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Introducing AAA-MS, a Rapid and Sensitive Method for Amino Acid Analysis Using Isotope Dilution and High-Resolution Mass Spectrometry

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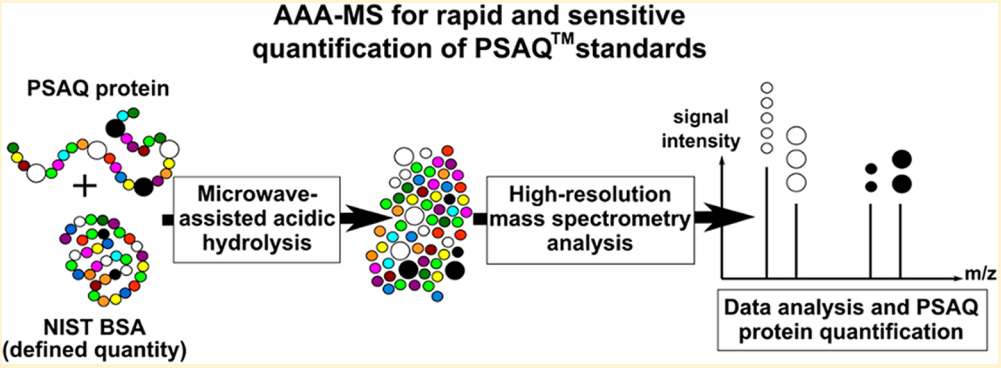
Supporting Information



ABSTRACT: Accurate quantification of pure peptides and proteins is essential for biotechnology, clinical chemistry, proteomics, and systems biology. The reference method to quantify peptides and proteins is amino acid analysis (AAA). This consists of an acidic hydrolysis followed by chromatographic separation and spectrophotometric detection of amino acids. Although widely used, this method displays some limitations, in particular the need for large amounts of starting material. Driven by the need to quantify isotope-dilution standards used for absolute quantitative proteomics, particularly stable isotope-labeled (SIL) peptides and PSAQ proteins, we developed a new AAA assay (AAA-MS). This method requires neither derivatization nor chromatographic separation of amino acids. It is based on rapid microwave-assisted acidic hydrolysis followed by high-resolution mass spectrometry analysis of amino acids. Quantification is performed by comparing MS signals from labeled amino acids (SIL peptide- and PSAQ-derived) with those of unlabeled amino acids originating from co-hydrolyzed NIST standard reference materials. For both SIL peptides and PSAQ standards, AAA-MS quantification results were consistent with classical AAA measurements. Compared to AAA assay, AAA-MS was much faster and was 100-fold more sensitive for peptide and protein quantification. Finally, thanks to the development of a labeled protein standard, we also extended AAA-MS analysis to the quantification of unlabeled proteins.

KEYWORDS: *amino acid analysis, quantification, protein, stable isotope-labeled peptide, PSAQ*

INTRODUCTION

Over the past decade, absolute protein quantification based on isotope dilution and mass spectrometry (MS) analysis has become widely used, in particular for biomarker and systems biology applications. In complex biological samples, absolute protein quantification generally involves trypsin digestion followed by LC−MS based analysis of signature peptides derived from the proteins of interest. Stable isotope-labeled (SIL) standards are used to quantify the proteins of interest. These can be peptides (SIL peptides), concatemers of peptides (QconCAT), or full-length proteins (PSAQ).[1,2] The analytical performance of assays using these standards depends closely on standard quality. Critical features for highly reliable quantification include the degree of purity, the isotope incorporation yield, and how accurately standards were quantified initially.[3] Other factors have also recently been shown to significantly influence quantification accuracy. These include stability of the standard over time, its solubilization properties, and its behavior during sample processing and digestion.[4−6]

The reference method for calibration of these isotope-labeled standards is amino acid analysis (AAA), which is based on determining the amino acid composition of polypeptides. AAA is widely used in the biotechnology industry for protein and

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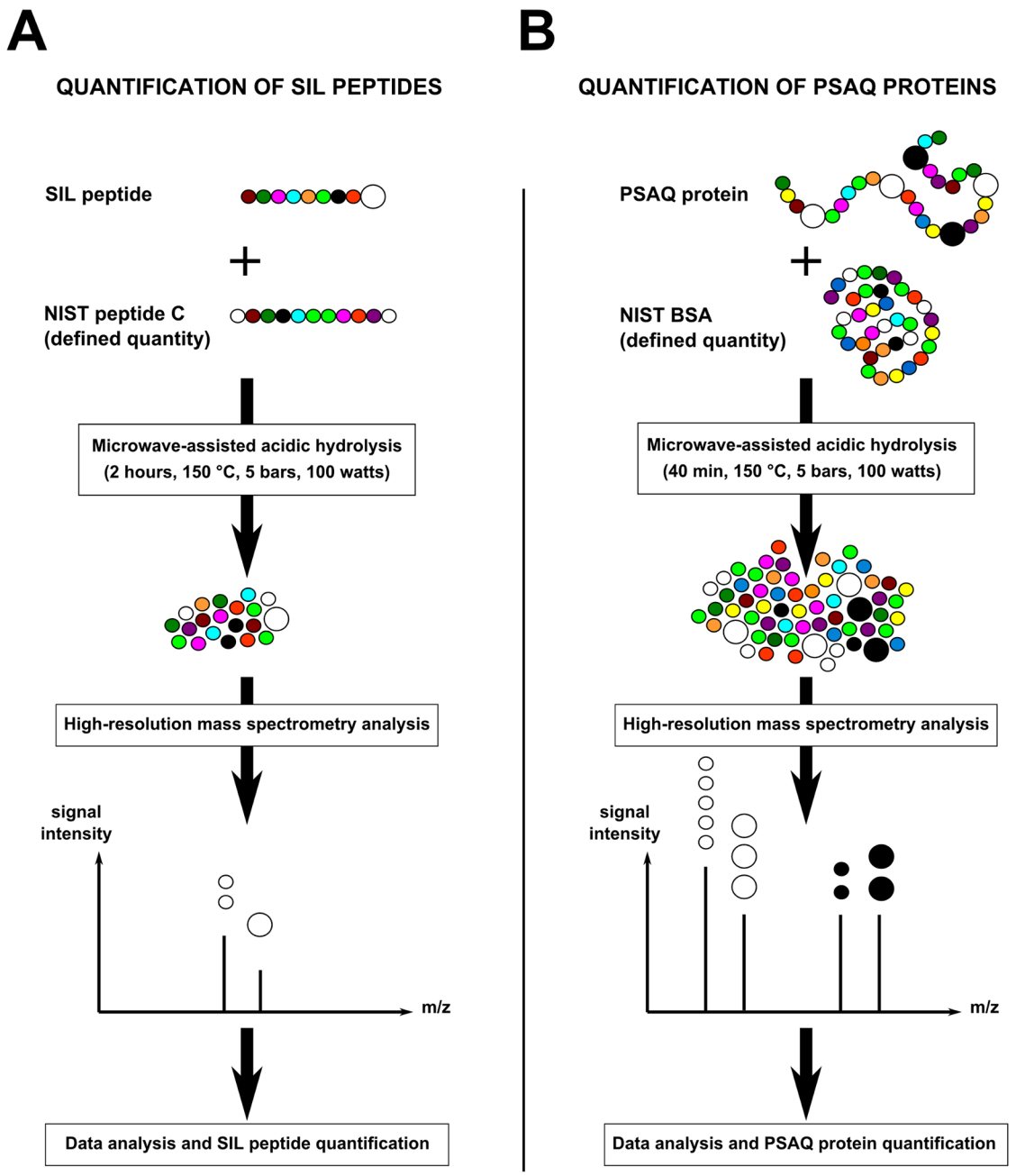

Figure 1. Schematic representation of the AAA-MS method. (A) AAA-MS quantification of SIL peptides. The SIL peptide to be quantified is mixed with defined amounts of NIST peptide C. After microwave-assisted hydrolysis, amino acids are analyzed directly by high-resolution MS. MS signals for the labeled amino acid (from SIL peptide) and its unlabeled version (from peptide C) are compared, their ratio is calculated, and the amount of hydrolyzed SIL peptide is calculated on the basis of this ratio. (B) AAA-MS quantification of PSAQ standards. The PSAQ standard to be quantified is mixed with defined amounts of NIST BSA standard. After microwave-assisted hydrolysis, amino acids are analyzed directly by high-resolution mass spectrometry. MS signals for labeled arginine and lysine (from PSAQ standard) are compared to those for unlabeled arginine and lysine (from NIST BSA). The corresponding ratios are used to calculate the amount of hydrolyzed PSAQ standard.

peptide quantification. The availability of international reference materials and standards makes this method highly reliable, and it is recommended by FDA and EMEA for bioproduct characterization (links to FDA and EMEA guidelines provided as Supporting Information). AAA is a a multistep analytical procedure consisting of (i) acid hydrolysis of peptides or proteins into amino acids, (ii) amino acid separation using liquid or gas chromatography, (iii) amino acid derivatization (which can be performed before or after chromatography), and (iv) spectrophotometric detection.[7] Conventional AAA assays are time-consuming (24 h) and require relatively large amounts of purified starting material (typically around 100 μg for a single protein analysis). In addition, the starting material must be solubilized in an appropriate buffer. Recently, AAA assays not requiring derivatization were developed, using MS to detect and quantify amino acid residues. These methods considerably enhanced sensitivity, reducing initial sample needs to a few nanograms for a single analysis.[3,8−10]

In 2007, we published the protein standard absolute quantification (PSAQ) strategy, which uses full-length isotope-labeled proteins as internal standards.[5] As throughput for PSAQ standard production increased, a need for rapid,

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sensitive, and reliable quantification of these proteins emerged. We therefore developed a derivatization-free and chromatography-free AAA assay to quantify our polypeptides. This method harnesses the capabilities of high-resolution mass spectrometers to discriminate between amino acid residues, eliminating the need for prior chromatographic separation. Assay speed was also increased by optimizing a microwave-assisted acidic hydrolysis step.[11] We have called this method AAA-MS. It was initially developed to calibrate purified SIL peptides and PSAQ standards, both of which are widely used in our laboratory (Figure 1), and has recently been extended to the quantification of purified unlabeled proteins.

EXPERIMENTAL SECTION

Chemical and Reagents

Bovine serum albumin (BSA) standard reference material 927d and "Peptide Reference Material for Molecular Mass and Purity Measurements" were obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). The latter is a standard reference material (SRM 2397), consisting of three peptides including peptide C (RQAKVLLYSGR). Labeled amino acids, i.e., [$^{13}C_6$,$^{15}N_4$] L-arginine, [$^{13}C_6$,$^{15}N_2$] L-lysine, [$^{13}C_6$,^{15}N] L-leucine, and [$^{13}C_9$] L-phenylalanine were obtained from Eurisotop (Saint-Aubin, France). Lyophilized SIL peptides were from Sigma Aldrich (Saint Quentin Fallavier, France), and solubilized SIL peptides were supplied by Thermo Fisher Scientific (Illkirch, France). Staphylococcal enterotoxin A (SEA), human creatine kinase M (CKM), and human creatine kinase B (CKB) PSAQ standards were produced in our laboratory as previously described.[5,12] These PSAQ standards were quantified using reference AAA analysis (SGS MScan, Plan les Ouates, Switzerland). BSA and human growth hormone (hGH) standards for pure unlabeled protein quantification were obtained from Thermo Fisher Scientific and NIBSC (Hertfordshire, United Kingdom), respectively. HCl 6 N used for acidic hydrolysis was purchased from Interchim (Montluçon, France). Phenol was obtained from Sigma Aldrich.

Microwave-Assisted Hydrolysis

Before acidic hydrolysis, 0.3 mL glass tubes (Thermo Fisher Scientific, reference W3843K) were pyrolyzed at 300 °C for 1 h. Peptide and protein samples were solubilized, transferred to vials, and spiked with defined amounts of NIST standards (peptide C or BSA) before drying in a vacuum centrifuge. Microwave-assisted acidic hydrolysis was performed using the Discover protein hydrolysis apparatus (CEM, Matthews, NC, USA), hydrolyzing up to 10 samples in parallel. Briefly, 10 mL of HCl 6 N was mixed with 0.1 mL of melted phenol and placed at the bottom of the reactor. Samples were then loaded into the vessel for hydrolysis. The reactor was sealed, and samples were hydrolyzed for the times indicated at 150 °C, at a pressure of 5 bar, with maximum microwave power of 100 W. For titration curves with labeled amino acid standards, samples were spiked with defined quantities of labeled amino acids after hydrolysis. Samples were dried in a vacuum centrifuge before MS analysis.

Mass Spectrometry Analysis

Samples were solubilized in 40 μL of 50% acetonitrile, 0.1% formic acid. The autosampler from an HPLC-Ultimate 3000 (Dionex, Voisins Le Bretonneux, France) was used to inject 10 μL of each sample (in a 20 μL injection loop) at a flow rate of 6 μL/min (mobile phase: 50% acetonitrile, 0.1% formic acid) directly into the nanosource of an LTQ-Orbitrap XL (Thermo Fisher Scientific). The nanoESI interface consisted of an uncoated PicoTip emitter (model FS360-50-30-N-20-C12, New Objective, Woburn, MA, USA). The mass spectrometer was operated in MS mode over a 70−400 m/z range to detect single-charged amino acids and diamino acids. Resolution was set to 60,000 (at m/z 400) except when labeled arginase-1 was used as standard. In this case, resolution was set to 100,000 to make it possible to distinguish between unlabeled arginine (m/z = 175.1189) and labeled [$^{13}C_9$] phenylalanine (m/z = 175.1164). MS signal was recorded for 6 min.

Data Analysis

Data analysis was performed using Xcalibur software (Thermo Fisher Scientific). MS signals from amino acids of interest (labeled and unlabeled versions) were extracted from the data file using the Qual Browser application. The mass window for data extraction was ±5 ppm. For each amino acid, MS signal intensity was averaged over 1 min, which corresponds to 69 scans at 60,000 resolution or 37 scans at 100,000 resolution. The number of residues of each amino acid in the standard and the analyte (peptide or protein) were taken into consideration in quantity calculations.

Synthesis of the Protein Standard

Isotopically labeled arginase-1 was synthesized as previously described.[13] Briefly, a liver cDNA library (Biochain Institute, Hayward, CA, USA) was used to amplify the *ARG1* gene by PCR. The PCR product was cloned into the pIVEX 2.4d expression vector (5 Prime, Hamburg, Germany) using the In-Fusion PCR cloning system (Clontech, Saint Germain en Laye, France). The pIVEX 2.4d vector provides a cleavable N-terminal hexahistidine purification tag. Recombinant plasmid was cloned into XL1-Blue cells (Agilent Technologies, Massy, France), purified, and sequenced (Cogenics, Meylan, France). Purified recombinant plasmid was used as a template for cell-free protein expression, performed using the RTS 500 Proteomaster *E. coli* HY kit (5 Prime). Isotope-labeling was achieved by adding [$^{13}C_9$] L-phenylalanine (Eurisotop) to the reaction mixture. The pIVEX 2.4d vector provides a cleavable N-terminal hexahistidine purification tag, which was used to purify labeled arginase-1 on a nickel affinity column (Ni Sepharose 6 Fast Flow resin, GE Healthcare, Orsay, France). Arginase-1 was eluted using an imidazole gradient. Purity was checked by SDS-PAGE and Coomassie staining (>95% purity). N-Terminal hexahistidine purification tag was removed using factor Xa (Novagen) according to the manufacturer's instructions. Isotope incorporation was verified by LC−MS analysis and was estimated to be greater than 99% (see Supporting Information). Isotopically labeled arginase-1 was quantified using the reference AAA method (SGS MScan).

RESULTS AND DISCUSSION

Quantification of Stable Isotope-Labeled (SIL) Peptide Standards

SIL peptides are widely used in proteomics for absolute protein quantification. These standards generally correspond to tryptic peptides, isotopically labeled on the C-terminal-arginine or -lysine residue. Isotope labeling of core amino acids such as leucine or valine is also frequent. To develop our AAA assay, we selected a model peptide from the NIST "Peptide Reference Material for Molecular Mass and Purity Measurements". NIST

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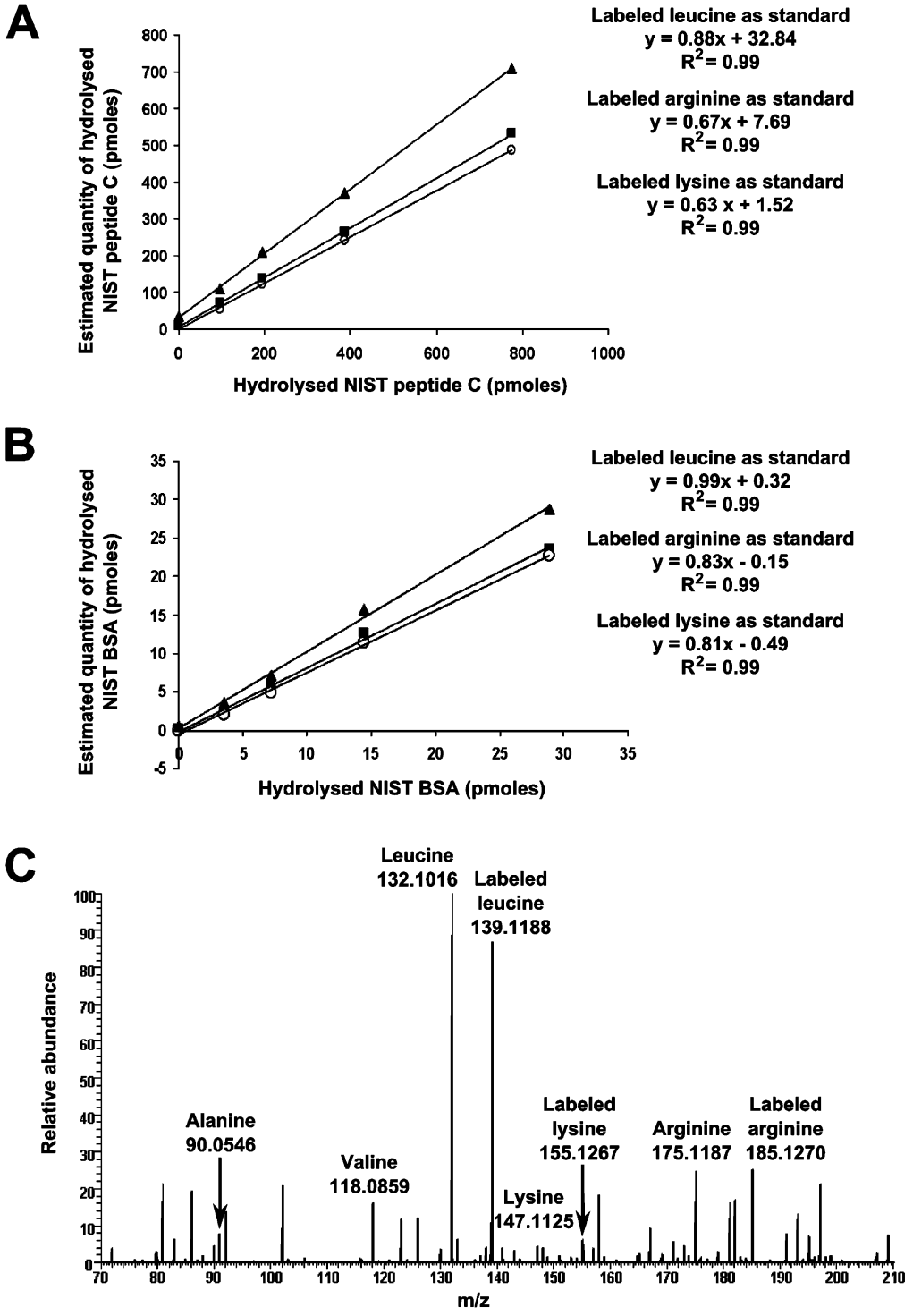

Figure 2. Titration using AAA-MS and isotope-labeled amino acids as standards. NIST peptide C or NIST BSA were hydrolyzed before adding labeled amino acids in defined quantities and analyzing samples by MS. Titration curves were designed with five points, each in duplicate, to take advantage of the 10 sample spaces in the microreactor. Each point on the graphs represents the mean from two replicates. (A) NIST peptide C titration with [$^{13}C_6$,$^{15}N_4$] L-arginine, [$^{13}C_6$,$^{15}N_2$] L-lysine, and [$^{13}C_6$,^{15}N] L-leucine. (B) NIST BSA titration with [$^{13}C_6$,$^{15}N_4$] L-arginine, [$^{13}C_6$,$^{15}N_2$] L-lysine, and [$^{13}C_6$,^{15}N] L-leucine. (C) High-resolution MS analysis of hydrolyzed BSA spiked with [$^{13}C_6$,$^{15}N_4$] L-arginine, [$^{13}C_6$,$^{15}N_2$] L-lysine, and [$^{13}C_6$,^{15}N] L-leucine. Monocharged amino acid signals, including those of labeled/unlabeled amino acid pairs, can be observed on the mass spectrum.

peptide C (RQAKVLLYSGR) was chosen as it shares many features with tryptic peptides, including length (11 amino acids) and an arginine residue at the C-terminal extremity. The sequence also includes amino acids that are frequently labeled in SIL peptides (i.e., K, L, V).

As the first step in developing our assay, hydrolysis conditions for peptide C were optimized for time, temperature, and pressure on a range of peptide quantities (0.125 to 1 μg corresponding to 97 to 775 pmol of peptide C). Isotopically labeled amino acids ([$^{13}C_6$,$^{15}N_4$] L-arginine, [$^{13}C_6$,$^{15}N_2$] L-lysine, and [$^{13}C_6$,^{15}N] L-leucine) were added to the samples in

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defined amounts before high-resolution MS analysis and were used to quantify the peptide. The presence of diamino acids, in particular leucine−leucine and leucine−valine pairs that tend to resist acidic hydrolysis,[7] was checked as a measure of hydrolysis completeness. As experiments were performed on a range of peptide quantities, it was possible to calculate a titration curve for peptide hydrolysis, comparing quantity added to quantity measured (Figure 2A). On the basis of these titration curves, we determined the hydrolysis conditions providing the best quantification performances over the entire range of peptide C quantities: 2 h at 150 °C, at a pressure of 5 bar and a maximum microwave power of 100 W.

We then used peptide C as a quantification standard to assay several SIL peptides (Figure 1A). SIL peptides from two different sources were supplied, either in lyophilized or solubilized form. Both suppliers provide data on classical AAA analysis for each SIL peptide. These data were compared to results obtained with our AAA-MS assay. After mixing the SIL peptide (500 pmol) with NIST peptide C (250 pmol) and acidic hydrolysis, MS signals for the unlabeled (peptide C-derived) and labeled amino acids (SIL peptide-derived) were compared. Based on the amino acid composition of the two peptides, the corresponding peptide quantity was deduced (Table 1). Results show that AAA-MS data correlate well with reference AAA results for lyophilized SIL peptides (labeled on leucine) and for SIL peptides delivered in solution (labeled on leucine or valine). Thanks to the use of microwaves to promote hydrolysis, up to 10 peptides can be hydrolyzed in 2 h (instead of 24 h for classical hydrolysis) and the whole experiment can be performed in less than 4 h.

In summary, our AAA-MS assay allows rapid and accurate quantification of SIL peptides using just 500 pmol of starting material. This quantity could be further reduced as only 125 pmol of peptide (1/8 of a 1 nmol vial, the quantity generally provided) was actually necessary for MS quantification. In the future, developing an accurately quantified standard peptide combining both unlabeled amino acids (typically R, K, L and V, which are frequently labeled in SIL standards) and labeled amino acids (alanine or glycine) would improve the robustness of SIL peptide quantification by making it possible to assess several amino acid pairs.

Table 1. SIL Peptide Quantification by AAA-MS

SIL peptide[a]	presentation	quantity used for hydrolysis[b] (pmol)	quantity measured by AAA-MS[c] (pmol) mean[d]	CV
L*GNYDNVR	lyophilized	500	590	0.19
QNTVPL*ETVK	lyophilized	500	798	0.04
VL*YDDNHVSAINVK	lyophilized	500	636	0.03[e]
L*PTPIELPLK	lyophilized	500	509	0.09
NVTVQEL*DLQAR	lyophilized	500	443	0.24
QNTVPL*ETVK	solubilized	500	598	0.02
GDL*SAFGAFFK	solubilized	500	490	0.11
SEFAYGSFV*R	solubilized	500	645	0.01
TVSL*PVGADEDDIK	solubilized	500	669	0.03[e]
VGDEVEI*VGIR	solubilized	500	447	0.03[e]

[a]Labeled amino acid is indicated with an asterisk. [b]Based on supplier information. [c]Using NIST peptide C as reference. [d]n = 3 full-process technical replicates. [e]Only two out of three technical replicates were analyzed because of technical problems during hydrolysis (condensation).

Quantification of Stable Isotope-Labeled Protein Standards (PSAQ Standards)

PSAQ is another type of standard with particular advantages for absolute quantification of biomarkers. These standards are full-length isotope-labeled proteins that are added to the samples to be quantified at an early stage, thus allowing highly accurate quantification.[5] For accurate quantification of biomarkers the typical quantities of PSAQ standard added to samples are in the 1−100 ng range. This is in stark contrast with the amounts required for calibration by reference AAA (50−100 μg). To help redress the balance, we developed an AAA-MS assay adapted to the quantification of PSAQ standards, which would require less than 1 μg of protein per analysis.

Hydrolysis and MS analysis conditions for proteins were optimized using NIST BSA as standard. A range of BSA quantities (0.25−2 μg corresponding to 3.61−28.86 pmol) were hydrolyzed under different temperature and pressure conditions, with various reaction times (see Supporting Information). SDS-PAGE and silver staining were used to assess completeness of hydrolysis (data not shown). MS analysis was used to detect any remaining diamino acids, in particular leucine−leucine and leucine−valine pairs. BSA was quantified by adding labeled amino acids to samples before MS analysis. For BSA, optimal hydrolysis conditions were 40 min at 150 °C at a pressure of 5 bar and 100 W maximum microwave power. These conditions allowed complete hydrolysis, and quantification was accurate over the quantity range tested (Figure 2B and C).

This method was then applied for the quantification of PSAQ standards (Figure 1B). Three different PSAQ standards that

Table 2. PSAQ Standard Quantification by AAA-MS

PSAQ standard	UniProt accession no.	estimated concn of PSAQ standard solution using AAA-MS (NIST BSA used as ref) (mg/mL) [1] based on labeled and unlabeled lysine MS signals[a] mean	CV	[2] based on labeled and unlabeled arginine MS signals[a] mean	CV	mean of [1] and [2]	concn of PSAQ standard solution by classical AAA analysis (mg/mL)[b]
Staphylococcal enterotoxin A	P0A0L2	0.64	0.04	0.58	0.07	0.61	0.68
Human creatine kinase M	P06732	0.28	0.04	0.34	0.08	0.31	0.39
Human creatine kinase B	P12277	0.50	0.06	0.55	0.05	0.53	0.45

[a]n = 4 full-process technical replicates. [b]For reasons of cost, a single classical AAA analysis was performed.

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had been previously calibrated by reference AAA were used. Each PSAQ protein (0.5 μg) was mixed with NIST BSA (1 μg). Samples were hydrolyzed in the optimal conditions for proteins, and the hydrolysate was analyzed by high-resolution MS. The signals corresponding to labeled (PSAQ-derived) and unlabeled (BSA-derived) arginine and leucine were analyzed to deduce the relative protein quantities. Quantification results with AAA-MS were consistent with those obtained with the classical AAA procedure (Table 2). AAA-MS hydrolysis was performed with 0.5 μg of PSAQ as this quantity corresponded to a volume that can be reproducibly pipetted while allowing complete hydrolysis. However, only 1/20 of the corresponding hydrolysate (25 ng of a 28 kDa protein) was needed for MS analysis. Thus, by adapting the method to allow for manipulation of smaller volumes of dilute samples, the quantities necessary for small-scale hydrolysis experiments could be further reduced. Microwave-assisted hydrolysis was completed in 40 min, meaning the whole quantification assay could be performed in less than 3 h (for up to 10 samples).

In summary, AAA-MS provides accurate quantification results, significantly speeds analysis, and dramatically lowers the quantities of proteins necessary for absolute quantification. Considering that quantification is generally performed in replicate for optimal reliability, this drastically reduces costs. Interestingly, AAA-MS with NIST BSA as reference can also be used for calibration of QconCAT,[2] PrEST,[14] FLEXIQuant[15] standards and ^{15}N uniformely labeled proteins,[16,17] increasingly used for quantitative proteomics experiments.

Quantification of Pure Unlabeled Proteins

We next extended this analytical procedure for the quantification of unlabeled and purified proteins, such as recombinant proteins. To reach this goal, a labeled protein was developed as quantification standard. As most of the proteins produced in our laboratory are human proteins, this protein quantification standard was designed with an amino acid composition close to that of human proteins. We chose arginase-1, which has an appropriate amino acid composition and can be easily synthesized in a cell-free system. The labeling strategy was chosen with care to make this standard suitable for the quantification of unlabeled proteins but also PSAQ standards. Thus, arginase-1 was labeled on phenylalanine, which is not sensitive to acid hydrolysis[7] and is not metabolically transformed to other types of amino acids in cell-free expression systems. The arginase-1 standard was synthesized and isotopically labeled using a cell-free expression; it was extensively purified before quantification by classical AAA analysis. Then, it was used to titrate NIST BSA standard over a 0.25−2 μg range (Figure 3). The hydrolysis and MS analysis conditions were similar to those described for whole proteins. As shown in Figure 3, phenylalanine MS signal allowed accurate quantification of NIST BSA.

We therefore used labeled arginase-1 to quantify unlabeled proteins. Pure unlabeled proteins included BSA standard from Thermo Fisher Scientific and hGH standard from NIBSC. A third protein was also quantified, the unlabeled form of staphylococcal enterotoxin A (SEA), which was produced and purified in the laboratory and was also quantified by classical AAA. The quantification results obtained using AAA-MS with phenylalanine MS signals correlated well with the classical AAA data for SEA and hGH proteins (Table 3). However, for BSA, AAA-MS estimated a concentration 25% lower than that

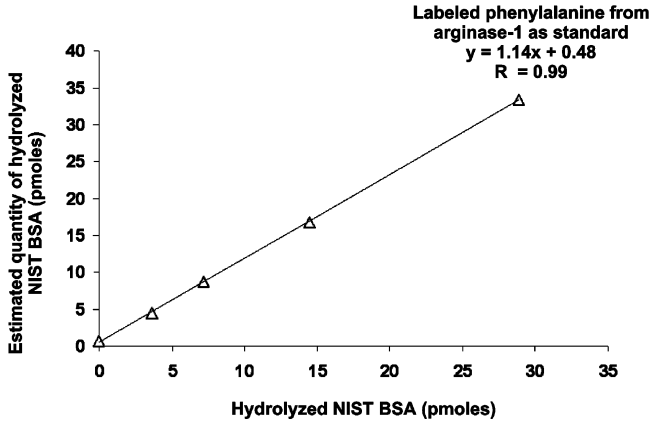

Figure 3. Titration using AAA-MS and isotope-labeled arginase-1 as standard. Arginase-1 was expressed and isotopically labeled with [$^{13}C_9$] L-phenylalanine. After purification and calibration by classical AAA analysis, it was used as a standard to titrate NIST BSA. The titration curve was designed with 5 points, each in duplicate, to take advantage of the 10 sample spaces in the microreactor. Each point on the graph represents the mean from two replicates.

indicated by the manufacturer (determined by an undisclosed method).

Labeled arginase-1 was also used as a reference standard to quantify PSAQ standards. Here, quantification relied on phenylalanine MS signal (labeled in arginase-1 and unlabeled in PSAQ standards) and also on arginine and lysine MS signals (unlabeled in arginase-1 and labeled in PSAQ standards). AAA-MS quantification of PSAQ standards once again correlated well with classical AAA data (Table 3).

In summary, AAA-MS combined with the use of a protein standard, labeled on phenylalanine residues, allows accurate quantification of purified proteins (natural or recombinant). Because it is labeled on phenylalanine, it can also be applied with PSAQ standards. Undoubtedly, the design of a protein standard labeled on phenylalanine and other residues (arginine, lysine, proline, glycine) could further improve the robustness of unlabeled protein quantification. This will be of interest in controlling the quality of protein reagents in the biotechnology and pharmaceutical industries. Another specific advantage of AAA-MS over classical AAA analysis is that AAA-MS shows a wider compatibility with buffers used for protein solubilization. For example, buffers containing Tris or detergents (such as SDS), which cannot be used for classical AAA analysis due to interference with derivatization or chromatography steps, pose no problems with AAA-MS. However, reagents that are not compatible with acidic hydrolysis (i.e., glycerol, trehalose, etc.) remain prohibited.

■ CONCLUSIONS

In 2004, Mirgorodskaya and co-workers developed an AAA assay for absolute protein quantification based on MALDI-ToF analysis and isotope dilution with labeled amino acids.[18] This assay was highly sensitive and highly accurate, and therefore we used it as a starting point in developing our AAA-MS assay. Like Mirgorodskaya et al., we circumvented amino acid derivatization and chromatography, but unlike them, we chose ESI rather than MALDI as ionization mechanism. This was because we wished to simultaneously detect lysine and arginine residues so as to robustly quantify PSAQ standards. Lysine and arginine cannot be detected using the same matrix

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Table 3. Purified Unlabeled Protein and PSAQ Protein Quantification by AAA-MS Using Labeled Arginase-1 as Standard

			estimated quantity of hydrolyzed PSAQ using AAA-MS (labeled arginase-1 used as ref) (mg/mL)							
			[1] based on labeled and unlabeled phenylalanine MS signals[a]		[2] based on labeled and unlabeled lysine MS signals[a]		[3] based on labeled and unlabeled arginine MS signals[a]			
protein name	type of protein	UniProt accession no.	mean	CV	mean	CV	mean	CV	mean of [1], [2] and [3]	quantity of hydrolyzed protein by classical AAA analysis (mg/mL)[b]
BSA (from NIST)	unlabeled protein	P02769	2.37	0.02					2.37	2.00
BSA (from Thermo Fisher Scientific)	unlabeled protein	P02769	1.52	0.09					1.52	2.00[c]
hGH (from NIBSC)	unlabeled protein	P01241	0.53	0.11					0.53	0.49
Staphylococcal enterotoxin A	unlabeled protein	P0A0L2	0.36	0.08					0.36	0.37
Human creatine kinase M	PSAQ standard	P06732	0.33	0.04	0.26	0.15	0.21	0.09	0.27	0.39
Human creatine kinase B	PSAQ standard	P12277	0.46	0.05	0.34	0.12	0.35	0.17	0.38	0.45

[a] n = 4 full-process technical replicates. [b] For reasons of cost, a single classical AAA analysis was performed. [c] BSA standard stock solution was calibrated by the manufacturer by an undisclosed method.

with MALDI, due to interferences from matrix components.[18] Thus, we performed high-resolution mass spectrometry using an OrbiTrap mass spectrometer and ESI to simultaneously detect all amino acids, including amino acids with similar m/z. Another difference between our assay and Mirgorodskaya's related to the standards used for quantification; we used NIST peptide C, NIST BSA, and labeled arginase-1 rather than labeled amino acids. These standards were added to samples before acidic hydrolysis, as early as possible during the analytical procedure. Finally, data from our AAA-MS was compared to data provided by reference AAA.

We demonstrated that the AAA-MS method is as accurate as the reference method for protein and peptide quantification, AAA. AAA-MS requires tiny amounts of starting purified material and is less affected by buffer composition. Although conventional acidic hydrolysis (i.e., protein hydrolysis with 6 M HCl at 110 °C during 24 h after vacuum removal of air) can be performed,[7] the specific use of microwave-assisted hydrolysis allows completing AAA-MS in a much shorter time frame. AAA-MS was initially developed for the quantification of isotope-dilution standards (SIL peptides and PSAQ proteins) widely used for quantitative proteomics experiments. In this domain, the use of AAA-MS will significantly lower the cost of standard calibration. Thanks to development of a labeled protein serving as reference, we also demonstrated the interest of AAA-MS for quantification of purified proteins (natural or recombinant). This will be of interest to the biotechnology and pharmaceutical industry, where protein reagent quality must be closely monitored.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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