Anal Bioanal Chem (2010) 397:511–519
DOI 10.1007/s00216-010-3616-9

ORIGINAL PAPER

Certification of NIST standard reference material 2389a, amino acids in 0.1 mol/L HCl—quantification by ID LC-MS/MS

Mark S. Lowenthal · James Yen · David M. Bunk · Karen W. Phinney

Received: 8 December 2009 / Revised: 21 February 2010 / Accepted: 23 February 2010 / Published online: 19 March 2010

Abstract An isotope-dilution liquid chromatography-tandem mass spectrometry (ID LC-MS/MS) measurement procedure was developed to accurately quantify amino acid concentrations in National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 2389a—amino acids in 0.1 mol/L hydrochloric acid. Seventeen amino acids were quantified using selected reaction monitoring on a triple quadrupole mass spectrometer. LC-MS/MS results were compared to gravimetric measurements from the preparation of SRM 2389a—a reference material developed at NIST and intended for use in intra-laboratory calibrations and quality control. Quantitative mass spectrometry results and gravimetric values were statistically combined into NIST-certified mass fraction values with associated uncertainty estimates. Coefficients of variation (CV) for the repeatability of the LC-MS/MS measurements among amino acids ranged from 0.33% to 2.7% with an average CV of 1.2%. Average relative expanded uncertainty of the certified values including Types A and B uncertainties was 3.5%. Mean accuracy of the LC-MS/MS measurements with gravimetric preparation values agreed to within |1.1|% for all amino acids. NIST SRM 2389a will be available for characterization of routine methods for amino acid analysis and serves as a standard for higher-order measurement traceability. This is the first time an ID LC-MS/MS methodology has been applied for quantifying amino acids in a NIST SRM material.

Keywords Amino acids · Standard reference material · Mass spectrometry · Isotope dilution · LC-MS/MS

Electronic supplementary material The online version of this article (doi:10.1007/s00216-010-3616-9) contains supplementary material, which is available to authorized users.

M. S. Lowenthal (✉) · D. M. Bunk · K. W. Phinney
Analytical Chemistry Division,
National Institute of Standards and Technology,
Gaithersburg, MD 20899-8392, USA
e-mail: mark.lowenthal@nist.gov

J. Yen
Statistical Engineering Division,
National Institute of Standards and Technology,
Gaithersburg, MD 20899-8980, USA

Introduction

A major focus of the National Institute of Standards and Technology (NIST) is the production of standard reference materials (SRMs) using well-characterized measurement procedures, thereby providing the scientific community with the means to demonstrate traceability through a higher-order reference standard to the SI. SRM 2389a consists of a solution of 17 amino acids in 0.1 mol/L HCl. A similar material was previously value assigned as SRM 2389 in 1993 through a combination of gravimetric preparation and a round-robin study using LC-UV. Since that time, the material has been depleted requiring a new preparation and certification—resulting in the current material described here, SRM 2389a. This manuscript describes quantification of amino acids in SRM 2389a using an isotope-dilution tandem mass spectrometry approach.

The use of ID in conjunction with liquid chromatography-tandem mass spectrometry (LC-MS/MS) is accepted as the "gold standard" for quantification of biomolecules [1]. LC-MS/MS provides excellent accuracy and precision, and high specificity for quantification of low abundant analytes in simple and complex matrices [2]. Advantages of using tandem mass spectrometry for amino acid quantification include improvements in selectivity, reproducibility, and repeatability [3]. Quantification using selected reaction

monitoring results in high selectivity through filtering of both precursor and product ions. Ultraviolet or fluorescent detection methods, which require derivatization using reagents such as phenylisothiocyanate, *o*-phthaldialdehyde, dansyl-Cl, or 9-fluorenylmethyl-chloroformate, are inherently more prone to measurement error compared with direct quantification of underivatized analytes [4–6].

NIST materials are characterized by defining a stringent traceability chain to the SI (Système International d'Unités). Because purity determinations are characteristically difficult for biomolecules, the propagation of measurement uncertainties typically results in larger expanded uncertainties than might be expected from highly precise data sets. This material is considered fit for purpose with relative expanded uncertainties in the range of 3%. The primary importance of a reference material to the scientific community is a focus on quality control and instrumental calibration. SRM 2389a will serve to benefit labs performing amino acid analysis (AAA), peptide/protein characterization and quantification, determination of peptide/protein identity based on amino acid composition, and support for structure analysis. Additionally, solution-based reference materials may also function as a foundation for value assignment for more complex matrix-based reference materials. SRM 2389a has aided quantitative assignment of amino acids in a related plasma-based certified reference material (SRM 1950) currently being developed at NIST. Ultimately, however, SRM 2389a serves the need for a common, traceable standard to define quantitative accuracy for determination of amino acids.

In this manuscript, we describe a measurement procedure for quantitative determination of amino acids in solution using ID LC-MS/MS and apply this method, in conjunction with gravimetric preparation measurements, towards certifying concentrations of 17 amino acids in NIST SRM 2389a. Development of this certified reference material provides the scientific community essential metrological traceability to routine analyses, establishing a common, stable and accurate baseline for instrument calibration, and thus linking exploratory and clinical analyses to an established higher-order standard, and to the SI.

Experimental

Materials All chemicals were obtained from commercial sources. SRM 2389a was prepared in-house (NIST, Gaithersburg, MD) and stored at −80 °C in amber glass ampoules. High-purity LC-MS grade CHROMASOLV water and acetonitrile (ACN) were purchased from Riedel-de Haën (Hanover, GE) through Sigma (St. Louis, MO). Trifluoroacetic acid (TFA) was purchased from Sigma (Fluka). Constant boiling hydrochloric acid (HCl) solution was purchased from Sigma. Labeled amino acids were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA) with a minimum isotopic enrichment of 3 Da. All labeled amino acids were tested for chemical purity at CIL and determined at >98%. Because the internal standard is added to both the samples and calibrants, purity is inconsequential (at high-purity levels). Amino acids were purchased with the following isotopic labels and enrichment: L-alanine (U-$^{13}C_3$ 98%, ^{15}N 98%), L-arginine (U-$^{13}C_6$ 98%), L-aspartic acid (U-$^{13}C_4$ 98%), L-cystine (U-$^{13}C_6$ 98%, $^{15}N_2$ 98%), L-glutamic acid (U-$^{13}C_5$ 98%), glycine (U-$^{13}C_2$ 97-99%, ^{15}N 97-99%), L-histidine (U-$^{13}C_6$ 98%, <5%D), L-isoleucine (U-$^{13}C_6$ 98%), L-leucine (U-$^{13}C_6$ 98%), L-lysine (U-$^{13}C_6$ 98%), L-methionine (U-$^{13}C_5$ 97-99%, ^{15}N 97-99%), L-phenylalanine (U-$^{13}C_9$ 97-99%, ^{15}N 97-99%), L-proline (U-$^{13}C_5$ 98%, ^{15}N 98%), L-serine (U-$^{13}C_3$ 98%, ^{15}N 98%), L-threonine (U-$^{13}C_4$ 97-99%), L-tyrosine (U-$^{13}C_9$ 98%, ^{15}N 98%), L-valine (U-$^{13}C_5$ 98%). Unlabeled amino acids were obtained from commercial vendors (Fluka, Sigma). Chemical purity was tested by the manufacturer and in-house.

Purity and moisture analysis Identical amino acid powders were used in the preparation of SRM 2389a and their respective calibrant solutions. Purity estimates were determined for each amino acid powder through an unweighted addition of water content estimates with organic impurity estimates. Water content was determined as a weighted average of multiple analyses, which are detailed in Electronic Supplementary Material Table SI. The moisture content was tested for each amino acid using Karl Fischer titration at Galbraith Laboratories (Knoxville, TN) and was confirmed in-house using Karl Fischer titration for selected amino acid powders. Organic purity was determined in-house using liquid chromatography with ultraviolet detection at 210 nm (LC-UV; data not shown) and using titration and TLC results (manufacturer's data). Elemental analysis was performed as confirmatory analyses. Contributions from moisture were determined to be significant at levels greater than 0.05%. Only lysine and arginine were measured to have significant levels of water impurity, and the necessary corrections were made for these amino acid. Several amino acids show evidence of organic impurities, as detailed in Electronic Supplementary Material Table SI. Concern about interferences by untargeted ions is limited due to the nature of the matrix. The consensus purity was multiplied directly with mass fractions (or molar concentrations) to yield purity corrected certified values.

Gravimetric preparation Weighing was performed to an accuracy of 10^{-5} grams using either a Mettler (Columbus, OH) AT201 or XP205 balance. Concentrations were calculated from mass fractions using a value for the

temperature-dependent density of SRM 2389a at 20 °C= 1.00123 g/cm^3. The density of SRM 2389a was determined experimentally in-house.

Experimental design Amino acids were analyzed in the following sets as labeled internal standards were acquired at different times—(1) proline, valine, isoleucine, leucine, phenylalanine; (2) aspartic acid, serine, tyrosine, lysine; (3) threonine, alanine, methionine, arginine; (4) glutamic acid, histidine; (5) glycine, cystine. Each set was analyzed from four unique ampoules distributed from all segments of the SRM 2389a production lot. Three aliquots were prepared from each of the four ampoules and analyzed in duplicate (two separate days). The precision of the concentration values is based on these 24 measurements. Amino acid sampling set 1 was analyzed in triplicate (three separate days); the precision of these concentration values is based on 36 replicates. Sample injections were structured in two groups of six with one of two sets of bracketing calibrants injected before and after each sample set. Blanks were run between samples and calibrant injections. Additionally, the injection order was changed within and between each grouping on different days.

Internal standard and calibrant preparation All isotopically labeled, and unlabeled, amino acid stock solutions were prepared gravimetrically in 0.1 mol/L HCl. Amino acids were solubilized overnight at 4°C. A working internal standard solution was prepared gravimetrically from all labeled stocks in an equal molar ratio to gravimetric amino acid levels determined in the SRM (≈50 μmol/amino acid/L). Two unique sets of four calibrant solutions (eight unique calibrants) were prepared gravimetrically for LC-MS/MS analyses from stock solutions. Calibrants were prepared at targeted analyte-to-internal standard molar ratios of 0.8, 0.9, 1.1, and 1.2 (≈40, 45, 55, and 60 μmol/L), one set from each of the two unique stocks. For amino acid sampling set 1, duplicate calibrants were prepared from independent stock solutions at concentrations of 45 and 55 μmol/L only. The identical internal standard solution added to the calibrants was also used for corresponding sample preparations. The linearity of mass spectrometry response was evaluated over a broad concentration range prior to sample analysis for each measurand.

Sample preparation Figure 1 below provides a schematic for the sample preparation and quantification. SRM 2389a was produced, ampouled, and stored (at 4 °C) in-house in amber glass ampoules. Four vials of SRM 2389a were selected from a stratified, random sample of the production lot and equilibrated to room temperature for 30 min. Three aliquots of ≈20 μL each were weighed into glass autosampler vials. Samples were lyophilized to dryness

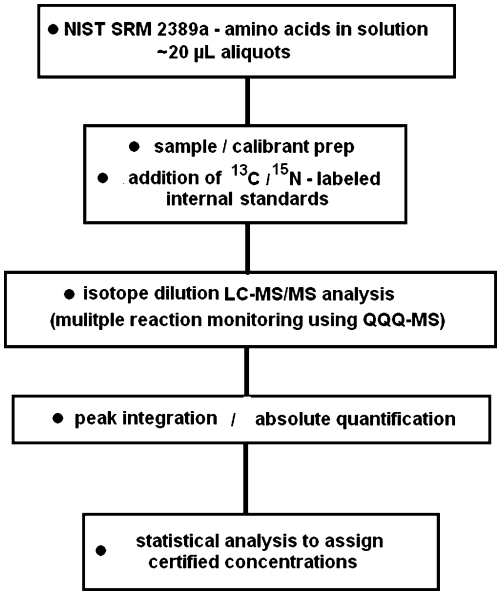

Fig. 1 Flow chart for quantification of amino acids in SRM 2389a

overnight (>16 h) without heating in a Model SPD 1010 SpeedVac (ThermoSavant). The following day, ≈1,000 μL of internal standard solution was added gravimetrically to each sample vial to yield final working concentrations ≈50 μmol/L for each labeled and unlabeled amino acid. Vials were lightly vortexed and stored overnight at 4 °C (>16 h) prior to MS analysis.

LC-MS/MS analysis Chromatographic separation was performed using an Agilent 1100 Series liquid chromatography system (Santa Clara, CA) coupled in-line to an Applied Biosystems API 4000 triple quadrupole mass spectrometer (Foster City, CA) equipped with a standard microflow source (for logistical reasons, sampling set 5 was analyzed on an Agilent 1200 Series LC system/API 5000 MS). Five-microliter injections were made onto a SIELC (Prospect Heights, IL) Primesep 100 mixed-mode LC column (2.1× 250 mm, 5 μm particle size, 10 nm pore size) with a Primesep 100 guard column (2.1×10 mm, 5 μm particle size, 100 Å pore size) to achieve chromatographic resolution of amino acids [7]. Primesep 100 is a mixed-mode analytical column with embedded acidic functional groups which separates acids by ion-exclusion and neutral compounds by reverse-phase interactions obviating the need for ion-pairing reagents. Mobile phases A and B consisted of 0.5 and 4.5 mL/L TFA, respectively in 0.3 L/L aqueous ACN. For sample set 1, requiring separation of Ile/Leu isomers, chromatographic separation was accomplished under isocratic conditions (100% A), followed by strong elution conditions (95% B) and re-equilibration. For sample sets 2 through 5, separation was achieved using an increasing linear gradient of organic solvent (ACN) over ≈30 min (starting at 0% B and ending at ≈50% B) in

tandem with a decreasing pH gradient (increasing TFA concentration) followed by a column wash and re-equilibration. Each sample set required minor variations of the gradient.

The column temperature was maintained at 30 °C for all experiments; the autosampler plate temperature control was set to 10 °C. Flow rates were maintained at a constant 200 μL/min. All analyses were performed with the following MS instrumental parameters (if different, settings for amino acid set 5 performed on an ABI 5000 are included in parenthesis): unit resolution in Q1 and Q3, collision gas = 41 kPa/6 psi, curtain gas (CUR)=69 kPa / 10 psi (275 kPa / 40 psi), ion source gas 1 (GS1) = 552 kPa/80 psi (207 kPa/30 psi), ion source gas 2 (GS2) = 345 kPa / 50 psi (276 kPa/40 psi), intensity threshold = 0, settling time = 10 ms, pause between mass ranges = 10 ms, *x*-axis spray position = 2 mm (0 mm), *y*-axis spray position = 5 mm (7 mm), ion spray voltage (IS) = 5,000 V, capillary temperature (TEM) = 500 °C, interface heater = ON, dwell time = 200 ms.

Details of the selected reaction monitoring functions used for tandem MS analysis from each set of amino acids are listed in the Electronic Supplementary Material Table SII. Data was acquired in separate periods specific to predetermined amino acid elution times whereby precursor-to-product ion transitions were detected only within that specified LC time period. Typically, each MS run was separated into three periods with no more than four transitions per period with each transition having a 200 ms dwell time. Traces of the total ion current (TIC) from representative samples are provided for each amino acid set, in Fig. 2(a–e). Fragmentation parameters were optimized for each amino acid transition by direct infusion prior to sample analysis. Source ionization and fragmentation parameters (declustering potential, entrance potential, collision energy, and collision cell exit potential) were optimized by monitoring MS-response over a broad range and noting maximum signal intensity. Labeled analogues of each amino acid were also infused for validation and to verify negligible isotope effect. For all MS analyses, these parameters were set equal for labeled/unlabeled pairs.

Data analysis Applied Biosystems Analyst software (v1.4 or v1.5) was used for peak selection and integration. Peaks were identified manually and peak areas were automatically integrated by Analyst using a bunching factor = 3, number of smooths = 1, and all other parameters set to default values. All peak integrations were visually inspected, and in some cases, manual integration was necessary. Peak area ratios were exported into Microsoft Excel for quantitative analysis. Unlabeled/labeled integrated peak area ratios were calculated from calibrant data and plotted against gravimetric mass ratios into eight-point calibration curves. For samples, molar mass ratios were extrapolated from the calibration curves according to the measured peak area ratios. From this data, amino acid concentrations were calculated and subjected to statistical evaluation.

Results and discussion

Concentrations of amino acids were calculated by interpolation through calibration curve plots created from matrix-matched calibrant data. Integrated peak area ratios (labeled/unlabeled) were plotted against gravimetric mass ratios (labeled/unlabeled) for each of eight unique calibrants, to create a calibration curve (Fig. 3). Regression model analysis of the data demonstrate linear responses within the target concentration range in all calibration curves using a $(y = mx + b)$ regression model; associated correlation coefficients (R^2) were ≥0.99 for all amino acid transitions, signifying a low fraction of variance and good predictability of sample concentration. Calibration curve slopes ranged between 0.6678 and 1.227.

Table 1 provides (a) mean experimental LC-MS/MS molar concentrations values, and (b) mass fractions of amino acids in SRM 2389a with corresponding uncertainty estimates. Unexpanded CVs (due to *Type A* uncertainty) of the overall repeatability ranged from 0.33% (threonine) to 2.7% (cystine). The mean unexpanded CV of all 17 amino acids was calculated as 1.2%, denoting excellent overall measurement precision. Gravimetric concentrations are calculated from the original SRM preparation data and are also provided in Table 1 along with their percent difference from LC-MS/MS data. Relative statistical deviations between gravimetric concentrations and LC-MS/MS data were calculated as percent differences of measurement means, as described according to the equation: $(\%\text{Diff} = [(x_1 - x_2)/((x_1 + x_2)/2)] \times 100)$. The mean percent difference among all amino acids was calculated using the absolute value of individual percent deviations. LC-MS/MS results and gravimetric preparation measurements agree well, with their percent differences ranging from within 0.071% (histidine) to 3.6% (serine). Further, the mean percent deviation between gravimetric and LC-MS/MS measurements for all amino acids was calculated as only 1.1%.

An overall purity estimate of each amino acid is provided below in Table 2 as determined by a combination of all known sources of impurities (Electronic Supplementary Material Table SI). Each LC-MS/MS and gravimetric measurement was corrected for by directly multiplying by its respective purity before statistical analysis. Table 2 provides purity corrected values of mean molar concentrations and mass fractions for each amino acid as determined by LC-MS/MS and gravimetric measurements.

A combination of LC-MS/MS and gravimetry measurements were statistically combined to obtain the certified

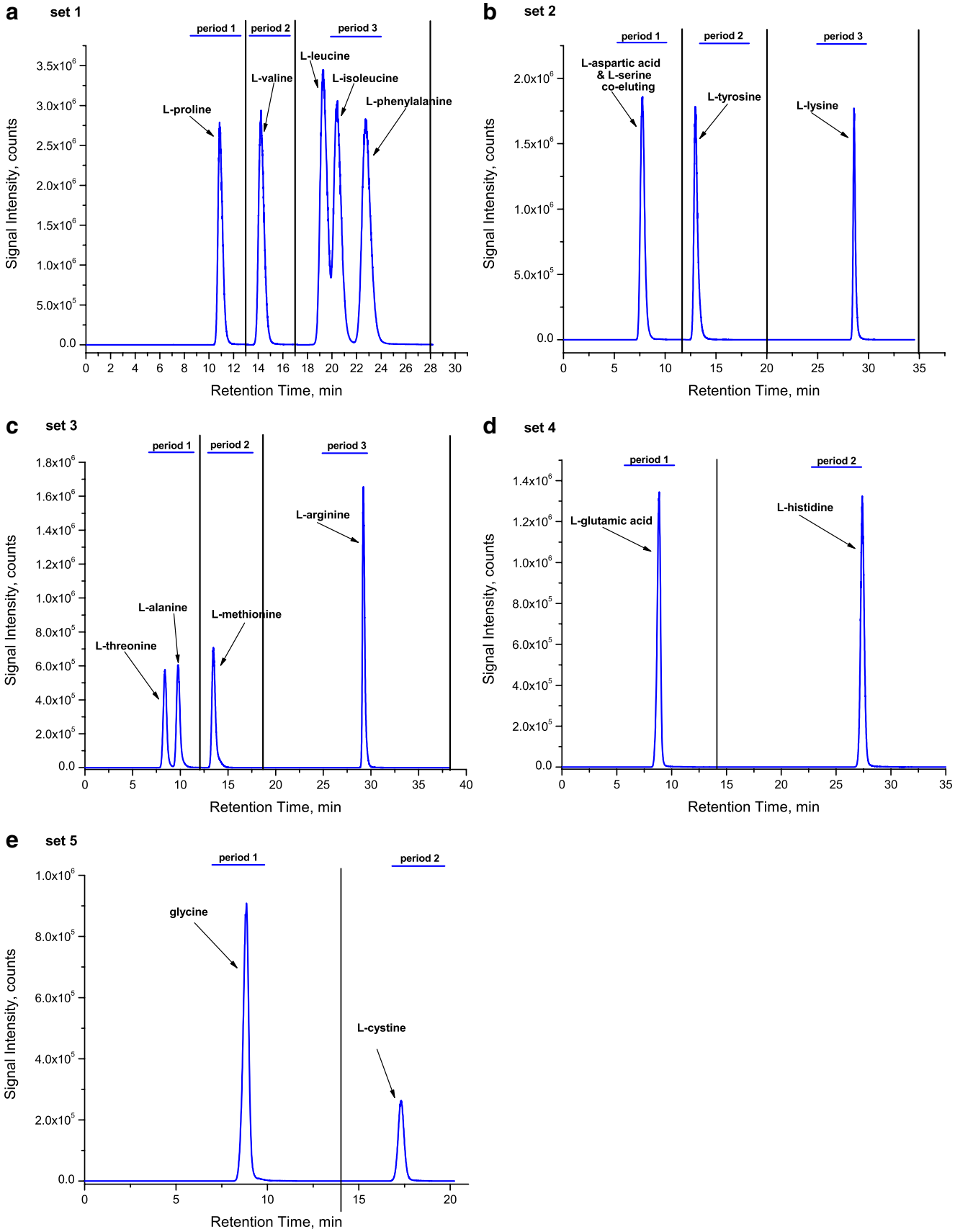

Fig. 2 Selected reaction monitoring chromatograms: TIC (total ion current) from the LC-MS/MS analysis of SRM 2389a for sets *1–5*

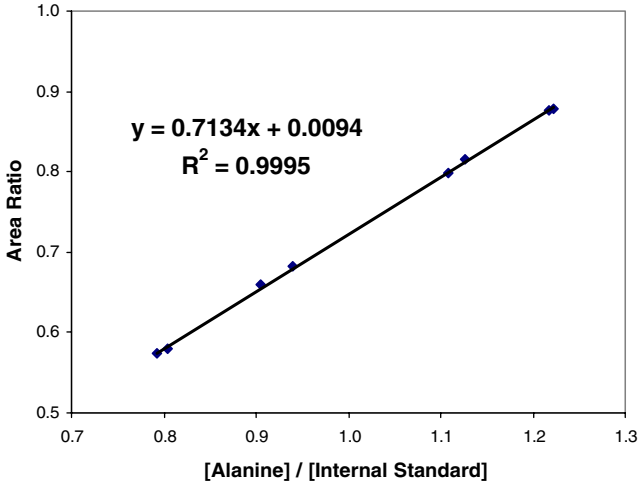

Fig. 3 Representative calibration curve generated for the amino acid alanine from eight independent points of integrated peak area ratio vs. gravimetric mass ratio

values with associated error for each amino acid in this material. Although the gravimetric measurements do not have an associated measurement precision error, the final certified values listed below (Table 3) are described by an expanded uncertainty estimate which includes a 1% Type B uncertainty for (among other sources of error) the gravimetric imprecision during sample preparation.

An expanded uncertainty (inclusive of *Types A* and *B* uncertainties) was calculated at the 95% confidence level for each amino acid as an unweighted average of LC-MS/MS measurements and gravimetry (Table 3). Relative expanded uncertainties ranged from 2.8% to 6.5%. Certified values are also provided in the Certificate of Analysis for SRM 2389a (http://ts.nist.gov/measurementservices/referencematerials/index.cfm). Certification of SRM 2389a provides a substantial reduction in overall expanded uncertainty from measurements presented for SRM 2389 resulting from the high precision of LC-MS/MS measurements. This reduction in expanded uncertainty has a positive impact on future protein-based certified reference materials produced at NIST (and at other NMIs) where SRM 2389a will be used for value assignment as part of the traceability scheme.

Statistical analysis of the data sets followed standard conventions as suggested by NIST [8] with uncertainty values conforming to International Organization for Standardization (ISO) guidelines [9]. *Type A* uncertainty from experimental data suggests measurement variability increased within factors as intra-ampoule<inter-ampoule< inter-day; however statistical significance at the 95% confidence level was not found between any of these sets. Measurement uncertainty from *Type B* components, as described above, was combined with *Type A* uncertainty estimates to yield a standard uncertainty, u_c. Because a normal distribution of the mean applies, u_c was multiplied by a coverage factor (k) to give the expanded uncertainty, U, as defining an interval having a level of confidence of approximately 95%. Relative expanded uncertainties are determined by the ratio of U to the measurement concentration mean.

Peak areas were measured for all amino acids. Automatic integration was demonstrated to be more consistent than manual integration, however all integrated peaks were visually inspected for systematic error. The unique integration of isoleucine and leucine can be explained by their isomeric properties. Because these measurands are partially unresolved chromatographically *and* have the same fragment ion transitions, the best quantitative measurement was determined by integrating between peak minima. This technique was found to be more precise as compared with using peak heights. Chromatography was reproducible to within ±10 s for peak maxima. Partially resolved or co-eluting amino acids within the same set (such as L-aspartic acid and L-serine) could be quantified over the same time period because peak widths were sufficiently wide (1–2 min) to allow alternating scans of multiple transitions while still acquiring enough data points over the curve for accurate quantification (for example, a 1-min-wide peak with a cycle time of 0.42 s would result in 142 points/peak—an absolute minimum of 10 points/peak is required for accurate quantification [10]). The selective detection of ions by selected reaction monitoring in a triple quadrupole MS combined with the simple nature of the sample matrix results in a low susceptibility to interference.

This measurement procedure was thoroughly tested for sources of bias and uncertainty. *Type A* uncertainty components were determined below 2.7% for all amino acid measurements signifying a high level of precision. Further, the accuracy of the LC-MS/MS measurements is closely confirmed by an orthogonal technique (gravimetric measurement) and is therefore considered also to be of high quality. Certified values for each amino acid were calculated as the unweighted average of LC-MS/MS means with gravimetry using an estimated uncertainty in the gravimetric value. The uncertainties for combining results from two analytical measurements are calculated using the "Type B on Bias" method [11]. Incorporated into the combined uncertainties are a 1% component related to purity of the standards for all amino acids except L-cystine (2%), L-isoleucine (2%), L-leucine (2%), L-lysine (3%), and L-proline (2%). Additionally, a 0.1% component of uncertainty was added to molar concentration values for the conversion from mass fractions, and a 1% component was added to all certified values for other Type B uncertainties including LC-MS/MS interferences and gravimetry error. Expanded uncertainties ranged from 0.0054 mg/g to 0.0229 mg/g (0.056 mmol/L to 0.157.1 mmol/L); all relative expanded uncertainties were calculated between 2.8% and 6.5%.

Table 1 Results of LC-MS/MS quantification and gravimetric preparation

Amino acid	Mean [LC-MS/MS]	Standard deviation	CV %	[Gravimetric preparation]	% Difference
Molar concentration values expressed as μmol/L—uncorrected for purity					
Alanine	2,490.8	15.3	0.61	2,511.9	0.84
Arginine	2,507.1	13.7	0.55	2,515.4	0.33
Aspartic acid	2,487.1	42.3	1.7	2,516.2	1.2
Cystine	1,255.1	34.3	2.7	1,257.9	0.23
Glutamic acid	2,491.1	13.3	0.53	2,516.1	1.0
Glycine	2,511.8	24.4	0.97	2,528.2	0.64
Histidine	2,517.1	12.1	0.48	2,515.3	0.071
Isoleucine	2,545.7	50.8	2.0	2,516.2	1.2
Leucine	2,520.6	40.4	1.6	2,514.0	0.26
Lysine	2,622.0	23.7	0.90	2,554.5	2.6
Methionine	2,494.6	32.2	1.3	2,515.4	0.83
Phenylalanine	2,571.1	28.3	1.1	2,526.5	1.7
Proline	2,519.3	28.1	1.1	2,512.5	0.27
Serine	2,426.1	34.5	1.4	2,515.9	3.6
Threonine	2,499.4	8.2	0.33	2,514.3	0.60
Tyrosine	2,549.7	49.9	1.9	2,527.7	0.86
Valine	2,584.4	30.5	1.2	2,514.5	2.7
Overall mean	–	–	1.2	–	|1.1|
Mass fractions expressed as mg/g—uncorrected for purity					
Alanine	0.22164	0.0014	0.61	0.22351	0.84
Arginine	0.43620	0.0024	0.55	0.43765	0.33
Aspartic acid	0.33063	0.0056	1.7	0.33450	1.2
Cystine	0.30121	0.0082	2.7	0.30191	0.23
Glutamic acid	0.36607	0.0020	0.53	0.36974	1.0
Glycine	0.18834	0.0018	0.97	0.18956	0.64
Histidine	0.39009	0.0019	0.48	0.38979	0.071
Isoleucine	0.33353	0.0067	2.0	0.32966	1.2
Leucine	0.33025	0.0053	1.6	0.32938	0.26
Lysine	0.38284	0.0035	0.90	0.37299	2.6
Methionine	0.37172	0.0048	1.3	0.37486	0.83
Phenylalanine	0.42420	0.0047	1.1	0.41684	1.7
Proline	0.28969	0.0032	1.1	0.28891	0.27
Serine	0.25465	0.0036	1.4	0.26407	3.6
Threonine	0.29732	0.00097	0.33	0.29914	0.60
Tyrosine	0.46140	0.0090	1.9	0.45743	0.86
Valine	0.30239	0.0036	1.2	0.29421	2.7
Overall mean	–	–	1.2	–	|1.1|

Isotope-dilution measurement procedures described here refer to the technique of adding isotopically labeled internal standards to both the samples and calibrants. In this work, we used the isotopically labeled analogue of each measurand as the internal standard. Stable isotopes act similarly during sample preparation, chromatographic retention, ionization, fragmentation, and detection. These mass spectrometry methods are characterized by high selectivity, the ability to limit interferences, accuracy over a large dynamic range, and the ease of adding stable isotope internal standards contingent on sample preparation. For quantification by LC-MS/MS, the ability to selectively detect ions in a triple quadrupole MS results in a low susceptibility to interferences. This approach satisfies all aspects of a well-characterized measurement procedure. Higher-order methods are typically too complex for routine use in most labs; however, this level of precision and accuracy is needed for value assignment of certified reference materials.

Table 2 Purity corrected values of LC-MS/MS quantification and gravimetric preparation

Amino acid	Overall estimated purity (%)	Mean [LC-MS/MS], μmol/L	[Gravimetric preparation], μmol/L	Mean LC-MS/MS, mg/g	Gravimetric preparation, mg/g
Alanine	100	2490.8	2511.9	0.22164	0.22351
Arginine	99.8098	2502.3	2510.6	0.43537	0.43682
Aspartic acid	100	2487.1	2516.2	0.33063	0.33450
Cystine	97.942	1229.2	1232.0	0.29501	0.29570
Glutamic acid	100	2491.1	2516.1	0.36607	0.36974
Glycine	100	2511.8	2528.2	0.18834	0.18956
Histidine	100	2517.1	2515.3	0.39009	0.38979
Isoleucine	96.408	2454.2	2425.8	0.32155	0.31782
Leucine	96.769	2439.1	2432.7	0.31958	0.31874
Lysine	93.2764	2445.7	2382.8	0.35710	0.34791
Methionine	100	2494.6	2515.4	0.37172	0.37486
Phenylalanine	100	2571.1	2526.5	0.42420	0.41684
Proline	97.623	2459.4	2452.8	0.28281	0.28204
Serine	98.802	2397.1	2485.8	0.25159	0.26091
Threonine	99.31	2482.1	2497.0	0.29527	0.29708
Tyrosine	100	2549.7	2527.7	0.46140	0.45743
Valine	98.31	2540.7	2472.0	0.29728	0.28924

Conclusion

NIST SRM 2389a will serve a number of purposes with uses ranging from instrument calibration for amino acid analysis, peptide mapping, or structural analysis to inter-laboratory comparisons and quality control. Seventeen amino acids in a buffered solution were accurately and precisely quantified using two orthogonal measurement techniques—isotope-dilution liquid chromatography-tandem mass spectrometry and gravimetric preparation. Amino acid concentrations were determined with high precision and accuracy for all measurands according to

Table 3 Certified concentrations of amino acids in SRM 2389a

Amino acid	Certified mass fraction ± expanded uncertainty (mg/g)	Relative expanded uncertainty (%)	Certified concentration ± expanded uncertainty [mmol/L]	Relative expanded uncertainty (%)
Alanine	0.2226±0.0064	2.9	2.501±0.072	2.9
Arginine	0.4361±0.0124	2.8	2.507±0.071	2.8
Aspartic acid	0.3326±0.0097	2.9	2.502±0.074	2.9
Cystine	0.2954±0.0133	4.5	1.231±0.056	4.5
Glutamic acid	0.3679±0.0106	2.9	2.504±0.073	2.9
Glycine	0.1889±0.0054	2.9	2.520±0.072	2.9
Histidine	0.3899±0.0110	2.8	2.516±0.071	2.8
Isoleucine	0.3197±0.0145	4.5	2.440±0.111	4.5
Leucine	0.3192±0.0143	4.5	2.436±0.109	4.5
Lysine	0.3525±0.0229	6.5	2.414±0.157	6.5
Methionine	0.3733±0.0108	2.9	2.505±0.072	2.9
Phenylalanine	0.4205±0.0127	3.0	2.549±0.077	3.0
Proline	0.2824±0.0127	4.5	2.456±0.110	4.5
Serine	0.2563±0.0091	3.5	2.441±0.086	3.5
Threonine	0.2962±0.0084	2.9	2.490±0.071	2.9
Tyrosine	0.4594±0.0133	2.9	2.539±0.074	2.9
Valine	0.2933±0.0095	3.2	2.506±0.082	3.3

NIST measurement procedures, and statistical evaluation has provided certified values for the material with appropriate uncertainty estimates. Development of a certified reference material for amino acids in solution provides the scientific community essential metrological traceability to routine analyses, establishing a common, stable, and accurate baseline for instrument calibrations and thus linking exploratory and clinical analyses to an established higher-order standard.

Acknowledgments The authors would like to thank Brandi Benford and Brian Lang (NIST) for their help in validating purity measurements for unlabeled amino acid powders.

Disclaimer Certain commercial equipment, instruments, 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.

References

1. Deleenheer AP, Thienpont LM (1992) Mass Spectrom Rev 11:249–307
2. Tai SSC, Xu B, Welch MJ, Phinney KW (2007) Anal Bioanal Chem 388:1087–1094
3. Bosch L, Alegria A, Farre R (2006) J Chromatogr B Biomed Sci Appl 831:176–183
4. Cohen SA, Michaud DP (1993) Anal Biochem 211:279–287
5. Sarwar G, Botting HG (1993) J Chromatogr 615:1–22
6. Strydom DJ, Cohen SA (1994) Anal Biochem 222:19–28
7. Burkitt WI, Pritchard C, Arsene C, Henrion A, Bunk D, O'Connor G (2008) Anal Biochem 376:242–251
8. KC Taylor BN. NIST Technical Note 1297. 1994
9. International Organization for Standardization. ISO (1993) Guide to the expression of uncertainty in measurement. 1993. Geneva, Switzerland
10. Meyer VR (1995) Error sources in the determination of chromatographic peak size ratios. Marcel Dekker, New York
11. Levenson MS, Banks DL, Eberhardt KR, Gill LM, Guthrie WF, Liu HK, Vangel MG, Yen JH, Zhang NF (2000) J Res Natl Inst Stand Technol 105:571–579