

Discovery and *De Novo* Sequencing of Insect Neuropeptides Using the LTQ Orbitrap XL™

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Key Words

- LTQ Orbitrap XL
- *De Novo* Sequencing
- HCD Higher Energy Collisional Dissociation
- Nano LC-MS/MS
- Neuropeptides

Introduction

Neuropeptides are important chemical messengers in animals. They are implicated in nearly all physiological processes, acting as neurotransmitters, neuromodulators or classical neurohormones. Recently, mass spectrometry (MS) has been used to localize neuropeptides in neuro-endocrine tissue^[1]. A sensitive and reliable identification of novel peptide sequences and post-translational modifications is of great interest, because bioactive (neuro)peptides may serve as drug leads for pharmaceutical development. The combination of on-line nano HPLC and high mass accuracy/high resolution mass spectrometry is ideally suited for *de novo* sequencing of peptides.

MS techniques such as MALDI-MS/MS give full structural elucidation only for a limited number of small peptides (< 20 amino acids). However, for larger peptides, the sequence coverage obtained is often incomplete. Analysis using QTOF instruments gives results which lack the necessary mass accuracy resulting in uncertainties or ambiguities for complete *de novo* sequence interpretation.

With the introduction of the LTQ Orbitrap XL (Figure 2) with its octapole collision cell based Higher Energy Collisional Dissociation (HCD), it is possible to carry out the most rigorous *de novo* sequencing, since high resolution, accurate mass data can be acquired over the entire mass range of m/z 50 to 2000. This is of considerable benefit especially because highly informative signals in the low mass range can be detected simultaneously with higher m/z b- and y-ions resulting in complete sequence coverage.

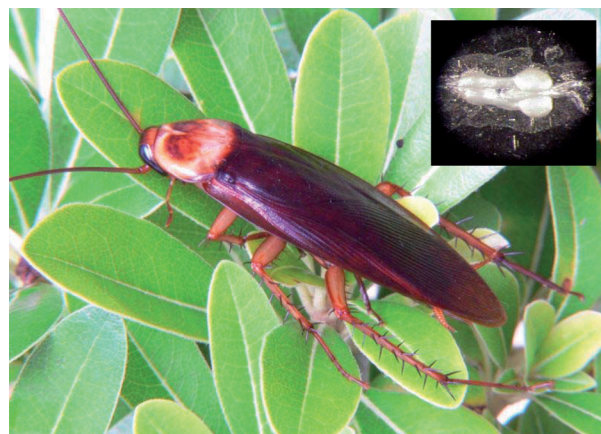


Figure 1: American cockroach (*Periplaneta americana*). The inset shows a microscope view of the corpora cardiaca/corpora allata complex (cc/ca).

For this study, the corpora cardiaca/corpora allata complex (cc/ca; the insect equivalent of the mammalian pituitary gland) of cockroaches (*Periplaneta americana* and *Periplaneta australasiae*; Figure 1) served as the source of sets of biological (neuro)peptides to be structurally elucidated by nano HPLC coupled to LTQ Orbitrap XL. The dataset combines information acquired in a single nano LC-MS/MS run using different types of fragmentation (both CID and HCD). The comprehensive information obtained led to the structural characterization of more than 20 known neuropeptides (including pyrokinins, sulfakinins, allatostatins, hyper-trehalosemic hormones, corazonin) by *de novo* sequencing as well as by database searching. These identifications provide a clear demonstration for the robustness of our *de novo* sequencing approach.

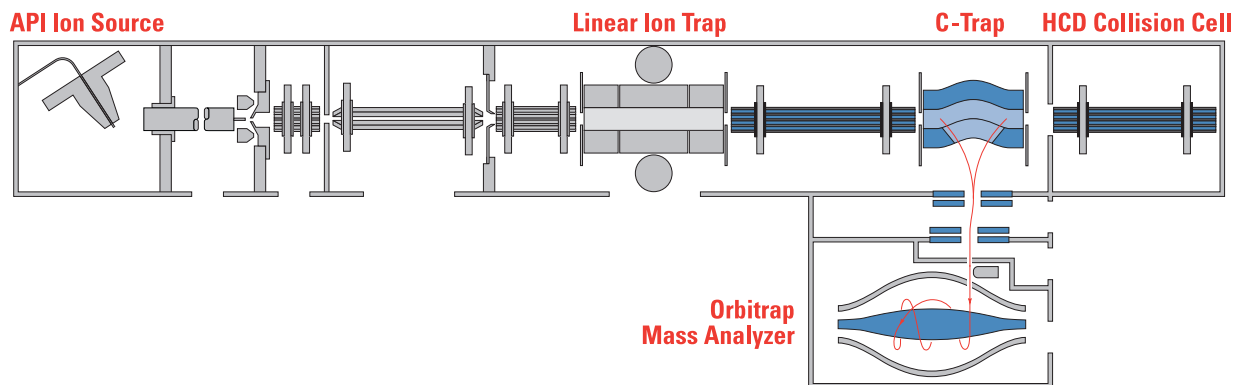


Figure 2: Schematic of LTQ Orbitrap XL. The additional octapole collision cell is placed behind the curved C-Trap. For Higher Energy Collisional Induced Dissociation (HCD) ions are transferred to the new octapole and, after fragmentation, they enter C-Trap again before being ejected into the Orbitrap mass analyzer.

Because of the high sample complexity (shown in Figure 3), *de novo* sequencing was setup in an automated fashion using the PEAKS Studio software, which incorporates also database and error tolerant search routines.

Methods

Cockroach species were obtained from local zoological gardens. Neuropeptides from isolated cc/ca were extracted in 1 min. using 50% MeOH, 0.1% formic acid (10 μ L per animal tissue equivalent, and after a 5-fold dilution with water (0.1% FA), injected into a nano-HPLC system using a 75 μ m ID (15 cm, C18, 5 μ m) analytical column. The injection volume of 10 μ L corresponded to the peptide equivalent of one fifth of a cc/ca-complex. Data were acquired with a hybrid linear ion trap - orbitrap mass spectrometer (LTQ Orbitrap XL, Thermo Fisher Scientific, Bremen) operating in a Data Dependent™ mode. Full scan MS spectra were acquired at 60,000 (FWHM) resolving power settings. The most abundant multiply charged ion was selected for collision-induced dissociation (CID) and HCD fragmentation of the same precursor both detected in the orbitrap mass analyzer.

Database search was performed by MASCOT™ 2.1.04 (Matrix Science, London, UK) on both SwissProt or nrNCBI databases. Tolerances were adjusted to 3 ppm (precursor) and 0.05 Da (fragment).

For *de novo* sequencing we used the PEAKS Studio 4.5 (BSI, Waterloo, Canada) software. PEAKS *de novo* sequencing as well as error tolerant searches and PEAKS protein identification were performed with tolerances of 3 ppm for the precursor and 0.05 Da for the fragments and with the same databases as used for MASCOT searches. For all data processing we used amidated C-terminus (X_{amide}), N-terminal pyroglutamic acid Q (q), and tyrosine sulfation (y) as variable modifications.

Instrument parameters:

Name	Value
Max. inject time (MS/MS ²) [ms]	100/500
AGC target value	2e ⁵
Normalized collision energy [arb]	40
Isolation width [Da]	3

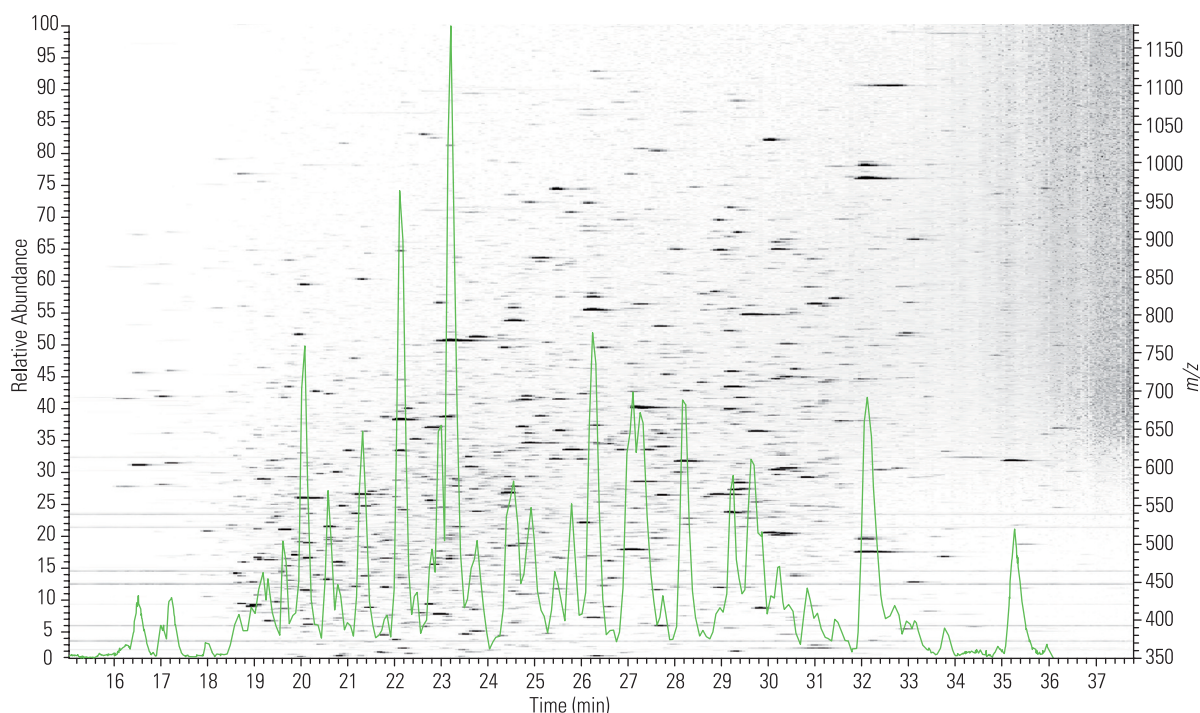


Figure 3: Peptide display with overlaid base peak chromatogram, showing the complexity of the sample (one fifth cc/ca equivalent of *Periplaneta americana*).

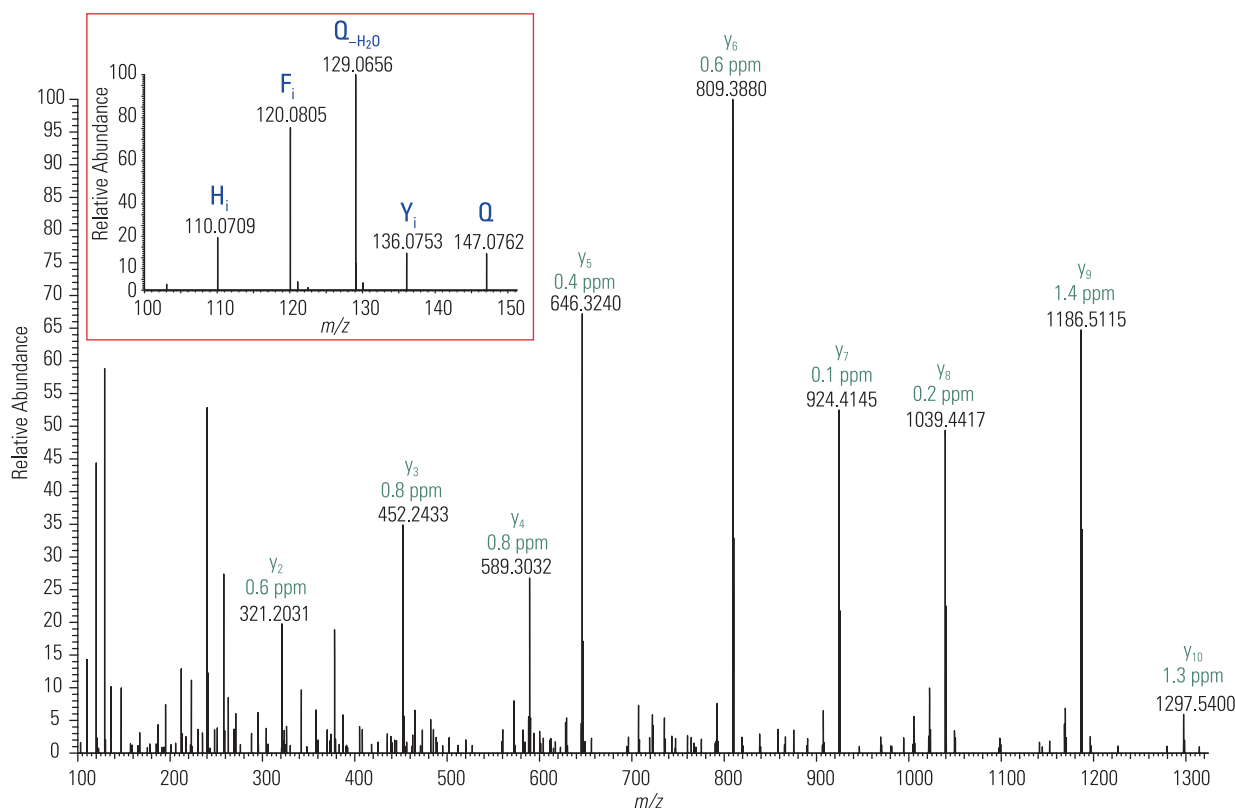


Figure 4: MS/MS Spectrum of perisulfakinin (Pea-SK-I) ($m/z=722.310552^*$). Mass accuracy is better than 3 ppm over the whole mass range. The insert shows the low m/z range with indicative immonium and other amino acid-specific ions (sequence: EQFDDYGHMRF_{amide}).

Results

In this work we have addressed the main requirements for *de novo* sequencing.

These are:

- High mass accuracy of both precursor and fragment ions (< 3 ppm)
- Sensitive and highly efficient generation of fragmentation spectra
- Generation of spectra with maximal interpretable information content
- High degree of automation

High mass accuracy is achieved by detecting MS/MS ions in the orbitrap mass analyser. As it is demonstrated in Figure 4, the mass accuracy for the parent and fragment ions is less than 3 ppm (with external calibration) for the complete mass range. In this example a virtually complete sequence of the peptide EQFDDYGHMRF_{amide} is determined (nearly complete y-ion series). The C-terminus is incompletely assigned as there are two possible alternatives: An oxidized and amidated methionine or an amidated phenylalanine. This information is directly available from the low mass range, where immonium and amino acid specific signals can be found. In this example we detect immonium ions for histidine, tyrosine and phenylalanine, as well as two glutamine specific ions., indicating the presence of phenylalanine at the C-terminal position.

Furthermore, the finding is supported by the fact that a potential oxidized and amidated methionine would have a m/z of 147.05866, which is a mass deviation of approximately 120 ppm compared to the measurement. We conclude, thus, the correct amino acid sequence contains a phenylalanine at its C-terminus, which is in excellent agreement with the precursor m/z of 722.310552* (mass deviation 2 ppm).

Another interesting finding from the dataset is depicted in Figure 5. CID fragmentation in the linear ion trap results in very low energy fragmentation pathways. This has many advantages, but in some cases the fragment spectrum consists only of a neutral loss ion, in particular when post-translational modifications, such as phosphorylations and tyrosine sulfations are present. The latter is a common modification in neuropeptides.

Figure 5 (upper panel) shows a typical CID MS/MS spectrum from the tyrosine sulfated neuropeptide leucosulfakinin-2 (qSDDyGHMRF_{amid}) indicating the neutral loss of 79.9578 Da, which corresponds to a loss of SO₃ (mass deviation of 2.1 ppm). In case of CID, an additional scan event, i.e. performing an MS³ experiment, is necessary to get sequence information. However, the lower panel in Figure 5 shows the same precursor, which has undergone HCD fragmentation. Due to the slightly higher collision energy, the sequence information can be directly deduced from the MS/MS experiment.

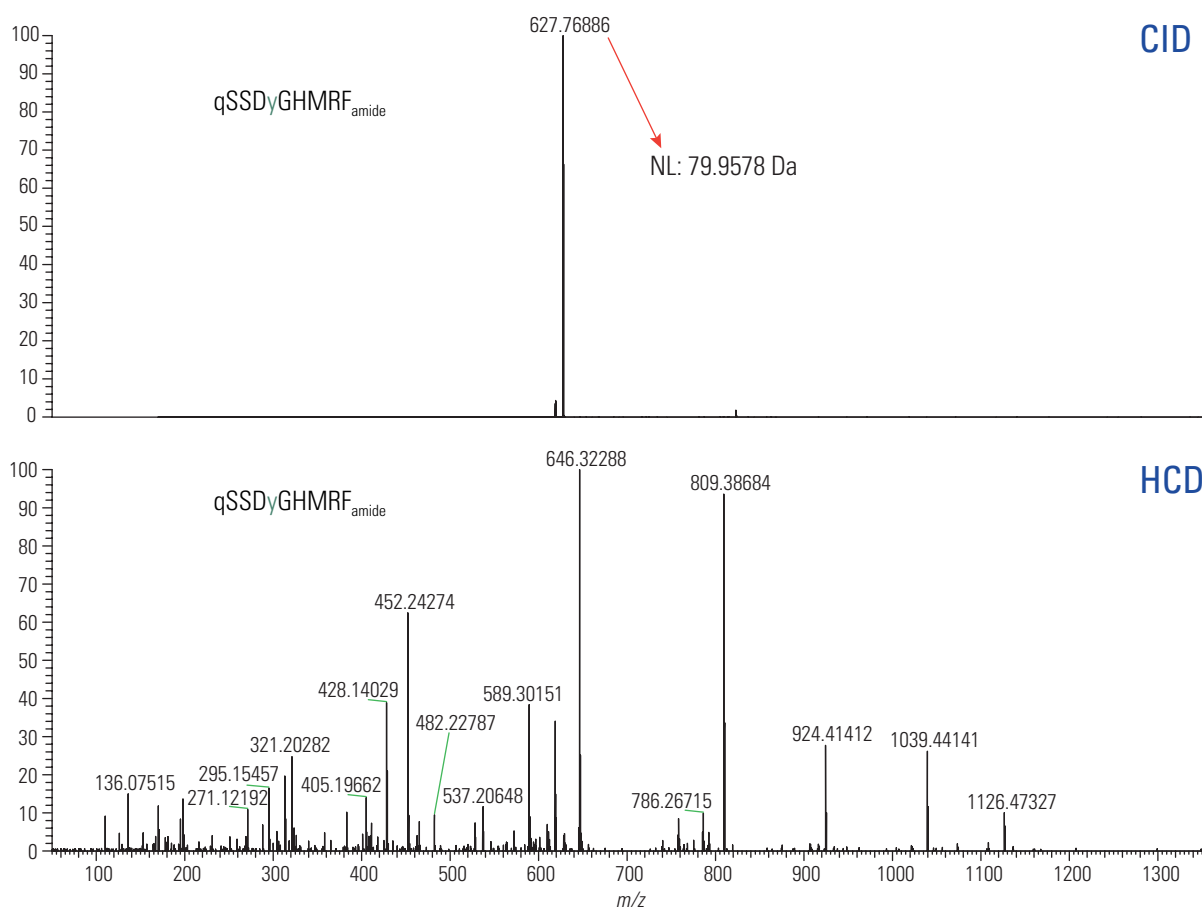


Figure 5: MS/MS fragmentation of a sulfated tyrosine neuropeptide from *P. americana*. The upper spectrum shows CID fragmentation with the neutral loss (NL) of 79.9578. Sequence information can not be obtained from this spectrum. The lower spectrum shows HCD fragmentation of the same precursor ion. Here the sequence can be obtained directly from the MS/MS spectrum.

AA-sequence	Accession	F-type		De novo search results [HCD]	
		CID	HCD	score	rank
SKYMYGFL _{amid}	(P12764) Allatostatin precursor	✓	✓	69	1
ARPYSFL _{amid}	(P12764) Allatostatin precursor	✓	✓	80	1
pQVNFSPNW _{amid}	(P84261) Hyper-trehalosamicfactor1	✓	✓	66	1
pQLTFPNW _{amid}	(P84256) Hyper-trehalosamicfactor2	–	✓	49	2
DDYGHMRF _{amid}	(P36885) Perisulfakinin	✓	✓	78	1
pQSDDYGHMRF _{amid}	(P67802) Leucosulfakinin-2	–	✓	99	1
QSDDYGHMRF _{amid}	(P67802) Leucosulfakinin-2	✓	✓	99	1
pQSDDYGHMRF _{amid}	(P67802) Leucosulfakinin-2	–	✓	99	1
QSDDYGHMRF _{amid}	(P67802) Leucosulfakinin-2	–	✓	99	1
EQFDDYGHMRF _{amid}	(P36885) Perisulfakinin	✓	✓	75	1
pQDQDVHFLRF _{amid}	(P21144) Leucomyosuppressin	✓	✓	67	1
QDQDVHFLRF _{amid}	(P21144) Leucomyosuppressin	✓	✓	99	1
DHLPHDVVSPRL _{amide}	(P84410) Pyrokinin4	✓	✓	99	1
DHLPHDVVSPRL	(P84410) Pyrokinin4	✓	✓	99	1
NDPEVPGMWFGPRL _{amide}	(P84368) Pyrokinin6	✓	✓	40	2
PGMWFGPRL _{amid}	(P84368) Pyrokinin6	✓	✓	50	2
HLPHDVVSPRL _{amid}	(P84410) Pyrokinin4	✓	✓	75	1
HTAGFIPRL _{amid}	(P82691) Pyrokinin1	–	✓	99	1
HLPHDVVSPRL _{amid}	(P84410) Pyrokinin4	✓	✓	25	1
PEVPGMWFGPRL _{amid}	(P84368) Pyrokinin6	✓	✓	99	1
pQTFQYSRGWTN _{amid}	(Q26377) Corazonin	–	✓	50	2

Table 1. Neuropeptides that are scored significantly high by database searching (MASCOT) are marked with ✓. De novo scores and ranking of spectra fragmented by means of HCD are shown in the last two columns. Database search and de novo sequencing was performed with tolerance windows of 3 ppm (precursor) and 0.05 Da (fragments).

Conclusions

We have carried out work specifically focused on automating the *de novo* sequencing. This included optimization of the settings for biologically relevant samples. In the course of this study we evaluated robustness and reliability by comparison to database searching. In addition we used *de novo* sequencing and error tolerant homology searching to find many new members of known neuropeptide families in cockroaches as well as two peptides previously not reported in the list of neuro-peptides from cockroaches.

Using HCD fragmentation in a new octapole collision cell improved considerably the *de novo* sequencing results compared to CID in linear ion trap or C-Trap.

We conclude that the method can be further improved by applying collision energy profiles to further improve fragmentation performance, or use base peak ejection to remove high intensity peaks prior to the detection to detect lower abundant peptide species.

Future work will concentrate on the new peptides, in particular on the characterization of their biological functions in cockroaches.

References

[1] Verhaert, P.D., Prieto Conaway, M.C., Pekar, T.M., Miller, K., Neuropeptide imaging on an LTQ with vMALDI source, The complete 'all-in-one' peptidome analysis. *Int. J. Mass Spec.* 2007, 260, 177–184.

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