

Non-targeted Identification of D-Amino Acid-Containing Peptides Through Enzymatic Screening, Chiral Amino Acid Analysis, and LC-MS

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Abstract

D-Amino acid-containing peptides (DAACPs) in animals are a class of bioactive molecules formed via the posttranslational modification of peptides consisting of all-L-amino acid residues. Amino acid residue isomerization greatly impacts the function of the resulting DAACP. However, because isomerization does not change the peptide's mass, this modification is difficult to detect by most mass spectrometry-based peptidomic approaches. Here we describe a method for the identification of DAACPs that can be used to systematically survey peptides extracted from a tissue sample in a non-targeted manner.

Key words D-Amino acid-containing peptides, Posttranslational modifications, Bioactive peptides, Peptide isomerization, Chirality

1 Introduction

While peptides derived from ribosomal synthesis are translated exclusively using L-amino acids, a bioactive group of D-amino acid-containing peptides (DAACPs) exists in diverse animal species [1, 2]. Formation of D-amino acid residues in animal peptides often appears to result from a posttranslational modification (PTM) in which an L-amino acid residue is enzymatically converted into a D-amino acid residue in the peptide chain. This modification has a profound impact on the structure and functional properties of a peptide, and often leads to enhanced biological activity and increased protease stability for the DAACP relative to its all-L-residue counterpart [3–8].

Despite the functional importance of peptide isomerization, this PTM is seldom explored in peptidomic studies. This is because L- to D-residue isomerization does not change the mass or chemical composition of a peptide, making it difficult to detect in mass spectrometry (MS)-based peptide characterizations. Although a number of analytical approaches have been developed for differentiating peptide diastereomers using

tandem MS (MS/MS), capillary electrophoresis, ion mobility MS, radical-directed dissociation MS, and other techniques [9–14], specific optimization of experimental conditions for a particular peptide of interest is often required. Therefore, these methods are useful for confirming the chirality of a peptide already suspected to exist as a DAACP, but are less efficient at identifying new DAACPs without prior knowledge. In this chapter we describe a non-targeted method to identify DAACPs extracted from complex biological samples [15]. This approach does not require prior knowledge about suspected DAACPs and can be applied to peptides extracted from various tissues or regions of interest across different animal models.

The first stage in this method involves a screening process based on the observation that DAACPs tend to have increased resistance to degradation by peptidases relative to their all-L-residue peptide analogs [16]. Since nearly all known DAACP neuropeptides and neurohormones have the D-residue near the N-terminus [2], an aminopeptidase should degrade these DAACPs at a slower rate than it degrades most peptides containing only L-residues. Aminopeptidase M (APM) is used in this protocol to assay complex mixtures of peptides extracted from animal tissues. By analyzing the APM reaction mixture over time using liquid chromatography coupled to mass spectrometry (LC-MS), peptides that are digested relatively slowly are marked as DAACP candidates and isolated for further study. In the second stage, purified DAACP candidates are hydrolyzed into their component amino acids, derivatized with Marfey's reagent to enhance chiral separation [17], and then analyzed with an LC-MS/MS system suitable for multiple reaction monitoring (MRM) to determine the chirality of the amino acids. If a peptide is found to contain a D-amino acid residue, the final stage is to chemically synthesize the proposed DAACP and compare its LC-MS retention time to that of the endogenous peptide. If the synthetic DAACP matches the retention time of the endogenous peptide obtained by LC-MS (and differs from that of the all-L-residue analog), then one can conclude that the endogenous peptide is indeed a DAACP. The method described is derived from our prior work characterizing DAACPs from the GFFD prohormone in *Aplysia californica* [8, 15, 18].

2 Materials

Prepare all solutions using LC-MS grade solvents, reagents and ultrapure water. Use low protein binding microcentrifuge tubes for peptide samples.

2.1 Aminopeptidase M Digestion

1. Aminopeptidase M, E.C. number 3.4.11.2, activity ≥ 50 units/mL.
2. Reaction buffer: 25 mM Tris-HCl, 0.5 M NaCl, pH 7.5.
3. pH paper.
4. 37 °C water bath or incubator.
5. Reversed-phase C18 solid-phase extraction columns for sample cleanup (e.g., ZipTip_{C18} pipette tips or C18 spin columns). Wetting solution: 100% acetonitrile (ACN). Equilibration solution: 0.1% trifluoroacetic acid (TFA) in water. Wash solution: 5% methanol in water, 0.1% TFA. Elution solution: 50% ACN in water, 0.1% formic acid.
6. LC-MS/MS system for peptide characterization and for structure confirmation; e.g., a nanoLC system (we use Dionex Ultimate, Thermo Fisher Scientific) coupled to a QTOF mass spectrometer (we use Impact, Bruker).
7. Data analysis software for peptide identification; e.g., PEAKS Studio (Bioinformatics Solutions Inc.).

2.2 Chiral Amino Acid Analysis

1. Microwave reactor, e.g., Discover (CEM Corporation), with fiber optic temperature probe for acid hydrolysis, valve panel connected to a nitrogen source and a vacuum source, with vacuum gauge and pressure sensor, and Teflon PFA vessel with microvial insert tray and tubing.
2. 300 μ L glass sample vials.
3. 200 μ L extra-long pipet tips.
4. 6 N DCl with 1% phenol: add 2.5 mL of concentrated DCl to 2.5 mL of 2% (w/w) phenol dissolved in D₂O (*see Note 1*).
5. Centrifugal evaporator; e.g., Savant SpeedVac (Thermo Scientific).
6. 0.5 M NaHCO₃ solution in water.
7. Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide), 1 mg/mL dissolved in ACN.
8. Standard amino acids mixture: a 200 μ M (total) mixture of glycine plus the L- and D-forms of the other 19 proteinogenic amino acids.
9. Mixing block with temperature control.
10. LC-MS/MS-MRM system; e.g., an UHPLC system (we use Advance, Bruker) coupled to a triple quadrupole mass spectrometer (we use EVOQ, Bruker).
11. LC column suitable for separating aromatic hydrocarbons; e.g., a phenyl-hexyl column (we use Kinetex, Phenomenex).
12. LC solvents: Solvent A: 25 mM ammonium formate in water; Solvent B: methanol.

3 Methods

3.1 Aminopeptidase M Digestion

1. Mix 15 μL reaction buffer with 5 μL peptide sample (5–10 μg in aqueous solution) in a low protein binding microcentrifuge tube. Check the pH of the solution with pH paper and adjust to $\sim\text{pH}$ 7.5 using 0.1 M HCl or 0.1 M NaOH solutions. Add 1 μL of APM (at 60 U/mL) and pipette up and down to mix. Save half of the mixture as the “0 h” sample and incubate the other half of the mixture at 37 $^{\circ}\text{C}$ for 24 h. For the 0 h sample, proceed immediately to the next step; for the 24 h sample, perform sample cleanup after incubation (*see Note 2*).
2. Sample cleanup by solid-phase extraction using reversed-phase C18 pipette tips: adjust sample to 0.1% TFA, $\text{pH} < 4$. Attach pipette tip to a pipettor set at 10 μL maximum volume. Wash the tip twice by slowly aspirating the wetting solution, then dispense to waste. Repeat twice with the equilibration solution. Aspirate and dispense the sample for 10 cycles to bind peptides to the pipette tip. Wash the tip twice with the wash solution to remove salts, contaminants, and unbound molecules. Elute the purified peptide sample in 5 μL of elution solution. Samples can be stored at -20°C (*see Note 3*).
3. Analyze samples with the LC-MS/MS system for peptide characterization. Use the data analysis software to identify peptide sequences through database searching and/or *de novo* sequencing.
4. Compare peptide content between the 0 and 24 h APM digestion samples to identify peptides that reproducibly show resistance to digestion by APM as possible DAACP candidates (Fig. 1) (*see Note 4*).
5. Isolate DAACP candidates using successive rounds of HPLC purifications. Detailed strategies vary with each specific peptide. Different LC gradients, solvents, and column binding properties are commonly employed to achieve peptide isolation [19, 20]. At least 5 nmol of each peptide should be isolated (purity $>80\%$) for the next stage of analysis (*see Note 5*).

3.2 Chiral Amino Acid Analysis

1. Place each glass vial inside a 1.5 mL microcentrifuge tube for easy handling. Transfer peptide sample (1–5 nmol in solution) into a glass vial, then evaporate the solvent using a centrifugal evaporator (*see Note 6*).
2. Add 5 mL of 6 N DCl with 1% phenol to the bottom of the Teflon PFA vessel. Place glass vials containing the dried peptide samples into the microvial insert tray, and place the tray inside the vessel body. Assemble the vessel and seal tightly, then connect the vessel to the valve panel and place it into the microwave

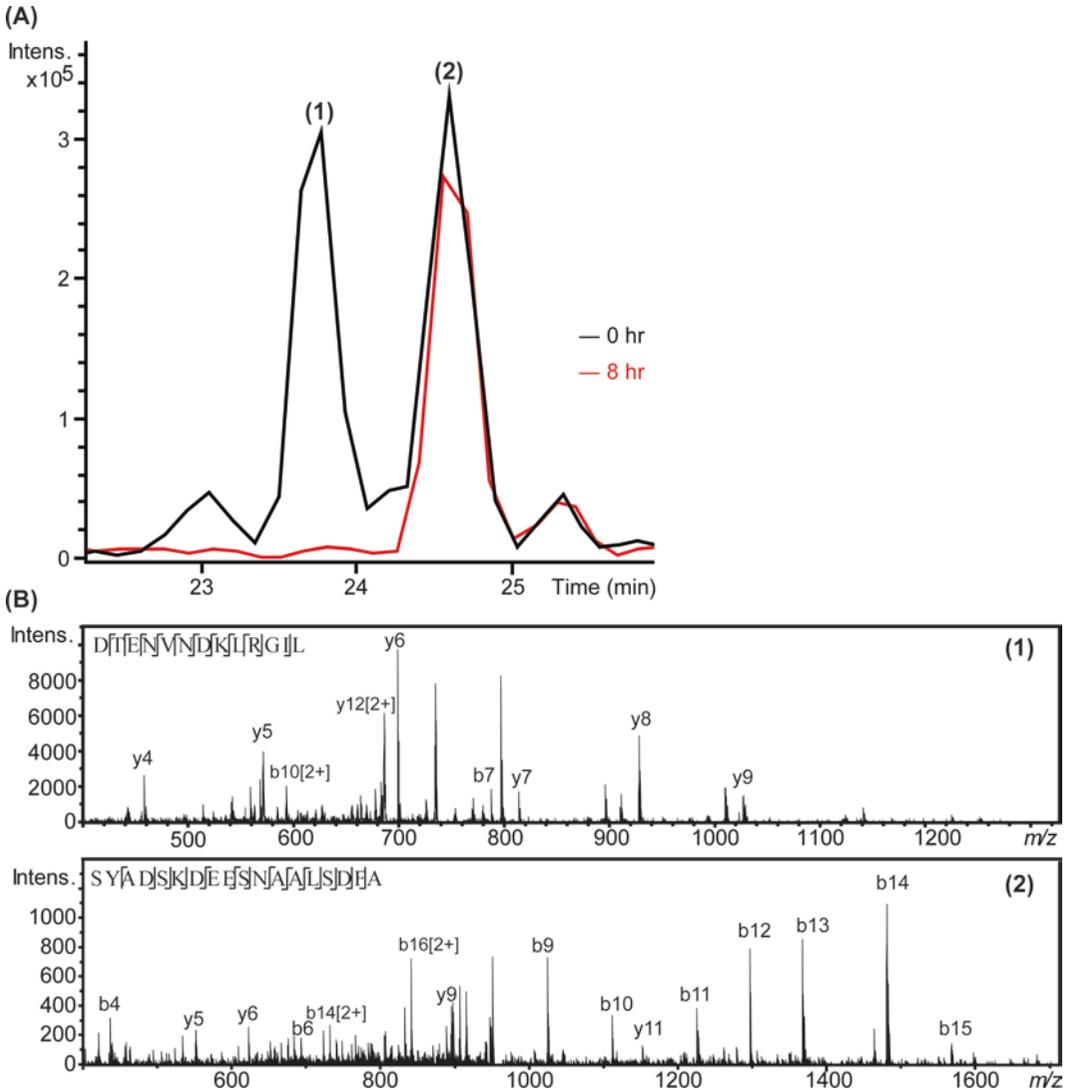


Fig. 1 Identify peptides that are resistant to APM by comparing peptide signals before (black chromatogram) and after (red chromatogram) digestion. In this example, an extracted peptide mixture from *Aplysia* ganglia was subjected to APM digestion for 8 h at 37 °C. (a) LC-MS results showed that peptide (1) was degraded while peptide (2) remained at similar levels throughout the APM digestion. Therefore, peptide (2) becomes a putative DAACP candidate and was isolated for further analysis. (b) The identities of peptides (1) and (2) were determined based on MS/MS data in a database search

reactor cavity. The valve panel should be connected to a nitrogen source (set at 15 psi) and a vacuum source. Flush the vessel with nitrogen for 10 s, then evacuate down to -20 mmHg. Repeat for three cycles. Adjust the final pressure to 5–10 psi of nitrogen in the vessel. Insert the fiber optic temperature probe (see **Note 7**).

- Run the microwave reactor using the following conditions: power = 200 W, pressure limit = 120 psi, temperature = 165 °C,

and hold time = 15 min (power and pressure settings may need to be adjusted based on the specific instrument used). At the end of the hydrolysis run, allow the vessel to cool before disassembling.

4. Remove glass vials from the vessel using forceps and place them back into 1.5 mL microcentrifuge tubes. Dry sample vials in the centrifugal evaporator to remove any solvent introduced to the sample during the hydrolysis.
5. Using extra-long pipet tips, add 25 μL of 0.5 M NaHCO_3 solution to each glass vial to redissolve the amino acid hydrolysate. Transfer samples to 0.5 mL microcentrifuge tubes and add in 20 μL of 1 mg/mL Marfey's reagent in ACN. In a separate vial, also prepare a sample consisting of 25 μL of the standard amino acids mixture and 20 μL of Marfey's reagent. Place tubes in a heated mixing block at 60 $^\circ\text{C}$ for 3 h under gentle mixing.
6. Separately prepare amino acid standards derivatized with Marfey's reagent and use them to establish MRM channels for each derivatized amino acid on the LC-MS-MRM system. Ensure that the LC method is capable of separating the L- and D-forms of every derivatized amino acid. Our setup uses a phenyl-hexyl column (2.6 μm particle size, 100 \AA pore size, 100 \times 2.1 mm, Phenomenex Kinetex) with a gradient elution using 25 mM ammonium formate as Solvent A and methanol as Solvent B, at a flow rate of 300 $\mu\text{L}/\text{min}$. The gradient is as follows: 5% B for 2 min, 5–15% B over 5 min, 15–60% B over 5 min, 60% B for 3 min, 60–100% B over 3 min, 100% B for 3 min, 100–5% B over 1 min, and 5% B for 2 min (*see Note 8*).
7. Analyze the derivatized amino acid hydrolysate samples on the LC-MS/MS-MRM system. Also include a run of the standard amino acid mixture in the same batch. Determine the chirality of each amino acid residue from a peptide sample by matching the peak retention time from the experimental sample to the retention times of the L- or D-amino acid standards (Fig. 2). For peptides that are suspected to contain a D-amino acid residue based on the chiral amino acid analysis, proceed to the next stage for final structure confirmation.

3.3 LC-MS for Structure Confirmation

1. Synthesize both the all-L-residue peptide and the proposed DAACP through commercial custom peptide synthesis or in-house synthesis (*see Note 9*).
2. Develop an LC method that separates the two peptide isomers on the LC-MS/MS system for structure confirmation.
3. Analyze each synthetic peptide separately on this LC-MS/MS system to establish retention times, MS, and MS/MS data for these standards. Analyze a sample of the endogenous peptide on the LC-MS/MS system. The existence of an endogenous

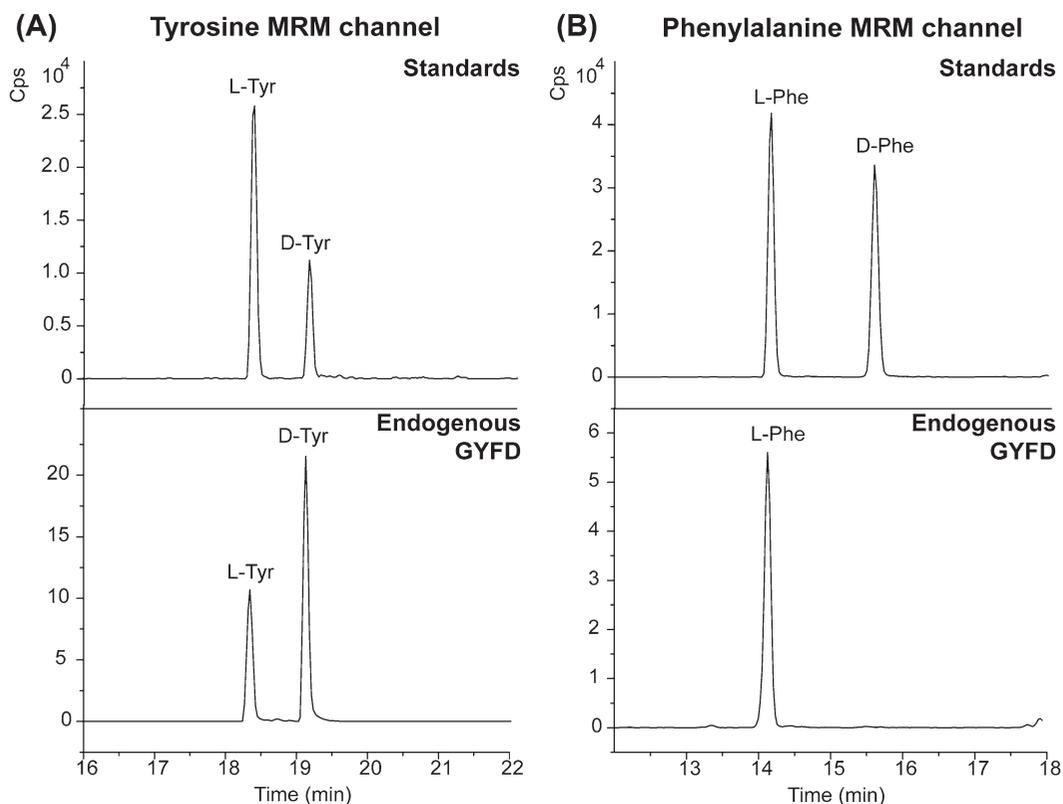


Fig. 2 Chiral amino acid analysis determines the chirality of amino acid residues in a candidate peptide. In this example of a chiral analysis on the extracted *Aplysia* peptide GYFD, D-tyrosine was detected in the tyrosine MRM channel (a) while only L-phenylalanine was detected in the phenylalanine MRM channel (b). This suggests that the endogenous peptide exists as GdYFD

DAACP can be confirmed if the retention time, mass, and MS/MS fragmentation pattern of the endogenous peptide matches that of the synthetic DAACP (Fig. 3) [15].

4 Notes

1. Prepare this solution immediately before use. “D” refers to deuterium (i.e., ^2H). DCl and D_2O are used in place of HCl and H_2O so that D-amino acids resulting from spontaneous racemization during acid hydrolysis would have a mass shift of +1 Dalton and thus be distinguished from any D-amino acid residue originally present in the peptide [21]. Phenol is added to prevent degradation of tryptophan and other amino acids [22].
2. The peptide sample should be prepared from peptides extracted from a target tissue using a suitable peptide extraction procedure, dried, and then reconstituted in aqueous solution. Peptide extraction protocols differ based on the tissue

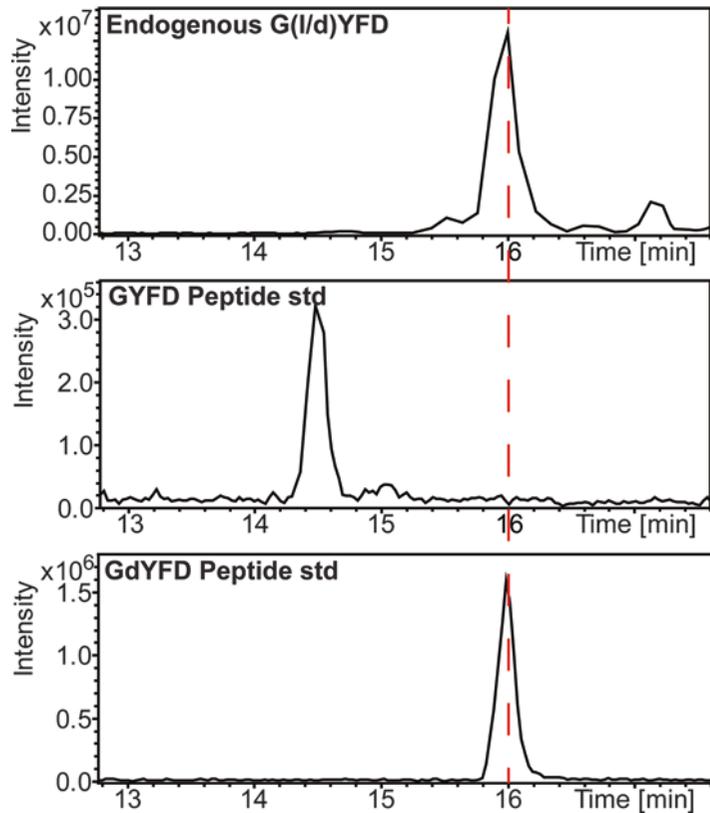


Fig. 3 Structure confirmation using synthetic peptide standards. LC-MS/MS analysis showed that the retention time (base peak chromatograms shown above) of the endogenous peptide, after 48 h of APM digestion, matches that of the synthetic DAACP standard, thereby confirming the structure of the endogenous peptide as GdYFD. (Adapted with permission from ref. [15], Copyright 2016 American Chemical Society)

type and are not described here. Total peptide concentration may be measured with a commercial kit (e.g., Pierce Quantitative Colorimetric Peptide Assay). One or more all-L-residue peptide standards (avoid peptides with pyroglutamate or proline near the N-terminus since these peptides may have higher resistance to APM) and DAACP standards (e.g., deltorphin) may be spiked into the sample as controls for enzyme activity. Choose peptides that are not endogenous to the biological sample for these controls. Each all-L-residue peptide standard should be present in the analysis of the 0 h time point but absent after 24 h. Failure to degrade all-L-residue standards indicates insufficient APM activity. Reaction conditions (APM concentration, digestion time) should then be adjusted accordingly. Each DAACP standard should be present at the 24 h time point at concentrations similar to

those seen at the 0 h time point. A dramatic loss of DAACP standards in the 24 h time point indicates excess APM or digestion time is being used. Test the activity of each batch of APM and adjust the enzyme concentration and reaction time to allow for full hydrolysis of all-L-residue standards while leading to minimal degradation of DAACP standards.

3. A 2% solution of aqueous TFA can be used to adjust the sample to a final concentration of 0.1% TFA. The low pH promotes peptide binding to the C18 stationary phase of the pipette tip, and also inactivates APM to stop the digestion reaction. Sample cleanup by solid-phase extraction is intended to desalt, purify, and concentrate peptide samples prior to LC-MS analysis. The capacity of a ZipTip_{C18} pipette tip is typically 5 µg. If higher binding capacity or higher loading volume (>15 µL) is needed, a good alternative is the Pierce C18 spin column, which can process samples of 10–150 µL and has a peptide binding capacity of up to 30 µg.
4. Note that not all APM-resistant peptides are expected to be DAACPs. Some N-terminal modifications (e.g., pyroglutamylation, acetylation) and certain sequences (e.g., proline residues near the N-terminus) can also increase a peptide's resistance to APM digestion [23]. It is also possible that some DAACPs may be degraded by APM, depending on the position of the D-residue in the peptide sequence. DAACPs with a D-residue far from the N-terminus may be degraded in this screening procedure (although they may give rise to truncated peptide fragments as APM stalls its digestion near the D-residue).
5. Peptide concentration can be estimated by UV absorbance at 280 nm (for peptides containing tryptophan or tyrosine), 214 nm, or 205 nm [24–26], or with a commercial peptide assay kit (e.g., Pierce Quantitative Colorimetric Peptide Assay). In addition, MALDI-TOF MS may be used to assess the integrity and purity of the isolated peptide. The peptide purity needed for chiral analysis is flexible and can be determined on a case by case basis. If other peptides in the sample do not contain the amino acid residue suspected to be a D-amino acid, then these impurities may not interfere with interpretation of the chiral analysis data and a lower purity is acceptable. On the other hand, if the residue in question is present in multiple peptides in the sample, a higher purity might be desired at this stage to avoid testing a large number of peptide conformations in the confirmation stage.
6. Transfer peptide sample in a solution of 20–100 µL so that the peptide sample is evenly distributed around the bottom of the vial rather than in a small clump; this allows for a more complete hydrolysis of the peptides.

7. To check if the hydrolysis vessel is sealed properly after connecting the tubing, fill the vessel with nitrogen (15 psi), then turn the valve to the “run” position. Make sure the pressure reading is stable (for 30 s) before moving on to the vacuum/nitrogen cycle. If the pressure drops while the valve is in the “run” position, check and tighten the ferrules, cap, and connections.
8. MRM parameters will vary with the MS instrument. Table 1, adapted from prior work [15], shows the MRM transition, collision energy, and ionization mode of each derivatized amino acid analyzed on a Bruker EVOQ triple quadrupole mass spectrometer using a heated electrospray ionization source with the following settings: spray voltage at 3500 V, cone temperature at 250 °C, cone gas flow at 20 units, probe

Table 1
MRM parameters for amino acids derivatized with Marfey's reagent

Amino acid	Parent ion (<i>m/z</i>)	Collision energy (eV)	Fragment ion (<i>m/z</i>)	Mode
Alanine	340.0	14	278.1	–
Arginine	426.9	10	70.1	+
Asparagine	382.9	33	175.9	–
Aspartic acid	384.0	24	267.9	–
Cysteine	372.1	21	284.9	–
Glutamic acid	398.0	24	202.0	–
Glutamine	396.9	20	353.0	–
Glycine	326.1	32	162.0	–
Histidine	658.2	30	549.1	–
Isoleucine/Leucine	382.0	18	319.8	–
Lysine	649.1	36	479.1	–
Methionine	400.0	17	337.9	–
Phenylalanine	416.0	20	337.1	–
Proline	365.9	15	321.9	–
Serine	355.9	16	263.9	–
Threonine	370.0	17	263.9	–
Tryptophan	457.1	10	188.0	+
Tyrosine(+2 Da) ^a	686.1	28	353.9	–
Valine	367.9	15	306.0	–

^aDerivatized tyrosine was observed with a mass shift of +2 Da following DCI hydrolysis (but not HCl hydrolysis), perhaps due to the incorporation of deuterons at positions *ortho* to the phenolic hydroxyl group [27]. (Table adapted with permission from ref. [15], Copyright 2016 American Chemical Society)

temperature at 400 °C, and probe gas flow at 45 units. These parameters may be used as a starting point for new method development.

9. Since chiral amino acid analysis does not provide information about the position of the D-residue in the peptide, multiple conformations may need to be synthesized. For example, if a D-phenylalanine signal was detected during chiral analysis, but the peptide sequence has more than one phenylalanine near the N-terminus, then peptide structures with D-phenylalanine at each position should be considered.

Acknowledgments

This work was supported by the National Institutes of Health, Award No. P30 DA018310 from the National Institute on Drug Abuse (NIDA) and Award No. 2 R01NS031609 from the National Institute of Neurological Disorders and Stroke (NINDS). J.W.C. was supported in part by a Beckman Institute Postdoctoral Fellowship, funded by a Beckman Foundation gift to the Beckman Institute for Advanced Science and Technology at the University of Illinois at Urbana-Champaign. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies.

References

1. Bai L, Sheeley S, Sweedler JV (2009) Analysis of endogenous D-amino acid-containing peptides in Metazoa. *Bioanal Rev* 1(1):7–24. <https://doi.org/10.1007/s12566-009-0001-2>
2. Ollivaux C, Soye D, Toullec JY (2014) Biogenesis of D-amino acid containing peptides/proteins: where, when and how? *J Pept Sci* 20(8):595–612. <https://doi.org/10.1002/psc.2637>
3. Richter K, Egger R, Kreil G (1987) D-alanine in the frog skin peptide dermorphin is derived from L-alanine in the precursor. *Science* 238(4824):200–202
4. Kamatani Y, Minakata H, Kenny PT, Iwashita T, Watanabe K, Funase K, Sun XP, Yongsiri A, Kim KH, Novales-Li P et al (1989) Achatin-I, an endogenous neuroexcitatory tetrapeptide from *Achatina fulica* Ferussac containing a D-amino acid residue. *Biochem Biophys Res Commun* 160(3):1015–1020
5. Ohta N, Kubota I, Takao T, Shimonishi Y, Yasuda-Kamatani Y, Minakata H, Nomoto K, Muneoka Y, Kobayashi M (1991) Fulicin, a novel neuropeptide containing a D-amino acid residue isolated from the ganglia of *Achatina fulica*. *Biochem Biophys Res Commun* 178(2):486–493. [https://doi.org/10.1016/0006-291X\(91\)90133-R](https://doi.org/10.1016/0006-291X(91)90133-R)
6. Buczek O, Yoshikami D, Bulaj G, Jimenez EC, Olivera BM (2005) Post-translational amino acid isomerization: a functionally important D-amino acid in an excitatory peptide. *J Biol Chem* 280(6):4247–4253. <https://doi.org/10.1074/jbc.M405835200>
7. Ollivaux C, Gallois D, Amiche M, Boscameric M, Soye D (2009) Molecular and cellular specificity of post-translational aminoacyl isomerization in the crustacean hyperglycaemic hormone family. *FEBS J* 276(17):4790–4802. <https://doi.org/10.1111/j.1742-4658.2009.07180.x>
8. Bai L, Livnat I, Romanova EV, Alexeeva V, Yau PM, Vilim FS, Weiss KR, Jing J, Sweedler JV (2013) Characterization of GdFFD, a D-amino acid-containing neuropeptide that functions as an extrinsic modulator of the *Aplysia* feeding circuit. *J Biol Chem* 288(46):32837–32851. <https://doi.org/10.1074/jbc.M113.486670>

9. Bai L, Romanova EV, Sweedler JV (2011) Distinguishing endogenous D-amino acid-containing neuropeptides in individual neurons using tandem mass spectrometry. *Anal Chem* 83(7):2794–2800. <https://doi.org/10.1021/ac200142m>
10. Sachon E, Clodic G, Galanth C, Amiche M, Ollivaux C, Soyez D, Bolbach G (2009) D-amino acid detection in peptides by MALDI-TOF-TOF. *Anal Chem* 81(11):4389–4396. <https://doi.org/10.1021/ac9002886>
11. Koehbach J, Gruber CW, Becker C, Kreil DP, Jilek A (2016) MALDI TOF/TOF-based approach for the identification of D-amino acids in biologically active peptides and proteins. *J Proteome Res* 15(5):1487–1496. <https://doi.org/10.1021/acs.jproteome.5b01067>
12. Sheeley SA, Miao H, Ewing MA, Rubakhin SS, Sweedler JV (2005) Measuring D-amino acid-containing neuropeptides with capillary electrophoresis. *Analyst* 130(8):1198–1203. <https://doi.org/10.1039/b504717j>
13. Jia C, Lietz CB, Yu Q, Li L (2014) Site-specific characterization of D-amino acid containing peptide epimers by ion mobility spectrometry. *Anal Chem* 86(6):2972–2981. <https://doi.org/10.1021/ac4033824>
14. Tao Y, Quebbemann NR, Julian RR (2012) Discriminating D-amino acid-containing peptide epimers by radical-directed dissociation mass spectrometry. *Anal Chem* 84(15):6814–6820. <https://doi.org/10.1021/ac3013434>
15. Livnat I, Tai HC, Jansson ET, Bai L, Romanova EV, Chen TT, Yu K, Chen SA, Zhang Y, Wang ZY, Liu DD, Weiss KR, Jing J, Sweedler JV (2016) A D-amino acid-containing neuropeptide discovery funnel. *Anal Chem* 88(23):11868–11876. <https://doi.org/10.1021/acs.analchem.6b03658>
16. Ewing MA, Wang J, Sheeley SA, Sweedler JV (2008) Detecting D-amino acid-containing neuropeptides using selective enzymatic digestion. *Anal Chem* 80(8):2874–2880. <https://doi.org/10.1021/ac7025173>
17. Bhushan R, Bruckner H (2004) Marfey's reagent for chiral amino acid analysis: a review. *Amino Acids* 27(3–4):231–247. <https://doi.org/10.1007/s00726-004-0118-0>
18. Yang CY, Yu K, Wang Y, Chen SA, Liu DD, Wang ZY, YN Su, Yang SZ, Chen TT, Livnat I, Vilim FS, Cropper EC, Weiss KR, Sweedler JV, Jing J (2016) Aplysia locomotion: network and behavioral actions of GdFFD, a D-amino acid-containing neuropeptide. *PLoS One* 11(1):e0147335. <https://doi.org/10.1371/journal.pone.0147335>
19. Finoulst I, Pinkse M, Van Dongen W, Verhaert P (2011) Sample preparation techniques for the untargeted LC-MS-based discovery of peptides in complex biological matrices. *J Biomed Biotechnol* 2011:245291. <https://doi.org/10.1155/2011/245291>
20. Conlon JM (2007) Purification of naturally occurring peptides by reversed-phase HPLC. *Nat Protoc* 2(1):191–197. <https://doi.org/10.1038/nprot.2006.437>
21. Liardon R, Ledermann S, Ott U (1981) Determination of D-amino acids by deuterium labelling and selected ion monitoring. *J Chromatogr A* 203:385–395. [https://doi.org/10.1016/S0021-9673\(00\)80309-X](https://doi.org/10.1016/S0021-9673(00)80309-X)
22. Fountoulakis M, Lahm HW (1998) Hydrolysis and amino acid composition of proteins. *J Chromatogr A* 826(2):109–134
23. Turner AJ (2013) Chapter 79—Aminopeptidase N In: Rawling ND, Salvesen G (eds) *Handbook of Proteolytic Enzymes*. Academic Press, London, pp 397–403. <https://doi.org/10.1016/B978-0-12-382219-2.00079-X>
24. Gill SC, von Hippel PH (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem* 182(2):319–326. [https://doi.org/10.1016/0003-2697\(89\)90602-7](https://doi.org/10.1016/0003-2697(89)90602-7)
25. Conibear AC, Daly NL, Craik DJ (2012) Quantification of small cyclic disulfide-rich peptides. *Biopolymers* 98(6):518–524. <https://doi.org/10.1002/bip.22121>
26. Anthis NJ, Clore GM (2013) Sequence-specific determination of protein and peptide concentrations by absorbance at 205 nm. *Protein Sci* 22(6):851–858. <https://doi.org/10.1002/pro.2253>
27. Murai Y, Wang L, Masuda K, Sakihama Y, Hashidoko Y, Hatanaka Y, Hashimoto M (2013) Rapid and controllable hydrogen/deuterium exchange on aromatic rings of α -amino acids and peptides. *Eur J Org Chem* 2013(23):5111–5116. <https://doi.org/10.1002/ejoc.201300405>