Journal of Visualized Experiments

Multi-Faceted Mass Spectrometric Investigation of Neuropeptides in Callinectes sapidus

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63322R2
Full Title:	Multi-Faceted Mass Spectrometric Investigation of Neuropeptides in Callinectes sapidus
Corresponding Author:	Lingjun Li UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	lingjun.li@wisc.edu
Order of Authors:	Ashley Phetsanthad
	Nhu Vu
	Lingjun Li
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Chemistry
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Madison, Wisconsin, USA
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TITLE:

Multi-Faceted Mass Spectrometric Investigation of Neuropeptides in *Callinectes sapidus*

AUTHORS AND AFFILIATIONS:

Ashley Phetsanthad[1,*], Nhu Q. Vu[1,*], Lingjun Li[1,2]

[1]Department of Chemistry, University of Wisconsin-Madison, 1101 University Avenue, Madison, WI, USA

[2]School of Pharmacy, University of Wisconsin-Madison, 777 Highland Avenue, Madison, WI, USA

[*]These authors contributed equally.

Email addresses of co-authors:

Ashley Phetsanthad (phetsanthad@wisc.edu)

Nhu Q. Vu (nqvu@wisc.edu)

Lingjun Li (lingjun.li@wisc.edu)

Corresponding author:

Lingjun Li (lingjun.li@wisc.edu)

KEYWORDS:

neuropeptide, mass spectrometry, peptide purification, isotopic labeling, *de novo* sequencing, quantitation, LC, MALDI, mass spectrometry imaging, database search, transcriptome mining

SUMMARY:

Mass spectrometric characterization of neuropeptides provides sequence, quantitation, and localization information. This optimized workflow is not only useful for neuropeptide studies, but also other endogenous peptides. The protocols provided here describe sample preparation, MS acquisition, MS analysis, and database generation of neuropeptides using LC-ESI-MS, MALDI-MS spotting, and MALDI-MS imaging.

ABSTRACT:

Neuropeptides are signaling molecules that regulate almost all physiological and behavioral processes, such as development, reproduction, food intake, and response to external stressors. Yet, the biochemical mechanisms and full complement of neuropeptides and their functional roles remain poorly understood. Characterization of these endogenous peptides is hindered by the immense diversity within this class of signaling molecules. Additionally, neuropeptides are bioactive at concentrations 100x – 1000x lower than that of neurotransmitters and are prone to enzymatic degradation after synaptic release. Mass spectrometry (MS) is a highly sensitive analytical tool that can identify, quantify, and localize analytes without comprehensive *a priori* knowledge. It is well-suited for globally profiling neuropeptides and aiding in the discovery of novel peptides. Due to the low abundance and high chemical diversity of this class of peptides, several sample preparation methods, MS acquisition parameters, and data analysis strategies

have been adapted from proteomics techniques to allow optimal neuropeptide characterization. Here, methods are described for isolating neuropeptides from complex biological tissues for sequence characterization, quantitation, and localization using liquid chromatography (LC)-MS and matrix-assisted laser desorption/ionization (MALDI)-MS. A protocol for preparing a neuropeptide database from the blue crab*, Callinectes sapidus*, an organism without comprehensive genomic information, is included. These workflows can be adapted to study other classes of endogenous peptides in different species using a variety of instruments.

INTRODUCTION:

The nervous system is complex and requires a network of neurons to transmit signals throughout an organism. The nervous system coordinates sensory information and biological response. The intricate and convoluted interactions involved in signal transmission require many different signaling molecules such as neurotransmitters, steroids, and neuropeptides. As neuropeptides are the most diverse and potent signaling molecules that play key roles in activating physiological responses to stress and other stimuli, it is of interest to determine their specific role in these physiological processes. Neuropeptide function is related to their amino acid structure, which determines mobility, receptor interaction, and affinity[1]. Techniques such as histochemistry, which is important because neuropeptides can be synthesized, stored, and released in different regions of the tissue, and electrophysiology has been employed to investigate neuropeptide structure and function[2–4], but these methods are limited by throughput and specificity to resolve the vast sequence diversity of neuropeptides.

Mass spectrometry (MS) enables the high throughput analysis of neuropeptide structure and abundance. This can be performed through different MS techniques, most commonly liquid chromatography-electrospray ionization MS (LC-ESI-MS)[5] and matrix-assisted laser desorption/ionization MS (MALDI-MS)[6]. Utilizing high accuracy mass measurements and MS fragmentation, MS provides the ability to assign amino acid sequence and post-translational modification (PTM) status to neuropeptides from complex mixtures without *a priori* knowledge to aid in ascertaining their function[7,8]. In addition to qualitative information, MS enables quantitative information of neuropeptides through label-free quantitation (LFQ) or label-based methods such as isotopic or isobaric labeling[9]. The main advantages of LFQ include its simplicity, low cost of analysis, and decreased sample preparation steps which can minimize sample loss. However, the disadvantages of LFQ include increased instrument time costs as it requires multiple technical replicates to address quantitative error from run-to-run variability. This also leads to a decreased ability to accurately quantify small variations. Label-based methods are subjected to less systematic variation as multiple samples can be differentially labeled using a variety of stable isotopes, combined into one sample, and analyzed through mass spectrometry simultaneously. This also increases throughput, although isotopic labels can be time consuming and costly to synthesize or purchase. Full scan mass spectra (MS1) spectral complexity also increases as multiplexing increases, which decreases the number of unique neuropeptides able to be fragmented and therefore, identified. Conversely, isobaric labeling does not increase spectral complexity at the MS1 level, although it introduces challenges for low abundance analytes such as neuropeptides. As isobaric quantitation is performed at the fragment ion mass spectra (MS2) level, low-abundance neuropeptides may be unable to be quantified as more

abundant matrix components may be selected for fragmentation and those selected may not have high enough abundance to be quantified. With isotopic labeling, quantitation can be performed on every identified peptide.

In addition to identification and quantification, localization information can be obtained by MS through MALDI-MS imaging (MALDI-MSI)[10]. By rastering a laser across a sample surface, MS spectra can be compiled into a heat map image for each *m/z* value. Mapping transient neuropeptide signal intensity in different regions across conditions can provide valuable information for function determination[11]. Localization of neuropeptides is especially important because neuropeptide function may differ depending on location[12].

Neuropeptides are found in lower abundance *in vivo* than other signaling molecules, such as neurotransmitters, and thus require sensitive methods for detection[13]. This can be achieved through the removal of higher abundance matrix components, such as lipids [11,14]. Additional considerations for the analysis of neuropeptides need to be made when compared to common proteomics workflows, mainly because most neuropeptidomic analyses omit enzymatic digestion. This limits software options for neuropeptide data analysis as most were built with algorithms based on proteomics data and protein matches informed by peptide detection. However, many software such as PEAKS[15] is more suited to neuropeptide analysis due to their *de novo* sequencing capabilities. Several factors need to be considered for the analysis of neuropeptides starting from extraction method to MS data analysis.

The protocols described here include methods for sample preparation and dimethyl isotopic labeling, data acquisition, and data analysis of neuropeptides by LC-ESI-MS, MALDI-MS, and MALDI-MSI. Through representative results from several experiments, the utility and ability of these methods to identify, quantify, and localize neuropeptides from blue crabs, *Callinectes sapidus*, is demonstrated. To better understand the nervous system, model systems are commonly used. Many organisms do not have a fully sequenced genome available, which prevents comprehensive neuropeptide discovery at the peptide level. In order to mitigate this challenge, a protocol for identifying novel neuropeptides and transcriptome mining to generate databases for organisms without complete genome information is included. All protocols presented here can be optimized for neuropeptide samples from any species, as well as applied for the analysis of any endogenous peptides.

PROTOCOL:

All tissue sampling described was performed in compliance with the University of Wisconsin-Madison guidelines.

1 LC-ESI-MS analysis of neuropeptides

1.1 Neuropeptide extraction and desalting

1.1.1 Prior to tissue acquisition, prepare acidified methanol (acMeOH) (90:9:1 MeOH:water:acetic acid) as described in[16].

1.1.2 Collect brain tissue from the crustacean[17] and use forceps to immediately place one tissue each in a 0.6 mL tube containing 20 µL of acMeOH.

NOTE: Tissue dissection protocols vary greatly for different animals and different tissue types, the reader is referred to protocol[17] for a detailed description on how to dissect brain tissue and multiple other tissue types from the crustacean. Samples can be stored at -80 °C until use (ideally within 6 months). The volumes described are used for a single brain from *Callinectes sapidus*. Volumes should be scaled for tissue size. Tissue may be flash frozen immediately without solvent, although this is not recommended as endogenous proteolytic enzymes will not be inhibited and remain active, though at a slower rate when cold.

1.1.3 Add 150 µL of acMeOH to the sample. Set the total sonication time to 24 s, pulse time to 8 s, pause time to 15 s, and amplitude to 50% on an ultrasonic homogenizer and homogenize the samples on ice.

NOTE: There are different homogenization systems available. Adjust the settings and conditions according to sample type and equipment.

1.1.4 Centrifuge the sample at 4 °C at 20,000 x *g* for 20 min. With a pipette, transfer the supernatant in a tube and dry it in a vacuum concentrator (266 x *g*, 1 x 10^{-4} Torr) at approximately 35 °C.

NOTE: The dried samples can be stored at -80 °C until use (ideally within 6 months). Heating the vacuum concentrator must be performed with caution. While heat shortens the dry time, the sample must be removed from the concentrator immediately after all the liquid has evaporated to minimize peptide degradation. To avoid this, heating may be omitted from this and all subsequent steps.

1.1.5 For desalting, reconstitute the extracted tissue sample in 20 µL of 0.1% formic acid (FA), vortex well, and sonicate in a 37 °C water bath for 1 min.

NOTE: There are different desalting materials available. Adjust the solutions and volumes according to the resin identity and neuropeptide amount. Total peptide amount may be estimated using a commercial peptide quantitation assay (see **Table of Materials**).

1.1.6 Apply 0.5 µL of sample to a pH strip to confirm that pH < 4. If the pH is higher, add 1 µL aliquots of 10% FA until pH < 4.

1.1.7 Obtain a 10 µL desalting tip with C18 resin (see **Table of Materials**).

1.1.8 Centrifuge at 4 °C and 20,000 x *g* for 30 s. Place the desalting tip on a 20 µL pipette that is set to 15 µL. Once the desalting tip is wet, prevent air from passing through by keeping the pipette depressed when out of solution until it will be discarded.

1.1.9 Follow manufacture protocol[18]. Prepare a wetting solution containing 100 µL of 50% acetonitrile (ACN), equilibration solution containing 100 µL of 0.1% FA, wash solution containing 100 µL of 0.1% FA, and elution solutions containing 20 µL of 25% ACN/0.1% FA, 20 µL of 50% ACN/0.1% FA, and 20 µL of 75% ACN/0.1% FA.

1.1.10 Aspirate the sample 10x with wetting solution followed by washing 3x in wash solution, discarding each wash. Elute by aspirating 10x in each of the elution solutions in order of increasing ACN.

NOTE: Elution fractions can be kept separate or combined for further analyses.

1.1.11 Discard the used desalting tip and dry the eluted neuropeptides in a vacuum concentrator (266 x *g*, 1 x 10^{-4} Torr) at approximately 35 °C.

NOTE: This can be stored at -80 °C until use (ideally within 6 months).

1.2 Isotopic labeling of neuropeptides in tissue extract

NOTE: This step is optional and only used when quantification is desired.

1.2.1 Prepare the 2-plex 1:1 isotopic dimethyl labeling solution in a fume hood: 1% CH_2OH_2 (13.5 µL of stock 37% weight/weight percentage (wt. %) in water solution in 486.5 µL of water), 1% CH_2OD_2 (25 µL of stock 20 wt. % in water solution in 475 µL of water), and 0.03 M borane pyridine (3.75 µL of stock 8 M solution in 996.25 µL of water).

CAUTION: Formaldehyde is toxic, so all solutions should be kept in a ventilated hood. Wear gloves, a lab coat, eye protection, and impervious footwear. Contact lenses should not be worn when working with this material.

NOTE: There are different isotopic reagents; select the appropriate ones based on sample type and number of labeling channels desired.

1.2.2 Dissolve crude neuropeptide extract in 10 µL of water and sonicate for 10 min.

1.2.3 Add 10 µL of a different isotopic formaldehyde solution (*i.e.*, CH_2OH_2, CH_2OD_2, etc.) to each different experimental condition to be measured quantitatively. Vortex to mix well and briefly centrifuge each sample at 2,000 x *g*.

1.2.4 Add 10 µL of 0.03 M borane pyridine to each sample tube. Vortex to mix well and briefly centrifuge each sample at 2,000 x *g*.

1.2.5 Incubate the samples for 15 min at 37 °C in a water bath.

1.2.6 Remove the samples from the water bath and add 10 µL of 100 mM ammonium bicarbonate. Vortex to mix well and briefly centrifuge each sample at 2,000 x *g*.

1.2.7 Combine the labeled samples for one 2-plex sample and dry the neuropeptides in a vacuum concentrator (266 x *g*, 1 x 10^{-4} Torr) at approximately 35 °C.

1.2.8 Desalt the labeled neuropeptides by reperforming steps 1.1.5 – 1.1.10 and store them until ready for data acquisition.

1.3 Data Acquisition

1.3.1 Reconstitute the dried desalted neuropeptides in 12 µL of 3% ACN/0.1% FA, vortex well, sonicate in 37 °C water bath for 1 min, and briefly centrifuge at 2,000 x *g*. Transfer each sample into autosampler vials.

NOTE: Adjust the sample volume according to neuropeptide amount to a concentration of ~1 µg peptide per µL. Total peptide amount may be estimated using a commercial peptide quantitation assay (see **Table of Materials**).

1.3.2 Use an autosampler to inject 1 µL of sample into a high-resolution nano-LC-MS/MS instrument (see **Table of Materials**).

1.3.3 Use an approximately 15 cm long reversed-phase (RP) C18 column (see **Table of Materials**) for running the sample with 0.1% FA in water as mobile phase A and 0.1% FA in ACN as mobile phase B. Run the samples with a gradient of 3% - 95% of B at a rate of 300 nL/min for over 11 min.

1.3.4 For the MS instrument used here, use common MS conditions of 2.00 kV for spray voltage and 275 °C for capillary temperature.

1.3.5 Acquire MS spectra in the range of 200 - 2,000 *m/z* with a resolution of 60,000, automatic gain control (AGC) target of 1 x 10^6, and max ion injection time (IT) of 150 ms.

1.3.6 Select the 15 most intense ions (minimum intensity of 3.2 x 10^4) for higher-energy collision dissociation (HCD) fragmentation using a normalized collision energy of 30, isolation window of 2.0 *m/z*, resolution of 15,000, an AGC target of 2 x 10^5, and max IT of 250 ms.

1.3.7 Set a dynamic exclusion window of 30 s. Exclude ions with a charge of 1 or ≥ 8 and ions with unrecognized charge states.

1.4 Neuropeptide identification and quantification

NOTE: Many software for database searching and peptide quantification (both open-source and commercial) are available. Here, PEAKS Studio (hereafter proteomics software)[15] will be used.

1.4.1 Perform database searching using the steps outlined in 1.4.2 – 1.4.6.

1.4.2 Create a new project and add the LC-MS data selecting **None** for the enzyme, **Orbitrap** for the instrument, **HCD** for the fragment, and data-dependent acquisition (**DDA**) for acquisition.

NOTE: Select the appropriate parameters based on data acquisition parameters.

1.4.3 Select **Identifications** and select **Correct Precursor [DDA]** and **Mass only**.

1.4.4 Select **Database Search** and set an error tolerance of **20.0 ppm** using monoisotopic mass for precursor mass and **0.02 Da** for fragment ion mass, **None** for enzyme type, **Unspecific** for digest mode, **100** for max missed cleavages, and the following variable PTMs with the max allowed variable PTM per peptide of **3**: **Amidation**, **Oxidation (M)**, **Pyro-glu from E**, and **Pyro-glu from Q**.

1.4.5 Select the **Neuropeptide Database**, estimate false discovery rate (FDR) with decoy-fusion.

NOTE: The mass tolerance error should be adjusted to match the data collected. Use the appropriate database for the sample type. When no enzyme is selected, the max missed cleavages parameter does not affect the search. However, a large number of missed cleavages is required if the software does not have the **No Enzyme** as an option.

1.4.6 If label-free quantification is desired, select **Quantification**, select **Label-Free** and set an error tolerance of **20.0 ppm** and retention time tolerance of **1.0 min**.

1.4.7 Perform precursor ion quantification if step 1.2 for isotopic labeling was performed.

1.4.7.1 Select **Quantification**, select **Precursor Ion Quantification**, use a retention time range of **1.0 min,** and use an FDR threshold of **1%**.

1.4.7.2 Select a preset or custom quantification method from the **Select Method** drop-down menu.

1.4.7.3 To create a new custom method, click **Window > Configuration > Label Q Method > New**. Name the new method and select **Precursor Ion Quantification** for Method Type. Select **Add Row** and select modification from the PTM Options list.

1.4.7.4 Add the LC-MS data and select **Reference Condition** to be the modification on neuropeptides from the control condition of the experiment.

1.4.8 Evaluate the search results as described in steps 1.4.9 – 1.4.11.

1.4.9 Filter the results through the summary tab for peptides and proteins with -10lgP ≥ **20**, select ≥ **1** unique peptide, and select box labeled **With Significant Peptides**.

1.4.10 Evaluate the database search results where Protein.csv represents neuropeptide identifications and Peptide.csv represents neuropeptide fragment identifications.

1.4.11 Inspect the database search for protein and peptide scores, mass accuracy, and sequence coverage.

NOTE: Each database search software uses unique scoring algorithms and may need to be evaluated accordingly. Identifications can be evaluated by manually inspecting the observed spectra for identified peptides containing the complete fragment ion series.

2 MALDI-MS spotting analysis of neuropeptides

2.1 Sample Preparation

2.1.1 Follow step 1.1 or steps 1.1 – 1.2 if quantification is desired, excluding step 1.2.8 (desalting after isotopic labeling is not required prior to MALDI-MS analysis).

2.1.2 Reconstitute the dried desalted neuropeptides in 5 µL of 0.1% FA, vortex well, sonicate in a 37 °C water bath for 1 min, and briefly centrifuge at 2,000 x *g*.

2.1.3 For spotting of neuropeptides in crustacean tissues, use 150 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 50% methanol (MeOH)/0.1% FA (v/v) as the matrix.

2.1.4 Pipette a 3 µL droplet of sample onto a hydrophobic film (see **Table of Materials**) and pipette 3 µL of the matrix directly on the sample droplet. Pipette up and down to mix.

2.1.5 Pipette 1 µL of the 1:1 sample: matrix mixture into a well of the MALDI stainless steel target plate. Use the pipette tip to spread each mixture out to the edges of the sample well. The sample must touch the edges of the well engraving to facilitate uniform distribution (**Figure 4A**).

2.1.6 Spot 1 µL of 1:1 calibrant: matrix mixture (commercial or custom calibration mix, polymer materials (*i.e.*, red phosphorus dissolved in MeOH), or common matrix cluster ions)[12] into a well near the sample.

NOTE: Red phosphorus does not need to be mixed with matrix before spotting.

2.2 Data acquisition

2.2.1 Insert target plate containing dried sample spots into MALDI Tandem Time-of-Flight (TOF/TOF) instrument (see **Table of Materials**).

2.2.2 Calibrate the instrument and optimize the percent laser power, detector gain, and select the appropriate sample carrier movement mode so that every acquisition covers roughly the entire spot and subsequent acquisitions do not go to the previous positions.

2.2.3 For DHB matrix set laser power to **95%**, select **Automatic Optimal Detector Gain**, and **Smart – Complete Sample** sample carrier movement mode. Acquire MS spectra in the range of 200 – 3200 *m/z* and add multiple spectra from each spot together to increase neuropeptide signal-to-noise ratio.

NOTE: Adjust the mass range to encompass desired neuropeptide range.

2.3 Data analysis

2.3.1 Open the MALDI-MS file in data analysis software (see **Table of Materials**) and click **Baseline Subtraction** for the software used here.

2.3.2 Perform peak picking by clicking **Find Mass List**. If there are too few peaks, edit the mass list manually by selecting **Edit Mass List** and click on peaks in the spectrum to add them to the mass list.

2.3.3 Perform accurate mass matching by comparing mass list with neuropeptide database containing $[M+H]^+$ *m/z* values (± 200 ppm error).

NOTE: Common salt adducts, such as $[M+K]^+$, $[M+Na]^+$, and $[M+NH_4]^+$, should also be included in the accurate mass matching target list.

2.3.4 To verify the identified peaks, generate a list of *m/z* of interest and perform MS/MS experiments.

3 MALDI-MS imaging analysis of neuropeptides

3.1 Sample preparation

NOTE: Embedding and sectioning steps are not necessary for tissues that are too thin to be sectioned.

3.1.1 Fill half a cryostat cup with gelatin (37 °C, 100 mg/mL in deionized water) and allow it to solidify at room temperature. Keep leftover liquid gelatin warm in a 37 °C water bath.

3.1.2 Collect desired neuronal tissue from the animal and use forceps to immediately dip the tissue into a 0.6 mL tube containing deionized water for 1 s.

NOTE: Refer to Step 1.1.2 NOTE for neuronal tissue dissection.

3.1.3 Place the tissue on top of the solid gelatin and fill the rest of the cryostat cup with liquid gelatin. Use forceps to position the tissue.

3.1.4 Place the cryostat cup on a flat surface and freeze with dry ice.

NOTE: Store samples at -80 °C until use (ideally within 6 months).

3.1.5 For sectioning preparation, separate the gelatin-embedded sample from the cryostat mold by cutting the mold away.

3.1.6 Mount the embedded tissue onto a cryostat chuck by pipetting a 1 mL droplet of deionized water onto the chuck and immediately pressing the embedded tissue onto the droplet.

3.1.7 Once frozen, pipette more deionized water around the tissue to further secure it to the chuck. Perform these steps inside the cryostat box (see **Table of Materials**) set at -20 °C.

3.1.8 Section the tissue at an approximate thickness of one cell (8-20 µm depending on the sample type) and thaw mount each section onto an indium tin oxide (ITO)-coated glass slide by placing one side of the slide near the section and placing a finger on the other side of the slide to slowly warm the glass and allow the section to stick to the slide.

NOTE: Tissue sections may also be thaw-mounted by picking up one edge of the gelatin with tweezers (chilled to -20 °C), placing it on the ITO-coated glass slide, and placing a finger on the other side of the slide to slowly warm the glass and allow the section to stick to the slide.

3.1.9 Spot the sample to be used as a calibrant (see step 2.1.6 for calibrant options) by drawing a small circle near the tissue section using a hydrophobic pen and spotting the calibrant inside the circle.

3.1.10 Mark each corner of the slide with a whiteout pen with a small shape containing sharp edges (*i.e.*, x) to be used as teach points.

3.1.11 Place the glass slide into the MALDI slide adapter plate and take a high resolution (≥2400 DPI) optical image scan using a scanner.

3.1.12 Spray matrix on the tissue section using an automated sprayer (see **Table of Materials** for sprayer details and instructions).

3.1.13 For MSI of neuropeptides in crustacean tissues use 40 mg/mL DHB in 50% methanol/0.1% FA (v/v) as the matrix, set the nozzle temperature to 80 °C, velocity to 1,250 mm/min, flow rate to 0.1 mL/min, number of passes to 12, and 30 s in between each pass for the automatic sprayer.

3.2 Data acquisition

3.2.1 Insert the completely dried target plate containing thaw-mounted tissue sections that were sprayed with the matrix.

3.2.2 Set up the MS imaging acquisition file parameters so that the laser diameter is smaller than the raster step size.

3.2.3 Load the scanned optical image and calibrate the sample plate using the x teach points. Define the tissue areas of interest to be measured slightly larger than the actual tissue section to also include areas containing only matrix.

3.2.4 Calibrate the instrument and acquire spectra in the range of 200 – 3200 *m/z.* Adjust the mass range to encompass desired neuropeptide range.

3.3 Data analysis

3.3.1 To process data, import MS imaging dataset into desired software, select a baseline removal algorithm, and normalize data using the **Total Ion Count.**

NOTE: Selection of different normalization algorithms, such as median, root mean square (RMS) value, or the intensity of a reference *m/z* value, will likely change the spatial distribution of many *m/z* values. Choose the normalization algorithm best suited for desired analytes.

3.3.2 To generate an image for each *m/z* value from a theoretical peak list, upload a comma-separated values (CSV) file containing neuropeptide $[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$, etc. Obtain *m/z* values by clicking **File** > **Import** > **Peak List**. Name the peak list.

3.3.3 To estimate the appropriate ppm error threshold, first manually identify a neuropeptide peak in the MS spectrum and compare it with the neuropeptide theoretical mass. Calculate the ppm error and click **File** > **File Properties** > **Interval Width** and input ppm error.

3.3.4 Select the peak list from the drop-down menu and click **Create *m/z* Images For Every Interval of The Peak List**.

3.3.5 Save each *m/z* image by clicking **Save Screenshot of Each *m/z* Image**.

NOTE: Putative neuropeptide identification can be performed by identifying *m/z* images where the analyte signal is only localized within the tissue and not in the surrounding matrix.

3.3.6 To verify peak identity, generate a list of *m/z* of interest and perform MS/MS experiments.

4 Discovering novel putative neuropeptides using *de novo* sequencing

4.1 Perform steps 1.4.2 – 1.4.5.

4.2 Export de novo only peptides.csv from peaks with an average of local confidence (ALC) score of≥ **75**.

NOTE: There are many software available to perform *de novo* sequencing, each with its own scoring algorithms and should be evaluated accordingly.

4.3 Search the peptide list for known sequence motifs indicative of neuropeptides belonging to specific neuropeptide families[19].

NOTE: While motifs are commonly well conserved across species, the motifs searched for should be selected with consideration to the sample organism.

5 Transcriptome mining for predicted neuropeptide sequences

NOTE: This step is optional and only used to add to an existing neuropeptide database or build a new neuropeptide database.

5.1 Choose a known preprohormone amino acid sequence of interest and use tBLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn&BLAST_PROGRAMS=tblastn&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome) to search query preprohormone sequence against databases including nr/nt, Refseq_genomes, EST and TSA.

NOTE: To search query sequences against protein database, use BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&BLAST_SPEC=&LINK_LOC=blasttab&LAST_PAGE=tblastn).

5.1.1 Select the target organism (tax id) and change **Expect Threshold** algorithm parameters to **1000** to include low score alignments.

5.1.2 Run **BLAST** program and then check the results for high homology scores between query and subject sequences producing significant alignments. Save FASTA file containing nucleotide sequence.

NOTE: If there are several subject sequences with similar homology scores, carry out a MAFFT alignment to narrow down putative sequences[20,21].

5.2 Translate preprohormone nucleotide sequence into preprohormone peptide sequences using **Expasy Translate** tool (https://web.expasy.org/translate/). For *C. sapidus*, select **Invertebrate Mitochondrial** for genetic code.

5.3 Check for signal peptide sequence and prohormone cleavage sites in the peptide sequences using **SignalP** (https://services.healthtech.dtu.dk/service.php?SignalP-5.0).

NOTE: Homology to known preprohormone processing schemes can also be used to identify prohormone cleavage sites. Possible post-translational modifications for signal peptides may be predicted if desired. Sulfinator (https://web.expasy.org/sulfinator/) can be used to predict sulfation state of tyrosine residues. DiANNA (http://clavius.bc.edu/~clotelab/DiANNA/) can be used to predict disulfide bond connectivity.

REPRESENTATIVE RESULTS:

The workflow for sample preparation and MS analysis is depicted in **Figure 1**. After the dissection of neuronal tissue, homogenization, extraction, and desalting are performed to purify neuropeptide samples. If isotopic label-based quantification is desired, samples are then labeled and desalted once again. The resulting sample is analyzed through LC-MS/MS for neuropeptide identification and quantification.

Neuropeptides identified through the proteomics software should have good peptide fragmentation sequence coverage, however, this is not globally defined or standardized. For absolute identification, every amino acid should produce a fragment ion that provides unambiguous identification and localization. This must also be compared with a synthesized peptide for confirmation of the intensity of each fragment ion. As the cost for performing this on every putative identification is not feasible, identification is commonly described based on confidence where more observed fragment ions increase peptide identification confidence. While **Figure 2A,B** depicts two neuropeptides that were both identified with 100% sequence coverage and a low mass error as defined by the max limit of 0.02 Da, the poor fragmentation coverage (from only three ions) observed only for the neuropeptide in **Figure 2B** decreases the confidence of identification of a specific isoform. **Figure 2C** depicts the extracted ion chromatograms (XICs), which is a plot containing the signal intensity of a selected *m/z* value as a function of retention time, of a neuropeptide detected in two samples used for LFQ. The retention times for the neuropeptide differ slightly because it was identified in two separate and consecutive runs; however, the difference is within the reliable threshold value of 1 min. Thus, the ratio between the software-calculated area under the curve from the XIC is used for the LFQ of this neuropeptide.

For the quantitation of dimethyl labeled neuropeptides, the MS1 spectrum should contain a peak at the theoretical neuropeptide *m/z* value and a peak at the *m/z* value with a mass shift that correlates to the mass difference between the isotopic labeling reagents used. In **Figure 2D**, the mass shift of this 2-plex dimethyl labeled sample is 4.025 Da. The area under the curve of the precursor ion from its XICs is then calculated by the software and used to calculate relative abundance ratios. A simplified version of the proteomics software export table containing identified neuropeptides and their LFQ ratios is shown in **Table 1**. Similar results are obtained for isotopically labeled neuropeptides.

Software algorithms enable the *de novo* sequencing of spectra to detect novel putative

neuropeptides. When claiming the detection of putative novel neuropeptides, high confidence identifications are ideal cases where all amino acids are identified and localized unambiguously, based on fragment ion observation. **Figure 3** depicts the spectrum of a *de novo* sequenced peptide containing the -RYamide motif at the C-terminal, a conserved sequence motif shared by known neuropeptides of the crustacean RYamide family[22]. A peptide was matched from the database with 100% sequence coverage, all amino acid forming fragment ions observed, low fragment ion mass error as defined by the max limit of 0.02 Da and contained a gaussian elution profile. These results indicate that an endogenous peptide belonging to the crustacean -RYamide neuropeptide family was observed.

MALDI-MS spot measurements can provide neuropeptide identifications that are complementary to LC-ESI-MS identification, as well as offer higher throughput capabilities. After crude tissue homogenate is extracted for neuropeptides, desalted, and labeled (if desired), the sample can be mixed with matrix and spotted on the MALDI stainless steel target plate, as shown in **Figure 4A**. Successful pipetting of homogenous sample spots produces clearly resolved peaks, especially within the calibration spectrum (**Figure 4B**). When using a MALDI-TOF instrument, the instrument must be calibrated at the beginning of each experiment. Any analytes with known masses can be used to calibrate the instrument if it is within the desired mass range of the sample. Here, red phosphorus is used for the positive ion mass calibration of the instrument. It has advantages over using peptide calibration mixes due to its stability at room temperature, cheap cost, abundant peaks due to its polymerization, high signal-to-noise ratio, and it does not require a matrix for ionization.

For MALDI-MS imaging of neuropeptides, the MALDI TOF/TOF instrument used requires manual image calibration of the ITO-coated slide (step 3.1.10) to correlate the optical image with the sample. The diagram in **Figure 5** shows the proper placement of the whiteout crosshairs to be used as teach points to allow the instrument to correlate the scanned optical image with the actual sample plate. It also illustrates areas of the ITO-coated slide that should be avoided by the user (*i.e.*, do not contain sample or matrix). For mass calibration, the placement of the calibration sample spot relative to the tissue sample on the ITO-coated slide directly impacts the mass error due to the inherent nature of time-of-flight mass analyzers, although the magnitude of this issue is dependent on the abundance of the target analytes. A solution to this problem is to manually check peaks from the MS1 spectra from different tissue sections for evidence of peak shifting. If there is peak shifting, consider adding additional mass calibration spots onto the ITO-coated slide right next to each tissue section. From there, the user can average the spectra from the calibration spots together or perform MS imaging on one tissue section at a time using only the calibration spot closest to the tissue section. After the sample is collected, verify that the signal from *m/z* values corresponding to neuropeptides is only localized within the tissue region (**Figure 6**) before assigning a putative neuropeptide identification.

FIGURE AND TABLE LEGENDS:

Figure 1: Neuropeptide sample preparation workflow for mass spectrometry analysis. For tissue extract analysis crude tissue homogenate is desalted, labeled with stable isotopic labels, desalted again, and analyzed by MS. For imaging analysis intact tissue is embedded,

cryosectioned, applied with matrix, and analyzed by MALDI-MSI.

Figure 2: Identification and quantification performed through the proteomics software. Neuropeptides are detected through spectra of ranging quality with (**A**) good or (**B**) poor MS2 fragmentation coverage. Fragment ion mass matching error is shown below the spectra. (**C**) XIC profile shapes and retention time (RT) can be manually inspected for neuropeptides quantified through LFQ. (**D**) MS1 spectra are used to detect and quantify dimethyl labeled neuropeptides.

Figure 3: *De novo* sequencing for novel neuropeptide detection. (**A**) The MS2 spectrum of a putative novel RYamide demonstrates good fragmentation coverage with low mass error for each fragment. (**B**) The identified fragment ions are listed for manual inspection. (**C**) The XIC of the novel neuropeptide is manually inspected for Gaussian peak shape. Abbreviations: RT = retention time.

Figure 4: MALDI-MS spots and calibration spectrum. (**A**) MS spectra quality relies on uniform matrix-peptide distribution in the MALDI stainless steel target well. The left three spots are examples of good spots that touch the edges of the well engraving and the right three spots are examples of bad spots. Both spots contain α-Cyano-4-hydroxycinnamic acid (CHCA) matrix and a peptide standard mix. (**B**) Calibration spectrum using red phosphorus clusters from 500 – 3200 *m/z*.

Figure 5: Depiction of ITO-coated glass slide. (**A**) Schematic with important areas noted: locations to place tissue sections (light blue rectangles), automatic teach points locations that should be avoided (red rectangles), example locations of where teach points may be drawn (white crosshairs), and where screws attach to the adapter plate and should be avoided (dark blue ovals). (**B**) Photo of glass slide containing two tissue sections, a spot containing a calibration mix, and crosshair marks. The location of the tissue section and calibration spots are outlined on the other side of the glass slide.

Figure 6: MS images of *C. sapidus* sinus glands. Neuropeptide $[M+H]^+$ ion distribution images of (**A**) HL/IGSL/IYRamide (*m/z* 844.48), (**B**) Allatostatin A-type NPYAFGLamide or GGPYAFGLamide (*m/z* 780.40), (**C**) Allatostatin A-type GQYAFGLamide (*m/z* 754.39), and (**D**) RFamide GRNFLRFamide (*m/z* 908.52) are shown. Images are generated using a ± 50 ppm window from the theoretical *m/z* value. Color bar indicates the range of signal intensity from 0 to 100%.

Table 1: Database search and LFQ results. Neuropeptides identified and quantified through LC-MS and proteomics software. Identified PTMs are listed along with the intensities of detected peptides in both samples for LFQ, along with the resulting LFQ ratio. The average masses and observed neuropeptide descriptions from the FASTA file are listed.

DISCUSSION:

The accurate identification, quantification, and localization of neuropeptides and endogenous peptides found in the nervous system are crucial toward understanding their function[23,24]. Mass spectrometry is a powerful technique that can allow all of this to be accomplished, even in

organisms without a fully sequenced genome. The ability of this protocol to detect, quantify, and localize neuropeptides from tissue collected from *C. sapidus* through a combination of LC-ESI- and MALDI- MS is demonstrated.

During sample preparation for LC-ESI-MS analysis, considerations must be made. While MS is a sensitive technique, the low peptide concentration of neuronal tissue (down to the femtomolar range[25]) poses a serious limitation. Careful sample preparation is required to not only remove more abundant and interfering matrix components, such as proteins and lipids but also minimize the loss of neuropeptides in each step[26,27]. For example, sample loss can be reduced by using microcentrifuge tubes that resist peptide adsorption. Depending on the composition of the tissue of interest, the use of various solvents (for extraction or precipitation) or solid-phase extraction materials can be used for separating biomolecules with different sizes and chemical properties. To ensure hydrophilic or hydrophobic peptides are present in the neuropeptide extract, multiple extraction solvent systems may be optionally used to target neuropeptides with different physicochemical properties. These, along with the use of protease inhibitors, may need to be modified for optimized purification to improve neuropeptide recovery[28]. The drawback of using multiple extraction systems is that several neuronal tissues need to be pooled to meet an overall higher peptide content requirement, as well as decreased throughput. Many steps such as desalting, isotopic labeling, and MS injection recommend certain starting peptide amounts. For the characterization of precious samples, such as neuropeptides, peptide assays are generally avoided to prevent extraneous peptide consumption. Additionally, peptide assays were developed to determine accurate peptide concentration from protein digests, which have different chemical properties than endogenous peptides. To overcome complications from unknown neuropeptide concentration, an initial peptide assay can be performed using pooled neuronal tissue extracts, where the results are used as an estimate for all subsequent analyses, although it must be kept in mind that all peptides in solution are measured, and not all of these are neuropeptides[29]. Other limitations of this method include potential biases to nonpolar and hydrophobic neuropeptides and the lack of native structure conservation. Modifications to solvent compositions and materials, such as using solutions that are more hydrophobic or omitting the use of organic solvents, may be performed to address this.

MALDI-MS measurements rely on careful sample preparation steps for consistent results and can have different sample considerations than for LC-ESI-MS measurements. Steps such as keeping neuropeptide extract on ice prior to MS analysis are still applied. Additional methods of preventing neuropeptide degradation include keeping glass slides containing thaw-mounted tissue sections in a desiccant box after dissection to prevent condensation from accumulating[30], although leaving the tissue sample in the desiccator after it has dried may result in sample degradation as well. Placing the tissue slides in a vacuum desiccator (final pressure: $1x10^{-4}$ Torr at room temperature) for 5–10 min immediately prior to matrix application is suggested. After the matrix is deposited onto the tissue slide, it can be kept in the refrigerator or freezer overnight and dried in the vacuum desiccator prior to MALDI-MS imaging measurement. For MALDI spot measurements, the matrix-peptide crystal structure is not equally distributed throughout the MALDI target well even if it appears so. There are ways to mitigate mass spectra variations from technical replicates due to this process. First, acquire an average spectrum from each well using

multiple laser shots (typically hundreds to thousands) where the laser is randomly rastered across the well (*i.e.*, selecting **SMART** or **Random** sample carrier movement in instrument parameters). Acquire at least five technical replicates for each sample type and select three technical replicates where the variation in signal intensity for desired peaks is the lowest. The tradeoff here is experimental throughput. It is necessary to keep parameters such as the number of laser shots, laser diameter, and other instrument settings consistent for all acquired spectra. Ideally, all technical replicates and biological replicates are analyzed at the same time using the same matrix solution and instrument calibration. Neuropeptide identification can be performed by accurate mass matching to a peak list containing theoretical $[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$, $[M+K]^+$, and other salt adducts producing singly charged ion *m/z* values. Normalization of data is critical for minimizing systematic artifacts from MALDI-MS experiments. Evaluation of reproducibility is especially important when MALDI-MS spot measurements are used for neuropeptide quantification by stable isotope labeling (SIL). Quality of sample preparation and data acquisition can be evaluated by taking a peptide standard or neuropeptide extract, splitting it into two equal aliquots, differentially labeling each sample by SIL, and analyzing the sample to ensure the relative intensity of paired peaks are 1:1. The variation reported from those ratios can be used to estimate the level of variance in the overall experiment attributed to user error.

It is important to note that MALDI-MS is also capable of providing sequence and quantitative information; however, the singly charged ions produced by MALDI limit peptide fragment detection, making it more difficult to obtain the complete sequence and inhibiting the quantitation methods discussed for LC-ESI-MS. Regardless, MALDI-MS is an attractive modality due to its capability for high throughput, as well as a higher tolerance for salts and impurities within the sample[12,31]. Additionally, MALDI-MS imaging has advantages over other conventional imaging techniques that require antibodies. In most MS modalities, lowering the mass resolution will boost sensitivity, enabling improved detection of low abundance neuropeptides. This strategy may be more practical for MALDI-TOF/TOF instruments than LC-ESI-MS instruments due to the ease of calibrating the MALDI instrument for each experiment. Another way that MALDI can be advantageous for neuropeptidomics is through spectra averaging. Typically, averaging spectra from LC-ESI-MS measurements is not used because it negates the benefits of LC separation; therefore, bioinformatics software is heavily relied upon to identify neuropeptides and the identifications are manually verified. However, there are fewer consequences for averaging spectra from MALDI-MS measurements because there was no initial separation; therefore, the raw data is smaller and easier for a user to manually comb through. Ease of data management is important as it changes the order in which the user can approach neuropeptide identification and verification strategies. While confidence in neuropeptide identifications from LC-ESI-MS could benefit from increasing the level of threshold stringency within data analysis software, this strategy is less likely to benefit MALDI-MS identifications. In the example of crustacean neuropeptides, the fact that there are less than 1000 entries from the lab-built database (*i.e.*, a maximum of <1000 spectral peaks or MS images to manually scan through), makes it possible to first filter the MALDI-MS data with a generous mass error threshold, and then manually verify those identifications by examining the isotopic envelopes at the MS1 level, as well as other verification methods discussed in the **Representative Results** section. Popular MS imaging data analysis software can perform accurate mass matching to a neuropeptide mass

database and extract the corresponding MS images. For clinical-based research questions, such as biomarker discovery, these software are able to extract *m/z* values unique to a tissue region of interest (typically called Region of Interest (ROI) analysis)[32] and perform statistical tests to quantify how different two tissue regions are. It is also worth noting there are also fewer data analysis software options for MS imaging than for LC-ESI-MS.

Performing LC-ESI-MS database searches for neuropeptides commonly entails the use of software algorithms built for the analysis of digested peptides. As such, there are limitations to software being able to perform nonspecific enzyme database searches or the amount of time it takes to complete the search. Currently, endogenous peptide searches are performed with the maximum number of missed cleavages, but this number is still limited, leading to potentially missed identifications. When the genome for a species is not fully sequenced, as with *C. Sapidus*, *de novo* sequencing can be performed to identify unknown/ novel neuropeptides through known conserved neuropeptide sequence motifs, although this method fails to identify neuropeptides that have unknown motifs or do not contain the motifs used as neuropeptide family identifiers[19]. For example, WSSMRGAWamide is a motif for the allatostatin B-type neuropeptide family[19]. Here lies the significance of transcriptome mining for predicted neuropeptide sequences for species without a completely decoded genome sequence[33]. Neuropeptide identifications are then confirmed by synthesizing the putative peptide sequence and comparing the MS/MS spectra from synthetic peptide and biological tissue[34]. Even after a sequence is verified, it is sometimes unknown whether it is the full neuropeptide sequence or a degradation product. It is worth noting that differences between MALDI and ESI-MS ionization sources (ESI is a softer ionization method than MALDI) may result in different rates of artificial degradation (i.e., in-source fragmentation). To distinguish between a truncated version of a peptide from an artificially induced (i.e., not *in vivo*) degradation product, the preprohormone processing pathway for that neuropeptide must be known. Since this is often not the case, a synthetic form of the peptide should be used in physiological assays and verified for biological activity.

Overall, the workflow exemplified here for neuropeptide analysis can benefit a variety of different fields. It fills a technical gap within middle-down MS analysis of peptides because it is optimized for endogenous peptides that are smaller than proteins typically analyzed by bottom-up or top-down MS analyses. Therefore, the sample preparation methods utilized by neuropeptidomics should be largely translatable to other bioactive endogenous peptides, such as those targeted by medicinal chemists for antibacterial properties[35]. A benefit of this protocol is that it utilizes common instruments from popular vendors offering an additional degree of translatability as well. In this way, the workflow can be used in academic and commercial settings, such as screening for pharmaceutical drug candidates or drug targets.

ACKNOWLEDGMENTS:

This research was supported by National Science Foundation (CHE-1710140 and CHE-2108223) and National Institutes of Health (NIH) through grant R01DK071801. A.P. was supported in part by the NIH Chemistry–Biology Interface Training Grant (T32 GM008505). N.V.Q. was supported in part by the National Institutes of Health, under the Ruth L. Kirschstein National Research Service Award from the National Heart Lung and Blood Institute to the University of Wisconsin-

Madison Cardiovascular Research Center (T32 HL007936). L.L. would like to acknowledge NIH grants R56 MH110215, S10RR029531, and S10OD025084, as well as funding support from a Vilas Distinguished Achievement Professorship and Charles Melbourne Johnson Professorship with funding provided by the Wisconsin Alumni Research Foundation and University of Wisconsin-Madison School of Pharmacy.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Figure1_workflow.pdf

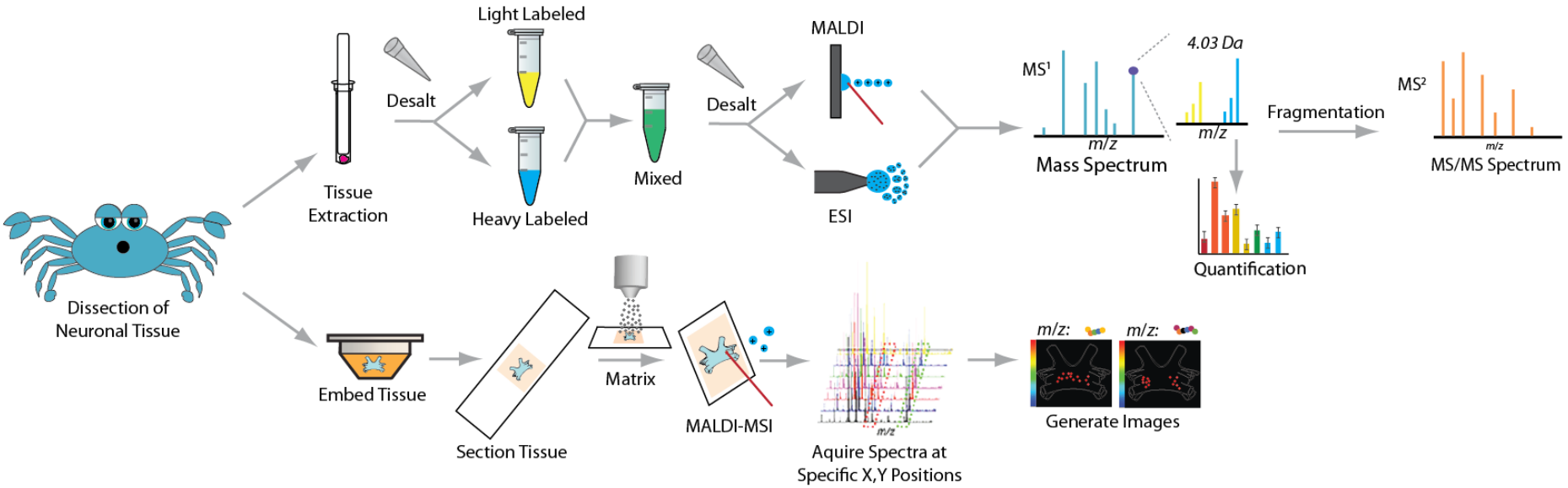


Figure 2

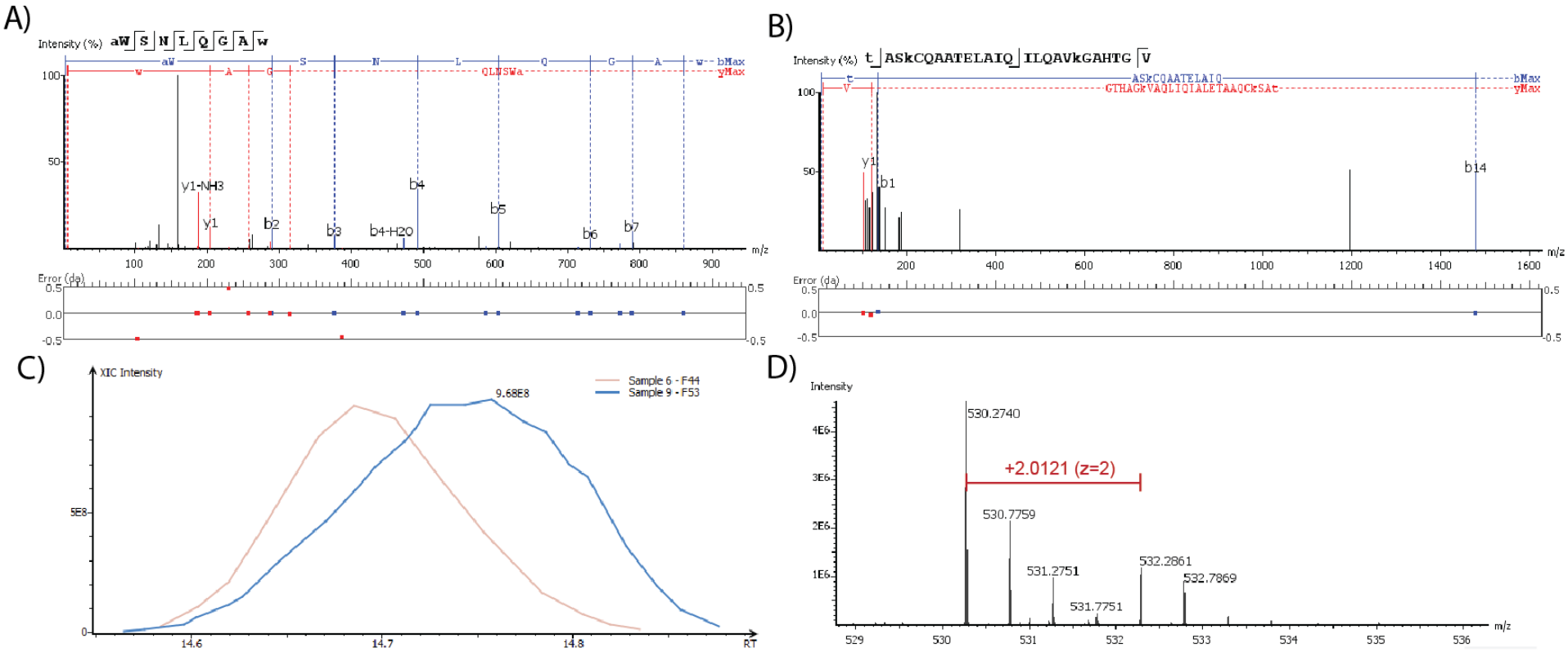
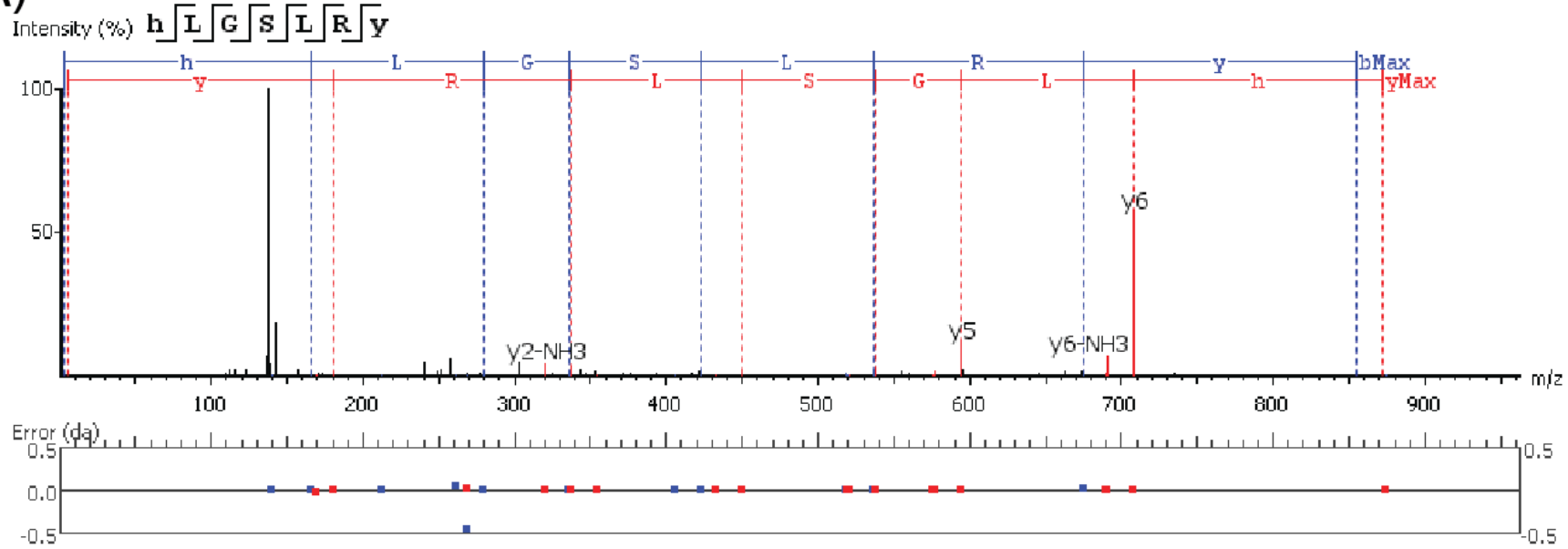


Figure 3

A)



B)

#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	166.10	148.09	149.07	83.55	H(+28.03)					7
2	279.18	261.12	262.16	140.08	L	707.42	689.41	690.40	354.22	6
3	336.20	318.19	319.18	168.60	G	594.34	576.32	577.31	297.67	5
4	423.24	405.22	406.21	212.14	S	537.32	519.31	520.29	269.13	4
5	536.32	518.31	519.31	269.13	L	450.28	432.27	433.26	225.64	3
6	692.42	674.38	675.39	346.71	R	337.20	319.19	320.17	169.13	2
7					Y(-.98)	181.10	163.09	164.07	91.05	1

C)

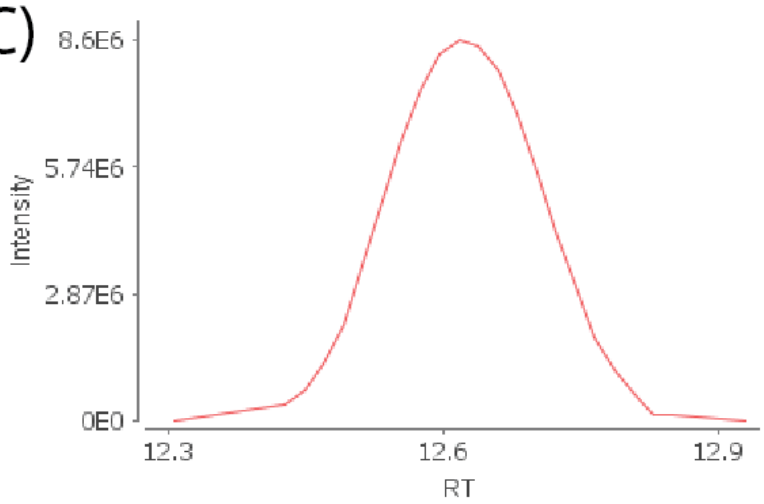
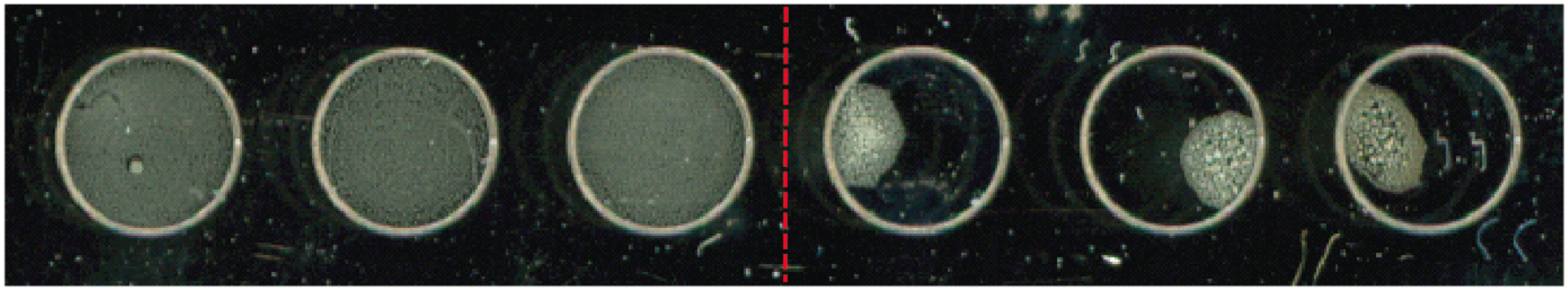


Figure 4

A)



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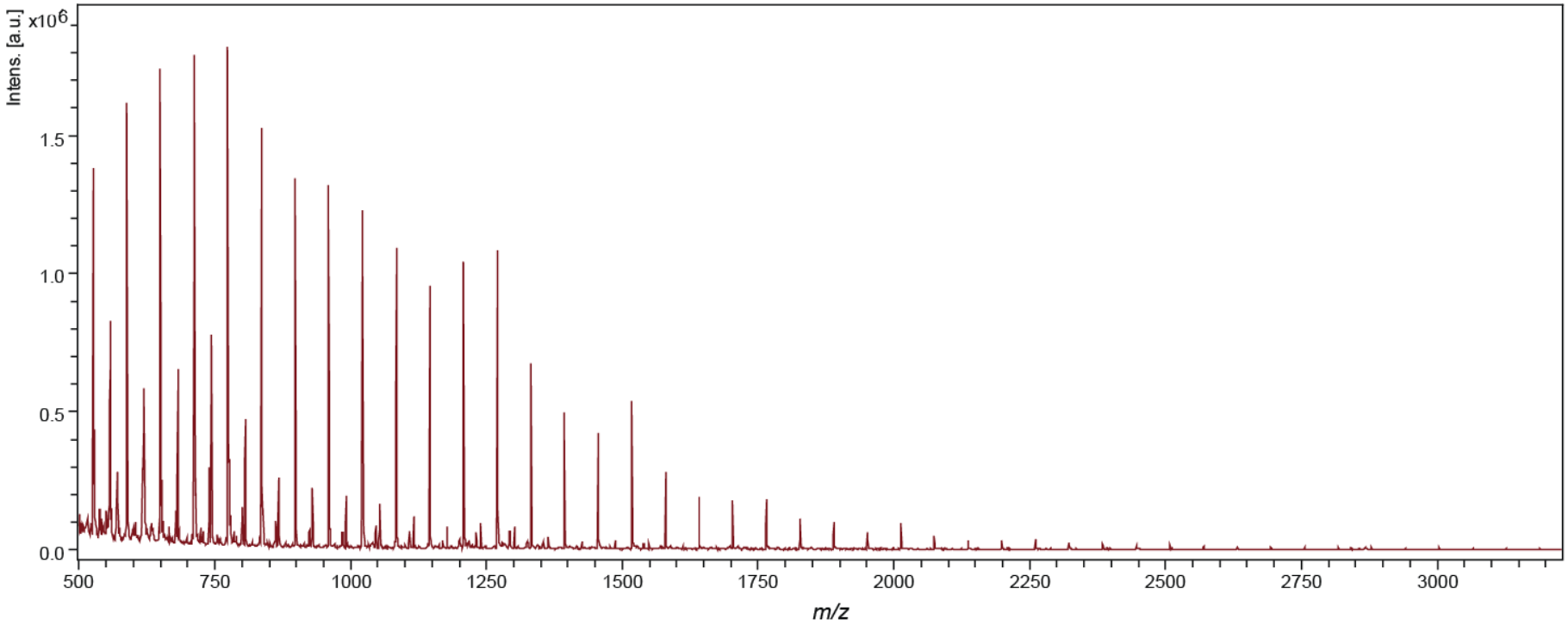


Figure 5

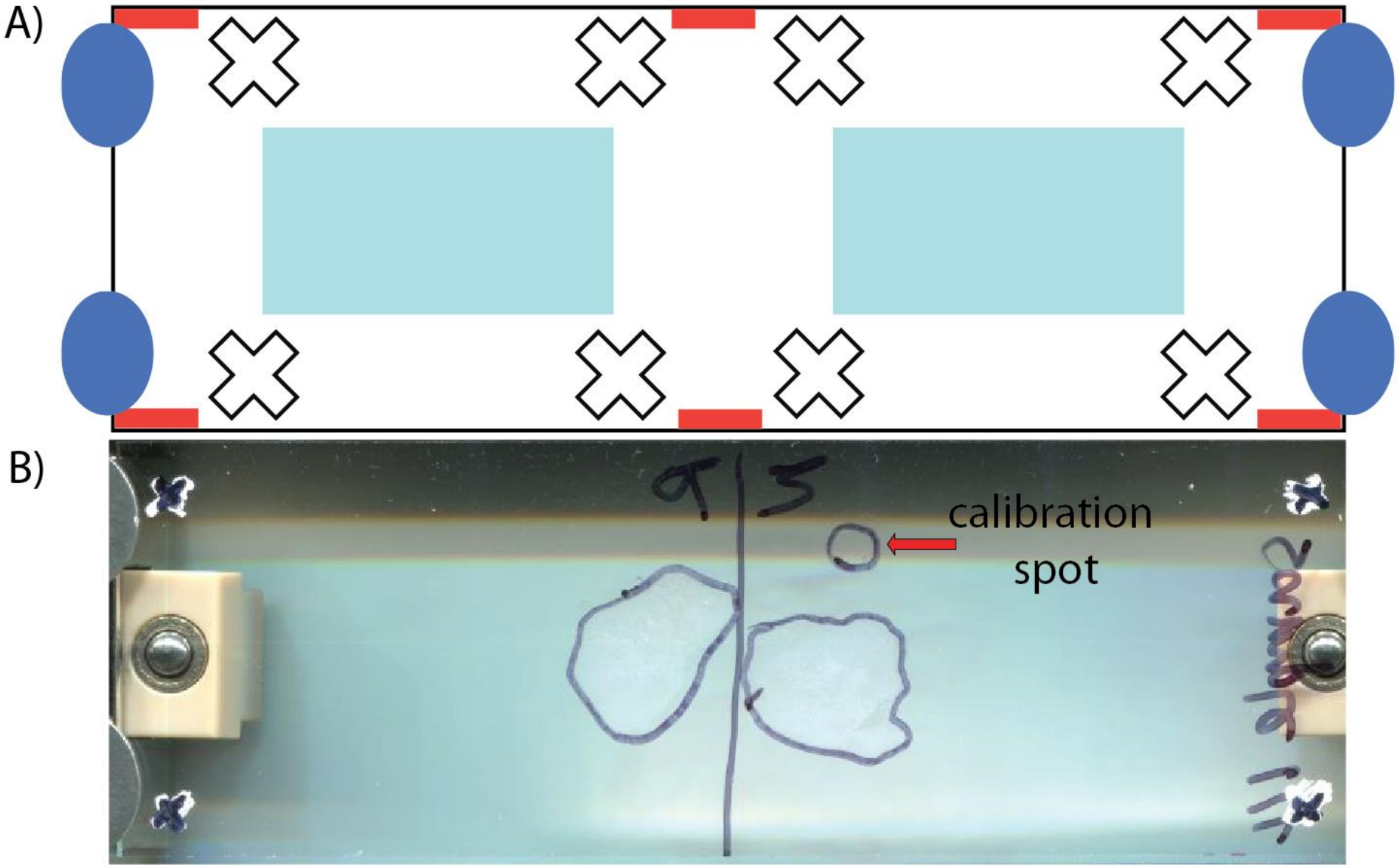
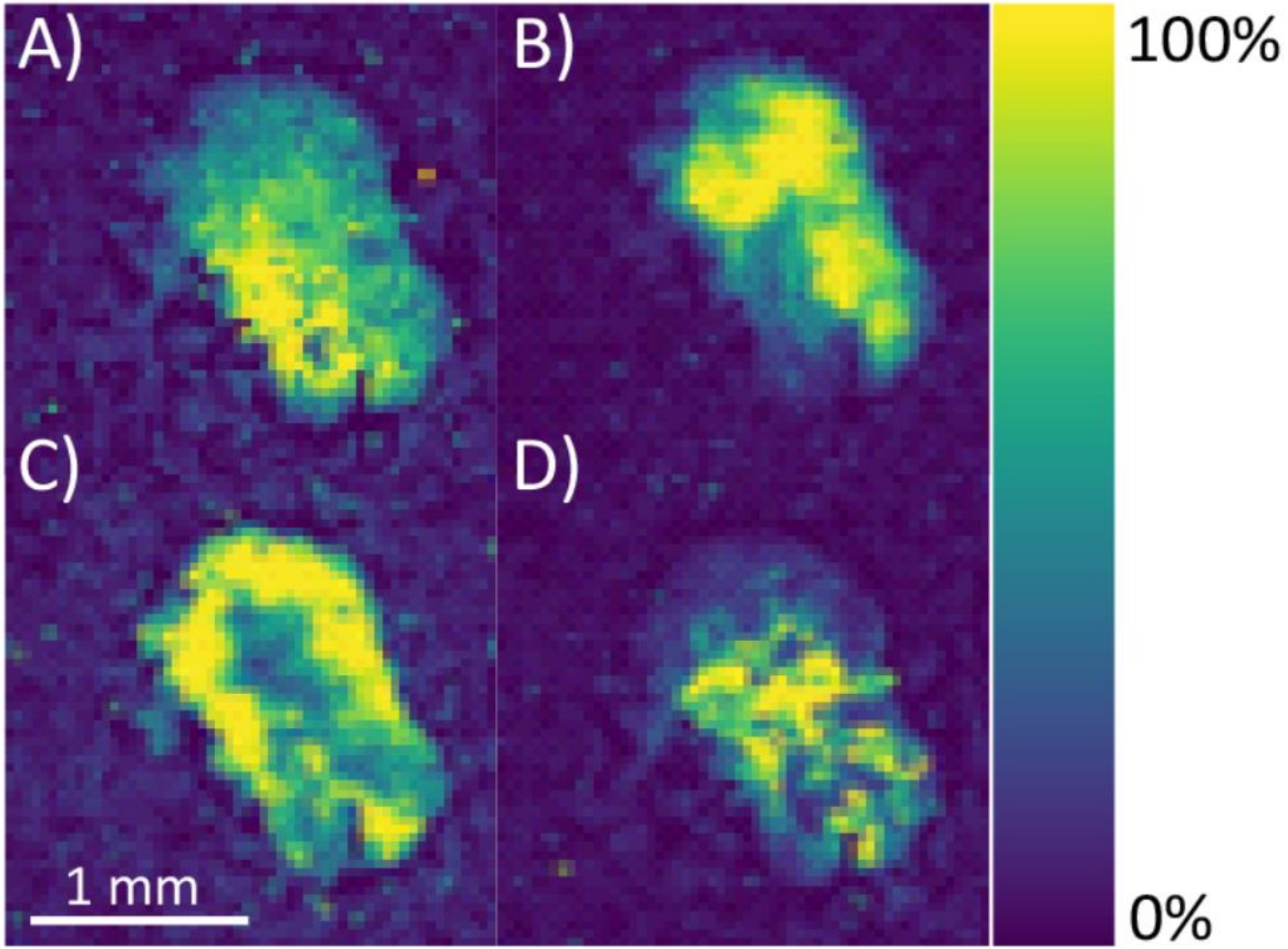


Figure 6



Table

Significance	Coverage (%)	Number of Peptides	PTM	Sample 1 Intensity Area
200	100	5		9.27E+07
200	100	1	Amidation	1.17E+08
200	100	2		1.28E+08
200	100	1	Amidation	2.85E+08
200	100	2	Amidation; Pyro-glu from Q	6.41E+08
200	100	3	Amidation	7.69E+08
200	91	4	Amidation	9.80E+08
200	100	4	Amidation	9.80E+08
200	100	3	Amidation	2.22E+09
200	100	1	Amidation	2.74E+09
200	100	1	Amidation	3.89E+09
200	100	2		9.56E+09
200	100	1	Amidation	1.29E+10
200	100	3	Amidation	2.61E+10
200	91	3	Amidation	2.61E+10
200	100	4	Amidation	2.71E+10
200	89	3	Amidation	7.50E+10
200	100	3	Amidation	7.50E+10
131.93	100	1	Amidation	2.48E+08
75.56	100	3	Amidation; Oxidation (M)	5.36E+09

Sample 2 Intensity Area	Sample Profile (Ratio)	Average Mass
9.31E+08	0.10:1.00	1230
2.04E+09	0.06:1.00	898
1.64E+09	0.08:1.00	1301
4.76E+09	0.06:1.00	845
8.39E+09	0.08:1.00	1048
7.19E+09	0.11:1.00	1183
4.57E+09	0.21:1.00	1336
4.57E+09	0.21:1.00	1223
1.33E+10	0.17:1.00	1294
2.78E+10	0.10:1.00	978
4.18E+10	0.09:1.00	1032
1.90E+11	0.05:1.00	1186
8.48E+10	0.15:1.00	1254
1.50E+11	0.17:1.00	1115
1.50E+11	0.17:1.00	1228
3.29E+11	0.08:1.00	1221
3.74E+11	0.20:1.00	1090
3.74E+11	0.20:1.00	977
3.76E+09	0.07:1.00	855
1.16E+10	0.46:1.00	1108

Description
gi|245|Li_Lab_DB|LL245|CPRP_(CHH_precursor-related_peptide)_SLKSDTVTPLLG_1230.69400_Csap_SG
gi|656|Li_Lab_DB|LL656|RYamide__L/IFVGGSRYamide_897.49411__Cp.PO
gi|441|Li_Lab_DB|LL441|Orcokinin__DFDEIDRSSFA_1301.56440_Csap_SG PO
gi|469|Li_Lab_DB|LL469|Others__HL/IGSL/IYRamide_844.47880__Csap_PO br_Cmaen_Br/SG/PO_Cb_br PO SG S
gi|667|Li_Lab_DB|LL667|RYamide__pQGFYSQRYamide_1030.47411__Csap_SG PO_Cmaen_PO_Cb_br PO SG STG
gi|160|Li_Lab_DB|LL160|Allatostatin_B-type_CbAST-B5_TSWGKFQGSWamide_1182.56907__Csap_PO br_Cmaer
gi|182|Li_Lab_DB|LL182|Allatostatin_B-type__LGNWNKFQGSWamide_1504.80000_Csap
gi|164|Li_Lab_DB|LL164|Allatostatin_B-type_CbAST-B6_GNWNKFQGSWamide_1222.57522__Csap_PO br_Cmae
gi|174|Li_Lab_DB|LL174|Allatostatin_B-type_CbAST-B8_STNWSSLRSAWamide_1293.63346__Csap_PO br_Cmae
gi|660|Li_Lab_DB|LL660|RYamide__SGFYADRYamide_977.44760_Cmaen_PO
gi|153|Li_Lab_DB|LL153|Allatostatin_B-type__AWSNLGQAWamide_1031.50570_Cmaen_PO
gi|465|Li_Lab_DB|LL465|Orcomyotropin__FDAFTTGFGHS_1186.51636__Csap_PO SG br_Cmaen_Br/VNC/SG/PO_
gi|168|Li_Lab_DB|LL168|Allatostatin_B-type__NDWSKFGQSWamide_1253.62000_Csap
gi|669|Li_Lab_DB|LL669|RYamide__SSRFVGGSRYamide_1114.57522__Csap_PO_Cmaen_PO_Cb_PO
gi|672|Li_Lab_DB|LL672|RYamide__LSSRFVGGSRYamide_1257.70000_Csap
gi|163|Li_Lab_DB|LL163|Allatostatin_B-type__SGDWSSLRGAWamide_1220.58070_Csap_PO br
gi|670|Li_Lab_DB|LL670|RYamide__LSGFYANRYamide_1117.58000_Csap
gi|659|Li_Lab_DB|LL659|RYamide__SGFYANRYamide_976.46354__Csap_PO_Cmaen_PO_Cb_PO
gi|054|Li_Lab_DB|LL054|Allatostatin_A-type__DGPYSFGLamide_854.40430_Csap_PO br_Cb_PO
gi|157|Li_Lab_DB|LL157|Allatostatin_B-type__AGWSSM(O)RGAWamide_1123.51470_Csap_PO

STG CoG
G CoG
n_PO_Cb_br PO STG

n_PO_Cb_br PO STG CoG
n_VNC/PO_Cb_PO

_Cb_br PO SG STG CoG

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Table of Materials

TableOfMaterials_RE.xlsx

Lingjun Li, Ph.D.
Vilas Distinguished Achievement Professor of Pharmaceutical Sciences and Chemistry

School of Pharmacy
and Department of Chemistry
University of Wisconsin
777 Highland Avenue
Madison, Wisconsin 53705
Email:lingjun.li@wisc.edu
Phone : (608) 265-8491
Fax : (608) 262-5345

December 5, 2021

Dr. Ling Hao
Guest Editor
Journal of Visualized Experiments
Email: linghao@email.gwu.edu

Nilanjana Saha, PhD
Review Editor
JoVE

Dear Dr. Saha and Dr. Hao,

Enclosed you will find a revised manuscript that we are submitting for publication in *JoVE* in a Focus Collection on Understanding the Central Nervous System by Mass Spectrometry. The article is titled "**Multi-Faceted Mass Spectrometric Investigation of Neuropeptides in *Callinectes sapidus***" and is contributed by Ashley Phetsanthad, Nhu Q. Vu, and Lingjun Li* (* corresponding author).

In addition to a clean version of the revised manuscript, we also provide a version with highlighted changes. We thank the reviewers for their valuable suggestions, and we feel that the revisions have served to greatly strengthen the article, especially in the discussions regarding critical considerations in the protocol. We hope that you find this revised manuscript acceptable for publication in *JoVE*.

Thank you for your consideration. Please do not hesitate to contact me if you have any questions or need additional information.

Best regards,

ingjun Li, Ph.D.
Charles Melbourne Johnson Distinguished Chair
Vilas Distinguished Achievement Professor of Pharmaceutical Sciences & Chemistry
School of Pharmacy & Department of Chemistry
University of Wisconsin-Madison
777 Highland Avenue
Madison, WI 53705-2222
Phone: 608-265-8491
Email: lingjun.li@wisc.edu

Response to Reviewers' Comments

Suggestions/Comments: In black
Response: In blue.

Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response: The manuscript has been proofread for spelling and grammar.
2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Response: The text has been revised.
3. Please define all abbreviations at the first usage.
Response: The text has been revised.
4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Zip tips, etc.
Response: The text has been revised.
5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Response: Additional clarification has been added throughout the protocol. Additional references have also been added throughout the protocol.
6. Please add more details to your protocol steps:
Step 1.1.1: please provide details of how the animal is sacrificed.
Response: A previous JoVE article that details this was cited.
https://www.jove.com/t/1207/cancer-borealis-stomatogastric-nervous-system-dissection
Step 1.1.2: Please provide action steps of dissecting the animal to extract neuronal tissue. Also please specify the neuronal tissue used for this work.
Response: See response above. Additionally, the brain was specified.
Step 1.1.4: Please specify how was the supernatant collected. Was a pipette used? How long this can be stored?
Response: The use of a pipette was included and a timeframe of 6 months was added.
Step 1.1.9: Please include a citation in place of manufacturer's instructions.
Response: This suggestion was incorporated.
Step 1.1.10: Please mention the parameters for the vacuum concentrator.
Response: The RPM and Torr for the vacuum concentrator was included.
Step 1.2.3/1.2.4: Please mention the centrifugation conditions.
Response: Additional centrifugation parameters were included.
Step 1.3.4: Please include all the settings for obtaining MS spectra.
Response: Several additional parameter settings were added.
Step 5.2/5.3: Are Expasy, Signal IP, sulfinator and DiANNA, are all open access?
Response: The text has been edited to specify that these software are all open access.

Steps 3.1.8 – 3.1.10: Please revise the text to avoid overlap with published material.
Response: The text has been revised and additional details have been added.
7. Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc.). Also please ensure that the button clicks are bolded throughout.
Response: All software steps have been revised to start with 'click' or 'select'. Button clicks have been bolded.
8. Please ensure that the highlighted steps are no more than 3 pages including headings and spacings.
Response: Highlighted steps were checked to make sure it constituted less than 3 pages.
9. Figure 4: x-axis label is missing from panel (B)
Response: The figure has been revised.
10. Figure 6: axis for the color bar is missing.
Response: The figure has been revised.

_____

Reviewers' comments:
Reviewer #1:
As peptides tend to adsorb to different surfaces, especially peptides at low concentrations, it would be interesting to know if special precautions are taken for this (for example Low Binding tubes, pipetting protocol, LC vials, solvent selection, etc). How can the authors ensure that both hydrophilic and hydrophobic peptides are still present?
Response: We thank the reviewer for their question. The text has been revised in the second paragraph of the discussion to address this.
Minor Concerns:
1.1.2:
- Could the authors indicate the maximum amount of tissue that can be used for sonication in the total volume of 170 µL?
Response: It was clarified in a note that this describes 1 brain tissue.
- Should the amount of tissue not be noted down in case of quantitation to get an idea of the amount of peptide/amount of tissue?
Response: It was clarified in a note that this describes 1 brain tissue.
- Why is the tissue stored at -80° in 20µL solvent?
Response: A note was added to include "Tissue may be flash frozen immediately without solvent, although this is not recommended as endogenous proteolytic enzymes will not be inhibited and remain active, though in a slower rate when cold."

1.1.4:
- What is meant with medium heat?
Response: Medium heat was replaced with "approximately 35 °C"

1.3.2:
- the dimensions of the nano LC column should be added
Response: The length of the column was added to the text and capillary dimensions were added as a comment to the capillary listed in the table of materials.

Reviewer #2:

Manuscript Summary:
A succinct description of a number of procedures for peptidomics of crab neural tissue.

Major Concerns:
This manuscript describes protocols that trainees spend months-years in lab to master. It is ambitious to describe in a journal article, and it is not realistic to expect an average reader to be able to follow the techniques unless they are already familiar with many of the procedures (e.g. a 'proteomics' background). However, this is not a concern about the specific manuscript, as it was an invited contribution.
Response:We thank the reviewer for their concern. Additional clarification throughout the protocol and the rationale behind some steps has been embedded within the protocol. The discussion section has been thoroughly revised to include additional details for critical considerations of the protocol.

Minor Concerns:
Introduction lines 72-76:
"Main advantages to LFQ include its simplicity, low cost of analysis, and decreased sample preparation steps which can minimize sample loss. However, label-based methods are subjected to less systematic variation as multiple samples can be differentially labeled using a variety of stable isotopes, combined into one sample, and analyzed through mass spectrometry simultaneously."
This will likely be confusing to the non-expert (e.g. the intended audience). Better to add another sentence between these two, that specifically outlines the disadvantages of label-free approaches (e.g. requires multiple technical replicates, prone to error, not accurate for small variations).
Response: We thank the reviewer for their suggestion. A sentence was added to include this additional information.

Introduction:
The authors briefly describe the concept of isotopic / isobaric labeling, and then at the end of the Intro say "We describe protocols for the sample preparation and isotopic labeling, data acquisition, and data analysis of neuropeptides by LC-ESI-MS, MALDI-MS, and MALDI-MSI."
Problem: they don't specifically define which labeling method they describe.
Response: Dimethyl labeling was included in the text to specify.

Protocol section 1.2.1 "Prepare the 2-plex 1:1 isotopic dimethyl labeling solutions in a fume hood: 1% FH2 (13.5 µL (37 wt. % in water) stock in 486.5 µL water), 1% FD2 (25 µL (20 wt. % in water) stock in 475 µL water), and 0.03 M borane pyridine (3.75 µL (8 M stock) in 996.25 µL water)."
Problem: what is FH2 and FD2? This is not a chemical name (F means fluorine). Presumably F is an abbreviation for 'formaldehyde' but I could not see where it was defined. Please define or better, use the chemical name (CH2O).
Response: We thank the reviewer for pointing that out. F was replaced with CH_2O in the text.

Reviewer #3:
Manuscript Summary:
#Comments to Multi-Faceted Mass Spectrometric Investigation of Neuropeptides in Callinectes sapidus submitted by Ashley Phetsanthad, Nhu Q. Vu and Lingjun Li

Phetsanthad et al. presents an optimized workflow for neuropeptiomic research based on the model system Callinectes sapidus. They described step by step each step for neuropeptide identification, confirmation, de novo sequencing and quantification by different mass spectrometric approaches. This analytical tool set contains very important and helpful hints for sample preparation, MS acquisition, MS analysis, and database generation of neuropeptides using LC-ESI-MS, MALDI-MS spotting, and MALDI-MS imaging which could also uses for other endogenous peptides. The manuscript is written very well. Each step is explaining clearly so that the providing pipeline can also use from scientists which are interested in this techniques, but not experts in this field.

I thoroughly enjoyed this manuscript and believe that the data are high quality. However, I have still a few questions and/or suggestions (particularly: discussion section) before I will propose the manuscript for publishing.
Response: We appreciate the positive comments and valuable suggestions to further improve our manuscript.

My Concerns:
Introduction:
Line 59-60: Techniques such as histochemistry and electrophysiology have been employed to investigate neuropeptide structure and function. How can histochemistry helps to investigate the function of a specific neuropeptide? Please explains this statement in more details.
Response: We thank the reviewer for their question. Neuropeptides can be synthesized, stored, and released in different regions of the tissue. The sentence has been modified to include this.

In general, the authors used the term MS1 in the manuscript, sometimes as an abbreviation for mass spectrometry; sometimes for fingerprint mass spectrum or MALDI-TOF MS mode measurements. Please use a consistent designation.
Response: MS1 and MS2 definitions were added for clarity.

Protocol:

1.1.2 What is the minimal sample size which could use in your represented experimental steps for a successful analysis?
Response: We believe that it would be misleading to state a minimal sample size to the readers as there are many variations that this depends on such as animal used, which tissue is collected, and the sensitivity of the mass spectrometer used. In the protocol we mention a suggested concentration of sample to be analyzed, but this number and the starting amount of tissue used is a general guideline for our specific animal model, tissue types, and instruments. We chose that suggested concentration of sample because it is likely to result in neuropeptide detection without causing instrumental errors (i.e., overloading the LC column). This is likely a looser definition than the reviewer's definition of a "successful analysis", and therefore the minimal sample size will vary depending on instrument, analyte, and the goal of the experiment. A qualitative analysis would require different amounts of starting material (i.e., number of sample types and replicates required) than a quantitative analysis. It would also depend on if a targeted or untargeted analysis is being performed.

1.1.10. Which temperature value is meaning with "medium heat"?
Response: Medium heat was replaced with the temperature for clarification.

1.3.1 What does "roughly 3 crustacean tissues mean? Please specify sample size?
Response: This was removed and only concentration is mentioned for unambiguity.

Representative results:

497: what does "should have goo peptide fragmentations" mean? Please specify this statement? Which coverage rate is needs to confirm a neuropeptide and which one is necessary for de novo sequencing? There are any rules? Regulations by the FDA or the mass spec consortium?
Response: We thank the reviewer for bringing up this perspective. Additional clarification was added to address these comments in the text.

499: What does "the poor fragmentation coverage" mean? See above. Please specify.
Response: Additional clarification was added to address these comments in the text.

501: Please explain "XICs?
Response: The acronym was defined for clarification.

506: For MALDI-MS, a matrix salt is needed for peptide analysis. Particularly by using DHB as matrix compound, the matrix-peptide crystal structure is not equal distributed throughout the sample spot. With other words: Depends on the position of the laser onto the sample spot, the resulting mass spectrum is slightly different. My question is: How do the authors deal with this issue? Is it possible to measure two synthetic peptides at the same concentration which are labelled as described for neuropeptide quantification in the manuscript with the same ion signal intensity in a resulting mass spectrum? How many laser shots, laser diameter, or other instrument settings are needed for these measurements? What is the control experiment for neuropeptide quantification by MALDI-TOF MS? How can the authors be sure that different neuropeptide concentrations in different sample sets are not results from matrix application procedure, different matrix crystal pattern, etc.? That are very important points for the evaluation and reproducibility of an experiment so that I would like to ask the authors to add more details about it.
Response: We thank the reviewer for these insightful questions. Additional clarification was added to address all of these comments in the discussion section.

509: Please add more details about "the precursor ion XIC intensity area"? What does it mean?
Response: This was reworded for clarity.

517-518: Please specify the statements "high sequence coverage", "low ion mass error", "high confidence". Please add specific values?
Response: These statements have been clarified in the text.

519: How can mass spec data suggesting a potential biological activity of a peptide to a specific neuropeptide family? Please specify.
Response: This was not the intended suggestion of the sentence. It was reworded for clarity.

525: figure 4a: good spots vs bad spots. Is matrix already applied onto spots 1-3 (left side)? Which matrix was used? How controls the authors such an equal distribution of matrix-analyte mixture onto the sample plate as shown for spot 1-3?
Response: The figure caption has been edited to include “CHCA matrix” and the steps to create

a uniform spot is written at Step 2.1.4 and the step has edited to include “Figure 4a”.

526: what is exactly calibrated with red phosphorus? And why? The ion pattern is different to the ion pattern of neuropeptides?
Response: We thank the reviewer for bringing this to our attention. When using a MALDI-TOF instrument, the instrument must be calibrated at the beginning of each experiment. Any analytes with known masses can be used to calibrate the instrument if it is within the desired mass range of the sample. Red phosphorus is used for the positive ion mass calibration of the instrument. It has advantages over using peptide calibration mixes due to its stability at room temperature, low cost, abundant peaks due to its polymerization, high signal-to-noise ratio, and does not require matrix. This text has been added to the manuscript.

530: How calibrated the authors the ITO-coated slides in IMS experiments?
Response: Step 3.1.8 describes how to calibrate the ITO-coated slide by drawing teach points using a whiteout pen. This has been noted at this part of the text.

531: The diagram is missing in Figure 5. There are only a schematic drawing of an ITO glass slide and a photograph of an ITO slide.
Response: The word schematic is more accurate for the figure and the Figure 5 caption has been updated.

531-538: Please rewrite this section and add more precise information that not only experts in IMS are able to follow this instruction.
Response: We thank the reviewer for this suggestion. This section has been re-written.

Discussion:

593: "Careful sample preparation is required to not only remove more abundant and interfering matrix components, such as proteins and lipids..." How you remove interfering matrix components like proteins and lipids from samples like neuronal tissue? A cell has a cell membrane which is a double lipid layer and there are many ion channels and receptors available. How you can remove these? Please specify.
Response: We thank the reviewer for these questions. Additional information was added to the text.

597: "For neuropeptide characterization in precious samples, peptide concentration is loosely estimated, as peptide assays require the consumption of more neuropeptides than the sample may contain." I do not understand this statement. Please rewriting.
Response: This statement was reworded for clarity.

602-605: To overcome this, an initial peptide assay can be performed using pooled neuronal tissue extracts, where the results are used as an estimate for all subsequent analyses, although it must be kept in mind that all peptides in solution are measured, and not all of these are bioactive neuropeptides.
How can the authors be distinguishing a bioactive neuropeptide from a not active neuropeptide in the samples by MS analysis? Is it possible to identify putative neuropeptide receptors in the same sample pool to suggest a neuropeptide function? What initial peptide assay has to be use? Please rewrite this statement.
Response: Neuropeptide was meant to describe a subset of bioactive molecules, not that

bioactive neuropeptides are distinguished from not active neuropeptides. The word bioactive was removed to alleviate any confusion.

606-607: Which one? Which modifications are necessary to address nonpolar and hydrophobic neuropeptides. Please add more details.
Response: More information was added to the text.

614: Please add the settings of the vacuum desiccator? Pressure, Temperature?
Response: The ultimate pressure is $1x10^{-4}$ Torr at room temperature. This has been added to the text.

615: Please remove: at
Response: Removed.

616: How long should the sample dried in the vacuum desiccator before IMS analysis? Is there a maximum time for such treatment?
Response: There has not been a study to determine the maximum time. The text has been edited to say that this step is important for preventing condensation, but it is not recommended to leave the tissue section in the desiccant system at room temperature after it is already dried.

645: The statement "more difficult" is very subjective. Please provide some ideas to overcome this challenge?
Response: This statement has been removed since it overlaps with points made in the next paragraph regarding LC-ESI-MS data analysis, which contains solutions to overcome those challenges (i.e., using software).

649: The authors are right. There are not many software packages available for IMS analysis, however, SCILS software package contains many useful tools for neuropeptide identification, even neuropeptide quantification. The discussion contains many information about MALDI-MS and LC-ESI-MS based neuropeptide analysis, however, very less for IMS-based neuropeptide investigation. Because of that, I would like asking the authors to improve the discussion by adding more detailed information to imaging MS neuropeptides analysis.
Response: We thank the reviewer for their suggestion. In addition to the information in Step 3.3, the discussion section has been edited to include more details. Popular MS imaging data analysis software can perform accurate mass matching to a neuropeptide mass database and extract the corresponding MS images. For clinical-based research questions, such as biomarker discovery, these software are able to extract *m/z* values unique to a tissue region of interest (typically called Region of Interest (ROI) analysis) and perform statistical tests to quantify how different two tissue regions are. Use of “SCiLS” is the text is not permitted.

658: "common motifs" Please add examples.
Response: The text has edited to add clarification and examples.

659: All species have a complete genome sequence. The complete genomes from a few species are decoded so far.
Response: The sentence has been edited.

661: Is a comparison of MSMS data obtained from a de novo sequenced neuropeptide with the MSMS pattern of the synthetic peptide the only way to confirm the amino acid structure of a

novel neuropeptide? This would be a very expensive strategy for neuro peptidomic of non-genomic model systems.
Response: We thank the reviewer for their question. We have added a discussion in the second paragraph of the representative results section to answer this question.

662: In the insect Manduca sexta, it was shown that the truncated version of a peptide has a higher biological activity in the physiological assay as the full version of the neuropeptide. Both, could be detected by MALDI-MS and LC-ESI-MS experiments. Is the degradation level of peptides comparable in MALDI and LC-ESI MS experiments? Do the peptide precursors containing information (e.g. potential cleavage sides) which helps to distinguish a truncated version of a peptide from a degradation-based detected neuropeptide?
Response: We thank the reviewer for their insightful question. We are not sure what study the reviewer is referring to since the paper is not referenced in our article. Regarding the degradation level between MALDI and LC-ESI-MS ionization sources, it is known that ESI is a "softer" ionization method than MALDI, so it is plausible to assume rates of artificial degradation (i.e., in-source fragmentation) is different. Regarding how to distinguish between a truncated version of a peptide from a degradation-based detected neuropeptide, this issue can be resolved if the preprohormone processing pathway for that neuropeptide is known. Since this is often not the case, the bioactivity of the neuropeptide can be assessed by physiological techniques. If a neuropeptide is shown to have bioactivity, it is likely not an artificial (i.e., not *in vivo*) degradation product. This has been added to the text.

Lingjun Li, Ph.D.
Vilas Distinguished Achievement Professor of
Pharmaceutical Sciences
and Chemistry

School of Pharmacy
and Department of Chemistry
University of Wisconsin
777 Highland Avenue
Madison, Wisconsin 53705-2222
Email: lingjun.li@wisc.edu
Phone : (608) 265-8491
Fax : (608) 262-5345

Neethu Abraham, PhD
Science Editor | JoVE
Email: neethu.abraham@jove.com

Ling Hao, PhD
Guest Editor
Journal of Visualized Experiments
Phone: 202-994-4492
Email: linghao@email.gwu.edu

September 17, 2021

Dear Neethu and Ling,

Thank you very much for the kind invitation to participate in a Focus Issue of *Journal of Visualized Experiments* (*JoVE*) on Understanding the Central Nervous System by Mass Spectrometry. Enclosed you will find a manuscript that we are submitting for publication in *JoVE* in response to this invitation. The article is titled "**Multi-Faceted Mass Spectrometric Investigation of Neuropeptides in *Callinectes sapidus***" and is contributed by Ashley Phetsanthad, Nhu Q. Vu, and Lingjun Li* (* corresponding author).

This manuscript describes general workflows and provides detailed protocols for mass spectrometry-based experiments for neuropeptide identification, quantitation, and localization. These protocols utilize popular vendors for instruments and consumable items, so we expect the protocol to be useful and accessible for a large number and a broad range of *JoVE* readers. The protocol is divided into three main sections: preparing tissue samples from a crustacean model organism for LC-MS, MALDI-MS (spot), and MALDI-MSI analysis; acquiring and analyzing the MS data; and building a neuropeptide database for model organisms without a complete known genome sequence, like our model organism *Callinectes sapidus*. Additionally, this manuscript contains a discussion about the critical steps in the protocol, especially considerations that are unique to endogenous peptides, and how to discern acceptable from unacceptable data. The manuscript concludes by comparing the different rationales involved in LC-MS and MALDI-MS analysis strategies, as well as addressing current challenges in neuropeptidomics, such as verifying neuropeptide bioactivity.

We believe this work will be informative and attractive to a large number of readers of *Journal of Visualized Experiments*, including anyone interested in developing more sophisticated methods for addressing challenges related to neuropeptide characterization, applying these methods to studying biomolecules such as endogenous peptides, or otherwise contributing to the field of LC-MS or MALDI-MS in any context.

We suggest the following as potential reviewers:

Susanne Neupert
Institute for Zoology
University of Cologne
Zülpicher Straße 47b
50674 Köln
Email: mail@susanne-neupert.de

Ron Heeren
Department of Health, Medicine and Life Sciences
Maastricht University
Minderbroedersberg 4-6
6211 LK Maastrict, The Netherlands
Email: r.heeren@maastrichtuniversity.nl

Dawn Blitz
Department of Biology
Miami University
274 Pearson Hall
Email: Dawn.Blitz@MiamiOH.edu

Per Andren
Department of Pharmaceutical Biosciences
Uppsala University
Box 591
751 24 UPPSALA
Email: per.andren@farmbio.uu.se

Jonathan Sweedler
Department of Chemistry
University of Illinois
600 South Mathews Avenue
Urbana, IL 61801
Email: sweedler@scs.illinois.edu

Peter Verhaert
Maastricht MultiModal Molecular Imaging (M4I) Institute
Maastricht University
Universiteitssingel 50, 6229ER Maastricht, The Netherlands
Email: p.verhaert@maastrichtuniversity.nl

Thank you for your consideration. Please do not hesitate to contact me if you have any questions or need additional information.

Best regards,

Lingjun Li, Ph.D.
Vilas Distinguished Achievement Professor of Pharmaceutical Sciences and Chemistry
Charles Melbourne Johnson Distinguished Chair in Pharmaceutical Sciences
School of Pharmacy and Department of Chemistry
University of Wisconsin-Madison
777 Highland Avenue
Madison, WI 53705-2222
Phone: 608-265-8491
Email: lingjun.li@wisc.edu

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Multi-Faceted Mass Spectrometric Investigation of Neuropeptides in Callinectes sapidus
Author(s):	Ashley Phetsanthad, Nhu Q. Vu, and Lingjun Li

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

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Cambridge, MA 02140
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CORRESPONDING AUTHOR

Name:	Lingjun Li
Department:	School of Pharmacy and Department of Chemistry
Institution:	University of Wisconsin-Madison
Title:	Professor

Signature:  Date: 9/17/2021

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