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Certification of Standard Reference Material® 1955a Homocysteine in Frozen Human Serum

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Abstract

Standard Reference Material (SRM) 1955a Homocysteine in Frozen Human Serum is intended for use in validating homocysteine measurement procedures used by *in vitro* diagnostics manufacturers, reference laboratories, and clinical laboratories in the United States and globally. A unit of SRM 1955a consists of three vials of frozen human serum, each of a different homocysteine concentration. This publication documents the production, analytical methods, and computations involved in characterizing this product.

Keywords

Homocysteine; Isotope Dilution Liquid Chromatography Mass Spectrometry (ID-LC-MS); Reference Measurement Procedure (RMP); Standard Reference Material (SRM).

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Acknowledgments

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1. Introduction

Homocysteine (Hcy) is a sulfur-containing non-proteinogenic amino acid derived from the metabolism of methionine. The chemical structure of homocysteine is depicted in Fig. 1.

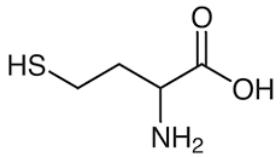


Fig. 1. Chemical Structure of Homocysteine.

Elevated concentrations of Hcy in blood are characteristic of a number of pathological conditions including vascular disease, cancer development, autoimmune diseases, and neurogenerative disease. While it remains unclear whether Hcy is a marker or a causative agent, Hcy concentration in blood is considered to be an important indicator of health status [1,2].

The National Institute of Standards and Technology (NIST) Standard Reference Material® (SRM®) 1955a Homocysteine in Frozen Human Serum is intended to support serum Hcy measurement processes of *in vitro* diagnostics manufacturers, reference laboratories, and clinical laboratories in the United States and globally. SRM 1955a is the second member of the SRM 1955 series. The original SRM 1955 Homocysteine and Folate in Frozen Human Serum [3] also provided certified values for folate. Folate measurements are now supported by SRM 3949 Folate Vitamers in Frozen Human Serum [4].

The sales history of SRM 1955 is displayed in Fig. 2 as a function of calendar year. The proportions of sales to customers in the US, Europe, Asia, and the rest of the world are displayed in Fig. 3, also as functions of calendar year.

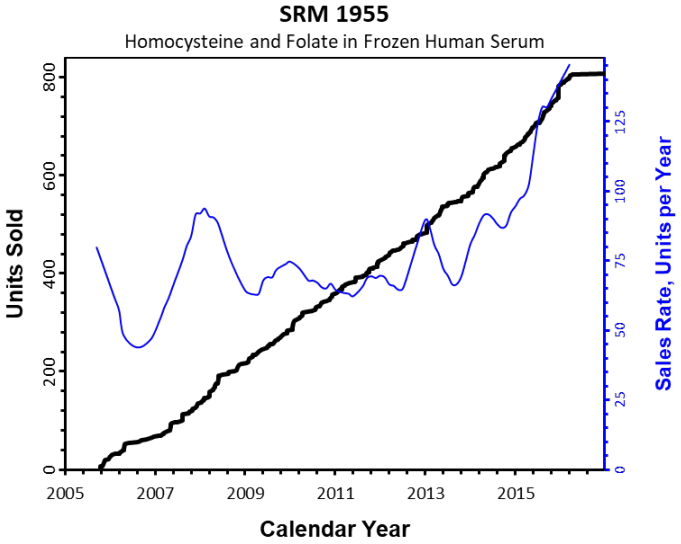


Fig. 2. Sales History of SRM 1955.

The thick black line depicts the cumulative distribution of sales as a function of the order date; it is plotted using the "Units Sold" axis at the left of the plot. The thin blue line depicts the total units sold per year; it is plotted using the "Sales Rate, Units per Year" axis to the right of the graph.

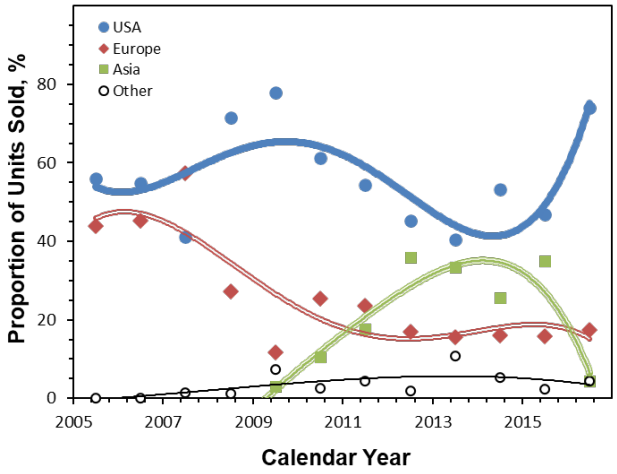


Fig. 3. Location of Customers for SRMs 1955.

The solid circles and the thick trendline display the proportion of sales to customers within the USA. Solid diamonds and the double-line trendline display the proportion of units sold to customers in Europe (including the United Kingdom); solid squares and the triple-line trendline display the proportion solid to customers in Asia. The open circles and thin single-line trendline display the proportion of units sold to customers elsewhere.

A unit of SRM 1955a consists of one vial each of three levels of Hcy: low, normal, and high. Each of the three vials contain approximately 1.1 mL of human serum. The Hcy concentration in these three materials was assigned using an optimized version of NIST's isotope-dilution liquid chromatography mass spectrometry (ID-LC-MS) method [5,6]. This method is recognized by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) as a reference measurement procedure (RMP) [7]. The RMP was validated on the current liquid chromatography-mass spectrometry (LC-MS) system.

The expected coefficient of variation expressed as a percentage (%CV, aka "relative standard deviation") for Hcy values assigned using the NIST RMP is (2 to 4) % [6]. The %CVs for the three levels of SRM 1955 range from (2.3 to 3.4) % [3], demonstrating that this uncertainty is fit-for-purpose.

Metrological traceability to the International System of Units (SI) of the assigned homocysteine values in the SRM 1955a materials is through calibration with a neat DL-homocystine that was purity assessed by ^{1}H-qNMR through determinations traceable to the NIST PS1 Primary Standard for qNMR (Benzoic Acid) [8,9]. The purity analysis characterized this material with respect to chemical components that yield Hcy entities when analyzed using the ID-LC-MS RMP.

Based on experience with SRM 1955, the Hcy levels of the SRM 1955a materials are expected to be stable for at least five years post-production. Stability will be evaluated within one year of the expiration date, with intermittent monitoring through its use as a control whenever Hcy in serum measurements are performed within NIST.

2. Production

In response to the following Statement of Work, a contract was awarded to Solomon Park Research Laboratories, Burien WA 98148, for the preparation of a minimum of 4,500 mL of pooled serum (1500 vials each of three levels) for SRM 1955a Homocysteine in Frozen Human Serum. The contractor packaged each set of 1500 vials into 30 boxes of 49 vials each and one box of 30 vials.

2.1. Statement of Work

2.1.1. Specific Tasks

Contractor shall produce 1,500 vials each of three serum materials at the concentrations specified below, with each vial containing (1.1 ± 0.1) mL of serum. The contractor shall acquire a minimum total of 4,500 mL of pooled serum according to the CLSI C37-A Guideline [10,11]. The lower level material (Level 1) can be diluted with phosphate buffered saline (PBS) to achieve the desired homocysteine target level. The normal level (Level 2) must consist of only pooled serum to achieve the target level. The high-level material (Level 3) can be attained by the addition of pure homocysteine to achieve the target homocysteine concentration. The homocysteine target value ranges for the materials are as follows:

Level 1: Pooled human serum, which can be diluted with PBS, containing homocysteine 3.7 µmol/L to 5.9 µmol/L.

Level 2: Pooled human serum containing homocysteine 8 µmol/L to 11 µmol/L.

Level 3: Pooled human serum, spiked with pure homocysteine, containing homocysteine 13 µmol/L to 18 µmol/L.

For the Level 1 material, PBS must be used to dilute the base serum to obtain the target level.

For the Level 3 material, a solution of 20 mmol/L homocysteine must be prepared first in base serum and then spiked into the pooled serum to obtain the target level.

The contractor is responsible for the screening analysis of homocysteine values, but the analysis can be subcontracted out. The results of these analyses must be submitted to NIST. Measured values for homocysteine shall be reported in units of µmol/L.

The contractor shall test the serum units for biosafety. All sera shall be demonstrated to be non-reactive when tested for hepatitis B surface antigen, hepatitis C virus, human immunodeficiency virus, and human immunodeficiency virus antigen 1 by tests licensed by the U.S. Food and Drug Administration. The contractor will provide written documentation stating the negative results of all donor units utilized for SRM 1955a preparation. Donor units testing positive for any of the stated infectious agents will not be included in the serum pools.

After the donor serum units are selected, they shall be pooled. The contractor shall thoroughly blend and filter the pools (0.22 µm polyvinylidene fluoride, PVDF) as defined in CLSI C37-A. The contractor shall dispense the serum into labeled amber glass serum vials capable of

withstanding ultracold temperatures (-80 °C), each containing nominally 1 mL of serum (1.1 mL dispensed with an accuracy better than 0.1 mL), and seal under nitrogen with a butyl rubber stopper and an aluminum crimp cap. The vials must be 3 mL amber serum vials, (17 × 37) mm, 13 mm crimp finish. The vials are available from Voigt Global Distribution (catalogue # 62413PV-3). The contractor must stress test the lot of serum vials prior to filling by picking 10 vials randomly from the lot and subjecting them to 5 freeze and thaw cycles at -80 °C to ensure that there is no breakage.

Prior to filling, the contractor shall label vials with labels that are appropriate for use at low temperature; these labels will be provided to the contractor by NIST within 60 days after award. The contractor shall use three different color aluminum crimp caps to differentiate among the three specified serum levels for easy identification. Vials shall be transferred, in fill order, from the bottling equipment to a box in a "Z" pattern, filling each row left to right. The location of the first vial in each box shall be noted on each side of the outside corner of the box, and boxes will be numbered sequentially. Boxes shall be labeled to indicate their contents. Materials shall be stored frozen (-80 °C) prior to overnight shipment on dry ice to NIST. Overnight shipments should not be sent on Friday/Saturday or before a Monday Federal Holiday. Delivery is expected within 180 days after award.

The Contractor shall provide NIST with details of the steps involved in material preparation not later than 180 days after award of this requirement to ensure documentation of any deviations as well as to provide information that shall be utilized in the NIST material acquisition Report of Analysis.

2.1.2. Deliverables and Deliverable Due Dates

The contractor shall deliver the vials and Biosafety report to NIST, using FOB Destination shipping terms not later than 180 days after award.

At the time of shipment, the contractor shall notify the NIST Technical Point of Contact (TPOC) by e-mail, providing the date the deliverables are shipped, shipping method, tracking number, method of delivery and the anticipated delivery date it will arrive at NIST.

2.1.3. Protection of Human Subjects

(a) Research involving human subjects is not permitted under this award unless expressly authorized in writing by the Contracting Officer. Such authorization will specify the details of the approved research involving human subjects and will be incorporated by reference into this contract.

(b) The Federal Policy for the Protection of Human Subjects (the “Common Rule”), adopted by the Department of Commerce at 15 CFR part 27, requires contractors to maintain appropriate policies and procedures for the protection of human subjects in research. The Common Rule defines a “human subject” as a living individual about whom an investigator conducting research obtains data through intervention or interaction with the individual, or identifiable private information. The term “research” means a systematic investigation,

including research development, testing and evaluation, designed to develop or contribute to generalizable knowledge. The Common Rule also sets forth categories of research that may be considered exempt from 15 CFR part 27. These categories may be found at 15 CFR 27.101(b).

(c) In the event that human subjects research involves pregnant women, prisoners, or children, the contractor is also required to follow the guidelines set forth at 45 CFR part 46 subpart B, C and D, as appropriate, for the protection of members of a protected class.

(d) Should research involving human subjects be included in the proposal, prior to issuance of an award, the contractor shall submit the following documentation to the Contracting Officer:

(1) Documentation to verify that contractor has established a relationship with an appropriate Institutional Review Board ("cognizant IRB"). An appropriate IRB is one that is located within the United States and within the community in which the human subjects research will be conducted;

(2) Documentation to verify that the cognizant IRB possesses a valid registration with the United States Department of Health and Human Services' Office for Human Research Protections ("OHRP");

(3) Documentation to verify that contractor has a valid Federal-wide Assurance (FWA) issued by OHRP.

(e) Prior to starting any research involving human subjects, the contractor shall submit appropriate documentation to the Contracting Officer for institutional review and approval. This documentation may include:

(1) Copies of the human subjects research protocol, all questionnaires, surveys, advertisements, and informed consent forms approved by the cognizant IRB;

(2) Documentation of approval for the human subjects research protocol, questionnaires, surveys, advertisements, and informed consent forms by the cognizant IRB;

(3) Documentation of continuing IRB approval by the cognizant IRB at appropriate intervals as designated by the IRB, but not less than annually; and/or

(4) Documentation to support an exemption for the project from the Common Rule [Note: this option is not available for activities that fall under 45 CFR part 46 subpart C].

(f) In addition, if the contractor modifies a human subjects research protocol, questionnaire, survey, advertisement, or informed consent form approved by the cognizant IRB, the contractor shall submit a copy of all modified material along with documentation of approval for said modification by the cognizant IRB to the Contracting Officer for institutional review and approval. The contractor shall not implement any IRB approved-modification without written approval by the Contracting Officer.

(g) No work involving human subjects may be undertaken, conducted, or costs incurred and/or charged to the project, until the Contracting Officer approves the required appropriate documentation in writing.

2.1.4. Acceptable Quality Level

The vials of all pooled serum with the associated physical and chemical properties specified in this Statement of Work must be suitable for use as reference materials. If deficiencies or inconsistencies between the material and the documentation listed in the Specific Tasks are found, or, if less than the stated number of vials are received intact, the contractor shall have 30 days to correct the deficiency at no additional cost to the Government.

2.1.5. Monitoring Method

The NIST TPOC will verify that the materials were successfully prepared and acceptable. Acceptance will be based upon the delivery of 1,500 vials of each pooled serum material (4,500 vials total) intact and unbroken, accompanied by screening results for homocysteine levels in each of the three pooled materials. This will be completed no later than 30 days after receipt and acceptance of the vials.

2.1.6. Minimum Contractor Qualifications

The Contractor shall provide documentation showing current certification for ISO 13485:2003, "*Medical devices -- Quality Management Systems -- Requirements for Regulatory Purposes*" and experience in analyzing homocysteine in human serum. Documentation may be a certificate from ISO or from a certification organization or a letter authorizing the Contractor to use the ISO logo.

Alternatively, if the Contractor is not ISO 13485:2003 certified, they must provide not less than three (3) each of the following records for homocysteine for a total of 15 records:

- Calibration records
- Quality control (QC) blank results
- QC spike amounts
- QC spike recovery
- Minimum detection limit determination data

The records shall be no older than three (3) years from the date of the solicitation. The Offerors shall provide the above records in hardcopy or as a pdf file.

2.1.7. Payment

One payment will be made for the final deliverables, which is the total value of the contract. NIST will accept the final deliverables no later than 30 days from receipt of the total quantity of 4,500 vials of blood serum. The Contractor shall invoice for the total price of the contract upon written acceptance.

2.1.8. Contractor's Certificate of Analysis

SOLOMONPARK
Improving the health of the Human Race

CERTIFICATE OF ANALYSIS

Client:	**NIST**
Purchase Order:	**1333ND21PNB640472**
Product:	**Homocysteine 1-3 SRM 1955a**
Lot Number:	**4310**
Production Date:	**Level 1: 02/22/2022** **Level 2: 02/23/2022** **Level 3: 02/24/2022**
Expiration Date:	**To be determined by client**
Source:	**Human Serum**
Form:	**Frozen serum produced by C37-A to pool and filter.** **1,500 vials x 1.1 mL per pool**
Final Values:	**Level 1: 6.5 µmol/L** **Level 2: 10.1 µmol/L** **Level 3: 14.3 µmol/L**
Preservative:	**None**
Storage:	**Keep at –70°C**
Shipping Temperature:	**Frozen (on dry ice)**

Tests performed on original donor units using Bio-Rad PR4100 plate reader and reagents from BioRad were non-reactive for HBsAg, HCV, HIV 1 (Groups M and O) and HIV 2. All materials consisting of, containing, or isolated from human sources should be considered potentially hazardous and handled with appropriate precautions. For In Vitro diagnostic use only.

Solomon Park Research Laboratories tel. 425.650.2000
658 S. 152nd St. Burien WA 98148 - email: info@solomon.org

2.2. Acceptance Measurements

Ninety three boxes (31 boxes for each level) containing the serum material for SRM 1955a Homocysteine in Frozen Human Serum were shipped on dry ice and received at NIST one day later. The materials were moved to -80 °C storage. The following day, four vials of each level were selected for acceptance measurements. Although the contractor's value for Level 1 was 0.6 µmol/L above the specified range, it was decided to still measure the material at NIST for acceptance since the level was sufficiently lower than Level 2, and close to the specifications.

Using the NIST RMP the amount concentration of Hcy (µmol/L) in each level of SRM 1955a was determined. SRM 1950 Metabolites in Frozen Human Plasma [12] was used as the control material. Table 1 compares the Contractor's and NIST's verification measurements with the specified ranges.

Table 1. Homocysteine Concentration Screening Estimates, µmol/L.

Material	Specification	Contractor	Verification
SRM 1950	8.3 to 8.7		8.78 ± 0.34
SRM 1955a Level $1_{original}$	3.7 to 5.9	6.5	7.65 ± 0.14
SRM 1955a Level $1_{replacement}$	3.7 to 5.9		5.70 ± 0.05
SRM 1955a Level 2	8.0 to 11.0	10.1	10.39 ± 0.18
SRM 1955a Level 3	13.0 to 18.0	14.3	16.37 ± 0.23

The verification measurements confirmed that the Level 2 and Level 3 materials had appropriate Hcy levels for SRM 1955a. The Level 1 material had a homocysteine level higher than the contractor's screening value and was deemed not acceptable for SRM 1955a. The contractor successfully prepared 1,500 vials of a replacement material which, when evaluated using the NIST RMP, was determined to be acceptable for use.

.

3. Method Optimization

The ID-LC-MS method described in [5,6] was reassessed and optimized with current liquid chromatography column technology. This section describes the improved separation of homocysteine (Hcy) using a hydrophilic bonded silica pentafluorophenyl (HS F5) and cyano bonded phase (CN) columns as well as the optimized conditions for sample preparation and analysis of Hcy. SRM 1950 Metabolites in Frozen Human Plasma was used as a control sample.

3.1. Materials

The DL-homocystine used to prepare calibrants was purchased from Fluka. The isotopically labeled internal standard (IS) was DL-homocystine-3,3,3′,3′,4,4,4′,4′-d_8, purchased from Cambridge Isotope Laboratories. DL-dithiothreitol (DTT), acetic acid, and formic acid (reagent grade) were purchased from Sigma-Aldrich. Hydrochloric acid was purchased from Taylor Scientific and sodium hydroxide pellets were purchased from Fisher Chemical. Solid phase anion extraction (SPAE) was achieved using Poly-Prep Prefilled Chromatography columns, purchased from BioRad. HPLC grade water and HPLC grade methanol were used in the preparation of calibration solutions, samples, and LC-MS mobile phases.

HPLC columns evaluated in this study included Discovery HS F5 25 cm × 4.6 mm, 5 µm particle size, Discovery HS F5 15 cm × 4 mm, 3 µm particle size, and Superlcosil LC-CN 7.5 cm × 4.6 mm, 3 µm particle size, which were all purchased from SupelCo (MilliporeSigma).

SRM 1950 Metabolites in Frozen Human Plasma was used as control samples. SRM 1950 is certified for Hcy at a concentration of (8.50 ± 0.20) µmol/L.

3.2. Material Preparation

3.2.1. Reagent Solution

The 1.5 % DTT aqueous solution was prepared by weighing 1.5 g DTT into a 100-mL volumetric flask, dissolving with approximately 60 mL of HPLC grade water, and then filling to the 100-mL mark with water. The 0.1 mol/L HCl solution was prepared by adding 0.305 mL HCl to approximately 70 mL HPLC grade water (in a 100-mL volumetric flask) then filling to the 100-mL mark with water. The 10 mol/L NaOH solution was prepared by weighing 4 g of NaOH pellets into a 100-mL volumetric flask, dissolving with approximately 70 mL of HPLC grade water, and then filling to the mark using water. The 1.5 % DTT in 0.15 mol/L NaOH solution was prepared by weighing 0.075 g of DTT into a 5-mL volumetric flask, dissolving with approximately 4 mL of HPLC grade water, adding 75 µL of 10 mol/L NaOH, then filling to the mark with water. The DTT solutions must be prepared fresh each day to ensure the proper reduction of serum samples. Mobile phases for HPLC were prepared by adding water or methanol into a 1 L volumetric flask, then adding 1 mL of formic acid before bringing the final volume to the mark with solvent.

3.2.2. Calibrant and Internal Standard

The Hcy calibration stock solutions were prepared by weighing 10 mg of commercially available homocystine, which was then reduced to Hcy with the addition of 10 g of 0.1 mol/L HCl, 100 µL of 10 mol/L NaOH, and 0.1 g of DTT. Three batches of Hcy stock solutions were prepared, each with an approximate concentration of 7400 µmol/L Hcy; one prepared and then a week later two more prepared. Each stock solution then divided into 1 mL aliquots and stored in 2 mL Eppendorf tubes at -80 °C. Homocysteine-d_4 (Hcy-d_4) was used as the IS; it was prepared by weighing (1 to 3) mg of homocystine-d_8 (lower weight due to the low solubility) and reducing with the same amount of HCl and neutralized with NaOH/DTT. The resulting Hcy-d_4 IS solution was then divided into 1 mL aliquots and stored in 2 mL Eppendorf tubes at -80 °C. Hcy calibrants were then prepared by a series of dilutions of the Hcy stock solution. First the stock was diluted to around 740 µmol/L, then to 74 µmol/L, and then to concentrations of (3.5, 5, 7, 10, 12, 15, 18, 25, and 50) µmol/L using 1.5 % DTT aqueous solution. The Hcy-d_4 IS stock was diluted to around 74 µmol/L and the appropriate amount was spiked into each calibrant to achieve a final concentration of approximately 10 µmol/L. All preparations were gravimetric using a Mettler Toledo XPE205 analytical balance.

3.2.3. Samples

The SRM 1950 samples were prepared following an established procedure where 200 µL of the serum sample is spiked with 10 µmol/L IS, reduced using 400 µL of 1.5 % DTT in 0.15 mol/L NaOH solution, heated to 40 °C for 15 min, then followed by clean-up step using a SPAE column. Samples were dried then reconstituted using 200 µL of 1.5 % DTT aqueous solution, transferred to a HPLC autosampler vial with 300-mL glass insert and stored at (2 to 8) °C until ready for LC-MS analysis.

3.3. LC-MS Method

All analyses were performed on an Agilent 1200 Series LC system with an Agilent 6130 Quadrupole LC-MS. This instrument was equipped with a binary pump, degasser, autosampler, column compartment, and a single quadrupole MS with electrospray ionization. The instrument was controlled using ChemStation software (Masshunter, Agilent). Separations were carried out on Supelco columns; (1) Discovery HS F5 25 cm × 4.6 mm, 5 µm particle size, (2) Discovery HS F5 15 cm × 4 mm, 3 µm, and (3) Superlcosil LC-CN 7.5 cm × 4.6 mm, 3 µm.

Mobile phases A was HPLC grade water with 0.1 % formic acid and mobile phase B was HPLC grade methanol with 0.1 % formic acid. The separation conditions included a 0.5 mL/min flow rate, 30 °C column temperature, and the mobile phase program summarized in Fig. 4.

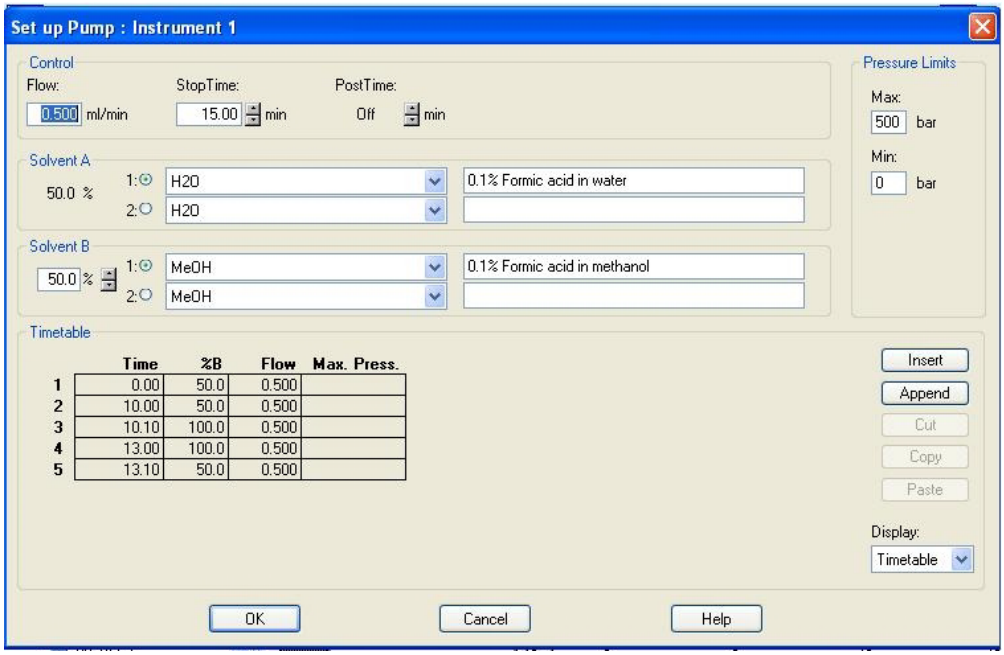


Fig. 4. Mobile Phase Gradient for the LC-MS Method.

All injection volumes were 10 µL. Detection was achieved using a single quadrupole MS with electrospray ionization in positive mode, set to detect ions at *m/z* 136 (Hcy), *m/z* 140 (Hcy-d_4), *m/z* 241 (homocysteine-cysteine disulfide), *m/z* 269 (homocystine), and *m/z* 277 (homocystine-d_8), as shown in Fig. 5.

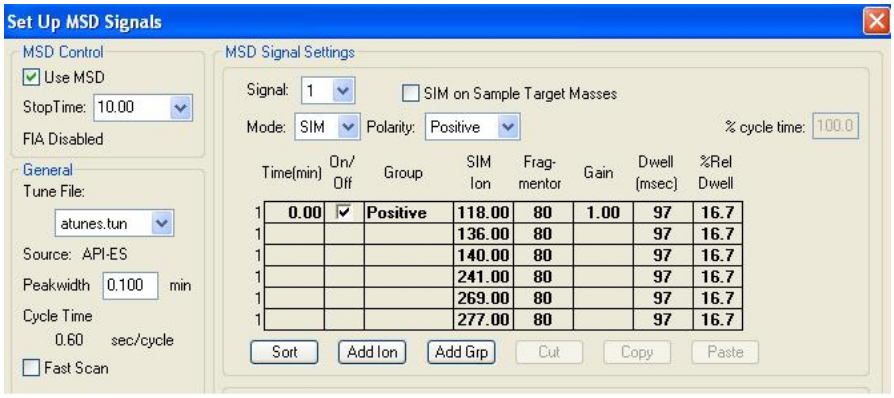


Fig. 5. MS Detection Parameters.

3.4. Column Comparison

The initial work was performed using a Discovery HS F5 (25 cm × 4.6 mm, 5 µm) column. Using SRM 1950 samples that were prepared (described in Section 3.2.3) but reconstituted in HPLC grade water only to reduce the interference of DTT, the percent mobile phase B was tested and compared. The results showed that at isocratic elution of (10, 20, and 30) % mobile phase B resulted in good peak shape with retention times of (10 to 13) min. Representative chromatograms are displayed in Fig. 6.

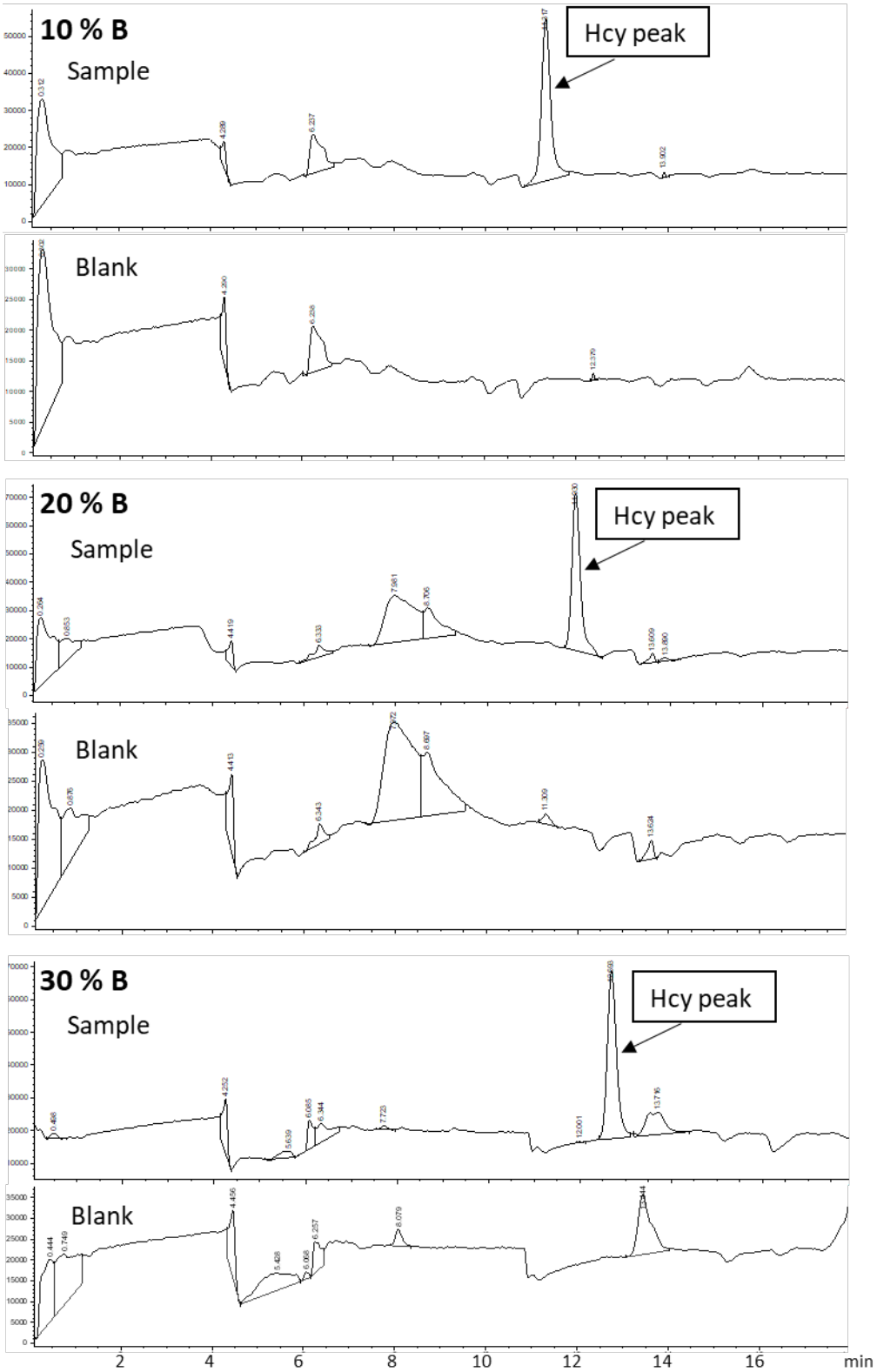


Fig. 6. Chromatograms on 25 cm HS F5 Column at (10, 20, 30) % Mobile Phase B.

A small peak was consistently present in these chromatograms for both sample and blank. At 10 % mobile phase B the peak eluted at around 6.2 min, at 20% mobile phase B it eluted at 7.9 min, and at 30 % it eluted right after the main Hcy peak at 13.1 min. However, since none of these peaks interfered with the separation and detection of the target peak, it seemed that the stationary phase of this column worked well for Hcy.

The Discovery HS F5 15 cm × 4 mm, 3 µm particle size was tested next to reduce the run time from 18 min to 15 min or less. Results revealed that the analysis time can be reduced to 10 min since the peaks after 10 min in the chromatogram were peaks that were also present in the water injection. Representative chromatograms are displayed in Fig. 7.

When comparing the different % B mobile phases on both F5 column configurations, the results are quite comparable. A similar pattern is seen where the sample preparation blank peak elutes earlier than the Hcy peak using 10 % or 20 % B, and elutes close to the Hcy peak using 30 % B. However, when using 40 % B, the sample preparation blank peak elutes at the same retention time as the Hcy peak. At 50 % B, the sample preparation blank peak is separated away from the Hcy peak, eluting later. Since both 20 % and 50 % B provide separation between the sample preparation blank peak and the Hcy peak, optimization of % B will be determined by the best target peak integrations and accurate peak area ratio calculations.

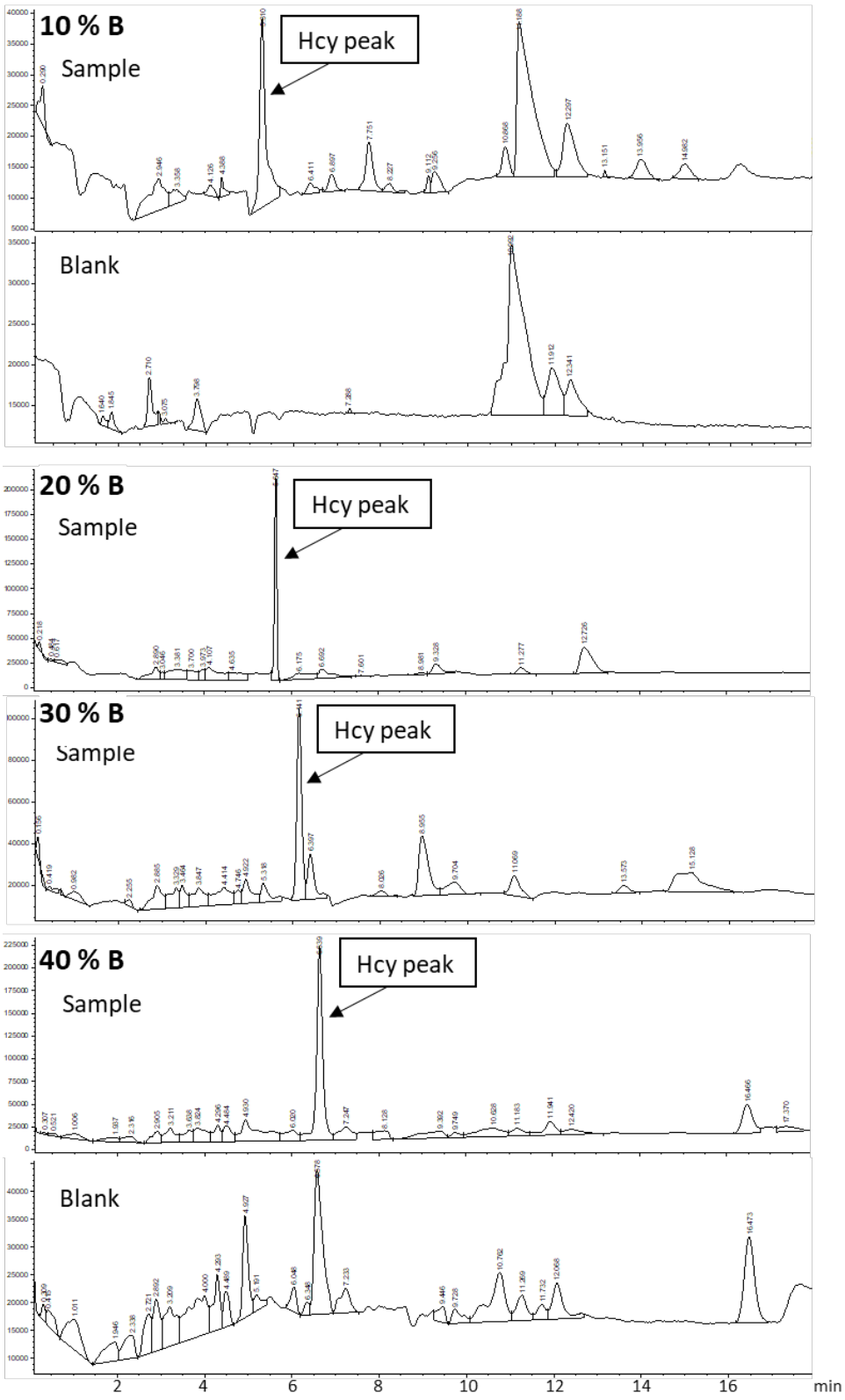


Fig. 7. Chromatograms on 15 cm HS F5 Column at 10 % Mobile Phase B.

The Supelcosil LC-CN (7.5 cm × 4.6 mm, 3 µm) column was then setup and the same comparison analysis was conducted. When using the same set of samples previously analyzed on the F5 columns, the Hcy peak was not clearly detectable as there were many other baseline changes. A previously prepared calibrant with a Hcy concentration of 35.2 µmol/L was used for further method development to ensure the highest concentration of Hcy injected onto the column. A clear peak was only detected at 50 % B, which eluted at 3.34 min. However, as seen in Fig. 8, the peak eluted in a section with a very noisy baseline.

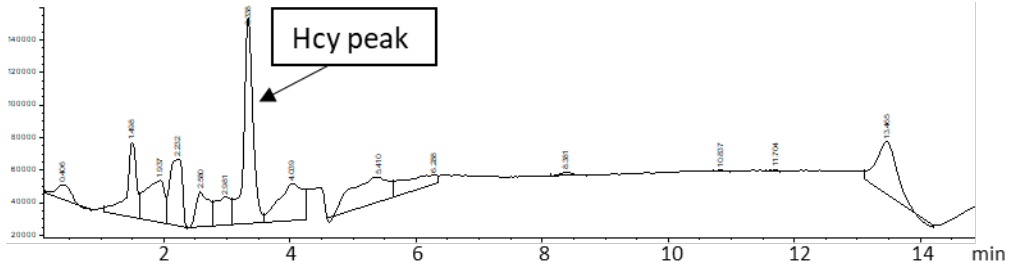


Fig. 8. Chromatogram on CN Column at 50 % Mobile Phase B.

Gradient elution conditions were tested on the Supelcosil LC-CN column using (4 to 95) % B in 10 min, (4 to 50) % B in 10 min and (4 to 30) % B in 10 min. As shown in Fig. 9, the Hcy peak eluted at a retention time at 2.76 minutes regardless of the gradient condition.

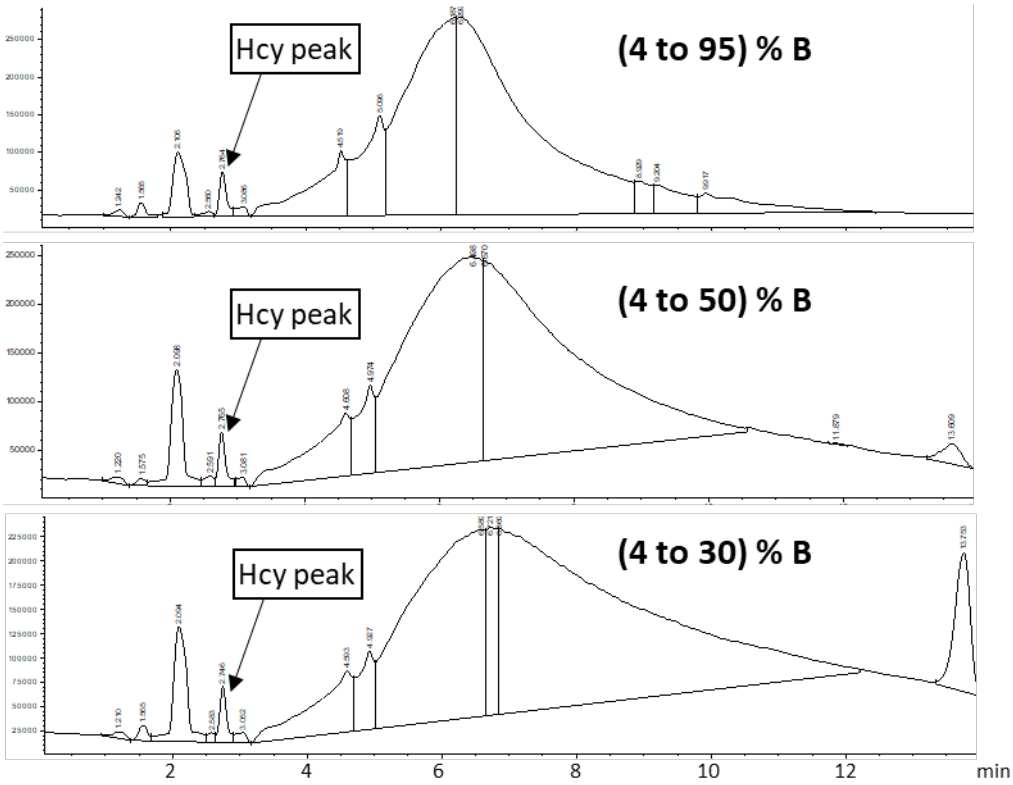


Fig. 9. Chromatograms on CN Column Using Several Gradient Elution Profiles.

Mass spectrum extracted at 2.76 min (Fig. 10) confirmed the identity of Hcy. The peak at 2.1 min was also observed in a DTT blank injection but was not associated with Hcy. These results suggest that Hcy did not interact with the stationary phase and was eluting near the injection peak. It was determined that the CN column was not suitable for Hcy measurements.

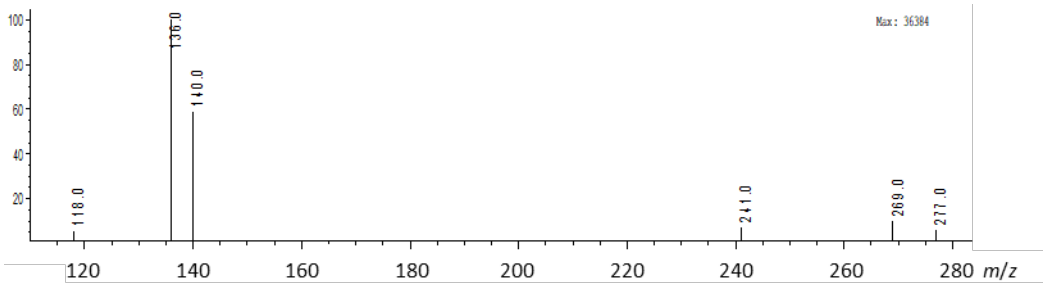


Fig. 10. Ion Extract Mass Spectrum at 2.765 min on CN column Using Gradient Elution.

3.5. Homocysteine Stability

The stability of the Hcy calibrant in HPLC grade water and in 1.5 % DTT aqueous solution was evaluated by observing peak intensities for the same samples run over time. The Hcy in water began to degrade, as seen by decreased peak intensities, after 1 week and was especially apparent at low concentrations (at or below 10 µmol/L). Compound degradation was not observed for Hcy in 1.5 % DTT aqueous solution after 3 weeks at 2 °C to 8 °C.

3.6. Mobile Phase Optimization

Calibrants were prepared at approximate concentrations of (3.5, 5, 10, 15, 20 and, 50) µmol/L and injected into the LC-MS system with the Discovery HS F5 (15 cm × 4 mm, 3 µm) column and isocratic elution at 20 % B. Ion monitoring was performed at *m*/*z* 136 and *m*/*z* 140. Peaks were integrated and the peak area ratios (Hcy/Hcy-d_4) were calculated. A calibration curve was determined by plotting the mass ratios versus peak area ratios and using linear regression to determine Eq. 1

$$\frac{A_{\text{analyte}}}{A_{\text{IS}}} = \beta_0 + \beta_1 \frac{m_{\text{analyte}}}{m_{\text{IS}}} \quad (1)$$

where A_{analyte} is the area of the *m*/*z* 136 (Hcy) peak, A_{IS} is the area of the *m*/*z* 140 (Hcy-d_4) peak, m_{analyte} is the mass of Hcy in the calibrant, and m_{IS} is the mass of Hcy-d_4 in the calibrant. β_0 and β_1 are the best estimates for the y-intercept and the slope, respectively.

Using 20 % B and injection volumes of (5 and 10) µL, the resulting calibration curve achieved an R^2 of 0.86, likely due to a small shoulder peak on the main *m*/*z* 136 peak (Fig. 11). Even with careful manual integration, the best R^2 achieved was 0.93.

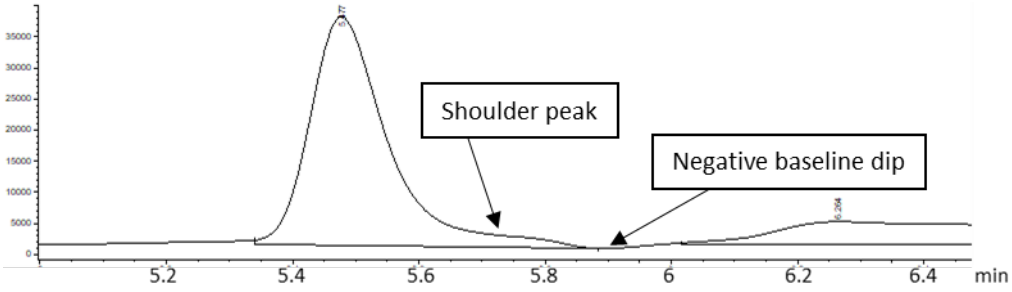


Fig. 11. Shoulder Peak and Baseline Dip Complicate Integration at 20 % B.

Mobile phase composition was again tested with (25, 30, 35, 40, 45, and 50) % B and isocratic elution. As the % B increased, the separation of the shoulder peak improved and the negative baseline dip was minimized (Fig. 12). With careful manual integration the R^2 could improve to 0.9995, even at 25 % B.

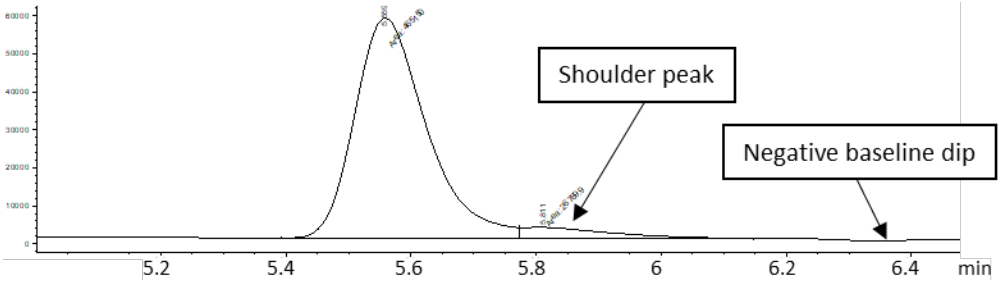


Fig. 12. Integration Improves at 25 % B.

Further investigation determined that the shoulder peak was due to the DTT aqueous solution used to reconstitute the sample and dilute calibrants from stock solution. At 30 % B, the shoulder peak eluted earlier than the Hcy peak. The negative dip into the baseline also moved and integrations improved. 10 µL injections provided good separation profiles and resulted in a calibration line with an R^2 = 0.999. However, the method was still not optimal for the separation of the interfering peak and the main Hcy peak in the samples.

SRM 1950 was prepared and analyzed for Hcy and, using 35 % B, a shoulder peak coeluted with the peak of interest. The interfering peak was observed in sample preparation blank and 1.5 % DTT aqueous solution injections. The separation improved as % B increased (Fig. 13), and the interfering peak (and negative baseline dip) was completely separated from the peak of interest at 50 % B. There were no *m*/*z* 136 and *m*/*z* 140 peaks detected in the window for the DTT and sample prep blank injections.

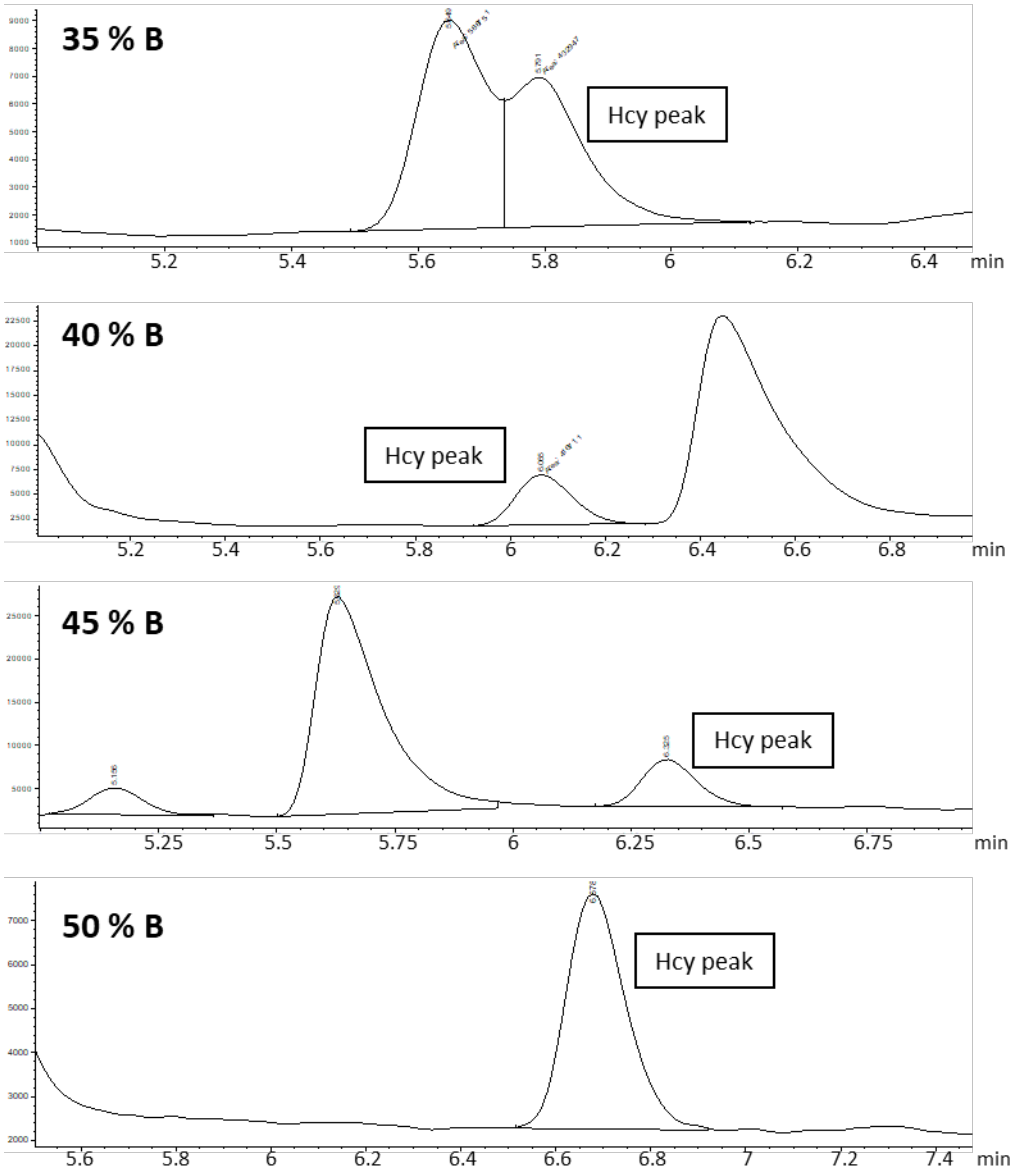


Fig. 13. With SRM 1950 Sample, m/z 136 Peak Separation is Best at 50 % B.

As shown in Fig. 14, the IS (Hcy-d_4, *m*/*z* 140) and Hcy (*m*/*z* 136) peaks eluted at the same time (6.2 min). All peaks in the calibration solutions were integrated easily from baseline to baseline, resulting in an R^2 of 0.9996. Therefore, the optimized LC method was determined to be an isocratic elution using 50 % B with an injection volume of 10 µL.

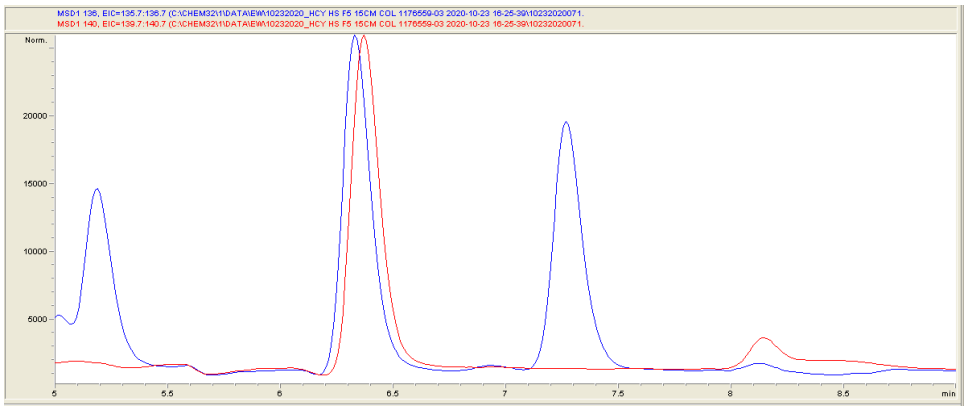


Fig. 14. The Hcy and Internal Standard Peaks Elute at the Same Time.

To further confirm the optimized separation method, calibrants were prepared using the three stock solutions prepared on three different days. Also, the calibrant concentration range was changed knowing that the highest potential Hcy concentration for the new serum material would be around 18 µmol/L. Calibrants were prepared at (3.5, 5, 7, 10, 12, 15, 18, 20, and 25) µmol/L. The concentrations (3.5, 10 and 18) µmol/L were prepared from stock solution 1, concentrations (5, 12 and 20) µmol/L were prepared from stock solution 2, and the remainder were prepared from stock solution 3. Two other sets of calibrants were also prepared, switching the order of the stock solutions used for the different concentration levels. All three calibration curves had $R^2 \geq 0.996$, confirming optimized LC-MS conditions and successful preparations of stock solutions and calibrants samples. The peak area ratios (Hcy/Hcy-d_4) for concentrations between 3.5 µmol/L to 25 µmol/L are shown in Fig. 15.

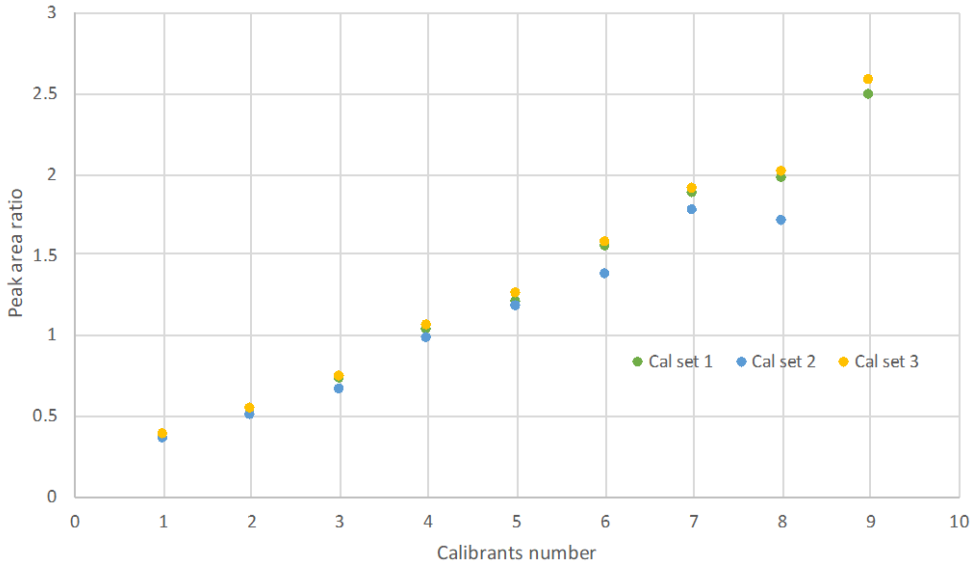


Fig. 15. Peak Area Ratios [(*m/z* 136)/(*m/z* 140)] for Three Sets of Calibration Solutions.

3.7. Sample Preparation Optimization

When preparing SRM 1950 plasma samples for the LC-MS optimization work, the sample was reduced, cleaned-up with SPAE, dried, and reconstituted with 1.5 % DTT prior to LC-MS analysis. Unfortunately, the sample recovery varied and resulting peak areas were smaller than desired. As a result, the sample preparation steps were investigated to improve the accuracy and reproducibility of the sample recovery.

Each SRM 1950 sample was removed from -80 °C storage, thawed to room temperature for at least 30 min, and vortex mixed. Then, a 200 µL aliquot was transferred into a 2-mL sample tube and spiked with the Hcy-d_4 stock solution to achieve a final concentration of approximately 10 µmol/L. All masses were recorded in each step. The homocystine reduction was achieved by adding 400 µL of 0.15 mol/L NaOH in 1.5 % DTT solution and heating the sample 40 °C for 15 min. For the initial optimization work, this step used a NaOH/DTT solution prepared ahead of time, which in some cases was prepared several months prior to sample preparation. When the results by LC-MS showed inconsistency, potential NaOH degradation with respect to time and exposure to air was investigated. A NaOH/DTT solution was prepared fresh on each sample preparation day, and the resulting Hcy peaks were more intense and consistent.

Another potential source of variability was the SPAE clean-up step. New SPAE columns were purchased, which did improve reproducibility. The SPAE elution composition and volume were also investigated to optimize sample recovery. SRM 1950 samples were reduced using freshly prepared NaOH/DTT solution, and allowed to cool to room temperature before loading onto preconditioned SPAE columns. The SPAE columns were preconditioned with 3 mL of methanol and 3 mL of water. The loaded sample was cleaned with 9 mL of 100 % water and 3 mL of 100 % methanol. Elution solvent was then added and allowed to sit in the column while changing to sample collection vials for final sample collection. Elution solvent composition was tested at (25, 50, 75, and 100) % methanol, all with freshly added 0.4 mol/L acetic acid. Three separate 1 mL volumes of elution solvent were added and collected individually for LC-MS analysis. Samples were dried under gentle N_2 flow and at 40 °C. Each sample was then reconstituted with 200 µL of 1.5 % DTT aqueous solution, vortexed, and transferred to amber HPLC autosampler vials with 300-µL inserts for LC-MS analysis.

The LC-MS results showed that the elution solvent compositions of (50, 75, and 100) % methanol all performed well. As seen by the peak heights in Fig. 16, the first and second collection steps (each of 1-mL elution solvent) contained a significant amount of Hcy, and the third elution step (another 1-mL elution solvent) also contained a smaller Hcy peak.

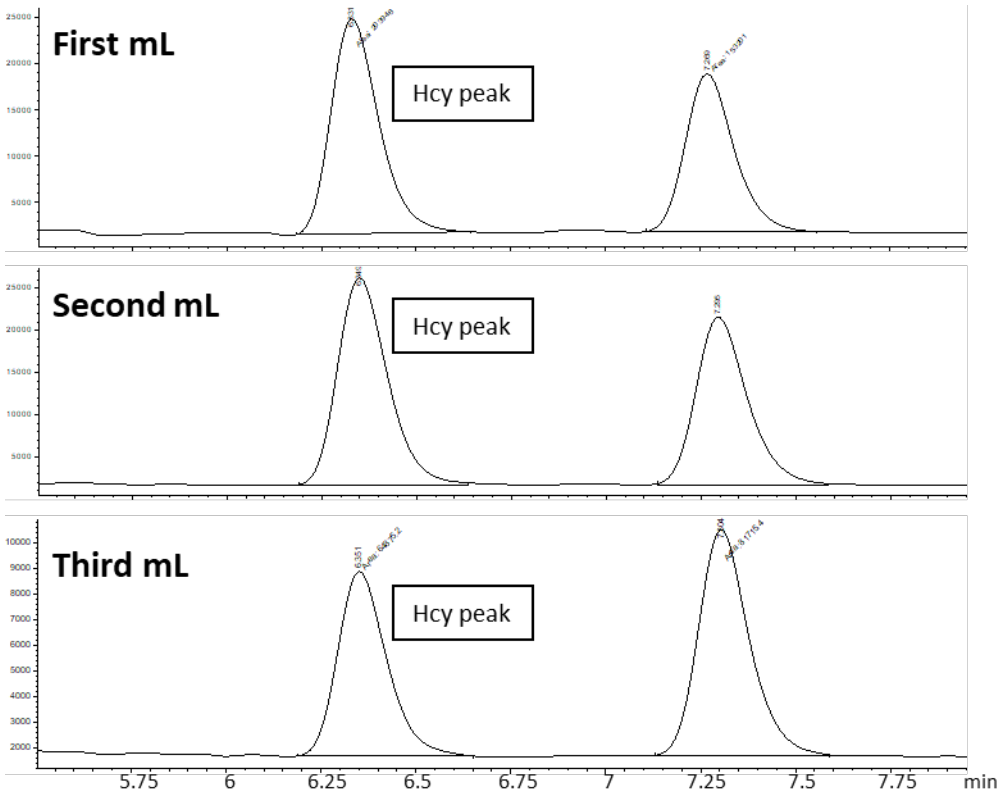


Fig. 16. Recovery of Hcy in SRM 1950 as a Function of Elution Volume, 100 % B.

Based on the calibration curve and the peak area ratio of SRM 1950 samples that were analyzed, the Hcy mass fractions were calculated:

$$w_{\text{analyte}} = \frac{\left(\frac{A_{\text{analyte}}}{A_{\text{IS}}} - \beta_0\right)}{\beta_1} \frac{m_{\text{IS}}}{m_{\text{sample}}}. \tag{2}$$

where β_0 and β_1 are the intercept and slope of the relevant calibration curve (see Eq. 1), A_{analyte} and A_{IS} are the peak areas of Hcy and Hcy-d_4 in the sample, m_{IS} is the mass of Hcy-d_4 in the sample, m_{sample} is the mass of the sample and w_{analyte} is the estimated mass fraction of Hcy in the sample.

To compare with the (8.50 ±0.20) µmol/L certified value of Hcy in SRM 1950, the amount-of-substance concentrations were calculated:

$$c_{\text{analyte}} \frac{\mu\text{mol}}{\text{L}} = \left(w_{\text{analyte}} \frac{\mu\text{g}}{\text{g}}\right)\left(\rho_{\text{serum}} \frac{\text{g}}{\text{mL}}\right)\left(\frac{1}{M_{\text{analyte}}} \frac{\text{mol}}{\text{g}}\right)\left(\frac{\text{g}}{10^6\,\mu\text{g}}\right)\left(\frac{10^3\text{mL}}{\text{L}}\right)\left(\frac{10^6\,\mu\text{mol}}{\text{mol}}\right) \tag{3}$$

where ρ_{serum} is the 1.02086 g/mL density of the serum, M is the 135.18 g/mol molar mass of Hcy. and c_{analyte} is the estimated amount-of-substance concentration of Hcy in the sample.

Note: transformation of the amount of substance concentrations to mass fractions is then

$$w_{\text{analyte}} \frac{\mu\text{g}}{\text{g}} = \left(c_{\text{analyte}} \frac{\mu\text{mol}}{\text{L}}\right)\left(\frac{1}{\rho_{\text{serum}}} \frac{\text{mL}}{\text{g}}\right)\left(\frac{M_{\text{analyte}}}{1} \frac{\text{g}}{\text{mol}}\right)\left(\frac{10^6\,\mu\text{g}}{\text{g}}\right)\left(\frac{\text{L}}{10^3\text{mL}}\right)\left(\frac{\text{mol}}{10^6\,\mu\text{mol}}\right) \tag{4}$$

Results for sample replicates of SRM 1950 extracted with (50 and 100) % methanol are summarized in Fig. 17. The mean amount-of-substance concentration estimates were between (8.21 and 9.75) µmol/L, with the estimates for the third mL collection much more variable and typically higher than those for the first and second mL. Since the results are comparable for elution solutions between (50 and 100) % methanol and the published RMP uses 100 % methanol, it was determined to use 100 % methanol. However, the volume of elution solvent was increased to 2 mL to ensure that most of the sample is recovered while avoiding the drying time required for 3 mL.

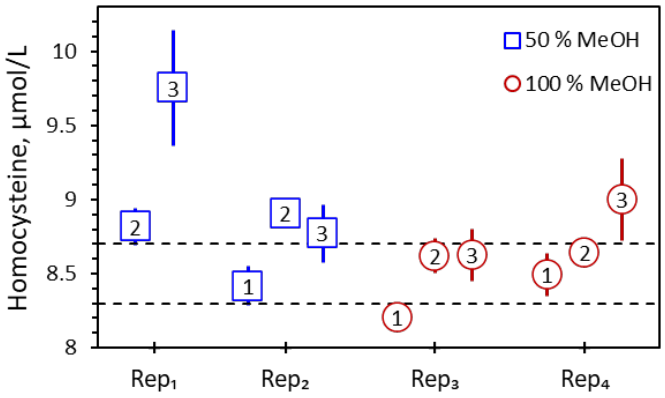


Fig. 17. Hcy as a Function of Elution Volume Using (50 and 100) % MeOH Elution Solvent.

Each symbol represents the mean of three injections of a 1 mL extract of SRM 1950; the error bars represent two standard deviations. The symbol labels indicate the order of collection for a given sample replicate. The first 1 mL collection for sample Rep_1 was lost. The dotted lines bound SRM 1950's certified (8.50 ±0.20) µmol/L 95 % confidence interval.

A sample of SRM 1950 was then prepared and collected with a 2 mL elution volume before drying and reconstitution. The first trial with this approach was poor; the elution collection step needs to be slower so that the acetic acid in the methanol can fully interact with the homocysteine anion. This was tested by slowing down the vacuum during sample collection step (by leaving three manifold holes open to the air) or allowing the sample to gravity drip for 20 minutes before applying vacuum to complete the sample collection. Both approaches gave good sample recovery, with an average amount-of-substance concentration for three SRM 1950 replicates of (8.70 ± 0.05) µmol/L, for a %CV of 0.58 %.

There was still detectable Hcy in a subsequent 1 mL of elution solvent, especially after the 20 min gravity drip, but the peak was quite small. It was decided that a 2 mL of 100 % methanol and a gravity drip of 10 min before applying vacuum for the elution step would be a good balance to ensure accurate and reproducible sample recovery.

3.8. Column Lot Comparison

A new lot of the Discovery HS F5 (15 cm × 4 mm, 3 µm) column was used to analyze a previous sample set. The same separation profile was achieved, the calibration line had an R^2 = 0.999, and comparable Hcy concentrations were determined for SRM 1950. However, as shown in Fig. 18 the retention time of the Hcy peak using the new column shifted to almost 3 min later than in the old column.

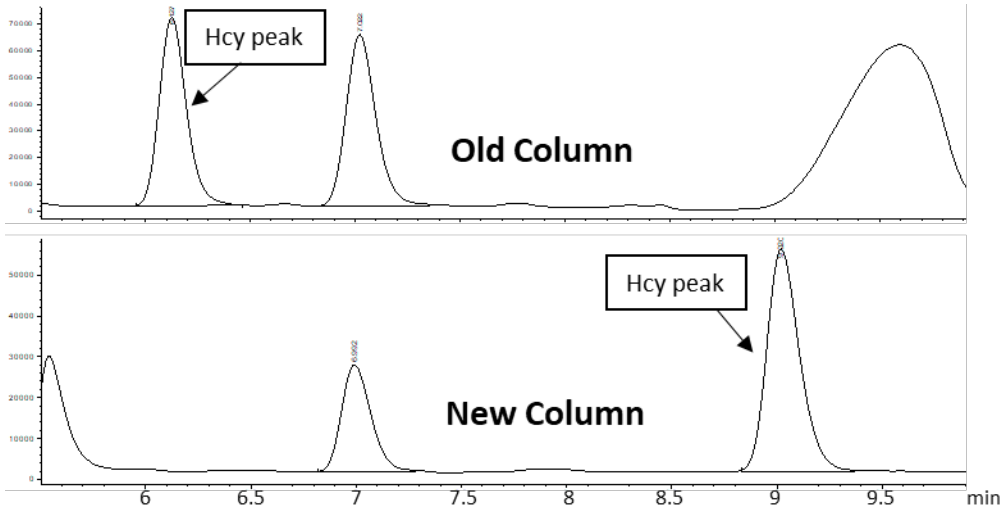


Fig. 18. m/z 136 SRM 1950 Chromatograms Using Old and New Columns.

As this new column was used for more sample analyses, the retention time for the Hcy peak slowly shifted to earlier retention times. The previous column has been used for method optimization and subjected to numerous samples injections with multiple mobile phases compositions. New columns may need more time to equilibrate, or more sample injections, to reach a steady retention time for the target analytes. As a result, the analysis time may need to be extended to more than 10 minutes with any new columns.

3.9. Unidentified Peaks in SRM 1950 Chromatograms

There were some unidentified peaks in the *m*/*z* 136 extracted ion chromatograms of SRM 1950 samples, observed using both the old and new columns. As seen in Fig. 19, these peaks are not present in the calibrant injections and they are likely associated with other components in human plasma. However, these peaks are baseline separated from the target peak and do not interfere with the calculation of the Hcy amount-of-substance concentration.

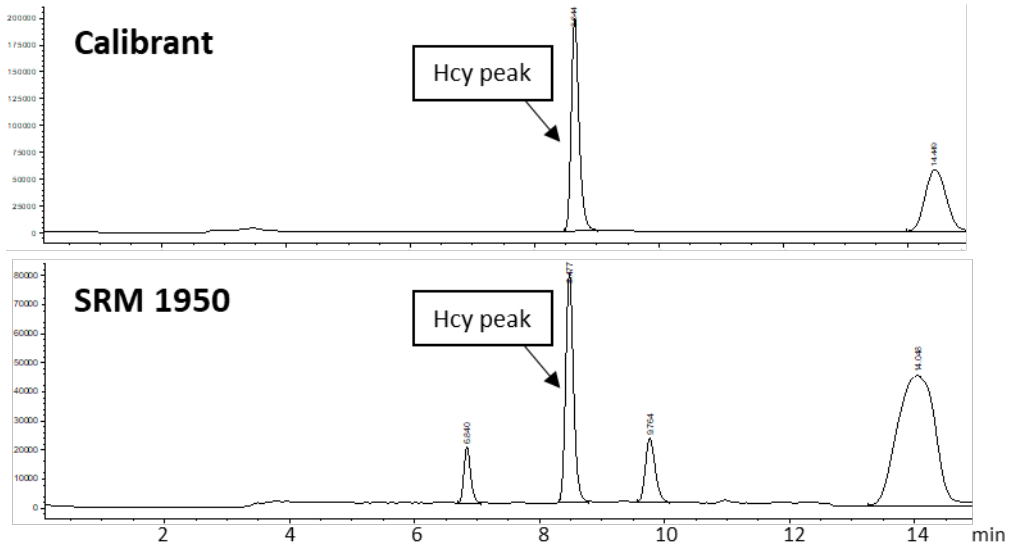


Fig. 19. m/z 136 Calibrant and SRM 1950 Chromatograms on New Column.

4. Serum Density Determinations

Density values are needed to express mass and amount-of-substance concentrations of homocysteine in the SRM 1955a materials. Density values at the reference temperature of 23 °C were determined from gravimetric mass measurements using the Lang-Levy pipet method [13] with sample volumes of approximately 500 µL.

4.1. Materials

HPLC grade water for calibration and pipet rinsing was obtained from JT Baker. Ethanol (200 proof) for rinsing pipets was obtained from Warner-Graham.

4.2. Method

A 500-µL Lang-Levy pipet was calibrated with water that had been equilibrated for at least 24 h at ambient conditions in the same room where the balance was located. The temperature of the room was measured using a 2626S Thermo-Hygrometer (Fluke Corporation) at the beginning and end of each set of measurements.

Using a Mettler Toledo XPE205 analytical balance, a metal pipette stand was tared, a clean and dry pipet was carefully placed on the stand, and the mass of the pipet was recorded. The pipet was then filled to the 500-µL mark with water, wiped with a lint-free cloth, and weighed. The same pipet was then cleaned and dried by attaching it to a vacuum trap, rinsing with several mL of water, followed by several mL of ethanol. The pipet remained attached to the vacuum trap and air was pulled through the pipet for 10 minutes to ensure it was completely dry. The water mass determinations were performed in triplicate.

The volume of the pipet at the reference 23 °C (V_{23}) was estimated from the measured mass of water (m_{water}) and a value of (0.99756 ±0.00001) g/mL for the density of water at 23 °C ($\rho_{23\text{water}}$):

$$V_{23} = m_{\text{water}}/\rho_{23\text{water}} \,. \tag{5}$$

The serum mass determinations were performed in triplicate. Between each weighing, the pipet was rinsed with water, then ethanol, and dried for 10 minutes under vacuum.

The density of the serum at the reference 23 °C ($\rho_{23\text{serum}}$) was calculated from the measured mass of serum (m_{serum}) and the estimated pipet volume at 23 °C:

$$\rho_{23\text{serum}} = m_{\text{serum}}/V_{23} = \frac{m_{\text{serum}}}{m_{water}}\rho_{23\text{water}} \,. \tag{6}$$

The mass concentration and amount-of-substance concentration values delivered by SRM 1955a was certified within the temperature range of (20 to 25) °C. Serum densities at temperatures (T) relatively close to the reference temperature of 23 °C can be estimated using the volumetric expansion formula given in [13].

$$\rho_{T\text{serum}} = \rho_{23\text{serum}}\big(1 - 0.00021(T - 23)\big) \,. \tag{7}$$

4.3. SRM 1955a Level 1

Three vials of SRM 1955a Level 1 (from boxes 7, 17, and 25) were removed from -80 °C storage and left undisturbed on the bench top to thaw at room temperature for 1.5 h each. The contents of the three vials were combined into a 4-mL glass vial.

The balance room temperature was 19.60 °C at the start of measurements and was 19.74 °C at the end, approximately 1.5 h later. Table 2 lists the measured masses, estimated mass differences, and summary statistics for the triplicate calibration and serum mass determinations.

Table 2. Mass Measurements for the Density Determination of SRM 1955a Level 1.

	HPLC Water				SRM 1955a Level 1			
	m_{empty}, g	m_{full}, g	m_{water}, g [a]		m_{empty}, g	m_{full}, g	m_{serum}, g [a]	
	8.38900	8.89673	0.50773	± 0.00001	8.38897	8.90722	0.51825	± 0.00001
	8.38899	8.89665	0.50766	± 0.00001	8.38900	8.90702	0.51802	± 0.00001
	8.38895	8.89669	0.50774	± 0.00001	8.38898	8.90714	0.51816	± 0.00001
$\bar{x}$ [b]			0.50771				0.51814	
s [b]			0.00004				0.00012	
$\bar{u}$ [b]				0.00001				0.00001
$u(\bar{x})$ [b]			0.00003				0.00007	

a) All direct mass measurements are assumed to be stable within ±0.00001 g, suggesting a standard uncertainty of 0.00001/√3 = 0.000006 g. The combined standard uncertainty of the $m_{full} - m_{empty}$ mass difference is then $\sqrt{0.000006^2 + 0.000006^2} = 0.000008 \approx 0.00001$ g.

b) $\bar{x}$: mean; s: standard deviation; $\bar{u}$: pooled standard uncertainty of individual values; $u(\bar{x})$: standard uncertainty of the mean, $u(\bar{x}) = \sqrt{(s^2 + \bar{u}^2)/3}$.

The density of SRM 1955a Level 1 at the reference 23 °C, including a temperature adjustment based on the initial and final temperature of the balance room, is:

$$\rho_{23\text{serum}} = \frac{(0.51814 \pm 0.00007)}{(0.50771 \pm 0.00003)}(0.99756 \pm 0.00001)\frac{(1.00071 \pm 0.00003)}{(1.00068 \pm 0.00003)}$$
$$= (1.01809 \pm 0.00018)\frac{\text{g}}{\text{mL}}.$$

This density is consistent with the expected nominal density of human serum (1.02 g/mL).

For two degrees of freedom, the Student's t_{95} expansion factor, k_{95}, is 4.3, for an approximate 95 % level of confidence expanded uncertainty, U_{95}, of:

$$U_{95} = k_{95}u \cong (4.3)(0.00018) = 0.00076\frac{\text{g}}{\text{mL}}.$$

The density estimates for SRM 1955a Level 1 at the endpoints of the (20 to 25) °C certification window are (1.01870 to 1.01766) g/mL. These are within the (1.01733 to 1.01885) g/mL estimated 95 % level of confidence interval for this material.

4.4. SRM 1955a Level 2

Three vials of SRM 1955a Level 2 (Boxes 11, 27, and 40) were removed from -80 °C storage and left undisturbed on the bench top to thaw at room temperature for 1.5 h each. The contents of the three vials were combined in a 4 mL glass vial.

The balance room temperature was 19.58 °C at the start of measurements and was 19.62 °C at the end approximately 1.5 h later. Table 3 lists the measured masses, estimated mass differences, and summary statistics for the triplicate calibration and serum mass determinations.

Table 3. Mass Measurements for the Density Determination of SRM 1955a Level 2.

	HPLC Water			SRM 1955a Level 2		
	m_{empty}, g	m_{full}, g	m_{water}, g [a]	m_{empty}, g	m_{full}, g	m_{serum}, g [a]
	8.38893	8.89636	0.50743 ± 0.00001	8.38904	8.90937	0.52033 ± 0.00001
	8.38907	8.89643	0.50736 ± 0.00001	8.38903	8.90955	0.52052 ± 0.00001
	8.38901	8.89656	0.50755 ± 0.00001	8.38902	8.90942	0.52040 ± 0.00001
$\bar{x}$ [b]			0.50745			0.52042
s [b]			0.00010			0.00010
$\bar{u}$ [b]			0.00001			0.00001
$u(\bar{x})$ [b]			0.00006			0.00006

a) All direct mass measurements are assumed to be stable within ±0.00001 g, suggesting a standard uncertainty of 0.00001/√3 = 0.000006 g. The combined standard uncertainty of the $m_{full} - m_{empty}$ mass difference is then $\sqrt{0.000006^2 + 0.000006^2} = 0.000008 \approx 0.00001$ g.

b) $\bar{x}$: mean; s: standard deviation; $\bar{u}$: pooled standard uncertainty of individual values; $u(\bar{x})$: standard uncertainty of the mean, $u(\bar{x}) = \sqrt{(s^2 + \bar{u}^2)/3}$.

The density of SRM 1955a Level 2 at the reference 23 °C, including a temperature adjustment based on the initial and final temperature of the balance room, is:

$$\rho_{23\text{serum}} = \frac{(0.52042 \pm 0.00006)}{(0.50745 \pm 0.00006)}(0.99756 \pm 0.00001)\frac{(1.00072 \pm 0.00003)}{(1.00071 \pm 0.00003)}$$
$$= (1.02307 \pm 0.00019)\frac{\text{g}}{\text{mL}}.$$

This density is consistent with the expected nominal density of human serum (1.02 g/mL).

For two degrees of freedom, the Student's t_{95} expansion factor, k_{95}, is 4.3, for an approximate 95 % level of confidence expanded uncertainty, U_{95}, of:

$$U_{95} = k_{95}u \cong (4.3)(0.00019) = 0.00083\frac{\text{g}}{\text{mL}}.$$

The density estimates for SRM 1955a Level 2 at the endpoints of the (20 to 25) °C certification window are (1.02370 to 1.02264) g/mL. These are within the (1.02223 to 1.02390) g/mL estimated 95 % level of confidence interval for this material.

4.5. SRM 1955a Level 3

Three vials of SRM 1955a Level 3 (Boxes 6, 23, and 26) were removed from -80 °C storage and left undisturbed on the bench top to thaw at room temperature for 1.5 h each. The balance room temperature was 19.69 °C at the start of measurements and was 19.75 °C at the end approximately 1.5 h later. Table 4 lists the measured masses, estimated mass differences, and summary statistics for the triplicate calibration and serum mass determinations.

Table 4. Mass Measurements for the Density Determination of SRM 1955a Level 3.

	HPLC Water				SRM 1955a Level 3			
	m_{empty}, g	m_{full}, g	m_{water}, g [a]		m_{empty}, g	m_{full}, g	m_{serum}, g [a]	
	8.38893	8.89678	0.50785	± 0.00001	8.38895	8.90966	0.52071	± 0.00001
	8.38895	8.89713	0.50818	± 0.00001	8.38892	8.90951	0.52059	± 0.00001
	8.38897	8.89667	0.50770	± 0.00001	8.38893	8.90995	0.52102	± 0.00001
$\bar{x}$ [b]			0.50791				0.52077	
s [b]			0.00025				0.00010	
$\bar{u}$ [b]				0.00001				0.00001
$u(\bar{x})$ [b]			0.00014				0.00013	

a) All direct mass measurements are assumed to be stable within ±0.00001 g, suggesting a standard uncertainty of 0.00001/√3 = 0.000006 g. The combined standard uncertainty of the $m_{full} - m_{empty}$ mass difference is then $\sqrt{0.000006^2 + 0.000006^2} = 0.000008 \approx 0.00001$ g.

b) $\bar{x}$: mean; s: standard deviation; $\bar{u}$: pooled standard uncertainty of individual values; $u(\bar{x})$: standard uncertainty of the mean, $u(\bar{x}) = \sqrt{(s^2 + \bar{u}^2)/3}$.

The density of SRM 1955a Level 3 at the reference 23 °C, including a temperature adjustment based on the initial and final temperature of the balance room, is:

$$\rho_{23\text{serum}} = \frac{(0.52077 \pm 0.00013)}{(0.50791 \pm 0.00014)}(0.99756 \pm 0.00001)\frac{(1.00070 \pm 0.00003)}{(1.00068 \pm 0.00003)}$$
$$= (1.02284 \pm 0.00040)\frac{\text{g}}{\text{mL}}.$$

This density is consistent with the expected nominal density of human serum (1.02 g/mL).

For two degrees of freedom, the Student's t_{95} expansion factor, k_{95}, is 4.3, for an approximate 95 % level of confidence expanded uncertainty, U_{95}, of:

$$U_{95} = k_{95}u \cong (4.3)(0.00040) = 0.0017\frac{\text{g}}{\text{mL}}.$$

The density estimates for SRM 1955a Level 3 at the endpoints of the (25 to 20) °C certification window are (1.02239 to 1.02348) g/mL. These are within the (1.02114 to 1.02453) g/mL estimated 95 % level of confidence interval for this material.

5. Homocysteine Molecular Weight

The molecular formula for homocysteine is $C_4H_9NO_2S$. Using the IUPAC Molecular Weight calculator [14,15], the molecular weight (molar mass) is (135.187 ±0.005) g/mol. The associated 95 % level of confidence interval is (135.177 to 135.197) g/mol. The calculator combines elemental atomic weights assuming that the weights are normally distributed across their stated uncertainty intervals; Fig. 20 displays the resulting probability density.

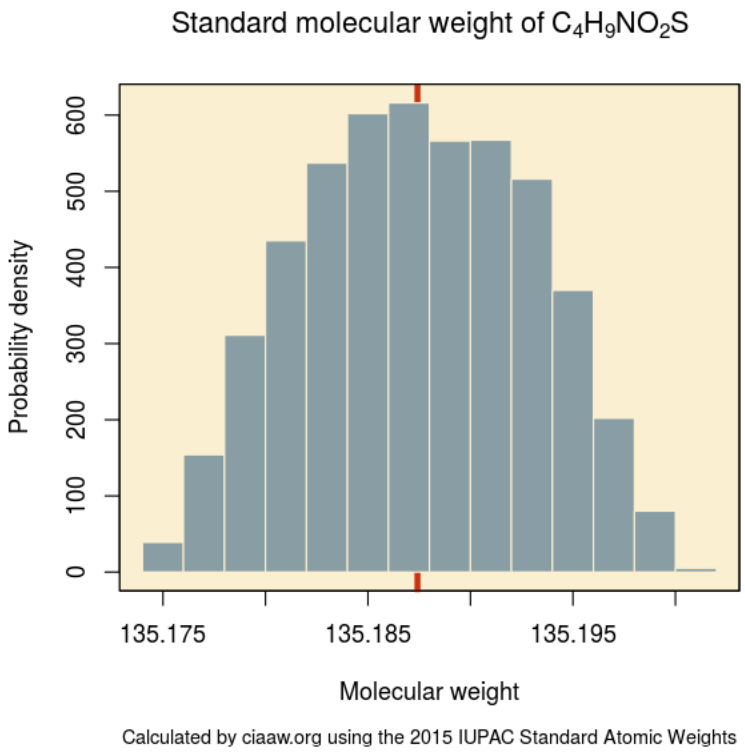


Fig. 20. Probability Density for the Molecular Weight of Homocysteine.

6. Calibrant Purity Assessment

Neat DL-homocystine was used as a calibration standard for measurement of homocysteine in human serum using the NIST isotope dilution-liquid chromatography-mass spectrometry (ID-LC-MS) reference measurement procedure (RMP). The purity analysis characterized the homocystine material with respect to the entities liberated by reaction with dithiothreitol (DTT) as depicted in Fig. 21, assuming that one mole of homocystine is completely reduced to two moles of homocysteine via reaction with DTT.

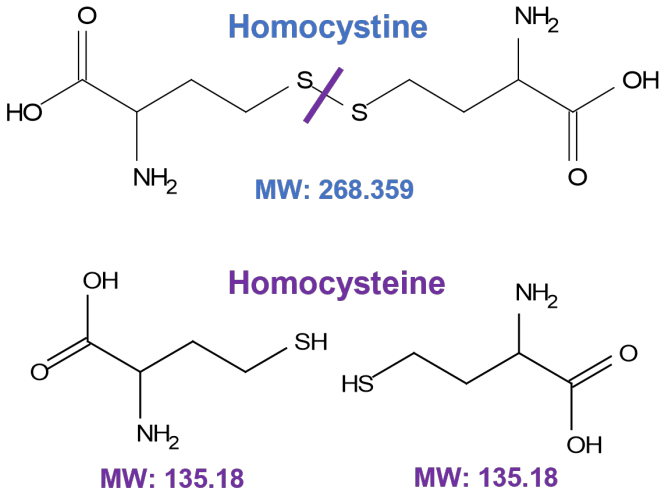


Fig. 21. Structures and molecular weights (g/mol) of homocystine and homocysteine.

The assessed purity is metrologically traceable to the International System of Units (SI) through ^{1}H NMR determinations of chemical structure and ^{1}H-qNMR measurements using a maleic acid internal standard (IS) of purity assigned against the NIST PS1 Primary Standard for qNMR (Benzoic Acid) [8,9].

6.1. Materials.

Purity was assessed for a 10 g bottle of neat DL-homocystine (Fluka, Lot #GA12481) stored at room temperature. Maleic acid (Sigma-Aldrich Lot BCBM8127V) of established purity [9] and stored at room temperature in a desiccator was used as the IS.

A 0.35 mol/L DCl in D_2O solution was the solvent used in the qNMR analyses. The solution was prepared from an ampoule of 20 % DCl (99.96 % D atom purity) in D_2O (Cambridge Isotope Laboratories). 1.2 mL of solution from the ampoule was removed with a glass pipette and further diluted with D_2O (99.9 % D atom purity, Cambridge Isotope Laboratories) to a total volume of 25 mL in a clean glass volumetric flask.

6.2. Sample Preparation.

Five DL-homocystine qNMR samples were prepared and analyzed. Maleic acid was selected as the IS because it was readily soluble in the 0.35 mol/L DCl buffer and its peaks in the NMR spectrum did not interfere with the analyte peaks.

Glassware used during sample preparation was rinsed with acetone, methanol, ethanol, and distilled water, and baked in a furnace at 450 ˚C. Clean Bruker 600 MHz NMR tubes (5 mm internal diameter, 17.8 cm (7 in) length) were used. Sample mass determinations and preparation of samples for ^{1}H-qNMR analysis were performed in accordance with established protocols. Neat material masses were determined using a calibrated ultra-microbalance (Mettler Toledo XPR2U, Columbus, OH). Samples were diluted using approximately 0.7 mL of the 0.35 mol/L DCl in D_2O solvent. Samples were sonicated several times and then vortexed to achieve complete dissolution while ensuring that no crystals of the neat materials adhered to the walls of the weigh bottles.

6.3. Analysis

NMR data was acquired by a Bruker Avance II 600 MHz spectrometer equipped with a 5-mm double resonance broadband probe with inverse coil configuration to optimize ^{1}H observation. The system was operated using Topspin (Version 3.2) software. The ^{1}H analyses, subsequent data processing, and chemical mass fraction purity quantifications were performed according to established protocols.

One dimensional ^{1}H NMR experiments were conducted using 90-degree excitation pulse widths, without ^{13}C decoupling. All experiments were conducted at 298 K. For each analysis, 96 acquired data scans were averaged, two dummy scans performed, the spectral sweep width was set to 20.0276 ppm, and the transmitter frequency offset for the ^{1}H (O1) channel was set to 6.175 ppm. Data acquisition time was 5.45 s for each scan to generate a free induction decay (FID) signal with 131072 data points. Signal digitization was performed using TopSpin default ‘digital’ mode. The spin lattice relaxation time (T1) for all analyzed analyte and IS ^{1}H resonances was determined using magnetization inversion recovery NMR experiments. The longest T1 among relevant resonances for all sample compositions was 5.5 s. The recycle delay, D1, was set to 60 s, allowing approximately 99.999 % recovery of the net magnetization equilibrium position between scans.

Tuning of the probe using the automated tuning function (“atma”) was much slower than usual due to the low pH of the aqueous DCl solution but was nevertheless successfully completed. However, experiments using ^{13}C decoupling (zgig) did not generate satisfactory spectra. It was unclear why the decoupling was problematic, but the spectra collected using the zg pulse program without decoupling were adequate for qNMR analyses.

6.3.1. Identification

The identity of primary chemical component (PC), DL-homocystine, was verified via ^{1}H-NMR (Fig. 22). The ^{1}H-NMR spectra also confirmed the presence of small amount of homocysteine.

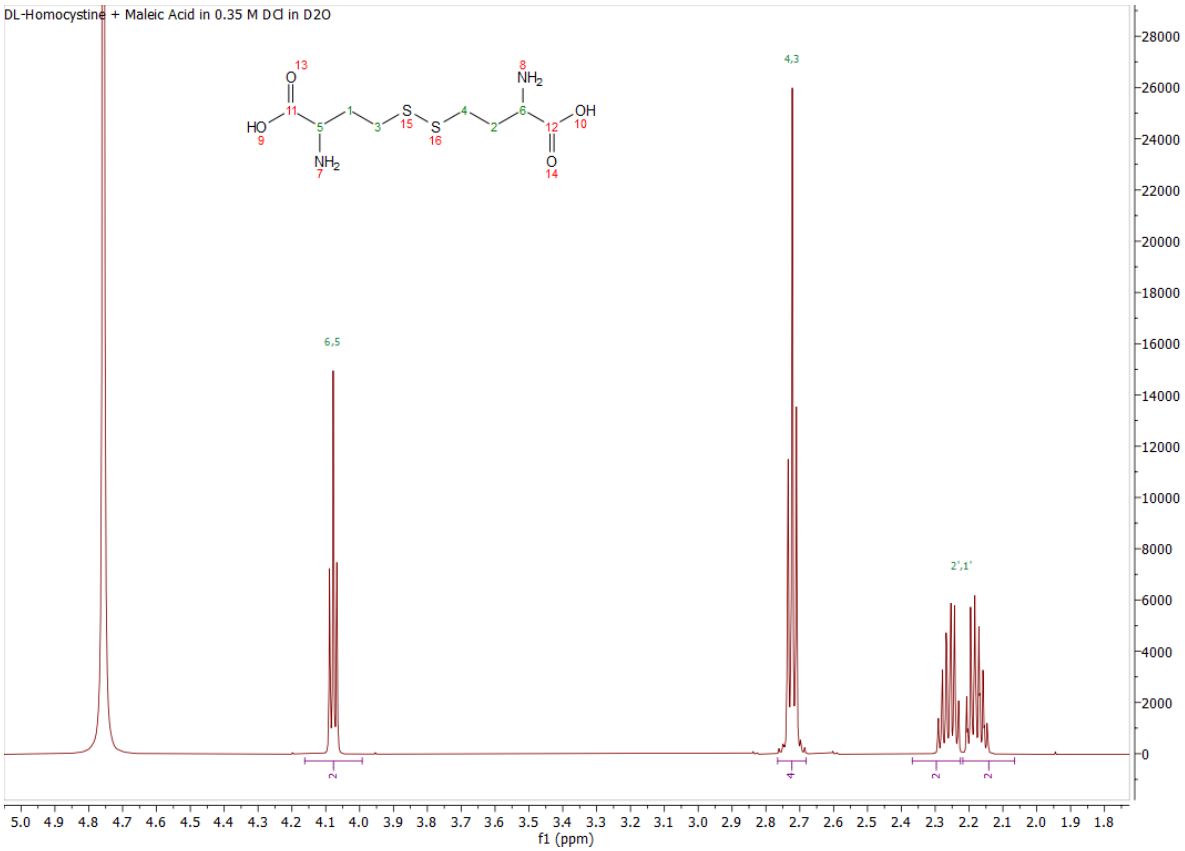


Fig. 22. ^{1}H-NMR Spectra for Homocystine HCl in 0.35 mol/L DCl in D2O.

6.3.2. Peak Selection

For purity assessments, distinct ^{1}H spectral regions were analyzed to determine integrals for the PC and IS, and a summary is presented in Table 5. The spectral regions of interest are displayed at high resolution in Fig. 23. Due to overlapping peaks, the integral for the DL-homocystine peak at 2.8 ppm was the only viable peak for quantitation.

Table 5. ^{1}H-NMR integral regions evaluated for ^{1}H-qNMRIS purity assessment.

Material	Chemical Shift (ppm)	Peak Type	^{1}H Structural Moiety [a]	Proton Multiplicity	T1 (s)
Maleic acid	6.0 to 6.5 6.7 to 6.8	singlet		2	5.5
DL-Homocystine	2.5 to 3.0	triplet	3,4	4	0.67
Homocysteine	2.6 to 2.7	multiplet		2	

a) ^{1}H chemical structure moiety numbering schemes are shown in Fig. 22.

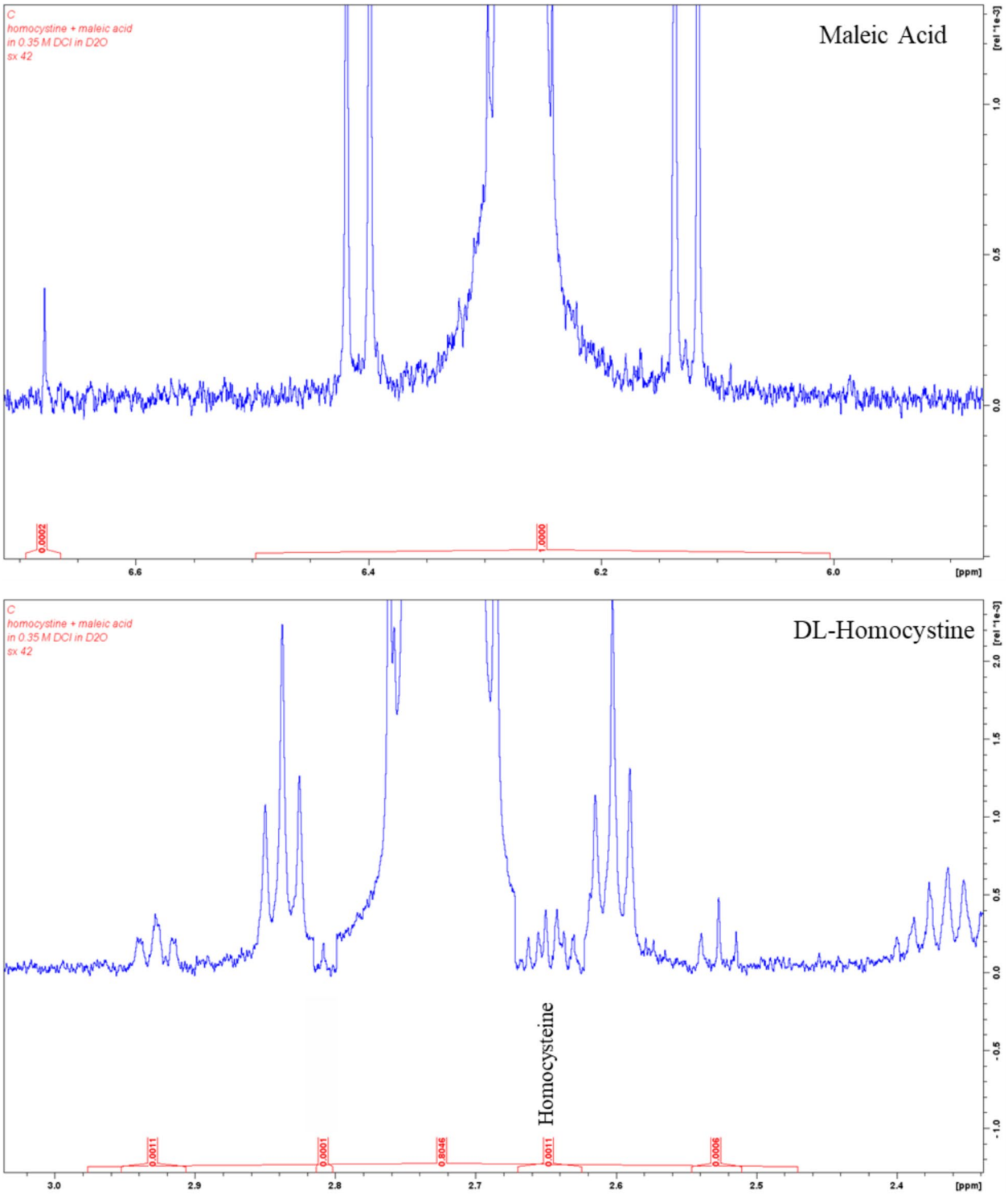


Fig. 23. ^{1}H-NMR Integration Regions for Maleic Acid and DL-Homocystine.

Spectra are shown following manual phase adjustment and baseline correction.

6.3.3. Quantitation

Measured values of mass fraction (g/g), w_{P}, of each PC chemical species (DL-homocystine and homocysteine) in the neat material were determined via ^{1}H-qNMR$_{IS}$ using the following measurement function:

$$w_{\mathrm{p}} = \left(\frac{N_{\mathrm{I}}}{N_{\mathrm{P}}}\right) \times \left(\frac{M_{\mathrm{P}}}{M_{\mathrm{I}}}\right) \times \left(\frac{A_{\mathrm{P}}}{A_{\mathrm{I}}}\right) \times \left(\frac{m_{\mathrm{I}}}{m_{\mathrm{C}}}\right) \times P_{\mathrm{I}} \quad (8)$$

where: N_{p} = multiplicity (# H/peak) of the PC species spectral peak,
N_{I} = multiplicity (# H/peak) of the IS peak,
M_{p} = relative molar mass (molecular weight, g/mol) of the PC species,
M_{I} = relative molar mass (molecular weight, g/mol) of the IS,
A_{p} = integrated area of the PC species ^{1}H peak,
A_{I} = integrated area of the IS ^{1}H peak,
m_{C} = mass (g) of the composite DL-homocystine material,
m_{I} = mass (g) of the IS, and
P_{I} = purity (g/g) of the IS.

The standard uncertainties associated with variables of Eq. 1 were evaluated as follows for each sample:

- the standard uncertainty of the homocystine and homocysteine ^{1}H-normalized peak integrals, $u(A_{\mathrm{P}})$, were estimated as a Type B relative uncertainty of 0.15 %.
- the standard uncertainties of the IS integral, $u(A_{\mathrm{I}})$, were assigned a Type B relative standard uncertainty of 0.05 %.
- measured masses of composite DL-homocystine material (m_{C}) and IS material (m_{I}) were assigned a Type B uncertainty of 0.5 µg.
- no uncertainty was considered for the ^{1}H multiplicities of the PC peaks (N_{P}) and IS peaks (N_{I}).
- the purity assignment of the maleic acid IS was treated as a normal N(0.9997, 0.0009) g/g distribution.
- normal distributions were specified for M_{P} and the M_{I}, based on the average relative molar masses and associated standard uncertainties calculated using the IUPAC Commission on Isotopic Abundances and Atomic Weights (CIAAW) Molecular Weight Calculator [16]. For homocystine, homocysteine, and maleic acid these distributions are specified as: N(268.359, 0.011) g/mol, N(135.18, 0.01 g/mol), and N(116.070, 0.003) g/mol, respectively.

The effective amount of homocysteine content (massic amount, mol/kg), k_{P}, that can be yielded from reduction of the DL-homocystine calibrant via dithiothreitol (DTT) during the calibration procedure specified in the RMP was also determined. This calculation was made given that both DL-homocystine and homocysteine are known components of the investigated material and contribute to the observed responses used to estimate a calibration function for ID-LC-MS procedures. This quantity was determined using the same model given that Eq. 8 can be reduced to:

$$w_{\mathrm{p}} = k_{\mathrm{p}} \times M_{\mathrm{p}} \times \frac{10^{-3}\ \mathrm{kg}}{1\ \mathrm{g}} \quad (9)$$

The measurands of interest were calculated using hierarchical Bayesian procedures modeled on observation equations based on the above equations and implemented in the OpenBUGS Markov Chain Monte Carlo (MCMC) empirical Bayesian analysis system [17]. A summary of the purity estimates via ^{1}H-qNMR$_{IS}$ is presented in Table 6.

Table 6. Purity Estimates [a].

Measurand	Mean ± *u*(Mean)	95 % Coverage	Units
w_{P1} (DL-homocystine)	0.9811 ± 0.0019	[0.9770, 0.9839]	g/g
w_{P2} (homocysteine)	0.0014 ± 0.0010	[0.0011, 0.0018]	g/g
k_P (amount of yieldable homocysteine) [b]	7.322 ± 0.016	[7.292, 7.350]	mol/kg
Effective homocysteine purity [c]	98.99 ± 0.016	[98.57, 99.37]	%

a Expressed as the mean value, the standard deviation (u), and the 95 % coverage interval of the posterior distribution estimated using the OpenBUGS models specified below.

b Massic amount of homocysteine content theoretically yieldable via reduction of DL-homocystine during the calibration procedure specified in the NIST RMP for homocysteine.

c Effective purity as a homocysteine calibration standard, determined as the mass fraction (in percent) of yieldable homocysteine (g) per gram of material, assuming that one mole of homocystine is completely (100 %) reduced to two moles of homocysteine by via reaction with dithiothreitol.

The OpenBUGS codes and ^{1}H-qNMR$_{IS}$ measurement (observation) data, including sample masses and integrals of the PC and IS, are provided below.

6.3.3.1. OpenBUGS Model

```
MODEL START{
### Mass fraction of homocystine ###
mws1~dnorm(116.07,111111)  #molar mass of maleic acid IS
mwb1~dnorm(268.359,8264.5) #molar mass of homocystine
ps1~dnorm(0.9997,1234568)  #purity of internal standard, g/g
px1<-1/(umx1*umx1)         #standard deviation transformation
#
#define beta prior for mass fraction of homocystine
mu1~dunif(0.8,1); sd1~dunif(0,0.1); c1<-mu1/(sd1*sd1); d1<-(1-mu1)/(sd1*sd1)
for(i in 1:n){pb1[i]~dbeta(c1,d1)}
#
#calculate homocystine mass fraction for n samples
for(i in 1:n){ms1[i]~dnorm(meanms1[i],px1); mb1[i]~dnorm(meanmb1[i],px1)}
for(i in 1:n){k1[i]~dunif(0,0.01)
  meanas1[i]<-ps1*ms1[i]/mws1/k1[i]
  pxa1[i]<-1/(uareas1[i]*uareas1[i])}
for(i in 1:n){areas1[i]~dt(meanas1[i],pxa1[i],2)}
for(i in 1:n){k.cut1[i]<-cut(k1[i])
  meanab1[i]<-pb1[i]*mb1[i]/mwb1/k.cut1[i]
  precareab1[i]<-1/(uareab1[i]*uareab1[i])}
for(i in 1:n){y1[i]~dnorm(meanab1[i],precareab1[i])}
#
### Mass fraction of homocysteine ###
mws2~dnorm(116.07,111111)  #molar mass of maleic acid IS
mwb2~dnorm(135.18,10000)   #molar mass of homocysteine
ps2~dnorm(0.9997,1234568)  #purity of internal standard, g/g
px2<-1/(umx2*umx2)         #standard deviation transformation
#
#define beta prior for mass fraction of homocysteine
```

```
mu2~dunif(0,0.2); sd2~dunif(0,0.1); c2<-mu2/(sd2*sd2); d2<-(1-mu2)/(sd2*sd2)
for(i in 1:n){pb2[i]~dbeta(c2,d2)}
#
#calculate homocysteine mass fraction for n samples
for(i in 1:n){ms2[i]~dnorm(meanms2[i],px2); mb2[i]~dnorm(meanmb2[i],px2)}
for(i in 1:n){k2[i]~dunif(0,0.01)
  meanas2[i]<-ps2*ms2[i]/mws2/k2[i]
  pxa2[i]<-1/(uareas2[i]*uareas2[i])}
for(i in 1:n){areas2[i]~dt(meanas2[i],pxa2[i],2)}
for(i in 1:n){k.cut2[i]<-cut(k2[i])
  meanab2[i]<-pb2[i]*mb2[i]/mwb2/k.cut2[i]
  precareab2[i]<-1/(uareab2[i]*uareab2[i])}
for(i in 1:n){y2[i]~dnorm(meanab2[i],precareab2[i])}
#
### Yieldable homocysteine ###
effcy<-1; yld<-effcy*2           #100% efficiency
mum1<-cut(mu1)/(mwb1/1000)*yld   #massic amount yieldable homocysteine
mum2<-cut(mu2)/(mwb2/1000)       #massic amount homocysteine
massicamt<-mum1+mum2             #massic amount total yieldable homocysteine
effcpurity<-massicamt*0.1*mwb2   #effective % mass fraction purity of homocysteine
}MODEL END
```

6.3.3.2. Observation Data for OpenBUGS Model

```
list(n=5, #number of samples
#1H-qNMR measurement data, homocystine
umx1=0.0000005, #standard uncertainty in mass determinations
meanms1=c(0.006450,0.003797,0.004974,0.006162,0.003595),#mass of IS
meanmb1=c(0.005128,0.003928,0.004713,0.006752,0.00421), #mass of homocystine
areas1=c(2.590785,1.567970,1.939517,2.859253,1.385285), #IS peak integrals
uareas1=c(0.001295,0.000784,0.000970,0.001430,0.000693),#IS standard uncertainty
y1=c(0.873591,0.688854,0.780464,1.329504,0.688996),     #homocystine peak integrals
uareab1=c(0.001310,0.001033,0.001171,0.001994,0.001033),#homocystine uncertainty
#
#1H-qNMR measurement data, homocysteine
umx2=0.0000005, #standard uncertainty in mass determinations
meanms2=c(0.006450,0.003797,0.004974,0.006162,0.003595),#mass of IS
meanmb2=c(0.005128,0.003928,0.004713,0.006752,0.00421), #mass of homocystine
areas2=c(2.590785,1.567970,1.939517,2.859253,1.385285), #IS peak integrals
uareas2=c(0.003886,0.002352,0.002909,0.004289,0.002078),#IS standard uncertainty
y2=c(0.002220,0.002010,0.002216,0.003074,0.001688),     #homocysteine peak integrals
uareab2=c(0.000003,0.000003,0.000003,0.000005,0.000003) #homocysteine uncertainty
)
```

7. Certification Measurements

7.1. Materials

The DL-homocystine used to prepare calibrants was purchased from Fluka. Purity was assessed and is previously described (Section 6). The internal standard (IS) was DL-homocystine-3,3,3′,3′,4,4,4′,4′-d_8 purchased from Cambridge Isotope Laboratories. DL-dithiothreitol, (DTT), hydrochloric acid and formic acid reagent grade were purchased from Sigma-Aldrich. Acetic acid was purchased from Supelco, and sodium hydroxide (NaOH) pellets were purchased from Fisher Chemical. HPLC grade water and HPLC grade methanol were used in the preparation of calibration solutions, samples, and LC-MS mobile phases. Poly-Prep Prefilled Chromatography columns (AG 1-X8, chloride form), purchased from BioRad were used for SPAE.

SRM 1950 Metabolites in Frozen Human Plasma was used as the quality control material. SRM 1950 is certified for Hcy at a concentration of (8.50 ± 0.20) µmol/L. One vial of SRM 1950 was evaluated in each measurement session.

Even though the Certificate of Analysis for SRM 1955 expired in December 2018 [3], units were available and the material was used as a pseudo control. The certified levels for Hcy were (3.98 ± 0.18) µmol/L for Level 1, (8.85 ± 0.6) µmol/L for Level 2, and (17.7 ± 1.1) µmol/L for Level 3. One vial of the appropriate level of SRM 1955 was evaluated in each measurement session.

The number of calibrant and sample preparations required to achieve a desired final expanded uncertainty of ≤6 % (similar to SRM 1955) was informed by results from the Design of Experiment application in the NIST ABACUS Chemical Analysis Package [18], ISO Guide 35 [19] recommendations, and the need to evaluate within-vial variability. The final experimental design required measuring ten vials of each level, with duplicate aliquots prepared from eight vials and single preparations from the remaining two vials.

Random stratified sampling was used to select the 10 vials across the batch of each level by first dividing the 31 total boxes into groups of 3, except the last group which included 4 boxes. The fill order was used to define vial number. One vial within each group of boxes was randomly selected.

The original design was to accomplish all measurements for each level in three sessions, each session completed in one day. Due to technical difficulties, additional vials of the Level 2 and Level 3 materials were required to replace or augment results from suspect measurements. The replacement vials were selected to have fill orders either just before or just after the vials that they replaced. Two additional one-day sessions were required to complete the Level 2 measurements; three additional sessions were required for Level 3. Table 7 lists the box number from which the vial was taken, the fill order, and the number of aliquots prepared. All aliquots of a given vial were measured during the same day.

Table 7. Selected Vials and Number Replicates.

	Level 1 (Low)			Level 2 (Normal)			Level 3 (High)		
Vial	Box	Fill Order	Aliquots	Box	Fill Order	Aliquots	Box	Fill Order	Aliquots
1	3	114	2	1	45	3	2	69	2
2	6	256	3	6	256	2	6	262	3
3	9	393	2	8	387	2	9	410	2
4	12	568	2	10	481	2	12	553	3
5	14	668	3	15	723	2	15	730	2
6	18	849	3	16	747	3	17	790	2
7	20	936	2	21	998	2	19	889	2
8	23	1112	3	22	1043	3	22	1078	3
9	25	1208	2	26	1254	3	26	1269	2
10	30	1458	2	29	1403	2	28	1337	3
1*				1	44	3	2	70	2
2*							6	261	2
3*				8	386	2	9	411	2
4*				10	482	2	12	552	3
5*				15	722	2	15	729	2
6*							17	789	3
7*				21	997	2	19	890	2
8*							22	1077	3
9*				26	1253	3	26	1268	2
10*				29	1402	2	28	1336	3

7.2. Reagent Solution Preparation

HPLC grade water was used for all solution preparations. A 1.5 % DTT aqueous solution was prepared by weighing 1.5 g of DTT into a 100-mL volumetric flask, adding approximately 70 mL of water, swirling until dissolved, and then filling to the mark with water. A 0.1 mol/L HCl solution was prepared by adding 0.305 mL HCl into a 100-mL volumetric flask and then filling to the mark with water. A 10 mol/L NaOH solution was prepared by weighing 4 g of NaOH pellets into a 100-mL volumetric flask, adding approximately 70 mL of water, swirling until dissolved, and then filling to the mark with water. Mobile phases for HPLC were prepared by adding water or methanol into a 1-L volumetric flask, then adding 1 mL of formic acid before bringing the final volume to the mark with solvent.

A 1.5 % DTT in 0.15 mol/L NaOH solution was prepared by weighing 0.15 g of DTT into a 10-mL volumetric flask, adding approximately 7 mL of water, swirling until dissolved, adding 150 µL of 10 mol/L NaOH, and then filling to the mark with water. Note: This solution must be prepared fresh each day to ensure the proper reduction of homocystine to Hcy.

A 0.4 M acetic acid in 100% MeOH was prepared by adding 1.15 mL of acetic acid into a 50-mL volumetric flask and filling to the mark with methanol. Note: This solution was prepared fresh each day to ensure the proper SPAE elution of Hcy.

7.3. Calibrant Preparation

Three independent Hcy stock solutions were prepared shortly before the first measurement session. An initial IS stock solution was prepared at the same time; a second IS stock solution was prepared shortly before the tenth session.

Homocystine stock solutions of about 7400 µmol/L Hcy were prepared by accurately weighing approximately 10 mg of homocystine into an aluminum weighing cup. The aluminum weighing cup and homocystine material was then transferred into a 15-mL Falcon centrifuge tube, tared on an analytical balance before the addition of 10 mL of 0.1 mol/L HCl, 100 µL of 10 mol/L NaOH, and 0.1 g of the 1 % DTT solution to convert the homocystine to Hcy. Masses of each volumetric addition were recorded. The stock solution was then divided into 0.4 mL aliquots and stored in 2 mL Eppendorf tubes at 80 °C. Table 8 lists the essential preparation information of the three stock solutions.

Table 8. Homocysteine Stock Solutions.

Stock Solution	Mass of Homocystine, mg	Final Mass of Solution, g	Purity-Adjusted Mass Fraction Homocysteine, mg/g [a]
HS1	10.3805	10.32988	0.9948
HS2	9.9985	10.27762	0.9630
HS3	10.0179	10.42479	0.9513

a Mass of homocystine divided by the final mass of the solution, multiplied by the estimated effective homocysteine purity mass fraction, 0.9899 g/g (Table 6).

The IS stock solutions were prepared in a similar manner. Approximately 1 mg of homocystine-d_8 was weighed into an aluminum weighing cup, transferred to a 10-mL volumetric flask, dissolved, and reduced to homocysteine-d_4 (Hcy-d_4) with 10 mL of 0.1 mol/L HCl, 100 µL of 10 mol/L NaOH, and 0.1 g of the 1 % DTT solution. The solution was then divided into 0.7 mL aliquots and stored in 2-mL Eppendorf tubes at -80 °C. Table 9 lists the essential preparation information of the two stock solutions.

Table 9. Internal Standard Stock Solutions.

Stock Solution	Mass of Homocystine-d_8, mg	Final Mass of Solution, g	Nominal Mass Fraction Homocysteine-d_4, mg/g [a]
IS1	1.2053	10.43221	0.1164
IS2	1.0683	10.26244	0.1049

a Mass of homocystine-d_8 divided by the final mass of solution, multiplied by the molar mass of two moles homocysteine-d_4 divided by the molar mass of one mole homocystine-d_8, 2×139.2/276.4 = 1.0073 g/g.

Prior to preparing calibrant mixtures, frozen stock solutions were removed from -80 °C storage and allowed to thaw at room temperature for at least half an hour. Hcy calibrants were then prepared using the stock solution by a series of dilutions (using 1.5 % DTT aqueous solution) to approximately 740 µmol/L, 74 µmol/L, then to (3, 8, 13, 18, 23, and 27.5) µmol/L. All volume masses were recorded to enable accurate concentration determinations. The Hcy-d_4 IS stock solution was diluted 1-to-1, to approximately 370 µmol/L, for calibrant preparation, and 1-to-20 (approximately 37 µmol/L) for sample preparation. The appropriate volume was spiked into each calibrant or sample to achieve a final Hcy-d_4 concentration of approximately 10 µmol/L. Approximately 1 mL of each of the calibrant mixtures was transferred into an amber HPLC autosampler vial for ID-LC-MS analysis. Details are shown in Table 10.

Table 10. Calibration Solution Mass Ratios.

	WS1				WS2				Calibration Solutions						
		m_{stock}	m_{total}	$w_{analyte}$		m_{WS1}	m_{total}	w_{Hcy}			m_{WSHcy}	m_{WSIS}	m_{Hcy}	m_{IS}	
	Stock	g	g	mg/g		g	g	µg/g		WS_{Hcy}	g	g	µg	µg	m_{Hcy}/m_{IS}
Day 1	HS1	0.20237	2.01852	0.09972		0.50438	5.02013	10.02	Cal1	WS2:3	0.12548	0.07121	1.20183	4.14539	0.28992
	HS2	0.20255	2.02038	0.09654		0.50579	5.00969	9.75	Cal2	WS2:2	0.33238	0.07160	3.23967	4.16809	0.77726
	HS3	0.20208	2.02050	0.09513		0.50505	5.01655	9.58	Cal3	WS2:1	0.52791	0.06678	5.28936	3.88750	1.36061
	IS1	0.50439	1.00858	0.05821					Cal4	WS2:3	0.76875	0.07151	7.36297	4.16285	1.76873
									Cal5	WS2:2	0.95850	0.07187	9.34238	4.18381	2.23299
									Cal6	WS2:1	1.11811	0.07229	11.20284	4.20826	2.66211
Day 2	HS1	0.20184	2.02023	0.09954		0.50527	5.00107	10.06	Cal1	WS2:3	0.12616	0.07100	1.20902	4.14778	0.29149
	HS2	0.20150	2.01598	0.09641		0.50457	5.01526	9.70	Cal2	WS2:2	0.34113	0.07117	3.30868	4.15771	0.79579
	HS3	0.20205	2.01515	0.09553		0.50553	5.03930	9.58	Cal3	WS2:1	0.52415	0.07144	5.27134	4.17349	1.26306
	IS1	0.50502	1.00628	0.05842					Cal4	WS2:3	0.76208	0.07181	7.30321	4.19510	1.74089
									Cal5	WS2:2	0.96197	0.07151	9.33032	4.17758	2.23343
									Cal6	WS2:1	1.11576	0.07168	11.22113	4.18751	2.67967
Day 3	HS1	0.20052	1.99630	0.09991		0.50582	5.00415	10.10	Cal1	WS2:3	0.12615	0.07199	1.21099	4.16377	0.29084
	HS2	0.20154	1.99657	0.09720		0.50457	5.03135	9.75	Cal2	WS2:2	0.33477	0.07232	3.26338	4.18286	0.78018
	HS3	0.20126	2.00725	0.09537		0.50632	5.03039	9.60	Cal3	WS2:1	0.52925	0.12796	5.34498	7.40097	0.72220
	IS1	0.49283	0.99186	0.05784					Cal4	WS2:3	0.76437	0.07235	7.33765	4.18459	1.75349
									Cal5	WS2:2	0.96066	0.07312	9.36462	4.22913	2.21432
									Cal6	WS2:1	1.11452	0.07265	11.25571	4.20194	2.67869
Day 4	HS1	0.20225	2.02091	0.09955		0.50775	5.00600	10.10	Cal1	WS2:2	0.12487	0.07257	1.21953	4.23367	0.28805
	HS2	0.20229	2.02069	0.09640		0.50679	5.00237	9.77	Cal2	WS2:3	0.34044	0.07189	3.26983	4.19400	0.77964
	HS3	0.20267	2.02164	0.09536		0.50681	5.03177	9.60	Cal3	WS2:1	0.52690	0.07197	5.32006	4.19867	1.26708
	IS1	0.50604	1.00970	0.05834					Cal4	WS2:2	0.75310	0.07220	7.35507	4.21209	1.74618
									Cal5	WS2:3	0.97791	0.07222	9.39254	4.21326	2.22928
									Cal6	WS2:1	1.11502	0.07213	11.25825	4.20800	2.67544
Day 5	HS1	0.20038	2.01175	0.09908		0.50622	5.02854	9.97	Cal1	WS2:2	0.12495	0.07091	1.20329	4.13298	0.29114
	HS2	0.20016	2.01077	0.09586		0.50517	5.02835	9.63	Cal2	WS2:3	0.33950	0.07201	3.22910	4.19709	0.76937
	HS3	0.20040	2.01458	0.09462		0.50558	5.02962	9.51	Cal3	WS2:1	0.52849	0.07112	5.27111	4.14522	1.27161
	IS1	0.50293	1.00443	0.05828					Cal4	WS2:2	0.76015	0.07177	7.32037	4.18310	1.74999
									Cal5	WS2:3	0.98935	0.07171	9.41005	4.17961	2.25142
									Cal6	WS2:1	1.12957	0.07231	11.26623	4.21458	2.67316

Table 10. Calibration Solution Mass Ratios (Continued).

	WS1				WS2				Calibration Solutions						
		m_{stock}	m_{total}	$w_{analyte}$		m_{WS1}	m_{total}	w_{Hcy}			m_{WSHcy}	m_{WSIS}	m_{Hcy}	m_{IS}	
	Stock	g	g	mg/g		g	g	µg/g		WS_{Hcy}	g	g	µg	µg	m_{Hcy}/m_{IS}
Day 6	HS1	0.20129	2.01099	0.09956		0.50464	5.02027	10.01	Cal1	WS2:2	0.12526	0.07047	1.20218	4.12063	0.29175
	HS2	0.19862	2.00871	0.09522		0.50451	5.00523	9.60	Cal2	WS2:3	0.34371	0.07060	3.25683	4.12824	0.78891
	HS3	0.19933	2.00664	0.09449		0.50438	5.02959	9.48	Cal3	WS2:1	0.52411	0.07097	5.24538	4.14987	1.26399
	IS1	0.50483	1.00497	0.05847					Cal4	WS2:2	0.75642	0.07129	7.25975	4.16858	1.74154
									Cal5	WS2:3	0.98034	0.07128	9.28922	4.16800	2.22870
									Cal6	WS2:1	1.11496	0.07166	11.15871	4.19022	2.66304
Day 7	HS1	0.20201	2.02227	0.09936		0.50403	5.08008	9.86	Cal1	WS2:3	0.12643	0.07101	1.20823	4.13021	0.29253
	HS2	0.20225	2.02226	0.09631		0.50388	5.02028	9.67	Cal2	WS2:1	0.32973	0.07167	3.25062	4.16860	0.77979
	HS3	0.20263	2.02378	0.09524		0.50389	5.02172	9.56	Cal3	WS2:2	0.54500	0.07197	5.26810	4.18605	1.25849
	IS1	0.50227	1.00520	0.05816					Cal4	WS2:3	0.76288	0.07236	7.29045	4.20873	1.73222
									Cal5	WS2:1	0.94714	0.07209	9.33730	4.19303	2.22686
									Cal6	WS2:2	1.16290	0.07212	11.24087	4.19477	2.67973
Day 8	HS1	0.20202	2.01874	0.09954		0.50374	5.02199	9.98	Cal1	WS2:3	0.12654	0.07110	1.20818	4.14086	0.29177
	HS2	0.20056	2.01801	0.09570		0.50500	5.03035	9.61	Cal2	WS2:1	0.32401	0.07165	3.23512	4.17290	0.77527
	HS3	0.20174	2.01956	0.09502		0.50558	5.03148	9.55	Cal3	WS2:2	0.54691	0.07184	5.25457	4.18396	1.25588
	IS1	0.50553	1.01040	0.05824					Cal4	WS2:3	0.76153	0.07212	7.27094	4.20027	1.73107
									Cal5	WS2:1	0.93481	0.07203	9.33374	4.19503	2.22495
									Cal6	WS2:2	1.16864	0.07214	11.22799	4.20143	2.67242
Day 9	HS1	0.20156	2.01818	0.09934		0.50449	5.01747	9.99	Cal1	WS2:3	0.12770	0.07225	1.21427	4.19986	0.28912
	HS2	0.20186	2.02075	0.09619		0.50361	5.06418	9.57	Cal2	WS2:1	0.32968	0.07206	3.29301	4.18882	0.78614
	HS3	0.20203	2.02234	0.09502		0.50347	5.03136	9.51	Cal3	WS2:2	0.55485	0.07171	5.30769	4.16847	1.27329
	IS1	0.50185	1.00495	0.05813					Cal4	WS2:3	0.77126	0.07215	7.33373	4.19405	1.74860
									Cal5	WS2:1	0.94032	0.07209	9.39238	4.19056	2.24132
									Cal6	WS2:2	1.18300	0.07219	11.31657	4.19638	2.69675
Day 10	HS1	0.20017	2.01109	0.09900		0.50310	5.02888	9.90	Cal1	WS2:3	0.12901	0.06437	1.22221	3.75438	0.32554
	HS2	0.19836	2.01286	0.09490		0.50386	5.02966	9.51	Cal2	WS2:1	0.33179	0.07216	3.28626	4.20873	0.78082
	HS3	0.20023	2.01760	0.09440		0.50357	5.01769	9.47	Cal3	WS2:2	0.56159	0.07249	5.33873	4.22798	1.26272
	IS1	0.50278	1.00344	0.05832					Cal4	WS2:3	0.77692	0.07265	7.36038	4.23731	1.73704
									Cal5	WS2:1	0.95178	0.07148	9.42703	4.16907	2.26118
									Cal6	WS2:2	1.18706	0.07155	11.28474	4.17315	2.70413

Table 10. Calibration Solution Mass Ratios (Continued).

	WS1				WS2				Calibration Solutions						
		m_{stock}	m_{total}	$w_{analyte}$	m_{WS1}	m_{total}	w_{Hcy}			m_{WSHcy}	m_{WSIS}	m_{Hcy}	m_{IS}	
	Stock	g	g	mg/g	g	g	µg/g		WS_{Hcy}	g	g	µg	µg	m_{Hcy}/m_{IS}
Day 11	HS1	0.20169	2.01866	0.09938	0.50408	5.01347	9.99	Cal1	WS2:3	0.12891	0.07203	1.23204	4.17963	0.29477
	HS2	0.19759	2.01122	0.09460	0.50410	5.01831	9.50	Cal2	WS2:1	0.32962	0.07222	3.29370	4.19066	0.78596
	HS3	0.20237	2.01740	0.09542	0.50363	5.02808	9.56	Cal3	WS2:2	0.56213	0.07181	5.34206	4.16687	1.28203
	IS1	0.50528	1.01362	0.05803				Cal4	WS2:3	0.76969	0.07190	7.35620	4.17209	1.76319
								Cal5	WS2:1	0.94114	0.07303	9.40425	4.23766	2.21921
								Cal6	WS2:2	1.19163	0.07186	11.32434	4.16977	2.71582
Day 12	HS1	0.20208	2.01765	0.09962	0.50328	5.08063	9.87	Cal1	WS2:2	0.12723	0.07989	1.22616	4.19132	0.29255
	HS2	0.20161	2.02123	0.09605	0.50491	5.03221	9.64	Cal2	WS2:3	0.34446	0.08004	3.28979	4.19918	0.78344
	HS3	0.20209	2.02030	0.09515	0.50519	5.03302	9.55	Cal3	WS2:1	0.53991	0.08003	5.32818	4.19866	1.26902
	IS1	0.50317	1.00578	0.05246				Cal4	WS2:2	0.76273	0.08028	7.35071	4.21178	1.74528
								Cal5	WS2:3	0.98729	0.08019	9.42918	4.20705	2.24128
								Cal6	WS2:1	1.16264	0.07951	11.47367	4.17138	2.75057
Day 13	HS1	0.20220	2.02156	0.09949	0.50456	5.00368	10.03	Cal1	WS2:2	0.12698	0.07974	1.22852	4.18339	0.29367
	HS2	0.20212	2.02224	0.09625	0.50512	5.02493	9.67	Cal2	WS2:3	0.34608	0.07964	3.31159	4.17815	0.79260
	HS3	0.20234	2.02238	0.09517	0.50508	5.02335	9.57	Cal3	WS2:1	0.53266	0.07998	5.34387	4.19599	1.27357
	IS1	0.50529	1.01003	0.05246				Cal4	WS2:2	0.76548	0.07963	7.40597	4.17762	1.77277
								Cal5	WS2:3	0.98802	0.08031	9.45422	4.21330	2.24390
								Cal6	WS2:1	1.14739	0.08014	11.51109	4.20438	2.73788
Day 14	HS1	0.20197	2.01986	0.09946	0.50467	5.06732	9.91	Cal1	WS2:3	0.13142	0.07955	1.22782	4.18539	0.29336
	HS2	0.20108	2.02207	0.09576	0.54059	5.17626	10.00	Cal2	WS2:1	0.33142	0.07969	3.28293	4.19275	0.78300
	HS3	0.20152	2.02148	0.09482	0.50446	5.12006	9.34	Cal3	WS2:2	0.53454	0.07909	5.34579	4.16118	1.28468
	IS1	0.50436	1.00529	0.05261				Cal4	WS2:3	0.78701	0.07951	7.35283	4.18328	1.75767
								Cal5	WS2:1	0.94898	0.07954	9.40025	4.18486	2.24625
								Cal6	WS2:2	1.13282	0.07927	11.32903	4.17066	2.71637

7.4. Instrumental Method

All analyses were performed on an Agilent 1260 Infinity II Series LC system equipped with a binary pump, degasser, autosampler, and column compartment, attached to an Agilent InfinityLab Quadrupole MS. The instrument was controlled using ChemStation software (Agilent). LC separation was achieved on a Discovery HS F5 column, 15 cm × 4 mm, 3 µm (Part 567532-U, SupelCo). Multiple columns were used throughout the analyses: three from batch lot BL-8795 and one from batch lot BL-10712. The sample injection volume was 10 µL, the column temperature was held at 30 °C, and mobile phase A was 0.1 % formic acid in HPLC grade water and mobile phase B 0.1 % formic acid in HPLC grade methanol. LC separation was achieved with isocratic conditions, with a column wash step, using the gradient program summarized in Table 11.

Table 11. Gradient Program for Homocysteine LC-MS Analysis.

Step	Time (min)	% A	% B	Flow (mL/min)
1	0.00	50.0	50.0	0.500
2	10.00	50.0	50.0	0.500
3	10.10	0.0	100.0	0.500
4	12.00	0.0	100.0	0.500
5	12.10	50.0	50.0	0.500
6	15.00	50.0	50.0	0.500

Detection was achieved using a single quadrupole MS with electrospray ionization in positive mode with selected ion monitoring (SIM) for *m*/*z* 136 (Hcy) and *m*/*z* 140 (Hcy-d_4). The fragmentor setpoint was 80 V, dwell time was 294 msec, and gain was 1. The drying gas flow rate was of 12 L/min at a temperature of 250 °C; the nebulizer pressure was 241 kPa (35 psig), and the capillary voltage was 3000 V. The SIM *m*/*z* 136 and *m*/*z* 140 peaks were manually integrated.

Representative LC-MS chromatograms for a calibrant, SRM 1950 control material, and each of the three SRM 1955 pseudo control materials are displayed in Fig. 24. Representative chromatograms for each of the three SRM 1955a materials are displayed in Fig. 25.

Column issues were observed throughout the measurement campaign. For both calibrants and samples, peak shapes would begin to broaden and eventually become a shoulder after two to three measurement sessions, where each measurement session was associated with 46 to 50 injections. Peak shapes deteriorated during the Day 3, Day 6, and Day 10 measurement sessions; new columns were used at the start of the next sessions. It was speculated that "gunk" accumulated at the front of the column and a reverse flush with acetonitrile at 1 mL/min for at least an hour after each batch sequence should be employed and that columns should be stored in acetonitrile to prolong the column life.

Another issue observed throughout the measurements involved the SPE process. Sample variability could be quite large due to the SPE columns, and it was also noted that the SPE columns must be shipped with the stationary phase fully immersed in the storage solution.

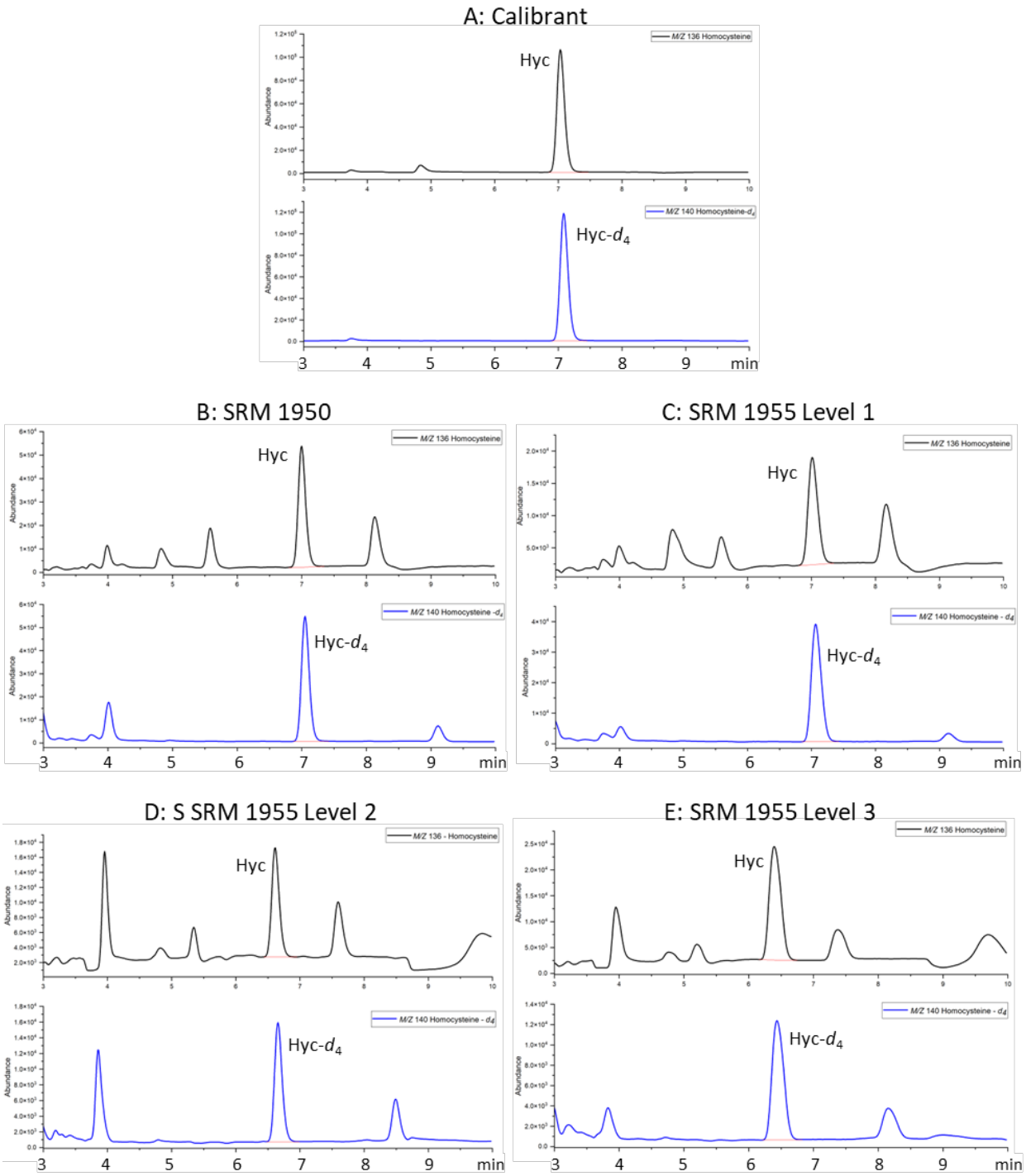


Fig. 24. Representative SIM Chromatograms for Calibrant and Control Materials.

Each panel displays chromatograms for m/z 136 (upper sub-panel) and m/z 140 (lower sub-panel): A) Calibrant, B) SRM 1950, C) SRM 1955 Level 1, D) SRM 1955 Level 2, and E) SRM 1955 Level 3.

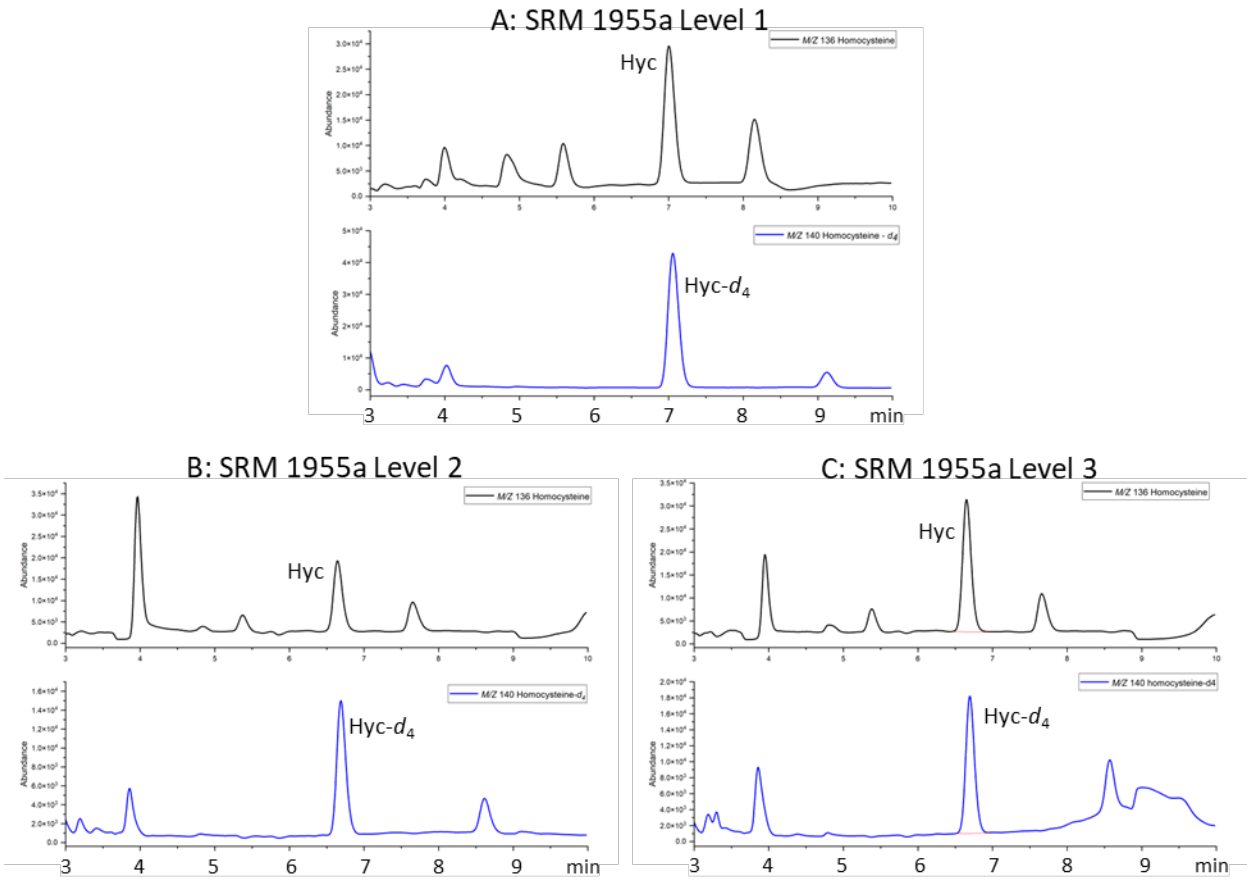


Fig. 25. Representative SIM Chromatograms for SRM 1955a Materials.

Each panel displays chromatograms for m/z 136 (upper sub-panel) and m/z 140 (lower sub-panel): A) SRM 1955a Level 1, B) SRM 1955a Level 2, and C) SRM 1955a Level 3.

7.5. Analysis

Calibrants, controls, and samples were prepared on the same day for each of the 14 measurement sessions. The LC-MS analysis sessions were typically organized as follows: water blanks, first injection of calibration solutions, water blank, first injection of controls and samples, water blank, second injection of controls and samples, water blank, second injection of calibrants, water blank, and DTT solution blanks. The number of control samples and the placing of the second calibrant injections varied somewhat across the measurement sessions. Table 12 details the analysis order for each session.

Table 12. Sample Analysis Order.

	Sample				
	Day 1	Day 2	Day 3	Day 4	Day 5
1	Water	Water	Water	Water	Water
2	Water	Water	Water	Water	Water
3	Water	Water	Water	Water	Water
4	Calibrant 1	Calibrant 1	Calibrant 1	Calibrant 1	Calibrant 1
5	Calibrant 2	Calibrant 2	Calibrant 2	Calibrant 2	Calibrant 2
6	Calibrant 3	Calibrant 3	Calibrant 3	Calibrant 3	Calibrant 3
7	Calibrant 4	Calibrant 4	Calibrant 4	Calibrant 4	Calibrant 4
8	Calibrant 5	Calibrant 5	Calibrant 5	Calibrant 5	Calibrant 5
9	Calibrant 6	Calibrant 6	Calibrant 6	Calibrant 6	Calibrant 6
10	Water	Water	Water	Water	Water
11	1950 V1 S2-1	1950 V2 S1-1	1950 V3 S1-1	1955L2 V1 S1-1	1950 V5 S2-1
12	1955L1 V1 S2-1	1955L1 V2 S1-1	1955aL1 #1458 S1-1	1950 V4 S1-1	1955L2 V2 S1-1
13	1955aL1 #849 S2-1	1955aL1 #936 S2-1	1955L1 V3 S1-1	1955aL2 #1043 S1-1	1955aL2 #1254 S3-1
14	1955aL1 #568 S1-1	1955aL1 #393 S1-1	1955aL1 #256 S1-1	1955aL2 #747 S3-1	1955aL2 #723 S1-1
15	1955aL1 #1112 S1-1	1955aL1 #1208 S1-1	1955aL1 #668 S1-1	1955aL2 #256 S1-1	1955aL2 #1254 S2-1
16	1955aL1 #1112 S2-1	1955aL1 #114 S1-1	1955aL1 #668 S3-1	1955aL2 #1043 S2-1	1955aL2 #45 S1-1
17	1955aL1 #849 S1-1	1955aL1 #1208 S2-1	1955aL1 #256 S2-1	1955aL2 #256 S2-1	1955aL2 #1254 S1-1
18	1955aL1 #568 S2-1	1955aL1 #114 S2-1	1955aL1 #256 S3-1	1955aL2 #747 S2-1	1955aL2 #45 S2-1
19	1955aL1 #849 S3-1	1955aL1 #393 S2-1	1955aL1 #668 S2-1	1955aL2 #747 S1-1	1955aL2 #45 S3-1
20	1955aL1 #1112 S3-1	1955aL1 #936 S1-1	1955aL1 #1458 S2-1	1955aL2 #1043 S3-1	1955aL2 #723 S2-1
21	1955L1 V1 S1-1	1955L1 V2 S2-1	1950 V3 S2-1	1950 V4 S2-1	1950 V5 S1-1
22	1950 V1 S1-1	1950 V2 S2-1	1955L1 V3 S2-1	1955L2 V1 S2-1	1955L2 V2 S1-2
23	Water	Water	Water	Water	Water
24	1950 V1 S1-2	1950 V2 S1-2	1950 V3 S2-2	1955L2 V1 S1-2	1950 V5 S1-2
25	1955L1 V1 S2-2	1955L1 V2 S1-2	1955L1 V3 S1-2	1950 V4 S2-2	1955L2 V2 S2-1
26	1955aL1 #849 S3-2	1955aL1 #1208 S2-2	1955aL1 #1458 S2-2	1955aL2 #256 S1-2	1955aL2 #1254 S3-2
27	1955aL1 #1112 S1-2	1955aL1 #936 S1-2	1955aL1 #668 S2-2	1955aL2 #747 S1-2	1955aL2 #1254 S1-2
28	1955aL1 #568 S1-2	1955aL1 #393 S1-2	1955aL1 #256 S2-2	1955aL2 #1043 S2-2	1955aL2 #723 S1-2
29	1955aL1 #849 S2-2	1955aL1 #393 S2-2	1955aL1 #256 S3-2	1955aL2 #747 S2-2	1955aL2 #45 S2-2
30	1955aL1 #568 S2-2	1955aL1 #936 S2-2	1955aL1 #1458 S1-2	1955aL2 #1043 S3-2	1955aL2 #45 S3-2
31	1955aL1 #1112 S2-2	1955aL1 #114 S1-2	1955aL1 #668 S1-2	1955aL2 #747 S3-2	1955aL2 #723 S2-2
32	1955aL1 #849 S1-2	1955aL1 #114 S2-2	1955aL1 #256 S1-2	1955aL2 #256 S2-2	1955aL2 #45 S1-2
33	1955aL1 #1112 S3-2	1955aL1 #1208 S1-2	1955aL1 #668 S3-2	1955aL2 #1043 S1-2	1955aL2 #1254 S2-2
34	1955L1 V1 S1-2	1955L1 V2 S2-2	1955L1 V3 S2-2	1950 V4 S1-2	1955L2 V2 S2-2
35	1950 V1 S2-2	1950 V2 S2-2	1950 V3 S1-2	1955L2 V1 S2-2	1950 V5 S2-2
36	Water	Water	Water	Water	Water
37	Calibrant 5	Calibrant 3	Calibrant 6	Calibrant 2	Calibrant 4
38	Calibrant 1	Calibrant 1	Calibrant 4	Calibrant 5	Calibrant 5
39	Calibrant 2	Calibrant 6	Calibrant 1	Calibrant 6	Calibrant 6
40	Calibrant 4	Calibrant 2	Calibrant 2	Calibrant 3	Calibrant 3
41	Calibrant 3	Calibrant 4	Calibrant 5	Calibrant 1	Calibrant 2
42	Calibrant 6	Calibrant 5	Calibrant 3	Calibrant 4	Calibrant 1
43	Water	Water	Water	Water	Water
44	DTT	DTT	DTT	DTT	DTT
45	DTT	DTT	DTT	DTT	DTT
46	Water	Water	Water	Water	Water

Table 12. Sample Analysis Order (Continued).

	Sample				
	Day 6	Day 7	Day 8	Day 9	Day 10
1	Water	Water	Water	Water	Water
2	Water	Water	Water	Water	Water
3	Water	Water	Water	Water	Water
4	Calibrant 1	Calibrant 1	Calibrant 1	Calibrant 1	Calibrant 1
5	Calibrant 2	Calibrant 2	Calibrant 2	Calibrant 2	Calibrant 2
6	Calibrant 3	Calibrant 3	Calibrant 3	Calibrant 3	Calibrant 3
7	Calibrant 4	Calibrant 4	Calibrant 4	Calibrant 4	Calibrant 4
8	Calibrant 5	Calibrant 5	Calibrant 5	Calibrant 5	Calibrant 5
9	Calibrant 6	Calibrant 6	Calibrant 6	Calibrant 6	Calibrant 6
10	Water	Water	Water	Water	Water
11	1950 V6 S2-1	1955L3 V1 S1-1	1950 V8 S1-1	1950 V9 S2-1	1950 V10 S1-1
12	1955L2 V3 S1-1	1950 V7 S2-1	1955L3 V2 S2-1	1950 V9 S1-1	1950 V10 S3-1
13	1955aL2 #387 S1-1	1950 V7 S3-1	1955aL3 #69 S2-1	1955L3 V3 S1-1	1955L3 V4 S2-1
14	1955aL2 #1403 S1-1	1955aL3 #262 S2-1	1955aL3 #1078 S3-1	1955aL3 #1269 S2-1	1955aL3 #70 S1-1
15	1955aL2 #998 S1-1	1955aL3 #1337 S3-1	1955aL3 #553 S1-1	1955aL3 #410 S1-1	1955aL3 #1077 S2-1
16	1955aL2 #387 S2-1	1955aL3 #262 S3-1	1955aL3 #1078 S2-1	1955aL3 #889 S2-1	1955aL3 #552 S3-1
17	1955aL2 #481 S2-1	1955aL3 #262 S1-1	1955aL3 #553 S2-1	1955aL3 #730 S1-1	1955aL3 #70 S2-1
18	1955aL2 #998 S2-1	1955aL3 #1337 S1-1	1955aL3 #553 S3-1	1955aL3 #730 S2-1	1955aL3 #1077 S1-1
19	1955aL2 #1403 S2-1	1955aL3 #790 S1-1	1955aL3 #69 S1-1	1955aL3 #410 S2-1	1955aL3 #552 S2-1
20	1955aL2 #481 S1-1	1955aL3 #790 S2-1	1955aL3 #1078 S1-1	1955aL3 #889 S1-1	1955aL3 #1077 S3-1
21	1955L2 V3 S2-1	1955aL3 #1337 S2-1	1950 V8 S3-1	1955aL3 #1269 S1-1	1955aL3 #552 S1-1
22	1950 V6 S1-1	1950 V7 S1-1	1955L3 V2 S1-1	1955aL3 #889 S2-2	1955L3 V4 S1-1
23	Water	1955L3 V1 S2-1	1950 V8 S2-1	1955L3 V3 S2-1	1950 V10 S2-1
24	1950 V6 S1-2	Water	Water	1950 V9 S3-1	Water
25	1955L2 V3 S1-2	1950 V7 S2-2	1955L3 V2 S1-2	Water	Calibrant 2
26	1955aL2 #998 S1-2	1955L3 V1 S1-2	1950 V8 S2-2	Calibrant 5	Calibrant 6
27	1955aL2 #1403 S2-2	1955aL3 #1337 S1-2	1955aL3 #1078 S1-2	Calibrant 2	Calibrant 3
28	1955aL2 #387 S2-2	1955aL3 #262 S1-2	1955aL3 #69 S1-2	Calibrant 3	Calibrant 1
29	1955aL2 #998 S2-2	1955aL3 #262 S3-2	1955aL3 #553 S1-2	Calibrant 1	Calibrant 5
30	1955aL2 #1403 S1-2	1955aL3 #790 S2-2	1955aL3 #553 S3-2	Calibrant 4	Calibrant 4
31	1955aL2 #481 S2-2	1955aL3 #790 S1-2	1955aL3 #1078 S2-2	Calibrant 6	Water
32	1955aL2 #387 S1-2	1955aL3 #1337 S2-2	1955aL3 #69 S2-2	Water	1950 V10 S2-2
33	1955aL2 #481 S1-2	1955aL3 #262 S2-2	1955aL3 #553 S2-2	1950 V9 S2-2	1950 V10 S3-2
34	1950 V6 S2-2	1955aL3 #1337 S3-2	1955aL3 #1078 S3-2	1955L3 V3 S2-2	1955L3 V4 S1-2
35	1955L2 V3 S2-2	1950 V7 S1-2	1950 V8 S1-2	1955aL3 #1269 S1-2	1955aL3 #552 S1-2
36	Water	1950 V7 S3-2	1955L3 V2 S2-2	1955aL3 #410 S2-2	1955aL3 #70 S2-2
37	Calibrant 4	Water	1950 V8 S3-2	1955aL3 #730 S1-2	1955aL3 #1077 S3-2
38	Calibrant 2	Calibrant 5	Water	1955aL3 #889 S2-3	1955aL3 #70 S1-2
39	Calibrant 6	Calibrant 3	Calibrant 6	1955aL3 #1269 S2-2	1955aL3 #552 S2-2
40	Calibrant 3	Calibrant 4	Calibrant 3	1955aL3 #889 S1-2	1955aL3 #1077 S1-2
41	Calibrant 5	Calibrant 6	Calibrant 1	1955aL3 #730 S2-2	1955aL3 #1077 S2-2
42	Calibrant 1	Calibrant 2	Calibrant 4	1955aL3 #410 S1-2	1955aL3 #552 S3-2
43	Water	Calibrant 1	Calibrant 5	1950 V9 S3-2	1955L3 V4 S2-2
44	DTT	Water	Calibrant 2	1955L3 V3 S1-2	1950 V10 S1-2
45	DTT	DTT	Water	1950 V9 S1-2	Water
46	Water	DTT	DTT	Water	DTT
47		Water	DTT	DTT	DTT
48			Water	DTT	Water
49				Water	

Table 12. Sample Analysis Order (Continued).

	Sample			
	Day 11	Day 12	Day 13	Day 14
1	Water	Water	Water	Water
2	Water	Water	Water	Water
3	Water	Water	Water	Water
4	Calibrant 1	Calibrant 1	Calibrant 1	Calibrant 1
5	Calibrant 2	Calibrant 2	Calibrant 2	Calibrant 2
6	Calibrant 3	Calibrant 3	Calibrant 3	Calibrant 3
7	Calibrant 4	Calibrant 4	Calibrant 4	Calibrant 4
8	Calibrant 5	Calibrant 5	Calibrant 5	Calibrant 5
9	Calibrant 6	Calibrant 6	Calibrant 6	Calibrant 6
10	Water	Water	Water	Water
11	1950 V11 S1-1	1950 V12 S1-1	1950 V13 S1-1	1950 V14 S2-1
12	1950 V11 S4-1	1950 V12 S2-1	1950 V13 S3-1	1950 V14 S4-1
13	1955L3 V5 S1-1	1950 V12 S3-1	1955L2 V5 S2-1	1955L3 V6 S1-1
14	1955aL3 #890 S2-1	1950 V12 S4-1	1955aL2 #722 S1-1	1955aL3 #261 S1-1
15	1955aL3 #411 S2-1	1955L2 V4 S1-1	1955aL2 #44 S1-1	1955aL3 #789 S3-1
16	1955aL3 #729 S2-1	1955L2 V4 S2-1	1955aL2 #44 S3-1	1955aL3 #261 S2-1
17	1955aL3 #890 S1-1	1955aL2 #386 S1-1	1955aL2 #1253 S3-1	1955aL3 #789 S2-1
18	1955aL3 #1268 S1-1	1955aL2 #386 S2-1	1955aL2 #722 S2-1	1955aL3 #1336 S3-1
19	1955aL3 #729 S1-1	1955aL2 #482 S1-1	1955aL2 #1253 S1-1	1955aL3 #1336 S1-1
20	1955aL3 #411 S1-1	1955aL2 #482 S2-1	1955aL2 #44 S2-1	1955aL3 #1336 S2-1
21	1955aL3 #1268 S2-1	1955aL2 #997 S1-1	1955aL2 #1253 S2-1	1955aL3 #789 S1-1
22	1955L3 V5 S2-1	1955aL2 #997 S2-1	1955L2 V5 S1-1	1955L3 V6 S2-1
23	1950 V11 S2-1	1955aL2 #1402 S1-1	1950 V13 S4-1	1950 V14 S1-1
24	1950 V11 S3-1	1955aL2 #1402 S2-1	1950 V13 S2-1	1950 V14 S3-1
25	Water	Water	Water	Water
26	Calibrant 5	1950 V12 S1-2	1950 V13 S2-2	1950 V14 S3-2
27	Calibrant 2	1950 V12 S2-2	1950 V13 S4-2	1950 V14 S4-2
28	Calibrant 3	1950 V12 S3-2	1955L2 V5 S2-2	1955L3 V6 S2-2
29	Calibrant 1	1950 V12 S4-2	1955aL2 #1253 S2-2	1955aL3 #1336 S1-2
30	Calibrant 4	1955L2 V4 S1-2	1955aL2 #44 S2-2	1955aL3 #261 S1-2
31	Calibrant 6	1955L2 V4 S2-2	1955aL2 #722 S1-2	1955aL3 #789 S3-2
32	Water	1955aL2 #386 S1-2	1955aL2 #1253 S1-2	1955aL3 #1336 S3-2
33	1950 V11 S2-2	1955aL2 #386 S2-2	1955aL2 #44 S3-2	1955aL3 #789 S1-2
34	1950 V11 S3-2	1955aL2 #482 S1-2	1955aL2 #722 S2-2	1955aL3 #261 S2-2
35	1955L3 V5 S2-2	1955aL2 #482 S2-2	1955aL2 #44 S1-2	1955aL3 #789 S2-2
36	1955aL3 #1268 S2-2	1955aL2 #997 S1-2	1955aL2 #1253 S3-2	1955aL3 #1336 S2-2
37	1955aL3 #411 S1-2	1955aL2 #997 S2-2	1950 V13 S3-2	1955L3 V6 S1-2
38	1955aL3 #890 S1-2	1955aL2 #1402 S1-2	1955L2 V5 S1-2	1950 V14 S2-2
39	1955aL3 #729 S1-2	1955aL2 #1402 S2-2	1950 V13 S1-2	1950 V14 S1-2
40	1955aL3 #890 S2-2	Water	Water	Water
41	1955aL3 #729 S2-2	Calibrant 5	Calibrant 4	Calibrant 4
42	1955aL3 #1268 S1-2	Calibrant 2	Calibrant 5	Calibrant 3
43	1955aL3 #411 S2-2	Calibrant 1	Calibrant 1	Calibrant 6
44	1950 V11 S4-2	Calibrant 6	Calibrant 6	Calibrant 5
45	1955L3 V5 S1-2	Calibrant 4	Calibrant 2	Calibrant 1
46	1950 V11 S1-2	Calibrant 3	Calibrant 3	Calibrant 2
47	Water	Water	Water	Water
48	DTT	DTT	DTT	DTT
49	DTT	DTT	DTT	DTT
50	Water	Water	Water	Water

7.5.1. **Calibration Functions**

The mass fraction of Hcy in the SRM 1950, SRM 1955, and SRM 1955a samples was determined using the internal standard approach described in Section 3. Calibration functions were generated using classical least-squares regression for each measurement session from the mass and peak area ratios of the calibration solutions (see Eq. 1). The mass fraction of Hcy in each sample was then calculated from the calibration function, the peak area ratio, and the sample mass using Eq. 2. Calibration functions for the first and second injections of the calibration solutions in each of the 14 measurement sessions are displayed in Fig. 26.

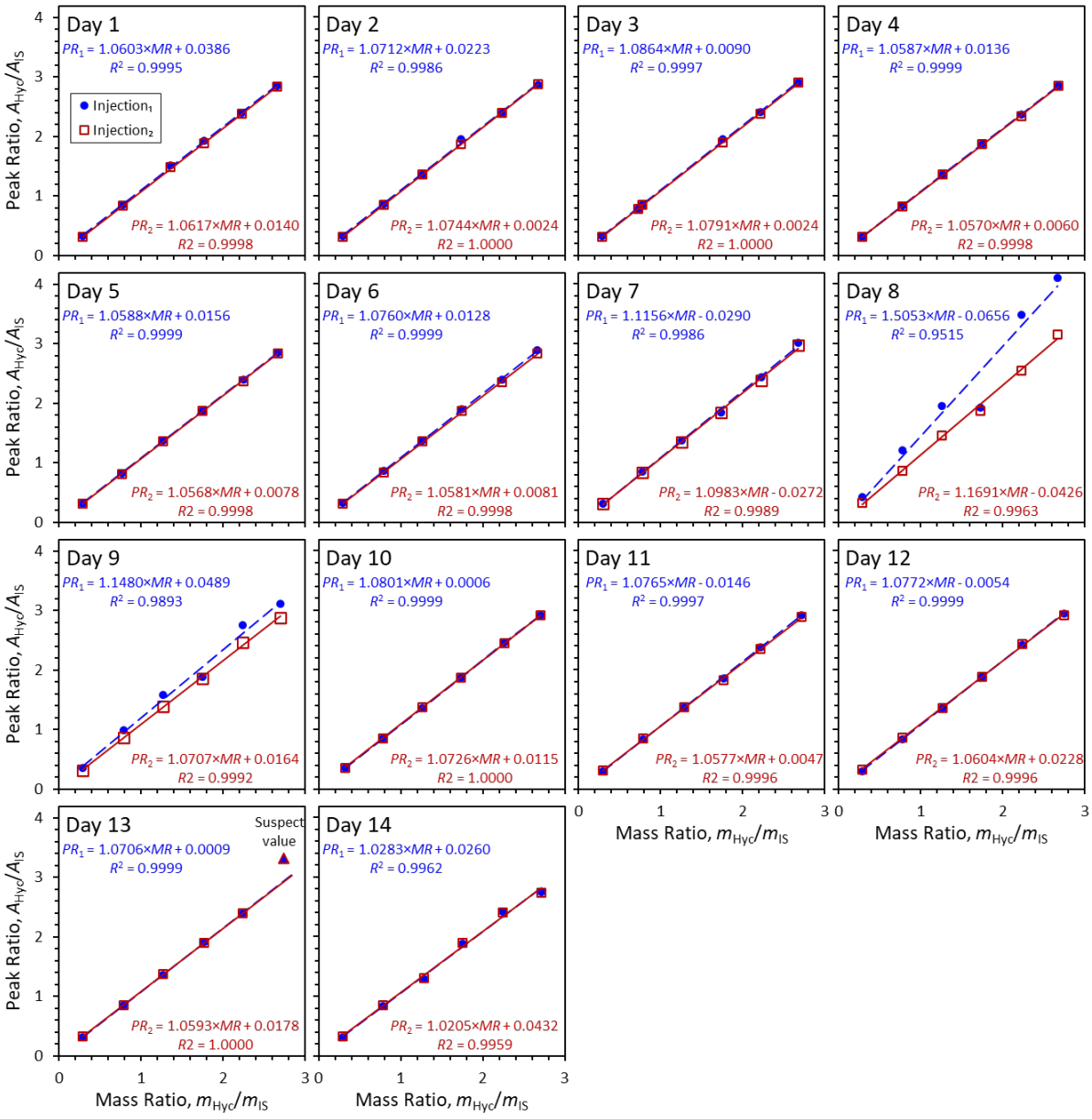


Fig. 26. Calibration Functions for First and Second Injection Peak Area Ratios.

Solid circles and dashed lines represent the first injection results and the estimated linear function (PR_1, upper left); open squares and solid line represent the second injection results and function (PR_2, lower right).

All of the functions are adequately linear and most of the first and second curves are superimposable, indicating that the preparation of calibrants was consistent, calibrants were stable, and that the peak area ratios were repeatable and reproducible over the experimental period. However, the functions for the first injections on Days 8 and 9 visibly differ from those for the second. The results for both injections of Calibrant 6 on Day 13 do not align with the function estimated using the other five calibrants.

Since the first and second injection peak area ratios for Calibrant 6 in Day 13 are nearly identical, the aberrant result is ascribed to an undetected preparation or recording error.

The greater than typical slope for the first results in the Day 8 and 9 sessions suggests that for some unexplained reason the conversion of homocystine-d_8 to Hcy-d_4 in the calibrants was somewhat slower than typical. The slopes for the results for the second injection, which were obtained at the end of each session, are unexceptional suggesting that the conversion proceeded to completion during the session. Since all sample injections came after the first round of calibrant injections, the second injection data are believed to be more representative of the samples.

The mass ratios for the calibration solutions are detailed in Table 10; the peak area ratios are detailed in Table 13. Excluding only the inconsistent peak area ratios for the Day 8, 9, and 13 sessions (listed in *red italic* font), the least square regression estimates for the calibration function intercept and slope in each measurement session are provided in Table 14.

Table 13. Calibration Solution Peak Area Ratios.

	Inj	Day	A_{Hcy}	A_{IS}	R_{area}	$\bar{x}_{area}$	s_{area}	Day	A_{Hcy}	A_{IS}	R_{area}	$\bar{x}_{area}$	s_{area}
Cal1	1	1	328082	995965	0.3294	0.3222	0.0102	2	328486	1021785	0.3215	0.3179	0.0050
	2		320989	1018939	0.3150				317193	1008927	0.3144		
Cal2	1		873632	1020466	0.8561	0.8467	0.0132		913743	1051671	0.8688	0.8607	0.0116
	2		858601	1025343	0.8374				867094	1017105	0.8525		
Cal3	1		1412545	931612	1.5162	1.4989	0.0245		1403574	1032557	1.3593	1.3606	0.0019
	2		1380593	931863	1.4815				1381785	1014573	1.3619		
Cal4	1		1927791	998983	1.9298	1.9049	0.0351		1860032	951082	1.9557	1.9138	0.0593
	2		1894774	1007783	1.8801				1855418	991244	1.8718		
Cal5	1		2404062	1006291	2.3890	2.3872	0.0026		2396335	1001999	2.3916	2.3947	0.0044
	2		2386556	1000495	2.3854				2386905	995446	2.3978		
Cal6	1		2832148	993229	2.8515	2.8445	0.0099		2874544	1002438	2.8676	2.8712	0.0051
	2		2806705	989154	2.8375				2866054	996950	2.8748		
Cal1	1	3	400380	1222879	0.3274	0.3232	0.0060	4	398778	1262688	0.3158	0.3132	0.0037
	2		321760	1008782	0.3190				412434	1327836	0.3106		
Cal2	1		1033139	1209829	0.8540	0.8512	0.0038		1029949	1233449	0.8350	0.8285	0.0092
	2		1082617	1275858	0.8485				1094829	1331898	0.8220		
Cal3	1		1679402	2157096	0.7785	0.7773	0.0018		1678320	1231904	1.3624	1.3587	0.0052
	2		1757647	2264966	0.7760				1777549	1311839	1.3550		
Cal4	1		2316547	1189182	1.9480	1.9224	0.0362		2267067	1209519	1.8744	1.8693	0.0071
	2		2420170	1275914	1.8968				2390044	1282014	1.8643		
Cal5	1		2934015	1216991	2.4109	2.3963	0.0205		2835394	1201907	2.3591	2.3494	0.0137
	2		3044673	1278299	2.3818				3036082	1297672	2.3396		
Cal6	1		3548336	1221247	2.9055	2.9031	0.0034		3378750	1185887	2.8491	2.8460	0.0045
	2		3680391	1268799	2.9007				3615947	1271973	2.8428		
Cal1	1	5	463987	1452833	0.3194	0.3168	0.0036	6	483758	1483555	0.3261	0.3208	0.0075
	2		464684	1478787	0.3142				461053	1461255	0.3155		
Cal2	1		1210668	1462816	0.8276	0.8222	0.0077		1251725	1460019	0.8573	0.8461	0.0159
	2		1213216	1485441	0.8167				1219607	1460947	0.8348		
Cal3	1		1975418	1440959	1.3709	1.3649	0.0084		2015691	1462466	1.3783	1.3678	0.0148
	2		1975446	1453632	1.3590				1945862	1433539	1.3574		
Cal4	1		2673944	1424685	1.8769	1.8722	0.0066		2730321	1439707	1.8964	1.8795	0.0239
	2		2713109	1452755	1.8676				2682528	1440219	1.8626		
Cal5	1		3316843	1387535	2.3905	2.3785	0.0169		3360983	1402716	2.3961	2.3717	0.0345
	2		3406567	1439487	2.3665				3285909	1399866	2.3473		
Cal6	1		3959843	1391493	2.8458	2.8437	0.0029		4031702	1398194	2.8835	2.8577	0.0365
	2		4014445	1412743	2.8416				3949849	1394804	2.8318		
Cal1	1	7	214800	674658	0.3184	0.3165	0.0026	8	*210055*	*498476*	*0.4214**	0.3284	
	2		212464	675245	0.3146				209245	637239	0.3284		
Cal2	1		562201	663374	0.8475	0.8416	0.0083		*553820*	*458533*	*1.2078**	0.8693	
	2		560921	671179	0.8357				544615	626526	0.8693		
Cal3	1		904555	656689	1.3774	1.3605	0.0239		*892603*	*458320*	*1.9476**	1.4501	
	2		894708	665897	1.3436				886448	611295	1.4501		
Cal4	1		1238064	671095	1.8448	1.8444	0.0006		*1216103*	*630775*	*1.9280**	1.8659	
	2		1229675	666875	1.8439				1200075	643163	1.8659		
Cal5	1		1578689	648539	2.4342	2.4098	0.0345		*1525033*	*438150*	*3.4806**	2.5437	
	2		1569432	657934	2.3854				1507678	592699	2.5437		
Cal6	1		1875857	622900	3.0115	2.9885	0.0326		*1846204*	*450617*	*4.0971**	3.1532	
	2		1862096	627934	2.9654				1830975	580669	3.1532		

Table 13. Calibration Solution Mass Ratios (Continued).

	Inj	Day	A_{Hcy}	A_{IS}	R_{area}	$\bar{x}$	s	Day	A_{Hcy}	A_{IS}	R_{area}	$\bar{x}$	s
	1		*193885*	*550373*	*0.3523**				117350	334020	0.3513		
Cal1	2		183579	583195	0.3148	0.3148			124755	346748	0.3598	0.3579	0.0059
	3								126612	349080	0.3627		
	1		*515215*	*521753*	*0.9875**				312829	369017	0.8477		
Cal2	2		489355	564884	0.8663	0.8663			330371	388920	0.8495	0.8488	0.0010
	3								332501	391494	0.8493		
	1		*817328*	*515468*	*1.5856**				506579	371726	1.3628		
Cal3	2		788226	565044	1.3950	1.3950			532781	389727	1.3671	1.3643	0.0024
		9						10	535809	393123	1.3630		
	1		*1108654*	*589669*	*1.8801**				701742	375630	1.8682		
Cal4	2		1073071	577685	1.8575	1.8575			731430	390646	1.8724	1.8698	0.0023
									730295	390796	1.8687		
	1		*1412424*	*512518*	*2.7559**				897742	365213	2.4581		
Cal5	2		1381281	561689	2.4592	2.4592			935224	382984	2.4419	2.4447	0.0122
	3								936865	384882	2.4342		
	1		*1671332*	*538167*	*3.1056**				1075657	369014	2.9149		
Cal6	2		1618344	561823	2.8805	2.8805			1118462	384480	2.9090	2.9054	0.0118
	3								1117572	386400	2.8923		
Cal1	1		95682	314072	0.3046	0.3088	0.0059		91990	302652	0.3039	0.3121	0.0115
	2		105215	336176	0.3130				104270	325620	0.3202		
Cal2	1		262083	312985	0.8374	0.8420	0.0065		249473	296288	0.8420	0.8530	0.0156
	2		283369	334734	0.8466				276035	319471	0.8640		
Cal3	1		426833	310322	1.3754	1.3759	0.0007		407437	300115	1.3576	1.3597	0.0029
	2		457132	332115	1.3764				439603	322818	1.3618		
Cal4	1	11	568711	307531	1.8493	1.8410	0.0118	12	566945	301640	1.8795	1.8801	0.0008
	2		602067	328524	1.8326				604921	321657	1.8806		
Cal5	1		749500	314762	2.3812	2.3684	0.0181		725371	299003	2.4260	2.4267	0.0010
	2		784587	333070	2.3556				776429	319863	2.4274		
Cal6	1		902902	309225	2.9199	2.9045	0.0218		870232	295773	2.9422	2.9282	0.0199
	2		940327	325474	2.8891				923222	316808	2.9141		
Cal1	1		93668	298196	0.3141	0.3195	0.0077		89507	282567	0.3168	0.3212	0.0063
	2		102390	315064	0.3250				97041	298034	0.3256		
Cal2	1		246149	290958	0.8460	0.8522	0.0088		236722	281232	0.8417	0.8476	0.0083
	2		265096	308824	0.8584				254525	298215	0.8535		
Cal3	1		396091	289892	1.3663	1.3687	0.0034		357988	276230	1.2960	1.3011	0.0072
	2		420550	306721	1.3711				382942	293188	1.3061		
Cal4	1	13	556768	291341	1.9110	1.9054	0.0080	14	522116	278210	1.8767	1.8844	0.0109
	2		586360	308654	1.8997				559517	295714	1.8921		
Cal5	1		699582	292207	2.3941	2.3917	0.0034		662350	274104	2.4164	2.4140	0.0034
	2		732707	306661	2.3893				697198	289099	2.4116		
Cal6	1		*843366*	*253800*	*3.3230*	*3.3188**	*0.0059**		754522	274653	2.7472	2.7428	0.0062
	2		*882683*	*266304*	*3.3146*				796382	290821	2.7384		

* Suspect value, not used in estimating the calibration function
Inj Injection sequence (1 to 2 or 3)
Day Measurement session (1 to 14)
A_{Hcy} Measured area of the m/z 136 (homocysteine) peak
A_{IS} Measured area of the m/z 140 (homocysteine-d_4 IS) peak
R_{area} Ratio of homocysteine and IS peak areas, $R_{area} = A_{Hcy}/A_{IS}$
$\bar{x}$ The mean of the R_{area} values for the first and second injections
s The standard deviation of the R_{area} values for the first and second injections

Table 14. "Classical" Linear Regression Calibration Functions.

			Slope		Intercept			
Session	Basis of R_{area}	v	B_1	$u(B_1)$	B_0	$u(B_0)$	R^2	RMSE
Day 1	Mean of two injections	4	1.0611	0.0084	0.0263	0.0144	0.9998	0.0166
Day 2	Mean of two injections	4	1.0711	0.0102	0.0123	0.0174	0.9996	0.0204
Day 3	Mean of two injections	4	1.0829	0.0053	0.0057	0.0087	0.9999	0.0112
Day 4	Mean of two injections	4	1.0579	0.0063	0.0098	0.0108	0.9999	0.0127
Day 5	Mean of two injections	4	1.0579	0.0050	0.0117	0.0086	0.9999	0.0101
Day 6	Mean of two injections	4	1.0671	0.0059	0.0105	0.0100	0.9999	0.0117
Day 7	Mean of two injections	4	1.1070	0.0190	-0.0281	0.0323	0.9988	0.0380
Day 8	Second injection only	4	1.1692	0.0355	-0.0426	0.0604	0.9963	0.0710
Day 9	Second injection only	4	1.0708	0.0153	0.0164	0.0262	0.9992	0.0308
Day 10	Mean of three injections	4	1.0728	0.0028	0.0099	0.0048	1.0000	0.0057
Day 11	Mean of two injections	4	1.0672	0.0101	-0.0049	0.0173	0.9996	0.0203
Day 12	Mean of two injections	4	1.0688	0.0082	0.0087	0.0141	0.9998	0.0167
Day 13	Mean, exclude Cal6	3	1.0650	0.0044	0.0093	0.0063	0.9999	0.0068
Day 14	Mean of two injections	4	1.0245	0.0320	0.0346	0.0552	0.9961	0.0649

R_{area} Ratio of homocysteine and IS peak areas, $R_{area} = A_{Hcy}/A_{IS}$
v Number of degrees of freedom for regression parameters, equal to the number of calibrants used minus the number of parameters estimated
B_1 Regression estimate of the slope of the linear model.
$u(B_1)$ Regression estimate of the standard uncertainty for the *slope* parameter.
B_0 Regression estimate of the intercept of the linear model.
$u(B_0)$ Regression estimate of the standard uncertainty for the intercept parameter.
R^2 Square of the Pearson's correlation between the measured and predicted R_{area}.
RMSE Root-mean sum of squares of the differences between the measured and predicted R_{area}, aka the standard error of the *Y* estimate.

7.5.2. **Measurements and Estimated values for SRM 1950**

Table 15 details the measured peak areas, sample and IS masses, calibration functions, and estimated Hcy mass fractions, w_{Hcy}, for all 80 analyses of the SRM 1950 control material. The mass fractions are displayed as a function of the SRM 1950 analysis sequence in Fig. 27.

Table 15. Peak Areas, Mass Measurements, and Estimated Values for SRM 1950.

					m_{sample}	m_{IS}			w_{Hcy}
Day	Sample	Seq	A_{Hcy}	A_{IS}	g	µg	b_1	b_0	µg/g
1	1950 V1 S1-1	22	358998	394994	0.20623	0.27473	1.0611	0.0263	1.1080
	1950 V1 S1-2	24	361709	396924					1.1110
	1950 V1 S2-1	11	353538	393274	0.20814	0.27903			1.1025
	1950 V1 S2-2	35	351531	390168					1.1051
2	1950 V2 S1-1	11	421127	459328	0.20810	0.27186	1.0711	0.0123	1.1032
	1950 V2 S1-2	24	418756	455916					1.1053
	1950 V2 S2-1	22	367646	409735	0.20990	0.28302			1.1140
	1950 V2 S2-2	35	371333	413723					1.1144
3	1950 V3 S1-1	11	407381	437723	0.20648	0.27551	1.0829	0.0057	1.1397
	1950 V3 S1-2	35	430611	465920					1.1318
	1950 V3 S2-1	21	402701	435920	0.20926	0.27674			1.1212
	1950 V3 S2-2	24	407222	444496					1.1119

Table 15. Peak Areas, Mass Measurements, and Estimated Values for SRM 1950 (Continued).

Day	Sample	Seq	A_{Hcy}	A_{IS}	m_{sample} g	m_{IS} µg	b_1	b_0	w_{Hcy} µg/g
4	1950 V4 S1-1	12	454905	511175	0.20262	0.27158	1.0579	0.0098	1.1151
	1950 V4 S1-2	34	473563	535172					1.1087
	1950 V4 S2-1	21	466371	514802	0.20955	0.27569			1.1144
	1950 V4 S2-2	25	463568	512802					1.1120
5	1950 V5 S1-1	21	501573	572274	0.20388	0.27215	1.0579	0.0117	1.0911
	1950 V5 S1-2	24	530841	600549					1.1006
	1950 V5 S2-1	11	498271	571926	0.20798	0.27403			1.0705
	1950 V5 S2-2	35	483446	550403					1.0794
6	1950 V6 S1-1	22	510642	581694	0.20762	0.27236	1.0671	0.0105	1.0663
	1950 V6 S1-2	24	516672	587074					1.0690
	1950 V6 S2-1	11	669480	772537	0.20908	0.28509			1.0939
	1950 V6 S2-2	34	628790	718951					1.1042
7	1950 V7 S1-1	22	231149	259365	0.20545	0.27515	1.1070	-0.0281	1.1122
	1950 V7 S1-2	35	233510	262571					1.1099
	1950 V7 S2-1	12	226363	249673	0.20902	0.27353			1.1050
	1950 V7 S2-2	25	223400	247309					1.1011
	1950 V7 S3-1	13	244164	268023	0.20911	0.27908			1.1322
	1950 V7 S3-2	36	241925	265726					1.1315
8	1950 V8 S1-1	11	217676	240286	0.20197	0.28094	1.1692	-0.0426	1.1284
	1950 V8 S1-2	35	212003	234406					1.1267
	1950 V8 S2-1	23	213363	233591	0.20551	0.28579			1.1371
	1950 V8 S2-2	26	213084	233624					1.1355
	1950 V8 S3-1	21	208565	229515	0.20821	0.27961			1.0927
	1950 V8 S3-2	37	205806	225915					1.0953
9	1950 V9 S1-1	12	232477	262380	0.20409	0.29514	1.0708	0.0164	1.1744
	1950 V9 S1-2	45	221954	251080					1.1717
	1950 V9 S2-1	11	208879	228477	0.21104	0.27271			1.0835
	1950 V9 S2-2	33	201743	223381					1.0701
	1950 V9 S3-1	24	210876	232457	0.21000	0.27080			1.0727
	1950 V9 S3-2	43	204729	227510					1.0639
10	1950 V10 S1-1	11	129405	143965	0.20382	0.27342	1.0728	0.0099	1.1116
	1950 V10 S1-2	44	132473	146930					1.1150
	1950 V10 S2-1	23	139272	149136	0.21034	0.26668			1.0920
	1950 V10 S2-2	32	140422	151101					1.0866
	1950 V10 S3-1	12	130383	142763	0.20834	0.27141			1.0970
	1950 V10 S3-2	33	135326	146497					1.1097
11	1950 V11 S1-1	11	113026	124140	0.20720	0.27297	1.0672	-0.0049	1.1300
	1950 V11 S1-2	46	122828	130585					1.1672
	1950 V11 S2-1	23	182502	200799	0.19749	0.25525			1.1067
	1950 V11 S2-2	33	184806	203327					1.1067
	1950 V11 S3-1	24	124230	131529	0.21532	0.24744			1.0223
	1950 V11 S3-2	34	125175	132933					1.0193
	1950 V11 S4-1	12	125348	136919	0.20679	0.27536			1.1484
	1950 V11 S4-2	44	136999	145250					1.1830

Table 15. Peak Areas, Mass Measurements, and Estimated Values for SRM 1950 (Continued).

Day	Sample	Seq	A_{Hcy}	A_{IS}	m_{sample} g	m_{IS} µg	b_1	b_0	w_{Hcy} µg/g
12	1950 V12 S1-1	11	111878	125309	0.20582	0.27816	1.0688	0.0087	1.1180
	1950 V12 S1-2	26	121827	132208					1.1542
	1950 V12 S2-1	12	115277	127222	0.20892	0.27759			1.1156
	1950 V12 S2-2	27	124778	135978					1.1299
	1950 V12 S3-1	13	112435	121744	0.20782	0.27460			1.1310
	1950 V12 S3-2	28	122365	129349					1.1588
	1950 V12 S4-1	14	116275	124559	0.20841	0.27858			1.1566
	1950 V12 S4-2	29	123788	130615					1.1744
13	1950 V13 S1-1	11	108663	117915	0.21089	0.26800	1.0650	0.0093	1.0885
	1950 V13 S1-2	39	116363	123780					1.1106
	1950 V13 S2-1	24	113422	122121	0.20780	0.27976			1.1623
	1950 V13 S2-2	26	116351	124131					1.1731
	1950 V13 S3-1	12	114675	123775	0.20801	0.27724			1.1478
	1950 V13 S3-2	37	120465	130072					1.1474
	1950 V13 S4-1	23	114580	120864	0.20651	0.27850			1.1887
	1950 V13 S4-2	27	115914	121965					1.1917
14	1950 V14 S1-1	23	106550	112627	0.21221	0.27760	1.0245	0.0346	1.1638
	1950 V14 S1-2	39	110770	116327					1.1717
	1950 V14 S2-1	11	99855	110259	0.20881	0.27640			1.1254
	1950 V14 S2-2	38	108231	114867					1.1727
	1950 V14 S3-1	24	109652	118546	0.20903	0.27985			1.1635
	1950 V14 S3-2	26	112102	120015					1.1754
	1950 V14 S4-1	12	97279	107578	0.21059	0.28100			1.1327
	1950 V14 S4-2	27	103809	111233					1.1704

Day — Measurement session
Sample — Sample name: SRM identifier (1950), vial index (one vial per session), subsample index (1 to 3 or 1 to 4), injection index (1 to 2)
Seq — Within measurement session sample injection sequence
A_{Hcy} — Measured area of the m/z 136 (homocysteine) peak
A_{IS} — Measured area of the m/z 140 (homocysteine-d_4 IS) peak
m_{sample} — Mass of sample in the analyzed extract
m_{IS} — Mass of the Hcy-d_4 IS in the analyzed extract
B_1 — Regression estimate of the slope of the linear model for the measurement session
B_0 — Regression estimate of the intercept of the linear model for the measurement session
w_{Hcy} — Estimated mass fraction of Hcy in the analyzed sample

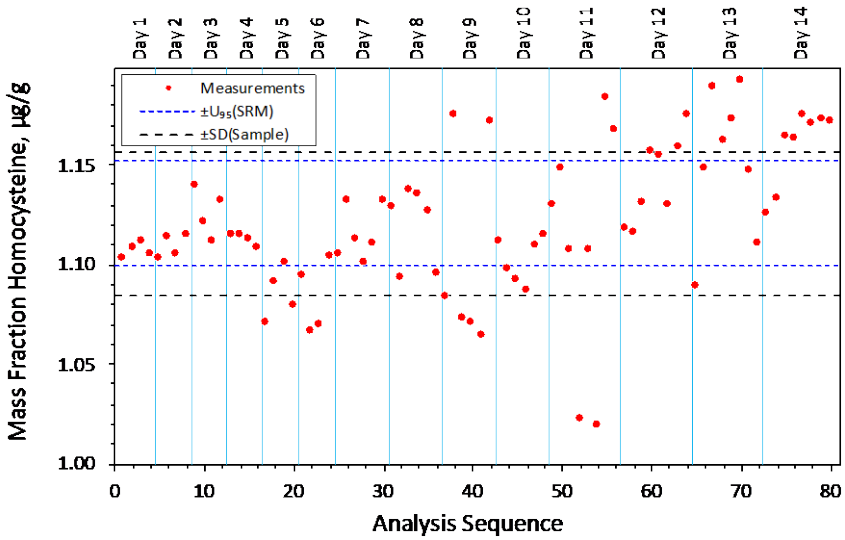


Fig. 27. Estimated Homocysteine Mass Fractions for the SRM 1950 Control Material.

Each symbol represents the estimated homocysteine mass fraction in one subsample from a vial of SRM 1950. A different vial was used in each of the 14 measurement sessions. The horizontal short-dash lines bound the 95 % level of confidence interval on the certified value. The horizontal long-dash lines bound the one standard deviation interval on the mean of the 80 subsamples. The light vertical lines separate the measurement sessions.

About 40 % of the 80 individual injection results for SRM 1950 are outside the 95 % level of confidence interval on the certified value. However, the mean mass fraction of 1.121 µg/g for these values is quite close to the mass fraction-transformed (1.126 ± 0.027) µg/g of the (8.50 ± 0.20) µmol/L certified value (see Eq. 4). Further, the relative standard deviation (% CV) of 100(0.036/1.121) = 3.2 % is within the RMP's expected (2 to 4) % [6].

While the peak shape and SPE column issues noted in Section 7.4 are likely the sources of the excessive variability, these issues do not appear to significantly bias the measurement process.

7.5.3. **Measurements and Estimated values for SRM 1955**

Table 16 details the measured peak areas, sample and IS masses, calibration functions, and estimated Hcy mass fractions, w_{Hcy}, for all analyses of the three SRM 1955 materials. The mass fractions are displayed as a function of the SRM 1955 analysis sequences in Fig. 28.

Table 16. Peak Areas, Mass Measurements, and Estimated Values for SRM 1955.

	Day	Sample	Seq	A_{Hcy}	A_{IS}	m_{sample} g	m_{IS} µg	b_1	b_0	w_{Hcy} µg/g
Level 1	1	1955L1 V1 S1-1	21	149331	356765	0.20816	0.27996	1.0611	0.0263	0.4972
		1955L1 V1 S1-2	34	150326	356565					0.5010
		1955L1 V1 S2-1	12	177199	429417	0.20787	0.26932			0.4717
		1955L1 V1 S2-2	25	178717	431538					0.4736
	2	1955L1 V2 S1-1	12	182722	439334	0.20696	0.27310	1.0711	0.0123	0.4972
		1955L1 V2 S1-2	25	180688	434598					0.4971
		1955L1 V2 S2-1	21	177128	417499	0.20767	0.27222			0.5042
		1955L1 V2 S2-2	34	176416	415352					0.5047
	3	1955L1 V3 S1-1	13	194836	461936	0.20673	0.27709	1.0829	0.0057	0.5150
		1955L1 V3 S1-2	25	202000	484895					0.5086
		1955L1 V3 S2-1	22	201100	478678	0.20819	0.27978			0.5143
		1955L1 V3 S2-2	34	206929	492527					0.5143
Level 2	4	1955L2 V1 S1-1	11	407107	453186	0.20782	0.28086	1.0579	0.0098	1.1351
		1955L2 V1 S1-2	24	430227	486329					1.1176
		1955L2 V1 S2-1	22	457665	516962	0.20899	0.28262			1.1192
		1955L2 V1 S2-2	35	449164	501624					1.1321
	5	1955L2 V2 S1-1	12	463338	506008	0.20999	0.27356	1.0579	0.0117	1.1132
		1955L2 V2 S1-2	22	467532	511193					1.1118
		1955L2 V2 S2-1	25	474175	647427	0.21035	0.33371			1.0808
		1955L2 V2 S2-2	34	478646	647271					1.0914
	6	1955L2 V3 S1-1	12	526192	595486	0.20823	0.27000	1.0671	0.0105	1.0610
		1955L2 V3 S1-2	25	499858	564150					1.0639
		1955L2 V3 S2-1	21	532020	596577	0.20902	0.27336			1.0801
		1955L2 V3 S2-2	35	531769	592275					1.0875
	12	1955L2 V4 S1-1	15	112132	124655	0.20949	0.27785	1.0688	0.0087	1.1055
		1955L2 V4 S1-2	30	120630	131877					1.1243
		1955L2 V4 S2-1	16	121609	127266	0.21024	0.27402			1.1547
		1955L2 V4 S2-2	31	129791	133953					1.1710
	13	1955L2 V5 S1-1	22	114227	115863	0.20940	0.27929	1.0650	0.0093	1.2230
		1955L2 V5 S1-2	38	116736	119199					1.2148
		1955L2 V5 S2-1	13	110882	118247	0.21001	0.28207			1.1709
		1955L2 V5 S2-2	28	118004	124133					1.1872
Level 3	7	1955L3 V1 S1-1	11	478848	272093	0.20716	0.28539	1.1070	-0.0281	2.2251
		1955L3 V1 S1-2	26	468797	267334					2.2173
		1955L3 V1 S2-1	23	505156	277763	0.20847	0.27457			2.1972
	8	1955L3 V2 S1-1	22	477121	262501	0.20848	0.27804	1.1692	-0.0426	2.1219
		1955L3 V2 S1-2	25	481867	264998					2.1228
		1955L3 V2 S2-1	12	437886	239992	0.20911	0.27793			2.1225
		1955L3 V2 S2-2	36	433334	236786					2.1288
	9	1955L3 V3 S1-1	13	589949	326475	0.19827	0.27034	1.0708	0.0164	2.2800
		1955L3 V3 S1-2	44	565200	313793					2.2726
		1955L3 V3 S2-1	23	403176	220462	0.21160	0.27988			2.2387
		1955L3 V3 S2-2	34	399543	220025					2.2228

Table 16. Peak Areas, Mass Measurements, and Estimated Values for SRM 1955 (Continued).

	Day	Sample	Seq	A_{Hcy}	A_{IS}	m_{sample} g	m_{IS} µg	B_1	B_0	w_{Hcy} µg/g
Level 3	10	1955L3 V4 S1-1	22	274336	147713	0.20933	0.27348	1.0728	0.0099	2.2497
		1955L3 V4 S1-2	34	275139	148089					2.2505
		1955L3 V4 S2-1	13	319183	174934	0.20952	0.27043			2.1833
		1955L3 V4 S2-2	43	329395	179513					2.1957
	11	1955L3 V5 S1-1	13	238200	127088	0.20790	0.28748	1.0672	-0.0049	2.4349
		1955L3 V5 S1-2	45	253514	133361					2.4694
		1955L3 V5 S2-1	22	249499	133678	0.20633	0.26178			2.2247
		1955L3 V5 S2-2	35	253773	135630					2.2302
	14	1955L3 V6 S1-1	13	227939	126309	0.20817	0.27672	1.0245	0.0346	2.2966
		1955L3 V6 S1-2	37	239361	131185					2.3225
		1955L3 V6 S2-1	22	232215	126092	0.20911	0.27974			2.3596
		1955L3 V6 S2-2	28	233428	127249					2.3502

Day — Measurement session
Sample — Sample name: SRM identifier (1955), level index (L1 to L3), vial index (one vial per session), subsample index (1 to 2), injection index (1 to 2)
Seq — Within measurement session sample injection sequence
A_{Hcy} — Measured area of the *m/z* 136 (homocysteine) peak
A_{IS} — Measured area of the *m/z* 140 (homocysteine-d_4 IS) peak
m_{sample} — Mass of sample in the analyzed extract
m_{IS} — Mass of the Hcy-d_4 IS in the analyzed extract
B_1 — Regression estimate of the slope of the linear model for the measurement session
B_0 — Regression estimate of the intercept of the linear model for the measurement session
w_{Hcy} — Estimated mass fraction of Hcy in the analyzed sample

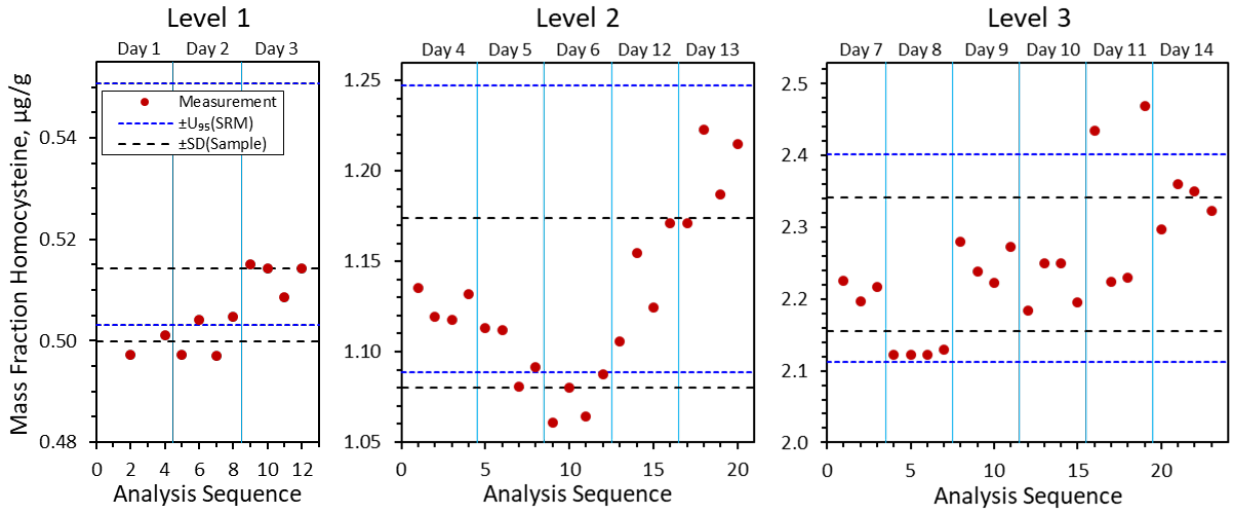


Fig. 28. Estimated Homocysteine Mass Fractions for the SRM 1955 Materials.

Each symbol represents the estimated homocysteine mass fraction in one subsample from a vial of one level of SRM 1955. A different vial was used in each measurement session. The horizontal short-dash lines bound the 95 % level of confidence interval on the certified value for that level. The horizontal long-dash lines bound the one standard deviation interval on the mean of the subsamples. The light vertical lines separate the measurement sessions.

Table 17 lists summary statistics for the three levels of the SRM 1955 homocysteine mass fraction measurements along with the mass fraction-transformations (see Eq. 4) of their certified values. Although the expiry date for SRM 1955 was December 2018 [3], the measured values are compatible with the certified values.

Table 17. Summary Statistics for SRM 1955 Measurements.

	SRM 1955 Certified Values			Measured Values					
SRM	Density (g/mL)	As Listed (μmol/L)	Transformed (μg/g)	n_{vial}	n_{sub}	n_{inj}	$\bar{x}$ (μg/g)	s (μg/g)	%CV %
1955 Lv1	1.01380	3.98 ± 0.18	0.527 ± 0.024	3	6	12	0.500	0.014	2.9
1955 Lv2	1.02414	8.85 ± 0.60	1.168 ± 0.079	5	10	20	1.127	0.047	4.2
1955 Lv3	1.02418	17.7 ± 1.1	2.257 ± 0.145	6	12	23	2.249	0.093	4.1

n_{vial} Number of vials of the SRM 1955 material analyzed
n_{sub} Number of subsamples analyzed
$\bar{x}$ Mean of the n_{inj} mass fraction estimates
s Standard deviation of the n_{inj} mass fraction estimates
%CV Relative standard deviation, 100(s/$\bar{x}$), of the mass fraction estimates stated as a percentage

7.5.4. **Measurements and Estimated values for SRM 1955a**

Table 18 details the measured peak areas, sample and IS masses, calibration functions, and estimated Hcy mass fractions, w_{Hcy}, for all analyses of the three SRM 1955a materials. The mass fractions as estimated using classical least-squares regression are displayed as a function of the SRM 1955a analysis sequences in Fig. 29.

Table 18. Peak Areas, Mass Measurements, and Estimated Values for SRM 1955a.

	Day	Sample	Seq	A_{Hcy}	A_{IS}	m_{sample} g	m_{IS} µg	b_1	b_0	w_{Hcy} µg/g
Level 1	1	1955aL1 #568 S1-1	14	263500	432169	0.20917	0.27717	1.0611	0.0263	0.7286
		1955aL1 #568 S1-2	28	266005	435077					0.7307
		1955aL1 #568 S2-1	18	236413	387914	0.20720	0.27821			0.7379
		1955aL1 #568 S2-2	30	238596	388667					0.7435
		1955aL1 #849 S1-1	17	245656	401652	0.20745	0.27653			0.7353
		1955aL1 #849 S1-2	32	247556	404913					0.7350
		1955aL1 #849 S2-1	13	253149	413523	0.20688	0.27356			0.7301
		1955aL1 #849 S2-2	29	253450	415241					0.7279
		1955aL1 #849 S3-1	19	259479	429309	0.20694	0.27519			0.7245
		1955aL1 #849 S3-2	26	259707	429205					0.7254
		1955aL1 #1112 S1-1	15	250802	409868	0.20743	0.27397			0.7289
		1955aL1 #1112 S1-2	27	253403	412479					0.7320
		1955aL1 #1112 S2-1	16	243609	403306	0.20680	0.27589			0.7264
		1955aL1 #1112 S2-2	31	245150	404312					0.7293
		1955aL1 #1112 S3-1	20	242809	409759	0.20750	0.27938			0.7185
		1955aL1 #1112 S3-2	33	245239	412515					0.7210
	2	1955aL1 #114 S1-1	16	269382	444215	0.20718	0.27938	1.0711	0.0123	0.7480
		1955aL1 #114 S1-2	31	272552	447805					0.7508
		1955aL1 #114 S2-1	18	258214	420166	0.20804	0.27398			0.7405
		1955aL1 #114 S2-2	32	263182	431384					0.7350
		1955aL1 #393 S1-1	14	258701	421319	0.20891	0.26987			0.7257
		1955aL1 #393 S1-2	28	259894	424690					0.7232
		1955aL1 #393 S2-1	19	269649	429718	0.20891	0.26869			0.7387
		1955aL1 #393 S2-2	29	275078	438313					0.7388
		1955aL1 #936 S1-1	20	268511	437445	0.20707	0.27415			0.7435
		1955aL1 #936 S1-2	27	266695	438925					0.7359
		1955aL1 #936 S2-1	13	272020	442020	0.20761	0.27433			0.7440
		1955aL1 #936 S2-2	30	273073	444162					0.7433
		1955aL1 #1208 S1-1	15	234365	380352	0.20818	0.27450			0.7434
		1955aL1 #1208 S1-2	33	235652	381119					0.7460
		1955aL1 #1208 S2-1	17	256659	414873	0.20854	0.26828			0.7283
		1955aL1 #1208 S2-2	26	260066	421458					0.7264

Table 18. Peak Areas, Mass Measurements, and Estimated Values for SRM 1955a (continued).

	Day	Sample	Seq	A_{Hcy}	A_{IS}	m_{sample} g	m_{IS} µg	b_1	b_0	w_{Hcy} µg/g
Level 1	3	1955aL1 #256 S1-1	14	258141	411353	0.20905	0.28025	1.0829	0.0057	0.7698
		1955aL1 #256 S1-2	32	270499	437356					0.7586
		1955aL1 #256 S2-1	17	270214	437666	0.20841	0.27703			0.7509
		1955aL1 #256 S2-2	28	281443	458197					0.7470
		1955aL1 #256 S3-1	18	232690	368503	0.20949	0.27803			0.7669
		1955aL1 #256 S3-2	29	240259	383077					0.7617
		1955aL1 #668 S1-1	15	286443	460881	0.20778	0.27627			0.7561
		1955aL1 #668 S1-2	31	301218	486341					0.7535
		1955aL1 #668 S2-1	19	148870	231900	0.20797	0.27902			0.7883
		1955aL1 #668 S2-2	27	151999	239952					0.7778
		1955aL1 #668 S3-1	16	132333	214603	0.20681	0.28276			0.7714
		1955aL1 #668 S3-2	33	272148	446488					0.7624
		1955aL1 #1458 S1-1	12	264847	422612	0.20460	0.27616			0.7740
		1955aL1 #1458 S1-2	30	279943	452123					0.7647
		1955aL1 #1458 S2-1	20	351554	571471	0.20819	0.28738			0.7769
		1955aL1 #1458 S2-2	26	358116	585857					0.7719
Level 2	4	1955aL2 #256 S1-1	15	479003	426124	0.20616	0.27857	1.0579	0.0098	1.4233
		1955aL2 #256 S1-2	26	500414	445811					1.4212
		1955aL2 #256 S2-1	17	546629	490280	0.20640	0.27434			1.3885
		1955aL2 #256 S2-2	32	537269	483850					1.3828
		1955aL2 #747 S1-1	19	492792	443380	0.20646	0.27399			1.3819
		1955aL2 #747 S1-2	27	489542	441670					1.3781
		1955aL2 #747 S2-1	18	538276	481798	0.20921	0.27581			1.3800
		1955aL2 #747 S2-2	29	537904	482414					1.3773
		1955aL2 #747 S3-1	14	584321	530898	0.20851	0.27639			1.3668
		1955aL2 #747 S3-2	31	593617	543849					1.3554
		1955aL2 #1043 S1-1	13	520165	469337	0.20893	0.26723			1.3281
		1955aL2 #1043 S1-2	33	528157	480137					1.3181
		1955aL2 #1043 S2-1	16	395162	481093	0.15391	0.27992			1.3953
		1955aL2 #1043 S2-2	28	398020	487286					1.3874
		1955aL2 #1043 S3-1	20	523333	477619	0.20791	0.27610			1.3631
		1955aL2 #1043 S3-2	30	515201	470510					1.3622
	5	1955aL2 #45 S1-1	16	555478	507957	0.20920	0.26598	1.0579	0.0117	1.3002
		1955aL2 #45 S1-2	32	552044	497496					1.3195
		1955aL2 #45 S2-1	18	598210	553277	0.20741	0.27473			1.3391
		1955aL2 #45 S2-2	29	599719	547586					1.3567
		1955aL2 #45 S3-1	19	590888	537743	0.20825	0.27115			1.3380
		1955aL2 #45 S3-2	30	594780	535826					1.3518
		1955aL2 #723 S1-1	14	579136	517502	0.20957	0.27655			1.3814
		1955aL2 #723 S1-2	28	582076	514878					1.3956
		1955aL2 #723 S2-1	20	412582	367393	0.20808	0.27585			1.3926
		1955aL2 #723 S2-2	31	413088	365348					1.4022
		1955aL2 #1254 S1-1	17	570555	511425	0.20913	0.27649			1.3796
		1955aL2 #1254 S1-2	27	571216	506775					1.3941
		1955aL2 #1254 S2-1	15	642894	600114	0.20883	0.27174			1.3033
		1955aL2 #1254 S2-2	33	645798	594166					1.3225
		1955aL2 #1254 S3-1	13	684736	563336	0.23423	0.27779			1.3495
		1955aL2 #1254 S3-2	26	693710	562669					1.3690

Table 18. Peak Areas, Mass Measurements, and Estimated Values for SRM 1955a (continued).

	Day	Sample	Seq	A_{Hcy}	A_{IS}	m_{sample} g	m_{IS} µg	b_1	b_0	w_{Hcy} µg/g
Level 2	6	1955aL2 #387 S1-1	13	890453	842248	0.20905	0.27489	1.0671	0.0105	1.2899
		1955aL2 #387 S1-2	32	874226	814359					1.3099
		1955aL2 #387 S2-1	16	886626	854120	0.20959	0.27306			1.2546
		1955aL2 #387 S2-2	28	850436	809231					1.2703
		1955aL2 #481 S1-1	20	706043	649106	0.20854	0.27513			1.3318
		1955aL2 #481 S1-2	33	719032	656289					1.3416
		1955aL2 #481 S2-1	17	713238	669640	0.20922	0.27666			1.3069
		1955aL2 #481 S2-2	31	713412	661459					1.3235
		1955aL2 #998 S1-1	15	725376	662723	0.20917	0.27112			1.3167
		1955aL2 #998 S1-2	26	683656	622841					1.3205
		1955aL2 #998 S2-1	18	588318	517400	0.21009	0.27660			1.3900
		1955aL2 #998 S2-2	29	600573	524555					1.3996
		1955aL2 #1403 S1-1	14	818569	783829	0.21001	0.27424			1.2651
		1955aL2 #1403 S1-2	30	778965	735293					1.2836
		1955aL2 #1403 S2-1	19	616432	572655	0.21594	0.27377			1.2664
		1955aL2 #1403 S2-2	27	623775	575006					1.2764
	12	1955aL2 #386 S1-1	17	137711	118208	0.20962	0.27622	1.0688	0.0087	1.4256
		1955aL2 #386 S1-2	32	148186	124913					1.4519
		1955aL2 #386 S2-1	18	140267	122129	0.20974	0.27633			1.4050
		1955aL2 #386 S2-2	33	148143	127659					1.4197
		1955aL2 #482 S1-1	19	143520	120074	0.20967	0.27801			1.4720
		1955aL2 #482 S1-2	34	151207	128573					1.4482
		1955aL2 #482 S2-1	20	142396	123255	0.20870	0.27648			1.4212
		1955aL2 #482 S2-2	35	152774	130739					1.4376
		1955aL2 #997 S1-1	21	149032	129245	0.20836	0.27711			1.4241
		1955aL2 #997 S1-2	36	158501	136446					1.4347
		1955aL2 #997 S2-1	22	157603	138085	0.20842	0.27753			1.4112
		1955aL2 #997 S2-2	37	164426	142612					1.4256
		1955aL2 #1402 S1-1	23	147356	128101	0.20905	0.28493			1.4559
		1955aL2 #1402 S1-2	38	152917	133978					1.4444
		1955aL2 #1402 S2-1	24	144748	122651	0.20861	0.27706			1.4557
		1955aL2 #1402 S2-2	39	147383	126729					1.4344
	13	1955aL2 #44 S1-1	15	124658	106220	0.20849	0.27824	1.0650	0.0093	1.4589
		1955aL2 #44 S1-2	35	130026	111732					1.4466
		1955aL2 #44 S2-1	20	138095	120342	0.20793	0.27803			1.4290
		1955aL2 #44 S2-2	30	146962	128560					1.4235
		1955aL2 #44 S3-1	16	129795	113789	0.20783	0.27645			1.4131
		1955aL2 #44 S3-2	33	134211	118732					1.4002
		1955aL2 #722 S1-1	14	134346	119253	0.20856	0.28475			1.4323
		1955aL2 #722 S1-2	31	141931	125149					1.4420
		1955aL2 #722 S2-1	18	140115	121404	0.20823	0.27545			1.4220
		1955aL2 #722 S2-2	34	140260	122265					1.4134
		1955aL2 #1253 S1-1	19	139899	121366	0.20848	0.27813			1.4323
		1955aL2 #1253 S1-2	32	144637	125182					1.4357
		1955aL2 #1253 S2-1	21	130930	111859	0.20865	0.27456			1.4347
		1955aL2 #1253 S2-2	29	134569	115163					1.4323
		1955aL2 #1253 S3-1	17	135190	116923	0.20838	0.27787			1.4361
		1955aL2 #1253 S3-2	36	140372	122485					1.4233

Table 18. Peak Areas, Mass Measurements, and Estimated Values for SRM 1955a (continued).

	Day	Sample	Seq	A_{Hcy}	A_{IS}	m_{sample} g	m_{IS} µg	b_1	b_0	w_{Hcy} µg/g
Level 3	7	1955aL3 #262 S1-1	17	417346	259060	0.20765	0.27740	1.1070	-0.0281	1.9781
		1955aL3 #262 S1-2	28	418722	260216					1.9758
		1955aL3 #262 S2-1	14	439319	274173	0.20789	0.27486			1.9473
		1955aL3 #262 S2-2	33	437408	271265					1.9594
		1955aL3 #262 S3-1	16	361318	222297	0.20982	0.27683			1.9707
		1955aL3 #262 S3-2	29	360986	222812					1.9644
		1955aL3 #790 S1-1	19	422104	261342	0.20795	0.28759			2.0529
		1955aL3 #790 S1-2	31	423030	260738					2.0620
		1955aL3 #790 S2-1	20	398878	247472	0.20841	0.28562			2.0302
		1955aL3 #790 S2-2	30	398973	248179					2.0250
		1955aL3 #1337 S1-1	18	380030	234706	0.20724	0.28059			2.0147
		1955aL3 #1337 S1-2	27	378819	234274					2.0120
		1955aL3 #1337 S2-1	21	419283	257714	0.20833	0.28041			2.0124
		1955aL3 #1337 S2-2	32	417471	257701					2.0039
		1955aL3 #1337 S3-1	15	444566	277522	0.20936	0.27949			1.9657
		1955aL3 #1337 S3-2	34	442167	276114					1.9651
	8	1955aL3 #69 S1-1	19	390706	239392	0.20851	0.28475	1.3638	-0.0196	1.9561
		1955aL3 #69 S1-2	28	391587	238508					1.9674
		1955aL3 #69 S2-1	13	394836	243553	0.20802	0.28134			1.9245
		1955aL3 #69 S2-2	32	389168	240444					1.9215
		1955aL3 #553 S1-1	15	435210	268513	0.20890	0.28655			1.9515
		1955aL3 #553 S1-2	29	432926	267258					1.9504
		1955aL3 #553 S2-1	17	373741	230110	0.20877	0.27428			1.8729
		1955aL3 #553 S2-2	33	371312	228789					1.8715
		1955aL3 #553 S3-1	18	417545	256765	0.20815	0.28013			1.9208
		1955aL3 #553 S3-2	30	412411	254489					1.9143
		1955aL3 #1078 S1-1	20	394222	243299	0.20773	0.27417			1.8772
		1955aL3 #1078 S1-2	27	394126	241880					1.8874
		1955aL3 #1078 S2-1	16	401630	247595	0.20929	0.27955			1.9018
		1955aL3 #1078 S2-2	31	401098	246933					1.9043
		1955aL3 #1078 S3-1	14	423191	260973	0.20793	0.27613			1.8903
		1955aL3 #1078 S3-2	34	418498	258011					1.8907
	9	1955aL3 #410 S1-1	15	356354	218083	0.20814	0.27698	1.1094	0.0326	2.0104
		1955aL3 #410 S1-2	42	345474	212390					2.0011
		1955aL3 #410 S2-1	19	493114	323425	0.20931	0.26866			1.8079
		1955aL3 #410 S2-2	36	486163	318352					1.8109
		1955aL3 #730 S1-1	17	382684	233880	0.20817	0.27421			1.9926
		1955aL3 #730 S1-2	37	375209	230551					1.9818
		1955aL3 #730 S2-1	18	362476	219016	0.20990	0.27461			2.0021
		1955aL3 #730 S2-2	41	346256	211397					1.9812
		1955aL3 #889 S1-1	20	392043	239373	0.20802	0.27623			2.0107
		1955aL3 #889 S1-2	40	382443	235472					1.9938
		1955aL3 #889 S2-1	16	453935	279708	0.21056	0.28011			1.9958
		1955aL3 #889 S2-2	22	452766	277455					2.0069
		1955aL3 #889 S2-3	38	444153	273930					1.9940
		1955aL3 #1269 S1-1	21	376181	229712	0.20847	0.27670			2.0095
		1955aL3 #1269 S1-2	35	374722	231531					1.9858
		1955aL3 #1269 S2-1	14	416526	256506	0.21021	0.27785			1.9842
		1955aL3 #1269 S2-2	39	403623	250121					1.9717

Table 18. Peak Areas, Mass Measurements, and Estimated Values for SRM 1955a (continued).

	Day	Sample	Seq	A_{Hcy}	A_{IS}	m_{sample} g	m_{IS} µg	b_1	b_0	w_{Hcy} µg/g
Level 3	10	1955aL3 #70 S1-1	14	251340	153697	0.20965	0.27630	1.0728	0.0099	1.9968
		1955aL3 #70 S1-2	38	259726	157154					2.0181
		1955aL3 #70 S2-1	17	244765	150118	0.20818	0.27768			2.0150
		1955aL3 #70 S2-2	36	250879	153765					2.0163
		1955aL3 #552 S1-1	21	200284	122290	0.20743	0.27722			2.0280
		1955aL3 #552 S1-2	35	202721	123373					2.0347
		1955aL3 #552 S2-1	19	257483	159303	0.20808	0.27590			1.9854
		1955aL3 #552 S2-2	39	263070	161900					1.9961
		1955aL3 #552 S3-1	16	359924	233086	0.20848	0.28166			1.9321
		1955aL3 #552 S3-2	42	367483	238117					1.9310
		1955aL3 #1077 S1-1	18	302223	195345	0.20818	0.28016			1.9284
		1955aL3 #1077 S1-2	40	306824	197517					1.9362
		1955aL3 #1077 S2-1	15	263277	163915	0.20818	0.27912			1.9950
		1955aL3 #1077 S2-2	41	271428	168475					2.0012
		1955aL3 #1077 S3-1	20	265177	160509	0.20808	0.27665			2.0352
		1955aL3 #1077 S3-2	37	267677	162267					2.0321
	11	1955aL3 #411 S1-1	20	217178	129512	0.21029	0.27571	1.0672	-0.0049	2.0661
		1955aL3 #411 S1-2	37	223316	132384					2.0784
		1955aL3 #411 S2-1	15	204094	122908	0.21095	0.26475			1.9586
		1955aL3 #411 S2-2	43	215131	128391					1.9763
		1955aL3 #729 S1-1	19	227238	136339	0.21003	0.30566			2.2795
		1955aL3 #729 S1-2	39	237368	142115					2.2844
		1955aL3 #729 S2-1	16	228061	136930	0.20572	0.29173			2.2197
		1955aL3 #729 S2-2	41	242933	144675					2.2378
		1955aL3 #890 S1-1	17	218283	132728	0.21233	0.27192			1.9794
		1955aL3 #890 S1-2	38	229222	138252					1.9955
		1955aL3 #890 S2-1	14	219326	131643	0.21184	0.25478			1.8832
		1955aL3 #890 S2-2	40	234813	138563					1.9154
		1955aL3 #1268 S1-1	18	215219	127048	0.21138	0.23340			1.7577
		1955aL3 #1268 S1-2	42	226280	133129					1.7636
		1955aL3 #1268 S2-1	21	218730	129389	0.19880	0.24925			1.9918
		1955aL3 #1268 S2-2	36	223201	131547					1.9991
	14	1955aL3 #261 S1-1	14	180625	111410	0.21987	0.22199	1.0245	0.0346	1.5637
		1955aL3 #261 S1-2	30	184827	114580					1.5556
		1955aL3 #261 S2-1	16	214528	130469	0.20676	0.27593			2.0969
		1955aL3 #261 S2-2	34	221604	134989					2.0934
		1955aL3 #789 S1-1	21	190203	115489	0.21312	0.23535			1.7379
		1955aL3 #789 S1-2	33	195900	119551					1.7290
		1955aL3 #789 S2-1	17	184644	112612	0.20790	0.27541			2.0754
		1955aL3 #789 S2-2	35	193896	117516					2.0888
		1955aL3 #789 S3-1	15	191669	114743	0.21203	0.29607			2.2296
		1955aL3 #789 S3-2	31	198419	120396					2.1991
		1955aL3 #1336 S1-1	19	170593	103433	0.20965	0.27108			2.0379
		1955aL3 #1336 S1-2	29	175582	106163					2.0437
		1955aL3 #1336 S2-1	20	194456	116806	0.20945	0.27270			2.0717
		1955aL3 #1336 S2-2	36	196971	118491					2.0686
		1955aL3 #1336 S3-1	18	201332	121900	0.21506	0.26753			1.9635
		1955aL3 #1336 S3-2	32	208883	126923					1.9563

Table 18. Peak Areas, Mass Measurements, and Estimated Values for SRM 1955a (continued).

Day	Measurement session
Sample	Sample name: SRM identifier (1955a), level index (L1 to L3), vial index (one vial per session), subsample index (1 to 3), injection index (1 to 2)
Seq	Within measurement session sample injection sequence
A_{Hcy}	Measured area of the m/z 136 (homocysteine) peak
A_{IS}	Measured area of the m/z 140 (homocysteine-d_4 IS) peak
m_{sample}	Mass of sample in the analyzed extract
m_{IS}	Mass of the Hcy-d_4 IS in the analyzed extract
B_1	Regression estimate of the slope of the linear model for the measurement session
B_0	Regression estimate of the intercept of the linear model for the measurement session
w_{Hcy}	Estimated mass fraction of Hcy in the analyzed sample

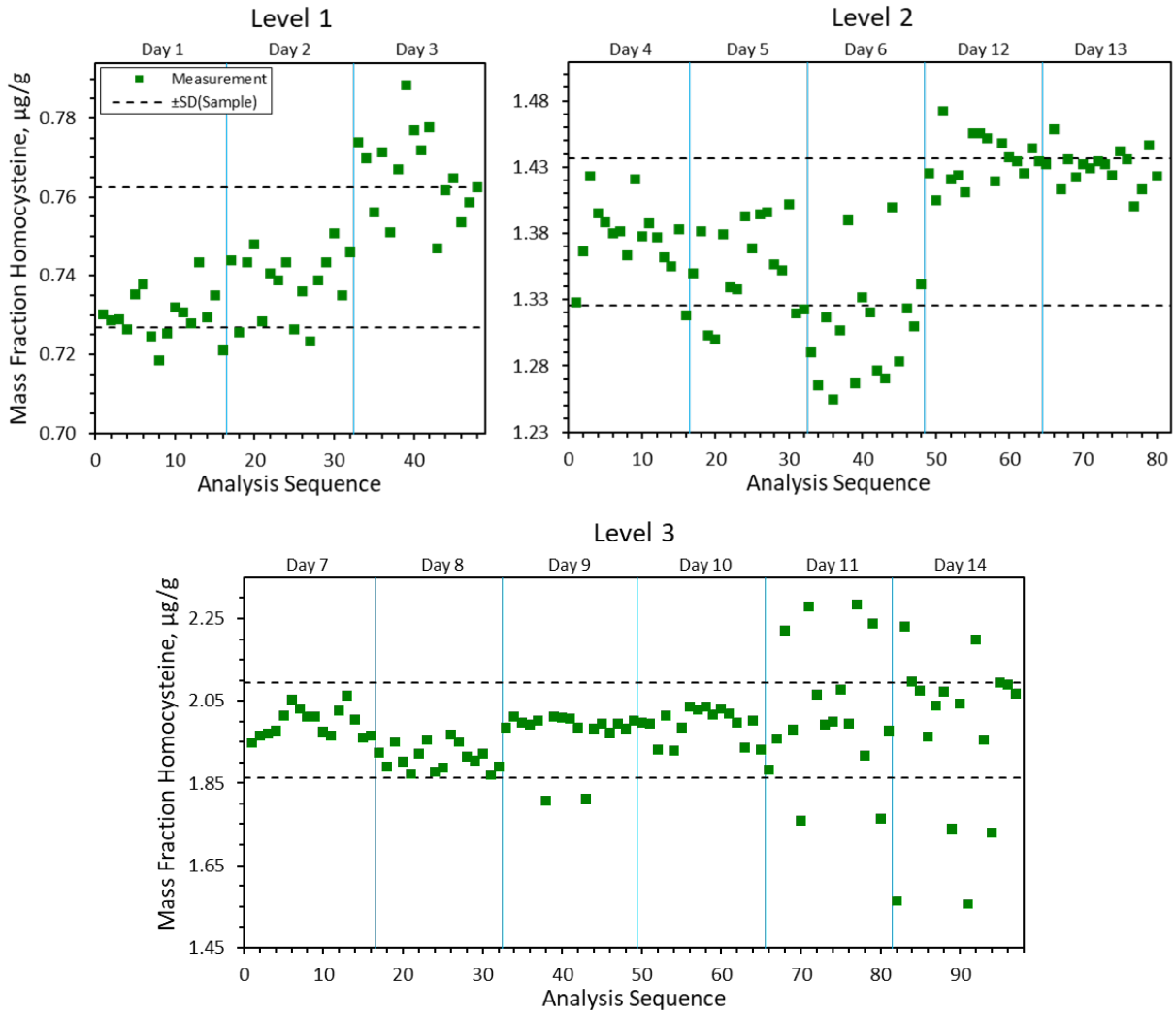


Fig. 29. Estimated Homocysteine Mass Fractions for the SRM 1955a Materials.

Each symbol represents the estimated homocysteine mass fraction in one subsample from a vial of one level of SRM 1955a. The horizontal long-dash lines bound the one standard deviation interval on the mean of the subsamples. The light vertical lines separate the measurement sessions.

7.5.5. **Homogeneity Assessment**

The variability in the level 2 and 3 materials is somewhat larger than desired and precludes direct visualization of any trends related to sample preparation or packaging. However, the close similarity of the analysis order trends between SRM 1955 and SRM 1955a visualized in Fig. 30 demonstrates that the observed variability is related to instrumental issues rather than differences among the vials. The SRM 1955a materials are adequately homogeneous.

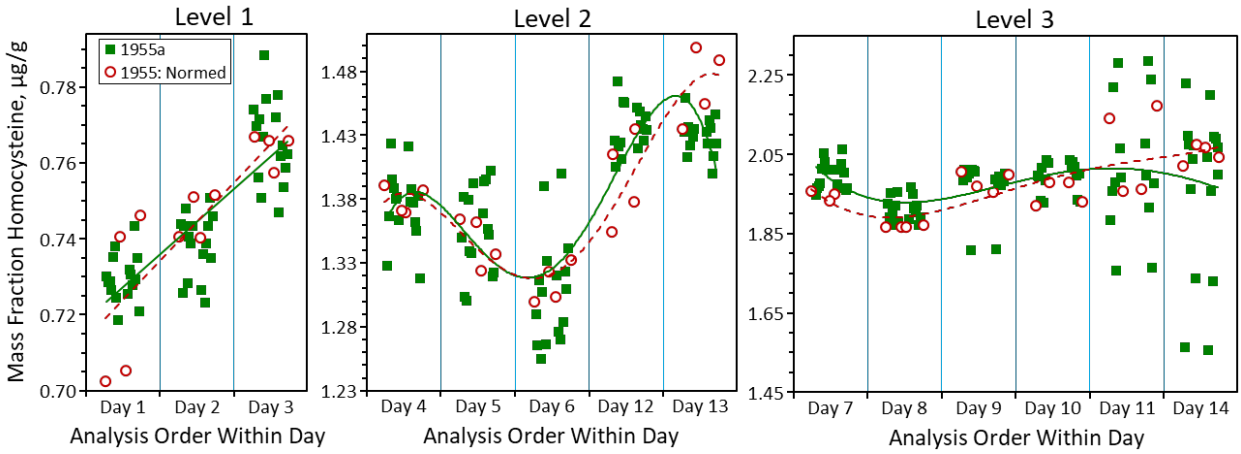


Fig. 30. Analysis Order Trends for SRMs 1955 and 1955a.

The solid squares represent the estimated homocysteine mass fraction in one subsample from a vial of one level of SRM 1955a. The open circles represent the estimated homocysteine mass fraction in one subsample from a vial of one level of SRM 1955, normed to have the mean value of the matching level of SRM 1955a. The solid lines represent an empirical polynomial fit to the SRM 1955a estimates. The dashed lines represent an empirical polynomial fit to the SRM 1955 estimates, where the polynomial is of the same order as used with SRM 1955a.

8. Statistical Evaluation of SRM 1955a

The analysis was accomplished using the linear regression with internal standard model implemented in the NIST “Apps for Bayesian Analysis of Chemical quantities Using Shiny)” (ABACUS) package [18]. The measurements were made at three levels, over several days for each level. Different three calibrants were used on each day. ABACUS was run separately for each day and the mass fraction (µg/g) results for each level were combined using the Bayesian Gauss – Gauss hierarchical model in the NIST Decision Tree (NDT) [20]. Mass concentration (µg/mL) and amount-of-substance concentration (mmol/L) were calculated from the measured mass fractions, serum densities (Section 4), and the molecular mass of Hcy (Section 5) using the NIST Uncertainty Machine (NUM) [21].

This ABACUS model implements Eqs. 1 and 2, using a regression procedure that recognizes the uncertainty in both the calibrant and peak area measurements. The model pools the injection values toward the box values and towards the grand mean of all samples in the set. The uncertainty in the grand mean is increased to accommodate any small apparent measurement differences among the boxes, subsamples, and injections. The use of the model is predicated upon the assumption that all of the input data has been critically evaluated and found to be adequately homogeneous.

8.1. ABACUS Inputs and Outputs

The data required to run the ABACUS system are provided in Section 7. For guidance, this section details the inputs required for the first set (Day 1) of results for SRM 1955a level 1 and the basic output from the analysis.

8.1.1. Calibration Standard and Sample Preparation Uncertainty Inputs

The six input fields that relate to calibrant and sample preparation are displayed in Fig. 31. The “Analyte working standard solution concentrations” are from column “w_{Hcy}” of Table 10. The “Uncertainties in working standard solution” are estimated as 0.5 % of the mass fraction based upon experience with similar preparations. When not otherwise specified the model assumes mass determinations have an associated uncertainty of 0.000015 g.

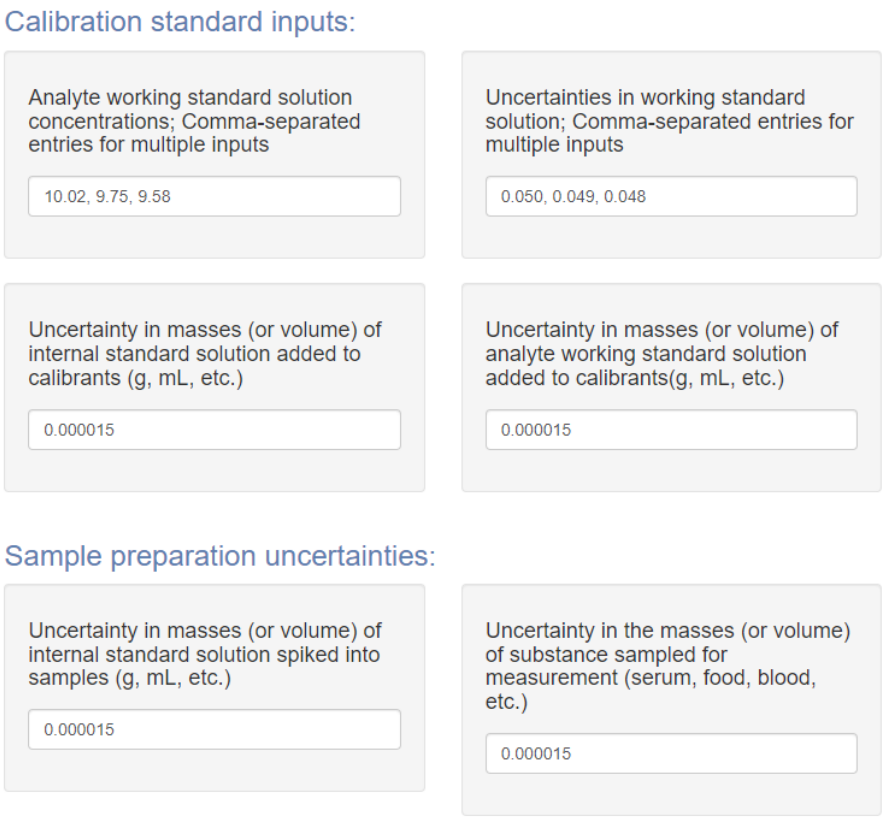


Fig. 31. ABACUS Calibration Standard and Sample Preparation Input Fields.

8.1.2. Calibration Standard and Sample Preparation Uncertainty Inputs

The calibration and sample data must be uploaded from simple files that have a quite rigidly defined structure.

The ABACUS prompt for calibration input is displayed in Fig. 32.

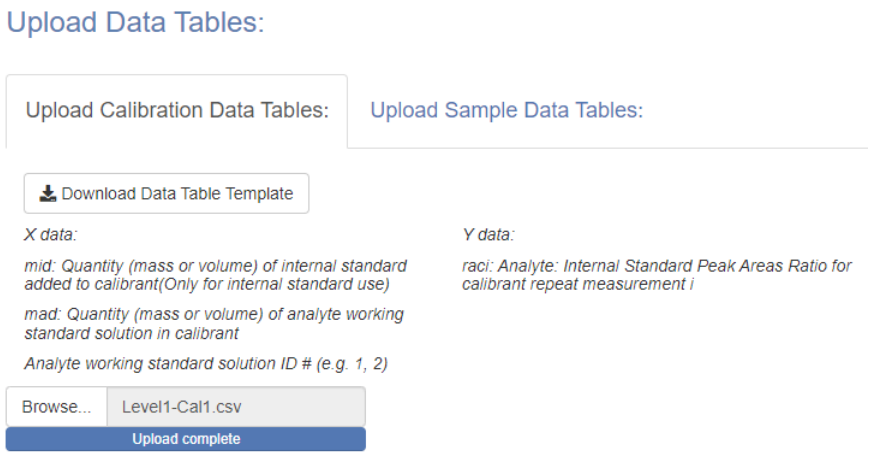


Fig. 32. ABACUS Calibration Data Input Prompt.

The contents of the comma-separated values (CSV) file of the calibration data for SRM 1955a Level 1 set 1, here named "Level1-Cal1.csv") are listed in Table 19. The "mid", "mad", and "wsol" values are from the m_{IS}, m_{WSHcy}, and WS_{Hcy} columns of Table 10. The "rac1" and "rac2" are from the R_{area} column of Table 13.

Table 19. Calibration Data for SRM 1955a Level 1 Set 1.

mid	mad	wsol	rac1	rac2
4.14539	0.12548	3	0.3294	0.3150
4.16809	0.33238	2	0.8561	0.8374
3.88750	0.52791	1	1.5162	1.4815
4.16285	0.76875	3	1.9298	1.8801
4.18381	0.95850	2	2.3890	2.3854
4.20826	1.11811	1	2.8515	2.8375

The ABACUS prompt for sample input is displayed in Fig. 33.

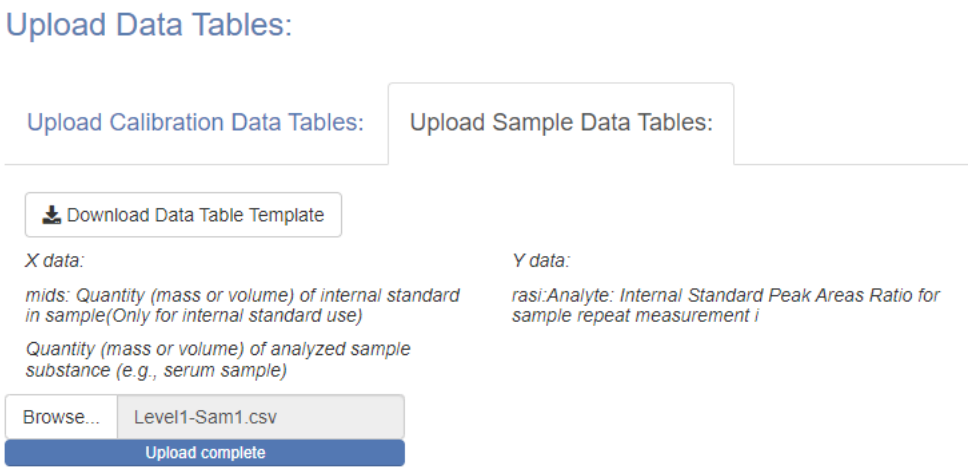


Fig. 33. ABACUS Sample Data Input Prompt.

The contents of the CSV file of the sample data for SRM 1955a Level 1 set 1, here named "Level1-Sam1.csv") are listed in Table 20. The "mids" and "mdi" values are from the m_{IS} and m_{sample} columns of Table 18. The "ras1" and "ras2" values are calculated from the values in the A_{Hcy} and A_{IS} columns of Table 18 as the A_{Hcy}/A_{IS} ratios.

Table 20. Sample Data for SRM 1955a Level 1 Set 1.

mids	mdi	ras1	ras2
0.27717	0.20917	0.60972	0.61140
0.27821	0.20720	0.60945	0.61388
0.27653	0.20745	0.61161	0.61138
0.27356	0.20688	0.61218	0.61037
0.27519	0.20694	0.60441	0.60509
0.27397	0.20743	0.61191	0.61434
0.27589	0.20680	0.60403	0.60634
0.27938	0.20750	0.59257	0.59450

8.1.3. Model Setting Inputs

The four input fields that relate to how the model is run are displayed in Fig. 34. Except for the "Number of decimal places for results", the values displayed are the defaults. Note: The "Number of decimal places" parameter only affects the display of the results and not the actual analysis. It can be changed at will after the analysis is complete.

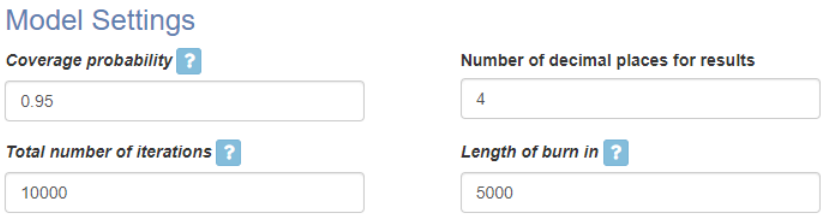
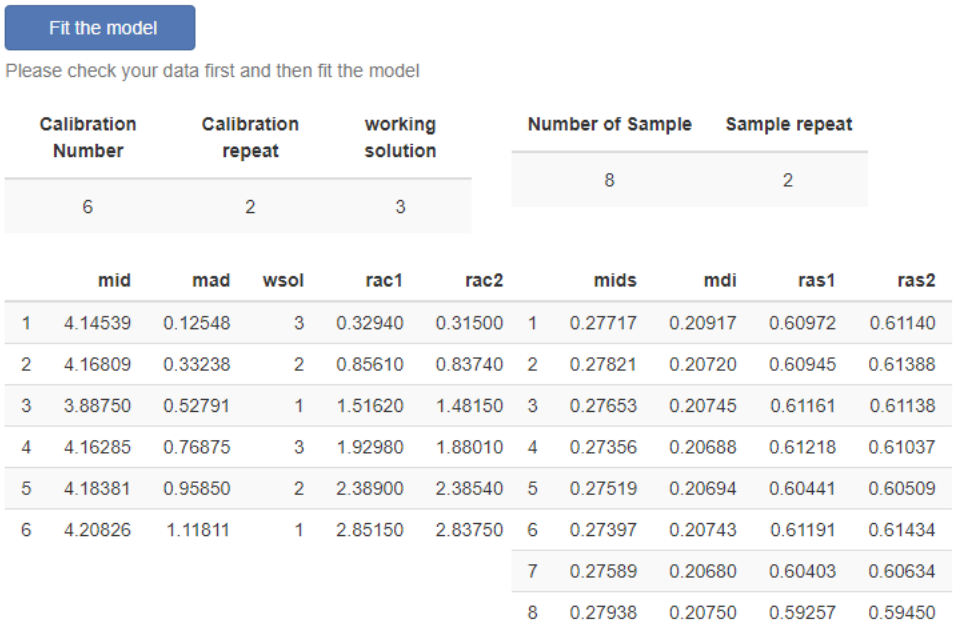


Fig. 34. ABACUS Model Settings Input Prompt.

8.1.4. Data Confirmation and Analysis Initiation Prompt

Prior to initiating the ABACUS analysis, as shown in Fig. 35 summary parameters and the calibration and sample input values are provided to allow confirmation that the calibration and sample inputs have been uploaded correctly. The analysis is not initiated until the "Fit the model" button is clicked.



Calibration Number	Calibration repeat	working solution
6	2	3

Number of Sample	Sample repeat
8	2

	mid	mad	wsol	rac1	rac2		mids	mdi	ras1	ras2
1	4.14539	0.12548	3	0.32940	0.31500	1	0.27717	0.20917	0.60972	0.61140
2	4.16809	0.33238	2	0.85610	0.83740	2	0.27821	0.20720	0.60945	0.61388
3	3.88750	0.52791	1	1.51620	1.48150	3	0.27653	0.20745	0.61161	0.61138
4	4.16285	0.76875	3	1.92980	1.88010	4	0.27356	0.20688	0.61218	0.61037
5	4.18381	0.95850	2	2.38900	2.38540	5	0.27519	0.20694	0.60441	0.60509
6	4.20826	1.11811	1	2.85150	2.83750	6	0.27397	0.20743	0.61191	0.61434
						7	0.27589	0.20680	0.60403	0.60634
						8	0.27938	0.20750	0.59257	0.59450

Fig. 35. ABACUS Analysis Initiation Prompt.

8.1.5. Results of ABACUS Analysis

The ABACUS analysis of the SRM 1955a Level 1 set 1 measurement data are displayed in Fig. 36. The values of greatest interest are the mean, standard uncertainty, and 95 % credible interval.

Model Results

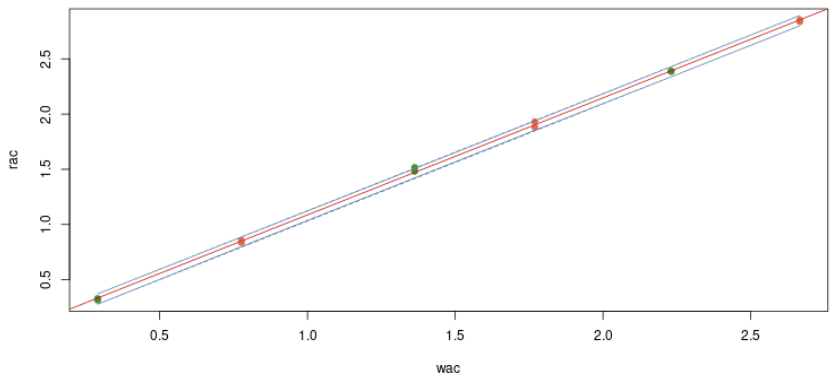
The posterior mean is: 0.7291

The standard uncertainty is: 0.019

The posterior median is: 0.7293

The 95% credible interval ranges from: 0.6921 to 0.7667025

Calibration Plot



R-squared:

data
0.99969

Intercept:

mean	sd
0.02702	0.01455

Slope:

mean	sd
1.06017	0.00920

heterogeneity variance as % of total y variance

mean
11.42450

Download PDF report

Posterior Distribution

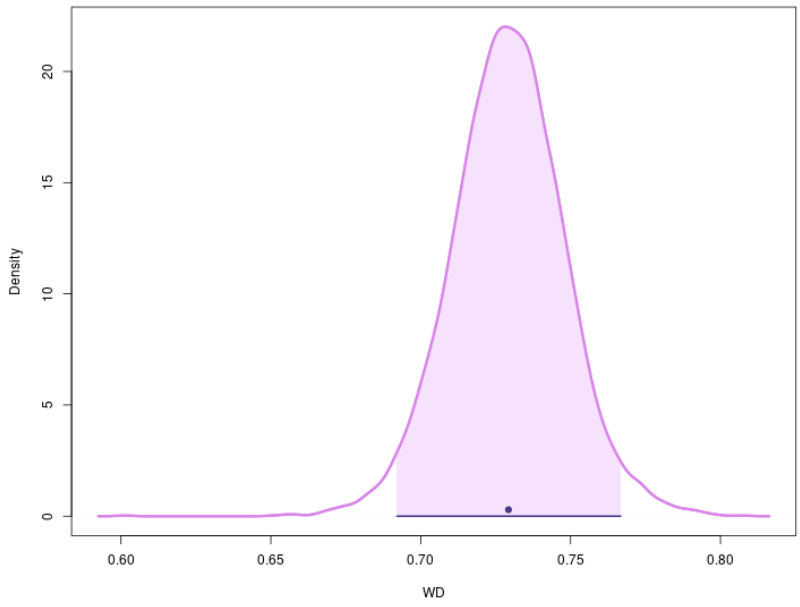


Fig. 36. Results of ABACUS Analysis of SRM 1955a Level 1 Set 1 Measurements.

8.2. Estimation of Mass Fraction Results

8.2.1. SRM 1955a Level 1

Table 21 lists the ABACUS estimates of the mass fraction Hcy in each of the SRM 1955a Level 1 measurement sessions and the NDT consensus estimate using its Hierarchical Gauss-Gauss (HGG) method (Fig. 37). HGG is the recommended method for data that is not assumed to be homogeneous but is assumed to be symmetric and normally distributed.

Table 21. Summary Statistics for SRM 1955a Level 1.

Day	Set	w_{Hcy} g/g	$u(w_{Hcy})$ g/g	$U_{95}(w_{Hcy})$ g/g	$\%CV_{95}$ %
1	1	0.729	0.016	0.034	4.7
2	2	0.738	0.020	0.040	5.4
3	3	0.766	0.012	0.025	3.3
Consensus:		0.746	0.009	0.018	2.4

Day — Project session index, 1 to 14
Set — Session index, 1 to 5 for Level 2
w_{Hcy} — Estimated mass fraction homocysteine
$u(w_{Hcy})$ — Estimated standard uncertainty for the mass fraction estimates
$U_{95}(w_{Hcy})$ — Estimated 95 % level of confidence expanded uncertainty for the mass fraction estimates
$\%CV_{95}$ — Expanded 95 % relative uncertainty, $100 \times U_{95}(w_{Hcy})/w_{Hcy}$, of the mass fraction estimates stated as a percentage
Consensus — Results provided by the NIST Decision Tree's Hierarchical Gauss-Gauss method

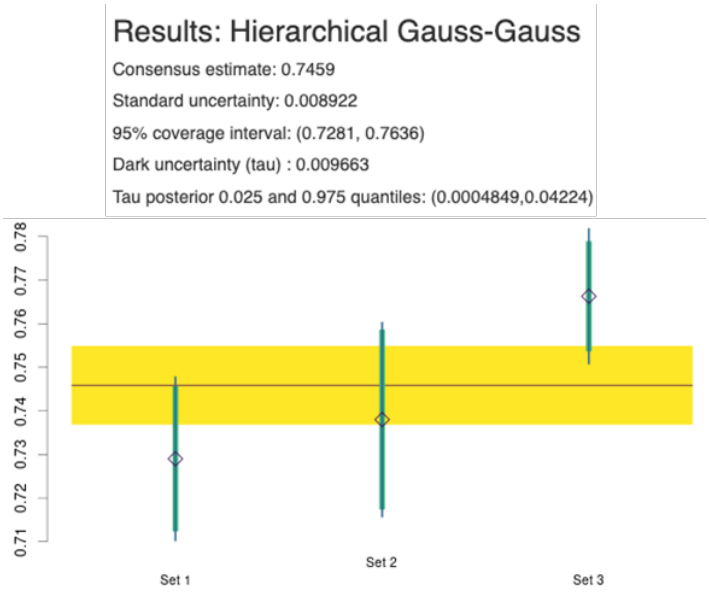


Fig. 37. NIST Decision Tree Analysis of ABACUS Results for SRM 1955a Level 1.

The black horizontal line represents the consensus estimate, $w_{Hcy,NDT}$, for the mass fraction homocysteine in SRM 1955a Level 1. The solid yellow region spans the 68 % central consensus interval, $w_{Hcy,NDT} \pm u(w_{Hcy,NDT})$. The open diamonds represent the ABACUS estimates of the mass fraction homocysteine in each measurement set of the material, $w_{Hcy,Set}$. The thick vertical lines through the diamonds represent $w_{Hcy,Set} \pm u(w_{Hcy,Set})$. The thin vertical lines through the diamonds represent a composite uncertainty interval that includes the estimated "dark uncertainty (tau)", $\tau(w_{Hcy,NDT})$: $w_{Hcy,Set} \pm (u(w_{Hcy,Set})^2 + \tau(w_{Hcy,NDT})^2)^{0.5}$.

8.2.2. SRM 1955a Level 2

Table 22 lists the ABACUS estimates of the mass fraction Hcy in each of the SRM 1955a measurement sessions and the NDT consensus estimate using its HGG method (Fig. 38).

Table 22. Summary Statistics for SRM 1955a Level 2.

Day	Set	w_{Hcy} g/g	$u(w_{Hcy})$ g/g	$U_{95}(w_{Hcy})$ g/g	$\%CV_{95}$ %
4	1	1.374	0.016	0.032	2.3
5	2	1.355	0.019	0.038	2.8
6	3	1.308	0.026	0.052	4.0
12	4	1.436	0.013	0.026	1.8
13	5	1.429	0.009	0.019	1.3
Consensus:		1.383	0.020	0.041	3.0

Day — Project session index, 1 to 14
Set — Session index, 1 to 5 for Level 2
w_{Hcy} — Estimated mass fraction homocysteine
$u(w_{Hcy})$ — Estimated standard uncertainty for the mass fraction estimates
$U_{95}(w_{Hcy})$ — Estimated 95 % level of confidence expanded uncertainty for the mass fraction estimates
$\%CV_{95}$ — Expanded 95 % relative uncertainty, $100 \times U_{95}(w_{Hcy})/w_{Hcy}$, of the mass fraction estimates stated as a percentage
Consensus — Results provided by the NIST Decision Tree's Hierarchical Gauss-Gauss method

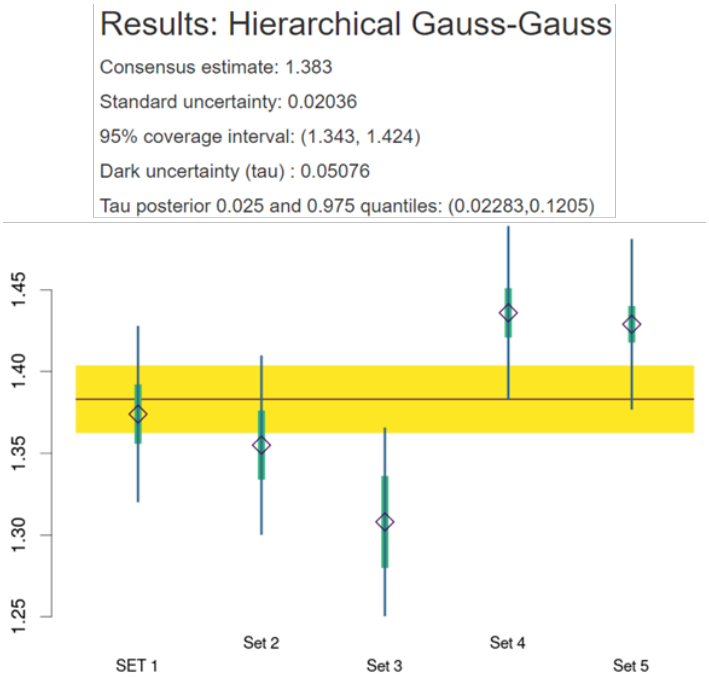


Fig. 38. NIST Decision Tree Analysis of ABACUS Results for SRM 1955a Level 2.

The black horizontal line represents the consensus estimate, $w_{Hcy,NDT}$, for the mass fraction homocysteine in SRM 1955a Level 2. The solid yellow region spans the 68 % central consensus interval, $w_{Hcy,NDT} \pm u(w_{Hcy,NDT})$. The open diamonds represent the ABACUS estimates of the mass fraction homocysteine in each measurement set of the material, $w_{Hcy,Set}$. The thick vertical lines through the diamonds represent $w_{Hcy,Set} \pm u(w_{Hcy,Set})$. The thin vertical lines through the diamonds represent a composite uncertainty interval that includes the estimated "dark uncertainty (tau)", $\tau(w_{Hcy,NDT})$: $w_{Hcy,Set} \pm (u(w_{Hcy,Set})^2 + \tau(w_{Hcy,NDT})^2)^{0.5}$.

8.2.3. SRM 1955a Level 3

Table 23 lists the ABACUS estimates of the mass fraction Hcy in each of the SRM 1955a measurement sessions and the NDT consensus estimate using its HGG method (Fig. 39).

Table 23. Summary Statistics for SRM 1955a Level 3.

Day	Set	w_{Hcy} g/g	$u(w_{Hcy})$ g/g	$U_{95}(w_{Hcy})$ g/g	$\%CV_{95}$ %
7	1	1.996	0.025	0.050	2.5
8	2	1.913	0.060	0.120	6.3
9	3	1.972	0.050	0.100	5.1
10	4	1.992	0.022	0.044	2.2
11	5	2.024	0.071	0.142	7.0
14	6	1.961	0.091	0.185	9.4
Consensus:		1.983	0.014	0.028	1.4

Day — Project session index, 1 to 14
Set — Session index, 1 to 5 for Level 2
w_{Hcy} — Estimated mass fraction homocysteine
$u(w_{Hcy})$ — Estimated standard uncertainty for the mass fraction estimates
$U_{95}(w_{Hcy})$ — Estimated 95 % level of confidence expanded uncertainty for the mass fraction estimates
$\%CV_{95}$ — Expanded 95 % relative uncertainty, $100 \times U_{95}(w_{Hcy})/w_{Hcy}$, of the mass fraction estimates stated as a percentage
Consensus — Results provided by the NIST Decision Tree's Hierarchical Gauss-Gauss method

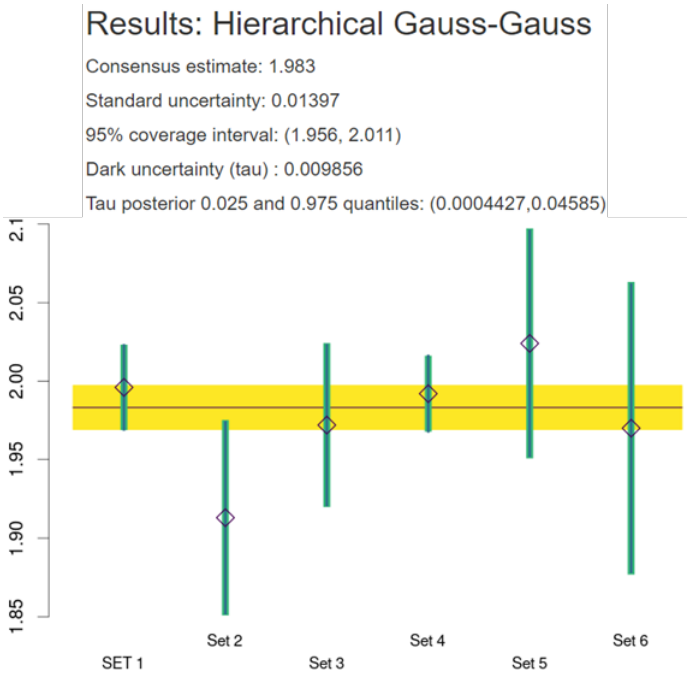


Fig. 39. NIST Decision Tree Analysis of ABACUS Results for SRM 1955a Level 3.

The black horizontal line represents the consensus estimate, $w_{Hcy,NDT}$, for the mass fraction homocysteine in SRM 1955a Level 3. The solid yellow region spans the 68 % central consensus interval, $w_{Hcy,NDT} \pm u(w_{Hcy,NDT})$. The open diamonds represent the ABACUS estimates of the mass fraction homocysteine in each measurement set of the material, $w_{Hcy,Set}$. The thick vertical lines through the diamonds represent $w_{Hcy,Set} \pm u(w_{Hcy,Set})$. The (barely visible) thin vertical lines through the diamonds represent a composite uncertainty interval that includes the estimated "dark uncertainty (tau)", $\tau(w_{Hcy,NDT})$: $w_{Hcy,Set} \pm (u(w_{Hcy,Set})^2 + \tau(w_{Hcy,NDT})^2)^{0.5}$.

8.3. Confirmation of Between-Box Homogeneity

To establish homogeneity across box numbers, mass fraction of homocysteine was calculated for each subsample in each box in each of the three materials using the linear regression with internal standard model as implemented in ABACUS but without pooling over boxes. While there is between-set heterogeneity and several discordant within-box subsamples, at a confidence level of about 95 %, there is no evidence of between-box heterogeneity for any of the three materials.

8.3.1. SRM 1955a Level 1

Table 24 lists the results and factors for SRM 1955a Level 1; the within- and between-box results are illustrated in Fig. 40. The Cochran Q [22] test of homogeneity with respect to boxes has *p*-value 0.22.

Table 24. Mass Fraction Results Per Box and Subsample for SRM 1955a Level 1.

w_{Hcy} µg/g	$u(w_{Hcy})$ µg/g	Vial	Box	Subsample	Set
0.729	0.044	568	12	1	1
0.740	0.044	568	12	2	1
0.735	0.045	849	18	1	1
0.728	0.044	849	18	2	1
0.725	0.045	849	18	3	1
0.730	0.044	1112	23	1	1
0.727	0.045	1112	23	2	1
0.719	0.045	1112	23	3	1
0.738	0.048	936	20	1	2
0.742	0.049	936	20	2	2
0.743	0.049	1208	25	1	2
0.726	0.049	1208	25	2	2
0.748	0.050	114	3	1	2
0.736	0.049	114	3	2	2
0.723	0.048	393	9	1	2
0.737	0.048	393	9	2	2
0.756	0.033	668	14	1	3
0.784	0.033	668	14	2	3
0.768	0.034	668	14	3	3
0.770	0.033	1458	30	1	3
0.776	0.034	1458	30	2	3
0.765	0.032	256	6	1	3
0.750	0.032	256	6	2	3
0.765	0.032	256	6	3	3

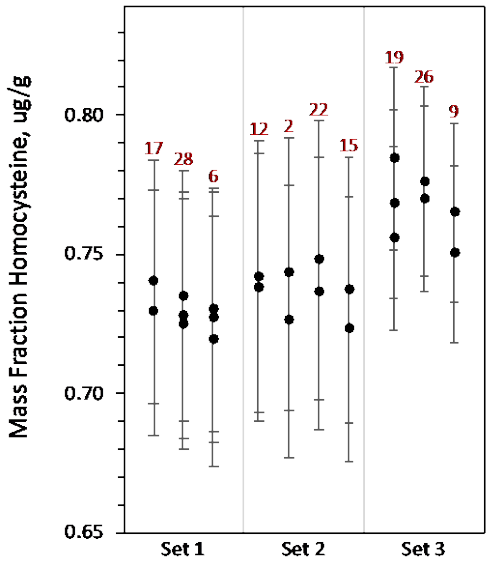


Fig. 40. Mass Fraction Results Per Box for SRM 1955a Level 1.

Each solid circle represents the mass fraction of homocysteine in one subsample in a vial drawn from one box. The error bars represent standard uncertainties. The box number is provided just above the error bars.

8.3.2. SRM 1955a Level 2

Table 25 lists the results and factors for SRM 1955a Level 2; the within- and between-box results are illustrated in Fig. 41. The Cochran Q [22] test of homogeneity with respect to boxes has *p*-value 0.69.

Table 25. Mass Fraction Results Per Box and Subsample for SRM 1955a Level 2.

w_{Hcy} µg/g	$u(w_{Hcy})$ µg/g	Vial	Box	Subsample	Set
1.379	0.019	747	16	1	1
1.378	0.019	747	16	2	1
1.360	0.019	747	16	3	1
1.323	0.018	1043	22	1	1
1.390	0.026	1043	22	2	1
1.362	0.019	1043	22	3	1
1.421	0.019	256	6	1	1
1.385	0.019	256	6	2	1
1.309	0.022	4	1	1	2
1.347	0.022	45	1	2	2
1.344	0.022	45	1	3	2
1.388	0.022	723	15	1	2
1.397	0.023	723	15	2	2
1.386	0.022	1254	26	1	2
1.312	0.022	1254	26	2	2
1.359	0.020	1254	26	3	2
1.336	0.016	481	10	1	3
1.314	0.016	481	10	2	3

1.318	0.015	998	21	1	3
1.394	0.015	998	21	2	3
1.273	0.016	1403	29	1	3
1.271	0.015	1403	29	2	3
1.299	0.016	387	8	1	3
1.262	0.016	387	8	2	3
1.460	0.028	482	10	1	4
1.430	0.028	482	10	2	4
1.429	0.028	997	21	1	4
1.419	0.028	997	21	2	4
1.450	0.029	1402	29	1	4
1.445	0.028	1402	29	2	4
1.438	0.028	386	8	1	4
1.413	0.028	586	8	2	4
1.432	0.054	44	1	1	5
1.408	0.055	44	1	2	5
1.390	0.055	44	1	3	5
1.420	0.056	722	15	1	5
1.399	0.054	722	15	2	5
1.415	0.055	1253	26	1	5
1.413	0.053	1253	26	2	5
1.411	0.055	1253	26	3	5

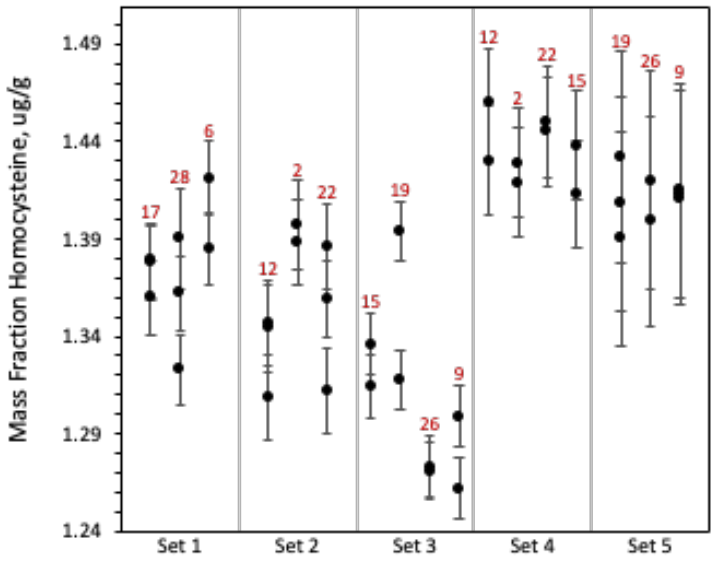


Fig. 41. Mass Fraction Results Per Box for SRM 1955a Level 2.

Each solid circle represents the mass fraction of homocysteine in one subsample in a vial drawn from one box. The error bars represent standard uncertainties. The box number is provided just above the error bars.

8.3.3. SRM 1955a Level 3

Table 26 lists the results and factors for SRM 1955a Level 3; the within- and between-box results are illustrated in Fig. 42. The Cochran Q [22] test of homogeneity with respect to boxes has *p*-value 0.27.

Table 26. Mass Fraction Results Per Box and Subsample for SRM 1955a Level 3.

w_{Hcy} µg/g	$u(w_{Hcy})$ µg/g	Vial	Box	Subsample	Set
2.057	0.018	790	17	1	1
2.028	0.018	790	17	2	1
2.013	0.018	1337	28	1	1
2.008	0.018	1337	28	2	1
1.965	0.017	1337	28	3	1
1.977	0.018	262	6	1	1
1.953	0.017	262	6	2	1
1.967	0.017	262	6	3	1
1.951	0.060	553	12	1	2
1.872	0.057	553	12	2	2
1.918	0.059	553	12	3	2
1.962	0.059	69	2	1	2
1.923	0.059	69	2	2	2
1.883	0.058	1078	22	1	2
1.903	0.058	1078	22	2	2
1.891	0.058	1078	22	3	2
1.987	0.037	730	15	1	3
1.992	0.036	730	15	2	3
2.002	0.037	889	19	1	3
2.001	0.037	889	19	2	3
1.998	0.037	1269	26	1	3
1.978	0.037	1269	26	2	3
2.006	0.037	410	9	1	3
1.809	0.036	410	9	2	3
2.030	0.014	552	12	1	4
1.990	0.014	552	12	2	4
1.931	0.014	552	12	3	4
2.006	0.014	70	2	1	4
2.015	0.014	70	2	2	4
1.931	0.014	1077	22	1	4
1.997	0.014	1077	22	2	4
2.033	0.014	1077	22	3	4
2.284	0.042	729	15	1	5
2.230	0.042	729	15	2	5
1.989	0.038	890	19	1	5
1.900	0.035	890	19	2	5
1.762	0.032	1268	26	1	5
1.997	0.036	1268	26	2	5
2.073	0.038	411	9	1	5
1.969	0.037	411	9	2	5
1.696	0.035	789	17	1	6

2.038	0.042	789	17	2	6
2.167	0.044	789	17	3	6
1.997	0.041	1336	28	1	6
2.025	0.041	1336	28	2	6
1.918	0.039	1336	28	3	6
1.527	0.032	261	6	1	6
2.050	0.042	261	6	2	6

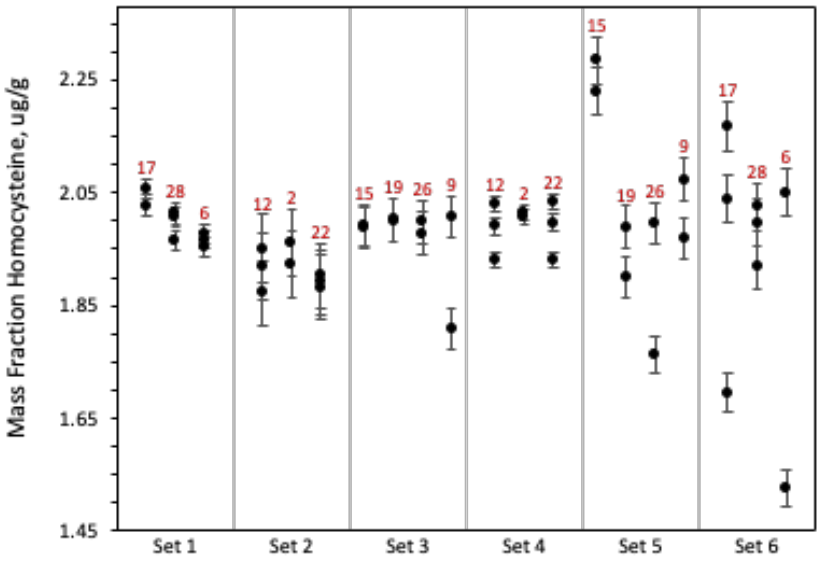


Fig. 42. Mass Fraction Results Per Box for SRM 1955a Level 3.

Each solid circle represents the mass fraction of homocysteine in one subsample in a vial drawn from one box. The error bars represent standard uncertainties. The box number is provided just above the error bars.

8.4. Mass Concentration

The mass fraction results are converted into mass concentration through multiplication by the serum density at the reference temperature, 23°C:

$$\gamma_{\text{analyte}}\,\frac{\mu\text{g}}{\text{dL}} = \left(w_{\text{analyte}}\,\frac{\mu\text{g}}{\text{g}}\right)\left(\rho_{23}\,\frac{\text{g}}{\text{mL}}\right)\left(\frac{100\ \text{mL}}{\text{dL}}\right). \qquad (10)$$

Table 27 lists the Hcy mass concentrations in the three SRM 1955a materials as estimated using NUM [21] to combine the Hcy mass fractions determined above with the serum densities estimated in Section 4.

Table 27. Homocysteine Mass Concentration in SRM 1955a Levels 1 to 3.

	Mass Fraction, µg/g		Density g/mL		Mass Concentration, µg/dL		
SRM 1955a	w_{Hcy}	$u(w_{\text{Hcy}})$	ρ_{23}	$u(\rho_{23})$	γ_{Hcy}	$u(\gamma_{\text{Hcy}})$	$U_{95}(\gamma_{\text{Hcy}})$
Level 1	0.746	0.009	1.01809	0.00018	0.759	0.009	0.018
Level 2	1.383	0.020	1.02307	0.00019	1.415	0.021	0.042
Level 3	1.983	0.014	1.02284	0.00040	2.028	0.014	0.028

8.5. Amount-of-Substance Concentration

The mass concentration results are converted into amount-of-substance concentration through division by the molecular weight (molar mass) of homocysteine:

$$c_{\text{analyte}}\,\frac{\mu\text{mol}}{\text{L}} = \left(\gamma_{\text{analyte}}\,\frac{\mu\text{g}}{\text{dL}}\right)\left(\frac{10\ \text{dL}}{\text{L}}\right)\left(\frac{\text{g}}{10^6\ \mu\text{g}}\right)\Big/\left(M_{\text{analyte}}\,\frac{\text{g}}{\text{mol}}\right)\left(\frac{\text{mol}}{10^6\ \mu\text{mol}}\right). \qquad (11)$$

Table 28 lists the Hcy amount-of-substance concentrations in the three SRM 1955a materials as estimated using NUM [21] to combine the Hcy mass concentrations determined above with the molar mass of homocysteine determined in Section 5.

Table 28. Homocysteine Amount of Substance Concentration in SRM 1955a Levels 1 to 3.

	Mass Concentration, µg/dL		Molar Mass, g/mol		Amount Concentration, µmol/L		
SRM 1955a	γ_{Hcy}	$u(\gamma_{\text{Hcy}})$	M_{Hcy}	$u(M_{\text{Hcy}})$	c_{Hcy}	$u(\gamma_{\text{Hcy}})$	$U_{95}(\gamma_{\text{Hcy}})$
Level 1	0.759	0.009			5.617	0.069	0.138
Level 2	1.415	0.021	135.187	0.005	10.47	0.15	0.30
Level 3	2.028	0.014			15.00	0.11	0.21

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Appendix A. List of Acronyms

ABACUS	Apps for Bayesian Analysis of Chemical quantities Using Shiny
CN	cyano bonded phase chromatographic column
DTT	dithiothreitol
Hcy	homocysteine
HGG	Hierarchical Gauss-Gauss
HS F5	hydrophilic bonded silica pentafluorophenyl chromatographic column
ID-LC-MS	isotope dilution-liquid chromatography-mass spectrometry
IS	internal standard
LC-MS	liquid chromatography-mass spectrometry
NDT	NIST Decision Tree
NIST	National Institute of Standards and Technology
NMR	nuclear magnetic resonance spectrometry
NUM	NIST Uncertainty Machine
OHRP	Department of Health and Human Services' Office for Human Research Protections
PC	primary chemical component
PBS	phosphate buffered saline
QC	quality control
^{1}H-qNMR	quantitative proton nuclear magnetic resonance spectrometry
RMP	reference measurement procedure
SI	International System of Units (Système international d'unités)
SPAE	solid phase anion extraction
SRM®	Standard Reference Material®
TPOC	Technical Point of Contact
%CV	coefficient of variation expressed as a percentage