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Characterization and Quantification of AST-C Peptides in Homarus americanus Using Mass Spectrometry

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Characterization and Quantification of AST-C Peptides
in *Homarus americanus* Using Mass Spectrometry

An Honors Paper for the Department of Chemistry

By Amanda Kelley Howard

Bowdoin College, 2015

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ABSTRACT

Neuropeptides are small signaling molecules found throughout the nervous system that influence animal behavior. Using the American lobster, Homarus americanus, as a model system, this research focused on an allatostatin type-C (AST-C) peptide, pQIRYHQCYFNPISCF (disulfide bond between underlined cysteine residues), and a structurally similar crustacean peptide, SYWKQCAFNAVSCFamide. These neuropeptides influence cardiac muscle contraction patterns and stomatogastric nervous system activity in the lobster. To understand their roles, this study sought to develop a method to quantify peptides in the pericardial organ (PO) and other crustacean tissues. Overall analysis involved microdissection to isolate tissues, tissue extraction, extract purification and concentration, and analysis by chip-based nano-electrospray ionization-liquid chromatography-mass spectrometry (nanoESI-LC-MS). In the present study, pQIRYHQCYFNPISCF was identified in the PO. To quantify target peptides, internal standards were tested as recovery and calibration references. However, experiments with pQIRYHQCYFNPISCF and other peptides showed evidence of adsorptive losses during sample preparation and analysis, with improvements in recovery resulting from the use of isopropanol-prewashed polypropylene vials. Preliminary results also suggested that introducing polyethylene glycol (PEG) in solution reduced adsorptive losses for hydrophobic peptides, but may have compromised hydrophilic peptide detection. Future directions include characterizing other sources of analyte loss and developing techniques to recover these signals. Since both target peptides as detected in the lobster are post-translationally modified, other directions include identifying modified and unmodified forms of these peptides in H. americanus. Ultimately, quantifying AST-C peptides and
identifying their modified and unmodified forms will help explain how neuropeptides regulate behavior within the lobster and more complex systems.
1.1 Context of study

Hundreds of signaling molecules called neuropeptides are responsible for
influencing behavior in humans and other animals. Neuropeptides are short α-amino acid
chains found throughout the nervous system. They originate from large precursor
proteins, or prepro-hormones, that are transcribed and translated from genomic genetic
material. These precursor proteins are post-translationally cleaved into several smaller
peptides by enzymatic processes. Like all amino acid chains, neuropeptides consist of a
series of amino acid residues linked by amide bonds (Fig. 1). The side of an unmodified
amino acid chain terminating with an amine group is called the N-terminus; the end with
a carboxylic acid group is the C-terminus. Structures and masses of the twenty amino
acids are presented in Table 1.

Fig. 1. Peptide structure. Amino acids are linked by amide bonds, with
the N-terminus given by the amine group and the C-terminus given by the
carboxylic acid. Amino acid identity given by unique side chain (R
group).

Active neuropeptides interact with membrane receptors to control behavior. Some
neuropeptides must be post-translationally modified by enzymatic processes in order to
become bioactive. Post-translational modifications may occur at the N- or C-terminus,
such as a C-terminal amidation (Fig. 2A). Other modifications may be internal, such as
the linking of two cysteine residues by a disulfide bond (Fig. 2B). Some neuropeptides
are active without being post-translationally modified or being minimally modified
(Perdew, 2007; Hou et al., 2012).

**Fig. 2. Examples of post-translational modifications.** (A) C-terminal
amidation via the partial cleavage of a terminal glycine to form a terminal
amide. (B) Disulfide bond formation between two cysteine residues. Both
conversions are mediated by enzymes in the organism.

Current research involves investigating neuropeptide interactions and the
mechanisms through which they regulate biological activity. Since the purpose of having
hundreds of neuropeptides in a given system is unknown, determining the unique
functions of a given peptide will help elucidate their functional roles within a system.
Whereas mammals have highly complex nervous systems, crustaceans have far fewer
neurons and neuropeptides and are appropriate model systems for neuropeptide research.
The American lobster, *Homarus americanus* has been used for studies of invertebrate
neural circuitry and peptide modulation in the stomatogastric and cardiac nervous
systems (Ma et al., 2009; Christie et al., 2010). In particular, after being removed from the animal, the stomatogastric nervous system and heart remain intact and functional. This is another advantage of using H. americanus as a model system and greatly facilitates the analysis of neuropeptide effects (DeKeyser and Li, 2006; Christie et al., 2010).

Within these systems, allatostatin type-C (AST-C) peptides are of particular interest due to their influence on cardiac muscle contraction patterns in H. americanus. Originally identified in insects as juvenile hormone production inhibitors in the corpora allata, the AST-C family is distinguished by a –PSCF sequence at the unblocked C-terminus, a pyroglutamate group at the N-terminus, and a disulfide bond linking the Cys$^7$ and Cys$^{14}$ residues (Christie et al., 2010).
Table 1. Amino acid abbreviations, residue masses, and side chain structures. Adapted from Luppino 2010.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Abbreviation</th>
<th>Code</th>
<th>Residue Mass (Da)</th>
<th>Side Chain Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Ala</td>
<td>A</td>
<td>71.03711</td>
<td>——CH₃</td>
</tr>
<tr>
<td>Arginine</td>
<td>Arg</td>
<td>R</td>
<td>156.10111</td>
<td>—NH₂</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>N</td>
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<td>—CH₂—CH₂—CH₂—NH₂—C—NH₂</td>
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<td>Aspartate</td>
<td>Asp</td>
<td>D</td>
<td>115.02694</td>
<td>——OH</td>
</tr>
<tr>
<td>Cystine</td>
<td>Cys</td>
<td>C</td>
<td>103.00918</td>
<td>—CH₂—SH</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Glu</td>
<td>E</td>
<td>129.04259</td>
<td>——OH</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln</td>
<td>Q</td>
<td>128.05858</td>
<td>—CH₂—CH₂—C—NH₂</td>
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<tr>
<td>Glycine</td>
<td>Gly</td>
<td>G</td>
<td>57.02146</td>
<td>—H</td>
</tr>
<tr>
<td>Histidine</td>
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<td>H</td>
<td>137.05891</td>
<td>—CH₂</td>
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<tr>
<td>Isoleucine</td>
<td>Ile</td>
<td>I</td>
<td>113.08406</td>
<td>—CH₃</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
<td>L</td>
<td>113.08406</td>
<td>—CH₂—CH₂—CH₂—CH₃</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
<td>K</td>
<td>128.09496</td>
<td>—CH₂—CH₂—CH₂—CH₃—NH₂</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met</td>
<td>M</td>
<td>131.04048</td>
<td>—CH₂—CH₂—S—CH₃</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
<td>F</td>
<td>147.06841</td>
<td>—CH₂</td>
</tr>
<tr>
<td>Proline</td>
<td>Pro</td>
<td>P</td>
<td>97.05276</td>
<td>—CH₂—OH</td>
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<tr>
<td>Serine</td>
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<td>S</td>
<td>87.03203</td>
<td>—CH₂—OH</td>
</tr>
<tr>
<td>Threonine</td>
<td>Thr</td>
<td>T</td>
<td>101.04768</td>
<td>—CH₂</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Trp</td>
<td>W</td>
<td>186.07931</td>
<td>—CH₂—NH—OH</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>Y</td>
<td>163.06333</td>
<td>—CH₂—OH</td>
</tr>
<tr>
<td>Valine</td>
<td>Val</td>
<td>V</td>
<td>99.06841</td>
<td>—CH—CH₃</td>
</tr>
</tbody>
</table>
1.2 Neuropeptides of interest

The neuropeptide pQIRYHQ_CYFNPISCF (disulfide bond between underlined Cys⁷ and Cys¹⁴) was the first type-C allatostatin to be identified in a non-insect and has been found to be widely distributed throughout the *H. americanus* nervous system (Stemmler et al., 2010). This peptide was found in the eyestalk, where many neuropeptides are manufactured and stored, and in the pericardial organ (PO), a tissue that delivers neuropeptides to cardiac muscle (Fig. 3) (Christie et al., 2010). Visualization by immunohistochemistry shows that pQIRYHQ_CYFNPISCF in the PO localizes to the nerve projecting from thoracic ganglion and the neurosecretory endings (Fig. 4).

![Fig. 3. Crustacean nervous system.](image)

(A) Whole body shown with (B) thoracic nervous system highlighted. This study focuses on the pericardial organ (PO) (dark orange), a tissue that delivers neuropeptides to cardiac muscle. Adapted from (Skiebe, 2003).
Similar to many other neuropeptides, pQIRYHQCYFNPISCF is post-translationally modified from its original form, QIRYHQCYFNPISCF (Fig. 5A). Through enzymatic processes, the N-terminal glutamine is converted to a pyroglutamate group and the hydrogens on the cysteine R-groups are lost to form a disulfide bridge linking these two internal Cys^6 and Cys^{13} residues. The final processed peptide is pQIRYHQCYFNPISCF (Fig. 5B). It is unknown whether this peptide is present in the animal in any unprocessed forms, such as one having an unmodified N-terminus or a reduced form lacking a disulfide bond, or whether these forms are bioactive.
Fig. 5. Target peptide post-translational modification. (A) unmodified QIRYHQCYFNPISCF and (B) pQIRYHQCYFNPISCF after the formation of pyroglutamate at the N-terminus and a disulfide bond linking Cys$^7$ and Cys$^{13}$. (C) unmodified SYWKQCAFNAVSCFG and (D) SYWKQCAFNAVSCFamide after C-terminal amidation and the formation of a disulfide bond linking Cys$^6$ and Cys$^{13}$.

In addition to pQIRYHQCYFNPISCF, a structurally similar peptide has been identified in *H. americanus* as SYWKQCAFNAVSCFamide. This peptide is initially translated as SYWKQCAFNAVSCFG (Fig. 5C). Like pQIRYHQCYFNPISCF, it undergoes enzymatic post-translational modification that converts the C-terminal glycine to an amide group and forms a disulfide bond bridging the internal Cys$^6$ and Cys$^{13}$ residues. Its N-terminus is left unblocked and the resulting processed form is SYWKQCAFNAVSCFamide (Fig. 5D). Both pQIRYHQCYFNPISCF and SYWKQCAFNAVSCFamide influence pyloric rhythm frequency in the stomatogastric nervous system and cardiac muscle contraction patterns (Dickinson et al., 2009; Ma et al.,
It is also unknown whether any unmodified forms, such as one having an unamidated C-terminus or a reduced form lacking a disulfide bond, are present in the animal or whether they are bioactive.

It has been observed that cardiac muscle contractions of different lobsters of the same species exhibit two different responses to applications of pQIRYHQCYFNPISCF and SYWKQCIAFNAVSCFamide. This response is unusual, as two animals of a single species generally respond similarly to a given treatment; however, when the semi-intact heart is exposed to pQIRYHQCYFNPISCF, the amplitude of cardiac muscle contractions increases in some lobsters and decreases in others. Cardiac muscle contraction frequency consistently decreases across preparations. This inter-animal variability and the structural similarity between pQIRYHQCYFNPISCF and SYWKQCIAFNAVSCFamide make these crustacean peptides interesting candidates for the study of neuropeptides (Wiwatpanit et al., 2012). Currently, post-translational modifications of pQIRYHQCYFNPISCF and SYWKQCIAFNAVSCFamide have been identified (Fig. 5), but other modified forms of these peptides are unknown.

1.3 Peptide characterization by liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (MS/MS)

In this study, we were working to develop a method to identify and quantify modified and unmodified forms of pQIRYHQCYFNPISCF and SYWKQCIAFNAVSCFamide in H. americanus eyestalk and PO tissue extracts using internal standards and chip-based nano-electrospray ionization-liquid chromatography-mass spectrometry (nanoESI-LC-MS). NanoESI LC-MS is a highly sensitive technique that allows for the detection of femtomolar amounts of peptides in small sample volumes.
while preserving detection accuracy, robustness, and precision (Karas et al., 2000; Yin and Killeen, 2007). Overall, samples may be separated by chromatography and subsequently identified by mass using mass spectrometry by measuring their mass-to-charge ratio ($m/z$). Peptides can also be sequenced by fragmenting target analytes and identifying the $m/z$ values associated with each fragment using tandem mass spectrometry (MS/MS).

The first step of this technique is high performance liquid chromatography (HPLC), which separates liquid sample components based on properties such as their size, charge, and interactions with the hydrophobic stationary phase column (Ho et al., 2003). In a conventional LC-MS instrument, the sample travels through a hydrophobic separation column to separate components. This column is connected to an ESI ionization source that nebulizes and ionizes the sample for MS analysis. However, the connections between the columns, capillaries, and valves involved in this process can leak and cause high sample loss (Yin and Killeen, 2007). To overcome such challenges posed by conventional LC-MS systems, an Agilent HPLC-Chip has been used for both HPLC sample separation and nanoelectrospray ionization (nanoESI) in this study. The chip is a multilayer polyimide film that eliminates leaky connectors and adapters, thereby maximizing sensitivity and ease of use. The liquid sample is injected and flows onto an enrichment column in the chip to be concentrated and washed to eliminate contaminants. When flow is directed from the enrichment column to the analytical column by rotation of a rotary valve, the hydrophobic analytical column separates the sample components, which travel through the column at different rates and elute at characteristic retention times. Lastly, a nanoESI tip is used for sample ionization before MS analysis (Yin and
Killeen, 2007). Liquid chromatography with MS detection yields a chromatogram of instrument response vs. retention time, which allows sample components to be distinguished based on elution time.

For mass analysis (Fig. 6), the liquid sample must be converted into gas phase ions by electrospray ionization. The liquid is nebulized from the nanoelectrospray tip on the HPLC-Chip using an electric field to yield highly charged droplets that are driven electrostatically to the mass analyzer inlet. Nitrogen gas is used to dry the sample so that the solvent evaporates and only the analyte ions reach the mass spectrometer. Since the mass spectrometer identifies analytes by their mass-to-charge ($m/z$) ratios, very large molecules can be detected by this technique because ESI allows for the formation of multiply charged ions. For example, a protein with molecular weight of 10,000 Da that is charged by one, two, three, four, or five protons would be detected as having an $m/z$ ratio of 10,001, 5001, 3334, 2501, or 2001 Da, respectively. This greatly widens the mass range of analytes that can be detected using this technique (Siuzdak, 1996).

Mass spectrometry uses a mass analyzer and detector to determine the $m/z$ ratio of the analyte ions. For the quadrupole time-of-flight (Q-TOF) mass analyzer used in this study, ions travel from the ionization region through eight metal rods called an octupole ion guide that ensures transmission of nearly all the target ions. The ions then enter the quadrupole mass filter, which consists of four rods that either can be used to transmit a wide range of ions to the TOF mass analyzer (MS mode), or can select a precursor ion for dissociation (MS/MS mode). In either mode, the ions are injected into the TOF mass analyzer, where they are accelerated to the same kinetic energy and mass analyzed by the differences in velocity resulting from differences in $m/z$. Using differences in flight times,
the mass analyzer determines the $m/z$ of each ion at high resolution. Ultimately, spectra of abundance vs. $m/z$ are collected at various retention times. Peak patterns in the MS spectrum reveal the charge state (number of added protons) for a given species and allow for the determination of the mass of the ion (Williamson and Bartlett, 2007); the exact mass measurements permit highly specific identifications based upon elemental compositional differences.

In order to determine the chemical identity and amino acid sequence of neuropeptides, tandem mass spectrometry (MS/MS) is used to sequence target molecules. After selecting a precursor using the quadrupole mass filter, the precursor ion is
accelerated into the hexapole collision cell (Fig. 6). Here, the accelerated ion collides with gas molecules (N₂) and the collisions produce product ions and uncharged fragments. The product ions are analyzed in TOF mass analyzer to determine their m/z and infer their masses (Kinter and Sherman, 2000).

To characterize pQIRYHQCYFNPISCFC and SYWKQCAFNAVSCFamide by MS and MS/MS, their measured m/z values will be compared to their expected exact masses and sequences. Based on the peptide sequences, their neutral exact masses are expected to be 1898.823 Da and 1649.712 Da, respectively. Using ESI, which allows for the formation of multiply charged species, all available basic sites on a peptide are expected to be protonated. Peptides are typically protonated at the N-terminal amine and at the basic residues lysine (K), arginine (R), and histidine (H) (Kinter and Sherman, 2000). Since a pyroglutamate group is not expected to be basic enough to be protonated, pQIRYHQCYFNPISCFC is expected to be protonated at the Arg³ and His⁵ residues. SYWKQCAFNAVSCFamide is expected to be protonated at the N-terminus and Lys⁴ residue. In addition to MS mass measurements, MS/MS analysis should confirm these amino acid sequences. Unmodified peptides will show retention time, m/z, and MS/MS mass spectral differences relative to the modified peptides.

1.4 Peptide quantification by internal standard

Internal standards have been used in previous studies to quantify peptides in biological tissues. Calibration using internal standards is a powerful quantification technique that can account for sample loss occurring throughout the tissue extraction and sample preparation when the internal standards are added at the beginning of the sample preparation process. Isotopically labeled standards are the most accurate because they
behave essentially identically to the target peptides. However, structurally-related analogue internal standards may also be used for quantification, as these are more accessible and economical and can be selected to mimic the behavior of the target peptides (Bronsema et al., 2013).

1.5 Quantifying AST-C peptides in *H. americanus*

Methods for quantifying pQIRYHQCYFNPISC\textsubscript{F} and SYWKQCAFNAVSCFamide will be explored using structurally similar internal standards. In order to make use of an internal standard, the amount of internal standard that must be added to the initial sample must be determined. Ideally, the amount of internal standard detected will be comparable to the amount of target peptide detected. However, the concentrations of AST-C peptides in crustacean tissue have not yet been quantified and may vary from animal to animal, making it difficult to apply internal standard techniques to the analysis. To address this challenge, a mixture of at least three internal standards will be added to the tissue extraction solvent. The concentration of each internal standard will differ by one order of magnitude. This way, the target peptide signal can be compared to the internal standard signal that is closest in intensity in order to maximize accuracy (Fig. 7).

To determine peptide concentrations following tissue extraction and sample preparation, the internal standard instrument responses will be compared to the known internal standard concentrations added to the extraction solvent. Using a calibration curve constructed from calibration data, the concentrations of pQIRYHQCYFNPISC\textsubscript{F} and SYWKQCAFNAVSCFamide originally present in the tissue will be determined from the detected concentrations of these target peptides. Optimal internal standard concentrations
will be determined empirically by estimating target peptide levels and testing for standard concentrations that maximize signal reliability and consistency. Ideally, the target peptide concentration will be near the center of the internal standard concentration range.

**Fig. 7. Peptide quantification by internal standard.**

Determination of pQIRYHQCYFNPI SCF and SYWKQCAFNAVSCFamide concentrations based on amounts of various internal standards. Chromatographic peak intensity for target peptides may vary over a wide range and will be compared to the internal standard peak with most similar intensity.

### 1.6 Project goals

In this study, peptide extraction and detection will be optimized and techniques will be tested to identify and quantify pQIRYHQCYFNPI SCF and SYWKQCAFNAVSCFamide in *H. americanus* tissue. Both peptides will be analyzed in the eyestalk and the former will also be analyzed in the PO. To identify target peptides, standards will first be characterized by liquid chromatography-mass spectrometry (LC-MS, described in more detail below). Eyestalk and PO tissue extracts will be analyzed for these peptides and delipidation techniques will be tested to reduce phospholipid.
contamination. Ultimately, techniques for quantifying peptides by internal standard and reducing peptide signal loss will be explored.

**EXPERIMENTAL**

2.1 Instrumentation

For chromatography and nano-electrospray ionization (nanoESI) for peptide standards and tissue extracts, a 1260 Chip Cube system (Agilent Technologies) and a ProtID-chip with a 40-nL enrichment column and a 150 mm x 75 μm analytical column (Agilent Technologies) were used. The enrichment and analytical columns were packed with 300-Å, 5-μm particles with C18 stationary phase. The mobile phases were 0.1% formic acid (FA) with 2% water in acetonitrile (ACN) and 0.1% FA in water, 300-nL/min flow rate; 0.01 to 1.0 μL injected. The mobile phase gradient was optimized for standard characterization and sample analysis.

A 6530 quadrupole time-of-flight (Q-TOF) mass analyzer (Agilent Technologies, Santa Clara, CA) was used for mass spectrometric analysis. Mass spectra were collected in positive (or negative) ion mode; the ionization voltage ranged from 1750 -1950 V, the fragmentor voltage was 175 V and the source temperature was held at 300 °C. Spectra were internally calibrated using reference compounds. Methyl stearate (C₁₇H₃₅CO₂CH₃) and hexakis (1H, 1H, 4H-hexafluorobutyloxy) phosphazine (HP-1221; C₂₄H₁₈O₆N₃P₃F₃₆), continuously infused and detected as [M+H]⁺, were used to internally calibrate all spectra in positive ion mode.

2.2 Animals

American lobsters (*Homarus americanus*) were purchased from Gurnet Trading Co. and kept in recirculating seawater tanks at 10-12 °C (Brunswick, ME).
2.3 Chemicals and sample storage and analysis vials

pQIRYHCYFNPISCF and SYWKQCAFNAVSCFamide were synthesized by GenScript (Piscataway, NJ, purities 95.7% and 95.1%, respectively). An extraction solvent of 85% acetone (SigmaAldrich, ≥ 99%), 13% deionized water, and 2% HCl (Fisherbrand; reagent grade) as a [%v/v] mixture was used for some tissue samples. For other samples, an extraction solvent of solvent 64% methanol (Fischer Scientific, ≥ 99%), 29% deionized water, and 7% acetic acid (SigmaAldrich, ≥ 99%) as a [%v/v] mixture was used. Dithiothreitol (DTT) (SigmaAldrich, ≥ 99%) was used to reduce disulfide bonds in peptide standards. Chloroform (Cambridge Isotope Laboratories; NMR-grade $^{13}$CDCl$_3$) was used for delipidation. [Arg$^8$]vasotocin, [Lys$^8$]vasopressin, [Arg$^8$]vasopressin, and oxytocin (American Peptide Co., ≥ 99%) were selected as internal standards.

2.4 Peptide standard preparation and characterization

To characterize pQIRYHCYFNPISCF and SYWKQCAFNAVSCFamide, 10 µM and 5 µM standards of each peptide were prepared using a serial dilution. First, $10^{-3}$ M stock solutions in water were diluted to 10 µM in plastic vials. These solutions were diluted to 5 µM in conical polypropylene vials for LC-MS analysis. Serial dilutions were also performed using low-retention plastic tubes and non-serial dilutions were prepared by diluting peptide standards directly into conical polypropylene vials for analysis.

2.5 Disulfide bond reduction by dithiothreitol (DTT)

To reduce the disulfide bond linking the cysteine residues in the target peptide, 66 µL of 0.2 M dithiothreitol (DTT) was added to 20 µL of 100 µM
SYWKQCAFNAVSCFamide in a polypropylene vial. The reaction was allowed to proceed at room temperature for 15 min.

2.6 Tissue extraction

Tissues (eyestalks and POs) were removed from the lobster using microdissection techniques and were immediately heated at 100 °C for 5 min in 50 µL of preheated extraction solvent in 0.6 mL low-retention tube (rinsed 3 x 200 µL of extraction solvent). Both an acetone solvent (85% acetone/13% deionized water/2% hydrochloric acid) and methanol solvent (64% methanol/29% water/7% acetic acid) were tested. The tissue was homogenized either by manually compressing and grinding the sample with a 0.2-mL low-retention tube (outside rinsed with acetone extraction solvent) or by using a polypropylene pellet pestle with cordless motor (SigmaAldrich). Tissues were sonicated for 5 min and centrifuged for 10 min at 10.1 g. The supernatant and an additional 100 µL of extraction solvent were filtered through a 0.45-µm filter (rinsed 3 x 300 µL of extraction solvent and centrifuged for 15 s per wash at 10.1 g) and centrifuged for 1 min at 10.1 g. An additional 100 µL of extraction solvent was drawn through the filter twice, centrifuged for 60 s, and the filtrate was vacuum dried. Samples not to be immediately analyzed were stored at -80 °C at this step. For analysis, vacuum dried samples were reconstituted in 50 µL of 25% acetonitrile (ACN), sonicated 5 min, and transferred to a conical polypropylene vial. Samples were analyzed by LC-MS.

2.7 Delipidation

A delipidation approach was tested by adding 50 µL of chloroform to the 350 µL filtrate before vacuum drying. The mixture was pipetted several times to mix, sonicated,
and let sit to separate. The bottom chloroform layer was removed and the resulting aqueous layer was vacuum dried and analyzed by LC-MS.

2.8 Detection limit assessment

Both target peptide standards were added to separate 40 µL aliquots of pooled eyestalk extracts at concentrations of 500, 100, 50, 10 and 1 nM. Samples were replicated so that two equivalent samples were analyzed for one target peptide at a single concentration. Serial dilutions were prepared from 10^{-3} M target peptide solutions in water in low-retention tubes. Chromatographic peaks were integrated and plotted as a function of peptide concentration to evaluate signal-to-concentration linearity.

2.9 Internal Standard Characterization

Solutions of [Arg^{8}]vasotocin, [Lys^{8}]vasopressin, [Arg^{8}]vasopressin, and oxytocin in water were prepared and characterized by chromatography and MS as described in Section 2.4. Mixtures of 1, 10, 100, and 1000 nM of each of these standards were then characterized, followed by mixtures of 100 nM of the four internal standards and the two target peptides. Multiple preparations of 100 and 10 nM mixtures of [Arg^{8}]vasotocin and the two target peptides were also analyzed at an injection volume of 1.0 µL. Lastly, the mixtures shown in Table 2 were analyzed by chromatography to determine an internal standard concentration gradient that would allow for the reliable detection of all peptides.
Table 2. Internal standard mixture concentrations for preliminary concentration gradient determination

<table>
<thead>
<tr>
<th>Mixture</th>
<th>[Arg⁸]vasotocin (M)</th>
<th>[Lys⁸]vasopressin (M)</th>
<th>[Arg⁸]vasopressin (M)</th>
<th>oxytocin (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>1.00 x 10⁻⁴</td>
<td>1.00 x 10⁻⁵</td>
<td>1.00 x 10⁻⁶</td>
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<tr>
<td>2</td>
<td>1.00 x 10⁻⁴</td>
<td>1.00 x 10⁻⁵</td>
<td>1.00 x 10⁻⁶</td>
<td>1.00 x 10⁻⁷</td>
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<tr>
<td>3</td>
<td>1.00 x 10⁻⁵</td>
<td>1.00 x 10⁻⁶</td>
<td>1.00 x 10⁻⁷</td>
<td>1.00 x 10⁻⁸</td>
</tr>
<tr>
<td>4</td>
<td>1.00 x 10⁻⁶</td>
<td>1.00 x 10⁻⁷</td>
<td>1.00 x 10⁻⁸</td>
<td>1.00 x 10⁻⁹</td>
</tr>
</tbody>
</table>

2.10 Dilution preparation with vial prewashing

Both polypropylene and glass vials were prewashed with water, 25% ACN, and isopropanol (all purchased from Fisher Scientific, LC-MS grade) to test the effectiveness of these solvents at preventing adsorptive losses. Each vial was washed 3 x 500 µL of each solvent and dried in open air overnight to ensure solvent evaporation. After these tests, all polypropylene vials used for analysis were prewashed by full submersion in isopropanol in a beaker and sonication for 5 min. The isopropanol was then discarded and this washing was repeated. Vials dried overnight in the beaker, which was partially covered in aluminum foil to prevent contamination while allowing evaporation. For subsequent experiments testing dilution preparation techniques, pipette tips and low-retention tubes were prewashed by repeated isopropanol submersion, sonication, and drying as described.

2.11 Dilution preparation to test signal recovery

Solutions of seven peptides at equal concentrations were prepared from standards synthesized by GenScript (Piscataway, NJ, purity shown in parentheses): RTVGGFA (99.3%), RAAFGFA (99.4%), AST-B (> 95%), RTVFGFA (99.8%), AFDEIDRSGFGFA (97.8%), CLDH (95.3%), and pQIRYHQCYFNPISC. All 10⁻³ M stock solutions were stored at –20°C and were fully thawed and sonicated for 5 min.
before diluting. Samples were prepared by diluting a $10^{-4}$ M mixture of all peptides to be analyzed in isopropanol-washed low-retention tubes.

**RESULTS AND DISCUSSION**

3.1 **Peptide standard characterization (oxidized and reduced forms)**

Standards of pQIRYHQCYFNPISC_F and SYWKQCAFNAVSC_Famide and their reduced forms were successfully characterized by chromatography, MS, and MS/MS. Based on the predicted target peptide sequences, the expected masses for the singly-, doubly-, and triply-charged states were calculated (Table 3). For 10 µM peptide solutions (prepared by diluting stock solutions directly into polypropylene vials), the chromatograms showed intense chromatographic peaks (Fig. 8A and C) whose MS spectra matched the expected masses (Fig. 8B and D) for each standard. Using the specified mobile phase gradient, pQIRYHQCYFNPI_SCF eluted at 7.02 min (Fig. 8A) and SYWKQC_AFNAVSC_Famide at 6.37 min (Fig. 8C). pQIRYHQCYFNPI_SCF consistently eluted after SYWKQC_AFNAVSC_Famide. Since modifications to the mobile phase gradient changed elution patterns, peptide retention times varied between analyses. Nonetheless, pQIRYHQCYFNPI_SCF consistently eluted after SYWKQC_AFNAVSC_Famide. This was consistent with expectations because pQIRYHQCYFNPI_SCF is the larger peptide, as indicated by its higher mass. This later elution time also suggests that pQIRYHQCYFNPI_SCF is more hydrophobic, which can be attributed to its pyroglutamate group. Upon pyroglutamate formation by the cyclization of the N-terminal glutamic acid, two hydrophilic amine groups are lost (Schlenzig et al., 2009). This increases the hydrophobicity of pQIRYHQCYFNPI_SCF
and, along with its larger size, causes greater retention by the hydrophobic C18 analytical column compared with SYWKQCAFNAVSCFamide.

**Table 3.** Target Peptide Sequences, Masses, and Single, Doubly, and Triply Charged State Mass-to-Charge (m/z) Ratios

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Sequence (possible protonation sites in bold)a</th>
<th>Mass [M] (Da)b</th>
<th>Expected mass-to-charge ratios, m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST-C</td>
<td>pQIRYHQCYFNPISCFC</td>
<td>1898.8234</td>
<td>[M+H]+ 1899.831 950.419 633.948</td>
</tr>
<tr>
<td></td>
<td>*SYWKQCAFNAVSCFamide</td>
<td>1649.712</td>
<td>[M+2H]+ 1650.719 825.863 550.911</td>
</tr>
<tr>
<td>AST-C reduced</td>
<td>pQIRYHQCYFNPISCFC</td>
<td>1900.838</td>
<td>[M+3H]+ 1901.845 951.426 634.620</td>
</tr>
<tr>
<td></td>
<td>*SYWKQCAFNAVSCFamide</td>
<td>1651.727</td>
<td></td>
</tr>
</tbody>
</table>

*a Asterisk indicates possible protonation at the N-terminal amine group  
b Monoisotopic mass

Although MS analysis does not indicate specific protonation sites, probable sites for protonation were predicted and are shown in bold in Table 3. Basic amino acid residues as well as unblocked N-termini were expected to be protonated. Both peptides were expected to be protonated at two sites: pQIRYHQCYFNPISCFC at the two basic residues (Arg^3 and His^5) and SYWKQCAFNAVSCFamide at the N-terminus and the basic Lys^4 residue. For both target peptides, the doubly-charged ion was most abundant, as expected. Furthermore, for pQIRYHQCYFNPISCFC, the measured m/z of 950.924 matched the theoretical m/z for the doubly-protonated peptide (Fig. 8B and Table 3). There were also significantly less intense peaks matching the singly- and triply-charged states. Similarly, the most abundant m/z peak for SYWKQCAFNAVSCFamide (m/z 825.866) matched the theoretical m/z for the doubly-protonated peptide (Fig. 8D and Table 3). A dramatically less intense peak matching the triply-charged state mass for pQIRYHQCYFNPISCFC was also observed.
Fig. 8. Chromatograms and MS spectra for pQIRYHQCYFNPISCF and SYWKQCAFNAVSCFamide. (A) Chromatogram for pQIRYHQCYFNPISCF, showing elution at 7.02 min. (B) MS spectrum for pQIRYHQCYFNPISCF shows [M+2H]+ ion with m/z 950.924 was most abundant. (C) Chromatogram for SYWKQCAFNAVSCFamide, showing elution at 6.37 min. (D) MS spectrum for SYWKQCAFNAVSCFamide shows [M+2H]+ ion with m/z 825.866 was most abundant.

To determine how the disulfide bond influenced chromatographic retention and mass spectral properties, the reduced forms of pQIRYHQCYFNPISCF and SYWKQCAFNAVSCFamide were generated. For both peptides, the reduced form eluted later than the oxidized, disulfide-bonded peptide (Fig. 9A). The increase in retention for the reduced peptide may result from the peptide being able to assume a more linear form with stronger interactions with the stationary phase. The MS spectra showed the expected mass shifts from the addition of two hydrogen atoms; the [M+2H]+ was the most abundant charge state for both reduced peptides (data not shown).

Although these MS peaks matched theoretical m/z values, peptide masses are insufficient to confirm amino acid sequences. Therefore, MS/MS data are needed to verify peptide identity. Using the known total peptide mass, differences between MS/MS fragment m/z values that corresponded to the m/z value of a particular amino acid residue
revealed where that amino acid was in the overall sequence. By compiling these fragment 
$m/z$ values, the total sequences for both peptides were confirmed.

![Graph](image)

**Fig. 9.** LC-MS/MS characterization of SYWKQCAFNAVSCFamide standard and SYWKQCAFNAVSCFamide. (A) SYWKQCAFNAVSCFamide eluted at 18.39 min and SYWKQCAFNAVSCFamide eluted slightly later at 19.24 min. (B) MS/MS spectrum collected for sequence confirmation. Peptide structure shown at top. Ions and residues shown in red were detected by MS/MS analysis. SYWKQCAFNAVSCFamide was mostly fragmented at sites outside the disulfide bond. (C) SYWKQCAFNAVSCFamide (reduced) allowed for fragmentation at many sites, as shown in red in the structure at the top.

MS/MS data were collected in order to characterize and confirm the sequences of oxidized SYWKQCAFNAVSCFamide (Fig. 9B) and reduced SYWKQCAFNAVSCFamide (Fig. 9C) forms. For MS/MS analysis, the abundant $[M+2H]^{2+}$ precursor ion was selected by the quadrupole mass filter and was dissociated in the collision cell. The product ions identified in the MS/MS spectra (Fig. 9) include $y$-
type ions, which include the C-terminus of the peptide, and b-type ions, which include the N-terminus. Low mass immonium ions, which are characteristic of different amino acids present in the sequence, appear at low mass and are identified by the one-letter amino acid codes.

The oxidized SYWKQCAFNAVSCFamide peptide was mostly fragmented at sites outside the disulfide bond and very little fragmentation was observed at sites between the cysteine residues (Fig. 9B). However, the reduced form of this peptide was highly fragmented at many sites and the mass spectrum showed evidence of most of the amino acid sequence. This suggested that the disulfide bond provided structural stability that prevented fragmentation. In the oxidized peptide, the breakage of a bond between the two cysteine residues would not produce fragmentation because the unbroken disulfide bond would keep the peptide intact. Similarly, breaking the disulfide bond without breaking a peptide bond would not produce any fragmentation. That is, in order for SYWKQCAFNAVSCFamide to fragment at a site between the cysteine residues, both the disulfide bond and a peptide bond must be broken. Therefore, few ions formed by fragmentation at sites between the cysteine residues were detected. Reducing the disulfide bond facilitated internal fragmentation, as supported by MS/MS data (Fig. 9C). MS/MS data for pQIRYHQCYFNPISC also showed high characteristic fragmentation outside the disulfide-bonded cysteine residues (data not shown).

3.2 Detection limit assessment in sample matrix

To evaluate detection limits, an eyestalk extract was used as a sample matrix and was spiked with increasing concentrations of target peptides to mimic the behavior of a biological tissue extract. To prepare the extract, an eyestalk ganglion was extracted into
an acidified methanol solvent. The tissue was heated at 100°C to denature enzymes that would attack the target peptides and was then homogenized and sonicated to release biological material into the extraction solvent (Stemmler et al., 2013). The sample was centrifuged to remove insoluble material and the supernatant was filtered to further eliminate insoluble contaminants. The diluted solution showed no pQIRYHQCYFNPISC\text{CF} peptide signals.

Each peptide-spiked tissue extract was analyzed by LC-MS, chromatographic peaks were integrated, and peak area was plotted as a function of peptide concentration (Fig. 10). pQIRYHQCYFNPISC\text{CF} showed higher signal-to-concentration linearity ($R^2 = 0.996$) than SYWKQCAFNAVSC\text{CFamide} ($R^2 = 0.802$), but both relationships were fairly linear. Based upon the 0.5 μL injection volume, the minimum amount of peptide detected was 25 fmol for pQIRYHQCYFNPISC\text{CF} and 5.0 fmol for SYWKQCAFNAVSC\text{CFamide}. This provided reassurance that when quantifying peptide levels, a linear calibration curve could be used to reliably determine the concentration of peptide present in a sample with a given target peptide signal intensity.

For a given change in peptide concentration, signal intensity increased more for pQIRYHQCYFNPISC\text{CF} than for SYWKQCAFNAVSC\text{CFamide}. This is indicated by the greater slope for the pQIRYHQCYFNPISC\text{CF} regression line and suggested that this peptide is more easily ionized and detected. Arg$^3$ is the most basic amino acid and is contained in pQIRYHQCYFNPISC\text{CF}, thereby making its affinity for protonation higher than that of SYWKQCAFNAVSC\text{CFamide}. In contrast, SYWKQCAFNAVSC\text{CFamide} only has two probable protonation sites at the N-terminus and the less basic Lys$^4$ residue.
Fig. 10. Peak area vs. peptide concentration for pQIRYHQCYFNPISCF and SYWKQCAFNAVSCFamide in sample matrix. Both relationships were linear (R² < 0.99 for pQIRYHQCYFNPISCF and R² = 0.80 for SYWKQCAFNAVSCFamide. Minimum amount of peptide detected was 25 fmol pQIRYHQCYFNPISCF and 5.0 fmol SYWKQCAFNAVSCFamide.

3.3 Peptide detection in PO and eyestalk

To determine if target peptides could be detected in a tissue extract, PO and eyestalk tissues from *H. americanus* were analyzed by LC-MS. pQIRYHQCYFNPISCF was detectable in a single PO extract. The chromatogram for the most abundant charge state of pQIRYHQCYFNPISCF showed an intense signal at 6.905 min (Fig. 11A). At this retention time, MS data shows an abundant doubly-charged ion with m/z 950.419 and a low-intensity singly-charged ion with m/z 1899.831 (Fig. 11B). MS/MS data (not shown) confirmed the identity of pQIRYHQCYFNPISCF. Neither MS nor MS/MS data showed evidence of the reduced form in this biological sample. Other abundant masses corresponded to peptides common to crustaceans including orcokinins, SIFamide, CPRPs, and crustacean hyperglycemic hormone (CHH) (Christie et al., 2010; Hui et al.,
Confirming the detection of pQIRYHQCYFNPISCF in a single PO extract gave confidence that quantification of very small peptide amounts was feasible; however, this analysis also highlighted challenges associated with the analysis.

Fig. 11. pQIRYHQCYFNPISCF detection in PO. (A) Total ion chromatogram (green) and extracted ion chromatogram (red) for expected most abundant m/z. Scaled to largest peak in each chromatogram. (B) MS data at peptide retention time showed an abundant doubly-charged ion with m/z 950.419 and an additional low-intensity peak for a singly-charged ion with m/z 1899.831. Contaminant peak shown has been seen in previous work and is known to coelute with pQIRYHQCYFNPISCF (E. Stemmler, unpublished data).

Some of these challenges included high levels of sample contamination (see total ion chromatogram in Fig. 11A showing many non-peptide peaks), most significantly phospholipids that can interfere with analysis. Variable peptide signal intensities may be associated with phospholipid contamination, dissection techniques, time of dissection, homogenization, or adsorptive losses. Specifically, when analyzing PO and eyestalk extracts, there seemed to be significant variability in crustacean peptide signal intensities.
There appeared to be no substantial signal intensity discrepancies between eyestalk extracts prepared by manual or motorized tissue homogenization or between dissections times of 10 min (short) and 20 min (long). Previous research has shown evidence that circadian rhythm in crayfish controls peptide manufacturing. Crustacean hyperglycemic hormone (CHH) levels are especially influenced by these patterns (Fanjul-Moles et al., 2010). Therefore, it is possible that neuropeptide expression patterns in *H. americanus* vary throughout the day. In addition, physiological conditions also affect peptide manufacturing (Strauss and Dircksen, 2010; Hou et al., 2012). In future experiments, these factors should be controlled for by extracting tissues at roughly the same times of day.

### 3.4 Delipidation

Phospholipids are known to hinder peptide ESI protonation, thereby interfering with peptide detection in biological samples (Van Eeckhaut et al., 2009). Since chloroform effectively dissolves large phospholipids and not peptides, it has served as a successful medium for removing lipids from aqueous tissue extracts (Folch et al., 1957). To determine if a micro liquid-liquid extraction with chloroform could be used to extract phospholipids from tissue extracts, a tissue extract was analyzed before and after chloroform extraction. The chloroform-methanol extraction successfully extracted lipids from neuropeptide material. Phospholipid signal intensity was much less when the chloroform extraction was performed, suggesting successful delipidation. However, there was essentially no difference in crustacean peptide signal intensity between samples. Furthermore, white precipitate formed upon extraction, raising concerns about sample loss. Therefore, this approach was not pursued as a useful method to improve peptide
recovery or detection. Whereas this technique involves liquid-liquid extraction, another student tested solid phase extraction using C18 spin columns for delipidation. The hydrophobic phospholipids should partition into the C18 stationary phase to separate components from other peptides. This may be a more effective alternative that will be explored in future experiments.

3.5 Internal standard selection and characterization

To quantify pQIRYHQCYFNPISCFCFamide and SYWKQCAFNAVSCFamide, [Arg⁸]vasotocin, [Lys⁸]vasopressin, [Arg⁸]vasopressin, and oxytocin were selected as potential internal standards. These internal standard sequences and masses are presented in Table 4, with basic residues and likely protonation sites shown in bold. These are non-crustacean neuropeptides not found in *H. americanus*. Due to the disulfide bond linking the cysteine residues in each internal standard, these peptides were expected to behave similarly to the target peptides. That is, any loss of pQIRYHQCYFNPISCFCFamide or SYWKQCAFNAVSCFamide due to heat treatment, loss to tubes or pipet tips during transfers, or losses when extracting or filtering, for instance, was also expected to affect the selected internal standards. The amidated C-terminus of each internal standard suggested they would behave especially similarly to SYWKQCAFNAVSCFamide. Therefore, [Arg⁸]vasotocin, [Lys⁸]vasopressin, [Arg⁸]vasopressin, and oxytocin were expected to serve as appropriate recovery references for quantification.

In the initial steps of quantification method development, the internal standards were characterized by LC-MS/MS as individual components and as mixtures of multiple standards. For all four internal standards, MS data showed that the doubly-charged ion was most abundant. This suggests each peptide was protonated at the unblocked N-
terminus. [Arg$^8$]vasotocin and [Arg$^8$]vasopressin were likely protonated at Arg$^8$, the most basic amino acid, and [Lys$^8$]vasopressin at the Lys$^8$, another highly basic amino acid. The most likely second protonation site on oxytocin is Pro$^7$ (Moret and Zebende, 2007).

When the four peptides were mixed together at equal concentrations of 10 µM and analyzed by LC-MS, they were chromatographically separated, as shown in Fig. 12.

**Table 4. Internal Standard Sequences, Masses, and Single, Doubly, and Triply Charged State Mass-to-Charge (m/z) Ratios**

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Sequence (possible protonation sites in bold)$^a$</th>
<th>Mass [M] (Da)$^b$</th>
<th>Expected mass-to-charge ratios, m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Arg$^8$]vasotocin</td>
<td>*CYIQNCPRGamide</td>
<td>1049.455</td>
<td>[M+H]$^+$ 1050.462 525.735 350.826</td>
</tr>
</tbody>
</table>

$^a$ Asterisk indicates possible protonation at the N-terminal amine group

$^b$ Monoisotopic mass

**Fig. 12. EIC overlays for internal standard mixtures.** All standards were at equal concentrations of 10 µM. The peak heights were scaled to the same value to show chromatographic peak shapes.
Because AST-C concentrations in tissue extracts may vary significantly, a multi-level internal standard calibration approach was defined. This requires mixtures of the four internal standards that each differ by an order of magnitude. In mixtures containing the highest concentrations, we expected that the more abundant peaks would be overloaded and unsymmetrical, and needed to ensure that these peaks did not interfere with lower concentration standards. In evaluating the appropriate internal standard concentration levels, mixtures with symmetric peptide chromatographic peaks for the peptide being used as the appropriate internal standard would be most reliable for quantification. In addition, target peptide chromatographic peak areas should be within the range of that of the internal standard peak areas. Having some internal standards elute before and some after the target peptides would also be ideal (Hou et al., 2012).

To evaluate how concentration changes impacted peak shapes, four internal standard mixtures were prepared and analyzed by LC-MS (Fig. 13). Mixtures #1 and #2 (Table 2) showed undesirable asymmetric chromatographic peaks for standards in high concentrations caused by column overloading. Mixture #3 showed symmetric chromatographic peaks and the three most concentrated internal standards were easily detectable. Since [Lys\(^8\)]vasopressin and [Arg\(^8\)]vasopressin elute at similar times, a combination of one of these two peptides, [Arg\(^8\)]vasotocin, and oxytocin would offer most reliable quantification.
**Fig. 13. Chromatogram overlays for internal standard mixtures.** The peak heights were scaled to the same value to show chromatographic peak shapes. Standards are labeled (1) [Arg$^8$]vasotocin, (2) [Lys$^8$]vasopressin, (3) [Arg$^8$]vasopressin, and (4) oxytocin. Adapted from a figure provided by E. Stemmler.

### 3.6 Internal standard mixtures with target peptides showed evidence of analyte loss

The target peptides, pQIRYHQCYFNPISCF and SYWKQCAFNAVSCFamide, were then mixed with these internal standards for further chromatographic analysis. For a solution of $10^{-4}$ M [Arg$^8$]vasotocin, $10^{-5}$ M [Lys$^8$]vasopressin, $10^{-6}$ M [Arg$^8$]vasopressin, and $10^{-7}$ M oxytocin with $10^{-5}$ M of each target peptide, the target peptide signals were comparable in intensity to that for $10^{-4}$ M [Arg$^8$]vasotocin (10 times more concentrated), suggesting that the target peptides had a higher nanoESI ionization efficiency. The mobile phase gradient was optimized to minimize chromatographic peak overlap between the target peptides and internal standards. Using the optimized gradient, [Arg$^8$]vasotocin...
eluted earliest followed by [Lys$^8$]vasopressin, [Arg$^8$]vasopressin, and oxytocin, respectively. The target peptides eluted later in the chromatogram than the internal standards (data not shown).

To investigate relative signal intensities as a function of concentration, several standard mixtures of a single internal standard, [Arg$^8$]vasotocin, mixed with SYWKQCAFNAVSCFamide and pQIRYHQCYFNPISCF were characterized. However, there were inconsistencies in signal strengths for the target peptides. For example, a 10 µM mixture of the target peptides and [Arg$^8$]vasotocin showed saturated signals for the two target peptides at this high concentration (Fig. 14A), while a 10-fold dilution of this solution showed SYWKQCAFNAVSCFamide and pQIRYHQCYFNPISCF peaks that were approximately 40 and 500 times lower in response, respectively (Fig. 14B). Multiple replicates showed that upon an additional 10-fold dilution, the peptides were undetectable. These decreases in signal strength are far greater than the approximate 10-fold decrease that would be conventionally expected.
Fig. 14. Target peptide signal losses upon sample dilution. (A) A 10 µM solution yields saturated signals for the two target peptides. (B) Upon 10-fold dilution of this solution, SYWKQCAFNAVSCFamide and pQIRYHQCYFNPISCF peaks were approximately 40 and 500 times lower in response, respectively.

All of these disproportionate losses in relative signal strength suggested possible peptide adsorption to the sample vial walls that was dependent on relative concentrations. During initial work directed at characterizing the target peptides, greater-than-expected losses of AST-C peptide signals were found following serial dilutions. For example, when pQIRYHQCYFNPISCF serial dilutions were prepared in standard plastic tubes before being transferred to polypropylene vials for analysis, chromatographic peaks decreased more dramatically than the expected dilution factor. In contrast, when dilutions were prepared directly in polypropylene LC-MS vials, the expected change in signal intensity was observed (Fig. 15). For the peaks shown in Fig. 15, both samples contained
10 µM pQIRYHQCYFNPISCF in water and 0.5 µL of each was injected for analysis. Therefore, the signals for the target peptide were expected to be equivalent. Contrary to expectations, the chromatographic peak area for the sample prepared in the polypropylene vial was about two orders of magnitude larger than that for the solution prepared in standard plastic (220 x 10^5 compared to 5 x 10^5). In contrast, SYWKQCAFNAVSCFamide did not show similar patterns and there did not seem to be any substantial sample loss in these early experiments.

![Graph](image)

**Fig. 15. pQIRYHQCYFNPISCF** sample loss. In this chromatogram overlay, 10 µM of solution prepared directly in polypropylene vials showed a much more intense signal (peak area 2 x 10^7) than for a solution of equivalent peptide concentration and injection volume prepared from a serial dilution in standard plastic tubes (peak area 5 x 10^6).

In this early work, it was hypothesized that this signal loss was due to the affinity of the peptide to the walls of the standard plastic vials. Previous studies have shown that some peptides exhibit this behavior when stored in glass or standard plastic, which can contribute to significant sample loss (Kraut et al., 2009). After observing such loss, all samples were prepared in low-retention plastic tubes to prevent peptide adsorption in standard plastic tubes. Furthermore, 25% acetonitrile (ACN) was included in dilution solvents to help prevent adsorptive losses. Such use of organic solvent has been shown to improve signal-to-concentration linearity (Warwood et al., 2013). In the present study,
we expected that diluting samples in organic solvent in low-retention tubes would prevent peptide loss. However, analyte loss was still observed in subsequent experiments. This led to a more detailed investigation of the causes of peptide signal loss.

3.7 Factors that may lead to analyte loss

Before proceeding with the development of a method for peptide quantification, the sources of these signal losses needed to be identified. Due to repeated evidence of sample loss, the focus of this study shifted to addressing factors leading to analyte loss. Factors that have been shown to negatively impact signal recovery include adsorptive losses to analysis vial walls, pipette tips, and various parts within the LC-MS instrument due to hydrophobic, hydrophilic, or electrostatic interactions between the analyte and adsorptive surface. Previous work has shown that at low concentrations, signal strength is highly compromised because a substantial proportion of peptide present is adsorbed. This can make the analyte barely detectable. At high concentrations, sample loss to adsorption is less drastic relative to the amount of peptide in solution (Maes et al., 2014). Since such research suggested that vial material could compromise signal recovery, a more rigorous systematic analysis of potential causes of these losses was performed in the present study.

3.8 Analyte loss to sample vials

To test whether loss was affected by analysis vial material, solutions of [Arg⁸]vasotocin and the target peptides at equal concentrations of 10⁻⁶ M were diluted directly into either a low-retention polypropylene or glass vial. Chromatography showed a dramatic reduction in pQIRYHQCYFNPI SCF signal intensity when using polypropylene (Fig. 16A) compared to glass (Fig. 16B). These results were consistent with a previous study finding that glass is preferred for storing hydrophobic peptides.
(Van Midwood et al. 2007). pQIRYHQCYFNPISC\textsubscript{F} contains hydrophobic residues (one Pro, two Ile, and two Phe residues) and elutes at a late retention time, suggesting that this is a particularly hydrophobic peptide. Ultimately, the results of the present study suggested that vial material affected signal recovery and that glass was preferred over polypropylene for detecting hydrophobic peptides.

![Graph showing SYWKQCAFNAVSCFamide reduction and pQIRYHQCYFNPISC\textsubscript{F} sample loss.](image)

**Fig. 16.** Evidence of SYWKQCAFNAVSCFamide reduction and pQIRYHQCYFNPISC\textsubscript{F} sample loss. (A) Low intensity signal for pQIRYHQCYFNPISC\textsubscript{F} when prepared in plastic. (B) pQIRYHQCYFNPISC\textsubscript{F} signal recovered when prepared in glass. Scaled to largest peak in each chromatogram.

However, hydrophobicity alone is not sufficient to explain why the pQIRYHQCYFNPISC\textsubscript{F} peak was most intense for the preliminary six-peptide mixture.
(four internal standards and both target peptides) and least intense for the three-peptide mixture ([Arg⁸]vasotocin and both target peptides). In the six-peptide mixture, [Arg⁸]vasotocin was 10 times more concentrated than the target peptides (10⁻⁵ M), making it possible that this internal standard occupied most of the adsorptive sites on the polypropylene vial walls. In contrast, for the three-peptide mixture, [Arg⁸]vasotocin and the target peptides were at equal concentrations of 10⁻⁵ M. Here, as explained by Maes et al., it is likely that the most hydrophobic peptide was unhindered by a more concentrated species from binding to the adsorptive sites. This suggests that adsorption may be concentration-dependent.

3.9 Prewashing analysis vials for signal recovery

In addition to vial material, contaminants or coatings inside the vials could have caused adsorptive losses. To test this, prewashing polypropylene and glass vials with water, 25% ACN, and isopropanol was explored as a way to eliminate unwanted contaminants. Across replicate experiments, prewashing polypropylene vials with isopropanol showed greater pQIRYHQCYFNPISSF signal recovery than any other treatment (Fig. 17). That is, the mean prewash-to-no prewash ratios were significantly higher for the isopropanol-washed polypropylene treatment than all other conditions (ANOVA, N = 2). These results suggested that prewashing polypropylene vials with isopropanol successfully conserved hydrophobic peptide signal intensity, possibly because isopropanol washed away any hydrophobic contaminants on the polypropylene vial walls.
Fig. 17. Prewash-to-no prewash ratios of pQIRYHQCYFNPISCF peak areas. 1 µM pQIRYHQCYFNPISCF analyzed in (A) polypropylene and (B) glass vials prewashed in H₂O, 25% ACN, and isopropanol. Prewash-to-no prewash ratio for isopropanol-washed polypropylene was significantly higher than the remaining treatments (ANOVA, N = 2). There were no significant differences between prewash-to-no prewash ratios for the other conditions (ANOVA, N = 2 for each condition).

Peptide standard solutions diluted using isopropanol-washed low-retention tubes and pipette tips did not have any apparent effect on pQIRYHQCYFNPISCF signal recovery. Sonication of samples before analysis also did not increase signal intensity (data not shown). Isopropanol-washed polypropylene vials were used for all subsequent experiments.

3.10 No initial evidence for sample loss within the instrument when varying injection volumes at constant peptide concentration

To test whether loss occurred within the instrument during analysis, injection volume was varied at constant peptide concentration. Loss at this point in the analysis could result from adsorptive losses to surfaces including, but not limited to, the injection needle, column, tubing, and mass spectrometer. Especially basic peptides can stick to metal surfaces while hydrophobic peptides tend to stick to plastic surfaces. pQIRYHQCYFNPISCF is particularly hydrophobic and contains basic residues, making
it prone to such losses. Any instrument loss would compromise the signal-to-injection linearity, especially at low concentrations where adsorptive losses are most evident (Maes et al., 2014).

A preliminary experiment showed that increasing injection volumes of the same peptide sample yielded pQIRYHQCYFNPISC\(_F\) signals that increased linearly. Multiple injections of 1 \(\mu\)M pQIRYHQCYFNPISC\(_F\) analyzed in isopropanol-washed polypropylene vials showed that the relationship between peak area and the amount of peptide injected was strongly linear, suggesting that sample loss in the instrument during analysis was negligible (Fig. 18). If there were sample loss here, signals for small injection volumes would be compromised. Greater injection volumes would be less affected because the amount of sample lost would be negligible compared to the amount of peptide injected. This would decrease the signal-to-injection volume linearity. However, these results showed that sample loss within the instrument did not affect peptide detection.
Fig. 18. pQIRYHQCYFNPISCF peak area vs. volume of 1 µM peptide injected. The relationship between pQIRYHQCYFNPISCF peak area and injection volume was strongly linear ($R^2 > 0.99$).

3.11 More in-depth investigation of concentration-dependent losses

In addition to the internal standard mixture analyses discussed in Sections 3.5 and 3.6, other preliminary data showed evidence of potential concentration-dependent signal loss. Signal intensity for a given molar amount of peptide injected was not expected to change between samples regardless of initial concentration. That is, signal intensity was expected to be the same when injecting 0.1 µL of 1 µM peptide and 1.0 µL of 0.1 µM peptide because 100 fmol of peptide was injected in both conditions. Contrary to this prediction, pQIRYHQCYFNPISCF was detectable from a 0.1 µL injection of 1 µM peptide (Fig. 19A), but was nearly undetectable from a 1.0 µL injection of 0.1 µM peptide (Fig. 19B). Since the same amount of peptide should have been injected in both conditions, these results suggested signal loss was concentration-dependent. This led to further exploration of the potential causes for this observed signal loss by analyzing additional peptides that differed in size and hydrophobicity.
Fig. 19. EICs for 100 fmol pQIRYHQCYFNPI SCF injections. (A) 0.1 µL injection of 1 µM peptide and (B) 1.0 µL injection of 0.1 µM peptide were injected. pQIRYHQCYFNPI SCF was detected from the 1 µM solution, but not the 0.1 µM solution.

3.12 Signal loss dependence on peptide size and hydrophobicity

Mixtures of seven peptides varying in size and hydrophobicity were characterized to determine whether signal loss was dependent on concentration or peptide-specific properties (Table 5 and Fig. 20). These peptides were selected based on availability, retention time, and chromatographic resolution. RTVGGFA, RAAFGFA, and RTVFGFA were relatively small in size and contained between three and five hydrophobic residues. AST-B and AFDEIDRSGFGFA were slightly larger in size and contained five and six hydrophobic residues, respectively. CLDH was very large and hydrophobic compared to the other peptides, including pQIRYHQCYFNPI SCF. In conjunction with the proportion of hydrophobic residues, retention time was used as the primary determinant of peptide hydrophobicity. The peptides that eluted latest were classified as most hydrophobic.
because they interacted most favorably with the hydrophobic stationary phase.

pQIRYHQCYFNPSCF appeared to be most difficult to detect (smallest peak area) and eluted latest.

**Table 5.** Peptide Sequences, Most Abundant m/z, and Retention Times

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Sequence (hydrophobic residues in bold)</th>
<th>Name</th>
<th>Mass (Da)a</th>
<th>Most abundant m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.8</td>
<td>RTVGGFA</td>
<td></td>
<td>706.3761</td>
<td>354.1954</td>
</tr>
<tr>
<td>13.8</td>
<td>RAADFgFA</td>
<td></td>
<td>738.3812</td>
<td>370.1979</td>
</tr>
<tr>
<td>14.8</td>
<td>TNWKNFQGSWamide</td>
<td>AST-B</td>
<td>1265.5940</td>
<td>633.8044</td>
</tr>
<tr>
<td>15.6</td>
<td>RTVFGFA</td>
<td></td>
<td>796.4231</td>
<td>399.2188</td>
</tr>
<tr>
<td>17.3</td>
<td>AFDEIDRSGFGFA</td>
<td></td>
<td>1430.6470</td>
<td>716.3306</td>
</tr>
<tr>
<td>20.1</td>
<td>GLDGGLGRGFSGSQAAXKLMGLAANFAGGPamide</td>
<td>CLDH</td>
<td>2939.5190</td>
<td>735.8870</td>
</tr>
<tr>
<td>21.7</td>
<td>pQIRYHQCYFNPSCF</td>
<td>AST-C</td>
<td>1898.8230</td>
<td>950.4190</td>
</tr>
</tbody>
</table>

*a Monoisotopic mass

**Fig. 20. Merged EIC of seven peptides in sample loss analysis.**
Chromatogram shows retention times and relative signal intensity of the seven peptides.

A mixture of these seven peptides was tested for signal loss at different concentrations and injection volumes. Concentration was varied to reveal whether any observed signal loss could be attributed to concentration-dependent sample loss. Injection volume was manipulated to determine whether any sample loss occurred within the
instrument. Detected peak areas were plotted as a function of the amount of peptide injected (Fig. 21); points originating from solutions of different concentrations were overlayed to determine if the same amount of injected peptide yielded the same peak area.

The relationship between peak area and the amount of peptide injected was highly linear for the small peptides: RTVGGFA (Fig. 21A, $R^2 = 0.99$), RAAFGFA (Fig. 21B, $R^2 = 0.98$) and RTVFGFA ($R^2 = 0.99$) for all three solution concentrations (0.4, 0.7 and 1.0 µM). Data for AST-B was also fairly linear ($R^2 = 0.91$). However, this pattern was not observed for the later eluting peptides, including CLDH (Fig. 21C) and pQIRYHQCYFNPISCF (Fig. 21D), which did not show evidence of strong signal-to-injection linearity.

The signal-to-injection nonlinearity for the two most hydrophobic peptides provided strong evidence for concentration-dependent sample losses during LC-MS analysis, with more analyte being lost when smaller injection volumes were used. In addition to the curvature observed as a function of injection volume, CLDH and pQIRYHQCYFNPISCF behaved differently at each mixture concentration, as shown by the lower peak areas for equivalent amounts of peptide injected from solutions of different concentration. This suggested that concentration-dependent sample loss could also have occurred when the solutions were diluted. For pQIRYHQCYFNPISCF, the signals were generally very weak or undetectable at 0.4 µM. Notably, CLDH and pQIRYHQCYFNPISCF are most impacted by the observed peptide loss. These peptides elute latest, and therefore, are presumably the most hydrophobic.
Fig. 21. Peak area vs. amount of peptide injected for (A) RTVGGFA, (B) RAAFGFA, (C) CLDH, and (D) pQIRYHQ_CYNPI_SCF. This relationship was highly linear for this RTVGGFA ($R^2 = 0.99$) and RAAFGFA ($R^2 = 0.98$). However, these curves were fit to second order polynomials for CLDH and pQIRYHQ_CYNPI_SCF. Injection volumes varied from 0.01–1.3 µL of seven-peptide mixtures varying from 0.4–1 µM in concentration. pQIRYHQ_CYNPI_SCF was not detected at 0.4 µM.

We also compared these most recent data with earlier experiments that showed a linear relationship between pQIRYHQ_CYNPI_SCF signal intensity and injection volume (Fig. 18). This discrepancy could have been influenced by factors such as samples run before performing these experiments that lingered in the instrument and interfered with
peptide detection. Nonetheless, this more extensive experiment testing multiple peptides at various concentrations and injection volumes showed convincing evidence of instrument loss for more hydrophobic peptides, which led to further investigation of techniques to reduce such losses.

### 3.13 Signal recovery in solution with PEG

Diluting peptide samples in polyethylene glycol (PEG, 0.001%) has previously been shown to increase signal recovery of various peptides by reducing adsorptive losses (Stejskal et al., 2013). This sample treatment was explored for solutions containing pQIRYHQCYFNPISC\(\text{CF}\). Consistent with the findings of Stejskal and colleagues, PEG successfully increased signal recovery for the peptide of interest, pQIRYHQCYFNPISC\(\text{CF}\) (Fig. 22). pQIRYHQCYFNPISC\(\text{CF}\) absolute peak areas for samples diluted in water were generally weak compared to those diluted in 0.001% PEG in water (Fig. 22A). There was a much greater difference in absolute peak area for the solutions at higher concentration (0.7 \(\mu\text{M}\)); however, the relative increase in peak area was much greater for solutions at the lower concentration (0.4 \(\mu\text{M}\)) (Fig. 22B). This suggested that PEG had a greater effect on signal recovery at lower concentrations. This is consistent with findings in both the present study and previous research showing that adsorptive losses most significantly affect solutions at low concentrations, whereas solutions at higher concentrations are less impacted.

In contrast, dilution in 0.001% PEG generally showed moderately decreased signal recovery for the remaining peptides at concentrations of 0.1, 0.4, and 0.7 \(\mu\text{M}\), with the exceptions of AST-B at 0.4 \(\mu\text{M}\) and RTVFGFA at 0.1 \(\mu\text{M}\) (data not shown). Therefore, PEG may have interfered with the detection of these smaller, more hydrophilic
peptides while enhancing signal recovery of pQIRYHQCYFNPI SF. Nonetheless, this served as a potential technique for analyzing hydrophobic peptides at low concentrations.

Fig. 22. Peak area vs. amount of pQIRYHQCYFNPI SF injected with and without 0.001% PEG in solution. (A) Adding 0.001% PEG increased absolute pQIRYHQCYFNPI SF peak areas most for samples at higher concentrations. (B) PEG-to-no PEG signal ratios were highest at low concentrations. Injection volumes varied from 0.05–1.0 µL of 0.1–1 µM solutions.
Although PEG successfully increased signal recovery for hydrophobic peptides, biological samples contain a broad range of peptides that vary greatly in size, hydrophobicity, and other properties. Therefore, optimizing detection of one category of peptide would prevent accurate quantification of other types of peptides. Moreover, peptides are found at very low concentrations in biological samples and their detection requires high sensitivity. However, results showed that signal recovery is most highly compromised for low analyte concentrations. Ultimately, the present study demonstrated that in order to minimize analyte loss, factors such as adsorptive losses during sample preparation and analysis that are especially problematic for hydrophobic peptides must be addressed.

**Future Work**

The source of peptide signal loss and techniques for signal recovery must be optimized in order to allow for successful peptide quantification in biological samples. Overall, the results of the present study show that there are likely multiple sources of sample loss, including adsorptive losses to vials and LC-MS components that most drastically affect hydrophobic peptides at low concentrations.

In the future, PEG should be tested as a prewash for analysis vials that would coat adsorptive sites on the vial walls without interfering with peptides in solution. More extensive experiments should be performed to analyze the target peptides across broader concentration ranges and injection volumes in continued effort to identify the conditions under which signals are most highly compromised. This will also help determine whether signal loss is primarily a function of peptide concentration, size, hydrophobicity, or other
factors such as temperature, which can particularly affect the detection of hydrophobic peptides (Maes et al., 2014).

After developing a method for optimal sample preparation, attention can be redirected at the goal of peptide quantification in biological samples by a multipoint internal standard mixture. This would involve determining the amount of internal standard that must be added at the beginning of the extraction process in order to recover the amounts that give the desired chromatographic signal intensity. This could be done experimentally by adding known concentrations of the internal standards to the extraction solvent before tissue removal from the animal. Following peptide extraction, the sample could be analyzed to determine the amount of internal standard and analyte recovered. Since the target peptides are found in very low concentrations in the eyestalk and PO, the number of tissues that might need to be pooled to detect the desired amount of peptide also must be determined experimentally. By pooling the desired number of tissues and adding the appropriate amount of internal standard, it would be possible to quantify the amount of target peptide in a single tissue.

Other future directions of this study could include identifying other modified and unmodified forms of pQIRYHQCYFNPISCFC and SYWKQCFAVFNSCFCamide. This information could be coupled with biological analyses to determine which forms are bioactive. Currently, there is little known information about how post-translational modifications contribute to bioactivity. This research will contribute to efforts to clarify the role of post-translational modifications in bioactivity and to identify differences between the functions of the structurally similar AST-C peptides. Above all, this
knowledge will help explain neuropeptide interactions and their influence on behavior in crustaceans and more complex systems.
APPENDIX I: COLOR FIGURES IN GRAYSCALE

Fig. 2B. Disulfide bond formation between two cysteine residues.

Fig. 3. Crustacean nervous system.
Fig. 4. Target peptide visualization in the PO.

Fig. 6B. Schematic diagram of Q-TOF mass analyzer. Adapted from Vollmer et al. 2011.
Fig. 7. Peptide quantification by internal standard.

Fig. 9B and C. LC-MS/MS characterization of SYWKQCAFNAVSCFamide standard and SYWKQCAFNAVSCFamide.

Fig. 11. pQIRYHQCYFNPISCF detection in PO. (A) Total ion chromatogram and extracted ion chromatogram for expected most abundant $m/z$. Scaled to largest peak in each chromatogram.
Fig. 16. Evidence of SYWKQC\textsubscript{AFNAVSC}Famide reduction and sample loss.
REFERENCES


