

Mass Spectrometry Methods and Applications for Functional Characterization of the Crustacean
Neuropeptidome

By

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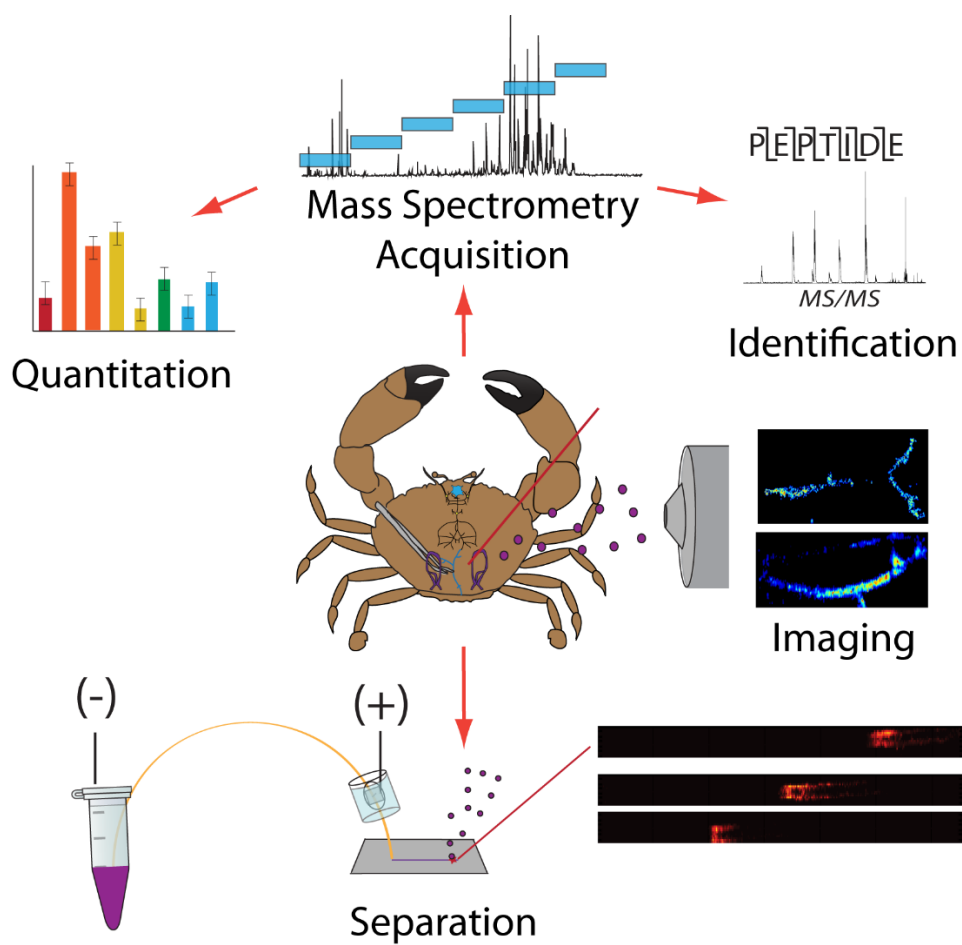
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Abstract



Abstract

Neuropeptides are a diverse class of signaling molecules that are responsible for modulating numerous functions within the nervous system. They are challenging to study because, like most signaling molecules, they are present in low abundances *in vivo* and prone to rapid degradation. In this dissertation, multifaceted mass spectrometry (MS) methods are developed and implemented to characterize neuropeptides within crustacean model organisms. Decapod crustaceans are a useful model for understanding circuit dynamics due to their simplified nervous system, robust central pattern generators, and homology to higher-order organisms, including mammals. This work involves developing methods to improve the coverage of neuropeptides detected by implementing complementary separation and ionization techniques, the identification and reproducibility of neuropeptide analysis with optimized mass spectral acquisition parameters, and the accuracy of sequence information with software specifically designed for neuropeptide analysis. These methods are utilized to profile the neuropeptides present in the cardiac neuromuscular system that may be responsible for modulating heartbeat, as well as feeding-related neuropeptides in tissue and circulating fluid that may be implicated in generating rhythms related to chewing and filtering of food. Overall, this dissertation research develops improved methods for analyzing neuropeptides, demonstrates the utility of these methods for better understanding their functional roles within crustacean model organisms, and provides deeper insight into how these neuropeptides may be involved in modulating key central pattern generators and influencing circuit dynamics.

Chapter 1

Introduction and Research Summary

Introduction

Neuropeptides are important signaling molecules responsible for modulating a wide variety of functions within the nervous and neuroendocrine system. These short-chain amino acid sequences are synthesized from larger preprohormones and can function both locally, on the same neuron they originate from or an adjacent neuron, as well as long range as circulating hormones.¹ Their various roles within circuit dynamics make them an important class of molecules to study, but their diversity makes these studies challenging.^{2, 3} Neuropeptides are the most diverse class of signaling molecules and, as with all signaling molecules, they are present in low abundances *in vivo* and prone to rapid degradation. Consequently, progress within the field of neuropeptidomics has been limited.⁴ The purpose of this work is to develop methods to overcome the challenges of neuropeptide studies and gain insight into their functional roles within the nervous system.

Decapod crustaceans, specifically the crab species *Cancer borealis*, *Callinectes sapidus*, and *Carcinus maenas*, are used as model organisms in all of these studies. Crabs, in addition to other decapod crustaceans, have long been used as a model organism for studying the circuit dynamics of the nervous system.⁵ These animals have simple yet elegant nervous systems comprised of a small number of easily-distinguishable neurons, making it possible to map individual neuron connections and perform accurate electrophysiological studies.⁶ However, crustaceans pose their own challenge. The genomes of most of these species are far from complete. Therefore, information about the neuropeptides present cannot be predicted and instead relies on sensitive mass spectrometry (MS) analysis facilitated by *de novo* sequencing.⁷ This research employs a multifaceted mass spectrometry approach in order to uncover more of these neuropeptides and gain a deeper understanding of their functional role within the nervous

system. This feat is accomplished by first improving neuropeptide analysis methods involving separation, ionization, MS acquisition, and identification. These methods are then implemented to obtain a deeper understanding of these neuropeptides within the tissue and circulating fluid and how they are affected by feeding behavior.

Research Summary

In order to fully understand how neuropeptides are involved in body process, behavior, and disease, in-depth knowledge must be gained on their structure, localization, and function. **Chapter 2** provides background and information about relevant literature of current methods to study neuropeptides.⁸ Information is provided on the importance of neuropeptides to understand biological mechanisms and circuits, as well as disease mechanisms. Structural elucidation has mostly been accomplished by MS techniques with assistance from nuclear magnetic resonance and other spectroscopy methods. Bioinformatics has greatly assisted in improving throughput and predictive power. Localization of neuropeptides within tissue has historically been performed mostly with targeted techniques such as immunohistochemistry and radioimmunoassays. However, there has been notable expansion in recent years in the field of MS imaging, providing untargeted spatial information of hundreds of molecules within a single analysis. Numerous approaches have been utilized to understand the function of neuropeptides, coupling behavioral studies with tissue-specific assays, electrophysiology, optogenetic tools, and quantitative mass spectrometry. All of these methods are described in the context of neuropeptide research, and a perspective of future frontiers within the field is also presented. This chapter highlights the importance of using multiple techniques together to gain a deeper

understanding of neuropeptides within the system as a whole, which is a theme highlighted throughout the rest of this dissertation.

The most common method for profiling neuropeptides is liquid chromatography (LC) coupled to MS with electrospray ionization (ESI). However, as neuropeptides are diverse and no single technique can provide complete coverage of all neuropeptides within a sample, **Chapter 3** describes the development of a complementary separation and ionization method using capillary electrophoresis (CE) and matrix-assisted laser desorption/ionization (MALDI).⁹ CE is a powerful separation technique that provides sharp, highly-resolved peaks in a short analysis time. MALDI possesses advantages as an ionization technique, such as simpler sample preparation, reduced dilution, and tolerance to salts. Most importantly, both CE and MALDI are complementary to LC and ESI, respectively, due to differences in their mechanisms. Therefore, by using both methods in parallel, the breadth of neuropeptide coverage can be drastically increased. With the CE-MALDI method, MS imaging is used to acquire MS spectra along a continuous trace, allowing the high separation resolution of CE to be retained. The addition of a coating to the inner wall of the capillary and a pre-concentration method helped to improve the sensitivity of the method. The CE-MALDI-MSI method demonstrated good reproducibility, with lower than 5% relative standard deviation for standards and enabled the identification of over 200 neuropeptides per run in both brain and sinus gland tissue extracts. While this number was similar to that of conventional LC-ESI-MS, most of the neuropeptides were unique to each method, indicating the orthogonality of the two approaches and the potential for the CE-MALDI-MSI method to uncover previously undetected neuropeptides within the crustacean nervous system. These results indicate the potential of CE-MALDI-MSI for routine use in neuropeptide research.

In addition to adequate separation and ionization, confident detection of neuropeptides requires suitable MS acquisition parameters. In order to ensure confident identification of neuropeptides, tandem MS (MS/MS) spectra must be collected. Typically, these spectra are collected using data dependent acquisition (DDA), in which the most abundant ions in each MS spectrum are fragmented for MS/MS. However, as neuropeptides are often present in very low abundances, they tend to get missed for fragmentation in favor of other artifacts in the sample (e.g. lipids and protein fragments). **Chapter 4** describes the development of a data independent acquisition (DIA) method for neuropeptides, in which instead of fragmenting individual ions, a range of ions are fragmented simultaneously.¹⁰ Parameters including mass range and window size were optimized specifically for crustacean neuropeptide samples, resulting in the detection of almost twice as many neuropeptides in brain and pericardial organ tissue. Furthermore, as identification was not dependent on relative ion abundances, the technical and biological reproducibility of the method were found to be greatly improved. With the improved detection capabilities of this method, 67 putative novel neuropeptides were detected and identified with *de novo* sequencing. The developed method is anticipated to be useful for enabling a deeper profiling of neuropeptides, especially those in low abundance, in a variety of sample types.

With the large number of spectra generated from a single MS experiment, identification of neuropeptides requires sophisticated software tools. Neuropeptide identification is challenging with the current tools available, as most are designed for bottom-up proteomic experiments, in which a digestion enzyme is used that yields a known pattern in the resulting peptides. Neuropeptides do not follow these patterns, but many of them possess their own pattern, as neuropeptides tend to belong to families with characteristic sequence motifs. **Chapter 5** describes a *de novo* sequencing software, titled PRESnovo, that takes advantage of these

sequence motifs.¹¹ With the software, neuropeptide sequences are broken into two parts: a motif with conserved amino acid residues and the remaining partial sequence. PRESnovo assigns motifs to each MS/MS spectrum based on characteristic fragment ions. A *de novo* sequencing software can then be used to identify the remaining part of the peptide sequence. Performance analysis showed high accuracy with the software and, when applied to tissue samples from *C. maenas* and *C. borealis*, led to the detection of a large number of neuropeptides, including 13 putative novel neuropeptides identified in *C. maenas* brain, 77 in *C. borealis* brain, and 47 in *C. borealis* sinus glands.

With advances in techniques for neuropeptide research, information can be gained about their role within various central pattern generators. One notable central pattern generator in crustaceans is the cardiac neuromuscular system. The muscles that contract the heart are controlled by the cardiac ganglion (CG). This ganglion contains only 9 neurons, 5 motor neurons and 4 pacemaker neurons, but is able to produce a robust rhythm. As the CG is neurogenic, determining what neuropeptides are present within this tissue can provide information into how this circuit works. **Chapter 6** describes a multi-platform MS approach to profile and localize neuropeptides within the general region of specific neurons in the CG. A comparison was made with two MALDI MS imaging platforms, using an Orbitrap and a time of flight (TOF) mass spectrometer. The Orbitrap provided greater mass resolution, offering more confident identifications, while the MALDI-TOF instrument was able to provide an improved spatial resolution. MS images were obtained for 235 putative neuropeptides in the CG, with the identification of 145 being confirmed by either complementary MS/MS data or accurate mass matching, a surprisingly high number for such a small tissue. Imaging results showed distinct localization patterns for neuropeptides in discrete areas that may be indicative of specific

neurons. These results highlight both the power of advanced MS imaging platforms and the neurochemical complexity of this small ganglion.

The other notable central pattern generator studied in crustaceans is within the stomatogastric nervous system (STNS), and it is also heavily modulated by neuropeptides. As a result, understanding how neuropeptides influence its output can provide critical information on circuit dynamics. **Chapter 7** describes an initial step at determining what neuropeptides are involved in generating these rhythms by studying the changes in abundance of neuropeptides during feeding in key neural tissue, the brain and pericardial organs (PO) in both *C. sapidus* and *C. maenas*.¹² Stable isotope labeling was used to label fed and unfed tissues, which were then combined and analyzed with CE for fractionation and high-specificity analysis in MALDI-MS. MALDI MS imaging was also used to map the spatial distribution of neuropeptides in the POs. A number of neuropeptides, including those belonging to the RFamide, B-type allatostatin, RYamide, and orcokinin families exhibited statistically-significant changes in abundance after feeding in this investigation. Peptides from the B-type allatostatin family found in PO tissue were shown to have both altered expression and localization changes after feeding, indicating that they might be a class of vital neuropeptide regulators involved in feeding behavior.

The rhythms generated from the STNS that are involved in feeding behavior (gastric mill and pyloric) are generated by the stomatogastric ganglion (STG), which receives input from neuropeptides both locally through direct neural projections from nervous system tissue and long-range from circulating hormones released from neuroendocrine tissue and transported through the hemolymph. **Chapter 8** and **Chapter 9** describe research related to uncovering more information regarding which neuropeptides are responsible for influencing these behaviors in tissue and circulating fluid of *C. borealis*. As hemolymph and tissue samples each carry their

unique challenges, different methods were employed for each. In **Chapter 8**, the neuropeptide content of hemolymph was profiled using the DIA-MS method described in **Chapter 4**, and it was shown to yield improvements in the number and reproducibility of detected neuropeptides. The method was used to study hemolymph collected from crabs at 5 time points, including unfed, 0 minutes post-feeding, 15 minutes post-feeding, 1 hour post-feeding, and 2 hours post-feeding. A total of 217 neuropeptides were detected, mostly from hormone families and neuropeptides commonly identified in the PO. Among these, many varied in their presence throughout the feeding process, and 8 showed statistically-significant changes in relative abundance throughout the feeding cycle. Of the peptides showing changes in either presence or abundance, many were involved in excitatory or inhibitory functions in the STNS, while others currently have unknown functions, but their changes observed in this study indicate that those functions may be related to feeding. Additionally, the high sensitivity of this method enabled the identification of 101 putative novel neuropeptides that may also have a functional role in feeding.

Chapter 9 describes an investigation of neuropeptides originating in tissue that may be involved in feeding modulation. To improve quantitative sensitivity, duplex reductive methylation was used as a chemical tag to differentially label samples from fed and unfed crabs. This method was used to profile neuropeptides in 5 different neural tissues, brain, POs, commissural ganglia, stomatogastric ganglion, and sinus glands, and 80 out of 278 detected neuropeptides were found to be significantly dysregulated in at least one tissue after feeding. MS imaging was also used to map the spatial distribution of neuropeptides in the brain and PO, leading to additional neuropeptides identified that may be involved in feeding. Additionally, *de novo* sequencing was employed to identify 69 putative novel neuropeptides, with 2 of these

being significantly upregulated in the PO. This research went a step further to assess the biological activity and function of these two neuropeptides. A key advantage of studying *C. borealis* is the robustness of its central pattern generators, enabling *ex vivo* analyses to be performed without disrupting the rhythms generated by these circuits. An *ex vivo* heart preparation was used to determine the effect of the two novel neuropeptides on heartbeat. One peptide was found to increase the contraction amplitude and frequency. The other peptide was found to decrease frequency of contractions. The fact that these two peptides elicited a change in heartbeat indicates that they may have important functional roles in modulation of circuits within the animal.

To summarize this research, **Chapter 10** provides an overview description of this thesis intended for a broader audience and was written in collaboration with the Wisconsin Institute for Scientific Literacy. **Chapter 11** provides general conclusions drawn from this research and speculation on future directions that may expand on this research, as well as how it can be extended to new directions to further contribute to neuroscience research. A list of publications and presentations resulting from this work is provided in **Appendix I**, and **Appendix II** details some of the protocols used in this research.¹³

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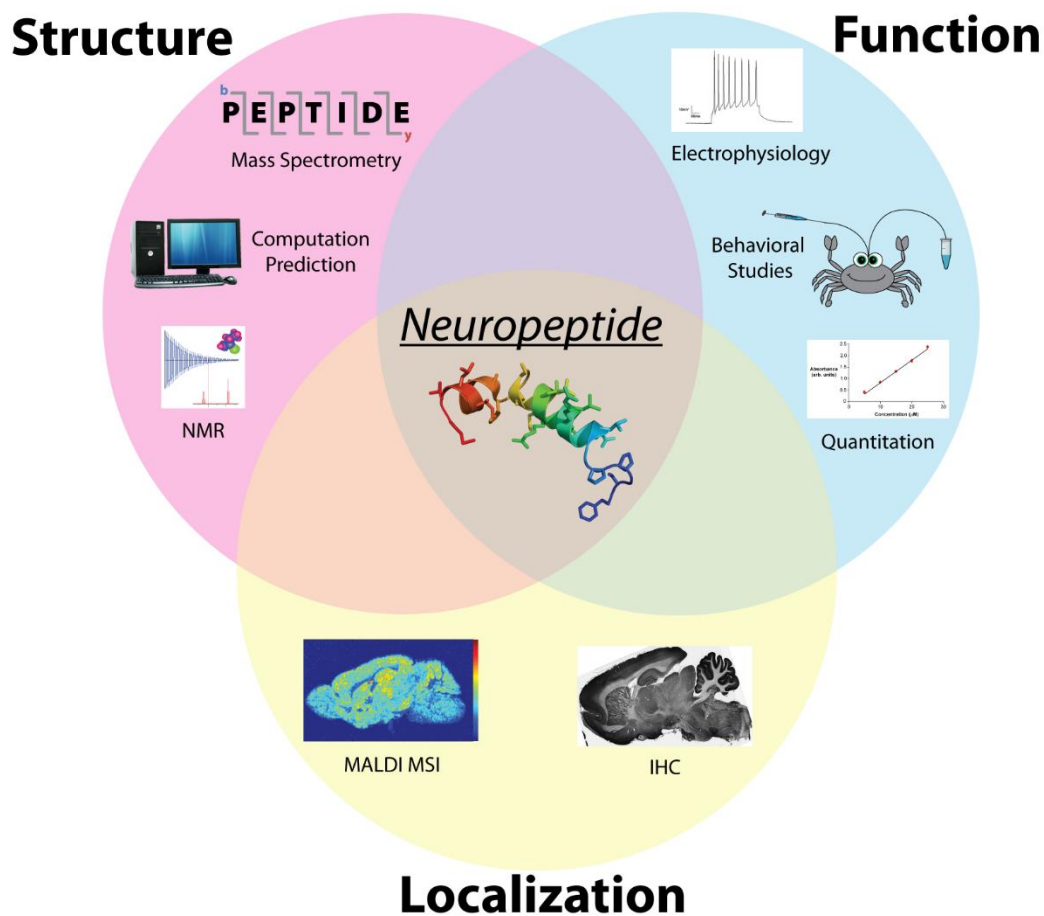
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Chapter 2

New Techniques, Applications, and Perspectives in Neuropeptide

Research



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Abstract

Neuropeptides are one of the most diverse classes of signaling molecules and have attracted great interest over the years owing to their roles in regulation of diverse physiological processes. However, there are unique challenges associated with neuropeptide studies stemming from the highly variable molecular sizes of the peptides, their low *in vivo* concentrations, high degree of structural diversity, and their large number of isoforms. As a result, much effort has been focused on developing new techniques for studying neuropeptides, as well as novel applications towards learning more about these endogenous peptides. The areas of importance for neuropeptide studies include structure, localization within tissues, interaction with their receptors, including ion channels, and physiological function. Here we discuss these aspects and their associated techniques, focusing on technologies that have demonstrated potential in advancing the field in recent years. Most identification and structural information has been gained by mass spectrometry, either alone or with confirmations from other techniques, such as nuclear magnetic resonance spectroscopy and other spectroscopy tools. While mass spectrometry and bioinformatics tools have proven to be the most powerful for large-scale analyses, they still rely heavily on complementary methods for confirmation. Localization within tissues, for example, can be probed by mass spectrometry imaging, immunohistochemistry, and radioimmunoassays. Functional information has been gained primarily from behavioral studies coupled with tissue-specific assays, electrophysiology, mass spectrometry, and optogenetic tools. Concerning the receptors for neuropeptides, the discovery of ion channels that are directly gated by neuropeptides opens up the possibility of developing a new generation of tools for neuroscience, which could be used to monitor neuropeptide release or to specifically change the

membrane potential of neurons. It is expected that future neuropeptide research will involve the integration of complementary bioanalytical technologies and functional assays.

Introduction

Neuropeptides are a class of endogenous peptides that act as long-lasting neurotransmitters in the nervous system and other target organs. By signaling via synapses or volume transmission via diffusion, as well as long-range signaling as circulating hormones, neuropeptides and their receptors play an important role in several key processes. When a neuron releases neuropeptides, the binding of the neuropeptide to its receptor on a receiving cell causes conformational changes within the receptor that, depending on the type of receptor, either open ion channels or activate coupled G proteins that can cause a series of downstream effects within the cell.¹ As neuropeptides are a highly diverse class of signaling molecules in the brain and other peripheral organs, their structures, function and localization are of great interest and relevance.² The extent of their implied roles in normal biological functions has been a point of focus in studies over the years.^{3, 4} Abnormalities in their expression can contribute to various neurological diseases by altering the function of specific neurons, and so understanding the mechanisms of how neuropeptides interact can help researchers to better understand these diseases and develop more focused and effective treatments.⁵ Furthermore, neuropeptides have been implicated in the regulation of normal biological functions, such as feeding regulation^{6, 7} and the adaptation to external factors, such as temperature fluctuation⁸ and internal stress factors, including depression, anxiety, and post-traumatic stress disorder.^{9, 10} As a result, understanding the specific role individual neuropeptides play in response to interactions with the environment and in the execution of biological functions can provide a greater understanding of the

underlying mechanisms at the cellular and systemic level. Investigations of the relationship between neuropeptides and their receptors are useful for the development of drug molecules for treating diseases or for otherwise manipulating interactions between peptidergic neurons, such as the treatment of specific symptoms (*e.g.*, chemotherapy-induced emesis).¹¹

While neuropeptides are interesting biomolecules and play important roles in regulating a wide range of physiological functions, they possess numerous characteristics that make them challenging to study. Because neuropeptides can be used as modulators for signaling locally between neurons as well as function as hormones that can travel long distance to target sites, the *in vivo* concentrations can vary dramatically. Furthermore, low concentrations of neuropeptides can have profound effects – these signaling molecules are typically present at low endogenous concentrations, up to 1000-fold or lower than classical neurotransmitters and other metabolites.¹² This challenge is exacerbated by the lack of a digesting enzyme for a typical neuropeptide analysis workflow as these molecules are products of proteolytic processing and post-translational modifications (PTMs) that occur inside the cells or during transportation. As such, there is only one opportunity for detecting each peptide, unlike in ‘bottom-up’ proteomic studies, where a single unique tryptic fragment is sufficient to detect a protein.¹³ This necessitates the development of highly sensitive detection methods in order to avoid large sample requirements. Additionally, sample processing methods need to be fast because, as with other signaling molecules, neuropeptides are prone to rapid degradation to prevent concentration increases with sequential release. Thus, it is often difficult to identify peptides as endogenous and not simply the product of a degraded larger protein, further complicating analysis.¹⁴ Additionally, there is a high amount of variability between different neuropeptides, either owing to possession of different sequences but the same mass or having numerous PTMs.¹⁵ Even when the structure is

identified, there are still complications. Neuropeptides can have the same structure but different functions or have different functions depending on the cell-type and nearby receptors.¹⁶

Furthermore, as many isoforms exist for various neuropeptide families, localization of specific neuropeptides can be challenging owing to difficulties assigning mass spectral peaks to specific peptides.¹⁷

Despite these difficulties, much progress has been made over the years to characterize neuropeptides, including gaining information about their structure, function, and localization within cells and the whole neuroendocrine system. Here we focus on reviewing recent advancements made in developing techniques and applications to study neuropeptides and their receptors, while pausing to offer insights into the direction in which the field is moving. The areas described include structural elucidation of neuropeptides, methods for their localization, and their functional assessment, as depicted in **Figure 1**, all of which are required to understand neuropeptide biology comprehensively. We also present a case study on the characterization of peptide-gated ion channels and how they might be developed into new tools for neuroscience. While space constraints mean that we do not intend to provide a comprehensive account of all recent publications, we nevertheless provide notable highlights of some key developments made within the past few years.

Elucidation of neuropeptide structures

Perhaps the most important information gained about neuropeptides relates to their structures (*e.g.*, amino acid sequence, folding pattern, binding sites, etc.), as these provide insights into their function and biological mechanisms. However, gaining this information can be challenging and cumbersome. While it has been almost a century since the first neuropeptide,

substance P, was discovered¹⁸ and 50 years since the sequence of that peptide was determined,¹⁹ technology has since developed impressively, and there are now records of almost 6000 neuropeptide sequences across all species.²⁰

Early work in structural elucidation relied on Edman degradation, a technique developed by Pehr Edman, in which peptides are reacted with phenyl isothiocyanate on the N-terminus and analyzed one amino acid at a time as each residue is removed.²¹ Successful sequencing using Edman degradation relies on the peptide being present in high concentrations (>1 picomolar) and high purity. While Edman degradation is a classic method that allowed for the sequencing of many neuropeptides early on, it is less widely used, as other, more high-throughput, methods have emerged in recent years. The technique is still used in some applications, although mostly coupled with mass spectrometry (MS). For example, it has been successfully used to sequence a novel neuropeptide, Y-HS, in leeches,²² to discover a novel arrangement of cysteine residues in a worm-hunting snail neuropeptide,²³ and to determine the sequence of human and mouse urocortin 2, a member of the corticotrophin-releasing factor neuropeptide family.²⁴ Although it has proven to be a useful addition to other techniques in these applications, the method has largely been replaced with higher-throughput and more-sensitive methods such as MS in the past decade.

Currently, MS serves as the method of choice for sequencing and determining PTMs of neuropeptides.^{12, 15, 25} MS has proven to be useful for detecting small amounts of peptides in complex biological samples, making it a high-throughput and versatile technique ideal for the study of endogenous neuropeptides. This advancement has enabled the emergence of ‘neuropeptidomics’, studying the entire neuropeptide complement as a whole either by comparing spectra to a database of known neuropeptides or *de novo* sequencing to discover new

neuropeptides.²⁶ **Figure 2** shows how MS spectra can be used to assign sequences by matching fragment ion masses to amino acids based on cleavage patterns and comparing the *de novo* sequences to those predicted based on genomic data.

Numerous studies have been performed using a variety of specific MS techniques. These techniques and their results have been comprehensively reviewed elsewhere,^{27 12} but noteworthy strides have been made within the past year, such as developments in analyzing neuropeptides rich in disulfide bonds, which in the past has been a particularly laborious task. A new method has recently been developed for identifying disulfide bonds by alkylating peptides and then performing targeted fragmentation on disulfide-bonded peptides.²⁸ Another method has integrated MS and nuclear magnetic resonance (NMR) techniques for rapid identification and characterization of disulfide bonds using a sample comprising only 4 ng. The method involved studying the mass differences between folded and unfolded neuropeptides. As disulfide bond formation results in a mass difference of about 2 Da due to the loss of 2 hydrogen atoms, and these disulfide bonds are not present in unfolded peptides, the mass differences can be used to assess the number of disulfide bonds present in a folded peptide. The presence of disulfide bonds was then confirmed with NMR.²⁹ Another challenge with neuropeptidomics is that neuropeptides degrade substantially over time. A new, high-throughput framework for neuropeptide identification has recently been developed for fast, high-throughput analysis, minimizing neuropeptide degradation. With this method, researchers were able to successfully identify thousands of neuropeptides and post-translational modifications.³⁰ Additionally, neuropeptide sequence coverage has been shown to improve when coupling several techniques of fragmentation (*e.g.*, high-energy collisional dissociation and electron-transfer dissociation),³¹⁻³³

especially when utilizing dual fragmentation with electron-transfer high-energy collisional dissociation (ET_hcD).^{34, 35}

Advances have also been made in the characterization of novel neuropeptides from model organisms, expanding our knowledge of existing neuropeptides. By combining matrix-assisted laser desorption ionization (MALDI-) and electrospray ionization (ESI-) MS to characterize the carpenter ant neuropeptidome, 39 neuropeptides were identified.³⁶ The beetle neuropeptidome has also been expanded within the past year, with novel neuropeptides from the adipokinetic hormone family sequenced with ESI-MS and tandem MS. These were confirmed by co-eluting each naturally existing neuropeptide with its synthetic neuropeptide by means of liquid chromatography (LC).^{37, 38} One other noteworthy group of organisms seeing significant growth in its neuropeptidome has been members of the subphylum crustacea. Multidimensional MS techniques have been successfully implemented in both defining neuropeptidomes, such as that of the spiny lobster,³⁹ and discovering numerous novel neuropeptides.^{40, 41} As a complementary separation technique, ion mobility spectrometry (IMS) is currently experiencing rapid growth, and its use in conjunction with MS has allowed for a more comprehensive study of peptide structure.⁴² Recently, IMS has been used for conformational studies investigating the role of the penultimate proline⁴³ and D-amino acid containing peptide epimers,^{44, 45} and it is expected that more structural knowledge will come from IMS in future studies.

The capabilities of MS for discovery and characterization of neuropeptides have been greatly assisted by the development of computational methods for predicting neuropeptides from precursor proteins and gene sequences. The area of *in silico* prediction has seen substantial growth in the past few years, with neuropeptidomes being predicted for a wide variety of organisms and novel neuropeptides being targeted for more-focused analyses.⁴⁶ Coupling these

informatics approaches to other methods of analysis, particularly MS, has allowed for an expanded coverage of neuropeptidomes, which is important for organisms or species without a fully sequenced genome, especially when one transcript can produce several neuropeptides.^{46, 47} For example, the crab *Cancer borealis* peptidome was doubled by mining its neural transcriptome.⁴⁸ In complement, researchers have also made use of *in silico* prediction methods to characterize neuropeptide receptors, which gives insight into both structural and functional properties by assessing similarities to previously characterized receptors.⁴⁹ Computational efforts have further been directed toward compiling databases of known neuropeptides to provide comprehensive coverage and compare neuropeptides detected in different species. The most recent of these, NeuroPep, contains almost 6000 entries.²⁰ Resampling approaches are being developed to improve database matching, allowing for better identifications in terms of both quality and quantity.⁵⁰

By combining information about structure gained from MS with other powerful analytical tools, researchers have been able to gain better insight into the overall structural composition of neuropeptides. Various types of NMR techniques have been implemented for studying neuropeptides, which are particularly valuable for characterizing folding patterns. **Figure 3** shows an example of how several complementary NMR experiments can be combined to assess the structural conformation of a neuropeptide, as was done to determine the conformational patterns of a hormone pheromonotropin that controls larval sex pheromone production.⁵¹ Several recent advances have been made in understanding the secondary structure of neuropeptides. As an example, a precursor protein existing in marine snail venom was investigated using solution NMR structural determination and found to have a disulfide-directed beta-hairpin fold, which initiates folding in other disulfide-containing areas of the peptide.⁵²

Some neuropeptides have been found to lack secondary structure, as in the case of an RFamide neuropeptide discovered in cone snail venom.⁵³ As structural characteristics are important to the interaction between neuropeptides and their receptors, many recent advances have used NMR to characterize these relationships, such as determining which conformations are important to biological activity, as has been investigated for various analogs of an allatostatin neuropeptide,⁵⁴ as well as determining which part of the receptor neuropeptides were bound to, as has been accomplished with solid-state NMR neuropeptide Y with its receptor.⁵⁵ The relationship with receptor sites has been extended to assess the structure of agonists and antagonists bound to neuropeptide receptors and study the respective conformations, for example of dynorphin bound to the human kappa opioid receptor.⁵⁶ These characterizations of neuropeptide and neuropeptide–receptor conformations should enable future advances in developing drugs to mimic or block neuropeptide binding.

Other spectroscopic methods have also been useful in the characterization of neuropeptides. Infrared (IR) spectroscopy has successfully been used to provide a quantitative estimate of secondary-structural elements. When analyzed with IR spectroscopy, peptides demonstrate characteristic peaks for different folding patterns, including alpha-helices, beta sheets, and turns, which were characterized in neuropeptide hPYY.⁵⁷ Circular dichroism (CD) spectra have also shown utility in the rapid determination of secondary structure, and have shown evidence for the existence of alpha helices in tachykinin-related peptides and beta sheets in MSH peptides that increase with increasing charge state.⁵⁸ X-ray crystallography benefits from low-angstrom resolution of key structural sites. The binding structures of neuropeptides with their receptors have been well characterized with X-ray crystallography, including studies of the receptor for neuropeptide S⁵⁹ and human OX₂ receptor.⁶⁰ Information from

crystallography can provide useful details about neuropeptide structure that might lead to insights about function.

Methods for neuropeptide localization

Neuropeptide localization in species and/or tissue(s) enables mapping in neuronal subtypes relative to structural components of the cell/tissue/whole organism, which can then be used to inform the function of a target neuropeptide and to direct functional biology experiments.⁶¹ Overall, the rapid increase in ‘omics’-derived neuropeptide sequence data has revolutionized our approach to the localization of neuropeptides and their signaling pathway components⁶² and facilitated our ability to construct species- and neuropeptide-specific ‘connectomes’.⁶³ The application of localization techniques across species, tissue- and cell-types is fundamental to understanding the complexity of neuropeptidergic signaling and has trans-disciplinary importance;⁶⁴ indeed these techniques have been applied to cell cultures (2D, 3D, or single cells)⁶⁵⁻⁶⁷ and entire organisms (whole and sections)^{68, 69}. Neuropeptide localization in thick tissues, such as whole organisms or invertebrate brain tissue, can be achieved by using 3D mapping and ion reconstruction of individual tissue sections to produce 3D representations of neuropeptide distributions.⁷⁰ The advances in the tools and techniques described here have facilitated exploration of neurocircuitry landscapes such that knowledge about neuropeptide localization/expression is accumulating rapidly.

Visualization or detection of neuropeptides at the cellular, tissue, whole organism, or bio-fluid levels has been enabled by the application of radioimmunoassays (RIAs), immunohistochemistry (IHC), immunocytochemistry (ICC), and immunoelectron microscopy to the extent that these techniques have provided most of our former⁷¹ and current knowledge on

the localization of neuropeptides.⁷¹ Compared with traditional histology-based approaches, these techniques enable enhanced specificity and sensitivity through the use of antibodies – for example for the detection of the specific psychostimulant neuropeptide CART (cocaine- and amphetamine-regulated transcript peptide).⁷² Antibodies can theoretically also be raised against virtually any peptide, however, many invertebrate neuropeptide genes encode more than one biologically active peptide that show high structural similarity to each other, leading to antibody cross-reactivity.⁷³⁻⁷⁵ Generation of N-terminally directed antisera, which can readily distinguish between peptides with highly similar C-terminal motifs, can help overcome cross reactivity issues.⁷⁶ Another limiting factor is the number of peptides (and peptide signaling pathway components) that can be co-localized at the same time through traditional IHC/ICC approaches, which is in contrast to MS-based peptidomics tools (see below) that enable the complete neuropeptide profile of the animal, tissue, organ or even a single cell to be deduced at any given time readily enabling the identification of multiple co-localization events.

In situ hybridization (ISH) methods facilitate target-specific expression mapping of neuropeptide-encoding genes at the whole animal, tissue and single-cell level by RNA localization for gene expression. This involves hybridization of a single-stranded RNA oligoprobe and the complementary tissue/cell native mRNA sequence. The field of ISH and fluorescence ISH (FISH) has advanced significantly to enable the high-sensitivity detection of multi-target RNAs simultaneously in multiple species coupled with automated data collection/analysis systems.⁷⁷ There are several different approaches for detection of hybridized probes, including non-radioactive and radioisotope strategies. Regardless of the approach, careful consideration should be given to transcript abundance, where the detection of low-level/single-copy transcripts (*e.g.*, of GPCRs) can benefit from the use of target, signal or probe

amplification techniques.⁷⁸ Whilst one caveat of ISH is that the information it provides on RNA localization gives no definite indication of translated peptide distribution, it can relate valuable spatio-temporal information to gene activity when used in conjunction with ICC/IHC.⁷⁶

Promoter::reporter gene constructs offer an alternative detection method to ISH that is useful for transcript detection in living cells/organisms. The method requires the promoter region of the neuropeptide gene and coding region of the reporter gene to be fused and inserted into the organism of choice for use as a reporter of gene expression. It is important that the reporter gene [which often encodes green fluorescent protein (GFP)] is non-native, assayed easily (*e.g.*, visual detection), and does not affect the normal physiology of the organism under study. The use of promoter::reporter gene constructs as localization tools is popular in model organisms such as the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, where transgenesis is readily achievable.⁷⁹⁻⁸¹ A cautionary note should be given to the reliance on transcriptional reporters however, as they do not always provide complete and reliable gene expression data in comparison to translational reporters that include important intron and 3'UTR regulatory elements.⁸²

Techniques for localization of the complete neuropeptidome of an organism have seen progress with the use of MS since its recent development as a molecular imaging tool.⁸³⁻⁸⁵ Because no prior knowledge of the molecules is needed for analysis, theoretically hundreds or thousands of molecules can be imaged in one sample run. Among the various ionization sources available, MALDI has been the most prominent in imaging peptides and neuropeptides,^{86, 87} although success of detecting or identifying the neuropeptides is dictated by sample preparation and the detector coupled to the MALDI source. Time-of-flight based instruments have a niche in analyzing larger neuropeptides, but the low resolution and sensitivity have motivated the

development of alternative ionization techniques for larger neuropeptides, including matrix-assisted ionization in vacuum and laserspray ionization, which can be accomplished with commercial MALDI sources.⁸⁸⁻⁹⁰ For example, Chen et al. was able to ionize an 18.7 kDa protein on a commercial MALDI-LTQ-Orbitrap XL, which is usually limited to molecules smaller than 4 kDa. It should be noted that some of the matrices required are more compatible with long imaging runs than others. Moving forward, refinements have been made recently in the optimization of sample preparation methods for MS imaging. For example, when sectioning tissue, only certain embedding materials are MS-compatible.^{91, 92} Optimal cutting temperature embedding material is commonly used for the classical histology staining, but due to its polymer structure, it tends to suppresses and mask analyte signal, especially in the mass range of most neuropeptides. Another major problem is that, prior to any histology analysis, samples tend to be passed through the fixation process to help maintain tissue structure and deactivate any degradation processes, which limits neuropeptide MS analysis.^{93, 94} Tissue fixation requires many washes, which may remove neuropeptides, and possibly a crosslinking step, which will make neuropeptides unavailable for extraction by the matrix and thus ionization. To complicate the situation further, the choice and application of the matrix is extremely important for proper extraction of neuropeptides, and extensive effort has been devoted to developing better, more effective methods.^{95, 96} For example, by utilizing electrospray deposition of the matrix α -cyano-4-hydroxycinnamic acid, researchers have imaged the FMRFamide neuropeptide family at a 5-micron spatial resolution in a snail, allowing confirmation of the localization found from immunohistochemical analysis.⁹⁷ For more details, readers may consult recent reviews for MS imaging and its application to neuropeptides.^{27, 83, 98, 99}

In general, the reliance of mass matching for compound identification in MS imaging poses limitations in identification confidence. Owing to the low abundance of neuropeptides, tandem MS during imaging is often challenging. Therefore, accurate mass matching is the easiest way to identify a putative neuropeptide. The incorporation of high-quality tandem MS in a hybrid linear ion-trap–orbitrap instrument has provided improved *in situ* neuropeptide identification.¹⁰⁰⁻¹⁰² The development of a spiral step method (**Figure 4**), instead of the standard raster step, has allowed for further enhancement of chemical information by improving the depth of profiling and producing higher-quality images on Orbitrap-based instruments.¹⁰³ Another technique, IMS, has the potential to be used before detection and to remove interfering molecules and thus increase the image quality.¹⁰⁴ Overall, the unparalleled chemical information and multiplexing capacity offered by MS imaging technology offers an attractive tool for high-throughput mapping the localization of neuropeptides, although the inherent limitation of laser beam size and matrix crystal size of the MSI technique prevent it from having the same spatial resolution offered by IHC/ICC approaches. Further technology development is needed to improve these aspects and sensitivity to allow MS imaging to become a central tool for neuropeptide localization in the nervous system.

Recent advances in bioimaging and microscopy tools in parallel with upgrades in computer processing and digital storage capabilities have significantly enhanced the ability to capture and describe the neuroanatomy of invertebrates. Traditionally, light microscopy has been used in invertebrate neuroscience research to map the coarse architecture of the nervous system, with electron microscopy being employed for fine ultrastructural analysis.¹⁰⁵ More recently, confocal and multiphoton microscopy have facilitated the generation of high-resolution 2D and 3D images of both thicker whole-mount and live specimens.¹⁰⁶ Laser microdissection tools

provide an alternative to labor-intensive antibody-based experiments by enabling the post-capture profiling of neuropeptides (e.g., via RNAseq) in specific neurons, or tissues embedded in heterogeneous samples.¹⁰⁷ Advances in image-analysis software programs make the comparative quantitation of neuropeptides in the nervous system more streamlined, and facilitates the integration of optical imaging technologies into the functional genomics ‘toolbox’.^{108, 109} Additionally, integrating multimodal imaging studies through MALDI-MS imaging and microscopy-based imaging would provide enhanced spatial and chemical information for neuropeptide localization.

Web-based databanks for curating neuropeptide data in invertebrates are a much-needed resource that will greatly facilitate invertebrate neuropeptide research and enable inter and cross-phyla comparative analyses, in addition to providing a ‘go to’ repository for researchers. These types of resources are currently available for a number of invertebrate phyla and provide a range of data-types in user-friendly formats;¹¹⁰ the database NeuroPep collates pan-phylum data and enables comparisons of neuropeptide structure, expression, and function.²⁰ Furthermore, the availability of species-specific anatomical maps of the nervous system is essential for the precise and comparable morphological description of peptidergic neurons in invertebrates. These data are currently available for only a few invertebrate species,¹¹¹ including key model organisms [for example, *C. elegans*: Wormatlas;¹¹² *Drosophila melanogaster*: Neurokernal, Virtual Fly Brain;¹¹³⁻¹¹⁵ zebrafish: Z-brain;¹¹⁶ and *Macrostomum lignano*¹¹⁷] – however, they are all at different stages of completion and vary in terms of their resolution, presentation, and data source. These data are extremely valuable to our understanding of invertebrate nervous system structure and function and will inform functional biology. Efforts to generate similar maps for other species of interest are under way, but significant attention and support should be directed to the

curation and maintenance of these resources, as there are many online databases that are no longer active owing to the termination of funding.¹¹⁸

Assessing the function of neuropeptides

While the identification of neuropeptides is important, understanding their role in the nervous system is key to further applications. Understanding function is extremely difficult, as neuropeptides can have completely different functions within different tissues. Furthermore, even slightly different neuropeptide isoforms from the same family can have drastically different effects. Interestingly, even with the development of new, technologically advanced alternatives, older, well-vetted methods are still present in the literature either as a method of analysis or to confirm the observed results.^{119, 120} Function can be explored at many levels, ranging from the macro (*e.g.*, behavioral) to the molecular scale (*e.g.*, signaling pathways). Localization can also aid elucidation of neuropeptide function, as the tissue(s) a peptide is localized in may provide key clues about its role in the organism.¹²¹ A variety of functional biology tools and techniques can be employed to determine the function of a neuropeptide, including those applied in either *in vivo* or *in vitro* settings. Two major approaches for functional analysis will be discussed below: altering the neuropeptide content and measuring neuropeptide levels.

Altering Neuropeptide Content

The most commonly performed and observed *in vivo* studies involve assessing behavioral/physical changes arising from the introduction of a neuropeptide into an organism or by using reverse genetics (RNA interference) to down-regulate a specific neuropeptide (*e.g.*, using siRNAs).^{122, 123} These approaches can provide a range of information from a general understanding of the physiological function of a neuropeptide to judging whether neuropeptides

are therapeutically active.^{124, 125} Neuropeptides or their antagonists (siRNAs) can be delivered to an organism in several different ways, including by injection,^{126, 127} media incubation,^{128, 129} and even microdialysis.¹³⁰ siRNA, RNA molecules that interfere with an expression of a gene, may require more sophisticated methods of delivery (*e.g.*, transfection) or can also be injected for the induction of gene-silencing and thus knockdown of the neuropeptide.^{131, 132} Beyond introducing a neuropeptide or a neuropeptide antagonist, neuropeptide production can be altered at the genetic level by using knockout or transgenic mice, or targeted genome editing approaches (*e.g.*, CRISPR/Cas systems).¹³³⁻¹³⁵ CRISPR/Cas has gained a lot of popularity for its speed, ease of use and efficiency compared to other methods used to knockout genes or create transgenic animals.¹³³ It should be noted that this technology is new and can be expensive to implement on a large scale. Furthermore, its application is not possible in non-model organisms. In all the cases described above, careful planning is required to determine the most appropriate and applicable technique to alter neuropeptide levels in an organism.

After alteration of the neuropeptide content, several behavioral observations or tests can be performed to assess change. Examples of behavior tests for animals (*e.g.*, Wistar rats) are open-field-based or maze-based,^{136, 137} and these tests are applicable to numerous species, including invertebrates such as planarians and *C. elegans*.^{138, 139} While these tests are easily performed and are normally the starting-point for functional studies,¹⁴⁰ behavioral studies are based on observations, meaning that data misinterpretation or choice of test to monitor changes can produce misleading data. Thus, care should be taken on choosing the most appropriate tests, methods, or animal models to assess behavioral changes attributable to application of neuropeptides.

As neurons transmit signals through electrical currents, another facet of function to consider is electrophysiology. By selective or global activation, researchers are able to understand synaptic mechanisms by which neurons communicate and modulate their electrical activities (**Figure 5**).¹⁴¹ These readings can be performed in a few ways depending on the goal of the study, including intracellular versus extracellular electrophysiological recording,¹⁴² whole-cell versus whole-network,¹⁴³⁻¹⁴⁶ or *in vitro* versus *in vivo*.^{144, 147-149} In terms of neurological studies, *in vitro* whole-cell recordings are the most common, although *in vivo* live-animal recordings, which are inherently more difficult, are becoming more refined.¹⁵⁰ Crustacean model systems have been used heavily for electrophysiological studies.¹⁵¹⁻¹⁵³ For example, the effects of neuromodulators on the same neuronal circuit was explored for the Jonah crab gastric mill motor pattern, which was interestingly explained using a mathematical model.¹⁵⁴ In general, to better understand neuronal modulation at the single-neuron and network level, crustaceans provide an excellent model to derive detailed knowledge about synaptic mechanism and neuronal connection owing to possession of a much simplified system compared with the mammalian system.¹⁵⁵ Interestingly, the coupling of electrophysiological probes for simultaneous monitoring of other chemicals has been incorporated recently. This could include selective applications (*e.g.*, oxygen, glucose, etc.)¹⁴⁷ or could be more global, such as microdialysis, which would allow for direct dosing of neuropeptides.¹⁵⁶ Notably, some researchers believe that electrophysiology, while never replaceable, might be overshadowed or combined with other optical imaging techniques that allow localization of the neurological signals.¹⁵⁰ One can postulate that combining the temporal resolution of classical electrophysiology and spatial resolution of optical imaging could lead to significant discoveries in neuroscience.

Measuring Neuropeptide Levels

Physiological changes related to neuropeptide actions are most commonly studied *in vivo* by performing quantitative analyses on the whole organism. It should be noted that this can be the first step in many cases to understanding the function of a peptide, as it might not be known that a neuropeptide is involved in a process until a neuropeptide is administered or a condition is applied (*e.g.*, a change in environment).¹²⁴ After the organism has been exposed to the neuropeptide or changed condition, it can be euthanized and the tissues of interest collected for quantitative comparison.¹⁵⁷ Alternatively, the tissue can be removed from the animal and incubated before analysis.¹⁵⁸ Classically, this has been performed for individual proteins using western blotting, which is still widely reported in the current literature.^{119, 123} Owing to the typically small size of neuropeptides, western blotting is normally used to assess other, related protein changes or expression of neuropeptide receptors.^{123, 159} Other complementary examples of a targeted technique are enzyme-linked immunosorbent assay (ELISA)^{120, 127} and use of radioactively labeled ligand and a gamma counter.¹⁶⁰ While these methods are excellent if one has a target of interest, a non-biased global view of all the changes is often needed to understand fully the role of neuropeptides and their possible function at the system level.

With the advancement of technology, MS is becoming a useful addition to functional studies as it is able to reveal changes in neuropeptide levels that might correlate with function. This technique is especially attractive for analysis of organisms without a sequenced genome, as no prior knowledge of the molecule, such as metabolite, protein, or peptide, is needed. To highlight some key areas of success, crustacean neuropeptide research has benefited from MS, allowing researchers to quantify several neuropeptide changes arising from stress caused by changes in salinity or temperature.^{8, 161} Microdialysis of neuropeptides has also been coupled to MS, and several reviews highlight considerations for coupling these two techniques.^{85, 99} Multiplexed

quantitation beyond duplex has been implemented in proteomics.¹⁶²⁻¹⁶⁴ In this technique, by using different combinations of stable isotopes (*i.e.*, ^{13}C , ^{15}N , ^2H , ^{18}O), samples are differentially labeled prior to being mixed and analyzed together in the MS. It is expected that multiplexing will be applied more commonly in neuropeptide analysis of multiple samples.^{165, 166} Although the use of MS is attractive, the depth at which it can profile depends upon many instrument characteristics, such as analysis time, resolution, and mass range. Using an analyte target list can increase neuropeptidomic coverage, although sensitivity and interfering species can introduce difficulties. Owing to the natural complexity of biological samples, the coupling of separations to MS has not only improved detection but also accurate quantitation. This coupling includes capillary electrophoresis (CE), LC, or IMS^{27, 104, 167} before MS detection. By reductive dimethylation comparative samples before CE separation, it has been shown that neuropeptides can be separated and quantified accurately, allowing for more-in-depth profiling.^{168, 169} Furthermore, new instrument methods, such as analysis at the MS3 level, have helped facilitate accurate quantitation.¹⁷⁰ It should be noted that, from these data, individual peptides can be selected for further analysis and validation by the above, targeted, methods. Finally, MS data are inherently more complicated, and the use and development of appropriate software to predict, identify or quantify is challenging, but necessary, for neuropeptidomics to continue progressing.^{3,}

171, 172

Upon understanding the peptide changes, gene analysis can be conducted to help provide information on the global impact on the plasticity of the system. This is most easily achieved by measuring the mRNA changes of a specific neuropeptide, its propeptide, or its pre-propeptides using a quantitative polymerase chain reaction (qPCR).^{158, 173} This approach differs from western blots and ELISAs, which measure the translated peptide, but does not require antibodies. Using

qPCR, it has been shown that dosing of amphetamine not only affects rat food intake but also affects hypothalamic mRNA levels of neuropeptide Y.¹⁴⁰ While more mRNA usually means enhanced gene expression, protein levels do not always correlate with the mRNA data, and the use of an orthogonal method (see above) should be performed to verify any conclusions. This is true for all of the methods above as all of them have complementary advantages and disadvantages.

Peptide-gated channels emerge as a tool for neuroscience

The function of a neuropeptide ultimately is determined by its receptor. While GPCRs mediate slow and more modulatory neurotransmission by changing the membrane potential, ion channel receptors by contrast mediate fast and transient neurotransmission, rapidly depolarizing or hyperpolarizing the postsynaptic membrane. It was common knowledge for decades that neuropeptides bind to and activate mainly GPCRs, but not ion channel receptors. There are now a few exceptions to this rule: ion channels directly activated by neuropeptides have been cloned and functionally characterized from different snails (molluscs) and the freshwater polyp *Hydra* (Cnidaria), unambiguously demonstrating the existence of ion channel receptors for neuropeptides in different animal phyla (**Figure 6**). Moreover, genomic data have revealed the presence of related channels in other phyla, mainly in basal lineages of the animal kingdom evolutionary tree, and electrophysiological data suggest the existence of a peptide-gated Cl⁻ channel in the nematode *Ascaris suum*.¹⁷⁴⁻¹⁷⁶ Thus, we speculate that the distribution of peptide-gated channels is at present vastly underestimated and that they might mediate some of the physiological functions of neuropeptides in several animals; maybe even in humans, although this is at present unlikely. Here, we will briefly describe the discovery of the known peptide-

gated channels, introduce their properties and then focus on how they might be developed into tools for neuroscience.

The first observations of a peptide-gated ion channel were made by Cottrell and co-workers, who showed that the cerebral C2 neuron of the snail *Helix aspersa* is rapidly excited by the neuropeptide FMRFamide.^{177, 178} Peptides related to FMRFamide, RFamide neuropeptides, are found in many animals. The excitation was fast and also observed in outside-out patches containing 5'-O-(2-thiophosphate), which blocks G-protein-coupled responses, in the patch pipette.¹⁷⁸ These results strongly suggested that FMRFamide directly activated ion channels in these neurons. The currents were Na⁺-selective and sensitive to the diuretic amiloride.¹⁷⁸ These biophysical and pharmacological properties are reminiscent of the epithelial Na⁺ channel (ENaC) from vertebrates, and, in 1995, by homology to ENaC, the FMRFamide-gated Na⁺ channel (FaNaC) was cloned from *H. aspersa*¹⁷⁹ – the first peptide-gated channel. A single FaNaC subunit is sufficient to produce functional channels with properties similar to the native channel in C2 neurons: they are Na⁺ selective and sensitive to amiloride (EC₅₀ = 0.6 μM; **Table 1**)¹⁷⁹. Although it was reported that FaNaC is a tetramer,¹⁸⁰ there is now compelling evidence from crystallization of closely related acid-sensing ion channels (ASICs),¹⁸¹ as well as from single-molecule imaging,^{182, 183} that channels of the DEG/ENaC gene family have a trimeric stoichiometry (**Figure 6A**). In addition, species orthologs of FaNaC have been cloned from three other molluscs, including *Aplysia*,¹⁸⁴⁻¹⁸⁶ but so far no additional subunits have been cloned. Thus, although it cannot be ruled out formally that the native channel contains other subunits, it is likely that FaNaC functions as a homotrimer. Table 1 provides an overview of the properties of known peptide-gated channels.

In 2007, by homology to ENaC and FaNaC, four related subunits were cloned from the freshwater polyp *Hydra*,¹⁸⁷ which belongs to the ancient phylum Cnidaria. It was found that two of them, when co-expressed in a heterologous expression system, formed an ion channel that was directly activated by two neuropeptides,¹⁸⁷ which had been previously isolated from the *Hydra* nervous system using a RIA.¹⁸⁸ Like FaNaC, the channel also conducts Na^+ and therefore was named Hydra Na^+ channel (HyNaC). These two neuropeptides, Hydra-RFamides I and II, share a C-terminal RFamide group with FMRFamide, the ligand of FaNaC. HyNaC is not the species ortholog of FaNaC, however, as it is more closely related to mammalian ASICs than to FaNaC or ENaC,¹⁸⁷ suggesting that peptide-gated channels are ancient and evolved before the cnidarian–bilaterian split. Three years later, another HyNaC subunit was cloned that assembles with the two previously cloned subunits, suggesting that the native channel is a heterotrimer containing three different subunits.¹⁸⁹ In contrast to FaNaC, HyNaC is an unselective cation channel with a high Ca^{2+} permeability.¹⁹⁰ Finally, all 12 DEG/ENaCs of *Hydra* were cloned, and it was shown that *Hydra* likely contains at least six different functional HyNaCs.¹⁹¹ All are heterotrimers consisting of three different subunits activated by Hydra-RFamides I and II, and all are unselective cation channels¹⁹¹ (**Table 1**). It is not clear why *Hydra* evolved such a variety of peptide-gated channels with similar properties, but differential targeting and differential ligand-affinity are two possibilities.

In situ hybridization revealed that two of the six HyNaCs are most likely expressed in epitheliomuscular cells at the oral side of the tentacle base, two at the aboral side and two in the foot region.¹⁹¹ Application of amiloride or diminazene, two inhibitors of HyNaCs (**Table 1**), delayed the feeding reaction of living *Hydra*,^{189, 191} which is characterized by a bending of the tentacles. Collectively, these results suggest that Hydra-RFamide peptides are released at

neuromuscular junctions and that HyNaCs contribute to fast neuromuscular transmission in *Hydra*.¹⁹²

Usually, ligand-gated ion channels desensitize in the continued presence of the ligand. This feature, together with rapid re-uptake or hydrolysis of small-molecule transmitters, makes transmission with ligand-gated channels transient. HyNaCs, by contrast, could also mediate longer-lasting depolarization of the postsynaptic membrane – they do not desensitize¹⁹⁰ (**Figure 6B**), and there is no known rapid re-uptake mechanism for their ligand. In combination with their high Ca^{2+} permeability, these features could endow HyNaC-expressing cells with an efficient entry path for extracellular Ca^{2+} , which could be important for muscle contraction.¹⁹²

DEG/ENaCs with high sequence similarity to either FaNaC or HyNaCs are present in several genomes, for example in that of *Nematostella vectensis*, a cnidarian that belongs to the subphylum anthozoa that is not closely related to Hydrozoans, and in that of the placozoan *Trichoplax adhaerens*.¹⁹² As *T. adhaerens* does not contain a nervous system, the presence of putative peptide-gated channels in this organism suggests that the channel-peptide ligand system predated the emergence of nervous systems and might have a role for example in paracrine signalling. Molecular cloning and functional analysis of these channels will improve our understanding of the physiological function of peptide-gated channels.

In addition to their importance in understanding neurotransmission in different organisms, peptide-gated ion channels are also interesting tools in neuroscience. For example, FaNaC has been used as a reporter of neuropeptide-release and achieves high temporal resolution.¹⁹³ FMRFa has been used to tag a neuropeptide prohormone, and FaNaC has acted as a reporter to monitor release of FMRFa and thereby also of the tagged neuropeptide.¹⁹³

In another example, it has been shown that heterologous expression of FaNaC in mammalian hippocampal neurons provides a means to depolarize the neurons and induce bursts of action potentials by focal application of FMRFa.¹⁹⁴ FaNaC had a somato-dendritic localization and was absent from axons.¹⁹⁴ As FMRFa is not present in the mammalian nervous system, and endogenous RFamides apparently do not activate FaNaC,¹⁹⁴ it is in principle possible to activate specific subsets of neurons selectively in intact nervous tissue. Transgenic expression of FaNaC under the control of specific promoters would enable driving of its expression only in specific subsets of neurons in living animals. Moreover, the possibility to ‘cage’ FMRFa chemically with a photolabile protecting group allows its release within milliseconds upon exposure to both single- and two-photon light sources¹⁹⁵ to rapidly excite cells expressing FaNaC. As HyNaCs are obligate heteromers, their heterologous expression in neurons is more difficult, but would allow expression of a foreign ion channel with high Ca^{2+} permeability. The cloning of further peptide-gated channels, such as the Cl^- channel from *A. suum*, will further increase the toolbox of peptide-gated channels.

A better understanding of the molecular binding site of peptide ligands on their ion channel receptors could also allow the future design of small molecules that gate the channels independently of peptides. This might allow peptide-gated channels to be employed, much like some GPCRs, as ‘designer’ receptors exclusively activated by designer drugs (DREADDs).¹⁹⁶

The identification of the peptide-binding site might also allow the covalent attachment of FMRFa (or other peptides) close to its binding site via a photoisomerizable molecule (a ‘photoswitch’) such that light would move the peptide in and out of its binding site to open and close the channel^{197, 198} (**Figure 6C**). Azobenzenes have been successfully used as such photoswitches, as they undergo fast trans-to-cis isomerization, much like retinal, upon

illumination with near-UV light.¹⁹⁸ They can be coupled via maleimides to single cysteine residues engineered into the primary sequence of a channel. High-resolution structures are not only useful for the identification of the peptide-binding site but also a pre-requisite for the identification of suitable attachment sites of peptide ligands close to the binding site. As ASIC1, a close homolog of HyNaCs, has been crystallized,¹⁸¹ appropriate homology models of the HyNaC structure, and perhaps also of the FaNaC structure, are feasible. Such photo-sensitive channels would allow to control the membrane potential of a neuron by light instead of a peptide ligand. Examples that such a synthetic optogenetics approach¹⁹⁸ is feasible have been provided, among others, for ionotropic glutamate and GABA_A receptors.^{199, 200} Clearly, peptide-gated ion channels have great potential to serve as useful tools for neuroscience.

Concluding remarks

It is clear that neuropeptide research has benefited tremendously from the substantial advancements of technology for neuropeptide structural elucidation, localization mapping, and functional understanding, although any single technique itself still does not provide us with all the answers we seek. A particularly promising technique is MS imaging with tandem MS but sensitivity issues might be limiting when single-cell resolution is needed. In general, the development of MS-based neuropeptidomics technique has proven to be the most influential technique for analyzing neuropeptides in a high-throughput and global manner, but other, often classical, methods provide validation and confirmation of all results generated by MS. Owing to its non-bias and global analysis, MS will likely be a central tool for all future neuropeptide studies, especially with the continued development of new methodology and technology.

Furthermore, another area that has proven to be extremely influential has been computational prediction and processing. Without sophisticated bioinformatics tools, not only would the identification of novel neuropeptides be slow, but MS data-sets, which are naturally large and complex, would be extremely difficult to process and understand. At the moment, the pace of investigating the neuropeptidome will continue to be set by the development of both of these areas, although new techniques that are complementary or capable of providing structure, function, and localization information are welcome additions to the study of neuropeptides. Also, peptide-gated ion channels are promising new tools for neuroscience. Finally, the integration of multiple bioanalytical techniques and molecular neuropharmacological tools will drive the field of neuropeptide research towards new frontiers.

Competing Interests

The authors have declared no competing interests.

Author Contributions

K.D. and A.R.B. contributed equally to writing this manuscript. L.A. contributed to writing a section of the manuscript. A.M., S.G. and L.L. conceived the outline and main topics of the manuscript, contributed sections and reviewed and edited the manuscript.

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Figure Legends

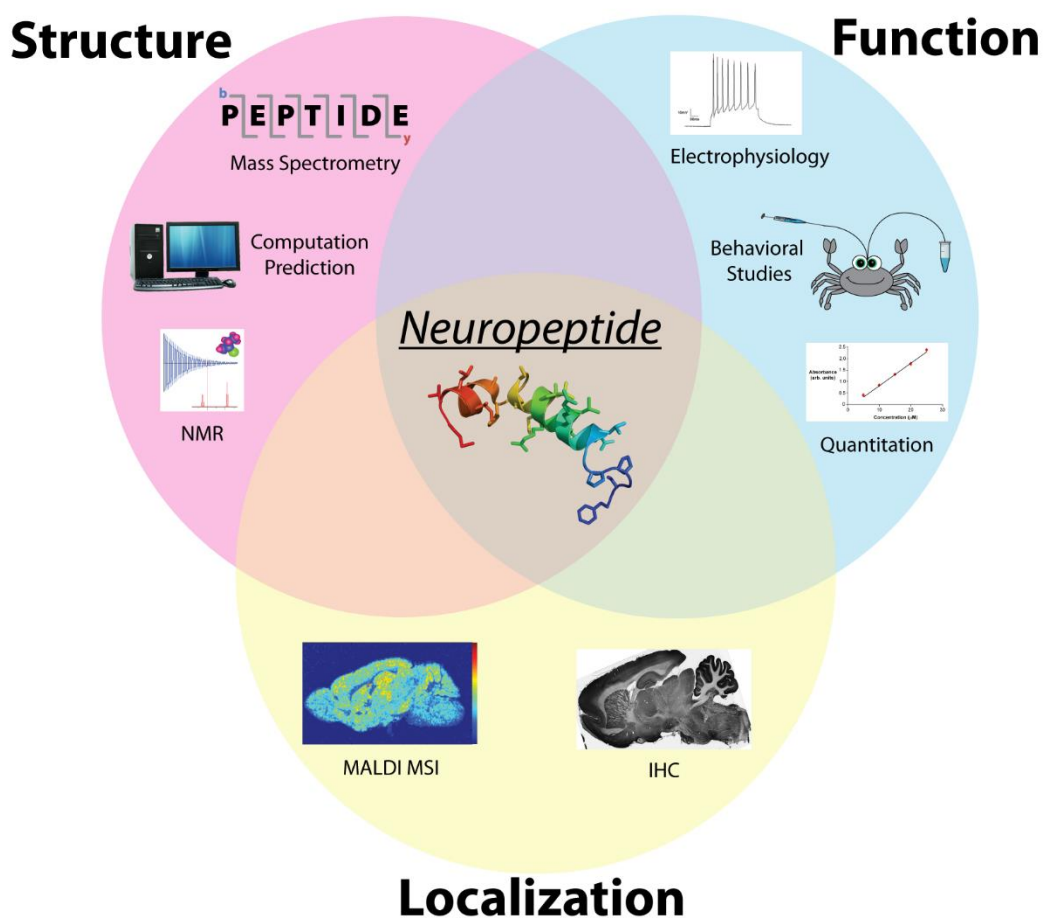


Figure 1. A general depiction of the importance of structure, function, and localization to provide key information about a neuropeptide. Several methods for each area of the Venn diagram are highlighted. For structure, mass spectrometry, computational prediction, and nuclear magnetic resonance (NMR) are shown. Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) and immunohistochemistry (IHC) are the examples depicted for localization. For understanding functionality, quantitation, behavioral studies, and electrophysiology are core techniques.

Table 1. A summary of notable techniques commonly used to provide information into the three major areas of neuropeptide research: structure, localization, and function. Each technique described has the potential to provide deep insight into neuropeptide biology, and often provide complementary information to other techniques. Several key references are indicated for each that demonstrate current trends in the field.

Area of Interest	Technique	Description	Key References
Structure	Mass spectrometry	Determines sequences, PTMs, and structural information	30, 28, 43
	<i>In silico</i> prediction	Predicts sequences and structure from precursor protein and gene sequences	46, 47, 49
	NMR	Gives information into conformations and folding patterns	52, 54, 55
	Spectroscopy	Uses characteristic peaks to identify folding patterns	57, 58
	X-ray crystallography	Characterizes key structural sites with high spatial resolution	59, 60
Localization	Immuno assays	Enables localization for virtually any peptide using antibodies	72, 73, 75
	<i>In situ</i> hybridization	Target-specific expression mapping of neuropeptide-encoding genes	76, 77, 78
	Promotor::reporter gene constructs	Enables transcript detection in living cells and organisms	79, 81, 82
	MSI	Capable of imaging entire neuropeptidomes without prior knowledge	89, 97, 103
	Bio imaging and microscopy	Maps the architecture of the nervous system	105, 106, 107

Function	Behavioral studies	Common first step to obtaining a general understanding of function to judging potential for disease treatment	124, 128, 131
	Electrophysiology	Provides understanding of synaptic mechanisms	145, 147, 151
	Quantitative analyses (Western blotting, ELISA, MS, etc)	Implies functions by measuring changes in neuropeptide levels due to specific behaviors or conditions	119, 120, 201

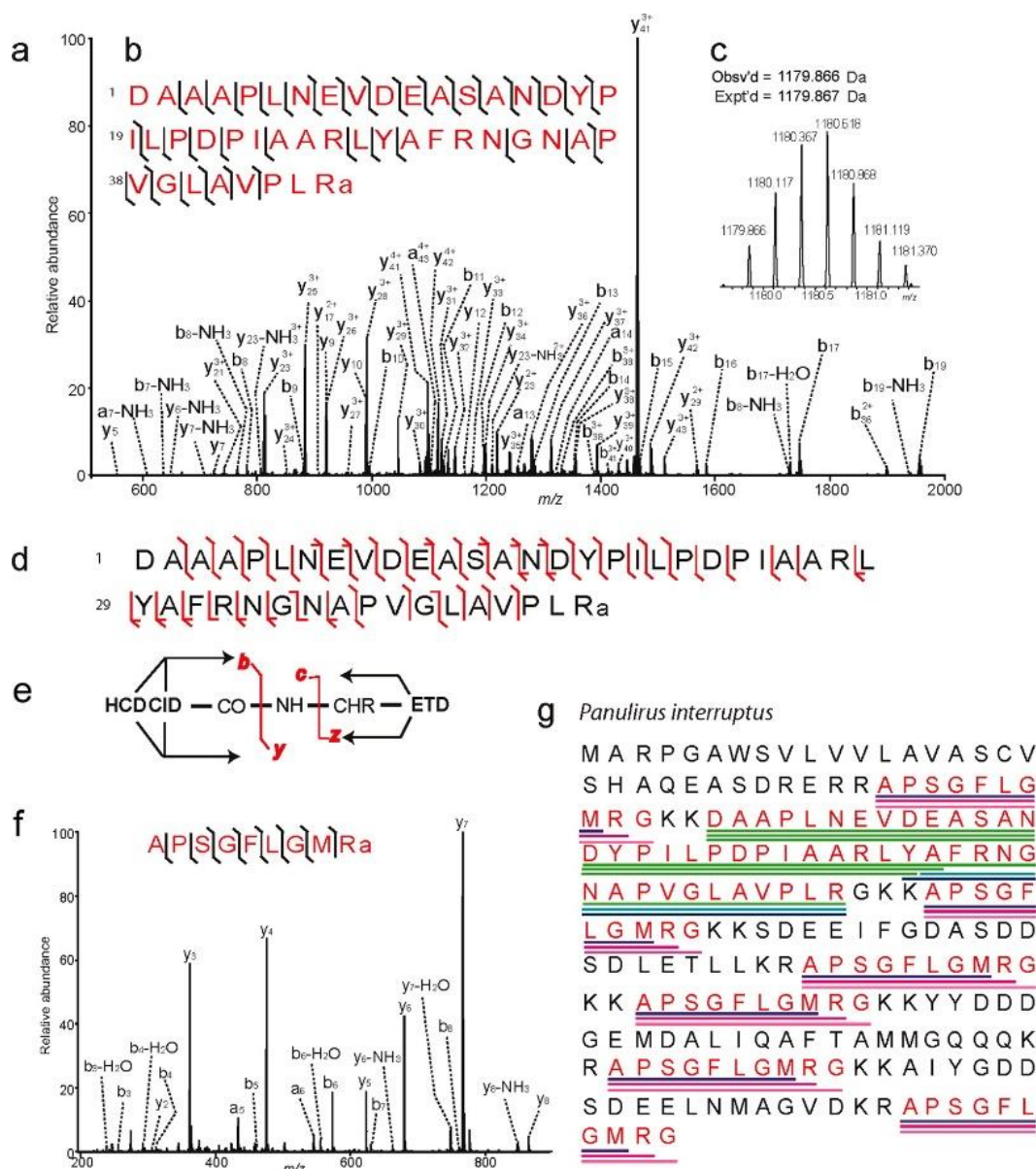


Figure 2. Depiction of *de novo* sequencing of peptides using mass spectroscopy (MS). (a) High-resolution tandem mass spectrum with sequence-specific fragment ions annotated. (b) Sequence assignment based on fragmentations, in which mass differences between adjacent fragment ion peaks are matched to amino acids. (c) Enlarged, high-resolution spectrum showing mass accuracy and isotopic distribution (necessary for measurements of differences between molecules with the same nominal mass). (d) Display of the **sequence coverage**. (e) Representation of the different types of ions produced in tandem MS depending on

fragmentation method used and bond cleavage sites. The cleavage sites are indicative of typical fragmentation patterns characteristic of the two common types of fragmentation methods. The b and y ions are produced during HCD and CID fragmentation, and c and z ions are produced during ETD fragmentation. (f) Tandem mass spectrum of another peptide (Jonah crab *Cancer borealis* tachykinin-related peptide) with fragments indicated. (g) Comparison between peptides detected with MS (different colored lines indicate different detected peptides) and those predicted based on the precursor cDNA sequence of the spiny lobster *Panulirus interruptus* (highlighted in red). Adapted with permission.³⁹ CID, collision-induced dissociation; ETD, electron transfer dissociation; HCD, higher-energy collisional dissociation.

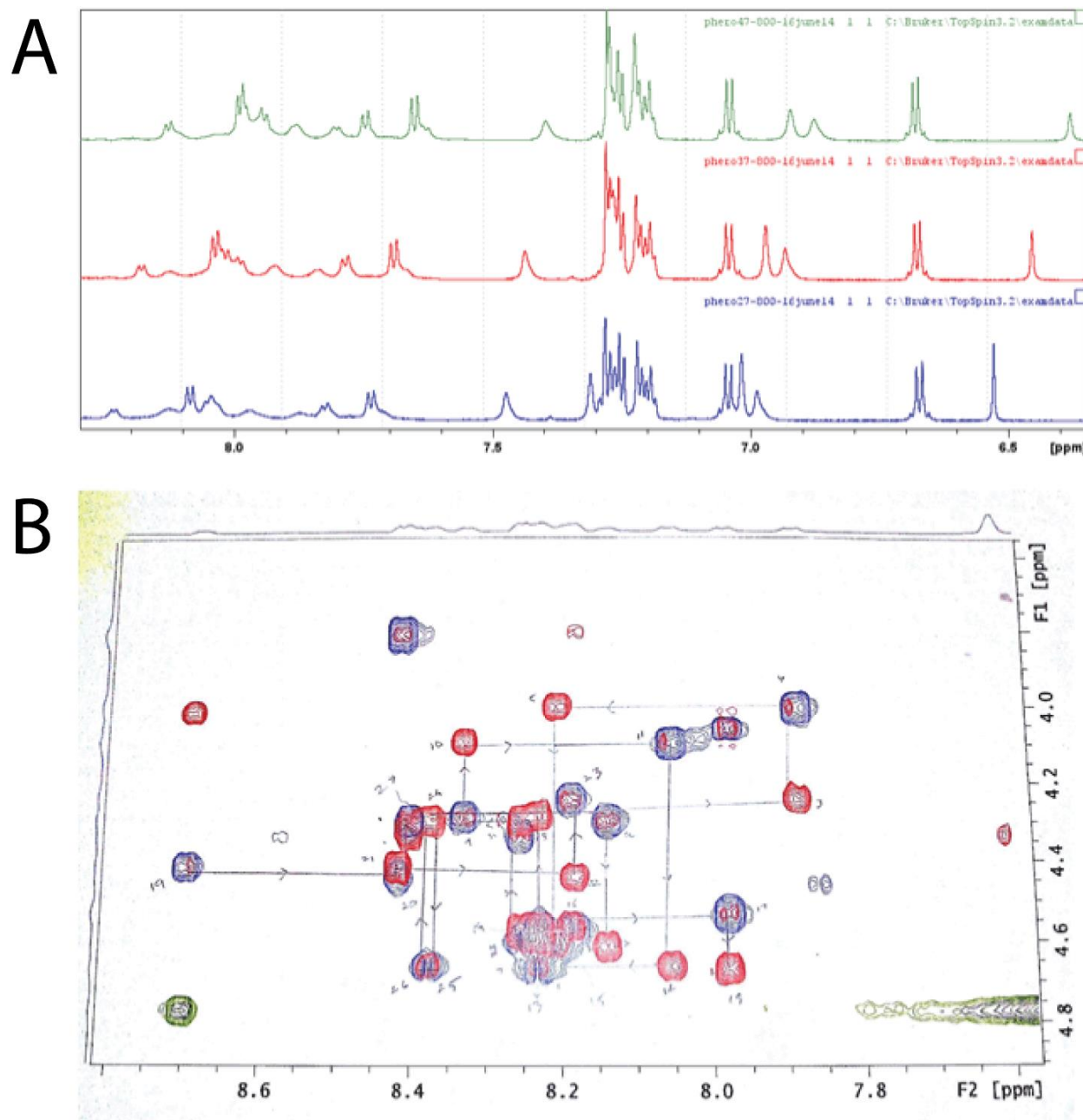


Figure 3. Nuclear magnetic resonance (NMR) spectra of a peptide standard of neuropeptide pheromotropin, originally discovered in an extract from the head of a *P. separata* larvae of *Bombyx*. (a) One-dimensional ^1H NMR spectra collected at different temperatures, showing differences in chemical shift of NH protons in the peptide. The dependence of chemical shift on temperature is indicative of the degree of hydrogen bonding. Values below 3.00 ppb (chemical

shift) per unit Kelvin indicate the presence of strong hydrogen bonds. As can be seen, the values for this peptide fall above that threshold, revealing that the protons are freely exposed to the solvent in this conformation. (b) Two-dimensional NMR spectra (TOCSY in blue, and ROESY in red), showing a sequential assignment walk. The TOCSY spectrum provided information on NH- α H cross peaks, while the ROESY spectrum's cross peaks represented NH_i - $\alpha\text{H}_{(i-1)}$. Adapted with permission.⁵¹

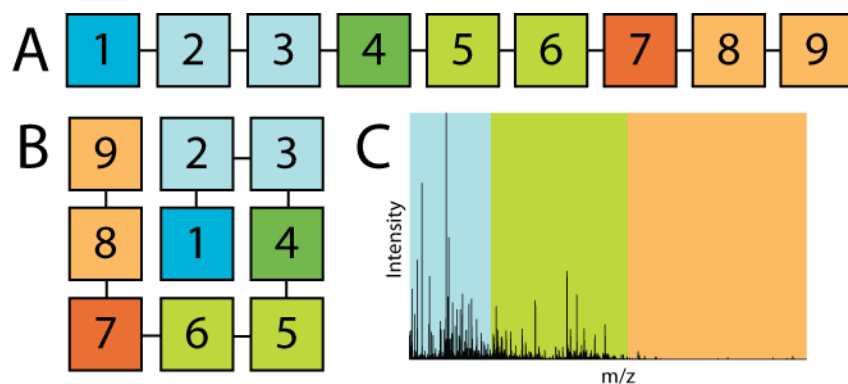


Figure 4. In order to achieve better profiling depth during MS imaging of neuropeptides, a spiral step method was developed. Instead of the classical raster step (a), a spiral square (b) is set up. In the example spiral, the square is broken into 9 individual steps. The first square is an MS scan (dark), while the two following scans are tandem MS scans. This repeats three times till all 9 steps in the spiral are completed. Each square is a raster step of 50 μm , with the whole spiral being 150 μm . This system can be customized to balance MS and tandem MS scans. For example, step one could be an MS scan, while squares 2-9 could be tandem MS scans if the user desires. Furthermore, this method can be targeted or used with data dependent acquisition (DDA). For DDA experiments, the highest intensity peaks are chosen for tandem MS analysis. Since neuropeptides tend to be in low abundance compared to lipids and have a wide mass range, we can segregate the spiral step method into multiple mass ranges (*e.g.*, three) to improve sampling of neuropeptides (c).¹⁰³

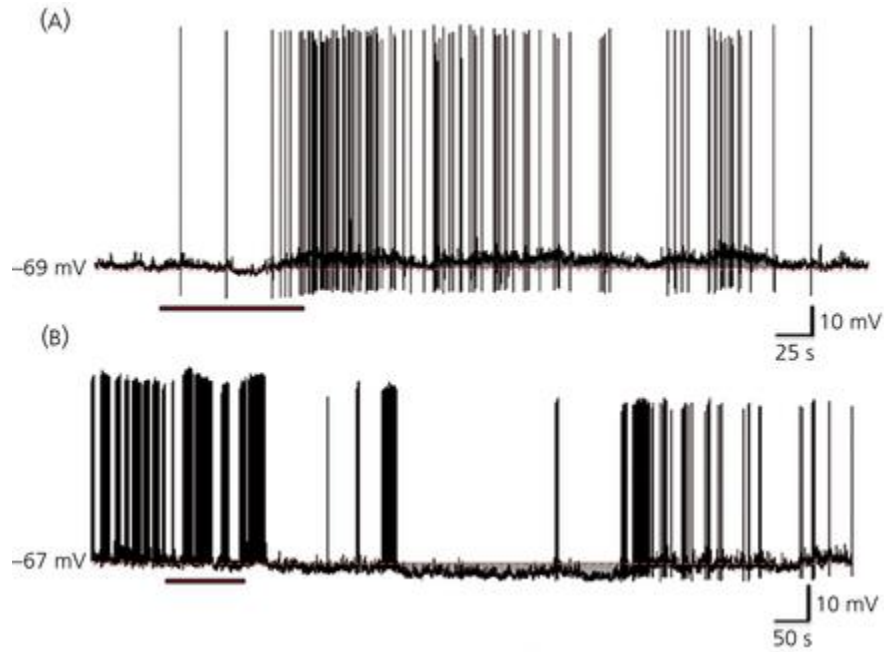


Figure 5. A graphical representation whole-cell patch clamp electrophysiology readings. In this image, subfornical organ neurons from rat brains are being exposed to 10 nM nesfatin-1, an anorexigenic neuropeptide, which is indicated by the line under the graph. When exposed, neurons can either become polarized (more signal) (a) or depolarized (less signal) (b). Adapted with permission.¹⁴¹

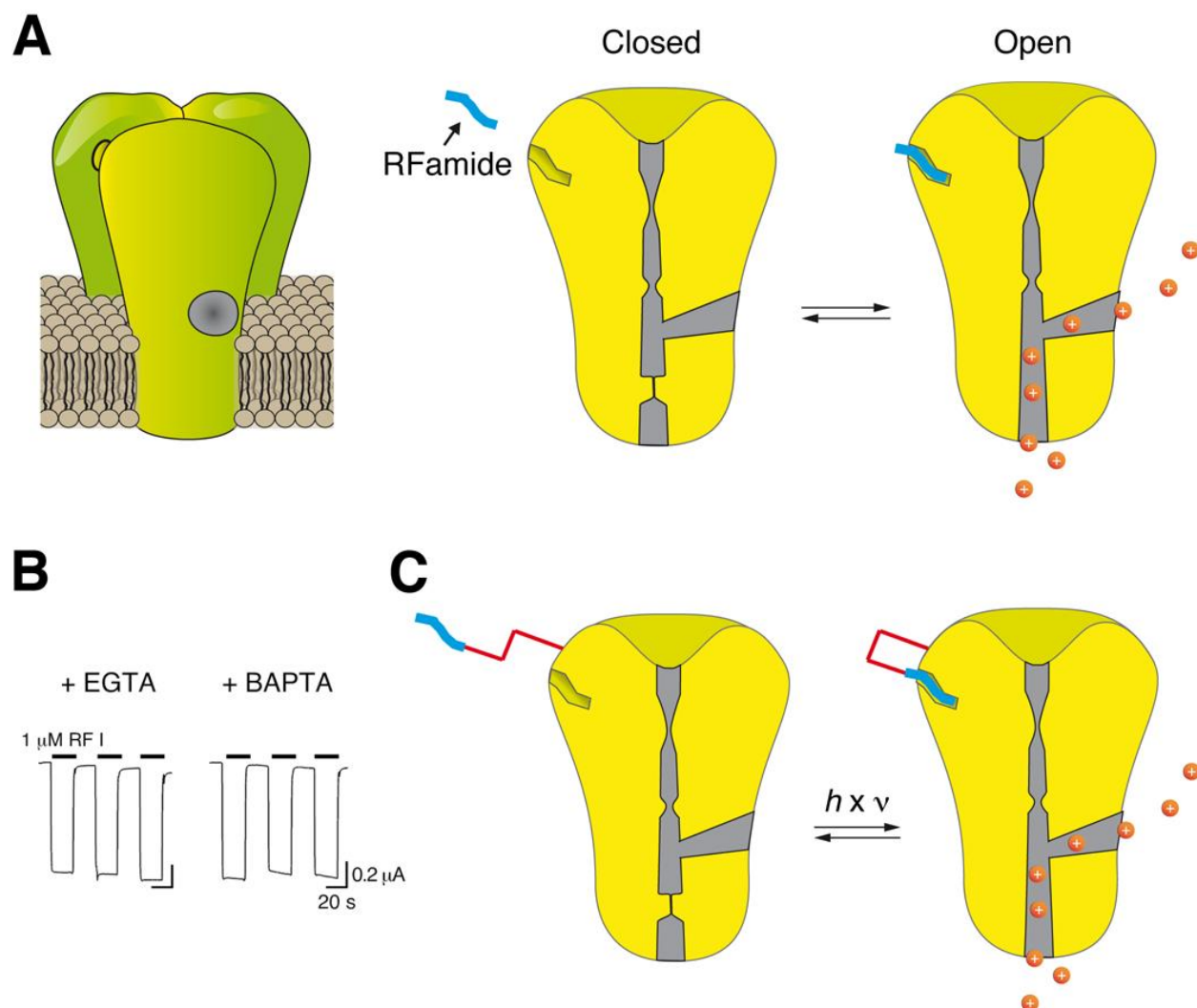


Figure 6. Properties of peptide-gated HyNaCs. (A) Left, cartoon illustrating the three-dimensional structure of a channel. The ligand binding site is unknown and is drawn here at the interface of two subunits for illustration. Right, HyNaCs can be either open or closed. The equilibrium between these two conformations is shifted by binding of a RFamide peptide (blue) to the extracellular domain. (B) HyNaCs can be repeatedly activated by their ligand, Hydra-RFamide I, and do not desensitize. The inward current is carried by Na^+ and Ca^{2+} . Used with

permission.¹⁹⁰ (C) Cartoon illustrating how a peptide covalently linked to the channel could be moved into and out of its binding site by application of light via a photoisomerizable linker (a “light-switch”, red).

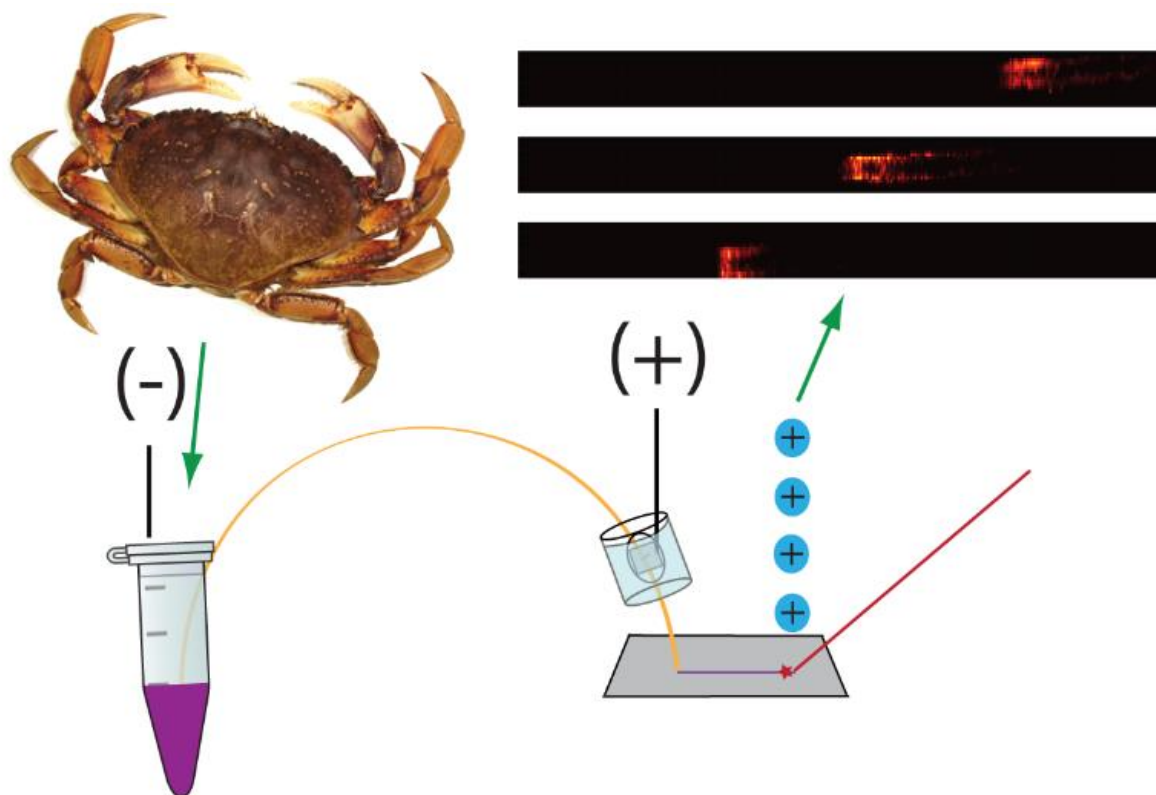
Table 2. Properties of peptide-gated ion channels ¹⁷⁵

Channel	Gene family	Stoichiometry	Ligand	Ligand affinity	Kinetics	Ion selectivity	Pharmacology
FaNaC	DEG/ENaC	Homo-trimer	FMRF-NH ₂	2–70 μ M ^{179,184}	Partially desensitizing ($\tau \sim 1$ min) ²⁰²	Na ⁺ -selective $P_{Na}/P_K > 10^{179}$	EC ₅₀ (amil) = 0.6 μ M ¹⁷⁹
HyNaCs	DEG/ENaC	Hetero-trimer ^{189,191}	pQWLGRF-NH ₂ pQWFNGRF-NH ₂	>30 μ M ^{189,191}	Non-Desensitizing ^{191,190}	Cation-unselective $P_{Na}/P_K = 3^{190}$ $P_{Ca}/P_{Na} = 4^{190}$	EC ₅₀ (amil) = 100 μ M ¹⁸⁹ EC ₅₀ (dimi) = 0.05 - 5 μ M ¹⁹¹
<i>A. suum</i> channel	Unknown	Unknown	KPNFLRF-NH ₂ (or similar)	0.001–0.1 μ M ^{174,175}	Non-Desensitizing ^{174,175}	Cl ⁻ -selective ^{174,175}	

Amil, amiloride; dimi, diminazene.

Chapter 3

Capillary Electrophoresis Coupled to MALDI Mass Spectrometry Imaging with Large Volume Sample Stacking Injection for Improved Coverage of *C. borealis* Neuropeptidome



Modified from:

DeLaney, K.; Li, L. Capillary Electrophoresis Coupled to MALDI Mass Spectrometry Imaging with Large Volume Sample Stacking Injection for Improved Coverage of *C. borealis* Neuropeptidome. *Analyst*. 2020, 145(1), 61-69. PMID 31723949.

Abstract:

Neuropeptides are important signaling molecules responsible for a wide range of functions within the nervous and neuroendocrine system. However, they are difficult to study due to numerous challenges, most notably their large degree of variability and low abundance *in vivo*. As a result, effective separation methods with sensitive detection capabilities are necessary for profiling neuropeptides in tissue samples, particularly those of simplified model organisms such as crustaceans. In order to address these challenges, this study utilized a capillary electrophoresis (CE)-matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry imaging (MSI) platform, building upon our previous design for improved neuropeptidomic coverage. The capillary was coated with polyethylenimine (PEI) to reduce peptide adsorption and reverse the electroosmotic flow, and large volume sample stacking (LVSS) was used to load and pre-concentrate 1 μ L of sample. The method demonstrated good reproducibility, with lower than 5% relative standard deviation for standards, and a limit of detection of approximately 100 pM for an allatostatin III peptide standard. The method was tested on brain and sinus gland (SG) tissue extracts and enabled detection of over 200 neuropeptides per run. When comparing the number detected in brain extracts in a direct spot, 60-second fractions, and 30-second fractions, the continuous trace collection afforded by the CE-MALDI-MSI platform yielded the largest number of detected neuropeptides. The method was compared to conventional LC-ESI-MS, and though the number of neuropeptides detected by LC-ESI-MS was slightly larger, the two methods were highly complementary, indicating the potential for the CE-MALDI-MSI method to uncover previously undetected neuropeptides in the crustacean nervous system. These results indicate the potential of CE-MALDI-MSI for routine use in neuropeptide research.

Introduction:

Neuropeptides are one of the most diverse classes of signaling molecules. These short-chain amino acid sequences are responsible for facilitating cell-to-cell signaling, driving central pattern generators, and modulating the neuroendocrine system as long-range circulating hormones.¹⁻⁴ However, studying neuropeptides remains challenging due to their low abundance *in vivo*, tendency toward rapid degradation, and signal suppression due to masking from other biomolecules present in their complex sample matrices.⁵ These challenges necessitate the development of rapid workflows, and having an effective separation method is critical to maximizing the number of detected neuropeptides. Furthermore, their large span of functions is a consequence of the extensive variability in their structures. As a result, finding a single technique able to reliably profile all neuropeptides is difficult.⁶ Model organisms are typically used in order to characterize the structure and function of neuropeptides. The crustacean model is commonly employed for neuropeptide research because crustaceans possess a simplified nervous system with many of their neuropeptides being either conserved in or homologous to those in mammals, such as RFamide and tachykinin families or the allatostatin/galanin family.⁷⁻⁹ However, even with this simplified model, coverage of the neuropeptidome remains incomplete, and it is expected that there are many more neuropeptides present that have not yet been detected and characterized.

Mass spectrometry is useful for probing the neuropeptides in these biological samples due to its sensitivity, fast acquisition rate, and ability to discriminate between different neuropeptide isoforms with different masses.¹⁰⁻¹² The development of robust reversed-phase liquid chromatography (LC)-mass spectrometry (MS) techniques made large-scale profiling of neuropeptides possible, vastly expanding neuropeptide identification and driving functional discovery of novel neuropeptides. Since that point, LC-MS has been the main-stream technology

for large-scale neuropeptide studies, and most routine workflows employ some variation of this method for neuropeptidomics, with very successful results.¹³⁻¹⁷ However, alternative separation methods have been largely under-explored. Most notable of these is capillary electrophoresis (CE), which is a highly-promising method due to its rapid and highly-efficient separation mechanism. Furthermore, it separates molecules based on electrophoretic mobility, making it orthogonal to reversed-phase LC, which separates based on hydrophobicity. Many recent works have demonstrated the impressive capabilities of CE, but it still has yet to be incorporated as a routine method of analysis like LC.¹⁸⁻²¹

Most MS workflows utilizing either LC or CE incorporate electrospray ionization (ESI) to introduce analytes to the MS.²²⁻²⁶ The other most commonly-used ionization method, matrix-assisted laser desorption/ionization (MALDI), has not seen quite as many developments in recent years.²⁷ As a solid-phase ionization method, MALDI cannot be easily coupled to online separation methods like ESI can. MALDI is a highly-suitable ionization method, though, because it is especially tolerant to salts and the formation of primarily singly-charged ions is useful for avoiding charge dilution, where the signal of a single peptide is spread out over several charge states. Furthermore, because MALDI uses a different ionization mechanism than ESI, the ionization efficiencies of molecules differ between the two sources, resulting in complementary detection of peptides.²⁸ In order to couple MALDI with separation methods, offline fraction collection is typically performed.²⁹⁻³¹ However, this limits the achievable separation resolution, which is particularly discouraging for CE, as one of the most notable benefits to CE is its sharp, highly-resolved peaks, which is compromised during fraction collection. A continuous collection and MS acquisition method is necessary to preserve the high resolution of CE and maximize separation of complex biological samples.

There have been several methods for achieving continuous analysis of CE samples with MALDI-MS in recent years. Many of these show highly promising results, including one online coupling with a rotating ball interface between the CE outlet and vacuum MALDI surface,³² and another implementing a vacuum deposition interface for continuous collection.^{33, 34} However, these technique requires sophisticated instrument modification, so cannot be easily incorporated into existing laboratory workflows. MS imaging (MSI) appears to be the most promising method for offline coupling of CE and MALDI, as it does not require additional instrumental modifications, and the only equipment required is a moving platform for continuous collection. The coupling of LC with MALDI-MSI has been demonstrated with a similar type of setup.^{35, 36} Previous studies in our group implementing CE-MALDI-MSI have shown excellent results with high separation resolution and detection sensitivity.³⁷⁻³⁹

Here, we improve upon our previous CE-MALDI-MSI method for the analysis of neuropeptides in crustacean tissue samples in order to increase the obtainable depth of neuropeptidomic coverage. This result was achieved by utilizing larger injection volumes and enhanced separation resolution with a positively-charged PEI capillary coating that reduces peptide adsorption and reverses electroosmotic flow (EOF). With this method, an increase in the number of detected neuropeptides in two tissue sample types was obtained with good reproducibility. When compared to LC-ESI-MS, the results yielded complementary identifications, showcasing the potential of the methods together to substantially improve neuropeptidomic profiling. These results indicate the potential of the CE-MALDI-MSI interface to address challenges associated with neuropeptide research.

Experimental Section:

Chemicals and Materials

Fused silica capillary with a 50 μm inner diameter and 360 μm outer diameter was purchased from Polymicro Technologies - Molex (Lisle, IL, USA). ACS-grade formic acid (FA), anhydrous methanol, and cellulose acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyethylenimine (50/50 v/v in isopropanol) was purchased from Gelest, Inc. (Morrisville, PA, USA). All other chemicals and solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). Acidified methanol was prepared with 90/9/1 v/v/v water/methanol/acetic acid. For LC-ESI-MS instrumental analysis, Optima-grade solvents were used.

Tissue collection and sample preparation

C. borealis crabs were purchased from the Fresh Lobster Company, LLC (Gloucester, MA, USA) and housed in artificial seawater tanks with an alternating light/dark cycle of 12 hours. Animals were packed in ice for 30 minutes prior to dissection for tissue collection. The dissection took place in chilled physiological saline (440 mM NaCl, 11 mM KCl, 26 mM MgCl_2 , 13 mM CaCl_2 , 11 mM Trizma base, 5 mM maleic acid, adjusted to pH 7.45 with NaOH) as described previously.⁴⁰ Brains and sinus glands (SG) were collected and stored in acidified methanol at -80°C until use. A total of 8 sinus glands and 10 brains were collected for the CE-MALDI-MSI experiments and comparison LC-ESI-MS experiment.

Tissues were extracted for neuropeptides with acidified methanol as the extraction solvent. Acidified methanol was added to each tissue in a glass homogenizer, and the tissue was manually homogenized, sonicated in a bath sonicator, and centrifuged for 15 minutes at 16.1 rcf. The supernatant was removed and the cycle repeated for two additional extractions. The supernatant was evaporated in a speedvac, reconstituted in 15 μL 0.1% FA, and desalted with

C18 ziptips (Agilent Technologies, Santa Clara, CA, USA). The SG sample and one of the brain samples were divided into two separate aliquots. Samples were then evaporated again in a speedvac and reconstituted in water for CE-MALDI-MSI experiments. The second aliquot of SG and brain extract were reconstituted in 0.1% FA for LC-ESI-MS runs.

All standards used in the CE-MALDI-MSI experiments were diluted from stock solutions (1 mg/mL) to the appropriate concentration with water.

CE experiments

For the CE-MALDI interface, a fracture was made 10 cm from the outlet of a 110 cm capillary with a glass cutter, as described elsewhere.⁴¹ Briefly, the glass cutter was used to scratch the coating off the capillary. The capillary was then gently bent to fracture the silica without breaking all the way through the capillary. The fractured part was affixed to a piece of plastic with glue, and a 120 mg/mL cellulose acetate solution was dripped over the fracture to create an ion-permeable membrane. The area was allowed to dry for 30 minutes prior to use. A Hewlett Packard 3D CE (Palo Alto, CA, USA) was used for all CE runs. The capillary was conditioned before the first used by flushing with each of the following for 4 minutes: methanol, water, 0.1 M NaOH, water, 0.1 M HCl, water, methanol. To coat the capillary with polyethylenimine (PEI) for a positively-charged surface, the capillary was flushed with 1 M NaOH for 30 minutes, followed by water for 10 minutes, and a 10% PEI solution (diluted with anhydrous methanol) for 2 hours. The coating was allowed to dry for 1 hour, and then the capillary was flushed with water for 25 minutes, followed by background electrolyte (BGE) solution for 20 minutes.⁴² The coating was replenished before each day's CE runs. As this coating generates a positive charge on the inner wall of the capillary, the electroosmotic flow

was reversed such that the bulk solution migrated from the negatively-charged cathode to the positively-charged anode.

Samples were injected using pressure injection such that the total volume injected was approximately 1 μ L, 12% of the total capillary volume. The inlet of the capillary was then moved to a vial of BGE solution and -20 kV was applied for 30 minutes for CZE separation. The BGE solution used was a 50 mM acetic acid buffer solution at pH 4.5, prepared by diluting glacial acetic acid with water and titrating the solution with 0.1 M sodium hydroxide to the desired pH. The outlet of the capillary was placed in a reservoir filled with BGE solution such that the fractured part was submerged in solution and the outlet tip was outside of the reservoir (**Figure 1A**). The capillary was flushed with BGE for 10 minutes between subsequent analyses. For fraction collection, the outlet tip of the capillary was placed on a spot on a MALDI target plate for either 30 seconds or 60 seconds and then moved to the adjacent spot. Each fraction was mixed on the spot with 0.2 μ L DHB matrix (150 mg/mL 2,5-dihydroxybenzoic acid in 50/50/0.1 water/methanol/FA). For continuous trace collection, the capillary tip was placed on a plain, stainless steel MALDI target plate attached to a syringe pump with a platform (**Figure 1B**). During the separation, the syringe pump was set to move at 4.2 mm/min, resulting in continuous collection of liquid emerging from the capillary tip. The plate was then coated with 12 layers of DHB matrix (40 mg/mL in 50/50/0.1 water/methanol/FA) using an automated TM sprayer set to 80° C with 30 s drying time between passes. The entire workflow is shown in **Figure 1C**.

MALDI-MSI analysis

Samples were run on a MALDI LTQ-Orbitrap XL (Thermo Scientific, Bremen, Germany) in positive mode with an m/z range of 500 to 2000 and resolution of 60,000. The MALDI source was equipped with a 337.1 nm, 60 Hz nitrogen laser, set to 20 μ J laser energy.

For spot analysis, 50 scans were acquired for each spot. For imaging, MS spectra were acquired at a raster size of 100 μm , with the exception of the images in **Figure 2C**, which were acquired at a raster size of 200 μm . MS imaging run times took approximately 6 hours to complete. LC-ESI-MS data was acquired on an Orbitrap Elite (Thermo Scientific, Bremen, Germany). Details of the parameters can be found in the **Supporting Information**.

Data Analysis

Spot data was processed by accurate mass-matching peaks to our lab's in-house crustacean neuropeptide database within ± 5 ppm mass error. Images were generated for the CE traces in ImageQuest (Thermo Scientific, Bremen, Germany). The image files were exported as centroid imzml files, which were uploaded into MSiReader.⁴³ MS heat map images were generated for each m/z in the neuropeptide database. All images were normalized to the total ion current (TIC), with a mass error set to ± 5 ppm, and 5th order linear smoothing applied. Electropherograms were constructed by exporting the intensity matrix in MSiReader to Excel for each detected neuropeptide m/z . For each x-axis coordinate, the y-value intensities were summed to make a 2-dimensional electropherogram, with different colors representing different m/z values. LC-ESI-MS data was processed in MaxQuant⁴⁴ for feature detection, and then accurate mass-matched to the neuropeptide database within ± 5 ppm mass error.

Results and Discussion

Optimization of separation conditions

CE separation of neuropeptides was employed with continuous collection of liquid coming out of the capillary for subsequent MALDI-MSI in order to maximize separation resolution. **Figure 1A** shows the overall setup of the CE, capillary outlet, and MALDI target

plate. **Figure 1B** shows a close-up of the CE outlet allowing for the interface with MALDI. The overall workflow of the method is depicted in **Figure 1C**. The purpose of this study was to improve upon our previous designs for the CE-MALDI interface in order to gain a more in-depth profiling of neuropeptides. The key aspects to achieve such improvements include the incorporation of a positively-charged coating and by increasing the injection volume with LVSS.

The capillaries used were bare fused silica, which carries a negative charge. The peptides traveling through the capillary are predominantly positively-charged, and so electrostatic interactions cause them to adhere to the capillary. This phenomenon results in peak broadening, which causes poorer separation resolution and detection sensitivity. To overcome this problem, we coated the capillary with PEI, creating a positive charge on the inner wall of the capillary. The positive charge repels the positive charge of the peptides, resulting in sharper, more resolved peaks. In addition to preventing inner-wall adsorption, the PEI coating improves separation by reversing the EOF in the capillary. Due to the reversed charge of the surface, the EOF migrates from the negatively-charged electrode (cathode) to the positively-charged electrode (anode), while the peptides still flow anode to cathode. As a result, the separation is lengthened when performed under reverse polarity, allowing the peptides longer distance to separate within the capillary, thus improving resolution. **Figure S1** (See **Supporting Information**) shows a comparison of electropherograms of a neuropeptide extract with and without the PEI coating, demonstrating the improvement in peak shape and resolution.

In order to increase the amount of peptides that could be loaded onto the capillary, we employed the LVSS injection and pre-concentration method. This method works with low-concentration samples dissolved in water, as the low concentration causes the sample plug to have a higher field strength than the surrounding BGE due to its lower conductivity. Instead of

dissolving the sample in 0.1% formic acid, as is done for LC analysis, the sample was dissolved in pure water. As the neuropeptides in crustacean tissue sample are present in low abundance, the method was well-suited for this analysis. After the sample plug is loaded on the capillary and a voltage applied, the more highly-concentrated BGE travels quickly through the capillary. As current needs to be uniform at all points in a closed circuit, ions in the dilute sample plug move at a faster rate until the ions reach the interface of the sample plug and BGE, thus concentrating the sample plug until it is at the same concentration as the BGE. At that point, the peptides in the sample plug separate based on their individual electrophoretic mobilities.⁴⁵⁻⁴⁷ Because the EOF and peptide migration were in opposite directions, the peptides moved toward the entrance of the capillary until they reached the BGE solution. At that point, their net migration was toward the capillary outlet as they were carried by the EOF. In this setup, the BGE was 50 mM pH 4.5 acetic acid/ammonium acetate buffer. This pH was selected because it reduced the EOF enough such that the peptides could separate within a 30-minute separation window. The LVSS method enabled an injection of 1 μ L of sample, making the method more comparable to nanoLC methods while still providing excellent separation and high-efficiency peak shapes. The MS images in **Figure 2A** show the separation of a mixture of 5 standard peptides (20 μ M concentration) in a single CE trace. An improvement in peak area of approximately 2-fold was observed for standard peptides compared to a conventional 20 nL injection at this concentration. It is likely that this improvement is even larger at lower concentrations, as the standards were not detected at lower concentrations with the 20 nL injection volume.

In order to demonstrate the reliability and reproducibility of the separation, three runs of the same standard peptides were performed on different days with different capillaries. **Figure 2B** shows heat map MS images of two standard peptides from each of the three runs. As can be

seen, the migration time of each peptide is approximately the same on different days. The relative standard deviation, based on the migration time of the peak maximum, was 0.67% for FMRamide and 2.31% for allatostatin III. While the peak migrations shifted a small amount from day to day, likely due to slight variations in capillary length, they remained generally consistent, and the reproducibility was found suitable for separating peptide mixtures.

With the increased loading capacity of the CE method, it was expected that the detection of low-abundance neuropeptides would be possible. In order to evaluate this expectation, the limit of detection was approximated using a standard peptide, allatostatin III. Subsequent runs were analyzed with varying concentrations. Solutions of 1 pM, 10 pM, 100pM, 1 nM, and 1 μ M were analyzed. The standard was able to be easily detected at levels down to 100 pM, but at 10 pM and 1 pM, no signal was observed. **Figure 2C** shows the MS images of the standard at the three concentrations of the standard peptide. The migration times changes slightly for lower concentrations of peptide due to the increased amount of stacking that occurs within the capillary. From these data, it was determined that the approximate limit of detection is 100 pM, though the actual concentration will likely vary based on ionization efficiencies of peptides and complexity of samples.

Application of the CE-MALDI-MSI method to profiling neuropeptides in crustacean tissue extracts

The CE-MALDI-MSI method was evaluated on two tissue extracts, brain and sinus gland. Approximately 1.72 μ g of SG extract was loaded onto the capillary. For the brain extract, approximately 0.44 μ g was loaded onto the capillary. For each sample, three replicate injections were run in order to obtain three technical replicates for each. Two additional biological replicates were performed with the brain extract on different days using different brain tissue

samples. All sample runs resulted in the detection of over 200 neuropeptides, a substantial improvement to the 67 neuropeptides detected with the previous MALDI-MSI method, with relatively low standard deviations. The results are summarized in **Figure 3**, which shows the number of neuropeptides detected in each sample, with error bars indicating the standard deviation between the three technical replicates. The number of detected neuropeptides in each sample had low relative standard deviations (3.9%, 12.4%, and 1.5% for the brain samples and 6.8% for the SG sample), indicating good technical reproducibility. The relative standard deviation of peaks areas between samples were also relatively low, 27.6%, 22.4%, and 31.8% for the brain samples and 32.3% for the SG sample. **Figure 4** shows electropherograms from representative runs of both the brain and tissue extract, with different colors indicating different neuropeptides detected. The electropherograms were constructed by exporting the intensity of each m/z value at each pixel. The intensities were summed across the Y axis for each m/z value to generate a plot of peak intensity as a function of the X axis. As can be seen, the separations led to highly-resolved peptide signals in the MS imaging trace. The narrowness of the peak shapes is likely due to the large degree of stacking that took place with neuropeptides in low abundance. Furthermore, the migration times of the neuropeptides appear to be evenly distributed throughout the entire separation time for both tissue samples, indicating that the optimized parameters are suitable for separating the neuropeptides within a relatively short separation window (30 minutes).

Comparison of different collection methods

While the CE-MALDI interface has been explored by numerous groups in a variety of contexts in the past, the coupling with MSI is a relatively recent technique. The improved number of identifications in tissue extract is largely attributed to the improved separation

resolution. Other methods often couple CE with MALDI using fraction collection. By continuously collecting liquid from the capillary onto the plate and performing MS imaging, the resolution is expanded drastically (with the limit being determined instead by the raster size of the instrument). In our method, the raster size was set to 100 μm , which corresponds to a time resolution of approximately 0.01 s. In order to determine if this enhanced resolution is beneficial, a comparison was made between different time resolutions, including a direct spot (no resolution), 60 s fraction collections, 30 s fraction collections, and the continuous trace. Brain tissue extract was used for all collections. The resulting number of detected neuropeptides with each method is shown in **Figure 5**. As is expected, the number of neuropeptides increases with increasing separation resolution. This observation was confirmed by performing a one-way ANOVA test ($p\text{-value} = 1.0 \times 10^{-4}$), followed by a Dunnett post-hoc test in which the continuous trace was compared to the three other methods. Each comparison was determined to be statistically significant below a threshold of 0.05. **Figure 6** shows the MS spectra acquired from 10 of the 60 s fractions collected of the brain sample showing the difference in peptides detected within each fraction of the CE separation.

Comparison of CE-MALDI-MSI to LC-ESI-MS

Online LC coupled to ESI-MS is the gold standard for peptide separations and is routinely performed with excellent results. In order to determine how the CE-MALDI-MSI method compared, brain and SG samples were run on an Orbitrap Elite MS coupled to a Waters nanoAcquity LC system. This instrument was chosen for comparison because it has the same geometry as the MALDI LTQ-Orbitrap XL instrument, with all aspects of the instrument being approximately the same except for the ionization source. A standard 2-hour LC gradient was used with a self-packed 14 cm C18 column. While the results showed a slightly greater number

of neuropeptides detected in the LC-ESI-MS samples, it is interesting to note that the results were highly complementary, with less than a third of the neuropeptides being detected with both methods. **Figure 7** shows the overlap of neuropeptides detected with the two methods. These results indicate that neither method is necessarily superior to the other, but combining the two may substantially improve neuropeptidomic coverage. Furthermore, applying the CE-MALDI-MSI method toward additional neuropeptide samples may result in the detection of neuropeptides that have not been previously detected.

The neuropeptides detected in LC and CE were compared in order to identify any trends in characteristics causing certain neuropeptides to be identified with one method over the other. The mass, m/z , family, and retention/migration times were compared. The range and average masses and m/z values were approximately the same for all samples with both methods, lying near the median value. The neuropeptide families were also compared, as neuropeptides are grouped into families based on shared sequence motifs. For all samples, similar numbers of neuropeptides were detected belonging to the different common neuropeptide families. The average LC retention times and CE migration times were close to the median values, indicating that there was no bias related to the time of separation. Therefore, it is assumed that the differences in detected neuropeptides between the two methods is due to the orthogonality of the separation mechanisms resulting in different cohorts of peptides to be better separated and more easily detected.

Conclusion

The purpose of this study was to address challenges in neuropeptide detection related to the large degree of diversity and low abundances *in vivo*. To overcome these challenges, an MS platform was established that is orthogonal and complementary to conventional LC-ESI-MS. The

developed CE-MALDI-MSI method further improved on our previous platform by utilizing larger injection volumes and enhanced separation resolution with a PEI capillary coating. The resulting method showed effective separation of both standards and tissue extracts with excellent reproducibility. Furthermore, the results yielded complementary identifications to that of LC-ESI-MS, indicating that combining the platforms can lead to substantial improvements in neuropeptidomic coverage and potentially novel, previously-undiscovered neuropeptides. While this method used accurate mass-matching to identify neuropeptides, it is expected that the incorporation of MS/MS acquisitions will result in more confident identifications, as well as the identification of novel neuropeptides with the assistance of *de novo* sequencing. This method demonstrates the potential of CE-MALDI-MSI as a routine method for neuropeptide profiling in a variety of samples.

Electronic supplementary information (ESI) is available for the online version of this article through publisher's web site.

Details of LC-ESI-MS method used for comparison, electropherograms of tissue extracted separated with uncoated and PEI-coated capillaries.

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Figures

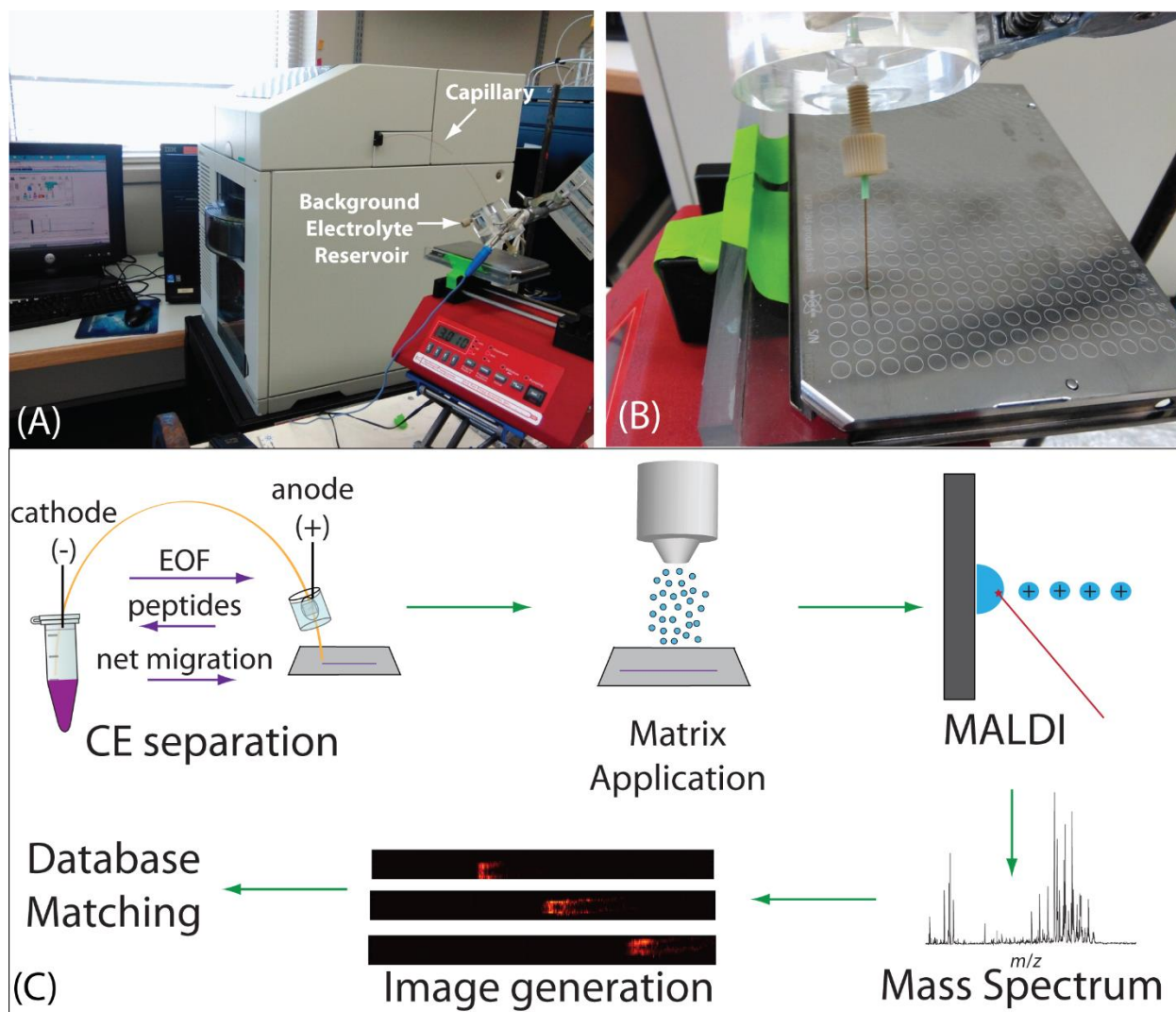


Figure 1. Depiction of the CE-MALDI-MSI set up, including a photograph of the interface of the capillary outlet with the MALDI plate (A), a close-up photograph of the capillary outlet and MALDI plate during trace collection (B), and a workflow showing the experimental steps, including CE separation (with arrows indicating direction of migration for electroosmotic flow (EOF), peptides, and net migration), matrix application, MALDI-MS analysis, generation of images, and database matching (C).

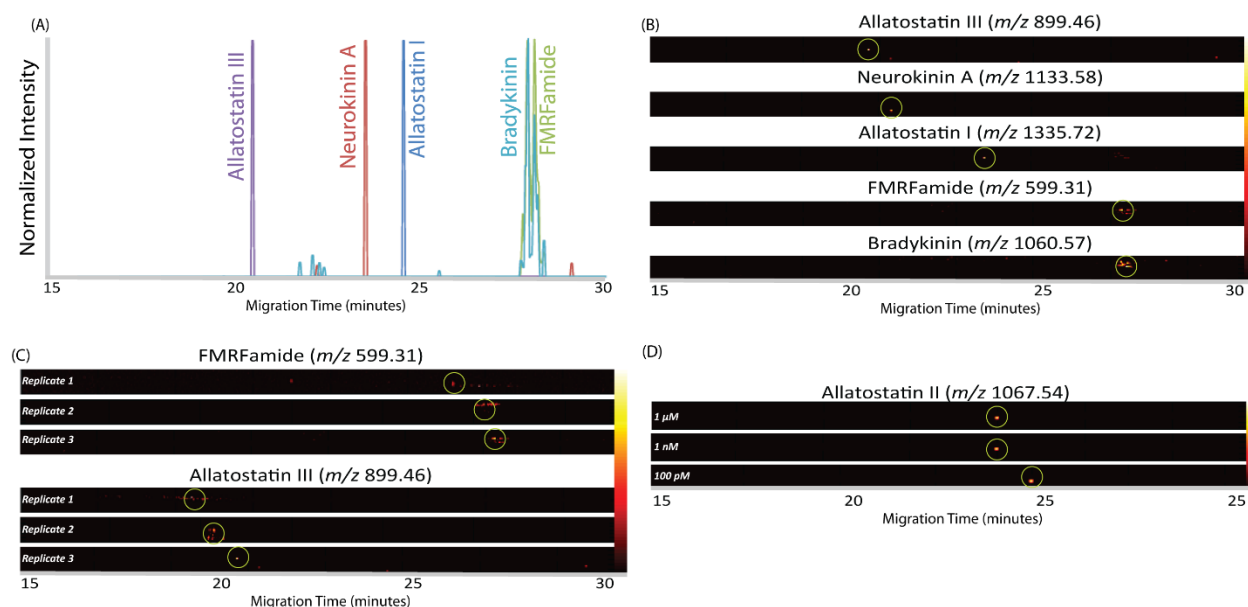


Figure 2. Electropherogram and MS images of CE traces of standard peptides, demonstrating the separation of a mixture of 5 standard peptides (A) with the corresponding MS images (B), the consistency of migration times of two standards run on different days (C), and the detection of a standard run at different concentrations (D). In each MS image, green circles indicate the location of the maximum peak intensity for the m/z value.

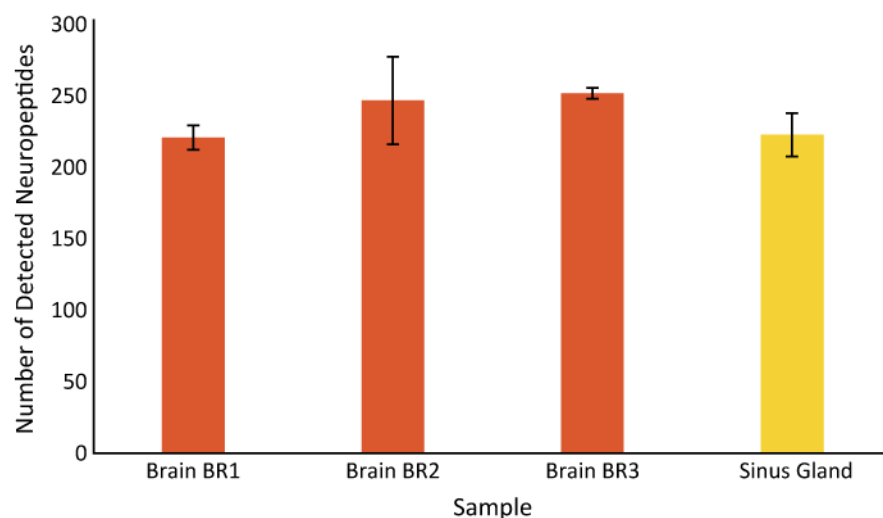


Figure 3. Number of detected neuropeptides in samples of neuropeptide tissue extract, with the total representing the average of three replicate injections of the same sample and error bars indicating the standard deviation.

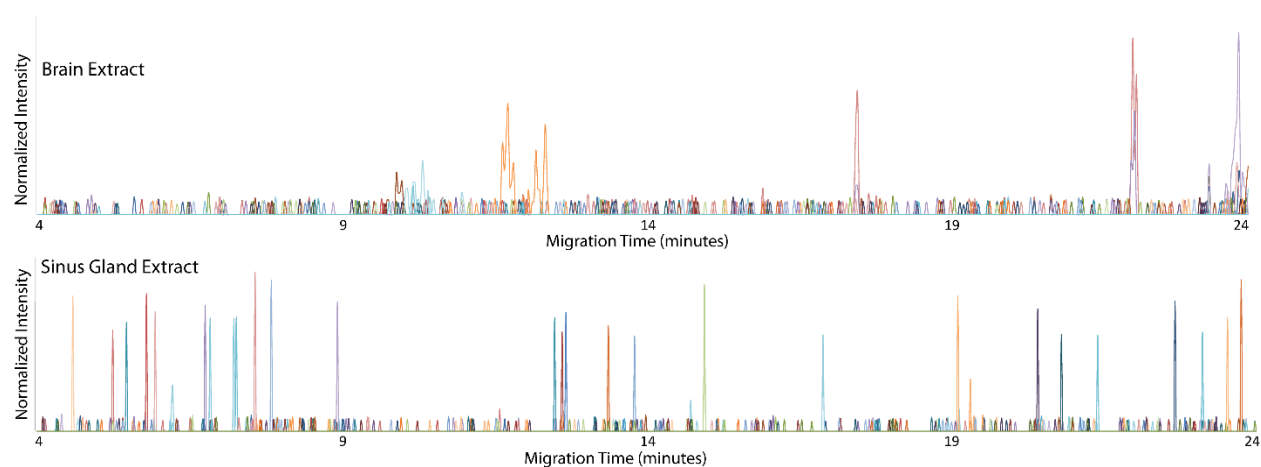


Figure 4. Electropherograms constructed from MS images of detected neuropeptides in representative brain (top) and sinus gland (bottom) CE-MALDI-MSI runs, with each color indicating a different neuropeptide.

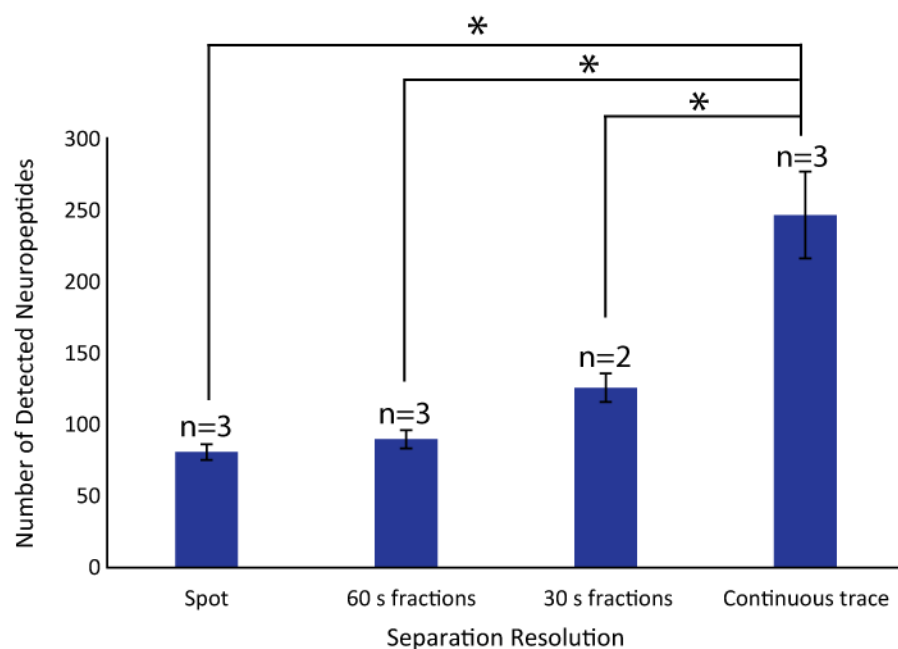


Figure 5. Bar graph indicating the number of neuropeptides detected in brain tissue extract samples when analyzing a direct spot, 60 s CE fractions, 30 s CE fractions, and a continuous CE trace using MSI. Error bars represent the standard deviation, and n corresponds to the number of replicates performed. An ANOVA test was performed (p -value 1.0×10^{-4}) followed by a Dunnett's post-hoc test comparing each method to the continuous trace. Asterisks indicate statistical significance at $\alpha < 0.05$.

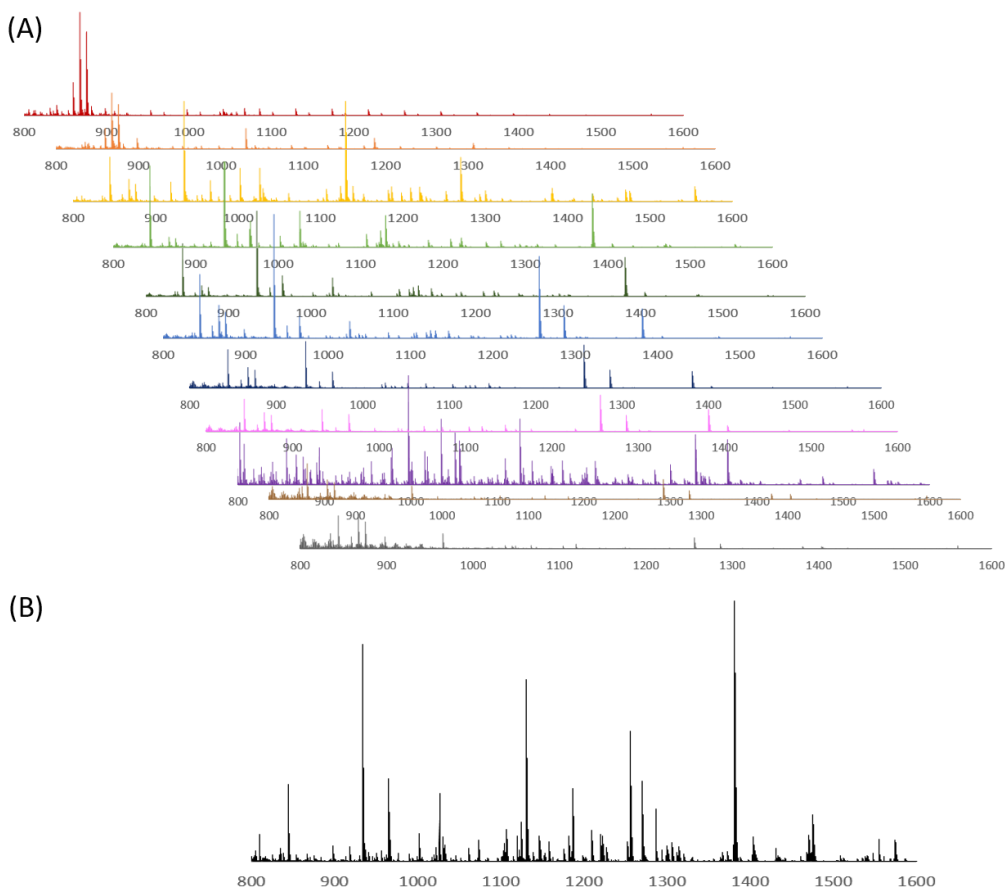


Figure 6. Mass spectra from CE separation of brain tissue extract collected as 60 second fractions and analyzed with MALDI-MSI. Shown are fractions 14 – 24, showing the difference in peptides identified in each fraction (A), as well as a mass spectrum of brain tissue extract without CE separation (B).

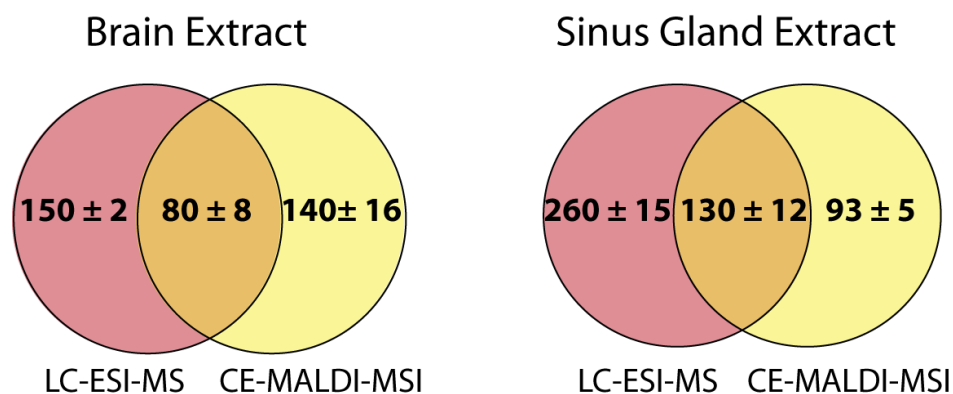


Figure 7. Venn diagrams indicating the overlap of neuropeptides detected in neuropeptide extracts from brain tissue (left) and sinus gland tissue (right) using LC-ESI-MS and CE-MALDI-MSI. Values indicate the number detected $\pm$ standard deviation ($n = 3$ technical replicates).

Methods

LC-ESI-MS parameters

For LC-ESI-MS analysis, an Orbitrap Elite (Thermo Scientific, Bremen, Germany) with a nano-ESI source coupled to a Waters nanoAcquity LC system (Waters Corp, Milford, MA, USA) was

used for analysis. Samples were dissolved in 15 μL 0.1% FA and 2 μL was injected onto a self-packed C18 column (75 μm inner diameter and 1.7 μm particle size). The flow rate was set to 0.300 $\mu\text{L}/\text{min}$. LC separation was carried out with water with 0.1% FA (A) and acetonitrile with 0.1% FA (B) as mobile phases. A two-hour gradient was implemented as follows: 0-1 min 3-10% B; 1-90 min 10-35% B; 90-92 min 35-95% B; 92-102 min 95% B; 102-105 min 95-3% B; 105-120 min 3% B. MS acquisition was carried out in positive mode from m/z 200 to 1600 with a resolution of 120,000.

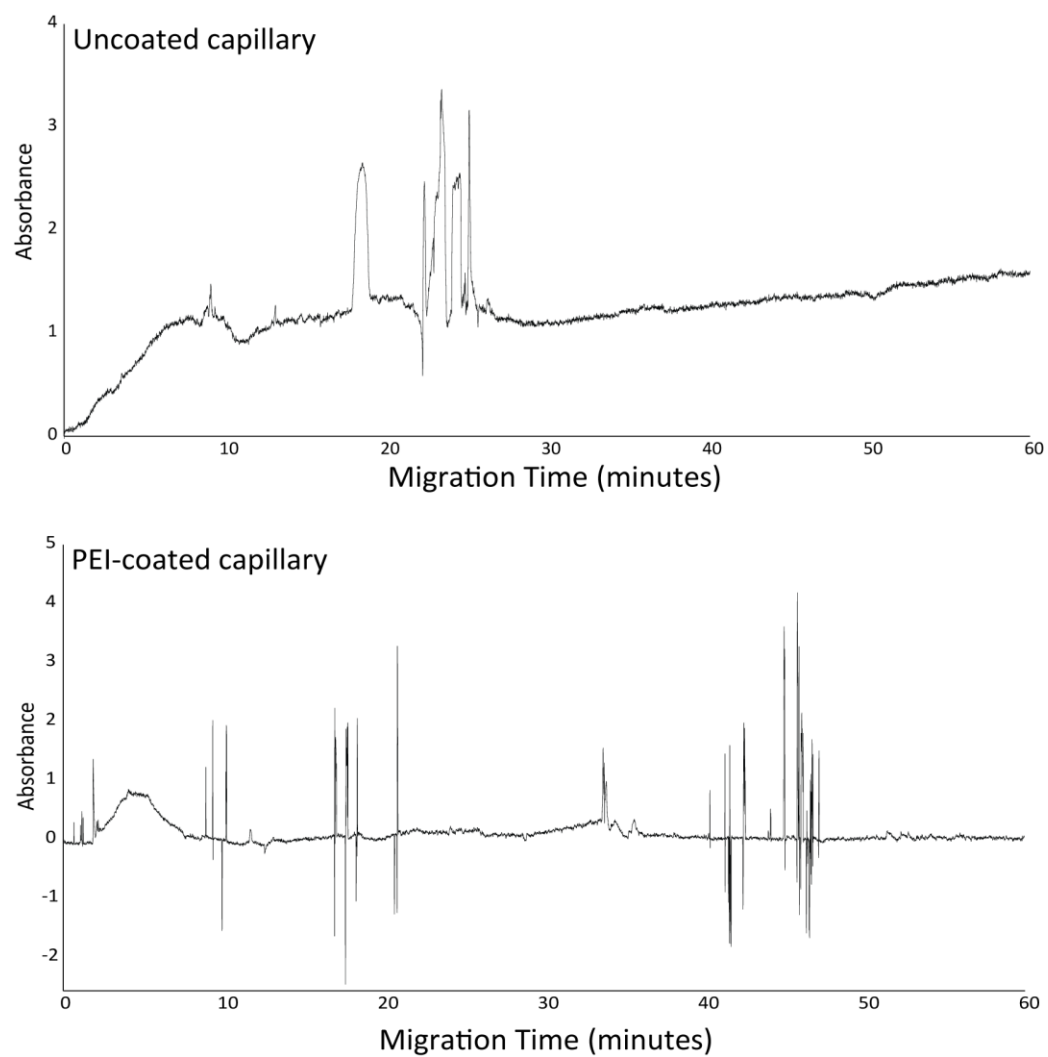
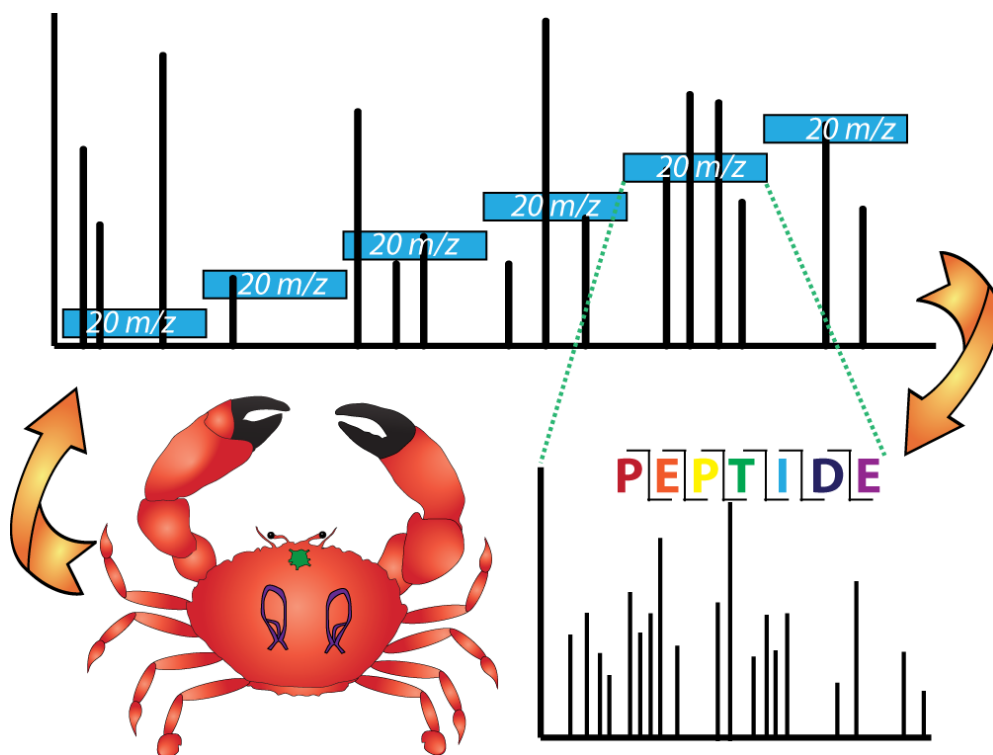


Figure S1. Electropherograms of brain tissue extract separated with an uncoated capillary (top) and a capillary coated with polyethylimine (PEI), using a UV detector at a wavelength of 240 nm.

Chapter 4

Data Independent Acquisition Mass Spectrometry Method for Improved Neuropeptidomic Coverage in Crustacean Neural Tissue Extracts



Modified from:

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Abstract:

Neuropeptides are an important class of signaling molecules in the nervous and neuroendocrine system, but they are challenging to study due to their low concentration *in vivo* in the presence of numerous interfering artifacts. Often the limitation of mass spectrometry analyses of neuropeptides in complex tissue extracts is not due to neuropeptides being below the detection limit, but due to ions not being selected for tandem mass spectrometry during the LC elution time and therefore not being identified. In this study, a data independent acquisition (DIA) method was developed to improve the coverage of neuropeptides in neural tissue from the model organism *C. borealis*. The optimal mass-to-charge ratio range and isolation window were determined and subsequently used to detect more neuropeptides in extracts from brain and pericardial organs than the conventional data dependent acquisition method. The DIA method led to the detection of almost twice as many neuropeptides in the brain and approximately 1.5-fold more neuropeptides in the pericardial organs. The technical and biological reproducibility were also explored and found to be improved over the original method, with 56% of neuropeptides detected in 3 out of 3 replicate injections and 62% in 3 out of 3 biological replicates. Furthermore, 67 putative novel neuropeptides were detected and identified with *de novo* sequencing. The quantitative accuracy of the method was also explored. The developed method is anticipated to be useful for gaining a deeper profiling of neuropeptides, especially those in low abundance, in a variety of sample types.

Introduction:

Neuropeptides are important signaling molecules in the nervous and neuroendocrine system that act both locally and long-range to modulate and regulate various biological activities.¹ These short-chain amino acid sequences represent a diverse class of biological molecules responsible for the modulation of various functions such as feeding^{2, 3} and adaptation to external stresses.^{4, 5} Dysregulation of the expression of neuropeptides can contribute to neurological diseases by altering the behavior of neurons, and so a more comprehensive characterization of neuropeptides is essential to the treatment of these diseases.⁶ However, the complexity and wide variety in characteristics of neuropeptides, in addition to their low abundance and propensity toward rapid degradation, make them difficult to study.

In order to characterize both sequence information and function of neuropeptides, it is necessary to use a model organism that simplifies the experimental matrix and enables samples to be reliably collected. The Jonah crab, *Cancer borealis*, provides a useful model for mass spectrometric investigation into the neuropeptidome and its changes in response to external stimuli, as its simple yet elegant nervous system has already been well-characterized with electrophysiological studies⁷⁻⁹ and many neuropeptides in crustaceans are direct homologues to mammalian neuropeptides (e.g., the allatostatin family is homologous to the mammalian galanin family¹⁰ and RFamide and tachykinin families are conserved across both invertebrates and vertebrates). Furthermore, despite not yet having a complete genome, an extensive database of neuropeptides has been compiled through a combination of multifaceted mass spectrometry (MS) and *in silico* prediction approaches.^{11, 12}

Despite these advantages, neuropeptides are difficult to study, even in model organisms such as crustaceans. Neuropeptides are highly variable, and there are numerous neuropeptide

isoforms belonging to each family, often differing only by a single amino acid. These isoforms can have identical or drastically different effects in the nervous and neuroendocrine system. For example, the C-type allatostatin (AST-C) neuropeptides I, II, and III have all been evaluated in the modulation of lobster heart rhythm in a recent study, and AST-C I and AST-C III were shown to have similar, though not identical, functionality, while AST-C II had largely contrasting effects, despite the high degree of sequence similarity.¹³ Moreover, the same neuropeptide can have varying effects in different parts of the nervous/neuroendocrine system or at different concentrations, such as pyrokinin neuropeptide PevPK2, whose effect on cardiac amplitude and frequency change with varying dosage concentrations.¹⁴

Mass spectrometry (MS) has been demonstrated to be effective at discriminating between similar isoforms of neuropeptides and providing relative changes in abundance between samples, and many studies have been successfully implemented to both profile neuropeptides in crustacean species and assess their changes in response to various stimuli.¹⁵⁻¹⁸ However, current methodologies are still limited in the depth at which they can profile the neuropeptidome and reproducibly detect low-abundance neuropeptides. Though these challenges are contributed by several factors, a major influence is inherent limitations in the way the samples are analyzed via MS. Up until now, data dependent acquisition (DDA) has been predominantly used to select ions for tandem MS (MS/MS) fragmentation, in which only the highest-abundance ions are selected and fragmented for subsequent identification. As a result, the method is largely biased toward high-abundance species, often selecting interfering artifacts such as lipids or protein fragments over low-abundance yet biologically-active neuropeptides, limiting the total number of neuropeptides detected. Furthermore, slight variations in relative abundance of ions, either due to

differing conditions, natural biological variability, or even slight differences between repeat injections, alter which ions are selected for MS/MS, limiting the reproducibility of the method.

Alternative methods have been investigated for addressing these limitations, such as using a targeted approach, in which ions are only fragmented if they are included on a list.¹⁹ This method works well if all neuropeptides of interest are known before analyzing the samples. However, this method is not suitable for discovery-based neuropeptidomic studies and largely limits the breadth of analysis. Data independent acquisition (DIA) is emerging as a promising alternative strategy that provides balance between the sensitivity and selectivity of targeted MS methods while retaining the breadth of coverage offered by DDA methods.²⁰⁻²⁸ In DIA methods, a large window of ions are simultaneously fragmented across an entire mass range, enabling theoretically comprehensive fragmentation of all ions in a sample in a reproducible and nonspecific manner. While the method is easy to implement on the instrument, data analysis has been difficult due to the lack of precursor ion information. The most common method of analysis has been to use spectral libraries that typically are generated by first analyzing the samples using a DDA method.²⁹ While this helps to reduce run-to-run variability, relying on DDA for spectral libraries retains the bias toward high-abundance neuropeptides.

Recently, an alternative method of analysis has emerged in which no spectral libraries are required for data analysis. These methods use sophisticated computational algorithms to parse complex DIA spectra that typically contain fragments from several ions. The two most prominent software for this are DIA-Umpire and PECAN. DIA-Umpire uses deconvolution of MS/MS spectra over time to map fragments to individual precursors, thereby creating “pseudo MS/MS spectra” that are similar to typical DDA MS/MS spectra.³⁰ PECAN is part of the open-source data analysis platform Skyline and uses a dot-product scoring method for database

searching.³¹ These methods offer the ability to perform discovery-based analyses in a reproducible, unbiased manner.

This study sought to develop a DIA method to improve the coverage of neuropeptides detected in *C. borealis* neural tissue in a manner that is reproducible both at the technical and biological level and does not rely on the generation of spectral libraries. The resulting method almost doubled the number of neuropeptides detected that are present in the database and demonstrated improved reproducibility when directly compared to the conventional DDA method. The method also demonstrated improved results compared to previous profiling of the *C. borealis* neuropeptidome, despite the previous study combining multiple MS methods.¹¹ Furthermore, 68 putative novel neuropeptides not currently present in the database were detected and identified with *de novo* sequencing. The method was also evaluated for quantitative accuracy and found to be acceptable for recognizing large changes in abundance between samples.

Methods:

Instrumental analysis

See **Supporting Information** for details of chemicals and materials, animal dissection, tissue collection, and sample preparation. Samples were reconstituted in 15 μ L of 0.1% FA in water (Optima-grade) for MS analysis, with the exception of the brain and pericardial organ (PO) samples for method optimization, which were reconstituted in 90 μ L so that all methods could be tested on injections from the same vial. Samples were analyzed on a Thermo Scientific Q Exactive instrument (Thermo Scientific, Bremen, Germany) coupled to a nano-ESI source connected to a Waters nanoAcquity LC system (Waters Corp, Milford, MA, USA). LC separation was carried out using a 15-cm self-packed C18 column with a 75 μ m internal

diameter and 1.7 μm particle size. Water with 0.1% FA and acetonitrile with 0.1% FA were used as mobile phases A and B, respectively. The flow rate was set to 0.300 $\mu\text{L}/\text{min}$. 2 μL of sample was injected onto the column and separated over a 120-minute gradient as follows: 0-1 min 3-10% B; 1-90 min 10-35% B; 90-92 min 35-95% B; 92-102 min 95% B; 102-105 min 95-3% B; 105-120 min 3% B.

For mass spectrometry analysis, 6 different acquisition methods were used: 2 DDA methods and 4 DIA methods. All MS acquisition methods used positive electrospray ionization (ESI) with a collision energy of 30 eV, with 70,000 resolution for MS scans and 17,500 resolution for MS/MS scans. The MS/MS scan range was adjusted depending on the parent mass or isolation window, with 50 m/z being the first fixed mass. The DDA methods were set to top 10 and top 20 for precursor ion selection, with an isolation window of 2 m/z and dynamic exclusion set to 40 seconds. The MS scan range was 300 – 2000 m/z . The DIA methods varied in their MS scan ranges and isolation widths: scan range of 250 to 850 m/z with a 20 m/z isolation width; scan range of 250 to 930 m/z with an isolation width of 20 m/z for 250 to 510 m/z and 60 m/z for 510 to 930 m/z ; scan range of 250 to 1000 m/z with an isolation width of 30 m/z for 250 to 700 m/z and 60 m/z for 700 to 1000 m/z ; scan range of 400 to 800 m/z with an isolation width of 20 m/z .

Data Analysis

Resulting MS spectra from the DIA methods were first converted to the mzXML file format using MSConvert³² and processed with DIA-Umpire³⁰ using the default parameters. The output mgf files from DIA-Umpire were converted to mzXML files again using MSConvert and processed in PEAKS 7.0 (Bioinformatics Solutions Inc., Waterloo, ON, CAN).³³ The resulting DDA files were directly processed in PEAKS with no initial processing. The parameters used for

PEAKS *de novo* sequencing and database searching were as follows: no enzyme cleavage specified, instrument orbi-orbi, HCD fragmentation, and precursor correction enabled.

Modifications were set to include amidation, pyroglutamate, dehydration, and oxidation. All other parameters were set to the default. An in-house crustacean neuropeptide database was used for searches. For quantification, abundances were obtained by taking the area under the curve of MS1 peaks from the raw file with no normalization used. For identification of putative neuropeptide sequences, *de novo* sequences exported from PEAKS were filtered using PepExplorer for sequence similarity to the neuropeptide database and then manually filtered for characteristic sequence motifs.³⁴ Only neuropeptides with an average local confidence (ALC) score greater than or equal to 75% were included.

Results and Discussion

Optimization of method isolation window and m/z range

DIA performs its acquisition in a cyclic manner in which it selects a window of ions at a time for fragmentation, sequentially working through an entire mass range, then cycles back and scans the range again, with intermittent MS scans within each loop. Therefore, the key factors affecting the detection efficacy of the method are the size of the mass range, the size of each isolation window, and the total number of scans in each loop. As the number of scans per loop is dependent upon the scan range and window size, this number was kept constant at 20 MS/MS scans in order to simplify the comparison of methods. The mass-to-charge ratio (m/z) scan range and isolation window were varied based on the frequency of m/z values commonly detected for neuropeptide samples. Histogram plots were constructed for several neuropeptide samples previously analyzed, and it was found that most detected neuropeptides fall within the m/z range

of 450 to 800, with a small number appearing outside this window and substantially decreasing in frequency further from this number. Very few neuropeptides were detected with m/z values greater than 1000, and so this number was the upper limit tested with the method. The window sizes were determined based on the mass range, with narrower windows for m/z values with a higher density and wider windows for lower density ranges.

A total of 4 DIA methods were tested for two neuropeptide sample types. The samples used were *C. borealis* brain and PO extracts, which typically have different neuropeptide compositions. The first method used a variable window optimized based on masses of commonly detected neuropeptides, mass range 250 to 930 m/z , window size 20 m/z for lower mass range (250 to 510 m/z) and window size 60 m/z for upper mass range (510 to 930 m/z). The second method also used a variable window but with a larger mass range that encompassed close to the entire range of m/z values detected for neuropeptide samples, 250 to 1000 m/z , with a window size of 30 m/z for the lower mass range (250 to 700 m/z) and 60 m/z for the upper mass range (700 to 1000 m/z). The third method used a uniform window size of 30 m/z with a slightly smaller m/z range of 250 to 850 m/z . The fourth method also used a uniform window size, but slightly smaller at 20 m/z , with the m/z range only encompassing the high-density m/z values detected for neuropeptide samples (400 to 800 m/z). For comparison, a conventional top-10 DDA method was also tested that reflected what has previously been optimized for neuropeptide samples. Additionally, a top-20 DDA method was also tested in order to have a more direct comparison with similar duty cycles between methods. A total of 20 isolation windows was used for method optimization because it coincides with the duty cycle typically employed on common mass spectrometers, thus enabling the method to be easily transferrable across instruments.

Overall, the results from the 4 DIA methods were largely similar to each other, but showed a noticeable improvement over the DDA methods (**Figure 1**). The methods were evaluated based on the number of neuropeptides detected, the number detected with full sequence coverage, and the average score of detected neuropeptides. The total number of detected neuropeptides improved substantially from DDA to DIA. The DDA methods resulted in the detection of 100 to 150 neuropeptides, while the DIA methods resulted in the detection of over 200 neuropeptides (with the exception of one of the DIA methods for the brain sample that only detected 151 neuropeptides). Furthermore, the number of neuropeptides with 100% sequence coverage improved substantially with 3 of the 4 DIA methods. The DIA method encompassing the scan range 250 to 930 m/z did not detect as many neuropeptides as the other methods and had a much lower number with full sequence coverage, which is likely attributed to its bias toward low m/z values. The method incorporated a small isolation width at low m/z values and a large isolation width for large m/z values, which likely resulted in more fragment peptides being detected and fewer intact neuropeptides, resulting in a low number of neuropeptides detected with full sequence coverage. The fourth method (scan range 400 to 800 m/z) showed the greatest improvement in detected neuropeptides (total and with full sequence coverage) for the PO samples and showed approximately the same results as the third method (scan range 250 to 850 m/z) for the brain samples. In order to ensure that the detected neuropeptides were reflecting quality identifications, the average PEAKS score of identified neuropeptides was also compared between the methods, and all were in close proximity to each other, between 65 and 90 $-10\log P$. Though all of the scores were similar, the fourth DIA method (scan range 400 to 800 m/z) had the highest average score for both the brain and PO samples (85.33 and 89.35, respectively). **Figure 2** shows three representative raw MS/MS spectra with

annotated fragmentation peaks. While there appears to be some co-isolation, as anticipated with DIA, the spectra are still very clear and most of the backbone fragmentation can be identified without excessive interference from co-isolating peaks. Based on these observations, it was concluded that the DIA method encompassing the scan range 400 to 800 m/z was the most appropriate to use for the data, and it was the one selected for all subsequent analyses. If the resulting MS/MS spectra acquired from DIA-MS analysis showed extensive co-isolation or the detected neuropeptides were low-scoring, a follow-up targeted DDA method could be employed in order to confirm the identification of those neuropeptides. However, the MS/MS spectra displayed clear fragment peaks and most peptides were high-scoring, so the incorporation of a follow-up DDA analysis does not appear to be necessary and would not be worth the increased analysis time required.

Comparison of neuropeptides detected in DIA and DDA methods

In order to ensure that the chosen DIA method was reliably detecting neuropeptides in each sample, the neuropeptides identified with the method were compared to those detected with the conventional top-10 DDA method. The total numbers of detected neuropeptides with each method were compared, as shown in **Figure 3**. As shown in the figure, there is a large amount of overlap in neuropeptides detected between the two methods, which indicates that the DIA method is comprehensive, not complementary to the DDA method.

However, while there was very good overlap between the two methods, there was still a small fraction that were detected by DDA but not DIA. To make sure that there was no bias against certain types of neuropeptides, the neuropeptides not in common between the two methods were compared. The average score of neuropeptides detected with only DDA was 55.93 and 42.55 in the brain and PO, respectively, while the average score detected with only DIA was

62.98 and 71.26 in the brain and PO, respectively, indicating that the additional neuropeptides detected by DIA were not just low-scoring peptides likely to be false-positives. The average mass of neuropeptides detected with only DDA was 1194.33 and 1688.56 in the brain and PO, respectively, and the average detected with only DIA was 2832.625 and 2266.798 in the brain and PO, respectively. These numbers initially suggest that DDA and DIA have differential bias toward neuropeptide masses. However, there were more low-molecular weight neuropeptides detected in both tissues with DIA only than with DDA only, which shows that DDA is not more likely to detect low-molecular weight neuropeptides than DIA.

The potential bias toward specific neuropeptide families or sequence motifs was also investigated. For each neuropeptide detected in DDA only, there were more neuropeptides from the same family detected in DIA only. For example, in the brain sample, 7 RFamide neuropeptides were only detected in DDA, but 27 RFamide neuropeptides were only detected in DIA. In addition, there were several neuropeptide families detected only in DIA, but no families detected exclusively in DDA. In summary, the DIA method appears to generally detect more neuropeptides than DDA without any apparent systematic bias. Those neuropeptides detected only in DDA are likely due to technical variation between analyses and not due to limitations of the method.

Evaluation of experimental and biological reproducibility

In addition to detecting a wider range and greater number of neuropeptides, DIA is useful for improving run-to-run reproducibility both within and between experiments. In order to assess this, the experimental (or technical) reproducibility of the DIA method was compared to that of the top-10 DDA method. Replicate injections of the brain extract sample were compared to determine the overlap in detected neuropeptides, as shown in **Figure S1** (see **Supporting**

Information). As can be seen, the DIA method showed much greater overlap between replicate injections, with 56% detected in every injection, compared to 32% detected in every injection with DDA.

The biological reproducibility of the DIA method was also explored. Neural tissue samples often contain a large amount of interfering biological matrices such as lipids, protein fragments, etc. These background interfering compounds can be present at different levels in different animals of the same species, which can affect which ions are selected for MS/MS fragmentation in samples from different animals. As a result, differences in identified neuropeptides may incorrectly be attributed to different neuropeptide compositions in different samples, which may not necessarily reflect the actual sample composition. It was suspected that using a DIA method would enable consistent identification of the same neuropeptides across different biological samples, with exceptions reflecting the true natural variability of individual animals. To test this, brains from 3 individual animals were extracted and analyzed separately using the DIA method and the resulting detected neuropeptides were compared. **Figure S2** (See **Supporting Information**) shows the overlap in neuropeptides detected in the three biological replicates. As can be seen, the results indicate excellent reproducibility at the biological level, with 62% of neuropeptides detected in every biological replicate.

Evaluation of quantitative accuracy and dynamic range

The data indicate that the method is very useful for improving the coverage of the neuropeptidome. To further evaluate its potential utility in neuropeptide analysis, the quantitative accuracy of the method was investigated. Pooled extracts from 15 *C. borealis* thoracic ganglia (TGs) were aliquoted into separate vials with concentration ratios of 0.5:1:1:2:5. The purpose of this experiment was to compare the abundance of neuropeptides across samples of different

concentrations. TGs were used for the analysis because they are a larger tissue than the brain or PO, and so offer a greater number of neuropeptides to be used for comparison. The samples were analyzed using the DIA method and quantified at the MS1 level by calculating the area under the curve of each MS1 peak. Ratios were calculated by dividing the peak area of each neuropeptide in each respective sample. The area of neuropeptides in the fifth sample were divided by those in the first sample to give a ratio of 1:10 without overloading the LC column. **Figure S3** (see **Supporting Information**) shows boxplots of the results obtained for each ratio. The experimental errors in the ratio for 1:1, 2:1, 5:1, and 10:1 were 18%, 23.5%, 32.8%, and 21.4%, respectively, indicating that this quantitation can be used to discriminate up and down regulation in the samples at large fold-changes. The quantitative accuracy can likely be substantially improved by the incorporation of chemical labeling, especially isotopic labeling where quantitation is performed at the MS1 level. However, labeling and combining samples increases spectral complexity, which would limit the ability of the DIA method to perform confident identifications. As a result, incorporating chemical labeling would require additional method optimization.

Detection of putative novel neuropeptides with de novo sequencing

A key advantage of using DIA software that converts DIA spectra into pseudo DDA spectra is that it does not require spectral libraries to process the data, which enables the possibility of discovery of novel peptides via *de novo* sequencing. As the *C. borealis* genome is not yet completely sequenced, ensuring a complete neuropeptide profile relies on *de novo* sequencing for detection of putative novel neuropeptides. The DIA method has been shown to be useful for obtaining a deeper neuropeptidomic profile of neural tissue samples, and so it was expected that the method could also detect neuropeptides not previously identified by MS

analysis and are therefore unknown. Putative, potentially-interesting/novel neuropeptides were determined by looking for common sequence motif characteristics of neuropeptide families. In the brain sample, 38 putative neuropeptides from 6 different families were detected, including orcomyotropin, orcokinin, pyrokinin, RFamide, tachykinin, and YRamide. There were 31 neuropeptides from 3 families in the PO sample, including B-type allatostatin, RFamide, and RYamide. **Table S1** lists all putative novel neuropeptides detected in the brain samples, and **Table S2** lists all putative novel neuropeptides that have been detected in PO samples (see **Supporting Information**). These results are consistent with what would be expected for each tissue. The brain is connected to the crustacean stomatogastric nervous system. Orcokinins are responsible for influencing hindgut contractions and modulating stomatogastric nervous system outputs, and therefore these neuropeptides tend to have a greater presence in the brain and stomatogastric nervous system.³⁵ Orcomyotropin neuropeptides also modulate hindgut contractions, and pyrokinin neuropeptides have a role in the gastric mill rhythm, and so it is expected that neuropeptides belonging to these families would also be identified in the brain.³⁵ B-type allatostatins function both locally and as secreted circulating hormones, and so they are commonly found in neurosecretory tissue such as the PO.³⁵ Neuropeptides ending in –RFamide can be classified into a variety of sub-families with various functions, and it is therefore not surprising that a large number of neuropeptides with this sequence motif were detected in both tissues.³⁵ One neuropeptide from this family, SENRDFLRFamide, was identified in both tissues, indicating that it may function both locally in the central nervous system and long-range as a circulating hormone. **Figure 4** shows three representative spectra of neuropeptides detected and identified with *de novo* sequencing with their key fragment ions annotated. Spectra of all *de novo* sequenced neuropeptides listed in **Tables S1** and **S2** are shown in **Figures S4 to S72**

(Supporting Information). As with the spectra in **Figure 2** for database-matched neuropeptides, there appears to be some co-isolation with other ions, which is to be expected with DIA analysis because the isolation window is large. Each neuropeptide was searched for in the comprehensive neuropeptide database NeuroPep³⁶, and no matches were found, indicating that these neuropeptides have not yet been identified in any species. The sequences did not match parts of longer sequences either, indicating that they are not simply degradation products from larger neuropeptides. Most of the putative novel neuropeptides listed possess the classic neuropeptide motif. The rest of the neuropeptides listed possess a similar motif but with the variation of one amino acid or variation in the presence of C-terminal amidation, indicating the possibility of a mutation that may alter the biological activity. Many other peptides were also sequenced with PEAKS software and did not possess similarity to common neuropeptide motifs. These peptides were not included in the list of putative neuropeptides, as they are likely degradation products from large proteins, but they may still be biologically active as non-classical neuropeptides.³⁷ Further studies of the precursor proteins and functional assessment of the peptides is necessary to properly classify them as non-classical neuropeptides.³⁷

Conclusions

In this study, DIA was explored as an alternative method to DDA to address current limitations in neuropeptide research. DIA's key advantage of unbiased precursor selection for MS/MS fragmentation analysis allowed for a greatly improved coverage of the crustacean neuropeptidome without sacrificing the quality of neuropeptide identifications, and the inclusion of most DDA-detected neuropeptides indicated that DIA can be used alone without being accompanied by DDA analyses. The Thermo Q Exactive Orbitrap MS used in this study enabled

a duty cycle time of 3.6 seconds, which was less than half the width of most peaks in the chromatogram. The breadth and depth of coverage may be increased by additional optimization on more sophisticated instruments with faster scan times, which would improve the duty cycle, but the method will still likely be viable on instruments with slower scan times as long as the duty cycle time does not exceed the width of the peaks in the chromatogram. With a faster scan time, more MS/MS acquisitions could be made in the same amount of time, enabling either a larger m/z range or narrower m/z windows to be obtained. However, the duty cycle was not found to be a limitation of DIA compared to DDA, as with identical duty cycles, DIA still outperformed DDA. The depth of coverage could also be improved upon by implementing a multi-dimensional separation platform, such as by the incorporation of ion mobility MS for gas-phase separation, or incorporating an orthogonal front-end separation such as capillary electrophoresis.³⁸⁻⁴¹ The additional level of separation would reduce the number of co-fragmented ions in each spectra, potentially improving the number of and quality of identifications.

The consistent fragmentation of all ions within a given m/z range allowed for reproducible identifications between both technical replicates and biological replicates, making the data more rigorous and reproducible for neuropeptidome profiling. Furthermore, through the use of PEAKS *de novo* sequencing, the method was able to identify putative neuropeptides that have previously gone undetected, likely due to their low *in vivo* abundance compared to other neuropeptides. The 67 novel neuropeptides identified here indicate the power of DIA for providing a more complete assessment of a biological sample without depending upon spectral libraries. Additional studies will need to be carried out in order to validate these neuropeptides, such as by performing a targeted analysis and matching to a synthetic standard. Functional

studies can also be implemented in order to assess the biological activity of the neuropeptides. The quantitative accuracy of the method was also evaluated and found to be satisfactory for obtaining general trends in up or down regulations. However, the accuracy of quantitation is limited, and can likely be improved with the use of an internal standard or more sophisticated computational methods, such as those available by software tools.^{30, 31} In general, DIA quantification would greatly benefit from the development of labeling methods using chemical tags, which would both improve quantitative accuracy and increase the multiplexing capabilities of the method.⁴²⁻⁴⁷ We anticipate that this DIA method will serve as a useful tool for profiling neuropeptides and other endogenous peptides in a variety of samples.

ACKNOWLEDGEMENT

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Supporting Information

Details of animal dissection, tissue collection, and sample preparation; tables of putative neuropeptides detected with *de novo* sequencing in brain and pericardial organs; figures showing MS/MS spectra of all *de novo* sequences

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Figures

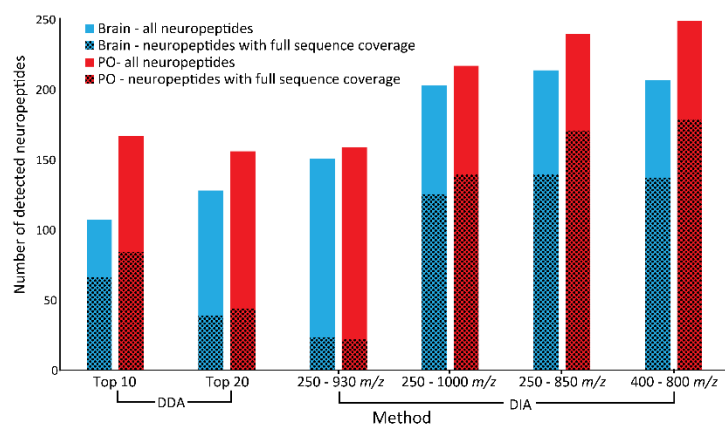


Figure 1. Comparison of the number of neuropeptides detected in brain (blue) and pericardial organ (PO) (red) using each acquisition method. The solid fills indicate the total number of neuropeptides detected, and the patterns indicate the number of neuropeptides detected with 100% sequence coverage.

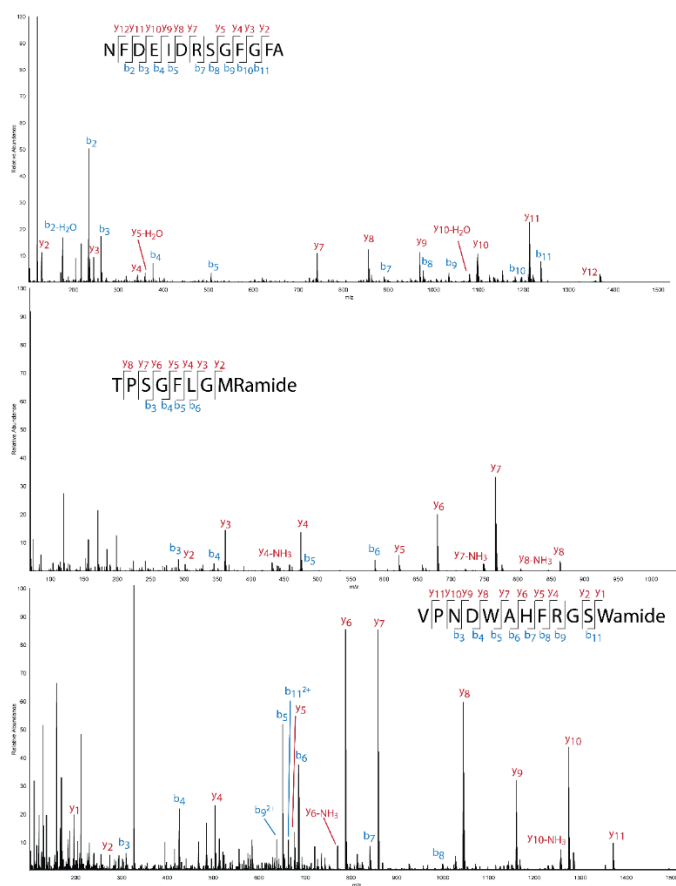


Figure 2. MS/MS spectra of three detected neuropeptides using the data independent acquisition (DIA) method, including (a) an orcokinin neuropeptide detected in the brain, (b) a tachykinin neuropeptide detected in the brain, and (c) an allatostatin B-type neuropeptide detected in the pericardial organ.

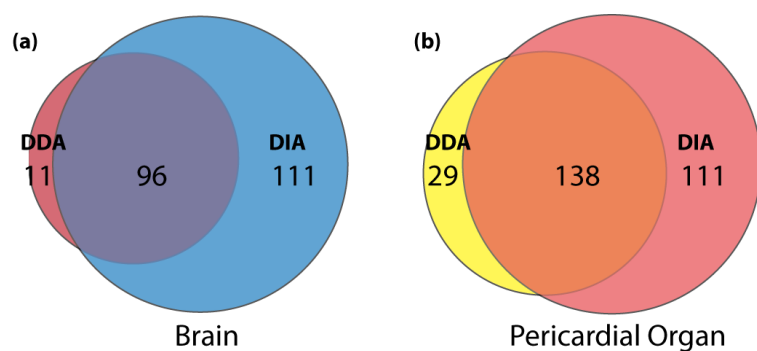


Figure 3. Overlap of neuropeptides detected using data dependent acquisition (DDA) and data independent acquisition (DIA) for (a) brain and (b) pericardial organ (PO) tissue extracts.

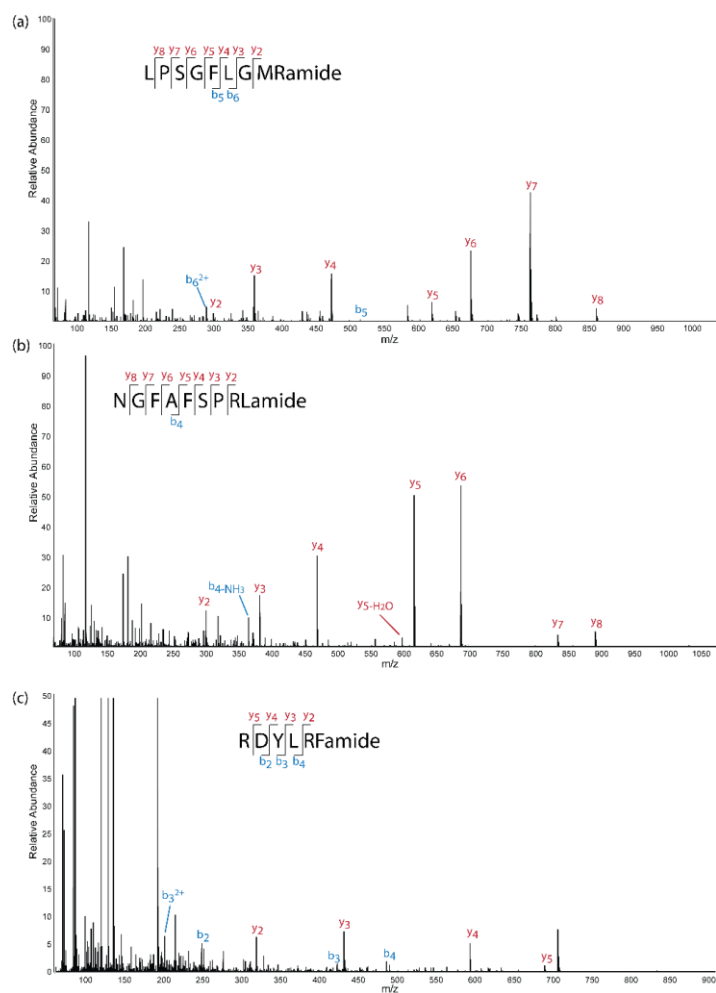


Figure 4. MS/MS spectra of three putative neuropeptides detected with the data independent acquisition (DIA) method, including (a) a tachykinin neuropeptide detected in the brain, (b) a pyrokinin neuropeptide detected in the brain, and (c) an RFamide neuropeptide detected in the pericardial organ.

Methods Supporting Information

Animals and tissue collection

C. borealis (Jonah crab) were purchased from the Fresh Lobster Company, LLC (Gloucester, MA, USA). The animals were housed in artificial seawater tanks kept at 12-13 °C with an alternating 12-hour light/dark cycle, and they were allowed to acclimate to their environment for a minimum of a week prior to tissue collection. For tissue collection, animals were cold-anesthetized in ice for 30 minutes and dissected in chilled physiological saline (440 mM NaCl, 11 mM KCl, 26 mM MgCl₂, 13 mM CaCl₂, 11 mM Trizma base, 5 mM maleic acid, adjusted to pH 7.45 with NaOH) as described previously.²⁶ Brains, pericardial organs (POs), and thoracic ganglia (TG) were collected and stored in extraction solvent (acidified methanol). For method optimization experiments, a total of 21 brains and 21 pairs of POs were collected. For the biological variability experiment, 3 brains were collected on the same day from animals of the

same shipment. For the quantitative accuracy experiment, 15 thoracic ganglia (TGs) were collected.

Sample preparation

Tissues were pooled for each respective experiment (with the exception of the biological variability experiment) and extracted for neuropeptides using chilled acidified methanol. Each sample was manually homogenized with 50 μ L acidified methanol per tissue using a glass homogenizer, sonicated for 5 minutes in a bath sonicator, and then centrifuged for 15 minutes at 16.1 rcf. The resulting supernatant was separated, 50 μ L of acidified methanol was added to the pellet, and the pellet was homogenized, sonicated, and centrifuged as previously stated. The supernatant was again removed, and the process repeated. The supernatants from the three extractions were combined and evaporated in a speedvac on medium heat.

Samples were reconstituted in 45 μ L of 0.1% formic acid (FA) in water and divided into three aliquots of 15 μ L. Each aliquot was desalted with 10 μ L C18 ziptips following package instructions, except that three elution solutions were used, 25/75 acetonitrile (ACN)/water with 0.1% FA, 50/50 ACN/water with 0.1% FA, and 75/25 ACN/water with 0.1% FA. After desalting, the elution solutions from all 3 aliquots were combined. For quantitative analysis, the TG sample was aliquoted into 5 tubes after desalting such that the volume ratios were 0.5:1:1:2:5. All samples were then evaporated in the speedvac on medium heat.

Table S1. Putative sequences of neuropeptides detected in brain with *de novo* sequencing. The score represents the average local confidence (ALC), with the cutoff threshold being 75%. * indicates peptide has a similar sequence to neuropeptides belonging to the respective family but does not follow the characteristic sequence motif.

Family	Sequence	Molecular Weight	Score
Orcomyotropin	FDAFTTGFGHE	1227.52	98
Orcokinin	QYDELDRSGFGFA*	1503.66	78
	QYDELDRSGFGFV*	1531.69	76
Pyrokinin	SYFAPRLamide	851.46	99
	EPFAFSPRLamide	1061.57	90
	NGFAFSPRLamide	1006.54	99
	TNFAFAPRPamide*	1018.54	93
	DCFAFSPRLamide	1053.51	97
	DTFAFSPRLamide	1051.55	81
RFamide	APPSMRLRFamide	1072.60	95
	SENDRFLRFamide	1181.60	99
	HHDRNFLRFamide	1239.64	98
	KENRNFLRFamide	1221.68	91
	AESDRNFLRFamide	1252.63	75
	LKNYLRFamide	951.57	98

	SPLNYLRFamide	1007.56	75
	EEDRNFLRFamide	1223.61	87
	RKDRNFLRFamide	1249.72	98
	CTSKNYLRFamide	1129.57	79
	EYDRNFLRFamide	1257.63	93
	LALKNYLRFamide	1135.69	80
	NSSKNYLRFamide	1126.59	87
	QKNYLRFamide	966.54	98
	THDRNFLRFamide	1203.63	97
	NRNFLRFDR*	1236.65	97
	NRNFLRFTR*	1222.67	97
Tachykinin	APSGFLGYR*	966.49	96
	DPSGFLGMRamide	977.48	96
	QLSGFLGMRamide	1006.54	88
	APEGFLGMRamide	975.50	97
	APSGFLGRY*	966.49	96
	EPSGFLGMRamide	991.49	97
	LPSGFLGMRamide	975.54	99
	YSSGFLGMRamide	1015.49	97
	KPSGFLGMRamide	990.55	86
	SPEGFLGMRamide	991.49	85
	TAEGFLGMRamide	979.49	83
YRamide	MLGSLYRamide	837.46	99

Table S2. Putative sequences of neuropeptides detected in PO with *de novo* sequencing. The score represents the average local confidence (ALC), with the cutoff threshold being 75%. * indicates peptide has a similar sequence to neuropeptides belonging to the respective family but does not follow the characteristic sequence motif.

Family	Sequence	Molecular Weight	Score
AST-B	KPQGSWamide*	700.37	80
	DNNWSKFQGSWamide	1366.61	87
RFamide	FRGNRNFLRFamide	1324.73	85
	GCNFLRFamide	854.43	78
	HDVSHVFLRFamide	1254.67	82
	KYYLRFamide	887.51	98
	MTERNFLRFamide	1211.63	80
	QNYLRFamide	838.45	96
	STNYLRFamide	898.47	81
	NRNFLRFG*	1022.54	99
	SENRDFLRFamide	1181.60	98
	YDRSFLRFamide	1101.58	95
	EDFLRFamide	824.42	92
	FSNFLRFamide	928.50	87
	TDLDHVFLRFamide	1260.66	80
	DRDFLRFamide	966.51	99
	RDYLRamide	867.47	99

	SKDYLRFamide	926.50	98
	FDRNFLRFamide	1112.59	97
	FNRNFLRFamide	1111.61	98
	HVNFLRFamide	930.52	81
	KTSKNYLRamide	1154.66	88
	MTNFLRFamide	926.48	82
	NHKNYLRamide	1089.59	99
	YDDMNYLRamide	1234.55	77
	GKNYLRamide	895.51	98
	HNYLRamide	847.45	97
	YNYLRamide	873.45	98
RYamide	QAFYSQRYamide	1060.51	88
	QFYADRYamide	960.45	92
	MFYANRY*	963.43	83

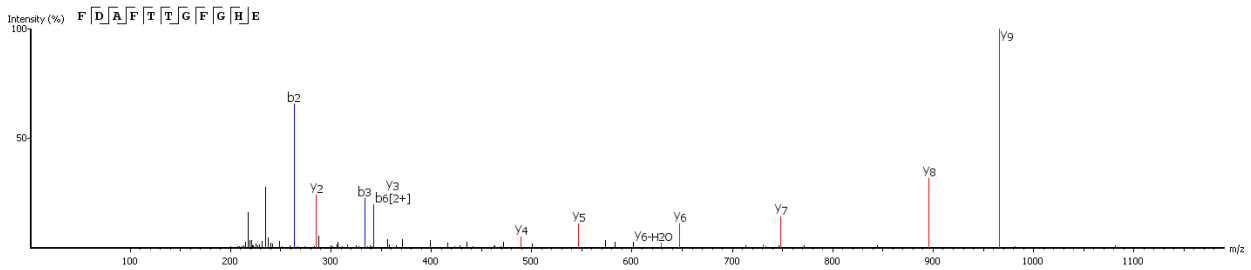


Figure S1. MS/MS spectrum from PEAKS software of a putative orcomyotropin neuropeptide with the sequence FDAFTTGFGHE detected in brain tissue extract with the data independent acquisition (DIA) method.

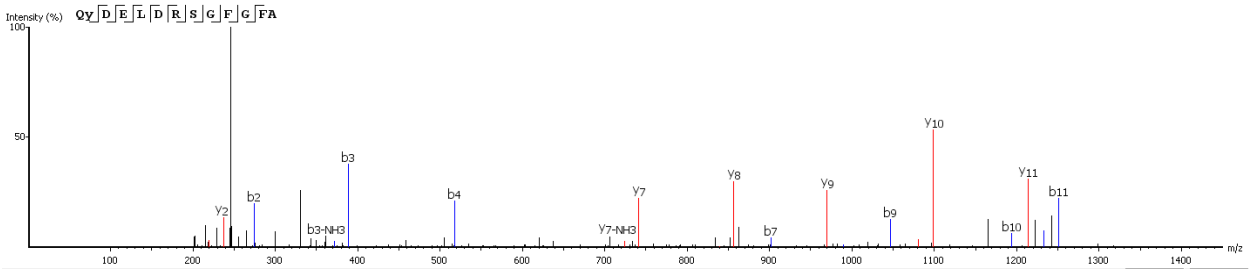


Figure S2. MS/MS spectrum from PEAKS software of a putative orcokinin-related neuropeptide with the sequence QYDEL/IDRSGFGFA detected in brain tissue extract with the data independent acquisition (DIA) method.

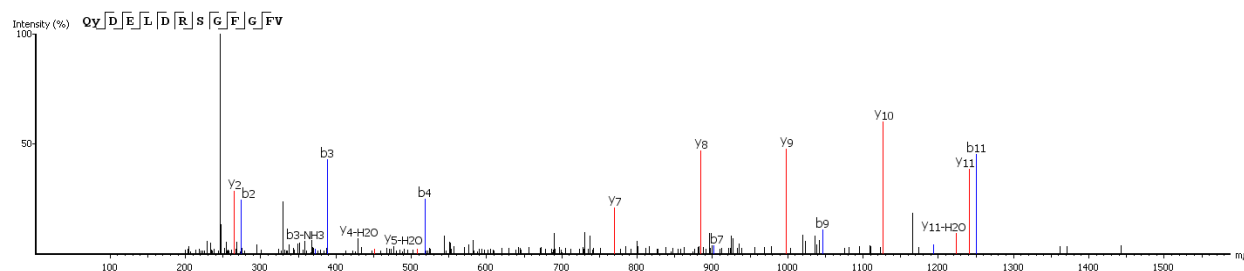


Figure S3. MS/MS spectrum from PEAKS software of a putative orckinin-related neuropeptide with the sequence QY(-H₂O)DELDRSGFGFV detected in brain tissue extract with the data independent acquisition (DIA) method.

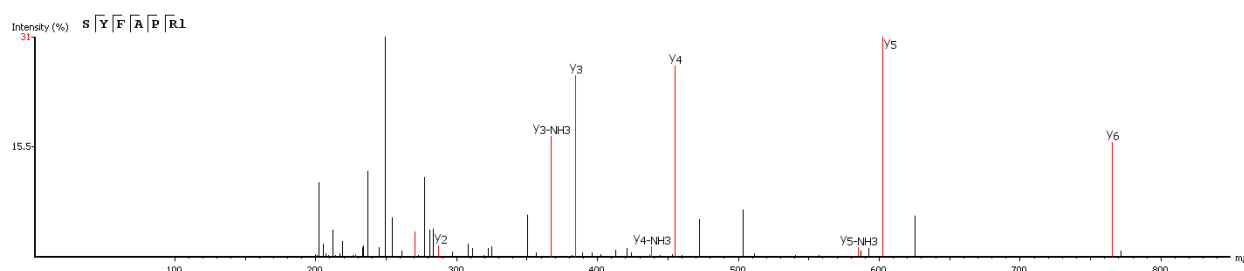


Figure S4. MS/MS spectrum from PEAKS software of a putative pyrokinin neuropeptide with the sequence SYFAPRLamide detected in brain tissue extract with the data independent acquisition (DIA) method.

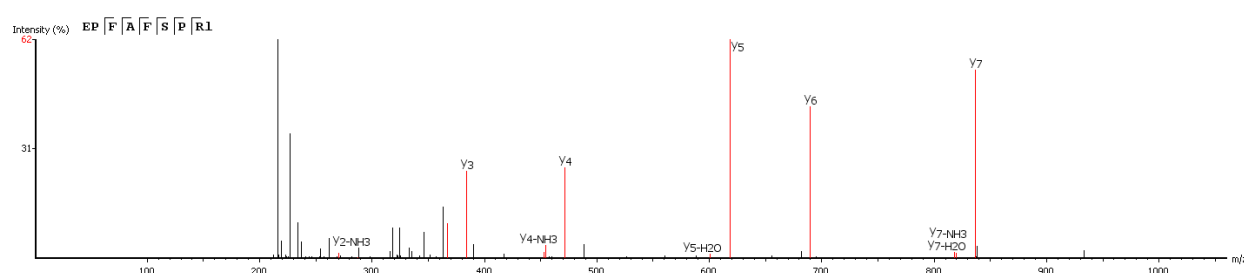


Figure S5. MS/MS spectrum from PEAKS software of a putative pyrokinin neuropeptide with the sequence EPFAFSPRLamide detected in brain tissue extract with the data independent acquisition (DIA) method.

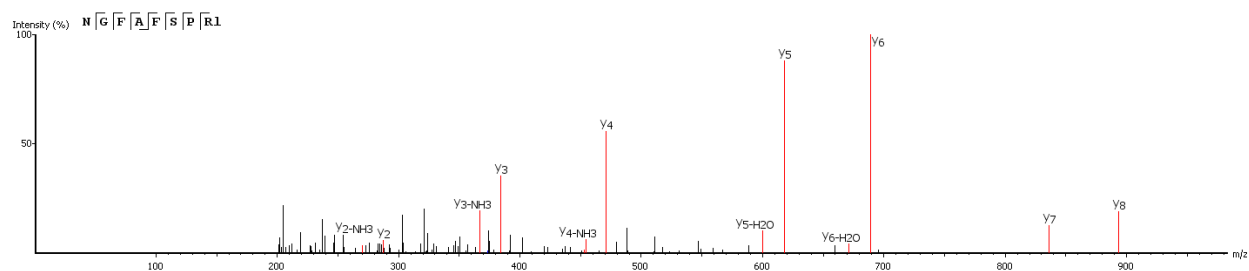


Figure S6. MS/MS spectrum from PEAKS software of a putative pyrokinin neuropeptide with the sequence NGFAFSPRLamide detected in brain tissue extract with the data independent acquisition (DIA) method.

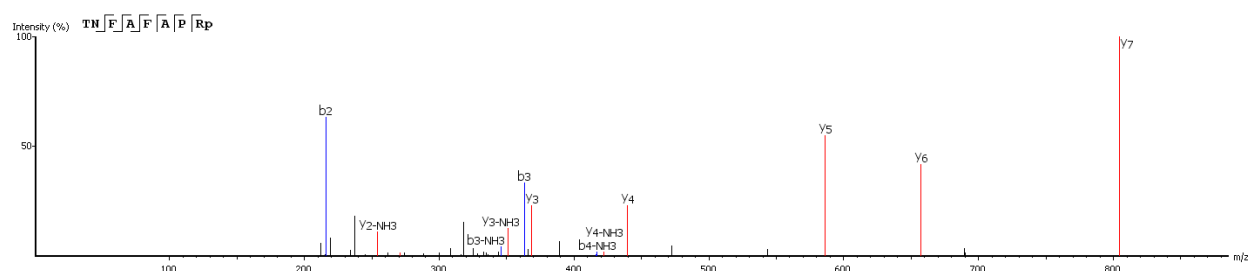


Figure S7. MS/MS spectrum from PEAKS software of a putative pyrokinin-related neuropeptide with the sequence TNFAFAPRPamide detected in brain tissue extract with the data independent acquisition (DIA) method.

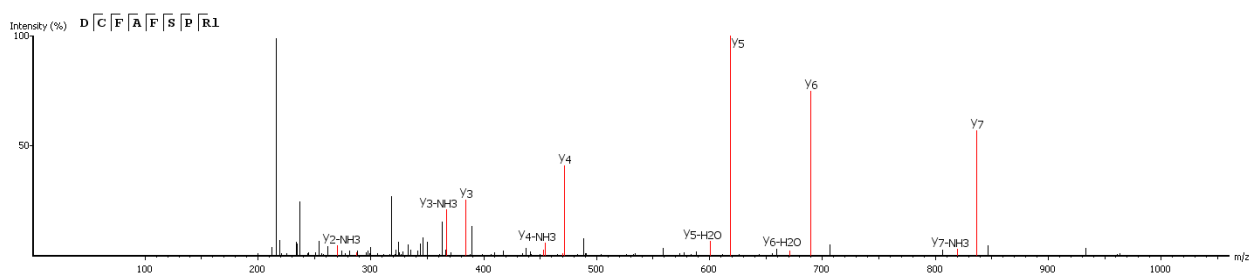


Figure S8. MS/MS spectrum from PEAKS software of a putative pyrokinin neuropeptide with the sequence DCFAFSPRLamide detected in brain tissue extract with the data independent acquisition (DIA) method.

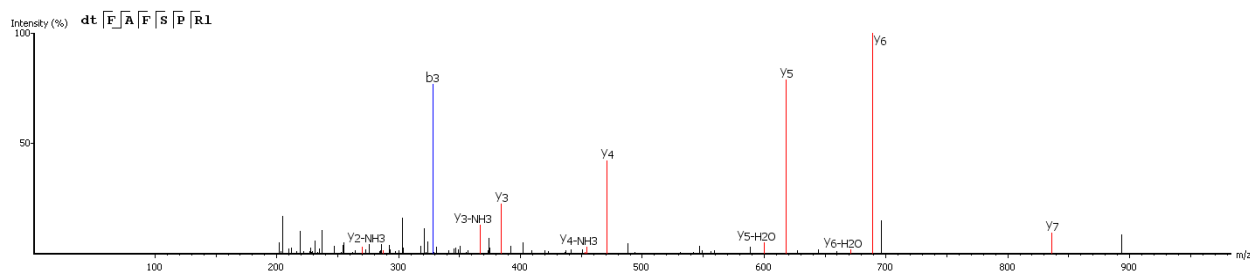


Figure S9. MS/MS spectrum from PEAKS software of a putative pyrokinin neuropeptide with the sequence D(-H₂O)T(-H₂O)FAFSPRLamide detected in brain tissue extract with the data independent acquisition (DIA) method.

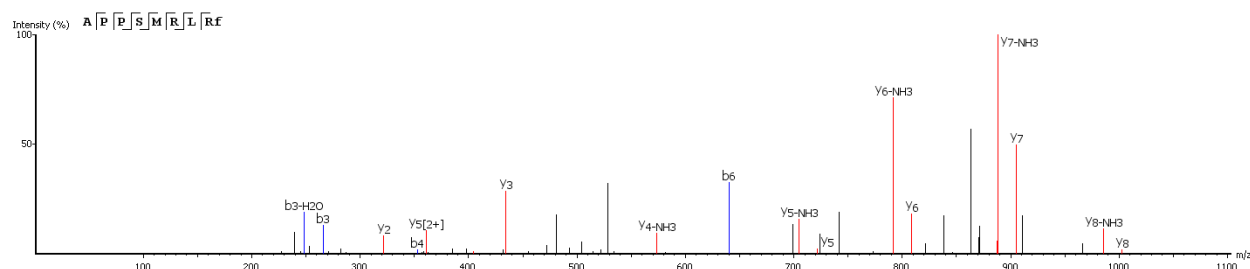


Figure S10. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence APPSMRLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

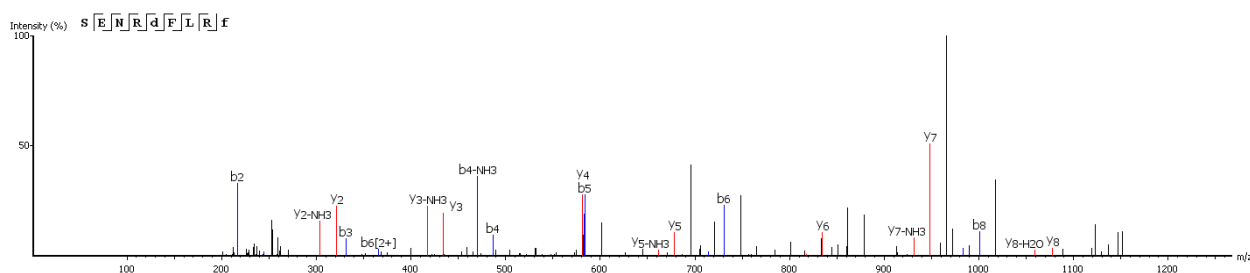


Figure S11. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SENRD(-H₂O)FLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

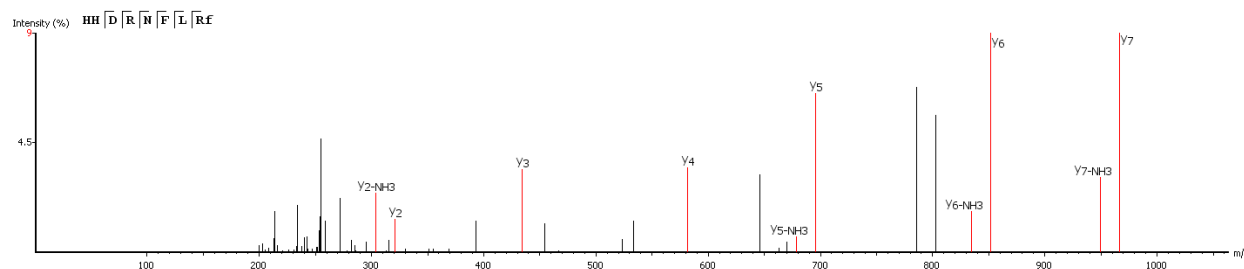


Figure S12. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence HHDRNFLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

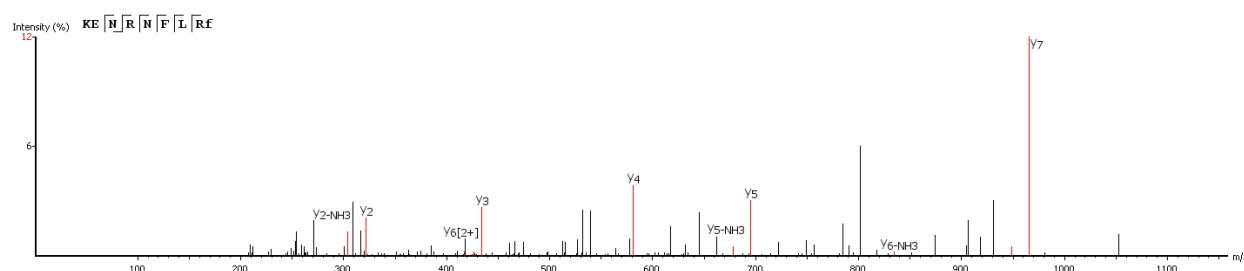


Figure S13. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence KENRNFLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

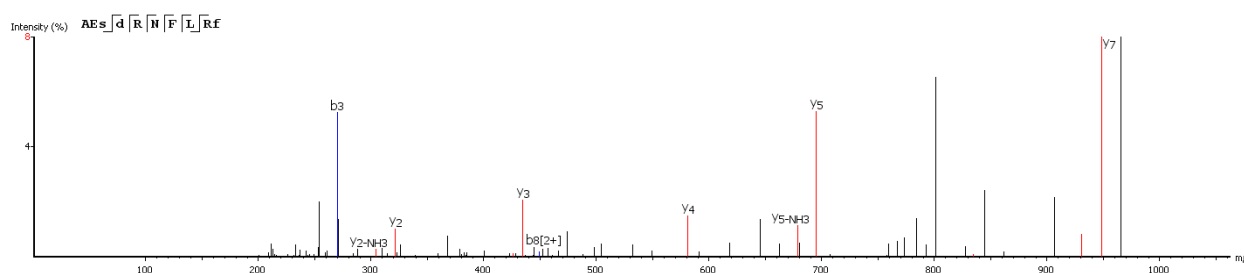


Figure S14. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence AES(-H₂O)D(-H₂O)RNFLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

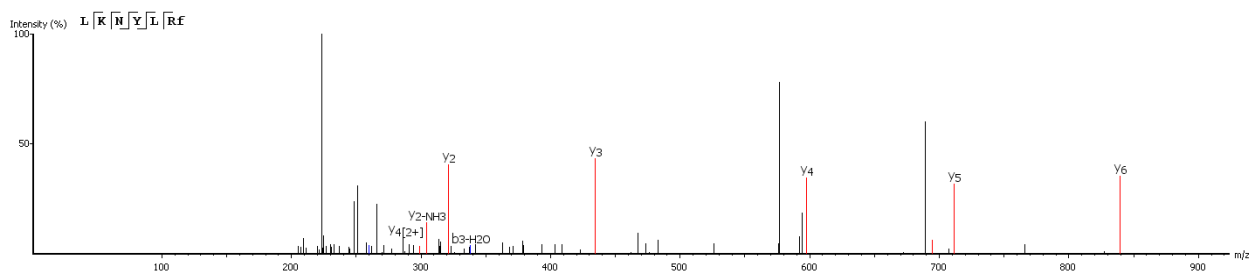


Figure S15. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence LKNYLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

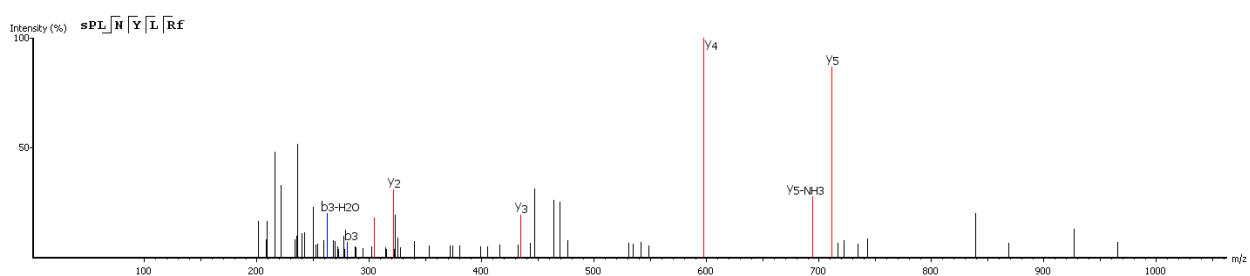


Figure S16. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence S(-H₂O)PLNYLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

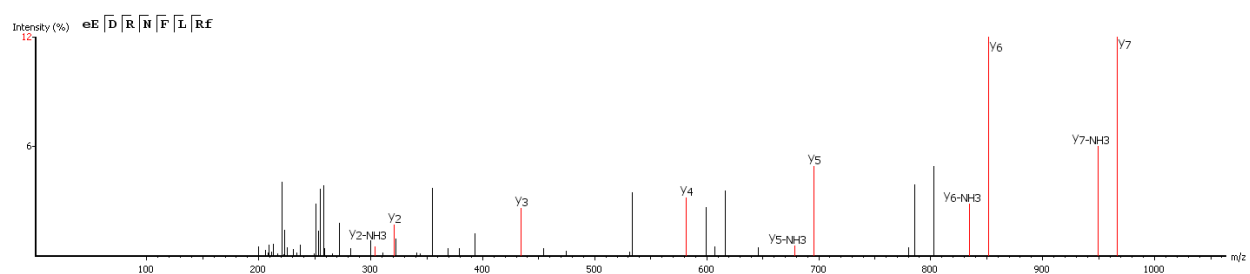


Figure S17. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence E(-H₂O)EDRNFLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

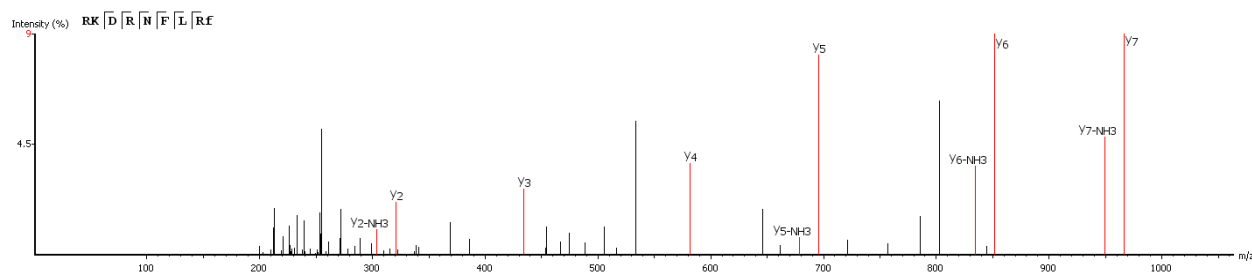


Figure S18. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence RKDRNFLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

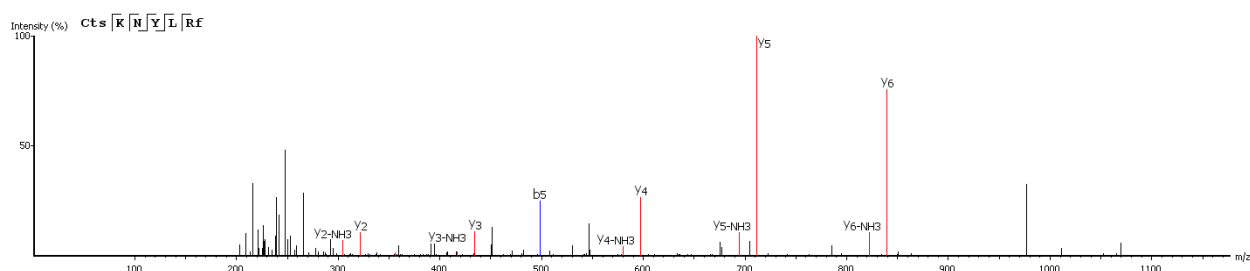


Figure S19. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence CT(-H₂O)S(-H₂O)KNYLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

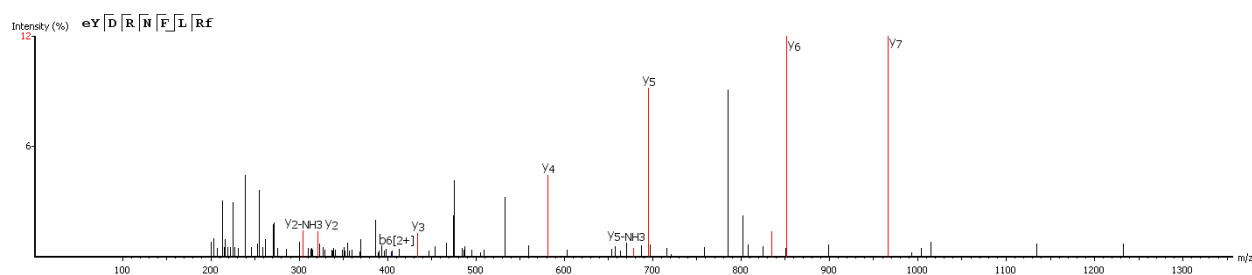


Figure S20. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence E(-H₂O)YDRNFLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

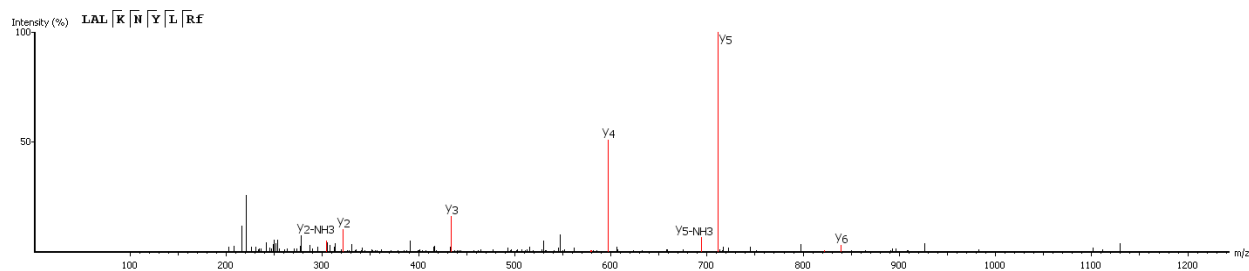


Figure S21. MS/MS spectrum from PEAKS software of a putative Rfamamide neuropeptide with the sequence LALKNYLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

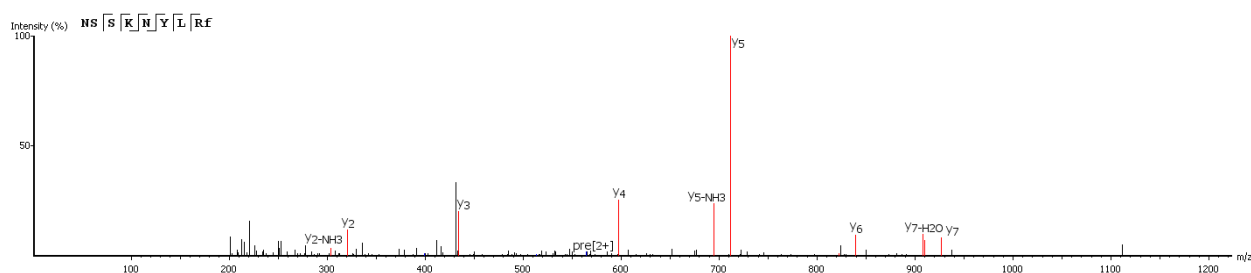


Figure S22. MS/MS spectrum from PEAKS software of a putative Rfamamide neuropeptide with the sequence NSSKNYLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

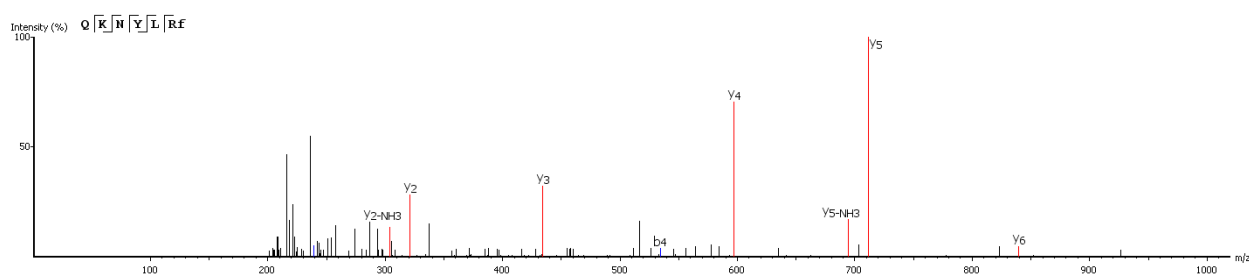


Figure S23. MS/MS spectrum from PEAKS software of a putative Rfamamide neuropeptide with the sequence QKNYLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

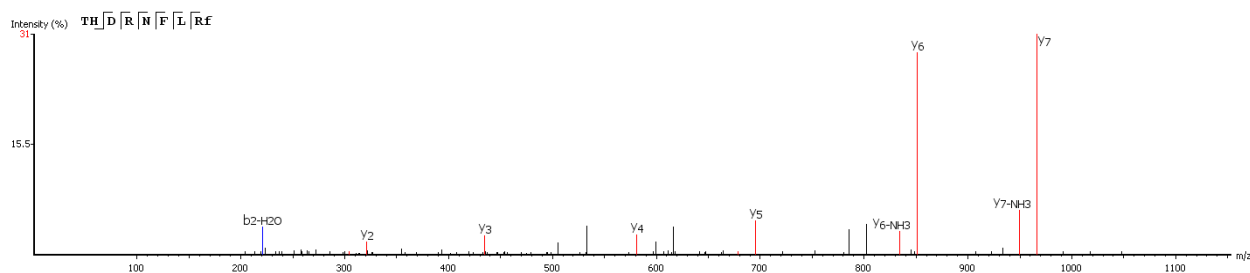


Figure S24. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence THDRNFLRFamide detected in brain tissue extract with the data independent acquisition (DIA) method.

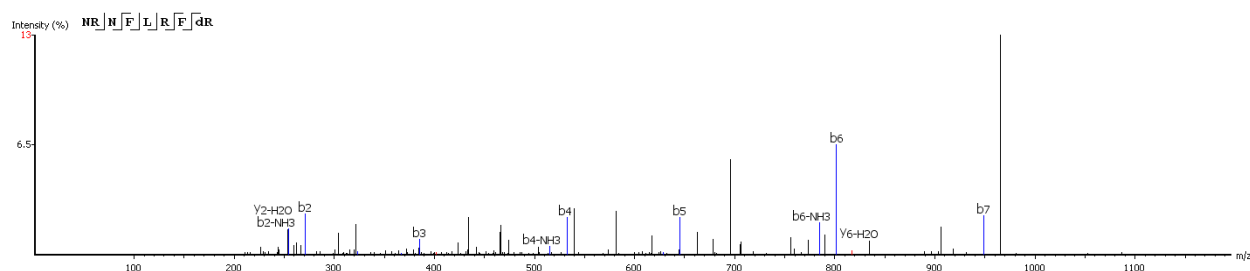


Figure S25. MS/MS spectrum from PEAKS software of a putative RFamide-related neuropeptide with the sequence NRNFLRFD(-H₂O)R detected in brain tissue extract with the data independent acquisition (DIA) method.

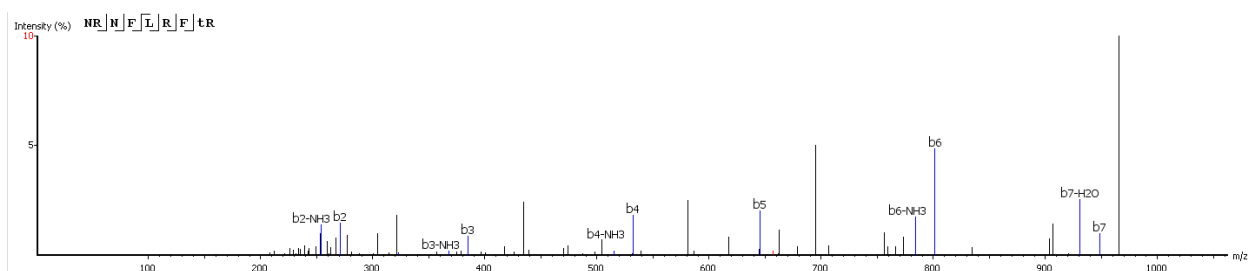


Figure S26. MS/MS spectrum from PEAKS software of a putative RFamide-related neuropeptide with the sequence NRNFLRFT(-H₂O)R detected in brain tissue extract with the data independent acquisition (DIA) method.

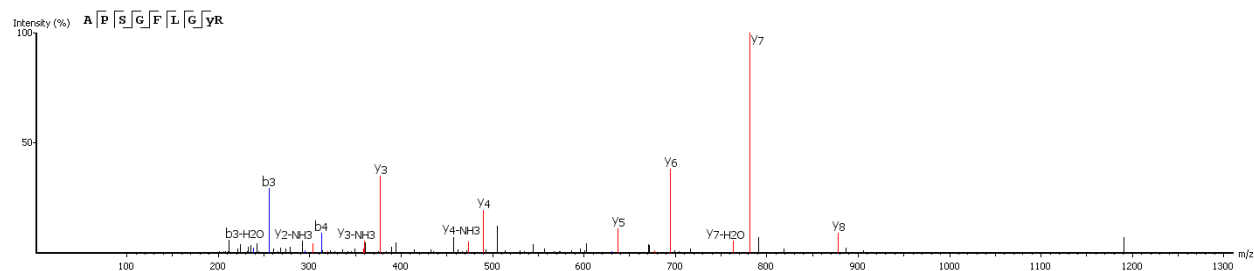


Figure S27. MS/MS spectrum from PEAKS software of a putative tachykinin-related neuropeptide with the sequence APSGFLGY(-H₂O)R detected in brain tissue extract with the data independent acquisition (DIA) method.

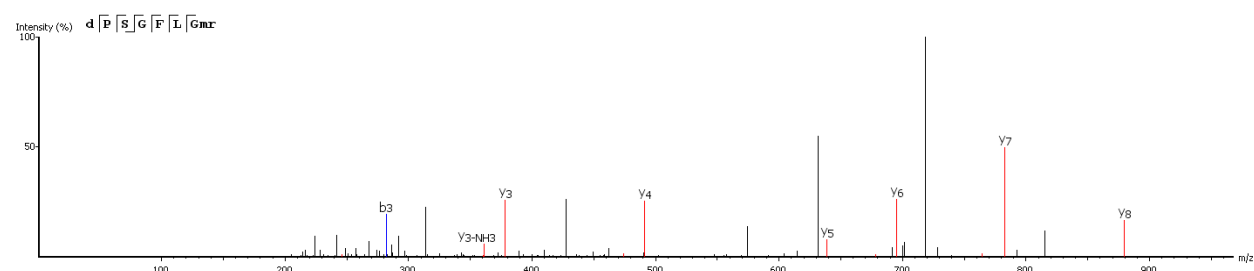


Figure S28. MS/MS spectrum from PEAKS software of a putative tachykinin neuropeptide with the sequence D(-H₂O)PSGFLGM(oxidation)Ramide detected in brain tissue extract with the data independent acquisition (DIA) method.

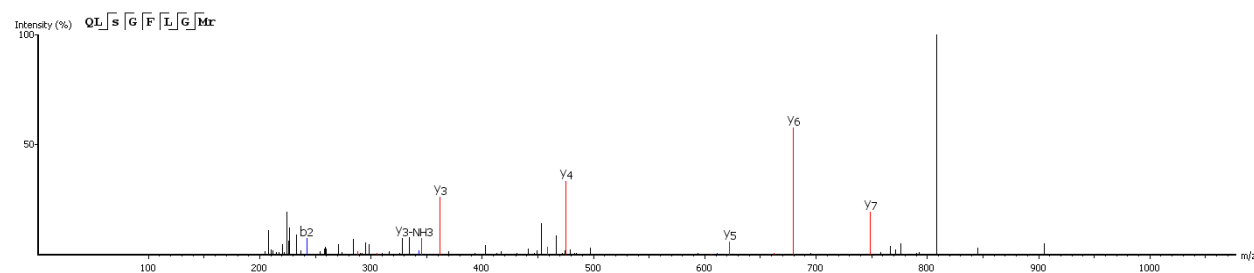


Figure S29. MS/MS spectrum from PEAKS software of a putative tachykinin neuropeptide with the sequence QLS(-H₂O)GFLGMRamide detected in brain tissue extract with the data independent acquisition (DIA) method.

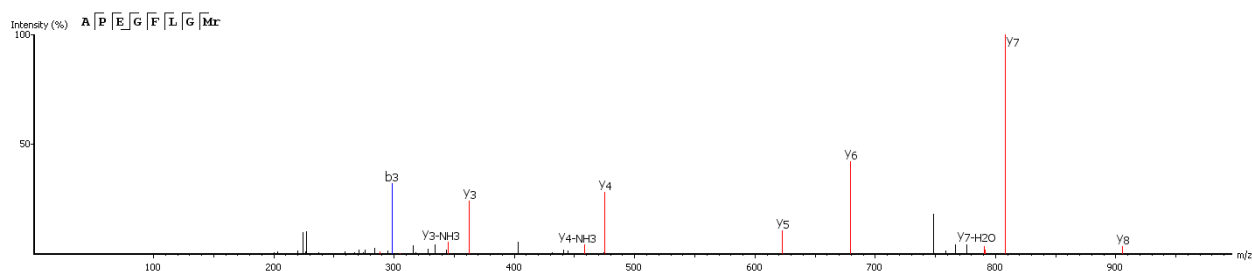


Figure S30. MS/MS spectrum from PEAKS software of a putative tachykinin neuropeptide with the sequence APEGFLGMRamide detected in brain tissue extract with the data independent acquisition (DIA) method.

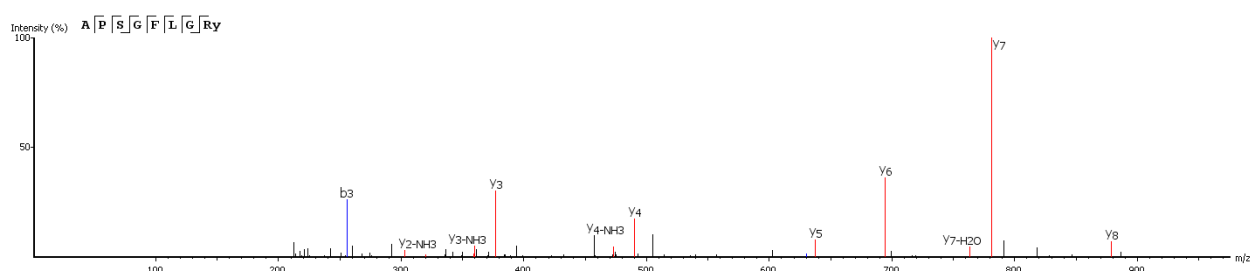


Figure S31. MS/MS spectrum from PEAKS software of a putative tachykinin-related neuropeptide with the sequence APSGFLGRY(-H₂O) detected in brain tissue extract with the data independent acquisition (DIA) method.

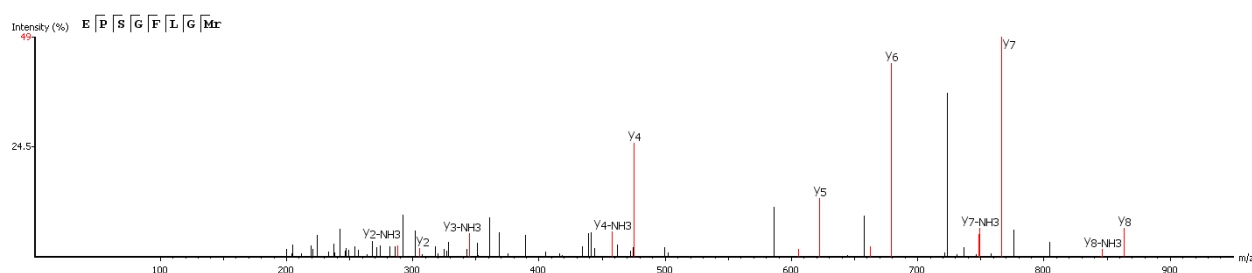


Figure S32. MS/MS spectrum from PEAKS software of a putative tachykinin neuropeptide with the sequence EPSGFLGMRamide detected in brain tissue extract with the data independent acquisition (DIA) method.

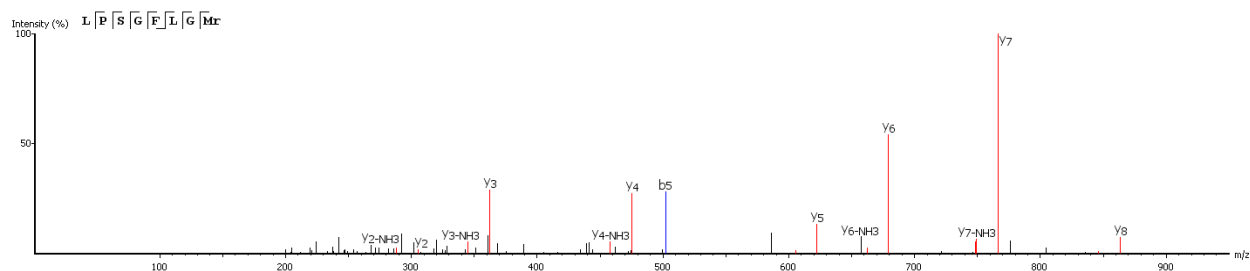


Figure S33. MS/MS spectrum from PEAKS software of a putative tachykinin neuropeptide with the sequence LPSGFLGMRamide detected in brain tissue extract with the data independent acquisition (DIA) method.

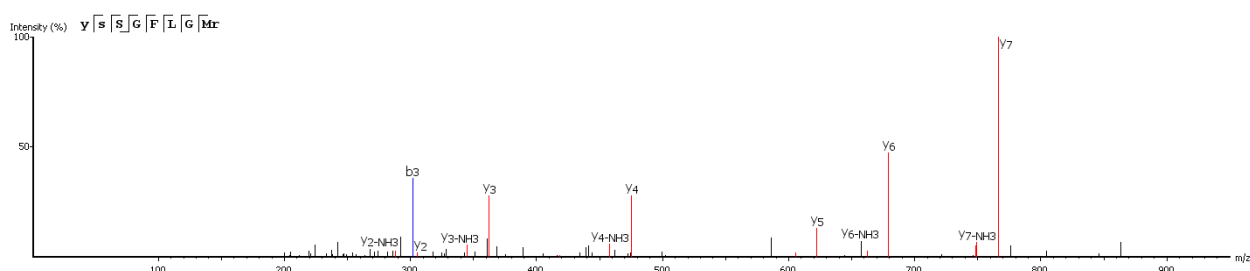


Figure S34. MS/MS spectrum from PEAKS software of a putative tachykinin neuropeptide with the sequence Y(-H₂O)S(-H₂O)SGFLGMRamide detected in brain tissue extract with the data independent acquisition (DIA) method.

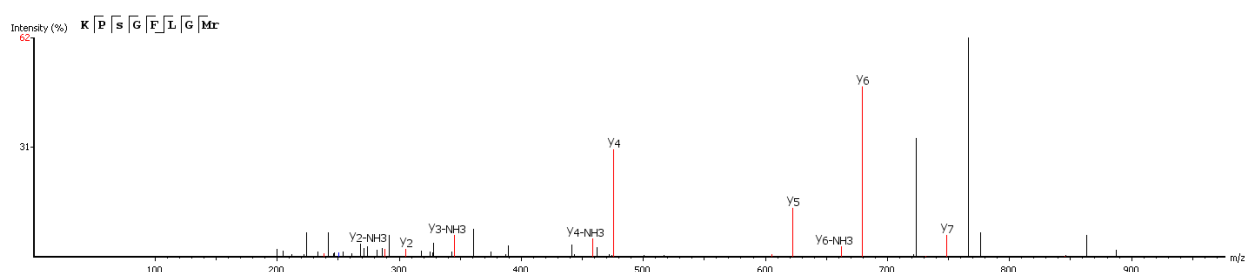


Figure S35. MS/MS spectrum from PEAKS software of a putative tachykinin neuropeptide with the sequence KPS(-H₂O)GFLGMRamide detected in brain tissue extract with the data independent acquisition (DIA) method.

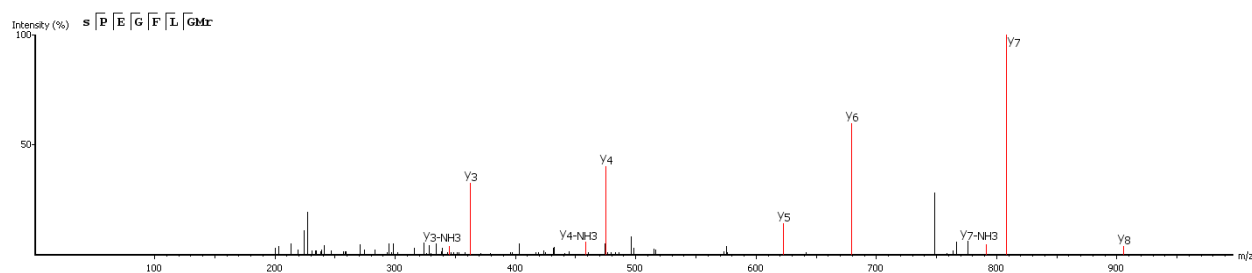


Figure S36. MS/MS spectrum from PEAKS software of a putative tachykinin neuropeptide with the sequence S(-H₂O)PEGFLGMRamide detected in brain tissue extract with the data independent acquisition (DIA) method.

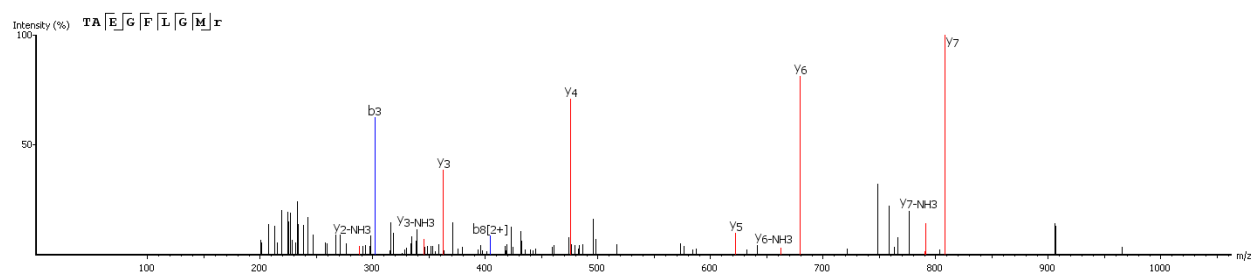


Figure S37. MS/MS spectrum from PEAKS software of a putative tachykinin neuropeptide with the sequence TAEGFLGMRamide detected in brain tissue extract with the data independent acquisition (DIA) method.

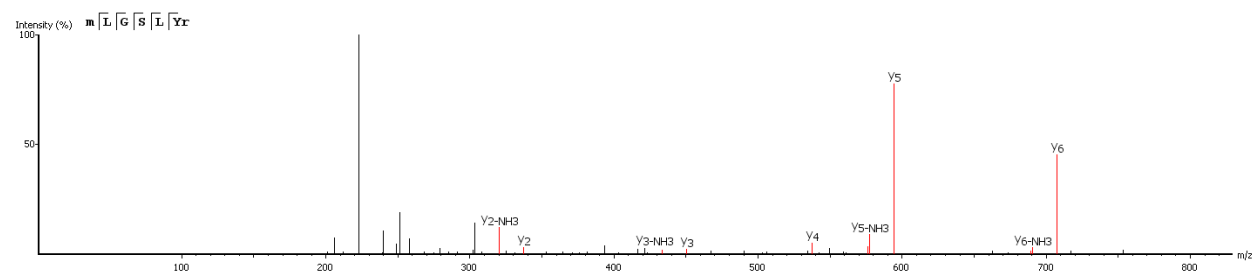


Figure S38. MS/MS spectrum from PEAKS software of a putative YRamide neuropeptide with the sequence M(oxidation)LGSLYRamide detected in brain tissue extract with the data independent acquisition (DIA) method.

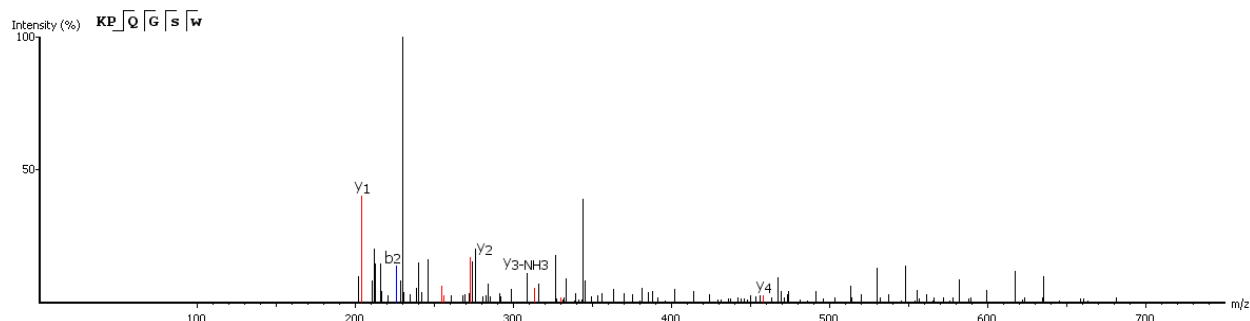


Figure S39. MS/MS spectrum from PEAKS software of a putative B-type allatostatin-related neuropeptide with the sequence KPGGS(-H₂O)Wamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

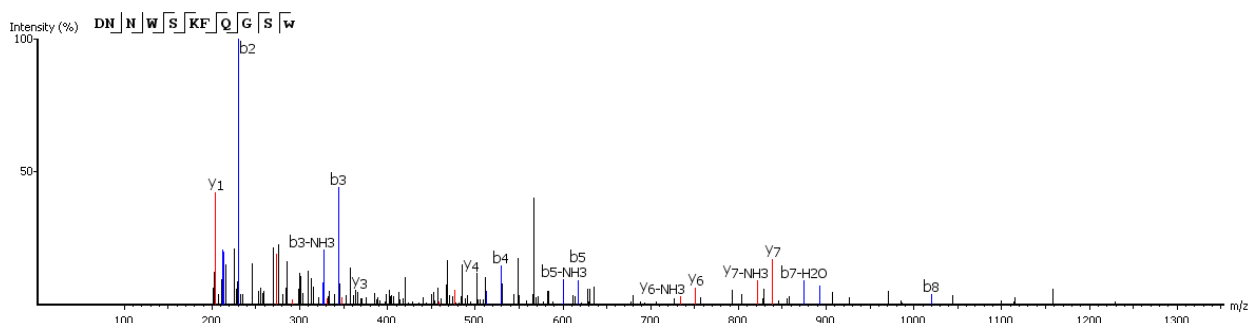


Figure S40. MS/MS spectrum from PEAKS software of a putative B-type allatostatin neuropeptide with the sequence DNNWSKFQGSWamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

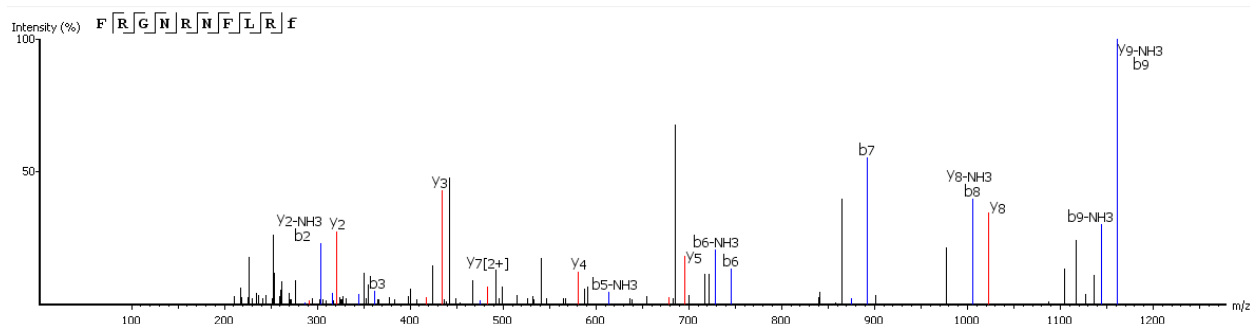


Figure S41. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence FRGNRNFLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

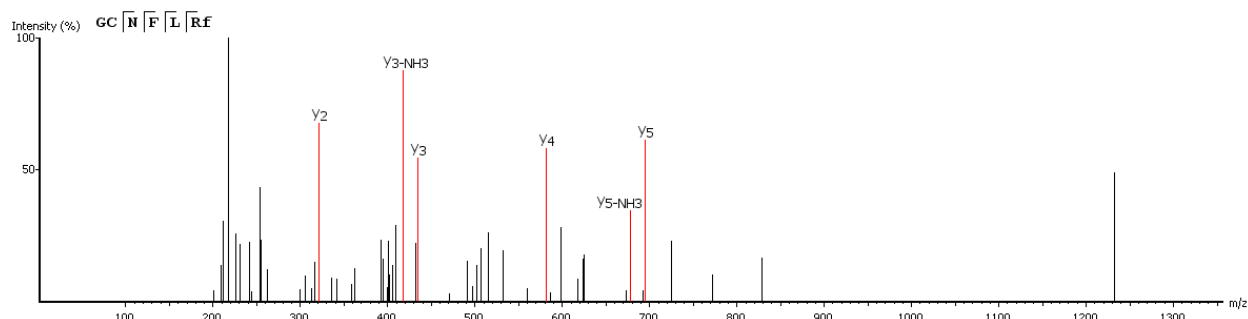


Figure S42. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence GCNFLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

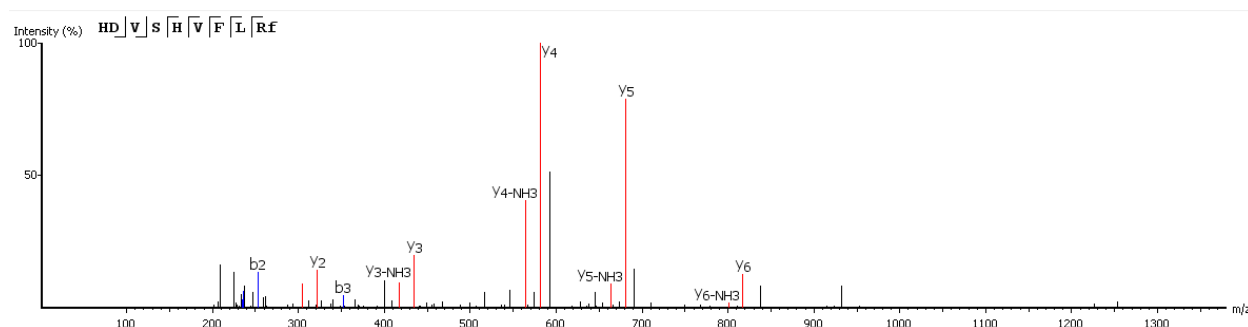


Figure S43. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence HDVSHVFLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

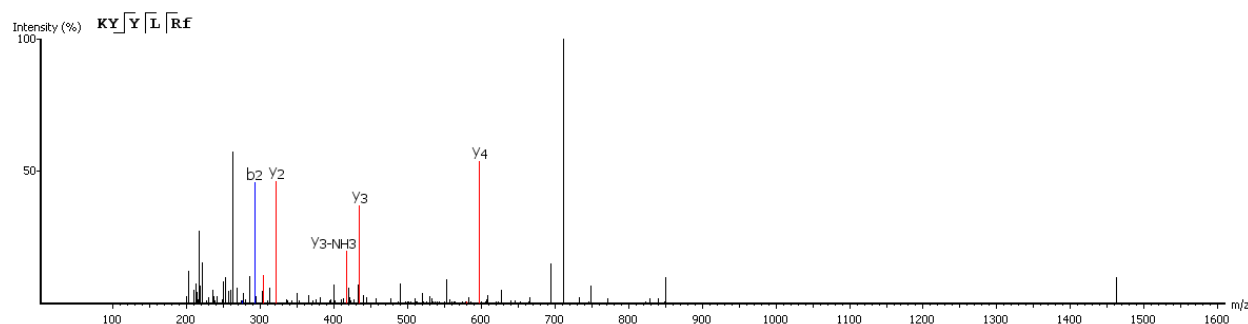


Figure S44. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence KYYLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

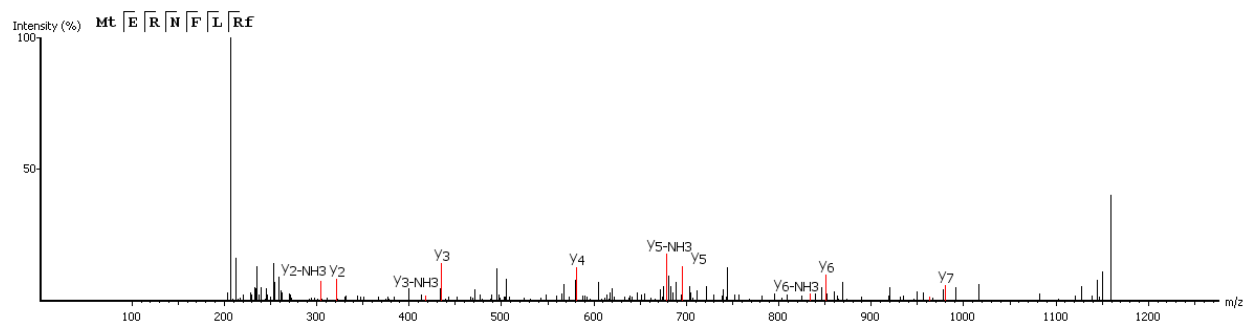


Figure S45. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence MT(-H₂O)ERNFLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

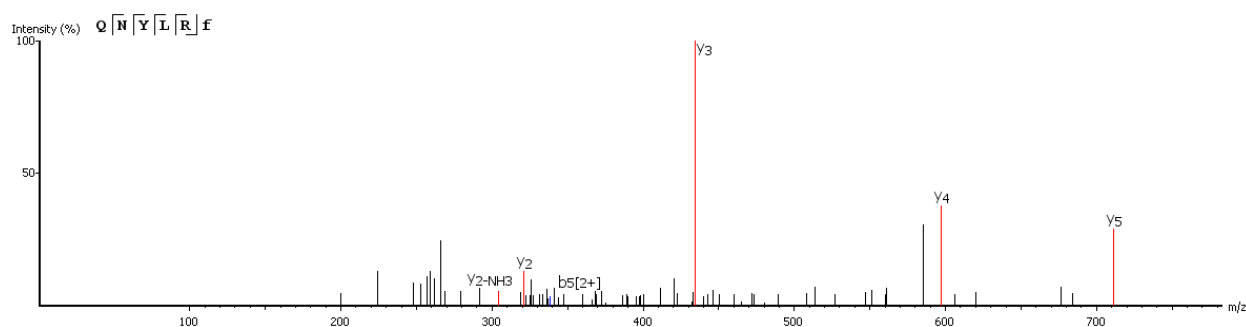


Figure S46. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence QNYLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

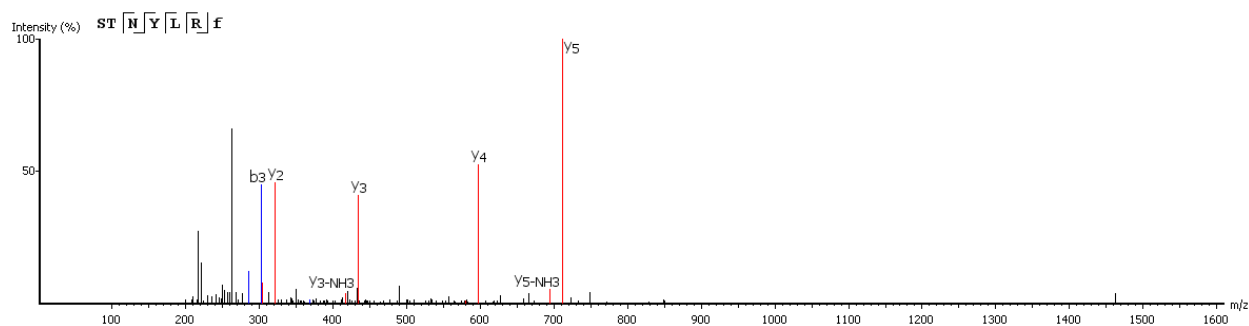


Figure S47. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence STNYLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

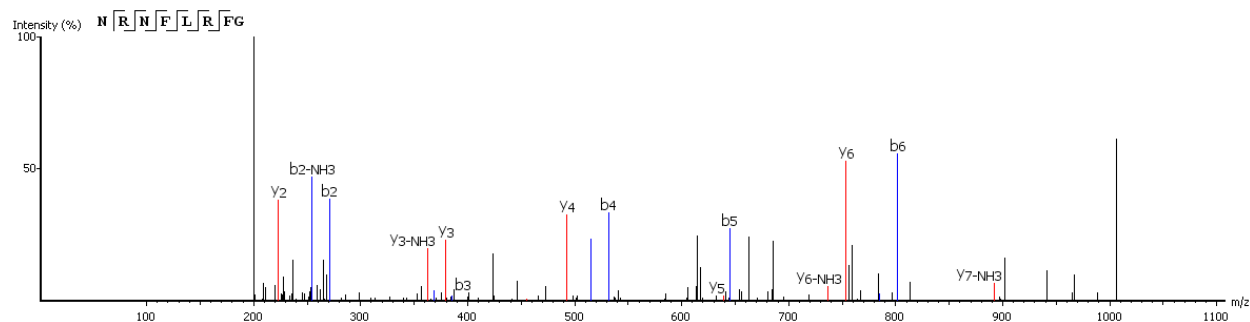


Figure S48. MS/MS spectrum from PEAKS software of a putative RFamide-related neuropeptide with the sequence NRNFLRFG detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

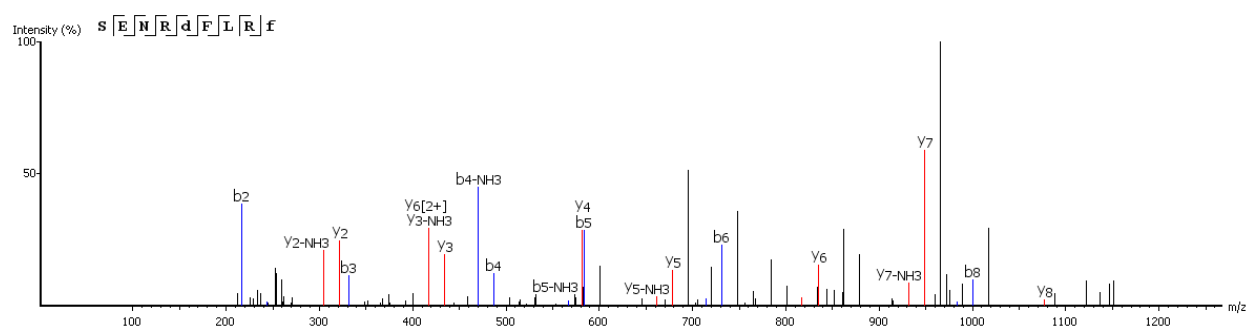


Figure S49. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SENRD(-H₂O)FLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

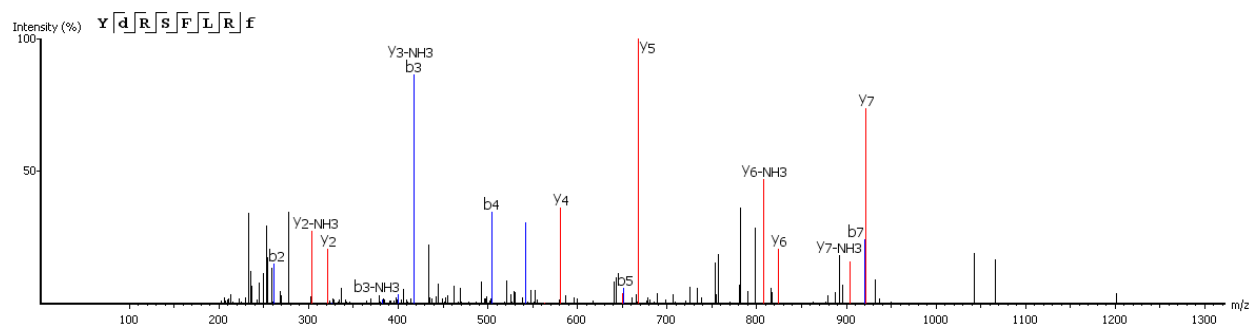


Figure S50. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence YD(-H₂O)RSFLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

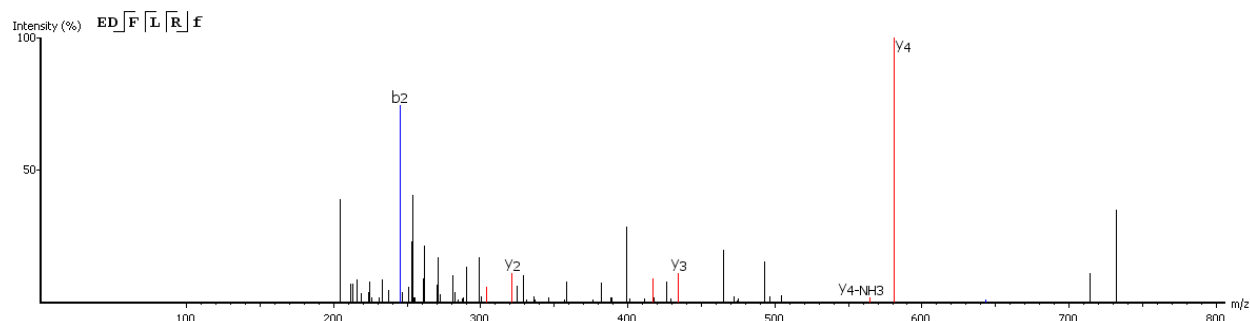


Figure S51. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence EDFLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

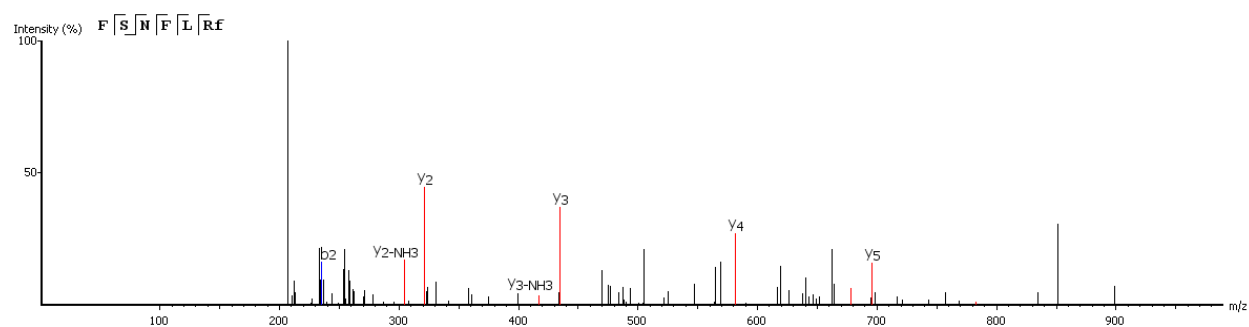


Figure S52. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence FSNFLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

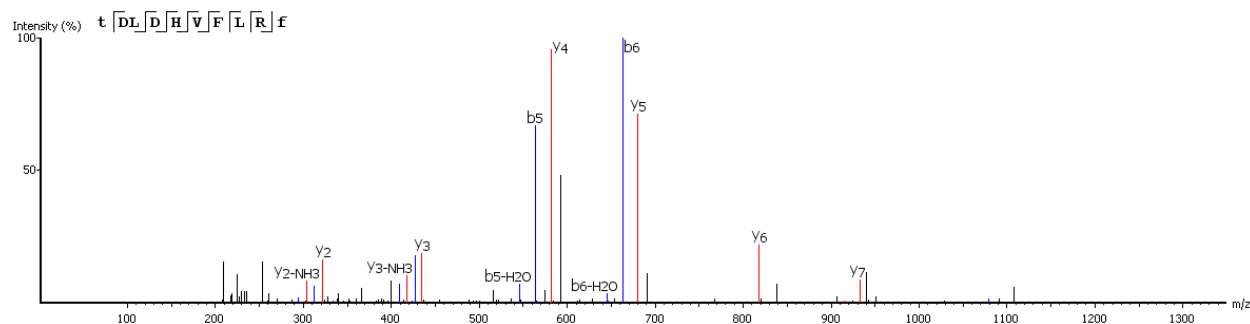


Figure S53. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence T(-H₂O)DLDHVFLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

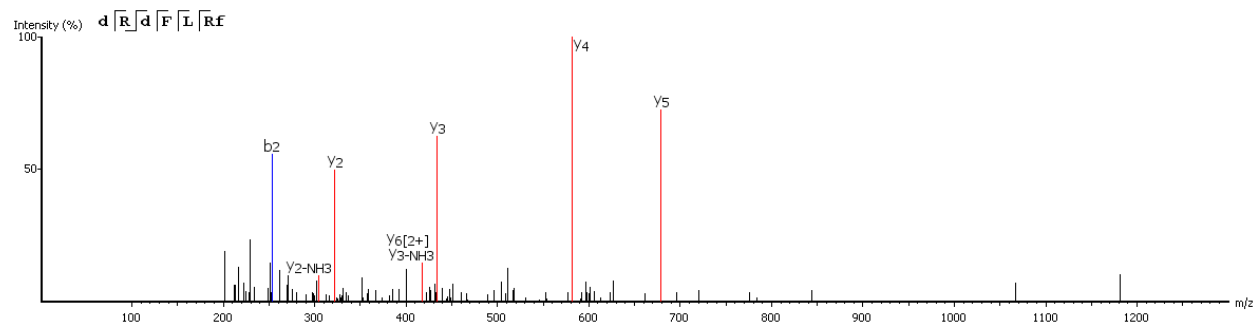


Figure S54. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence D(-H₂O)RD(-H₂O)FLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

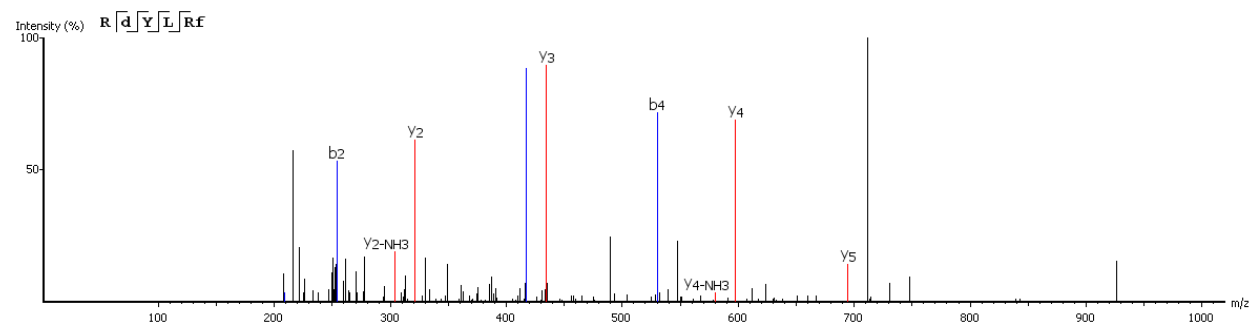


Figure S55. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence RD(-H₂O)YLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

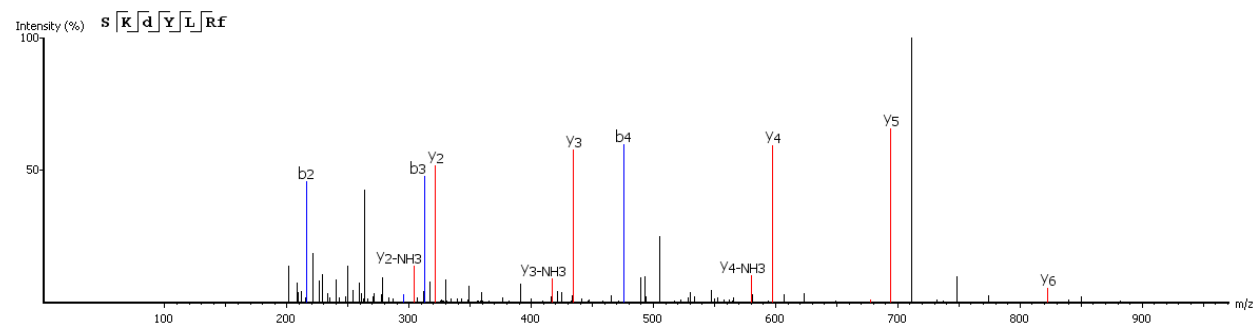


Figure S56. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SKD(-H₂O)YLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

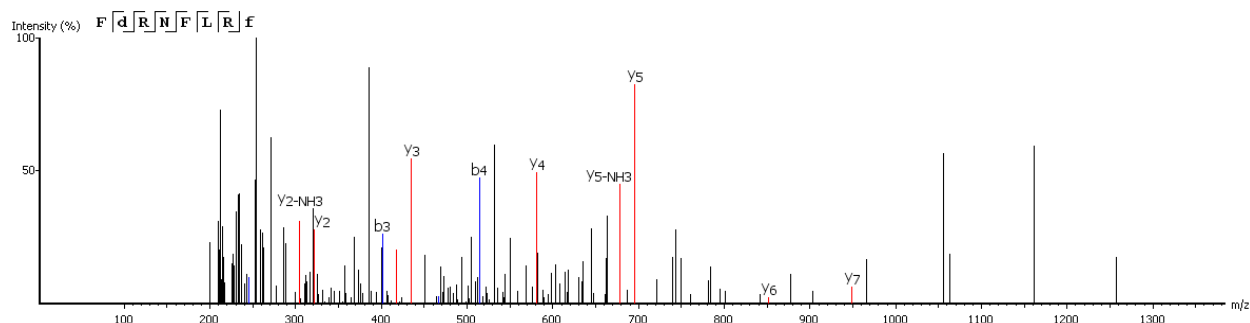


Figure S57. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence FD(-H₂O)RNFLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

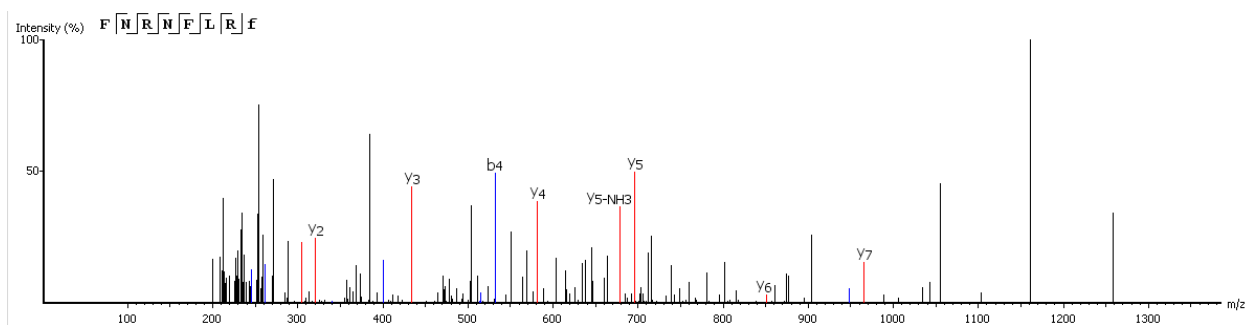


Figure S58. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence FNRNFLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

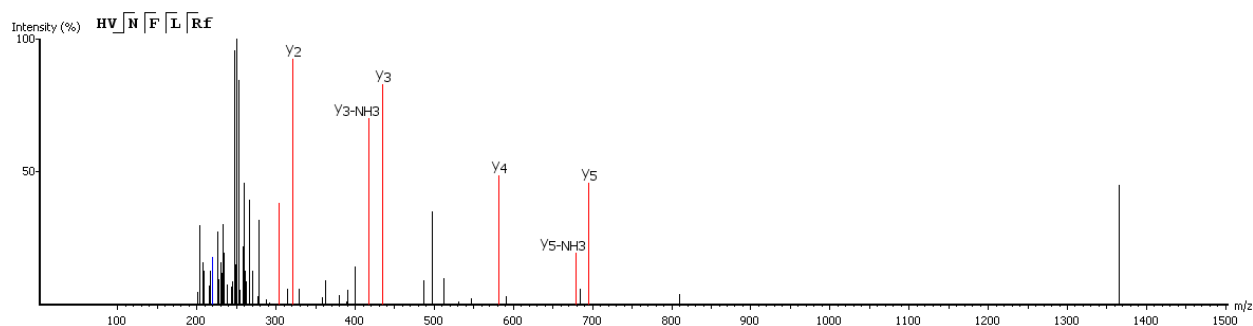


Figure S59. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence HVNFLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

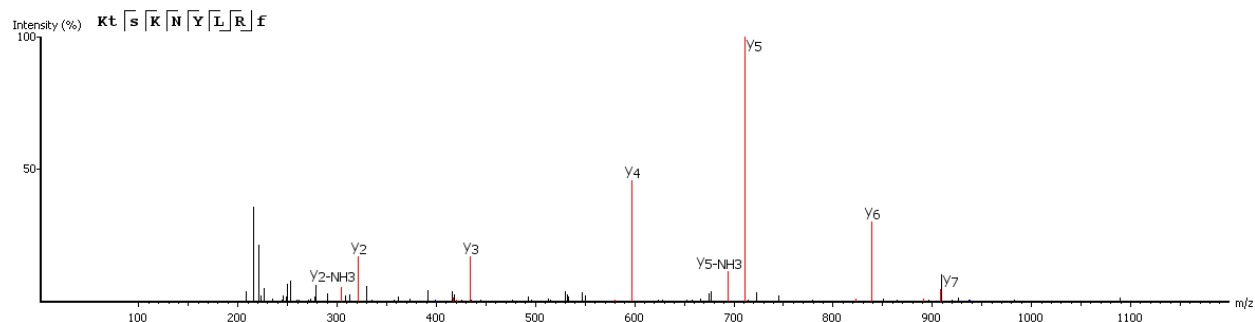


Figure S60. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence KT(-H₂O)S(-H₂O)KNYLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

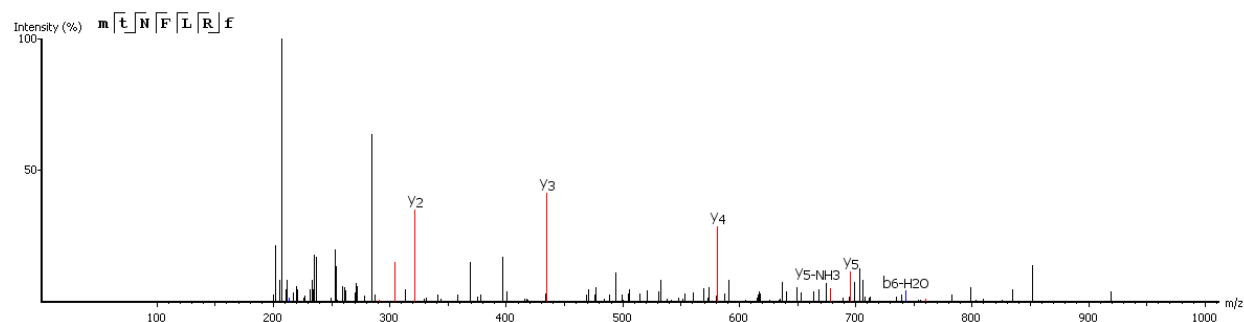


Figure S61. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence M(oxidation)T(-H₂O)NFLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

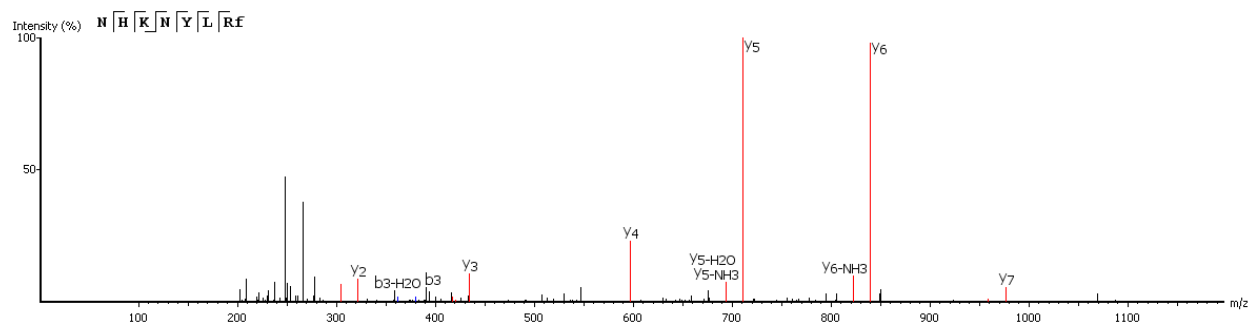


Figure S62. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence NHKNYLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

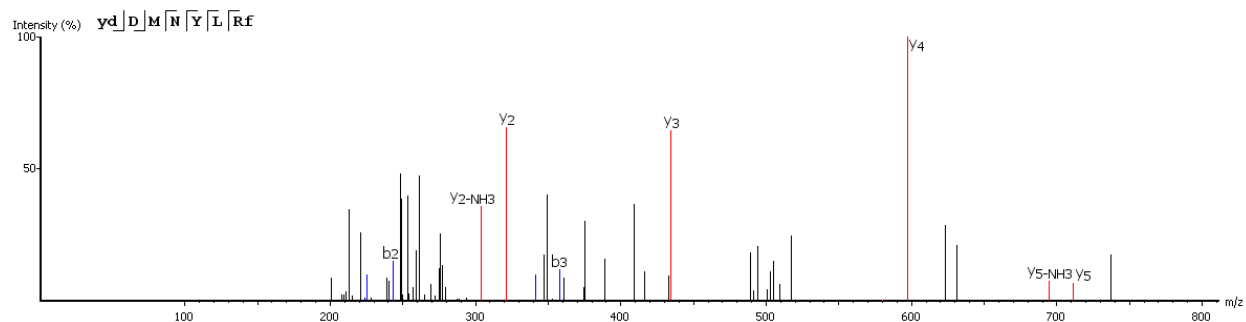


Figure S63. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence Y(-H₂O)D(-H₂O)DMNYLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

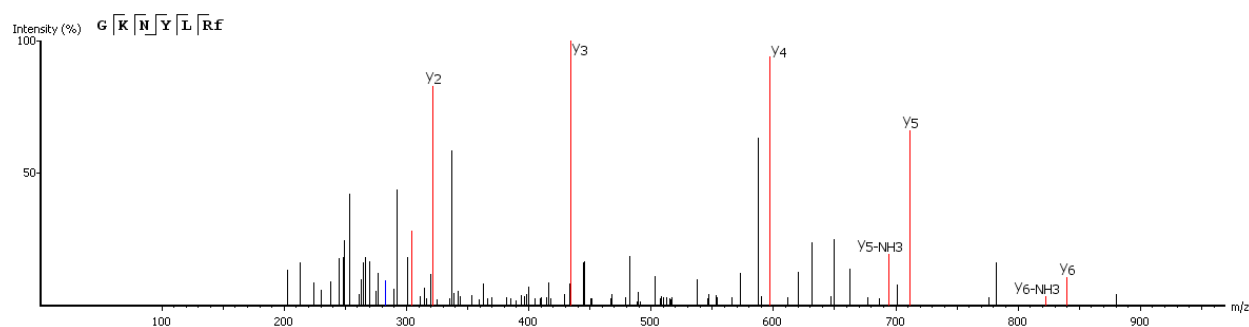


Figure S64. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence GKNYLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

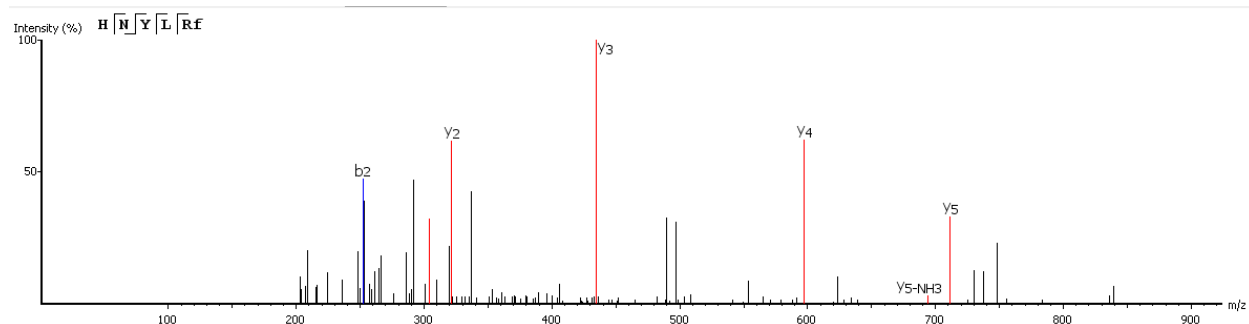


Figure S65. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence HNYLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

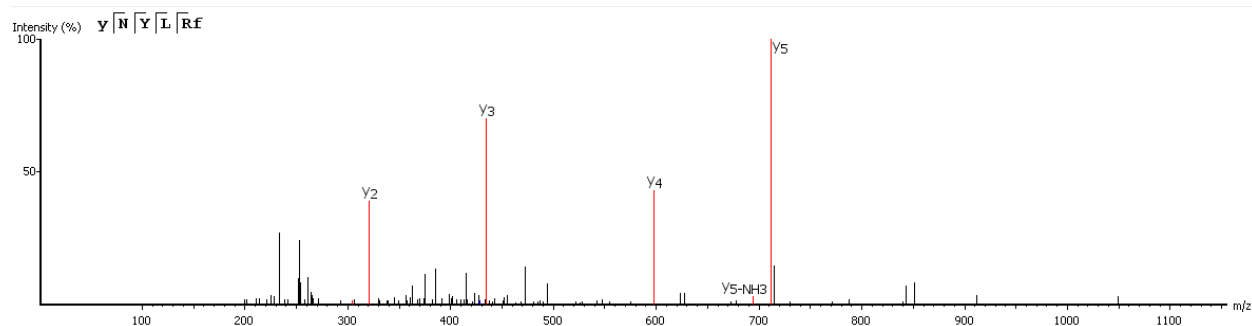


Figure S66. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence Y(-H₂O)NYLRFamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

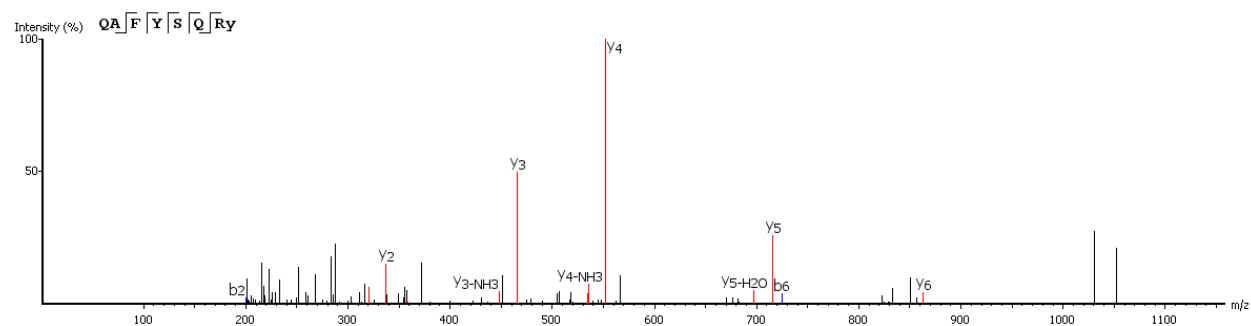


Figure S67. MS/MS spectrum from PEAKS software of a putative RYamide neuropeptide with the sequence QAFYSQRYamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

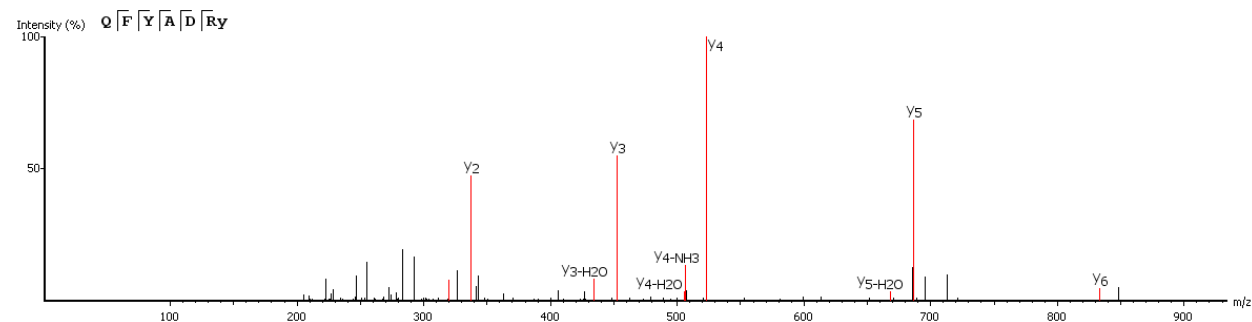


Figure S68. MS/MS spectrum from PEAKS software of a putative RYamide neuropeptide with the sequence QFYADRYamide detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

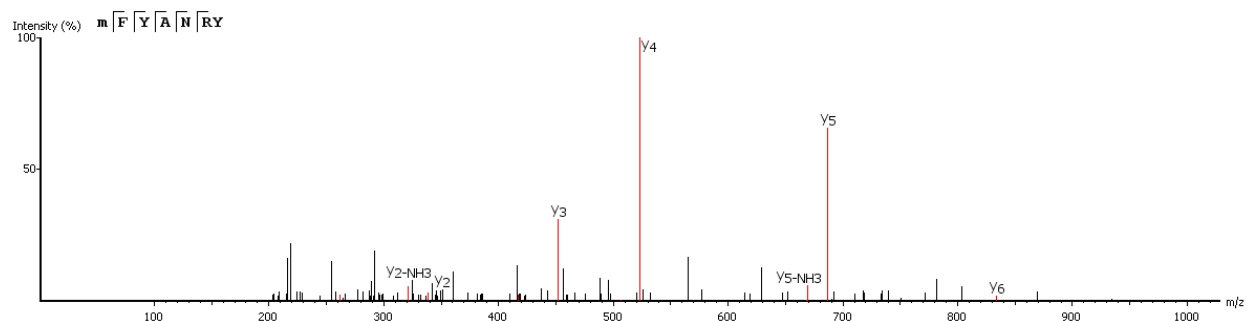
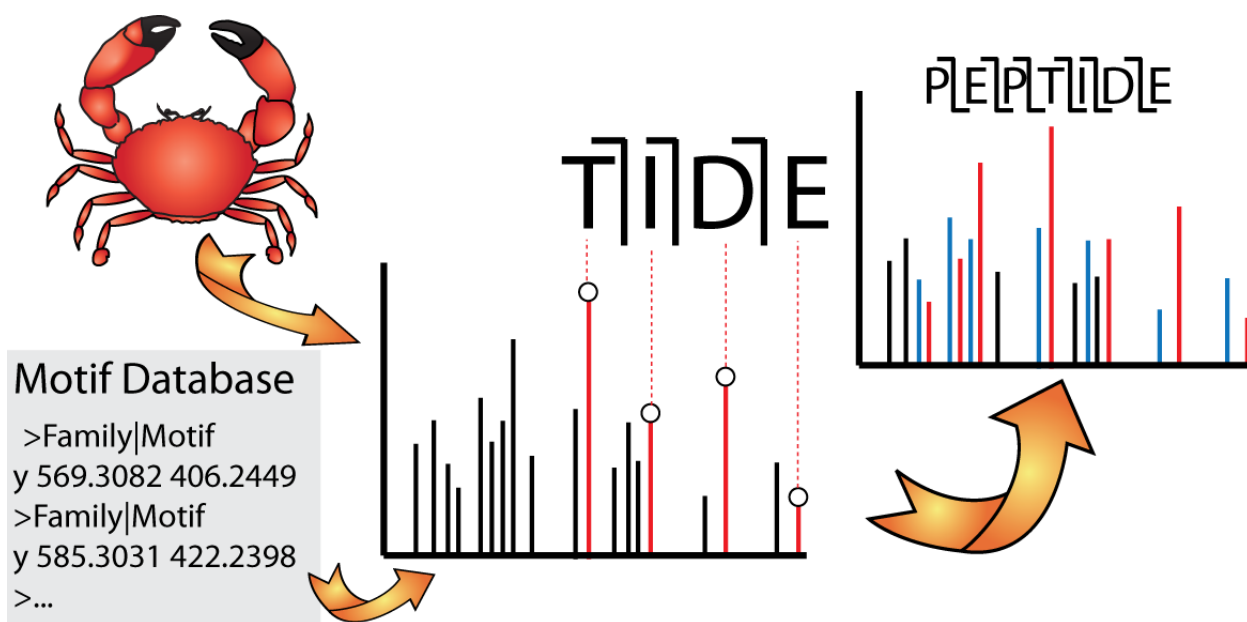


Figure S69. MS/MS spectrum from PEAKS software of a putative RYamide-related neuropeptide with the sequence M(oxidation)FYANRY detected in pericardial organ tissue extract with the data independent acquisition (DIA) method.

Chapter 5

PRESnovo: Prescreening Prior to *de novo* Sequencing to Improve

Accuracy and Sensitivity of Neuropeptide Identification



Modified from:

DeLaney, K.*; Cao, W.*; Ma, Y.; Ma, M.; Zhang, Y.; Li, L. PRESnovo: Prescreening Prior to *de novo* Sequencing to Improve Accuracy and Sensitivity of Crustacean Neuropeptide Identification. *Journal of the American Society for Mass Spectrometry*. 2020. Accepted.

Abstract

Identification of peptides in species lacking fully-sequenced genomes is challenging due to the lack of prior knowledge. *De novo* sequencing is the method of choice, but its performance is less than satisfactory due to algorithmic bias and interference in complex MS/MS spectra. The task becomes even more challenging for endogenous peptides that do not involve an enzymatic digestion step, such as neuropeptides. However, neuropeptides possess common sequence motifs that are conserved across members of the same family. Taking advantage of this feature to improve *de novo* sequencing of neuropeptides, we have developed a method named PRESnovo (prescreening precursors prior to *de novo* sequencing) to predict the motif from a MS/MS spectrum. A neuropeptide sequence is broken into a motif with conserved amino acid residues and the remaining partial sequence. By searching against a predefined motif database constructed from known homologous sequences, PRESnovo assigns the most probable motif to each precursor via a sophisticated scoring function. Performance analysis was conducted with 15 neuropeptide standards, and 11 neuropeptides were correctly identified with PRESnovo compared to 1 identification by PEAKS only. We applied PRESnovo to assign motifs to peptide sequences in conjunction with PEAKS for assigning the rest of the peptide sequence in order to discover neuropeptides in tissue samples of green crab, *C. maenas*, and Jonah crab, *C. borealis*. Collectively, a large number of neuropeptides were identified, including 13 putative neuropeptides identified in green crab brain, 77 in Jonah crab brain, and 47 in Jonah crab sinus glands for the first time. This PRESnovo strategy greatly simplifies *de novo* sequencing and enhances the accuracy and sensitivity of neuropeptide identification when common motifs are present.

Introduction

With the advancement of high-throughput mass spectrometry (MS), shotgun proteomics has been employed as the major tool for performing large-scale analysis of biological samples. With current MS instruments and workflows, thousands of MS and MS/MS spectra are produced from a single analysis.¹⁻³ With the large numbers of spectra being generated, traditional manual analysis is no longer feasible, and numerous data analysis strategies have been developed to identify proteins from the high volume of MS/MS spectra. The methods of choice for annotating these spectra mainly include spectral matching, database searching, and *de novo* sequencing. Spectral matching can be performed with a variety of software platforms, including SpectraST,⁴ X1Hunter,⁵ and Bibliospec.⁶ However, this method of analysis requires the generation of spectral libraries, which can be time consuming and costly. Database searching employs search engines such as Mascot, SEQUEST, OMSSA, MSFragger and X!Tandem⁷⁻¹¹ to interpret tandem MS spectra by scoring the similarities between the experimental and theoretical spectra generated through *in silico* prediction. The matches are then ranked such that the match with the highest score is the best predicted peptide spectrum match. While this method has proven to be highly effective for large-scale identification, it requires prior knowledge of the sequences of peptides in the sample, typically through the use of genomic data. This limits its applicability to organisms with a fully-sequenced genome.

For those species without a fully-sequenced genome, no protein database is available, and so *de novo* sequencing is the main approach employed for peptide identification. With *de novo* sequencing, peptide sequences are derived from the masses of their fragment ions as shown in a MS/MS spectrum. When performing *de novo* sequencing, no protein sequence database is used for reference, and so no prior knowledge is required of peptides in the sample. A number of

algorithms and software packages have been developed recently for *de novo* peptide sequencing.¹²⁻¹⁵ Most software use “spectral graph” or “probabilistic model” to interpret MS/MS spectra. A spectral graph is constructed from a MS/MS spectrum in which nodes represent fragment peaks and two nodes are connected by an edge if the mass difference between these two nodes matches a known amino acid residue. The algorithm tries to find the longest possible path of connected edges from the N-terminus to the C-terminus in order to determine the combination of amino acids that best represents the peptide sequence. However, any incompleteness of fragmentation can cause gaps which disconnect the longest path from N- to C-termini. In some cases, the longest path fails to represent the correct sequence or partial sequences due to interfering fragments. Instead of constructing spectral graphs, some algorithms such as PEAKS¹⁵ compute peptide sequences among all possible amino acid combinations and then map these sequences directly onto each MS/MS spectrum to find the best sequence match, which improves the likelihood of a correct sequence assignment despite interfering ions. While this method is more computationally expensive, dynamic programming is always employed to increase the computing efficiency. As a result, this method has been employed successfully for numerous *de novo* sequencing methods. However, in comparison to the database search strategy, the accuracy and sensitivity of *de novo* sequencing are far less satisfactory.¹⁶ The combination of database searching and *de novo* sequencing to some extent improves the accuracy of prediction.^{16,17} Although these hybrid methods enhance peptide prediction, they require protein databases available for the species of interest. For species without available genomes, improving peptide identification remains an unmet challenge.

One example of a group of species without a complete genome is crustaceans, which are important model organisms for studying dynamic neural networks and neuromodulation.

Neuropeptides, or short endogenous peptides involved in neuronal signaling, are an important class of molecules related to this system. Much effort has been placed on characterizing the neuropeptides involved in these signaling pathways with MS¹⁸⁻²⁴ and *in silico* prediction.²⁵ However, this class of molecules is particularly challenging to study because their workflow does not involve a digestion step.²⁶ With tryptic digests from proteins, the search space is able to be reduced based on how trypsin digests the proteins with well-defined amino acid residue at the carboxyl-end of resulting tryptic peptides. With neuropeptides, no enzymatic digestion parameters can be used to reduce the search space. However, highly-conserved peptide sequences like neuropeptides share conserved motifs, which can aid *de novo* sequencing. Herein, we focus on interpreting a part of a tandem MS spectrum to extract the conserved motif instead of sequencing the entire MS/MS fragmentation spectrum. The determination of a motif will simplify *de novo* sequencing of the rest of the sequence and increase the accuracy of peptide identification. In order to determine the neuropeptide motif, a database search method is employed similar to commonly-used methods previously described. First, motifs are derived from known peptide sequences from homologous species. Second, theoretical *in silico* fragmentation is performed for these motifs. The resulting b- and y- ions as well as their neutral loss fragments together with the corresponding motif are used to construct a motif database. This database is then searched by inputting experimental fragments to find the best matched motif for each precursor. A sophisticated scoring function, based on the sum of ratios of lengths of fragments to total motif lengths, ensures the correct assignment of motif to a given precursor. This strategy, named PRESnovo, simplifies the subsequent *de novo* sequencing step and increases the accuracy and sensitivity of peptide identifications by performing a preprocessing function prior to *de novo* sequencing.

Methods

Construction of Motif Database.

A motif database is required to run PRESnovo. Here, motif refers to a representation of the similarity of different sequences in the same peptide family. For accurate results, it is recommended that all possible motifs for the peptides of interest are included. In this study, previously known neuropeptides from invertebrate species, mainly crustaceans, were collected from public databases including Uniprot knowledgebase and publications.^{25, 27-29} In order to estimate the false positive rate, some vertebrate motifs were also included as decoy motifs because these motifs are not observed in crustacean neuropeptides. As the targeted neuropeptide sequences in most cases contain fewer than 20 amino acid residues, longer sequences such as proteins or receptors were excluded from the list. **Figure 1** shows how a motif database is constructed. The known neuropeptides collected from the various sources were clustered according to their family names followed by a multiple alignment procedure with ClustALW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) for each family. Afterwards, the most aligned region was truncated to extract motifs with WebLogo3 (<http://weblogo.threeplusone.com/>). Longer peptides whose motifs were not easily determined were removed from the list, such as Bursicon, crustacean hyperglycemic hormone (CHH), molt inhibiting hormone (MIH), etc. When using WebLogo3, multiple motifs were adopted to represent each family. For example, all 5 C-terminal motifs, YAFGL, YDFGL, YNFGL, YEFGL, and YSFGL, were used to represent allatostatin A-type (AST-A) peptides.

The PRESnovo method works by mapping tandem MS spectra onto a home-built motif database in order to find the best suitable motif for each precursor. The practical implementation is to

compare the experimental fragments associated with a precursor to those theoretical fragments associated with a motif. Therefore, a list of theoretical fragments must be created for each motif. The extracted motifs were *in silico* fragmented by MS-Product (<http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct>), and the resulting b ion set (including b ions and neutral losses, b- H₂O and b-NH₃) were adopted as the theoretical fragments for N-terminal motifs while y ion set (including y ions and neutral losses, y- H₂O and y-NH₃) were adopted as the theoretical fragments for C-terminal motif. Post-translational modifications (PTM) were also included when fragmenting these motifs. For example, for motif YXFGLamide, C-terminal amidation was considered for the production of theoretical fragments. These were selected based on common PTMs observed for neuropeptide families.^{27, 30} We used these extracted motifs and the associated theoretical fragments to construct our motif database (*see supplemental file*) in which 87 motifs covering 25 families were compiled for crustacean neuropeptides (2 families of 7 mammalian neuropeptides (Angiotensinogen (5) and Arg_vasopressin (2) were also included for test only, to be used as decoys to assess the false positive rate). The distribution of the motifs is shown in **Figure 2**.

Scoring Function and Motif Assignment.

Accurately assigning a motif to a given precursor is crucial. A well-defined scoring function can ensure the unbiased assignment of a motif in most cases. As such, a score is needed to evaluate the comparison between experimental and theoretical fragments within PRESnovo. Several considerations must be taken to generate a scoring function. These factors include significance of different-length theoretical fragments, the number of matches between experimental and theoretical fragments, and the percentage of the total number of experimental fragments with respect to the total number of theoretical fragments. We first define the score of a theoretical

fragment via dividing the length of fragment by the total length of the motif. This definition is suitable because the significance of longer fragments is higher than that of shorter fragments.³¹

This score definition is also beneficial to the interpretation of non-informative tandem MS spectra because any high-scoring longer fragments can ensure a reliable motif assignment without consideration of gaps. After computing scores for fragments, these fragment scores were used to evaluate the motif assignment. Given that the more theoretical fragments are matched by experimental fragments the more reliable the corresponding motif assignment is, the score for a motif assignment is defined by the following formula:

$$S(P(\vec{F}_E) \sim M(\vec{F}_T)) = \frac{\sum S_E(\vec{F}_E | M(\vec{F}_T))}{\sum S_T(\vec{F}_T | M(\vec{F}_T))}$$

$S(P(\vec{F}_E) \sim M(\vec{F}_T))$ is the score for motif $M(\vec{F}_T)$ assigned to a precursor $(P(\vec{F}_E))$,

$\sum S_E(\vec{F}_E | M(\vec{F}_T))$ is the sum of the scores of all matched experimental fragments while

$\sum S_T(\vec{F}_T | M(\vec{F}_T))$ is the sum of the scores of all theoretical fragments. In our motif database,

the scores of fragments were calculated in advance and stored along with motifs. The neutral loss fragments share the same score as the associated b or y ions.

The workflow for using PRESnovo to assign the best matched motif to a given precursor is shown in **Figure 3**. The user-defined mass tolerance was set to 0.5 Da for QTOF data and 0.02 Da for Orbitrap data for both precursor and fragments. The experimental fragments were compared to the theoretical fragments associated with each motif and the matched pairs were stored. If a neutral loss ion pair and the corresponding b or y ion pair were both found, only one pair was kept for the following calculation. In the list of matched fragment pairs, all the matched theoretical fragment scores were used to calculate the score of each motif assignment. As a

result, for a given precursor, all motifs were tentatively assigned to it with calculated scores. These motifs were then ranked in descending order based on the score for each motif assignment and the first five ranked motifs were reported.

NanoLC-ESI-QTOF Analysis for Peptide Standards and Tissue Sample.

A peptide standard mixture containing 15 neuropeptides (Supplemental Table S1) was subjected to nano-LC-ESI-QTOF (Waters Corp., Milford, MA) and the resulting MS data were used to test the performance of PRESnovo. Furthermore, PRESnovo was used for real tissue sample to identify endogenous neuropeptides. Tissue samples were extracted from the brain of European green crabs, *C. maenas* and brain and sinus glands of Jonah crabs, *C. borealis*. Green crab tissue was offline HPLC fractionated, and the collected fractions were analyzed on a nano-LC-ESI-QTOF (Waters Corp., Milford, MA). The Jonah crab tissue was analyzed on a nano-LC-ESI Q Exactive (Thermo Scientific, Bremen, Germany). The details about experimental protocols including animal dissection, tissue extraction, offline HPLC fractionation and MS analysis can be found in the supporting information and followed those previously described.³² After MS analysis, the QTOF raw data were converted into pkl formatted data with ProteinLynx (Waters Corp., Milford, MA). Parameters for ProteinLynx were set with default settings except noise reduction threshold at 10%. The Orbitrap raw data was converted into mzXML format using MSConvert with default settings.³³

De novo Sequencing with the Aid of PRESnovo.

The resulting pkl and mzXML files were used as input to PRESnovo. The current version of PRESnovo supports pkl and mzXML formats. Parsing of mzXML data was performed with jmxReader.³⁴ Other data formats need to be converted into one of these two formats. Mass tolerances for both precursor and fragments were set to 0.5 Da for QTOF data and 0.02 Da for

Orbitrap data. PRESnovo outputs a .csv file in which each precursor is assigned five motifs as the default setting according to a descending order of score. The number of motif assignments can be changed by the user. The scored fragments associated with each motif are also included in the output. By setting a motif score threshold, the user can filter out low-confidence motif assignments. Once a motif is obtained, the rest of the sequence can be determined by either manual sequencing or PEAKS. Given that PEAKS can report high-confidence sequence tags with individual positional confidence scores,¹⁵ it is very useful to combine PEAKS and PRESnovo together to sequence a peptide.

As a comparison, raw MS/MS data was also processed with PEAKS. The raw data were directly loaded into PEAKS (PEAKS 7, Bioinformatics Solutions Inc., Waterloo, ON) for *de novo* sequencing. The setting for PEAKS were as follows: mass tolerances for both precursor and fragments 0.5 Da for QTOF data and 0.02 Da for Orbitrap data, no enzyme digestion, and pyro-glutamine (pQ), pyro-glutamic acid (pE), oxidation of methionine, and C-terminal amidation as variable PTMs.

Results and Discussion

Performance of PRESnovo.

In order to evaluate the performance of PRESnovo, a standard mixture comprised of 15 peptides was analyzed with LC-MS/MS on a Waters QTOF instrument. The data were processed with both PRESnovo and PEAKS *de novo* sequencing to compare performance. As five predictions were output from both software packages, it was important to differentiate which was the most suitable. The predicted sequences from each software were compared to the actual sequences of the standards. Interestingly, in most cases, the first prediction was the best one in the PRESnovo

results. In the PEAKS results, this was not always the case, as other sequence predictions rather than the first one were the best prediction for some peptides. **Table 1** summarizes the results obtained from PRESnovo and PEAKS. Five output sequences are provided from both software, and the sequence matching the standard the closest is displayed. As shown, 11 out of 15 peptides were predicted correctly by PRESnovo while only 1 was computed correctly by PEAKS with at least six consecutively correct residues. The 11 peptides identified by PRESnovo produced doubly-charged precursors leading to an almost even number of b and y ions, which facilitated the detection of fragments associated with their motifs by PRESnovo. Two examples are shown in **Figure 4**, from which it can be seen that almost all motif-related fragments were produced, although some of them were at low intensities. Consequently, two motifs were confidently assigned to the precursors, respectively. However, PEAKS lacks specificity for identification of these endogenous peptides because PEAKS is originally designed for general use in bottom-up proteomics where tryptic peptides are the main targets. With tryptic peptides, certain patterns exist that inform the resulting *de novo* sequences obtained. As a result, PEAKS by itself is not optimal for non-tryptic peptide prediction.¹⁵ Because PRESnovo is designed to recognize patterns specific to endogenous neuropeptides, it provides improved accuracy in sequencing these signaling peptides when used in conjunction with PEAKS.

The 4 peptides not identified correctly by PRESnovo fall into two categories: AST-A peptides (GDGRLYAFGLa and APSGAQRLYGFGLa) and disulfide bond bridging peptides (PFCNAFTGCa and CYFQNCPRGa). AST-A peptides are generally ionized as singly charged precursors which tend to produce predominantly N-terminal (primarily b-series) fragments. These fragments (a/b/c ions) dominate the MS/MS spectrum, as shown in **Figure 5A**. However, the characteristic motif of AST-A peptides, YXFGLamide (X is a variable residue), is located at

the C-terminus, which causes the motif database to deduce y-series fragments from this motif. As a result, the mismatch between the experimental b-series fragments and *in silico* y-series fragments leads to misidentification of these peptides. Although this demonstrates a limitation of PRESnovo's predicting power, it is not difficult to manually sequence AST-A peptides with the aid of PEAKS, given that most peptides belonging to this family have a simple pattern of b-ions dominating fragmentation. Disulfide bond bridging peptides are prone to producing complicated internal fragments that suppress the production of b/y ions (**Figure 5B**). As a result, not enough motif-associated b/y ions are available for PRESnovo to determine the motif for the precursor. While the inability to predict disulfide bond bridging peptide sequences is a limitation of PRESnovo, the same is true across many *de novo* sequencing software. Disulfide bond bridging peptides are relatively uncommon amongst neuropeptides, and only 4 possible sequences with a characteristic motif are present in the crustacean neuropeptide database. Therefore, this limitation is not expected to substantially impede the identification of crustacean neuropeptides.

The Factors Impacting on the Performance of PRESnovo

The performance of PRESnovo is impacted mainly by the motif database being searched and the quality of tandem MS spectra being queried. The construction of a motif database is crucial to PRESnovo, as accurate, detailed compilation of motifs improves the likelihood of confident identifications being made. A motif database consists of two parts: a string of amino acids comprising each motif and its associated fragments. Motifs are generally collected from known peptide sequences present in homologous species. There is a tradeoff between the number of motifs incorporated in a motif database and the prediction power of PRESnovo. If too few motifs are included such that all forms of a neuropeptide motif aren't sufficiently represented, the

search may return results with low specificity or inaccurate identifications. Conversely, having an excess number of motifs included in the database inflates the false positive rate and decreases the sensitivity of the assignment.³⁵ In order to maximize the detection of neuropeptides in tissue samples, a motif library at least 80 motifs is satisfactory, as indicated in **Figure S1a**. To avoid these issues, only biologically-related species should be considered when extracting motifs for a species of interest. For example, in this study, motifs were only extracted from known peptide sequences in other crustacean species. If the interest is in peptide identifications from a vertebrate species such as human or mouse, a vertebrate peptide motif database can be created specifically for the species of interest that only includes those from similar species. In this way, effort can be made to exclude interference from other motifs that are not likely to be present in the species of interest. Another possible solution is to use long motifs, which would reduce the likelihood of false positive prediction and would also simplify the following *de novo* sequencing. *In silico* fragments associated with motifs also impact on the performance of PRESnovo. In our current motif database, only b and y series ions (i.e., b, y, and neutral loss ions) are included for each motif while no a/c or x/z ions are considered. The reason for this specification is that b and y ions are sufficient for determining a motif even though some of them may have low abundance in some cases.

The quality of tandem MS spectra also has significant impact on the performance of PRESnovo. Different from many automated *de novo* algorithms, PRESnovo can predict correct motifs for relatively low-quality MS/MS spectra provided that enough motif-associated fragments are present in the spectra. However, in the case of processing less informative spectra, if the experimental fragments associated with motif are insufficient or high-score fragments (i.e. large fragments) are missed, PRESnovo will report low-confidence motifs, resulting in high false

positive rate for peptide prediction. On the other hand, if a tandem MS spectrum contains too much noise and these interferences are not effectively removed, it will lead to the wrong motif assignment and thus increase the false positive rate.³⁶⁻³⁸ Therefore, an efficient and effective preprocessing algorithm is needed to clean up tandem MS spectra. In this study we used ProteinLynx (Waters Co.) to remove noise or background signals from QTOF data prior to processing. ProteinLynx adopts Savitzky-Golay method to smooth the data and thus ensure fewer interfering signals are contained in the final pkl data files. While the quality of MS/MS spectra is important, the mass accuracy of MS/MS spectra does not appear to substantially impact the performance of PRESnovo, as shown in **Figure S1b**, where the search was run with alternating mass tolerances and, except for very high mass errors (e.g. greater than 1,000 ppm), the results remained consistent.

Application of PRESnovo in conjunction with PEAKS to identify neuropeptides in C. maenas and C. borealis.

While PRESnovo accurately predicts the sequence motif of a neuropeptide, its use in conjunction with PEAKS *de novo* software enables improved detection of neuropeptides present in real biological samples. An important characteristic of PEAKS is its ability to report the positional confidence for each amino acid in the predicted sequence.¹¹ This feature can be used to identify the rest of the sequence after PRESnovo predicts the motif. For instance, peptide TNFAFSPRLa shown in **Figure 2** was predicted with high confidence to possess the motif of FSPRLa (score: 0.93) by PRESnovo. Meanwhile, PEAKS reports a confident N-terminal sequence tag TNFAFSP (as in **Table 1**) for the same peptide. By combining these two predictions, one can easily determine the sequence with manual verification.

PRESnovo and PEAKS were employed collectively to identify neuropeptides in tissue samples of green crab, *C. maenas*, and Jonah crab, *C. borealis*. These animals were chosen because they are well-characterized model organisms whose nervous systems have been previously studied using electrophysiology and immunohistochemistry.³⁹⁻⁴² However, while their dynamic neural networks are well-understood, there is currently no fully-sequenced genome available for either species, and so their neuropeptidomes are not yet fully characterized. Furthermore, with the inherent difficulties associated with MS characterization of neuropeptides, even detecting known neuropeptides remains challenging. To address these challenges and uncover more of the neuropeptides underlying neural modulation in these organisms, PRESnovo was employed in conjunction with PEAKS. First, PRESnovo was used to identify a motif for each precursor. The motif score threshold was set to 0.60 to filter out low-scoring motif assignments and the corresponding precursors. For high-score precursors, the PRESnovo results were compared to their corresponding PEAKS predictions. Manual sequencing was then used to combine this information and determine the final sequence for each precursor.

Brain extract from green crab was analyzed on a Waters QTOF instrument and pre-processed with ProteinLynx prior to PRESnovo analysis. The resulting data included 77 neuropeptides, summarized in **Table 2**, of which 13 sequences are putative neuropeptides that have never before been identified in green crab.⁴³ Of the 13 novel sequences, 7 of them have previously been identified in other invertebrate species, such as *Callinectes sapidus* and *Homarus americanus*, and all were only identified with the assistance of PRESnovo. Both brain and sinus gland tissue extracts from Jonah crab were analyzed on a Thermo Q Exactive Orbitrap instrument, and the raw files were converted to the open-source mzXML format. After analysis with PRESnovo and PEAKS, 100 neuropeptides were identified in the brain sample, 77 of these being putative novel

neuropeptides never before identified in previous literature. The repeatability across technical replicates and overlap between biological replicates from distinct brain samples are shown in **Figure S2**. A total of 76 neuropeptides were identified in the sinus gland extract, with 47 of these sequences being putative novel neuropeptides not previously identified in any species. **Table 3** summarizes the neuropeptides detected in Jonah crab that match the crustacean database, while **Table 4** lists the putative novel neuropeptides identified in Jonah crab, 24 of which were only identified with the assistance of PRESnovo. **Figures 6a** and **6b** show MS/MS spectra of novel neuropeptides identified in brain and sinus gland tissue, respectively. **Figures S3-S5** show the MS/MS spectra of all novel neuropeptides identified. As can be seen, PRESnovo predicted the characteristic sequence motif that led to the full neuropeptide sequence shown. **Figure 6c** shows a neuropeptide identified that is present in the crustacean database but was incorrectly assigned by PEAKS, demonstrating the improvement in identification afforded by PRESnovo pre-screening. The originally-predicted sequence in PEAKS scrambled the last three amino acids, but PRESnovo was able to assign them based on mapping the fragment ions to a common motif. This example demonstrates the improved accuracy offered by combining PRESnovo with PEAKS for *de novo* sequencing, as identifying fragment ions characteristic of neuropeptide motifs increases the likelihood of correct identifications.

Of the neuropeptides identified in the two species, the most common families were AST-A, FMRFamide-related peptide (FaRP), RYamide, orcokinin, tachykinin, and pyrokinin. AST-A and AST-B neuropeptides are distributed throughout the nervous and neuroendocrine system of crustaceans and have been found to be inhibitors of neuromodulation.^{44, 45} Several novel peptides belonging to these families were identified in both the brain and sinus glands, indicating that these peptides may also exhibit inhibitory effects. FaRP neuropeptides have been found to

have a variety of functions within the nervous and neuroendocrine system, including as autocrines, paracrines, and circulating hormones.⁴⁶⁻⁴⁸ Therefore, it is expected that these neuropeptides would be identified in both the sinus glands where they may be released as circulating hormones and in the brain, where they may serve a more local function within the neuropil. While there have not been many studies on the function of pyrokinins in crustaceans, they were found to have an effect on the gastric mill.⁴⁹ As the brain is located within the stomach of the crab, pyrokinin neuropeptides were mostly identified in brain tissue and the putatively identified novel pyrokinin peptides may also have a role in gastric activity. Tachykinin and orcokinin peptides are also more prominent in the stomatogastric nervous system, with a variety of functions including hindgut contractions.⁵⁰⁻⁵² In this study, putative novel tachykinin and orcokinin peptides were identified in the brain that may also have a role in modulating some stomach activity. Additionally, 3 tachykinin peptides were identified in the sinus gland, indicating that they may have a different modulatory role. Putative novel RYamide neuropeptides were identified in the brain and sinus glands as well. The biological activity of RYamides is not fully understood, but they have been previously identified in neuroendocrine tissue and central neuropil, suggesting functions both locally and as circulating hormones.^{53,54} Follow-up experiments will need to be performed in order to confirm the putative peptide identifications and determine their biological activity. However, these results demonstrate the great potential PRESnovo has for both facilitating the discovery of novel neuropeptides and improving detection coverage of the crustacean neuropeptidome identified in analyses. This application provided here indicates that PRESnovo can greatly improve the discovery of neuropeptides across a variety of other species and indicates potential for improving identification of other endogenous peptides across a variety of other sample types, provided that

commonly-shared sequence motifs exist.

Conclusions

In this work, a prescreening strategy, namely PRESnovo, was developed to improve the accuracy, specificity and sensitivity of peptide identification. In conjunction with *de novo* sequencing algorithms such as PEAKS, this method is powerful for identification of highly conserved peptides such as neuropeptides. The strategy we proposed in this manuscript can be easily extended to other species of interest, provided that a well-constructed motif database is obtained. Future directions may include incorporation of more sophisticated algorithms for sequencing disulfide bond bridging peptides and peptides with motifs that are difficult to detect, such as AST-A. The software and motif database used in this work can be freely downloaded via the following link: <https://www.lilabs.org/resources>.

Abbreviations:

AKH/RPCH: Adipokinetic hormone/red pigment concentrating hormone; AST-A: Allatostatin A; AST-B: Allatostatin B; AVP: Arginine vasopressin; CCAP: Crustacean cardioactive peptide; FaLP: FMRFamide-related peptide

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Supporting Material Available

Experimental details for crustacean tissue collection, preparation, and MS analysis, table of peptide standards used for performance analysis, and MS/MS spectra of all putative novel neuropeptides are included in the supporting material.

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Table 1. The identified peptide standards by PRESnovo and PEAKS 7, with the displayed sequence being the output from each software that most closely matches the correct sequence.

Family	Mass	Real Sequence	PRESnovo (Motif)	PEAKS 7
CCAP	955.37	PFCNAFTGCa	FIRFa	MCSAAGACAAT
RYamide	975.44	SGFYANRYa	FYANRYa	GRNTAAGGGDT
Pyrokinin	1036.53	SGGFAFSPRLa	FSPRLa	<u>SGGFA</u> FM(O)AFFFGT
Pyrokinin	1050.55	TNFAFSPRLa	FSPRLa	GS <u>F</u> A FSPVGla
AST-A	1066.54	GDGRLYAFGLa	FGPRLa	TGGAQPEQLPAa
AVP	1083.44	CYFQNCPRGa	NSELINSILG	M(O)SQEPAAHM(O) Ga
FLP	1104.57	GAHKNYLRF	NYLRFa	HQGAGGVPMRPa
AST-B	1106.50	QWSSMRGAWa	WSSMRGAWa	TGRSSGAAAGADS
AST-B	1259.64	SGKWSNLRGAWa	WSNLRGAWa	QCARSVAGGSASAPa
Angiotension	1281.65	DRVYVHPFHL	DRVYVHPF	SAGPVEGGDLMLH
AST-A	1334.70	APSGAQRLYGFGLa	pQVNFSPNWa	<u>APSGA</u> TCSSMGVGVLa
AST-B	1469.68	VPNDWAHFRGSWa	WAHFRGSWa	VMDLGSAGSGNGMQM(O)
Orcokinin	1472.66	NFDEIDRSFGFGAa	NFDEIDR	<u>NFDELD</u> AG SGSFG GT
Orcokinin	1473.64	NFDEIDRSFGFGA	NFDEIDR	CTG <u>DEL</u> DAGPGGAPGGT
Orcokinin	1516.65	NFDEIDRSFGGFN	NFDEIDR	GCT <u>DE</u> NNNM(O)EGGCGT

*the bold refers to the correct identifications. a: amide. The underlined residues are the correctly identified by PEAKS 7. 11 were identified by PRESnovo while 1 by PEAKS 7 (NFDEIDRSFGFGAa, based on criteria of six consecutively correct residues)

Table 2. Identified neuropeptides in brain tissue of green crab, *C. maenas*

Family	Mass	Sequence
AST-A	768.38	EAYAGFLa
AST-A	779.39	NPYAFGLa
AST-A	779.39	GGPYAFGLa
AST-A	780.38	DPYAFGLa
AST-A	793.41	AGPYAFGLa
AST-A	794.39	EPYAFGLa
AST-A	807.43	AAPYAFGLa
AST-A	809.40	AGPYSFGLa
AST-A	823.42	ASPYAFGLa
AST-A	850.47	GKPYAFGLa
AST-A	852.40	EPYEFGLa
AST-A	878.47	RGPYAFGLa
AST-A	896.44	FSGASPYGLa
AST-A	908.48	ARPYSFGLa
AST-A	924.50	LKAYDFGLa
AST-A	925.46	ATGQYAFGLa
AST-A	938.49	TRPYSFGLa
AST-A	922.52	KLPYSFGLa
AST-B	1106.57	QWSSMRGAWa
AST-B	1259.70	SGKWSNLRGAWa
AST-B	1292.62	STNWSSLRSAWa
AST-B	1469.69	VPNDWAHFRGSWa
CCAP	956.38	PFCNAFTGCa
FaRPs	734.40	GPFLRFa
FaRPs	850.49	RNFLRFa
FaRPs	886.55	PSLRLRFa
FaRPs	904.50	PSMRLRFa
FaRPs	920.50	PSM(O)RLRFa
FaRPs	937.52	NRSFLRFa
FaRPs	953.52	SRNYLRFa
FaRPs	964.53	NRNFLRFa
FaRPs	965.52	DRNFLRFa
FaRPs	976.51	PQGNFLRFa
FaRPs	1021.55	GNRNFLRFa
FaRPs	1022.53	GDRNFLRFa
FaRPs	1047.56	APQGNFLRFa
FaRPs	1103.60	GAHKNFLRFa
FaRPs	1104.62	SMPSLRLRFa
FaRPs	1123.62	GLSRNYLRFa
FaRPs	1136.58	DGNRNFLRFa

FaRPs	1157.61	YGNRSFLRFa
FaRPs	1207.62	DQNRNFLRFa
FaRPs	1270.64	pQDLDHVFLRFa
FaRPs	1270.67	PELDHVFLRFa
FaRPs	1287.67	QDLDHVFLRFa
FaRPs	1288.62	QDNDHVFLRFa
FaRPs	1313.77	DARTAPLRLRFa
Orcokinin	936.42	DEIDRSGFa
Orcokinin	1197.54	NFDEIDRSGFa
Orcokinin	1227.55	NFDEIDRSSFa
Orcokinin	1255.54	NFDEIDRSGFG
Orcokinin	1269.55	NFDEIDRSGFA
Orcokinin	1285.55	NFDEIDRSSFG
Orcokinin	1299.57	NFDEIDRSSFa
Orcokinin	1473.65	NFDEIDRSGFGFA
Orcokinin	1546.67	NFDEIDRSSFGFN
Orcomyotropin	1185.51	FDAFTTGFGHS
Others	843.47	HIGSLYRa
Others	915.53	KIFEPLVA
Others	1371.78	KIFEPLRDKNL
PDH	1926.01	NSELINSLLGIPKVMNDAA
Pyrokinin	877.51	LYFAPRLa
Pyrokinin	1023.55	TSFAFSPRLa
Pyrokinin	1108.56	TDGFAFSPRLa
RPCH	929.43	pQLNFSPGWa
SIFamide	1160.64	RKPPFNGSIFa
SIFamide	1380.73	GYRKPPFNGSIFa
Tachykinin	765.39	SGFLGMRa
Tachykinin	862.45	PSGFLGMRa
Tachykinin	878.44	PSGFLGM(O)Ra
Tachykinin	933.48	APSGFLGMRa
Tachykinin	934.46	APSGFLGMR
Tachykinin	949.48	APSGFLGM(O)Ra
Tachykinin	963.49	TPSGFLGMRa
Tachykinin	979.49	TPSGFLGM(O)Ra
Tachykinin	991.49	APSGFLGMRG
Tachykinin	1007.48	APSGFLGM(O)RG

*Red color represents novel neuropeptides to green crab. “a” indicates C-terminal amidation. “p” indicates pyroglutamate Gln. “O” stands for oxidation of Met.

Table 3. Identified neuropeptides matching to the database in brain and sinus gland tissue of Jonah crab, *C. borealis*

Family	Mass	Sequence	Tissue
AST-B	1106.5081	AGWSSMRGAWa	Brain, SG
AST-B	1106.5081	QWSSMRGAWa	Brain, SG
AST-B	1292.6262	STNWSSLRSAWa	Brain, SG
Corazonin	1368.6211	pQTFQYSRGWTNa	Brain
FaRP	1146.6411	APQRNFLRFa	SG
FaRP	965.5195	DRNFLRFa	Brain, SG
FaRP	961.5246	ERNFLRFa	Brain
FaRP	1103.5989	GAHKNYLRFa	SG
FaRP	1021.5569	GNRNFLRFa	Brain, SG
FaRP	1145.5981	GYSKNYLRFa	Brain, SG
FaRP	816.4758	HVFLRFa	Brain
FaRP	1103.6353	KHKNYLRFa	Brain
FaRP	694.3915	NFLRFa	Brain, SG
FaRP	964.5355	NRNFLRFa	Brain, SG
FaRP	1270.6458	pQDLDHVFLRFa	Brain, SG
FaRP	1287.6724	QDLDHVFLRFa	Brain, SG
FaRP	850.4926	RNFLRFa	Brain
FaRP	1180.6101	SENRNFLRFa	Brain, SG
Proctolin	648.3595	RYLPT	SG
Pyrokinin	835.4704	FAFSPRLa	Brain
Pyrokinin	877.5174	LYFAPRLa	Brain, SG
Pyrokinin	1036.5454	SGGFAFSPRLa	SG
Pyrokinin	1050.561	TNFAFSPRLa	Brain
Ryamide	1029.4668	EGFYSQRYa	SG
RYamide	783.4028	FVGGSRYa	SG
RYamide	861.4133	FYSQRYa	SG
RYamide	1113.5679	RSSFVGGsRYa	SG
RYamide	975.4562	SGFYANRYa	Brain, SG
Ryamide	1113.5679	SSRFVGGsRYa	SG
Tachykinin	933.4854	APSGFLGMRa	Brain, SG
Tachykinin	621.3421	FLGMRa	SG
Tachykinin	765.3956	SGFLGMRa	Brain, SG
Tachykinin	963.496	TPSGFLGMRa	Brain, SG

“a” represents C-terminal amidation. “p” means pyroglutamate Gln or Glu. “SG” indicates sinus gland.

Table 4. Identified putative novel neuropeptides neuropeptides in brain and sinus gland tissue of Jonah crab, *C. borealis*

Family	Mass	sequence	Tissue
AST-A	1065.55	APTDLYAFGLa	Brain
AST-A	1065.55	PATDLYAFGLa	Brain
AST-A	983.4824	QRDYSFGLa	Brain
AST-A	939.4926	RQAYSFGLa	SG
AST-B	1259.652	KGSWSNLRGAWa	Brain
AST-B	1292.608	MGNWSSLRSAWa	Brain
FaRP	964.5242	AAQNFLRFa	SG
FaRP	1103.599	AGHKNYLRFa	SG
FaRP	1146.641	APAGRNFLRFa	Brain
FaRP	1146.641	APGARNFLRFa	Brain, SG
FaRP	1146.641	APGRANFLRFa	Brain, SG
FaRP	1006.535	APGSNFLRFa	Brain
FaRP	976.5242	APNNFLRFa	Brain
FaRP	1267.624	CAENRNFLRFa	Brain
FaRP	1267.607	CCPGGRNFLRFa	Brain
FaRP	1267.607	CCPNRNFLRFa	Brain
FaRP	1324.646	DGMGNRNFLRFa	Brain
FaRP	1270.639	DHVCHVFLRFa	SG
FaRP	1394.567	DSGPDDYGHMRFa	SG
FaRP	1394.567	DSPGDDYGHMRFa	Brain
FaRP	1180.61	DTNRNFLRFa	SG
FaRP	1394.567	pEGTSDDYGHMRFa	SG
FaRP	1267.624	EACNRNFLRFa	Brain
FaRP	1222.621	EERNNFLRFa	Brain
FaRP	1532.762	EESAEVPPNFLRFa	Brain
FaRP	1022.53	EGAANFLRFa	Brain
FaRP	1233.589	EQANDNFLRFa	Brain
FaRP	1769.896	EQQPHAGLSAGNFLRFa	Brain
FaRP	1180.61	ESNRNFLRFa	SG
FaRP	1180.61	ESRNNFLRFa	Brain, SG
FaRP	1324.664	ESSNGRNFLRFa	Brain
FaRP	1032.598	FALAGRPRFa	Brain
FaRP	1180.614	FGAPNNFLRFa	Brain
FaRP	1103.599	HAGKNYLRFa	Brain, SG
FaRP	1270.639	HDVCHVFLRFa	Brain
FaRP	1146.594	HEVSNFLRFa	Brain
FaRP	1249.647	HFDRNFLRFa	Brain
FaRP	1248.663	HFNRNFLRFa	Brain

FaRP	1146.594	HSDLNFLRFa	Brain
FaRP	1532.832	KAAPSNRNNFLRFa	Brain
FaRP	1146.666	KAPRNFLRFa	SG
FaRP	1498.746	KCSTDGRGNFLRFa	Brain
FaRP	1146.666	KGAPVNFLRFa	SG
FaRP	966.5399	KGSNFLRFa	Brain
FaRP	1103.599	KHAGNYLRFa	SG
FaRP	1103.599	KHQNYLRFa	SG
FaRP	1146.666	KLPNNFLRFa	Brain
FaRP	1146.678	KPARNFLRFa	SG
FaRP	1248.709	KQQLGNFLRFa	Brain
FaRP	1146.678	KRAPNFLRFa	SG
FaRP	1305.749	KRMVPNFLRFa	Brain
FaRP	1145.598	KSGYNYLRFa	Brain, SG
FaRP	1333.737	KSPNGRNFLRFa	Brain
FaRP	1145.598	KSYGNYLRFa	SG
FaRP	1146.666	KVPQNFLRFa	SG
FaRP	1333.737	LQAGNRNFLRFa	Brain
FaRP	1021.553	MVPNFLRFa	Brain
FaRP	976.5242	NPANFLRFa	Brain
FaRP	992.5192	NSPNFLRFa	Brain
FaRP	1544.748	NSYSERNNFLRFa	Brain
FaRP	1146.641	PAGARNFLRFa	SG
FaRP	976.5242	PAGGNFLRFa	Brain
FaRP	1006.535	PNTNFLRFa	Brain
FaRP	1146.605	pQGQRNFLRFa	SG
FaRP	1222.625	pQQAANFLRFa	Brain
FaRP	1551.78	PVMEMRNNFLRFa	Brain
FaRP	1103.599	QHKNYLRFa	Brain, SG
FaRP	1180.599	QKDDNFLRFa	Brain
FaRP	1333.737	QLAGNRNFLRFa	Brain
FaRP	1146.641	QPARNFLRFa	SG
FaRP	1248.695	QRNRNFLRFa	Brain
FaRP	1146.641	RAGAPNFLRFa	SG
FaRP	965.5195	RDNFLRFa	Brain, SG
FaRP	964.5355	RGGNFLRFa	Brain, SG
FaRP	1021.557	RGNNFLRFa	SG
FaRP	1180.61	RNDTNFLRFa	Brain
FaRP	1180.61	RNESNFLRFa	Brain, SG
FaRP	1021.557	RNGNFLRFa	Brain, SG
FaRP	964.5355	RNNFLRFa	Brain, SG
FaRP	1180.61	RNSENFLRFa	SG
FaRP	3692.954	RPGQLLLAEASSWLPTQQEGTKRGYSKNYLRFa	Brain

FaRP	1248.695	RQNRNFLRFa	Brain
FaRP	1276.679	SAGPNRNFLRFa	Brain
FaRP	1219.658	SAPNRNFLRFa	Brain
FaRP	1394.567	SDGPDDYGHMRFa	Brain
FaRP	1180.61	SERNNFLRFa	SG
FaRP	1483.71	STDEYPNFLRFa	Brain
FaRP	1180.599	STPNSNFLRFa	SG
FaRP	1180.61	TDRNNFLRFa	SG
FaRP	1180.635	TSAQVNFLRFa	SG
FaRP	1544.729	TSDELTTCNFLRFa	Brain
FaRP	1180.599	TSPNSNFLRFa	SG
FaRP	1180.599	TSSNPNFLRFa	SG
FaRP	1532.836	VPGFAPNRNFLRFa	Brain
FaRP	1004.523	YFNFLRFa	Brain
FaRP	1145.598	YGSKNYLRFa	SG
Pyrokinin	2380.269	FNGPKPLAKYVDTNFAFSPRLa	Brain
Pyrokinin	2379.252	FNPGKLPKSQMTTNFAFSPRLa	Brain
Pyrokinin	1036.545	GSGFAFSPRLa	Brain
Pyrokinin	1050.561	QSFAFSPRLa	Brain, SG
RYamide	1029.467	pEGFYSQRYa	SG
RYamide	975.4562	GSFYANRYa	Brain, SG
RYamide	1029.467	pQGFYSQRYa	Brain
RYamide	2133.035	SADRTQLTERSGFYANRYa	Brain
RYamide	1113.568	SRSFVGGSRYa	SG
RYamide	2133.035	TGARDGTLTERSGFYANRYa	Brain
Tachykinin	1143.622	APPLSGFLGMRa	SG
Tachykinin	1175.623	KNAPSGFLGMRa	Brain
Tachykinin	1145.613	KSAHTFLGMRa	SG
Tachykinin	1145.576	KSDHGFLGMRa	SG
Tachykinin	1253.547	NCCAPSGFLGMRa	Brain

“a” represents C-terminal amidation. “p” means pyroglutamate Gln or Glu. “SG” indicates sinus gland.

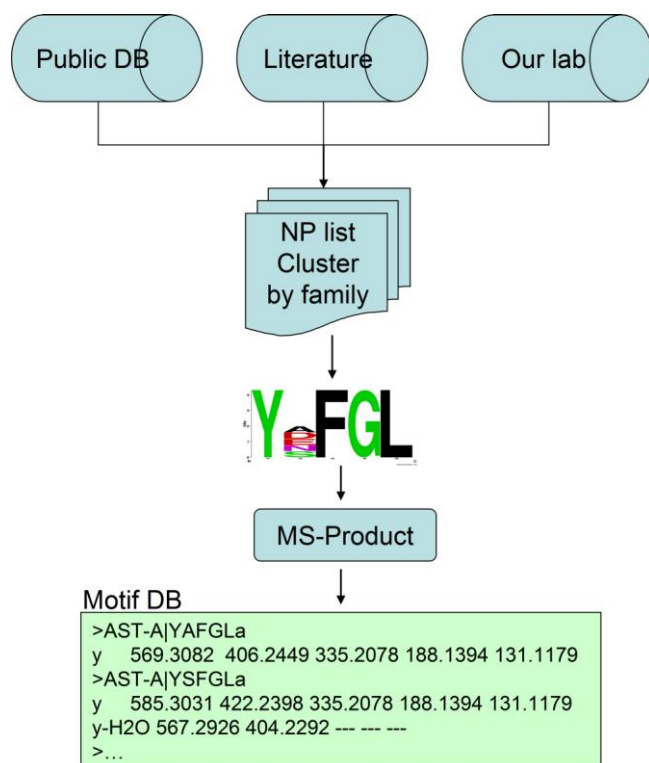


Figure 1. The diagram illustrates the construction of motif database. Three resources including public databases (e.g. NCBI), previous publications and the neuropeptide discoveries by our lab are used to generate a collection of crustacean neuropeptides which are clustered into families according to their conserved motifs. Then each family of neuropeptides are aligned with WebLogo (version 3.0, <http://weblogo.threeplusone.com/>) to extract detailed motifs followed by *in silico* fragmentation of these extracted motifs with MS-product (<http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct>). Finally, the motif and the corresponding b- or y- series fragments are compiled into a motif database.

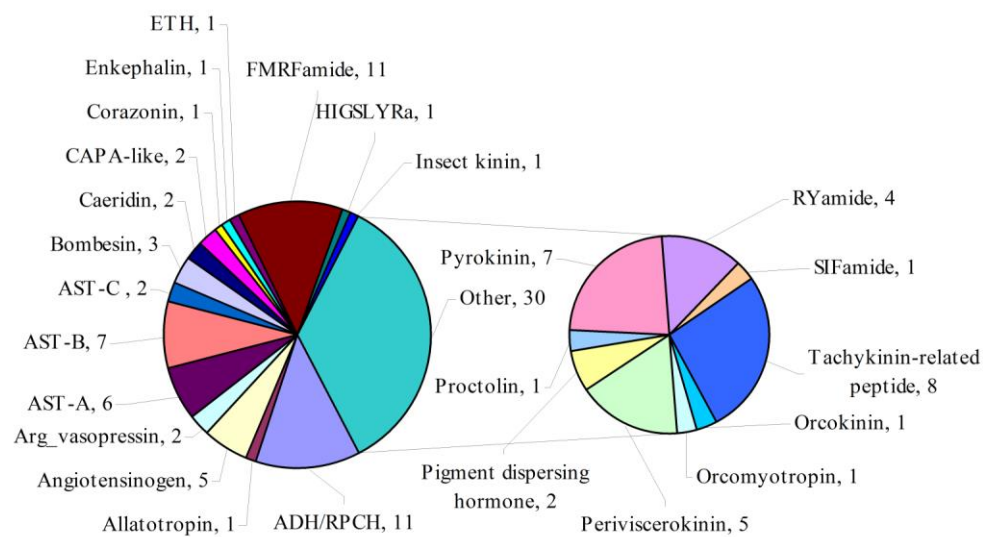


Figure 2. The distribution of motifs according to their families. The number represents the total number of motifs associated with each family.

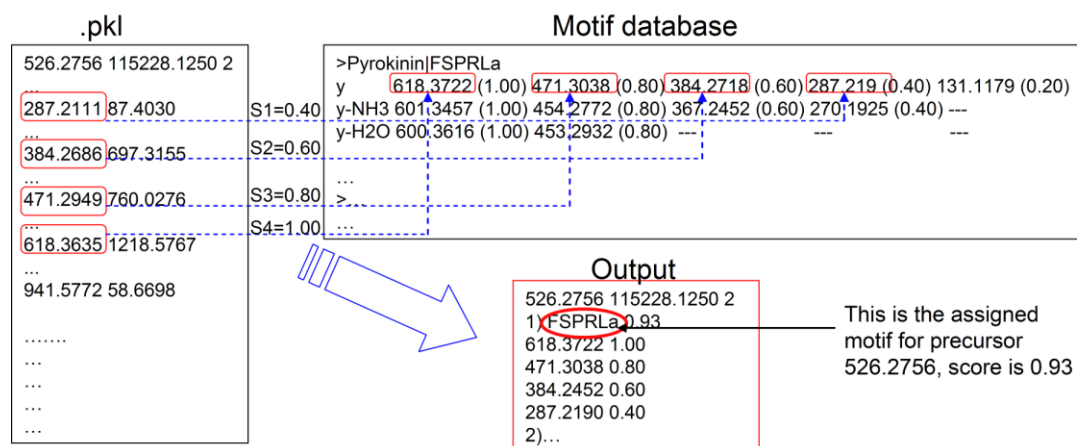


Figure 3. The schematic representation of motif assignment. The experimental fragments associated with the precursor (in .pkl file) are searched against a predefined motif database in which each motif contains y-series ions (y, y-NH₃, y-H₂O) or b-series ions (b, b-NH₃, -H₂O). The scores from the matched experimental and theoretical fragments (S1, S2, S3, S4) are used to calculate the overall score for motif assignment as shown in Output file. The motif with the highest score is assigned to the precursor.

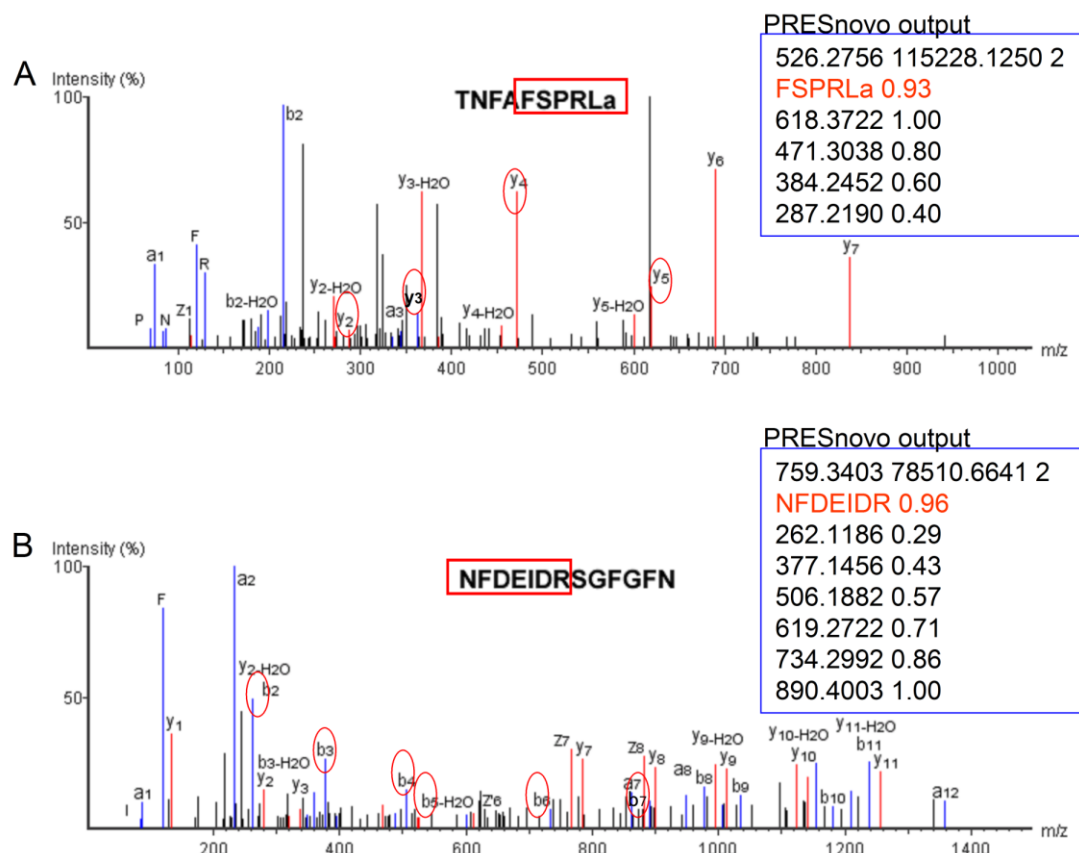


Figure 4. The tandem MS spectra and PRESnovo output. A) MS/MS spectrum of TNFAFSPRLa. PRESnovo found four motif-associated fragments (y2, y3, y4 and y5) and then assigned C-terminal motif of FSPRLa to this precursor; B) MS/MS spectrum of NFDEIDRSGFGFN. PRESnovo found six motif-associated fragments (b2, b3, b4, b5, b6 and b7) and then assigned N-terminal motif of NFDEIDR to this precursor.

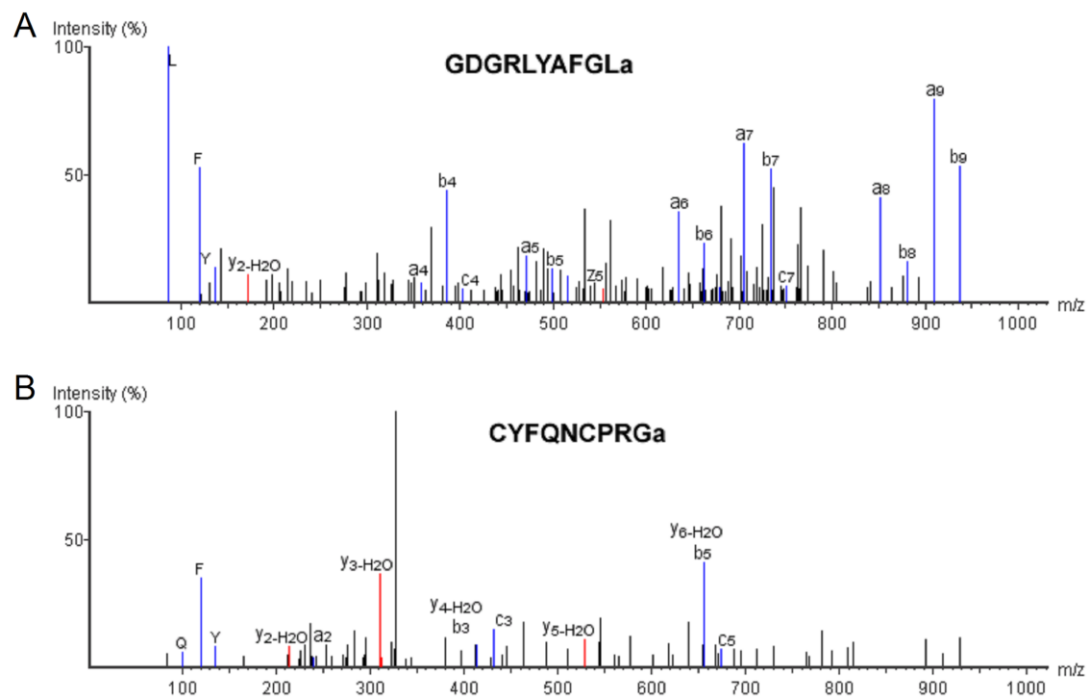


Figure 5. PRESnovo fails to interpret tandem MS spectra of AST-A peptide and disulfide bond bridging peptide. A) a/b/c ions dominate in MS/MS spectrum of GDGRLYAFGLa; B) incomplete motif-associated fragments in MS/MS spectrum of CYFQNCPRGa.

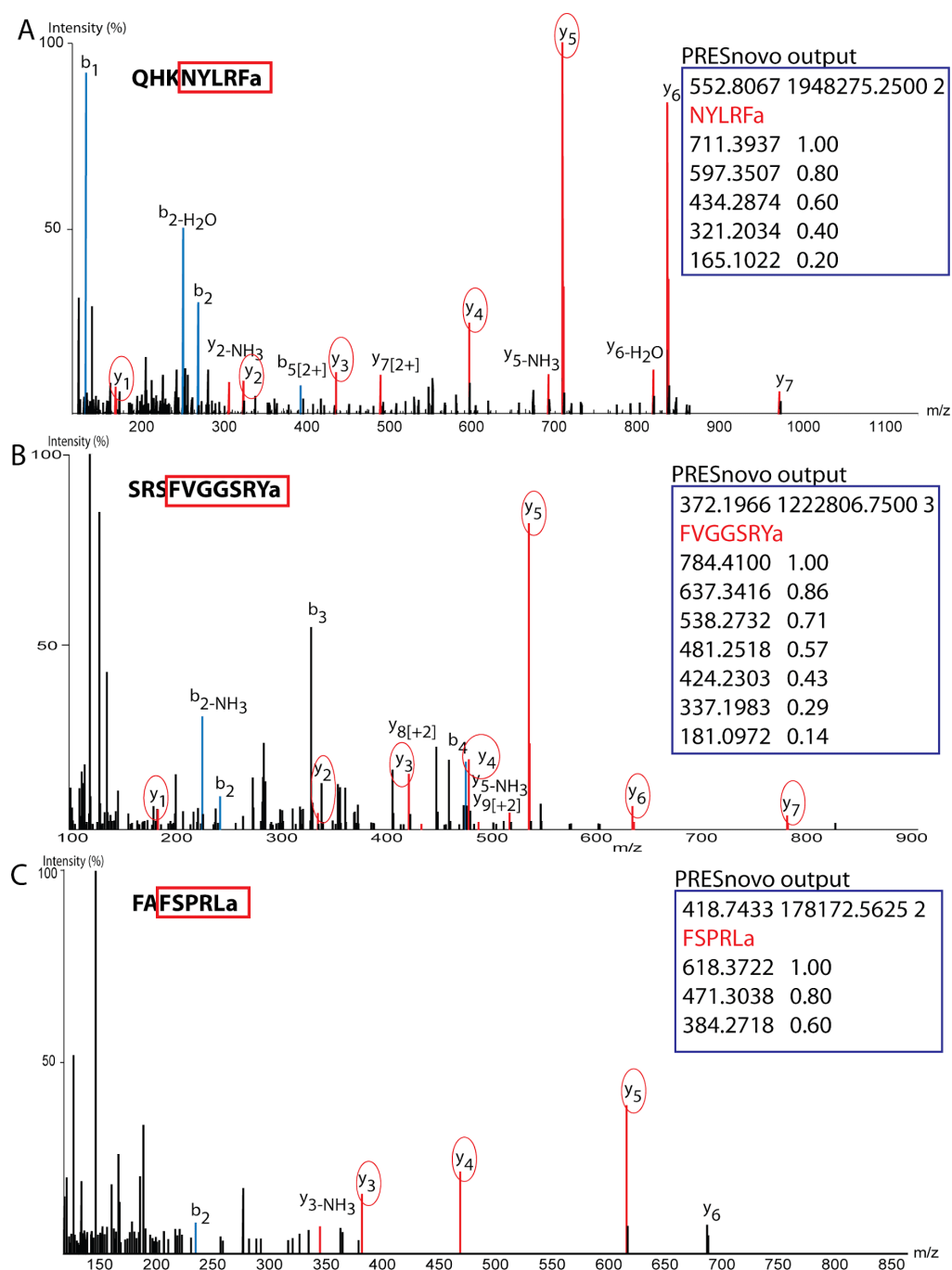


Figure 6. MS/MS spectra of putative novel neuropeptides detected in (A) brain and (B) sinus gland of Jonah crab, *C.borealis*, as well as a neuropeptide matching the crustacean neuropeptide database that PEAKS failed to assign correctly. In all 3 cases, PRESnovo was able to identify fragment ions indicative of common motifs.

Supporting Information

Protocol for mass spectrometric analysis:

Chemicals and Materials. ACS-grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). 10 μ L C18 zip tips were purchased from Agilent Technologies (Santa Clara, CA, USA). Acidified methanol for extraction was prepared with 90/9/1 water/methanol/acetic acid. For sample preparation, ACS-grade solvents and Milli-Q water (Merck Millipore, Billerica, MA, USA) were used. For instrumental analysis, Optima-grade solvents were used.

Tissue Collection. European green crabs, *C. maenas*, were collected by hand from multiple locations on Mount Desert Island, Maine, USA. Jonah crabs, *C. borealis*, were purchased from The Fresh Lobster Company (Gloucester, MA, USA). Crabs were maintained without food in an artificial seawater tank at 10–12 °C. Animals were cold-anesthetized by packing in ice for 15–30 min prior to dissection. They were then dissected and the brain and sinus glands were collected. All dissections were carried out in chilled physiological saline (composition in mM: NaCl, 440; KCl, 11; MgCl₂, 26; CaCl₂, 13; Trizma base, 11; maleic acid, 5; pH 7.45).

Tissue Extraction, Off-line HPLC Fractionation. Tissues were separately pooled, homogenized, and extracted with acidified methanol: 90% methanol (Fisher Scientific, Pittsburgh, PA), 9% glacial acetic acid (Fisher Scientific), and 1% deionized water. Extracts were dried in a Speed-Vac concentrator (Thermo Electron) and resuspended with minimum amount of 0.1% formic acid (Fisher Scientific). The resuspended extracts were then vortexed and briefly centrifuged. The resulting supernatants from green crab were subsequently fractionated via high-performance liquid chromatography (HPLC). HPLC separations were performed using a Rainin

Dynamax HPLC system equipped with a Dynamax UV-D II absorbance detector (Rainin Instrument, Inc., Woburn, MA). The mobile phases included (Solution A) deionized water containing 0.1% formic acid, and (Solution B) acetonitrile (ACN; HPLC grade, Fisher Scientific) containing 0.1% formic acid. About 20 μ L of extract was injected onto a Macrosphere C18 column (2.1 mm i.d. $\times$ 250 mm length, 5 μ m particle size; Alltech Assoc. Inc., Deerfield, IL). The separations consisted of a 120 min gradient of 5%–95% Solution B. Fractions were automatically collected every 2 min using a Rainin Dynamax FC-4 fraction collector. The resulting supernatants from Jonah crab were suspended in 0.1% FA and desalted using 10 μ L C18 ziptips following package instructions, except that three sequential elution solutions were used consisting of 25/75 ACN/water 0.1% formic acid, 50/50 ACN/water 0.1% formic acid, and 75/25 ACN/water with 0.1% formic acid.

nanoLC-ESI-QTOF MS/MS. nanoLC-ESI-QTOF MS/MS was performed using a Waters capillary LC system coupled to a Q-TOF Micro mass spectrometer (Waters Corp., Milford, MA). Chromatographic separations were performed on a C18 reverse phase capillary column (75 μ m internal diameter $\times$ 150 mm length, 3 μ m particle size; Micro-Tech Scientific Inc., Vista, CA). Three mobile phases were used: deionized water with 5% acetonitrile and 0.1% formic acid (A), acetonitrile with 5% deionized water and 0.1% formic acid (B) and deionized water with 0.1% formic acid (C). A 6.0 μ L aliquot of an HPLC fraction was injected and loaded onto the trap column (PepMap: C18; 300 μ m column internal diameter $\times$ 1 mm, 5 μ m particle size; LC Packings, Sunnyvale, CA) using mobile phase C at a flow rate of 30 μ L/min for 3 min. Following injection, the stream select module was switched to a position in which the trap column became in line with the analytical capillary column, and a linear gradient of mobile phases A and B was initiated. A splitter was added between the mobile phase mixer and the

stream select module to reduce the flow rate from 15 $\mu\text{L}/\text{min}$ to 200 nL/min . The nanoflow ESI source conditions were set as follows: capillary voltage 3200 V, sample cone voltage 35 V, extraction cone voltage 1 V, source temperature 120°C, cone gas (N_2) 10 l/h. A data-dependent acquisition (DDA) was employed for the MS survey scan and the selection of precursor ions and subsequent MS/MS of the selected parent ions. The MS scan range was from m/z 300 to 2000 and the MS/MS scan was from m/z 50 to 1800.

nanoLC-ESI-Orbitrap MS/MS. Jonah crab samples were reconstituted in 0.1% FA in Optima-grade water for MS analysis. Samples were analyzed on a Thermo Scientific Q Exactive instrument (Thermo Scientific, Bremen, Germany) coupled to a nan-ESI source connected to a Waters nanoAcquity LC system (Waters Corp, Milford, MA, USA). LC separation was carried out using a self-packed C18 column (15 cm length, 75 μm internal diameter, 1.7 μm particle size). Mobile phases consisted of water with 0.1% FA (A) and acetonitrile with 0.1% FA (B). The flow rate was 0.300 $\mu\text{L}/\text{min}$, and μL of sample was injected onto the column per run and separated over a 120-minute gradient as follows: 0-1 min 3-10% B; 1-90 min 10-35% B; 90-92 min 35-95% B; 92-102 min 95% B; 102-105 min 95-3% B; 105-120 min 3% B. The MS acquisition parameters were as follows: positive ionization mode, collision energy of 30 eV, 70,000 resolution for MS scans and 17,500 resolution for MS/MS scans, MS scan range 300 – 2,000 m/z , isolation window 2 m/z . The DDA parameters were set to top 10 precursor ions, 2 m/z isolation window, 40 s dynamic exclusion, and the MS/MS scan range was adjusted depending on the parent mass, with 50 m/z being the first fixed mass.

Table S1. The peptide standard used for performance analysis of PRESnovo.

SGGFAFSPRLamide and GAHKNYLRF were synthesized at the Biotechnology Center of the University of Wisconsin at Madison. The remaining were purchased from the American Peptide Company (Sunnyvale, CA).

Family	Monoisotopic Mass	Sequence
CCAP	955.365	PFCNAFTGCa*
RYamide	975.445	SGFYANRYa
Pyrokinin	1036.534	SGGFAFSPRLa
Pyrokinin	1050.55	TNFAFSPRLa
AST-A	1066.545	GDGRLYAFGLa
AVP	1083.435	CYFQNCPRGa
FLP	1104.572	GAHKNYLRF
AST-B	1106.497	QWSSMRGAWa
AST-B	1259.641	SGKWSNLRGAWa
Angiotension	1281.651	DRVYVHPFHL
AST-A	1334.699	APSGAQRLYGFGLa
AST-B	1469.684	VPNDWAHFRGSWa
Orcokinin	1472.657	NFDEIDRSGFGFAa
Orcokinin	1473.641	NFDEIDRSGFGFA
Orcokinin	1516.647	NFDEIDRSGFGFN

*a: C-terminal amidation

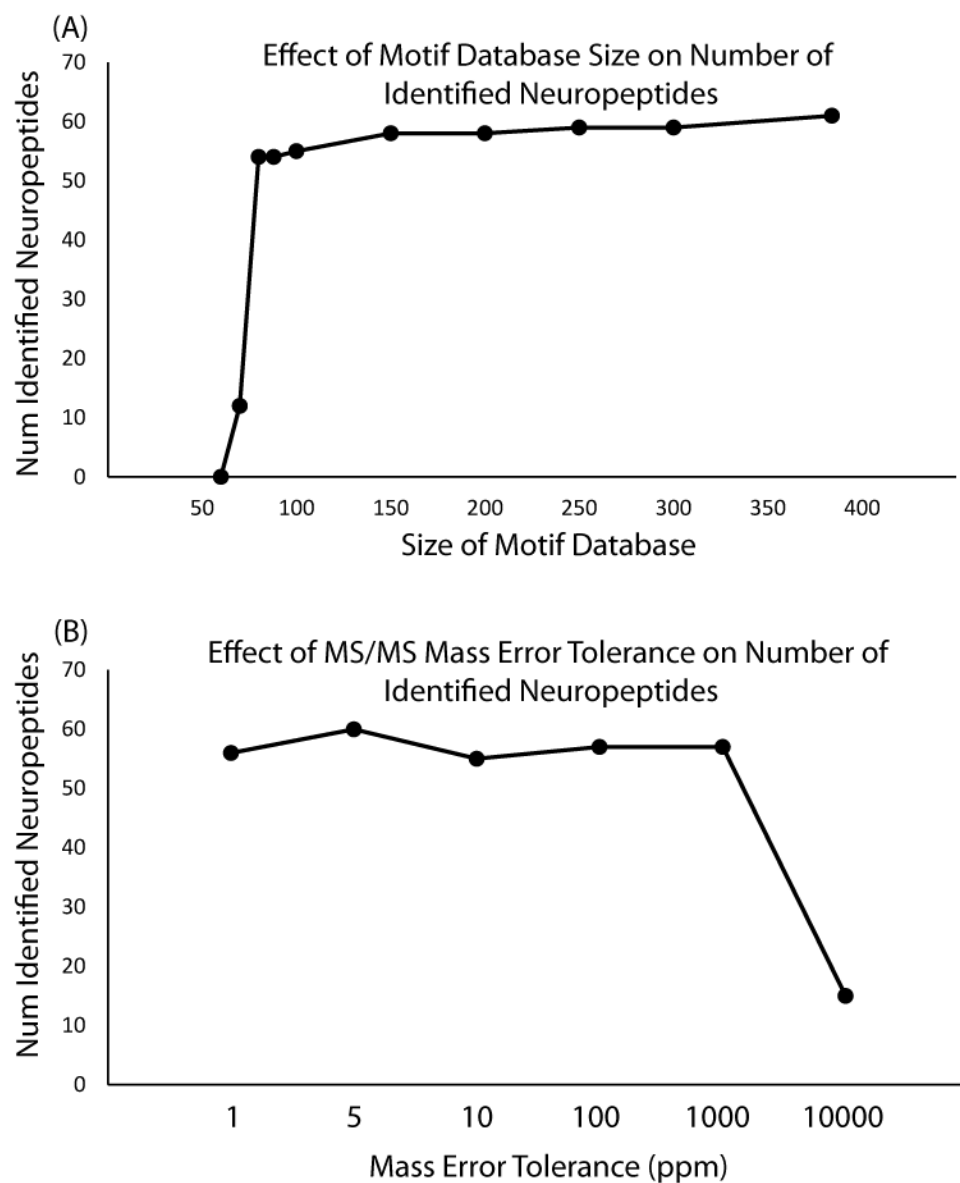


Figure S1. Effect of motif database size and mass error tolerance on number of identified neuropeptides in tissue, including (A) the effect of changing the size of the motif database on the resulting number of identified neuropeptides and (B) the effect of changing the MS/MS error tolerance on the resulting number of identified neuropeptides.

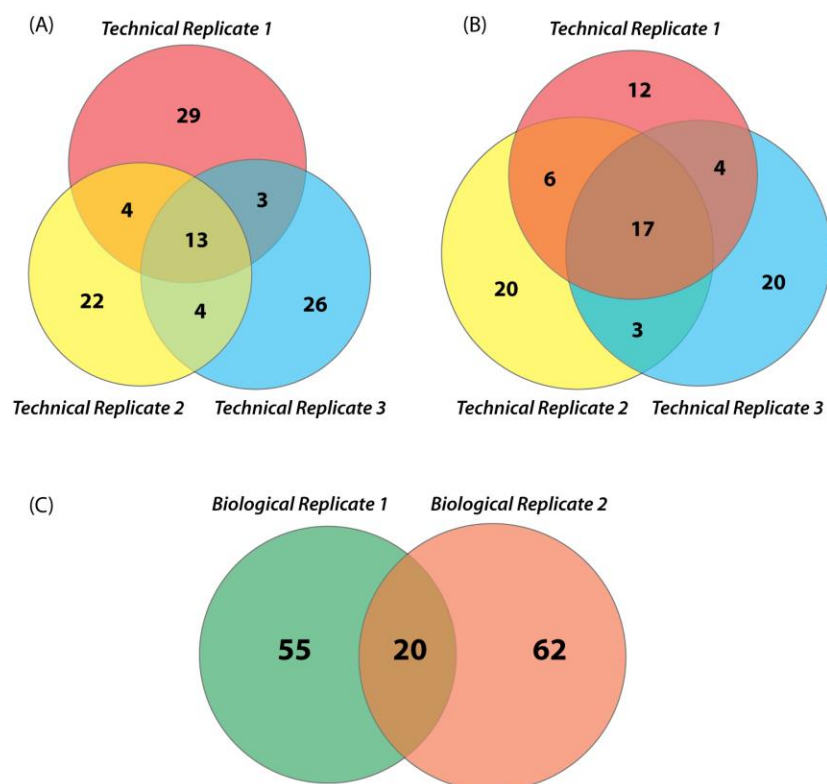
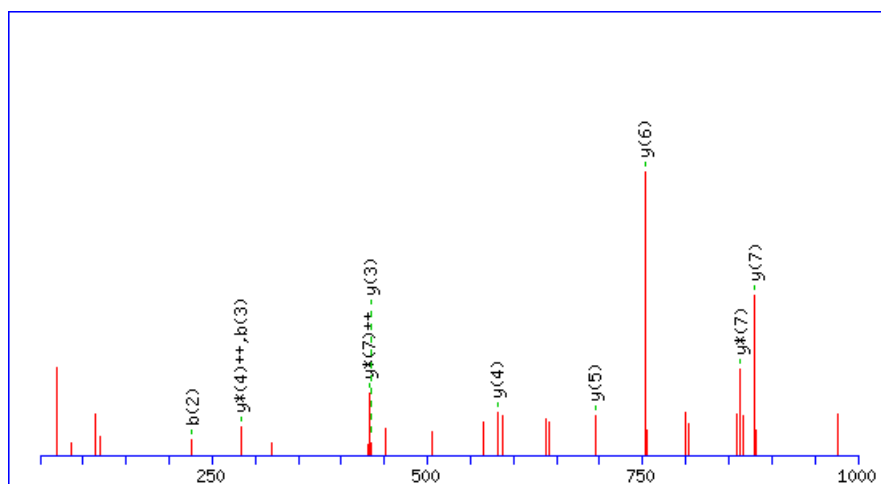


Figure S2. Overlap of identified neuropeptides in (A and B) technical replicates consisting of 3 replicate injections of the same sample, with (A) and (B) representing distinct biological replicates, and (C) biological replicates in which brain extracts came from distinct animals.

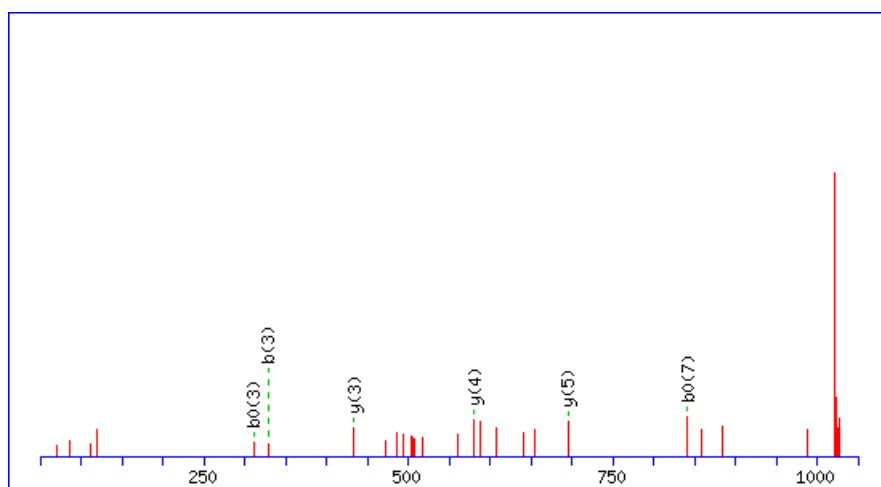
Figure S3. MS/MS spectra for novel neuropeptides identified in brain extract from green crab.

b*: b-NH₃; b0: b-H₂O; y*: y-NH₃; y0: y-H₂O.

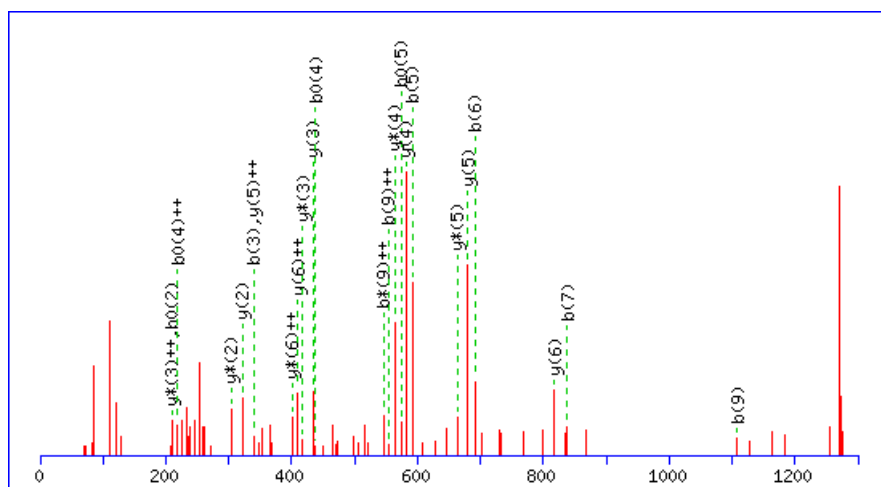
1. PQGNFLRFa:



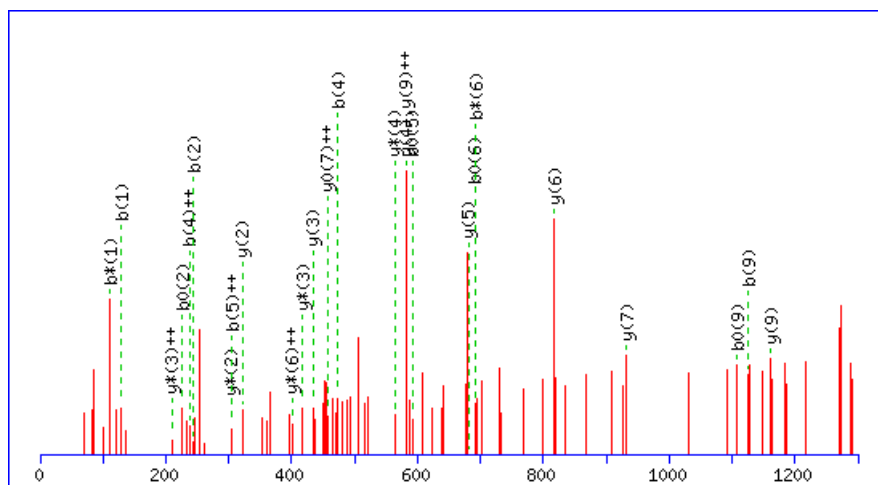
2. GDRNFLRFa:



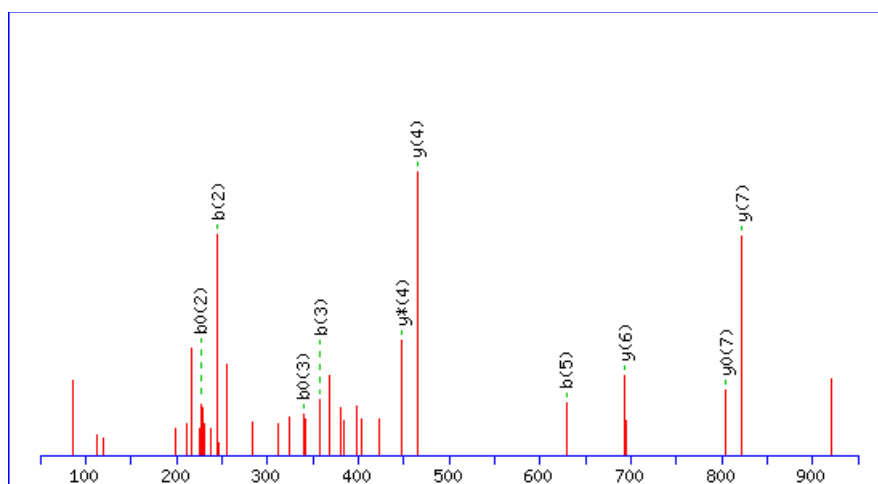
3. PELDHVFLRFa:



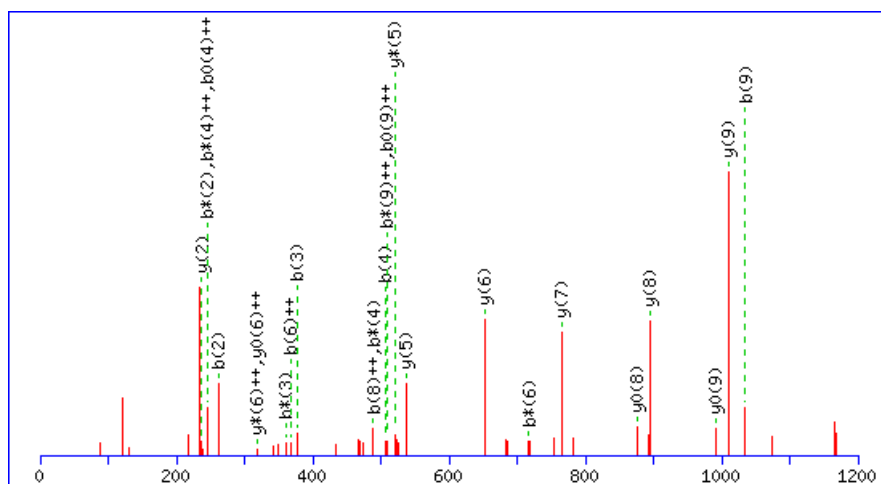
4. QDNDHVFLRFa:



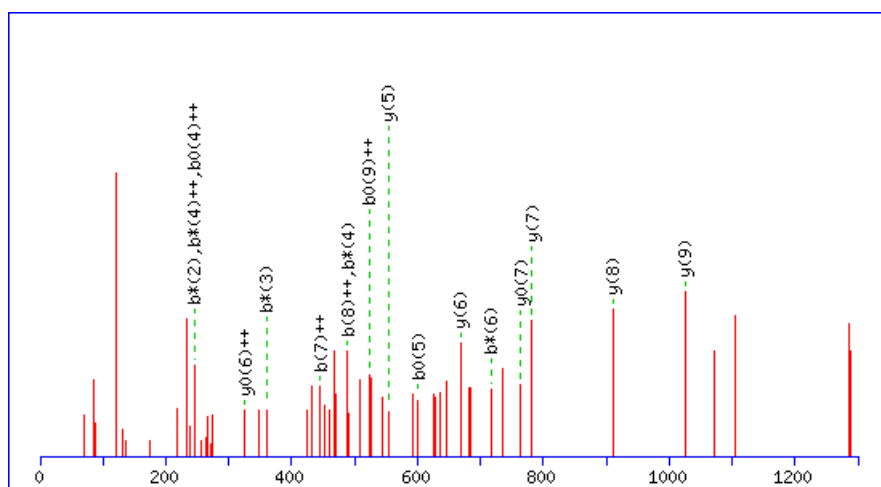
5. DEIDRSGFa:



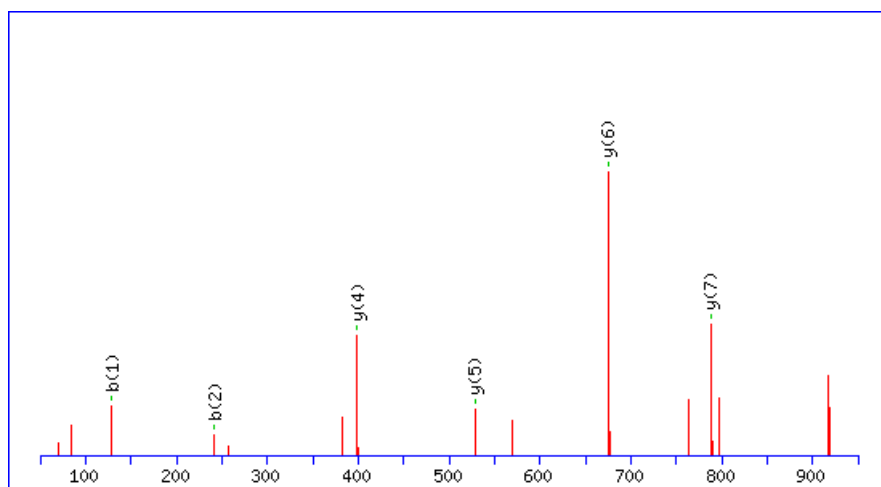
6. NFDEIDRSGFa:



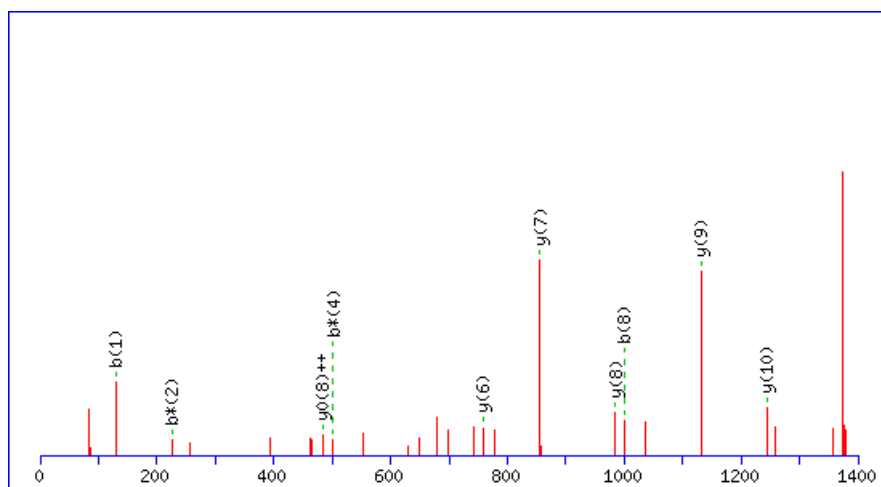
7. NFDEIDRSSFG:



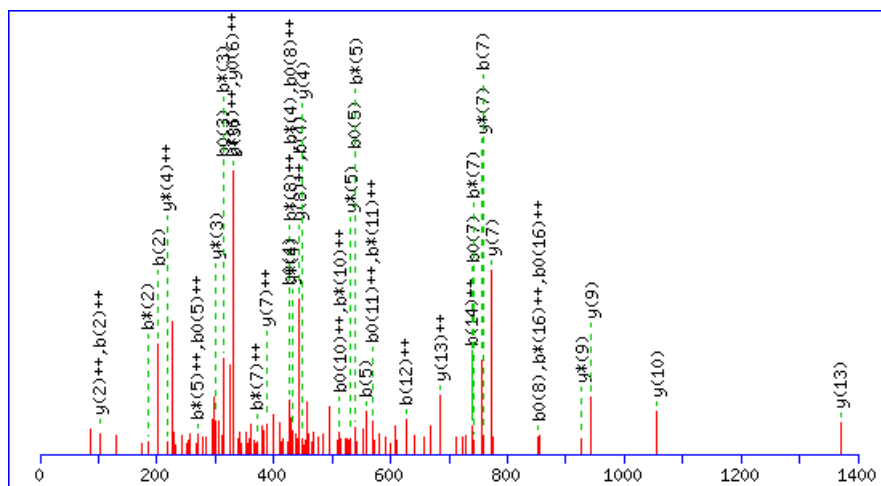
8. KIFEPLVA:



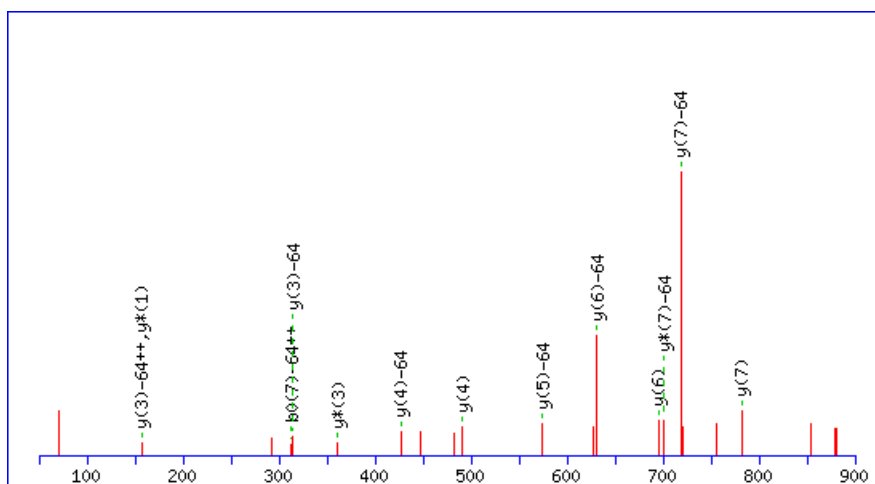
9. KIFEPLRDKNL



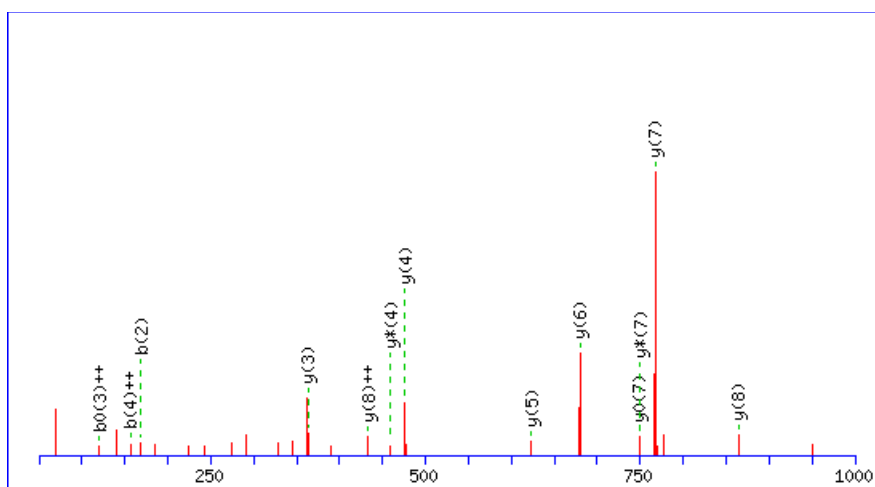
10. NSELINSLGIPKVMNDAA



11. PSGFLGM(O)Ra



12. APSGFLGMR



13. APSGFLGM(O)RG

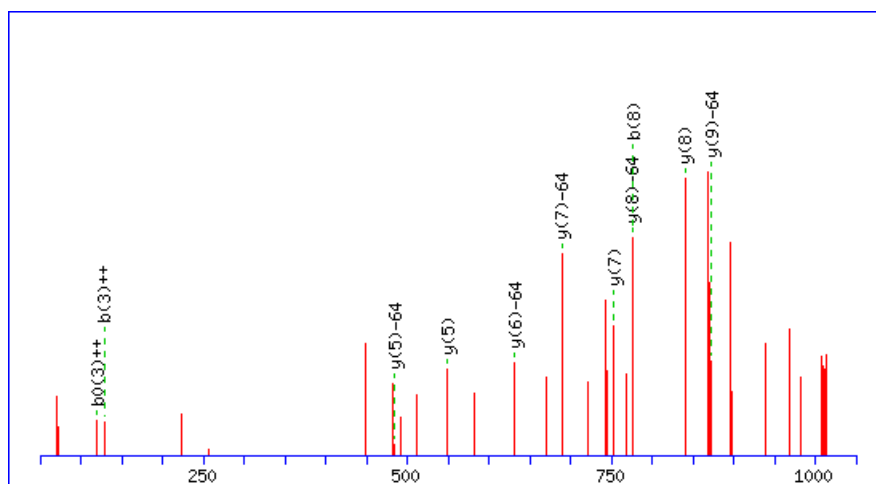
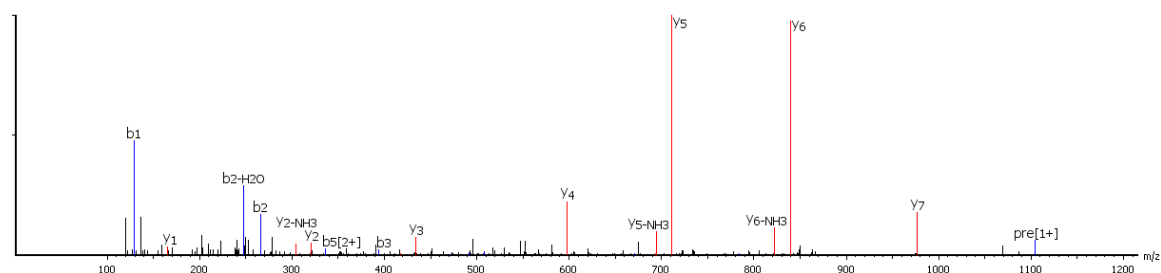
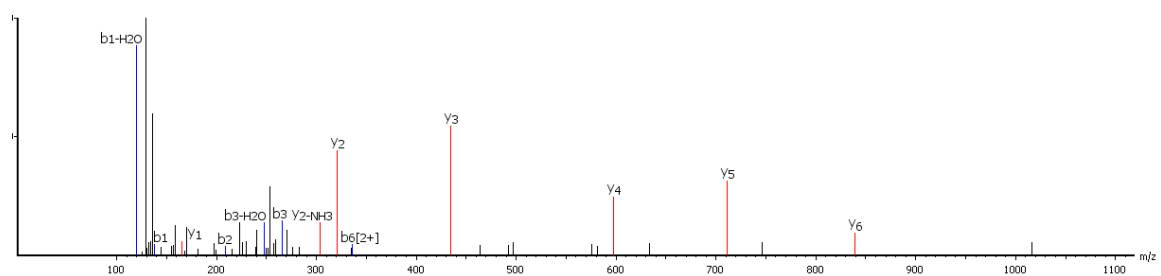


Figure S4. MS/MS spectra for novel neuropeptides identified in brain extract from Jonah crab.

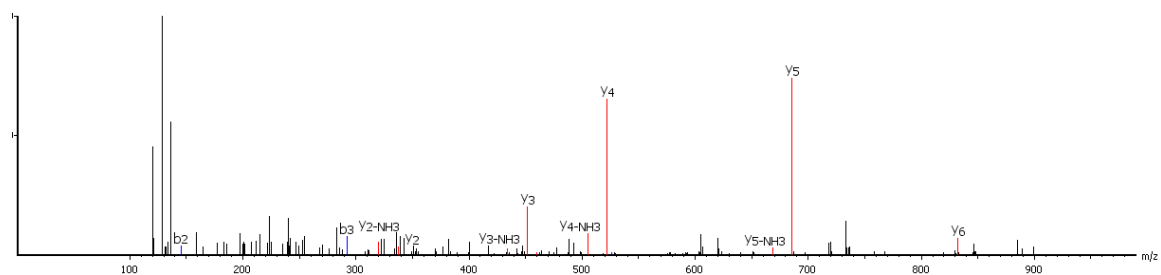
1. QHKNYLRFa



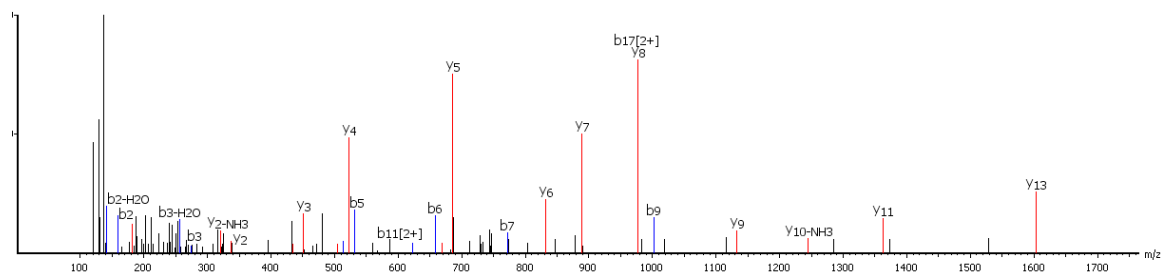
2. HAGKNYLRFa



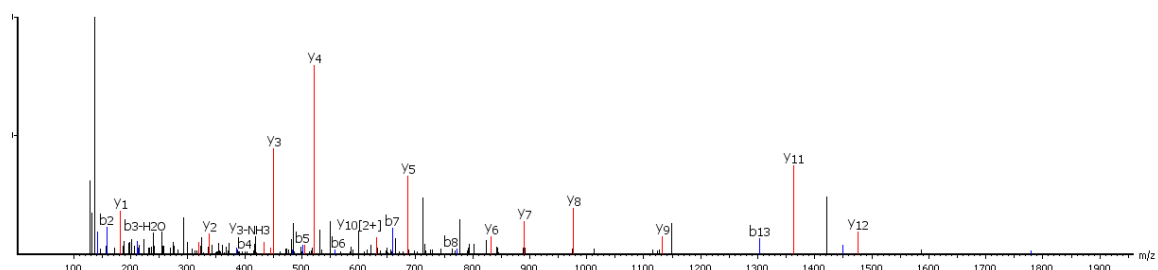
3. GSFYANRYa



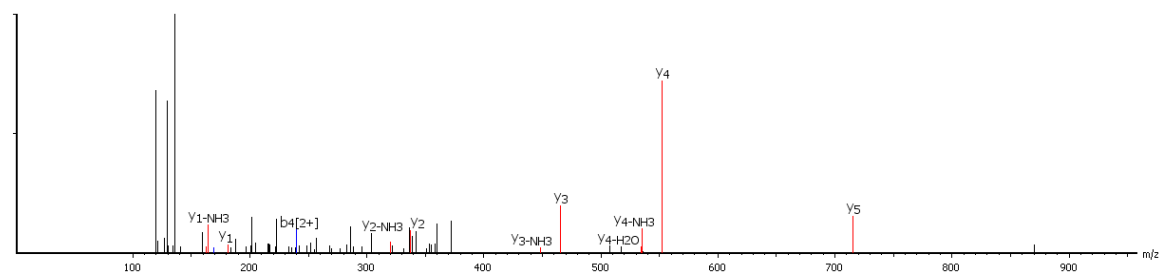
4. SADRTQLTERSGFYANRYa



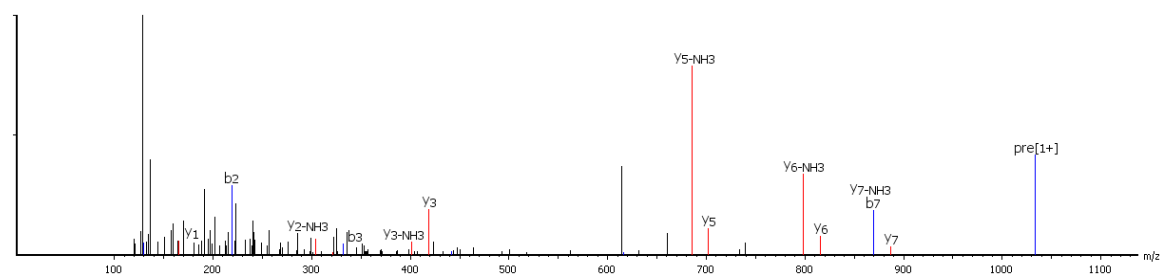
5. TGARDGTLTERSGFYANRYa



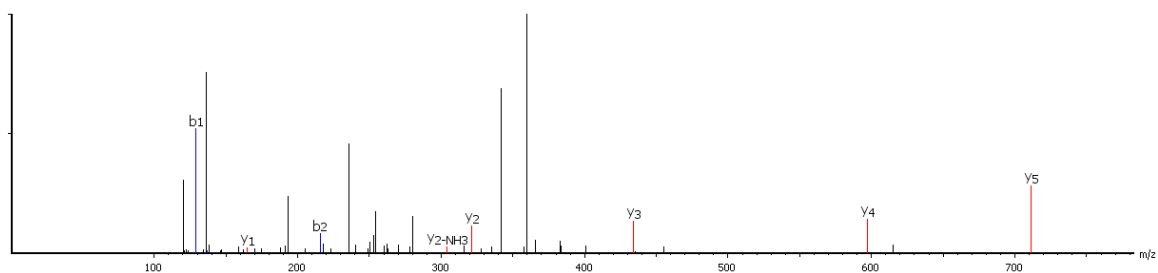
6. pQGFYSQRYa



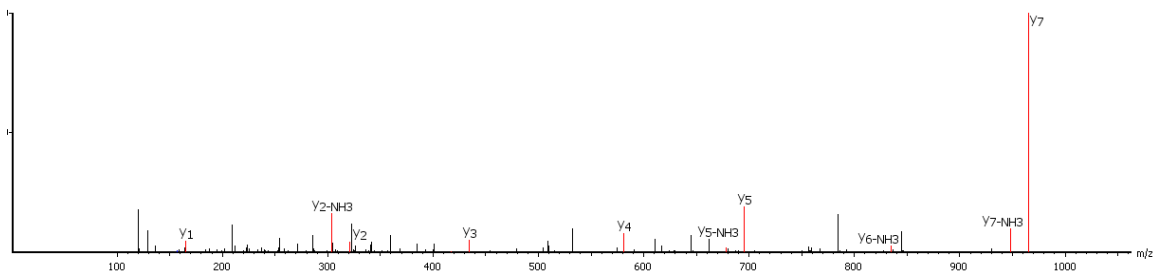
7. FALAGRPRFa



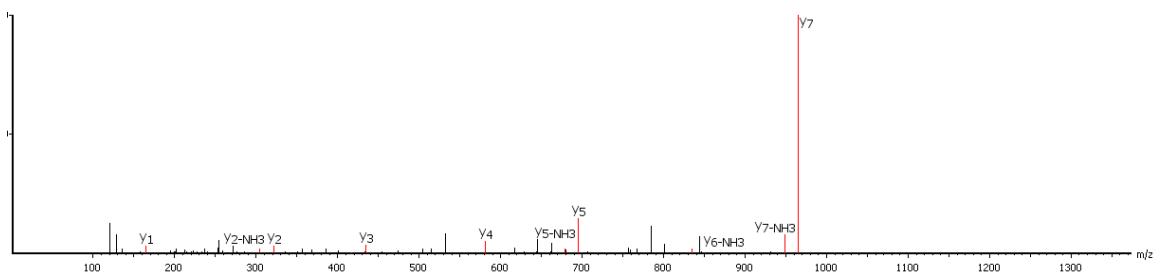
8. KSGYNYLRFa



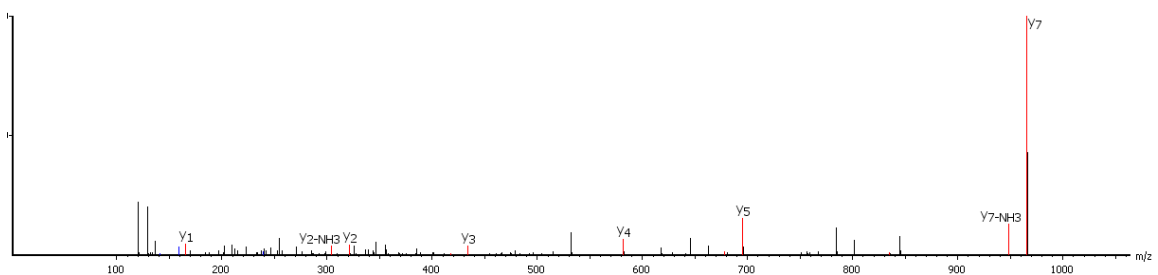
9. RQNRNFLRFa



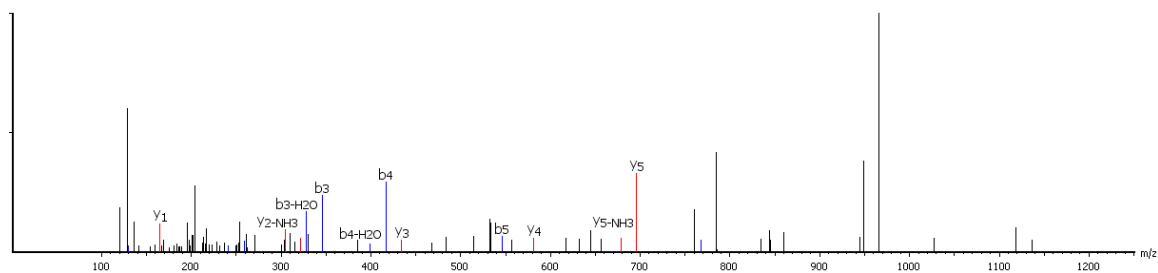
10. CCPNRNFLRFa



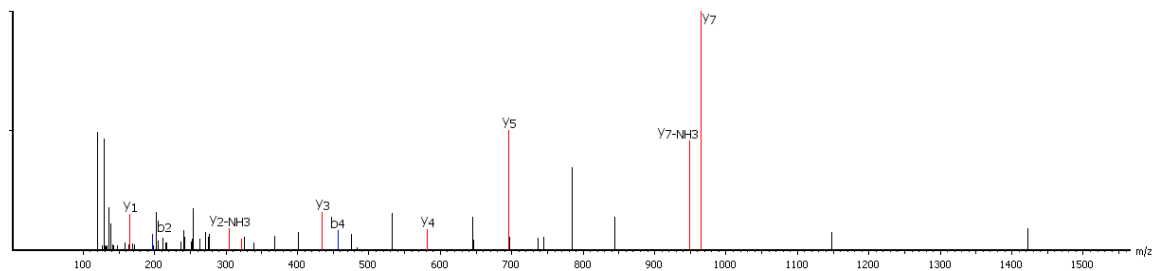
11. SAPNRNFLRFa



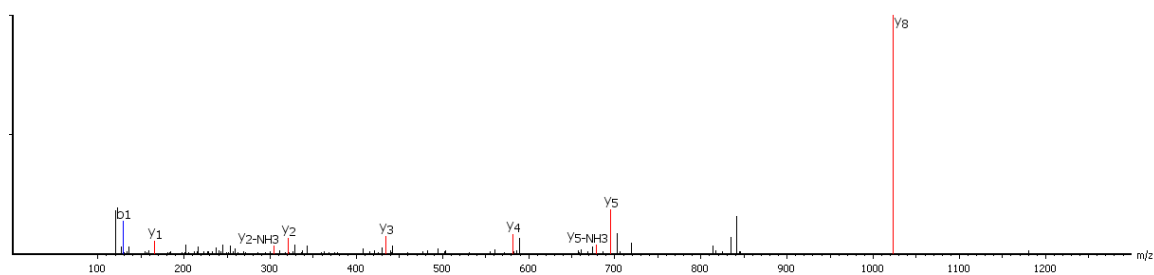
12. EESA EVPPNFLRFa



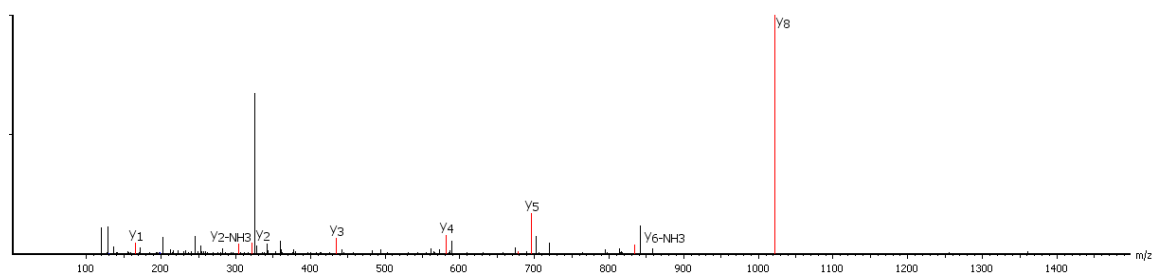
13. PVMEMRNNFLRFa



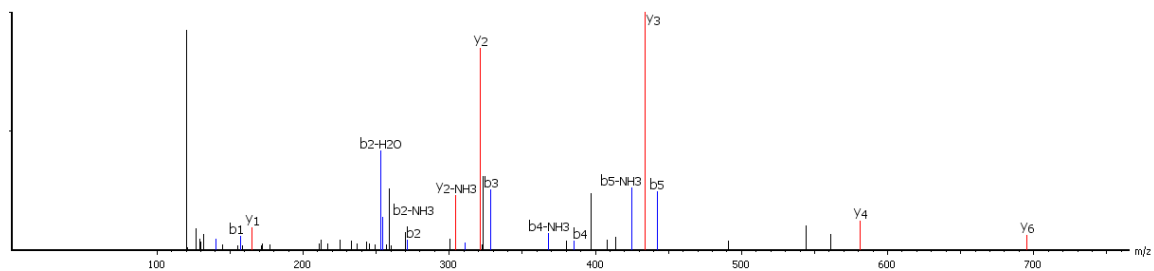
14. KRMVPNFLRFa



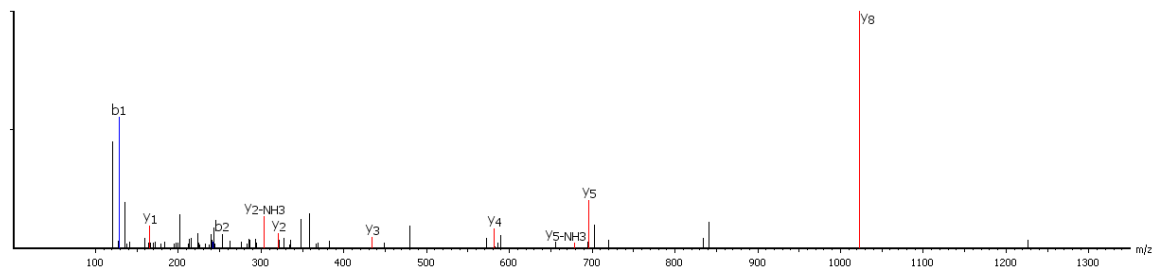
15. ESSNGRNFLRFa



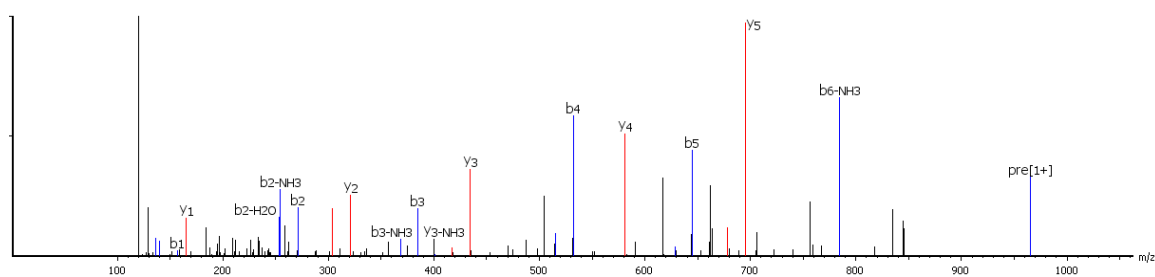
16. RNGNFLRFa



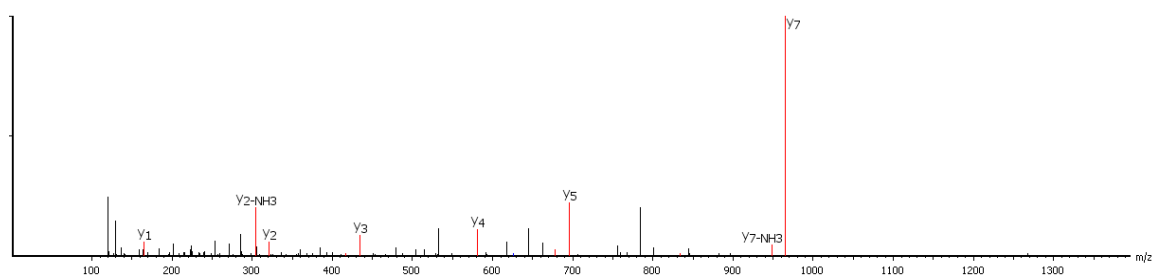
17. QLAGNRNFLRFa



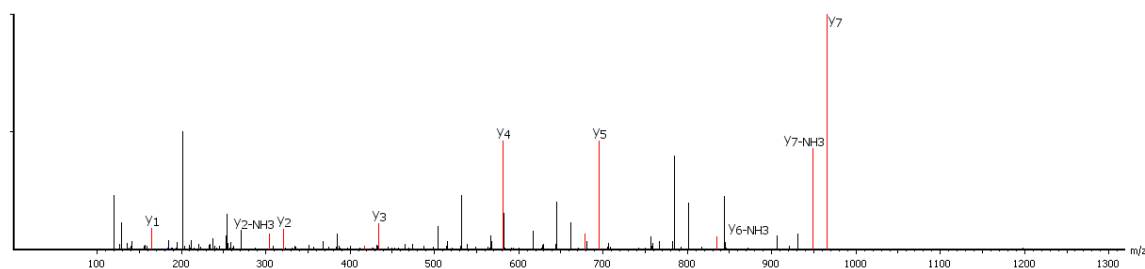
18. RNNFLRFa



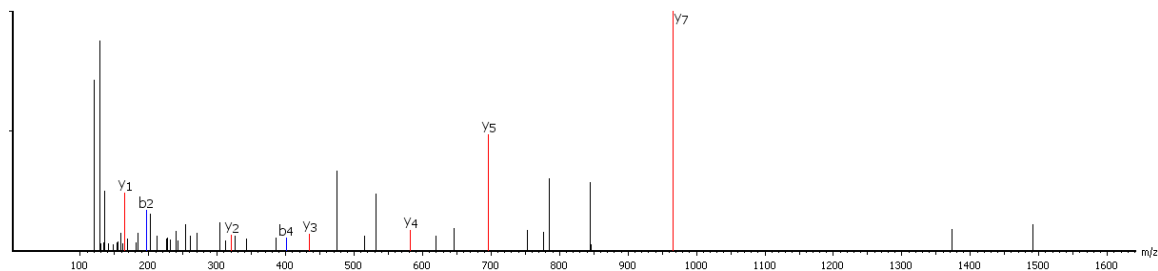
19. QRNRNFLRFa



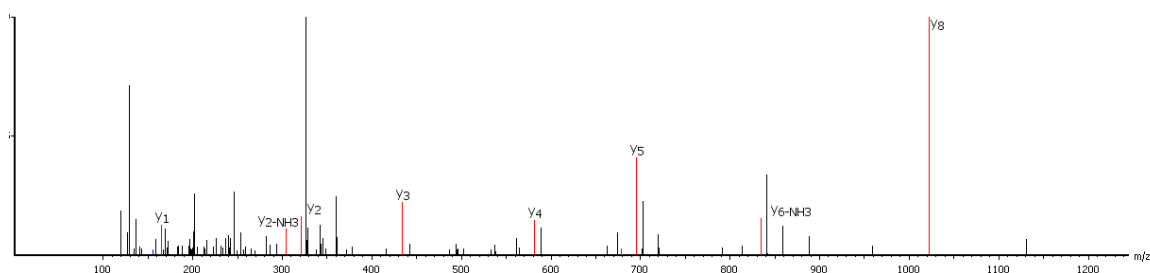
20. EACNRNFLRFa



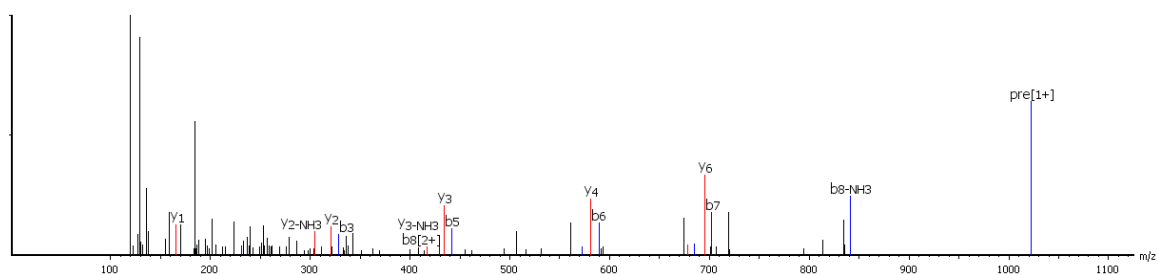
21. VPGFAPNRNFLRFa



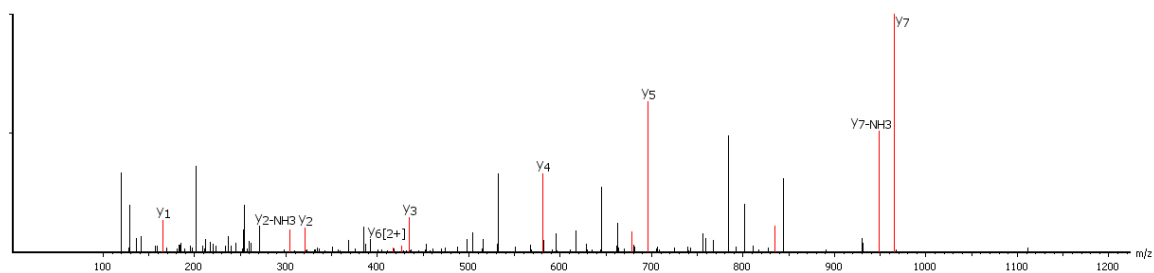
22. DGMGNRNFLRFa



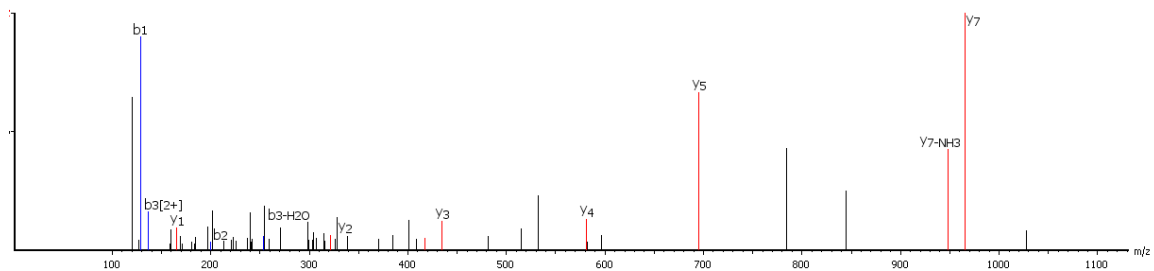
23. MVPNFLRFa



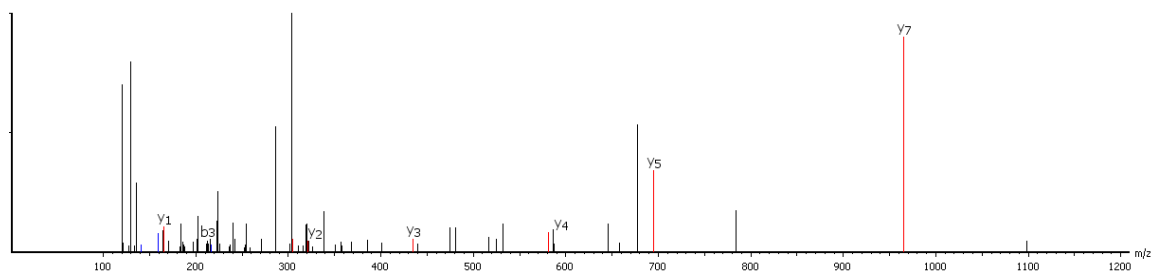
24. CAENRNFLRFa



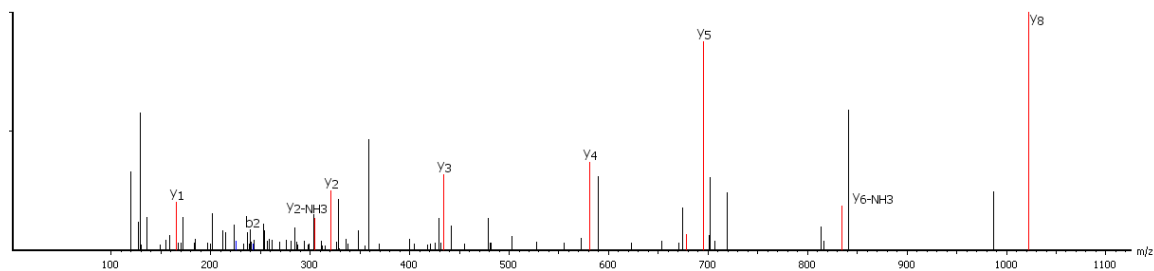
25. KAAPSNRNNFLRFa



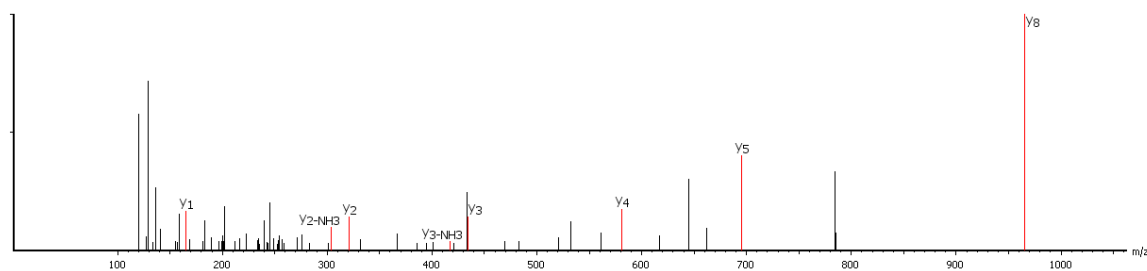
26. SAGPNRNNFLRFa



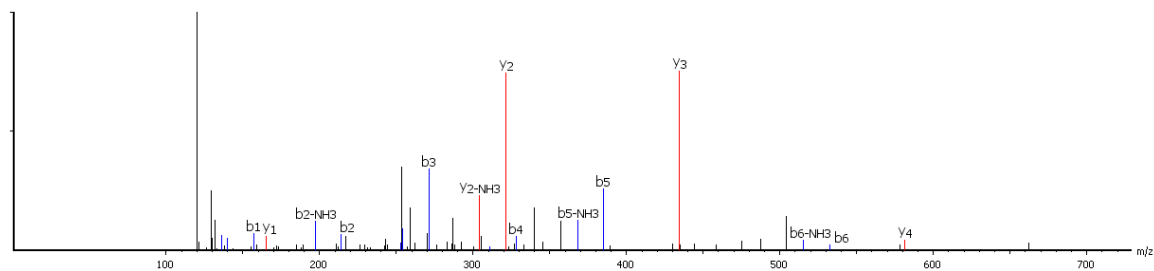
27. LQAGNRNNFLRFa



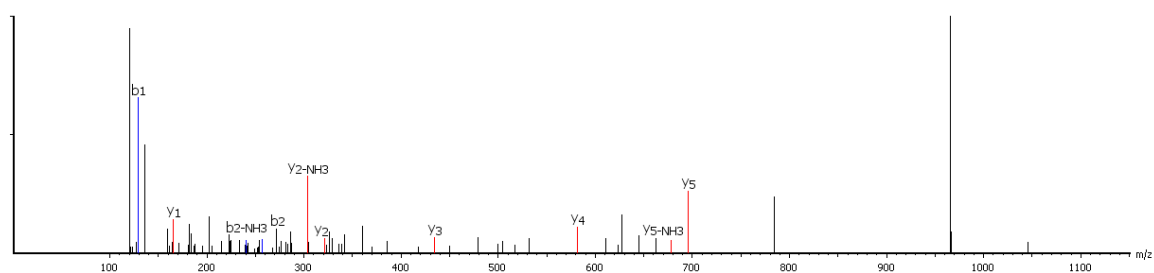
28. CCPGGRNFLRFa



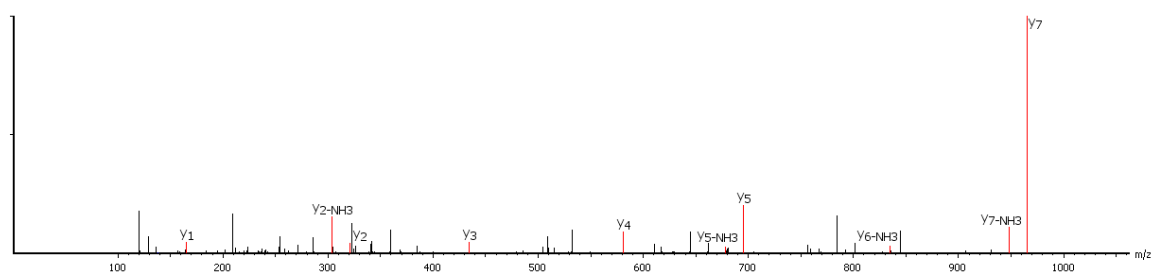
29. RGGNFLRFa



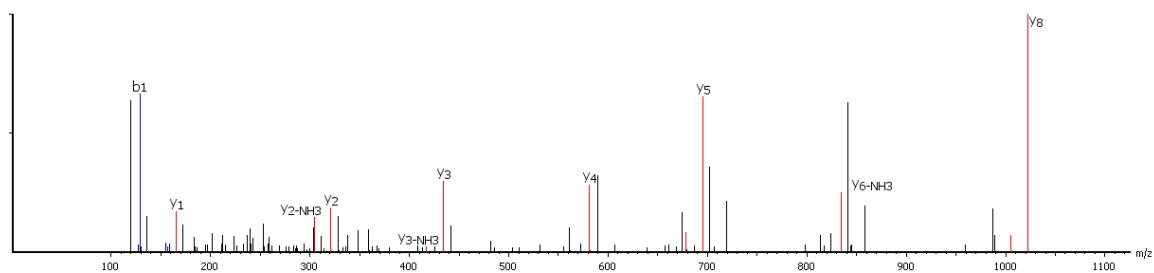
30. KQQLGNFLRFa



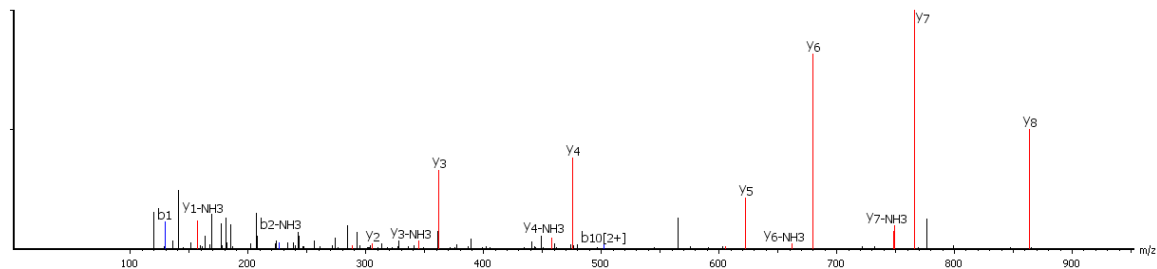
31. HFNRNFLRFa



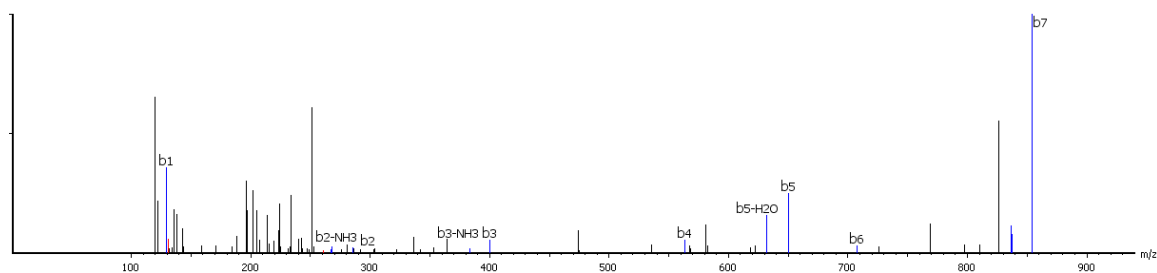
32. KSPNGRNFLRFa



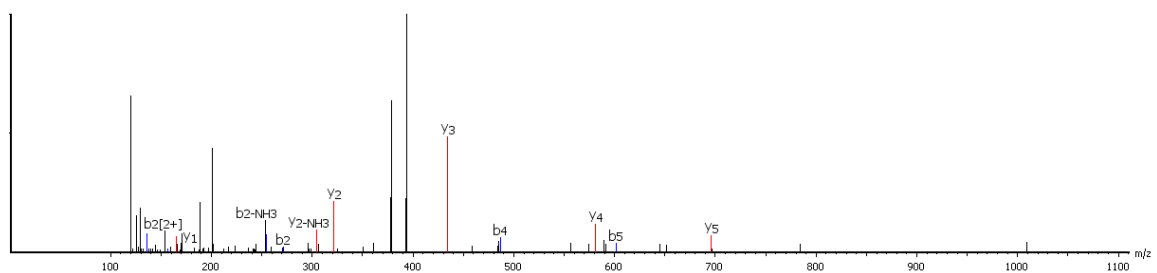
33. KNAPSGFLGMRa



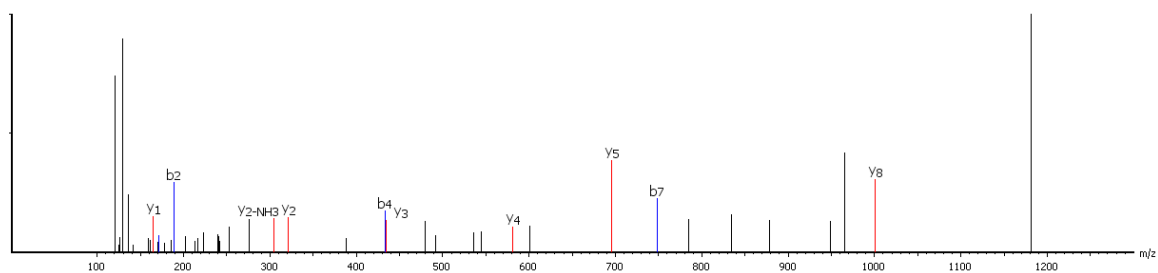
34. QRDYSFGLa



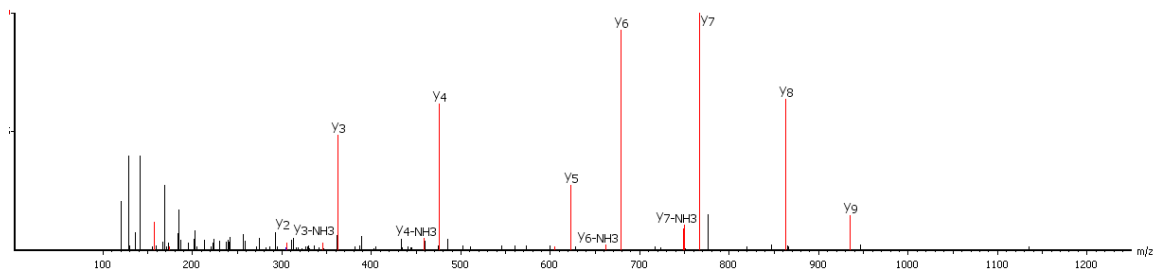
35. RNESNFLRFa



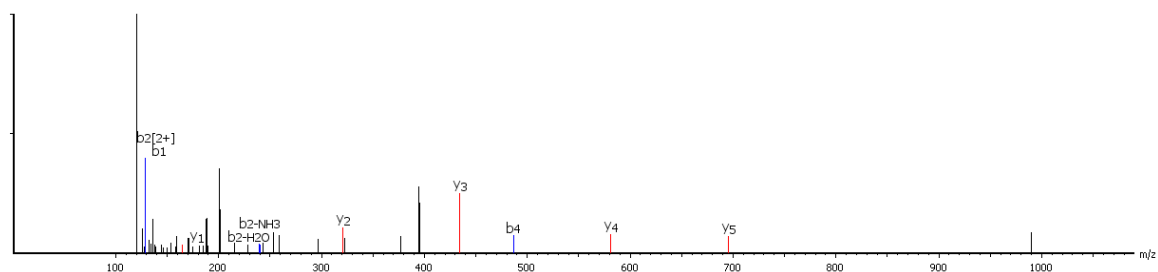
36. TSELTTCNFLRFa



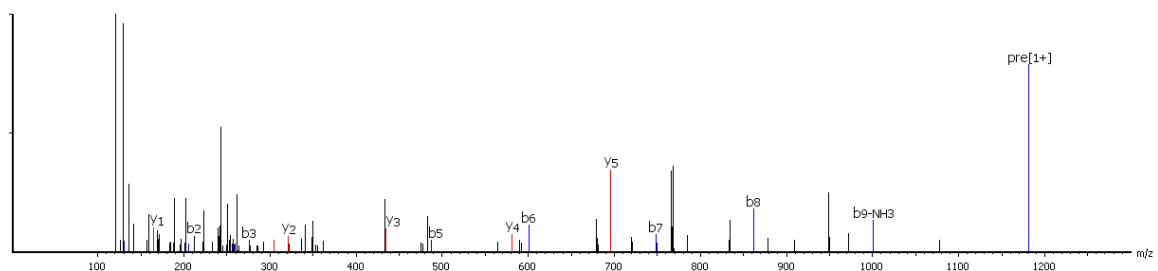
37. NCCAPSGFLGMRa



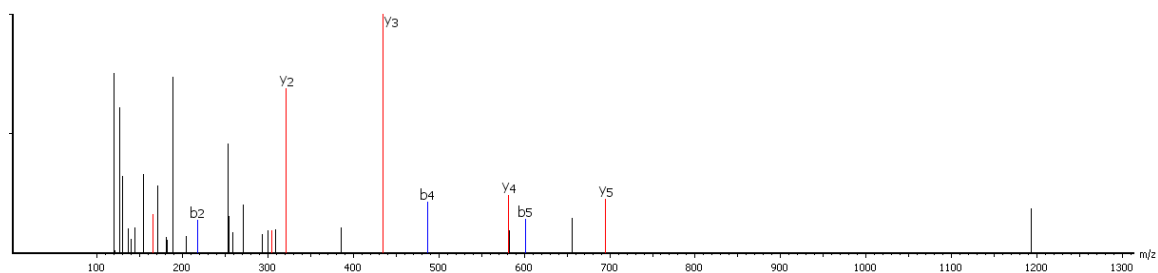
38. QKDDNFLRFa



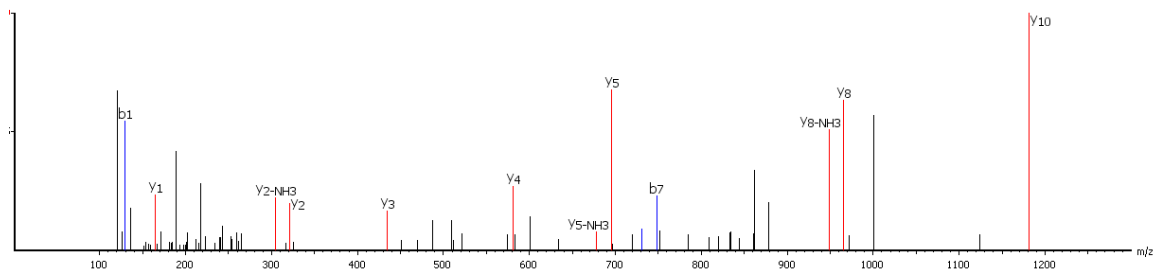
39. FGAPNNFLRFa



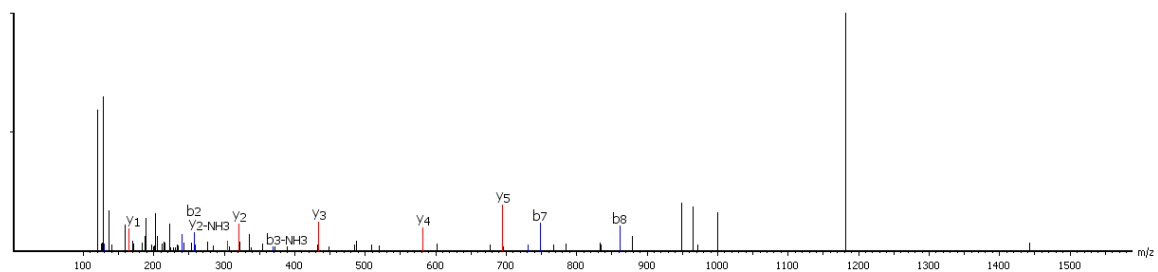
40. ESRNNFLRFa



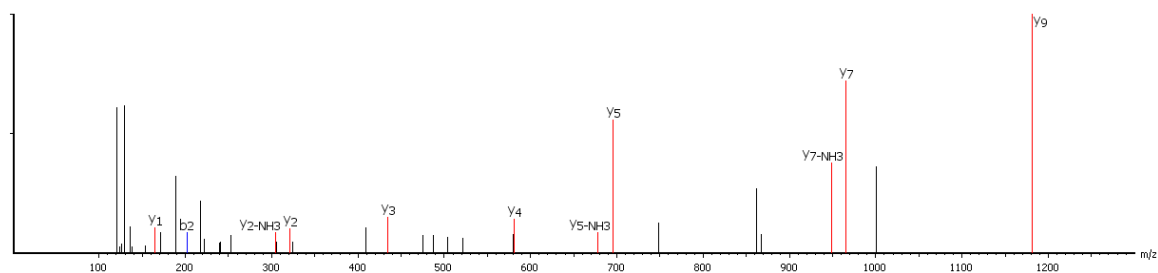
41. KCSTDGRGNFLRFa



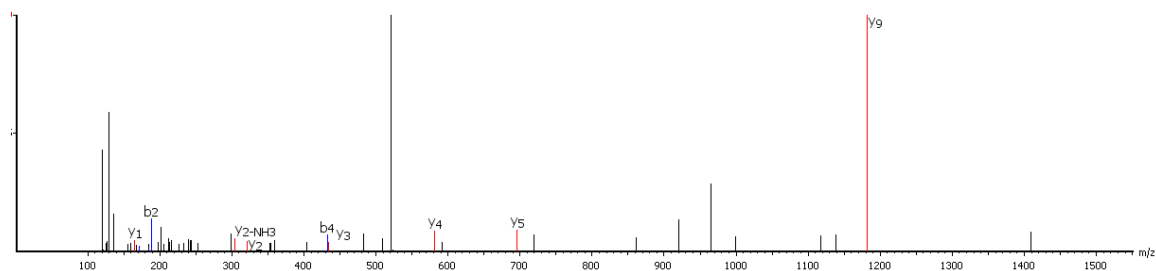
42. EQQPHAGLSAGNFLRFa



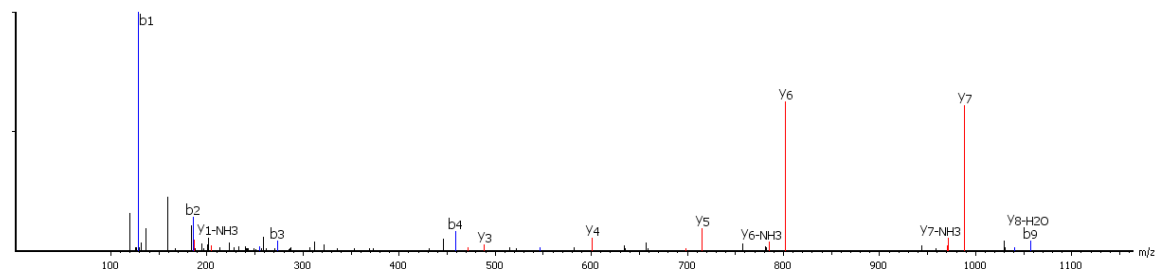
43. NSYSERNNFLRFa



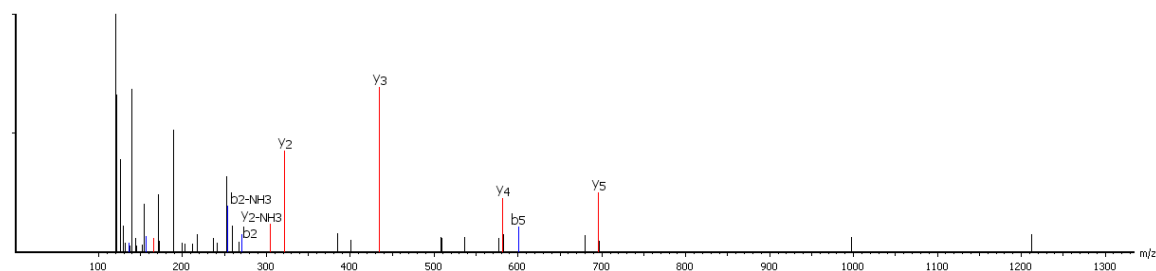
44. STDEPYPNFLRFa



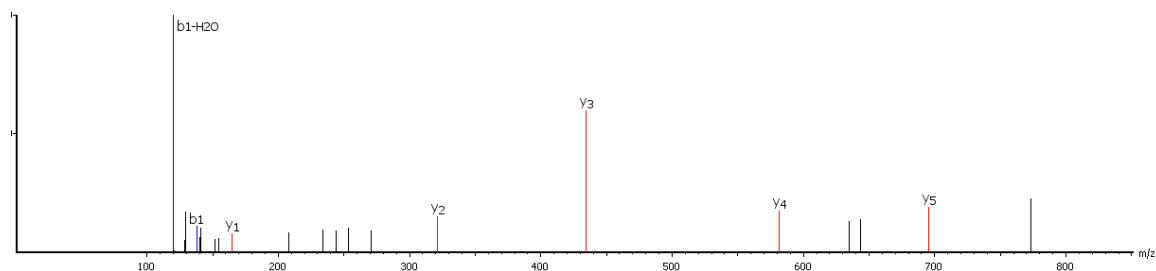
45. KGSWSNLRGAWa



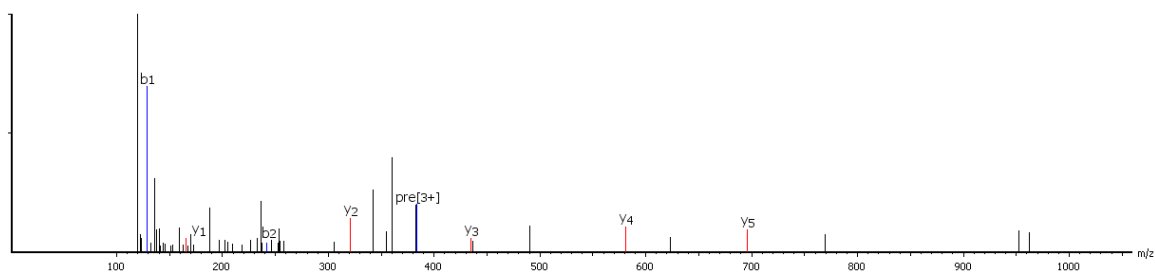
46. RNDTNFLRFa



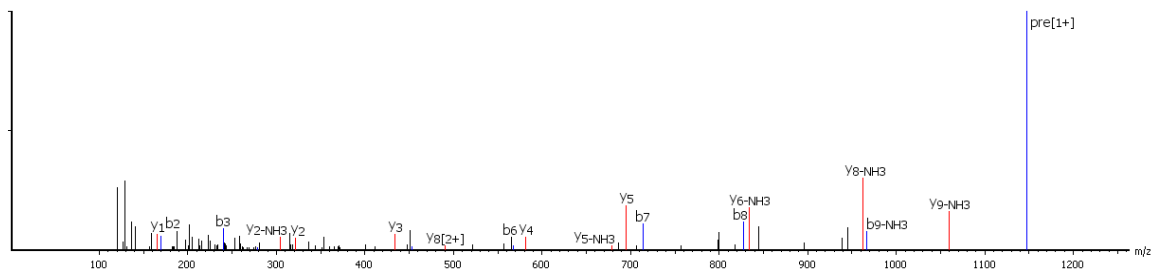
47. HSDLNFLRFa



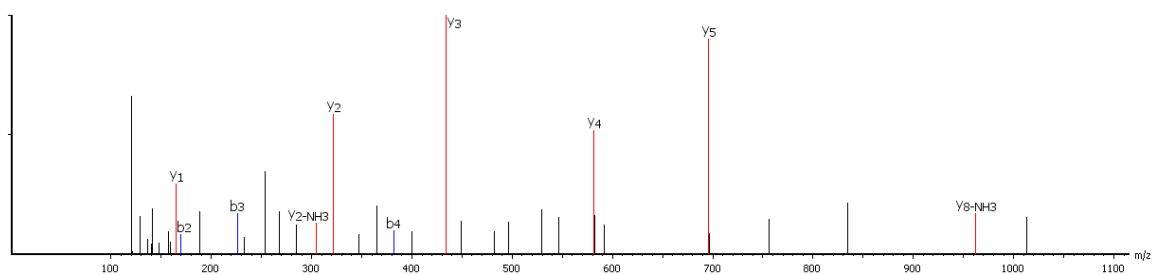
48. KLPNNFLRFa



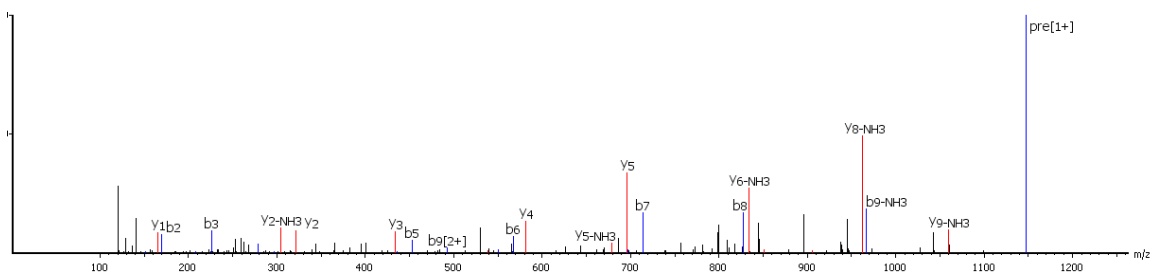
49. APAGRNFRLRFa



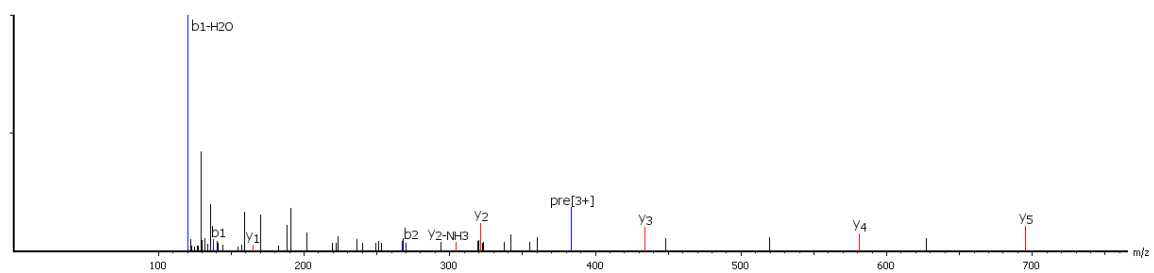
50. APGRANFLRFa



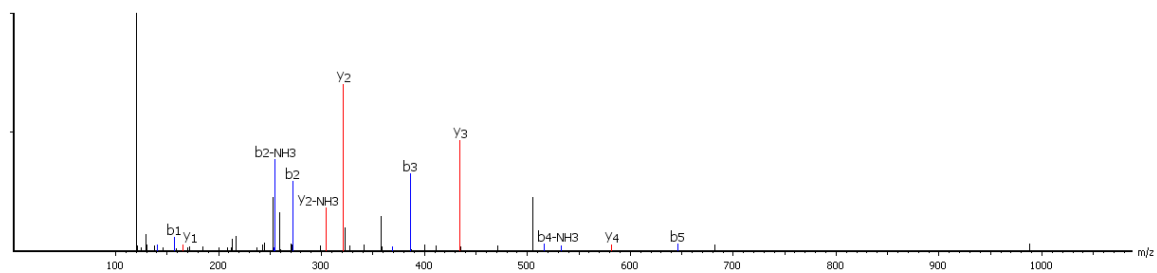
51. APGARNFRLRFa



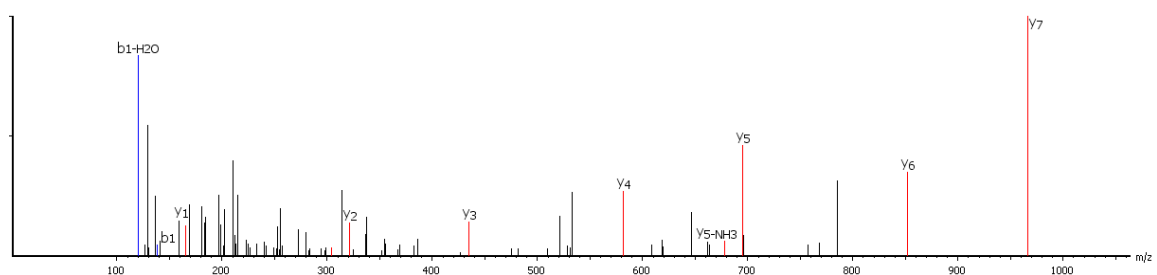
52. HEVSNFLRFa



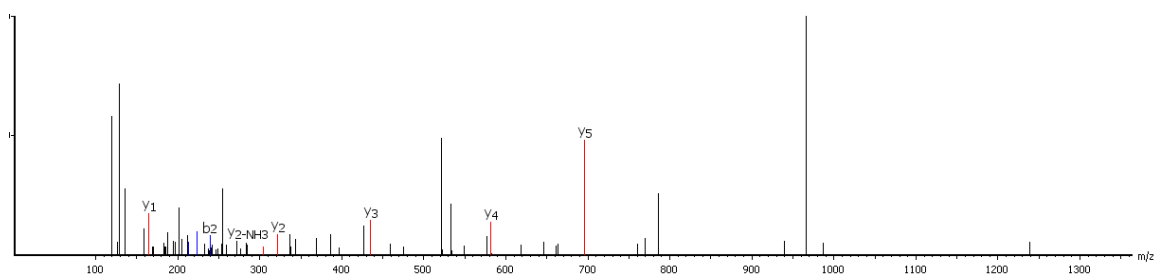
53. RDNFLRFa



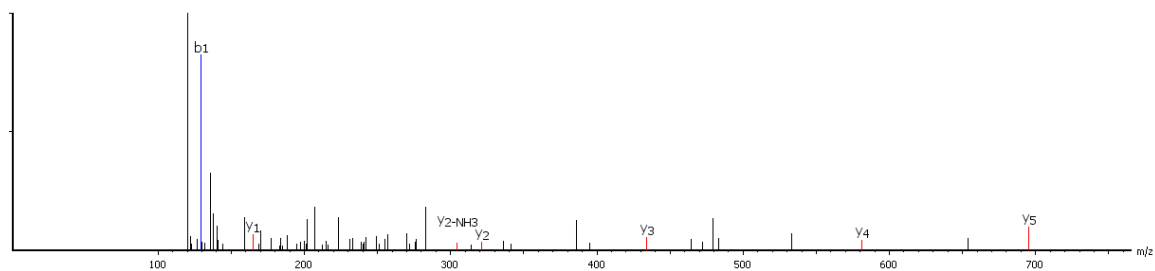
54. HFDRNFLRFa



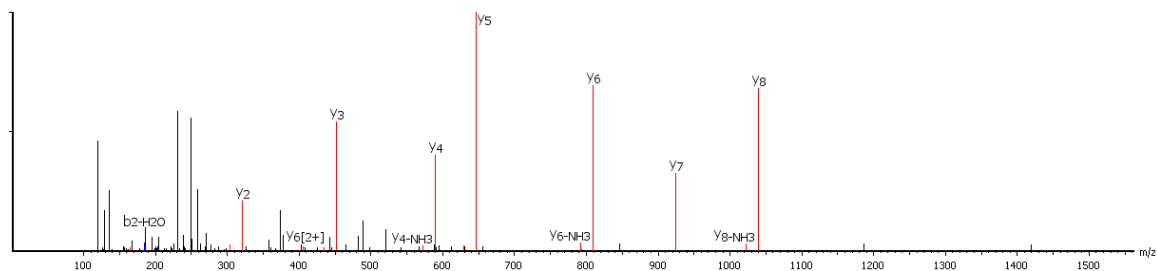
55. EQANDNFLRFa



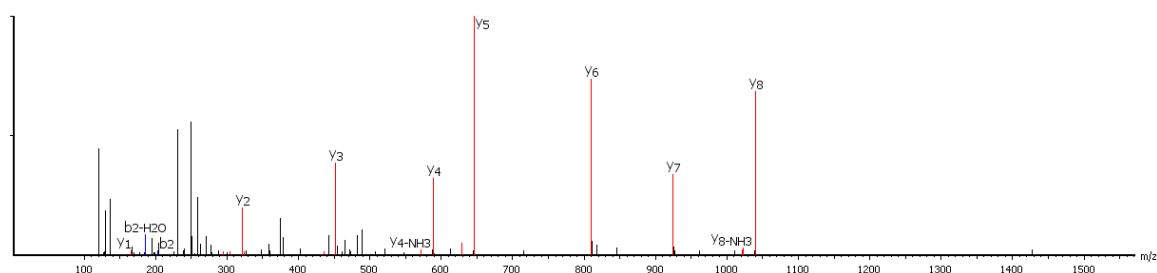
56. KGSNFLRFa



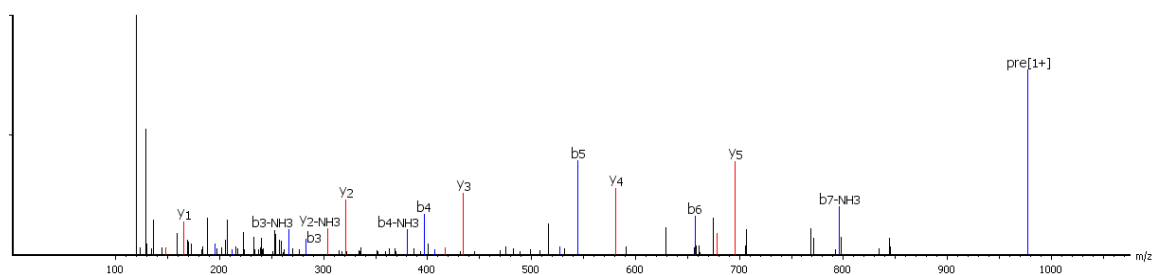
57. DSPGDDYGHMRFa



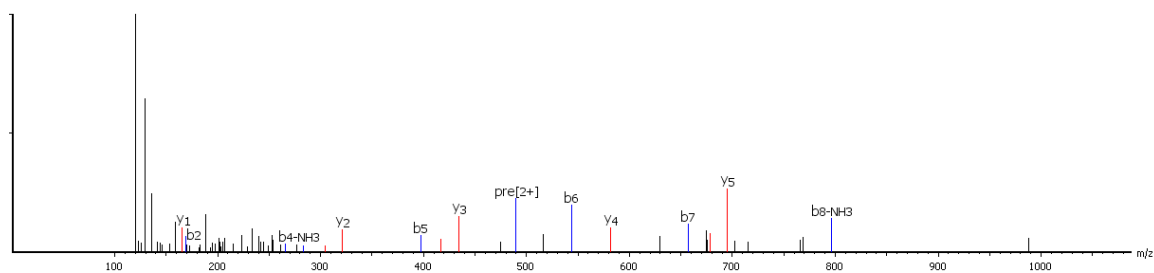
58. SDGPDDYGHMRFa



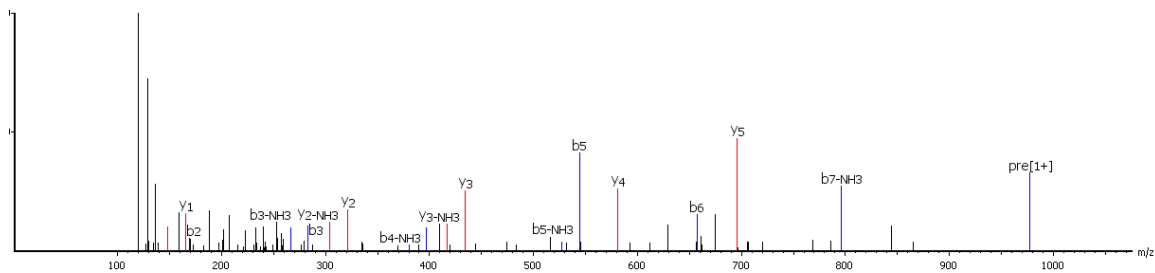
59. NPANFLRFa



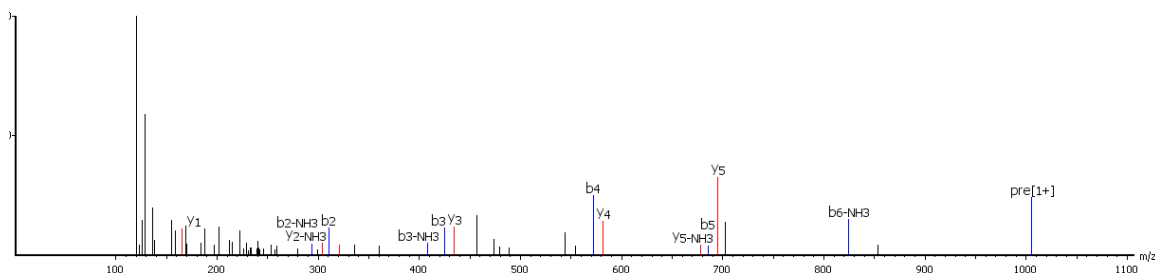
60. PAGGNFLRFa



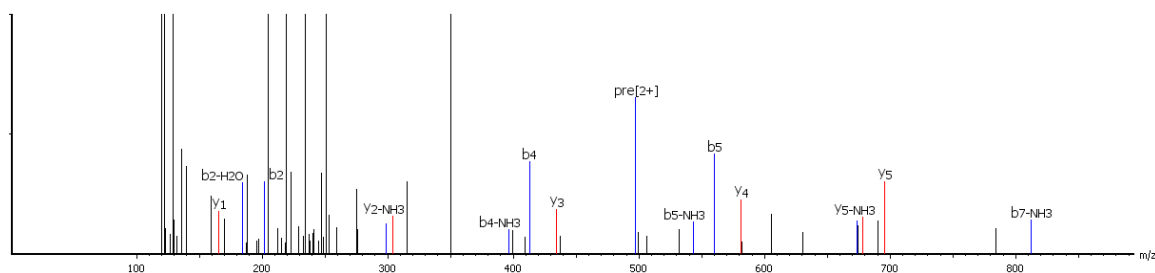
61. APNNFLRFa



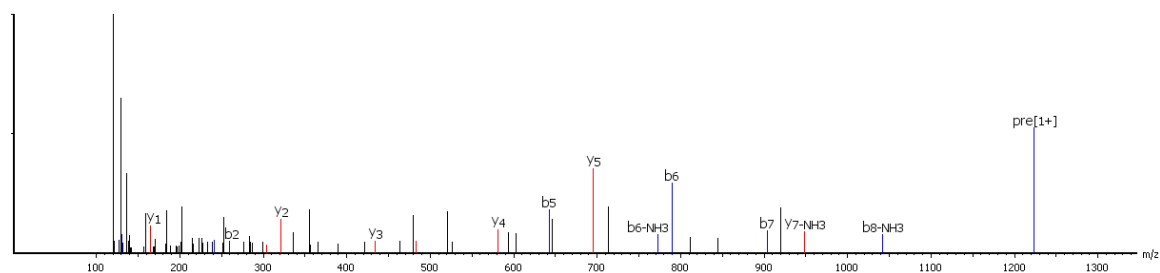
62. YFNFLRFa



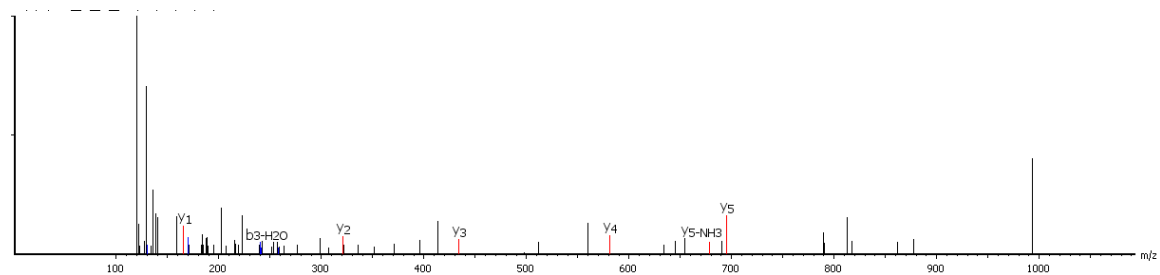
63. NSPNFLRFa



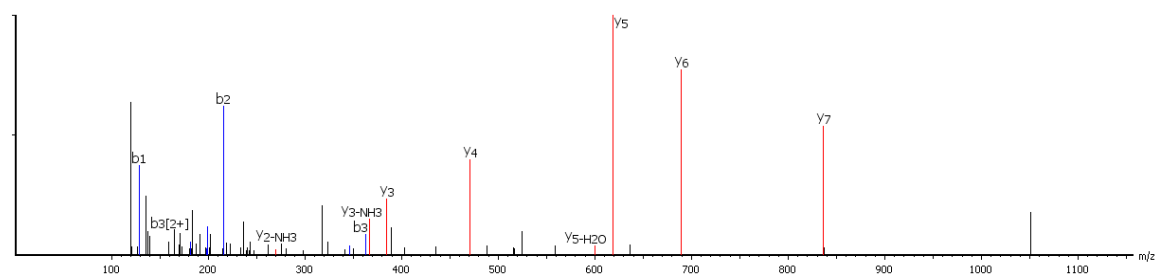
64. EERNFLRFa



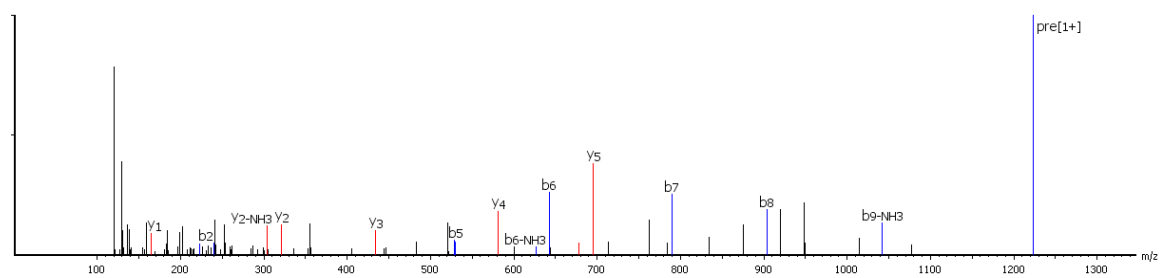
65. EGAANFLRFa



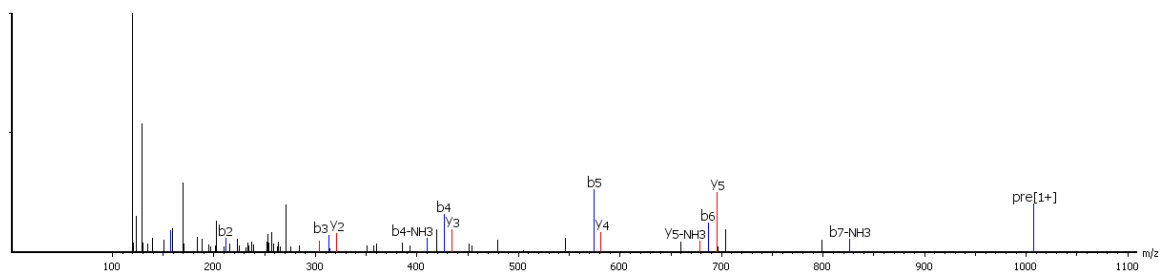
66. QSFAFSPRLa



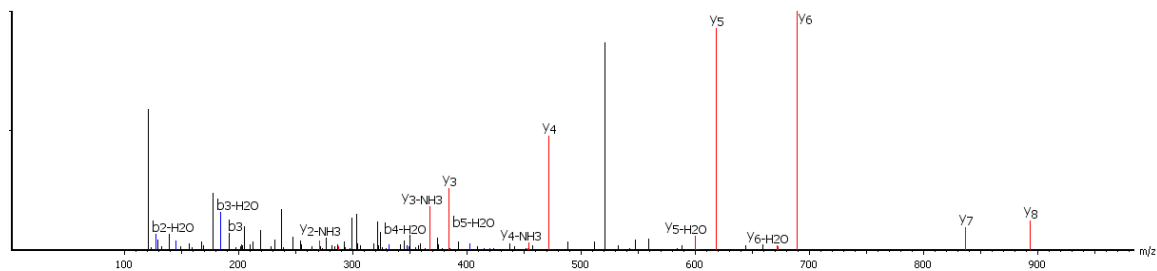
67. pQQAAFNFLRFa



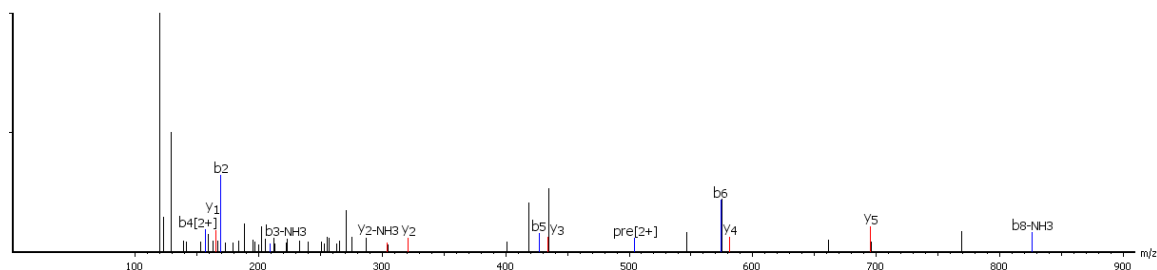
68. PNTNFLRFa



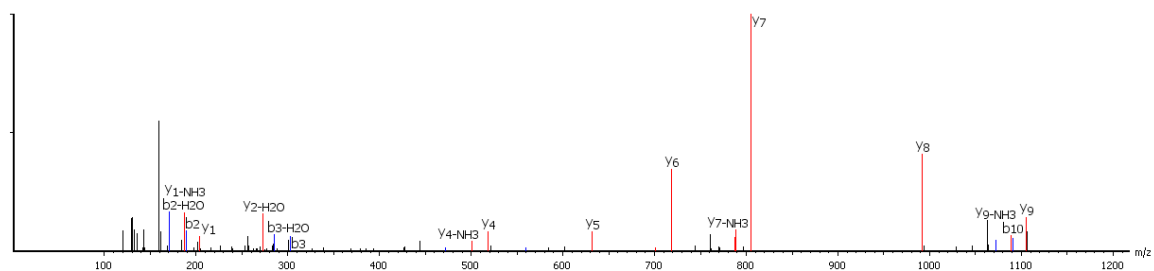
69. GSGFAFSPRLa



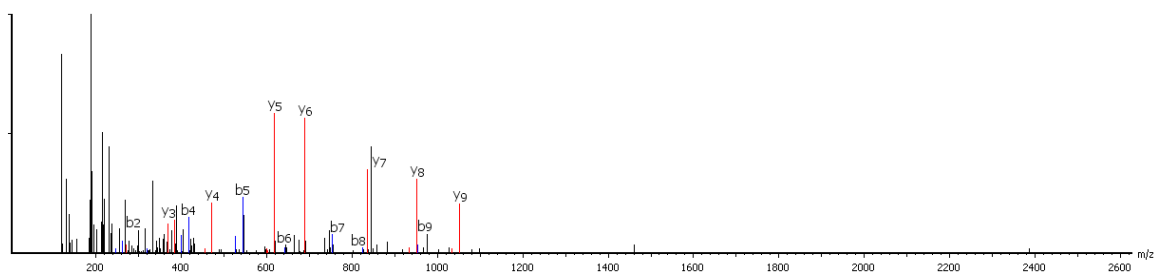
70. APGSNFLRFa



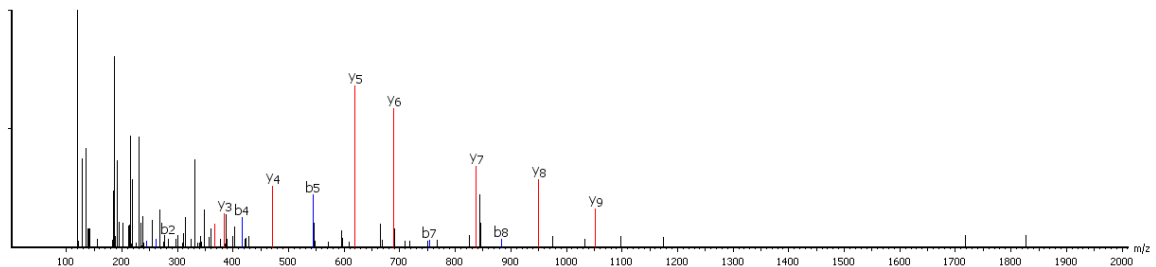
71. MGNWSSLRSAWa



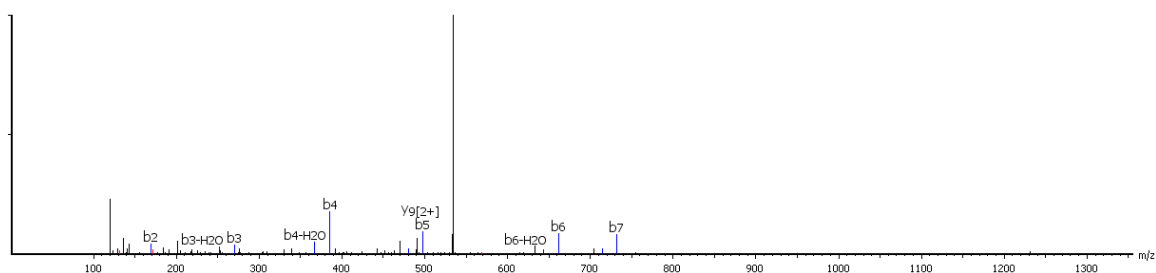
72. FNGPKPLAKYVDTNFAFSPRLa



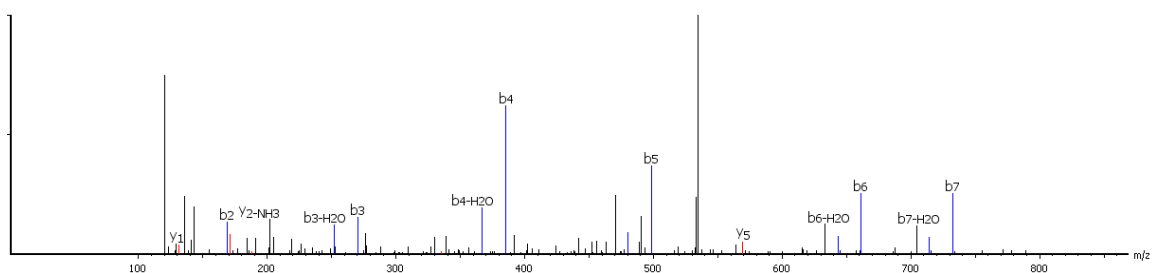
73. FNPGLPKSQMTTNFAFSPRLa



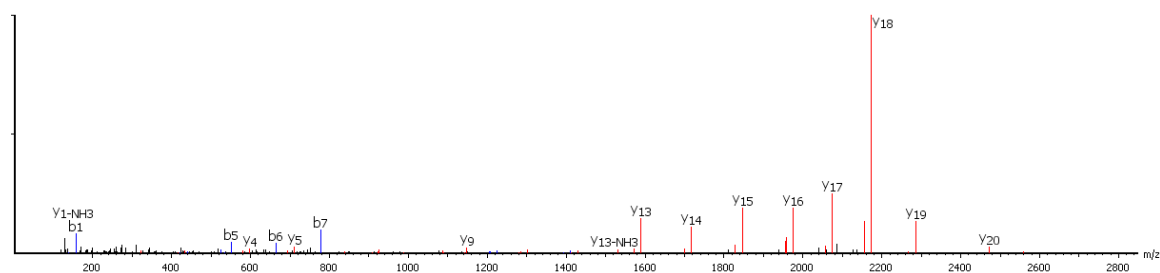
74. APTDLYAFGLa



75. PATDLYAFGLa



76. RPGQLLLAEASSWLPTQQEGTKRGYSKNYLRFa



77. HDVCHVFLRFa

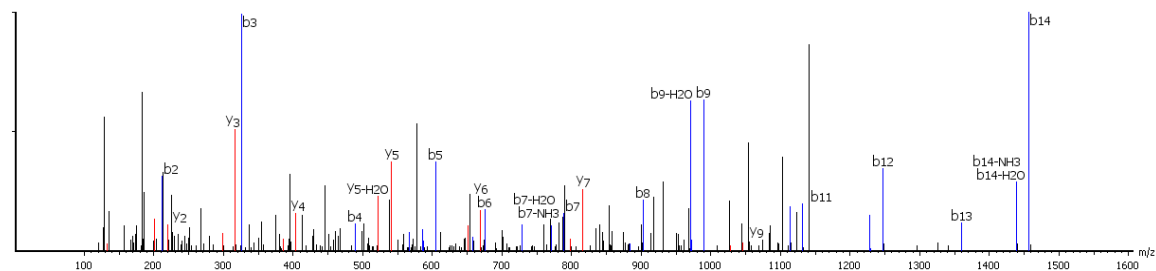
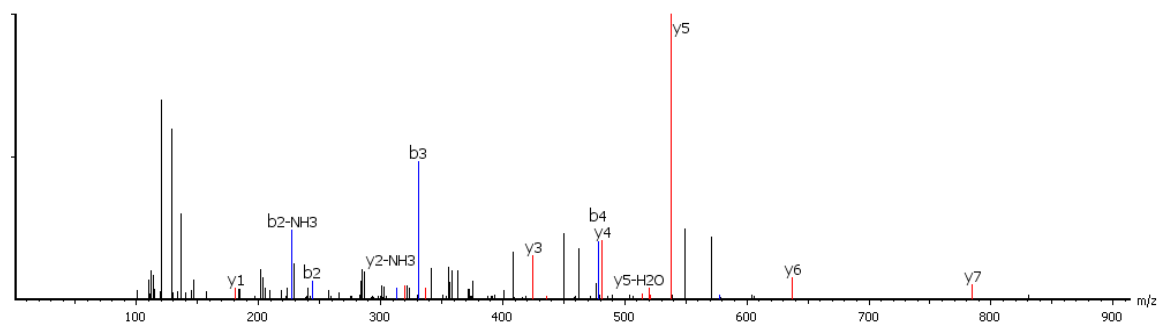
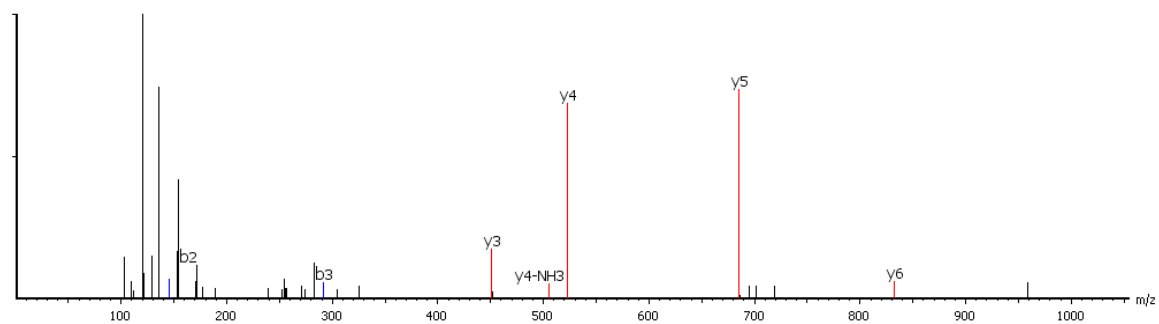


Figure S5. MS/MS spectra for novel neuropeptides identified in sinus gland extract from Jonah crab.

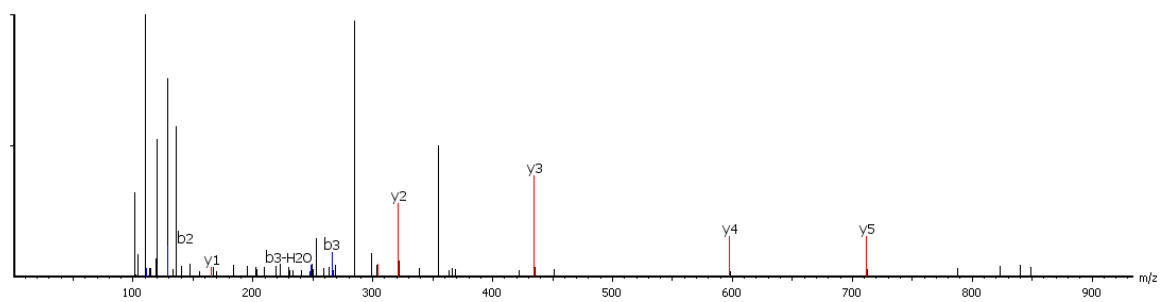
1. SRSFVGGSRYa



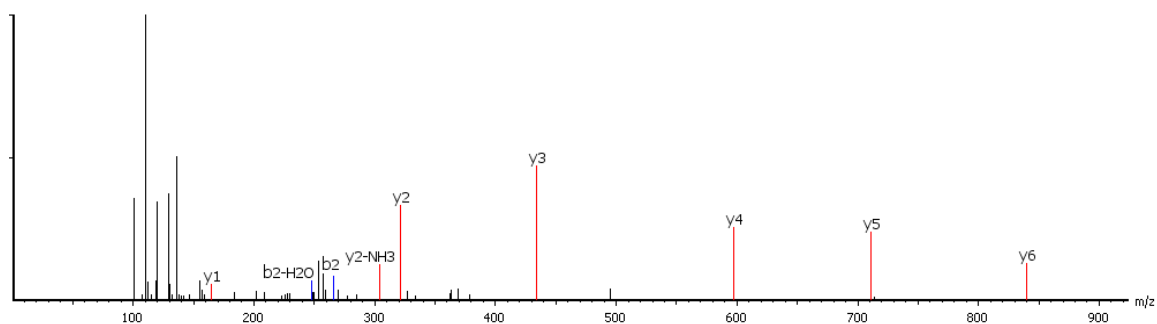
2. GSFYANRYa



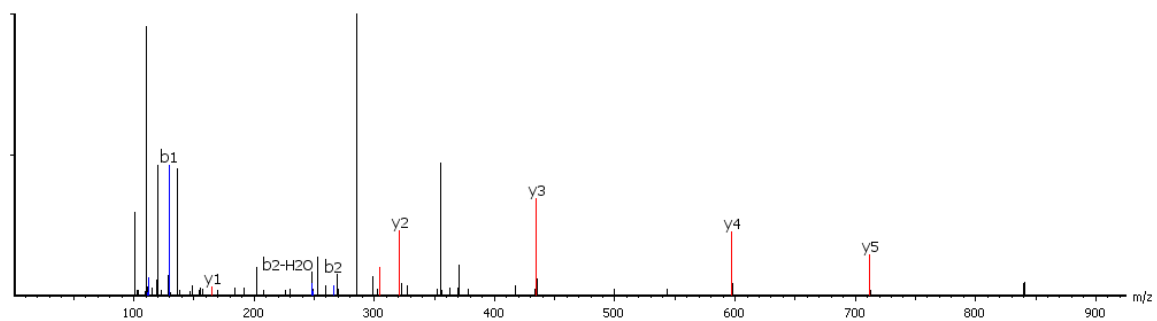
3. AGHKNYLRFa



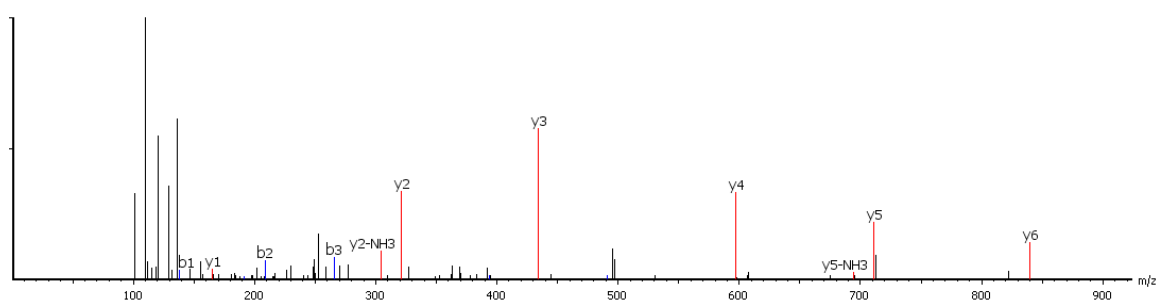
4. QHKNYLRFa



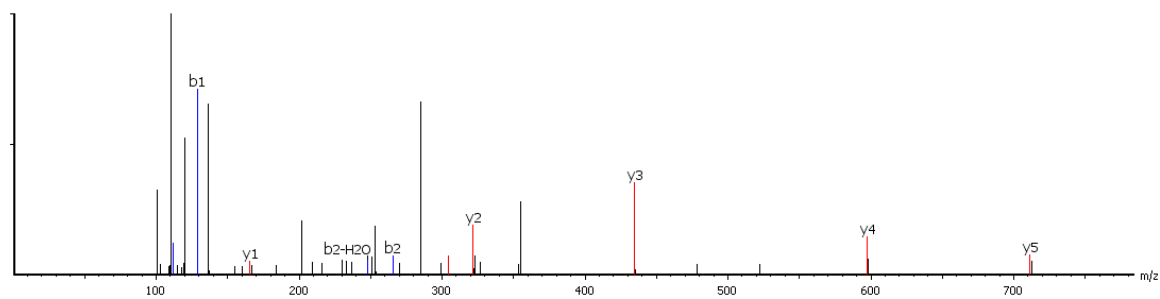
5. KHQNYLRFa



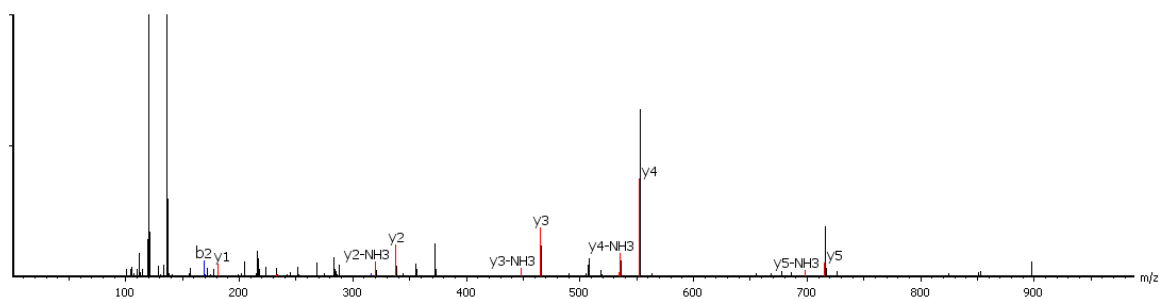
6. HAGKNYLRFa



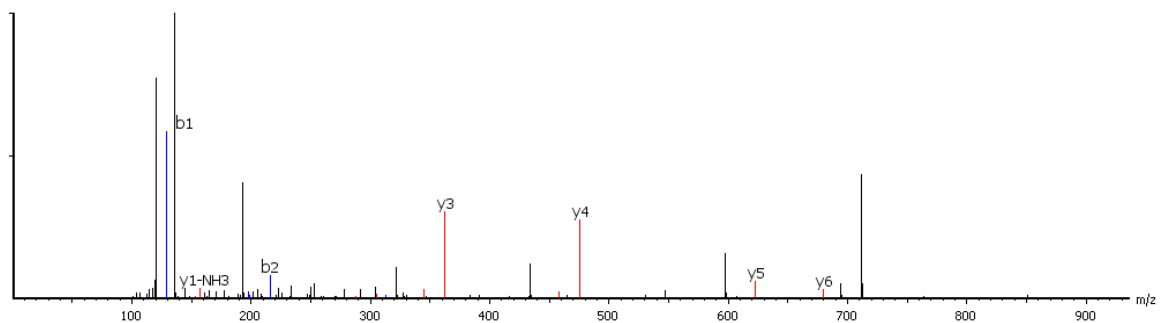
7. KHAGNYLRFa



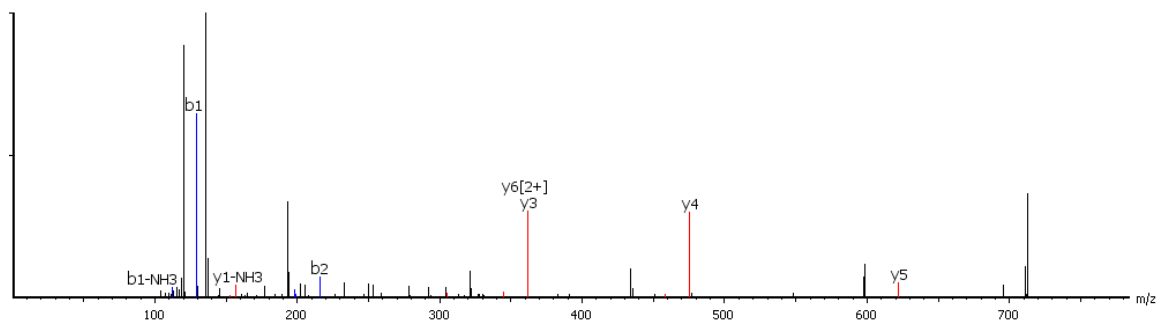
8. pEGFYSQLRYa



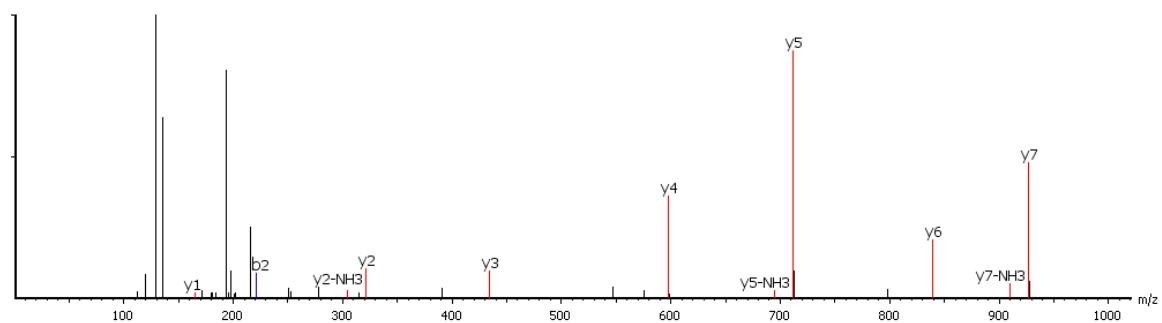
9. KSDHGFLGMRa



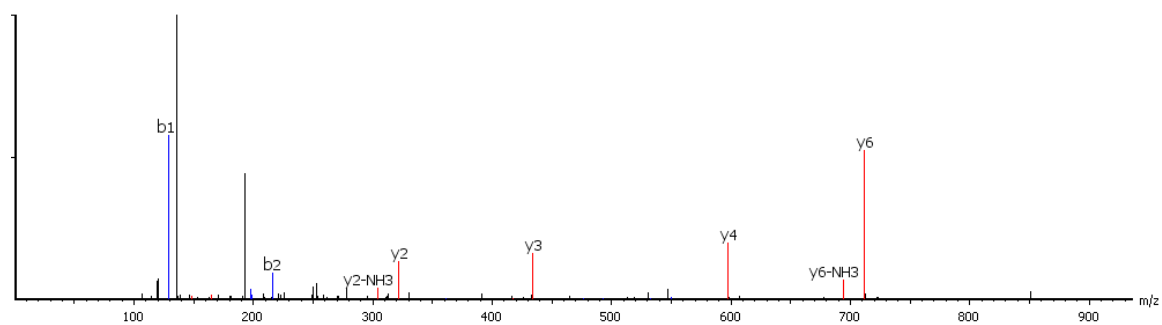
10. KSAHTFLGMRa



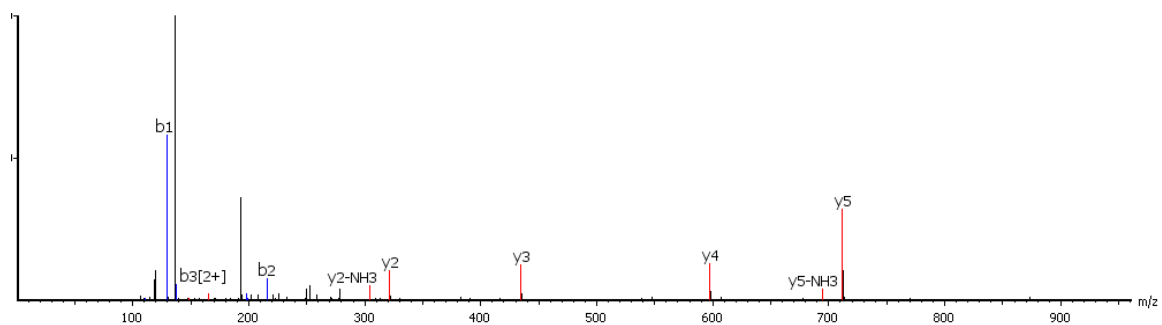
11. YGSKNYLRFa



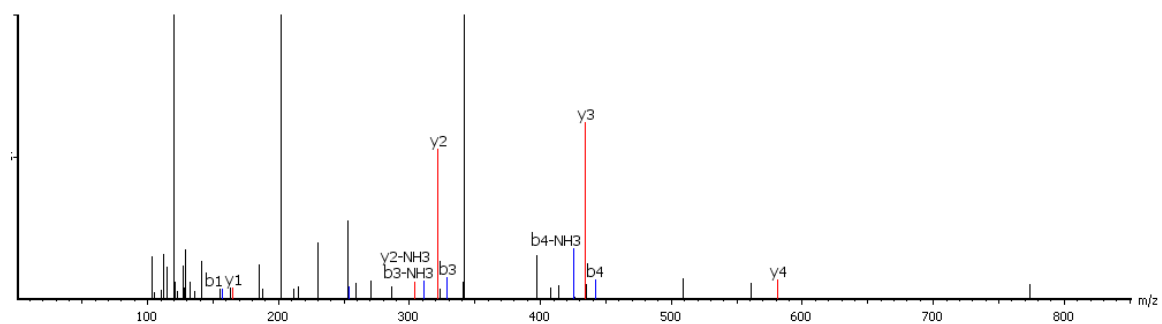
12. KSYGNYLRFa



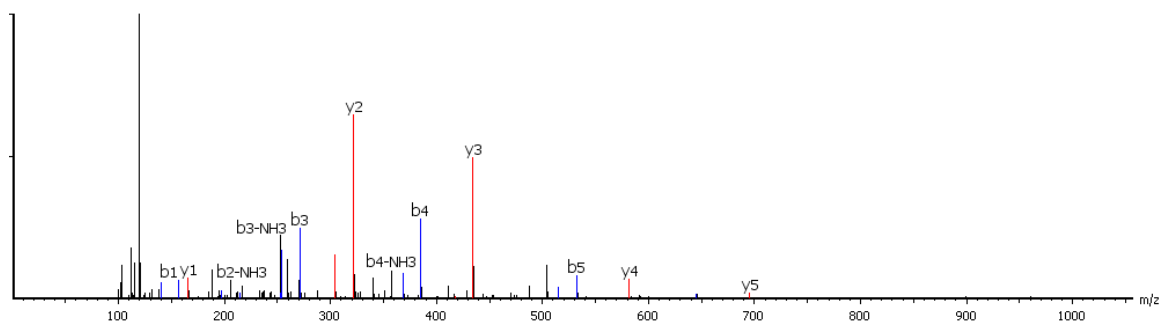
13. KSGYNYLRFa



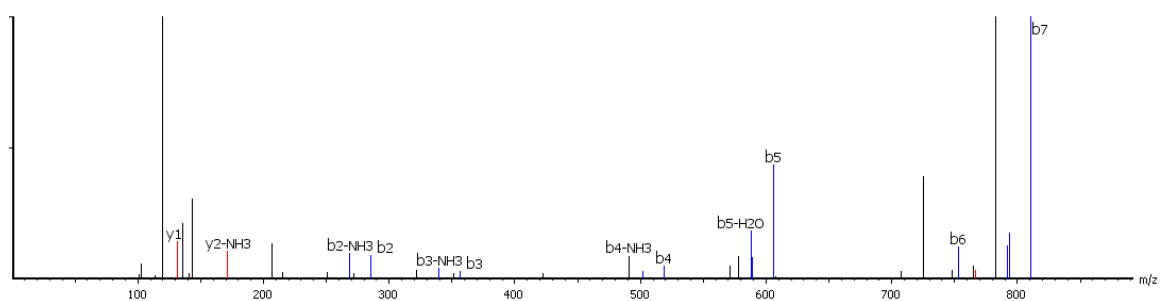
14. RNGNFLRFa



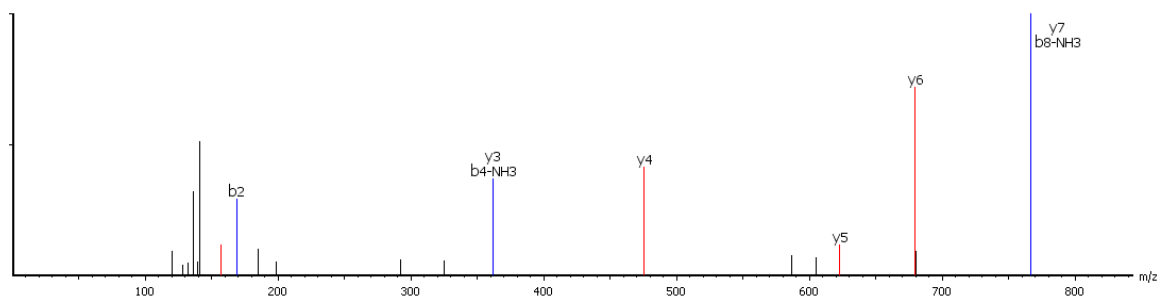
15. RGGNFLRFa



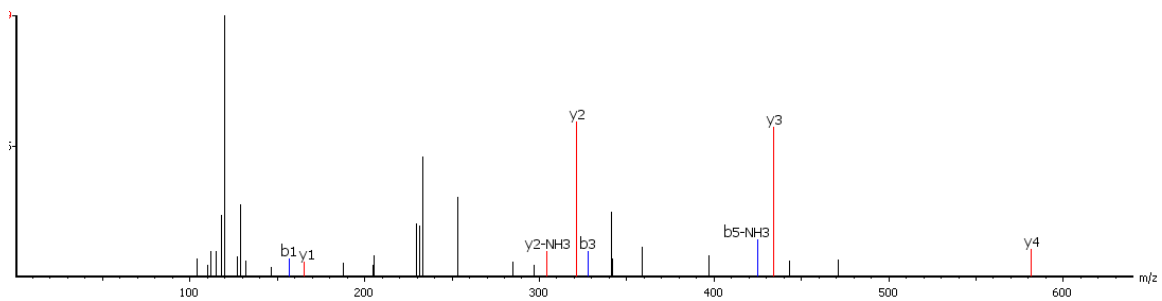
16. RQAYSFGLa



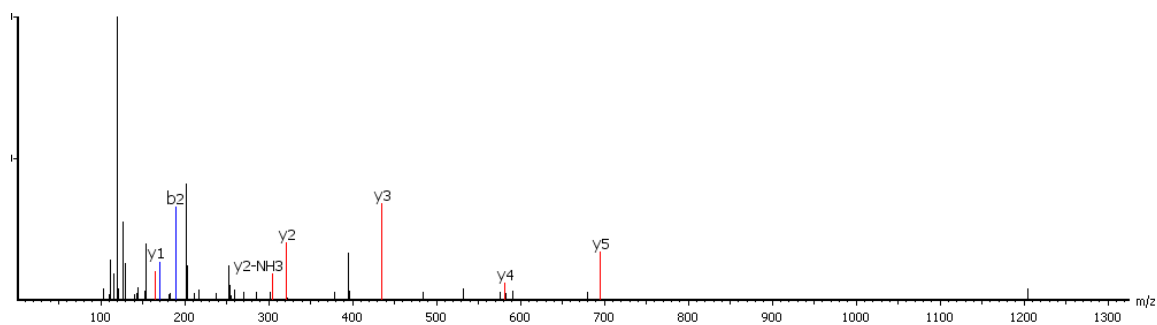
17. APPLSGFLGMRa



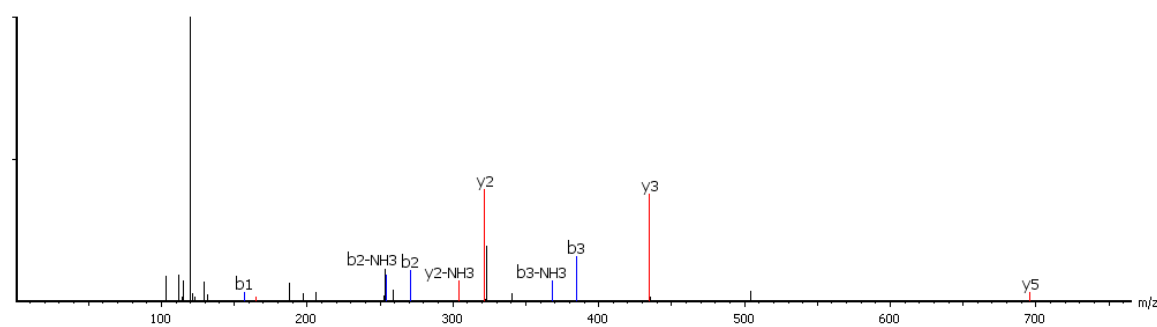
18. RGNNFLRFa



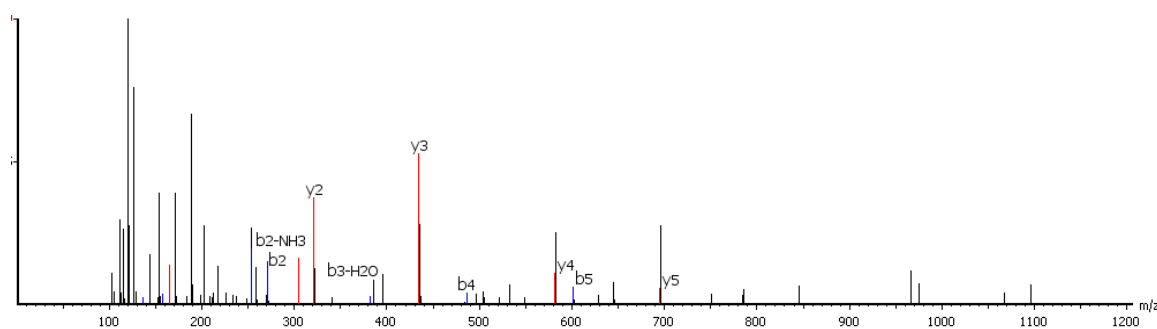
19. STPNSNFLRFa



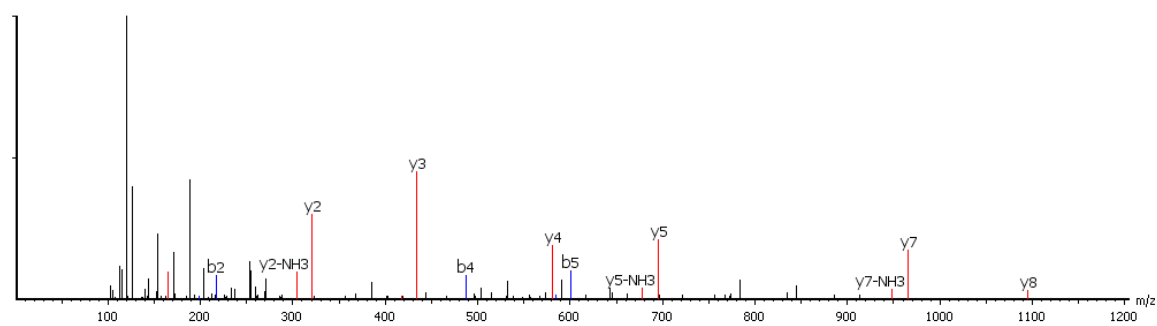
20. RNNFLRFa



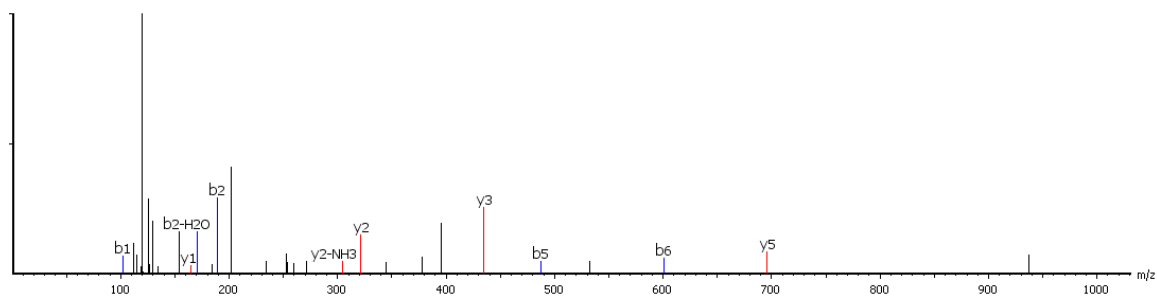
21. RNESNFLRFa



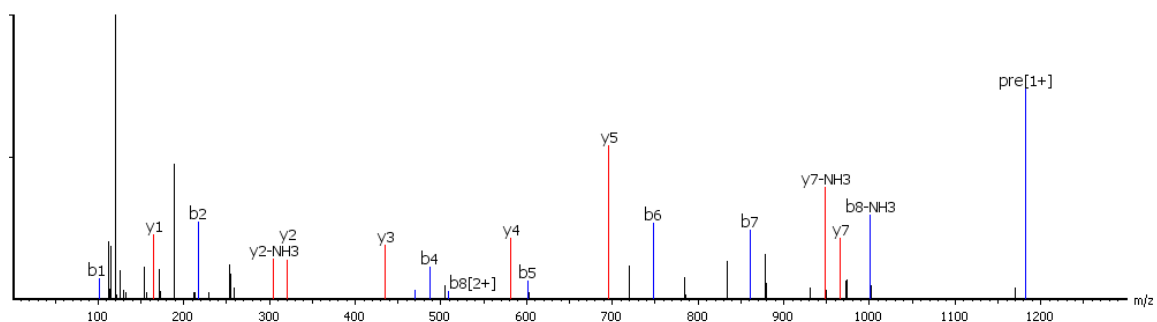
22. SERNNFLRFa



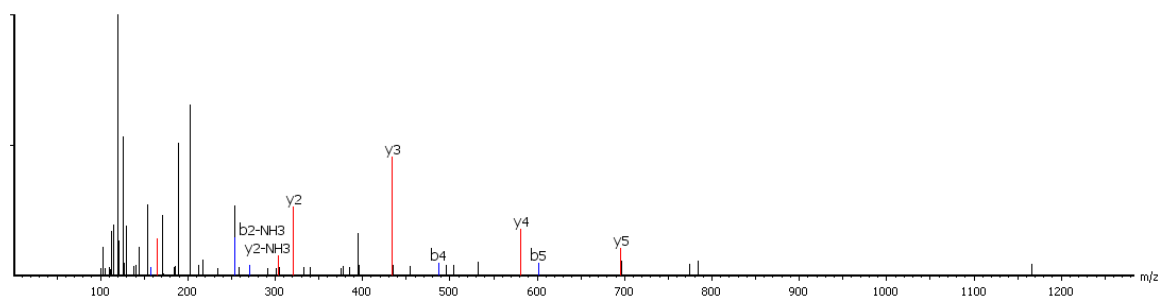
23. TSSNPNFLRFa



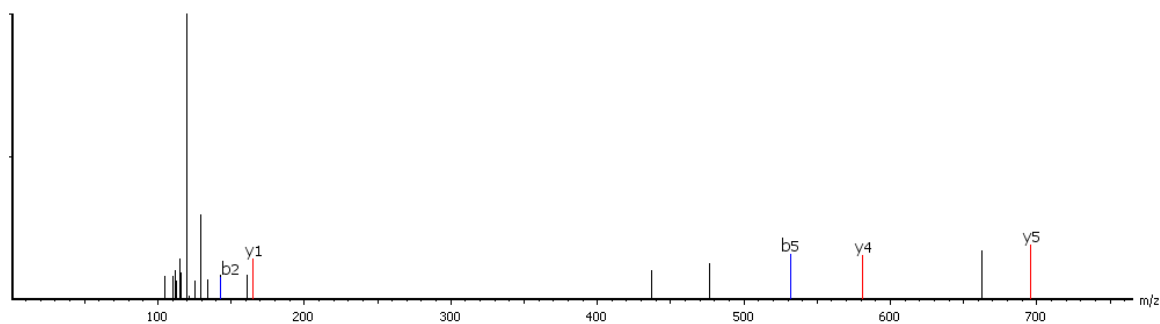
24. TDRNNFLRFa



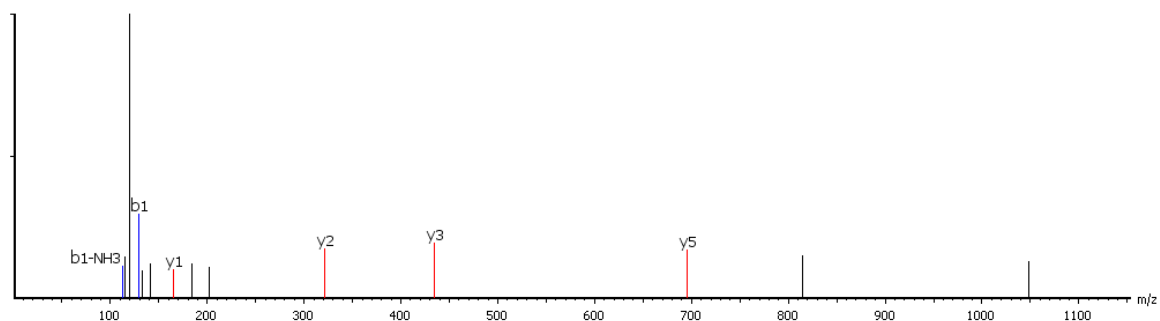
25. RNSNFLRFa



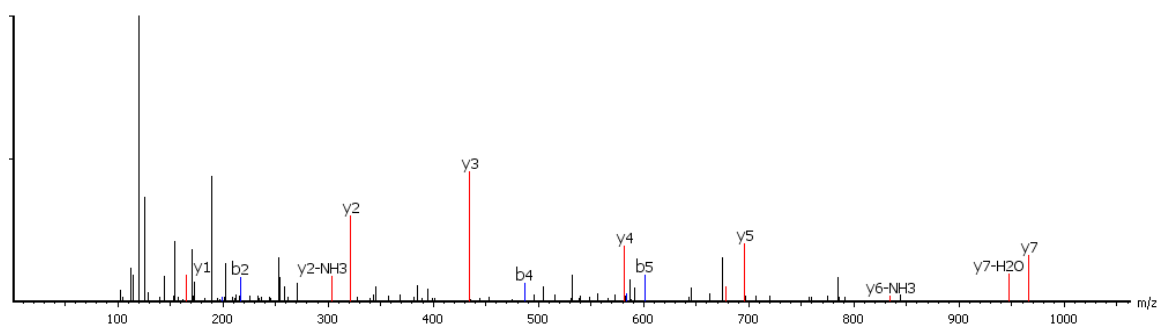
26. AAQNFLRFa



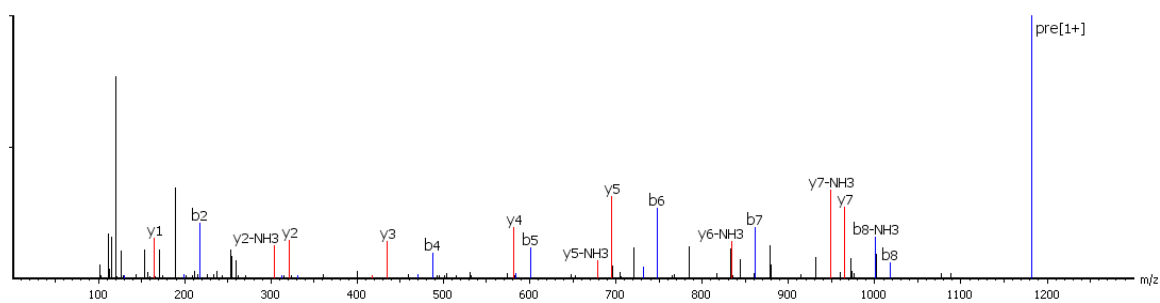
27. KAPRNFLRFa



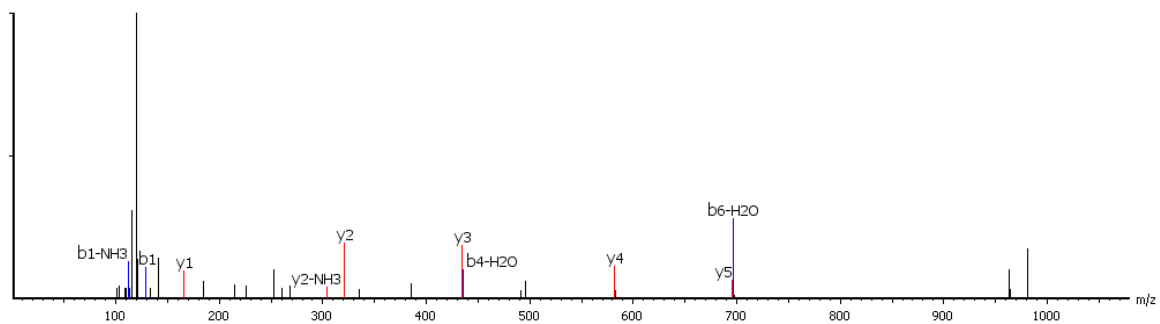
28. DTNRNFLRFa



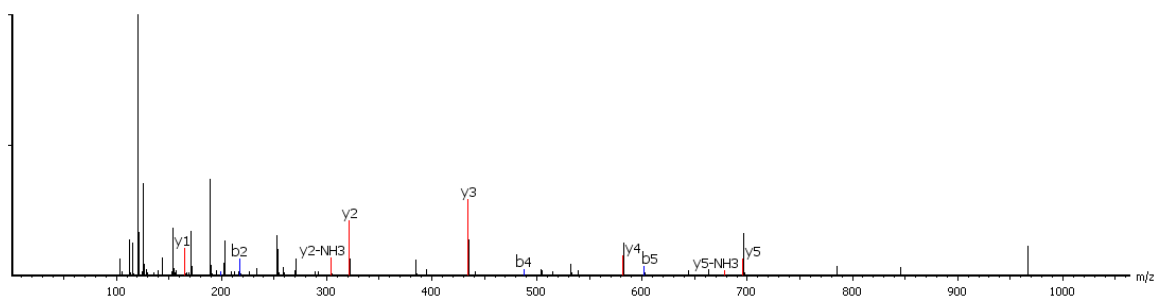
29. ESNRNFLRFa



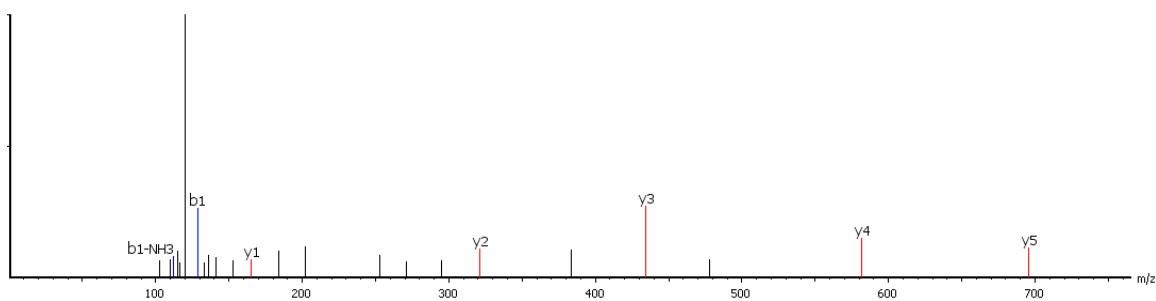
30. KRAPNFLRFa



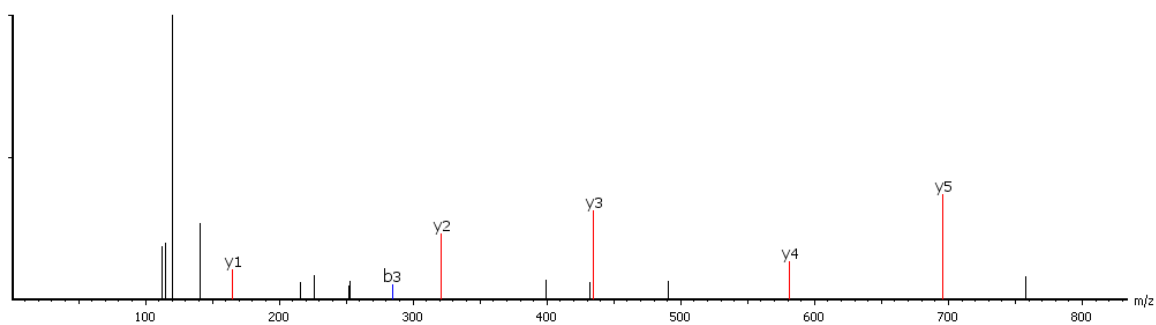
31. ESRNNFLRFa



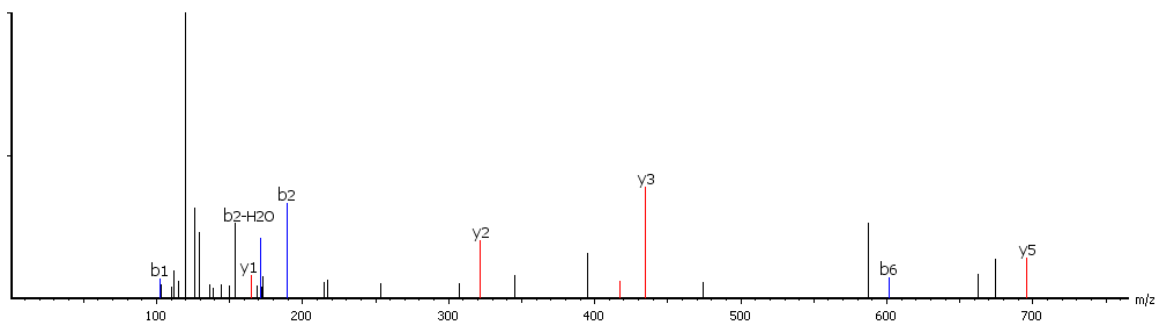
32. KPARNFLRFa



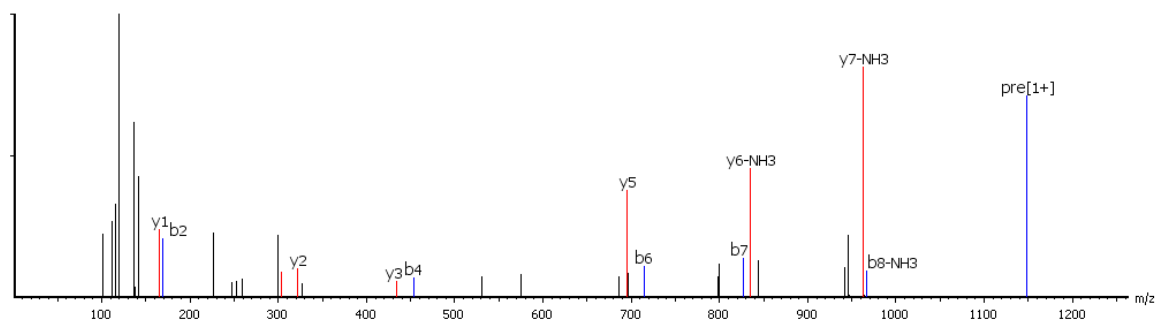
33. RAGAPNFLRFa



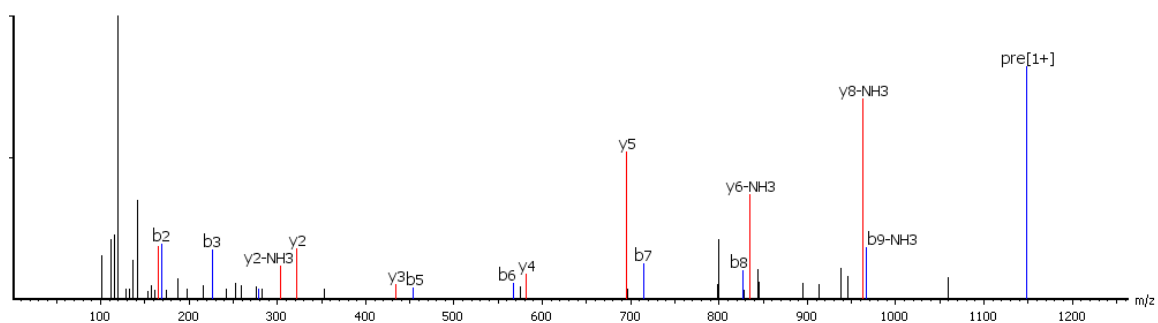
34. TSAQVNFLRFa



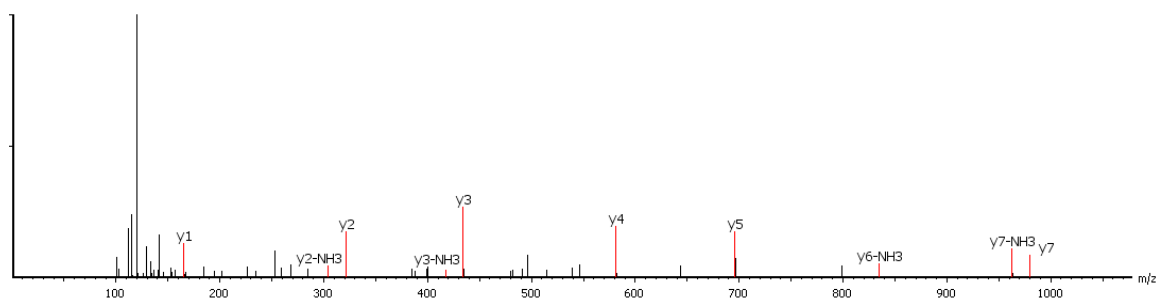
35. APGARNFLRFa



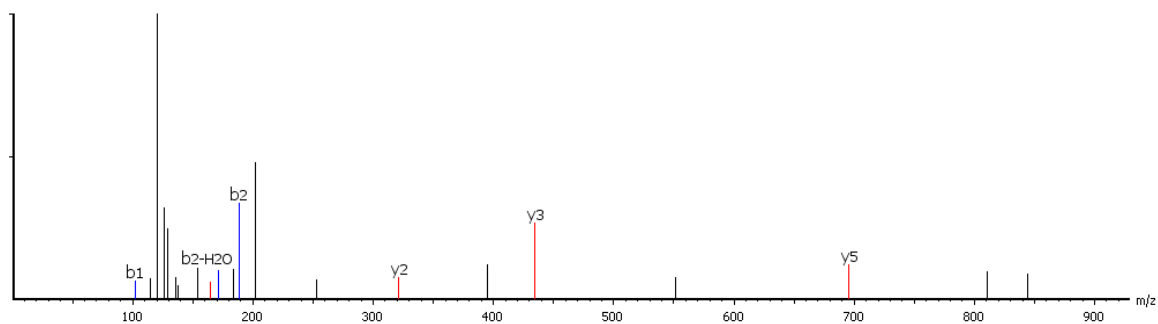
36. PAGARNFLRFa



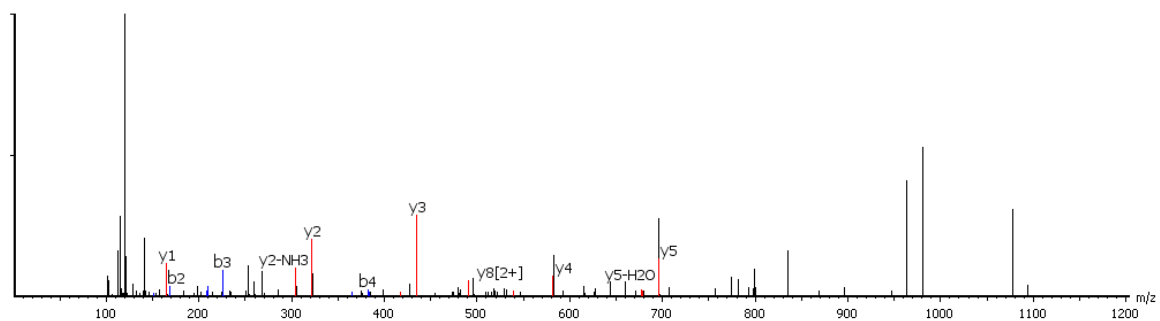
37. pQGQRNFLRFa



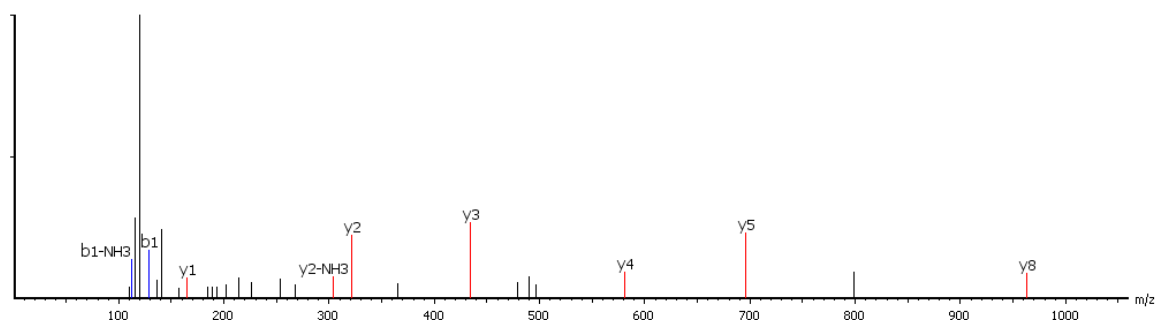
38. TSPNSNFLRFa



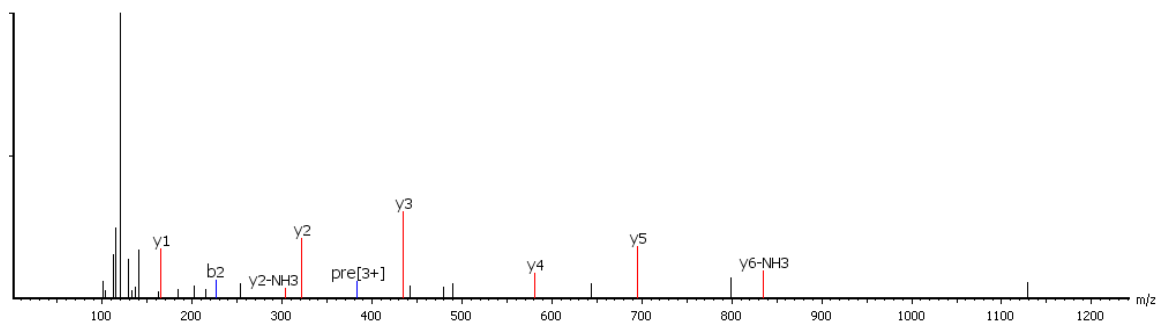
39. APGRANFLRFa



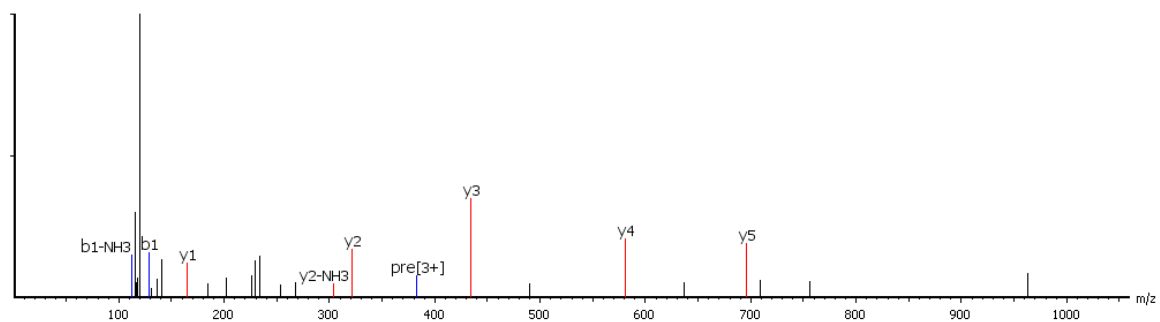
40. KGAPVNFLRFa



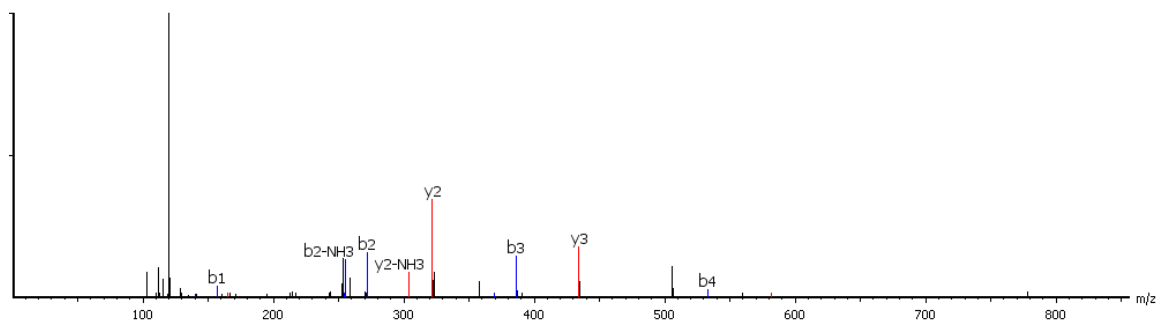
41. QPARNFLRFa



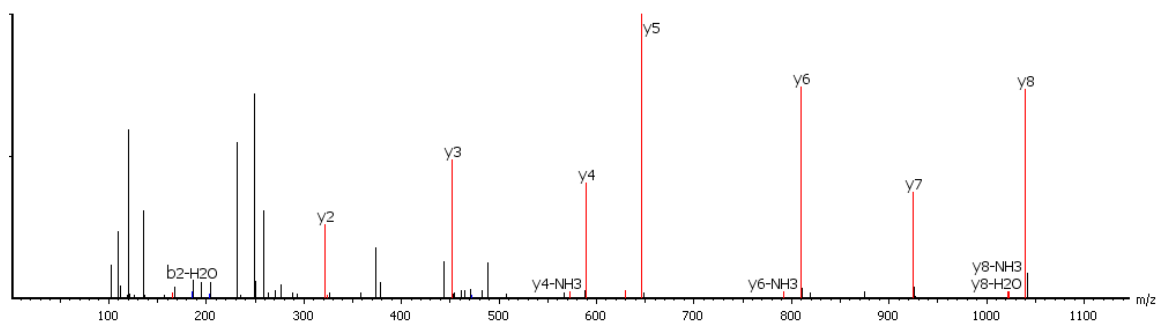
42. KVPQNFLRFa



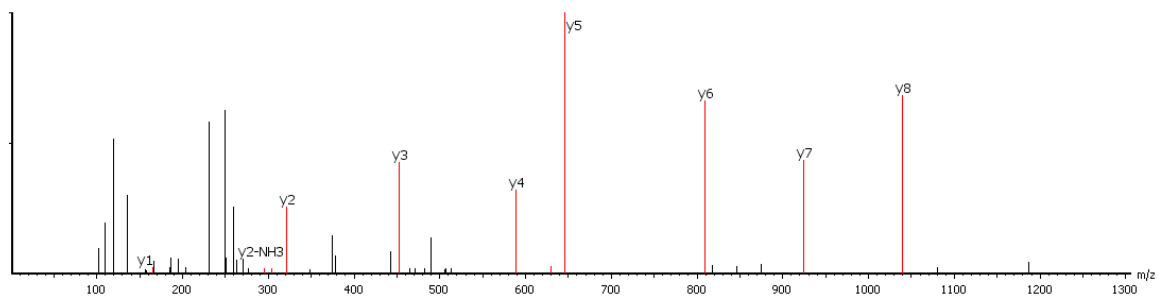
43. RDNFLRFa



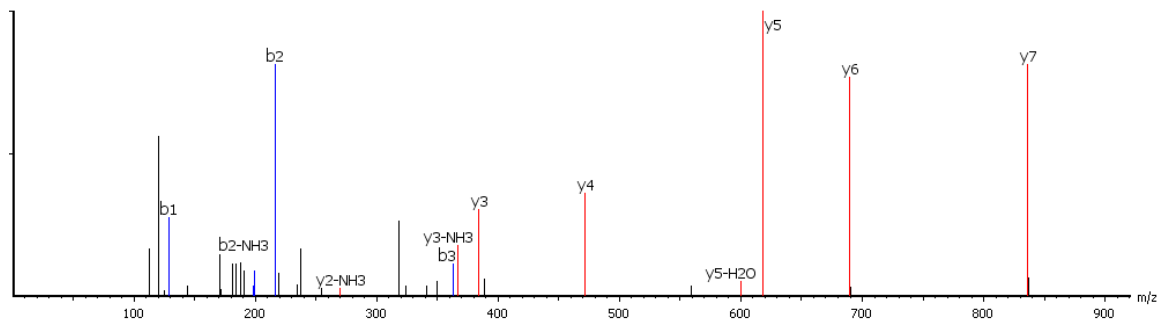
44. DSGPDDYGHMRFa



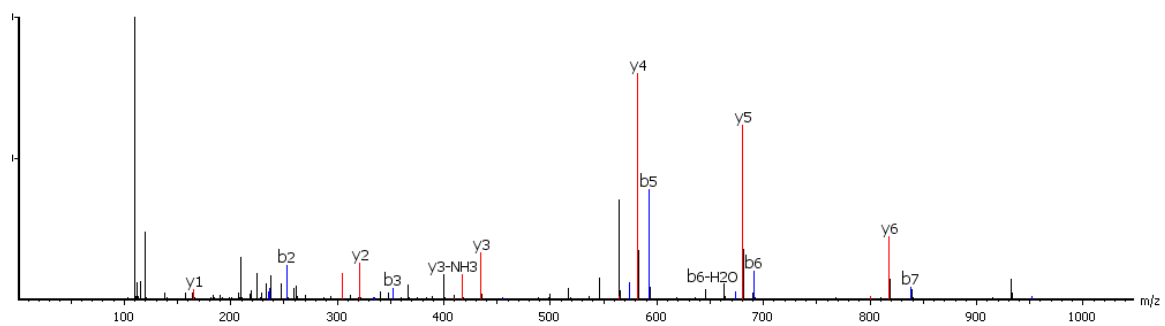
45. pEGTSDDYGHMRFa



46. QSFAFSPRLa

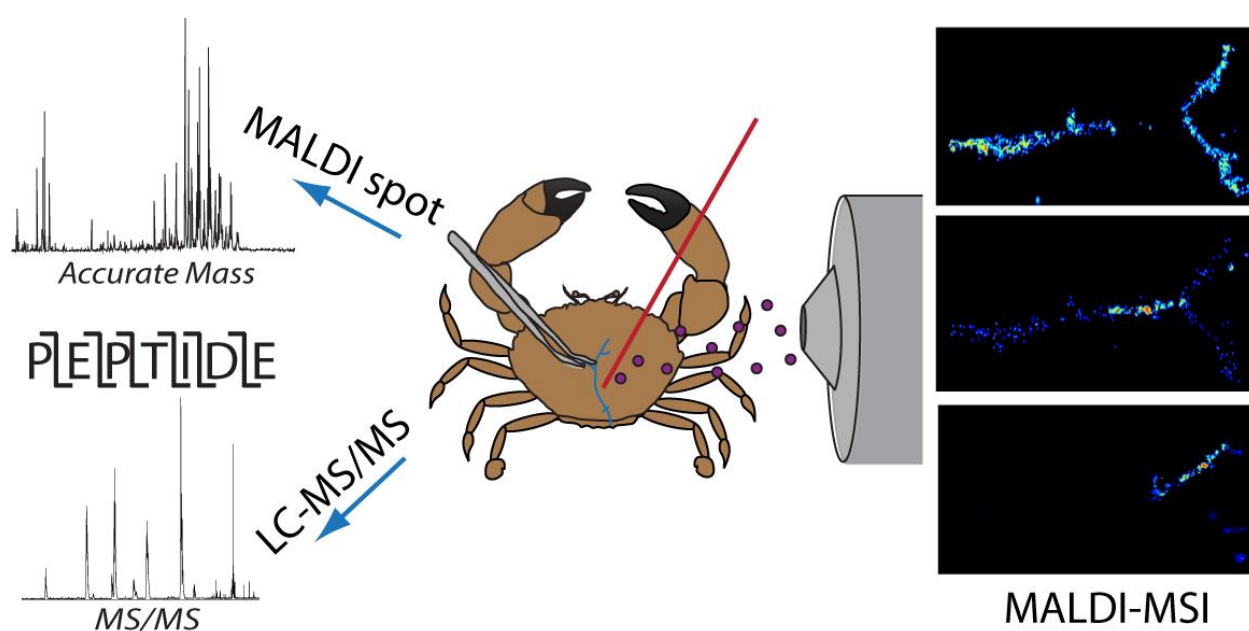


47. DHVCHVFLRFa



Chapter 6

Neuropeptidomic Profiling of the Crustacean Cardiac Ganglion and Localization Using Mass Spectrometry Imaging with Multiple Platforms



Modified from:

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Abstract

The crustacean cardiac neuromuscular system is a useful model for studying how neural circuits generate behavior, as it is comprised of a simple ganglion containing nine neurons, yet acts as a robust central pattern generator. The crustacean heart is neurogenic, meaning that it receives input from neuropeptides. However, the specific effects of neuropeptides on cardiac output is not fully understood, and the large degree of comodulation between multiple neuropeptides makes studying these effects more challenging. To address this challenge, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) imaging was used to localize neuropeptides within the cardiac ganglion (CG), providing information both of the identity of neuropeptides are present and their specific regions. CG extracts were also profiled using liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) with a data independent acquisition (DIA) method, resulting in the confirmation of 316 neuropeptides. Two imaging platforms were compared in order to provide comprehensive results, including a MALDI-Orbitrap instrument for high mass resolution for accurate identifications and a MALDI-TOF/TOF for improved spatial resolution and sensitivity, providing more descriptive MS images. MS images for 235 putative neuropeptides were obtained, with the identification of 145 of these being confirmed by either complementary MS/MS data or accurate mass matching. Imaging results show distinct localization patterns for neuropeptides in specific areas, including regions where pacemaker neurons are present or anterior/posterior motor neurons are located. The MSI studies demonstrate the sensitivity and power of MALDI-based in situ analytical strategy for unraveling the chemical complexity present in a small 9-cell neuronal system. The results of this study will enable more informative assays of the functions of neuropeptides within this important neural circuit.

Introduction

Central pattern generators have long been studied in order to understand how neural circuits generate behavior. Crustaceans have been particularly useful for studying the chemical modulation of neural circuits because of their simplicity [1]. Circuits driving behavior in decapod crustaceans consist of a small number of easily-distinguishable neurons, making it possible to map how neurons connect to each other and form feedback loops [2]. Furthermore, the effect of substances modulating this activity, such as neuropeptides and neurotransmitters, can be more easily studied to determine how they affect the bursting patterns of specific neurons [3, 4].

The crustacean cardiac neuromuscular system is of great utility for studying circuit dynamics, as it is one of the simplest central pattern generators [5, 6]. Crustaceans have an open-circulatory system, in which tissues are bathed in hemolymph that is circulated by means of a single-chambered heart [7]. The heart's cardiac neuromuscular system consists of the cardiac ganglion (CG) and surrounding muscular tissue. While the specific neuronal makeup of the CG varies, in *Cancer borealis*, it is comprised of 9 neurons, including 4 pacemaker neurons and 5 motor neurons. These neurons are connected in a manner to drive the heartbeat, signaling to the muscle tissue to drive contractions, pushing hemolymph throughout the rest of the crab [6].

As crustacean hearts are neurogenic, neuropeptides, along with neurotransmitters, are responsible for modulating the heartbeat. There have been numerous studies investigating the roles of specific neuropeptides on the CG of both crabs and lobsters, including those belonging to the following families: pyrokinin [8], allatostatin [9], tachykinin [6], and SIFamide [10]. These findings have unveiled how individual neuropeptides alter heart contractions, both amplitude and frequency, and how these effects change as a function of other factors such as concentration or temperature [8, 11, 12]. Studies have also illuminated the intricate complexity

within neuropeptide families, as neuropeptides possessing common sequence motifs often elicit contrasting functions. For example, neuropeptides belonging to the pyrokinin family possess the common motif –FXPRLamide, but when 5 of these were tested for effect on heart activity, only one had an effect, increasing both contraction amplitude and frequency [8, 13]. Similarly, 3 isoforms of allatostatin C-type were found to elicit distinct cardiac outputs in lobster hearts despite their sequence similarity [9]. Immunohistology has also been employed in order to localize these neuropeptides to discrete areas of the ganglion [4, 9].

The results from these studies have been helpful in determining the functions of specific neuropeptide isoforms within the cardiac neuromuscular system. However, these studies have been limited to individual neuropeptides. It has been found that neuropeptides often co-modulate one another to elicit or alter functions within circuits [14]. This finding suggests that the function of neuropeptides studied in artificial saline may be different than *in vivo* because of the presence of other neuropeptides within or surrounding the ganglion. In order to fully understand the function of neuropeptides, their effects must be studied in relation to other co-modulating substances.

Currently, little is known about what neuropeptides are present in the *C. borealis* cardiac ganglion that may be affecting its activity. There have been extensive studies profiling and localizing the neuropeptides in other neural tissue, such as members of the stomatogastric nervous system, another robust central pattern generating circuit in the crab, as well as neurosecretory tissue that release neuropeptides into the heart, most notably the pericardial organs that are located laterally on either side of the heart [15-19]. However, there has not yet been an in-depth profiling of the neuropeptides present in the CG.

The purpose of this study is to implement a multifaceted MS method to image neuropeptides present in the *C. borealis* CG and localize these neuropeptides to specific regions of the tissue. MS imaging method was employed to localize these neuropeptides to discrete regions of this small tissue. A multi-platform approach was used to identify and localize neuropeptides, including matrix-assisted laser desorption ionization (MALDI) MS imaging on an Orbitrap MS for high mass resolution and a time of flight (ToF) MS for high spatial resolution and speed for mapping these peptides, and LC-electrospray ionization (ESI)-MS/MS for confident identification. The combined approach enabled the identification of 316 neuropeptides across 19 families and the localization of 235 neuropeptides to discrete cellular regions of the CG, demonstrating the powerful results of this method, both in terms of sensitivity and specificity, for studies of small, yet complex neuronal systems.

Materials and Methods

Chemicals and Materials

ACS-grade formic acid (FA) was purchased from Sigma-Aldrich (St. Louis, Mo, USA). Gelatin was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). All other chemicals and solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). ACS-grade solvents were used for sample preparation, and Optima-grade solvents were used for MS analysis. Acidified methanol was prepared using 90/9/1 water/methanol/acetic acid. 10 µL C18 ziptips were purchased from Merck Millipore (Billerica, MA, USA).

Animal dissection, tissue collection, and sample preparation

Male *Cancer borealis* animals purchased from the Fresh Lobster Company, LLC (Gloucester, MA, USA) were housed in artificial seawater tanks maintained at 12-13°C with an

alternating 12-hour light/dark cycle. Animals were allowed to adjust to their environment for a minimum of one week prior to use. For tissue dissection, animals were cold-anesthetized for 30 minutes on packed ice and dissected in chilled artificial crab saline (440 mM NaCl, 11 mM KCl, 26 mM MgCl₂, 13 mM CaCl₂, 11 mM Trizma base, 5 mM maleic acid, adjusted to pH 7.45 with NaOH).

For LC-MS/MS experiments, tissues were collected and placed in chilled acidified methanol (90/9/1 methanol/water/acetic acid). Tissues were manually homogenized with a glass homogenizer in chilled acidified methanol, sonicated in a bath sonicator, and centrifuged at 16.1 rcf for 10 minutes. The supernatant was collected, acidified methanol was added to the pellet, and the process was repeated for two more extractions. The combined supernatants were evaporated in a speedvac on medium heat and reconstituted in 10 µL of 0.1% FA in water. The samples were then desalted using C18 ziptips following package instructions, and the elutions were evaporated in the speedvac on medium heat. Extracts from 12 CGs were pooled for subsequent LC-MS/MS analysis.

For imaging of the CG and PO, tissues were collected, dipped in water to desalt, placed on a plain glass or indium tin oxide (ITO)-coated glass microscope slide (Bruker, Billerica, MA, USA), and dried in a dessicator box. For imaging of the brain, the tissue was collected, dipped in water to desalt, and embedded in gelatin and flash frozen on dry ice. The brain was then sectioned at 12 µm thickness and placed on a plain glass or ITO-coated glass microscope slides. Each tissue was coated with 12 passes of 40 mg/mL 2,5-dihydroxy benzoic acid (DHB) using an automated TM sprayer (HTX Technologies LLC, Chapel Hill, NC, USA).

LC-MS/MS analysis

The CG extract was reconstituted in 15 μL of 0.1% FA in water and was analyzed on a Thermo Scientific Q Exactive HF instrument coupled to an online Dionex UltiMate 3000 nanoLC (Thermo Scientific, Bremen, Germany). A 15-cm self-packed C18 column (75 μm internal diameter, 1.7 μm particle size) was used for LC separation. Mobile phases consisted of water with 0.1% FA (A) and acetonitrile with 0.1% FA (B). The flow rate was set to 0.300 $\mu\text{L}/\text{min}$, and a μL injection volume was used. The LC run consisted of a 120-minute gradient as follows: 0-1 min 3-10% B; 1-90 min 10-35% B; 90-92 min 35-95% B; 92-102 min 95% B; 102-105 min 95-3% B; 105-120 min 3% B. For MS analysis, a data independent acquisition (DIA) method was utilized, as has previously been optimized [19]. Positive ESI was used with a collection energy of 30 eV, 70,000 resolution for MS scans, and 17,500 resolution for MS/MS scans. The MS scan range was 400 to 800 m/z with an isolation width of 20 m/z .

MALDI-MS analysis

MALDI-MS imaging was performed on a Thermo Scientific MALDI-LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany) and a Bruker RapifleX MALDI TissueTyper ToF/ToF (Bruker, Billerica, MA, USA). Optical images were acquired with an HP scanner and imported into each instrument's tune page. The Orbitrap mass spectrometer was equipped with a 337.1 nm, 60 Hz nitrogen laser. Samples were analyzed in positive-ion mode with an m/z range of 500 to 2000, 20 kJ laser energy, and 75 μm raster size. The RapifleX mass spectrometer was equipped with a smart-beam 3D 10 kHz laser. Samples were analyzed in positive-ion mode with an m/z range of 200 to 3200, 80% laser energy, and 20 μm raster size.

The MALDI-Orbitrap instrument was also used for spot analysis of an extract containing 12 pooled CG tissues. The sample was dissolved in 2.5 μL 0.1% FA, mixed with equal volume of 120 mg/mL DHB in 50/50 water/methanol (v/v) with 0.1% FA, and spotted on a MALDI

target plate, 1 μ L per spot, 5 spots total. Crystal positioning system (CPS) plate motion was used to acquire a total of 50 scans (2 microscans/scan) for each spot.

Data analysis

MS spectra from LC-MS/MS analysis were converted to the open-source mzXML file format using MSConvert [20] and processed with DIA-Umpire [21] using the default parameters. Output mgf files were converted to mzXML and processed with PEAKS 7.0 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) [22, 23] using the following parameters for *de novo* sequencing: no enzyme cleavage specified, instrument orbi-orbi, HCD fragmentation, and precursor correction enabled. Modifications were set to include amidation, pyroglutamate, dehydration, and oxidation. All other parameters were set to the default. An in-house crustacean neuropeptide database was used for searches. MALDI-MS spot data was processed using accurate mass-matching to the same crustacean neuropeptide database using a mass error of ± 5 ppm.

MALDI MS imaging files were converted into either imzML (Orbitrap) or img (RapifleX) format using vendor-specific software. Data were loaded into MSiReader, and images were generated of neuropeptides in the crustacean neuropeptide database [24]. The mass error was set to 5 ppm for Orbitrap data and 0.1 Da for RapifleX data. All images were normalized to the total ion current (TIC). For the RapifleX data of CG images, 5th order linear smoothing was applied.

Results and Discussion

The crustacean CG is an important circuit in the crustacean neuromuscular system that modulates the contractions of the heart, circulating hemolymph throughout the animal. As the

heart is neurogenic, knowing what neuropeptides are present within the tissue and where they are localized provides important insight into how the rhythmic activity of the CG correlates with behavioral outputs. To address this gap in knowledge, this study used a multi-platform MS approach to profile the neuropeptide content within the tissue and localize neuropeptides to specific areas of the tissue, providing insight into their biological activity.

Comparison of MS imaging platforms

As the CG is a small tissue and localization of neuropeptides to regions of specific neuronal tissue was desired, spatial resolution was critical to gaining quality MS images. The Bruker RapifleX instrument offers high spatial resolution in addition to greater acquisition speeds. However, the mass resolution of the TOF mass analyzer is notably lower compared to Orbitrap instruments, making high-confidence mass measurements more difficult. Furthermore, neuropeptide analysis of crustacean tissue samples has never before been performed on a Rapiflex instrument. In order to ensure data quality, a comparison was made on the two MS imaging platforms used for neuropeptide analysis, MALDI-LTQ-Orbitrap XL and MALDI RapifleX MALDI Tissue typer ToF/ToF.

In order to compare the two platforms in their ability to detect neuropeptides in crustacean tissue, serial sections of crab brain tissue were analyzed on each platform. A total of 10 sections were analyzed, with 5 being on each respective platform. Brain tissue was used because the tissue is large enough to section such that technical replicates could be analyzed on each platform. Overall, 314 neuropeptides were putatively detected with the RapifleX TOF/TOF instrument, while only 19 were detected with the Orbitrap instrument. While the mass error tolerance was greater for the RapifleX than the Orbitrap, 14 neuropeptides were detected with both platforms, and these in general showed improvement in ion localization with the RapifleX.

Figure 1 shows examples of 6 neuropeptides detected with both platforms, demonstrating the improvement in ion images that was offered by the RapifleX. These results were observed for neuropeptides from a variety of families, i.e. sequence motifs, indicating that there was not bias toward specific neuropeptide families. While the Orbitrap MS imaging experiments show MS images as scattered signals, the improved resolution of RapifleX images enable visualizing discrete localization within the tissue, indicating clustering of neuropeptides to specific ganglia within the brain.

Pericardial organs (POs) were also used to compare the performance of each of the MS imaging platforms. While both the CG and PO are too thin to section for multiple replicates, the PO is a paired tissue, with one located on either side of the heart. Therefore, a comparison could be made in which POs from the same animal were analyzed on each platform, e.g. the left on the RapifleX and the right on the Orbitrap. As POs release neuropeptides into the heart of the crab, they are also suitable for testing the platforms because the neuropeptide content is likely similar to that of the CG, located in the heart. When analyzing neuropeptides from POs from the same crab, The RapifleX enabled the detection of 190 neuropeptides, while the Orbitrap detected 128 neuropeptides, with 52 of these neuropeptides detected with both platforms. While the numbers were much more comparable to one another in the POs than in the brain tissue, the resulting RapifleX MS images in general had greater ion intensities than the Orbitrap MS images, indicating greater sensitivity of the RapifleX instrument, though several neuropeptides showed higher ion intensity in the Orbitrap MS images **Figure 2** shows a comparison of 5 neuropeptides identified with both platforms, showing the general improvement in spatial localization and ion images of most images acquired from the RapifleX.

Finally, CGs were imaged on both platforms in order to compare performance. CGs were collected from distinct crabs on the same day, with one being analyzed on the Orbitrap and one on the RapifleX. Again, the RapifleX demonstrated superior performance, with 119 neuropeptides putatively detected, while the Orbitrap enabled the detection of only 24 neuropeptides. Based on the comparative results, it was determined that the sensitivity and spatial resolution offered by the RapifleX provided better performance for imaging the CG, while the Orbitrap platform would be used for complementary analysis to validate putative identifications due to its higher mass spectral resolution and accurate mass measurement.

Localization of neuropeptides within cardiac ganglion

The cardiac ganglion consists of 9 neurons distributed asymmetrically across a tissue approximately 5 mm in length. The posterior side of the tissue connects to the posterior artery and extends to a T-shaped branch. Near this branch are 4 small pacemaker neurons that are electrically coupled and provide excitatory synaptic inputs to the 5 motor neurons. The motor neurons are located throughout the rest of the tissue, with 2 on the posterior side, near the pacemaker neurons. The anterior side of the CG branches into a Y shape, with 2 motor neurons on one side and 1 on the other side. The motor neurons work to produce depolarizing excitatory postsynaptic potentials on the heart muscle cells, thus driving the rhythmic contractions of the heart. The bursting of these neurons is controlled by Ca^{2+} , K^{+} , and neuropeptide modulators [4]. By determining which neuropeptides are localized to specific regions of the ganglion, insight into how these neuropeptides modulate heart activity can be obtained.

A total of 235 neuropeptides were localized in 3 biological replicates of MS imaging of the CG, comprising 24 neuropeptide families. Confirmation of these identifications was performed by analyzing CG extract with MALDI-MS and LC-ESI-MS/MS. Of the 235

identifications, 104 were confirmed with MS/MS and 65 with accurate mass matching within a 5 ppm mass tolerance. **Table S1** lists all the neuropeptides identified in the CG with MSI, along with the method of confirmation for identification.

MS images of the distribution of several neuropeptides are shown in **Figure 3**, along with the optical image of the tissue. The neuropeptides shown displayed discrete localization patterns that correlate with specific neuron locations within the tissue. The neuropeptide A-type allatostatin SPYAFGLamide (m/z 753.4) and RFamide ASNNLRFamide (m/z 820.4) appear to be localized to the posterior cells, including both the pacemaker and motor neurons in this region, while two A-type allatostatins, NVYSFGLamide (m/z 798.4) and NGSYPFGLamide (m/z 853.4), appear to be more concentrated to the pacemaker neurons, with a small amount in the posterior motor neurons. As RFamides have been shown to have excitatory functions and allatostatin peptides elicit inhibitory effects, their localization here indicates that these neuropeptides may also serve modulatory roles in the CG. The peptide HIGSLRYamide (m/z 844.5) is also localized to the posterior region of the CG, but is more broadly distributed, indicating potential presence in the axons of the neurons as well. While the functional role of HIGSLRYamide is not currently known, it has been widely detected throughout other tissue with previous MS studies, indicating it may have a variety of functions, both locally and as a circulating hormone [40-42]. There were also neuropeptides detected that were localized predominantly on the anterior side of the CG in the location of the anterior motor neurons, including A-type allatostatin GPYEFGLamide (m/z 781.4), CCHamide precursor-related peptide SVDKTEE (m/z 807.4), and IGSEFLamide (m/z 664.4). While these three peptides have been detected in the anterior motor neurons, they have distinct localization patterns from each other,

indicating that they may all serve modulatory functions in these neurons, but their specific functions may differ from one another.

Profiling results

In order to validate the neuropeptides localized in the CG, tissues collected from 12 animals were extracted, pooled together, and analyzed with LC-ESI-MS/MS. As the tissue contains only 9 neurons, the *in vivo* concentration of neuropeptides was expected to be low. In order to detect more low-abundance neuropeptides within the tissue, a DIA method was employed, as DIA has been previously demonstrated to improve the detection of neuropeptides in crustacean tissue [19]. The CG contains lipids and other interfering species that are present in high abundances. In contrast, if a conventional data dependent acquisition (DDA) method is implemented, in which only the top most abundant ions are selected for fragmentation, then fewer neuropeptides would be selected for fragmentation, resulting in a smaller number of identifications.

To demonstrate this difference, the same CG sample was analyzed with a top-10 DDA method, and the number of neuropeptides was compared. With the DDA method, 56 neuropeptides were identified, a considerably high number for such a small neuronal ganglion containing only nine neurons. However, with the DIA method, a total of 316 neuropeptides were identified, approximately six-fold improvement from the DDA method, including most of the neuropeptides identified with DDA. The drastic improvement in identification number is attributed to the ability to obtain fragment ions for all species in the sample spanning the given mass range of 400 to 800 *m/z*, regardless of relative abundance, as long as the abundance is above the limit of detection of the instrument. **Figure 4C** shows the overlap of neuropeptides

identified with DIA and DDA methods, with results being similar to those found previously in brain and pericardial organ tissue from *C. borealis* [19].

In addition to LC-ESI-MS/MS analysis, analysis of extracts with MALDI-MS was also employed. Numerous previous studies have shown the complementary nature of ESI and MALDI as ionization methods [25-27]. MALDI was used here to identify additional neuropeptides that may not be easily detectable with ESI. By directly analyzing tissue extract from 12 pooled CGs, 239 neuropeptides were identified, a similar number to that identified by ESI. The lower number is likely due to signal suppression within the sample, as no separation step was used prior to MALDI MS analysis.

With the LC-ESI-MS/MS DIA method, a total of 316 neuropeptides were identified, belonging to 31 different neuropeptide families. With the MALDI-MS method, 239 neuropeptides were identified over 23 neuropeptide families. Of these neuropeptides, 49 were detected in both platforms, and the representation of neuropeptides within each family were similar between each, as shown in the pie charts in **Figure 4A** and **4B**. These numbers are considerably large for a small, relatively simple tissue, indicating the underlying complexity within the circuitry involved in this system. The breakdown of identification confirmations for all putative identifications made with MS imaging is shown in the pie chart in **Figure 4D**.

Neuropeptides possess common motifs that are characteristic of different groups, or families. The most commonly represented neuropeptide families in the CG are allatostatin, RFamide, crustacean hyperglycemic hormone (CHH) and its precursor related peptide (CPRP), orcokinin, and pyrokinin. Allatostatins are divided into three types: A-type, B-type, and C-type, all of which have been found to have inhibitory effects on neural circuits [28, 29]. The physiological effects of C-type allatostatins have already been characterized in the cardiac

neuromuscular system, most causing a decrease in heart rate [9]. Allatostatin peptides have also been commonly identified in previous studies of the pericardial organs, which are located laterally on either side of the heart and are believed to release neuropeptides into the pericardial sinus [17, 30]. Therefore, it is expected that this family would be highly represented within the CG as well. Neuropeptides from the CHH superfamily have been implicated in numerous functions, including metabolism, ion transport, uptake of water, reproduction, and molting, mostly serving as endocrine signaling agents [31, 32]. As heart rate has been found to change with other physiological functions such as feeding, it is possible that CHH could also act as a signaling agent for cardiac output as well. While little is known about the function of CPRPs, they originate from the same precursors as CHH, so it is not surprising that they are present in the CG with CHH peptides. RFamide neuropeptides are broadly distributed throughout the nervous and neuroendocrine systems, functioning locally as autocrines and paracrines, as well as long range as circulating hormones [33-35]. Numerous RFamide neuropeptides have been found to modulate cardiac activity, and given the large number of RFamide peptide isoforms present in the CG, it is likely that additional RFamide neuropeptides play a physiological role in this tissue [36, 37]. The large presence of orcokinin neuropeptides in the CG is unexpected, as these neuropeptides are most frequently detected in nervous system tissue, such as the brain, and they have been implicated in modulating hindgut contractions and output of the stomatogastric nervous system [38, 39]. However, their presence in the CG indicates that they may also perform some modulatory role within the cardiac neuromuscular system. Pyrokinin neuropeptides have also been mostly studied in the stomatogastric nervous system, but there has been at least one study demonstrating an excitatory role in the lobster cardiac neuromuscular system [8].

Conclusions

This study sought to identify and localize neuropeptides within the crustacean cardiac ganglion, a small yet chemically complex neural circuit comprised of a small number of neurons. LC-MS/MS with a DIA method resulted in the identification of 316 neuropeptides, a surprisingly large number of peptides present in a small neuronal ganglion. After comparing MALDI-MSI platforms, a Bruker RapifleX TOF/TOF instrument was used to localize 235 neuropeptides to discrete regions of the CG with a high spatial resolution. Overall, the large number of neuropeptides able to be identified and localized within this tissue highlights the complexity of the modulatory circuits taking place within the crustacean cardiac neuromuscular system, and the results presented here demonstrate the powerful capabilities of MALDI-MS imaging with a multifaceted approach for studying small neural networks. While the specific functions of these peptides within the CG are largely unknown, their presence within the tissue indicates that they may function as a molecular regulator in modulating heartbeat. An important future direction will be to assess the function of these neuropeptides on cardiac output in both *ex vivo* and *in vivo* conditions in order to determine the biological activity of each neuropeptide.

Abbreviations: 2,5 dihydroxybenzoic acid (DHB), cardiac ganglion (CG), crustacean hyperglycemic hormone (CHH), crustacean hyperglycemic hormone precursor related peptide (CPRP), Crystal positioning system (CPS), data dependent acquisition (DDA), data independent acquisition (DIA), electrospray ionization (ESI), formic acid (FA), indium tin oxide (ITO), liquid chromatography (LC), mass spectrometry (MS), mass spectrometry imaging (MSI), matrix-assisted laser desorption/ionization (MALDI), pericardial organ (PO), total ion current (TIC), tandem mass spectrometry (MS/MS), time of flight (TOF)

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Figures

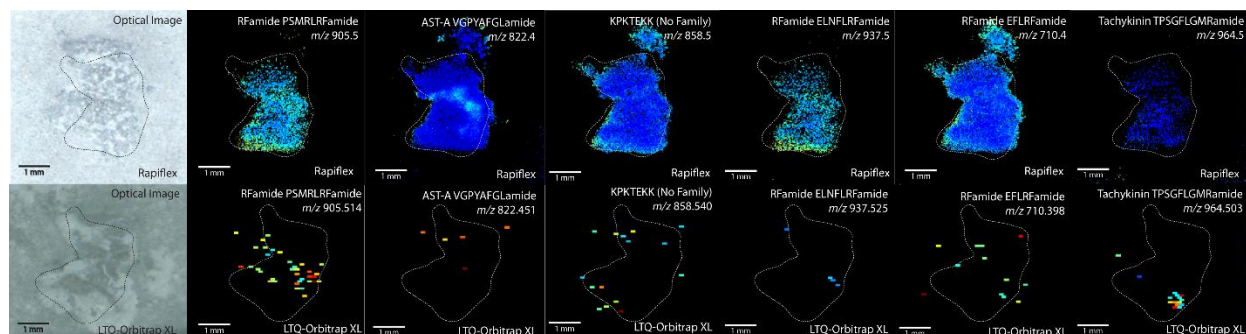


Figure 1. MALDI-MS images of *C. borealis* brain serial sections analyzed on a RapifleX TOF/TOF instrument (top) and LTQ-Orbitrap XL instrument (bottom). The optical images are shown on the far left. The comparison shows an improvement in spatial resolution for images acquired on the RapifleX. The image intensity was in general stronger for ions on the RapifleX, but several showed stronger signal on the Orbitrap, such as Tachykinin TPSGFLGMRamide (m/z 964.503).

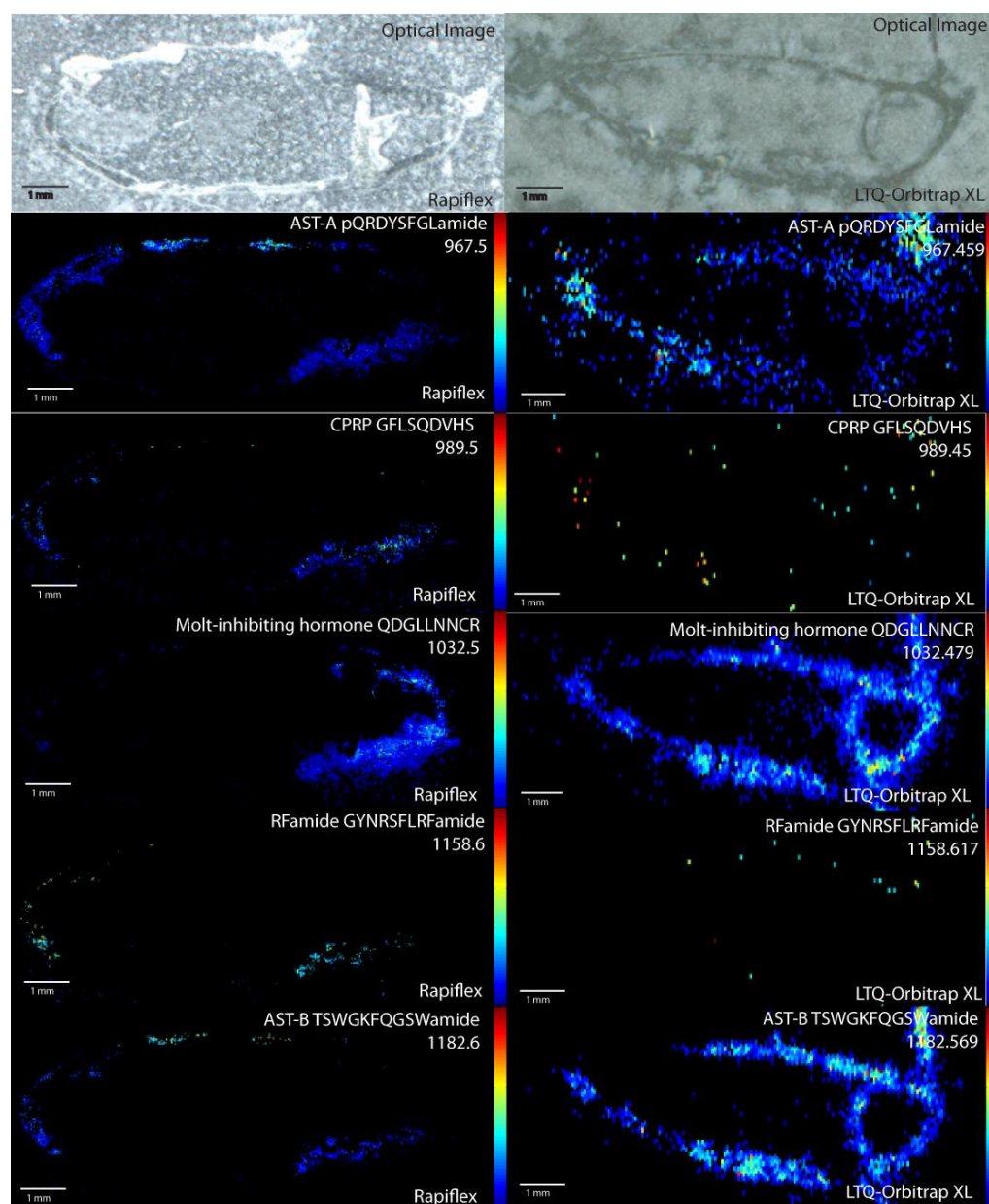


Figure 2. MALDI-MS images of *C. borealis* pericardial organs (POs) analyzed on a RapifleX TOF/TOF instrument (left) and LTQ-Orbitrap XL instrument (right). POs were collected from the same animal and the left was analyzed on the RapifleX and the right on the Orbitrap. The optical images are shown at the top of the figure. The comparison shows an improvement in spatial resolution for images acquired on the RapifleX. The image intensity was stronger for some ions on the RapifleX and others on the Orbitrap.

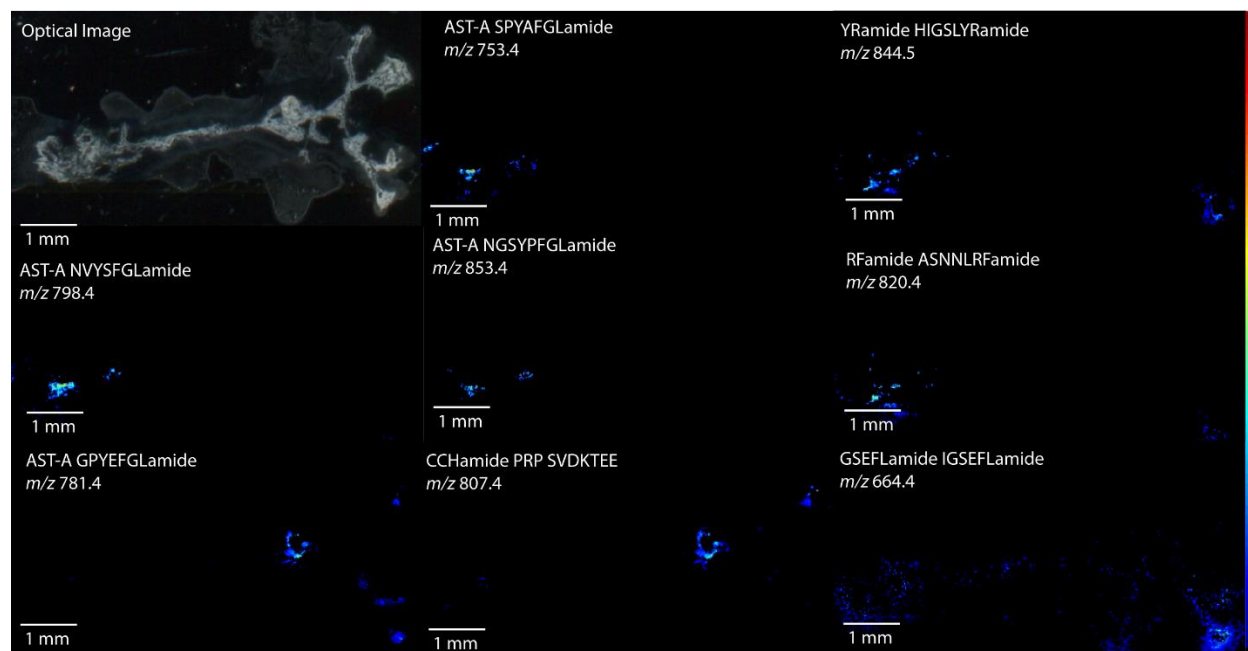


Figure 3. MALDI-MS images of *C. borealis* cardiac ganglion acquired on the RapifleX TOF/TOF instrument. Images represent neuropeptides localized to distinct regions of the tissue. The first image shows the optical image obtained of the tissue.

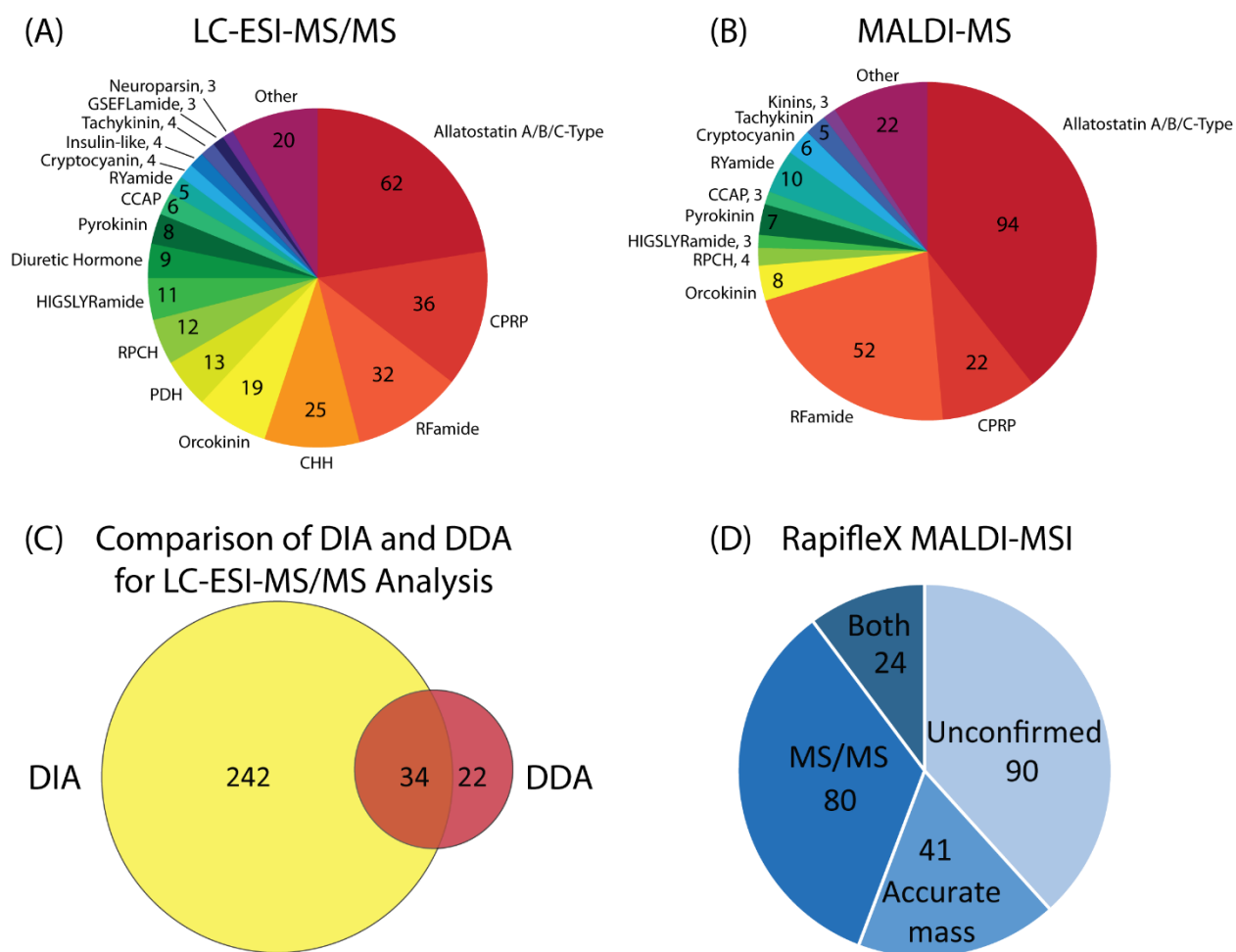


Figure 4. Representation of neuropeptides identified in the cardiac ganglion (CG), including the distribution of families neuropeptides belonged to that were identified with (A) LC-ESI-MS/MS with DIA analysis and (B) MALDI-MS with accurate mass matching. Also shown is (C) a comparison of two LC-MS/MS acquisition methods, DIA and DDA, showing the superior performance of DIA for the current analysis, and (D) a breakdown of the validation of neuropeptides detected with MSI on the RapifleX instrument, including those with confident MS/MS spectra, high-resolution accurate masses matching to the known database, and those with neither, listed as unconfirmed identifications.

Supporting Information

Table S1. Neuropeptides localized in the CG with MALDI-MSI and the method used to confirm their identity, if applicable. AMM indicates identification made by accurate mass matching, P represents pyroglutamate/pyroglutamic acid, O represents oxidation, sulf represents sulfation.

Family	Sequence	m/z	MS/MS	AMM
AST Combos	EPYAFGLGKRPATDL	1634.854	X	
AST Combos	DPYAFGLGKRPADLYEFGlamide	2128.086	X	
AST-A	YAFGLamide	569.308	X	
AST-A	YSFGLamide	585.303		
AST-A	YTFGLamide	599.319		
AST-A	SYDFGLamide	700.330		
AST-A	GIYGFGLamide	725.388		
AST-A	SGYSFGLamide	729.357		X
AST-A	GPYSFGLamide	739.377		
AST-A	SPYAFGLamide	753.393		
AST-A	GQYAFGLamide	754.388		
AST-A	EAYAFGLamide	769.388	X	
AST-A	GGAYSFGLamide	770.383		X
AST-A	SDYAFGLamide	771.367		X
AST-A	NPYAFGLamide	780.404		
AST-A	GPYEFGLamide	781.388		X
AST-A	DPYAFGLamide	781.389		X
AST-A	DDDSDVE	794.269		X
AST-A	AGPYAFGLamide	794.420		
AST-A	EPYAFGLamide	795.404		X
AST-A	NPYSFGLamide	796.399		
AST-A	NVYSFGLamide	798.404		
AST-A	GRYSFGLamide	798.426	X	X
AST-A	SGAYSFGLamide	800.394		
AST-A	GHYNFGLamide	806.394		X
AST-A	AAPYAFGLamide	808.435	X	
AST-A	AGPYSFGLamide	810.415		X
AST-A	SPYEFGLamide	811.399		
AST-A	GGPYSYGLamide	812.430	X	X
AST-A	NSYDFGLamide	814.373		
AST-A	SQYTFGLamide	814.409	X	
AST-A	ERPYSGL	820.421	X	
AST-A	VGPYAFGLamide	822.451	X	X
AST-A	AGQYAFGLamide	825.425	X	X
AST-A	GDPYAFGLamide	838.409	X	X
AST-A	GGPYEFGLamide	838.410	X	X
AST-A	TAPYAFGLamide	838.446		

AST-A	AGGAYSFGLamide	841.420		
AST-A	AGQYSFGLamide	841.421		
AST-A	SGSYNFGlamide	843.400		X
AST-A	GKPYAFGLamide	851.477		
AST-A	AGPYEFGlamide	852.425		
AST-A	EPYEFGlamide	853.409	X	
AST-A	NGSYPFGlamide	853.410	X	
AST-A	DGPYSFGLamide	854.404		X
AST-A	TPSYAFGLamide	854.441	X	X
AST-A	SGQYSFGLamide	857.415		X
AST-A	PNPYAFGLamide	877.457		X
AST-A	ANQYAFGLamide	882.447	X	
AST-A	SNPYSFGLamide	883.431	X	
AST-A	PSMYAFGLamide	884.434	X	X
AST-A	ADLYSFGLamide	884.451		
AST-A	ASQYTFGLamide	885.447	X	
AST-A	GSGQYAFGLamide	898.441		
AST-A	AKSYNFGlamide	898.478	X	
AST-A	QGMYSFGLamide	901.424	X	X
AST-A	ADMYSFGLamide	902.408		
AST-A	KLPYSFGLamide	923.535	X	
AST-A	ATGQYAFGLamide	926.473	X	
AST-A	PRTYSFGLamide	939.505		X
AST-A	pQNMYSFGLamide	941.419		X
AST-A	ERAYSFGLamide	941.474		
AST-A	SRQYSFGLamide	956.495	X	X
AST-A	DRTYSFGLamide	957.479		X
AST-A	APQPYAFGLamide	962.509		X
AST-A	YSKFNFGLamide	974.509		
AST-A	AGLSALYSFG	985.489	X	
AST-A	RNMYSFGLamide	986.477		
AST-A	AWSALHGAWamide	997.500	X	
AST-A	APRPYSFGLamide	1006.537	X	
AST-A	HGTEGPYPFGLamide	1173.620	X	
AST-A	ANEDEDAASLFAFGLamide	1568.723	X	
AST-A	EYDDMYTEKRPKVYAFGLamide	2224.074	X	
AST-A PRP	QEDVGFS	781.337		
AST-A PRP	DQDSDLE	821.316		
AST-A PRP	EDDSIE	822.300		
AST-A PRP	DY(SO)DDSDVE	957.333		
AST-A PRP	AWKEASNRLI	1187.654	X	X
AST-A PRP	SVRDVGSEEDK	1349.618	X	
AST-A PRP	EEAAEIEDEYDQLGFamide	1756.755	X	

AST-B	AGWSSLQ	748.363		
AST-B	VTWGKFQGSWamide	1194.606	X	X
AST-B	TNWNKFQGSWamide	1266.600		
AST-B	SKWNKFQGSWamide	1266.638		
AST-B	NNNWSKFQGSWamide	1366.629		X
AST-B	NNNWTKFQGSWamide	1380.644	X	
AST-B	LNWNKFQGSWamide	1447.760	X	
AST-B	MFAPLAWPKGGARWamide	1586.841		
AST-B PRP	TPDTLISA	817.431	X	
AST-B PRP	GEEIQDAEE	1019.417		
AST-C PRP	LPA	300.192		
AST-C PRP	MFAPLSGLPGNLRTI	1586.873	X	X
CCAP	PFNAFTG	753.346		
CCAP	PFCNAFTGCamide	956.375	X	X
CCAP PRP	RPVTL	585.372		X
CCAP PRP	IDGRPTY	821.416	X	
CCHamide PRP	SVDKTEE	807.374	X	
CPRP	GFLSQDVHS	989.450	X	
CPRP	RSVEGVSRME	1149.530		
CPRP	RGFEGETGHPN	1199.555	X	
CPRP	PLGFLSQDHSV	1199.620		
CPRP	SLKSDTVTPLLG	1230.694	X	
CPRP	ASLKSPTVTPLR	1269.753	X	
CPRP	ASLKSPTVTPLR	1287.727		
CPRP	KLLSSSNSPSSTP	1304.640		
CPRP	RSAQGMGKMERL	1363.690		
CPRP	RSAEGLGRVGRL	1383.818	X	
CPRP	RSVEGVSRMEKL	1390.690		
CPRP	KLLSSSNSPSSTPLG	1474.780		
CPRP	KLLSSISPSSTPLGF	1533.820		
CPRP	RSTQGYGRMDPIL	1552.830		
CPRP	RSVEGASRMEKLLS	1562.830		
CPRP	RSVEGASRMEKLLT	1576.850	X	
CPRP	SISPSSTPLGFLSQDHSV	1972.890		
CPRP	SSSNSPSSTPLGFLSQDHSV	2147.870	X	
CPRP	LLSSSNSPSSTPLGFLSQDHSV	2260.140		
Cryptocyanin	YKIFEPLRESN	1395.727		X
DH44 PRP	NPRYM	680.319		
DH44 PRP	AWPHSFS	831.379		
Enkephalin	YGGFM	574.233		X
GSEFLamide	IGSEFLamide	664.367		
GSEFLamide PRP	DEGVGEVA	775.347		
GSEFLamide PRP	YLSLLMN	853.449	X	X

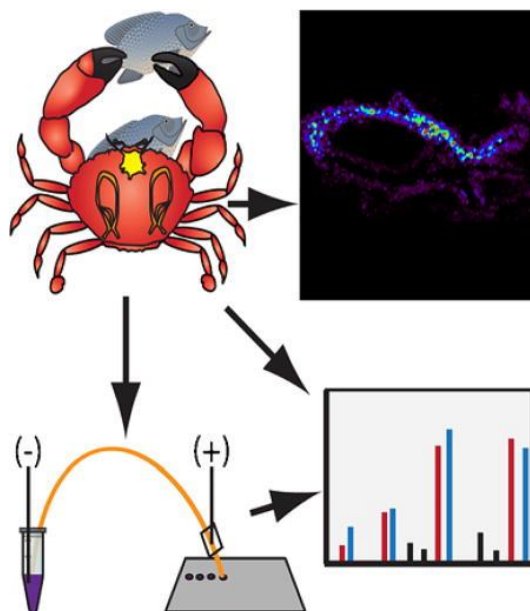
GSEFLamide PRP	GGGSSRDPMML	976.452	X	
GSEFLamide PRP	VLGALSRSLA	1142.701	X	
HIGSLYRamide	HIGSLRYamide	844.4789		X
HIGSLYRamide	HIGSLYRD	960.490	X	
HIGSLYRamide	HIGSLYLED	1133.548	X	X
HIGSLYRamide	EDNLY(SO3)HDLSED	1349.550	X	
HIGSLYRamide PRP	DEDNSYYDLNED	1491.540	X	
Insulin-like PRP	SPRTLPEGEAVSEGE	1557.740	X	
Intocin PRP	PRFDLLSTSRDSWEEQ	1965.931	X	
Kinin	AFSPWAamide	677.341		X
Kinin	ASFSPWGamide	750.357		X
Kinin	YFSPWGamide	755.351		
Kinin	PAFSPWGamide	760.378		
Kinin	VAFSPWGamide	762.393	X	
Kinin	pEAFSPWAamide	788.373	X	
Kinin	VRFSPWGamide	847.500	X	
Leucokinin	SAWAamide	433.220		
Leucokinin	FNAWAamide	607.299		
MIH	DCANIFR	838.377		
MIH	NTGMATLCR	966.439		
MIH	VINDDCPNLIGNR	1442.695		
MIH	VDWICKDCANIFR	1582.740		
MIH	VEWICEDCSNIFR	1613.698		
Orcokinin	FDEIDRSGFA	1156.527		
Orcokinin	EIDRSSFGFN	1171.538		
Orcokinin	NFDEIDRSGF	1199.533		
Orcokinin	SSEDMDRLGFG	1213.504		
Orcokinin	SSEDMDRLGFA	1227.520		
Orcokinin	VYGPRDIANLY	1280.663	X	
Orcokinin	DFDEIDRSSFG	1287.549		
Orcokinin	NFDEIDRSGFGF	1403.623		
Orcokinin	DFDEIDRSSFGFV	1533.686	X	
Orcokinin	NFDELDMSGFGFH	1540.671	X	
Orcokinin	NFDEIDRSGFGFH	1540.682	X	
Orcokinin	NFDEIDRSSFGFN	1547.676	X	
Orcokinin	NFDEIDRTGFGFH	1554.697	X	
Other	KPKTEKK	858.540	X	X
Other	EVEEPEAPAPPAK	1363.670	X	X
PDH	pQELHVPAREA	1190.576		
PDH	NSELINSLLGISRLMNEAamide	1973.048	X	
PDH	NSELINSLLGLSRLMNEA	1974.022	X	
Proctolin	RYLPT	649.367	X	X
Pyrokinin	SSFSPRPamide	776.406	X	X

Pyrokinin	ATFAFAPRPamide	976.537		X
Pyrokinin	ANFAFAPRPamide	989.532		
RFamide	EFLRFamide	710.398		
RFamide	GPFLRFamide	735.430		
RFamide	RARPRFamide	801.496		
RFamide	HVFLRFamide	817.516		X
RFamide	LRNLRFamide	817.515		X
RFamide	ASNNLRFamide	820.442		X
RFamide	RNFLRFamide	851.500		X
RFamide	GRNFLRFamide	908.521		
RFamide	SKNYLRFamide	926.521		X
RFamide	APALRLRFamide	942.600		
RFamide	PGVNFLRFamide	948.541		X
RFamide	FDDFLRFamide	958.478		
RFamide	pQRNFLRFamide	962.532		
RFamide	GGRNFLRFamide	965.533		
RFamide	NRNFLRFamide	965.543		X
RFamide	DRNFLRFamide	966.527		X
RFamide	GPKNFLRFamide	977.490	X	
RFamide	PSDNFLRFamide	994.500		
RFamide	NPSDFLRFamide	994.511		
RFamide	SGRNFLRFamide	995.553		
RFamide	GNRNFLRFamide	1022.565		X
RFamide	TNRNFLRFamide	1066.590		
RFamide	LETNFLRFamide	1066.600	X	X
RFamide	LPGVNFLRFamide	1090.660		
RFamide	ENRNFLRFamide	1094.585	X	X
RFamide	GAHKNYLRFamide	1104.606	X	X
RFamide	AQPSMRLRFamide	1104.609	X	X
RFamide	KHKNYLRFamide	1104.632	X	
RFamide	SQPSMRLRFamide	1120.604	X	
RFamide	SQGLNSDLRFamide	1135.585	X	
RFamide	EFVDPNLRamide	1135.589	X	
RFamide	YGNRSFLRFamide	1158.606	X	
RFamide	GYNRSFLRFamide	1158.617		
RFamide	YGNRPHLRFamide	1158.620	X	
RFamide	AYNRSFLRFamide	1172.632	X	X
RFamide	ARPRNFLRFamide	1175.691	X	
RFamide	LNQPNFLRFamide	1176.600	X	
RFamide	LSPRNFLRFamide	1177.620	X	
RFamide	DQNRNFLRFamide	1208.628	X	
RFamide	NYDKNFLRFamide	1215.627		
RFamide	YGSDRNFLRFamide	1273.644		

RFamide	LDGPLAPFLRFamide	1273.660	X	
RFamide	AYSDRNFLRFamide	1287.659	X	
RFamide	GYSVGLNYLRFamide	1287.684	X	
RFamide	LAPQRNFLRFamide	1288.810	X	
RFamide	pQFDEY(sulf)GHMRamide	1391.514	X	
RFamide	GDSSSVEDMDVLPETEDTIE	2167.908	X	
RPCH	pELNFSSGWamide	920.426	X	
RPCH	pEVNFSPSWamide	946.442	X	
RPCH	pELNFSPSWamide	960.457	X	X
RYamide	FVGGSRYamide	784.410		X
RYamide	GFVSNRYamide	841.432		
RYamide	FYSQRYamide	862.421		X
RYamide	RFVGGSRYamide	940.511		
RYamide	SGYNANRYamide	943.428		
RYamide	SGFYAPRYamide	959.473	X	X
RYamide	SGFYANRYamide	976.464		X
RYamide	SGFYADRYamide	977.448		
RYamide	LEWYSQRYamide	1171.560	X	
SIFamide	AYRKPPFNGSIFamide	1395.753	X	
Tachykinin	APSGFQamide	605.310	X	
Tachykinin	APSGFQ	606.278		
Tachykinin	GFLGMRamide	679.370		
Tachykinin	SGFLGMRamide	766.403		X
Tachykinin	APSGFLGM	779.376		
Tachykinin	TPSGFLGMRamide	964.503	X	
Tachykinin	TPSGFLGM(O)Ramide	980.498	X	

Chapter 7

A multifaceted mass spectrometric method to probe feeding related neuropeptide changes in *Callinectes sapidus* and *Carcinus maenas*



Modified from:

Zhang, Y.*; DeLaney, K.*; Hui, L.; Wang, J.; Sturm, R.; Li, L. A Multifaceted Mass Spectrometric Method to Probe Feeding Related Neuropeptide Changes in *Callinectes sapidus* and *Carcinus maenas*. *Journal of the American Society for Mass Spectrometry*. 2018, 29(5), 948-960. PMID 29435768. (*Co-first authors)

Abstract

Food intake is regulated by various neuromodulators, including numerous neuropeptides. However, it remains elusive at the molecular and cellular level as to how these important chemicals regulate internal processes and which regions of the neuronal organs are responsible for regulating the behavior. Here we report a comparative neuropeptidomic analysis of the brain and pericardial organ (PO) in response to feeding in two well-studied crustacean physiology model organisms, *Callinectes sapidus* and *Carcinus maenas*, using mass spectrometry (MS) techniques. A multifaceted MS-based approach has been developed to obtain complementary information on the expression changes of a large array of neuropeptides in the brain and PO. The method employs stable isotope labeling of brain and PO extracts for relative MS quantitation, capillary electrophoresis (CE)-MS for fractionation and high-specificity analysis, and mass spectrometric imaging (MSI) for *in-situ* molecular mapping of peptides. A number of neuropeptides, including RFamides, B-type allatostatins (AST-B), RYamides, and orcokininins exhibit significant changes in abundance after feeding in this investigation. Peptides from the AST-B family found in PO tissue were shown to have both altered expression and localization changes after feeding, indicating that they may be a class of vital neuropeptide regulators involved in feeding behavior.

Introduction

The uptake of energy from the environment is essential to all organisms, as it is necessary for performing biological functions within the body. As organisms continuously consume energy to carry out functions necessary for survival, both maintaining a reliable energy influx and functioning internal mechanisms for processing the energy are crucial to survival. In animals, the supply of energy comes from food intake, and, as such, its dysfunction may be correlated with diseases such as obesity, which in turn could lead to an increased risk of heart disease, diabetes, and stroke.¹ Food intake regulation utilizes an integrated response performed by neural circuits spanning from the central nervous system to peripheral signals, utilizing a wide variety of signaling molecules.²⁻⁴ The complexity of this physiological process makes it difficult to study, and the identity and function of specific signaling molecules involved in the process remain elusive. Members of one of the most important and complex classes of signaling molecules, neuropeptides, have been shown to play a critical role in regulating the food intake process. Neuropeptides are short-chain amino acid sequences that function as neuromodulators or neurohormones and are essential to normal chemical signaling within the body. Recent efforts have focused on characterizing neuropeptides and their signaling pathways in a variety of species.⁵⁻⁹ Furthermore, there have been numerous studies connecting specific neuropeptides with feeding behavior. For example, orexigenic neuropeptides galanin, neuropeptide Y (NPY), orexin, melanin-concentrating hormone, and corticotropin-releasing factor cause an increase in food intake, while proopiomelanocortin, neurotensin, cholecystokinin (CCK), bombesin, tachykinin, and serotonin decrease food intake.^{1, 10-14} However, the enormous technical challenges of studying the complex mammalian nervous system has prevented a more detailed understanding of the interplay between these neurotransmitters and hormones.

Invertebrate nervous systems are relatively simple and well-characterized. Consequently, they are used extensively to gain insights into the functional roles of endogenous peptides, especially neuropeptides, in food intake.¹⁵⁻¹⁹ Invertebrate models are also highly relevant, as increasing evidence suggests that many of the signaling molecules and pathways underlying complex behaviors such as feeding are conserved across species. For example, the conserved NPY signaling pathway has been strongly implicated in the stimulation of food intake in vertebrates as well as in the regulation of the food-conditioned foraging behaviors of *Caenorhabditis elegans*.²⁰ ²¹ More recently, *Drosophila* neuropeptide F, a human NPY homologue, was reported to mediate food signaling through a conserved pathway.²² Another neuropeptide family involved in the regulation of feeding behavior is composed of peptides terminating in Arg-Phe-NH₂, which belong to the RFamide family. A large number of RFamides have been characterized in invertebrates,²³ ²⁴ and members of the RFamide family have been demonstrated to be involved in feeding behavior in both vertebrates and invertebrates.²⁵ The conservation of peptide structure in the nervous systems of various animals and the previously demonstrated involvement of neuropeptides in feeding behavior enforce the validity of using invertebrate model systems for the identification of new regulatory peptides in feeding.

Callinectes sapidus, blue crab, and *Carcinus maenas*, green crab, were chosen as experimental models in this study because the neuropeptide complements of these two species have been extensively studied by immunohistochemistry²⁶⁻³⁰ and mass spectrometry.^{31, 32} Furthermore, the electrophysiology and anatomical connections of these systems are well-established.³³⁻³⁵ The stomatogastric nervous system (STNS) neural network of crustaceans contains several central pattern-generating neural circuits that control the motion of gut and foregut. The STNS is composed of four ganglia, namely the stomatogastric ganglion (STG),

esophageal ganglion (OG), and the paired commissural ganglia (CoG). The brain communicates with the STNS via the unpaired inferior ventricular nerve (*ivn*) and the paired circumesophageal commissures (CoCs) through CoGs.³⁶ The pericardial organs (POs), paired neurosecretory organs that surround the heart, function as major release sites of hormones and neuromodulators. By studying several key tissues in each species, we can gain insight into the overall mechanisms and signaling pathways involved in feeding behavior.

In this study, we employed stable isotope labeling to investigate the relative quantitative changes of diverse neuropeptide families in crustacean brains and pericardial organs (PO) between unfed and fed subjects. The results indicate the up-regulation of several neuropeptide families located in the crab brain after food intake, including tachykinins, orcokininins, and YRamides. Other families, such as RYamides, B-type allatostatins (AST-B), and RFamides, are significantly reduced in the PO. We also utilized matrix-assisted laser desorption/ionization (MALDI)-MS imaging (MSI) to examine the distribution of neuropeptides of interest in the POs and observed neuropeptide localization changes induced by feeding. Overall, this study further demonstrates that crustaceans provide useful model systems to study the neuroendocrine regulation of feeding, and that our multifaceted MS-based platform offers a powerful tool to directly examine neuropeptide expression changes under different physiological states.

Methods

Materials

Methanol, acetonitrile, formic acid, acetic acid, borane pyridine, and formaldehyde (FH₂) were purchased from Fisher Scientific (Pittsburgh, PA). Deuterium formaldehyde (FD₂) was purchased from Isotech (Miamisburg, OH). 2,5-dihydroxybenzoic acid (DHB) was obtained from MP Biomedicals, Inc. (Solon, OH). α -cyano-4-hydroxy-cinnamic acid (CHCA) was

purchased from Sigma-Aldrich (St. Louis, MO). Acidified methanol was prepared using 90% methanol, 9% glacial acetic acid, and 1% water. All water used in this study was doubly distilled on a Millipore filtration system (Bedford, MA). C18 ziptips were purchased from Millipore (Billerica, MA).

Animals and Feeding Experiments

Blue crabs *Callinectes sapidus* were purchased from a local grocery store, and green crabs *Carcinus maenas* were purchased from Marine Biological Lab (Massachusetts, USA). All animals were maintained without food in an artificial seawater tank at 12-13 °C for 5 days before use. In the feeding experiments, crabs were fed pieces of either fish or shrimp. The food was placed in the tank and the crabs were allowed to eat until they stopped, which usually took 45 minutes. Control crabs remained unfed prior to dissection. Crabs were then cold-anesthetized by being kept on ice for 15 minutes. Dissections were performed in chilled (approximately 10 °C) physiological saline (composition: 440 mM NaCl, 11 mM KCl, 13 mM CaCl₂, 26 mM MgCl₂, 10 mM trizmaHCl, pH 7.4 [adjusted with NaOH]). The details of dissection were described previously³⁷.

Tissue Extraction

To extract neuropeptides, the tissue samples were homogenized with a tissue homogenizer in 40 µL of acidified methanol (90/9/1 methanol/acetic acid/water). The homogenate was then transferred to a 1000 µL microcentrifuge tube. The solution was sonicated for 10 min to improve the extraction efficiency, and the resulting homogenate was centrifuged at 13,200 rpm for 10 minutes. The supernatant was transferred to another 1000 µL microcentrifuge tube and placed on ice. The tissue homogenizer was rinsed with 50 µL acidified methanol, and the resulting solution was used to wash the pellet in the microcentrifuge tube. The homogenate was centrifuged for 8

minutes and then added to the supernatant tube. The rinse and wash steps were repeated 3 times, and the combined supernatant was desiccated using a Savant SC 110 SpeedVac concentrator (Thermo Electron Corporation, West Palm Beach, FL). The sample was then resuspended in 8 μ L of Millipore water containing 0.1% formic acid (v/v). The crude extract was desalted using C18 ziptips according to the product instruction in order to remove lipids and salts before performing the formaldehyde labeling reaction.

In-Solution Formaldehyde Labeling

A 3 μ L aliquot of tissue extract from brain or PO was labeled in solution by adding 0.7 μ L borane pyridine (C_5H_8BN , 120 mM in 10% methanol) and mixing with formaldehyde (FH_2 , 4% in H_2O , 0.5 μ L) for unfed samples and deuterium formaldehyde (FD_2 , 4% in H_2O , 0.5 μ L) for fed samples. The samples were then left at room temperature for 15 minutes to allow the labeling reaction to go to completion. Aliquots of 4 μ L from each solution were combined and mixed in a 1:1 ratio. The resulting mixture was spotted on a target plate and analyzed using MALDI-FTMS or MALDI-TOF/TOF.

Off-line CE-MALDI MS Analysis

Off-line CE separation was performed on a customized CE apparatus equipped with a capillary of 75 cm in length (50 μ m i.d. $\times$ 360 μ m o.d.). The CE runs were carried out with -18 kV applied to the capillary inlet while the outlet was connected to ground. An ammonium formate buffer (50 mM, 2.5% MeOH (v/v) pH 3.5) was used and the runs were conducted at room temperature, 25 $^{\circ}C$. The CE effluent was deposited every sixty seconds onto a MALDI target plate coated with parafilm with predeposited DHB matrix spots. The apparatus and procedure are described previously.²⁶ The collected CE effluents from 5 to 35 min were subsequently analyzed by MALDI-FTMS.

Mass Spectrometry and MALDI Imaging

MALDI-TOF/TOF

A model 4800 MALDI TOF/TOF MS analyzer (Applied Biosystems, Framingham, MA) equipped with a 200 Hz, 355 nm Nd:YAG laser was used for brain and PO extract quantitation and MALDI imaging. For sample spotting, 0.4 μL of sample was spotted on the stainless steel MALDI plate first, allowed to dry, and followed by the addition of 0.4 μL of matrix solution. The matrix used was 5 mg/mL of α -cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile (v/v). Each sample was spotted twice onto the MALDI plate, and one spectrum was obtained for each pair of spots. Acquisitions were performed in positive ion reflectron mode. Instrument parameters were set using the 4000 Series Explorer software (Applied Biosystems). Mass spectra were obtained by averaging 900 laser shots covering a mass range of m/z 500 to 4000.

MALDI-FTMS

A Varian Fourier transform mass spectrometry (MALDI-FTMS) instrument (Lake Forest, CA) equipped with a 7.0 T actively-shielded superconducting magnet was used for CE separation analysis. All mass spectra were collected in the positive ion mode. The matrix was comprised of 150 mg/mL DHB in 50% MeOH (v/v). A 355 nm Nd:YAG laser (Laser Science, Inc., Franklin, MA) was used to create ions that can be accumulated in the external hexapole storage trap before being transferred through a quadrupole ion guide to the ion cyclotron resonance (ICR) cell. The ions were excited prior to detection with an RF sweep beginning at 7050 ms with a width of 4 ms and amplitude of 150 V from base to peak. The filament and quadrupole trapping plates were initialized to 15 V, and both were ramped to 1 V from 6500 to 7000 ms to reduce baseline distortion of the peaks. Detection was performed in the broadband mode from m/z 108.00 to 2500.00.

MALDI Imaging

MALDI imaging of *C. sapidus* and *C. maenas* PO was performed as previously described.³⁸ Briefly, the PO was rinsed in water immediately following dissection to eliminate salt content. It was then placed onto a MALDI plate and placed in a desiccator to dry on the plate without being frozen or sectioned. Before imaging acquisition, five coats of DHB (150 mg/mL in 50% methanol, v/v) were applied on the tissue surface using an airbrush (Paasche airbrush company, Chicago, IL) with 30 seconds of drying time between each coat. Imaging acquisition was performed on the model 4800 MALDI TOF/TOF analyzer (Applied Biosystems, Framingham, MA, USA) controlled using the 4800 Imaging application (Novartis, Basel, Switzerland) available through the MALDI MSI website (www.maldi-msi.org). To generate images, spectra were collected at 100 μm intervals in both the x and y dimensions across the surface of the sample. Each mass spectrum was generated by averaging 200 laser shots over the mass range m/z 800-2000. Individual spectra were acquired using 1.0 ns binning to yield 27812 data points per spectrum. Image files were then processed and extracted ion images were created using the TissueView software package (Applied Biosystems, Framingham, MA, USA).

Data Analysis of Quantitative Experiments

The spectra were analyzed manually using accurate-mass matching and the peak pairs corresponding to known crustacean neuropeptides were selected. The monoisotopic peak intensities of FH₂ (light)- and FD₂ (heavy)-labeled peak pairs were determined from these spectra. As the fed and unfed extracts for each replicate were run together in a single acquisition, the abundance ratio for each neuropeptide in fed crab versus unfed crab was determined by dividing the heavy-labeled peak intensity by the light-labeled peak intensity within the spectrum. Average ratios were calculated based on spectra from two duplicate experiments (technical replicates) in order to measure neuropeptide expression differences between the two samples in each acquisition.

A student's two-tailed unequal variance test was also performed using Microsoft Excel in order to evaluate the differences between fed and unfed samples without assuming equal population variances. In the t-test, the intensities of FH₂- and FD₂- labeled peaks were normalized by dividing the intensity of each peak by the sum of the intensities of both peaks within the FH₂/FD₂-labeled peak pair to eliminate differences of ionization efficiency between different acquisitions (technical replicates).

Results and Discussion

This study sought to develop and implement an analytical method to characterize changes in neuropeptide regulation within the brain and PO of *C. sapidus* and *C. maenas*. Using stable isotope labeling with formaldehyde, we examined the quantitative changes of neuropeptide expression in fed animals versus unfed animals. As a control experiment, we examined isotopic formaldehyde labeled brain extracts from unfed animals showing approximately 1:1 ratio of light and heavy labeled peptide pairs with the same physiological state (**Figure S1**). To investigate the correlation between neuropeptide expression and localization, MALDI MS imaging was used to map the spatial distribution of several neuropeptides of interest in the PO. It has been shown that there is an observable and measurable change in both expression levels and localization patterns within the tissue for some neuropeptides, while others remain unchanged. These findings provide insight into potential functions of these neuropeptides, indicating that certain neuropeptides participate to some extent in the regulation of feeding within crustaceans, while others do not appear to play a detectable role. As crustacean neuropeptides have numerous homologues in humans, this information may translate to the human neuropeptidome as well.

Quantitative Expression Changes of Neuropeptides in the Brain from Feeding

Quantitative changes in neuropeptide expression were examined in brains from 4 fed-unfed pairs of *C. maenas* and brains from 5 fed-unfed pairs of *C. sapidus*. **Figure 1A** shows a representative MALDI-TOF/TOF mass spectrum from one pair of brain extracts from *C. maenas*. The peak pairs of labeled neuropeptides are indicated with their corresponding masses. The abundance ratios (fed to unfed) of several families of neuropeptides, including CabTRPs, orcokinins, RFamides, YRamides, and others, exhibited an increase. **Table 1** lists the average abundance ratios from the 4 experiments for 18 neuropeptides detected in the *C. maenas* brain. Each ratio is calculated from two replicate MS spectra. The mean ratios averaged from the four groups of data and their corresponding p-values are also included. A representative MALDI-TOF/TOF mass spectrum comparing a pair of control animals is shown in **Figure 1B**. As can be seen, the ratios do not change appreciably between the control crabs, indicating that biovariability is not a substantial contributor to ratios deviating from unity in fed versus unfed animals. Therefore, it can be assumed that the ratios observed between fed and unfed animals represent changes due to feeding and not natural biovariability.

In *C. sapidus*, neuropeptides in the brain were relatively quantified in 5 groups of animals. **Figure 2** shows a bar graph representation of abundance ratios (fed to unfed) for 16 neuropeptides detected in the brain, including neuropeptides from the YRamide, CabTRP, RFamide, orcokinin, and SIFamide families. The y-axis represents the neuropeptide abundance ratios, and the x-axis provides the neuropeptide sequences. The error bars indicate standard error, and the corresponding p-values are displayed on the graph. Most of the neuropeptides showing significance differences suggest an increase in expression in the brain resulting from feeding, which is consistent with results found in previous studies on other species.³⁹⁻⁴³

Significant changes ($p < 0.05$) are observed for members of several neuropeptide families, indicated by bold and italicized font in **Table 1** for *C. maenas* and **Figure 2** for *C. sapidus*. Of all of the neuropeptide families, YRamides are consistently increased by a relatively large amount in both species. In our previous study, YRamides showed elevation after food intake in *C. borealis*,³⁹ and a similar trend was observed in *C. maenas* and *C. sapidus*. The YRamide HIGSLYRa (m/z 844.48) is increased by 1.5-fold in the fed *C. maenas* brain and 1.6-fold in the *C. sapidus* brain. These results suggest that YRamides may have a function in brain processes associated with crustacean feeding regulation.

Members of the orcokinin family, one of the most abundant neuropeptide families present in crustacean brain, showed significant increases in fed *C. maenas* brain (approximately 1.2-fold, $p < 0.05$). This includes NFDEIDRSGFGFA (m/z 1474.63), NFDEIDRSGFGFV (m/z 1502.69), NFDEIDRSSFGFV (m/z 1532.70), and NFDEIDRSSFGFN (m/z 1547.68). However, one of the isoforms detected, NFDEIDRSGFa (m/z 1198.55), did not show a consistent expression change ($p > 0.05$). Two isoforms from the orcokinin family, NFDEIDRSSFGFN (m/z 1547.68) and NFDEIDRTGFGFH (m/z 1554.70), showed higher expression levels after food intake in *C. sapidus*, while isoforms NFDEIDRSGFGFA (m/z 1474.63) and NFDEIDRSSFGFV (m/z 1532.70) did not change significantly. Orcokinin was first discovered by Stangier and colleagues in the crayfish *Orconectes limosus* and was reported to be a powerful stimulator of hindgut contractility.⁴⁰ A variety of other orcokinin isoforms were later discovered not only in decapods, but also in insects.^{41, 44-46} In previous research, orcokinins were found to function as stimulators of gut muscles,⁴⁰ as well as neuromodulators in crustaceans.⁴¹ Interestingly, however, it was found that the same orcokinin isoform could have different functions in different species.⁴⁷ The quantitation data in this study shows that orcokinin isoforms increase in crab brain after eating,

suggesting that orckinins may also be involved in the regulation of feeding processes in crustaceans.

The RFamides are a neuropeptide family conserved in both invertebrate and vertebrate animal nervous systems. RFamides have long been known to regulate feeding as anorexigenic signaling molecules in mice,⁴⁸ as well as in other animals.⁴³ Recent reports have shown that RFamides convert feeding motor programs from ingestive to egestive and depress feeding muscle contractions.^{42, 43} Our study further confirms the anorexigenic functional roles of some RFamides involved in feeding in crustacean. Three RFamide isoforms, NRNFLRFa (m/z 965.54), DRNFLRFa (m/z 966.53), and APQGNFLRFa (m/z 1048.57), exhibited a small increase (approximately 1.15-fold) in the *C. maenas* brain after feeding, whereas the remaining RFamides did not consistently increase after food intake. Similarly, two RFamides, NRNFLRFa (m/z 965.54) and TNYGGFLRFa (m/z 1073.55) in *C. sapidus* showed a small elevation after feeding, while other RFamides, GLSRNYLRFa (m/z 1124.61), GYSKNYLRFa (m/z 1146.61), and YGNRSFLRFa (m/z 1158.62), did not change significantly.

Two neuropeptides from the CabTRP family, APSGFLGMRa (m/z 934.49), detected in both species, and TPSGFLGMRa (m/z 964.50) detectable in *C. maenas* only, showed significant increases after feeding. Other types of neuropeptides, such as SIFamide and proctolin, did not exhibit significant changes in either species' brain after feeding. In summary, multiple families of neuropeptides present in animal brains were studied by MS-based quantitation methods. Various neuropeptide families including CabTRPs, RFamides, YRamide, and orckinins are likely involved in feeding regulation based on their changes in expression. This data suggests that food intake behavior is a complex process regulated by multiple signaling molecules, indicated by the

variety of responses from the detected neuropeptides, and the brain plays a substantial role in such regulation.

Neuropeptide Release from the Pericardial Organs upon Feeding

The pericardial organ is an important neuroendocrine tissue and has long been known to be a major source of circulating hormones. Moreover, it has been shown that many of the hormones present in the PO can modulate the neural circuits in the STG.⁴⁹ Thus, it can be expected that this important neuroendocrine organ is involved in feeding regulation by releasing neuropeptides and hormones into the hemolymph, or circulating fluid. Our previous research used *Cancer borealis* as an experimental model to test this hypothesis³⁹ and discovered that after food-intake, a variety of neuropeptide families were detected in the hemolymph, presumably released from the PO. To further correlate neuropeptide release with the feeding process, we performed a quantitative study of neuropeptides in the POs of fed and unfed *C. sapidus* and *C. maenas*. **Figure 3** shows representative MALDI TOF/TOF mass spectra of 4 pairs of PO extracts from unfed and fed *C. maenas*. The details of the trends in neuropeptide expression changes in *C. maenas* are shown in **Table 2**. **Figure 4** shows the abundance ratios of each detected neuropeptide in 5 pairs of PO extracts from unfed and fed *C. sapidus*. We were able to examine 18 neuropeptides from 6 families in *C. maenas* and 13 neuropeptides from 5 families in *C. sapidus*. In contrast to the quantitation data from the brain, most neuropeptides in the POs exhibited a reduction in expression levels after food intake, suggesting that feeding might trigger the release of these neuropeptides from the PO into the animal's hemolymph. This result is to be expected, as the PO is a prominent neurosecretory tissue that releases neuropeptides into the hemolymph to modulate the STG.

The RYamide family consists of neuropeptides with sequences highly conserved across different species. As described previously, RYamides have been found to be important to food-

intake in various animals. Therefore, it is not surprising that these peptides are likely involved in the feeding process in crustaceans. All RYamides in *C. maenas* PO, FVGGSRYa (m/z 784.41), SGFYANRYa (m/z 976.46), and SSRFVGGSRYa (m/z 1114.47), and one RYamide in *C. sapidus* PO, SSRFVGGSRYa (m/z 1114.47), were found to decrease after feeding.

AST-B family members have been reported to exert physiological actions on the pyloric neural circuit in the stomach where they have inhibitory effects on the pyloric rhythm.⁵⁰ In this study, all of the detected AST-B family members showed a significant decrease after food intake in both species, with the exception of VPNDWAHFRGSWamide (m/z 1470.70). This further ties the role of AST-B neuropeptides to the feeding process. Neuropeptides from the AST-B family, including QWSSMRGAWa (m/z 1107.56), SGDWSSLRGAWa (m/z 1220.63), STNWSSLRSAWa (m/z 1293.65), and VPNDWAHFRGSWa (m/z 1470.74), exhibited reduced expression levels in *C. sapidus* PO extract. In *C. maenas*, the AST-B neuropeptides QWSSMRGAWa (m/z 1107.5) and STNWSSLRSAWa (m/z 1293.63) also showed reduced expression levels. However, the AST-B neuropeptide VPNDWAHFRGSWa (m/z 1470.7), which exists in most crustacean species, had a highly inconsistent response.

Other neuropeptides, including orcokinins NFDEIDRSGFGFV (m/z 1502.69) and NFDEIDRSSFGFV (m/z 1532.70) in *C. maenas*, and YRamide HIGSLYRa (m/z 844.48) and proctolin RYLPT (m/z 649.47) in *C. sapidus* were reduced in the PO extracts of fed animals. The remaining detected neuropeptides did not exhibit significant changes.

CE Separation for Higher Peptidomic Coverage and Analytical Specificity

In blue crab *C. sapidus* brain, the AST-B family peptides were not detected, likely due to their abundance falling below the detection limit with direct MALDI-MS analysis. One possible reason is that the brain extracts are highly complex, containing numerous biological molecules,

such as lipids, protein fragments, and other endogenous peptides of high abundances. Another reason may be that the AST-B peptides are at lower abundances in *C. sapidus* compared to other species. To explore this, microscale separation utilizing CE was implemented in order to provide separation, effectively decreasing the complexity of the sample for MALDI-MS analysis. The low abundance AST-B isoforms, TSWGKFQGSWa (m/z 1182.57), TGWNKFQGSWa (m/z 1209.58), and GNWNKFQGSWa (m/z 1222.58) that were barely seen in direct MALDI TOF/TOF analysis are readily detected after CE separation (Supporting information **Figures S2, S3, and S4**). The TGWNKFQGSWa (labeled m/z 1237.61 and 1241.64) and GNWNKFQGSWa (labeled m/z 1250.61 and 1254.64) peak pairs indicate a small increase after food intake, while the pair of TSWGKFQGSWa (labeled m/z 1210.60 and 1214.63) did not exhibit this trend.

Feeding Regulation Associated with Neuropeptide Localization

Compared to traditional methods like immunohistochemistry, MALDI-MSI can be used to study the localization of multiple molecules simultaneously in a high throughput manner. MALDI-MSI has been applied to map many different types of molecules in tissues, including lipids, proteins, and peptides.⁵¹⁻⁵⁴ In this study, MALDI-MSI was used to examine the differential distribution of numerous neuropeptides in both fed and unfed *C. maenas* and *C. sapidus*. The structure of the POs in *C. sapidus* and *C. maenas* is similar to that of *C. borealis*, an already well-characterized species, as they contains a dorsal trunk, ventral trunk, anterior bar, and posterior bar. This is shown in **Figure 5** which displays the optical images of the unfed and fed *C. sapidus* POs on a MALDI plate without matrix coating. Areas of the PO referenced are labeled on the optical images. A total of 20 neuropeptides from 7 families were studied in *C. sapidus*, and 11 neuropeptides from 5 families were studied in *C. maenas* for location information, and example ion images for each unfed and fed pair are shown in **Figures 5 and 6**. In the quantitation study,

AST-B, orcokinin, RYamide, and some RFamide peptides were found to have different expression levels after food intake in both species. Therefore, the localization of these neuropeptides was carefully examined in order to provide more information about neuropeptide secretion in relation to feeding. **Figure 5** represents neuropeptide distribution in the POs of unfed and fed *C. sapidus*. Although RYamide and orcokinin families exhibit different expression levels after feeding behavior, the localizations of these two neuropeptide families remain unchanged in the dorsal trunk after feeding. Thus, it is possible that these neuropeptide families regulate feeding behavior by releasing neuropeptides into hemolymph but not through transport within tissue. **Figure 6** shows the spatial distribution of neuropeptides in the POs from fed and unfed *C. maenas*. In unfed *C. maenas*, RYamides are present extensively in the entire PO. After feeding, they become more concentrated in the posterior bar region. Our group also conducted MS imaging experiments for *C. borealis* PO and found that RYamides were present in the anterior bar and adjacent nerves ³⁸. These observations suggest that the same isoforms can have different spatial distribution patterns in the tissue and are thus likely to play different roles in different species.

The results for MS imaging of *C. maenas* PO are shown in **Figure 6**. **Figure 6i** shows the optical images of the unfed and fed POs before matrix coating, with areas of the PO labeled. **Figures 6ii. – vi.** show the neuropeptide localization in unfed and fed *C. maenas* POs. AST-B (**Figure 6 ii.** and **Figure S5ii. – v.**) is the most abundant neuropeptide family in the *C. maenas* PO. The AST-B neuropeptides QWSSMRGAWa (m/z 1107.52) (**Figure 6ii.**) and GSNWSNLRGAWa (m/z 1246.61) (**Figure S5v.**) have especially high abundances in the PO of unfed *C. maenas* and are prevalent throughout the whole PO, following the distribution trend of the entire family. The presence of these AST-Bs decreases substantially after feeding, and they become more localized to the posterior and anterior bars. The RYamides (**Figure 6iii.** and **Figure S5vi. – viii.**) are present

throughout the unfed PO, with higher concentrations in trunk parts. However, these neuropeptides appear more localized to the posterior bar in the fed PO. The abundance of RFamides (**Figure 6vi.** and **Figure S5ix.** and **x.**) and CCAP (**Figure 6v.** and **Figure S5xi.**) in *C. maenas* PO is relatively low in both fed and unfed compared to other families but is higher in the posterior and anterior parts. The most interesting phenomenon is an expression level change for SIFamide (**Figure 6vi.** and **Figure S5xii.**). Although this neuropeptide does not show a significant change in the extraction experiment, it exhibited much stronger signal after feeding in the imaging MS study.

Several example ion images for neuropeptides existing in both unfed and fed *C. sapidus* are shown in **Figures 5ii. – viii.**, as well as **Figure S4**, including 20 neuropeptides from 7 families. In the unfed PO, all RYamides (**Figure 5ii.** and **Figure S4ii. – v.**) are in half of the anterior bar and posterior bar while isoforms FVGGSRYa (m/z 784.41) and pEGFYSQRYa (m/z 1030.45) are also abundant in the dorsal trunk. In the fed PO, these same neuropeptides appear to be concentrated to primarily dorsal trunk. All AST-Bs (**Figure 5iii.** and **Figure S4vi. – ix.**) in the unfed PO are present extensively in the entire anterior bar, while STNWSSLRSAWa (m/z 1293.65) is distributed throughout the whole PO tissue. In the fed PO, the neuropeptides appeared to be more distributed throughout the whole tissue, while still being more intense in the anterior bar. The CCAP PFCNAFTGCa (m/z 956.38) (**Figure 5iv.** and **Figure S4x.**) is localized to the posterior bar of both the unfed and fed POs. The two AST-As shown (**Figure 5v.** and **Figure S4xi.** and **xii.**) are present throughout the entire unfed PO tissue, but are most concentrated in the anterior bar. In the fed PO, these AST-As became more evenly distributed over the entire tissue. All detected RFamides (**Figure 5vi.** and **Figure S4 xiii. – xvi.**) are abundant in the anterior portion of both the unfed and fed POs. Orcokinin neuropeptides (**Figure 5vii.** and **Figure S4xvii. – xx.**) share the similar distribution patterns among isoforms and are most concentrated in the dorsal trunk of both

unfed and fed POs. Tachykinin APSGFLGMRG (m/z 992.50) (**Figure 5 viii.** and **Figure S4xxi.**) is most abundant at the junction of the dorsal trunk and anterior part of the unfed PO and does not change substantially after feeding.

The AST-B neuropeptides exhibit consistent quantitative changes in expression level in both species, as evident by analyses of the PO extracts. Imaging studies reveal that this family also exhibits a change in localization after food intake in both species. As shown in **Figure 5** all members of the AST-B family are prevalent in the anterior bar in unfed *C. sapidus* PO, while these isoforms become more widely distributed throughout the whole tissue after food intake. In *C. maenas*, AST-B isoforms are present extensively across the whole tissue, but after feeding they are more concentrated in the posterior and anterior parts. This observation suggests potential regulatory roles of AST-B peptides in feeding behavior.

Tachykinin is a neuropeptide family conserved across many species, and a large number of studies have been conducted to characterize their various physiological functions.⁵⁵⁻⁵⁷ Tachykinin has been reported to regulate food intake behavior in rats and goldfish⁵⁸⁻⁶⁰ as well as feeding behavior in other animals.⁶¹⁻⁶³ It also has been found to affect olfactory and locomotive behavior in the fruit fly, *Drosophila melanogaster*.⁶¹⁻⁶⁴ The concentration of tachykinin is low in the PO tissue; therefore it is difficult to obtain accurate quantitation from extraction experiments. However, the imaging data reveals the distinct location of this peptide in the PO tissue of unfed and fed *C. sapidus*, which warrants further study to explore its functional role.

Conclusion

In summary, we employed isotopic formaldehyde labeling and MALDI MS-based methods to quantitatively study neuropeptide regulation of feeding behavior. Multiple neuropeptide families have been shown to be involved in the regulation of feeding behavior in

both *C. sapidus* and *C. maenas*. MALDI-TOF/TOF imaging was utilized to map the spatial distribution of a multitude of neuropeptides from various peptide families. The combined isotopic labeling and mass spectrometric imaging analysis revealed that in addition to neuropeptide expression level changes after feeding, the locations of several neuropeptides also changed. For example, both the tissue expression level and distribution pattern of the AST-B family showed distinct changes in response to food intake. This multifaceted approach provides a list of potential neuropeptide candidates involved in feeding whose functional roles will be further investigated.

Supporting Information

Three example MALDI-FTMS spectra of fractions collected from CE separation at 18, 19, and 20 minutes of elution time, showing peak pairs of labeled neuropeptides detected in each fraction. Additional MALDI-MS images of POs from *C. sapidus* and *C. maenas* are also included.

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Figure Legend

Figure 1. (A) Representative MALDI-TOF/TOF spectrum of isotopic formaldehyde-labeled mixture of fed crab brain extract and unfed brain extract. Each extract was comprised of one unfed and one fed crab brain. Unfed sample was labeled with FH₂ and fed sample was labeled with FD₂. (B) Representative MALDI-TOF/TOF spectrum of formaldehyde labeled mixture of unfed crab brain extracts. Each extract was comprised of two unfed crab brains. One unfed sample was labeled with FH₂ and the other unfed sample was labeled with FD₂. The heavy labeled peaks are labeled with open circles, and the light labeled peaks are labeled with closed

circles. The peak pairs from several abundant neuropeptides are indicated in each figure and annotated with their amino acid sequences.

Figure 2. Abundance ratios of 16 neuropeptides in *C. sapidus* fed brain versus unfed brain from five groups of feeding experiments. The sequence of each neuropeptide is labeled on the x-axis, and they are organized by families. The y-axis represents the abundance ratio of each neuropeptide between fed and unfed crab. * indicates $p < 0.05$, ** indicates $p < 0.005$.

Figure 3. Representative MALDI-TOF/TOF spectrum of a formaldehyde-labeled mixture of PO extract from fed and unfed *C. maenas*. Each extract was comprised of two POs from the same crab. Unfed sample was labeled with FH₂ and fed sample was labeled with FD₂. The heavy-labeled peaks are indicated with open circles, and the light-labeled peaks are indicated with closed circles. The peak pairs from several abundant neuropeptides are indicated and masses.

Figure 4. Abundance ratios of 13 neuropeptides in the POs from fed *C. sapidus* versus unfed crabs from five groups of feeding experiments. The sequence of each neuropeptide is labeled on the x-axis, and they are organized in the order of families. The y-axis represents the abundance ratio of each neuropeptide between fed and unfed crab. * indicates $p < 0.05$, ** indicates $p < 0.005$ (n=5).

Figure 5. Neuropeptide localization in unfed and fed *C. sapidus* PO. (i.) Optical image of a PO tissue on a MALDI plate before being coated with DHB matrix. (ii. – xxi.) MALDI-MS images of several neuropeptides of interest from seven families: four RYamides, four AST-B peptides, one CCAP, two AST-A peptides, four RFamides, four orcokinins, and one tachykinin. The orientation of all the figures was the same.

Figure 6. Neuropeptide localization in unfed and fed *C. maenas* PO. (i.) Optical image of a PO tissue on a MALDI plate before being coated with DHB matrix. (ii. – ix.) MALDI-MS images of several neuropeptides of interest from six families: including four AST-Bs, three RYamides, two RFamides, one CCAP, and one SIFamide. The orientation of all the figures was the same.

Table 1. Ratios of neuropeptide expression in fed and unfed *Carcinus maenas* brain from four groups of feeding experiments, the average ratios of each neuropeptide, and the corresponding p-value.^a

Neuropeptide Family	m/z	Sequence	Experiment				R _{average}	p-value
			1	2	3	4		
RFamide	965.54	<i>NRNFLRFamide</i>	1.05	1.18	1.14	1.25	1.15	0.033
	966.53	<i>DRNFLRFamide</i>	1.1	1.1	1.23	1.17	1.15	0.013
	1022.57	GNRNFLRFamide	1.02	1.15	1.03	1.13	1.08	0.085
	1048.57	APQGNFLRFamide	1.22	1.04	1.19	1.26	1.18	0.031
	1105.63	SMPSLRLRFamide	1.02	1.02	1.27	1.17	1.12	0.054
	1124.63	GLSRNYLRFamide	1	1.12	1.22	1.13	1.12	0.083
	1137.59	DGNRNFLRFamide	1.2	1.09	1.26	1.03	1.15	0.058
	1158.62	YGNRSFLRFamide	1.01	1.05	1.05	1.29	1.1	0.2
Orcokinin	1198.55	NFDEIDRSGFamide	1.54	1.08	0.89	1.07	1.15	0.381
	1474.63	<i>NFDEIDRSGFGFA</i>	1.66	1.41	1.26	1.15	1.37	0.029
	1502.7	<i>NFDEIDRSGFGFV</i>	1.13	1.18	1.27	1.24	1.2	0.006
	1532.7	<i>NFDEIDRSSFGFV</i>	1.19	1.18	1.1	1.13	1.15	0.004
	1547.68	<i>NFDEIDRSSFGFN</i>	1.2	1.15	1.05	1.11	1.13	0.024
CabTRPs	934.49	<i>APSGFLGMRamide</i>	1.45	1.26	1.25	1.29	1.31	0.004
	964.5	<i>TPSGFLGMRamide</i>	1.84	1.54	1.57	1.66	1.65	0.001
Proctolin	649.37	RYLPT	0.94	1.07	1.24	1.26	1.13	0.195
YRamide	844.48	<i>HIGSLYRamide</i>	1.33	1.74	1.44	1.37	1.47	0.008
SIFamide	1381.74	GYRKPPFNGSIFamide	1.03	1.01	1.16	1.24	1.11	0.133

a. Statistically significant ratios (p-value < 0.05) are indicated by bold and italicized font.

Table 2. Ratios of neuropeptide expression in fed and unfed *Carcinus maenas* pericardial organs (POs)

from four groups of feeding experiments, the average ratio of each neuropeptide, and the corresponding p-value.^a

Neuropeptide Family	m/z	Sequence	Experiment				R _{average}	p-value
			1	2	3	4		
Protocollin	649.37	RYLPT	0.97	0.8	0.62	0.9 4	0.83	0.033
RYamide	784.41	<i>FVGGSRYamide</i>	0.61	0.6 3	0.57	0.7 2	0.63	<0.001
	976.46	<i>SGFYANRYamide</i>	0.56	0.6 1	0.6	0.6 7	0.61	<0.001
	1114.47	<i>SSRFVGGSRYa</i>	0.57	0.7	0.63	0.6 5	0.64	<0.001
RFamide	965.54	NRNFLRFamide	1.17	0.8 7	0.72	0.4 2	0.79	0.097
	966.53	DRNFLRFa	1.12	0.7 7	0.71	0.3 7	0.74	0.06
	1022.57	GNRNFLRFamide	1.19	0.8 7	0.87	0.4 1	0.84	0.136
	1048.57	<i>APQGNFLRFa</i>	0.98	0.6 4	0.62	0.5 2	0.69	0.005
	1124.63	<i>GLSPNYLRFa</i>	0.57	0.7 9	0.7	0.3 8	0.61	0.003
AST-B	1137.89	DGNRNFLRFa	1.31	1.0 6	0.88	0.7 3	1	0.773
	1158.62	YGNRSRLRFa	1.1	0.8 2	0.67	0.4 2	0.75	0.051
	1107.58	<i>QWSSMRGAWa</i>	0.85	0.7 7	0.77	0.7 1	0.77	<0.001
	1293.63	<i>STNWSSLRSAWamide</i>	0.98	0.7 2	0.55	0.6 8	0.73	0.007
	1470.7	VPNDWAHFRGSWamide	1.62	0.2 7	0.25	1.1 8	0.83	0.217
Orcokinin	1502.69	<i>NFDEIDRSGFGFV</i>	0.91	0.9 2	0.76	0.8 2	0.85	0.003
	1532.7	<i>NFDEIDRSSFGFV</i>	0.7	0.7 7	0.65	0.6 2	0.68	<0.001
	1547.68	NFDEIDRSSFGFN	0.86	1.1	0.89	0.6 5	0.88	0.1

a. Statistically significant ratios (p-value < 0.05) are indicated by bold and italicized font.

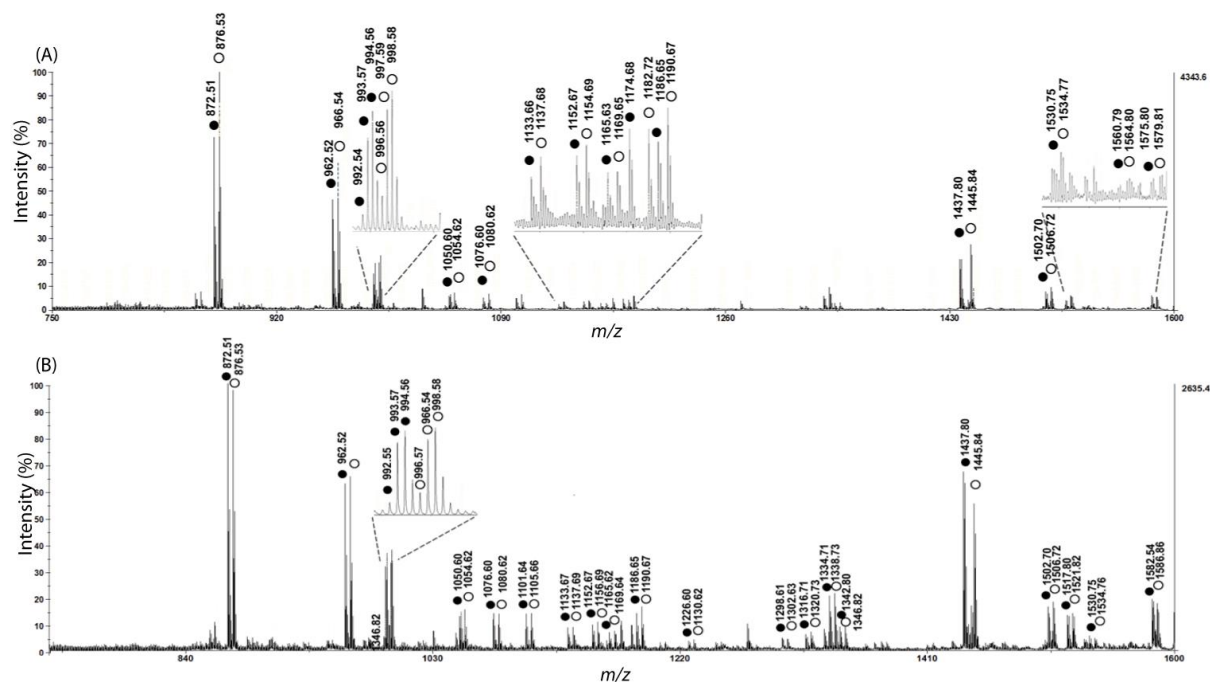


Figure 1. (A) Representative MALDI-TOF/TOF spectrum of formaldehyde-labeled mixture of fed crab brain extract and unfed brain extract. Each extract is comprised of one unfed and one fed crab brain. Unfed sample is labeled with FH₂ and fed sample is labeled with FD₂. (B) Representative MALDI-TOF/TOF spectrum of formaldehyde labeled mixture of unfed crab brain extracts. Each extract is comprised of two unfed crab brains. One unfed sample is labeled with FH₂ and the other unfed sample is labeled with FD₂. The heavy labeled peaks are labeled with blank circles, and the light labeled peaks are labeled with solid circles. The peak pairs from several abundant neuropeptides are indicated in each figure and labeled with their masses. The spectrum of FH₂ and FD₂ unfed brain extracts indicates that there is no apparent labeling bias, as the ratios are similar each peak pair.

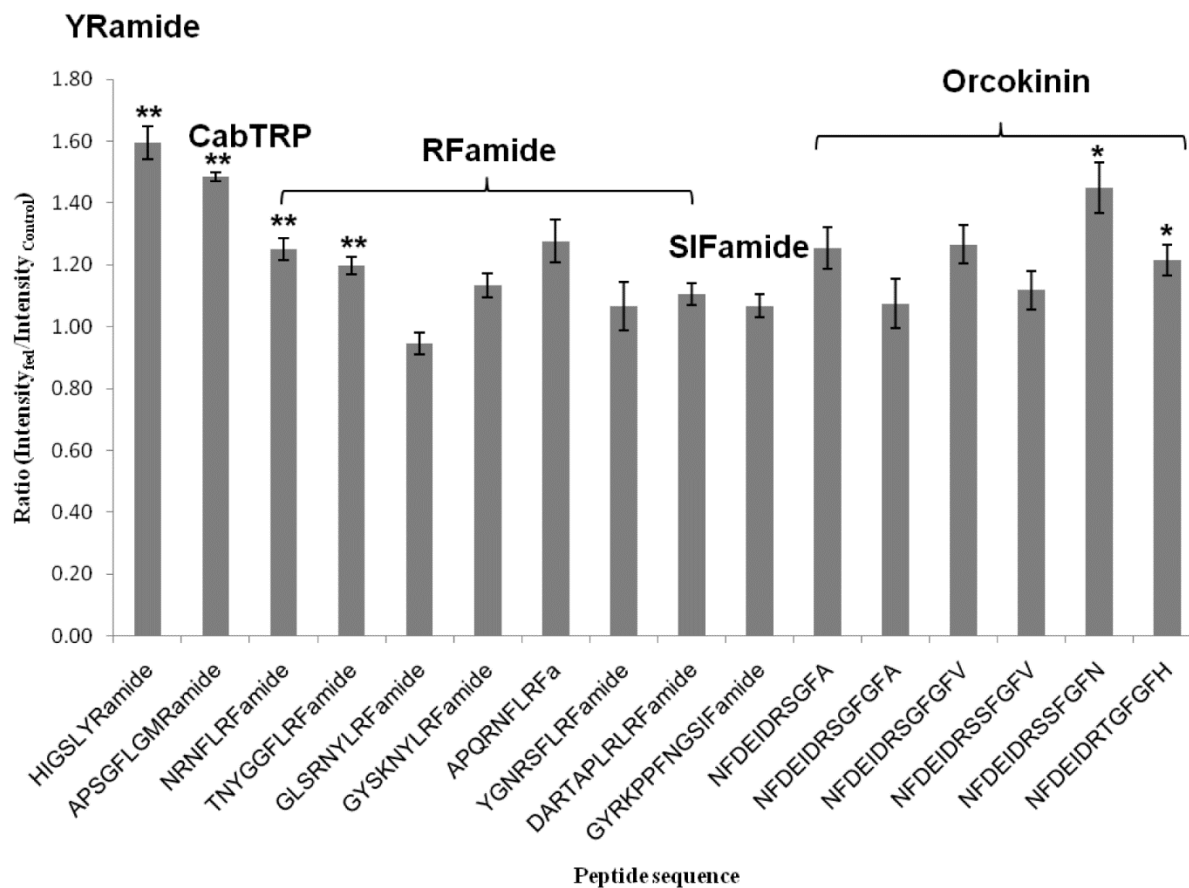


Figure 2. Abundance ratios of 16 neuropeptides in *C. sapidus* fed brain versus unfed brain from five groups of feeding experiments. The sequence of each neuropeptide is labeled on the x-axis, and they are organized by families. The y-axis represents the abundance ratio of each neuropeptide between fed and unfed crab. * indicates $p < 0.05$, ** indicates $p < 0.005$.

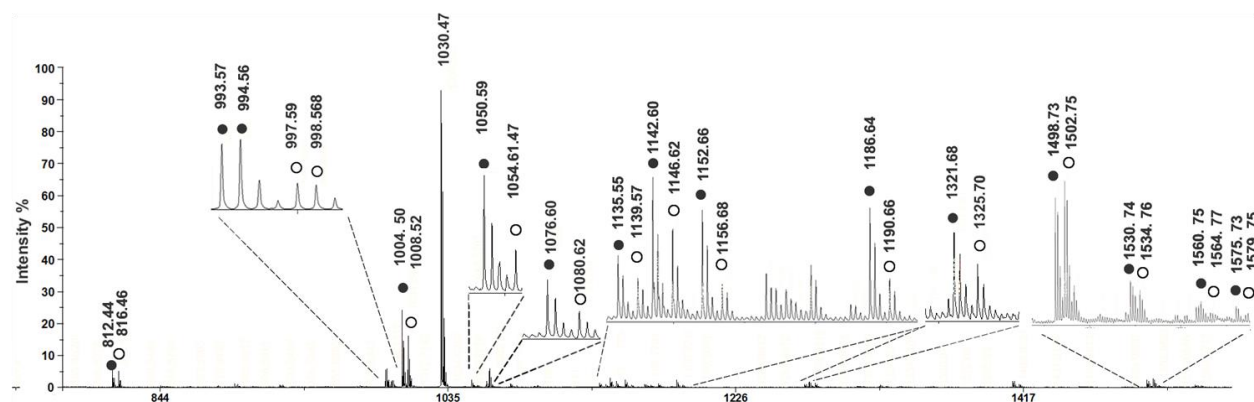


Figure 3. Representative MALDI-TOF/TOF spectrum of a formaldehyde-labeled mixture of *C. maenas* fed PO extract and unfed PO extract. Each extract is comprised of 2 POs from the same crab. Unfed sample is labeled with FH₂ and fed sample is labeled with FD₂. The heavy-labeled peaks are indicated with blank circles, and the light-labeled peaks are indicated with solid circles. The peak pairs from several abundant neuropeptides are indicated and labeled with their amino acid sequences.

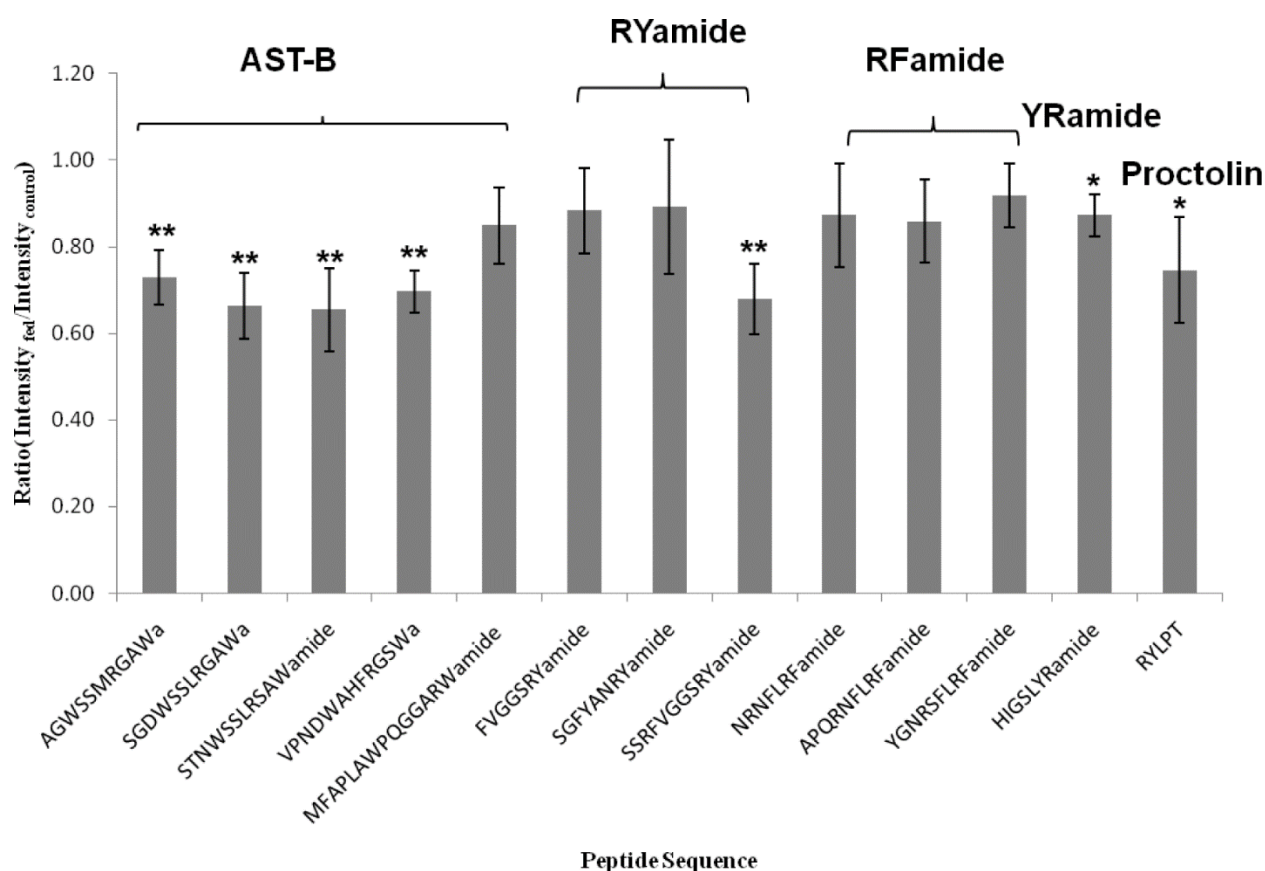


Figure 4. Abundance ratios of 13 neuropeptides in *C. sapidus* fed PO versus unfed PO from five groups of feeding experiments. The sequence of each neuropeptide is labeled on the x-axis, and they are organized in the order of families. The y-axis represents the abundance ratio of each neuropeptide between fed and unfed crab. * indicates $p < 0.05$, ** indicates $p < 0.005$.

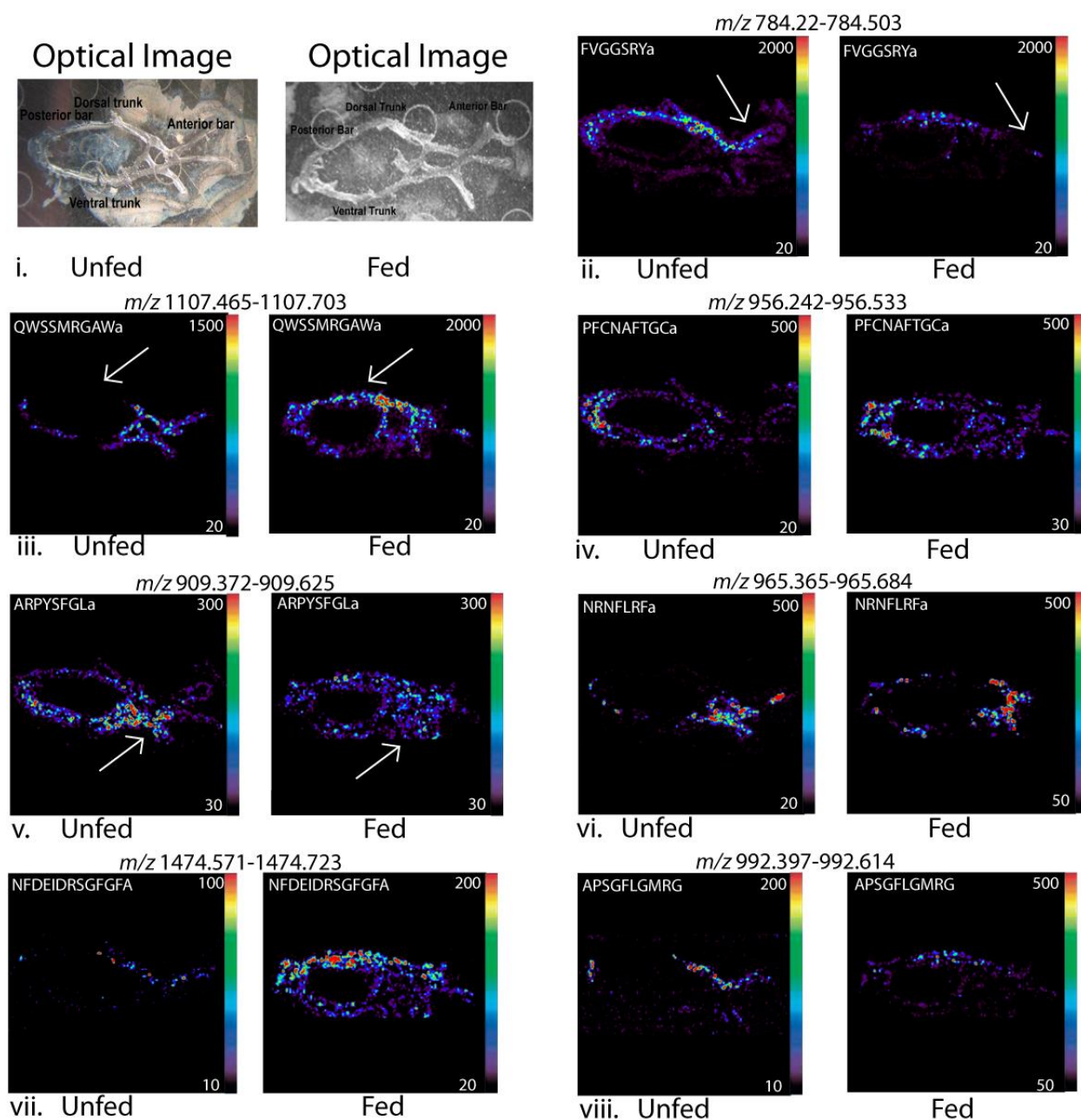


Figure 5. Neuropeptide localization in unfed and fed *C. sapidus* PO. (i.) Optical image of a PO tissue on a MALDI plate before being coated with DHB matrix. (ii. – viii.) MALDI-MS images of several neuropeptides of interest from each of seven families: RYamide, AST-B, CCAP, AST-

A, RFamide, orcokinin, and tachykinin. The orientation of the all figures is the same, and color bar reflects relative intensity. Arrows indicate areas where there is observed change in localization between fed and unfed.

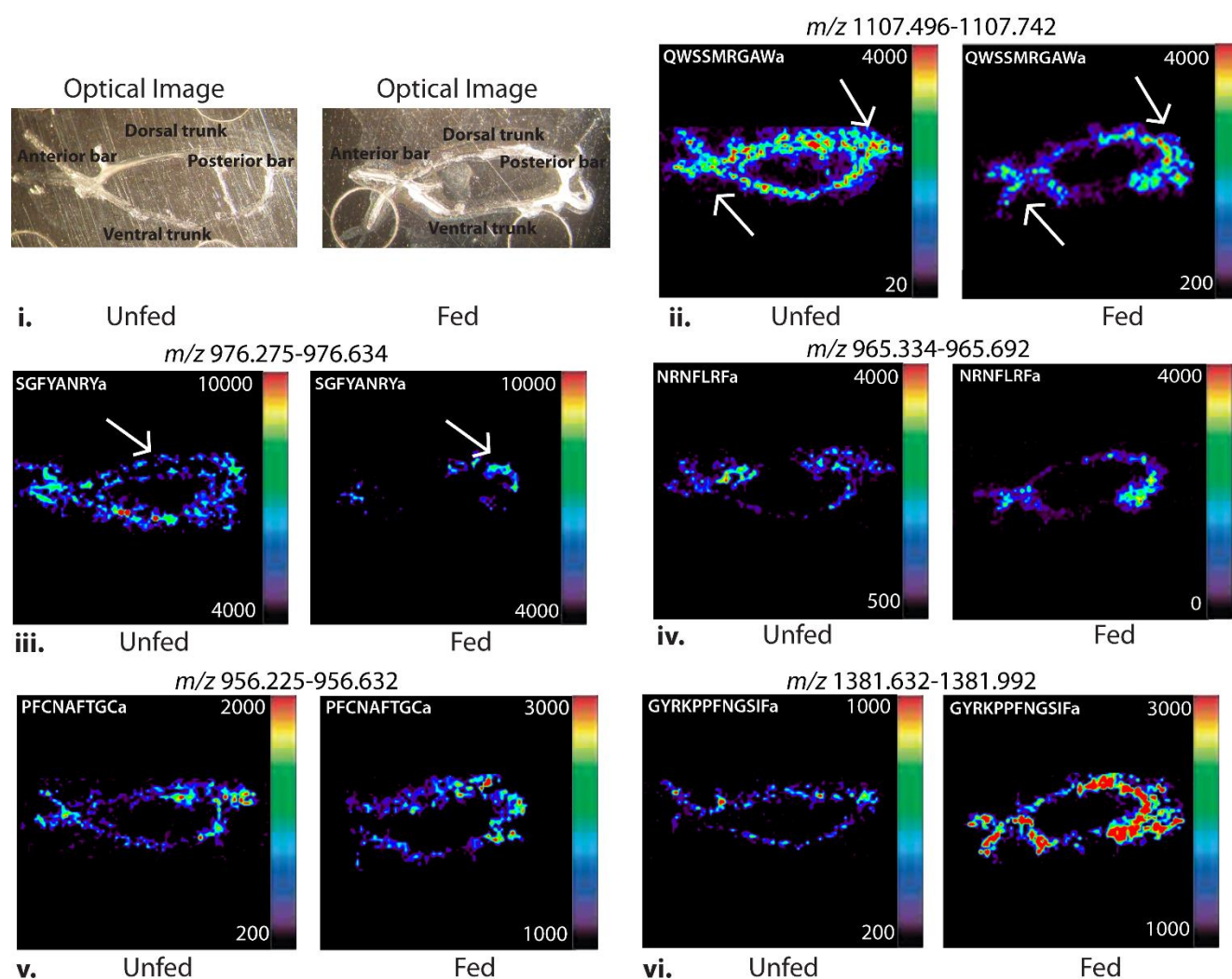


Figure 6. Neuropeptide localization in unfed and fed *C. maenas* PO. (i.) Optical image of a PO tissue on a MALDI plate before being coated with DHB matrix. (ii. – vi.) MALDI-MS images of several neuropeptides of interest from each of six families: including AST-B, RYamide, RFamide, one CCAP, and SIFamide. The orientation of the all figures is the same, and color bar reflects relative intensity. Arrows indicate areas where there is observed change in localization between fed and unfed.

Supporting Information

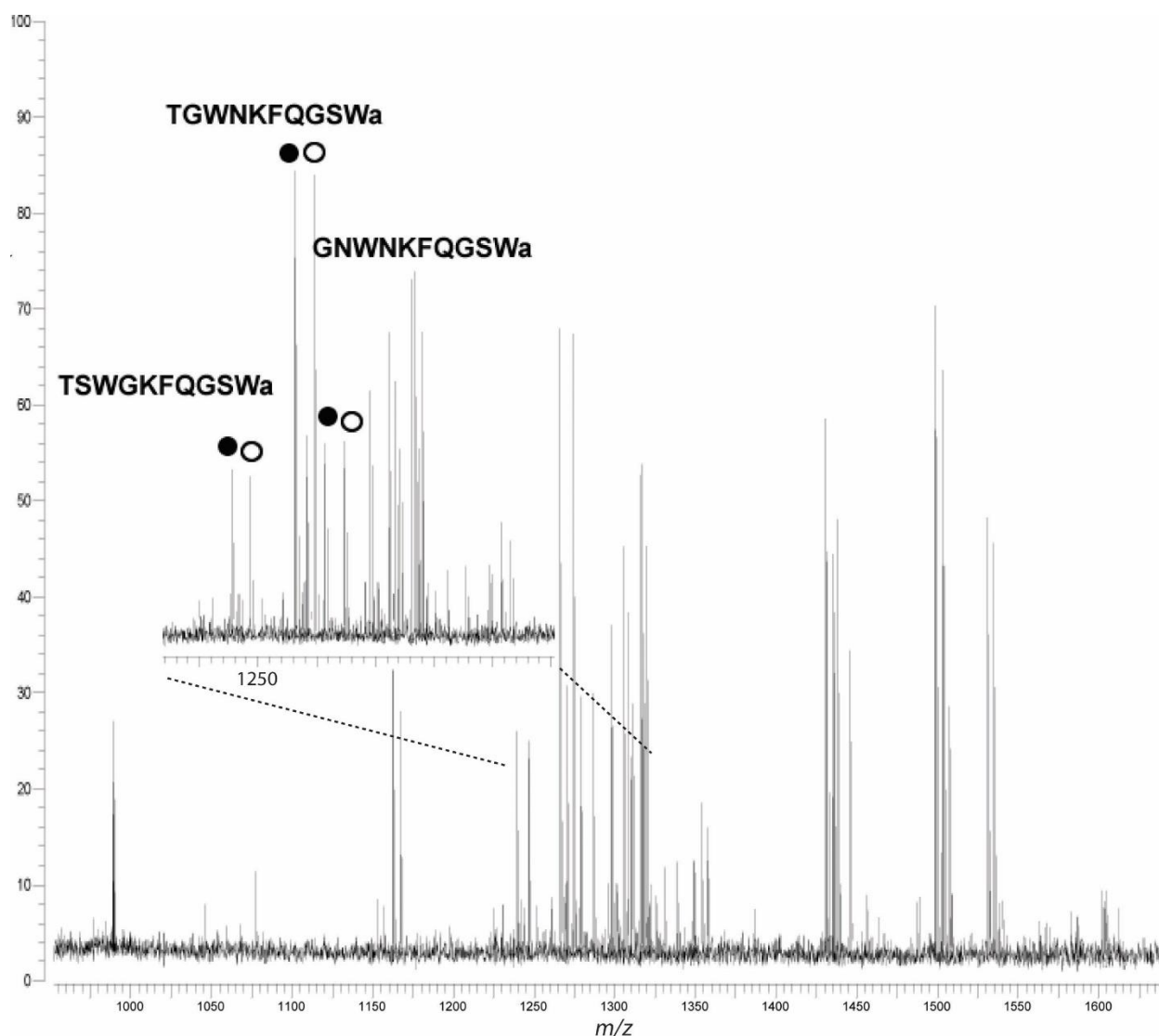


Figure S1. CE-MALDI-FTMS profiling of brain from *C. sapidus* in response to food intake.

Spectrum was acquired from the sample collected at 18 minutes of CE separation time. Circles indicate labeled peak pairs (peaks separated by 4.03 m/z). Closed circles represent light-labeled peptide peaks, and open circles represent heavy-labeled peptide peaks. The zoomed-in spectrum displays peak pairs of AST-B isoforms within this specific CE fraction.

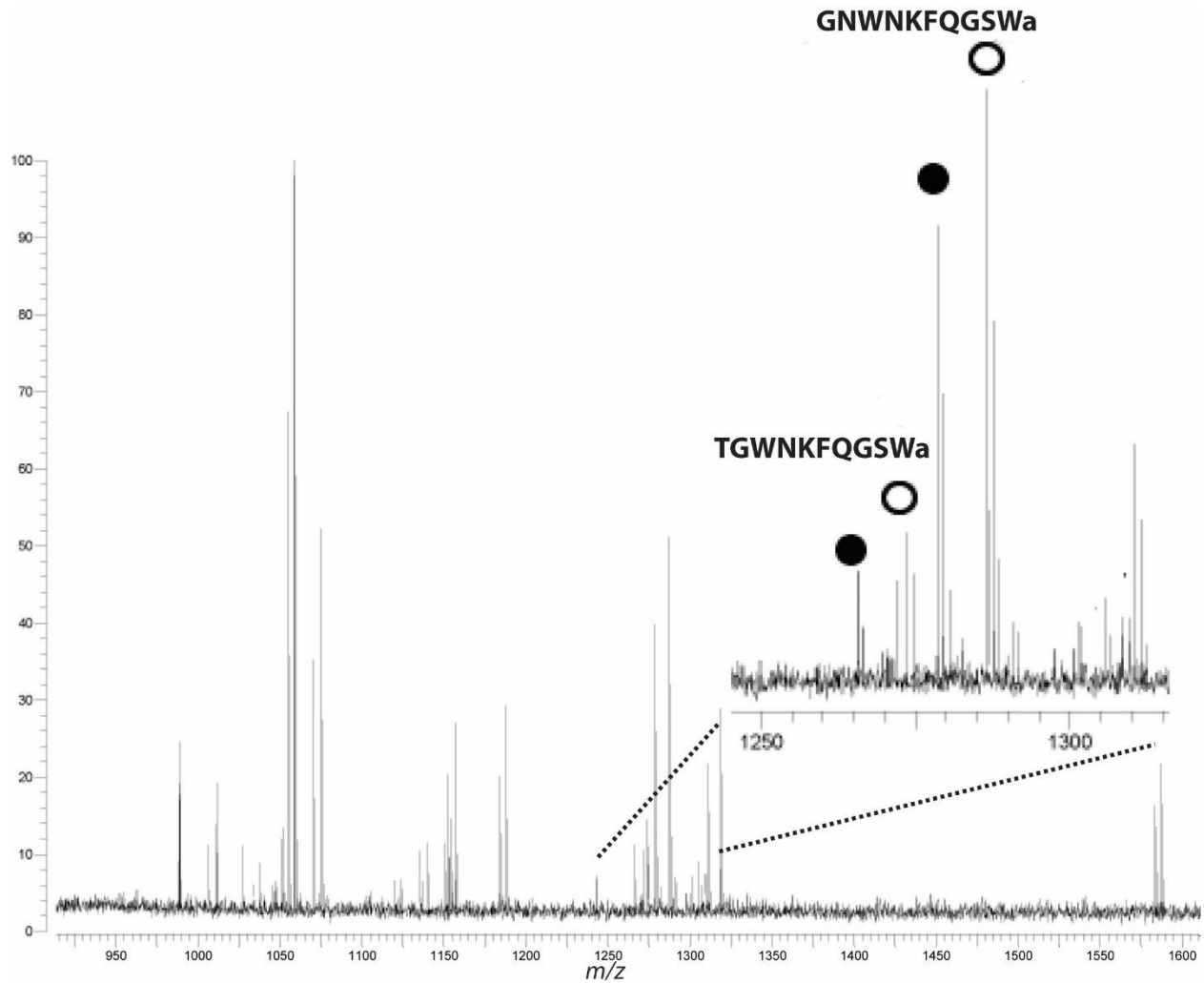


Figure S2. CE-MALDI-FTMS profiling of brain from *C. sapidus* in response to food intake.

Spectrum was acquired from the sample collected at 19 minutes of CE fractionation. Circles indicate labeled peak pairs (peaks separated by 4.03 m/z). Closed circles represent light-labeled peptide peaks, and open circles represent heavy-labeled peptide peaks. The enlarged spectrum displays peak pairs of AST-B isoforms within this specific CE fraction.

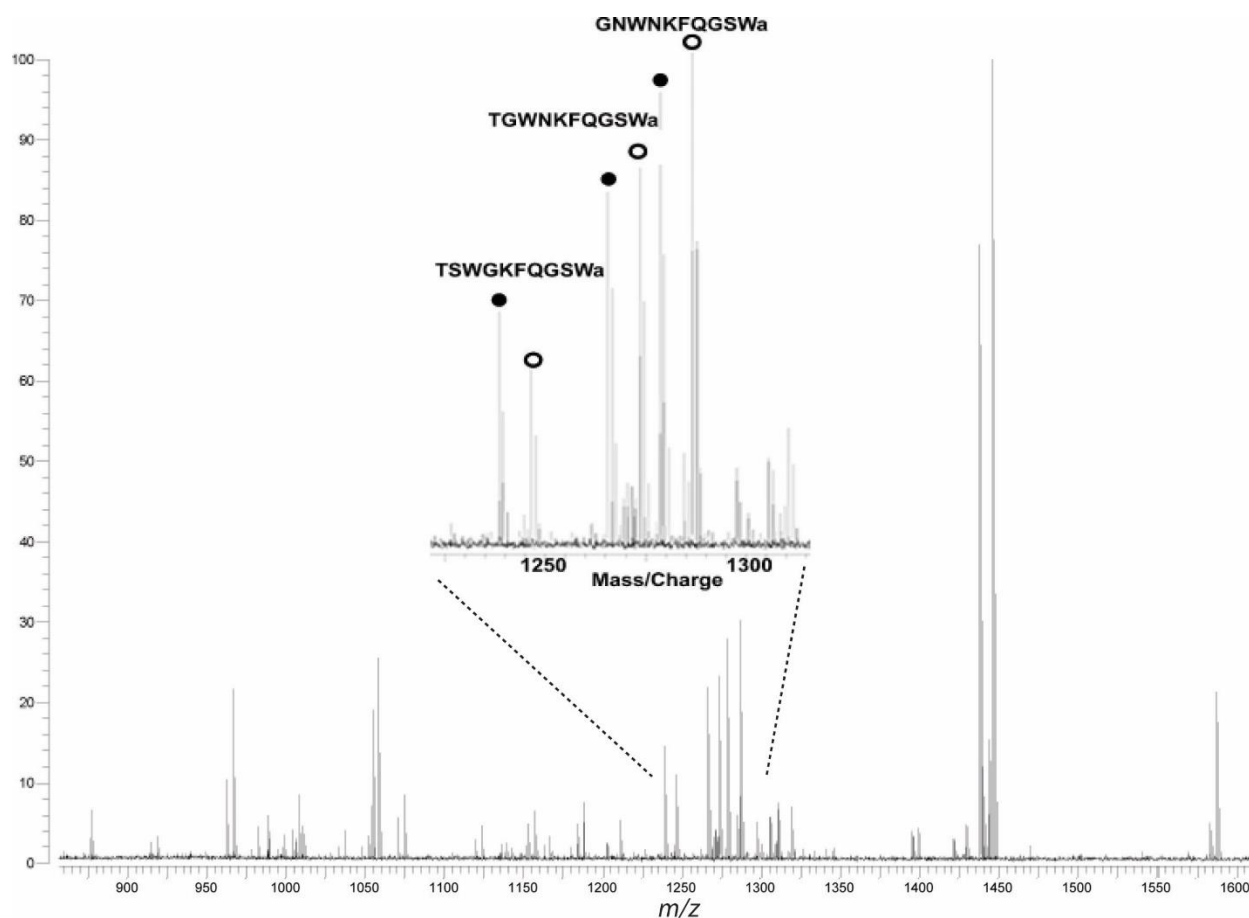


Figure S3. CE-MALDI-FTMS profiling of brain from *C. sapidus* in response to food intake. Spectrum was acquired from the sample collected at 20 minutes of CE fractionation. Circles indicate labeled peak pairs (peaks separated by 4.03 m/z). Closed circles represent light-labeled peptide peaks, and open circles represent heavy-labeled peptide peaks. The zoomed-in spectrum displays peak pairs of AST-B isoforms within this specific CE fraction.

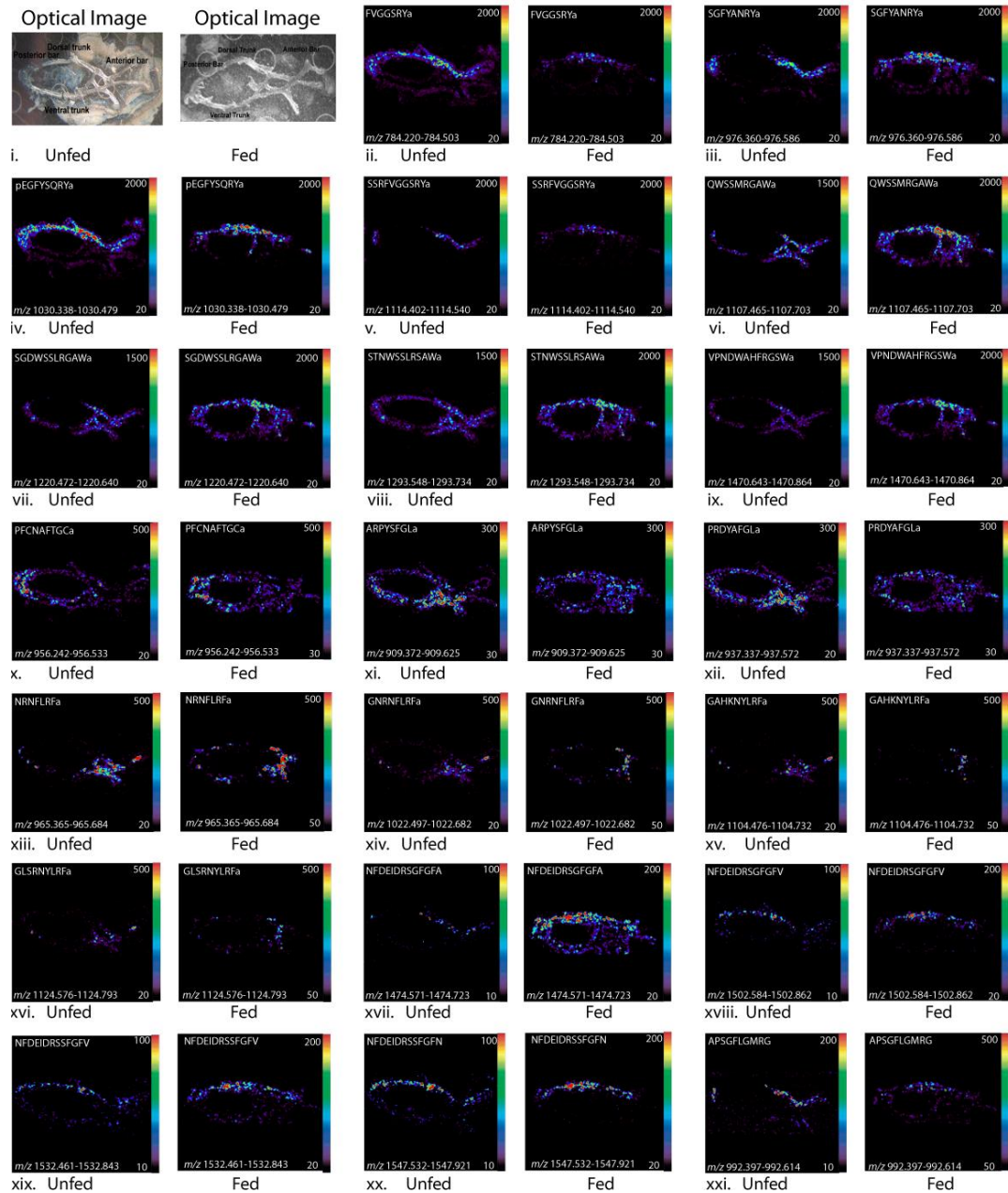


Figure S4. Neuropeptide localization in unfed and fed *C. sapidus* PO. (i.) Optical image of a PO tissue on a MALDI plate before being coated with DHB matrix. (ii. – xxi.) MALDI-MS images of several neuropeptides of interest from seven families: four RYamides, four AST-B, one CCAP, two AST-A, four RFamide, four orcokinin, and one tachykinin. The orientation of the all figures is the same, and color bar reflects relative intensity.

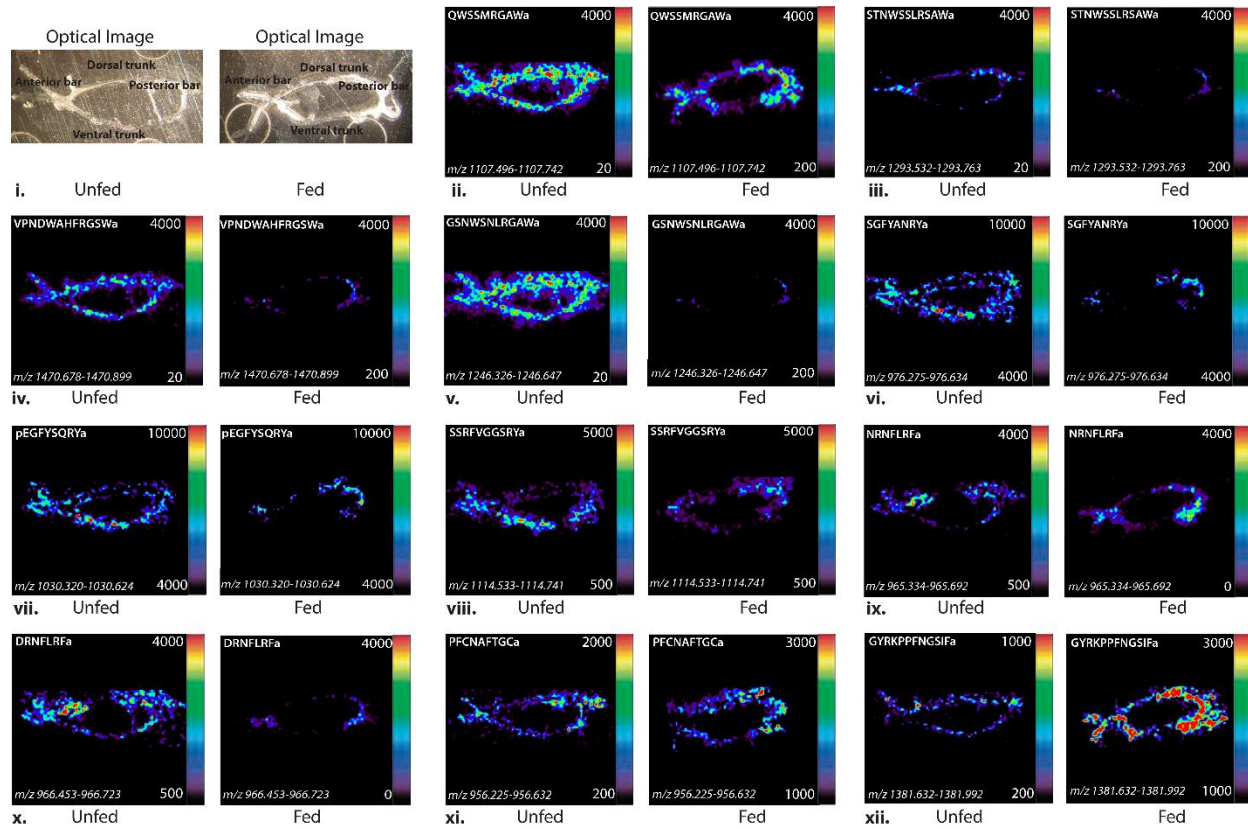
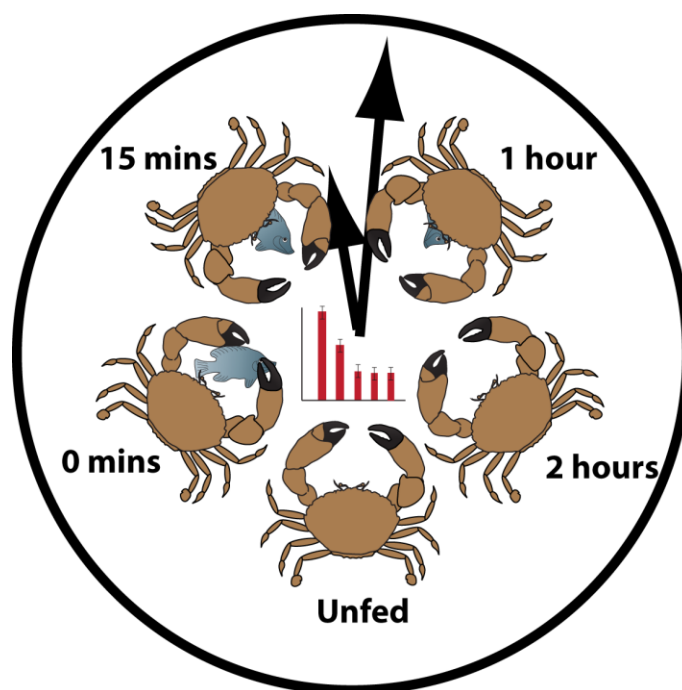


Figure S5. Neuropeptide localization in unfed and fed *C. maenas* PO. (i.) Optical image of a PO tissue on a MALDI plate before being coated with DHB matrix. (ii. – ix.) MALDI-MS images of several neuropeptides of interest from six families: including four AST-Bs, three RYamides, two RFamides, one CCAP, and one SIFamide. The orientation of the all figures is the same, and color bar reflects relative intensity.

Chapter 8

Mass Spectrometry Profiling and Quantitation of Changes in Circulating Hormones Secreted over Time in *Cancer borealis* Hemolymph Due to Feeding Behavior



Modified from:

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Abstract

The crustacean stomatogastric nervous system (STNS) is a valuable model for understanding circuit dynamics in neuroscience, as it contains a small number of easily-distinguishable neurons. These neurons receive modulation by neuropeptides, which act in part as hormones transported through the hemolymph. Previous work characterizing neuropeptides in the hemolymph has been limited due to their low abundance *in vivo* in the presence of a complex biological matrix and their propensity toward rapid degradation. To improve the detection of neuropeptides, a data independent acquisition (DIA) mass spectrometry (MS) method was implemented, which improved the number of neuropeptides detected by approximately two-fold and showed greater reproducibility between experimental and biological replicates. The method was used to profile neuropeptides at different stages of the feeding process, including unfed, 0 min post-feeding, 15 min post-feeding, 1 hour post-feeding, and 2 hour post-feeding. The results show differences both in the presence and relative abundance of neuropeptides at the various time points. Additionally, 101 putative neuropeptide sequences were identified with *de novo* sequencing, indicating there may be more key modulators within this circuit than is currently known. The results indicate a cohort of neuropeptides that may elicit a function on the STNS throughout the feeding process and provide groundwork for targeted follow-up electrophysiological studies to obtain a more detailed understanding of their functional role.

Introduction

Crustaceans have long been used in neuroscience to study circuit dynamics.¹ Their simplified system, comprised of a small number of easily-distinguishable neurons, makes mapping connectivity between neurons a feasible task. A particularly interesting circuit is the stomatogastric nervous system (STNS), located in the stomach of crabs and lobsters.² This

system is responsible for modulating feeding-related activities, such as the gastric mill rhythm for chewing and pyloric rhythm for filtering of food. The rhythmic output of these circuits is controlled by the stomatogastric ganglion (STG), which is made up of 25-26 neurons in *Cancer borealis*.³ The STG is modulated by neurotransmitters and neuropeptides from multiple sources, including sensory neurons and descending modulatory projection neurons.⁴ Neuropeptides released as circulating hormones from neurosecretory tissue have also been shown to influence the STG.^{5, 6} These neurosecretory tissues include predominantly the pericardial organs (PO) located laterally on either side of the heart, and the sinus glands (SG) found in the eyestalks.

When molecules are released from neurosecretory tissue, they are transported throughout the body via hemolymph. Hemolymph, a combination of blood and lymph, acts as circulatory fluid, providing oxygen and nutrients to various parts of the body. As crustaceans have an open circulatory system, this transportation is done through fluid pressure, in which the heart contracts to push hemolymph to various tissues that are bathed in the fluid.⁷ The contents of hemolymph are complex, containing degradation enzymes, proteases, clotting factors other abundant proteins and protein fragments, lipids, metabolites, and neuropeptide hormones.⁸ Through the hemolymph, molecules released from the secretory organs such as POs and sinus glands (SGs) are able to reach the STG in order to exhibit a modulatory effect.

Neuropeptides are short chains of amino acids synthesized from preprohormones that act most commonly on G-protein coupled receptors.⁹ Despite their small size, neuropeptides are the most diverse class of signaling molecules, responsible for modulating a variety of functions within the nervous system. They are present in numerous isoforms, and often times these isoforms differ by only a single amino acid but can have the same or highly contrasting functions. Furthermore, neuropeptides, as with all signaling molecules, are present in low

abundances *in vivo* and are prone to rapid degradation. These characteristics make studying neuropeptides challenging.⁹ As a result, the extent to which these molecules modulate biological activities is not fully understood.

Many recent developments have been made to improve our capabilities of studying neuropeptides. While targeted assays such as histology, nuclear magnetic resonance (NMR), and antibody assays can provide substantial detail about individual neuropeptides, mass spectrometry (MS) has been an invaluable tool for large-scale profiling of neuropeptides in biological samples. It is advantageous for untargeted, discovery-based experiments, as not only can it identify large numbers of neuropeptides in a single analysis, but it also does not require prior knowledge about the neuropeptides. This is particularly advantageous for model organisms without a completely sequenced genome, as is the case with *Cancer borealis*.¹⁰ With the use of sophisticated *de novo* sequencing software available, information about the sequence of neuropeptides can be obtained, facilitating the discovery of neuropeptides not previously identified or characterized within these organisms.

Previous studies have investigated the role of neuropeptides in tissue and hemolymph due to various perturbations, such as stress caused by alterations in salinity,¹¹ hypoxia,¹² temperature,¹³ and pH,¹⁴ leading to information regarding how neuropeptides are involved in adaptations of the animal. There have also been studies investigating the role of neuropeptides in feeding, focusing on the tissue responsible.^{15, 16} However, there has been relatively little research done into the contents of hemolymph that are modulating feeding-related activity.¹⁷ Moreover, these studies have mostly been limited to two discrete conditions (e.g. unfed and fed). Feeding is a cyclic process. Rather than involving simply a before state and an after state, there are numerous stages that a crab undergoes throughout the process of food intake. A thorough

investigation of multiple time points throughout the feeding process is crucial to our understanding of how circulating molecules influence the STNS.

Profiling neuropeptides in hemolymph is particularly challenging. Compared to tissue, hemolymph neuropeptides are present in even smaller amounts *in vivo*, often on the order of 1 to 100 pM, and are in the presence of numerous interfering artifacts such as lipids, enzymes, and protein fragments.⁸ Rapid and sophisticated sample preparation is required prior to MS analysis, as hemolymph clots substantially after withdrawal from the animal without proper treatment.¹⁸ Furthermore, with the complexity of hemolymph samples, sufficiently sensitive MS acquisition methods are necessary in order to detect neuropeptides in the presence of other interfering biological species.

In order to address these challenges and better understand the influence of circulating neuropeptide hormones on the STNS, this study uses a label-free data independent acquisition (DIA) MS approach to profile and relatively quantify neuropeptides in hemolymph at five time points over the course of feeding: before feeding, 0 mins after feeding, 15 mins after feeding, 1 hour after feeding, and 2 hours after feeding. Additionally, *de novo* sequencing is employed to identify putative novel neuropeptides that may also be implicated in the feeding process.

Methods

Chemicals and materials

ACS-grade formic acid (FA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). Amicon Ultra 10 kDa 0.5 mL molecular weight cutoff (MWCO) devices and 10 μ L C18 ziptips were purchased from Merck Millipore (Billerica, MA USA). Acidified methanol was

prepared using 90/9/1 water/methanol/acetic acid. ACS-grade solvents were used for sample preparation, and Optima-grade solvents were used for MS analysis.

Animals and Feeding Experiments

Jonah crabs, *Cancer borealis*, were purchased from the Fresh Lobster Company, LLC (Gloucester, MA USA) and housed in artificial seawater tanks maintained at 12-13°C with an alternating 12-hour light/dark cycle. Animals were allowed to adjust to their environment for two weeks without food prior to feeding experiments. For feeding experiments, crabs were given 4 g of thawed tilapia. Time after feeding was counted from when the crab finished eating, which typically took 10 to 15 minutes. The following time points were analyzed: unfed (crab was not given tilapia), 0 minutes post-feeding, 15 minutes post-feeding, 1 hour post-feeding, and 2 hour-post feeding. After the appropriate amount of time elapsed for each time point, crabs were cold-anesthetized on packed ice for 10 minutes. A 1 mL plastic syringe with a 23 gauge needle was used to withdraw 1 mL of hemolymph from the pericardial sinus (open chamber above the heart) via the soft leg joint of the leg closest to the claw. Hemolymph was immediately added to an equal volume of chilled acidified methanol containing 1×10^{-9} M bradykinin as an internal standard. A total of 6 biological replicates from distinct animals were performed. All hemolymph was withdrawn from the crabs at approximately the same time of day (between 9am and 11am local time).

Sample preparation

Neuropeptide extraction of hemolymph was performed immediately after withdrawal from animals. Samples were vortexed, bath-sonicated for 5 minutes, and centrifuged at 16.1 rcf for 5 minutes. The supernatant from each sample was collected, and 0.5 mL of acidified methanol was added to the pellet. The pellet was then manually homogenized, and again

vortexed, sonicated, and centrifuged. The resulting supernatant was collected, and the process was repeated for a total of 3 extractions. The combined supernatant was evaporated in a speedvac on medium heat and reconstituted in 1 mL 70/30 water/methanol.

Each sample was then separated into 2 aliquots of 0.5 mL and subjected to molecular weight cutoff. For MWCO of hemolymph, the device was rinsed with 0.2 mL of 0.1 M NaOH by centrifuging at 14 rcf for 4 minutes. The device was then rinsed with 0.5 mL of 50/50 methanol/water for 8 minutes at 14 rcf. The hemolymph sample was then loaded into the device and centrifuged at 14 rcf until most of the liquid passed through the filter. The device was then rinsed with 0.1 mL 70/30 water/methanol for 20 minutes at 14 rcf, and the flow-through from the sample and rinse were combined and evaporated in the speedvac on medium heat.

Samples were reconstituted in 45 μ L 0.1% FA and divided into 3 aliquots of 15 μ L. Each aliquot was desalted with C18 ziptips following package instructions. The eluted samples were then evaporated in the speedvac on medium heat and stored at -80°C until MS analysis.

Mass Spectrometry Analysis

Samples were constituted in 12 μ L of 0.1% FA and 2 μ L per technical replicate was loaded onto a 14 cm self-packed C18 column (75 μ m inner diameter, 1.7 μ m particle size) for LC separation on a Waters nanoAcquity LC system (Waters Corp, Milford, MA, USA) with a flow rate of 0.300 μ L/min. Water with 0.1% FA (A) and acetonitrile with 0.1% FA (B) were used as mobile phases, and the following gradient was used for separation: 0-1 min 3-10% B; 1-90 min 10-35% B; 90-92 min 35-95% B; 92-102 min 95% B; 102-105 min 95-3% B; 105-120 min 3% B. The LC system was coupled online to a Thermo Scientific Q Exactive instrument (Thermo Scientific, Bremen, Germany) with a nano-ESI source. MS spectra were acquired with positive ESI, 30 eV collision energy, 70,000 resolution for MS scans and 17,500 resolution for

MS/MS scans. 20 MS/MS spectra were acquired from 400 to 800 m/z per cycle with a DIA method using 20 m/z isolation width.

Data Analysis

Raw MS files were converted to the vendor-neutral mzXML format using MSConvert¹⁹ and processed with DIA-Umpire²⁰ using the default parameters. Output files from DIA-Umpire were converted from mgf to mzXML using MSConvert and processed in PEAKS 7.0 (Bioinformatics Solutions Inc., Waterloo, ON, CAN) with a list of known crustacean neuropeptides used as the database.²¹ The following parameters were specified for PEAKS *de novo* and database searches: no enzyme cleavage specified, instrument orbi-orbi, HCD fragmentation, and precursor correction enabled. The following modifications were included in the search parameters: amidation, pyroglutamate, dehydration, and oxidation. All other parameters were set to the default. Search results were exported from PEAKS for subsequent MS1 quantitation with Skyline with Orbitrap settings, unspecific enzymatic cleavage, and all other parameters set to default. [ref] Statistical analysis was performed with Perseus, including log₂(x) transformation, imputation from normal distribution, one-way ANOVA, and Tukey HSD post-hoc test. For identification of putative novel neuropeptides, *de novo* sequences from PEAKS were filtered for an average local confidence (ALC) score threshold of 75% or greater and were manually searched for characteristic neuropeptide sequence motifs.²²

Results and Discussion

The STNS is an interesting nervous system that is active throughout the feeding process and elicits phasic, patterned outputs at various stages throughout the process, controlling both the

gastric mill and pyloric rhythms.¹ As the stomatogastric ganglion neuronal circuit is modulated by neuropeptides, partly through long-range effects as circulating hormones, understanding how these neuropeptides change both in their presence and relative abundance is important for understanding their effect on feeding behavior. This research focused on understanding these changes by implemented a DIA-MS method to detect and relatively quantify neuropeptides at the different time points: unfed, 0 min post-feeding, 15 min post-feeding, 1 hour post-feeding, and 2-hour post-feeding. Overall, over 200 neuropeptides were identified that match those previously-characterized. Furthermore, with the high sensitivity of the method implemented, 101 putative sequences of peptides that possess characteristic sequence motifs of common neuropeptides were identified, suggesting additional molecular players involved in the feeding process.

Assessment of DIA method

Neuropeptide studies are uniquely challenging due to the characteristics of signaling molecules, low abundance *in vivo*, rapid degradation, high variability. These challenges are exacerbated with fluid samples due to the even lower *in vivo* concentrations because of the complex biological matrix containing numerous other species. As a result, previous studies detecting neuropeptides in hemolymph have been limited. In order to improve coverage and reproducibility of neuropeptides in these samples, a DIA method that had previously been optimized for neuropeptides in tissue samples was applied to the hemolymph samples.²³ Because this was the first time using this method for crustacean fluid samples, a comparison was performed between the DIA method and the typical data dependent acquisition (DDA) method normally used for these samples.

For the comparison, 6 mL of hemolymph was pooled and aliquoted into 6 separate

sample vials for LC-MS/MS analysis. Of these aliquots, 3 were analyzed with a conventional top-10 DDA method as is normally done and 3 were analyzed with a DIA method. When the methods were compared, the DIA method resulted in the detection of over twice as many neuropeptides (an average of 97 compared to 44). Furthermore, as shown in **Figure 1A**, almost all of the neuropeptides detected with DDA were also detected with DIA, confirming that the DIA method is not missing a substantial number of neuropeptides that DDA is able to detect. This demonstrates that DIA is a suitable replacement for DDA in analyzing hemolymph samples and shows promise at providing a more in-depth analysis of neuropeptides present.

In addition to detecting more neuropeptides, the DIA method offered improved reproducibility in neuropeptide detection. Reproducibility is a challenge with hemolymph because the contents are continuously circulating throughout the animal and so fluctuate in their relative abundance, particularly in relation to other molecules in the sample. When detection relies on relative abundance of an ion, as is the case with DDA, these variations impact what is detectable in each sample. With DIA, the same ions are fragmented in each run regardless of abundance. As a result, DIA offers more reproducibility. **Figure 1B** shows the overlap in detected neuropeptides with DDA and DIA between experimental replicates that originate from the same hemolymph sample. **Figure 1C** shows the overlap in detected neuropeptides in biological replicates, originating from hemolymph samples from distinct animals. As can be seen in both cases, the percentage of neuropeptides detected consistently in all 3 replicates is greater in the DIA samples than in the DDA samples, demonstrating the improved robustness and reliability of the DIA method. Based on these comparisons, it was determined that the DIA method was more suitable for hemolymph analysis and was used to analyze feeding-induced changes.

Changes in presence of neuropeptides in hemolymph at various stages of feeding

The neuropeptide contents were profiled at each time point over the course of feeding from 0 to 2 hours post-feeding, as well as before feeding. A total of 217 neuropeptides were detected in at least half of the biological replicates ($n=3/6$) at one or more time points, a substantial improvement over previous profiling studies. Of the detected neuropeptides, 120 were identified in all five time points. These neuropeptides are similar to those previously identified in hemolymph or neurosecretory tissue.

The families of neuropeptides detected in all time points are shown in **Figure 2B**, which indicates the family and corresponding number of isoforms detected. A large portion of these are hormones, such as diuretic hormones (DH), pigment-dispersing hormones (PDH), and crustacean hyperglycemic hormone (CHH) fragments and its precursor-related peptides (CPRP). DH peptides have been extensively characterized in insects, including insect hemolymph. Homologs present in crustaceans, such as the isoforms identified here, have been found in neuroendocrine and cardiac tissue and have been found to elicit an effect on the lobster cardiac neuromuscular system.²⁴⁻²⁶ Their presence in the hemolymph suggests they may also act as long-range circulating hormones outside of the heart. PDH peptides are present in numerous isoforms and abundant in the SGs, many of them acting as circulating hormones in many species, and so it is unsurprising that they are well-represented in the hemolymph. However, PDH peptides were not found to be biologically active within the STNS and are suspected to be involved in circadian rhythm.²⁷ Their ubiquitous presence that is unperturbed by the feeding process is in agreement with this finding. CHH corresponds to a superfamily of large peptides with a variety of functions, such as carbohydrate metabolism and hydration.²⁸ However, as the CHH peptides

identified in this study are all fragments of intact CHH isoforms, it cannot be determined if CHH is present and biologically active in hemolymph or if the detected peptides are degradation byproducts. While CPRPs are derived from the same preprohormone as CHH, nothing is currently known about their function, though they are known to be present in hemolymph. There are also many neuropeptides present at all of the five time points that are commonly identified in the pericardial organs, such as those belonging to the allatostatin, RFamide, and pyrokinin families. This finding is to be expected, as hemolymph is sampled from the pericardial sinus, which is in close proximity to the pericardial organs. Other notable neuropeptide families identified across all time points include HIGSLYRamide, Neuroparsin, whose functions are not currently known, and crustacean cardioactive peptide (CCAP), whose function has only been studied in the heart.²⁹

The rest of the neuropeptides detected varied between samples. **Figure 2A** shows the number of neuropeptides detected and corresponding families at the various time points, with those detected in all time points excluded in order to make differences easier to observe. Those identified in all time points are shown in **Figure 2B**. Interestingly, samples from unfed crabs had the largest number of detected neuropeptides. This finding may be a result of many of these neuropeptides being consistently circulated in the hemolymph and up-taken by tissue during the course of feeding to elicit an effect, which may also indicate why the fewest number of neuropeptides were detected in animals 15 min after feeding. Many of the neuropeptides unique to the unfed time point are allatostatin peptides, including type A, B, and C. Neuropeptides from these families generally have inhibitory effects on the pyloric rhythm,³⁰ and so their absence at the onset of feeding may indicate that they function to inhibit the pyloric and gastric mill rhythms when feeding is not actively taking place. The other families prominent in unfed

hemolymph include orcokinin, tachykinin, and pyrokinin, all of which contain isoforms that have been shown to be active in the STNS.³¹⁻³³ Therefore, it is surprising that neuropeptides from these families are present in only the unfed hemolymph, but their absence in later time points could be due to an uptake into tissue in the STNS, where they may exert an effect, or it could be due to having a shorter half-life in the circulating fluid or being more sensitive to protease activities.

There were also neuropeptides that appeared only at other notable time points. An orcomyotropin peptide, FPAFTTGFGSH (m/z 1168.542) was only present at the time point 0 mins after feeding, suggesting that release of this peptide may be triggered by the act of food intake and that the peptide is later absorbed by tissue to exert a function, though there is currently no published work to suggest this mechanism. At least one member of the orcomyotropin family has previously been found to have an excitatory role in modulating hindgut contractions.³⁴ A C-type allatostatin precursor-related peptide (MFAPLSGLPGNLRTI, m/z 1586.873) was detected only 0 mins and 1 hour after feeding, and an RFamide peptide (HDLVQVFLRFamide, m/z 1271.64) was detected at 0 mins and 2 hours after feeding. The presence of these neuropeptides only at certain time points indicates they may have roles related to specific stages in the feeding process. A complete list of neuropeptides and the time points at which they were detected is shown in **Table S1**.

Changes in relative abundance of neuropeptides in hemolymph throughout feeding process

While there were many neuropeptides whose presence altered throughout the feeding process, there were also neuropeptides that were present at all 5 time points, but varied in abundance. These neuropeptides may also elicit a function, as changes in abundance can be

indicative of secretion or uptake by tissue. These peptides are shown in **Figure 3**. As previously noted, many A-type allatostatins were detected at the various time points. One A-type allatostatin, YSKFNFGLa_{amide} (m/z 974.509), showed a statistically significant decrease 15 mins, 1 hour, and 2 hours post-feeding compared to 0 min after feeding, as shown in **Figure 3A**. This change may indicate absorption of this peptide into tissue after feeding. A B-type allatostatin, TNWNKFQGSWa_{amide} (m/z 1266.600) showed a gradual increase at time points following feeding, with the increase being statistically significant between unfed and 2 hours post-feeding, shown in **Figure 3B**, which may indicate a slow release of this peptide after feeding. A CPRP peptide, EKLLSSISPSSTPLGFLSQDHSV_N (m/z 2543.230) significantly increased from 1 hour to 2 hours post-feeding, demonstrating secretion possibly related to termination of the feeding process (**Figure 3C**). Orcokinin NFDEIDRSGSFGFV (m/z 1502.691), **Figure 3D**, and RFa_{amide} YSQVSRPRFa_{amide} (m/z 1138.612), **Figure 3E**, both showed a decrease from earlier time points to later time points, indicating possible late-stage uptake into the tissue for modulation of processes such as hindgut contractions. A HIGSLYRa_{amide} precursor-related peptide, HFGSLLKSPSYRAISIPa_{amide} (m/z 1872.050), significantly increased 1 hour post-feeding compared to unfed (**Figure 3F**). The functional role of these peptides is not currently known, but these results indicate it may be feeding-related. Additionally, two cryptocyanin peptides showed changes, with one of them (KIFEPLRDKN, m/z 1259.711) showing decrease from 15 minutes to 1 hour post-feeding and the other (KIFEPLVA, m/z 916.550) showing increase at 2 hour post-feeding, as shown, in **Figures 3G** and **3H**. These peptides were previously attributed to degradation products, but their feeding-induced changes may be indicative of a functional role.³⁵

Discovery of putative novel neuropeptides

In addition to identifying many previously-reported neuropeptides in hemolymph, the enhanced detection capabilities of the DIA method enabled the discovery of putative novel neuropeptides. A total of 101 putative sequences were identified with *de novo* sequencing that have not previously been reported in the literature but possess characteristic sequence motifs of common neuropeptide families. A summary of the families is shown in **Table 1**, along with how many sequences were identified in samples from each time point. A complete list of all sequences is shown in **Table S2**, and MS/MS spectra of each sequence are shown in **Figures S1-S101**. Interestingly, more sequences were identified in samples from post-feeding time points than in the unfed sample. This finding is likely a consequence of previously studies focusing on the hemolymph of either control crabs or crabs exposed to other perturbations, such as pH stress.¹⁴ These peptides may only be released into the hemolymph after feeding, indicating potential feeding-related functions. Most of these peptides correspond to the RFamide and RYamide families, which is expected, as these are diverse families already containing many known isoform varieties.²² There were also many neuropeptides identified corresponding to A and C-type allatostatins. Again, this observation is expected because many known neuropeptides from these families were also identified in the hemolymph. The sequences of these neuropeptides will need to be confirmed with follow-up analyses and the biological activity tested for specific functions before any conclusive statements can be made. However, the large number that were able to be identified demonstrates both the capabilities of this method and the large degree of complexity of the neuropeptidomic profile of crustacean circulating fluid.

Conclusion

This study examined the neuropeptide contents of hemolymph and how they change over the course of the feeding process in *C. borealis*, including changes in presence and relative abundance of neuropeptides. By implementing a DIA MS analysis, a greater number of neuropeptides were able to be reproducibly detected, compared to a conventional DDA MS analysis. With the improved method, a total of 217 neuropeptides were detected in hemolymph samples, a larger number than had previously been achieved in a single study. Of these, 120 were consistently detected across all 5 time points. The remaining were present only at certain time points, which may be indicative of feeding-triggered release or uptake of these neuropeptides from tissue. Of the neuropeptides detected over the 5 time points, 8 showed relative changes in abundance that were statistically significant at specific time points. Collectively, these results indicate a cohort of neuropeptide hormones that may have a functional role within the STNS. Additionally, 101 putative neuropeptides were identified with *de novo* sequencing, some of which may also elicit an effect on the STNS. Follow-up studies confirming the sequence of these neuropeptides and electrophysiological studies can be performed to elucidate a more detailed understanding of the functional role of these neuropeptides. Overall, this study identifies neuropeptides that may be implicated in hormonal STNS modulation and uncovers more of the complexity of the crustacean neuropeptidome.

Materials and Correspondence

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Supporting Information

Table of all detected neuropeptides at each time point, table of putative neuropeptide sequences identified with *de novo* sequencing, MS/MS spectra of each putative neuropeptide sequence.

Data availability

The data used in this study are available from the corresponding author upon reasonable request.

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Author Contributions

KD, MPN, and LL all contributed to conception and design of experiments. KD, MH, and WW contributed to performing all experiments and analyzing resulting data. KD wrote the manuscript. All authors contributed to review and revision of the manuscript.

Competing Interests

The authors declare no competing interests.

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Figures and Tables

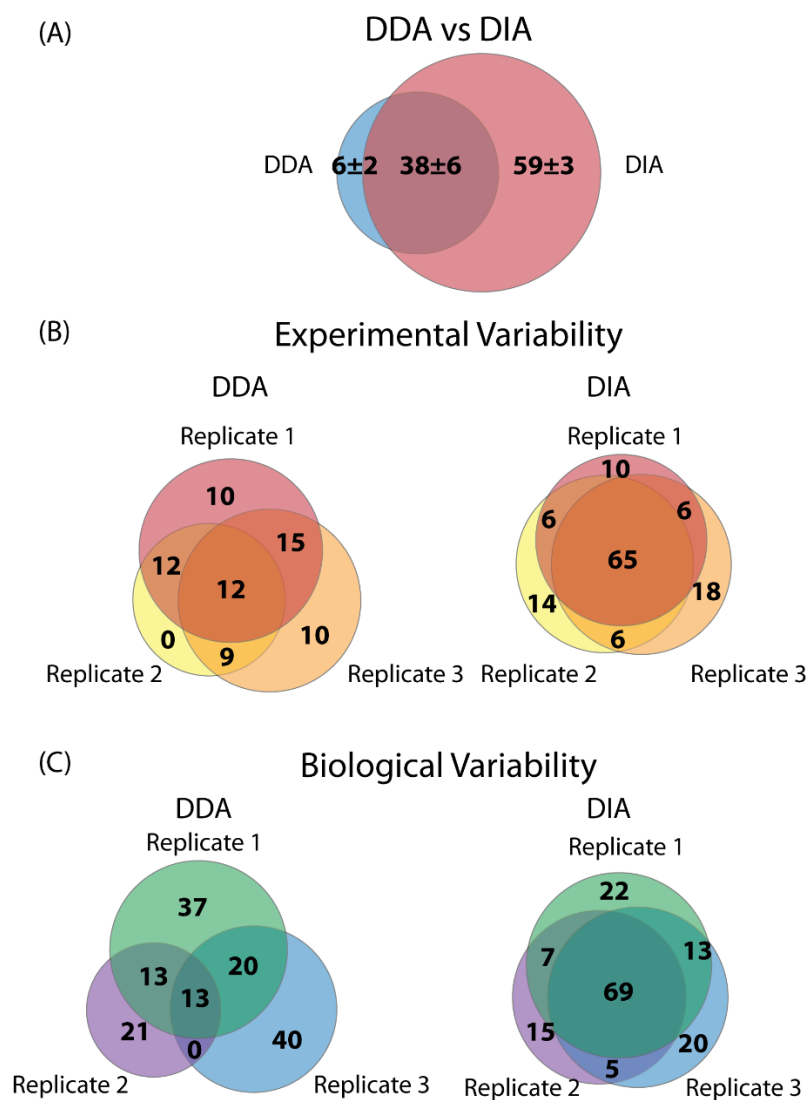


Figure 1. Comparison of data dependent acquisition (DDA) and data independent acquisition (DIA) methods for detecting neuropeptides in hemolymph, including (A) comparison of number of detected neuropeptides with each method (n=3), (B) comparison of overlap of detected neuropeptides between experimental replicates of identical hemolymph samples (n=3), and (C) comparison of overlap of detected neuropeptides between biological replicates originating from distinct animals (n=3).

Family Distribution of Neuropeptides Detected in Hemolymph

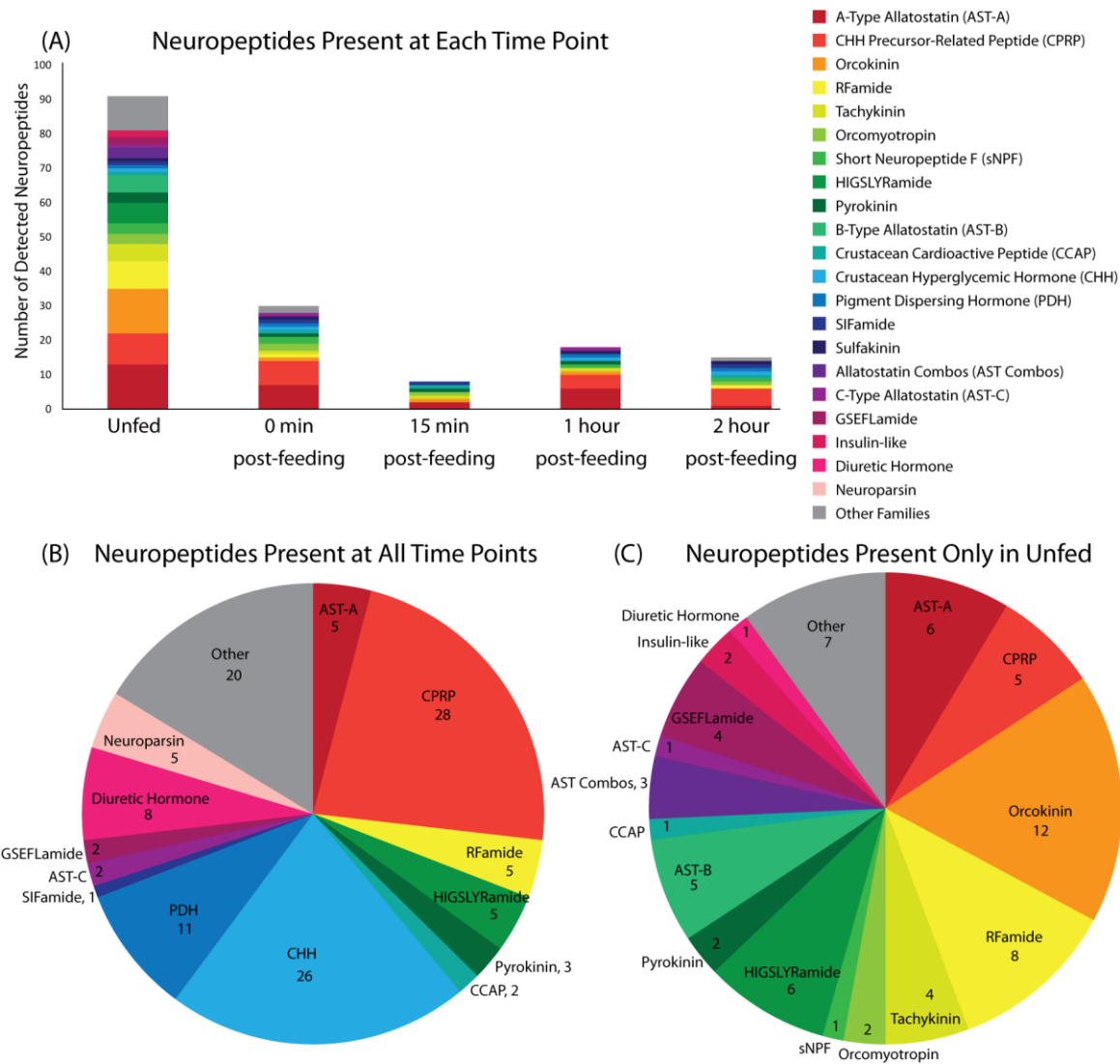


Figure 2. Distribution of neuropeptides detected in each sample of hemolymph belonging to each neuropeptide family, including (A) distribution of families detected at each time point, excluding neuropeptides detected at all time points, (B) family distribution of neuropeptides detected in all time points, and (C) family distribution of neuropeptides detected only in unfed hemolymph (n=6). Abbreviations are defined in the key.

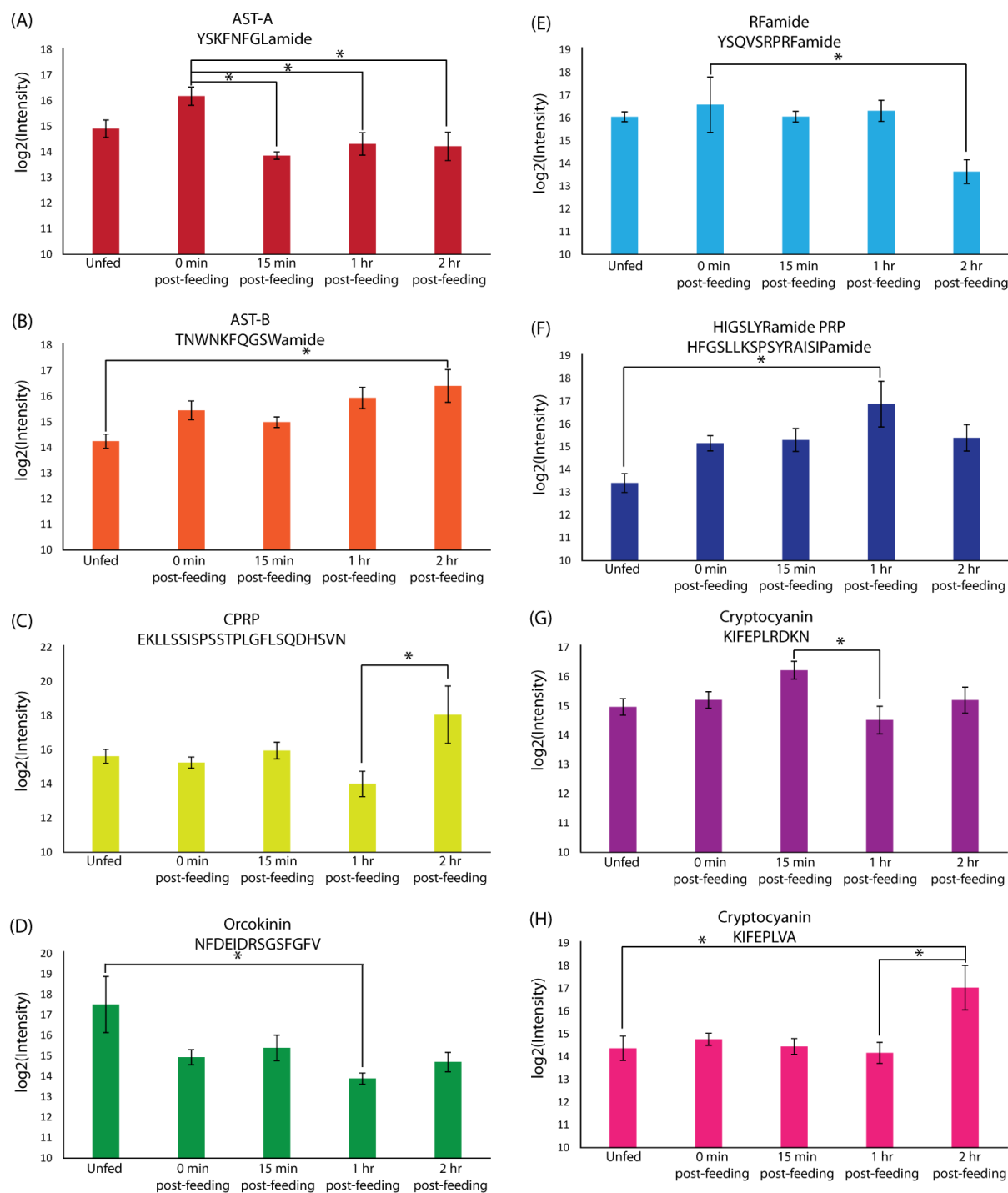


Figure 3. Relative changes in abundance of neuropeptides across all 5 time points for 8 different neuropeptides, * indicates statistical significance (p-value < 0.05 based on one-way ANOVA followed by Tukey HSD post-hoc test), error bars indicate standard error of the mean (SEM) (n=6).

Table 1. Number of putative novel neuropeptide sequences belonging to each family identified and the time point they were identified in.

Family	Post-feeding Time Point					Total
	Unfed	0 min	15 min	1 hr	2 hr	
A-type Allatostatin	1	2	5	6	3	15
B-type Allatostatin	0	2	0	1	2	5
C-type Allatostatin	7	8	3	2	7	16
Cryptocyanin	0	0	0	3	2	5
Insect Kinin	1	0	0	1	2	3
Pyrokinin	0	0	0	1	0	1
RFamide	3	5	5	5	8	21
RYamide	7	13	6	5	2	30
SIFamide	1	0	2	2	0	4
Tachykinin	0	0	1	0	0	1

Supporting Information

Table S1. List of all neuropeptides detected at each time point, precursor-related peptide (PRP), sulfation (sulf), oxidation (O).

Family	Sequence	m/z	Post-feeding Time Point				
			Unfed	0 min	15 min	1 hr	2 hr
Actin	LRVAPEESPVL	1209.684	X	X	X	X	X
Actin	LRVAPEEHPVLL	1372.790	X	X	X	X	X
Allatostatin A-type	TAPYAFGLamide	838.446	X				
Allatostatin A-type	LVKYSFGLamide	925.551	X				
Allatostatin A-type	ADSYGLAFGNGGDALEMGLamide	1856.848	X	X		X	
Allatostatin A-type	EVPDPETERNSYDFGLamide	1866.850	X	X			
Allatostatin A-type	PDAEESNKRDRLYAFGLamide	1979.993	X				
Allatostatin A-type	NEVPDPETERNSYDFGLamide	1980.893	X	X		X	
Allatostatin A-type	AYSYVSEYKRLPVYNFGLamide	2168.118	X	X	X	X	
Allatostatin A-type	DPYAFGLRHTSFVLYAFGLamide	2173.210	X	X	X	X	X
Allatostatin A-type	EYDDMYTEKRPKVYAFGLamide	2224.074	X	X		X	
Allatostatin A-type	TARGALDLQSPAYASDLGKRIGSAYSFGLamide	3099.586	X	X	X	X	X
Allatostatin A-type	DARGALDLQSPAYASDLGKRIGSAYSFGLamide	3113.565	X	X		X	X
Allatostatin A-type	GYEDEDEDPRPFYALGLGKRPRTYSFGLamide	3150.528	X				
Allatostatin A-type	ALGVYGDSRQNYDFGLa	1773.856	X				
Allatostatin A-type PRP	ETIEEDEDHTKMAADNKALPYPPSSSSSSSSSS	3599.545	X	X	X	X	X
Allatostatin A-type PRP	ADEEDDEEEEEEEDEEGLDQDVVEDLA	3224.230	X	X	X	X	X
Allatostatin A-type PRP	EPYGWSLLLWAWQERTLCI	2364.185	X	X	X	X	
Allatostatin A-type PRP	PLRFRAWQTTC	1378.705	X				
Allatostatin B-type	SGKWSNLRGAWamide	1260.660	X				
Allatostatin B-type	LMFAPLAWPKGGARWamide	1728.040	X				
Allatostatin B-type PRP	QDEGSGLAGAQA	1103.497	X				
Allatostatin B-type PRP	TPDDTPEHGLQVSED	1639.709	X				
Allatostatin B-type PRP	QDDLIHLQDLED	1453.681	X				
Allatostatin Combos	DPYAFGLGKRPDMYGFGLamide	2002.984	X				

Allatostatin Combos	DPYAFGLGKRPDMYAFGLamide	2017.000	X					
Allatostatin Combos	EPYAFGLGKRPATDLYAFGLamide	2185.144	X					
Allatostatin C-type PRP	VPDAPKEVSKGHEGGRLQ	1903.999	X					
Allatostatin C-type PRP	AAASSASDTSEEEAALKDIILARLAAELDDSWQDLPSF	4120.025	X	X	X	X	X	X
Allatostatin C-type PRP	MFAPLSGLPGNLRTI	1586.873		X			X	
Allatostatin C-type PRP	LEDDGTLDAALINYLFQKQLVQRLRTPSEVSREAQ	3960.083	X	X	X	X	X	X
Allatotropin	GFKNVEMMTARGFamide	1486.729	X					
A-type Alltostatin	MGIAARTMISGLVRETPMPLGLENLVLTPSGLERENHMAG PYSFGLa	5024.599	X	X	X	X	X	X
bursicon	RSYGECETLPSTIHISKEEYDDTGRLVRVCEEDVAVNKCEGAC VSKVQPSVNTPSGFLKDCRCCREVHLRARDITLTHCYDGDG ARLSGAKATQHVKLREPADCQCFKCGDSTR	12618.981	X	X	X	X	X	X
bursicon	DECSLRPVIHILSYPGCTSKPIPSFACQGRCTSYVQVSGSKLW QTERSMCCQESGEREAAITLNCPPKPRGPKEKKVLTRAPID CMCRPCTDVEEGTVLAQKIANFIQDSMPDSVPFLK	13257.451	X	X	X	X	X	X
CCHamide	HGPVKVGCLNYGHSCGLGAHa	1947.943	X					
CCHamide	GSWPAPSAPRTPATALLAPFLQGLATPRSPARSWQDKVMTQ HRQHHQDQHLHHLEQQQQQQQQQVFPVAVYDEVALPRI PASLH	9476.819	X	X	X	X	X	X
CCHamide	APIVDSSCKGQYSMYIWNKLYFVCECTNIFRTDGFEAQCR QGCFDIDIFAKCLRELSKDVNEYQIMATSLRGS	8520.940	X	X	X	X	X	X
Crustacean Cardioactive Peptide PRP	DIGDLLEGKD	1074.531	X					
Crustacean Cardioactive Peptide PRP	SDPKLEGLASGSELNDNKNVLAEARLWEQLQSEMETMRL AS	4673.319	X	X	X	X	X	X
Crustacean Cardioactive Peptide PRP	SLNHPQHDCTHSAQTIDHKVTCTQL	2814.305	X	X	X			X
Crustacean Hyperglycemic Hormone	SLFDPSCSGVFDRELLGRLNRVCDDCYNVFRDPKVAMECKS NCFLNPAFIQCLEYLLPEDLHEEYQSHQVVamide	8255.878	X	X	X	X	X	X
Crustacean Hyperglycemic Hormone	pEVFDQACKGIYDRAIFKKLDRVCEDCYNLYRKPYVATTCRQ NCYANSVFRQCLDLDLLIDVLDEYISGVQTVamide	8402.110	X	X	X	X	X	X

Crustacean	pEIYDTSCCKGVYDRGLFSDLEHVCDDCYNLYRNSYVASACRS	8419.819	X	X	X	X	X
Hyperglycemic Hormone	NCYSNVVFRQCMEEELLMDEFDKYARAVQIV						
Crustacean	pEIYDTSCCKGVYDRGLFSDLEHVCDDCYNLYRNSYVASACRS	8428.910	X	X	X	X	X
Hyperglycemic Hormone	NCYSNVVFRQVMEELLMEEFDKYARAVQIVamide						
Crustacean	QIYDSSCKGVYDRGLFSDLEHVCDDCYNLYRNSYVASACRSN	8431.921	X	X	X	X	X
Hyperglycemic Hormone	CYSNVVFRQVMEELLMEEFDKYARAVQIVamide						
Crustacean	QIYDSSCKGVYDRGLFSDLEHVCDDCYNLYRNSYVASACRSN	8436.846	X	X	X	X	X
Hyperglycemic Hormone	CYSNVVFRQCMEEELLMEEFDKYARAVQIV						
Crustacean	QIYDTSCCKGVYDRGLFSDLEHVCDDCYNLYRNSYVASACRSN	8436.846	X	X	X	X	X
Hyperglycemic Hormone	CYSNVVFRQCMEEELLMDEFDKYARAVQIV						
Crustacean	QIYDTSCCKGVYDRGLFSDLEHVCDDCYNLYRNSYVASACRSN	8445.937	X	X	X	X	X
Hyperglycemic Hormone	CYSNVVFRQVMEELLMEEFDKYARAVQIVamide						
Crustacean	QIYDTSCCKGVYDRGLFSDLEHVCDDCYNLYRNSYVASACRSN	8450.862	X	X		X	X
Hyperglycemic Hormone	CYSNVVFRQCMEEELLMEEFDKYARAVQIV						
Crustacean	SFFDIQCKGNYDKSIFARLDRICEDCYNLFREPQLHSLCRSDFK	8471.136	X	X	X	X	X
Hyperglycemic Hormone	SPYFKGCLQALLLIDEEEFKNQMVEILamide						
Crustacean	pEVFDQACKGVYDRNFLFKKLDRVCEDCYNLYRKPFVATTCRE	8479.010	X	X	X	X	X
Hyperglycemic Hormone	NCYSNWVFRQCCLDDLLSNVIDEYVSNVQM						
Crustacean	pQIYDTSCCKGVYDRALFNDLEHVCDDCYNLYRTSYVASACRS	8525.785	X	X	X	X	X
Hyperglycemic Hormone	NCYSNLVFRQCMDDLLMMDEFDQYARKVQMVamide						
Crustacean	pEVFDQACKGVYDRNFLFKKLDRVCEDCYNLYRKPFVATTCRE	8578.078	X	X	X	X	X
Hyperglycemic Hormone	NCYSNWVFRQCCLDDLLSDVIDEYVSNVQMVamide						
Crustacean	pQIYDTSCCKGVYDRALFNDLEHVCDDCYNLYRTSYVASACRN	8631.867	X	X	X	X	X
Hyperglycemic Hormone	NCFENEVFDVCVYQLYFPNHEEYLRSDGLKG						
Crustacean	pEVFDQACKGVYDRNFLFKKLNRVCEDCYNLYRKPFIVTTCRE	8633.172	X	X	X	X	X
Hyperglycemic Hormone	NCYSNRVFRQCCLDLLMIDVIDEYVSNVQMVamide						
Crustacean	RYVFEPCPGVMGNRAVHGKVTRVCEDCYNVFRDTDVLAGC	8652.176	X	X	X	X	X
Hyperglycemic Hormone	RKGCFSSSEMFKLCLLAMERVEEFPDFKRWIGLLNAamide						
Crustacean	pQIYDTSCCKGVYDRALFSDLEHVCDDCYNLYRSSYVASECRR	8660.014	X	X	X	X	X
Hyperglycemic Hormone	NCYSNVVFRQCMEEELLMEEFDKYARAVQIVamide						

Crustacean	SYIENTCRGVMGNRDIYKKVVRVCECTNIFRLPGLDGMCR	8987.411	X	X	X	X	X
Hyperglycemic Hormone	DRCFNNEWFLVCLKAANRDELDKFKVWISILNPGL						
Crustacean	SFIDNRCRGVMGNRDIYKKVVRVCECTNIFRLGLDGMCRN	9022.449	X	X	X	X	X
Hyperglycemic Hormone	RCFYNEWFLICLKAANREDEIEKFRVWISILNAGQ						
Crustacean	ASAWFTNDECPGVMGNRDLYEKVAWVCNDCANIFRNND	9136.226	X	X	X	X	X
Hyperglycemic Hormone	VGVMCKKDCFHTMDFLWCYATERHGEIDQFRKWVSILRA amide						
Crustacean	ASAWFTNDECPGVMGNRDLYEKVAWVCNDCANIFRNND	9137.226	X	X	X	X	X
Hyperglycemic Hormone	VGVMCKKDCFHTMWFLWCYATERHGEIDQFRKWVSILR						
Crustacean	RVINDECPNLIGNRDLYKKVEWICEDCSNIFRKTGMASLCRR	9154.315	X	X	X	X	X
Hyperglycemic Hormone	NCCFNEDFVWCVHATERSEELEDLEEWVGILGAGRD						
Crustacean	RVINDDCPNLIGNRDLYKKVEWICEDCSNIFRNTGMATLCRK	9195.360	X	X	X	X	X
Hyperglycemic Hormone	NCCFNEDFLWCYATERTEEMSQRQWVGILGAGRE						
Crustacean	RVINDDCPNLIGNRDLYKRVEWICEDCSNIFRNTGMATLCRK	9223.366	X	X	X	X	X
Hyperglycemic Hormone	NCCFNEDFLWCYATERTEEMSQRQWVGILGAGRE						
Crustacean	RRINNDCQNFIGNRAMYEKVDWICKDCANIFRQDGLLNNC	9236.296	X	X	X	X	X
Hyperglycemic Hormone	RSNCFYNTEFLWCIDATENTRNKEQLEQWAAILGAGWN						
Crustacean	RRINNDCQNFIGNRAMYEKVDWICKDCANIFRKDGLLNNC	9236.333	X	X	X	X	X
Hyperglycemic Hormone	RSNCFYNTEFLWCIDATENTRNKEQLEQWAAILGAGWN						
Crustacean	SFFEDINCKGLYDKSIFARLDRIQCDCYSLYREPELHTLCRKNC	9491.711	X	X	X	X	X
Hyperglycemic Hormone	FTTNYFKGCLDALLINDEKDQIRVMKDISIIHQIPI						
Crustacean	GFLSQDVHS	989.450	X				
Hyperglycemic Hormone							
PRP							
Crustacean	RGFEGETGHPN	1199.555		X			
Hyperglycemic Hormone							
PRP							
Crustacean	DLKSDTVTPLR	1244.685		X		X	
Hyperglycemic Hormone							
PRP							

Crustacean Hyperglycemic Hormone PRP	ASLKSPTVTPLR	1269.753	X	X		X	X
Crustacean Hyperglycemic Hormone PRP	RSTQGYGRMDPIL	1552.830	X				
Crustacean Hyperglycemic Hormone PRP	RSVEGASRMEKLLT	1576.850	X				
Crustacean Hyperglycemic Hormone PRP	RSAQGLGKYLRLLASY	1796.000	X				
Crustacean Hyperglycemic Hormone PRP	WSVDGLARIEKLLSTSSSASAASPTRGQALNL	3286.739	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	WSLDGLARIEKLLSTSSSASAASPTRGQALNL	3300.755	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSVEGVSRMEKLLSSIPSTPLGFLSQDHSV	3430.700	X	X		X	X
Crustacean Hyperglycemic Hormone PRP	RSVEGVSRMEKLLSSIPSSMPLGFLSQDHSV	3460.560	X	X		X	X
Crustacean Hyperglycemic Hormone PRP	RSVEGASRMEKLLSSSNPSSTPLGFLSQDHSV	3490.630	X				
Crustacean Hyperglycemic Hormone PRP	RSVEGSSRMERLLSSGSSSSEPLSFLSQDQSVS	3516.687	X	X	X	X	X

Crustacean Hyperglycemic Hormone PRP	RSVEGASRMEKLLSSISPSSTPLGFLSQDHSVN	3516.775	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSVEGSSRMERLLSSGSSSSEPLSFLSQDHSVS	3525.687	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSVEGASRMEKLLSSISPSSTPLGFLSQEHSVN	3530.791	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSVEGSSRMERLLSSGSSSSEPLSFLSQDQSVN	3543.698	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSVEGVSRMEKLLSSISPSSTPLGFLSQDHSVN	3544.806	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSVEGVSRMEKLLSSISPSSMPLGFLSQDHSVN	3574.799	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSVEGASRMEKLLSSSNPSSTPLGFLSQDHSVN	3604.766	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSVEGASRMEKLLSSSNPSSTPLGFLSQEHSVN	3618.782	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	AQGLGKMERLLASYRGALEPNTPLGDLGSLGHPVE	3733.930		X			X
Crustacean Hyperglycemic Hormone PRP	SAQGLGKMERLLASYRGALEPNTPLGDLGSLGHPVE	3820.980	X	X			X

Crustacean Hyperglycemic Hormone PRP	RSAEGFGRMGRLASLKADSLGPVQDFGVEGAHPVE	3824.950	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSAEGLGRMGRLASLKSDTVPLRGFEGETGHPLE	3838.003	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSAQGLGKMEHLLASYRGALEPNTPLGDLSGSLGHPVE	3958.024	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSAQGLGKMERLLASYRGALEPNTPLGDLSGSGVHPVE	3963.051	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSAQGMGKMERLLASYRGALEPSTPLGDLSGSLGHPVE	3968.012	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSAQGMGKMEHLLASYRGALESNTPTGDLPGGVLHPVE	3975.980	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSAQGLGKMERLLASYRGALEPNTPLGDLSGSLGHPVE	3977.066	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSAQGMGKMEHLLASYRGALESNTPLGDLPGGVLHPVE	3988.017	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSAQGLGKMERLLVSYRGAVEPNTPLGDLSGSLGHPVE	3991.082	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSAQGMGKMERLLASYRGAVEPNTPLGDLPGGVLHPVE	4003.064	X	X	X	X	X

Crustacean Hyperglycemic Hormone PRP	RSAQGMGKMERLLASYRAAVEPNTPLGDLPGGLVHPVE	4017.080	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSTPGYGRMDRILAALKTSPMEPSAALAVEHGTTHPLE	4061.070	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSTQGYGRMDRILAALKTSPMEPSAALAVENGTTTHPLE	4069.059	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSTQGYGRMDRILAALKTSPMEPSAALAVQHGTTHPLE	4091.091	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSTQGYGRMDRILAALKTSPMEPSAALAVEHGTTHPLE	4092.075	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	RSAEGFGRMERLLASIRGGADSMGHLGELTGAGEGAGHPL E	4150.031	X	X	X	X	X
Crustacean Hyperglycemic Hormone PRP	GPVYPQHTASPVQSQALPGEAKQL	2503.295	X	X	X	X	X
Cryptocyanin	YKIFEPLRESN	1395.727	X				
Cryptocyanin	YKIFEPLRESNL	1508.811	X	X	X	X	X
Diuretic Hormone	GLDLGLGRGFSGSQAAKHLMGLAAANFANFAGGPamide	3272.675	X	X	X	X	X
Diuretic Hormone	GLDMGLGRGFSGSQAAKHLMGIAAANYAGGPα	2974.478	X	X	X	X	X
Diuretic Hormone	SSGLSLSIDASMKVLREALYLEIARKKQRQQLQRAQHKNKALL NTIα	5118.880	X	X	X	X	X
Diuretic Hormone	FTEYQPRNSAEANNNSNNRKA	2425.125	X				
Diuretic Hormone	PLSLPPHRLAPHNPDLIPDDQALALMSDPASRYEEGLLNEL ENPHSSSSYHLKQLISELADAITAAEELEGGAYGAPLPAEIAEA IVAAEGIEGGADGRPLPSDVAEIAEAEAAVMGGGLARP WED	13353.593	X	X	X	X	X

Diuretic Hormone PRP	ESEAATIDQHDDHHLAVQQHSAAAAAAPAGLSPHSS	3835.777	X	X	X	X	X
Diuretic Hormone PRP	SVTNNTNNNNNNNSLTSTSTGTSTTPNNNNNNNNKLA NNHNSGMF	4982.158	X	X	X	X	X
Diuretic Hormone PRP	DVASQLVGVDQGVALQNARN	2054.063	X	X	X	X	X
Diuretic Hormone PRP	SPINREPSRAVVEIDDPDY(SO)VLELLTRFSNSII	3657.913	X	X	X	X	X
Ecdysis-triggering	DPSPEFPNPYNRFRQKIPRIamide	2584.353	X				
Eclosion hormone	AVAANRKVSICIKNCGQCKKMYTDYFNGGLCGDFCLQTEGR FIPDCNRPDILIPFFLQRL	6932.389	X	X	X	X	X
FMRamide-like	GDSSSVEDMDVLPETEDTIE	2167.908	X				
FMRamide-like	SDEDDASDMMMDMPASYNRYV	2442.920	X				
GSEFLamide	YLLVAMAQPGPRYAAPQILSRGV	2471.360	X				
GSEFLamide	VLGALSRSRLA	1142.701	X				
GSEFLamide PRP	SPVVESPEVVESPEPGKTCESGSCVTEEDHDHADDL	3809.628	X	X	X	X	X
GSEFLamide PRP	EQLSFTGQYNEQEGSDGLQDEPD	2586.076	X	X	X	X	X
HIGSLYRamide	FLGSVLRQDGRLPWGF	1961.076	X				
HIGSLYRamide	HLSSLARNNAYPKDLRE	1984.036	X				
HIGSLYRamide	HYSSLRNGPLPFIQD	1856.966	X				
HIGSLYRamide	HFGSLLKSPSYRAISIPa	1872.050	X				
HIGSLYRamide	DEDSLYQDLNED	1455.576	X				
HIGSLYRamide	DEDNLY(SO3)HELSED	1478.592	X				
HIGSLYRamide PRP	SFGVQQDSDDSDHNDALTLPAASVPEGIPSQSPQTQALE	3936.838	X	X	X	X	X
HIGSLYRamide PRP	YFASLLKSRAFGDDSKLIPHNAAGDSEPHLQ	3384.698	X	X	X	X	X
HIGSLYRamide PRP	FYASLLKSDSPQTAYLNSMFYRQD	2942.403	X	X	X	X	X
HIGSLYRamide PRP	SPNAVEDPEDNPVRELEDIRDLSGFDGDLHDRLVAAGVI SPGLSVRGDTQDLEALLTAWYAAATSGQD	7534.644	X	X	X	X	X
HIGSLYRamide PRP	EDNLY(SO3)HDLSED	1349.550	X	X	X	X	X
Insulin-like	LCGWKLANELNRVCKGVYNKPTVSTNALFYLKERGa	3984.110	X	X	X	X	X
Insulin-like	GLSAECCRKACSVSELAGYCY	2212.950	X				
Insulin-like	SPRTLPEGEAVSEGE	1557.740	X				
Insulin-like	VDLWPLGIELQFPPWTQAPADE	2522.261	X	X	X	X	X
Insulin-like	ANQIIESHQPDKPMEQSLGESQRLVFLTGPEASQVVSGLPRV	4572.352	X	X	X	X	X

Intocin	TENMVPITCFNSDLKPCGRMQEa	2512.146	X					
Intocin PRP	SGGLMSQLGRARTCTSCGPGLLGRCIGPDICCGPRIGCFLGS RET	4556.170	X	X	X	X	X	X
Leucokinin	SDDNKSQKFSGGTENHNNEENQDQFLW	3281.437	X	X	X	X	X	X
Leucokinin	DDAFHSPESRFHPWLa	1839.857	X					
MIH	RVINDDCPNLIGNRDLYKKVEWICDDCANIYRSTGMASLCRK DCFFNEDFLWCVRATERSEDLAQLKQWVTILGAGRI	9072.408	X	X	X	X	X	X
Neuroparsin	APRCDRHDEEAPKNCKYGTQDWCKNGVCAKGPGETCGG YRWSEGKCGEGTPCSCGICGGCSPFDGKCGPTSIC	7708.210	X	X	X	X	X	X
Neuroparsin	APRCEKYDQPPPKHCKFGSTVDWCNNGICAKGPRESGGY RWVDGKCGEGTFCSCGVCAGCSPFGGDCPVISPC	7777.324	X	X	X	X	X	X
Neuroparsin	APRCAEYDREPPSDCKYGTAKDWCHNGICAQGPGDICGGY HDEKGVCGEGTYCDCGRRCRGCSFDTSCHPGQIC	7905.197	X	X	X	X	X	X
Neuroparsin	TPLCPERNEIAPEDLSQCKYGVVLGWCGNAACGKGPDEPCG GRWEENGICGEGMYCVCGYCAGCTSTLECVLGRFC	8027.422	X	X	X	X	X	X
Neuroparsin	GPVCASLNEVLPEMLPPCRHGVVRDYCDNAMCMKGPGET CGGRWNENGFCGKGLYCVCGYCAGCSKDLQCALGRFC	8111.514	X	X	X	X	X	X
Orcokinin	NFDEIDRSFGA	1270.570	X					
Orcokinin	NFDEIDRSSFG	1286.565	X					
Orcokinin	NFDEIDRSSFGF	1433.633	X					
Orcokinin	NFDEIDRAGFGFA	1458.665	X					
Orcokinin	SSEDMPSSLGFGFN	1474.615	X					
Orcokinin	NFDEIDRSFGFGFA	1474.660	X	X	X	X	X	
Orcokinin	SSEDM(o)PSSLGFGFN	1490.622	X					
Orcokinin	NFDEIDRSFGFGV	1502.691	X					
Orcokinin	DFEDIERSGFGFV	1503.680	X					
Orcokinin	NFDEIDRSSFGFA	1504.670	X					
Orcokinin	NFDEIDRSSFGFV	1532.702	X					
Orcokinin	NFDEIDRSSFGFN	1547.676	X					
Orcokinin	NFDEIDRTGFGFH	1554.697	X					
Orcomyotropin	FPAFTTGFGSH	1168.542		X				

Orcomyotropin	FDAFTTGFGHS	1186.516	X	X	X		X
Orcomyotropin	FDAFTTGFGHN	1213.527	X				
Orcomyotropin	FPAFTTGFGHS	1168.532	X				
Other	KPKTEKK	858.540	X	X			X
Other	DLPKVDTALK	1099.640	X	X	X	X	X
Other	AVLLPKKTEKK	1254.811	X	X	X	X	X
Other	EVEEPEAPAPPAK	1363.670	X	X			
Pigment Dispersing Hormone	NSGMINSILGIPRVMTEAamide	1901.994	X	X		X	X
Pigment Dispersing Hormone	QEVNVSEREAVATLAAHILKVVCAPLEGAGGLPHKRNSELIN SILGLPKVMNEAamide	5685.073	X	X	X	X	X
Pigment Dispersing Hormone	QELHVPEREAVANLAARILKIVHAPHDAAGVPHKRNSELINS LLGISALMNEAamide	5712.103	X	X	X	X	X
Pigment Dispersing Hormone	QREPTASKCQAATELAIQILQAVKGAHTGVAAGPHKRNSELI NSLLGLPKFMIDAamide	5792.122	X	X	X	X	X
Pigment Dispersing Hormone	QDLKYQAREMVAELAQQIYRVAQAPQAGAVGPHKRNSELI NSILGLPKVMNDAamide	5829.080	X	X	X	X	X
Pigment Dispersing Hormone	DSSLKYFEREVVSELAQQILRVAQGSAFVAGPHKRNSELINS LLGIPKVMTDAamide	5851.133	X	X	X	X	X
Pigment Dispersing Hormone	QELKYPEREVVAELAQQIYRVAQAPWAGAVGPHKRNSELIN SILGLPKVMNEAamide	5853.139	X	X	X	X	X
Pigment Dispersing Hormone	QDLKYQEREMVAELAQQIYRVAQAPWAGAVGPHKRNSELI NSILGLPKVMNDAamide	5945.107	X	X	X	X	X
Pigment Dispersing Hormone	QEDLKYFEREVVSELAQQILRVAQGSAFVAGPHKRNSELIN SLLGIPKVMNDAamide	5947.165	X	X	X	X	X
Pigment Dispersing Hormone	QELKYQEREMVAELAQQIYRVAQAPWAAAVGPHKRNSELI NSILGLPKVMNDAamide	5973.138	X	X	X	X	X
Pigment Dispersing Hormone	QDLNPTEKEVLSNMLDFLQRHSRTTYMFPLLESKRNSELIN SLLGAPRVLNNAamide	6199.218	X	X	X	X	X
Pigment Dispersing Hormone	QSRDFSISEREIVASLAKQLLRVARMGYVPEGDLPRKRNAELI NSLLGVPRVMSDAamide	6250.382	X	X	X	X	X
Pyrokinin	TSFAFSPRLamide	1024.557	X				

Pyrokinin	GSGFAFSPRLa	1037.553	X					
Pyrokinin PRP	VDEdGVSSFPSSPLLHRLGSSAPWGHVAANPMTSLLAGHLN LQPPMHTIQERMS	5772.848	X	X	X	X	X	X
Pyrokinin PRP	DEITNLEEPEDTTSIPYRWWWPLVSV	3175.526	X	X	X	X		
Pyrokinin PRP	AEEQTEVDLPYDYVAGDLYDTLDDDEEEIEDEEDEEGNAAVL QD	5008.071	X	X	X	X	X	X
Pyrokinin PRP	ADSSSETEQTQAGQTTWWFGAEAGTPATTLPPFLPSRLE	4122.921	X	X	X	X	X	X
Red Pigment Concentrating Hormone PRP	NEAVRLVQCDEEY(SO3)La	1679.806	X	X	X	X	X	X
RFamide	I/LNFTHKFamide	905.490	X	X	X	X	X	X
RFamide	pEDVVHSFLRFamide	1229.600	X					
RFamide	QDVVHSFLRFamide	1246.700	X					
RFamide	HDLVQVFLRFamide	1271.640		X				X
RFamide	EPLDHVFLRFamide	1271.660	X					
RFamide	HPLSFVSALRFamide	1271.774	X					
RFamide	DARTAPLRLRFamide	1314.775	X					
RFamide	KPDPSQLANMAEALKYLEQELDKYYSQVSRPRFamide	3913.991	X	X	X	X	X	X
RFamide	pEDVVHSFLRFamide	1229.643	X					
RFamide PRP	SVDRQLSEQKTRDAPVAPTATIHSPAKTQESHRS	3728.907	X	X	X	X	X	X
RFamide PRP	SVDRQLSEQKTRDAPVAPTATIHSPAKTQESHRS	4268.932	X	X	X	X	X	X
RFamide PRP	SDAEEFGLPGGPLAFDNNVQEDDLTDY(SO)PMEE	3414.448	X	X	X	X	X	X
RPCH PRP	AAAASGGGGGVGEAVSALHPSVVGAPGGVMPPGSSSSGD SCGPIPVSAVMHIYRLI	5098.519	X	X	X	X	X	X
Short Neuropeptide F	DMGWQVAQRSMPSLRLRFa	2177.122	X					
Short Neuropeptide F PRP	NVDQADPILDHDLV	1563.765	X	X			X	X
Short Neuropeptide F PRP	GATFGEEDMASQEQ	1499.596	X	X				
SIFamide	DLDSVLDPISIFamide	1219.610	X	X	X			X
SIFamide PRP	SGTDAVYEPGKSQALASVCQMAMEACTLWFPGAE	3548.585	X	X	X		X	X

Sulfakinin	TSGGEDDY(sulf)GQDDYGHLRFamide	2109.786	X	X		X	X
Tahykinin	APSGFLGMRamide-NH3-loss	917.462	X				
Tahykinin	APSGFLGMRamide	934.493	X				
Tahykinin	APSGFLGMR	935.477	X				
Tahykinin	APSGFLGM(O)Ramide	950.488	X				
Tahykinin	APSGFLGMRG	992.498	X	X	X	X	

Table S2. Putative sequences of neuropeptides detected in hemolymph with PEAKS *de novo* sequencing. ALC stands for average local confidence, with the cutoff threshold being 75%.

“Like” indicates peptide has a similar sequence to neuropeptides belonging to the related family but does not follow the characteristic sequence motif.

Family	Peptide	Molecular Weight	ALC
AST-A	EKADLEAQLNEVGLamide	1508.7834	87
	ELAACGLamide	674.3421	78
	EQPDKGLamide	766.3973	81
	HKGGGGGGGLYGGGSGLamide	1367.6694	85
	KMKLDRVNPGLamide	1284.7336	79
	LEEESFRDGLamide	1192.5723	91
	LKTRKSGFGLamide	1086.6663	75
	LRGGTGGWPLGGNNGLamide	1523.7957	76
	LTSGGGGLYGGGSGLamide	1214.6042	82
	MLLTGESGAGKTETTGLamide	1663.845	83
	SVTSESGLamide	741.3657	93
	TEEFGLamide	693.3333	86
	VPSGGGGLMAGGSGLamide	1214.6077	75
	VQAADPGLamide	768.413	93
AST-B	AWPPGYSRWamide	1117.5457	78
	QAMWQLAAKWWamide	1416.7124	78
	WLQGLYLWamide	1058.5701	79
	WLQLVCLWamide	1058.5735	75

	WLQPAFLWamide	1058.5701	75
AST-C	AKLGGCamide	693.3632	83
	DPDLKTLPQCamide	1274.6328	79
	EVKCCamide	605.2996	85
	GGLGGCamide	608.274	76
	GGLGSMCCamide	751.3146	88
	GNGGLRGTGSGGCamide	1237.5623	80
	HKQPLTKLCamide	1212.6802	76
	HWTFNPSVSLCCamide	1435.6707	76
	KLVHLCamide	857.4946	96
	KVDYCCamide	754.3472	98
	NLGGCamide	608.274	93
	PMDCamide	610.2244	76
	pQKTCamide	607.2788	82
	VEAVACamide	736.3578	75
Cryptocyanin	CGKLFEPRES	1277.6438	91
	CGKLFEPRTD	1277.6438	91
	EKLFEPRESDamide	1360.6987	98
	WCYKLFEPRE	1482.7329	87
	YKLFEPRESDKamide	1522.8142	75
Insect kinin	FLSWGamide	1750.875	85
	FLVWGamide	619.3482	79
Pyrokinin	SEGEHASSPPRLamide	1228.5974	85

RFamide	AFKRFamide	666.3965	77
	DEGRFamide	621.287	78
	DNLRFamide	662.35	88
	EGNRFamide	602.2925	90
	FGLLTGTNQLGHPRFamide	1655.8896	77
	HDVRFamide	671.3503	78
	KKSRFamide	645.4074	81
	KTMRFamide	680.3792	79
	KYRFamide	611.3543	86
	LDWVRFamide	833.4548	84
	LHEEEEQRFamide	1214.5679	94
	LYLRFamide	709.4275	82
	MTSRFamide	603.2951	80
	NDLRFamide	662.35	87
	PHPNLLDRFamide	1088.5879	87
	PLMSRFamide	748.4054	76
	SHLRFamide	639.3605	75
	SNFGDNVQDRFamide	1296.5847	85
	SPERFamide	615.3129	77
	VTGLLRFamide	803.5018	79
RYamide	DDQSLAHDGRYamide	1274.564	78
	DELRamide	693.3445	96
	DSKYSQRYamide	1044.4988	79

EANQHGRYamide	954.442	80
ELKDRYamide	803.429	77
HNQHGRYamide	909.4318	90
HQLLDRYamide	942.5035	80
KMWKSDVRYamide	1174.6069	77
KRMLEKTGHNQHGRYamide	1852.959	83
NHDTGHNQHGRYamide	1433.6296	90
NPTGRHRYamide	998.5158	90
NTTRYamide	616.3082	77
PEGVSNDMAYGGSGAYNVDGRYamide	2276.9756	82
PEGVSNMDAYGGSGAYNVDGRYamide	2276.9756	80
PEGVSNVMAYGGSGAYNVDGRYamide	2261.0171	80
PEGVSNVMAYNSGAYNVDGRYamide	2261.0171	76
PEGVSNYQKHSGAYNVDGRYamide	2239.0405	76
pQDARYamide	633.287	82
pQTPTGHNQHGRYamide	1376.6333	87
pQVARYamide	617.3286	78
RQRYamide	602.3401	76
SFKRYamide	698.3864	95
SKPPRYamide	727.4129	80
SWYRYamide	736.3445	78
TTTRYamide	603.3129	84
VLVYSQRYamide	1025.5658	84

	YLLERYamide	931.528	80
	YNVGSGAYNVDGRYamide	1532.7007	95
	YTYGHNQHGRYamide	1393.6277	95
	YVVHGRYamide	891.4715	78
SIFamide	EEESLFamide	733.3238	93
	EESLFamide	622.2962	81
	EESSLFamide	691.3177	88
	pQEESLFamide	733.3282	82
Tachykinin	LPETVPSGFLGMRamide	1401.7439	84

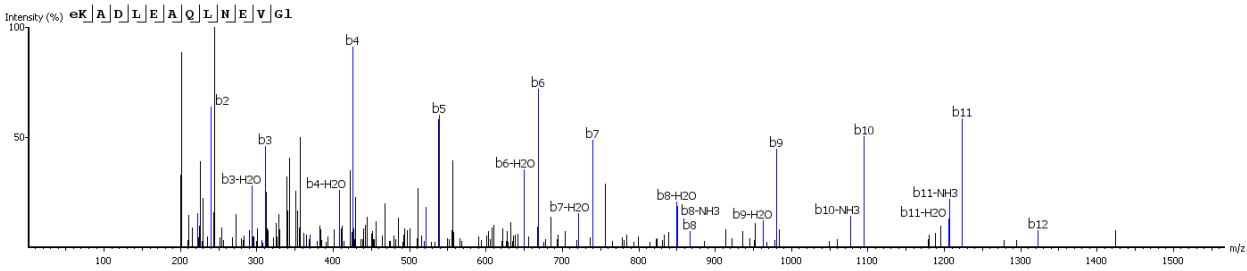


Figure S1. MS/MS spectrum of a putative AST-A neuropeptide with the sequence E(-18.01)KADLEAQLNEVGLamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

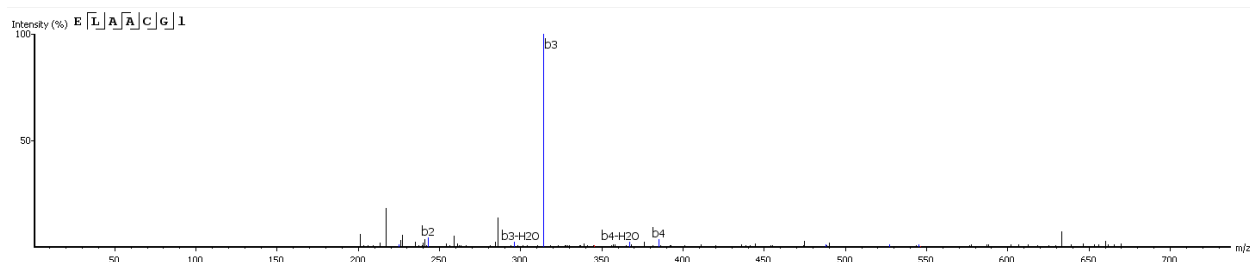


Figure S2. MS/MS spectrum of a putative AST-A neuropeptide with the sequence ELAACGLamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

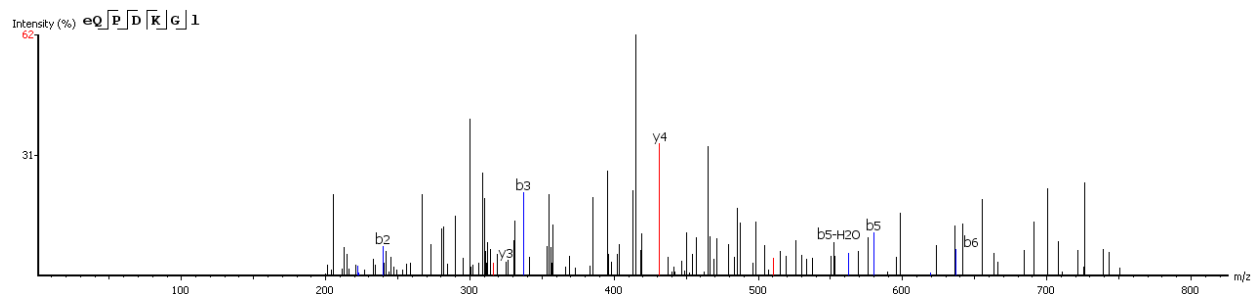


Figure S3. MS/MS spectrum of a putative AST-A neuropeptide with the sequence E(-18.01)QPKDKGLamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

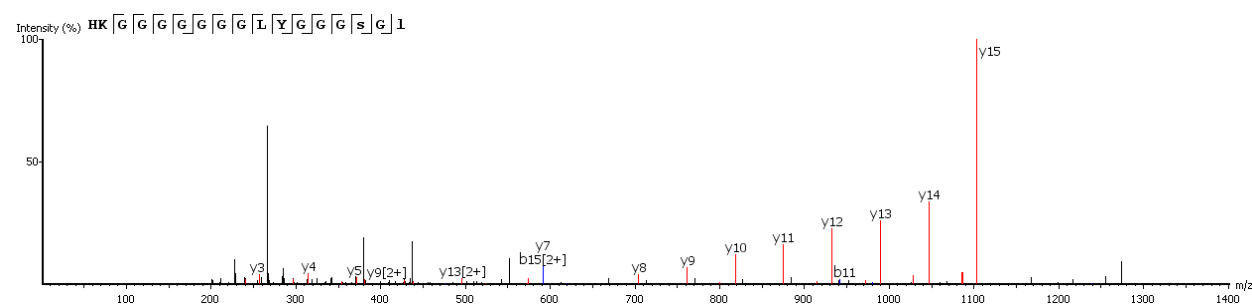


Figure S4. MS/MS spectrum of a putative AST-A neuropeptide with the sequence HKGGGGGGGLYGGGS(-18.01)GLamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

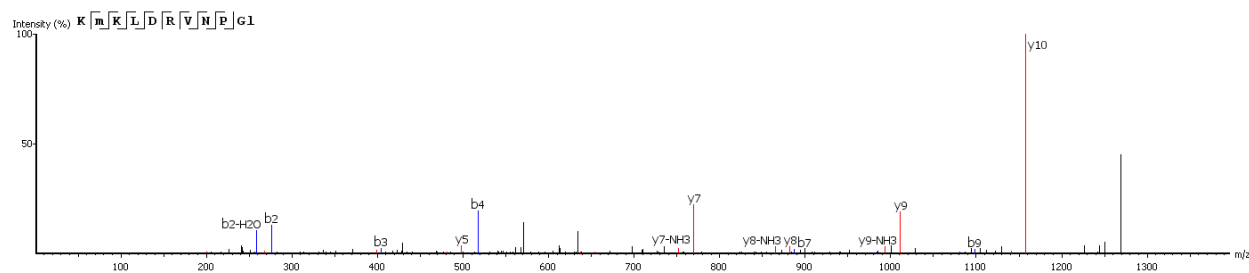


Figure S5. MS/MS spectrum of a putative AST-A neuropeptide with the sequence KM(Oxidation)KLDRVNPGLamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

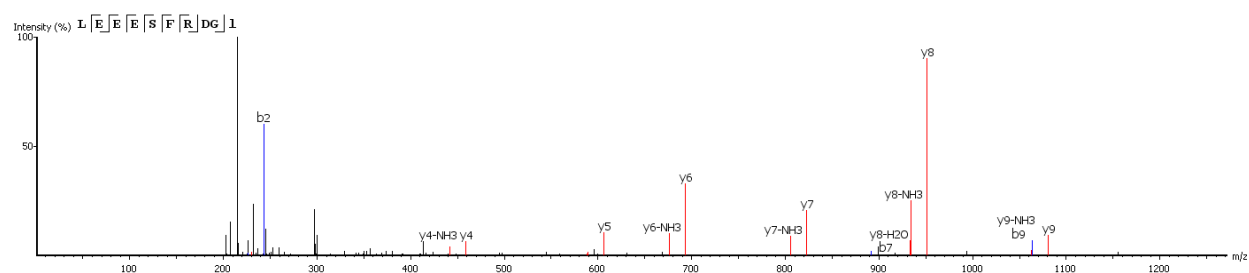


Figure S6. MS/MS spectrum of a putative AST-A neuropeptide with the sequence LEEESFRDGLamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

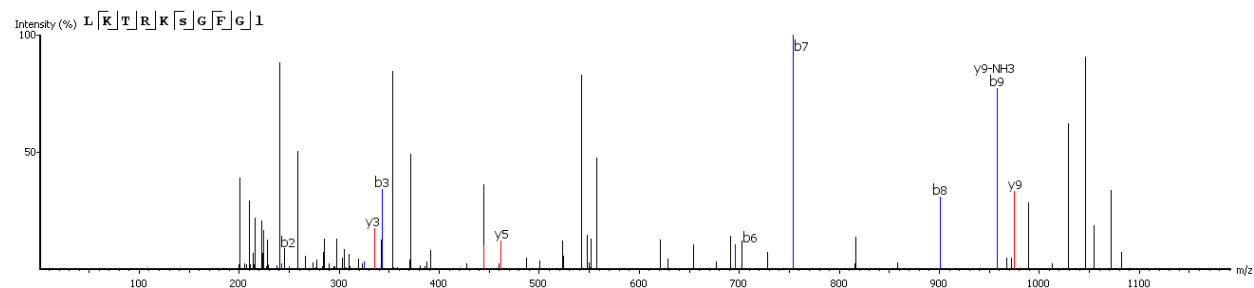


Figure S7. MS/MS spectrum of a putative AST-A neuropeptide with the sequence LKTRKS(-18.01)GFGlamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

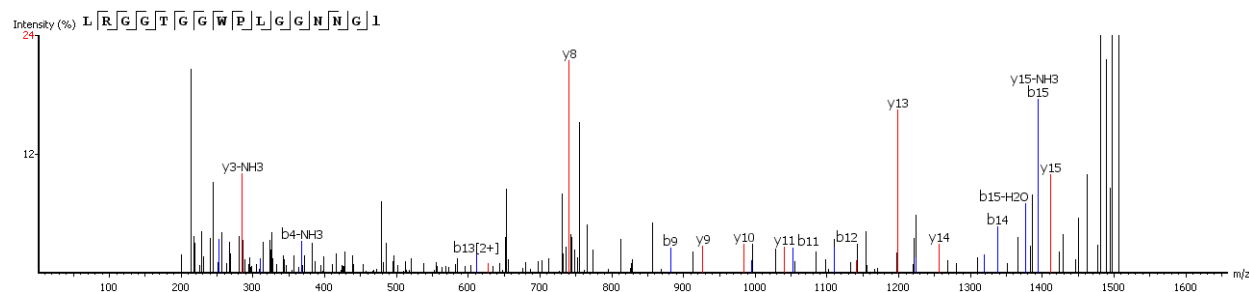


Figure S8. MS/MS spectrum of a putative AST-A neuropeptide with the sequence LRGGTGGWPLGGNNGLamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

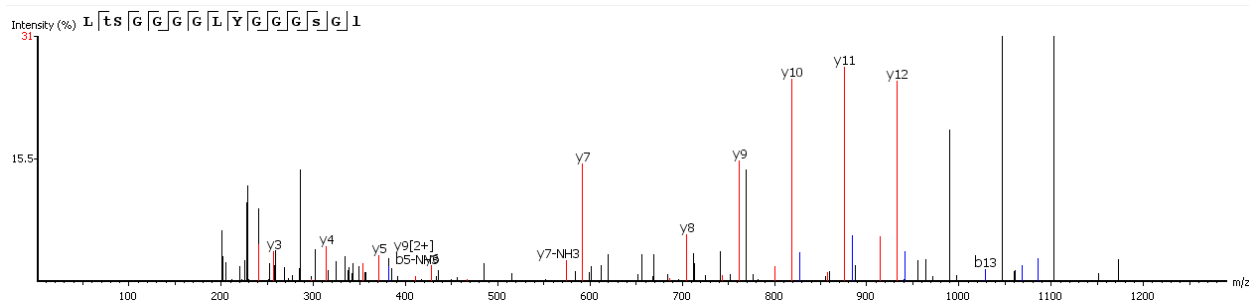


Figure S9. MS/MS spectrum of a putative AST-A neuropeptide with the sequence LT(-18.01)SGGGGLYGGGS(-18.01)GLamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

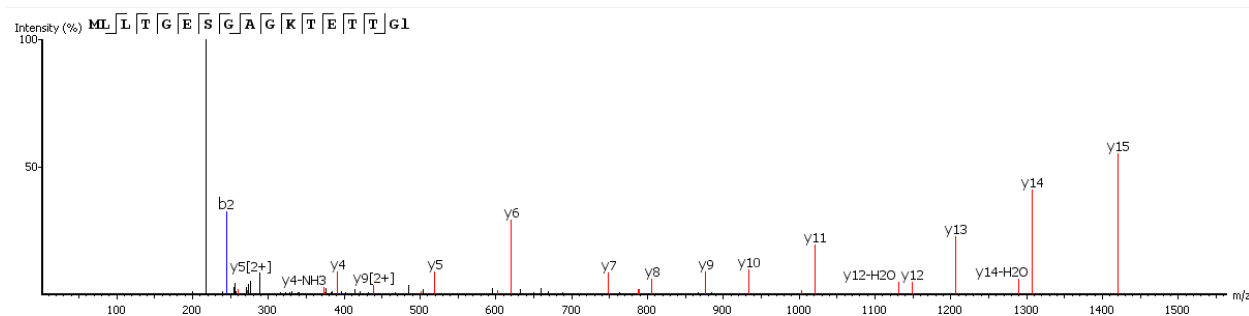


Figure S10. MS/MS spectrum of a putative AST-A neuropeptide with the sequence MLLTGESGAGKTETTGLamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

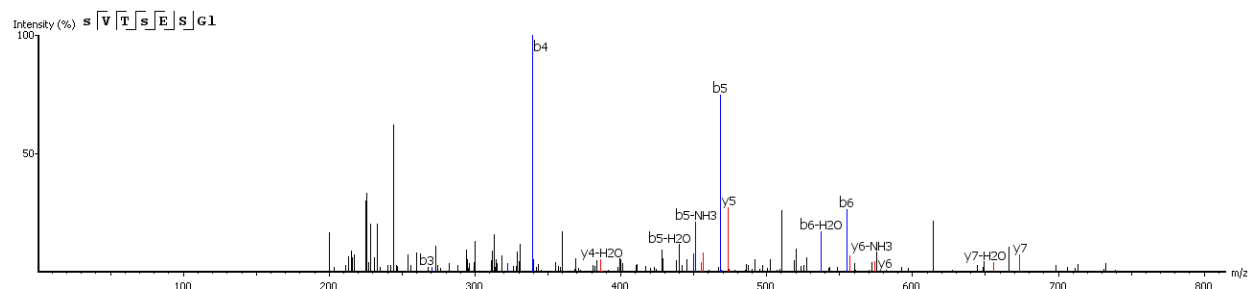


Figure S11. MS/MS spectrum of a putative AST-A neuropeptide with the sequence S(-18.01)VT(-18.01)ESGLamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

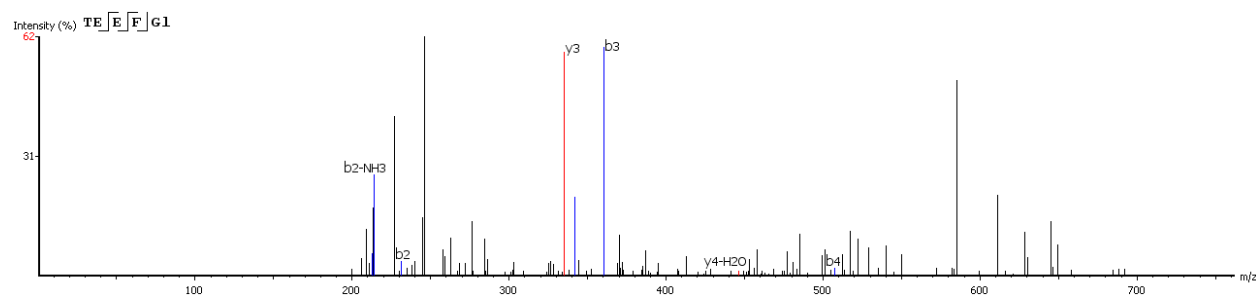


Figure S12. MS/MS spectrum of a putative AST-A neuropeptide with the sequence TEEFGLamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

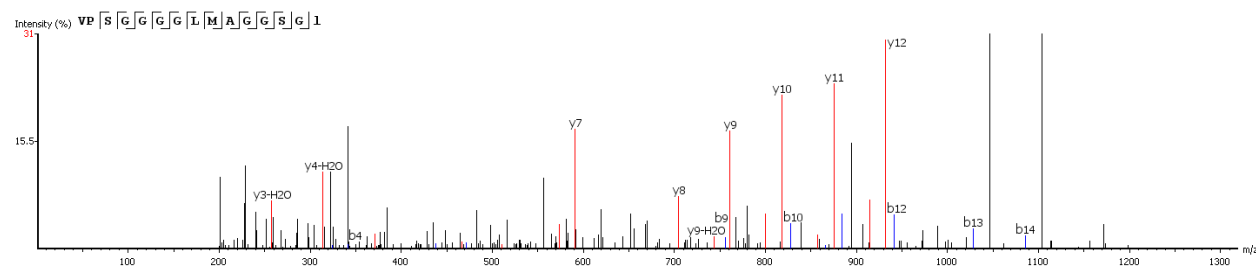


Figure S13. MS/MS spectrum of a putative AST-A neuropeptide with the sequence VPSGGGGLMAGGSGLa₁ detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

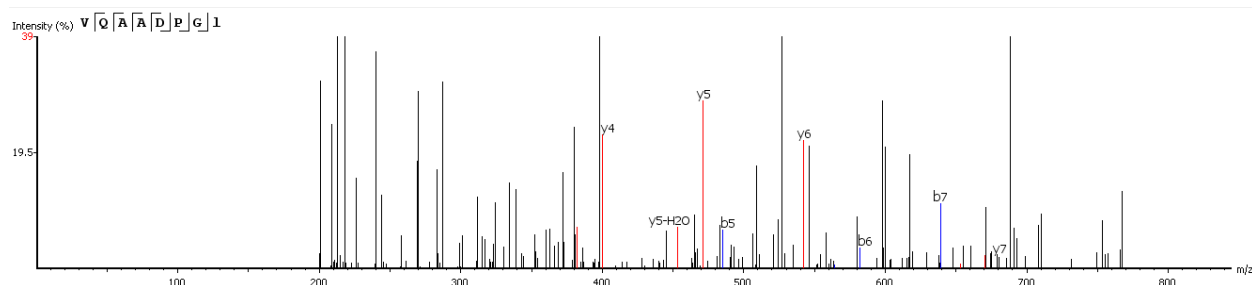


Figure S14. MS/MS spectrum of a putative AST-A neuropeptide with the sequence VQAADPGLamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

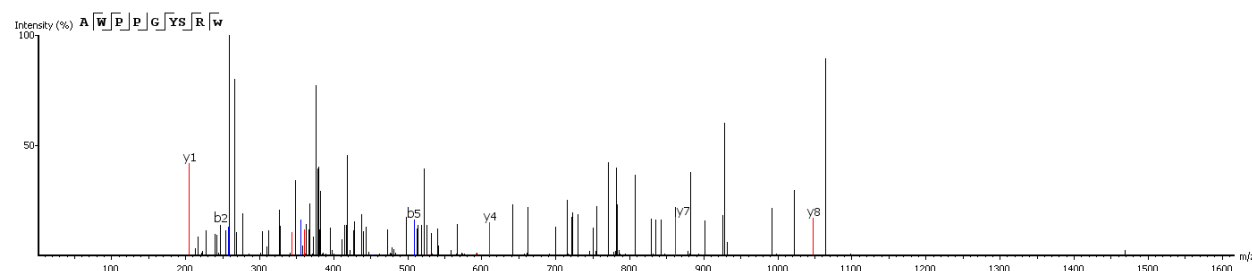


Figure S15. MS/MS spectrum of a putative AST-B neuropeptide with the sequence AWPPGYSRWamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

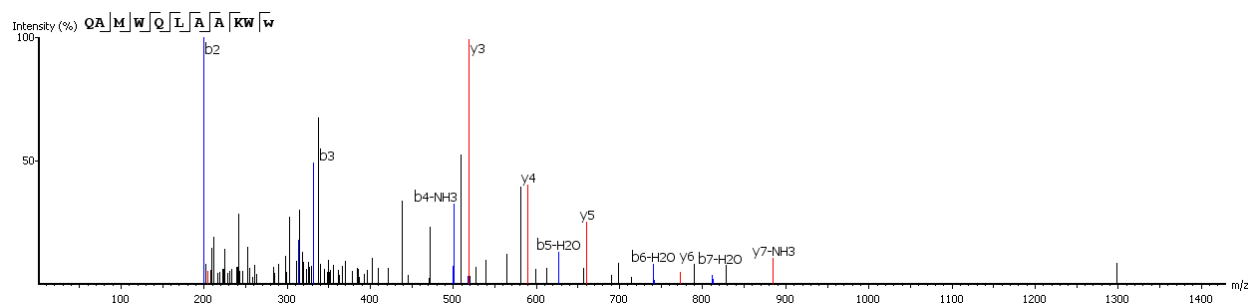


Figure S16. MS/MS spectrum of a putative AST-B neuropeptide with the sequence QAMWQLAAKWWamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

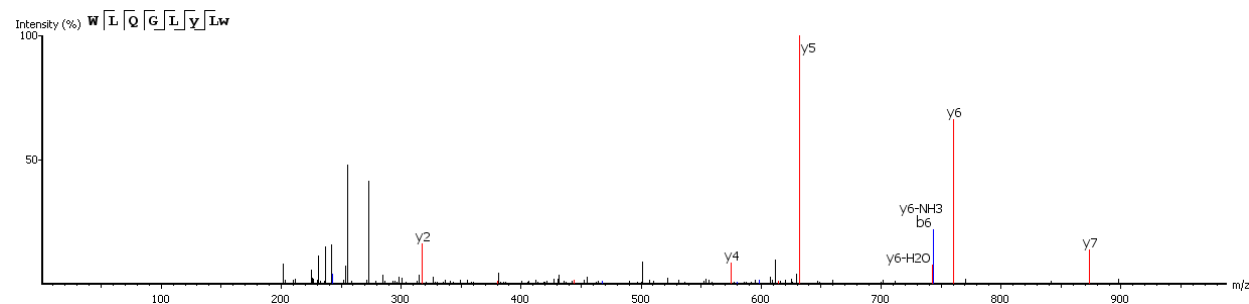


Figure S17. MS/MS spectrum of a putative AST-B neuropeptide with the sequence WLQGLY(-18.01)LWamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

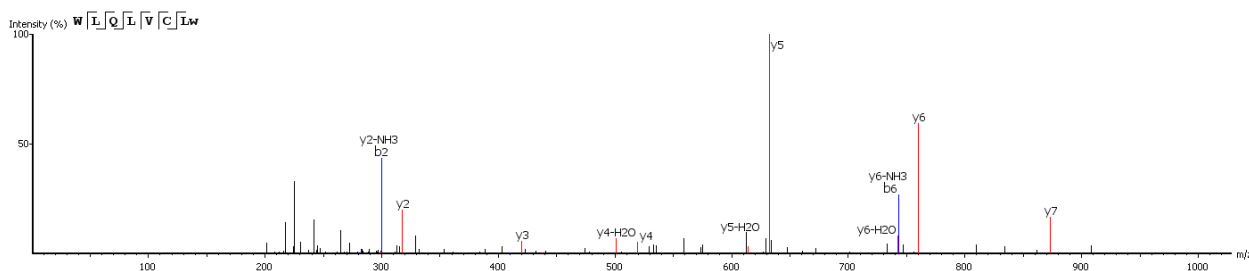


Figure S18. MS/MS spectrum of a putative AST-B neuropeptide with the sequence WLQLVCLWamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

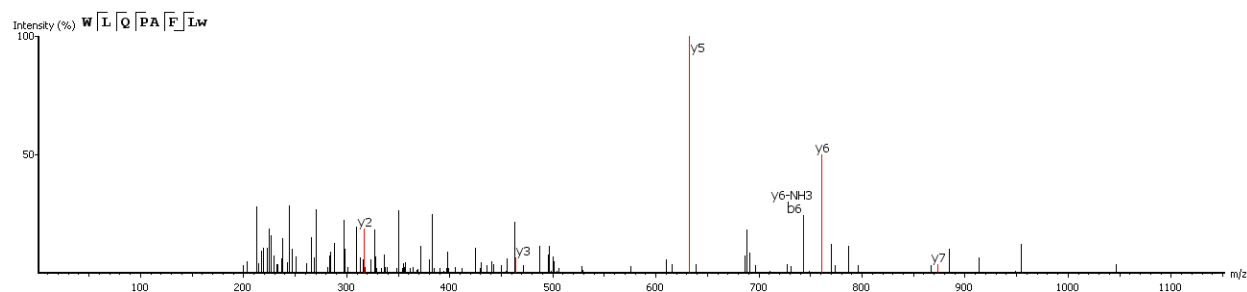


Figure S19. MS/MS spectrum of a putative AST-B neuropeptide with the sequence WLQPAFLWamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

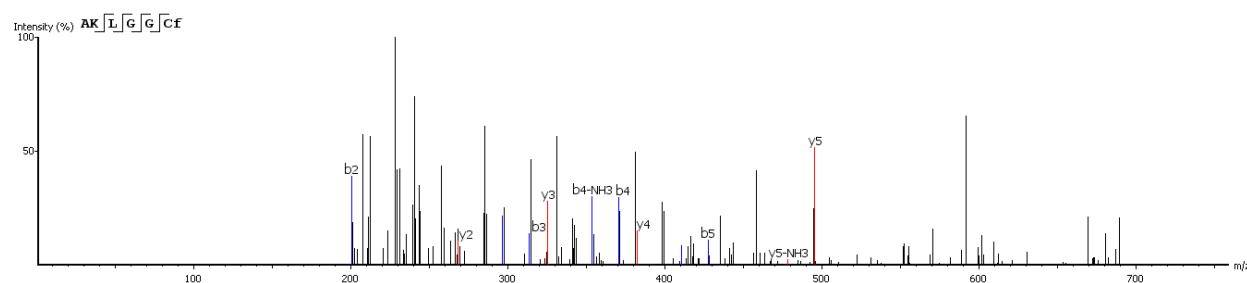


Figure S20. MS/MS spectrum of a putative AST-C neuropeptide with the sequence AKLGGCfamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

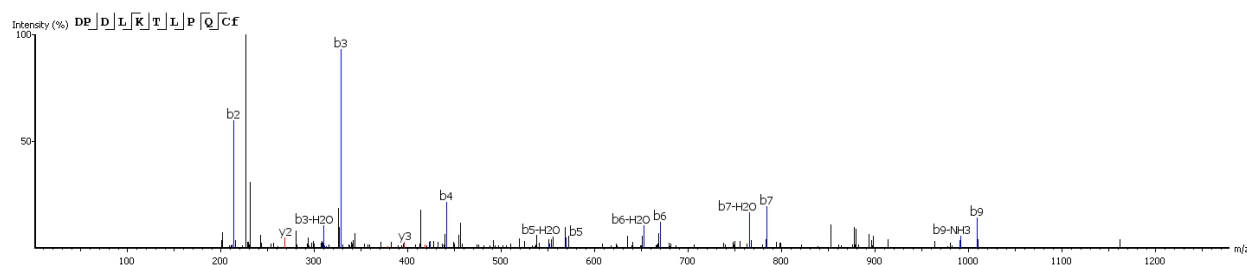


Figure S21. MS/MS spectrum of a putative AST-C neuropeptide with the sequence DPDLKTLPQCFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

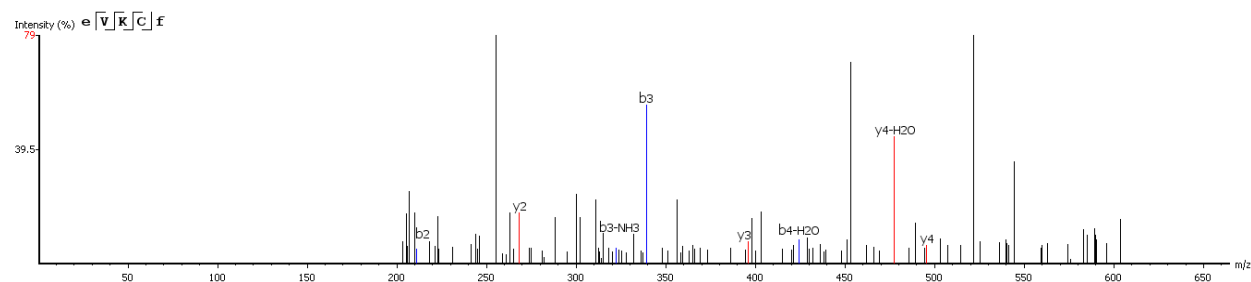


Figure S22. MS/MS spectrum of a putative AST-C neuropeptide with the sequence E(-18.01)VKCFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

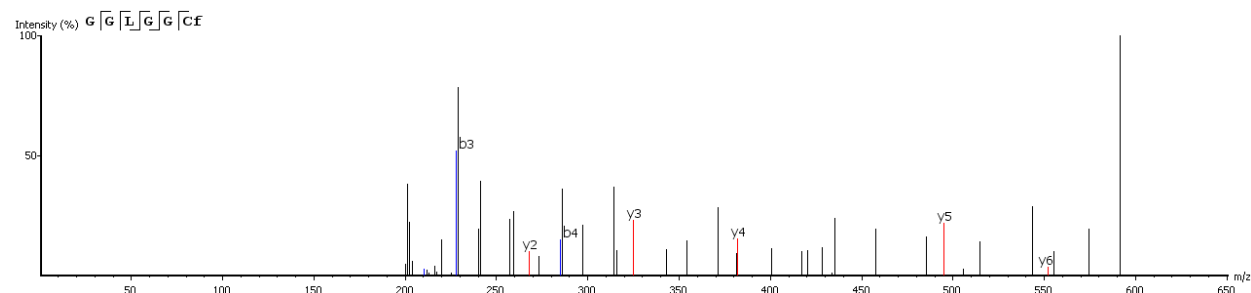


Figure S23. MS/MS spectrum of a putative AST-C neuropeptide with the sequence GGLGGCFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

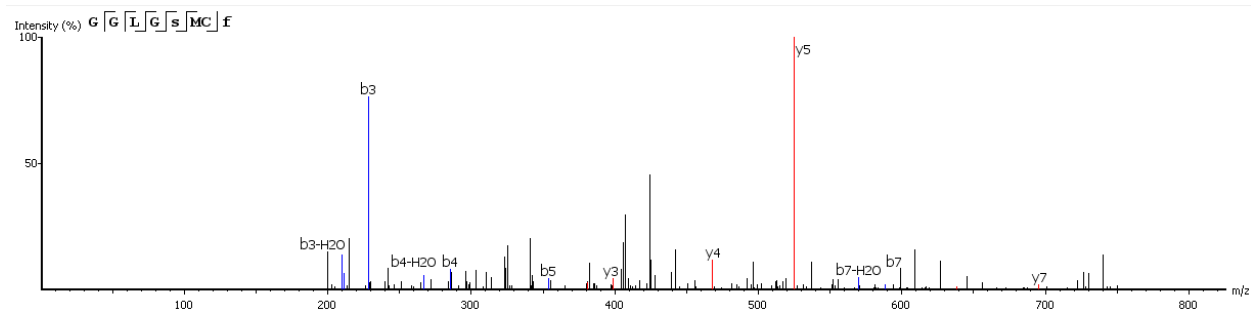


Figure S24. MS/MS spectrum of a putative AST-C neuropeptide with the sequence GGLGS(-18.01)MCFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

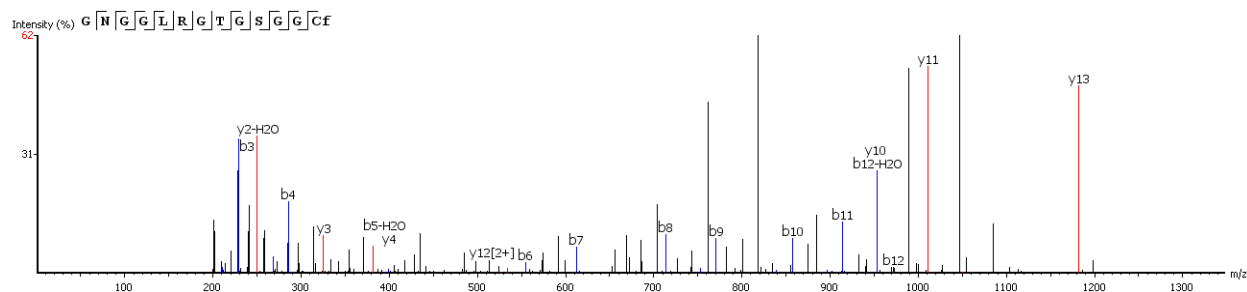


Figure S25. MS/MS spectrum of a putative AST-C neuropeptide with the sequence GNGGLRGTGSGGCFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

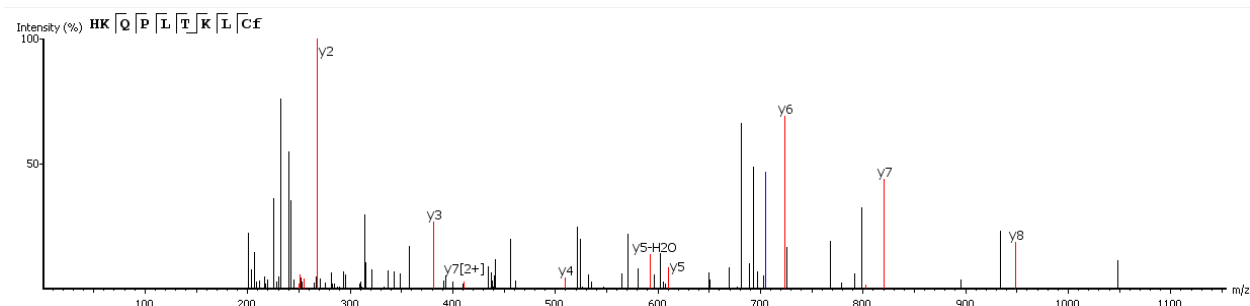


Figure S26. MS/MS spectrum of a putative AST-C neuropeptide with the sequence HKQPLTKLCFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

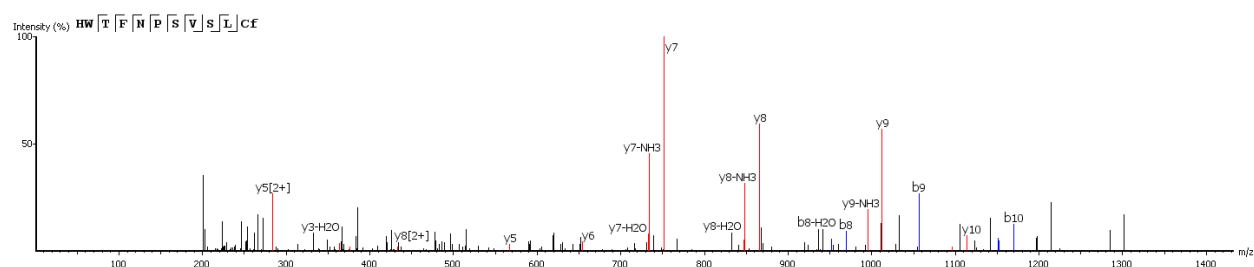


Figure S27. MS/MS spectrum of a putative AST-C neuropeptide with the sequence HWTFNPSVSLCFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

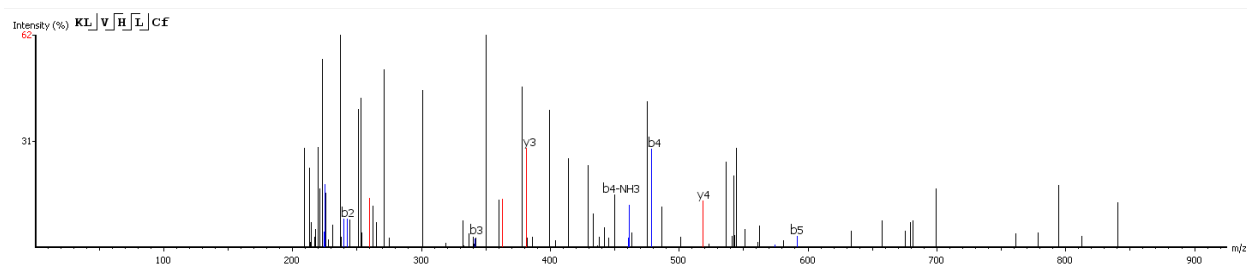


Figure S28. MS/MS spectrum of a putative AST-C neuropeptide with the sequence KLVHLCFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

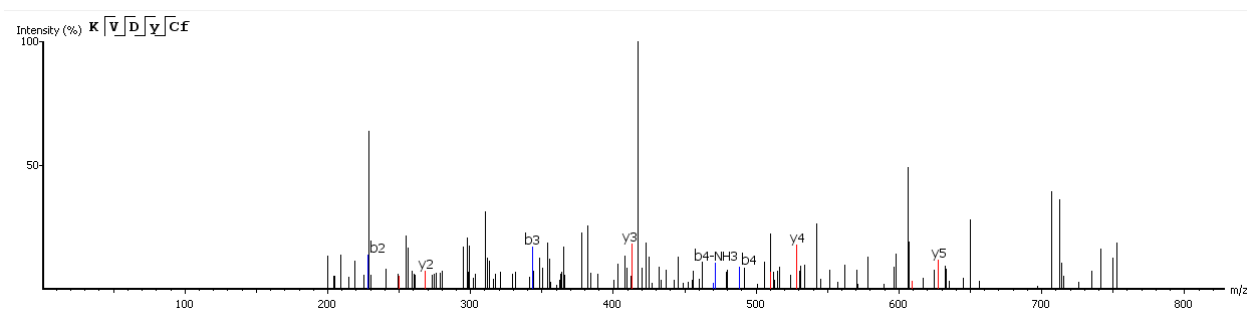


Figure S29. MS/MS spectrum of a putative AST-C neuropeptide with the sequence KVDY(-18.01)CFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

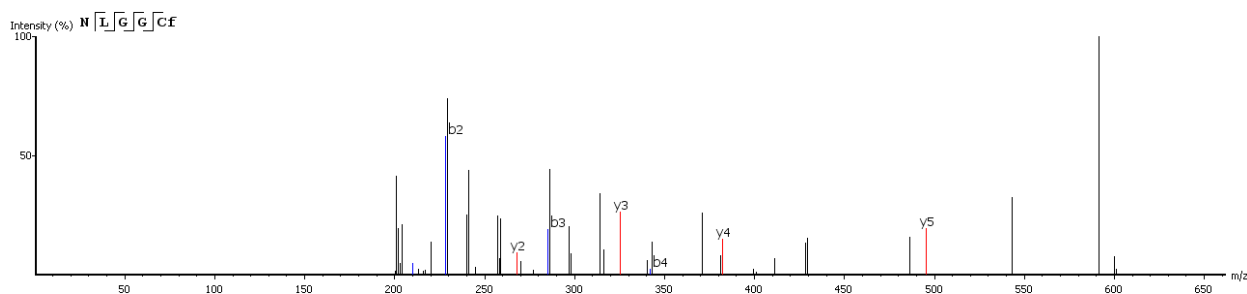


Figure S30. MS/MS spectrum of a putative AST-C neuropeptide with the sequence NLGGCFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

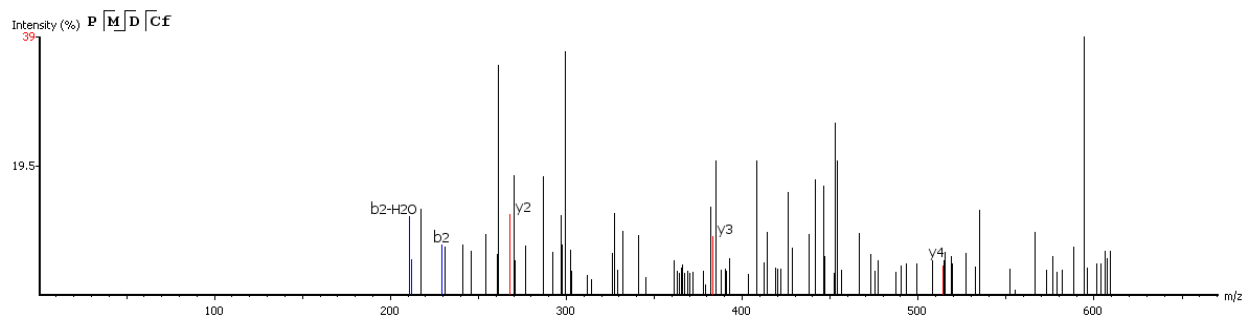


Figure S31. MS/MS spectrum of a putative AST-C neuropeptide with the sequence PMDCFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

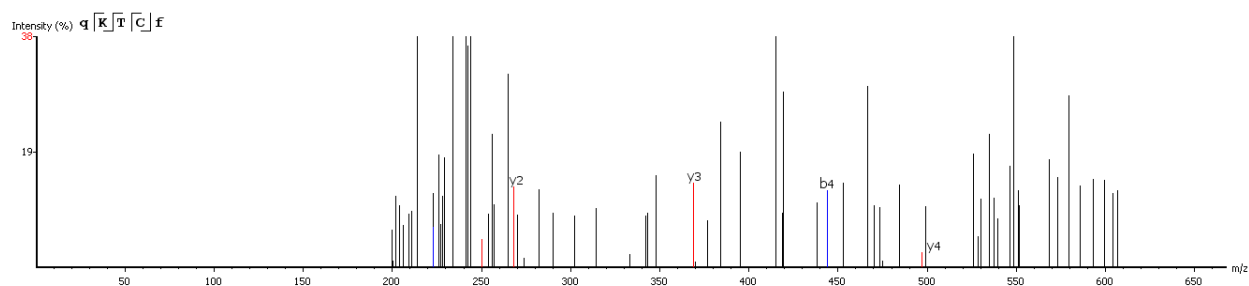


Figure S32. MS/MS spectrum of a putative AST-C neuropeptide with the sequence Q(-17.03)KTCFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

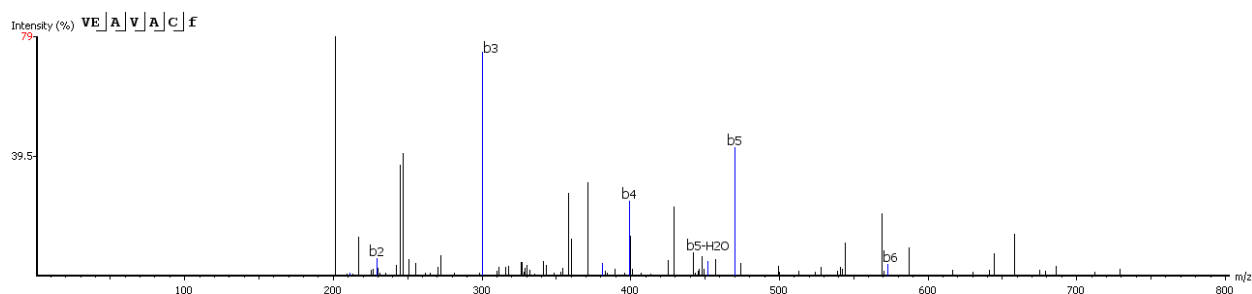


Figure S33. MS/MS spectrum of a putative AST-C neuropeptide with the sequence VEAVACFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

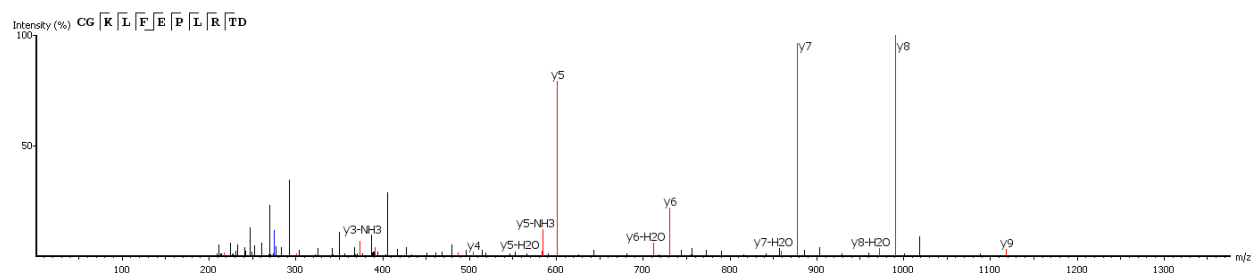


Figure S34. MS/MS spectrum of a putative cryptocyanin like neuropeptide with the sequence CGKLFELRES detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

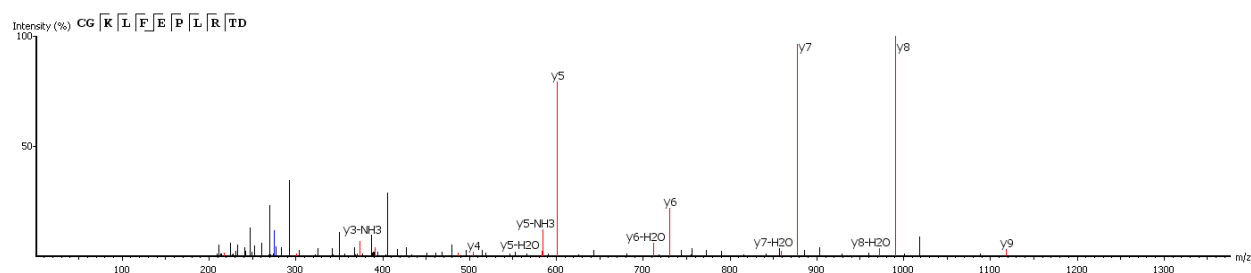


Figure S35. MS/MS spectrum of a putative cryptocyanin like neuropeptide with the sequence CGKLFELRTD detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

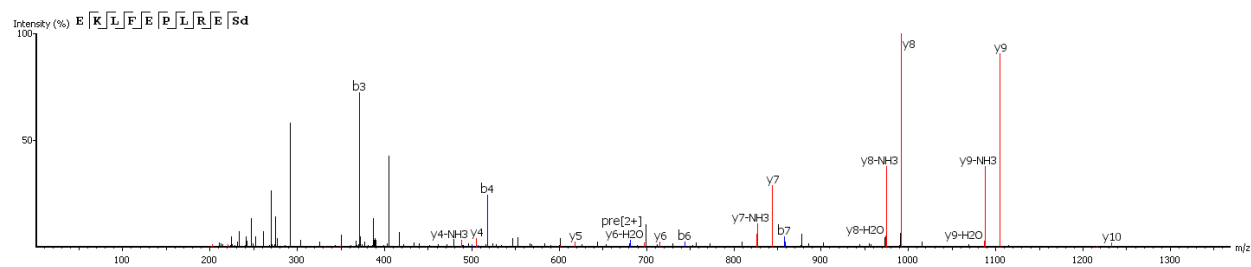


Figure S36. MS/MS spectrum of a putative cryptocyanin like neuropeptide with the sequence EKLFEPLRESDamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

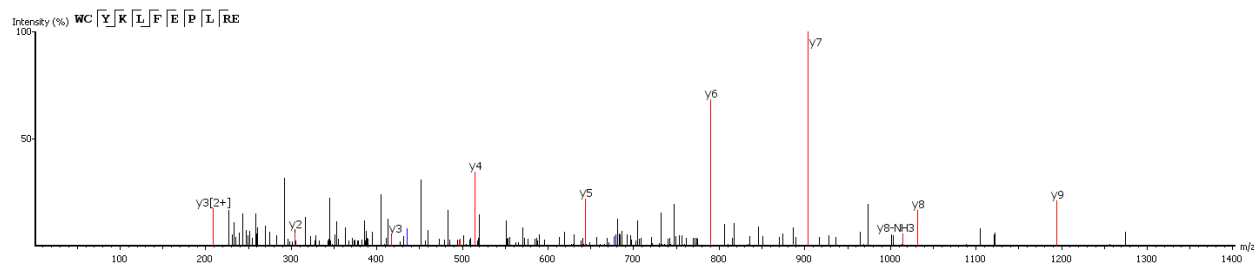


Figure S37. MS/MS spectrum of a putative cryptocyanin like neuropeptide with the sequence WCYKLFEPRE detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

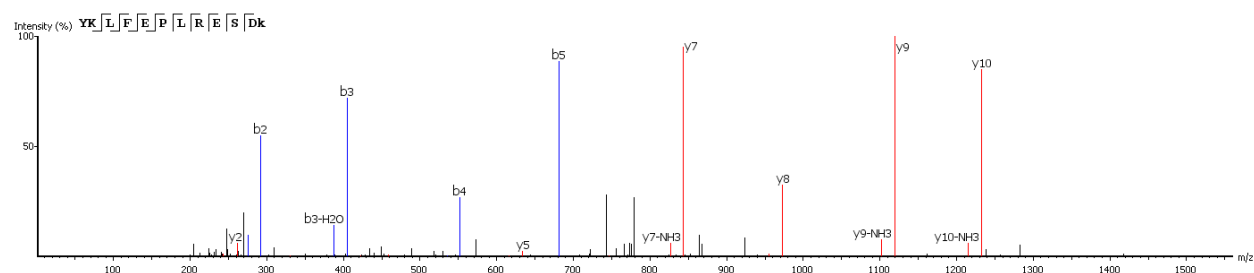


Figure S38. MS/MS spectrum of a putative cryptocyanin like neuropeptide with the sequence YKLFEPRESDKamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

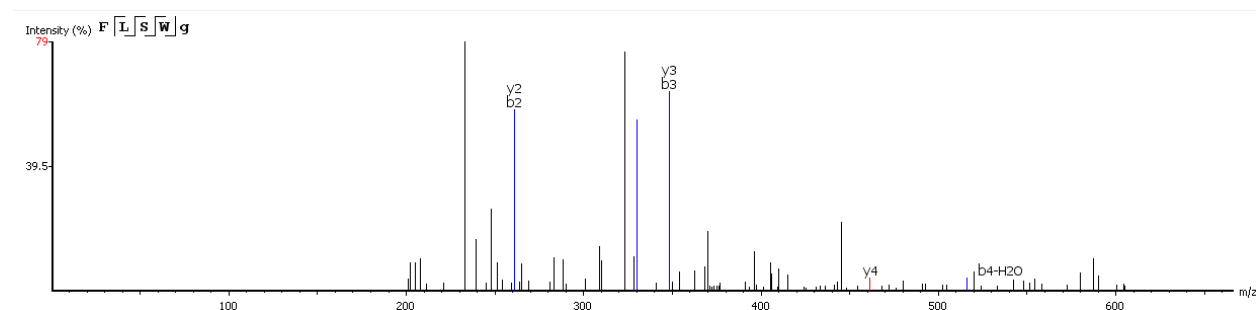


Figure S39. MS/MS spectrum of a putative insect kinin like neuropeptide with the sequence FLSWGamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

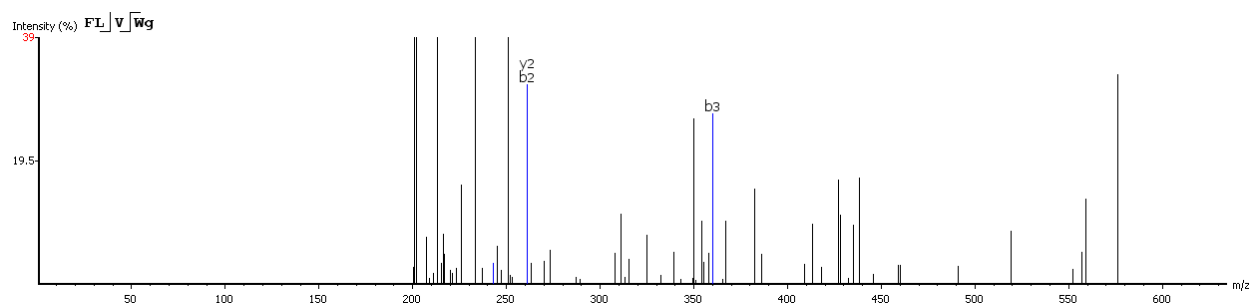


Figure S40. MS/MS spectrum of a putative cryptocyanin like neuropeptide with the sequence FLVWGamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

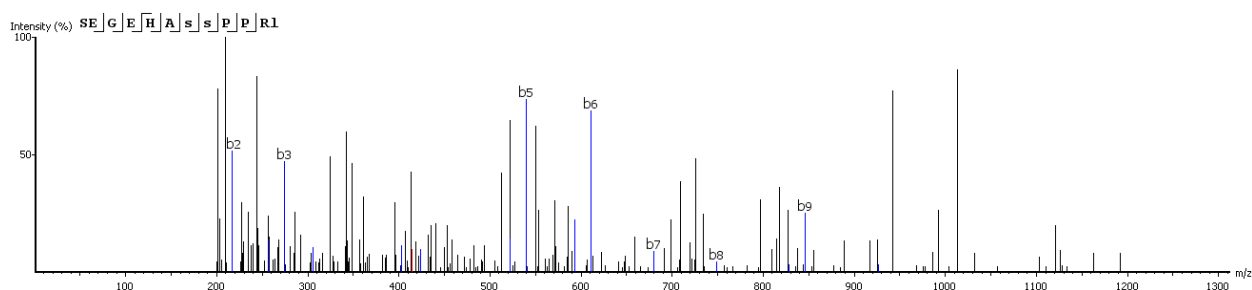


Figure S41. MS/MS spectrum of a putative pyrokinin like neuropeptide with the sequence SEGEHAS(-18.01)S(-18.01)PPRLamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

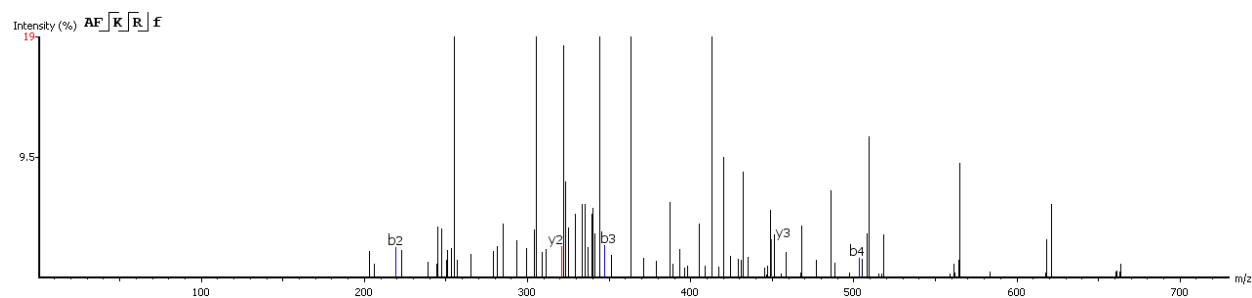


Figure S42. MS/MS spectrum of a putative RFamide neuropeptide with the sequence AFKRFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

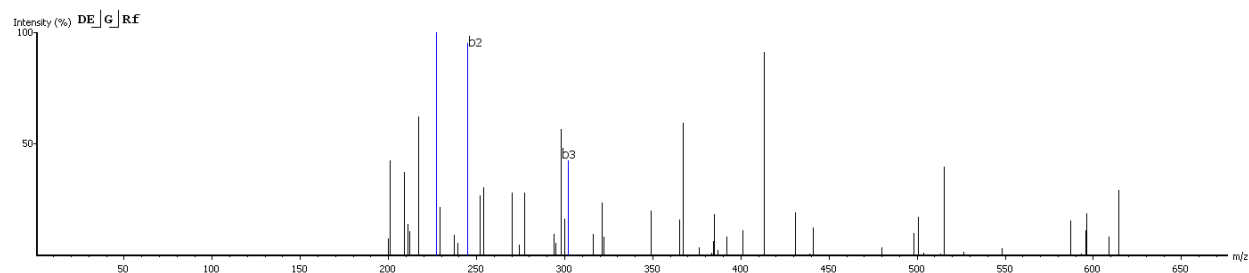


Figure S43. MS/MS spectrum of a putative RFamide neuropeptide with the sequence DEGRFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

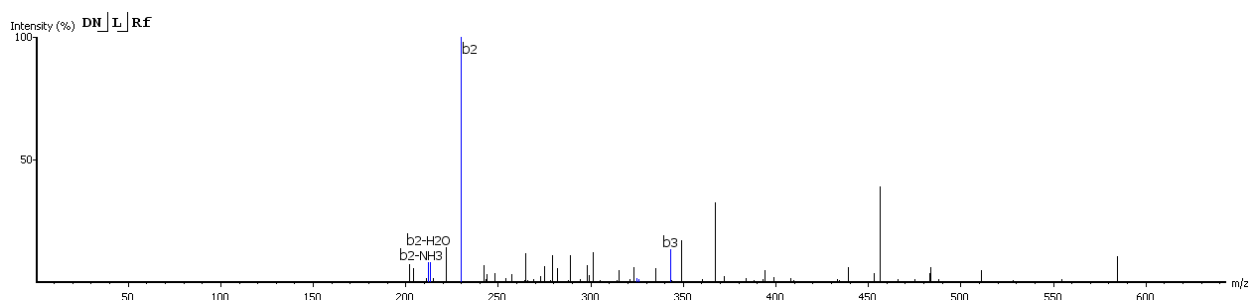


Figure S44. MS/MS spectrum of a putative RFamide neuropeptide with the sequence DNLRamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

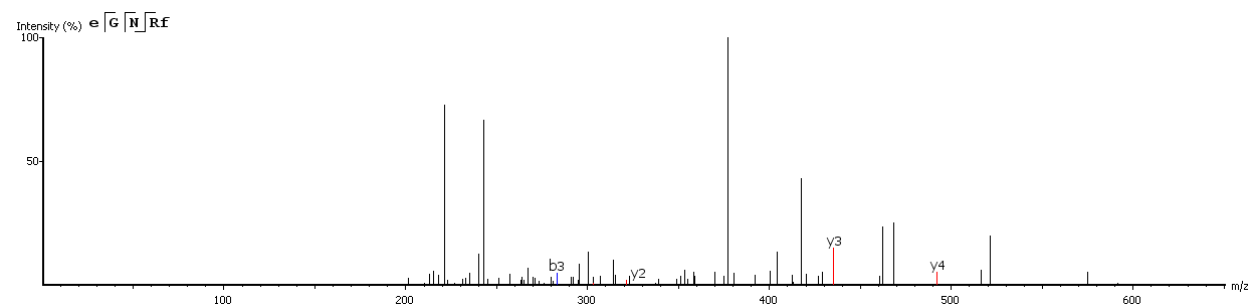


Figure S45. MS/MS spectrum of a putative RFamide neuropeptide with the sequence E(-18.01)GNRFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

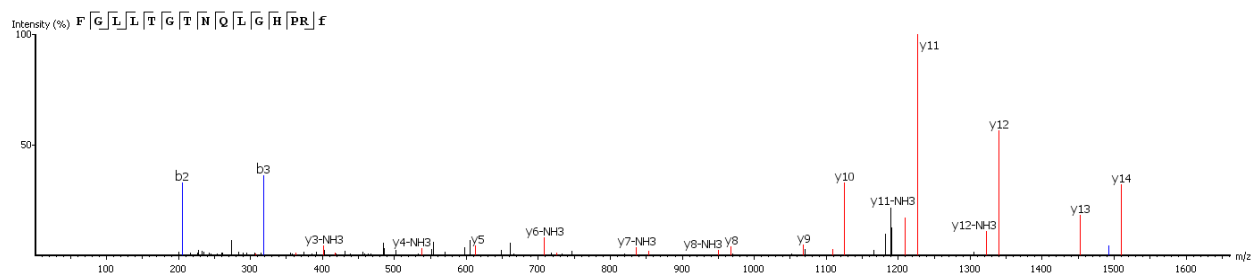


Figure S46. MS/MS spectrum of a putative RFamide neuropeptide with the sequence FGLLTGTNQLGHPRFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

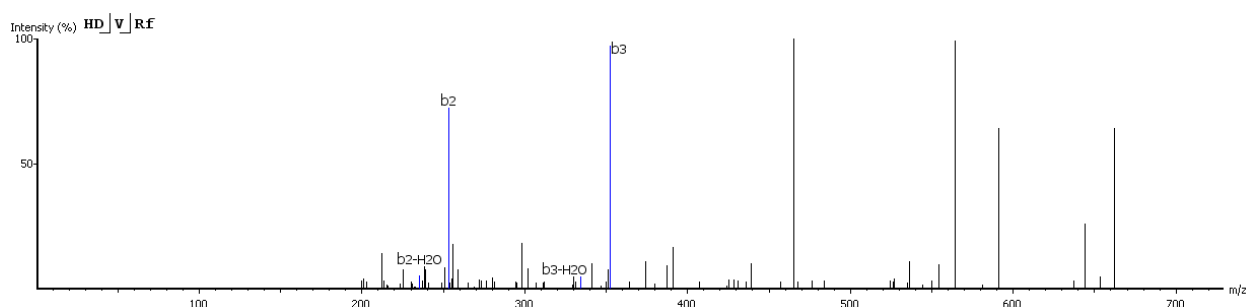


Figure S47. MS/MS spectrum of a putative RFamide neuropeptide with the sequence HDVRFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

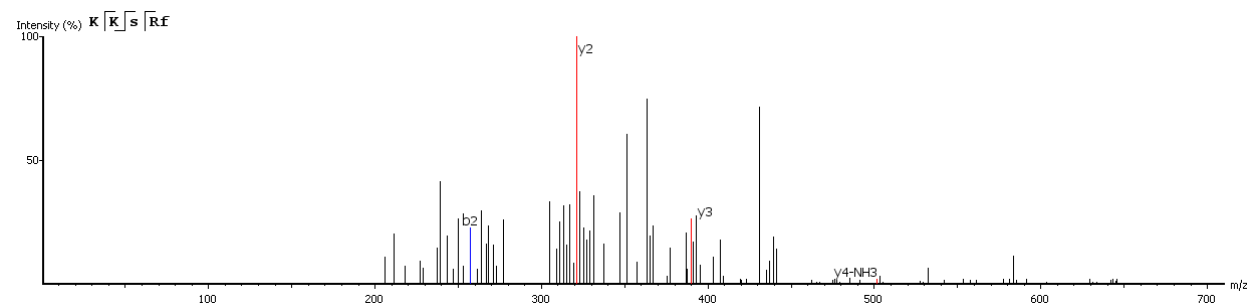


Figure S48. MS/MS spectrum of a putative RFamide neuropeptide with the sequence KKS(-18.01)RFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

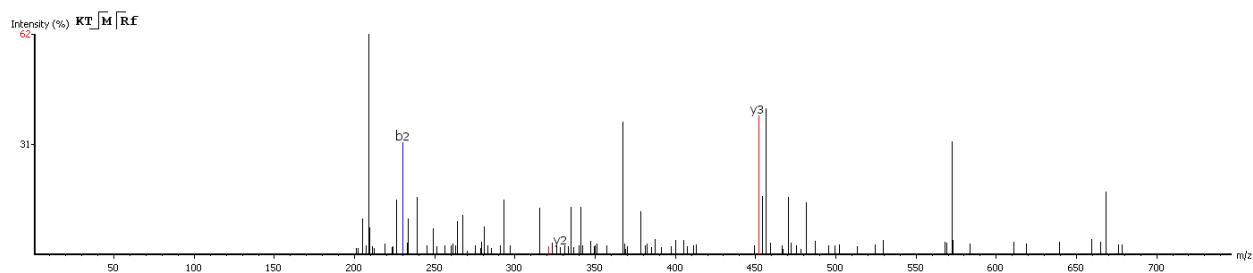


Figure S49. MS/MS spectrum of a putative RFamide neuropeptide with the sequence KTMRamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

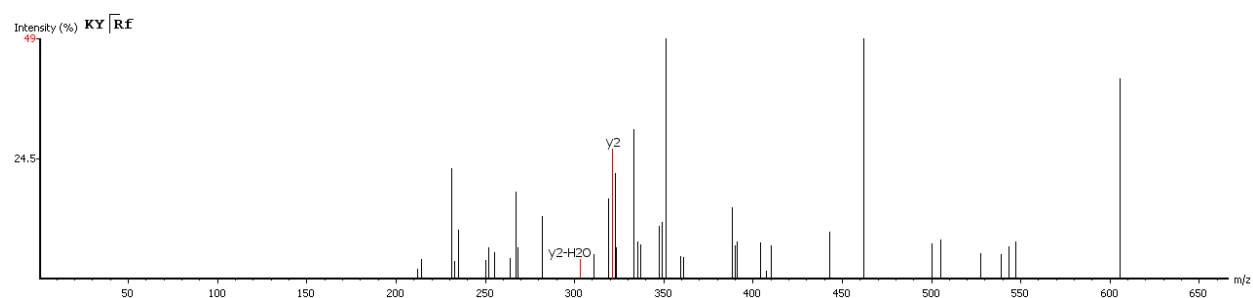


Figure S50. MS/MS spectrum of a putative RFamide neuropeptide with the sequence KYRamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

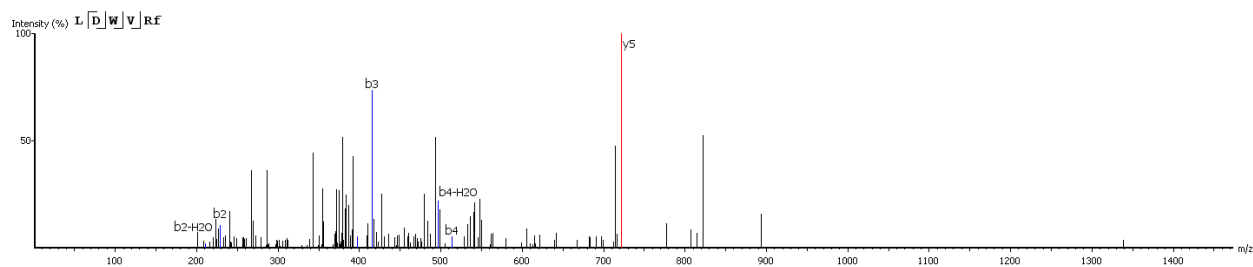


Figure S51. MS/MS spectrum of a putative RFamide neuropeptide with the sequence LDWVRamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

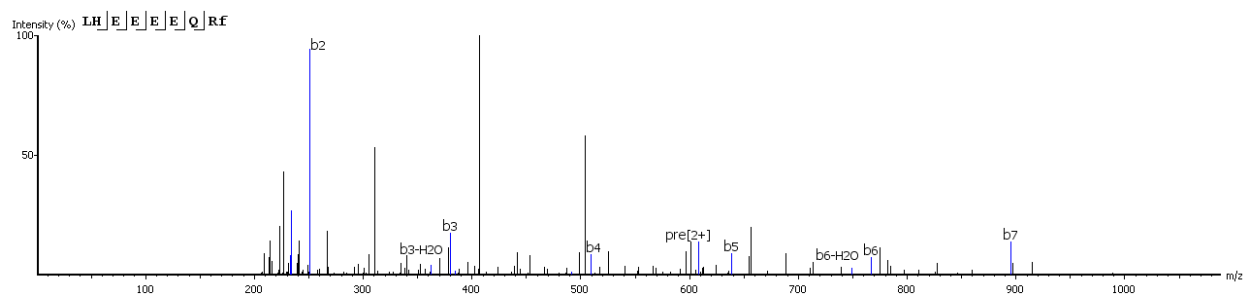


Figure S52. MS/MS spectrum of a putative RFamide neuropeptide with the sequence LHEEEQRfamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

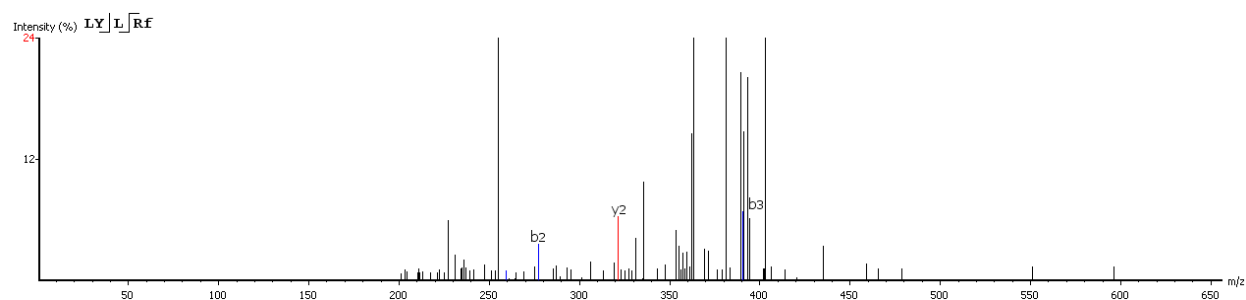


Figure S53. MS/MS spectrum of a putative RFamide neuropeptide with the sequence LYLRfamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

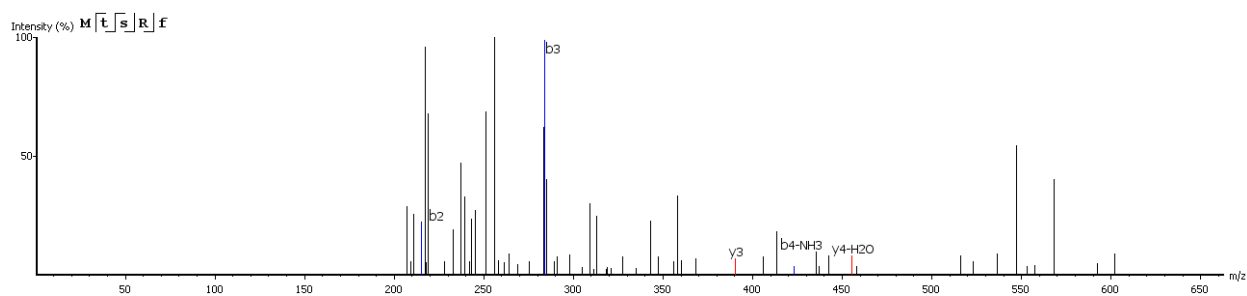


Figure S54. MS/MS spectrum of a putative RFamide neuropeptide with the sequence MT(-18.01)S(-18.01)Rfamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

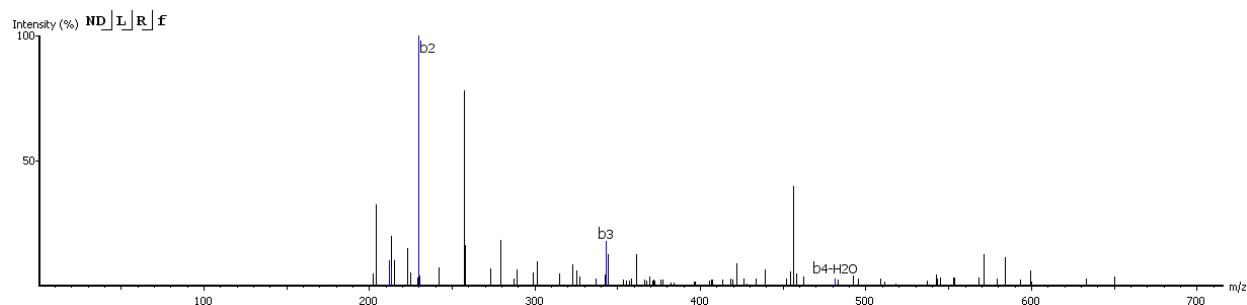


Figure S55. MS/MS spectrum of a putative RFamide neuropeptide with the sequence ND[L]Rfamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

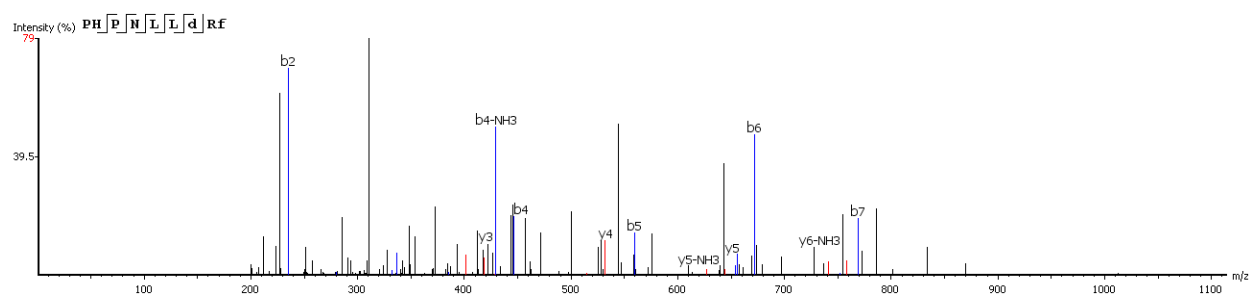


Figure S56. MS/MS spectrum of a putative RFamide neuropeptide with the sequence PHPNLLD(-18.01)Rfamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

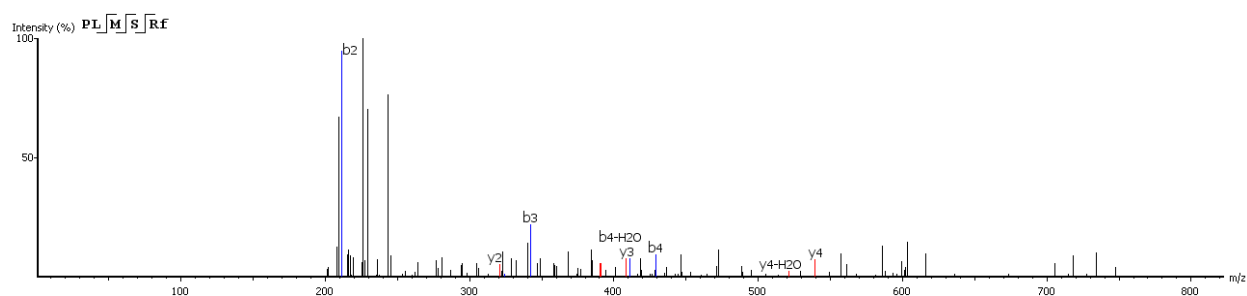


Figure S57. MS/MS spectrum of a putative RFamide neuropeptide with the sequence PLMSRfamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

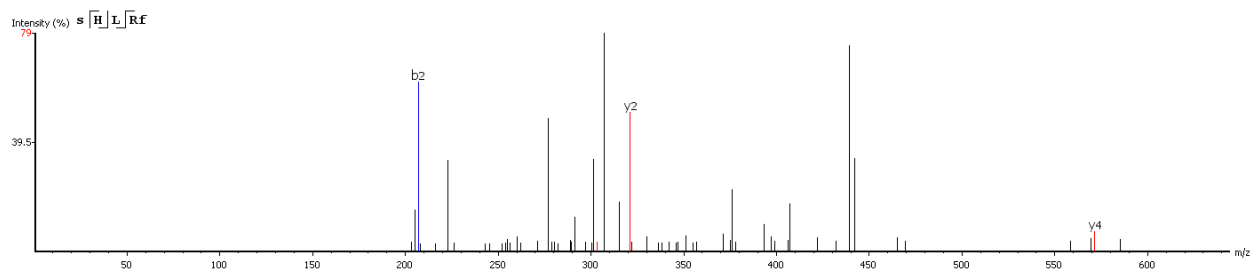


Figure S58. MS/MS spectrum of a putative RFamide neuropeptide with the sequence S(-18.01)HLRFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

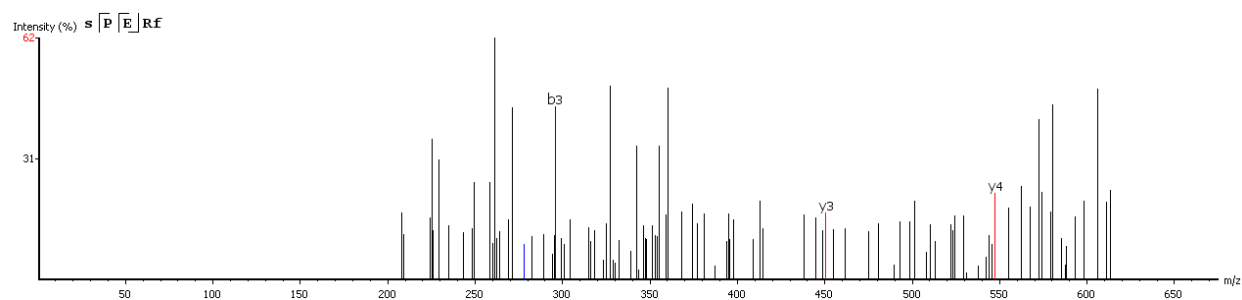


Figure S59. MS/MS spectrum of a putative RFamide neuropeptide with the sequence S(-18.01)PERFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

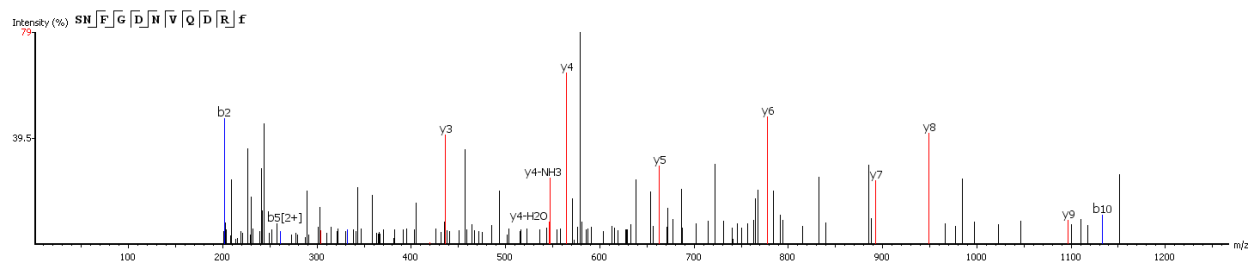


Figure S60. MS/MS spectrum of a putative RFamide neuropeptide with the sequence SNFGDENVQDRFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

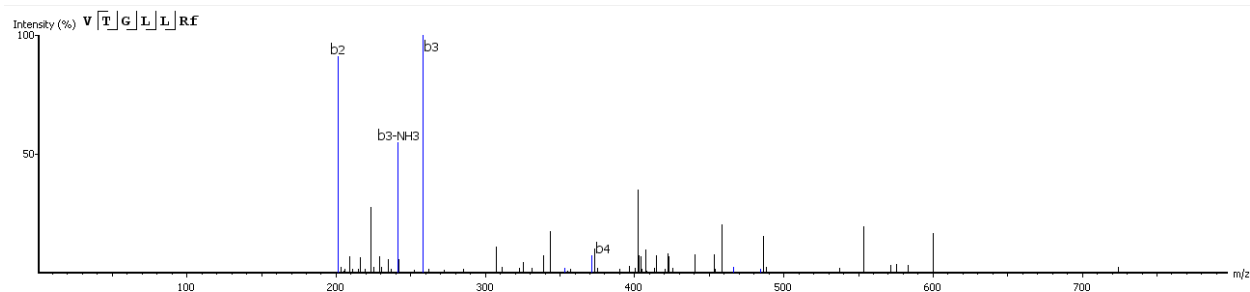


Figure S61. MS/MS spectrum of a putative RFamide neuropeptide with the sequence VTGLLRamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

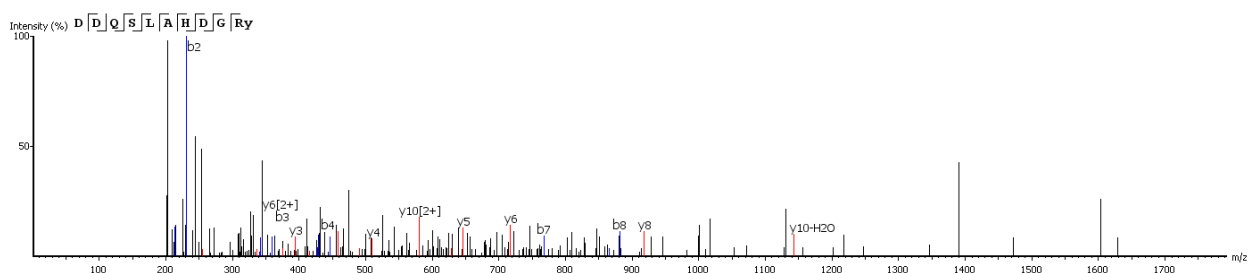


Figure S62. MS/MS spectrum of a putative RYamide neuropeptide with the sequence DDQSLAHDGRamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

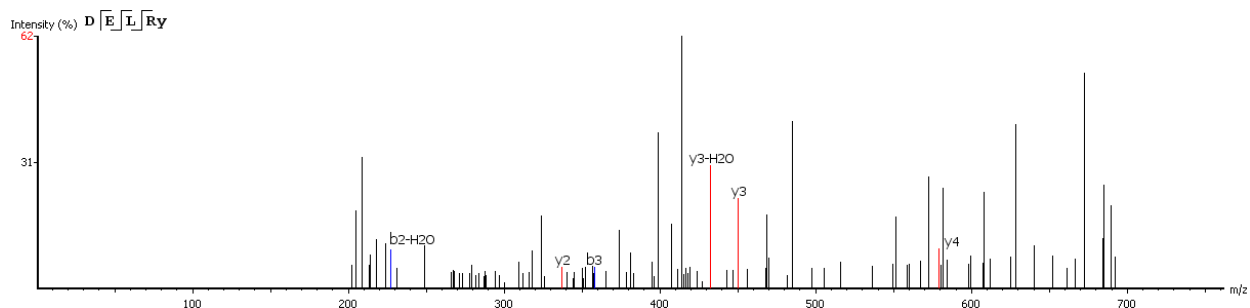


Figure S63. MS/MS spectrum of a putative RYamide neuropeptide with the sequence DELRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

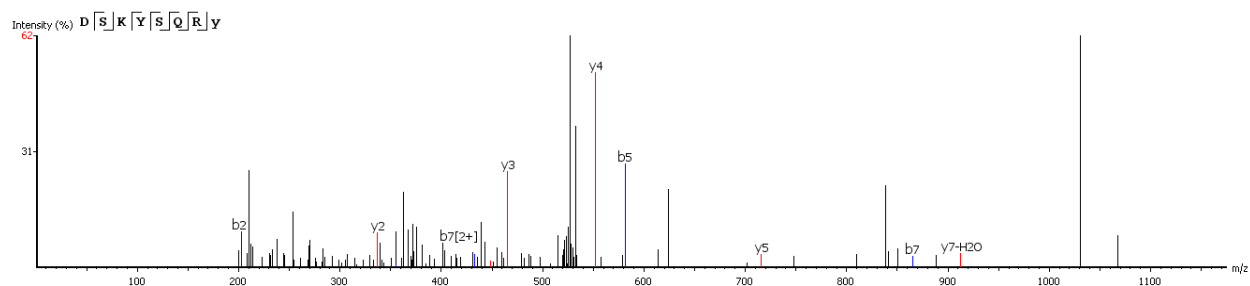


Figure S64. MS/MS spectrum of a putative RYamide neuropeptide with the sequence DSKYSQRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

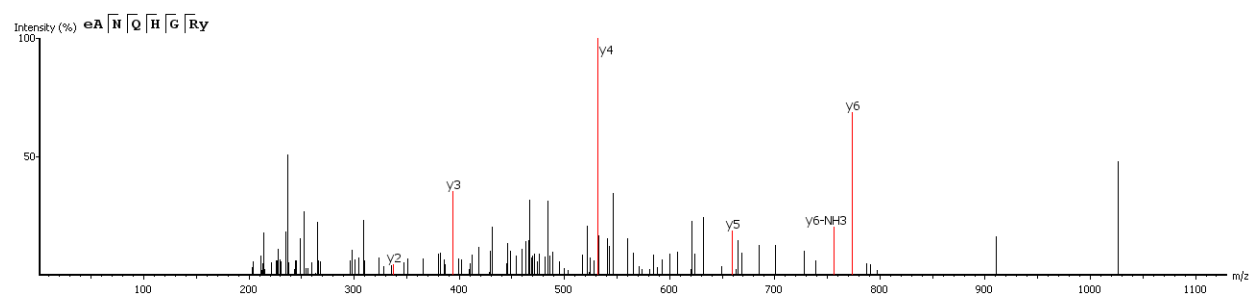


Figure S65. MS/MS spectrum of a putative RYamide neuropeptide with the sequence E(-18.01)ANQHGRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

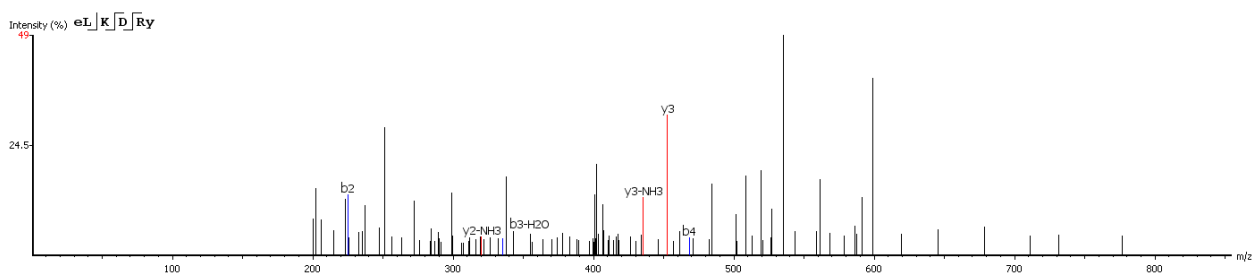


Figure S66. MS/MS spectrum of a putative RYamide neuropeptide with the sequence E(-18.01)LKDRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

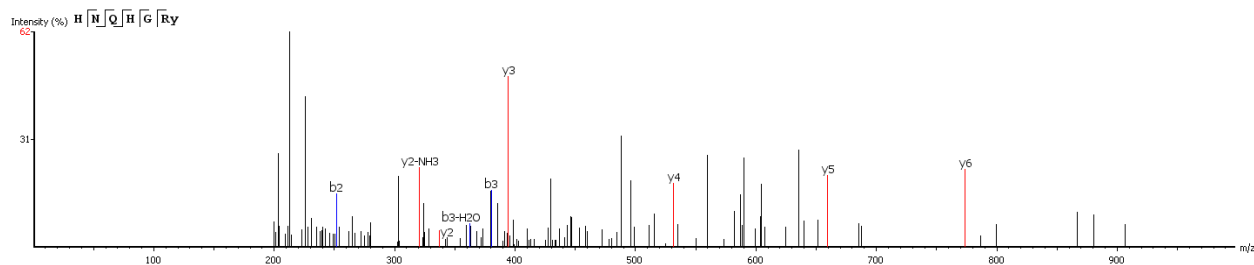


Figure S67. MS/MS spectrum of a putative RYamide neuropeptide with the sequence HNQHGRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

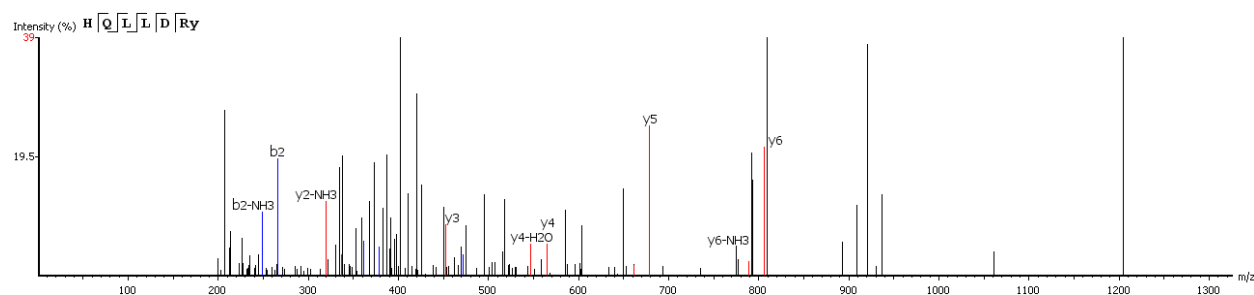


Figure S68. MS/MS spectrum of a putative RYamide neuropeptide with the sequence HQLLDTRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

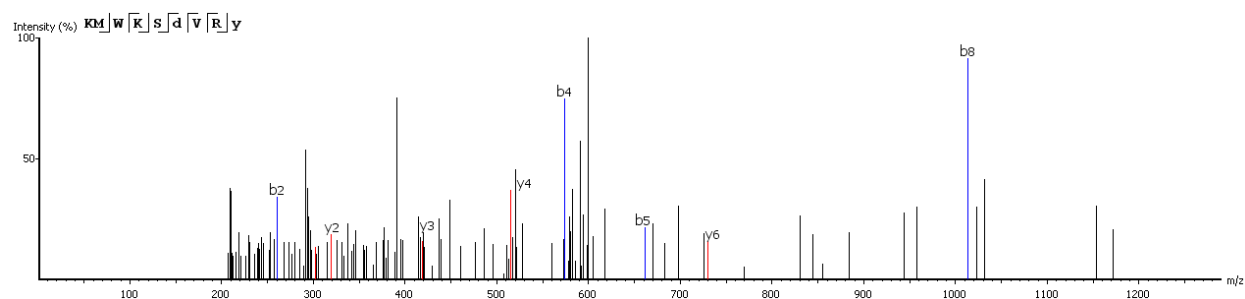


Figure S69. MS/MS spectrum of a putative RYamide neuropeptide with the sequence KMWKSD(-18.01)VRamide(-18.01) detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

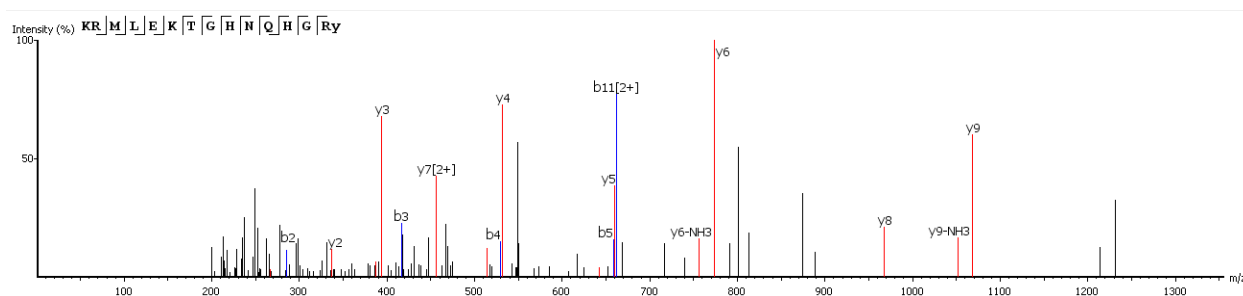


Figure S70. MS/MS spectrum of a putative RYamide neuropeptide with the sequence KRMLEKTGHNQHGRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

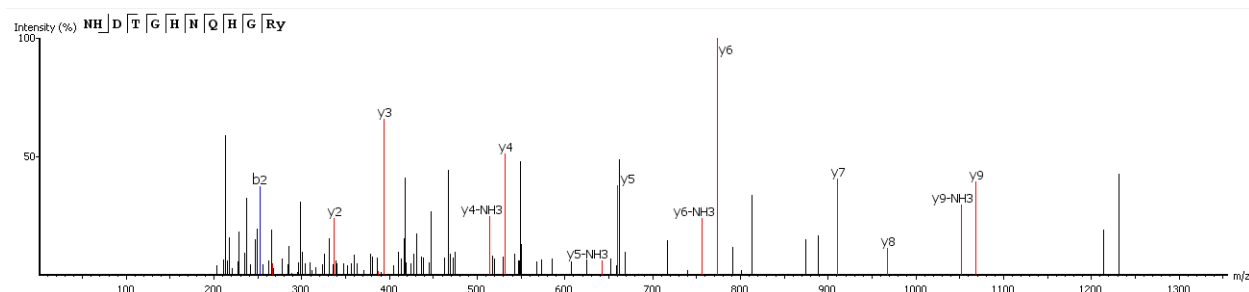


Figure S71. MS/MS spectrum of a putative RYamide neuropeptide with the sequence NHDTGHNQHGRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

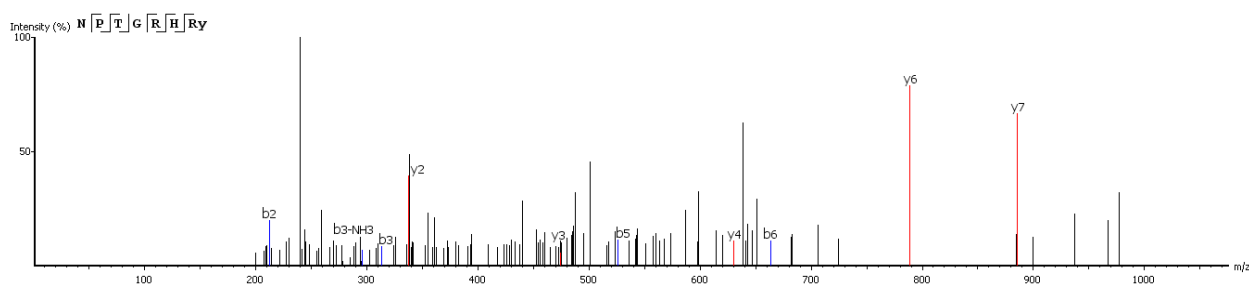


Figure S72. MS/MS spectrum of a putative RYamide neuropeptide with the sequence NPTGRHRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

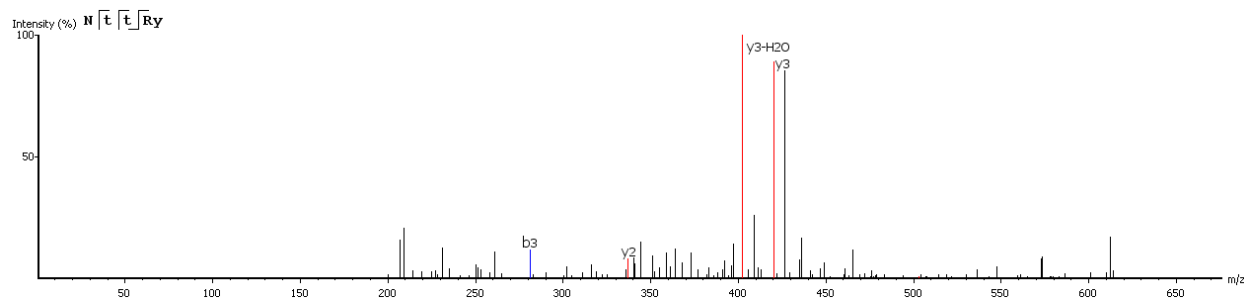


Figure S73. MS/MS spectrum of a putative RYamide neuropeptide with the sequence NT(-18.01)T(-18.01)RYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

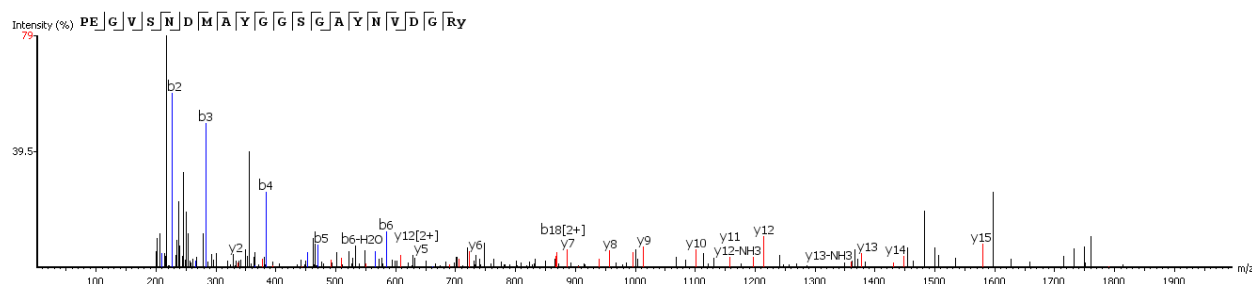


Figure S74. MS/MS spectrum of a putative RYamide neuropeptide with the sequence PEGVSNDMAYGGSGAYNV DGRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

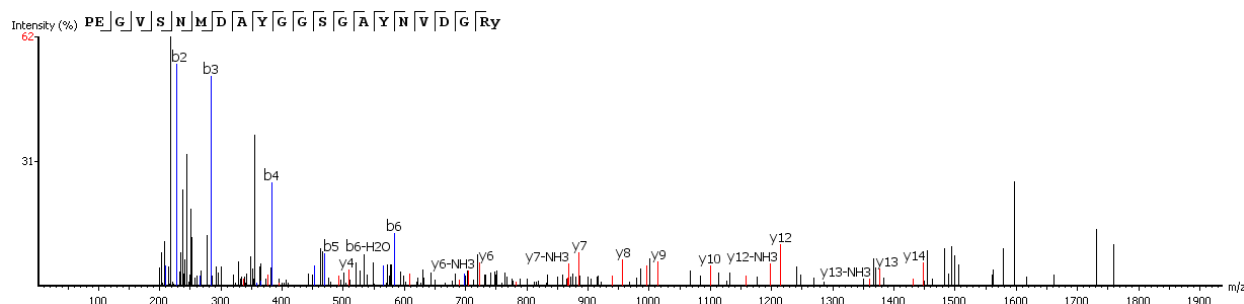


Figure S75. MS/MS spectrum of a putative RYamide neuropeptide with the sequence PEGVSNDMAYGGSGAYNV DGRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

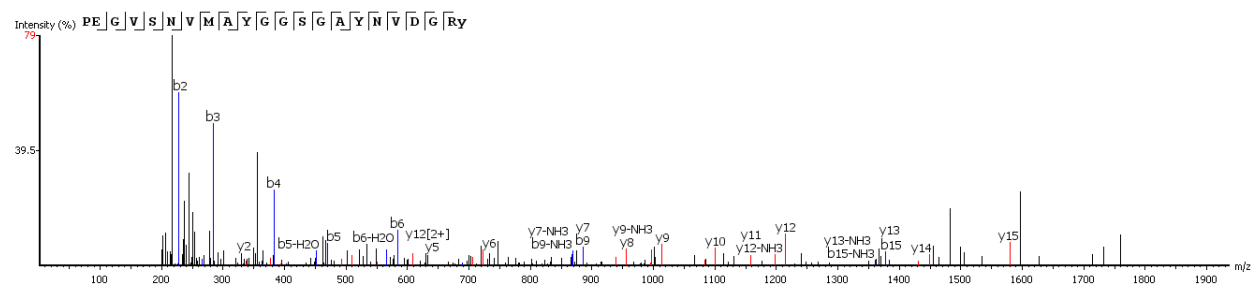


Figure S76. MS/MS spectrum of a putative RYamide neuropeptide with the sequence PEGVSNVMAYGGSGAYNVDGRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

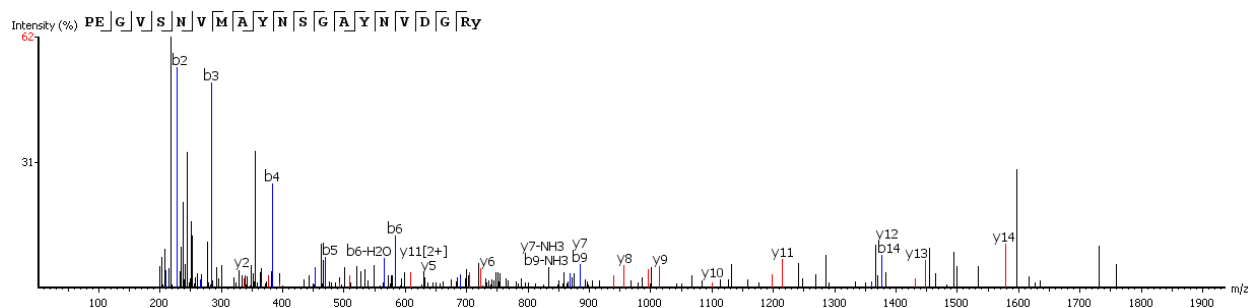


Figure S77. MS/MS spectrum of a putative RYamide neuropeptide with the sequence amide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

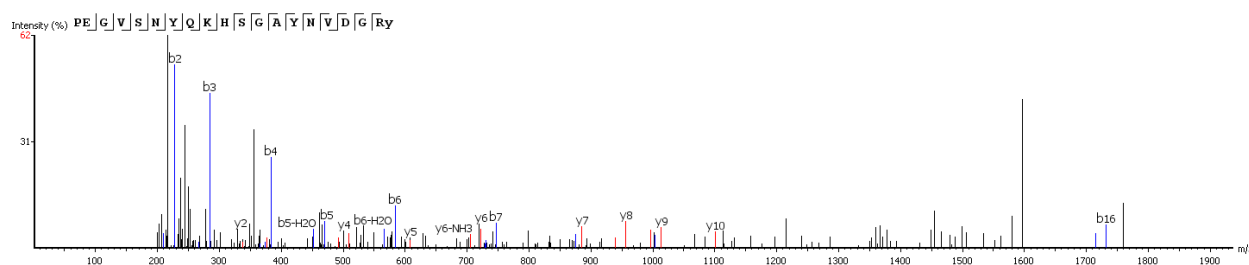


Figure S78. MS/MS spectrum of a putative RYamide neuropeptide with the sequence PEGVSNYQKHSGAYNVDGRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

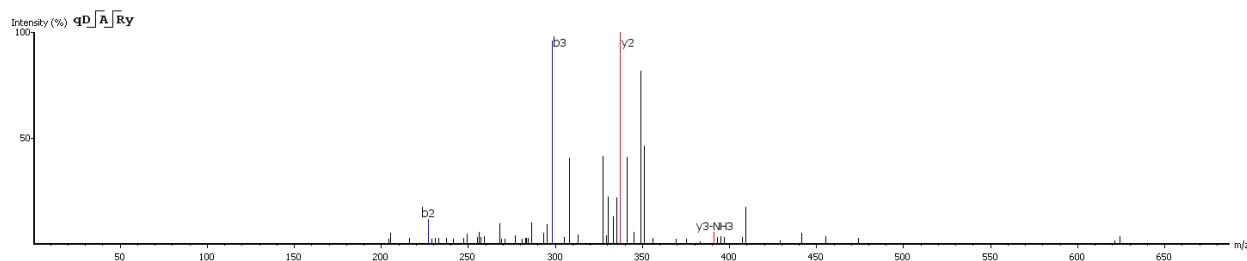


Figure S79. MS/MS spectrum of a putative RYamide neuropeptide with the sequence Q(-17.03)DARYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

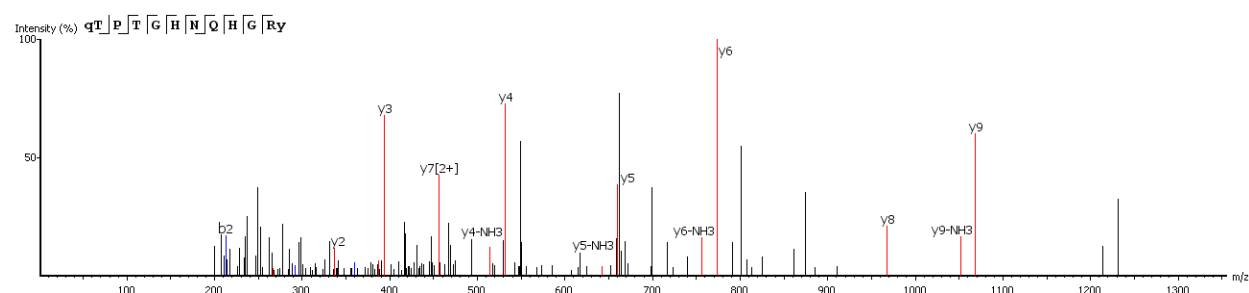


Figure S80. MS/MS spectrum of a putative RYamide neuropeptide with the sequence Q(-17.03)TPTGHNQHGRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

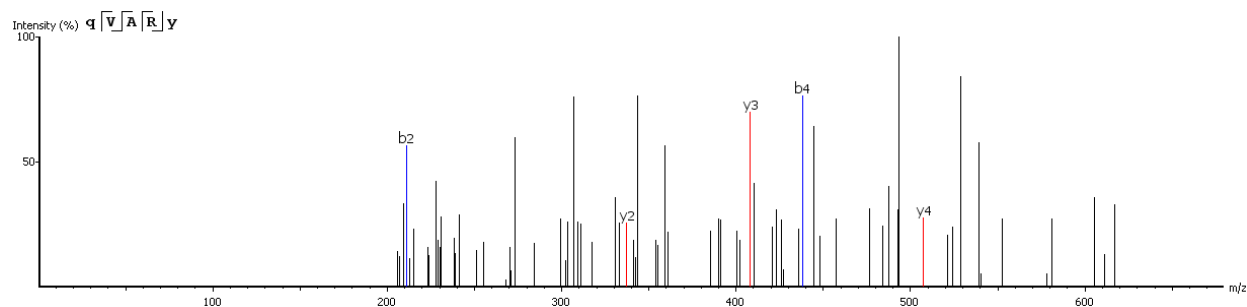


Figure S81. MS/MS spectrum of a putative RYamide neuropeptide with the sequence Q(-17.03)VARYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

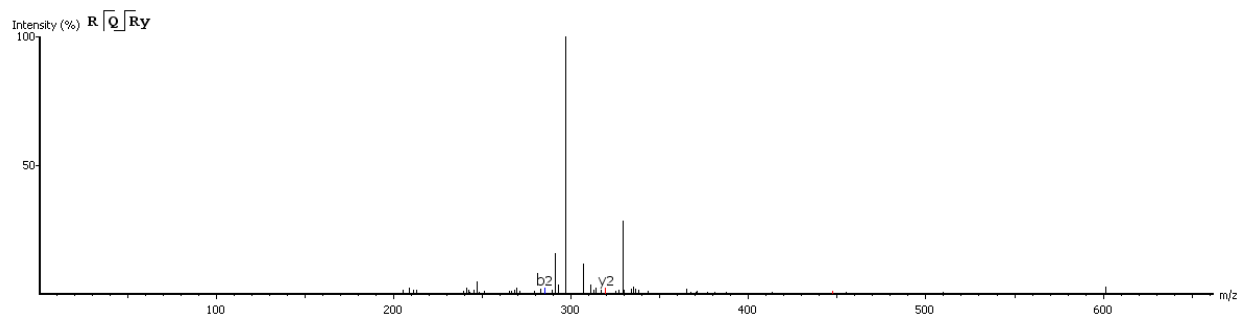


Figure S82. MS/MS spectrum of a putative RYamide neuropeptide with the sequence RQRYamide(-18.01) detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

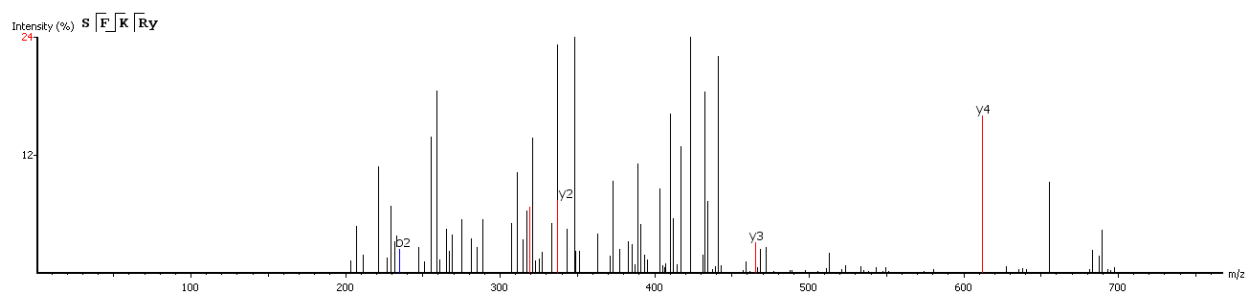


Figure S83. MS/MS spectrum of a putative RYamide neuropeptide with the sequence SFKRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

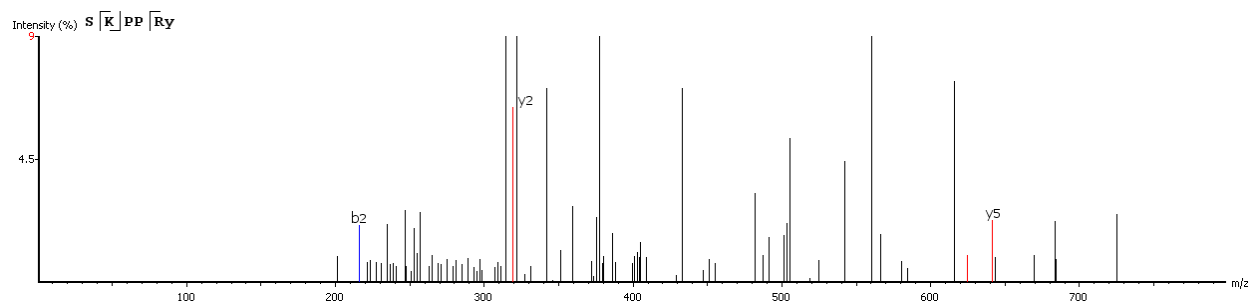


Figure S84. MS/MS spectrum of a putative RYamide neuropeptide with the sequence SKPPRYamide(-18.01) detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

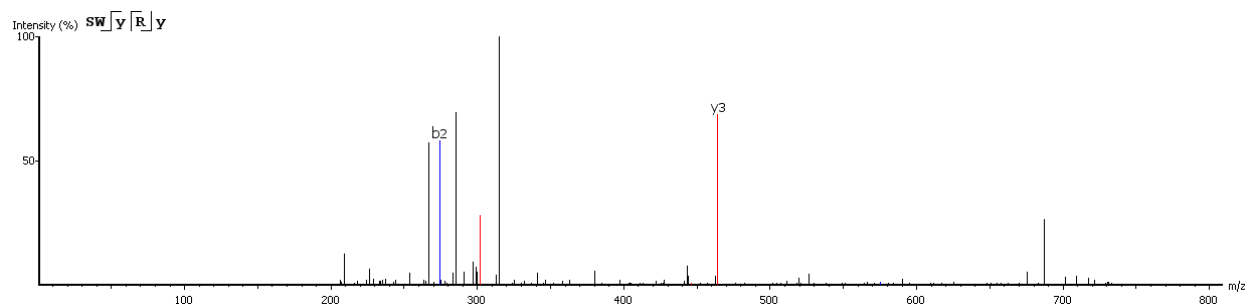


Figure S85. MS/MS spectrum of a putative RYamide neuropeptide with the sequence SWY(-18.01)RYamide(-18.01) detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

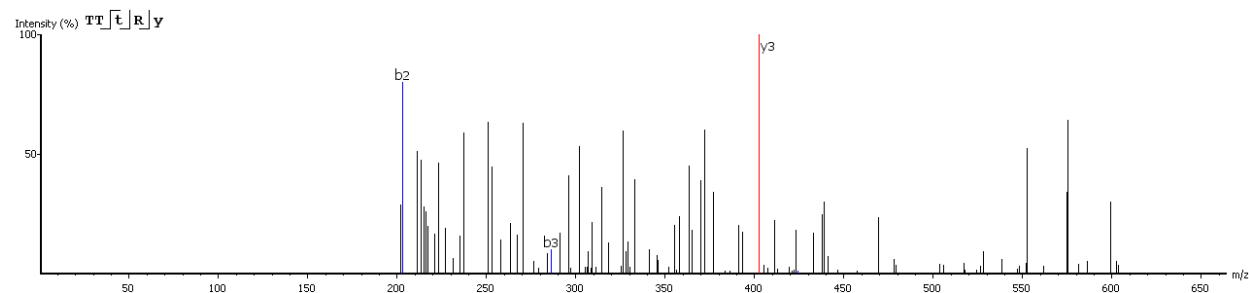


Figure S86. MS/MS spectrum of a putative RYamide neuropeptide with the sequence TTT(-18.01)RYamide(-18.01) detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

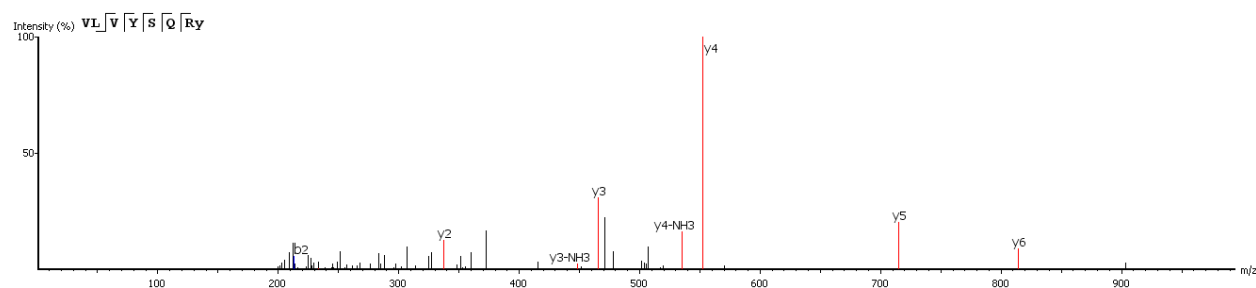


Figure S87. MS/MS spectrum of a putative RYamide neuropeptide with the sequence VLVYSQRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

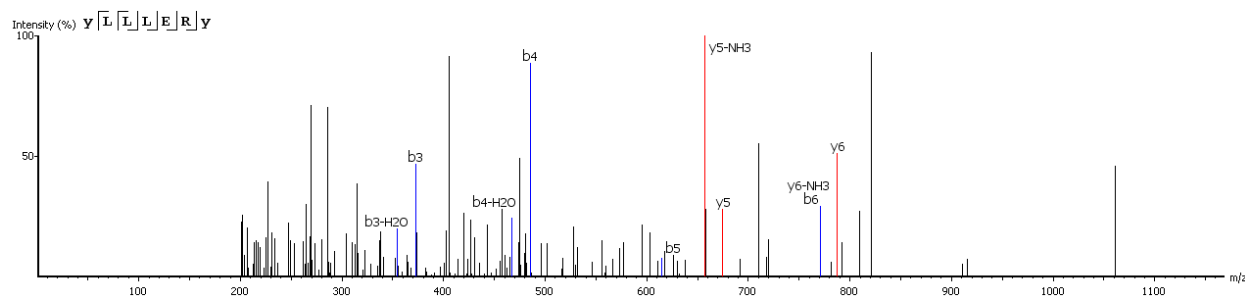


Figure S88. MS/MS spectrum of a putative RYamide neuropeptide with the sequence Y(-18.01)LLERYamide(-18.01) detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

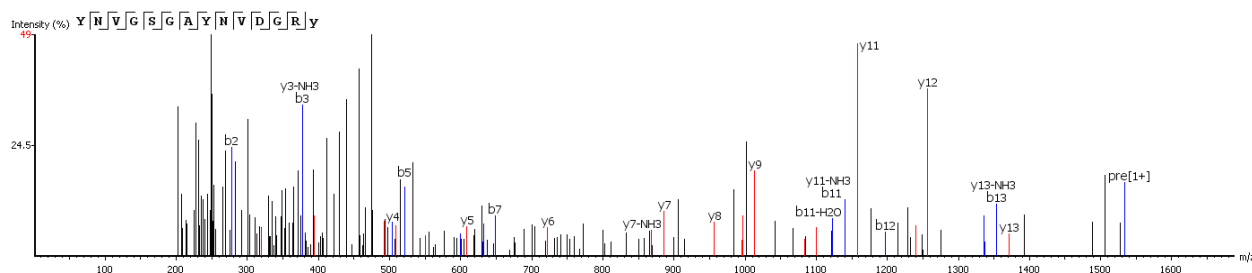


Figure S89. MS/MS spectrum of a putative RYamide neuropeptide with the sequence YNVGSGAYNVDGRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

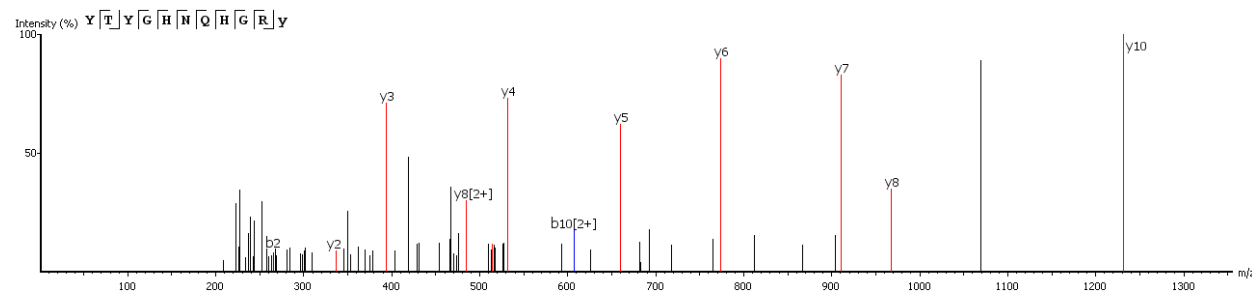


Figure S90. MS/MS spectrum of a putative RYamide neuropeptide with the sequence YTYGHNQHGRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

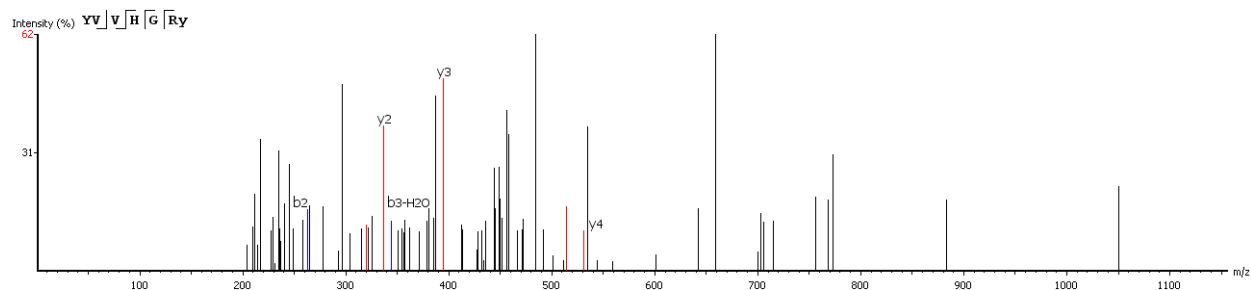


Figure S91. MS/MS spectrum of a putative RYamide neuropeptide with the sequence YVVHGRYamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

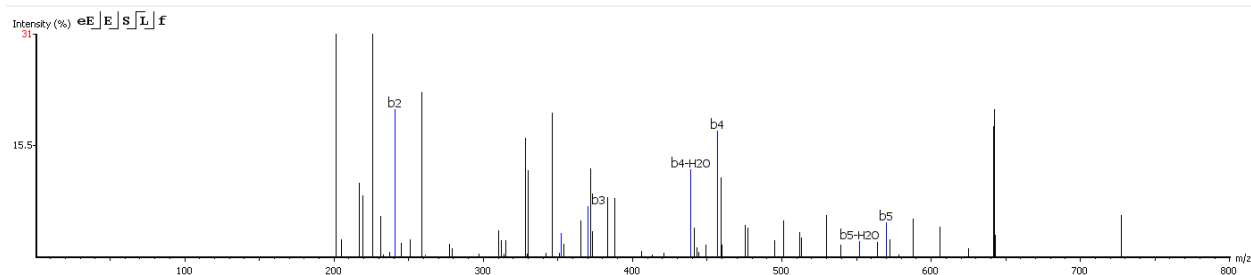


Figure S92. MS/MS spectrum of a putative SIFamide like neuropeptide with the sequence E(-18.01)EESLFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

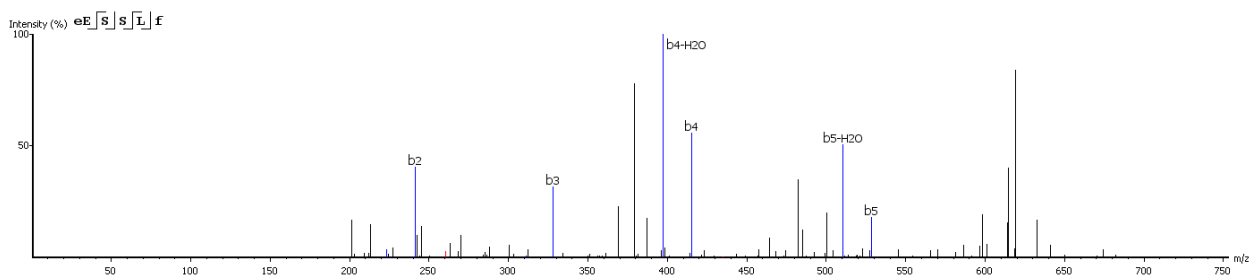


Figure S93. MS/MS spectrum of a putative SIFamide like neuropeptide with the sequence E(-18.01)ESSLFamide detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

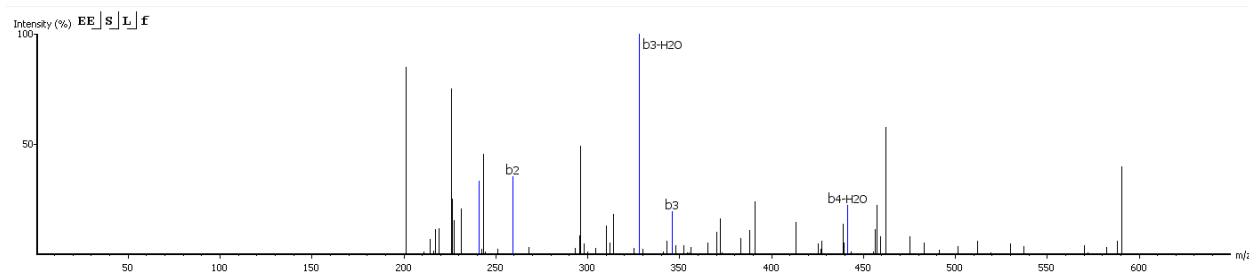


Figure S94. MS/MS spectrum of a putative SIFamide like neuropeptide with the sequence EE|S|L|f detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

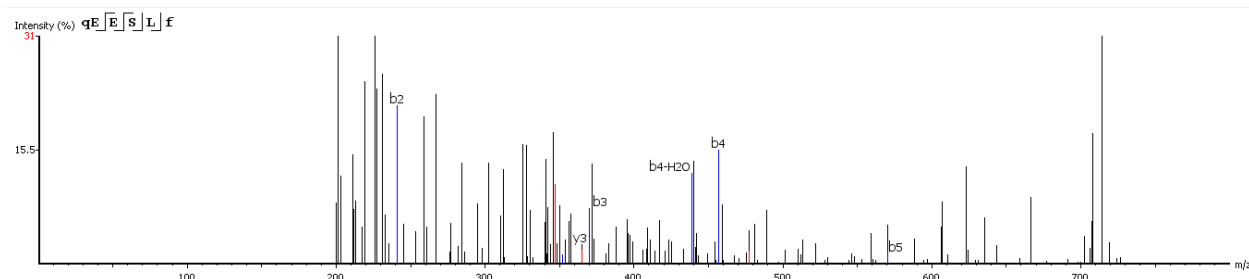


Figure S95. MS/MS spectrum of a putative SIFamide like neuropeptide with the sequence Q(-17.03)EE|S|L|f detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

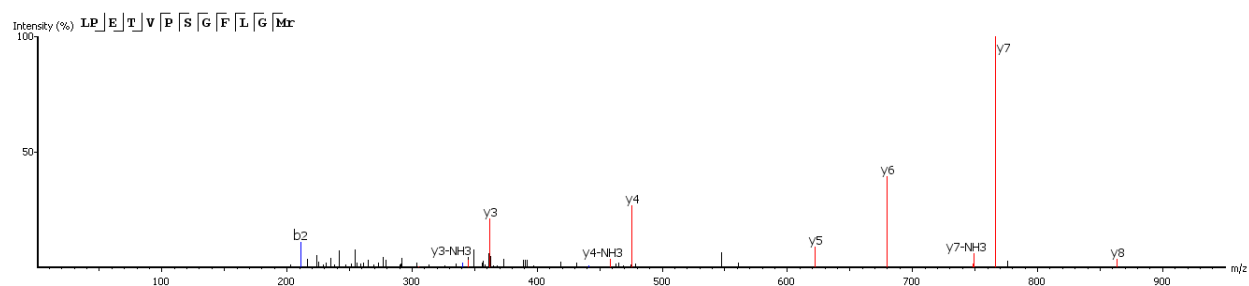
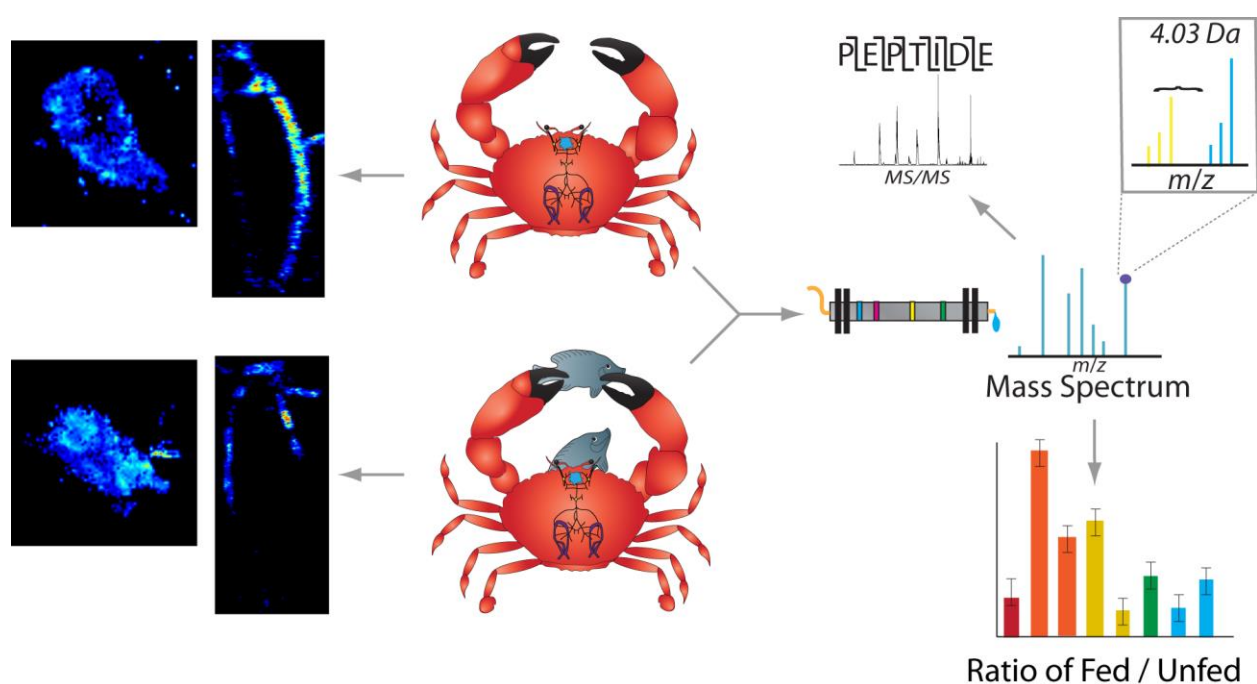


Figure S96. MS/MS spectrum of a putative tachykinin neuropeptide with the sequence LP|E|T|V|P|S|G|F|L|G|Mr detected in hemolymph extract generated by PEAKS software using data independent acquisition (DIA) method.

Chapter 9

Mass Spectrometry Quantification, Localization, and Discovery of Feeding-Related Neuropeptides in *Cancer borealis*



Modified from:

DeLaney, K.; Hu, M.; Hellenbrand, T.; Dickinson, P.S.; Nusbaum, M.P.; Li, L. Mass Spectrometric Quantification, Localization, and Discovery of Feeding-Related Neuropeptides in *Cancer borealis*. *ACS Chemical Neuroscience* 2020. Submitted.

Abstract:

The crab *Cancer borealis* nervous system is an important model for understanding circuit dynamics and its modulation, but the influence of neuromodulatory substances on circuit dynamics remains incomplete, particularly for behavioral state-dependent modulation. Therefore, we used a multifaceted mass spectrometry (MS) method to identify neuropeptides that differentiate the unfed and fed states. Duplex stable isotope labeling revealed that the abundance of 80 of 278 identified neuropeptides was distinct in ganglia and neurohemal tissue from fed vs. unfed animals. Additionally, MS imaging revealed that 7 and 11 neuropeptides showed altered spatial distributions in the brain and pericardial organs (POs), respectively, during these two feeding states. Furthermore, *de novo* sequencing identified 72 putative newly identified neuropeptides that may influence feeding state-related neuromodulation. Two of these latter neuropeptides were determined to be upregulated in PO tissue from fed crabs and these peptides influenced heartbeat in *ex vivo* preparations. Overall, the research presented here identifies a cohort of neuropeptides that may be implicated in the feeding process, providing valuable insight for future electrophysiological investigations.

Introduction:

The crustacean stomatogastric nervous system (STNS) has been used for over 50 years as a model for understanding how neuronal circuits generate rhythmic motor patterns. This numerically small yet elegant extension of the central nervous system (CNS) has enabled researchers to gain extensive knowledge on neural circuit dynamics, including how individual neurons contribute to circuit output.¹ The STNS is composed of four linked ganglia, including the stomatogastric ganglion (STG), oesophageal ganglion (OG), and paired commissural ganglia

(CoG).² In addition to the neuronal interactions between these ganglia, the STNS is influenced by other key neural tissues such as the brain (i.e. supraoesophageal ganglion) and by circulating peptide and small molecule hormones released from the neuroendocrine system, which includes the sinus glands (SGs) in the eye stalks and the paired pericardial organs (POs).

In the crab, *Cancer borealis*, the stomatogastric ganglion (STG) contains only 26 neurons, but overlapping sets of them are sufficient to comprise the gastric mill (chewing) and pyloric (pumping/filtering of chewed food) motor circuits. Due to their small number, presence of most components as single copies, large soma size (30-120 μm), and ability of both circuits to continue operating in the isolated STNS, the associated connectomes and how they operate under particular conditions are well-defined.³ The ability of applied- and neurally-released modulators to influence these circuits has also received considerable attention,³⁻⁵ but the complete repertoire of modulators in this system and how these modulatory systems act collectively, in a behavioral state-dependent manner, to influence the STG circuits remains to be determined.

Neuropeptides are the most diverse class of signaling molecules and the complete array of mechanisms by which they affect the STG circuits remains elusive. The primary structure of these neuropeptides can vary widely, and many of these peptides include numerous isoforms. These isoforms can differ by as few as a single amino acid, yet in most cases it remains to be determined whether they have conserved or distinct functions. In one established example, however, three neuropeptides with conserved sequence homology, AST-C I, II, and III, had distinct effects and spatial distribution within the cardiac neuromuscular system of lobsters.⁶ Furthermore, the same neuropeptide can have different functions in different neurons or at different concentrations, such as PevPK2, which affects the contraction frequency, duration, and fall time of the lobster heart in a concentration-dependent manner.⁷ In addition to the high degree

of complexity of neuropeptide function, these molecules are challenging to study due to their low abundances *in vivo* and propensity toward rapid degradation.⁸⁻¹⁰

To understand specifically how neuropeptides function in the nervous system, electrophysiological recordings must be made of each neuropeptide in an isolated system. However, this is a daunting task when there are currently hundreds of known neuropeptides and likely many more that are not yet identified, and the function of neuropeptides are affected by other neuropeptides that are co-transmitted.³ To gain a better understanding of which subset of neuropeptides is influencing the STNS, large-scale, untargeted profiling has been successfully employed using mass spectrometry (MS). MS is a highly suitable tool for identifying the key neuropeptides responsible for behavioral outputs because it is fast, sensitive, and can theoretically detect all analytes above the limit of detection in a sample, and can do so in a single analysis without prior knowledge about the specific analytes. As a result, MS has been used to profile the neuropeptides present in key tissues¹¹⁻¹³ and to identify the neuropeptides involved in numerous biological processes such as adaptation to temperature stress,¹⁴ salinity stress,¹⁵ and pH stress.¹⁶ The role of neuropeptides in feeding behavior has also been previously explored, both in various tissues and circulating fluid.¹⁷⁻¹⁹

In this study, a multifaceted MS method using advanced techniques was employed to gain deeper insight into the neuropeptides involved in feeding behavior in the crab *Cancer borealis*.¹ Quantitative analysis, using duplex formaldehyde chemical labeling, was performed to compare the relative abundance of neuropeptides in five major neural tissues, including the STG, CoGs, brain, SGs, and POs, in the unfed and fed states. Additionally, MS imaging was used to spatially localize neuropeptides within the brain and POs, with the goal of understanding how neuropeptide organization changed within these tissue during feeding. Finally, *de novo*

sequencing was utilized to identify putative novel neuropeptides that are significantly dysregulated during the feeding process and may have critical roles in neuromodulatory functions within *C. borealis*. The results of this study reveal a collection of neuropeptides that show changes in either abundance or localization in various tissue and thus may alter the feeding-related circuit output within the STNS. Furthermore, two novel neuropeptides were identified and their sequences confirmed with custom-synthesized standards. These neuropeptides were found to have an effect on the cardiac neuromuscular system, which has been previously shown to be influenced by the feeding process.²⁰

Results and Discussion

Feeding regulation is an important biological process that is controlled by multiple neural circuits.²¹⁻²⁴ To gain insight into the role(s) of specific neuropeptides in the feeding process, we analyzed neuropeptide expression levels in unfed and recently fed (1 hr post-feeding, see Methods) crabs in several nervous system (brain, STG, CoGs) and neuroendocrine (PO, SG) tissues. These tissues were chosen for their key roles in neuropeptide secretion or transmission during feeding.^{25, 26} In parallel, we spatially mapped neuropeptide localization within the brain and POs in the unfed and recently fed conditions, using MALDI-MSI (matrix-assisted laser desorption/ionization-mass spectrometry imaging).

Changes in Tissue Expression

Neuropeptide samples from the aforementioned 7 tissues were acquired from fed and unfed crabs and then differentially labeled using reductive methylation. For complementary coverage, two different methods, MALDI-MS and LC-ESI-MS/MS (liquid chromatography-electrospray ionization-tandem mass spectrometry), were used to study the tissues. For both approaches 8 biological replicates were collected for all tissues. Across all 5 tissues, 278

neuropeptides were detected, as listed in **Supporting Information II**. As presented below, neuropeptide abundance post-feeding either increased, decreased, or did not change relative to that in unfed animals. Increased abundance suggests that these peptides accumulated in the studied tissue(s) after feeding, perhaps because their release was suppressed due to inhibition of the peptide containing neurons. In contrast, decreased abundance suggests that these peptides exhibited increased release after feeding, presumably due to increased excitatory drive to the peptide-releasing neurons. Those that did not show changes may not have a role in feeding processes at this early time point, but may still affect the feeding process at later time points, such as those previously characterized with physiological recordings.

The stomatogastric nervous system. Despite the small size of the STG, 44 neuropeptides were identified in both unfed and fed tissue, listed in **Supporting Information II**. Of these 44 peptides, 6 were significantly up-regulated by feeding, including 2 pyrokinins (PKs), a tachykinin-related peptide (TRP), a N-terminally extended RFamide, an allatostatin (AST) A-type, and a diuretic hormone 44 precursor-related peptide (DH 44-PRP) (**Fig. 1A**). PKs, TRP, RFamide and AST peptides are all present in the STNS and modulate the gastric mill and/or pyloric rhythms.²⁷⁻³² The two PK peptides that exhibited an increased abundance, TNFAFAPRPamide and ANFAFAPRPamide, are novel identifications for *C. borealis*. However, ANFAFAPRPamide was originally predicted using *in silico* mining of the *C. borealis* transcriptome,³³ while TNFAFAPRPamide was recently identified in *C. irroratus*.³⁴ While other, previously identified PK peptides were not detected in the STG, they were identified in other tissue.

The ~400 neurons in each CoG include ~20 projection neurons that innervate the STG.³⁵ In this tissue, 58 neuropeptides belonging to 20 different families were detected. Of these

neuropeptides, 8 were significantly up-regulated and one was significantly down-regulated in fed tissue (**Fig. 1B**). Among these were three members of the orcokinin family, which activate hindgut contractions in several species and modulate the STNS output.³⁶⁻³⁹ Interestingly, one of these peptides (FGGDELDKQYGFN) exhibited a large increase in abundance in fed tissues, while another one (SSFDELDRSGFGFA) exhibited a 2-fold decrease in abundance. These differences suggest that, despite their sequence similarity, these peptides may have different feeding-related functions. Two TRPs, APSFGQamide and APSGFLGMRamide (aka CabTRP Ia),⁴⁰ also showed an increased abundance in fed tissue. CabTRP Ia is present in MCN1, an identified CoG projection neuron that innervates the STG and modulates the gastric mill and pyloric rhythms.⁴⁰⁻⁴²

The brain. The OG and CoGs are directly linked to the brain.⁴³ Additionally, one projection neuron (IVN) in the brain innervates both the CoGs and STG.⁴⁴ Therefore, the brain likely plays a pivotal role in modulating feeding behavior. In this study, 138 neuropeptides were detected in fed and unfed brain tissue, with 24 exhibiting changes in abundance in fed tissue (**Fig. 1C**). Only one of these peptides, ocrokinin EIDRSGFGFA, exhibited a decreased abundance post-feeding, with the other 23 peptides displaying increased abundance. Among this latter cohort were 4 A-type and 4 B-type ASTs. A-type ASTs are well-characterized in crustaceans as inhibitors of various activities, including the pyloric rhythm.^{31, 45, 46} This peptide is also present in the gastro-pyloric receptor (GPR) neurons, a set of muscle stretch-sensitive sensory neurons that influence the STG circuits both directly and via actions on CoG projection neurons.⁴⁷ B-type ASTs, while less documented, also decrease pyloric rhythm activity.⁴⁸ The accumulation of these neuropeptides in the tissue may indicate their preparation for release to terminate feeding behavior or inhibition of their release to allow feeding to take place, though the

exact reason is unknown. Eight members of the RFamide family also showed increased abundance after feeding. Members of this family are broadly distributed throughout the crustacean CNS and neuroendocrine system, but only some of their functions are known. For example, bath-applying different family members modulates the gastric mill and pyloric rhythms.^{32, 49} Two orcokinin peptides (DFDEIDRSGFGFV, NFDEIDRSGF), and one TRP (APSGFLGM) also increased in abundance in fed tissue. As described above, these families have been implicated in STNS activity and their observed feeding-related changes support those findings for these specific isoforms.

Neuroendocrine system. In addition to acting on the STG through direct neural projections, neuropeptides that are released from neuroendocrine tissue such as the paired POs, and paired SGs also act as circulating hormones. Neuropeptides released from these tissues are transported via the hemolymph, which is pumped from the pericardial sinus. Interestingly, the STG is located within the dorsal ophthalmic artery and so is continually receiving circulating hormones.⁵⁰

The PO is a paired neurohemal site located in the lateral interior walls of the pericardial cavity. It contains the terminals of thoracic ganglion neurons, which release neuropeptides directly into the pericardial sinus from where they are transported within hemolymph throughout the animal. Among the first points of post-release contact is the STG. A total of 137 neuropeptides was detected in both fed and unfed POs; 22 of them differed in abundance in fed and unfed tissue (**Fig. 2A**). Of these 22 peptides, 6 were down-regulated, including 2 B-type AST (AGWSSLKAWamide, NNNWTKFQGSWamide), an arthropod CHH/MIH/GIH/VIH hormone (RVINDDCPNLIGNR), an RFamide (DGGRNFLRFamide), and an RYamide (QGFYSQRYamide). The effect of RYamide and CHH/MIH/GIH/VIH peptides on the STG

circuits is unknown. However, the decrease in their abundance after feeding suggests that they play a role in hormonal modulation of the STG or some other feeding-related activity. While members of the B-type AST family and RFamide family showed decreases in abundance in fed tissue, other isoforms from these families showed increases in abundance in fed tissue, indicating again the diversity of functions even between neuropeptides within the same family.

The SGs, located in the crab eyestalks, are another major source for circulating hormones. In this tissue, 171 neuropeptides were detected, of which the abundance of 25 was distinct between the unfed and fed states (**Fig. 2B**). Unlike the other tissues, where most neuropeptides were observed to increase in abundance, 16 of the 25 significant peptides detected in the SG decreased in abundance. These peptides belong to the A-type AST, orcokinin, YRamide precursor-related peptide, pyrokinin (PK), sulfakinin (SK), and WXXXnRamide families. While these neuropeptides were identified in other tissue, only one of them exhibited a change in abundance in another tissue that was statistically significant. EIDRSGFGFA (m/z 1097.503) belonging to the orcokinin family was lower in abundance in fed brain and SG tissue. SG peptides that exhibited an increase in abundance belonged to the A-type and B-type ASTs, orcokinin, YRamide precursor-related peptide, and SIFamide families. While the various isoforms of SIFamide have been widely characterized,⁵¹⁻⁵⁴ functional studies have only recently been explored in crab species.^{54, 55} The significant decrease in abundance suggests an immediate release of these SG peptides into the hemolymph to potentially act long-range on circuits within the animal, while the increase in abundance of other peptides is unknown. It may indicate inhibition of neuronal release, leading to an accumulation in preparation for later release to modulate subsequent feeding-related behavior, or inhibition of release in order to initial feeding processes.

Changes in tissue localization

In addition to measuring changes in peptide abundance between the fed and unfed states, we can visualize neuropeptide distribution within different ganglia and neurohemal organs in a feeding state-dependent manner, to provide additional clues for the function of these peptides. Therefore, we implemented MALDI-MS imaging to spatially localize neuropeptides in brain tissue sections and POs. Twenty-nine neuropeptides were reproducibly detected in brain sections across biological replicates, and 59 neuropeptides were consistently detected in PO biological replicates. Of these, a subset in each tissue exhibited notable differences between unfed and fed animals.

In the brain tissue sections, 7 neuropeptides had distinct spatial distributions in unfed and fed animals (**Fig. 3**). These distributions were conserved across 4 fed and 4 unfed biological replicates (**Fig. S1**). The optical images of two representative brain sections are shown in **Figure 3a**. Two of the detected neuropeptides, including the tachykinin APSGFLGMRamide (**Fig. 3b**) and the YRamide HL/IGSL/IYRamide (**Fig. 3d**), were localized more towards the outer regions of the brain in unfed tissue; this localization was more prominent in fed tissue. Another neuropeptide, KPKTEKK (**Fig. 3c**), showed the opposite trend, where the unfed section localized toward the perimeter of the brain whereas the fed tissue showed a more centralized distribution. The 4 other neuropeptides, all members of the RFamide super family including GRPNFLRFamide, YGAHVFLRFamide, GNSFLRFamide, and RARPRFamide, showed an overall decrease in signal within the brain in fed animals compared to unfed. These localization patterns may suggest a relatively rapid release or accumulation of these peptides during or after feeding, perhaps to influence neuronal systems that regulate some aspect of the feeding process, such as the STNS-generated rhythms, though the exact role cannot be determined until

downstream physiological analyses are performed.

Figure 4 shows representative MALDI-MSI results for the POs, including the optical image of two representative POs, with all images oriented with the top being the anterior- and the bottom being the posterior side. Eleven detected neuropeptides showed distinctive localization patterns in fed and unfed POs. The results for both pairs of all 3 biological replicates are shown in **Figure S2**. The optical image of two representative POs are shown in **Figure 4a**, which is focused on the anterior bar, which extends across the PO on its anterior side. Three AST A-type neuropeptides, GHYNFGLamide, PKTYSFGLamide, and PRAYSFGLamide (**Fig. 4b-d**), localized to the side of the PO in the unfed tissue, but showed an increased abundance at the posterior and anterior junctions in the fed tissue. Neuropeptides from the RFamide family (**Fig. 4i-l**) exhibited a similar trend in that the abundance appeared to decrease on the sides of the tissue and increase at the posterior and especially the anterior junction. These differences indicate a potential functional role of AST-A and RFamides that may be region-specific within the POs. Four additional neuropeptides that were seen in the POs did not show any trends within their families but exhibited distinctions in the fed and unfed animals, such as a change in localization from the anterior to the posterior side, as occurred the tachykinin SGFLGMRamide (**Fig. 4f**), KPKTEKK (**Fig. 4g**), and the RYamide FVGGSRYamide (**Fig. 4h**). Proctolin (RYLPT), a neuropeptide well characterized in the STNS and cardiac neuromuscular system, also displayed increased abundance at the posterior and anterior junctions in fed animals compared to unfed. This observation suggests that, in addition to its release from projection neurons directly into the STG neuropil to modulate the pyloric rhythm, proctolin plays a modulatory role as a circulating hormone related to the feeding process.⁵⁶⁻⁶⁰ Overall, these observations in localization of specific neuropeptides in the brain and PO complement the

quantitative data assessing total peptide content to provide more detail as to which neuropeptides contribute to the behavioral outputs involved in the feeding process.

Putative novel neuropeptides identified with *de novo* sequencing

In addition to observing changes in abundance and localization of known neuropeptides in various tissues, we also searched the data for putative neuropeptides not currently present in the database. To this end, with the use of PEAKS *de novo* sequencing, peptide sequences of MS/MS spectra were obtained. These sequences were mined for peptides that either possessed the sequence motifs characteristic of common crustacean neuropeptide families or exhibited a large degree of sequence similarity to those already present in the database. After filtering through neuropeptide sequences, each MS/MS spectrum was manually checked to make sure it had a score $\geq 75\%$ average local confidence (ALC), representing an average of the confidence of each amino acid within the sequence, and cleavage between every amino acid to ensure proper sequence assignment. We discovered 72 peptides that adhered to these specifications, with motifs similar to those belonging to 4 different families including AST A-type, orcomyotropin, RFamide, and RYamide. An “other” category was also included for neuropeptides not matching a sequence motif, but having high sequence similarity to database neuropeptides. The sequences are listed in **Table S2** (see **Supporting Information**), along with their molecular weight, tissue in which they were detected, and ALC. The MS/MS spectra for the *de novo* sequences are shown in **Figures S3-71**.

Figure 5a shows the number of putative novel neuropeptides identified in each tissue. As expected, the largest numbers of neuropeptides were identified in the SGs, POs, and brain, with 32, 29, and 26 neuropeptides identified in each, respectively. These tissues play key neuromodulatory functions, either by releasing circulating hormones or through direct neural

projections onto other tissues. A small number of putative novel neuropeptides were also detected in the CoG and TG, 6 and 4, respectively. Most notably, the tissues with the smallest number of neurons, the STG and OG, also contained putative neuropeptides not present in the database. Two RFamides, SPANFLRFamide and EHNFLRFamide, were identified in the STG. None of these neuropeptides appear to have been previously identified in a crustacean species. However, one neuropeptide identified in the SGs, neuropeptide PF1 SDPNFLRFamide, has been previously identified in several other invertebrate species, including 2 species of roundworm, *Ascaridia galli*⁶¹ and *Ascaris suum*⁶², land snail, *Helix aspersa*⁶³, and 3 species of nematode, *Caenorhabditis briggsae*⁶⁴, *Caenorhabditis elegans*⁶⁵, and *Panagrellus redivivus*⁶⁶. Previous studies of this neuropeptide revealed a role in inhibiting muscle contractions in *A. galli* and *A. suum*, but it is currently unclear if the peptide possesses the same function in *C. borealis*^{61, 67-69}.

While it is unclear whether these neuropeptides are biologically active, two of them (YKLFNPLRESN and RFamide FDRQNFLRFamide) were up-regulated in the POs of fed- compared to unfed animals ($p < 0.05$), as shown in **Figure 5b**. These results indicate that the feeding process triggers an accumulation of these two neuropeptides in POs, which may be in anticipation for release into the circulating fluid, though the specific effects are unknown. The MS/MS spectra of these two peptides are shown in **Figures 5c** and **5d**. As can be observed, both spectra appear to be good quality with complete backbone fragmentation and sufficient ion intensity. MS/MS spectra of all other neuropeptides are shown in **Figures S3-71** (see **Supporting Information**).

Assessment of Function of novel neuropeptides on cardiac neuromuscular system

To determine the biological activity of the two putative novel neuropeptides up-regulated in the PO of fed crabs, we custom synthesized these two peptides for use in functional studies.

First, we confirmed the sequence accuracy of these two novel peptides. **Figures 5c** and **5d** show a mirror plot of the MS/MS spectrum obtained for each peptide in PO extract compared to the MS/MS spectrum of the respective synthetic standard, confirming the de novo sequencing results and identity of each peptide. The peptides were each perfused through the cardiac sinus within the intact crab heart at a concentration of 10^{-6} M. In all preparations, perfusion of FDRQNFLRFamide elicited a reversible, substantial increase in both amplitude and frequency of heart contractions ($p < 0.05$, $n = 10$, Paired Student's t-Test) (**Fig. 6a,b**). The second peptide, YKLFNPLRESN, elicited a reversible decrease in contraction frequency in all heart preparations except for one ($p < 0.05$, $n = 5$, Paired Student's t-Test) (**Fig. 6c,d**). One biological replicate was removed after determining it to be an outlier based on the Grubbs Test. This heart preparation elicited an increase in frequency, while the other 5 preparations elicited a decrease in frequency. While this difference could indicate inter-animal variability in response to this peptide, it was omitted from statistical calculations in order to determine the significance of the decrease observed in the other 5 preparations. Unlike the change in contraction frequency, applying YKLFNPLRESN did not change the contraction amplitude ($p > 0.05$, $n = 5$). These changes in heartbeat are summarized in **Figure 6e** and indicate that these novel neuropeptides can modulate heartbeat, thus demonstrating their biological activity. However, as the concentrations of peptides tested in this study are relatively high, a threshold concentration would need to be determined in order to assess whether these effects are behaviorally relevant.

Conclusions

This study examined neuropeptide abundances and distributions in key neural and neuroendocrine tissues of *C. borealis* in a feeding state-dependent manner. By employing a multifaceted mass spectrometric approach using complementary ionization- and acquisition

methods, we detected 278 neuropeptides, based on our in-house crustacean neuropeptide database that was compiled from various sources.^{33, 70, 71} Through the use of duplex formaldehyde labeling, we determined that a subset of these peptides exhibited either increased or decreased abundance in the analyzed tissues of fed compared to unfed animals. Additionally, using MALDI-MSI, we found changes in the spatial distribution of some peptides during these two feeding states. By combining the information gained from these two methodologies, we have obtained a list of neuropeptides that are positioned to influence one or more aspects of feeding behavior. Follow-up functional studies (e.g. electrophysiological recordings) should shed light on the feeding-related role of each peptide. We also identified 72 new *C. borealis* neuropeptides as part of this study. While the biological activity of these neuropeptides is currently unknown, two of them were up-regulated in the POs of fed crabs compared to unfed crabs and modulated cardiac activity when applied to the isolated crab heart. Overall, this study provides deeper insight into the complexity of neuropeptide modulation in the STNS via the brain and neuroendocrine structures. The success of these experimental approaches in the crustacean nervous system offers promise for their successful use in studying neuropeptides in the larger and more complex vertebrate nervous system. Furthermore, the findings here in dysregulated neuropeptides provides a target cohort of feeding-related peptides that can be investigated with electrophysiological measurements. These results also provide groundwork for studies in other, more complex central pattern generators.

Methods:

Chemicals and Materials

Formaldehyde (FH₂), deuterated formaldehyde (FD₂), and ACS-grade formic acid (FA)

were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gelatin was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). All other chemicals and solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). Amicon Ultra 10 kDa 0.5 mL molecular weight cutoff (MWCO) devices and 10 μ L C18 ziptips were purchased from Merck Millipore (Billerica, MA, USA), and 100 μ L C18 zip tips were purchased from Agilent Technologies (Santa Clara, CA, USA). Acidified methanol was prepared using 90/9/1 water/methanol/acetic acid. ACS-grade solvents and Milli-Q water (Merck Millipore, Billerica, MA, USA) were used for sample preparation, and Optima-grade solvents were used for MS analysis. Peptide standards were custom synthesized by GenScript Corporation (Piscataway, NJ, USA).

Animals and Feeding Experiments

C. borealis were purchased from the Fresh Lobster Company, LLC (Gloucester, MA, USA). Animals were adjusted to their environment for two weeks prior to experiments. Animals were housed in artificial seawater tanks (12° – 13° C) with an alternating 12-hour light/dark cycle. All animals were kept in an unfed state for 2 weeks prior to feeding experiments.

For tissue experiments, 3 control (unfed) animals and 3 experimental (fed) animals were used per biological replicate. Experimental animals were fed an excess of tilapia for 30 minutes and control animals were left in an unfed state. Animals were then cold-anesthetized on packed ice for 30 minutes, and dissected in chilled physiological saline (440 mM NaCl, 11 mM KCl, 26 mM MgCl₂, 13 mM CaCl₂, 11 mM Trizma base, 5 mM maleic acid, adjusted to pH 7.45 with NaOH) as described elsewhere.⁷² The SGs, POs, brain, commissural ganglia, and STG were collected separately and placed in chilled acidified methanol, with 5 biological replicates total (each biological replicate comprised of tissue from 3 fed and 3 unfed animals). For imaging of the brain and POs, the experiment was conducted as described above except that the tissue was

quickly dipped in water to desalt after dissection. The brain was embedded in gelatin and flash-frozen in dry ice. The POs were placed directly on a glass microscope slide and placed in a dessicator box. The tissue was then stored at -80°C. A total of 3 control and experimental biological replicates were performed for MS imaging of each tissue.

Extraction and Desalting

Tissues were extracted in chilled acidified methanol. For each tissue type, samples from the three experimental animals were pooled, as were those from the control animals. The sets of tissue were manually homogenized with 150 µL acidified methanol (50 µL per tissue), sonicated using a bath sonicator, and then centrifuged for 10 minutes at 16.1 rcf. The supernatant was transferred to a new tube, and 50 µL of acidified methanol was added to the pellet. The pellet was then homogenized, sonicated, and centrifuged as previously. The resulting supernatant was again kept, and the process was repeated a second time. The combined supernatant from the three extractions was evaporated in a SpeedVac on medium heat and stored at -80° C.

Tissue samples were then desalted with 10 µL C18 ziptips using package instructions, except that a three-step elution was used, consisting of 25/75 acetonitrile/water with 0.1% FA, followed by 50/50 acetonitrile/water with 0.1% FA, and finally 75/25 acetonitrile/water with 0.1% FA. The three elutions were then combined and evaporated in the SpeedVac on medium heat.

In-Solution Formaldehyde Labeling

Control and experimental samples were differentially labeled using 2-plex isotopic formaldehyde. Samples were suspended in 10 µL of water, after which 10 µL of 1% formaldehyde solution (FH₂ or FD₂) was added to each, followed by 10 µL borane pyridine. The samples were then incubated in a 37° C water bath for 15 minutes, after which 10 µL of 100 mM

ammonium bicarbonate was added to quench the labeling reaction. Light and heavy-labeled samples were then combined and evaporated in the SpeedVac on medium heat. The labels used for control and experimental samples were alternated for different bioreplicates to account for possible labeling bias.

Samples were reconstituted in 15 μ L of 0.1% formic acid (FA) in water. A 2.5 μ L-aliquot was removed from each sample and combined with 2.5 μ L of 150 mg/mL 2,5-dihydroxy benzoic acid (DHB) matrix. The mixture was then spotted on a stainless steel MALDI plate, 1 μ L per spot, for MALDI-MS analysis. The remaining 12.5 μ L of sample was desalted using 10 μ L C18 zip tips with the three-step gradient described above and evaporated in the SpeedVac on medium heat. The sample was then dissolved in 15 μ L of 0.1% FA in water for LC-ESI-MS/MS analysis.

Brain Sectioning and Matrix Application for Imaging

Brain tissues that were embedded in gelatin were sectioned on a cryostat at -23° C. Sections of 12 μ m thickness were thaw-mounted on a glass microscope slide. An automated matrix deposition sprayer (TM sprayer, HTX Technologies LLC, Chapel Hill, NC, USA) was used to apply 12 layers of 40 mg/mL DHB matrix to brain tissue sections and POs, with 30 seconds of drying time between each coating.

Mass Spectrometry and MALDI Imaging

MALDI-MS analysis was performed using a Thermo Scientific MALDI-LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a 337.1 nm, 60 Hz nitrogen laser for analysis of spots of tissue and hemolymph extract. Spots and tissue sections were analyzed in positive-ion mode with an m/z range of 500 to 2000 and 20 kJ laser energy. Crystal positioning system (CPS) plate motion was used to acquire a total of 50 scans (2 microscans/scan) for each spot of extract (tissue and hemolymph). Three spots were analyzed for

each sample, making a total of 3 technical replicates per sample. For imaging of brain tissues, optical images were acquired with an HP scanner and imported into the LTQ tune page. A raster plate motion was used to obtain spectra over the tissue section area with a step size of 75 μm .

For acquisition of MS/MS spectra, a Thermo Scientific Q Exactive instrument (Thermo Scientific, Bremen, Germany) coupled to a nano-ESI source was used with a Waters nanoAcquity LC system (Waters Corp, Milford, MA, USA). A 15 cm self-packed C18 column (75 μm internal diameter, 1.7 μm particle size) was used for LC separation. Mobile phases consisted of water with 0.1% FA (solution A) and acetonitrile with 0.1% FA (solution B), and the flow rate was set to either 0.300 or 0.350 $\mu\text{L}/\text{min}$ (to ensure a pressure of approximately 7000 psi at 100% solution A). Different instrumentation methods were used for hemolymph and tissue due to differences in sample composition. For hemolymph, 3 μL samples were injected onto the column and separated over a 108 minute gradient as follows: 0-0.5 min 0-4% B; 0.5-70 min 4-35% B; 70-80 min 35-75% B; 80-81 min 75% B; 81-82 min 75-95% B; 82-92 min 95% B; 92-93 min 95-0% B; 93-108 min 0% B. MS acquisition took place in positive ESI mode with a top 10 data dependent acquisition (DDA), in which the top 10 most abundant precursor ions were selected for HCD fragmentation with an isolation window of 2 m/z and a collision energy of 30 eV. The MS scan range was 300 – 1800 m/z at 35,000 resolution, and the MS/MS scan range was adjusted depending on the parent mass with a fixed first mass of 120 m/z at 17,500 resolution. For tissue, 2 μL of sample was injected onto the column and separated over a 60 minute gradient as follows: 0-0.1 min 0-4% B; 0.1-30 min 4-40% B; 30-40 min 40-95% B; 40-50 min 95% B; 50-50.5 min 95-0% B; 50.5-60 0% B. MS acquisition took place in positive ESI mode with a top 15 data dependent acquisition (DDA) with an isolation window of 2 m/z and a collision energy of 30 eV. The MS scan range was 200 – 2000 m/z at 70,000 resolution, and the MS/MS scan range

was adjusted depending on the parent mass with a fixed first mass of 100 m/z at 17,500 resolution.

Whole Heart Functional Assessment of Neuropeptides on Cardiac Neuromuscular System

The function of two newly identified neuropeptides was tested on the isolated whole heart of *C. borealis*, as described in previous literature^{6, 7, 73}. Briefly, crabs were anesthetized on ice for 30 minutes, after which the heart and carapace above the heart were removed and placed in a dissecting dish filled with chilled artificial saline. The heart remained attached to the carapace such that the amount of stretch placed on the heart was identical to *in vivo* conditions. Artificial saline at 12° C was perfused both through the heart via the posterior artery and over the heart to maintain temperature, at a flow rate of 2.5 mL/min using a Peltier temperature control system (CL100 bipolar temperature controller and SC-20 solution heater/cooler; Warner Instruments, Hamden, CT, USA). The anterior arteries were tied with suture silk to a Grass FT03 force-displacement transducer (Astro-Med, Inc., West Warwick, RI, USA) at a 45° degree angle for heart contraction measurements. Heart preparations were left to stabilize for at least 1 hour, and then peptide standards at 1×10^{-6} M in saline were perfused into the heart. The force displacement transducer was connected to an ETH-250 Bridge amplifier (CB Sciences, Dover, NH, USA). Data were acquired on a computer using a Micro 1401 data acquisition unit with Spike2 version 9 software (Cambridge Electronic Design Limited, Cambridge, UK).

Data Analysis

For analysis of MALDI-MS data, neuropeptides were identified by accurate-mass matching to an in-house crustacean neuropeptide database within a 5 ppm mass tolerance. The database included neuropeptides previously identified in our lab, those predicted by the *C. borealis* transcriptome³³, and crustacean neuropeptides reported in NeuroPep⁷¹. For LC-ESI-

MS/MS data analysis, raw data acquired from the Q Exactive instrument was processed using PEAKS *de novo* software (Bioinformatics Solutions Inc., Waterloo, ON, CAN)⁷⁴. PEAKS parameters were set as follows: no enzyme cleavage specified, instrument orbi-orbi, HCD fragmentation, and precursor correction. For *de novo* sequencing and database search, modifications were set to light and heavy demethylation, amidation, pyroglutamate, and oxidation. All other parameters were set to the default. For quantitative analysis, neuropeptides were only included if both the light and heavy-labeled peaks were detected. Ratios were calculated by dividing the experimental intensity by the control intensity. For putative neuropeptide identifications, results from PEAKS were filtered using PepExplorer⁷⁵ for sequence similarity to our crustacean neuropeptide database, and peptides were selected that had an average local confidence (ALC) score of 75% or greater. MS/MS spectra were manually inspected for fragment ions indicative of cleavage at every amino acid to ensure the appropriate sequence was assigned. Each peptide sequence was searched in the NeuroPep database to determine if it had been previously identified in other species⁷¹.

MALDI-MS images of brain and pericardial organ tissue were generated and exported as imzml files using Thermo Scientific ImageQuest software. Data were then loaded into MSiReader to generate images of neuropeptides in the crustacean neuropeptide database with a 5 ppm mass error⁷⁶. All images were normalized to the TIC and 5th order linear smoothing was applied.

Statistical Information

For quantitative analysis, ratios (fed / unfed) were averaged across 8 biological replicates for MALDI-MS and 5 biological replicates for ESI-LC-MS/MS, each with 3 technical replicates, and the standard error of the mean (SEM) was calculated. Statistical analysis was done using a

Student's Unpaired Two-Tailed t-Test, with $p < 0.05$ indicating statistical significance. For analysis of whole-heart preparations, a Student's Paired Two-Tailed t-Test was used to compare the average heart contraction amplitude and frequency between baseline and peptide conditions. For peptide FDRQNFLRFamide, 10 biological replicates were used. For YKLFNPLRESN, one biological replicate was removed after being determined to be an outlier based on the Grubbs Test, and 5 biological replicates were used for determining significance.

Supporting Information

- I. MALDI-MS images of all biological replicates of brain and POs, table of putative neuropeptides detected with *de novo* sequencing, MS/MS spectra of all *de novo* sequences.
- II. Spreadsheet of all detected neuropeptides across all tissues.

Data Availability

The data used in this study are available from the corresponding author upon reasonable request.

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Author Contributions

K.D., P.S.D., M.P.N., and L.L. all contributed to conception and design of experiments. K.D., M.H., and T.H. contributed to performing all experiments and analyzing resulting data. K.D. wrote the manuscript. All authors contributed to review and revision of the manuscript.

Competing Interests

The authors declare no competing interests.

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Figures

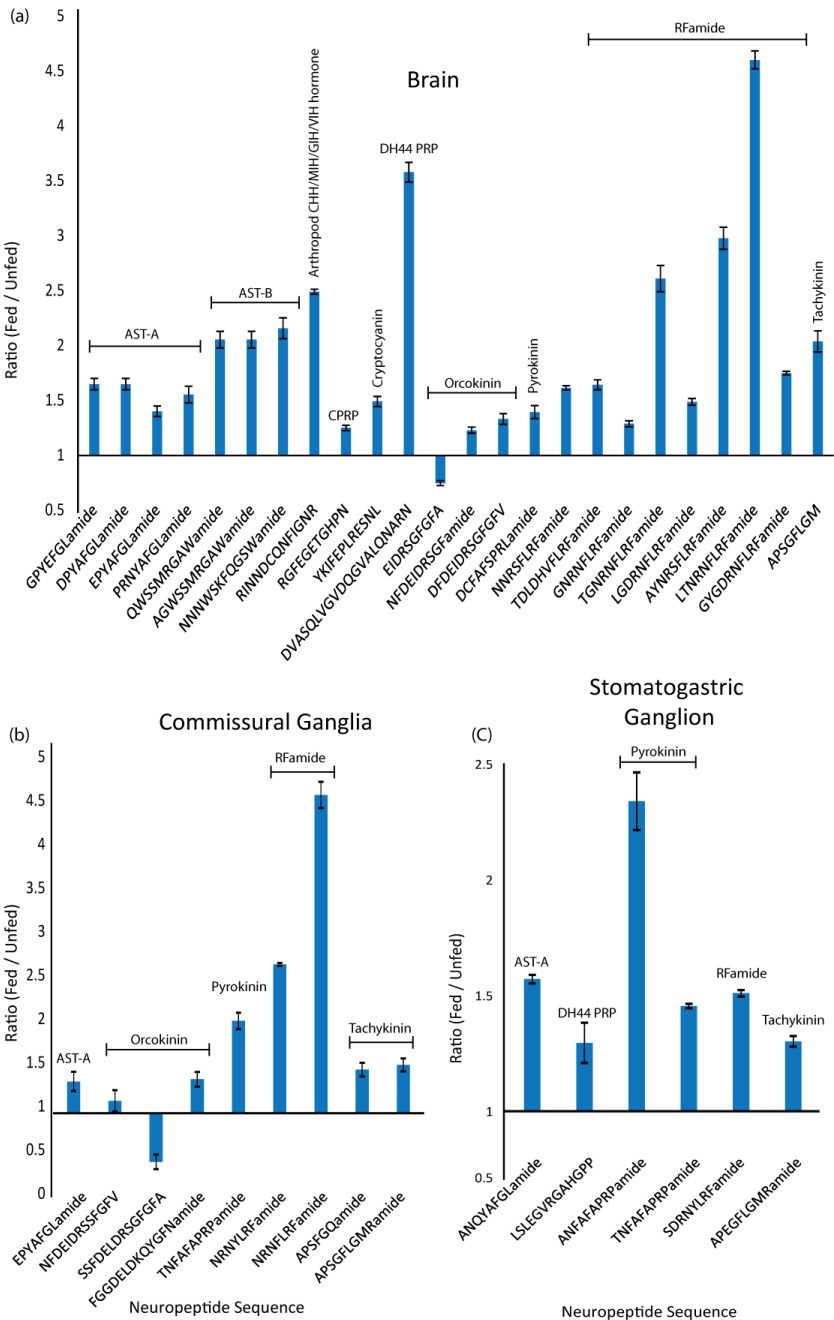


Figure 1. Neuropeptides exhibiting changes in abundance after feeding, in the brain, commissural ganglion and stomatogastric ganglion of *C. borealis*. Bar graphs show neuropeptides displaying significantly increased or decreased abundance in post-feeding tissues compared to unfed tissue. The y-axis represents the ratio of fed- to unfed abundance.

Significance indicated by $p < 0.05$ based on an Unpaired, Two-Tailed Student's t-Test. Error bars represent SEM. Neuropeptide families are indicated, allatostatin (AST), crustacean hyperglycemic hormone precursor related peptide (CPRP), precursor related peptide (PRP), diuretic hormone (DH).

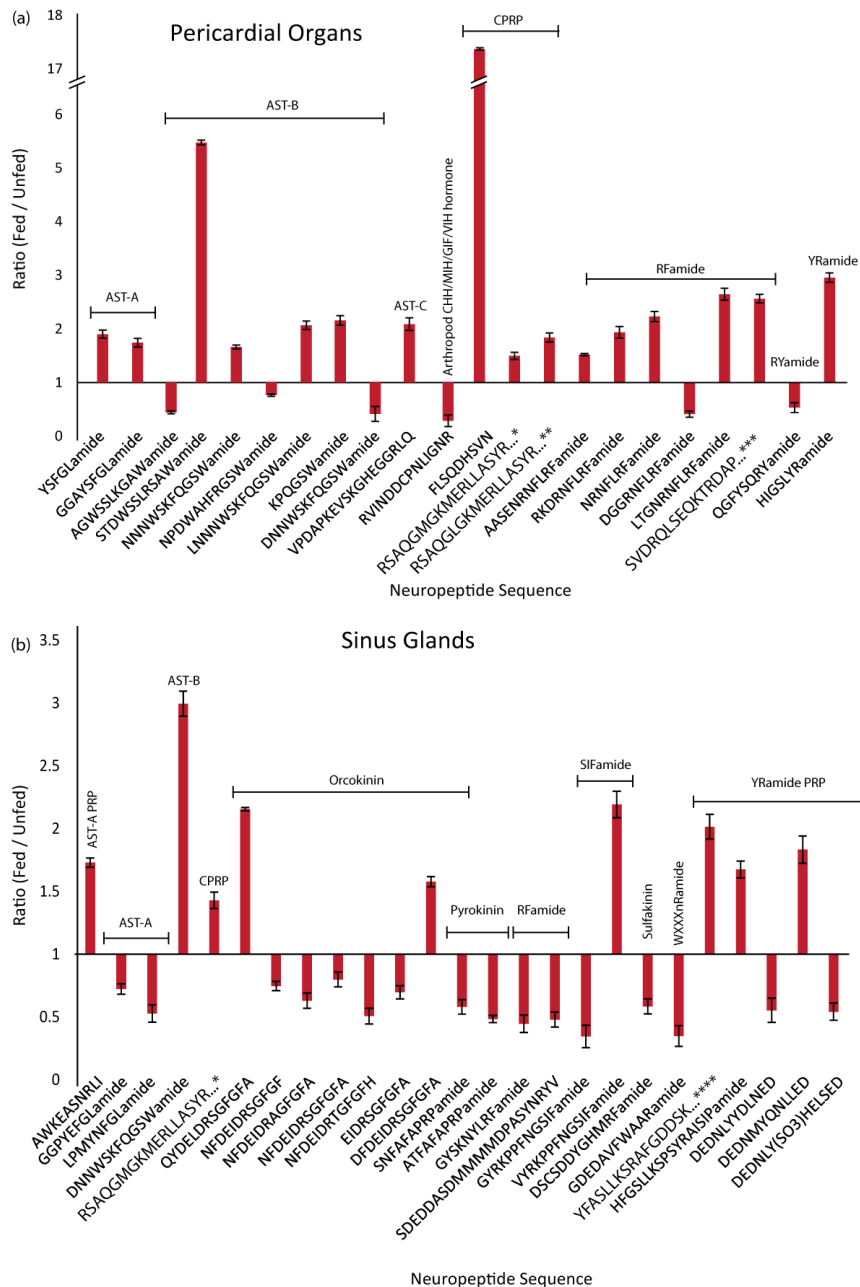


Figure 2. Feeding state-related changes in neuropeptide abundance in the POs and SGs, two regions of the *C. borealis* neuroendocrine system. Bar graphs show neuropeptides that were significantly increased or decreased in fed- compared to unfed tissue. The y-axis represents the

ratio of fed- to unfed abundance. Significance indicated by $p < 0.05$, based on the Unpaired Two-Tailed Student's t-Test. Error bars represent SEM. Neuropeptide families are indicated, allatostatin (AST), crustacean hyperglycemic hormone precursor related peptide (CPRP), precursor related peptide (PRP), diuretic hormone (DH), pigment dispersing hormone (PDH).

Full sequences: *RSAQGLGKMERLLASYRGALEPNTPLGDLSGSLGHPVE,

**RSAQGLGKMEHLLASYRGALEPNTPLGDLSGSLGHPVE,

***SVDRLSEQKTRDAPVAPTATIHSPAKTQESHRS,

****YFASLLKSRAFGDDSKLIPHNAAGDSEPHLQ

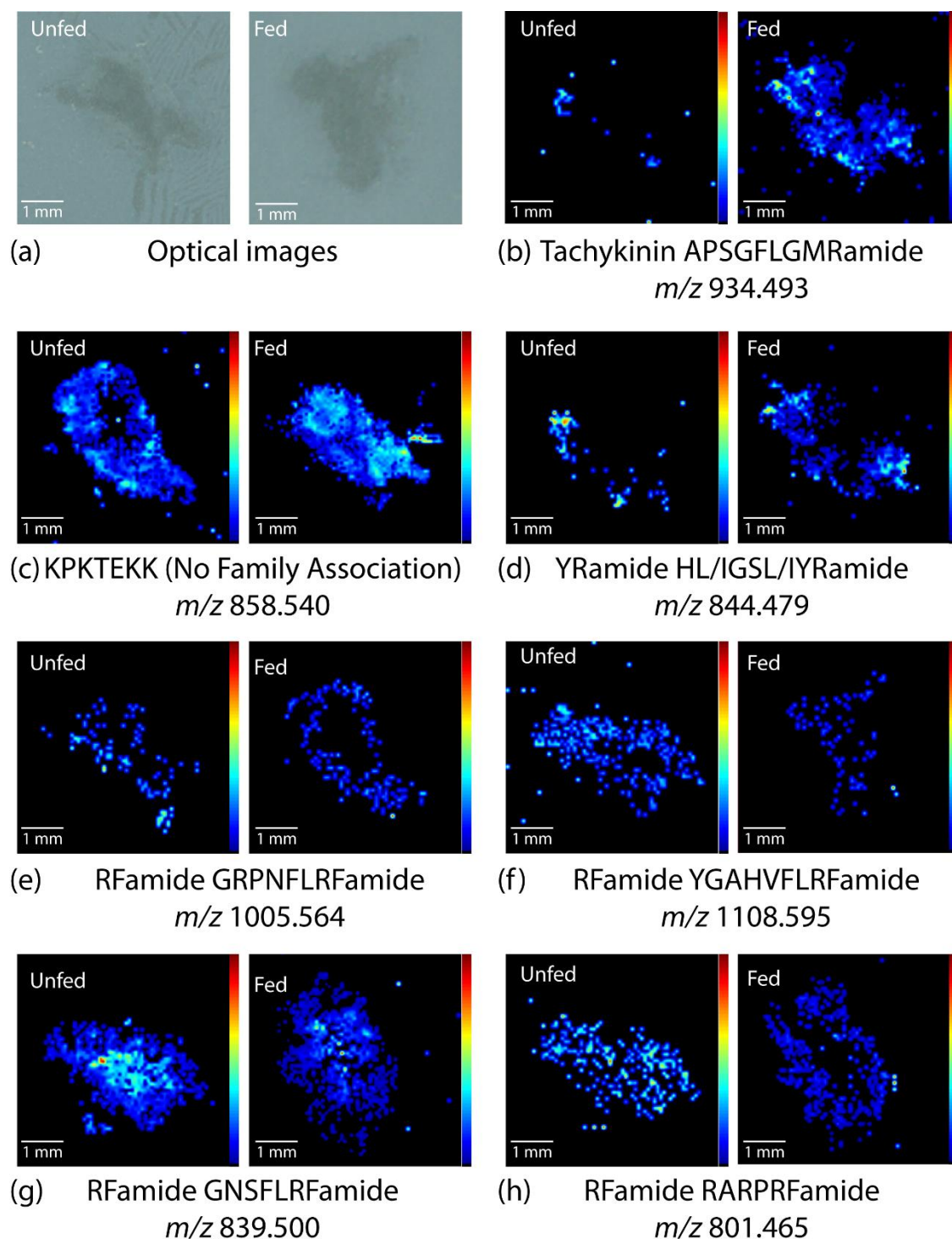


Figure 3. Representative MALDI-MS images of unfed and fed brain sections, including (a) the optical images of the brain section and (b-h) heatmap images of intensity distribution of specific neuropeptides within each tissue. All images are normalized to the total ion current (TIC) and oriented such that the anterior side is top right and posterior side is bottom left. The images show differences in neuropeptide distribution between unfed and fed brain tissue for most

neuropeptides.

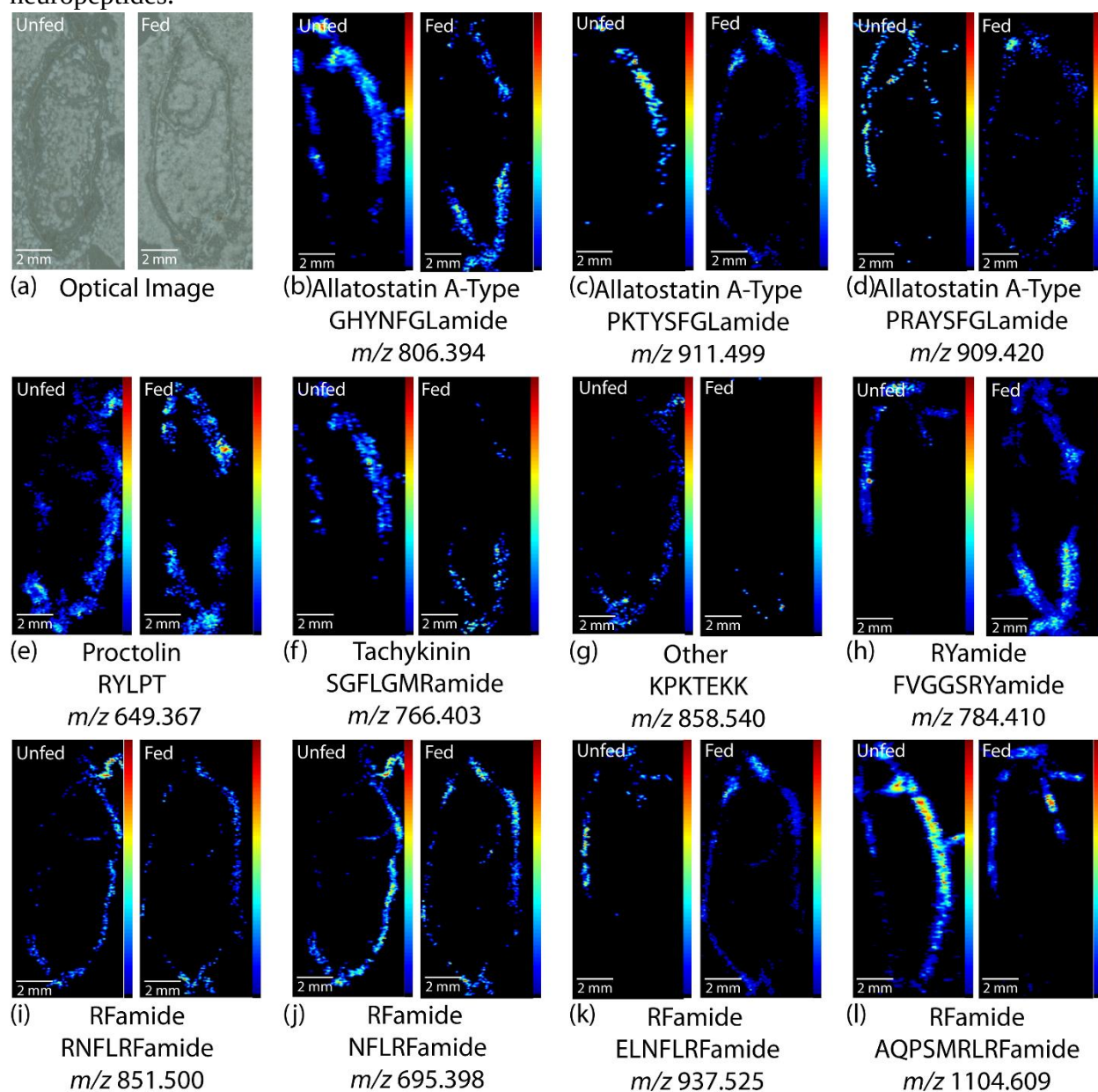


Figure 4. Representative MALDI-MS images of unfed and fed pericardial organs, including (a)

the optical images of the PO and (b-l) heatmap images of intensity distribution of specific neuropeptides within each tissue. All images are normalized to the TIC and oriented such that the anterior side is on top and posterior side is on the bottom. The images show differences between unfed and fed tissue for various neuropeptides, such as distinct differences in presence within the tissue or relative abundance in various areas of the tissue.

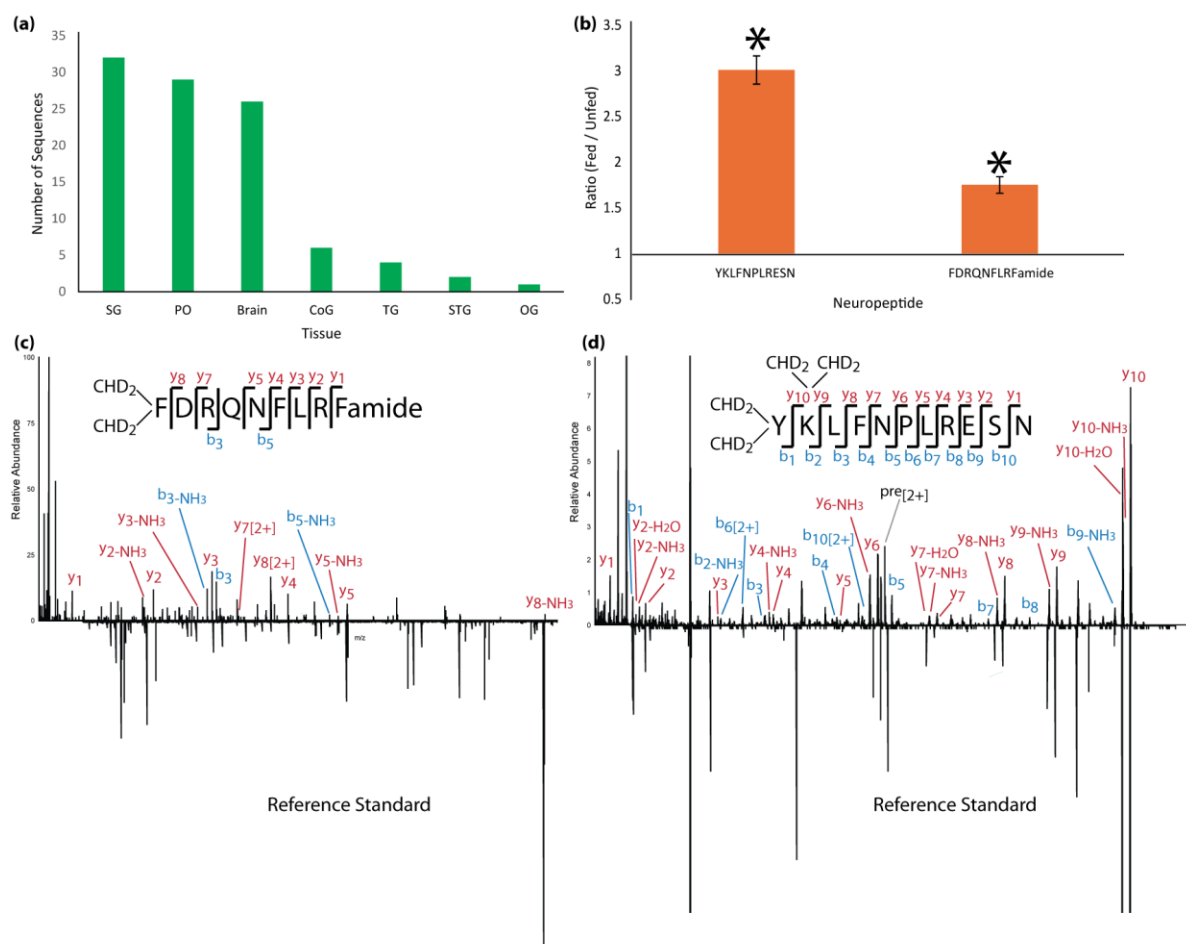


Figure 5. Summary of results of putative novel neuropeptide sequences identified, including (a) the number of neuropeptides identified in each tissue and (b) the relative ratio of two of these neuropeptides in fed and unfed POs that were significantly different ($p < 0.05$). No other novel sequences showed changes that were statistically significant. (c,d) MS/MS spectra mirror plots of the two novel neuropeptides identified with *de novo* sequencing, including (c) RFamide peptide FDRQNFLRFamide and (d) Cryptocyanin-like peptide YKLFNPLRESN. The top spectrum shows the experimentally-obtained spectrum of each from tissue extract and the bottom mirrored spectrum shows the spectrum obtained from a commercially-synthesized standard.

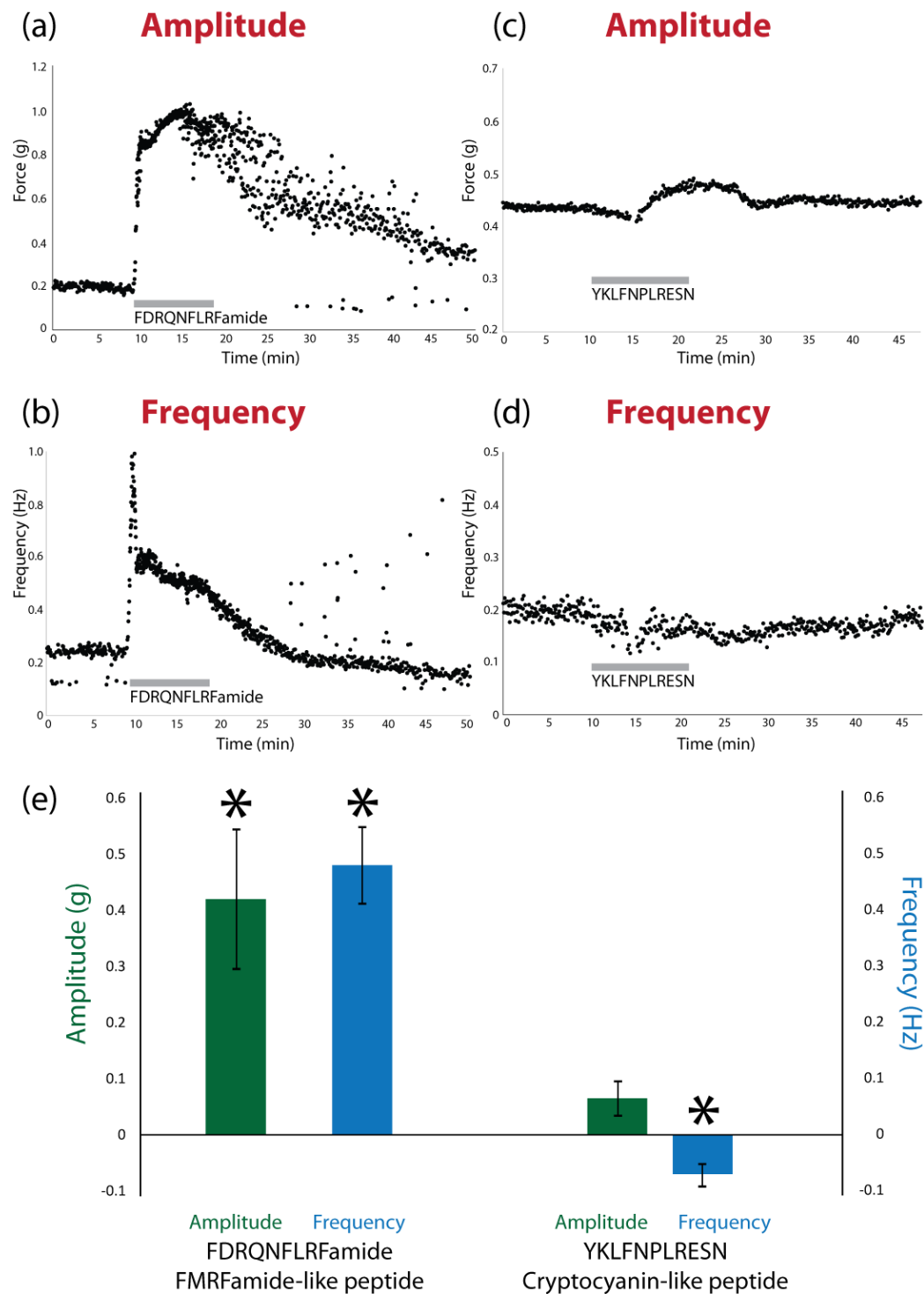


Figure 6. Results from functional assessment of synthesized peptides on ex vivo whole heart preparations, including examples of the change in amplitude and frequency during perfusion of each peptide (a-d) and the average change in amplitude and frequency for each peptide, relative to controls, across all biological replicates (* $p < 0.05$, Student's Paired Two-Tailed T-Test; $n =$

10 for FDRQNFLRFamide, $n = 5$ for YKLFNPLRESN). The average control amplitude was 0.17 g and the average control frequency was 0.20 Hz across all replicates ($n = 15$).

Supporting Information I

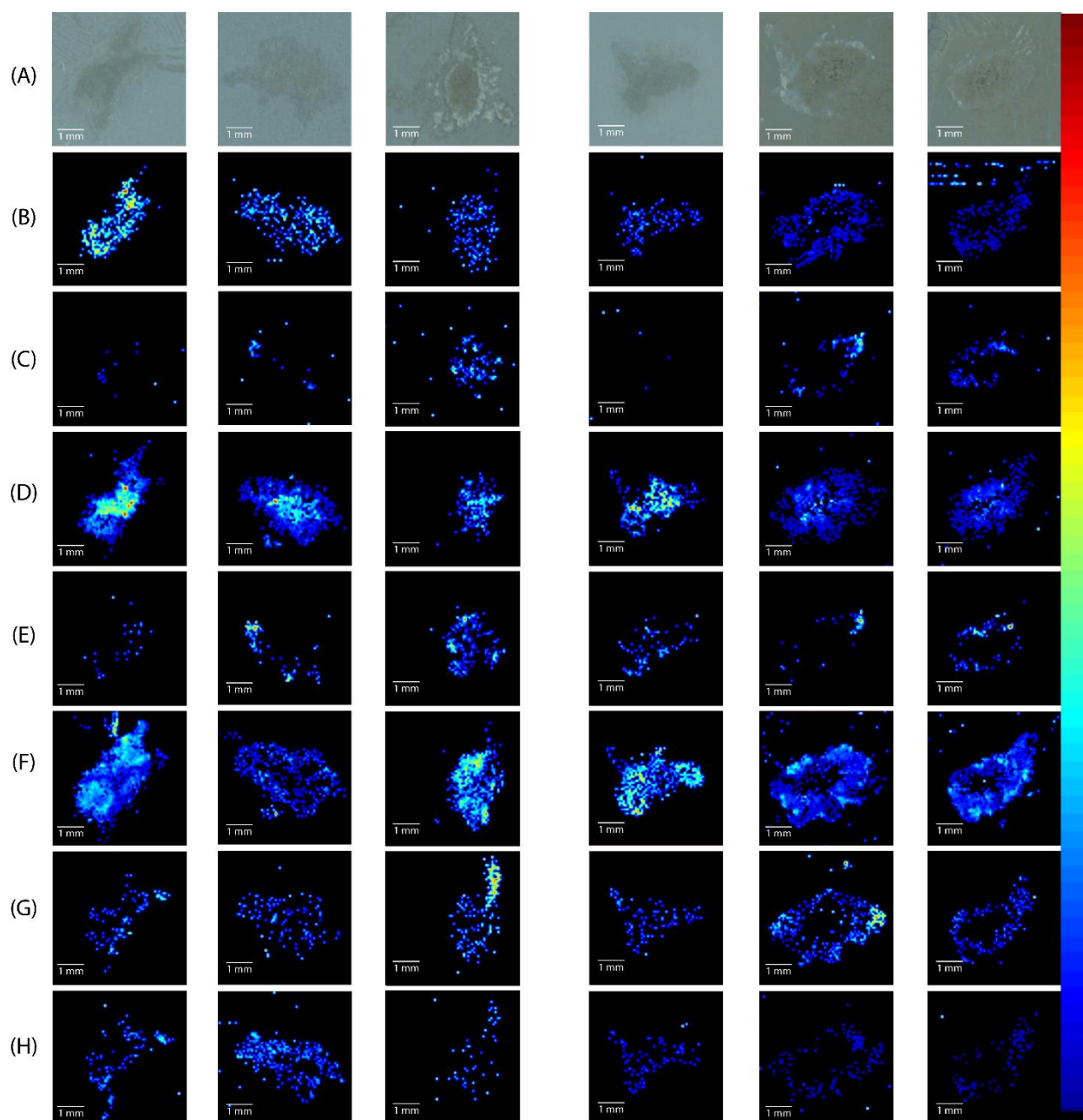


Figure S1. MALDI-MS images of brain sections from all biological replicates from fed and unfed animals, including (A) optical images and MS images of neuropeptides (B) APSGFLGMRamide, (C) KPKTEKK, (D) HL/IGSL/IYRamide, (E) GRPNFLRFamide, (F) YGAHVFLRFamide, (G) GNSFLRFamide, and (H) RARPRFamide. The first three rows represent the three biological replicates from unfed animals and the second three rows represent the three biological replicates from fed animals.

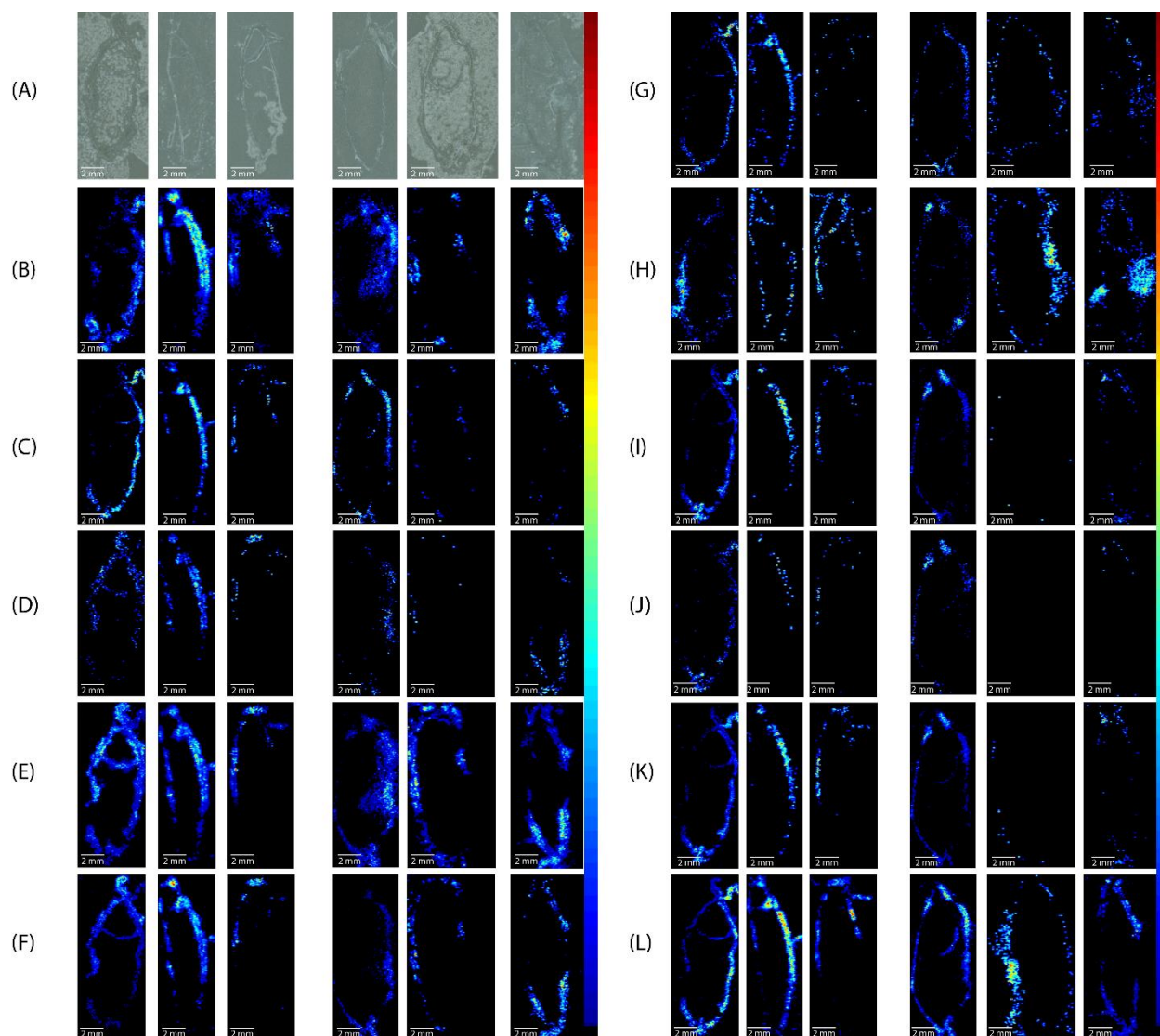


Figure S2. MALDI-MS images of pericardial organs from all biological replicates from fed and unfed animals, including (A) optical images and MS images of neuropeptides (B) GHYNFGLamide, (C) PKTYSFGLamide, (D) PRAYSFGLamide, (E) RYLPT, (F) SGFLGMRamide, (G) KPKTEKK, (H) FVGGSRYamide, (I) RNFLRFamide, (J) NFLRFamide, (K) ELNFLRFamide, and (L) AQPSMRLRFamide. The first three rows represent the three biological replicates from unfed animals and the second three rows represent the three biological replicates from fed animals.

Table S1. Putative sequences of neuropeptides detected in tissue extracts with *de novo* sequencing. Peptides are grouped by the families based on their characteristic sequence motifs, and peptides listed as “other” are ones that don’t have a sequence motif characteristic of a specific family but have a high degree of sequence homology to previously-identified neuropeptides. The tissue that each peptide was identified in is indicated with the average local confidence (ALC) listed in parentheses. * represents peptide does not have the exact sequence motif of that family but is highly similar to other neuropeptides belonging to that family. Pericardial organ (PO), sinus gland (SG), oesophagul ganglion (OG), thoracic ganglion (TG), stomatogastric ganglion (STG), commissural ganglion (CoG).

Family	Sequence	Molecular	
		Weight	Tissue (% ALC)
AST-A	TADLYEFGlamide	1026.491	SG (76%) PO (75%)
	LWAYSFGlamide	924.507	PO (76%)
	SVKYSFGlamide	898.48	Brain (78%)
	KSKYSFGlamide	927.507	Brain (82%)
	KDYSFGlamide	827.407	PO (76%)
	YKLFEPGL*	1078.595	PO (81%)
RFamide	STFLRFamide	768.417	CoG (78%)
	QDLHDNFLRFamide	1302.636	CoG (76%)
	SNKNFLRFamide	1023.55	CoG (80%) SG (78%)
	AYDKSFLRFamide	1144.592	PO (83%) SG (77%)
	AYKDSFLRFamide	1144.592	Brain (85%) PO (83%)
	SKSNFLRFamide	996.539	Brain (84%) PO (79%) SG (81%)

SNNNFLRFamide	1009.498	Brain (78%) CoG (75%) SG (78%)
TNRDFLRFamide	1066.556	SG (97%)
SDPNFLRFamide	993.492	SG (77%)
RDLNFLRFamide	107.593	Brain (80%)
SPNNFLRFamide	992.508	Brain (77%)
SPTNFLRFamide	979.513	SG (78%)
SPDNFLRFamide	993.492	SG (78%)
WPQRDFLRFamide	1262.656	SG (97%)
SGFLRFamide	724.391	Brain (92%) PO (82%) SG (89%)
EHNFLRFamide	960.482	STG (77%)
RPPNFLRFamide	1044.587	Brain (76%)
SEQRDFLRFamide	1195.599	Brain (88%) SG (90%)
SHKNFLRFamide	1046.566	Brain (82%) PO (75%) SG (75%)
RSPSNFLRFamide	1121.598	PO (77%)
RLNFLRFamide	963.566	PO (77%)
GNSNFLRFamide	952.477	PO (80%)
CPEKNFLRFamide	1151.58	PO (80%)
RFNFLRFamide	997.55	SG (79%)
NPRKNFLRFamide	1189.672	PO (75%)
RKNFLRFamide	978.577	PO (79%)
SPPNFLRFamide	975.518	PO (81%)
GTFLRFamide	738.407	PO (95%)
QKNFLRFamide	950.534	PO (78%)
SGWNFLRFamide	1024.513	Brain (75%)
WKNFLRFamide	1008.555	Brain (86%) SG (81%)

	SPYNFLRFamide	1041.529	Brain (75%)
	RPSKDYLRFamide	1179.64	Brain (76%)
	LPFLRFamide	790.474	Brain (75%)
	KWNFLRFamide	1008.555	Brain (85%) CoG (84%) SG (83%) PO (80%)
	LPPQRDFLRFamide	1286.714	PO (77%)
	VKSNFLRFamide	1008.576	SG (81%)
	ACKNFLRFamide	996.522	SG (82%)
	LDRQNFLRFamide	1206.651	Brain (87%) SG (86%)
	AKSNFLRFamide	980.545	SG (78%)
	SPFNFLRFamide	1025.534	Brain (75%) SG (75%) PO (75%)
	SMNNFLRFamide	1026.496	SG (82%)
	YKNFLRFamide	985.539	SG (86%)
	THKNFLRFamide	1060.582	SG (75%)
	KELNFLRFamide	1064.602	SG (75%)
	SYNNFLRFamide	1058.519	SG (78%)
	NGRDNFLRFamide	1136.573	SG (77%)
	FDRQNFLRFamide	1240.635	PO (81%)
	HGDVNFLRFamide	1102.556	SG (78%)
RYamide	YYANRYamide	847.387	PO (89%)
	SGKYANRYamide	956.472	PO (82%)
	GEWYSQRYamide	1086.477	PO (80%)
	MWYSQRYamide	1034.454	PO (91%)
	TWYSQRYamide	1001.461	SG (89%)
Other	YKLFEPLRESEamide	1408.724	PO (96%)
	YKLFNPLRE	1178.634	Brain (92%) PO (82%)

		Brain (99%) CoG (84%) PO (96%) SG (98%) TG
DLPKVDSVLK	1112.633	(99%)
DLPKVE	699.369	Brain (93%) TG (97%)
YKLFEPLWL	1207.653	Brain (97%) PO (91%)
YKLFEPLW	1207.653	PO (91%)
YKLFNPLRESN	1379.709	PO (82%)
DLPKVDVSLK	1112.633	Brain (91%) SG (90%) TG (90%) OG (90%)
YKLFNPLR	1049.591	Brain (92%) TG (96%)

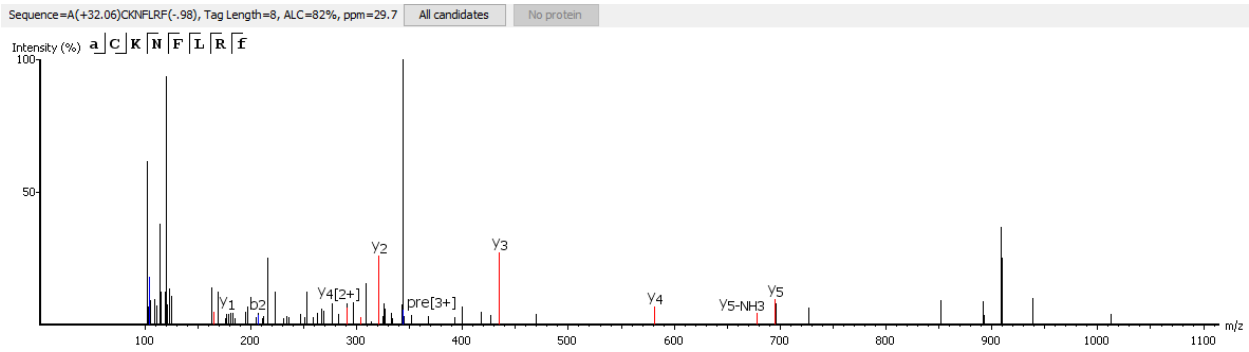


Figure S3. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence ACKNFLRFamide.

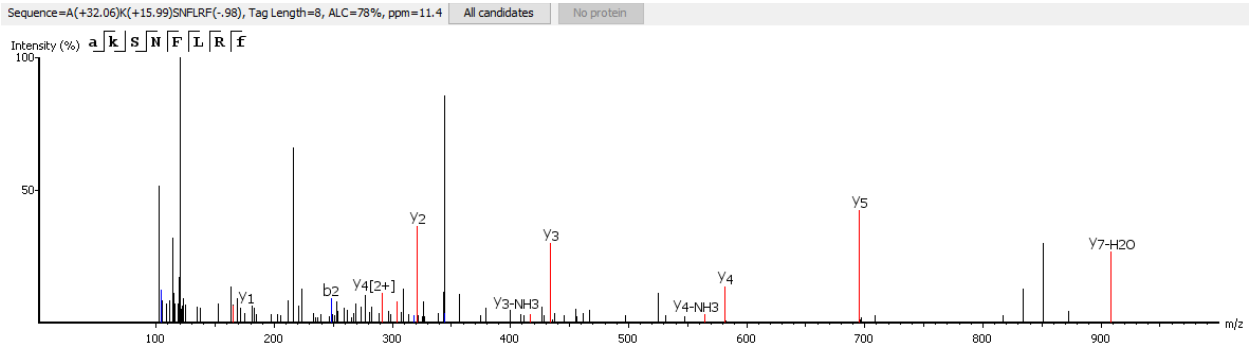


Figure S4. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence AKSNFLRFamide.

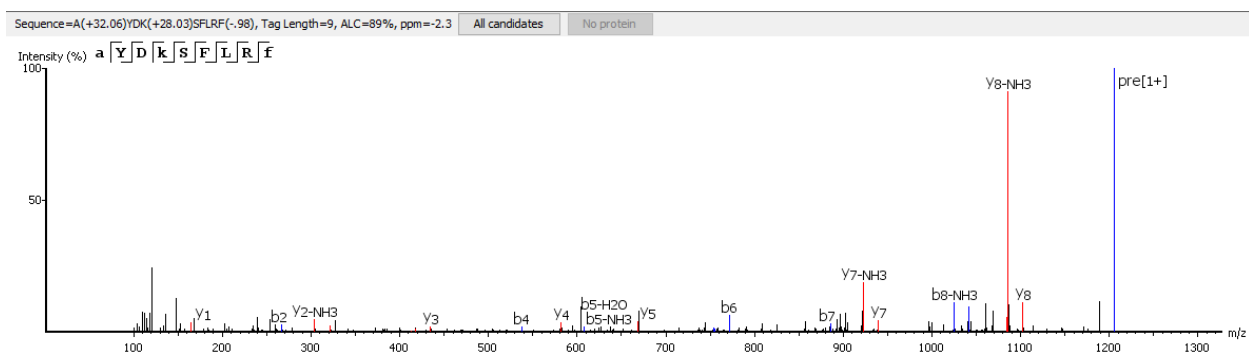


Figure S5. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence AYDKSFLRFamide.

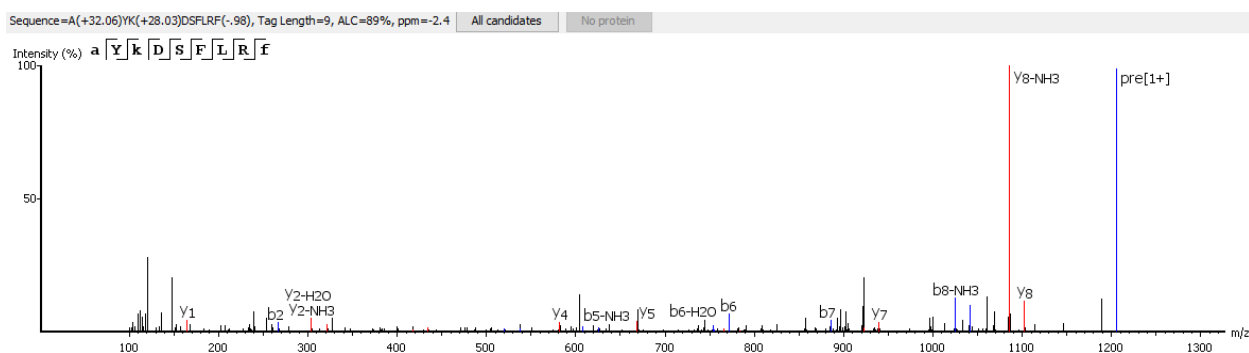


Figure S6. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence AYKDSFLRFamide.

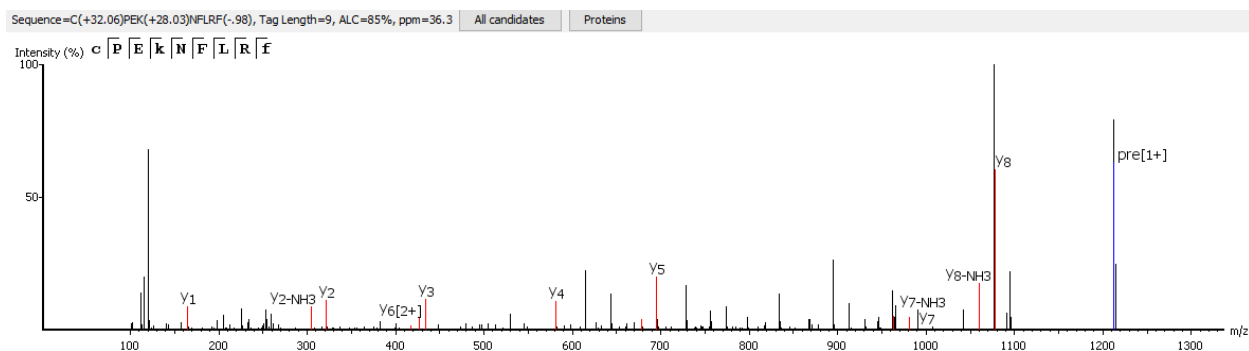


Figure S7. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence CPEKNFLRFamide.

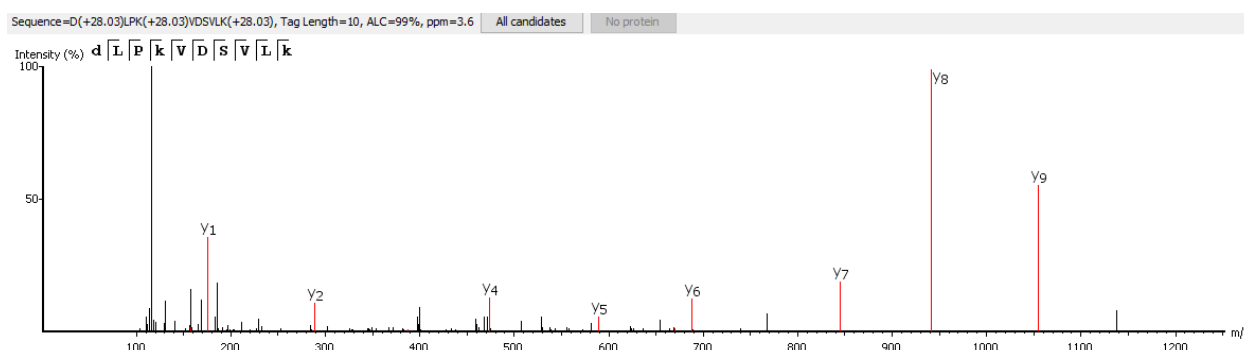


Figure S8. MS/MS spectrum from PEAKS software of a putative neuropeptide with the sequence DLPKVDSVLK that does not match the sequence motifs of common neuropeptide families.

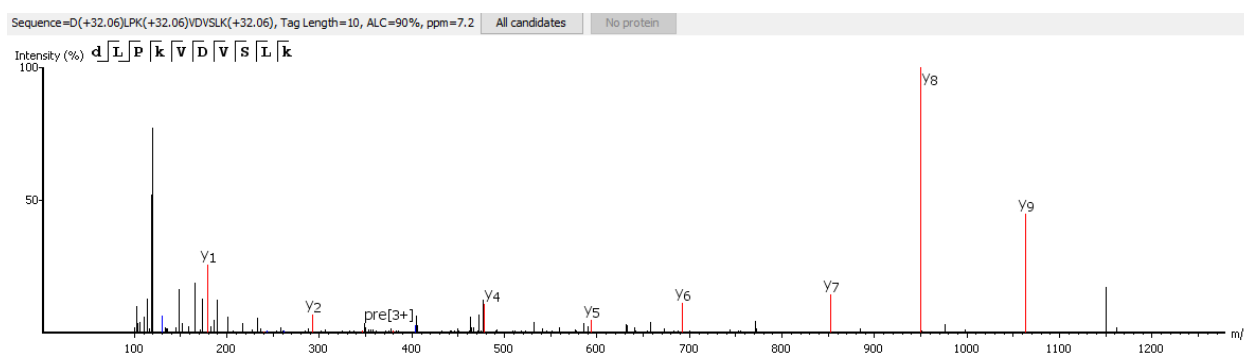


Figure S9. MS/MS spectrum from PEAKS software of a putative neuropeptide with the sequence DLPKVDVSLK that does not match the sequence motifs of common neuropeptide families.

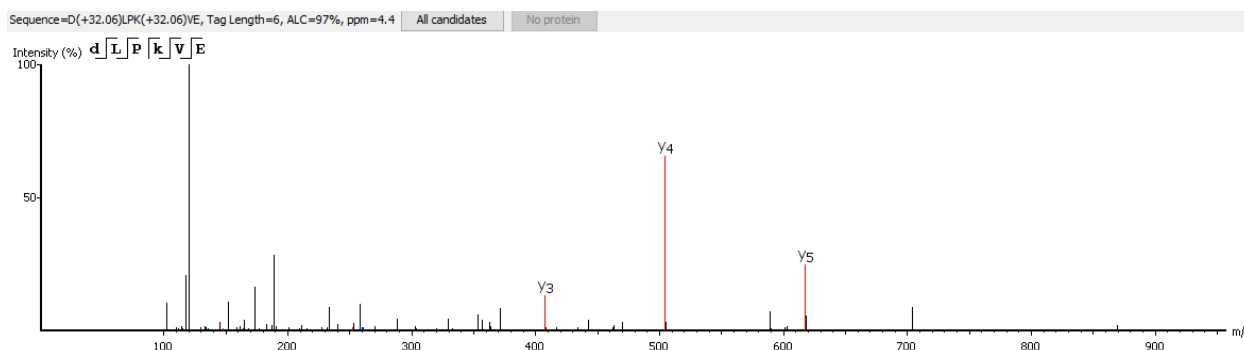


Figure S10. MS/MS spectrum from PEAKS software of a putative neuropeptide with the sequence DLPKVE that does not match the sequence motifs of common neuropeptide families.

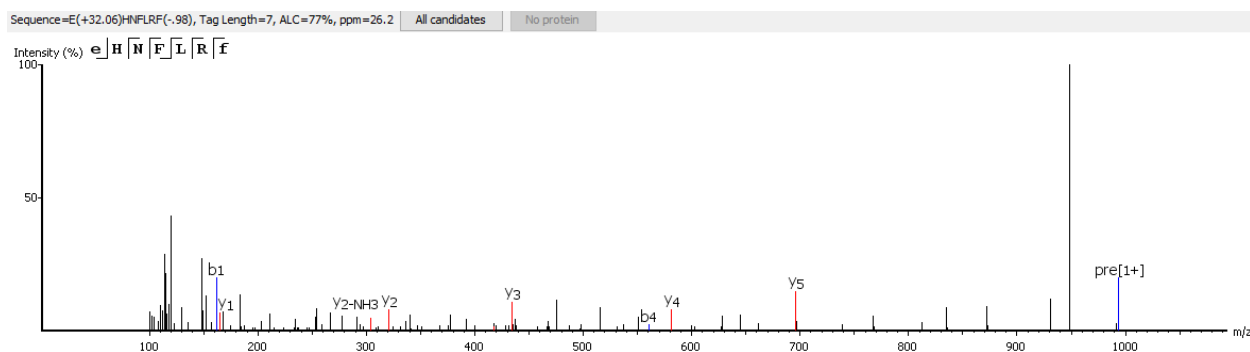


Figure S11. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence EHNFLRFamide.

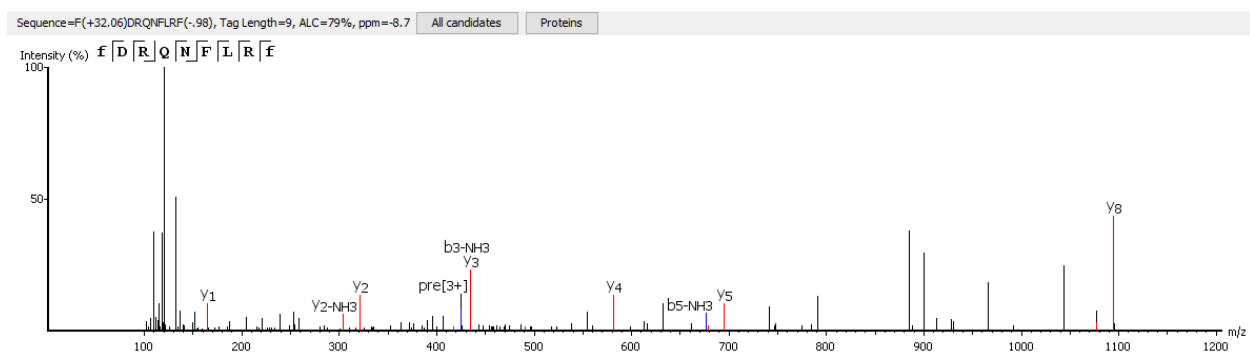


Figure S12. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence FDRQNFLRFamide.

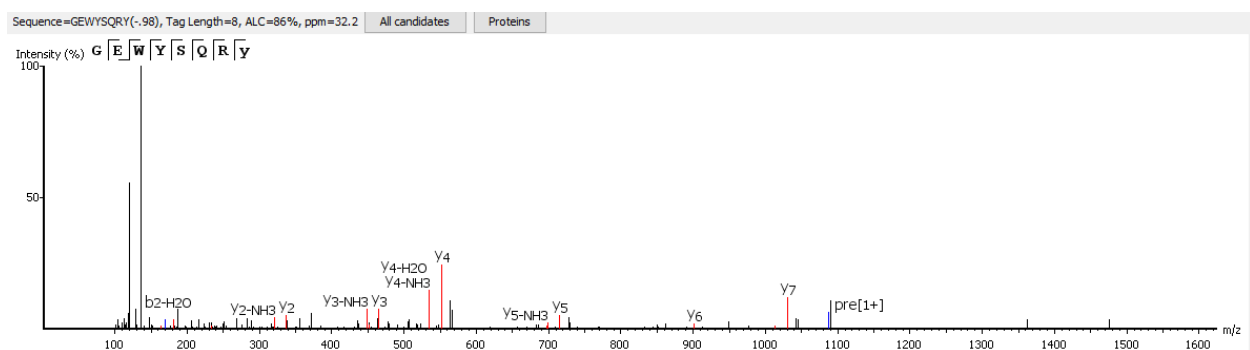


Figure S13. MS/MS spectrum from PEAKS software of a putative RYamide neuropeptide with the sequence GEWYSQRYamide.

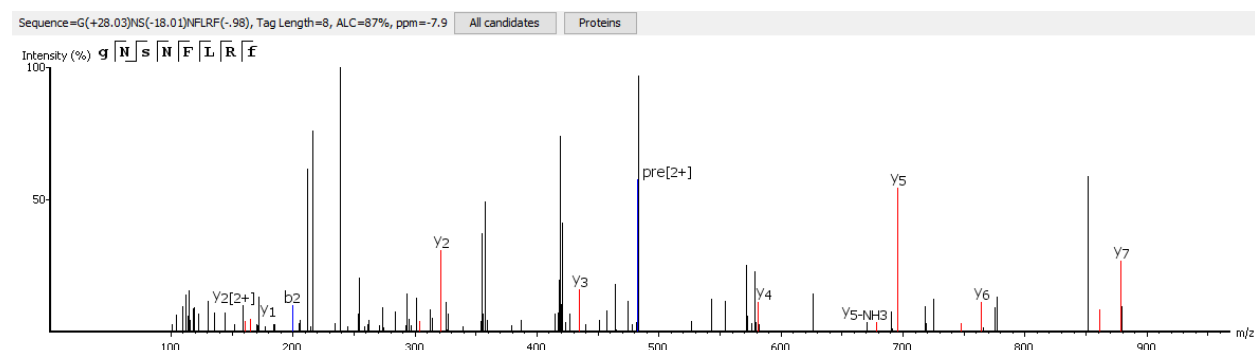


Figure S14. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence GNSNFLRFamide.

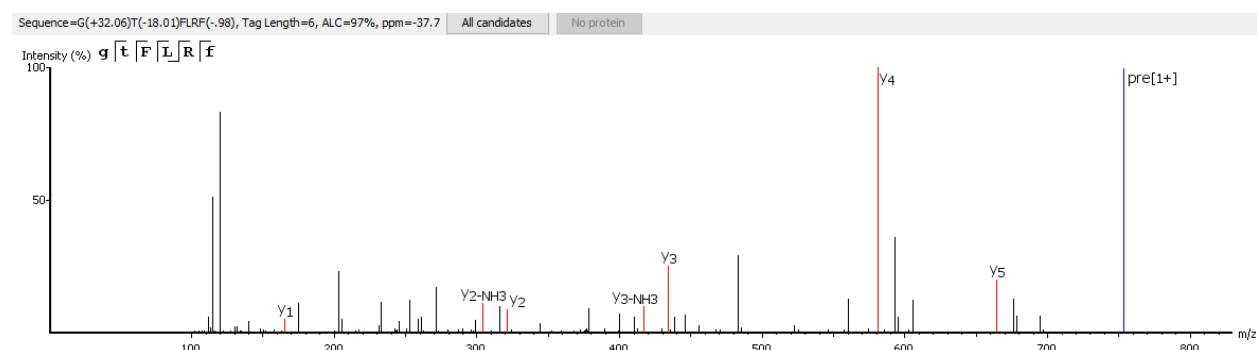


Figure S15. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence GTFLRFamide.

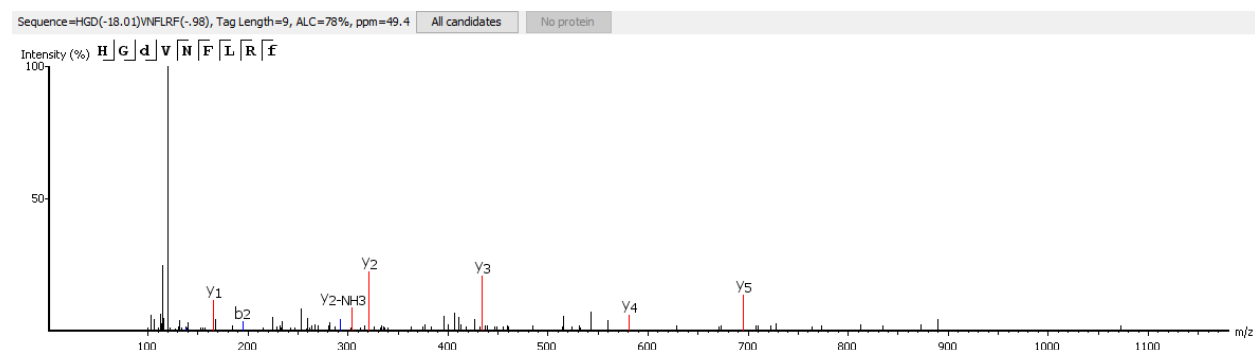


Figure S16. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence HGDVNFLRFamide.

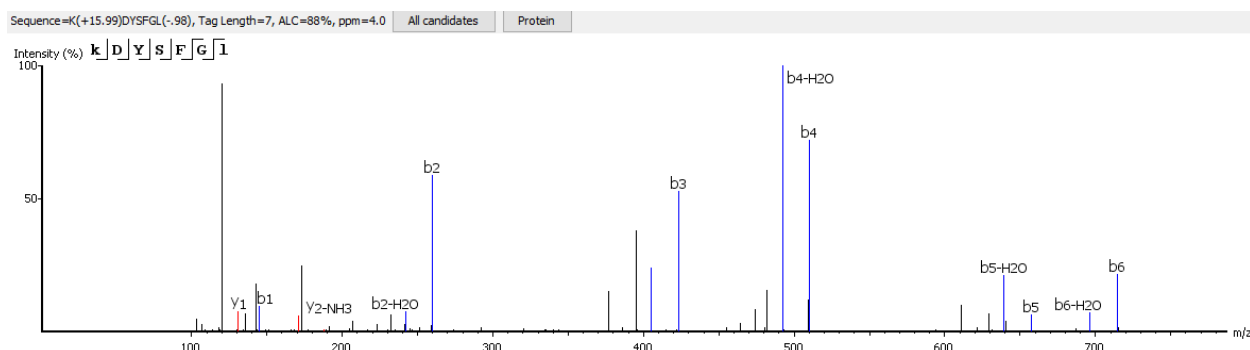


Figure S17. MS/MS spectrum from PEAKS software of a putative allatostatin A-type neuropeptide with the sequence KDYSFGLamide.

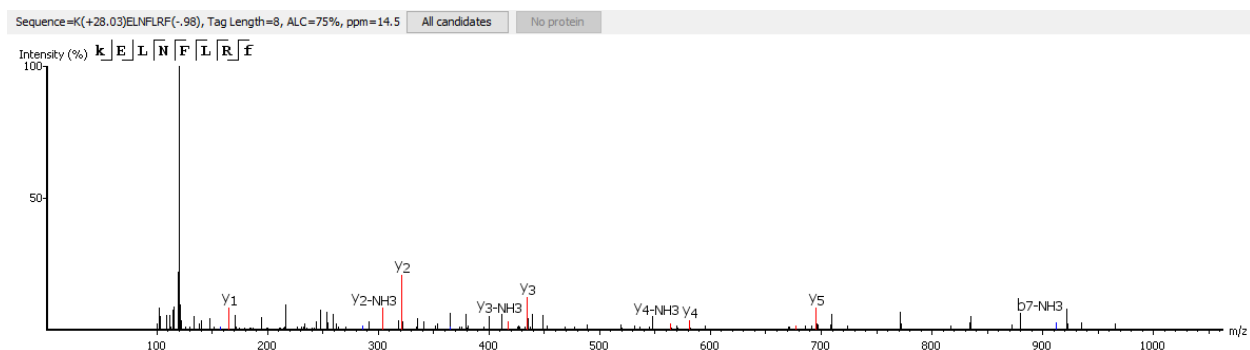


Figure S18. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence KELNFLRFamide.

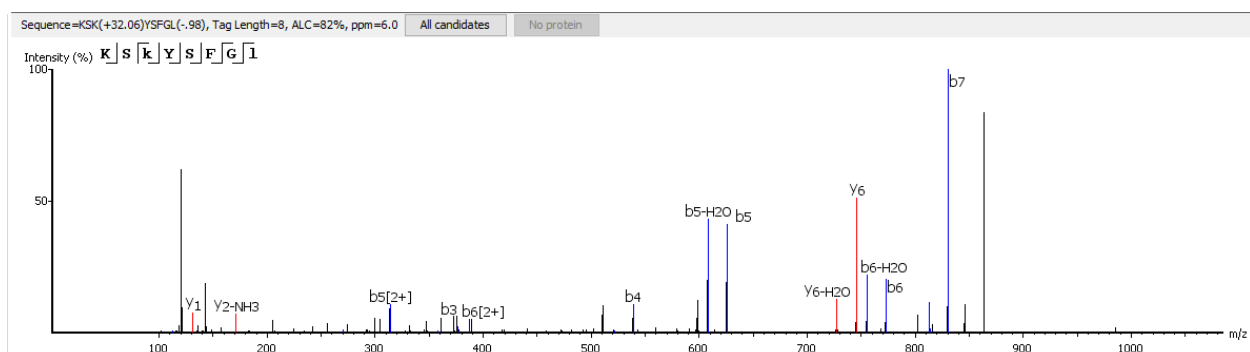


Figure S19. MS/MS spectrum from PEAKS software of a putative allatostatin A-type

neuropeptide with the sequence KSKYSFGLamide.

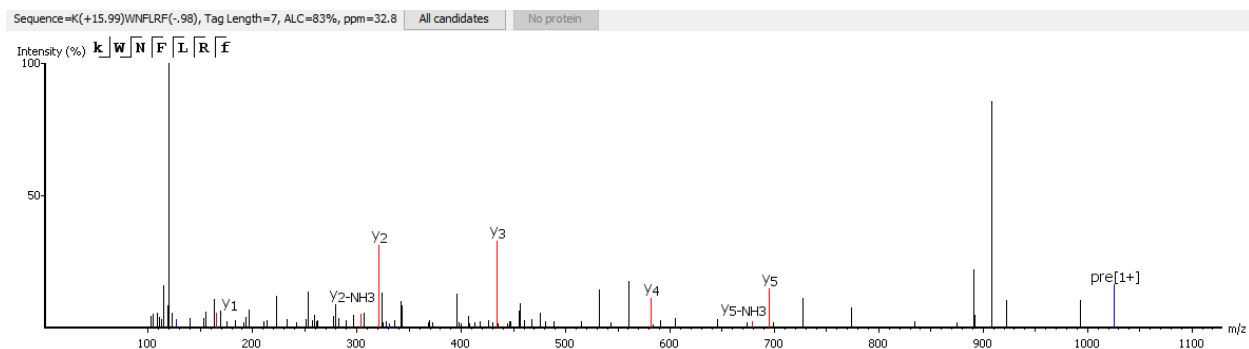


Figure S20. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence KWNFLRFamide.

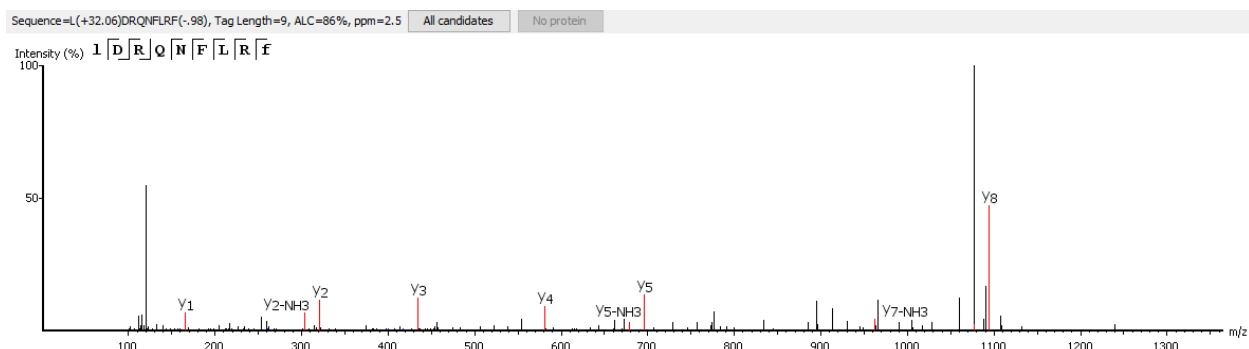


Figure S21. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence LDRQNFLRFamide.

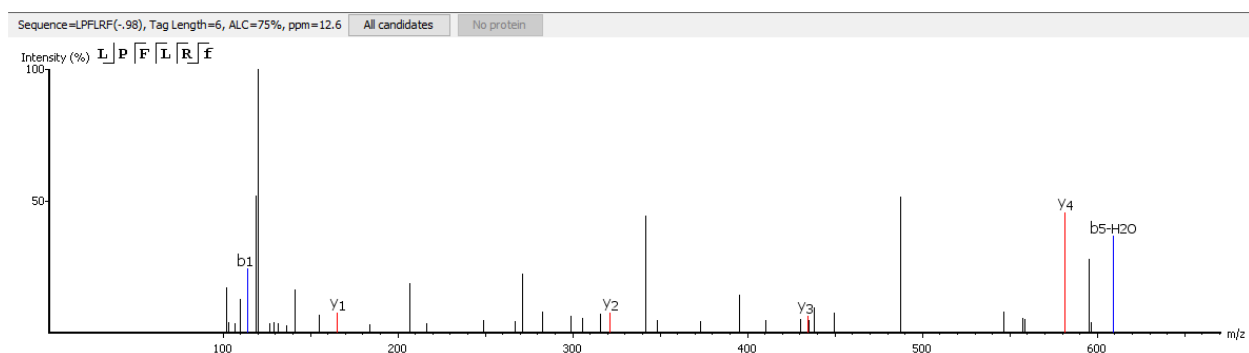


Figure S22. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence LPFLRFamide.

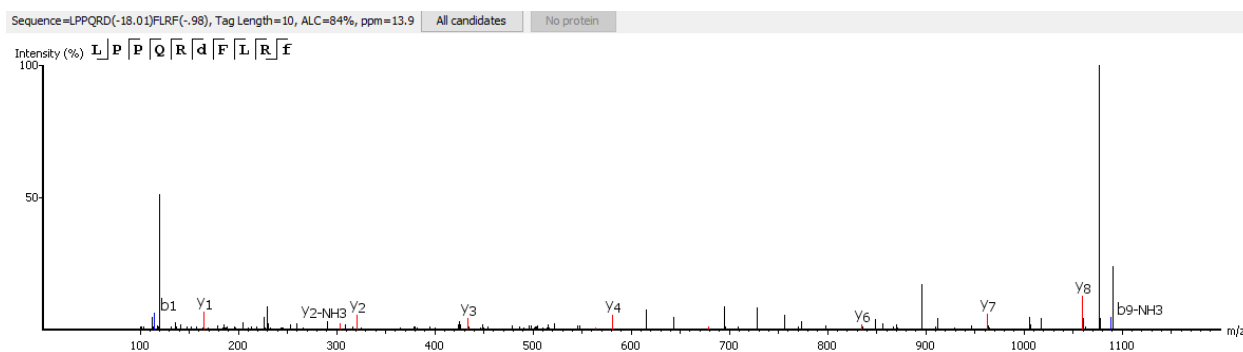


Figure S23. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence LPPQRDFLRamide.

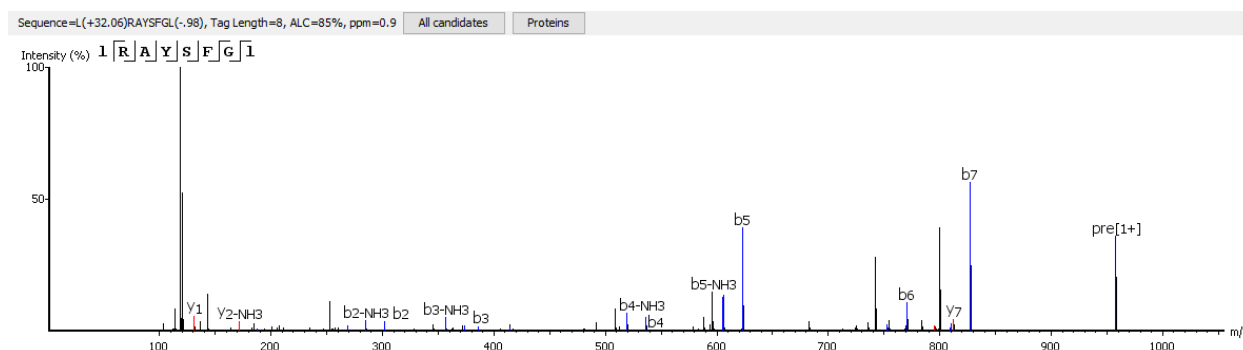


Figure S24. MS/MS spectrum from PEAKS software of a putative allatostatin A-type neuropeptide with the sequence LRAYSFGLamide.

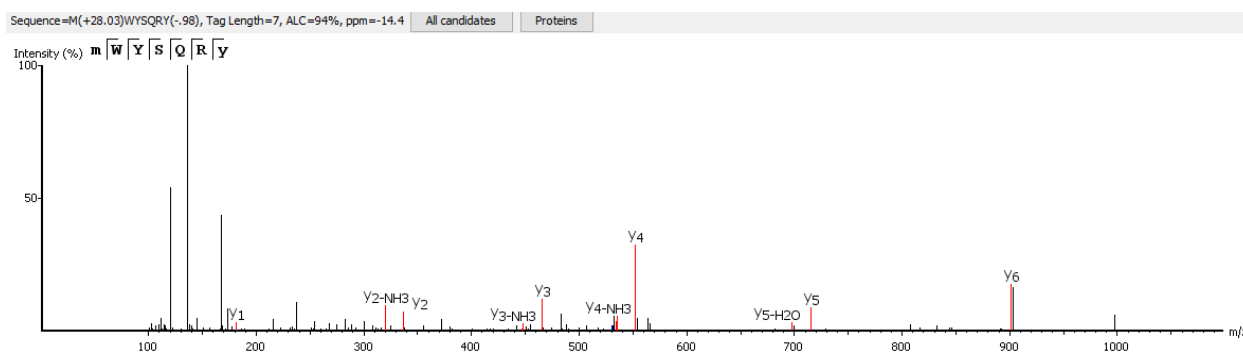


Figure S25. MS/MS spectrum from PEAKS software of a putative RYamide neuropeptide with the sequence MWYSQRYamide.

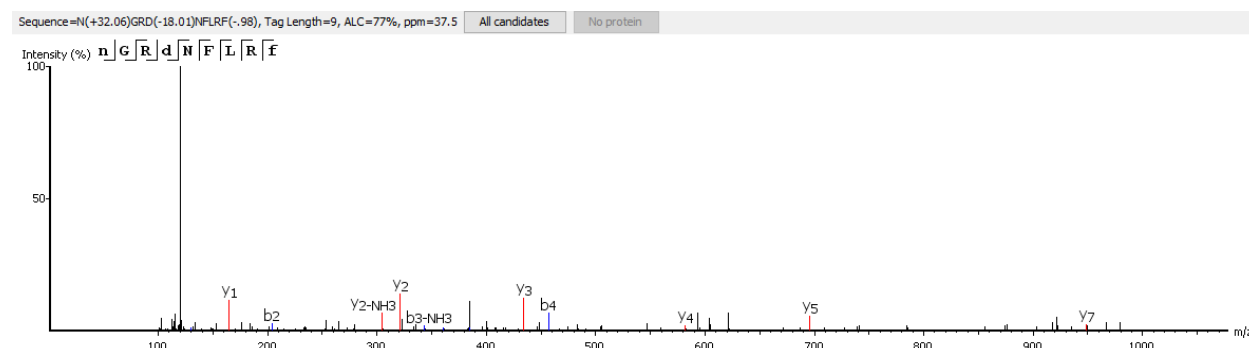


Figure S26. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence NGRDNFLRFamide.

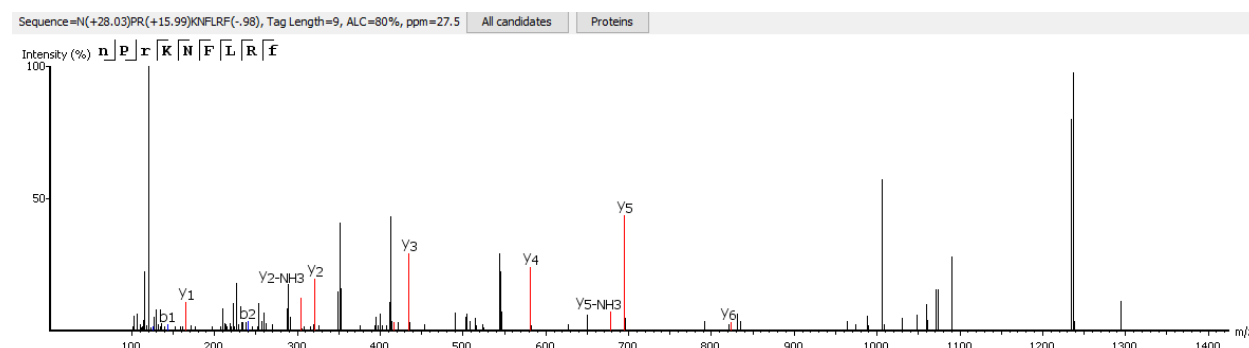


Figure S27. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence NPRKNFLRFamide.

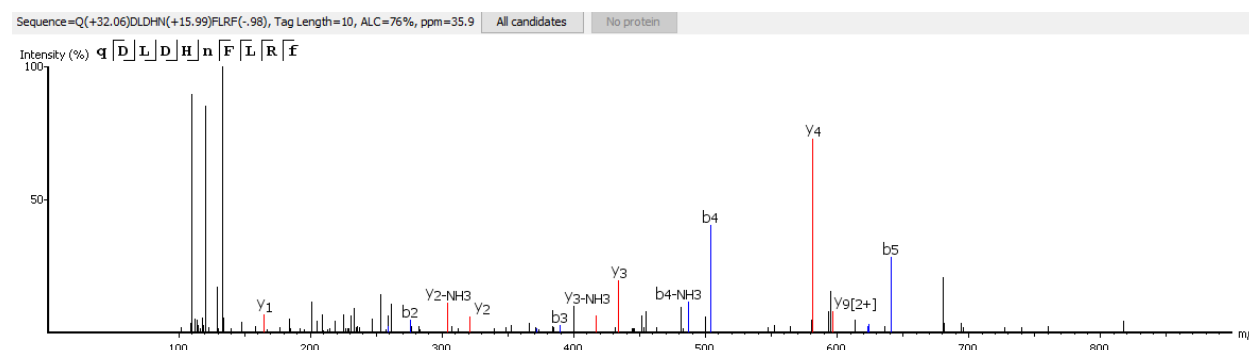


Figure S28. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence QDLHDHNFLRFamide.

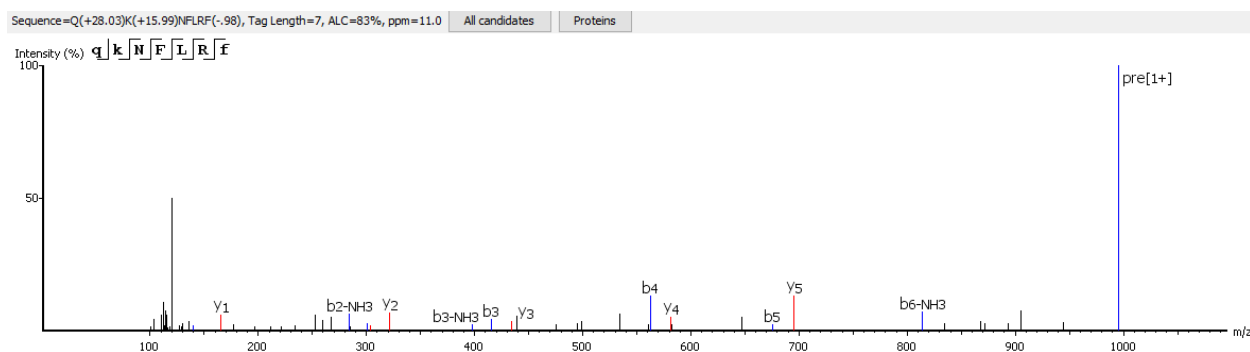


Figure S29. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence QKNFLRFamide.

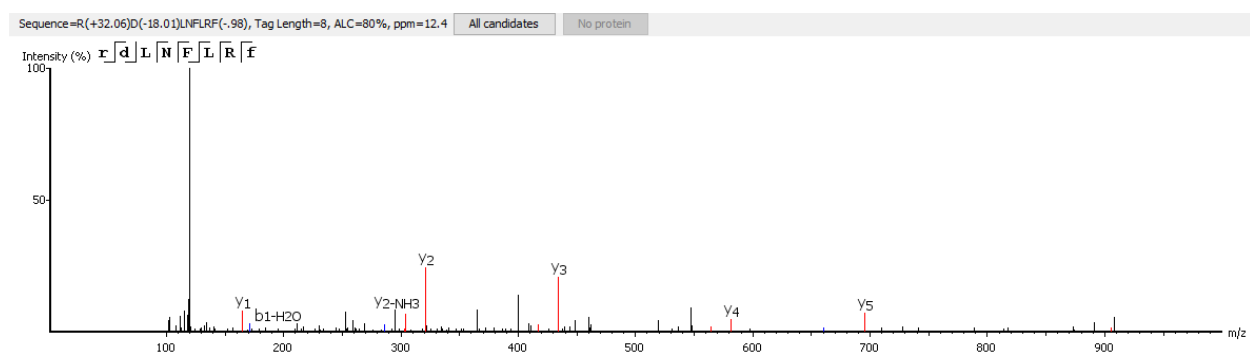


Figure S30. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence RDLNFLRFamide.

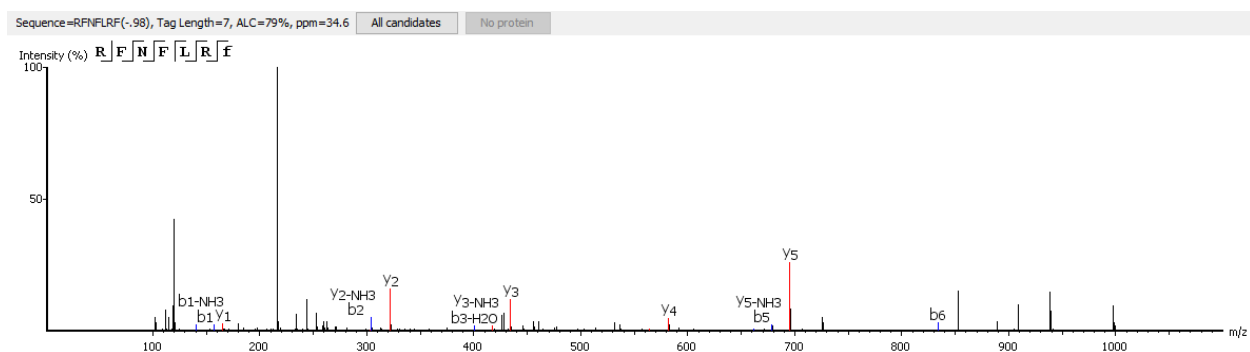


Figure S31. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence RNF₁LRFamide.

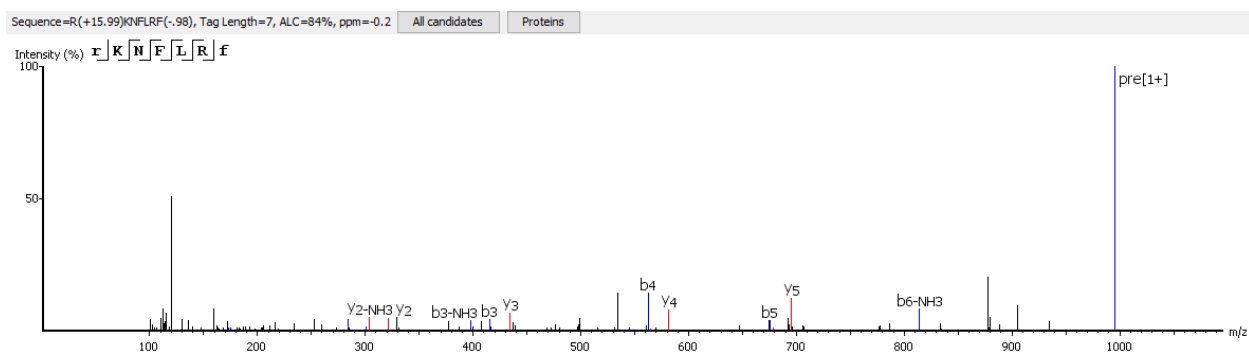


Figure S32. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence RKN₁FLRFamide.

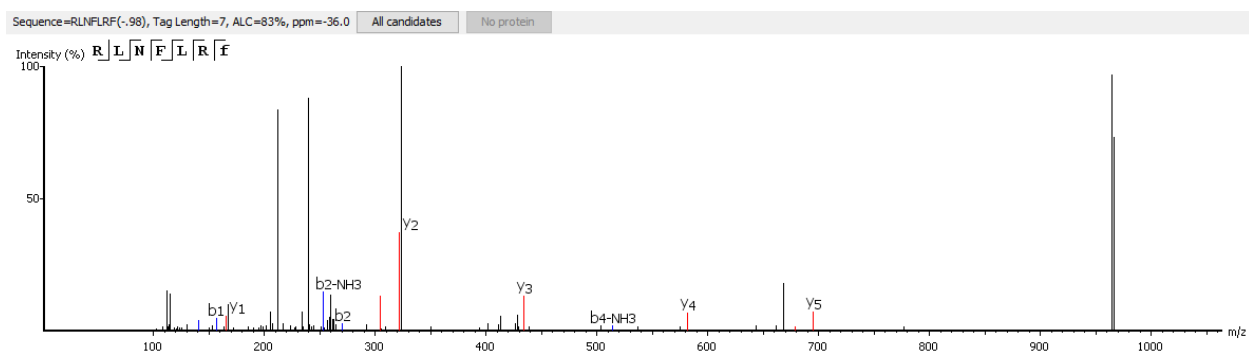


Figure S33. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence RLN₁FLRFamide.

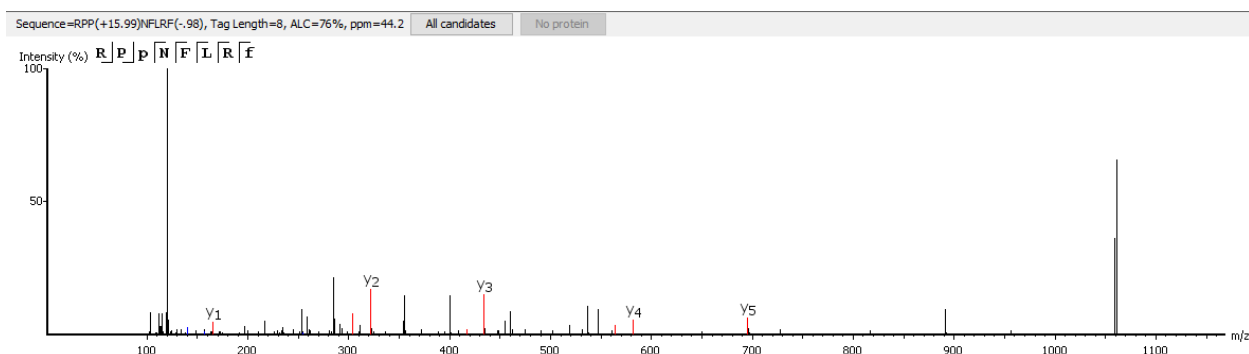


Figure S34. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence RPPN₁FLRFamide.

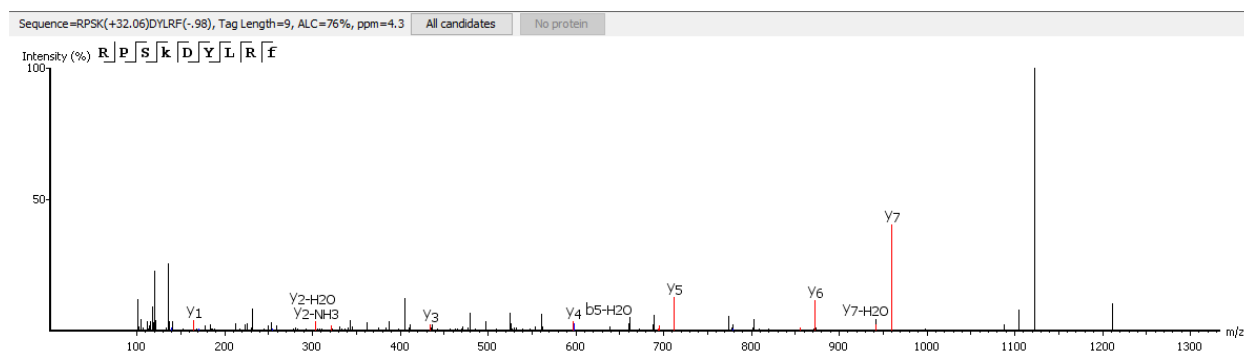


Figure S35. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence RPSKDYLRamide.

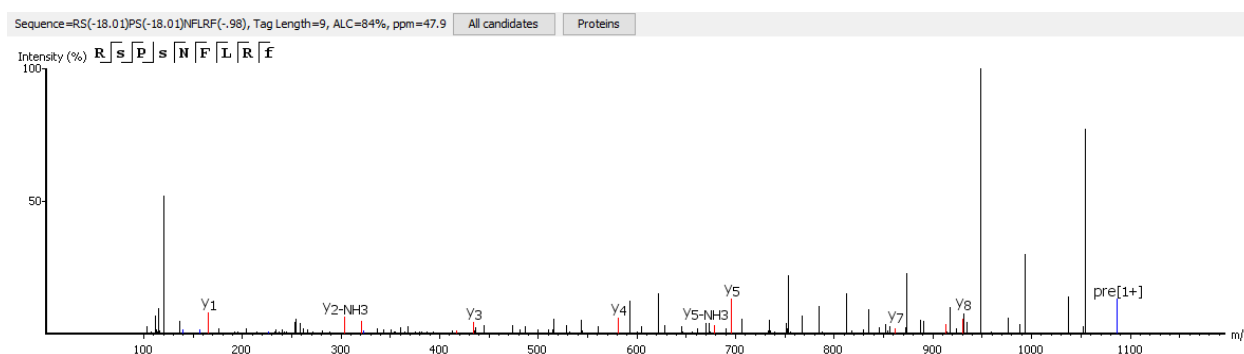


Figure S36. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence RSPSNFLRamide.

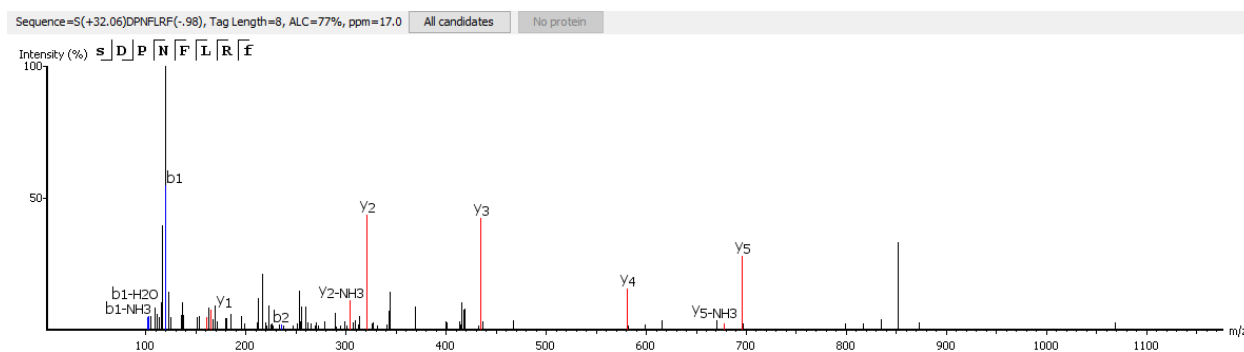


Figure S37. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SDPNFLRamide.

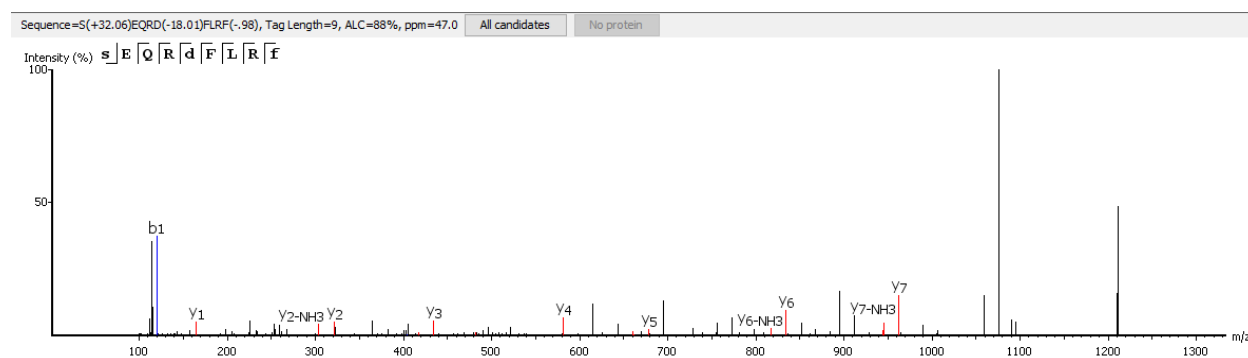


Figure S38. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SEQRDFLRFamide.

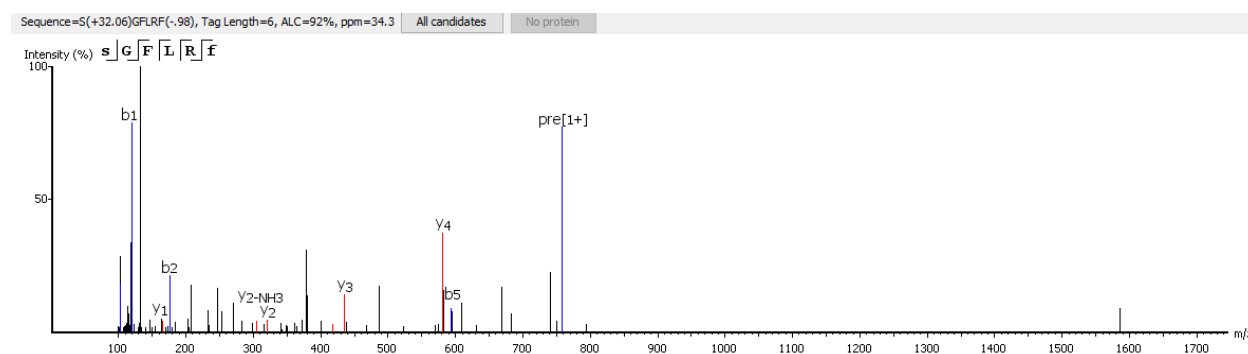


Figure S39. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SGFLRFamide.

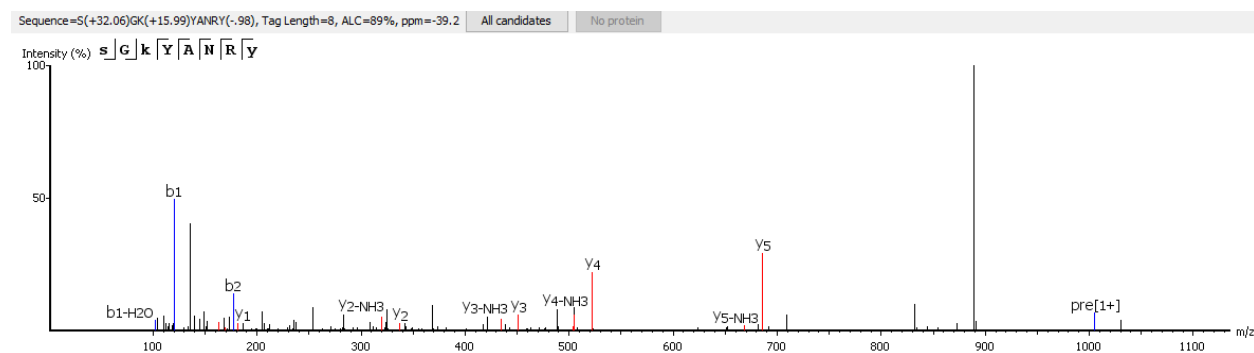


Figure S40. MS/MS spectrum from PEAKS software of a putative RYamide neuropeptide with the sequence SGKYANRYamide.

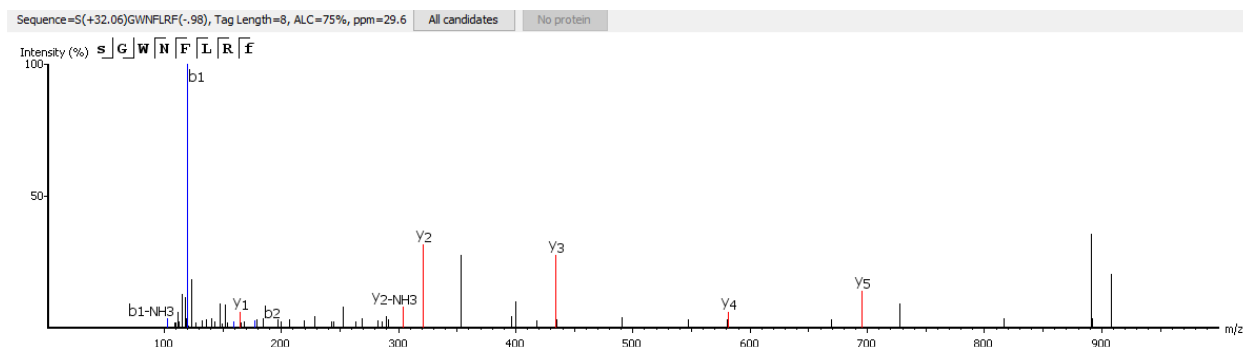


Figure S41. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SGWNFLRFamide.

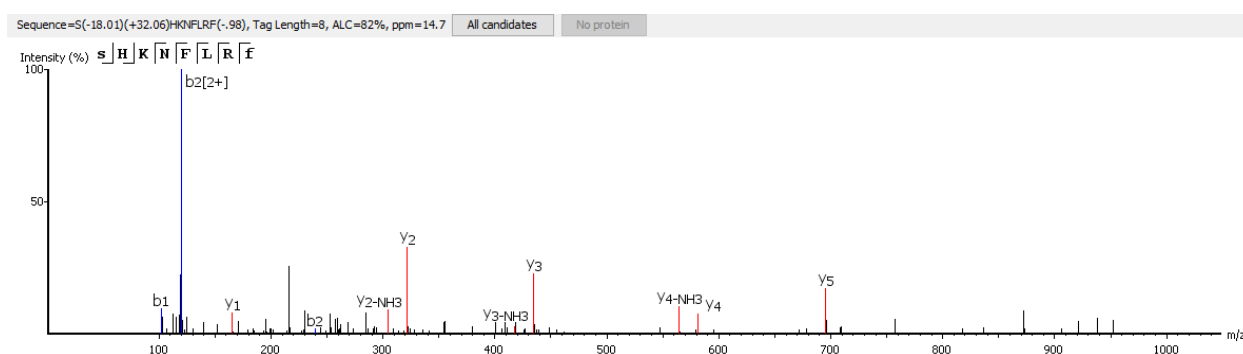


Figure S42. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SHKNFLRFamide.

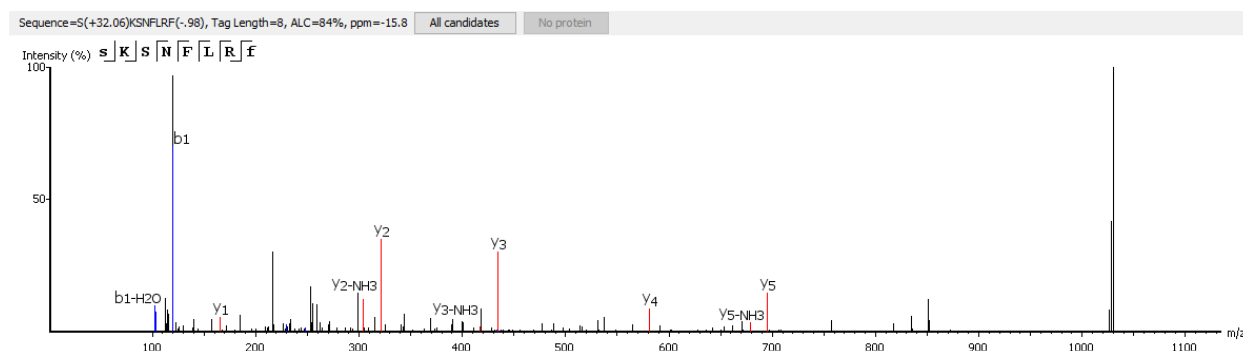


Figure S43. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SKSNFLRFamide.

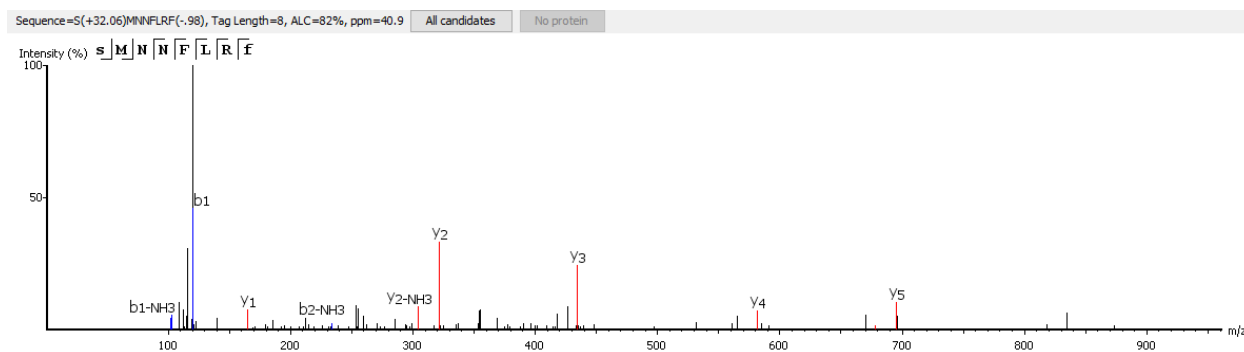


Figure S44. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SMNNFLRFamide.

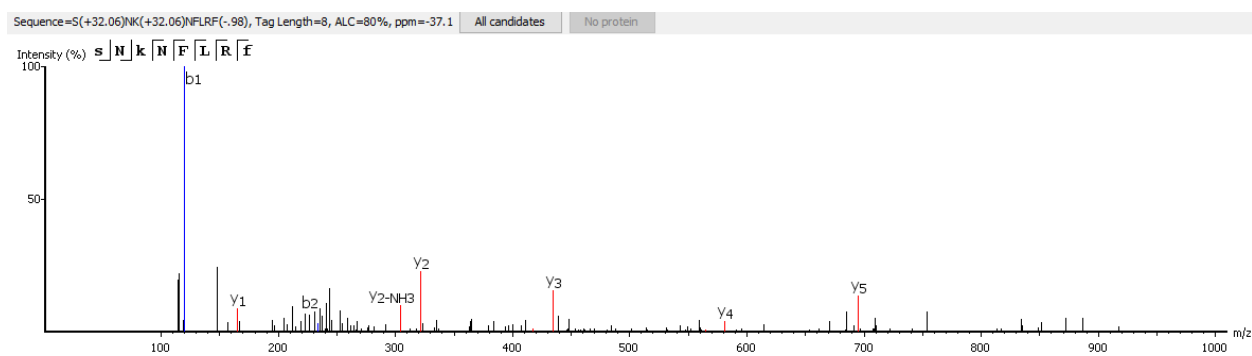


Figure S45. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SNKNFLRFamide.

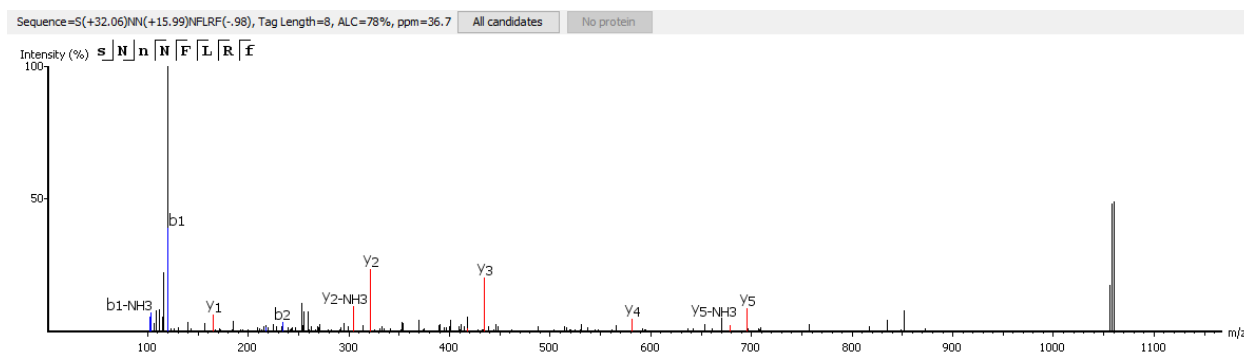


Figure S46. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SNNNFLRFamide.

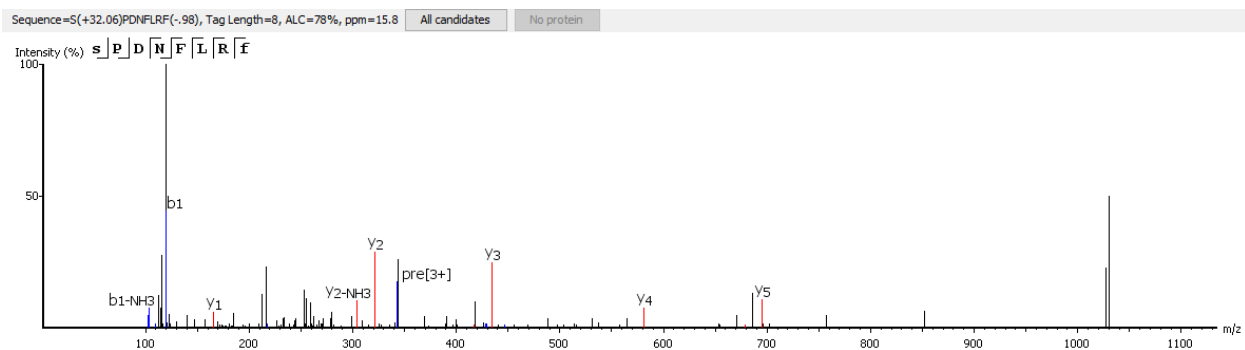


Figure S47. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SPDNFLRFamide.

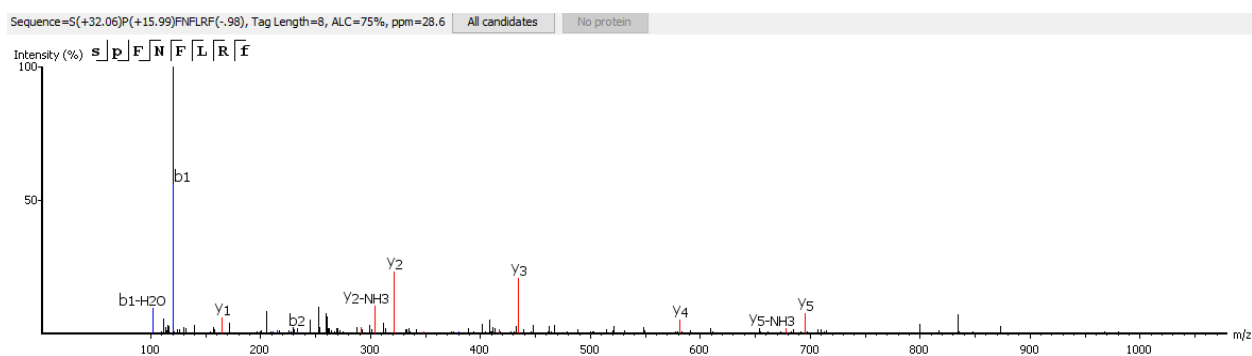


Figure S48. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SPFNFLRFamide.

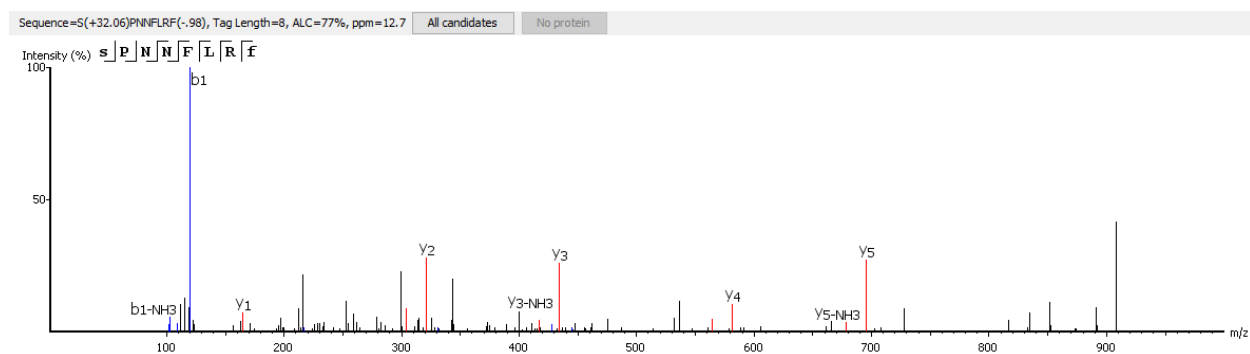


Figure S49. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SPNNFLRFamide.

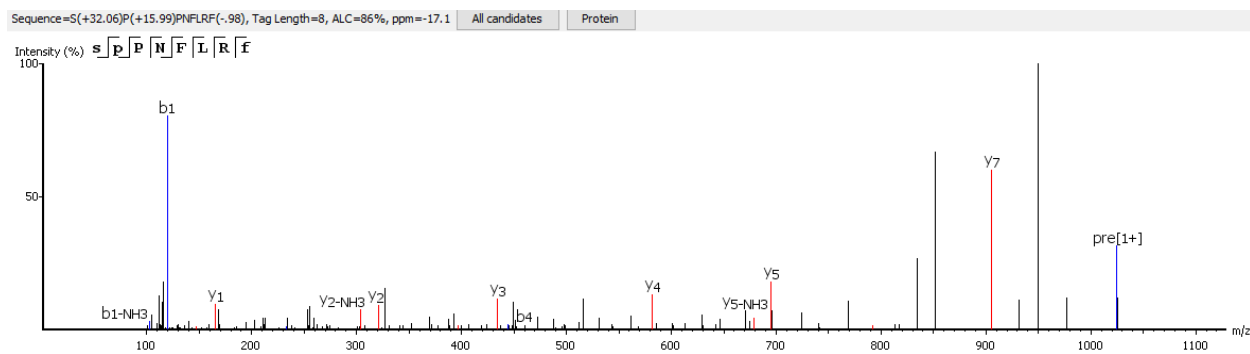


Figure S50. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SPPNFLRFamide.

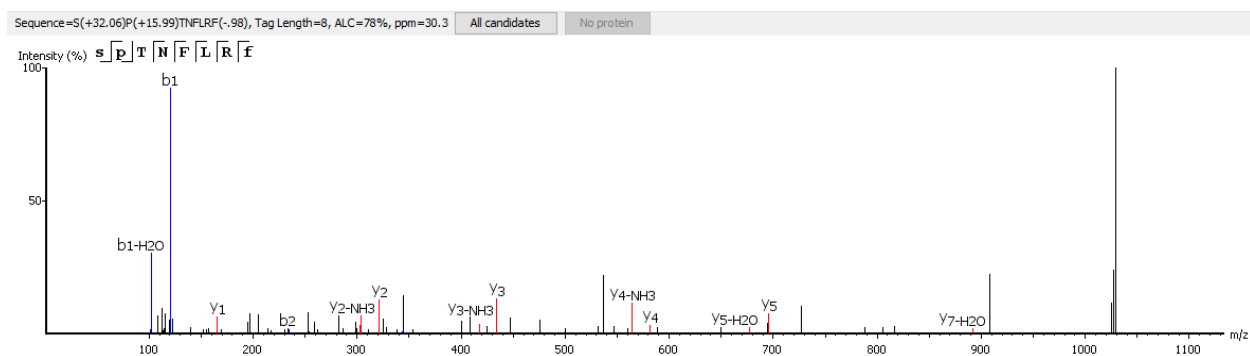


Figure S51. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SPTNFLRFamide.

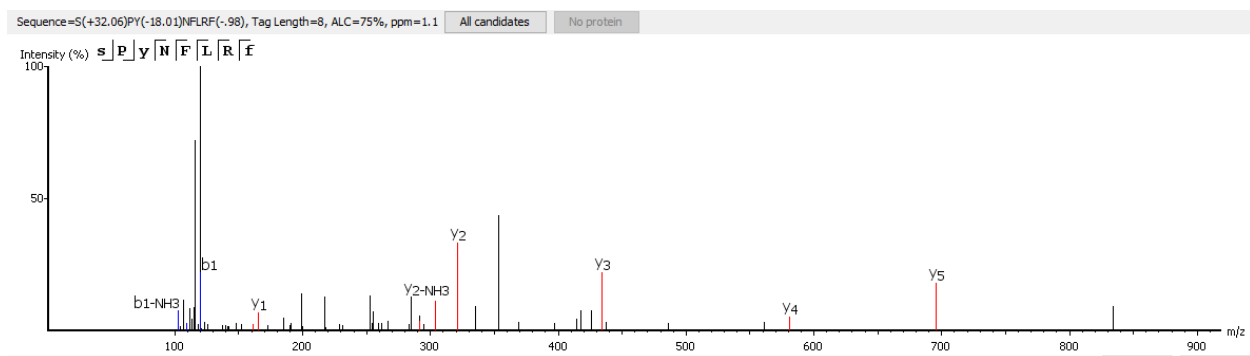


Figure S52. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SPYNFLRFamide.

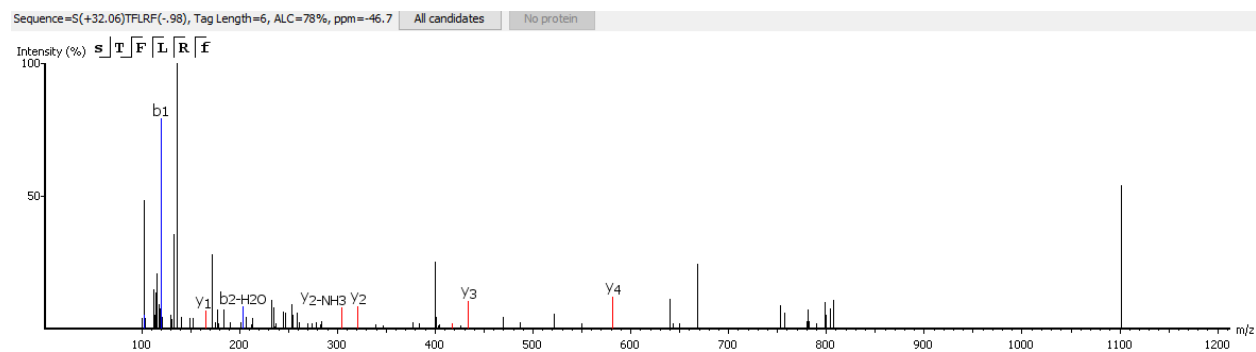


Figure S53. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence STFLRFamide.

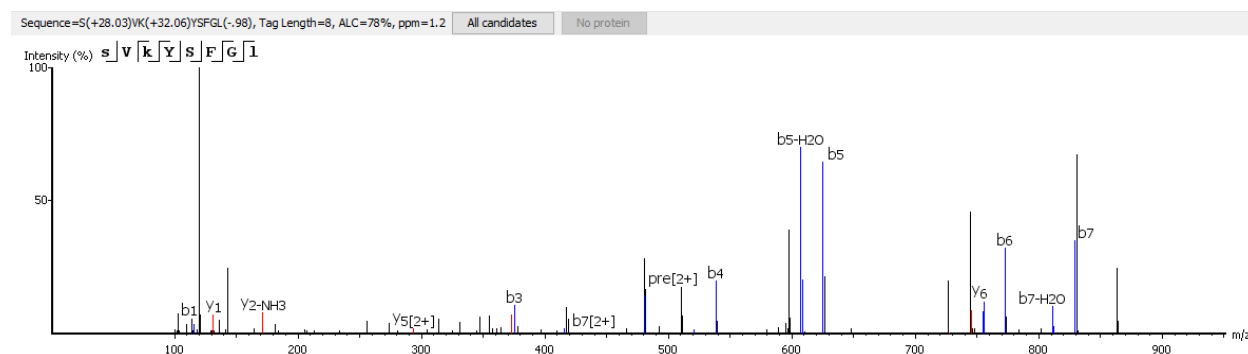


Figure S54. MS/MS spectrum from PEAKS software of a putative allatostatin A-type neuropeptide with the sequence SVKYSFGLamide.

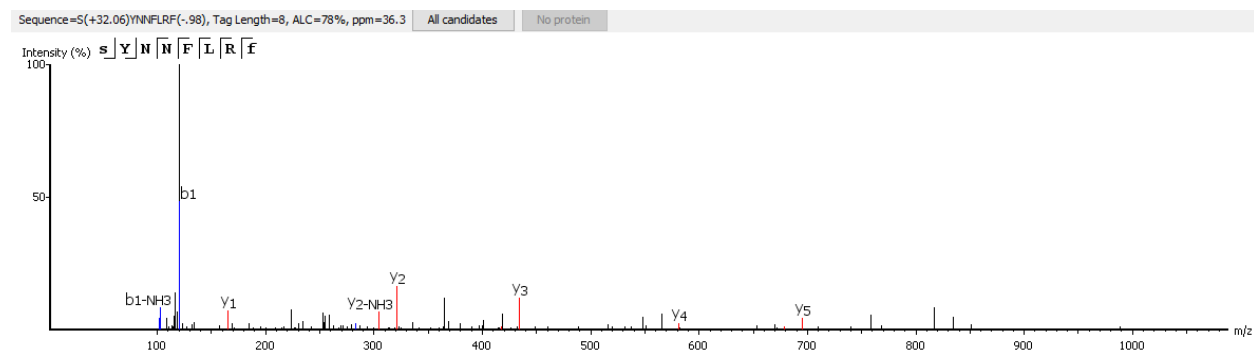


Figure S55. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence SYNNFLRFamide.

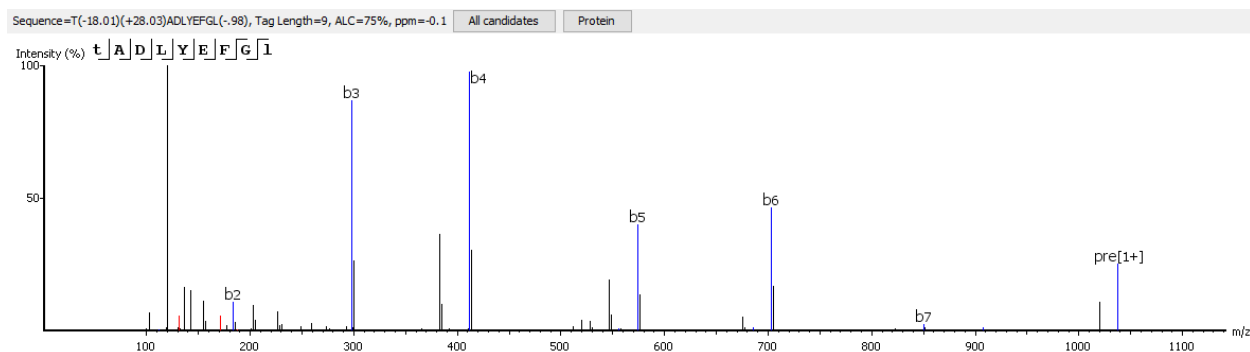


Figure S56. MS/MS spectrum from PEAKS software of a putative allatostatin A-type neuropeptide with the sequence TADLYEFGamide.

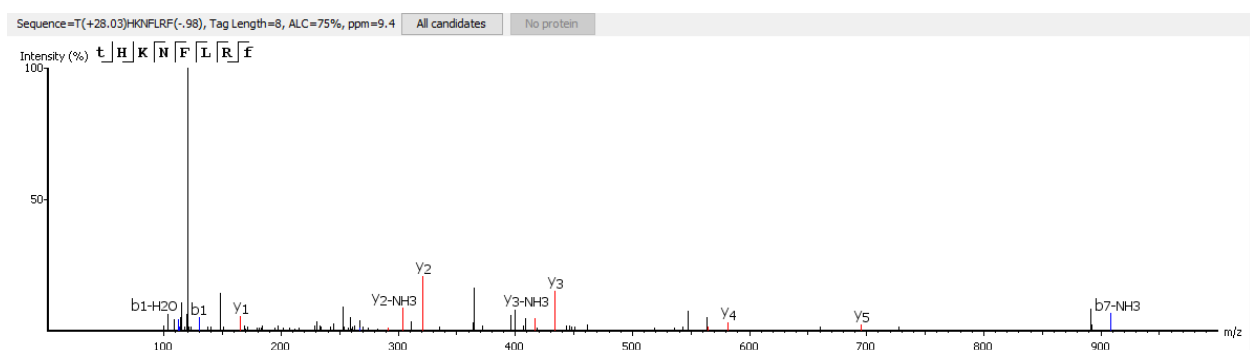


Figure S57. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence THKNFLRFamide.

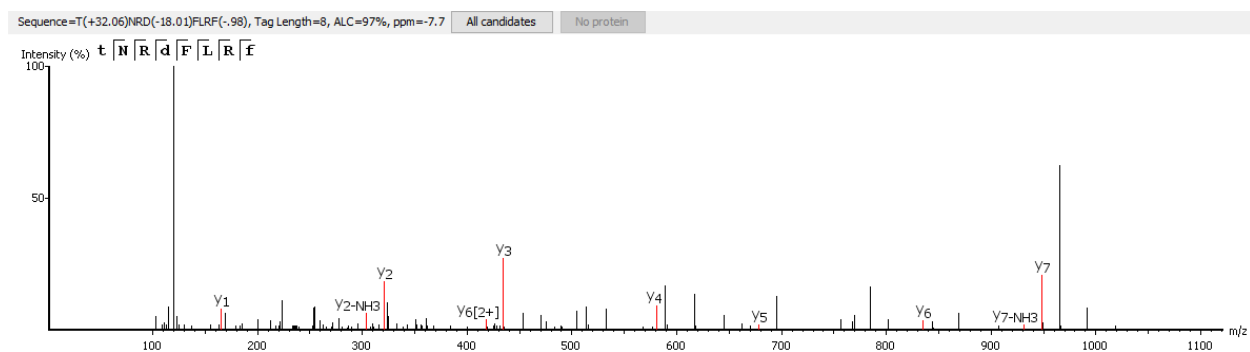


Figure S58. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence TNRDFLRFamide.

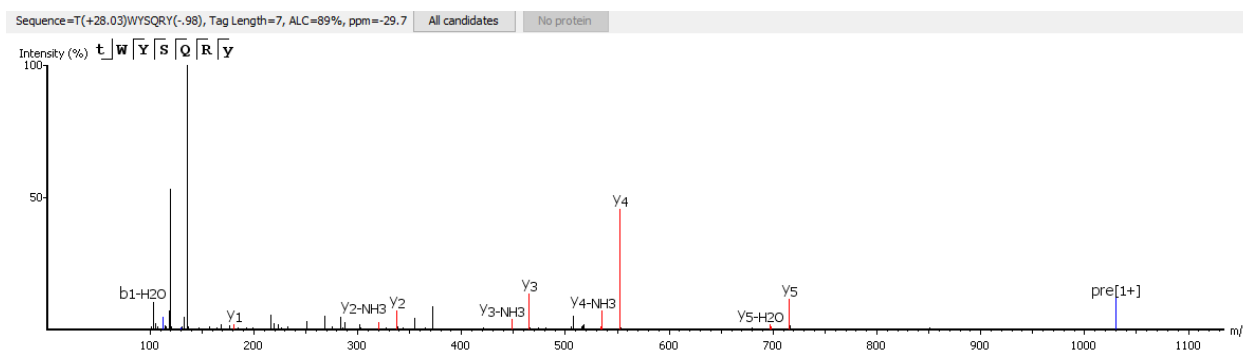


Figure S59. MS/MS spectrum from PEAKS software of a putative RYamide neuropeptide with the sequence TWYSQRYamide.

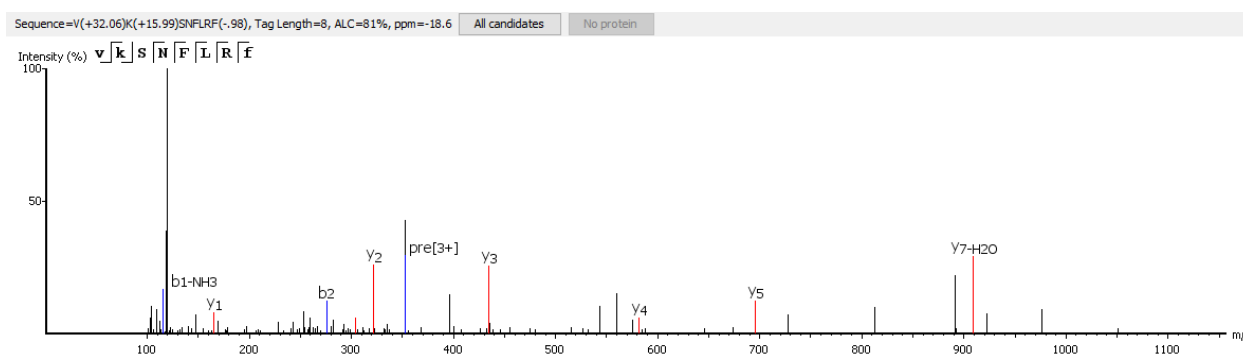


Figure S60. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence VKSNFLRFamide.

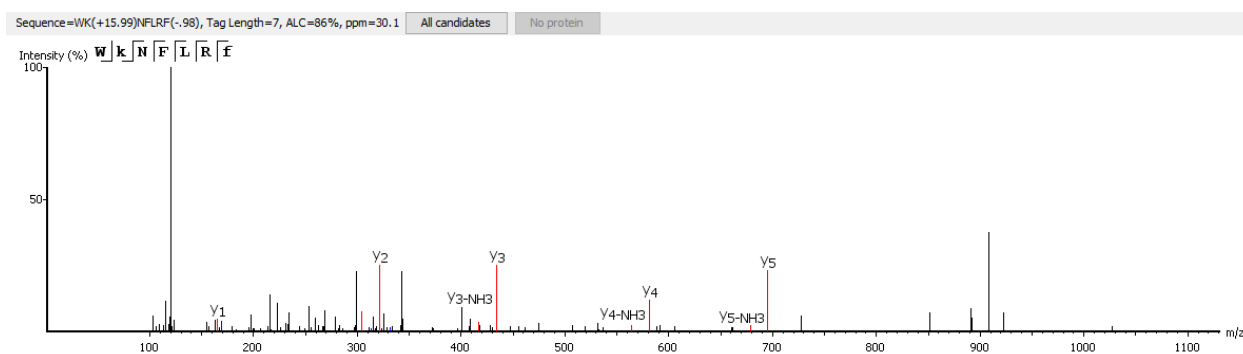


Figure S61. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence WKNFLRFamide.

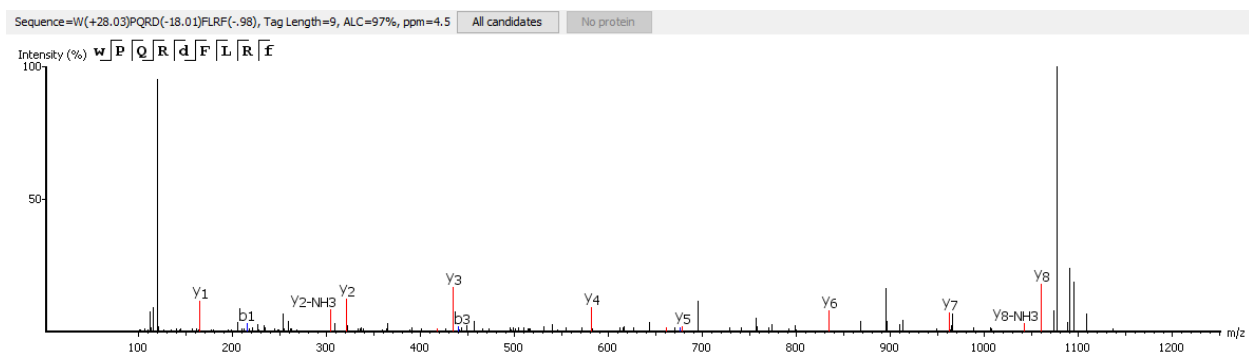


Figure S62. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence WPQRDFLRamide.

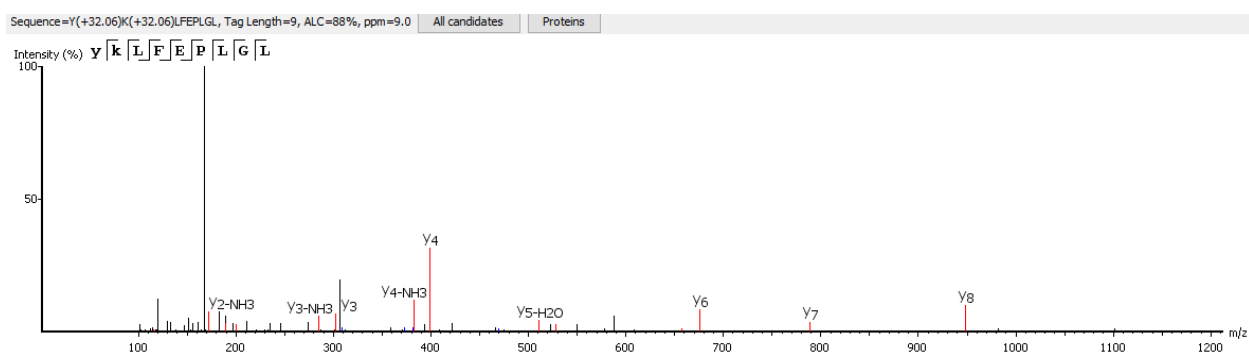


Figure S63. MS/MS spectrum from PEAKS software of an allatostatin A-type putative neuropeptide with the sequence YKLFEPGL.

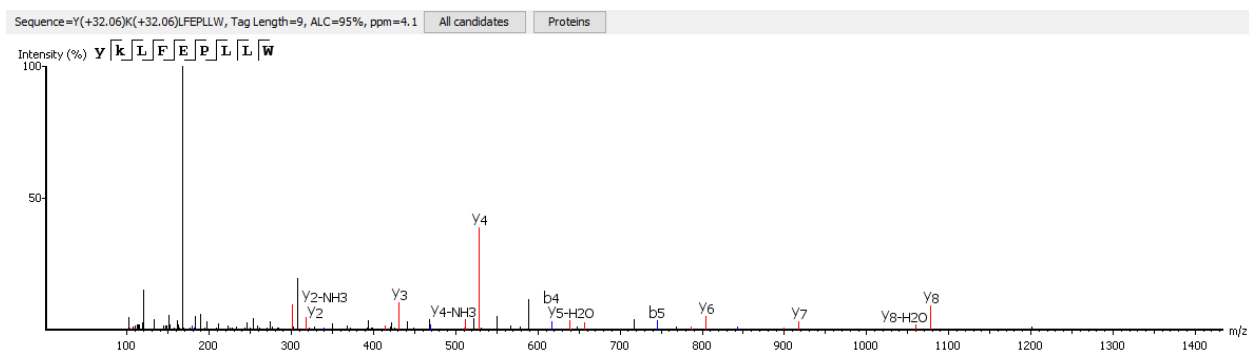


Figure S64. MS/MS spectrum from PEAKS software of a putative neuropeptide with the sequence YKLFEP LLW that does not match the sequence motifs of common neuropeptide families.

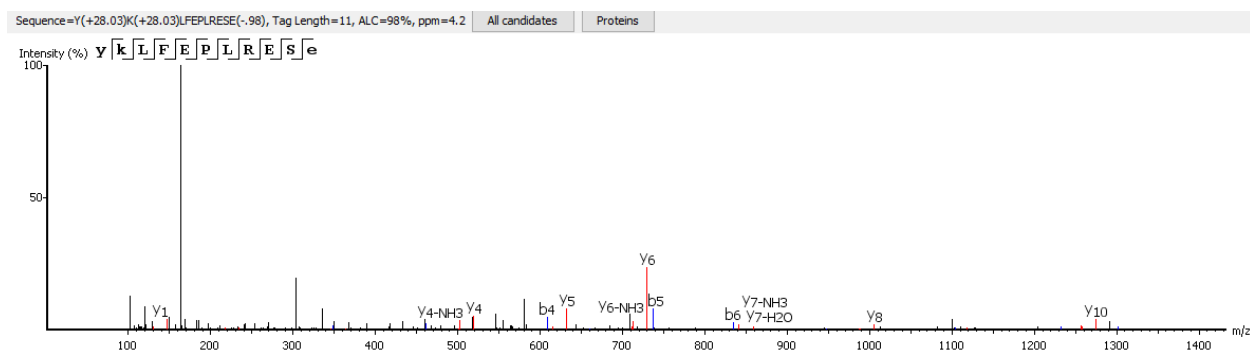


Figure S65. MS/MS spectrum from PEAKS software of a putative neuropeptide with the sequence YKLFEPLRESEamide that does not match the sequence motifs of common neuropeptide families.

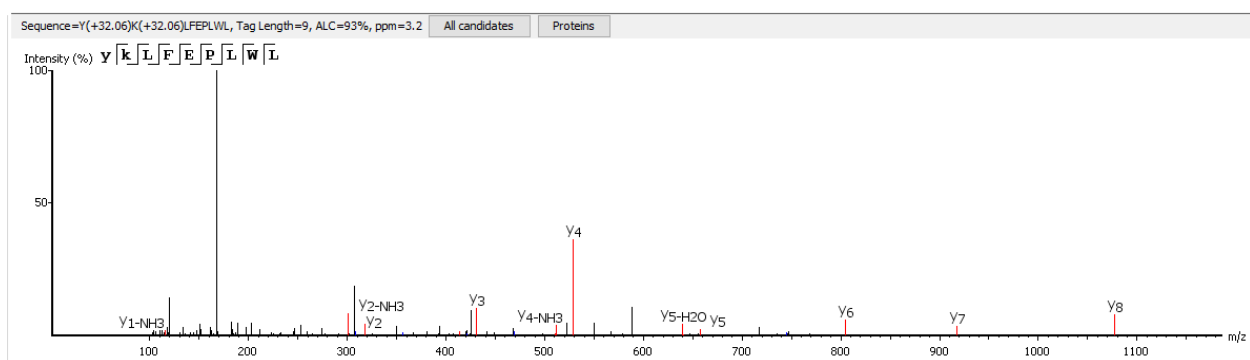


Figure S66. MS/MS spectrum from PEAKS software of a putative neuropeptide with the sequence YKLFEPLWL that does not match the sequence motifs of common neuropeptide families.

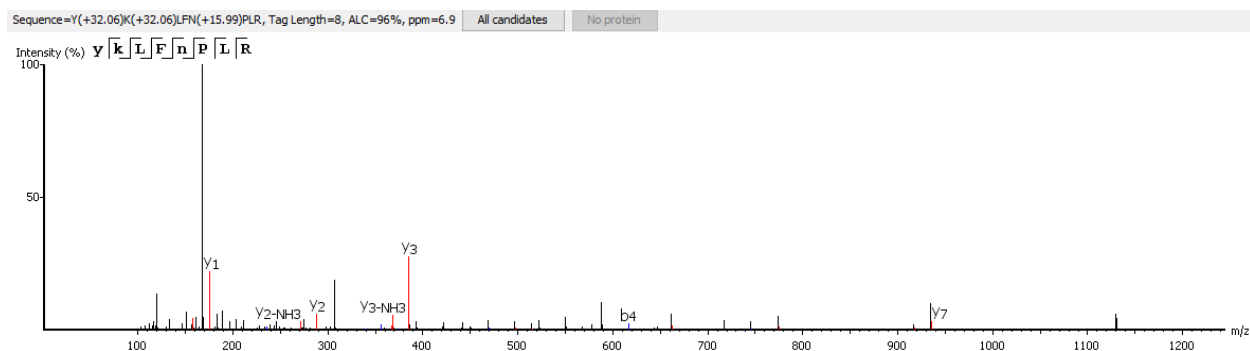


Figure S67. MS/MS spectrum from PEAKS software of a putative neuropeptide with the

sequence YKLFNPLR that does not match the sequence motifs of common neuropeptide families.

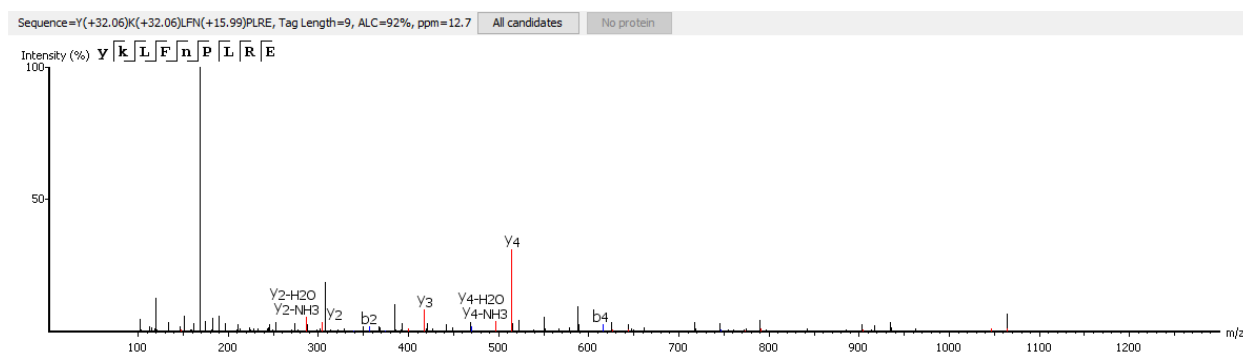


Figure S68. MS/MS spectrum from PEAKS software of a putative neuropeptide with the sequence YKLFNPLRE that does not match the sequence motifs of common neuropeptide families.

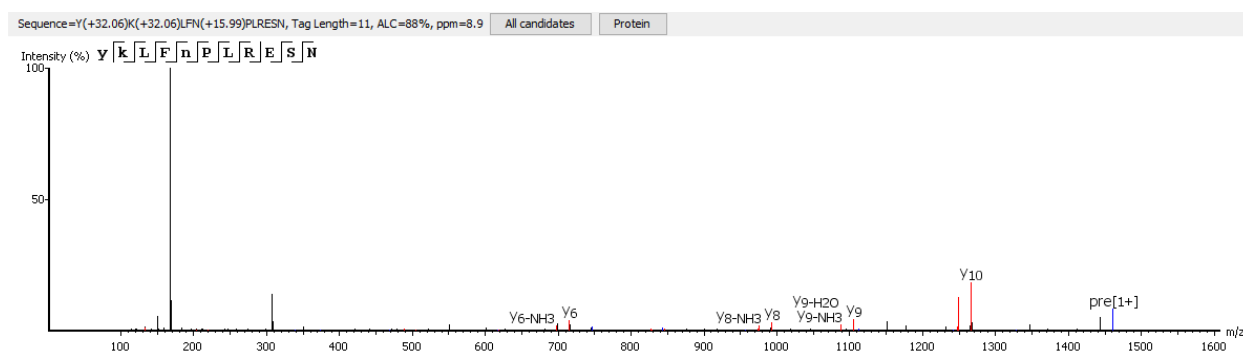


Figure S69. MS/MS spectrum from PEAKS software of a putative neuropeptide with the sequence YKLFNPLRESN that does not match the sequence motifs of common neuropeptide families.

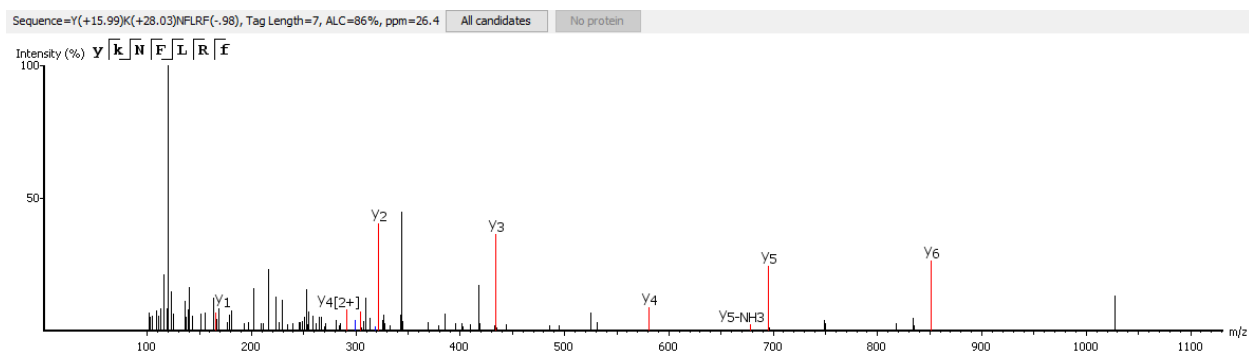


Figure S70. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence YKNFLRFamide.

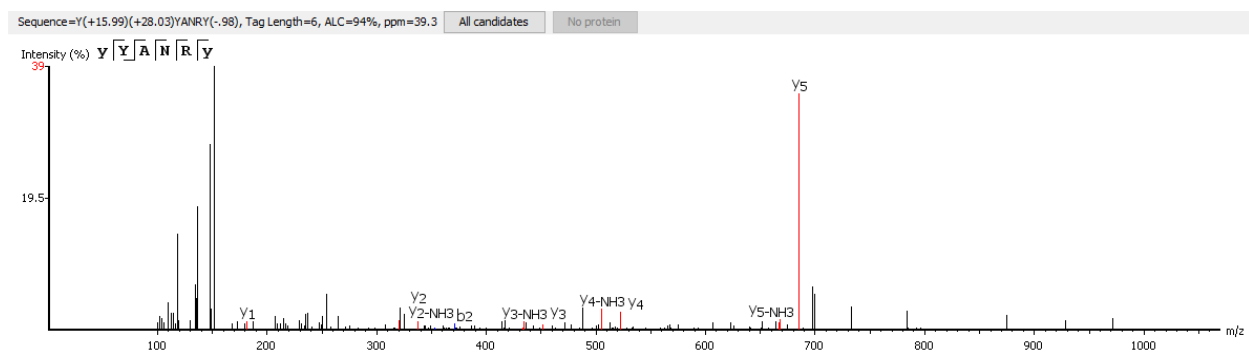


Figure S71. MS/MS spectrum from PEAKS software of a putative RFamide neuropeptide with the sequence YYANRYamide.

Supporting Information II

Family	Sequence	Mass	Brain				PO				SG				CoG				STG			
			Ratio	SEM	p-value	Method	Ratio	SEM	p-value	Method	Ratio	SEM	p-value	Method	Ratio	SEM	p-value	Method	Ratio	SEM	p-value	Method
AST A-type	YSFGLa	584.285	2.71	1.26	0.65 71	MALDI- MS	2.19	0.56	0.01 2	MALDI- MS	1.59	0.61	0.77 2	MALDI- MS	2.60	0.88	0.47 1	MALDI- MS	4.1 7	2. 34	0.25 4	MALDI -MS
AST A-type	GGAYSFGLa	769.365					2.60	0.97	0.04 9	MALDI- MS												
AST A-type	GPYEFGLa	780.37	1.78	0.32	0.00 64	MALDI- MS	10.4 9	7.93	0.31 9	MALDI- MS	0.88	0.11	0.07 6	MALDI- MS	3.67	2.01	0.89 7	MALDI- MS				
AST A-type	DPYAFGLa	780.37	1.78	0.32	0.00 64	MALDI- MS	10.4 9	7.93	0.31 9	MALDI- MS	0.88	0.11	0.07 6	MALDI- MS	3.67	2.01	0.89 7	MALDI- MS				
AST A-type	EPYAFGLa	794.385	1.46	0.27	0.04 67	MALDI- MS	1.39	0.41	0.65 2	MALDI- MS	1.09	0.22	0.55 8	MALDI- MS	1.46	0.28	0.02 4	MALDI- MS				
AST A-type	GRYSFGLa	797.407					2.26 19.4	1.01 18.3	0.91 9	MALDI- MS												
AST A-type	GHYNFGLa	805.376					7	9	0.72 7	MALDI- MS												
AST A-type	SGPYAFGLa	809.396	1.23	0.16	0.17 68	MALDI- MS	0.98	0.31	0.35 7	MALDI- MS	2.07	1.02	0.38 0	MALDI- MS					4.1 2	3. 62	0.96 8	MALDI -MS
AST A-type	NSYDFGLa	813.355									1.54	1.22	0.59 5	MALDI- MS								
AST A-type	AGQYAFGLa	824.407									27.47	13.92	0.51 8	MALDI- MS								
AST A-type	GDPYAFGLa	837.391	1.37	0.35	0.49 72	Both	2.63	1.47	0.79 6	Both	0.93	0.21	0.34 3	Both	2.23	0.78	0.08 9	MALDI- MS				
AST A-type	GGPYEFGLa	837.391	1.66	0.53	0.13 45	MALDI- MS	1.25	0.29	0.78 3	MALDI- MS	0.76	0.12	0.01 5	MALDI- MS	2.23	0.78	0.08 9	MALDI- MS				
AST A-type	AGGAYSFGLa	840.402					2.00	0.70	0.23 9	MALDI- MS									2.3 5	1. 36	0.70 3	MALDI -MS
AST A-type	AGQYSFGLa	840.402					2.00	0.70	0.23 9	MALDI- MS									2.3 5	1. 36	0.70 3	MALDI -MS
AST A-type	AGLSYGLa	841.422					0.97	0.25	0.16 2	LC-ESI- MS/MS	1.61	0.68	0.96 7	LC-ESI- MS/MS								
AST A-type	AGHSYFGLa	849.402									1.00	0.23	0.74 7	MALDI- MS					1.2 5	0. 23	0.21 8	MALDI -MS
AST A-type	ANQYAFGLa	881.429									1.08	0.04	0.08 3	MALDI- MS					1.5 1	0. 09	0.00 1	MALDI -MS
AST A-type	ANQYTFGLa	911.439	0.85	0.28	0.23 23	MALDI- MS					1.09	0.15	0.68 6	MALDI- MS					1.3 4	0. 21	0.06 0	MALDI -MS
AST A-type	SDMYSFGLa	917.384	2.67	0.91	0.31 20	LC-ESI- MS/MS	2.07	0.78	0.59 3	LC-ESI- MS/MS												
AST A-type	pQRAYSFGLa	922.455 45					1.01	0.34	0.60 3	LC-ESI- MS/MS	1.46	1.06	0.33 7	LC-ESI- MS/MS	1.03	0.35	0.73 1	MALDI- MS	2.2 8	1. 46	0.60 1	MALDI -MS
AST A-type	SRPYSFGLa	924.471					8.03	4.33	0.67 7	MALDI- MS												
AST A-type	SSGQYAFGLa	927.434					0.79	0.21	0.06 3	LC-ESI- MS/MS	0.90	0.16	0.72 2	LC-ESI- MS/MS								
AST A-type	PRNYAFGLa	935.487	2.13	0.56	0.01 88	MALDI- MS					1.21	0.31	0.63 9	MALDI- MS	1.18	0.35	0.87 3	MALDI- MS				
AST A-type	QRAYSFGLa	939.482					1.28	0.30	0.33 8	Both	0.82	0.24	0.09 9	Both								
AST A-type	DRPYSFGLa	952.466	1.76	0.53	0.13 14	MALDI- MS	1.87	0.82	0.33 0	MALDI- MS	0.86	0.21	0.07 8	MALDI- MS	1.45	0.38	0.18 5	MALDI- MS				
AST A-type	pQRTYSFGLa	952.465 45	1.76	0.53	0.13 14	MALDI- MS	1.87	0.82	0.33 0	MALDI- MS	0.86	0.21	0.07 8	MALDI- MS	1.45	0.38	0.18 5	MALDI- MS				

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RFamide PRP	EDNLY(SO3)HDLSED	1331.50	3.82	2.94	0.50	LC-ESI-	373.	165.	0.15	LC-ESI-	7.90	6.17	0.39	Both	2.32	0.75	0.35	LC-ESI-	1.1	0.	0.42	MALDI
		445			62	MS/MS	43	02	0	MS/MS			2				6	MS/MS				
RPCH	pQLNFSPGWa	929.428																	1.1	0.	0.42	MALDI
		45																	2	21	8	-MS
RYamide	FVGSGFYANRYa	1278.61					65.4	26.2	0.12	LC-ESI-												
		45					0	3	0	MS												
	DTGTRQLTERSGFYAN	2133.03					57.7	30.7	0.20	LC-ESI-			0.10	LC-ESI-								
RYamide	RYa	52					3	1	9	MS	0.78	0.24	3	MS/MS								
	TSDRTQLTERSGFYAN	2163.04					25.3	11.6	0.68	LC-ESI-												
RYamide	RYa	57					0	2	3	MS												
	ESGTRQLTERSGFYAN	2133.03					31.3	17.3	0.10	LC-ESI-			0.50	LC-ESI-								
RYamide	RYa	52					4	8	9	MS	1.11	0.37	3	MS/MS								
		1129.57					117.	91.9	0.39	LC-ESI-												
RYamide	KMSFVGGSRYa	03					42	1	1	MS												
		960.448							0.74	MALDI-												
RYamide	QFYADRYa	8322					1.47	0.58	4	MS												
		963.426																				
RYamide	MFYANRYa	7237																				
									0.48	MALDI-	6.13	5.47	4	MS			0.29	MALDI-				
RYamide	FVGGSRYa	783.392					2.32	0.78	1	MS	1.62	0.71	0.48	MALDI-	0.86	0.54	5	MS				
									0.05	MALDI-			7	MS	1.88	1.30	0.85	MALDI-				
RYamide	FYANRYa	831.392					1.55	0.30	7	MS												
									0.37	MALDI-												
RYamide	FYSQRYa	861.402					1.77	0.61	9	MS												
									0.30													
RYamide	SGFYANRYa	975.445					3.20	1.70	8	Both	1.86	1.18	0.73	MALDI-	1.31	0.62	0.99	MALDI-				
		1046.48							0.03	LC-ESI-			9	MS			0	MS				
RYamide	QGFYSQRYa	2					0.65	0.21	2	MS/MS												
		1113.55																				
RYamide	SSRFVGGSRYa	7																				
		1088.52							0.38	LC-ESI-	3.90	2.76	0.52	MALDI-								
RYamide	LSGFYANRYa	9					8.17	5.85	5	MS/MS			5	MS								
SIFamide	GYRKPPFNGSIFa	1380.71	12.3		0.15	LC-ESI-							0.00	LC-ESI-								
		9	0	8.18	00	MS/MS							1	MS/MS								
		1422.76											0.02	MALDI-								
SIFamide	VYRKPPFNGSIFa	6									3.43	1.67	2	MS								
		1218.60											0.78	MALDI-								
SIFamide	DLDSVLDPSIFa	2									1.12	0.50	5	MS					1.7	0.	0.37	MALDI
		1562.74			0.72								0.54	LC-ESI-					9	85	6	-MS
sNPF PRP	NVDQADPILDHDLV	7	1.39	0.63	14	Both					1.67	0.96	0	MS/MS								
		1498.57																				
sNPF PRP	GATFGEEDMASQEQ	7															0.37	LC-ESI-				
		1394.51			0.58	LC-ESI-							0.02	LC-ESI-	0.97	0.52	4	MS/MS				
Sulfakinin	DSCSDDYGHMRFa	32	0.96	0.26	77	MS/MS					0.70	0.08	4	MS/MS								
					0.41	MALDI-			0.43	MALDI-			0.61	MALDI-			0.01	MALDI-	1.5	0.	0.05	MALDI
Tachykinin	APSGQa	604.286	1.33	0.24	17	MS	1.58	0.66	9	MS	1.09	0.25	8	MS	1.76	0.36	0	MS	9	33	1	-MS
					0.02	MALDI-							0.80	MALDI-								
Tachykinin	APSGFLGM	778.357	3.29	1.53	10	MS					4.05	3.19	8	MS								
					0.27								0.71	MALDI-			0.56	MALDI-				
Tachykinin	TPSGFLGMRa	963.485	2.18	1.03	92	Both					2.99	2.10	9	MS	1.52	0.42	5	MS				
					0.93	LC-ESI-							0.42									
Tachykinin	APSGFLGMRG	991.48	1.11	0.33	66	MS/MS					2.23	1.14	8	Both								
		1025.50																				
Tachykinin	YPSGFLGMRa	1			0.06				0.66	MALDI-			0.67				0.11	MALDI-	2.3	0.	0.24	MALDI
					65	Both	1.04	0.21	1	MS	2.84	1.73	8	Both	0.60	0.38	5	MS	5	94	6	-MS
					0.55	MALDI-							0.14	MALDI-	2.53	0.64	0.01	MALDI-	1.2	0.	0.00	MALDI
Tachykinin	APSGFLGMRa	933.475	1.63	0.31													2	MS	7	08	2	-MS
Tachykinin	APEGFLGMRa	975.496	1.03	0.50	26	MS					0.73	0.22	7	MS								

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Chapter 10

Using Crabs to Study Neuropeptides and Their Role in the Nervous System with Mass Spectrometry Methods

Written by Kellen DeLaney, Lingjun Li Research Group



Written for the Wisconsin Initiative for Scientific Literacy in order to describe this thesis for a broader audience

This chapter was written to explain the research I have conducted to a broad, non-scientific audience. All scientific research is performed with the goal of being useful to the greater population. As such, it is critical that our findings are communicated with others. Science communication is a fundamental component of our responsibilities as scientists. I acknowledge the Wisconsin Initiative for Science Literacy at the University of Wisconsin-Madison for providing this platform and for sponsoring and supporting the creation of this chapter.

Abstract

The human nervous system is comprised of billions of cells that work together to transport messages throughout the body. These messages are transported partly by molecules called neuropeptides. My thesis focuses on developing and applying tools to study these neuropeptides. I use crabs as a model organism because their nervous system is much simpler than that of humans but is still similar. By changing various ways the neuropeptides are analyzed, we were able to identify more neuropeptides and even new, never-before-detected neuropeptides. These neuropeptides were tested to see if they had a function in the nervous system related to feeding, a universal process, and heartbeat. Those neuropeptides that did have a function may provide useful insight into how the nervous system works in the body and information on their function may lead to improvements in how we treat neurological disorders.

Crabs as a model for the nervous system

The nervous system is a beautifully complex system within the human body that includes the brain, spinal cord, and nerves running through every part of the body. Overall, the nervous system is made up of 86 billion cells, called neurons, that transport messages throughout the body. While this vast complexity enables the body to perceive and respond to our surroundings in a variety of sophisticated ways, it makes studying this system difficult. If we can understand the way neurons communicate within the body, we can be better equipped to understand and treat neurological disorders, such as Parkinson's disease and Alzheimer's disease. However, in order to understand specifically how the neurons communicate with each other in this system, we would have to map connections between each of these 86 billion neurons, and each can have multiple connections to various other neurons.

To address this daunting task, we look to crabs, specifically species such as *Cancer borealis*, or Jonah crab. These ten-legged, shelled critters seem far removed from humans on the surface, but the basic workings of their nervous system is surprisingly similar to that of humans. The difference is that the crab nervous system is much simpler. While a human nervous system has billions of neurons, a crab has only one hundred thousand neurons, which is one ten-thousandth of a percent of the number in humans. The small number of neurons in the crab nervous system means that it is easier to map how neurons connect to each other and influence each other. For example, twenty-five neurons are involved in controlling a crab's chewing and are connected as shown in **Figure 1**, where circles represent neurons and lines represent connections.¹ The arrows represent the direction molecules move between the neurons. The connections between neurons typically act as gates; sometimes they are open and let things pass through and sometimes they are closed so nothing can pass through. These gates are selective, in

that they only let certain molecules pass through, similar to how different keys allow access to different doors in an apartment building.

Neuropeptides: The molecules that make things happen

Just like gates, the connections between neurons can open with the right “key.” Some molecules are shaped in a way that they can fit into a part of the gate and tell it to open. One group of molecules that can do this is neuropeptides, which are made out of the same substance as proteins, but are smaller in size. These neuropeptides act as messengers, entering and leaving neurons throughout the body to change the way they behave. As a result, neuropeptides are incredibly important for helping to control virtually all processes within the body. Because of this, understanding how they work not only improves our understanding of the nervous system, but it can help us develop treatments for neurological diseases by designing drugs that mimic the shape and function of neuropeptides.

However, the characteristics that make them great signaling molecules are also the reason they are difficult to study. They are present in very small amounts and degrade rapidly over time. This is helpful for conveying messages. For example, if you touch a hot burner with your hand, your hand releases a burst of neuropeptides to communicate this to your brain, telling your hand to move away from the hot burner. If you then decide for whatever reason to immediately touch that burner again, another burst of neuropeptides will be released. If the previous burst is still circulating around in the body, the next message won’t be as easily recognized and it will take longer to tell your hand to move away from the burner. In other words, by having a small number of something, differences are more noticeable and so the response is more striking. Additionally, neuropeptides come in a wide variety of forms, which is useful for conveying more specialized

messages, but also makes them challenging to study because there are more of them to keep track of.²

Because neuropeptides are present in small amounts, do not last long outside the body, and are present in many different varieties, finding a way to study what each one is and how it works is difficult.

Developing ways to detect neuropeptides

Most methods of studying neuropeptides can only look at one neuropeptide at a time. These methods give lots of helpful details, but can be tedious when there are hundreds of neuropeptides in the body, and impossible when we don't know which neuropeptides we're looking for. In order to study all of the neuropeptides at once, we use mass spectrometry, which is a technique that detects neuropeptides (and other molecules) based on their mass-to-charge ratio, or how big they are versus how much charge they have on them. This method is good because we see almost all neuropeptides at once and we don't need to know what we're looking for ahead of time. However, we still encounter challenges with neuropeptides being present in low amounts, making it hard to detect them with a mass spectrometer.

In order to better detect neuropeptides, we need to separate them from each other, like spreading out a crowd of people to see the individuals more clearly. We also need to make sure they have a charge because if a molecule has no charge, the mass spectrometer cannot detect it. An overview of the general steps involved in neuropeptide analysis, including separation, charging, and mass spectrometry analysis, is shown in **Figure 2**. Most of the time, mass spectrometry is used with a separation method called liquid chromatography. Molecules are pushed through a tube filled with a substance. The speed at which they travel through the

substance depends on how much they stick to the substance in the tube. This is shown in **Figure 2B**. They are then given a charge by applying a voltage as they are being emitted from the tip of the tube. This method works to detect some neuropeptides, but not all neuropeptides can be separated from each other this way, and not all neuropeptides can become charged in liquid form this way. As neuropeptides come in many varieties, there isn't one perfect way to separate them. Just like how you can separate people in a crowd in multiple ways (e.g. how tall they are or what color shirt they're wearing), molecules can be separated in multiple ways that makes it easier to see different ones, and we can give them a charge in different ways as well.

To increase the number of neuropeptides detected, we developed a method that used a different separation mechanism, called capillary electrophoresis, shown in **Figure 2B**, and combined it with a method that gave molecules a charge in a different way. With this method, molecules were separated as they moved through a tube based on how quickly they could move in the presence of an electric field, similar to electricity in a wire. When they leave the tube, they are deposited on a plate attached to a continuously moving platform and allowed to dry. Instead of giving charge to the neuropeptides in liquid form with a voltage, the charge is given to the neuropeptides in solid form with a laser. The overall setup is shown in **Figure 3**.³ By changing both the separation method and the method of giving charge, different neuropeptides are able to be detected within the same sample. In this way, complementary methods are combined to increase the overall number of neuropeptides able to be detected.

Developing ways to characterize neuropeptides

In addition to simply detecting the presence of neuropeptides in a sample, we need to know more about the details of the neuropeptides, such as what components they are made of.

This can be done by performing a second round of mass spectrometry after the first. The initial mass spectrometry measurement identifies the mass-to-charge ratio of the neuropeptides. We can then select individual neuropeptides, break them into pieces, and see the mass-to-charge ratio of the individual pieces. This tells us more about what pieces make up the neuropeptide. However, selecting every individual neuropeptide to be broken apart would take too long, so it isn't feasible. Typically, only the most prominent mass-to-charge ratios are selected and broken into pieces. This method is called data dependent acquisition (DDA) because what is selected to be broken depends on the relative amount of molecules in the data. As a result, many molecules that are present in low amounts, such as neuropeptides, get overlooked and we end up not knowing what pieces they are made of.

To address this problem, we used a method that selected a wide range of mass-to-charge ratios to be broken into pieces simultaneously. This method is called data independent acquisition (DIA) because it doesn't depend on the amounts of each molecule in the data. The difference between DDA and DIA can be thought of like picking apples, where each apple is a mass spectrometry scan, and ripe apples are neuropeptides. The DDA method is like picking the 5 apples on each tree that are closest to you and putting them each in its own basket. The DIA method is like picking all of the apples that you can reach and putting them each in one of the 5 baskets. At the end of the day, you look through the baskets and determine which ones are actually ripe. With the DDA method that only has 1 apple per basket, it's faster and easier to determine apple ripeness. With the DIA method, there are maybe 10 to 20 apples in each basket that have to be filtered for ripeness. It takes longer to find the ripe apples in the basket, but you ultimately end up with more ripe apples. **Figure 4** shows a depiction of the two different methods.

While this new method made the secondary mass spectrometry measurements more complicated, it ensured that all molecules were getting broken into pieces. We then used software developed in another lab that could match the individual pieces back to the original mass-to-charge ratio they came from.⁴ In this way, we were able to get detailed information about more of the neuropeptides that were in lower amounts in the sample, resulting in information about twice as many neuropeptides compared to the original method with good reproducibility, as shown in **Figure 5**.⁵ In this way, we can increase the number of neuropeptides we're able to characterize to get a deeper understanding of what neuropeptides are present within the nervous system.

How neuropeptides influence feeding behavior

Seeing what neuropeptides are present in the nervous system is important, but we also need to know what these neuropeptides do (if they even do anything). There are numerous ways to get an idea of what a neuropeptide does. The simplest way is to compare the neuropeptides between two conditions, such as before or after a crab eats food. Looking at neuropeptides involved in feeding behavior is particularly interesting in crabs because their nervous system is located in their stomach. This system controls the chewing and filtering of food and receives instruction from neuropeptides. We compared the neuropeptides in crabs that were fed tilapia for 30 minutes to those that were not fed anything, and we found that, in general, the amount of neuropeptide increased in many parts of the nervous system, such as the brain. However, in other parts of the crab that release neuropeptides as hormones, like the sinus glands, the amount of many neuropeptides decreased. Some of these results are shown in **Figure 6**. The fact that certain neuropeptides change when the crab is fed indicates that they may have an influence on

the feeding process in some respect. This finding is helpful because instead of trying to get functional information on hundreds of neuropeptides, we can focus on the subset that do change, reducing the workload and making studies more meaningful.

The discovery of never-before-seen neuropeptides and their effect on heart rate

With the improvements in detection and characterization of neuropeptides, we made an exciting discovery. Over the course of studying these neuropeptides, we found numerous neuropeptides that have never been detected before. These neuropeptides looked similar to those previously identified in that some of the pieces were the same. However, each had unique pieces that made it different from anything we've seen before. Furthermore, the amount of two of these neuropeptides increased in the crab after feeding, indicating that they may have a biological role. This is exciting because we're uncovering more of the unknown parts of the crab nervous system and gaining a deeper understanding of all the molecules working together.

These two neuropeptides were identified in the tissue on either side of the heart that typically releases neuropeptides into the heart, called the pericardial organs. In order to get a better idea of how these two neuropeptides are involved in the crab's bodily functioning, we tested their effect on crab hearts. A notable benefit to using crabs as a model organism is that they have robust central pattern generators. This means that you can remove the heart from the crab and it will continue to beat. We removed the heart, placed it in a dish filled with saline, and infused saline through the major artery on the tail-side. The arteries on the other side of the heart were tied to a force displacement transducer, a small instrument that measures force, or how hard something pulls on it. Every time the heart contracted, it pulled on the force displacement transducer, which measured the strength of the heartbeat.⁶ Using this setup, shown in **Figure 7A**,

we could measure the normal heartbeat of the crab, then infuse a neuropeptide into the heart and see how the heartbeat changed. When we infused the first neuropeptide, the heartbeat became stronger and faster, as shown in **Figure 7B** and **C**. When we infused the second neuropeptide, the strength of the contractions didn't change much, but the heartbeat became slower. These results indicate that these two neuropeptides are biologically active and may have a role in modulating heartbeat that is feeding-related.

What this means for neuroscience

Throughout the course of my Ph.D. research, I developed improved tools for studying neuropeptides and have applied these methods to identify which neuropeptides are biologically active. While my research focused on a global understanding of neuropeptides, more detailed functional analyses can be performed on the neuropeptides that we identified to be biologically active. Once a better understanding of these neuropeptides is achieved, the findings can be extended to more complex nervous systems, such as that of insects, mice, and even humans. Ultimately, understanding how neurons use these neuropeptide messengers to communicate throughout the body can improve our ability to generate treatments for neurological diseases and disorders.

Acknowledgements

The research described here was conducted with funding from a predoctoral fellowship supported by the National Institutes of Health, under Ruth L. Kirschstein National Research Service Award T32 HL 007936 from the National Heart Lung and Blood Institute to the University of Wisconsin-Madison Cardiovascular Research Center and the National Institutes of

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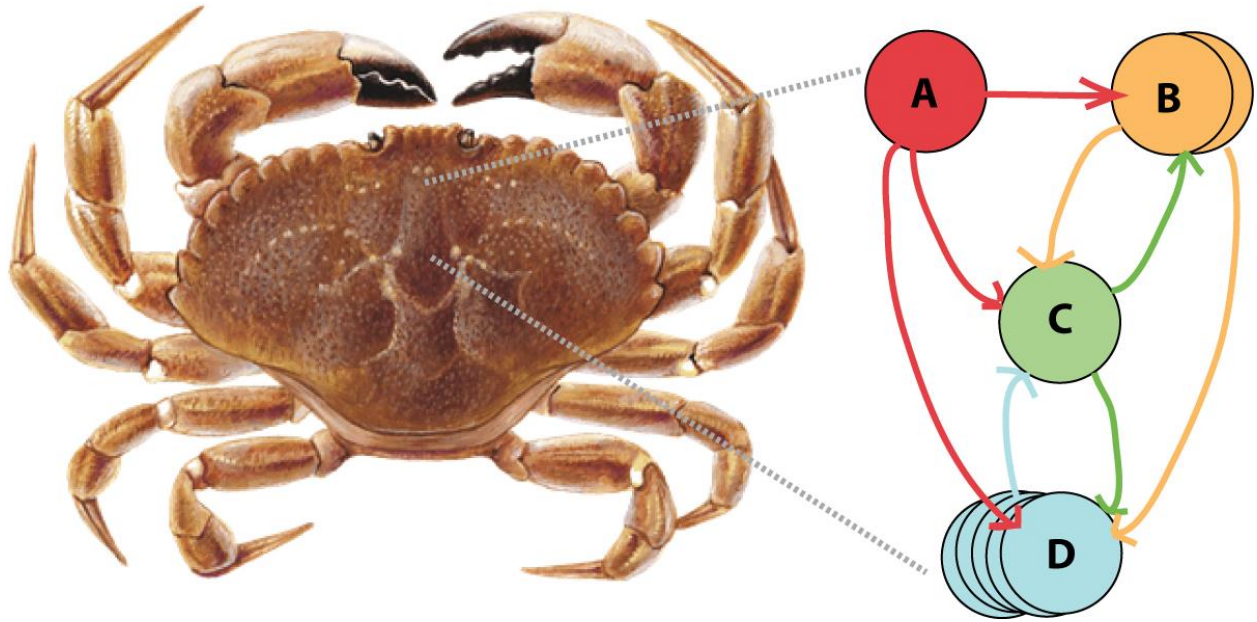
Figures

Figure 1. Depiction of the neurons in the crab nervous system that are responsible for controlling chewing. Circles indicate neurons, with different neurons labeled by different letters and colors, and lines indicate connections between neurons.¹

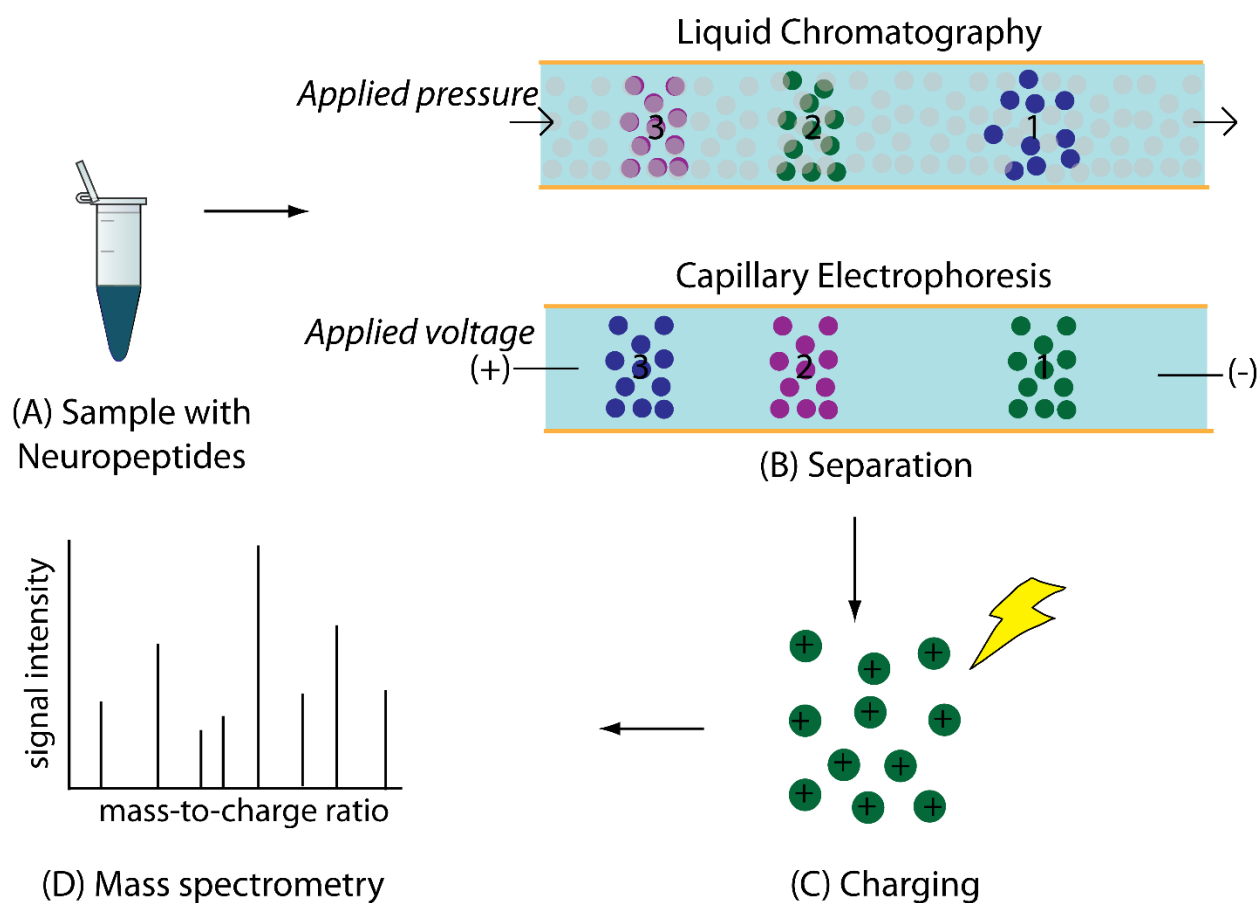


Figure 2. Overview of a typical mass spectrometry workflow, (A) starting with a sample containing a mixture of neuropeptides, (B) separation using two common methods, (C) providing a charge to the molecules so that they can be detected, and (D) mass-to-charge ratio measurements of the molecules using mass spectrometry.

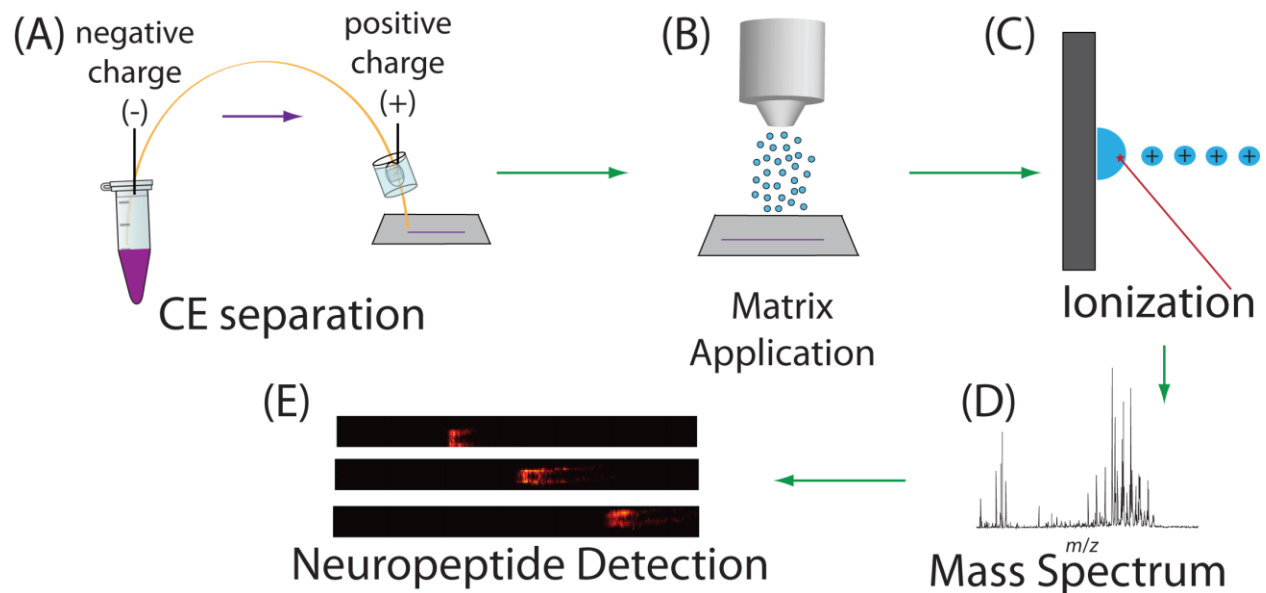


Figure 3. Depiction of the alternative separation and charging method for detection of neuropeptides. (A) A voltage is applied to a tube with neuropeptides, allowing them to move through the tube at different speeds and land on a metal plate in a process called capillary electrophoresis (CE). The metal plate is attached to a continuously moving platform so that neuropeptides are deposited in a continuous line. (B) A special molecule (matrix) is sprayed on the plate to help absorb the laser energy. (C) A laser is shot at the dry neuropeptide and matrix, causing the molecules to ionize, or obtain a charge. (D) The molecules are analyzed with mass spectrometry and detected based on their mass-to-charge ratio (m/z) in the mass spectrum. (E) Neuropeptides are detected at different points along the line so that their signals are separated from each other.³

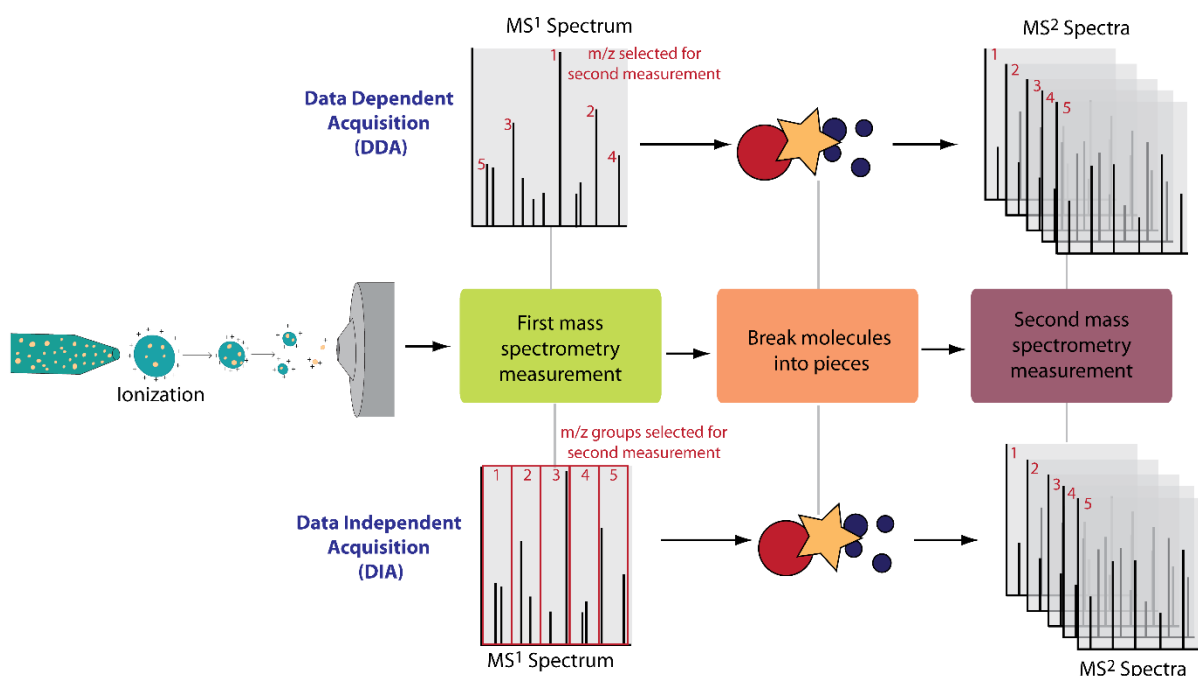


Figure 4. Depiction of different methods of identifying neuropeptides in a mass spectrometry experiment, including (top) data dependent acquisition (DDA) and (bottom) data independent acquisition (DIA). First, molecules are ionized, or given a charge. Next, an initial mass spectrometry measurement is made to determine the mass-to-charge ratio (m/z) of each molecule. Then, certain m/z values or groups of m/z values are selected and broken into pieces. The resulting pieces are detected by their m/z values in a second mass spectrometry measurement.

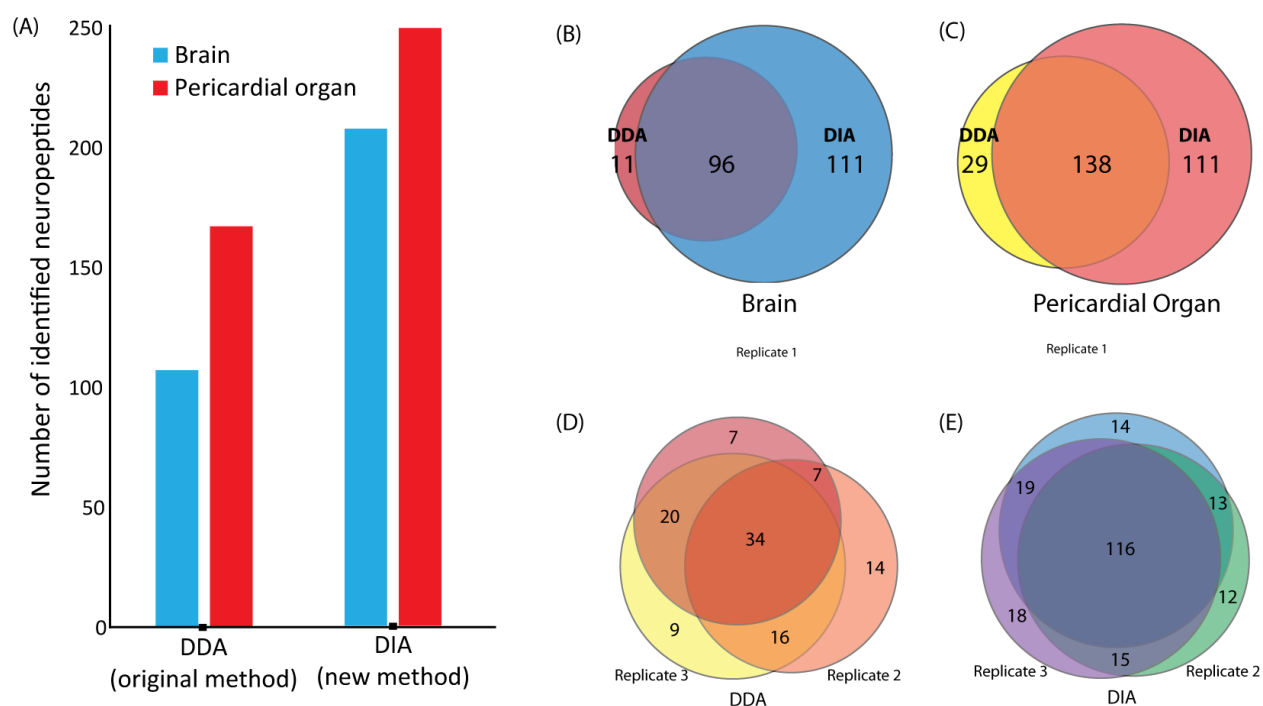


Figure 5. Resulting data comparing the original data dependent acquisition (DDA) identification method with the new data independent acquisition (DIA) identification method for mass spectrometry. (A) The bar graph shows the number of neuropeptides identified with each method for two different tissues, the brain and pericardial organs. Pericardial organs are located on either side of the crab heart and release neuropeptides into the heart. The overlap of neuropeptides identified in the (B) brain and (C) pericardial organs show that most neuropeptides identified with DDA were also identified with DIA, indicating that the new method wasn't missing neuropeptides the original method detected. The overlap of neuropeptides identified in replicate samples in (D) the DDA method was lower than that of the (E) DIA method, showing that the results of the new method were reproducible.⁵

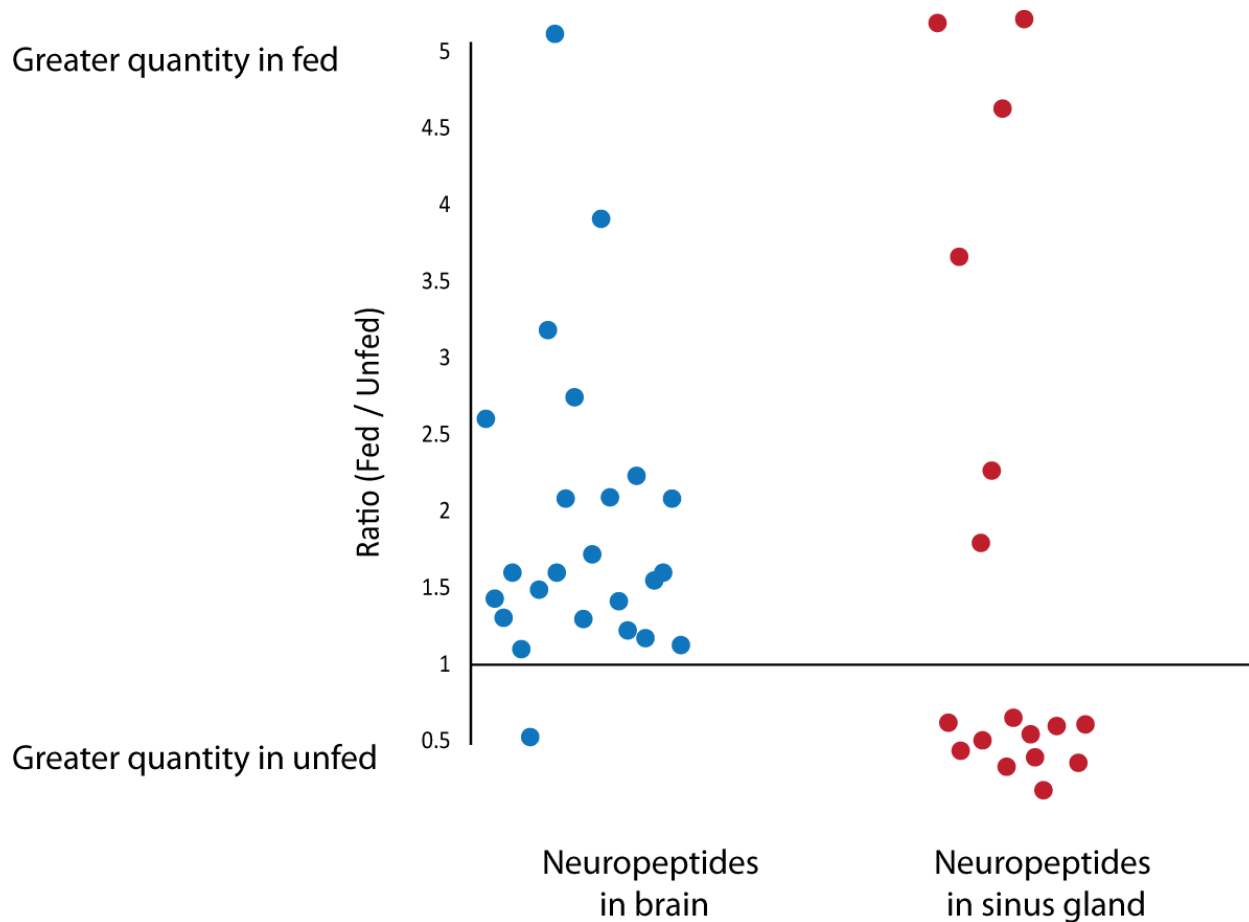


Figure 6. Differences in the amount of neuropeptide in tissue in crab between fed and unfed state in brain and sinus gland tissue. Each circle represents a different neuropeptide, and the value shown for each is the ratio of amount in fed crab vs amount in unfed crab. Ratios greater than 1 indicate an increase in neuropeptide in fed tissue, and ratios less than one indicate a decrease in neuropeptide in fed tissue.

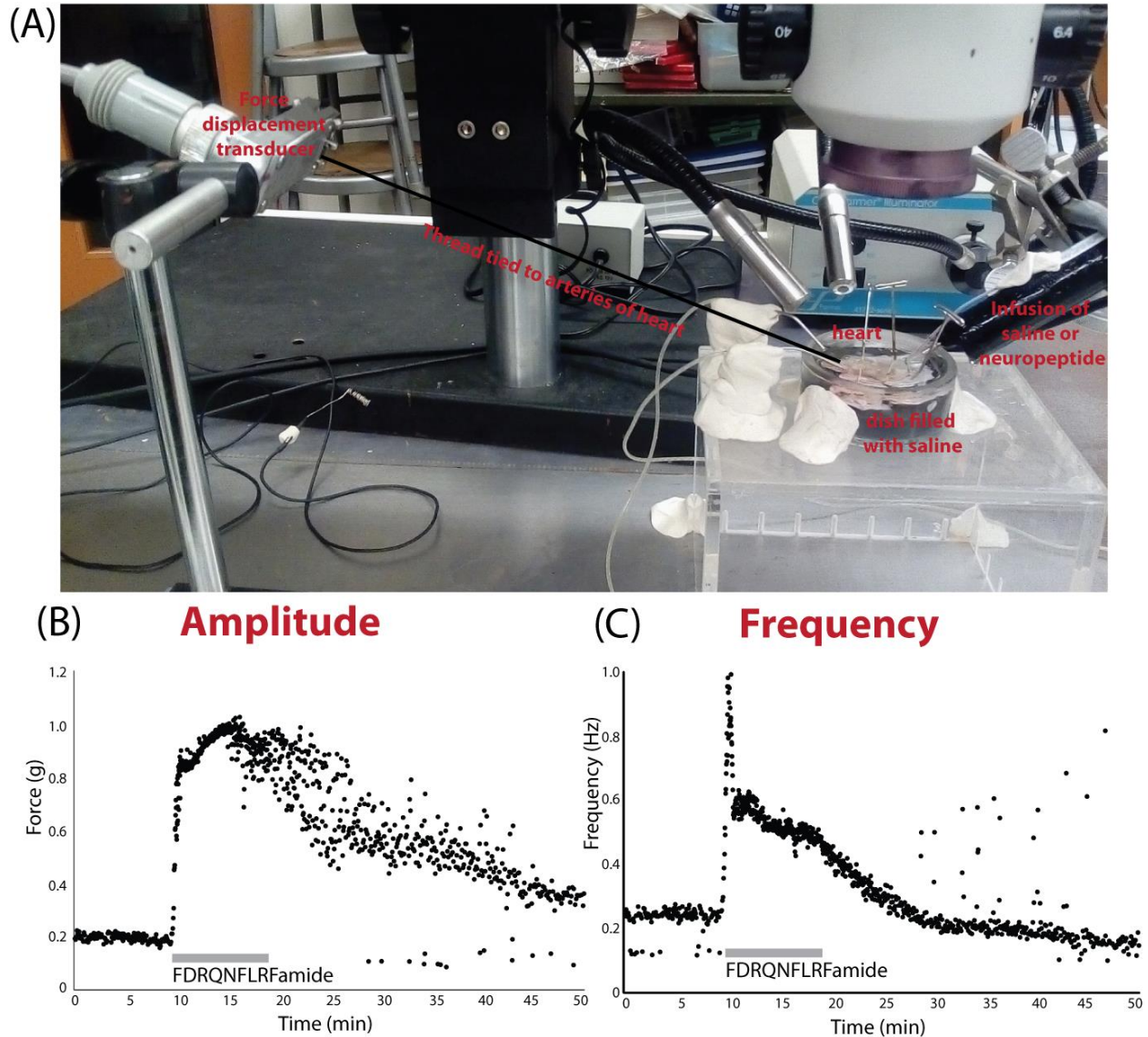


Figure 7. Measurements were made on the effects of neuropeptides on crab heartbeat. (A) The heart is removed from the crab and placed in a dish filled with chilled saline. Saline and neuropeptides are infused into the heart through the tail-side artery. The head-side arteries are tied with thread to a force displacement transducer that measures the strength at which the heart pulls on the thread when it contracts. Recordings of the (B) force and (C) frequency are collected over the course of time as a neuropeptide is infused into the heart. In this way, we can see how the presence of a neuropeptide changes the heartbeat.

Chapter 11

Conclusions and Future Directions

Conclusions

The research presented here sought to overcome challenges limiting neuropeptide research and uncover the identity and functional role of neuropeptides in circuit dynamics. As neuropeptides are diverse and complex in their functional roles, no single technique can capture all information. The importance of a multifaceted approach is emphasized in **Chapter 2** and supported by the work presented in subsequent chapters, which all rely on more than one approach to answer a question within, and in one case beyond, the field of mass spectrometry (MS).

In order to improve the breadth of information able to be gleaned from a single experiment, developments have been made to the traditional MS workflow, including separation/ionization (**Chapter 3**), spectral acquisition (**Chapter 4**), and data analysis (**Chapter 5**). Using orthogonal separation and complementary ionization techniques, namely capillary electrophoresis (CE)-matrix assisted laser desorption/ionization (MALDI) MS imaging and liquid chromatography (LC)-electrospray ionization (ESI) MS, the number of detectable neuropeptides increases, enabling a deeper profiling of the neuropeptidome of a particular sample, as demonstrated in **Chapter 3**. Adjusting the tandem MS (MS/MS) acquisition parameters to be consistent and unbiased and optimizing these parameters specifically for neuropeptides increases the number of confidently-identified neuropeptides and improves the reproducibility of the method, making the results more reliable, as evidenced by results presented in **Chapter 4**. Recognizing patterns in the sequence motifs common to neuropeptides has further improved confident identifications when combined with sophisticated *de novo* sequencing tools, which is described in **Chapter 5**. These techniques together expand the capabilities of neuropeptide research toward understanding their functional consequences.

In addition to the development of advanced tools for studying neuropeptides, this research includes application to important central pattern generators within the model organism *Cancer borealis*. One of these central pattern generators is the cardiac neuromuscular system, which is responsible for heart contractions and transportation of hemolymph throughout the animal. In **Chapter 6**, a multi-platform MS approach was used to profile and localize neuropeptides within the cardiac ganglion, a small tissue comprised of 9 neurons that control heartbeat. The results of this study provide insight into what neuropeptides are responsible for modulating heartbeat. Furthermore, functional studies were performed on two novel neuropeptides identified in **Chapter 9** that elicit changes in heartbeat. Together, these findings highlight the complexity of this small circuit and provide foundational information for more detailed electrophysiological studies.

The other central pattern generator studied in this research is within the stomatogastric nervous system (STNS), which is responsible for driving the gastric mill and pyloric rhythms. To provide a better understanding of the neuropeptide modulation of this circuit, changes in neuropeptide expression, both in abundance and spatial distribution, was explored in key neural tissue, including the brain and pericardial organs of *Callinectes sapidus* and *Carcinus maenas* (**Chapter 7**) and the brain, pericardial organs, sinus glands, stomatogastric ganglion, and commissural ganglia of *C. borealis* (**Chapter 9**). As neuropeptides are often transported long-range to act on the stomatogastric ganglion via the hemolymph, changes in neuropeptides over the course of feeding in *C. borealis* was investigated and are described in **Chapter 8**. The collective results from these feeding studies identify a cohort of neuropeptides that may be involved in modulation of feeding-related circuits driving the gastric mill and pyloric rhythms.

Overall, the conclusions reached in the research presented here present improved methods for analyzing neuropeptides, demonstrate the utility of these methods for better understanding of neuropeptides within the model organisms including *C. borealis*, *C. sapidus*, and *C. maenas*, and provide deeper insight into how these neuropeptides may be involved in modulating key central pattern generators and influencing circuit dynamics. The methods described here can easily be applied to the study of neuropeptides within other organisms, and the biological findings may be translatable to homologous neuropeptides in higher-order species.

Future Directions

While the methods described here have demonstrated improvements in neuropeptide detection and identification, there are still limitations that have yet to be overcome that can further expand our understanding of the nervous system. These include improving the confidence of identifications with MS/MS, combining these techniques with more sophisticated quantitation methods for functional assessment, and using these methods with samples closer to *in vivo* conditions in order to obtain a more biologically relevant assessment.

Confident identification of neuropeptides relies on obtaining high quality MS/MS spectra that can confirm the sequence and modifications of the peptide. While the LC-ESI-MS methods described in this work all successfully used MS/MS for neuropeptide identifications, the CE-MALDI-MSI method described in **Chapter 3** relied on accurate mass matching to within a 5 ppm tolerance. Obtaining quality MS/MS with MALDI is more challenging than ESI due to the formation of predominantly singly-charged ions that do not fragment well. Additionally, the limited sample available with MALDI-MS imaging makes obtaining MS/MS spectra even more challenging. **Figure 1** shows an example of the MS/MS spectrum offered by the current CE-

MALDI-MSI platform on a Thermo LTQ-Orbitrap XL mass spectrometer. While some fragment ions were present in the spectra, they were present in low abundances and difficult to distinguish from noise. While there has been previous success in developing a DDA method for MS imaging of tissue sections, the method has not yet been optimized for the CE-MALDI-MSI platform.¹ Exploring alternative acquisition parameters, matrices, and instrument platforms may improve the quality of MS/MS spectra such that the detected neuropeptides with the platform can be confidently analyzed. Furthermore, as the platform is highly complementary to LC-ESI-MS, it is expected that many more neuropeptides can be identified that have previously been undetected with conventional methods. High quality, unambiguous MS/MS spectra would facilitate the discovery of these neuropeptides and improve the completeness of the neuropeptide database for crustaceans.

In addition to improving detection and identification of neuropeptides, accurate quantitation of the neuropeptides is important for understanding how these peptides are perturbed due to environmental and behavioral changes. As quantifying changes in abundance of neuropeptides is often the first step in functional assessment, improving these techniques can enable a more sensitive assay of neuropeptides involved in various processes. The DIA method described in **Chapter 4** and implemented in **Chapter 8** utilized label-free quantitation to determine relative changes in abundance. While the method is suitable for detecting large-fold changes in abundance, it is less sensitive to subtle changes in abundance. The incorporation of chemical tags, either isotopic or isobaric, would improve the quantitative accuracy of the method. However, as these isotopic tags increase spectral complexity, their coupling with DIA is challenging. Preliminary experiments were performed to evaluate the use of duplex reductive methylation labeling with DIA-MS, and the results were promising, particularly when taking

advantage of a hybrid mass analyzer, as shown in **Figure 2**. Additional optimization of parameters may enable more accurate quantitation and improve instrument throughput.

The cardiac (**Chapter 6**) and feeding-related (**Chapters 7-9**) studies presented in this research identified a cohort of neuropeptides that may be responsible for modulating various circuit dynamics. These samples were analyzed at discrete time points from distinct animals. In order to better understand how these neuropeptides change over the course of time, *in vivo* studies within the same animal would be highly beneficial. Microdialysis has been shown to be a suitable technique for analyzing the change in abundance of molecules over the course of time in *C. borealis*.²⁻⁵ By coupling this technique with the methods described in this research, more detailed information about how neuropeptides change over the course of feeding can be obtained. A challenge with this technique is the small sample volume, typically 10 μ L, but the advantages include sampling from the same animal over the course of time with minimal stress inflicted and minimal sample cleanup required. Preliminary studies measuring the secretion of neuropeptides throughout the feeding process are shown in **Figure 3**.

While quantifying the changes in abundance of these neuropeptides suggests the possibility of biological functions, additional functional studies are needed in order to fully characterize these neuropeptides. The *ex vivo* heart preparation described and utilized in **Chapter 9** can be used to characterize the function of more neuropeptides identified with *de novo* sequencing in the other chapters (**Chapters 4, 5, 8, and 9**) in order to determine their biological activity. Follow-up electrophysiology studies can then be employed to determine their exact function within the cardiac neuromuscular system or stomatogastric nervous system.

A final future direction for this research is to take the entire process *in vivo* by combining microdialysis with cardiac and pyloric measurements that have been described previously.⁶

Placing infrared sensors on the carapace of the crab above the stomach and heart enable the outputs of each of these circuits to be measured in real time without inflicting stress on the animal. Peptides can be perfused into the crab through the microdialysis probe inlet, and secreted neuropeptides can be collected from the outlet. In this way, the neuropeptide content can be analyzed and quantified, the functional effects on the two central pattern generators can be measured, and changes due to biological processes such as feeding can be assessed in actual biological conditions. A depiction of the combined approach is shown in **Figure 4**.

Ultimately, the findings of this research can be used to further our understanding of how neuropeptides modulate circuit dynamics and how these changes are related to the behavioral outputs we observe. The methods described herein can be applied to additional physiological conditions or biological processes, organisms, and molecule types. The biological findings can be used to guide more in-depth studies of crustaceans or higher-order organisms such as mammals and humans. The research described in this dissertation serves as a stepping stone in the ultimate goal of understanding the deep complexities of the nervous system.

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Figures

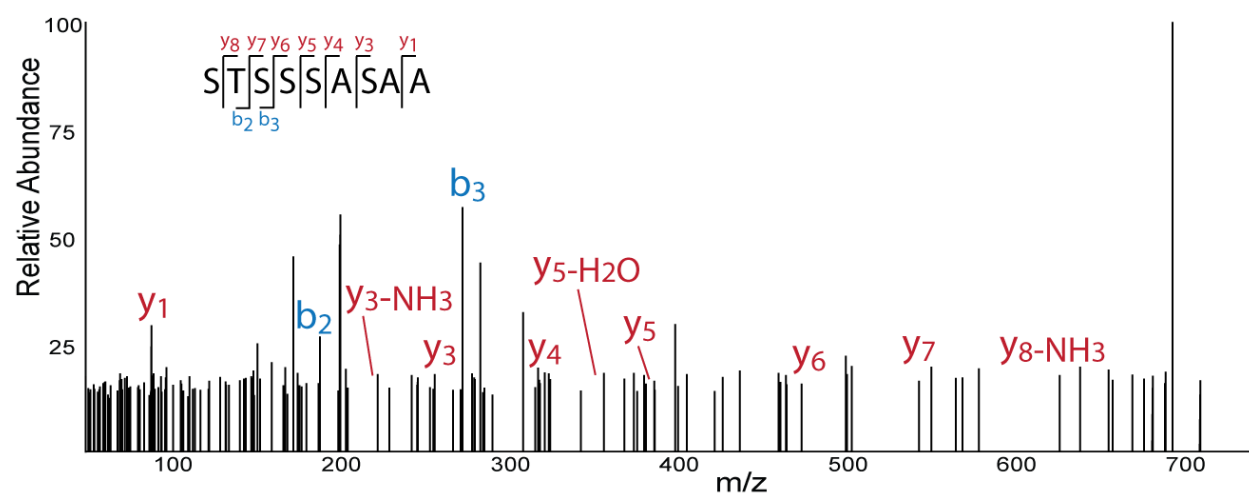


Figure 1. MS/MS spectrum of a neuropeptide detected in *C. borealis* thoracic ganglion using the CE-MALDI-MSI platform (unpublished data).

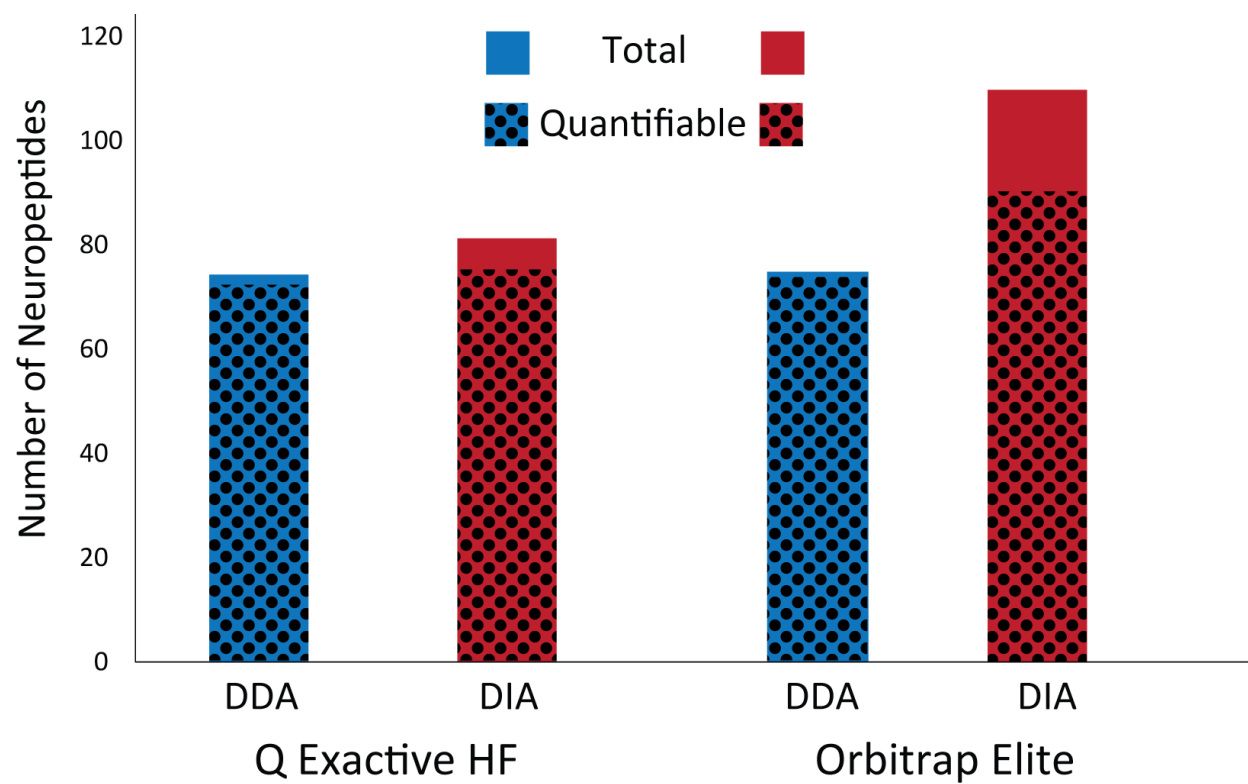


Figure 2. Comparison of number of duplex reductive methylation-labeled neuropeptides with DDA and DIA methods on two different MS platforms (unpublished data).

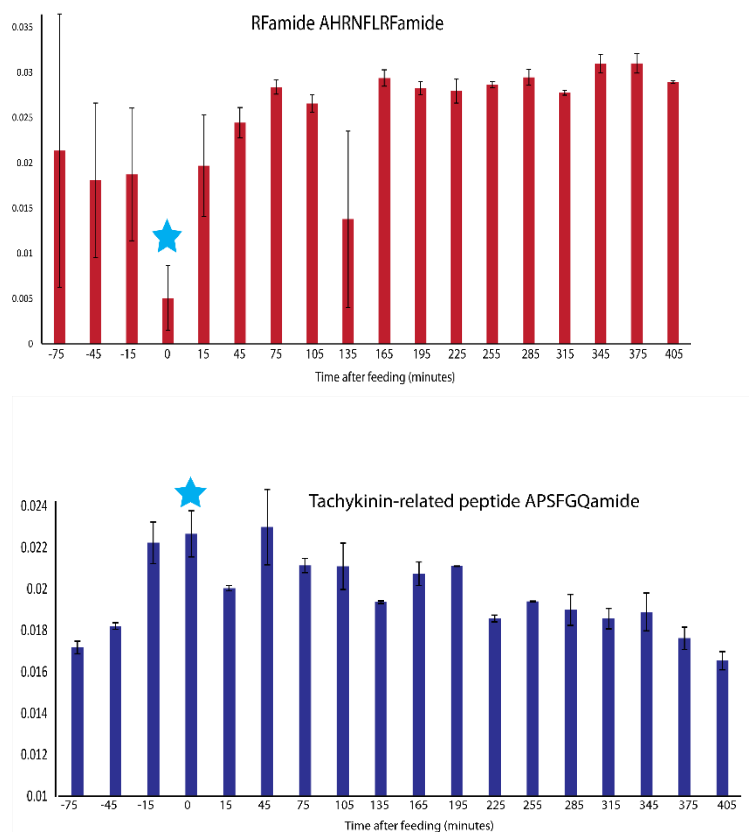


Figure 3. Changes in abundance of two neuropeptides over the course of feeding, sampled using *in vivo* microdialysis. The star indicates the time point at which the crab was fed (unpublished data).

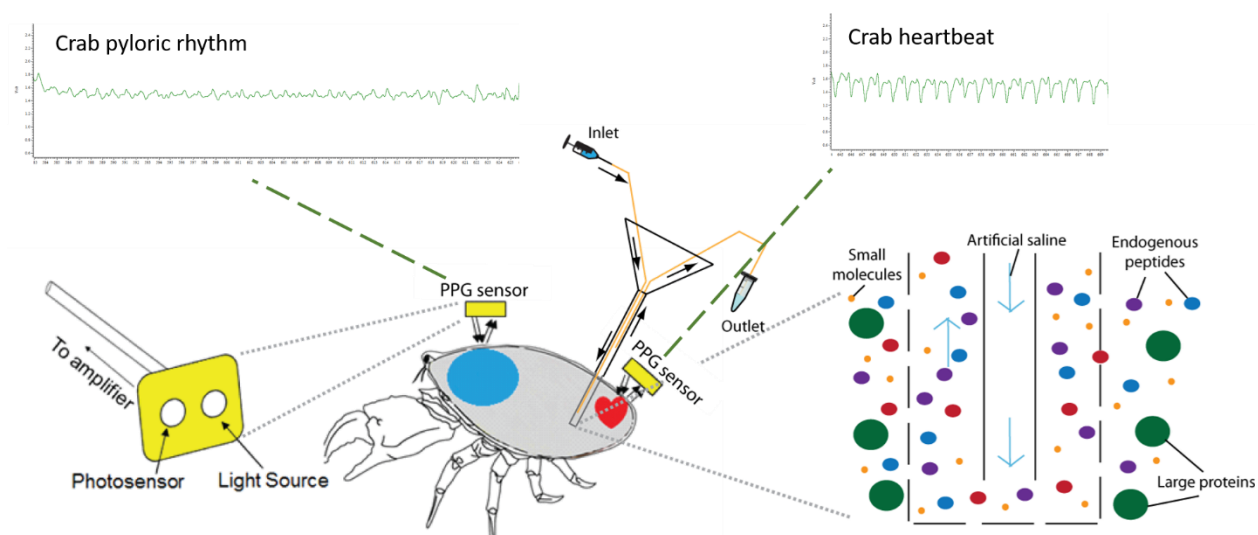


Figure 4. Depiction of setup for *in vivo* functional analysis of neuropeptides with infrared sensing of pyloric and cardiac output and microdialysis sampling of circulating fluid.

Appendix I

List of Publications, Presentations, and Grants

PUBLICATIONS

1. DeLaney, K.; Li, L. Neuropeptidomic Profiling of the Crustacean Cardiac Ganglion and Localization Using Mass Spectrometry Imaging with Multiple Platforms. *Journal of the American Society for Mass Spectrometry*. 2020. Submitted.
2. DeLaney, K.; Hu, M.; Hellenbrand, T.; Dickinson, P.S.; Nusbaum, M.P.; Li, L. Mass Spectrometric Quantification, Localization, and Discovery of Feeding-Related Neuropeptides in *Cancer borealis*. 2020. To be submitted.
3. DeLaney, K.*; Li, G.*; Li, L. Improvements in the Separation and Detection of Neuropeptides with Liquid Chromatography, Capillary Electrophoresis, and Ion Mobility Mass Spectrometry. *Journal of the American Society for Mass Spectrometry*. 2019. Invited Critical Insight, to be submitted.
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12. Vu, N.Q.; DeLaney, K.; Li, L. Neuropeptidomics: Improvements in Mass Spectrometry Imaging Analysis and Recent Advancements. *Current Protein and Peptide Science*. Submitted.
 13. Li, G.; Phetsanthad, A.; Ma, M.; Yu, Q.; Nair, A.; Zheng, Z.; DeLaney, K.; Ma, F.; Hong, S.; Li, L. Expedited Structural Mapping of Glycoprotein Transferrin Sialylation Associated with Alzheimer's Disease. *Journal of the American Chemical Society*. Submitted.
 14. Buchberger, A.R.; DeLaney, K.; Liu, Y.; Helfenbein, K.; Vu, N.; Li, L. Mass Spectrometric Profiling of Neuropeptides in *Callinectes sapidus* during Hypoxia Stress. *ACS Chemical Neuroscience*. Submitted.
 15. Thomas, S.; Hao, L.; DeLaney, K.; McLean, D.; Steinke, L.; Marker, P.C.; Vezina, C.M.; Li, L.; Ricke, W.A. Spatiotemporal Proteomics Reveals the Molecular Consequences of Hormone Treatment in a Mouse Model of Lower Urinary Tract Dysfunction. *Journal of Proteome Research*. 2020. Accepted.
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PROFESSIONAL PRESENTATIONS

1. DeLaney, K.; Hellenbrand, H.; Li, L. Profiling, imaging, and functional assessment of neuropeptides in the crustacean cardiac neuromuscular system using DIA-MS. Poster. 68th Annual ASMS Conference on Mass Spectrometry and Allied Topics. Houston, TX. June 2020. Abstract accepted.
2. DeLaney, K.; Li, L. Integrating group activities into traditionally lecture-based methods course for graduate students using primary literature. Poster. Teaching and Learning Symposium. Madison, WI. May 2020. Abstract accepted.
3. DeLaney, K.; Li, L. Profiling, imaging, and functional assessment of neuropeptides in the crustacean cardiac neuromuscular system. Oral Presentation. Pittcon 2020. Chicago, IL. March 2020.
4. DeLaney, K.; Li, L. Profiling and functional assessment of neuropeptides in *Cancer borealis* using mass spectrometry techniques. Oral Presentation. 2019 STG Meeting (Society for Neuroscience Satellite Meeting). Chicago, IL. October 2019.
5. DeLaney, K.; Li, L. DIA and DDA MS for Profiling the *Cancer borealis* Neuropeptidome and Peptidomic Changes Resulting from Food Intake. Oral Presentation. 67th Annual ASMS Conference on Mass Spectrometry and Allied Topics. Atlanta, GA. June 2019.
6. DeLaney, K.; Li, L. DIA and DDA MS for Profiling the *Cancer borealis* Neuropeptidome and Peptidomic Changes Resulting from Food Intake. Oral Presentation. Pittcon 2019. Philadelphia, PA. March 2019.
7. DeLaney, K.; Li, L. Enhanced detection of neuropeptides through CE-MALDI-MS imaging and data independent acquisition (DIA) LC-ESI-MS analyses. Poster.

Midwestern Universities Analytical Chemistry Conference. East Lansing, MI. November 2018.

8. DeLaney, K.; Li, L. Improving neuropeptidomic coverage of the stomatogastric nervous system with mass spectrometry techniques. Oral presentation. 2018 STG Meeting (Society for Neuroscience Satellite Meeting). San Diego, CA. November 2018.
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10. DeLaney, K.; Li, L. **Multifaceted Quantitation and Imaging of Changes in Crustacean Neuropeptidome Resulting from Food Intake. Poster. Graduate Student Faculty Liason Committee Annual Poster Session. Madison, WI. April 2018.**
11. DeLaney, K.; Li, L. Profiling secreted neuropeptides in the pericardial sinus and neurosecretory tissue in response to feeding behavior using mass spectrometry. Poster. UW Cardiovascular Research Center 23rd Annual Scientific Poster Fair. Madison, WI. December 2017.
12. DeLaney, K.; Li, L. Profiling secreted neuropeptides in the stomatogastric nervous system using mass spectrometry. Oral presentation. 2017 STG Meeting (Society for Neuroscience Satellite Meeting). Washington, D.C. November 2017.
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14. DeLaney, K.; Li, L. Mass Spectrometric Quantitation and Imaging of Neuropeptide Expression Levels in Crustacean Tissue and Circulating Fluid Resulting from Food Intake. Oral presentation and poster. 29th Sanibel Conference on Mass Spectrometry—Peptidomics: Bridging the Gap between Proteomics and Metabolomics by MS. Clearwater Beach, FL. January 2017.
15. DeLaney, K.; Buchberger, A.; Li, L. Mass Spectrometric Quantification and Imaging of Changes in Crustacean Neuropeptide Expression Levels Resulting from Hypoxia Stress. Poster. 64th Annual ASMS Conference on Mass Spectrometry and Allied Topics. San Antonio, TX. June 2016.

GRANTS

1. Mass spectrometry profiling of secreted neuropeptides in the stomatogastric nervous system in response to feeding
Funding Agency: NIH/NIGMS
Type: NRSA F31 Predoctoral Fellowship

Duration: September 1st 2018 – August 31st 2020
Received an impact score of 20 and ranked at 8%.

Appendix II

Protocols for Sample Preparation

Modified from:

DeLaney, K.; Buchberger A.; Li, L. Identification, Quantitation, and Imaging of the Crustacean Peptidome. In: Schrader M., Fricker, L. (eds) *Peptidomics. Methods in Molecular Biology*, 2018, 1719, Humana Press, New York, NY.

Abstract

Crustaceans serve as a useful, simplified model for studying peptides and neuromodulation, as they contain numerous neuropeptide homologs to mammals and enable electrophysiological studies at the single-cell and neural circuit levels. In particular, crustaceans contain well-defined neural networks, including the stomatogastric ganglion, oesophageal ganglion, commissural ganglia, and several neuropeptide-rich organs, such as the brain, pericardial organs, and sinus glands. Due to the lack of a genomic database for crustacean peptides, an important step of crustacean peptidomics involves the discovery and identification of novel peptides and the construction of a database, more recently with the aid of mass spectrometry (MS). Herein, we present a general workflow and detailed methods for MS-based peptidomic analysis of crustacean tissue samples and circulating fluids. In conjunction with profiling, quantitation can also be performed with isotopic or isobaric labeling. Information regarding the localization patterns and changes of peptides can be studied via mass spectrometry imaging. Combining these sample preparation strategies and MS analytical techniques allows for a multi-faceted approach to obtaining a deep knowledge of crustacean peptidergic signaling pathways.

1. Introduction

Crustacea is a diverse subphyla of arthropods including invertebrate animals that often live in aquatic environments. The most common members of this group include crabs, lobsters, crayfish, and shrimp. Crustaceans are a useful model for peptidomic studies because they possess a relatively simple nervous system with a rich repertoire of signaling peptides that modulate diverse functions of the animal. With numerous homologues to mammalian

neuropeptides, crustaceans can provide a useful test-bed to determine how networks of neurons generate behavior under the actions of various peptide modulators and hormones. The crustacean stomatogastric nervous system includes several key tissues that are rich in neuropeptides and are the focal point of crustacean peptidomic studies. These tissues include the stomatogastric ganglion (STG), oesophageal ganglion (OG), and a pair of commissural ganglia (CoG), which are all connected in series by motor nerves. The brain (*e.g.* the central nervous system) is connected to the OG by the inferior ventricular nerve (*ivn*). Other notable organs that play a crucial role in the neuronal signaling pathway are the sinus glands (SG), located in the eyestalks, and the pericardial organs (PO), located on either side of the heart. **Figure 1** shows the location of important tissues in *Cancer borealis*, a commonly studied crustacean species. The neuroendocrine cells within these tissues release neuropeptide hormones into the circulating fluid (*e.g.* hemolymph) of the crustacean^{1,2}. By studying the chemical content of these tissues and the transporting hemolymph, a deep understanding of the simplified signaling pathways can be acquired, which can be later applied to more complex organisms.

While crustaceans serve as a useful model for understanding complex neuronal networks, they pose a challenge for peptidomic studies due to the lack of a genomic database. Extensive efforts have been put forth to generate in-house peptide databases based on experimental data and predicted sequences from transcriptomic data³⁻⁵. In recent years, extensive profiling has been performed on crustaceans, as well as *in silico* predictions based on transcriptomes⁶, with novel peptides frequently being added to the growing database of crustacean peptides. Furthermore, BLAST homology searches are often performed to determine if identified peptide sequences are conserved across many species, allowing for possible functional information.

These efforts have been further driven forward by the development of mass spectrometry (MS)-based techniques for probing the crustacean peptidome. MS is an attractive choice for studying crustacean peptides because of its speed, selectivity, and sensitivity. High-resolution MS can even detect minute mass differences of peptide isoforms, which can have varying functions within the crustacean nervous system. Furthermore, with MS, there is no need for prior knowledge about the analytes, making it ideal for discovery of novel peptides and building a peptide database.

In addition to identification methods, efforts have been directed toward studying the functions of crustacean neuropeptides. These species have been shown to be highly resilient to stress, making them ideal candidates for comparative studies involving environmental or biological stresses (*e.g.* temperature ⁷ and salinity changes ⁸) as well as normal regulatory phenomena (*e.g.* feeding ⁹). MS-imaging has also been shown to be a useful strategy for mapping the location of neuropeptides within tissue and studying how these localization patterns differ in both two and three dimensions ^{10, 11}.

Here we describe methods for profiling neuropeptides and quantifying dynamic changes of neuropeptidomes under physiological perturbations. These methods can be used to identify novel neuropeptides to expand a database, quantify changes in peptide expression as a result of biological activities, or explore the localization of neuropeptides via MS imaging techniques. Methods for compiling a database and detecting peptides will be discussed as well as quantitation methods, such as label-free, isotopic, and isobaric labeling strategies. Methods for mapping the location of peptides within tissue will also be discussed. A generic workflow is shown in **Figure 2**. Details for preparation and handling of neuropeptides from collection to instrumental analysis and data analysis methods are described.

2. Materials

2.1 Chemicals and Equipment

1. Ultrapure water, used for all of the solutions listed below
2. Methanol
3. Acetonitrile
4. Formic acid
5. Acidified methanol (90:9:1 methanol: water: glacial acetic acid)
6. Isotopic formaldehyde solution (1% v/v)
7. Borane pyridine solution (30 mM)
8. Ammonium bicarbonate solution (100 mM)
9. Gelatin (100 mg/mL dissolved in water)
10. Matrix-assisted laser desorption/ionization (MALDI) matrix
 - a. 2,5-dihydroxybenzoic acid (DHB) dissolved in 50:50 methanol:water with 0.1% formic acid (150 mg/mL for spots; 40 mg/mL for imaging)
 - b. α -cyano-4-hydroxycinnamic acid (CHCA) dissolved in 50:50 acetonitrile:water with 0.1% formic acid (10 mg/mL for spots; 5 mg/mL for imaging)
11. Glass manual homogenizer (*e.g.* Wheaten 1 mL Tissue Grinder, Tenbroeck)
12. Teflon pestle (*e.g.* Wheaten 1 mL Tissue Grinder, Tapered Pestle)
13. Bath sonicator
14. Centrifuge capable of up to 16,000 g and able to fit microfuge tubes and microfilters (*e.g.* Eppendorf 5424 R)

15. Vacuum centrifuge/concentrator, such as a SpeedVac
16. 3 kDa or 10 kDa Molecular Weight Cutoff (MWCO) device (*e.g.* Amicon Ultra)
17. Reversed-phase C18-packed pipette tips (*e.g.* Omix 100 μ L Tips or Millipore P10 ZipTips)
18. Sprayer for MALDI imaging (*e.g.* HTX Technologies)
19. Crab saline (440 mM NaCl, 11 mM KCl, 26 mM MgCl₂, 13 mM CaCl₂, 11 mM TRIS, 5 mM maleic acid, adjusted to pH 7.45 with HCl or NaOH. Stored at 4°C)
20. Tweezers
21. Plastic Cup (1 inch Height x 1 inch Diameter)
22. Razor blade
23. Syringe Pump
24. Syringe
25. Automated sample collector with temperature control

2.2 Instrumentation and Software

For quantitation and profiling, a Hybrid Quadrupole-Orbitrap Mass Spectrometer (we use Thermo Q-Exactive) with a nano-electrospray ionization (ESI) source can be coupled to a UPLC system (we use Waters nanoAQUITY) for separation of peptides with MS and MS/MS analysis for identification and quantitation. For imaging and complementary profiling, we use a hybrid MALDI- Ion Trap-Orbitrap Mass Spectrometer (Thermo MALDI-LTQ-Orbitrap XL). Other mass spectrometers with a MALDI source and an ESI source may also be used. The associated vendor software Xcalibur and ImageQuest can be used for data analysis and image processing. For MS/MS identification, *de novo* sequencing software can be used, such as PEAKS ¹²,

MaxQuant¹³, or PepNovo¹⁴. Additional image processing can be performed using freely available software, such as MSiReader¹⁵ and Image J.

3 Methods

3.1 Identification and Quantitation

1.) Sample Collection and Extraction

a. Tissues

i. Collection

1. Collect the tissue of interest through dissection^{16,17}.
2. Place tissues in a 0.6 mL microfuge tube with 10 to 20 microliters of acidified methanol.
3. Store at -80° C until ready to use (*see Note 1*).

ii. Extraction

1. Add 100 microliters of acidified methanol per tissue to a glass manual homogenizer (*see Notes 2, 3, 4, and 5*).
2. Move the tissues to the homogenizer using tweezers. Transfer the storage solution as well.
3. Homogenize the tissue until no large particles are visible.
Transfer the liquid to a clean 1.5 mL microfuge tube.
4. Add 100 microliters of acidified methanol to the homogenizer, breaking up any residual particles. Transfer to same microfuge tube. Repeat once more.
5. Sonicate mixture for 10 minutes.

6. Centrifuge mixture for 20 minutes at $>16,000$ g.
7. Transfer the supernatant to a new 1.5 mL tube.
8. Rinse the pellet with 100 microliter of acidified methanol and break it up with a Teflon pestle.
9. Repeat steps 5-7. Discard the pellet.
10. Dry down supernatant in a vacuum centrifuge on medium heat (see **Note 6**).

b. Hemolymph

i. Collection

1. Place the crab on ice for 5-10 minutes. Afterwards, place it in a metal dishpan on its back with its tail pointing towards you.
2. Prepare a 25g needle by connecting it to a 1 mL plastic syringe.
3. Place the needle into an exposed leg joint of the crab.
4. Pull up on the syringe to create a vacuum. Hemolymph should come out immediately. If not, wiggle the needle at different angles until the liquid is withdrawn.
5. Add an equal amount of acidified methanol to the collected hemolymph in a 1.5 or 2 mL microfuge tube (see **Note 7**). A protein precipitate will be produced.
6. Store at -80°C till ready to use (see **Note 1**).

ii. Extraction

1. Using the Teflon pestle, break up the precipitate in the microfuge tube to produce a homogenous solution.

2. Sonicate tube for 10 minutes.
3. Centrifuge mixture for 20 minutes at >16,000 g.
4. Transfer the supernatant to a new 2 mL microfuge tube.
5. Rinse the pellet with 500 microliters of acidified methanol and break it up with a Teflon pestle.
6. Repeat steps 2-5. Discard the pellet.
7. Dry down supernatant in vacuum centrifuge on medium heat (see **Note 6 and 8**).

iii. Microfiltration

1. Add 200 microliters of 0.1 M NaOH solution to the 3 kDa or 10 kDa MWCO device (see **Note 9**). Centrifuge at >14,000 g for 5 minutes.
2. Rinse MWCO device with 500 microliters of 50:50 water:methanol. Centrifuge for 10 minutes at >14,000 g.
3. Dissolve sample in 500 microliters of 30:70 water:methanol, vortex, and sonicate for 10 minutes.
4. Add the sample to the MWCO device and run it through the membrane by centrifuging for 30 minutes at >14,000 g. Save the flow-through from this step by placing it in a 1.5 mL microfuge tube (see **Note 10**).
5. Rinse the membrane with 100 microliters of 30:70 water:methanol. Collect the flow-through and add it to the tube from the previous step.

6. Dry down the flow-through in vacuum centrifuge on medium heat.

c. Microdialysis (see **Notes 11 and 12**)

i. Collection

1. Rinse probe with water and then crab saline using a 3 mL plastic syringe and a syringe pump set to 0.5 microliters per minute.
2. Surgically implant the probe into the animal directly above its heart¹⁸. **Figure 1** indicates the location of the heart in *Cancer borealis*.
3. Allow the animal to recover for 24 to 48 hours (see **Note 13**).
4. Collect samples at desired time points. Collection can be done manually with a 0.6 mL centrifuge tube on ice or using an automated sample collector set at 4°C (see **Notes 14, 15, and 16**).
5. Add enough formic acid to make the total concentration 0.1% (v/v).
6. Store samples at -80°C if they are not being analyzed immediately. However, samples should be used as soon as possible to avoid degradation (see **Note 17**).

2.) Desalting

- a. Dissolve each sample in 0.1% formic acid (10 microliters for tissues, 200 microliters for hemolymph) by vortexing and sonicating for 10 minutes.

(Microdialysis samples do not need to be redissolved.) If the pH is greater than 3, add small amounts of diluted formic acid (*e.g.* 10%) until the pH is below 3.

- b. Using the appropriately sized reversed-phase C18 pipette tip and respective volume (*see Note 18*) (10 microliter for tissues and microdialysate, 100 microliter for hemolymph), flush the packing material with pure acetonitrile at least 3 times.
- c. Flush the packing material with 0.1% formic acid 3 times.
- d. Bind the sample of interest to the packing material by flushing it with dissolved sample at least 15 times.
- e. Wash the packing material with 0.1% formic acid 3 times to wash away salts and other unbound contaminants. Add the first wash to its own tube or to the original sample vial as a precaution in the event that nothing binds to the packing material.
- f. Elute the sample into a 0.6 mL tube by flushing 10 microliters of 50:50 acetonitrile:water up and down through the packing material 10 times.
- g. Dry down the eluate in a vacuum centrifuge on medium heat.

3.) Quantitative Labeling

- a. Dissolve each of the samples (*e.g.* of stressed and control) in 10 microliters of water by vortexing and then sonicating for 10 minutes.
- b. Add in 10 microliters of the isotopic formaldehyde to its designated channel (*see Notes 19 and 20*).
- c. Add in 10 microliters of borane pyridine to each microfuge tube.

- d. Place the tubes in a 37°C water bath for 15 minutes to allow complete labeling.
- e. Quench the reaction with 10 microliters of ammonium bicarbonate solution.
- f. Mix equal amounts of each channel into a 0.6 mL tube. Do this for each set of channels.
- g. Dry down mixture in a vacuum centrifuge on medium heat.

4.) Tissue Analysis and Data Acquisition

a. MALDI-MS

i. Matrix Application

1. Dissolve samples in 5 microliters of 0.1% formic acid for each tissue used by vortexing and sonicating for 10 minutes (*see Note 21*).
2. In a separate 0.6 mL tube, mix equal amounts of matrix (*see Note 22*) and sample (*see Note 23*) solutions, vortexing them to ensure complete mixing.
3. Take 1 microliter out of the tube using a pipette and spot it on the MALDI stainless steel plate. Rub the tip of the pipette along the edges of the spot circle to make sure the whole circle is filled.
4. Allow the matrix to crystalize fully before placing in the instrument.

ii. Analysis on hybrid MALDI- Ion Trap-Orbitrap-MS

1. If necessary, attach a backing plate to the MALDI stainless steel spot plate (MALDI-LTQ-Orbitrap XL requires this), and insert the plate set into the instrument by placing the plate in the correct position and pressing the appropriate button on the vendor's tune page (e.g. the "Insert MALDI sample plate" button on the "MALDI source" window of the instrumental LTQ tune page).
2. Once the plate has inserted, confirm the spot in which you placed your sample and shoot the spot with an appropriate laser energy to obtain a signal intensity (approximately $1E7$ for MALDI-LTQ-Orbitrap XL, *see* **Note 24**).
3. Open Thermo Xcalibur Sequence Setup, and create a new sequence (or use alternative vendor's software with regard to its specifications).
4. Fill the sequence, including the title, path (for saving the data), instrumental method (*see* **Note 25**), and spot position (*see* **Note 26**).
5. Start automated or manual acquisition of every spot of the sequence (in Xcalibur: Select each line to be run and press the "Run Sequence" button).

b. ESI-MS (*see* **Notes 27, 28, and 29**)

i. Sample Prep

1. Dissolve samples (approximately 5 micrograms on average) in 5 microliters of 0.1% formic acid per tissue used or per 0.25 mL hemolymph by vortexing and then sonicating for 10 minutes.
 2. Transfer the volume into a LC vial with a screw top septum (we use Waters vials), making sure to get all the volume to the bottom with no bubbles.
- ii. MS analysis (we use Q-Exactive, Thermo)
1. Place the LC vial into the chilled sample compartment of the LC system (we use Waters NanoAquity). Remember the tray number and tray position in which the samples were placed.
 2. Make sure the column system has been equilibrated to the starting conditions of the gradient you will be using.
 3. On the instrument profile, set up the sequence as described above (see **Note 25**).
 4. Select each sample in the sequence (see **Note 30**) to be run (in Xcalibur: press the “Run Sequence” button).

5.) Data Analysis

a. MALDI-MS

- i. Average all the MS spectra collected by left-clicking and dragging across the chromatogram.

- ii. Copy the raw data into your vendor's software using the exact masses (Xcalibur: by right-clicking on the spectrum, selecting "Export" and "Clipboard (exact mass))."
- iii. Paste data in blank spreadsheet file (we use Excel, Microsoft).
- iv. Compare the masses found to the in-house database (unlabeled or labeled) (*see Note 31 and 32*). See **Figure 3** for an example spectrum.

b. ESI-MS

- i. Load the data into a *de-novo* sequencing software (we use PEAKS), creating a new project, and loading the raw data into the program (*see Note 33*).
- ii. Process the samples (using the "PEAKS search" button), indicating any enzyme used, any PTMs expected (*see Note 34*), and choosing the in-house database. *De novo* sequencing will be done with the same parameters (*see Notes 35*).
- iii. Once the search is complete, quantitative information can be mined (*see Note 36*), in PEAKS by using the "Quantification" icon.

3.2 Localization by Mass Spectrometric Imaging

1.) Sample Preparation

- a. Small or thin tissue (*e.g.* PO, STNS, STG as in Fig. 1)
 - i. Collect the tissue of interest by means of dissection^{17, 19}, being mindful of tissue orientation (*see Note 37*).
 - ii. Hold onto tissue with tweezers and briefly submerge it in a microfuge tube of water to desalt (*see Note 38*).

- iii. Place the clean tissue directly on a glass slide. Use a marker to label the orientation of the tissue (*i.e.* which area is closest to the head, tail, etc.). Stretch the tissue out on the slide to ensure that it is lying flat and easily visible.
 - iv. Tissue should be analyzed immediately (*see Note 39*).
- b. Large tissue (*e.g.* brain, TG)
- i. Prepare gelatin solution, vortex, and place it in a 37°C water bath until fully dissolved. Keep gelatin warm while not in use.
 - ii. Pour enough gelatin into a plastic cup to cover the bottom of the cup. Allow the gelatin to solidify at room temperature.
 - iii. Collect the tissue and desalt as described above for small tissue.
 - iv. Place the tissue on top of the gelatin layer, making note of the tissue orientation. Fill the cup with warm gelatin. The tissue will float toward the top. Reorient the tissue if necessary to make sure it is centered in the cup.
 - v. Immediately place the cup with tissue in dry ice to flash freeze it (*see Note 39*).
 - vi. Store tissue at -80°C until use.
 - vii. Cross-section tissue using a cryostat.
 - 1. Remove tissue-embedded gelatin block from plastic cup and trim excess gelatin with a clean razor blade.
 - 2. Place small droplet of water on cryostat chuck and place tissue block on top. Surround the rest of the block with water, making sure to not get any below the chuck.
 - 3. Keep the block/chuck in the cryostat to allow the water to freeze, attaching the tissue block on the chuck (approximately 15 minutes).

4. Attach the chuck to cryostat and align tissue block so that even slices can be made through tissue.
5. Obtain several 12 – 16 micron sections from throughout the tissue. Thaw-mount each section to a glass slide by warming the glass slide slightly and placing it directly above section. Section will adhere to slide (*see Note 40*).
6. Store slides at -80° C until ready for analysis (*see Note 41*).

2.) Image Acquisition (MALDI-MS)

a. Matrix Application via Sprayer (*see Note 42*).

- i. Turn on matrix sprayer (we use TM-Sprayer, all subsequent experimental details refer to that) and solvent syringe pump, setting desired solvent flow rate.
- ii. Set pressurized air to 10 psi.
- iii. Open program and set temperature to 80°C for DHB (*see Notes 43 and 22*).
- iv. Set up the desired method (*see Note 44*).
- v. Switch the injection loop to “Load” and load matrix using a syringe pump.
Switch the injection loop to “Spray” and ensure that matrix is being sprayed. It may take several minutes for matrix to reach the spray nozzle.
- vi. Click “Start” on the Sprayer software (*see Note 45 and 46*).
- vii. When method has finished, flush the matrix loop with solvent three times and set the temperature of the sprayer to 30°C. When the sprayer temperature has lowered to this temperature, turn off sprayer, syringe pump, pressurized air, and software.

b. Analysis on MALDI-LTQ-Orbitrap

- i. Insert one or two slides into MALDI imaging plate.

- ii. Place plate face-down in scanner, and scan image of entire plate. Ensure that the white crosses in the corners of the plate are clearly visible in the scanned image; otherwise, the image cannot be aligned to the plate in the instrument.
 - iii. Attach the backing plate to the MALDI imaging plate and insert the plate set into the instrument.
 - iv. Once the plate has been inserted, click on the “Tissue Imaging” tab of the MALDI Source page and check the box titled “Use tissue imaging feature.”
 - v. Under “Position File,” check “Import Image” and upload the scanned image of the MALDI plate (*see Note 47*)
 - vi. Enter the appropriate raster size (*see Note 48*) and ensure that the size “rectangular” is selected. (Rectangles are the preferred shape for methods.)
 - vii. Select “View Plate,” and click on the square selection tool. Select the area of the tissue. Close the “View Plate” window and save the position file (*see Notes 49-51*).
 - viii. Click on the “Control” tab and shoot the laser at an area of matrix. Adjust the laser energy as needed in order to obtain sufficient signal (with our instrument on the order of $1E7$).
 - ix. Open the software setup (Thermo Xcalibur Sequence Setup), and create a new sequence as previously described. For the position, copy and paste the directory of the position file for each tissue into its respective row.
 - x. Select each line to be run and press the “Run Sequence” button.
- c. Data Analysis
- i. ImageQuest

1. Open raw data in ImageQuest.
2. Click the “New data set” icon and type in the mass of interest and the tolerance window with the “Base Peak” plot type. Under plot type, select the desired normalization, if any (see **Note 52**). Click “OK.”
3. To save or export individual images, select the image and click “Copy” in the Edit tab. Paste the image in the desired location (see **Note 53**). See **Figure 4** for example MS images.

ii. MSiReader (see **Note 54**) (12)

1. Open the raw data in MSiReader using the appropriate open-access format (see **Note 55**).
2. Enter an m/z value from ImageQuest that displays a clear distribution in the tissue.
3. Enter the appropriate parameters for the m/z window, normalization (see **Note 52**), and color map.
4. Use the image overlay feature to upload a scanned image of the tissue and align the image to the MS image of the tissue. Set the transparency to around 50% to ensure both optical and MS images are clearly visible.
5. Generate an image of each neuropeptide signal of interest from a database using “generate an image for each peak in a list” button.
6. Manually examine images for detected neuropeptides and distinct spatial distributions.
7. Search for novel neuropeptides and m/z values outside of the database by using the peak finding tool.

iii. 3D image generation (Image J)

1. Open grayscale images of consecutive tissue slices with the same m/z value (saved from ImageQuest).
2. Align the images using the functions under “image.”
3. Combine the images into a three-dimensional stack using the “images to stack” button.
4. View the three-dimensional image by clicking “image,” “stacks,” and then “3D project.” Viewing parameters can be adjusted in the 3D projection window.

4. Notes

1. While storage at -80°C minimizes postmortem degradation, tissues should only be stored for up to six months before use. There are several ways to decrease postmortem degradation for both tissues and hemolymph, which would extend their storage life. For tissues, the use of a Denator heat stabilizer system or boiling of the tissues for heat stabilization has been shown to be effective ²⁰. For hemolymph, the addition of protease inhibitor cocktail or EDTA can reduce protease activity.
2. Several different extraction solvents have been tested for their ability to extract neuropeptides from tissues, including acidified organic solvents ²¹. A method termed “mixing on column” (MOC), where 4 different extraction solvents are incorporated sequentially, has been shown to be extremely effective for mammalian neuropeptides ²², but this technique’s usefulness is still being tested for crustacean peptidomics.

3. While a manual homogenizer has been shown to be effective for the small crustacean tissues, an electronic sonicator may be needed to fully break down the tissue walls of large organs. These should be used with the tissues on ice to help with heat dissipation.
4. When deciding how many tissues to pool, it is important to balance biological variance with the amount of tissues required for the study. In most stress-related studies, at least 3 animals' tissues are pooled for each biological replicate.
5. Direct profiling is alternative strategy to extraction. The tissue of interest can be placed on the stainless steel spotting plate, with matrix being spotted directly onto the tissue area of interest. This method has become phased out with the development of new imaging techniques (see "Localization using Imaging" section in this book chapter).
6. Many crustacean peptides vary widely in their molecular masses. For example, crustacean neuropeptides can range from 0.5 to 9 kDa. With many orbitrap-based instruments, it is difficult to analyze the larger peptides without taking a bottom-up approach. This means some sort of digestion prior to analysis may be required to get a full picture of a crustacean peptidome. For crustacean peptides, the digestion is usually done on the initial extract. After reducing all disulfide bonds with dithiothreitol and alkylating with iodoacetamide, an enzyme (*e.g.* trypsin) is added at a 25-50:1 peptide: trypsin ratio. Peptide content can be determined using the bicinchoninic acid assay or a similar assay.
7. Each crab has a limited amount of hemolymph stored in its body. For example, *Callinectes sapidus* has approximately 5 mL. After removal, hemolymph is restored over a period of time. Thus, the amount of hemolymph removed depends on the species of crab and whether it is to survive afterwards. Replacing the volume of removed

hemolymph with an equal volume of crab saline can increase the crab's chance at survival.

8. After drying down any hemolymph samples (initially to fully processed), they should be resuspended prior to storage in the -80°C freezer to ensure full dissolution.
9. Depending on the size of the peptides of interest, two versions of MWCO filters are available: 3K or 10K Daltons. 10K is normally used to purify a wide mass range of crustacean peptides (see **Note 5**). After digestion, 3K MWCO filters can be used to simplify the sample and allow for a targeted analysis.
10. Many times, the MWCO filters become clogged with proteins that (a) were not fully pelleted in the extraction step or (b) were not dissolved in the methanol:water mixture that was added to the extract. In order to lessen the chance of this happening, (a) centrifuge the extract at >16,000 g for 15-20 minutes before putting the supernatant through the filter, (b) separate the extract into two fractions with two MWCO filters, or (c) centrifuge the extract through the filter for a longer period of time (45+ minutes).
11. There are several types of commercial probes that can be used for microdialysis. Alternatively, homemade probes can be implemented at a much lower cost. Whether purchasing or making probes, there are several factors worth considering, including membrane material, the molecular weight cutoff (the expected MWCO is approximately a third of the reported MWCO), area of membrane, and use of polymer coatings²³.
12. The microdialysis technique tends to have low recovery *in vivo*. The recovery can be enhanced through the use of affinity agents²⁴.

13. It is important to closely monitor the crab while it recovers from surgery, as the majority of post-surgery complications (*i.e.* crab dying, pulling out probe, clogging probe) typically occur within the first twenty-four hours.
14. Collection windows are typically within the range of 30 minutes to 2 hours. This is dependent on the necessary temporal resolution and recovery of sample. Shorter collection durations provide higher resolutions, but yield lower sample volumes. Collection times of several minutes have been shown feasible, often using online microdialysis collection couple to ESI. However, this method also comes with drawbacks ²⁵.
15. The optimum infusion rate for microdialysis is a tradeoff between relative recovery and absolute recovery. Lower flow rates allow for more diffusion of sample through the membrane, but result in less sample volume, while higher flow rates enable the collection of more sample overall but run the risk of disturbing the crab. We have found the ideal flow rate to be 0.5 microliters per minute.
16. When collecting samples, it is important to account for the dead volume between the tip of the probe and the end of the tubing (and collection needle, if one is being used). The delay from sample diffusing into the probe to reaching the end of the tubing could be an hour or greater, depending on the length of tubing and infusion rate.
17. Microdialysis samples have been shown to degrade rapidly, even within several hours. Therefore, samples should be analyzed as soon as possible after collection. If longer storage time is necessary, there are several methods for improving the lifetime of the samples ^{25, 26}.

18. Many different varieties of separations can be done to increase peptide purity, including strong cation exchange (SCX) and C18 pipette tips (i.e. ZipTips). Success has also been found in increasing peptide coverage in fractionation using C18 pipette tips or an HPLC system ²⁷.
19. Formaldehyde labeling is commonly used to produce 2 or 3 different isotopic forms that differ in mass ²⁸⁻³⁰, although up to 5 different isotopic forms can be generated with different combinations of heavy and light formaldehyde and reducing agent (see Dasgupta et al chapter in this volume). Other labeling methods exist, such as isotopic N,N-dimethyl leucine (iDiLeu), which boasts 5-plex labeling capabilities, which allows for relative or absolute quantitation by an in-solution calibration curve ³¹. Absolute quantitation of a single peptide can also be done by adding a deuterated-version of the peptide to the original extract of the sample.
20. For crustacean tissues, formaldehyde labeling has been shown to be extremely effective due to its quick and complete labeling of peptides. While MS-based quantitation strategies are simple, they have limitations, especially when analyzing more than 5 samples or samples with high spectral complexity. On the other hand, MS/MS-based quantitation strategies, such as iTRAQ, TMT, or N,N-dimethyl leucine (DiLeu), allow for higher multiplexing (up to 12 sample comparisons at once have been demonstrated, but up to 18 may be possible) with lower MS spectral complexity ^{32, 33}. Unfortunately, quantitative depth may suffer because, in order to become quantified, the peptide needs to be selected for MS/MS.
21. Prior to spotting with matrix, an offline separation can be done, for example with capillary electrophoresis or high-pressure liquid chromatography (HPLC). Success has

been seen for separating tryptic peptides with both high-pH and strong-cation-exchange chromatography^{34,35}.

22. Several matrices work well for crustacean peptide analysis, including DHB and CHCA. While DHB extracts peptides well, CHCA, known for being a “universal matrix,” tends to provide a more homogenous layer.
23. Several spotting techniques exist, including the suggested premixing, alternatives are sandwiching, or individual mixing on plate prior to recrystallization.
24. Depending on the instrument, lifetime of the laser, instrumental setup, and the matrix of choice, the laser energy used will need to be optimized for each sample.
25. In order to acquire more confident identifications or perform *de novo* sequencing, tandem MS is necessary. For MALDI instruments, collision-induced dissociation and high-energy collisional dissociation are the main commercially-available options. Unfortunately, MALDI ionization mainly produces singly charged ions, which lead to poor fragmentation. This means that most identifications for MALDI are done through accurate mass matching. ESI provides much higher-quality fragmentation spectra, meaning that tandem MS can be used for high-confidence identification and discovery of novel peptides. As another fragmentation option, electron transfer dissociation, which is better for post-translational modification analysis, is now being more readily available. Even with high quality tandem MS spectra, *de novo* sequencing of peptides can be challenging. The use of chemical derivatization with a non-isotopic version of formaldehyde (see **Note 19**) has allowed for more complete coverage when *de novo* sequencing putative peptides³⁰. Other methods, such as thiol reduction and alkylation

have also been used to achieve high-resolution sequencing of larger, disulfide-bonded crustacean peptides ³⁶.

26. Xcalibur software only recognizes plates as having 96 wells, so it will only allow you pick spots that are within that plate size, even if you are using a 384-well plate.
27. Besides classic LC separation, several other complementary options exist. Capillary electrophoresis is compatible offline for both MALDI and ESI analysis for enhanced separation of crustacean neuropeptides ^{37, 38}. Ion mobility has also been shown to be effective at separating D/L-epimeric crustacean peptides ³⁹.
28. The use of either a commercial or homemade column should be considered. Homemade packing lowers the cost of each column and allows for customization, which may improve peptide separation and thus coverage. However, low uniformity can cause inconsistency between columns in comparison to commercially available columns. Although, these columns can be much more expensive in comparison.
29. For peptides, the reverse-phase C18 column setup uses a gradient of water with 0.1% FA and acetonitrile with 0.1% FA to elute the sample. Depending on the complexity of the sample, the time of gradient, highest percent acetonitrile added, and instrumental parameters (*e.g.* dynamic exclusion) will need to be adjusted.
30. When beginning any LC-MS experiment, a quality control sample should be run to determine if the instrument is working optimally. Also, blanks should be run between each new sample type to decrease sample overlap.
31. For orbitrap instruments, the standard mass tolerance is +/- 5 ppm.
32. This can be done manually or with a simple peak-picking program.

33. Several software packages are capable of performing *de novo* sequencing, database searching, and peak peaking for quantitation besides PEAKS (*e.g.* Proteome Discoverer).
34. Common post-translational modifications (PTMs) for crustacean neuropeptides include amidation and dehydration. If any labeling is done, it is important to include the tag or modification during the search (*e.g.* dimethylation).
35. PEAKS software package works by first *de novo* sequencing all of the raw data, which will then be matched to the provided database of precursor masses and proposed sequences. It is important to note that, while other databases come from sequenced genomes, the crustacean neuropeptide database has been developed in-house, as there is no genomic database for crustaceans. When looking at peptides with no digestion, PEAKS shows the peptide searched as a “protein” in the program. The “peptides” section is the individual peptides that make up all the crustacean peptides, which can include cleaved or degraded derivatives. Anything that doesn’t match is placed in a “de novo only” tab, which can provide you with possible novel peptide groups. For neuropeptides, these “de novo only” peptides are compared to current family sequences, from which certain sequence themes can be found to identify novel neuropeptides. Confident identification can then be done by synthesizing standards to confirm MS/MS and LC retention patterns.
36. Both MS and MS/MS-based quantitation can be done, depending on the labeling used in the quantitation step. Label-free quantitation is also possible.
37. It may be helpful to label dissection dishes with the orientation of the tissues to avoid uncertainties after the tissue is dissected.

38. Delicate tissue such as the PO can be transferred to the water by either carefully folding the tissue into quarters or by holding the tissue at either end with separate sets of tweezers.
39. To prevent degradation, a Denator heat stabilizer can be implemented immediately after desalting. However, caution must be taken to avoid melting delicate tissue.
40. It is common for slices to fold, tear, or become distorted during the process of thaw mounting. Once the slice is on the slide, thoroughly check it to ensure the integrity of the tissue.
41. For best results, slides should be analyzed as soon as possible. During storage, slides can be wrapped in tin foil to protect them from damage. When removing the slides from the freezer, water will condense on the slides, which can cause diffusion, due to the major temperature change. Place them in a desiccator to minimize this effect.
42. There are several methods for applying matrix to a sample, including airbrush, automated sprayer, inkjet printer, sublimation, etc. The method described here utilizes an automated sprayer (*e.g.* TM-Sprayer) which has been found to be the most reproducible ⁴⁰.
43. The sprayer temperature should be high enough to evaporate the matrix/solvent mixture. For the TM-Sprayer, turn up the temperature slowly until you hear a “puffing” noise. Reduce the temperature by 5°C for the final method.
44. Parameters to consider when choosing a method include the number of times the sprayer passes over and coats the slide, the drying time between coats, the direction the sprayer moves in to coat the slide (*i.e.* horizontal or vertical), and the system flow rate. Our preferred method includes 12 passes with a 30-second dry time between passes, and the orientation alternating between horizontal and vertical with each pass to provide an even

coating at a flow rate of 0.1 mL/min. Methods should be optimized to the peptide group of interest and matrix to minimize diffusion while increasing peptide extraction.

45. The matrix loop only has a finite volume it can hold, and so it may be necessary to reload it with matrix during the course of the method. If this is necessary, wait until the sprayer is done with its current pass, switch the injection loop to “Load,” load matrix, and switch the injection loop back to “Spray” before it starts the next pass.
46. After clicking “Start,” the system may say the temperature is unstable due to the sensitivity of the system. Since the temperature will never fully stabilize, click “Start Now” to start the method.
47. The MALDI-Orbitrap is also capable of scanning in the whole plate or each individual slide at varying image qualities. Scanning the plate with an external system only takes a few minutes, while scanning one slide at “normal” resolution takes ~25 minutes. For other instruments, images need to be scanned by an external scanner.
48. Raster size determines the spatial resolution of the tissue being imaged. While small raster sizes provide better image resolution, they can drastically increase the analysis time of the instrument. The MALDI-Orbitrap allows for a raster size of 75 microns without oversampling, but other MALDI instruments boast spatial resolution down to a few microns.
49. After selecting the final area of interest, saving this imaging in the “View Plate” window allows for easier image overlay during data processing.
50. The software tends to shift the selected area unpredictably after closing the window. Therefore, it is recommended to close and reopen the “View Plate” window after a selection has been made to ensure that the area of interest is still within the selection box.

51. It is good practice to include a small amount of area outside of the tissue when selecting the area to be analyzed in order for it to be used as a means to distinguish signal from random noise.
52. Typically, normalization is performed in reference to the total ion current (TIC), in which each mass spectrum is divided by its TIC. This ensures that all spectra have the same integrated area under their curves. Other normalization strategies are emerging ⁴¹, but TIC continues to be the most widely used.
53. When comparing multiple images, it is often helpful to set them to the same intensity scale. This can be done by entering the Min and Max Plot Values in the “Scale” tab.
54. MSiReader is a freely-available, open-access software that can be downloaded from <http://www.msireader.com/> ¹⁵.
55. To convert to imzML in ImageQuest, click File, Export, imzML. Either all peaks or centroids only can be exported.

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Figures

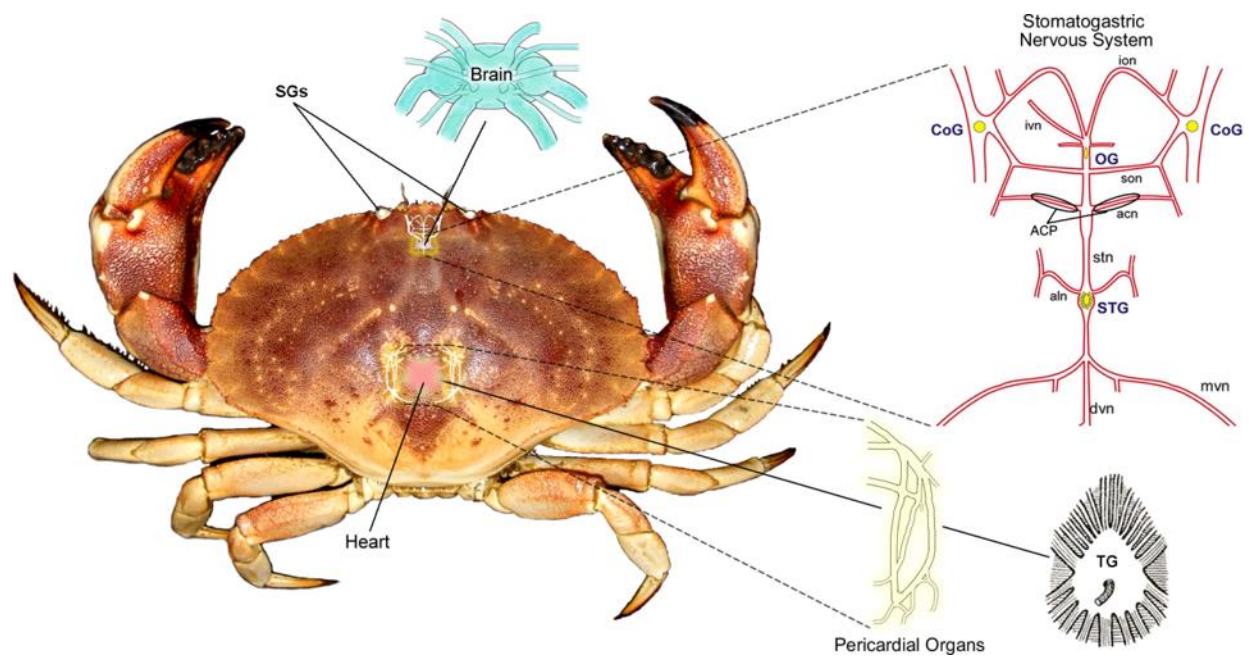


Figure 1. Schematic diagram of the location of tissues in *Cancer borealis* that are used for peptidomic studies, including the brain, CoGs, OG, STG, TG, POs, and SGs. The location of the heart is also indicated, which is important for probe placement in microdialysis studies.

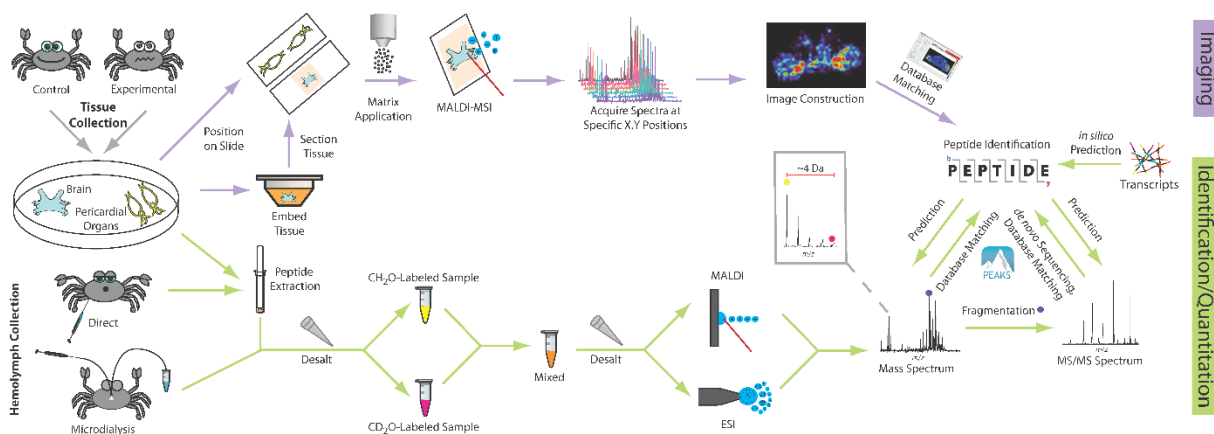


Figure 2. Workflow indicating the steps for sample collection, preparation, and data acquisition and analysis. The workflow contains steps for analysis of tissues, crude hemolymph, and microdialysis samples and describes the processes for both imaging and quantitation. For relative quantitation, duplex formaldehyde labeling is indicated, with CH₂O indicating nonisotopic formaldehyde and CD₂O indicating deuterated formaldehyde used.

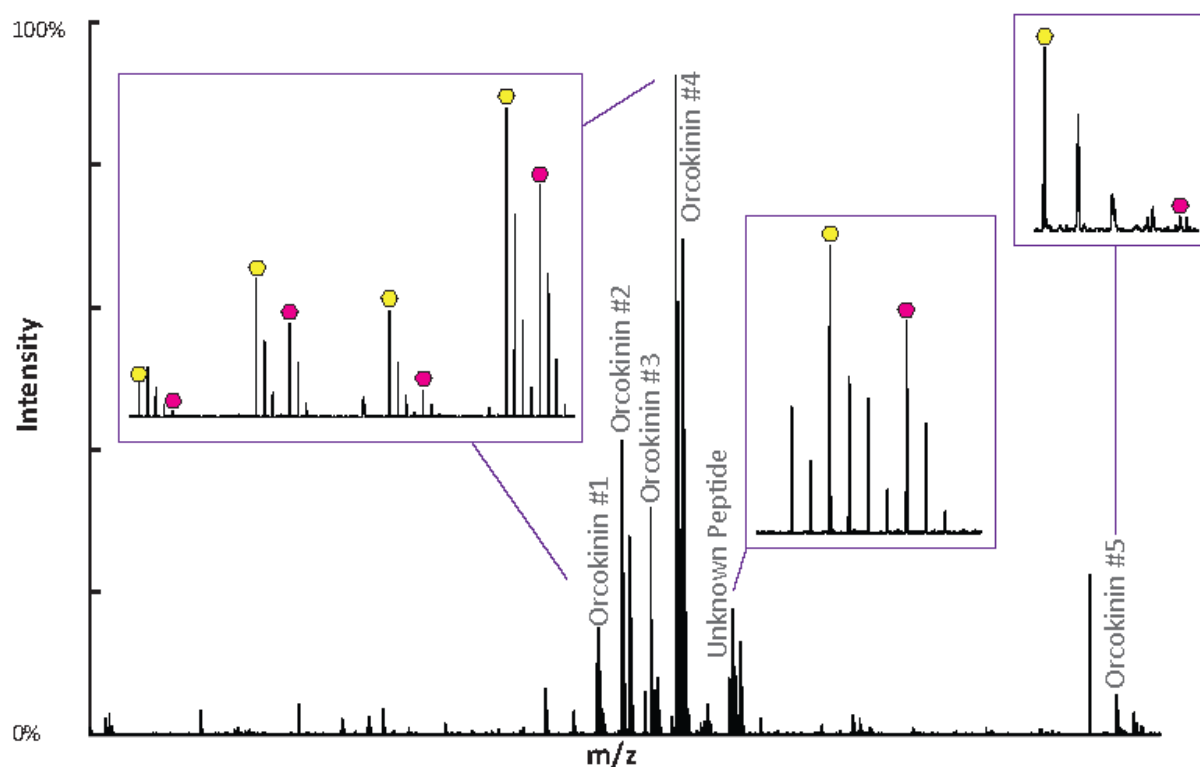


Figure 3. Example spectrum of results obtained from tissue analysis of blue crab, *Callinectes sapidus* brain using a MALDI-LTQ-Orbitrap XL. Neuropeptides from the Orcokinin family are indicated, as well as an unknown peptide. Enlarged images show the mass shift between light and heavy-labeled peptides, which enables relative quantitation.

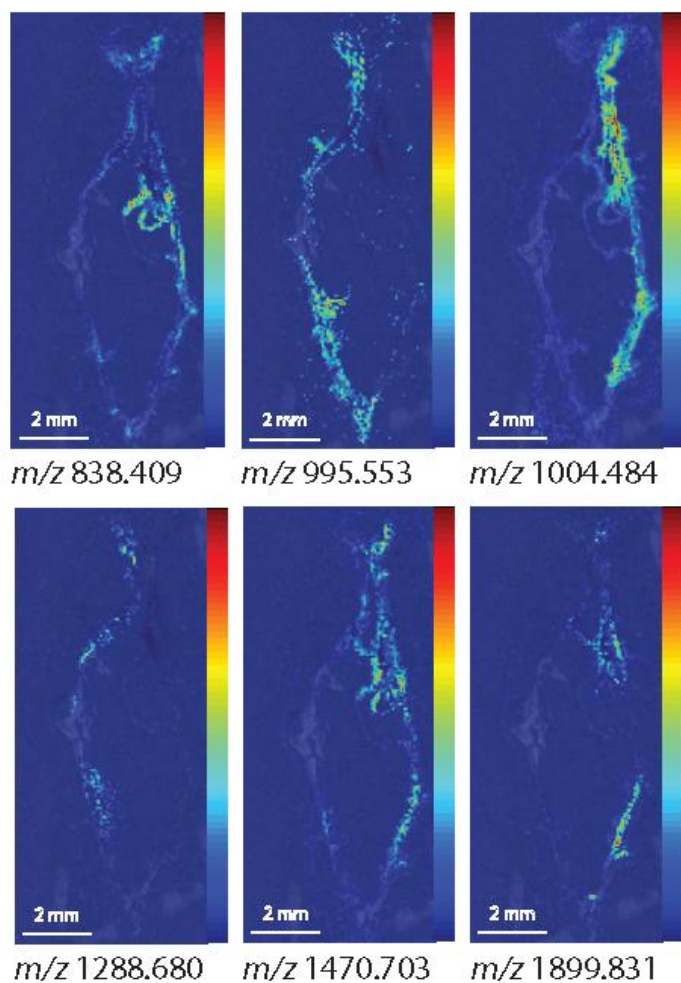


Figure 4. Example MS images of a blue crab, *Callinectes sapidus*, PO. Images were obtained on a MALDI-LTQ-Orbitrap XL and show the spatial distribution of six selected m/z values. All images are normalized to the TIC and overlaid on the optical image of the tissue.