Research Article

Rapid Communications in Mass Spectrometry

Received: 11 September 2011 Revised: 12 January 2012 Accepted: 16 February 2012 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2012**, *26*, 1165–1174
(wileyonlinelibrary.com) DOI: 10.1002/rcm.6204

Determination of isotopic labeling of proteins by precursor ion scanning liquid chromatography/tandem mass spectrometry of derivatized amino acids applied to nuclear magnetic resonance studies

André LeBlanc[1], Alexandre A. Arnold[1], Bertrand Genard[2], Jean-Bruno Nadalini[2], Marc-Olivier Séguin Heine[1], Isabelle Marcotte[1], Réjean Tremblay[2] and Lekha Sleno[1]*

[1]Université du Québec à Montréal, Chemistry Department, Montréal, QC, Canada
[2]Université du Québec à Rimouski, Institut des sciences de la mer de Rimouski (ISMER), Rimouski, QC, Canada

RATIONALE: A method has been developed for the quantitation of isotopic labeling of proteins using liquid chromatography/tandem mass spectrometry (LC/MS/MS) for the application of protein nuclear magnetic resonance (NMR) studies. NMR relies on specific isotopic nuclei, such as ^{13}C and ^{15}N, for detection and, therefore, isotopic labeling is an important sample preparation step prior to in-depth structural characterization of proteins. The goal of this study was to develop a robust quantitative assay for assessing isotopic labeling in proteins while retaining information on the extent of labeling for individual amino acids.

METHODS: Complete digestion of proteins by acid hydrolysis was followed by derivatization of free amino acids with 6-aminoquinolyl *N*-hydroxysuccinimidyl carbamate (AQC) forming derivatives having identical MS/MS fragmentation behavior. Precursor ion scanning on a hybrid quadrupole-linear ion trap platform was used for amino acid analysis and determining isotopic labeling of proteins.

RESULTS: Using a set of isotope-labeled amino acid standards mixed with their unlabeled counterparts, the method was validated for accurately measuring % isotopic contribution. We then applied the method for determining the ^{13}C isotopic content of algal proteins during a feeding study using $^{13}C_6$-glucose- or ^{13}C-bicarbonate-supplemented culture media as well as the level of labeling in mussel byssal threads obtained after feeding with labeled algae.

CONCLUSIONS: This method is ideally suited for assessing the extent of protein labeling prior to NMR studies, where the isotopic labeling is a determining factor in the quality of resulting protein spectra, and can be applied to a multitude of different biological samples.

Protein fibers such as silk, collagen or mussel byssal threads are complex biopolymers which have unique mechanical properties exquisitely tuned to the tasks that they perform.[1,2] These macroscopic properties are a consequence of the structure of these proteins at a molecular level. It is thus desirable to unravel their structure in order to produce bio-inspired synthetic materials, which can be achieved using solid-state nuclear magnetic resonance (SS-NMR). The major drawback of NMR is its lack of sensitivity, a weakness which can be offset by isotopic labeling. This strategy is routinely used in the study of large soluble proteins and becomes a compulsory step in the study of insoluble proteins such as silk or mussel byssal threads by high-resolution SS-NMR of carbon or nitrogen nuclei. The natural abundances of the NMR-active isotopes of these nuclei (^{13}C and ^{15}N) are only 1.1 and 0.37%, respectively, thus leading to very weak NMR signals. Isotopic enrichment not only directly increases the signal-to-noise ratio of the NMR spectrum; it has also a much more marked effect on the number of NMR acquisitions needed for a similar quality spectrum. A 10-fold increase in isotopic enrichment, for example, is thus equivalent to a 100-fold increase in the number of averaged acquisitions. This reduction in acquisition time becomes a critical step when performing the two-dimensional NMR experiments required for the structural study of proteins. For these types of experiments, a 10-fold increase in isotopic labeling will reduce the total experimental time to a couple of days and the experiments thus become feasible.

In the case of silks or byssal threads, animals are fed a labeled diet to incorporate stable isotopes into the protein fibers. In order to optimize the fiber labeling, the efficiency and kinetics of the transfer between labeled food to the secreted fibers must first be determined. Experimentally, these two parameters (efficiency and kinetics) appear to

* *Correspondence to:* L. Sleno, Laboratory for Bioanalytical Mass Spectrometry, Chemistry Department, Université du Québec à Montréal, C.P. 8888 Succ. Centre-Ville, Montréal, Québec, Canada, H3C 3P8.
E-mail: sleno.lekha@uqam.ca

vary with the organism species, type of food and environmental conditions.[3,4] We have seen that, in the case of mussels, the most efficient and less costly strategy consists in producing labeled microalgae which will subsequently be fed to the mussels (unpublished data). Consequently, a method for determining the isotopic labeling of total protein mass is required. To be useful for protein NMR studies, the method should be amenable to the measurement of ^{13}C or ^{15}N enrichment and allow labeling levels to be distinguished between different amino acids as well as be able to discriminate between partial and total labeling. Indeed, partially labeled amino acids can yield ambiguous NMR patterns which can lead to wrong assignments and thus render an accurate NMR analysis impossible.

Several techniques exist that can measure isotopic enrichment of molecules, including isotope ratio mass spectrometry (IRMS), gas chromatography/mass spectrometry (GC/MS) and, most recently, liquid chromatography/mass spectrometry (LC/MS). The choice of analytical method for measuring isotope enrichment is highly dependent on the needs of the experiment. In this study, in order to obtain an accurate estimate of isotopic labeling of amino acids from algal and byssal protein extracts, the main priorities for the analytical method were speed, cost effectiveness and the flexibility to determine either ^{13}C or ^{15}N enrichment. IRMS experiments yield very accurate results for isotope measurements; however, they do not allow any structural information to be extracted from the data since all molecules are transformed into combustion products prior to entering the ion source, also necessitating highly specialized instrumentation,[5] and, furthermore, would not be easily amenable for simultaneous determination of ^{15}N enrichment. GC/MS is a common technique used for isotope analysis and has often been applied to the study of amino acid isotopic enrichment.[6–9] Most GC/MS analyses use time-consuming derivatization steps in order to transform analytes into volatile species and resulting electron ionization (EI) spectra are often very difficult to interpret when many isotopomers are present at once.[10] LC/MS for isotope analysis of amino acids has previously been reported, demonstrating the power and applicability of this technique.[11–14] LC/MS is also amenable to the evaluation of the extent of labeling within a given amino acid.[15] Previous methods have relied on full scan or single ion monitoring detection. In this study, we have developed an LC/MS/MS-based method for determining isotopic labeling of proteins following acid hydrolysis and rapid amino acid derivatization. A related method has been described by Hess *et al.*[4] for the determination of isotopic enrichment in silk proteins by derivatization of hydrolyzed amino acids with Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (FDAA) and single quadrupole MS analysis, with results being shown for four amino acids (Ala, Gly, Pro, Glu). The present study, using a selective precursor ion scan for detecting 6-aminoquinolyl *N*-hydroxysuccinimidyl carbamate (AQC)-derivatized amino acids, presents data from a majority of protein amino acids and is shown for determining isotopic enrichment of both algal proteins and mussel byssus thread samples. The method allows the evaluation of feeding protocols of both the microalgae and mussels, for further structural investigation of suitably labeled mussel byssal threads by NMR.

EXPERIMENTAL

Materials

Alanine, arginine, aspartic acid, asparagine, glutamic acid, cysteine, glutamine, glycine, histidine, isoleucine, lysine (HCl salt), methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine, $^{13}C_9^{15}N$-tyrosine, $^{13}C_6$(ring-labeled)-phenylalanine, $^{13}C_5^{15}N$-methionine, $^{13}C_5^{15}N_2$-glutamine, glucose, $^{13}C_6$-glucose, di(*N*-succinimidyl) carbonate and formic acid (ultrapure) were all obtained from Sigma-Aldrich (Oakville, ON, Canada). Sodium [^{13}C]-bicarbonate was purchased from Cambridge Isotope Laboratories (Cambridge, MA, USA). Concentrated hydrochloric acid was supplied by Fisher Scientific (Ottawa, ON, Canada). Sodium tetraborate decahydrate and 6-aminoquinoline were purchased from Alfa Aesar (Ward Hill, MA, USA). HPLC grade methanol and acetonitrile were obtained from Caledon Laboratories Ltd. (Georgetown, ON, Canada). Ultrapure water was supplied by a Synergy purification system (Millipore, Billerica, Massachusetts, USA).

Production of ^{13}C-labeled microalgae

Glucose diet

Enrichment of microalgae with isotope-labeled glucose was performed using *Amphora* sp. obtained from the Centre for Culture of Marine phytoplankton (CCMP) at Bigelow Laboratory for Ocean Sciences (West Boothbay Harbor, ME, USA). Algae were cultured in seawater enriched with f/2 medium[16] at pH 8 and temperature $20.6 \pm 0.4°C$, under lighting supplied by fluorescent grow lights (intensity of 191.6 ± 9.9 μE/m^{2}s). Seawater was filtered and sterilized prior to use. A 10 mL volume of the microalgae solution was used to inoculate a 250 mL Erlenmeyer flask filled with f/2 culture media supplemented with 5 g/L glucose (50% mixture of unlabeled and $^{13}C_6$ labeled glucose). Triplicate cultures were maintained for 19 days and cell concentrations were measured each day with a Z2 Coulter counter fitted with a 70-μm orifice tube (Beckman Coulter Canada, Mississauga, ON, Canada). Stationary phase growth was achieved at day 19 and the triplicate cultures had a mean concentration of $1.38 \pm 0.24 \times 10^6$ cells/mL. Sample aliquots were removed every 2 days from day 1 to day 19, lyophilized and stored (at 4°C) prior to analysis.

Sodium bicarbonate diet

Microalgae (*Nannochloropsisocculata*) were batch-cultured at 20°C and under continuous illumination during 10 days in f/2 seawater medium[16] supplemented with 25 mM sodium [^{13}C]-bicarbonate. f/2 medium was transferred into four glass bottles (250 mL) then autoclaved with specific seawater setting to avoid salt precipitation. After the addition of labeled sodium bicarbonate, the culture system was sealed and the medium was purged with nitrogen gas to eliminate atmospheric CO_2. The culture system was built to ensure the elimination of oxygen produced by algae and avoid gas exchange with the ambient atmosphere. Culture bottles were inoculated with 10 mL of a growing culture (±20 cells/mL) using sterile syringes. Sample aliquots were taken after the 10-day culture period, lyophilized and stored (at 4°C) prior to analysis.

Production of ^{13}C-labeled mussel byssus

Mytilus edulis were fed with ^{13}C-labeled *Nannochloropsisocculata* microalgae (from bicarbonate-labeling experiment) for 2 days in a 40 L tank filled with UV-treated oxygenated seawater. Water temperature was maintained at 18°C with a salinity ratio of 26. Every 12 h, 250 mL of ^{13}C-labeled microalgae culture was added to the seawater. Subsequently, mussels were placed on a vertical polyvinyl chloride (PVC) rod using cyanoacrylate glue suspended 10 mm above a PVC plate for byssus production. Mussels were submerged in an experimental flume[17] and submitted to a current velocity of 12 cm/s to stimulate byssus production. Byssi were sampled every second day for analysis.

Protein hydrolysis

Lyophilized microalgae (1–1.5 mg) or mussel byssal thread (0.4–0.6 mg) samples were accurately weighed and dissolved in 1.0 mL of 6 M HCl. The samples were hydrolyzed for 24 h at 110°C, using a VWR Digital Heatblock (VWR International, Mississauga, ON, Canada). Sample tubes were opened and the acid was evaporated, rinsed with 1 mL water, and further evaporated using a SpeedVac concentrator (Fisher Scientific, Ottawa, ON, Canada) before reconstituting the sample in a final volume of 500 µL of water.

Standard preparation

Standard mixtures of 0, 1, 2, 3, 4, 5, 10, 20, 40, 50, 60, 80, 90, 95, 96, 97, 98, 99 and 100% (labeled/unlabeled concentrations) of glutamine, methionine, phenylalanine and tyrosine were prepared at a total (labeled + unlabeled) concentration of 1 µM for each amino acid in water.

Derivatization and LC/MS analysis

The reconstituted algal and byssal hydrolysates were centrifuged at 13000 rpm for 30 s to sediment particulates prior to derivatization and diluted 10-fold in water. Amino acids were derivatized in a rapid reaction with 6-aminoquinolyl *N*-hydroxy-succinimidylcarbamate (AQC) (see Fig. 1). AQC was synthesized according to the literature[18] in a one-step procedure combining di(*N*-succinimidyl) carbonate and 6-aminoquinoline. Amino acids were derivatized by adding 10 µL of a saturated solution of AQC (approximately 3 mg/mL) dissolved in anhydrous acetonitrile to a mixture of 20 µL amino acid standard or protein hydrolysate and 70 µL of borate buffer (25 mM borate, pH 8.8).

All samples (1 µL injections) were separated on a Zorbax Eclipse Plus C18 column (2.1 × 100 mm, 1.8 µm) using a Shimadzu Prominence HPLC system (including two LC20AD-XR pumps with in-line degasser, a SIL20AC autosampler, and a CTO20A column compartment) with the following gradient: 5% B with a 2 min hold, up to 70% B at 15 min, 95% B at 15.5 min held until 20 min before re-equilibration, where mobile phases A and B were water and methanol (both containing 0.1% formic acid), respectively, at a flow rate of 0.2 mL/min and a column temperature of 50°C. Mass spectrometric analysis was performed on a QTRAP 5500 hybrid triple quadrupole-linear ion trap (AB Sciex, Concord, ON, Canada) in positive ion mode. The TurboIonSpray® source was operated with an ionspray voltage of 5000 V at 450°C. The curtain gas was maintained at 35 psi and GS1 and GS2 gases were held at 55 and 60 psi, respectively. The declustering potential (DP) and exit potential (EP) were set to 120 and 10 V, respectively. A precursor ion scan of the fragment ion at *m/z* 171.1 was performed with collision energy (CE) of 30V and at a scan rate of 200 Da/s with a 0.1 Da step size between *m/z* 235 and 500. The collision cell exit potential (CXP) was maintained at 13 V. The offset values in the resolution tables for both quadrupoles were adjusted to obtain baseline resolution between consecutive ^{13}C and/or ^{15}N isotope peaks, reflecting slightly higher than unit resolution. All raw data was processed with Analyst® software (version 1.5.1) and MultiQuant™ (version 2.0.2) software for signal integration.

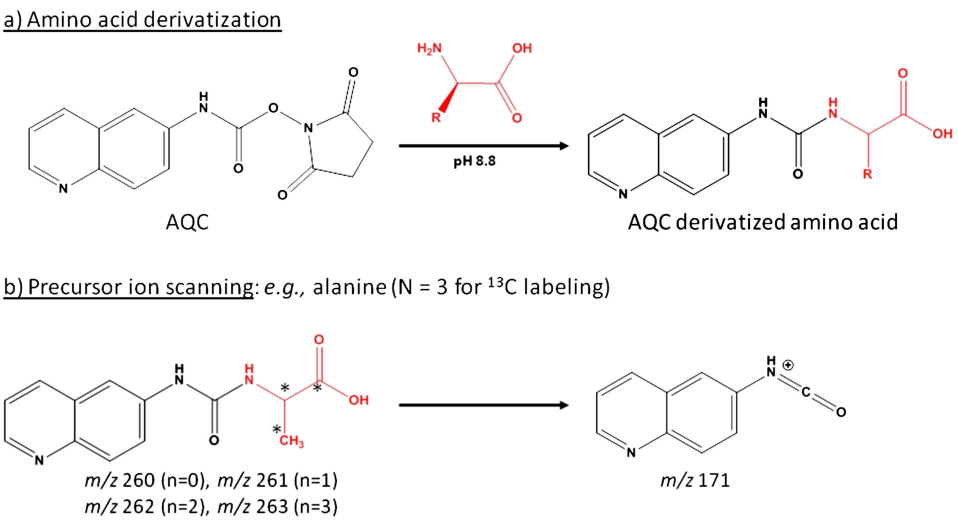

Figure 1. AQC derivatization reaction of amino acids (a) and example of extracted precursor ions in the case of a ^{13}C-labeling experiment for monitoring alanine (b).

Data analysis

Extracted ion chromatograms (EICs) were integrated from precursor ion scan data using a mass selection window of ±0.4 *m/z* relative to the theoretical *m/z* value for each isotopomer precursor ion. The area measured for each isotopomer was divided by the sum of areas for all possible isotopomers (up to fully labeled) of a given amino acid. The resulting ratio was expressed as a percentage in order to obtain a measure of the relative contribution from each isotopomer to the total amount of amino acid, as shown in the following equation:

$$\%\ contribution\ of\ isotopomer\ (C) = \left(\frac{A_{M_n}}{\sum_{n=0}^{N} A_{M_n}}\right) \times 100\% \quad (1)$$

where A represents the area of the EIC peak, M_n is the isotopomer from amino acid 'M' with 'n' labeled atoms, and N is the maximum number of labeled atoms for amino acid 'M'.

From this data, the total labeling percentage was calculated by weighting each isotopomer according to the number of labeled atoms they contain, then dividing by the maximum number of labeled atoms, which corresponds to the atom percent (AP) labeled, as shown below:

$$labeled\ AP\ (of\ amino\ acid\ M) = \frac{\sum_{n=1}^{N}(C_{M_n})n}{N} \quad (2)$$

For example, in a ^{13}C-labeling experiment, the isotopomer masses of AQC-derivatized alanine are shown in Fig. 1. If the extracted precursor ion at *m/z* 260 (n = 0) were twice the size of the peak area for fully labeled alanine (n = 3) at *m/z* 263, and no other isotopomer peak was detected, then the total label atom percent (AP) would be calculated as 33%, since exactly one-third of the total pool of alanine carbons would be ^{13}C-labeled.

RESULTS AND DISCUSSION

Calculation of % isotope labeling

Amino acid separation by reversed-phase chromatography normally requires derivatization due to the polar nature of the analytes. This poses an interesting problem for amino acid isotopomer analysis by reversed-phase LC/MS since the resulting derivatized amino acids will have an altered isotope pattern compared to the free amino acids, thus complicating data analysis. Our derivatization method using AQC, a common reagent used for reversed-phase amino acid analysis and designed for fluorescence detection,[18] permits the use of a selective precursor ion scanning method that addresses this problem. The fragmentation of AQC-derivatized amino acids yields a prominent, and thus highly sensitive, product ion at *m/z* 171 that results from the loss of the AQC moiety.[19] Since the ion at *m/z* 171 is the loss of the entire monoisotopic derivatization agent, the isotopic pattern detected from the precursor ion scan corresponding to the AQC-derivatized amino acids is dictated exclusively by the free amino acids. Moreover, the precursor ion scan adds a level of selectivity that non-MS/MS methods, such as single ion monitoring, do not provide. The possibility of mass interference during the analysis of all isotopomers for multiple amino acids from a relatively complex biological sample is a legitimate concern. Fortunately, the precursor ion scan selectively detects AQC-derivatized free amine-containing molecules. Furthermore, the AQC-derivatization reaction is a one-step process that can be performed directly in the injection vial and is complete within a few minutes. In short, AQC derivatization is ideal for isotopomer analysis of amino acids since it is a very fast and easy reaction that allows for separation of amino acids by reversed-phase HPLC and the use of a selective and sensitive precursor ion scanning method that does not include any isotope contribution from the derivatizing agent.

The remaining concern in the development of a method for accurate and reproducible measurement of amino acid isotope enrichment was cross-isotope spectral interference. Quadrupoles are most commonly operated at unit resolution which does not provide baseline separation of spectral peaks separated by a difference of 1 Da. For our method, both filtering quadrupoles (Q1 and Q3) were tuned for increased resolution in order to obtain baseline separation of neighboring isotopomers separated by 1 Da, as well as to assure that there would be no isotope pattern skewing due to a contribution from non-monoisotopic (AQC) fragment ions. Setting the instrument parameters for increased resolution in both quadrupoles does decrease its sensitivity; however, this is generally not a problem for these types of applications since, typically, a few milligrams of dried protein extract are easily available which represents a large excess considering the sensitivity of modern day mass spectrometry. To further insure that there would be no interference when calculating the relative areas of isotope peaks of each amino acid, the mass window used for each EIC was carefully chosen to avoid any contribution from neighboring isotopomers. By choosing mass windows of ±0.4 *m/z* relative to the theoretical *m/z* value for the isotopomer of interest, we cover nearly the entire spectral width of each peak while avoiding any contribution from its neighbors.

The calculation of the percentage of isotope label for a given amino acid is based on the ratio of the EIC peak area of each isotopomer relative to the sum of all isotopomer signals. The quantitative information is therefore extracted from a chromatographic peak rather than from a spectral peak, removing any possibility of skewed results due to chromatographic shifts from isotopically labeled ions. In order to calculate the total isotopic contribution, the areas corresponding to each isotopomer are weighted according to the number of labeled atoms they contain, resulting in an absolute percentage of labeling, or atom percent (AP). The data can then be treated according to the needs of the experiment, for example a correction factor can be applied to account for natural abundance, thus yielding a % enrichment value. Isotopomers can also be analyzed individually, since the data from each is available, preserving the opportunity for determining the distribution of labels incorporated in any amino acid for a given experiment.

Method validation using isotope-labeled standards

In order to test our method of measuring isotopic enrichment, amino acid standards prepared at varying unlabeled to labeled ratios were tested. The standards were prepared by mixing four unlabeled amino acids (phenylalanine, methionine, glutamine and tyrosine) with their corresponding fully ^{13}C- and ^{15}N-labeled counterparts (with the exception of phenylalanine, which was $^{13}C_6$ only) in precise ratios. It is difficult to prepare standards that will perfectly reflect biological samples, which

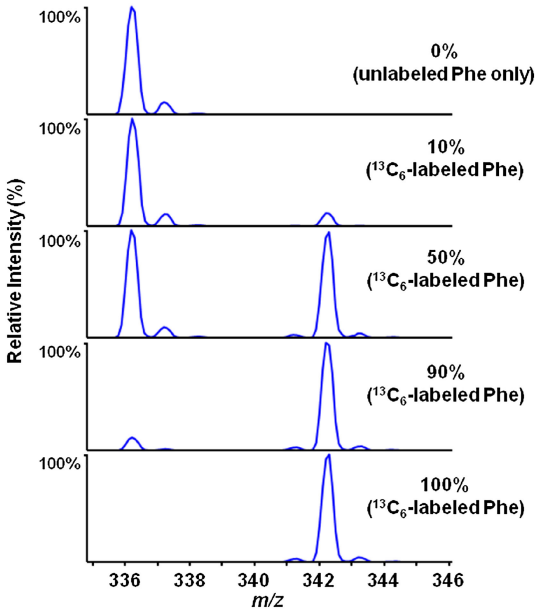

Figure 2. Mass spectra (from *m/z* 335 to 346) from different standard mixtures of ring-labeled ($^{13}C_6$) phenylalanine.

will contain various degrees of isotope labeling, since commercially available standards are manufactured to be isotopically uniform. These standards suit our purpose of validating the method by testing our ability to correctly determine isotope ratios and to calculate a percentage of isotope enrichment from these ratios. As an example of the results obtained with these standards, Fig. 2 shows the mass spectra from different standard mixtures of phenylalanine. The spectra clearly show the typical baseline resolution obtained between isotopomers using our method. In the case of the standard mixtures, the total percentage of labeled atoms was corrected for the natural isotopic abundance for each amino acid by adjusting the targeted percentage based on the theoretical isotope distributions of the standard mixtures and the isotopic purity of the labeled amino acids. The results show excellent linearity and consistent results for all standard amino acids (Fig. 3 and Table 1). The precision of the method is excellent, with all standard samples injected having a coefficient of variance (CV) within 20%. The accuracy of the method is also very good, with all standard samples having a calculated AP within ±2.5% and a percent accuracy within ±20% of the expected theoretical value, including the vast

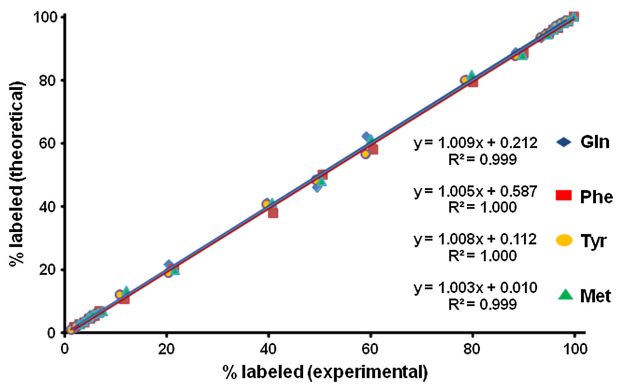

Figure 3. Calibration curves of standard amino acids (Gln, Phe, Tyr, Met) at different labeling percentage mixtures.

majority (89.5%) of the samples being within ±10% of the expected value. In order to obtain these standard curves, a final concentration of 200 nM derivatized amino acid standard representing between 29 and 36 pg of each pre-derivatized amino acid (from the four standard tested) were injected onto the column. The performance and sensitivity of the method is clearly suitable for the application described since we would always be starting with an initial protein sample of at least 0.1 mg prior to hydrolysis.

Amino acid analysis and protein hydrolysis

In order to obtain a good measurement of the degree of isotopic enrichment in proteins, we have opted for one of the simplest and most commonly used methods: acid hydrolysis using concentrated hydrochloric acid. Like most other hydrolysis methods, this one does not permit the analysis of all amino acids. By combining different hydrolysis methods, it is possible to recover every amino acid and it is important to note that our derivatization and LC/MS methods can be adapted to be compatible with any hydrolysis method.

There are six amino acids that we cannot directly measure using our combination of acid hydrolysis and LC/MS method, as shown in Table 2. Cysteine and tryptophan are known to be destroyed by acid hydrolysis and asparagine and glutamine are deamidated to their carboxylic acid counterparts, aspartic acid and glutamic acid, respectively.[20] Even though we cannot directly analyze asparagine or glutamine, all of their ^{13}C-labeling information is incorporated into the data obtained from aspartic acid and glutamic acid. This complicates the individual analysis of these four amino acids and renders the measurement of side-chain ^{15}N-labeling for asparagine and glutamine impossible when using acid hydrolysis. It is best to use other hydrolysis methods[16] if the precise labeling information for these amino acids is required. Two more amino acids, histidine and arginine, are not sensitive enough to be analyzed using our method, mainly due to the highly polar nature of their AQC derivatives, and therefore very early elution of these derivatives using an acidic mobile phase. If these amino acids are specifically of interest, simply using a more basic mobile phase, such as ammonium acetate, would increase their retention on a C18 column, thus increasing their sensitivity. The formic acid mobile phase, however, was chosen as the best choice since, overall, we obtain very good resolution and sensitivity of a majority of the amino acid derivatives.

Representative chromatograms of overlaid monoisotopic EICs from a mixture of all 20 standard amino acids (without hydrolysis), each at a concentration of 0.5 μM, and a hydrolyzed algal protein sample (from an initial amount of 1 mg lyophilized algae) are illustrated in Fig. 4. Using the precursor ion scanning method from *m/z* 235–500, we did not observe peaks in the standard mix for cysteine, arginine and histidine, since most of the ion intensities for these amino acid AQC derivatives are doubly charged and therefore below the mass range detected (see Table 2). However, if cysteine is protected to remove the free thiol group prior to protein hydrolysis, the corresponding protected AQC derivative will be measurable with the same precursor ion scanning method as described here. Basic amino acids, such as lysine, arginine and histidine, since they are doubly charged will not have complete resolution between isotopomers, and therefore will not be taken into account in the final assay for protein

Table 1. Atom percent labeling for mixtures of unlabeled and labeled amino acid standards

% labeled std	Gln			Phe			Met			Tyr		
	Calc% (Theo)	%Δ	%CV	Calc% (Theo)	%Δ	%CV	Calc% (Theo)	%Δ	%CV	Calc% (Theo)	%Δ	%CV
0	1.1 (1.1)	0.9	19.7	1.9 (1.8)	4.1	1.9	2.7 (2.6)	5.0	11.5	1.1 (1.1)	−0.6	3.9
1	1.7 (2.0)	−14.8	11.4	2.8 (2.8)	0.3	8.5	3.7 (3.6)	5.0	14.6	2.2 (2.1)	5.9	6.8
2	2.4 (3.0)	−19.0	13.4	3.3 (3.8)	−10.9	8.2	4.9 (4.5)	8.7	6.2	2.7 (3.0)	−9.4	8.2
3	3.7 (3.9)	−5.6	7.5	4.4 (4.7)	−5.9	8.5	6.1 (5.4)	11.9	16.3	4.0 (4.0)	−0.1	19.9
4	5.8 (4.9)	18.8	18.0	5.4 (5.7)	−5.6	3.5	6.4 (6.4)	0.4	18.6	4.5 (4.9)	−8.4	19.3
5	5.3 (5.9)	−9.7	6.9	6.8 (6.7)	1.8	10.7	6.9 (7.3)	−5.0	13.6	6.0 (5.9)	2.6	17.2
10	12.4 (10.7)	16.0	3.2	10.9 (11.5)	−5.0	2.1	13.5 (12.0)	12.4	4.8	12.1 (10.7)	14.0	14.5
20	21.8 (20.3)	7.6	15.9	20.3 (21.2)	−4.4	12.4	19.9 (21.5)	−7.2	5.8	19.1 (20.2)	−5.4	6.4
40	41.4 (39.6)	4.5	5.0	37.9 (40.7)	−6.8	4.2	41.3 (40.6)	1.8	0.6	40.8 (39.5)	3.5	8.5
50	46.2 (49.3)	−6.3	3.6	50.3 (50.5)	−0.4	1.8	48.2 (50.2)	−4.1	4.5	48.6 (49.1)	−1.1	4.5
60	62.4 (59.0)	5.6	1.3	58.2 (60.3)	−3.4	9.4	61.5 (60.0)	2.5	2.6	56.8 (58.9)	−3.5	2.9
80	80.4 (78.6)	2.3	4.1	79.7 (80.0)	−0.3	0.3	81.9 (79.7)	2.8	3.5	80.2 (78.4)	2.3	1.8
90	89.1 (88.4)	0.8	0.6	88.9 (89.8)	−1.0	0.9	88.2 (89.6)	−1.6	1.5	88.0 (88.3)	−0.3	0.7
95	93.6 (93.3)	0.4	3.1	95.1 (94.8)	0.4	0.4	94.6 (94.7)	0.0	1.7	93.9 (93.2)	0.7	1.1
96	95.1 (94.3)	0.9	1.3	96.2 (95.7)	0.4	0.3	96.4 (95.7)	0.8	1.2	94.7 (94.2)	0.6	0.6
97	96.0 (95.3)	0.8	0.5	96.7 (96.7)	0.0	0.4	97.3 (96.7)	0.6	0.3	95.6 (95.2)	0.4	0.8
98	97.6 (96.2)	1.4	0.3	98.2 (97.7)	0.5	0.1	98.2 (97.7)	0.6	0.2	97.5 (96.2)	1.4	0.4
99	98.5 (97.2)	1.3	0.3	99.0 (98.7)	0.3	0.0	99.2 (98.7)	0.5	0.3	98.3 (97.2)	1.2	0.1
100	99.2 (98.2)	1.0	0.1	100.3 (99.7)	0.6	0.2	100.2 (99.7)	0.5	0.1	99.3 (98.1)	1.2	0.0

%Δ: Percent difference between the calculated AP labeling and the theoretical value.
%CV: Standard deviation as a percentage from triplicate injections.

Table 2. Amino acid characteristics following protein hydrolysis, AQC derivatization and LC/MS analysis

Amino acid	# C atoms	# N atoms	MW (g/mol)	*m/z* (AQC derivative)	RT (min)	Comment
His[a]	6	3	155.1	163.6, 326.1	3.3	Low sensitivity (charge/early elution)
Asn	4	2	132.1	303.1	4.9	Acid hydrolysed to aspartic acid
Arg[a]	6	4	174.1	173.1, 345.2	5.3	Low sensitivity (charge/fragmentation)
Gln	5	2	146.1	317.1	6.4	Acid hydrolysed to glutamic acid
Ser	3	1	105.0	276.1	6.5	
Gly	2	1	75.0	246.1	6.8	
Asp	4	1	133.0	304.1	7.4	
Glu	5	1	147.1	318.1	7.8	
Thr	4	1	119.1	290.1	8.2	
Ala	3	1	89.0	260.1	8.7	
Pro	5	1	115.1	286.1	9.2	
Lys[ab]	6	2	146.1	244.1, 487.1	10.6	Doubly derivatized and doubly charged
Tyr	9	1	181.1	352.1	11.0	
Cys[ab]	3	1	121.0	231.6, 462.1	11.1	Degraded during acid hydrolysis
Met	5	1	149.1	320.1	11.3	
Val	5	1	117.1	288.1	11.8	
Trp	11	2	204.1	375.1	13.4	Degraded during acid hydrolysis
Ile/Leu	6	1	131.1	302.1	13.5	Coelution of isobaric species
Phe	9	1	165.1	336.1	13.6	

[a]Doubly charged species as base peak.
[b]Doubly derivatized amino acid as major species.

hydrolysates. One alternative for measuring the isotopic contributions for these two amino acids, as well as lysine, is to use a mobile phase that is less acidic and therefore would more likely form the 1+ charge state. The acidic mobile phase is much more sensitive for the detection of AQC derivatives of amino acids and therefore was chosen for this study.

One advantage of this approach is that baseline resolution is not needed for all amino acids; however, it is crucial that all amino acids with the potential to share isobaric isotopomers are well resolved chromatographically. For hydrolyzed protein samples, our method therefore has the ability to measure ^{13}C isotope enrichment with data originating from 15 of the 20

Rapid Communications in Mass Spectrometry

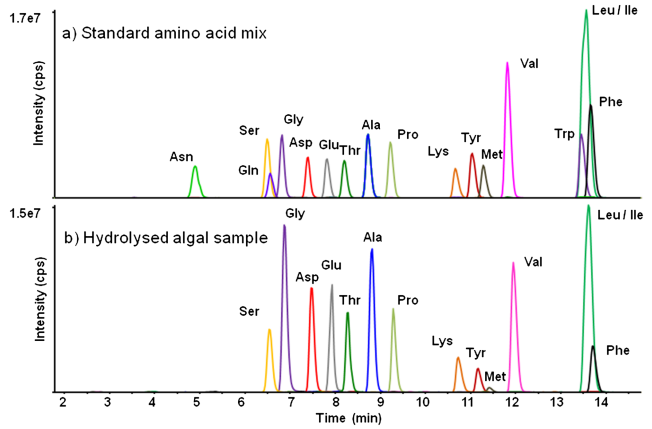

Figure 4. Extracted ion chromatograms of monoisotopic peaks for AQC-derivatized amino acid standard mix (at 0.5 μM amino acid concentration) (a) and protein hydrolysate from representative algal sample (b).

amino acids, removing the doubly charged derivatives (Lys, His, Arg) and those that are destroyed by acid hydrolysis (Cys, Trp), providing enough coverage to obtain a reliable estimate of the total ^{13}C enrichment of the protein sample.

Analysis of ^{13}C enrichment in proteins from glucose-fed algae

A cost-effective strategy to obtain labeled byssal threads is to first label algae which will subsequently be used to feed the mussels. In the current study, we have tested two possibilities for labeling of algae: feeding with labeled glucose, as well as growing algae in the presence of labeled salts. Our method was first tested on protein extracts from algae that were fed with isotopically labeled $^{13}C_6$-glucose (50%) for a period of 19 days. Algae were taken from the growth medium every second day for analysis and the total percentage of ^{13}C-labeled carbon atoms was calculated (see Table 3). The labeling kinetics is thus established and the optimal time for algae harvesting prior to mussel feeding determined.

The results do not include data for methionine, since it is both one of the lesser sensitive of the amino acids and one of the least abundant in proteins; therefore, the resulting signal obtained from our samples was not sufficient for reliable data to be extracted, resulting in data quantified from 14 amino acids.

In order to compare the mass spectra originating from a standard algal sample with those from the labeled samples, threonine is used as an example in Fig. 5. The mass spectrum of threonine obtained from an algal sample taken on the first day of labeling is, as expected, very similar to that observed from the standard. A difference is clearly seen, however, in the mass spectra between the first (day 1) and last day (day 19) of the feeding experiment. The relative areas of the labeled threonine isotopomers are visibly larger compared to the unlabeled threonine in the day 19 sample, as we can start to see a fully labeled threonine peak starting to emerge. Since the algae were continuously fed ^{13}C-labeled glucose during the experiment, we expect protein extracts to have increasing ^{13}C content throughout the 19 days of sampling. When the average ^{13}C-labeling percentage of amino acids was calculated, we indeed observed a continuous increase in labeling throughout the sampling period (Fig. 6), reaching a plateau of labeling when the algae entered the stationary phase of

Table 3. % Isotope enrichment in algal samples fed with $^{13}C_6$-glucose over 19 days for individual amino acids

Amino acid	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13	Day 15	Day 17	Day 19
Ser	3.9 ± 0.3 (9.6)	5.2 ± 0.7 (7.7)	6.5 ± 0.2 (7.2)	8.1 ± 1.4 (4.8)	9.6 ± 1.4 (7.6)	10.3 ± 0.9 (4.6)	11.8 ± 1.8 (4.9)	14.1 ± 2.1 (3.1)	15.7 ± 1.7 (3.5)	15.2 ± 1.3 (3.4)
Gly	4.7 ± 0.7 (7.3)	5.5 ± 0.8 (5.4)	6.4 ± 0.9 (4.5)	7.4 ± 1.2 (2.9)	9.3 ± 1.6 (4.4)	9.8 ± 0.8 (2.3)	11.2 ± 1.9 (3.2)	13.5 ± 2.1 (3.2)	14.5 ± 1.3 (5.8)	14.2 ± 1.6 (1.6)
Asp*	3.3 ± 0.3 (8.6)	5.0 ± 0.9 (6.6)	7.2 ± 0.7 (7.1)	8.5 ± 1.4 (3.0)	10.2 ± 1.5 (4.8)	10.9 ± 1.3 (2.8)	12.7 ± 2.0 (2.5)	14.4 ± 1.5 (3.6)	15.3 ± 1.8 (2.6)	15.3 ± 1.4 (1.9)
Glu*	2.9 ± 0.3 (10.9)	6.0 ± 1.1 (5.5)	8.4 ± 0.5(4.3)	9.4 ± 1.5 (4.8)	11.0 ± 1.9 (1.1)	11.4 ± 1.3 (1.4)	13.4 ± 2.2 (2.2)	15.1 ± 2.3 (2.8)	16.0 ± 2.2 (1.7)	16.2 ± 1.0 (1.1)
Thr	2.0 ± 0.2 (12.6)	3.2 ± 0.3 (11.2)	5.0 ± 0.7 (4.2)	6.2 ± 1.0 (3.8)	7.8 ± 1.3 (1.6)	8.3 ± 0.8 (4.9)	9.8 ± 1.7 (1.5)	11.1 ± 1.5 (3.8)	11.7 ± 1.3 (3.2)	11.9 ± 1.2 (3.0)
Ala	5.5 ± 0.3 (13.2)	13.2 ± 4.0 (3.1)	17.4 ± 4.0 (2.3)	15.6 ± 3.8 (1.2)	11.9 ± 1.7 (4.0)	13.4 ± 1.8 (3.1)	14.2 ± 2.4 (7.5)	16.2 ± 2.1 (2.8)	17.5 ± 2.0 (3.9)	17.4 ± 1.4 (4.1)
Pro	2.4 ± 0.2 (11.3)	3.3 ± 0.6 (15.3)	4.7 ± 0.9 (8.9)	5.7 ± 0.9 (2.8)	7.0 ± 1.0 (6.9)	7.7 ± 0.9 (1.4)	8.7 ± 1.6 (2.1)	9.1 ± 1.1 (6.5)	10.0 ± 1.5 (5.0)	10.0 ± 1.6 (3.4)
Tyr	1.6 ± 0.3 (17.8)	2.2 ± 0.8 (10.6)	3.6 ± 0.6 (6.9)	5.6 ± 1.2 (6.8)	7.1 ± 1.4 (2.8)	8.0 ± 1.0 (4.8)	9.2 ± 1.4 (8.1)	11.4 ± 1.4 (2.4)	12.0 ± 1.5 (2.0)	11.8 ± 1.2 (3.9)
Val	2.3 ± 0.2 (7.4)	3.4 ± 0.5 (4.7)	5.2 ± 0.7 (2.9)	6.3 ± 0.9 (4.3)	7.7 ± 1.4 (3.9)	9.0 ± 1.0 (2.7)	10.4 ± 1.7 (5.6)	12.1 ± 1.4 (3.2)	13.1 ± 1.5 (1.0)	13.1 ± 1.5 (2.3)
Leu/Ile	2.4 ± 0.3 (5.1)	3.3 ± 0.5 (4.0)	4.7 ± 0.7 (3.7)	5.8 ± 0.9 (1.4)	7.0 ± 1.2 (2.0)	7.9 ± 0.9 (3.2)	9.1 ± 1.5 (2.1)	10.7 ± 1.4 (1.5)	11.7 ± 1.5 (0.9)	11.7 ± 1.1 (1.3)
Phe	1.9 ± 0.5 (11.0)	2.5 ± 0.5 (11.7)	3.9 ± 0.5 (8.5)	5.1 ± 0.8 (4.8)	6.4 ± 1.0 (2.7)	7.7 ± 1.1 (4.3)	9.4 ± 1.9 (5.9)	10.6 ± 1.1 (1.0)	11.3 ± 1.1 (1.1)	11.0 ± 0.9 (1.6)

± Values represent biological variability (absolute values).
Values in parentheses are %CV for triplicate analysis of the same biological sample.

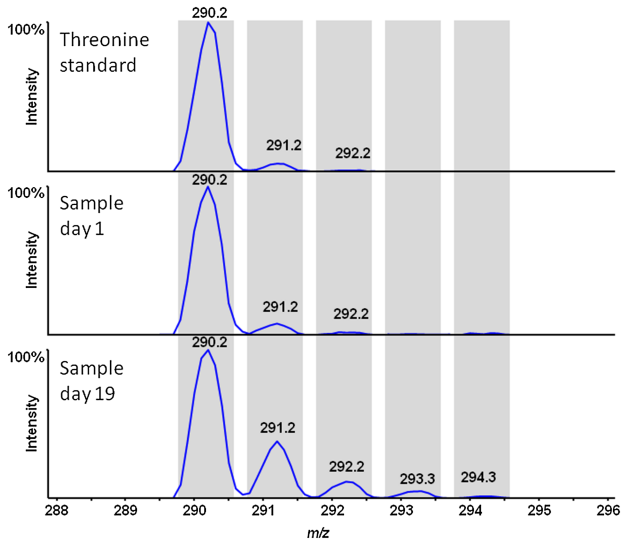

Figure 5. Mass spectra (from *m/z* 288 to 296) and integration windows from AQC-derivatized threonine from standard (a), algal sample on day 1 (b), and algal sample day 19 (c).

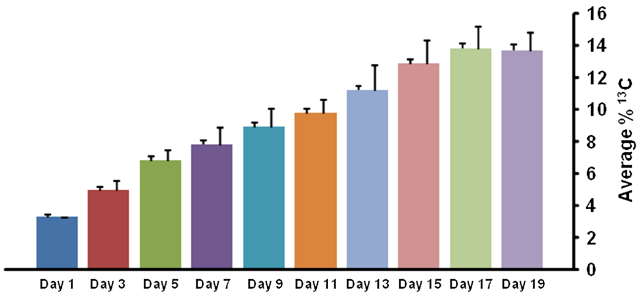

Figure 6. Average percentage of ^{13}C labeling in algal protein hydrolysates from time-course $^{13}C_6$-glucose feeding experiment, with error bars showing analytical reproducibility (n = 3) (left) and biological variability (n = 3) (right).

growth at day 19. We can clearly see a nice trend in the overall data even if the percentage change is subtle between samples from consecutive sampling days, demonstrating that the method can reliably differentiate samples which differ by about 2% ^{13}C-labeling (AP). The precision of the method for the analysis of algal samples is clearly consistent with the data from the standard curves. Three samples were taken at each time point (biological replicates) and each one of these samples was injected three times (analytical replicates). The %CV of the analytical replicates was again excellent: all samples were under 20%, including over 90% of samples with %CV under 10% (see Table 3). The standard deviations between different biological replicates suggest that the accuracy of our method also reflects the results of our standard curves since 91.8% of the samples are within 2% AP.

An important aspect of this method is that the data pertaining to all isotopes are available, allowing for deeper analysis of the data. We can obtain relevant biological information from this type of analysis, since we can monitor the degree of labeling for each amino acid and the relative abundance of each isotopomer, as opposed to only having access to a total amount of ^{13}C labeling. As previously mentioned, it is very useful for subsequent NMR studies to be able to discriminate partial and total labeling of a given amino acid.

While the glucose-feeding experiment does serve as an excellent example of the applicability of our method, the overall percentage of labeling of the algae is not sufficient to obtain a satisfactory labeling of byssal threads for NMR studies; therefore, we assessed a second method of algal production using labeled inorganic salts as carbon source.

Analysis of ^{13}C enrichment in protein from sodium bicarbonate-fed algae and mussel byssus

Sodium bicarbonate is a common carbon source for microalgae and therefore it was expected that a ^{13}C-labeled bicarbonate diet would allow a more efficient incorporation of ^{13}C into algal proteins. In order to maximize labeling efficiency through the labeled salt diet, a culture system was designed to ensure the elimination of oxygen produced by algae and avoid gas exchange with the ambient atmosphere. Indeed, this experimental approach yields algae with significantly higher ^{13}C labeling than with the previous glucose-feeding experiment, with a ^{13}C labeling of 84.9% (AP). Figure 7 shows the extracted ion chromatograms (EICs) corresponding to each of the ^{13}C isotopomers of alanine, comparing the bicarbonate-fed labeled algae to unlabeled (control) algae. The areas of each of these peaks represent the raw data used to calculate the ^{13}C atom percent labeled (AP). The efficiency of this feeding experiment is clearly illustrated since the predominant isotopomer peak from the labeled algae corresponds to the fully labeled ($^{13}C_3$-alanine at *m/z* 263) alanine peak.

The amount of algae labeling provided by this feeding technique is sufficient in order to move on to the next step and assess the labeling incorporation into mussel byssus. This algal culture was used to study the efficiency of the mussel feeding and byssus production protocols. Normally, byssus production is stimulated by transferring the mussels in a tank designed to produce a current which forces the mussels to produce byssal thread as a means to immobilize themselves on a solid support. Due to the volume of this tank and its configuration having relatively high currents, the mussels were pre-fed with labeled algae and then transferred into the tank for stimulated byssus production. Every second day, the byssal thread was removed. The optimal sampling day was then determined by our LC/MS/MS method in order to maximize the labeling of the byssus sample. From our data, the optimal labeling was determined to be at day 5, and the mass spectra in the window of the ^{13}C isotopomers of valine from these ^{13}C-labeled byssus threads are shown in Fig. 8. By comparing the labeled byssal thread sample to unlabeled and labeled algae samples, it is clearly seen that some ^{13}C labels have been incorporated into the byssus, notably with the appearance of the fully labeled valine isotopomer peak at *m/z* 293. While the byssus that was sampled on day 5 proved to have the highest ^{13}C-isotopic enrichment, it is still relatively low at 8.6% (AP), illustrated by the fact that unlabeled valine still remains the major isotopomer peak. Considering that the major isotopomer peak for all amino acids in the bicarbonate-fed algal sample corresponds to the fully labeled amino acid (as shown for valine), the feeding and sampling methods can still be improved significantly if feeding is continued during byssus production. This modest amount of label incorporation, however, is still enough to reduce the acquisition time ~60-fold compared to unlabeled byssal thread, allowing for preliminary NMR structural studies to begin while the mussel feeding and byssus production protocols are further optimized.

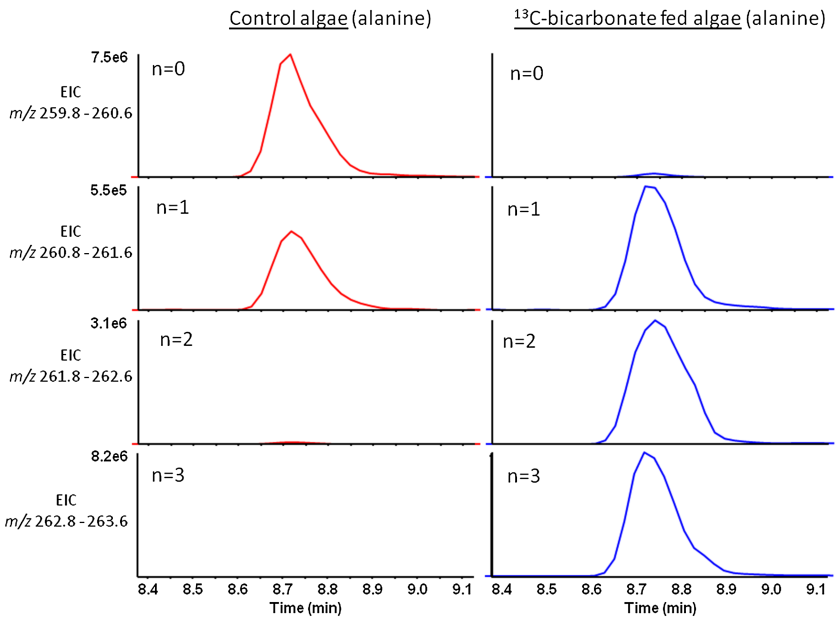

Figure 7. Extracted ion chromatograms from precursor ion scanning experiment for alanine ^{13}C isotopomers in unlabeled and ^{13}C-bicarbonate-fed algal samples.

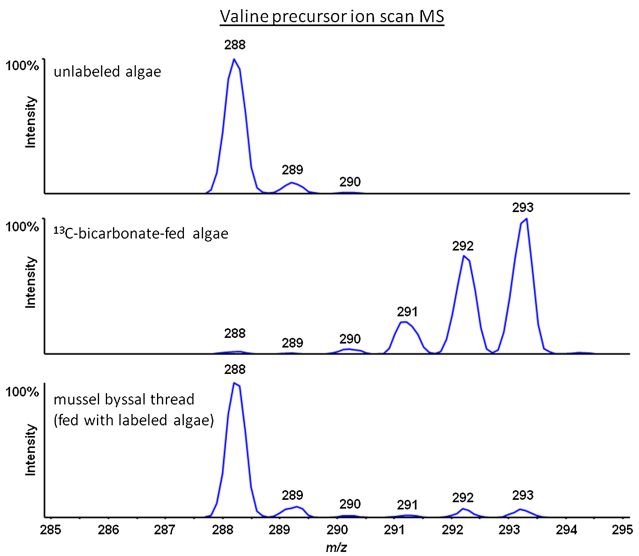

Figure 8. Mass spectra (from *m/z* 285 to 295) showing valine isotopomers from ^{13}C-bicarbonate-fed algae and labeled mussel byssus sample.

CONCLUSIONS

We have developed an efficient method for measuring isotopic enrichment of amino acids from protein extracts using a LC/MS/MS platform based on a simple derivatization procedure that is compatible with most protein hydrolysis methods. Furthermore, the use of a precursor ion scan allows us to take advantage of an added level of selectivity not present in non-MS/MS approaches that are more frequently used for this type of isotopomer analysis. The method was validated using standard curves from four different amino acids, obtaining excellent reproducibility and very good accuracy allowing us to distinguish between samples that differ by 2% (AP) with a very modest amounts of amino acid (≤36 pg of pre-derivatized amino acid) injected onto the column. In short, the performance of the method, its sensitivity and its accessibility render it an ideal approach for assessing protein labeling. Within the context of protein structural studies by NMR, this is especially true since the method is adaptable for the measurement of either ^{13}C or ^{15}N enrichment and allows one to evaluate labeling levels for individual amino acids, while also being able to discriminate between partial and total labeling of a given amino acid.

This new method was used for the measurement of ^{13}C labeling in algal protein and mussel byssus thread proteins, demonstrating that we were able to obtain reliable ^{13}C-labeling data originating from 14 amino acids using the combination of a simple acid hydrolysis method and our analytical approach for two different types of biological samples. The method has allowed us to evaluate two different feeding protocols for microalgae and subsequently monitor the optimization of mussel feeding protocols for selecting suitable mussel byssal threads for further structural investigation.

REFERENCES

[1] J. Gosline, M. Lillie, E. Carrington, P. Guerette, C. Ortlepp, K. Savage. Elastic proteins: biological roles and mechanical properties. *Phil. Trans. R. Soc. Lond. B* **2002**, *357*, 121.
[2] A. A. Arnold, I. Marcotte. Studying natural structural protein fibers by solid-state nuclear magnetic resonance. *Concepts Magn. Reson. Part A* **2009**, *34A*, 24.
[3] M. Osanai, M. Okudaira, J. Naito, M. Demura, T. Asakura. Biosynthesis of L-alanine, a major amino acid of fibroin in *Samia cynthia* ricini. *Insect Biochem. Mol. Biol* **2000**, *30*, 225.
[4] S. Hess, J. van Beek, L. K. Pannell. Acid hydrolysis of silk fibroins and determination of the enrichment of isotopically labeled amino acids using precolumn derivatization and high-performance liquid chromatography–electrospray ionization–mass spectrometry. *Anal. Biochem.* **2002**, *311*, 19.

[5] M. K. Hellerstein, R. A. Neese. Mass isotopomer distribution analysis at eight years: theoretical, analytic, and experimental considerations. *Am. J. Physiol. – Endocrinol. Metab.* **1999**, *276*, E1146.
[6] T. Shen, W. Shen, Y. Xiong, H. Liu, H. Zheng, H. Zhou, B. Rui, J. Liu, J. Wu, Y. Shi. Increasing the accuracy of mass isotopomer analysis through calibration curves constructed using biologically synthesized compounds. *J. Mass Spectrom.* **2009**, *44*, 1066.
[7] B. E. Patterson, X.-J. Zhang, Y. Chen, S. Klein, R. R. Wolfe. Measurement of very low stable isotope enrichments by gas chromatography/mass spectrometry: Application to measurement of muscle protein synthesis. *Metabolism* **1997**, *46*, 943.
[8] G. Molero, I. Aranjuelo, P. Teixidor, J. L. Araus, S. Nogués. Measurement of ^{13}C and ^{15}N isotope labeling by gas chromatography/combustion/isotope ratio mass spectrometry to study amino acid fluxes in a plant–microbe symbiotic association. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 599.
[9] M. E. Jennings, D. E. Matthews. Determination of complex isotopomer patterns in isotopically labeled compounds by mass spectrometry. *Anal. Chem.* **2005**, *77*, 6435.
[10] M. R. Antoniewicz, J. K. Kelleher, G. Stephanopoulos. Accurate assessment of amino acid mass isotopomer distributions for metabolic flux analysis. *Anal. Chem.* **2007**, *79*, 7554.
[11] H. M. H. vanEijk, D. R. Rooyakkers, A. J. M. Wagenmakers, P. B. Soeters, N. E. P. Deutz. Isolation and quantitation of isotopically labeled amino acids from biological samples. *J. Chromatogr. B* **1997**, 691, 287.
[12] H. M. H. vanEijk, D. R. Rooyakkers, P. B. Soeters, N. E. P. Deutz. Determination of amino acid isotope enrichment using liquid chromatography-mass spectrometry. *Anal. Biochem.* **1999**, *271*, 8.
[13] H. M. H. vanEijk, D. P. L. Suylen, C. H. C. Dejong, Y. C. Luiking, N. E. P. Deutz. Measurement of amino acid isotope enrichment by liquid chromatography mass spectroscopy after derivatization with 9-fluorenylmethylchloroformate. *J. Chromatogr. B* **2007**, *856*, 48.
[14] M. B. Goshe, V. E. Anderson. Determination of amino acid isotope ratios by electrospray ionization- mass spectrometry. *Anal. Biochem.* **1995**, *231*, 387.
[15] J.-P. Godin, L.-B. Fay, G. Hopfgartner. Liquid chromatography combined with mass spectrometry for ^{13}C isotopic analysis in life science research. *Mass Spectrom. Rev.* **2007**, *26*, 751.
[16] R. R. L. Guillard. Culture of phytoplankton for feeding marine invertebrates, in *Culture of Marine Invertebrate Animals*, (Eds: W. L. Smith, M. H. Chanley), Plenum, New York, **1975**, pp. 29–60.
[17] I. Redjah, F. Olivier, R. Tremblay, B. Myrand, F. Pernet, U. Neumeier, L. Chevarie. The importance of turbulent kinetic energy on transport of juvenile clams (*Mya arenaria*). *Aquaculture* **2010**, *307*, 20.
[18] S. A. Cohen, D. P. Michaud. Synthesis of a fluorescent derivatizing reagent 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high performance liquid chromatography. *Anal. Biochem.* **1993**, *211*, 279.
[19] N. Mitrea, A. LeBlanc, M. St-Onge, L. Sleno. Assessing covalent binding of reactive drug metabolites by complete protein digestion and LC–MS analysis. *Bioanalysis* **2010**, *2*, 1211.
[20] M. Fountoulakis, H.-W. Lahm. Hydrolysis and amino acid composition analysis of proteins. *J. Chromatogr. A* **1998**, *826*, 109.

