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APPLICATION NOTE 53

Selection and Synthesis to Analysis: Stable Isotope-Labeled Peptides for Proteomic Applications

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Highlights

- Guidelines and considerations for peptide selection, synthesis, and analysis in bottom-up proteomic applications are described
- Isotopically labeled peptide standards from two published research articles were selected for reference and relevance
- Outlined synthesis procedures utilized CIL's stable isotope-labeled protected amino acids and were performed on CEM's Liberty PRIME™ 2.0 peptide synthesizer
- Qualitative and quantitative applications of the spotlighted proteomic articles are summarized

Introduction

Proteomics is among the core 'omics technologies commonly implemented in the scientific field to study the molecular processes that drive cellular function and disease pathogenesis. While this enabling technology can be employed independently, it is being increasingly combined today with other 'omics technologies, such as metabolomics, to capture a more comprehensive view of systems biology.[1,2] Regardless of the 'omics technology deployed, mass spectrometry (MS) is the ideal tool for analyte detection (peptides being the focus here) due to its wide-spanning array of analytical benefits. At the sample analysis stage, a mass spectrometer can be operated in a variety of modes (e.g., multiple reaction monitoring, MRM; parallel reaction monitoring, PRM) and is commonly fronted by a separation technique (e.g., liquid chromatography or LC) for enhanced specificity and sensitivity of measurement.

One of the critical elements of method development is the sample preparation. The method used should incorporate isotopically labeled standards (e.g., ^{13}C and/or ^{15}N) as these can help compensate for matrix-induced suppression and procedural/instrumental variation. Additional benefits of these standards are that they assist in correctly identifying endogenous analytes, detecting chemical interference, and improving analytical precision. In a proteomics application, the choice of normalizer (i.e., peptide or protein labeled standard) is user-based, with each carrying its inherent advantages and disadvantages that should be weighed. The stable isotope-labeled peptide standard approach is common in MS proteomics, particularly in targeted biomarker research,[3] and has proven through the years to be a reliable means to qualify and quantify peptides (and proteins by inference). To ensure robust assay workflows, certain considerations must be borne in the experimental design and application development. This pertains, for example, to the selection, synthesis, and analysis of the stable isotope-labeled peptide standards.

In this article, two stable isotope-labeled peptide standards from two recent (and dissimilar) publications from the proteomics field were selected. The case examples were peptides ATHVGNDTTLAQIV**K** ($^{13}C_6/^{15}N_2$ at K or L-lysine) and AYNVTQ**A**FG**R** ($^{13}C_3$ at A or L-alanine and $^{13}C_6/^{15}N_4$ at R or L-arginine). In their applications, the ATHVGNDTTLAQIV**K** labeled standard was used to quantify the ATP7B protein in dried blood spot samples of Wilson's disease patients.[4] The latter AYNVTQ**A**FG**R** standard was used in the targeted LC-PRM/MS analysis of clinical nasopharyngeal swab samples of COVID-19 patients.[5] Example methods for their synthesis and characterization is described herein along with a summary of the labeled standard application in MS-based proteomics research.

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Experimental Overview

Chemicals and Reagents

All reagents (e.g., diisopropylcarbodiimide, pyrrolidine, 3,6-dioxa-1,8-octanedithiol) were of the highest analytical grade. Dichloromethane (DCM), *N,N*-dimethylformamide (DMF), anhydrous diethyl ether, and acetic acid were obtained from VWR (West Chester, PA USA). LC-MS-grade water and acetonitrile were obtained from Fisher Scientific (Waltham, MA USA). Rink Amide ProTideTM LL resin (0.20 meq/g substitution) was obtained from CEM Corporation. The following unlabeled protected amino acids were used in the solid phase peptide synthesis (side chain protecting groups noted in parenthesis; CEM Corporation): L-Ala, -Asn(Trt), -Arg(Pbf), -Asp(OMpe), -Gln(Trt), Gly, -His(Boc), -Ile, -Leu, -Lys(Boc), -Phe, -Thr(tBu), -Tyr(tBu), and -Val. Peptide labeling was achieved using the following stable isotope-labeled protected amino acids (see **Figure 1** for structures):

- $^{13}C_3$ L-Alanine-*N*-Fmoc (CIL catalog no. CLM-7785)
- $^{13}C_6/^{15}N_4$ L-Arginine-*N*-Fmoc, Pbf-OH (CIL catalog no. CNLM-8474-H)
- $^{13}C_6/^{15}N_2$ L-Lysine-α-*N*-Fmoc, ε-*N*-*t*-Boc (CIL catalog no. CNLM-4754-H)

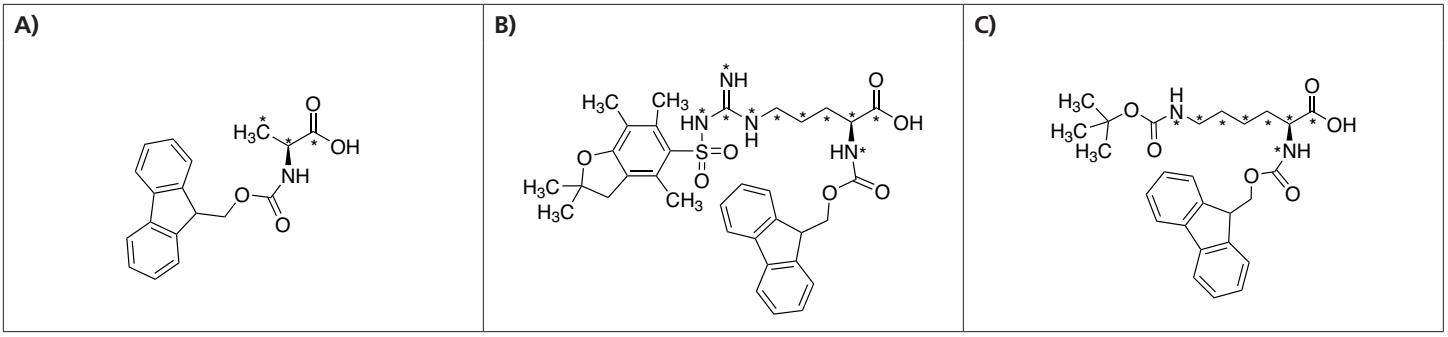


Figure 1. Protected amino acid structures. Their abbreviated descriptions are $^{13}C_3$ L-Ala Fmoc in **A)**, $^{13}C_6/^{15}N_4$ L-Arg Fmoc(Pbf) in **B)**, and $^{13}C_6/^{15}N_2$ L-Lys Fmoc/Boc in **C)**. The asterisks represent the site of isotopic labeling.

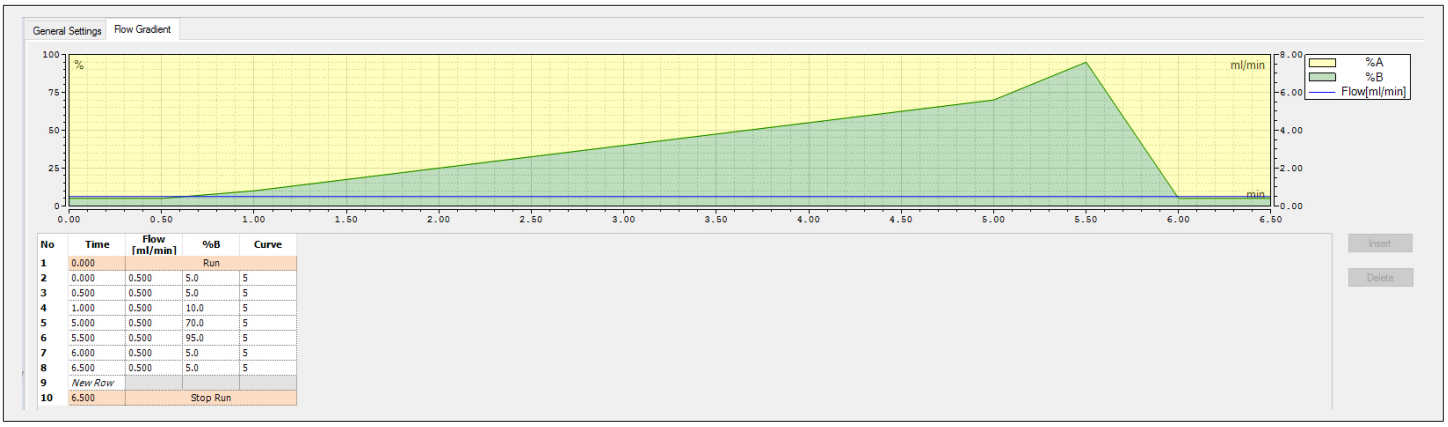
The labeled protected amino acids were stored refrigerated (-5 to 5°C) under desiccated and/or light-protected conditions, while the unlabeled protected amino acids were stored at ambient temperature.

Peptide Synthesis

Peptides ATHVGNDTTLAQIV**K** (isotopes: $^{13}C_6/^{15}N_2$ at the C-terminal L-Lys) and AYNVTQ**A**FG**R** (isotopes: $^{13}C_3$ at L-Ala and $^{13}C_6/^{15}N_4$ at L-Arg) were synthesized using Fmoc-standard solid phase synthesis. These were synthesized on the 0.1 mmol scale using the Liberty PRIME 2.0 automated microwave peptide synthesizer (CEM Corporation) and Rink Amide ProTide LL resin. Deprotection was performed with pyrrolidine in DMF. Coupling reactions were conducted with five equivalents of Fmoc-AA-OH, diisopropylcarbodiimide, and Oxyma, while labeled amino acids were coupled using two equivalents. Cleavage was performed at 38°C using a cocktail of reagents (trifluoroacetic acid, water, triisopropylsilane, and 3,6-dioxa-1,8-octanedithiol). Following cleavage, the peptides were precipitated with diethyl ether and lyophilized overnight. Aliquots of the stable isotope-labeled peptides were stored at -80°C until use.

Peptide Characterization

At the time of sample processing, a lyophilized aliquot was dissolved in deionized water (~2 mg/mL peptide concentration) and the solution placed in an autosampler vial on a Vanquish UPLC system (Thermo Fisher; Waltham, MA USA). An Acquity UPLC BEH C8 reversed-phase column (100 × 2.1 mm i.d., 1.7 µm, 130 Å; Waters Corporation, Milford, MA USA) was coupled to a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo) via an ESI source (operated in positive polarity). The column and autosampler were maintained at 40 and 4°C, respectively. Separations were performed at 0.5 mL/min with gradient elution of 0.05% trifluoroacetic acid in (i) water and (ii) acetonitrile (see **Figure 2**).



No	Time	Flow [ml/min]	%B	Curve
1	0.000	Run		
2	0.000	0.500	5.0	5
3	0.500	0.500	5.0	5
4	1.000	0.500	10.0	5
5	5.000	0.500	70.0	5
6	5.500	0.500	95.0	5
7	6.000	0.500	5.0	5
8	6.500	0.500	5.0	5
9	New Row			
10	6.500	Stop Run		

Figure 2. Screenshot of flow gradient from Chromeleon software.

Peptide Analysis
Peak data analysis was achieved with Chromeleon software (Ver. 7.0).

Guided Methods and Discussion

Peptide Selection and Labeled Standards
One of the critical aspects of experimental design in targeted bottom-up proteomic studies is the selection of proteotypic peptides as these typically function as molecular surrogates for the proteins of interest. Peptide selection can arise from literature review, *in silico* digestion, and/or empirical MS/MS measurements. Among other factors, the appropriateness of the target peptide(s) should be based on a series of sequence (e.g., be unique within a given proteome, devoid of missed tryptic cleavages, 6-25 residues in length) and residue (e.g., absence of *N*-terminal glutamine, sequential prolines, cysteine and methionine modifications) specific selection rules.[3] These guiding filters are designed to yield peptides with the greatest probability of success in synthesis, solubility, storage, proteolysis, and LC-MS processing. For improved detection confidence and measurement precision, the peptides should be stable isotope-labeled (e.g., with ^{13}C and/or ^{15}N at high isotopic/chemical purity). In application, these labeled peptides function as standards for system suitability testing and/or quantitative measurement determinations. In the two case examples spotlighted here, peptides ATHVGNDTTLAQIV**K** and AYNVTQ**A**FG**R** were selected from empirical MS/MS measurements and adhered to the abovementioned rules. These targets were synthesized in their stable isotope-labeled form (with ^{13}C and/or ^{15}N) and were used to quantify proteins[4] or qualify peptides[5] in two types of MS proteomic applications. The following sections describe an example process for their synthesis and characterization. The applications that the resultant stable isotope-labeled peptide standards were used in are briefly summarized thereafter.

Peptide Synthesis Workflow

Liberty PRIME 2.0 Synthesizer
CEM offers a complete suite of peptide synthesis reagents for optimized solid-phase peptide synthesis (SPPS) using microwave irradiation. This includes a library of standard and unique protected amino acids (unlabeled), PEG and polystyrene resins, and the powerful activator Oxyma. Using CEM's high-quality unlabeled reagents together with CIL's high-quality stable isotope-labeled protected amino acids produces high-quality labeled peptides when used in SPPS with CEM's innovative methodology and instrumentation (e.g., Liberty PRIME 2.0; see Figure 3).

Figure 3. Liberty PRIME™ 2.0 with HT24.

The Liberty PRIME 2.0 system features a unique one-pot deprotection and coupling process allowing for an ultra-fast, automated synthesis workflow that can dramatically outperform traditional parallel peptide synthesizers. The Liberty PRIME 2.0 provides the ultimate flexibility for long, difficult, or complex peptides. Additionally, The Liberty PRIME 2.0 can be utilized in cGMP peptide production. Some of the other features include:

- precise calibration-free delivery of reagents
- true internal fiber-optic temperature control
- 0.005 – 5 mmol scale range with digital N_2 control
- 30 amino acid positions
- integrated reaction vessel (RV) camera
- high-throughput upgrade options (HT4, HT12, HT24)

Peptide Synthesis on Liberty PRIME 2.0

1. Open Method Editor on the Liberty PRIME 2.0 main software screen and input the peptide sequences (e.g., ATHVGNDTTLAQIV**K** and AYNVTQ**A**FG**R** in the case here), synthesis scale (0.10 mmol) and resin type (Rink Amide ProTide LL).
2. Load the method into the resin slot of the main software screen. From there, the software automatically calculates the total amounts of reagent and wash solvent (i.e., DMF) required to synthesize a peptide (see "Usage Calculator" tab).
3. Prepare the following reagent solutions for 0.10 mmol scale peptide synthesis: Fmoc-amino acids (0.50 M in DMF), labeled Fmoc-amino acids (0.20 M in DMF), *N,N'*-diisopropylcarbodiimide (DIC; 0.75 M in DMF), Oxyma (0.26 M in DMF), and pyrrolidine (25% *v/v* in DMF).
4. Connect amino acid centrifuge tubes and reagent bottles (DIC and Oxyma) to their appropriate positions on the system. Fill the pyrrolidine and DMF bottles with the volumes specified by the Usage Calculator.
5. Weigh Fmoc-Rink amide ProTide resin (0.20 mmol/g substitution, 0.5 g) into a 50 mL centrifuge tube and load it on the high-throughput resin transfer module of the Liberty PRIME automated microwave peptide synthesizer.
6. Click the Start button on the Liberty PRIME 2.0 software main screen. **Note that peptide synthesis on the Liberty PRIME system uses CarboMAX coupling with one-pot coupling and deprotection.** The first Fmoc-deprotection step is initiated by adding 3.5 mL of DMF and 0.75 mL of 25% pyrrolidine/DMF to the resin. The reaction is continued for 40 s at 110°C before draining. This is followed by a 2 × 4 mL DMF wash. The system then performs coupling with Fmoc-amino acid (1.0 mL, 0.5 M in DMF, five equivalents) or labeled Fmoc-amino acid (1.0 mL, 0.2 M in DMF, two equivalents), DIC (1.0 mL, 0.75 M in DMF, 7.5 equivalents) and Oxyma (1.5 mL, 0.26 M in DMF, four equivalents) for 60 s at 105°C. This is followed by the initiation of deprotection by adding 0.75 mL of 25% pyrrolidine/DMF directly to the undrained post-coupling solution for 40 s at 110°C before draining. This is followed again by a 2 × 4 mL DMF wash. A special coupling cycle is used for labeled amino acid residues that includes additional 3 × 4 mL post-deprotection washings and an increased coupling time of 2 min at 105°C. The cycle involving deprotection-washing-coupling is automatically performed for all amino acid residues in the peptide sequence. A final deprotection step removes the Fmoc protecting group from the last amino acid in the sequence.
7. After the synthesis is complete, disconnect the 50 mL centrifuge tube containing the peptidyl resins from the resin transfer module and prepare for cleavage.

Peptide Cleavage on Razor Cleavage System

1. Set the Razor cleavage system temperature to 40-42°C.
2. Transfer the peptidyl resins into the reaction tube on the Razor system. Move the Drain valve knob to the OPEN position and turn the vacuum pump ON to drain any solvent from the resin.
3. Rinse the resin three times with 5 mL DCM, leaving the pump ON and the Drain knob OPEN to drain the liquid. After rinsing, turn the vacuum pump OFF and CLOSE the Drain knob.
4. Move the reaction tube containing the rinsed resin to an empty, clean position on the Razor hot block and turn the numbered valve knob to the CLOSED position.
5. Add 5 mL of cleavage cocktail [prepared by mixing TFA/Water/TIS/DODT (at 92.5%/2.5%/2.5%/2.5% *v/v/v/v*)] to the resin. Allow the resin with cleavage cocktail to remain on the Razor hot block for 30 min at 40-42°C.
6. After cleavage, turn the appropriate numbered valve knob to the OPEN position and turn the vacuum pump ON to begin draining the cleaved peptide solution into a clean 50 mL centrifuge tube.

Peptide Precipitation and Lyophilization

1. To precipitate the cleaved peptide, add up to 40 mL (or eight times the amount of drained liquid from the cleavage step) of ice-cold ether.
2. Centrifuge the peptide solution for 5 min at 3500 rpm, or until a white (or clear) peptide pellet forms on the bottom of the tube.
3. Decant the ether, leaving the precipitated peptide in the centrifuge tube.
4. The peptide can be resuspended in ice cold ether and centrifuged again. **Note that this second centrifugation step with cold ether helps remove traces of cleavage cocktail reagents and cleaved protecting groups from the crude peptide material.**
5. Solubilize the peptide gel in 10% acetic acid/deionized water and freeze with liquid nitrogen before placing on the lyophilizer overnight.

Peptide Characterization

Using the methods documented above, the Liberty PRIME 2.0 synthesizer produced labeled peptides ATHVGNDTTLAQIV**K** ($^{13}C_6/^{15}N_2$ at the C-terminal L-Lys) and AYNVTQ**A**FG**R** ($^{13}C_3$ at L-Ala and $^{13}C_6/^{15}N_4$ at L-Arg). **Figures 4** and **5** illustrate the high quality UPLC-MS results.

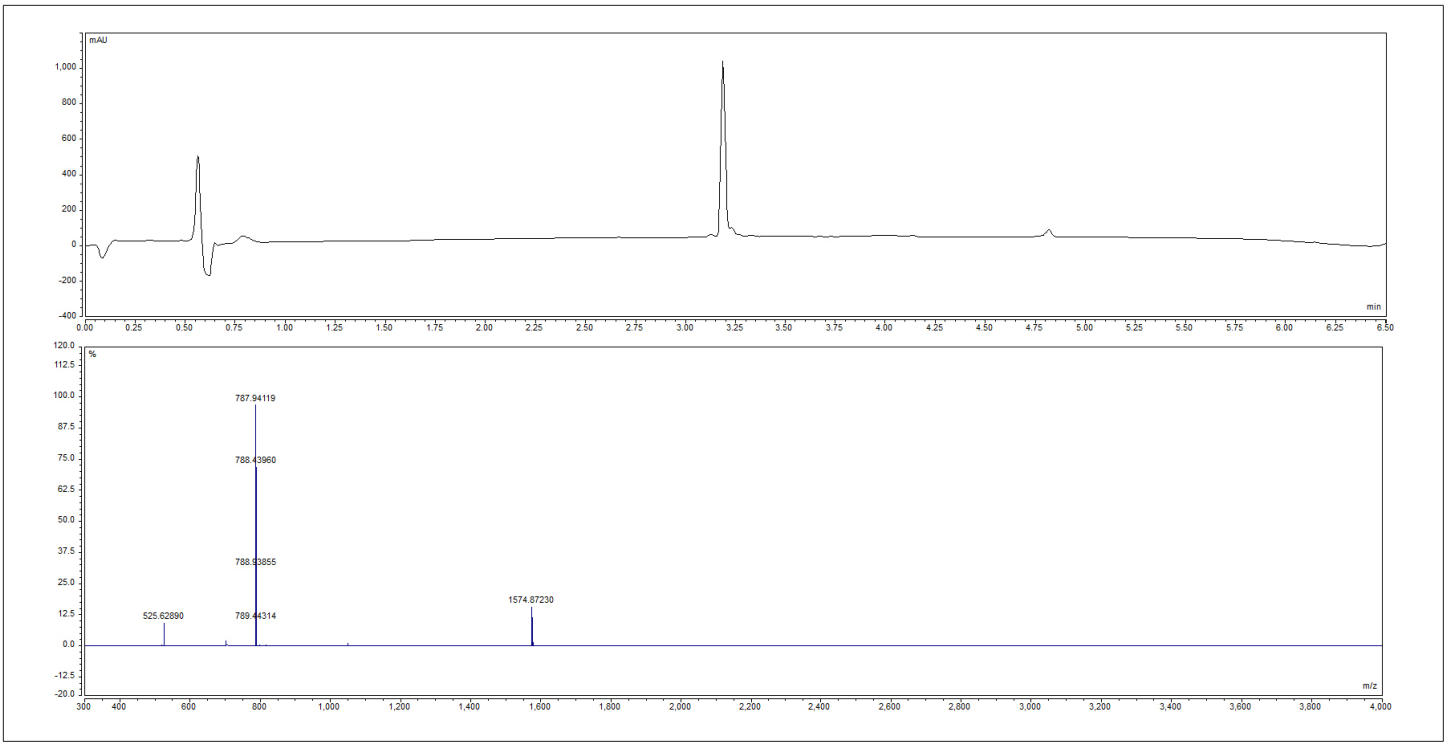


Figure 4. UPLC-MS of crude ATHVGNDTTLAQIV**K** ($^{13}C_6/^{15}N_2$ at K). The isotope-labeled peptide MW is 1574.71 Da having *m/z* values of 788.35 (2+ ion) and 525.90 (3+ ion).

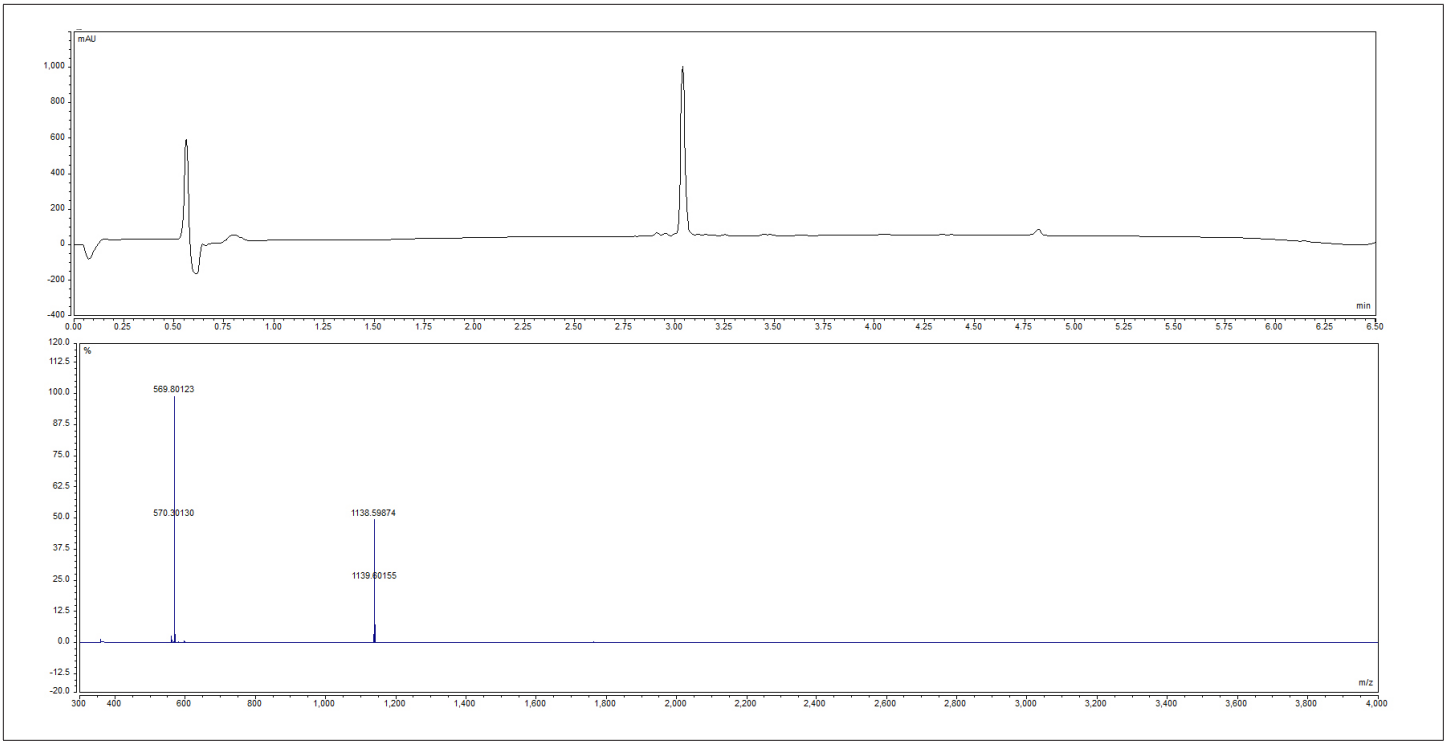


Figure 5. UPLC-MS of crude AYNVTQ**A**FG**R** ($^{13}C_3$ at A and $^{13}C_6/^{15}N_4$ at R). The isotope-labeled peptide MW is 1138.14 Da having a *m/z* value of 570.07 (2+ ion).

Spotlighted Proteomic Applications

The synthesized isotope-enriched peptide standards can be implemented in proteomic applications in different ways. A common deployment is as an internal standard (IS) in matrix-based studies for protein concentration determination. Quantitation can be performed by varying strategies, with single-point measurement and calibration curves (be it internal or external generated in a forward or reverse manner) being the two general categories.[6] In the Wilson's disease quantitative proteomics article,[4] the surrogate ATHVGNDTTLAQIV**K** peptide (residues 887-101 from copper-transporting ATPase 2) was measured by an immunoaffinity-based LC-MRM/MS method to quantify the ATP7B protein. Here, quantification was performed by peptide ratio signal analysis (i.e., of endogenous/labeled together with blood volume and IS concentration) through "single point" calculations of whole blood samples (n = 264). The results demonstrated this method to serve as a noninvasive diagnostic approach to screen Wilson's disease with high sensitivity and specificity. The accuracy of this proteomic assay helps overcome the ambiguities associated with current tests making it a viable complementary (or alternative) strategy for clinical screening laboratories.

In the COVID-19 article,[5] Pandey and colleagues developed an analytical approach involving immunoaffinity enrichment and targeted MS proteomics for the qualitative analysis of the SARS-CoV-2 nucleocapsid protein in nasopharyngeal swab samples. Two peptides, an example being AYNVTQ**A**FG**R**, served as standard surrogates in their targeted LC-PRM/MS assay. These peptides were found to be top performers (based on endogenous detectability and lack of matrix interference) in the untargeted discovery experiments and additionally were found to obey the peptide selection rules stated earlier. Stable isotope-labeled peptides were used for instrument parameter optimizations and as standard controls in retention monitoring and peak integration of the response data. As the aim of this study was qualitative, the use of the labeled peptide standards was in quality control (QC). This represents a different type of application that the synthetically produced labeled peptides can be implemented in. For further background on the use of synthetically labeled peptides in performance evaluations, the reader is referred to the Smit et al. article.[7] It should be noted that a follow-up study from the Pandey laboratory on a larger number subjects used the labeled peptide standards in calibration curves to quantify the viral nucleocapsid protein in clinical swab samples.[8]

Conclusions

The requirements and considerations behind peptide selection, synthesis, and analysis are detailed herein using peptide standard examples (specifically ATHVGNDTTLAQIV**K** and AYNVTQ**A**FG**R**; bolded residues are isotope-labeled) from two manuscripts from the proteomics field.[4,5] The methods utilized CILs stable isotope-labeled protected amino acids, with peptide synthesis performed on CEM's Liberty PRIME 2.0 synthesizer. The application of the labeled peptides in the spotlighted studies was summarized here as case examples. This article can serve as a guide to the production of proteotypic, high-quality, stable isotope-labeled standards for MS proteomic applications.

References

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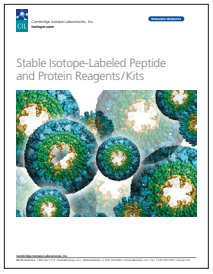
Related Products

Please refer to the proteomics catalog shown here and isotope.com for a listing of CIL's protected amino acids and preloaded resins.



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