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Chapter Title	Isotope Dilution Liquid Chromatography-Tandem Mass Spectrometry for Quantitative Amino Acid Analysis	
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Abstract	The role of amino acid analysis in bioanalysis has changed from a qualitative to a quantitative technique. With the discovery of both electrospray ionization and matrix-assisted laser desorption ionization in the early 1990s, the use of amino acid analysis for qualitative analysis of proteins and peptides has been replaced by mass spectrometry. Accurate measurement of the relative molecular masses of proteins and peptides, peptide mapping, and sequencing by tandem mass spectrometry provide significantly better qualitative information than can be achieved from amino acid analysis. At NIST, amino acid analysis is used to assign concentration values to protein and peptide standard reference materials (SRMs) which, subsequently, will be used in the calibration of a wide variety of protein and peptide assays, such as those used in clinical diagnostics. It is critical that the amino acid analysis method used at NIST for assigning concentration values in SRM deliver the highest accuracy and precision possible. Therefore, we have developed an amino acid analysis method that uses isotope dilution LC-MS/MS—the analytical technique routinely used at NIST to certify analyte concentrations in SRMs for a wide variety of analytes. We present here our most recent method for the quantification of amino acids using isotope dilution LC-MS/MS.	
Keywords (separated by '-')	Isotope dilution - Liquid chromatography - Tandem mass spectrometry - Quantification - Amino acid analysis	

Chapter 12

Isotope Dilution Liquid Chromatography-Tandem Mass Spectrometry for Quantitative Amino Acid Analysis

David M. Bunk and Mark S. Lowenthal

Abstract

The role of amino acid analysis in bioanalysis has changed from a qualitative to a quantitative technique. With the discovery of both electrospray ionization and matrix-assisted laser desorption ionization in the early 1990s, the use of amino acid analysis for qualitative analysis of proteins and peptides has been replaced by mass spectrometry. Accurate measurement of the relative molecular masses of proteins and peptides, peptide mapping, and sequencing by tandem mass spectrometry provide significantly better qualitative information than can be achieved from amino acid analysis. At NIST, amino acid analysis is used to assign concentration values to protein and peptide standard reference materials (SRMs) which, subsequently, will be used in the calibration of a wide variety of protein and peptide assays, such as those used in clinical diagnostics. It is critical that the amino acid analysis method used at NIST for assigning concentration values in SRM deliver the highest accuracy and precision possible. Therefore, we have developed an amino acid analysis method that uses isotope dilution LC-MS/MS—the analytical technique routinely used at NIST to certify analyte concentrations in SRMs for a wide variety of analytes. We present here our most recent method for the quantification of amino acids using isotope dilution LC-MS/MS.

Key words Isotope dilution, Liquid chromatography, Tandem mass spectrometry, Quantification, Amino acid analysis

1 Introduction

The role of amino acid analysis has changed in bioanalysis from a qualitative to a quantitative technique. Amino acid analysis was originally used qualitatively to validate the identity of a purified protein or peptide. After hydrolysis of the protein or peptide into constituent amino acids, the relative ratios of all amino acids were determined and compared to expected ratios. In this way, the pattern of amino acid ratios could be used as a fingerprint for identification. With the discovery of both electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) in the early 1990s, the use of amino acid analysis for qualitative analysis of proteins and peptides has been replaced by mass spectrometry. Accurate measurement of the relative molecular masses of

Michail Alterman (ed.), *Amino Acid Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2030,
https://doi.org/10.1007/978-1-4939-9639-1_12,

proteins and peptides, peptide mapping, and sequencing by tandem mass spectrometry provide significantly better qualitative information than can be achieved from amino acid analysis. While mass spectrometry has replaced amino acid analysis as a qualitative technique, the role of amino acid analysis has shifted to that of an important quantitative technique.

The analytical sensitivity and specificity of mass spectrometry makes it one of the most powerful techniques for the analysis of proteins and peptides, for both qualitative measurement and, most recently, quantification [1]. Accuracy in quantitative mass spectrometry requires accuracy in the concentration of the peptide and protein calibrants used; yet, this is not always easy to achieve. Accuracy in the concentration of the calibration materials used in the analysis of other chemical analytes is typically achieved through gravimetry—the accurate weighing of solid analyte standards of known purity. Gravimetry is typically not feasible for protein and peptide analytes because often there is not a sufficient amount of solid protein or peptide standard available (if at all) for accurate weighing and, quite often, purity assessments of protein and peptide standards are crude. Because of the limitations of gravimetry to directly prepare accurate protein and peptide calibrants, amino acid analysis has become an important technique to assign concentration values to these calibrants.

Amino acid analysis used for quantitative analysis of protein and peptide solutions requires absolute quantification following hydrolysis of the protein or peptide into constituent amino acids. Amino acids are available in bulk with accurately assigned purity, allowing accurate preparation of calibrants to be produced using gravimetry for the amino acid analysis (i.e., solutions of amino acids). This allows protein and peptide quantification to be metrologically traceable [2] to gravimetrically prepared calibrants, producing the highest degree of accuracy [3].

For the accuracy and precision of gravimetrically prepared amino acid calibrants to results in accurate and precise peptide or protein calibration solutions, the quantitative amino acid analysis method should have high precision and be free of bias (or have a known and constant bias). Of course, the required precision and accuracy of the amino acid analysis depend[s] on the accuracy and precision needed in the final measurement result of the protein or peptide analyte. At NIST, amino acid analysis is used to assign concentration values to protein and peptide standard reference materials (SRMs) which, subsequently, will be used in the calibration of a wide variety of protein and peptide assays, such as those used in clinical diagnostics. It is critical that the amino acid analysis method used at NIST for SRM measurement deliver the highest accuracy and precision possible. Therefore, we have developed an amino acid analysis method that uses isotope dilution LC-MS/MS [4]—the analytical technique routinely used at NIST to certify

analyte concentrations in SRMs for a wide variety of analytes. Amino acid analysis by isotope dilution LC-MS/MS was first used to measure the concentration of bovine serum albumin in NIST SRM 927d ("bovine serum albumin, 7% solution") and, for the subsequent lot, SRM 927e. We have recently refined our isotope dilution LC-MS/MS amino acid analysis method to certify the concentration of 17 amino acids in NIST SRM 2389a ("amino acids in 0.1 mol/L hydrochloric acid") and 16 amino acids in SRM 1950 ("Metabolites in Human Plasma") and to measure the protein concentration in SRM 2924 ("C-Reactive Protein Solution"). We present here our most recent method for the quantification of amino acids using isotope dilution LC-MS/MS.

2 Materials

Solvents used are all high-purity "LC-MS" grade and reagents are analytical grade or better (*see* **Note 1**).

2.1 LC Components

1. LC mobile phase A: 0.5 mL/L trifluoroacetic acid in 0.2 L/L aqueous acetonitrile. To 200 mL "LC-MS" grade acetonitrile, add "LC-MS" grade water to a total volume of 1 L. Add 0.5 mL of trifluoroacetic acid. Store at room temperature (*see* **Note 2**).
2. LC mobile phase B: 4.5 mL/L trifluoroacetic acid in 0.2 L/L aqueous acetonitrile. To 200 mL "LC-MS" grade acetonitrile, add "LC-MS" grade water to a total volume of 1 L. Add 4.5 mL of trifluoroacetic acid. Store at room temperature (*see* **Note 2**).
3. LC column: Primesep 100, 2.1 mm × 250 mm, 3 μm particle size, 10 nm pore size (SIELC Technologies, Wheeling, IL).
4. LC instrumentation: 1290 Infinity liquid chromatography system (Agilent Technologies, Santa Clara, CA) consisting of a binary LC pump, well-plate autosampler with temperature control, and thermostatted column compartment.

2.2 MS Components

MS instrumentation: Agilent 6460A triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with an ESI source.

2.3 Labeled and Unlabeled Amino Acid Standards

1. Labeled amino acids: L-alanine (U-$^{13}C_3$ 98 %, ^{15}N 98 %), L-arginine (U-$^{13}C_6$ 98 %, $^{15}N_4$ 98 %), L-aspartic acid (U-$^{13}C_4$ 98 %), L-glutamic acid (U-$^{13}C_5$ 98 %), L-histidine (U-$^{13}C_6$ 98 %), 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 %), and L-valine (U-$^{13}C_5$ 98 %) (Cambridge Isotope Labs, Andover, MA) (*see* **Note 3**).

2. Unlabeled amino acids: all L-isoforms were used. Chemical purity of each amino acid standard was evaluated by the manufacturer and confirmed through in-house measurements, if necessary (*see* **Note 4**). Certified reference materials for L-alanine, L-arginine, L-isoleucine, L-leucine, L-phenylalanine, and L-valine were purchased from the National Metrology Institute of Japan. All other amino acids were obtained from MilliporeSigma (St. Louis, MO) at the highest purity available.

3 Methods

3.1 Calibrant and Sample Preparation

1. Stock solutions of each isotopically labeled amino acid are prepared by weighing out the solid amino acid and dissolving in aqueous 0.1 mol/L hydrochloric acid. These stock solutions are stored at 5 °C.
2. An internal standard solution is prepared by volumetrically blending the individual labeled amino acid stock solutions. The concentrations of the labeled amino acids in the internal standard solution should match as closely as possible the concentrations of amino acids in the hydrolysate of the protein or peptide solution being quantified. Aqueous 0.1 mol/L hydrochloric acid is used as the diluent, if necessary, to achieve the desired concentrations. The internal standard solution is stored at 5 °C.
3. Stock solutions of each unlabeled amino acid are prepared by weighing out the solid amino acid and dissolving in aqueous 0.1 mol/L hydrochloric acid. To achieve the most accurate gravimetric weights, the solid amino acids should be stored in a desiccator for several days prior to use and should be equilibrated for at least several hours with the temperature of the room where they will be weighed. These stock solutions are stored at 5 °C.
4. Calibration solutions are prepared by gravimetrically (preferred, for highest accuracy and precision) or volumetrically blending the individual unlabeled amino acid stock solutions and the internal standard solution (containing the isotopically labeled amino acids). Several calibration solutions are prepared which contain varying molar concentration ratios of the unlabeled and labeled amino acids. For example, for preliminary measurements, calibration solutions with unlabeled-to-labeled amino acid molar concentrations of approximately 10, 3, 1, 0.3, and 0.1 are typically prepared. After preliminary

measurement of the amino acid concentrations are made, higher accuracy and precision in the concentration measurement can be achieved using the technique of double exact matching isotope dilution mass spectrometry [5, 6].

5. Hydrolysate samples (*see* **Note 5**) are first lyophilized to dryness overnight (>16 h) without heating in a SPD 1010 SpeedVac (Savant/Thermo, Rockford, IL). The lyophilized hydrolysate is resolubilized with the internal standard solution and equilibrated overnight (≥16 h) at 5 °C. If not analyzed immediately, samples are stored at 5 °C.

3.2 LC-MS/MS Measurements

1. For the chromatographic separation of amino acids, gradient elution with mobile phases A and B is used. The elution starts at 100 % mobile phase A and increases linearly to 50 % mobile phase B in 20 min. The mobile phase composition ramps to 100 % mobile phase B over 2 min and washes the column at 100 % B for 3 min. Next, the column is re-equilibrated with the starting conditions (100 % mobile phase A) for 15 min. A constant mobile phase flow rate of 200 μL/min is used. The Primesep 100 column is maintained at 15 °C in the thermostatted column compartment. Samples are maintained at 5 °C in the autosampler.
2. The triple quadrupole mass spectrometer is operated in positive ion mode using dynamic multiple reaction monitoring (dMRM) scans with delta retention times varying between 1 and 2 min depending on the amino acid. All analyses are performed with the following Agilent 6460A MS instrumental parameters: unit resolution for MS1 and MS2, source gas temperature = 300 °C, source gas flow = 6 L/min, nebulizer = 45 psi, sheath gas temperature = 250 °C, sheath gas flow = 11 L/min, capillary voltage = 4000 V, and nozzle voltage = 500 V.
3. The instrument parameters for the dMRM scans used in the measurement of each set of labeled and unlabeled amino acids are listed in Table 1.
4. Agilent MassHunter Workstation software (version B.07.01) is used for peak selection and integration. Peak retention times and integrated peak areas were automatically determined by MassHunter. All peak integrations are visually inspected, and in some cases, manual integration is necessary. Peak area ratios are exported into Microsoft Excel for quantitative analysis. Unlabeled/labeled integrated peak area ratios are calculated from calibrant data and plotted against gravimetric mass ratios into calibration curves. For samples, molar mass ratios are extrapolated from the calibration curves according to the measured peak area ratios. From this data, amino acid concentrations are

Table 1
Mass spectrometric instrument parameters for the dynamic MRM scans of amino acids measured using the Agilent Technologies 6460A Triple Quad mass spectrometer

Amino acid	Formula	Precursor *m/z*	Product *m/z*	CE (V)	Fragmentor (V)
Asp	$C_4H_7NO_4$	134.0	74.0	10	60
*Asp	$^{13}C_4H_7NO_4$	138.0	76.0		
Ser	$C_3H_7NO_3$	106.1	60.1	6	50
*Ser	$^{13}C_3H_7{}^{15}NO_3$	110.1	63.0		
Thr	$C_4H_9NO_3$	120.1	56.1	10	90
*Thr	$^{13}C_4H_9NO_3$	124.1	59.0		
Glu	$C_5H_9NO_4$	148.1	84.0	10	70
*Glu	$^{13}C_5H_9NO_4$	153.1	88.0		
Ala	$C_3H_7NO_2$	90.1	44.1	6	50
*Ala	$^{13}C_3H_7{}^{15}N0_2$	94.1	47.0		
Pro	$C_5H_9NO_2$	116.1	70.1	10	80
*Pro	$^{13}C_5H_9{}^{15}NO_2$	121.1	74.0		
Val	$C_5H_{11}NO_2$	118.1	72.1	6	60
*Val	$^{13}C_5H_{11}NO_2$	123.1	76.0		
Met	$C_5H_{11}NO_2S$	150.1	56.1	10	70
*Met	$^{13}C_5H_{11}{}^{15}NO_2S$	156.1	60.0		
Tyr	$C_9H_{11}NO_3$	182.1	136.0	6	90
*Tyr	$^{13}C_9H_{11}{}^{15}NO_3$	192.1	145.0		
Ile	$C_6H_{13}NO_2$	132.1	86.1	6	60
*Ile	$^{13}C_6H_{13}NO_2$	138.1	91.0		
Leu	$C_6H_{13}NO_2$	132.1	86.1	6	60
*Leu	$^{13}C_6H_{13}NO_2$	138.1	91.0		
Phe	$C_9H_{11}NO_2$	166.1	120.0	6	70
*Phe	$^{13}C_9H_{11}{}^{15}NO_2$	176.1	129.0		
His	$C_6H_9N_3O_2$	156.1	110.0	6	90
*His	$^{13}C_6H_9N_3O_2$	162.1	115.0		
Lys	$C_6H_{14}N_2O_2$	147.1	84.1	10	80
*Lys	$^{13}C_6H_{14}N_2O_2$	153.1	89.0		
Arg	$C_6H_{14}N_4O_2$	175.1	70.1	22	90
*Arg	$^{13}C_6H_{14}{}^{15}N_4O_2$	185.1	75.0		

CE collision energy
The amino acids are listed in their chromatographic retention order, from first to elute to the last amino acid retained.

Table 2
Typical measurement CVs observed for peak area ratios of unlabeled-to-labeled amino acids at an approximate concentration of 5 μmol/L using isotope dilution LC-MS/MS ($N = 10$ replicate injection)

Amino acid	Measurement CV (%)
Alanine	0.6
Arginine	0.2
Aspartic acid	0.7
Glutamic acid	0.6
Histidine	0.6
Isoleucine	0.5
Leucine	0.7
Lysine	0.5
Methionine	0.3
Phenylalanine	0.2
Proline	0.4
Serine	0.3
Threonine	0.6
Tyrosine	0.4
Valine	0.2

calculated and subjected to statistical evaluation. The repeatability of measured unlabeled-to-labeled amino acid peak area ratios using this LC-MS/MS method are listed in Table 2.

5. Figure 1 shows the separation of 15 amino acids using this LC-MS/MS method.

4 Notes

1. Certain commercial equipment, instruments, or materials are identified in this chapter 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.
2. Solutions containing trifluoroacetic acid have limited stability, and the breakdown products can increase the chemical background seen in mass spectrometry. Because of this, mobile phases containing trifluoroacetic acid are prepared each week.

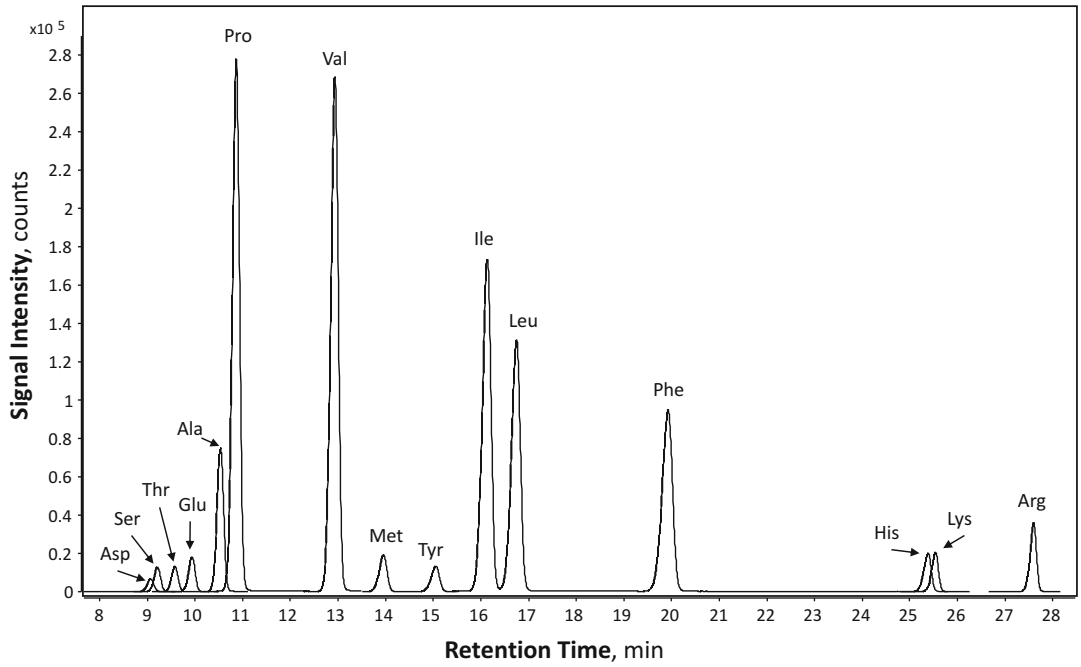

Fig. 1 Total ion chromatograms for the isotope dilution LC-MS/MS measurements of amino acids grouped into four sets: (1) L-proline, L-valine, L-leucine, L-isoleucine, and L-phenylalanine; (2) L-aspartic acid, L-serine, L-tyrosine, L-lysine; (3) L-threonine, L-alanine, L-methionine, L-arginine; (4) L-glutamic acid, L-histidine

3. When choosing an isotopically labeled internal standard for isotope dilution mass spectrometry, we aim to have the labeled internal standard to be at least 3 Da greater in molecular mass than the unlabeled analyte. When there is at least a 3 Da mass difference, there will be minimal overlap of the isotopic distribution of the unlabeled analyte with the monoisotopic peak of the labeled internal standard; with greater mass difference, the overlap will be even less. Isotopically labeled internal standards containing ^{13}C and/or ^{15}N are preferable to those which are labeled with deuterium. Deuterium-labeled molecules may occasionally have slightly different chromatographic retention times than the unlabeled molecule which can impact quantification by isotope dilution.
4. The purity assessment performed by the manufacturer of small organic molecules, such as amino acids, is generally reliable. We have seen occasional discrepancies between the results of purity assessments performed at NIST and those of manufacturers which is what prompts us to perform in-house purity measurements when we are striving for high measurement accuracy. For the amino acids, the measurements performed at NIST for L-lysine indicated slightly lower purities than those reported by the manufacturer.

5. The success of the isotope dilution LC-MS/MS method discussed in this chapter to quantify proteins and peptides depends significantly on the successful hydrolysis of the protein or peptide into constituent amino acids. Incomplete hydrolysis or the degradation of amino acids during hydrolysis will adversely affect the accuracy of quantification by amino acid analysis. In our experience, the hydrolysis conditions must be validated for each protein or peptide analyte. It is important to note that for quantification of proteins or peptide by amino acid analysis, we choose to quantify only the amino acids that display stability under routine gas-phase hydrochloric acid hydrolysis conditions (110 °C for 48 h). These amino acids are alanine, arginine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, and valine.

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