



# HHS Public Access

Author manuscript

*Anal Bioanal Chem.* Author manuscript; available in PMC 2024 July 29.

Published in final edited form as:

*Anal Bioanal Chem.* 2023 February ; 415(5): 809–821. doi:10.1007/s00216-022-04474-w.

## Development of an Improved Standard Reference Material for Folate Vitamers in Human Serum

Johanna E. Camara<sup>1</sup>, Jeanita S. Pritchett<sup>1</sup>, Yasmine C. Daniels<sup>1</sup>, Mary Bedner<sup>1</sup>, Michael A. Nelson<sup>1</sup>, Mark S. Lowenthal<sup>1</sup>, Zia Fazili<sup>2</sup>, Christine M. Pfeiffer<sup>2</sup>, Karen W. Phinney<sup>1</sup>, Katherine E. Sharpless<sup>1</sup>, Lane C. Sander<sup>1</sup>, Katrice A. Lippa<sup>1</sup>, James H. Yen<sup>1</sup>, Adam J. Kuszak<sup>3</sup>, Stephen A. Wise<sup>3</sup>

<sup>1</sup>National Institute of Standards and Technology, Gaithersburg, MD, USA

<sup>2</sup>Centers for Disease Control and Prevention, National Center for Environmental Health, Atlanta, GA, USA

<sup>3</sup>National Institutes of Health, Office of Dietary Supplements, Bethesda, MD, USA

### Abstract

The U.S. National Institute of Standards and Technology (NIST) developed a Standard Reference Material® (SRM®) 3949 Folate Vitamers in Frozen Human Serum to replace SRM 1955 Homocysteine and Folate in Human Serum. The presence of increased endogenous levels of folic acid and 5-methyltetrahydrofolate (5mTHF) in SRM 3949, enhanced folate stability via addition of ascorbic acid, and inclusion of values for additional minor folates are improvements over SRM 1955 that should better serve the clinical folate measurement community. The new SRM contains folates at three levels. To produce SRM 3949, pilot sera were collected from 15 individual donors, 5 of whom were given a 400- $\mu$ g folic acid supplement 1 hour prior to blood draw to increase serum levels of 5mTHF and folic acid for the high-level material. To stabilize the folates, 0.5 % (mass concentration) ascorbic acid was added as soon as possible after preparation of serum. These pilot sera were screened for five folates plus the pyrazino-s-triazine derivative of 4- $\alpha$ -hydroxy-5-methyltetrahydrofolate (MeFox) at the U.S. Centers for Disease Control and Prevention (CDC) by isotope dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS). Based on these results, a blending protocol was specified to obtain the three desired folate concentrations for SRM 3949. ID-LC-MS/MS analysis at the CDC and NIST was utilized to assign values for folic acid and 5mTHF, as well as several minor folates.

---

Corresponding author: Johanna E. Camara, johanna.camara@nist.gov.

**Conflict of Interest:** S.A. Wise is an Editor of the journal of *Analytical and Bioanalytical Chemistry* and was not involved in peer reviewing this manuscript. There are no financial or nonfinancial competing interests for any of the remaining coauthors.

Human subjects ethics

The National Institute of Standards and Technology Research Protections Office reviewed the protocol for this project and determined it is “not human subjects research” as defined in 15 CFR 27, the Common Rule for the Protection of Human Subjects.

Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention. Certain commercial equipment or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

## Keywords

folate; reference material; human serum; liquid chromatography-tandem mass spectrometry

---

## Introduction

Folate is an essential nutrient, functioning as a co-substrate in one-carbon transfers in nucleic acid synthesis and amino acid metabolism (1). In 1998 U.S. and Canadian governments began requiring folic acid fortification of certain grain products to reduce the risk of neural tube defects in newborns (2, 3), and similar mandatory fortification programs have since been established in several other countries (4). Current research suggests folate status might also be associated with several additional health outcomes including risk of certain cancers, cardiovascular disease and stroke, and autism spectrum disorder (5–9). However, a clear understanding of folate's possible biological role in these conditions, and the relationship between folate intake and status and risk factors, has been difficult to ascertain from epidemiological studies and clinical trials to date (10, 11). Therefore, there is continued interest in monitoring the folate status of individuals and populations due to the possibility of adverse health effects from both inadequate and excessive folate intake (10, 12–14).

The National Institute of Standards and Technology (NIST) provides a variety of Standard Reference Materials® (SRMs) for the determination of nutrient levels in clinical matrices. To facilitate assessment of folate status, NIST had offered SRM 1955 Homocysteine and Folate in Human Serum since 2005, which contained three concentration levels with certified values assigned for homocysteine and 5-methyltetrahydrofolate (5mTHF), and reference values assigned for folic acid, the fully oxidized monoglutamate form of folate. The preparation of SRM 1955 Level 1 required that pooled human serum be diluted with phosphate buffered saline, while Level 3 required spiking of a serum pool with exogenous homocysteine and 5mTHF to achieve the target concentrations. SRM 1955 Level 2 consisted of a serum pool without further manipulation.

Since its issuance in 2005, additional status assessment needs have emerged in the context of folic acid fortification and supplementation, which were not fully addressed by SRM 1955. For example, concentrations of the folate forms 5mTHF and folic acid currently detected in serum samples may well exceed the highest concentration of these species found in SRM 1955 ( $\approx 37$  nmol/L for 5mTHF and  $\approx 1$  nmol/L for folic acid), as reported in recent National Health and Nutrition Examination Survey (NHANES) data (15, 16). In addition, other folate species (vitamers) such as tetrahydrofolate (THF), 5-formyltetrahydrofolate (5fTHF), and 5,10-methenyltetrahydrofolate (5,10-methenylTHF) may also be present in serum samples and contribute to biological activity. Furthermore, the biologically inactive pyrazino-s-triazine derivative of 4- $\alpha$ -hydroxy-5-methyltetrahydrofolate (MeFox), which is an oxidation product of 5mTHF, may also be present. Increased knowledge of folate stability and the advancement of liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the determination of folate vitamers necessitated the development of an updated SRM design. In response to these folate measurement needs, NIST, in collaboration with the National

Institutes of Health–Office of Dietary Supplements (NIH-ODS) and the Centers for Disease Control and Prevention (CDC), developed an improved material, designed specifically for the determination of folate vitamers, to replace SRM 1955. With endogenous 5mTHF and folic acid levels covering a greater range of biologically observed concentrations, certified values for both 5mTHF and folic acid, and enhanced folate viter stability via ascorbic acid addition, SRM 3949 is a significant improvement over the original SRM 1955 in supporting serum folate measurement needs, further empowering epidemiological studies and research on folate metabolism. This new SRM 3949 Folate Vitamers in Frozen Human Serum, which provides three levels of folates, became available from NIST in October 2018.

## Materials and Methods

### Reference Material

The National Institute of Standards and Technology Research Protections Office reviewed the protocol for this project and determined it is “not human subjects research” as defined in 15 CFR 27, the Common Rule for the Protection of Human Subjects. Serum used in preparation of SRM 3949 was acquired from Aalto Scientific (Eatonton, GA). To produce SRM 3949, approximately 250 mL to 300 mL pilot sera were collected from each of 15 individual donors. Each donor serum unit contained a 2 mL side tube to allow for screening of donor folate levels prior to pooling. Five donors were given a 400- $\mu$ g folic acid supplement one hour prior to blood draw to increase serum levels of 5mTHF and folic acid for the high-level material (Level 2). Total serum volume from five donors was expected to be sufficient to produce 1 L of this level. To stabilize the folates, 0.5 % (mass concentration) ascorbic acid was added as soon as possible after preparation of serum. These pilot sera were screened for five folate forms and MeFox at the CDC by ID-LC-MS/MS (17). The distribution of folate values for all 15 donors allowed for the creation of a blending protocol specified by NIST that was utilized by the contractor to produce approximately equal volumes of three levels of SRM 3949 to achieve the desired folic acid and 5mTHF values. The folic acid target concentrations for Levels 1, 2, and 3 were  $(1 \pm 0.5)$  nmol/L,  $(10 \pm 4)$  nmol/L, and  $(5 \pm 3)$  nmol/L, respectively. The 5mTHF target concentrations for Levels 1, 2, and 3 were  $(10 \pm 5)$  nmol/L,  $(50 \pm 5)$  nmol/L, and  $(30 \pm 5)$  nmol/L, respectively. The target concentrations for THF, 5fTHF, 5,10-methenylTHF, and MeFox in Level 3 were all  $(5 \pm 3)$  nmol/L. As is typical, the minor folates 5fTHF and 5,10-methenylTHF were undetectable by the CDC ID-LC-MS/MS method in the 15 individual donor samples. Per the blending protocol specified by NIST, 5fTHF (MilliporeSigma) and 5,10-methenylTHF (Merck & Cie) were spiked into Level 3 of SRM 3949 by the contractor to achieve the desired final concentrations. Four of the five pilot sera from donors who were administered a folic acid supplement displayed significantly elevated levels of both 5mTHF and folic acid. For 5fTHF and 5,10-methenylTHF, the contractor obtained the chemicals and spiked the Level 3 serum pool, based on the suggestion from NIST to aim for the higher end of the desired concentration range [ $(5 \pm 3)$  nmol/L each]. This was accomplished by exogenous spiking with a high-concentration, low-volume addition of 5fTHF and 5,10-methenylTHF in aqueous solvent.

## Calibration Reference Standards

For the preparation of calibration solutions for the NIST methods, a neat folic acid material was obtained from LGC Standards (Manchester, NH) and characterized at NIST. 5mTHF was obtained from Toronto Research Chemicals (Ontario, Canada).  $^{13}\text{C}_5$ -labeled folic acid and 5mTHF were obtained from Merck & Cie (Schaffhausen, Switzerland), as were unlabeled and  $^{13}\text{C}_5$ -labeled versions of 5fTHF, THF, and 5,10-methenylTHF. Unlabeled and  $^{13}\text{C}_5$ -labeled MeFox were kindly provided by Z. Fazili and C. Pfeiffer of the CDC and were originally obtained from Merck & Cie. For preparation of calibration solutions for the CDC method, all unlabeled and  $^{13}\text{C}_5$ -labeled folates were obtained from Merck & Cie.

## Reagents

Ascorbic acid, mono- and di-basic potassium phosphate, L-cysteine, hydrochloric acid, LC grade acetone, acetonitrile, methanol, glacial acetic acid (all obtained from MilliporeSigma), and ammonium hydroxide (30 %, volume fraction) and formic acid (both obtained from Fluka) were used in sample and mobile phase preparation. For NIST methods, solid phase extraction (SPE) cartridges (BondElut phenyl, 1 mL capacity, 100 mg bed size, 40  $\mu\text{m}$  particle size) were purchased from Agilent Technologies (Lexington, MA). The CDC method used BondElut phenyl, 1 mL capacity, 50 mg bed size, 40  $\mu\text{m}$  particle size SPE cartridges from Agilent Technologies for both screening and final characterization sample preparation.

**NIST ID-LC-MS/MS Method 1:** An isotope-dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) method was used to simultaneously quantitate multiple folate forms in SRM 3949. The method quantifies folic acid, 5mTHF, THF, 5fTHF, 5,10-methenylTHF, and MeFox. Unlabeled and labeled folate vitamer stock solutions for calibrants and internal standards were prepared according to procedures previously developed by the CDC (17), unless otherwise stated. Purity of the neat unlabeled folic acid calibration standard was determined at NIST by quantitative  $^1\text{H}$  nuclear magnetic resonance spectroscopy ( $q^1\text{H-NMR}$ ), and all folic acid stock solutions were prepared gravimetrically. 5mTHF stock solution concentrations were determined by UV absorption at 290 nm and purity was further adjusted by LC-UV analysis with detection at 290 nm (*vide infra*). No additional purity determinations were made for the remaining neat folate materials or stock solutions, with stock concentrations determined by UV absorption. The wavelengths and absorptivity coefficients for each vitamer are listed in Online Resource 1 (18). Stock solutions were stored at  $-80\text{ }^\circ\text{C}$  when not in use. Serum vials were thawed in the dark and thoroughly vortex mixed. All calibrants and samples were prepared under subdued lighting. To prepare samples for analysis, serum (275  $\mu\text{L}$ ) was mixed with ammonium formate buffer (1 % formic acid, 0.5 % formic acid, pH 3.2) (770  $\mu\text{L}$ ) and an internal standard mixture (55  $\mu\text{L}$ ) that contained  $^{13}\text{C}_5$ -labeled folate forms. Conditioning of 100 mg phenyl sorbent SPE cartridges on a vacuum manifold system was performed with acetonitrile, methanol, and 1 % formic acid, pH 3.2. After the sample mixture was added to the cartridges, they were washed with 0.05 % ammonium formate, pH 3.4 and samples were eluted from the SPE cartridges with 49 % water, 40 % methanol, 10 % acetonitrile, 1 % acetic acid, and 0.5 % ascorbic acid and analyzed by ID-LC-MS/MS in positive ion mode using electrospray ionization on an Agilent 6460 Triple Quadrupole Mass Spectrometer coupled to an Agilent 1200 LC system.

Chromatographic separation was achieved using a Luna C8(2) HPLC column (150 mm × 3 mm, 5 μm, Phenomenex) with an isocratic premixed mobile phase [49.5 % deionized water, 40 % methanol, 10 % acetonitrile, 0.5 % acetic acid (volume fractions)] and a total run time of 10 min. The flow rate was 250 μL/min, the column temperature was 30 °C, and the injection volume was 20 μL. Optimized MS/MS transitions and instrument settings are listed in Online Resource 2. For each SRM level, duplicate preparations from ten vials (n=20), chosen in a stratified random sampling across the entire batch, were analyzed with two injections per preparation. Values were calculated based on two independent sets of calibration standards. Quantitation was performed by LC-MS/MS peak area ratio (analyte to internal standard) and based on a  $y=mx+b$  linear regression model using a six-point calibration curve, including a zero-point calibrant, where calibrants were carried through all sample preparation steps.

The measurements were performed as two separate sets on two separate days. Each set contained duplicate independent sample preparations from SRM 1955 Homocysteine and Folate in Frozen Human Serum (Level 2 and Level 3) as controls for folic acid and 5mTHF, duplicate independent sample preparations from five independent vials for each level of SRM 3949 (total of 20 independent preparations per level), and two sets of six calibrants prepared from independently prepared stock solutions. All samples were prepared and analyzed under within-laboratory reproducibility conditions. The ten vials analyzed by this method were selected based on a stratified sampling approach to account for homogeneity across the batches of each level.

**NIST ID-LC-MS/MS Method 2:** A second approach was used to measure only folic acid in SRM 3949. Serum vials were thawed in the dark and thoroughly vortex mixed. All calibrants and samples were prepared under subdued lighting. To prepare samples for analysis, serum (300 μL) was mixed with ammonium formate buffer (1 % formic acid, 0.5 % formic acid, pH 3.2) and an internal standard mixture that contained <sup>13</sup>C<sub>5</sub>-labeled folic acid (100 μL). Conditioning of 100 mg phenyl sorbent SPE cartridges on a vacuum manifold system was performed with acetonitrile, methanol, and 1 % formic acid, pH 3.2. After the sample mixture was added to the cartridges, they were washed with 0.05 % ammonium formate, pH 3.4 and samples were eluted from the SPE cartridges with 49 % water, 40 % methanol, 10 % acetonitrile, 1 % acetic acid, and 0.5 % ascorbic acid. The SPE procedure was performed manually for each sample, control, and calibrant with positive pressure using an SPE adapter and a 3-mL plastic syringe barrel. Eluted samples were analyzed by ID-LC-MS/MS (Sciex Qtrap 6500+ triple quadrupole/linear ion trap mass spectrometer coupled to an Agilent 1290 Infinity II LC) in positive ion mode using electrospray ionization. Optimized MS/MS transitions and fragmentation settings are listed in Online Resource 3. Chromatographic separation was achieved using a Zorbax SB-C18 reversed-phase analytical column (2.1 mm × 150 mm, 3.5 μm, Agilent) with a gradient mobile phase and a total run time of 20 min (see Online Resource 4), which is a variation of a previously published NIST LC method (19). The flow rate was 400 μL/min, the column temperature was 35 °C, and the injection volume was 20 μL. Quantitation was performed by LC-MS/MS peak area ratio (analyte to internal standard) and based on an average response factor from calibrants prepared at concentration levels similar to those expected in the SRM samples and carried

through all sample preparation steps. The measurements were performed as four separate sets on four separate days. Each set contained duplicate independent sample preparations from SRM 1955 Homocysteine and Folate in Frozen Human Serum (Level 2 and/or Level 3) as controls, single independent sample preparations from six independent vials of SRM 3949 (total of 24 independent preparations per level) under within-laboratory reproducibility conditions, and three calibrants prepared from independently prepared stock solutions.

**CDC ID-LC-MS/MS Method:** An ID-LC-MS/MS method was used to measure multiple folate forms in SRM 3949 (17, 20–22). The method quantifies folic acid, 5mTHF, THF, 5fTHF, 5,10-methenylTHF, and MeFox. The calibrant solutions were prepared as previously described (17, 20) and were value assigned by UV spectroscopy (Online Resource 1). The calibration range is 1 nmol/L to 100 nmol/L for 5mTHF and 0.5 nmol/L to 50 nmol/L for all other folate forms. To prepare samples for analysis, serum (150  $\mu$ L) was mixed with ammonium formate buffer and an internal standard mixture that contained  $^{13}\text{C}_5$ -labeled folate forms. Sample clean-up was performed using a 50 mg phenyl SPE 96-well plate and an automated 96-probe SPE system (Caliper-Zephyr; Perkin Elmer Inc.) Samples were eluted from the SPE plate with an organic elution solvent containing ascorbic acid and acetic acid and analyzed by ID-LC-MS/MS in positive ion mode using electrospray ionization on an AB Sciex 6500 triple-quadrupole MS system coupled to an HP1200C LC system (Agilent Technologies). Chromatographic separation was achieved using a Luna C8(2) analytical HPLC column (150 mm  $\times$  3 mm, 5  $\mu$ m) with an isocratic mobile phase and a total run time of 7 min. Quantitation was performed by peak area ratio (analyte to internal standard) and based on linear regression using a five-point calibration curve weighted  $1/x^2$  where calibrants were carried through all sample preparation steps. For each SRM 3949 level, independent duplicate preparations from seven vials ( $n=14$ ) were carried independently through the entire sample processing and each analyzed by single injection. The CDC measurement campaign was designed based on consultation between NIST and CDC, with final approval by NIST.

### Folic acid purity determination by $q^1\text{H-NMR}$

The chemical purity (mass fraction) of the neat folic acid calibration standard was evaluated using a  $q^1\text{H-NMR}$  primary measurement procedure calibrated via internal standard (23–25). Three samples containing accurately weighed quantities of folic acid material (4.68 mg to 5.03 mg) and dimethyl sulfone internal standard (2.70 mg to 4.52 mg) were prepared in  $\text{D}_2\text{O}$  phosphate buffer solution ( $\text{pH} \approx 7.2$ ). Purity of the dimethyl sulfone internal standard was previously determined by  $q^1\text{H-NMR}$  comparison to the NIST PS1 Primary Standard for quantitative NMR (Benzoic Acid) (26) and is treated as 1.001 g/g,  $u=0.001$ . The purity of dimethyl sulfone was calculated using an average value for relative molar mass of dimethyl sulfone (based on standard atomic weights). Treatment in the purity calculations, whereby the limit of 1 g/g is not observed, is appropriate and does not contribute to bias when the dimethyl sulfone is used as a calibrant for  $q\text{NMR}$  measurement of the folic acid.  $^1\text{H-NMR}$  measurement of folic acid samples was conducted using a Bruker AVANCE II spectrometer operating at 600.14 MHz with a double-resonance inverse broadband probe (BBI) optimized for  $^1\text{H}$  observation. Experiments were performed using a  $90^\circ$  single-pulse sequence with the following parameters: 12 019 Hz (20.028 ppm) spectral width; 3 706 Hz (6.175 ppm)

transmitter frequency offset; 4 dummy scans; signal averaging of 128 scans; 60 s relaxation delay; and 5.453 s data acquisition time. Data files were apodized using an exponentially decaying window function for 0.3 Hz line broadening and processed with Bruker TopSpin Version 3.2 software. Confirmation of folic acid chemical structure and determination of purity were assessed through properties of Fourier transformed  $^1\text{H}$  spectra and the respective integral ratios for folic acid and internal standard signals. The folic acid purity results are metrologically traceable to the International System of Units (SI) unit for mass, expressed as mass fraction of folic acid in the calibration standard, through linkage of the known purity value of the dimethyl sulfone internal standard to that of the NIST PS1 Primary Standard for quantitative NMR (Benzoic Acid).

### 5mTHF purity determination by LC-UV for NIST measurements

Three independent samples of 5mTHF powder ranging from 0.8 mg to 2.4 mg were weighed and dissolved in  $\approx 10$  g of 20 mmol/L potassium phosphate buffer, pH 7.2. Final 5mTHF solutions ranged in mass fraction from 0.07 mg/g to 0.21 mg/g. The LC method for purity determination of 5mTHF was an extended version of a method previously used by NIST to determine folates in food materials (19). LC-UV absorbance analysis of all samples was performed on an Agilent 1290 LC system equipped with a UV absorbance detector. Separation was achieved using a Zorbax SB-C18 reversed-phase analytical column (2.1 mm  $\times$  150 mm, 3.5  $\mu\text{m}$  particle size, Agilent). The solvent system is detailed in Online Resource 5. Additional LC parameters were as follows: flow rate, 0.2 mL/min; column temperature, 30  $^\circ\text{C}$ ; autosampler tray temperature, 5  $^\circ\text{C}$ ; injection volume, 10  $\mu\text{L}$  with UV detection performed at 290 nm. Three injections were performed for each independently prepared 5mTHF solution. Purity was determined for each injection as the peak area of the 5mTHF ratioed to the sum of the peak areas from integrated peaks observable in the chromatogram. The purity estimate for the material was determined as the average purity determined from all injections, and the uncertainty was estimated by the standard deviation.

## Results and Discussion

### Preparation of SRM 3949

To achieve high levels of endogenous folic acid and 5mTHF for this serum material, 5 of the 15 individual donors were supplemented with 400  $\mu\text{g}$  of folic acid 1 hour prior to blood draw. This level of supplementation is consistent with the adult Recommended Daily Allowance for folate and is expected to cause a temporary increase in serum 5mTHF and folic acid. Folate screening values from the CDC ID-LC-MS/MS method indicated that four out of those five supplemented donors displayed elevated levels of folic acid and 5mTHF. The distribution of folate values for all 15 donors allowed for a blending protocol that resulted in approximately equal volumes of each SRM level that achieved the desired folic acid and 5mTHF target value ranges. The 5mTHF value (value  $\pm$  expanded uncertainty) for Level 2 [(45.71  $\pm$  4.07) nmol/L] is higher than the highest level in the predecessor SRM 1955 [(37.1  $\pm$  1.4) nmol/L]. While this value is significantly lower than some values reported in recent NHANES ( $>80$  nmol/L) (15), it was a realistic target that could be achieved without the need to screen hundreds of donors, which was not feasible. It was also achieved by mixing endogenous serum pools, and not through exogenous spiking, which was used for

the high level of SRM 1955. The folic acid value (value  $\pm$  expanded uncertainty) for Level 2 [(6.75  $\pm$  1.54) nmol/L] is significantly higher than the highest level in SRM 1955 [(1.07  $\pm$  0.24) nmol/L] and more similar to higher fasting values reported in recent NHANES (15). As is typical, the minor folates 5fTHF and 5,10-methenylTHF were undetectable by the CDC ID-LC-MS/MS method during the screening of all individual donor serum. These two folates were spiked into SRM 3949 Level 3 using the prescribed blending protocol to achieve the desired final concentrations. While a measurable level of 5fTHF was present in the final, spiked pool of Level 3, 5,10-methenylTHF was not detected after spiking. This is possibly due to instability of 5,10-methenylTHF in the solution used for spiking if it was not prepared at acidic pH (18).

### Value Assignment of folic acid, 5mTHF, 5fTHF, THF, MeFox, and total folate

**General quality assessment**—All folates were analyzed simultaneously by NIST ID-LC-MS/MS Method 1 and the CDC ID-LC-MS/MS method. For NIST Method 1, example chromatograms for a NIST calibration mix solution and SRM 3949 Level 2 are shown in Fig. 1 and Fig. 2, respectively. The folates elute in two clusters. The first occurs at  $\approx$ 2.2 min and consists of 5mTHF, THF, and 5,10-methenylTHF, while the second cluster elutes at  $\approx$ 3.2 min and consists of folic acid, 5fTHF, and MeFox. For the CDC ID-LC-MS/MS method, example chromatograms for a folate calibration mix and SRM 3949 Level 2 are shown in Fig. 3 and Fig. 4, respectively. The folates elute in two clusters. The first occurs at  $\approx$ 2.4 min and consists of 5mTHF, THF, and 5,10-methenylTHF, while the second cluster elutes at  $\approx$ 3.1 min and consists of folic acid, 5fTHF, and MeFox. Co-eluting folates from both ID-LC-MS/MS methods were further resolved by MS/MS detection of their specific transitions. Only folic acid was analyzed using NIST ID-LC-MS/MS Method 2 and elutes at  $\approx$ 10 min as shown in the example chromatograms for a folic acid calibrant and SRM 3949 Level 2 in Fig. 5 and Fig. 6, respectively.

To assess any inhomogeneity of SRM 3949, analyses of variance (ANOVA) with 5 % significance level and graphical analyses were run on NIST data where fill order information was available from vials chosen in a stratified random sampling of the entire batch for each level. In general, there was not significant inhomogeneity and no uncertainty component for inhomogeneity was included in the combined uncertainty calculations. For folic acid, some of the ANOVA tests did turn out to be statistically significant, but whatever box order effects may have been detected were not consistent either in the box differences or in scale when comparing data from NIST methods. This indicated that the variability is due to other factors like measurement variability rather than inhomogeneity of the materials. Homogeneity plots for all folates based on NIST measurements are displayed in Online Resource 6.

SRM 1955 Level 2 and Level 3 were analyzed as controls along with all NIST measurements (Table 1). For NIST method 1, the folic acid values for SRM 1955 Level 2 and Level 3 overlap the reference values within one standard deviation. For 5mTHF, the SRM 1955 Level 2 value was slightly lower than the certified range. However, the SRM 1955 Level 3 value overlaps the certified value within one standard deviation. NIST Method 2, which focused solely on folic acid measurements, resulted in SRM 1955 values that fell within the reference range for both control levels. SRM 1955 Level 2 and Level 3



were also analyzed as controls along with all CDC measurements. For SRM 1955 Level 2, the CDC 5mTHF value was slightly above the certified range and the folic acid value was slightly above the reference range. However, the mean folic acid value was similar to that measured by NIST. For SRM 1955 Level 3, the CDC 5mTHF value and the folic acid value both overlap the certified value and reference value within one standard deviation, respectively. Since the initial value assignments of SRM 1955, NIST has updated its approach to determining purity of neat folate reference standards and both NIST and the CDC have updated their ID-LC-MS/MS methods, which may account for some bias observed in control measurements.

### Folic acid in SRM 3949

All NIST folic acid measurement values were corrected for the purity of the calibration standard [ $90.51\% \pm 0.40\%$ , value (mass fraction)  $\pm U_{95}$ ], as determined by  $q^1\text{H-NMR}$ . Given that CDC assigned calibrator stock concentrations spectrophotometrically, folic acid measurements were not further corrected for purity.

For NIST method 1, the response for Level 1 was determined to be below the limit of quantitation and was not utilized in the determination of the certified value. Although the NIST method was modeled after the CDC method, the NIST folic acid values for Levels 2 and 3 were  $\approx 20\%$  lower than those of CDC (Table 2). An additional measurement campaign at NIST focusing only on the measurement of folic acid was conducted in response to the measurement discrepancy. A second analyst conducted measurements with a variation of the initial ID-LC-MS/MS method. This second NIST method attempted to address the possibilities that the spiking of other folate internal standards and/or that added time required for gravimetric preparation of multi-folate calibrants and samples were introducing a negative bias into the final NIST folic acid values compared to CDC values. In addition, the second NIST analyst provided additional method orthogonality to NIST Method 1 and the CDC Method through (1) the use of an internal standard/matching calibration scheme, (2) the use of different LC-MS/MS instrumentation, and (3) the use of a manual, positive pressure syringe solid-phase extraction process. The mean results of the second NIST method were consistent with first NIST method (Table 2). However, the second NIST method had a lower limit of quantitation and was able to measure the folic acid in Level 1. Overall, the %RSD of the second NIST method was lower than that of the first method, with values ranging from 1.0% to 4.1% versus 8.6% to 12%, respectively. For folic acid, the CDC method displayed %RSDs ranging from 5.8% to 9.5%. For all methods, the highest %RSD values were associated with measurements of the lower folic acid levels as would be expected. In an independent study, CDC had also confirmed that there was no difference in their method results for measurements of folic acid as a single analyte or if it was measured as part of the established multi-folate analyte method.

The final certified folic acid values are based on the mean of the CDC method and NIST method estimate (mean of means of the two NIST methods) (Table 3). As the source of the  $\approx 20\%$  bias among CDC and NIST folic acid values remains elusive, the CDC estimate and the mean NIST estimate were weighted equally in the combined value. Each method estimate is the mean of the measurements for that analyte using that method, with

the uncertainty being the standard error of that mean. For the NIST mean folic acid, an additional (very small) uncertainty related to purity estimation is incorporated. An additional estimate in the units ng/mL is given by multiplying by the density (in g/mL) for that level and incorporating the (very small) uncertainty associated with density estimation (Table 3). The within-method variability is dominated by the between-method variability. The uncertainty of the combined mean is estimated using a bootstrap procedure based on a Gaussian random effects model for the between-method effects (27–31). The certified folic acid values are metrologically traceable to the SI measurement units through the  $q^1\text{H-NMR}$  purity determination of the primary folic acid standard employed in the NIST methods and to mass attenuation (extinction) coefficient values and results from spectrophotometric procedures in methods used by CDC.

### 5mTHF in SRM 3949

For NIST Method 1 5mTHF measurements, the calibration stock solution concentrations were initially calculated based on absorbance at 290 nm and an absorptivity coefficient of  $31\,700\text{ L mol}^{-1}\text{ cm}^{-1}$  (18), but were then further corrected using an LC-UV absorbance peak area approach at 290 nm. The purity estimate of 5mTHF at 290 nm was determined to be  $95.80\% \pm 0.50\%$  (mean  $\pm$  standard deviation,  $n=3$ ). CDC assigned calibrator stock concentrations spectrophotometrically without additional adjustments. NIST measured 5mTHF values were 8.6 % to 15 % lower than those of CDC (Table 2). While a portion of this low bias could be associated with the additional correction to NIST calibration stocks, it would not account for the total bias. For 5mTHF, the NIST method %RSDs ranged from 6.6 % to 13 %, while the CDC method %RSDs ranged from 2.2 % to 3.8 %. The final certified 5mTHF values are based on the mean of the CDC method mean and NIST method mean (Table 3), which were weighted equally. Each method estimate is the mean of the measurements for that analyte using that method, with the uncertainty being the standard error of that mean. For the NIST mean 5mTHF, an additional (very small) uncertainty related to purity estimation is incorporated. An additional estimate in the units ng/mL is given by multiplying by the density (in g/mL) for that level and incorporating the (very small) uncertainty associated with density estimation (Table 3). The within-method variability is dominated by the between-method variability. The uncertainty of the combined mean is estimated using a bootstrap procedure based on a Gaussian random effects model for the between-method effects (27–31). Metrological traceability of the certified 5mTHF values is to the measurement units realized through purity determination of the primary 5mTHF standard employed in the NIST method.

### 5fTHF, THF, and MeFox in SRM 3949

Calibrator stock concentrations for 5fTHF, THF, and MeFox were determined spectrophotometrically without additional adjustments by both NIST and the CDC. For THF in Level 1, the CDC measurements were below the limit of quantitation. Therefore, the assigned value for Level 1 (Table 3) is based only on the mean and expanded uncertainty of NIST ID-LC-MS/MS results (Table 2).

NIST measured THF values were  $\approx 100\%$  higher than those of CDC (Table 2). For THF, the NIST method %RSDs ranged from 44 % to 63 %, while the CDC method %RSDs ranged from 9.4 % to 15 %. The very high %RSD values for all NIST measurements may be due to issues of reproducibility in extracting THF and/or the quality of peak shape or interferences.

5fTHF was only detected in the Level 3 material, as expected, based on the initial CDC screening of individual donor serum and specification that it be spiked into the Level 3 pool by the contractor. The NIST 5fTHF value was biased 75 % high compared to that of CDC (Table 2). For 5fTHF, the NIST method %RSD was 16 %, while the CDC method %RSD was 3.6 %. The contractor was instructed to spike Level 3 to a final concentration of  $(5 \pm 3)$  nmol/L, along with a general suggestion to target the higher side of the acceptable range. The CDC value was within the target range ( $\approx 5$  nmol/L,  $\approx 2.4$  ng/g), while the NIST value was high compared to the target ( $\approx 9$  nmol/L,  $\approx 4.3$  ng/g) (Table 2).

NIST measured MeFox values were biased 13 % to 62 % high compared to those of CDC (Table 2). For MeFox, the NIST method %RSDs ranged from 23 % to 52 %, while the CDC method %RSDs ranged from 4.7 % to 6.8 %. Similar to THF, the very high %RSD values for NIST MeFox measurements may be due to issues of reproducibility extracting MeFox and/or the quality of peak shape or interferences.

The final non-certified 5fTHF, THF, and MeFox values are based on the mean of the CDC method mean and NIST method mean, except for THF in Level 1 (Table 3). For THF in Level 1, the value is based on only one method and the uncertainty is the uncertainty of the single method estimate used. For the other non-certified values, NIST mean and the CDC mean were weighted equally. For the NIST mean of the non-certified folates, an additional estimate in the units ng/mL is given by multiplying by the density (in g/mL) for that level and incorporating the (very small) uncertainty associated with density estimation (Table 3). The within-method variability is dominated by the between-method variability. The uncertainty of the combined mean was estimated using a bootstrap procedure based on a Gaussian random effects model for the between-method effects (27–31). Non-certified values for 5fTHF, THF, and MeFox are metrologically traceable to the measurement procedures utilized for value assignment, as previously described.

### Total folate in SRM 3949

The non-certified total folate values for all three levels of SRM 3949 are based on the sum of the individual folate measurements obtained at the CDC by ID-LC-MS/MS (Table 3) and are reported using amount concentration units (nmol/L). Only measured folate values at or above the limit of quantitation were summed. Non-certified values for total folate are metrologically traceable to the CDC ID-LC-MS/MS method. While LC-MS/MS methods are developed to determine levels of individual folate vitamers, laboratories that use methods such as microbiological assays, which report total folate, may still be able to utilize SRM 3949 as validation materials for these methods.

## Additional studies

NIST and CDC collaborated on additional studies to investigate the possibility of calibration bias in the final measured values. In the first study, NIST provided prepared folate stock solutions to CDC for analysis and use in the folate measurements of quality control (QC) and reference materials. The results of that study suggested that differences between CDC and NIST results were not a result of calibration bias for folic acid and MeFox. Results did however suggest that the differences for 5mTHF and 5fTHF concentrations may be due, in part, to calibration bias. For 5mTHF, NIST performed a purity correction to the original stock concentrations determined through UV absorbance, which could account for a portion of the observed bias. In a second study, NIST provided powder stock of folic acid to CDC, which had been procured from a different source than that of CDC. CDC prepared a calibrator stock from NIST powder stock and compared folic acid results for QC and reference materials. Similar to the previous study, folic acid results were similar when either NIST or CDC calibrators were used. While calibration bias may account for some of the overall bias for a subset of folates, it does not appear to be the dominating factor. While NIST endeavored to reproduce the CDC LC-MS/MS method in NIST laboratories, there were still notable differences in the sample preparation. Several aspects of the NIST methodology extend the length of time for sample preparation. Additional time is needed for spiking internal standards into calibrants and samples, which is performed gravimetrically at NIST. Also, at NIST, the SPE sample preparation step was performed using either a 12-port vacuum manifold (NIST Method 1) or manual positive pressure syringe (NIST Method 2), versus an automated 96-probe SPE system at CDC. The extended time of sample preparation at ambient temperature could allow for degradation or interconversion of folates.

## Conclusion

The protocol used to design SRM 3949 Folate Vitamers in Frozen Human Serum resulted in the production of a new SRM that provides three folate concentration levels, including a high level that better reflects folic acid and 5mTHF levels recently observed in the U.S. population. In addition to certified values assigned for both 5mTHF and folic acid, SRM 3949 is also characterized with non-certified values for the minor folate metabolites THF, 5fTHF, and MeFox, which had not been assigned in the previous SRM 1955. Except for 5,10-methenylTHF, which was likely added in an unstable manner to Level 3, the original folate target values were achieved. The presence of endogenous levels of folic acid and 5mTHF, enhanced folate stability via ascorbic acid addition during serum processing, and the addition of minor folate values are improvements over SRM 1955 that are expected to better serve the folate measurement community. The observed measurement biases that persist between NIST and CDC folate measurements resulted in the assignment of an equally weighted certified value and with an uncertainty representative of the best estimate of the true concentration range in SRM 3949. While the certified uncertainties of folic acid and 5mTHF are wider compared to many other clinical matrix NIST SRMs, they are similar or narrower than the ranges of median %CVs (2.0 % to 23 % and 1.7 % to 9.8 % for folic acid and 5mTHF, respectively) and mean relative % biases (-47 % to 578 % and -24 % to 30 % for folic acid and 5mTHF, respectively) reported across 14 laboratories measuring folates in serum by LC-MS/MS in a round robin study administered by the CDC (32). SRM

3949 with certified values for folic acid and 5mTHF can support the need for improved accuracy among LC-MS/MS methods and comparability across laboratories.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

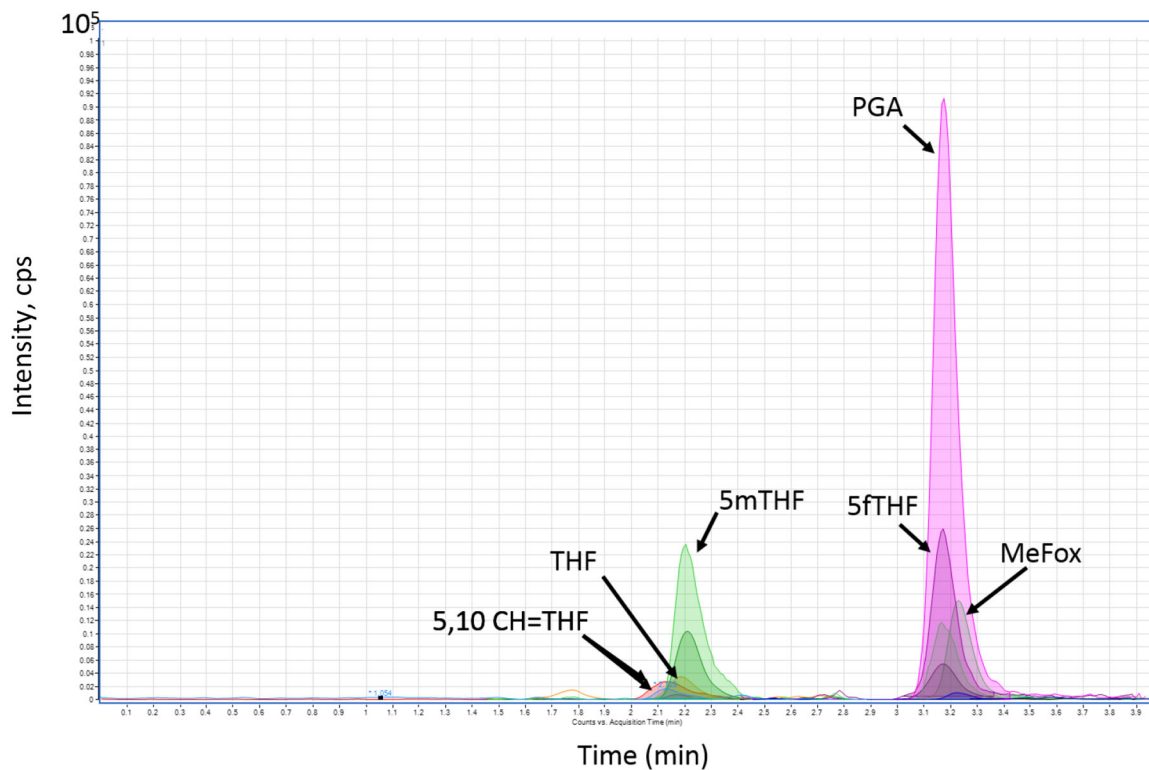
## Funding:

This study was supported in part by funding provided by the Office of Dietary Supplements at the National Institutes of Health (NIH-ODS) to the National Institute of Standards and Technology (NIST).

## Reference List

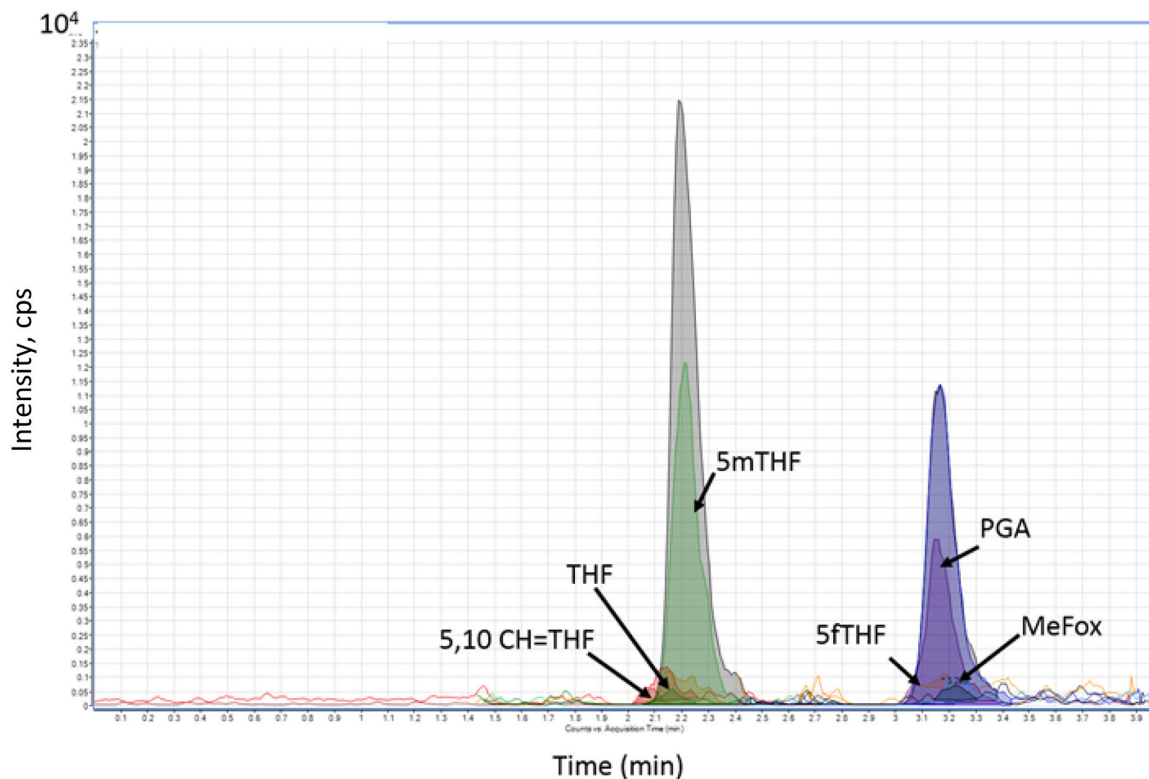
1. Bailey LB, Caudill MA. Folate. In: Erdman JWJ, Macdonald, Ian A, Zeisel, Steven H, editor. Present Knowledge in Nutrition. 10th ed 2012. p. 321–42.
2. FDA U. Food Standards: Amendment of Standards of Identity for Enriched Grain Products to Require Addition of Folic Acid. In: Services HaH, editor. Federal Register 1996. p. 8781–97.
3. Canada Go. Regulations amending the food and drug regulations. Canada Gazette 1998.
4. Crider KS, Bailey LB, Berry RJ. Folic acid food fortification-its history, effect, concerns, and future directions. *Nutrients*. 2011;3(3):370–84. [PubMed: 22254102]
5. Kim Y-I. Folate and cancer: a tale of Dr. Jekyll and Mr. Hyde? *The American Journal of Clinical Nutrition*. 2018;107(2):139–42. [PubMed: 29529163]
6. Kim Y-I. Will mandatory folic acid fortification prevent or promote cancer? *The American Journal of Clinical Nutrition*. 2004;80(5):1123–8. [PubMed: 15531657]
7. Martí-Carvajal AJ, Solà I, Lathyris D, Dayer M. Homocysteine-lowering interventions for preventing cardiovascular events. *Cochrane Database of Systematic Reviews*. 2017(8).
8. Tian T, Yang K-Q, Cui J-G, Zhou L-L, Zhou X-L. Folic Acid Supplementation for Stroke Prevention in Patients With Cardiovascular Disease. *The American Journal of the Medical Sciences*. 2017;354(4):379–87. [PubMed: 29078842]
9. DeVilbiss EA, Gardner RM, Newschaffer CJ, Lee BK. Maternal folate status as a risk factor for autism spectrum disorders: a review of existing evidence. *British Journal of Nutrition*. 2015;114(5):663–72. [PubMed: 26243379]
10. NIH ODS Folate Fact Sheet for Health Professionals 2018 [Available from: <https://ods.od.nih.gov/factsheets/Folate-HealthProfessional/>.]
11. Colapinto CK, O'Connor DL, Sampson M, Williams B, Tremblay MS. Systematic review of adverse health outcomes associated with high serum or red blood cell folate concentrations. *Journal of public health (Oxford, England)*. 2016;38(2):e84–e97. [PubMed: 26160024]
12. Sobczyk ska-Malefora A, Harrington DJ. Laboratory assessment of folate (vitamin B9) status. *Journal of Clinical Pathology*. 2018;71(11):949. [PubMed: 30228213]
13. NTP MONOGRAPH: IDENTIFYING RESEARCH NEEDS FOR ASSESSING SAFE USE OF HIGH INTAKES OF FOLIC ACID. In: Services UDoHaH, editor. 2015.
14. Yetley EA, Pfeiffer CM, Phinney KW, Fazili Z, Lacher DA, Bailey RL, et al. Biomarkers of folate status in NHANES: a roundtable summary. *The American journal of clinical nutrition*. 2011;94(1):303S–12S. [PubMed: 21593502]
15. Fazili Z, Sternberg MR, Potischman N, Wang C-Y, Storandt RJ, Yeung L, et al. Demographic, Physiologic, and Lifestyle Characteristics Observed with Serum Total Folate Differ Among Folate Forms: Cross-Sectional Data from Fasting Samples in the NHANES 2011–2016. *The Journal of Nutrition*. 2020;150(4):851–60. [PubMed: 31875475]
16. Pfeiffer CM, Sternberg MR, Fazili Z, Lacher DA, Zhang M, Johnson CL, et al. Folate status and concentrations of serum folate forms in the US population: National Health and Nutrition Examination Survey 2011–2. *The British journal of nutrition*. 2015;113(12):1965–77. [PubMed: 25917925]

17. Fazili Z, Whitehead RD Jr., Paladugula N, Pfeiffer CM. A high-throughput LC-MS/MS method suitable for population biomonitoring measures five serum folate vitamers and one oxidation product. *Analytical and bioanalytical chemistry*. 2013;405(13):4549–60. [PubMed: 23462981]
18. Blakley RL. The biochemistry of folic acid and related pteridines. In: Neuberger A, Tatum EL, editors. *Frontiers of Biology*. Amsterdam: North Holland Publishing Company; 1969. pp 92–94.
19. Camara JE, Lowenthal MS, Phinney KW. Determination of fortified and endogenous folates in food-based Standard Reference Materials by liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*. 2013;405(13):4561–8. [PubMed: 23354578]
20. Fazili Z, Pfeiffer CM. Accounting for an isobaric interference allows correct determination of folate vitamers in serum by isotope dilution-liquid chromatography-tandem MS. *The Journal of nutrition*. 2013;143(1):108–13. [PubMed: 23173171]
21. Pfeiffer CM, Fazili Z, McCoy L, Zhang M, Gunter EW. Determination of Folate Vitamers in Human Serum by Stable-Isotope-Dilution Tandem Mass Spectrometry and Comparison with Radioassay and Microbiologic Assay. *Clinical Chemistry*. 2004;50(2):423. [PubMed: 14670827]
22. Fazili Z, Pfeiffer CM. Measurement of Folates in Serum and Conventionally Prepared Whole Blood Lysates: Application of an Automated 96-Well Plate Isotope-Dilution Tandem Mass Spectrometry Method. *Clinical Chemistry*. 2004;50(12):2378. [PubMed: 15459090]
23. Jancke H NMR spectroscopy as a primary analytical method of measurement. *Nachrichten aus Chemie, Technik und Laboratorium*. 1198;46(7–8):722.
24. Malz F, Jancke H. Validation of quantitative NMR. *Journal of Pharmaceutical and Biomedical Analysis*. 2005;38(5):813–23. [PubMed: 15893442]
25. Schoenberger T Determination of standard sample purity using the high-precision <sup>1</sup>H-NMR process. *Analytical and Bioanalytical Chemistry*. 2012;403(1):247–54. [PubMed: 22327969]
26. Nelson MA, Waters JF, Toman B, Lang BE, Rück A, Breitruck K, et al. A New Realization of SI for Organic Chemical Measurement: NIST PS1 Primary Standard for Quantitative NMR (Benzoic Acid). *Analytical Chemistry*. 2018;90(17):10510–7. [PubMed: 30058803]
27. JCGM 100:2008; Evaluation of Measurement Data -- Guide to the Expression of Uncertainty in Measurement (ISO GUM 1995 with Minor Corrections); Joint Committee for Guides in Metrology. 2008.
28. Evaluation of measurement data--Supplement 1 to the "Guide to the expression of uncertainty in measurement"--Propagation of distributions using a Monte Carlo method; Joint Committee for Guides in Metrology (BIPM, IEC, IFCC, ILAC, IUPAC, IUPAP and OIML), JCGM 101:2008, International Bureau of Weights and Measures (BIPM). Sevres, France 2008.
29. Taylor BNK, C. E Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results; NIST Technical Note 1297. Washington, DC: U.S. Government Printing Office; 1994.
30. Efron BT, R. J An Introduction to the Bootstrap. UK: Chapman & Hall; 1993.
31. Searle SC G; McCulloch C Variance Components. Hoboken, NJ: John Wiley; 1992.
32. Fazili Z, Sternberg MR, Paladugula N, Pfeiffer CM. Two International Round-Robin Studies Showed Good Comparability of 5-Methyltetrahydrofolate but Poor Comparability of Folic Acid Measured in Serum by Different High-Performance Liquid Chromatography-Tandem Mass Spectrometry Methods. *J Nutr*. 2017;147(9):1815–25. [PubMed: 28768831]
33. Sniegoski LT, Moody JR. Determination of serum and blood densities. *Analytical Chemistry*. 1979;51(9):1577–8. [PubMed: 484867]



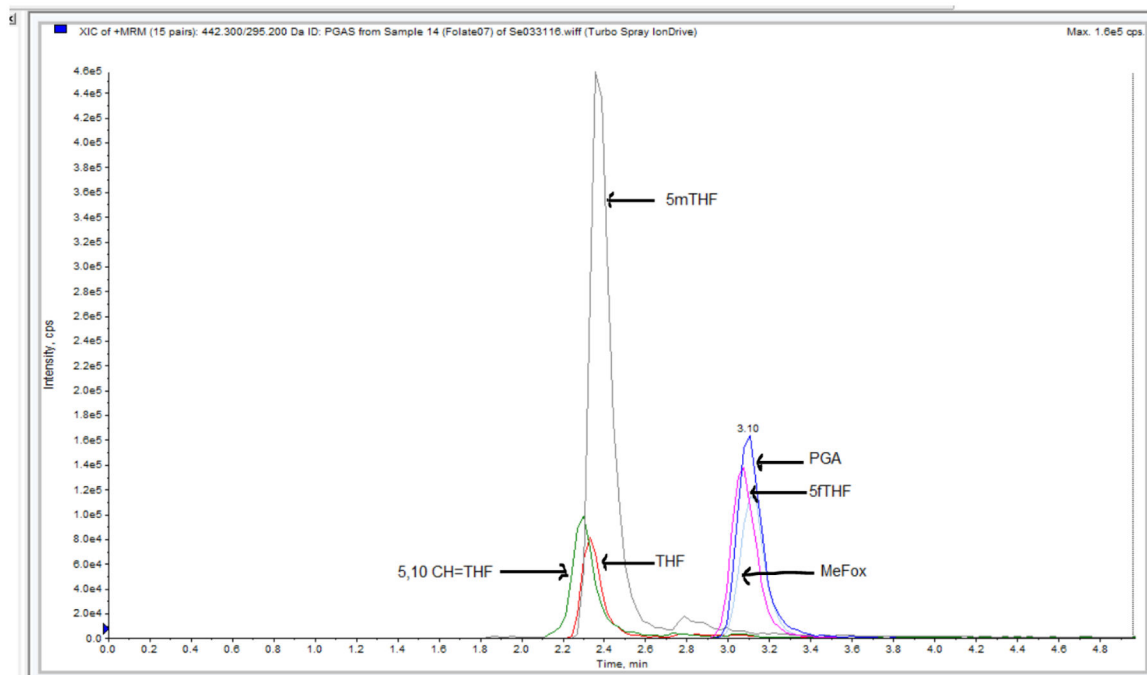
**Fig. 1.**

Representative MRM chromatograms (quantifier) for calibrant mixture (NIST ID-LC-MS/MS Method 1) with all folates at  $\approx 14$  nmol/L. The folates are indicated as folic acid (also known as pteroylglutamic acid, PGA) in light pink, 5fTHF in dark pink, MeFox in light green over pink, 5mTHF as light green, THF in light orange over green, and 5,10-methenylTHF in light orange (additional co-eluting peaks represent the  $^{13}\text{C}_5$ -labeled analogs for each folate vitamer indicated)

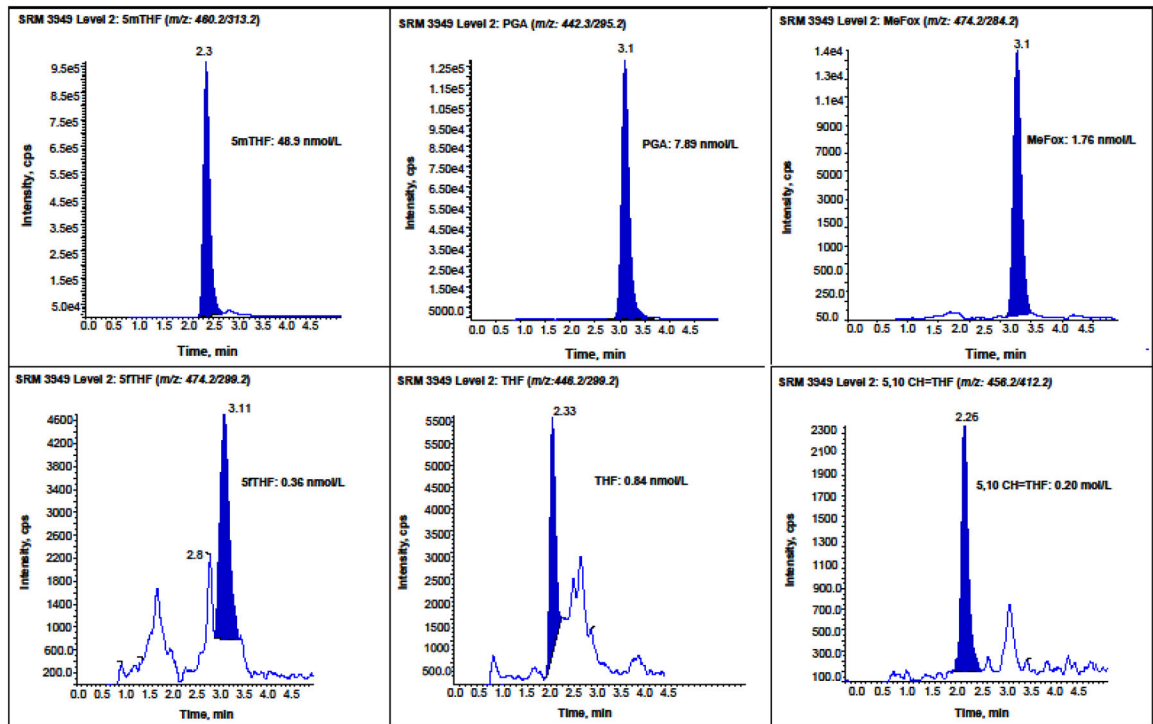


**Fig. 2.** Representative MRM chromatograms (quantifier) for SRM 3949 Level 2 (NIST ID-LC-MS/MS Method 1). The folates are indicated as folic acid (also known as pteroylglutamic acid, PGA) in dark purple, 5fTHF in orange over purple, MeFox in blue, 5mTHF as light green, THF in green over orange, and 5,10-methenylTHF (5,10 CH=THF) in red (additional co-eluting peaks represent the <sup>13</sup>C<sub>5</sub>-labeled analogs for each folate vitamier indicated)

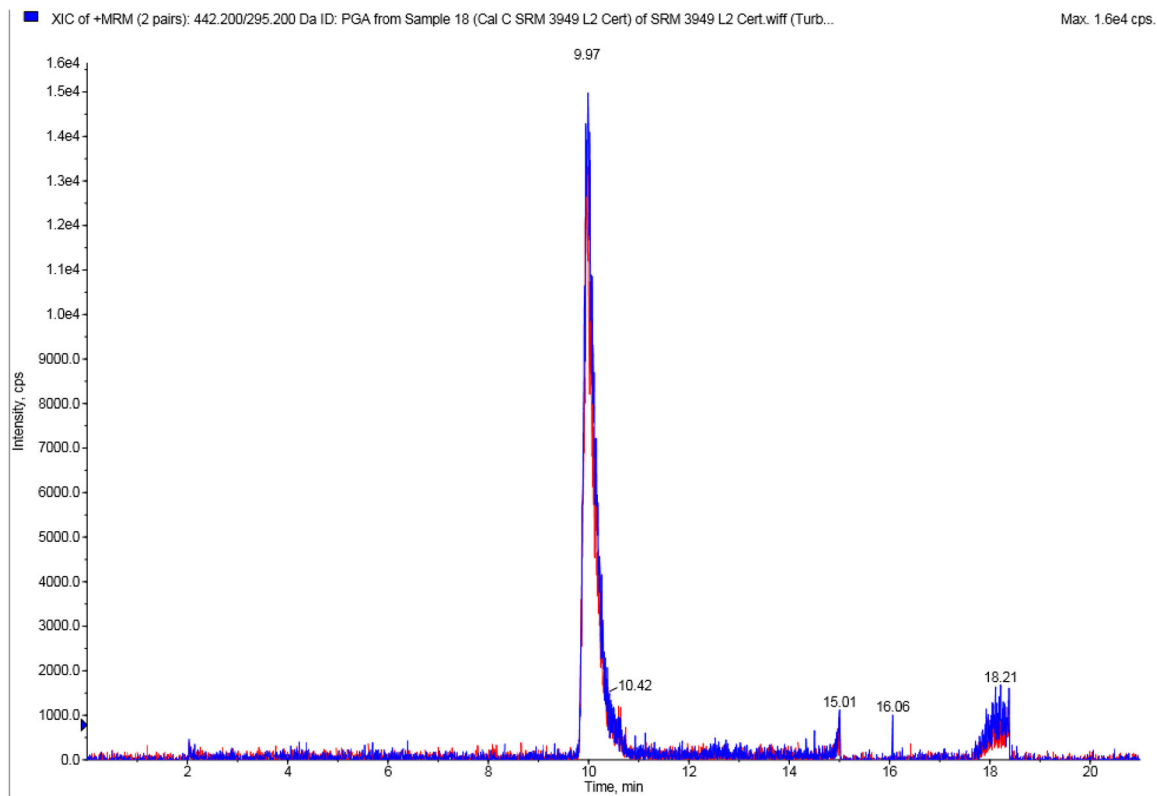


**Fig. 3.**

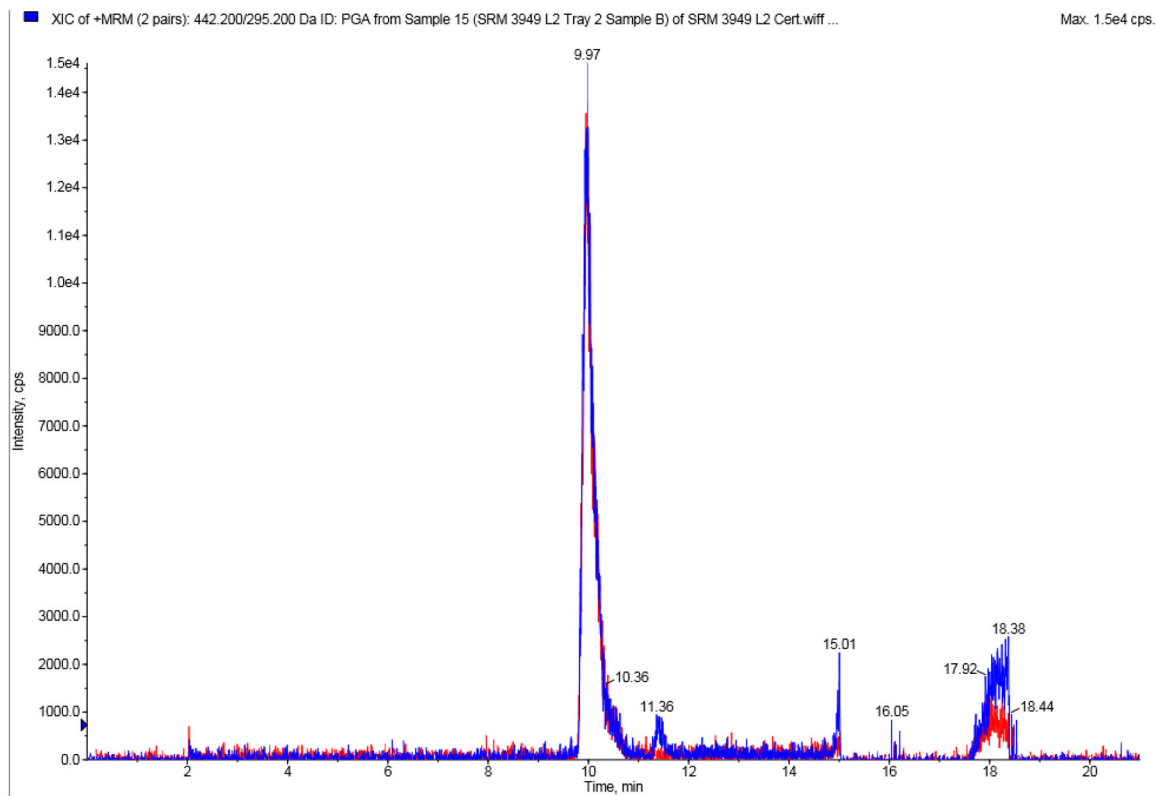
Representative MRM chromatograms for folate calibration mixture (CDC ID-LC-MS/MS Method) with 20 nmol/L for 5mTHF and 10 nmol/L for all other folate forms. The folates are indicated as folic acid (also known as pteroylglutamic acid, PGA) in dark blue, 5fTHF in purple, MeFox in light blue, 5mTHF as gray, THF in red, and 5,10-methenylTHF (5,10 CH=THF) in green



**Fig. 4.**  
Representative MRM chromatograms for folate forms in SRM 3949 Level 2 (CDC ID-LC-MS/MS Method)



**Fig. 5.** Representative chromatograms for calibrant (NIST ID-LC-MS/MS Method 2). The blue trace represents the transition for folic acid ( $\approx 1$  ng/g final concentration), whereas the red trace represents the transition for  $^{13}\text{C}_5$ -folic acid



**Fig. 6.** Representative chromatograms for a sample of SRM 3949 L2 (NIST ID-LC-MS/MS Method 2). The blue trace represents the transition for folic acid, whereas the red trace represents the transition for  $^{13}\text{C}_5$ -folic acid

**Table 1**Method-specific results for folate vitamers in SRM 1955 controls (mean  $\pm$  standard deviation)

	<b>CDC ID-LC-MS/MS</b>	<b>NIST 1 ID-LC-MS/MS</b>	<b>NIST 2 ID-LC-MS/MS</b>	<b>Reference Value</b>
<b>Folic acid</b>	<b>(nmol/L)</b>	<b>(nmol/L)</b>	<b>(nmol/L)</b>	<b>(nmol/L)</b>
Level 2	1.32 $\pm$ 0.06	1.25 $\pm$ 0.20	1.03 $\pm$ 0.04	1.05 $\pm$ 0.16
Level 3	1.31 $\pm$ 0.07	1.47 $\pm$ 0.22	1.01 $\pm$ 0.03	1.07 $\pm$ 0.24
<b>5mtdF</b>				<b>Certified Value</b>
	<b>(nmol/L)</b>	<b>(nmol/L)</b>	<b>(nmol/L)</b>	<b>(nmol/L)</b>
Level 2	10.8 $\pm$ 0.29	8.87 $\pm$ 0.57	N/A <sup>a</sup>	9.73 $\pm$ 0.24
Level 3	38.4 $\pm$ 0.29	33.5 $\pm$ 2.3	N/A	37.1 $\pm$ 1.4

<sup>a</sup>N/A: not determined

**Table 2**

Method-specific results for folate vitamers in SRM 3949. Relative standard deviations (% RSD) are given in parentheses

	CDC ID-LC-MS/MS	NIST 1 ID-LC-MS/MS	NIST 2 ID-LC-MS/MS
<b>Folic acid</b>	(ng/g)	(ng/g)	(ng/g)
Level 1	0.50 (9.5)	ND <sup>a</sup>	0.36 (4.1)
Level 2	3.24 (5.8)	2.55 (8.6)	2.62 (1.0)
Level 3	2.24 (6.0)	1.81 (12)	1.77 (1.5)
<b>5mTHF</b>			
Level 1	7.09 (3.8)	6.10 (13)	N/A <sup>b</sup>
Level 2	21.42 (2.2)	19.61 (7.0)	N/A
Level 3	13.97 (2.3)	12.29 (6.6)	N/A
<b>THF</b>			
Level 1	ND	0.50 (44)	N/A
Level 2	0.42 (15)	0.91 (64)	N/A
Level 3	0.39 (9.4)	0.83 (63)	N/A
<b>5THF</b>			
Level 1	ND	ND	N/A
Level 2	ND	ND	N/A
Level 3	2.46 (3.6)	4.31 (16)	N/A
<b>MeFox</b>			
Level 1	0.55 (6.8)	0.91 (24)	N/A
Level 2	0.81 (4.7)	1.00 (23)	N/A
Level 3	0.90 (5.9)	1.15 (52)	N/A
<b>Total folate</b>	(nmol/L)	(nmol/L)	(nmol/L)
Level 1	16.95 (3.8)	N/A	N/A
Level 2	56.01 (2.5)	N/A	N/A
Level 3	41.80 (1.8)	N/A	N/A

<sup>a</sup>ND: not detected; below limit of detection

<sup>b</sup>N/A: not determined

Table 3

Certified (in bold) and non-certified values for folates in SRM 3949

Folate	Level 1		Level 2		Level 3				
	(ng/g) <sup>(d)</sup>	(ng/mL) <sup>(b)</sup>	(nmol/L) <sup>(c)</sup>	(ng/g) <sup>(d)</sup>	(ng/mL) <sup>(b)</sup>	(nmol/L) <sup>(c)</sup>	(ng/g) <sup>(d)</sup>	(ng/mL) <sup>(b)</sup>	(nmol/L) <sup>(c)</sup>
Folic acid	<b>0.43 ± 0.13</b>	<b>0.44 ± 0.14</b>	<b>1.00 ± 0.32</b>	<b>2.91 ± 0.67</b>	<b>2.98 ± 0.68</b>	<b>6.75 ± 1.54</b>	<b>2.01 ± 0.44</b>	<b>2.06 ± 0.45</b>	<b>4.67 ± 1.01</b>
5mTHF	<b>6.60 ± 0.97</b>	<b>6.75 ± 1.00</b>	<b>14.69 ± 2.18</b>	<b>20.52 ± 1.83</b>	<b>21.00 ± 1.87</b>	<b>45.71 ± 4.07</b>	<b>13.13 ± 1.69</b>	<b>13.45 ± 1.73</b>	<b>29.27 ± 3.77</b>
THF	0.50 ± 0.08	0.51 ± 0.08	1.14 ± 0.18	0.67 ± 0.49	0.68 ± 0.50	1.53 ± 1.12	0.61 ± 0.43	0.62 ± 0.44	1.39 ± 0.99
5THF	ND	ND	ND	ND	ND	ND	3.39 ± 1.86	3.47 ± 1.90	7.33 ± 4.01
MeFox	0.73 ± 0.36	0.75 ± 0.37	1.58 ± 0.78	0.90 ± 0.18	0.93 ± 0.19	1.96 ± 0.40	1.02 ± 0.26	1.05 ± 0.26	2.22 ± 0.55
Total Folate	N/A	N/A	17.0 ± 0.4	N/A	N/A	56.0 ± 0.8	N/A	N/A	41.8 ± 0.5

ND: not detected; below limit of detection

N/A: not determined

<sup>a</sup> Values are expressed as  $x \pm U_{95\%}(x)$ , where  $x$  is the estimated value and  $U_{95\%}(x)$  is the expanded uncertainty of the value. As measured by the specific method(s), the true value of the analyte is believed to lie within the interval  $x \pm U_{95\%}(x)$  with about 95 % confidence.

<sup>b</sup> Mass concentration values were calculated from mass fractions using measured serum densities (mean  $\pm$  standard deviation, n=3): Level 1, (1.02332  $\pm$  0.00032) g/mL; Level 2, (1.02376  $\pm$  0.00031) g/mL; Level 3, (1.02435  $\pm$  0.00047) g/mL (33).

<sup>c</sup> Amount concentration values, nmol/L, are calculated from mass concentration results, nanogram per milliliter, via multiplication by 1000/ $M$ , where  $M$  is the molar mass, grams per mole, of the analyte. Relevant molar masses are:  $M_{\text{folic acid}} = 441.40$  g/mol,  $M_{5\text{-methyltetrahydrofolate}} = 459.46$  g/mol,  $M_{\text{tetrahydrofolate}} = 445.43$  g/mol,  $M_{5\text{-formyltetrahydrofolate}} = 473.44$  g/mol,  $M_{\text{MeFox}} = 473.44$  g/mol.