

Accurate Mass Mapping and Sequencing of Corpus Cardiacum Neuropeptides by means of Nanoscale LC-MALDI-Tof MS/MS

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Abstract

This application note shows an LC-MALDI MS/MS based study of a biological mixture of natural peptides freshly collected from living tissue.

Introduction

The combination of off-line nanoscale LC separation – including direct spotting on a stainless steel target plate – with MALDI MS/MS offers an alternative separation and identification strategy compared to the more “traditional” on-line electrospray data directed analysis for complex protein/peptide mixtures.

With the advent of novel orthogonal acceleration Time-of-Flight (Tof) mass spectrometers exhibiting ever increasing sensitivity, mass accuracy and resolution, it is becoming more and more evident that naturally occurring neuropeptides which can be readily analyzed from biological tissue sources are far

more complex than could previously be imagined.

This is illustrated in this note, where we show an LC-MALDI MS/MS based study of a biological mixture of natural peptides freshly collected from living tissue. Whereas in principle any living tissue releases peptides into its environment, here we focus on an easily accessible and particularly rich source of biological peptides, *i.e.* the insect master neurosecretory gland (equivalent to the mammalian pituitary). The corpus cardiacum of a large cockroach species (*Blaberus giganteus*) was used as model tissue.



Blaberus giganteus, large cockroach species.

The method employs the separation of freshly prepared extracts with a MALDI spotting device utilizing reversed phase chromatography, microfraction collection and direct spotting on a standard MALDI target plate including co-deposition of a matrix solution. Subsequently to an MS acquisition, data files were processed with MALDI-merge (Waters Corp.) software that generates precursor ion information based on ion intensities detected across all fractions deposited on the MALDI target. The most intense precursor ions were selected and exported as an include list for automatic MS/MS fragmentation experiments.

Verhaert and co-workers previously demonstrated that neuropeptides can be analyzed by MALDI instruments, requiring very little, if any, sample manipulations of the neurosecretory tissue prior to the actual spectrum acquisition.¹ Using this approach, neuropeptide spectra are typically generated directly from the tissue placed on a MALDI target, or after a brief one-minute extraction.

Identification of peptides is based on exact precursor mass as well as exact fragmentation data considering specific modifications that frequently occur in (insect) neuropeptides. This study was performed with

two freshly prepared tissue extracts and three independent LC-MALDI MS/MS experiments. The results of one of the latter experiments are explained in more detail in this application note.

Experimental

Sample Preparation

Giant cockroaches, *Blaberus giganteus*, were obtained from a standard culture maintained at Antwerp Zoo. Corpora cardiaca were rapidly micro-dissected and briefly (1 min) extracted in 10 μ L of a 50% MeOH, 0.1% TFA solution. 5 μ L of tissue extract was 10 fold diluted with an 0.1% TFA solution and loaded on a Symmetry 5 mm x 300 μ m C₁₈, 5 μ m Pre-column at flow rate of 20 μ L/min. 60 fractions of 1 min were directed onto a MALDI standard target plate with a CTC PAL microfraction collector (Figure 1).



Figure 1. CTC PAL (CTC Analytics, Switzerland) MALDI spotter/microfraction collector.

A 2 mg/mL α -cyano-4-hydroxycinnamic acid solution (plus 5mM ammonium dihydrogenphosphate) was added post-column via a y-connector and co-deposited during sample collection (0.8 μ L/min flow rate) utilizing a syringe pump.

LC Fractionation and MS Conditions

Nanoscale LC fractionation experiments were conducted using a 1 hr reversed-phase gradient at 300 nL/min (5 to 40% acetonitrile over 60 minutes) conducted with a nanoACQUITY System (Waters Corp.) utilizing an Atlantis 3 μ m C₁₈ NanoEase 75 μ m x 15 cm Column (Waters Corp).

The MALDI Q-ToF Premier Mass Spectrometer (Waters Corp.) was externally calibrated with a polyethyleneglycol mixture (m/z 50 to m/z 3000). During acquisition the data were lock mass corrected using the monoisotopic mass of [Glu1]-Fibrinopeptide B on the near-point lock mass spot on the MALDI target plate. Data were acquired

automatically in MS and MS/MS mode. MALDI Merge Software² allows merging of all MS information and peptide sorting according to their mass and intensity. This information was used for defining an include list of all precursors in order to perform unattended, automated MS/MS fragmentation experiment with pre-defined collision energies. Deisotoping of the fragmentation spectra was conducted with MaxEnt3 prior to *de novo* sequence annotation with PepSeq Software.

Results and Discussion

Figure 3 shows an MS spectrum of the sample mixture before separation, *i.e.* direct tissue analysis utilizing 160 mg/mL DHB as a matrix. Although the information content of the spectrum is dense, gathering sequence information from selected precursors – applying a data directed analysis experiment – would be challenged due to ion suppression effects and rapid sample consumption.

Therefore, an off-line LC-MALDI MS/MS experiment using a precursor inclusion list consisting only of potentially interesting neuropeptides detected in a preceding MS run is a suitable approach, as demonstrated here. Figure 2 illustrates the MALDI merge software tool that allows handling MS data acquired in a first instance. Several different sorting strategies are possible, but the most common strategy was employed in this study – analyzing the most intense precursors first.

LC MALDI Merge

File Settings Grid Process Refresh Grid Help

#	Select	Mass	Intensity	Well	Path	File	Gain	Rel Int	Offset	Row	Col	LC Time
139	x	1362.7689	8135.938	B10	tion-29March06-MS_022.raw	deisotoped	-	-	-	2	10	31m30s
98	x	1164.6328	6626.375	B3	tion-29March06-MS_015.raw	deisotoped	-	-	-	2	3	26m15s
86	x	1150.6162	5369.75	A12	tion-29March06-MS_012.raw	deisotoped	-	-	-	1	12	24m0s
114	x	996.6424	5167.126	B5	tion-29March06-MS_017.raw	deisotoped	-	-	-	2	5	27m45s
144	x	1369.6225	5139.501	B11	tion-29March06-MS_023.raw	deisotoped	-	-	-	2	11	32m15s
116	x	883.5101	4960.813	B6	tion-29March06-MS_018.raw	deisotoped	-	-	-	2	6	28m30s
115	x	1237.5047	3834.875	B5	tion-29March06-MS_017.raw	deisotoped	-	-	-	2	5	27m45s
78	x	1672.9258	3575.438	A10	tion-29March06-MS_010.raw	deisotoped	-	-	-	1	10	22m30s
135	x	1457.6199	3449.125	B9	tion-29March06-MS_021.raw	deisotoped	-	-	-	2	9	30m45s
50	x	909.4755	2711.813	A6	tion-29March06-MS_006.raw	deisotoped	-	-	-	1	6	19m30s
143	-	1362.7669	2698.625	B11	tion-29March06-MS_023.raw	deisotoped	-	-	-	2	11	32m15s
147	x	1384.6273	2494.875	B12	tion-29March06-MS_024.raw	deisotoped	-	-	-	2	12	33m0s
150	x	1897.9608	2407.25	B12	tion-29March06-MS_024.raw	deisotoped	-	-	-	2	12	33m0s
47	x	822.4429	2398.813	A6	tion-29March06-MS_006.raw	deisotoped	-	-	-	1	6	19m30s
56	x	1272.6105	2051	A7	tion-29March06-MS_007.raw	deisotoped	-	-	-	1	7	20m15s
119	-	1237.5074	1888.875	B6	tion-29March06-MS_018.raw	deisotoped	-	-	-	2	6	28m30s
138	x	1352.7502	1774.938	B10	tion-29March06-MS_022.raw	deisotoped	-	-	-	2	10	31m30s
140	-	1369.6289	1757.688	B10	tion-29March06-MS_022.raw	deisotoped	-	-	-	2	10	31m30s
72	x	1853.9627	1753.75	A9	tion-29March06-MS_009.raw	deisotoped	-	-	-	1	9	21m45s
128	x	1452.7534	1684.688	B7	tion-29March06-MS_019.raw	deisotoped	-	-	-	2	7	29m15s
42	x	1948.9777	1657.813	A4	tion-29March06-MS_004.raw	deisotoped	-	-	-	1	4	18m0s
91	x	1275.6927	1626.625	B1	tion-29March06-MS_013.raw	deisotoped	-	-	-	2	1	24m45s
1	x	1329.6945	1603.751	A1	tion-29March06-MS_001.raw	deisotoped	-	-	-	1	1	15m45s
148	x	1624.7479	1550.375	B12	tion-29March06-MS_024.raw	deisotoped	-	-	-	2	12	33m0s
45	x	2368.0963	1512.063	A5	tion-29March06-MS_005.raw	deisotoped	-	-	-	1	5	18m45s
85	x	1867.9118	1493.063	A11	tion-29March06-MS_011.raw	deisotoped	-	-	-	1	11	23m15s
80	x	1140.5959	1484.75	A11	tion-29March06-MS_011.raw	deisotoped	-	-	-	1	11	23m15s
95	x	1154.6149	1483.5	B2	tion-29March06-MS_014.raw	deisotoped	-	-	-	2	2	25m30s
81	x	1286.6267	1477.063	A11	tion-29March06-MS_011.raw	deisotoped	-	-	-	1	11	23m15s
61	x	1143.5687	1394.563	A8	tion-29March06-MS_008.raw	deisotoped	-	-	-	1	8	21m0s
77	x	1655.9049	1355.125	A10	tion-29March06-MS_010.raw	deisotoped	-	-	-	1	10	22m30s
48	x	868.4940	1296.813	A6	tion-29March06-MS_006.raw	deisotoped	-	-	-	1	6	19m30s
149	x	1639.9534	1286.688	B12	tion-29March06-MS_024.raw	deisotoped	-	-	-	2	12	33m0s
87	x	1157.5832	1282.063	A12	tion-29March06-MS_012.raw	deisotoped	-	-	-	1	12	24m0s
113	x	979.6199	1265.125	B5	tion-29March06-MS_017.raw	deisotoped	-	-	-	2	5	27m45s
35	x	1342.6644	1252.25	A3	tion-29March06-MS_003.raw	deisotoped	-	-	-	1	3	17m15s
132	x	931.4990	1240.688	B8	tion-29March06-MS_020.raw	deisotoped	-	-	-	2	8	30m0s
105	x	1271.7568	1233.25	B4	tion-29March06-MS_016.raw	deisotoped	-	-	-	2	4	27m0s
100	x	862.4697	1230.125	B4	tion-29March06-MS_016.raw	deisotoped	-	-	-	2	4	27m0s
90	-	1150.6194	1206.625	B1	tion-29March06-MS_013.raw	deisotoped	-	-	-	2	1	24m45s
124	x	1190.5371	1194.438	B7	tion-29March06-MS_019.raw	deisotoped	-	-	-	2	7	29m15s
55	-	822.4461	1173.313	A7	tion-29March06-MS_007.raw	deisotoped	-	-	-	1	7	20m15s
103	-	1164.6373	1171.875	B4	tion-29March06-MS_016.raw	deisotoped	-	-	-	2	4	27m0s
41	x	1260.7799	1118	A4	tion-29March06-MS_004.raw	deisotoped	-	-	-	1	4	18m0s
134	x	1263.7007	1087.25	B9	tion-29March06-MS_021.raw	deisotoped	-	-	-	2	9	30m45s
63	x	861.4867	1086.875	A9	tion-29March06-MS_009.raw	deisotoped	-	-	-	1	9	21m45s
94	x	2278.1275	1071.125	B1	tion-29March06-MS_013.raw	deisotoped	-	-	-	2	1	24m45s

Data Grid Mass Difference Exclude List

Select Mass 1170.5 Select Mass Find Mass Difference 162 Find Diff

134 peaks selected

Figure 2. MALDI Merge Software Tool listing peptides intensity sorted after an MS acquisition.

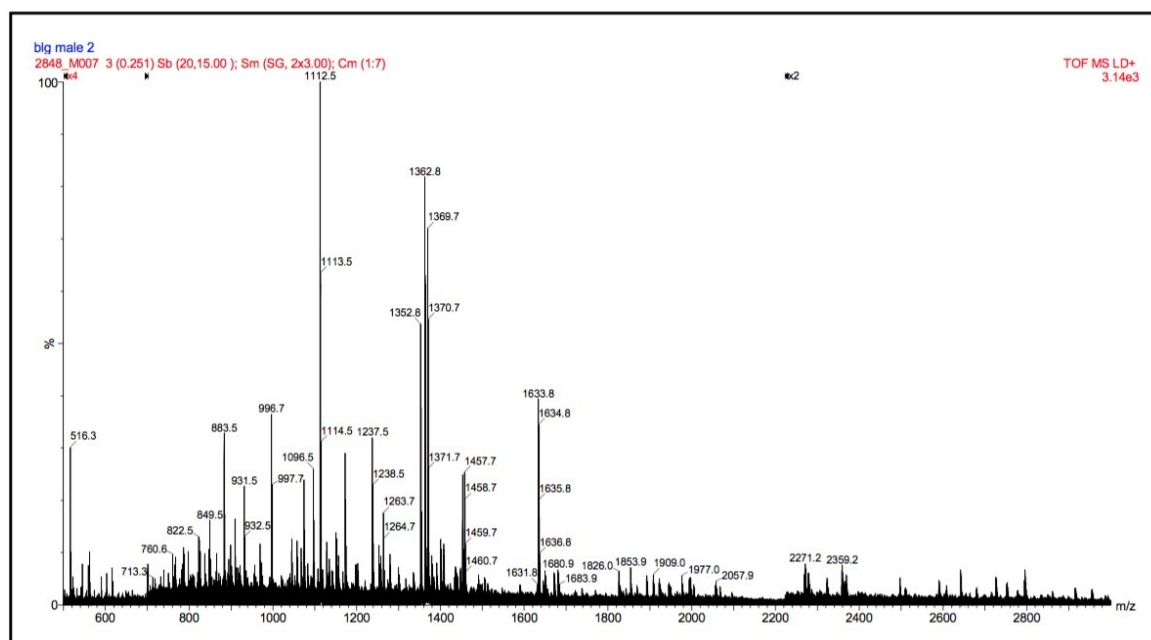


Figure 3. MS MALDI ToF spectrum of neurosecretory gland of *Blaberus giganteus*, direct tissue analysis.

Sequence Motives

The results of the two sequence annotation experiments – shown in Figures 4 and 5 – confirm the presence of a common sequence motive $-(\text{PF})\text{XPRI}/\text{L-NH}_2$ previously observed by Verhaert *et al.*¹ These common sequence motives often give rise to signature/fingerprint MS/MS profiles, which reflect various neuropeptide classes sharing ‘base’ amino acid sequences, but also distinct in a large number of possible post-translational modifications (PTMs) and a complex combination thereof. Several of these modifications comprise easily identifiable known mass differences. A considerable number, however, are defined by mass differences which have not been characterized before.

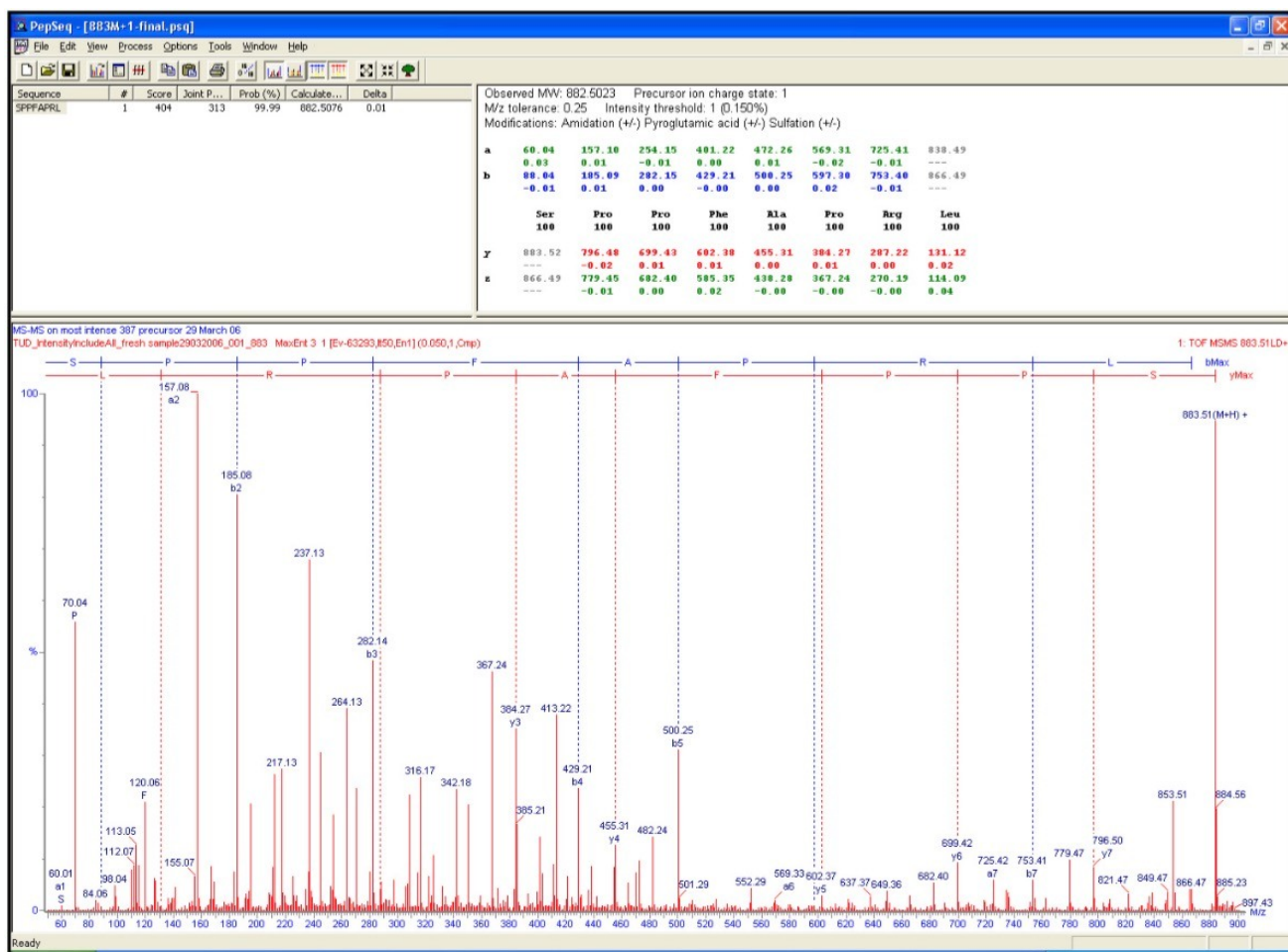


Figure 4. De novo sequence result from precursor m/z 883.5 (L or I, C-terminal amide).

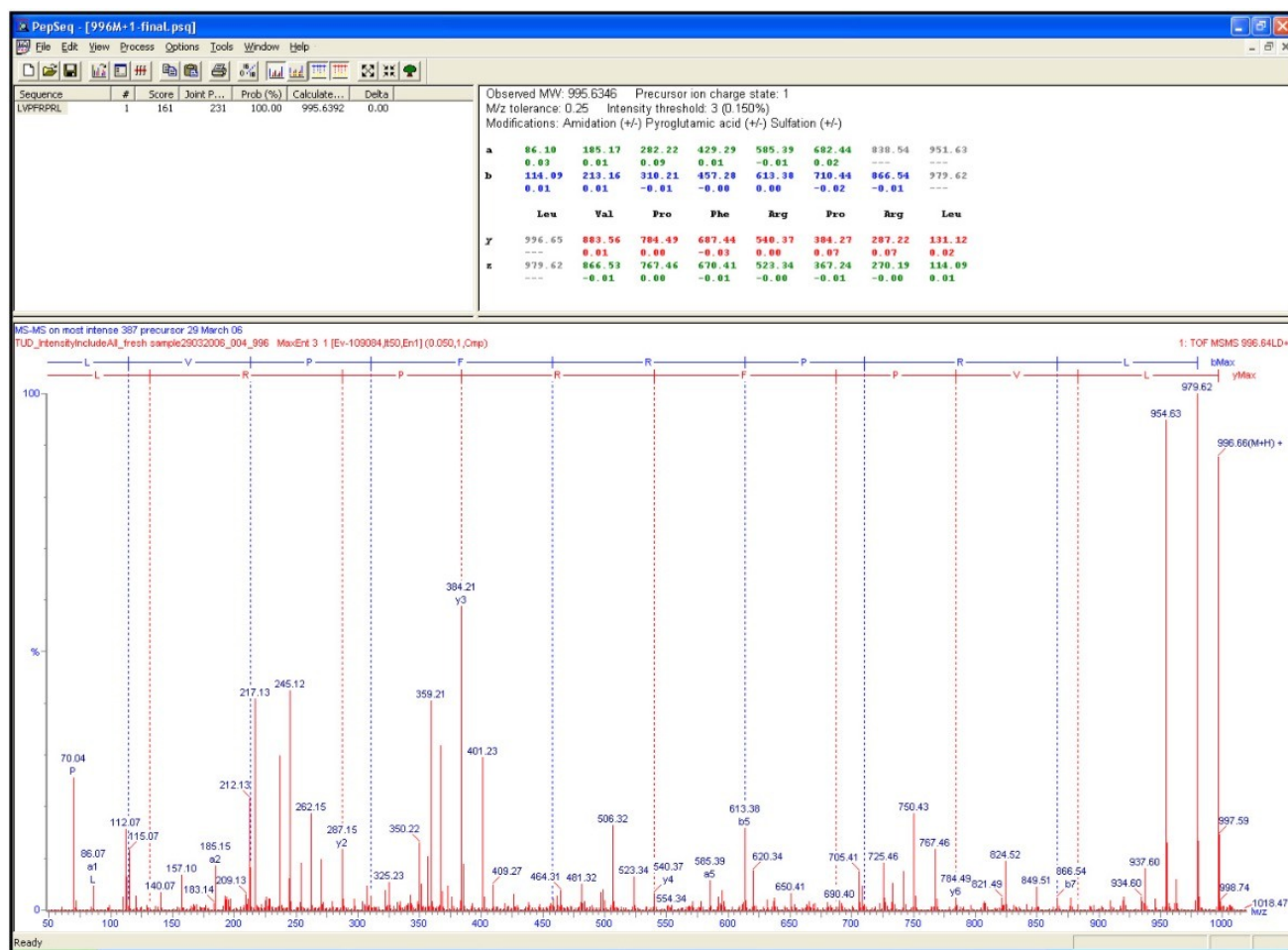


Figure 5. De novo sequence result of precursor m/z 996.6 (L or I, C-terminal amide).

The biological and physiological implications of these findings are intriguing. We believe that the presence of many variants of neuropeptides synthesized *in vivo* comprise PTMs representing numerous subtle regulation steps modulating the peptide's biological (in)activity.

Thus, the strategy of decoupling a separation step from MS acquisition allows a more in depth selection for targeted precursors, avoiding redundant MS/MS acquisition in subsequent runs.

Over 80 useful MALDI-MS/MS spectra were automatically collected with the presented approach, which is a significant improvement compared to direct tissue analysis neuropeptide approaches. This is a result of reduced ion suppression as the components are separated in time and space from each other. The main challenge, however, still remains the (automated) annotation of the MS/MS spectra. Development of software that allows for

the sequencing of non-tryptic peptides – taking into account the analysis of internal fragments, such as the intense fragments with an aminoterminal proline as observed here, as well as possible rearrangements – would greatly facilitate *de novo* sequencing of 'native' neuropeptides.

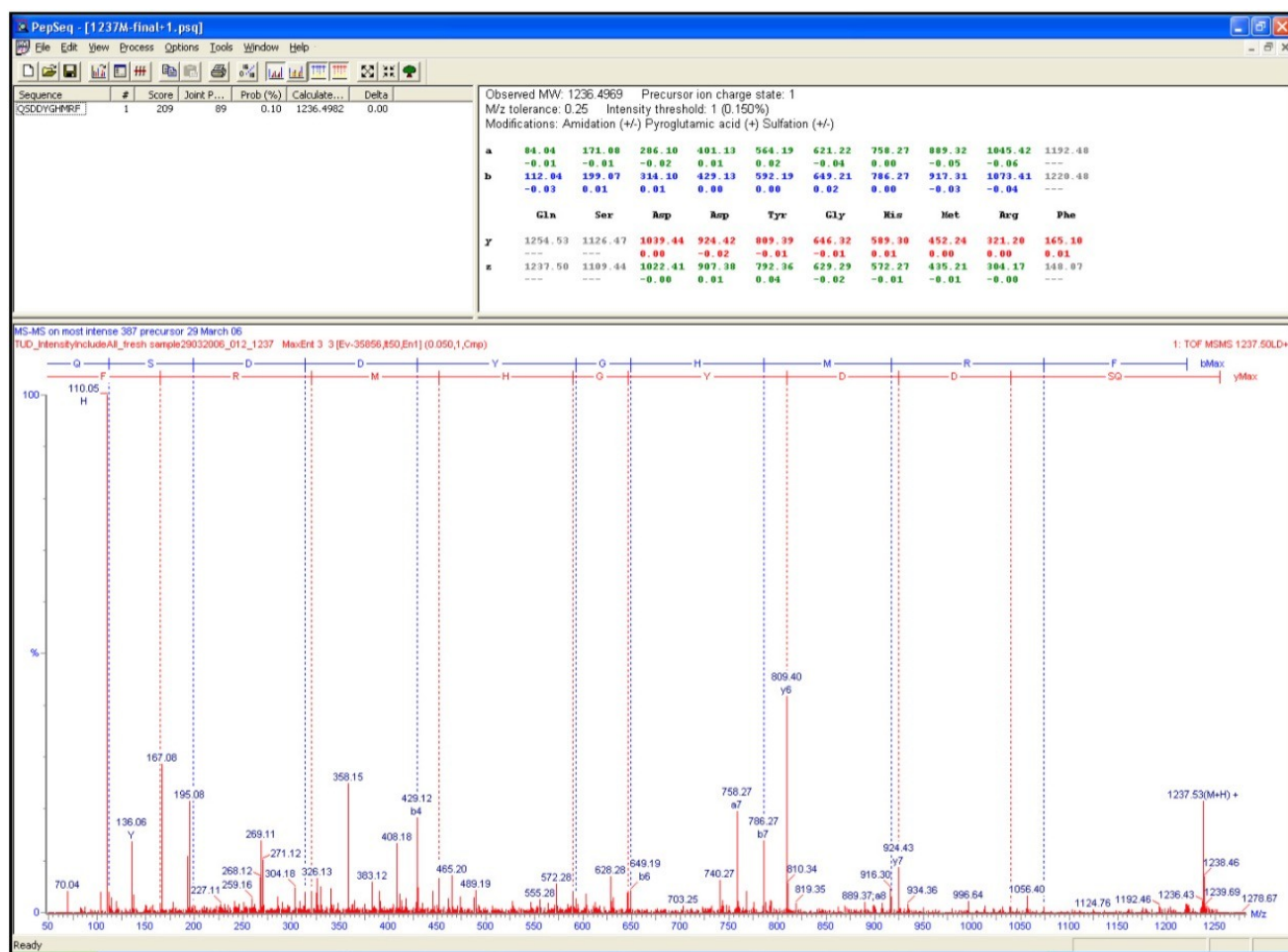


Figure 6. *De novo* sequence result of precursor m/z 1237.5 (N-terminal pyroglutamic acid, C-terminal amide).

Conclusion

- Nanoscale LC allows for the easy, robust and unattended fractionation and off-line collection of peptides prior to MALDI-MS/MS analysis.

- MALDI-MS/MS is a rapid and sensitive sequencing technique leading to unambiguous peptide identification.
- The processing of LC-MALDI data using automated software tools, allows a non-redundant list of peptides to be generated for each well position.
- MS/MS fragmentation information from more than 80 precursors were automatically obtained and selected from the intensity sorted merge list.

References

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Future Work

Future work will focus on optimization of separation and spotting with the MALDI spotter in order to increase system versatility. In addition, comparison with electrospray data will reveal whether the identified sequences are unique to the applied nanoscale LC-MALDI MS/MS approach.

Acknowledgements

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