/627), using the same non-polar GC column used for the principal measurements.

Total glycerides and triglycerides

Total glycerides and triglycerides were determined for the first time in SRM 909b, using newly developed DMs [9]. Total glycerides are defined as the sum of free glycerol and the mono-, di-, and triglycerides. Triglycerides are defined as the pure triglyceride species [4]. Briefly, the total glycerides method consisted of spiking a weighed aliquot of serum with a known mass of ^{13}C-tripalmitin. Alkaline hydrolysis converted the glyceride species to glycerol, and each sample was then passed through a deionizing resin column. The glycerol in the eluate was converted first to a butylboronic ester, then converted to the TMS ether of the butylboronic ester.

For the measurement of triglycerides, the hydrolysis and derivatization steps were the same as for total glycerides. The triglycerides were isolated for separate measurement by chloroform : methanol extraction of the spiked serum, followed by solid phase extraction to remove any mono- or diglycerides. The $[M-15]^+$ EI ions were monitored at m/z 215 and 218. Confirmatory measurements were made on a subset of the samples using a moderate polarity GC column and a different pair of EI ions (m/z 185/187) than were used for the principal measurements.

Determination of inorganic analytes

Lithium, magnesium, potassium, calcium, and chloride – TI-IDMS

The theory and application of TI-IDMS is well established [20–22]. Quantification was accomplished by adding a known amount of enriched isotope (spike) to the sample, equilibrating isotopes, and measuring the altered mass ratio with a thermal ionization mass spectrometer. The analyte was chemically purified to achieve a stable ion beam and reproducible fractionation pattern for measurement of mass ratios. Analytical blank was assessed by processing a small amount of enriched isotope in a manner similar to the samples. The sample concentration was calculated from the amount of spike added, the measured isotopic composition of the natural and enriched materials, and the measured altered sample ratio after subtraction for analytical blank.

Samples from eight vials of each level of SRM 909b were analyzed. Two vials of each level of SRM 909a served as control samples. Sample manipulation was done in a class 100 clean room. Weight aliquots of the appropriate enriched isotope solution listed in Table 1 were added individually to each subsample. For the determination of Mg, a spike mix containing enriched ^{25}Mg and ^{26}Mg with resulting isotopic composition of $^{24}Mg = 0.7\%$, $^{25}Mg = 50.0\%$, and $^{26}Mg = 49.3\%$ was used. This allowed for quantification using either the $^{24}Mg/^{25}Mg$ ratio or the $^{24}Mg/^{26}Mg$ ratio and for investigations related to internal normalization. Results reported here were calculated using the $^{24}Mg/^{25}Mg$ mass ratio after an external correction for fractionation. The concentration of enriched isotope in each solution was determined by reverse IDMS against two gravimetrically prepared solutions of the appropriate primary standard listed in Table 1.

For the determination of Li, Mg, K, and Ca, 2.5 g sub-samples were processed. Equilibration was achieved by wet ashing with HNO_3 and $HClO_4$ until a clear solution was observed. Samples were taken to near dryness several times with H_2O and HCl in preparation for separations. The residual salts were redissolved in a minimum volume of water and transferred to clean, conditioned cation exchange columns (AG50W-X8, 100–200 mesh, 0.7 cm × 10 cm). Potassium, Mg, and Ca were purified and separated by gradient elution with HCl alone. In contrast to previous serum determinations [11–13], Li was separated using 0.5 mol/L HCl in a volume fraction of 80% methanol to achieve better separation of Li from Na impurities. The elution profile for each sample was followed in real time by use of a flame test. Separated fractions were taken to dryness with $HClO_4$ to decompose residual organics from the column and converted to the appropriate salt. Magnesium and Ca were converted to nitrates and K to the chloride. Li was converted to LiOH by passing the salts through an anion exchange column (AG 1X8) conditioned with high purity ammonium hydroxide. At least three analytical blank samples were processed with each level.

For the determination of chloride, subsamples of approximately 0.3 g were processed in clean polyethylene centrifuge tubes. Samples were diluted to approximately 6 mL with dilute nitric acid. One mL of ammonium molybdate (50 g/L) was added to each tube to precipitate protein material that was then removed by centrifugation. Chloride was separated from the supernatant by the addi-

tion of 1 mL of freshly prepared silver nitrate (20 g/L). The tubes were allowed to stand in the dark for 10 min, following which the silver chloride precipitate was separated by centrifugation. The precipitate was thoroughly washed with dilute nitric acid and redissolved in 1 mL 50% (v/v) ammonium hydroxide solution. The precipitation step was repeated by the drop-wise addition of 1% (v/v) nitric acid and the silver chloride was finally dissolved in 0.75 mL 50% (v/v) ammonium hydroxide.

Isotope ratios were measured on solid source mass spectrometers built at NIST with 30 cm radius of curvature, and 90° magnetic sectors. Samples were thermally ionized from degassed, high purity Re filaments. Signal intensities were measured by Faraday cup collection. Mass spectrometric procedures for the determination of K, Ca, and Cl have been described in detail [14, 10, 12]. Triple filament procedures were used for the determination of K and Ca, loading 10 µg and 1 µg, respectively. Measured $^{41}K/^{39}K$ ratios were externally corrected for fractionation by measurement of SRM 999, however for the determination of Ca, data were internally normalized using the measured $^{44}Ca/^{40}Ca$ and $^{42}Ca/^{40}Ca$ ratios. Chloride was determined using a negative ion Re triple filament procedure. A total of 15 µg of sample was loaded, and fractionation of the $^{35}Cl/^{37}Cl$ ratio was corrected externally by measurement of SRM 975.

Mass spectrometric procedures applied to the determination of Mg and Li were different from previous work [11, 13]. Magnesium was determined using a silica gel procedure. Three µL of sample solution containing 3 µg of Mg in the nitrate form was dried onto the Re sample filament with 3 µL of silica gel and 5 µL of 2.25 mol/L phosphoric acid. The filament was brought to red heat in air for 5 s and loaded into the mass spectrometer. The sample was heated to 1500° C for 30 min before the start of data collection. During this period, the $^{23}Na^+$ signal was monitored. Excess $^{23}Na^+$ causes the formation of secondary electrons resulting in a dip in the Mg baseline. Two blocks of ratios consisting of two sets each of $^{25}Mg/^{24}Mg$ and $^{26}Mg/^{24}Mg$ ratios were taken. The baseline signal was measured before and after each block of ratios to monitor the Na signal. Measured ratios were externally corrected for fractionation by normalizing the measured composition of an unspiked Mg standard to that determined by Catanzaro et al. [23].

The mass spectrometric determination of Li involved use of a positive ion Re triple-filament procedure, heating only the ionizing filament. To compensate for the increase in the peak width due to the effects of mass dispersion, the resolution of the mass spectrometer was decreased to approximately 200. The procedure consisted of loading 2.5 µL of 0.25 mol/L H_3PO_4 followed by 500 ng of LiOH onto each of two outgassed Re side filaments. The side filaments were dried under an infrared heat lamp for 15 min. The assembled filaments were mounted into the mass spectrometer, and the center filament heated in stages until a stable ion beam was obtained. After a 60 min conditioning and background stabilization, multiple blocks of $^7Li/^6Li$ isotope ratios were measured. Baseline measurements were made at a single position approximately midway between the mass 6 and 7 peaks. External fractionation corrections were determined by measurement of IRM 016, a Li isotopic standard, and applied to spiked and natural samples.

Chloride – Coulometry

Chloride was measured by coulometry as well as by IDMS. The coulometric determination of chloride is based on the titration of electrogenerated Ag^+, which reacts with the Cl^- in the serum sample to form AgCl. Biamperometric detection detects excess Ag^+ present after the endpoint. Systematic sources of bias are eliminated by performing a pre-titration in the presence of the serum matrix prior to the titration using the same parameters used to detect the analytical endpoint. The amount of Ag^+ generated between these two endpoints is calculated from Faraday's Laws and is equivalent to the amount of Cl^- present in the sample.

For the coulometric analyses, 16 vials of candidate SRM 909b, eight at level I and eight at level II, were analyzed in randomized order. SRMs 909 (one level) and 909a (levels I and II) were used as controls. Chloride determinations were performed on a coulometric analyzer. Five aliquots were analyzed from each sample and control vial. The supporting electrolyte consisted of 0.9 g polyvinyl alcohol, 0.63 g HNO_3, 10.5 g CH_3COOH, and 0.58 g NaCl per 100 mL solution.

Sodium – Gravimetry

Sodium, which is monoisotopic, was determined by gravimetry. Sodium was isolated from the serum matrix using cation exchange chromatography. Sulfuric acid was added to the resulting solution which was evaporated to dryness and ignited to sodium sulfate. Solutions eluting before and after the collected sodium fraction were analyzed for sodium by flame atomic emission spectrometry (FAES) to ensure complete recovery.

Sodium was determined in four samples of 909b level I and five samples of 909b level II using the methods described in Moody and Vetter [15] with a modification of the wet ashing procedure. For the Na determinations the entire 10 mL reconstituted sample was taken. Nominal 10 g aliquots of reconstituted serum were wet ashed with a mixture of 10 mL of high purity nitric acid [24] and 2 mL of hydrogen peroxide in microwave-oven digestion vessels. The digested samples were transferred to Teflon beakers and evaporated to near dryness. The salts were redissolved with water and eluted through an ion-exchange column with 0.4 mol/L HCl to recover the Na fraction. Solutions eluting before and after the collected Na fraction were analyzed for Na by flame atomic emission spectrometry (FAES). After a H_2SO_4 solution was added to the collected Na fraction, the solution was evaporated to fumes of sulfur trioxide and then to dryness. Addition of $(NH_3)_2CO_3$ to the precipitate was followed by ignition of the sample to constant mass as Na_2SO_4 at 900° C. The Na was determined by dividing the blank corrected mass of Na_2SO_4 by a gravimetric factor of 3.08927 [25] to determine the mass of Na. Sodium determined gravimetrically was added to the amount of Na determined by FAES to calculate the total Na.

Density

Density was determined in a pooled sample consisting of five serum samples. A mass of diluent water, corrected for air buoyancy and equal to 10.00 mL of water at ambient temperature (measured to the nearest 0.1° C), was added to the freeze-dried serum. A pycnometer was filled with serum and capped with a ground glass thermometer. Excess liquid was removed from the pycnometer exterior, the sidearm was capped and the temperature of the mass of serum contained in the pycnometer was determined. The pycnometer was calibrated with distilled water in a similar manner. The volume of the pycnometer was determined using a density value for water of 0.9977683 g/mL at 22.0° C. The determined density values were 1.0221 g/mL with an uncertainty of 0.0002 g/mL for level I and 1.0370 g/mL with an uncertainty of 0.00003 g/mL for level II.

Results

The combined concentrations and uncertainties of the inorganic and organic analytes for SRM 909b are given in Table 3. Each certified value is the mean of the DM determinations and represents the analyte concentration when the freeze-dried serum is reconstituted with 10.00 ± 0.02 mL of the provided diluent. Concentrations were assessed on a mass basis and converted to a volumetric basis using the determined density. Table 3 lists concentrations in units of mmol/L and mmol/L/g to accommodate the two different analytical protocols available for the use of SRM

Table 3 Certified Concentrations and Uncertainties in SRM 909b, Human Serum

Analyte	Level	Concentration (mmol/L)	Expanded Uncertainty (95% / 95%)[a]	Concentration (mmol/L/g)	Expanded Uncertainty (99%)[b]
Calcium	I	2.218	± 0.016	2.5289	± 0.0080
	II	3.532	± 0.028	2.3416	± 0.0067
Chloride	I	89.11	± 0.57	101.65	± 0.25
	II	119.43	± 0.85	79.40	± 0.17
Cholesterol	I	3.787	± 0.047	4.315	± 0.031
	II	6.084	± 0.077	4.030	± 0.026
Creatinine	I	0.05618	± 0.00055	0.06397	± 0.00036
	II	0.4674	± 0.0053	0.3110	± 0.0019
Glucose[c]	I	5.40	± 0.28	6.16	± 0.21
	II	15.0	± 1.1	9.97	± 0.50
Lithium	I	0.6145	± 0.0050	0.6997	± 0.0027
	II	2.600	± 0.023	1.7235	± 0.0061
Magnesium	I	0.7634	± 0.0050	0.8703	± 0.0022
	II	1.918	± 0.021	1.2723	± 0.0062
Potassium	I	3.424	± 0.025	3.903	± 0.011
	II	6.278	± 0.052	4.166	± 0.012
Sodium	I	120.76	± 0.92	137.15	± 0.45
	II	141.0	± 1.3	93.30	± 0.31
Glycerides (Total)	I	0.949	± 0.061	1.080	± 0.046
	II	1.529	± 0.035	1.014	± 0.013
Triglycerides	I	0.804	± 0.011	0.9153	± 0.0074
	II	1.271	± 0.014	0.8428	± 0.0049
Urea	I	5.51	± 0.15	6.29	± 0.11
	II	30.75	± 0.32	20.376	± 0.089
Uric Acid	I	0.2809	± 0.0051	0.3194	± 0.0036
	II	0.7579	± 0.0090	0.5019	± 0.0029

[a] Each expanded uncertainty derived from the 95% confidence, 95% coverage statistical tolerance intervals were computed as a multiplier times the material standard deviation [26]
[b] Each expanded uncertainty, computed according to the CIPM method [27], is at the 99% level of confidence
[c] Glucose was de-certified by SRMP 31 Oct 97

909b. Figure 1 shows results obtained for Mg given both on a mg/dL basis and after normalization for vial fill mass (mmol/L/g). The figure demonstrates the lower uncertainty obtained, particularly for level I determinations, by normalizing to the vial fill mass.

The expanded uncertainty listed in Table 3 for results expressed on a mmol/L basis is a 95% confidence, 95% coverage statistical tolerance interval, and reflects the combined effects of measurement replication and the variability of the mass of dry serum among vials. The distribution of the vial fill masses for level I was Gaussian whereas for level II the distribution was slightly skewed. The relative standard deviation of the fill masses was the estimate used for the material standard deviation of each level. Expanded uncertainties derived from the 95% confidence, 95% coverage statistical tolerance intervals were computed as a multiplier times the material standard deviation [26]. Since the material variance was the same for all analytes from each level, the expanded uncertainty in Table 3 was smaller when measurement replication was more precise. The interval defined by the certified value and expanded uncertainty includes the concentrations for 95% of all the vials of SRM 909b, at a confidence level of 95%, when reconstituted with 10.00 ± 0.02 mL of diluent.

To meet high precision end-user needs, concentrations normalized to the fill mass of the vial are given. In this manner measurement uncertainty becomes the main component of the total uncertainty estimate. Normalized concentrations and the associated uncertainty estimate are given in columns 5 and 6 of Table 3. Each expanded uncertainty, computed according to the CIPM method [27], is at the 99% level of confidence. Each certified value and expanded uncertainty defines a range of values within which the true concentration is expected to lie with approximately 99% confidence.

Discussion

Measurement uncertainty was computed from Type A (random) and Type B (systematic) components of uncertainty. The following discussion enumerates the effort to minimize and adequately assess components of uncertainty in each DM measurement used for the certification of SRM 909b.

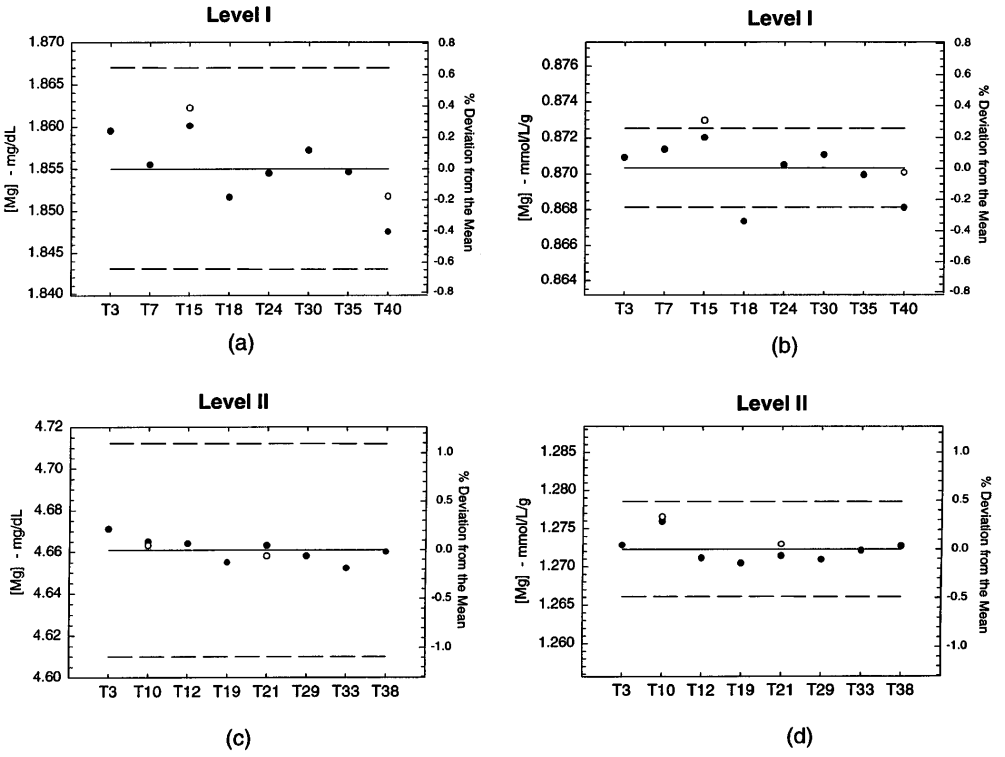

Fig. 1 a Concentration of Mg (mg/dL) in the eight different vials used for certification of Level I. The open circles represent replicate analyses drawn from the respective vials, while the dashed lines represent the 95%/95% statistical tolerance interval reflecting the combined effects of measurement imprecision and the variability of the mass of dry serum among vials. The right hand scale shows the percent deviation from the certified concentration represented by the solid line. **b** Concentration of Mg (mmol/L/g) in the eight different vials used for certification of Level I, corrected for variations in the fill weight of the dry serum. The open circles represent replicate analyses drawn from the respective vials, while the dashed lines represent the 99% confidence interval. This expanded uncertainty defines the range of values within which the true concentration is expected to lie with approximately 99% confidence. The right hand scale shows the percent deviation from the certified concentration represented by the solid line. **c** As Fig. 1 a, but for Level II. **d** As Fig. 1 b, but for Level II

Organics

The GC-IDMS methods used for the determination of organic analytes in SRM 909b fulfill the stringent requirements for National Reference System in Clinical Laboratories (NRSCL) DMs [28]. Generally, bias in the sample preparation steps are tested for by preparing multiple sets of samples and standards independently. The presence of measurement interferences is investigated by measuring sample/internal standard ratios using prominent mass peaks from electron impact ionization, followed by measurement of a subset of samples using other prominent electron impact ion pairs, using dissimilar GC columns, and/or by chemical ionization. The choice of the labeled analog used for spiking the serum was investigated for evidence of isotope effects. During the development of the DM for creatinine, for instance, the choice of labeled material was found to be critical to achievement of measurements with very low uncertainty. Use of creatinine-d_3 failed to yield reproducible ratios, likely due to isotope effects in the derivitization reaction. It was necessary to synthesize creatinine $^{13}C_2$ in-house [29], and use of this material subsequently yielded highly reproducible results. In addition, the derivative chosen for a given analyte was investigated to determine if it yields reproducible behavior in the GC system as well as measurement results with low uncertainty. For instance, the TMS derivative of cholesterol was tested for chromatographic system memory effects by sequentially injecting the unlabeled cholesterol derivative, the labeled derivative, and the unlabeled derivative, and measuring each corresponding isotope ratio. The ratios for the injections of the unlabeled material were not significantly different, proving the absence of memory effects. In summary, these GC-IDMS methods have been exhaustively investigated and found to be suitable as DMs.

For the measurements of the organic analytes in SRM 909b, standard cross checks and confirmatory measurements all demonstrated that the methods were performing properly. Tests for consistency between independent sets of standards, [(measured ratio – mass ratio) × 100/mass ratio] range from 0.10% to 0.18%. In addition, control measurements of SRM 909a were included in each set of 909b samples. Results for the control material determinations (Table 4) demonstrate good agreement with the certified values. Confirmatory measurements were also made as required to confirm the absence of error in the analytical measurements.

For most of the organic analytes, uncertainties were about as low or lower for 909b as they were for 909 or

909a. The uncertainties for glucose were somewhat higher, however. Glucose is the least stable of the organic analytes, yielding the highest relative expanded uncertainty (5.04% for level II mmol/L/g measurements), and showing evidence of degradation over time in similar natural matrix reference materials. Control (SRM 909a) values for glucose were 94.3% and 95.1% of the level I and level II certificate values (determined in October 1990), respectively. These values were consistent with in-house studies which have shown glucose to degrade at approximately 1% per year. The stability of glucose in SRM 909b will therefore be monitored at two-year intervals. Studies are underway at NIST to determine the stability of glucose in fresh-frozen human serum materials. Another influence that can increase uncertainties is set to set differences. Probabilities for set-to-set differences were high for both levels of glucose and uric acid in SRM 909b. Confidence intervals for these analytes were therefore calculated based upon the normalized means from each of the three sets.

Inorganics

TI-IDMS

Components of a TI-IDMS analysis which affect the accuracy of results include, isotope equilibration, spike concentration, analytical blank correction, spectral interference, and mass fractionation [2]. Isotope equilibration is difficult to confirm experimentally, however upon addition of spike, samples were subjected to procedures resulting in complete dissolution. Spike isotope concentrations were determined by reverse IDMS against primary standards. Agreement among four spike calibration mixes was better than 0.1% (1 s) for Ca and K, and better than 0.2% (1 s) for Mg and Li. Level I and II samples were processed at different times, and a spike calibration performed with each determination. Agreement between repeat calibrations was better than 0.1% for Li, K, and Mg, but a 0.26% increase in the concentration of the Ca spike solution was observed. This was probably due to evaporative loss.

Analytical blank was assessed by processing a small amount of spike in a manner exactly the same as the sample. Though 100% analyte recovery is not necessary for accurate quantification with isotope dilution, the accuracy of the blank correction depends on the blank to sample ratio. Therefore, the amount of sample recovered after dissolution and separation was experimentally determined and found to be greater than 95% in all cases. The magnitude of the correction for analytical blank was less than 0.05% for each analyte except level I Ca and level II Mg. In both cases the magnitude of the correction was 0.2%, and the observed variability in the samples can be explained by the variability in the measured blank.

To provide a measure of the magnitude and variability of analyte added to the dry serum upon reconstitution with the provided diluent, a 10 mL aliquot of the diluent was spiked and processed with the samples. The concentration of K, Ca, and Mg measured in the diluent water was significantly higher than measured in our laboratory water. The measured diluent water blank, though relatively constant, was 0.1% and 0.04% of the total Mg in the vial, and 0.09% and 0.06% of the total Ca in the vial for level I and level II samples, respectively. Since these amounts are included in the certified value, it is important that the diluent provided be used.

Systematic uncertainties in mass ratio measurement result from interferences in the mass spectrum and from mass fractionation in the spectrometer. Fractionation corrections were applied using isotopic standards and carefully controlling analysis conditions. However, contaminants have been shown to alter the fractionation pattern of samples relative to that obtained for the standards [14, 30]. Therefore, preliminary investigations on unspiked samples of SRM 909b were performed to assess the purity of separated analyte fractions. Potassium, Mg and Ca fractions in separated serum samples were analyzed by inductively coupled plasma mass spectrometry (ICP-MS)

Table 4 Control Concentrations (SRM 909 a) During Certification of SRM 909 b

Analyte	Level	Measured Concentration (mg/dL)	Certified Concentration (mg/dL)
Lithium	I	0.32	0.323 ± 0.006
	II	1.83	1.844 ± 0.026
Potassium	I	14.28	14.3 ± 0.3
	II	24.27	24.3 ± 0.4
Magnesium	I	2.11	2.10 ± 0.04
	II	4.45	4.49 ± 0.07
Calcium	I	9.32	9.31 ± 0.16
	II	13.41	13.4 ± 0.2
Chloride (IDMS)	I	326.2	328 ± 6
	II	426.2	422.2 ± 7.8
Chloride (Coul.)	I	328.43	328 ± 6
	II	425.26	422.2 ± 7.8
Sodium	I	341.4	341.4 ± 5.5
	II	290.7	291 ± 5
Cholesterol	I	188.97	189.2 ± 2.4
	II	172.73	172.6 ± 2.8
Creatinine	I	0.95	0.95 ± 0.01
	II	5.29	5.24 ± 0.07
Glucose	I	92.23	96.7 ± 2.3
	II	287.21	297.4 ± 9.5
Urea	I	33.6	33.2 ± 0.4
	II	117.1	116.9 ± 1.5
Uric acid	I	3.99[a]	3.93 ± 0.05
	II	9.01[a]	8.83 ± 0.15

Note: Triglycerides and total glycerides were not certified in SRM 909 a

[a] Uncertainties associated with these control measurements overlap the certified values

using the semi-quantitative scan mode and quantification with an In internal standard. The fractions were established to be sufficiently pure. Isobaric interference for K and Ca at mass 40 was determined to be less than 0.01% of the respective sample signal. Comparison of isotopic ratios in separated unspiked serum sample with high purity standards provided further confirmation of the absence of systematic errors in the measured ratios. Isotopic ratios for K, Mg, and Ca measured in separated, unspiked serum samples were not statistically different from isotopic ratios measured in the appropriate high purity standard. Such a comparison cannot be made for Li, as the natural isotopic composition of Li is variable [11]. However, a natural Li isotopic standard was separated in the same manner as the samples and found to be in agreement with the known composition.

As a final measure of analytical control, two subsamples from two vials of each level of SRM 909a were analyzed. The results are given in Table 4. All SRM 909a determinations are in agreement with the certified value. Total uncertainty estimates for TI-IDMS measurements were calculated from the standard error of the samples, spike calibration, and blank correction (Type A uncertainties). Uncertainty in knowledge of the calibrant assay was included as a Type B error. Uncertainty intervals for certification of all inorganic analytes in SRM 909b were lower than for SRM 909, and SRM 909a (Table 5), clearly demonstrating control of measurement process, and improvement of material homogeneity.

Gravimetry

The components of uncertainty for Na in SRM 909b were calculated as described in Moody and Vetter [15]. The relative combined Type A uncertainty, calculated from replication of standard recovery, blanks and measurements was 0.097% for level I and 0.078% for level II. The relative combined Type B uncertainty, calculated from mechanical loss, sample volume, ignition mass and the FAES measurement was 0.073% for level I and 0.059% for level II.

Comparison of coulometry and TI-IDMS for the determination of Cl

Agreement was obtained between the results for chloride determined by IDMS and by coulometry. Without correction for variations in fill mass, chloride was determined for level I at (315.39 ± 1.04) mg/mL by IDMS and (315.99 ± 0.35) mg/mL by coulometry. For level II, chloride was determined as (423.44 ± 0.97) mg/mL by IDMS and (423.34 ± 1.17) mg/mL by coulometry. On a mmol/L/g basis (corrected for variations in fill mass), the results for level I were (101.72 ± 0.49) mmol/L/g by IDMS and (101.59 ± 0.12) mmol/L/g by coulometry. For level II, the results were (79.57 ± 0.32) mmol/L/g for IDMS and (79.24 ± 0.18) mmol/L/g for coulometry. The variation between the two techniques likely results from slight variations in

Table 5 Improvement in Human Serum Standard SRM 909 Series

Analyte	Concentration (mmol/L) ± % Uncertainty[a]		
	909[b]	909a (level I)[c, e]	909b (level I)[d, e]
Calcium	3.02 + (5.6%) 3.02 – (2.0%)	2.322 ± (1.8%)	2.218 ± (0.72%)
Chloride	108 + (6.5%) 108 – (2.8%)	92.4 ± (1.8%)	89.11 ± (0.64%)
Cholesterol	3.68 + (6.0%) 3.68 – (2.2%)	4.892 ± (1.2%)	3.787 ± (1.2%)
Creatinine	–	0.084 ± (1.2%)	0.05618 ± (0.98%)
Glucose	6.62 + (6.6%) 6.62 – (3.0%)	4.95 ± (6.1%)	5.40 ± (5.2%)
Lithium	1.65 + (7.2%) 1.65 – (3.6%)	0.465 ± (1.7%)	0.6145 ± (0.81%)
Magnesium	1.21 + (11.6%) 1.21 – (8.3%)	0.868 ± (1.8%)	0.7634 ± (0.65%)
Potassium	3.52 + (5.4%) 3.53 – (1.7%)	3.656 ± (2.0%)	3.424 ± (0.73%)
Sodium	–	148.5 ± (1.6%)	120.76 ± (0.76%)
Total Glycerides	–	–	0.949 ± (6.4%)
Triglycerides	–	–	0.804 ± (1.4%)
Urea	–	5.535 ± (1.3%)	5.51 ± (2.7%)
Uric Acid	0.481 + (6.4%) 0.481 – (2.5%)	0.234 ± (1.3%)	0.281 ± (1.8%)

[a] Uncertainties are expressed as 95% confidence, 95% coverage statistical tolerance limits
[b] NBS (NIST) Certificate SRM 909, January 13, 1981 (first revision)
[c] NIST Certificate SRM 909a, July 24, 1991
[d] NIST Certificate SRM 909b, June 7, 1996 (first revision)
[e] 909a and 909b are supplied in low (level I) and high (level II) concentrations

the measurement procedure for the fill mass. The tighter relative uncertainties for level I demonstrate the higher precision which is attainable by correcting the analytical results for variations in fill mass. The SRM 909a results for Cl by both IDMS and coulometry lie within the certified confidence interval for this SRM and function as an effective check against gross error. The certified value for chloride is the mean of the TIMS and coulometry results.

Conclusion

NIST has successfully used DMs, primarily IDMS, to develop a series of materials with measurements of clinically significant analytes that most closely approach true value. The determinations by the DMs described in this discussion allowed for the certification of thirteen clinically significant analytes in SRM 909b, the most recent issue in a series of lyophilized human serum standards. Comparison of the certification results for level I of this standard with the previous releases of this material, SRM 909a and SRM 909, presented in Table 5, demonstrate that continual improvement in this series has been successfully realized. Improvement has been made in the number of analytes certified, from eight in 1981 to thirteen in 1996. Additional utility of these materials has been realized by the development of "high" and "low" level materials. Finally, NIST scientists have successfully identified and reduced components of variability related to both the manufacture of the serum materials, and the certification of the analytes.

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Note added in proof Glucose was decertified by SRMP on 31 October 1997 [31]. In previous SRM 909 Human Serum lots, the rate of glucose degradation has been approximately 1% per year. However, this current lot, SRM 909b Level II, was found to have a significantly higher rate of degradation of ca. 3% per year. The reason for this higher rate of degradation is not known; however, Level II has higher concentrations of other analytes including some proteins that may play a role. Because the rate is much higher than expected, certified values for glucose are no longer supplied for this material. If a serum-based reference material for glucose is needed, SRM 965 Glucose in Frozen Human Serum can be used.